ARTEMIA

RESEARCH AND ITS APPLICATIONS

volume 1

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RESEARCH AND ITS APPLICATIONS

VOLUME 1

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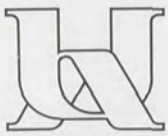
Editors

Proceedings of the
Second International Symposium on the
brine shrimp *Artemia*, organised under the
patronage of His Majesty the King of Belgium



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Welcome address

Prof. Walter Decleir
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Mr. Chairman of the University of Antwerp, Representatives of the Ministries of Foreign Development and Cooperation, Agriculture, and Education, of the European Economic Community, and the Universities of Antwerp, Ghent and Brussels, Your Excellencies representing the Countries of Brazil, India, Israel, Malaysia, Panama, Peru, Spain, Thailand, and the United States of America, dear Colleagues, Ladies and Gentlemen,

It is both a great honour and a pleasure to welcome you to the University and City of Antwerp to participate in the Second International Symposium on the Brine Shrimp Artemia. This symposium has been honoured by the patronage of His Majesty the King of the Belgians. During a recent visit to Professor Persoone's Laboratory for Mariculture in Ghent and to the associated Artemia Reference Center, of which Dr. Sorgeloos is the Director, the King has already shown his keen interest in the research into and the applications of the brine shrimp.

As you may know, the first International Symposium on Artemia was held in 1979 in Corpus Christi, Texas, USA, and we are very happy to be able to organize this second symposium on the same subject here in Antwerp. This was made possible thanks to the generous help of many institutions. Therefore I want to thank the Antwerp State University Center (RUCA) and the Antwerp University Institution (UIA), the Belgian National Science Foundation, the Ministry of Education, the Belgian Administration for Development Cooperation, the Belgian Center for Oceanography, the Artemia Reference Center and the Institute for Marine Scientific Research (IZWO). The assistance of colleagues who kindly accepted to serve as Chairmen, Moderators, and Rapporteurs has been greatly appreciated, as has the help of all those members of the staff who have contributed to the organization of this symposium. Last but not least I wish to thank the Ambassadors and Consuls-General of Canada, France, Germany, Malaysia, Spain, and Thailand, who kindly contributed to the banquet by offering drinks typical of their country.

I am greatly indebted to the Antwerp State University Center (RUCA) for the use of the university's premises for this symposium and to the UIA, the second of the three bodies which together constitute the University of Antwerp, for making its facilities available in order to organize a workshop.

There were three reasons why we decided to organize this second symposium on the brine shrimp in Belgium. The first is the generous support of the Belgian National Science Foundation, which four years ago agreed to sponsor a research program in which various departments of the Universities of Ghent, Antwerp, and Leuven have participated. The organization of this symposium seemed to be the appropriate climax to this initiative. The second reason is that for many years the Artemia Reference Center of the State University of Ghent has played a central role in the coordination of research into *Artemia* and has created the 'International Study on Artemia' group, in which research laboratories from Italy, Spain, Great Britain, the USA, and Belgium are

participating. And thirdly, Belgium through its Administration for Development Cooperation is actively promoting Belgian know-how with regard to *Artemia* applications in third world countries. This is carried out by supporting research, sponsoring training courses, and setting up demonstration projects in various countries in Africa, Asia, and South America. It is not only Belgian governmental organizations which support *Artemia* research and development. The private sector too has recently shown an interest and has set up the Belgian joint venture company 'Artemia Systems'.

All this is sufficient proof of the key role which Belgium has played so far and is still continuing to fulfil in *Artemia* research. The organization of this symposium with 250 participants from 38 countries is an excellent illustration of this.

I should like to finish this short welcome address by expressing my sincere hope that you will enjoy your stay in our City of Antwerp and at our University. Furthermore, I hope that your participation in this symposium will be very fruitful and that this meeting will be a milestone on the way towards a growing knowledge about that tiny crustacean which we call *Artemia* and which seems to be predestined to play such an important role in future world food production.

Opening address

Mr. J. P. Goyens
Director-General of the Belgian Administration for Development Cooperation.

Mr. Chairman, Excellencies, Ladies and Gentlemen,

At a time when industrialized nations combine efforts to boost food productions in developing countries, when in 1985 alone an estimated amount of 3 billion US dollars will be spent on emergency food to only some 20 of the most effected countries, it is encouraging to realize that the scientific community contributes to these efforts by developing more pressing strategies and also inventing new resources.

Aquaculture is one of the fields where opportunities to increase food production in a relatively short time and at reasonable cost, seem very promising. Developing countries which are willing and able to exploit intensively their salt lakes and saltworks can obtain very profitable results by taking *Artemia* developing initiatives and introducing new techniques. In the first place, this food source creates a potential for improving local aquaculture production, and in most cases and at the same time, a better quality of the salt produced by solar evaporation. Secondly, in a number of third world countries, climatological and geological conditions prevail which favor mass production of *Artemia* cysts. Export of high quality cysts can become an important source of income. The promising results of past research initiated by the Belgian Artemia Reference Center, and implemented in collaboration with numerous national and international research and development organizations, have incited the Belgian Government to include in its national development cooperation programme a chapter on *Artemia* which aims at rendering the acquired know-how in the field of selection and reproduction of *Artemia* strains accessible to third world countries.

Each year, the Belgian Administration for Development Cooperation offers a number of fellowships to enable citizens of developing countries, interested in *Artemia* production, to participate in a special training course organized by Belgian universities. Since two years the same administration provides the necessary funds for a project of applied research in view of optimizing the use of *Artemia*, standardizing inoculation techniques, and selecting strains appropriate for inoculation in saltponds. More recently, two *Artemia* projects were started in the field. One aims at the inoculation of *Artemia* in saltponds in Malindi near Mombasa in Kenya, in view of intensive production in a region where *Artemia* are not present under natural conditions. A second project should help Thailand to master and improve the brine shrimp production techniques and coordinate its inoculation programmes. At the present moment, it is too early to evaluate these development cooperation activities in all their aspects. One conclusion we can draw already is that these *Artemia* projects, directed to practical applications in well-chosen environments, do not need important investments, as they are based for the major

part on results obtained by relatively simple techniques. We can also conclude that the exchange and most of all the pooling of numerous and different experiments on this micro-shrimp can only lead to new progress in the knowledge of *Artemia.*

Mr. Chairman, Excellencies, Ladies and Gentlemen, on behalf of the Belgian Administration for Development Cooperation, I wish the participants of this second international Symposium on *Artemia* fertile discussions and work, in view of improving this new resource for the benefit of the third world countries which ultimately is to the interest of the industrialized nations.

Speakers at the opening session (from left to right) : Dr. Patrick Sorgeloos, Prof. Dr. Walter Decleir (Symposium Organizers) and Mr. J. P. Goyens (Director-General of the Belgian Administration for Development Cooperation).

Group picture of participants

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Morphology

The majority of morphology papers presented at the First International Symposium on *Artemia* reported on electron microscopic studies of individual structures or organs of adult *Artemia.*

At the Second Symposium, the emphasis had changed to developmental studies, of the whole animal (1-2) or of individual structures (3-4).

(1) A. Schrehardt.
A scanning electron-microscope study of the post-embryonic development of *Artemia.*

(2) A. Schrehardt.
Ultrastructural investigations of the filter-feeding apparatus and the alimentary canal of *Artemia.*

(3) C. E. Blanchard.
A scanning electron-microscope study of the development of the phyllopods in *Artemia.*

(4) C. E. Blanchard.
Pioneer neurons and the early development of the nervous system in *Artemia.*

Artemia *Research and its Applications. 1987. Vol. 1. Morphology, Genetics, Strain characterization, Toxicology*
P. Sorgeloos, D. A. Bengtson, W. Decleir, and E. Jaspers (Eds). Universa Press, Wetteren, Belgium. 380 p.

A scanning electron-microscope study of the post-embryonic development of *Artemia*[1]

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Abstract

The post-embryonic development of the brine shrimp has been studied using scanning electron microscopical methods.

The hatching stage of *Artemia* is a true nauplius with three pairs of cephalic appendages, which undergo morphological transformations resulting in the sexual dimorphism of the antennae, the total reduction of the larval mandibles, and the new formation of the adult mandibles. During the meta-naupliar and post-metanaupliar periods of development, the maxillules, the maxillae, and 11 pairs of multifunctional thoracopods develop in the post-mandibular region replacing the larval filtering apparatus of the larval antennae and the larval mandibles.

Based on the post-embryonic data, the morphology of the adult *Artemia* is reviewed and the phylogenetic level of the Euanostraca is discussed.

Introduction

Despite the increasing scientific interest in *Artemia*, morphological investigations of the brine shrimp are rare. Ontogenetical studies on the post-embryonic development of *Artemia* are mostly restricted to histological (Joly, 1840 ; Claus, 1886 ; Fränsemeier, 1940 ; Weisz, 1947 ; Anderson, 1967) or morphometrical examinations (Weisz, 1946 ; Gilchrist, 1960 ; Blake, 1979 ; Olson, 1979), whereas only a few contradictory morphological results (Heath, 1924 ; Baid, 1967 ; Hentschel, 1968) exist.

Contradictory reports about the number of instars of the post-embryonic development of *Artemia* have been presented and the majority of the development stages are unnamed. Only Weisz (1947) and Kaestner (1967) suggested a classification of the Euanostracan post-embryonic development in different "phases" and "periods", respectively. While the division of the life cycle into an embryonal, a thoracal, a genital, and an abdominal phase (Weisz, 1947) does not allow an exact delimitation of the different phases of development, the grouping into a naupliar, a meta-naupory rep oost-metanaupliar, and a post-larval period (Kaestner, 1967) is exactly defined and used for the present study.

In accordance with previous authors, Kaestner (1967) and Siewing (1969) defined the end of the naupliar stage with the beginning formation of post-mandibular somites and limb buds.

[1] Paper dedicated in memory of Prof. Dr. Rolf Siewing.

The functioning of the first pair of thoracopods marks the transition from the metanaupliar period to the post-metanaupliar period, whereas the complete segmentation of the trunk and the loss of the natatory function of the antennae indicate the beginning of the post-larval period (Kaestner, 1967). For clarity, the instar numbers are specified in parentheses together with the single developmental stages and figures.

The present paper describes the morphology of the post-embryonic development of the bisexual Macau strain of *Artemia* studied with scanning electron microscopical (SEM) methods, reviews the morphology of the adult organization based on the post-embryonic data, and discusses the phylogenetic level of the morphological organization of the Euanostraca.

Materials and methods

Cysts of the Macau, Brazil strain were obtained from the Artemia Reference Center and incubated at a temperature of 25 °C and a salinity of 30 ‰. The hatched larvae were cultured in the same medium. Larvae, juveniles, and adults were fed a mixture of *Dunaliella* sp. and *Phaeodactylum* sp.

For the SEM studies, *Artemia* in different stages of development were fixed in a 2 % OsO_4-seawater-solution for 12 h at 4 °C. An overnight rinsing in cold seawater was followed by gradual dehydration in acetone and critical-point-drying in CO_2. All specimens were mounted on aluminum stubs, coated with gold in a sputtering device, and examined in an ETEC-autoscan.

Results

The naupliar period

Nauplius (L 1)

The hatching stage of *Artemia* is egg-shaped, about 350 µm in length with three pairs of larval appendages (Fig. 1 ; the list of abbreviations used in the figures is given in Table I). At the procephalon the uniramous, morphologically unjointed antennules arise on both sides of the nauplius eye. The tip of each antennule bears three antennula-setae-type I (Tyson and Sullivan, 1979), the middle one being longest. One to three of the antennular setae possess short spines, which are sparsely distributed over the whole surface of the seta.

Ventral to the base of the antennules the large labrum extends to the mandibles covering the stoma, which is located parantennal to the antennae (Fig. 2). On the dorsal side of the larva the osmoregulatory, dome-shaped neck organ (Hootman *et al.*, 1972 ; Hootman and Conte, 1975) vaults from the antennae to the mandibles (Fig. 3). This larval salt gland becomes reduced during the following stages of development.

The limbs of the tritocephalon, the antennae, are biramous and function as the primary structures of locomotion (Fig. 4). The protopodite of each antenna bears two endites with long setae : the gnathobasenseta and the hook bristle (Fig. 4). The endopodite presents one short and two long setae, the subconical exopodite ten terminal setae (Fig. 4). Rows of cuticular spines about 2 µm in length are located on the ventral surface of the protopodite and the endopodite (Fig. 5).

The biramous larval mandibles are divided into a protopodite with two endites bearing one bristle each, an endopodite with two and an exopodite with three terminal setae (Fig. 4). Similar

TABLE I

List of abbreviations used in Fig. 1-92

a	antennule	cc	crystal cone	fg	food groove
ab	abdomen	cp	compound eye	fk	frontal knob
abs	abdominal segment			fs	filter seta
am	adult mandible	e	endite	fu	furca
an	antenna	en	endopodite	fub	furcal bristle
as 1	antennula-seta-type I	ep	epipodite		
as 2	antennula-seta-type II	es	eye stalk	gb	gnathobase seta
au	anus	ex	exopodite	gs	genital segment
hb	hook bristle	la	labrum	m	maxillule
		lm	larval mandible	ma	maxilla
is	intersegmental furrow			mg	midgut
				mr	mechanoreceptor
jo	joint			s	seta
		p	protuberance	se	setula
ne	nauplius eye	pa	paragnath	sh	shaft
no	neck organ	pc	proctodaeum	sp	spine
		pe	penis	st	stomodaeum
om	ommatidium	prm	post-mandibular region		
ov	ovisac	po	pore	te	telopodite
		pr	protopodite	tel	telson
		pxr	post-maxillar region	th	thoracopod
				ts	thoracomer = thoracal segment

to the antennae, the mandibles form cuticular spines on their ventral surface, but without any regular arrangement.

The post-mandibular region with the terminal anus is unsegmented without any rudiments of limb buds. The three germ layers are monolayered and undifferentiated (Fig. 6). By the definition of Kaestner (1967) and Siewing (1969) the hatching stage of *Artemia* is an orthonauplius characterized by three cephalic segments bearing larval appendages, an unsegmented post-mandibular region, a nauplius eye, and an extensive labrum.

THE METANAUPLIAR PERIOD

The first molt finishes the naupliar period and the larva of *Artemia* enters the metanaupliar period, which comprises four stages of development.

Metanauplius I (L 2)

The 2nd instar (Fig. 7) differs only in a few characters from the nauplius. Each antennule of the metanauplius I forms two buds of antennula-setae-type II (Tyson and Sullivan, 1979) oriented basally to the setae type I. The short terminal bristle of the endopodite of each antenna elongates and a fourth seta develops. The terminal setae of the antennae and of the mandibular exopodites differentiate setulae about 2.5 μm in length arranged in single rows with an inter-setular distance of about 350 nm. Those setulae enhance the efficiency of the locomotory motions ot the antennae and the mandibles and are a part of the larval filtering apparatus, because the

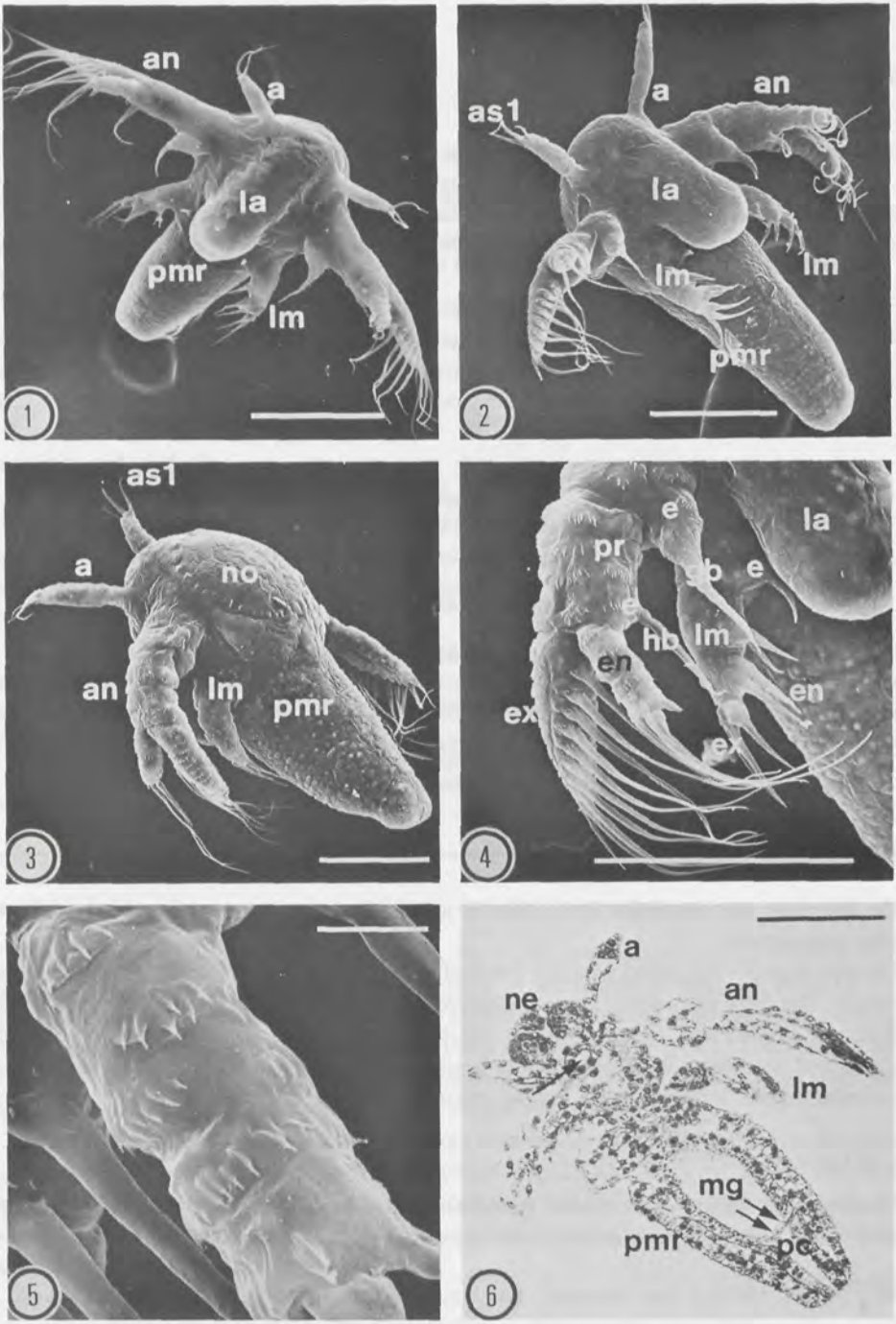


FIG. 1. Ventral view of the hatching stage of *Artemia* (L 1). Scale : 100 µm.

FIG. 2. Ventral view of a nauplius just before moulting (L 1). Scale : 100 µm.

FIG. 3. Dorso-lateral view of a nauplius. Note the dome-shaped neck organ and the unsegmented post-mandibular region (L 1). Scale : 100 µm.

FIG. 4. Larval antenna and larval mandible of the nauplius stage. Both extremities are biramous and divided into a protopodite, an endopodite, and an exopodite. (L 1). Scale : 100 µm.

FIG. 5. Endopodite of the larval antenna. Note the rows of cuticular spines. (L 1). Scale : 10 µm.

FIG. 6. Horizontal section through a nauplius. The post-mandibular region is unsegmented and limb buds are not visible. Note the stomodaeum (arrow) and the two plate epithelia (double arrow), which separate the midgut from the proctodaeum. (L 1). Scale : 100 µm.

metanauplius I starts food ingestion by filter-feeding. The hook bristles and the gnathobasensetae (Fig. 8) and the setae of the mandibular endites and endopodites become feathered at their distal tips by spirally arranged setulae, which are about 15 μm in length. Those setae also belong to the larval filtering apparatus and transfer food particles to the mouth. In spite of the similar organization of the antennae and the mandibles, only the biramous antennae contribute significantly to swimming and filter-feeding of the larva (Barlow and Sleigh, 1980). The movements of the mandibles are usually restricted to a comparatively small angle of 30° to 40°, while the antennae move about 180° in the propulsive stroke (Gauld, 1959 ; Barlow and Sleigh, 1980).

The post-mandibular region of the metanauplius I expands and shows the beginning of segmentation. Two intersegmental furrows indicate the borders of the maxillular and the maxillar segments (Fig. 9).

Metanauplius II (L 3)

The 3rd stage of development of *Artemia* (Fig. 10) is distinguished by several morphological characters. The two rudiments at the tip of each antennule develop into two antennula-setae-type II, which are differentiated in a short, thickened base and a flagellum-like shaft (Fig. 11). The upper lip of the larva starts flattening (Fig. 10) and monopodial ramified bristles develop lateral to the sagittal axis of the larva on the dorsal part of the labrum. The bifurcate tips of the gnathobasensetae of the antennal endites (Fig. 12) are a dependable criterion for the distinction of the late metanauplius I and the early metanauplius II.

Before any reduction of the biramous mandibles is apparent, the buds of the adult mandibles vault dome-like over the attachment sites of the larval appendages (Fig. 14), which remain on the adult structures until they are totally reabsorbed during the subsequent development.

Compared with the structures for filter-feeding of the preceeding stage of development, the larval filtering apparatus of the metanauplius II appears to be more complicated. The setular borders of the terminal setae (Fig. 13) of the endopodites and the exopodites of the antennae and of the mandibular exopodites are active in gathering food particles, while the protopodial setae of the antennae and the bristles of the endites and endopodites of the larval mandibles (Fig. 15) transfer the food towards the mouth, which is situated beneath the labrum. Those transfer setae develop second-order setulae, which narrow the setular mesh. Food particles are transported mainly from the ventral side into the vestibule (Gauld, 1959) or "atrium oris" (Erikson, 1934 in Gauld, 1959) by water currents, which are caused by the effective stroke of the antennae and the transfer motions of the mandibles and the protopodial setae of the antennae. The vestibule of the larva is formed between the postero-dorsal surface of the labrum and the ventral surface of the body and is limited laterally by the ramified bristles of the upper lip (Fig. 16).

The post-mandibular region of the larva is more elongated and the thoracomers I and II, as well as the buds of the maxillules, maxillae and the thoracopods I are visible (Fig. 17). On the dorsal part of the post-maxillary region the segmentally arranged setae of the thoracomers I and II appear and during the following developmental stages a pair of dorsal setae develops on each trunk segment. The single pair may be replaced by three setae in some cases.

Metanauplius III (L 4)

Compared with the foregoing stage of development the procephalon of the metanauplius III (Fig. 18) shows no prominent morphological changes. At the gnathocephalon of the larva the

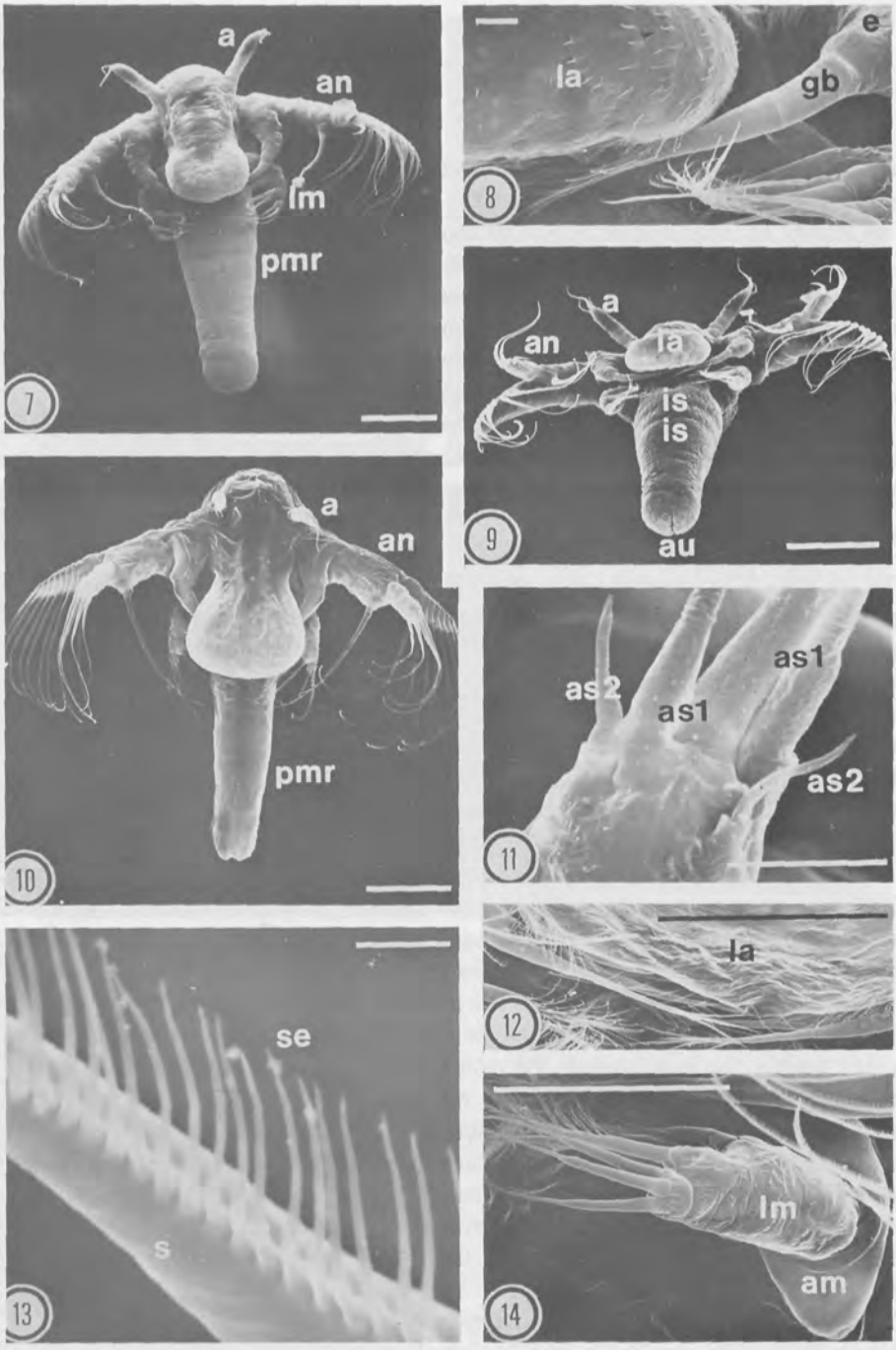


FIG. 7. Ventral view of a metanauplius I (L 2). Scale : 100 µm.

FIG. 8. Gnathobasenseta of a metanauplius I ; the tip of seta bears setulae. (L 2). Scale : 10 µm.

FIG. 9. Ventro-caudal view of a metanauplius I. The post-mandibular region shows a beginning segmentation. (L 2). Scale : 100 µm.

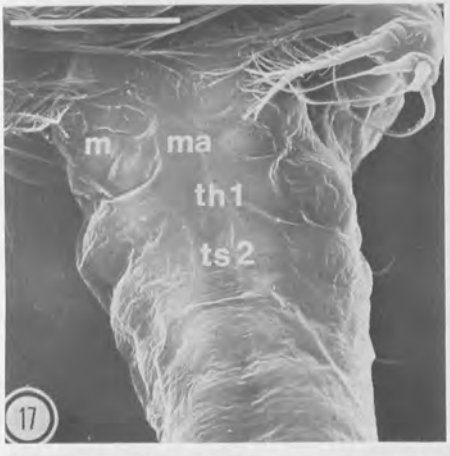
FIG. 10. Ventral view of a late metanauplius II (L 3). Scale : 100 µm.

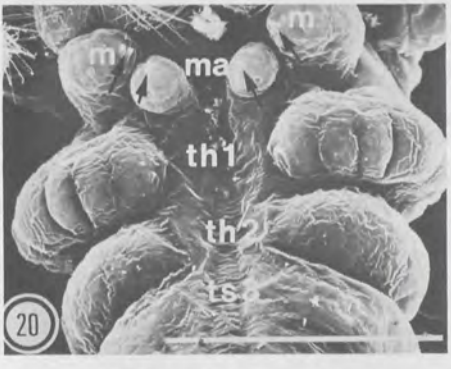
FIG. 11. The tip of each antennule of the metanauplius II bears three antennula-setae-type I and two antennula-setae-type II. (L 3).Scale : 10 µm.

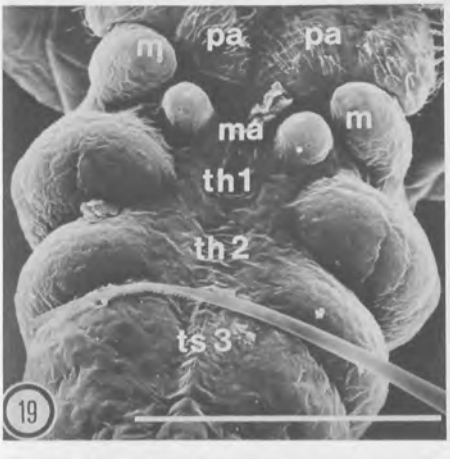
FIG. 12. The tips of the gnathobasensetae of the metanauplius II split bifurcate (L 3). Scale : 10 µm.

FIG. 13. Detail of a terminal seta of the antennal exopodite. Note the setular border. (L 3). Scale : 1 µm.

FIG. 14. Limb bud of an adult mandible with the remaining larval extremity. (L 3). Scale : 100 µm.







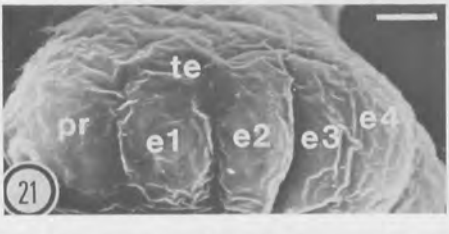


FIG. 15. Setae of the mandibular endopodite of the metanauplius II. Note the second-order setulae. (L 3). Scale : 10 µm.

FIG. 16. Monopodially ramified bristles on the dorsal surface of the labrum. (L 3). Scale : 10 µm.

FIG. 17. Ventral view of the post-mandibular region of a late metanauplius II (L 3). Scale : 100 µm.

FIG. 18. Ventral view of a metanauplius III (L 4). Scale : 100 µm.

FIG. 19. Ventral view of the post-mandibular region just after the 3rd exuvation of the larva (L 4). Scale : 100 µm.

FIG. 20. Ventral view of the post-mandibular region just before the 4th moult of the larva. Note the beginning bristle formation of the maxillules and the maxillae and the articulation of the thoracopods (L 4). Scale : 100 µm.

FIG. 21. Left thoracopod I of the metanauplius III (L 4). Scale : 10 µm.

rudiments of the adult mandibles expand without any further differentiation, while the lar mandibles still remain on the adult structures. Near the sagittal longitudinal axis of metanauplius III haired paragnaths develop in the form of globular protrusions forming a fo channel toward the mouth (Fig. 19). The maxillules and the maxillae enlarge and just before 4th exuvation some individuals show a beginning bristle formation at the distal tips of unjointed maxillules and maxillae (Fig. 20). Despite their morphological changes the maxillu and the maxillae play no part in food ingestion by the larva.

At the expanded post-maxillar region of the metanauplius III (Fig. 18) the thoracomers and IV are distinguishable by intersegmental furrows. Furthermore the rudiments of the thora pods II can be distinguished (Fig. 19 and 20), while the buds of the thoracopods I st articulation (Fig. 20). Until the 4th molt the extremities of the 1st trunk segment are divided i a protopodite and the telopodial endites (Fig. 21).

Metanauplius IV (L 5)

The procephalon of the metanauplius IV reveals the rudiments of the compound eyes rounded elevations dorsal to the antennules (Fig. 22). A bud of the 3rd antennula-seta-type develops at the tip of each antennule and the large labrum becomes increasingly disc-shap (Fig. 22). While the antennae of the larva exactly retain their organization, the larval mandib decrease in size and the rudiments of the adult mandibles bend toward the sagittal axis of larva. The stoma, which is covered by the flattened upper lip, shifts continuously from parantennal into a parmandibular position.

At the gnathocephalon the maxillules and the maxillae enlarge while their distal setae start formation of fine setulae (Fig. 23). In the post-maxillar region the thoracomers V and VI limited by intersegmental furrows, while the thoracomers III and IV develop limb buds (Fig. 2 The thoracopods II and in some cases the vestigial thoracopods III show a beginning articulati into the protopodite and the six telopodial endites, but only the 1st pair of trunk extremit possesses an exopodite and an epipodite.

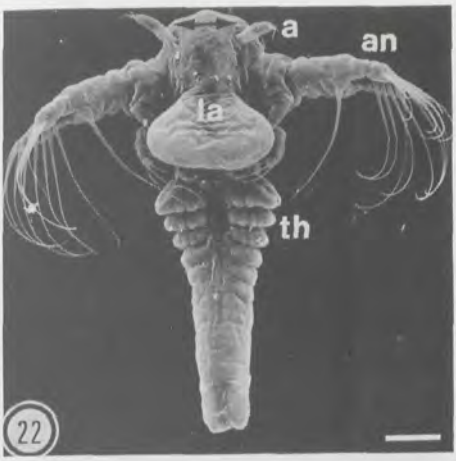
THE POST-METANAUPLIAR PERIOD

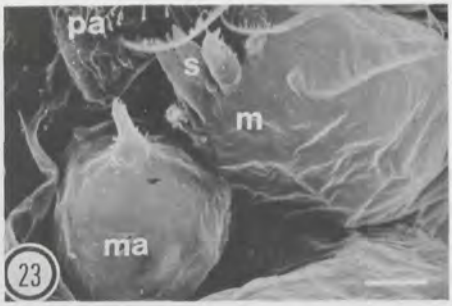
According to the definition of Kaestner (1967) the 5th exuvation finishes the metanaupl period and the larva of *Artemia* enters the post-metanaupliar period, which comprises sev developmental stages.

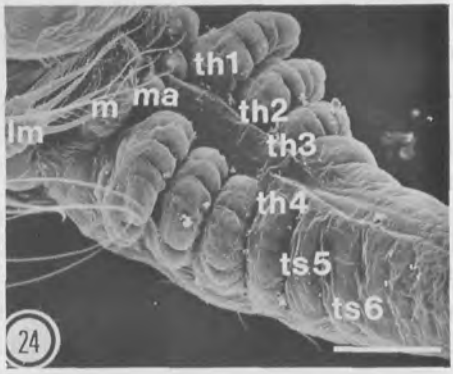
Post-metanauplius I (L 6)

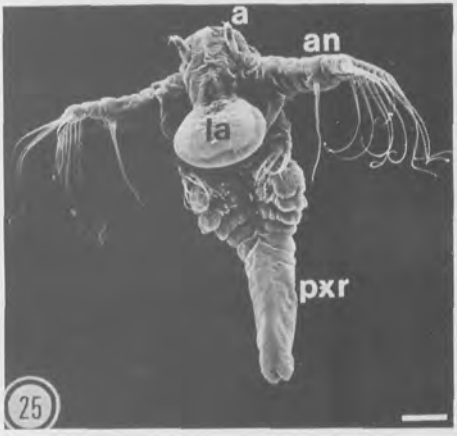
In the cephalon of the post-metanauplius I (Fig. 25) the rudiments of the 4th antennu seta-type II are visible at the tip of each antennule, while the 3rd setae type II start differentiati in a short, thickened base and a flagellum-like shaft. The reduction of the second-order setu of the protopodial gnathobasensetae and hook bristles of the antennae (Fig. 26) indicate beginning transformation of those extremities, which effects only insignificant morphologi changes during the following four stages of development.

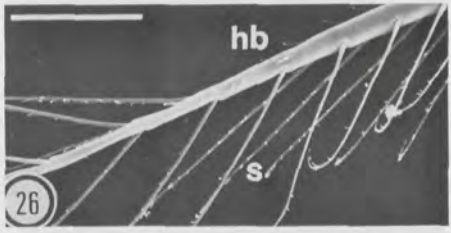
The larval mandibles decrease more and more in size while the adult extremities form cutt edges. Compared with the foregoing stage of development the maxillules and the maxillae ha strengthened and are active in transporting the filtered food particles through the paragna channel toward the mouth. In the post-maxillar region the thoracomers VII and VIII

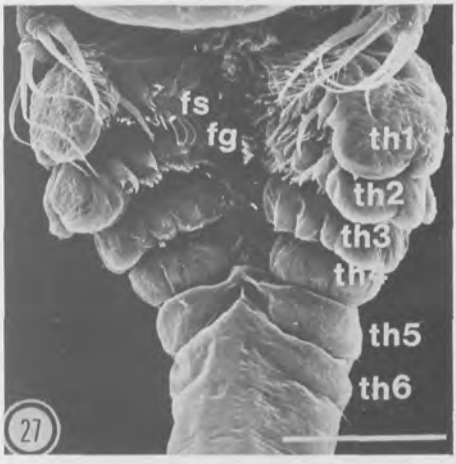












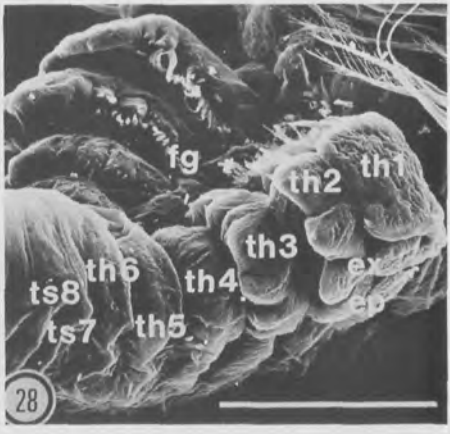


FIG. 22. Ventral view of a late metanauplius IV (L 5). Note the rudiments of the compound eyes (arrows). Scale : 100 μm.

FIG. 23. Maxillule and maxilla of a metanauplius IV (L 5). Scale : 10 μm.

FIG. 24. Ventro-lateral view of the post-mandibular region of a late metanauplius IV (L 5). Scale : 100 μm.

FIG. 25. Ventral view of an early post-metanauplius I (L 6). Scale : 100 μm.

FIG. 26. Distal portion of the hook bristle of the antenna. Note the beginning reduction of the second-order setulae. (L 6). Scale : 10 μm.

FIG. 27. Ventral view of the food groove of an early post-metanauplius I (L 6). Scale : 100 μm.

FIG. 28. Lateral view of the anterior portion of the post-maxillar region of an early post-metanauplius I (L 6). Scale : 100 μm.

distinguishable and the rudiments of the thoracopods V and VI can be seen. The thoracopods I and II are completely jointed and their protopodites and endites develop feathered filter-setae (Fig. 27 and 28). At the thoracopods III and IV the protopodites and endites are identifiable while the limb buds of the thoracomers V and VI still lack articulation. The thoracopods and the limb buds delimit a mid-ventral channel, the food groove (Fig. 27). Despite the activity of the first two pairs of trunk extremities in the production of swimming-feeding currents, the larval filtering apparatus is mainly responsible for the food collecting of the larva.

Post-metanauplius II (L 7)

The antennules of the post-metanauplius II (Fig. 29) are characterized by a complete supply of antennular setae. The tip of each antennule is provided with three antennula-setae-type I and four antennula-setae-type II similar in number to those of the adult organization. Despite the total reduction of the second-order setulae, the antennae are still active in locomotion and food gathering. The larval mandibles decrease in size, while the adult extremities develop cuticular teeth for a mechanical treatment of the filtered food (Schrehardt, 1987). The maxillules and the maxillae assume their final shape with seven to nine distal bristles and one distal seta, respectively (Fig. 30) ; during subsequent stages of development those extremities show only an increase in size without morphological changes.

The post-maxillar region presents 10 thoracomers with the first eight segments bearing extremities and limb buds (Fig. 31). The thoracopods I to IV are completely articulated and delimit the food groove. The filter-setae of those thoracopods form setular borders, a character which is exemplified in the development of each trunk extremity. The thoracopods V and VI are articulated into a protopodite with six and three to four endites, respectively, while the rudiments of the thoracopods VII and VIII are unjointed limb buds. The first four pairs of trunk extremities constitute multifunctional phyllopods, which are active in locomotion, in filter-feeding (Barlow and Sleigh, 1980), in osmoregulation (Copeland, 1967), and in respiration (Schrehardt, in prep.) (Fig. 32). Compared with the morphometric relations of the thoracopods of the post-metanauplius I the gills (=epipodites) increase in size. At the posterior terminus of the post-maxillar region a slight indentation indicates the formation of the furca ; the tip of each furcal branch bears a short seta.

Post-metanauplius III (L 8)

The compound eyes of the post-metanauplius III (Fig. 33) enlarge, but ommatidia as seen with the light microscope are not yet identifiable with the SEM. The upper lip of the larva changes in shape forming a trapezoid border. The antennae and the larval mandibles are characterized by an initial reduction of the setulae, while the other cephalic extremities show no remarkable changes in morphological characters.

In the post-maxillar region the 11th thoracomer and the 1st genital segment are delimited by intersegmental furrows so that the segmentation of the thorax is finished. The thoracopods V are completely jointed, while the rudiments of the 6th and 7th pairs of extremities differ in their degree of articulation. The thoracomers VIII to X bear undifferentiated limb buds.

Post-metanauplius IV (L 9)

The 4th post-metanaupliar stage (Fig. 35) is distinguished by several morphological characters. The median nose-shaped portion of the labrum is surrounded by a trapezoid border

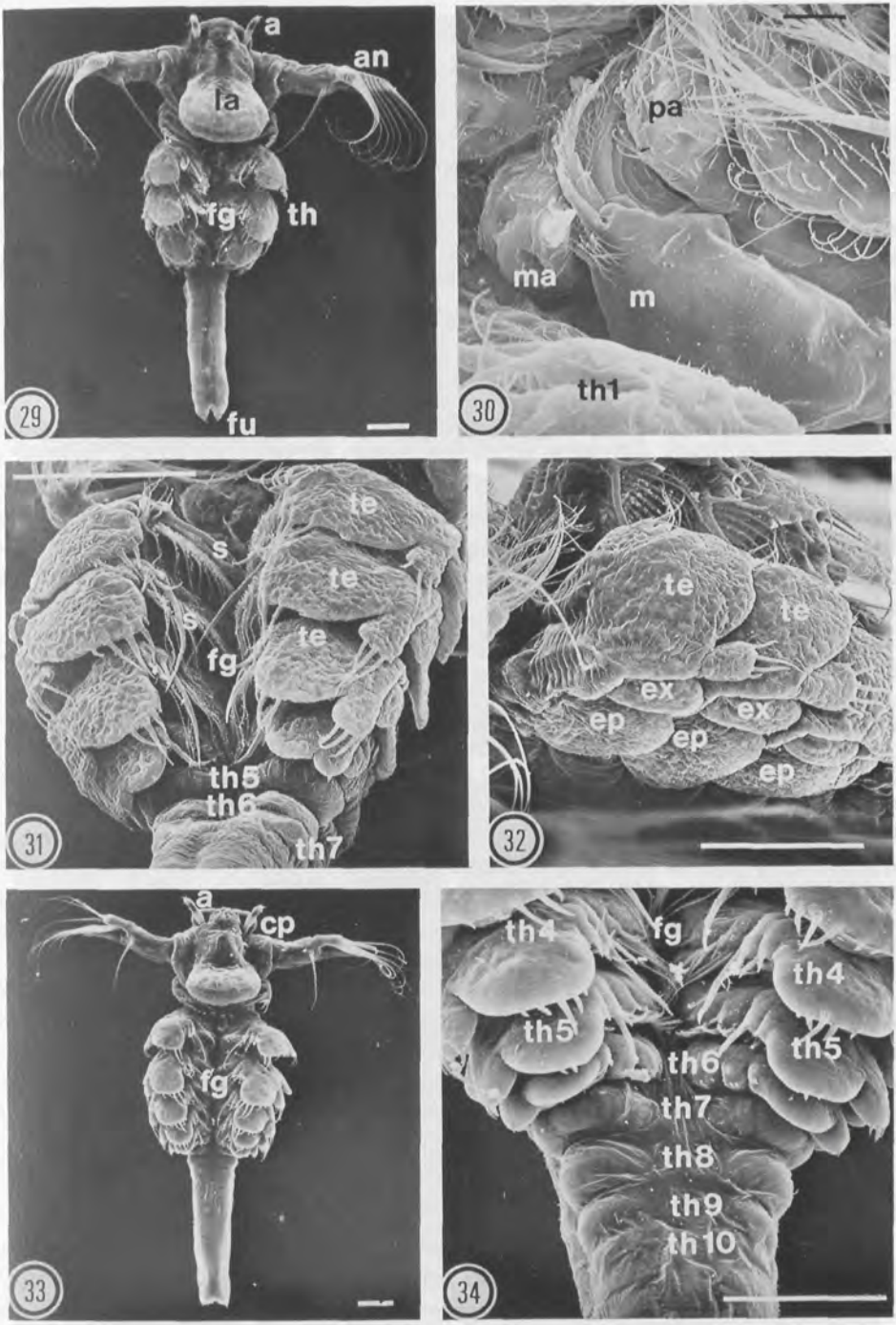


FIG. 29. Ventral view of a post-metanauplius II (L 7). Scale : 100 μm.
FIG. 30. Maxillule, maxilla, and paragnath of the gnathocephalon. (L 7). Scale : 10 μm.
FIG. 31. Food groove of a post-metanauplius II (L 7). Scale : 100 μm.
FIG. 32. Lateral view of the first three thoracopods. Note the increasing size of the gills (epipodites). (L 7). Scale : 100 μm.
FIG. 33. Ventral view of an early post-metanauplius III (L 8). Scale : 100 μm.
FIG. 34. Posterior portion of the thorax. Note the different stages of development of the thoracopods. (L 8). Scale : 100 μm.

(Fig. 36). At the tip of each antennule the shaft of the setae-type II increasingly attains the form of a bottle with a distal pore (Fig. 37). The rudiments of the compound eyes protrude and single ommatidia can be distinguished (Fig. 38). The thoracopods VI and VII finish their differentiation, while the 8th and 9th pairs of extremities start articulation and the thoracomer XI develops limb buds (Fig. 39). In the abdominal region the 2nd (=2nd genital segment) and 3rd abdominal segments are distinguishable.

Post-metanauplius V (L 10)

The ommatidia of the compound eyes of the larva (Fig. 40) multiply and initial reabsorption of the trapezoid border tissue of the labrum can be observed. Although the antennae of the post-metanauplius V are still active in the production of swimming-feeding currents, the reduction of the setulae of the endite setae and of the terminal bristles advances. The larval mandibles reduce in size accompanied by the reabsorption of setulae. The thoracopods VI and VII of the larva are completely supplied with feathered filter-setae, while the thoracopods VIII and IX finish articulation and the limb buds of the thoracomers X and XI start differentiation into a protopodite and the telopodial endites.

The abdominal segments IV and V are delimited by the formation of intersegmental furrows and each furcal ramus develops three to four feathered setae.

Post-metanauplius VI (L 11)

The 6th post-metanaupliar stage (Fig. 41) is characterized by the advanced reduction of larval structures. At the cephalon of the larva (Fig. 42) the trapezoid border tissue of the upper lip collapses, indicating an increasing reabsorption. The endites of the antennae are vestigial (Fig. 43) and the terminal setae, the gnathobasensetae and the hook bristles (Fig. 44) deteriorate in basal funnels (Fig. 45) so that the antennae cease their locomotory and filtering functions. The larval mandibles are only rudimentary extremities remaining on the adult structures (Fig. 46 and 47) and a distinction of the endopodite and the exopodite is no longer possible (Fig. 47).

The thoracopods X and XI finish articulation while the extremities of the thoracomers VIII and IX complete their supply of filter-setae. At the abdomen the segments VI and VII develop (Fig. 48 and 49) and, similar to the dorsal portion of the trunk segments, the abdominal segments III and IV start the formation of a pair of setae on their ventral surfaces. During subsequent development a pair of ventral setae develops on each abdominal segment. The furcal rami of the larva elongate (Fig. 50).

Post-metanauplius VII (L 12)

The 12th instar represents the last post-metanaupliar stage of development of *Artemia* (Fig. 51). The border tissue of the labrum is almost completely reabsorbed and the upper lip increasingly attains its definitive tongue-shape as seen in the adult organization. The terminal setae of the vestigial endopodite (Fig. 52) and exopodite (Fig. 53) of the antennae deteriorate, the gnathobasensetae are only rudimentary (Fig. 54), and the hook bristles are no longer demonstrable. The rudiments of the larval mandibles are only button-shaped tissue remnants on the adult structures (Fig. 55). The thoracopods X and XI present a complete supply of filter-setae and abdominal segment VIII and the telson are separated by the formation of an intersegmental furrow. Covered by the thoracopods, the buds of the penes (Fig. 56) appear as globular swellings

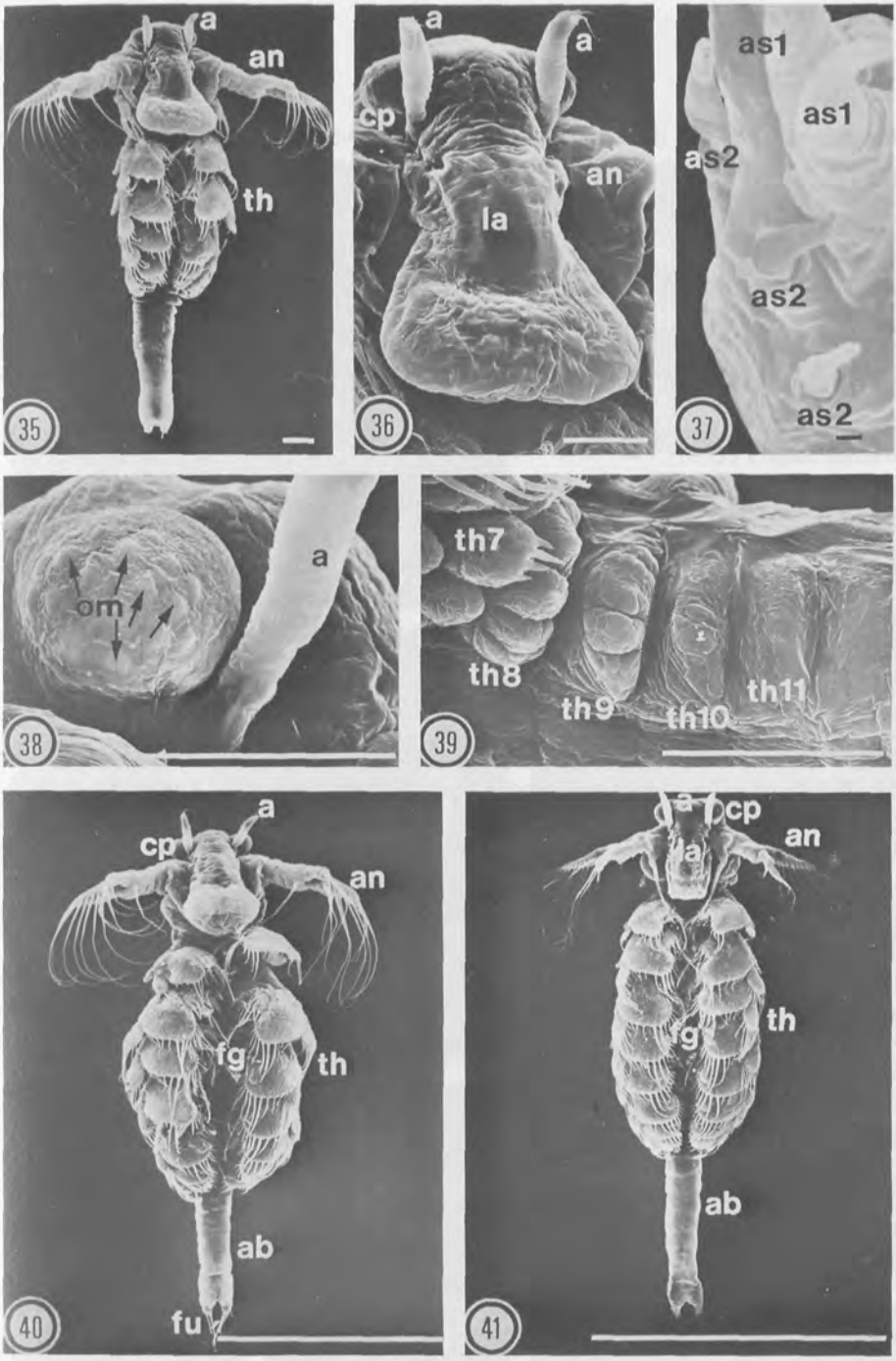


FIG. 35. Ventral view of a post-metanauplius IV (L 9). Scale : 100 μm.
FIG. 36. Cephalon of a post-metanauplius IV. Note the shape of the labrum. (L 9). Scale : 100 μm.
FIG. 37. Tip of an antennule. Note the two different types of antennular setae. (L 9). Scale : 1 μm.
FIG. 38. Rudiment of a compound eye of a post-metanauplius IV. Note the single ommatidia. (L 9). Scale : 100 μm.
FIG. 39. Lateral view of the posterior portion of the thorax. (L 9). Scale : 100 μm.
FIG. 40. Ventral view of a post-metanauplius V (L 10). Scale : 700 μm.
FIG. 41. Ventral view of a post-metanauplius VI (L 11). Scale : 1 mm.

on the ventral portion of the genital segments, while the ovisac is built by the fusion of ventral tissue lobes of the two genital segments (Fig. 57).

