# THE BRINE SHRIMP
# ARTEMIA

volume 1

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# VOLUME 1

MORPHOLOGY

GENETICS

RADIOBIOLOGY

TOXICOLOGY

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MORPHOLOGY

GENETICS

RADIOBIOLOGY

TOXICOLOGY

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# Foreword

*The "International Symposium on the brine shrimp* Artemia salina*" was convened at the La Quinta Royale Motor Inn in Corpus Christi (Texas, USA) in August 1979 and was attended by approximately 200 participants from 26 countries of the five continents.*

*This symposium was entirely devoted to all research and application aspects of an aquatic invertebrate which is used worldwide as a most suitable study object for fundamental research in practically all biological disciplines, and which moreover constitutes an indispensable source of live food for larval crustaceans and fishes mass cultured for commercial purposes.*

*The purpose of the Convention was three-fold :*

- *To bring together all those working or interested in* Artemia *to exchange their findings on this unique crustacean ;*
- *To promote contacts among specialists in different* Artemia *research areas and to stimulate interdisciplinary research on brine shrimp ;*
- *To publish the reviews, the contributed papers and the syntheses and recommendations of the workshops as a reference book giving the state of the art of the present knowledge on* Artemia.

*Because the response to the call for papers was overwhelming, the program was divided in concurrent sessions with the following topics :*

*Morphology – Radiobiology – Genetics*
*Physiology – Toxicology*
*Biochemistry – Molecular Biology*
*Ecology – Culturing – Use in Aquaculture*

*During these sessions seven reviews covering the major areas of* Artemia *research were presented by invited authorities and complemented by approximately 100 contributed papers.*

*In order to make a synopsis of the progresses reported at the Symposium and to define particular areas of* Artemia *research deserving urgent attention, the last day of the Symposium was devoted to four workshops on the following themes :*

- *Characterization of* Artemia *strains for application in aquaculture ;*
- *Commercial aspects of* Artemia *exploitation ;*
- *Species characterization in* Artemia *;*
- *Proposal for an intercalibration exercise for a standard* Artemia *toxicity test.*

*According to most participants, this interbreeding of "fundamentalists" with "applicants" and of "researchers" with "commercially oriented people" has been extremely stimulating. Everybody went home convinced of the following facts :*

*1)* Artemia *and* Artemia *are two ;*
*2) Despite the tremendous amount of literature on* Artemia *(nearly 3 000 references) there are large gaps in our knowledge of this unique crustacean ;*
*3) The advantages of* Artemia *as a study object for fundamental research are not yet fully exploited and the potential of brine shrimp as a direct or indirect source of food for man becomes more and more obvious ;*
*4) Interdisciplinary research on* Artemia *which was embryonic so far is gradually developing and deserves full attention and stimulation.*

*The Editors*


Welcome
International Symposium on Brine Shrimp
"ARTEMIA SALINA"

# Group picture of participants

*Despite all efforts and help we have not been able to identify all the people on the picture. We apologize for the "blanks" and list the names of those we recognized.*

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136. Al Warner
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140. Harold Webber
141. Bill Shaw
142. William Tobias
143. Giuseppe Baudo
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148. Paul Sandifer
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150. Oswald Roels
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# Editorial note on the taxonomy of *Artemia*

*The binomen* Artemia salina *L. is taxonomically no longer valid (Bowen and Sterling, 1978).*

*Crossing experiments of different* Artemia *populations revealed reproductive isolation of several groups of populations (Halfer-Cervini* et al., *1968 ; Barigozzi, 1972, 1974 ; Clark and Bowen, 1976) and led to the recognition of sibling species to which different names have been given according to the International Conventions of Taxonomical Nomenclature (Bowen and Sterling, 1978). So far 20 bisexual strains have been classified into five sibling species (Bowen* et al., *1978).*

*Theoretically the conventional name* Artemia salina *L. can only be used for the original material from salt ponds in Lymington, England employed by Schlosser in 1755 to make the first drawing and by Linnaeus in 1758 to make the first description of the species (Kuenen and Baas-Becking, 1938). Because these salt ponds have disappeared, and* Artemia *no longer occurs in England, the species name* salina *should no longer be used.*

*In view of the important genetical differences that exist between parthenogenetical strains of brine shrimp (Abreu-Grobois and Beardmore, 1980) species definition in the genus* Artemia *has become confusing.*

*It is suggested that unless the exact sibling species of a bisexual* Artemia *strain can be identified* (cf. *Bowen* et al., *1978, 1980) and until speciation in brine shrimp is more clearly understood, only the genus designation* Artemia *should be used.*

## Literature cited


Abreu-Grobois F. A. and J. A. Beardmore. 1980. International Study on *Artemia*. II. Genetic characterization of *Artemia* populations – an electrophoretic approach. p. 133-146. *In* : The brine shrimp *Artemia*. Vol. 1. Morphology, Genetics, Radiobiology, Toxicology. Persoone G., P. Sorgeloos, O. Roels, and E. Jaspers. Universa Press, Wetteren, Belgium. 345 p.

Barigozzi C. 1972. Problems of speciation in the genus *Artemia*. p. 61-66. *In* : Proc. 5th European Marine Biology Symposium, Battaglia B. (Ed.). Piccin Editore, Padova, Italy. 348 p.

Barigozzi C. 1974. *Artemia* : a survey of its significance in genetic problems. p. 221-252. *In* : Evolutionary biology. Vol. 7. Dobzhansky T., M. K. Hecht, and W. C. Steere (Eds). Plenum Press, New York, 314 p.

Bowen S. T., M. L. Davis, S. R. Fenster, and G. A. Lindwall. 1980. Sibling species of *Artemia*. p. 155-167. *In* : The brine shrimp *Artemia*. Vol. 1. Morphology, Genetics, Radiobiology, Toxicology. Persoone G., P. Sorgeloos, O. Roels, and E. Jaspers (Eds). Universa Press, Wetteren, Belgium. 345 p.

Bowen S. T., J. P. Durkin, G. Sterling, and L. S. Clark. 1978. *Artemia* hemoglobins : genetic variation in parthenogenetic and zygogenetic populations. *Biol. Bull.* 155:273-287.

Bowen S. T. and G. Sterling. 1978. Esterase and malate dehydrogenase isozyme polymorphisms in 15 *Artemia* populations. *Comp. Biochem. Physiol.* 61B:593-595.

Clark L. S. and S. T. Bowen. 1976. The genetics of *Artemia salina*. VII. Reproductive isolation. *J. Hered.* 67(6): 385-388.

Halfer-Cervini A. M., M. Piccinelli, T. Prosdocimi, and L. Baratelli-Zambruni. 1968. Sibling species in *Artemia* (Crustacea, Branchiopoda). *Evolution* 22:373-381.

Kuenen D. J. and L. G. M. Baas-Becking. 1938. Historical notes on *Artemia salina* (L.). *Zool. Med.* 20:222-230.

# Life history of the brine shrimp *Artemia*

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*The first written record of the existence of the brine shrimp dates back to 1755 (Schlosser in Kuenen and Baas-Becking, 1938). Nonetheless this "filtering animal" was known since much longer times by different ethnic groups who attributed a better salt production in brine pools to the presence of* Artemia *; hence its popular names such as brineworm, Salztierchen, verme de sale, sóféreg, Bahar el dud, Fezzanwurm,* etc.

*Despite the primitive optical equipment available at that time, Schlosser's drawings were very detailed (Fig. 1) and rightly gave the adult animal 11 pairs of thoracopods. Several other scientists, including Linnaeus (1758), later described adult* Artemia *with only 10 thoracopods. This controversy lasted until 1836 when finally Audouin confirmed the observations of Schlosser.*

*From the second half of the 19th century on, several studies were published dealing with the morphology and taxonomy of this Anostracan crustacean. Soon* Artemia *was used as a most suitable test-object in the most diverse disciplines of biological sciences : histology, genetics, radiobiology, toxicology, biochemistry, molecular biology, ecology,* etc.

*Salt lakes and brine ponds with* Artemia *populations are found worldwide. The ecological conditions in these biotopes are extreme* (e.g. *the salinity can exceed 300 g salts/l water), and as a result only a small number of bacterial and algal species can survive. As a consequence of the often occurring blooms of monocultures of specific algal species, these waters are colored red, blue or green. One of the very few invertebrates that could adapt to such an extreme habitat is the brine shrimp* Artemia. *Favored by the absence of predators and food competitors,* Artemia *mostly develops into very dense populations in the salinas.*

*At certain moments of the year, enormous quantities of minuscule brown particles (200-300 µm in diameter) are floating at the lake's surface and are finally thrown ashore by wind and waves (Fig. 2. 1). These apparently inert particles are in fact the inactive dry cysts of the brine shrimp which remain in diapause as long as they are kept dry or under anaerobic conditions. Upon immersion in seawater, the cysts hydrate, become spherical and within their shell the metabolism of the embryo is activated. A number of hours later, the outer membranes of the cyst burst (= "breaking" or E-1 stage) and the embryo appears, surrounded by the hatching membrane. The only structural feature which can be observed is the nauplius eye (Fig. 2. 2). During the following hours the embryo leaves the cyst's shell (E-2 stage ; Fig. 2. 3). Inside the hatching membrane, the newly differentiated antennae and mandibles start moving ; within a short period of time the hatching membrane is ruptured and the free-swimming nauplius is*

[1] "Bevoegdverklaard Navorser" at the Belgian National Foundation for Scientific Research (NFWO).

*born. This first instar larva which is colored brownish-orange due to the presence of yolk, has three pairs of appendages : the antennae which have a locomotory function, the sensorial antennulae and the rudimentary mandibles (Fig. 2. 3). An unpaired red ocellus is situated in the head region between the antennulae. The ventral side of the animal is covered by a large labrum.*

*The larva grows and differentiates through about 15 molts : the trunk and abdomen are elongating ; the digestive tract becomes functional ; food particles are collected from the medium by the setae of the antennae ; paired lobular appendages which will differentiate into the thoracopods are budding in the thrunk-region ; lateral complex eyes are developing on both sides of the ocellus ; etc. (Fig. 2. 4 and 5).*

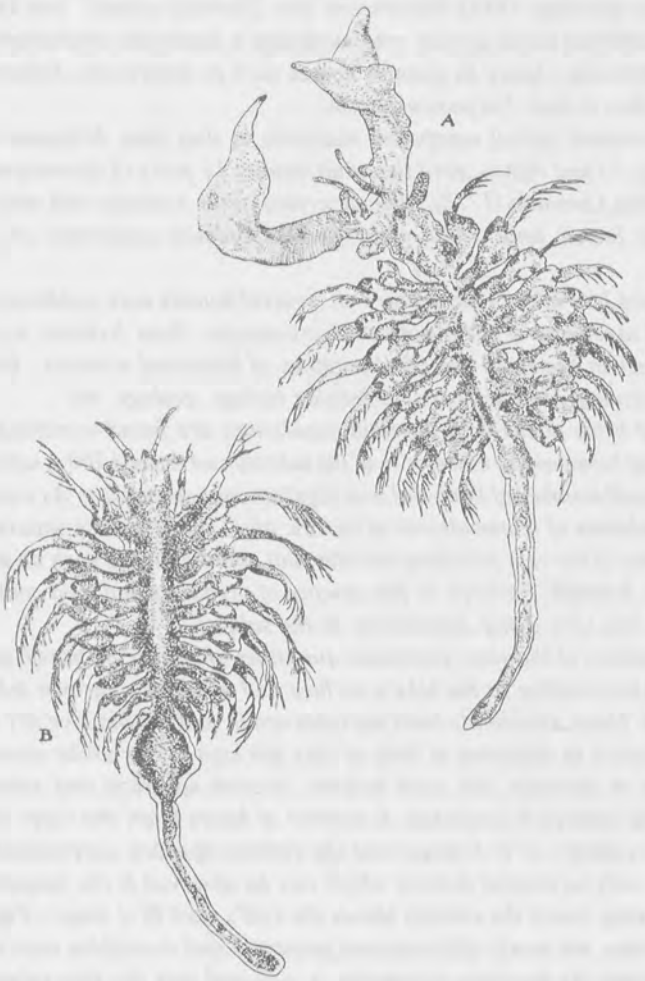


FIG. 1. Schlosser's drawing of a male (A) and a female (B) brine shrimp (From Kuenen and Baas-Becking, 1938).

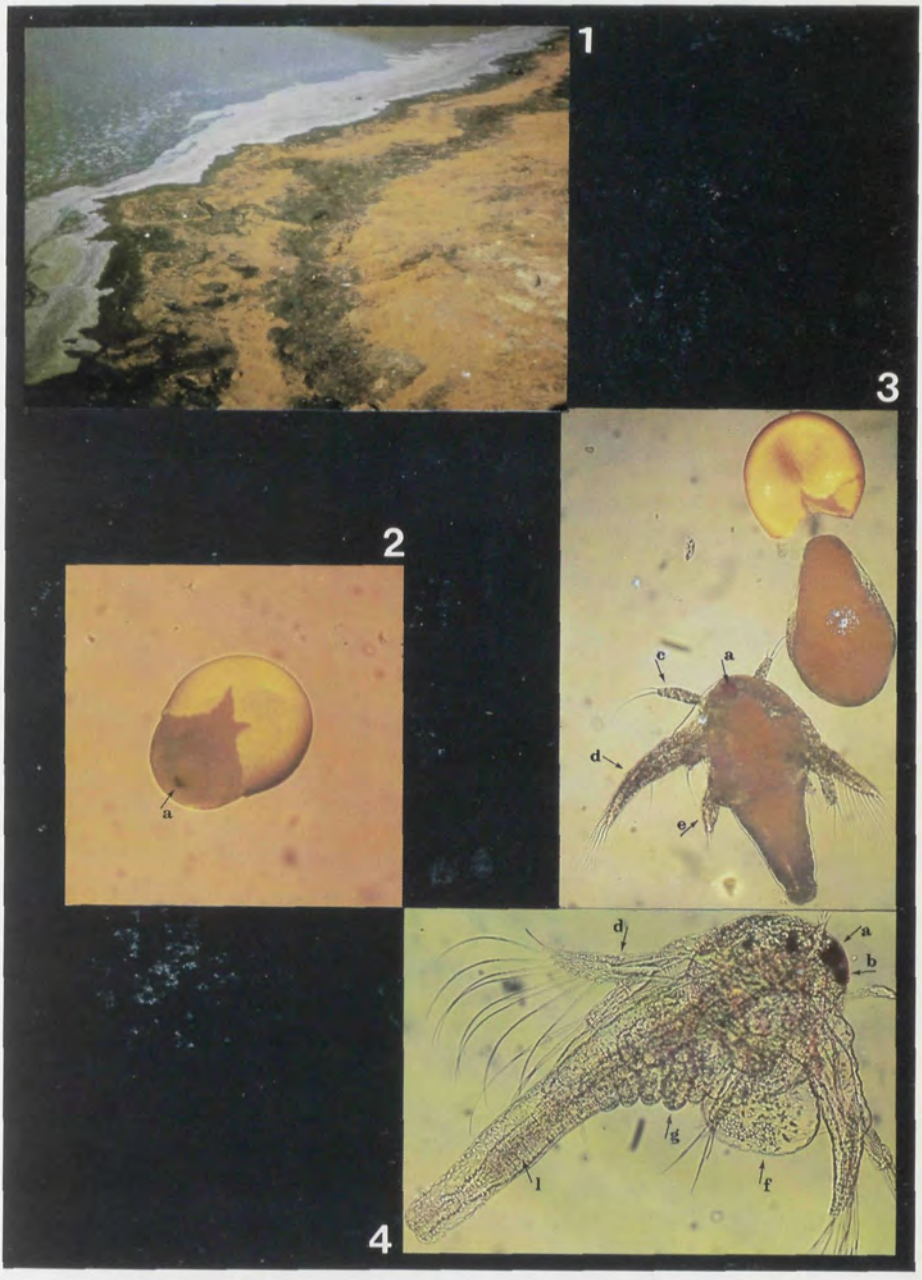


FIG. 2. 1. Brown layer of brine shrimp cysts accumulated on the shore of a salina ; 2. Pre-nauplius in E-1 stage ; 3. Pre-nauplius in E-2 stage and freshly hatched instar I nauplius ; 4. Instar V larva. a. nauplius eye ; b. lateral complex eye ; c. antennula ; d. antenna ; e. mandible ; f. labrum ; g. budding of thoracopods ; h. digestive tract.


FIG. 3. 5. Head and anterior thoracic region of instar XII ; 6. Head and anterior thoracic region of young male (instar XV) ; 7. Posterior thoracic region and uterus of fertile female ; 8. Head of adult male ; 9. *Artemia*-couple in riding position. a. nauplius eye ; b. lateral complex eye ; c. antennula ; d. antenna ; e. mandible ; h. exopodite ; i. telopodite ; j. endopodite ; k. frontal knob ; m. inactive ovary ; n. ripe eggs in oviduct ; o. uterus ; p. penis.

*From the 10th instar on, important morphological changes are taking place : the antennae loose their primitive locomotory function ;* i.e. *they loose their long setae and undergo sexual differentiation. In the future males they develop into hooked graspers, while in the females the antennae degenerate into sensorial appendages (Fig. 3. 5, 6, and 8). The thoracopods are now differentiated into three functional parts : the telopodites acting as a filter, the oarlike endopodites having a locomotory activity, and the membranous exopodites functioning as gills (Fig. 3. 5 and 6).*

*The adult animal 8-10 mm long, is characterized by the stalked lateral (complex) eye, the sensorial antennulae, the linear digestive tract, and the 11 pairs of functional thoracopods (Fig. 3. 6 and 9). In the male* Artemia *the antennae are transformed into muscular graspers which have a frontal knob at their inner side (Fig. 3. 8). In the posterior part of the trunk region a paired penis can be observed (Fig. 3. 9).*

*Female* Artemia *have very primitive antennae with sensorial function ; their paired ovaries are situated on both sides of the digestive tract behind the thoracopods. The ripe oocytes are transported from the ovaries into the unpaired brood pouch or uterus via two oviducts (Fig. 3. 7).*

*Precopulation in adult brine shrimp is initiated by the male in grasping the female with its antennae between the uterus and the last pair of thoracopods. In this "riding position" the couples can swim around for long periods (Fig. 3. 9).*

*Copulation itself is a very fast reflex : the male abdomen is bent forward and one penis is introduced into the uterus aperture. The fertilized eggs develop into either free-swimming nauplii (ovoviviparous reproduction) which are set free by the mother, or when reaching the gastrula stage, they are surrounded by a thick shell and are deposited as cysts, which are in diapause (oviparous reproduction).*

*[Morphology of* Artemia *after Heath (1924), Nakanishi* et al. *(1962), Anderson (1967), Benesch (1969), and Wolfe (1973)].*

## Acknowledgements

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## Literature cited

Anderson D. T. 1967. Larval development and segment formation in the branchiopod crustaceans *Limnadia stanlejana* (Conchostraca) and *Artemia salina*. *Austr. J. Zool.* 15:47-91.

Audouin A. 1836. Examen des Crustacés rapportés de la saline de Marignane. *Annales sciences naturelles, Zoologie, Paris, Sér. 2*, 6:226-231.

Benesch R. 1969. Zur Ontogenie und Morphologie von *Artemia salina* L. *Zool. Jb. Anat.* 86:307-458.

Heath H. 1924. The external development of certain phyllopods. *J. Morph.* 38(4):453-483.

Kuenen D. J. and L. G. M. Baas-Becking. 1938. Historical notes on *Artemia salina* (L.). *Zool. Med.* 20:222-230.

Linnaeus C. 1758. Systema naturae. Ed. X., Hafniae, 634 p.

Nakanishi Y. H., T. Iwasaki, T. Okigaki, and H. Kato. 1962. Cytological studies of *Artemia salina*. I. Embryonic development without cell multiplication after the blastula stage in encysted dry eggs. *Annot. Zool. Japon.* 35:223-228.

Wolfe A. F. 1973. Observations on the clasping behavior of *Artemia salina*. *Amer. Zool.* 13(4):472.

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# REVIEW

*The Brine Shrimp* Artemia. *Vol. 1. Morphology, Genetics, Radiobiology, Toxicology*
*G. Persoone, P. Sorgeloos, O. Roels, and E. Jaspers (Eds). Universa Press, Wetteren, Belgium. 345 p.*

# Review on the radiobiology of *Artemia*

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**Content**

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Diapause embryos
Short- and long-term somatic effects
Female germ-cells of parthenogenetic strains
Quantitative traits and fitness components
Comments and conclusions
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## Introduction

Several reasons have been offered from time to time to justify experimental research on the biological effects of ionizing radiation. Limiting our interests to animals and animal cells, there are still two important justifications for radiation biology that are both relevant to large-scale practical problems, which are immediately appreciated not only by scientists, but also by the general public : 1. the medical uses of radiation, particularly for the radiotherapy of cancer and for diagnostic purposes, and 2. radiation protection. A third reason may be found in the large "fall-out" of basic information and data, new techniques and methodologies that was generated by radiation biology and that has stimulated so much fundamental research in the last 30 years. One can say on a retrospective basis that in the 1950s and early 60s radiobiological studies have given positive contributions to the advancement of practically all fields of biological science.

Yet, the two reasons mentioned at the beginning, radiotherapy and radiation protection, are still with us and in my opinion will remain with us for quite some time. Both of them are related directly to man, and indirectly to the general environment: so then, why radiation biology of the little brine shrimp, that is naturally confined to small and extreme ecosystems, almost irrelevant to the general economy of our universe ? If I may quote here a personal experience, I remember that one of the reasons to propose a few pilot radiation experiments on *Artemia* at Professor Barigozzi's Institute in Milan, back in 1959, was that *Artemia* appeared to be the only animal model system with polyploid forms available for laboratory studies. At that time polyploid tumor cells were thought to be more radiation resistant than diploid cells, thus contributing to the overall radiation resistance of tumors, and perhaps responsible for at least some cases of tumor recurrence after radiotherapy. Truly polyploid

tumor cell lines were not yet developed, and the effect of polyploidy on radiosensitivity was measured only on yeasts and on haploid *vs.* diploid *Habrobracon* (Metalli and Ballardin, 1972). The idea was accepted and a line of research was developed with results of some interest in different fields. In fact we know now that the problems posed by radioresistance of tumors and of neoplastic cells are much more complex than it was thought 20 years ago, but the results of *Artemia* radiobiology have contributed positively to the general problem.

All research in radiobiology should be designed in such a way as to generate quantitative data for the analysis of dose-effect relationships for defined effects. Qualitative data, or the effects of only one dose, are usually meaningless at the present state of knowledge in this field, and a wide range of radiation doses should be explored in order to construct dose-effect relationships. Peculiar to radiobiology is the concept of "radiation dose", which has evolved very rapidly, if compared to other quantities identified and used in physical and natural sciences. There is still some confusion in terminology, particularly among non-specialists, and some points of clarification are needed here. 1. The term "dose" in this paper is used strictly to mean the "radiation absorbed dose" with dimensions = energy $\times$ mass$^{-1}$, the unit of which is the "rad" ($= 100$ erg $\times$ gram$^{-1}$). Recently, the new SI unit "Gray" (symbol: Gy) has been defined (1 Gy = 1 J $\times$ Kg$^{-1}$; 1 Gy = 100 rad), and should be used according to International Conventions (ICRV Report, 1980). However, I have preferred the old unit rad because it may be more familiar to non-specialists. 2. Dosimetric methodology allows correct measurements of the absorbed dose in rad units, and therefore reliable and reproducible results, only under well defined physical conditions; this is usually the case with X- and gamma-radiation beams and the *Artemia* in water. 3. Under different conditions (e.g. dry or wet cysts laid on a sheet of paper), measurement of the absorbed dose may be much more difficult and comparisons of results from different laboratories may just be meaningless, except for orders of magnitude. 4. For the latter reason the use of the old unit of the quantity "exposure" (the "roentgen", symbol: R, more familiar to most people) may be preferred, but it should be born in mind that it is not a unit of dose; it refers, rather, to the characterization of the beam, it is defined only in free air, and can be used only for comparisons with the same experiment under the same physical conditions.

The last point of general importance that should be mentioned here is the rather loose concept of "radiation sensitivity". No general agreement has been reached up to date on a definition of this concept (Metalli, 1979). It can be defined operationally and estimated quantitatively only under particular circumstances and therefore the general use of this concept should be avoided, except again for gross comparisons and differences of orders of magnitude in dose or exposure.

**Diapause embryos**

Some of the biological characteristics of the life cycle of *Artemia* have attracted the attention of radiation biologists since a long time. The radiation response of diapause embryos has been particularly studied mainly because they mimick bacterial spores, and the effects of some modifying agents under extreme conditions can be investigated in an animal cell model (water content, gas phase, temperature, ionization density, etc.). The early data have been reviewed by Metalli and Ballardin (1972). In more recent times the brine shrimp eggs have also been used for biophysical research ((Iwasaki *et al.*, 1980 ; Gaubin *et al.*, 1980), and for studies on

the effects of space radiation (Planel *et al.*, 1980). In general, it can now be said that the astonishingly high radioresistance of these cysts, as compared to practically all animal systems, remains largely unexplained. Although some contributing factors have been identified, such as the dry state, the high glycerol content and the genotype, we still have no ideas about a possible physical or biological target to be hit by radiation in order to inhibit the emergence and hatching of nauplii. A possible approach to this problem could be the use of radiations with different ionization density, as it was done as early as in 1961 (Easter and Hutchinson, 1961) and is continued up to date (Gaubin *et al.*, 1980). But the scattered data obtained under very different conditions can hardly allow calculations of target size with results of general validity. Furthermore, since the biological processes leading to emergence and hatching do not depend on cell division, and the frequency of success in hatching may be an intermediate endpoint of minor radiobiological significance in cellular terms, my personal opinion is that *Artemia* cysts are a most suitable test system for biophysical studies rather than for radiobiological research. The hypothesis of a formidable capacity to repair radiation damage is also appealing, and would really deserve more attention at the molecular level.

**Short- and long-term somatic effects**

The quantitative study of somatic effects of radiation require an optimal and standardized culture method. We have chosen to carry out all our experiments with artificial sea water and algae as exclusive food, the algae being obtained by monoxenic mass culture techniques. This can be achieved on a laboratory scale, although it is rather expensive. It may be of some interest to mention here that a 4-week old diploid parthenogenetic *Artemia*, just beginning reproduction, has cleared from the medium something like $10^9$ cells, which were obtained on the average from one liter of algal culture. The cost of consumable material to get an adult animal, normalized to 1968 prices (before the inflation rate started to grow faster) was of the order of U.S. $ 0.10 (Metalli, 1968). This would also be the average cost of maintenance for every subsequent week of life.

Under reasonably controlled and uniform conditions, the problem of life-span shortening by radiation was studied on a scale large enough to quantify this effect and to correlate it with strain and genotype. An example of the observational data is given in Fig. 1, where the simple method of probit plot shows that the normalized cumulative mortality is linearly related to post-radiation time up to relatively high doses, and displaced to shorter times with increasing doses. Grosch and Erdman (1955) obtained similar data as early as in 1955, but their maximum control life span was about 45 days, as compared to over 200 days under our experimental conditions. Incidentally, there is some indication from the experiments in our laboratory that longevity may be critically dependent upon the concentration of iron ions in the medium.

About 70 plots similar to that of Fig. 1 were obtained, and by simple statistical treatments the median or mean post-radiation survival time was analyzed as a function of dose, as exemplified in Fig. 2 for one of our laboratory strains. The mean survival time is a negative exponential function of radiation dose for doses up to about 50 Krad, the extrapolation number is not significantly different from 1.0 (in fact it is slightly less than 1.0), and the inverse slope is of the order of 27 Krad. This means that the life-span shortening effect of radiation in *Artemia* has no threshold, and that any dose increment takes away a constant fraction of the average expected remaining life span.

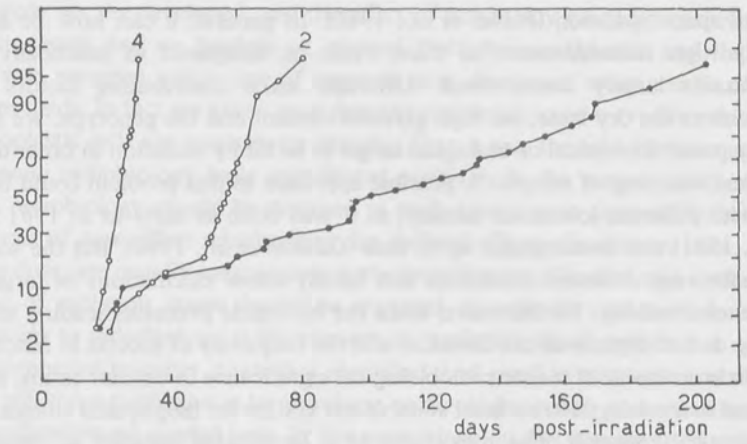


FIG. 1. Probit transformation of the per cent cumulative mortality of tetraploid parthenogenetic *Artemia* from Comacchio (Italy), as a function of post-treatment time in days. 0 = group of unirradiated controls ; 2 and 4 = groups irradiated with 20 and 40 Krad single acute doses, respectively, at 14 days of age (irradiation in water under conditions of full backscatter ; X-rays, 240 KVcp, HVL = 0.54 mmCu, 1240 rad/min, Simplex PTW dose-rate meter, calibration against secondary standard). 30 animals per group, feeding exclusively on algae. All the animals used in the experiments of Figs. 1, 2, and 3 were random samples from a laboratory strain derived from the progeny of a single *Artemia* hatched from a cyst collected in the Comacchio lagoon ; details can be found in Ballardin and Metalli (1963) and Metalli and Ballardin (1972).

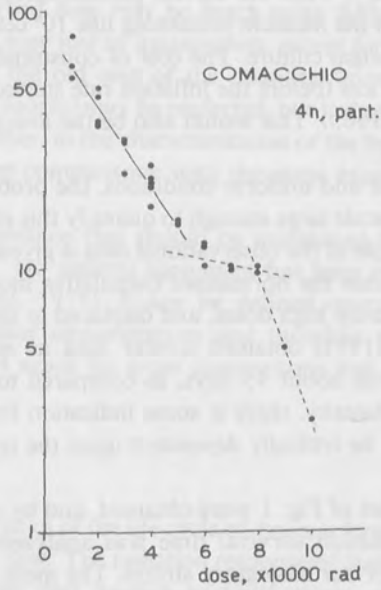


FIG. 2. Logarithm of mean post-treatment survival time in per cent of unirradiated controls, of tetraploid parthenogenetic *Artemia* from Comacchio (Italy), as a function of radiation dose. Each point represents the mean of two or more experimental groups of 30 animals each from independent experiments, irradiated with the same dose level. Solid line = interpolated best fit from linear regression analysis ; dotted lines = interpolation by eye. Control data are pooled from 12 independent experiments.

Such a dose-effect relationship has some interesting implications : 1. With respect to diapause embryos the radiosensitivity is increased by a factor of at least 10 ; 2. The absence of threshold would predict that no repair of radiation damage should take place, and this was confirmed by appropriate dose fractionation experiments ; 3. Under conditions of chronic exposure the overall effect should be expected as additive and cumulative with dose, although to the best of my knowledge no experiments have yet been done to verify this hypothesis ; 4. The type of radiation response of Figs. 1 and 2 should have some consequences on the total productivity of an irradiated *Artemia* population, and hence may have important implications for its population dynamics.

I shall not discuss the first three points in terms of fundamental mechanisms of radiation action, and would rather like to draw attention to the last point which is of more interest for practical reasons. From our observations we can conclude that at least under laboratory conditions the brine shrimp do continue to reproduce for all their life span, with very few exceptions in extremely old animals [cf. Tables B.11 and B.15 of Ballardin and Metalli (1963)]. We also observed that the maximum productivity in terms of number of eggs per brood is attained only at the third of fourth brood, approximately at the age of 7 weeks, and keeps constant for the rest of the life. From Fig. 1 it should then be expected that radiation takes out the proportional fraction of life span as predicted by the relationship of Fig. 2 at the age of maximum productivity for any level of dose, including low and very low doses. Thus a somatic effect of radiation, sometimes overlooked in modelling the predicted dynamic response of a population (IAEA Technical Report Series 1975, 1976, 1979) may be additive to the direct genetic effects in terms of decreased reproductive potential. In my opinion this point should be carefully considered in any case where methodologies are available, not only for radiation but also for other toxic agents of potential relevance to aquatic ecosystems.

Another main observation from our experiments was that the relationship of Fig. 2 holds for all the *Artemia* strains tested, independently of genotype, mode of reproduction, sex and ploidy, with the exception of the males from a California diploid bisexual sample, which were slightly more resistant; this exception remains so far unexplained.

Table I reports the final estimates of the radiosensitivity parameters from most of our experiments. These conclusions indicate that for late somatic effects of radition the basic mechanisms of action must be different from those underlying the single-cell response (see below), where the ploidy effect is prominent. As to the nature of these mechanisms, very little, if anything is known ; a major limitation is due to the lack of information on the causes of death and on the physio-pathological syndromes associated with somatic radiation damage in *Artemia*, and in lower organisms in general. Only at very high doses, practically above 60 Krad, does Fig. 2 show that the monotonic relationship already described breaks down into different slopes; the survival time is so short that it may be regarded as indicative of acute lethality, and the inflections are suggestive of different modes of action. In fact, it has been shown (unpublished) that the molting cycle is at first deeply altered and the animals will invariably die within the second post-radiation intermolt phase, the molting time delay being dependent on dose. But it has also been shown that at lower doses the molting cycle remains unaffected for all the remaining life span; this suggests that both the systems directly involved in the production of the exoskeleton and their regulatory mechanisms may not be damaged by radiation, thus contributing very little to the shortening of life.

TABLE I

Summary of the date from the linear regression analyses of the log mean post-radiation survival time of irradiated groups, normalized for the control values, as a function of dose.

| Laboratory strain | No. of experiments | Intercept at 0 dose (± st. error) (a) | Log slope × $10^{-3}$ (± st. error) (b) | |
|---|---|---|---|---|
| Comacchio (Italy) | | | | |
| tetraploid, parthenogenetic (*ameiotic*) | 28 | 0.88 ± 0.05 | −15.6 ± 1.8 | |
| Sète (France) | | | | |
| diploid, parthenogenetic (meiotic) | 8 | 0.91 ± 0.06 | −15.4 ± 1.8 | |
| San Bartolomeo (Sardinia, Italy) | 9 | 0.96 ± 0.08 | −15.5 ± 2.6 | Females |
| diploid, bisexual | | 1.05 ± 0.03 | −15.6 ± 0.3 | Males |
| San Francisco (California, USA) | 6 | 1.02 ± 0.10 | −15.6 ± 3.0 | Females |
| diploid, bisexual | | 1.21 ± 0.11 | −10.0 ± 2.6 | Males |

*Notes* : Test for parallelism on all strains, except males from San Francisco : $F < 1$ ; combined log slope : −15.5 ± 1.6. Two groups of animals from a sample of bisexual tetraploid or highly heteroploid animals from the Great Salt Lake (Utah, USA) collected in 1952 as dry cysts, fitted exactly the combined slope after a dose of 40 Krad ; this sample is now extinct (see considerations in the Report of Workshop III and the final recommendations !).

(a) The extrapolation number not significantly different from 1 (or from 100 % on the scale of Fig. 2) is evidence for non-threshold affects.

(b) The inverse slope (on appropriate scale and with transformation required by simple algebra) is the dose required to get $1/e = 0.37$ effect, and is commonly referred to as $D_0$.

I shall not leave this subject without suggesting a possible indirect approach to the study of life span in lower organisms, which has been proven to be very useful in mammalian systems (Metalli, 1979). The simple methods of the type exemplified in Fig. 1 extract a single estimate (e.g. the mean, or the slope) from a complex dose-time relationship. Attempts have been carried out to apply to shorter survival times and smaller experimental groups some methods of calculation of the mortality intensity, that is the approximation to the Gompertz function. Fig. 3 shows an example of these attempts obtained with the method proposed by Kimball (1960), using a computer program. The data were from a dose fractionation experiment already mentioned, and the simple analysis failed to show any significant effect of the radiation-free interval between the two half-doses. Essentially the same conclusions are reached by the new method ; in addition, a small but systematic difference is suggested between the two fractionation times although its significance cannot be properly tested. But the most interesting result is the biphasic wave of death rate revealed for the single-dose group at around 20 days, which is also present in both split-dose groups, consistently shifted to an earlier time. At present this method does not help much in the interpretation of the mechanisms, essentially because of the lack of biological and pathological data to be related to the "accidents" shown by the death rate analysis. However, it confirms that some information may be lost with simple methods, and that finer analyses may provide hints for further research. Fig. 3 suggests, for example, that radiation may be able to introduce a sort of "modulation" of the mortality intensity, which is not seen in unirradiated animals, and may be dependent on a purely physical variable, such as dose fractionation in time.

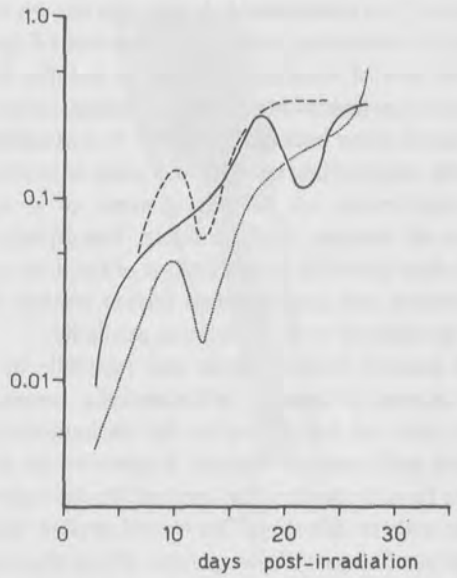


FIG. 3. Logarithm of standardized death rate computed according to Kimball (1960) in probability of death/animal/day, as a function of post-treatment time in days. Smoothing of the curves was obtained directly by the computer program superimposing estimates obtained with different time intervals over which the death rate was computed. Solid line = 40 Krad single acute dose, pooled data from groups irradiated at 14, 15, and 16 days of age ; dotted line = 20 Krad at 14 days + 20 Krad at 15 days (40 Krad split by 24 hrs interval), pooled data from two experiments ; interrupted line = 20 Krad at 14 days + 20 Krad at 16 days (40 Krad split by 48 hrs interval), pooled data from two experiments.

## Female germ-cells of parthenogenetic strains

The oocytes of parthenogenetic strains have been the subject of extensive research which have been fully published (Metalli and Ballardin, 1972). The results will only be briefly summarized here. The basic observation was that all the oocytes of a single brood are synchronous in their maturation process, and the macroscopic events of female reproduction as they may be seen by the naked eye, can be strictly correlated with the cytological events. This led to the development of relatively simple methods by which large number of oocytes could be irradiated in defined meiotic stages to construct and analyze dose-effect relationships, taking the total dominant embryonic lethality as the measure of radiation damage. The results were collected in Table 4 of our 1972 paper (Metalli and Ballardin, 1972), and to the best of my knowledge no further observations have been published.

It can be concluded that: 1. tetraploid oocytes are more resistant than diploid ones; 2. the radiation resistance increases from early to late meiotic stages, being maximum at diakinesis-metaphase and irrespective of the mechanisms of meiosis in different strains. The differential radiosensitivity as a function of ploidy can be estimated by the ratio of the inverse slopes (doses to give 0.37 survival), which is almost exactly 2 for early meiotic phases (diplotene ?),

1.8-2.5 at diakinesis and 3.1-3.7 at metaphase I. It was also shown that oocytes at diakinesis-prometaphase are capable of recovering only about one-half of the potentially recoverable sub-lethal damage, and this lack of complete recovery is not due to possible damage to the repair systems. Most of these conclusions are rather surprising, since they are at variance with the majority of data collected in other biological systems. In mechanistic terms, only one point deserves emphasis: up to the present time the best and most economical explanation of all the results from radiation experiments on cellular systems of *Artemia* is consistent with chromosomal mechanisms of damage. Unfortunately, this hypothesis cannot be properly tested because of the difficulties posed by investigations of the *Artemia* karyotype, but may be important for general problems and can stimulate further studies, if and when appropriate techniques of genetic exploration of *Artemia* become available.

A further comment of general validity seems also justified: we have already seen that *Artemia* shows different degrees of general radiosensitivity depending on the life stage at which it is irradiated, and now we can add to the list the sensitivity of developing oocytes. 0.37 inactivation of diploid early meiotic oocytes is achieved by doses as low as 250 rad, which is 100-fold less than for a corresponding level of life shortening, and more than 1000-fold less than for diapause embryo lethality. This would predict that at the population level both direct somatic and direct first-generation genetic effects should be considered to affect substantially the productivity, and hence the population dynamics.

Quantitative estimates of the gonial sensitivity of radiation (female or male germ stem-cells) have been much more difficult to obtain, although they are extremely important for continuous, low-level irradiation. A large number of experiments have been carried out in our laboratory, but no consistent and reproducible results were obtained. A recent re-evaluation of this kind of data indicated that some bisexual female animals irradiated at the first or second brood are unable to initiate the maturation of the following brood, being sterile for all the rest of their life. In contrast, other animals of the same irradiated group and under identical conditions continue reproduction with extremely variable degrees of egg production and hatch rates. The phenomenon appears to be strain- and dose-dependent, some diploids from California being the most sensitive ones; on the average, 10 to 15% of the females are permanently sterilized. These observations would suggest an all-or-none type of effect, with extreme individual sensitivities; whether it can be reproduced in other laboratories and generalized to other strains, and whether it can be extended to other toxic agents is of some interest not only for descriptive reasons, but also for methodological purposes.

## Quantitative traits and fitness components

The last subject that I would like to review briefly is an attempt at the study of genetic effects of radiation, taking advantage of parthenogenetic strains and of the previous demonstration that the quantitative character "number of setae of the furca" is under genetic control, very probably polygenic. Most of the basic data have already been reviewed (Ballardin and Metalli, 1972) in sufficient detail to give the necessary background of information. In summary, the diploid parthenogenetic strain of Sète was chosen for radiation experiments because: 1. the chromosome mechanisms of oocyte maturation are such that both segregation and recombination events are present and constitute possible sources of

FIG. 4. Diploid parthenogenetic *Artemia* from Sète (France) : example of "low" expression of the character "number of setae of the furca", obtained by selection (see text). The original unselected laboratory strain was derived from a large sample of cysts, and maintained through ovoviviparity by random sampling ; average number of setae was about 6, and stabilized "high" lines reached values as high as 16.

spontaneous genetic variability by reassortment ; 2. by the simple selection of only extreme phenotypes to reproduce the next generation, it was shown that a clear divergent phenotypic response was obtained rapidly, as well as stabilized "low" and "high" lines. It was then clear that any new genetic variability, as for instance introduced by irradiation, would have been detected by applying a selection pressure on irradiated lines, taking into account the appropriate controls. Since the character may be dependent on the environmental conditions, a special large-scale culture method was developed in such a way that all the experimental groups were living for generations in the same culture medium, being taken out only for irradiation and for setae counting. Irradiation was delivered on the 14th day of age, at least a week before initiation of oocyte maturation, to insure that gonial stages were irradiated. 500 and 1000 rad per generation were used as dose levels, and the experimental design included unirradiated unselected, irradiated unselected, and irradiated selectes lines. By the appropriate comparisons the following results were obtained : 1. Under random propagation (no selection), the quantitative expression of the character was unaffected by radiation for seven generations, up to total accumulated doses of 3500 and 7000 rad respectively in the treated lines. At the 8th and 9th generation a slight drop of the average number of setae was noted, indicating some drift in favour of "low" genotypes. 2. After the 7th generation large samples

were drawn from all the lines, irradiation on these samples was interrupted and a selection for extreme phenotypes was applied. At the second generation of selection, when the unirradiated control lines did not yet show any indication of a divergent response, the previously irradiated lines selected for "low" expression began to decrease the average number of setae, showing an initial response, but the experiment could not be continued because the selected extreme phenotypes (both "low" and "high") resulted permanently sterile. 3. The control lines of the selection experiment, that is the previously irradiated lines propagated by random sampling, could be maintained, but the average estimates of some components of fitness measured on random samples were substantially decreased, with some animals being unable to let their progeny be represented in the following generation. Of those animals in the samples which did give offspring the proportion of surviving adult descendants relative to unirradiated unselected control lines was reduced to about one half and one quarter after accumulated genome doses of 3500 and 7000 rad, respectively (Ballardin and Metalli, 1968). The combined evidence from these complex experiments shows that radiation may induce some genetic variability in specific polygenic systems, which may be detected by divergent selection, but the sensitivity of other polygenic systems controlling important components of darwinian fitness is so high that sufficiently extended observations to measure the selection response (and the corresponding induced genetic variance) are practically impossible. Finally some correlations between the two blocks of polygenic control is suggested, but it does not seem to be sufficient to explain the sudden and complete drop of fertility observed in lines subjected to selection.

## Comments and conclusions

In this review I have tried to collect some of the observations on the radiobiology of *Artemia*, pointing mainly to those results which may have some bearing on present-day problems and may stimulate interest in this animal species for further research in environmental toxicology. Most of the other basic questions about the fundamental mechanisms of radiation action, for which *Artemia* can be a very useful material, have been overshadowed by the feeling of the scientific community that we are now in great need for choosing model systems suitable for quantitative research on the effects of potentially harmful manipulations of aquatic ecosystems. Large efforts have been made at the international level to foster this type of research, most of them being confined, however, to radiation and radioactivity (IAEA Technical Report Series 1975, 1976, 1979). The availability of new methods of culture, the technological developments in the field of automated measurements and monitoring, and some recent advancements in coordinated international actions are all positive contributions to a renewed interest in this small, but interesting and promising animal.

## Literature cited

Ballardin E. and P. Metalli. 1963. Osservazioni sull biologia di *Artemia salina* Leach. Tecniche di colture e fenomeni riproduttivi. Istituto Lombardo (Rend. Sci.) B. 97:194-254.

Ballardin E. and P. Metalli. 1968. Stima di alcune componenti della fitness in *Artemia salina Partenogenetica* diploide irradiata per piu generazioni. *Atti Ass. Genet. Ital.* 13:341-345.

BALLARDIN E. and P. METALLI. 1972. Osservazioni sulla trasmissione di caratteri in *Artemia salina Parthenogenetica*. *Boll. Zool.* 39:551-564.

EASTER S. S. and HUTCHINSON F. 1961. Effects of radiations of different LET on *Artemia* eggs. *Radiation Research* 15:333-340.

GAUBIN Y., H. PLANEL, E. KOVALEV, B. PIANEZZI, and J. C. BESS. 1980. Effects of proton and neutron irradiation on *Artemia* eggs. p. 171-180. *In* : The brine shrimp *Artemia*. Vol. 1. Morphology, Genetics, Radiobiology, Toxicology. Persoone G., P. Sorgeloos, O. Roels and E. Jaspers (Eds). Universa Press, Wetteren, Belgium. 345 p.

GROSCH D. S. and H. E. ERDMAN. 1955. X-ray effects on adult *Artemia*. *Biol. Bull.* 108:277-282.

INTERNATIONAL ATOMIC ENERGY AGENCY. 1975. Design of radiotracer experiments in marine biological systems. Technical Report Series N° 167, IAEA, Vienna, 292 p.

INTERNATIONAL ATOMIC ENERGY AGENCY. 1976. Effects of ionizing radiation on aquatic organisms and ecosystems. Technical Report Series N° 172, IAEA, Vienna, 131 p.

INTERNATIONAL ATOMIC ENERGY AGENCY. 1979. Methodology for assessing impacts of radioactivity on aquatic ecosystems. Technical Report Series N° 190, IAEA, Vienna, 416 p.

INTERNATIONAL COMMISSION ON RADIATION UNITS AND MEASUREMENTS. 1980. Radiation Quantities and Units. ICRU Report N° 33, Washington DC, USA, 25 p.

IWASAKI T., T. INADA, K. KAWACHI, T. KANAI, and T. YAMADA. 1980. Usefulness of *Artemia* in radiobiology: the effects of 60 MeV protons and of synchrotron orbital radiation on the eggs. p. 181-187. *In* : The brine shrimp *Artemia*. Vol. 1. Morphology, Genetics, Radiobiology, Toxicology. Persoone G., P. Sorgeloos, O. Roels and E. Jaspers (Eds). Universa Press, Wetteren, Belgium. 345 p.

KIMBALL A. W. 1960. Estimation of mortality intensities in animal experiments. *Biometrics* 16:505-521.

METALLI P. 1968. The laboratory culture of the brine shrimp *Artemia salina*. II. The culture methods and the use of flagellates for feeding *Artemia*. Comitato Nazionale Energia Nucleare, Technical Report RT/BIO(68)43, Roma.

METALLI P. 1979. Life span shortening. Proc. Joint USA-Italy Seminar on : Radiation Sensitivity : Facts and Models. *Int. J. Radiation Oncology Biol. Phys.* 5:1123-1130.

METALLI P. and E. BALLARDIN. 1972. Radiobiology of *Artemia* : Radiation effects and ploidy. *Current Topics in Radiation Research Quarterly* 7:181-240.

PLANEL H., Y. GAUBIN, R. KAISER, and B. PIANEZZI. 1980. Effects of space environmental factors on *Artemia* eggs. p. 189-198. *In*: The brine shrimp *Artemia*. Vol. 1. Morphology, Genetics, Radiobiology, Toxicology. Persoone G., P. Sorgeloos, O. Roels and E. Jaspers (Eds). Universa Press, Wetteren, Belgium. 345 p.

# PAPERS

# Morphology

*The Brine Shrimp* Artemia. *1980. Vol. 1. Morphology, Genetics, Radiobiology, Toxicology*
*G. Persoone, P. Sorgeloos, O. Roels, and E. Jaspers (Eds). Universa Press, Wetteren, Belgium. 345 p.*

# Differentiation in *Artemia* strains from Spain[1]

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**Abstract**

A total of 36 different *Artemia* strains, found in the Iberian peninsula, have been characterized with regard to their mode of reproduction and ploidy.

A detailed biometrical analysis of 17 spanish and 4 foreign strains (7 bisexual and 14 parthenogenetic) has been performed on animals harvested from wild populations or cultured under laboratory conditions. Most of the distinctive morphological characteristics found, appear to be single consequences of allometric phenomena, and are also related to a different degree of sexuality, ploidy, and size of nucleus in different strains.

Data are given on the biometrics of the telopodite filtering-apparatus, larval growth rate, and adult fertility under different environmental conditions, as a first step in the determination of the relative value of different *Artemia* strains for aquaculture purposes.

The data presented suggest the existence of a specific North-American strain which has to be considered as a different species as well as various Spanish and Mediterranean autochtonous strains.

## Introduction

The brine shrimp *Artemia* has become a species that is cultured worldwide as a source of food in aquaculture projects. As a result, the strictly scientific problem of the existence of a variety of strains, which was a study object of genetics, evolution, biogeography, and ecology, is now suscitating more and more interest from the practical point of view. Different strains indeed possess different characteristics and are as a consequence better or less suited for particular uses. The best known and most used strain, namely the one from San Francisco Bay, California, USA, can now be replaced by specific strains available locally in various countries. In this regard Spain has undoubtedly a large potential because a variety of *Artemia* strains have been discovered at different sites. In the near future this will hopefully permit to select the appropriate strain among the geographical races of Spanish brine shrimp in function of particular nutritional or other prerequisites for specific purposes and uses.

[1] This paper is a synthesis of the Ph. D. thesis "Diferenciacion y distribucion de las poblaciones de *Artemia* (Crustaceo Branquiopoda) de Espanã" by Amat Domenech (1979).

**Distribution of *Artemia* in the Iberian peninsula and neighboring islands**

The geographical distribution of 36 different strains of *Artemia* occurring naturally in Spain is given in Fig. 1. Initial strain-differentiation is based on morphological comparison of the Spanish strains with foreign ones previously studied by Artom (1922, 1925, 1926, 1929), Stella (1933), Barigozzi (1934, 1941, 1957, 1974), Barigozzi and Tosi (1959), Clark and Bowen (1976), Goldschmidt (1952) and kindly made available to us by several scientists from different countries. The strains which were found are generally diploid bisexual, though several diploid and tetraploid parthenogenetic races and several intermediate (also parthenogenetic) forms have been encountered. It is interesting to note that at some locations mixed populations of bisexual and parthenogenetic *Artemia* are co-existing.


FIG. 1. Geographical distribution of *Artemia* strains in the Iberian peninsula and neighboring islands.

**Morphological analysis**

Juvenile and adult *Artemia* from 21 different locations (17 spanish, 4 foreign, 7 bisexual, 14 parthenogenetic, Table I) were narcotized in a chloroform-saturated water solution and measured under a dissection microscope. The following measurements were performed (Fig. 2): total length, length of abdomen, maximal width of brood pouch, width of 3rd abdominal segment, length of furca, number of setae on each branch of the furca, width of head, length of 1st antenna, maximal diameter and distance between the compound eyes, total length of and number of filtering setae on the telopodite of the 6th thoracopod. Measurements were performed on animals harvested from nature (Spanish strains) and on animals cultured from wild cysts (Spanish and foreign strains) under standard laboratory conditions (*Tetraselmis* food, 25 °C, and seawater of 30-32 ‰ salinity).

TABLE I

List of geographical strains studied

| Strain | | Origin |
|---|---|---|
| Bisexual (diploid) | | |
| San Francisco Bay, California, USA | | Metaframe, San Francisco California, USA |
| San Félix | | San Fernando, Cádiz, Spain |
| San Fernando | | |
| Salinera Española | | Santa Pola, Alicante, Spain |
| Bras de Port | | |
| San Pedro del Pinatar | | San Pedro del Pinatar, Murcia, Spain |
| Ibiza | | La Canal de San José, Ibiza, Spain |
| Parthenogenetic | | |
| Gerri de la Sal | (diploid) | Gerri de la Sal, Lérida, Spain |
| Calpe | (diploid) | Calpe, Alicante, Spain |
| Bras de Port | (diploid) | Santa Pola, Alicante, Spain |
| Bonmatí | (diploid) | Santa Pola, Alicante, Spain |
| Cabo de Gata | (diploid) | Cabo de Gata, Almería, Spain |
| Ayamonte | (diploid) | Ayamonte, Huelva, Spain |
| Isla Cristina | (diploid) | Isla Cristina, Huelva, Spain |
| San Fernando | (diploid) | San Fernando, Cádiz, Spain |
| Janubio | (diploid) | Yaiza, Lanzarote, Spain |
| Sanlucar | (diploid and tetraploid) | Sanlucar de Barrameda, Cádiz, Spain |
| San Antonio | (tetraploid) | River Ebro delta, Tarragona, Spain |
| Alcochete | (tetraploid) | Alcochete, south of Lisbon, Portugal |
| Larache | (diploid and tetraploid) | Larache, atlantic coast, Morocco |
| Comacchio | (tetraploid) | Comacchio, Emilia Romaña, Italy |

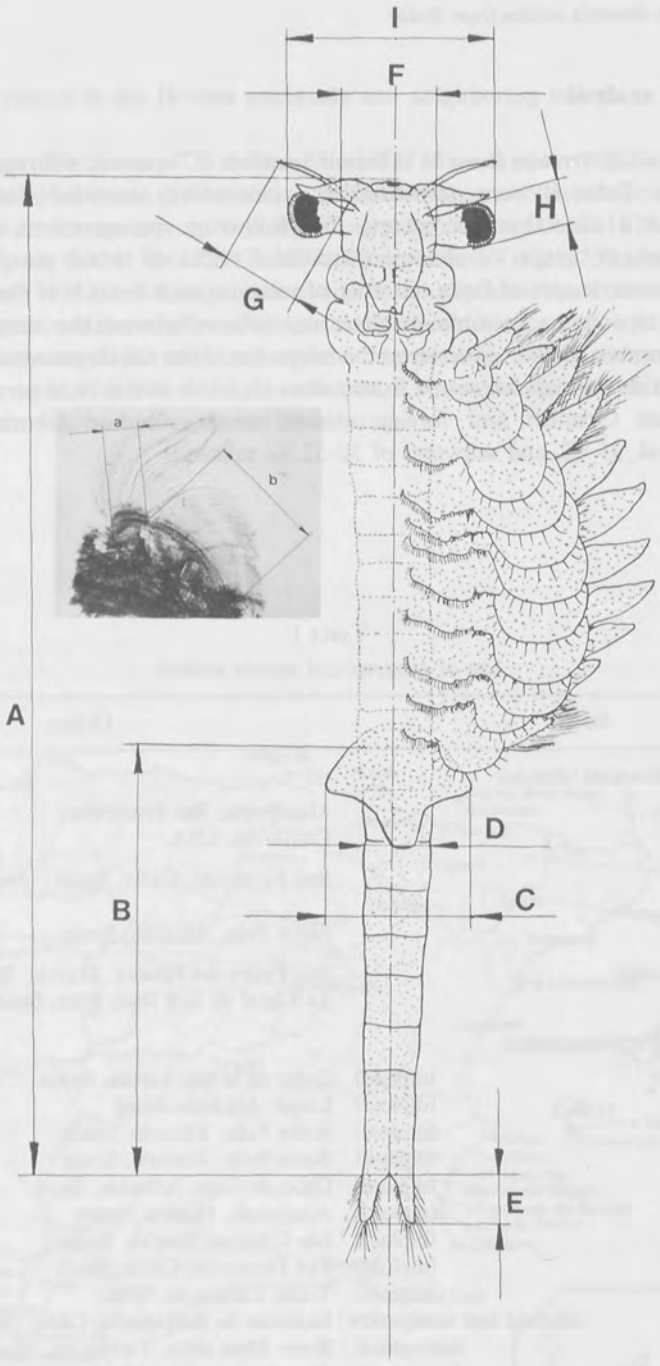


FIG. 2. Schematic drawing of adult *Artemia* and microscopic detail of the telopodite of the 6th thoracopod with indications of the various size measurements. A : total length ; B : abdomen length ; C : maximal width of brood pouch ; D : width of 3rd abdominal segment ; E : furca length ; F : width of head ; G : length of 1st antenna ; H : maximal diameter of complex eye ; I : distance between complex eyes ; a + b = total telopodite length of 6th thoracopod.

BIOMETRICS OF ANIMALS CULTURED UNDER STANDARD LABORATORY CONDITIONS

1. From Fig. 3 and 4 it appears that there is a positive correlation between abdomen length and total individual length. This correlation is more obvious in females than in males, especially in the bisexual individuals of Spanish origin.
2. The maximal width of the brood pouch (ovisac) and of the 3rd abdominal segment are both proportional to the total individual length (Fig. 5 and 6). It was furthermore noted that the shape of the ovisac can be used to distinguish Spanish strains from San Francisco Bay *Artemia* (Fig. 7).
3. The length of the furca as well as the number of setae on each furcal branch increase with the size of the animals (Fig. 8 and 9). The morphology of the furca is different in the strains studied (Fig. 10).
4. As shown in Fig. 11 the width of the head increases in all *Artemia* strains, proportionally with the total individual length. In San Francisco Bay *Artemia* the head is elliptical, while it is smaller and of a more irregular shape in the Spanish strains. San Francisco Bay and Spanish bisexual strains can be differentiated through the male claspers : *i.e.* their relative size is larger in San Francisco Bay *Artemia*, the shape of the frontal knob is subspherical in San Francisco Bay, but subconical in Spanish *Artemia* (Fig. 12).
5. In all *Artemia* strains studied a positive correlation was noted between the length of the animals and the length of their 1st antenna, the diameter of and the distance between the compound eyes (Fig. 13, 14 and 15). Antennulae in San Francisco Bay *Artemia* are shorter than in the bisexual strains from Spain. The compound eyes appear to be larger in males than in females, especially in San Francisco Bay *Artemia*.

BIOMETRICS OF ANIMALS HARVESTED FROM THEIR NATURAL HABITAT OR CULTURED IN THE LABORATORY

1. Increasing salinities induce a reduction in size of the length of the furca (Fig. 16) and the number of setae on the furca (Fig. 17).
2. The eye diameter and the width of the ovisac do not change much in function of the salinity of the medium (Fig. 18, 19 and 20). However, it appears that in bisexual *Artemia* (Fig. 19) sexual maturity is reached at a smaller width of the brood sac than in parthenogenetic animals (Fig. 20).
3. The relative length of the abdomen, on the contrary, increases with the salinity of the medium (Fig. 21).
4. Whereas in San Francisco Bay *Artemia* the ratio of the total number of filter setae to the telopodite length is constant, it appears that in all Spanish strains studied, there is a positive correlation between the number of setae and the telopodite length (Fig. 22). It is interesting to note that animals harvested from high salinity salt ponds have smaller telopodites and reduced numbers of setae as compared to controls cultured in the laboratory under standard conditions (*cf.* stripped areas in Fig. 22). This could be an adaptation to the decreased size of food particles in media of high salinity (Erhardt *et al.*, 1971).

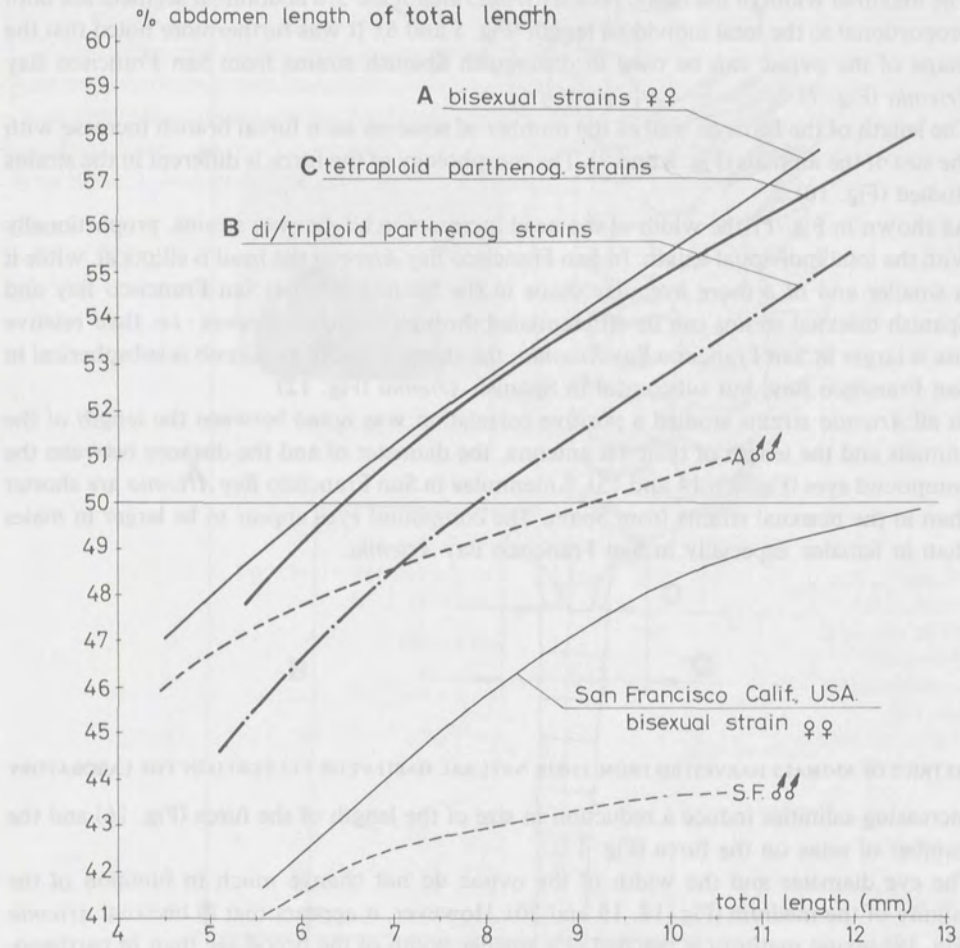


FIG. 3. Abdomen length expressed as percent of total length in San Francisco Bay and Spanish *Artemia* cultured under standard conditions. S.F. : San Francisco Bay strain ; A : bisexual ; B : parthenogenetic di/triploid and C : parthenogenetic tetraploid strains of Spanish origin.

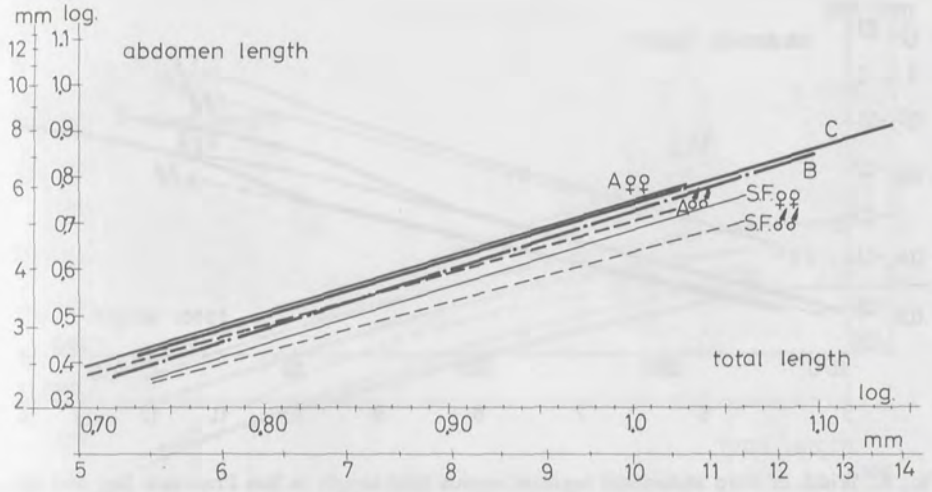


FIG. 4. Abdomen length *versus* total length in San Francisco Bay and Spanish *Artemia* cultured under standard conditions (legend to letters A, B, and C see Fig. 3).

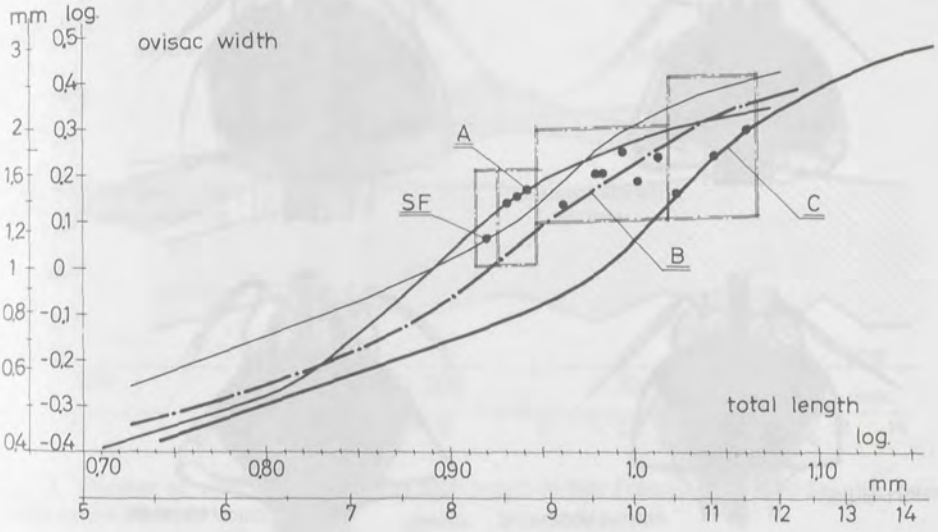


FIG. 5. Width of ovisac *versus* total length in San Francisco Bay and Spanish *Artemia* cultured under standard conditions (full circles represent a population of which 50% of the females carry their first offspring ; legend to letters A, B, and C see Fig. 3).

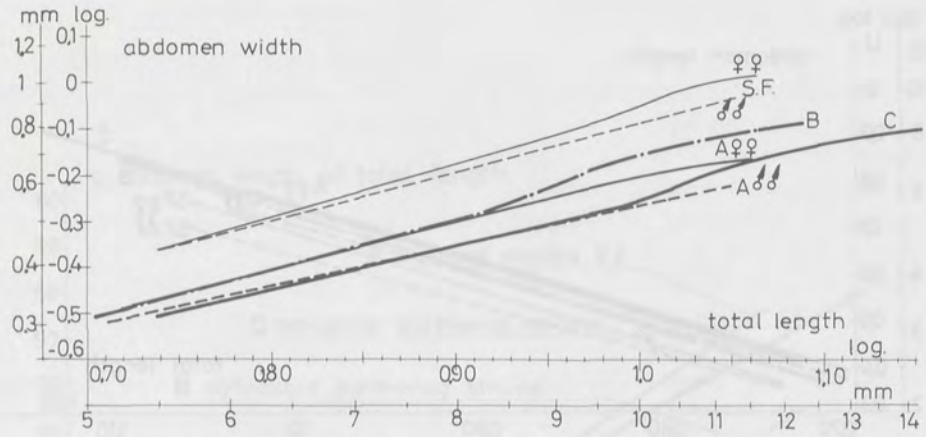


FIG. 6. Width of third abdominal segment versus total length in San Francisco Bay and Spanish *Artemia* cultured under standard conditions.

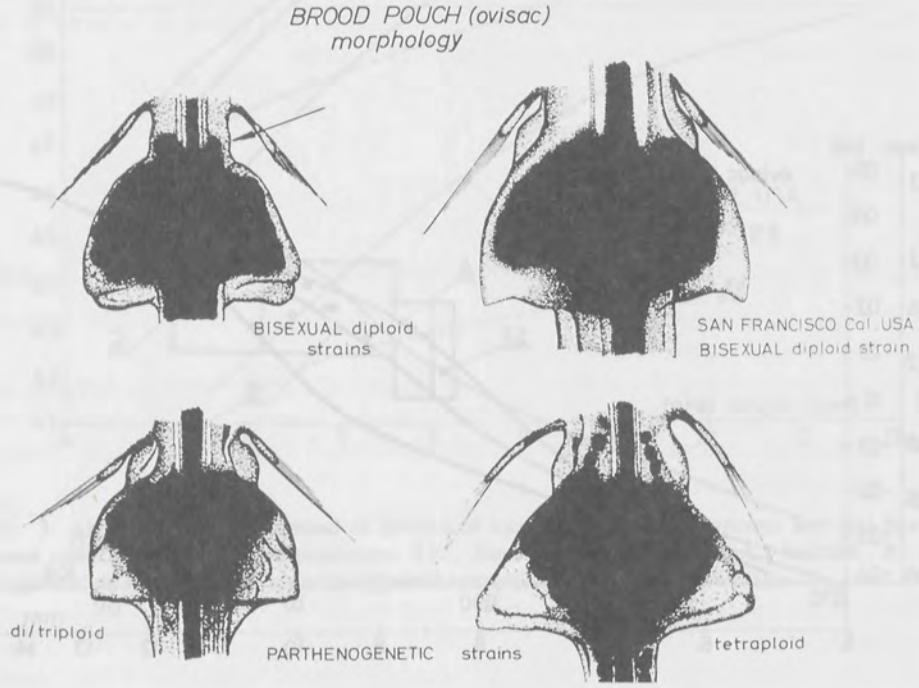


FIG. 7. Morphology of ovisac in San Francisco Bay and Spanish (bisexual and parthenogenetic) strains.

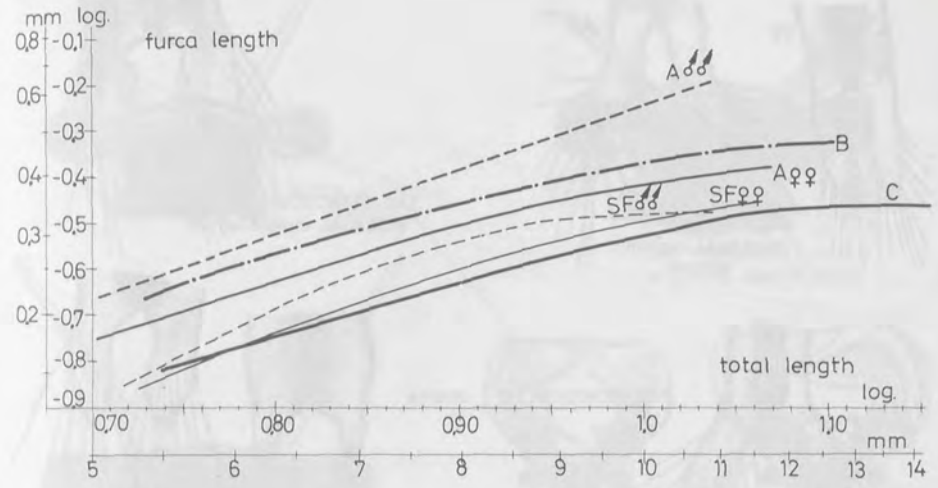


FIG. 8. Furca length *versus* total length in San Francisco Bay and Spanish *Artemia* cultured under standard conditions.

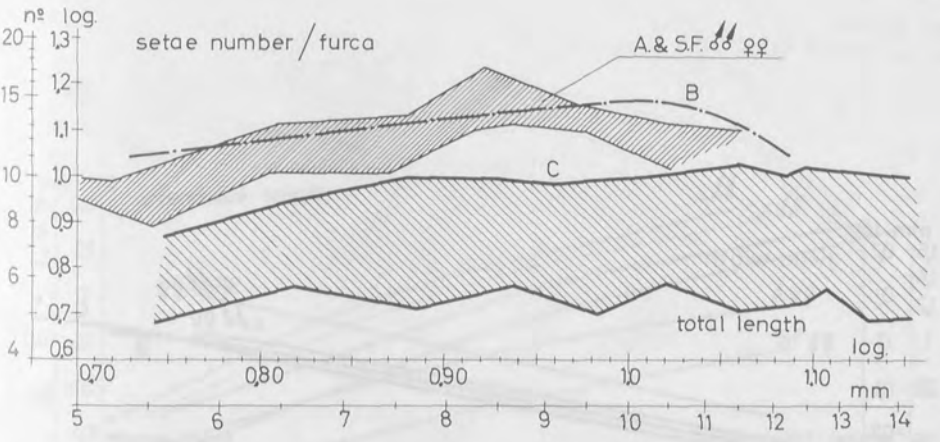


FIG. 9. Number of setae per furca *versus* total length in San Francisco Bay and Spanish *Artemia* cultured under standard conditions.

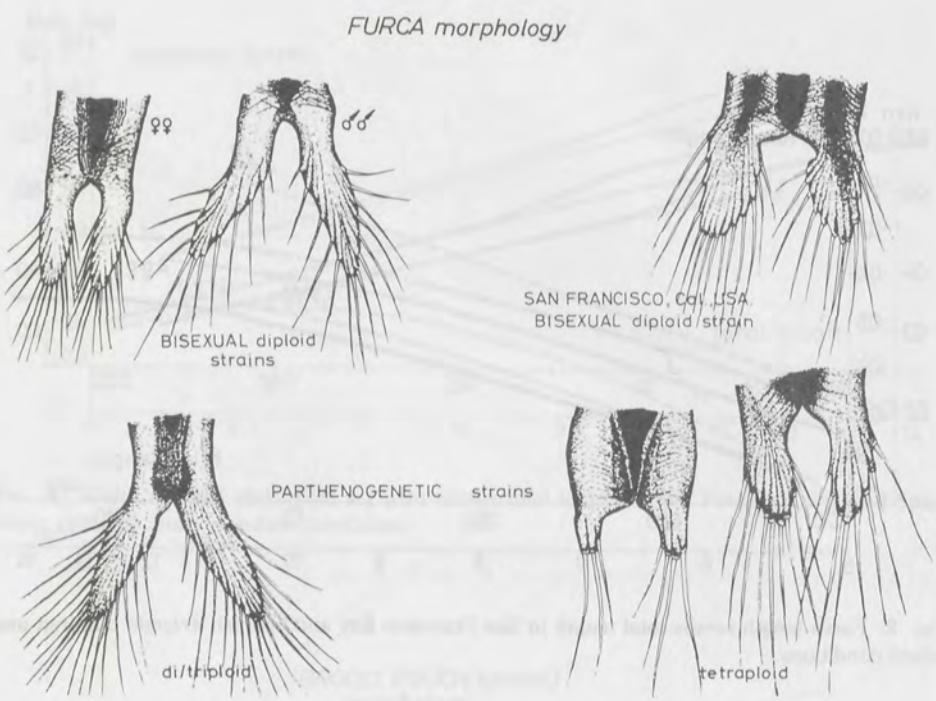


FIG. 10. Morphology of the furca in San Francisco Bay and Spanish (bisexual and parthenogenetic) strains.

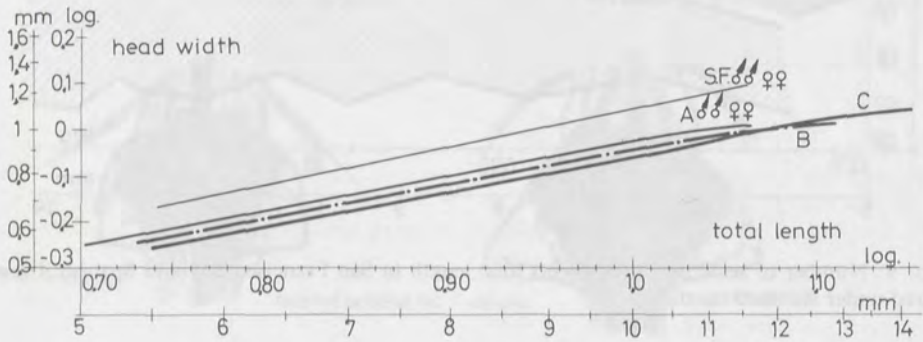


FIG. 11. Width of head *versus* total length in San Francisco Bay and Spanish *Artemia* cultured under standard conditions.

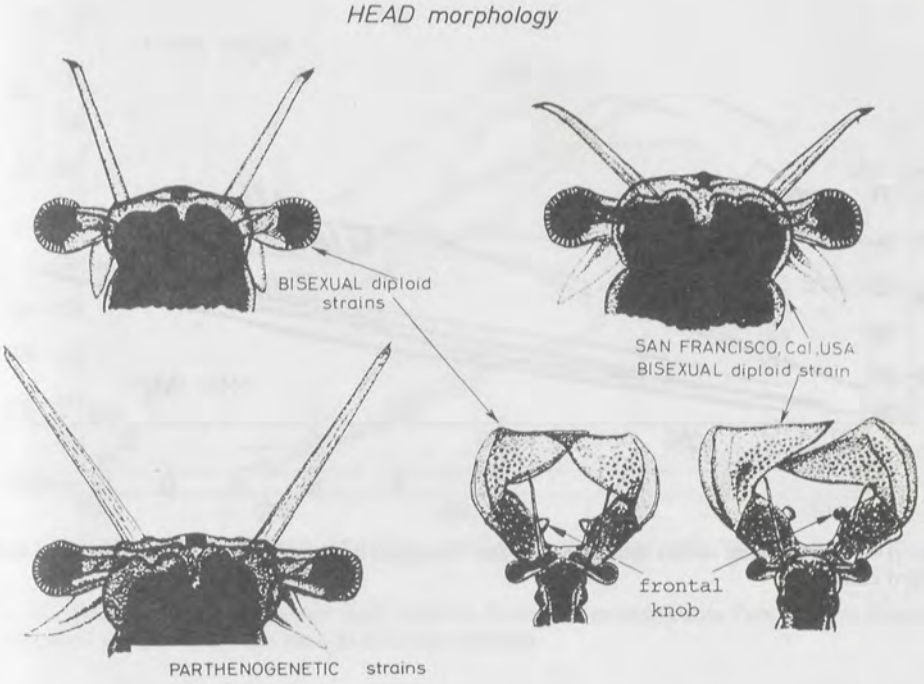


FIG. 12. Morphology of the head in San Francisco Bay and Spanish (bisexual and parthenogenetic) strains.

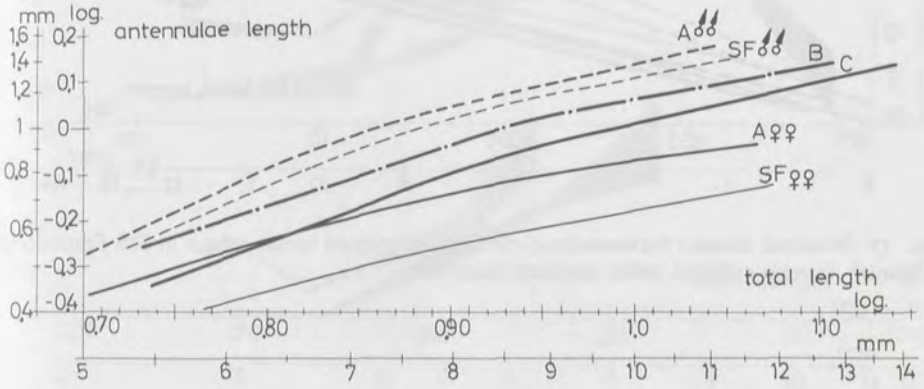


FIG. 13. Length of antennulae (1st antennae) *versus* total length in San Francisco Bay and Spanish *Artemia* cultured under standard conditions.

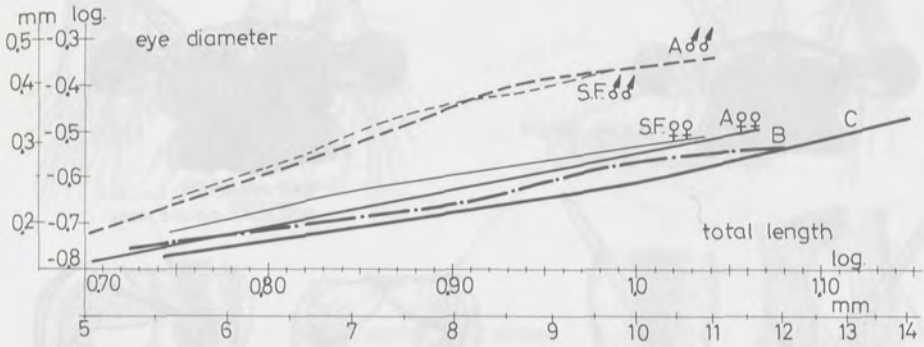


FIG. 14. Eye diameter *versus* total length in San Francisco Bay and Spanish *Artemia* cultured under standard conditions.

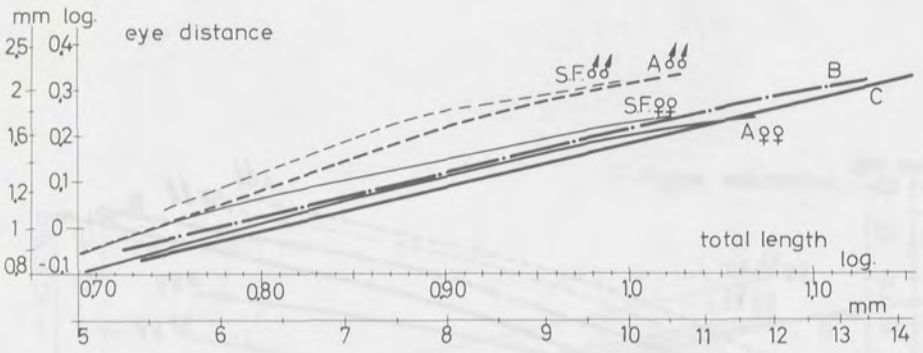


FIG. 15. Maximal distance between the eye's external contour *versus* length in San Francisco Bay and Spanish *Artemia* cultured under standard conditions.

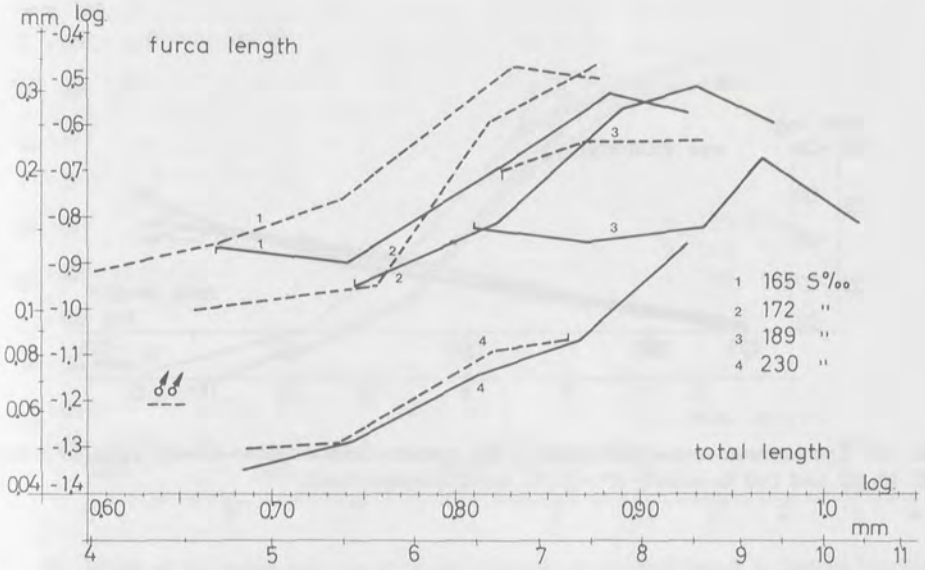


FIG. 16. Length of the furca *versus* total length in Salinera Espanola-Santa Pola *Artemia* (bisexual diploid strain) sampled from salt pans at different salinities.

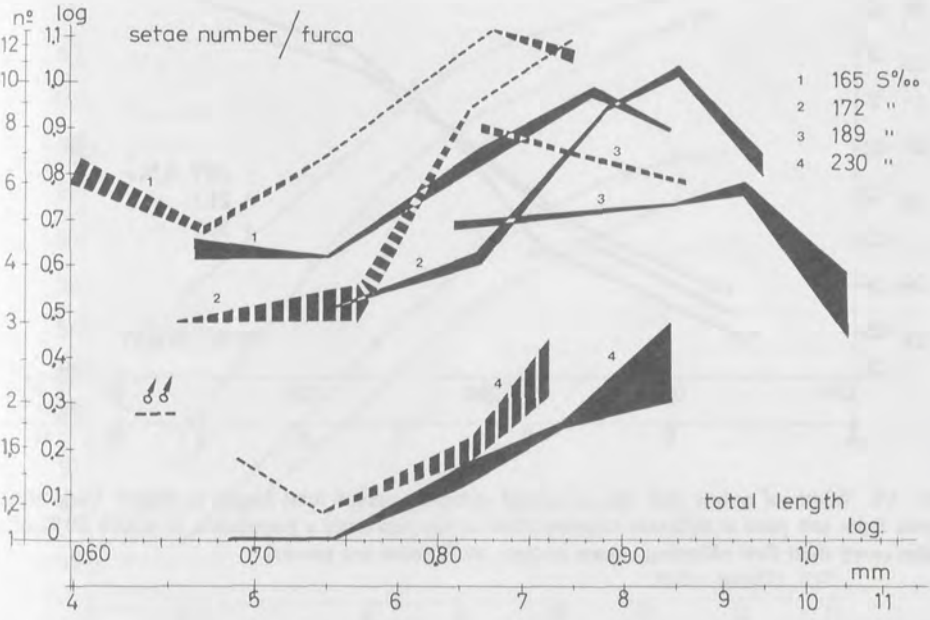


FIG. 17. Number of setae per furca *versus* total length in Salinera Espanola-Santa Pola *Artemia* (bisexual diploid strain) sampled from salt pans at different salinities.

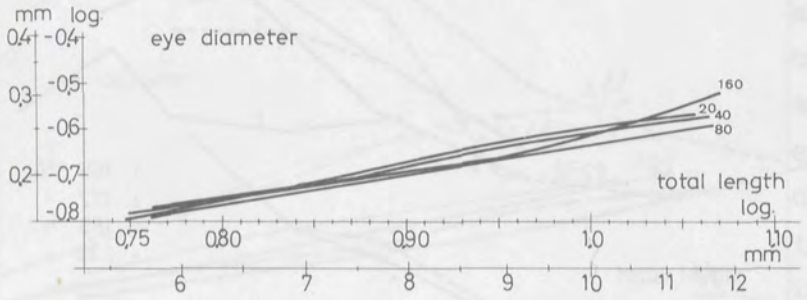


FIG. 18. Eye diameter *versus* total length in San Antonio-Delta del Ebro-*Artemia* cultured in media of 20, 40, 80, and 160 ‰ salinity (t° = 25 °C, dried *Spirulina* food).

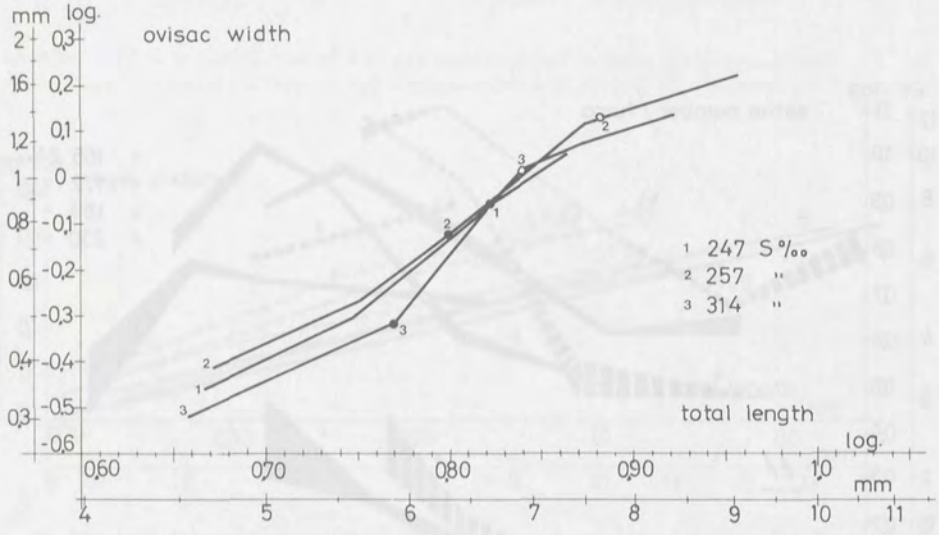


FIG. 19. Width of ovisac and rate of sexual maturity versus total length in Salero Viejo-Villena *Artemia* from salt pans at different salinities (full circles represent a population of which 50 % of the females carry their first offspring ; open circles : all females are gravid).

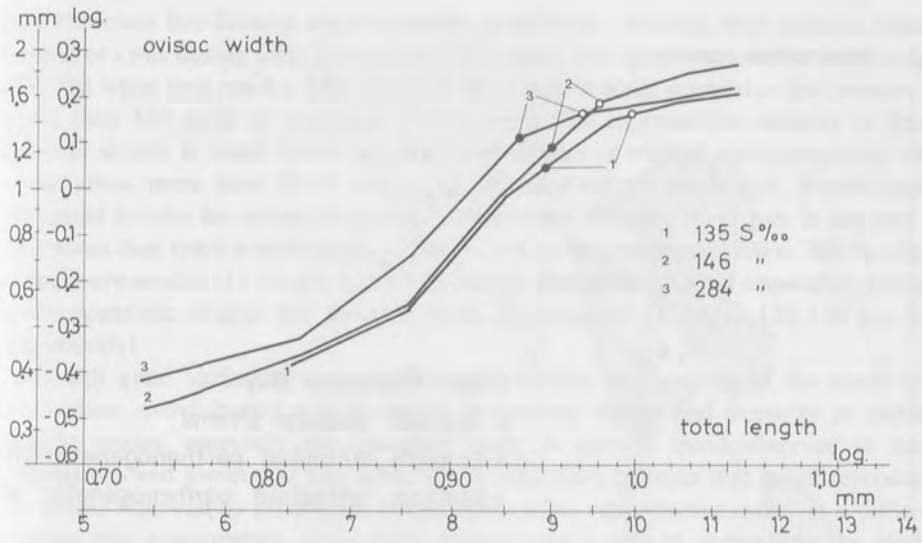


FIG. 20. Width of the ovisac and rate of sexual maturity *versus* total length in Salinas Maritimas-Calpe *Artemia* sampled from salt pans at different salinities (full circles represent a population of which 50 % of the females carry their first offspring ; open circles : all females are gravid).

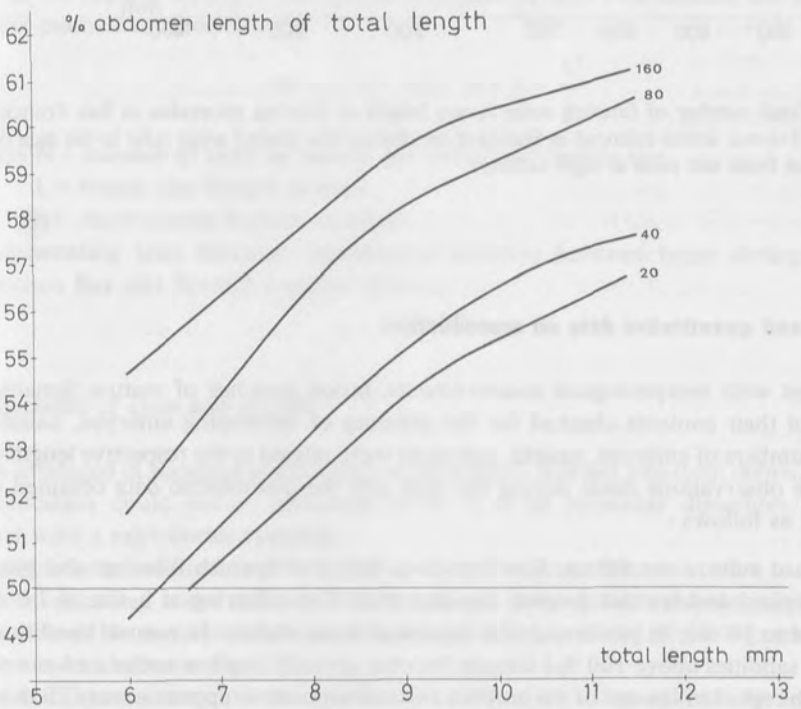


FIG. 21. Abdomen length expressed as percent of total length in San Antonio-Delta del Ebro-*Artemia* cultured in media of 20, 40, 80, and 160 ‰ salinity (t° = 25 °C, dried *Spirulina* food).

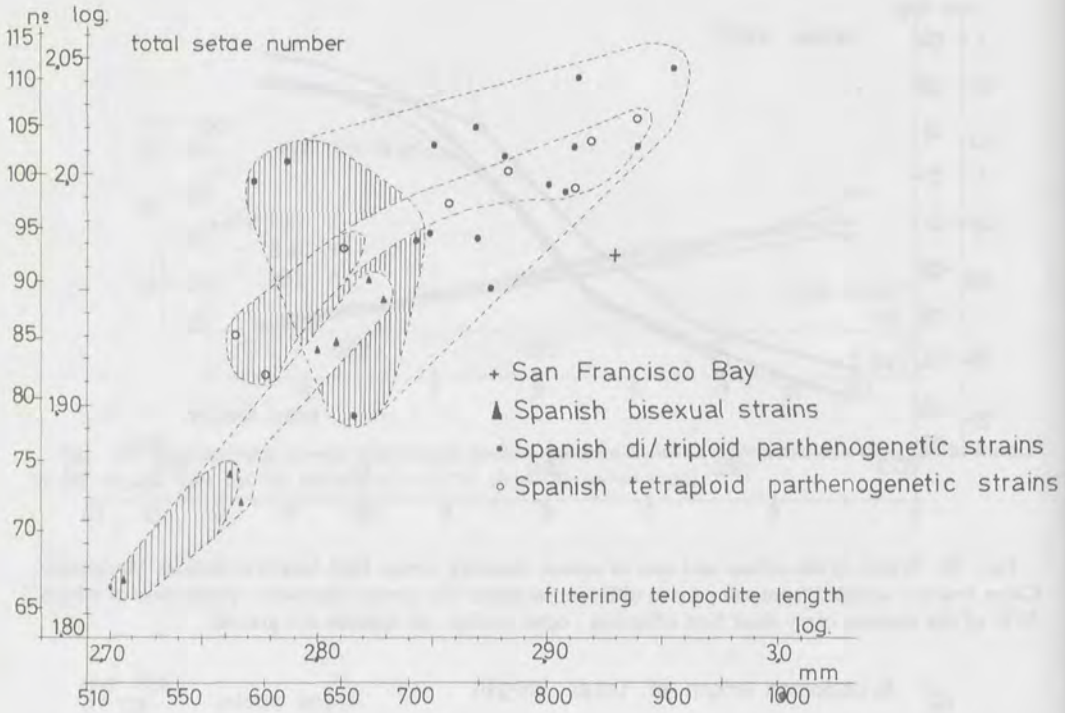


FIG. 22. Total number of filtering setae *versus* length of filtering telopodite in San Francisco Bay and Spanish *Artemia* adults cultured in Standard conditions (the shaded areas refer to the data obtained for individuals from salt pans at high salinity).

**Qualitative and quantitative data on reproduction**

Concurrent with morphological measurements, brood pouches of mature females were dissected and their contents checked for the presence of developing embryos, nauplii, and cysts. The numbers of embryos, nauplii, and cysts were related to the respective lengths of the females. The observations made during the tests and the quantitative data obtained can be summarized as follows :

1. In standard culture conditions. San Francisco Bay and Spanish bisexual and parthenogenetic diploid and triploid *Artemia* produce their first offspring at a size of 7-8 mm as compared to 10 mm in parthenogentic tetraploid brine shrimp. In natural conditions (salt ponds at salinities above 100 ‰) females become sexually mature earlier and at a smaller size. With regard to the age of the animals this corresponds to approximately 22 days after hatching for San Francisco Bay *Artemia*, 28 days for the bisexual Spanish strains, and a little more for parthenogenetic *Artemia* ; tetraploid strains are the slowest to reproduce.

2. San Francisco Bay females are remarkably proliferous : whereas they produce about 50 nauplii or cysts during their first reproductive cycle, this figure increases to an average of 300-350 when they reach a total length of 14-15 mm (it is not unusual to find females with more than 500 cysts or nauplii in their ovisac). The reproductive capacity in Spanish *Artemia* strains is much lower : bisexual and diploid or triploid parthenogenetic strains never attain more than 12-13 mm under standard culture conditions. Parthenogenetic tetraploid females lay about 30 nauplii or cysts when they are 10-11 mm in size and 150-160 when they reach a total length of 16-17 mm. In natural brines (100 to 300 ‰ salinity) animals are smaller (11-12 mm and 13-16 mm in diploid and triploid respectivey tetraploid parthenogenetic strains) but produce more offspring (45-50 up to 120-130 per brood respectively).
3. Although great variation was noted among strains with regard to the mode of reproduction, ovoviviparity was dominant in bisexual strains and oviparity in parthenogenetic strains, especially the tetraploid ones. A general trend observed in natural populations and sometimes also noted in the laboratory cultures was initial reproduction by nauplii followed by production of cysts after a few reproductive cycles. It is difficult to explain this phenomenon since many factors play a role in controlling the mode of reproduction, *e.g.* salinity of the medium, food quality, environmental conditions, intrinsic factors related to the genetical constitution of the strains studied, *etc.*
4. The number of cysts or nauplii per brood can be allometrically related to the female's size and to the haploid chromosome number (Margalef, 1953). This relation can be expressed by the mathematical equations :

$$N = a \times L^3 \quad \text{and} \quad N = b \times \frac{L^3}{n}$$

where N = number of cysts or nauplii per brood and female size
L = female size (length in mm)
n = chromosome haploid number.

5. Cross-breeding tests revealed reproductive isolation between brine shrimp from San Francisco Bay and Spanish bisexual *Artemia*.

## Characteristics of cysts and nauplii

Cysts produced in standard culture tests were isolated, washed free from debris, and before any dessication could occur, measured under a Wild binocular dissection microscope equipped with a micrometer eyepiece.

Nauplii collected within 2 hr after hatching or removal from the ovisac were narcotized in a chloroform-saturated water solution and their length determined under the microscope.

The results of the measurements are summarized in Tables II and III. Histograms of the size of the cysts are shown in Fig. 23.

The size differences are obvious : San Francisco Bay cysts and nauplii are the smallest of all strains studied. In the Spanish strains bisexual *Artemia* are smaller than parthenogenetic brine shrimp ; in the latter group the tetraploid strains score the highest figures.

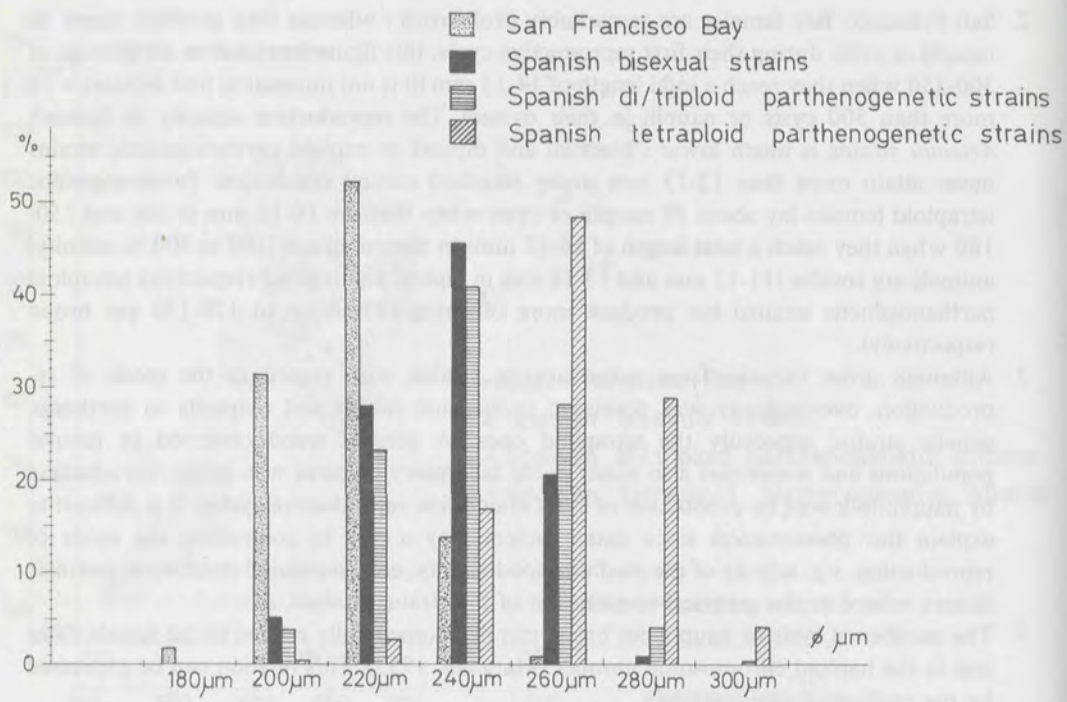


FIG. 23. Size frequency distribution of cysts harvested from standard culture tests of San Francisco Bay and Spanish *Artemia* strains.

It is interesting to note that ovoviviparous nauplii are larger than nauplii hatched from cysts ; this can perhaps be explained by the more energy-expensive mode of reproduction in oviparity as compared to ovoviviparity (Clegg, 1964, 1974 ; Morris, 1971).