THE POST-LARVAL PERIOD

According to Kaestner (1967) the end of the post-metanaupliar period is fixed by the completion of the number of trunk somites and the beginning transformation of the antennae toward their definitive shape and function, so that the post-larval period, which comprises five stages of development, is characterized by the development of the antennae and the genital structures.

Post-larval stage I (L 13)

The compound eyes of the juvenile *Artemia* (Fig. 58) multiply their ommatidia and initial extension of the eye stalks can be distinguished. The antennae start shifting from a lateral into a ventro-lateral position and the vestigial terminal bristles of the antennae, which are still visible after the 12th exuvation, are totally reduced until the 13th molt (Fig. 59).

The buds of the penes and the rudiment of the ovisac enlarge and the furca develops more feathered setae, which multiply during subsequent developmental stages.

Post-larval stage II (L 14)

The juveniles of this developmental stage (Fig. 60) are characterized by the definitive tongue-shape of the upper lip and an advanced extension of the eye stalks. The protopodites of the antennae, which attain their final ventral position, are no longer visible and the endopodites are only stump-like tissue remnants at the bases of the exopodites. At the inner side of each antennal exopodite of the males the rudiment of a frontal knob develops (Fig. 61) and the basal portions of the antennal exopodites in both sexes are provided with setae (Fig. 62), which are similar in morphology to those of the trunk segments.

The ovisacs of the females (Fig. 64) and the penes of the males increase in size and the genitals of the males show the orifices of the vasa deferentia (Fig. 63). The ventral portion of the intersegmental furrow separating the genital segments is reduced, so that a ventro-lateral segment fusion is demonstrable (Fig. 65).

→

FIG. 42. Ventral view of the cephalon of a post-metanauplius VI. Note the shape of the labrum and the rudimental larval mandibles. (L 11). Scale : 100 µm.

FIG. 43. Left antenna of a post-metanauplius VI. Note the reduction of the gnathobasenseta and the hook bristle. (L 11). Scale : 100 µm.

FIG. 44. Hook bristle of the antenna. (L 11). Scale : 10 µm.

FIG. 45. "Basal funnel" of the hook bristle. (L 11). Scale : 1 µm.

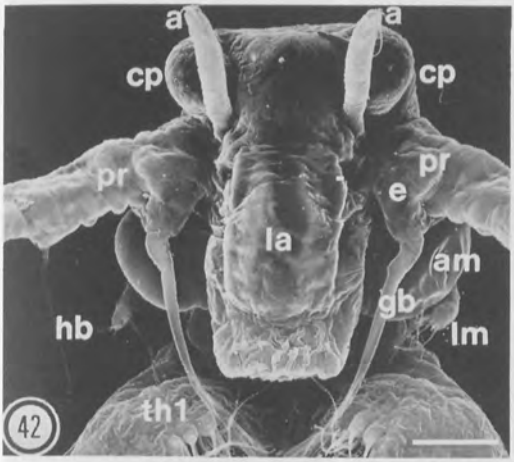
FIG. 46. Adult and vestigial larval mandibles. (L 11). Scale : 100 µm.

FIG. 47. Rudimentary larval mandible ; a distinction of the endopodite and the exopodite is impossible. (L 11). Scale : 10 µm.

FIG. 48. Lateral view of the abdomen. (L 11). Scale : 100 µm.

FIG. 49. Ventral view of the abdomen. (L 11). Scale : 100 µm.

FIG. 50. Telson and furca of a post-metanauplius VI. (L 11). Scale : 100 µm.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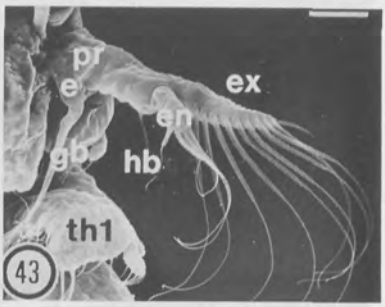
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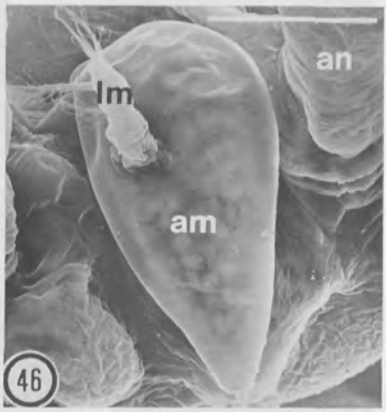
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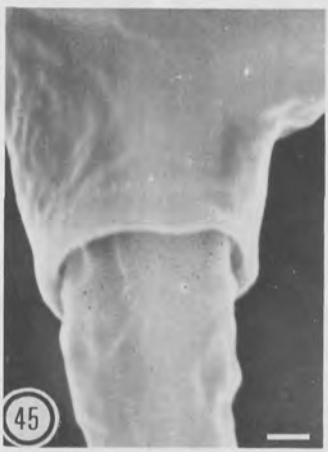
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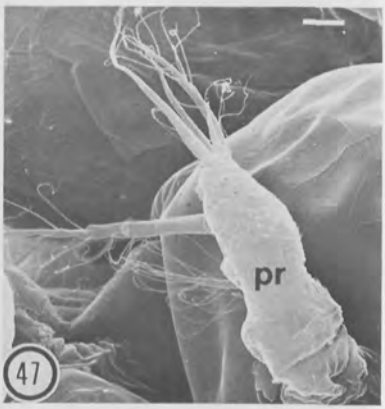
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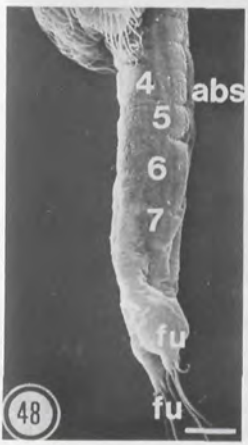
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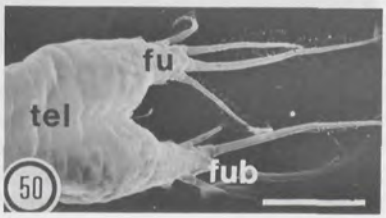
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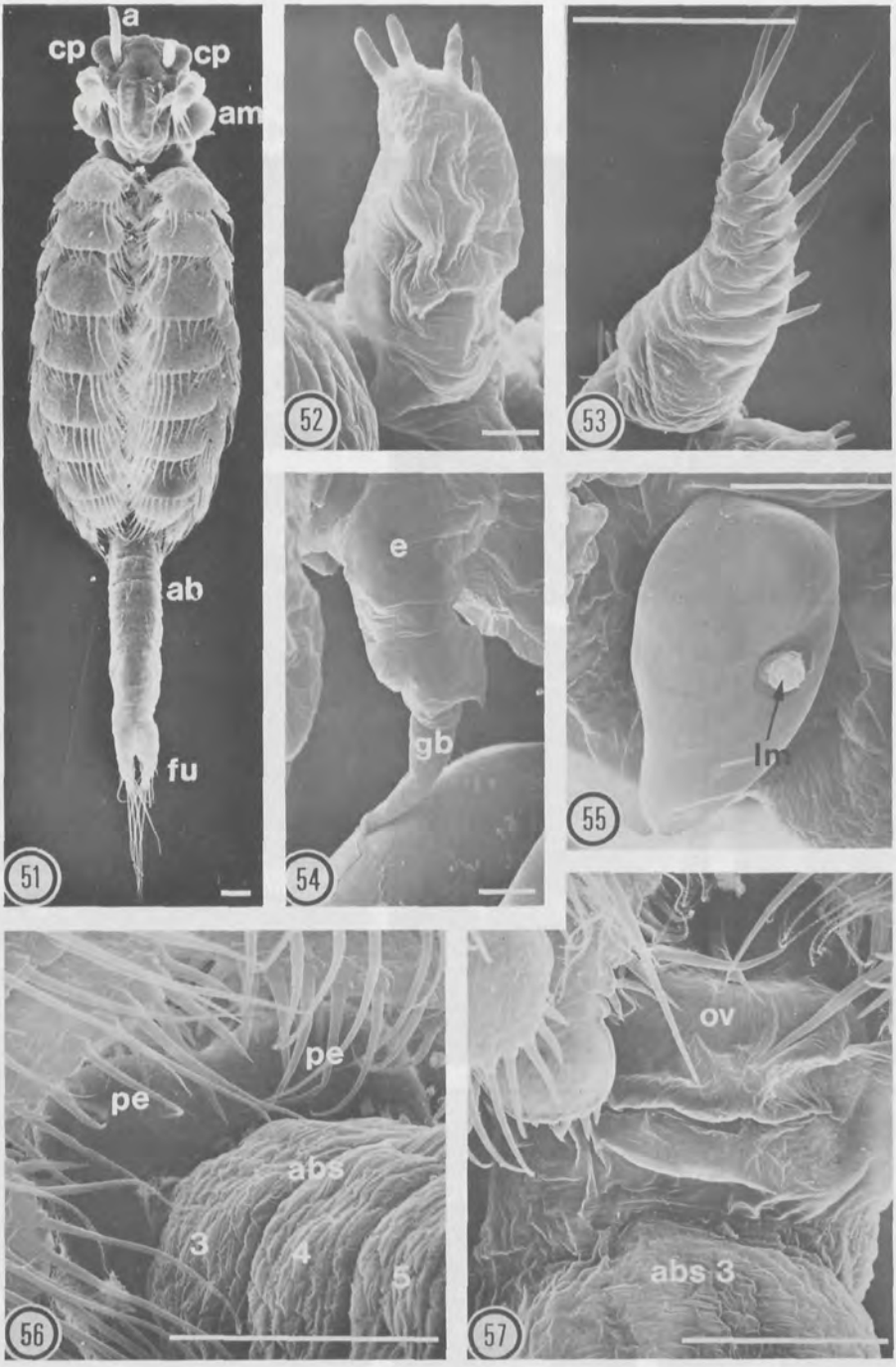

FIG. 51. Ventral view of a post-metanauplius VII (L 12). Scale : 100 µm.

FIG. 52. Endopodite of the antenna. (L 12). Scale : 10 µm.

FIG. 53. Exopodite of the antenna. Note the reduction of the terminal setae. (L 12). Scale : 100 µm.

FIG. 54. Protopodial endite of the antenna. Note the vestigial gnathobasenseta. (L 12). Scale : 10 µm.

FIG. 55. Adult mandible with a button-shaped tissue rudiment of the larval mandible. (L 12). Scale : 100 µm.

FIG. 56. Buds of the penes of a male post-metanauplius VII (L 12). Scale : 100 µm.

FIG. 57. Female post-metanauplius VII : formation of the ovisac. (L 12). Scale : 100 µm.

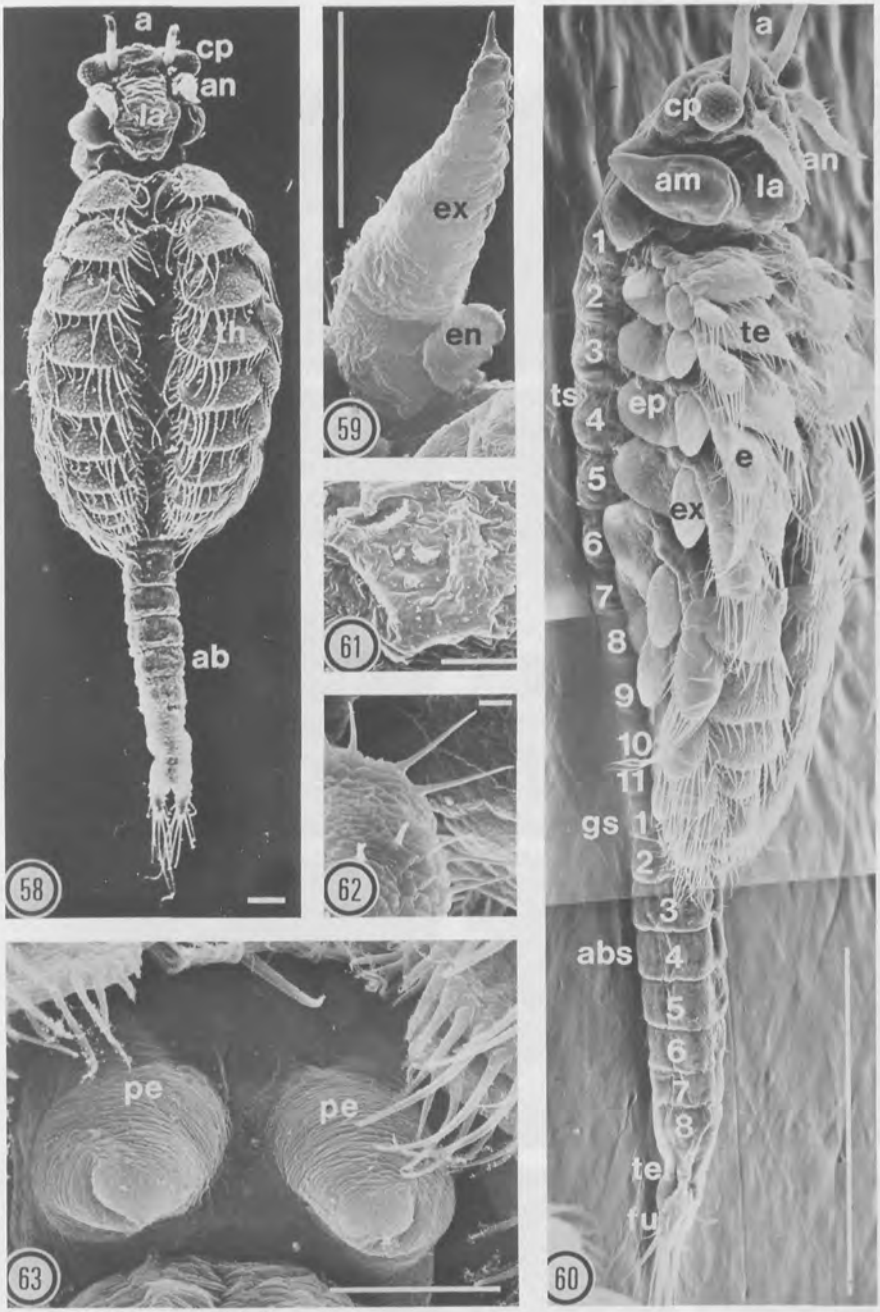

FIG. 58. Ventral view of the 1st post-larval stage (L 13). Scale : 100 µm.
FIG. 59. Antenna of the 1st post-larval stage (L 13). Scale : 100 µm.
FIG. 60. Lateral view of the 2nd post-larval stage (L 14). Scale : 1 mm.
FIG. 61. Rudiment of the frontal knob of a male. (L 14). Scale : 10 µm.
FIG. 62. Setae of the antenna. (L 14). Scale : 10 µm.
FIG. 63. Penes of a male. Note the openings of the vasa deferentia. (L 14). Scale : 100 µm.

Post-larval stage III (L 15)

Compared with females, males of the 3rd post-larval stage are characterized by sickle-shaped antennal exopodites (Fig. 66). In both sexes the antennae are tied together by a basal tissue pons covering the basal portion of the labrum. The frontal knob of the males enlarges and initial formation of cuticular spines can be noticed, while the endopodite stump decreases in size (Fig. 67).

Post-larval stage IV (L 16)

A part of the dorsal surface of the cephalon deepens to form a transverse cervical groove joining the basal portions of the adult mandibles. Its morphological significance is the strengthening of the attachment site for the dorsal suspension of tendons and muscles.

The antennal exopodites of the males develop a secondary hindge joint (Fig. 68) which is absent in females. The frontal knobs of the males (Fig. 70) enlarge and cuticular spines (Fig. 71) and the rudiments of mechanoreceptors (Wolfe, 1980) appear. In both sexes the antennal setae (Fig. 69) increase in number and the endopodites of the antennae are totally reduced.

Post-larval stage V (L 17)

The 5th post-larval stage differs only in a few morphological characters from the adult organization. The eye stalks of the compound eyes extend and the antennal exopodites of the males increase rapidly in size forming the typical claspers (Fig. 72), while the antennal exopodites of the females show no noteworthy morphological changes. The frontal knobs of the males enlarge and develop ramified and non-ramified spines, which occur singly or in pairs ; occasionally three spines arise from the same region. Only a few individuals show tissue remnants of the larval mandibles laterally on the adult extremities.

The genitals of males and females expand and the ovisacs of the females form a spine and an ovisac button on each lateral side.

THE ADULT ORGANIZATION

The 17th molt finishes the post-embryonic development and both sexes obtain the adult organization (Fig. 73 and 74).

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FIG. 64. Ovisac of a female. (L 14). Scale : 100 µm.

FIG. 65. Lateral view of the anterior portion of the abdomen. The arrow indicates the ventro-lateral segment fusion of both genital segments. (L 14). Scale : 100 µm.

FIG. 66. Cephalon of a male post-larval stage III. Note the shape of the antennae. (L 15). Scale : 100 µm.

FIG. 67. Basal portion of the antenna of a male. Note the frontal knob and the rudiment of the endopodite. (L 15). Scale : 10 µm.

FIG. 68. Cephalon of a male post-larval stage IV. Note the formation of secondary joints at the antennae. (L 16). Scale : 100 µm.

FIG. 69. Setae of the antennae. (L 16). Scale : 10 µm.

FIG. 70. Frontal knob of a male (L 16). Scale : 10 µm.

FIG. 71. Cuticular spines of the frontal knob. (L 16). Scale : 1 µm.


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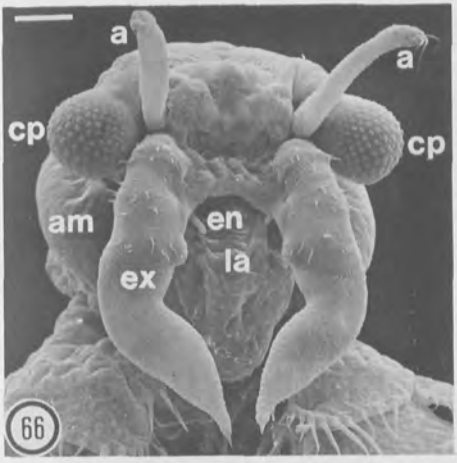
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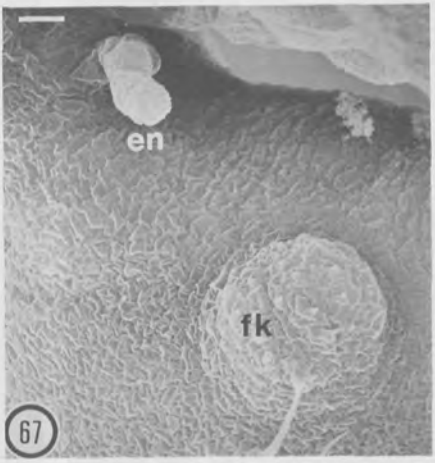
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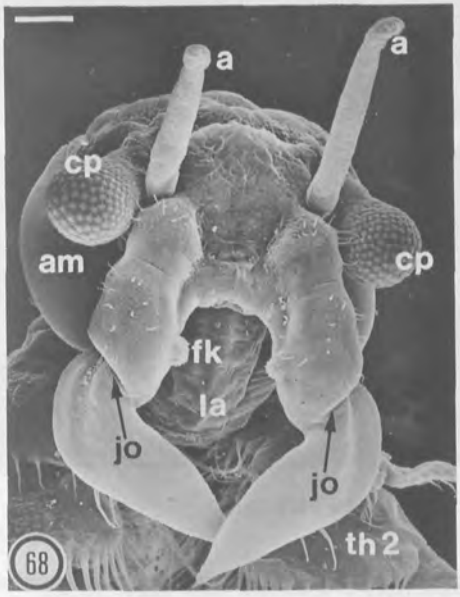
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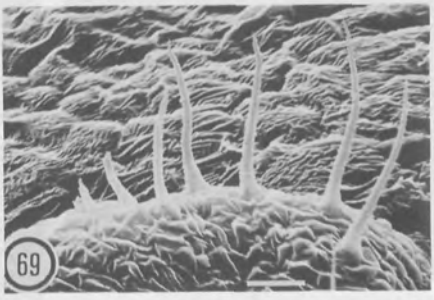
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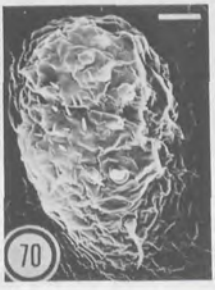
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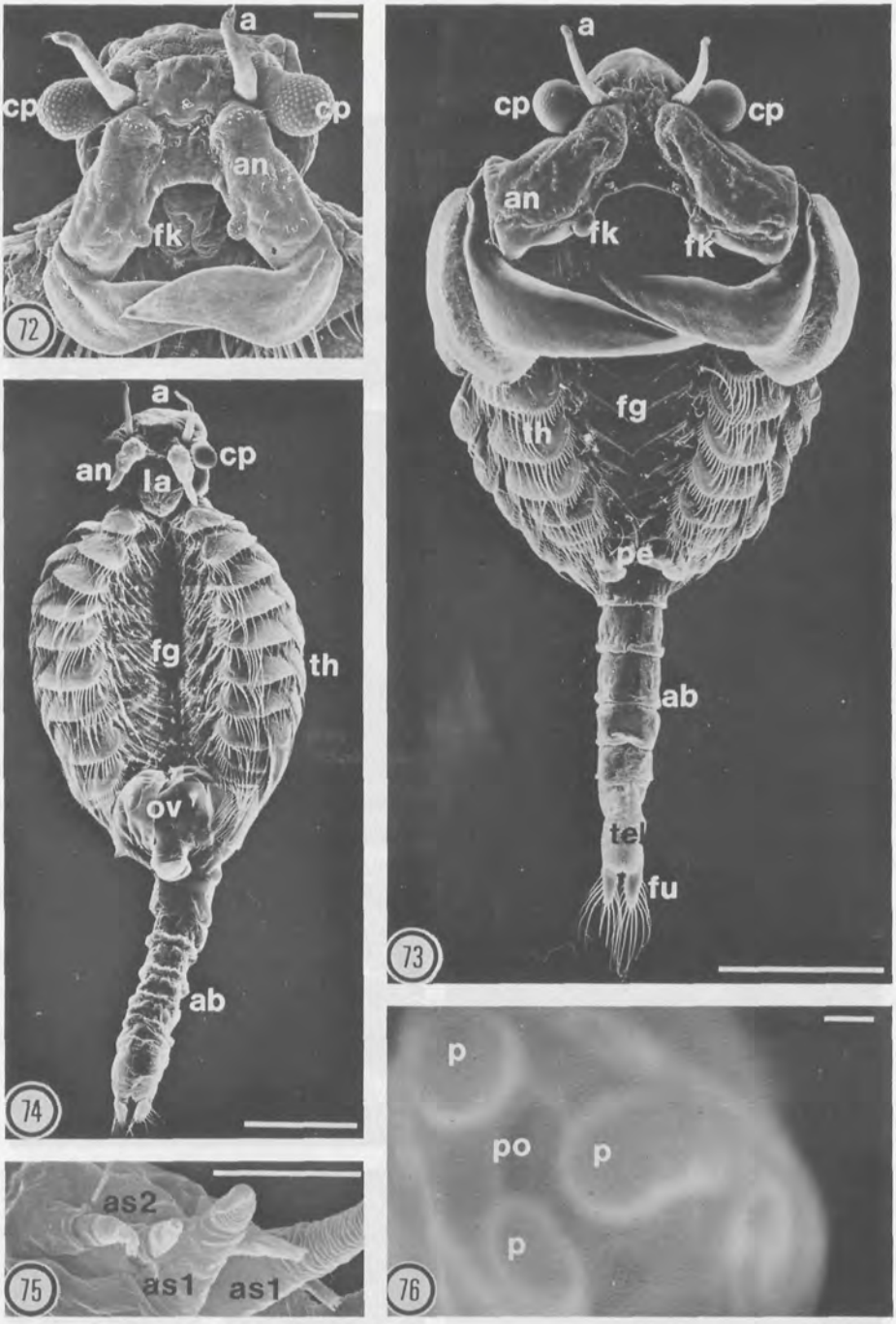


FIG. 72. Cephalon of a male post-larval stage V (L 17). Scale : 100 µm.

FIG. 73. Ventral view of an adult male. Scale : 1 mm.

FIG. 74. Ventral view of an adult female. Scale : 1 mm.

FIG. 75. Tip of an antennule. Note the two different types of antennula setae. Scale : 10 µm.

FIG. 76. Tip of an antennula-seta-type II. Note the pore which is surrounded by three protuberances. Scale : 100 nm.

The cephalon of *Artemia* presents five pairs of extremities : the antennules, the antennae, the mandibles, the maxillules, and the maxillae. The antennules are unjointed, uniramous appendages each bearing three setae-type I and four setae-type II at its tip (Fig. 75). While the antennula-setae-type I are possible mechanoreceptors, a distal pore (Fig. 76) indicates a possible chemoreceptive function of the antennula-setae-type II (Tyson and Sullivan, 1979). At the base of the antennules the eye stalks arise (Fig. 77) bearing distally the compound eyes which are composed of about 200 ommatidia each (Fig. 78 ; Hertel, 1980). The antennae of *Artemia* are sexually dimorphic, forming the typical claspers with an hinge joint in males, while inconspicuous in females. In both sexes the basal portions of the antennae are tied together by a basal tissue pons and bordered with antennal setae, which are composed of a central shaft surrounded by one or two circles of protuberances (Fig. 79). In males each antenna shows a basal frontal knob (Fig. 80) oriented toward the sagittal axis of the body. Each frontal knob forms cuticular spines (Fig. 82) and mechanoreceptors (Wolfe, 1980), which play a role in the precopulatory and the copulatory activities.

The nose-shaped labrum covers the extremities of the gnathocephalon. The large mandibles (Fig. 83) grind the filtered food between the cuticular teeth of their molar surfaces, while the uniramous maxillules and maxillae transport food particles through the paragnath channel (Fig. 84) toward the mandibles. Each maxilla and each maxillule bear one and seven to nine distal feathered setae, respectively (Fig. 84).

The thorax of *Artemia* comprises 11 thoracomers bearing one pair of extremities each. The thoracopods are multifunctional phyllopods (Fig. 85 and 86) showing a division of labour : they are active in locomotion, food gathering (protopodites, telopodial endites ; Barlow and Sleigh, 1980 ; Schrehardt, 1987), osmoregulation (exopodites ; Copeland, 1967), and respiration (epipodites ; Schrehardt, in prep.).

The abdomen contains eight segments and the telson ; the first two abdominal segments are the genital segments showing a ventro-lateral segment fusion. The paired penes (Fig. 87) and the ovisac (Fig. 88 and 89) arise from the ventral surface of the genital segments. The ovisac can treble its volume during the storage of eggs and cysts (Fig. 88). The ovisac bears two cuticular spines (Fig. 90) and two "ovisac buttons" (Fig. 91) forming cuticular ledges (Fig. 92). Both structures attach at the lateral sides of the ovisac (Fig. 88). The terminal telson exhibits two furcal rami bearing 12 to 15 feathered setae each. As a result of the mobility of the abdomen the furca functions as a hydroplane during locomotion.

The dorsal surface of each thoracomer and abdominal segment gives rise to one pair of segmentally arranged setae. Those paired setae occur also on the ventral surface of the abdominal segments III to VIII and are similar in morphology to the setae of the basal portions of the antennae. A central shaft without pores is surrounded by up to seven protuberances. Tyson and Sullivan (1980) discuss a sensory function of those setae but the ultrastructural evidence is still lacking.

Discussion

Cephalon

The morphological organization of the hatching stage of *Artemia* is similar to the nauplii of other Euanostracan genera. The procephalon of the larva bears two pairs of cephalic appendages

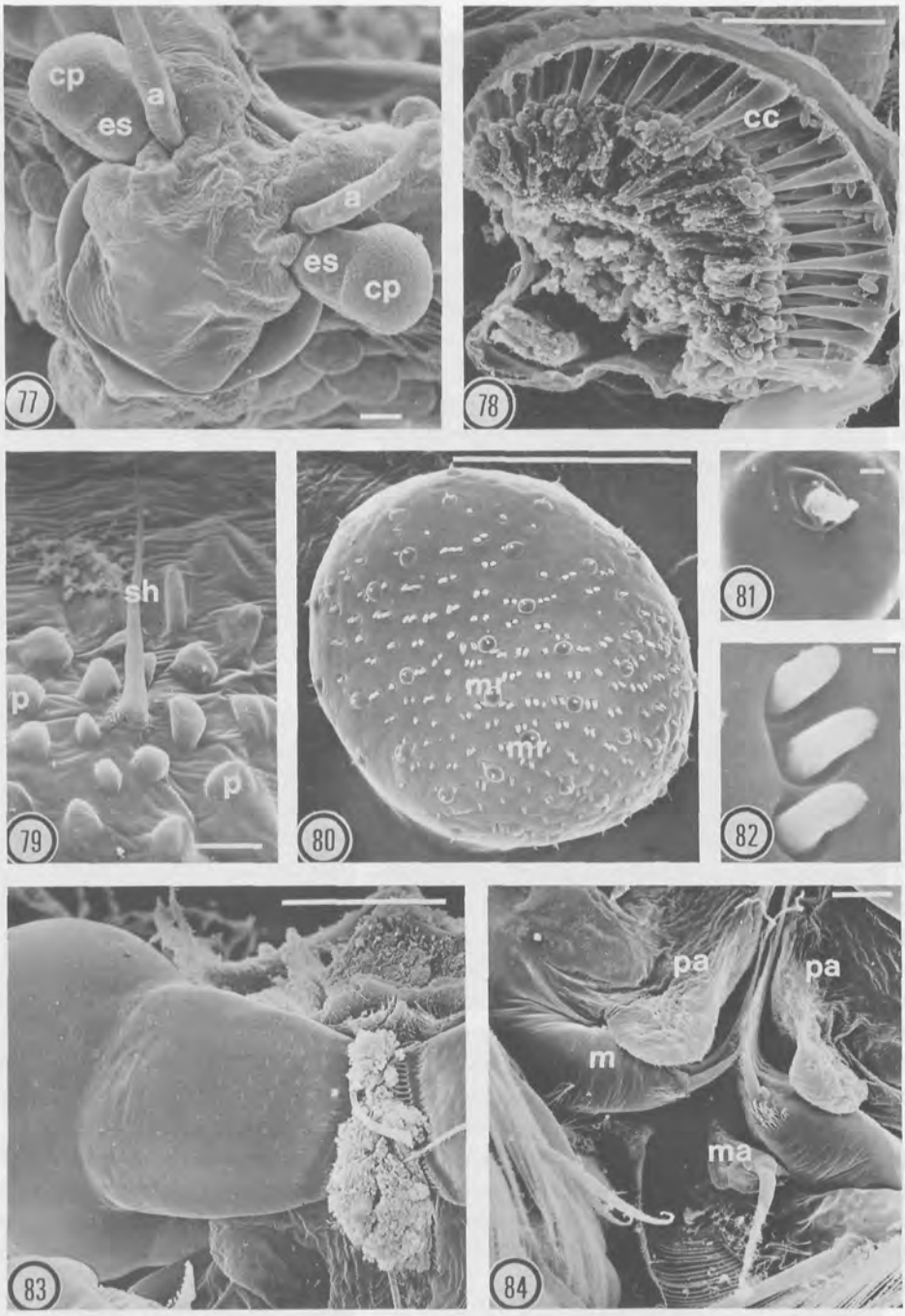
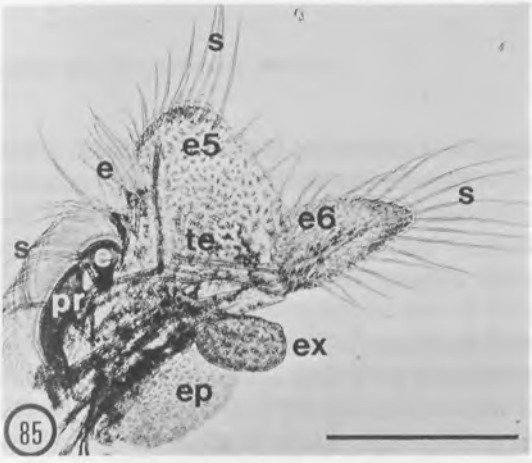
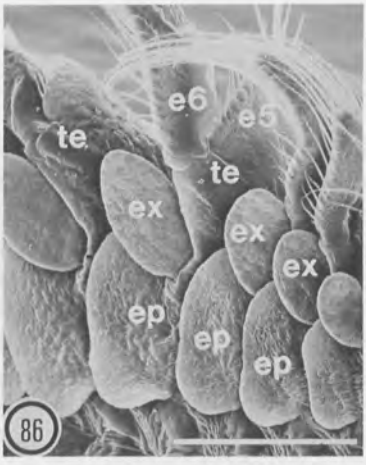
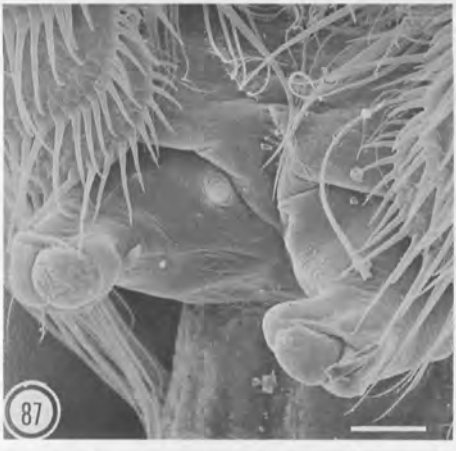
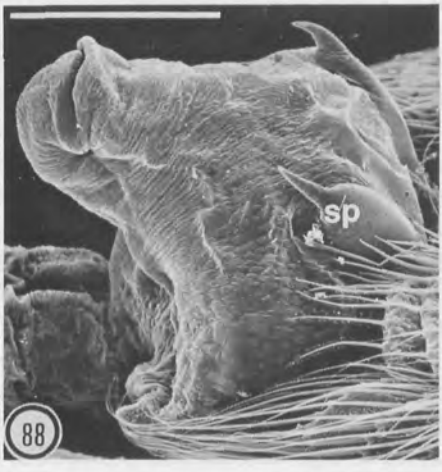


FIG. 77. Frontal view of the cephalon of an adult male. Note the eye stalks. Scale : 100 µm.
FIG. 78. Fracture through the compound eye of *Artemia*. Note the crystal cones. Scale : 100 µm.
FIG. 79. Seta of the basal portion of the antenna. Scale : 10 µm.
FIG. 80. Frontal knob of an adult male. Scale : 100 µm.
FIG. 81. Mechanoreceptor of the frontal knob. Scale : 1 µm.
FIG. 82. Cuticular spines of the frontal knob. Scale : 1 µm.
FIG. 83. Mandibles after removal of the labrum. Scale : 100 µm.
FIG. 84. Maxillules, maxillae, and paragnaths channel of an adult female. Scale : 10 µm.









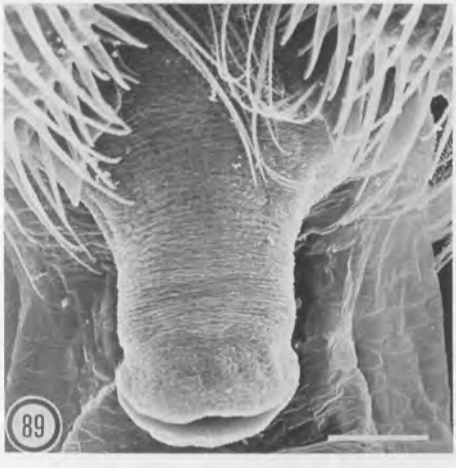


FIG. 85. Thoracopod. Scale : 500 μm.

FIG. 86. Lateral view of the gills (epipodites) and the exopodites, which are active in osmoregulation. Scale : 500 μm.

FIG. 87. Paired penes of an adult male. Scale : 100 μm.

FIG. 88. Ovisac of an adult female. Note the spines of the ovisac. Scale : 500 μm.

FIG. 89. Empty ovisac of an adult female. Scale : 100 μm.

FIG. 90. Ovisac spine. Scale : 100 μm.

FIG. 91. Ovisac button. Scale : 100 μm.

FIG. 92. Cuticular ledges of an ovisac button. Scale : 10 μm.

(antennules, antennae), while the gnathocephalon presents only the larval mandibles. According to Siewing (1963) the procephalon of the arthropods comprises three cephalic segments : the protocephalon (=preantennal segment), the deutocephalon, and the tritocephalon. In the nauplius of *Artemia* there are no morphological characters indicating the preantennal segment, which was demonstrated as an embryonal segment in *Artemia* by Benesch (1969) using histological methods.

The uniramous deutocephalic antennules are common in Euanostracan nauplii. Those jointed extremities bear two (Gauld, 1959 ; Baid, 1967) or three distal antennular setae (Heath, 1924 ; Hentschel, 1968), which are described as antennula-setae-type I by Tyson and Sullivan (1979). The antennular setae lack any innervation both in nauplii and in subsequent stages of development of *Streptocephalus seali* (Baquai, 1963), while Tyson (unpubl., cited in Tyson and Sullivan, 1979) found an innervation of those setae in *Artemia*. Tyson and Sullivan (1979) proposed a mechanoreceptive function for the setae type I, but detailed physiological and TEM-studies are required to clarify the functional significance of those structures. During the metanaupliar and the early post-metanaupliar period, *Artemia* develops four antennula-setae-type II characterized by a distal pore, which might be active in chemoreception (Tyson and Sullivan, 1979). The formation of setae similar to the antennula-setae-type II of *Artemia* was described in *Chirocephalus nankinensis* by Hsü (1933) and in *Streptocephalus seali* by Baquai (1963), who demonstrated an innervation of those structures. Despite the histological (Baquai, 1963) and the morphological data (Tyson and Sullivan, 1979), the physiological significance of the antennal setae type II is yet unknown.

The tritocephalic antennae of *Artemia* shows morphological and functional changes during the post-embryonic development. The biramous larval structures are divided into a protopodite, an endopodite, and an exopodite. Heath (1924), Anderson (1967), and Hentschel (1968) described an unjointed antennal protopodite in *Artemia* similar in morphology to the antennal protopodites in the larvae of *Branchinecta occidentalis* (Heath, 1924), *Chirocephalus sybillae* (Mura and Calzecchi-Onesti, 1981), *Streptocephalus dichotomus* (Pai, 1958 ; Bernice, 1972), and *Streptocephalus seali* (Baquai, 1963). Only Gauld (1959) and Baid (1967) demonstrated an articulated protopodite in the nauplius of *Artemia*. Contradictory to the findings of Gauld (1959) and Baid (1967), the present study reveals that the protopodite, the endopodite, and the exopodite of the larval antenna in *Artemia* lack true limb limits, but the cuticular spines of those extremities might be morphological characters of a vestigial articulation. Following that explanation, the antennal protopodite of the *Artemia* nauplius is divided into a praecoxa, a coxa, and a basis, while the endopodite comprises ischium, merus, carpus, propodus, and dactylus.

During the metanaupliar period the larval antennae differentiate into a part of the larval filtering apparatus characterized by the formation of setulae of first and second order. Those morphological changes are followed by the reduction of the protopodite and the endopodite, the shift of the "antennae" from a lateral into a ventro-lateral position, and a morphological and functional transformation of the exopodite. In males the antennal exopodites increase rapidly in size and develop a secondary hinge-joint resulting in the typical claspers. These findings differ from those of Heath (1924) and Baquai (1963) for *Artemia* and *Streptocephalus seali*, respectively, who described remaining protopodites representing the basal portions of the male claspers. Based on the unbroken post-embryonic data, the present study disproves the results of Heath (1924) and evidences the total reduction of the protopodites and the secondary formation of the hinge joints of the male claspers. Those morphological transformations are combined with a

functional change from filtering to clasping. In contrast to males, the antennal exopodites of the females show only a minor enlargement forming supports for receptive structures.

Contradictory opinions exist furthermore about the morphology and the development of the mandibles. Most authors have described the larval mandibles of *Artemia* as uniramous extremities (Heath, 1924 ; Gauld, 1959 ; Anderson, 1967 ; Baid, 1967), while Hentschel (1968) demonstrated a typical biramous appendage as seen in the present study. The terminal setae of the mandibular endopodite do not attach at the main axis of the extremity, so that a vestigial endopodite can be observed. In contrast to the antennae, the larval mandibles are totally reduced and, during the metanaupliar period, the adult extremities develop to form cutting edges provided with cuticular teeth. Although Heath (1924) described the adult mandibles in *Artemia*, his study lacked developmental data for those extremities, so that only the accounts of Oehmichen (1921) and Baquai (1963) on *Branchipus grubei* and *Streptocephalus seali*, respectively, are available. Both authors pointed out a transformation of the protopodial coxae of the larval mandibles into the adult extremities. The present developmental succession shows the transitory nature of the larval mandibles and the new formation and differentiation of the adult extremities, so that the findings of Oehmichen (1921) and Baquai (1963) seem to be doubtful. Correlated with the continuous exchange of the larval and the adult mandibles the function of the extremities changes from filtering to grasping of food particles.

The 2nd and 3rd gnathocephalic segments bear the uniramous maxillules and maxillae. A few authors have described the bisection of the maxillae in *Artemia* (Heath, 1924 ; Anderson, 1967 ; Baid, 1967), in *Chirocephalus nankinensis* (Hsü, 1933), in *Branchipus grubei* (Oehmichen, 1921) and in two species of *Streptocephalus* (Pai, 1958 ; Baquai, 1963). This bisection has been disproved by detailed studies on 12 different Euanostracan genera (various authors cited by Linder, 1941), which possess unjointed maxillae, consistent with the findings of the present paper.

Thorax

The thorax of *Artemia* comprises 11 thoracomers bearing one pair of thoracopods each. Despite the concurring data on the number of thoracal segments and extremities, the mode and the timing of their post-embryonic development are still discussed. Fränsemeier (1940) supported an ectodermal activity in the segment formation, while Weisz (1947) and Anderson (1967) attributed the development of the thoracomers to the mesoderm. The detailed ontogenetic study of Benesch (1969) showed that the segment formation cannot be explained by a single germ layer, *i.e.* both the ectoderm and the mesoderm are responsible for the segment formation in *Artemia*. In spite of the multitude of histological findings, data on the timing of the post-embryonic development of the thoracomers and the thoracopods in *Artemia* vary widely. Baid (1967) described a complete differentiation of the thoracopods until the 5th molt, whereas Heath (1924) pointed out that the differentiation of the extremities is finished at the 7th developmental stage. According to Hentschel (1968), the last pair of thoracopods is completely developed at the post-metanaupliar stage VII, which is equivalent to the 12th instar. The results of Hentschel (1968) are supported by the results of the present study. A detailed comparison of the data on the timing of developmental events in *Artemia* seems to be impossible, because temperature (Hentschel, 1968) and salinity (Weisz, 1946) of the culture medium influence the post-embryonic progress. Most of the studies cited lack, however, information on the culture

conditions, the geographical origin of the "*Artemia* species" used, and the mode of determination of the single stages of development.

Each thoracopod of *Artemia* is articulated in a protopodite, a telopodite with six endites, an exopodite and usually one epipodite (Siewing, 1959/60 ; Schrehardt, in prep.). The Euanostracan thoracopods are turgid extremities, which are quite different from the thoracopods of the Phyllopoda (Preuss, 1957). The thoracic appendages of *Artemia* are multifunctional extremities showing a division of labor : the protopodites and the endites are used for food gathering (Barlow and Sleigh, 1980 ; Schrehardt, 1987), the exopodite is active in osmoregulation (Copeland, 1967) and the epipodite functions as a gill (Schrehardt, in prep.). The unbroken post-embryonic data show that the larval filtering apparatus is continuously replaced by the filter-mechanism of the thoracopods. Larval antennae and mandibles are used to assist the developing trunk extremities in locomotion and filter-feeding up to and including the time when all thoracopods are functional as described for *Branchinecta ferox* (Fryer, 1983). These results disprove the findings of Anderson (1967), who pointed out that the first six pairs of thoracopods become functional simultaneously.

Abdomen

There exist contradictory data about the number of abdominal segments in the adult organization of the Euanostraca. Baid (1967) described seven abdominal segments in *Artemia*, while Hsü (1933) and Baquai (1963) pointed out nine abdominal segments in *Chirocephalus nankinensis* and *Streptocephalus seali*, respectively. Corresponding with Heath (1924), Linder (1941), Siewing (1959/60), and Hentschel (1968), the present study identifies eight abdominal segments and the telson. The ventro-lateral fusion of the first two abdominal segments (=genital segments) and the distinct intersegmental furrow delimiting the eighth abdominal segment and the telson might be the reasons for the misleading interpretations of Hsü (1933), Baquai (1963), and Baid (1967).

Phylogenetic level of the adult organization

The majority of previous authors (*e.g.*, Tasch, 1963) united the Euanostraca, the Cephalocarida, and the Phyllopoda to the superorder Branchiopoda or described the Euanostraca as a subgroup of the Phyllopoda. The comparative morphological studies of Preuss (1951, 1957) and the detailed analysis of the relationships within those crustacean groups of Siewing (1959/60) showed the systematic position of the Euanostraca as the most developed Gnathostraca. These phylogenetic conclusions were critized, *e.g.* by Kaestner (1967), who pointed out the characters that indicated the primitive organization of the Euanostraca : the uniform segmentation of the trunk, the segmental nervous system, and the long heart with the segmentally arranged ostia.

Vehstedt (1941) studied the heart of *Artemia* at the light microscopical level and emphasized the primitive character of that tube-shaped organ. Electron microscopical investigations of the hearts of four Euanostracan species by Økland *et al.* (1982) disproved the conclusions of Vehstedt (1941). Økland *et al.* (1982) showed that the heart constitutes a pseudo-tube along most of its length and the ventral and the lateral parts of the heart are built up by a single layer, the myocardium, while the organ lacks an endocardium and an epicardium. Furthermore, these authors proved the dorsal part of the Euanostracan heart to be formed for most of its length by the basal lamina of the epidermis. Based upon the ultrastructural data, Økland *et al.* (1982) pointed out that the heart of the Euanostraca is more strictly organized than the heart of

Phyllopod crustaceans, *e.g.* the Notostraca. Following the homology criterion of structure and the homology criterion of transitional forms of Remane (in Siewing, 1969), the ultrastructure of the Euanostracan heart represents a character indicating the phylogenetically derived organization of that crustacean group.

The statement of Kaestner (1967) that the uniform segmentation of the Euanostraca indicates their phylogenetically primitive organization was disproved by the results of Linder (1941), Siewing (1959/60), and the post-embryonic data of the present study, which show a division of the Euanostracan body into cephalon, thorax, and abdomen.

The last primitive character cited by Kaestner (1967) is the nervous system, which was studied by Warren (1930). The lack of recent investigations of the nervous system allow no comparative evaluation of that character.

To summarize the above data, the complicated post-embryonic development, especially the transformations of the antennae and the mandibles, the highly differentiated thoracopods, and the ultrastructure of the heart, all argue for the phylogenetically derived organization of the Euanostraca.

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Ultrastructural investigations of the filter-feeding apparatus and the alimentary canal of *Artemia*

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Abstract

Scanning and transmission electron-microscopy were used to study the filtering apparatus and the alimentary canal of the adult brine schrimp. *Artemia* collects bacteria and unicellular algae by filter-feeding, the setular mesh of the trunk limbs functioning as a sifter. The filtered food is transported toward the mandibles, ground by those mouth parts and ingested through the stoma.

The alimentary canal of *Artemia* is composed of three major sections. The oesophagus and the hindgut are lined by a thin cuticle, while the midgut forms an apical brush border. All epithelial cells of the midgut, including the cells forming the hepatopancreas, are of a single basic type. The midgut epithelium is active in absorption, storage of glycogen and lipid, osmoregulation, and the secretion of digestive enzymes. The possible mechanism of enzyme secretion is discussed.

Introduction

The brine shrimp *Artemia* is a typical inhabitant of salt lakes and brine ponds, which are found all over the world. *Artemia* feeds on suspended bacteria and unicellular algae by filter-feeding, a mechanism which has been carefully studied (*e.g.* Cannon, 1933 ; Barlow and Sleigh, 1980). However, ultrastructural data only exist for the feeding mechanism of the Euanostracan *Branchinecta ferox* (Fryer, 1983). The histology of the gut of *Artemia* is known in detail at the light microscopical level (Claus, 1886 ; Frenzel, 1892 ; Kuenen, 1939), but only the naupliar alimentary epithelium (Hootman and Conte, 1974) and the midgut of the adult brine shrimp (Kikuchi, 1971) have been studied with transmission electron-microscopical methods.

This paper describes the ultrastructural fundamentals of the filter-feeding apparatus and the ultrastructure of the alimentary canal of the adult brine shrimp.

Materials and methods

Adult males and females of *Artemia* (Macau strain, Brazil) were reared in the laboratory. For scanning electron-microscopy whole individuals and excised guts were fixed by immersion with a 2 % OsO_4-seawater solution at 4 °C for 12 h. Rinsing with seawater was followed by gradual dehydration in acetone and critical point drying. After mounting on aluminum stubs the objects were coated with gold-palladium and were examined with an ETEC-autoscan.

For transmission electron microscopy the animals were cut into pieces and fixed by immersion in 5 % glutaraldehyde in cacodylate buffer (pH 7.6 ; 0.2 m) at 4 °C for 5 h. Rinsing with chilled buffer was followed by postfixation with 2 % OsO_4 in the same buffer at 4 °C for 2 h. The objects were dehydrated in ethanol and embedded in Durcupan. Ultrathin sections were stained with uranyl acetate for 30 min and lead citrate for 10 min ; appropriate sections were examined with a ZEISS EM 9S and a ZEISS EM 10B electron microscope.

Results

FILTERING APPARATUS AND MOUTH PARTS

The metanauplius I (=instar II) of *Artemia* starts food ingestion by filter feeding using the larval antennae and the larval mandibles as filtering organs (Barlow and Sleigh, 1980 ; Schrehardt, 1987). During the post-embryonic development the morphology and the function of the antennae and the mandibles change so that the mechanism of filter feeding of juvenile and adult brine shrimp is quite different from the metanaupliar filter feeding. The metanaupliar and post-metanaupliar stages of development form thoracopods, which undertake gradually the function of filter feeding.

Juvenile and adult brine shrimp (Fig. 1 ; the list of abbreviations used in the figures is given in Table I) possess 11 pairs of trunk extremities, which are multifunctional phyllopods used for locomotion, osmoregulation (exopodites ; Copeland, 1967), respiration (epipodites ; Schrehardt, in prep.), and nutrition (Cannon, 1933 ; Barlow and Sleigh, 1980). The thoracic limbs limit a mid-ventral channel, the food groove (Fig. 2).

The protopodial and the endite setae of each limb bear setulae of about 3 µm in length arranged in borders, forming an effective filtering apparatus with an inter-setular distance of about 500 nm (Fig. 3). In swimming adults the thoracopods beat at an average of 3 to 4 Hz at 25 °C with a cycle phase difference of about 1/9 of a complete oscillation. The metachronial movement of the thoracic limbs causes a flow by which nutrients are sucked into the food groove mainly from the ventral side. Inside the food groove the nutrient flow passes the setular filter and suspended bacteria and algae adhere to the setulae (Fig. 3). The filtered water current is pressed out through the inter-limb spaces of the trunk (Barlow and Sleigh, 1980).

During the recovery stroke of the limbs the protopodial and the endite setae of one pair of thoracopods comb the setae of the following posterior extremities and collect the filtered bacteria and algae (Fig. 4), concentrating them with their own filtrate during the next effective stroke. As a result of the metachronial rhythm of the thoracopod movement, the collected and concentrated food is transported by the protopodial and the endite setae within the food groove toward the mouth.

The frontal delimitation of the food groove is marked by the uniramous maxillae and the uniramous maxillules (Fig. 5), whose feathered setae carry the food particles through the paragnaths channel to the mandibles covered by the labrum. The mandibles of the brine shrimp (Fig. 6) are phylogenetically derived extremities (Schrehardt, 1987) which bear several rows of different cuticular teeth (Fig. 7-9) used for the mechanical treatment of the filtered bacteria and algae. Each mandible possesses peripheral incisors (Fig. 7 and 8) and blunt-crowned teeth which form the molar surface of those mouth parts (Fig. 9). The dentition of the molar region

TABLE I

List of abbreviations used in Fig. 1-46

a	antennule	b	bacterium	cb	cytoplasmic body
ab	abdomen	ba	basal labyrinth	cm	circular muscle
an	antenna	bc	blood cell	cp	compound eye
ax	axon	bl	basal lamina	cu	cuticle
dc	degenerating cell	fg	food groove	g	Golgi-complex
		fk	frontal knob	GE	gland epithelium
eg	electron-dense granule	fp	food particle	gm	granular matrix
ER	endoplasmic reticulum	fs	filter seta	gy	glycogen
		fu	furca		
HC	hepatopancreatic caecum	l	lipid	m	mitochondrium
he	hemocoel	lu	lumen	ma	maxillule
hc	heterochromatin	ly	lysosome	man	mandible
				mb	myelin body
is	intercellular space			Md	Musculus dilator
				mv	microvilli
jo	joint			mx	maxilla
				my	mesentery
				myf	*myofilaments*
n	nucleus	oe	oesophagus	pa	paragnath
ne	neuron			pc	pericaryon
nm	nuclear membrane			pe	penis
np	nuclear pore			po	polysome
nu	nucleolus			pv	pinocytotic vesicle
				px	peroxisome
rER	rough ER	sc	secretory cell	tel	telson
r	ribosome	se	setula	th	thoracopod
				tw	terminal web

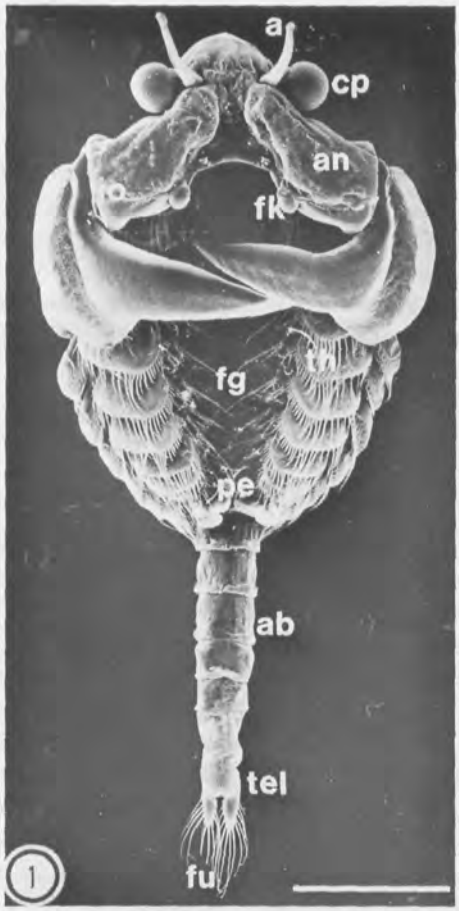
of each mandible is a mirror image of the other. Finally the ground food is ingested through the mouth into the gut.

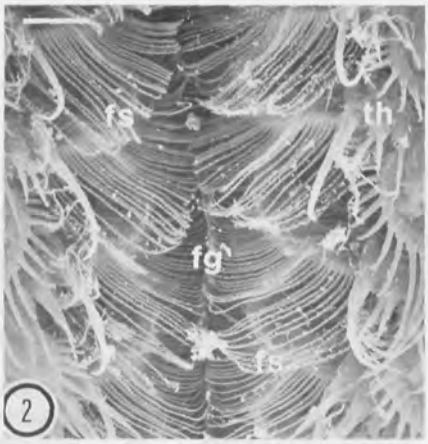
ALIMENTARY CANAL

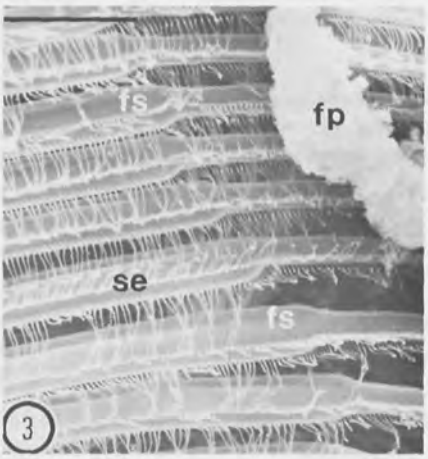
The hook-shaped alimentary canal of the adult brine shrimp is composed of three major sections : the oesophagus, the midgut, and the hindgut.

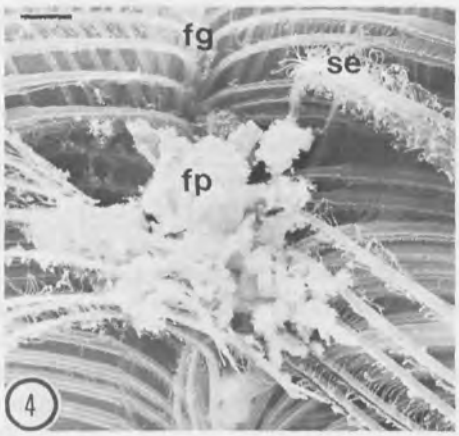
Oesophagus

The mouth is situated ventrally beneath the labrum and leads into the oesophagus which is derived from stomodeal ectoderm (Fig. 10). The epithelial cells of the oesophagus are cuboidal anteriorly and become more columnar posteriorly toward the junction of the oesophagus and the midgut. The oesophagus epithelium is lined with a cuticle (Fig. 10), which is very thin (0.25 µm) in comparison with the cuticle of the body wall (1 µm), but is composed of the same layers : a homogeneous electron-dense epicuticle and a broad electron-transparent endocuticle. Only the









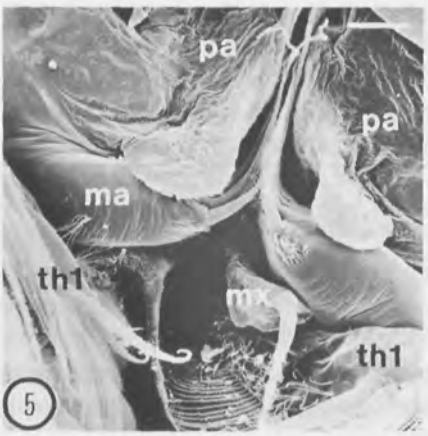


FIG. 1. Ventral view of an adult male of *Artemia*. The large antennae are used for clasping a female during precopulation and copulation. The 11 pairs of thoracopods delimit the food groove. Scale : 1 mm.

FIG. 2. The 11 pairs of thoracopods are multifunctional extremities used for locomotion, osmoregulation, respiration, and filter-feeding. The protopodite and the endites of each thoracopod bear filter setae with setular borders. Scale : 100 μm.

FIG. 3. Filter setae of a trunk limb. Each seta shows setular borders with an intersetular distance of about 500 nm. Scale : 10 μm.

FIG. 4. The collected and concentrated food particles are transported within the food groove by the metachronial rhythm of the thoracopod movement. Scale : 10 μm.

FIG. 5. The maxillae and the maxillules delimit the anterior end of the food groove. Those mouth parts take over the food particles from the thoracopods and transport them into the paragnaths channel toward the mandibles. Scale : 10 μm.

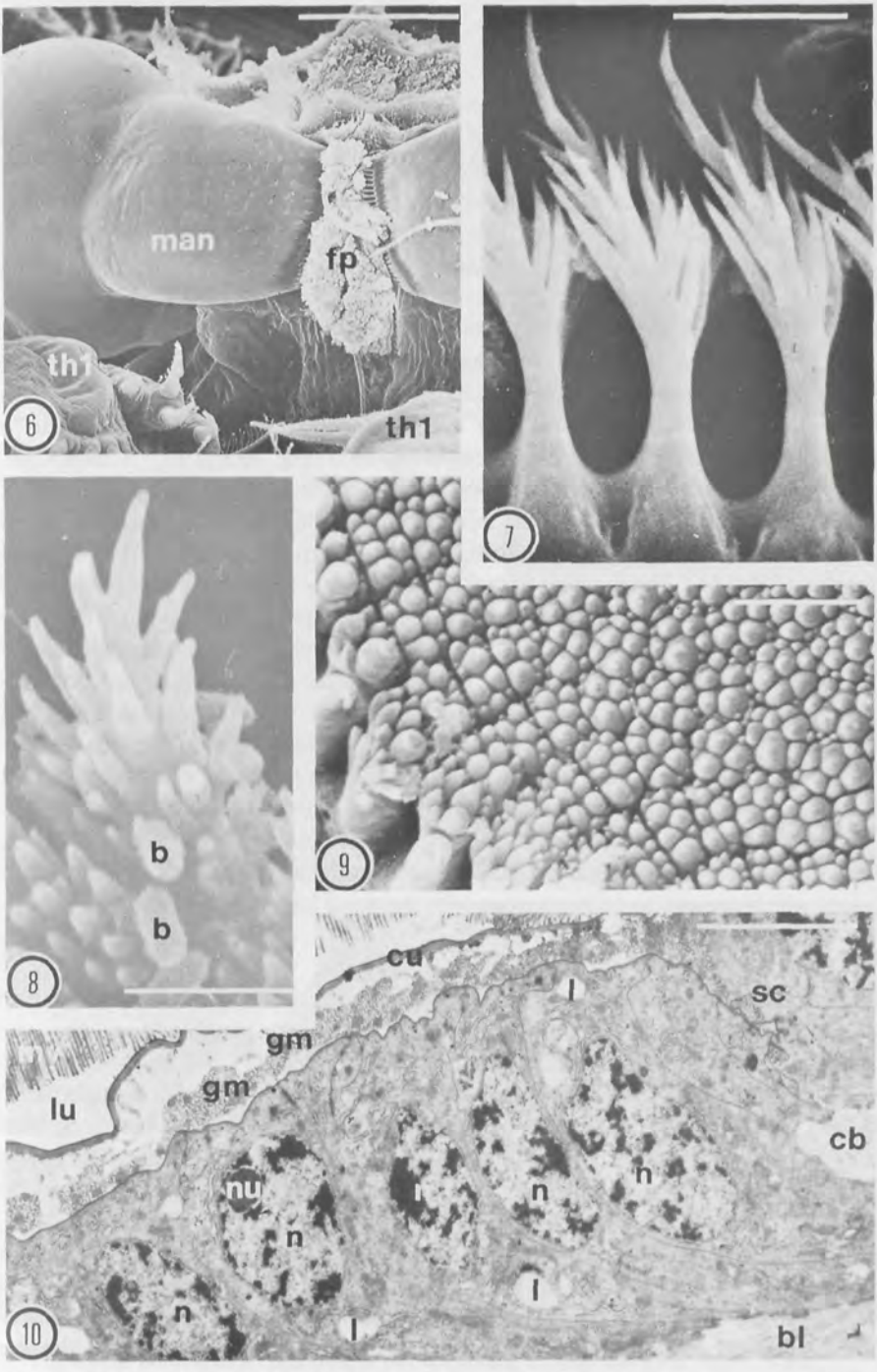


FIG. 6. Mandibles of an adult female after removal of the labrum. Scale : 100 μm.
FIG. 7. Cuticular teeth from the ventral margin of the mandible. Scale : 5 μm.
FIG. 8. Cuticular tooth from the ventral margin of the mandible. Scale : 1 μm.
FIG. 9. General view of the molar surface of the mandible. Scale : 5 μm.
FIG. 10. Columnar epithelium of the oesophagus near the junction of the midgut and the oesophagus. ote the secretory cell. Scale : 5 μm.

epithelia of the hindgut and the gills of *Artemia* show a comparably thin cuticle (Schrehardt, in prep.). Cuticular knobs and ledges as seen in the foregut of other Gnathostracan crustacea (Schlecht, 1979) are absent in the oesophagus of *Artemia*.

Each epithelial cell contains a central elongated nucleus of about 6 to 7 µm in length with usually one conspicuous nucleolus (Fig. 10). Clumps of heterochromatin are sparsely scattered throughout the nucleoplasm and along the inner nuclear membrane (Fig. 10). The cytoplasm of the epithelial cells is considerably electron-dense and ribosomes are the predominant cell organelles. Those organelles stud the outer nuclear membrane, but there are also free ribosomes (Fig. 12) and numerous cisternae of the rough endoplasmic reticulum. Mitochondria (Fig. 11 and 12) and Golgi-bodies (Fig. 11) are included within the cytoplasm. The Golgi-bodies are formed of 5 to 7 flattened cisternae with a few associated vesicles. Epithelial cells of the oesophagus store only small amounts of glycogen and lipid (Fig. 10 and 16). Adjacent epithelial cells form apical zonulae adhaerentes followed by basal tight junctions.

The epithelial cells of the oesophagus near the junction of the oesophagus and the midgut secrete apically a fine granular matrix into the space between the cuticle and the cell apices of the epithelium (Fig. 14). The function of that granular matrix is still unknown, but a connection with the synthesis of the cuticle might be possible. The epithelium rests on a fine granular, monolayered basal lamina bordering the hemocoel. Muscle cells circle the oesophagus at regular intervals along most of its length (Fig. 17). They contain the typical striated myofibrils and are innervated by mostly multipolar neurons. Mitochondria, an elongated nucleus, a few cisternae of the smooth endoplasmic reticulum, small amounts of glycogen, and often large lipid drops (Fig. 13) are included within the cytoplasm of the muscle cells in the circular muscles. Those muscle cells control the movement of the oesophagus, but there are also mighty dilator muscles (Fig. 15 and 17), similar in ultrastructure, which insert at right angles to the axis of the circular muscles and function as antagonists. Circular and dilator muscles are covered by a basement membrane, which appears to be continuous with the basal lamina of the epithelium.

Midgut

The oesophagus leads into the midgut (Fig. 16) which is derived from the entodermal germ layer. The midgut forms, at its anterior margin just above the optic ganglion, two lateral, globular protrusions : the hepatopancreatic caeca or gastric caeca (Fig. 17). All epithelial cells of the midgut including the cells forming the hepatopancreas epithelium belong to a single basic type. The luminal surfaces of the columnar epithelial cells of the hepatopancreas bear long microvilli with round tips which measure up to 8 µm in length and 0.15 µm in diameter (Fig. 18). Those cylindrical microvilli are regularly arranged in a hexagonal pattern with a center-to-center spacing of about 0.25 µm (Fig. 21). Each microvillus is limited by a thick unit membrane coated with a fine fibrous material which can be stained with ruthenium red (Kikuchi, 1971) and alcian blue indicating mucopolysaccharides. In each microvillus bundles of fine filaments extend from the center of the microvillus into the apical cytoplasm. The bundles are oriented in the longitudinal axis of the microvilli and form the terminal web (Fig. 19) under the brush border. Between the microvilli, invaginations of the apical cell membrane indicate pinocytotic pits with a diameter up to 250 nm (Fig. 20).

The centrally located nuclei of the hepatopancreatic cells are oval in shape with an average diameter of 8 µm. Most of the nuclei have two conspicuous nucleoli (Fig. 22) and only small amounts of heterochromatin scattered throughout the nucleoplasm and the inner nuclear

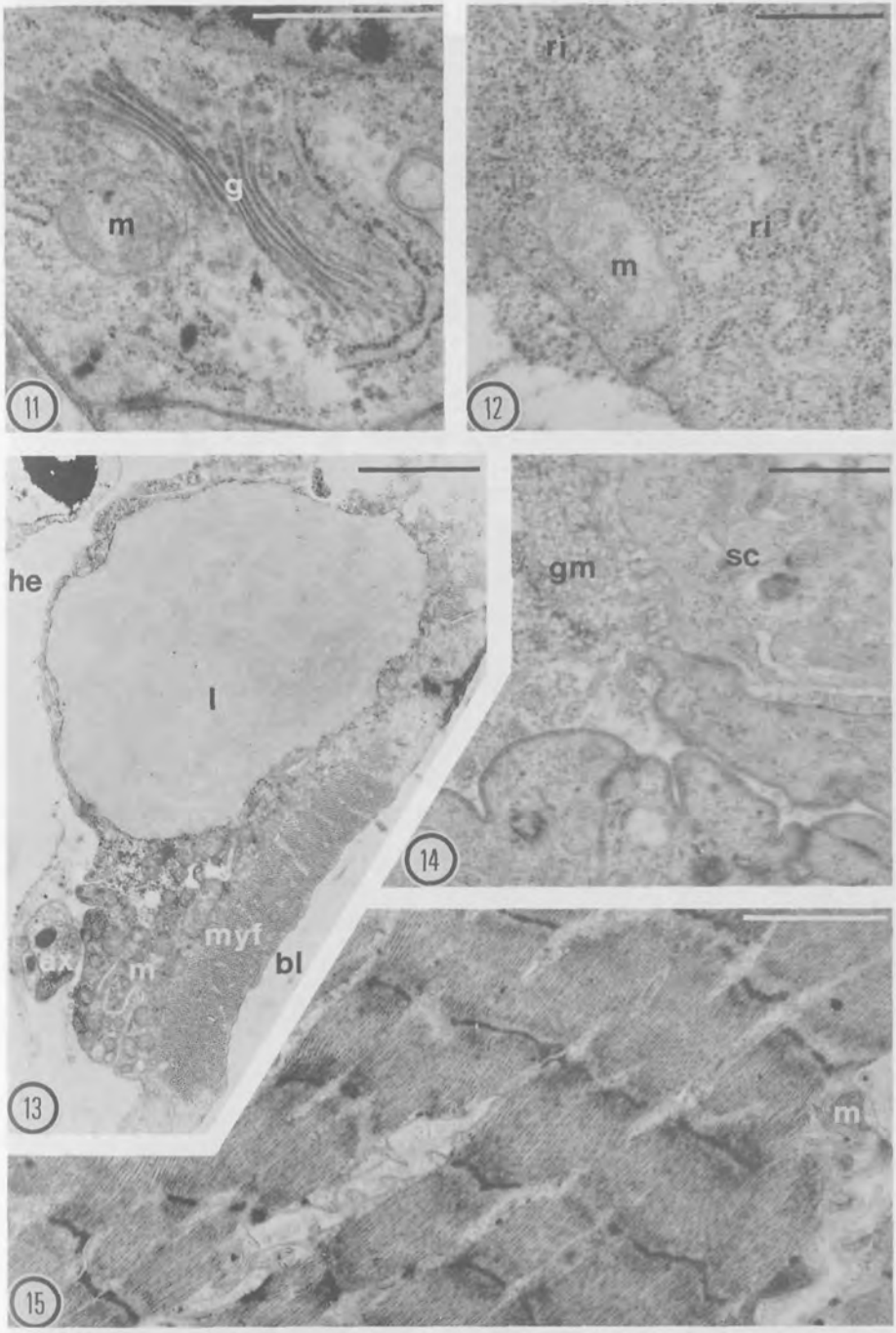


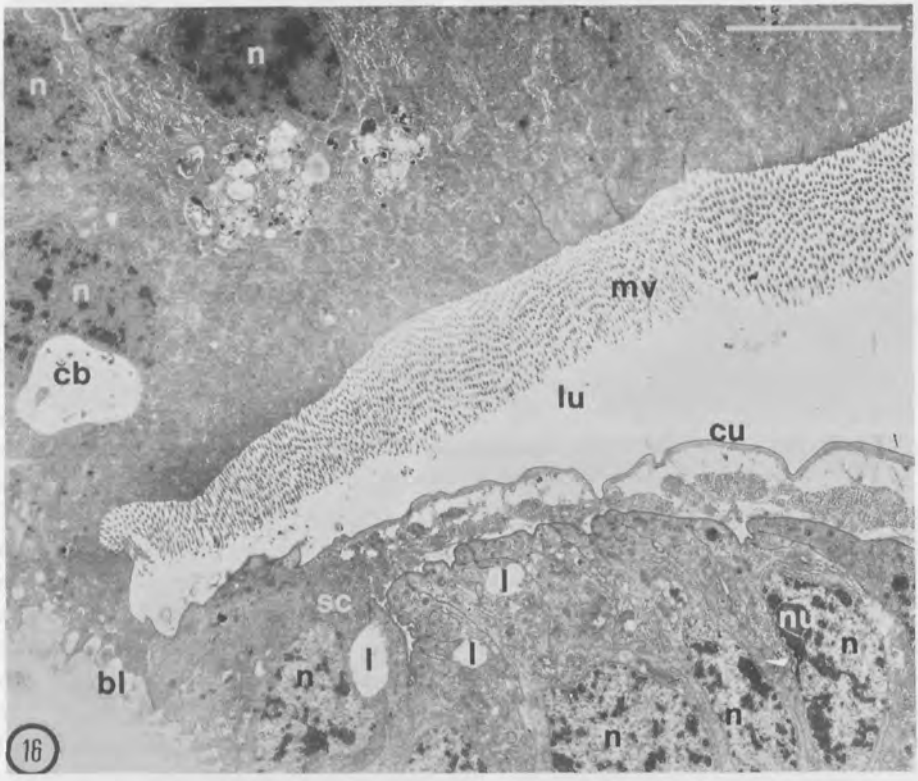
FIG. 11. Apical portion of an oesophagus cell containing a Golgi-complex. Scale : 1 µm.

FIG. 12. Ribosome field of the apical portion of an oesophagus cell. Scale : 0.5 µm.

FIG. 13. Transverse section through an axon and an adjacent circular muscle cell which is separated om the oesophagus epithelium by the basal lamina. Note the synaptic vesicles of the axon and the large pid drop of the muscle cell. Scale : 2 µm.

FIG. 14. Apical portion of a secretory oesophagus cell near the junction of the midgut and the sophagus. Note the secretion of granular matrix. Scale : 1 µm.

FIG. 15. Tangential section through the dilator muscle of the oesophagus. Scale : 2 µm.



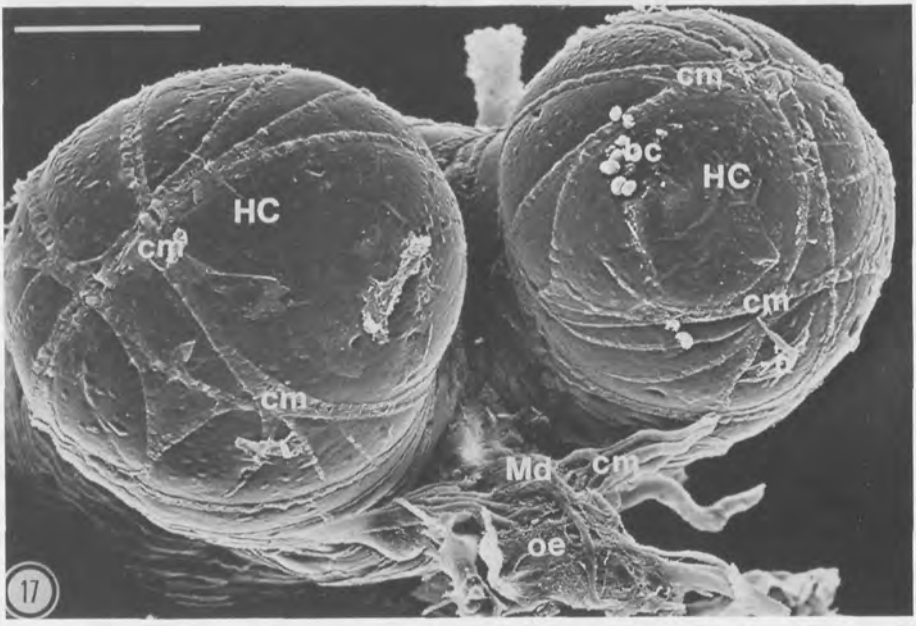


FIG. 16. Junction of the foregut and the midgut. The transition to the midgut is evidenced by the cuticularization of the foregut cells and by the "brush border" of the midgut epithelium. Scale : 5 µm.

FIG. 17. The hepatopancreatic caeca are globular protrusions from the anterior margin of the midgut. Note the circular muscles without any innervation. Scale : 100 µm.

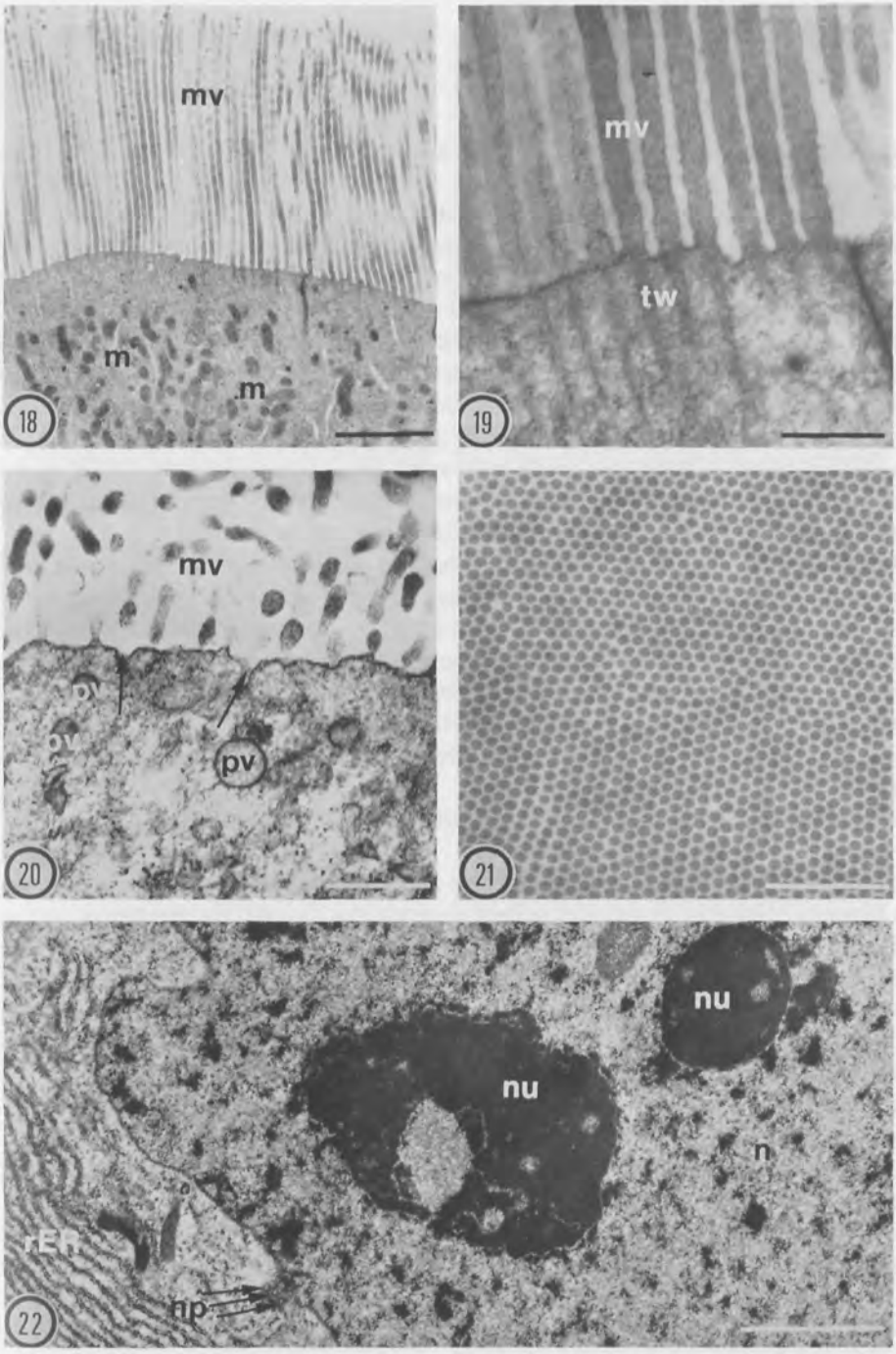


FIG. 18. Apical portion of two midgut cells. Mitochondria are numerous in the subapical cytoplasm. ale : 2 µm.

FIG. 19. The microvilli contain bundles of fine filaments extending from the centers of the microvilli o the apical cytoplasm forming the terminal web. Scale : 0.5 µm.

FIG. 20. Apical portion of a midgut gland cell with pinocytotic vesicles. Note the formation of the sicles (arrows). Scale : 0.5 µm.

FIG. 21. Tangential section through the microvilli border of a hepatopancreatic cell ; the arrangement the microvilli is regular and hexagonal. Scale : 2 µm.

FIG. 22. Central portion of a midgut gland cell. The nucleus contains two conspicuous nucleoli and only all amounts of heterochromatin. Note the nuclear pores (arrows). Scale : 2 µm.

membrane. Well-fed animals sometimes show a deformation of the nuclei under the pressure of large lipid drops situated in the cytoplasm.

The subapical cytoplasm beneath the terminal web is rich with mitochondria, but multivesicular bodies, ribosomes and sometimes glycogen particles are also abundant. The cisternae of the rough endoplasmic reticulum are distributed in the supranuclear region (Fig. 23), but there are also ribosomes studding the outer nuclear membrane and free ribosomes, which are often aggregated into polysomes (Fig. 25). The epithelial cells of the hepatopancreas near the junction of the oesophagus and the midgut contain electron-dense granules up to 300 nm in diameter with an unknown function (Fig. 26). Those granules are located close to the nucleus and surrounded by great amounts of ribosomes. Golgi bodies prevail in the supranuclear region and consist of three to five cisternae, which are usually quite small with an average diameter of 0.5 to 0.7 µm. Only a few cells of the hepatopancreas epithelium contain peroxisomes with a fine granular matrix, but crystalline inclusions of urate oxidase could not be observed (Fig. 27).

Lipid is the main storage substance in the hepatopancreas of *Artemia*, either arranged in the form of circular drops up to 12 µm in diameter or of irregular lipid fields (Fig. 28). Single epithelial cells also store small amounts of glycogen (Fig. 29). Most of the columnar cells possess a cytoplasmic body (Kikuchi, 1971) in the supranuclear region. Those polymorphic bodies are limited by a unit membrane and contain different components, *e.g.* myelin figures (Fig. 30), degenerated mitochondria, lipid drops, etc. The basal portion of the epithelial cells forms, by deep infoldings of the basal cell membrane, a basal labyrinth (Fig. 31) whose channels are often associated with mitochondria (Fig. 24).

Adjacent cells of the hepatopancreas epithelium are joined by septate desmosomes and tight junctions.

The epithelium of the gastric caeca rests on a fine-granular, bilayered basal lamina bordering the hemocoel (Fig. 32). Bundles of striated muscle (Fig. 33), without any innervation as seen in SEM and TEM, circle the midgut glands. Those muscle cells contain an elongated nucleus, mitochondria, glycogen granules, cisternae of the smooth endoplasmic reticulum and typical striated myofibrils, and are covered by a thin basement membrane, which appears to be continuous with the basal layer of the basal lamina of the epithelial cells.

In the midgut of *Artemia* a degeneration of epithelial cells is demonstrable and is more frequent in the hepatopancreatic caeca than in the posterior portion of the midgut. The initial phase of degeneration is characterized by intrusion of the cells into the gut lumen (Fig. 34). The microvilli border and the cisternae of the endoplasmic reticulum are decomposed, the nucleus becomes irregular in shape and the cells store only small amounts of glycogen and lipid (Fig. 34). Lysosomes are seen in the cytoplasm and are surrounded by an increasingly electron-transparent cytoplasmic border (Fig. 34). These ultrastructural changes are followed by an advanced thrusting of the degenerating cells into the gut lumen (Fig. 35). The cisternae of the endoplasmic reticulum, the rudiments of the microvilli, the nuclear membrane and the nuclear chromatin, the Golgi-bodies and the lysosomes disintegrate and only the nucleoli, a few mitochondria, and lipid drops are still detectable (Fig. 35). Simultaneously the cytoplasm becomes more electron-transparent. The degeneration of the epithelial cells progresses with the decay of the nucleolus, the mitochondria and the lipid drops accompanied by an increased thrusting of the cells into the gut lumen (Fig. 37), followed by the disintegration of the apical cell membrane and release of the cytoplasm into the gut lumen (Fig. 36 and 37). After the degeneration and release of the cells only cytoplasmic rudiments remain (Fig. 38) which are eventually shed into the lumen of the gut

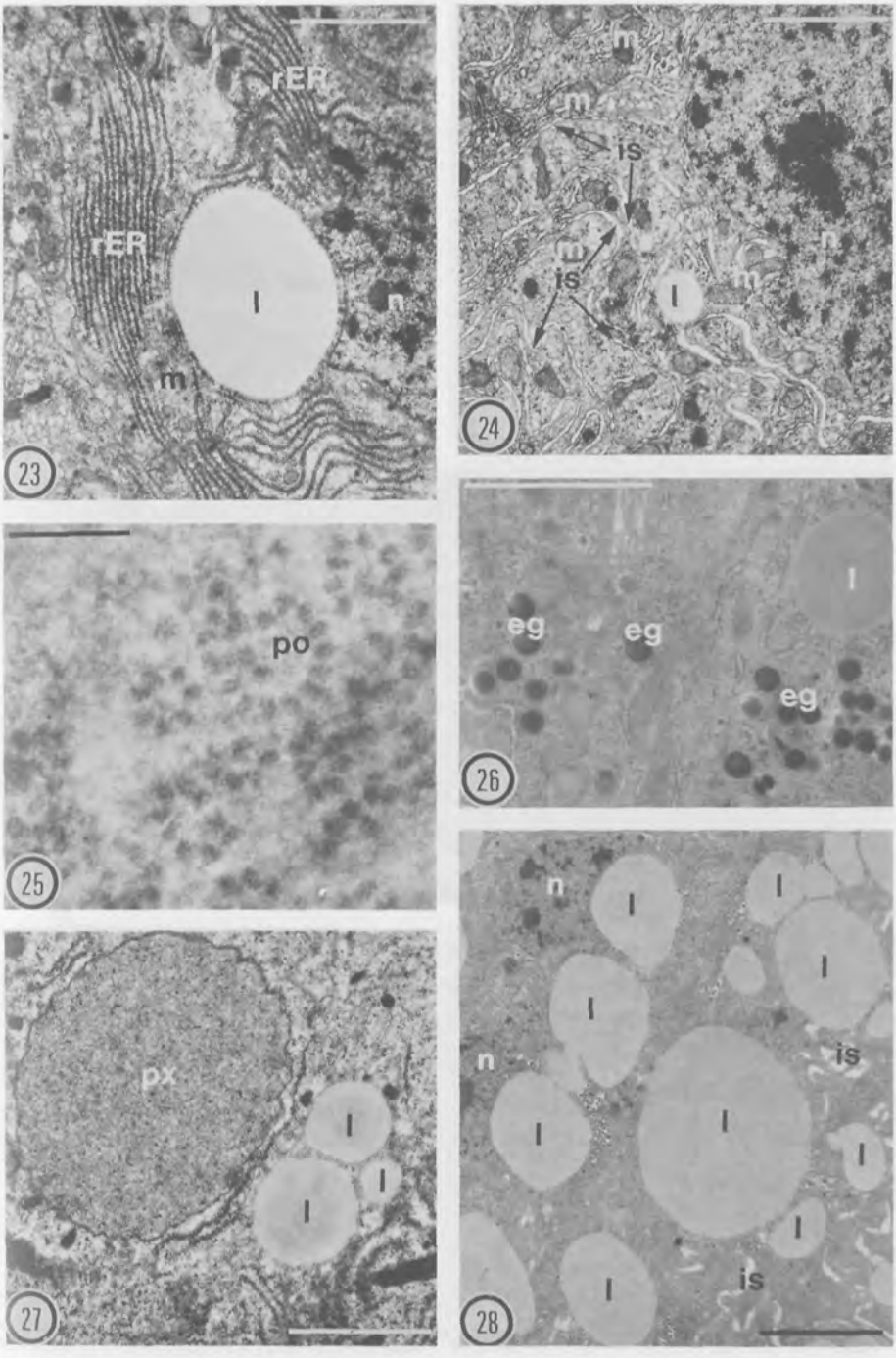


FIG. 23. Rough endoplasmic reticulum in the central cytoplasm of a midgut cell. Scale : 2 µm.
FIG. 24. Transverse section through the basal portion of a hepatopancreatic cell. Mitochondria are ciated with the infoldings of the basal labyrinth. Scale : 2 µm.
FIG. 25. The ribosomes of the midgut gland cells are often aggregated into polysomes. Scale : 100 nm.
FIG. 26. The hepatopancreatic cells nearby the junction of the oesophagus and the midgut contain iron-dense granules, only visible in these cells. Scale : 2 µm.
FIG. 27. Peroxisome in the central portion of a hepatopancreatic cell. Scale : 2 µm.
FIG. 28. Lipid drops of two midgut gland cells. Scale : 5 µm.

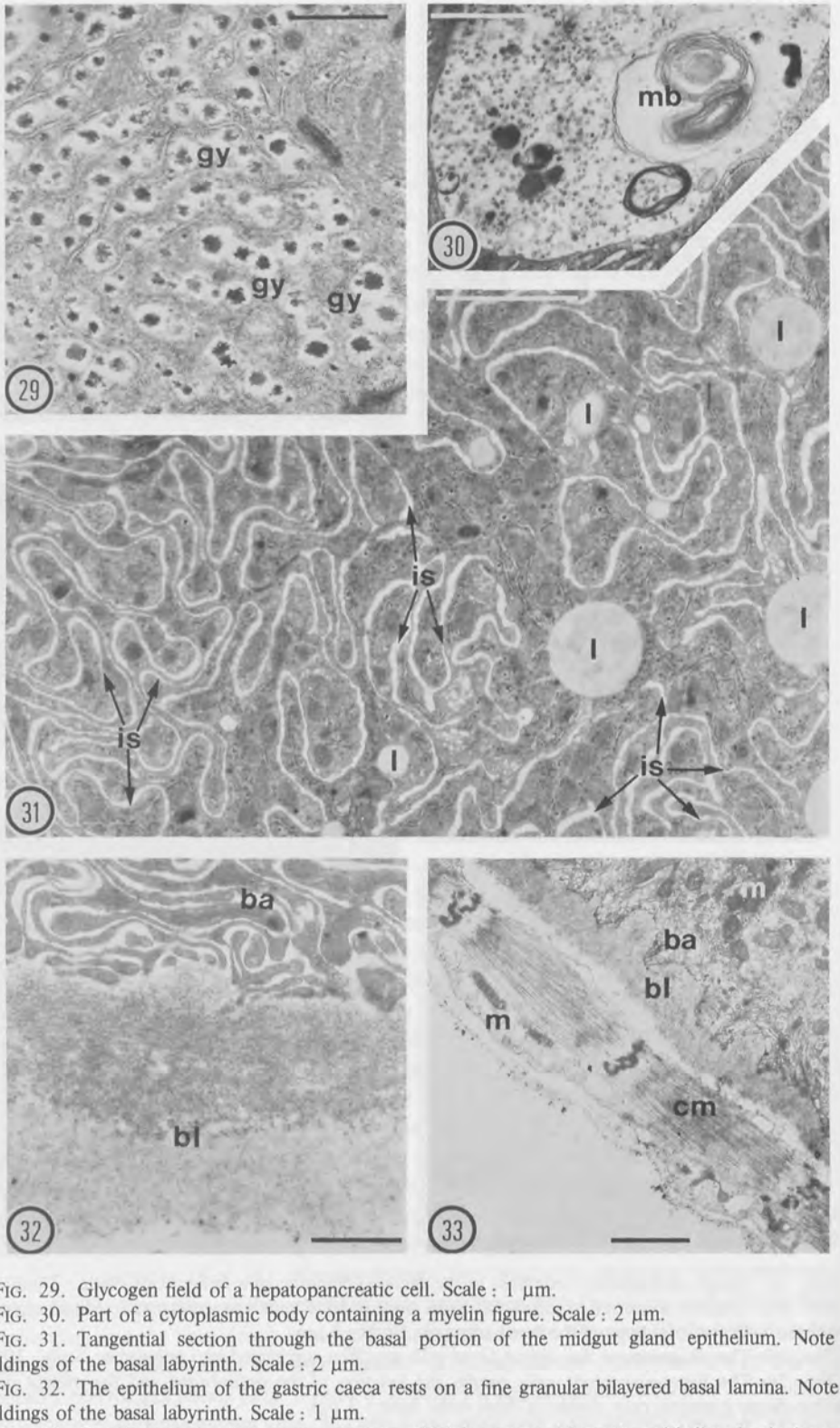


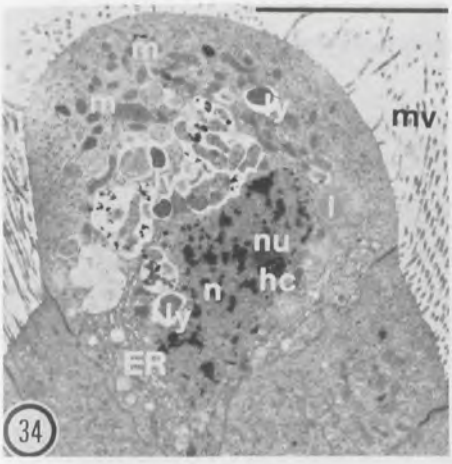
FIG. 29. Glycogen field of a hepatopancreatic cell. Scale : 1 µm.

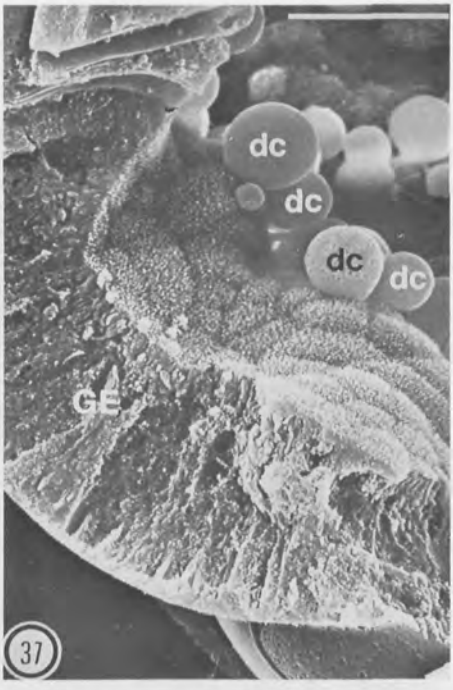
FIG. 30. Part of a cytoplasmic body containing a myelin figure. Scale : 2 µm.

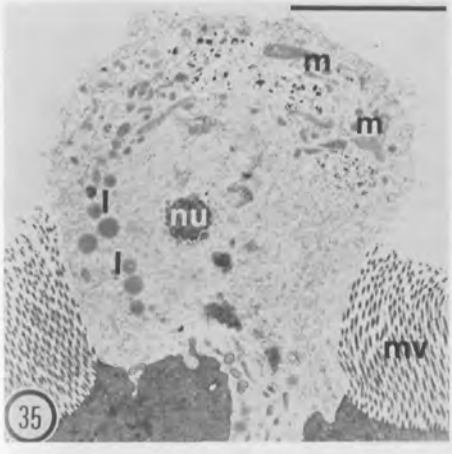
FIG. 31. Tangential section through the basal portion of the midgut gland epithelium. Note t infoldings of the basal labyrinth. Scale : 2 µm.

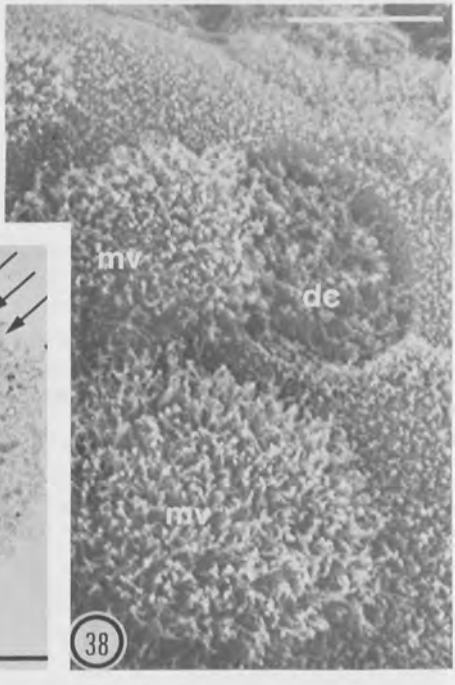
FIG. 32. The epithelium of the gastric caeca rests on a fine granular bilayered basal lamina. Note t infoldings of the basal labyrinth. Scale : 1 µm.

FIG. 33. Circular muscle of the hepatopancreas which is separated from the epithelium by the basem membrane. Note the basal labyrinth. Scale : 2 µm.









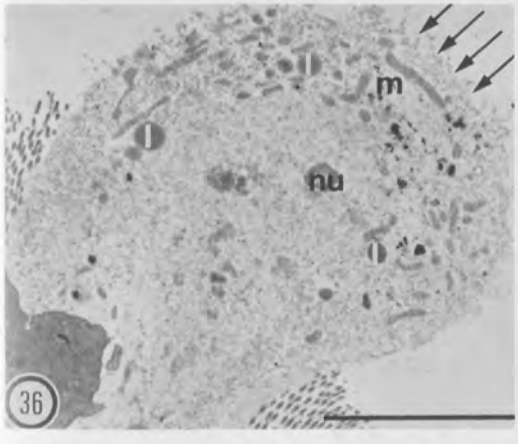
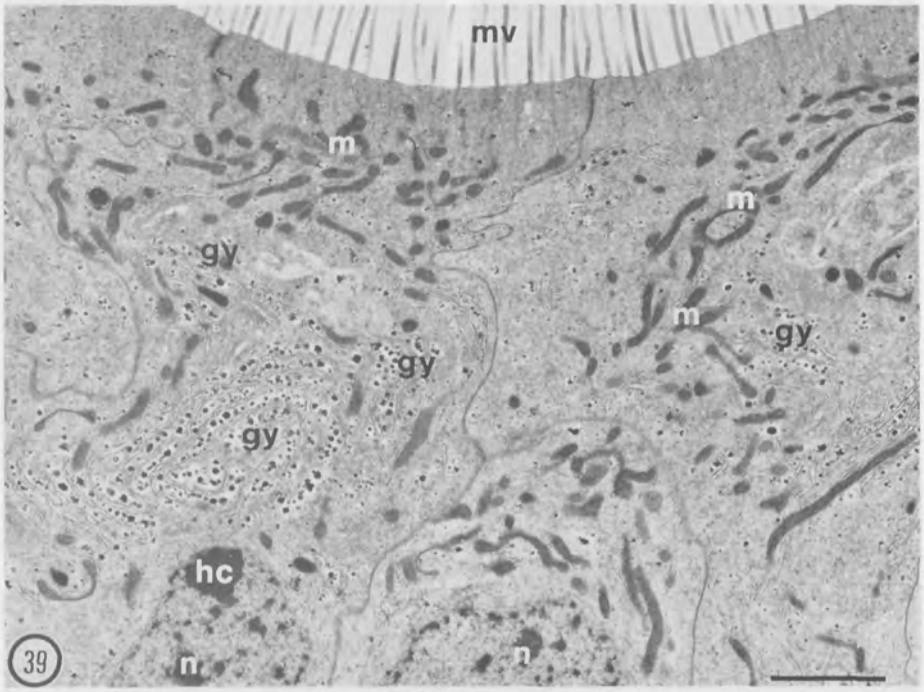


FIG. 34. Beginning degeneration of a hepatopancreatic cell. Scale : 5 µm.
FIG. 35. Advanced degeneration of a hepatopancreatic cell. Note the electron-transparent cytoplasm the degeneration of the cell organelles. Scale : 5 µm.
FIG. 36. More advanced degenerated epithelial cell of the gastric caeca. Arrows : disintegration of the al cell membrane. Scale : 5 µm.
FIG. 37. Degenerating cells of the hepatopancreas as seen with the SEM. Scale : 40 µm.
FIG. 38. Cytoplasmic rudiments of a degenerated hepatopancreatic cell after the releasing of the cell . Scale : 10 µm.



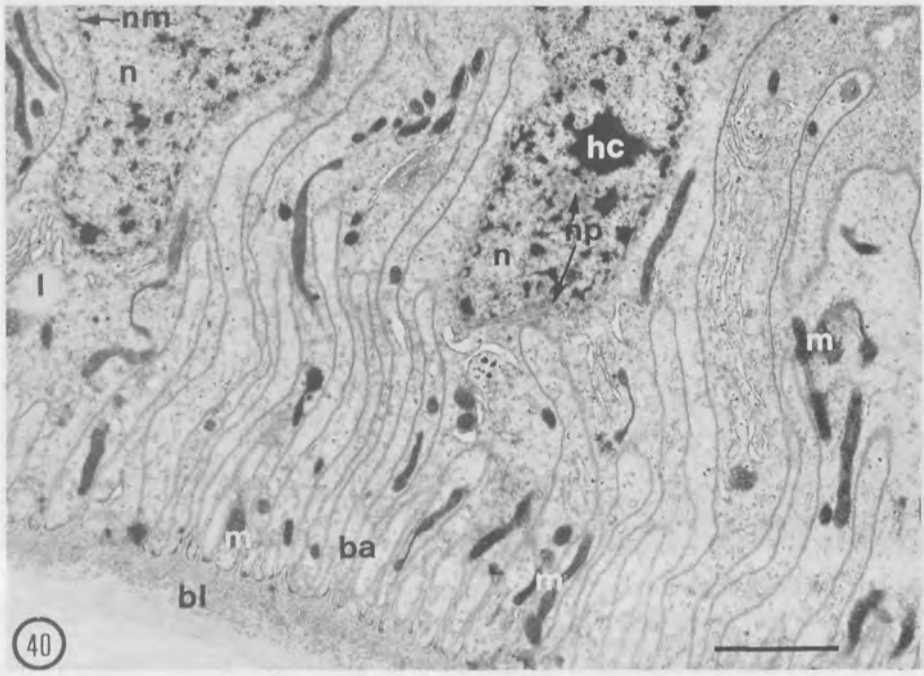


FIG. 39. Apical portion of the midgut epithelium. Scale : 2 µm.
FIG. 40. Basal portion of the midgut epithelium. Scale : 2 µm.