TABLE II

Mean diameter and cyst volume of the San Francisco Bay strain and Spanish strains (harvested from standard cultures at 30-32 ‰ salinity)

| Strains | Mean diameter ($\mu$m) | Mean volume ($10^6$ $\mu m^3$) | Number of cysts measured |
|---|---|---|---|
| San Francisco Bay | 216 | 5.27 | 1 080 |
| Spanish | | | |
| Bisexual diploid | 236 | 6.88 | 4 625 |
| Parthenogenetic diploid and triploid | 240 | 7.23 | 7 498 |
| Parthenogenetic tetraploid | 262 | 9.41 | 4 558 |

TABLE III

Average length of ovi- and ovoviviparous nauplii from the San Francisco Bay strain and from Spanish strains

| Strains | Oviparous nauplii ($\mu$m) | Number measured | Ovoviviparous nauplii ($\mu$m) | Number measured |
|---|---|---|---|---|
| San Francisco Bay | 412.27 | 417 | 421.57 | 114 |
| Spanish | | | | |
| Bisexual diploid | 445.95 | 366 | 474.57 | 328 |
| Parthenogentic diploid or triploid | 447.72 | 1 075 | 476.48 | 1 455 |
| Parthenogentic tetraploid | 469.70 | 431 | 501.19 | 134 |

**Cytogenetical characteristics**

To obtain more information to classify the strains studied into bisexual diploid, parthenogenetic diploid or triploid and parthenogenetic tetraploid, either chromosome counting or nuclei surface area measurements are necessary. Since the classic squash and orcein staining method proved to be laborious and unreliable, a method was worked out to measure the surface area of nuclei in epithelium cells of the digestive tract extending from the 3rd to the 5th abdominal segment. Dissected abdomens were fixed in Bouin's solution, dehydrated, inbedded in parafin, cut in sections of 7 $\mu$m, stained with Weighert's and Mallory's hematoxylin, and finally mounted in Canada balsam. Elliptically outlined sections of nuclei were measured under a Wild microscope, with a calibrated eyepiece, at a magnification of 1 500 X. 200 measurements were performed for each strain or group of strains.

The following strains were studied :

- San Francisco Bay, California, USA ;
- bisexual strains from San Felix, Spain and San Fernando, Spain ;
- parthenogenetic strains from the Spanish salinas Maritimas-Calpe, Cabo de Gata, and Ayamonte ;
- parthenogenetic strains from San Antonio-Delta del Ebro, Spain and Comacchio, Italy.

The numerical data obtained are summarized in Table IV. The bisexual diploid San Francisco Bay strain has the smallest nuclei of all strains studied ; bisexual *Artemia* from Spain have a slightly larger nucleus and a wide variation in nucleus size is found among the parthenogenetic groups. The data allow to make a distinction between diploid bisexual and parthenogenetic strains (16 to 21 $\mu m^2$) and the tetraploid parthenogenetic group of strains from San Antonio-Delta del Ebro, Alcochete, Larache, and Comacchio. In fact the ploidy nature of the Comacchio strain was already known from the work by Artom (1922), Stella (1933) and Barigozzi (1957).

The small differences in nuclei surface areas are considerably increased when the data are converted into nuclei volumes :

TABLE IV

Nuclei area of epithelium cells of the digestive tract of bisexual and parthenogenetic *Artemia* groups

| Strains | Mean nuclei surface area ± s.d. ($\mu m^2$) |
|---|---|
| San Francisco Bay | 16.41 ± 2.52 |
| Bisexual strains from San Felix and San Fernando | 17.96 ± 3.26 |
| Parthenogenetic strains from Salinas Maritima-Calpe | 19.05 ± 3.27 |
| Cabo de Gata | 19.89 ± 2.61 |
| Ayamonte | 21.67 ± 3.85 |
| Parthenogenetic strains from San Antonio-Delta del Ebro and Comacchio | 37.77 ± 6.77 |

- the Ayamonte strain has a nucleus volume which is 1.5 times larger than that calculated for the Spanish bisexual strains ; this suggests triploidy in the Ayamonte strain (to be confirmed by chromosomal counts) ;
- the San Antonio strain has a nucleus volume 3 times that of the Ayamonte strain, suggesting the existence of a higher ploidy.

Such wide variation in ploidy is very common in parthenogenetic animals and plants (Margalef, 1974).

## Conclusion

All the data gathered in this study with *Artemia* strains from Nearctic and Palearctic regions allow to distinguish at least two different species :

- *Artemia salina* (Linnaeus, 1758) Leach 1819, bisexual diploid, distributed all around the Palearctic region (North Africa, Spain, Mediterranean area in general)
- *Artemia gracilis* (Verrill, 1869), bisexual diploid, distributed in the Nearctic region, mainly the United States of America, the Caribbean Sea islands and the northern part of South America.

The parthenogenetic strains in the Palearctic region probably have their origin in the bisexual diploid strain as a result of chromosome mutations (Stefani, 1964).

It is difficult to say when or how these mutations occurred ; they have resulted, however, in a genetical barrier between bisexual and parthenogenetic forms that precluded their further crossing.

## Literature cited

AMAT DOMENECH F. 1979. Diferenciacion y distribucion de las poblaciones de *Artemia* (Crustaceo Branquiopoda) de Espana. Thesis, University of Barcelona, Spain. 251 p.

ARTOM C. 1922. Nuovi datti sulla distribuzione geografica e sulla biologia delle due specie (micropirenica e macropirenica) del genere *Artemia*. *Atti della Reale Academia Nazionale dei Lincei. Rendiconti. Classe di scienze fisiche, matematiche e naturali. Roma*, Ser. 5, 31:529-532.

ARTOM C. 1925. Le correlazioni obbligatorie del vero tetraploidismo (Gigantismo e grandezza cellulare). *Atti della Reale Accademia Nazionale dei Lincei. Rendiconti. Classe di scienze fisiche, matematiche e naturali. Roma*, Ser. 6, 2(9):355-358.

ARTOM C. 1926. Tetraploidismo e gigantismo. Essame comparativo degli stadi postembrionali dell'*Artemia salina* diploide e tetraploide. *Internationale Revue der gesamten Hydrobiologie und Hydrographie, Leipzig* 16(1/2): 51-80.

ARTOM C. 1929. Il diploidismo dell'*Artemia salina* di Cette. *Bolletino della Società Italiana di Biologia Sperimentali, Milano* 4:862-864.

BARIGOZZI C. 1934. Diploidismo e tetraploidismo in *Artemia salina* di Margherita di Savoia. *Bolletino della Società Italiana di Biologia Sperimentali* 9(2):195-197.

BARIGOZZI C. 1941. Cytogenetical analysis of two wild populations in *Artemia salina* in connection with polyploidism. p. 57-58. *In* : Proc. 7th Intern. Genetical Congress. Edinburgh, Scotland, August 23-30, 1939. Cambridge University press.

BARIGOZZI C. 1957. Differentiation des génotypes et distribution géographique d'*Artemia salina* Leach : données et problèmes. *L'année biologique, Paris*, Ser. 3, 33:241-250.

BARIGOZZI C. 1974. *Artemia*. A survey of its significance in genetic problems. p. 221-252. *In* : Evolutionary biology. Vol. 7. Dobzhansky T. (Ed.). Plenum Press, New York. 314 p.

BARIGOZZI C. and M. TOSI. 1959. New data on tetraploidy of amphigonic *A. salina* Leach and on triploids resulting from crosses between tetraploids and diploids. *Conv. Gen. Ric. Sci. Suppl.* 29:3-6.

CLARK L. S. and S. T. BOWEN. 1976. The genetics of *Artemia salina* VII. Reproductive isolation. *The Journal of Heredity* 67:385-388.

CLEGG J. S. 1964. The control of emergence and metabolism by external osmotic pressure and the role of free glycerol in developing cysts of *Artemia salina*. *J. Exp. Biol.* 41:879-892.

CLEGG J. S. 1974. Biochemical adaptations associated with the embryonic dormancy of *Artemia salina*. *Trans. Amer. Micros. Soc.* 93(4):481-490.

ERHARDT J. P., R. MONCOULON, and P. NIAUSSAT. 1971. Comportement in vitro de la chlorophycée *Dunaliella salina* dans les milieux a salinité différente. Determination d'un optimum de salinité. *Vie et milieu*. Suppl. 22(1):203-217.

GOLDSCHMIDT E. 1952. Fluctuation in chromosome number in *Artemia salina*. *J. Morph.* 91:111-133.

LEACH W. E. 1819. Entomostraca. p. 524-543. *In* : Dictionnaire des sciences naturelles. Vol. 14.

LINNAEUS C. 1758. Systema naturae. Ed. X., Hafniae. 634 p.

MARGALEF R. 1953. Caracteres ligados a las magnitudes absolutas de los organismos y su significado sistematico y evolutivo. *P. Inst. Biol. Apl.* XII:111-121.

MARGALEF R. 1974. Ecologia. Editorial Omega, Barcelona. 951 p.

MORRIS J. E. 1971. Hydration, its reversibility and the beginning of development in the brine shrimp *Artemia salina*. *Comp. Biochem. Physiol.* 39A:843-857.

STEFANI R. 1964. L'origine dei maschi nelle popolazioni partenogenetiche di *Artemia salina*. *Rivista di Biologia*. LVII (2/3):147-162.

STELLA E. 1933. Phenotypical characteristics and geographical distribution of several biotypes of *Artemia salina* L. *Z. ind. Abst. Vererbugslehre* 65:412-446.

VERRILL A. E. 1869. Observations on phyllopod Crustacea of the family Branchipodidae, with descriptions of some new genera and species from America. p. 230-247. *In* : Proc. American Association for the Advancement of Science. 18th Meeting.

*The Brine Shrimp* Artemia. *1980. Vol. 1. Morphology, Genetics, Radiobiology, Toxicology*
*G. Persoone, P. Sorgeloos, O. Roels, and E. Jaspers (Eds). Universa Press, Wetteren, Belgium. 345 p.*

# Nauplius eye and adjacent organs of adult *Artemia*

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**Abstract**

The structure of the three ocelli of the nauplius eye is studied in adult *Artemia*, particularly the location of the nerves which end at a neuropile located in front of the eye. A specimen was accurately reconstructed, and variability in total morphology and in the cells determined. Retinula cells were studied with regard to their important characteristics of axon origin, rhabdom position and structure, and different cytoplasmic areas. A distal sphere of microvilli was observed in the median ocellus of some specimens. Pigment cells and vision capacities of the nauplius eye were briefly analyzed. Multivesicular bodies and spheroidal bodies were described.

The ventral frontal organ appeared to be composed of isolated groups of photosensitive cells dispersed at levels ventral to the nauplius eye ; these groups were adjacent to the tegument, and to the cerebrum or an hemocoel.

## Introduction

The existence of a nauplius eye of *Artemia* has already been cited by Leydig (1851), and since then numerous authors have studied the nauplius eye and the frontal organs of *Artemia* and other anostracan crustaceans (Claus, 1873, 1885, 1886, 1891 ; Zograf, 1904 ; Nowikoff, 1905, 1906 ; Moroff, 1912 ; Vaissière, 1956 ; Lochhead and Resner, 1958). Elofsson (1966) found that anostracans have a nauplius eye-type which differs from that of other groups of crustaceans ; it has indeed three ocelli and only two types of component cells. He also described the ventral frontal organs as paired photosensory organs with rhabdom, ventral to the nauplius eye, and with nerves running to the nauplius eye centre. The organ originally called "dorsal frontal organ" and later "X-organ" (Elofsson, 1966), was redescribed as a "cavity receptor organ" by Elofsson and Lake (1971) using the electron microscope. Our findings confirm the statements of the latter authors, so that it is not necessary to report on them in this paper. This cavity receptor organ is a paired sensory organ, immediately adjacent to the dorsal tips of the nauplius eye, composed of bipolar neurons whose dendrites, after piercing through two giant "accompanying cells", end with modified cilia in a cavity limited by an epidermal cell in the tegument. Rasmussen (1971) studied the nauplius eye and ventral frontal organ of *Artemia* with the electron microscope, but her results were not conclusive. We therefore undertook a deeper study with particular emphasis on the anatomy of both organs.

## Materials and methods

Adut brine shrimps were obtained by culturing the nauplii hatched from cysts obtained from SeRa 5138 (Heinsberg, Fed. Rep. Germany) ; the cultures were fed with live *Chlorella*.

Whole heads were stained *in toto* with boracic carmine (Romeis, 1936) to observe sections with a light microscope. The embeddings for electron microscopy were done in Durcupan ACM. Fixation was as described by Hootman and Conte (1975). Ultrathin sections were stained with lead citrate (Reynolds, 1963) and 2% uranyl acetate in water. These sections were observed and photographed with a Phillips EM-300 electron microscope.

Serial sections of the organs were made. Photographic maps of the nauplius eye region of some of those sections were obtained by mounting successive pictures. These maps were used to draw the cell profiles, and the organ was reconstructed to study its anatomy.

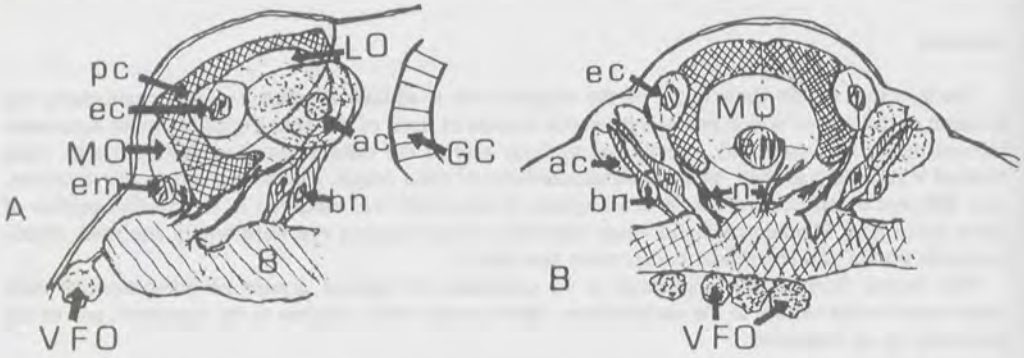



FIG. 1. Lateral (A) and frontal (B) views of the nauplius eye, cavity receptor organ (CRO) and ventral frontal organ (VFO) ; GC, gastric caeca ; LO, lateral ocellus ; MO, median ocellus ; em, sphere of microvilli ; ec, epidermal cell ; ac, accompanying cell ; bn, bipolar neuron ; B, brain ; n, optic nerves ; pc, pigment cell.

## Results

### Nauplius eye

The nauplius eye is partially situated in a frontal prominence on the sagittal plane of the brine shrimp. Lateral to and at the dorsal level of the nauplius eye is the paired cavity receptor organ (Fig. 1). The nauplius eye has variable dimensions ; usually the lateral diameter is the longest, measuring up to 120 $\mu$m. The other diameters range from 50 to 120 $\mu$m. The eye is sometimes broader anteriorly, at the level of the anterior arms of the pigment cells, as was the case in the eye we examined most thoroughly, but in other cases the pigment mass has large ventral expansions. The nauplius eye is composed of only two types of cells : two giant, central pigment cells, and numerous retinula cells. The eye has three ocelli : one is median (MO) and anterior, while the others are symmetrical, and lateral or dorsolateral (LO). The MO has 8-12 retinula cells, the LO 17-29. The two LO of one specimen may have the same or quite different numbers of cells. Ventral to the nauplius eye, and sometimes partially immersing it, is the mass of protocerebrum which also extends in front of the eye. Posterior to and on a

dorsal level with the eye are the gastric caeca. The nauplius eye is surrounded by a wide hemocoel, except where it is adjacent to the cuticular epidermis, the cavity receptor organ and the cerebrum.

*Lateral ocelli*

We studied in detail the anatomy of a specimen (E1), and particularly the retinula cells of the left LO (LLO). This ocellus was found to have 25 retinula cells (Fig. 2), the right LO had 29, and the MO had 11.


FIG. 2. Reconstruction of contiguous portions of all retinula cells of the left lateral ocellus (LLO) of E1 as seen from a lateral position. Each cell has a number ; nuclei and rhabdomeric surfaces as seen directly (large dots) and by transparence (fine dots) are drawn.

The retinula cell surface is adjacent in certain areas to the pigment cell. This appears to be a characteristic pattern. However, it was observed that the LLO had at least one small cell (19) reduced to the periphery of the ocellus. Similar cells were found in the other two ocelli. We have considered these to be cells that had not fully developed as photoreceptor cells, since they did not have any rhabdomere.

The shape of the cells is variable ; some show a polarity between the periphery and the pigment cup, but others, particularly the border cells, are largely contiguous to the pigment cup and do not show such polarity. The disposition of the retinula cells is irregular. Very few cells run along the whole cup ; more frequently several cells, or unequal rows of cells, suceed one another dorsoventrally (Fig. 3). Many cells are radially situated with respect to the cup ; dorsal cells usually have a more dorsal exterior surface, while ventral cells have a more ventral one. Consequently, in horizontal sections of an eye it appears as if two layers of retinula cells might exist at some places, which is not the case.

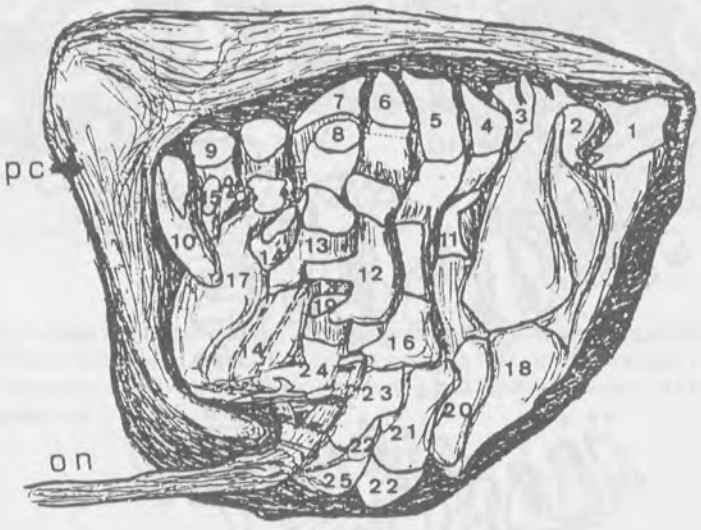



FIG. 3. Lateral view of the surface of the LLO. Pigment cup is represented, as well as the area where axons gather, and the axon origin of some retinula cells ; on, optic nerve ; pc, pigment cell.

*Median ocellus*

The median ocellus (MO) always has less retinula cells than the LO. The cells are approximately perpendicular to the pigment cup, and consequently their orientation is different from those of the LO. In some specimens an inclination of cells was noticed, in addition to the presence of a great mass of microvilli. The difference in size, orientation, and the appearance of the sphere of microvilli conferred a special character to the MO (Fig. 4 and 5).

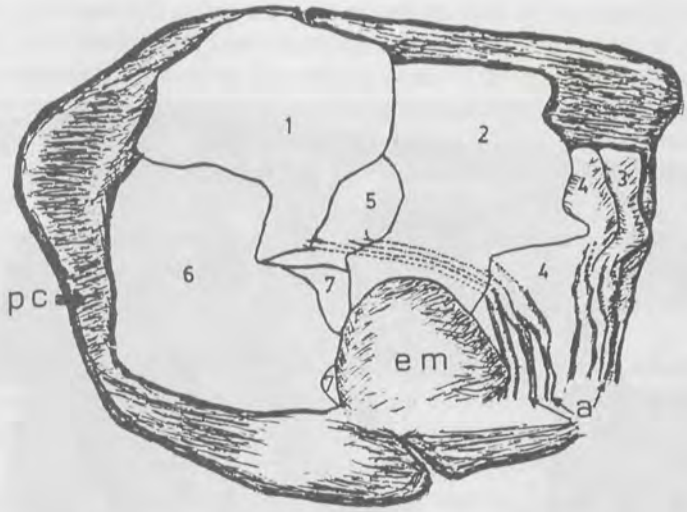



FIG. 4. Frontal view of the median ocellus. Pigment and retinula cells, and axons (a) and their origin are drawn. em, sphere of microvilli.

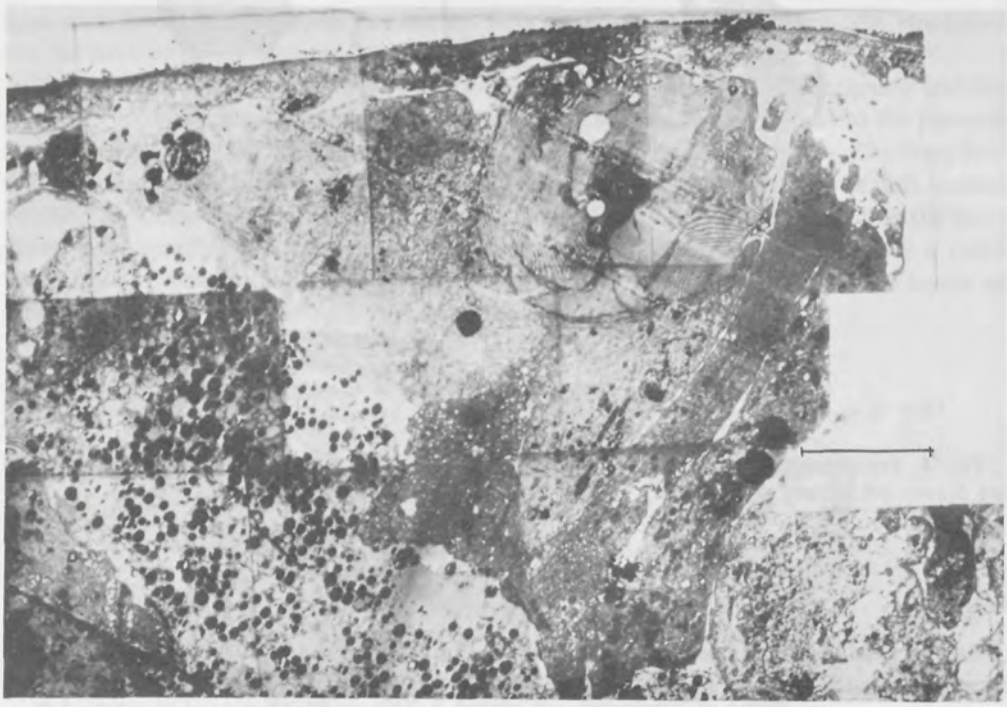
*Innervation*

Each ocellus has an independent nerve (Fig. 1). The three nerves run usually horizontally to a centre, situated anterior to the nauplius eye. The nerves have no other cellular element between the axons. In some specimens, we found, near the optic nerve origin of the LO, a region that may be considered as a neuropile. This could imply that each nerve has specific contact areas, independent of the others. The existence of three optic neuropiles for the nauplius eye was indicated by Benesch (1969) contrary to the opinion of other authors, who thought there was only one. There clearly is a neuropile, anterior to the nauplius eye, where all the nerves finally end. Sometimes the neuropile is not exactly at the sagittal plane of the animal, but laterally displaced, in which case the nerve of the LO describes a curve in front of the eye to reach the neuropile. We can confirm with our observations that there is variability or irregularity in the innervation of the nauplius eye of *Artemia*.

*Retinula cells*

The shape of retinula cells is irregular. The cells on the edge of the LO have, at certain points, equidiametral proportions, while the cells in the middle of the ocellus are more elongated, regular, tightly disposed and have smoother surfaces. The volume is not uniformly distributed among the cells of the ocellus.

Despite the lack of polarity in the general shape of certain cells, all the cells have a morphological and physiological polarity. This polarity is determined by the proximity or the distal position in relation to the pigment cup. The rhabdomeres are always near the pigment cup, and axons originate on the peripheral face. We determined the axon origin of some cells of the LLO of E1 (Fig. 2), and the place where all the axons gather. We observed, in horizontal



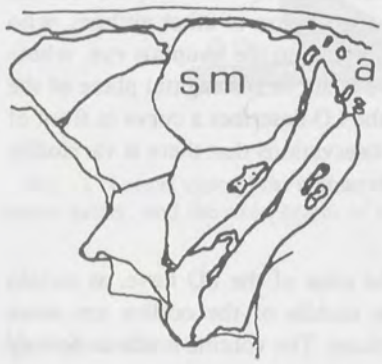



FIG. 5. Map of a section of the median ocellus. Inset, drawing of cell profiles of the map; sm, sphere of microvilli. Bar = 10 $\mu$m.

sections, that axons of each LO gather at a vertical axis situated on the anterior surfaces of the lateral ocelli (Fig. 6). Usually these axes run along the periphery of the retinula cells, but they are sometimes observed to lean upon the external arms of the pigment cells, near the most anterior retinula cells. At the points where the optic nerve is not yet differentiated, the axoplasm is very similar to the cytoplasm and contains mitochondria ; the axons are so close to the cells that they are hardly distinct from, and can be confused with, areas of cytoplasm limited by the ER. At certain levels of the axis, the axons describe a curve and leave the ocellus, thus forming the optic nerve. At this point the axoplasm becomes very dielectronic, and mitochondria become dark and elongated ; numerous neurotubuli run through the axoplasm and cross the fenestrated cisternae of the ER, which have a transversal position in the axon (Fig. 7).

Elofsson, working on crustaceans (1963, 1966) defines the eyes as inverse or everse according to the position of the axon origin, while authors studying Arachnida do this according to the position of the rhabdomeres. Following these criteria the ocellar cups of the nauplius eye of *Artemia* and the majority of Crustacea, except Malacostraca, are inverse. The origin of the axons is on the peripheral face of each cell, asymmetrical to it and always on the side nearest the gathering axis, as can be seen in Fig. 2.

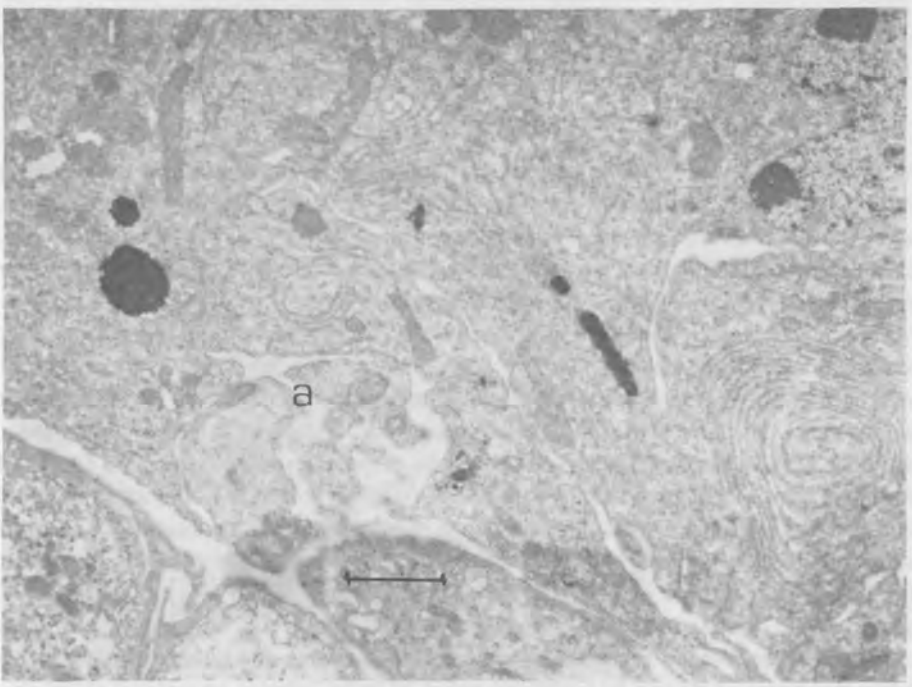



FIG. 6. Peripheral area of a lateral ocellus where axons (a) gather and one originates. ER, dense granules and mitochondria in the periaxonic area are visible. Bar = 2 $\mu$m.

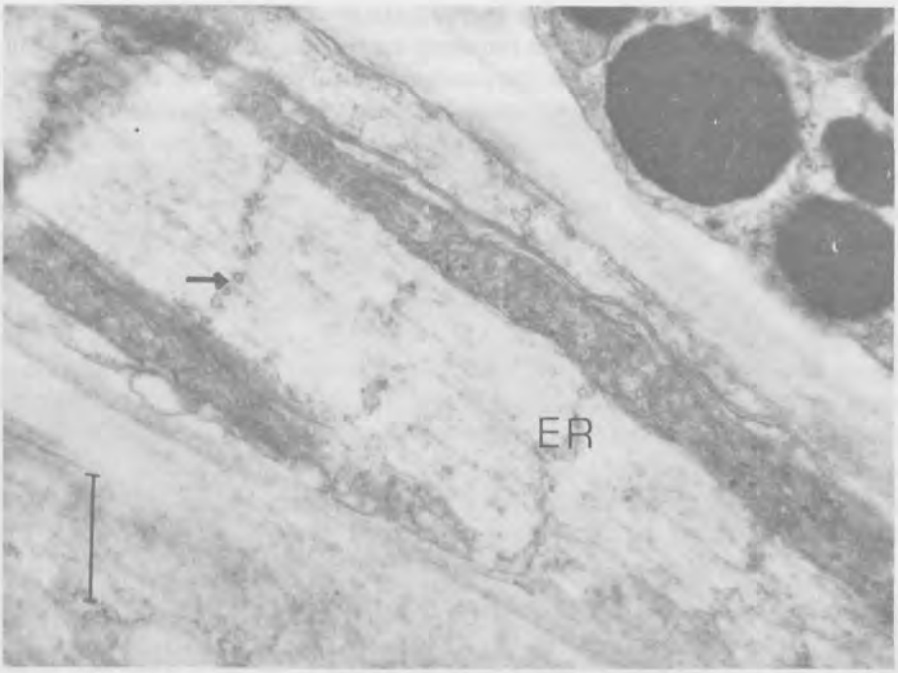



FIG. 7. Characteristic aspect of axons at a level where optic nerve is already differentiated. ER, endoplasmic reticulum ; neurotubuli (arrow). Bar = 0.5 $\mu$m.

The rhabdomeres of the nauplius eye are developed on the surface of the retinula cell faces which end in front of the pigment cup (Fig. 8). Only in particular instances are rhabdomeric surfaces developed directly on the faces adjacent to the pigment cup. A rhabdomere is a cell membrane differentiation composed of microvilli, which in these ocelli are 50-65 nm in diameter. The microvilli of the different cells, except those in rhabdomeres touching the pigment cup, are interdigitated and do not leave any intersticial space, thus forming a closed rhabdom. Most of the microvilli are longitudinally cut in horizontal sections, and the rhabdom present between two cells has a wedge shape, of which the wide part is situated next to the cup, and the narrow end is in a distal position, ending in a zonula adhaerens. The rhabdom, therefore, has a polarity, and the length of the microvilli varies with the width of the wedge. The wedges are usually not parallel, and their axes are not straight. These facts imply that there are not a few definite directions of microvilli, but a continuum of directions. We call "rhabdom" the entire rhabdom areas of the ocellus, as well as each definite portion of it. The concepts of rhabdom and rhabdomere, as used in ommatidia, are not useful here, since rhabdomeres can not be morphologically identified, and since a rhabdom is not a very definite unit. The entire rhabdom in the ocelli of adult *Artemia* consists of a series of surfaces forming a net-like structure. There can be a reduced group of cells, forming an isolated rhabdom, but this may be something aleatory rather than physiological. Nearly all the rhabdomeric surfaces (Fig. 2) are located centrally in the ocellus, next to the pigment cup.

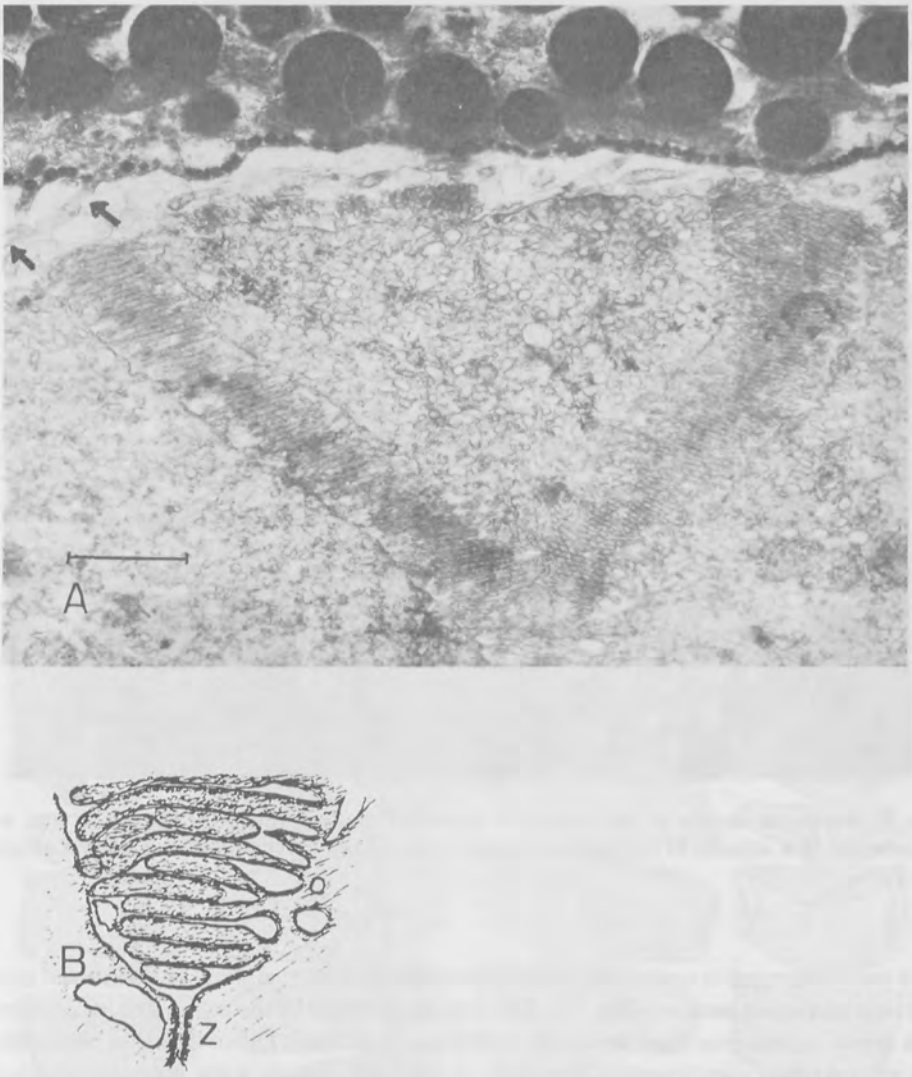



FIG. 8. A. Confluent rhabdom areas. Vesicularization of perirhabdomal areas is visible. The adjacent pigment cell has large pigment granules and a layer of finer and superficial granules ; the cell also develops stereocilia (arrows). B. Diagram of the structure of the rhabdom showing the disposition of microvilli and zonula adhaerens (z). Bar = 1 $\mu$m.

In some specimens we found a sphere of microvilli in a distal portion of the MO, next to the tegument (Fig. 5 and 9). Its connections with the surrounding cells are not clear in many places (Fig. 10). The microvilli seem to flatten at their tips, forming masses of lamellae that often occupy central places in the sphere. The sphere has a complicated orientation of microvilli with a diameter of 120-130 nm (Fig. 11), approximately twice the diameter of microvilli in the other rhabdom. Their content is very dielectronic.

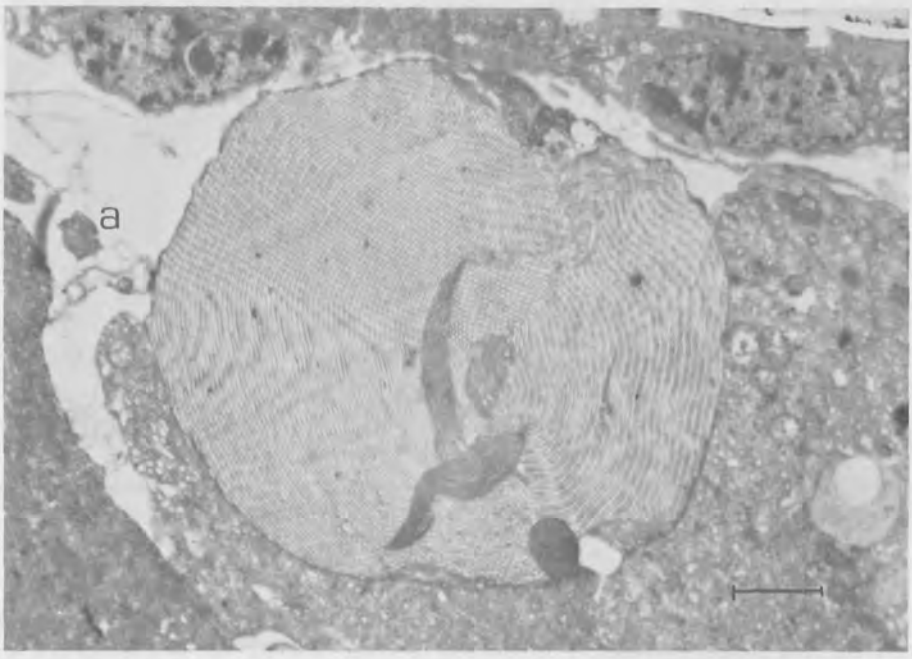



FIG. 9. Horizontal section of the sphere of microvilli showing microvilli disposition and areas with lamellae. It is adjacent to the tegument (upper position) and to retinula cells. a, section of axons. Bar = 2 $\mu$m.

We were interested in understanding the complete cell and, therefore, reconstructed one by observing successive sections (Fig. 12). The capricious shape of the cell is striking. A ventral and a dorsal prominence bear important rhabdomeric surfaces. Other very thin prominences have an unknown significance. Other cells of the LLO, which were not reconstructed in detail, are more regular, with the rhabdomeres on simple surfaces rather than on prominences.

The nucleus of the elongated cells is also elongated and is not always located at the peripheral side of the ocellus. The chromatin appears to be finely granulated, and with a few scattered spots, usually associated with or in the periphery of, the nucleoli. A single nucleus often has more than one vacuolated nucleolus which appears in different positions. Golgi bodies are visible near the nucleus ; they contain two or three dielectronic sacculi and vacuoles or dilatations, as well as some dense vesicles.

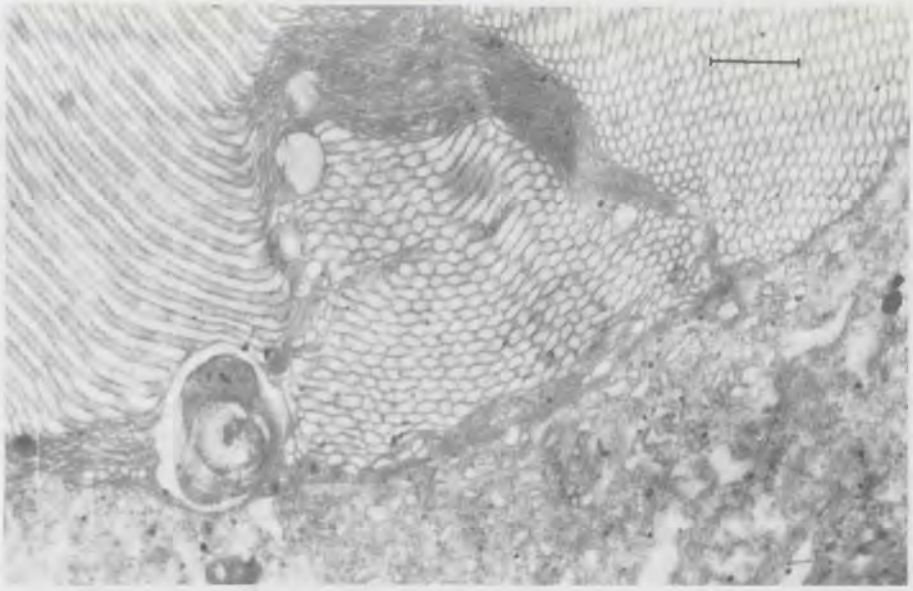


FIG. 10. Detail of the sphere of microvilli and its relation with adjacent retinula cells. The origin of the membranous element of the sphere is clearly visible at the left side. Bar = 1 $\mu$m.

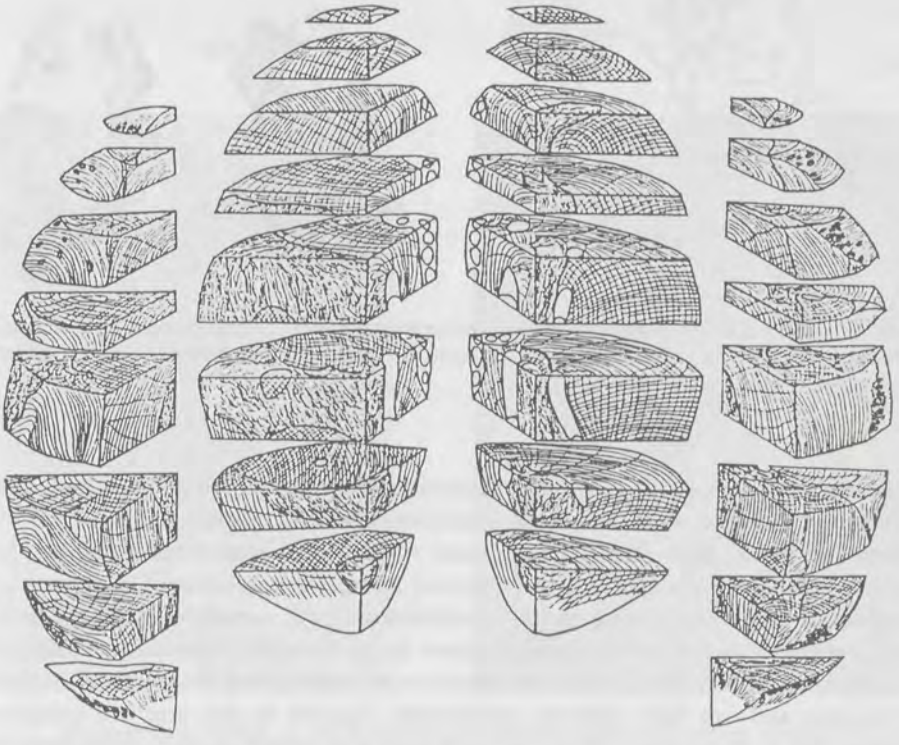


FIG. 11. Reconstruction of proximal view of the sphere of microvilli divided in quarters. At the periphery of the lateral quarters small circlets or points indicate places where the connections between sphere and retinula cells were more distinct.

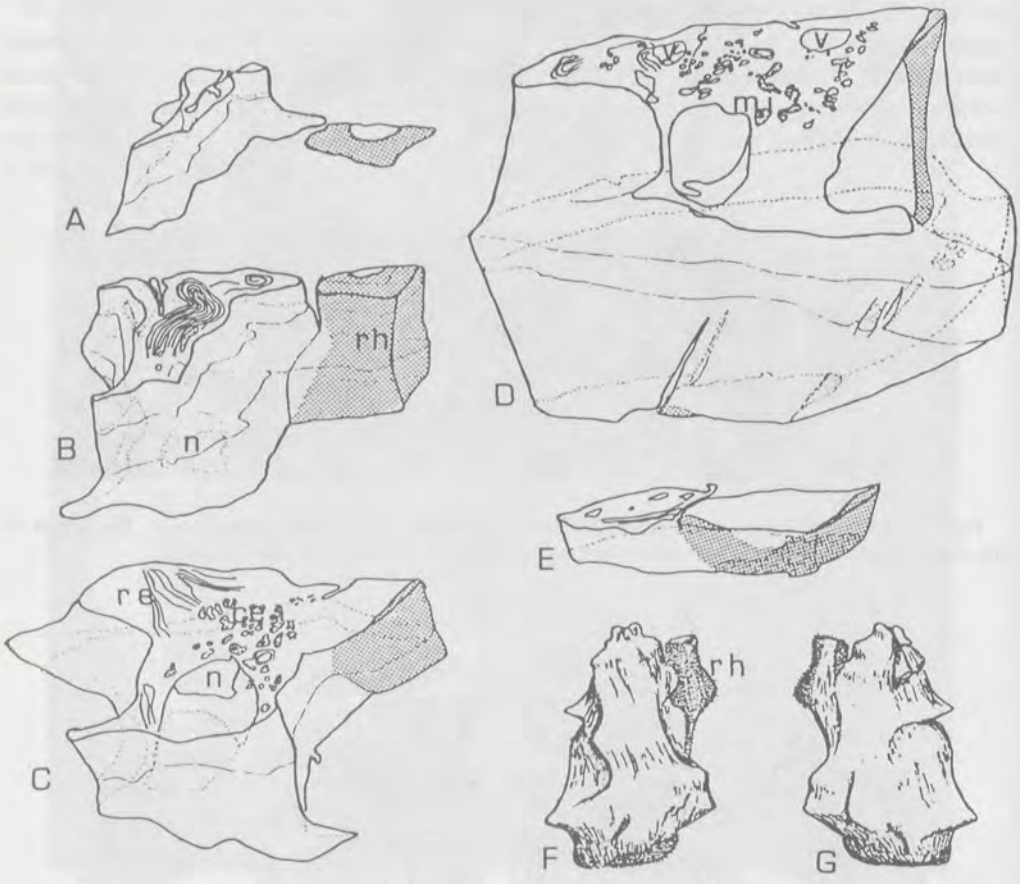



FIG. 12. Cell 3 (Fig. 2). A-E lateral view of successive portions. rh and pointed area, rhabdomere ; n, nucleus ; re, ER ; mi, mitochondria ; v, vacuole (D) ; ce, spheroidal bodies (C). F, G total cell from opposite points of view.

The cytoplasm of each cell has a particular distribution of organules and elements. Mitochondria are the most ubiquitous organules, and we observed that they are branch-shaped and reach great sizes. They contain tubular, and sometimes triangular, cristae. Retinula cells have very typical perirhabdomal and periaxonic areas. The former contain many vesicles which are round, small and adielectronic, but sometimes elongated or slightly dense. Perirhabdomal vacuoles can appear next to the rhabdom, but even in a single ocellus areas exist where they do no occur. Microtubules are usually very abundant in the perirhabdomal area, and can have different orientations. Vesicles in this area are considered as agranular ER by Rasmussen (1971). The periaxonic area usually contains three characteristic elements : mitochondria, ER and dense granules (Fig. 6). All these can be found in other areas, however. The ER is present in the cell as flattened and varicose cisternae. These cisternae may

be dispersed, constituting an irregular and conspicuous net, or parallel and concentric, filling a compact zone of the cell. This latter disposition is present in periaxonic areas.

In many cells, a portion of the cytoplasm contains a characteristic grouping of elements : multivesicular bodies (MVB), lamellar bodies, mitochondria, and sometimes spheroidal bodies. Some elements may be lacking and others may be abundant ; sometimes the elements are found alone in various different areas, but usually they are all present together. There are MVB with equidiametral vesicles and convex limits, while others have flattened, compact and curved vesicles, and have a smaller size and irregular and concave limits (Fig. 13). Sometimes both types of vesicles coexist. We believe that the MVB with flattened vesicles derive from the MVB with round vesicles. We define the spheroidal bodies as a characteristic element of the retinula cells (Fig. 14). They measure 0.3-0.6 $\mu$m in diameter, and have a limiting membrane, and an amorphous, rather dense and homogeneous content. They always occupy a unique position in the cell, forming a compact group that excludes any other element or organule. They represent a conspicuous component, not precisely described in previous literature. Small dielectronic vesicles appear frequently between adjacent spheroidal bodies. Various types of vacuoles can also be found in the retinula cells.

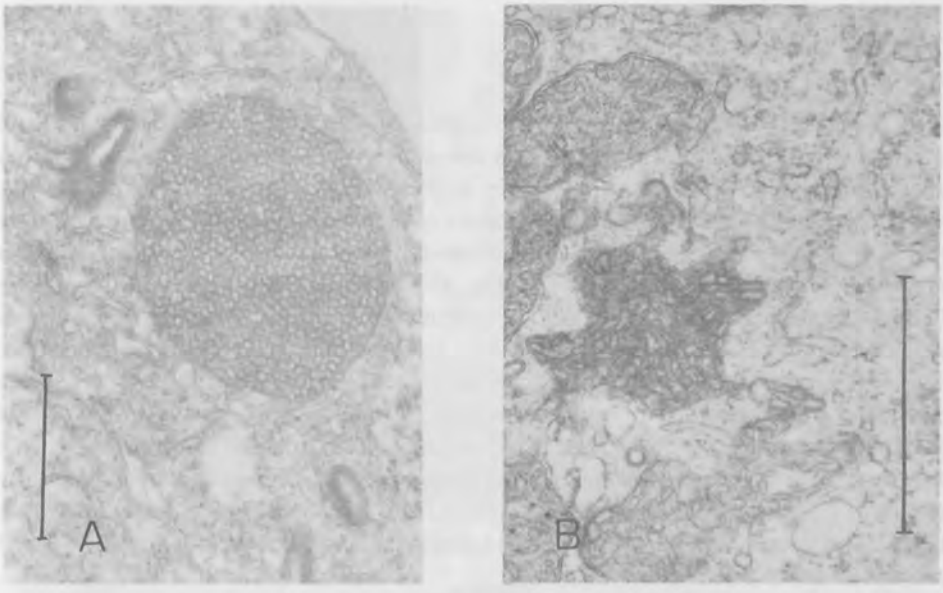



FIG. 13. Multivesicular bodies. A, MVB with equidiametral vesicles and convex surface of a photosensitive cell. Bar 1 $\mu$m. B, MVB with flattened vesicles and irregular and concave surfaces at the center of a retinula cell, on the middle. Lower position, MVB of intermediate or unusual characteristics. Bar = 1 $\mu$m.

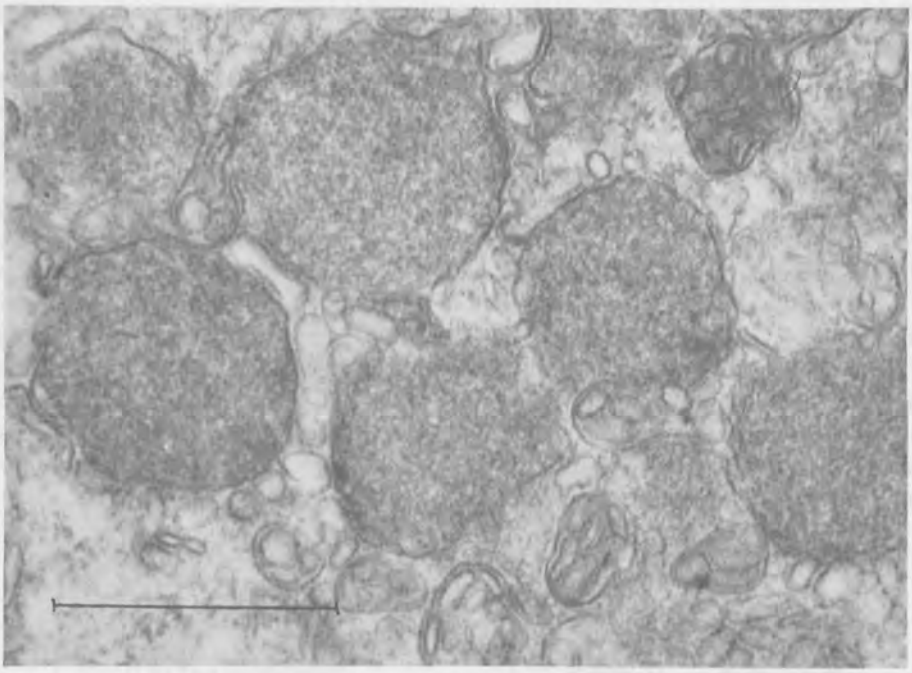


FIG. 14. Spheroidal bodies. Light vesicles are present between them. Bar = 0.5 μm.

*Pigment cells*

The shape of the pigment cells is very variable (Fig. 15). The nucleus is lobated and is ventrally located. It has several nucleoli : in one sample we found as many as five nucleoli. Pigment granules are elipsoidal, 0.5-1.1 μm in their longest diameter. They are orientated parallel to the sagittal plane of the animal, and are bordered by a membrane. Bordering the cell membrane of the pigment cells there is sometimes a simple layer of another type of dense granules, with a diameter of 120-250 nm (Fig. 8). There are also some stereocilia which are differentiated at the cell membrane of the pigment cells, on surfaces contiguous to rhabdom areas. The scarcity of ER cisternae reveals little synthetic activity in the pigment cells. Some cytoplasmic elements are very frequent. They are difficult to describe, since they have varying characteristics. They consist of irregular masses, sometimes spherical, partially or totally covered by a membrane, measuring 1 μm or more in diameter, being denser than the cytoplasm. These cytoplasmic elements of the pigment cells sometimes contained adielectronic granules of 0.2 μm or less in diameter, as well as light vesicles or parallel membrane systems (Fig. 16). The frequency of their presence and other characteristics imply a possible role in the formation of the pigment granules.

The cell membrane between both pigment cells and other surfaces of the nauplius eye is extremely digitated, increasing the cell surface and thus perhaps facilitating the metabolic exchange of the pigment cells. At some places zonula adhaerens were observed joining these digitations.

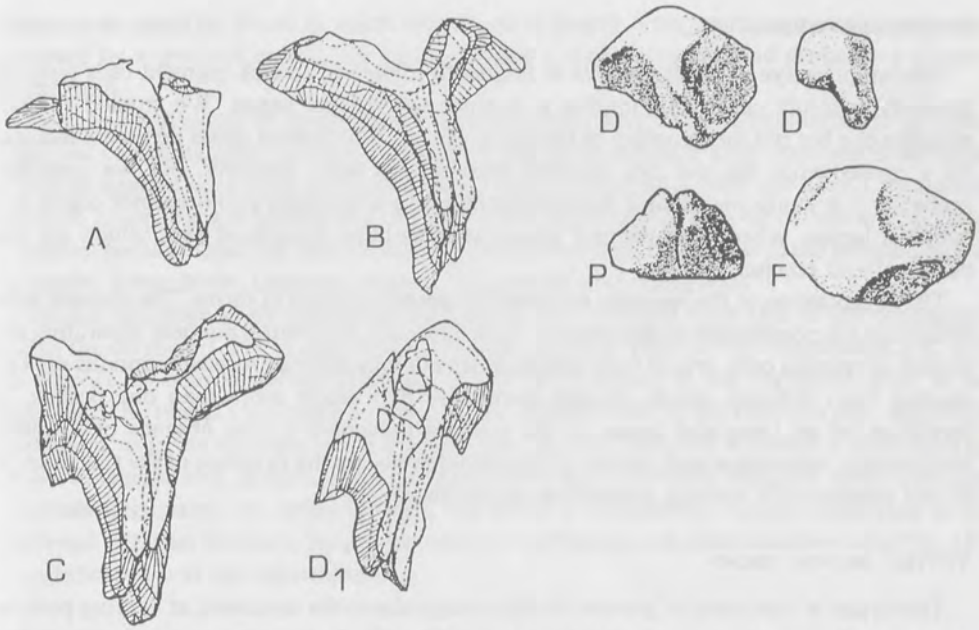



FIG. 15. A-$D_1$ successive approximately horizontal portions of the pigment cells of E1. D, P, F dorsal (D), posterior (P) and frontal (F) views of total reconstructions of the pigment cells of different specimens.

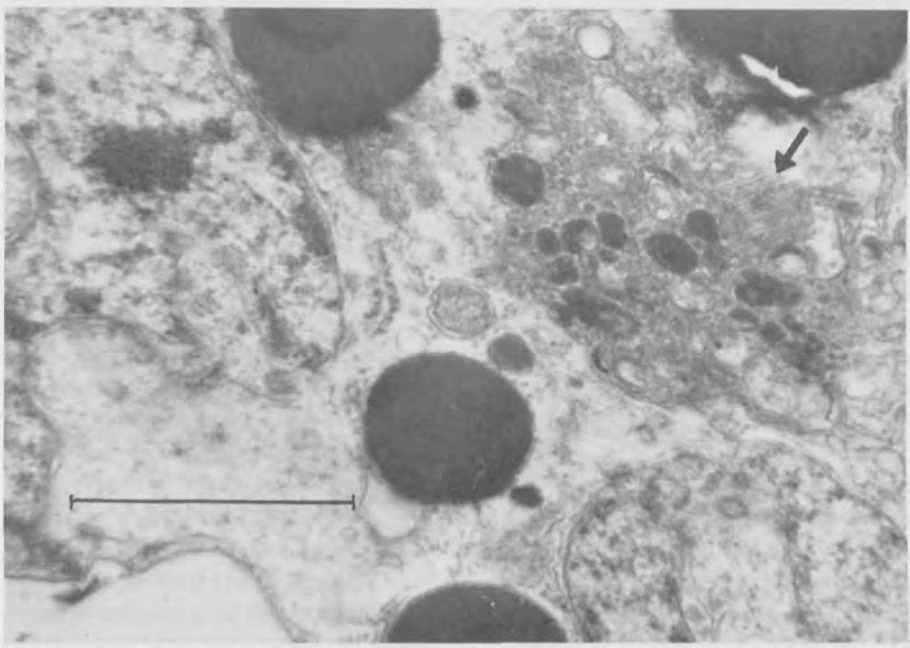


FIG. 16. Cytoplasmic element of pigment cell showing parallel membranes (arrow) and adi-electronic granules. Bar = 1 $\mu$m.

*General observations*

The nauplius eye of adult *Artemia* is frequently observed to lack pigment cells and, very probably, retinula cells. This implies a degeneration of the organ. We studied the adult nauplius eye but not the structure of the larval eye, on which there exists no recent literature. As a consequence we can not confirm degeneration with certainty, but we consider it probable. It is worth mentioning that the nauplius eye is the main photoreceptor organ in the nauplius larvae, whereas adults and young animals have compound eyes which are more perfected and precise.

The organization of the nauplius eye does not permit a vision of forms. The element which determine the possibilities of discernment is the presence of a central pigment separating three groups of retinula cells, which have different orientations and can receive luminous rays proceeding from different points, though some rays may reach more than one ocellus. The formation of an integrated image at the ocelli is prevented by the absence of a uniform morphology, orientation and pattern of the retinula cells, by the structure of the rhabdom, and by the continuously varying orientation of the microvilli.

VENTRAL FRONTAL ORGAN

This organ is composed of groups of cells contiguous to the tegument, at varying positions, ventral to the nauplius eye. Each group has 5-10 cells. It was observed in horizontal and sagittal sections that the groups were dispersed in both directions. We could not conclude the exact number of components, as it is not always the same. In the specimens examined with EM we found four groups of cells. In one specimen, horizontal sections revealed two groups, situated on both sides of the nauplius eye and as if they were immersed in the cerebrum. The two other groups, located lateral to the anterior ones, were immersed in the hemocoel. Other specimens revealed these two types of situations as well, often with certain groups at places where the tegument describes curves or angles. The groups have only one type of cell, considered to be photosensitive as a result of the presence of a rhabdomere (Fig. 17). They

FIG. 17. Partial reconstruction of a group of photosensitive cells adjacent to the tegument (posterior part) and open to the hemocoel (anterior); n, nucleus; r, rhabdom, represented as a wall between cells.

measured as much as 30 μm in width and 40 μm in length. Two groups appeared sometimes separated by a space of only 8 μm. Each group is a morphological, and probably a physiological, unit.

The cells are disposed in relation to the groups centre, where the rhabdomeres are differentiated and constitute plain surfaces. "x" and "y" forms of rhabdom were observed in sections, as well as straight lines with attenuated edges. The photosensitive cells can have a maximum length of 23 μm, but are usually smaller. Parts of the periphery of a group of cells are irregular in outline, perhaps spherical, and do not stay in close contact, in contrast to the morphology at the centre, towards the rhabdom, where cells are flat and contiguous.

The fine structure of the photosensitive cells is very different from that of the contiguous cells in the cerebrum. The presence of MVB with spherical vesicles, the conspicuous ER, and the absence of neurosecretory granules, which are frequent in the cerebrum cells, is remarkable. The nuclei are similar to those in the retinula cells of the nauplius eye. Microvilli orientation, as in the nauplius eye, is a continuum, and the rhabdom is limited at both sides by zonula adhaerens and, as such, is bipolar. The rhabdom is orientated obliquely to the cuticular epidermis (tegument). In certain groups, we found a cytoplasmic lamina, belonging to an epidermal cell, that formed a border between the hemocoel and photosensitive cells (Fig. 18). Its significance was not determined.

**Discussion**

Some characteristics of the adult nauplius eye of *Artemia* were found to be different from previous descriptions. The MO is located anteriorly, in agreement with a drawing of Dahl (1959) in which the position of the gastric caeca reveals a horizontal section. The MO, however, was termed "ventral eye" or "posterior". The exact position of the optic nerves had never been determined. The presence of one nerve for each ocellus had been determined, but the descriptions indicated a nerve position posterior to the nauplius eye. The most recent paper, of Rasmussen (1971), did not describe the axons or the nerve trajectory. Our results indicate that the axons and the innervation do not confirm the observations of Elofsson (1966) in *Branchinecta paludosa*, which he considered similar to *Artemia*.

The rhabdom structure generally agrees with EM observations of other nauplius eye (Fahrenbach, 1964 ; Rasmussen, 1971). Rasmussen (1971), however, indicates that microvilli of opposed cells may interdigitate, or be coincident at a medium distance, whereas we have only found the first situation. Fahrenbach (1964), examining the eye of the copepod *Macrocyclops albidus*, does not describe this aspect. It is very important to emphasize the results of a paper by Krebs and Schaten (1976) on the lateral photoreceptor of the barnacle *Balanus eburneus* (Cirripedia). This eye is supposed to originate directly from the larval nauplius eye, which becomes three separate eyes in the adult, as is frequently the case in certain nauplius eyes such as those of Copepoda and Ostracoda. These authors determined the existence in this eye of one type of glia cells, intermingled with the retinula cells and particularly with their rhabdomeres. The rhabdomeres of this photoreceptor were situated on dendritic prominences of the retinula cells and were not continuous. Dendrites and glia cells are elements which were observed neither in other works cited, nor in studies of many authors on the nauplius eye of Crustacea using the light microscope. Nevertheless the cell of *Artemia* reconstructed in Fig. 12 has true prominences bearing rhabdomeres, what could be

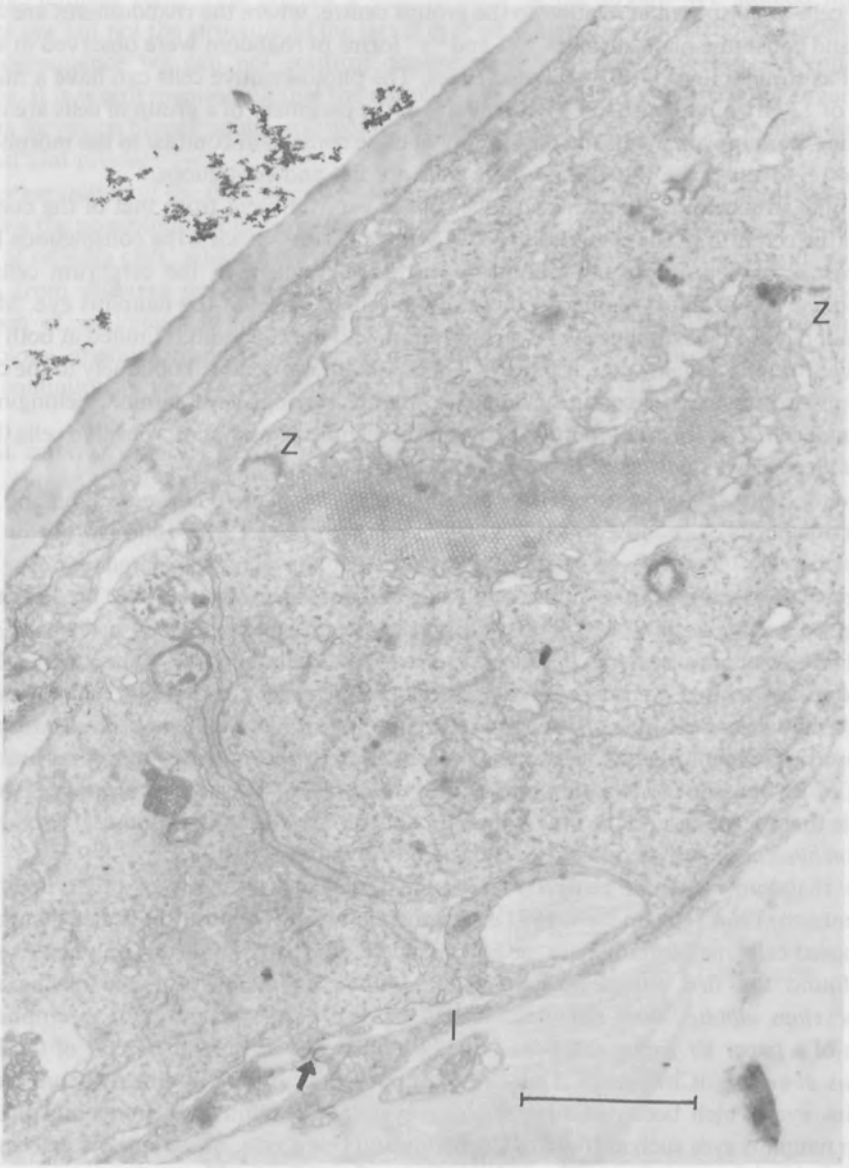



FIG. 18. Ventral frontal organ. Rhabdom is bipolar and limited by zonula adhaerens (z); l, epidermal lamina with thickenings (arrow). Bar = 2 $\mu$m.

considered as dendrites, though that is not usual. The structure of related eyes should be studied to confirm the differences observed.

In relation to the rhabdom, the most striking fact is that the sphere of microvilli is not always present. The presence of a distal rhabdom whose microvilli are twice as thick as those of the proximal rhabdom was determined in the ommatidia of the nocturnal beetle *Sericesthis* by Meyer-Rochow (1977), who described its functional significance. Distal position and double diameter of microvilli have been also found independently (Horridge, 1969 ; Dow and Eaton, 1976). However, the obviously spherical shape, the presence of piles of membranes, the length of microvilli, and above all the origin of the microvilli, are not easy to explain. Perhaps the role of the sphere is to function as a lens, and the relation with contiguous retinula cells is virtual. This is suggested by the fact that the sphere is an element not constant in the eye.

Multivesicular bodies have various characteristics in retinula cells. A photograph of a MVB of *Artemia* (Rasmussen, 1971) does not show the closely disposed vesicles which we observed. We believe this may be due to an imperfect fixation, since, in general, the cytoplasm appears very vesicularized. In *Macrocyclops*, Fahrenbach (1964) described elements similar to the "fenestrated bodies" of Fernández-Morán (1958), but not similar to MVB. The morphological evolution of MVB was accurately studied by White (1968) in the larvae of the mosquito *Aedes aegypti*. He described a series of events which took place in these bodies from their exposition to light, with varying results. Bocquet (1976), studying the photoreceptor of the polychaete *Autolytus pictus*, found primary lysosomes, secondary or autolytic vacuoles (demonstrating the presence of acid phosphatase) and residual bodies, whose morphology was similar to that of the final bodies observed by White. Types of *Artemia* lamellar bodies and vacuoles correspond with certain types described. The types of MVB in our results and their possible development are not the same as those of *Aedes aegypti*. Spheroidal bodies constitute a very characteristic element whose function has not been determined.

The structure of the ventral frontal organ (VFO) as described here, *i.e.* some groups of cells with an apparently irregular distribution, does not agree with Elofsson (1966) and Rasmussen (1971). They found a pair of VFO adjacent to the MO of the nauplius eye. Elofsson described a complicated organ and its innervation. Rasmussen mentions a nerve regularly found in sections between the nauplius eye and the VFO, but did not precisely determine the innervation. We found small sections presumed to be axons of photosensitive cells near the bodies of these cells. In certain groups they were present in substantial numbers, but we did not follow their trajectory. We admit we do not know the possible changes the VFO may undergo with age, but, due to their individual variability, different descriptions based on different specimens are probable.

## Literature cited

Benesch R. 1969. Zur Ontogenie und Morphologie von *Artemia salina* L.. *Zool. Jb. Anat.* 86:307-458.

Bocquet M. 1976. Ultrastructure de l'organe photorécepteur d'*Autolytus pictus* (Annélide Polychètes). I. Etude chez la souche, le stolon parvenu à maturité sexuelle et la tête régénérée. *J. Microsc. et Biol. Cell.* 25:61-66.

Claus C. 1873. Zur Kenntnis des Baues und der Entwicklung von *Branchipus stagnalis* und *Apus cancriformis*. *Abh. Ges. Wiss. Gottingen* 18:93-136.

Claus C. 1885. Neue Beitrage zur Morphologie der Crustaceen. *Arb. zool. Inst. Univ. Wien* 6:1-108.

Claus C. 1886. Untersuchungen über die Organisation und Entwicklung von *Branchipus* und *Artemia* nebst vergleichenden Bemerkungen über andere Phyllopoden. *Arb. zool. Inst. Univ. Wien* 6:267-358.

Claus C. 1891. Das Medianauge der Crustaceen. *Arb. zool. Inst. Univ. Wien* 9:225-266.

Dahl E. 1959. The ontogeny and comparative anatomy of some protocerebral sense organs in Notostracan phyllopods. *Q. Jl. microsc. Sci.* 100(3):445-462.

Dow M. A. and J. L. Eaton. 1976. Fine structure of the ocellus of the cabbage looper moth (*Trichoplusia ni*). *Cell. Tiss. Res.* 171:523-533.

Elofsson R. 1963. The nauplius eye and frontal organs in Decapoda (Crustacea). *Sarsia* 12:1-68.

Elofsson R. 1966. The nauplius eye and frontal organs of the non-Malacostraca (Crustacea). *Sarsia* 25:1-128.

Elofsson R. and P. Lake. 1971. Cavity receptor organ (X-organ or organ of Bellonci) of *Artemia salina* (Crustacea : Anostraca). *Z. Zell. Mikr.* 121:319-326.

Fahrenbach W. H. 1964. The fine structure of a nauplius eye. *Z. Zellforsch.* 62:182-197.

Fernández-Morán H. 1958. Fine structure of the light receptors in the compound eyes of insects. *Exp. Cell Res.*, Suppl. 5:586-644.

Hootman S. R. and F. P. Conte. 1975. Functional morphology of the neck organ in *Artemia salina* nauplii. *J. Morphol.* 145(3):371-386.

Horridge G. A. 1969. The eye of the firefly *Photuris*. *Proc. Royal Soc. (London)* B 171:445-463.

Krebs W. and B. Schaten. 1976. The lateral photoreceptor of the barnacle, *Balanus eburneus*. Quantitative morphology and fine structure. *Cell Tiss. Res.* 168:193-207.

Leydig F. 1851. Ueber *Artemia salina* und *Branchipus stagnalis*. Beitrag zur anatomischen Kenntnis dieser Thiere. *Z. wiss. Zool.* 3. 266 p.

Lochhead J. D. and R. Resner. 1958. Functions of the eyes and neurosecretion in Crustacea Anostraca. p. 397-399. *In* : Proc. XVth Int. Congr. Zool. London.

Meyer-Rochow V. B. 1977. A tri-directional microvillus orientation in the mono-cellular, distal rhabdom of a nocturnal beetle. *Cytobiologie* 13(3):476-481.

Moroff T. 1912. Entwicklung und phylogenetische Bedeutung des Medianauges bei Crustaceen. *Zool. Anz.* 40:11-25.

Nowikoff M. 1905. Über die Augen und die Frontalorgane der Branchiopoden. *Z. wiss. Zool.* 79:432-464.

Nowikoff M. 1906. Einige Bemerkungen über das Medianauge und die Frontalorgane von *Artemia salina*. *Z. wiss. Zool.* 81:691-698.

Rasmussen S. 1971. Die Feinstruktur des Mittelauges und des ventralen Frontalorganes von *Artemia salina L.* (Crustacea : Anostraca). *Z. Zellforsch.* 117:576-596.

Reynolds E. S. 1963. The use of lead citrate at high pH as an electronopaque stain in electron microscopy. *J. Cell Biol.* 17:208-212.

Romeis B. 1936. *Guia formulario de técnica histológica*. Labor S. A. (Ed.). Barcelona. 722 p.

Vaissière R. 1956. Evolution de l'œil médian d'*Artemia salina* Leach (Crustacé branchiopode phyllopode) au cours de ses stades post-embryonnaires. *C. r. hebd. Séanc. Acad. Sci. Paris* 242:2051-2054.

White R. H. 1968. The effect of light and light deprivation upon the ultrastructure of the larval mosquito eye. III. Multivesicular bodies and protein uptake. *J. Exp. Zool.* 169:261-278.

Zograf N. V. 1904. Das umpaare Auge, die Frontalorgane und das Nackeorgan einiger Branchiopoden. Friedländer and So., Berlin. 44 p.

*The Brine Shrimp* Artemia. *1980. Vol. 1. Morphology, Genetics, Radiobiology, Toxicology*
*G. Persoone, P. Sorgeloos, O. Roels, and E. Jaspers (Eds). Universa Press, Wetteren, Belgium. 345 p.*

# The propulsion and use of water currents for swimming and feeding in larval and adult *Artemia*

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**Abstract**

Propulsion of water by *Artemia* has been studied using high speed cinemicrography. Nauplii use one pair of limbs for swimming and in later stages also for feeding ; this system is inefficient compared to that found in copepod nauplii and may be a compromise dictated by environmental and developmental factors.

Pairs of thoracic limbs develop at successive moults. Older larval stages and adults use a metachronally beating system of thoracic limbs to produce water currents for both swimming and feeding. Antennae and thoracic limbs function together in mid-development stages. The metachronal system of the adult is of a type that occurs widely in branchiopods especially in the Anostraca.

## Introduction

Interest in the swimming and feeding mechanisms of the Branchiopoda seems to have declined over the last 40 years, if one can judge from the number of publications. Recent descriptions of the limbs and mouthparts of *Branchinecta* (Fryer, 1966) relate more to the taxonomic significance of the structures than to their functional operation. Earlier work on the Branchiopoda was aimed at understanding feeding mechanisms in various component groups, notably on *Apus* (now *Triops*) (Lankester, 1881), *Estheria* (Cannon, 1924), *Chirocephalus* (Cannon, 1928), *Sida* (Storch, 1929) and various anostracans, conchostracans and notostracans (Lundblad, 1920). Storch (1929) was the only one of this group to use high speed cinemicrography. The detailed work by Cannon (1928) on *Chirocephalus* included descriptions of the water currents created by the adult. This was followed by a discussion of feeding mechanisms in all orders of the Branchiopoda, including a brief comment on the nauplii of *Estheria* and *Chirocephalus* (Cannon, 1933). The relationship between the limb beat and the production of various water currents concerned with feeding of adult *Chirocephalus* was discussed by Lowndes (1933). The structure and movement of the limbs of *Balanus* (Cirripedia) nauplii and the water currents they produce was described by Lochhead (1936), and Gauld (1959) has more recently compared the feeding of nauplii of *Balanus*, *Artemia* and copepods.

In the account which follows the changes in activity of the limbs of *Artemia* during development from first stage nauplius to adult are described and related with the way in which the water currents created are used in swimming and feeding.

## Materials and methods

*Artemia* cysts purchased from Interpet Ltd. (Dorking, Surrey, England) were hatched at 20 °C ± 2 °C in the laboratory in normal seawater (s.g. 1.030). Cultures were reared in crystallising dishes of 19 cm diameter and 10 cm depth. Animals fed on microscopic algae growing in the dishes, which were kept by a well lit window, but this was supplemented by brewers yeast and added phytoflagellates. Cultures were aerated and the water was changed whenever it became cloudy. Cultures, once established, were self sustaining for many months with several generations completing their life cycles. This gave rise to a mixed culture with all developmental stages present.

Animals were examined either in the free-swimming state or else tethered to fine drawn glass needles. Individuals were glued to the needles using a butyl ester cyano-acrylate adhesive from Avdel Ltd. (Eastleigh, Hampshire, England). For successful attachment animals were anaesthetized by placing them in $CO_2$ saturated seawater until all movements ceased. Specimens were allowed to recover for at least 2 hr in fresh seawater before being used. Survival of animals tethered in such a way varied with development. Stage 1 nauplii (Anderson, 1967) would swim actively for 3-8 hr after the operation, older stages would survive for progressively longer times. Adults were sometimes kept attached to needles for weeks, being very rarely killed by the process but often managing to free themselves. Survival time for early stages probably depends on how much time there is before the next moult, which may be prevented by the presence of the adhesive.

Animals were filmed under low power (× 10 to × 40) bright field optics on a Reichert Zetopan microscope. To record limb and particle movements, a Mitchell high speed camera was used driving a Chadwick-Helmuth strobe flash at frame rates up to 400 fps.

## Results and discussion

Staging of animals is based on the descriptions of Heath (1924) and Anderson (1967). Anderson (1967) describes three moults as occurring within Heath's stage 1 and we have found diagnostic differences in antennal setation to correspond with Anderson's staging. Anderson's stage 4 corresponds with Heath's stage 2. Since Heath's stage 1 represents the true nauplius, whilst stage 2 larvae are metanauplii, we have designated the first 3 naupliar stages as stages 1a, 1b and 1c so as to maintain compatibility with Heath's staging for subsequent development. Propulsive antennae persist as thoracic limbs develop during metanaupliar stages 2 and 3 and later larval stages 4-7, but the antennae of post-larval stages 8-12 and adults serve other functions.

### Naupliar locomotion

Although all three pairs of naupliar limbs, the antennules, antennae and mandibles, show a co-ordinated action during swimming, only the antennae contribute significantly to

swimming in *Artemia* nauplii. Copepod nauplii derive a certain amount of propulsion from all three pairs of naupliar appendages (unpublished observation).

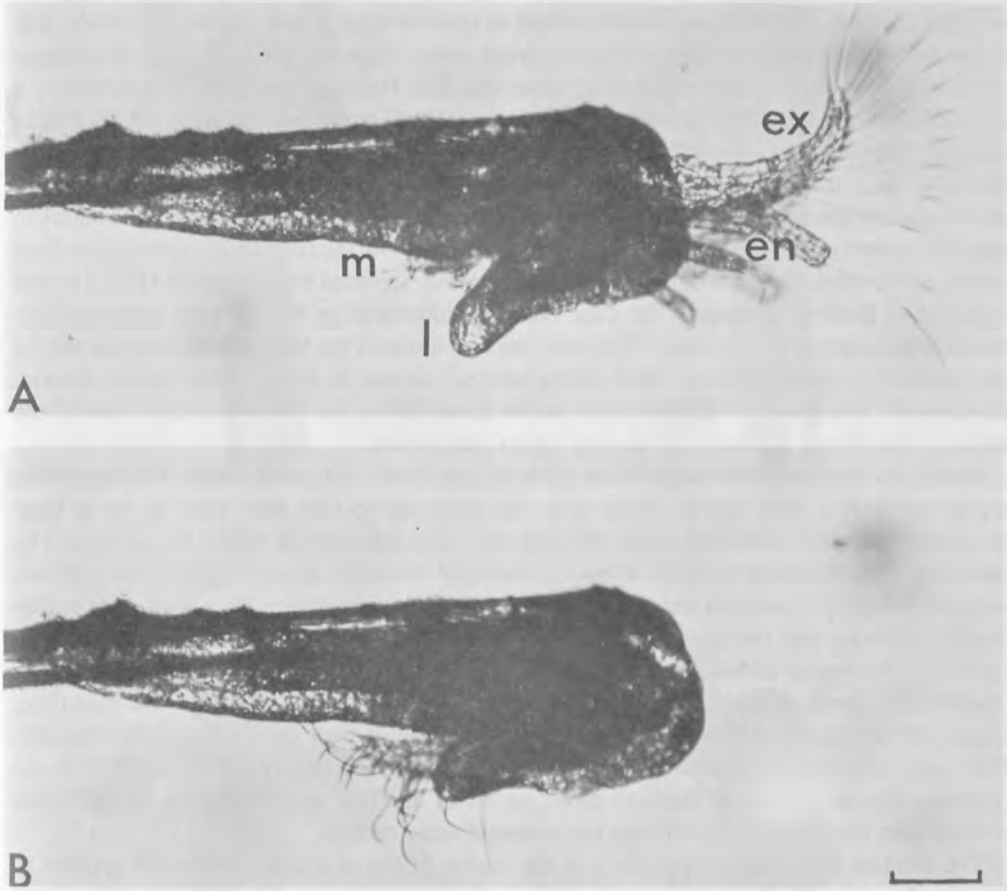


FIG. 1. Profile view of a stage 1b animal tethered with its ventral side down. The needle is visible on its dorsal surface. A) The start of the effective stroke. The setae of the exopodite (ex) and endopodite (en) are spread in a fan. The labrum (l) is starting to open and the mandibles (m) are just visible. B) The end of the effective stroke. The antennal setae are passing between the body and labrum. Scale bar: 0.1 mm.

In *Artemia* nauplii the antennal exopodite usually carries eight or nine setae at stage 1a increasing to nine or ten by stage 1b. The endopodite starts with two well formed plus one rudimentary seta in stage 1a. These develop a so called 'hinge' by stage 1b and by stage 1c the endopodite possesses three full setae and a fourth rudimentary one. By stage 2 the last has grown to give four large setae on the endopodite. The exopodite and endopodite setae together with their endites form a large propulsive fan.

The beat cycle of the antennae of excysted nauplii starts with both limbs directed anteriorly. The exopodites and endopodites and their setae are actively spread into fans (Fig. 1a) and the

two limbs move in phase through about 180° in the propulsive or effective stroke. After approximately 150° the straight limbs start to move inwards towards the body. Once the limb tips have passed the 180° mark the effective stroke has finished, but in at least the stage 1c nauplius the limb tips continue to curve round so that the setae project across the ventral side of the body and under the labrum which drops away from the body during the effective stroke and then closes over the antennal setae (Fig. 1b). The setal tips come to a halt having travelled through about 260° from the start of the stroke. The limbs recover flexed with setae bunched and trailing and initially with the endopodite leading the exopodite, but by the time the limbs reach their most anterior position the exopodite is leading. Thus the limb probably rotates slightly towards the end of the effective stroke to bring the exopodite under the labrum from the spread position seen during the effective stroke. During the recovery stroke the limb rotates in the opposite direction. A similar effect was reported by Lochhead (1936) in the antennae of *Balanus*. Stages of the beat cycle are illustrated in Fig. 2. This represents the maximum excursion of the limbs. However, the amplitude of the beat of each antenna can be independantly varied although both limbs always remain in phase. This allows turning movements. The plane of the limb beat lies at about 20° to the anteroposterior axis of the animal ; this allows the limb tips to pass under the labrum.

During the beat the antennules follow the antennae about 1/3 cycle behind. The mandibles appear to be 1/2 cycle out of phase with the antennae so that they seem to be at their maximum sideways extension when the antennal setae are curving under the labrum. The mandible setae interlock with the antennal ones and these are drawn between the coarsely setulated mandibule setae as the antennae begin the recovery stroke. This was observed by Gauld (1959) as was the grooming of the antennae by the mandibles when nauplii were placed in very heavy suspensions of particles. We found that if we glued the antennal setae together they became repeatedly entangled in the mandible setae. Although the mandibles appear to be co-ordinated with antennae, the amplitude of their beat seems to be variable. They can move through a fairly large angle, about 30° according to Gauld (1959) or on occasons we have observed them to move hardly at all. This would give the animal some control over the amount of combing the antennal setae receive.

The greatest acceleration of water is in the region of the setal fan, but the fan appears to 'leak' badly, at least in tethered animals, with the water reaching only some 40% of the speed of the setae. This leakiness persists through development up to about stage 5. Gauld (1959) has said that the antennae act as a casting net drawing particles towards the mouth area. From stage 1c onwards, this is probably true, because the antennae of free swimming animals can be seen to travel through the water drawing particles under the labrum and catching them on the antennal setae themselves. In heavy particle suspensions many particles can be seen clogging the antennal and mandible setae. However, in stage 1a and 1b nauplii filmed in the free swimming situation we find that the setae of the antenna do not travel through the water but rather seem to anchor themselves within it and lever the animal through. Water movement at the limb tips of the animal is virtually zero during the effective stroke and so the limb merely moves away from and back towards the body of the animal. Combined with this the body of the animal appears to be surrounded by a thick boundary layer and drags a large amount of water forward with it during the effective stroke. During recovery the animal drags a good deal of water forward with the recovering limbs and this results in the whole body being pulled backwards.

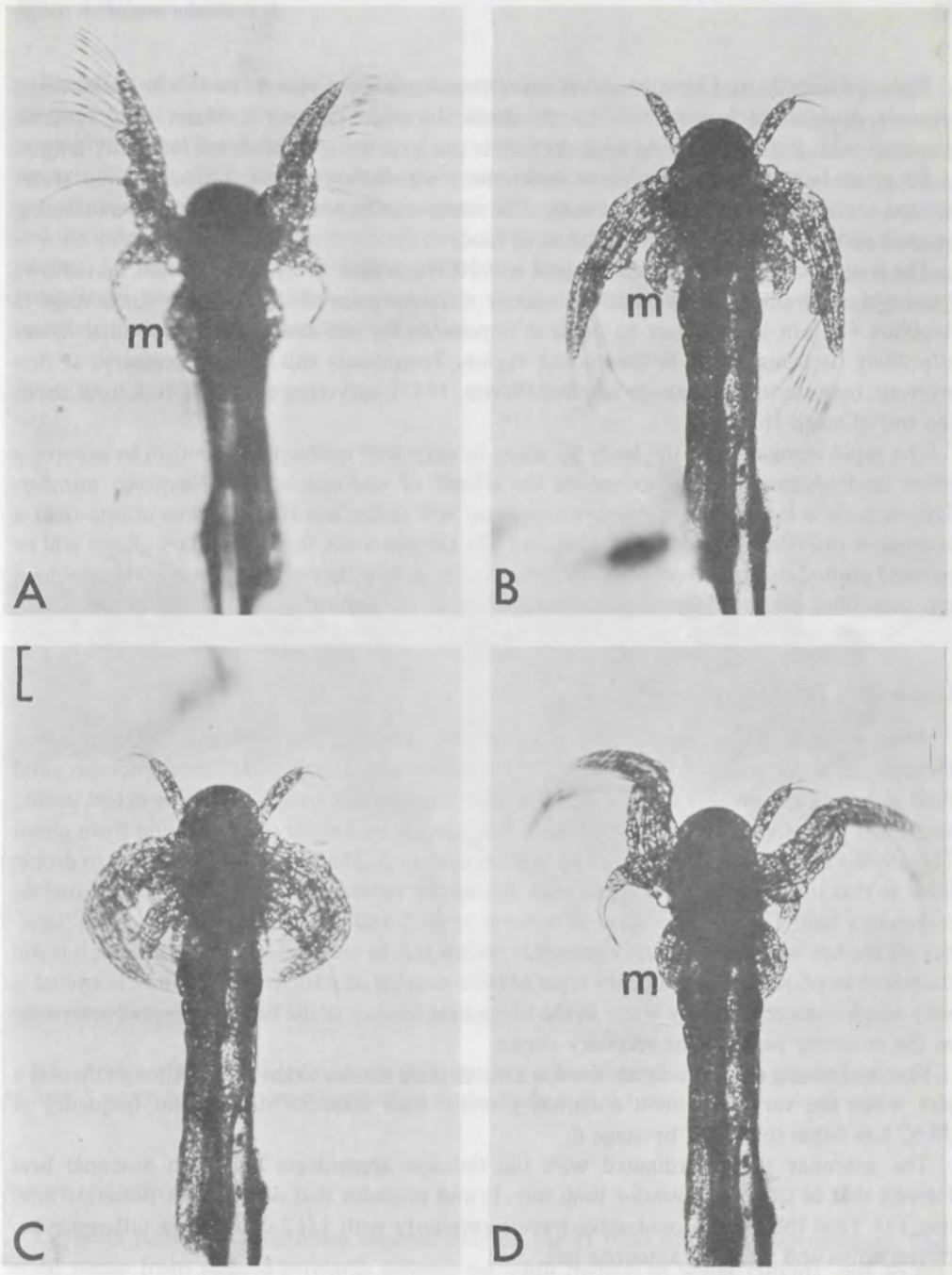


FIG. 2. The beat cycle in a stage 1c animal viewed from the dorsal aspect. A) The antennae are opening prior to the effective stroke. B) End of the effective stroke. C) Recovery stroke with setae under labrum. D) Flexed antennae and setae in mid recovery stroke. The right hand limb is prescribing a smaller amplitude recovery beat than the left. This will cause the animal to turn to the right with its next effective stroke. The mandibles (m) can be seen moving out to meet the antennae and then back under the labrum. Scale bar : 0.5 mm.

Tethered stage 1a and 1b animals are also characterized by a viscous flow with water being strongly dragged back and forth by the limbs. In stage 1a nauplii, water flow reverses direction with the limbs during each half cycle out to a distance of about one body length.

By stage 1c the animal is able to make more satisfactory forward progress and water around the animal flows less viscously. The setae can be seen to 'leak' in free-swimming animals which is necessary for filtration of food.

The frequency at which the limbs beat is 8-10 Hz, which is about the lowest that allows reasonable movement. However,the viscosity characteristics of the situation for a stage 1a nauplius 450 $\mu$m long appear to make it impossible for the antennal setae to filter water effectively or bring water to the mouth region. Fortunately this is not necessary, at first anyway, because the animals do not feed (Weisz, 1947), surviving on stored yolk until about the end of stage 1b.

The rapid elongation of the body by stage 1c may well enable the organism to acheive a better hydrodynamic shape to reduce the effects of viscosity at low Reynolds number. Theoretically a longer, faster moving organism will suffer less from viscous effects than a short slow one (Blake and Sleigh, 1974). In a less viscous water flow, boundary effects will be reduced around the limbs and body, allowing water to flow between the antennal setae which can then filter out food particles and bring them to the mouth.

### Locomotion in later larval stages

After stage 2, when thoracic segmentation first appears, the movement of the animal through the water becomes less erratic. The antennae remain the only effective propulsive and food gathering appendages until stage 6. The antennae develop to a considerable extent during stages 2-5 with the setae becoming bigger ; the longest exopodite setae growing from about 150 $\mu$m in a stage 1a nauplius to around 500 $\mu$m at stage 5. The stage 2 animal is able to propel itself so that the body is not dragged back during the recovery phase of the limb but merely comes to a halt. The antennal setae of tethered stage 2 and 3 specimens still appear to 'leak', but off the tips water flow is fairly smoothly backwards in tethered specimens although it still fluctuates in velocity. The boundary layer of slow moving or static water around the animal is very much reduced and only water in the immediate vicinity of the limbs is dragged anteriorly as the antennae perform the recovery stroke.

Free-swimming stage 6 animals show a smooth glide similar to the adult although there is a jerk when the very prominent antennae perform their effective stroke. Beat frequency at 20 °C has fallen to 6-7 Hz by stage 6.

The antennae are co-ordinated with the thoracic appendages and each antennal beat follows that of the most anterior limb (no. 1) and precedes that of the most posterior limb (no. 11). Thus the metachronal wave travels anteriorly with 1/12 cycle phase difference between limbs and with the antennae last.

The major change in antennal beat, visible at this time, is that these limbs no longer curve over the ventral surface to bring water and filtered particles to the mouth. Instead, the setal tips come to rest after travelling through 180° and then recover with limbs flexed and setae bunched and trailing as in the nauplius. Exopodite and endopodite still appear to rotate during the beat cycle as described for the nauplius.

The effect of this is that water that previously went to the mouth is pushed down the side of the body to the region of limbs 2-3 which at this point are finishing their recovery strokes and proceed to propel the water posteriorly. The more posterior limbs are already in their effective strokes (Fig. 3). This is probably a good sign that from stage 6 onwards the thoracic appendages have developed enough to take over the adult functions of feeding and propulsion and the antennae have become auxiliary propulsion units. By stage 7 the antennae are already starting to regress and make little contribution to propulsion ; by stage 8 the thoracic appendages have fully taken over the adult function whilst the antennae have regressed completely and play no propulsive role.

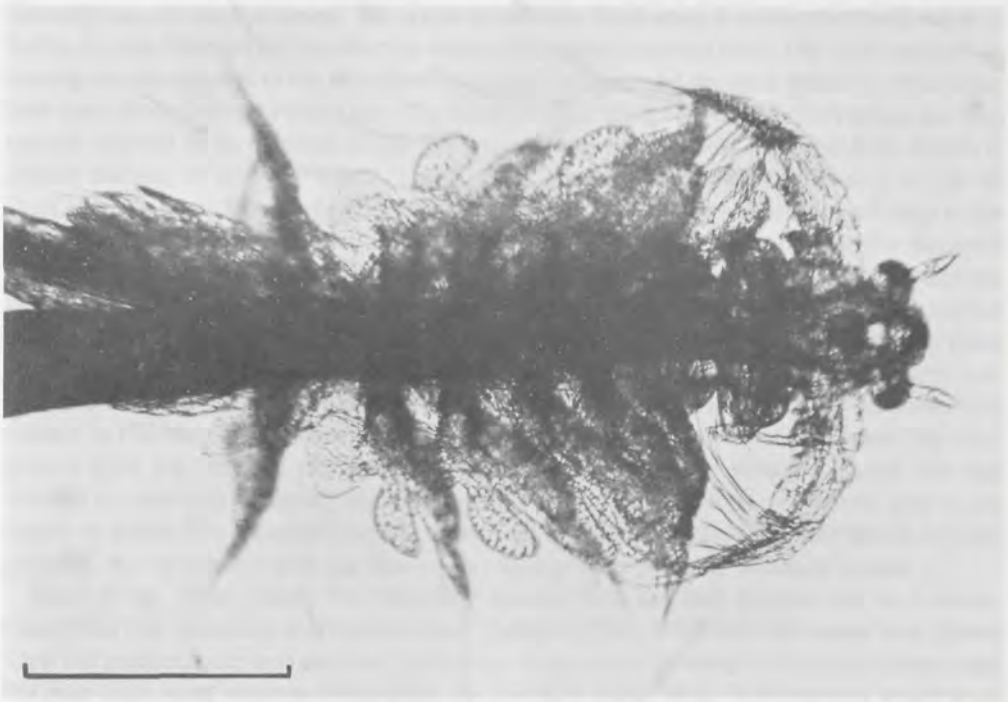

FIG. 3. Stage 6 larva. The antenna is at the end of the effective stroke and its tip is level with the tip of limb 3 which is about to start its effective stroke. Scale bar : 0.5 mm.

ADULT LOCOMOTION

The adult pattern of propulsion depends only on the 11 pairs of thoracic appendages. The larval stages from stage 5 onwards develop an adult pattern of water curents, due to the thoracic appendages, which soon dominates the flow around the animals. The description here is for adults of 9-11 mm in length, but seems to apply in general from stage 5 onwards.

In cruising adults the 11 pairs of thoracic limbs beat at an average of 3 Hz at 20 °C but the frequency can vary in the same individual from 2-4 Hz depending on conditions. The phase difference changes such that limbs 11 and 1 are beating in phase although the phase difference

can shorten slightly in faster beating individuals where 11 and 2 beat in phase. Thus there is an 1/10 to 1/9 cycle phase difference. The largest limbs travel through about 120° during the effective stroke.

Fig. 4 shows the major currents observed around a tethered male animal. However, it can be seen that little of the water flow shown in the ventral view (A) actually comes into contact with the limbs. The lateral view shows only a small flow of water past the dorsal side of the animal and a major flow on the ventral side travelling right into the limbs.

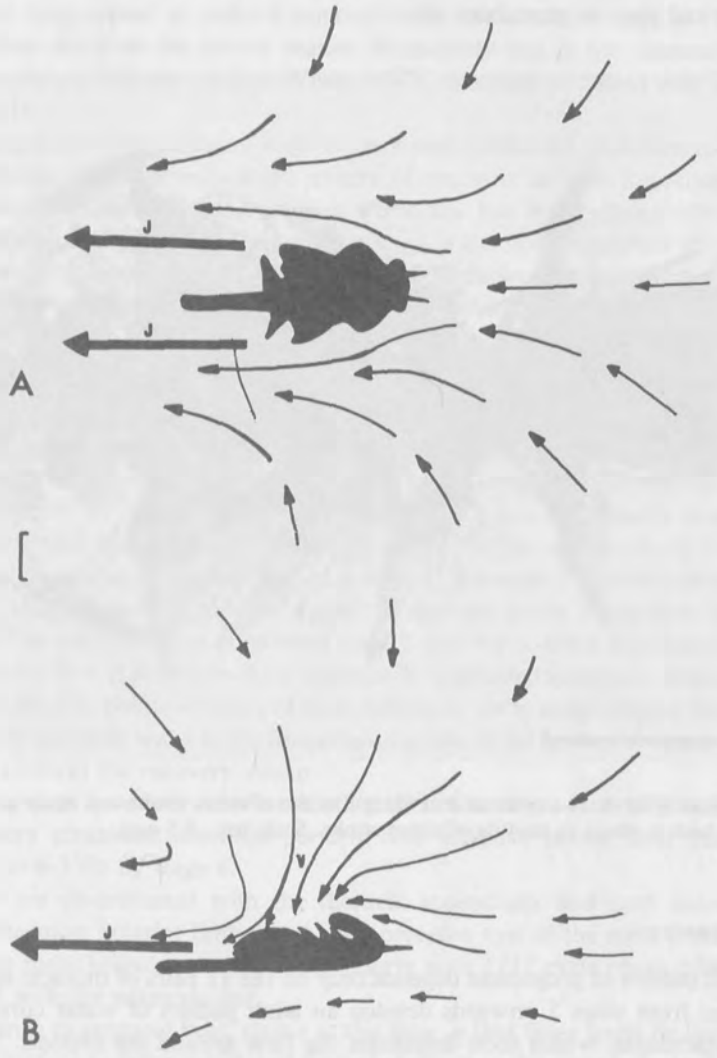


FIG. 4. A) Ventral view and B) lateral view of general water flow around a tethered adult male *Artemia*. Longer arrows represent areas of fastest flow. This occurs off the limb tips and in the two outflow jets (j). Little flow is generated on the dorsal side of the animal. The ventral current (v) can be detected over a body length away from the animal. Scale bar : 2 mm.

In free-swimming adults little water movement to the lateral and dorsal side of the animal is visible compared to the prominence of the ventrally directed current. There are two prominent jets of water leaving the posterior of the animal from the rows of limbs, one down each side. Fig. 5 shows in detail how the water is drawn through the limb system. The view is towards the side from the mid ventral line of the animal. During the recovery phase of the largest limbs water in the median space comes virtually to a halt, but as the limbs start to break away from the anteriorly directed recovery wave and perform their posteriorly directed effective stroke, the inter-limb space that forms anterior to each limb as it moves increases rapidly in volume. The result of this is for water to be accelerated laterally from the median space ; this gives rise to the ventral current (v in Fig. 5) which draws food particles perpendicularly into the median space. The water in the inter-limb space is accelerated backwards (a in Fig. 5) with the start of the effective stroke of the next anterior limb. The limb similarly is causing an enlargement of the inter-limb space anterior to it and drawing water into this inter-limb space from the mid ventral line. The third phase of the acceleration occurs when the limb reaches the end of its effective stroke and closes down on the next posterior limb which is already starting its recovery stroke. The water is jetted out of the closing space (j in Fig. 5). Thus all water used for propulsion travels from the v current to the a current and finally to the j current. The water undergoes a progressive acceleration as it travels through the sequence and is travelling at its fastest as it leaves the limbs in j. The two streams of ejected water are visible behind the animal in tethered and free-swimming animals. Water from the ventral current is filtered as it passes into the inter-limb space to be accelerated by the a current. Food particles caught on the filter setae appear to be caught up in an anteriorly directed current in the base of the food groove and carried to the mouthparts. Cannon (1928) described this current in *Chirocephalus*, believing it to be caused by water being blown back into the food groove from the bases of the limbs at the end of each effective stroke. Cannon said this resulted in anteriorly directed pulses of water. Our own observations on *Artemia* give us no reason to doubt that a similar system operates in *Artemia*. The rapid anterior travel of small particles, not in contact with the base of the food groove or setae, is clearly visible.

Most of the water enters the inter-limb spaces from the mid ventral line in *Artemia*. Describing this operation in *Chirocephalus*, Cannon (1928, 1933) said the water was drawn from the median space into the inter-limb space throughout recovery of the major limbs since the inter-limb space enlarges throughout the recovery phase. (Fig. 5). Physically water must fill this space and is quite probably drawn from the median space. However, in *Artemia* at least, the quantity of water drawn into the inter-limb space during the passage of the recovery wave is small compared to the water drawn in at the start of the breaking away of the effective strokes from the posterior face of the recovery wave.

An observation which supports the idea that propulsive water comes from the ventral surface and median space is that animals cruising with the median space in very close contact with the meniscus at the water surface, so that no water can get into the median space, come to a standstill ; this effect is most noticeable in males where the presence of the claspers severely restricts the flow of water from ahead. Females can draw some water from ahead and thus move slowly forward even when very close to the surface.

This situation is the closest *Artemia* comes to hovering ; in midwater the animal will always swim forwards. Lowndes (1933) described how the anostracan *Chirocephalus* rotates the exopodite in a propellor action during the limb beat cycle and this allows this animal to

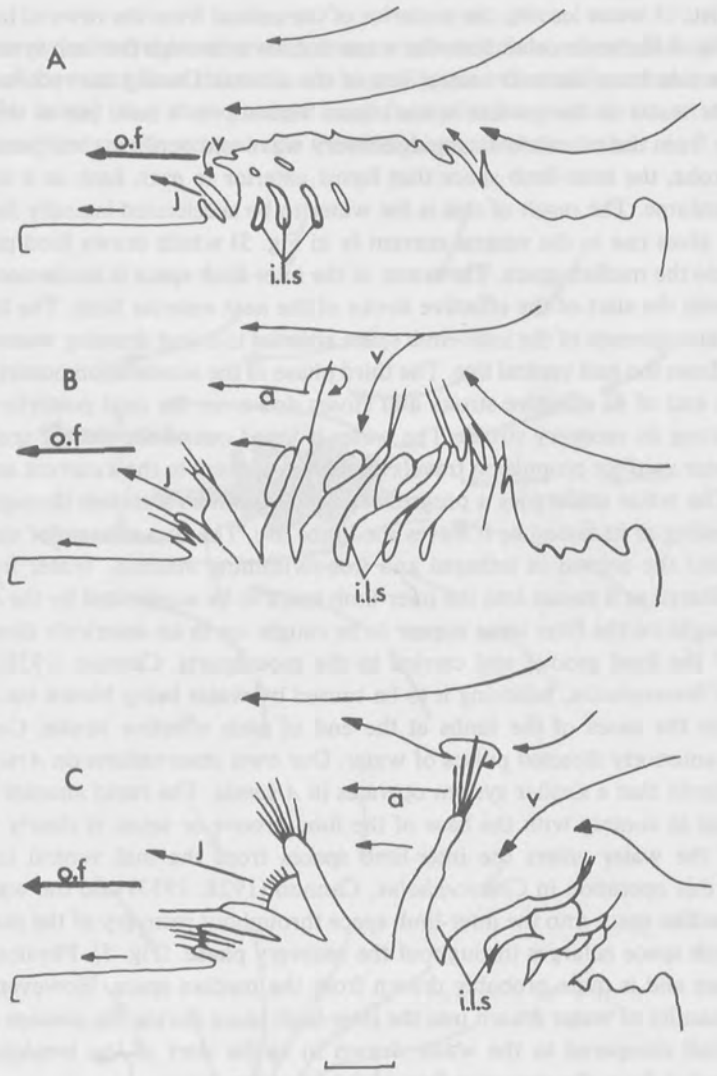


FIG. 5. Three stages, A-C, during the beat cycle of the adult limbs. The view is from the midline of the animal with ventral (v) acceleration (a) and jet (j) water flows marked. The inter-limb spaces (i.l.s.) can be seen enlarging as the limbs pass from the recovery to the effective phase (Cannon, 1928). Arrows represent direction of water flow, but not relative velocities, which are fastest in the j current. The j current marks the start of the outflow jet (o.f.) visible in Fig. 4. The ventral current has a component of flow into the plane of the diagram as well as the parallel component marked. Also note the counter-flow through the setal tips at the start of the effective stroke. Scale bar : 1 mm.

perform hovering manœuvres even in mid water. Such manœuvres do not occur in *Artemia* which does not seem to rotate its exopodite in the way described for *Chirocephalus*. To perform its normal manœuvres *Artemia* depends on bending its body and tail into a curve, thus using its tail as a rudder. The animal turns its body so that the propulsive jets of water point in the direction towards which it will turn. For left and right turns the amplitude of the limbs on the outside of the curve is increased and that of the limbs on the inside of the curve decreased. However, the pair of limbs on each segment always remain in phase. This results in a differential in the limb tip speeds of limbs on the same segment and in turn results in a relatively higher water speed in the propulsive stream on the outside of the curve of the body as compared to the inside, thus turning the animal.