In spite of the single basic type of the epithelial cells, the posterior midgut epithelium differs in a few ultrastructural characters from the hepatopancreas epithelium. The microvilli of the luminal cell surfaces are shorter than those of the hepatopancreatic cells. The centrally located nuclei of the posterior midgut epithelium are more elongated with an average length of about 10 to 11 µm and contain usually only one nucleolus. In contrast to the epithelium of the midgut glands, glycogen is the main storage substance scattered throughout the cytoplasm, whereas only small lipid drops are present (Fig. 39). Ribosomes, Golgi-bodies, and the cisternae of the endoplasmic reticulum are less frequent. The basal cell membranes of the posterior midgut are folded into narrow channels which penetrate the cytoplasm up to the nuclear region (Fig. 40). Similar to the basal labyrinth of the hepatopancreas, mitochondria are associated with the infoldings of the basal cell membrane (Fig. 40). Muscle cells circle the midgut at regular intervals lying directly on the surface of the bilayered basal lamina (Fig. 41). Each muscle cell contains an elongated nucleus, typical striated myofibrils, cisternae of the smooth endoplasmic reticulum, and few mitochondria. In contrast to the musculature of the gastric caeca, the circular muscles of the posterior midgut are innervated by multipolar neurons (Fig. 42-44). The circular muscles and the basal surface of the whole midgut are covered by a fine connective tissue bordering the hemocoel. The connective tissue contains only a few organelles, *e.g.* mitochondria, ribosomes, etc. and forms mesenteries which stabilize the position of the alimentary canal (Fig. 45).

Hindgut

At the posterior terminus of the midgut there is an abrupt transition from the columnar midgut epithelium to the cuboidal hindgut epithelium which is derived from proctodeal ectoderm. The hindgut is lined by a cuticle which is like the cuticle of the oesophagus and the gills, being very thin (0.25 µm) and built up by the same layers : a homogeneous electron-dense epicuticle and a broad electron-transparent endocuticle. The hindgut epithelium rests on a fine-granular, monolayered basal lamina and is circled by muscle cells containing the typical striated myofibrils. Each epithelial cell contains an elongated nucleus up to 8 µm in length with usually one nucleolus and large amounts of heterochromatin (Fig. 46). Not only a few mitochondria and Golgi-bodies, but also myelin figures, are present in the hindgut epithelium. Ribosomes are the predominant cell organelles which stud the outer nuclear membrane and the cisternae of the rough endoplasmic reticulum, but free ribosomes associated with long-chained polysomes are also seen. Neighbouring cells of the hindgut epithelium come into contact forming apical zonulae adhaerentes followed by basal tight junctions. The hindgut ends with the terminal anus.

Discussion

According to Cannon (1933) the feeding process of the Euanostraca is subdivided into three main parts : 1) the production of swimming-feeding currents ; 2) the collection of food particles from those currents ; 3) the transfer of the food so collected to the mouth. In nauplii and metanauplii of *Artemia* the antennules, antennae and mandibles show co-ordinated movements, but only the biramous antennae contribute significantly to the locomotion of the larvae (Barlow and Sleigh, 1980). During swimming the larvae collect and transfer food to the mouth by the larval antennae and the larval mandibles, respectively (Schrehardt, 1987). The larval filtering apparatus is replaced continuously by the filter mechanism of the trunk limbs during the

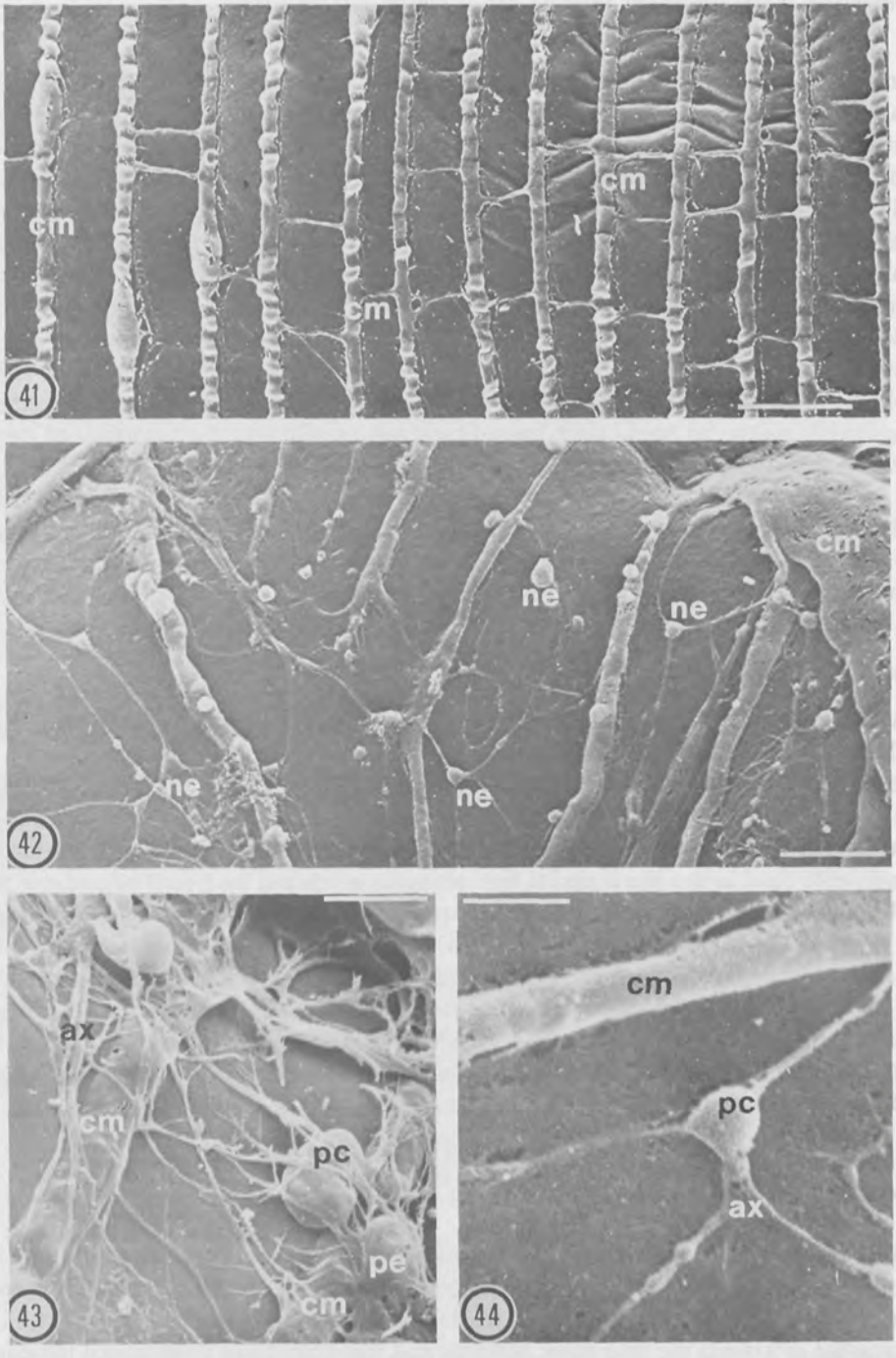


FIG. 41. Circular muscles surrounding the midgut ; these muscles are responsible for the peristal ic motions of the gut. Scale : 150 µm.

FIG. 42. The circular muscles of the midgut are innervated by multipolar neurons. Scale : 25 µm.

FIG. 43. Neuronal web between two circular muscles of the midgut. Scale : 10 µm.

FIG. 44. Single multipolar neuron of the midgut. Scale : 5 µm.

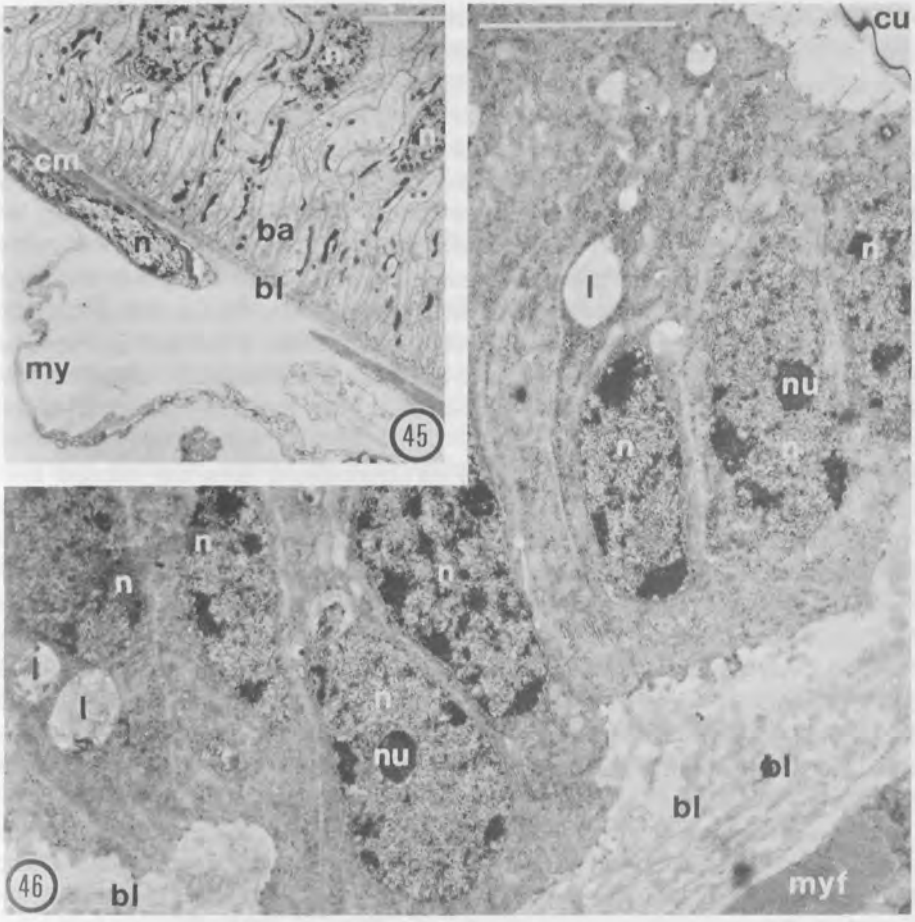


FIG. 45. Basal portion of the midgut epithelium with an underlying muscle cell covered by a thin nective tissue, which form a mesentery. Scale : 5 µm.
FIG. 46. Tangential section through the hindgut epithelium. Scale : 5 µm.

t-embryonic development (Barlow and Sleigh, 1980 ; Schrehardt, 1987). Larval antennae and ndibles are used to assist the developing thoracopods in locomotion and filter-feeding up to including the time when all thoracopods are functional, as described for *Branchinecta ferox* yer, 1983). Those results do not conform to the data described by Anderson (1967) for *mia.* Anderson (1967) showed in a chart (Fig. 17, p. 79) that the first six pairs of acopods become functional simultaneously. Those data seem to be doubtful, because detailed lies on the post-embryonic development of *Branchinecta ferox* (Fryer, 1983) and *Artemia* hrehardt, 1987) showed a continuous functional development of the thoracopods of those nostracan species. In adult specimens of *Artemia* only the trunk limbs produce swimming- ing currents (Cannon, 1933 ; Menner, 1938 ; Barlow and Sleigh, 1980) whereby a nutrient is sucked ventrally into the food groove and filtered by the setular filter apparatus. Cannon 28) described an anteriorly directed water current at the bottom of the food groove of

Chirocephalus diaphanus carrying the food particles to the mouth parts. The observations of Cannon (1928) were confirmed for *Artemia* by Barlow and Sleigh (1980). The presence of a limb gland at the base of each thoracopod (Claus, 1886 ; Cannon, 1933 ; various authors, see Fryer, 1983) and the length of the thoracopodial setae (Fig. 2 and 4) argue against a water current as a single factor carrying the food particles toward the mouth. The limb glands produce a mucous secretion (Lundblad, 1920 ; Dornesco and Steopoe, 1958 ; as cited in Fryer, 1983), probably functioning as an organic cement which adheres the food particles in clumps. According to this explanation a mechanical pressure of the trunk limbs is necessary to join the collected food particles together with each other and with the mucous secretion of the limb glands. Furthermore, the protopodial setae of the thoracopods overlap at the base of the food groove (Fig. 4) so that "an anterior travel of small particles, not in contact with the base of the food groove or setae" (Barlow and Sleigh, 1980) seems to be doubtful. For the detailed clarification of that mechanism, the ultrastructure of the limb glands and the mode of secretion transfer, which are yet unknown, must be studied.

According to the studies of Fryer (1983) on *Branchinecta ferox* the maxillae and the maxillules, which are the frontal delimitation of the food groove, bridge the gap between the 1st pair of thoracopods and the mandibles carrying the food particles through the paragnath channel to the mandibles. The oesophagus of *Artemia* lacks structures for mechanical treatment of the ingested food like a gizzard, as in the Malacostraca (Dennhöfer, 1985), or cuticular ledges, as in the Conchostraca (Schlecht, 1979). The luminal surfaces of the oesophagus and the hindgut are lined by a very thin cuticle indicating a respiratory function which was discussed by Claus (1886). The cuticle of the anterior and posterior portions of the alimentary canal is as thin as the cuticle of the gills (Schrehardt, in prep.), but the epithelia of the oesophagus and the hindgut are about 20 times thicker than the respiratory epithelia of the gills. A respiratory function of the oesophagus and the hindgut therefore seems to be improbable.

The cells of the midgut epithelium, including those which comprise the hepatopancreas, are of a single basic type, which seems to be similar to the R-cells in the gastric caeca of Decapods (Gibson and Barker, 1979). The long microvilli and the pinocytotic vesicles of the epithelial cells in the midgut of *Artemia* indicate a resorptive function (Kikuchi, 1971). Contrary to the division of labour of the four cell types in the hepatopancreas of Decapods, the uniform cells in the midgut of *Artemia* undertake various functions like resorption, storage of glycogen and lipid, and the production of digestive enzymes. Previous authors studying the alimentary canal of *Artemia* with light microscopical techniques described extrusion cells with a possible function of secreting digestive enzymes (Frenzel, 1892 ; Kuenen, 1939). Kikuchi (1971) observed at the electron microscopical level the extrusion of cytoplasm on the luminal surface of the columnar epithelium at the anterior region of the midgut. Those cytoplasmic extrusions are most probably artefacts. During fixation by immersion from the hemocoel surface, the circular muscles of the hepatopancreas contract and epithelial cells are passively pressed into the gut lumen. When the same fixative is injected into the gut lumen so that fixation by immersion starts from the luminal surface, no cytoplasmic extrusions are seen. The thesis of the artificial character of those cytoplasmic extrusions is supported by the observation of Kuenen (1939) that the extrusion frequency is independent of the amount of food present in the intestine. The extrusion of epithelial cells described by Frenzel (1892), Kuenen (1939), and Kikuchi (1971) is quite different from the degeneration of epithelial cells. This process seems to be similar to the second mechanism of cell extrusion described by Frenzel (1892), who observed the degeneration of the

nucleus and the cytoplasm and the development of a "secretory vesicle". It is yet unknown whether the degeneration of epithelial cells in the midgut of *Artemia* is correlated with the production and secretion of digestive enzymes. Schultz and Kennedy (1976) described the degeneration of epithelial cells in the midgut of *Daphnia pulex* as enzyme secretion of the holocrine type.

Furthermore, intracellular digestion in the midgut of *Artemia* has been discussed. Kikuchi (1971) described a possible lysosomal nature of the cytoplasmic bodies in the supranuclear region indicated by intensive activities of the β-D-glucuronidase and the N-acetyl-β-glucosaminidase demonstrated in the same region by enzyme histochemical techniques at the light microscopical level (Kikuchi and Shiraishi, 1969). Although the development of the cytoplasmic bodies is yet unknown and various components like myelin figures, etc., are not typical for a secretory nature of those organelles (*cf.* Kikuchi, 1971), there are some characteristics similar to those of degenerating cells of the midgut epithelium : the matrix inside the cytoplasmic bodies is electron-transparent and mitochondria and other typical cell organelles are not present without degenerating marks. A detailed enzyme-cytochemical study will be necessary to determine the secretory activity of the degenerating epithelial cells and the cytoplasmic bodies.

Enteroendocrine cells as in the anterior midgut of *Leptestheria dahalacensis* (Rieder *et al.*, 1984) have not been demonstrated in the alimentary canal of *Artemia.* In addition to the resorptive and storage function the midgut epithelium of *Artemia* seems to be active in osmoregulation. Croghan (1958) showed an active uptake of Na^+- and Cl^--ions across the gut epithelium of *Artemia.* Those data are supported by ultrastructural findings, *e.g.* the accumulation of mitochondria in the subapical cytoplasm of the midgut as seen in metanauplii (Hootman and Conte, 1974) and adult specimens, the extensive basal labyrinth representing a surface magnification, and the mitochondria associated with the channels of the basal labyrinth. Those infoldings associated with mitochondria are also present in the maxillary gland of *Artemia,* which is active in the transport of water and ions (Tyson, 1968, 1969).

Acknowledgements

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A scanning electron-microscope study of the development of the phyllopods in *Artemia*

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Abstract

The morphogenesis of the thoracic appendages (phyllopods) has been studied, from their first appearance as smooth bulges on the ventro-lateral surfaces of the larva, to the complex, lobed appendages of the adult.

The newly-hatched nauplius of *Artemia* is undifferentiated in the post-mandibular region. The 11 thoracic segments gradually develop during the subsequent larval instars, appearing and differentiating in an antero-posterior sequence. The first lobe of the developing phyllopod to become distinct is the exopodite, which is the most distal part of the larval appendage. This is also the first part of the phyllopod to show the appearance of rudimentary setae, which eventually develop as a thick fringe on all the inner, ventral lobes of the phyllopods.

Metamorphosis from nauplius larva to young adult occurs at the 9th moult by which time six thoracic segments bear a pair of phyllopods of the adult form. The remaining five phyllopods finish differentiating after metamorphosis while the first six continue to grow in size and amount of setation.

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Pioneer neurons and the early development of the nervous system in *Artemia*

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Abstract

Early development of the nervous system of *Artemia* has been studied using light and electron microscopy.

Two longitudinal axon tracts are present along the entire length of the undifferentiated 1st instar larva, on either side of the ventral midline. They are derived from the outgrowth of axons from two terminally situated pairs of pioneer neurons. These pairs of axons also branch medially in an antero-posterior sequence as the development of the body segments proceeds gradually in this direction. These branches form the basis for a pair of transverse commissures in each segment.

The first sensory structures to differentiate on the *Artemia* thoracic segment are a pair of dorsal spines that lie on either side of the ecdysial line. These appear just as the segment becomes visible externally as a slight bulge. They have a pair of neurons associated with them that send axons round the side of the animal to join the longitudinal axon pairs. One of the dorsal spine axons bifurcates and sends a branch anteriorly and another posteriorly along the longitudinal axon pair. The other dorsal spine axon turns anteriorly when it joins the longitudinal axon pair.

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Genetics

The review by Abreu-Grobois (1) summarizes well the advances in *Artemia* genetics that have occurred since the First International Symposium on *Artemia*. The papers that follow this review are organized in the same manner as the review, *i.e.* from cytogenetic (2-4) to evolutionary (5) in approach.

The interested reader should also consult the Concluding Remarks of Prof. Beardmore at the end of this volume for further comments on *Artemia* genetic research.

(1) F. A. Abreu-Grobois.
A review of the genetics of *Artemia*.

(2) L. Baratelli.
First metrical data on the length of the prophasic chromosomes of diploid and tetraploid parthenogenetic *Artemia*.

(3) C. Barigozzi, P. Valsasnini, E. Ginelli, G. Badaracco, P. Plevani, and L. Baratelli.
Further data on repetitive DNA and speciation in *Artemia*.

(4) Th. J. Abatzopoulos, C. D. Triantaphyllidis, and C. D. Kastritsis.
Preliminary studies on some *Artemia* populations from northern Greece.

(5) R. A. Browne and M. H. Spencer.
Intrapopulational differences in life history traits of obligately parthenogenetic clones of the brine shrimp *Artemia*.

Artemia *Research and its Applications. 1987. Vol. 1. Morphology, Genetics, Strain characterization, Toxicology*
P. Sorgeloos, D. A. Bengtson, W. Decleir, and E. Jaspers (Eds). Universa Press, Wetteren, Belgium. 380 p.

A review of the genetics of *Artemia*

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ntroduction

The earliest genetic studies of the genus *Artemia* were published over 75 years ago (Brauer, 893 ; Artom, 1906, 1907) when the existence of two distinct, stable reproductive modes parthenogenesis and bisexuality) were recognised. Additionally, several polyploid states were und in asexual forms besides the euploid form with 42 chromosomes. Subsequent work ucidated parthenogenetic oogenesis, the extent of phenotypic variation and the sex determiting mechanism in this organism, all of which has been already thoroughly reviewed by arigozzi (1974).

Much of the substantial advance in the field of genetics occurring within the last decade has en possible through the greater availability of cysts collected from an ever increasing number known natural and artificially-inoculated populations found throughout the world (see rsoone and Sorgeloos, 1980 ; Browne and MacDonald, 1982). The ease with which these rmant cysts can be transported and the hatched animals cultured singly or in large populations s permitted broader interpopulational comparative research on such aspects as reproductive rformance (Browne, 1980ab, 1982, 1983a), reproductive and ecological isolation between pulations adapted to different water chemistry (Bowen *et al.*, 1985), and cytological charac-

teristics (Barigozzi and Baratelli-Zambruni, 1982, 1983 ; Abreu-Grobois and Beardmore, 1982 ; Abreu-Grobois, 1983).

Electrophoretic studies of *Artemia* populations (Abreu-Grobois and Beardmore, 1980, 1982 ; Abreu-Grobois, 1983) has permitted not only an estimation of the genetic relationships between the species, but also an assessment of the extent of differentiation between local populations of both bisexual and parthenogenetic forms. This approach has also allowed a solution to a long-standing question, difficult to elucidate utilising traditional methods : from which branch of this genus did parthenogenesis originate?

The present review concentrates on genetic studies which have been carried out during the years subsequent to the first International Symposium on *Artemia* (Corpus Christi, Texas, USA, August 1979). The advances obtained attest to the potential of this organism for becoming a sort of "marine *Drosophila*".

Cytogenetics

Chromosome numbers

It is by now well known that the euploid (2n) chromosome number in the bisexual species of the genus is 42 (*e.g.* in *A. tunisiana* and in *A. franciscana*) except in *A. persimilis* where it is 44 (Barigozzi, 1974). Within the parthenogenetic forms, various ploidies exist : diploidy, triploidy, tetraploidy, and pentaploidy. A decaploid population was also reported (Haas and Goldschmidt, 1946) but was later demonstrated to be pentaploid (Goldschmidt, 1952). Reproduction in diploid parthenogenetic *Artemia* involves normal meiosis and the chromosomal number is restored through fusion of 1st and 2nd division products (this mechanism is also termed "automixis"). In all polyploid forms meiosis is not evident and oogenesis involves a sort of mitosis ("apomixis", see Barigozzi, 1974).

Development of convenient and rapid methods for preparations of somatic chromosomes derived from freshly-hatched nauplii (Barigozzi and Baratelli-Zambruni, 1982 ; Abreu-Grobois, 1983) which avoid the lengthy and laborious fixation, sectioning and squashing used formerly, together with the availability of samples from the much increased list of known populations of *Artemia*, has allowed important advances in the study of the karyology of brine shrimp.

The more recent surveys have confirmed the widespread occurrence of the basic euploid chromosome number of 42 in all bisexual species except *A. persimilis* (Table I). Furthermore, the coexistence of two or more species has been recorded in some localities where natural ranges overlap, *e.g.* between 1) *A. tunisiana* and various parthenogenetic forms of different ploidies, and 2) purely asexual individuals of a number of different ploidies (Barigozzi, 1974 ; Amat Domenech, 1980 ; Abreu-Grobois and Beardmore, 1982 ; Browne and MacDonald, 1982).

A conspicuous feature found in some of the taxonomic groups is a distinct tendency towards aneuploidy. One form presents fluctuating chromosome numbers but includes even as well as odd numbers. This condition appears to be concentrated characteristically within polyploid forms particularly in tri- and pentaploids (Abreu-Grobois, 1983) in a manner akin to that found by Goldschmidt (1952) and discussed in Barigozzi (1974). This type of aneuploidy has also been found in *A. franciscana* material from the Great Salt Lake (Iwasaki, 1969 ; Abreu-Grobois, 1983) though only in a very small proportion.

TABLE I

Chromosome numbers in *Artemia*

Species	Total number of populations studied	Modal chromosome number	Number of populations containing aneuploids	Chromosome numbers observed	References[a]
Bisexuals					
– New World					
A. franciscana	19	42	1	21-48	1,2,3,7
A. monica	1	42	0	42	1
A. persimilis	2	44	0	44	1,4,5,7
– Old World					
A. tunisiana	7	42	1	42,44,46,48,50	1,2,4,5,7
A. urmiana	1	42	0	42	1
Parthenogenetic					
– Diploid	11	42	4	40,42,44,46,48, 50,52,54,56,63	1,2,7
– Triploid	5	63	3	57,58,63,64,65	1,2,6,7
– Tetraploid	11	84	1	84,89	1,2,7
– Pentaploid	3	105	2	104,105,106, 107-111	1,6,7

[a] 1=Abreu-Grobois (1983), 2=Barigozzi and Baratelli-Zambruni (1983), 3=Iwasaki (1969), 4=Piccinelli and Rosdocimi (1968), 5=Halfer-Cervini *et al.* (1968), 6=Goldschmidt (1952), 7=Barigozzi (1974).

The second aneuploid condition, observable in *A. tunisiana* and some diploid parthenogenetic populations, exhibits a very discrete series of chromosome numbers, with the most common being the euploid number of 42 and increasing stepwise by 2 units up to a maximum of 56 (Barigozzi and Baratelli-Zambruni, 1983). An exceptional population, found among parthenogenetic nauplii from Gerri de la Sal (Spain), also contained some individuals with 40 chromosomes.

CHROMOCENTERS

Prior to Barigozzi and Baratelli-Zambruni's (1982) report of the presence of heavily staining heterochromatic areas (or "chromocenters") in the resting nuclei of *A. franciscana* nauplii, the interphase cells, known only from studies of *A. tunisiana* and parthenogenetic *Artemia* material, were known to contain thin filaments of chromatin with one or two segments of highly condensed regions only in exceptional cases (Barigozzi, 1941). When present, the chromocenters can be shown to stain heavily with dyes such as quinacrine (Barigozzi and Baratelli-Zambruni, 1982) or Giemsa (Abreu-Grobois and Beardmore, 1982) and, thus, must be highly repetitive and rich in adenine and thymine residues. All of the *A. franciscana* populations studied from the USA and Canada exhibit a high mean number of chromocenters per nucleus (14-17, Table II) which is also the case for *A. monica*. Caribbean populations of *A. franciscana* characteristically contain somewhat fewer (about seven) and smaller chromocenters, while the sole Mexican population

TABLE II

Chromocenters in *Artemia*

Species	Population(s)	Number of populations surveyed	Average number of chromocenters per nucleus	Reference
A. franciscana	San Francisco Bay, California, USA Great Salt Lake, Utah, USA	2	14.7	Barigozzi and Baratelli-Zambruni (1982)
	USA and Canada[a]	11	17.1	
	Caribbean	4	7.2	
	Yavaros, Mexico	1	3.9	
A. monica	Mono Lake, California, USA	1	16.7	Beardmore and Abreu-Grobois (1983), Abreu-Grobois (1983)
A. persimilis	Buenos Aires, Argentina	1	3.0	
A. urmiana	Lake Urmia, Iran	1	0	
A. tunisiana	Continental Europe and Mediterranean	5	0	
A. parthenogenetica	Europe, Mediterranean and Asia	28[b]	0	

[a] Includes populations transplanted to Brasil and Australia from San Francisco Bay material, as well as samples treated independently collected in different years from a same locality.

[b] A sample containing n ploidies was counted as n "populations".

so far studied exhibits an even lower mean number, comparable to that found for *A. persimilis.* Recent research on repetitive DNA suggests that the heterochromatic blocks observed cytologically are rich in Alu I DNA fragments (Barigozzi *et al.*, 1984) and has demonstrated the presence of these structures in *A. tunisiana* as well (Barigozzi *et al.*, 1987).

As mentioned by Barigozzi and Baratelli-Zambruni (1982), the chromocenters observed could correspond either to entire heterochromatic chromosomes or to heterochromatic regions of some of the chromosomes. Although insufficient evidence is available at the moment, it is probable that the second alternative is the more accurate as some prophase chromosomes show heavily stained distal areas (pers. observ., and Barigozzi *et al.*, 1984).

Chromosomal evolution

The cytological results demonstrate great diversity, containing a range of chromosomal mutations rarely seen within a single taxonomic group. Nonetheless, in spite of the complexity, it has become possible to suggest mechanisms capable of leading to most of the conditions observed.

Table III

Frequency of aneuploids in some populations of *A. tunisiana* and parthenogenetic *Artemia* (from Barigozzi and Baratelli-Zambruni, 1983)

cality	Reproductive method	Chromosome numbers observed	Frequency (%)	N Total
ri de la Sal, Spain	parthenogenetic	40	31	29
		42	62	
		44	3.5	
		46	3.5	
pe, Spain	parthenogenetic	42	38	29
		44	14	
		46	21	
		48	7	
		50	10	
		56	3	
		63	7	
ochète, Portugal	parthenogenetic	42	40	20
		44	10	
		46	30	
		48	10	
		52	5	
		54	5	
nera, Spain	bisexual	42	70	27
		44	15	
		46	7	
		48	4	
		50	4	

For example, mechanisms capable of producing the aneuploids seen in polyploid *Artemia* have already been reviewed by Barigozzi (1974): *e.g.* misdistribution of chromosomes at mitosis and the repeated loss of chromosomes from a polyploid set. It is feasible that the presence of multiple copies of each chromosome affords a "genetic buffering" to the loss of genetic material.

The second aneuploid type, where a discrete series of even numbers of chromosomes exist within a population, is far more interesting. The observed consistency in the numbers observed and the decreasing frequencies for numbers above 42 (Table III) suggest the existence of a persistent, and possibly analogous, mechanism generating aneuploids in both bisexual *A. tunisiana* and diploid parthenogenetic forms. Potential pathways involved here may also be applicable to the evolution of *A. persimilis* which contains a duplicated set of chromosomes.

For bisexual *Artemia* two alternative routes can be considered, with non-disjunctional events occurring at different phases of the life cycle (Fig. 1a). One such event could occur during the mitosis of the 1st division of a blastomere (Fig. 1-a.1) generating a mosaic individual with half of its cells trisomic (2n+1) and the other half monosomic (2n-1). If the cells later generating the gonadal tissue were to derive from the 2n+1 cells, gametes with n and n+1 complements would be produced at reproduction. Crossing between this anomalous individual and normal (euploid) organisms would produce 2n and 2n+1 progeny. If, further, the 2n+1 individuals were fertile and interbreeding between siblings were possible, a second generation of organisms with 2n, 2n-1 and 2n+2 composition would result with relative expected frequencies of 3:1:1 respectively. As, on the whole, tetrasomics and other even-numbered extra chromosome types behave in more stable and regular fashion at meiosis than do odd-numbered types, it would be expected that the trisomic condition (2n+1=43) would be less capable of survival. The tetrasomic individuals would also be eliminated unless 1) this chromosomal condition had no drastic effect on fertility, or 2) diploidisation of the chromosomes were to be established, thus overcoming the detrimental effects on fertility that complicated chromosome pairing normally brings about, or 3) the tetrasomic condition, when compared to the parental euploid type was either neutrally advantageous (the two chromosome number types would coexist) or selectively advantageous (the tetrasomics would replace the euploid types).

A second possible route to tetrasomy involves non-disjunctional events during gamete formation (Fig. 1a.2). If this occurred during only one meiotic division (Fig. 1a.2.1), gametes containing n+1 and n-1 haploid numbers would ensue, leading to a sequence of events very similar to that described above. If, on the other hand, two non-disjunctional events (during both meiotic divisions) occurred sequentially in a single line leading to germ cells (Fig. 1a.2.2) gametes with n+2 and n-1 chromosomal complements would be generated. Providing low viability for the n-1 condition prevailed, the only cross possible (that between the n+2 gamete and normal haploids n) would produce directly true tetrasomic individuals (2n+2, not 2n+1+1). This would automatically duplicate a particular homologous chromosome pair if both non-disjunctional events involved the same chromosomes. The first route to the formation of 2n+2 individuals would also produce true tetrasomy, but only if the trisomic individuals interbreeding were full sublings derived from the same mosaic parent.

If double non-disjunctional events were sufficiently frequent in a population and occurred in both sexes, it is also conceivable that double tetrasomic individuals (2n+4, or 2n+2+2=46) could be generated from crosses between n+2 gametes.

Confirmation of whether homologous chromosomes are duplicated in aneuploid *Artemia* individuals would be possible if detailed banding maps or electrophoretic markers were available

which discriminated between chromosomes. In *A. persimilis*, a tetrasomic (2n+2) where we have an electrophoretically assayable locus (PGI, Abreu-Grobois and Beardmore, 1982) marking its duplicated chromosome pair, analysis of test crosses strongly suggests that the duplicated loci are unlinked and under disomic control (Abreu-Grobois, 1983). Hence, one of the conditions for the establishment of stable tetrasomy has occurred. This phenomenon is consistent with an evolutionary trend found also in other organisms which have undergone extensive duplication of chromosomes, such as members of the salmonid family (Allendorf and Utter, 1973 ; Allendorf *et al.*, 1975 ; Engel *et al.*, 1975).

The establishment of a pure aneuploid population (such as a progenitor of *A. persimilis*) would be more likely if the events suggested leading to this condition occurred in a very small group of animals or even derived from a single, pregnant, colonising female. This scheme contains two elements which have been implicated in speciation under certain circumstances : founder-flush events (Carson, 1971, 1982 ; Templeton, 1980) and chromosomal restructuring (White, 1978 ; Templeton, 1981). Whether these elements, by themselves, caused speciation of *A. persimilis* cannot, of course, be demonstrated. Yet, the establishment of such a chromosomal discontinuity within a previously continuous species range would normally be expected to reinforce reproductive isolation.

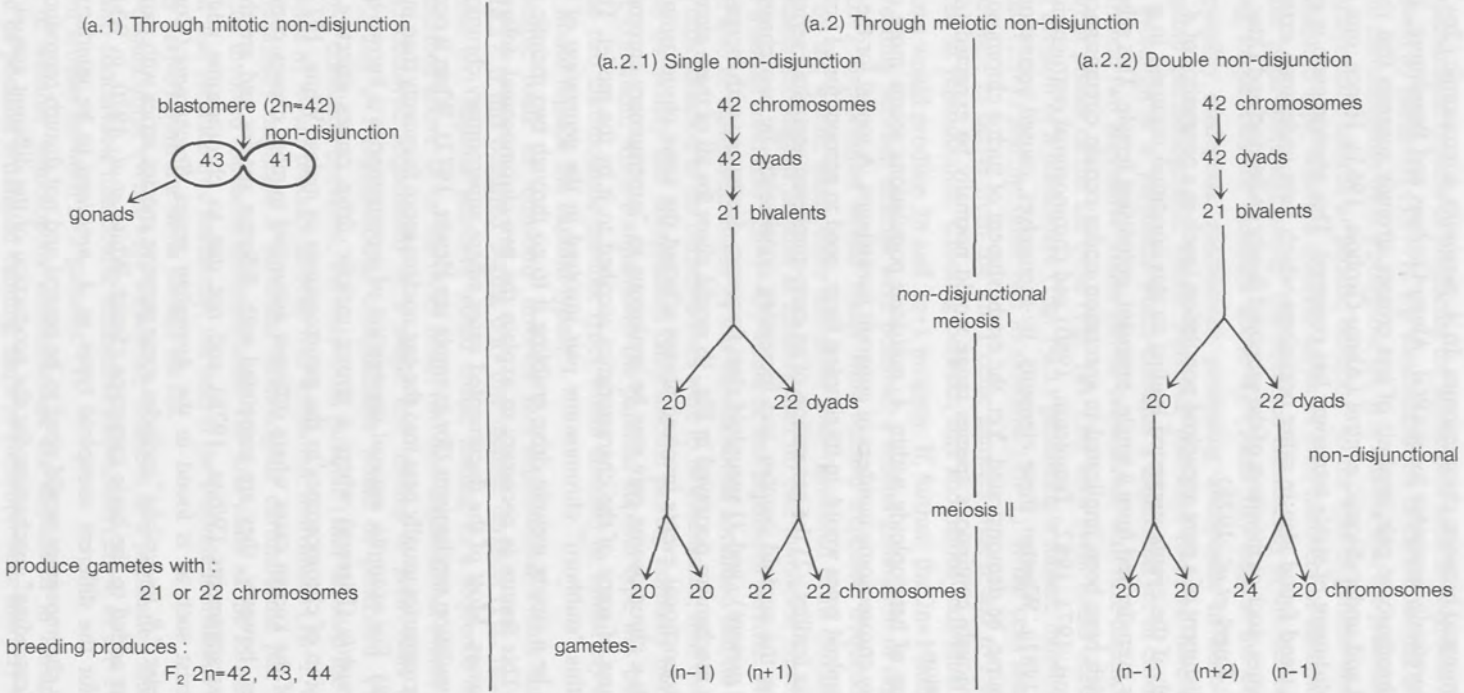
The finding of heteroploidy within *A. tunisiana* populations seems more complicated as it involves many chromosome numbers in apparent coexistence. A model for the generation of the different aneuploid types would, in its simplest form, need to account for : 1) complete sympatry and inter-class fertility ; 2) the generation of an even-numbered aneuploid series, with the lowest number being the euploid number and increasing consistently by two chromosomes (*i.e.* no monosomics present) ; and 3) aneuploid class frequency decreasing with increasing chromosome number. The mechanism portrayed in Fig. 1b would allow for all of these elements, particularly the non-disjunctional events involved always affected the same chromosome pair within the species. Such a chromosome pair may be analogous to "supernumary chromosomes" in other species because of some of the characteristics ascribed to it by the model. The most dramatic property of this "stubborn" chromosome pair involved in the generation of the supernumary series would be a strong meiotic drive enabling it to go through two meiotic divisions without segregation. This feature is necessary to explain the two-chromosomal difference between the euploid classes. Most of the documented cases where supernumary chromosomes are found possess accumulation mechanisms (for example see Hewitt, 1973). When it occurs in the female , the supernumaries usually pass into the egg nucleus more frequently than into the polar body (White, 1978). For example, unusual segregation of supernumaries is known in the fungus gnat *Sciaria coprophila* (Diptera) where a strong meiotic drive causes selective segregation and unequal division of chromosomes in the gametogenesis of males (White, 1973).

In most of the known cases where different aneuploid number classes contain supernumary chromosomes, however, they are associated with different species and are the result of chromosomal rearrangements (White, 1978) and not due to chromosome additions. However, exceptions exist such as is found in the Australian genus *Brachycome* (Compositae) whose species complex "*B. lineariloba*" includes some putative species series with pairs of homologous chromosomes added to the basic karyotype (Smith-White *et al.*, 1970, in White, 1978).

In order for the different aneuploid types in *A. tunisiana* to be interfertile, however, the supernumary chromosomes would need to be neutral and not disturb reproduction. If this were the case, a "cascading" mechanism for the production of the different aneuploid classes would

HYPOTHETICAL SCHEMES FOR THE DERIVATION OF ANEUPLOIDS

(a) *IN BISEXUAL FORMS*



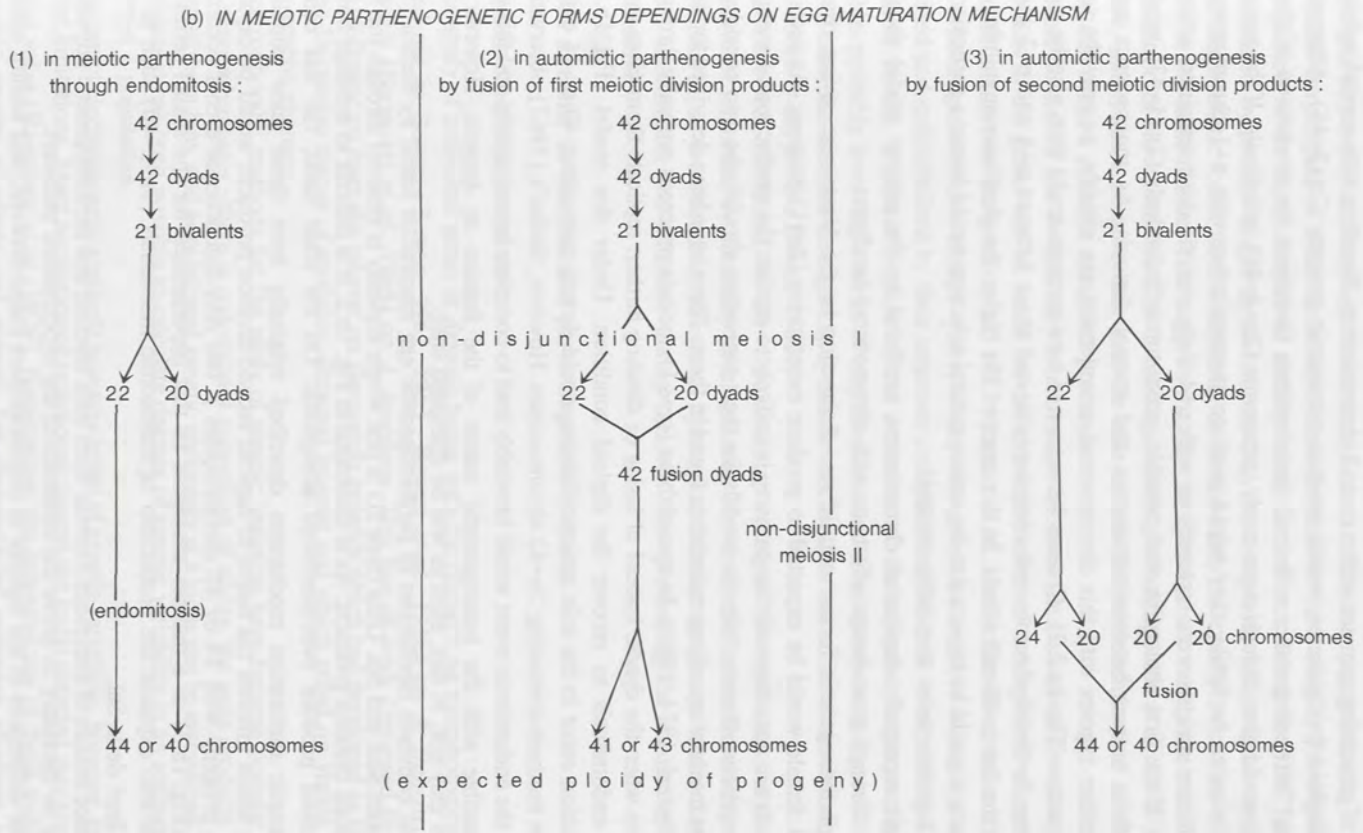


FIG. 1. Hypothetical schemes for the derivation of aneuploids in *Artemia* (see text).

be possible : those (presumably rare) diploid individuals with the "stubborn" chromosome would be capable of generating gametes with n or n+2 chromosomes. Breeding with normal individuals providing haploid (n) gametes, would produce tetrasomic progeny (2n+2=44). Alternatively, "in-breeding" between gametes with n+2 chromosomes (assuming the production of these in both sexes) would give, though more rarely, octasomic (2n+4=46) individuals. If the aneuploid individuals were to be fertile, they would produce gametes either with n+1 chromosomes (if normal reductive meiosis were restored) or with n+4 if the meiotic drive remained active and cumulative. If the first alternative was possible, monosomic individuals (2n+1=43) would be produced from crosses between tetrasomics and normal diploids. As monosomics are not observed, either zygotes with this chromosomal complement are inviable, or only the second pathway is active (Fig. 1a.2.2). Crosses between n+4 type gametes could then also be capable of generating 2n+8=50 chromosome complements, and those between n+4 and n+2 gametes would give the 2n+6=48 individuals. In this manner, the higher the chromosome number class, the more rare it would be expected to be, unless natural selection would favour a particular class (*i.e.* if the supernumaries stop being neutral).

The single repeatedly duplicated chromosome predicted by the present model should be identifiable through gene dosage effects or with chromosome bandings.

In the parthenogenetic forms, mitotic non-disjunction in the blastomere giving rise to a 2n+1/2n-1 female would be expected to produce monosomic (2n+1) progeny. As such types of aneuploids are not observed, this pathway is unlikely to explain the production of aneuploidy in parthenogenetic *Artemia*. Meiotic models for their generation should take into consideration the different mechanisms of egg maturation found in these. Three have been described for diploid forms (Barigozzi, 1974, 1980). In one of these (type I), meiosis proceeds normally through the first division when the dyads, instead of fusing or dividing further, replicate themselves through a sort of endomitosis to recover the diploid condition. Under this model (Fig. 1b.1), a non-disjunction event in the sole meiotic division would directly give either 40 or 44 chromosomes from mothers containing 2n=42 chromosomes. However, Stefani's (1967) observation is still valid : the endomitotic event would inevitably lead to complete homozygosity and this would be incompatible with the heterogametic nature of the females in *Artemia*. However, this mechanism may not, in fact, exist as will be discussed later.

The most common mechanism of parthenogenetic egg maturation found by Stefani (1960, 1967) in both Sète and Sta. Gilla (type II) is one where diploidy is retained through the fusion of first meiotic division products. As is illustrated in Fig. 1b.2, it is difficult to envisage how this pathway could produce even-numbered aneuploids. On the other hand, the less common parthenogenetic maturation mechanism described originally from these same populations (type III), which involves the fusion of haploid second division products, would be capable of generating progeny with 44 or 40 chromosomes if two non-disjunctional events occurred in sequence (Fig. 1b.3) in a manner analogous to that hypothesised for the sexual series. This mechanism also explains the production of nullosomic individuals (2n-2=40) such as those found in Gerri de la Sal.

Inheritance studies of aneuploid *Artemia*, from both bisexual and parthenogenetic populations, will clearly be necessary to prove the existence of the hypothesised pathways.

The clear differences in the number of chromocenters found between, and within, species of *Artemia* may have important evolutionary significance reflecting important population differentiation. For example, differences in mean number of chromocenters closely parallels the results from

electrophoretic surveys (see further) which can distinguish between Old and New World bisexual species and between Caribbean and North American *A. franciscana* populations.

Detailed karyotyping of *Artemia* chromosomes, an essential next step for the detailed study of the evolution of its chromosomes is already being carried out. Baratelli (1987) has already demonstrated by precise length measurements that the tetraploid chromosome complement of the parthenogenetic forms from Margherita di Savoia (Italy) is exactly twice that of its diploid relative in the same habitat. This suggests that the tetraploid is not merely the result of random accumulation of chromosomes. The G-banding results of Abatzopoulos *et al.* (1987) also show promise, additionally because they may be able to aid in the determination of the route to pentaploidy from a possible tetraploid precursor.

Deeper studies of the evolutionary relationships between the chromocenter complements of the strains in the genus will be based on the characterisation of the repeated sequences in this heterochromatin (see Barigozzi *et al.*, 1987 ; Renart *et al.*, 1987). However, heterochromatin is often a heterogeneous assemblage where, in any one genome a number of regions can each be described and individualised by their sequence composition (Peacock *et al.*, 1981). Drawing on the latest results, a clearer picture of the evolution of the repeated sequences will become possible, probably involving a series of amplifications and inter-chromosomal spreading from an original common ancestor through highly mobile mechanisms not unlike those described recently (see Moore *et al.*, 1978 ; Dover, 1982).

The observed differences in the chromocenter contents between and within species is suggestive of important evolutionary developments. However, the heterochromatic regions may be involved in, as yet unstudied, functional cytological roles in the groups where they are found such as stabilisation and guidance during meiosis, thus preventing the accumulation of stray chromosomes.

Genetic diversity in bisexual and parthenogenetic *Artemia*

EXTENT OF GENETIC VARIABILITY

The study of individual genes in *Artemia* began with the research on morphological variations carried out by Bowen (Bowen, 1962, 1963 ; Bowen *et al.*, 1966) on *A. franciscana* and by Barigozzi (Barigozzi *et al.*, 1969) on *A. tunisiana*. This line of work (reviewed by Barigozzi, 1974) followed on from the interest on *Drosophila* genetics which had developed as the synthetic or neo-Darwinian theory of evolution attracted interest and which relied on visible mutants to study genetic changes.

Electrophoretic methods for the study of genetic variation have become popular because of their many advantages : there is codominant expression of alleles, the gene products studied are chosen without bias, and populational genetic uniformity at a locus can be ascertained. With these techniques it has been possible to calculate degrees of genetic variability in an average individual within a population.

The use of electrophoretic methods to assess levels of genetic variability, however, has not been without limitations. More sophisticated techniques suggest the levels detected may underestimate the true value (Ramshaw *et al.*, 1979). Nonetheless, the extensive use of these methods across taxonomic groups of plants and animals has demonstrated pervasive allozyme variation in virtually all natural populations (see reviews by Powell, 1975 ; Selander, 1976). Furthermore,

there are significant differences in the levels of genetic variability among taxonomic groups (Nevo, 1978). Various reviews have appeared (Lewontin, 1974 ; Harris and Hopkinson, 1976) which provide excellent information regarding methodology, interpretation and overall evolutionary significance of the results for organisms in general, or specifically for Crustacea (Hedgecock *et al.*, 1982).

Following Gilchrist's (1954) work, the first genetic study of haemoglobins (with its biochemically-detected polymorphism) was carried out (Bowen *et al.*, 1969) and genetic variation in the gene loci coding for them was later discovered among parthenogenetic and bisexual *Artemia* (Bowen *et al.*, 1978). Biochemical characterisation (Bowen *et al.*, 1976 ; Sterling and Bowen, 1977) coupled with inheritance studies (Bowen *et al.*, 1977) have provided data for a model describing the genetic control of *Artemia* haemoglobins, based on two polyallelic loci, alpha and beta, giving rise to three dimeric haemoglobins with composition $(alpha)_2$, (alpha)-(beta), and $(beta)_2$, respectively (Ferry *et al.*, 1983).