### Metachronism and its functional significance

The metachronal beat of the limbs is vital to the maintenance of the type of water flow described here. The a current depends on one limb and this stage of acceleration on its own is of little use to the organism, as is illustrated by the naupliar stages. The v current, apart from providing the source of propulsive water for a and j, also provides the source of food for the filter mechanism. The j current produces the two streams of high velocity water on either side of the animal. The direction of these two jets can be controlled by means of bending the body and the power by the amplitude of the limb beat. The tail is also involved as a rudder being especially useful for dorsal or ventral turning. Thus the j current along with the tail enables the adult *Artemia* to effect manœuvres. Both v and j currents depend on the existence of phase differences between at least two limbs.

The existence of a metachronal beat may represent a real energy saving since no individual limb has to accelerate water from zero ; at any instant there will always be an energy input to keep the flow going. Energy losses between successive inputs are minimised and the speed of water flow can be maintained at the level of the limb-tip velocity, as is observed in adult *Artemia*.

The naupliar antennae manage to propel water to a mere 40% of the speed of the setal tips. It is interesting, therefore, to find that by stage 6 water flowing in similar regions around the antenna is reaching around 60% to 70% of the setal tip velocity. However by this time the antennae have become integrated members of a metachronal system and no longer function to filter particles from the flow.

Metachronism may also reduce the net forces acting on individual limbs, requiring lighter, less muscular appendages with a saving in weight and in the energy required to drive them. The thoracic appendages of *Artemia* are much more delicately built than antennae of a corresponding size.

The nauplius is extremely inefficient in terms of propulsion. The slow rate of beating of the limbs is just sufficient to allow enough movement for simple orientation responses but little else. This makes the nauplius completely defenceless and unable to feed until it grows. This may represent an adaptation to the temporary pond environment. Since the early nauplii do not feed but utilize yolk reserves they must save energy to grow and develop. Also a brine pond, especially a freshly flooded one, allows few organisms to survive and so *Artemia* nauplii possibly have fewer problems with predators than most animals. Thus a high rate of limb beat and high manœuvrability might well expend energy for no purpose. Freshwater

Anostraca all have similar slow moving nauplii and all survive in the temporary pond habitat. The nauplii hatch quickly after flooding and grow much faster than other potentially predatory crustaceans such as copepods. Insect predators also colonize temporary ponds and pose a similar threat. However, adult stages are well able to avoid such predators being much larger and more manœuvrable than *Artemia* nauplii. Copepod nauplii, on the other hand, survive in permanent bodies of water by moving fast with limbs beating at very high frequencies and they can move rapidly through the water to avoid predators. Although copepod nauplii are considerably smaller than nauplii of *Artemia* our observations show that they are able to swim and manœuvre with considerable agility.

The limited, low frequency, naupliar propulsion system may well be a compromise with other developmental factors which gives way to the multi-limbed metachronal mechanism of the adult as soon as possible. This endows the animal with greater manœuvrability and a more efficient energy expenditure which covers three major life functions ; swimming, feeding and respiration.

Our continuing studies on propulsion are very much concerned with the question of the advantages in energy expenditure and hydrodynamic efficiency of metachronal systems in aquatic propulsion in crustacean limbs and protozoan cilia.

A full quantitative analysis of the movie films from which these descriptions are drawn is in progress.

## Summary

1. Swimming movements of development stages of *Artemia* were filmed by high speed cinemicrography.

2. Animals were studied in the free-swimming state and also tethered to glass needles.

3. Naupliar locomotion utilises one pair of propulsive appendages, the antennae. These both propel the animal and filter food particles upon which it feeds. At the earliest stage viscous effects of the water prevent filtration of water in free-swimming animals and would thus prevent feeding until stage 1c.

4. The successive moults allow the development of thoracic appendages. The antennae appear to lose their feeding function by stage 6 and become incorporated into a metachronal propulsion system with the thoracic appendages.

5. The adult locomotion utilises 11 pairs of paddlelike limbs co-ordinated in a metachronal rhythm. These give rise to a continuous water flow which both draws food into the filter system and creates two propulsive water streams at the rear of the animal. These can be directed and independently controlled in power to enable manœuvres to be executed.

6. The relevance of metachronism is discussed, as is the possible importance of developmental and environmental factors in determining the form of naupliar locomotion in Anostraca.

## Acknowledgements

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## Literature cited

ANDERSON D. T. 1967. Larval development and segment formation in the branchiopod crustacean *Limnadia stanleyana* (Conchostraca) and *Artemia salina* (Anostraca). *Aust. J. Zool.* 15:47-91.

BLAKE J. R. and M. A. SLEIGH, 1974. Mechanics of ciliary locomotion. *Biol. Rev.* 49:85-125.

CANNON H. G. 1924. On the development of an Estherid crustacean. *Phil. Trans. Roy. Soc. Lond. B* 212:395-430.

CANNON H. G. 1928. On the feeding mechanism of the fairy shrimp, *Chirocephalus diaphanus* Prévost. *Trans. Roy. Soc. Edinb.* 55:807-822.

CANNON H. G. 1933. On the feeding mechanism of the Branchiopoda. *Phil. Trans. Roy. Soc. Lond. B* 222:267-339.

FRYER G. 1966. *Branchinecta gigas* Lynch, a non filterfeeding raptatory anostracan, with notes on the feeding habits of certain other anostracans. *Proc. Linn. Soc. Lond.* 177:19-34.

GAULD D. T. 1959. Swimming and feeding in crustacean larvae : the nauplius larva. *Proc. Zool. Soc. Lond.* 132:31-50.

HEATH H. 1924. The external development of certain phyllopods. *J. Morph.* 38:453-483.

LANKESTER E. R. 1881. Observations and reflections on the appendages and on the nervous system of *Apus cancriformis*. *Q. Jl. microsc. Sci.* 21:343-376.

LOCHHEAD J. H. 1936. On the feeding mechanism of the nauplius of *Balanus perforatus* Bruguière. *J. Linn. Soc. (Zool)* 39:429-442.

LOWNDES A. G. 1933. The feeding mechanism of *Chirocephalus diaphanus* Prévost, the fairy shrimp. *Proc. Zool. Soc. Lond.* 103:1093-1118.

LUNDBLAD O. 1920. Vergleichende Studien über die Nahrungsaufnahme einiger schwedischer Phyllopoden. *Ark Zool.* 16:1-114.

STORCH O. 1929. Analyse der Fangapparate neiderer Krabse auf Grund von Micro-zeitlupenaufnahmen 1. Mittl. der Gangapparat von *Sida crystallina* O. F. Muller. *Biol. Generalis. Wien* 5:1-62.

WEISZ P. G. 1947. The histological pattern of metameric development in *Artemia salina*. *J. Morph.* 81:45-96.

*The Brine Shrimp* Artemia. *1980. Vol. 1. Morphology, Genetics, Radiobiology, Toxicology*
*G. Persoone, P. Sorgeloos, O. Roels, and E. Jaspers (Eds). Universa Press, Wetteren, Belgium. 345 p.*

# Morphology of the female genital apparatus of *Artemia* : a review

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**Abstract**

A description of the different parts of the female genital tract of the brine shrimp *Artemia* is given, including results of recent electron microscopic studies.

The ripening of the oocytes in the ovaries is discussed. A secretory cycle is demonstrated in the oviducts, whose secretion seems to affect the course of the first meiotic division. In the shell glands, three types of secretory cycles are discerned which may be related to the different modes of reproduction. The attention is drawn on a virus-like infection which occurs three times as much in animals with brown shell glands as in animals with white shell glands.

**Review**

Although the scientific interest in *Artemia* has increased considerably in recent years, most studies are dealing with the biochemical, genetical or ecological aspects of this fickle animal and morphological studies are still scarce.

To our knowledge the first histological description of *Artemia*'s genital tract was given by Claus in 1886. A later study by Cassel (1937) did not yield much more information. The ontogeny of the genital tract was studied by Benesch (1969). Fautrez-Firlefyn (1949, 1950, 1951, 1957), Fautrez-Firlefyn *et al.* (1963), and Fautrez and Fautrez-Firlefyn (1961, 1964, 1977) made extensive cytochemical studies of the ovaries. Anteunis (1964), Anteunis *et al.* (1964, 1966ab), Roels (1970ab) and Roels and Wisse (1973) further elucidated some interesting aspects of the ovaries with the electron microscope. The shell glands were studied more extensively (Anderson *et al.*, 1970 ; Criel, 1972, 1977 ; De Maeyer-Criel, 1970ab, 1971, 1973). This is probably due to their presumed function in the determination of ovoviviparity *versus* oviparity. Only rudimentary descriptions can be found of the other parts of the genital tract. The purpose of this review is to make up the state of the art of our knowledge on this subject, based on the available literature data and unpublished personal observations.

To locate the different parts of the genital tract Claus' diagram (1886) of the female abdomen is well suited (Fig. 1). According to Cassel (1937) the cuticular structure formed by the two fused genital segments will be called ovisac whereas the internal pouch in which the fertilized eggs further develop is called uterus.

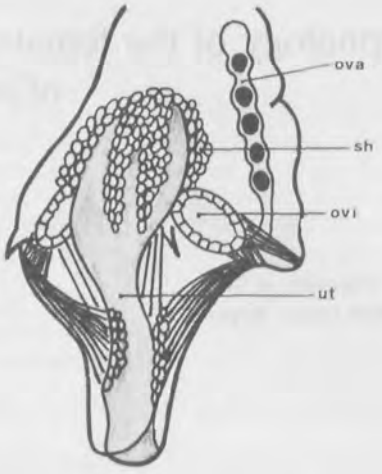


FIG. 1. Schematic drawing (after Claus, 1886) of the ovisac of an *Artemia* with an empty uterus. (ova) ovary with ripening eggs (the caudal part of the ovary lays outside the ovisac and is not drawn here) ; (sh) shellgland ; (ovi) oviduct ; (ut) uterus.

The ovaries extend from the 11th thoracic segment up to the posterior part of the sixth abdominal segment (Cassel, 1937). They lie on both sides of the gut in the abdomen. Right after entering the genital segment they debouch into the oviducts. The ovaries are surrounded by a thin basement membrane.

With the aid of various histochemical techniques Fautrez-Firlefyn (1949, 1950, 1951, 1957) studied the evolution of the germinal cell *nuclei* during oogenesis (Fig. 2). The germinal cells located on the ventral side of the ovaries, move dorsally and medially while maturing. The oogonia are small cells with a relatively large nucleus and numerous nucleoli. Four or five oocytes form a curved ribbon. The nucleus lies excentrally at the convex side of the curvation. Blocks of chromatin lie close to the nuclear membrane. The large nucleoli are often vacuolated. With the electron microscope (Fig. 3) it can be seen how the cytoplasm mainly consists of free ribosomes, a dispersed small Golgi apparatus, few mitochondria and some cisternae of rough endoplasmic reticulum. Clusters of dense material often stick to the cytoplasmic side of the nuclear membrane (Anteunis *et al.*, 1968). "Open" and "closed" cytoplasmic bridges can be noticed between the oocytes of the ribbon. They are supposed to be remnants of incomplete cytodieresis (Anteunis *et al.*, 1966a). The ribbons are separated from each other by follicle cells which can be easily recognized by their clear nucleus and their abundant smooth endoplasmic reticulum. In the growing oocytes a cytoplasmic increase precedes the vitellogenesis. The nucleus swells up to form a germinal vesicle without any visible chromatin. Cytoplasmic basophilia increases at first, then diminishes leaving only a small basophilic cape which at first surrounds the nucleus but then slips to one side, creating in this way the typical bilateral symmetry of primary oocytes.

Vitellogenesis starts in the centre of the oocyte where an atypical vitelline nucleus is found (Petrunkewitsch, 1902 ; Artom, 1907 ; Fautrez-Firlefyn, 1957 ; Fautrez-Firlefyn *et al.*, 1963 ;

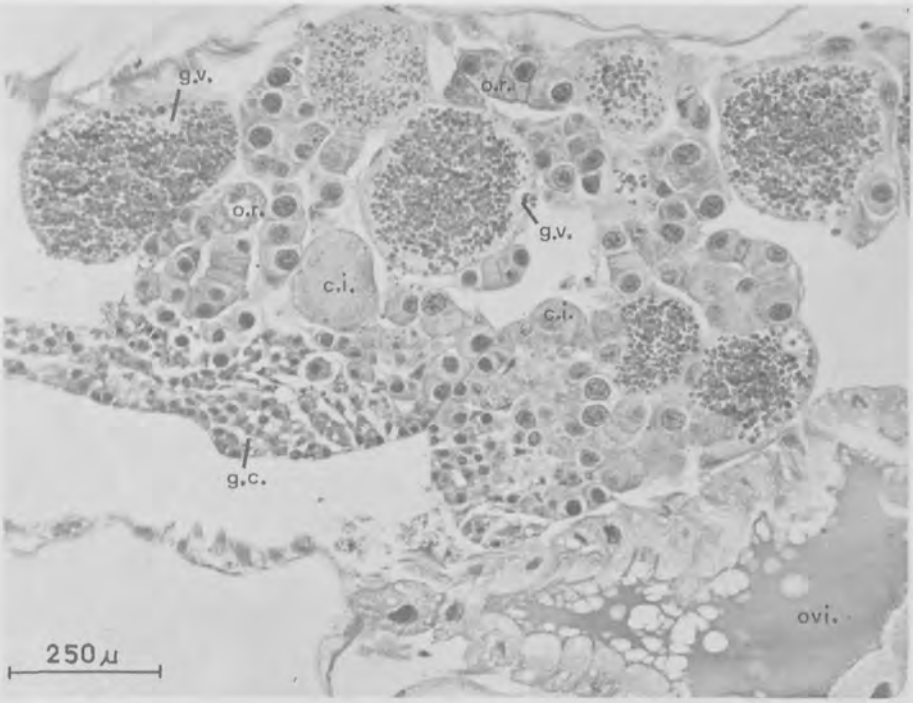


FIG. 2. Longitudinal section through the ovary and oviduct (ovi). (g.c.) small germinal cells ; (o.r.) oocyte ribbon ; (c.i.) oocytes showing the cytoplasmic increase preceeding the vitellogenesis ; (g.v.) germinal vesicle of oocytes in vitellogenesis.

Fautrez and Fautrez-Firlefyn, 1964). Fautrez-Firlefyn *et al.* (1963) demonstrated that this nucleus contains acid phosphatases. The electron microscopical study of Anteunis *et al.* (1964) showed the vitelline nucleus to be composed of multivesicular bodies, dense bodies, microvesicles and free ribosomes (Fig. 4). The vitelline nucleus is thought to form the proteic vitelline granules. On the other hand the origin of lipoid vitellus is less clear. Thanks to its phosphatasic activity Fautrez and Fautrez-Firlefyn (1964) were able to follow this nucleus which persists in one or the other blastomere during cleavage till the beginning of gastrulation. They found the nucleus again in the first invaginating cells, and because these are considered the germinal initials, both investigators wondered if this nucleus did not also act as a germinative determinant. At the same phase of vitellogenesis Anteunis *et al.* (1966c) found a tubulo-mitochondrial apparatus formed by a paracrystalline apposition of mitochondria and endoplasmic reticulum cisternae clumped together by an electron dense cement. This apparatus too is thought to cooperate in some way to the yolk formation.

Also in this phase bodies appear bound by a single membrane suspected to be peroxisomes because of their peroxidatic activity (Roels, 1970b) ; later it was concluded that they contain a peroxidase, not a catalase, (Roels and Wisse, 1973). Fautrez-Firlefyn (1949, 1950, 1951) and Anteunis *et al.* (1966b) showed the phagocytosis of surrounding nurse cells by the growing oocytes.

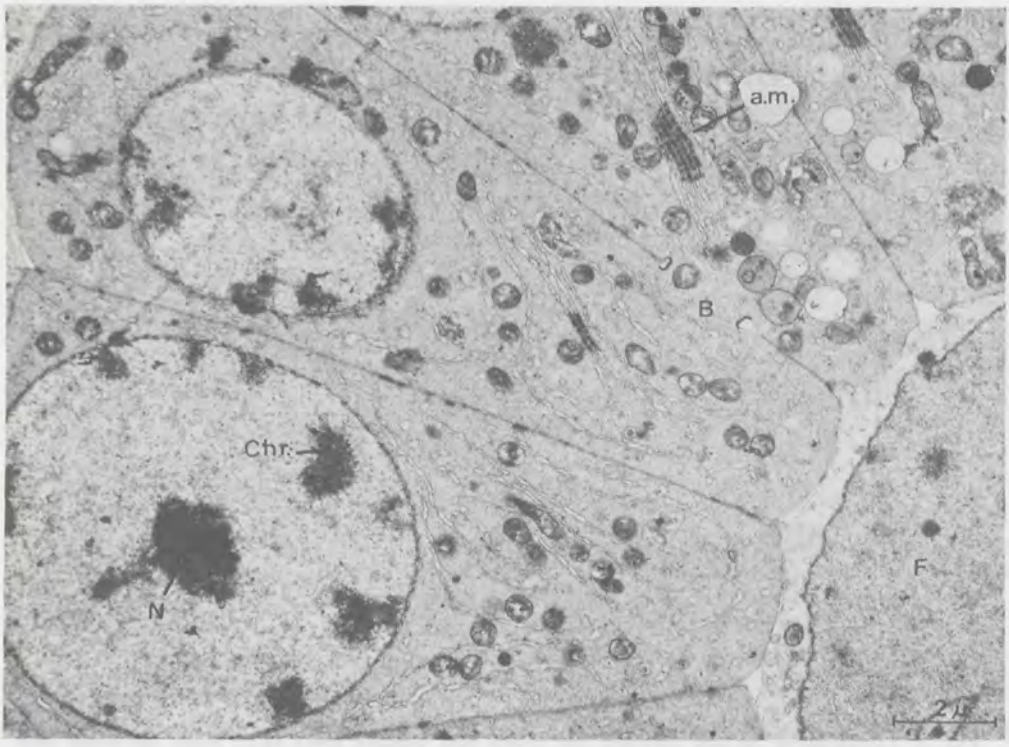


FIG. 3. Electron micrograph of a ribbon of young oocytes. (N) nucleolus ; (Chr) chromatin blocks close to the nuclear membrane ; (B) open cytoplasmic bridge between two oocytes ; (a.m.) annulate membranes ; (F) follicle cell with a large clear nucleus.

In many ovaries degenerating young oocytes extrude lumps of DNA (Fautrez-Firlefyn, 1949, 1950, 1951). This DNA seems to originate from endonuclear polyploidy leading to the death of the cell (Lison and Fautrez-Firlefyn, 1950).

At the end of vitellogenesis chromatin reappears in the germinal vesicle and forms dyads, (Fautrez-Firlefyn, 1949) ; in the cytoplasm the smooth endoplasmic reticulum cisternae of the cortex acquire peroxidatic activity (Roels, 1970b).

After ovulation the eggs enter the oviducts where they are stored for some hours, sometimes for some days, bathing in the oviductal secretion. The expanded oviducts form the so called lateral pouches. The cells lining the pouches are so stretched that they are hardly recognizable as oviductal cells. The pouches are separated from the uterus by a shutter which is partially cellular, partially fibrous (Fig. 5a,b). We wonder why Claus (1886) and Cassel (1937) paid no attention to this. Forty minutes to an hour after copulation the shutters open and the eggs are released into the uterus where they are immediately fertilized (Fig. 5a and Fig. 6). During the time lapse between the copulation and the opening of the shutters the medial and frontal part of the uterus function as a seminal receptacle : the spermatozoids indeed further ripen in the female organism, some of them acquire peripheral spines while others disintegrate.

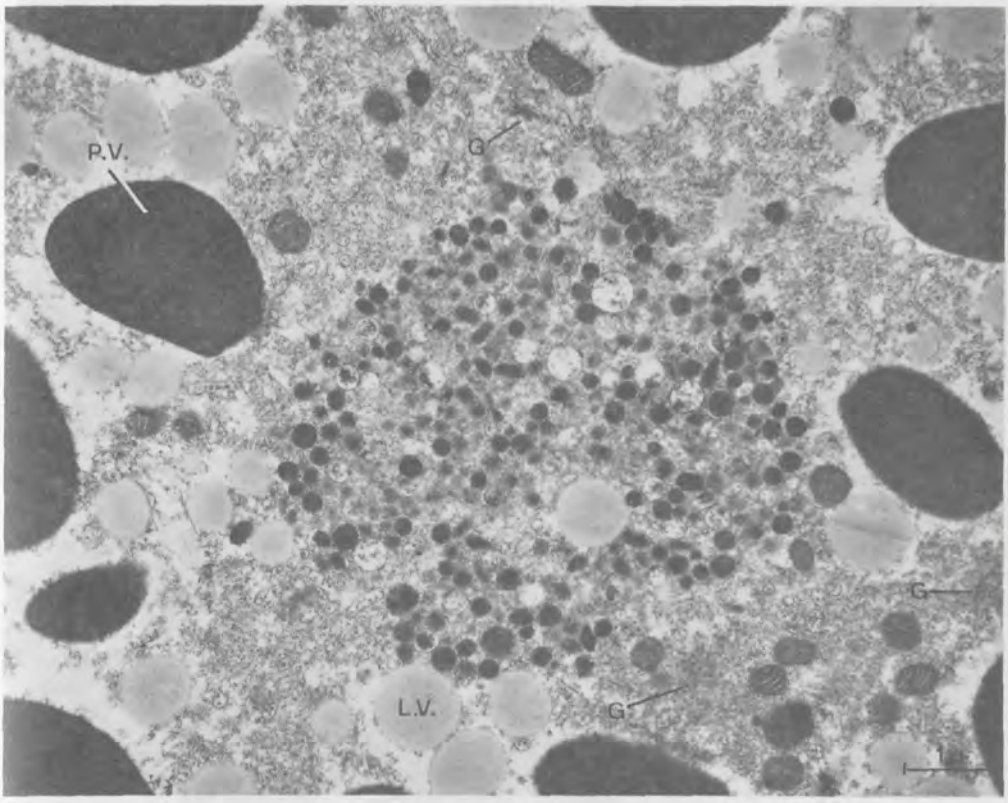


FIG. 4. Electron micrograph of the atypical vitelline nucleus in an egg just after the descent into the uterus. (G) small Golgi complexes ; (L.V.) lipid vitellus ; (P.V.) proteinaceous vitellus.

Under the lateral horns of the ovisac the oviducts can be seen as two folded pouches surrounded by a looze but regular network of circular and longitudinal muscle fibers. The oviductal cells show a secretory cycle which has been studied at the light and electron microscopical level. It begins when the eggs embedded in the oviductal secretion leave the lateral pouches and enter the uterus. The extended oviduct then slowly shrinks to its normal dimensions and the flatenned cells gradually become cubic and cylindrical (Fig. 7a,b). At first only empty vesicles of hitherto unknown origin are found in the lumen of the oviduct, but from the second day on a PAS positive secretion is accumulating there although no secretion granules can be seen in the cells (Fig. 7c,d). A clear rim separates the secretion from the apical parts of the cell. Cassel (1937) thought it was a chitinous membrane that kept the secretion away from the cells but an ultrastructural study shows that the rim is formed by cell tops pinched off in an apocrine secretion.

The oviductal secretion has a peculiar composition as the first meiotic division of the eggs proceeds till metaphasis when the eggs enter the oviducts (Gross, 1936) but this metaphasis can only be completed when the eggs are released or taken from the oviduct (Fautrez and

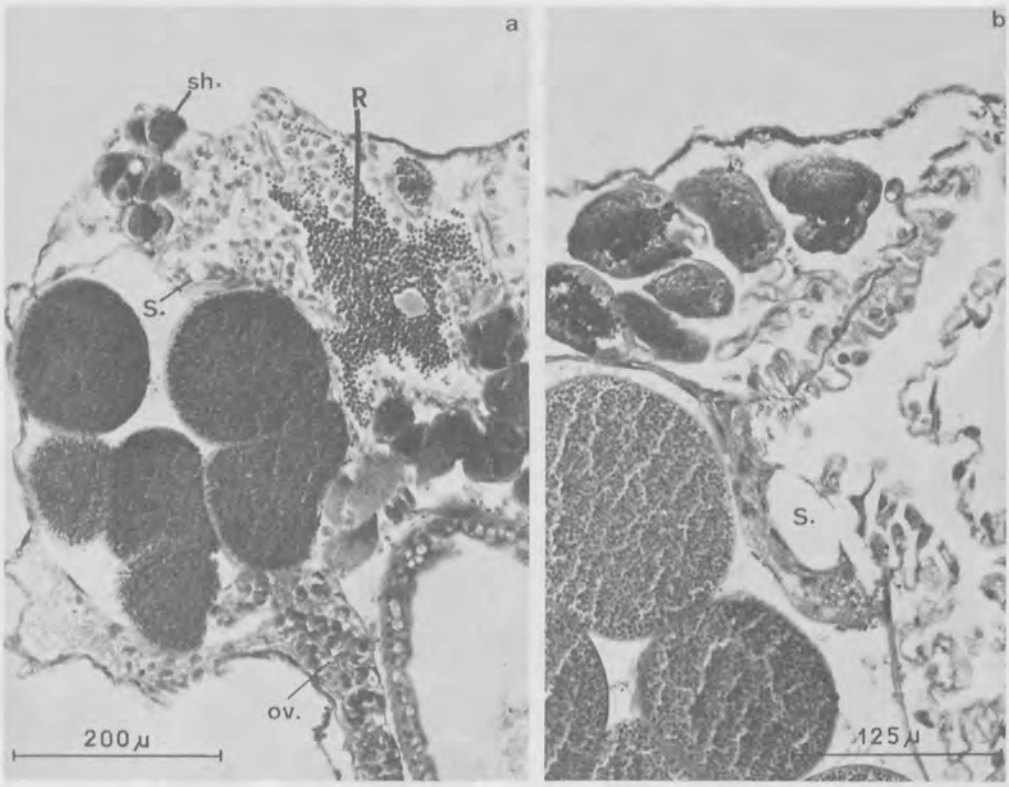


FIG. 5. a. Frontal section through the ovisac of an animal with eggs in the lateral pouches. (ov) ovary ; (sh) shell gland ; (S) shutter separating the pouches from the uterus ; (R) median pouch of the uterus (with numerous spermatozoids) which functions as a seminal receptacle. b. Enlargement of the shutter between the lateral pouches and the uterus.

Fautrez-Firlefyn, 1961). An electron microscopic study revealed furthermore that the formation of the vitelline membrane starts immediately after the descent of the eggs in the uterus even in virgin females.

The shell glands consist of several clusters of glandular cells. The excretory ducts of the lateral clusters open near to the shutter between the oviduct and the uterus, the median clusters excrete in the middle pouches of the uterus. The color of the gland cells varies in different reproductive cycles : all intermediate colours between dark brown and white and even colorless gland cells may occur. Already in 1873 Von Siebold pointed to the role of the shell glands in the determination of ovoviviparity and oviparity, but to this day nobody has exactly elucidated this complex mechanism. Thusfar we can only say that most animals with brown shell glands are oviparous, most animals animals with white shell glands ovoviviparous.

Fautrez and Fautrez-Firlefyn (1971) made a comprehensive study with the light microscope of the shell gland. Each glandular unit consists of two or four opposed cells. The secretion is released between the two cells and taken up by the excretory duct originating at the basis of

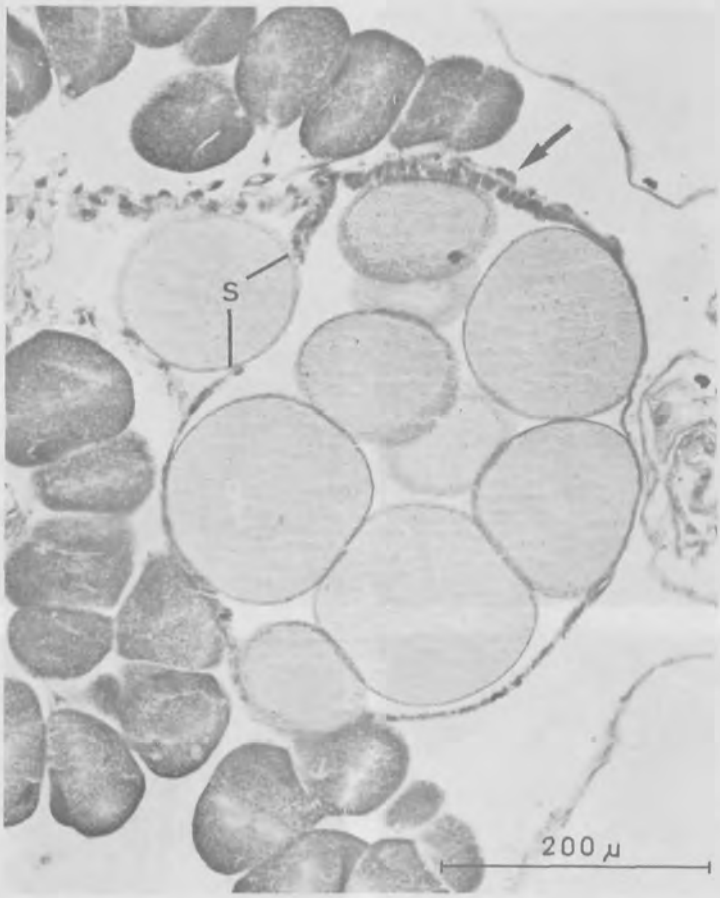


FIG. 6. Release of the eggs from the lateral pouches : the shutter (S) has opened and the extended oviduct gradually retracts (arrow).

the two cells. The chitinous membrane bordering the lumen of the excretory ducts is continuous with the chitinous membrane of the uterus. A similar organization of glandular cells is found in the tegumental glands and this strengthened Benesch (1969) to claim the ectodermal origin of this gland. The secretory granules contain mucopolysaccharide, proteins and lipids ; in the brown shell glands hemoglobin was localized by histochemical techniques. This is in accordance with Dutrieu's (1960) hypothesis that the brown shell glands excrete the superfluous hemoglobin of the blood.

As far as the morphological appearance of the secretory granules is concerned (Fig. 8) we distinguished two types of white glands (Criel, 1972, 1977). The secretory granules of one type completely differ from those in the brown glands, therefore we thought they might be related to the formation of nauplii. In the other type the granules resemble those of the brown glands ; we think that they could contribute to the envelope either of eggs hatching immediately after oviposition or of thin-shelled eggs. As we observed that the secretion of the

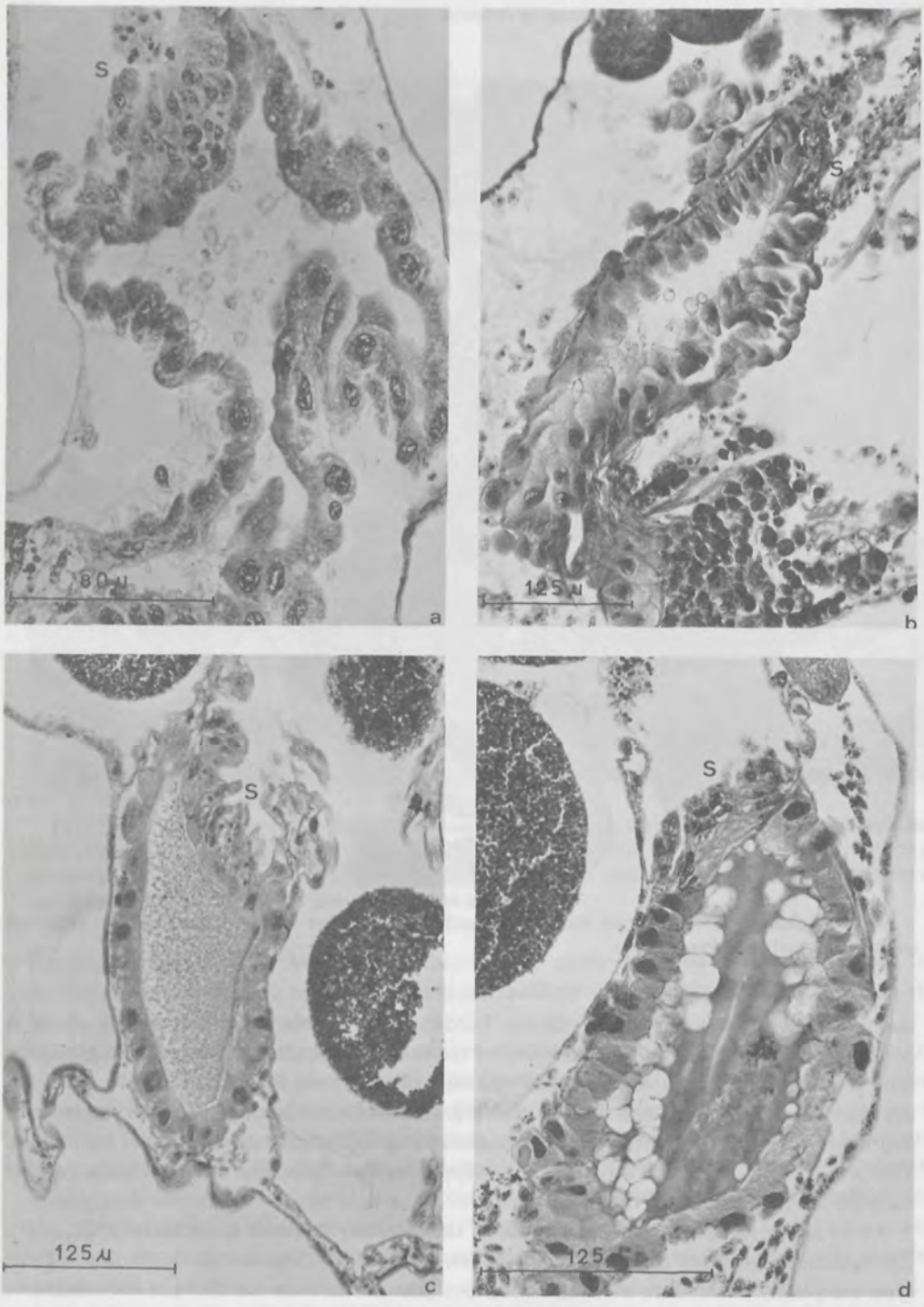


FIG. 7. Oviduct at various stages of the reproductive cycle ; (S) shutter. a) 10 min after the descent of the eggs into the uterus ; b) 1 hr after the descent ; c) 12 hr after the descent ; d) 72 hr after the descent.

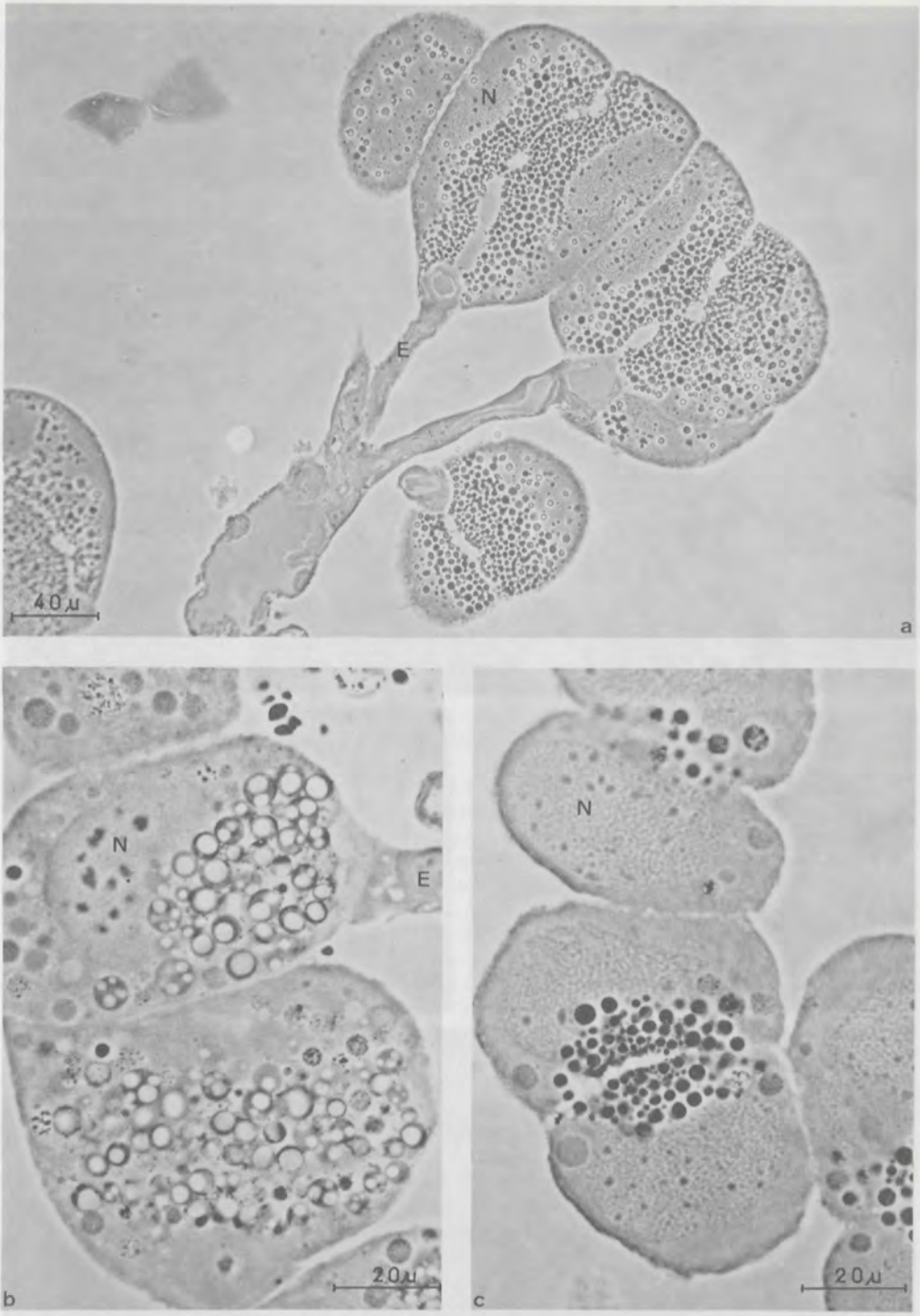


FIG. 8. Light microscope pictures of epon-embedded shell glands ; (E) excretory channel ; (N) nucleus. a) brown shell gland ; b) typical white shell gland whose secretory granules differ from those in the grown gland cells ; c) a typical while shell gland in which most of the secretory granules are similar to those in the brown gland cells.

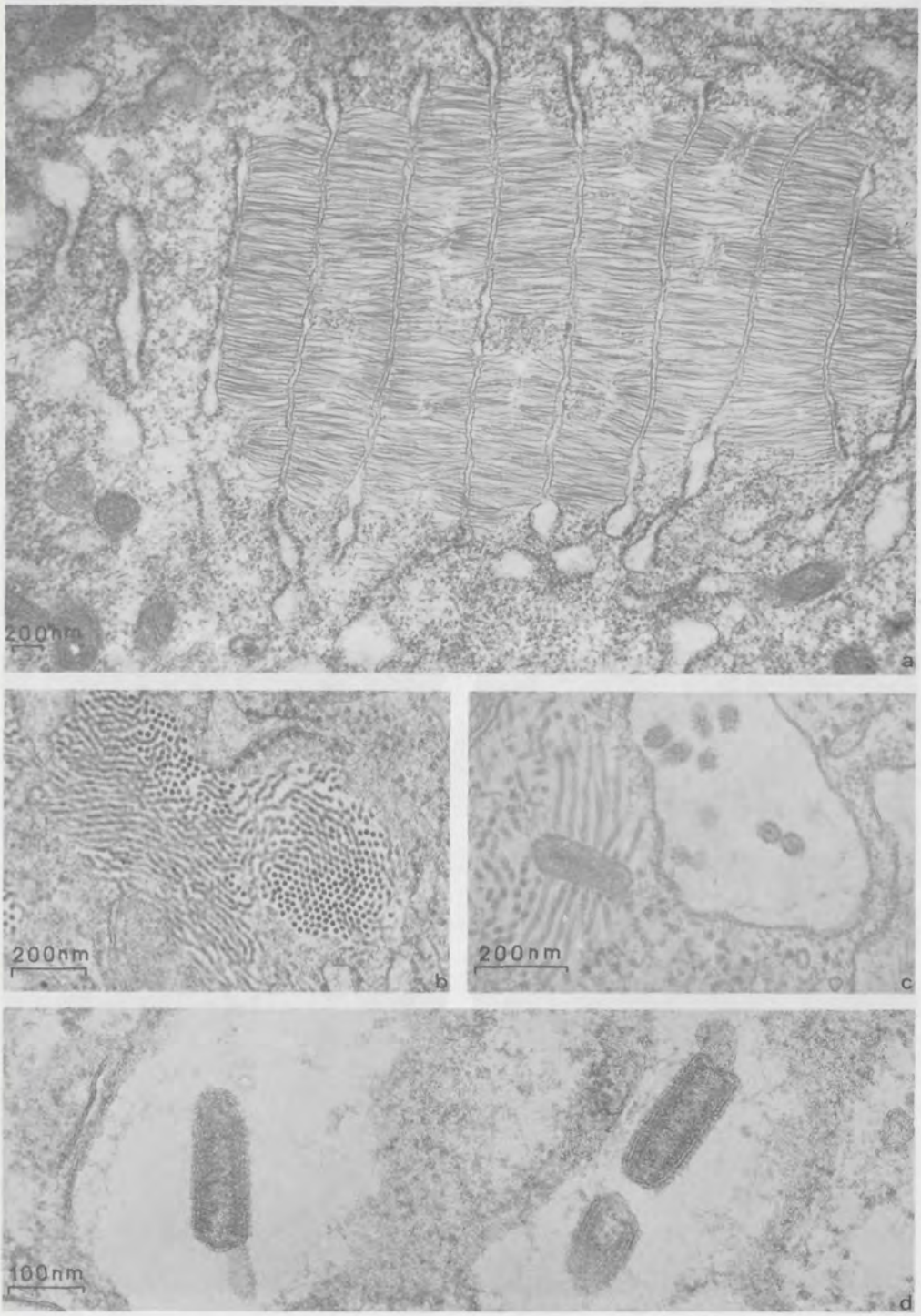


FIG. 9. Electron microscope pictures, tentatively outlining the development of virus like inclusions in the shell gland (a, b, c : in the cells ; d : in the basement membrane).

typical white glands, although less striking, lasted longer than that of the brown glands (De Maeyer-Criel, 1973) we thought that it might provide the developing nauplii with the substances needed for succesfull development in utero.

In the shell gland, the ovaries and the oviducts we regularly found ultrastructural alterations which we think are viral infections. They occur on average three times as much in animals with brown shell glands as in those with white glands. In a first stage fibrils replace the ribosomes on some of the rough surfaced endoplasmic reticulum cisternae (Fig. 9a). Later on clusters of these fibrils lie in the cytoplasm (Fig. 9b) and form viruslike rods (Fig. 9c) which eventually enter the secretion granules and which can also be found in the basement membrane (Fig. 9d).

## Acknowledgement

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## Literature cited

Anderson E., J. H. Lochead, M. S. Lochead, and E. Huebner, 1970. The origin and structure of the tertiary envelope in thick-shelled eggs of the brine shrimp *Artemia*. *J. Ultrastruct. Res.* 32:497-525.

Anteunis A. 1964. Les membranes annelées dans l'œuf d'*Artemia salina*. Etude au microscope électronique. *Bull. Ass. Anat.* 125 (XLIV[e] Réunion, Madrid): 168-173.

Anteunis A., N. Fautrez-Firlefyn, J. Fautrez, and A. Lagasse. 1964. l'Ultrastructure du noyau vitellin de l'œuf d'*Artemia salina*. *Exp. Cell Res.* 36:239-247.

Anteunis A., N. Fautrez-Firlefyn, and J. Fautrez. 1968. Ultrastructure du nucléole expulsé dans le cytoplasme de l'oocyte d'*Artemia salina*. *C.R. Acad. Sc. Paris* 266:1862-1863.

Anteunis A., N. Fautrez-Firlefyn, and J. Fautrez. 1966a. La structure des ponts intercellulaires "obturés" et "ouverts" entre les oogonies et les oocytes dans l'ovaire d'*Artemia salina*. *Arch. Biol.* 77:645-664.

Anteunis A., N. Fautrez-Firlefyn, and J. Fautrez. 1966b. L'incorporation de cellules nourricières par l'oocyte d'*Artemia salina*. Etude au microscope électronique. *Arch. Biol.* 77:665-676.

Anteunis A., N. Fautrez-Firlefyn, and J. Fautrez, 1966c. A propos d'un complexe tubulo mitochondrial ordonné dans la jeune oocyte d'*Artemia salina*. *J. Ultrastruct. Res.* 15:122-130.

Artom C. 1907. La maturazione, la fecondazione e i primi stadii di sviluppo dell'uovo dell'*Artemia salina*. *Lin. di Cacliari Bijologica – Raccolta di scritti di Biologia, Torino* 1:495-515.

Benesch R. 1969. Zur Ontogenie und Morphologie von *Artemia salina* L. *Zool. Jb. Anat.* 86:307-458.

Cassel J. D. 1937. The morphology of *Artemia salina*. M. A. Thesis, Stanford University, California. 108 p.

Claus C. 1886. Untersuchungen über die Organisation und Entwicklung von Branchipus und *Artemia* nebst vergleichenden Bemerkungen über andere Phyllopoden. *Arbeiten Zoologischen Institute Wien* 8:267-370.

Criel G. 1972. Elektronenmikroskopisch onderzoek van de schaalklier bij *Artemia salina* Leach. Thesis, State University of Gent. 220 p.

Criel G. 1977. An electron microscope study of the while and brown shell glands of *Artemia salina*. p. 47-48. *In*: Fundamental and applied research on the brine shrimp *Artemia salina* (L.) in Belgium. European Mariculture Society Special Publication No. 2. Jaspers E., and G. Persoone (Eds). European Mariculture Society, Bredene, Belgium. 110 p.

De Maeyer-Criel G. 1970a. Sur la présence de microfibrilles associées au reticulum endoplasmique des cellules des glandes coquillières chez *Artemia salina*. *Arch. Biol.* 81:61-82.

De Maeyer-Criel G. 1970b. Contribution de vésicules rugueuses à la formation des grains de sécrétion dans la glande coquillière d'*Artemia salina*. *Arch. Biol.* 81:492-494.

De Maeyer-Criel G. 1971. Localisation de la phosphatase acide au niveau des cloisons intercellulaires dans la glande coquillière d'*Artemia salina*. *Arch. Biol.* 82:163-165.

De Maeyer-Criel G. 1973. La glande coquillière non pigmentée d'*Artemia salina* Leach. *Z. Zellforsch. Mikrosk. Anat.* 144:299-308.

Dutrieu J. 1960. Observations biochimiques et physiologiques sur le développement d'*Artema salina* Leach. *Arch. Zool. exp. gén.* 99:1-134.

Fautrez J. and N. Fautrez-Firlefyn. 1961. Activation expérimentale des œufs d'une race amphigonique d'*Artemia salina*. *Arch. Biol.* 72:611-626.

Fautrez J. and N. Fautrez-Firlefyn. 1964. Sur la présence et la persistance d'un noyau vitellin atypique dans l'œuf d'*Artemia salina*. *Developm. Biol.* 9:81-91.

Fautrez J. and N. Fautrez-Firlefyn. 1971. Contribution à l'étude de la glande coquillière et des coques de l'œuf d'*Artemia salina*. *Arch. Biol.* 82:41-83.

Fautrez J. and N. Fautrez-Firlefyn. 1977. Ovogenesis and cleavage of the egg of *Artemia salina*. p. 9-14. *In* : Fundamental and applied research on the brine shrimp *Artemia salina* (L.) in Belgium. European Mariculture Society Special Publication No. 2. Jaspers E., and G. Persoone (Eds). European Mariculture Society, Bredene, Belgium. 110 p.

Fautrez-Firlefyn N. 1949. Etude cytochimique des acides nucléiques au cours de l'ovogénèse chez *Artemia salina*. 3e Belgisch-Nederlandse Cyto-Embryologische Dagen te Gent, 26-28 Mei. *Natuurwetenschappelijk Tijdschrift Gent* (Ed.). 127 p.

Fautrez-Firlefyn N. 1950. Expulsion d'acide thymonucléique hors du noyau de certaines cellules de l'ovaire d'*Artemia salina*. *C.R. Soc. Biol.* 144:1127-1128.

Fautrez-Firlefyn N. 1951. Etude cytochimique des acides nucléiques au cours de la gamétogénèse et des premiers stades du développement embryonnaire chez *Artemia salina*. *Arch. Biol.* 62:391-438.

Fautrez-Firlefyn N. 1957. Proteines, lipides et glucides dans l'œuf d'*Artemia salina*. *Arch. Biol.* 68:249-296.

Fautrez-Firlefyn N., J. Fautrez, and M. Th. Labis. 1963. L'activité phosphatasique acide dans l'œuf d'*Artemia salina*. *Biologisch Jaarboek Dodonaea* 31:301-312.

Gross F. 1936. Die Reifungs- und Furchungsteilungen von *Artemia salina*. Zusammenhang mit dem Problem des Kernteilungsmechanismus. *Z. Zellforsch.* 23:523-565.

Lison L. and N. Fautrez-Firlefyn. 1950. Desoxyribonucleic acid content of ovarian cells in *Artemia salina*. *Nature* 166:610-611.

Petrunkewitsch A. 1902. Die Reifung der parthenogenetischen Eier von *Artemia salina*. *Anat. Anz.* 21:256-265.

Roels F. 1970a. Localisation ultrastructurale du cytochrome c dans l'ovocyte d'*Artemia salina* par la 3,3'-diaminobenzidine c pH 9. *C.R. Acad. Sc.* 270:2322-2324.

Roels F. 1970b. Localisation d'activités peroxidasiques dans l'œuf d'*Artemia salina* à l'aide de 3,3'-diaminobenzidine et le pyrogallol. *Arch. Biol.* 81:229-274.

Roels F. and E. Wisse. 1973. Distinction cytochimique entre catalase et peroxidases. *C.R. Acad. Sc.* 276:391-393.

Von Siebold C. T. E. 1873. Ueber parthenogenesis der *Artemia salina*. *Sitzungsbericht des Mathematische naturwissenschaftliche Classe der k.b. Akademie der Wissenschaften zu München* 3:168-196.

*The Brine Shrimp* Artemia. *1980. Vol. 1. Morphology, Genetics, Radiobiology, Toxicology*
*G. Persoone, P. Sorgeloos, O. Roels, and E. Jaspers (Eds). Universa Press, Wetteren, Belgium. 345 p.*

# Ultrastructural observations on the oviduct of *Artemia*

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**Abstract**

A study of the oviduct during a complete ovulation cycle shows the existence of a secretory cycle. The results of electron microscopic investigation of the various phases of this cycle are described.

Two types of cells can be discerned. Cells containing small secretory granules are scattered among a majority of cells in which the absence of secretory granules is striking. In both types of cells expanded rough endoplasmic reticulum cisternae are filled with a flocculent material, and the Golgi apparatus is mostly bypassed in the elaboration of the secretion as it only produces a few tiny granules. In the cell apex the cisternae coalesce into a granular mass, whereafter the cell tops are pinched off in an apocrine-like secretion.

## Introduction

The oviducts of the brine shrimp are pouchlike structures lying underneath the lateral horns of the ovisac. After ovulation the eggs are temporarily stored in these oviducts which extend to form the so-called lateral pouches. From there the eggs are released into the uterus about one hour after copulation.

Despite the studies of Claus (1886) and Cassel (1937) the morphology of the oviduct has not been thoroughly unraveled and hitherto the nature of the secretion has not been elucidated. It is thought among others to have an influence on the first meiotic division (Gross, 1936 ; Fautrez and Fautrez-Firlefyn, 1961).

The present paper describes the oviductal cells and the changes they undergo during the reproductive cycle.

## Materials and methods

Mature female *Artemia* of the Californian strain were processed in different stages of the reproductive cycle either for light microscopy or for electron microscopy analysis.

For light microscopy, whole animals were fixed in an alcohol-formol-acetic acid mixture, dehydrated and embedded in paraffin. Serial, 4 to 5 $\mu$m thick, sections were stained with toluidine blue at pH 5.2 or with Masson's trichrome stain.

For electron microscopy the uteri were excised and fixed by immersion in 2.5% glutaraldehyde in cacodylate buffer at pH 7.2. An overnight rinsing in cold buffer was followed by a

postfixation in 1 % $OsO_4$ in the same buffer. The tissue was stained in block with uranyl-acetate (Farquhar and Palade, 1965) dehydrated and embedded in epon. Serial sections of approximatey 70 nm were taken up on formvar-coated single slot grids and stained with uranylacetate and lead citrate.

## Results

### Light microscopy

For details on the light microscopy see Fig. 7 of the other paper by Criel, presented at this Symposium (Criel, 1980).

The oviducts can be seen as oval or lozenge shaped tubes separated from the lateral horns of the ovisac by the hemocoel, except in the period of the so-called lateral pouches where the extreme stretching caused by the ovulated eggs, makes their structure hardly recognizable.

The secretory cells form a monolayer of flattened to cylindrical cells surrounded by a single layer of longitudinal muscle cells around which some circular muscle cells lie at regular interspace. The longitudinal layer is interrupted in places by the bases of the glandular cells.

The frontal part of the oviduct forms a shutter on which the uterus epithelium is attached on both sides. This shutter is partly fibrous, partly cellular ; some blood cells often seem to be caught between its elements. A lump of necrotic cells protrudes from the shutter into the uterus. When the oviduct is stretched by the descending eggs, after the ovulation, the shape of the shutter remains unchanged.

The glandular cells of the oviducts undergo cyclical changes related to the reproductive cycle. Immediately after the descent of the eggs into the uterus, the cells display a club-shaped appearance caused by their rounded cell tops while clear vesicles appear in the empty lumen. This image remains at least till the developing eggs are reaching the four celled stage but often subsists during the first day of embryonic development. Lateron a small rim of clear vesicles appears on the apical surfaces of the cells whereas in the lumen a fine granular secretion is seen. Finally the oviduct acquires the typical appearance which it has during the major part of the reproductive cycle : the cubical glandular cells with a basal nucleus show conspicious apical blebs protruding in an homogeneous opaque secretion.

### Electron microscopy

During the reproductive cycle the changes in the glandular cells are mainly restricted to the apical portions of the cell. The lateral and basal portions have features that remain constant in morphology.

The highly indented bases of the secretory cells are separated by a thin basement membrane (Fig. 2) from the edentations of the monolayer of longitudinal muscle cells (Fig. 1). A few cells pierce through this muscular layer and become sandglass-shaped.

The secretory cells are linked together by zonulae adhaerentes, gap and septate junctions. The nucleus, usually located in the basal third of the cells, varies in shape from ovoid to irregular. The cytoplasm is rich in free ribosomes. The extended vesicular elements of the granular endoplasmic reticulum containing a fine granular secretion are the most prominent feature of the cytoplasm. Short cisternal elements are only found in the basal portions of the cell. Golgi complexes are dispersed throughout the cell. Each stack is composed of four to five

saccules from which mainly clear vesicles are pinched off. However some small dense cored vesicles are formed in some of the secretory stages. The mitochondria are ovoid with irregular cristae and a coarse granular matrix. No mitochondrial granules can be seen. Many clear and moderately dense vacuoles containing irregular membranous material are found especially in the apical part of the cell. Recent cytochemical findings indicate the presence of acid phosphatases in these vacuoles.

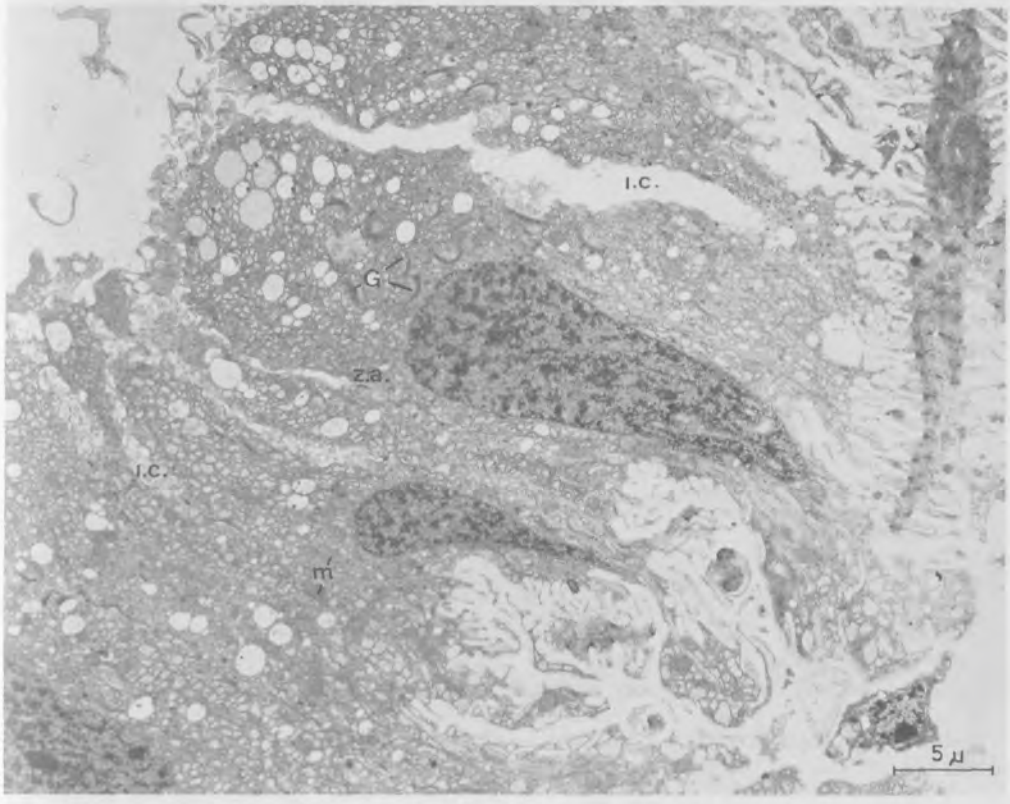


FIG. 1. Part of the oviduct shortly after the descent of the eggs. (G.) Golgi stack ; (m.) mitochondria ; (z.a) zonula adhaerens ; (i.c.) intercellular canaliculus.

Soon after the descent of the eggs into the uterus the irregularly digitated luminal surfaces of the club-shaped cells point into an apparently empty oviduct (Fig. 1). Intercellular canaliculi lined with villi extend the lumen to the basal third of the secretory cells. These channels in which some small dense cores vesicles originating from adjacent Golgi complexes are released, run an irregular course between the secretory cells and end on a zonula adhaerens (Fig. 2). The lateral surfaces not participating in the formation of these channels are interdigitated and separated by a uniform intercellular space, interrupted by occasional gap and

septate junctions. Irregular masses of heterochromatin are dispersed in the nucleus. The clear vacuoles are mainly located in supranuclear position. A few disseminated secretory cells are filled with small dense cored vesicles.

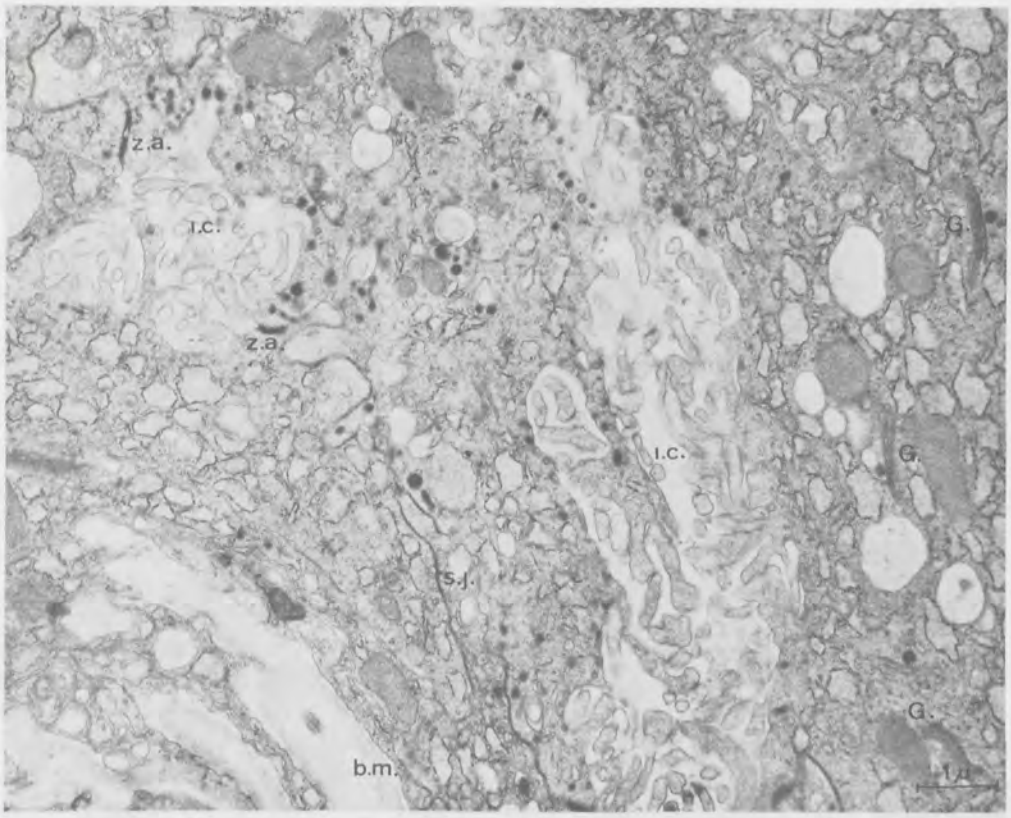


FIG. 2. Intercellular canaliculus (i.c.) wherein dense cored vesicles produced by nearby lying Golgi stacks (G.) are released ; (z.a.) zonula adhaerens ; (s.j.) septate junction ; (b.m.) basement membrane.

After a few hours the cells broaden (Fig. 3) ; the intercellular channels seem to spread open, villi appear on the luminal surface where the irregular digitations have disappeared. Consequently the dense cored vesicles are released at the cell tops. Cells full of dense cored vesicles are rarely found; Beneath the villi a small cytoplasmic rim without cell organelles is formed. Homogeneous dense patches appear in the fine granular secretion product which fills the lumen of the oviduct.

After the first day of uterine development (Fig. 4), the villi of the cell tops swell up and form ballooned apical projections which contain almost no organelles. The villi are confined to some shallow crypts which are sometimes found between adjacent cells. At this moment the density of the secretion equals the density of the cell tops. Apically several vesicles of the

granular reticulum fuse with each other and probably also with the clear vacuoles. Some dense cored vesicles are scattered throughout the cell. In the nucleus the heterochromatin gradually disperses.

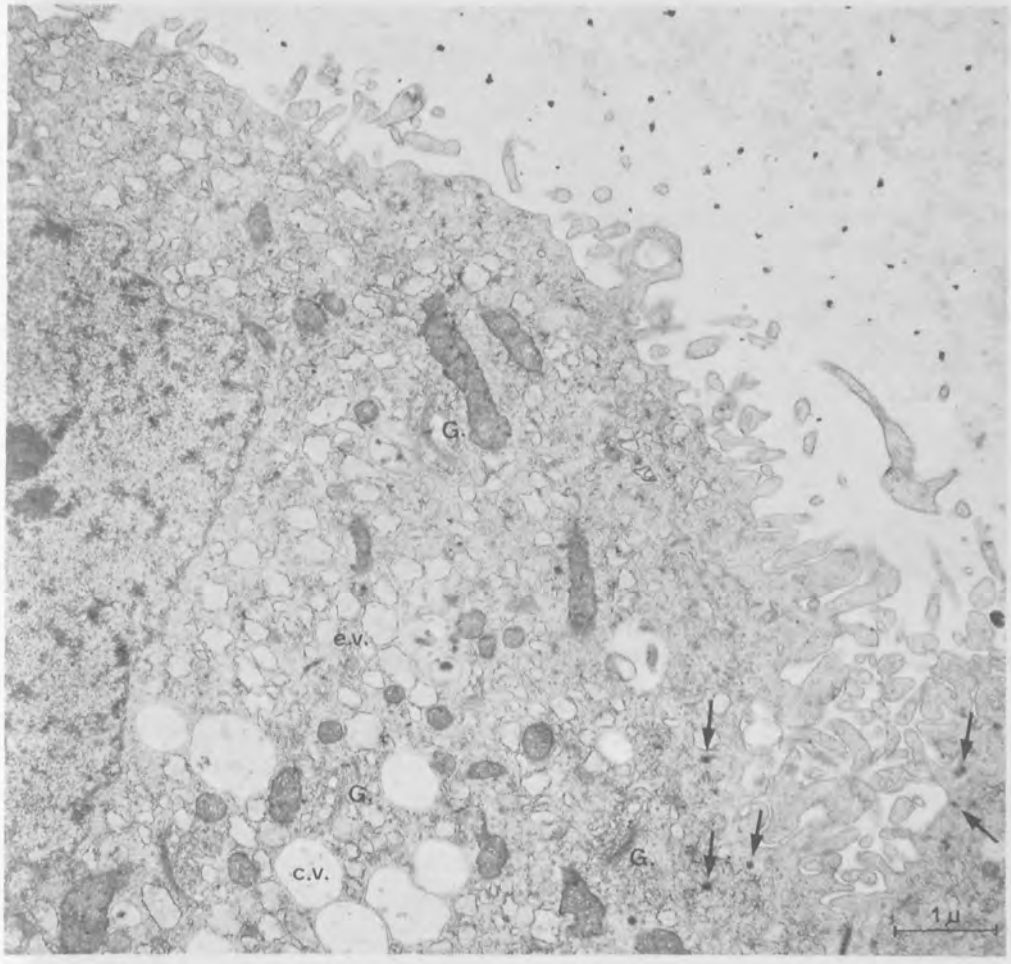


FIG. 3. Oviductal cells a few hours after the release of the eggs. At this magnification the swollen ergastoplasmic vesicles are clear (e.v.). Some dense cored visicles are excreted in the broadened intercellular canaliculus (arrow). (c.v.) clear vacuoles ; (G.) Golgi apparatus.

At a later stage of the secretion a constriction develops between the cell body and the apical projection. Flattened vesicles and dense cored vesicles formed by nearby Golgi stacks align at the base of the apical projection (Fig. 5). The fusion of these vesicles may cause the separation of the top from the intact body of the cell. In the lumen of the oviduct the pinched-off cell tops coalesce to a homogeneous dense opaque secretion.

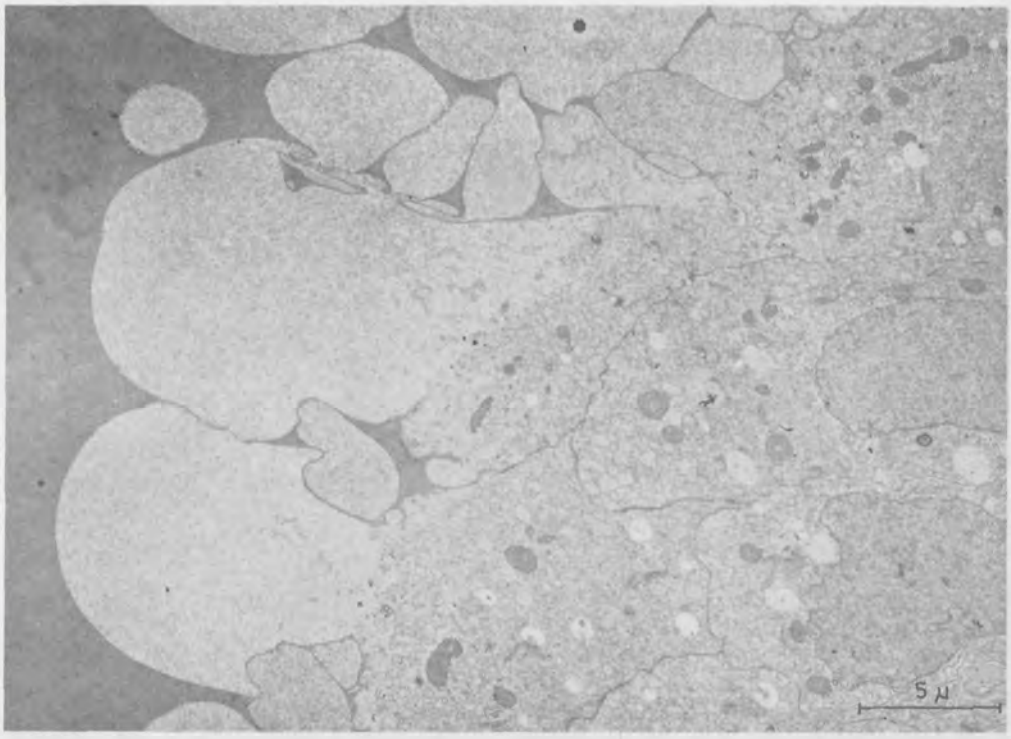


FIG. 4. Ballooned apical protrusions on oviductal cells 76 hr after the release of the eggs into the uterus. Note the demarcation line between the projection and the cell body.

From now on until the ovulation, the morphology apparently remains unchanged.

After the ovulation when the oviducts extend to form the lateral pouches the cells are flattened to a cubical form (Fig. 6). On the apical surfaces the ballooned apical projections are replaced by irregular digitations while on the lateral surfaces villous intercellular canaliculi end on extensive zonulae adhaerentes. No dense cored vesicles are found.

**Discussion**

Invertebrate oviducts are seldom described even in light microscopical studies.

A brief description of *Artemia* oviduct was given by Claus (1886) and by Cassel (1937). These authors report a cilindrical epithelium lying on a muscular layer, but do not mention any secretory activity in the lumen nor did they notice the shutter between the oviduct and the uterus.

The light microscopical appearance is reminiscent of an apocrine secretion although the aspect only does not represent a conclusive evidence. Electron microscopic studies on glands, generally accepted to be apocrine, have indeed shown that most of them are in fact merocrine. In so far even that some authors doubt whether apocrine secretion is not an artifact (Hibbs, 1962 ; Biempica and Montes, 1965 ; Munger, 1965ab ; Ellis, 1967). On the contrary some

authors accept apocrine secretion (Kurosomi *et al.*, 1961, Kneeland, 1966 ; Bell, 1974 ; Schaumberg-Lever and Lever, 1975 ; Smith and Hearn, 1979) and still others even distinguish different mechanisms of apocrine secretion (Hashimoto *et al.*, 1966 ; Riemann and Thorson, 1976). Munger (1965ab) ascribes the apical blebbing causing a false impression of apocrine secretion in some apocrine sweet glands to the absence of a terminal web. Although in the oviduct a terminal web is not found we consider that in this case the strictures on the border between the apical projections and the cell body and the concentrated flattened vesicles in this region are indicative for a factual apocrine secretion. A similar mechanism of apocrine secretion has been described in the rabbit submandibular gland by Kurosomi *et al.* (1961), in the human apocrine sweet gland by Schaumberg-Lever and Lever (1975) and in the anal scent gland of the wood chuck by Smith and Hearn (1979).

The microvilli fringed intercellular canaliculi and the merocrine excretion of dense cored granules in these canaliculi are other features which the glandular cells of the oviduct share with other apocrine glands (Kneeland, 1966) and with the human submaxillary gland (Tandler, 1962). Presumably the study of other apocrine gland cells will make it possible to define some general characteristics of apocrine cells.

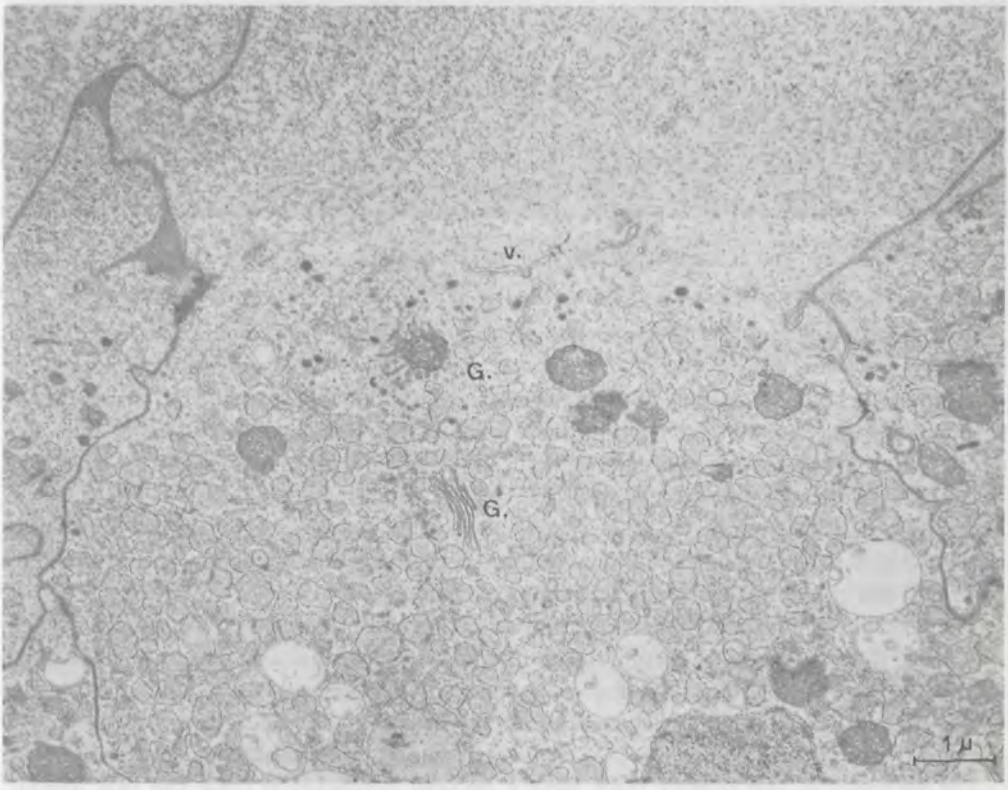


FIG. 5. Higher magnification of the demarcation line between the apical protrusion and the cell body. (G.) Golgi apparatus ; (v.) flattened vesicles.

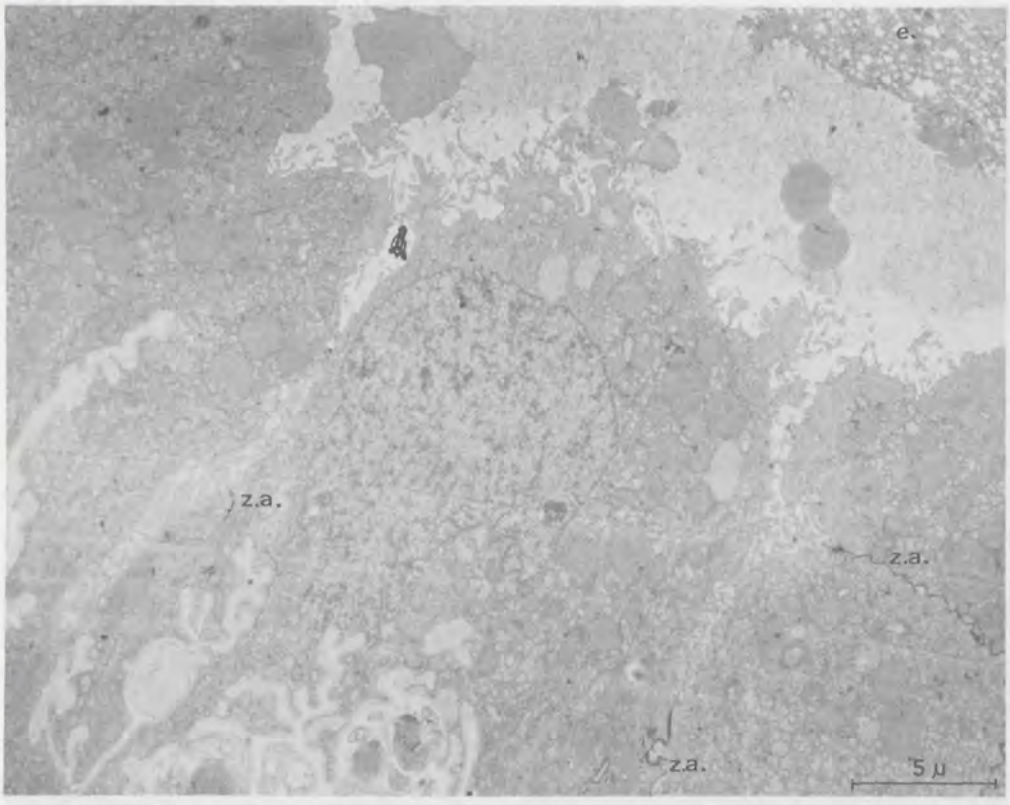


FIG. 6. Oviductal cell of a lateral pouch. (z.a.) zonula adhaerens ; (e.) egg in the lateral pouch.

In contrast with most above-mentioned apocrine gland cells, the oviduct has no definite secretory granules but is filled with vesicles of rough surfaces endoplasmic reticulum containing a granular secretion. The fact that these vesicles enlarge gradually while moving to the apex and never show any relationship with the Golgi apparatus suggests that they are directly related to the secretion process as in the case of the mouse prostate gland (Brandes and Portela, 1960), the albumen-secreting cells of the hen oviduct (Hendler *et al.*, 1957) and the rat mammary gland (Murad, 1970). In the oviduct the function of the Golgi apparatus thus seems to be restricted to the elaboration of the small dense cored vesicles released in the intercellular canaliculi and probably also to the formation of the clear vacuoles.

The proteinaceous secretion of the endoplasmic reticulum vesicles may be modified when they loose their surrounding membranes in the apical protrusions and are possibly blended with the clear vacuoles. This modification continues in the lumen of the oviduct as is shown by its gradual densification and opacification. What part the dense cored vesicles, that are released by a merocrine secretion, play in this still has to be elucidated.

The secretion of the oviduct must have a peculiar composition since the first meiotic division of the eggs stops at metaphasis on entering the oviduct, ascribed to its physiological condition by Gross (1936), and proceeds whenever the eggs are released from the oviducts

even without fertilization (Fautrez and Fautrez-Firlefyn, 1961). The formation of the vitelline membrane also starts at the release from the oviducts even in virgin females (personal unpublished observations). As the scantinesss of the secretion probably excludes the determination of its components by biochemical means, further research with cytochemical methods is necessary to estimate the nature and function of the diverse secretions.

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**Literature cited**

Bell M. 1974. The ultrastructure of human axillary apocrine glands after epinephrine injection. *J. Invest. Derm.* 63:149-159.

Biempica L. and L. F. Montes. 1965. The secretory epithelium of the large axillary sweat glands. A cytochemical and electron microscopic study. *Amer. J. Anat.* 117:47-72.

Brandes D. and A. Portela. 1960. The fine structure of the epithelial cells of the mouse prostate. II. The ventral lobe epithelium. *J. Biophys. Biochem. Cytol.* 7:511-514.

Cassel J. D. 1937. The morphology of *Artemia salina*. M.A. Thesis, Stanford University, California. 108 p.

Claus C. 1886. Untersuchungen über die Organisation und Entwicklung von *Branchipus* und *Artemia* nebst vergleichenden Bemerkungen über andere Phyllopoden. *Arbeiten Zoologischen Institute Wien* 8:267-370.

Criel G. 1980. Morphology of the female genital apparatus of *Artemia* : a review. p. 75-86. *In* : The brine shrimp *Artemia*. Vol. 1. Morphology, Genetics, Radiobiology, Toxicology. Persoone G., P. Sorgeloos, O. Roels, and E. Jaspers (Eds). Universa Press, Wetteren, Belgium. 345 p.

Ellis R. A. 1967. Ecrine sebaceous and apocrine glands. p. 132-162. *In* : Ultrastructure of the normal and abnormal skin. A. S. Zelickson (Ed.). Lea and Febiger. Philadelphia. 431 p.

Farquhar M. and G. Palade. 1965. Cell junctions in the amphibian skin. *J. Cell Biol.* 26:263-291.

Fautrez J. and N. Fautrez-Firlefyn. 1961. Ativation expérimentale des œufs d'une race amphigonique d'*Artemia salina*. *Arch. Biol.* 72:611-626.

Gross F. 1936. Die Reifungs- und Furchungsteilungen von *Artemia salina* im Zusammenhang mit dem Problem des Kernteilungsmechanismus. *Z. Zellforsch.* 23:523-565.

Hashimoto K., B. G. Gross, and W. F. Lever. 1966. Electron microscopic study of apocrine secretion. *J. Invest. Derm.* 46:378-390.

Hendler R. W., A. J. Dalton, and G. G. Glenner. 1957. Cytological study of the albumen-secreting cells of the hen oviduct. *J. Biophys. Biochem. Cytol.* 3:325-330.

Hibbs R. G. 1962. Electron microscopy of human apocrine sweat glands. *J. Invest. Derm.* 38:77-84.

Kneeland J. E. 1966. Fine structure of the sweat glands of the antebrachial organ of *Lemur catta*. *Z. Zellforsch.* 73:521-533.

Kurosomi K., M. Yamagashi, and M. Sekine. 1961. Mitochondrial deformation and apocrine secretory mechanism in the rabbit submandibular organ as revealed by electron microscopy. *Z. Zellforsch.* 55:297-312.

Munger B. L. 1965a. The cytology of apocrine sweat glands. I. Cat and monkey. *Z. Zellforsch* 67:373-389.

Munger B. L. 1965b. The cytology of apocrine sweat glands. II. Human. *Z. Zellforsch.* 68:837-851.

Murad T. H. 1970. Ultrastructural study of rat mammary gland during pregnancy. *Anat. Rec.* 167:17-36.

Riemann J. G. and G. J. Thorson. 1976. Ultrastructure of the ductus ejaculatorius duplex of the mediterranean flour moth *Anagasta kühniella* (Zeller) (Lepidoptera : Pyralidae). *Int. J. Insect. Morphol. Embryol.* 5:227-240.

Schaumberg-Lever G. and W. F. Lever. 1975. Secretion from human apocrine glands : an electron microscopic study. *J. Invest. Derm.* 64:38-41.

Smith J. D. and G. W. Hearn. 1979. Ultrastructure of the apocrine-sebaceous anal scent gland of the woodchuck *Marmotta monax* : evidence in apocrine and merocrine secretion by a single cell type. *Anat. Rec.* 193:269-292.

Tandler B. 1962. Ultrastructure of the human submaxillary gland. I. Architecture and histological relationship of the secretory cells. *Amer. J. Anat.* 111:287-308.

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*G. Persoone, P. Sorgeloos, O. Roels, and E. Jaspers (Eds). Universa Press, Wetteren, Belgium. 345 p.*

# Structure and ultrastructure of the egg shell of *Artemia*

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**Abstract**

The present study deals with the analysis of the structure and ultrastructure of the egg shell of *Artemia*.

The shell is built up of two major regions : an outer chorion and an inner embryonic cuticle, with inbetween a third layer, the outer cuticular membrane. The chorion (6-8 $\mu$m thick) is differentiated into two parts, a peripheral layer (consisting of an outer membrane and a cortical layer) and an alveolar layer. The outer cuticular membrane (0.5 $\mu$m thick) is a triple-layered biological membrane. The embryonic cuticle is approximately 1.8-2.2 $\mu$m thick and can be devided into an upper fibrous layer and inner cuticular membrane.

The findings of the present study are similar to those reported by Morris and Afzelius (1967). We observed, however, that the outer membrane is prominent and structurally different from the cortical layer.

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# Scanning electron microscopy of cuticular sensilla of *Artemia* : setae of the adult trunk segments

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**Abstract**

Scanning electron microscopy was used to map the distribution of segmentally arranged sensory setae found on the trunk of adult brine shrimp. Such setae are very simple in external morphology. Each possesses a gradually tapered shaft (27-52 $\mu$m long) that appears to lack pores, and near the base of each shaft there is a circlet of three to seven distinctive protuberances. On the dorsal surface of the trunk there is one pair of setae per segment, *i.e.* 19 pairs in all. Similar setae also occur on the ventral surface of the abdominal region (trunk segments 14 to 19), but the number of setae per segment is more variable here. The variability that exists within the ventral setal series is characterized, and possible functions of the trunk setae are discussed.

## Introduction

Segmentally arranged setae, which are probably sensory in function, have long been known to occur on the abdomen of adult brine shrimp (Packard, 1883). Indeed, the presence or absence of such setae was thought at one time to be a useful taxonomic character to distinguish species within the genus *Artemia*. Kuenen (1939) reported that such setae were absent from California shrimp, but present on the abdominal segments of European animals. Later investigators, however, found similar setae to be present on California brine shrimp as well (Lochhead, 1941 ; Gilchrist, 1960).

During the course of scanning electron microscopical studies of certain cephalic sense organs (Tyson and Sullivan, 1979, 1980), incidental observations were made of body segments posterior to the head, and it was found that the segmental setae of the trunk are not in fact confined to the abdominal region but also occur on the genital and limb-bearing segments. The present investigation aims to describe the external morphology of such setae, as well as to map their distribution on the trunk.

## Materials and methods

Live brine shrimp, *Artemia*, were obtained from a commercial supplier (Living World, San Francisco Bay Brand, Newark, California). Two different fixation procedures were used to preserve adult males and females for study by scanning electron microscopy. With one

method, whole shrimp were fixed initially in a mixture of 4% acrolein and 2% glutaraldehyde in 0.1 M sodium cacodylate for 90 min at room temperature and then stored for months in cold 2% glutaraldehyde in 0.1 M sodium cacodylate. With the second method, whole animals were fixed in 10% formalin in 0.1 M sodium cacodylate for two days at room temperature and stored for months in 5% formalin in 0.05 M sodium cacodylate. Prior to osmication, specimens prepared by both methods were rinsed in buffer or buffered sucrose and then dissected in the same solution. In the latter step each animal was decapitated and parts of the swimming appendages were cut off, in order to facilitate entry of subsequent processing solutions. Dissected specimens were post-fixed in 2% osmium tetroxide in 0.1 M sodium cacodylate for 2 to 3 hr at room temperature. In some instances, further osmication was carried out by means of a thiocarbohydrazide procedure (for details, see Tyson and Sullivan, 1979). All specimens were dehydrated in ethanol, critical-point dried in carbon dioxide, and mounted on aluminum stubs with either the dorsal or ventral body surface visible. Mounted specimens were coated with gold in a sputtering device (Technics, Inc.) and examined in a Hitachi HHS-2R scanning electron microscope. In order to map the position of setae on trunk segments, photographs (150×) were assembled into montages that showed either the dorsal surface of the complete trunk or the ventral surface of the abdomen. In all, 10 montages of dorsal views (eight females, two males) and 24 montages of ventral views (13 females, 11 males) were made. Since the ovisac or penes often obscured setae situated on the first abdominal segment, photographs were first taken with these reproductive organs in place, and then these structures were removed with forceps. The specimens were recoated with gold and the first abdominal segment examined again.

## Results

Because of variations in terminology in the published literature, it is necessary to explain the terms to be used. In the present paper, the body of a brine shrimp is considered to consist of two main parts, *i.e.* a head and a trunk. The trunk consists of 19 segments : 11 segments that bear swimming appendages, two genital segments, and six limbless segments. The latter six segments are also called the abdomen.

The external morphology of the trunk setae, as seen in scanning electron micrographs (Fig. 1, 2, and 3), is quite simple. Each seta possesses a shaft that is widest at the base and gradually tapers to a slender, rounded tip (Fig. 2). The surface of the shaft exhibits a crinkled texture except near the tip, where the cuticle usually appears to be smooth, and near the base, where there are circularly oriented ridges (Fig. 3). No pores capable of being resolved by scanning electron microscopy are visible. The length of the shaft was measured on 33 setae and was found to vary from 27 $\mu$m to 52 $\mu$m. Around the base of each seta there was always a circlet of distinctive protuberances that varied in number from three to seven (Fig. 1 and 3). The presence of this group of protuberances made it feasible to identify the position of a seta, even when the shaft had broken off during processing.

The distribution of setae on the dorsal surface of the trunk was similar in all ten animals examined. As shown in Fig. 4 and 5 the dorsal series consists of 19 pairs of setae, *i.e.* one pair per trunk segment. Although some minor variability was noted from one animal to another with respect to the relative distances between members of setal pairs, the most commonly seen arrangement is shown in Fig. 5.

On the last six segments of the trunk a ventral series of setae is also present (Fig. 6). In marked contrast to the dorsal series, the number of ventral setae per abdominal segment differed from one individual to another. The basic pattern that emerged from the examination of 24 animals is that either one or two pairs of ventral setae may be present on any of the first five abdominal segments (trunk segments 14-18), while a single ventral pair is typical for the final body segment (trunk segment 19). However, the variability encountered is further increased by the fact that the number of setae on the left side of a segment may not be the same as on the right and, more rarely, setae may be entirely absent from one side or the other of a particular segment. To illustrate this variability, ten of the setal patterns observed are listed in Table I.

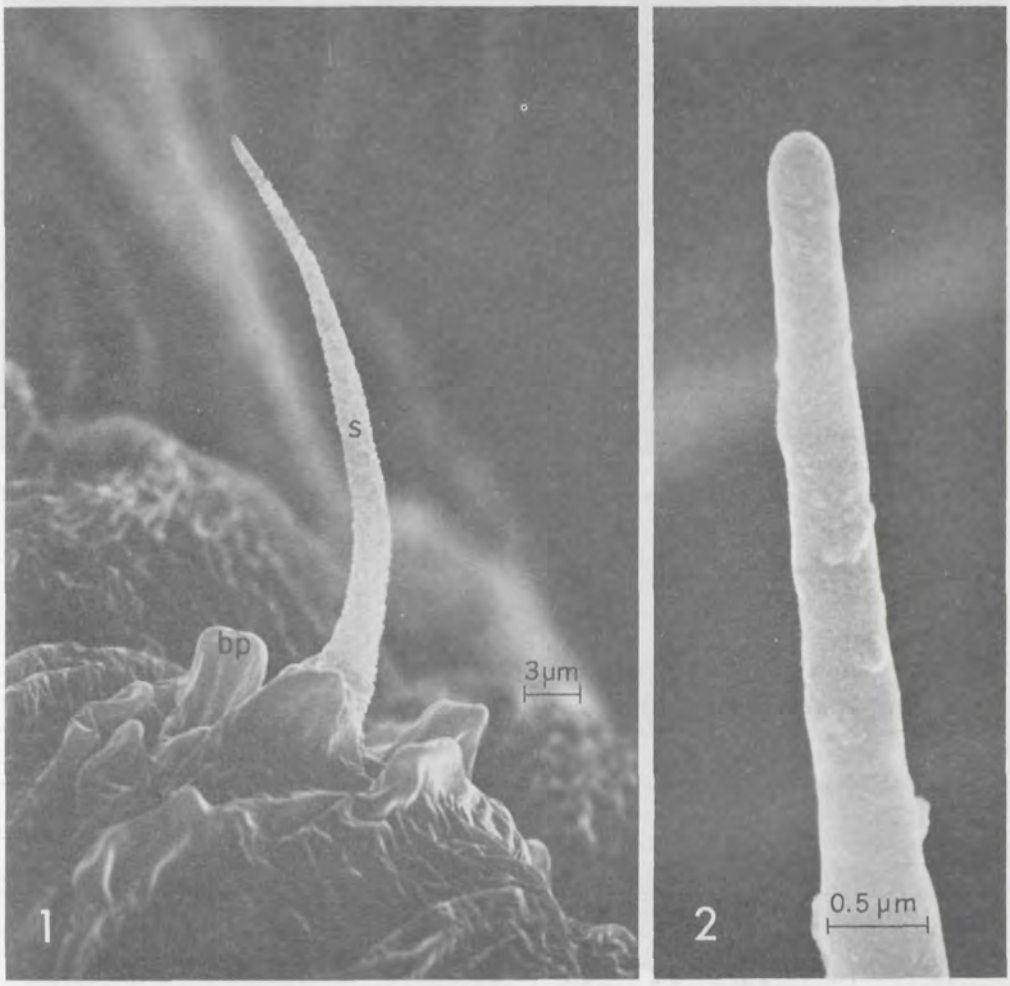



FIG. 1. Scanning electron micrograph showing profile view of trunk seta. Note shaft (s) and circlet of basal protuberances (bp).

FIG. 2. High magnification micrograph of tip of trunk seta. Note apparent absence of pore(s).

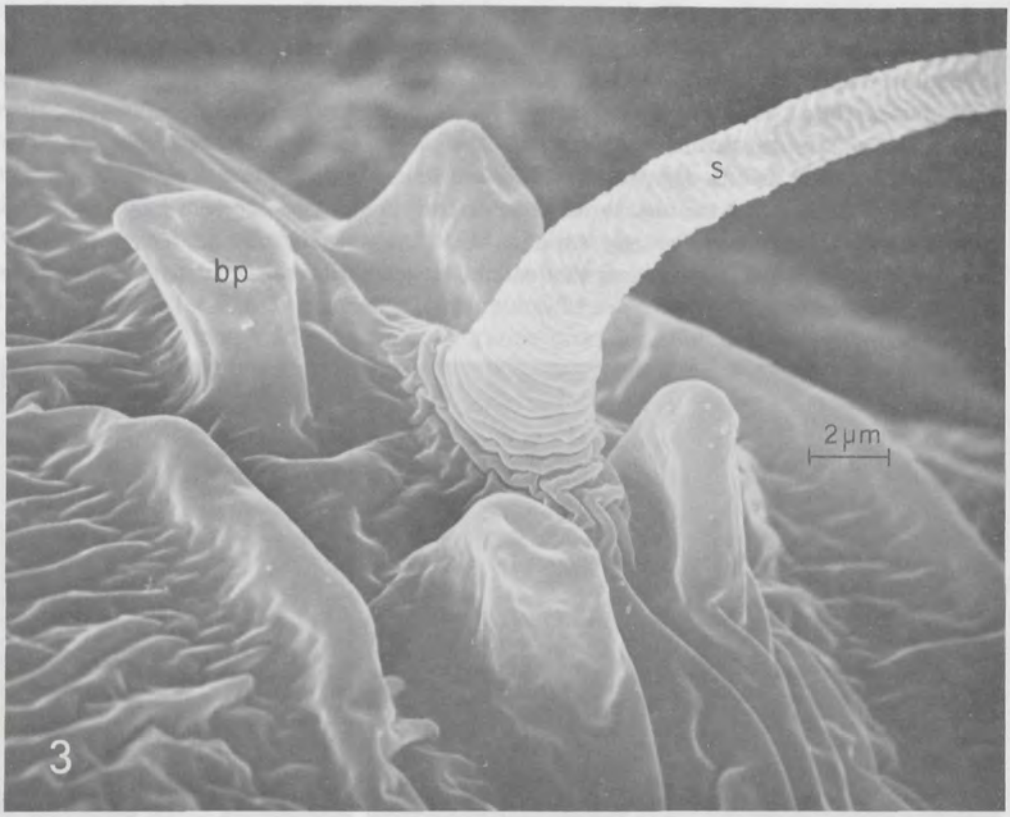



FIG. 3. Base of trunk seta, showing circlet of basal protuberances (bp) and texture of cuticle of shaft (s).

TABLE I

Examples of variability within the ventral series of abdominal setae.
(Number of setae on the right (R) and left (L) sides of abdominal segments of ten animals studied)

| Trunk segment | Animals | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
| | R L | R L | R L | R L | R L | R L | R L | R L | R L | R L |
| 14 | 1-1 | 1-0 | 2-2 | 1-1 | 1-1 | 1-1 | 1-1 | 1-1 | 1-1 | 1-1 |
| 15 | 1-1 | 1-1 | 2-2 | 1-1 | 2-2 | 1-1 | 1-1 | 1-1 | 1-1 | 1-1 |
| 16 | 1-1 | 1-1 | 2-2 | 2-1 | 2-2 | 1-2 | 1-1 | 2-2 | 1-1 | 1-2 |
| 17 | 1-1 | 1-1 | 2-1 | 2-2 | 2-2 | 1-1 | 1-1 | 1-1 | 1-2 | 2-2 |
| 18 | 1-1 | 1-1 | 2-2 | 2-2 | 2-2 | 1-2 | 2-1 | 1-1 | 1-1 | 1-2 |
| 19 | 1-1 | 1-1 | 1-1 | 1-1 | 1-1 | 1-1 | 1-1 | 1-1 | 1-1 | 1-1 |

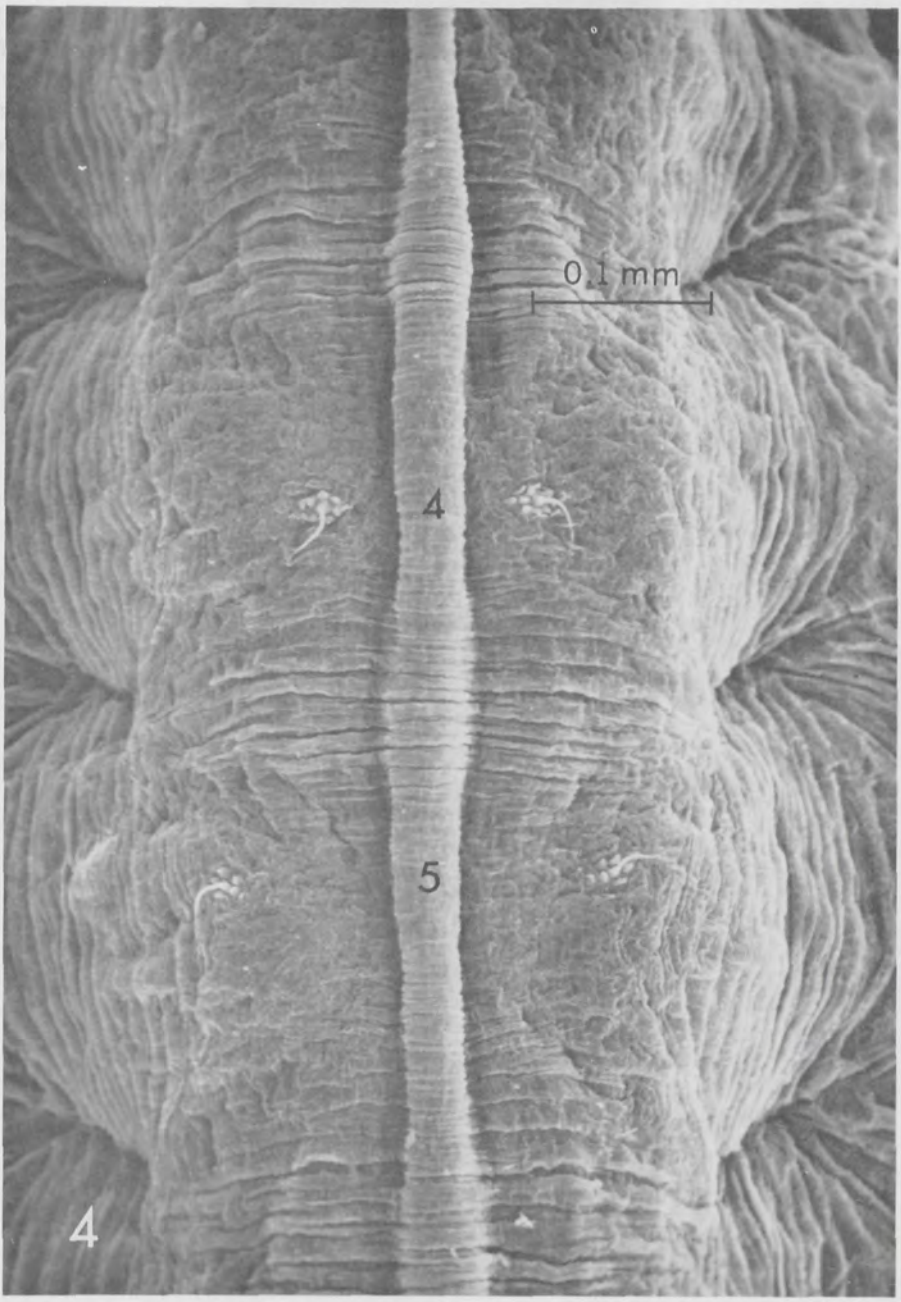
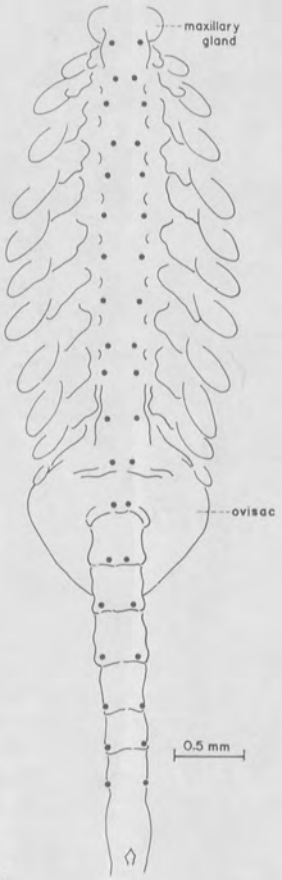



FIG. 4. Dorsal surface of trunk, showing position of setae of trunk segments 4 and 5. Compare to Fig. 5.

## Discussion

The segmental setae of the trunk are most likely sense organs, but the external morphological features observed provide no helpful clues about the nature of effective stimuli. In this regard, it could be argued that the apparent absence of pores suggests the setae are not chemosensory. In fact, the setae may possess pores through which appropriate chemical substances enter, but the openings may be too small to be resolved by means of the techniques used in the present study. The examination of ultrathin sections by transmission electron microscopy could provide a more reliable answer to the question of whether or not pores exist.




5

FIG. 5. Diagram of dorsal surface of trunk illustrating position of individual dorsal setae (black dots). In this diagram only basal parts of the swimming appendages are shown. For orientation, the last head segment (second maxillary) is included in the illustration.

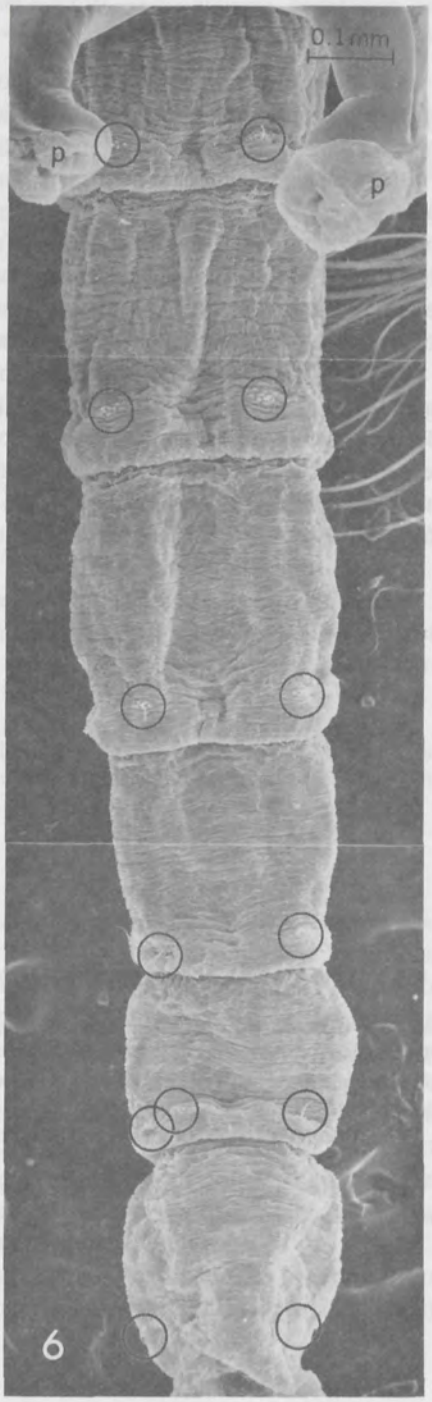



FIG. 6. Montage showing position of individual ventral setae (circled) on the abdomen of one of the animals studied. In this individual the ventral series consists of one pair of setae per segment, except for segment 18, which possesses two setae on the right side and one on the left. p, penes.