In further protein loci assayed on *Artemia* populations derived from six continents (Bowen and Sterling, 1978 ; Bowen *et al.*, 1980) interspecific differences were visible. More extensive surveys of allozyme polymorphisms (Abreu-Grobois and Beardmore, 1980, 1982 ; Abreu-Grobois, 1983) have demonstrated the presence of large amounts of genetic variation in the genus. The values can be compared with analogous estimates in other taxa and, in particular, with other crustaceans using the more common indices which have been employed for quantification, *i.e.* 1) proportion of polymorphic loci (P) which is calculated as the fraction of all loci where the frequency of the most common allele does not exceed 0.99 (sometimes 0.95 is used alternatively) ; 2) expected heterozygosity over all loci (H_e), this is calculated from single loci heterozygosty (h_e) defined as $1-(x_1^2)$ where the x_1's are allele frequencies, the overall heterozygosity is derived from the arithmetic mean of the values over all the loci scored (this value will be a measure of the proportion of heterozygotes in a randomly interbreeding population) ; 3) average number of alleles per locus (n_e).

Table IV summarises the observed levels of genetic variability in various crustacean taxonomic subgroups (data from Hedgecock *et al.*, 1982) utilising the various measures mentioned above and compares them with the results for the *Artemia* bisexual species (Abreu-Grobois, 1983). It is evident that, within Crustacea, the levels of genetic variation found in this genus are among the highest. They are also comparable to the mean obtained from various invertebrates (mean P=0.40, mean H=0.11) which are, in turn, higher than those obtained from plants (mean P=0.26, mean H=0.07) and those from vertebrates (mean P=0.17, mean H=0.05) as compiled by Nevo (1978).

The P value (Table IV) for *A. franciscana* is slightly misleading for it was computed as a mean per population. In fact, 73 % of the loci assayed in the species as a whole showed polymorphism in at least one population. The analogous figure for *A. tunisiana* is 50 % (Abreu-Grobois, 1983).

When dealing with asexual organisms, however, the calculation of these measures of genetic variability can be slightly misleading as the unit of segregation is the composite genotype and not single genes as in bisexuals. Additionally, polyploid populations having multiple copies of some genes can express intralocus variation which causes dosage deviations from typical diploid electrophoretic phenotypes (Abreu-Grobois, 1983). Yet, although the calculation of H_e for parthenogenetic genotypes will not relate directly to the frequency of observed heterozygotes, it is a reflection of genetic diversity easily calculated from gene frequencies and can be universally applied regardless of reproductive system (Nei, 1975).

TABLE IV

Allozyme variation in bisexual species of *Artemia* compared to that in various other crustacean taxonomic subgroups (data for *Artemia* from Abreu-Grobois, 1983, rest from Hedgecock *et al.*, 1982)

Taxonomic group	Number of species (s) or population (p)	Average number of loci per species or population $\bar{x}$ (S.D.)	Average number of alleles per locus n_a (S.D.)	Average proportion of loci polymorphic per species or population $\bar{P}$ (S.D.)	Average expected proportion of heterozygosity per individual $\bar{H}_e$ (S.D.)	Average expected proportion of heterozygosity per population $\bar{H}_e$ (S.D.)
Crustacea						
Diplostraca	3 s	14.3 (2.1)	1.56	0.385 (0.140)	0.135 (0.044)	
Maxillopoda	20 s	19.1 (2.5)	1.81 (0.37)	0.483 (0.191)	0.131 (0.069)	
Hoplocarida	2 s	22.5	1.21	0.193	0.024	
Peracarida	4 s	18.5 (4.7)	1.47 (0.24)	0.214 (0.075)	0.073 (0.067)	
Eucarida	3 s	31.3 (4.2)	2.52 (0.69)	0.625 (0.233)	0.137 (0.078)	
Decapoda	65 s	24.5 (6.7)	1.38 (0.23)	0.231 (0.085)	0.052 (0.027)	
Totals for Crustacea	97 s	22.8 (6.9)	1.50 (0.39)	0.305 (0.177)	0.073 (0.052)	
Artemia						
A. franciscana	21 p	22.0	1.40 (0.70)	0.303 (0.126)		0.091 (0.037)
A. monica	1 p	22.0	1.82 (1.05)	0.500		0.185 (0.054)
A. persimilis	1 p	23.0	1.74 (0.92)	0.478		0.130 (0.037)
A. tunisiana	7 p	21.3	1.48 (0.97)	0.260 (0.072)		0.095 (0.024)
A. urmiana	1 p	22.0	2.09 (1.11)	0.636		0.141 (0.039)
Totals for *Artemia*	31 p	22.1 (0.6)	1.71 (0.96)	0.435 (0.154)		0.128 (0.039)

TABLE V

Summary of allozymic variation found in parthenogenetic *Artemia* (from Abreu-Grobois, 1983)

Ploidy group	Number of genotypes	Average number of loci assayed per genotype	Average proportion of heterozygous loci per genotype (S.D.)	Average genotype $\bar{H}_e$ (S.D.)	Average number of genotypes per population (S.D.)
Diploid	42	21.9	0.133 (0.043)	0.064 (0.019)	2.9 (3.4)
Triploid	3	22.0	0.227 (0.047)	0.100 (0.020)	1.0 (0.0)
Tetraploid	11	21.9	0.494 (0.015)	0.210 (0.006)	1.1 (0.3)
Pentaploid	3	22.0	0.424 (0.027)	0.182 (0.0)	1.5 (0.7)

The available data of genetic variability levels in parthenogenetic *Artemia* are summarised in Table V which can be compared to the bisexual results. For example, we can take the value of P in bisexual forms and the average proportion of loci heterozygous per genotype (PLH) of the parthenogens as analogous measures (although the former is a population or species parameter, while the latter is a clonal one). The PLH of parthenogenetic *Artemia* appears to increase with increasing ploidy and only the higher ploidies present values comparable to the P of bisexual counterparts. H_e, lower in di- and triploid asexuals than in the bisexual species, increase to higher values in the two highest ploidy groups. Nonetheless, measures of H_e in parthenogenetic forms do not fully describe their levels of genetic variation. Some measure of genotype diversity is also necessary as will be discussed later. In this case the values for average number of genotypes per population show an opposite trend to H_e with the diploid asexual populations, in general, containing about twice as many as their polyploid relatives. The probable basis for these results will be discussed in the following section.

Bases for the observed genetic variation

As with surveys of genetic variation in other taxa, the factors that may be responsible for maintaining the levels and patterns of diversity within populations must be considered. The body of literature dealing with this subject is divided largely between two opposing views. One of these, termed "neutral mutation" or "random genetic drift" (*e.g.* Crow and Kimura, 1963 ; Kimura, 1968a) asserts that observed genetic variation at the molecular level in populations of organisms is caused by the random drift of selectively neutral or nearly neutral gene mutants, altering the relative frequencies of the alleles but not the Darwinian fitness of their carriers. Changes in allele frequencies are, thus, governed by stochastic processes rather than by adaptive events derived from the action of balancing natural selection. According to neutral theory average heterozygosity is largely dependent on the rate at which selectively neutral alleles appear in each generation and on the effective population size of the reproducing population (Kimura, 1968b). In a system of differentiated populations, immigration can also augment variability (Maruyama, 1970). Neutral theory does not deny the existence of adaptive changes though it insists that the selective differential for alternative allele products at the molecular level is exceedingly small and natural selection occurs largely at the phenotypic level.

Opposed to this view is the theory that natural selection intervenes to preserve genetic variation in a population (see Lewontin, 1974) by operating directly upon specific assayable loci or upon alleles at neighbouring, tightly-linked loci. Selection, in order to be effective at maintaining genetic variability can take one of three major forms : 1) heterosis or over-dominance in which heterozygotes have higher fitness than homozygotes ; 2) frequency-dependent selection whereby genotypes will have increased fitness the rarer they are ; and 3) selection values varying across space or time in such a manner that alternative genotypes are better adapted to particular (alternative) portions of the species niche.

Excellent recent reviews have appeared to which the reader is advised to turn (cf. Kimura, 1982ab ; Nevo, 1983) including one where data on crustaceans are used to demonstrate strong discrepancies with the predictions of the theory of selective neutrality and supportive evidence for several forms of balancing natural selection using numerous studies of single and multiple loci in various species (Hedgecock *et al.*, 1982).

It would be fair to say, however, judging from the results from the very numerous studies, that "a synthetic view of the major forces maintaining genetic diversity, *i.e.* natural selection, mutation, migration and random drift, may be more realistic in explaining the structure of organic nature" (Nevo, 1983). In other words, there may be an interplay between these forces with the relative importance of each varying between loci and between species and ecosystems.

The available data for genetic variability in *Artemia* lend themselves for the testing of the applicability of some corollaries of the theories mentioned above. Five hypotheses have been tested in order to explain the electrophoretically determined genetic diversity (Abreu-Grobois, 1983), *i.e.* 1) levels of allozymic variability are determined by the molecular dimensions (Koehn and Eanes, 1978 ; Nei *et al.*, 1978) and quaternary structure (Zouros, 1976 ; Ward, 1977) of the proteins ; 2) the location of subcellular isozymes impose different levels of constraints on the amounts of "permissible" variation in the genes coding for them (Gottlieb and Weeden, 1981) ; 3) there is a positive association between the degree of environmental heterogeneity and levels of genetic variability (Hedrick *et al.*, 1976 ; Nevo, 1978) ; 4) there is a functional relationship between effective population size, rates of immigration and genetic diversity (Maruyama, 1970 ; Nei, 1975 ; Fuller and Lester, 1980) ; 5) there is a positive correlation between ploidy levels and heterozygosity in parthenogenetic forms, and the mode of reproduction (sexual *vs.* parthenogenetic, and apomictic *vs.* automictic asexual) affects the level of observable genetic variation (Lokki, 1983).

The results can be summarised as follows :

1) Utilising pooled results from all *Artemia* species, no significant association was found between number of protomers in enzyme and H_e ($r=0.10$, $P<0.25$) or n_a ($r=0.11$, $P<0.25$) though a positive correlation was found between subunit molecular weight (SMW) and H_e ($r=0.26$, $P<0.005$) and between SMW and n_a ($r=0.22$, $P<0.01$).

These results, consistent with analyses of data derived from many taxa (Koehn and Eanes, 1978) would be predicted by mutation drift hypothesis (Nei *et al.*, 1978). They can be described by a model which incorporates a mutation rate which is dependent on size but becomes lowered with increased quaternary structure as a result of the reduction of accessible surface area in the oligomers (Koehn and Eanes, 1978).

2) Of the three loci assayed in *Artemia* with subcellular segregation of isozymes (GOT, IDH and MDH), only the MDH system demonstrated significantly different total number of subcellular isozyme variants with the form believed to correspond to mitochondrial MDH having significantly more variability. This result was tentatively explained by the important role the cytosolic MDH has in osmoregulation (Conte *et al.*, 1980 ; Hand and Conte, 1982ab) thus possibly increasing the constraints on the permitted levels of variability for this isozyme compared to its less crucial homologue in the mitochondria.

3) Utilising a crude scoring system for descriptors of environmental heterogeneity (spatial and temporal differences) a negative Kendall's coefficient of rank correlation ($tau=-0.54$, $P<0.05$) was found between H_e and the overall environmental score for seven populations of *A. franciscana* and one of *A. monica* for which habitat data were available. This negative correlation would argue against the existence of an overriding influence of the environmental heterogeneity on the preservation of genetic variability in *Artemia*.

4) The very high levels of genetic variability seen in the *A. monica* sample (Table IV), both in terms of the H_e and of n_a, were difficult to explain. An alternative hypothesis would suggest

the passage of the remaining populations through bottlenecks in the past. For example, Bradbury (1971) reported drastic fluctuations in the size of the population in Zuñi Salt Lake caused by unpredictable explosive rainfalls leading to dramatic collapses of salinity, but no evidence exists for similar events in other habitats. Another explanation for the retention of high heterozygosity in the Mono population would be that it is a response to difficult ecological conditions in a manner analogous to that suggested by Parsons (1973) for *Drosophila.*

Of considerable interest, though controversial, is the possibility of input of genes from other populations into Mono Lake. Mono Lake lies on the flight paths of migratory bird species some of which visit other *Artemia* habitats. California gulls, eared grebes, northern and Wilson's phalaropes use the habitat as a "staging post", migrating in their thousands and feeding extensively on *Artemia* (Winkler *et al.*, 1977 ; Lenz, 1980 ; Jehl, 1981 ; Cooper *et al.*, 1984) and most probably bring millions of cysts from other populations attached to their bodies. Admitting this goes against the accepted wisdom that Mono Lake water, high in all three major anions (chloride, carbonate and sulphate), is not tolerated by other strains (Bowen, 1964 ; Clark and Bowen, 1976), the electrophoretic results may be providing circumstantial evidence that immigration has occurred, at least in the recent past.

That Mono Lake *Artemia* is interfertile with *A. franciscana* strains has been demonstrated by Bowen *et al.* (1985) by means of an artificial culture medium. If there is gene flow from *A. franciscana* strains into the Mono Lake population, enhancing n_a and N_e, it could either be through very rare pre-adapted genotypes or, perhaps more plausibly, through routes entering intermediate ecosystems whose populations contain genes for tolerance of both chloride and triple ion waters. One such system is exemplified by Fallon Lake (Nevada, USA), whose *Artemia* can be reared in either source water (Lenz, 1980) thus potentially being able to act as intermediary between populations from the two types of habitats.

5) A study of the patterns of genetic variation in parthenogenetic populations is important, particularly since for some time these were considered "evolutionary dead-ends", with little potential for genetic change. To test this assumption, a consideration of the theoretical relationship between the different modes of reproduction and their long-term consequences on genetic diversity is necessary. Whereas bisexual populations can increase their levels of genetic variation through various mechanisms such as sexual mixis, immigration of novel genes, meiotic recombination and mutation, only the latter two are available to automictic parthenogens, and only the last one to apomicts. On the other hand, while some variation will be removed by selection against deleterious mutations in both sexual and diploid parthenogenetic forms, polyploid relatives are largely "immune", as the increased number of gene copies buffers against detrimental mutations which become established. One would, therefore, expect heterozygosity to accumulate to a greater extent in polyploids than in their diploid relatives. Initially heterozygous loci in automictic populations will become homozygous in some of the clones generated through the action of meiotic crossing-over events. Based on these considerations, the following predictions can be made regarding the expected relative levels of genetic variability in bisexual and parthenogenetic populations :

Mode of reproduction	Genotype diversity	Heterozygosity
Bisexuality	Maximal	High
Automixis	High	Low
Apomixis	Low	Maximal

As was mentioned earlier, both apo- and automictic parthenogenesis are found in *Artemia*. All polyploid brine shrimp reproduce apomictically (Brauer, 1893 ; Artom, 1906 ; Barigozzi, 1974) and are therefore expected to accumulate heterozygosity as was explained above. Automictic forms (all of them diploid) will have their expected heterozygosity levels affected depending on the egg maturation pathway followed. Type I mechanism (see above) would be expected to lead to complete homozygosity very quickly even at sex loci. On the other hand, types II and III will be expected to retain heterozygosity at loci not involved in crossing over and as far as heterozygosity is concerned would be expected to be equivalent.

Results of electrophoretic surveys of parthenogenetic *Artemia* do not allow us to distinguish between maturation types, although no totally homozygous genotype has been reported which would fit the expectations arising from the endomitotic mechanism described above.

What can be observed from the large array of reproductive modes is a close agreement with the predicted levels of genetic variation. Genotypic diversity is, of course, much greater in bisexual forms, and among parthenogens it is consistently greater in the automictic diploids than in the apomictic polyploids (Table V). Heterozygosity, on the other hand, is strongly positively correlated with ploidy although the bisexuals have, on average, levels intermediate between those found in tri- and tetraploid asexuals. The heterozygosity-ploidy relationship is very consistent with data obtained from various taxa which contain related sexual and parthenogenetic forms of various ploidies (Lokki *et al.*, 1976ab ; Abreu-Grobois, 1983 ; Lokki, 1983).

The genetic variation levels observed in parthenogenetic *Artemia* attest to their evolutionary potential. Yet, when dealing with genetic diversity measures it is necessary to distinguish between genotype diversity and heterozygosity which, as has been explained above, are not equivalent under this mode of reproduction. Furthermore, even within diploid parthenogenetic populations, the magnitudes of these values are not constant ; some localities showing much larger numbers of related genotypes than others (Fig. 5) reflecting differences in rates of evolution between populations.

Evolutionary divergence and speciation

Morphological differentiation

The strong effect which the salinity of the medium exerts on the morphological development of *Artemia* is now widely known. Unfortunately, oversight of this phenomenon during studies in the latter part of the nineteenth and early twentieth century resulted in a proliferation of species, subspecies and varieties described for the genus (*e.g.* Daday de Dées, 1910). Most probably they were all *A. tunisiana* growing under different salinities. The confusion seems unnecessary for Schmankewitsch (1875, 1877) had already demonstrated how different salinities affect the morphology of the brine shrimp. Yet, even he persisted in accepting and supporting the existence of the various taxonomic entities which could be distinguished only by slight environmentally-modified details.

Consistent morphological differences between the species, however, are visible if they are grown under standardised conditions. Measures of length of furca, setae per furca, ratio of abdominal length to total length, shape of ovisac, shape of the clasper knobs, presence or absence of penis spines, etc. are the basis for the systematics of the bisexual forms (Gilchrist, 1960 ; Piccinelli and Prosdocimi, 1968 ; Amat Domenech, 1980). An updated list of the most

discriminatory morphological characters are given in Table VI, together with cytological data of relevance. These features are not all as widely known as they should be. Many morphological differences also exist between parthenogenetic populations but these have not been studied fully.

Considering the significant interpopulational differentiation found by other methods, it is unfortunate that modern cladistic and numerical taxonomic analyses have not been applied to the available morphological differences. It is highly probable that this information would provide complementary results which could aid the study of its intraspecific evolution.

Qualitative rare traits governed by single genes have already been mentioned. However, with the exception of the two- or three-lobed protuberance on the antennae of most females in *A. franciscana* from Zuñi Lake (Bowen, 1964) they are not useful as discriminators of populational differentiation.

Reproductive characteristics and ecological isolation

Two general classes of reproductive isolation mechanisms have been recognised : "premating", containing mechanisms which will prevent interspecific crosses and which include ecological, ethological and mechanical mechanisms ; and "postmating", which includes processes which reduce or prevent the viability during part of the life cycle of progeny resulting from hybrid crosses. Determination of "species" normally relies on the demonstration that the different entities are reproductively isolated from each other while within each one there is actual or potential interbreeding. This is the definition according to the "biological species concept" (Mayr, 1963).

In *Artemia*, demonstration of reproductive isolation between sexual populations provided the key for the proper classification of the species. The results of various studies are summarised in Table VII and have been reviewed by Bowen *et al.* (1980). Because brine shrimp of different species clasp and appear to copulate readily in trial matings, the mechanisms of reproductive isolation must contain weak or no ethological and structural incompatibilities and must rely largely on postmating elements.

Finding of mixtures of reproductively isolated species (*e.g. A. persimilis* with *A. tunisiana* in Italian and Argentinian salterns ; Halfer-Cervini *et al.*, 1968) led to the belief that additional species and subspecies would be found as better characterisation became established (Bowen *et al.*, 1980). However, the available information has not warranted changes in the systematics of the genus in the last six years, with the possible exception of subdivisions of *A. franciscana* in North America.

In contrast to the habitat of Old World *Artemia* species, the variation in the relative ionic composition of the media inhabited by *franciscana* populations is exceptionally large, exceeding that found in the range of any other aquatic metazoan (Cole and Brown, 1967). It is not surprising, therefore, that physiological adaptations have developed in *franciscana*. Mono Lake habitat water is lethal to shrimp from high chloride lakes and Mono Lake *Artemia* have reduced viability in seawater or other high chloride waters (Bowen, 1964 ; Mason, 1967 ; Lenz, 1980). Because of the existence of ecological barriers to gene exchange in the natural environment, Bowen (1964) suggested the retention of full species status for this population : *A. monica* (after Verrill, 1869). A similar case may be made for Jesse Lake *Artemia* (*A.* sp.) which has a more extreme ionic composition (Bowen *et al.*, 1980).

TABLE VI

Summary of the characteristics of bisexual *Artemia* species

	Species	*A. franciscana*[a]	*A. monica*[b,e]	*A. persimilis*[c]	*A. tunisiana*[a,c]	*A. urmiana*[b,d,e]
(a)	Both sexes :					
	Natural distribution	American continent and Caribbean	Mono Lake, California, USA	Argentina	Europe and N. Africa	Lake Urmia, Iran
	Diploid chromosome no.[b]	42	42	44	42	42
	Chromocenters[b]	+/+++	+++	+	Absent	Absent
	Furca morphology	Two lobed ; many setae ; basal constriction	As *franciscana*	Rudimentary lobed ; few setae	Two lobed ; many setae ; without basal constriction	Rudimentary lobed ; very few setae
(b)	Females :					
	Ovisac	Laterally pointed	As *franciscana*	Laterally pointed with spines	Laterally round	Laterally ellipsoidal
	Lower thoracic protuberance[a]	Present	As *franciscana*	Present	Absent	Present
(c)	Males :					
	Clasper Knob shape	Subspherical	As *franciscana*	Subspherical	Subconical	Subspherical
	Penes	With spine	As *franciscana*	With spine	Without spine	With spine

[a] Amat Domenech (1980), [b] Beardmore and Abreu-Grobois (1983), [c] Piccinelli and Prosdocimi (1968), [d] Günther (1900), [e] Abreu-Grobois (1983).

TABLE VII

Results of test for reproductive and ecological isolation between and within species of *Artemia* (diagonal and above the diagonal) and the reported existence of mixtures of species in natural habitats (below the diagonal)

	A. franciscana	*A. monica*	*A.* sp.	*A. persimilis*	*A. tunisiana*	*A. urmiana*	parthenogenetics[a]	References[b]
A. franciscana	0	0/+	0/+	+	+	+	+	1,2,3,4,5,8,11,14
A. monica	n.i.	0	n.d.	+	+	+	n.d.	6,8,10,11,14
A. sp. (Jesse Lake)	n.i.	n.i.	n.d.	n.d.	n.d.	n.d.	n.d.	11,14
A. persimilis	yes/no	no	no	0	+	+	+	7,8
A. tunisiana	no	no	no	yes/no	0	+	+	1,2,4,7,8,12
A. urmiana	no	no	no	no	no	0	0	9,13
parthenogenetic	yes	no	no	no	yes	no	+	9,13
References[b]	4/13	13	13	7/13	7,13	13		

Key to abbreviations :

n.i. = not identifiable.

\+ = crosses between these pairs are either sterile or impossible because of different media requirements.

0 = crosses between these pairs are fertile.

0/+ = though some natural habitat media present ecological barrier to reproduction between some populations, artificial media allow cross-breeding in the laboratory.

n.d. = not documented.

[a] Attempted crosses here refer to the use of bisexual females mated to rare males derived parthenogenetically (see text).

[b] References to the right of the table concern reproductive and ecological isolation studies, those below the table concern mixtures of species.

References : 1=Kuenen (1939), 2=Gilchrist (1960), 3=Bowen (1964), 4=Bowen (1965), 5=Amat Domenech (1980), 6=Mason (1967), 7=Halfer-Cervini *et al.* (1968), 8=Clark and Bowen (1976), 9=Bowen *et al.* (1978), 10=Lenz (1980), 11=Bowen *et al.* (1980), 12=Browne (1983b), 13=Abreu-Grobois (1983), 14=Bowen *et al.* (1985).

The types of waters where *Artemia* is found can be classified into three main categories based on the anion with the highest relative concentration : chloride, sulphate, or carbonate. Tolerance of anions by North American brine shrimp has been recently studied by Bowen *et al.* (1985) who were able to distinguish population clusters based on their capacity to tolerate chloride. In general, high chloride media were selective against populations from low chloride habitats ; high carbonate media were selective against shrimp from high chloride habitats, but was tolerated by those from low chloride habitats.

The genetic adaptations underlying the ecological adaptations are difficult to assay, although differences in the viabilities between populations in single media are attributable to genotypic differentiation (Bowen *et al.*, 1985). Further insights into the inheritance of the tolerance capacities are possible from this study, particularly from the results of exceptional populations. For example, Penley Lake *Artemia*, though inhabiting a system with a relatively low carbonate content (<5 %), is capable of surviving high carbonate media (22 % CO_3) ; Fallon Lake *Artemia* which normally live in low carbonate lake (25 % CO_3) is capable of surviving both low and high carbonate concentrations and can be successfully reared in seawater or in carbonate waters (Lenz, 1980). These characteristics are probably adaptations inherited from ancestral populations which were adapted to carbonate systems such as could be found in the past when the chemical composition of the water was different than that at present (Bowen *et al.*, 1985).

Reproductive isolation was also tested under controlled conditions, with carefully contrived artificial mixtures of salts permitting growth of different ecotypes in common media. Thus, populations substantially ecologically isolated in nature, can be shown to be cross-fertile with each other or with third populations (Bowen *et al.*, 1985). This is shown diagrammatically in Fig. 2 where the most extreme examples of the *franciscana* populations are selected. The solid lines represent the theoretically expected level of habitat barrier to gene flow if no other obstacles existed, based on the reported media viabilities (data from Bowen *et al.*, 1985). If the habitat conditions fit within the tolerated concentrations, an arrow is used. This arrow is drawn unidirectional (if only a one-way flow is deduced) or bidirectional (if exchange in either direction could be possible). Broken lines represent the results of Bowen's crossbreeding experiments using artificial media. Thus, Mono Lake *Artemia* can be crossed with high chloride shrimp (such as those from San Francisco) producing fertile F_1 and viable F_2 progeny. On the other hand, Jesse and San Francisco shrimp, which cannot be intercrossed because of remaining differences in tolerance to anion concentrations, are both crossfertile with Fallon *Artemia* with intermediate tolerance.

Differences in survival rates of different *Artemia* strains under various combinations of temperature and salinity (Sorgeloos *et al.*, 1976 ; Vanhaecke *et al.*, 1984) are also evidence for genetic differences governing ecological adaptations. Differentiation at loci affecting these traits is reflected not only when comparing between species but also between populations from a single species. For example, though all strains demonstrate tolerance to a wide range of temperatures (20-34 °C), Chaplin nauplii characteristically demonstrated higher survivals at conditions with temperatures lower than 20 °C, reflecting adaptations to its colder habitat.

A deeper understanding of the genetic basis for these ecological adaptations will necessarily involve more sophisticated quantitative analysis of breeding studies between different ecotypes. Nonetheless, the ecological isolation found in *franciscana* has immediate consequences on the systematics of the genus which will be discussed in a later section.

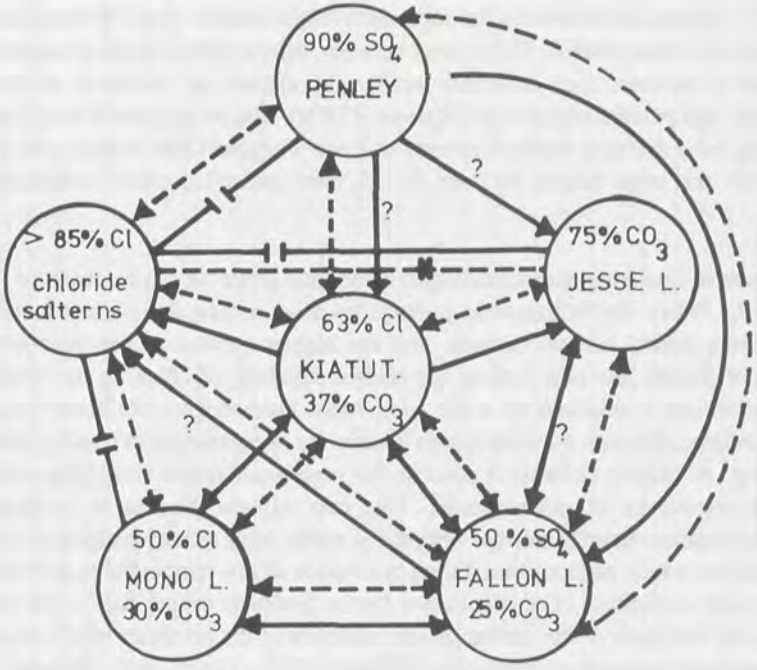


FIG. 2. Reproductive and ecological isolation among selected populations of *A. franciscana*. Percentages inside circles indicate approximate relative molar concentrations of the principal anions in each habitat (for Giatuthlanna the figures represent the average between Green and Red pond values). Solid arrows point towards anionic conditions acceptable to populations on the basis of their tolerances. Solid lines with cut indicate intolerance of conditions by populations. Broken arrows indicate results of crossing experiments in permissive media. Question marks indicate untested theoretical expectations. See text for details (all data derived from Bowen *et al.*, 1985).

Quantitative analysis of reproductive characteristics of *Artemia* has given insight into evolutionary questions that have been difficult to test with other animals not amenable to cultivation. The cost of reproduction (trade-off between early reproduction or high reproductive output with longevity) is significant in the brine shrimp but only under low food conditions (Browne, 1982; Browne *et al.*, 1984). The relative advantages in reproduction and growth when comparing sexual and parthenogenetic organisms has long been a subject of debate (Bell, 1982). There are difficulties in demonstrating the theoretically inherent advantage of uniparental reproduction. The results are affected both by the food concentration and the population density. Under high food and high animal densities, sexual strains displace parthenogens, yet under low food and low densities neither reproductive mode is superior (Browne, 1980b). The superiority of the bisexuals under high food conditions would seem to contradict the theory and even seems unexpected for parthenogenetic brine shrimp which are at least twice as fecund as sexual populations when calculated on a per adult basis (Browne *et al.*, 1984). Other factors must be affecting the outcome in these competition experiments, possibly including some reproductive advantages in bisexuals, such as a shorter prereproductive period than in the parthenogenetics.

Significant interstrain differences in reproductive parameters may be responsible for the variable results in these studies. Differences between strains within a single species have been demonstrated in variables such as broods per female, zygotes per brood or per female reproductive period, and percent encystment (Browne, 1980a). The proportion of encystment has also been found to have diverged between present day San Francisco Bay *Artemia* and a population originally from the same habitat but kept for 25 years under laboratory conditions (Browne, 1983a).

Some degree of clustering corresponding to taxonomic grouping can be observed (Table VIII ; Browne *et al.*, 1984). Parthenogenetic populations have among the longest female lifespans, longest recovery period between broods, and the highest percent of live born broods. Some populations of *franciscana* rank among the highest numbers of offspring per brood and total offspring per female. Calculated on a per adult basis, parthenogens are more fecund than the sexual populations, although the *franciscana* females are as productive in terms of daily rates and total offspring. A striking contrast is seen in the *tunisiana* females which demonstrated weak performance relative to all other species. This may explain the rise in dominance by the parthenogens in some Spanish salterns during the warm, high salinity periods taking over from bisexual *tunisiana* which predominate during conditions of low temperatures and salinity (Amat Domenech, pers. commun.). The dominance by the bisexuals during part of the season is not clear. It may be the result of the greater genetic diversity of the bisexuals which enables them to track harsh and/or uncertain environments (Williams, 1975 ; Lloyd, 1980 ; Browne *et al.*, 1984) or causes them to respond to environmental cues that trigger hatching at different times.

Partitioning of the genetic and the environmental components of the reproductive characteristics in *Artemia* has been attempted (Browne *et al.*, 1984). If the parthenogenetic samples were sufficiently homogeneous genetically (*i.e.* strictly monoclonal), the observed variance levels of the reproductive traits should be the result of environmental influences alone. On the other hand, variance levels within sexual populations will be caused by both environmental and genetic variations (or from a combined effect). Therefore, determination of the environmental contributions to total variation should be possible by dividing the average coefficient of variation (cv) of the asexuals by that of the sexuals. Unfortunately, even with 12 populations, strong interstrain differences between parthenogenetics affected the calculations (Browne *et al.*, 1984), leading at times to results contradicting earlier conclusions (Browne, 1980b). The assumption of genetic homogeneity among parthenogenetic populations was also not borne out by the high levels of genotypic diversity reported elsewhere (Abreu-Grobois and Beardmore, 1982). This led to their overestimation of the environmental contribution. Unbiased estimates of the relative genetic variation may only be obtained by utilising offspring from a single laboratory parthenogenetic female, as the authors suggest (Browne, 1980b).

Correlations between life history traits in many species of *Artemia* have been useful in determining the trade-offs between the different reproductive characters (Browne, 1980b). Recovery time between broods has been found to be positively correlated with number of broods per female ($r=0.75$, $P<.01$), number of offspring ($r=0.50$, n.s.), female lifespan ($r=0.75$, $P<.01$), and female reproductive period ($r=0.86$, $P<.01$). These results, together with the negative correlation between female postreproductive period and reproductive output (mean $r=-0.62$) indicate an exhaustion of energy reserves in *Artemia* subsequent to high reproductive output, leading to quicker postreproduction senescence.

TABLE VIII

Comparative reproductive performance for bisexual and parthenogenetic *Artemia*

Populations with no significant difference between the mean score for each characteristic have been joined with an underline.
Figures at extreme left and right of each characteristic represent the mean values of the populations with the worst, respectively the best performance.
Population lists for each characteristic are ordered according to performance, with highest values towards the right
(for methods and original data see Browne *et al.*, 1984).

Characteristic	Populations												
Offspring per brood	20.7	T(tun)	T(cy)	T(sp)	A(ca)	A(gi)	F(cr)	A(ku)	P(pe)	A(tur)	F(ch)	A(ma)	F(sf)	111.4
Broods per female	3.9	P(pe)	T(cy)	T(sp)	T(tun)	A(gi)	F(ch)	A(ca)	F(cr)	A(ma)	F(sf)	A(tur)	A(ku)	19.1
Offspring per day per female	4.5	T(tun)	T(cy)	A(ca)	T(sp)	A(tur)	A(ku)	A(gi)	F(cr)	A(ma)	F(ch)	F(sf)	P(pe)	31.6
Time between broods (in days)	2.9	P(pe)	T(sp)	T(cy)	F(sf)	F(cr)	A(gi)	F(ch)	T(tun)	A(ma)	A(ca)	A(ku)	A(tur)	6.0
Percent offspring encysted	3.0	A(ku)	A(gi)	A(ma)	A(tur)	F(ch)	F(sf)	F(cr)	P(pe)	A(ca)	T(sp)	T(tun)	T(cy)	100.0
Total offspring per female	112	T(cy)	T(tun)	T(sp)	P(pe)	A(gi)	A(ca)	F(cr)	F(ch)	A(tur)	A(ma)	A(ku)	F(sf)	1619
Female prereproductive period (in days)	28.9	F(cr)	F(sf)	A(ku)	F(ch)	T(tun)	P(pe)	A(ma)	A(gi)	A(tur)	T(sp)	T(cy)	A(ca)	43.4
Female reproductive period (in days)	12.2	P(pe)	T(cy)	T(sp)	A(gi)	T(tun)	F(ch)	F(cr)	F(sf)	A(ca)	A(ma)	A(tur)	A(ku)	82.0
Total female lifespan (in days)	57.7	P(pe)	T(cy)	T(sp)	F(cr)	T(tun)	A(gi)	F(ch)	F(sf)	A(ma)	A(ca)	A(tur)	A(ku)	146.6

Identification codes : F=*A. franciscana* ; P=*A. persimilis* ; T=*A. tunisiana* ; A=parthenogenetic strain ; ca=Cadiz, Spain ; gi=Giraud, France ; tu=Izmir, Turkey ; ma=Madras, India ; ku=Kutch, India ; sp=Santa Pola, Spain ; tun=Chott Ariana, Tunisia ; cy=Larnaca Lake, Cyprus ; sf=San Francisco Bay, California, USA ; ch=Lake Chaplin, Saskatchewan, Canada ; cr=Cabo Rojo, Puerto Rico ; pe=Hidalgo, Argentina.

The comparisons of reproductive traits between strains of *Artemia* not only allow a closer scrutiny of evolutionary theories, but also give greater insight into the relative performance of strains and species.

Molecular phylogeny and the origin of parthenogenesis

Prior to the arrival of biochemical methods for the study of genetic differentiation in the early 60's, phylogenetic studies among taxa relied on studies of comparative morphology and reproductive isolation. The complexities of the changes accompanying speciation were difficult to interpret genetically and could not be related to time of divergence. In particular, though the different bisexual species in *Artemia* were distinguishable on morphological and cytological grounds, and were reproductively isolated, the evolutionary relationships between parthenogenetic and bisexual forms were almost impossible to elucidate as they could not be intercrossed.

Electrophoretic assay, although lacking the precision of amino acid and nucleotide sequence information, is at present the best method for routine measurements of genetic differences involving a large number of populations. In comparison to orthodox techniques, enzyme electrophoresis has the advantages of objectivity, constancy of characters through development, and a measure which is directly comparable between studies of different taxa (Avise, 1974 ; Ferguson, 1980).

The most widely used measures of genetic differences, developed by Nei (1972) and Nei and Roychoudhury (1974), are derived from estimates of allelic frequencies in populations. Both genetic identity (I) and genetic distance (D) measures can be derived. The latter is proportional to divergence time between pairs of populations and estimates the number of codon substitutions per locus between them. Studies cover a wide range of taxons, at different levels of taxonomic separation and have been generally consistent across taxa and in accord with accepted classifications based on morphological traits (Ayala, 1983 ; Thorpe, 1983).

Genetic identities have been calculated for *Artemia* at single loci (Abreu-Grobois and Beardmore, 1982 ; Abreu-Grobois, 1983), demonstrating different patterns among groups of enzyme loci. Distribution of single locus I values describe the common U shape found in other studies, with more than 75 % of the loci I values falling in the 0.9-1.0 range for intraspecific comparisons, while for interspecific comparisons the majority (>85 %) of the I values lie within 0.0-0.1. The results are almost the same when parthenogenetic populations are compared. The values, which are lower than those reported by Ayala *et al.* (1974) for the *Drosophila willistoni* complex, reflect an untypically high level of interpopulational differentiation.

Overall genetic distances have been estimated between a large number of populations (Abreu-Grobois and Beardmore, 1980, 1982 ; Abreu-Grobois, 1983 ; the matrix of D values for 90 pairwise comparisons is available from the author upon request). The magnitude of D for different taxonomic level comparisons (Table IX) is larger than expected from comparable studies of other taxa (see Ayala, 1983) and is another reflection of the strong tendencies towards differentiation in this genus. For example, average congenetic estimates in *Artemia* are not only higher than analogous measures for other organisms but are similar in magnitude to intergenetic comparisons. Mean D values between *A. tunisiana* and *A. franciscana* are even higher (mean D=2.77). Thus, on the basis of electrophoretic results alone, this species pair should not be referred to as "sibling species".

TABLE IX

Mean overall genetic distances (Nei's $\bar{D}$) in the genus *Artemia* (from Abreu-Grobois, 1983)

Species	$\bar{D}$	Standard error
Conspecific comparisons		
A. franciscana	0.126	0.067
A. salina	0.091	0.061
All parthenogenetics	0.189	0.092
Mean conspecific value = 0.135 (with parthenogenetics) ; 0.109 (without parthenogenetics)		
Congeneric comparisons		
A. franciscana vs. *A. monica*	0.098	0.050
A. franciscana vs. *A. persimilis*	1.073	0.299
A. franciscana vs. *A. tunisiana*	2.767	0.871
A. franciscana vs. *A. urmiana*	1.497	0.413
A. franciscana vs. parthenogenetics	1.952	0.542
A. monica vs. *A. persimilis*	1.025	0.282
A. monica vs. *A. tunisiana*	2.436	'.687
A. monica vs. *A. urmiana*	1.529	0.385
A. monica vs. parthenogenetics	2.022	0.525
A. persimilis vs. *A. tunisiana*	1.892	0.542
A. persimilis vs. *A. urmiana*	1.381	0.384
A. persimilis vs. parthenogenetics	2.258	0.659
A. tunisiana vs. *A. urmiana*	0.949	0.277
A. tunisiana vs. parthenogenetics	1.023	0.295
A. urmiana vs. parthenogenetics	0.498	0.170
Mean congeneric value = 1.493 (with parthenogenetics) ; 1.465 (without parthenogenetics)		

Application of cluster analysis to the genetic distance matrix of *Artemia* populations (Abreu-Grobois and Beardmore, 1980, 1982) clearly demonstrates the genetic separation between Old World species (*tunisiana, urmiana,* and parthenogenetic forms) and their New World relatives (*franciscana, monica,* and *persimilis*) at one level (mean $D>2.0$), and between each of the species at another (mean $D<1.0$). Within species, patterns of differentiation are only discernible within the *franciscana* group, with sets of geographically-proximal populations clustering together (Abreu-Grobois and Beardmore, 1982). This is the case for the populations in the Caribbean, the Canadian sulphate lakes (Chaplin and Little Manitou), and the two carbonate ponds at Kiatuthlanna (Green and Red Pond). The ecological isolation found between these groups has already been discussed. The genetic distance data corroborates the presence of significant differentiation at the level of structural gene loci. The remaining strongly ecologically isolated populations are Jesse Lake and Mono Lake. The former does, in fact, demonstrate both a marked genetic distance from the rest of the *franciscana* clusters and a geographic isolation. Mono Lake, on the other hand, does not show significant levels of differentiation from *franciscana* as a whole (typical D=0.06-0.09). This result can be interpreted in one of two ways : either there is no complete genetic isolation in Mono Lake and some gene flow can occur or has

occurred in the recent past at least through intermediate habitats (see previous section), or the isolation is effective and very few loci are involved in the ecological adaptation (cf. Beardmore and Abreu-Grobois, 1983 ; Bowen *et al.*, 1985).

The population subdivision of those species within which more than one population sampled has been estimated by the G_{ST} statistic (Nei, 1975) which calculates the proportional magnitude of genetic differentiation between subpopulations. The results for *franciscana* suggest significant subdivision (G_{ST}=0.24, P<0.01), while for *tunisiana* this is not the case (G_{ST}=0.12, n.s.).

Overall measures of D have allowed for the first time an objective assessment of the degree of relationship between the parthenogenetic and the bisexual species in this genus. The data strongly suggest that the closest extant relative to the asexuals is *A. urmiana* with a mean D=0.5. The clustering together of all the parthenogens, further, is evidence for a monophyletic origin to parthenogenesis. These results have also been suggested by studies of haemoglobins (Bowen *et al.*, 1978, 1980).

Among the parthenogenetic populations, some patterns are discernible which are of evolutionary significance. The tetra- and pentaploids cluster together with very low genetic distances (mean D between tetrapoids =0.02 ; between pentaploids =0.01 ; between tetra- and pentaploids =0.04 ; Abreu-Grobois, 1983) suggesting a very close relationship. Recent cytological observations of oogenesis in Greek tetraploids show the production of aberrant pentaploid cells (Abatzopoulos, pers. commun.) corroborating the possible existence of a route to the derivation of pentaploidy directly from a tetraploid ancestor.

In contrast, triploid parthenogens tend to cluster apart and have relatively high genetic distances not only between themselves and other asexuals, but also between each other. This suggests that the generation of this ploidy has probably occurred more than once in independent events.

The more common ploidy group among parthenogenetic brine shrimp, diploids, contains a heterogeneous assemblage. Some populations contain a large number of clones, all closely related and probably derived from the same colonising female through mutation and/or recombination events. Some electrophoretically equivalent or very similar clones are found in more than one locality. These suggest a pattern of cyst transport between habitats and a strong tendency towards local differentiation and diversification (Abreu-Grobois, 1983). The ecological significance of the different electrophoretic clones is not known although there are differences in reproductive performance between different uniparentally derived lines of Salin de Giraud *Artemia* (Browne and Spencer, 1987). The equivalence of the electrophoretic clones and these lines has yet to be determined.

Measures of genetic distances for *Artemia* comparisons have been converted to divergence times using various formulas (Beardmore and Abreu-Grobois, 1983). Although this approach is controversial because of the large error involved if no palaeontological record is available to calibrate the "molecular clock", it does allow useful approximations. A simplified version of the dendrogram of genetic distances in the genus (Fig. 3) summarises the overall D values and the calculated date of branching, as well as genetic events characterising various lineages. Thus, it is possible to speculate that the evolution of parthenogenesis occurred about 5.4 million years ago in the Mediterranean area. What is more interesting about this time estimate is that geological evidence suggests that approximately 6 million years ago the whole Mediterranean basin underwent a dramatic increase in salinity and subdivision of habitats (Hsü, 1972). Such an event would effectively have opened up a vast area for colonisation to *Artemia*. This would have placed

a premium on genotypes capable of self-fertilization particularly in the leading edges of the population where the rapid expansion of the range may have thinned out the density. This hypothesis is not unlike the one for the evolution of parthenogenetic insects during recolonisation after the retreat of the Ice Age in Northern Europe (Suomalainen *et al.*, 1976) and finds favour in Bell's (1982) conclusions when analysing current theories on the evolution of sex in animals.

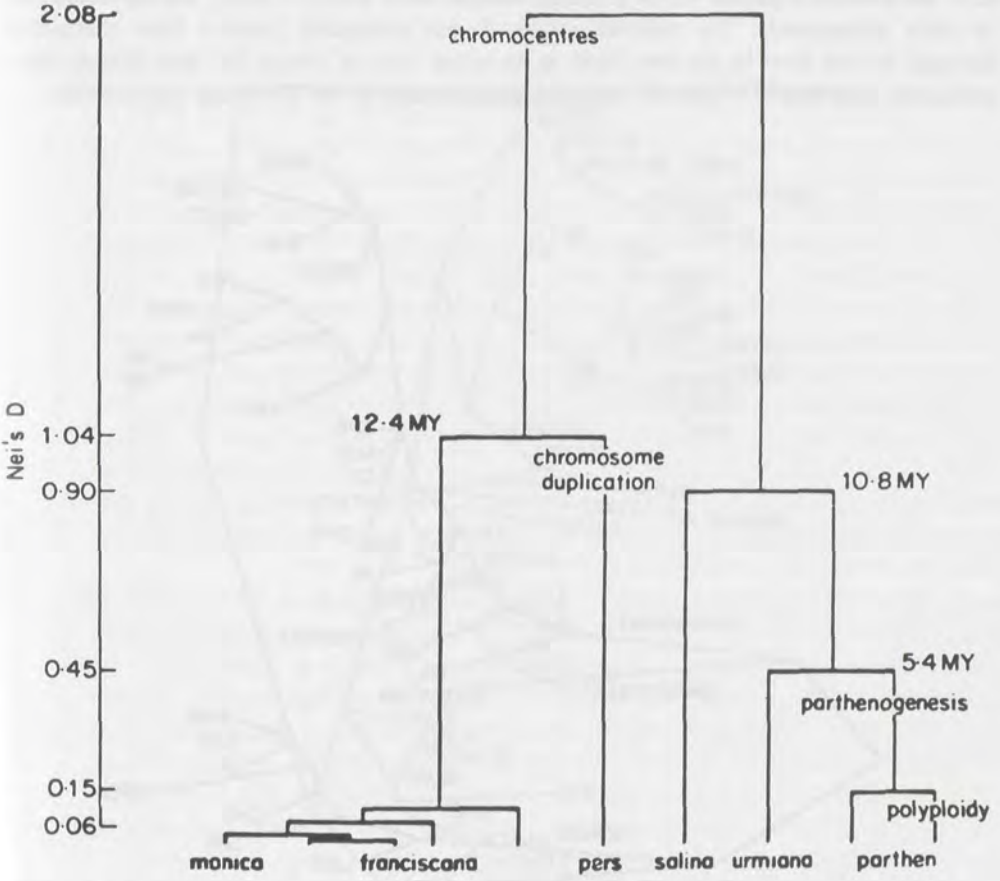


FIG. 3. Genetic distance and evolution in *Artemia.* Simplified version of dendrogram of genetic stances in the genus (from Beardmore and Abreu-Grobois, 1983) (in this figure "salina" denotes *A. nisiana*).

The resolution of the evolution of polyploidy has also been difficult. A simple endomitosis in rm cells or the fusion of polar body spindles in a diploid automictic maturation with the ppression of polar body extrusion can lead to a tetraploid condition. However, a further step ould be required as diploid parthenogenetic *Artemia* retain meiosis while polyploids do not. vo schemes can be considered : apomixis evolving from an intermediate automictic polyploid m, or a macromutation leading directly to polyploidy and apomixis from an automictic diploid imitive condition. The available data, while strongly suggesting a single line to tetraploidy, do

not allow us to distinguish between the alternatives. In contrast, as was mentioned, triploidy probably evolved more than once. A number of mechanisms for its evolution can be considered : 1) automixis between a pronucleus (2n) and a reduced polar body (n) ; 2) a triple fusion of haploid nuclei, as is found to occur occasionally in *Drosophila parthenogenetica* (Stalker, 1954), or 3) an allopolyploid event resulting from a successful fusion between a parthenogenetic egg (2n) and a reduced gamete (n) of a related bisexual male. Loss of meiotic pairing would need to occur subsequently. The available results do not distinguish between these alternatives, although the last must be the least likely as no extant bisexual species has been studied with a sufficiently high degree of genetic similarity to correspond to the theoretical expectations.

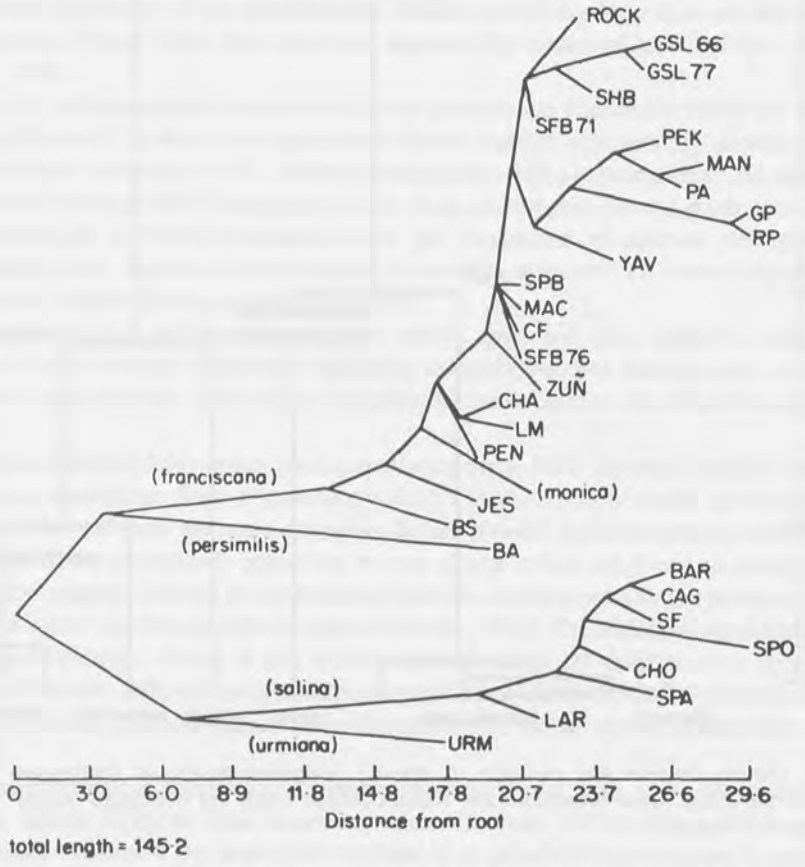


FIG. 4. Phylogenetic tree of bisexual *Artemia*. BA : Buenos Aires, Argentina ; BAR : Barbanera, Spain ; BS : Bahia Salina, Puerto Rico ; CAG : Cagliari, Italy ; CHA : Chaplin Lake, Canada ; CHO : Chott Ariana, Tunisia ; CF : Cabo Frio, Brazil ; GP : Green Pond, USA ; GSL : Great Salt Lake, USA ; JES : Jesse Lake, USA ; LAR : Larnaca, Cyprus ; LM : Little Manitou Lake, Canada ; MAC : Macau, Brazil ; MAN : Manaure, Colombia ; PA : Punta Araya, Venezuela ; PEK : Pekelmeer, Bonaire ; PEN : Penley Lake, USA ; ROCK : Rockhampton, Australia ; RP : Red Pond, USA ; SF : San Fernando, Spain ; SFB : San Francisco Bay, USA ; SHB : Shark Bay, Australia ; SPA : San Pablo, Spain ; SPB : San Pablo Bay, USA ; SPO : Santa Pola, Spain ; URM : Lake Urmia, Iran ; YAV : Yavaros, Mexico ; ZUÑ : Zuñi Lake, USA (in this figure salina denotes *A. tunisiana*, from Beardmore and Abreu-Grobois, 1983).