The possibility that the trunk setae may function as mechanoreceptors should also be mentioned. Their wide distribution on the dorsal and ventral surfaces of the trunk could be suited to the detection of water currents and/or the reception of tactile stimuli. With respect to possible tactile stimulation, it may be significant that adult *Artemia* normally swim with the dorsal surface down (Lochhead, 1936). With this upside down orientation, many of the dorsal trunk setae are in a position to be stimulated, should the animal's back graze or strike the bottom of the pool in which it swims. All of the preceding suggestions about possible functions of the trunk setae are, of course, entirely speculative. Elucidation of the true functional significance of these organs awaits future studies of their ultrastructure and physiology.

The abdominal setae of the trunk were called "lateral hairs" or "lateral setae" by some previous investigators (Kuenen, 1939 ; Lochhead, 1941 ; Gilchrist, 1960). Indeed, some of the setae of both the dorsal and ventral series are relatively lateral in position and as a result may be seen in profile view when one looks squarely (or nearly so) at either the dorsal or ventral body surfaces. However, the term "lateral" has been avoided in the present paper because the overall pattern of setal distribution is more consistent with the interpretation that there are basically two (dorsal and ventral) series of segmental sensory setae on the trunk. The relationships among trunk setae with respect to serial homologies will no doubt be better understood once information is available about the innervation of the setae and their anatomical connections to the central nervous system.

**Acknowledgement**

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**Literature cited**

Gilchrist B. M. 1960. Growth and form of the brine shrimp *Artemia salina* (L.). *Proc. zool. Soc. London* 134: 221-235.

Kuenen D. J. 1939. Systematical and physiological notes on the brine shrimp, *Artemia*. *Arch. néerl. Zool. (Leiden)* 3:365-449.

Lochhead J. H. 1936. Body orientation of the lower crustacea (Branchiopoda). *Nature (London)* 138:(3484):232-233.

Lochhead J. H. 1941. *Artemia*, the "brine shrimp". *Turtox News* 19:41-44.

Packard A. S. 1883. A monograph of the phyllopod Crustacea of North America, with remarks on the order Phyllocarida. p. 295-593. *In* : 12th Ann. Rept. US Geol. Survey.

Tyson G. E. and M. L. Sullivan. 1979. Antennular sensilla of the brine shrimp, *Artemia salina*. *Biol. Bull. (Woods Hole)* 156:382-392.

Tyson G. E. and M. L. Sullivan. 1980. Scanning electron microscopy of the frontal knobs of the male brine shrimp, *Artemia salina* (L.). *Trans. Amer. Micros. Soc.* 99:167-172.

*The Brine Shrimp* Artemia. *1980. Vol. 1. Morphology, Genetics, Radiobiology, Toxicology*
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# International Study on *Artemia*[1] III. The use of Coulter Counter® equipment for the biometrical analysis of *Artemia* cysts. Methodology and mathematics

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**Abstract**

Geographical strains of the brine shrimp, *Artemia*, can be characterized by the diameter of their cysts, their cyst volume and chorion thickness. This paper describes the routine procedure that has been worked out for an accurate size-analysis of *Artemia* cyst batches using Coulter Counter® equipment.

## Introduction

Within the framework of the international and interdisciplinary study on various geographical strains of the brine shrimp, *Artemia*, the Artemia Reference Center contributes to the overall characterization of over 50 different geographical strains (Sorgeloos *et al.*, 1976); among other studies this task includes a detailed quantitative analysis of the size of the cysts, as characterized by their diameter, volume and chorion thickness. From preliminary studies performed in our laboratory (Claus *et al.*, 1977) it appeared indeed that there are considerable size differences from one geographical strain to another. A statistical analysis of the data gathered is, however, necessary in order to check the size constancy within batches from the same geographical origin and the size differences between genotypically or phenotypically identical or different strains.

For this purpose we have worked out a routine procedure to measure processed *Artemia* cysts using Coulter Counter® equipment and to statistically analyse the data obtained.

[1] International interdisciplinary study on *Artemia* strains coordinated by the *Artemia* Reference Center, State University of Ghent, Belgium.

[4] "Bevoegdverklaard Navorser" at the Belgian National Foundation for Scientific Research (NFWO).

**Preparation of the cysts**

Since most of the cyst-batches, which we have received from all over the world, contain a certain amount of debris (sand, empty shells, etc.), the samples to be analyzed (1-2 g per strain) are subjected to the washing and cleaning procedure as described by Sorgeloos *et al.* (1978). The remaining full cysts are hydrated for 2 hr at 30 °C in cylindrical-conical tubes containing 100 ml electrolyte solution filtered on a 0.2 $\mu$m filter. This solution consists of natural seawater diluted with distilled water to a salinity of 10 ‰, and 1 % of lugol's solution. The addition of lugol's solution inhibits the metabolic activity within the cyst embryo so that only a physical swelling occurs. During the 2 hr hydration period, the cysts are kept in suspension by a continuous aeration from the bottom of the tube. One ml of lugol's solution is then added and the cyst suspension is poured into a 250 ml container, stoppered, and stored at room temperature in darkness, in order to maintain the activity of the lugol.

Microscopic measurements revealed that for an at random sample of four strains, the maximum hydration volume is reached within 2 hr of incubation. Nevertheless the cysts are kept for a total of 24 hr in the hydration medium since D'Agostino (1965) and Collins (personal communication) reported that for some strains from the USA more than 2 hr are needed for full hydration.

Previous work in our laboratory had already shown that neither the addition of the lugol's solution – which allows to store the cysts for a period of several weeks – nor the storage temperature have a significant influence on the final volume of the cysts.

For size analysis on decapsulated cysts 1-2 cyst material is processed according to the procedure described by Bruggeman *et al.* (1979). The decapsulation treatment is followed under the microscope to verify that no parts of the chorion are left. The broken cysts and the light debris are removed according to the flotation procedure outlined by Sorgeloos *et al.* (1978). The remaining cyst-product is then resuspended in a brine solution in a cylindrical-conical tube. After 5 min the heavy debris has settled and is siphoned off. The intact embryos are finally hydrated and prepared for size analysis following the hydration procedure described above.

**Size-analysis with Coulter Counter® equipment**

The measurements are performed with a counter ZB equipped with a channelyzer C-1000 and a P64 X-Y recorder.

One hour before the measurements the cysts are filtered off on a 110 $\mu$m screen. The lugol is washed out with electrolyte solution and the cysts resuspended in 50 ml electrolyte solution. Subsamples of 4 ml are taken and transferred to the measuring beaker.

The operational settings on the Coulter Counter® are as follows :

- tube orifice : 560 $\mu$m
- 1/aperture current : 4
- 1/amplification : 16
- base channel threshold setting : 10
- window width setting : 100
- count range : 400

- count control switch : stop at full scale
- edit : off

Since it appeared that the reference channel number for a specific volume-range is affected by varying vacuum pressures in the ZB measuring unit, the vacuum pressure is kept constant at $20 \times 10^3$ Pa. Prior to start a series of measurements, the calibration for channel-size analysis has to be performed following the procedure outlined in the Instruction Manual for the Coulter Channelyzer C-1000, (Coulter Electronics Ltd, 1973).

**Data analysis**

The numerical data obtained with the Coulter Counter® provide a frequency distribution (Table I).

TABLE I

Frequency distribution of the numerical data obtained for *Artemia* cysts from Adelaide (Australia)

| Channel no. | Frequency | Channel no. | Frequency |
|---|---|---|---|
| 4 | 2 | 27 | 348 |
| 5 | 0 | 28 | 273 |
| 6 | 0 | 29 | 228 |
| 7 | 0 | 30 | 207 |
| 8 | 1 | 31 | 118 |
| 9 | 2 | 32 | 121 |
| 10 | 2 | 33 | 89 |
| 11 | 2 | 34 | 56 |
| 12 | 0 | 35 | 49 |
| 13 | 9 | 36 | 20 |
| 14 | 15 | 37 | 35 |
| 15 | 20 | 38 | 14 |
| 16 | 40 | 39 | 11 |
| 17 | 52 | 40 | 10 |
| 18 | 90 | 41 | 9 |
| 19 | 146 | 42 | 6 |
| 20 | 189 | 43 | 4 |
| 21 | 253 | 44 | 2 |
| 22 | 337 | 45 | 1 |
| 23 | 386 | 46 | 13 |
| 24 | 399 | 47 | 6 |
| 25 | 397 | 48 | 2 |
| 26 | 351 | 49 | 3 |

From these data and the total number of cysts analysed – given by the C-1000 integrator – the mean, variance, and standard deviation of the distribution can be calculated.

The distribution can also be represented graphically by an X-Y plot on the recorder (Fig. 1).

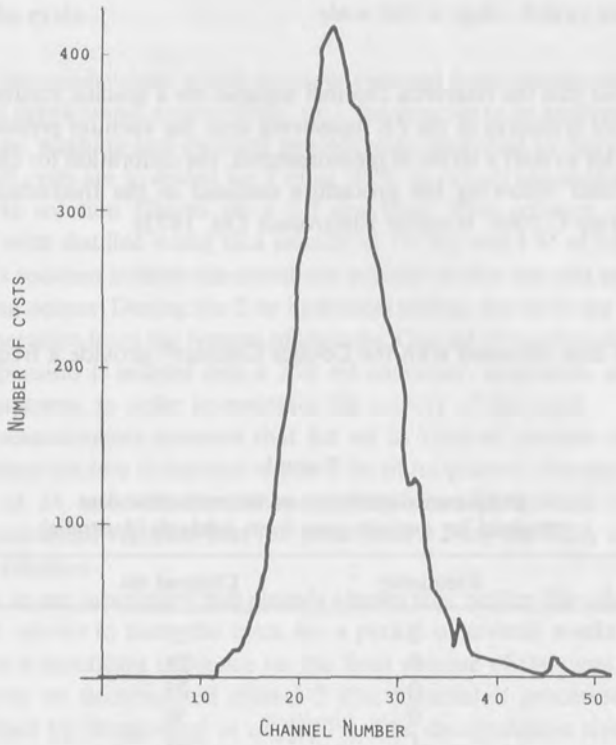


FIG. 1. Size distribution of *Artemia* cysts from Adelaide (Australia).

The specific individual cyst volume per channel ($V_N$) can be calculated with the following formula from the Instruction Manual for the Coulter Channelyzer C-1000 (Coulter Electronics Ltd, 1973):

$$[(C_N \times \frac{WW}{100}) + BCT] \times TF = V_N \quad (1)$$

where $C_N$ : channel of a particular size class
WW : window width setting = 100
BCT : base channel threshold setting = 10
TF : threshold factor (see further)

$C_N$ and $V_N$ are in our case the specific channel number and the specific volume in cubic microns of the mean of the frequency distribution curve. The threshold factor (TF), which has to be determined for each new series of analyses, is determined with the aid of a sample of calibration material of well known volume. Since there is no specific calibration material available on the market within the size-range of *Artemia* cysts, we decided to use the commercial cyst batch San Francisco Bay, no 288-2596 to calibrate the Coulter Counter®. The mean volume $V_C$ of this cyst sample was assessed from over 600 microscopic measurements.

The threshold factor can now be calculated as follows :

$$TF = \frac{V_C}{C_C + BCT} \tag{2}$$

where $V_C$ : volume in $\mu m^3$ of the calibration material
$C_C$ : channel number of the mean of the distribution for the calibration material

The cyst diameter is calculated from the cyst volume using the formula :

$$D = \sqrt[3]{\frac{6V}{\pi}} \tag{3}$$

To calculate the variance $\sigma^2_v$ and the standard deviation $\sigma_v$ of the volume, the variance $\sigma^2$ and the standard deviation $\sigma$ of the distribution – given as channels – are multiplied with respectively the square TF value and the TF value.

$$\sigma^2_v = \sigma^2 \times (TF)^2 \tag{4}$$

$$\sigma_v = \sigma \times TF \tag{5}$$

Using $\sigma^2\{f(x)\} = (\frac{\delta f}{\delta x})^2 . \sigma^2 x$ with $f(x) = d = \sqrt[3]{\frac{6V}{\pi}}$
$x = V$

the variance of the diameter is given by the formula :

$$\sigma^2_d = \sigma^2_v \left(\frac{2}{9\pi V^2}\right)^{2/3} \tag{6}$$

Upon comparison of the mean volumes and diameters of replicate cyst samples from the same batch, significant differences were found at the 0.01 level. Statistical analysis of the data obtained following the methods of the maximum likelihood and the weighed sum of squared deviates revealed that the real variance of the mean is about 10 times higher than the estimated value. This appears to be due to the $\pm 1\%$ accuracy of the channelyzer. As a consequence 10 measurements were performed for each batch in order to be able to compare the Coulter data on a statistical basis. This way a mean channelnumber $\bar{C}_N$ with variance $\sigma^2_{\bar{C}_N}$ can be determined out of the 10 replicates of each batch. The TF value is also calculated from five replicates. Since the TF value is not constant, but determined from the mean channelnumber $\bar{C}_N$ of five replicates, this should be included in the calculation of the variances.

The mean volume of a batch is then given by :

$$\bar{V} = (\bar{C}_N + BCT) \times TF \tag{7}$$

or

$$\bar{V} = (\bar{C}_N + BCT) \times \frac{\bar{V}_C}{\bar{C}_C + BCT}$$

Using $\sigma^2\{f(x,y)\} = \sigma^2_x \left(\frac{\delta f}{\delta x}\right)^2 + \sigma^2_y \left(\frac{\delta f}{\delta y}\right)^2$ with $f(x,y) = V$
$x = \bar{C}_N$
$y = \bar{C}_C$

$$\text{we obtain : } \sigma^2_v \simeq \sigma^2_{\bar{C}_N} \times (TF)^2 + \sigma^2_{\bar{C}_c} \left[\frac{(\bar{C}_N + BCT)\bar{V}_C}{(\bar{C}_C + BCT)^2}\right]^2 \quad (8)$$

A t-test can be performed to compare the mean volumes of different batches and strains using the data from each analysis for the mean volume $\bar{V}$, its variance and the number of replicates.

In order to compare the mean diameters, the mean and the variance of the diameter has to be calculated using the equations (3) and (6).

Provided data are available for both non decapsulated and decapsulated cysts, one can calculate the volume and the diameter of the chorion (the alveolar and cortical layer as described by Morris and Afzelius (1967) from the differences between the respective values for untreated and decapsulated cysts.

An u-test is used to compare the chorion-volume of two strains :

$$u = \frac{(\bar{V}_1 - \bar{V}_1') - (\bar{V}_2 - \bar{V}_2')}{\sqrt{\frac{s^2_{v1}}{n} + \frac{s^{2'}_{v1}}{n} + \frac{s^2_{v2}}{n} + \frac{s^{2'}_{v2}}{n}}} \quad (9)$$

with $\bar{V}_1$ and $s^2_{v1}$ = mean volume and variance for the non decapsulated cyts of the 1st strain
$\bar{V}_1'$ and $s^{2'}_{v1}$ = mean volume and variance for the decapsulated cysts of the 1st strain
$\bar{V}_2$ and $s^2_{v2}$ = mean volume and variance for the non decapsulated cysts of the 2nd strain
$\bar{V}_2'$ and $s^{2'}_{v2}$ = mean volume and variance for the decaptulated cysts of the 2nd strain
n = number of replicates.

The calculated value of u has to be compared with the percentage of the Gaussian distribution or the value of t for $\infty$ degrees of freedom in the Fisher-table. The same formula can be used for the comparison of the chorion diameter of two strains if the mean diameters and the variances of the diameters are calculated.

**Practical example**

The cyst volume, cyst diameter, chorion volume and chorion thickness have been determined for batches from three geographical strains of *Artemia*.

The channel numbers for the mean of the frequency distribution and the corresponding estimation of the variance for the 10 replicates of untreated and decapsulated cyst samples are summarized in Table II.

Since the strains were not all analyzed on the same day, a slightly different TF value was obtained. Table III contains the channel numbers for the mean of the frequency distribution of the calibration material. Calibration 1 was used for SFBB 2606 untreated, Shark Bay decapsulated and Adelaide decapsulated. Calibration 2 was used for SFBB 2606 decapsulated and calibration 3 was used for Shark Bay untreated and Adelaide untreated. Since the volume of the calibration material is 5 937 913 $\mu m^3$ the TF value is given by :

$$TF = \frac{5\,937\,913}{\bar{C}_c + 10}$$

TABLE II

Channel numbers ($C_N$) for the mean of the frequency distribution and corresponding estimation of the variance ($s^2$) for the 10 replicates of untreated (A) and decapsulated (B) cyst-samples

| San Francisco Bay, California, USA (SFBB 2606) | | | | Shark Bay, Australia | | | | Adelaide, Australia | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| A | | B | | A | | B | | A | | B | |
| $C_N$ | $s^2$ | $C_N$ | $s^2$ | $C_N$ | $s^2$ | $C_N$ | $s^2$ | $C_N$ | $s^2$ | $C_N$ | $s^2$ |
| 24.3 | 27.5 | 18.2 | 26.9 | 43.3 | 36.3 | 33.5 | 27.5 | 25.5 | 24.8 | 18.5 | 16.0 |
| 24.5 | 28.1 | 18.0 | 24.6 | 43.4 | 33.7 | 33.7 | 27.8 | 25.7 | 28.6 | 18.1 | 15.8 |
| 24.6 | 29.2 | 18.3 | 24.6 | 43.3 | 37.3 | 33.3 | 27.9 | 25.2 | 25.4 | 18.1 | 14.3 |
| 24.6 | 33.8 | 17.9 | 23.2 | 43.4 | 37.2 | 33.3 | 27.2 | 25.2 | 27.1 | 18.0 | 13.5 |
| 24.5 | 29.5 | 18.1 | 22.9 | 43.4 | 35.0 | 33.0 | 26.4 | 25.4 | 25.1 | 17.8 | 14.3 |
| 24.7 | 29.4 | 18.1 | 24.0 | 43.8 | 35.4 | 33.5 | 30.2 | 24.9 | 25.1 | 18.0 | 14.6 |
| 24.7 | 30.7 | 18.2 | 24.1 | 43.7 | 36.5 | 34.4 | 30.7 | 25.3 | 26.0 | 18.0 | 14.4 |
| 24.7 | 30.1 | 18.1 | 23.9 | 44.3 | 36.1 | 34.3 | 32.3 | 25.3 | 26.1 | 18.3 | 14.1 |
| 24.5 | 29.3 | 18.0 | 23.8 | 43.7 | 35.8 | 34.0 | 34.8 | 24.4 | 27.0 | 18.3 | 14.4 |
| 24.3 | 31.9 | 18.1 | 24.0 | 43.6 | 35.6 | 34.1 | 30.6 | 25.2 | 25.5 | 18.3 | 14.3 |
| $\bar{C}_N$ | $\bar{s}^2$ | $\bar{C}_N$ | $\bar{s}^2$ | $\bar{C}_N$ | $\bar{s}^2$ | $\bar{C}_N$ | $\bar{s}^2$ | $\bar{C}_N$ | $\bar{s}^2$ | $\bar{C}_N$ | $\bar{s}^2$ |
| 24.54 | 29.95 | 18.10 | 24.20 | 43.59 | 35.89 | 33.71 | 29.54 | 25.21 | 26.07 | 18.14 | 14.57 |

TABLE III

Channel numbers for the mean of the frequency distribution of the calibration material

| Calibration 1 | Calibration 2 | Calibration 3 |
|---|---|---|
| $C_c$ | $C_c$ | $C_c$ |
| 24.4 | 24.3 | 24.9 |
| 24.7 | 24.2 | 24.9 |
| 24.6 | 24.0 | 24.2 |
| 24.7 | 23.9 | 24.8 |
| 24.5 | 24.4 | 24.7 |
| $\bar{C}_c$ 24.58 | 24.16 | 24.70 |

The data for the cyst volume, cyst diameter, chorion volume, and chorion thickness of the three *Artemia* strains are given in Table IV.

The three figures for the variance $\sigma^2_{\bar{\varepsilon}_n}$ of the different cyst batches are given in Table V. The variance of the calibration material for calibration 1 is 0.017 ; for calibration 2: 0.043, and for calibration 3: 0.085.

Since the TF values, $\sigma^2_{\bar{C}_N}$, $\sigma^2_{\bar{C}_c}$, $\bar{C}_N$, $\bar{C}_C$ and $\bar{V}_C$ are known, it is possible to calculate $\sigma^2_v$ and $\sigma^2_d$ using the equations (8) and (6) ; the data obtained for the different cyst batches are given in Table VI.

A Student t-test reveals that the untreated cysts of Adelaide are significantly larger than the San Francisco Bay cysts at the 0.01 confidence level, whereas the mean diameters of the decapsulated cysts for the San Francisco Bay and Adelaide batches are not significantly different.

TABLE IV

Cyst volume, cyst diameter, chorion volume and chorion thickness of the three *Artemia* strains

| | San Francisco Bay | | Shark Bay | | Adelaide | |
|---|---|---|---|---|---|---|
| | A | B | A | B | A | B |
| Volume ($\mu m^3$) | 5 931 036 | 4 884 511 | 9 170 374 | 7 505 633 | 6 025 170 | 4 832 060 |
| s | 939 738 | 855 111 | 1 025 156 | 933 283 | 873 723 | 655 447 |
| Diameter ($\mu m$) | 224.6 | 210.5 | 259.7 | 242.9 | 225.8 | 209.8 |
| s | 11.9 | 12.3 | 9.7 | 10.1 | 10.9 | 9.5 |
| Chorion volume ($\mu m^3$) | 1 046 525 | | 1 664 712 | | 1 193 110 | |
| Chorion thickness ($\mu m$) | 7.05 | | 8.40 | | 8.00 | |

TABLE V

Variance data of untreated (A) and decapsulated (B) cyst batches

| Strain | $\sigma^2_{C_N}$ | |
|---|---|---|
| | A | B |
| San Francisco Bay | 0.02267 | 0.01333 |
| Shark Bay | 0.09433 | 0.22100 |
| Adelaide | 0.12544 | 0.04267 |

TABLE VI

$\sigma^2_v$ and $\sigma^2_d$ for the three *Artemia* strains

| | $\sigma^2_v$ | | $\sigma^2_d$ | |
|---|---|---|---|---|
| | A | B | A | B |
| San Francisco Bay | $1.16865 \times 10^9$ | $1.28195 \times 10^9$ | 0.1862 | 0.2646 |
| Shark Bay | $8.69890 \times 10^9$ | $7.31732 \times 10^9$ | 0.7751 | 0.8516 |
| Adelaide | $6.23590 \times 10^9$ | $1.59011 \times 10^9$ | 0.9728 | 0.3329 |

The cysts from Shark Bay, untreated and decapsulated, are significantly larger than the respective cyst preparations of the two other strains.

An u-test, using equation (9) performed on the date for these strains reveals that at the 0.01 confidence level the chorion volume of the Shark Bay cysts is significantly larger than that of the Adelaide cysts, which in turn have a significantly larger chorion volume than the San Francisco Bay cysts.

With regard to the chorion thickness there appears to be no difference between the Adelaide and the Shark Bay strain. The chorion of the San Francisco Bay cysts seems, however, to be significantly thinner than the corresponding value for the two other strains.

**Acknowledgement**

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**Literature cited**

BRUGGEMAN E., M. BAEZA-MESA, E. BOSSUYT, and P. SORGELOOS. 1979. Improvements in the decapsulation of *Artemia* cysts. p. 309-315. *In* : Cultivation of fish fry and its live food. Styczynska-Jurewicz E., T. Backiel, E. Jaspers and G. Persoone (Eds). European Mariculture Society Special Publication No. 4. EMS, Bredene, Belgium. 534 p.

CLAUS C., F. BENIJTS, and P. SORGELOOS. 1977. Comparative study of different geographical strains of the brine shrimp : *Artemia salina*. p. 91-105. *In* : Fundamental and applied research on the brine shrimp, *Artemia salina* (L.) in Belgium. E. Jaspers and G. Persoone (Eds). European Mariculture Society Special Publication No. 2. EMS, Bredene, Belgium. 110 p.

Coulter Electronics Ltd. 1973. Introduction manual for Coulter channelyzer C-1000. Coulter Electronics Limited, Bedfordshire, England. 37 p.

D'AGOSTINO A. S. 1965. Comparative studies of *Artemia salina* (development and physiology). Thesis. New York University. 83 p. University Microfilms, 66-5653.

MORRIS J. E., B. A. AFZELIUS. 1967. The structure of the shell and outer membranes in encysted *Artemia salina* embryos during cryptobiosis and development. *J. Ultrastruct. Res.* 20:244-259.

SORGELOOS P., M. BAEZA-MESA, F. BENIJTS, and G. PERSOONE. 1976. Current research on the culturing of the brine shrimp *Artemia salina* L. at the State University of Ghent, Belgium. p. 473-495. *In* : Proc. 10th European Symp. Marine Biology. Vol. 1. Mariculture. Persoone G. and E. Jaspers (Eds). Universa Press, Wetteren, Belgium. 620 p.

SORGELOOS P., G. PERSOONE, M. BAEZA-MESA, E. BOSSUYT, and E. BRUGGEMAN. 1978. The use of *Artemia* cysts in aquaculture : the concept 'hatching efficiency' and description of a new method for cysts processing. p. 715-721. *In* : Proc. 9th Annual Meeting World Mariculture Society. Avault J. W., Jr. (Ed). Louisiana State University, Baton Rouge, Louisiana, USA. 807 p.

*The Brine Shrimp* Artemia. *1980. Vol. 1. Morphology, Genetics, Radiobiology, Toxicology*
*G. Persoone, P. Sorgeloos, O. Roels, and E. Jaspers (Eds). Universa Press, Wetteren, Belgium. 345 p.*

# A light and electron microscopic study of the frontal knob of *Artemia* (Crustacea, Branchipoda)

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**Abstract**

The frontal knob, a protuberance located along the anteromedial surface of each protopodite, is peculiar to the second antenna or clasper of male artemiae. The frontal knob and its associated processes were studied by scanning and transmission electron microscopy as well as light and phase microscopy. Two types of processes project from the knob giving it the general appearance of a pin cushion. The smaller, more numerous, non-cellular spinous processes are conical and frequently occur in pairs. The larger cellular processes are the outgrowths of three cells. One cell forms a dome-shaped collar through which a seta, containing a sensory neuron, projects. The frontal knobs, as well as other processes of the protopodite, facilitate grasping and holding, especially during clasping and copulating behavior. The non-cellular spines help to insure a firmer grip by the male during these reproductive activities while the role of the sensory setae remains obscure. Although their function might be completely unrelated to the reproductive process, the juxtaposition of these setae to the ovisac during clasping might provide the male with reproductive cues.

## Introduction

The structure of arthropod cuticular receptors has received considerable attention (Cohen and Dijkgraaf, 1961 ; Horridge, 1965 ; Thurm, 1965 ; McIver, 1975). Usually these studies or reviews have dealt with the appendages associated with the head, especially the antennal sensory receptors. Although the receptors of all of the major groups of arthropods have been studied, much of the information as well as the various classifications of sensory receptor types has been obtained from studies of insects (Slifer, *et al.*, 1957 ; Thurm, 1965 ; Lewis, 1970 ; Schwartzkopff, 1974 ; Toh, 1977).

Although Leydig (1851) first described setae in the anostracan, *Artemia* in 1851, little histological or ultrastructural information has been available on cuticular receptors in crustaceans until recently. Most of these studies have concentrated on the decapods ; however, Strickler and Bal (1973) and Tyson and Sullivan (1978) have described cuticular receptors located on the first antennae of *Cyclops* and *Artemia*, respectively. In crustaceans, which typically possess two pairs of antennae, the first antennae are utilized primarily for sensory reception while the second antennae might be modified for locomotion, attachment to hosts, capturing food, clinging to vegetation, or reproductive activity (Meglitsch, 1972).

The second antennae of male *Artemia* are greatly enlarged and modified as claspers which are used for grasping and holding the female during clasping and copulation. Located along

the anteromedial surface of the muscular protopodite is the frontal knob ; it was reported first by Leydig (1851) and its development was studied by Heath (1924). Lochhead (1950) suggested that the knob facilitates holding the female during clasping and copulation. Periodically workers have suggested that the frontal knob is sensory in function and Heath (1924), Cassel (1937), and Wolfe (1968) have reported the presence of two different types of processes on the knob. This paper describes the frontal knob and its associated processes, the non-cellular spines and the sensory setae.

## Materials and methods

### Animals

The *Artemia* used in this study were obtained commercially (Carolina Biological Supply Co.) in the form of dried eggs which were hatched in boiled, filtered reconstituted seawater ("Instant Ocean"). The cultures were maintained at room temperature in gallon jars. A suspension of beef liver paste was added once a week to the cultures and deionized water was added as needed to compensate for evaporation (specific gravity 1.022-1.054).

Mature animals were used throughout the study and were defined as males possessing sperm within the vas deferens or females possessing large ova within the ovary or ovisac. All of these mature animals ranged from 6 to 12 mm in length and the males possessed well developed claspers.

### Preparation of tissue

#### *Light microscopy*

Entire animals or male heads including the claspers were immersed in Carnoy's fixative (6:3:1) at room temperature, washed and dehydrated in absolute ethanol, cleared in toluene, embedded in Tissue Prep (M.P. 56.5 °C), and sectioned at 6 or 10 $\mu$m. For routine observations and general morphology the sections were stained with Delafield's hematoxylin and eosin, periodic acid Schiff and hematoxylin, or Mallory's triple stain (Humason, 1972). Sevier-Munger's method was used to identify the location and distribution of nerve fibers (Sevier and Munger, 1965).

Living animals were observed by phase microscopy as well as light and dark field microscopy. These animals were narcotized lightly with a drop of chloroform added to the medium. Some of the animals were stained with a 1% aqueous nigrosine solution, a 1% aqueous toluidine blue solution or a 0.5% aqueous crystal violet solution to enhance the contrast.

#### *Transmission electron microscopy*

Male artemiae heads including the claspers were fixed in formaldehyde (2%) and glutaraldehyde (2%) in a 0.1 M phosphate buffer for 2 hr at room temperature (Forssmann *et al.*, 1977). The animals were post-fixed in 1% $OsO_4$ in 0.1 M S-collidine buffer for 2 hr at 4 °C. The animals were embedded in Durcupan ACM and sectioned on a Sorvall (Porter-Blum) MT-2 ultramicrotome. Thick sections (1 or 2 $\mu$m) were stained with 1% toluidine blue. Thin sections were stained on the grids with 2% aqueous uranyl acetate followed by a 1% aqueous

lead citrate (Venable and Coggeshall, 1965) and appropriate sections were examined with an RCA EMU 4 electron microscope.

*Scanning electron microscopy*

The animals were fixed in formaldehyde (2%) and glutaraldehyde (2%) in a 0.1 M phosphate buffer for 2 hr at room temperature followed by immersion in 1% aqueous $OsO_4$, for 2 hr at 4 °C, 1% aqueous thiocarbohydrazide for 20 min at room temperature and 1% aqueous $OsO_4$ for 1 hr at room temperature (Kelley *et al.*, 1973). The animals were dehydrated in ethanol, critical point dried, mounted on aluminium stubs, and coated with gold-palladium alloy. The preparations were examined with an AMR 900 scanning electron microscope.

## Results

The frontal knobs are situated along the anteromedial surface of each muscular protopodite and are located within the first third of its length (Fig. 1). Each knob is spherical, has a diameter of approximately 170 $\mu$m and projects from a short stalk. The surface of the stalk as well as the underside of the knob are smooth while the upper surface of the knob resembles a pin cushion containing two different types of processes, spines and sensory setae.

The spines are more numerous than the sensory setae and are distributed most densely around the center of the frontal knob (Fig. 2). No definite pattern or specific number of spines is apparent ; however, the anterior and lateral surfaces are practically devoid of spines (Fig. 3). The spines usually occur singly or in pairs, and very rarely three spines arise from the same region (Fig. 4).

The spines project from slight depressions in the general surface of the exoskeleton. The base of the spine is 1 $\mu$m in diamter, is somewhat irregular in outline and tapers distally for approximately 5 $\mu$m ending in a tip that is curved slightly posteriorly (Fig. 5). The spine consists only of cuticular material that is probably secreted by the underlying hypodermal cell and the entire spine is replaced at each molt (Fig. 6 and 7). There is no evidence of any innervation associated with the spines.

The sensory setae are arranged in rows along the entire upper surface of the frontal knob (Fig. 2). They are located approximately 25 $\mu$m apart and extend further into the anterior and lateral regions than the spines (Fig. 3).

The sensory seta unit consists of a large dome-shaped supporting cell, the seta-producing cell and a sensory neuron (Fig. 8). Projecting from the surface of the knob, the dome-shaped cell has a diameter of 5 $\mu$m and protrudes 3 $\mu$m above the surface. Occasionally the shape of the supporting cell varies from dome-shaped to tubular but at present no other evidence suggests that they are different cell types (Fig. 9). The seta extends through the supporting cell approximately 4 $\mu$m above the surface of the dome (Fig. 10 and 11). Usually a single seta projects through each supporting cell ; however, occasionally a pair of setae project from the same supporting cell (Fig. 9 and 12).

The exoskeleton covering the supporting cell is conspicuously thinner than the general surface of the frontal knob. During molting the old exoskeleton is raised from the new exoskeleton and the supporting cell surface as well as the seta surface are lost (Fig. 6). Observations of exuvia from recently molted *Artemia* revealed that well defined dome-shaped surfaces and very finely tapered setae are shed at each molt.

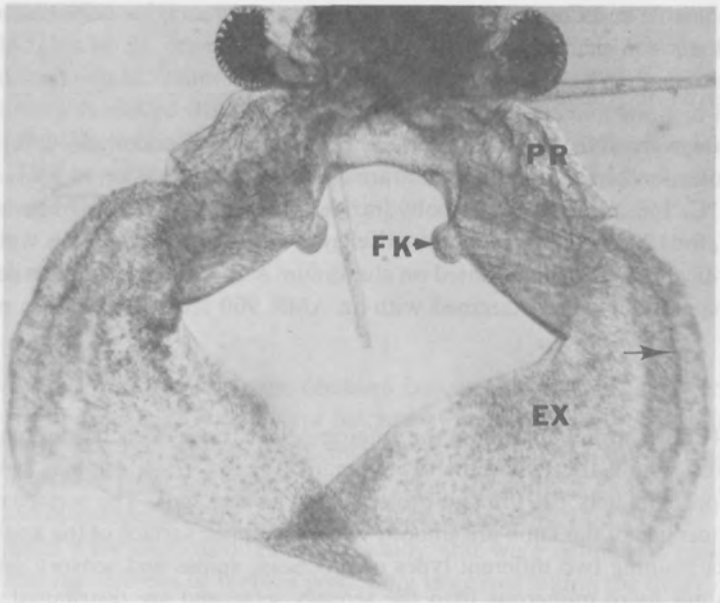



FIG. 1. Head of male *Artemia* showing the position of the frontal knob (FK) on the muscular protopodite (PR). The exopodite (EX) is hollow and curled along the lateral edge (arrow). × 20.

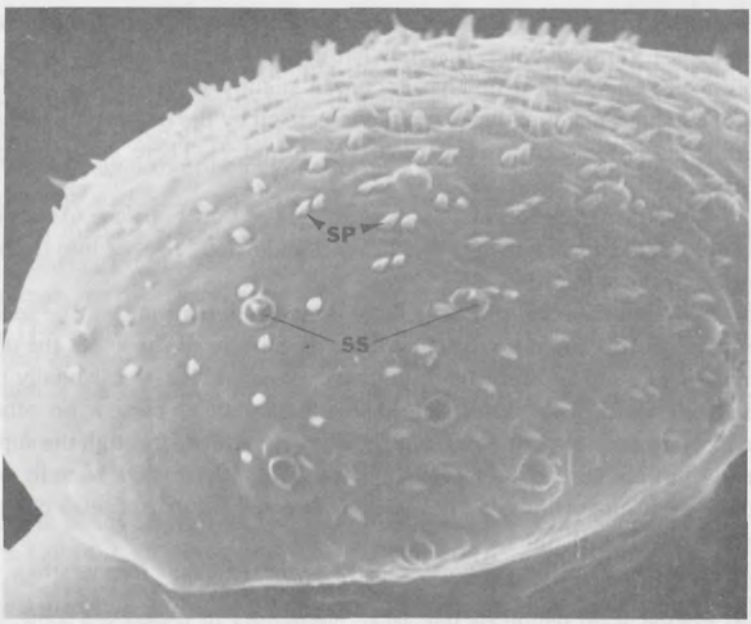
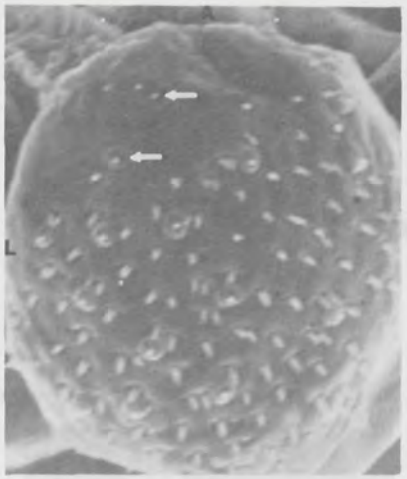



FIG. 2. Scanning electron micrograph of the entire frontal knob showing the arrangement and distribution of spines (SP) and sensory setae (SS). × 1100.

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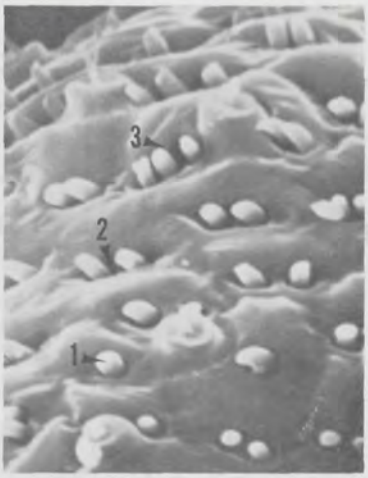


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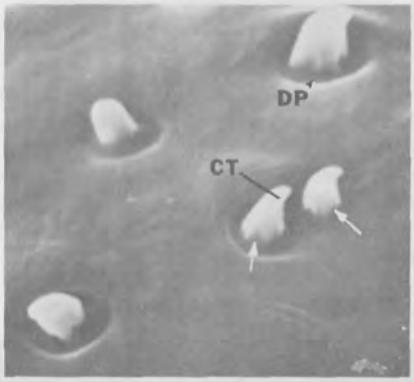


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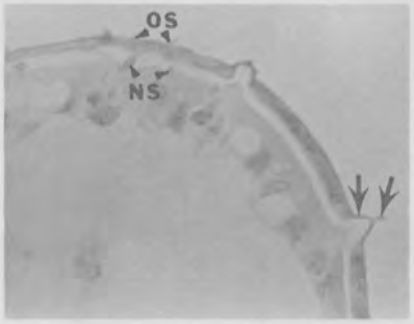


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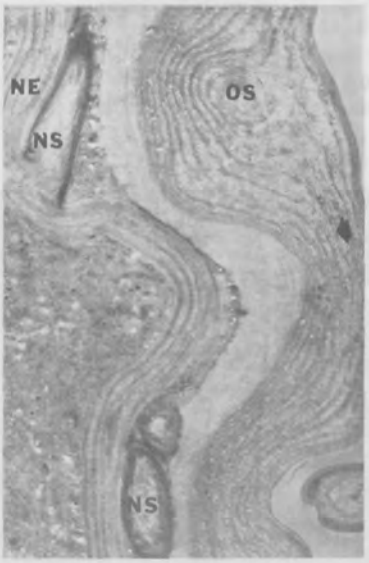


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FIG. 3. Scanning electron micrograph of the entire frontal knob illustrating the sparse distribution of spines along the anterior (A) and lateral (L) surfaces. Sensory setae (arrows) do appear in these regions. × 700.

FIG. 4. Numbers (1,2,3) indicate the various arrangements of spines arising from a single depression in the exoskeleton. × 2000.

FIG. 5. Spines project from a depression (DP) in the exoskeleton and have an irregular base (arrows) and a curved tip (CT). × 3400.

FIG. 6. The old exoskeleton is separated from the body surface and both the old spines (OS) and the new spines (NS) are evident. At molting the thin, dome-shaped supporting cell exoskeleton and seta are shed (arrows). × 550.

FIG. 7. Newly secreted spines (NS) are lying flat against the new exoskeleton (NE) while the old spine (OS) and the old exoskeleton (arrow) are still intact. × 10 200.

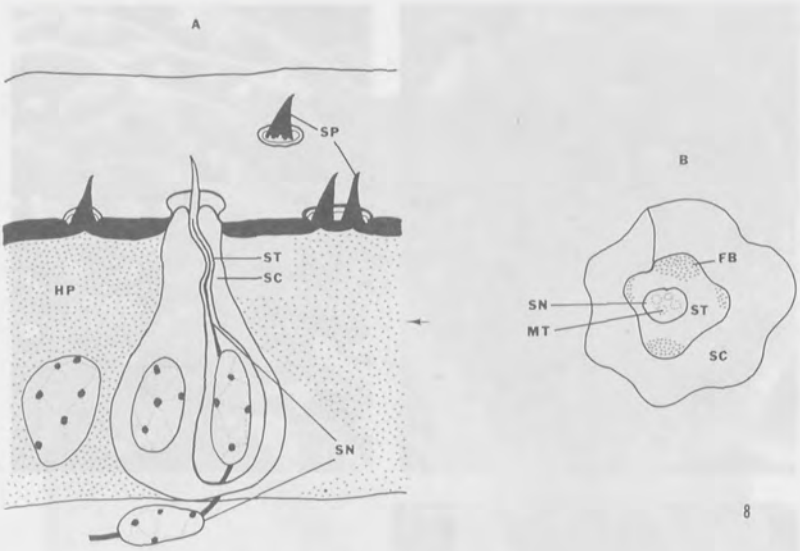



FIG. 8. A. Diagram of spines (SP) and sensory seta on the frontal knob of *Artemia*. The sensory seta unit consists of a supporting cell (SC), setal cell (ST), and sensory neuron (SN). These sensory setae are surrounded by the hypodermis (HP).

B. Diagram of a cross section through the sensory seta unit of Fig. 8A at the level of the arrow. The single supporting cell (SC) surrounds the setal cell (ST) which contains the fibrillar material (FB) arranged in bands. The sensory neuron, completely surrounded by the setal cell, contains four groups of microtubules (MT) that appear to be modified ciliary processes.

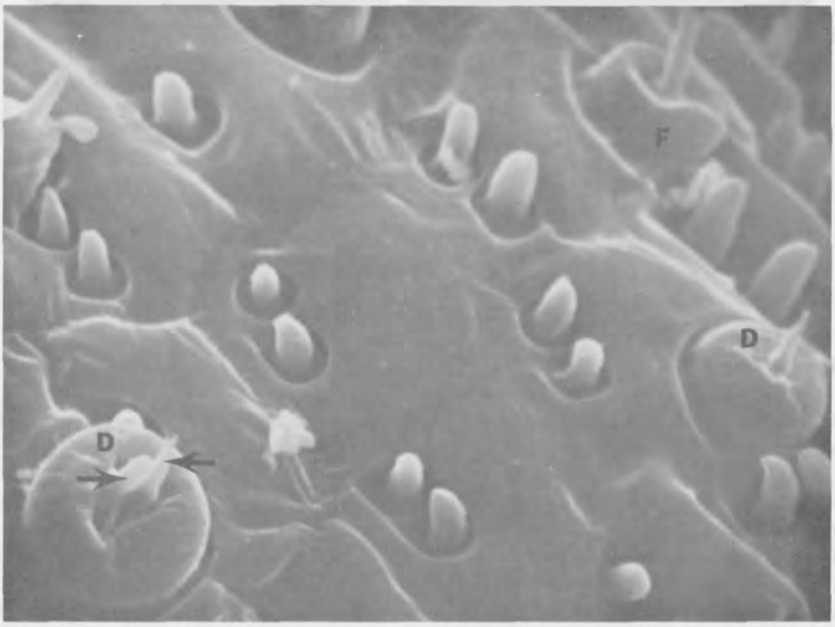
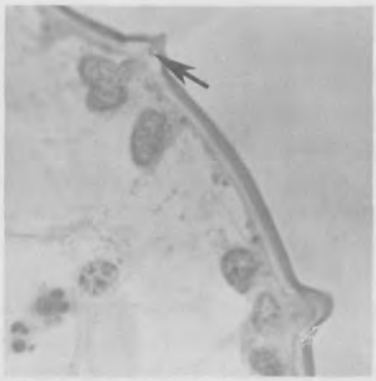



FIG. 9. Outer surface of supporting cells usually appears dome-shaped (D) but occasionally the surface is flat (F). Arrows indicate two setae arising from a single supporting cell. × 3 200.



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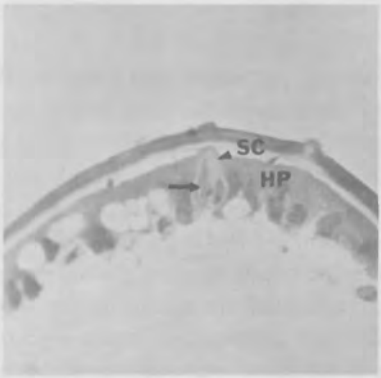


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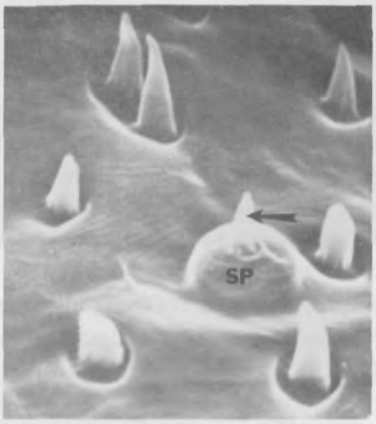


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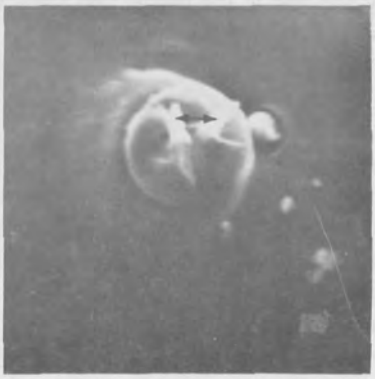

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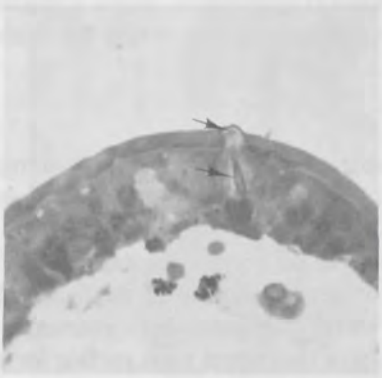

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FIG. 10. Opening in the supporting cell (arrow) through which the seta passes. × 1 400.

FIG. 11. Single seta (arrow) arising from the dome-shaped supporting cell (SP). × 3 400.

FIG. 12. Pair of setae (arrows) projecting from the same dome-shaped supporting cell. × 2 550.

FIG. 13. Goblet-shaped supporting cell (SC) containing the seta (arrow). Note the clear cytoplasm of the supporting cell compared to the cytoplasm of the hypodermis (HP). × 1 200.

FIG. 14. Setal cell is located to side of the supporting cell (SC). Darkly staining material is visible deep within the seta (arrow). × 1 400.

FIG. 15. Setal cell surface (arrows) is stained darkly along most of its length deep within the supporting cell and near the dome of the supporting cell. The apparent discontinuity of the seta is due to its irregular orientation. × 1 250.

The supporting cell stains less intensely than the surrounding hypodermal cells and is shaped like a goblet with the basal end of the cell broader than the apical end (Fig. 13). The setal cell is surrounded by the supporting cell and throughout most of its length the setal cell is displaced to one side of the supporting cell (Fig. 13, 14, and 15). The cytoplasm of the supporting cell contains many mitochondria but other organelles, especially the rough endoplasmic reticulum, are less numerous than in the hypodermis (Fig. 16 and 17).

The setal cell extends beneath the surface and is approximately 15 $\mu$m in overall length. Also extending beneath the surface of the supporting cell is a darkly staining material that is very irregular and appears continuous with the outer surface of the seta (Fig. 15). At higher magnification this darkly staining material is fibrillar and the fibers are oriented parallel to the long axis of the seta (Fig. 18). These fibers do not completely delimit the circumference of the seta but occur in two large bands and several smaller bands (Fig. 19).

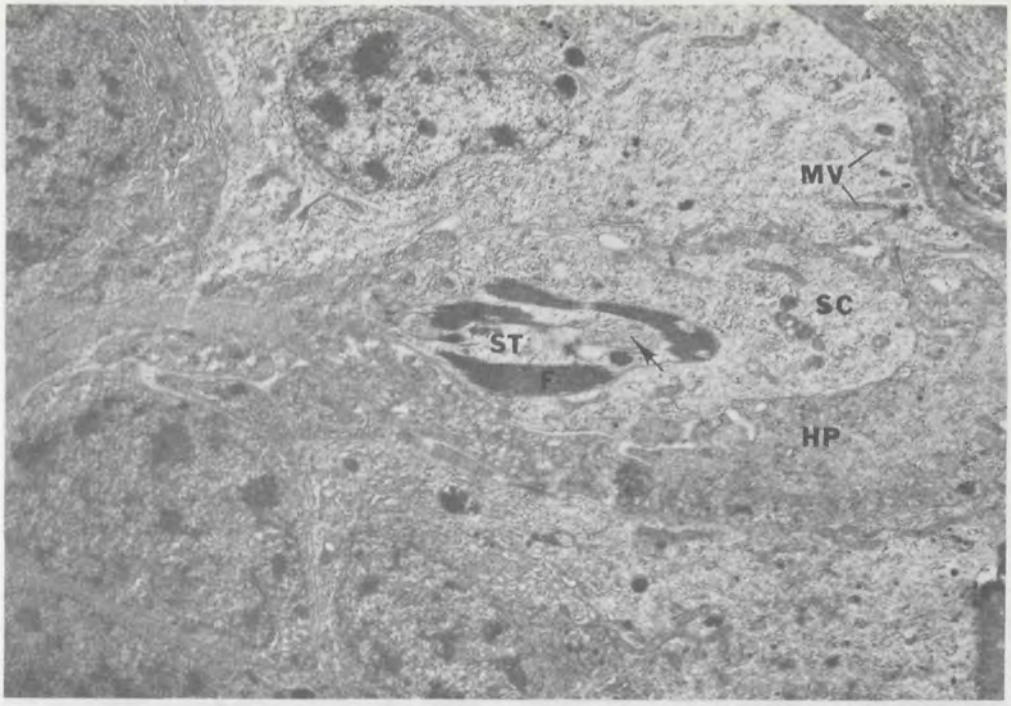
Throughout most of its length, the seta completely surrounds the sensory neuron (Fig. 17 and 19). The neuron penetrates the proximal end of the seta and extends at least to the region where the supporting cell begins to expand into its dome-shaped pattern (Fig. 20). The neuron contains four groups of microtubules that are positioned parallel to each other. Each group resembles a modified ciliary process containing eight or nine microtubules arranged peripherally with no tubules in the center (Fig. 19). No other organelles are visible in the neuron at its distal end.

Although this investigation was designed to study the structure of the frontal knobs of male artemiae, a brief description of the exact site on the female where these knobs are placed during reproductive behavior should be helpful. During copulation the frontal knobs are positioned into two depressions, the copulation cups, located along the lateral surfaces of first genital segment (Fig. 21). This region is slightly anterior to the ovisac and these cup-shaped depressions are near the area where the oviduct passes into the ovisac.

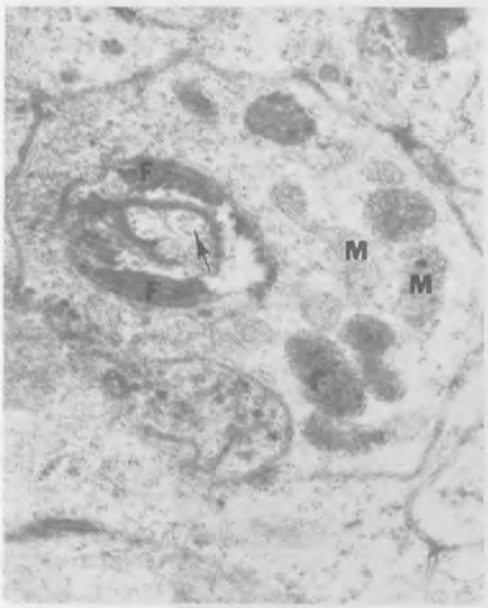
## Discussion

The association of the male clasper with reproductive activity in the anostracans has been reported by Leydig (1851), Jensen (1918), Heath (1924), Lochhead (1950), Bowen (1962), and Wolfe (1973) for *Artemia* and by Pearse (1912) and Moore and Ogren (1962) for *Eubranchipus*. In the normal clasping position the frontal knobs are brought into close contact with the first genital segment of the female. Since the spines consist entirely of exoskeletal material with no associated neural elements, these numerous, short processes are probably modifications of the frontal knob surface for grasping. Such processes are not uncommon in the arthropods (Barrington, 1979). The posterior curvature of these spines and their distribution toward the center and posterior medial surface of the knob should facilitate grasping and holding the female, especially during copulation.

In terms of its cellular arrangement, the sensory setae of *Artemia* consist of the same basic components as most arthropod mechanoreceptors, a supporting cell ( = tormogen cell), a setal cell ( = trichogen cell), and a sensory neuron. Similar cell types have been reported in the crustaceans, *Daphnia* (Agar, 1950), *Orchestia* (Charniaux-Cotton, 1957), and *Cyclops* (Strickler and Bal, 1973); in insects (Thurm, 1965; Schwartzkopff, 1974); and in the arachnids, *Ciniflo* (Harris and Mill, 1973) and *Microcaeculus* (Haupt and Coineau, 1978). Unlike the type 2 sensilla of the *Artemia* antennule (Tyson and Sullivan, 1978), the frontal knob setae do not




16

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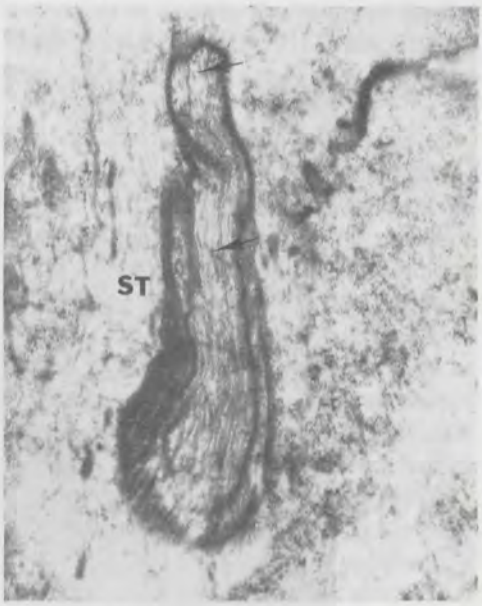


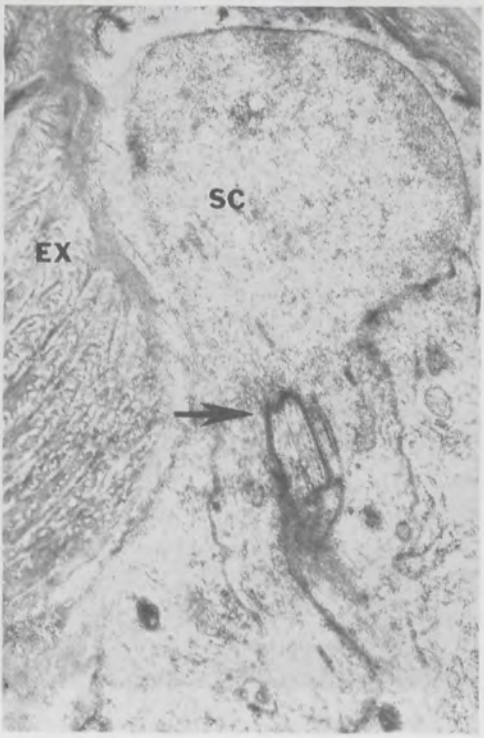

18

FIG. 16. Electron micrograph through the sensory seta unit showing the lightly stained cytoplasm of the supporting cell (SC) and darkly stained cytoplasm and microvilli (MV) of hypodermis (HP). The setal cell (ST) contains fibrillar material (F) and surrounds the sensory neuron in which microtubules (arrow) appear in longitudinal sections. × 16 200.

FIG. 17. Cross section through the sensory seta unit showing the mitochondria (M) of the supporting cell, the fibrillar bands (F) of the seta, and microtubules within the neuron (arrow). × 43 660.

FIG. 18. Longitudinal section through the setal cell (ST) and the microtubules (arrows) of the sensory neuron. The twisted nature of the seta and neuron are evident by the different planes of these elements in this section. × 35 400.


19

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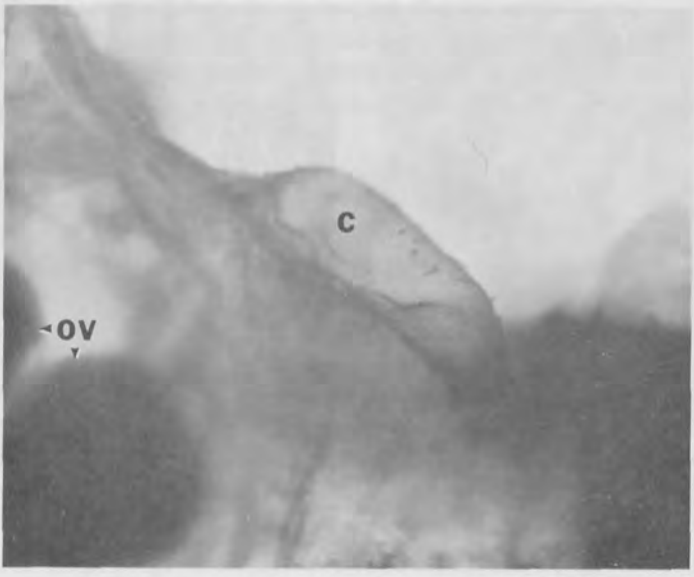


21

FIG. 19. Cross section through the setal cell showing the fibrillar bands (F) and through the neuron showing the four ciliary-like processes (arrow). × 21 600.

FIG. 20. Longitudinal section of supporting cell (SC) indicating the level to which the sensory neuron penetrates (arrow). The thick exoskeleton (EX) of the general surface of the frontal knob appears to the left. × 28 700.

FIG. 21. Depression, copulation cup (C), in the first genital segment of the female in which the frontal knobs are positioned during copulation. Ova (OV) are visible to the left of the figure, which is the posterior ventral surface of the animal. × 120.

stain with crystal violet, a technique that usually indicates a chemoreceptor. In the absence of any physiological data at this time, the morphological evidence strongly suggests that the frontal knob setae are mechanoreceptors.

The dome-shaped supporting cell of the frontal knob of *Artemia* generally resembles the supporting cells ( = tormogen cells, envelope cells) of some insects (Hawke *et al.*, 1973 ; Schwartzkopff, 1974 ; Toh, 1977) and arachnids (Harris and Mill, 1973 ; Haupt and Coineau, 1978) except that in *Artemia* only a single supporting cell is present. Also, in many insects the apical surface of the supporting cell is usually sunken below the general surface of the exoskeleton and not raised as in *Artemia* (Thurm, 1965 ; Lewis, 1970 ; Gaffal and Hansen, 1972). Although some of the supporting cells have a tubular instead of a dome-shape, no other evidence warrants the consideration of these structures as different cell types at this time.

The shape, length and degree of motility of setae vary considerably within the arthropods, suggesting a wide variation in mechanoreceptive function (Schwartzkopff, 1974). Despite such structural variation each seta must possess a stiff shaft whose deflection transmits the stimulus to the underlying neuron (Thurm, 1964, 1965). In addition to the presence of such a seta in *Artemia*, the rigid cuticle appears to extend well beneath the surface of the setal cell. Such a cuticular sheath associated with an underlying sensory neuron has been reported in insects (Thurm, 1965 ; Schwartzkopff, 1974), arachnids (Harris and Mill, 1973 ; Haupt and Coineau, 1978) and crustaceans (Debaisieux, 1949 ; Horridge, 1965 ; Strickler and Bal, 1973). Usually these sheaths completely surround the neuron but in *Artemia* the bands of fibrillar material comprising the sheath are interrupted. Gnatzy and Schmidt (1971) found that the chitinous sheath is produced by the internal enveloping cell in insects which would be comparable to the setal cell in *Artemia*.

In most insects the mechanoreceptor neuron terminates at the base of the seta as a conspicuous tubular body (Thurm, 1965). In *Cyclops* the exact point of attachment of the neuron to the seta was not shown ; however, Strickler and Bal (1973) reported that no nervous tissue was found within the seta. Although the sensory setae of *Artemia* possess a single sensory neuron, no tubular body was evident and the sensory neuron was not found in the seta above the general exoskeletal surface ; the latter may have been the result of sectioning. The neuron extends at least to the region where the thinner dome-shaped exoskeleton begins to form, suggesting that the neuron may terminate in the dome surface or penetrate to the base of seta. Unlike the eccentric position of many arthropod mechanoreceptor neurons (Haupt and Coineau, 1978) the neuron in *Artemia* is found within the center of the setal cell. The modified ciliary structures of the neuron are a common component of most arthropod cuticular receptors (Thurm, 1965 ; Harris and Mill, 1973 ; Strickler and Bal, 1973 ; Schwartzkopff, 1974). The presence of four modified ciliary structures in the *Artemia* neuron differs from the number in other arthropods, but that probably just represents a species variation. The present study confirmed the reports of Warren (1930) and Cassel (1937) that the sensory neurons of the clasper arise from the antennal nerve whose origin is the circumesophageal connective.

Since the surfaces of both the spines and the sensory setae are covered by the cuticular exoskeleton, the entire covering of both processes are lost at ecdysis. The loss of chitinous parts of sensory units has been reported by Slifer *et al.* (1957) ; Zacharuk (1962) ; Walcott and Salpeter (1966) ; Moran (1971). Zacharuk (1962) stated further that not only is the outer cuticular exoskeleton shed, but also the cuticular sheath which extends along the neuron.

The morphological evidence indicates that the sensory setae could perceive tactile stimuli and the positioning of the frontal knobs into the depressions along the lateral surfaces of the female during copulation suggests a possible reproductive function. Schlegel (1967) reported the presence of tactile bristles in the drone sex organ which is used as the clasper in insects. Schwartzkopff (1974) discussed the importance of tactile stimuli in the initiation and maintenance of copulation. Wolfe (1973) described the normal and abnormal clasping behavior, as well as the copulatory behavior of *Artemia* ; however, no copulatory behavior was reported unless a male was clasped to a female. At least two possibilities exist for the initiation of the copulatory response of the male involving the sensory setae on the frontal knob – 1) either the positioning of the frontal knobs within these depressions along the sides of the female is sufficient stimulus for the initiation of this behavior, or 2) since these lateral depressions in the first genital segment of the female area in the proximity of the area where the oviduct enters the ovisac, the movement of the ova might provide the necessary reproductive cue to the male.

**Summary**

1. The frontal knobs which are spherical protuberances located along the anteromedial surface of the protopodite of each male second antenna, bear two types of processes, spines and sensory setae, along their upper surface.

2. The numerous, short spines usually occur singly or in pairs and consist of cuticular material only.

3. The sensory setae consist of a dome-shaped supporting cell, a setal cell and a sensory neuron.

a. The supporting cell is goblet-shaped, has faintly staining cytoplasm, contains many mitochondria and surrounds the seta.

b. The setal cell is eccentric in its position within the supporting cell, has a series of darkly staining, fibrillar material aggregated into bands around its circumference and surrounds the sensory neuron.

c. The sensory neuron penetrates the seta at least to the level of the dome-shaped pattern of the supporting cell. The neuron contains four groups of microtubules with each group resembling a modified ciliary process.

4. The dome-shaped surface of the supporting cell as well as the distal part of the seta are lost during molting.

5. During copulation these frotal knobs are positioned into two depressions on the lateral surfaces of female. The spines probably help to insure a firmer grip by the male during these reproductive activities. The sensory setae could function as mechanoreceptors and quite possibly provide the male with important reproductive cues. The proximity of these lateral depressions on the female to the region where oviduct enters the ovisac support such a suggestion.

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## Literature cited

AGAR W. E. 1950. The swimming setae of *Daphnia carinata*. *Quart. J. Micr. Sci.* 91:353-368.

BARRINGTON E. J. W. 1979. Movement and arthropodization. p. 172-216. *In* : Invertebrate structure and function. (Second edit.). Barrington, E. J. W. John Wiley and Sons, New York. 765 p.

BOWEN S. T. 1962. The genetics of *Artemia salina*. I. The reproductive cycle. *Biol. Bull.* 122:25-32.

CASSEL J. D. 1937. The morphology of *Artemia salina* (Linnaeus). Unpublished Master's thesis, Stanford University, Palo Alto, California. 109 p.

CHARNIAUX-COTTON H. 1957. Croissance, régénération et déterminisme endocrinien des caractères sexuels d'*Orchestia gammarella* (Pallas), Crustacé Amphipode. *Ann. sci. nat. Zool. et biol. animale* 19:411-559.

COHEN M. J. and S. DIJKGRAAF. 1961. Mechanoreception. p. 65-108. *In* : The Physiology of Crustacea. Vol. 2. Sense organs, integration and behavior. Waterman T. H. (Ed.). Academic Press, New York. 670 p.

DEBAISIEUX P. 1949. Les poils sensoriels d'arthropodes et l'histologie nerveuse. *Cellule* 52(3):311-360.

FORSSMANN W. G., S. ITO, E. WEIHE, A. AOKI, M. DYM, and D. W. FAWCETT. 1977. An improved perfusion fixation method for the testis. *Anat. Rec.* 188:307-314.

GAFFAL K. P. and K. HANSEN. 1972. Mechanorezeptive Strukturen der antennalen Haarsensillen der Baumwollwanze *Dysdercus intermedius* Dist. *Z. Zellforsch.* 132:79-94.

GNATZY W. and K. SCHMIDT. 1971. Die Feinstruktur der Sinneshaare auf den Cerci von *Gryllus bimaculatus* Deg. I. Faden und Keulenhaare. *Z. Zellforsch.* 122:190-209.

HARRIS D. J. and P. J. MILL. 1973. The ultrastructure of chemoreceptor sensilla in *Ciniflo* (Araneida, Arachnida). *Tissue and Cell* 5(4):679-689.

HAUPT J. and Y. COINEAU. 1978. Moulting and morphogenesis of sensilla in a prostigmate mite (Acari, Actinotrichida, Actinedida : Caeculidae). I. Mechanoreceptive bristles. *Cell Tiss. Res.* 186:63-79.

HAWKE S. D., R. D. FARLEY, and P. D. GREANY. 1973. The fine structure of sense organs in the ovipositor of the parasitic wasp, *Orgilus lepidus* Muesebeck. *Tiss. and Cell* 5(1):171-184.

HEATH H. 1924. The external development of certain phyllopods. *J. Morphol.* 38:453-483.

HORRIDGE G. A. 1965. Arthropoda : receptors other than eyes. p. 1005-1061. *In* : Structure and function in the nervous systems of invertebrates. Vol. 2. Bullock T. H. and G. A. Horridge (Eds). Freeman W. H., San Francisco. 1719 p.

HUMASON G. L. 1972. Animal tissue techniques. (Third edit.). Freeman W. H., San Francisco. 641 p.

JENSON A. C. 1918. Some observations on *Artemia gracilis*, the brine shrimp of Great Salt Lake. *Biol. Bull.* 34:18-32.

KELLEY R. O., R. A. F. DEKKER, and J. G. BLUEMINK. 1973. Ligand-mediated osmium binding : its application in coating biological specimens for scanning electron microscopy. *J. Ultrastr. Res.* 45:254-258.

LEWIS C. T. 1970. Structure and function in some external receptors. *Symp. roy. ent. Soc. London* 5:59-76.

LEYDIG F. 1851. Über *Artemia salina* und *Brachipus stagnalis*. *Zeit. f. wiss. Zool.* 3:280-307.

LOCHHEAD J. H. 1950. *Artemia*. p. 384-389. *In* : Selected invertebrate types. Arthropoda. Brown F. A. (Ed.). Wiley, New York. 597 p.

MCIVER S. B. 1975. Structure of cuticular mechanoreceptors of arthropods. *Ann. Rev. Entomol.* 20:381-397.

MEGLITSCH P. A. 1972. The aquatic mandibulates-Crustacea. p. 516-602. *In* : Invertebrate zoology. (Second edit.). Meglitsch P. A. Oxford, New York. 834 p.

MOORE W. G. and L. H. OGREN. 1962. Notes on the breeding behavior of *Eubranchipus holmani* (Ryder). *Tulane Studies in Zoology* 9(5):315-318.

MORAN D. T. 1971. Loss of the sensory process of an insect receptor at ecdysis. *Nature* 234:476-477.

PEARSE A. S. 1912. Observations on the behavior of *Eubranchipus dadayi*. *Bull. Wisc. Nat. Hist. Soc.* 10:109-117.

SCHLEGEL P. 1967. Electrophysiologische Beobachtungen an den Borstenfeld-Sensillen des ausseren Geschlechtsapparates der Drohne (*Apis mellifica*). *Naturwissenschaften* 54:26.

Schwartzkopff J. 1974. Mechanoreception. p. 273-352. *In* : The physiology of the insecta. Vol. 2. (Second edit.). The insect and the external environment. Rockstein M. (Ed.). Academic Press, New York. 568 p.

Sevier A. C. and B. L. Munger. 1965. A silver method for paraffin sections of neural tissue. *J. Neuropath. and exp. Neurol.* 24:130-135.

Slifer E. H., J. J. Prestage, and H. W. Beams. 1957. The fine structure of the long basiconic sensory pegs of the grasshopper (Orthoptera, Acrididae) with special reference to those on the antenna. *J. Morphol.* 101:359-397.

Strickler J. R. and A. K. Bal. 1973. Setae of the first antennae of the copepod *Cyclops scutifer* (Sars) : their structure and importance. *Proc. Nat. Acad. Sci. USA* 70:2656-2659.

Thurm U. 1964. Mechanoreceptors in the cuticle of the honeybee. Fine structure and stimulus mechanism. *Science* 145:1063-1065.

Thurm U. 1965. An insect mechanoreceptor. Part I. Fine structure and adequate stimulus. *Cold Spring Harb. Symp. quant. Biol.* 30:75-82.

Toh Y. 1977. The structure of antennal sense organs of the male cockroach, *Periplaneta americana*. *J. Ultrastr. Res.* 60:373-394.

Tyson G. E. and M. L. Sullivan. 1978. Scanning electron microscopy of antennular sensilla of brine shrimp. *Am. Zool.* 18:632. (Abstract 368).

Venable J. H. and R. Coggeshall. 1965. A simplified lead citrate stain for use in electron microscopy. *J. Cell Biol.* 25:407-408.

Walcott C. and M. M. Salpeter. 1966. The effect of molting upon the vibration receptor of the spider (Achaearanea tepidariorum). *J. Morphol.* 119:383-392.

Warren H. S. 1930. The central nervous system of the adult *Artemia salina*. *Trans. Am. Microsc. Soc.* 49:189-209.

Wolfe A. F. 1968. A histological and histochemical study of the male reproductive system of *Artemia* (Crustacea, Branchiopoda). Unpublished doctoral thesis, The University of Vermont, Burlington, Vermont. 81 p.

Wolfe A. F. 1973. Observations on the clasping behavior of *Artemia salina*. *Am. Zool.* 13:1340. (Abstract 472).

Zacharuk R. Y. 1962. Exuvial sheaths of sensory neurons in the larva of *Ctenicera destructor* (Brown). *J. Morphol.* 111:35-47.

# Genetics

*The Brine Shrimp* Artemia. *1980. Vol. 1. Morphology, Genetics, Radiobiology, Toxicology*
*G. Persoone, P. Sorgeloos, O. Roels, and E. Jaspers (Eds). Universa Press, Wetteren, Belgium. 345 p.*

# International Study on *Artemia*[1] II. Genetic characterization of *Artemia* populations – an electrophoretic approach

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**Abstract**

Horizontal starch gel eletrophoresis of bisexual and parthenogenetic *Artemia* populations has been used in a systematic study of genetic differentiation between allopatric populations within the genus. Estimates of genetic distances between bisexual populations are in general agreement with the presently accepted taxonomy of the genus.

Evidence is given for the evolution of the parthenogenetic forms in Europe from the European bisexual species.

Genetic heterogeneity found in the diploid parthenogenetic *Artemia* is discussed in relation to the known aspects of their cytology.

## Introduction

The systematics of the brine shrimp have remained a problem for over ninety years. Following the original description (Linnaeus, 1758) the species was divided into different varieties according to the salinity regimes of their habitats (Schmankewitsch, 1877). Eventually, the effects of salinity on morphology became recognised as non-heritable (Sampter and Heymons, 1902) and true genetic differences began to be studied. Classic work by Artom (Barigozzi, 1974) demostrated the existence of polyploidy in the parthenogenetic forms, contrasting with the diploidy of the bisexual shrimps with 42 chromosomes. His surveys led him to classify the genus into different 'biotypes' based on ploidy and reproductive mechanism. He also distinguished, within the diploid parthenogenetic shrimps, a number of variations in the maturation process of the oocytes. Later studies extended his work to other localities in Europe. (Barigozzi, 1935, 1944, 1974 ; Stefani, 1960, 1967). However, lack of suitable biological criteria made the problem of systematics of parthenogenetic *Artemia* an intractable problem.

[1] International Interdisciplinary Study on *Artemia* Strains coordinated by the Artemia Reference Center, State University of Ghent, Belgium.

[2] Currently under scholarship from the National Council for Science and Technology (CONACYT), Mexico.

Reproductive isolation between bisexual Mediterranean and North American bisexual *Artemia* was reported by Gilchrist (1960) and by Bowen (1965). A third species was described by Halfer-Cervini *et al.* (1968), Piccinelli *et al.* (1968) and by Piccinelli and Prosdocimi (1968). This species was sexually isolated from the first two, had 44 chromosomes but found sympatrically with the European bisexual form in San Bartolomeo, Sardinia and in Salinas Grandes de Hidalgo, Argentina. A more extensive survey by Clark and Bowen (1976) recognized two additional isolated populations in Mono Lake, California, USA and in Lake Urmia, Iran.

TABLE I

*Artemia* populations sampled

| Locality | Country/Region | Abbreviation |
|---|---|---|
| *Bisexual* | | |
| Barbarena-San Fernando | Spain | BAR |
| Bonaire | Carribbean | BON |
| Buenos Aires | Argentina | BA |
| Cabo Frio | Brazil | CF |
| Chaplin Lake | Canada | CH |
| Larnaca Lake | Cyprus | CYP |
| Great Salt Lake 1966[1] | USA | GSL66 |
| Great Salt Lake 1977 | USA | GSL77 |
| Macau | Brazil | MAC |
| Manaure | Colombia | MG |
| Port Araya | Venezuela | PA |
| San Francisco Bay 1971 | USA | SFB71 |
| San Francisco Bay ref. no. 1628[2,3] | USA | SPB |
| San Francisco Bay ref. no. 2265[2] | USA | SFB2265 |
| Adelaide | Australia | SHB |
| Yavaros | Mexico | YAV |
| *Parthenogenetic* | | |
| Burgas Pomerije | Bulgaria | BP |
| Cadiz-San Lucar | Spain | CAD |
| Calpe | Spain | CAL |
| Comacchio | Italy | COM |
| Delta del Ebro | Spain | DE |
| Lake Techirghiol | Rumania | LT |
| Margherita di Savoia | Italy | MS |
| Santa Pola | Spain | SP |
| Sète | France | SET |

[1] Year of processing.

[2] Batch number stamped on can.

[3] Now known to be cysts harvested from San Pablo Bay, north of San Francisco Bay.

Building on the pioneering work of Harris (1966) and Lewontin and Hubby (1966) protein electrophoresis has become a powerful tool with which to examine the genetic composition of biological systems. Various statistical methods have been developed which derive, from

electrophoretic data, estimates of genetic similarities between populations (Nei, 1972 ; Rogers, 1972 ; Thorpe, 1979). This approach has become extremely valuable in systematics and, while deductions closely parallel taxonomy in some better studied groups (Avise, 1974 ; Thorpe, 1979), in some cases new taxa have come to light. The major advantages offered by this technique over orthodox methods are : 1) it relies on objective data ; 2) electrophoretic phenotypes are scored as the direct expression of gene loci and are not susceptible to environmental differences ; 3) there is no need for lengthy breeding tests.

Application of electrophoretic techniques to the systematics of *Artemia* has already been demonstrated by Bowen *et al.* (1978), using haemoglobins,and by Bowen and Sterling (1978) using esterase and malate dehydrogenase. This approach allowed the authors to distinguish between some parthenogenetic populations and between parthenogenetic and bisexual shrimps. We have been interested in measuring genetic distances using electrophoresis and the present work describes data derived from 16 bisexual and 25 parthenogenetic populations (Table I). Twelve enzymes, coded for by a maximum total of 20 loci were assayed. The main purpose of this work was to systematically elucidate the genetical relationships within and between the different *Artemia* biotypes and species.

However, we are also concerned with 1) the connection between the genetic classification of the different *Artemia* races and comparative experiments by other members of the International Study on *Artemia* in the fields of mariculture and biometrics and 2) providing basic genetic data to permit work on adaptation to natural and artificial environments and on selection for characters of economic interest. The results described here will be reported in greater detail elsewhere.

## Materials and methods

### Cultures

As far as possible, cultures larger than 5 000 individuals were kept in modified raceway systems and fed on a suspension of micronised rice bran according to the techniques of Sorgeloos *et al.* (1977, 1980). However, some strains do not respond well to this regime. Difficult strains were kept in smaller populations, of less than 500, and were fed instead with live *Dunaliella tertiolecta* cells. Both culture systems were kept at constant temperature (26-28 °C) and salinity (32-35 ‰). Tests showed that electrophoretic results were not affected by different culture methods.

### Electrophoresis

Homogenates from fresh adults were utilised for horizontal starch gel (12 %) electrophoresis. Three buffer systems were used depending on the enzyme assayed : Tris-EDTA-boric acid (60.57 g, 5.99 g, and 14.0 g/l respectively, pH 8.5 for the electrode compartment, diluted 1:10 for the gel) for phosphoglucomutase (PGM), phosphoglucose isomerase (PGI) and leucine amino peptidase (LAP) ; Tris-EDTA-citric acid (16.35 g, 0.46 g, and 8.26 g/l respectively, pH 7.1 for the electrode ; 16.35 g, 6.9 g, and 9.035 g/l respectively, pH 7.1 for the gel) of F. J. Ayala, (personal communication) for malate dehydrogenase (MDH), lactate dehydrogenase (LDH), 6-phosphogluconate dehydrogenase (6-PGDH), isocitrate dehydroge-

nase (IDH), esterase (EST) and esterase-D (EST-D) ; Poulik and Bearn's (1962) discontinuous tris-citrate for PGI, peptidases (PEP), malic enzyme (ME), tetrazolium oxidase (TO) and catalase (CAT). Staining methods were taken from Shaw and Prasad (1970) and Harris and Hopkinson (1976).

### Methods for calculation of genetic similarities between populations

There are various methods available which convert allele and genotype frequencies, derived from electrophoretic data, into a common 'meter' of genetic relationships between populations. The one proposed by Nei (1972) was chosen for this survey mainly because it has become so widely employed that it allowed us to make comparisons between our results and those previously published for various other taxa. According to this method, the normalised genetic identity of genes between two populations at the *j*th locus is defined as :

$$Ij = \frac{\Sigma xiyi}{(\Sigma xi^2 yi^2)^{\frac{1}{2}}}$$

wher $x_i$ and $y_i$ represent the frequencies of the ith allele in populations X and Y respectively. For all loci in a survey, the overall genetic identity of X and Y becomes :

$$I = \frac{Jxy}{(JxJy)^{\frac{1}{2}}}$$

where $J_x$, $J_y$ and $J_{xy}$ are the arithmetic means over all loci of $x_i$, $y_i$, and $x_iy_i$, respectively.

This method was used for both the bisexual and the parthenogenetic populations comparisons. However, when comparing parthenogenetic populations it became apparent that a simpler and, perhaps, more obvious approach was possible. This consisted in calculating the theoretical minimum number of mutations which would be necessary to interconvert two genotypes. This technique, admittedly also gives an underestimate of genetic distance particularly as no allowances are made for different levels of ploidy. However, in the electrophoretic phenotypes of the two known tetraploids in our sample the same alleles at all loci were present as in strains known to be diploids. An overall measure of genetic distance can then be calculated from the sum of the values from all of the loci studied. Fig. 1 describes the method.

The resulting matrices for either method were fed into a computer program system devised by Wishart (1970) which performs an unweighted pair-group cluster analysis (Sokal and Sneath, 1963). Similarity or distance phenetic dendrograms were constructed from the results. In order to be able to take these as representing evolutionary trees, it was necessary to assume that the overall rate of evolutionary divergence is homogeneous over the phyletic lines.

## Results

The similarity dendrogram between the *Artemia* populations studied (Fig. 2) clearly distinguishes three major branches : the Argentinian form, the remaining American races, together with the Australian sample ; and the European populations.

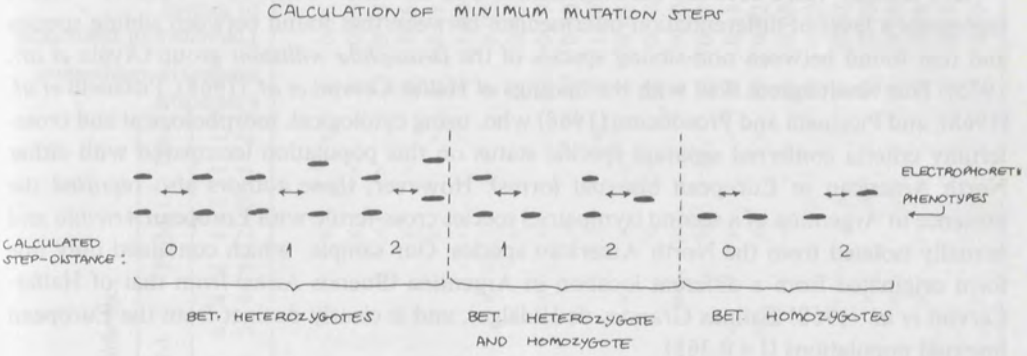


FIG. 1. Scheme for calculating the minimum number of mutations necessary to interconvert two observed genotypes.

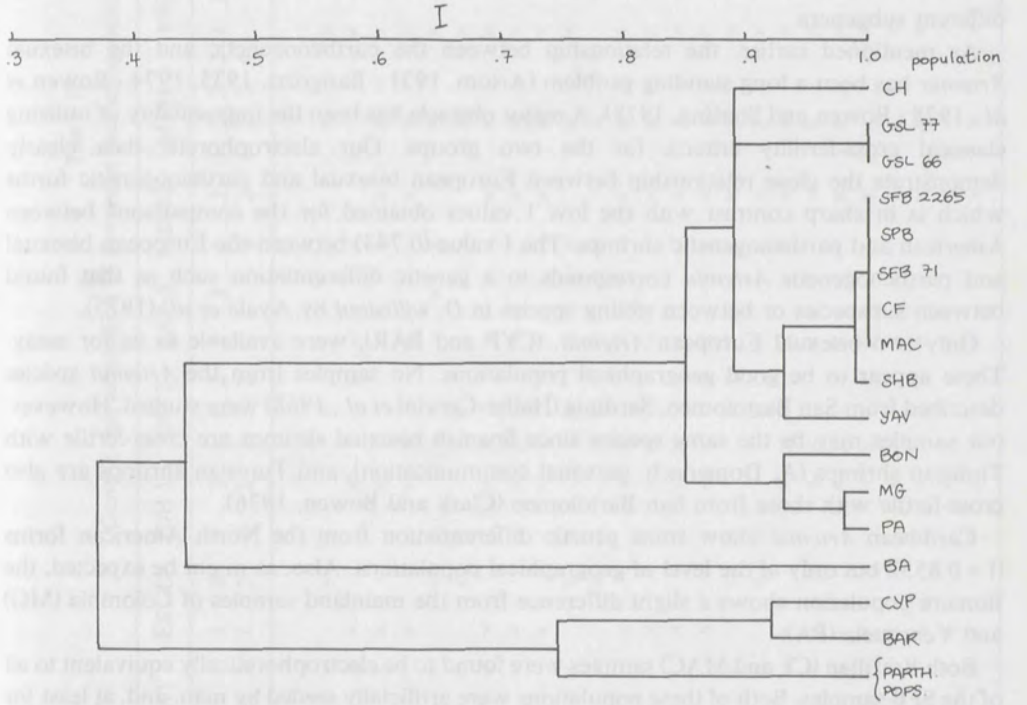


FIG. 2. Similarity dendrogram of *Artemia* populations based on unweighted pair-group average cluster analysis of genetic identities (I's, after Nei, 1972).

Nei's identity value between the Argentinian and the other American samples (I = 0.434) represents a level of differentiation intermediate between that found between sibling species and that found between non-sibling species of the *Drosophila willistoni* group (Ayala *et al.*, 1975). This result agrees well with the findings of Halfer-Cervini *et al.* (1968), Piccinelli *et al.* (1968), and Piccinelli and Prosdocimi (1968) who, using cytological, morphological and cross-fertility criteria conferred separate specific status on this population (compared with either North American or European bisexual forms). However, these authors also reported the presence in Argentina of a second (sympatric) species cross-fertile with European *Artemia* and sexually isolated from the North American species. Our sample, which contained only one form originated from a different location in Argentina (Buenos Aires) from that of Halfer-Cervini *et al.* (1968) (Salinas Grandes de Hidalgo), and is clearly distinct from the European bisexual populations (I = 0.368).

One further interesting aspect about the results for the BA sample was that it was the only population to show two PGI loci. It would seem quite likely, considering the evidence of Halfer-Cervini *et al.* (1968) for the existence of an extra chromosome pair in the Argentinian shrimps, that the PGI locus is on this particular chromosome pair.

The very low values of I between European and American populations indicate a degree of genetic divergence corresponding in other groups to different non-sibling species or even to different subgenera.

As mentioned earlier, the relationship between the parthenogenetic and the bisexual *Artemia* has been a long standing problem (Artom, 1931 ; Barigozzi, 1935, 1974 ; Bowen *et al.*, 1978 ; Bowen and Sterling, 1978). A major obstacle has been the impossibility of utilizing classical cross-fertility criteria for the two groups. Our electrophoretic data clearly demonstrate the close relationship between European bisexual and parthenogenetic forms which is in sharp contrast with the low I values obtained for the comparisons between American and parthenogenetic shrimps. The I value (0.744) between the European bisexual and parthenogenetic *Artemia* corresponds to a genetic differentiation such as that found between subspecies or between sibling species in *D. willistoni* by Ayala *et al.* (1975).

Only two bisexual European *Artemia*, (CYP and BAR), were available to us for assay. These appear to be good geographical populations. No samples from the *Artemia* species described from San Bartolomeo, Sardinia (Halfer-Cervini *et al.*, 1968) were studied. However, our samples may be the same species since Spanish bisexual shrimps are cross-fertile with Tunisian shrimps (A. Domenech, personal communication), and Tunisian shrimps are also cross-fertile with those from San Bartolomeo (Clark and Bowen, 1976).

Caribbean *Artemia* show some genetic differentiation from the North American forms (I = 0.853), but only at the level of geographical populations. Also, as might be expected, the Bonaire population shows a slight difference from the mainland samples of Colombia (MG) and Venezuela (PA).

Both Brazilian (CF and MAC) samples were found to be electrophoretically equivalent to all of the SFB samples. Both of these populations were artificially seeded by man, and, at least for the MAC population there is evidence of this. (P. Sorgeloos, personal communication).

Differentiation from SFB by the Mexican (YAV) and the Canadian (CH) populations has been mainly due to fixation of one allele at five normally highly polymorphic loci (CAT, 6PGDH, PGI, IDH-1, and PGM) in the YAV locality and due to complete fixation of a unique

TABLE II

Genetic characterisation of European parthenogenetic populations – Variable loci

| Locus | | CAT | | | EST-D | | IDH-1 | | IDH-2 | | | LAP-3 | | MDH-1 | | MDH-2 | | | | ME | | 6PGDH | | | | PGI-1 | | | PGM-1 | | | TO | | Composite phenotype designation | Frequency of phenotype in locality | Proportion of total loci heterozygous | Putative ploidy |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Phenotype | | 1 1 | 1 2 | 1 3 | 1 1 | 1 2 | 1 3 | 2 2 | 1 1 | 1 3 | 2 2 | 1 | 2 | 1 2 | 2 2 | 1 1 | 2 2 | 3 3 | 2 4 | 1 | 2 | 1 2 | 2 3 | 2 2 | 2 2 | 3 3 | 2 4 | 1 5 | 4 4 | 1 1 | 1 2 | 1 3 | 1 1 | 1 2 | | | |
| Population | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| MS | I | | | × | | × | × | | | × | | × | | × | | | × | | | × | | × | | | | × | | | × | | | | × | A | 0.95 | 0.47 | 2n |
| | II | | | × | × | | | × | | | × | × | | × | | × | | | | × | | | | × | | × | | | × | | × | | | B | 0.05 | 0.16 | 2n |
| SET | I | | | × | × | | | × | × | | | × | | | × | | × | | | × | | × | | | | × | | | × | | × | | | C | 0.53 | 0.16 | 2n |
| | II | | | × | × | | | × | × | | | × | | | × | | × | | | × | | × | | | | × | | | × | | × | | | D | 0.26 | 0.16 | 2n |
| | III | | | × | | × | × | | | × | | | × | × | | | × | | | × | | × | | | | × | | | × | | | | × | E | 0.21 | 0.47 | 2n |
| COM | | | | × | | × | × | | | × | | × | | × | | | × | | | × | | × | | | | × | | | × | | | | × | A | 1.00 | 0.47 | 4n |
| CAD | I | | | × | × | | | × | × | | | × | | | × | | × | | | × | | × | | | | | | × | | × | | | | F | 0.05 | 0.11 | 2n |
| | II | | | × | × | | | × | × | | | × | | | × | | × | | | × | | × | | | × | | | | | × | | | | G | 0.95 | 0.16 | 2n |
| SP | I | × | | | × | | | × | × | | | × | | | × | | × | | | × | | × | | | | | | | × | | × | | | | K? | 0.10 | 0.11 | 2n |
| | II | | | × | | × | × | | | × | | × | | × | | | × | | | × | | × | | | | × | | | | | × | | | | A? | 0.90 | 0.44 | 2n |
| DE | | | | × | | × | × | | | × | | × | | × | | | × | | | | × | × | | | | × | | | | | × | | | × | H | 1.00 | 0.47 | 4n |
| CAL | I | × | | | × | | | × | × | | | × | | | × | | × | | | × | | | | × | | | | × | | × | | × | | | I | 0.05 | 0.05 | 2n |
| | II | × | | | × | | | × | × | | | × | | | × | | × | | | × | | | | × | | | × | | | × | | × | | | J | 0.10 | 0.11 | 2n |
| | III | × | | | × | | | × | × | | | × | | | × | | × | | | × | | × | | | | | | × | | × | | | | | K | 0.70 | 0.11 | 2n |
| | IV | × | | | × | | | × | × | | | × | | | × | | × | | | × | | × | | | | × | | | | × | | × | | | L | 0.15 | 0.16 | 2n |
| BP | I | | | × | | × | × | | | × | | × | | | × | | × | | | × | | | | × | | | × | | | × | | | | × | M | 0.18 | 0.37 | ? |
| | II | | | × | | × | × | | | × | | × | | | × | | × | | | × | | × | | | | | × | | | × | | | | × | N | 0.82 | 0.42 | ? |
| LT | I | | | × | × | | | × | × | | | × | | | × | | × | | | × | | × | | | × | | | | | × | | | | | G | 0.05 | 0.17 | ? |
| | II | | | × | × | | | × | × | | | × | | | × | | × | | | × | | × | | | × | | | | | | × | | | | O | 0.03 | 0.17 | ? |
| | III | | | × | × | | | × | × | | | × | | | × | | × | | | × | | × | | | | × | | | | | × | | | | P | 0.63 | 0.22 | ? |
| | IV | | × | | × | | | × | × | | | × | | | × | | | | × | × | | | × | | | | | | × | | | × | | | Q | 0.03 | 0.22 | ? |
| | V | | × | | × | | | × | × | | | × | | | × | | | × | | × | | × | | | | | | | × | | | × | | | R | 0.08 | 0.17 | ? |
| | VI | | × | | × | | | × | × | | | × | | | × | | × | | | × | | | × | | | | | | × | | | × | | | S | 0.08 | 0.17 | ? |
| | VII | | | × | × | | | × | × | | | × | | | × | | × | | | × | | | × | | × | | | | | | | × | | | T | 0.05 | 0.17 | ? |
| | VIII | | | × | × | | | × | × | | | × | | | × | | × | | | × | | × | | | | | | | × | | | × | | | U | 0.05 | 0.17 | ? |

allele at the 6PGDH locus in the CH sample. Both of these peculiarities could be the result of founder effects or genetic drift.

Differentiation between the GSL and the SFB shrimps, on the other hand, seems to have occurred through greater genetic variability in the former. For example, 40% of the loci in the GSL samples are polymorphic (frequency of the common allele less than or equal to 0.99), compared with only 30% in the SFB samples ; the average heterozygosity (Nei, 1975) of the GSL samples is $0.124 \pm 0.044$ while that of the SFB samples is $0.089 \pm 0.039$. (Abreu-Grobois and Beardmore, in preparation).

Five of the loci (IDH-1, IDH-2, LAP-3, PGM-1, TO) were found to be capble of distinguishing (with frequency overlap of less than 0.01) between the three main branches of our dendrogram. Ayala and Powell (1972) in a similar work studying 28 loci in *Drosophila* found 15-35% of the loci to be diagnostic for any two sibling species.

### Parthenogenetic *Artemia*

Of the nine localities sampled for parthenogenetic *Artemia*, only two did not contain more than one genotype (Table II). Both of these, COM and DE, were tetraploid forms (Barigozzi, 1974 and Amat Domenech, personal communication respectively) had the highest levels of heterozygous loci (47%) and no observed genetic variation. Other populations contained at least two clones, though as many as eight were observed in the Rumanian sample (LT). Quite possibly more will be found in this last population with a larger sample size. The localities with more clones had, in general, lower proportions of fixed heterozygosity (17 to 22%).

Cluster analysis of the mutation-step analysis clearly shows three major clusters (Fig. 3). Some clones (for example in LT and CAL) appeared to be more closely related to clones in the same locality, while others (*e.g.* MSI, SPII, DE, COM, SETIII), were found to be closer to those of geographically distant localities. In fact, the MSI, COM and SPII clones appeared to be identical at the loci tested. Two points should be emphasized here, however, 1) electrophoretic bands of a given mobility may not be entirely homogeneous and may represent more than one allele, consequently leading to an underestimate of genetic distance (Avise, 1974 ; Lewontin, 1974) and 2) only qualitative distinctions were made in gel scoring, no consideration being given to effects caused by polyploidy on relative intensities of allozyme bands in heterozygotes (*i.e.* $A_3B_1$ would have been classified as $A_2B_2$, *etc.*).

The far left cluster contains both tetraploid populations and all of the clones with the highest proportion of loci fixed for heterozygotes. The second, and most numerous cluster, contains the most variable populations and the clones with the lowest proportion of loci fixed for heterozygotes. The I value between these two clusters is 0.841, corresponding to a genetic differentiation between subpopulations as seen in *D. willistoni* (Ayala *et al.*, 1975). The last cluster contains only one genotype wich was observed in the MS sample at a very low frequency (0.05).

Two males found in the LT population were also assayed. These showed genotypes consistent with those in the otherwise all-female population. These results are confirmation that parthenogenetic populations are capable of producing males, perhaps through a homogenetic fusion of second division haploid nuclei as proposed by Stefani (1964).

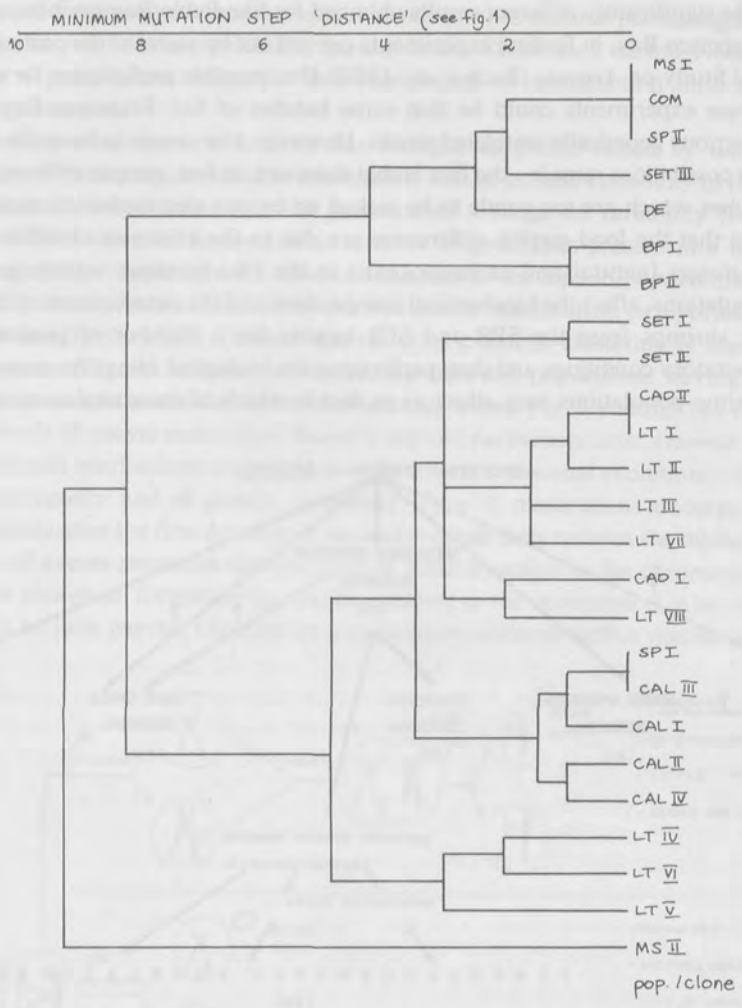


FIG. 3. Distance dendrogram of parthenogenetic *Artemia* clones based on unweighted pairgroup average analysis of 'mutational steps' between them (see Materials and methods).

## Discussion and conclusions

The very high genetic identity value obtained for the comparison between the Australian and the San Francisco Bay samples is good evidence in favor of the common belief that *Artemia* was artificially introduced to that continent from North America. Clark and Bowen (1976) made reference to this problem and showed that Australian bisexual *Artemia* were cross-fertile with SFB *Artemia*.

Virtual genetic identity was also seen between the San Pablo Bay and the San Francisco Bay shrimps from two different years, 1971 and 1976. These results are extremely interesting

because of the significantly different results obtained for San Pablo Bay samples, as compared with San Francisco Bay, in feeding experiments carried out by some of the participants of the International Study on *Artemia* (Beck *et al.*, 1980). One possible explanation for the different results in these experiments could be that some batches of San Francisco Bay cysts are a mixture of various genetically unrelated stocks. However, this seems to be unlikely from our results. Two possibilities remain : the first is that there are, in fact, genetic differences between the two batches which are too subtle to be picked up by our electrophoretic technique ; and the second is that the food quality differences are due to the existence of different environmental parameters (natural and anthropogenic) in the two localities which, acting on the parental populations, affect the biochemical composition and the development of the embryos.

Culturing shrimps from the SPB and SFB locality for a number of generations under standard laboratory conditions and then performing the biological assays on samples from the resulting shrimp populations may allow us to decide which of these explanations is correct.

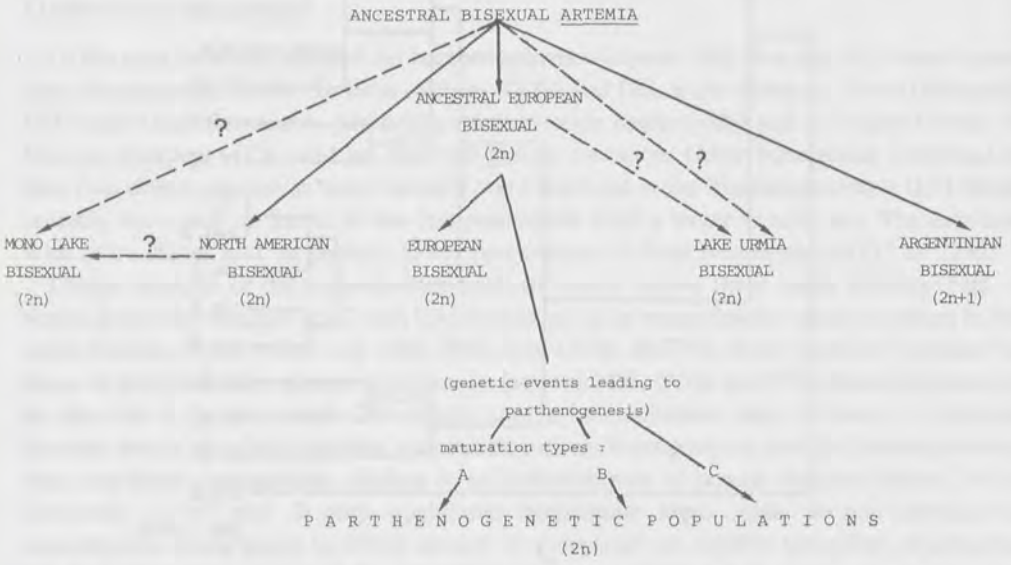


FIG. 4. Proposed scheme for the evolution of the genus *Artemia* (broken lines link populations not studied by us and whose relationship to the others is still unquantified).

Comparison of our results for bisexual *Artemia* with previous work by Clark and Bowen (1976), Bowen and Sterling (1978), and Bowen *et al.* (1978), and Bowen *et al.* (1980) demonstrates very close agreement in the genetic classification of the populations sampled. However, we cannot categorically state that our European samples are identical with the '*A. tunisiana*' of Bowen's terminology until we compare San Bartolomeo and Tunisian shrimp through our assay methods. We have derived from our data a modification of Artom's (1931) scheme of the evolution of the various forms in *Artemia* (Fig. 4) integrating the recent findings of the speciation of the genus. In this tree we describe the origin of parthenogenesis as monophyletic, derived, as we have given evidence for, from European bisexual shrimps. However,

we only sampled European parthenogenetic *Artemia* and, as natural parthenogenetic populations are also known to exist outside Europe (*e.g.* India, China, *etc.*) we must leave open the possibility for a polyphyletic origin for this mechanism of reproduction until these other populations are assayed.

Our work on parthenogenetic *Artemia* has brought up points raised by Ballardin and Metalli (1972) concerning the levels of heterozygosity and of genetic variability in these forms. The electrophoretic data demonstrate the presence of a range of variability levels in the different populations sampled (measured as number of genotypes present in a locality, see Table II). The genotypic heterogeneity observed in some of the diploid populations may well be due to the existence of more than one oocyte maturation mechanism, as reported by Artom (1931) and Stefani (1960, 1967). Further, the resulting phenetic clustering of the parthenogenetic populations (Fig. 3) indicates a relationship between populations having particular heterozygosity levels and particular genotypic heterogeneities. Fig. 5 summarises the various described methods of oocyte maturation found in diploid parthenogenetic *Artemia* (Barigozzi, 1974). The different mechanisms may lead to different chromosomal evolutions affecting the levels of heterozygosity and of genetic variability. Type A maturations undergo fusion of nuclei immediately after the first division. A second division then restores the diploid number. This sequence of events promotes the retention of heterozygosity in the chromosomal areas not involved in chiasmata formation (*i.e.* the loci nearest to the centromere). Also, one would expect there to be little genetic variation in a population utilising such a mechanism.

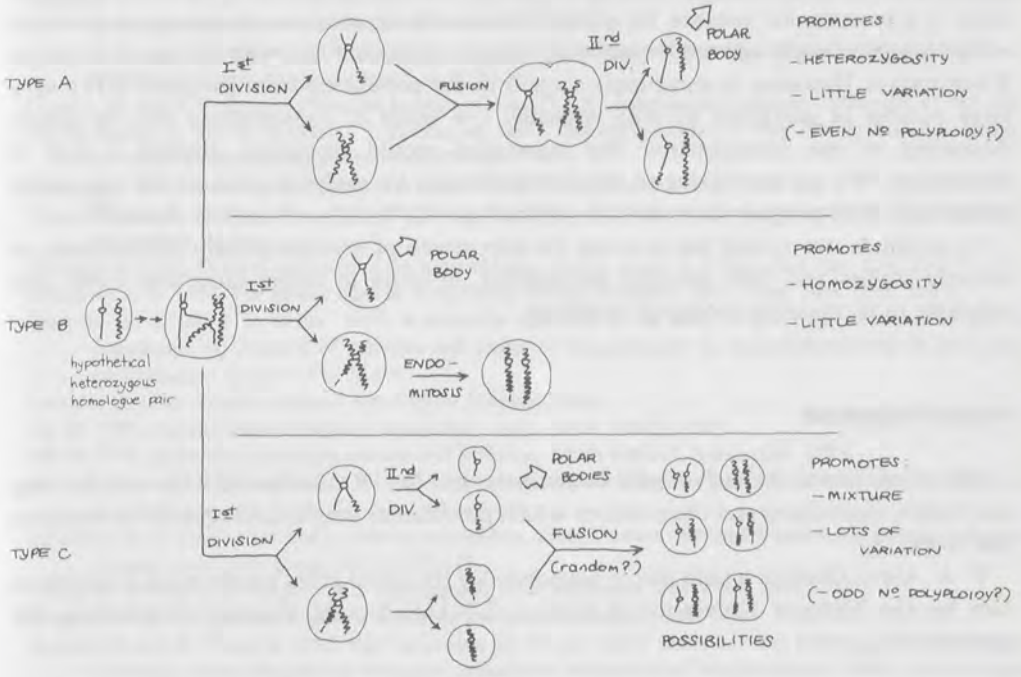


FIG. 5. Described mechanisms of oocyte maturation found in diploid parthenogenetic *Artemia* (Barigozzi, 1974).

Type B, on the other hand, restores the normal diploid number by an endomitotic process following the first division. This forces an obligatory homozygosity in the chromosome areas outside the chiasmata and, again, leads to low levels of genetic variability.

The last maturation mechanism undergoes the two divisions without fusion, producing four haploid oocyte nuclei. Fusion of a pair will produce the developing embryo while the rest are shed as polar bodies. In this case, however, there is a possibility for a much greater genetic heterogeneity than the previous two and also the levels of heterozygosity of a population using this maturation type would be expected to be much more variable.

Putting together our data on heterozygosity and genetic variability for the parthenogenetic populations and the schemes described above, we have derived a working hypothesis allowing us to utilise electrophoretic results to distinguish between the maturation types in any specific population of diploid asexual *Artemia*.

Those diploid genotypes which cluster together with the tetraploid populations (DE and COM) all have high levels of heterozygosity. We suggest that these shrimps undergo type A egg maturation which theoretically promotes the preservation of heterozygosity. Furthermore, as polyploidy must necessarily pass through a diploid phase and considering the close genetic relationship between the diploid forms of this cluster and the tetraploids, we would also tentatively advance the idea that this type of mechanism is the one likely to engender tetraploidy.

The remaining populations, we would suggest, undergo either type B or type C maturations. In most cases, our data do not permit us to ditinguish clearly between these two types. It is possible, for instance, for a type C mechanism to produce a homozygous genotype which is subsequently selected for and may henceforth be confused with the results of a type B maturation. However, in some cases such as in our population from Rumania (LT) a very large number of genotypes all with relatively low levels of homozygosity may be found. According to our interpretation this population would reproduce through a type C mechanism. We are now going to test our deductions by studying cytologically the oocyte maturation from progeny derived from parthenogenetic females of specific clusters.

To conclude, we would like to stress the importance of interdisciplinary collaboration in *Artemia* studies and their practical applications, in order to obtain integrated views and solutions to its manifold biological problems.

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## Literature cited


ARTOM C. 1931. L'origine e l'evoluzione della partenogenesi attraverso i differenti biotipi di una specie collettiva (*Artemia salina* L.) con speciale riferimento al biotipo diploide partenogenetico di Sète. *Mem. Reale Accad. Ital. Cl. Sci. Fis. Mat. Nat.* 2:1-57.

AVISE J. C. 1974. Systematic value of electrophoretic data. *Syst. Zool.* 23:465-481.

AYALA F. J. and J. R. POWELL. 1972. Allozymes as diagnostic charactersof sibling species of *Drosophila*. *Proc. Natn. Acad. Sci. USA* 69:1094-1096.

AYALA F. J., M. L. TRACEY, D. HEDGECOCK, and R. C. RICHMOND. 1975. Genetic differentiation during the speciation process in *Drosophila*. *Evolution* 28:576-592.

BALLARDIN E. and P. METALLI. 1972. Osservazioni sulla trasmissione di caratteri in *Artemia salina* partenogenetic. *Boll. Zool.* 39(4):551-564.

BARIGOZZI C. 1935. Il legame genetico fra i biotipi partenogenetici di *Artemia salina*. *Arch. Zool. Ital.* 22:33-77.

BARIGOZZI C. 1944. I fenomeni cromosomici delle cellule gerinali in *Artemia salina* Leach. *Chromosoma* 2:549-575.

BARIGOZZI C. 1974. *Artemia* : a survey of its significance in genetic problems. *Evol. Biol.* 7:221-252.

BECK A. D., D. A. BENGTSON, and W. H. HOWELL. 1980. International Study on *Artemia*. V. Nutritional value of five geographical strains of *Artemia* : effects on survival and growth of larval Atlantic silverside *Menidia menidia*. p. 249-259. *In* : The brine shrimp *Artemia*. Vol. 3. Ecology, Culturing, Use in Aquaculture. Persoone G., P. Sorgeloos, O. Roels, and E. Jaspers (Eds). Universa Press, Wetteren, Belgium. 456 p.

BOWEN S. T. 1965. The genetics of *Artemia salina*. V. Crossing over between x and y chromosomes. *Genetics* 52(3):695-710.

BOWEN S. T., M. L. DAVIS, S. R. FENSTER, and G. A. LINDWALL. 1980. Sibling species of *Artemia*. p. 155-167. *In* : The brine shrimp *Artemia*. Vol. 1. Morphology, Genetics, Radiobiology, Toxicology. Persoone G., P. Sorgeloos, O. Roels, and E. Jaspers (Eds). Universa Press, Wetteren, Belgium. 345 p.

BOWEN S. T., J. P. DURKIN, G. STERLING, and L. S. CLARK. 1978. *Artemia* haemoglobins : genetic variation in parthenogenetic and zygogenetic populations. *Biol. Bull.* 155-273-287.

BOWEN S. T. and G. STERLING. 1978. Esterase and malate dehydrogenase isozyme polymorphisms in 15 *Artemia* populations. *Comp. Biochem. Physiol.* 61 B:593-595.

CLARK L. S. and S. T. BOWEN. 1976. The genetics of *Artemia*. VII. Reproductive isolation. *J. Heredity* 67:385-388.

HALFER-CERVINI A. M., M. PICCINELLI, T. PROSDOCIMI, and L. BARATELLI ZAMBRUNI. 1968. Sibling species in *Artemia* (Crustacea Branchiopoda). *Evolution* 22:373-381.

HARRIS H. 1966. Enzyme polymorphisms in man. *Proc. Roy. Soc. Ser. B.* 164:298-310.

HARRIS H. and D. H. HOPKINSON. 1976. Handbook of enzyme electrophoresis in human genetics. North-Holland, Amsterdam. 354 p.

GILCHRIST B. 1960. Growth and form of the brine shrimp, *Artemia salina* (L.). *Proc. Zool. Soc.* 134:221-235.

KEWONTIN R. C. 1974. The genetic basis of evolutionary change; Columbia Univ. Press, New York. 346 p.

LEWONTIN R. C. and J. L. HUBBY. 1966. A molecular approach to the study of genic heterozygosity in natural populations. II. Amount of variation and degree of heterozygosity in natural populations of *Drosophila pseudoobscura*. *Genetics* 54:595-609.

LINNAEUS C. 1758. *Systema naturae*. Xth Edition. Hafniae. 634 p.

NEI M. 1972. Genetic distance between populations. *Amer. Natur.* 106:283-292.

NEI M. 1975. Molecular population genetics and evolution. North-Holland, Amsterdam. 279 p.

PICCINELLI M. and T. PROSDOCIMI. 1968. Descrizione tassonomica delle due specie *Artemia salina* L. e *Artemia persimilis n.sp. Inst. Lomb. Sci. Lett.* 102:113-118.

PICCINELLI M., T. PROSDOCIMI, and L. BARATELLI ZAMBRUNI. 1968. Ulteriori ricerche sull'isolamento genetico nel gerne *Artemia*. *Atti. Assoc. Genet. Ital.* 13:170-179.

POULIK M. D. and A. G. BEARN. 1962. Heterogeneity of ceruloplasmin. *Clin. Chim. Acta* 7:374.

ROGERS J. S. 1972. Measures of genetic similarity and genetic distances. *Univ. Texas. Publ.* 7213:145-153.

SAMPTER M. and R. HEYMONS. 1902. Die Variationen bei *Artemia salina* Leach und ihre Abhangigkeit von ausseren Einflüssen. *Abhandlungen der königlich preussischen Akademie der Wissenschaften, Berin. Abhandlungen nicht zur Akademie gehöriger*. Gelehter 3:1-62.

SCHMANKEWITSCH W. J. 1877. Zur Kenntniss des Einflusses der ausseren Lebensbedingungen auf die Organisation der Tiere. *Z. Wiss. Zool., Leipzig* 29(4):429-494.

Shaw C. R. and R. Prasad. 1970. Starch gel electrophoresis of enzymes – a compilation of recipes. *Biochem. Genetics* 4:297-320.

Sokal R. R. and P. H. A. Sneath. 1963. Principles of numerical taxonomy. W. H. Freeman, San Francisco. 573 p.

Sorgeloos P., M. Baeza-Mesa, E. Bossuyt, E. Bruggeman, J. Dobbeleir, D. Versichele, E. Laviña, and A. Bernardino. 1980. The culture of *Artemia* on rice bran : the conversion of a waste-product into highly nutritive animal protein. *Aquaculture*. (in press).

Sorgeloos P., M. Baeza-Mesa, C. Claus, G. Vandeputte, F. Benijts, E. Bossuyt, E. Bruggeman, G. Persoone, and D. Versichele. 1977. *Artemia salina* as live food in aquaculture. p. 37-46. *In*: Fundamental and applied research on the brine shrimp *Artemia salina* L. in Belgium. European Mariculture Society Special Publication No. 2. Jaspers E., and G. Persoone (Eds). EMS, Bredene, Belgium. 110 p.

Stefani R. 1960. L'*Artemia salina partenogenetica* a Cagliari. *Riv. Biol.* 52:463-491.

Stefani R. 1964. L'origine del maschi nelle popolazioni partenogenetiche di *Artemia salina*. *Riv. Biol.* : 147-162.

Stefani R. 1967. La maturazione dell'uovo nell'*Artemia salina* di Sète. *Riv. Biol.* 60:599-615.

Thorpe J. P. 1979. Enzyme variation and taxonomy : the estimation of sampling errors of measurements of interspecific genetic similarity. *Biol. J. Linn. Soc.* 11(4):369-386.

Wishart D. 1970. Clustan IA computer programs, Computing Lab., Univ. of St. Andrews, Five, Scotland. 119 p.

*The Brine Shrimp* Artemia. *1980. Vol. 1. Morphology, Genetics, Radiobiology, Toxicology*
*G. Persoone, P. Sorgeloos, O. Roels, and E. Jaspers (Eds). Universa Press, Wetteren, Belgium. 345 p.*

# Genus *Artemia* : problems of systematics

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**Abstract**

*Artemia salina* Leach has for a long time been the only species belonging to the genus, described on material collected at Lymington (England). The observation of morphological characters and of the mode of reproduction gave rise first to the recognition of a number of variants and to a complicated nomenclature with many synonymous names. Later, the study of the chromosomes opened new possibilities to the systematics of the genus. More recently, the analysis of crosses brought about in the laboratory revealed the existence of several sibling species. With each species a polymorphism of haemoglobins, slow proteins and enzymes has been found.

All these data lead the genus *Artemia* to be considered as subdivided into a number of bisexual sibling species and a parthenogenetic entity, which is not homogenous as far as the chromosomal phenomena are concerned.

The author concludes that the sibling species must be named according to the international convention and that the denomination *A. salina* should be used only for the original material upon which the first description was made (Lymington, England) and for that studied by Artom in Cagliari (Sardinia).

It is proposed that the parthenogenetic complex to called *Artemia parthenogenetica*, followed by the indication of locality where the sample has been collected.

This paper does not present new data, but aims to discuss several problems with regard to the genus *Artemia* and to propose a reorganization of its systematics, taking advantage of the many contributions obtained using several techniques. I wish to say at once that my conclusions support the view already expressed by Bowen *et al.* (1978) and by Bowen and Sterling (1978).

Since the interest of biologists in the brine shrimp is not new, I think it useful to summarize in Table I the techniques used and the problems they trew light on in the course of two centuries. The first period begins with the first description of the species in 1755 by Schlosser, named by Linné *Cancer salinus* in 1778, and renamed *Artemia salina* by Leach in 1819 (quoted from Artom, 1931). For many years, *A. salina* was known as a bisexual species and many morphological observations led to distinguish different entities and variations under the influence of salt concentration. Joly (quoted from Artom, 1931) found in 1840 that the brine shrimps living in Villeneuve (southern France) were parthenogenetic. Von Siebold (1873) found the same mode of reproduction in the brine-shrimps of Capodistria, and, a few years later, in 1877 (Von Siebold, 1977), started by experimental study of *Artemia*, by breeding in the laboratory material from the Great Salt Lake of Utah (called *A. fertilis*). Von Siebold (1877) proved that non fertilized eggs of the Utah *Artemia* do not develop. This interesting

experimental approach, however, was not resumed until much later. The morphological observations led to make distinctions between specimens of different origin, and this gave rise to many synonymous terms, one of which deserves to be quoted here : *A. gracilis*, used as early as 1869 by Verrill (1869), atributed to the Californian *Artemia*, keeping the name *salina* for the European brine shrimp. Verrill (1869) also described *A. monica*, the brine shrimps of Mono Lake, California. All these names did not lead to split the species, all the variants being included under the denomination of *A. salina*.

TABLE I

Overview of *Artemia* research

| Techniques | Main Authors | Approximate time period | Main biological problems approached |
|---|---|---|---|
| Microscopy : description of morphological characters, reproduction | Schlosser<br>Linné<br>Leach<br>Von Siebold<br>Verrill | 1775<br><br><br><br>1865 | Morphology and its variation, modes of reproduction |
| Cytology and genetics on collected and cultivated shrimps | Brauer<br>Artom<br>Kuenen<br>Barigozzi's group<br>Stefani<br>Bowen | 1893<br><br><br>(Piccinelli *et al.*)<br><br>1976 | Polyploidy, meiosis in bisexual parthenogenetic strains, chromosomal control of cell size, sex determination, mutants, reproductive isolation |
| Biochemical techniques : electrophoresis of hemoglobins and enzymes | Bowen's group<br>Moens and Kondo | 1969<br>1978 | Polymorphisms, determination of gene flow within and between populations, fine analysis of the genotype |

It is to be noticed that the characters considered are more or less strongly influenced by the environment, thus they are not very reliable to describe systematic entities.

A turning point in *Artemia* research came with the study of the chromosomes. Brauer (1893) found that parthenogenetic *Artemia* from Capodistria (Adriatic Sea) had 84 chromosomes, but only Artom (1905, 1906, 1907, 1911, 1912) saw that both chromosome number and mode of reproduction could provide new elements for a revision of the systematics of *Artemia*. After having discovered that the bisexual *Artemia* of Cagliari was diploid since it has 42 chromosomes (this being the first case of polyploidy found in animals), he also found a correlation between chromosome number and both nuclear and cellular size. The parthenogenetic shrimps (the tetraploid ones) with 84 chromosomes have larger nuclei and cells and exhibit also a moderate body gigantism in comparison with the bisexual form, which has 42

chromosomes. Thus Artom proposed to distinguish, within the species *A. salina*, two subspecies : *A. salina univalens* with 42 chromosomes and *A. salina bivalens* with 84. This distinction, however, was never accepted and Artom himself did not insist to use it. In fact the later discovery, again by Artom (1931), of a diploid parthenogenetic *Artemia* living at Sète (southern France) and the possible (and never clarified) existence of bisexual tetraploids at Odessa, made the distinction between *univalens* and *bivalens* almost meaningless, because it fails to show any connection with the mode of reproduction which is clearly important for a definition of the species based on genetic elements. Artom (1931), however, has the merit of having considered the species *A. salina* as a collective species ; he called "biotypes" the different forms distinguishable according to both chromosome number and mode of reproduction.

We can disregard, for the moment, the analysis of the different types of meiosis in parthenogenetic shrimps (Barigozzi, 1944 ; Stefani, 1960), the chromosomal mechanism of sex determination (Stefani, 1963 ; Bowen, 1963ab, 1965) and the discovery of several mutants (Bowen *et al.* 1966 ; Barigozzi, 1974) because they are not related to the aim of the present paper. On the other hand, the paper by Kuenen (1939) is very important because it provides the first example of sexual isolation within *A. salina* between two bisexual forms, *i.e.* the Californian and the Sardinian. This study required the breeding of *Artemia* in the laboratory, which is now a routine procedure. After Kuenen's (1939) work, the splitting of the genus *Artemia* (or, if you prefer, of the species *A. salina*) into sibling species has appeared to be the most important phenomenon in the speciation of the brine shrimp. Gilchrist (1960) found sexual isolation between *Artemia* from California and *Artemia* from North Africa. Bowen (1965) found sexual isolation between Californian and Sardinian *Artemia*. Halfer Cervini *et al.* (1968) found sexual isolation between shrimps living in the same salterns (San Bartolomeo, Sardinia and Hidalgo, Mexico). The new sibling species was described by Piccinelli and Prosdocimi (1968), distinguished by 44 chromosomes instead of 42, but morphologically almost indistinguishable, it was called *Artemia persimilis*. New data relating to interpopulational crosses were given by Bowen *et al.* (1978), thus increasing the list of the sibling species.

Within the species, polymorphisms have been found for hemoglobins, enzymes and slow proteins (Bowen *et al.*, 1978 ; Bowen and Sterling, 1978).

At the end of this review, it is clear that *Artemia* comprises a number of sibling species, the majority of which have 42 chromosomes, and a number of parthenogenetic variants, with different types of meiosis and different degrees of ploidy.

Integrating the results of the investigation carried out so far and considering them critically from the standpoint of the internationally accepted taxonomic rules, we can now proceed to reorganize the genus *Artemia*, principally in order to define in which case the use of the denomination *salina* is justified. The genus seems to be composed of two sections. One comprises the bisexual sibling species, which can be determined primarily by means of crosses and of chromosome analysis. The other section, the parthenogenetic complex, obviously is not open to the first method, and has to be considered in a different manner.

The present stage of the situation is shown in Table II. The sibling species described and named so far are seven in number one of them (*A. odessensis*) being problematic. The remainders have 42 chromosomes, except *A. persimilis* with 44. *A. urmiana* and *A. monica* have never been submitted (as far as I know) to cytological investigation.

TABLE II

Systematics of genus *Artemia*

| Group | Name | Localities | Chromosome number |
|---|---|---|---|
| I. Bisexual diploid, subdiploid or tetraploid | *A. salina* Leach | Lymington (Engl.) | 42 |
| | *A. franciscana* Kellogg | North America (California *etc.*) | 42 |
| | *A. tunisiana* Bowen | Northern Africa Cagliari (Sardinia) | 42 |
| | *A. urmiana* Gunther | Lake Urmia (Iran) | ? |
| | *A. persimilis* Prosdocimi and Piccinelli | S. Bartolomeo (Cagliari), Hidalgo (Mexico) | 44 |
| | *A. monica* Verrill | Mono Lake (California) | ? |
| | *A. odessensis* New name | Odessa (USSR) | 84 ? |
| II. Parthenogenetic with different degrees of ploidy and different mechanisms of chromosome number regulation | *A. parthenogenetica* of Istria | Istria | predominantly 84 |
| | *A. pathenogenetica* of Sta Gilla | Sta Gilla (Cagliari) | 42 |
| | *A. parthenogenetica* of Apulia | Margherita di Savoia (Italy) | 42, 84 |
| | *A. parthenogenetica* of Comacchio | Comacchio (Italy) | 84 |
| | *A. parthenogenetica* of Sète | Sète (France) | 42 |
| | *A. parthenogenetica* of Odessa | Odessa (USSR) | 84 |
| | *A. parthenogenetica* of India | Madras (Kutch) | ? |
| | *A. parthenogenetica* of Australia | Port Hedland (Australia) | ? |
| | *A. parthenogenetica* of Japan | Yamaguchi (Japan) | ? |
| | *A. parthenogenetica* of the Dead Sea | Dead Sea | 63, 105 |

The problem of the taxonomical nomenclature seems fairly simple. Since the number of sibling species has increased and, at least in two localities (San Bartolomeo, Sardinia, and Hidalgo, Mexico), two are sympatric, there is no reason to retain the denomination *A. salina* except for those shrimps living two centuries ago in Lymington, England.

A special problem is raised by the shrimps studied by several authors and especially by Artom (1931), collected from the salterns of San Bartolomeo, Cagliari (Sardinia). There is no indication whatsoever as to whether the Lymington shrimps and the San Bartolomeo shrimps were of the same species or not. Artom excluded to call his material *A. calaritana* and adopted

the denomination *A. salina*. On the other hand the San Bartolomeo salterns do not exist any more, and the problem is thus solved for ever.

The number of the sibling species (the phenotypes of which are still far from being adequately known) is likely to increase in the future.

With regard to the phenotype, it is necessary to point out that an interesting part of it consists of biochemical differences. These are certainly useful for descriptive purposes, but I do not think tha they would be good to clarify the philogenetic connection between sibling species, except in special cases : *e.g.* the same or similar frequencies of biochemical markers in a diploid and i a tetraploid strain may point to the derivation of a tetraploid from the diploid.

The section comprising the partenogenetic shrimps (10 up to now) is certainly derived from the bisexual ones. Here the problem of denomination requires a solution which is entirely different from that adopted for the previous section. Following the system indicated by Mayr (1969) and in agreement with Bowen *et al.* (1978), I propose, as the denomination of all parthenogenetic *Artemia*, *A. parthenogenetica* followed by the vernacular or latin name of the locality of origin. This solution has the advantage of simplifying the taxonomy, but fails to take into consideration either the chromosomal mechanisms or the degree of ploidy. In other words, this denomination system will in many cases correspond more to a population than to a systematic entity. The complete description in these terms would, however, be too long.

For example : the denomination of the parthenogenetic *Artemia* of Sta Gilla would be : *Artemia parthenogenetica diploidis non automictica Sanctae Gillae*. It seems more expedient to add the indications needed (ploidy, presence of automixis during oogenesis) to the simpler denomination *A. parthenogenetica Sanctae Gillae* or of Santa Gilla (Sardinia).

Before going further, it seems necessary to point out that it is not the ploidy *per se*, but the chromosomal phenomena during oogenesis, which are of importance for a genetic evaluation of the parthenogenetic variants.

Four types of meiosis have been described so far in parthenogenetic *Artemia* (Barigozzi, 1974) :

1) Meiosis with pairing, crossing-over, extrusion of the first polar body, fusion between the second spindles or between the two second polar bodies (*e.g.* Sta Gilla).
2) Meiosis with pairing, crossing-over and extrusion of only the first polar body, or of only the second one (*e.g.* Sète).
3) Transient chromosome pairing, followed by the extrusion of only one polar body, *i.e.* the first (*e.g.* Dead Sea).
4) Pairing not observed : only one mitosis which extrudes the first and only polar body (ameiosis) (*e.g.* tetraploids at Margherita di Savoia).

The last two mechanisms could be pratically the same because it is difficult to completely exclude the possibility of chromosome pairing during prophase, when there are as many as 84 chromosomes. Genetic markers can be helpful in this connection. These facts give rise to the following consequences : a) whenever a marker (*e.g.* an hemoglobin) is fixed *i.e.* homozygous in all individuals, no changes are to be expected in the transmission, even in the long term, except as an effect of mutation ; b) whenever a marker is present in a heterozygous condition, the retention of heterozygosity is to be expected in the highest degree in type 4, in a lesser degree in the others. The mechanism which can maintain heterozygosity in a population is merely selective advantage.

These considerations should be taken into account with regard to the extent to which the term "clone" is justfied. With regard to the shrimps of the salterns of Sète, where there is a variety of meiotic mechanisms (Artom, 1931), the word clone would be used only when a single female produces offspring exhibiting only one type of meiosis. The stability of the meiotic phenomena in a "clone" is not known.

Another problem, which can be discussed only as hypothesis concerns the mechanism responsible for polyploidy in parthenogenetic shrimps. The step from diploidy to tetraploidy, or from tetraploidy to octoploidy, could be a simple endomitosis in germ cells. For the triploids, one could hypothesize fusion between the pronucleus and one of the polar bodies whenever the second division of the second polar body occurs. Thus 42 + 21 = 63. It is more difficult to explain pentaploidy. This problem is, however, of secondary importance for this paper.

We can now consider the last item, *i.e.* the philogenetic link between bisexual species and the parthenogenetic complex. The only certain element, when discussing this point, is that parthenogenesis is a derivative of bisexuality. It must also be pointed out that the gap between parthenogenetic and bisexual shrimps is rather wide, doubtless wider than in *Solenobia*, where parthenogenetic females can be mated with males to produce gynandromorphs. In *Artemia* copulations between parthenogenetical females and males are totally fruitless. The passage from bisexuality to parthenogenesis must have a mutational basis, and we do not know any case where the derivation can be established. This could also be of a taxonomical significance because as Bowen *et al.* (1978) pointed out, it is important to know whether the *Artemia parthenogenetica urmiana* is in fact *Artemia urmiana parthenogenetica*. This could be demonstrated by using a suitable number of markers. If this approach will be fruitfully followed in the future, a revision of the nomenclature should become necessary.

For the moment, and apart from possible experimental approaches, I wish to recommend the study of populations where males are rare,such as those according to Anikin (1898 quoted from Artom 1931) of Odessa and Mormyschansk (Siberia) if they still exist, or of those parthenogenetic populations kept in culture in which males occur unexpectedly (Ballardin and Metalli, 1963 ; Stefani, 1964 ; Bowen *et al.* 1978).

At the end of this presentation, I must recognize that most of the facts and problems discussed here are of mere theoretical significance, but some of them may also be relevant for the practical purposes of this Symposium. If *Artemia* were to become important for practical purposes in aquaculture, proper stocks would have to be selected.

Characters which should be considered are : high fecundity and fertility, viviparity, and precocity in reaching sexual maturity, high content of aminoacids, large body size, based on large cell size and resistance to diseases.

In order to create stocks of superior value, a knowledge of the genetical background of the systematics of the genus *Artemia* is necessary, especially in view of selection, whenever there is possibility to practice it.

**Literature cited**

Artom C. 1905. Osservazioni generali sull'artemia salina delle saline di Cagliari. *Zoologischer Anzeiger* 29(9):284-291.
Artom C. 1906. Ricerche sperientali sul modo di riprodursi dell'*Artemia salina* Lin. di Cagliari *Biol. Zentralblatt* 26:26-32.

Artom C. 1907. La maturazione, la fecondazione e i primi stadi di sviluppo dell'uovo dell'*Artemia salina* Lin. di Cagliari. *Biologia (Racc. Scr. Biol.)* 1:495-515.

Artom C. 1911. Analisi comparativa della sostanza cromatica nelle mitosi di maturazione e nelle prime mitosi di segmentazione dell'uovo dell'*Artemia* sessuate di Cagliari (univelens) e dell'uovo dell'artemia partenogenetica di Capodistria (bivalens). *Arch. f. Zell.* 7:277-295.

Artom C. 1912. Le basi citologiche di una nuova sistematica del genere *Artemia*. *Arch. f. Zellf.* 9:87-113.

Artom C. 1931. L'origine e l'evoluzione della partenogenesi attraverso i differenti biotipi di una specie collettiva (*A. salina*) con speciale riferimento al biotipo diploide partenogenetico di Sète. *Mem. R. Accad. Ital. Cl. Sci. Fis. Mat. Nat.* 2:1-57.

Ballardin E. and P. Metalli. 1963. Osservazioni sulla biologia di *Artemia salina* Leach. Tecniche di cultura e fenomeni riproduttivi. *Rend. Ist. Lomb. Sc. Lett. B* 97:194-254.

Barigozzi C. 1944. I fenomeni cromosomici delle cellule germinali in *Artemia salina* Leach. *Chromosoma (Berl.)* 2:549-575.

Barigozzi C. 1974. *Artemia* : a survey of its significance in genetic problems. *Evolutionary Biology* 7 : 221-252.

Bowen S. T. 1963a. The genetics of *A. salina*. II. *Biol. Bull.* 124:17-23.

Bowen S. T. 1963b. The genetics of *A. salina*. III. *Biol. Bull.* 125:431-440.

Bowen S. T. 1965. The genetics of *Artemia salina*. II. Crossing over between X and Y chromosomes. *Genetics* 52: 695-710.

Bowen S. T., J. P. Durking, G. Sterling, and L. S. Clark. 1978. *Artemia* hemoglobins : genetic variation in parthenogenetic and zygogenetic populations. *Biol. Bull.* 155:273-287.

Bowen S. T., J. Hanson, P. Dowling, and M. Poon. 1966. The genetics of *Artemia salina*. IV. *Biol. Bull.* 131:733-747.

Bowen S. T. and G. Sterling. 1978. Esterase and malate dehidrogenase isozyme polymorphism in 15 *Artemia* populations. *Comp. Biochem. Physiol.* 61B:593-595.

Brauer A. 1893. Zur Kenntnis der Reifung des parthenogenetisch sich entwicklenden Eies von *Artemia salina*. *Arch. Mikr. Anat.* 43:162-222.

Halfer Cervini A. M., M. Piccinelli, T. Prosdocimi, and L. Baratelli Zambruni. 1968. Sibling species in *Artemia* (Crust. Branchiopoda) *Evolution* 22:373-381.

Kuenen D. J. 1939. Systematical and physiological notes on the brine shrimp *Artemia*. *Arch. Neerl. Zool.* 3:365-449.

Mayr E. 1969. Principles of systematic zoology. Mc Graw-Hill, New York. 428 p.

Piccinelli M. and T. Prosdocimi. 1968. Descrizione tassonomica delle due specie *Artemia salina* L. e *Artemia persimilis n. sp. Rend. Ist. Lomb. Sci. Lett.* B102:170-179.

Stefani R. 1960. L'*Artemia salina* partenogenetica a Cagliari. *Riv. Biol.* 52:463-491.

Stefani R. 1963. La digametia femminile in *Artemia slaina* Leach. e la costituzione del corredo cromosomico nei biotipi diploide anfigonico e diploide partenogenetico. *Caryologia* 16:625-636.

Stefani R. 1964. L'origine dei maschi nelle popolazioni partenogenetiche di *Artemia salina*. *Riv. Biol.* : 147-162.

Verrill A. E. 1869. Observations on phyllopod Crustacea of the family Branchiopodidae, with description some new genera and species from America. *Proc. Am. Ass. Advanc. Sci.* 18:230-247.

Von Siebold C. 1873. Ueber die Parthenogenese der *Artemia salina*. (quoted from Artom C., 1931).

Von Siebold C. 1877. Ueber in München ges. *Artemia fertilis* aus dem grossen Salzsee von Utah. (quoted from Artom C., 1931).

*The Brine Shrimp* Artemia. *1980. Vol. 1. Morphology, Genetics, Radiobiology, Toxicology*
*G. Persoone, P. Sorgeloos, O. Roels, and E. Jaspers (Eds). Universa Press, Wetteren, Belgium, 345 p.*

# Sibling species of *Artemia*

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**Abstract**

Homogenates of individual adult brine shrimps were analyzed by starch gel electrophoresis to demonstrate five isozymes : lactate dehydrogenases (LDH), esterases (EST), NAD-dependent malate dehydrogenases (MDH), phosphoglucose isomerases (PGI), and alkaline phosphatases (ALP). Hemolymphs of other adults were analyzed by polyacrylamide electrophoresis to separate the hemoglobins. Although parthenogenetic *Artemia* from different locations had different genotypes, our data are in accord with the hypothesis of monophyletic origin of the five parthenogenetic populations. In 27 populations, the hemoglobins and PGI isozymes provided diagnostic characters for populations within species. Three other proteins (EST-1, EST-2, and ALP-1) fulfilled the seven requirements for species diagnostic characters. In the 12 populations in which all three of these enzymes were studied, they defined five categories (based on protein polymorphism profiles). The first four categories each contained one (and only one) species. The fifth category contained the remaining two of the six species previously established by the criterion of reproductive isolation in nature. We review the requirements for valid tests of reproductive isolation of *Artemia* populations. We report on our preliminary studies of habitat isolation among shrimps from three carbonate lakes : Mono, Jesse, and Kiatuthlanna.

## Introduction

When aquaculturists transport *Artemia* cysts to new habitats, gene exchange may occur between populations with different gene pools. Therefore, the most useful criterion for assignment of *Artemia* populations to different species is the presence of barriers to gene exchange. By this criterion, there are at least six sibling species of *Artemia*. (Sibling species are reproductively isolated in nature but identical or very similar in outward appearance.) The six *Artemia* species are listed in Table I along with citations of the evidence for their reproductive isolation.

There have been numerous reports of two different *Artemia* species in adjacent ponds or in the same pond (reviewed by Barigozzi, 1974). Before making a test for reproductive isolation between *Artemia* from two locations, one must be assured that each population consists of only one species. Some means of estimating within-population genetic heterogeneity is needed : descriptions of morphology, karyotype, or biochemical variation. These would also be essential for clarity of communication between scientists working on *Artemia* from the same locale when there is a possibility that each may have a cyst collection containing different proportions of two species.

TABLE I

Eighteen *Artemia* populations : geographic location and citation of evidence fo reproductive isolation of each of the five zygogenetic sibling species and for the monophyletic origin of the parthenogenetic populations. Species designation of Jesse Lake population is uncertain. Detailed descriptions of most locations given by Clark and Bowen, 1976[a]. Authors of species names cited by Bowen *et al.* (1978)

| Species and population | Location | Evidence for reproductive isolation of zygogenetic species and monophyletic origin of *A. parthenogenetica* |
|---|---|---|
| 1. *Artemia tunisiana* | | Bowen, 1965 ; Clark and Bowen, |
| TUN and SBT | Tunisia ; San Bartolomeo, Italy | 1976 ; Bowen and Sterling, 1978 |
| 2. *A. parthenogenetica* | | Bowen *et al.*, 1978 ; this paper |
| PHD | Port Hedland, Australia | |
| KUT | Gulf of Kutch, India | |
| YMG and SET | Japan ; Sète, France | |
| 3. *A. urmiana* | | Clark and Bowen, 1976 ; |
| URM | Lake Urmia, Iran | Bowen and Sterling, 1978 |
| 4. *A. persimilis* | | Halfer-Cervini *et al.*, 1968[a] ; |
| CRU and HDL | Carahue and Hidalgo, Argentina | Clark and Bowen, 1976 |
| 5. *A. franciscana* | | |
| GSL and ZUN | Great Salt Lake ; Zuni Salt Lake ; | Bowen, 1964 ; Clark and Bowen, |
| SFR and PCH | San Francisco, USA ; Pichilingue, Mexico | 1976 ; Bowen *et al.*, 1978 |
| ING, TLL | Caribbean : Inagua and Tallaboa | |
| KRD | Kiathuthlanna Red Pond, Arizona, USA | |
| 6. *A. monica* | | |
| MNO | Mono Lake, California, USA | Bowen, 1964 |
| 7. *Artemia* sp. (?) | | |
| JSS | Jesse Lake, Sheridan Co., Nebraska, USA | This paper |

[a] Possible identities of these cyst collections with those used in previous cross-fertility studies : TUN may be "Algerian" of Gilchrist (1960). SBT may be the same as those from Cagliari studied by Kuenen (1939) and the same as a portion of a mixture of sibling species at San Bartolomeo described by Halfer-Cervini *et al.* (1968). The "Californian" *Artemia* used by Kuenen (1939) and Gilchrist (1960) were from Monterey and San Diego, respectively. These were shown to be crossfertile with San Francisco shrimps and therefore are *A. franciscana*. (Bowen *et al.*, 1978).

We will report on electromorph variation in six different proteins and their relative usefulness in characterizing populations representing six species of *Artemia*. We will show that the phosphoglucose isomerases (PGI) and the hemoglobins provide population diagnostic characters which allow discrimination of populations within species. Two other proteins, esterases (EST) and alkaline phosphatases (ALP), provide species diagnostic characters which can be used in a taxonomic key to species. We will review the evidence that the 18 "populations" (shrimps hatched from cysts collected at one location) listed in Table I are not mixtures of sibling species : the zygogenetic populations are panmictic Mendelian populations and the parthenogenetic populations show no within-population variation despite the presence of between-population differences (with regard to hemoglobin electromorphs).

Because these 18 samples of shrimps are drawn from the same cyst collections tested in our reproductive isolation experiments, this lends support to apportionment of populations among the five sibling species : *Artemia tunisiana*, *A. urmiana*, *A. parthenogenetica*, *A. franciscana*, and *A. persimilis*.

We will discuss the additional taxonomic problems encountered in *Artemia* populations in terminal lakes with ionic composition different from that found in seawater. We will report our preliminary studies on habitat isolation in three carbonate lakes : Jesse, Mono, and Kiatuthlanna.

## Materials and methods

### *Artemia* collections

Adult Mono Lake shrimps were collected by Gayle Dana. Jesse Lake cysts were collected by Dr. Nicholas C. Collins. Ionic composition of the two lakes has been described by Cole and Brown (1967), McCarraher (1970), Collins (1977) and by Collins and Stirling (1980). The other habitats were reviewed by Clark and Bowen (1976). Our *Artemia* "populations" (collections of adults from Mono Lake and collections of cysts from the other locations) are designated by a three letter code (defined in Table I). ZUN is a newer code for QMD : Zuni Lake near Quemado, New Mexico.

### Culture methods

Mono shrimps were maintained in Mono Lake water. All other shrimps were hatched from cysts collected in the natural habitat and grown to adulthood in the laboratory. Jesse shrimps were cultured in Medium J which was obtained by mixing equal amounts of seawater and medium E (40 g $Na_2CO_3$, 32 g $NaHCO_3$, 8 g $K_2SO_4$/1), then filtering. Shrimps other than Jesse and Mono were maintained in culture Medium D (1 g sodium tetraborate, 50 g NaCl, and 40 g of the solid component of "Instant Ocean"/l), boiled and filtered before use. All shrimps were maintained in 5 ml of medium in shell vials, two shrimps/vial, in the dark. The culture technique has been described by Clark and Bowen (1976). The data in Tables II and III were obtained from adults 3-6 weeks of age maintained at 23 °C.

### Sample preparation

KUT and SFR cysts were hatched in seawater at 28 °C. Nauplii were cultured at 17 °C, 23 °C, or 28 °C for 0-48 hr, then ground with a cold mortar and pestle and analyzed immediately by starch gel electrophoresis.

To collect the date in Table II, each adult shrimp was placed in a drop of starch gel buffer and all tissues crushed with a pestle in a mortar (2 cm I.D., 4 °C). For each population, comparisons were made between unfrozen and frozen (-70 °C) samples and between homogenized whole shrimps and homogenized shrimps from which the guts had been removed. We concluded that freezing or the presence of the gut did not affect the mobility of the bands described in Table II. However, when the gut was removed, the EST-1 and EST-2 bands of SFR and CRU shrimps showed better resolution. The slower esterase bands and slower ALP bands were often not detectable in shrimps from which the guts were removed.

### Starch gel electrophoresis

The data in Table II were obtained from electrophoresis on 12% starch gels. The Tris-citrate buffer and the esterase and MDH staining techniques were detailed by Bowen and Sterling (1978). The two fastest esterases could be demonstrated with three substrates : alpha-naphthyl butyrate, alpha-naphthyl propionate, and beta-naphthyl acetate. The slower esterases could not hydolyze the last substrate. Other staining techniques were taken from Selander, *et al.* (1971).

### Polyacrylamide gel electrophoresis

The Tris-glycinate discontinuous buffer system, slab gels, and techniques used to obtain molecular weights of *Artemia* enzymes were described by Bucks and Bowen (1979). The hemoglobins of individual shrimps were analyzed on a water-cooled slab gel (0.75 mm thickness) by Bowen *et al.*, 1978. Because the hemoglobins were taken from one set of shrimps and the isozymes were determined on another set, the number of independent genotypes assayed from each population can be obtained by adding together the number of hemoglobin analyses with the number of assays of any one enzyme (Table II).

## Results

### Lactate dehydrogenases (LDH)

LDH isozymes were assayed in 15 populations (those shown in Table II, with the exception of KRD, MNO, and JSS). Only one band was usually present, in accord with the report of Ewing and Clegg (1972). The same band was fixed or at high frequency in all populations. Polymorphism was detected only in the SFR and ZUN populations where frequency of the rare slower allozyme was 0.2 and 0.1, respectively.

### Phosphoglucose isomerases (PGI)

PGI is also known as glucose phosphate isomerase (5.3.1.9). No intrapopulation polymorphism was observed in *A. urmiana*, *A. tunisiana*, nor in *A. parthenogenetica* (populations and numbers of shrimps listed in Table II). Two PGI isozymes were present in PHD shrimps ; the same two bands were seen in URM shrimps. In the five shrimps from Tunisia, only the faster band was seen. Extensive intrapopulation variation was seen in *A. franciscana*, *A. monica*, and the JSS population. These three populations lacked the slowest band found in *A. urmiana* and *A. parthenogenetica*.

### Malate dehydrogenases (MDH)

The R, S, and T patterns cited in Table II were described by Bowen and Sterling (1978). MDH bands vary in intensity with age and culture temperature.

TABLE II

Electrophoretic patterns of 18 populations representing six or seven sibling species of *Artemia*. Each analysis made on a single adult hatched from a cyst collected in the natural habitat. An asterisk (*) indicates that the phenotype is unknown (not tested). All patterns or alleles had frequencies of 1.00 except those designated as "polymorphic" and the EST patterns in the ING, TLL, and KRD populations. In the latter, C, C', and C" represent the 100/100, 100/104, and 104/104 genotypes at the Est-1 locus (shown as conventional allele frequencies in Table III)

| Species and populations | EST pattern | MDH pattern | Presence of PGI slowest band | | Relative mobility of ALP-1 | Number of analyses one adult/analysis | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | Known | Unknown | | EST | MDH | PGI | ALP | Hemoglobin |
| 1. *Artemia tunisiana* | | | | | | | | | | |
| TUN | A | R | – | | 112 | 26 | 19 | 5 | 9 | 19 |
| SBT | A | R | | * | * | 7 | 5 | 0 | 0 | 10 |
| 2. *A. parthenogenetica* | | | | | | | | | | |
| PHD | A | S | + | | 98 | 22 | 10 | 7 | 19 | 8 |
| KUT | A | T | + | | 102 | 106 | 83 | 42 | 76 | 87 |
| YMG | A | T | | * | * | 11 | 4 | 0 | 0 | 26 |
| SET | A | T | | * | * | 9 | 6 | 0 | 0 | 51 |
| 3. *A. urmiana* | | | | | | | | | | |
| URM | B | T | + | | 108 | 19 | 14 | 2 | 10 | 17 |
| 4. *A. persimilis* | | | | | | | | | | |
| CRU | D | T | | * | polymorphic | 25 | 28 | 0 | 15 | 31 |
| HDL | D | T | | * | * | 4 | 4 | 0 | 0 | 15 |
| 5. *A. franciscana* | | | | | | | | | | |
| GSL | C | T | – | | 100 | 61 | 52 | 5 | 40 | 51 |
| ZUN | C | T | – | | polymorphic | 68 | 56 | 4 | 43 | 66 |
| SFR | C | T | – | | 100 | 167 | 132 | 16 | 81 | 151 |
| PCH | C | T | | * | * | 2 | 2 | 0 | 0 | 28 |
| ING | C, C' | T | | * | * | 13 | 27 | 0 | 0 | 9 |
| TLL | C, C' | T | – | | 100 | 37 | 30 | 7 | 26 | 42 |
| KRD | C, C', C" | T | – | | 94 | 12 | 2 | 2 | 6 | 61 |
| 6. *Artemia* sp. (?) | | | | | | | | | | |
| JSS | C" | T | – | | 94 | 27 | 7 | 25 | 24 | 0 |
| 7. *A. monica* | | | | | | | | | | |
| MNO | C | T | – | | polymorphic | 41 | 41 | 40 | 40 | 16 |

TABLE III

Distribution of alleles at two presumptive loci governing the two fastest esterases (demonstrated with alpha-naphthyl butyrate). The 18 *Artemia* populations represent six or seven sibling species (by the criterion of reproductive isolation). JSS shrimps show some degree of habitat isolation from the others but may represent a semispecies. The three letter code for each population is given in Table I

| | | Allele frequencies at two presumptive gene loci | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Est-1 alleles | | | | Est-2 alleles | | | CODE |
| | Number of analyses one adult/analysis | 100 | 104 | 106 | 108 | 96 | 100 | 104 | used in Table II |
| Old world populations | | | | | | | | | |
| 1. *Artemia tunisiana* | | | | | | | | | |
| TUN | 26 | | | 1.00 | | | 1.00 | | A |
| SBT | 7 | | | 1.00 | | | 1.00 | | A |
| 2. *A. parthenogenetica* | | | | | | | | | |
| PHD | 22 | | | 1.00 | | | 1.00 | | A |
| KUT | 106 | | | 1.00 | | | 1.00 | | A |
| YMG | 11 | | | 1.00 | | | 1.00 | | A |
| SET | 9 | | | 1.00 | | | 1.00 | | A |
| 3. *A. urmiana* | | | | | | | | | |
| URM | 19 | | | | 1.00 | | | | B |
| New world populations | | | | | | | | | |
| 4. *A. persimilis* | | | | | | | | | |
| CRU | 25 | 1.00 | | | | 1.00 | | | D |
| HDL | 4 | 1.00 | | | | 1.00 | | | D |
| 5. *A. franciscana* | | | | | | | | | |
| GSL | 61 | 1.00 | | | | | 1.00 | | C |
| ZUN | 68 | 1.00 | | | | | 1.00 | | C |
| SFR | 167 | 1.00 | | | | | 1.00 | | C |
| PCH | 2 | 1.00 | | | | | 1.00 | | C |
| ING | 13 | 0.96 | 0.04 | | | | 1.00 | | C, C' |
| TLL | 37 | 0.97 | 0.03 | | | | 1.00 | | C, C' |
| KRD | 12 | 0.50 | 0.50 | | | | 1.00 | | C, C', C'' |
| 6. *Artemia* sp. (?) | | | | | | | | | |
| JSS | 27 | | 1.00 | | | | 1.00 | | C'' |
| 7. *A. monica* | | | | | | | | | |
| MNO | 41 | 1.00 | | | | | 1.00 | | C |
| Total : | 657 | | | | | | | | |

### Alkaline phosphatases (ALP)

The fastest alkaline phosphatase band (ALP-1) was easily demonstrated in every adult and showed no intrapopulation polymorphism is ten of the 13 populations in which it was studied (Table II).

### Esterases (EST)

EST-1 is the most anodal esterase (Bowen and Sterling, 1978). The A, B, C, and D patterns cited in Table II are defined in Table III by combinations of the relative mobilities of the two fastest esterases (EST-1 and EST-2). An EST-2 genotype of 100/100 along with EST-1 genotypes 100/100, 100/104, and 104/104 are coded as C, C', and C", respectively. The two fastest esterases were present in every adult and stained more intensely than the slower esterases in adults. However, in nauplii within 6 hr after hatching, EST-1 and EST-2 stained less intensely than the slower esterases (alpha-naphthyl butyrate as substrate).

### Estimates of molecular weights of EST-1, EST-2, and ALP-1

Ferguson plots of the logarithm of relative mobility against concentration of polyacrylamide gave estimates of the molecular weights of EST-1, EST-2, and ALP-1 of 1.1, 1.1 and $1.6 \times 10^5$ daltons (Bucks and Bowen, 1979).

### Comparison of isozyme expression in adults and nauplii

The two fastest esterases, EST-1 and EST-2, and the fastest ALP band, ALP-1, were faintly staining or absent in nauplii within six hours after hatching from cysts. In nauplii maintained at 23 °C or 28 °C for 48 hr post-hatching, the three bands showed intense staining. These results on Madras and San Francisco shrimps (*A. parthenogenetica* and *A. franciscana*) are in accord with the studies of Schwab (1974) on one population of *Artemia*.

### Hemoglobins

The hemoglobins of 27 populations have been characterized on polyacrylamide electrophoresis (Bowen, *et al.*, 1978). We now report that even on starch gels, differences in relative mobility of some hemoglobins can be detected. The hemoglobins may interfere with staining of bands demonstrated by tetrazolium dyes. When present in large amounts, they alter the mobility of adjacent proteins (regardless of mode of staining). In separations on 12% starch gels in our Tris-citrate buffer, the most anodal hemoglobin (Hb1) lies behind the LDH band, ahead of MDH patterns, and far ahead of PGI. Hb2 and Hb3 often interfere with MDH bands and Hb3 may interfere with rare PGI bands.

### Viability of Jesse and Mono shrimps in a common culture medium

Cysts collected from Jesse Lake were hatched in seawater. The JSS nauplii were reared to adulthood in Medium J, then transferred to other media. JSS adults had good viability and fertility in Medium J and in other solutions made by mixing artificial seawater with Medium J in varying proportions. JSS adults died within 4 days when transferred to Mono Lake water.

Mono Make adults had good viability in Medium J but did not reproduce in either J or in Mono Lake water.

## Discussion

### Genetic analyses

The hemoglobins are the only biochemical traits which we have analyzed in $F_2$ and back-cross progeny of Mendelian crosses. Our data minimize intrapopulation variation because we have analyzed electromorph differences in only one buffer system and our report does not cite obvious differences in staining intensity, heat sensitivity, and width of bands. We have not calculated genetic distances (method of Nei, 1972) for these reasons and also because there is a bias in our selection of isozymes which fit the criteria in Table IV. Our goal has been the development of a practical method of using 4-8 stains which demonstrate species diagnostic characters which may be used on the four slices of a starch gel. These could be used to demonstrate covariation of diagnostic characters (correlated variation among loci). The importance of the use of covariation in taxonomy has been stressed by Throckmorton (1978) and Wake *et al.* (1978).

### Diagnostic characters

A species diagnostic character is defined as one that discriminates between two species. That is, an individual can correctly be assigned to one of the two species with a probability of 99% or higher (Ayala and Powell, 1972). Table IV lists the seven attributes of an ideal species diagnostic character. We find that EST-1, EST-2, and ALP-1 are the three genetic markers which best fit the ideal. Although there were some losses in band intensity in some populations when reared at higher culture temperatures (30 °C) or with increased age (12 weeks of age), the three bands were demonstrable in every adult. They were absent in newly emerged nauplii but detectable within 48 hr after emergence. Each discriminates between more than one pair of species. Used conjointly, the EST-1, EST-2, and ALP-1 enzymes group the populations into five categories. The first four categories contain one (and only one) species (those numbered 1-4 in Table II). The fifth category contains two species (*A. franciscana* and *A. monica*) and the putative semispecies at Jesse Lake.

Table IV

List of attributes of the most useful electromorph to be selected as a "species diagnostic character" (defined in Discussion section)

1. Expressed in every adult ; relatively unaffected by extremes of environment.
2. Intrapopulation variation low.
3. Interpopulation variation high.
4. Unaffected by presence of hemoglobins. Mobility not altered by proximity to hemoglobins. Variant electromorphs not obscured by hemoglobins.
5. Present in juvenile and adult. (Advantage of short-term experiments).
6. Absent in nauplius. (In the assay of whole animal homogenates, gravid females do not express naupliar genotypes.)
7. Interpopulation variation correlated with patterns of reproductive isolation. The most useful character discriminates between populations of more than one pair of species.

The ideal population diagnostic character possesses attributes no. 1, 4, 5 and 6 in Table IV. The PGI isozymes and hemoglobins show great interpopulation differences within species and are of value for tests of panmixis and identification of populations (in case of mistaken identity of cyst collections).

### Hemoglobins

The absolute amounts and relative proportions of the four hemoglobins (Hb1, Hb2, HbX, and Hb3) change with age and oxygen concentration. In gels with low molecular sieving capacity, HbX and Hb3 of the San Francisco population may overlap (Bucks and Bowen, 1979). However, conditions may be chosen in which all four hemoglobins are demonstrable in every shrimp. Hb1 and HbX are homopolymers ; Hb2 is a heteropolymer containing the two polypeptides found in Hb1 and HbX (Bowen *et al.*, 1977). Hb3 contains neither of these polypeptides. Thus, three gene loci account for the four hemoglobins. *Artemia* hemoglobins have been extensively reviewed elsewhere (Bucks and Bowen, 1979 ; Heip *et al.*, 1980).

By means of electrophoresis on a water-cooled polyacrylamide slab (0.75 mm thickness), Bowen *et al.* (1978) showed that 23 of 27 populations had unique hemoglobin profiles (different prevailing electromorphs for one or more of three hemoglobins). The remaining two pairs could be discriminated on the basis of Hb1 electromorph frequencies.

### Monophyletic origin of parthenogenetic *Artemia*

An earlier study on the hemoglobins (Bowen *et al.*, 1978) suggested that European and Asian parthenogenetic *Artemia* had Hb1 electromorphs of the same relative mobility which differed from that of PHD (Australian) parthenogenetic *Artemia*. The new data in Table II lend further support to the hypothesis of a single origin of parthenogenesis with PHD shrimps diverging earlier from the clone from which the Asian and European parthenogenetic *Artemia* are derived. Because we have examined only a few proteins, we cannot state whether the parthenogenetic shrimps are more closely related to *A. urmiana* or to *A. tunisiana*.

### Tests for barriers to gene exchange

When the $F_1$ progeny of non-inbred parents are mated, some of these matings fail to produce offspring. Because a substantial percentage of within-population crosses are sterile, the question arises whether the "population" might be a mixture of sibling species. Therefore, the first step in a valid test of reproductive isolation is the demonstration of the homogeneity of the populations (Table V).

In our studies of the 18 populations listed in Tables I and II, we found that in highly polymorphic populations, there was no correlation of rare electromorphs of different isozymes. For example, although there was great genetic variation in ALP and PGI isozymes in the CRU population, a shrimp with the rare ALP had the prevalent PGI and *vice versa*. The frequencies of hemoglobin alleles in zygogenetic populations approximate a Hardy-Weinberg distribution (Bowen *et al.*, 1978). Evidence from isozymes and hemoglobins suggest that the shrimps within each parthenogenetic population have the same genotype (for the few loci studied). We conclude that the 18 populations in Table II are not mixtures of sibling species.

TABLE V

List of requirements for a valid test of reproductive isolation of two *Artemia* populations (of same level of polyploidy)

1. Both populations must be shown to be homogeneous (rather than a mixture of sibling species).
   A) Rare variants of diagnostic characters must show absence of correlated variation among unlinked loci.
   B) Zygogenetic populations must show an approximation to a Hardy-Weinberg distribution for variant electromorphs of two population diagnostic characters (*e.g.*, hemoglobin or PGI loci).
2. All intrapopulation and interpopulation matings must be made using shrimps hatched from cysts from the natural habitat (rather than inbred laboratory stocks) to insure a sample of independent genotypes.
3. Test for habitat isolation :
   A) If both populations have high intrapopulation fertility in the same medium :
      1) Reproductive isolation is excluded by production of fertile $F_1$ and viable $F_2$ adults with segregation of genetic markers to exclude gynogenesis. Viable backcross progeny will exclude hybrid breakdown.*
      2) Reproductive isolation is demonstrated if viable $F_2$ or backcross progeny are not produced. There is a possibility of a false negative result if the hybrid $F_1$ shows viability lower than that of nauplii from the two parental populations.*
   B) If the two populations cannot be grown in the same culture medium (with high intrapopulation fertility in both), there is reproductive isolation due to habitat adaptation.* There is a possibility of a false negative result.

* Incomplete reproductive isolation may be present. In 3A, there may be reduced amounts of gene exchange rather than absence of exchange between populations. In 3B, two populations may be unable to exist together in either natural habitat but may coexist in a medium of intermediate composition.

In our studies of reproductive isolation (cited in Table I), the second and third requirements (Table V) were fulfilled. Interpopulation matings were made with adults hatched from cysts collected in the natural habitat and reared in the laboratory. This is preferable to use of adults from many generations of mass laboratory culture where one genotype may have a selective advantage and displace many others. If there was no apparent barrier to gene exchange, a genetic marker was followed into the $F_2$ and backcross generation to exclude the possibilities of gynogenesis and hybrid breakdown. In a test of interfertility, a "false negative result" may be obtained. For example, in our first matings of San Bartolomeo (SBT) and Chott Ariana (TUN) shrimps, the $F_1$ viability was low and $F_2$ progeny were not detected. After the culture technique was changed, we succeeded in demonstrating viable fertile $F_2$ offspring. The conclusion that TUN and SBT were conspecific was affirmed by discovery that both have the same unique MDH pattern (Bowen and Sterling, 1978) and prevalent mobilities of three hemoglobins (Bowen *et al.*, 1978).

### HABITAT ISOLATION

Ecological or habitat isolation (a type of reproductive isolation) will lead to spirited discussions of *Artemia* taxonomy. *Artemia* populations in inland salt lakes are adapted to specific ratios of carbonate/chloride, or chloride/sulfate, etc. (Collins, 1980). Most biologists would agree that if two shrimp populations canot be grown to adulthood in a common

medium, they would be different species. The controversies will arise when isolation is incomplete. For example, each of two populations might be inviable in the natural lake water of the other but be viable (and fertile) in an artificial laboratory medium. A second example of a complex situation might be the terminal populations in a carbonate-chloride cline which are not viable in any one common medium but which might be crossfertile with an intermediate population in an artificial medium.

Adult shrimps from San Francisco salterns and from Great Salt Lake, Utah cannot live in Mono Lake water. Mono Lake adults have reduced viability when transferred to sea water or sea water enriched with NaCl (Bowen, 1964). Lenz (1980) reports greatly reduced viability when Mono Lake nauplii are reared in sea water or when San Francisco nauplii are reared in Mono Lake water. Because these facts are evidence for substantial reproductive isolation in the natural habitat, Mono Lake *Artemia* are designated *A. monica* in Table I. We anticipate that further studies may reveal that genes might be exchanged if matings were made in an artificial medium or through an intermediate population.

### Anticipated taxonomic problems

As scientists characterize *Artemia* from other terminal lakes, we can anticipate the discovery of additional species and semispecies. Genetic identity may be greater among species which have diverged recently due to habitat isolation. Species diagnostic characters may make up a smaller proportion of the characters than in the species described thus far.

Although taxonomic problems will become more complex, new opportunities will arise for study of the process of speciation. Because the ecological parameters of the *Artemia* habitat are easily determined (which is not the case for the *Drosophila* habitat), speciation may be shown to be associated with certain alleles or duplicate loci.

Although the *Artemia* sibling species are not adequately characterized at the present time, we must give them serious consideration. We list some problems : 1) a collection of cysts obtained from another investigator or from a commercial supplier may be incorrectly labeled in regard to place of origin ; 2) there are five known non-parthenogenetic sibling species and we anticipate the discovery of additional species and semispecies ; 3) there have been two instances of transportation of *Artemia* to different continents for improvement of salt production or for aquaculture ; we anticipate an increased rate of dispersal for aquaculture enterprises ; 4) there have been many reports of a mixture of sibling species in one pond and we can expect that the frequency of mixed species will increase. Two scientists might take aliquots of the same lot of cysts which (unknown to them) contains a mixture of *Artemia* species. If they rear the nauplii to adulthood under different culture methods, they might select for different species, then discover a substantial communication problem about the results of their experiments. A model for natural selection in intergeneric competition is presented in another paper given at this symposium by Geddes (1980) who reports that *Artemia* and *Parartemia* occur in a series of Australian ponds of increasing salinity. One genus dominates the low salinity ponds, the other the high salinity ponds, with both present in the middle ranges.

There is an abundance of San Francisco and Argentinian cysts available now from commercial sources. Two non-parthenogenetic species might be introduced at the same location. If present in equal numbers, 50% of the "matings" (clasping pairs) would be unpro-

ductive. In the future, mass *Artemia* culture for use in aquaculture might be threatened not only by industrial pollution but by introduction of many sibling species at one site.

*Artemia salina* is not one species but a complex of sibling species. If investigators are not certain which species is present (if the population has not been biochemically characterized), we suggest they use the designation *Artemia sp.* or *Artemia salina*. While the former is taxonomically correct, the latter may be preferred by those scientists who do not need to contrast sibling species. It is better to call a population *Artemia sp.* than to risk assignment of the "wrong" name (a binomen associated with previous descriptions of morphology, karyotpe, or isozymes applied to a population not characterized in the same manner).

To determine which population or species is present in a commercial shipment of cysts, one requires a constellation of characters which discriminate between populations or which group populations into species. Four papers presented at this symposium approach this problem. Amat Domenech (1980) describes the external morphology of adults. Abreu-Grobois and Beardmore (1980) estimates genetic distances based on polymorphisms in many adult isozymes. Our paper describes a few proteins of value as diagnostic characters in adults from populations previously characterized by studies of reproductive isolation. Seidel *et al.* (1980) make the initial step toward devising a naupliar taxonomic key by isoelectric focusing of proteins from pooled nauplii. These preliminary studies may serve as models for the more extensive work which must be done.

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## Literature cited

Abreu-Grobois F. A. and J. A. Beardmore. 1980. International Study on *Artemia*. II. Genetic characterization of *Artemia* populations – an electrophoretic approach. p. 133-146. *In* : The brine shrimp *Artemia*. Vol. 1. Morphology, Genetics, Radiobiology, Toxicology. Persoone G., P. Sorgeloos, O. Roels, and E. Jaspers (Eds). Universa Press, Wetteren, Belgium. 345 p.

Amat Domenech F. 1980. Differentiation and distribution of *Artemia* populations in Spain. p. 19-39. *In* : The brine shrimp *Artemia*. Vol. 1. Morphology, Genetics, Radiobiology, Toxicology. Persoone G., P. Sorgeloos, O. Roels, and E. Jaspers (Eds). Universa Press, Wetteren, Belgium. 345 p.

Ayala F. J. and J. R. Powell. 1972. Allozymes as diagnostic characters of sibling species of *Drosophila*. *Proc. Nat. Acad. Sci.* (USA) 69:1094-1096.

Barigozzi C. 1974. *Artemia* : a survey of its significance in genetic problems. p. 221-252. *In* : Evolutionary biology. Vol. 7. Dobzhansky T., M. K. Hecht, and W. C. Steere. Plenum Press, New York, 314 p.

Bowen S. T. 1964. The genetics of *Artemia salina*. IV. Hybridization of wild populations with mutant stocks. *Biol. Bull.* 126:333-344.

Bowen S. T. 1965. The genetics of *Artemia salina*. Crossing over between the X and the Y chromosomes. *Genetics* 52:695-710.

Bowen S. T., J. P. Durkin, G. Sterling, and L. S. Clark. 1978. *Artemia* hemoglobins : genetic variation in parthenogenetic and zygogenetic populations. *Biol. Bull.* 155:273-287.

Bowen S. T. and G. Sterling. 1978. Esterase and malate dehydrogenase isozyme polymorphisms in 15 *Artemia* populations. *Comp. Biochem. Physiol.* 61B:593-595.

Bowen S. T., G. Sterling, and J. Barkan. 1977. The hemoglobins of *Artemia salina*. IV. A model for genetic control of hemoglobin-1, hemoglobin-2, and hemoglobin-X. *Biochemical Genetics* 15:409-422.

Bucks D. A. W. and S. T. Bowen. 1979. The naupliar hemoglobins of three populations of *Artemia*. p. 100-112. *In* : Proceedings of the Symposium on Biochemistry of *Artemia* Development. Bagshaw, J. C. and A. H. Warner (Eds). University Microfilms, Ann Arbor, Michigan. 240 p.

Clark L. S. and S. T. Bowen. 1976. The genetics of *Artemia salina*. VII. Reproductive isolation. *The Jour. Heredity* 67:385-388.

Cole G. A. and R. J. Brown. 1967. The chemistry of *Artemia* habitats. *Ecology* 48:858-861.

Collins N. C. 1977. Ecological studies of terminal lakes : their relevance to problems in limnology and population biology. pp.411-420. *In* : Desertic terminal lakes. Greer D. C. (Ed.). Utah Water Research Laboratory, Utah State University, Logan Utah. 436 p.

Collins N. C. 1980. Comparison of *Artemia* strains : survival and growth of nauplii as a function of ionic composition, osmosity, and temperature of the medium. (Abstract) p. 123. *In* : The brine shrimp *Artemia*. Vol. 2. Physiology, Biochemistry, Molecular Biology. Persoone G., P. Sorgeloos, O. Roels, and E. Jaspers (Eds). Universa Press, Wetteren, Belgium. 664 p.

Collins N. C. and G. Stirling. 1980. Relationships among total dissolved solids, conductivity, and osmosity for five *Artemia* habitats (Anostraca : Artemiidae). Great Basin Naturalist. 39:(4). (In press).

Ewing R. D. and J. S. Clegg. 1972. Evidence for a single macromolecular form of lactate dehydrogenase in *Artemia salina*. *Arch. Biochem. and Biophysics* 150:566-572.

Geddes M. C. 1980. The brine shrimps *Artemia* and *Parartemia* in Australia. p. 57-65. *In* : The brine shrimp *Artemia*. Vol. 3. Ecology, Culturing, Use in Aquaculture. Persoone G., P. Sorgeloos, O. Roels, and E. Jaspers (Eds). Universa Press, Wetteren, Belgium. 456 p.

Gilchrist B. 1960. Growth and form of the brine shrimp, *Artemia salina* (L.). *Proc. Zool. Soc. Lond.* 134:221-235.

Halfer-Cervini A. M., M. Piccinelli, T. Prosdocimi, and L. Baratelli-Zambruni. 1968. Sibling species in *Artemia* (Crustacea : Branchiopoda). *Evolution* 22:373-381.

Heip J., L. Moens, R. Hertsens, E. J. Wood, H. Heyligen, A. Van Broekhoven, R. Vrints, D. De Chaffoy, and M. Kondo. 1980. *Artemia* extracellular hemoglobins : ontogeny, structure and *in vivo* radiolabeling. p. 427-448. *In* : The brine shrimp *Artemia*. Vol. 2. Physiology, Biochemistry, Molecular Biology. Persoone G., P. Sorgeloos, O. Roels, and E. Jaspers (Eds). Universa Press, Wetteren, Belgium. 664 p.

Lenz P. H. 1980. Ecology of an alkali-adapted variety of *Artemia* from Mono Lake, California. p. 79-96. *In* : The brine shrimp *Artemia*. Vol. 3. Ecology, Culturing, Use in Aquaculture. Persoone G., P. Sorgeloos, O. Roels, and E. Jaspers (Eds). Universa Press, Wetteren, Belgium. 456 p.

McCarraher D. B., 1970. Some ecological relations of fairy shrimps in alkaline habitats of Nebraska. *The American Midland Naturalist* 84:59-68.

Nei M. 1972. Genetic distance between populations. *Amer. Natur.* 106:283-292.

Schwab W. E. 1974. Sex-specific proteins in *Artemia salina* and their possible function in cryptobiosis. *Comparat. Biochem. Physiol.* 48B:175-180.

Seidel C. R., J. Kryznowek, and K. L. Simpson. 1980. International study on *Artemia*. XI. Amino acid composition and electrophoretic protein patterns of *Artemia* from five geographical locations. p. 375-382. *In* : The brine shrimp *Artemia*. Vol. 3. Ecology, Culturing, Use in Aquaculture. Persoone G., P. Sorgeloos, O. Roels, and E. Jaspers (Eds). Universa Press, Wetteren, Belgium. 456 p.

Selander R. K., M. H. Smith, S. Y. Yang, W. E. Johnson, and J. B. Gentry. 1971. Biochemical polymorphism and systematics in the genus *Peromyscus*. I. Variation in the old-field mouse (*Peromyscus polionotus*). *Studies in Genetics VI. Univ. Texas Publ.* 7103:49-90.

Throckmorton L. 1978. Molecular phylogenetics. p. 361-368. *In* : Beltsville Symposium in Agricultural Research. Vol. II. Biosystematics. Romberger J. A. (Ed.). Wiley. 475 p.

Wake D. B., L. R. Maxson, and G. Z. Wurst. 1978. Genetic differentiation, albumin evolution, and their biogeographic implications in Plethodontid salamanders of California and Southern Europe. *Evolution* 32(3): 529-539.

# Radiobiology

*The Brine Shrimp* Artemia. *1980. Vol. 1. Morphology, Genetics, Radiobiology, Toxicology*
*G. Persoone, P. Sorgeloos, O. Roels, and E. Jaspers (Eds). Universa Press, Wetteren, Belgium. 345 p.*

# Effects of proton and neutron irradiation on *Artemia* eggs

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**Abstract**

Dry *Artemia* eggs were exposed to various doses of 645 MeV and 9.2 GeV protons or to fast neutrons. Results on emergence- and hatching rates and nauplius survival were compared to the effects of gamma irradiation. RBE values, calculated for nauplius survival at the 50% effect level are 2.3 for 645 MeV protons, 1.5 for 9.2 GeV protons, and about 10 for neutrons. A slightly better survival was observed in *Artemia* hatched from eggs exposed to low doses of high energy protons.

## Introduction

In previous space experiments, effects of cosmic rays were investigated in *Artemia* eggs flown aboard Apollo or Cosmos spacecrafts. Cosmic rays are made up of protons, $\alpha$ particles and heavy ions, protons being the main component. Inside the spacecraft biological objects are exposed to secundary radiations, in particular to neutron irradiation.

Effects of proton irradiation on *Artemia* eggs have not been studied yet. Iwasaki *et al.* (1971) reported the effects of neutron irradiation on brine shrimp. Departing from these data, we have investigated the effects of 645 MeV and 9.2 GeV protons and fast neutrons on dry *Artemia* eggs.

This paper deals with irradiation effects on the first stages of development and on larval survival, as compared to the effects induced by gamma irradiation.

## Material and methods

### Irradiation conditions

Irradiations were performed on dry eggs of a Californian *Artemia* strain. The eggs were put in small airtight plastic bags and exposed to 65 up to 675 Krad of gamma rays, in comparison with proton irradiation, from a $^{60}$Co source at a dose-rate of 1 Krad/min. They were exposed to 1 to 300 Krad of gamma rays, in comparison with neutron irradiation.

Irradiation by plateau region protons was performed in Dubna (Gaubin *et al.*, 1979). For 645 MeV protons, the doses applied were : 1, 10, 100, 200, 400, and 600 Krad (dose-rate 35

rad/sec) and for 9.2 GeV protons : 1, 10, 100, 200, and 320 Krad (dose-rate 3 rad/sec). Dosimetry was performed with the aid of a tissue equivalent ionization chamber and by the carbon activation method.

The neutron irradiation was carried out on the experimental horizontal tangential channel of the water cooled and water moderated IRT-2000 reactor of the Institute of Physics of the Academy of Sciences of the Latvia, USSR (Belogurov *et al.*, 1979). The objects were placed in a cassette of organic glass with 3 mm thick walls, which did not noticeably attenuate the flux of incident neutrons. Monitoring of the exposure was performed with $^{32}S$ activation detectors calibrated with homogeneous tissue equivalent thimble ionization chambers (Isayev and Bregadze, 1967). The dose resulting from the gamma radiation present in the neutron beam was measured with detectors of aluminophosphate glass, which have a very low sensitivity to neutrons of the fission spectrum.

The mean energy of the neutron spectrum was 1.5 MeV ; that of the gamma quants 1.1 MeV. The tissue dose rates due to neutrons were $12.7 \pm 1.2$ rad/min ; those due to gamma radiation $1.3 \pm 0.15$ rad/min. Total absorbed doses were 1.6, 5.5, 13, 19, 29, 46, and 70 Krad.

Control eggs were placed in similar plastic bags for the same period of time.

### Culture conditions

Samples, consisting of 30 irradiated or control eggs in each analysis, were transferred to petri dishes containing 4 ml artificial seawater, at pH 7 and maintained at a temperature of 27 °C. Endpoints of analysis of radiation effectiveness were emergence, hatching, and survival rates of 4-5 day old larvae. For long duration investigations, freshly hatched nauplii were transferred to culture vessels (20 nauplii per vessel) containing 200 ml artificial seawater at pH 7, and kept at 25 °C. Survival capacity was studied from the first day after hatching to the 21th day (neutron irradiation) or to the 10th week (proton irradiation).

## Results

### Emergence, hatchability and naupLius survival

#### *Proton irradiation*

Effect of proton irradiation on the percentages of emergence and hatching, and larval survival on the 4-5th day, compared to those of gamma ray irradiation, are shown in Fig. 1, 2 and 3 respectively. For each dose, 600 to 900 eggs were tested. The results are plotted on a semi-logarithmic scale and the curves fitted by eye. Standard deviations are estimated from pooled results. For the three types of radiations a plateau is observed in the emergence and hatching curves, followed by a shoulder. For nauplius survival curves, the shoulder occurs at doses higher than 10 Krad.

It is obvious that the effects of proton irradiation are more pronounced than those of gamma rays. The long plateau, observed in the emergence and hatching curves after gamma irradiation, is remarkably reduced in 645 MeV and 9.2 GeV proton irradiated eggs ; after the shoulders, curve slopes are steeper in the proton irradiated set.

Moreover, irradiation effects progressively increase with the development of the egg as shown in Table I.

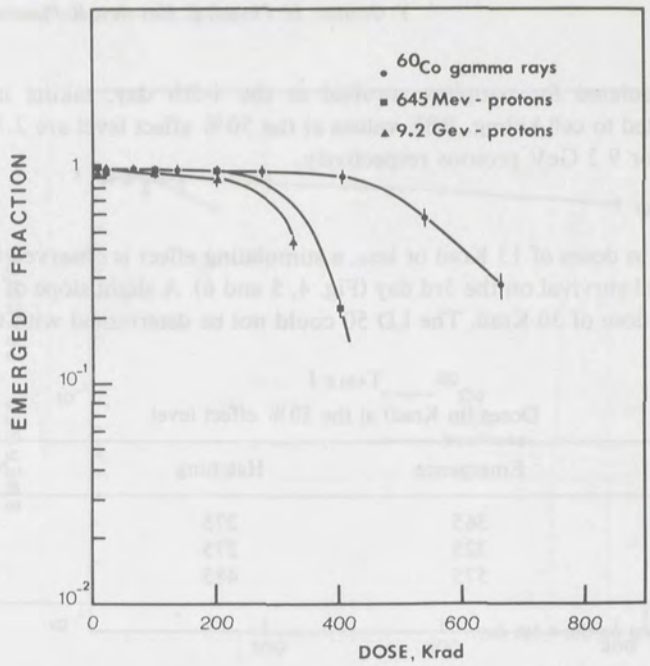


FIG. 1. Emerged fraction as a function of proton and gamma-ray dose. Error bars indicate standard deviation of mean values.

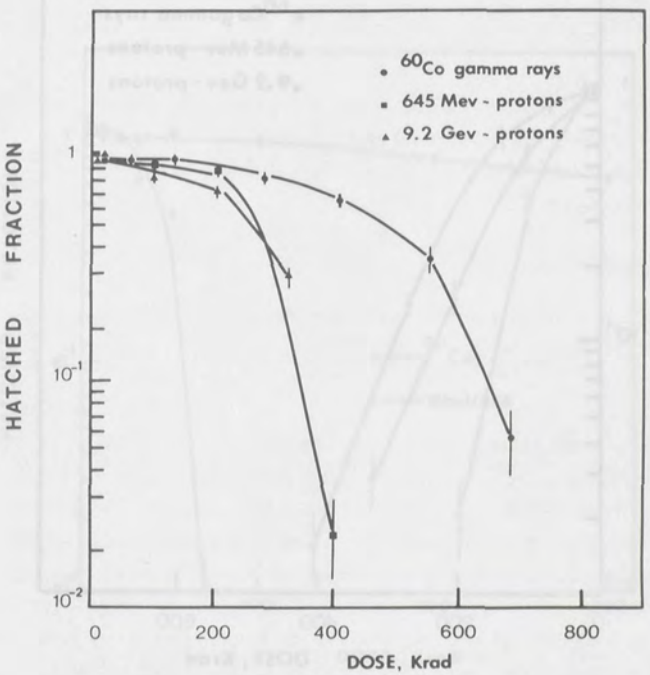


FIG. 2. Hatched fraction as a function of proton and gamma-ray dose.

RBE were calculated for nauplius survival at the 4-5th day, taking into account a phenomenon related to cell killing. RBE values at the 50% effect level are 2.3 for 645 MeV protons and 1.5 for 9.2 GeV protons respectively.

*Neutron irradiation*

After exposure to doses of 13 Krad or less, a stimulating effect is observed for emergence, hatching and larval survival on the 3rd day (Fig. 4, 5 and 6). A slight slope of the emergence curve occurs at a dose of 30 Krad. The LD 50 could not be determined with the doses used.

TABLE I

Doses (in Krad) at the 50% effect level

| Radiations | Emergence | Hatching | Nauplii[1] |
|---|---|---|---|
| 645 MeV Protons | 365 | 275 | 65 |
| 9.2 GeV Protons | 325 | 275 | 120 |
| $\gamma$ Rays | 575 | 485 | 175 |

[1] 50% nauplius survival on the 4-5th day.

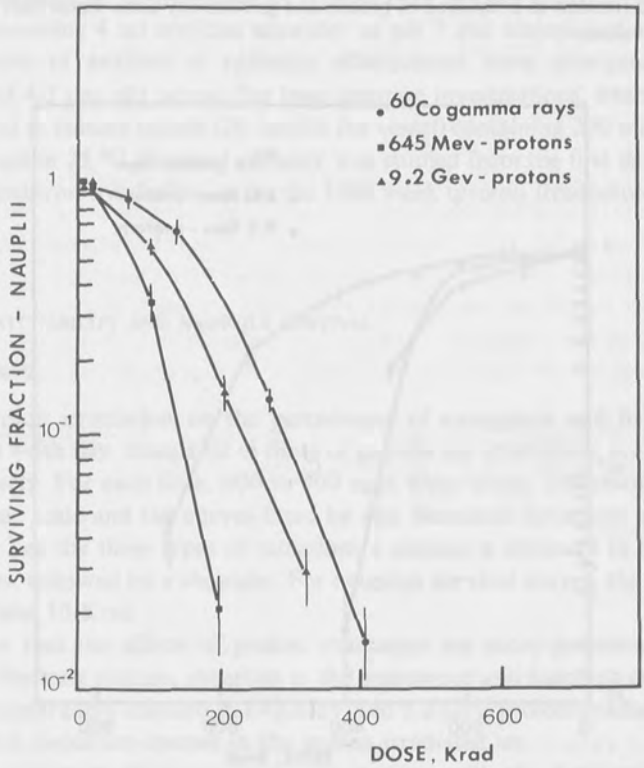


FIG. 3. Survival of 4-5 day old larvae as a function of proton and gamma-ray dose.

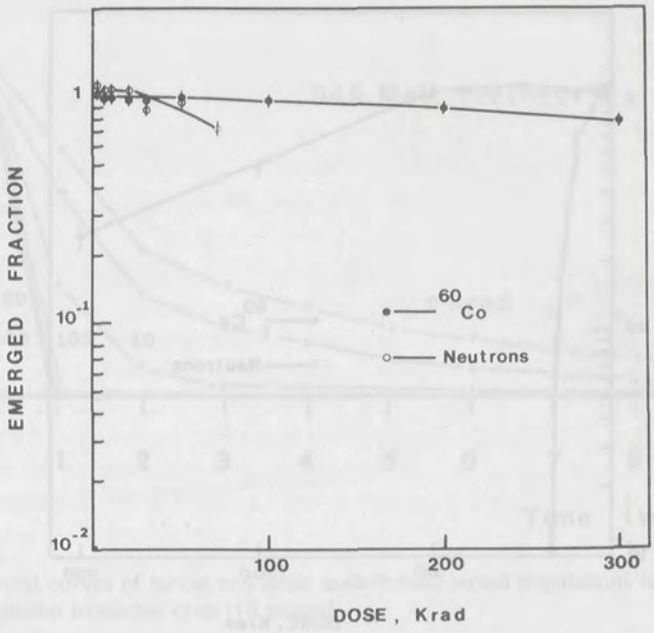


FIG. 4. Emerged fraction as a function of neutron and gamma ray dose.

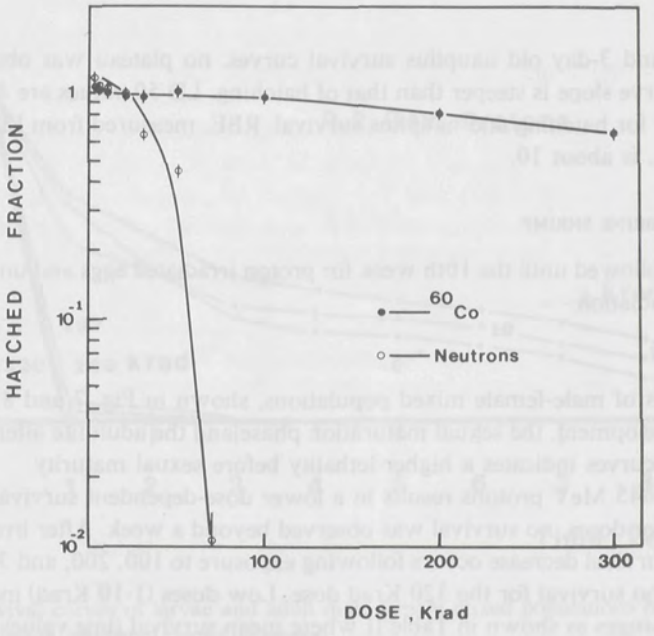


FIG. 5. Hatched fraction as a function of neutron and gamma ray dose.

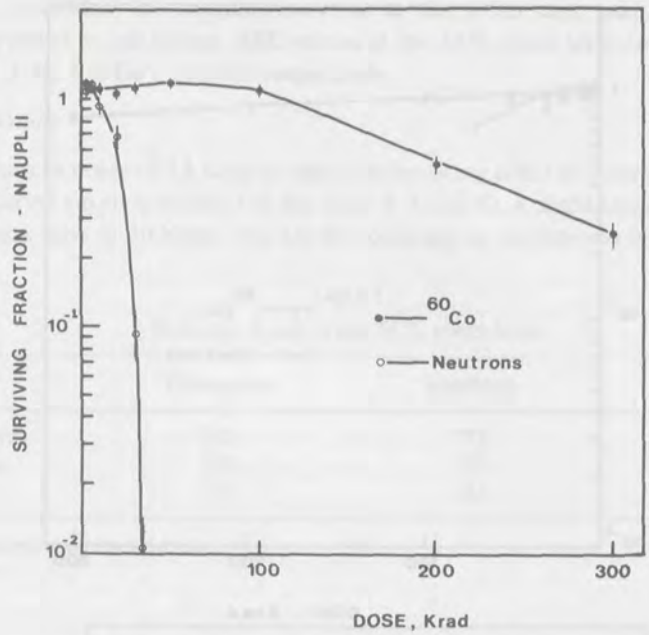


FIG. 6. Survival of 3 day-old larvae as a function of neutron and gamma-ray dose.

For hatching and 3-day old nauplius survival curves, no plateau was observed and the larval survival curve slope is steeper than that of hatching. LD 50 values are 40 Krad and 20 Krad respectively for hatching and nauplius survival. RBE, measured from LD 50 values for nauplius survival, is about 10.

### SURVIVAL OF THE BRINE SHRIMP

Survival was followed until the 10th week for proton irradiated eggs and until the 21st day after neutron irradiation.

#### *Proton irradiation*

Survival curves of male-female mixed populations, shown in Fig. 7 and 8, deal with the first stages of development, the sexual maturation phase and the adult life after the 3rd week. The slope of the curves indicates a higher lethality before sexual maturity.

Irradiation by 645 MeV protons results in a lower dose-dependent survival capacity. For 100 Krad or higher doses, no survival was observed beyond a week. After irradiation by 9.2 GeV protons, a survival decrease occurs following exposure to 100, 200, and 320 Krad. After 1 week, there is no survival for the 320 Krad dose. Low doses (1-10 Krad) induce slight but not significant changes as shown in Table II where mean survival time values, calculated for 10 weeks, are reported.

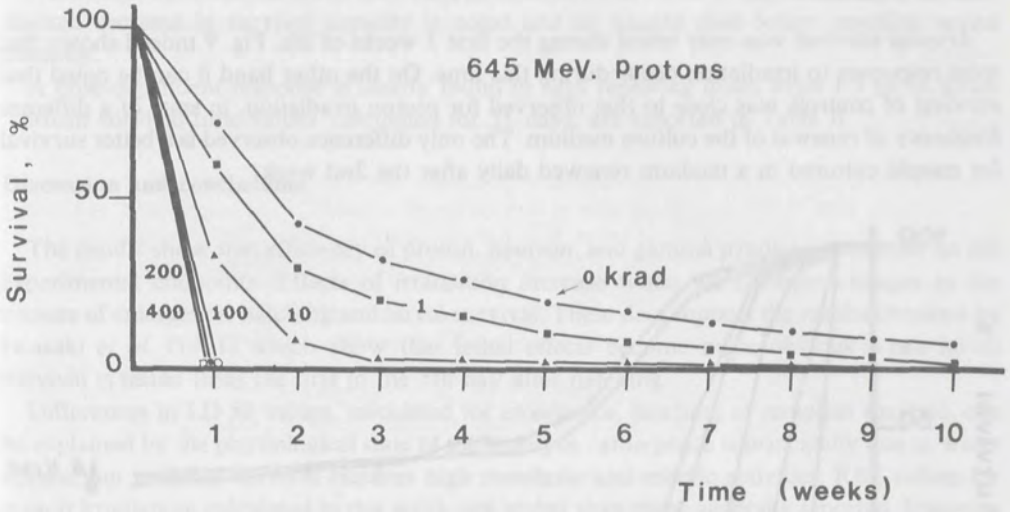


FIG. 7. Survival curves of larvae and adult male-female mixed populations hatched from control and 645 MeV proton irradiated cysts (10 weeks).

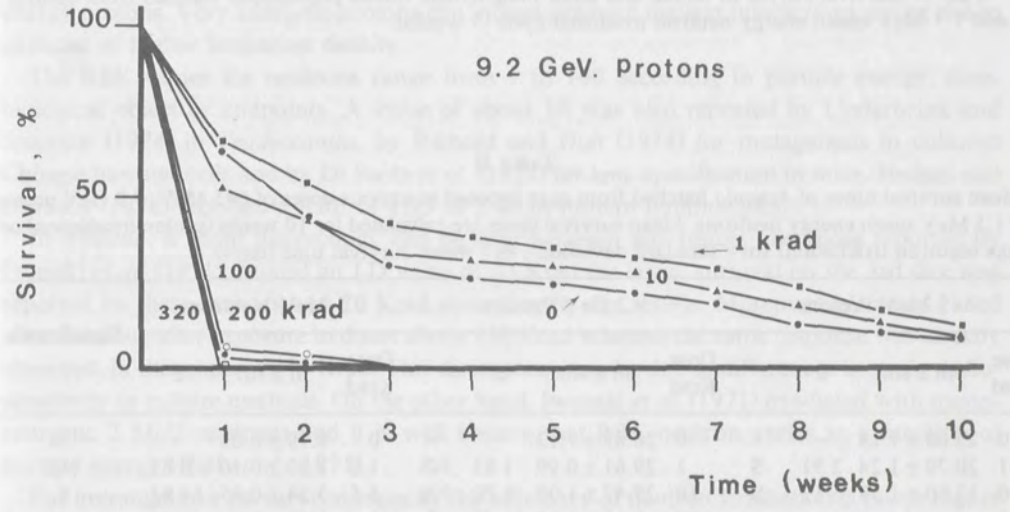


FIG. 8. Survival curves of larvae and adult male-female mixed populations hatched from control and 9.2 GeV proton irradiated cysts (10 weeks).

*Neutron irradiation*

*Artemia* survival was only tested during the first 3 weeks of life. Fig. 9 indeed shows that most responses to irradiation occur during this time. On the other hand it can be noted that survival of controls was close to that observed for proton irradiation, in spite of a different frequency of renewal of the culture medium. The only difference observed is a better survival for nauplii cultured in a medium renewed daily after the 2nd week.

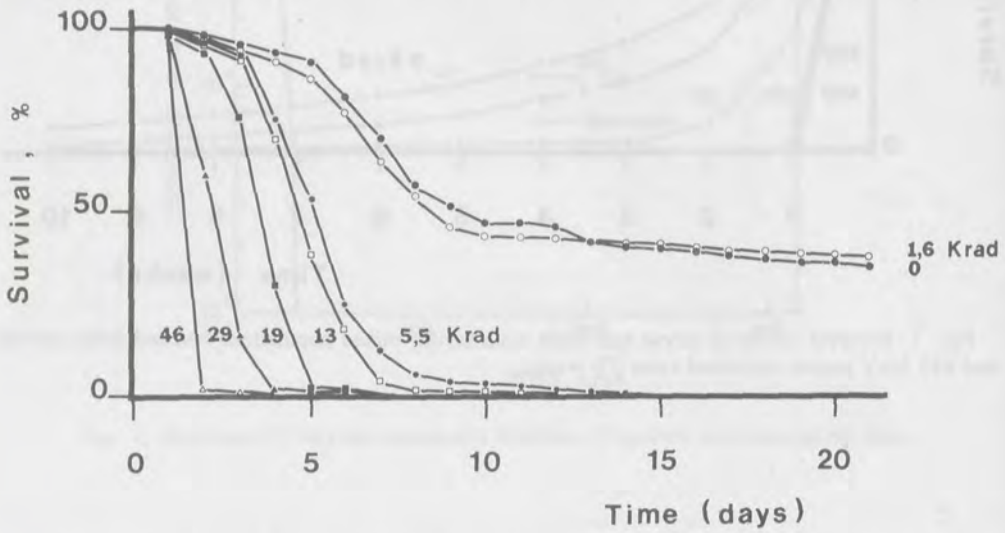


FIG. 9. Survival curves of larvae and adult male-female mixed populations hatched from control and 1.5 MeV mean energy neutron irradiated cysts (3 weeks).

TABLE II

Mean survival times of *Artemia* hatched from eggs exposed to various doses of 645 MeV, 9.2 GeV protons and 1.5 MeV mean energy neutrons. Mean survival times are calculated for 10 weeks (proton irradiation) or 3 weeks (neutron irradiation) sm = standard deviation ; m = mean survival time (days).

| 645 MeV Protons | | | | 9.2 GeV Protons | | | | 1.5 MeV Neutrons | | | Significance |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Dose Krad | m ± sm | ε | | Dose Krad | m ± sm | ε | | Dose Krad | m ± sm | ε | |
| 0 | 25.60 ± 1.14 | – | – | 0 | 26.82 ± 1.13 | – | – | 0 | 8.79 ± 0.09 | – | – |
| 1 | 20.70 ± 1.24 | 2.91 | S | 1 | 29.61 ± 0.99 | 1.85 | NS | 1.6 | 8.90 ± 0.10 | 0.83 | NS |
| 10 | 13.60 ± 1.59 | 6.12 | S | 10 | 29.47 ± 1.08 | 1.70 | NS | 5.5 | 3.24 ± 0.05 | 54.83 | S |
| 100 | < 7 | – | – | 100 | 8.17 ± 1.17 | 11.47 | S | 13 | 2.85 ± 0.04 | 60.89 | S |
| 200 | < 7 | – | – | 200 | 8.75 ± 1.75 | 8.66 | S | 19 | 2.14 ± 0.03 | 70.62 | S |
| 320 | – | – | – | 320 | < 7 | – | – | 29 | 1.54 ± 0.03 | 77.75 | S |
| 400 | < 7 | – | – | – | – | – | – | 46 | 1.01 ± 0.01 | 87.28 | S |

No change occurs in the survival of nauplii hatched from 1.6 Krad eggs. For higher doses, a marked decrease in survival capacity is noted and all nauplii died before reaching sexual maturity.

A dose-dependent response is clearly found in eggs receiving doses from 1.5 to 46 Krad. Mean survival time values, calculated for 21 days, are reported in Table II.

**Discussion and conclusions**

The results show that efficiency of proton, neutron, and gamma irradiation depends on the experimental endpoints. Effects of irradiation increase when we consider changes in the success of emergence, hatching and larval survival. These data support the results obtained by Iwasaki *et al.* (1971) which show that lethal effects become more obvious when larval survival is tested from the first to the 5th day after hatching.

Differences in LD 50 values, calculated for emergence, hatching or nauplius survival, can be explained by the physiological state of the embryos : emergence is principally due to water uptake, but nauplius survival requires high metabolic and mitotic activities. RBE values for proton irradiation, calculated in this work, are higher than those generally reported. Indeed, a value close to 1.0 is seen more often (Clapp *et al.*, 1974 ; Robertson *et al.*, 1975). Grigoriev *et al.* (1964) determined that the RBE of 125-510 MeV protons was about 2.5 in dogs. RBE was measured by Clearly *et al.* (1973) with respect to cataract formation in rabbits. These authors have reported a RBE value of 2.0 for 110 MeV protons and 2.6 for 20 MeV protons. More recently, using 70 GeV protons and taking into account abnormal mitosis frequency in *Vicia faba*, Akoev *et al.* (1974) found a RBE up to 2.1.

Values of RBE higher than 1, as reported in this paper or by authors using high energy protons, can be explained by an energy loss into biological material different from that of low energy protons. Very energetic protons can indeed generate nuclear interactions giving rise to particles of higher ionization density.

The RBE values for neutrons range from 1 to 100 according to particle energy, dose, biological object or endpoints. A value of about 10 was also reported by Underbrink and Sparrow (1974) in *Tradescantia*, by Richold and Holt (1974) for mutagenesis in cultured Chinese hamster cells and by Di Paola *et al.* (1978) for lens opacification in mice. Hedges and Hornsey (1978) reported a RBE of 1.95 to 2.46 in human lymphocytes.

In *Artemia*, a slight discrepancy can be seen between our results and those reported by Iwasaki *et al.* (1971). Indeed an LD value of 33 Krad for larval survival on the 3rd day was reported by these authors and 20 Krad according to our results. Moreover, all nauplii died after hatching after exposure to doses above 150 Krad whereas the same response was already observed, in this paper, at 46 Krad. This discrepancy can be due to differences in strain radiosensitivity or culture methods. On the other hand, Iwasaki *et al.* (1971) irradiated with monoenergetic 2 MeV neutrons and it is well known that RBE neutron varies as a function of particle energy (Hall *et al.*, 1975).

For investigations on survival capacity the efficiency of neutron irradiation is much higher than that of protons but effects on lifespan were not determined in this paper. Differences between 645 MeV and 9.2 GeV protons are in good agreement with RBE values which we have reported for young nauplii. Furthermore, the slight but non significant increase in adult survival after exposure to 1 or 10 Krad of 9.2 GeV protons could be compared to the

lengthening in lifespan already described in insects after exposure to various doses of radiations (Giess, 1978). It can be noted that this effect has not been observed after neutron irradiation in *Artemia* eggs.

## Literature cited


Akoev J. G., B. S. Fomenko, A. K. H. Akhmadieva, V. N. Lebedev, and V. S. Lukanin. 1974. BRE of secundary radiation from 70 GeV protons according to the experimental endpoints. *Radiobiologija USSR* 14:631-634.

Belogurov V. N., Y. Ya Bondars, A. A. Lapenas, R. S. Reznikov, and P. E. Senkov. 1979. Tangential channel for nuclear gamma-resonance spectrometry during thermal neutron capture. *Izvestya An. Latv. USSR, seriya physicheskih i technichestkih nauk* 1:11-15.

Clapp N. K., E. G. Darden, Jr. and M. C. Jernigan. 1974. Relative effects of whole-body sublethal doses of 60 MeV protons and 300 kVp X-Rays on disease incidences in RF mice. *Radiat. Res.* 57:158-186.

Clearly S. F., W. J. Geeraets, R. C. Williams, H. A. Mueller, and W.T. Ham. 1973. Lens changes in the rabbit from fractionated X ray and proton irradiations. *Health Phys.* 24(3):269-276.

Di Paola M., M. Bianchi, and J. Baarli. 1978. Lens opacification in mice exposed to 14 MeV neutrons. *Radiat. Res.* 73:340-350.

Gaubin Y., H. Planel, B. Pianezzi, E. E. Kovalev, and V. I. Popov. 1979. Effects of 645 MeV and 9.2 GeV protons on *Artemia* eggs. *Intern. Journ. Radiation Biology* 36:489-497.

Giess M. C. 1978. Etude expérimentale sur la longévité et le vieillissement de *Drosophila melanogaster* : influence du sexe et de l'activité sexuelle. Thèse Doctorat ès Sciences, Université P. Sabatier, Toulouse, No. 830.

Grigoriev Yu G., N. G. Darenskia, M. M. Domshlak, A. V. Lebendinsky, Y. G. Nefedov, and N. I. Rizov. 1964. Caractéristique des effets biologiques et de l'EBR des protons de haute énergie. p. 223-230. *In* : Biological effects proton, neutron irradiation. Proc. Symp. Upton 1963, Vienna, Intern. Atom. Energy Agency. (in Russian).

Hall E. J., J. K. Novak, A. M. Kellefer, H. H. Rossi, S. Marino, and J. Goodman. 1975. RBE as a function of neutron energy. I. Experimental observations. *Radiat. Res.* 64:245-255.

Hedges M. J. and S. Hornsey. 1978. The effect of X-rays and neutrons on lymphocyte death and transformation. *Int. J. Radiat. Biol.* 33:291-300.

Isayev B. M. and Yu. I. Bregadze. 1967. Neutrons in the biological experiment. *Nauka M.* 118:210-218.

Iwasaki T., T. Maruyama, Y. Kumamoto, and Y. Kato. 1971. Effects of fast neutrons and $^{60}$Co $\gamma$ rays on *Artemia*. *Radiat. Res.* 45:288-298.

Richold M. and P. D. Holt. 1974. The effects of differing neutron energies of mutagenesis in cultured Chinese hamster cells. p. 237-243. *In* : Biological effects of neutron iradiation. Proc. Symp. Neuherberg, 1973. Intern. Atom. Energy Agency, Vienna.

Robertson J. M., J. R. Williams, R. A. Schmidt, J. P. Little, D. F. Flynn, and H. D. Suit. 1975. Radiobiological studies of a high energy modulated proton beam utilizing culturated mammalian cells. *Cancer* 35:1664-1677.

Underbrink A. G. and A. H. Sparrow. 1974. The influence of experimental endpoints, dose, dose-rate, neutron energy, nitrogen ions, hypoxia, chromosome volume and ploidy level on RBE in *Tradescantia* stamen hairs and pollen. p. 185-213. *In* : Biological effects of neutron irradiation. Proc. Symp. Neuherberg, 1973. Intern. Atom. Energy Agency, Vienna.

*The Brine Shrimp* Artemia. *1980. Vol. 1. Morphology, Genetics, Radiobiology, Toxicology*
*G. Persoone, P. Sorgeloos, O. Roels, and E. Jaspers (Eds). Universa Press, Wetteren, Belgium. 345 p.*

# Usefulness of *Artemia* in radiobiology : the effects of 60 MeV protons and of synchrotron orbital radiation on the eggs

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**Abstract**

Dry eggs of *Artemia* have several specific characteristics : they are extremely dehydrated, they tolerate a high vacuum and they are quite small in size (200 $\mu$m). Taking into account these particular features, the following experiments in fundamental radiation biology were carried out.

Proton beam irradiation

Proton beams are of interest in radiotherapy because of their precise localization in the stopping region. The biological effectiveness of proton ion beams with regard to depth of penetration was measured on *Artemia* eggs as study material. Dry eggs of *Artemia* were irradiated with proton beams from a cyclotron under vacuum condition ($10^{-6}$ Torr). A good correlation was found between depth-dose of proton beams and the biological damage as indicated by the decrease of hatchability.

Synchrotron orbital radiation (SOR) irradiation

The use of SOR (290 nm >) in radiation biology as a new photon source requires many technical developments both in the instrumentation and in the handling of biological specimens. Dry eggs of *Artemia* were used in the experiments under two forms : untreated eggs and decapsulated eggs in which the shell has been removed by antiformin treatment. The eggs were attached to scotch tape in a monolayer and irradiated with SOR under vacuum ($10^{-6}$ Torr). The hatchability of untreated cysts was not influenced by the maximum irradiation dose applied in the present experiment. The radiosensitivity of decapsulated cysts, on the contrary, increased dramatically by irradiation. When irradiation was administered through a quartz filter, the radiosensitivity decreased remarkably.

## Introduction

Many extensive research studies of the physiological effects of radiation on *Artemia* have been reported. Of these studies on the role of free radicals produced by irradiation (Iwasaki, 1965, 1966), the modification of the radiosensitivity by change of water content (Iwasaki, 1964b), and the oxygen effect on the radiosensitivity (Iwasaki and Kumamoto, 1976) using the dry eggs of *Artemia* have provided invaluable information on fundamental radiobiology.