A derivation of pentaploidy has already been suggested, although as the tetraploid apomixis extrudes only one polar body with 84 chromosomes (Barigozzi, 1974) and is therefore difficult to envisage an anomaly that could elevate the number of chromosomes to 105.

Phylogenetic trees which have been derived in the analysis of electrophoretic data from *Artemia* (Beardmore and Abreu-Grobois, 1983) are included to summarise the branching patterns observable in the evolution of the genus (Fig. 4, 5) and already fully discussed in terms

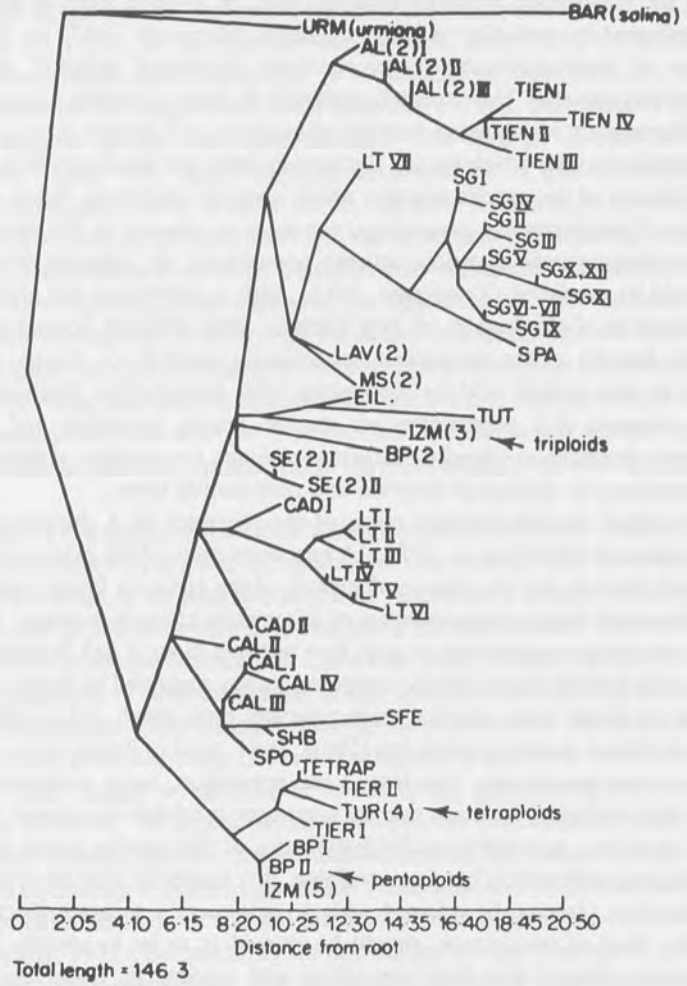


FIG. 5. Phylogenetic tree of representative Eurasian bisexual and parthenogenetic *Artemia*. AL : cochète, Portugal ; BP : Burgas Pomerije, Bulgaria ; CAD : Cadiz, Spain ; CAL : Calpe, Spain ; EIL : lat, Israel ; IZM : Izmir, Turkey ; LAV : Lake Lavalduc, France ; LT : Lake Techirgiol, Rumania ; MS : argherita di Savoia, Italy ; SE : Sète, France ; SFE : San Felix, Spain ; SG : Salin de Giraud, France ; TRAP : tetraploids ; TIEN : Tientsin, P.R. China ; TIER : Tierzo, Spain ; TUR : Turkey ; TUT : ticorin, India (for other abbreviations see legend to Fig. 4 ; in this figure salina denotes *A. tunisiana* ; from ardmore an Abreu-Grobois, 1983).

of genetic distances. This method of representation places emphasis on the changes in single characters along phylads and the pattern of branchings. The length of the branches in the tree are proportional to the evolutionary rate of change.

Speciation and systematics

The apparent diversity in the modes of speciation inferred from studies of various organisms and the time scales involved in the process have been partly responsible for the slow progress in elucidating the mechanism or mechanisms involved. In *Artemia*, many of the elements which have been implicated in speciation in other organisms (Barigozzi, 1982) are observable : clines in the content of heterochromatin (chromocenters), ecological isolation, distinct aneuploid classes, and parthenogenesis. Unfortunately, although we have information regarding the amount of genetic differentiation which exists between populations and species in the genus, and a large selection of characters with which we can distinguish them, we know very little about the events and the architecture of the genetic changes which underlie speciation. Some inferences can be made, however. Considering the pronounced tendency in *Artemia* for the development of local adaptations and large genetic distances between populations, an "adaptive divergence" mode of speciation could be predicted (Templeton, 1981), with a continuous and gradual development of isolating barriers. Colonisation of new habitats with different ecological characteristics, however, relies heavily on the remarkable physiological plasticity of *Artemia* while the genetic contributions to this process and its association with reproductive incompatibilities are unstudied. The absence of a dependence on specific mating behaviour and in particular no conspecific mate recognition capacity, furthermore, limits the number of possible reproductive isolating mechanisms to ecological isolation and post-mating types.

In fact, ecological barriers separate many of the members of *A. franciscana* and incipient speciation is apparent (Bowen *et al.*, 1985). A new assessment of the extent of transport of cysts between natural habitats and the degree of survival of the larvae in foreign waters is suggested, as has been discussed, before the systematics of this species group is reviewed. If populations are confirmed to be totally isolated with no gene flow between them, it will be necessary to consider subdivisions using subspecific or specific categories as was suggested by Bowen *et al.* (1985). An exception may be Mono Lake *Artemia* which produces cysts which sink in lake water and their hatching rate is highest at low temperatures (Dana, 1981, cited in Bowen *et al.*, 1985) in contrast to those from other populations. The Jesse Lake population, being more extreme in its ionic requirements and ecological isolation may be a stronger candidate for species rank on the basis of ecological isolation. A revision of the systematics of this species group is premature until sufficient additional information becomes available. It is suggested that the existing taxonomy of the North American *Artemia* be retained with *A. monica* as a separate species. Reproductive incompatibility, itself of importance, should be studied in order to identify the nature of the associated genetic changes and their connection with cytogenetic characters which were discussed. This group of populations offers a unique opportunity to study these processes among closely related and very tractable populations in the laboratory.

To the extent that ecological isolation appears to be the driving force for the process of speciation in *Artemia* with little ethological differences between species, it is instructive to contrast this genus with a more specious one, *Drosophila*, where ethological changes are critical in the development of reproductive isolation. Indeed, here there is experimental evidence that

suggests mate recognition changes to be the first isolating mechanisms which evolve during speciation events (Powell, 1978). This series of events are important in the evolution of *Drosophila* for they allow the isolation of developing genetic adaptations to novel ecologies which would otherwise be affected by interbreeding.

In the *tunisiana* species, although extensive heteroploidy is found, it is not associated with reproductive isolation (Browne, 1983a). Nonetheless, more research is necessary to determine the effect of the different chromosome numbers on the fertility of adult brine shrimp. The systematics of this species has become slightly confused. *A. salina*, the original name for the European bisexual brine shrimp, has been invalidated (Bowen and Sterling, 1978 ; Barigozzi, 1980 ; Bowen *et al.*, 1985) on the grounds that the type specimen is not available, the type population is non-existent and therefore it is impossible to determine the equivalence of present-day species with the original sample of Schlosser (1756). The difficulty in deducing the identity of the original species was compounded by the findings of mixtures of species in European salterns (Table VII). Electrophoretic determination of genetic distances, however, suggests that *persimilis* and *salina*, believed because of these reports to be naturally sympatric in Europe, have evolved in separate continents and therefore only one bisexual form (the original *salina*) is native. Unfortunately, artificial transport into eighteenth century Europe cannot be ruled out. Only by comparing DNA sequences (as Bowen *et al.*, 1985 suggest) or diagnostic characters in preserved material with those of the extant species in question can the equivalence be established or ruled out. The usefulness of morphological characters, however, is questionable, unless the type specimen was taken at relatively low salinities. If this assessment is not possible, taxonomical nomenclature rules indicate the invalidation of *salina* and accepts *tunisiana* Bowen and Sterling as a replacement. At present this is the most satisfactory solution to the systematics of this species.

Parthenogenesis is one mode of speciation which becomes established quickly. The conditions permitting its development in *Artemia* are speculative (see earlier), and have evidently occurred only once. Further, there is no interconversion of reproductive modes. This is known for all bisexual species, except *urmiana* which, besides being the least studied from this point of view, is the closest relative to the parthenogenetic forms (see Table IX and Fig. 3). For these reasons, the possible development of parthenogenesis in this species should be studied more closely.

As far as the systematics of parthenogenetic *Artemia* is concerned, the fertile mating between a parthenogenetically-produced male (from a Yamaguchi population) and an *urmiana* female (Bowen *et al.*, 1978) would be evidence in favour of the adoption of the name *A. urmiana parthenogenetica*. However, the genetic distance between the two branches (mean D=0.5) places them intermediate between incipient species and full species (cf. values in Ayala, 1983). Also, the passage from bisexuality to parthenogenesis is irreversible and the genetic assciation with the bisexual ancestor is broken. Use of a single category *A. parthenogenetica*, a proposed alternative (Bowen and Sterling, 1978 ; Barigozzi, 1980) also suffers the disadvantage of grouping together sets of organisms with very distinct and genetically important characteristics. Cole (1985) analysed the taxonomic treatment of parthenogenetic animals since they are quite unconventional in many aspects and there are different views on how to treat them. The adoption of a systematic category separate from the bisexuals is suggested by his work as, in *Artemia* 1) they have distinctive characters that can be identified and are perpetuated generation after generation independent of interaction with any ancestral population, such that the ancestral form could become extinct while the unisexual group perpetuates itself ; and 2) these forms are reproduc-

tively isolated entities that can undergo independent dispersal, mutation, natural selection and evolution. These traits endow the different forms specific properties of genetic importance already detailed and should be reflected in their systematic placements. In unisexual *Artemia* there are also important evolutionary differences between the different ploidies. Cole's (1985) proposal that such forms should be treated as different species on the grounds that they indicate different evolutionary potential and backgrounds is also acceptable although a more thorough study of useful morphological and oogenetic diagnostic characters should be developed, permitting a more complete classification. Thus, establishment of tentatively separate parthenogenetic taxa at subspecific levels according to their ploidy is supported by the available genetic studies of evolution within this group : *A. parthenogenetica diploidis, A. parthenogenetica triploidis, A. parthenogenetica tetraploidis*, and *A. parthenogenetica pentaploidis.*

Conclusions

It is clear that much progress has been made in the study of the genetics of the genus *Artemia* in the intervening years since the last symposium in 1979 in Corpus Christi. Mostly we have been able to understand the evolutionary relationships and reproductive characteristics varying between and within species, and the factors which affect them.

We would like to suggest how the various areas of research with this organism could be brought together for mutual benefit. Although we know of the extent of genotypic variety in bisexuals and parthenogenetics, we have little knowledge of the ecological values of the various forms. It may become possible to use markers at various levels of the genetic architecture (chromocenters and allozymes) for the study of characteristics associated with or linked to the process of adaptation to particular ecological parameters such as the ionic composition of the medium. Quantitative genetics will be essential in determining the genetic contribution to the adaptations.

An understanding of the genetic basis for characters that are of importance for the culture of *Artemia* will be required in order to artificially select particular qualities. Some characteristics already identified as desirable for aquaculture and possible amenable to genetic manipulation are : reduction in the mean size of the nauplii, increased growth rate and fecundity, tolerance to higher (or lower) temperatures. It will be necessary to determine the genetic contribution to the variance in their expression.

Inoculation into habitats with high pH may become a possibility if tolerance can be cross bred from populations inhabiting carbonate waters.

These research lines will not only aid in the characterisation of the genetic system in the brine shrimp but also provide us with important practical applications of the results. An essential basis for these plans, however, is the existence of a diversity of genotypes. For this reason, it is imperative that natural populations of *Artemia* be protected from extinction by habitat destruction or by indiscriminate introduction of more competitive species when inoculating brine shrimp-free salterns.

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First metrical data on the length of the prophasic chromosomes of diploid and tetraploid parthenogenetic *Artemia*

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Abstract

A metrical comparison has been made between diploid and tetraploid parthenogenetic *Artemia* from Margherita di Savoia (Apulia, Italy). The data obtained show that chromosome lengths in diploid animals do not differ from the chromosome lengths in tetraploid animals, *i.e.* the total length of the prophasic chromosomes in tetraploid *Artemia* is just twice that in diploid animals.

Introduction

Metrical data on the chromosomes of *Artemia* are still lacking. As in every case of paleoploidy a quantitative comparison between the chromosomes of diploid and tetraploid forms is necessary to ascertain whether, besides numerical variation (Barigozzi and Baratelli, 1982), also structural changes occurred in the history of the species.

Materials and methods

Nauplii were used of the parthenogenetic population of Margherita di Savoia (Apulia, Italy) which is composed of diploid and tetraploid females.

Orcein stained prophases, selected for similar packing, were photographed and drawn on transparent paper. The drawn chromosomes were measured with a graphical digitalizer Videoplan. The measurements were performed on prophases of somatic cells, where length differences are detectable.

Results

Eleven prophases of diploid and ten prophases of tetraploid *Artemia* have been analyzed. The lengths of single chromosomes within each prophase and the total lengths of prophasic chromosomes within both diploid and tetraploid set have been recorded and statistically elaborated (ANOVA).

The data obtained show no statistical difference between the length of the chromosomes of diploid and tetraploid prophases : $\bar{y}_{42} = 10.31 \pm 1.10$; $\bar{y}_{84} = 10.37 \pm 0.76$.

As a result the total length of tetraploid prophasic chromosomes is just twice the total length of diploid prophasic chromosomes.

Conclusions

In this parthenogenetic population no detectable structural changes differentiate diploids and tetraploids.

These first data should be used for further comparisons between chromosomal sets of *Artemia* populations living in different geographical areas.

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Further data on repetitive DNA and speciation in *Artemia*

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Abstract

Using the technique of DNA hybridization by the dot-blot method, the presence of the highly repetitive DNA family (*Alu*I 110 bp) has been studied in different *Artemia* populations.

This technique allows a more refined analysis of the phenomenon of genetic differentiation in a number of bisexual and parthenogenetic, diploid and polyploid populations from Europe, Asia and America.

The results are interpreted as a contribution to the study of speciation of *Artemia* based on the amount of repetitive DNA.

Introduction

In view of the presence of heterochromatin in *A. franciscana* and of its absence in an *Artemia* population living in Tsing-Tao, People's Republic of China (Barigozzi *et al.*, 1984) the study of repetitive DNA in *Artemia* has been performed on different sibling species of the genus *Artemia*, as well as on populations within the genus, but not safely attributable to a taxon, which differ in the mode of reproduction (parthenogenesis *vs* bisexuality) and the chromosome number (diploidy, triploidy, tetraploidy, pentaploidy). So far, none of these geographically distinct strains have been studied with the techniques described below.

This investigation consists mainly of a comparison between the presence or absence of chromocenters in the interphasic nuclei, and presence or absence of repetitive DNA fragments of 110 bp. The base sequence of the fragments has also been determined.

Materials and methods

The list of the strains studied is given in Table I. Nauplii hatched from cysts were used as study material. The aceto-orcein staining technique of Barigozzi *et al.* (1984) was used to study the presence of chromocenters in the interphase nuclei and to determine the chromosome numbers in the prophases. DNA sequencing was carried out by the dideoxy-chain termination methods of Sanger *et al.* (1977).

The techniques for extraction of genomic DNA, nick translation of the recombinant pUA41 plasmid containing the cloned repetitive fragment, and DNA hybridization have been described earlier (Barigozzi *et al.*, 1984).

Analysis of genomic DNA with regard to presence or absence of the *Alu*I repetitive DNA was carried out with the dot blot technique (Kafatos *et al.*, 1979).

TABLE I

Origin and biological characteristics of strains studied

Sibling species	Geographical origin	Mode of reproduction[1]	Ploidy level[2]
NEW WORLD			
A. franciscana	San Francisco Bay, California, USA	B	2n
A. sp.	Yucatan, Mexico	B	2n
	Sonora, Mexico	B	2n
	Puerto Araya, Venezuela	B	2n
OLD WORLD			
A. tunisiana	B	2n	
A. urmiana	Lake Urmia, Iran	B	2n, 5, subdiploid
A. spp.	Trapani, Italy	B	2n
	Comacchio, Italy	P	4n
	Margherita di Savoia, Italy	P	2n, 4n
	Lake Kalatoz, USSR	P	4n
	Lake Burlju, USSR	P	2n
	Altai, USSR	P	2n
	Eilat, Israel	P	3n
	Tsing-Tao, PR China	P	2n
	Tian-Jin, PR China	P	2n, 4n

[1] B : bisexual ; P : parthenogenetic.

[2] 2n : diploid ; 3n : triploid ; 4n : tetraploid ; 5n : pentaploid.

Results

NEW WORLD *ARTEMIA*

High numbers of chromocenters corresponding with high amounts of repetitive DNA were found in *Artemia franciscana*. The base sequence of its monomer is given in Table II.

Similar results were obtained for the other three American populations, however, the chromocenter frequencies are lower than in *A. franciscana* and repetitive DNA is present, but in a lower amount.

TABLE II

Sequence of the repetitive DNA in *A. franciscana*

5′ G T A C G T A T G T[10]T G G A A A A A T G[20]A A G A T A T T A G[30]
A C T A T T T T A T[40]T A T T G C A C A T[50]G G T A A A G A A T[60]
G T G A T T A A A G[70]G C T C T T T T C T[80]A T G T A A A A G T[90]
T T T A G T T T T G[100]A G G G T A T A G 3′

OLD WORLD *ARTEMIA*

A. tunisiana has a mean number of 4.4 chromocenters per nucleus. *A. urmiana* shows small chromocenters difficult to count and no repetitive DNA, both in pentaploid (105 chromosomes) and in diploid (42) or subdiploid (44) individuals.

Chromocenters and repetitive DNA are missing in the populations of Eilat (Israel) ; Trapani, Comacchio and Margherita di Savoia (Italy) ; Lake Kalatoz, Lake Burlju and Altai (USSR), and Tian-Jin and Tsing-Tao (PR China). No heterochromatin was found in detectable amount in species or populations living in the Old World.

Discussion

The results obtained confirm earlier published data (Barigozzi *et al.*, 1984) and allow for the first time to study the distribution of repetitive DNA in various sibling species and populations of the brine shrimp *Artemia.* Apparently no correlation can be made with the mode of reproduction and the ploidy level.

The finding in *A. franciscana* of the *Alu*I 110 bp repetitive DNA can provide a powerful tool to study speciation in the genus *Artemia.*

The approach is clearly a preliminary attempt to connect the presence or absence of repetitive DNA with speciation. As far as the geographical distribution of the samples studied is concerned it must be recognized that much more localities in critical positions need to be studied. A necessary complement to this study will be the determination of the genetic connection between sibling species and populations.

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Preliminary studies on some *Artemia* populations from northern Greece

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Abstract

Three *Artemia* populations, found in northern Greece, have been characterized with regard to their mode of reproduction and ploidy. All three populations were found to be parthenogenetic and tetraploid (4n=84). We have been able to construct both diploid and tetraploid karyotypes, and have attempted to obtain C-banding patterns of the chromosomes of *Artemia* sp. The construction of a C-banding karyotype was accomplished from strain GSL 185 (*A. franciscana*). Although it was not possible to construct a Giemsa C-banding karyotype from our tetraploid populations, we do have definite evidence of chromosomal homologies between these population and the above mentioned diploid strain.

Preliminary studies of DNA reassociation kinetics have shown that the C_0t curve of one of our parthenogenetic populations is very similar to that of the diploid and bisexual strain from San Francisco (*A. franciscana*).

Our data on 13 systems of enzymatic polymorphisms of the northern Greek populations indicate that they belong, most likely, to the same species; further experimentation is needed to determine their relationship to other *Artemia* populations.

Biometrical analysis of cysts and adults of the northern Greek populations shows a high degree of similarity with tetraploid parthenogenetic strains from Spain.

ntroduction

We have recently started a study of some northern Greek populations of *Artemia* and are now n the position to report our preliminary data concerning some of the cytogenetic, biochemical, nolecular and biometrical parameters of these animals. We hope that our continuing study of hese and other Greek populations will contribute toward the more general understanding of this nimal and, at the same time, will provide Greece with the opportunity to explore the possibility f the practical use of the Greek populations for the country's needs.

laterials and methods

Three different populations of northern Greek *Artemia* have been used for the various proaches described in this preliminary report, *i.e.* a population from the saltworks of Citros ieria), a population from the saltworks of M. Embolon (Thessaloniki), and a population from e saltworks of Kalloni (island of Lesbos).

In addition to the above animals, we have used as reference material a Great Salt Lake (Utah, SA) strain (GSL-185), which has been kindly provided to us by Dr. P. Sorgeloos.

Biometry

Up to now, biometry was mainly performed on the Citros populations, as well as on cysts from the M. Embolon population. Hydrated cysts produced by cultured animals from both populations were also measured.

Adult shrimps were narcotized in chloroform-saturated water solution and measured under a dissection microscope. The following measurements were performed : total length, abdominal length, maximal width of brood pouch, width of 3rd abdominal segment, width of head, length of 1st antenna, maximal diameter of complex eye, and distance between complex eyes. Measurements were performed on animals cultured from wild cysts (Greek strains) under standard laboratory conditions (yeast as food, 25±1 °C and seawater of 32-35 ‰ salinity).

Cytogenetics

Cells were obtained from live instar I nauplii, mainly from the Citros and the M. Embolon populations. Due to scarcity of material, only seven nauplii were examined from the Kalloni population. Two different techniques were followed for chromosomal analysis :

Orcein staining

The technique of Oster and Balaban (1963) was used, but instead of putting the slides on dry ice for 30 min they were immersed in liquid nitrogen for several seconds.

Giemsa staining (C-banding)

The technique of Hsu (1971) was used after the following modifications : instead of incubating in 0.07 N NaOH solution we used 0.1 N NaOH at 60 °C for 20 or 40 s. After the dehydration steps, the slides were stored for 5 days at 37 °C. The stage of incubation in 6×SSC was omitted. Giemsa 3-4 % in phosphate buffer 1M (pH 6.8) was used for staining for approximately 7 min.

All preparations were studied under a Zeiss phase contrast microscope, and photographed on Kodak Panatomic-X film.

DNA analysis

Cysts from the M. Embolon population were hatched in seawater (35 ‰ salinity) in 10 g batches at room temperature (25±1 °C) in a 20 l aquarium under vigorous aeration. After 48 h the swimming larvae were collected and used for DNA extraction and purification according to the technique described by Lagowski *et al.* (1973).

Pure DNA in 0.12 M sodium phosphate buffer (pH 6.8) was broken into small fragments and used for DNA-DNA renaturation kinetics from C_0t_0 to C_0t_{100} according to Vaughn (1977).

Isozymes

Large samples of adult shrimps (just after reaching sexual maturity) from the Citros and M. Embolon populations were used for the 12 % starch gel horizontal electrophoresis. Four buffers were used for the separation of the various isozymes :

- Tris-citric acid buffer (pH 8.0) according to Bowen and Sterling (1978) for MDH, LDH, Est-1, Est-2 and Aph ;
- Tris-citric acid buffer (pH 8.5) according to Ashton *et al.* (1961) for LAP, Acph and EsD ;
- Tris-EDTA-borate buffer (pH 8.0) according to Smith (1968) for ME, 6-PGD and SOD ;
- Tris-citric acid buffer (pH 8.5) according to Steiner *et al.* (1977) for ME, a-GPDH and PGM.

The staining of the gels was carried out according to classical techniques (*e.g.* Harris and Hopkinson, 1976).

Results and discussion

Biometry

The following results were obtained (mean values for 50 individuals ± standard deviation of the mean) :

- total length : 12.428 ± 0.0829 mm ;
- abdominal length : 6.550 ± 0.0468 mm ;
- maximal width of brood pouch : 1.862 ± 0.0218 mm ;
- width of 3rd abdominal segment : 0.573 ± 0.0056 mm ;
- width of the head : 0.828 ± 0.0102 mm ;
- length of 1st antenna : 1.147 ± 0.0150 mm ;
- maximal diameter of complex eye : 0.304 ± 0.0045 mm ;
- distance between complex eyes : 1.628 ± 0.0164 mm.

The mean diameter of 500 cysts collected from the Citros and M. Embolon populations were found to be 260.19 µm respectively 264.73 µm. The mean diameter of cysts obtained from laboratory cultures of the same populations were 270.13 µm respectively 279.36 µm.

All biometrical data of the Greek populations studied appear to be very close to the data of Amat Domenech (1980) for Spanish tetraploid parthenogenetic strains. The same holds true for the correlations between the values of the different parameters.

Cytogenetics

So far karyotypes for *Artemia* species have been presented only in a few cases, *e.g.* some Russian populations by Mitrofanov *et al.* (1976, 1982). On the other hand, several workers (*e.g.* Barigozzi, 1941, 1944 ; Goldschmidt, 1952 ; Barigozzi and Tozi, 1959 ; Stefani, 1963 ; Iwasaki, 1969) have presented data on the chromosomes of *Artemia* spp.

The nuclei of the cells from the Greek populations did not exhibit any distinct chromocenters like those observed in the Great Salt Lake and other strains (Barigozzi and Baratelli, 1982 ; Abreu-Grobois and Beardmore, 1982 ; Abreu-Grobois, 1983).

For purposes of comparison of the chromosomes of our populations with those of a standard strain, we have constructed both a routine and a Giemsa C-banding karyotype of the GSL strain (see Fig. 1 and 2).

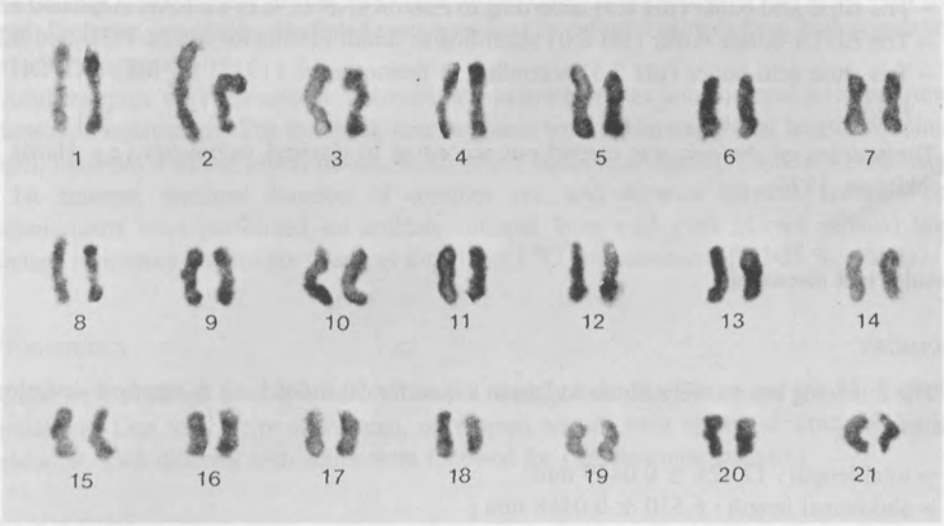

FIG. 1. Diploid karyotype of *A. franciscana* (Great Salt Lake strain).

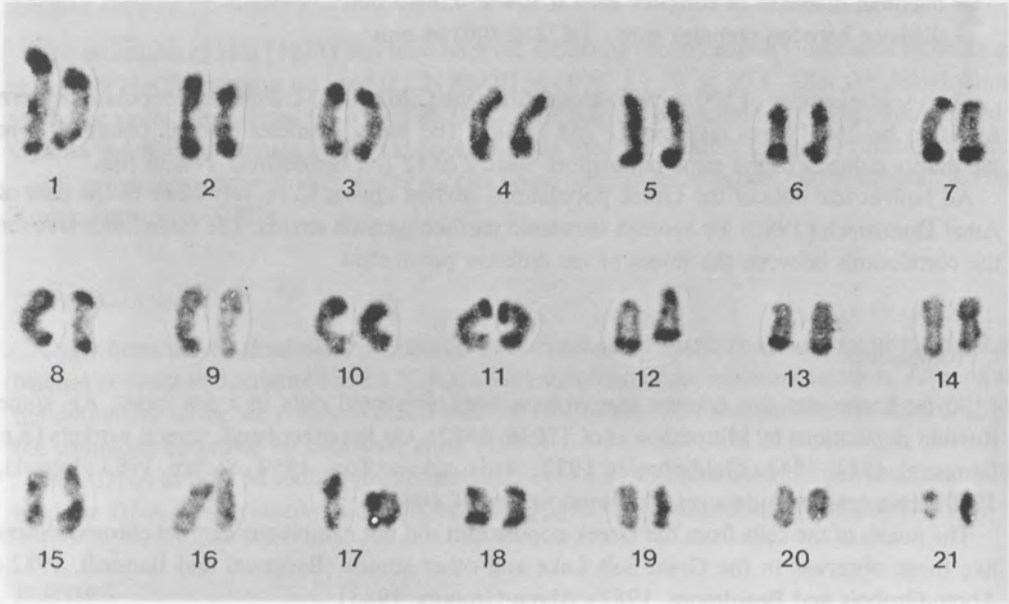

FIG. 2. C-banded diploid karyotype of *A. franciscana* (Great Salt Lake strain).

Fig. 3 shows a tetraploid karyotype from the Citros population and this karyotype shows no differences from that of the M. Embolon population. Unfortunately no Giemsa C-banding karyotype could be constructed from these populations but certain C-banding patterns for some of the chromosomes (Fig. 4) were obtained. We cannot be certain that this situation indicates that there are no bands in the remaining chromosomes, as in the Tsing-Tao (China) population studied by Barigozzi *et al.* (1984). Our technique might not have been sufficiently successful.

From the few chromosomes of our strains that show C-banding patterns, it is impossible to establish any homologies with the Great Salt Lake strain. Undoubtedly, both the lack of chromocenters and the lack of bands in most of the chromosomes indicate at least a different pattern of heterochromatin distribution in the Greek populations. However, the comparison of karyotypes of the diploid Great Salt Lake strain and the tetraploid populations from Greece reveal some morphological similarities between the chromosomes.

FIG. 3. Tetraploid karyotype of the parthenogenetic *Artemia* from Citros.

NA ANALYSIS

The curve for DNA-DNA renaturation kinetics coincides with the one of Vaughn (1977), nstructed for a diploid bisexual strain from San Francisco (California, USA). Our data,

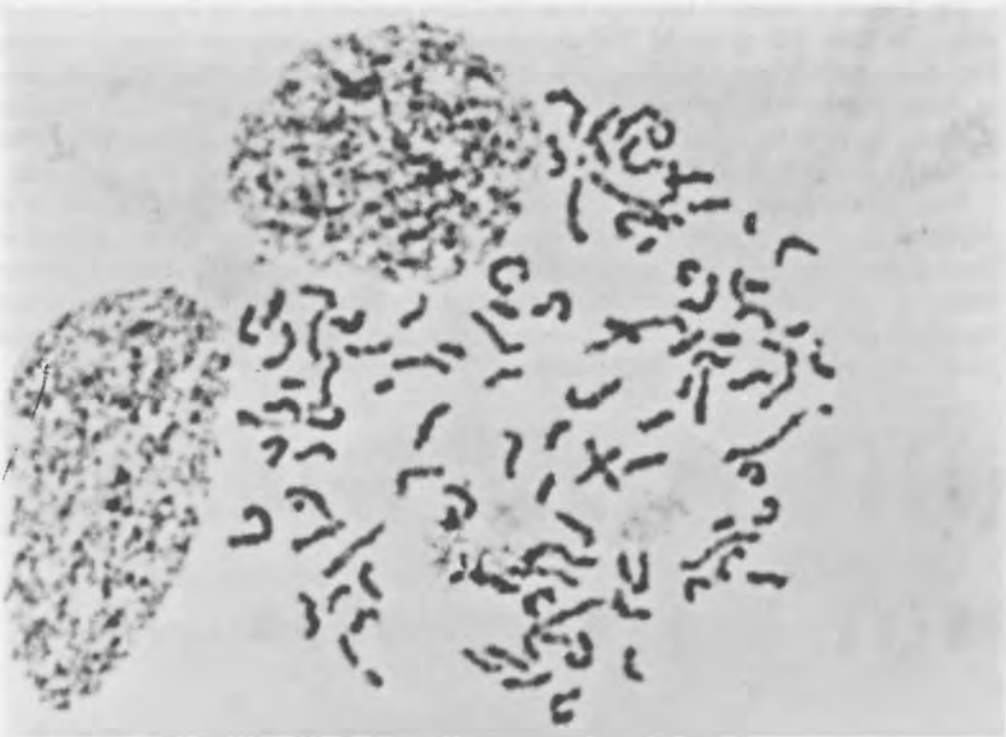

FIG. 4. C-banded chromosomes of tetraploid parthenogenetic *Artemia* from Citros.

therefore, support the theory of Vaughn that polyploidy does not change the relative concentration of any DNA sequences involved, so that the DNA renaturation kinetics are identical in polyploids as in diploids.

ISOZYMES

The following results were obtained from the electrophoretic analysis of 13 enzyme systems :

- SOD, ME and a-GPDH : one zone, monomorphic. No variation was observed for the SOD and ME enzymatic systems by Abreu-Grobois (1983). The data for a-GPDH reveals the expression of a single locus.
- 6-PGD : two zones, monomorphic. Abreu-Grobois (1983) reports that this enzyme is dimeric, but satellite bands are prominent in each phenotype, giving the tetraploid heterozygotes five banded patterns. The enzymatic pattern which we obtained indicates that all individuals studied are probably homozygotes.
- LDH, one or no zones. This is a potentially interesting phenomenon, the further investigation of which might show whether or not it involves regulatory processes (Ewing and Clegg, 1969) or a silent mutation.
- MDH : three to four zones, polymorphic. A total of four phenotypes were observed but the inability to perform crosses does not allow the determination of the number of loci involved.

- PGM : three or four zones, polymorphic. Three or four alleles are apparently expressed in the tetraploid individuals studied.
- Est-1 and Est-2 : one zone each with quantitative differences between animals. This pattern is similar to the one described by Bowen and Sterling (1978).
- EsD : one major and one minor band. No variation was observed. The data indicate that all individuals studied are homozygotes.
- Aph : three zones with quantitative differences between animals. The three banded pattern is characteristic of a dimeric enzyme. The densities of the bands could be due to the existence of different combinations of alleles in the genotypes of the individuals.
- LAP and Acph : various numbers of diffuse and inconsistent zones. Abreu-Grobois (1983) described that part of the LAP variation as an artefact.

The similarity of the above mentioned enzyme systems for the Citros and M. Embolon populations indicates that these strains are genetically identical. Only further experimentation will show the relationship of the Greek populations to other known populations.

cknowledegments

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Intrapopulational differences in life history traits of obligately parthenogenetic clones of the brine shrimp *Artemia*

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Abstract

Nine clonal lines were established from single parthenogenetic females originating at Salin de Giraud, France. Offspring from each clonal line were then reared under identical standardized laboratory conditions. Significant differences were found between five clonal groups for a number of life history traits. The total number of offspring per female varied 4 fold between clones, ranging from 274 to 1 162. The length of the reproductive period varied 2.5 fold, ranging from 16.3 days to 42.2 days. Other traits that were found to vary significantly were number of offspring per brood, number of broods per female, mean number of offspring per day, and the encystment-viviparity ratio.

Previous electrophoretic analysis (Abreu-Grobois and Beardmore, 1980, 1982) has shown that there are very small genetic distances between clones ($D<0.05$). The fact that such large differences in life history traits are found within a geographically isolated parthenogenetic population, despite the small genetic distances between clones, is highly relevant to the evolutionary potential of parthenogenetic organisms.

Introduction

Parthenogenesis in animals is an important evolutionary phenomenon and provides a unique opportunity for studies in evolutionary processes. However, the potential for evolutionary change within a parthenogenetic population is still widely debated on the basis of only a relatively small amount of pertinent experimental data. Points of significance for evolutionary theory, therefore, may be undermined by this lack of knowledge of the evolutionary processes in parthenogens (*e.g.* Williamson, 1981 ; Mayr, 1982).

Most investigators consider the principal advantage of sexual reproduction to be that the offspring have functional capabilities not shared by asexual progeny and that sexual reproduction increases the fitness of parents through genetically diverse offspring. This allows continued adaptation of a lineage to a changing biotic and abiotic environment (Lloyd, 1980). In contrast, because of the limitations associated with parthenogenesis, many biologists have labeled asexual organisms as evolutionary dead ends (*e.g.* Stalker, 1956 ; Mayr, 1970 ; Maynard Smith, 1978). This lower level of genetic variance in asexual lineages is expected to render them exceptionally insitive to environmental perturbations and to hinder them in interactions with sexual organisms .g. Selander and Hudson, 1976 ; Glesener and Tilman, 1978 ; Hamilton *et al.*, 1981). owever, this latter view is increasingly being reexamined. Mutation, mitotic recombination, ra-cistronic recombination, mitotic non-disjunction, transposition of regulatory genes, hetero- romatization and polyploidization help generate variance in asexual populations (Lynch,

1984ac ; also see McClintlock, 1984). Many of these factors may play important roles in parthenogenetic populations.

Most parthenogenetic populations examined using electrophoretic procedure, tissue grafting or cytology are composed of multiple clones. Parker (1979) reviewed the literature and found this to be true in taxa as diverse as crustaceans, sea anemones, oligochaete worms, beetles and other insects, and fish. The only exceptions are the lizards *Cnemidophorus larodoensis* (McKinney *et al.*, 1973) and *C. uniparens* (Cuellar, 1976) and the snail, *Campeloma*, where samples taken from a limited area of its range were found to be uniclonal, although two distinct clones occurred in the alternative populations (Selander *et al.*, 1978).

Parthenogenetic populations of *Artemia* may be composed entirely of one genotype (monoclonal) or of two or more clones. Abreu-Grobois and Beardmore (1980, 1982) looked at 16 systems representing 23 loci and found a large degree of interclonal variation within the majority of the parthenogenetic populations they examined. Many of the asexual populations were polyclonal, with the population from Salin de Giraud (France) consisting of the greatest number, with at least 13 electrophoretically identifiable clones. In order to fully understand the evolutionary potential of organisms employing parthenogenetic reproduction, it is now becoming clear that we need to understand multiclonality. The purpose of this study is to discern whether clones from a parthenogenetic population differ not only genotypically (electrophoretically) but also in terms of fitness variables such as life history traits.

Materials and methods

Cysts from Salin de Giraud (collected by Gene MacDonald of Wake Forest University in November 1980) were mass hatched in Instant Ocean and then transferred after 1 day into brine (90 ‰). When ovarian development had begun (at day 15) 25 parthenogenetic females (hereafter referred to as P1) were removed from the mass cultures and placed in individual glass brood jars containing 500 ml of brine and reared in standardized conditions (Browne, 1980 ; Browne *et al.*, 1984). *Artemia* consumed algal cultures present in each jar (primarily *Dunaliella*) along with the maximum amount of yeast (Fleischmann's) that could be cleared daily (±1.0 mg/adult/day). All cultures were maintained under 24 h cool white fluorescent lighting, and temperature was maintained at 24 °C±2 °C. Initial pH of the brine was 8.1. Approximately 100 ml of culture water containing feces and excess algal clumps was removed from each jar weekly and replaced by an equal amount of fresh brine. At least every other day, offspring were removed from each brood jar and counted.

Ten life history traits were recorded for each individual : offspring per brood, broods per female, offspring per day per female, time between broods, percent offspring encysted, total offspring per female, female prereproductive period, female reproductive period, female postreproductive period, and total female lifespan. Length of female prereproductive life was scored from day of hatch until the first brood parturition day ; female reproductive span from day of birth of the first brood to the last brood ; female postreproductive span from the last brood date until death.

Clones A, B, C, D, E, and G were isolated in the following manner. P1 females which were found to have outlier life-history (*i.e.* those furthest removed from the mean ; for example, those females that take the least or most amount of time to reach sexual maturity or those that have the least or most amount of offspring) were isolated and separate clonal lines derived using their

offspring. Clonal lines A and B represent an internal control because both lines are descended from the same female. Clones SGI, SGII and BGII were established from offspring from females taken from 3 l laboratory stock jars that had initially contained a multiclonal population started from Salin de Giraud cysts but had been continuously reared in the laboratory for the past 2 years. SGI and SGII were taken from one jar, BGII from another. In all cases, each clonal line could be traced back to a single female.

From 20 to 25 individuals from each clonal line were reared in individual brood jars using the same standardized conditions as described for the P1 group. Similarly, the ten life history traits recorded for the P1 group were recorded for the individuals in each clonal line.

The analysis of variance tests used were single factor ANOVAs where homoscedasticity was confirmed via F-max tests (Sokal and Rohlf, 1981). If significant heteroscedasticity was found, Games and Howell (1976) tests for Unplanned Comparisons of Means were utilized.

Results

Five reproductive characteristics and five lifespan characteristics for the nine lineages are summarized in Table I. Standard errors are in most cases small, reflecting small intraclonal variance levels. Significant differences were found for all characteristics examined except the length of the post reproductive period. The results of the Games and Howell (1976) tests showed that the nine lineages could be separated into five significantly different groups, *i.e.* A and B ; SGI, SGII, and BGII ; D and E ; C ; and G. Clonal lines A and B showed no significant differences for any trait, as expected, since they served as internal controls.

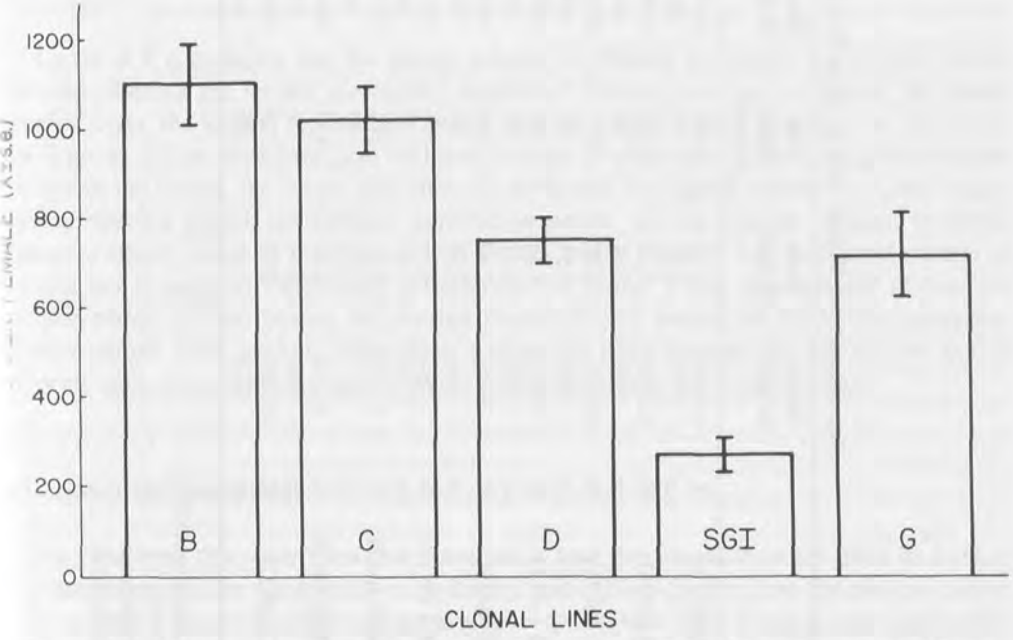

FIG. 1. Variation in the number of offspring per female between five clones of the *Artemia parthenogenetica* population from Salin de Giraud (France).

TABLE I

Reproduction and lifespan traits of clones from the Salin de Giraud population of *Artemia parthenogenetica*

		Offspring per brood[a]	Broods per female[a]	Total offspring per female[a]	Offspring per day[a]	Days between brood[b]	Percent cysts[a]	Prereproductive period (days)[a]	Reproductive period (days)[a]	Postreproductive period (days)[c]	Total lifespan[a]
Clone A (n=22)	$\bar{X}$	110.50	9.73	1088	26.31	4.21	0	30.41	40.91	4.23	76.00
	s.e.[d]	3.75	0.51	77	0.81	0.10	0	0.30	2.35	0.54	2.27
Clone B (n=25)	$\bar{X}$	116.80	9.32	1162	25.99	4.36	1.17	31.08	42.24	4.96	78.28
	s.e.	7.20	0.69	93	1.63	0.25	0.67	0.52	3.25	2.61	2.67
Clone C (n=25)	$\bar{X}$	102.07	9.80	1021	24.59	4.15	2.75	27.00	41.52	3.36	71.28
	s.e.	4.39	0.59	78	0.98	0.10	1.03	0.65	2.54	0.64	2.52
Clone D (n=25)	$\bar{X}$	76.71	9.28	746	17.85	4.41	4.76	28.96	41.04	4.32	74.52
	s.e.	4.50	0.54	58	1.14	0.24	3.99	0.43	2.87	1.22	3.30
Clone E (n=25)	$\bar{X}$	70.74	7.80	556	14.99	4.89	7.09	29.52	37.12	2.48	69.92
	s.e.	3.14	0.59	52	0.97	0.20	3.24	0.81	2.65	0.78	2.77
Clone SGI (n=25)	$\bar{X}$	71.72	4.44	336	16.53	4.36	15.05	34.38	20.52	5.75	59.52
	s.e.	5.88	0.43	39	1.43	0.29	4.97	0.21	2.26	0.93	2.75
Clone SGII (n=24)	$\bar{X}$	65.10	2.56	274	15.79	3.70	12.28	33.71	16.29	6.10	54.04
	s.e.	6.17	0.52	37	1.63	0.35	6.09	0.24	2.68	1.77	3.58
Clone BGII (n=20)	$\bar{X}$	67.41	6.15	459	16.44	4.22	23.16	33.80	27.30	4.95	66.05
	s.e.	4.98	0.72	76	1.36	0.20	1.36	0.69	3.76	1.11	3.92
Clone G (n=22)	$\bar{X}$	109.79	8.42	719	24.05	5.48	5.26	14.26	41.11	12.05	66.55
	s.e.	9.32	1.66	88	4.46	0.80	1.93	1.82	5.69	2.88	4.82

[a] $P < 0.001$ by ANOVA.
[b] $P < 0.05$.
[c] Not significant.
[d] Standard error.

Differences between clones are significant and by no means trivial. For example, total offspring per female varied more than 4× among the five clonal groups (Fig. 1) as did the length of the reproductive period. Percent of offspring encysted also varied significantly between the clonal groups (Fig. 2).

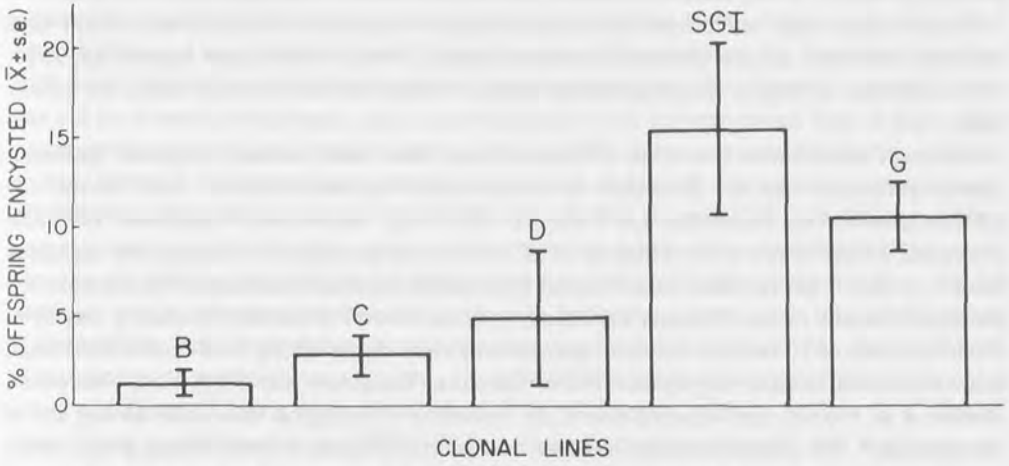

FIG. 2. Variation in the percent of offspring encysted between five clones of the *Artemia parthenogenetica* population from Salin de Giraud (France).

Group A-B consistently had the highest number of offspring per brood, the highest number of total offspring per female, the highest number of offspring per day per female, the lowest percent cysts, the longest reproductive period and the longest female lifespan. The SGI-SGII-BGII group, on the other hand, had the lowest number of offspring per brood, the lowest number of broods per female, the lowest total offspring per female, the highest percent cysts, the longest prereproductive period, the shortest reproductive period, and the shortest lifespan. However, maternal effects cannot be ruled out entirely for this group. Clone C had the highest number of broods per female and the shortest postreproductive period. Clone G was found to have the longest period between broods, the shortest prereproductive period and the longest postreproductive period. Thus, a strong relationship is frequently found between the reproductive and/or lifespan traits of an individual and to which of the five groups the clone belongs.

Discussion and conclusions

The data from this study show that there are at least five clones from the Salin de Giraud population which differ significantly in life history traits. This is the first time that multiple clones from a single population, which appear to stably coexist, have been found to vary significantly in life history traits. Intrapopulational differences between clones for some traits approach those found for differences between populations belonging to the entire *Artemia* complex. In a study

of 12 populations from three different species found on five continents (raised using standardized conditions ; Browne *et al.*, 1984), the mean number of total offspring per female ranged from 112 to 1 619. This is only slightly greater than the range of 274 for Clone SGI to 1 162 for Clone B. The mean number of broods per female ranged from 4.0 to 19.1 for the interpopulational study, and from 2.6 to 9.8 for the clonal groups. The interclonal range for the prereproductive period (20 days ; 14-34) actually exceeded that found for the 12 populations (29-43).

Despite these large interclonal differences in fitness variables, electrophoretic studies have revealed only small genetic differences between clones (Abreu-Grobois and Beardmore, 1980, 1982 ; Browne, in prep.). Interpopulational genetic differences, on the other hand, are usually large.

Although pronounced ecological differences have been found among sympatric parthenogenetic genotypes that are generated by recent sexual reproduction and then "frozen" by parthenogenesis (see Harshman and Futuyma, 1985 ; *e.g. Poeciliopsis*, Vrijenhoek, 1979 and *Alsophilia*, Mitter *et al.*, 1979, Futuyma *et al.*, 1981) and probably for *Daphnia* (as explained below), to date it has not been demonstrated when parthenogenesis is obligate. For example, the genotype diversity of the obligately parthenogenetic earthworm *Octolasion tyraeum* is very low ; electrophoresis of 10 variable enzymes revealed only eight clones among 2 197 individuals taken from numerous habitats throughout North Carolina, Tennessee and New York. Moreover, Jaenike *et al.* (1980) could discern almost no indication of ecological differences among any of the genotypes. The impression then has been that genetic diversity is considerably greater when asexual lineages are recurrently recruited from sexual populations than when parthenogenesis is obligate (Harshman and Futuyma, 1985). The data on obligately parthenogenetic *Artemia* presented here challenges this belief since the preliminary evidence points to a high degree of both inter- and intrapopulational clonal diversity, accompanied by major differences in ecologically important life history traits among clones.