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As previously reported, dry eggs of *Artemia* have several specific characteristics (Whitaker, 1940 ; Hinton, 1954 ; Iwasaki, 1973-5) ; they are quite small in size (200 $\mu$m) and tolerate severe condition such as an extremely dehydrated condition, a high temperature (several hours at 100 °C) or high vacuum condition ($10^{-6}$ Torr). Taking into account these particular features, the following two preliminary experiments in fundamental radiation biology were performed.

## Effect of proton beams

In recent years proton beams have become of increasing interest in radiotherapy of cancer because of their good localization characteristics and well defined range. The use of X-rays and $\gamma$-rays in cancer therapy causes some damage to normal tissues before reaching the tumor tissues, since the energy is gradually lost as it passes through the tissues. On the other hand, the bulk of the energy of protons is released at the end of the path and the dose can be concentrated in the tumor tissue. Therefore, protons as well as heavy particles may inflict small damage on the normal tissues through which they pass. In other words, failure in the precise assessment of the location and size of the tumor tissue in the body might give rise to severe damage to the normal tissue. For this reason, the dose distribution and the biological effects associated with the penetration of the proton beam in the tissue should be thoroughly studied before clinical trials.

To measure the radiobiological damage as a function of depth dose, the method of gelatin containing uniformally suspended cells was developed by Skarsgard (1974). It is, however, a very sophisticated technique. In this regard we tested whether the dry eggs of *Artemia* could be a suitable material for this purpose, since the cysts are quite small in size and because they can be easily set in a given position accompanied with the rapid variation of the proton LET in the range of energies studied.

### Biological material

Approximately 500 dry eggs of *Artemia* were attached to scotch tape in a monolayer of 5 mm in diameter. The tapes with the attached eggs were inserted between each layer of Cu and Al absorber as shown in Fig. 1, and irradiated in a vacuum irradiation chamber. After irradiation, the scotch tapes were immersed in culture medium. The eggs separate from the tape within a few minutes, and their subsequent development proceeds normally. The biological damage was estimated on the basis of hatchability of the eggs as already reported in a previous paper (Iwasaki, 1964a).

### Irradiation

The homogeneity of the beam at the position of the monolayer and the beam spread to a useful area were measured by determining the decoloring of blue cellophane. The stacks of Cu and Al absorber of various thickness were used for changing the depth dose desired. The experiments were performed using the 60 MeV proton beam of the cyclotron of the National Institute of Radiological Sciences at Chiba in Japan. The dose average LET of protons in such a steep Bragg peak was estimated by an expedient calculation to be 13.5 KeV/$\mu$m while that in

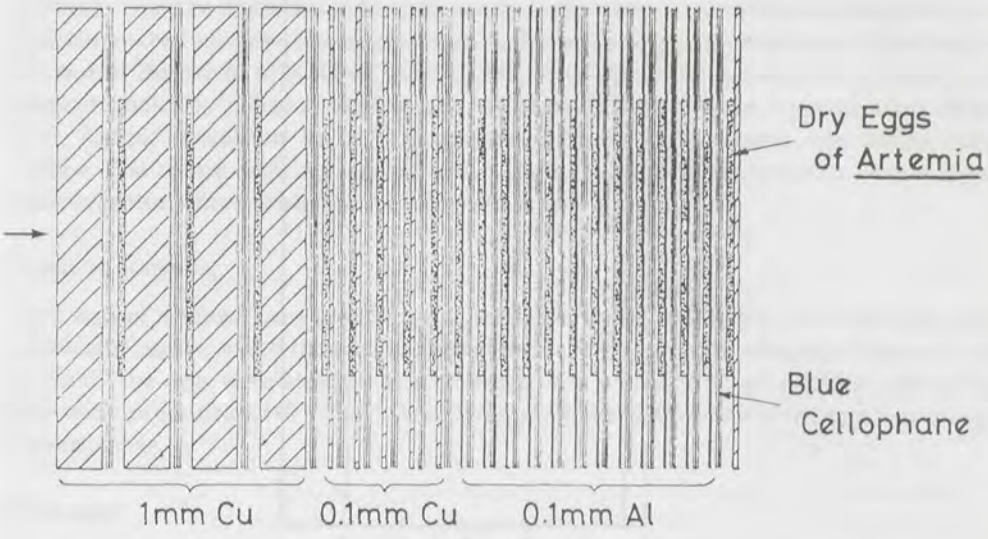


FIG. 1. Setup of the sample for proton irradiation. The arrows show the incident direction of proton beams.

the plateau region was 1.3 keV/$\mu$m. Proton beam intensity was measured by a Faraday cup device in the down stream at the chamber end, the dose-rate being $10^4$-$10^5$ rad/min.

RESULTS AND REMARKS

A depth-dose profile for a 60 MeV proton beam is shown in Fig. 2. The hatchability of the irradiated eggs obtained corresponds to the various depths from the plateau to the peak of the Bragg curve in function of the proton dose (Fig. 3). The greatly increased killing efficiency near the Bragg ionization peak is very noticeable. It is also important to note that the killing effect is almost nil at depths beyond the range of the primary proton beam.

The curves obtained in these experiments are quite similar to those obtained with mammalian cells (Tobias, 1973, Dertinger *et al.*, 1976 ; Davies *et al.*, 1977). Although at present it is not possible to draw a more definite conclusion on the relative biological effectiveness of protons, it can be strongly suggested that *Artemia* dry eggs are a suitable indicator material for radiosensitivity and also a biodosimetrical material comparing favourably with culture cells.

**Effect of SOR irradiation**

Many studies have been carried out on the biological effects of the radiations with shorter wavelengths than soft X-rays or longer ones than near UV. However, studies of the radiation ranging from 6.5 eV to several KeV have never been performed, because of the lack of a suitable light source which can deliver this special range of wavelengths. Studies on these

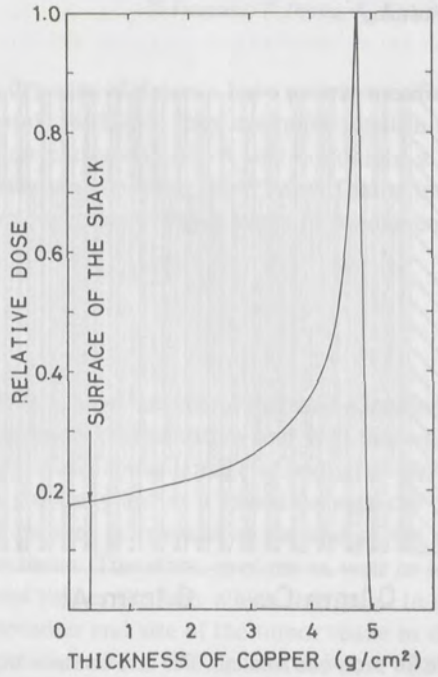


FIG. 2. Physical dose distribution in Cu stacks.

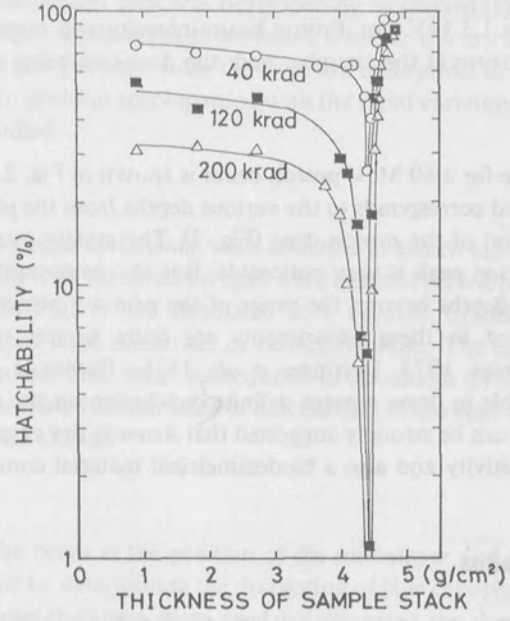


FIG. 3. Biological dose distribution in each stack. Irradiation doses (40, 120, and 200 krad) indicate the dose at the position of beam incidence. The surface of the stack in Fig. 2 corresponds to the zero point in the abscissa (the thickness of sample stack) in this Figure.

wavelenths could be important in order to elucidate the relative contribution of excitation and ionization on the radiation-induced inactivation. Recent technical developments of the photon generator of the use of SOR-RING have allowed to perform the fundamental physical and biological studies by radiation with shorter wavelengths than 290 nm (vacuum ultra violet VUV). Sample irradiation by SOR is performed under high vacuum and temperature variation. The reason why dry eggs of *Artemia* were used in this experiment, is that these special organisms have proved to tolerate such severe conditions.

### Biological material

Dry eggs of *Artemia* were used in the experiments under two forms ; untreated eggs and decapsulated eggs in which the shell has been removed by antiformin treatment (Nakanishi *et al.*, 1963). The eggs were attached to scotch tape in a monolayer and irradiated with SOR under vacuum condition ($10^{-6}$ Torr). After irradiation, the hatchability of the eggs was tested, as stated above.

### Irradiation

Irradiation with VUV was carried out at the Institute for Nuclear Study, University of Tokyo, using the SOR-RING, the characteristics of which have already been published in detail (Ito *et al.*, 1977ab). The usable range of wavelengths was limited to 115 nm by using a $MgF_2$ window, by which the introduction of water vapor or of other compounds from biological materials into the ultra-high vacuum system is avoided. Photon intensity was determined by measurement of the amount of ozone generated from oxygen. Dose measurement has been descried in detail elsewhere (Ito *et al.*, 1977ab). The dose was expressed in mA-min (ring current times exposure time).

### Results and remarks

The hatchability of the untreated eggs was not influenced by the maximum irradiation dose applied in the present experiment. The radiosensitivity of decapsulated eggs, on the contrary, increased with increasing irradiation dose of SOR. In other words, the hatchability decreased up to 30% by 250 mA-min irradiation. If irradiation was carried out through a quartz filter cuts off wavelengths shorter than 190 nm, the killing effect was remarkably reduced (Fig. 4).

Both types of eggs were also irradiated with 254 nm far UV light and $^{60}Co$ $\gamma$-rays for comparison. Hatchability of the eggs with shell did not decrease by UV irradiation but that of the decapsulated eggs decreased remarkably with increased irradiation dose (Fig. 5). The dose required to reduce hatchability of the decapsulated eggs to 50% was 4 000 erg/mm$^2$. For $^{60}Co$ $\gamma$-rays, it was 288 krads in the eggs with shell as well as for decapsulated ones (Fig. 6).

From the resent data, it may be concluded that SOR and UV light can not penetrate the shell of *Artemia* eggs, and that inactivation of eggs seems to be restricted to the range of wavelengths shorter than 190 nm. Similar experiments using the same SOR apparatus were also carried out with other living materials. The dose required to reduce survival of yeast cells to 50% was around 85 mA-min with no filter and 107 mA-min with a quartz filter (Hieda, 1977). The survival of bacterial spores decreased by 90% by exposure to doses of 40-80 mA-min ; the killing effect was considerably reduced when a quartz filter was used (Kada and

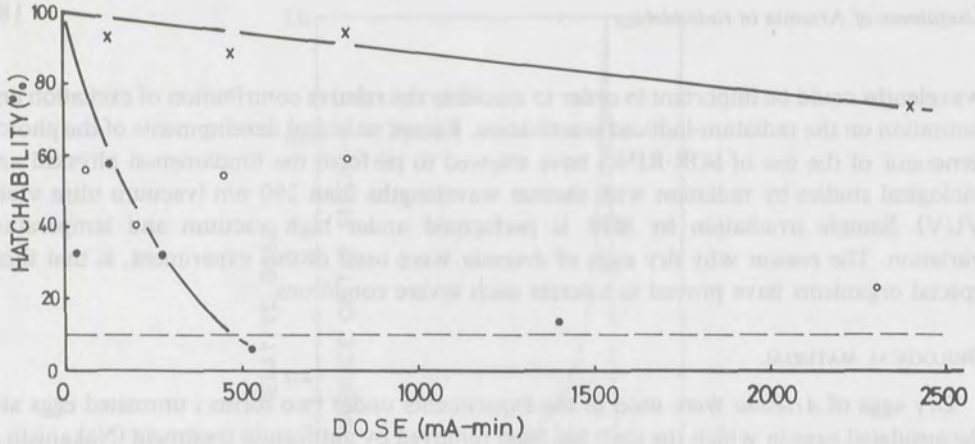


FIG. 4. Hatchability of decapsulated dry eggs of *Artemia* after SOR irradiation. x ——— x : Quartz filter + $M_gF_2$ window. o ——— o : $M_gF_2$ window only. The dotted base line indicates the UV-resistant fraction of eggs after removal of the shell by antiformin treatment. Around 10% of the eggs do not have the shall thoroughly removed by the treatment.

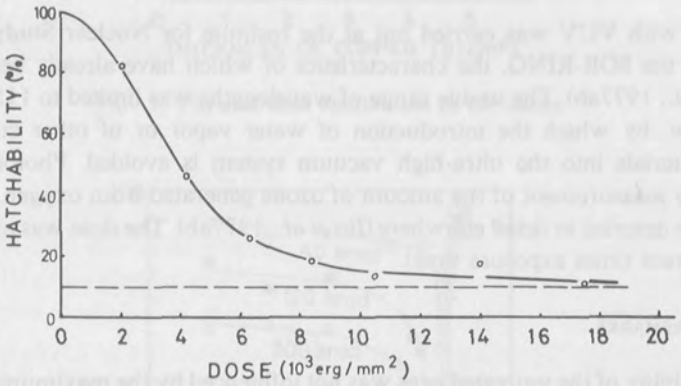


FIG. 5. Hatchability curve of decapsulated dry eggs of *Artemia* after UV irradiation.

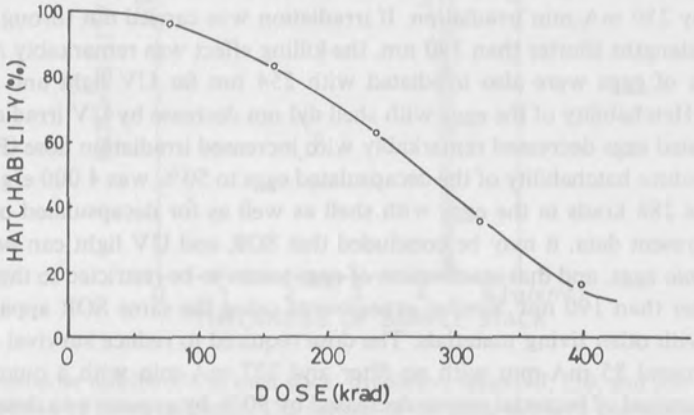


FIG. 6. Hatchability curve of decapsulated dry eggs of *Artemia* after $^{60}$Co $\gamma$-rays.

Ohta, 1977). For comparison with these data, the 50% hatching dose of *Artemia* eggs was 160 mA-min as calculated from the hatchability-effective curve in Fig. 4. It can be concluded that *Artemia* eggs are very resistant to VUV compared to other living materials.

**Acknowledgements**

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**Literature cited**

Davies J. D., C. J. Batty, and K. Green. 1977. The multi-disciplinary role of "pion factories". *Nature* 270:667-676.

Dertinger H., C. Lücke-Huhle, H. Schlag, and K. F. Weibezahn. 1976. Negative pion irradiation of mammalian cells. I. Survival characteristics of monolayers and spheroids of Chinese hamster lung cells. *Int. J. Radiat. Biol.* 29:271-277.

Hieda K. 1977. A note on the dose distribution and the quality of SOR after $MgF_2$ window as measured by inactivation of yeast cells. *Inst. Nuclear Study, Univ. Tokyo, Annual Report 1977* : 119-120.

Hinton H. E. 1954. Resistance of the dry eggs of *Artemia salina* L. to high temperatures. *Ann. Mag. Nat. Hist.* 7: 158-160.

Ito T. and K. Kobayashi. 1977a. Radiation biology with synchrotron radiation : some characteristics of SOR. Inst. Nuclear Study, Univ. Tokyo, Annual Report 1977. p. 118-119.

Ito T., K. Kobayashi, Y. Hatano, K. Ito, A. Yokoyama, and K. Hieda. 1977b. Radiation biology with synchrotron radiation : Experimental setup. Inst. Nuclear Study, University of Tokyo, Annual Report 1977. p. 117-118.

Iwasaki T. 1964a. Sensitivity of *Artemia* eggs to the $\gamma$-irradiation. I. Hatchability of encysted dry eggs. *J. Radiat. Res.* 5:69-75.

Iwasaki T. 1964b. Sensitivity of *Artemia* eggs to the $\gamma$-irradiation. II. Effects of water content. *J. Radiat. Res.* 5:76-81.

Iwasaki T. 1965. Sensitivity of *Artemia* eggs to $\gamma$-irradiation. VII. Relationship between the degree of biological damage and the decay of free radicals in irradiated eggs. *Int. J. Radiat. Biol.* 9:573-580.

Iwasaki T. 1966. Sensitivity of *Artemia* eggs to $\gamma$-irradiation. VIII. Modification of amount of radiation-induced free radicals by post-irradiation treatments. *Int. J. Radiat. Biol.* 11:153-159.

Iwasaki T. 1973-5. Tolerance of *Artemia* dry eggs for temperature, vacuum and radiation. *I.I.F.-I.I.R.-Commission C1-Sapporo* : 79-88.

Iwasaki T. and Y. Kumamoto. 1976. The effect of oxygen on the radiation sensitivity of *Artemia* eggs : a preliminary result of OER on dry eggs. *Radiat. Res.* 67:168-172.

Kada T. and Y. Ohta. 1977. Experiments on killing and mutation by synchrotron radiation in *Bacillus subtilis* spores. Inst. Nuclear Study, Univ. Tokyo, Annual Report 1977, p. 123-124.

Nakanishi Y. H., T. Iwasaki, H. Kato, and T. Okigaki. 1963. Effects of temperature, gamma-rays, and Carzinophilin on encysted dry eggs of *Artemia salina*. *Symposia Cell. Chem.* 13:365-374.

Skarsgard L. D. 1974. Pretherapeutic research programmes at $\pi$-mesons facilities. p. 447-454. *In* : Proc. XIIIth Int. Congress Radiology. Int. Congress Series No. 339, Radiology, Vol. 2, Excepta Medica, Amsterdam.

Tobias C. A. 1973. Pretherapeutic investigations with accelerated heavy ions. *Radiology* 108:145-158.

Whitaker D. M. 1940. The tolerance of *Artemia* cysts for cold and high vacuum. *J. Exp. Zool.* 83:391-399.

*The Brine Shrimp* Artemia. *1980. Vol. 1. Morphology, Genetics, Radiobiology, Toxicology*
*G. Persoone, P. Sorgeloos, O. Roels, and E. Jaspers (Eds). Universa Press, Wetteren, Belgium. 345 p.*

# Effects of space environmental factors on *Artemia* eggs

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**Abstract**

Effects of space environmental factors were investigated on *Artemia* eggs, flown aboard Apollo and Cosmos spacecrafs. A special correlative method between biological objects and cosmic particle tracks was developed in order to study individual effects of HEZ particles (high energy and high atomic number particles, *i.e.*, particles of $Z > 2$). Eggs hit by a single heavy ion can be inactivated. A developmental inactivation also occurs in the non-hit eggs ; the mechanism of this response is discussed.

## Introduction

For several years, we have been working on the effects of space environmental factors in the *Artemia* egg. Indeed, the dry *Artemia* egg is a very suitable material : a motionless, small-sized object, without nutritional requirement. Such as seeds, it can be easily used in passive experiments, which do not require an energy supply. Up to now *Artemia* eggs flew aboard Apollo 16 (Biostack I, Planel *et al.*, 1974, 1975), Apollo 17 (Biostack II), ASTP or Rendez-Vous Apollo-Soyouz (Biostack III), Cosmos 782 (Biobloc 1, Blanquet *et al.*, 1977) Cosmos 936 (Biobloc 2, Gaubin *et al.*, 1979). Some experiments were also performed in airborne stratospheric balloons.

The objective of these investigations was to obtain information on the biological effects of cosmic rays. Primary cosmic rays are made up of protons (80 %), $\alpha$ particles (19 %) and heavy ions or HEZ particles (high energy and high atomic number). When these cosmis particles cross the earth atmosphere, they give rise to very complex radiations. Space experiments were mainly devoted to cosmic heavy ions. Their biological effects must be well known : indeed, if the total absorbed dose, from primary cosmic rays and secundary radiations inside the space-craft, is low (about 15 to 30 mrad/day, measured by thermoluminescent detectors or ionization chamber), the local dose deposited along the heavy tracks can reach several thousands rads. Estimation of space flight radiation hazard must be mainly based on the effects of these particles. However, their flux is low and only small parts of living organisms are directly hit by the cosmic ions during a space flight. Under these conditions, it was necessary to develop a special correlative method in order to separate eggs hit by the heavy particles from the non-hit ones.

## Material and methods

*Artemia* eggs (Californian bisexual and diploid strain) were either embedded in polyvinyl alcohol or placed in circular holes drilled in Makrofol plates. Biological monolayers were sandwiched with Ilford nuclear emulsions of various sensitivity : after recovery, the stack was disassembled and each biological layer with its contiguous nuclear emulsion was covered with a millimetric grid. An exposure to weak light allowed to obtain egg and grid shadows. After processing, heavy ion tracks were observed with a light microscope.

In order to determine if an egg was hit or not hit by the heavy ions, the tracks must be geometrically defined. The coordonates x, y, and z are measured (Fig. 1). This process is followed by a shrinking in the z component of the nuclear emulsion ; tracks must be reconstructed according to the measure of thickness before and after development. The thickness of each emulsion is not constant and calculation of track orientations must be based on mean values of thickness. At the egg level, track positions cannot be determined with an accuracy higher than 10 $\mu$m. Determination of hit and not-hit eggs is made taking in account the accuracy of the correlative method.

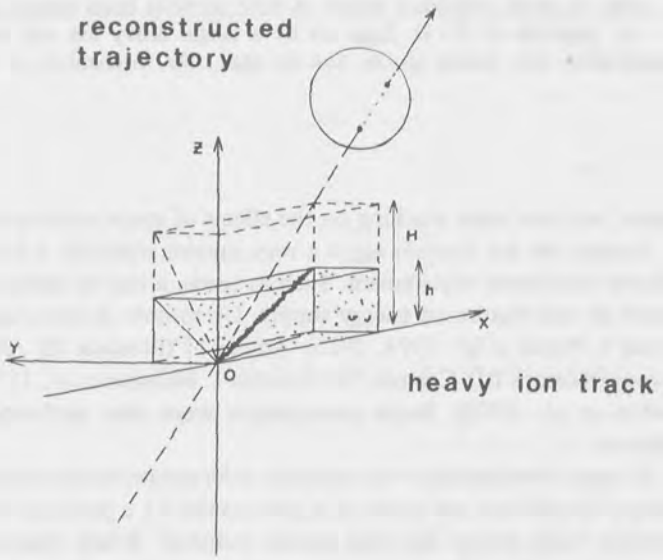


FIG. 1. Correlation between *Artemia* eggs and passage of cosmic heavy ions. Reconstruction of ion trajectory in nuclear emulsion. (H) nuclear emulsion thickness before processing ; (h) after processing.

Based upon nuclear emulsion scanning and using the same millimetric grid, hit and non-hit eggs were picked up, individually placed in crystal dishes with 2 ml of culture medium (artificial seawater at a pH of 7) and incubated at 27 °C. After hatching, algae (*Dunaliella viridis*) were added to the culture medium. Emergence rates, hatching rates and the number of nauplii alive on the 4-5th day were determined.

Dosimetry was made by Fli thermoluminescent detectors, located in the containers, at the egg level. Total absorbed dose is given by the light, protonic component, by the heavy component of the primary cosmic rays and by the secundary radiations. Biostack experiments were performed during lunar flights ; that means that the spacecraft had to cross the Van Allen's belts, made up of protons (internal belt) and electrons (external belt) trapped in the geomagnetic field. The passage through the Van Allen's belt increases the total absorbed dose during the spaceflight.

For Biobloc II experiment, two containers were used : one inside the spacecraft and the other one outside. Eggs in bulk were placed in small plastic bags. The eggs were placed outside the spacecraft in order to directly expose the biological objects to the cosmic rays.

These "outside eggs" were submitted to a temperature ranging from 0 to 30 °C, to a pressure of $10^{-5}$ mm Hg but were shielded by a thin aluminium sheet against solar UV.

Biostack experiments were carried out with different biological objects by members of a working group of Space Biophysics of the Council of Europe ; Biobloc experiments were performed in cooperation with the Institut for Biological and Medical Problems in Moscow.

## Results

### Hit eggs

Fig. 2 shows results of Biostack I experiment flown aboard Apollo 16. For 400 ground control eggs, 263 eggs emerged and 242 nauplii were still alive 4-5 days after the cultures had been set up. For 400 in-flight hit eggs, 38 underwent a beginning of hatching. However, total emergence was obtained for 25 specimens only and six larvae later died.

Percentages of emergences, hatching and nauplii alive on the 4-5th day are plotted in histograms of Fig. 3. Similar results were found in Biostack II experiment, flown aboard Apollo 17.

Space flight also resulted in an emergence delay : on the 18th hr, 20 % of the control eggs from Biostack I had emerged while emergences were only starting for the in-flight specimens (Fig. 4).

The inactivation of the hit eggs can be explained by the energy loss due to heavy ions passing through the eggs. Values of energy losses inside the eggs approximatively range from a few MeV to 130 MeV. Most hit eggs could not develop (Fig. 5).

A total inactivation by a single heavy particle was also found after egg irradiation by carbon or oxygen ions (1.1 or 2.2 MeV incident energy per nucleon), performed with Berkeley's accelerator (Fig. 6). In these experiments, irradiated and control eggs were embedded in PVA and placed in the same stacks than that of Biostack experiment. Energy losses by a single particle, within the eggs, ranged from 1.6 to 3.9 MeV.

### Non-hit eggs

The most striking finding after a space flight was seen in the non-hit eggs but, in this case, results of lunar missions, *i.e.* outside the Van Allen's belts, must be distinguished from those of orbital flights, *i.e.* below the Van Allen's belts.

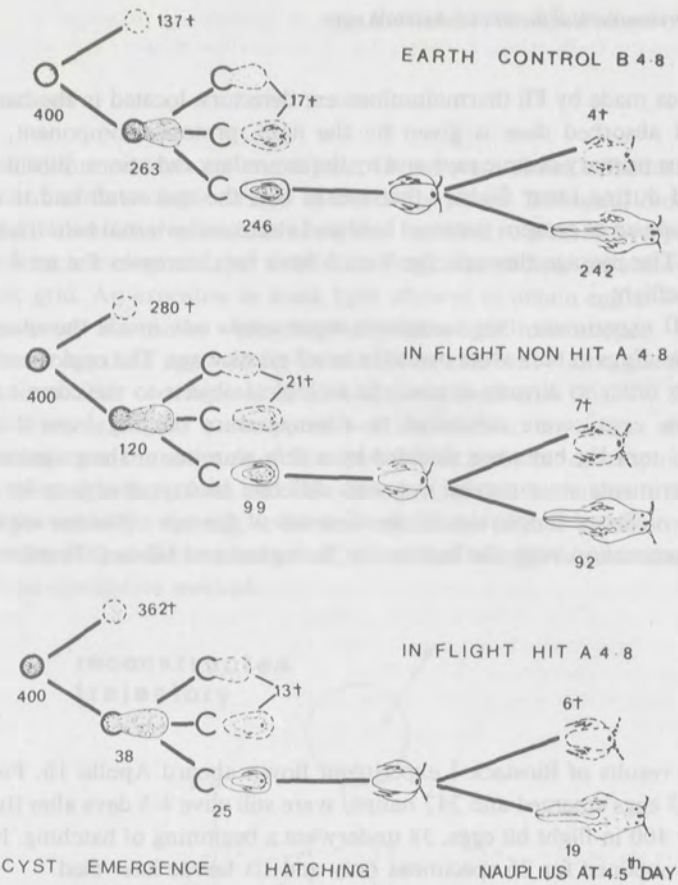


FIG. 2. Developmental capacity of *Artemia* eggs, flown aboard Apollo 16, hit or non-hit by cosmic heavy ions.

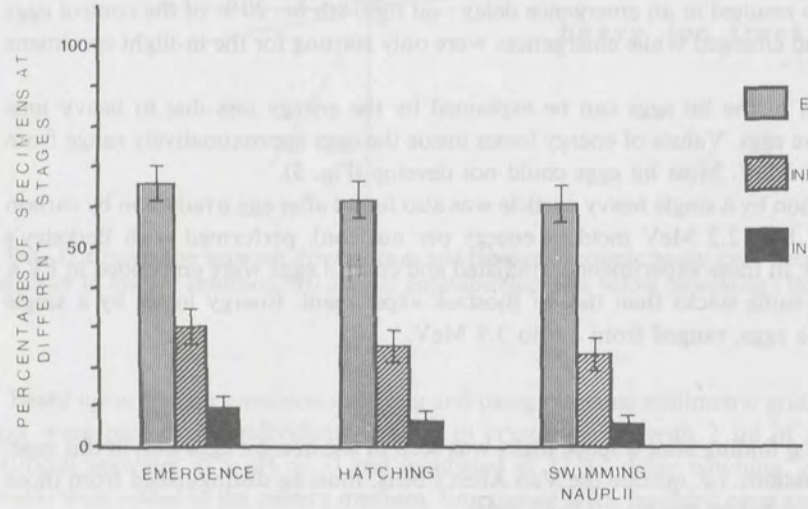


FIG. 3. Variations of developmental capacity of *Artemia* eggs flown aboard Apollo 16.

*Lunar missions*

If we consider results of Biostack I experiment (Fig. 2 and 3) it is obvious that the space flight resulted in a lower hatchability of the non-hit eggs ; however, this decrease in developmental capacity was less pronounced than for the hit eggs.

Emergence delay was also lower in the non-hit eggs (Fig. 4). These biological changes were observed about one month after the recovery (Biostack I and Biostack II experiments).

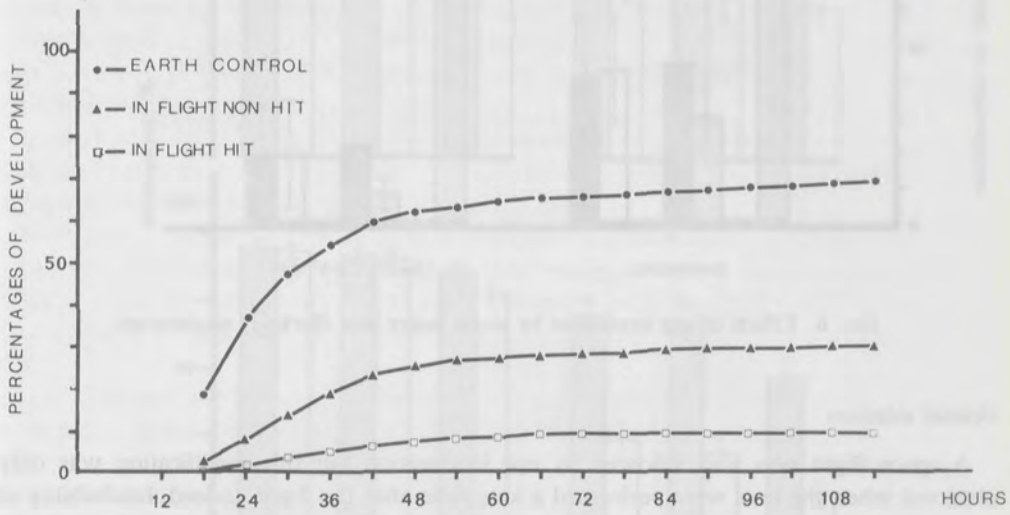


FIG. 4. Time course of emergence of *Artemia* eggs flown aboard Apollo 16.

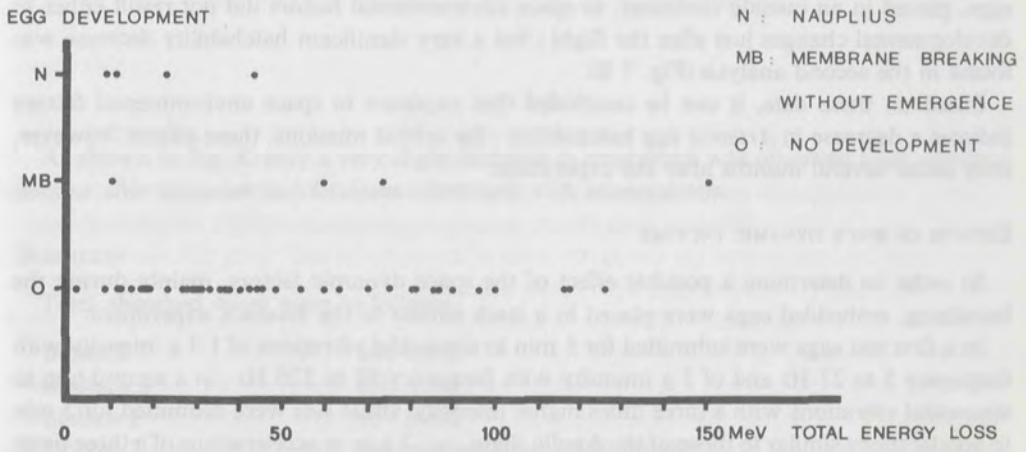


FIG. 5. Correlation between egg hatchability and total energy loss.

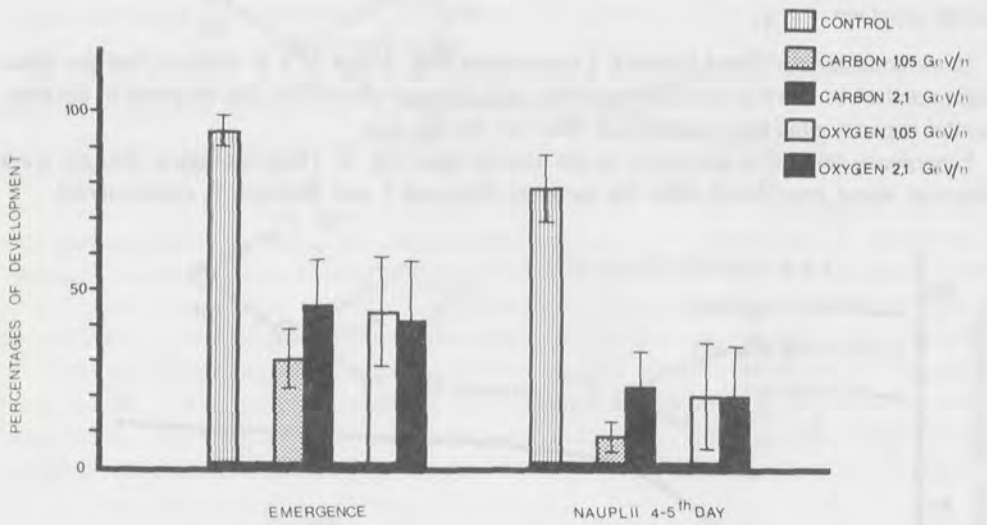


FIG. 6. Effects of egg irradiation by single heavy ions (Berkeley accelerator).

*Orbital missions*

A space flight was also followed by egg inactivation but this modification was only observed when the tests were performed a long time after the flight. Indeed, hatchability of eggs, placed in small plastic bags, without visual detectors and flown aboard the Soviet Cosmos 936 (Biobloc 2 experiment), was normal when egg developmental capacity was checked 1-2 months after the space flight (Fig. 7A).

A very significant decrease in emergence and hatchability rates and larval survival could, however, be demonstrated when the eggs were tested five months later. Exposure of *Artemia* eggs, placed in an outside container, to space environmental factors did not result either in developmental changes just after the flight ; but a very significant hatchability decrease was found in the second analysis (Fig. 7 B).

Based on these data, it can be concluded that exposure to space environmental factors induces a decrease in *Artemia* egg hatchability : for orbital missions, these effects, however, only occur several months after the experiment.

EFFECTS OF SPACE DYNAMIC FACTORS

In order to determine a possible effect of the space dynamic factors, mainly during the launching, embedded eggs were placed in a stack similar to the Biostack experiment.

In a first test eggs were submitted for 5 min to sinusoïdal vibrations of 1.3 g intensity with frequency 5 to 27 Hz and of 5 g intensity with frequency 52 to 520 Hz ; in a second test, to sinusoïdal vibrations with a three times higher intensity. Other sets were submitted for 3 min to accelerations similar to those of the Apollo flight, *i.e.*, 7 g or to accelerations of a three times higher intensity. Other eggs were submitted to vibrations followed by accelerations at the Apollo level.

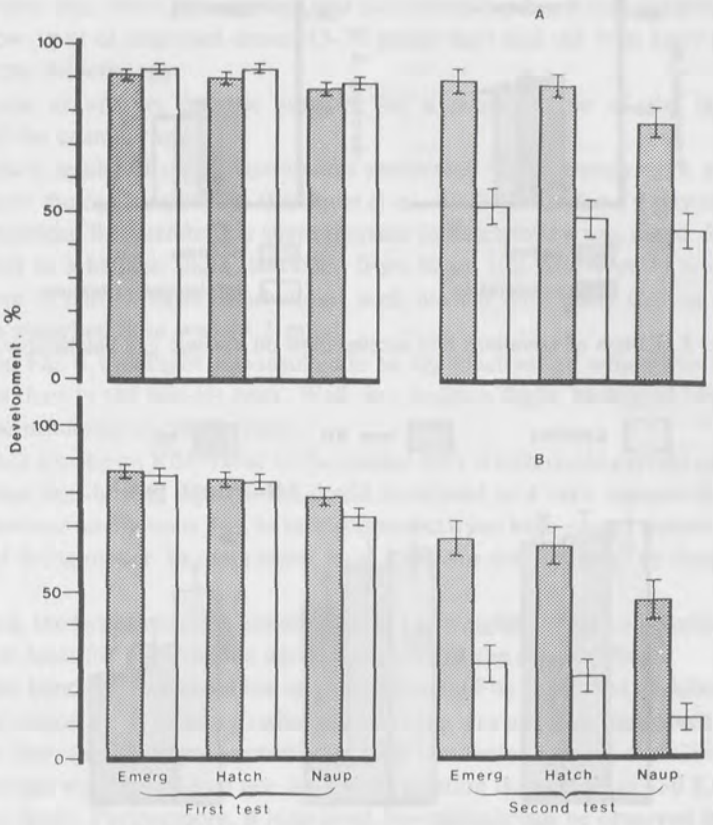


FIG. 7. Influence of time on hatchability of *Artemia* eggs flown aboard Cosmos 936. (A) container located inside the spacecraft ; (B) container located outside the spacecraft.

As shown in Fig. 8, only a very slight decrease in emergence was observed after vibration tests or after exposure to vibrations combined with accelerations.

DOSIMETRY

Total absorbed doses were as follows :

- Biostack 1 : 600 mrad
- Biostack 2 : 850 mrad
- Biobloc 1 : 450 mrad
- Biobloc 2 : internal container : 650 mrad
  external container : 30 rad.

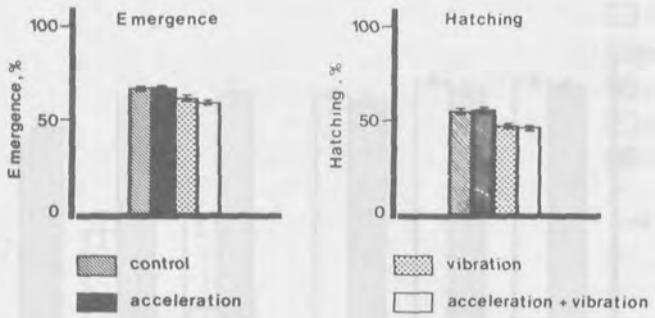


FIG. 8. Effects of vibrations and accelerations on *Artemia* egg hatchability.

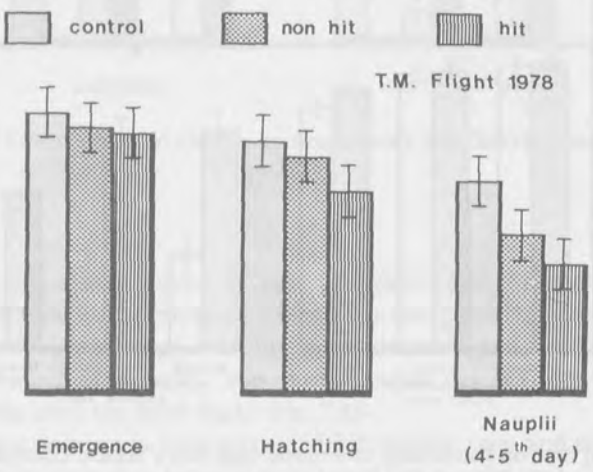


FIG. 9. Developmental capacity of eggs after a transmediterranean balloon flight.

## Discussion

Our results demonstrate that irradiation by a single heavy ion can inactivate the developmental capacity of the *Artemia* egg. The same results are obtained with accelerated heavy ions. However, irraditions on the earth surface were performed with particles of energy higher than that of most cosmic heavy ions (about 300 MeV/nucleon). Nevertheless these data are in good agreement with the assumption formulated by Easter and Hutchinson (1961) : "the passage of a single heavy ion can lead to *Artemia* egg death".

On the other hand, a non predicted effect was observed in the non hit eggs. The origin of this phenomenon is not clear : there is, however, no evidence suggesting that non hit egg inactivation could be induced by vibration or acceleration. Furthermore, it seems unlikely that the non-hit egg response could only be induced by weightlessness. This seems to be more probable in organisms with a high metabolic activity than in those in a resting state.

Based on these data, it can be suggested that our results can be related to the cosmic rays in spite of the low level of absorbed doses (15-30 mrad/day) and the well known high radioresistance of the *Artemia* egg.

Indeed, some arguments provide support for a possible role of the light, protonic component of the cosmic rays.

If we compare results of space experiments performed with *Artemia* eggs, the longer the flight the greater the egg inactivation (Blanquet *et al.*, 1977). Even for a 2 day duration flight aboard the American Biosatellite II, a slight increase in hatchability was noted. Recently, eggs were sumbitted to a balloon flight, launched from Sicily and recovered in Southern Spain. Eggs were kept in plastic plates, sandwiched with nuclear emulsions. Ceiling duration was 22 hr and the absorbed dose was 15.2 mrad.

As shown in Fig. 8, this flight also resulted in an egg inactivation which was more obvious in the hit eggs than in the non-hit ones. Well, in a balloon flight, biological objects are only exposed to the influence of cosmic rays.

As we cannot ascribe an RBE value to the cosmic rays which could explain our results, we can assume that non-hit egg inactivation could be related to $\delta$ rays, created along particule tracks, or to nuclear interactions, *i.e.*, to interactions between high energy cosmic particles and atom nuclei of living matter. In these cases, local doses are not expressed by the global dosimetry.

Furthermore, the hypothesis of a combined effect of weightlessness and cosmic rays cannot be excluded, at least for experiments carried out aboard the spacecrafts.

On the other hand, late effects of the space flights could be compared to delayed responses occuring after exposure to ionizing radiations. For the *Artemia* egg, responses after storage can be higher than that observed immediately after irradiation (Snipes and Gordy, 1963). In this paper, storage was limited to a few days and radiation doses, 150 to 450 Kr, were much higher than in space. Furthermore, if long-lived free radicals can be observed in dry objects, we know that their survival is always limited to a few days (Snipes and Gordy, 1963 ; Iwasaki, 1965). Thus, the origin of delayed effects of space flights is still unknown.

## Conclusions

The *Artemia* egg appears to be a suitable organism to investigate space biological effects. Using this material, non predicted responses were observed and complementary experiments, carried out in space or in earth-based accelerators, will be necessary to explain the mechanism of the phenomena which were demonstrated in this first series of experiments.

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## Literature cited

BLANQUET Y., H. PLANEL, H. G. GREGORIEV, G. GASSET, V. A. BENEVOLENSKY, M. DELPOUX, C. JACQUOT, V. J. POPOV, B. PIANEZZI, and A. A. MARENNY. 1977. Preliminary results of the Biobloc experiment on the Cosmos 782 flight : effets of cosmic rays on brine shrimp eggs and tobacco seeds. p. 165-168. *In* : Life Sciences and Space Research XV. Holmquist R. (Ed.). Pergamon Press.

EASTER S. S. and F. HUTCHINSON 1961. Effects of radiations of different LET on *Artemia* eggs. *Radiation Research* 15:333-340.

GAUBIN Y., E. E. KOVALEV, H. PLANEL, L. V. NEVZGODINA, G. GASSET, and B. PIANEZZI. 1979. Development capacity of *Artemia* cysts and lettuce seeds flown in Cosmos 936 and directly exposed to cosmic rays. *Aerospace and Environmental Medicine* 50:134-138.

IWASAKI T. 1965. Sensitivity of *Artemia* eggs to $\gamma$-irradiation. *Int. J. Radiat. Biol.* 9:573-580.

PLANEL H., Y. BLANQUET, J. P. SOLEILHAVOUP, R. KAISER, and B. PIANEZZI. 1975. Effects of cosmic heavy ions on *Artemia* egg development. p. 1152-1163. *In* : Radiation research. Biomedical, chemical and physical perspectives. Nygaard O. F., H. I. Adler, and W. K. Sinclair (Eds). Academic Press.

PLANEL H., J. P. SOLEILHAVOUP, R. KAISER, and Y. BLANQUET. 1974. Study of cosmic ray effects on *Artemia salina* eggs during the Apollo 16 and 17 flights. p. 85-89. *In* : Life sciences and space research XII. Holmquist R. (Ed.). Akademie-Verlag, Berlin.

SNIPES W. C. and W. GORDY. 1963. Radiation damage to *Artemia* cysts : effects of water vapor. *Science* 142:503-504.

# Toxicity

*The Brine Shrimp* Artemia. *1980. Vol. 1. Morphology, Genetics, Radiobiology, Toxicology*
*G. Persoone, P. Sorgeloos, O. Roels, and E. Jaspers (Eds). Universa Press, Wetteren, Belgium. 345 p.*

# Alterations to the reproductive performance of *Artemia* caused by antifouling paints, algaecides, and an aquatic herbicide

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**Abstract**

This investigation was concerned with metal compounds and pesticides which contaminate the aquatic environment. *Artemia* serves well as an assay organism for total lifetime reproductive performance because running seawater is not required for maintenance or rearing. Preliminary tests served to identify a sublethal exposure which would allow adults to survive at least 2 weeks post treatment. Then data were collected on all components of reproductive fitness.

Copper proved more toxic than the other fourth period metals when adults were exposed to either the inorganic sulfate or the naphthenate form. Reciprocal-cross pair matings revealed that more damage was incurred by the females than the males. As cuprous oxide, released from antifouling paint and oxidized to cupric ions, a subtle influence results which allows continuous exposure. This mode of treatment was not possible with antifouling paints in which tri-n-butyl tin resinate is the toxicant. Even very small painted areas release enough organotin to cause adult death by interference with ATP production and electron transport. Evidently the ionic antagonisms of excess copper is less drastic.

The metal compounds influenced fecundity primarily by decreasing life span, while two organic pesticides were found to influence the number of zygotes without curtailing brood number. These were nitrogenous compounds, a triazole herbicide and a quatenary organic ammonium algaecide, but modes of action may differ. The herbicide probably inhibits metalloprotein enzymes and the algaecide has surfactant properties. None of the treatments seriously altered sex ratio of offspring and encystment of broods was rare. The bearing of these matters on population extinction was discussed, but definitive predictions for natural populations cannot be made without information of possible interactions between contaminants.

## Introduction

The purpose of this investigation was to study the effects of a variety of chemicals commonly used in ways that introduce them into the aquatic environment. The approach was to determine a sublethal level of exposure which allowed adults to survive long enough to produce more than one brood. Then data on reproductive performance was collected until the death of the treated adults.

The toxic levels identified for a variety of agents are regarded as typical of the strain tested under laboratory conditions, and not necessarily characteristic for the species. However, some general principles emerged. A decrease in the life span, particularly of the female, had an

important influence upon the number of broods and their size. On the other hand, two of the agents were able to decrease the numbers of zygotes without curtailing the number of broods.

This report includes tests in which the toxicant was mixed with the water, and tests in which toxic painted surfaces were immersed in it. The latter type of exposure can be just as serious as the former when the toxic action of an antifouling paint depends upon the release of a toxicant.

**Materials and methods**

The adult *Artemia* used for these experiments were reared from cysts of San Francisco ancestry. Most of the tests were performed with shrimp from a mass population maintained in the laboratory for over 20 years. The adults exposed to the organic algaecide were grown from cysts collected and vacuum packed 10 years ago.

The plan was to have 10 pairs of reproducing adults representing each treatment group. Simultaneously, data on the reproductive performance of an equal number of control pairs were recorded. Upon the death of one member of a pair, a replacement from the same treatment group was supplied as soon as possible.

Details of exposure will be used to identify experiments in the Results. The characteristics of each agent has to be considered in devising methods for exposure. Most of the exposures lasted no more than 4 days, as could occur with an occasional pesticide application, industrial discharge, or ship mooring; Continuous exposure to a toxicant was feasible only with small areas of cuprous oxide antifouling paint.

After exposure, pairs were maintained in separate jars containing 500 ml of a standard brine solution (Grosch, 1966) except those of the organic algaecide test which survived better in artificial seawater. As they appeared, larvae were counted and transferred to a separate jar of brine before their parent's daily feeding of 0.5 ml of yeast suspension. Upon the rare occasion that cysts were produced, they were filtered, dried, and resuspended in seawater to determine the proportion from which larvae could emerge. Both pair-mating jars and rearing jars were kept under constant fluorescent light. Under these conditions a brood was produced by each pair every 3-5 days, an average of 4 days. Volunteer algae grew in all pair mating jars and brood rearing jars.

At higher concentrations copper sulfate is used for algae control as well as in fungicidal and insecticidal preparations. Also copper sulfate appears among the components of industrial wastes along with zinc, cobalt and nickel sulfates. In my experiments a dilution series of each metal sulfate was used, starting with saturated aqueous solutions of the crystalline forms of divalent Cu, Co, Ni, and Zn sulfates. From each of these a small measured amount was added to a liter of seawater and stirred by hand. From this, further dilutions were made as necessary. In order to qualify for a reproductive performance test the exposed individuals had to survive 4 days of treatment and at least 2 weeks post treatment. During this time they were expected to begin producing offspring. For this set of experiments a reciprocal cross design was used for the pair matings : treated males mated to untreated females and *vice versa*. The adults were withdrawn from the stock culture just after they reached maturity.

Naphthenates of the same fourth period cations have been put to diverse uses in Central and Eastern Europe where there is a high naphthenic content of the crude oils. In the United States, only copper and zinc naphthenates are commonly used,primarily as wood and textile

preservatives, but these are stocked by most hardware and building supply stores. By nature the preparations are not water soluble. At first I tried the horizontal surface approach used to test the anti-settling effect of paints on bryozoan larvae (Wisely, 1963). This proved unsatisfactory partly because brine shrimp browse the bottom surface. Painted panels which could be clamped vertically in a treatment jar was more satisfactory. The size of the panel and volume of water was suggested by an apparatus for testing the leaching rate of antifouling paints (Anonymous, 1967).

Currently the boat bottom paints in general use employ one of two toxicants. The active agent in the long time favorite has been cuprous oxide. More recent formulations employ organotin as the toxin ingredient. Both are available in the stores near boating facilities. Antifouling paints are formulated to release their toxicant at an adequate rate in a designated time per unit area. Therefore the smaller the painted area the lower is the amount of toxicant delivered. Successively smaller areas painted with an antifoulant functioned well in providing a graded series of exposures for *Artemia*. Pine garden stakes about 2 mm thick were painted with square or rectangular area. Before use they were hung up to dry. Areas providing enough toxicant to kill adult *Artemia* did not interfere with algae growth in the treatment jar.

Amitrole (3-amino-1, 2, 4-triazole) is marketed for use on pasture weeds as well as on rushes and other aquatic vegetation. In preliminary survival tests levels of treatment ranged from 1 000 ppm to 5 ppm. In the higher concentrations all adults died within 4 days. After removal to brine from 50 ppm on the fourth day adults died within a week. Shrimp from the 5 ppm jar treated similarly produced broods, so a full scale experiment was set up at this level.

Algaecide (Cal Jet™) liquid contains 7.5% of alkyl dimethyl benzyl ammonium chloride. The recommended initial treatment of swimming pool water is above 30 ppm. In preliminary tests this killed all shrimp within 24 hr. Higher doses killed in 12 hr. Serial dilutions were tested down to 0.06 ppm which shortened adult life span to about 2 weeks for shrimp maintained in standard brine. Within that period only half the females produced a brood. During these tests the laboratory reached a temperature of 30 °C during the daytime. Another series of dilutions down to 1 part/$10^9$ was then used for *Artemia* exposures at 25 °C. However, even at the lowest dose, deaths occurred soon after the treated adults were transferred to standard brine. A feasible level for studying reproductive performance over an appreciable period post-treatment was identified only when we modified the usual procedure and transferred treated shrimp to artificial seawater.

## Results

### Fourth period cations

Table I summarizes data on reproductive performance of *Artemia* in which only one sex of the tested pairs was exposed to a fourth period metal sulfate. The level of tolerance was that which allowed exposed individuals to survive at least 2 weeks post-treatment. As shown, this resulted in life spans approaching or exceeding those of controls.

In all the tests males lived longer than the respective females, and in the $CuSO_4$ and $ZnSO_4$ tests there is evidence that females were more vulnerable. With Cu a more dilute solution had to be used to treat females. With Zn the identical dilution for each sex shortened female life span and decreased the number of broods produced. Otherwise the average number of broods

per pair approached or exceeded the control value. With all four of the agents the mean number of offspring per brood,and survival to adulthood was good.

TABLE I

Adult *Artemia* life span and the components of their reproductive performance summarized as means and standard errors. Data were for pairs in which one sex was treated

| Treatment | Parent treated | ppt | Life span (days) | Number of broods | Offspring per brood | Survival to adult (%) | Sex ratio |
|---|---|---|---|---|---|---|---|
| None | ♀ | | 32 ± 4 | 5.5 | 150 ± 17 | 60.4 | 1.00 |
| | ♂ | | 42 ± 1 | | | | |
| $CuSO_4$ | ♀ | 0.0003 | 31 ± 5 | 6.5 | 168 ± 12 | 62.1 | 0.92 |
| | ♂ | 0.006 | 39 ± 8 | 8.8 | 154 ± 21 | 66.6 | 1.18 |
| $ZnSO_4$ | ♀ | 0.01 | 24 ± 5 | 3.7 | 133 ± 15 | 59.7 | 1.05 |
| | ♂ | 0.01 | 48 ± 7 | 8.1 | 154 ± 11 | 46.3 | 0.99 |
| $CoSO_4$ | ♀ | 0.03 | 30 ± 4 | 6.7 | 141 ± 11 | 70.2 | 0.88 |
| | ♂ | 0.03 | 38 ± 4 | 5.7 | 176 ± 8 | 56.5 | 0.87 |
| $NiSO_4$ | ♀ | 0.3 | 31 ± 4 | 5.4 | 168 ± 20 | 56.0 | 1.22 |
| | ♂ | 0.2 | 35 ± 4 | 5.1 | 132 ± 11 | 67.4 | 1.04 |

Primarly on the basis of adult toleration in ppt, the order of toxicity as tabulated was Cu, Zn, Co, Ni. At levels higher than could be used for testing reproductive performance a copper green color appeared in the gut contents on the second day of exposure. At subsequent death some of these shrimp exhibited exceptionally dark green appendicular tissues. Some of the dead females in higher level cobalt exposures showed unusually deep orange-red somatic tissues.

METAL NAPHTHENATES

Their insolubility in water, a utilitarian aspect of copper and zinc naphthenate wood preservatives, posed a problem for treating shrimp. Initially I coated the bottoms of quart jars with copper naphthenate and allowed it to dry. Pairs of adults in 900 ml of seawater swam slowly after 2 days exposure. After removal to brine these adults survived only a day. Additional pairs of adults exposed for only 1 day survived 2 weeks post-treatment. The mean number of broods produced was 2.2 ± 0.6. Meanwhile a similar experiment was begun using a thin layer of zinc naphthenate. These pairs survived for a month in the presence of the agent and produced a large number of larvae. Finally an extreme measure succeeded in killing the adults after a few days of exposure to zinc naphthenate : 100 ml of the commercial product containing 13.5 % of the active ingredient was evaporated down to a gummy residue which coated the 5 000 $mm^2$ bottom of a jar. Adults maintained in 900 ml of seawater over this residue declined in vigor over 3 days and died within a week without reproducing.

At this point I shifted to using painted wood panels as a device for exposing shrimp to agents supplied as suspensions in organic solvents. Adults of both sexes tolerated 4 000 $mm^2$ areas painted with either zinc, ferric, cobalt, or nickel napthenate. Neither life span nor

reproductive performance was decreased significantly. Testing larger areas seemed impractical and likely to be unproductive.

BOAT BOTTOM PAINTS

*Cuprous oxide*

Vertical painted surfaces are used in standard tests of antifouling ability as well as physical-chemical properties. Here they were used for exposing shrimp swimming in the vicinity of paints which release a toxicant.

In the traditional favorite the active ingredient is cuprous oxide which must leach at a rate greater than 8 $\mu g/cm^2/day$ to serve as an effective antifoulant. Typically this requires more than 13.8% dry weight (Anonymous 1967). We used a formulation containing 24% cuprous oxide dry weight. Table II summarizes the components of reproductive fitness for adult shrimp exposed to a relatively large area of wood painted with a standard copper antifouling paint. Life spans were decreased as much by a 5 day exposure as they were when the painted surface remained in the treatment jar until all shrimp had died. After a 3 day exposure, females survived for an average of 22.7 days. However the number of broods produced and the number of offspring was low.

TABLE II

Adult *Artemia* life span and components of reproductive fitness after exposure to a 4 000 $mm^2$ surface of cuprous oxide antifouling paint

| Length of exposure | Life span (days) | | Number of broods | Total no. zygotes | Survival to adult (%) | Sex ratio |
|---|---|---|---|---|---|---|
| | ♀ | ♂ | | | | |
| Continuous | 7.2 ± 0.2 | 7.4 ± 0.2 | 1.2 | 47.8 ± 10.3 | 55 | 0.9 |
| 5 days | 6.7 ± 0.7 | 7.1 ± 0.8 | 0.6 | 74.2 ± 11.1 | 81 | 1.0 |
| 3 days | 22.7 ± 7.3 | 17.4 ± 5.7 | 1.2 | 69.2 ± 21.1 | 64 | 1.2 |
| Control | 50.4 ± 4.1 | 51.6 ± 3.5 | 9.2 | 1 238.7 ± 214.7 | 61 | 0.9 |

Fig. 1 compares the pattern of fecundity for controls with that of shrimp exposed until death to relatively small areas of copper oxide paint. Table III summarizes various components of their reproductive fitness. The control values are given on Table II. Life spans were decreased by the exposure and proportionately fewer broods were produced. On the other hand, the growth of adults was sufficient for the females to give birth to some broods containing more than 100 larvae. Survival to adulthood was good.

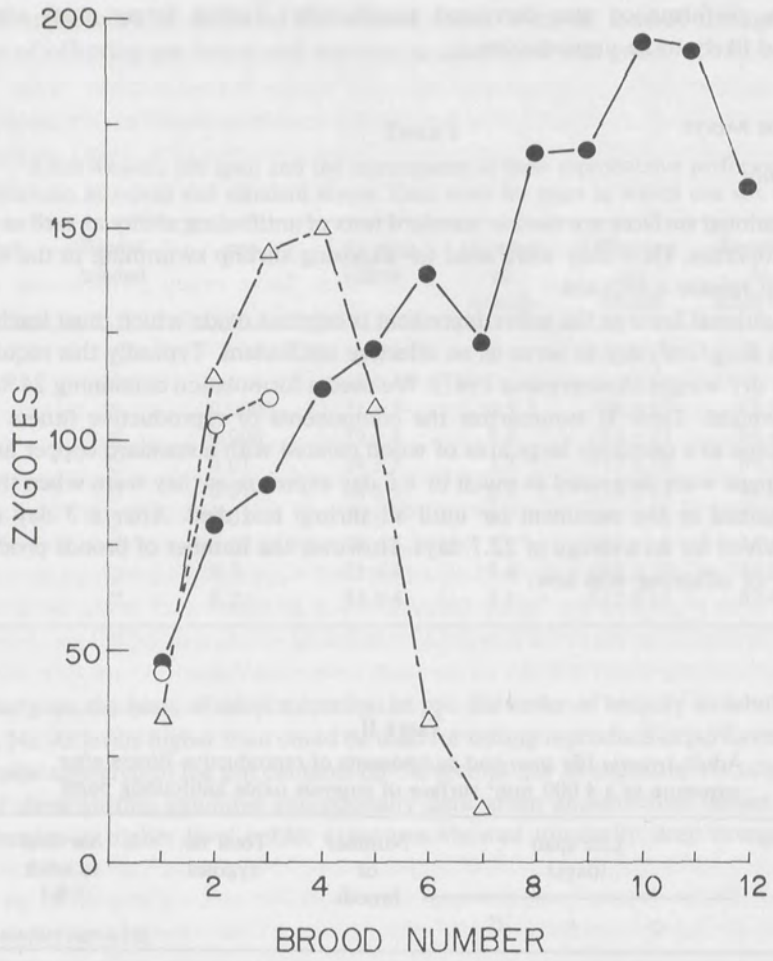


FIG. 1. The fecundity of controls (dots) compared with that of adults in the presence of copper oxide bottom paint. Results from areas of two sizes were plotted : circles – 160 sq. mm. ; triangles – 80 mm².

TABLE III

Adult *Artemia* life span and components of reproductive fitness during exposure to small areas of cuprous oxide antifouling paint

| Painted area (mm²) | Life span (days) | | No. of broods | Total no. zygotes | Survival to adult (%) | Sex ratio |
|---|---|---|---|---|---|---|
| | ♀ | ♂ | | | | |
| 160 | 17 ± 1 | 17 ± 1 | 2.4 | 201 ± 42 | 63 | 1.2 |
| 80 | 37 ± 3 | 31 ± 1 | 4.6 | 505 ± 30 | 69 | 0.8 |

*Organotin*

A red, hard-finish for racing craft containing n-tributyl tin resinate was tested, also by using vertical painted surfaces. Fig. 2 gives some of the mortality curves obtained by exposing samples of adult *Artemia* to decreasing aread of the red paint. Those drawn are representative of the family of curves obtained from 11 tests, and served as a basis for selecting a 4 day exposure to a 9 mm² area for a full scale study of reproductive performance.

The means of Table IV provide a basis for comparing the experimental results with their controls. Despite a longer average life for females and an extra two broods, treated pairs did not outproduce controls. The cumulative total number of zygotes reflects a consistent slight deficit of offspring in each successive broad.

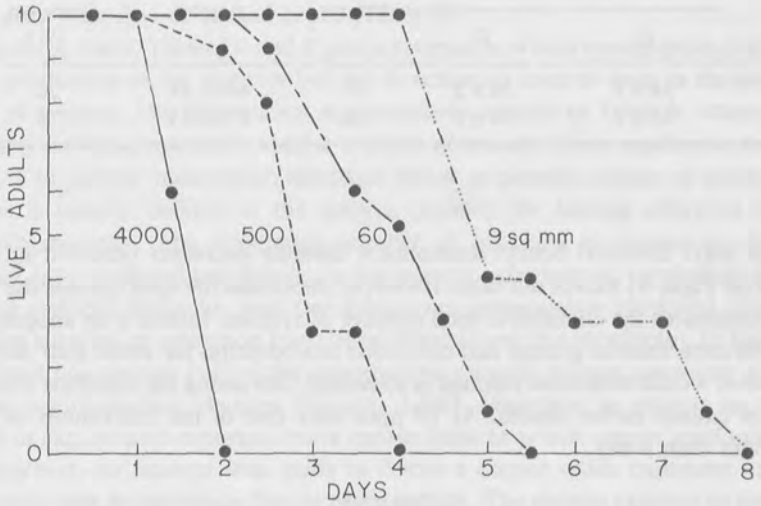


FIG. 2. Mortality curves for *Artemia* maintained in the presence of organotin boat bottom paint. The area of the painted surface is shown along each curve.

TABLE IV

Adult *Artemia* life span and components of reproductive fitness after four days of exposure to a 9 mm² surface of butyl tin antifouling paint

| Group | Life span | | No. of broods | Total no. zygotes | Larval survival (%) | Sex ratio |
|---|---|---|---|---|---|---|
| | ♀ | ♂ | | | | |
| Control | 53 ± 5 | 72 ± 7 | 10.4 | 1 208 ± 244 | 55 | 1.1 |
| Treated | 66 ± 3 | 61 ± 7 | 12.1 | 1 028 ± 214 | 57 | 1.3 |

AQUATIC HERBICIDE

Another example of results which differ in fecundity is presented by Table V. All other components of reproductive fitness are similar to the control values. A slightly higher survival to adulthood reflects the less crowded condition of brood jars containing lower numbers of larvae from treated mothers. Statistically the difference in survival is not significant. Standard errors for the control and treated groups were 4.6 and 3.8% respectively. In this experiment nine of the ten control pairs and eight of the ten treated pairs had ceased reproduction after seven broods. For an optimum comparison the tabulation is based on seven broods.

TABLE V

Adult *Artemia* life span and components of reproductive fitness after a sublethal exposure to triazole aquatic herbicide

| Group | Life span | | No. of broods | Total no. zygotes | Larval survival (%) | Sex ratio |
|---|---|---|---|---|---|---|
| | ♀ | ♂ | | | | |
| Control | 34 ± 3 | 36 ± 2 | 5.1 | 404 ± 84 | 70 | 1.0 |
| Treated | 33 ± 3 | 36 ± 5 | 5.6 | 270 ± 61 | 75 | 1.1 |

ALGAECIDE

In tests of alkyl dimethyl benzyl ammonium chloride decreases occurred in all criteria summarized on Table VI except sex ratio. However, shortened life span curtails the number of broods and influences the cumulative total number of zygotes. Indeed if an adequate number of pairs in the experimental groups had continued reproduction for about four more broods, the control level would have been reached or exceeded. Increasing the algaecide concentration above 2 ppm caused earlier deaths. At 10 ppm only one of ten individuals of either sex survived longer than a day.

TABLE VI

Adult *Artemia* life span and components of reproductive fitness after exposure to an algaecide (alkyl dimethyl benzyl ammonium chloride)

| Group | Life span | | No. of broods | Total no. zygotes | Larval survival (%) | Sex ratio |
|---|---|---|---|---|---|---|
| | ♀ | ♂ | | | | |
| Control | 68 ± 9 | 101 ± 11 | 13.2 | 1 183 ± 234 | 72 | 0.9 |
| 1 ppm | 39 ± 12 | 85 ± 11 | 8.6 | 1 060 ± 350 | 63 | 0.9 |
| 2 ppm | 46 ± 12 | 50 ± 15 | 9.6 | 876 ± 365 | 68 | 0.9 |

## Discussion and conclusions

The pair mating experiments reported above revealed that decreases in reproductive performance could be caused by every toxicant tested. However, obvious decreases in

fecundity were assocated with impending early death of the parents. When post-treatment survival was adequate to allow production of more than one brood, nearly normal reproductive performance resulted. In cases where life span was curtailed, a concomitant decrease in broods and total offspring resulted. Examples include the data for zinc sulfate treated females of Table I, for copper antifoulant exposed adults of Tables II and III, and for the algaecide treated adults of Table VI.

Usually life span of the female was the limiting factor for two reasons. Once started, broods tend to be voided regularly until death. In addition, size is indeterminate for shrimp. The longer they live the larger they grow, and the more zygotes per brood a female can produce. Fig. 1 shows the general pattern of fecundity for untreated *Artemia* : a gradual increase in offspring for nearly every successive brood until senility. During investigations over more than 20 years, peak production of about 200 zygotes per brood had been common (Grosch, 1966, 1973). Some experiments peaked at 300, as occurred for the controls of Table IV. Early broods are expecially small and the cumulative total for zygotes is low if the females die after voiding only their first or second brood (Table II).

On the other hand, Tables IV and V give examples in which treated pairs lived long enough to be as productive as the controls but fail to achieve a control level in the cumulative total number of zygotes. The discrepancy is particularly notable in Table V where experimental and control females produced a similar number of broods. These results can be explained by the defects in cellular metabolism identified below as possible modes of action.

Fitness is usually defined as the relative capacity for leaving offspring that attain reproductive maturity. The maximum number of offspring is limited by the number of functional eggs produced per female. In the present experiments, survival to adulthood was good and reduced fecundity was the deleterious consequence identified. The question of population survival or extinction must be addressed from this standpoint. In long term studies of irradiated populations half of the reproductive capacity seemed necessary as a reserve for buffering environmental changes (Grosch, 1966). Decreases as serious as this were not obtained in experiments reported above except those in which copper antifouling paint were used. However, no attempt was made to devise a copper oxide treatment mild enough to permit every pair to reproduce five or more broods. The shrimp exposed to the copper paint simply did not perform like the adults surviving 2 weeks after exposure to other agents. In particular, the butyl tin paint either killed the adult or barely hindered its performance. The continuous exposures to copper paint were obtained out of curiosity. At appreciable levels, none of the other agents were tolerated more than a few days.

Along with many other organisms, *Artemia* have adopted the reproductive strategy of investing maximum energy in gamete production, but in addition to quantity, the quality or type of zygote is significant for the survival of shrimp populations. Shunting offspring into a dormant, encysted state is a highly effective *Artemia* response to unfavorable external factors. However, this time, cyst production was unexpectedly meager. Most of the treated pairs gave no cysts although control pairs produced at least one encysted brood late in life. Thus treated *Artemia* could be vulnerable to additional environmental alterations.

The mode of action of the various agents appears due to cytosomal rather than to nuclear damage. Sex ratios were not altered as they would be from sex linked recessive lethals. Also good survival to adulthood argues against dominant lethals that are expressed in larval stages. Therefore, at doses that alter lifespan and decrease fecundity these toxicants would act as

selective agents upon the genotypes present in the population of shrimp, rather than as inducers of additional genetic variability. If the heritable potentiality exists in the population, a more tolerant strain could emerge from the selective process.

The toxicity of fourth period cations traditionally is attributed to antagonisms resulting from a maladjustment in the balance of trace metals essential to the functioning of enzymes and other aspects of cell physiology (Albert, 1965). Table I shows that *Artemia* adults tolerated less divalent copper than any of the other three cations tested. The values are more meaningful in the relative rather than the absolute sense. The concept of a species toxicity value loses credance because Browne (unpublished) has demonstrated significantly different tolerances of $CuSO_4$ for each of the six different strains maintained in my laboratory.

Finding copper more toxic than zinc is consistent with reports for other organisms. Wisely and Blick (1967) demonstrated this for the larvae of five different marine invertebrates, and MacInnes and Calabrese (1978) reported it for adult oysters, clams, and sea urchins. Trout tolerate less copper than zinc in either hard or soft water (Lloyd, 1961). The LD 50 for copper in laboratory strains of rats is well established but the lethal level for dietary zinc is elusive. One recently identified mechanism involved in copper and zinc tolerance of marine organisms is the endocytotic formation of vesicles which can prevent contact of excess metal with vital cellular constituents (Coombs and George, 1978). However, the sex difference revealed in the reciprocal crosses of treated parents (Table I) may have a different basis, related to the metabolic investment in the impressive fecundity of the females. Less copper had to be used and less zinc should have been used for the females.

The toxicity of divalent copper is also a factor in the effectiveness of antifouling paints. Although they contain the cuprous form, much of the copper released into the sea is rapidly oxidized to its cupric state. This explains the pale band of cupric coloration which developed on the unpainted area of my test panels. The situation becomes complicated when complexes are formed with the ubiquitous chloride ions (Anonymous, 1967) and with various organic molecules (Steeman-Nielsen and Wium-Anderson, 1970). Accordingly in natural waters, the toxicity of copper depends on the free ions, and not necessarily on the amount of dissolved copper (Engel and Sunda, 1979).

Somewhat related in toxic action to metals may be the triazole herbicide. *In vitro*, amitrole inhibits catalase and several other metalloprotein enzymes. However, in animals, retention is brief and tissue incorporation is negligible (Carter, 1975). In plants the herbicide interferes with carotinoid accumulation in the plastids.

Inorganic tin compounds are relatively insoluble and poorly absorbed, but the alkyl organotin compounds developed in recent decades are highly toxic to animals (Kimbrough, 1976). They can be incorporated into biomembranes (Andrews *et al.*, 1978) and at very low concentrations inhibit ATP formation and coupled electron transport (Gould, 1976). The action is conceptualized as blockage of a membrane-bound carrier or channel. If the leaching rates are at all similar, tri-n-butyl tin antifouling paint is more toxic than a standard cuprous oxide formulation. A sublethal dose was not attained until a much smaller area was used for the exposure. In any event devising a chronic exposure to organotin paint does not seem biologically significant.

The mode of action of the organic ammonium salt is not clear. In addition to the evidence of algicidal effectiveness, Chemical Abstracts list reports of quatenary ammonium salts useful as a disinfectant for stables, as a sanitizer of poultry drinking water, and as antibacterial fabric

softener. Thus the descriptive evidence of its toxicity is accumulating. However, a definitive reference on molecular mode of action has eluded me. In my shrimp experiments foaming was observed even at extreme dilutions. This together with a loss of the shrimp ability to survive in brine suggests a surfactant influence.

**Summary**

The reproductive performance was determined for *Artemia* adults after sublethal exposure to fourth period metal sulfates and naphtheates, cuprous oxide and organotin antifouling paints, a triazole herbicide, and a quatenary organic ammonium algaecide. The metal agents influenced fecundity through decreased life span, while the organic pesticides were able to influence the number of zygotes without curtailing brood number. None of the agents evoked encystment of broods. The modes of toxic action and the question of population extinction were discussed.

**Acknowledgement**

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**Literature cited**

ALBERT A. 1965. Selective toxicity. Methuen and Co. Ltd., London. 394 p.

ANDREWS S. B., J. W. FALLER, R. J. BARRNETT, and V. MIZUHIRA. 1978. Organometallic fatty acid and phospholipid analogs. Synthesis and incorporation and detection in model membranes and biomembranes. *Bioch. Biophys. Acta* 506:1-17.

ANONYMOUS. 1967. Marine fouling and its prevention. Woods Hole Oceanographic Institution Contribution No. 580. 3rd printing. George Banta Co., Menasha, Wisconsin. 388 p.

CARTER M. C. 1975. Amitrole. p. 377-398. *In* : Herbicides. Vol. 1. Kearney P. C. and D. D. Kaufman (Eds). Marcel Dekker Inc., New York, Basel. 500 p.

COOMBS T. L. and S. G. GEORGE. 1978. Mechanisms of immobilization and detoxication of metals in marine organisms. p. 179-187. *In* : Physiology and behaviour of marine organisms. Proc. 12th Eur. Symp. Mar. Biol. McLusky D. S. and A. J. Berry (Eds). 388 p.

ENGEL D. W. and W. G. SUNDA. 1979. Toxicity of cupric ion to eggs of the spot *Leiostomus xanthurus* and the Atlantic silverside Minidia menidia. *Mar. Biol.* 50:121-126.

GOULD J. M. 1976. Inhibition by triphenyltin chloride of a tightly-bound membrane component involved in photophosphorylation. *Eur. J. Biochem.* 62:567-575.

GROSCH D. S. 1966. The reproductive capicity of *Artemia* subjected to successive contaminations with radiophosphorus. *Biol. Bull.* 131:261-271.

GROSCH D. S. 1973. Reproduction tests : the toxicity for *Artemia* of derivatives from non-persistent pesticides. *Biol. Bull.* 145:340-351.

KIMBROUGH R. D. 1976. Toxicity and health effects of selected organotin compounds : a review. *Environmental Health Perspectives* 14:51-56.

LLOYD R. 1961. The toxicity of mixtures of zinc and copper sulfate to rainbow trout. *Ann. Appl. Biol.* 49:535-538.

MACINNES J. R. and A. CALABRESE. 1978. Response of embryos of the American oyster, *Crassostrea virginicia*, to heavy metals at different temperatures. p. 195-202. *In* : Physiology and behaviour of marine organisms. Proc. 12th Eur. Symp. Mar. Biol. McLusky D. S. and A. J. Berry (Eds). 388 p.

STEEMAN-NIELSEN E. and S. WIUM-ANDERSON. 1970. Copper ions as poison in the sea and freshwater. *Mar. Biol.* 6:93-97.

WISELY B. 1963. Effects of antifouling paints on settling larvae of the bryozoan *Bugula neritina* L. *Aust. J. Mar. Freshwat. Res.* 14:44-59.

WISELY B. and R. A. P. BLICK. 1967. Mortality of marine invertebrate larvae in mercury, copper, and zinc solutions. *Aust. J. Mar. Freshwat. Res.* 18:63-72.

*The Brine Shrimp* Artemia. *1980. Vol. 1. Morphology, Genetics, Radiobiology, Toxicology*
*G. Persoone, P. Sorgeloos, O. Roels, and E. Jaspers (Eds). Universa Press, Wetteren, Belgium. 345 p.*

# The interaction of *Vibrio* with *Artemia* nauplii

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**Abstract**

The interaction of three species of *Vibrio* with nauplii of the brine shrimp *Artemia* was studied. Dry *Artemia* cysts were soaked in merthiolate to kill bacteria on the outer surface and were then hatched in sterilized artificial seawater. Bacteria-free algae (*Dunaliella*) were fed to the young shrimp. Challenge-survival tests were run using three concentrations of each of three *Vibrio* species : *V. alginolyticus*, *V. parahaemolyticus*, and *V. anguillarum*. Two of the three species of bacteria coated the shrimp's body and completely inhibited swimming when tested at concentrations above $10^8$ cells/ml. This coating required 2 hr for *V. alginolyticus* and 8 hr for *V. parahaemolyticus*. *Vibrio anguillarum* did not coat the shrimp and appeared to have no negative effects on *Artemia* nauplii. Scanning electron micrographs of the bacteria showed distinctive projections on the two fouling bacteria, which were tentatively identified as fimbriae or unsheathed lateral flagella. These might be involved in the mechanism for bacterial adhesion to the shrimp. No lateral projections were found on *V. anguillarum*.

## Introduction

From a public health standpoint, *Vibrio* is a very important bacterial genus in the field of seafood microbiology. One species, *V. parahaemolyticus*, is both a human and crustacean pathogen. It causes as much as 70% of the gastroenteritis in Japan (Sakazaki, 1965, cited by Vanderzant *et al.*, 1970). The practice of eating raw marine fish is one possible cause of the high incidence of *Vibrio* food poisoning in Japan. This bacterium is a common marine organism and has been studied extensively in Puget Sound (Baross and Liston, 1968) and in Chesapeake Bay (Kaneko and Colwell, 1978). Vanderzant *et al.* (1970) found that *V. parahaemolyticus* was pathogenic for brown shrimp (*Penaeus aztecus*) at concentrations of $10^4$ to $10^5$ cells/ml, and Kaneko and Colwell (1978) found it attached to copepods in Chesapeake Bay during warm months of the year. Concentrations as high as $3 \times 10^9$ bacteria per gram wet weight of copepod were reported for samples taken in late summer.

Another species, *Vibrio alginolyticus*, is not usually considered to be responsible for gastroenteritis ; however it has been isolated from infections of the extremities, eyes, and ears of individuals in contact with the marine environment (Joklik and Willett, 1976). It has been implicated in *Vibrio* epizootics in blue shrimp (*Penaeus stylirostris*) by Lightner (1977) and

since this species can grow in 11% sodium chloride it could survive in some of the solar evaporation ponds which *Artemia* inhabit.

A third species, *Vibrio anguillarum*, is a pathogen for salmon (Wood, 1974). Vibriosis caused by this species is most likely to become epizootic at water temperatures above 13.3 °C (McNeil and Bailey, 1975). This bacterium has not been reported as a crustacean pathogen although it has the ability to metabolize chitin, a major component in crustacean exoskeletons.

The three species of *Vibrio* used in our experiments are all able to metabolize chitin. It was thought that chitin degradation might be an important aspect of shrimp-bacteria interactions.

The human pathogenicity of some species of *Vibrio*, combined with the fact that they are comman marine organisms, makes the study of this genus worthwhile. Its presence in sea-foods should be measured and understood as much as possible. With the evidence from Vanderzant *et al.* (1970) and Lightner (1977), on penaeid shrimp, it seems that some species of *Vibrio* are crustacean pathogens. The question thus arises if it is possible to find general features for crustacean bacterial pathogens. For example, is chitinoclastic ability or the ability to stick to a crustacean exoskeleton common to such pathogens ?

*Artemia* are used as live feed for many cultured aquatic animals. Is it possible to transmit *Vibrio* pathogens to larval fish or crustaceans grown in aquaculture systems by feeding *Artemia* contaminated with *Vibrio* ? These are the questions that guided the research reported in this paper. *Artemia* nauplii thus served as representative crustaceans and also were studied for their own sake, since they are used so extensively as live feed for other animals.

The dry cysts of *Artemia* can be surface sterilized and the nauplii which hatch out are bacteria-free. This allows experiments with *Vibrio* to be performed without the interference of any other bacteria. The ability to simplify the interactions between bacteria and *Artemia* in this way was considered important and useful for the study of pathology in this very important crustacean. The overall procedure followed was to hatch and culture *Artemia* in bacteria-free conditions and then add pure cultures of *Vibrio* bacteria. Attachment of the *Vibrio* to the nauplii proved to be an important part of the interaction, so scanning electron microscopy was used to study the *Artemia* nauplii closely.

## Materials and methods

Artificial seawater, called M100, was prepared by extensively modifying the ASP formula of Provasoli *et al.* (1957). This artificial seawater was essentially like seawater in the composition of its major salts but was enriched with nitrate, phosphate, iron and included a trace mineral mixture. The exclusion of bicarbonate buffering agent allowed the medium to be autoclaved without consequent precipitation (Table I).

A second medium was made for cases in which bacterial contamination was to be detected. The medium, called sterility test medium, was simply the artificial seawater to which bacterial culture medium (Difco plate count broth mix) was added, so that if bacteria got into the test tubes the water would rapidly turn cloudy from bacterial growth.

A diet of axenic *Dunaliella* was made available to the *Artemia* so that the shrimp would have food available as soon as they were able to eat it. *Artemia* larvae start feeding after their second molt (Provasoli and Shiraishi, 1959). The alga was made bacteria-free by an osmotic shock series whereby it was grown in a sequence of culture tubes which increased in salinity to NaCl saturation and then decreased back to sea water strength. This series eliminated

bacteria, but the alga was able to tolerate it. Algal concentrations in the challenge tests were $10^6$ cells/ml in each test tube.

TABLE I

Concentration of salts in M100 medium

| Compound | mg/l | millimolarity |
|---|---|---|
| NaCl | $2.8 \times 10^4$ | 484.7 |
| $MgSO_4.7H_2O$ | $6.7 \times 10^3$ | 27.0 |
| $MgCL_2.6H_2O$ | $5.4 \times 10^3$ | 26.5 |
| $CaCl_2.2H_2O$ | $1.5 \times 10^3$ | 10.2 |
| KCl | $7.3 \times 10^2$ | 9.8 |
| $NaNO_3$ | $3.5 \times 10^2$ | 4.1 |
| $NaH_2PO_4.H_2O$ | $2.8 \times 10^1$ | $2.0 \times 10^{-1}$ |
| $Na_2EDTA$ | $3.5 \times 10^1$ | $1.0 \times 10^{-1}$ |
| $FeSO_4.7H_2O$ | $1.2 \times 10^1$ | $4.3 \times 10^{-2}$ |
| $H_3BO_3$ | 5.7 | $9.2 \times 10^{-2}$ |
| $FeCl_3.6H_2O$ | $2.5 \times 10^{-1}$ | $1.0 \times 10^{-3}$ |
| $MnSO_4.7H_2O$ | $8.2 \times 10^{-1}$ | $3.7 \times 10^{-3}$ |
| $ZnSO_4.7H_2O$ | $1.1 \times 10^{-1}$ | $3.8 \times 10^{-4}$ |
| $CoSO_4.7H_2O$ | $2.4 \times 10^{-2}$ | $8.5 \times 10^{-5}$ |

Dry *Artemia* cysts were soaked in distilled water and then treated with 1/1 000 dilution of merthiolate, to sterilize them, a procedure used by Gibor (1956). Treated cysts were hatched in sterility test medium and those tubes which did not become turbid after 72 hr were considered as bacteria-free.

*Artemia* nauplii were prepared for scanning electron microscopy by fixation in an osmium tetroxide and glutaraldehyde mixture, wash in distilled water, acetone dehydration, critical point drying, and gold coating.

The first step of a challenge test consisted of growing axenic *Dunaliella* in test tubes and then adding one axenic *Artemia* per test tube. The nauplii were subsequently challenged by exposure to approximately $10^6$ cells/ml, $10^7$ cells/ml, and $10^8$ cells/ml of each of the three *Vibrio* species. Twenty-five replicates of each bacterial concentration were run. Control shrimp not exposed to bacteria were kept in both artificial seawater and sterility test medium. The shrimp were examined four times in a 22 hr period. The number of normally swimming nauplii was recorded. The major effect of the bacteria was to stick and cause abnormal swimming movements, so that visual assessment of swimming activity provided a measure of bacterial attachment to the shrimp. The entire test series was repeated twice with similar results.

## Results

The challenge tests showed that *V. alginolyticus* and *V. parahaemolyticus* readily attached to *Artemia* nauplii and completely inhibited normal swimming activity. Axenic *Artemia* (Fig. 1) from control test tubes, swam normally throughout the test period. *Vibrio alginolyticus*

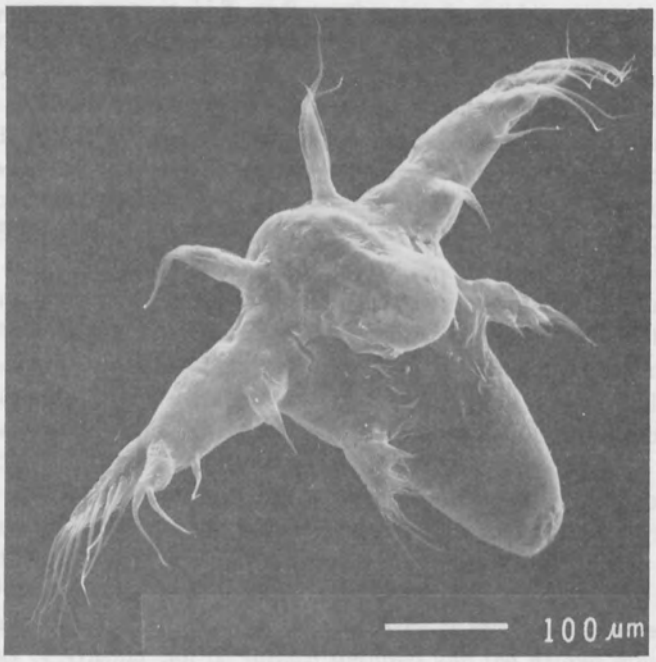


FIG. 1. Normal *Artemia* nauplius.

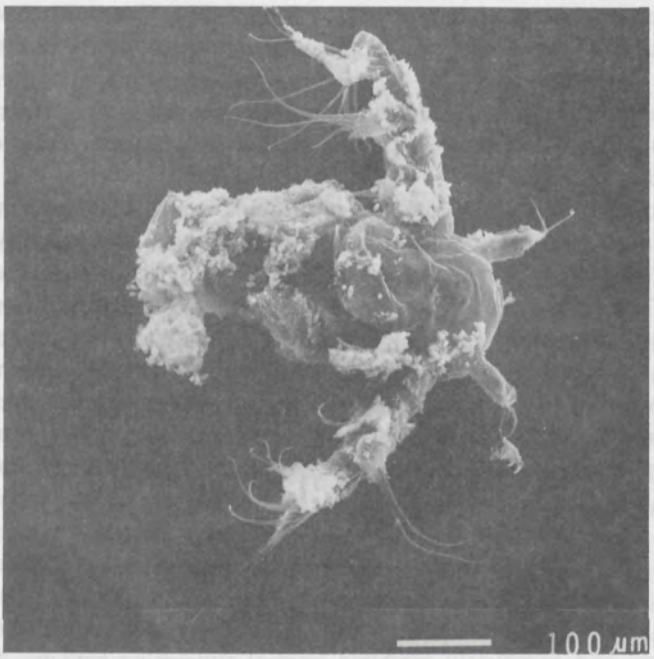


FIG. 2. Nauplius coated with *Vibrio alginolyticus*.

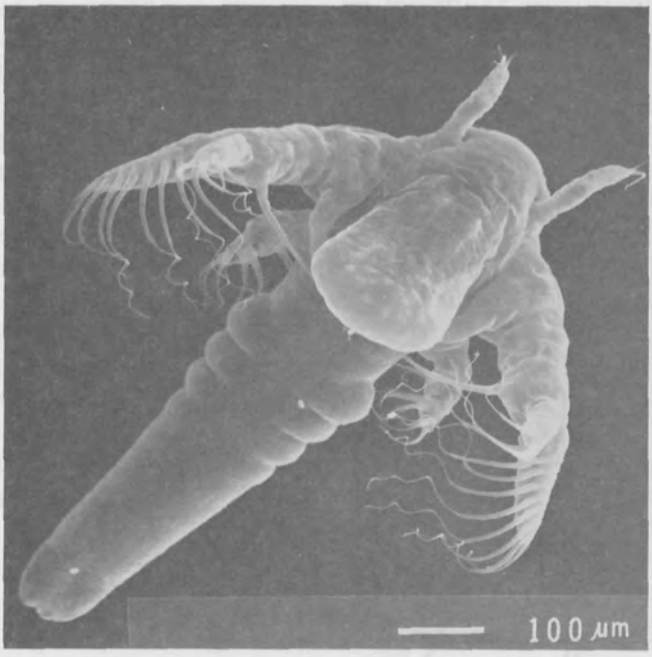


FIG. 3. Nauplius exposed to *Vibrio anguillarum* for several hours.

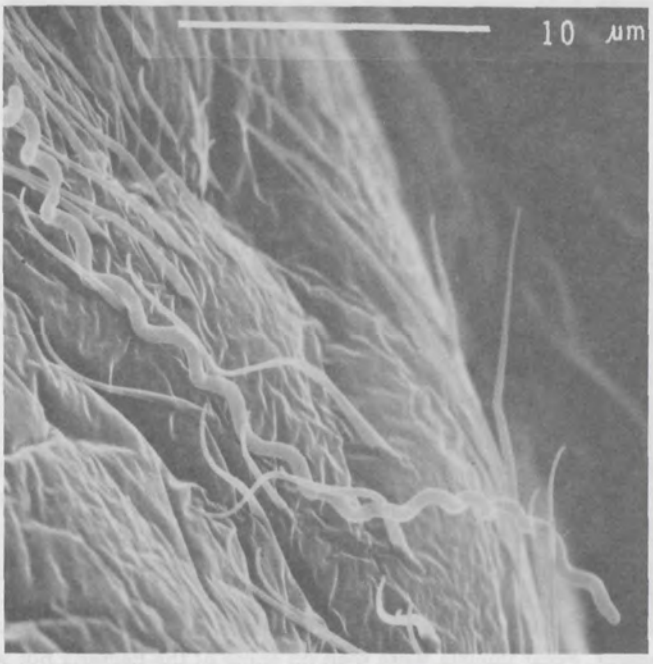


FIG. 4. Higher magnification of mid-section of nauplius shown in Fig. 3, showing a long filament of *V. anguillarum* entangled in nauplius surface spines.

coated the nauplii in less than 2 hr (Fig. 2). *Vibrio anguillarum* did not seem to harm the brine shrimp in any way even at concentrations as high as $7.5 \times 10^7$ cells/ml. Fig. 3 and 4 show an *Artemia* that had been exposed to *V. anguillarum* for several hours. Although these bacteria did not coat the shrimp and prevent them from swimming, there were filaments composed of incompletely divided bacterial cells, found stuck in the spines on the *Artemia*. The bacteria produce these filaments under special environmental conditions, especially mature cultures that are not rapidly growing.

Fig. 5 summarizes the results of the challenge test in graphic form. There was a rapid decrease in the number of nauplii swimming normally in the *V. alginolyticus* challenge at $1.2 \times 10^7$ cells/ml. Results were similar at $1.2 \times 10^8$ cells/ml, whereas there was little or no change in their swimming behavior at $1.2 \times 10^6$ cells/ml. *Vibrio parahaemolyticus* coated nauplii slower than did *V. alginolyticus*, even when the concentration was eight times higher. The bacterial coating looked the same, however, in the light and scanning electron microscopes for these two species of bacteria. The high survival percentage for nauplii exposed to *V. anguillarum* supports the idea that high bacterial concentrations were not in themselves harmful to the nauplii, but rather that some vibrios were pathogens and others were not.

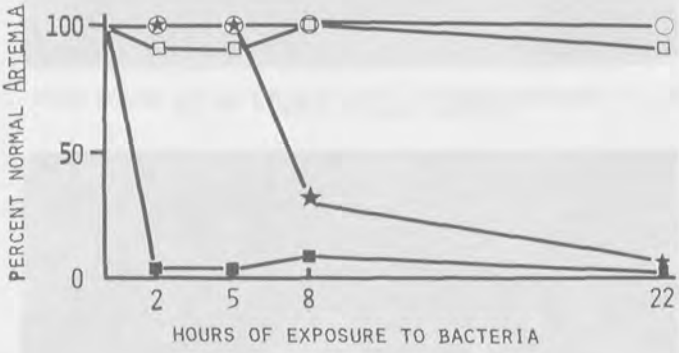


FIG. 5. Challenge test results. (○) Bacteria-free control *Artemia*; (□) $7.5 \times 10^7$ *V. anguillarum*/ml; (★) $1.0 \times 10^8$ *V. parahaemolyticus*/ml; (■) $1.2 \times 10^7$ *V. alginolyticus*/ml. The percentage of nauplii which were swimming normally was plotted because bacterial coatings on nauplii affected swimming ability very noticably.

The scanning electron microscope (SEM) was used to study more closely the bacteria which coated the nauplii. Evidence of chitinoclastic activity or special attachment mechanisms might have become visible at higher magnification if they were present. Fig. 6 shows *V. alginolyticus* attached to the naupliar exoskeleton, and Fig. 7 shows *V. parahaemolyticus* attached to the fine structures of the larval swimming appendages. Although it is clear that the bacteria in both these pictures had attached in high numbers, there is no clear evidence for the exact method of attachment. Polar flagella were seen on some of the bacteria but were not clearly involved in anchoring the bacteria to the nauplii.

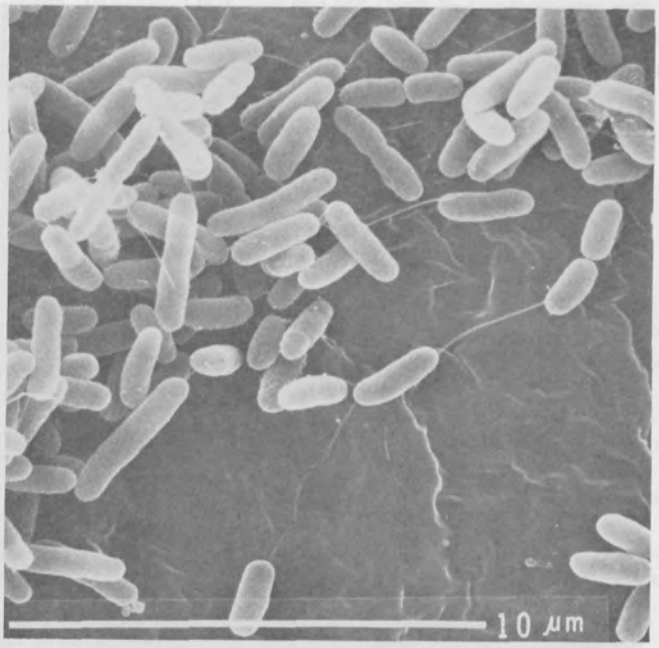


FIG. 6. *Vibrio alginolyticus* cells attached to the naupliar exoskeleton.

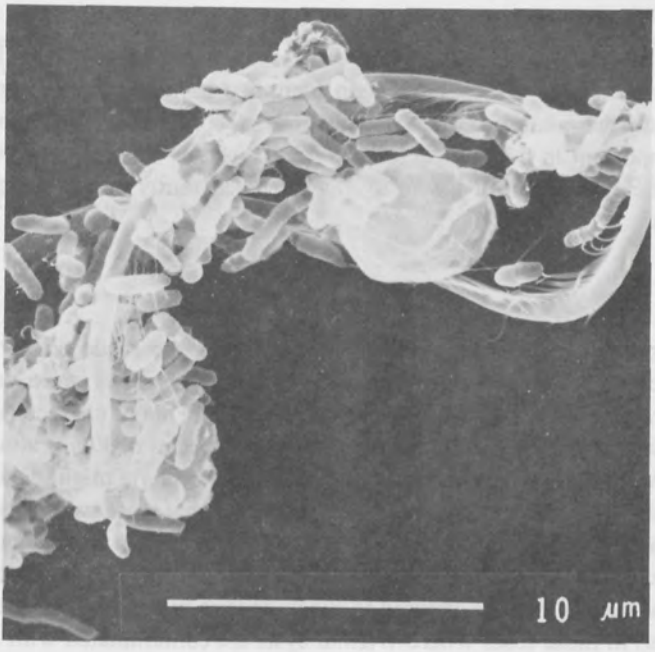


FIG. 7. *Vibrio parahaemolyticus* attached to the fine structure of the larval swimming appendages. The large oval object is a *Dunaliella* algal cell.

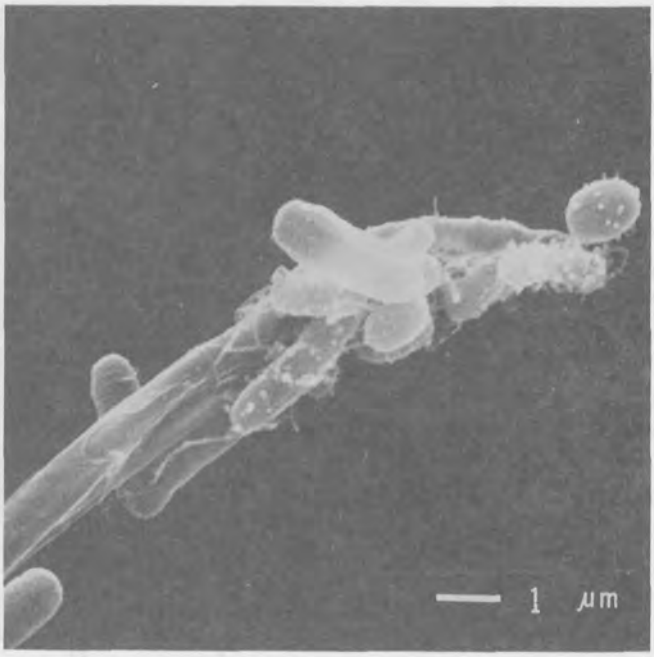


FIG. 8. Surface structure of *Vibrio parahaemolyticus* cells attached to *Artemia* nauplius appendage.

Fig. 8 shows *V. parahaemolyticus* with structures extending laterally from the bacterial cell. These resemble structures which have been identified as unsheathed lateral flagella in *V. alginolyticus* and *V. parahaemolyticus* by de Boer *et al.* (1975), who hypothesized that lateral flagella were important in holding bacteria to solid surfaces. The importance of these lateral structures for the attachment of *Vibrio* to *Artemia* is currently being studied by one of the authors (Catena).

**Discussion**

The results presented here allow some conclusions to be made concerning *Vibrio* and *Artemia* interactions. *Vibrio alginolyticus* and *V. parahemolyticus* were pathogenic at the higher concentrations tested but not at the lowest doses. The major characteristic that could be documented with the SEM studies was that these two species of *Vibrio* attach readily to the nauplii. No SEM pictures were obtained to indicate if the bacteria might display chitinoclastic activity, but preliminary light microscope studies showed that these two species did invade the shrimp body cavity and metabolized all the shrimp tissue within a few hours.

In conclusion it can be extrapolated from this study that *Artemia* hatched and kept in sea water of normal salinity may be exposed to *Vibrio* species which may not be present in brine ponds. However, in those cases where *Artemia* cysts are contaminated with *Vibrio*, the use of a decapsulation method (Bruggeman *et al.*, 1980) which reduces the microbial population, may be a distinct advantage. *Vibrio alginolyticus* and *V. parahemolyticus* are able to multiply

very rapidly (Ulitzur, 1974) and it is possible for them to reach pathogenic concentrations in the time it takes *Artemia* cysts to hatch, especially if the hatch water is warm. The ability of these species to stick to nauplii allows them to be transmitted as possible pathogens to other species grown in aquaculture systems which use live nauplii as feed.

## Literature cited


Baross J. and J. Liston. 1968. Isolation of *Vibrio parahaemolyticus* from the Northwest Pacific. *Nature* 217: 1263-1264.

Bruggeman E., P. Sorgeloos, and P. Vanhaecke. 1980. Improvements in the decapsulation technique of *Artemia* cysts. p. 261-269. *In* : The brine shrimp *Artemia*. Vol. 3. Ecology, Culturing, Use in Aquaculture. Persoone G., P. Sorgeloos, O. Roels, and E. Jaspers (Eds). Universa Press, Wetteren, Belgium. 456 p.

de Boer W. E., C. Golten, and W. A. Scheffers. 1975. Effects of some chemical factors of flagellation and swarming of *Vibrio alginolyticus*. *Anton Van Leeuwenhoek J. Microb. Serol.* 41:385-403.

Gibor A. 1956. Some ecological relationships between phyto- and zooplankton. *Biol. Bull.* 11:230-234.

Joklik W. K. and H. P. Willett (Eds). 1976. Zinsser microbiology. 16th ed. Appleton-Century-Crofts, New York. 1223 p.

Kaneko T. and R. R. Colwell. 1978. The annual cycle of *Vibrio parahaemolyticus* in Chesapeake Bay. *Microbial Ecology* 4:135-155.

Lightner D. V. 1977. *Vibrio* disease in shrimps. p. 19-26. *In* : Disease diagnosis and control in North American marine aquaculture. Sinderman C. J. (Ed.) Elsevier Scientific Publishing Co., New York. 329 p.

McNeil W. J. and J. E. Bailey. 1975. Salmon rancher's manual. Northwest Fisheries Center, Seattle, Washington. 95 p.

Provasoli L., J. J. A. McLaughlin, and M. R. Droop. 1957. The development of artificial media for marine algae. *Arch. Mikrobiol.* 25:392-428.

Provasoli L. and K. Shiraishi. 1959. Axenic cultivation of the brine shrimp *Artemia salina*. *Biol. Bull.* 117:347-355.

Sakazaki R. 1965. *Vibrio parahaemolyticus* : a non-choleragenic enteropathogenic vibrio. p. 30-34. *In* : Proc. of the Cholera Research Symposium 1965. US Dept. Health, Education, and Welfare, Washington, D.C. 397 p.

Ulitzur S. 1974. *Vibrio parahaemolyticus* and *Vibrio alginolyticus* : short generation-time marine bacteria. *Microbial Ecology* 1:127-135.

Vanderzant C., R. Nickelson, and J. C. Parker. 1970. Isolation of *Vibrio parahaemolyticus* from gulf coast shrimp. *J. Milk Food Tech.* 33:161-162.