The existence of multiple clonal groups within the same population that differ widely in life history traits suggests that the evolutionary potential for asexually reproducing organisms can be much higher than previously thought. If genotypically different clones within the same population vary significantly in key fitness variables such as age at first reproduction, reproductive rate and output, and lifespan components, then the evolutionary potential of parthenogens is greater than previously considered by most investigators. The existence of multiple clonal lines, along with the introduction of new genetic material via mutation (both on single locus and polygenic traits) offers a considerable amount of genetic and phenotypic variability upon which natural selection can act. Multiclonality may be one method of avoiding the evolutionary dead end that has been so widely applied to describe parthenogenetic organisms.

One factor that could contribute to a high evolutionary rate in parthenogenetic clones is that selection necessarily perceives clones as complete linkage groups and acts upon the composite properties of the genotype including interactions within and between loci (Lynch, 1984ab). Thus any new mutation that arises in a clone will be carried for the life of the clone (except when back and secondary mutations occur), even though its effects may become masked by balancing mutations on accompanying genes (Lynch, 1984a). High potential for evolutionary change in natural populations of parthenogens has been found by several authors : Atchley (1977) for the grasshopper *Warramaba virgo*, Parker (1979) for *Cnemidophorus* clones, and Oliver and Herrin (1976) for *Haemaphysalis longicornia*.

Extremely rapid clonal diversity may occur in cyclically parthenogenetic organisms such as *Daphnia* (Lynch, 1983), and in these cases there is evidence suggesting that the clones are not ecological equivalents (Hebert and Crease, 1980). Additional cases of clonal diversity in cyclic organisms have been found for rotifers (Snell, 1979) and for aphids (Frazer, 1972).

In a few species of *Daphnia*, obligate parthenogenesis has been reported, with some ecological differentiation found between clones (Loaring and Hebert, 1981). However, there are significant genetic and ecological differences between *Daphnia* and *Artemia*. In the *Artemia* population from Salin de Giraud, a much larger number of genotypically different clones, 13, have been identified while there is a much smaller number in the *Daphnia* populations. The *Daphnia* populations also may not be in stable coexistence, since the immigration rates between ponds may be high, while the temporal period of clonal coexistence may be short. For *Artemia*, the electrophoretic evidence strongly points to a single origin for the clonal complex at Salin de Giraud that is not the result of immigration from outside clones (Abreu-Grobois and Beardmore, 1982). Predation pressures have also been postulated to play a key role in structuring freshwater zooplankton communities, allowing congeneric zooplankton of similar size to coexist. Similar pressures may play an important role in allowing clonal types to coexist in freshwater systems, thus limiting the effects of competition, but are probably not important in the hypersaline ponds that *Artemia* inhabit where almost all predators are excluded. In addition, the parthenogenetic origins of *Daphnia* clones are probably much more recent than those of *Artemia*, perhaps as little as a decade. Abreu-Grobois and Beardmore (1980, 1982) estimate from electrophoretic data that parthenogenesis in *Artemia* is of monophyletic origin and occurred approximately 6×10^6 years ago. Multiple clones have thus had time to exist in relatively long-term equilibrium. In addition, as Hebert (1978) has pointed out, since male reproduction is retained by obligately parthenogenetic *Daphnia* clones, it may serve as a primary factor in the displacement of clones reproducing by cyclical parthenogenesis. Via this method, the genes responsible for obligate parthenogenesis could enter a wide variety of genetic backgrounds and clones of newly obligate parthenogens may very rapidly diverge and evolve. Thus, the situation in *Daphnia* is quite different from that of *Artemia*, where clonal divergence is probably not nearly so recent and there is no continual genetic variation via sexual recombination. The situation in *Artemia* is therefore more analogous to the majority of species that reproduce by obligate parthenogenesis.

Although most parthenogenetic populations are multiclonal (Parker, 1979), Lynch (1984a) has advanced the concept of a general purpose genotype hypothesis, which states that clonal selection over time will promote the evolution of highly generalized genotypes which have broad tolerance ranges and low fitness variance for relevant physical, chemical, and biotic gradients. However, the evidence from the present study on *Artemia* does not initially support the general purpose genotype hypothesis since there are highly significant differences in life history traits between clones inhabiting the same site. There is the possibility, however, that different clones partition the habitat on either a microgeographic or temporal (*e.g.* seasonal) scale. These possibilities are currently being investigated through electrophoretic and field studies of the Salin de Giraud population.

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Strain characterization

In 1978, the International Study on *Artemia* (ISA) was created to establish an interdisciplinary approach to the characterization of *Artemia* strains. The characteristics selected for study were : chemical composition (both nutrient levels and pollutant concentrations), value as a food for aquaculture species (fish and crustacean), general biology (biometrics, hatching characteristics, growth in culture) and genetics (both electrophoretic and cytogenetic information). The approach proved successful and 10 papers were presented at the First International Symposium on *Artemia* to describe the ISA results.

The task of strain characterization continues on a worldwide basis, as evidenced by the following papers : Vanhaecke *et al.* (1) first update the inventory of known *Artemia* populations and attempt to explain the distribution of *Artemia* with a global perspective. The next two papers (2-3) cover different „fingerprinting" techniques for possible identification of strains. The remaining papers are arranged in the order given above, from chemical composition (4-8) through general biology (6-12) to genetics (13-14). Some of those papers, especially those of Van Ballaer *et al.* (7) and Vilela and Castelo Branco (8) have adopted the ISA approach and present interdisciplinary information.

(1) P. Vanhaecke, W. Tackaert, and P. Sorgeloos.
The biogeography of *Artemia* : an updated review.

(2) P. J. D. Requintina and K. L. Simpson.
International Study on *Artemia*. XLV. Geographical and developmental changes in isozymes of *Artemia* as separated by isoelectrofocusing.

(3) A. Schimmelmann, P. Lavens, and P. Sorgeloos.
Carbon, nitrogen, oxygen, and hydrogen stable isotope ratios in *Artemia* from different geographical origin.

(4) U. M. Cowgill, H. W. Emmel, G. U. Boggs, P. G. Murphy, F. M. Gersich, I. T. Takahashi, and D. A. Bengtson.
Variations in chemical composition of *Artemia* cysts from three geographical locations.

(5) R. P. Kandiuk, V. J. Lisovskaya, and Y. N. Makarov.
Biochemical evaluation of the brine shrimp *Artemia* from the Kuyalnitsky liman (USSR).

(6) S. C. Bhargava, G. R. Jakher, M. M. Saxena, and R. K. Sinha.
Laboratory culture and nutritional assessment of *Artemia* from Didwana Salt Lake (India).

(7) E. Van Ballaer, D. Versichele, P. Vanhaecke, Ph. Léger, N. Ben Abdelkader, S. Turki, and P. Sorgeloos.
Characterization of *Artemia* from different localities in Tunisia with regard to their use in local aquaculture.

(8) M. H. Vilela and M. A. Castelo Branco.
Characterization of Portuguese *Artemia* strains.

(9) J. Castritsi-Catharios, M. Christodoulopoulou, A. Aivatzidou, and B. Kiortsis.
Biometrics of *Artemia* from Milos (Greece)

(10) N. Lysenko de Tejeda
Preliminary characterization of four populations of *Artemia* from the Dominican Republic.

(11) C. Yaneng
Observations on parthenogenetic and bisexual brine shrimp from the People's Republic of China.

(12) C. Thoeye, A. Van der Linden, F. Bernaerts, R. Blust, and W. Decleir.
The effect of diurnal temperature cycles on survival of *Artemia* from different geographical origin.

(13) W. Tackaert, P. Vanhaecke, and P. Sorgeloos.
Preliminary data on the heritability of some quantitative characteristics in *Artemia.*

(14) C. Gallardo and J. Castro.
Reproduction and genetics of Mexican *Artemia.*

Artemia *Research and its Applications. 1987. Vol. 1. Morphology, Genetics, Strain characterization, Toxicology*
P. Sorgeloos, D. A. Bengtson, W. Decleir, and E. Jaspers (Eds). Universa Press, Wetteren, Belgium. 380 p.

The biogeography of *Artemia*: an updated review

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Abstract

An updated list of more than 350 *Artemia* sites is provided. It includes geographical coordinates of the sites and available data on the mode of reproduction of the locally occurring brine shrimp.

The geographical distribution of *Artemia* is related to different types of climate. The classification of climate according to Thornthwaite provides a good correlation with the geographical occurrence of *Artemia*, as a result of which the world distribution pattern of *Artemia* can be predicted. Moreover, areas with possible *Artemia* sites and areas suited for transplantation and/or inoculation with *Artemia* can be identified on the basis of water balance data. This is illustrated for three case studies (Peru, the Philippines and Sri Lanka).

The relation between the biogeographical distribution of *Artemia* and its speciation in different sibling species as well as its reproduction mode is discussed.

Introduction

It is generally known that the brine shrimp *Artemia* is widely distributed on the five continents in many salt lakes, coastal lagoons, and solar saltworks. In 1915 Abonyi already published a list of 80 *Artemia* sites located in 21 different countries. Later on, some less extensive distribution lists were reported by Artom (1922) (18 sites), Stella (1933) (28 sites), and Barigozzi (1946) (29 sites). The most recent review of the distribution of *Artemia* (Persoone and Sorgeloos, 1980) included a list of 243 sites distributed over 48 countries.

In recent years interest in *Artemia* has increased steadily and as a result the occurrence of *Artemia* has been studied and recorded in a growing number of countries.

The present paper provides an updated list of *Artemia* sites resulting from a thorough literature study (Vanhaecke, 1983) and numerous personal communications received at the Artemia Reference Center. The distribution lists have been completed with the geographical coordinates of the sites and the available data on the mode of reproduction and the sibling species.

The present distribution list of *Artemia* sites was further used to determine a logical trend in the distribution pattern of *Artemia* sites. This in order to allow the identification of areas where *Artemia* might occur, or areas that might be suitable for brine shrimp transplantation or inoculation. With this aim correlations were evaluated between the geographical distribution of *Artemia* and different classifications of climate.

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Finally the biogeography of *Artemia* is discussed in relation to the differentiation between sibling species and in relation to the reproduction mode of *Artemia*.

The world distribution of *Artemia* sites

The listings of *Artemia* sites in the Tables I through VIII only include those biotopes in which the population reappears each year, and do not cover temporal *Artemia* populations mostly introduced through inoculation in seasonal salt operations (*e.g.* Panama, Costa Rica, Burma, Thailand, Philippines, Viet Nam, Indonesia). In view of favorable climatic conditions and/or specific management allowing for year-round storage of brine, some of these inoculated strains might, however, become established as natural strains and should be added to the listings in due time, *e.g.* Cam Ranh Bay, Viet Nam (Vu Do Quynh and Nguyen Ngoc Lam, 1987) and Aguadulce, Panama (Pang, pers. commun.).

The world distribution of *Artemia* is presented in Fig. 1. Although about 360 *Artemia* sites could be traced back, the present distribution list remains provisional. It is interesting to note that within a time period of not more than 6 years 117 new *Artemia* biotopes have been recorded. This observation not only indicates that an increasing number of investigations have been carried out recently, but also confirms our hypothesis that more extensive survey work should lead to the discover of many more *Artemia* biotopes in different parts of the world. On the other hand, as history has already proved, brine shrimp populations and biotopes might disappear not only as a consequence of human intervention but also from natural causes such as temporal climate changes, *e.g.* Lymington, UK ; Capodistria, Yugoslavia).

The common feature of all *Artemia* biotopes is their high salinity. Salinity is without any doubt the predominant abiotic factor determining the presence of *Artemia* and consequently limiting its geographical distribution. This was clearly illustrated by Hammer (1978) who studied the salinity and presence of *Artemia* in 60 salt lakes in the Saskatchewan region. Whereas salinity ranged from 2.4 up to 370 ‰, *Artemia* was only found in those five lakes with salinities over 94 ‰. An analogous distribution of *Artemia* in relation to salinity has been reported by McCarraher (1970) for Nebraska lakes. The impact of other parameters such as temperature, light intensity, primary food production, *etc.* on the *Artemia* distribution is limited to the quantitative population development of brine shrimp or may cause only a temporary absence of *Artemia*.

Although high salinity is imperative to allow brine shrimp to persist, not all salt lakes or highly saline biotopes are populated with *Artemia*, *e.g.* for the continental USA McCarraher (1972) listed over 30 highly saline lakes (> 100 ‰) not inhabited by *Artemia*, in West Victoria (Australia) *Artemia* is absent in 15 natural salt lakes (> 100 ‰) (Williams, 1981). Apparently, none of the distribution vectors wind, birds nor man allowed *Artemia* to colonize these biotopes. In the particular case of Australia, however, it is not unlikely that the endemic genus *Parartemia* which is better adapted to the specific conditions of Australian salt lakes minimizes the chance of an introduced *Artemia*-population to develop (Geddes, 1980ab, 1981).

Although *Artemia* is widely distributed throughout the world, these observations clearly illustrate that it is not ubiquitous nor can it even be considered a cosmopolitan organism, as it is not present in every suitable biotope.

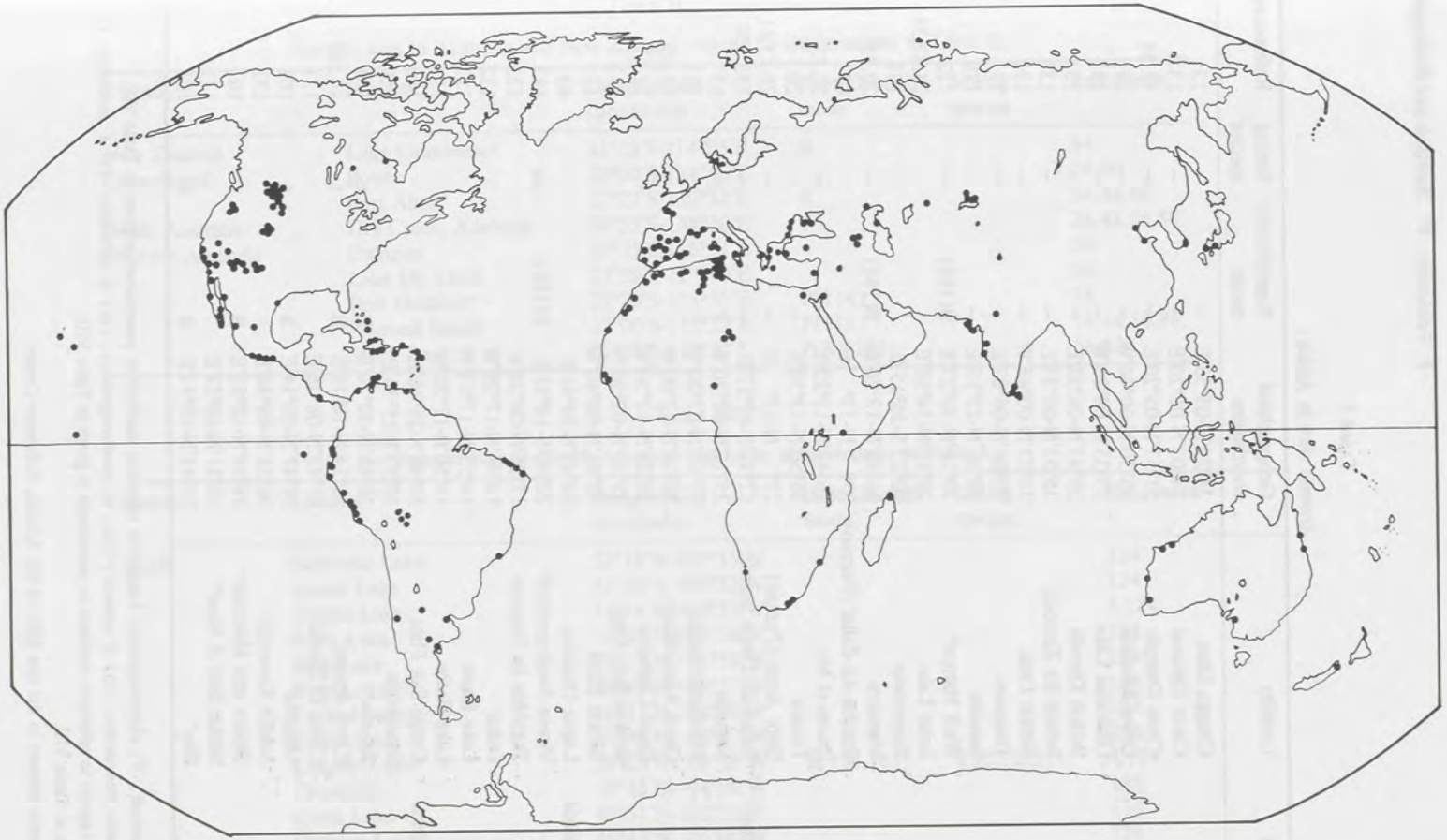

FIG. 1. The world distribution of *Artemia*.

TABLE I

Artemia sites in Africa

Country	Locality	Geographical coordinates	Reproduction mode[1]	Sibling species[2]	References[3]
Algeria	Chegga Oase	34°29'N-05°53'E	–	–	131
	Chott Djeloud	34°03'N-06°20'E	–	–	131
	Chott Ouargla	31°57'N-05°20'E	–	–	12,24
	Dayet Morselli	35°30'N-00°46'W	–	–	24
	Gharabas Lake	35°35'N-00°25'W	–	–	131
	Sebket Djendli	35°43'N-06°32'E	–	–	131
	Sebket Ez Zemouk	35°53'N-06°33'E	–	–	131
	Sebket Oran	35°32'N-00°48'W	–	–	131
	Tougourt	33°06'N-06°07'E	–	–	73
Egypt	Ismailia	30°36'N-22°15'E	–	–	179
	Wadi Natron*	30°10'N-30°27'E	B(180)	–	122
	Solar Lake	29°10'N-34°50'E			24,37,38
Kenya	Elmenteita	00°27'S-36°15'E			121
Libya	Mandara	26°40'N-13°20'E	B(180)	–	20,73
	Ramba-Az-Zallaf (Fezzan)	27° N-13° E			83
	Quem el Ma	26°41'N-13°22'E	–	–	20,73
	Trouna	26°50'N-13°30'E	–	–	20
	Gabr Acun (Fezzan)	27° N-13° E	–	–	20,73,81
Madagascar	Salins de Diego Suarez	12°19'S-49°17'E	–	–	133,150
Marocco	Larache	35°12'N-02°20'W	P	pa	63
	Moulaya estuary	35°07'N-02°20'W			131
	Qued Ammafatma	28°18'N-12°00'W			73
	Qued Chebeica	28°25'N-11°50'W			73
	Sebket Bon Areg	35°10'N-02°50'W			131
	Sebket Zima	32°05'N-08°40'W			131
Mozambique	Lagua Quissico	24°41'S-34°46'E			40
Namibia	Vineta Swakopmund*	22°40'S-14°34'E	P(180)	pa	181
Niger	Teguidda In Tessoun	17°26'N-06°39'E			122
Senegal	Dakar	14°34'N-17°29'W			122
	Lake Kayar	14°55'N-17°11'W			122
	Lake Retba	14°50'B-17°20'W			73
South Africa	Coega Salt Flats	33°46'S-25°40'E			159
	Swartkops	33°52'S-25°36'E			159
Tunisia	Bekalta*	36°48'N-10°20'E	B	t	101
	Chott Ariana*	36°54'N-10°18'E	B	t	44,101
	Chott El Djerid	33°42'N-08°26'E			131
	Megrine*	36°47'N-10°14'E	B	t	101
	Sebket Kowezia*	36°26'N-09°46'E			131
	Sebket mta Moknine*	35°39'N-10°53'E	B	t	101
	Sebket Sidi el Hani*	35°31'N-10°27'E			131
	Sfax*	35°45'N-10°43'E	B	t	101

[1] (B) : bisexual ; (P) parthenogenetic. Legend to reference numbers in parentheses is given in Table VIII.

[2] (f) *Artemia franciscana* ; (m) *A. monica* ; (pa) : *A. parthenogenetica* ; (pe) *A. persimilis* ; (t) *A. tunisiana* ; (u) *A. urmania*. Legend to reference numbers in parenthesis is given in Table VIII.

[3] See list in Table VIII.

* Cyst sample present in the cyst bank of the Artemia Reference Center.

TABLE II

Artemia sites in Australia and New Zealand (legend to abbreviations in Table I)

Country	Locality	Geographical coordinates	Reproduction mode	Sibling species	References
New Zealand	Lake Grassmere*	41°38'S-174°05'E	B		94
Queensland	Bowen	20°00'S-184°16'E			56,80
	Port Alma*	23°22'S-150°32'E	B		56,44,80
South Australia	Dry Creek, Adelaide	34°55'S-138°20'E			26,48,56,80
Western Australia	Dampier	20°35'S-116°51'E			56
	Lake Mc Leod	23°59'S-113°40'E			56
	Port Hedland*	20°25'S-118°35'E	P(114)		56
	Rottnest Island	32°00'S-115°27'E	P(114)		56,44,80,86
	Shark Bay*	25°15'S-113°20'E	P(B)(103)		56,80

TABLE III

Artemia sites in North America (legend to abbreviations in Table I)

Country	Locality	Geographical coordinates	Reproduction mode	Sibling species	References
Canada	Akerlund Lake	52°18'N-109°15'W			124
	Alsask Lake	51°20'N-109°52'W			124
	Aroma Lake	51°18'N-108°33'W			4,124
	Berry Lake	52°07'N-105°30'W			124
	Boat Lake	50°17'N-109°59'W			124
	Burn Lake	49°49'N-105°27'W			124
	Ceylon Lake	49°27'N-104°36'W			124
	Chain Lake	50°30'N-108°43'W			124
	Chaplin Lake*	50°25'N-106°38'W	B(104,105)	f(102,103)	4,124
	Churchill	58°45'N- 94°00'W			182
	Coral Lake	49°51'N-102°21'W			124
	Drybore Lake	49°43'N-105°30'W			124
	Enis Lake	52°10'N-108°19'W			124
	Frederick Lake	49°59'N-105°38'W			124
	Fusilier Lake	51°50'N-109°44'W			124

TABLE III. Continued

Country	Locality	Geographical coordinates	Reproduction mode	Sibling species	References
Canada	Grandora Lake	52°06'N-107°00'W			124
	Gull Lake	50°06'N-108°27'W			124
	Hatton Lake	50°02'W-109°50'W			124
	Horizon Lake	49°32'N-105°17'W			124
	Ingerbright Nath	50°22'N-109°19'W			124
	Landis Lake	52°13'N-108°27'W			124
	La Perouse	55°14'N- 98°00'W			182
	Little Manitou Lake	51°48'N-105°30'W	B	f(105)	4,24,44,46,90,124
	Lydden Lake	52°09'N-108°13'W			124
	Mawer Lake	50°46'N-106°22'W			124
	Meacham Lake	52°07'N-105°47'W			124
	Muskiki Lake	52°20'N-105°45'W			124
	Neola Lake	52°02'N-107°49'W			124
	Oban Lake	52°09'N-108°09'W			124
	Richmond Lake	52°01'N-108°01'W			124
	Shoe Lake	49°55'N-105°27'W			124
	Snakehole Lake	50°30'N-108°30'W			124
	Sybouts Lake-East	49°02'N-104°24'W			124
	Sybouts Lake-West	49°02'N-104°27'W			124
	Verlo West	50°19'N-108°37'W			124
	Vincent Lake	50°13'N-108°57'W			124
	Wheatstone Lake	49°49'N-105°24'W			124
	Whiteshore Lake	52°08'N-108°17'W			124
USA-Arizona	Kiatuthlana Red Pond	34°50'N-109°26'W	B(114)	f(102,105)	11,12,13,24,44
	Kiatuthlana Green Pond	34°50'N-109°26'W	B(114)	f(102,105)	11,12,13,24,44
-California	Carpinteria Slough	34°24'N-119°30'W			43
	Chula Vista	32°36'N-117°05'W			112
	Mono Lake*	38°00'N-119°00'W	B(44)	m(105,120)	6,12,22,24,58,66
	Moss Landing, Monterey Bay	36°42'N-121°49'W			44,87
	Owens Lake	36°25'N-117°56'W			24
	San Diego	32°50'N-117°10'W			44
	San Francisco Bay*	37°28'N-122°30'W	B(44,180)	f(102,105)	21,44,76,90
	San Pablo Bay*	38°00'N-122°16'W	B(104)	f(105)	77,123
	Vallejo West Pond	38°12'N-122°15'W			21,44

USA-Hawaii	Christmas Islands*	01°50'N-157°20'W			97,98
	Hanapepe	21°54'N-159°30'W			23
	Laysan Atoll	25°30'N-167°00'W			23
-Nebraska	Alkali Lake	43°32'N-100°38'W			24
	Ashenburger Lake	42° N-102° W			24
	Cook Lake	42° N-102° W			12,24,49
	East Valley Lake	42° N-102° W			24
	Grubny Lake	42° N-102° W			24
	Homestead Lake	42° N-102° W			24
	Jesse Lake*	42°06'N-102°39'W	B	f(102)	11,12,49
	Johnson Lake	42° N-102° W			12,24
	Lilly Lake	42° N-102° W			11,12,49
	Reno Lake	42° N-102° W			24
	Richardson Lake	42° N-102° W			12,24,49
	Ryan Lake	42° N-102° W			12
	Sheridan County Lakes	42° N-102° W			12
-Nevada	Fallon	39°31'N-118°52'W			58,95
-North Dakota	Miller Lake	–			24
	Stink (Williams) Lake	–			24
-New Mexico	Laguna del Perro*	34°32'N-106°01'W			126
	Loving Salt Lake	32°17'N-104°04'W	B(114)		129
	Quemado*	34°17'N-108°28'W	B	f(105)	44
	Zuni Salt Lake	34°27'N-108°46'W	B(114)	f(102)	5,11,12
-Oregon	Lake Abert*	42°35'N-120°15'W	B(130)		125
-Texas	Cedar Playa	32°49'N-102°07'W			75
	McKenzies Playa	32°41'N-102°10'W	B(180)		75
	Mound Playa	33°10'N-101°56'W			75
	Playa Thahoka*	33°12'N-101°34'W	B(180)		24,75
	Raymondville*	26°10'N- 97°48'W	B(180)		124
	Rich Playa	33°13'N-102°03'W			75
	Snow drop Playa	32°59'N-101°40'W			75
-Utah	Great Salt Lake*	41°00'N-112°30'W	B(44,104,106)	f(102,105)	11,12,18,19,24, 30,31,32,42,90
-Washington	Hot Lake	48°58'N-119°29'W			3,7
	Omak Plateau	48°25'N-119°24'W			7,11
	Soap Lake	47°33'N-119°25'W	B(114)		44

TABLE IV

Artemia sites in Central America (legend to abbreviations in Table I)

Country	Locality	Geographical coordinates	Reproduction mode	Sibling species	References
Bahamas	Great Inagua*	21°00'N- 75°20'W	B		44
	Long Island*	23°20'N- 75°07'W	B(180)		124
	San Salvador	24°00'N- 74°35'W			128
British Virgin Islands	Anegada	18°45'N- 64°24'W			160
Caribbean Islands	Antigua	17° 0'N-61°45'W			144
	St. Kitts	17°20'N- 62°45'W			144
	St. Martin	18°04'N- 63°06'W			160
Costa Rica	Gulfo Nicoya	10°00'N- 84°49'W			146
Dominican Republic	Isla Cabra	19°53'N- 71°40'W	B		115
	Las Calderas		B		115
	Monte Cristi	19°52'N- 71°39'W	B		115
	Puerto Alejandro		B		115
	Punta Salinas*	18°20'N- 71°04'W	B(180)		130
Haiti	Grandes salines	18° N- 72° W	B	f	180
Mexico	Bahia de Cueta*	24°05'N-107°00'W	B(180)		29
	Carretas, Pereyra	15°30'N- 93°13'W			107
	Chanchuto Panzacola				107
	Chiapas	15°56'N- 93°30'W			29
	Guerrero Negro	28°06'N-114°03'W			29
	Isla del Carmen	26°00'N-111°40'W			107
	Laguna der Mar Muerto	16° N- 94° W			107
	La Joya, Buenavista	27°27'N-106°15'W			107
	Las Salinas	22°40'N-101°42'W			140
	Los Palos, Solo Dios				107
	Pichilingue Island	24°17'N-110°20'W	B(114)		29,44
	Salina cruz	16°10'N- 95°10'W			29
	San Jose Island	25°00'N-110°50'W	B(114)		151
	San Quintin	30°28'N-115°58'W			44
	Yavaros	26°43'N-109°33'W	B	f(102,104)	29
Netherlands Antilles	Aruba	12°30'N- 70°00'W			15
	Bonaire Duinmeer*	12°04'N- 68°13'W	B	f(102,104)	33
	Gotomeer	12°14'N- 68°23'W			15,33
	Pekelmeer	12°04'N- 68°16'W			15,33,84
	Martinus	12°09'N- 68°17'W			14,15
	Slagbaai	12°16'N- 68°25'W			14,15
	Curaçao Fuik	12°03'N- 68°51'W			14,15
	Rifwater	12°08'N- 68°57'W			14,15
Puerto Rico	Bahia Salinas*	17°57'N- 67°12'W	B(180)	f(102)	170
	Bogueron*	18°01'N- 67°10'W			170
	Cabo Rojo*	17°56'N- 67°08'W	B(114)		85
	La Parguera	17°59'N- 67°03'W			52
	Ponce	18°00'N- 66°38'W			170
	Tallaboa	18°00'N- 66°42'W			44

TABLE V

Artemia sites in South America (legend to abbreviations in Table I)

Country	Locality	Geographical coordinates	Reproduction mode	Sibling species	References
Argentina	Bahia Blanca	38°43'S-62°15'W			120
	Buenos Aires*	34°30'S-58°20'W	B	pe(102,104)	
	Hidalgo	37°10'S-63°32'W			44,65,88,89
	Mar Chiquita	30°39'S-62°30'W			172
Bolivia	Lake Canapa		B		173
	Lake Chulluncani	16°22'S-67°30'W			173
	Lake Hedonia				173
	Lake Poopo	18°23'S-66°58'W	B		173
Brazil	Aracati	4°32'S-37°45'W			108
	Cabo Frio*	22°51'S-42°03'W	B	f(102)	16
	Fortaleza	3°45'S-38°35'W			108
	Icapui	4°42'S-37°21'W			154
	Macau*	5°00'S-36°40'W	B	f(102,104)	68
	Mundau	3°15'S-39°24'W			108
Chili	Atacama Lake	23°30'N-68°10'W			184
	Pichelimu	34°22'S-79°09'W			70
Colombia	Galerazamba*	10°25'S-74°40'W	B	f(102)	128
	Manaure*	12°09'S-71°55'W	B	f(102,104)	128
Ecuador	Galapagos	0° S-89° W			44
	Pacoa*	2°00'S-80°50'W	B(180)		156
	Salinas	2°20'S-80°58'W			156
Peru	Caucato	13°40'S-76°05'W			148
	Chicama	7°42'S-79°27'W			148
	Chilca*	12°35'S-76°41'W	B(180)		157,148
	Estuario de Virrila*	5°50'S-80°50'W	B(180)		148
	Guadalupe	7°17'S-79°28'W			148
	Pampa de Salinas	11°14'S-77°35'W			148
	Pampa Playa Chica	11°14'S-77°35'W			148
	Puerto Huarmey	10°03'S-78°08'W			148
	Tumbes*	3°37'S-80°27'W	B(180)		148
Venezuela	Boca Chica	10°57'N-64°26'W			60,166
	Coya Sal	10°56'N-68°15'W			166
	Coche	10°41'N-63°58'W			166
	Coro Coastline	11°30'N-69°45'W			152
	La Orchila	11°49'N-66°00'W			166
	Las Aves	12°00'N-67°17'W			166
	Los Roques	11°50'N-66°38'W			164,166
	Port Araya*	10°39'N-64°17'W	B(102,104)		165
	Tucacas*	10°48'N-68°19'W	B(180)		166

TABLE VI

Artemia sites in Asia (legend to abbreviations in Table I)

Country	Locality	Geographical coordinates	Reproduction mode	Sibling species	References
China	Aibi Lake*				175
	Tientsin*	39°10'N-117°00'E	P	pa(102)	127,145
	Tsingtao*	36°00'N-120°25'E			127,147
	Urumuchi Lake	43°43'N- 87°38'E			175
India	Bhayander, Bombay*	18°55'N- 72°50'E	P(180)		34,61,62
	Didwana	27°17'N- 74°25'E			135
	Jamnagar	22°30'N- 70°08'E			28,62
	Karsewar Island	8°50'N- 78°10'E			1
	Kutch*	23°20'N- 71°00'E	P	pa(44)	28,137
	Mithapur*	23°00'N- 70°10'E	P(180)		28,62,139
	Pattanamaruthur	8°55'N- 78°08'E			59
	Spic Nagar	8°50'N- 78°08'E			59
	Thirispuram	8°50'N- 78°08'E			59
	Tuticorin	8°50'N- 78°08'E	P	pa(102)	26,27,57,59,62
	Vadala, Bombay	18°55'N- 72°50'E			26,27
	Vedaranyam	10°01'N- 79°50'E			110
	Veppalodai	8°59'N- 78°08'E			59
	Vivar, Bombay	18°55'N- 72°50'E			136
Iraq	Abu-Graib, Baghdad	33°20'N- 44°30'E	P(114)		141
	Basra	30°25'N- 47°51'E			2
	Dayala	33°30'N- 44°30'E			2
	Mahmoodia	33° N- 44° E			2
Iran	Ormia*	37°20'N- 45°40'E	B	u(44)	12,24,45
	Schor-Gol	37°03'N- 45°32'E			12,24,45
	Shurabil	48°17'N- 38°15'E			109
	Athlit	32°42'N- 34°56'E			96
Israel	Eilat North*	29°32'N- 34°56'E	P	pa(44)	142
	Eilat South	29°28'N- 34°56'E			142
Japan	Chang Dao	34° N-132° E			55
	Tamano	34°35'N-133°59'E			51
	Yamaguchi	34°10'N-131°32'E	P(114)		44,51
Kuwait		29° N- 47° E			149
Korea	Pusan	35°05'N-129°02'E			176
Sri Lanka	Bundala	6°12'N- 81°15'E			180
	Hambantota*	6°07'N- 81°07'E			180
	Palavi	7°58'N- 79°51'E			180
	Putallam	8°02'N- 79°50'E	P(180)	pa(180)	153
Taiwan	Peinan Salina				177
Turkey	Aivalik				134
	Izmir (Camalti)*	38°25'N- 27°08'E	P	pa(102)	35
	Tuz Golii	38°45'N- 33°30'E			10

TABLE VII

Artemia sites in Europe (legend to abbreviations in Table I)

Country	Locality	Geographical coordinates	Reproduction mode	Sibling species	References
Bulgaria	Burgas*	42°33'N-27°29'E	P	pa(102)	8,47
	Pomorye	42°26'N-27°41'E			8,47
Cyprus	Akrotiri lake	34°34'N-32°58'E			17
	Larnaca lake*	34°56'N-33°35'E	B(111)	t(102,104)	17
France	Aigues Mortes*	43°34'N- 4°11'E	P(180)		132
	Carnac-Trinité sur Mer	47°36'N- 3°05'W			82
	Guérande-le Croisic*	47°20'N- 2°26'W	P(180)		82
	La Palme	42°59'N- 3°00'E			154
	Lavalduc*	43°24'N- 4°56'E	P	pa(102,104)	132
	Mesquer-Assérac	47°26'N- 2°29'W			82
	Porte La Nouvelle	42°57'N- 3°02'E			112
	Salin de Berre*	43°24'N- 5°05'E	P(180)	pa(102)	155
	Salin de Fos	43°26'N- 4°56'E			155
	Salin de Giraud*	43°24'N- 4°44'E	P	pa(102)	154
	Salins d'Hyères	43°07'N- 6°12'E			155
	Salin des Pesquiers	43°07'N- 6°12'O			155
	Sète*	43°25'N- 3°42'E		pa(144,102)	78,79
Greece	Embolon	40°38'N-22°58'E	P		117
	Kalloni	39°16'N-26°16'E	P		117
	Katerini	40°15'N-22°30'E	P		161
	Kitros	40°22'N-22°34'E	P		117
	Mesolongi*	38°21'N-21°26'E	P		161
	Milos	36°44'N-24°25'E	P		116,161
	Porto		P		117
Italy	Cagliari, Sardinia	39°13'N- 9°08'E			118
	Carloforte Sardinia	39°08'N- 8°17'E			118
	Cervia	44°16'N-12°21'E			180
	Commachio*	44°41'N-12°10'E	P	pa(63)	78,79
	Margherita di Savoia*	41°25'N-16°05'E	P	pa(102)	167
	San Antioco, Sardinia	39°02'N- 8°30'E			118
	Santa Gilla, Sardinia	39°14'N- 9°06'E	P(114)		78,79
	Siracuse, Sicily	37°04'N-15°18'E			143
	Tarquinia	42°29'N-11°45'E			118
	Trapani, Sicily	38°01'N-12°30'E			168
Portugal	Alcochete	38°45'N- 8°57'W	P	pa(102)	63
	Tejo estuary	38°50'N- 9°00'W			168
	Sado estuary	38°25'N- 8°43'W			168
	Ria de Aveiro	40°37'N- 8°38'W			168
	Ria de Farc	37°02'N- 7°55'W			168
Rumania	Lake Techirghiol*	43°04'N-28°34'E	P(114)		9,36,39
Spain	Armalla*	40°54'N- 1°59'W			63
	Ayamonte	37°13'N- 7°24'W	P		63
	Cabo de Gata*	36°48'N- 2°14'W	P	pa	63
	Buyaraloz	41°29'N- 0°10'W			63,71
	Cadiz*-San Felix	36°30'N- 6°20'W	B		41,63,67,71
	-San Fernando	36°22'N- 6°17'W	B		41,63,67,71
	Calpe*	38°39'N- 0°03'E	P	pa(102)	63
	Campos del Puerto, Mallorca	39°26'N- 3°01'E			63,71
	Delta de Ebro*	36°25'N- 6°18'W	P	pa	63
	Gerri de la Sal	42°20'N- 1°04'E	P	pa	63,71
	Imon	41°10'N- 2°45'W			63

TABLE VII. Continued

Country	Locality	Geographical coordinates	Reproduction mode	Sibling species	References
Spain	Isla Cristina	37°13'N- 7°19'W	P	pa	63
	Janubio, Lanzarote	28°56'N-13°50'W	P	pa	63
	Laguna de Quero	39°34'N- 3°17'W			63
	Las Palmas	28°10'N-15°28'W			96
	Lepe	37°15'N- 7°12'W			63
	Lerin	42°29'N-10°59'W			63
	Medacineli	41°12'N- 2°30'W			63
	Molina del Segura	38°03'N- 1°11'W			63
	Peralta de la Sal	42°00'N- 0°24'E			63
	Poza de la Sal	42°40'N- 3°30'W			63,71
	Rienda	41°06'N- 2°34'W			63
	Roquetas	40°50'N- 0°30'E			63
	Saelices	39°55'N- 2°49'W			63
	Salinera Catalena	37°37'N- 0°51'W			63
	Salinera Espanola, Formentera	38°40'N- 1°26'E	B(114)		63
	Salinera Espanola, Ibiza	38°55'N- 1°35'E	B		63,71
	Salinera Punta Galera	37°42'N- 0°54'W			63
	San Juan del Puerto	37°20'N- 6°50'W			63
	Sanlucar de Barrameda*	36°43'N- 6°23'W	PB		63
	San Pedro del Pinatar	37°50'N- 0°50'W	B	t	63
	Santa Pola-Bonmati*	38°13'N- 0°35'W	PB	pa(102)	63
	-Bras de Port	38°13'N- 0°35'W	P	pa	63
	-Salinera Espanola	38°13'N- 0°35'W	B		63
	Siguenza	41°04'N- 2°38'W			63
	Villena	38°39'N- 0°52'W			63
USSR	Bolshoe Otar Mojnakshoe	45° N-33° E			53
	Bolshoe Yarovoe*	53°00'N-78°30'E	P(180)		143
	Burlinskoe ozero	53°12'N-78°30'E			24,178,183
	Szharylgach	45°35'N-32°56'E			53,54
	Ghenicheskoe Lake	46°15'N-35°00'E			74
	Karachi Lake	41°16'N-72°00'E			92
	Kazakhstan*	49°00'N-50°00'E			69
	Kuchukskoe	52°40'N-79°40'E			24
	Kujalnic estuarium	46°43'N-30°40'E	P(180)		9,119
	Kyzyl-Jar Lake (Primorsk)	40°14'N-49°33'E			92
	Mangyshlak penisula*	43°40'N-52°30'E			149
	Moekba*				178
	Odessa*	46°30'N-30°45'E	P(111)	pa(44)	69
	Ontario Lake				92,93
	Petukhovshoe	52°10'N-78°40'E			24
	Popovskoe Lake	45° N-33° E			54
	Sakshoe	45°10'N-33°30'E			24,53,72
	Sasyk Lake*	45°15'N-33°25'E			50,53,72,92
	Sasykol Lake	53°40'N-61°40'E			64
	Seitenj*				178
	Tambukan				24
	Tinaki Lake*		P(180)		169
	Tobechicskoe Lake	45°10'N-36°05'E			53,162
	Turkomama				171
	Yalovoye*				169
Yugoslavia	Portoroz*	45°39'N-13°36'E	P	pa	113
	Strunjan	45°32'N-13°36'E	P	pa	113
	Ulcinj	41°55'N-19°12'E	P	pa	113

TABLE VIII

List of references given in Tables I through VII

Literature references

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Personal communications to the Artemia Reference Center

120. Martinez ; 121. Coomans ; 122. Dumont ; 123. Smith ; 124. Atton ; 125. Conte ; 126. Gaffney ; 127. Li Maotang ; 128. Pedini ; 129. Browne ; 130. Jakowska ; 131. Morgan ; 132. Richard ; 133. Woyarovich ; 134. Davis ; 135. Bhargava ; 136. Bohra ; 137. Dave ; 138. Robichaux ; 139. Thomas ; 140. Castro ; 141. Khalaf ; 142. Dimentman ; 143. Barigozzi ; 144. Nunn ; 145. Ishiyama ; 146. Webber ; 147. Satoh ; 148. Carrera ; 149. Farmer ; 150. Collart ; 151. Ehnis ; 152. Urosa ; 153. Ferdinando ; 154. Mc Donald ; 155. Lavens ; 156. Haxby ; 157. Nava ; 158. Figueriredo ; 159. Emmerson ; 160. Walkers ; 161. Castritsi-Catharios ; 162. Ivleva ; 163. Spectorova ; 164. Perez ; 165. Teruel ; 166. Brownell ; 167. Trotta ; 168. Morales ; 169. Spectorova ; 170. Waldner ; 171. Spitchak ; 172. Rakocz ; 173. Risacher ; 174. Marinho ; 175. Ming Ren Li ; 176. Sung Bum Hur ; 177. Ting Lang Wang ; 178. Zoobedineni ; 179. Saad El-Sheriff ; 180. Own observation ; 181. Harrison ; 182. Barsfield ; 183. Polirpov ; 184. Wilson.

The geographical distribution of *Artemia* in relation to climate

Since habitat salinity is function of local water supply (*i.e.* precipitation, ground water seepage and surface water runoff) and of local evaporation rates, it is obvious that climate will have an important effect on the geographical distribution pattern of *Artemia* biotopes. In this regard, we have studied the distribution of *Artemia* sites in relation to the classification of the different types of climate. We have used the classification system of Thornthwaite (1948), and Thornthwaite and Mather (1955, 1957) which is based on the calculation of the water balance of the soil taking into account monthly data on precipitation and potential evaporation. The system of Thornthwaite makes use of the parameter "precipitation efficiency" which is determined by the index I_m.

$$I_m = I_p - I_a$$

$$\text{with } I_p = \frac{100\ S}{n} \text{ and } I_a = \frac{100\ d}{n}$$

whereby S = yearly surplus
d = yearly deficit
n = yearly need of water.

According to the numerical value of I_m, six climate types can be distinguished A = perhumid, B_4 - B_1 = humid, C_2 = humid-subhumid, C_1 = dry subhumid, D = semiarid, E = arid (Thornthwaite and Mather, 1955). This system furthermore allows characterization of the seasonal aspect of humidity or aridity. In analogy to the classification based upon the „precipitation efficiency" the so-called "adapted potential evapotranspiration" can be calculated to consider the "temperature efficiency" classification. This provides five climate types : A'_1 = megathermal, B'_4 - B'_1 = mesothermal, C'_2 - C'_1 = microthermal, D' = tundra, E' = frost. Seasonal characters of temperature efficiency can also be determined.

We have determined the type of climate corresponding to each *Artemia* site by using the monthly and yearly data on potential evapotranspiration, precipitation, water deficit, and surplus (Thornthwaite Associates 1962, 1963abc, 1964abc, 1965) or by use of climatic maps (Burgos and Vidal, 1951 ; Carter, 1954 ; Carter and Mather, 1966).

The distribution of the *Artemia* biotopes over the different types of climate (Table IX) reveals that the geographical distribution of brine shrimp is limited by climatological conditions, *i.e.* no

TABLE IX

The classification of *Artemia* sites (in %) in relation to the climate types according to Thornthwaite

Precipitation efficiency	Temperature efficiency						
	A'	B'_{3-4}	B'_{1-2}	C'_{1-2}	D'	E'	Total
E	15	6	2	0	0	0	23
D	9	12	12	1	0	0	34
C_1	5	6	17	12	0	0	40
C_2	1	0	2	0	0	0	3
B_4-B_1	0	0	0	0	0	0	0
A	0	0	0	0	0	0	0
Total	30	24	33	13	0	0	100

Artemia is found in perhumid (A) or humid (B_4 - B_1) climate types, and 97 % of the biotopes are located in areas where yearly evaporation exceeds yearly precipitation (negative I_m index : C_1, D, E).

The predominant effect of aridity on the distribution pattern of *Artemia* is further stressed when considering the seasonal aspect. Indeed, more than 97 % of the *Artemia* sites are located in areas where not just on a yearly basis, but also during the different seasons of the year, no or a very small water surplus can be noted. This is only a logical consequence of the limitation of *Artemia* presence to those biotopes where the salinity remains sufficiently high on a year-round basis. The 3 % of biotopes found in the humid sub-humid, C_2 climate type are all operational saltworks (three in France, four in India, one in Argentina, and one in Japan). Human interventions to keep high salinity levels do allow *Artemia* to survive in these biotopes, *e.g.* drainage or pumping of the freshwater stratification layer (Jones *et al.*, 1981), storage of brine in deep reservoirs (Richard, pers. commun.).

Aside from humidity the "efficiency of temperature" seems also to affect the distribution pattern of *Artemia*. No *Artemia* are found in the cold tundra and frost climate types (D' and E') as the prevailing low temperatures preclude *Artemia* development. Potential evaporation will also be very limited in these regions. Nonetheless a few salt lakes have been reported in Antarctica (Burton, 1981).

The proportional distribution of *Artemia* biotopes in the different climatic types (Table IX) is not correct. The number of *Artemia* sites in the arid (E) climates is probably underestimated since prospecting in those deserted and hardly accessible areas has been limited so far.

In view of the good correlation between the climate types, in particular aridity, and the geographical distribution of *Artemia*, we have outlined the potential distribution pattern of brine shrimp on a global level (Fig. 2). It is obvious, however, that this map only provides a general view of the potential distribution of *Artemia*. Climate types may vary significantly even within relatively short distances. Furthermore specific local conditions may result in isolated micro climates eventually (un)suitable for *Artemia*. Comparison of the potential distribution pattern with the actual known locations of *Artemia* sites reveals that on almost every continent extended areas exist which may contain many more *Artemia* biotopes. Africa in particular seems to be promising in this regard, *i.e.* Mauretania, Egypt, Somalia, Ethiopia, Sudan, etc. In Asia more in-depth searches may lead to the discovery of *Artemia* sites in Iraq, Iran, Afghanistan, Pakistan, and Turkey. It is expected that in the USSR as well as along the coasts of Mexico, Peru, Chile, and Argentina many more salt lakes or lagoons are inhabited by *Artemia* or might be suitable for *Artemia* but have not been colonized yet by lack of dispersion, e.g. the many saltworks in Northeast Brazil are now populated with *Artemia* since the human intervention in Macau in 1977, followed by dispersion by wind and local waterbirds over an area of more than 1 000 km.

The positive correlation between aridity and the presence of *Artemia* is not only obvious at the world level but even at the scale of small islands characterized by different climate types. In Sri Lanka, for instance, a preliminary investigation led to the discovery of one *Artemia* biotope in Puttalam (Ferdinando, pers. commun). From the climate map of Sri Lanka (Fig. 3, after Carter, 1954) it appeared that only the northwest (near Puttalam) and the southern tip of the island might permit the occurrence of *Artemia*. A recent survey resulted in three other *Artemia* sites precisely located in the few suitable climatic zones.

The study of the climate constitutes a fundamental guideline not only for the search of existing *Artemia* biotopes, but also for the planning and site selection for inoculation and/or transplan-

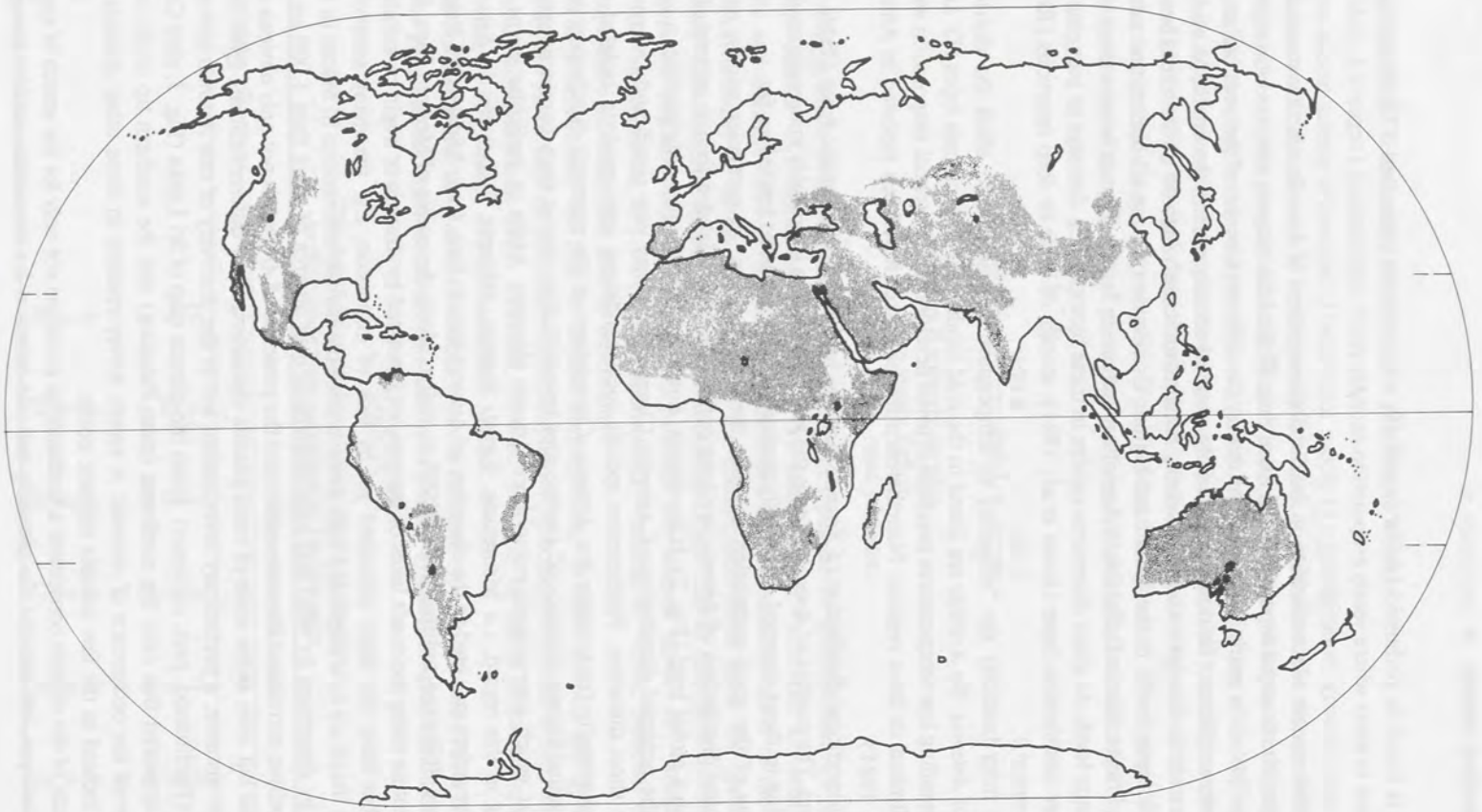

FIG. 2. The potential distribution pattern of *Artemia* at the world level.