Wood J. W. 1974. Diseases of pacific salmon : their prevention and treatment. 2nd ed. State of Washington, Dept. of Fisheries, Olympia, Washington. 82 p.

*The Brine Shrimp* Artemia. *1980. Vol. 1. Morphology, Genetics, Radiobiology, Toxicology*
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# The brine shrimp *Artemia* as a laboratory bioassay organism I. The effects of the heavy metal cadmium on reproduction

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**Abstract**

Brine shrimp are widely used in toxicity tests conducted by pharmaceutical, agricultural, and petrochemical industries.

The Freshwater Institute stocks are obtained from Chaplin Lake, Saskatchewan, Canada as resting eggs (cysts) and are maintained in our laboratory through successive generations in a reconstituted medium, using a defined food source. Reproduction in these cultures is oviparous or ovoviviparous ; the cysts being stored under various conditions for months. Rehydration of these cysts in a saline medium provides the laboratory stock for bioassays.

Acute tests are conducted during particular stages of the life cycle and chronic tests on the whole life span of the animals. The delay in embryological development, the hatching efficiency of the cysts as the per cent nauplii produced after defined intervals of incubation, the mortality of the nauplii and adults, the fecundity of the adults and the sensitivity to pre-exposure to the toxicant, are the parameters measured to evaluate effects of a toxicant applied at several concentrations.

*The Brine Shrimp* Artemia. *1980. Vol. 1. Morphology, Genetics, Radiobiology, Toxicology*
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# Susceptibility of four geographical strains of adult *Artemia* to *Ptychodiscus brevis* toxin(s)

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**Abstract**

Four strains of the brine shrimp, *Artemia* were used as the bioassay organism to detect concentrations of *Ptychodiscus brevis* toxin(s) in culture media. *P. brevis* is the unarmored dinoflagellate responsible for Florida red tides. The four strains indicated differential susceptibility to the extracted crude toxin(s) as demonstrated by $LD_{50}$ values ranging from 5.1 $\mu$g/ml (ppm) to 9.4 $\mu$g/ml. Statistical analyses of linear regressions indicated no significant differences between replicates. Shark Bay strain had the lowest $LD_{50}$ (highest sensitivity) and was the easiest to raise. The bioassay procedure is suggested as a standard method for determining *P. brevis* toxin(s) in water.

## Introduction

Red tides in the Gulf of Mexico are the result of population increases or 'blooms' of the unarmored dinoflagellate *Ptychodiscus brevis* (Davis) Steidinger ( = *Gymnodinium breve*). The toxic effects (*e.g.*, fish kills, toxic shellfish) associated with these blooms have instigated numerous studies concerning the toxigenic properties of this species. Martin and Chatterjee (1969) published the first characterization of the chemical and physical properties of this toxin(s). Subsequent characterization studies often produced conflicting results due to different purification schemes. However, recently developed purification procedures have produced very pure toxic components which are being chemically analyzed (Baden *et al.*, 1979 ; Risk *et al.*, 1979). *Ptychodiscus brevis* possesses at least two major toxic components (McFarren *et al.*, 1965 ; Padilla *et al.*, 1978 ; Baden *et al.*, 1979 ; Risk *et al.*, 1979). The most abundant fraction of *P. brevis* toxins is slower acting and slightly less potent than the second toxic component (Baden *et al.*, 1979 ; Risk *et al.*, 1979). A multi-component toxin increases the assay's complexity because all components should be measured.

McFarren *et al.* (1965) were the first to document human illness due to consumption of toxic shellfish ; they extracted an oil soluble crude toxin from these shellfish and developed a mouse bioassay procedure. Cummins and Hill (1969) later standardized the assay. Since the toxin(s) is concentrated by filter-feeding bivalves, the mouse bioassay is adequately sensitive for its purpose. However, low levels of toxin in natural seawater, cultures or purified toxic fractions cannot be quantified using this technique. Most researchers use various small fish bioassays based on death time for toxin purification analyses. These bioassay procedures are not standardized and some utilize acclimated freshwater species.

The first detailed report on the use of *Artemia* as a bioassay organism in toxicological studies (Michael *et al.*, 1956) stimulated research involving various toxins and procedures which used adult and naupliar stages (Harwich and Scott, 1971 ; Grosch, 1973 ; Curtis *et al.*, 1974 ; Price *et al.*, 1974 ; Granada *et al.*, 1976). Trieff *et al.* (1973) suggested the use of mature brine shrimp as a possible alternative bioassay organism for *P. brevis* toxin(s) but did not establish a standard procedure. Researchers have recently pointed out the importance of using one strain and thoroughly defining culture conditions (Zillioux *et al.*, 1973 ; Sorgeloos *et al.*, 1978). Sorgeloos *et al.* (1978) demonstrated differential susceptibility of *Artemia* nauplii of different instar stages to Lugol's solution. They also cited hatching condition requirements, stage identification, and time limitations of stage metamorphosis as problems associated with bioassays using nauplii.

This study examines the differential susceptibility of four geographical strains of adult *Artemia* to *P. brevis* toxin(s) and demonstrates advantages of using adults instead of nauplii. A standardized procedure for quantifying this natural biotoxin(s) is presented.

## Materials and methods

### Assay organism

Strains of *Artemia* cysts from Great Salt Lake (USA), North/South San Francisco Bay (USA), Shark Bay (Australia) and Puerto Rico were used in the bioassay experiments. Instant Ocean (synthetic seawater) was the sole medium used. Salinity was maintained at 35 ‰ ± 1 ‰, temperature at 25 °C ± 2 °C, and pH at 8.10 ± 0.20. Cysts hatched between 24 and 48 hr after being placed in a 2 l conical flask aerated with an air stone placed near the bottom to keep all cysts in continual motion (Sorgeloos and Persoone, 1975). After 48 hr motile nauplii were separated from unhatched cysts and debris and raised to adults in 19 l aquaria. The developmental period from nauplii to adult (as evidenced by copulation) averaged 21 days. *Dunaliella* sp., grown in modified 'f/2' medium ('f' medium of Guillard and Ryther (1962), diluted 1:2 with seawater), was maintained in the brine shrimp culture at concentrations between $2.40 \times 10^3$ and $1.07 \times 10^6$ cells/ml and served as the only live food source.

### Crude toxin preparation

About 80 l of *Ptychodiscus brevis* culture were grown in NH-15 artificial medium (modified from Gates and Wilson, 1960) over a 3 month period in 12 l carboys. As each carboy reached approximately $16 \times 10^6$ cells/l in log growth phase, the cultures were frozen, thawed, and refrozen in order to lyse cells and release the endotoxin(s). When sufficient culture material was available, all carboys were thawed and mixed thoroughly.

One liter aliquots of lysed culture material were adjusted to pH 4.5 with approximately 2 ml 10% HCl to stabilize the toxin(s), then extracted with 200 ml of anhydrous ethyl ether in a 2 l separatory funnel by moderate shaking for 10 min on a mechanical shaker. After the layers separated, the lower water layer was discarded, and the middle emulsion layer and upper ether layer were drained into a pre-weighed 400 ml beaker and evaporated under a hood. Three additional 1 l quantities of lysed culture were extracted using the same procedure and all were combined in the same beaker. The ether was evaporated to a volume of about 40 ml, poured into a conical centrifuge tube, and centrifuged at approximately 1 000 G for 5 min to

separate cell debris and water from the toxic ether layer. The ether was returned to the 400 ml beaker and evaporated to dryness. The beaker containing the crude towin extract was weighed and stored frozen up to two months for later assay. Crude toxin extracts averaged 0.02 g. The residue yielded enough crude toxin extract for one bioassay replicate. A total of 12 extracts was prepared in this manner.

### Bioassay procedure

The bioassay procedure is a modification of Trieff *et al.* (1973). Two ml of ethyl ether and one drop (0.05 ml) of the solubilizer Tween 80 were added to each beaker containing crude toxin to resuspend the toxin(s). The ether was evaporated to dryness, then 10 ml of 35 ‰ Instant Ocean were added. The crude toxin suspension was dispensed by volumetric pipets in a range of concentrations between 0.5 to 30 $\mu$g crude toxin/ml into 100 ml beakers containing 98 ml of Instant Ocean. Six concentrations were prepared for each replicate trial. Approximately 2 ml of water were added to the beakers during transfer of the brine shrimp so the total volume equaled about 100 ml. Three beakers were used for each concentration with four brine shrimp per beaker (total 72 brine shrimp per replicate). Three replicates were assayed for each strain (total 216 brine shrimp per strain). Twenty-four individuals were used as controls both in Instant Ocean and Instant Ocean plus Tween 80 in conjunction with each strain's triplicate series. Individual brine shrimp were carefully transferred from culture aquaria to beakers using a thumb-operated repipet. The end of the glass repipet tube had been enlarged to 4 mm to prevent injury to the brine shrimp during handling. During the assay, beakers were covered with an inverted watch glass to reduce evaporation. After 48 hr the number of dead adults was recorded at each toxin concentration. Death included individuals swimming in a fixed position (Tarpley, 1958).

### Mathematical and statistical analyses

Toxin concentrations were transformed to logarithmic values. $LD_{50}$ values were calculated from a computed linear regression of unweighted probit values *versus* log concentration of toxin. Probit values for 0 and 100% mortality were determined using Bartlett's procedure (Miller and Tainter, 1944). When more than one concentration per trial replicate resulted in 0% mortality, only the highest of the toxin concentrations was used in the linear regression calculation. If multiple values of 100% mortality had occurred, only the lowest concentration value would have been used.

An analysis of variance was performed to determine if significant differences occurred between $LD_{50}$'s of the four strains. Tukey's $w$ procedure was applied to determine where differences occurred. An analysis of covariance was used to determine if significant differences occurred between computed regression lines of replicates within strains. Significance was determined at the $P = 0.05$ level.

## Results

Analysis of convariance of the linear regressions computed from results of each trial indicated that no significant differences occurred between the replicates within a strain. The correlation coefficient for the linear regression of each trial was high with all values above

r = 0.93. A high positive correlation between increases in toxin(s) concentration and increases in mortality is essential for a reliable bioassay experiment. Graphical representation of each regression replicate by strain is presented in Fig. 1.

The four geographical strains exhibited differential susceptibility to *P. brevis* toxin(s) as indicated by significant differences between $LD_{50}$ values. $LD_{50}$ values for each trial with means and standard deviations are given in Table I. The statistical relationships between the four strains are indicated in the table with underlining denoting statistical similarity.

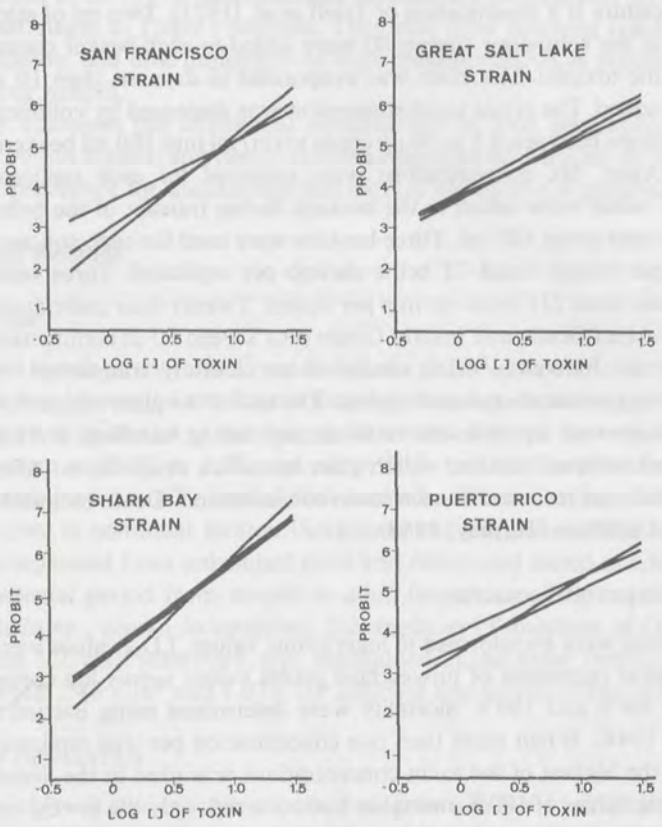


FIG. 1. Trial replicate linear regressions of four strains of *Artemia* exposed to *P. brevis* toxin(s).

## Discussion and conclusions

Direct quantitative analyses such as atomic absorption spectrophotometry or gas chromatography are preferable to bioassays which introduce variables, such as individual organism response, that reduce precision. However, when the substance to be tested is unknown or uncharacterized, no standard for comparison may be available and bioassays may be the only suitable alternative.

TABLE I

$LD_{50}$ values ($\mu$g of crude toxin/ml) of four strains of *Artemia* resulting from exposure to *Ptychodiscus brevis* toxin(s)

| | Strain | | | |
|---|---|---|---|---|
| | San Francisco | Puerto Rico | Great Salt Lake | Shark Bay |
| 1 | 8.9 | 9.7 | 5.4 | 4.5 |
| 2 | 10.0 | 6.8 | 6.1 | 5.0 |
| 3 | 9.5 | 7.3 | 7.1 | 5.7 |
| $\bar{x}$ | 9.5 | 7.9 | 6.2 | 5.1 |
| Standard deviation ± | 0.55 | 1.55 | 0.85 | 0.60 |
| Statistical Similarity* | | | | |

* as determined using Tukey's *w* procedure.

Because *P. brevis* crude toxin extracts are multi-component complexes with fast and slow acting toxin, bioassay design must attempt to measure complete toxicity. In this instance, a short experimental period, such as 2 hr, may only measure the minor toxic fraction of a *P. brevis* extract. First and second instar nauplii stages moult in approximately 15 hr (Anderson, 1967 ; Sorgeloos *et al.*, 1978), which limits the effectiveness of using nauplii as a long term bioassay organism and necessitates the use of adults to ensure extended toxicity measurements. Previous studies involving *P. brevis* toxin(s) have shown that an experimental interval of 48 hr is sufficient to measure slow toxin effects (Medlyn, unpublished).

The use of adult brine shrimp instead of nauplii as a bioassay organism has advantages and disadvantages. The main disadantage in using adults is the culturing process, which requires additional laboratory space, food source maintenance, and extended time. However, adults provide several advantages; First, the need to identify and use only one naupliar stage is eliminated. Also, adults are easier to see and handle. Finally, the researcher can avoid using abnormal, unhealthy individuals.

This study demonstrates that adult *Artemia* of different strains are differentially susceptible to *P. brevis* toxin(s) using $LD_{50}$ values. The results support conclusions of previous researchers using intermediate stages that bioassay experimentation should be conducted with cysts of known origin (*e.g.*, Zillioux *et al.*, 1973 ; Sorgeloos *et al.*, 1978).

For toxicological studies testing the suitability of different strains or species, statistical analyses performed on the bioassay regressions provide the researcher with a method to evaluate the response of a strain or species over a range of toxin concentrations rather than at a single point. Similarity of replication within strains suggests that *Artemia* is a reliable assay organism for *P. brevis* toxin(s).

Adult brine shrimp are very sensitive to *P. brevis* toxin(s). $LD_{50}$ values of these four strains ranged from 5.1 to 9.4 $\mu$g crude toxin/ml (ppm). Further experiments are currently in progress to determine minimal *P. brevis* cell concentrations detectable with this assay. Currently mouse bioassay is the only standardized assay to detect *P. brevis* toxin(s) (Cummins

and Hill, 1969). It was designed for testing shellfish meats and is unsuitable for toxicological studies involving low toxin concentrations or limited toxin quantities. Until the chemical structure of this toxin(s) is completely characterized, quantitative chemical assay procedures remain remote. Therefore, the bioassay described in this study is suggested as a standard method for quantifying *P. brevis* toxin(s) in water. Shark Bay strain had the lowest $LD_{50}$ value (highest sensitivity), was the easiest to raise, and is the recommended strain for future assays.

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**Literature cited**

Anderson D. T. 1967. Larval development and segment formation in the branchiopod crustaceans *Limnadia stanleyana* King (Conchostraca) and *Artemia salina* (L.) (Anostraca). *Aust. J. Zool.* 15:47-91.

Baden D. G., T. J. Mende, and R. E. Block. 1979. Two similar toxins isolated from *Gymnodinium breve*. p. 327-334. *In* : Toxic dinoflagellate blooms. Taylor D. L. and H. H. Seliger (Eds). Elsevier North Holland, New York. 505 p.

Cummins J. M. and W. F. Hill, Jr. 1969. Method for the bioassay of *Gymnodinium breve* toxin(s) in shellfish. *Gulf Coast Mar. Health Sci. Lab. Spec. Rep.* 69(3):1-6.

Curtis R. F., D. T. Coxon, and G. Levett. 1974. Toxicity of fatty acids in assays for mycotoxins using the brine shrimp (*Artemia salina*). *Food Cosmet. Toxicol.* 12(2):233-235.

Gates J. A. and W. B. Wilson. 1960. The toxicity of *Gonyaulax monilata* Howell to *Mugil cephalis*. *Limnol. Oceanogr.* 5:171-174.

Granada H. R., P. C. Cheng, and N. J. Doorenbos. 1976. Ciguatera. I. Brine shrimp (*Artemia salina* L.) larval assay for ciguatera toxins. *J. Pharm. Sci.* 65(9):1414-1415.

Grosch D. D. 1973. Reproduction tests : the toxicity for *Artemia* of derivates from non-persistent pesticides. *Biol. Bull.* 145:340-351.

Guillard R. R. L. and J. H. Ryther. 1962. Studies of marine planktonic diatoms. I. *Cyclotella nanna* Hustedt and *Detonula confervacea* (Cleve) Gran. *Can. J. Microbiol.* 8:229-239.

Harwich J. and P. M. Scott. 1971. Brine shrimp (*Artemia salina* L.) larvae as a screening system for fungal toxins. *Appl. Microbiol.* 21(6):1011-1016.

Martin D. F. and A. B. Chatterjee. 1969. Isolation and characterization of a toxin from the Florida red tide organism. *Nature (Lond.)* 221:51.

McFarren E. F., H. Tanabe, F. J. Silva, W. B. Wilson, J. E. Campbell, and K. H. Lewis. 1965. The occurrence of a ciguatera-like poison in oysters, clams and *Gymnodinium breve* cultures. *Toxicon* 3:111-123.

Michael A. S., C. G. Thompson, and M. Abramovitz. 1956. *Artemia salina* as a test organism for bioassay. *Science* 123:464.

Miller L. C. and M. L. Tainter. 1944. Estimation of the $ED^{50}$ and its error by means of logarithmic-probit graph paper. *Proc. Soc. Biol. Med.* 57:261-264.

Padilla G. M., Y. S. Kim, M. Westerfield, E. Rauckman, and J. W. Moore. 1978. Pharmacological activities of purified toxins from *Gymnodinium breve* and *Prymnesium parvum*. p. 271-283. *In* : Marine natural products chemistry. Faulkner D. J. and W. H. Fenical (Eds). Plenum, New York. 433 p.

Price K. S., G. T. Waggy, and R. A. Conway. 1974. Brine shrimp bioassay and seawater BOD of petrochemicals. *J. Water Pollut. Control Fed.* 46:63-77.

Risk M., Y. Y. Lin, P. D. MacFarlane, V. M. Sadagopa Ramanujam, L. L. Smith, and N. M. Trieff. 1979. Purification and chemical studies on a major toxin from *Gymnodinium breve*. p. 335-344. *In* : Toxic dinoflagellate blooms. Taylor D. L. and H. H. Seliger (Eds). Elsevier North Holland, New York. 505 p.

Sorgeloos P. and G. Persoone. 1975. Technological improvements for the cultivation of invertebrates as food for fishes and crutaceans. II. Hatching and culturing of the brine shrimp, *Artemia salina* L. *Aquaculture* 6: 303-317.

Sorgeloos P., C. Remiche-Van Der Wielen, and G. Persoone. 1978. The use of *Artemia* nauplii for toxicity tests – a critical analysis. *Ecotoxicology and Environ. Safety* 2:249-255.

Tarpley W. A. 1958. Studies of the use of the brine shrimp *Artemia salina* (Leach) as a test organism for bioassay. *J. Econ. Entomol.* 51(6):780-783.

Trieff N. M., M. McShan, D. Grajcer, and M. Alam. 1973. Biological assay of *Gymnodinium breve* toxin using brine shrimp (*Artemia salina*). *Tex. Rep. Biol. Med.* 31(3):409-422.

Zillioux E. J., H. R. Foulk, J. C. Prager, and J. A. Cardin. 1973. Using *Artemia* to assay oil dispersant toxicities. *J. Water Poll. Control Fed.* 45(11):2389-2396.

*The Brine Shrimp* Artemia. *1980. Vol. 1. Morphology, Genetics, Radiobiology, Toxicology*
*G. Persoone, P. Sorgeloos, O. Roels, and E. Jaspers (Eds). Universa Press, Wetteren, Belgium. 345 p.*

# Effects of asbestos on survival of *Artemia*

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**Abstract**

The effect of asbestos on survival rate of *Artemia* was studied using a randomized block statistical design. There is a decrease in survival rate of *Artemia* larvae exposed to asbestos fibers. The effect is concentration dependant, with a minimum survival in 400 mg/l of short fiber chrysotile asbestos variety. Beyond this concentration no increase in mortality was documented. Turbulence interacts with asbestos to cause greater mortality at each asbestos concentration. This effect results from an increased number of fibers in suspension and therefore in contact with the *Artemia*.

Mortality is related to fiber length of asbestos. Short fiber chrysotile asbestos causes higher mortality than medium or long fiber chrysotile. However, at the same fiber length and concentrations, short fiber crocidolite asbestos causes the same mortality as chrysotile. This indicates that mortality from asbestos is caused by the fiber size and shape, not by asbestos chemistry. Asbestos types used in this research are those of commercial, ecological and public health importance.

## Introduction

Although it has been known for the last seventy years that asbestos causes asbestosis, it has only recently been recognized as a carcinogen (Selikoff, 1969). This discovery has prompted research into levels of asbestos in the environment and their effects on mammals with an emphasis on human health. A few recent studies (Nicholson, 1974 ; Flickinger and Standridge, 1976 ; Lawrence and Zimmerman, 1977 ; Oliver, 1977 ; Schmitt *et al.*, 1977) have measured the concentrations of asbestos in certain waters, but as yet no studies have been published on the effects of asbestos on aquatic organisms. In view of the fact that high concentrations of asbestos can occur in aquatic habitats, such as waste tailings from taconite mines being dumped into Lake Superior, research should be focused on the various organisms in the environment. The present study will begin to remedy this lack of information by examining the effects of asbestos on a planktonic filter feeding crustacean. *Artemia* larvae proved to be an ideal experimental animal for this research.

### The occurrence of asbestos

Asbestos is a generic term for a variety of fibrous minerals having the common feature of a length-to-width ratio equal to or greater than three-to-one (Speil and Leineweber, 1969 ; Ampian, 1976). There are at present six recognized varieties of asbestos. One of these is the

fibrous form of serpentine known as chrysotile, which comprises 90 %-95 % of the asbestos used industrially in the United States (Speil and Leineweber, 1969 ; Speil, 1974 ; Ampian, 1976). The other five asbestos varieties are fibrous forms of the amphibole group ; amosite, tremolite, anthophyllite, actinolite, and crocidolite (Speil and Leineweber 1969 ; Ampian 1976 ; Campbell *et al.*, 177). Veblen *et al.* (1977) also recognize four new amphibole asbestos varieties and two new pyroxene asbestos varieties.

## Environmental transmission of asbestos

There are a variety of ways asbestos can enter the environment. Since asbestos minerals are naturaly occurring and are fairly widespread there will usually be a background level of asbestos in any area. The background levels of asbestos in Canada and the northern United States are reported to be in the range of $10^5$ to $10^7$ fibers per liter (Lawrence and Zimmerman, 1977). These background levels are the result of weathering of the parent rock and should result in higher levels in aquatic habitats in regions containing asbestos in the parent rock.

Man's use of naturally occuring asbestos has increased the environmental load of this material in both aerial and aquatic situations. A direct contribution to the aquatic environment is the result of waste tailings from taconite concentration operations in many areas (Lawrence and Zimmerman, 1977 ; Schmitt *et al.*, 197).

There are approximately 3 000 different commercial uses for asbestos based on their properties of fire resistance and insulating qualities (Harington *et al.*, 1975 ; Schmitt *et al.*, 1977).

The mining and processing of asbestos releases quantities of fiber into the air. Dust can be controlled in processing plants relatively easily by installation of dust filters. Control in mining operations poses more serious problems in dust disposal.

Fibers can still be released into the air after the asbestos is processed into a finished product. The construction industry in particular has a problem with asbestos fiber contamination due to the looseness of the fiber in insulation and the cutting of fiber board (Sawyer 1977).

The release of asbestos into the environment has changed the role of asbestos related diseases from a strictly occuptional problem to one of general concern for society. Asbestos bodies are being found routinely in lung tissue samples in many cities such as New York (Langer, 1974a). Asbestos fibers are also being found in many commercial products which are consumed. Some of this contamination is the result of spraying foods with pesticides which contain asbestos (Kay, 1974). Other contamination results from the use of talc which contains asbestos impurities as an antisticking agent for foods and as a dusting powder on chewing gum and coated rice (Eisenberg, 1974). Talc is also used for talcum powders and these have been found to contain varying quantities of asbestos (Snider *et al.*, 1972 ; Rohl and Langer, 1974). Asbestos filters have been employed in the beer and wine industries.

It can be seen that asbestos fibers can enter the air in various ways. These fibers may eventually end up in the waterways when they are washed out of the air by rain and thereby increase the asbestos concentrations in the aquatic environment.

## Biological effects of asbestos

Asbestos has recently been linked to a variety of diseases in man. The first to be recognized was asbestosis or fibrosis (Selikoff, 1969).

Studies conducted in fibrosis include in vitro studies of hemolysis and cytotoxicity and in vivo studies in experimental animals.

All asbestos varieties have been found to produce fibrosis. Continued experimentation, as indicated by Langer (1974b), on the extent of ambient levels in the environment and on the biological effects of asbestos is needed. Studies on the effectsof asbestos on aquatic organisms can serve as an indicator of environmental degradation and also as a measure of water quality for human use.

### Purpose of the study

This study aims at showing the effects of asbestos on the experimental species. By generalizing with other similar species we may add to the knowledge of the environmental effects of asbestos in aquatic systems. The central focus of this study was the effect of increasing concentrations of asbestos on survival rates in *Artemia*. A short fiber chrysotile was used for this aspect of the study, as it is the most readily available and the most abundant in commercial use in the United States. A second aspect studied was the effect of age of the *Artemia* on mortality caused by short fiber chrysotile. A third aspect examined was the effect of turbulence in keeping the asbestos fibers suspended, a condition potentially more lethal to this planktonic crustacean. A fourth question studied was the differential survival of *Artemia* caused by medium and long length chrysotile fibers. The survival of *Artemia* with increasing concentrations of these fibers was compared with the survival of *Artemia* with increasing concentrations of short fibers.

A final aspect studied was the differential survival effect caused by an amphibole asbestos of the same fiber length as the chrysotile asbestos.

In summary, the purpose of this study concerning the differential survival rates of *Artemia*, was to examine : 1) the effect of increasing concentrations of asbestos, 2) the effect of different fiber lengths, 3) the effect of two different varieties of asbestos, 4) the effect of turbulence, and 5) the effect of larval age.

## Materials and methods

The experimental organism used in this study was the larval stage of *Artemia*, a saline planktonic filter feeding crustacean. The advantage of using *Artemia* is the availability of eggs and the consequential abundance of research organisms which can be hatched and raised under standardized conditions.

*Artemia* is a non-selective filter feeder, taking both organic and inorganic particles from the water column (Reeve, 1963a). The use of *Artemia* allows generalizations to other non-selective filter feeding crustaceans, particularly some of the cladoceran and copepod representatives that can be found in the vicinity of asbestos and taconite mines where they will be exposed to high concentrations of asbestos. Some cladocerans and copepods have also been found to be non-selective filter feeders (Ryther, 1954 ; Marshall and Orr, 1955). Ecological equivalents of these crustacea function in food chains where the higher trophic levels may be consumed by man.

*Artemia* cultures were prepared daily by placing eggs from San Francisco Bay (Living World Brand) in $ASW_{10}$ (Artificial Sea Water, Instant Ocean) at a salinity of 32-34 ‰ and a temperature of 20°-23 °C.

The cultures were aereated and used between 2 and 3 days from the initial set-up for the two day culture and the three day culture runs respectively.

The trials for the different tests were prepared in the same maner. Five or ten jars were filled with 50 ml of $ASW_{10}$ at a salinity of 31-34 ‰ and temperature of 20°-23 °C. Individual *Artemia* nauplii of the appropriate age were transferred to each test jar by Pasteur pipette until there were 100/jar.

Asbestos was weighed on a Mettler H3/AR balance and then added to the jars to obtain the various concentrations and a control. In order to keep the asbestos in suspension, the jars (including the control) were aerated by air stone. In the turbulence study, however, no aeriation was employed. All jars were covered to reduce evaporation. After a period of 22 to 26 hr, the surviving *Artemia* were counted.

The effect of increasing concentrations of short fiber chrysotile on 3-day old *Artemia* was carried out in 30 replications with each air stone used three times at each concentration in order to remove any possible effect from the air stones. This is a randomized block statistical design. The short fiber chrysotile asbestos was supplied courtesy of the Johns-Manville Corporation from their Quebec mine.

The effect on younger larvae was studied with ten replications of 2-day old *Artemia*. All other conditions were the same except each asbestos concentration was run with each air stone in rotation to remove any possible variation from the stones.

Tests of the effect of turbulence were conducted using 2-day old cultures of *Artemia* and short fiber chrysotile. The runs were prepared the same way as for the short fiber chrysotile, 2-day old *Artemia* culture runs, except that after stirring no air stones were added. Only five replicates were run because there was no need to remove the variability caused by the air stones.

The study, testing the effect of length of fibers from medium and long chrysotile, was done with the same weights of asbestos and this resulted in fewer fibers per liter. Since only six replications were run for each length, a complete rotation of air stones was not possible. No air stone was used twice at the same concentration in order to prevent bias from this source.

Medium and long chrysotile fibers for the experiments were provided by the Bowling Green State University Geology Department from samples of the Johns-Manville corporation. The sources of asbestos were essentially the same as for the previous experiments.

Amphibole asbestos was obtained from the Bowling Green State University Geology Department. This specimen was ground and then sieved through a 74 $\mu$m screen. The fibers that were deposited in the pan were used for tests in the same way as for the chrysotile. These samples had to be stirred longer, as the fibers did not disperse as easily into the water. Six replicates were made using the air stones only once for each concentration.

A two way analysis of variance was computed on the 3-day *Artemia* culture – short fiber chrysotile data. The five concentrations of asbestos were used as one factor and the ten air stones were used as the second factor. These data would reveal interaction between the air stones and the different concentrations of asbestos. The data were run on a Univac 1100 computer using the BMD program 02V.

Since no interaction was found, a one way analysis of variance was run on the three day *Artemia* culture – short fiber chrysotile data on the Univac 1100 computer using the BMD program 01V. A one way analysis of variance was also preformed on each set of data for the two day *Artemia* culture – short fiber chrysotile runs, the turbulence turn, the medium fiber

runs, the long fiber runs, and the amphibole asbestos runs using the same program and computer.

When significance was found on any of the above ANOVAs, the Duncan Multiple Range test was applied to determine which concentrations were significantly different.

Four sets of t-tests were employed for the comparison of the three days *Artemia* culture – short fiber chrysotile data to the 2-day *Artemia* culture – short fiber chrysotile, the medium fiber chrysotile, the long fiber chrysotile, and the amphibole asbestos data sets, as well as the two day *Artemia* culture with short fiber chrysotile and the turbulence data.

These tests used the unequal sample size method as the number of replicates varied from one experiment to the other. All t-tests were calculated using the Call OS system on an IBM Model 75 computer. The program was written on the basis of the t-test. Samples were placed on a stub and allowed to evaporate to dryness. They were then placed in a desiccator at 60 °C for 6 hr to remove all moisture. The stubs were then gold coated and examined with a Hitachi Model HHS-2R scanning electron microscope at a magnification at 200 X. Ten fields from each stub were selected at random. If asbestos fibers were present in the field a polaroid picture was taken. The numbers of fibers for each fiber length and type were counted and averaged for ten fields. The scanning scope was also used to verify the identification of the asbestos type by morphology. The pictures from the electron microscope were also employed to calculate the frequency distribution of fiber length for each fiber type. Fifty fibers from each asbestos type and length were selected at random and measured.

To verify the types of asbestos used, samples of short, medium, and long fiber chrysotile and the amphibole asbestos were X-ray analyzed utilizing a Phillips Model 12045, 60 cycle X-ray diffractometer. The samples were run by using a packed chamber.

Scanning electron microscopy was used to determine the concentrations of the various length and types of asbestos in fibers per liter. A 3 ml sample was taken from a 200 mg/l concentration for each fiber length and the amphibole fiber. (Actual size and concentration of asbestos fibers are given in the Appendix Fig. 4 and Table XI.)

## Results

### 3-DAY *ARTEMIA* CULTURE – SHORT FIBER CHRYSOTILE

The average number of *Artemia* surviving after 24 hr in different concentrations of asbestos with aeration are given in Table I,A and Fig. 1. An analysis of variance was run on these data (Table I,B) which demonstrated that results of the various concentrations were different at the $P < 0.01$ level. Since significance was found, the Duncan Multiple Range test was applied to determine which concentrations were significantly different from the others (Table I,C). As can be seen from the table, the results at all concentrations of asbestos are significantly different from the control but not from each other. It should be noted that while the results at the different concentrations are not significantly different, there is a trend for the mortality to level out after a certain concentration is reached (Fig. 1).

### 2-DAY *ARTEMIA* CULTURE – SHORT FIBER CHRYSOTILE

The means (Table II,A, Fig. 1) in this set of runs based on ten replicates are higher in all cases than in the preceding set of data. A significant difference at the $P < 0.05$ level was found

between the results at different concentrations of asbestos (Table II.B). The Duncan Multiple Range test shows that all concentrations of asbestos are significantly different from the control and that these concentrations fall into two groups. Table II.C). The effect of age was determined by comparing the 2-day culture data to the three day culture data by means of t-tests run for each concentration level (Table III). The results show that age causes a significant increase in survival of *Artemia* at all levels of asbestos concentrations except for the highest one.

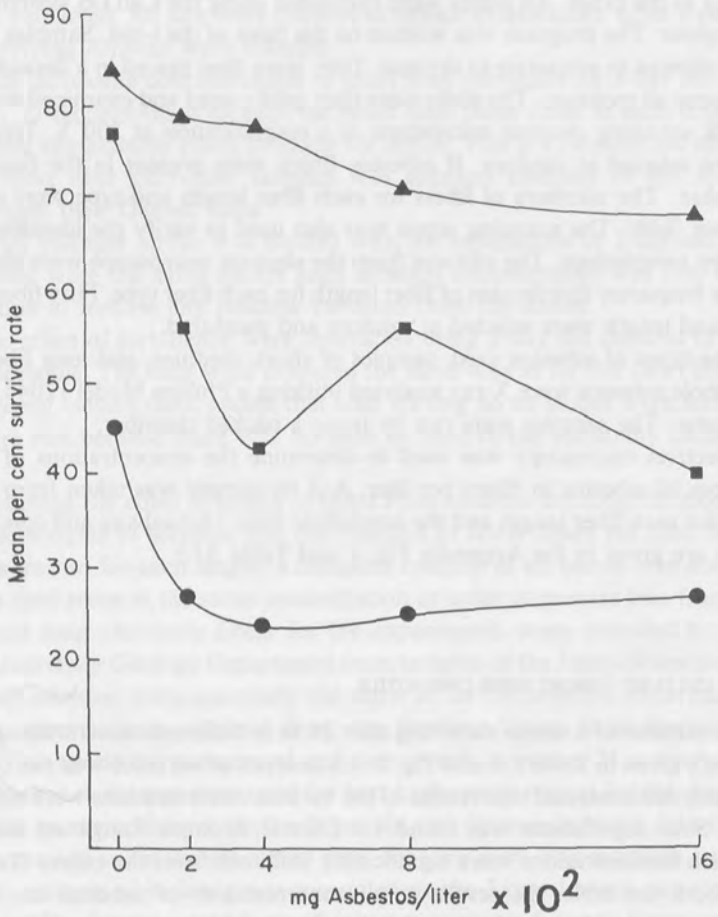


FIG. 1. Linear plots of the mean per cent survival rates of 3-day old *Artemia* nauplii in short fiber chrysotile (●——●), 2-day old nauplii in short fiber chrysotile (■——■), and 2-day old nauplii in short fiber chrysotile with no turbulence (▲——▲).

TABLE I

Survival of 3-day old *Artemia* in short fiber chrysotile

A. Means and standard deviations

| Concentrations of asbestos (mg/l) × $10^2$ | Mean survival | Standard deviation |
|---|---|---|
| 0 | 45.13 | 29.15 |
| 2 | 26.97 | 20.82 |
| 4 | 23.97 | 21.97 |
| 8 | 24.93 | 21.68 |
| 16 | 27.30 | 19.22 |

B. ANOVA

| Source of variation | SS | DF | MS | F ratio |
|---|---|---|---|---|
| Between groups | 9210.0937 | 4 | 2302.5234 | 4.4192** |
| Within groups | 75549.5605 | 145 | 521.0314 | |
| Total | 84759.6543 | 149 | | |

** Significant at the $P < 0.01$ level.

C. Duncan Multiple Range test

| Concentration of asbestos (mg/l) × $10^2$ | 4 | 8 | 2 | 16 | 0 |
|---|---|---|---|---|---|
| Means | 23.97 | 24.93 | 26.97 | 27.30 | 45.13 |

Any two means not underscored by the same line are significantly different.
Any two means underscored by the same line are not significantly different.

TURBULENCE

The means and standard deviations for *Artemia* survival in jars with no turbulence and different levels of asbestos concentration are presented in Table IV,A and Fig. 1. An analysis of variance did not reveal any significant difference between the survival in different concentrations of asbestos (Table IV,B). Since the turbulence data was collected using 2-day old *Artemia* cultures, the results could be compared to the ten replicates of the 2-day old *Artemia* in short fiber chrysotile using t-tests (Table V). Significance was found in two cases and was approached in the two other ones where asbestos was present.

3-DAY *ARTEMIA* CULTURE – MEDIUM FIBER AND LONG FIBER CHRYSOTILE

The mean survival of *Artemia* in different concentrations of long and medium length chrysotile fiber is presented in Table VI and Fig. 2. ANOVAs were run to determine if the

TABLE II

Survival of 2-day olf *Artemia* in short fiber chrysotile

A. Means and standard deviations

| Concentrations of asbestos (mg/l) × $10^2$ | Mean survival | Standard deviation |
|---|---|---|
| 0 | 76.60 | 17.30 |
| 2 | 56.10 | 25.03 |
| 4 | 43.30 | 36.69 |
| 8 | 56.20 | 28.32 |
| 16 | 39.50 | 31.84 |

B. ANOVA

| Source of variation | SS | DF | MS | F ratio |
|---|---|---|---|---|
| Between groups | 8441.7187 | 4 | 2110.4297 | 2.5817* |
| Within groups | 36785.4990 | 45 | 817.4555 | |
| Total | 45227.2178 | 49 | | |

* Significant at the $P < 0.05$ level.

C. Duncan Multiple Range test

| Concentration of asbestos (mg/l) × $10^2$ | 16 | 4 | 2 | 8 | 0 |
|---|---|---|---|---|---|
| Means | 39.50 | 43.30 | 56.10 | 56.20 | 76.60 |

Any two means not underscored by the same line are significantly different.
Any two means underscored by the same line are not significantly different.

TABLE III

T-tests comparing 2-day *Artemia* cultures to 3-day *Artemia* cultures in short fiber chrysotile

| Concentrations of asbestos (mg/l) × $10^2$ | $t_s$ | Significance level |
|---|---|---|
| 0 | -5.0845 | 0.001 |
| 2 | -4.9018 | 0.001 |
| 4 | -2.4764 | 0.05 |
| 8 | -4.8082 | 0.001 |
| 16 | -1.8257 | NS |

various concentrations were significantly different ; these results are given in Tables VII,A and B for medium and long fibers respectively. No significant differences were found for either the medium or the long fibers. Unequal sample size t-tests were run using the 30 replicates of the 3-day *Artemia* culture – short fiber chrysotile in comparison with the medium and the long fiber chrysotile runs (Table VIII,A and B, respectivey). No significant differences were observed except for the 200 mg/l long fiber chrysotile concentration.

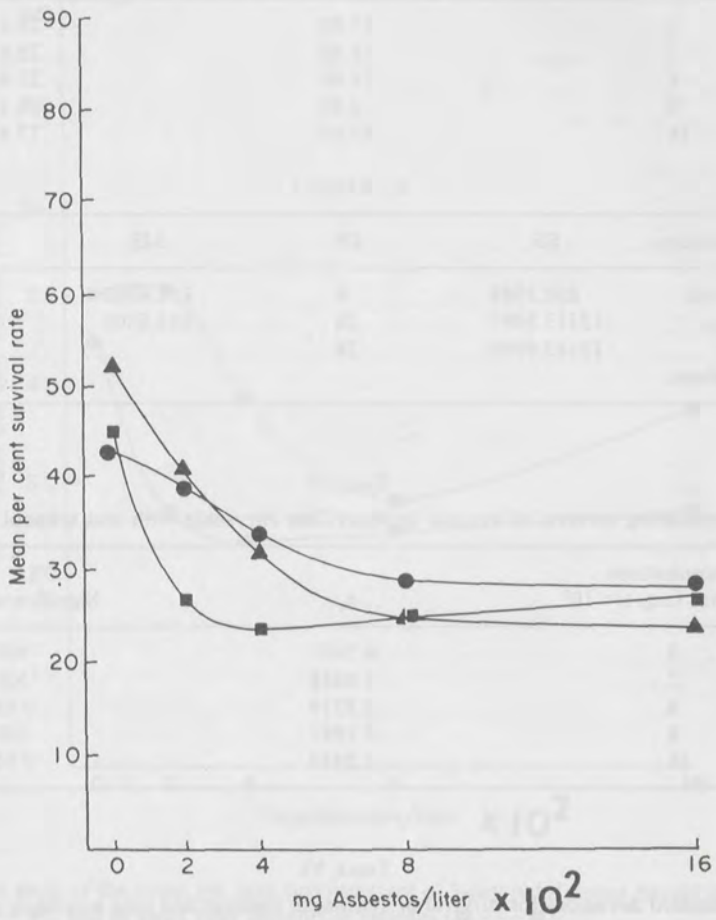


FIG. 2. Linear plots of the mean per cent survival rates of 3-day old *Artemia* nauplii in short fiber chrysotile (■——■), medium fiber chrysotile (●——●), and long fiber chrysotile (▲——▲).

3-DAY *ARTEMIA* CULTURES – SHORT FIBER AMPHIBOLE ASBESTOS

The mean survival for the six replicate runs with amphibole asbestos is presented in Table IX,A and Fig. 3. The analysis of variance (Table IX,B) showed no significant difference between the concentrations. The t-tests comparing the effect of short fiber chrysotile to the

TABLE IV

Survival of 2-day old *Artemia* in short fiber chrysotile in jars with no turbulence

A. Means and standard deviations

| Concentrations of asbestos (mg/l) × $10^2$ | Mean survival | Standard deviation |
|---|---|---|
| 0 | 83.80 | 25.41 |
| 2 | 78.80 | 25.61 |
| 4 | 78.00 | 23.40 |
| 8 | 70.80 | 26.10 |
| 16 | 67.60 | 23.42 |

B. ANOVA

| Source of variation | SS | DF | MS | F ratio |
|---|---|---|---|---|
| Between groups | 850.3999 | 4 | 212.6000 | 0.3453NS |
| Within groups | 12313.5997 | 20 | 615.6800 | |
| Total | 13163.9996 | 24 | | |

NS = Not significant.

TABLE V

T-tests comparing survival of *Artemia* in short fiber chrysotile with and without turbulence

| Concentrations of asbestos (mg/l) × $10^2$ | $t_s$ | Significance level |
|---|---|---|
| 0 | -0.7407 | NS |
| 2 | -2.0048 | NS |
| 4 | -2.5219 | 0.05 |
| 8 | -1.1987 | NS |
| 16 | -2.2483 | 0.05 |

TABLE VI

Means and standard deviations for survival of *Artemia* in medium and long lengths of chrysotile fibers

| Concentration of asbestos (mg/l) × $10^2$ | Medium fiber | | Long fiber | |
|---|---|---|---|---|
| | Mean | Stand. dev. | Mean | Stand. dev. |
| 0 | 42.67 | 30.32 | 52.33 | 24.25 |
| 2 | 39.16 | 30.26 | 41.33 | 25.43 |
| 4 | 33.50 | 33.48 | 32.17 | 21.52 |
| 8 | 28.67 | 24.51 | 24.67 | 26.24 |
| 16 | 28.33 | 16.51 | 24.00 | 22.24 |

effect of short fiber amphibole showed a significant difference at the 200 mg/l and the 400 mg/l concentrations, but not at the 800 mg/l and the 1 600 mg/l concentrations nor between the controls (Table X).

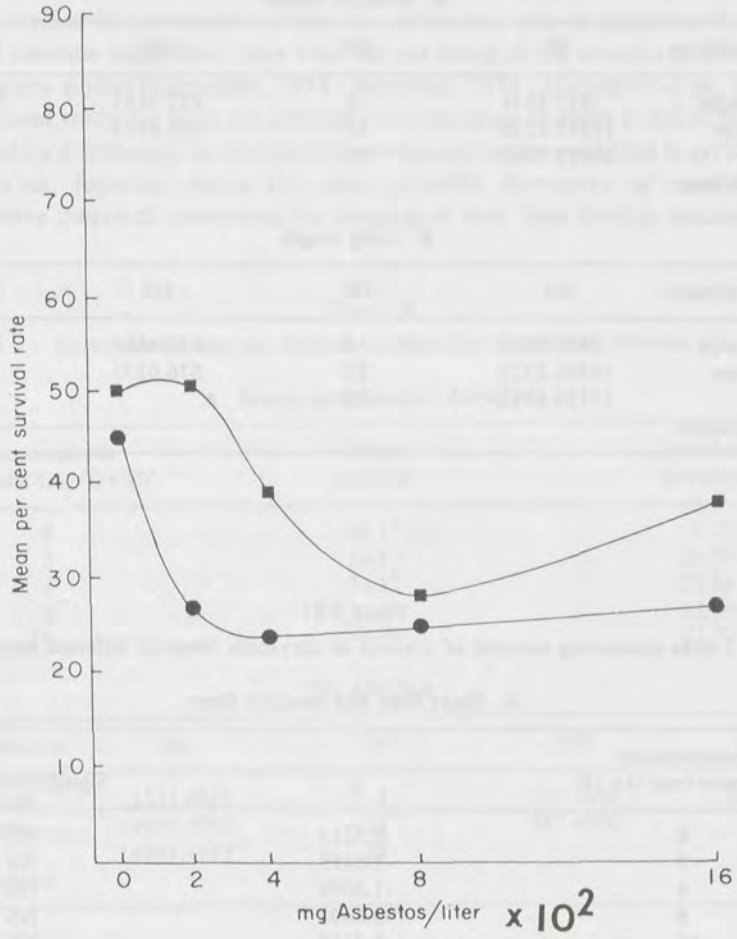


FIG. 3. Linear plots of the mean per cent survival rates of 3-day old *Artemia* nauplii in short fiber chrysotile (●——●), and in short fiber amphibole asbestos (■——■).

There is evidence that maximum filtration rate in feeding is maintained over a wide range of particle concentrations (Reeve, 1963ab). These studies show that the filtration rate is independent of the particle concentration until a certain high concentration is reached beyond which the filtration rate falls. Where this occurs depends on the nature of the particle. These studies indicate that behaviorily the *Artemia* are filtering at a rate independant of the particle concentration. Reeve (1963b) reported that the filtration rate dropped off after a concentration of $1 \times 10^6$ sand particles/cm$^3$ was reached. In this study the death rate dropped after $2 \times 10^5$

TABLE VII

Anova for survival of *Artemia* in chrysotile fibers of different length

A. Medium length

| Source of variation | SS | DF | MS | F ratio |
|---|---|---|---|---|
| Between groups | 933.5334 | 4 | 233.3833 | 0.3048NS |
| Within groups | 19141.8330 | 25 | 765.6733 | |
| Total | 20075.3662 | 29 | | |

NS = Not significant.

B. Long length

| Source of variation | SS | DF | MS | F ratio |
|---|---|---|---|---|
| Between groups | 3457.8663 | 4 | 864.4666 | 1.5007NS |
| Within groups | 14400.8329 | 25 | 576.0333 | |
| Total | 17858.6992 | 29 | | |

NS = Not significant.

TABLE VIII

T-tests comparing survival of *Artemia* in chrysotile fibers of different length

A. Short fiber and medium fiber

| Concentration of asbestos (mg/l) $\times 10^2$ | $t_s$ | Significance level |
|---|---|---|
| 0 | 0.3212 | NS |
| 2 | -1.8195 | NS |
| 4 | -1.3040 | NS |
| 8 | -0.6403 | NS |
| 16 | -0.3315 | NS |

B. Short fiber and long fiber

| Concentration of asbestos (mg/l) $\times 10^2$ | $t_s$ | Significance level |
|---|---|---|
| 0 | -1.0180 | NS |
| 2 | -2.3944 | 0.05 |
| 4 | -1.4663 | NS |
| 8 | 0.0268 | NS |
| 16 | 0.6150 | NS |

fibers/cm$^3$ was reached. This figure is actually higher in total numbers of particles as only the asbestos fibers were counted and there were other particles present. Death due to clogging of the filter-feeding mechanism could then result in a curve similar to that found in this study.

If ingestion of asbestos is the cause of death, then there would be an increase in mortality with each increase in concentration until the maximum rate of ingestion is reached. The ingestion of asbestos might then react with the gut lining of the *Artemia* as in the hemolytic and cytotoxicity studies (Harington, 1974 ; Schnitzer, 1974 ; Harington *et al.*, 1975).

In the present study we have not determined if the cause of death is due to the clogging of the filter feeding mechanism or ingestion of asbestos, as both would tend to produce the same survival curves. Ingestion seems the more probable alternative as most filter feeding organisms have means of preventing the clogging of their filter feeding mechanism.

TABLE IX

Survival of 3-day old *Artemia* in short fiber amphibole asbestos

A. Means and standard deviations

| Concentrations of asbestos (mg/l) × $10^2$ | Mean survival | Standard deviation |
|---|---|---|
| 0 | 50.17 | 9.77 |
| 2 | 50.83 | 21.77 |
| 4 | 39.17 | 28.66 |
| 8 | 27.50 | 32.39 |
| 16 | 38.00 | 22.32 |

B. ANOVA

| Source of variation | SS | DF | MS | F ratio |
|---|---|---|---|---|
| Between groups | 2251.4666 | 4 | 562.8666 | 0.9579NS |
| Within groups | 14689.9998 | 25 | 587.6000 | |
| Total | 16941.4663 | 29 | | |

NS = Not significant.

TABLE X

T-tests comparing 3-day old *Artemia* survival in short fiber chrysotile and short fiber amphibole asbestos

| Concentration of asbestos (mg/l) × $10^2$ | $t_s$ | Significance level |
|---|---|---|
| 0 | -0.8967 | NS |
| 2 | -4.3402 | 0.001 |
| 4 | -2.3500 | 0.05 |
| 8 | -0.3742 | NS |
| 16 | -1.9896 | NS |

### 2-DAY *ARTEMIA* CULTURE – SHORT FIBER CHRYSOTILE

The 2-day *Artemia* culture exhibited the same trend as in the 3-day *Artemia* cultures in that the mean percent survival curve tended to level out (Fig. 1). The 800 mg/l concentration is somewhat out of line, but this could be due to random variation as only ten replicates were run. A significant difference was again found between the groups with all asbestos concentrations having a greater mortality than the control.

The t-tests comparing the three day *Artemia* cultures to the 2-day cultures (Table III) in short fiber chrysotile showed a significant difference in four out of the five cases, the fifth case also approaching significance. This indicates an increased mortality with increased age for all concentrations. There appears to be no interaction between age and asbestos, as the greatest difference occurred between the control series.

The increase in mortality with increased age may be due to lack of food and consequent reduction in vigor, abrasion of the cuticle from the asbestos needles and clogging of the mouth by the longer fibers.

### TURBULENCE

Absence of turbulence resulted in higher survival rates of *Artemia* at all levels of asbestos tested. The analysis of variance for turbulence showed no difference between the various levels of asbestos, though the curve of the mean percent survival rate (Fig. 1), shows a decrease of the survival rate with increasing asbestos concentrations. The fact that the jars were stirred at the beginning of each run and therefore the asbestos started out in suspension is, in our opinion, a possible explanation for this fact. According to Lawrence and Zimmerman (1977) asbestos settles out of suspension readily at concentrations greater than $5 \times 10^{10}$ fibers/l. While asbestos is settling the *Artemia* would be in contact with more asbestos at higher concentrations that at the lower concentrations, resulting in abrasion of the delicate larval cuticle by the asbestos fibers under conditions of high turbulence.

The t-tests comparing the two day *Artemia* cultures in short fiber chrysotile with and without turbulence showed variability in significance. All concentrations of asbestos, however, showed a greater $t_s$ value than the control indicating an interaction between turbulence and asbestos in causing greater mortality. This interaction is probably due to the asbestos being kept in suspension and therefore in contact with the *Artemia*.

### 3-DAY *ARTEMIA* CULTURE – MEDIUM FIBER AND LONG FIBER CHRYSOTILE

In both of these series there was a greater mortality in the jars containing asbestos than in the controls, even though the individual differences were not significant in either case. As in the short fiber chrysotile runs the curves of the mean percent survival rate had a tendency to level out (Fig. 2), but the leveling out takes place at higher concentrations. This may be due to the smaller concentration of short fibers in the filter feeding range of the *Artemia*, thus not until higher levels are reached does the concentration of the short fiber asbestos approach the point where no increase in mortality occurs. This may be the case as there are short fibers present in the long and medium fiber samples, but the correlation is not precise as it would be expected that long fiber chrysotile samples would not level out until a concentration of 1 600 mg/l of asbestos is reached. This expectation is based on the distribution of fiber lengths in the

samples. This lack of precision in the correlation could be the result of the small number of replicates or the preparation of the samples and the counting of the fibers with the electron microscope.

The lack of significance in the ANOVAs for both the medium and the long fibers may be a consequence of the smaller number of replications used in these runs than in the short fiber runs. The difference between the controls and where the curve levels out is essentially the same in all three cases, so a greater number of replications would obtain significance. As in the demonstration of carcinogensis, a very large number of experimental units may be necessary to separate the effect statistically. Another hypothesis would be that there may be chemical differences between these asbestos types, in addition to the difference in fiber length.

Examination of the t-tests comparing the short fiber chrysotile to the medium and the long fiber chrysotile (Table VIII,A and B) do not reveal significant differences. There was, however, a tendency for the t statistic to decrease at each succeeding higher concentration of asbestos. These data confirm the trend that the surviva of *Artemia* approaches the same point with higher concentrations for all fiber lengths. This indicates that the long and medium fibers may not be lethal, but the concentration of short fibers associated with them is. Again this is evidence that mortality is not due to associated metals or ions, but is dependant of fiber length, which in turn is related to the range of particle sizes filtered by *Artemia*.

### 3-DAY *ARTEMIA* CULTURES – SHORT FIBER AMPHIBOLE ASBESTOS

The amphibole asbestos was identified as crocidolite on the basis of the X-ray diffraction patterns and the morphology of the fibers as indicated in the scanning electron microphotographs. Identification was complicated by the presence of a quantity of talc which shows an X-ray diffraction pattern similar to the amphibole asbestos varieties.

Crocidolite allows a higher survival rate of *Artemia* at all concentrations than the short fiber chrysotile asbestos. The crocidolite was difficult to get into suspension and had a tendency to float on top of the water. This may be due to the negative surface charge, the presence of talc or simply the dryness of the sample. Once in suspension, however, there was a greater tendency for the crocidolite sample to drop to the bottom. The same weight of amphibole asbestos occupied a smaller volume than chrysotile indicating that it is denser.

Although the analysis of variance did not show significance, the curve for the mean percent survival rate (Fig. 3) showed the same general condition of higher mortality at higher concentrations. The curve did not drop off as rapidly as in the chrysotile curve and the highest concentration showed greater survival than expected. This may be due to random variation, as only six replicates were conducted. The crocidolite curve resembles the curves for the medium and long fiber chrysotile asbestos in that the survival rate does not level off until higher concentrations are reached. The treshold concentration of crocidolite fibers remaining in suspension is probably but reached at higher concentrations. Since the scanning electron microphotographs showed that the amphibole asbestos was contaminated with talc, the concentration in fibers per liter only reached the same level as with the short fiber chrysotile at the 800 mg/l concentration, at which point the mortalities for the two types of asbestos were similar. This would indicate that there is no difference caused by the different chemical composition of the two asbestos types and that mortality is related to fiber size and shape.

The t-tests comparing crocidolite to the short fiber chrysotile asbestos show a significant difference at low concentrations, but the survival rates for the two types of asbestos approach

unity at high concentrations. If mortality were due to chemical differences between the two asbestos types the curves would not resemble each other since an analogous trend indicates a similar chemical effect which only differs in magnitude. While this cannot be ruled out, the above data obtained from the microphotographs indicate that the observed difference in mortality at the lower concentrations is due to differences in the concentrations of the fibers.

### Environmental implications

Schmitt *et al.* (1977) found that unfiltered water from Lake Superior contained from 0.85 to 9.6 mg of asbestos/l. This is equivalent to $8.3 \times 10^6$ fibers/l at the high end of the range and $8.0 \times 10^5$ fibers/l at the low end. Flickinger and Standridge (1976) reported essentially the same concentrations for Lake Superior, while Lawrence and Zimmerman (1977) reported concentrations of up to 100 000 times these figures in the vicinity of asbestos and iron are mines. The concentrations used in this study therefore are somewhat above the range that can be found in the natural environment, but lower than in heavily polluted environments. Since Lake Superior is a deep lake, we can expect longer and larger fibers to settle to the bottom, while short and small fibers would be most likely to be generally distributed. The small and short fibers are most toxic according to the present study.

If other filter-feeding crustaceans react the same way to the asbestos as *Artemia* did in this study, then there could be a drastic reduction in the zooplankton population in areas polluted with asbestos. This reduction of zooplankton would in turn cause a reduction in associated communities. Ryther (1954) showed that *Daphnia magna*, a cladoceran, is a non-selective filter feeder, as is *Artemia*. Marshall and Orr (1955) also describe a salt water copepod, *Calanus finmarchus*, as a non-selective filter feeder and this conclusion may tentatively be extended to the freshwater copepods. These two types of crustaceans are among the main components of the freshwater zooplankton community and may be expected to react to asbestos in the same way as *Artemia*.

One of the aspect of this study which may not be found to a great an extent in the environment is turbulence. Turbulence will certainly exist in nature as moderate or heavy wave action in the upper water layer or in flowing streams. Some of the asbestos would drop out of suspension in lakes and thereby reduce the concentration in natural waters. This would however simply shift the problem to the benthic community.

## Literature cited

Ampian S. G. 1976. Asbestos minerals and their nonasbestos analogs. For review of Mineral Fibers Session of Electron Microscopy of Microfibers. 30 p.

Campbell W. J., R. L. Blake, L. L. Brown, E. E. Catlar, and J. J. Sjoberg. 1977. Selected silicate minerals and their asbestiform varities : mineralogical definitions and their identification-characterization. US Dept. Interior, Bureau of Mines. Information circular 8751. 56 p.

Eisenberg W. V. 1974. Inorganic particle content of foods and drugs. *Environ. Hlth. Perspec.* 9:183-191.

Flickinger J. and J. Standridge. 1976. Identification of fibrous material in two public water supplies. *Environ. Sci. Tech.* 10:1028-1032.

Harington J. S. 1974. Fibrogenesis. *Environ. Hlth. Perspec.* 9:271-280.

Harington J. S., A. C. Allison, and D. V. Badami. 1975. Mineral fibers : chemical, physiochemical and biological properties. *Advan. Pharmacol. Chemother.* 12:291-402.

Kay K. 1974. Inorganic particles of agricultural origin. *Environ. Hlth. Perspec.* 9:193-196.

LANGER A. M. 1974a. Inorganic particles in human tissues and their association with neoplastic disease. *Environ. Hlth. Perspec.* 9:229-234.

LANGER A. M. 1974b. Research perspectives concerning asbestos minerals and their effects on biological systems. *Environ. Hlth. Perspec.* 9:335-340.

LAWRENCE J. and H. W. ZIMMERMAN. 1977. Asbestos in water : mining and processing effluent treatment. *Water Poll. Cont. Fed. J.* 49:156-160.

MARSHALL S. M. and A. P. ORR. 1955. On the biology of *Calanus finmarchicus*. VIII. Food uptake, assimilation, and excretion in adult stage V. *Calanus. J. Mar. biol. Ass. UK* 34:495-529.

NICHOLSON W. J. 1974. Analysis of amphibole asbestiform fibers in municipal water supplies. *Environ. Hlth. Perspec.* 9:165-172.

OLIVER T. 1977. An electron microscope study of asbestiform fiber concentrations in Rio Grande Valley supplies. *Amer. Water Works Ass. J.* 69:428-431.

REEVE M. R. 1963a. The filter-feeding of *Artemia*. I. In pure cultures of plant cells. *J. Exp. Biol.* 40:195-205.

REEVE M. R. 1963b. The filter-feeding of *Artemia*. II. In suspensions of various particles. *J. Exp. Biol.* 40:207-214.

ROHL A. N., A. M. LANGER. 1974. Identification and quantitation of asbestos in talc. *Environ. Hlth. Perspec.* 9:95-109.

RYTHER J. H. 1954. Inhibitory effects of phytoplankton upon the feeding of *Daphnia magna* with reference to growth, reproduction, and survival. *Ecology* 35:522-533.

SAWYER R. N. 1977. Asbestos exposure in a Yale building. *Environ. Res.* 13:146-169.

SCHMITT R. P., D. C. LINDSTEN, and T. F. SHANNON. 1977. Decontaminating Lake Superior of asbestos fibers. *Environ. Sci. Tech.* 11:462-465.

SCHNITZER R. J. 1974. Modification of biological surface activity of particles. *Environ. Hlth. Perspec.* 9:261-266.

SELIKOFF I. J. 1969. Asbestos. *Environment* 11:2-7.

SICK L. V. 1976. Nutritional effect of five species of marine algae on growth, development, and survival of the brine shrimp *Artemia salina*. *Mar. Biol.* 35:69-78.

SNIDER D. W., D. E. PFEIFFER, and J. J. MANCUSO. 1972. Asbestiform impurities in commercial talcum powders. *Compass* 49:65-67.

SPEIL S. 1974. Chrysotile in water. *Environ. Hlth. Perspec.* 9:161-163.

SPEIL S. and J. P. LEINEWEBER. 1969. Asbestos minerals in modern technology. *Environ. Res.* 2:166-208.

VEBLEN D. R., P. R. BUSECK, and C. W. BURNHAM. 1977. Asbestiform chain silicates : new minerals and structural groups. *Science* 198:359-365.

## Appendix

### X-RAY DIFFRACTION DATA

X-ray diffraction patterns revealed that the three lengths of asbestos believed to be chrysotile were in fact chrysotile asbestos fibers. Each had matching peaks at 12.7° 2 $\ominus$, 19.7°-20.1° 2 $\ominus$, and at 24.6° 2 $\ominus$, wich are the primary diffraction peaks of chrysotile. The short fiber chrysotile had in addition a few minor peaks in the 8°-10° 2 $\ominus$ range which could be the result of the crushing of the fibers during the milling process.

The amphibole asbestos pattern was somewhat ambiguous due primarily to contamination by talc and secondarily because the amphiboles are a continuous mineral series group and the X-ray diffraction patterns are fairly similar in all the different varieties. The peaks at 10.5° 2 $\ominus$ and 32.2° 2 $\ominus$ show the asbestos present may possibly be crocidolite, the asbestiform variety of riebeckite. The scanning electron microphotographs confirmed that the amphibole asbestos was crocidolite as noted by the irregular cleavage ends which are the result of cleaving in a stepwise fashion.

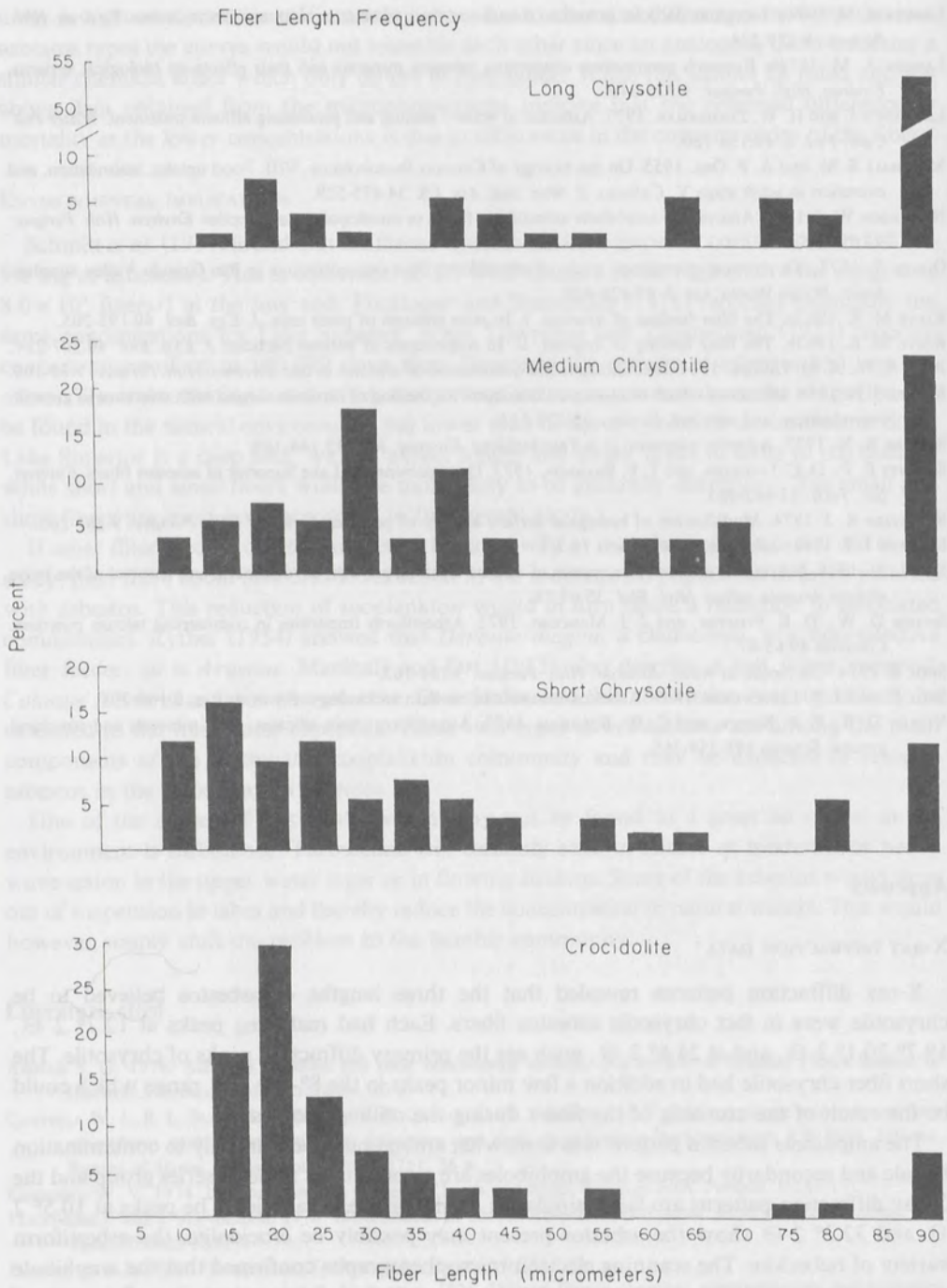


FIG. 4. Length frequency distributions of short, medium, and long chrysotile fiber samples and amphibole asbestos fiber samples.

FIBER CONCENTRATION AND LENGTH DISTRIBUTION

Scanning electron microphotographs were used to determine the number of fibers per liter. The average numbers of fibers for each type of asbestos was determined by counting all the fibers in each microphotograph and dividing by the number of fields which were randomily selected. In each case ten fields had been selected, but only those fields with fibers present had photographs taken of them. In dividing by ten, all fields were taken into account whether they had fibers present or not. The average number of fibers for each type was converted to fibers per liter. The 200 mg/l concentrations were used to determine fibers/liter for the short and medium length chrysotile fibers, while the 800 mg/l concentrations were used for the long length chrysotile and the crocidolite asbestos. These concentrations were either multiplied or divided by the appropriate factor to determine the number of fibers per liter for the other concentrations (Fig. 4, Table XI).

TABLE XI

Concentrations in fibers per liter for the different types of asbestos

| Asbestos type | Concentration (mg/l) | | | |
|---|---|---|---|---|
| | 200 | 400 | 800 | 1600 |
| Chrysotile | | | | |
| Short | $1.2 \times 10^8$ | $2.4 \times 10^8$ | $4.8 \times 10^8$ | $9.6 \times 10^8$ |
| Medium | $6.1 \times 10^7$ | $1.2 \times 10^8$ | $2.4 \times 10^8$ | $4.8 \times 10^8$ |
| Long | $2.2 \times 10^7$ | $4.4 \times 10^7$ | $8.9 \times 10^7$ | $1.8 \times 10^8$ |
| Crocidolite | $4.4 \times 10^7$ | $8.8 \times 10^7$ | $1.7 \times 10^8$ | $3.5 \times 10^8$ |

*The Brine Shrimp* Artemia. *1980. Vol. 1. Morphology, Genetics, Radiobiology, Toxicology*
*G. Persoone, P. Sorgeloos, O. Roels, and E. Jaspers (Eds). Universa Press, Wetteren, Belgium. 345 p.*

# Toxicity of heavy metals, oils and other organics on *Artemia*

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**Abstract**

A bioassay using *Artemia* has permitted the determination of 24 hr $LD_{50}$'s for several heavy metals in Instant Ocean® artificial seawater : $Cu^{+2}$ (2.33 mg/l) > $Hg^{+2}$(5.34 mg/l) > $As^{+3}$(32.1 mg/l) > $Zn^{+2}$(63.2 mg/l) > $Cd^{+2}$(68.8 mg/l) > $Pb^{+2}$(207.2 mg/l). The *Artemia* were found to be considerably more resistant to the toxicity of heavy metals, when weight is considered, than are fish. While in both cases -SH binding is likely to be involved, in fish, the reaction is with albuminoid compounds in he mucous secreted by the fish gills (Carpenter, 1927) while in *Artemia* it is probably with -SH groups on the active transport filter system, the peleopods or external gills. Several oils were also tested and a crude fuel oil blend and H-coal distillate found to be most toxic, while the shale oil-derived crude and processed oils appear to be less toxic. Advantages and disadvantages to the method are given.

## Introduction

The present study arose out of some work that we were doing on isolation and purification of toxins from marine dinoflagellates, such as those from *Gymnodinium breve* ("red-tide"). During this work a biological assay was developed utilizing brine shrimp (Trieff *et al.*, 1973). It was noted that the brine shrimp ware quite resistant to the effects of the crude toxin from *G. breve* compared with fish such as *Gambusia affinis*. Thus, a brine shrimp weighing about 6 mg can withstand more than 10 times the lethal concentration for one fish weighing 60 mg. We wondered whether this resistance to dinoflagellate toxins extended to other types of substances such as heavy metals.

Advantages of the use of *Artemia* as a bioassay are that this species is available year-round underthe form of dry cysts, from which the animals can be hatched and cultured very easily (Needham *et al.*, 1937 ; Sorgeloos, 1974 ; Vanhaecke *et al.*, 1980).

Feeding *Artemia* is easy both on live algae – *Tetraselmis* and *Dunaliella* – as on inert feeds – rice bran, whey, lactoserum – (Sorgeloos, 1980).

Perhaps the greatest advantage is the fact that adult brine shrimp are sufficiently small so that some 3-10 of them can be accomodated in a 100 ml beaker permitting easy observation of their movement and subsequent mortality. We have found that five to six *Artemia* per beaker represents the optimal number. Thus, the use of three beakers per dose of substance, each containing five *Artemia* will result in 15 brine shrimp per dose, permitting a reasonable

number for statistical purposes per dose. Compared to rodents or even fish, the amount of space consumed is miniscule.

Disadvantages of the method are first, that the brine shrimp tend to be fairly resistant to toxic substances ; second, that either filtered ocean water (if this is accessible to the laboratory) or Instant Ocean ® artificial seawater, needs to be used. The latter presents some problems as will be noted in the Results and Discussion sections of this paper.

Okibo (1957) has utilized the 48 hr *Artemia* larvae as a bioassay tool and found the mean lethal toxicity, TLm ($LD_{50}$) to be fairly consistent. He observed that the responses of the *Artemia* larvae were somewhat insensitive compared with those of other marine animals with regards to effects of some pollutants. The use of brine shrimp for toxicity bioassays has been reported by a large number of investigators (see review by Vanhaecke *et al.*, 1980).

Trieff *et al.* (1973) developed a bioassay method using adult *Artemia* directed, particularly, at determining toxicities of dinoflagellate toxins, such as those from *Gymnodinium breve*. Effects of the parameters – container, number of brine shrimp per unit volume, temperature, and solvent or dispersing agent – were studied, quantitatively on the assay for *G. breve* toxin. $LD_{50}$ values were obtained for the various solvents – methanol, ethanol, ethylene glycol, Tween 80 (Polysorbate 80), and PG 420 – as well as for *G. breve* toxin.

## Materials and methods

*Artemia* cysts from Macau, Brazil were kindly provided to us by the National Marine Fisheries Service, NOAA, Galveston, Texas and hatched according to standard procedures. After hatching, the nauplii were fed for approximately 2 weeks on thawed, frozen *Tetraselmis* algae, and then on alternate days with rice bran and *Tetraselmis* algae. The rice bran and frozen *Tetraselmis* (5 000 cells/ml) were also kindly donated by the National Marine Fisheries Service. The brine shrimp were maintained on Instant Ocean® (I.O.) Synthetic Sea Salts (Aquarium Systems 1978) at a specific gravity of about 1.021 (not measured) at 25 °C by making up approximately 100 ml of I.O. to 4 l with tap water as per instructions. Whenever the aquarium water level fell through evaporation, it was made up again to the original mark. I.O., made up as indicated, was also used for the solvent in the bioassay studies.

### Heavy metals

The following heavy metals were all reagent-grade and were made up in stock solutions to the concentrations indicated in distilled water, unless indicated otherwise : cadmium (II) chloride ($CdCl_2.2\ 1/2H_2O$, $10^{-3}$M) ; mercury (II) chloride ($HgCl_2$, anhydrous, $10^{-3}$M) ; copper (II) sulfate ($CuSO_4.5H_2O$, $10^{-2}$M) ; sodium arsenite (III) ($AS_2O_3$, anhydrous, dissolved in minimal smount of NaOH, $10^{-2}$M) ; lead (II) chloride ($PbCl_2$, anhydrous, $10^{-3}$M) ; zinc (II) sulfate ($ZnSO_4.7H_2O$, $10^{-2}$M).

### Oils

The following oils, which were obtained from the Department of Energy, were all tested in the bioassay system. Sample No. 1701.15, SRC – II Fuel Oil Blend ; 4614.26 – Petroleum-Derived JP-5 Product ; 4608.38 Shale Derived JP-5 Product ; 4601-42 – Crude Shale Oil ; 1601.13-H-Coal Distillate (Raw).

### *Artemia* assay

Selection of the brine shrimp was done by suction using a Propipette® bulb attached to the bottom (narrow) end of a 10 ml graduated pipette. Adult brine shrimp were sucked up through the wide end of the pipette and then five *Artemia* expelled gently into each beaker[1]. For the $LD_{50}$ determinations on the heavy metal salts, at least five concentrations and a control were used for each metal, with three beakers containing 50 ml of the appropriate concentration per dose. If necessary, additional concentrations were tested if it were found that insufficient points on the linear portion of the dose response curve were used. Death was indicated by complete cessation of movement of *Artemia*.

For the oils, the tests were less exact, three levels of concentration being used per oil and five *Artemia* per concentration. Because most of the oils tested were relatively viscous a dispo® pipette was used to measure out varying number of drops – 1, 5, or 10 – which contain a volume of about 0.025 ml/drop.

### Determination of $LD_{50}$'s from metal experiments

In most instances, the Reed-Muench (1938) method of cumulative toxicity was used ; the Miller-Tainter (1944) probit method was also employed and gave essentially the same results as indicated in the results section.

## Results

### Heavy metal toxicity

Sample raw data for cadmium chloride toxicity to *Artemia* in terms of mortality fraction at both 24 and 48 hr appear in Tables I and II.

If the data in Table II are utilized to obtain cumulative mortality and survivals by applying the Reed-Muench method and utilizing the assumptions that any *Artemia* dying at one concentration will also die at a higher concentrations, and similarly that any *Artemia* surviving at one concentration will survive at a lower concentration, we obtain the resulting mortality date in Table III. A linear interpopulation between 81% and 18.2% mortality in Table III is used to find the concentration at which the 50% accumulated mortality occurs ; this is found to be $3.02 \times 10^{-4}$M, thus, by the Reed-Muench (1938) method.

By the Miller-Tainter (1944) probit technique, the data can be arranged as in Table IV. The resulting $LD_{50}$ is $2.28 \times 10^{-4}$M corresponding to a probit of 5.0 if only the data at $5.0 \times 10^{-4}$M and $1.0 \times 10^{-4}$M are employed. This is in reasonable agreement to the value obtained by the Reed-Muench (1938) method. Probably closer agreement might be obtained if a plot of probit *versus* log concentration were used from $7.5 \times 10^{-4}$M to $5 \times 10^{-5}$M.

Most of the data were more amenable to treatment by the Reed-Muench cumulative mortality method (1938). Calculated $LD_{50}$'s for the heavy metals tested on *Artemia* appear in Table V.

[1] No attempt was made to select either all males or females or an equal distribution of sexes : the *Artemia* were pipetted at random and the sex-ratio probably showed a random variation throughout the experiments. The difference in response between sexes towards the different chemical toxins in this study has not been investigated.

TABLE I

Mortality data for cadmium chloride to *Artemia* (adults) at 24 hr (temperature = 25 ± 2 °C)

| Concentration $Cd^{++}$(M) | Mortality fraction beaker no. | | | Total mortality fraction |
|---|---|---|---|---|
| | 1 | 2 | 3 | |
| 1) $7.5 \times 10^{-4}$ | 5/5 | 2/5 | 3/5 | 10/15 |
| 2) $5.0 \times 10^{-4}$ | 1/5 | 2/5 | 2/5 | 5/15 |
| 3) $1.0 \times 10^{-4}$ | 0/5 | 0/5 | 0/6 | 0/16 |
| 4) $5.0 \times 10^{-5}$ | 0/5 | 0/5 | 0/5 | 0/15 |
| 5) $1.0 \times 10^{-5}$ | 0/5 | 0/5 | 0/5 | 0/15 |
| 6) $5.0 \times 10^{-6}$ | 0/5 | 0/5 | 0/5 | 0/15 |
| 7) 0-Control | 0/5 | 0/5 | 0/5 | 0/15 |

TABLE II

Mortality data for cadmium chloride to *Artemia* (adults) at 48 hr (temperature -25 ± 2 °C)

| Concentration $Cd^{++}$(M) | Mortality fraction beaker no. | | | Total mortality fraction |
|---|---|---|---|---|
| | 1 | 2 | 3 | |
| 1) $7.5 \times 10^{-4}$ | 5/5 | 4/5 | 4/5 | 13/15 |
| 2) $5.0 \times 10^{-4}$ | 5/5 | 5/5 | 3/5 | 13/15 |
| 3) $1.0 \times 10^{-4}$ | 1/5 | 1/5 | 0/6 | 2/16 |
| 4) $5.0 \times 10^{-5}$ | 0/5 | 1/5 | 0/5 | 1/15 |
| 5) $1.0 \times 10^{-5}$ | 1/5 | 0/5 | 0/5 | 1/15 |
| 6) $5.0 \times 10^{-6}$ | 0/5 | 0/5 | 0/5 | 0/15 |
| 7) 0-Control | 0/5 | 0/5 | 0/5 | 0/15 |

TABLE III

Adjusted cumulative mortality data for cadmium chloride to *Artemia* (adults) at 48 hr (temperature = 25 ± 2 °C)

| Concentration $Cd^{++}$(M) | Observed mortality fraction (from Table II) | Deaths | Survived | Accumulated | | | Accumulated % morality |
|---|---|---|---|---|---|---|---|
| | | | | Deaths | Survived | Total | |
| 1) $7.5 \times 10^{-4}$ | 13/15 | 13 | 2 | 30 | 2 | 32 | 93.8 |
| 2) $5.0 \times 10^{-4}$ | 13/15 | 13 | 2 | 17 | 4 | 21 | 81.0 |
| 3) $1.0 \times 10^{-4}$ | 2/16 | 2 | 14 | 4 | 18 | 22 | 18.2 |
| 4) $5.0 \times 10^{-5}$ | 1/15 | 1 | 14 | 2 | 32 | 34 | 5.88 |
| 5) $1.0 \times 10^{-5}$ | 1/15 | 1 | 14 | 1 | 46 | 47 | 2.13 |
| 6) $5.0 \times 10^{-6}$ | 0/15 | 0 | 15 | 0 | 61 | 61 | 0 |
| 7) 0-Control | 0/15 | 0 | 15 | 0 | 76 | 76 | 0 |

TABLE IV

Mortality data for cadmium chloride to *Artemia* at 48 hr adjusted for Miller-Tainter (1944) method (temperature = 25 ± 2 °C)

| Concentration $Cd^{++}$(M) | log concentration | % mortality | Probit |
|---|---|---|---|
| $5.0 \times 10^{-4}$ | -3.301 | 86.7 | 6.11 |
| | x | | 5.0 |
| $1.0 \times 10^{-4}$ | -4.000 | 12.5 | 3.84 |
| | $\Delta x$ | | |

TABLE V

$LD_{50}$'s (24 and 48 hr) to *Artemia* from exposure to various heavy metal salts (Instant Ocean, temperature = 25 ± 2 °C)

| Metal Ion | $LD_{50}$-24 hr | $LD_{50}$-48 hr |
|---|---|---|
| $Cd^{++}$ | $6.12 \times 10^{-4}$ M (M-T) | $3.02 \times 10^{-4}$ M (R-M)[1]<br>$2.28 \times 10^{-4}$ M (M-T)[2] |
| $Hg^{++}$ | $2.66 \times 10^{-5}$ M (R-M) | |
| $As^{+3}$ | $4.28 \times 10^{-4}$ M (R-M) | |
| $Cu^{++}$ | $3.66 \times 10^{-5}$ M (R-M) | |
| $Pb^{++}$ | $1.0 \times 10^{-3}$ M (extroplation from 48 hr $LD_{50}$)[4] | $5.00 \times 10^{-4}$ M (R-M)[3] |
| $Zn^{++}$ | $9.66 \times 10^{-4}$ (R-M)[5] | |

[1] R-M = Reed-Muench (1938) cumulative mortality method.

[2] M-T = Miller-Tainter probit method (1944).

[3] Done in 1:5 I.O. to avoid precipitation of $PbSO_4$.

[4] Extrapolating using inverse relationship between time and dose (Haber's Law), *i.e.*, ($LD_{50}$24 hr)(24 hr) = ($LD_{50}$48 hr)(48 hr).

[5] Measured at 25 hr.

### TOXICITY TO OILS

The data for toxicity of the oils noted under Materials and methods appear in Table VI.

### TOXICITY OF MISCELLANEOUS ORGANICS

In an effort to see the effect of chelating agents on heavy metals toxicity to *Artemia*, $10^{-2}$M $Na_2EDTA$ in I.O. was tested. It was found that after 15 hours, mortality of the brine shrimp was 100% (15/15).

A preliminary test of an anti-schistosomal agent, N-butyl, N-2-chloroethylethanolamine acetate, an analogue of acetylcholine was made. When 5 ml of $1.38 \times 10^{-3}$M concentration of this substance was added to 25 ml of I.O. there was no observable effect on two *Artemia*. This concentration would have been lethal to schistosomes.

TABLE VI

Mortality data of *Artemia* exposed to various oils[1]
(five *Artemia* per concentratin of each oil unless otherwise indicated
(temperature = 25 ± 2 °C)

| Oil fraction | Mortality fraction | | |
|---|---|---|---|
| | 1 drop (0.025 ml per 50 ml) | 5 drops (0.125 ml per 50 ml) | 10 drops (0.25 ml per 50 ml) |
| 1) No. 1601.13 H-coal distillate raw (18 hr) | 5/5 | 5/5 | 5/5[3] |
| 2) No.1701.15 SRC – II fuel oil blend (18 hr) | 5/5 | 5/5 | 5/5[2] |
| 3) No. 4614.26 petroleum-derived JP-5 product (18 hr) | 1/10 | 1/5 | 3/5 |
| 4) No. 4608.38 shale-derived JP-5 product (18 hr) | 0/4 | 0/5 | 2/5 |
| 5) No. 4601.42 crude shale oil (23 hr) | 0/5 | 1/5 | 1/5 |

[1] Obtained from EPA/DOE Fossil Fuels – Research Materials Facility.
[2] All dead in 3 hr.
[3] Three dead in 3 hr.

## Discussion

### TOXICITY OF HEAVY METALS TO *ARTEMIA*

The results for $LD_{50}$'s at 24 hr in molarity may be ordered in decreasing toxicity from mercury (II) to Zn (II) and Pb (II) as follows (in units of metallic ion molarity):

$LD_{50}$-24 hr (M):

$$\underset{(2.66\times10^{-5})}{Hg^{+2}} > \underset{(3.66\times10^{-5})}{Cu^{+2}} > \underset{(4.28\times10^{-4})}{As^{+3}} > \underset{(6.12\times10^{-4})}{Cd^{+2}} > \underset{(9.66\times10^{-4})}{Zn^{+2}} > \underset{(1.0\times10^{-3})}{Pb^{+2}}$$

If 24 hr $LD_{50}$'s are given in the units of mg/l by multiplying by atomic weight of metal (g/mole) and by 1 000 (mg/g) we obtain the sequence in decreasing toxicity:

$LD_{50}$-24 hr (mg/l):

$$\underset{(2.33)}{Cu^{+2}} > \underset{(5.34)}{Hg^{+2}} > \underset{(32.1)}{As^{+3}} > \underset{(63.2)}{Zn^{+2}} > \underset{(68.8)}{Cd^{+2}} > \underset{(207.2)}{Pb^{+2}}$$

The molarity sequence thus is reversed, because of introduction of atomic weight, between $Cu^{+2}$ and $Hg^{+2}$ and between $Cd^{+2}$ and $Zn^{+2}$.

Earlier results for 24 hr $LD_{50}$'s (in mg/l) from our laboratory (Trieff, 1974) were considerably lower thus:

$LD_{50}$-24 hr (mg/l) (old study): $Hg^{+2}$ (0.106) > $Cu^{+2}$ (0.785) > $Zn^{+2}$ (9.65) > $Pb^{+2}$ (28.8)

For the ratios of new/old study we have :

| | $Hg^{+2}$, | $Cu^{+2}$, | $Zn^{+2}$, | $Pb^{+2}$ |
|---|---|---|---|---|
| New $LD_{50}$-24 hr / Old $LD_{50}$-24 hr | 50.38, | 2.97, | 6.54, | 7.19 |

This discrepancy was disturbing to us, although it was recognized that there were several differences between the manners in which the old and new studies were conducted. First, the manner of feeding had changed. In the earlier study, the *Artemia* grazed on algae, primarily *Tetraselmis* grown from sewage using the technique described by Songer *et al.* (1974), while in the present study, as noted, the *Artemia* were fed on a combination of thawed, frozen cultured *Tetraselmis* and rice bran. Secondly, the manufactured Instant Ocean® had changed in the interim. At the time the earlier study was done, the I.O. had the composition listed in Table VII, while for the present study no composition is given nor is there an accompanying trace element solution. Finally, the composition of the tap water in Galveston has changed in the interim, becoming softer.

TABLE VII

Composition of Instant Ocean (SpGrav = 1.025 at 15 °C) in ppm (as reported in 1974)

| | | | | | |
|---|---|---|---|---|---|
| $Cl^-$ | 18 400 | $Br^-$ | 20 | [1]$I^-$ | 0.07 |
| $Na^+$ | 10 200 | $Sr^{+2}$ | 8 | [1]EDTA | 0.05 |
| $SO_4^{-2}$ | 2 500 | $Si^{+4}$ | 3 | [1]$Al^{+3}$ | 0.04 |
| $Mg^{+2}$ | 1 200 | $PO_4^{-3}$ | 1 | [1]$Zn^{+2}$ | 0.02 |
| $K^+$ | 370 | $Mn^{+3}$ | 1 | [1]$V^{+5}$ | 0.02 |
| $Ca^{+2}$ | 370 | $MoO_4^{-3}$ | 0.7 | [1]$Co^{+2}$ | 0.01 |
| $HCO_3^-$ | 140 | $S_2O_3^{-2}$ | 0.4 | [1]$Fe^{+3}$ | 0.01 |
| $H_3BO_3$ | 25 | $Li^+$ | 0.1 | [1]$Cu^{+2}$ | 0.003 |
| | | [1]$Rb^+$ | 0.1 | | |

[1] Present in trace-elements solution.

It is possible that the *Artemia* grown from sewage-*Tetraselmis* were, in fact, in a nutritionally marginal state and, therefore, more susceptible to the toxic effects of heavy metal ions. It is also possible that between the harder tap water and sewage the *Artemia* had a higher heavy metal burden in their bodies already. It has been reported by Linman *et al.* (1973) that heavy metal ions such as $Cd^{+2}$, $Pb^{+2}$, *etc.* are concentrated in sewage sludge.

For toxicity of fish to heavy metals there is a great disparity in results, depending on the species of fish used. Nevertheless, as shown in selected results from Table VIII, in most instances, for the metals studied, *Artemia* are considerably more resistant to heavy metals than are fish, particularly considering the disparity in weight.

Our brine shrimp were found to have an average weight of 2.0 mg dry weight (average weight of 10 adults brine shrimp, dried between filter papers and desiccated for 1 hr). If we assume for $Cu^{++}$, that the minnow studies has a 24 hr $LD_{50}$ essentially equal to that for *Artemia* and if the minnow has a weight of 10 g, then on a per-weight basis, the $Cu^{++}$ is 500 times more toxic to minnows than it is to *Artemia*. For the case of rainbow trout and $Hg^{++}$, the difference is considerably greater.

TABLE VIII

Comparison of 24 hr $LD_{50}$'s for *Artemia* (present study) and fish

| Metal | $LD_{50}$ *Artemia* 24 hr (mg/l) | $LD_{50}$ fish (in mg/l) |
|---|---|---|
| $Hg^{++}$ | 5.34 | 0.01 (rainbow trout-204 hr) Jones (1939) |
| $Pb^{++}$ | 207.2 | 0.33 (minnow, stickleback, brown trout) Carpenter (1927) |
| $Cu^{++}$ | 2.33 | 0.2 (blue gill 96 hr) Tarzwell and Henderson (1960) |
| | | 1.0 (minnow, brown trout 80 hr) Liepolt and Weber (1958) |
| $Zn^{++}$ | 63.2 | 0.3 (stickleback-204 hr) Jones (1939) |
| | | 100 (goldfish-120 hr) Ellis (1937) |
| | | 0.5 (rainbow trout-64 hr) Lloyd (1960) |

This large difference in heavy metal toxicity to fish and *Artemia* suggest a different mechanism of toxicity between the two species.

The theory of heavy metal toxicity to fish is explored by Erichsen Jones (1964) in which he discussed the "coagulation film anoxia" theory of Carpenter (1927). In fish exposed to heavy metals such as lead, zinc, and copper (and probably mercury and cadmium as well) the body and gills becomes covered with a veil-like film, appearing as coagulated mucus. The film has been shown to contain heavy metal. Death appears to be the result of asphyxiation brought on by the heavy metal ions reacting with some constituent of the mucus secreted by the gills. The mucus is albuminous in nature and has the function of reducing body friction in swimming and protecting the fish from the attacks of parasites and microorganisms. It also precipitates fine suspended matter, permitting a fish to exist in muddy water. Since heavy metals are known to bind on sulfhydryl groups of proteins such as serum albumin, and eventually precipitate them it is, not surprising that they precipitate the mucus film on fish. The gills on the fish are also damaged by the heavy metals.

Because of the substantially greater resistance of *Artemia* to the heavy metals, as compared with fish, one would suspect a different toxic mechanism. *Artemia* is a filter feeder (Gauld, 1959 ; Reeve, 1963). The animal is capable of regulating its feeding in such a way that as the food concentration increases, the filtration rate maintains a constant maximum value while the ingestion rate increases. When the concentration reaches a value at which a constant maximum ingestion rate is attained, the filtration rate falls off (Reeve, 1963). Such capability of control of filtration suggests an active transport mechanism. It is very likely that the heavy metals in question may combine with sulfhydryl groups on enzymes connected with the filtration system.

Other sites for attack are on the peleopods which would tend to immobilize the brine shrimp and possibly the external gills on the legs.

### TOXICITY OF OILS TO *ARTEMIA*

Of the five oils obtained from the EPA/DOE Fossil Fuels research material facility, the observed toxicity studies, while preliminary, showed the order of toxicity to be :

SRC-II fuel oil blend > H-coal distillate raw > petroleum-derived JP-5 product > shale-derived JP-5 product > crude shale oil. The first two – fuel oil blend and H-coal distillate raw – were clearly the most toxic. It is interesting that the shale-oil-derived materials, whether crude or processed, appear to be less toxic than petroleum or coal-derived materials. Clearly, many more samples will have to be tested to ensure that this observation is, indeed, correct. How much of the toxicity is due to the non-specific increase in biochemical oxygen demand and how much due to specific toxicants present in the oil mixtures in unclear at this point.

Price *et al.* (1974) have considered both acute brine shrimp bioassays on numerous organic chemicals as well as the % biodegradability of the substances in 20 days. They note that if a substance is held below the *Artemia* toxicity level and at the same time, it is rapidly biodegraded, the concern about its chronic effects on the eco-system should be negligible. *Artemia* represents an excellent organism for testing toxiciy of oils in view of the fact that most spills are in a saline environment and because of the advantages inherent to the use of *Artemia* as test organism.

### Toxicity of miscellaneous organics

While EDTA (0.01 M) is highly toxic to *Artemia*, the obtaining of a dose-response curve for this chelating agent as well as other chelating agents (penicillamine, BAL, *etc.*) would be of interest. Once a slightly toxic chelating agent were found it could be used to test efficacy for reduction of toxicity of particular heavy metal, providing that it was an efficacious chelator of it.

The lack of toxicity of the anti-schistosomal agent, N-butyl, N-2-chloroethylethanolamine acetate suggested that this was because its highly polar nature (positively charged nitrogen and negatively charged carbonyl in the acetate moiety) prelude its entering the filtration system of the *Artemia*.

## Summary

*Artemia* has been used for a bioassay and 24 hr $LD_{50}$'s determined for several heavy metals in Instant Ocean®: $Cu^{+2}$(2.33 mg/l) > $Hg^{+2}$(5.34 mg/l) > $As^{+3}$(32.1 mg/l) > $Zn^{+2}$(63.2 mg/l) > $Cd^{+2}$(68.8 mg/l) > $Pb^{+2}$(207.2 mg/l).

*Artemia* is considerably more resistant to the toxic manifestations of heavy metals than are fish. While the mechanism of toxicity, no doubt, involves -SH binding in both cases, in the case of fish the heavy metals probably bind with albuminoid molecules in the mucous secreted by the gills of the fish (Carpenter, 1927); for *Artemia*, the binding probably occurs in the filtering system or possibly on the peleopods or external gills. Five oils were tested for toxicity and the method clearly differentiated between the oils in terms of toxicity. A fuel oil blend and H-coal distillate were the most toxic with a shale-derived and crude shale oil being least toxic. Toxicity may be due to a non-specific increase in BOD or toxicants present or both.

The *Artemia* bioassay method is a most promising and simple technique for determining the toxicity of environmental pollutants.

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**Literature cited**

Carpenter K. E. 1927. The lethal action of soluble metallic salts on fishes. *Brit. J. Exp. Biol.* 4:378-90.

Ellis M. M. 1937. Detection and measurement of stream pollution. *Bull. US Bur. Fish* 48:365-437.

Erichsen Jones J. R. 1964. Fish and river pollution. Butterworths, London. 203 p.

Gauld D. T. 1959. Swimming and feeding in crustacean larvae : the nauplius larva. *Proc. Zool. Soc. London* 132(1):31-50.

Jones J. R. E. 1939. The relation between the electrolytic solution pressures of the metals and their toxicity to the stickleback (*Gastersteus aculeatus* L.). *J. Exp. Biol.* 16:425-437.

Liepolt R. and E. Weber. 1958. Die Giftwirkung von Kupfersulfat auf Wasserorganismen. *Wass. U. Abwass.* : 335-363.

Linman L. S., A. Anderson, and K. O. Nilsson *et al.* 1973. Cadmium uptake by wheat from sewage sludge used as a plant nutrient source. *Arch. Environ. Health* 27:45-47.

Lloyd R. 1960. The toxicity of zinc sulphate to rainbow trout. *Ann. Appl. Biol.* 48:84-94.

Miller L. C. and M. L. Tainter. 1944. Estimation of $ED_{50}$ and its error by means of logarithmic probit graph paper. *Proc. Soc. Exp. Biol. (NY)* 57:261-264.

Needham J. G., P. S. Galtsoff, F. E. Lutz, and P. S. Welch. 1937. Culture methods for invertebrate animals. Dover Publications, Inc., New York.

Okibo K. 1957. Study on the bio-assay method for the evaluation of water pollution. I. An attempt to use brine shrimp as a test animal. *Bull. Tokai Reg. Fisheries Res. Lab.* 18:31-36.

Price K. S., G. T. Waggy, and R. A. Conway. 1974. Brine shrimp bioassay and seawater BOD of petrochemicals. *Journal WPCF* 46(1):63-77.

Reed L. J. and H. Muench. 1938. Simple method of estimating 50 per cent endpoints. *Am. J. Hygiene* 27:493-497.

Reeve M. R. 1963. The filter-feeding of *Artemia* L. in pure cultures of plant cells. *J. Exptl. Biol.* 40(1):195-205.

Songer J. G., R. F. Smith, and N. M. Trieff. 1974. Sewage treatment by controlled eutrophication : bacterial study. *Applied Microbiology* 28(3):359-361.

Sorgeloos P. 1974. The influence of algal food preparation on its nutritional efficiency for *Artemia salina* L. larvae. Thallasia Yugoslavica 10(1,2):313-320.

Sorgeloos P. 1980. The use of the brine shrimp *Artemia* in aquaculture. p. 25-46. *In* : The brine shrimp *Artemia*, Vol. 3. Ecology, Culturing, Use in Aquaculture. Persoone G., P. Sorgeloos, O. Roels, and E. Jaspers (Eds). Universa Press, Wetteren, Belgium. 456 p.

Tarzwell C. M. and C. Henderson. 1960. Toxicity of less common metals to fishes. *Industr. Wastes* 5:12.

Trieff N. M., M. McShan, D. Grajcer, and M. Alam. 1973. Biological assay of *Gymnodinium breve* toxin using brine shrimp (*Artemia salina*). *Texas Rep. Biol. Med.* 31(3):409-422.

Trieff N. M. 1974. Toxicity of heavy metals on brine shrimp (*Artemia salina*). Presented at the 77th Annual Meeting of the Texas Academy of Science (March).

Vanhaecke P., G. Persoone, C. Claus, and P. Sorgeloos. 1980. Research on the development of a short term standard toxicity test with *Artemia* nauplii. p. 263-285. *In* : The brine shrimp *Artemia*. Vol. 1. Morphology, Genetics, Radiobiology, Toxicology. Persoone G., P. Sorgeloos, O. Roels, and E. Jaspers (Eds). Universa Press, Wetteren, Belgium. 345 p.

*The Brine Shrimp* Artemia. *1980. Vol. 1. Morphology, Genetics, Radiobiology, Toxicology*
*G. Persoone, P. Sorgeloos, O. Roels, and E. Jaspers (Eds). Universa Press, Wetteren, Belgium, 345 p.*

# Research on the development of a short term standard toxicity test with *Artemia* nauplii

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**Abstract**

Standardization of toxicity tests on aquatic organisms to detect the impact of chemicals on freshwater or marine ecosystems is an urgent necessity.

From the variety of methods, criteria, and test species proposed, simple standardized tests for algae, crustaceans, and fish are now close to being adopted at the international level for the freshwater environment. Despite the same urgency, very little has been achieved for the marine environment.

*Artemia* is an extremely suitable test-species since, contrary to all other organisms, it does not require continuous maintenance of stock cultures. Unfortunately none of the *Artemia* toxicity tests that have been worked out so far can be adopted as such as a representative standard test, because they attribute too little importance to parameters, which influence the results and the repeatability.

Starting from the pertinent literature information on the use of *Artemia* as a test species for toxicity studies and from literature data on the factors influencing the hatching and molting of brine shrimp, we studied the parameters of importance for a routine short-term toxicity test with an acceptable reproducibility, which are :

- selection of the instar stage of the nauplii
- determination of the test's duration
- sensitivity of early *versus* late hatching nauplii
- influence of the storage conditions of the cysts
- sensitivity of different geographical strains and batches of *Artemia*
- selection of a standard reference toxicant
- determination of the accuracy and repeatability of the test

## Introduction

The increasing concern of reducing the adverse effects of chemicals on the ecosystems has led to the development of a large number of ecotoxicological tests for the predictive assessment of the potential effects of pollutants on the biota.

In view of the regulatory measures which are now taken at the national as well as at the international level, it is obvious that standardization of the methods which will be witheld or recommended at the different levels of complexity of testing is highly desirable.

[3] "Bevoegdverklaard Navorser" at the Belgian National Foundation for Scientific Research (NFWO).

This is especially the case for routine tests, which must be as reproducible, reliable, and simple as possible. This automically means that the tests should not require sophisticated equipment nor specialized personnel.

Among the numerous species that have been used for toxicity studies in the marine environment (Wisely and Blick, 1967 ; Connor, 1972 ; Standard Methods, 1975 ; Stephan, 1975), the brine shrimp appears to be a very suitable candidate to develop a standard bioassay for worldwide utilization. Dry *Artemia* cysts are indeed available year-round and can be shipped very easily in small packages to any point of the world. This means that the test can be carried out wherever and whenever, starting from biological material stored "on the shelf". As a result the cumbersome and expensive maintenance of stock cultures is completely eliminated.

These advantages of *Artemia* have led to a marked increase of the use of this organism for bioassay purposes during the past two decades. Unfortunately, a study of the literature reveals, however, that there is no uniformity in the methodologies nor in the criteria used to assess the dose-effect relationship.

Departing from the urgent necessity of standardizing a few bioassay methodologies representative for the marine environment, and considering the advantages inherent to the use of *Artemia* as a suitable candidate, we studied a number of parameters of importance to develop a routine short term toxicity test with this particular species. A thorough review of the existing literature dealing with the use of *Artemia* in toxicity testing was at the basis of our studies.

## Literature review

The dose-effect criteria used to assess toxicity on brine shrimp can be classified in three categories according to the life-stage of the test-species :

a) cysts
b) nauplii
c) adults

### The hatching process as criterion

As far as we know, Delcambe (1955) was the first scientist who used the hatching success as a bioassay criterion. His method has been followed by that of Pham-Huu-Chanh and Mamy (1963). De Brabander and Vandeputte (1967) took several criteria into consideration : the percentage of unhatched cysts, that of the cysts in the breaking stage, and the percentage of free-swimming nauplii. Jensen (1975) also proposed the use of the hatching rate as a standard bioassay technique. His method, slightly modified, has been used by Jörgensen and Jensen (1977, 1978). Finally, Saliba and Krzyz (1976a) studied the influence of heavy metals on the hatching of *Artemia* cysts.

An intercomparison of the dose-effect relationship obtained by different authors using the hatchability criterion is hardly possible. Indeed, the hatching conditions used varied widely. Temperature for example varied from 19 up to 28 °C and the duration of the test from 48 hr up to 5 days. Moreover, in most cases abiotic factors such as pH and oxygen levels of the

incubation medium were either not taken into consideration or at least not described properly. Jennings and Whitaker (1941), Nimura (1968), Jones (1972), and Metalli and Ballardin (1972) have nevertheless demonstrated that pH should not be lower than 7 in order to obtain a good hatching ; the use of unbuffered NaCl solutions may lead to a hatching efficiency which can be very low even in the controls. The toxicity of products causing a pH drop is thus overestimated. An adequate aeration of the medium is another prerequisite to obtain successful hatching, a fact underlined by Nimura (1968), Von Hentig (1971), and Sorgeloos (1975).

The major restriction, however, to use hatching as a standard bioassay criterion is the variability of the hatching efficiency and the hatching rate among different geographical strains of *Artemia* (D'Agostino, 1965 ; Sorgeloos and Persoone, 1975 ; Person-Le Ruyet and Salaun, 1977 ; Smith *et al.*, 1978) and among different batches of a specific strain (Smith *et al.*, 1978) ; these factors are also influenced by the storage conditions of the cysts (Sorgeloos, 1979).

## The use of nauplii for toxicity studies

Since the publication of Corner and Sparrow (1956), *Artemia* nauplii have become a popular test object to study the toxicity of a wide array of chemical compounds : pesticides (Tarpley, 1958 ; Ludemann and Neumann, 1961 ; Springer and Legge, 1966 ; Caserio *et al.*, 1970 ; Nelson and Matsumura, 1975a ; Claus, 1976), petrochemicals and oil dispersants (Tarzwell, 1969 ; Anonymous 1973 ; Zillioux *et al.*, 1973 ; Price *et al.*, 1974), heavy metal salts (Corner and Sparrow, 1957 ; Corner and Rigler, 1958 ; Herald and Dempster, 1965 ; Wisely and Blick, 1967 ; Brown and Ahsanullah, 1971 ; Saliba and Ahsanullah, 1973 ; Cox, 1974), mycotoxins and microbial and fungal metabolites (Brown *et al.*, 1968 ; Harwig and Scott, 1971 ; De Waart *et al.*, 1972 ; Reiss, 1972 ; Durackova *et al.*, 1977), carcinogenic products (Morgan and Warshawsky, 1977) etc.

The criteria which have been taken into consideration for the dose-effect relationship differ widely. In most cases the life-death criterion, which is the most easy to detect, has been used. Some authors, however, preconize more sensitive criteria such as immobilization (Robinson *et al.*, 1965 ; Morgan and Warshawsky, 1977), osmoregulation (Schmidt-Nielsen, 1974) and oxygen consumption (Hood *et al.*, 1960). Others studied the larval growth in chronic toxicity tests (Brown and Ahsanullah, 1971 ; Saliba and Ahsanullah, 1973 ; Benijts and Versichele, 1975 ; Saliba and Krzyz, 1976b).

In most cases very little emphasis was given nor attention paid to the hatching conditions. In Cox (1974), Curtis *et al.* (1974), Granade *et al.* (1976) and Hudson and Bagshaw (1978) no details can be found on the hatching conditions. Several authors are very vague in indicating the hatching temperature, using terms such as "about" or "approximately", whereas precisely this factor influences, to a very large extent, both the hatching rate of the cysts and the molting rate of the nauplii (Sorgeloos *et al.*, 1978).

The importance of starting the tests with nauplii of exactly the same age has already been pointed out by Tarzwell (1969). With regard to this, Sorgeloos (1975), Claus (1976), and Sorgeloos *et al.* (1978) demonstrated that instar II larvae are more sensitive than instar I larvae. As a result, the data obtained by all those who do not follow a very strict procedure with regard to the hatching conditions (and thus do not know the exact age of the nauplii at the start of the tests) can fluctuate from one experiment to another.

Sorgeloos *et al.* (1978) did not observe significant differences in sensitivity between instar II and instar III nauplii and Tarpley (1958) found no significant differences among the instar III – V – VII – IX and XI stages.

As pointed out above, another factor responsible for differences in dose-effect results is the geographical origin of the cysts. Sorgeloos *et al.* (1978) found different tolerance ranges for several environmental factors between some geographical *Artemia* populations. Already in 1969, Tarzwell followed by Zillioux *et al.* (1973) emphasized the importance of the selection of one specific *Artemia* strain for bioassays.

### The use of adults for toxicity studies

Results of bioassays made on adult brine shrimp have been published by many scientists : Shackell (1925), Michael *et al.* (1956), Tarpley (1958), Grosch (1966, 1967, 1970, 1973), Hallopeau (1969), Brown and Ahsanullah (1971), Knauf and Schulze (1973), Trieff *et al.* (1973), Cunningham (1976a,b), Knöfel (1976), and Saliba and Krzyz (1976b).

The following dose-effect criteria have been taken into consideration by these authors : death, life span, fecundity, reproduction capacity, survival of zygotes, and accumulation of the toxicant.

Adult *Artemia* are, however, less frequently used for toxicity studies than nauplii. Because the use of adults implies the culturing of the organisms, implying technological and biological difficulties. As a consequence, adults are less suited for short term standard toxicity tests than nauplii not the least from the economical point of view. Moreover Tarpley (1958), Brown and Ahsanullah (1971) and Saliba and Krzyz (1976b) found that the adults are less sensitive than the nauplii. Literature data revealed, however, that adult *Artemia* are very well suited for long term chronic bioassays.

### Synthesis and conclusions

From the literature information cited above and data published on the sensitivity of *Artemia* as compared to other aquatic organisms (Doudoroff and Katz, 1953 ; Corner and Sparrow, 1956 ; Sanders and Cope, 1966 ; Wisely and Blick, 1967 ; Connor, 1972 ; Knauf and Schulze, 1973 ; Jung, 1975 ; and Claus, 1976) we made the following deductions with regard to the use of *Artemia* as a potential candidate species for a short-term standard bioassay :

1. To date *Artemia* is the only animal species available which, because of the commercial availability of cysts, can be used for bio-assays at any place in the world and at any moment without the necessity of maintenance of stocks.
2. *Artemia* is a suitable test species to evaluate the relative toxicity of a broad range of chemical compounds.
3. *Artemia* does not belong to the most sensitive species of marine organisms.
4. Hatchability of the cysts does not seem to be a very good criterion.
5. The early larval stages of *Artemia*, which can survive for a few days without feeding, are most suited for acute toxicity tests.
6. The sensitivity varies with the geographical origin and the age of the animals :
   - adults are less sensitive than nauplii
   - the first instar stage is less sensitive than the later instars (which all have approximately the same sensitivity).

7. In order to optimize the reproducibility of the results, the larvae should be hatched out under strictly controlled conditions of temperature, salinity, aeration, light, and pH.
8. The larvae must be of exactly the same age at the start of every experiment.

**Research**

Based on the premises outlined above we have endavored to work out a standardized test-procedure based on the use of *Artemia* nauplii.

The major biological, technological, and physicochemical parameters which could exert an influence on the variability of the results have been checked out one by one in different series of experiments.

Each time a choice was made and a decision taken at the end, to determine a precise experimental condition to be strictly observed or a procedure to be followed in order to obtain a maximal reproducibility in the final standard procedure.

BASIC METHODOLOGY

The following technical procedure for which we finally settled is the result of a lot of trial and error experimentation based to a considerable extent on the experience existing at the Artemia Reference Center with regard to hatching and culturing of brine shrimp.

*Hatching of the cysts and collection of the nauplii*

The cysts were always incubated under identical, strictly controlled conditions. For each test, 100 mg of cysts were put in 100 ml artificial seawater (35 ‰) in a cylindroconical tube at a temperature of 25 ± 1 °C. The seawater was prepared according to the formula of Dietrich and Kalle (in Kinne, 1971); after filtration (0.2 $\mu$m) and aeration, the pH was 7.5.

The cysts and hatching nauplii were kept in suspension by a gentle aeration from the bottom of the tube. Since light intensity has an influence on the hatching rate (Vanhaecke *et al.*, in preparation) the hatching tubes were placed nearby a light source (intensity of 1 000 lux).

To harvest the hatched nauplii, the aeration was stopped and the nauplii, which concentrate at the bottom of the tube, were sucked out by pipeting and transferred to a vial containing fresh seawater.

*Preparation of the tests*

All the experiments were carried out in glass petri dishes (diameter 60 mm ; height 12 mm). Petri dishes indeed proved to be very handy to check the mortality under a dissection microscope. The nauplii were transferred to the petri dishes with a Pasteur pipet which carried over less than 0.05 ml seawater into the dishes. The dishes were then filled with 10 ml of the respective concentrations of toxicant in seawater. Then they were closed and placed in darkness in an incubation chamber (temperature of 25 ± 1 °C) for the respective test periods. The animals were not fed during the bioassays. The concentrations of toxicant to be tested were chosen from the logarithmic scale of Doudoroff *et al.* (1951).

Preliminary trials revealed that bioassays in triplicate with 10 nauplii per petri dish appeared to be a good compromise between the precision of the test and economic imperatives such as labor and time.

At the end of the experimental period the number of dead larvae was checked in each petri dish. The nauplii were considered dead when no moving of the appendages was observed within a few seconds.

The LC50, the 95% confidence limits, and the slope function were calculated following Litchfield and Wilcoxon (1949). We also used the method developed by these authors for the statistical comparison of the LC50 values obtained. Mean values of replicates were compared at the aid of a Student's t-test.

## Experiments

### *Selection of the instar stage of the nauplii and determination of the duration of the test*

From the literature review outlined above it is clear that the nauplii must have exactly the same age at the start of every experiment because there is a marked difference in sensitivity between instar I and instar II-III larvae. From the practical point of view of handling and reproducibility it is, however, not known which stage of development is the most suited for short-term standard toxicity tests. The only information found in this regard was that by Wisely and Blick (1967), who noted that the LC50 values obtained are relatively more constant using older larvae (20-80 hr).

We therefore decided to compare both stages of development from the viewpoint of sensitivity and of practicality.

*Artemia* cysts from the San Francisco Bay Brand Company (batch number 1628) were incubated under the conditions outlined above. In order to obtain a population consisting of first instar nauplii only, the hatched larvae were harvested after 18 hr (the first larvae appeared 14 hr after the start of the incubation). One half of the population was used immediately for the test on instar I larvae. The other half of the population was incubated at 25 °C in an Erlenmeyer in gently aerated seawater ; regular checks of the morphological stage of development showed that after 24 hr, more than 99% of the nauplii had molted into the instar II or even instar III stage. The larvae were then collected to carry out bioassays on *Artemia* nauplii which are in the instar II-III stage.

With regard to the test period we settled in both series for 6 hr and 24 hr. The 6 hr period was selected because during that period of time instar I larvae do not yet molt.

Sodium lauryl sulphate was chosen as toxicant. After determination of the critical range in a preliminary experiment, the following concentrations were selected for further experimentation :

- instar I larvae : 10 – 13.5 – 18 – 24 – 32 – 42 – 56 – 75 – 87 – 100 mg/l ;
- instar II larvae : 5.6 – 10 – 13.5 – 18 – 24 – 32 – 42 – 56 – 75 – 100 mg/l.

All bioassays were carried out in eight replicates.

In Table I the means of the LC50 values, their 95% confidence limits, and the slope function (S) are summarized. This Table also gives the standard deviation and the coefficient of variation of the LC50 values and the standard deviation of the slope function. The mortality curves computed from the mean LC50 values and the mean S values of the replicates are represented graphically in Fig. 1.

TABLE I

Mean LC 50 values, (with standard deviation and variation coefficient), slope function (with standard deviation), and 95% confidence limits of the LC 50 data obtained with instar I, respectively instar II-III larvae for 6 hr and 24 hr exposure to sodium lauryl sulphate

| | Instar I | | Instar II-III | |
|---|---|---|---|---|
| | 6 hr | 24 hr | 6 hr | 24 hr |
| Mean LC50 (ppm) | 77.4 | 33.9 | 44.2 | 17.8 |
| Standard deviation (ppm) | 5.9 | 5.2 | 4.4 | 0.9 |
| Variation coefficient (%) | 7.7 | 15.3 | 10.0 | 5.1 |
| Slope function (S) | 1.16 | 1.23 | 1.31 | 1.35 |
| Standard deviation | 0.05 | 0.04 | 0.05 | 0.06 |
| 95% confidence limits of LC50's (ppm) | 73.6-81.8 | 30.8-37.4 | 39.9-48.9 | 15.9-20.0 |

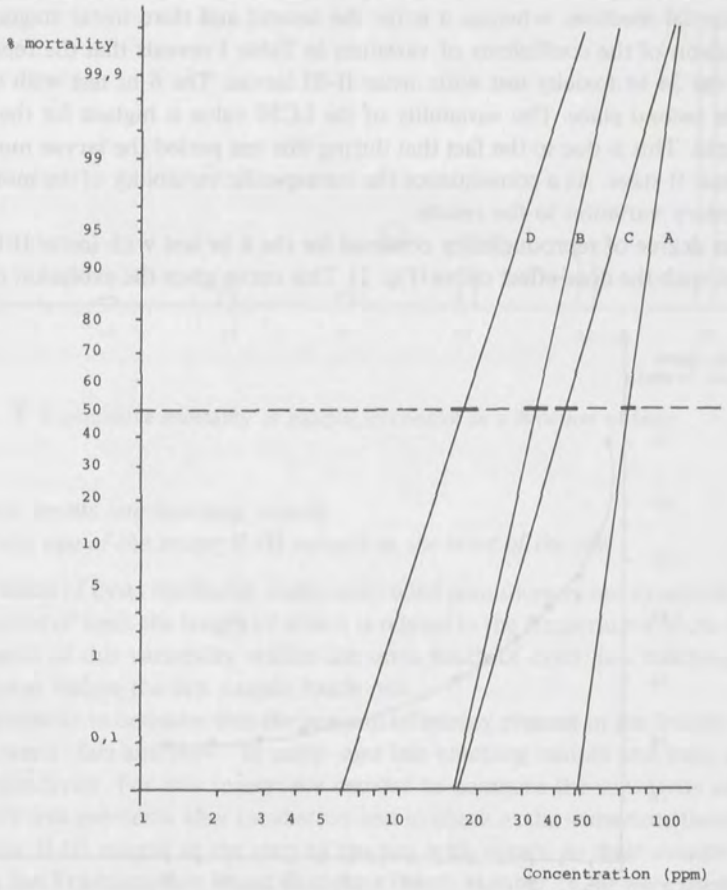


FIG. 1. Mortality curves calculated from the mean LC50's (6 hr and 24 hr) and their slope function, for instar I and instar II and III larvae respectively. The 95% confidence limits of the mean LC50's are also shown. (A) instar I larvae – 6 hr test ; (B) instar I larvae – 24 hr test ; (C) instar II-III larvae – 6 hr test ; (D) instar II-III larvae – 24 hr test.

From the results it is clear that instar II-III larvae are significantly more sensitive than instar I larvae ; the ratios are 1.75 for 6 hr incubation and 1.90 for 24 hr respectively. These results confirm the findings of Wisely and Blick (1967), Claus (1976) and Sorgeloos *et al.* (1978).

In both cases, the value of the slope function for instar I larvae appears to be significantly different (at the $P < 0.01$ level) from the S values obtained for instar II-III larvae. The slope function for the 6 hr test with instar I nauplii also differs significantly from the value for the 24 hr test with these larvae. Since the S value varies with the mode of action of the toxicant (Bliss, 1957), the differences observed reveal that this mode of action is not the same for the instar I and instar II-III larvae. The intermediate value obtained for the 24 hr tests on instar I nauplii can be explained by the fact that the nauplii molted into the instar II stage during the test. An explanation for these findings can be found in the work of Sorgeloos *et al.* (1978) ; these authors impute the difference in sensitivity between instar I and instar II-III nauplii to the fact that in freshly hatched nauplii the epithelium of the digestive tract is not in contact with the external medium, whereas it is for the second and third instar stages.

A comparison of the coefficients of variation in Table I reveals that the reproducibility is highest for the 24 hr toxicity test with instar II-III larvae. The 6 hr test with instar I larvae comes in the second place. The variability of the LC50 value is highest for the 24 hr test on instar I nauplii. This is due to the fact that during this test period the larvae molt to the more sensitive instar II stage. As a consequence the intraspecific variability of the molting rate adds a supplementary variation to the results.

The lower degree of reproducibility obtained for the 6 hr test with instar II-III nauplii can be explained with the dose-effect curve (Fig. 2). This curve gives the evolution of the LC50 in

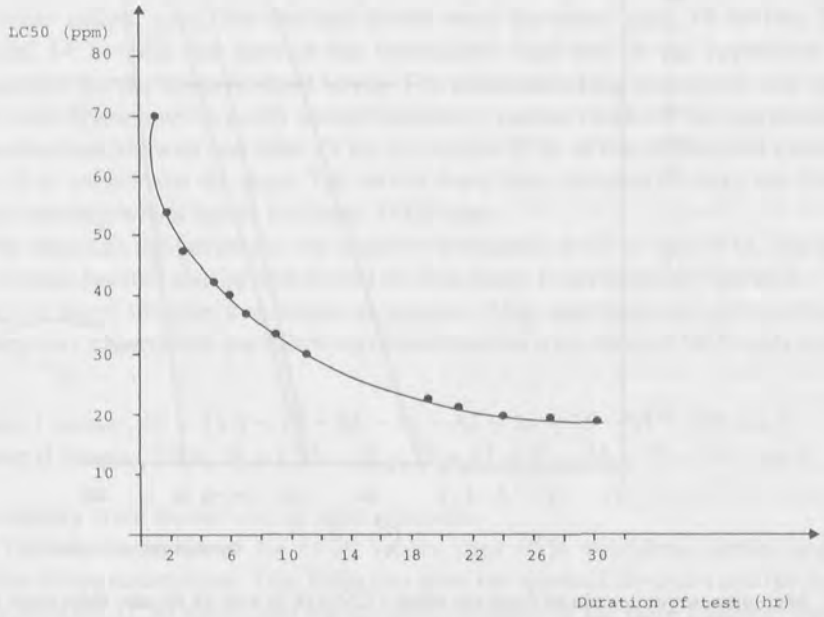


FIG. 2. Dose-effect curve for bioassays on instar II-III nauplii of *Artemia* with sodium lauryl sulphate as toxicant.

function of the duration of the test (Sprague, 1969). From Fig. 2 it is clear that after 6 hr the horizontal part of the dose-effect curve is not yet reached. As a result the 6 hr LC50 values are liable to larger fluctuations than the 24 hr LC50 values.

Prolongation of the test period beyond 24 hr does not make sense because the unfed test animals start to die in the controls. In Fig. 3 the procentual mortality of the nauplii in the controls is given in function of the length of the test period. From this figure it is clear that the mortality is already 36 % 72 hr after the harvest of the nauplii. Therefore it is impossible to carry out 48 hr toxicity tests with instar II-III nauplii, except by complicating the test by feeding the animals.

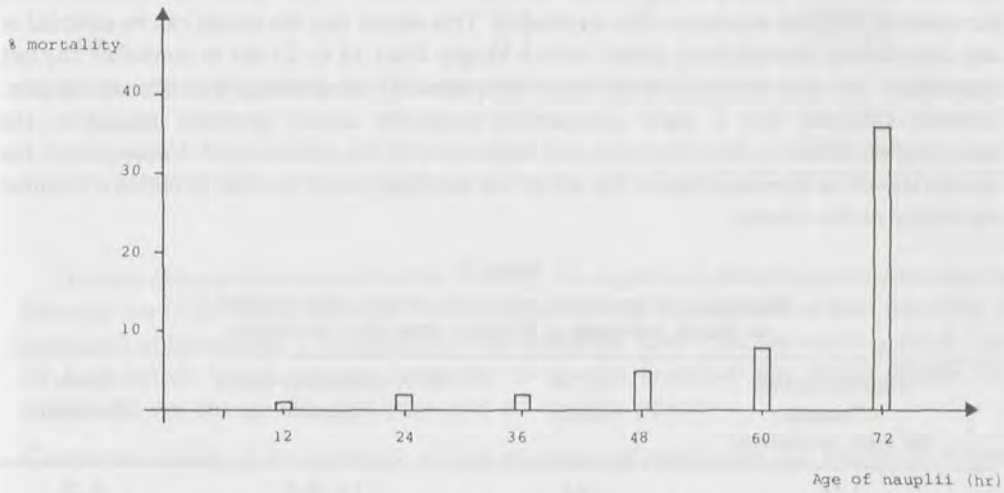


FIG. 3. Cumulative mortality of nauplii in control as a function of time.

*Sensitivity of early* versus *late hatching nauplii*
*and influence of the age of the instar II-III nauplii at the start of the test*

Hatching of a batch of cysts incubated under controlled conditions is not synchronous, but extends over a period of time, the length of which is related to the temperature of the hatching medium. As a result of this variability within the same batch of cysts, fast hatching nauplii appear several hours before the last nauplii hatch out.

It is therefore possible to consider that the amount of energy present in the freshly hatched larvae differs between "fast and slow" or early- and late-hatching nauplii and may influence their respective sensitivity. For this reason we decided to compare the sensitivity of nauplii hatched out at different moments after incubation and to check at the same time the influence of the age of instar II-III nauplii at the start of the test with regard to their sensitivity.

Cysts from the San Francisco Bay Brand Company (batch number 1628) were used. Sixteen hours after incubation, the hatched nauplii were separated from the unhatched cysts with a separator box (Persoone and Sorgeloos, 1972). At that moment about one third of the cysts had hatched. Two hours later a part of the embryo's in the umbrella stage were sampled and again 2 hr later the nauplii hatched out of these embryo's were collected. Twenty two hours

after incubation, when almost all cysts had hatched, one third of the remaining naupliar population was removed from the hatching tube. The last two samples of nauplii were collected 26 and 30 hr after incubation respectively.

The nauplii sampled at different moments were all incubated for exactly 24 hr at 25 °C, after which bioassays were started with sodium lauryl sulphate as toxicant. The experimental test period was 24 hr. The concentrations tested out were : 5.6 – 10 – 13.5 – 18 – 24 – 32 – 42 mg/l.

From the results (Table II) it is clear that there is no difference in sensitivity between larvae that hatch out first and those that hatch out later. A statistical comparison of the data furthermore revealed that there is no significant difference (at the $P < 0.05$ level) between the larvae harvested at different moments after incubation. This means that the larvae can be sampled at any time during the hatching period (which ranges from 14 to 24 hr) to constitute the test population. The data obtained for the larvae harvested 26, respectively 30 hr after incubation, however, indicate that a slight increase in sensitivity occurs probably related to the consumption of energy (for swimming and respiration) of the unfed nauplii. Consequently the nauplii should be harvested before the end of the hatching period in order to obtain a minimal variability of the results.

TABLE II

Results of 24 hr toxicity tests with sodium lauryl suphate on nauplii harvested at different times after incubation

| Time of harvest of nauplii (hr after incubation) | LC50 (ppm) | 95% confidence limits (ppm) | Slope function |
|---|---|---|---|
| 16 | 19.0 | 17.6-20.6 | 1.29 |
| 20 | 19.0 | 17.6-20.5 | 1.27 |
| 22 | 18.8 | 17.2-20.5 | 1.33 |
| 26 | 17.5 | 16.3-18.8 | 1.18 |
| 30 | 17.4 | 15.9-19.0 | 1.20 |

*Influence of the storage conditions of the cysts*

Rakowicz (1975) noted that the viability of *Artemia* cysts decreases upon storage under humid conditions and Sorgeloos (1979) demonstrated that the hatching efficiency varies with different storage conditions. These findings indicate that biochemical changes within the embryo can occur depending upon specific storage conditions.

In order to find out whether the sensitivity of the nauplii is influenced by storage time and storage conditions of the cysts, toxicity tests were performed on instar II-III nauplii hatched out from cysts stored under air, oxygen, vacuum, nitrogen, and in brine respectively. The cysts originated from salt ponds in the San Francisco Bay and the San Pablo Bay (San Francisco Bay Brand Company, batch numbers 288-2596 and 1628 respectively).

In each case the nauplii were harvested 20 hr after incubation ; 24 hr later the bioassays were started. Sodium lauryl sulphate was used as toxicant ; the test duration was 24 hr.

TABLE III

Influence of storage period and storage conditions on sensivity of *Artemia* nauplii (24 hr-LC50 with sodium lauryl sulphate)

| Storage conditions | LC50 (ppm) | 95% confidence limits (ppm) |
|---|---|---|
| a) San Francisco Bay strain (batch 288-2596) storage period : 1 year | | |
| Vacuum | 18.5 | 16.8-20.4 |
| Air | 20.0 | 18.3-21.9 |
| $O_2$ | 20.4 | 18.7-22.2 |
| $N_2$ | 19.5 | 18.2-20.9 |
| Brine | 18.7 | 17.1-20.4 |
| b) San Pablo Bay strain (batch 1628) storage period : 6 months | | |
| Vacuum | 17.0 | 15.4-18.8 |
| Air | 16.5 | 14.9-18.3 |

The data obtained are summarized in Table III. No significant differences could be observed between the LC50 values obtained. This indicates that the storage method does not affect the sensitivity of the nauplii. A comparison of the data from Table I and the results given in Table III furthermore reveals that the sensitivity of the San Francisco Bay Brand sample 1628 sample did not change due to storage over a 6 months period.

*Comparative study of the sensitivity of some geographical populations and batches of* Artemia

The following geographical populations and batches were studied :

- San Francisco Bay (San Francisco Bay Brand Company), USA
  - batch number 288-2596
  - batch number 288-2606
- San Pablo Bay (San Francisco Bay Brand Company – batch number 1628), USA
- Great Salt Lake (Sanders Brine Shrimp Company), USA
  - harvest 1966
  - harvest 1977
- Macau (Cirne Brand), Brazil
  - harvest May 1978
  - batch number 87500
  - batch number 871172
- Shark Bay (World Ocean – batch number 114), Australia
- Buenos Aires (Aquarium Products – harvest 1977), Argentina
- Margherita di Savoia (harvest 1977), Italy.

All samples were incubated under the standard conditions outlined above (see basic methodology). Since the various populations and batches have different hatching rates, the freshly-hatched nauplii were separated from the unhatched cysts and hatching debris when a 50% hatching success was reached.

The nauplii were then incubated for 24 hr at 25 °C. After this period, in all the populations studied, more than 99% of the larvae had molted to the instar II-III stage except the Shark Bay strain, which still contained 4-5% instar I nauplii.

Sodium lauryl sulphate and potassium dichromate were used as toxicants. The concentrations tested out were 5.6 – 10 – 13.5 – 18 – 24 – 32 – 42 – 56 mg/l for sodium lauryl sulphate and 5.6 – 10 – 18 – 32 – 56 – 100 ppm for potassium dichromate.

The results of the 24 hr toxicity tests are summarized in Tables IV and V. Fig. 4 gives the statistical comparison (at the $P < 0.05$ level) of the LC50 values obtained ; from the data it is clear that there are significant differences between the geographical strains studied.

TABLE IV

24 hr-LC50 with 95% confidence limits and slope function of instar II-III larvae from different geographical strains or batches of *Artemia*, with sodium lauryl sulphate as toxicant

| Origin of sample | LC50 (ppm) | 95% confidence limits (ppm) | Slope function |
|---|---|---|---|
| San Pablo Bay 1628 | 16.0 | 14.9-17.2 | 1.27 |
| San Pablo Bay 1628 | 16.7 | 15.6-17.9 | 1.25 |
| San Francisco Bay 288-2596 | 16.5 | 15.4-17.7 | 1.26 |
| San Francisco Bay 288-2606 | 15.9 | 14.4-17.6 | 1.26 |
| Macau May 1978 | 14.8 | 13.7-16.0 | 1.29 |
| Macau 87500 | 15.5 | 14.0-17.1 | 1.38 |
| Macau 871172 | 15.8 | 14.1-17.7 | 1.45 |
| Shark Bay | 21.2 | 19.7-22.8 | 1.18 |
| Buenos Aires | (13.0)* | | |
| Margherita di Savoia | 22.7 | 20.0-25.7 | 1.50 |
| Great Salt Lake 1966 | 24.8 | 23.1-26.7 | 1.18 |
| Great Salt Lake 1977 | 23.8 | 22.7-24.9 | 1.11 |

* Mortality in control : 30%.

TABLE V

24 hr-LC50 with 95% confidence limits and slope function of instar II-III larvae from different geographical strains or batches of *Artemia* with potassium dichromate as toxicant

| Origin of sample | LC50 (ppm) | 95% confidence limits (ppm) | Slope function |
|---|---|---|---|
| San Pablo Bay 1628 | 28 | 23.5-33.4 | 1.50 |
| San Pablo Bay 1628 | 29 | 24.3-34.5 | 1.66 |
| San Francisco Bay 288-2596 | 41 | 36.3-46.3 | 1.48 |
| San Francisco Bay 288-2606 | 40 | 35.1-45.6 | 1.53 |
| Macau May 1978 | 46 | 38.9-54.4 | 1.60 |
| Macau 87500 | 48 | 40.8-56.5 | 1.45 |
| Macau 871172 | 45 | 38.5-52.6 | 1.43 |
| Shark Bay | 64 | 56.7-72.3 | 1.32 |
| Margherita di Savoia | 58 | 48.3-69.7 | 1.52 |
| Great Salt Lake 1966 | 35 | 30.3-40.4 | 1.39 |
| Great Salt Lake 1977 | 32.5 | 27.4-38.6 | 1.48 |

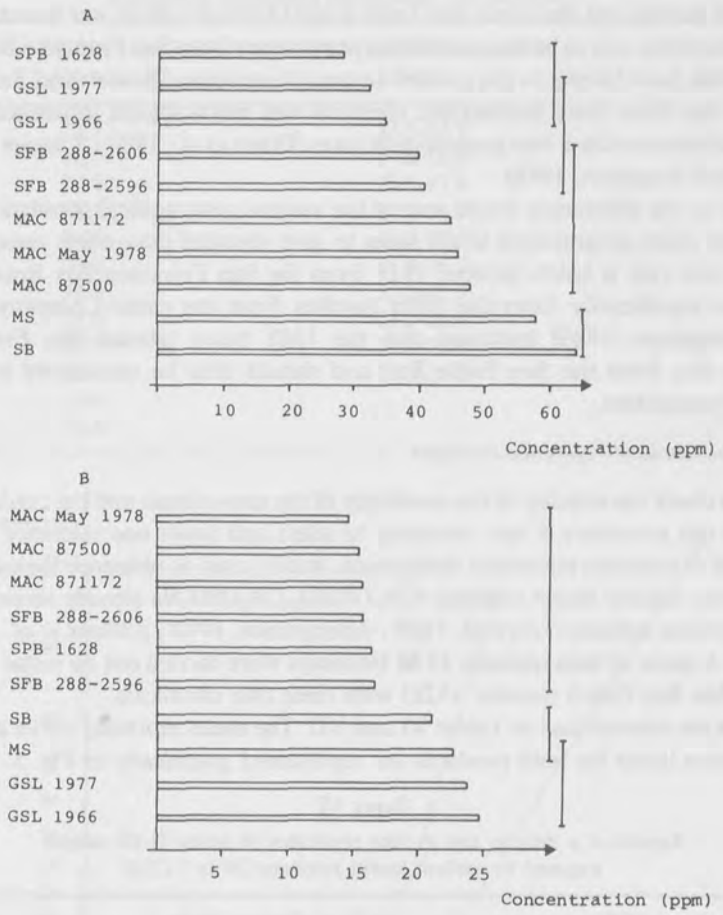


FIG. 4. 24 hr LC50 values of instar II-III larvae of different strains or batches of *Artemia* for potassium dichromate (A) and sodium lauryl sulphate (B), and statistical analysis of differences in sensitivity ($P < 0.05$ level). Any strain or batch connected by the same line was not statistically different. SFB = San Francisco Bay ; SPB = San Pablo Bay ; MAC = Macau ; MS = Margherita di Savoia ; SB = Shark Bay ; GSL = Great Salt Lake.

We have to be aware of the fact, however, that the strain with the lowest LC50 value is not necessarily the most sensitive one. Sorgeloos *et al.* (1976) and Claus *et al.* (1977) indeed indicated that the temperature-salinity tolerances vary between geographical populations. As a consequence, the LC50 values obtained are only relevant for the specific test conditions used here. The temperature-salinity combination defined and adopted earlier when working exclusively with the San Francisco Bay strain is for instance not suited for the race from Buenos Aires, for which a 30% mortality was noted in the control !

The data obtained also show that the sequence of sensitivity of different strains varies according to the product tested. This shifting points to physiological differences between these populations. It is known for example that there is an important genetic difference between the

Margherita di Savoia and the Great Salt Lake strain (Abreu-Grobois and Beardmore, 1980). This seems, however, not to be the case for the populations from San Francisco Bay and Great Salt Lake which both belong to the species *Artemia franciscana* (Bowen and Sterling, 1978), although on the other hand biometrical, chemical and physiological differences have been demonstrated between these two geographical races (Olney *et al.*, 1980 ; Schauer *et al.*, 1980 ; Vanhaecke and Sorgeloos, 1980).

In contrast to the differences found among the various geographical populations, different batches of the same geographical strain seem to give identical dose-effect results. The only exception to this rule is batch number 1628 from the San Francisco Bay Brand Company which differs significantly from the other batches from the same Company studied for $K_2 Cr_2 O_7$. Sorgeloos (1980) indicated that the 1628 batch labeled San Francisco Bay, originates in fact from the San Pablo Bay and should thus be considered as a different geographical population.

*Selection of a standard reference toxicant*

In order to check the stability of the sensitivity of the test-animals and the conformity of the experimental test procedure it was necessary to select and adopt one reference toxicant.

We decided to compare potassium dichromate, widely used as reference toxicant in aquatic toxicology with sodium lauryl sulphate $(CH_3)(CH_2)_{10}CH_2OSO_3Na$ already recommended for *Artemia* by several authors (Tarzwell, 1969 ; Anonymous, 1973 ; Zillioux *et al.*, 1973 ; Price *et al.*, 1974). A series of nine replicate 24 hr bioassays were carried out on instar II-III nauplii from San Pablo Bay (batch number 1628) with these two chemicals.

The results are summarized in Tables VI and VII. The mean mortality curve and the mean 95% confidence limits for both products are represented graphically in Fig. 5.

TABLE VI

Results of a toxicity test in nine replicates on instar II-III nauplii exposed to sodium lauryl sulphate (24 hr LC50)

| Statistics | LC50 (ppm) | 95% confidence limits (ppm) | Slope function |
|---|---|---|---|
| | 15.8 | 14.5-17.2 | 1.32 |
| | 15.5 | 14.3-16.8 | 1.29 |
| | 18.5 | 17.1-20.1 | 1.30 |
| | 18.4 | 16.7-20.3 | 1.47 |
| | 18.1 | 16.7-19.6 | 1.38 |
| | 15.6 | 14.3-17.0 | 1.33 |
| | 19.0 | 17.7-20.4 | 1.26 |
| | 18.8 | 17.2-20.5 | 1.33 |
| | 17.0 | 15.6-18.5 | 1.32 |
| $\bar{X}$ | 17.4 | 16.0-18.9 | 1.33 |
| s | 1.45 | | 0.06 |
| %s | 8.3 | | 4.6 |

From the LC50 values it is clear that *Artemia* larvae are more sensitive to sodium lauryl sulphate than to potassium dichromate. A comparison of the two slope functions furthermore

TABLE VII

Results of a toxicity test in nine replicates on instar II-III nauplii exposed to potassium dichromate (24 hr-LC50)

| Statistics | LC50 (ppm) | 96% confidence limits (ppm) | Slope function |
|---|---|---|---|
| | 27.9 | 23.5-33.1 | 1.73 |
| | 30.0 | 24.6-36.5 | 1.89 |
| | 36.0 | 29.6-43.8 | 2.18 |
| | 28.1 | 23.1-34.2 | 1.88 |
| | 34.5 | 29.5-40.3 | 1.65 |
| | 33.0 | 27.8-39.2 | 1.97 |
| | 39.0 | 33.2-45.9 | 1.69 |
| | 28.0 | 23.5-33.4 | 1.50 |
| | 31.0 | 26.0-36.9 | 1.76 |
| $\bar{X}$ | 31.9 | 26.6-28.3 | 1.81 |
| s | 3.96 | | 0.20 |
| %s | 12.4 | | 11.0 |

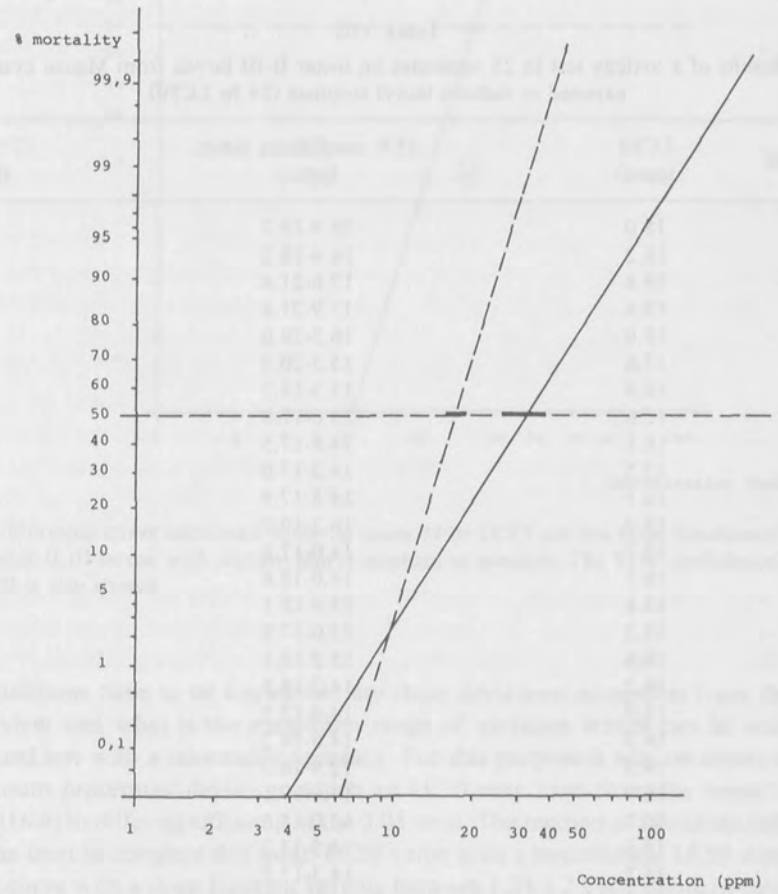


FIG. 5. Mortality curves calculated from the mean 24 hr LC50's and their slope function for instar II-III larvae, exposed to sodium lauryl sulphate (– –) and potassium dichromate (—). The 95% confidence limits of the mean LC50's are also shown.

indicates a different mode of action for both products. Sodium lauryl sulphate acts within a narrower range of concentrations. As a result the 95% confidence interval with this product is closer to the LC50 (± 9%) than it is for $K_2 Cr_2 O_7$ (± 20%). Moreover, a comparison of the variation coëfficients shows that the reproducibility of the bioassays is much better with sodium lauryl sulphate. On the basis of these results we decided to advise sodium lauryl sulphate as reference toxicant.

*Determination of the accuracy and repeatability of the test ; establishment of the acceptable limits of the LC50 range for sodium lauryl sulphate*

A 24 hr toxicity test has been carried out in 25 replicates on instar II-III larvae from the geographical population of Macau (harvest May 1978). For each replicate a new cyst sample was incubated and a freshly made solution of sodium lauryl sulphate used. The following concentrations of the reference toxicant were tested : 10 – 13.5 – 18 – 24 – 32 mg/l.

The LC50 values of the 25 replicates, the 95% confidence limits and the S values are summarized in Table VIII. The mean mortality curve is represented in Fig. 6. The 95%

TABLE VIII

Results of a toxicity test in 25 replicates on instar II-III larvae from Macau cysts exposed to sodium lauryl sulphate (24 hr LC50)

| Statistics | LC50 (ppm) | 95% confidence limits (ppm) | Slope function |
|---|---|---|---|
| | 18.0 | 16.4-19.7 | 1.37 |
| | 16.2 | 14.4-18.3 | 1.40 |
| | 19.4 | 17.6-21.4 | 1.32 |
| | 19.6 | 17.9-21.4 | 1.28 |
| | 18.0 | 16.2-20.0 | 1.43 |
| | 17.6 | 15.3-20.3 | 1.35 |
| | 16.9 | 15.3-18.7 | 1.33 |
| | 15.6 | 13.6-17.9 | 1.46 |
| | 16.1 | 14.8-17.5 | 1.27 |
| | 15.5 | 14.2-17.0 | 1.29 |
| | 16.1 | 14.5-17.9 | 1.35 |
| | 18.0 | 16.2-19.9 | 1.42 |
| | 15.7 | 14.0-17.6 | 1.37 |
| | 16.1 | 14.0-18.6 | 1.32 |
| | 15.6 | 13.4-18.1 | 1.26 |
| | 15.2 | 13.0-17.8 | 1.25 |
| | 16.6 | 15.2-18.1 | 1.28 |
| | 16.2 | 14.2-18.5 | 1.45 |
| | 15.9 | 14.4-17.5 | 1.31 |
| | 16.3 | 14.6-18.2 | 1.37 |
| | 14.5 | 12.9-16.3 | 1.38 |
| | 16.9 | 14.6-19.5 | 1.50 |
| | 16.7 | 15.0-18.6 | 1.35 |
| | 15.8 | 14.5-17.2 | 1.27 |
| | 15.7 | 14.3-17.2 | 1.29 |
| $\bar{X}$ | 16.6 | 14.8-18.5 | 1.35 |
| s | 1.25 | | 0.07 |
| %s | 7.5 | | 5.1 |

confidence limits are within 11 % of the mean LC50 value. The extreme LC50 values recorded are 87 % and 118 % respectively of the mean LC50.

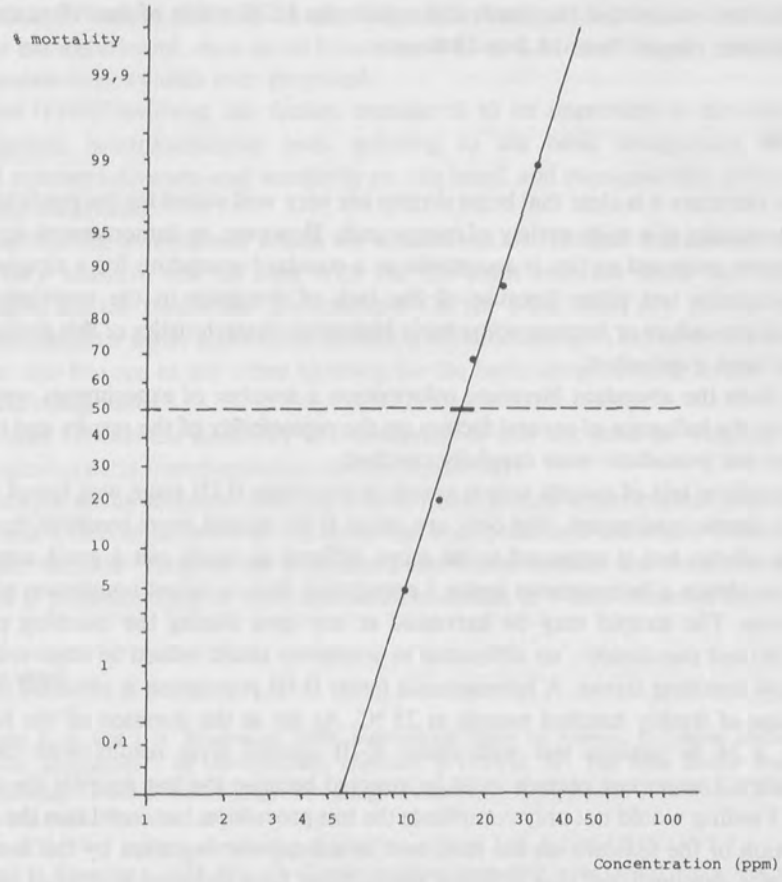


FIG. 6. Mortality curve calculated from the mean 24 hr LC50 and the slope function of 25 replicate tests on instar II-III larvae with sodium lauryl sulphate as toxicant. The 95 % confidence limits of the mean LC50 is also shown.

Two questions have to be answered : are these deviations acceptable from the statistical point of view and what is the maximum range of variation which can be accepted for a standardized test with a reasonable accuracy. For this purpose it was necessary to calculate the minimum procentual deviation which an LC50 may have from the mean LC50 value obtained (16.6) to differ significantly at the 0.05 level. The method of Litchfield and Wilcoxon (1949) was used to compare this mean LC50 value with a hypothetical LC50 derived from a mortality curve with a slope function varying between $1.35 \pm 2\ s$ (s = standard deviation of S). This calculation revealed that a ratio $\frac{\text{LC50 m}}{\text{LC50 h}} = 1.195$ (with LC50m = 16.6 and LC50h = the

hypothetical LC50) is necessary in order to obtain a significant difference at the $P < 0.05$ level between the hypothetical LC50 value and the mean. All LC50 values within a range of 20% of the mean LC50 value are thus acceptable.

In practice this means that the interval in which the LC50 value of the reference toxicant must be situated, ranges from 13.3 to 19.9 ppm.

## Conclusions

From the literature it is clear that brine shrimp are very well suited for the predictive assessment of the toxicity of a wide variety of compounds. However, as demonstrated above, none of the methods proposed so far, is acceptable as a standard procedure for a simple, routine, short-term toxicity test either because of the lack of precision in the description of the experimental procedure or because some basic biological characteristics of this particular test-species had been overlooked.

Starting from the abundant literature information a number of experiments were carried out in which the influence of several factors on the repeatibility of the results and the practicality of the test procedure were carefully checked.

A 24 hr toxicity test of nauplii which are all in the instar II-III stage was found to be best suited for a simple routine test. Not only are instar II-III nauplii more sensitive than instar I nauplii and adults, but it appeared to be more difficult to work out a good experimental procedure to obtain a homogenous instar I population than a mixed population of instar II and III larvae. The nauplii may be harvested at any time during the hatching process to constitute the test population ; no difference in sensitivity could indeed be observed between fast and slow hatching larvae. A homogenous instar II-III population is obtained easily by a 24 hr storage of freshly hatched nauplii at 25 °C. As far as the duration of the bioassay is concerned, a 24 hr toxicity test with instar II-III nauplii gives results with the highest reproducibility. Longer test periods must be rejected because the test animals die off due to starvation. Feeding would not only complicate the test procedure, but could bias the results by the adsorption of the toxicant on the food and its subsequent ingestion by the test species.

Since it was demonstrated that different populations have different ecological optima and different sensitivities, one geographical strain had to be selected for the standard bioassay. Presently, the geographical population from the salt ponds of Macau (Brazil) seems to be the most suited. The Brazilian cysts are indeed commercially available worldwide and the exact origin of these cysts is guaranteed. The pesticide and heavy metal level of this strain is much lower than that of some other populations (Olney *et al.*, 1980) and no differences in sensitivity could be observed between various batches of this population.

The major advantage of *Artemia* for toxicity studies is the overall availability of the dry cysts ; one and the same sample of cysts can be used for at least 1 year. The sensitivity of the nauplii is not affected by storage of the cysts under different conditions. Storage under dry conditions is, however, highly recommended to maintain a high degree of hatching.

From the comparison of the data for repeatability of bioassays with *Daphnia* and *Brachydanio* (Cabridenc, 1979) and the values which we obtained for *Artemia* nauplii (Tables VI, VII and VIII) it is clear that the reproducibility of this standard *Artemia* test equals at least that of the internationally adopted tests on *Daphnia* and *Brachydanio*.

A standard short-term test with *Artemia* nauplii is attractive for many reasons. It does not require elaborate equipment nor much labor and time. The preparations to hatch the cysts are made in a few minutes, the pipeting of the nauplii into the petri dishes takes, with a little skill, not more than 30 sec per dish and the counting of the dead nauplii takes to the most a quarter of an hour per experiment. As a result it can be considered as a very handy and certainly one of the cheapest toxicity tests ever proposed.

Persoone (1980) reviewed the factors considered to be important in the elaboration of routine aquatic eco-toxicological tests, pointing to the basic antagonism between the ecological representativeness and sensitivity on one hand, and manageability, practicality, and costs on the otherhand.

It is clear that the *Artemia* test which we worked out also fits into this antagonism, since it is not a very sensitive test (at least with the life-death criterion used) and certainly not representative for the freshwater environment. On the other hand it is most suited for the marine and brackish-water ecosystem, including effluent testing in estuaries and dumpings at sea ; it can also be used as any other bioassay for the basic, range-finding level of the toxicity of chemical compounds.

In the latter context the simplicity and cheapness of this test must be weighed against the two biological criteria (representativeness and sensitivity).

To conclude let us mention that the detailed and precise experimental procedure to be followed and which is the result of this study has been published elsewhere (Vanhaecke *et al.*, 1980) under the title "Proposal for a standard short-term toxicity test with *Artemia* nauplii" and that it is presently used in intercalibration exercises in North-America and Europe.

## Literature cited


Abreu-Grobois F. A. and J. A. Beardmore. 1980. International Study on *Artemia*. II. Genetic characterization of *Artemia* populations – an electrophoretic approach. p. 133-146. *In* : The brine shrimp *Artemia*. Vol. 1. Morphology, Genetics, Radiobiology, Toxicology. Persoone G., P. Sorgeloos, O. Roels, and E. Jaspers (Eds). Universa Press, Wetteren, Belgium. 345 p.

Anonymous. 1973. Navy military specification. Emulsifier, oil-slick. MIL-E-22864 B (NAVY). 8 p.

Benijts F. and D. Versichele. 1975. The role of water-sediment interaction in the Ostend Sluice-dock with regard to the toxicity of polluants to invertebrate larvae. p. 221-234. *In* : Sublethal effects of toxic chemicals on aquatic animals. Koeman J. H. and J.J. T. W. A. Strik (Eds). Elsevier Scientific Publishing Co., Amsterdam. 234 p.

Bliss C. I. 1957. Some principles of bioassay. *Am. Sci.* 45:449-466.

Bowen S. T. and G. Sterling. 1978. Esterase and malate dehydrogenase isozyme polymorphisms in 15 *Artemia* populations. *Comp. Biochem. Physiol.* 61B:593-595.

Brown B. and M. Ahsanullah. 1971. Effect of heavy metals on mortality and growth. *Mar. Pollut. Bull.* 2(12): 182-187.

Brown R. F., J. D. Williams and R. M. Eppley. 1968. Temperature-dose relationship with aflatoxin on the brine shrimp, *Artemia salina*. *J. Ass. off. agric. Chem.* 51:905-906.

Cabridenc R. 1979a. Inter-laboratory test programme concerning the study of the ecotoxicity of a chemical substance with respect to the *Daphnia*. Commission of the European Communities, Study D. 8368. 19 p.

Cabridenc R. 1979b. Inter-laboratory test programme concerning the study of the ecotoxicity of a chemical substance with respect to the fish. Commission of the European Communities, Study D. 8368. 20 p.

Caserio G., G. Mascherpa, and E. Marubini. 1970. Possibilita di utilizzazione di *Artemia salina* come animale test per la evidenziazione di pesticidi organo-fosforati nelle carni. *Arch. Veter. Ital.* 21(1):49-61.

Claus C. 1976. Onderzoek naar de toxiciteit van drie herbiciden op hat aquatisch ecosysteem. Thesis, State University of Ghent. 197 p.

CLAUS C., F. BENIJTS, and P. SORGELOOS. 1977. Comparative study of different geographical strains of the brine shrimp *Artemia salina*. p. 91-105. *In* : Fundamental and applied research on the brine shrimp, *Artemia salina* (L.) in Belgium. Jaspers E. and G. Persoone (Eds). European Mariculture Society Special Publication No. 2. EMS. Bredene, Belgium. 110 p.

CONNOR P. M. 1972. Acute toxicity of heavy metals to some marine larvae. *Mar. Pollut. Bull.* 3(12):190-192.

CORNER E. D. S. and F. H. RIGLER. 1958. The modes of action of toxic agents. III. Mercuric chloride and N-amylmercuric chloride on crustaceans. *J. mar. biol. Ass. UK* 37:85-96.

CORNER E. D. S. and B. W. SPARROW. 1956. The modes of action of toxic agents. I. Observations on the poisoning of certain crustaceans by copper and mercury. *J. mar. biol. Ass. UK* 35(3):531-548.

CORNER E. D. S. and B. W. SPARROW. 1957. The modes of action of toxic agents. II. Factors influencing the toxicities of mercury compounds to certain Crustacea. *J. mar. biol. Ass. UK* 36:459-472.

COX J. L. 1974. The use of the dilution water effect as a water quality criterion. *Bull. Environmental Contamination and Toxicology* 11(3):256-257.

CUNNINGHAM P. A. 1976a. Effects of Dimilin on reproduction in brine shrimp. *Am. Zool.* 16(2):15-30.

CUNNINGHAM P. A. 1976b. Effects of Dimilin (TH 6040) on reproduction in the brine shrimp *Artemia salina*. *Environ. Entomol.* 5(4):701-706.

CURTIS R. F., D. T. COXON, and G. LEVETT. 1974. Toxicity of fatty acids in assays for mycotoxins using the brine shrimp (*Artemia salina*). *Food Cosm. Toxicol.*, 12(2):233-235.

D'AGOSTINO A. S. 1965. Comparative studies of *Artemia salina* (development and physiology). Thesis, New York University. 83 p. University Microfilms, 66-5653.

DE BRABANDER K. and H. VANDEPUTTE. 1967. Emploi d'*Artemia salina* pour la mesure de la toxicite de certains produits atteignant les eaux de surface. *Centre Belge d'Etude et de Documentation des Eaux* 282:1-12.

DELCAMBE L. 1955. Activités biologiques des actinomycines de Streptomyces S-67. *Arch. Int. Pharmacodyn.* 101: 358-566.

DE WAART J., F. VAN AKEN and H. POUW. 1972. Detection of orally toxic microbial metabolites in foods with bioassay systems. *Zbl. Bakt. Hyg.* 222:96-114.

DOUDOROFF P., B. G. ANDERSON, G. E. BURDICK, P. S. GALTSOFF, W. B. HART, R. PATRICK, E. R. STRONG, E. W. SURBER, and W. M. VAN HORN. 1951. Bio-assay methods for the evaluation of acute toxicity of industrial wastes to fish. *Sewage Industrial Wastes* 23:1380-1397.

DOUDOROF P. and M. KATZ. 1953. Critical review of literature on the toxicity of industrial wastes and their components to fish. II. The metals, as salts. *Sewage Indistrial Wastes* 25(7):802-839.

DURACKOVA Z., V. BETINA, B. HORNIKOVA, and P. NEMEC. 1977. Toxicity of mycotoxins and other fungal metabolites to *Artemia salina* larvae. *Zentralblad Bakteriol. Parasitenkd. Infektionshr. Hyg. Zweite Naturwiss. Abt. Allg. Landwirtsch. Tech. Mikrobiol.* 132(4):294-299.

GRANADE H. R., P. C. CHENG, and N. J. DOORENBOS. 1976. Ciguatera. I. Brine shrimp *Artemia salina* larval assay for Ciguatera toxins. *J. Pharm. Sci.* 65(9):1414-1415.

GROSCH D. S. 1966. The reproductive capacity of *Artemia* subjected to successive contaminations with radio-phosphorus. *Biol. Bull.* 131(2):261-271.

GROSCH D. S. 1967. Poisoning with DDT : effect on reproductive performance of *Artemia*. *Science* 155(3762) 592-593.

GROSCH D. S. 1970. Poisoning with DDT : second and third-year reproductive performance of *Artemia*. *Bio Science* 20:913.

GROSCH D. S. 1973. Reproduction tests : the toxicity for *Artemia* of derivates from non-persistent pesticides. *Biol. Bull.* 145:340-351.

HALLOPEAU C. 1969. Growth and reproduction of *Artemia salina* in the presence of products of fission. *Bull. Mus. Nat. Hist. Natur.* 41:1238-1243. (in French).

HARWIG, J. and P. M. SCOTT. 1971. Brine shrimp (*Artemia salina* L.) larvae as a screening system for fungal toxins. *Appl. Microbiol.* 21(6):1011-1016.

HERALD E. S. and R. P. DEMPSTER. 1965. Brine shrimp versus copper solutions. *Aquar. J.* 36:334-336.

HOOD D. H., T. W. DUKE, and B. STEVENSON. 1960. Measurement of toxicity of organic wastes to marine organisms. *Water Pollution Control Federation* 32(9):982-993.

HUDSON R. A. and J. C. BAGSHAW. 1978. Toxicity of Di-N-butyl phthalate for developing larvae of the brine shrimp, *Artemia salina*. *Fed. Proc.* 37:1702.

JENNINGS R. H. and D. M. WHITAKER. 1941. The effect of salinity upon the rate of excystment of *Artemia*. *Biol. Bull.* 80(2):194-201.

JENSEN K. 1975. Hatching rate as bioassay. Proposal for a standard technique. *Bull. Environ. Contam. Toxicol.* 14(5):562-564.

JONES A. J. 1972. An inexpensive apparatus for the large scale hatching of *Artemia salina* L. *J. Cons. perm. int. Explor. Mer* 34(3):351-356.

JORGENSEN K. F. and K. JENSEN. 1977. Effect of copper (II) chloride on the hatching rate of *Artemia salina* (L.). *Chemosphere* 6:287-291.

JORGENSEN K. F. and K. JENSEN. 1978. Effect of zinc chloride on the hatching rate of *Artemia salina* (L.). *Biokon. Rep.* 6:1-5.

JUNG K. D. 1975. Bibliographic study of the sensitivity of crustaceans (*Daphnia*, *Gammarus*, *Artemia*) to chemical products. *Trib. CEBEDEAU* 28(381-382):301-303. (in French).

KINNE, O. (Ed.). 1971. Marine ecology. Vol. 1. Environmental factors. Part 2. John Wiley and Sons. New York (USA). 561 p.

KNAUF W. and E. F. SCHULZE. 1973. New findings on the toxicity of endosulfan and its metabolites to aquatic organisms. *Meded. Fac. Landbouwwetensch. Rijksuniversiteit Gent* 38(3):717-732.

KNÖFEL B. 1976. Untersuchungen zur Methodik der Verwendung von *Artemia salina* (Leach) in einer Testkette zur Messung der Akkumulation von Pestiziden. Thesis. University of Hamburg. 67 p.

LITCHFIELD J. T., Jr. and F. WILCOXON. 1949. A simplified method of evaluating dose-effect experiments. *J. Pharmac. exp. Ther.* 96:99-113.

LUDEMANN D. and H. NEUMANN. 1961. Studien uber die Verwendung von *Artemia salina* L. als Testtier zur Nachweis von Kontaktinsektiziden. *Z. angew. Zool.* 48(3):325-332.

METALLI P. and E. BALLARDIN. 1972. Radiobiology of *Artemia* : radiation effects and ploidy. *Current Topics in Rad. Res. Quart.* 7(2):181-240.

MICHAEL A. S., C. G. THOMPSON, and M. ABRAMOVITZ. 1956. "*Artemia salina*" as a test organism for bioassay. *Science* 123:464.

MORGAN D. D. and D. WARSHAWSKY. 1977. The photodynamic immobilization of *Artemia salina* nauplii by polycyclic aromatic hydrocarbons and its relationship to carcinogenic activity. *Photochem. Photobiol.* 25(1):39-46.

NELSON J. O. and F. MATSUMURA. 1975a. A simplified appoach to studies of toxic toxaphene components. *Bull. Envir. Contam. Toxicol.* 13:464-470.

NELSON J. O. and F. MATSUMURA. 1975b. Separation and comparative toxicity of toxaphene components. *J. Agric. Food Chem.* 23(5):984.

NIMURA Y. 1968. Note on hatching the cyst of *Artemia*. *Aquiculture* 16(2):105-115. (in Japanese).

OLNEY C. E., P. S. SCHAUER, S. MCLEAN, You LU, K. L. SIMPSON. 1980. International Study on *Artemia*. VIII. Comparison of the chlorinated hydrocarbons and heavy metals in five different strains of newly hatched *Artemia* and a laboratory-reared marine fish. p. 343-352. *In* : The brine shrimp *Artemia*. Vol. 3. Ecology, Culturing, Use in Aquaculture. Persoone G., P. Sorgeloos, O. Roels, and E. Jaspers (Eds). Universa Press. Wetteren, Belgium. 456 p.

PERSON-LE RUYET J. and A. SALAUN. 1977. Etude comparative des possibilités d'élevage larvaire de quelques poissons marins avec une souche d'œufs d'*Artemia salina* de Chypre. ICES-CM 32. 13 p.

PERSOONE G. 1980. Standardization of aquatic bio-assays : compromises between biological and economical criteria. Proc. 6th Ann. Aquatic Toxicity Workshop. Winnipeg Manitoba, Canada. (in press).

PERSOONE G. and P. SORGELOOS. 1972. An improved separator box for *Artemia* nauplii and other phototactic invertebrates. *Helgolander wiss. Meeresunters.* 23:243-247.

PHAM-HUU-CHANH and G. MAMY. 1963. A simple biological reagent for toxicity : the eggs of "*Artemia salina*". *Revue Agressologie* 4(6):599-602.

PRICE K. S., G. T. WAGGY, and R. A. CONWAY. 1974. Brine shrimp bioassay and seawater BOD of petrochemicals. *Journ. Water Poll. Control Fed.* 46(1):63-77.

RAKOWICZ M. 1975. Notes on *Artemia salina*. Prawn Farming Systems Techniques. June 25-Sept. 5, 1975. Food Institute, East-West Center, Honolulu, HI, USA. 13 p.

REISS J. 1972. Vergleichende Untersuchungen uber die Toxizitat einiger Mycotoxine gegenüber den Larven des Salinenkrebses (*Artemia salina* L.) *Zbl. Bakt. I. Orig.* B155:531-534.

ROBINSON A. B., K. F. MANLY, M. P. ANTHONY, J. F. CATCHPOOL, and L. PAULING. 1965. Anesthesia of *Artemia* larvae ; method for quantitative study. *Science* 149:1255-1258.

Saliba L. J. and M. Ahsanullah. 1973. Acclimation and tolerance of *Artemia salina* and *Ophryotrocha labronica* to copper sulphate. *Mar. Biol.* 23:297-302.

Saliba L. J. and R. N. Krzyz. 1976a. Effects of heavy metals on hatching of brine shrimp. *Mar. Poll. Bull.* 7(10): 181-182.

Saliba L. J. and R. N. Krzyz. 1976b. Acclimation and tolerance of *Artemia salina* and *Ophryotrocha labronica* to copper sulphate. *Mar. Biol.* 23:297-302.

Sanders H. O. and O. B. Cope. 1966. Toxicities of several pesticides to two species of Cladocerans. *Trans. Am. Fish. Soc.* 95:165-169.

Schauer P. S., D. M. Johns, C. E. Olney, and K. L. Simpson. 1980. International Study on *Artemia*. IX. Lipid level, energy content and fatty acid composition of the cysts and newly hatched nauplii from five geographical strains of *Artemia*. p. 365-373. *In* : The brine shrimp *Artemia*. Vol. 3. Ecology, Culturing, Use in Aquaculture. Persoone G., P. Sorgeloos, O. Roels, and E. Jaspers (Eds). Universa Press, Wetteren, Belgium. 456 p.

Schmidt-Nielsen B. 1974. Osmoregualtion : effect of salinity and heavy metals. *Fed. Proc.* 33(10):2137-2146.

Shackell L. F. 1925. The relation of dosage to effect. II. *Jour. Pharm. Exper. Ther.* 25:275-288.

Smith T. I. J., J. S. Hopkins, and P. A. Sandifer. 1978. Development of a large scale *Artemia* hatching system utilizing reciculated water. p. 701-714. *In* : Proc. 9th Annual Meeting World Mariculture Society, Avault, J. W., Jr. (Ed.). Louisiana State University, Baton Rouge, LA, USA. 807 p.

Sorgeloos P. 1975. De invloed van abiotische en biotische faktoren op de levenscyclus van het pekelkreeftje, *Artemia salina* L. Thesis, State University of Ghent. 235 p.

Sorgeloos P. 1979. Het gebruik van het pekelkreeftje *Artemia* spec. in de aquakultuur. Thesis, State University of Ghent. 319 p.

Sorgeloos P. 1980. The use of the brine shrimp *Artemia* in aquaculture. p. 25-46. *In* : The brine shrimp *Artemia*. Vol. 3. Ecology, Culturing, Use in Aquaculture. Persoone G., P. Sorgeloos, O. Roels, and E. Jaspers (Eds). Universa Press, Wetteren, Belgium. 456 p.

Sorgeloos P., M. Baeza-Meza, F. Benijts, and G. Persoone. 1976. Current research on the culturing of the brine shrimp *Artemia salina* L. at the State University of Ghent, Belgium. p. 473-495. *In* : Proc. 10th European Symposium Marine Biology, Vol. 1. Mariculture. Persoone G. and E. Jaspers (Eds). Universa Press, Wetteren, Belgium. 620 p.

Sorgeloos P. and G. Persoone. 1975. Technological improvements for the cultivation of invertebrates as food for fished and crustaceans. II. Hatching and culturing of the brine shrimp, *Artemia salina* L. *Aquaculture* 6: 303-317.

Sorgeloos P., C. Remiche-Van der Wielen, and G. Persoone. 1978. The use of *Artemia* nauplii for toxicity tests – a critical analysis. *Ecotoxicology and Environmental Safety* 2:249-255.

Sprague J. B. 1969. Measurement of pollutant toxicity to fish. I. Bioassay methods for acute toxicity. *Water Research* 3:793-821.

Springer A. and A. P. Legge. 1966. On the use of the brine shrimp *Artemia salina* L. as a test organism for the bioassay of insecticides (DDT, benzine, hexachloride, dieldrin). *Long. Ashton. Hort. Res. Sta. Ann. Rep.* : 229-232.

Standard Methods for Examination of Water and Wastewater. 1975. Greenberg A. C., M. C. Rand, and M. J. Taras (Eds). 14th Edit. American Public Health Association, American Water Works. Association Water Pollution Control Federation, Washington, USA. 1193 p.

Stephan, C. E. 1975. Methods for acute toxicity tests with fish, macroinvertebrates and amphibians. EPA-660/3-75.009. National Environmental Research Center. Office of Research and development. US Environmental Protection Agency Corvallis, Oregon. 61 p.

Tarpley W. A. 1958. Studies on the use of the brine shrimp, *Artemia salina* (Leach) as a test organism for bioassay. *J. Econ. Ent.* 51(6):780-783.

Tarzwell C. M. 1969. Standard methods for the determination of relative toxicity of oil dispersants and mixtures of dispersants and various oils to aquatic organisms. p. 179-186. *In* : Proc. Joint Conference Prevention and Control of Oil Spills. API and FWPCA.

Trieff N. M., M. McShan, D. Grajcer, and M. Alam. 1973. Biological assay of *Gymnodinium breve* toxin using brine shrimp (*Artemia salina*). *Texas Rep. Biol. Med.* 31(3):409-422.

Vanhaecke P. and P. Sorgeloos. 1980. International Study on *Artemia*. IV. The biometrics of *Artemia* strains from different geographical origin. p. 393-405. *In* : The brine shrimp *Artemia*. Vol. 3. Ecology, Culturing, Use in

Aquaculture. Persoone G., P. Sorgeloos, O. Roels, and E. Jaspers (Eds). Universa Press, Wetteren, Belgium. 456 p.

Vanhaecke P., A. Cooreman, and P. Sorgeloos. International Study on *Artemia*. XV. The effect of light intensity on the hatching rate of *Artemia* cysts from various geographical origin. *Mar. Biol.* (submitted for publication).

Von Hentig R. 1971. Einfluss von Salzgehalt und Temperatur auf Entwicklung, Wachstum, Fortpflanzung und Energiebilanz von *Artemia salina*. *Mar. Biol.* 9:145-182.

Wisely B. and R. A. P. Blick. 1967. Mortality of marine invertebrates larvae in mercury, copper and zinc solutions. *Austr. J. mar. Freshwater Res.* 18:63-72.

Zillioux E. J., H. R. Foulk, J. C. Prager, and J. A. Cardin. 1973. Using *Artemia* to assay oil disperant toxicities. *J. Wat. Pollut. Control. Fed.* 45(11):2389-2396.

# Biology education

*The Brine Shrimp* Artemia. *1980. Vol. 1. Morphology, Genetics, Radiobiology, Toxicology*
*G. Persoone, P. Sorgeloos, O. Roels, and E. Jaspers (Eds). Universa Press, Wetteren, Belgium. 345 p.*

# *Artemia* as a multipurpose biomaterial for biology education

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**Abstract**

Both laboratory and field exercises are essential to biology education at any level. However, it is not easy to find proper biomaterials for the exercices. The brine shrimp *Artemia* can be cultured easily in the laboratory and can be obtained year-round at any stage of development for these exercises. The brine shrimp is utilized for fundamental research in various disciplines and can also serve as a very useful and multipurpose biomaterial for biology education at the college level as well as at the high school level.

On the basis of studies on the brine shrimp by the present author and others, the senior author has developed many practical exercises in which the brine shrimp is utilized as a biomaterial.

The present paper outlines a number of these exercises for students and pupils. They cover gross and microscopic anatomy, histology, embryology, physiology, biochemistry, and phototactic behavior.

## Introduction

Both laboratory and field exercises dealing with living materials are essential to biology education at any level. Because of the inconstant supply of proprer biomaterials it is not so easy to organize such exercises. In fact, during the past decade in Japan, natural populations of many familiar animals and plants useful for biology research and education have diminished rapidly, due to the destruction of the natural environment by the development of industries and cities. Such a situation may also exist in other industrialized countries or become their fate in the next future. Therefore efforts should be made to keep the balance between preservation and supply of living beings useful for biology education and to increase the array of potential candidate species for teaching.

The brine shrimp, *Artemia* which can be easily cultured in the laboratory is one of those species. Fed dry yeast and green algae, larvae hatched from dried eggs grow up to adults, year-round and thrive very well, generation after generation, even in 1 l beakers. This means that we can obtain as many *Artemia's* as we want when we wish them and at any stage of development, without any destruction of the natural biota. Since brine shrimps are utilized worldwide for research in various disciplines, *Artemia* is useful as a multipurpose biomaterial for biology education. We have developed practical exercises in which *Artemia* is used as a

teaching material. The present paper outlines several exercises for students and pupils as application aspects of fundamental research on *Artemia*.

## Exercises dealing with animal morphology

### Methods and procedures

To observe living adults of *Artemia* it is helpful to narcotize them by adding a small amount of soda water or ether to a culture medium (a few drops to 10 ml of medium) ; it is also useful to starve them for several days before observation to empty the contents of the gut. Chloroform is a better narcotic but more dangerous to pupils.

For microscopic observation under high power magnification, the sections of adults and larvae are prepared by fixing the animals in Carnoy's or Bouin's fluid, embedding in paraffin, and staining with Ehrlich's hematoxylin and eosin.

Using both living adults and larvae and/or prepared sections, the following laboratory exercises can be considered :

1) Observation of anatomical features of the *Artemia* larvae, as a typical nauplius.
2) Observation of the adult body plan, appendages and other external structures.
3) Observation of sexual dimorphism in the external appearance of the adult.
4) Microscopic anatomy of internal organ systems, especially the central nervous system, the alimentary tract, the vascular system and others.
5) Histological observations of organs and tissues, such as nauplius eye and compound eye, the striated muscle fiber showing A, I, Z, and H bands at low magnifications, the gut epithelium (especially the flat foregut or hindgut epithelial cells provided with cuticle covering), the columnar midgut epithelial cells provided with a brush border, and pseudopodium-like cytoplasmic extrusions found in the paired pouches of the midgut cytoplasmic fragments (probably derived from the extrusions) suggestive of secretion of digestive enzymes from the paired pouches, etc.
6) Observation of superficial cleavage and early development of embryos found in the ovisac.
7) Observation of late development of nauplii and metanauplii.

### Discussion and comments

*Artemia* is a primitive crustacean, belonging to the Order Anostraca characterized by an elongated trunk without carapace. Although the anterior part of the trunk is covered by a carapace in most crustaceans, *Artemia* is still an adequate biomaterial typical for a crustacean body plan.

The body of *Artemia* is divided into a head and trunk. The head has five pairs of appendages : first the antennules next the antennae modified as claspers in the adult males, then the mandibles, maxillules and maxillae. These structures are typical for crustaceans. The stalked compound eyes are well separated on each side of the head. In the adult the distinct nauplius eye is persistent, although it degenerates in most adult malacostracans. The trunk is composed of a series of similar segments and bears eleven pairs of flattened leaf-like appendages : the phyllopodia. A typical phyllodium is biramous, and consists of a median branch, or endopodite, and a lateral branch, or exopodite, each of which is provided with setae along the

margin. The coxa, or coxopodite, bears a fleshy process, or metepipodite, serving as a gill. Lochhead's article (1950) is a compact reference for *Artemia* anatomy.

The exoskeleton of *Artemia* is so transparent that we can observe internal features of a living (eventually narcotized) whole intact specimen through a dissection microscope or a low power microscope. Moreover, the exoskeleton is rather soft and paraffin sections can be prepared for histology exercises (Koshida, unpublished).

The eggs are fertilized and undergo early embryonic development within the ovisac and sections cut through the ovisac of the mature female are most appropriate to study the standard embryonic development of crustaceans. In fact, patterns of the superficial cleavage are often shown schematically in biology textbooks, but it is not so easy to observe embryos showing superficial cleavage, unless *Artemia* is used as a material (Fig. 1).

Dr. Barigozzi suggested that *Artemia* should be used for cytological or chromosomal exercises, but such exercices are still to be developed.

## Exercises dealing with animal physiology

Osmoregulation, measurement of $O_2$ consumption, enzymes in tissues and organs, heart pulsation, blood circulation, and others can be proposed for physiology exercises with *Artemia*. Exercises on osmoregulation and tyrosinase will be outlined hereunder as examples.

Various aspects of osmotic regulation of *Artemia* have been studied extensively by Croghan (1958abc). This author showed that *Artemia* can adapt to seawater media varying from 0.26% NaCl to crystallizing brine, that *Artemia* continuously swallows its medium to take up water from the gut lumen to control its water balance and prevent dehydration in hypertonic media, and that *Artemia* excretes NaCl from its haemolymph through the first ten pairs of metepipodites.

Croghan's findings can be easily demonstrated in class exercises, employing glassware and several ordinary chemicals without the need for special instruments. The following two exercises were developed initially by Mizukoshi (1974) and were revised by the present authors. They are recommended to reach a better understanding of osmoregulation in animals living in concentrated media.

### Water uptake through the gut

*Methods and procedures*

Put adult *Artemia* for 2-3 hr in a culture medium containing dissolved trypan blue (final concentration of 0.002-0.005%) and observe them in a normal medium after rinsing away the dye.

*Results and discussion*

The contents of the gut are stained blue, while the body except the lumen of the gut remains unstained. The result indicates that *Artemia* takes up culture medium, and that the water in the medium is absorbed through the gut epithelium, while the dissolved trypan blue in the medium is not absorbed and accumulates in the lumen of the gut.

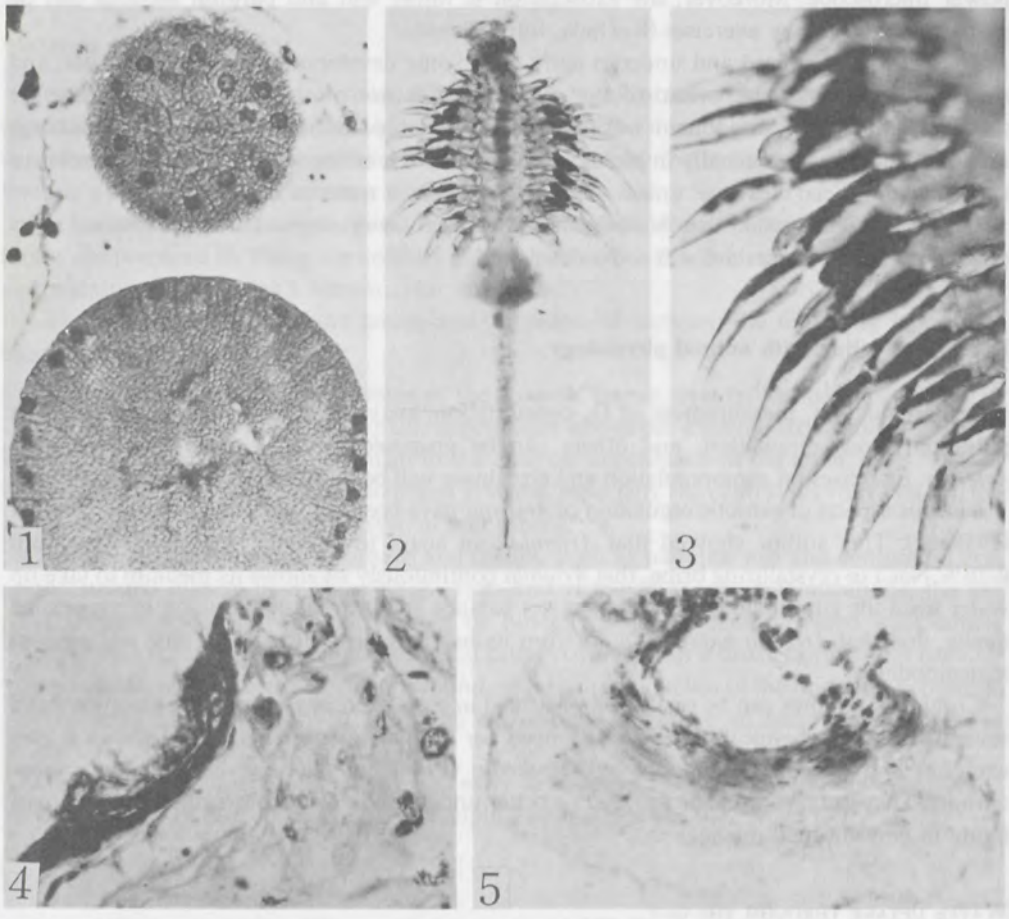


Fig. 1. *Artemia* embryos : early cleavage stage in the ovisac.

Fig. 2. An adult *Artemia* female first put into a solution of silver nitrate and then into a photographic developer.

Fig. 3. Black stained metepipodites of the female shown in Fig. 2.

Fig. 4. Cross section through a wound of the eyestalk of an adult *Artemia* placed in a normal medium 6 hr after ablation of a compound eye.

Fig. 5. A similar section of an adult *Artemia* placed for 6 hr in a medium containing phenylthiourea after the ablation.

### Salt excretion through the gills

*Methods and procedures*

Thoroughly wash off salts from the body surface of adult *Artemia* with distilled water and put them into 1 % silver nitrate solution. After 2-3 min, rinse the animals in distilled water for 1-2 min and put them into a photographic developer for 30-60 sec, and observe them under a microscope.

*Results and comments*

The first ten pairs of metepipodites are stained black, while the eleventh pair and the other parts of the appendages remain unstained (Fig. 2 and Fig. 3).

This black staining can be interpreted as follows : the silver ions diffuse into the cuticle, and combine with chloride ions derived from the haemolymph through the epithelium underlying the cuticle ; consequently a silver chloride precipitate is formed within the cuticle and the precipitate is reduced to the black metallic state by the photographic developer. This black staining is purely a passive process, therefore it is concluded that the cuticle stained black is quite permeable while the unstained cuticle is impermeable.

The results shown in Fig. 2 and Fig. 3 indicate that, in *Artemia*, the first ten pairs of metepipodites are the sites capable of actively excreting sodium chloride from the haemolymph.

### Appearance of tyrosinase activity at the wounded site on the eye stalk

*Methods and procedures*

Cut off, with Wickel's scissors under a dissection microscope, the compound eyes of adult narcotized *Artemia* at about mid level of the eye stalks. After the recovery from narcotization, divide the operated *Artemia* into two groups. Place one group in a normal medium and the other one in a medium containing phenylthiourea, an inhibitor of tyrosinase. Observe the wounds at proper intervals and compare the wound aspects between the two cases.

*Results and comments*

In the operated *Artemia* placed in a normal medium, the edges of the wound on the stump become black within 2-4 hr after amputation ; but in a medium containing to 0.1 % phenylthiourea this blackening hardly shows in 24 hr after amputation (Fig. 4-Fig. 7).

However, the blackening occurs immediately in the colorless stump when the animals are transferred back to the normal medium. The blackening is found in the stumps of head and trunk appendages when they are cut off.

This blackening is due to melanin deposited within the cuticle and melanin formation seems to begin as the result of tyrosinase activation induced probably by amputation (Koshida, unpublished). As shown in amphibian larvae, it is very likely also in *Artemia* that phenylthiourea enters into the stump and inhibits tyrosinase reaction ; consequently melanin is hardly produced (Koshida, 1955, 1956).

The swimming of *Artemia* placed in a medium containing 0.1 % penylthiourea is affected to a large extent after 12-15 hr and all the animals die within 36 hr. *Artemia* can survive longer



FIG. 6. Anterior part of an adult *Artemia* male placed in a normal medium 12 hr after ablation of the left compound eye. Blackening is distinct at the tip of the stump.

FIG. 7. Anterior part of an adult *Artemia* female placed in a medium containing 0.1% phenylthiourea 12 hr after the ablation. No blackening is observed in the stump.

in a medium with a lower concentration of phenylthiourea, but the effect on tyrosinase inhibition also decreases. Incidentally, in 3-4 days after amputation of the eye stalk, the cut-surface of the stump is covered with a new epithelium underlying new and blackened cuticles. Later the blackened cuticle falls off and the wound heals.

**Exercises on phototactic behavior**

*Artemia* shows distinct behavioral responses to light (Seifert, 1932 ; Hiroki, 1974, 1979 ; Hiroki and Koshida, 1976, 1977 ; HOSHIKAWA *et al.*, 1979). These responses can be demonstrated and studied with a simple apparatus. Examples of such exercises on phototactic behavior are given below.

APPARATUS AND EQUIPMENT

The exercises are to be performed in a dark room, but if not available, a class room provided with black curtains is sufficient to do a few exercises.

A general arrangement of the apparatus needed is shown in Fig. 8. The observation chamber, a rectangular vessel made of acrylic resin, is placed on top of a dark box with a shutter. A piece of photographic paper is inserted in this box just beneath the shutter. Three light sources or lamps are provided, called L1, L2, and L3, respectively. L1 is a dim red lap, L2 is hung on the ceiling above the observation chamber and is used to record the distribution of *Artemia* ; L3 is placed on one side of the chamber to trigger the phototactic response in *Artemia*. To record the distribution of the *Artemia*, turn off L3, open the shutter, and flash L2 to photograph the shadows of the *Artemia* on a piece of photographic paper. The photographic paper is then processed to obtain the record.

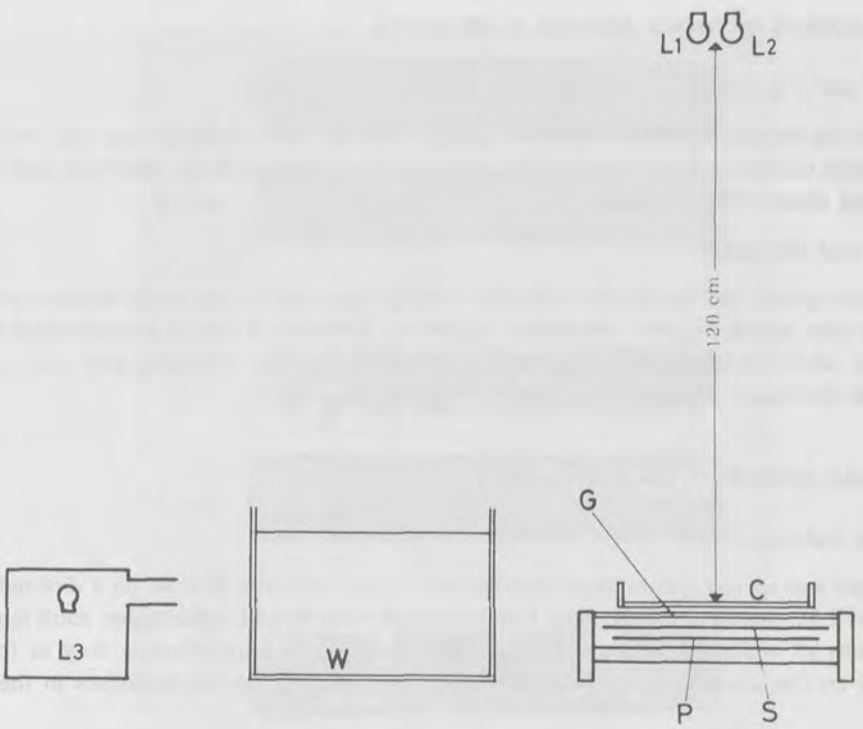


FIG. 8. Diagram showing the arrangement of the apparatus for exercises on phototactic behavior. (C) observation chamber ; (G) glass holder of the observation chamber ; (L1) dim red lamp ; (L2) flash lamp ; (L3) light source to induce phototactic behavior in *Artemia* ; (P) photographic paper ; (S) shutter ; (W) water reservoir to avoid thermal effect caused by L3.

### BEHAVIORAL RESPONSE TO DIFFUSE LIGHT

*Methods and procedures*

Put about 100 nauplii in the observation chamber and leave them for 5 min either in the dark or under a dim red lamp. Let diffuse light shine through the side wall of the chamber, and observe the response of the nauplii. Carry out a similar experiment with 25 adults and compare the results.

*Results and discussion*

The nauplii, are dispersed all over the chamber in the dark or under a dim red lamp ; as soon as diffuse light shines, about half of the population moves in the direction of the light, but the other half moves in the opposite direction. Nauplii thus split into two separate groups, one crowding close to the wall facing the light and the other close to the opposite wall. This pattern of distribution remains for a long time under a diffuse light.

Contrary to the nauplii, the adults move in the direction opposite to the light, but soon they disperse all over the chamber. It is thus evident that nauplii and adults show different behavioral responses to diffuse light.

### Alternation of phototacic behavior in the nauplii

*Method and procedures*

When the nauplii have split into two groups, transfer each of the groups into two new observation chambers. Leave them in the dark or under a dim red lamp, let diffuse light shine again and observe the responses.

*Results and discussion*

In either group, the nauplii split again into two groups, that is, one group displays positive and the other group negative phototactic migration. Therefore it can be demonstrated that a nauplius often switches from positive to negative phototactic behavior and *vice versa* ; although the trigger of the switch is still unknown.

### Behavioral response to a directed light beam

*Methods and procedures*

Prepare and set the apparatus as described in the first exercise, and put on a directed light beam (by L3), instead of diffuse light. Carry out tests with nauplii, adult males, adult females, and adults of which both compound eyes have been ablated in the same way as for the exercise on the appearance of tyrosinase. Observe and compare the responses in the four cases.

*Results and discussion*

When the chamber is illuminated by the directed light beam all nauplii display negative phototactic behavior, contrary to the case of diffuse light. The intact adults, either males or females, also display negative phototactic migration (Fig. 9). The adult males, however, move rapidly in the direction opposite to the light and towards the opposite wall (back wall) of the chamber and soon show an inclination to disperse again all over the chamber. On the other hand, the adult females move more slowsly toward the back wall of the chamber and stay near the wall longer than the adult males.

Thus between adult males and adult females there are differences in behavioral patterns to a directed light beam. As for the adults without both compound eyes, no phototactic response is expressed at all, although they are still provided with a nauplius eye which is better developed than in the nauplius stages. An example of a series of photographic records is given in Fig. 9.

### Behavioral response to a gradient of illumination intensity

*Methods and procedures*

A triangular vessel made of acrylic resin and filled with a solution of black ink is set as an optical wedge. Two observation chambers are placed at right angle with each other, as shown in Fig. 10. Put the adults into both chambers, apply a directed light beam and observe the response of the animals.

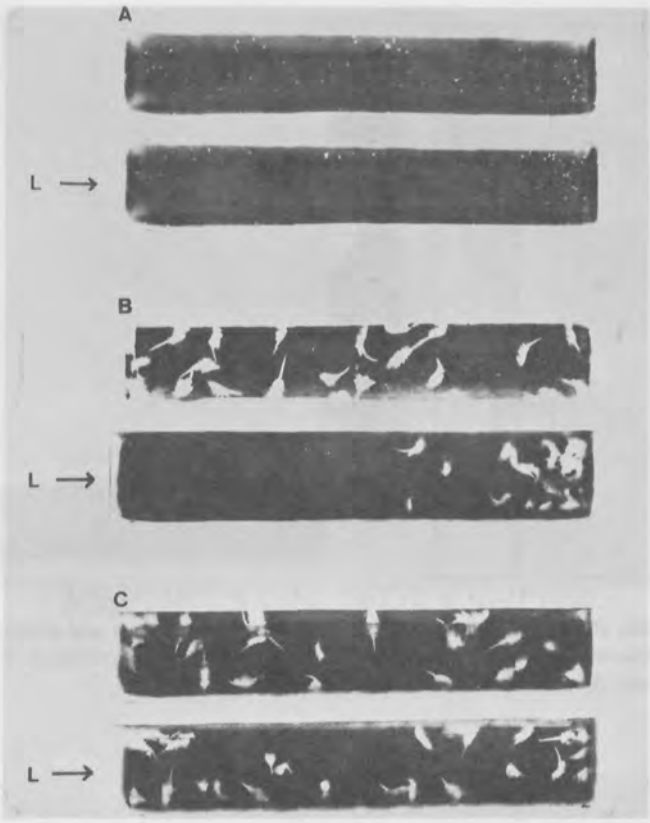


FIG. 9. Photographic records showing the distribution of *Artemia* in the observation chamber before (top) and 180 sec after the start of illumination (bottom). (A) nauplii ; (B) intact adult females ; (C) adults with both compound eyes ablated. Arrow shows direction of light.

*Results and discussion*

Fig. 10 is a record obtained 30 sec after the start of illumination. In both chambers, a majority of *Artemia* gather near the side opposite to the light source. It is clear that the adult *Artemia* respond to the direction of the light beam, but not to the gradient of illumination intensity.

**General discussion**

It is very worthy to find a teaching biomaterial which is suited for many fields of biology. *Artemia* is apparently one of the living beings satisfying many of our present requirements for biological exercises. In addition, utilization of a large number of cultured *Artemia* is by no means affecting the natural biota since the cysts of this species are now harvested by the tons in many places of the world. *Artemia* has become familiar as a multipurpose living material for biology education at the tertiary as well as at the secondary level in Japan. The use of this species for biology exercises should be promoted throughout the world and should stimulate the development of other pertinent exercises in different biological disciplines.

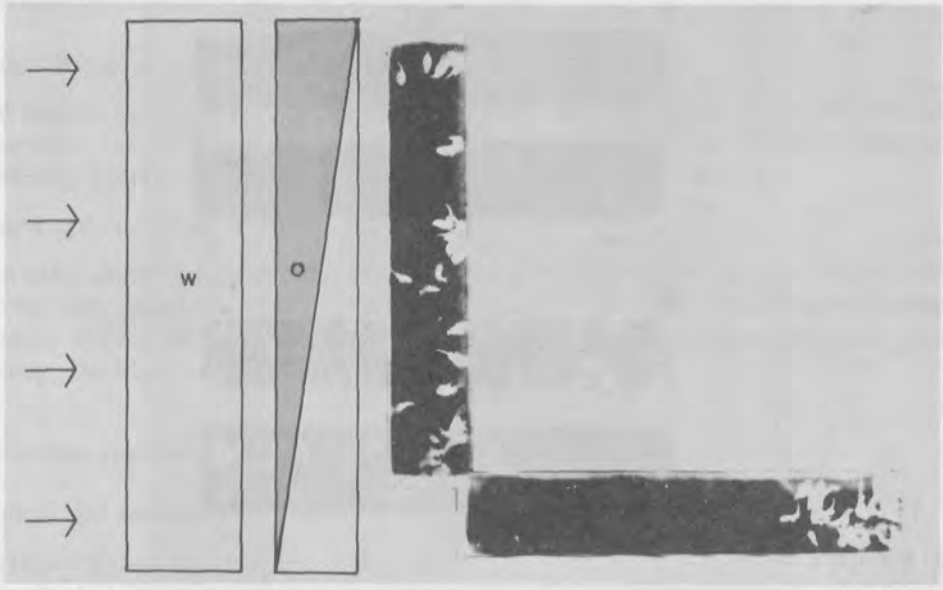


FIG. 10. Diagram showing optical wedge and observation chambers and photographic records of distributions of *Artemia* in the chambers 30 sec after the start of illumination. (O) optical wedge ; (W) water reservoir. Arrow shows the light direction.

## Literature cited

CROGHAN P. C. 1958a. The osmotic and ionic regulation of *Artemia salina* (L.). *J. Exp. Biol.* 35:219-233.

CROGHAN P. C. 1958b. The mechanism of osmotic regulation in *Artemia salina* (L.) : the physiology of the branchiae. *J. Exp. Biol.* 35:234-242.

CROGHAN P. C. 1958c. The mechanism of osmotic regulation in *Artemia salina* (L.) : the physiology of the gut. *J. Exp. Biol.* 35:243-249.

HIROKI M. 1974. Sex difference and the effect of crowding on the phototactic behavior of *Artemia salina*. *Zool. Magazine (Tokyo)* 83(1):83-90. (in Japanese, English abstract).

HIROKI M. 1979. Utilization of *Artemia* for biology education. (I) Phototaxis and visual function. *Ann. Rep. Res. Sc. Ed. Kyoto Univ. of Ed.* 9:7-15. (in Japanese).

HIROKI M. and Y. KOSHIDA. 1976. Nauplian eye and its role in the phototactic behavior of *Artemia salina*. *Zool. Magazine (Tokyo)* 85(1):78-83. (in Japanese, English abstract).

HIROKI M. and Y. KOSHIDA. 1977. *Artemia* as a material for biology education. *Iden* 31(2):41-47. (in Japanese).

HOSHIKAWA C., S. KURITA, I. KUMAMOTO, S. KONOMI, A. OKADA, H. KONDO, and H. FUJISHIMA. 1979. Phototactic behavior in *Artemia*. *Live Teaching Material Information Service* 43:643-647. (in Japanese).

KOSHIDA Y. 1955. The effects of phenylthiourea on the melanogenesis of amphibian larvae I. *Zool. Magazine (Tokyo)* 64:286-290. (in Japanese, English abstract).

KOSHIDA Y. 1956. The effects of phenylthiourea on the melanogenesis of amphibian larvae II. *Zool. Magazine (Tokyo)* 65:397-403. (in Japanese, English abstract).

LOCHHEAD J. H. 1950. *Artemia*. p. 394-399. *In* : Selected invertebrate types. Brown F. A., Jr. (Ed.). John Wiley and Sons Inc. New York and London. 597 p.

MIZUKOSHI K. 1974. Utilization of *Artemia* as an experimental material for biology education. *Iden* 28(1):87-91. (in Japanese).

SEIFERT R. 1932. Raumorientierung und Phototaxis der anostraken Euphyllopoden. *Z. f. vergl. Physiol.* 16:111-184.

# REPORTS ON WORKSHOPS

FIG. 10. Diagram showing optical wedge and observation chambers and histographic records of distribution of *Artemia* in the chambers. (A) no other (the dark of illumination), (B) optical wedge, (W) water reservoir. Arrow shows the light direction.

*The Brine Shrimp* Artemia. *1980. Vol. 1. Morphology, Genetics, Radiobiology, Toxicology*
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# Workshop III. Species characterization in *Artemia*

**Chairman**

Claudio Barigozzi, Universita di Milano
(Italy)

**Rapporteur**

Pietro Metalli, CNEN-CSN Casaccia, Rome
(Italy)

At the end of the International Symposium on *Artemia* it should be recognized first that a high level of integration has been attained by a wide spectrum of scientific disciplines on one side, and of rapidly developing techniques for its exploitation in aquaculture on the other side. The latter aspect is already in evidence in both developed and developing countries.

It has long been known that *Artemia* comprises numerous genotypes of widely different characteristics, spread out all over the world. The problem of species characterization of *Artemia* has been the subject of several scientific papers in the Symposium, and some topics have been taken up again in the discussion held at the workshop. Emphasis was given, however, to collecting suggestions on priorities for the development of future programs. It may not be useful to report fully on the proceedings of the workshop ; rather, it may be convenient to record the major points on which some sort of general consensus was reached. For instance, everyone could notice that chromosome studies on *Artemia* were not reported in the Symposium, and very few new information appeared in the literature of recent times. This classic field of genetic research has limited applications, due to the difficulties posed by investigations on *Artemia* caryotype, but it may give results of the utmost importance for the problems considered at the workshop. The simple determination of chromosome number (either on the somatic cells of nauplii, or on the male and/or female germ cells) is one of the first and necessary steps in species and strain characterization. It was strongly suggested that the few scientists having experience in this field should make it available to other investigators, by training and dissemination of appropriate techniques. As a second suggestion it was also pointed out that the characterization of present-day populations of *Artemia* should on one side collect data according to "categorized" levels (e.g. : 1. mode of reproduction and sex ratio, 2. sexual isolation, 3. chromosome number, 4. isozyme diagnostic loci and hemoglobins, etc.), and on the other side should possibly focus attention to traits of potential practical interest for aquaculture (nuclear size/body size, productivity, heritability of quantitative characters, etc.). The levels, and the order of levels of the above examples reflect practical problems related to the local availability of specific techniques and methodologies. Some data can be collected directly in the field, others require laboratory work with techniques of increasing complexity, consideration being also given to the actual costs of the more sofisticated approaches.

Finally, it was recognized that several manipulations of *Artemia* biotopes, as well as some transfers of "strains" from place to place, had already taken place, causing both potential damage to the natural populations and admixtures of animals of possibly different genotypes. The group was then prompted to consider the problem of preservation of *Artemia*, a concept taken in the broadest possible sense. After a lively discussion of several proposals and contributions, the workshop agreed on a draft of recommendations, which is published here, in the hope that at least some of the ideas and proposed actions will contribute positively to the general scope. It should be emphasized that this draft, worked out by the chairman of this workshop, is not intended to be complete ; it represents a sort of tentative and minimum proposal open to verification by experts and scientists on the occasion of International Meetings to follow.

**Proposal for the preservation of *Artemia* germoplasm : the inventory of *Artemia* types**

The new techniques, which make *Artemia* an animal of increasing economical interest, may cause (and in fact have already caused) transfer of *Artemia* samples from place to place. These transfers, as well as the alterations or destruction of the original habitats may result in the risk, within few years, to be unable to recognize the wild-types in the original biotopes, and to see the disappearing of some of them.

Recognition of wild-types, presumably genetically more or less differentiated, is a need in order to select the best ones for mass culture and to build up "superlines", as it has been done in all cases of useful plants and domesticated animals.

The inventory should give for each biotope (habitat) some basic *Artemia* characters (mode of reproduction, sex ratio in case of bisexuality, chromosome number, morphological peculiarities, etc.), as well as habitat characteristics.

For selecting the basic characters, a coordination of phases is needed between different countries, in order to obtain coordinated inventories for larger geographical areas. Such an inventory recalls the "gene banks" already set up for a number of useful plants.

It is also recommended that any mass transfer or inoculation from place to place be communicated to the Artemia Reference Center at the State University of Ghent in Belgium, and that a record be kept of all such actions.

However, an "*Artemia* bank", when not restricted to the conservation of cysts and of fixed material, is hard to conceive and to materialize, except for a small number of genotypes of particular interest. In a more general way, and taking into account feasibility problems, one could better think of a continuing and systematic watching of the salterns and lagoons considered in the inventory, in order to control the preservation of the genotypes described in the inventory itself.

Finally, taking advantage of this Symposium it is recommended that a first step be taken as soon as possible.

*The Brine Shrimp* Artemia. *1980. Vol. 1. Morphology, Genetics, Radiobiology, Toxicology*
*G. Persoone, P. Sorgeloos, O. Roels, and E. Jaspers (Eds). Universa Press, Wetteren, Belgium. 345 p.*

# Workshop IV. Proposal for an intercalibration exercise for a standard *Artemia* toxicity test

**Chairman**

G. Persoone, Laboratory for Mariculture,
State University of Ghent (Belgium)

**Rapporteur**

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(New York, USA)

From the different papers presented during the session on Toxicology it appeared that :

1) *Artemia* offers unique possibilities for toxicological studies because of the permanent availability of dried *Artemia* cysts and the facility of hatching and culturing brine shrimp ;
2) the dose-effect relationship of pollutants on brine shrimp is assessed by different scientists, with different criteria, on *Artemia* from different geographical origin, and in different stages of development.

As a result, and although these bio-assays are carried out on the same species, the data are in most cases not intercomparable and there is a need for standardization, not the least for short-term routine bio-assays.

During the workshop a motion was proposed and passed that it was highly desirable that an intercalibration exercise be carried out at the international level on a short-term toxicity test on *Artemia* as a first step in the standardization of marine bio-assays.

The parameters and criteria for the practical execution of such a short-term test were then discussed in detail, departing from a proposal worked out by the Artemia Reference Center at the State University of Ghent in Belgium and presented during the toxicity session in the Symposium (Vanhaecke *et al.*).

It was agreed that this test should be a 24 hr TL 50, carried out on a mixture of second and third instar nauplii of *Artemia* from a particular, well known geographical origin (preference being given for the moment to the strain from Macau, Brazil), in artificial seawater (preferably "Instant Ocean Seawater"), strictly following well defined practical procedures.

Potassium dichromate and sodium sulphate were selected as reference toxicants.

All laboratories wishing to participate will be provided with the materials and instructions, and a deadline will be fixed to turn in the results.

The results will be analyzed on a statistical basis to determine the accuracy of the proposed testing procedure.

All participants will receive a report of the results and the conclusions.

If it appears that the actual test procedure is satisfactory from the point of view of reproducibility, steps will be taken to propose it for adoption by the International Standardization Organization as the first standard short-term bio-assay for marine biota.

**Note by the Editors**

*Since the* Artemia *Symposium in Corpus Christi the proposed intercalibration exercise is in progress in North America (with participation of approximately one hundred laboratories) under the joint supervision of the Toxicology Section of the Freshwater Institute in Winnipeg (Canada) and the Artemia Reference Center at the State University of Ghent, Belgium.*

*An analogous exercise will be started shortly in Europe, coordinated by the Artemia Reference Center and sponsored by the Commission of the European Economic Communities.*

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