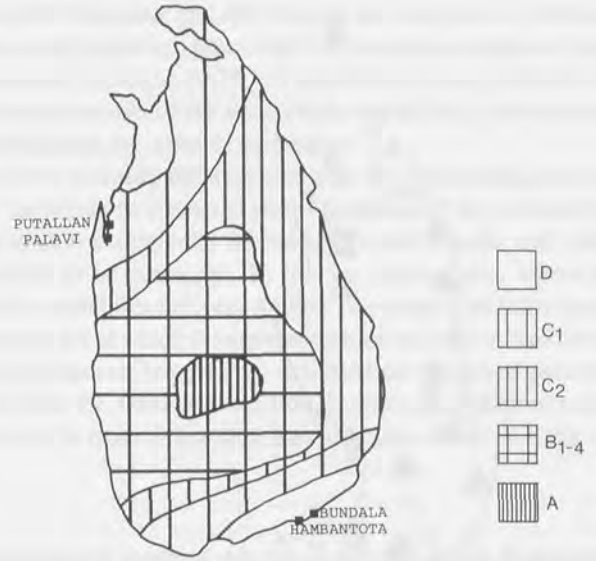

FIG. 3. The humidity types of Sri Lanka.

tion of *Artemia*. Yearly and seasonal evaporation-precipitation ratios will indicate if a given area ay be suited for *Artemia* production, and whether one single inoculation (then better called nsplantation) may be sufficient to create a persistent *Artemia* population or whether inocula- ns have to be repeated on a yearly basis to achieve seasonal *Artemia* production. The D and types of Thornthwaite are most suitable for transplantations. Dry subhumid areas (C_1) are vertheless promising provided, however, that no yearly period of water surplus occurs. As a sult potential areas for *Artemia* transplantation correspond to the regions in Fig. 2 where *temia* biotopes may be found.

Artemia production is still possible, however, in areas not characterized by a C_1, D, or E mate.

Generalizing maps such as Fig. 2 need to be used with great caution. Besides aridity, seasonal tribution of precipitation and evaporation are also important. This means that in some cases re detailed climatological conditions and water balance data have to be used (Thornthwaite sociates 1962, 1963abc, 1964abc, 1965). This is illustrated for two case studies: the lippines and Peru. The Philippines are characterized by a tropical humid climate. On a yearly is precipitation always exceeds evaporation. As a result no natural biotopes can be located transplantation of *Artemia* will fail to be successful. However, Fig. 4 reveals that in several s evaporation exceeds precipitation for a consecutive number of months. The duration of this season varies considerably from site to site. It is evident that longer dry seasons offer better sibilities for *Artemia* biomass and cyst production. Furthermore, stratified low-salinity waters y eventually be drained during periods of rainfall resulting in opportunities to extend *Artemia* mass) production far into (or even throughout) the rainy season (Wongrat for Thailand, s. commun.; Jumalon for the Philippines, pers. commun.). From the climatological point of

FIG. 4. Seasonal distribution of precipitation in the Philippines. (●) > 6 months dry season (■) 2-3 months dry season ; (▲) 4-5 months dry season ; (▮) no dry season.

view the areas in SW-Mindanao and SE-Negros are best suited. Several other regions, *i.e.* W.-Luzon, Mindoro, and Panay, are also suited for inoculation purposes, taking into account that high water temperatures (mostly over 25 °C) guarantee a short generation time (De los Santos *et al.*, 1980). The eastern coasts of the Philippines, which lack a distinct dry season, should not be taken into consideration for *Artemia* inoculation.

The situation in Peru is totally different (see Fig. 5). The coastal area is characterized by an arid climate ; as a consequence this zone seems to be suited for *Artemia* transplantation. In the interior, on the contrary, a subhumid or humid climate prevails and transplantation or even inoculation is unlikely to be successful. In the few interior sites where a dry season occurs, *Artemia* production possibilities are very limited since mean monthly temperatures maximally reach 15 °C, a temperature at which the generation time of *Artemia* is more than 2 months (Lenz, 1980). Next to water balance, temperature is indeed an important parameter to be taken into account for site selection for *Artemia* production. In this regard the Peruvian coast north of Lima (20-30 °C year-round) is more interesting than the zone south of Lima (20-30 °C for 6 to 9 months a year).

Speciation and reproductive mode of *Artemia* in relation to its biogeography

Several authors have searched for a correlation between the different "types" or sibling species of *Artemia* and their geographical distribution pattern. Stella (1933) failed to find a relation between the *Artemia* "biotypes" and the geographical location of their biotopes. Barigozzi (1946, 1957) could not correlate the different "forms" of *Artemia* to specific geographical areas. Bowen *et al.* (1978) reported that "... a search for geographical patterns and latitudinal lines of alleles also yielded negative results". The latter authors could not find a relation between the electromorphs of the different *Artemia* populations and the salinity (variations often larger within a biotope than between biotopes ; Vanhaecke, 1983) or ionic composition of their biotopes.

More recent studies reveal a certain pattern in the distribution of different *Artemia* sibling species. As pointed out by Browne and MacDonald (1982) it is striking that reproduction is exclusively sexual in the Americas. With the exception of the geographically isolated *Artemia persimilis* sibling species in Argentina, all *Artemia* in the New World belong to the *Artemia franciscana* sibling species. On the other hand, all Asian brine shrimp are parthenogenetic (*Artemia parthenogenetica*) with the exception of one *Artemia urmiana* population in Iran. In Europe, Africa, and Australia apparently both parthenogenetic and bisexual forms occur. However, the few bisexual populations in Europe and Africa (only limited data available) belong to the *Artemia tunisiana* sibling species.

Based on genetic studies, Abreu-Grobois and Beardmore (1980, 1982) postulated that the ancestral bisexual form originated in the Mediterranean and the Middle East. Furthermore, on the basis of genetic distances they concluded that the following evolutionary steps may have occurred : first separation between the bisexual form of the Old and the New World, after which the parthenogenetic form arose from the European bisexual form, finally *Artemia persimilis* arose from *Artemia franciscana*. The distribution of *Artemia franciscana* in the New World may largely be explained through dispersion by birds. *Artemia* biotopes are known to be feeding grounds for many birds, in particular flamingos (Rooth, 1976). Furthermore important migration routes in the Americas, *i.e.* the Pacific route from the Middle West of the USA along the Mexican coast,

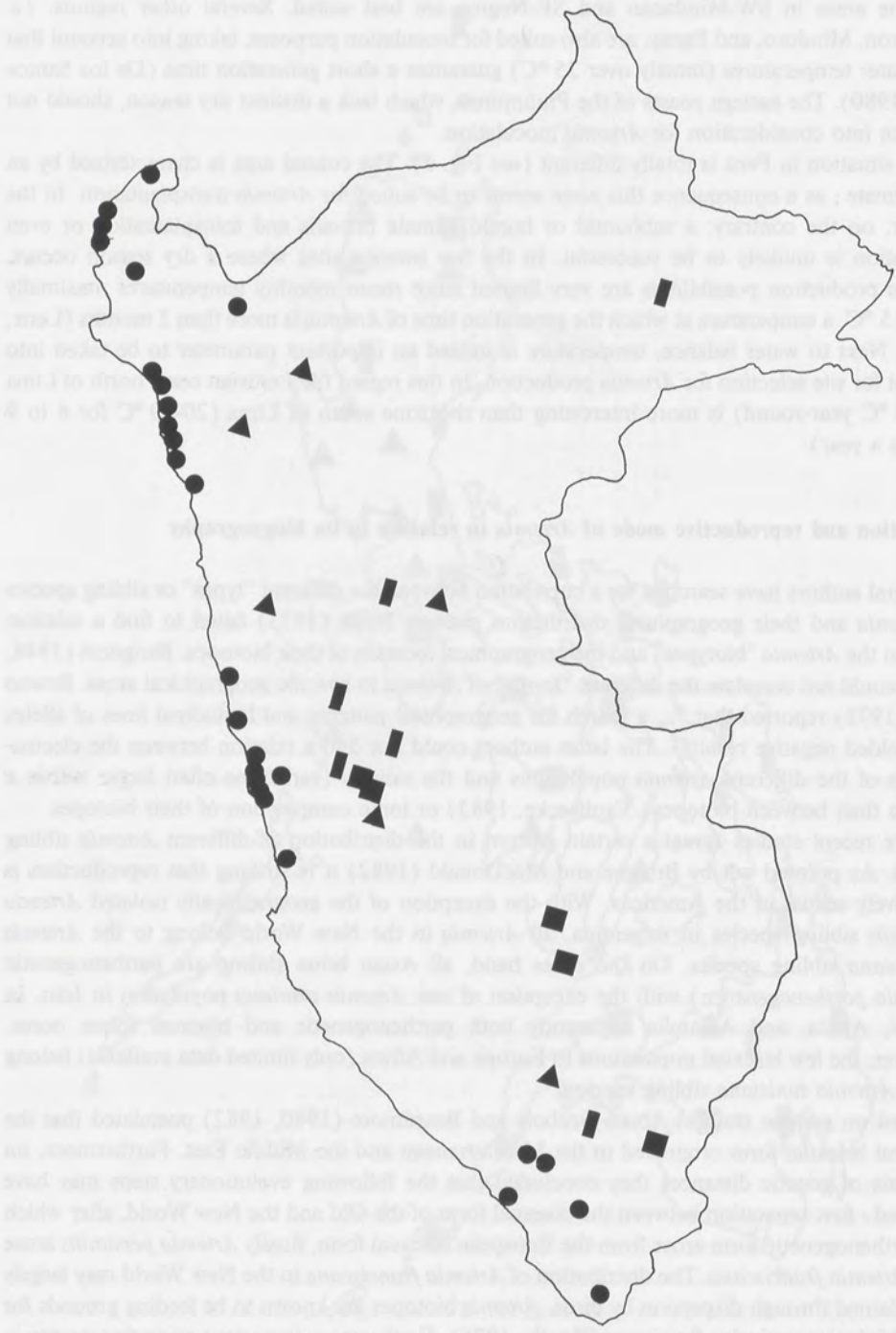

FIG. 5. Seasonal distribution of precipitation in Peru. (●) no water surplus ; (■) 3 months dry season ; (▲) 5-6 months dry season ; (▮) no dry season.

as well as the route form the West Indies through Venezuela and Colombia (Dorst, 1962 ; Rooth, 1976) cover many existing *Artemia* biotopes. Taking into account that cysts remain viable for 1 to 3 days upon ingestion by birds (Löffler, 1964 ; Proctor *et al.*, 1967 ; MacDonald, 1980) and that migrating birds can reach a speed of 50-100 km/h (Dorst, 1962), it is obvious that they can play an important role in dispersing *Artemia* over large distances. Birds are also thought to be responsible for the north-south transfer of *Artemia* in Europa and Africa and for the *Artemia* distribution in India (Royan *et al.*, 1970 ; Achari, 1971). In Australia, both *Artemia parthenogenetica* and *Artemia franciscana* were introduced by man (Clark and Bowen, 1976 ; Geddes 1980ab ; Jones *et al.*, 1981).

Contrary to the hypothesis of Browne and MacDonald (1982) it appears from our data that there is no correlation between the mode of reproduction and the appearance of *Artemia* in either inland salt lakes or coastal salt operations. Not only *Artemia franciscana* but also European and African bisexual brine shrimp are found in both types of biotopes. Furthermore neither climate nor latitude seem to have a major influence on the distribution pattern of the different sibling species. This can be explained by the fact that among populations from the same sibling species substantial genetic differentiation occurs (Abreu-Grobois and Beardmore 1980, 1982). This is expressed in terms of different tolerance towards abiotic conditions such as temperature and salinity (Vanhaecke *et al.*, 1984). In the case of *Artemia parthenogenetica* polyploidy may account for higher heterozygosity and genetic variability (Abreu Grobois and Beardmore, 1980, 1982). It is interesting to note that in Europe no bisexual populations are found north of 39° latitude (see also Browne and MacDonald, 1982). This is rather surprising since European *Artemia* populations studied so far seem to be the least tolerant to high temperatures (Vanhaecke *et al.*, 1984).

Finally, it should be stressed that uncontrolled introduction of *Artemia* in different biotopes may not only disturb the geographic distribution pattern but also lead to a decrease of natural variability. In this regard transplantation trials need to be planned very carefully and whenever possible with locally available *Artemia*.

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International Study on *Artemia*[1] XLV. Geographical and developmental changes in isozymes of *Artemia* as separated by isoelectrofocusing

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Abstract

The isozyme patterns of *Artemia* nauplii of different geographical origins were analyzed using isoelectrofocusing on polyacrylamide gels. Results indicate isozyme polymorphisms can differentiate bisexual from parthenogenetic populations. Differences in the isozyme patterns among the bisexual and parthenogenetic populations are reported. *Artemia* nauplii of different developmental stages were found to have changing isozyme patterns which indicate the importance of developmental stage in the identification of sibling species. The advantage of isoelectrofocusing as a method for the identification of different *Artemia* populations is discussed.

Introduction

Artemia's widespread distribution is due to natural habitation and to accidental or deliberate transfer by man and birds. The success of inoculating *Artemia* in different locations around the world is due to the ability of the organism to tolerate a wide range of environmental parameters such as salinity, pH, and dissolved oxygen.

Identification of groups within the genus *Artemia* involved morphological descriptions (Amat Domenech, 1980), actual laboratory mating studies (Halfer-Cervini *et al.*, 1968, Clark and Bowen, 1976), and biochemical variation studies (Bowen and Sterling, 1978 ; Bowen *et al.*, 1980 ; Seidel *et al.*, 1980 ; Abreu-Grobois and Beardmore, 1982 ; Seidel and Simpson, 1984). Some of these studies showed that sexual isolation exists among groups and matings between sexually isolated groups may be totally fruitless.

Studies on the biochemical aspect of morphogenesis of *Artemia* showed that changes occur in several biochemical parameters as a function of ontogeny and environmental factors (Finamore and Clegg, 1969 ; Heip *et al.*, 1977 ; Bagshaw, 1980 ; Clegg and Conte, 1980). The levels of glucose, trehalose, glycerol, and glycogen change during development (Boulton and Huggins, 1977). Lactate dehydrogenase activity during the emergence and subsequent transformations of *Artemia* embryos into nauplii also change with levels of anaerobiosis (Ewing and Clegg, 1969).

Seidel and Simpson (1984) found unique protein patterns for each of the seven *Artemia* populations when separated by isoelectrofocusing in the pH range of 4-6.5. They also found that

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

generally, the parthenogenetic populations have protein patterns with fewer major bands than the bisexuals. Variations in the protein patterns were also found among bisexuals. Differences in the protein patterns were found when they compared three developmental stages, *i.e.* nauplii, 7 days post hatch and 14 days post hatch.

The present study evaluates isoelectrofocusing (IEF) on thin layer polyacrylamide gels as a method for easy and rapid identification of different *Artemia* populations. Two isozyme systems namely, phosphoglucose isomerase (PGI) and esterase (EST) were used in differentiating the populations. This study also examined the changes in the isozyme patterns as *Artemia* undergoes naupliar development during the 72 h period after cyst hydration.

Materials and methods

Artemia populations collected from different locations in the world (Table I) were examined for esterase and phosphoglucose isomerase isozyme activity using IEF on polyacrylamide gels.

TABLE I

Description of *Artemia* populations used

Geographical origin	Year of harvest	Symbol	Current classification (after Abreu-Grobois and Beardmore, 1982)
Geat Salt Lake South Arm, USA	1977	GSL 77	*A. franciscana*
Geat Salt Lake North Arm, USA	1983	GSL 85	–
San Francisco Bay USA	1980	SFB	*A. franciscana*
San Pablo Bay USA	1978	SPB	*A. franciscana*
Chaplin Lake Canada	–	CHAP LK	*A. franciscana*
Macau, Brazil	1981	MACAU	*A. franciscana*
Cholburi Province Thailand	1983	THAI A	–
Chachoengsao Province Thailand	1983	THAI B	–
Virgin Islands, USA	1984	VI	–
Salinas, Ecuador	1985	ECUAD	–
Reference *Artemia* Cysts	1982	RAC II	–
Bohai Bay, China	–	CHI-BO	–
Tientsin, China	–	CHI-TSN	*A. parthenogenetica*
Lavalduc, France	1979	LAV	*A. parthenogenetica*
Comacchio, Italy	–	COM	*A. parthenogenetica*
Margherita di Savoia, Italy	–	MAR di SAV	*A. parthenogenetica*
Spain (unknown sample)	–	SPAIN A	–

HATCHING PROCEDURE

Of each population, 20 g of cysts were hatched in 2 l separatory funnels containing 1.8 l filtered seawater which was vigorously aerated. Approximately 1/3 of the nauplii were harvested from the hatching container at 24, 48, and 72 h after hydration. Table II shows the size range of the three developmental stages with corresponding naupliar stages as classified according to Olson (1979). The harvested nauplii were kept frozen until analyses.

TABLE II

Size ranges and stages of nauplii used

Age (h)	Size range (mm)	Stage of nauplii (after Olson, 1979)
24	0.39-0.45	I
48	0.65-0.79	III
72	0.85-1.00	IV

SAMPLE PREPARATION

Samples were prepared by homogenizing 1 g of wet *Artemia* nauplii in 3 ml 1 % cold glycine solution (pH 7.0) using a Polytron Ultrasonic homogenizer (Brickman Instruments, Westbury, NY, USA). Each homogenate was centrifuged for 10 min and the supernatant was filtered through Hyflo-Super Cel. Samples were analyzed for protein concentration using a Sigma Protein Determination Kit (Sigma Chemicals Co., St. Louis, MO, USA).

IEF PROCEDURE

IEF was performed at 4 °C with 25 Watts constant power. Approximately 40 µg protein (about 10 µl) of each sample was applied to the gel. IEF was performed until the proteins attained equilibrium and the isozyme bands were properly focused. This took approximately 2 h. After IEF, the pH gradients of the gel were determined using a surface pH electrode (Ingold electrode, Andover, MA, USA).

STAINING PROCEDURE

After IEF, the gels were stained in the following staining solutions (the chemicals were obtained from Sigma Chemicals Co.):

- EST staining solution (Bonte and Bode, 1981): α-naphthyl acetate, 37.5 mg; Fast Blue R, 125 mg; phosphate buffer, pH 7.4, 0.1 M, 125 ml
- PGI staining solution (Harris and Hopkinson, 1976): fructose-6-phosphate, 80 mg; NADP, 16 mg; $MgCl_2.6H_2O$, 80 mg, glucose-6-phosphate dehydrogenase, 0.3 mg; phenazine methosulfate (PMS), 5 mg; 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide thiazol blue (MTT), 28 mg; Tris-HCl buffer, pH 8, 0.1 M, 120 ml.

Quantitative densitometric scans of stained gels were made using an Ultro Scan Laser densitometer (LKB Instruments, Inc.) and recorded by a reporting integrator (Hewlett Packard, model 3390 A, Avondale, PA, USA). Isozyme bands were also examined visually and photographed for proper documentation.

Results and discussions

Isoelectrofocusing of the EST and PGI isozymes of pooled *Artemia* nauplii resulted in well-defined patterns for both systems. pH gradient in the gel of 3-10 and 4-6.5 range had linear distribution (Fig. 1) allowing distinction of bands with pI difference of up to 0.05 pH units.

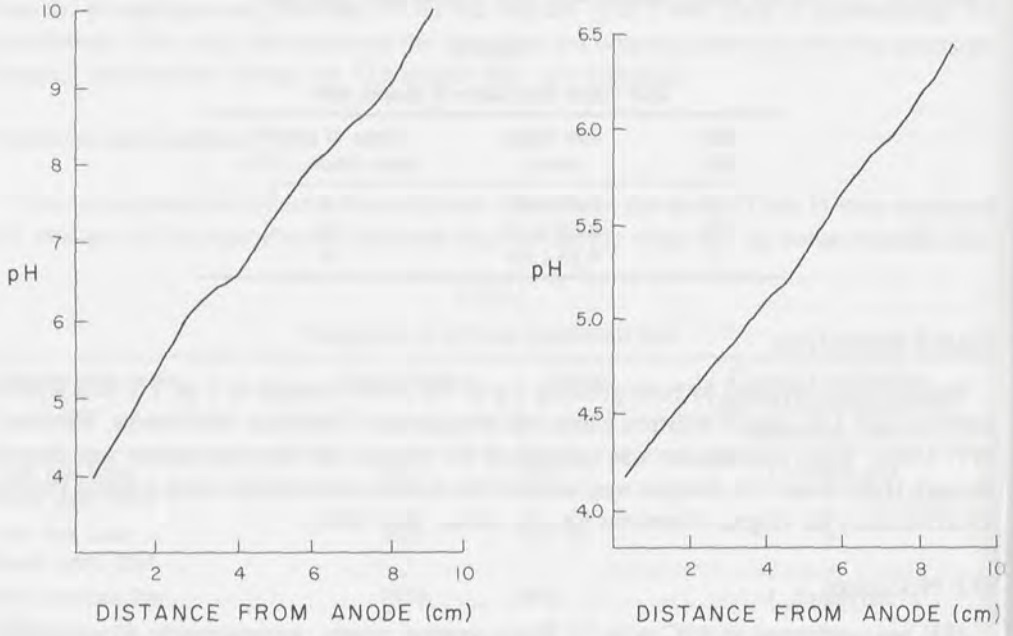

FIG. 1. Resulting pH gradients for ampholytes with pH range of 3-10 and 4-6.5.

GEOGRAPHICAL STUDY

Phosphoglucose isomerase

Fig. 2 is a diagram of the PGI isozyme patterns of all *Artemia* populations studied with the corresponding pI of the major bands. The PGI isozyme bands of the bisexuals have different pI's from that of the parthenogenetics. Differences among the bisexuals can also be detected. Virgin Islands and Ecuador samples had distinctly different patterns from the other bisexuals.

Esterase

The EST isozymes separated in the gel with pH gradients in the range of 3-10 resulted in a very densely stained band which was further partitioned when a gel with a narrow range of 4-6.5 pH gradient was used. The EST bands of the bisexuals have different pI's from that of the parthenogenetics.

The bisexual populations examined had basically the same major bands ; the minor bands, however, showed some variations (Fig. 3). The parthenogenetic populations examined can be differentiated further when the EST band of pI 5.03 was considered. That of Bohai Bay and

Tientsin was composed of one heavily stained band while that of Lavaduc, Comacchio, and Margherita di Savoia had two bands.

Isozymes are multiple forms of enzymes catalyzing the same reaction in the same cells or organism, and encoded by multiple genetic loci. Therefore, isozyme polymorphisms have been extensively used in the study of the genetic structure of species.

Bowen and Sterling (1978) divided *Artemia* populations into four groups based on the EST and malate dehydrogenase isozyme patterns of adults as separated by starch gel electrophoresis. The present study, using IEF to separate the EST and PGI isozymes of pooled *Artemia* nauplii, differentiated parthenogenetic groups from the bisexuals. Further examination of the isozymes revealed some differences among the bisexual and parthenogenetic populations.

The use of isozymes as separated by IEF may be important in the easy and rapid identification of the sibling species of *Artemia*. Sibling species are sexually isolated from each other but identical or very similar in outward appearance (Bowen, 1962 ; Barigozzi, 1974, Bowen *et al.*, 1980). These findings may have important implications on aquaculture since the inoculation of foreign *Artemia* into salt ponds is becoming popular.

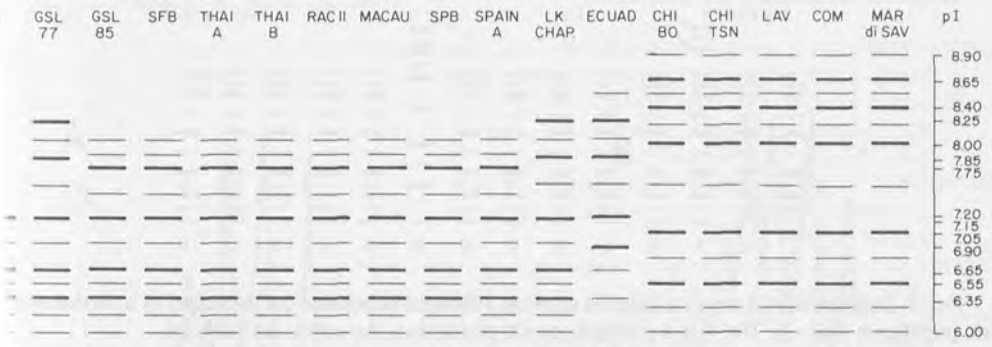

FIG. 2. Diagram of PGI isozyme patterns of *Artemia* from different geographical locations (legend to abbreviations in Table I).

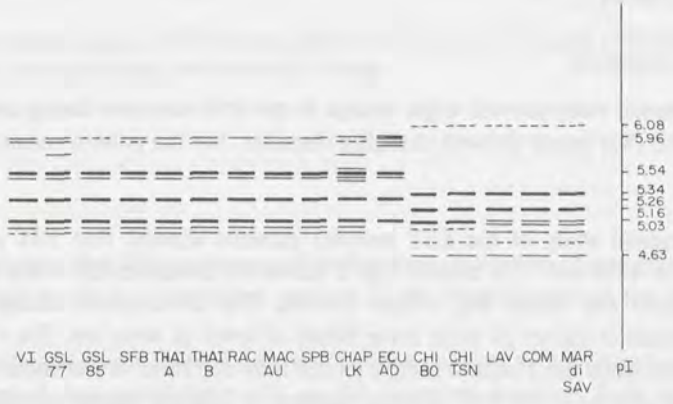

FIG. 3. Diagram of the esterase isozyme patterns of 48 h *Artemia* nauplii from different geographical locations (legend to abbreviations in Table I).

The results of this study also point out that isozyme polymorphisms as separated by IEF can differentiate *Artemia* populations more efficiently than the general protein polymorphisms as was previously reported (Seidel and Simpson, 1984). Isozymes are more specific and less complicated. The resolution of the bands can be improved further by varying the range of the pH gradients of the gel by using different ampholytes. Checks and counterchecks of the classification can be done by using several isozyme systems.

Fig. 4 shows the PGI isozyme pattern of *Artemia* samples from the ponds of the Southeast Asian Fisheries Development Center in Leganes, Iloilo, Philippines. The population in pond no. 3 was dominantly bisexual but a contamination by a parthenogenetic is revealed in the PGI isozyme pattern.

Such finding can be important so that aquaculturists will have a check on the maintenance of a pure stock of *Artemia* in their ponds. Furthermore, this method, being able to detect even slight contamination, will be of great use to aquaculturists and food scientists in their study of *Artemia* quality as feed for different aquaculture species.

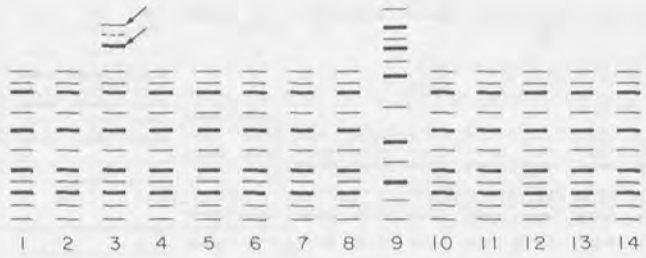

FIG. 4. Diagram of PGI isozyme patterns showing a bisexual population contaminated by a parthenogenetic population (No. 3). No. 9 is a parthenogenetic population, the others are bisexual.

DEVELOPMENTAL STUDY

Phosphoglucose isomerase

The developmental study showed slight change in the PGI isozymes during the 72 h naupliar development. The PGI bands showed changing densities, but the pattern remained the same.

Esterase

The developmental study of the EST isozyme patterns showed that EST activity became prominent only in 48 h and 72 h nauplii. Fig. 5 shows the densitometric scans of 48 and 72 h nauplii of Thailand and Bohai Bay, China *Artemia*. The development change involved the appearance of bands of higher pI while some bands of lower pI were lost. The Chinese sample gained three bands while the Thailand sample gained only one band. It was apparent that the rate of developmental change in the EST isozyme patterns is different among populations.

Olson (1979) showed that at 25 °C, the *Artemia* naupliar development from stage I to IV occurs within 4 days after hatching. Such developmental processes would mean changing

metabolic needs of the nauplii as indicated in this study by their EST isozyme system. Changes in the isozyme patterns may be also indicative of the switching on and off of genes (Masters and Holmes, 1975).

The difference in the rates of development among populations, if investigated further, can give some information on the role of the environment in the developmental genetics of this organism.

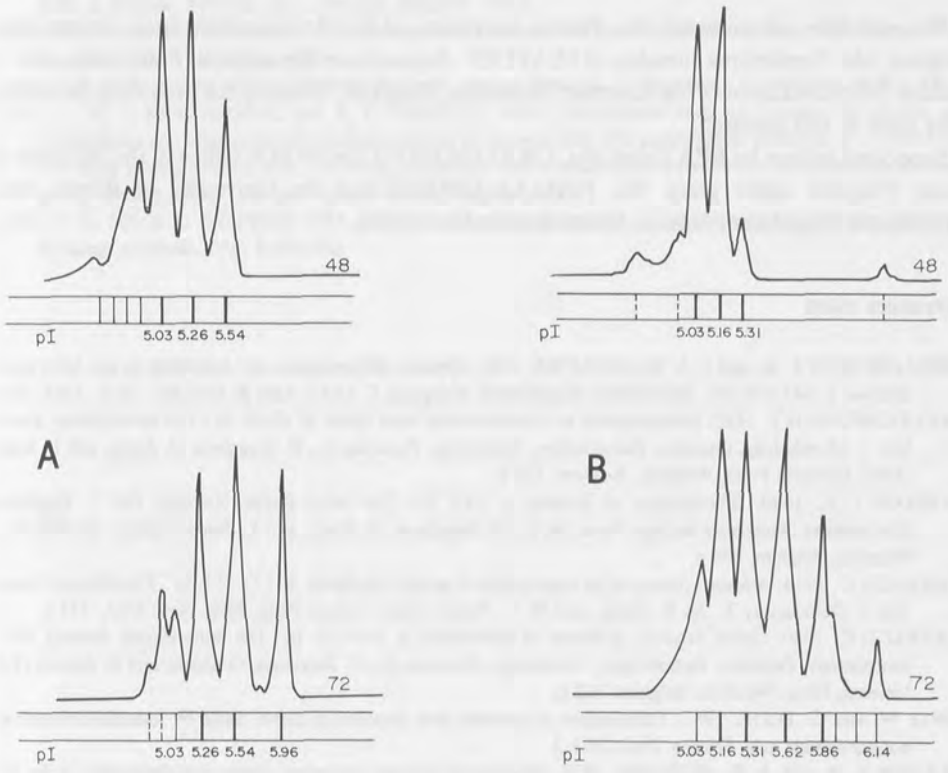

FIG. 5. Densitometric scans of EST isozyme patterns of 48 h and 72 h nauplii of Thailand (A) and Bohai ay, China (B) *Artemia* showing developmental change.

nclusions

This study showed that IEF can be a good tool in the examination of isozymic variation among temia populations. It is a promising method in the identification of the sibling species of temia.

The developmental stages showed different isozyme patterns which may be due to changes in ir physiological and metabolic needs as the organism develops from nauplius to adult. erefore, it is necessary when comparing several *Artemia* populations, to always use nauplii of same developmental stage.

This study also indicates that the method is sensitive enough to detect even slight contamination in the bisexual population by a parthenogenetic. Aquaculturists, therefore, have a method for checking the existing *Artemia* populations in their ponds.

Acknowledgements

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Carbon, nitrogen, oxygen, and hydrogen-stable isotope ratios in *Artemia* from different geographical origin

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Abstract

Stable isotope ratios $^{13}C/^{12}C$, $^{15}N/^{14}N$, $^{18}O/^{16}O$, and D/H have been determined in *Artemia* biomass and its chitin-derived substrates of various wild and laboratory-cultured strains of *Artemia monica* and *A. franciscana.*

The comparison of the isotopic data from a given *Artemia* population of unknown origin with the isotopic fingerprint of well-characterized *Artemia* populations from known origin, allows identification of the environment of origin and might make strain identification possible. The confidence of identification can be increased by utilizing four independent isotopic parameters of chitin-derived compounds rather than using only two parameters of the entire biomass as such.

To ensure positive identification with this new technique, further isotopic measurements are to be performed to evaluate seasonal and other factors contributing to the isotopic variability in brine shrimp from various environments.

Introduction

Stable isotopes of carbon, nitrogen, and hydrogen in different types of animals have been used in many ecological, environmental, physiological, and climatic studies (see reviews by Fritz and Fontes, 1980 ; Fry and Sherr, 1984). The most extensive compilation of stable isotopic data from 75 arthropod species, including *Artemia*, collected in 59 localities and grown in the laboratory, mainly utilized the poly-amino-sugar chitin from arthropod exoskeletons. This amino-sugar is well-defined chemically and allows for higher accuracy in interspecies comparisons than with ill-defined whole biomass of selected tissues (Schimmelmann, 1985). Briefly, it was shown that the $^{13}C/^{12}C$ ratios in marine arthropod chitins are different from those of terrestrial chitins, whereas chitin levels from brackishwater environments are intermediate. The $^{18}O/^{16}O$ ratios largely relate to the ratios of the waters available to primary biomass producers, which, in case of terrestrial meteoric waters, can be correlated with the climate. The D/H ratios of carbon-bound hydrogen reflect dietary influences that, for a given genus like *Artemia*, can also be climatically interpreted in the light of the D/H ratios of meteoric and ambient waters available to the local

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biota. Finally, $^{15}N/^{14}N$ ratios are relatively species-independent and therefore characteristics for a given environment.

Natural populations of *Artemia* inhabit aquatic environments which due to high rates of evaporation and/or closed-basin character, display extreme salinities, chemical compositions of dissolved salts, pH, etc., that are likely to cause large variabilities of the environmentally and climatically sensitive stable isotope ratios in *Artemia*.

The stable isotope ratios of laboratory cultured brine shrimp populations are largely influenced by the respective isotopic compositions of their diets and ambient waters (DeNiro and Epstein, 1978, 1981 ; Schimmelmann 1985).

The objective of this study was to evaluate the potential of stable isotope ratios in chitin-derived compounds and whole biomass from *Artemia* to serve as an analytical tool to identify brine shrimp from certain saline ecosystems.

Materials and methods

Two natural *Artemia* populations of different species were examined. *Artemia monica* was collected at Mono Lake, USA in June 1983, and *Artemia franciscana* from the San Francisco Bay area was supplied as commercial product by the San Francisco Bay Brand Co. (purchased in 1983).

Furthermore, cultures of brine shrimp (*A. franciscana*) from San Francisco Bay (one culture) and Great Salt Lake (three cultures) were raised in a closed flow-through culture system as described in Lavens *et al.* (1985). Temperature and salinity were kept at 25 ± 1 °C and 35 ± 1 ‰ respectively. The population density was 10 individuals/l. With algal growth being excluded, the composition of the diet (50 % corn bran, 50 % rice bran ; overall $\delta^{13}C = -19.6$ ‰, $\delta^{15}N = +4.8$ ‰ ; δ values are defined below) was kept constant for all populations raised.

We used the chromatographically purified D-glucosamine ("deacetylated chitin-monomer") in its hydrochloride form (GlcN · HCl) and dehydrated chitose instead of macromolecular chitin isolates, which still may be contaminated by covalently bounded proteinaceous compounds or may show compositional and isotopic heterogeneities due to deacetylation and adsorption effects (Muzzarelli, 1977 ; Schimmelmann, 1985). The methods for processing whole biomass, chitin, and chitin-derived compounds for stable isotope analyses have been described in detail elsewhere (Northfelt *et al.*, 1981 ; Schimmelmann, 1985 ; Schimmelmann and DeNiro, 1986). The results are expressed in δ-notation, *i.e.* in per mil enrichment [δ value > zero] or depletion [δ value < zero] of the heavy isotope in the sample relative to a standard :

$$\delta\ {}^{x}A = \frac{({}^{x}A/{}^{z}A)\ \text{sample} - ({}^{x}A/{}^{z}A)\ \text{standard}}{({}^{x}A/{}^{z}A)\ \text{standard}} \cdot 1\,000\ ‰\ ;$$

and ${}^{x}A = {}^{18}O$, ${}^{15}N$, ${}^{13}C$, or D, and ${}^{z}A = {}^{16}O$, ${}^{14}N$, ${}^{12}C$, or H.

The standards are "Vienna Standard Mean Ocean Water" (V-SMOW) for $\delta\ {}^{18}O$ values and δD values, the Peedee Belemnite (PDB) carbonate for $\delta\ {}^{13}C$ values and atmospheric nitrogen (AIR) for $\delta\ {}^{15}C$ values. The precisions of the measurements, given as standard deviations of the means calculated for 20 replicate analyses, are ± 0.2 ‰ for $\delta\ {}^{18}O$ values, ± 0.1 ‰ for $\delta\ {}^{15}N$ and $\delta\ {}^{13}C$ values, and ± 3 ‰ for δD values.

Results and discussion

The use of GlcN · HCl (for $\delta\ ^{13}C$, $\delta\ ^{15}N$, and $\delta\ ^{18}O$ values) and of dehydrated chitose (for δ D values) from chitin permits the determination of four independent stable isotope ratios. Whole biomass allows only for the determination of $\delta\ ^{13}C$ and $\delta\ ^{15}N$ values, because the presence of sulfur in complex organic matter interferes with the determination of the $\delta\ ^{18}O$ value, and the exchangeability of non-carbon-bound hydrogen (*e.g.* OH-and NH_2- hydrogen) with ambient water hydrogen during processing of the sample renders D/H ratios in whole biomass and in macromolecular chitin isolates unreliable.

Fig. 1 and 2 show *Artemia* chitin-derived δ values. Dashed lines compare ranges of δ values of natural fully marine arthropods with δ values of not fully marine chitins. Data from marine arthropods cluster within relatively small ranges reflecting the largely thermally and isotopically homogeneous environmental and climatic conditions in the marine environment. In contrast, *Artemia* data, and those from non-marine arthropods in general (Schimmelmann, 1985), fall within larger ranges due to the extreme variance of their habitats.

Taking the precisions of the measurements into account, we observed no overlapping between the data among natural *Artemia* populations and those among *Artemia franciscana* grown in the laboratory. The two strains of *A. franciscana* that were raised under controlled conditions showed only minor isotopic variances that can be related to small variances in overall dietary and water isotopic compositions during growth, but do not give sufficient evidence for biochemical chitin-metabolic differences among strains of *Artemia.*

We noted that the four independent isotopic parameters measured for a given chitin represent a four-dimensional vector characterizing a given *Artemia* population. Two-dimensional graphic display used in Fig. 1 and 2 can be completed by similar graphs $\delta\ ^{13}C$ *versus* δD, $\delta\ ^{13}C$ *versus* $\delta\ ^{18}O$, $\delta\ ^{15}N$ *versus* D, and $\delta\ ^{15}N$ *versus* $\delta\ ^{18}O$. Measurements on whole biomass, on the contrary, yield only two independent stable isotope ratios, $\delta\ ^{13}C$ and $\delta\ ^{15}N$, with only one graph possible.

Table I presents δ values of whole biomass and GlcN · HCl from cultured strains of *Artemia franciscana* that can be compared with the δ values of the diet given above. The metabolic isotope fractionation between diet and GlcN · HCl for carbon of 2.1 ± 0.3 ‰ (n = 4) and for nitrogen

TABLE I

Carbon and nitrogen stable isotopic compositions of whole biomass and of D-glucosamine hydrochloride from cultured *Artemia*

Whole biomass		D-glucosamine hydrochloride	
$\delta\ ^{13}C$ (‰)	$\delta\ ^{15}N$ (‰)	$\delta\ ^{13}C$ (‰)	$\delta\ ^{15}N$ (‰)
	Artemia franciscana San Francisco Bay strain		
- 19.2	+ 5.3	- 21.8	- 6.4
	Artemia franciscana Great Salt Lake strain, three populations measured		
- 19.2	+ 4.4	- 21.1	- 4.8
- 19.3	+ 4.8	- 21.8	- 4.5
- 19.0	+ 4.6	- 21.9	- 5.6

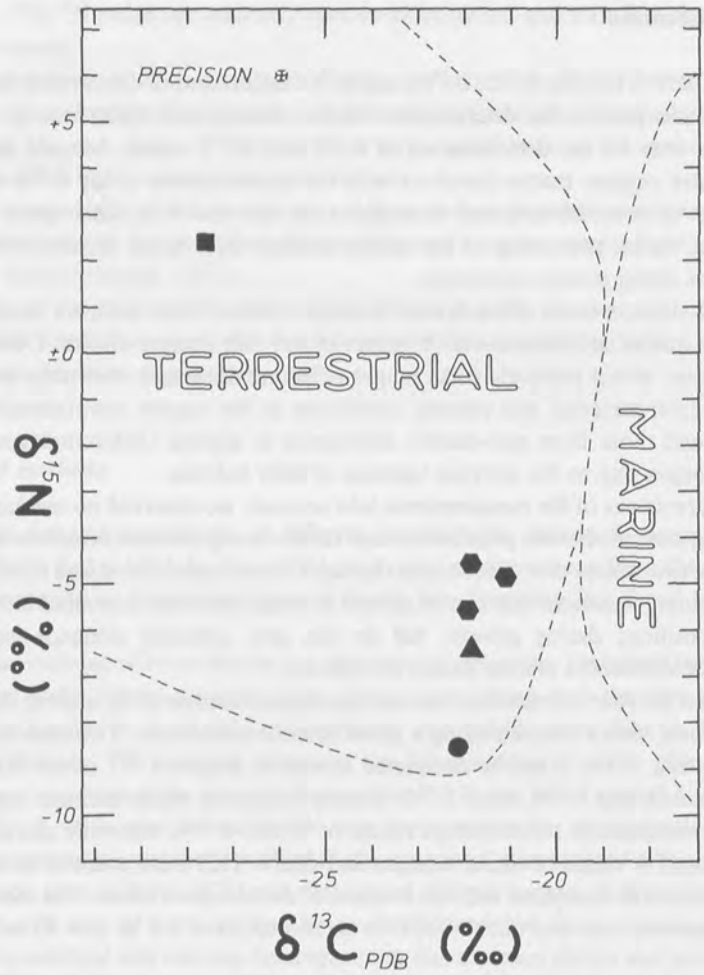

FIG. 1. Carbon and nitrogen stable isotope ratios of D-glucosamine hydrochloride from chitin. The ranges of marine and terrestrial arthropod chitins are indicated (after Schimmelmann, 1985).
(■) *Artemia monica* from Mono Lake ; (●) *Artemia franciscana* from San Francisco Bay area ; (▲) *Artemia franciscana*, San Francisco Bay strain, grown in the laboratory ; (■) *Artemia franciscana*, Great Salt Lake strain, grown in the laboratory, three populations measured.

of 10.1 ± 0.7 ‰ (n = 4), and the isotopic differences between whole biomass and diet averaging for carbon 0.4 ± 0.1 ‰ (n = 4) and for nitrogen 0.0 ± 0.3 ‰ (n = 4) are quantitatively similar to results from other arthropods (Schimmelmann and DeNiro, 1985) and emphasize systematic isotopic differences between amino-sugar (chitin, GlcN · HCl) and whole biomass (largely proteinaceous). When free from other organic debris, total biomass of *Artemia* appears to be a reliable substrate for infra-genus comparisons, but its limitations to $\delta\ ^{13}C$ and $\delta\ ^{15}N$ make it an inferior substrate when compared to chitin-derived compounds. In any case, due to the isotopic differences between the two types of organic substrates, comparisons are valid only among δ values of whole biomass, or among chitin-derived δ values.

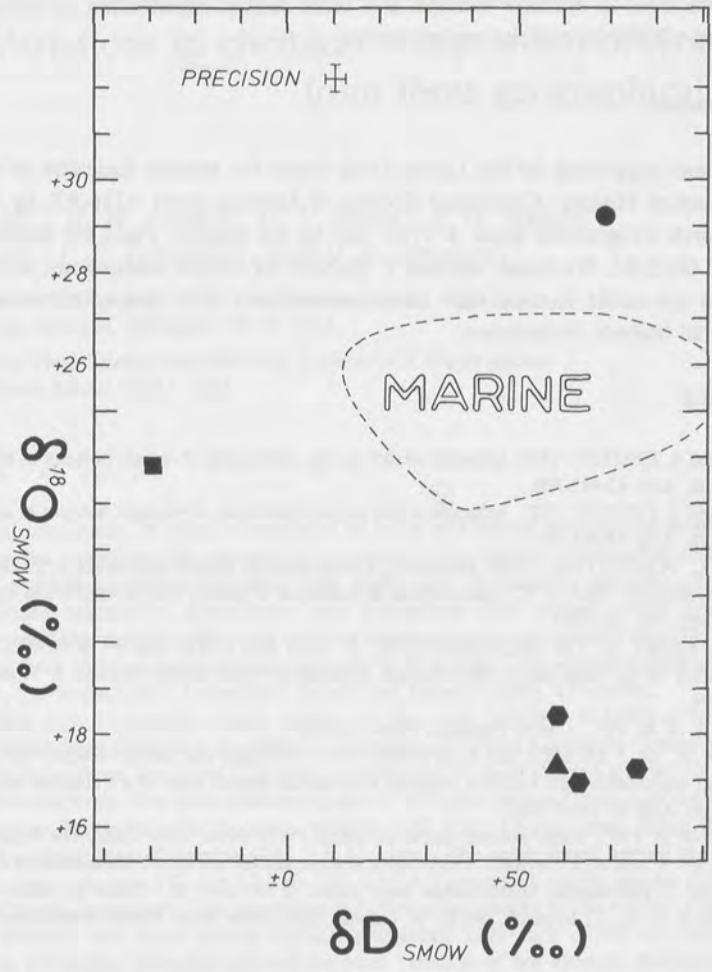

FIG. 2. Hydrogen and oxygen stable isotope ratios in D-glucosamine hydrochloride and dehydrated chitose. The range of marine arthropod chitins is indicated (after Schimmelmann, 1985). For definition of symbols, see Fig. 1.

Conclusions

Stable isotope ratios in whole biomass and in chitin-derived substrates from *Artemia* are highly sensitive environmental indicators. The comparison of isotopic data from a given *Artemia* population of unknown origin with the isotopic signatures of well-characterized *Artemia* populations from known source areas, allows analytical identification, regardless of the strain involved. The confidence of identification can be increased by utilizing four independent isotopic parameters in chitin-derived compounds rather than using only two parameters in whole biomass. Positive identification should be preceded by isotopic measurements of *Artemia* populations from

potential source areas to evaluate seasonal and other factors contributing to isotopic variability in brine shrimp within the various environments.

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Variations in chemical composition of *Artemia* cysts from three geographical locations

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Abstract

The chemical consistency of *Artemia* cysts from lot to lot and among geographical locations has become important since the nauplii are the sole source of nutrition for a variety of laboratory-maintained animals. Variations in physical variables, detectable fatty acids, and 18 essential elements (selenium, sodium, potassium, calcium, magnesium, phosphorus, iron, manganese, zinc, copper, cobalt, molybdenum, chromium, nickel, vanadium, arsenic, sulfur, and chlorine) within lots of commercially available cysts and among lots originating from different geographical locations were studied. The sources examined were from San Francisco Bay, California-USA ; Guanabara, Brazil, and Galera Zamba, Colombia.

Aliquots taken from thoroughly mixed samples of the same lot (No. 503-48) of San Francisco Bay *Artemia* cysts which were shipped in six different containers exhibited a moisture content of 5.70 ± 2.39 % ($\bar{x} \pm$ SD) while those from Brazil and Colombia (one sample for each source) yielded 10.51 ± 0.21 % and 4.32 ± 0.29 % respectively. The mean diameter (µm) of 100 cysts removed from each sample indicated that the San Francisco Bay cysts were far more variable (237.4 ± 14.6 µm) than either those from Brazil (216.2 ± 8.5) or those from Colombia (241.3 ± 11.1 µm). The range found among the six containers of San Francisco Bay cysts was 212.4 ± 12.2 to 255.8 ± 14.9 µm which might suggest different origins for the contents of the six containers. This suggestion is further substantiated by the results of elemental analysis. The greatest variation was found among triplicates analyzed from each of the six containers that were purchased from California. Selenium showed the most variation of any element investigated regardless of origin. Data on the fatty acids suggested the same degree of variability within samples from the three geographical regions. It is possible that rearing problems often reported with mysids may have originated from the absence or insufficient quantities of essential fatty acids as well as selenium. Clearly, the most expedient solution to fatty acid and selenium deficiencies is algal supplementation.

Introduction

Many investigators employ *Artemia* Leach 1819 as the only sustenance for *Mysidopsis bahia* Molenock 1969 and various species of fish larvae. As a result of this practice, the chemical consistency of brine shrimp cysts from lot to lot and among geographical locations becomes important since their nauplii are the sole source of nutrition for a variety of laboratory-maintained animals. Recently, it has been shown (Watanabe *et al.*, 1978 ; 1980 ; Fujita *et al.*, 1980 ; Schauer *et al.*, 1980 ; Seidel *et al.*, 1982) that *Artemia* may be divided into two categories : those that contain high quantities of the fatty acid, linolenic acid, 18:3ω3, an essential fatty acid for freshwater fishes, and those that have high concentrations of the fatty acid, cis-5,8,11,14,17-eico-

sapentaenoic acid, 20:5ω3, an essential fatty acid for marine organisms. It has been suggested (Millamena *et al.*, 1985) that the cause of this phenomenon is that the fatty acid composition of *Artemia* closely resembles that of their diet. *Artemia* nauplii from various geographical locations differ significantly in their ability to sustain marine crustacean larvae (Bookhout and Costlow 1970 ; Johns *et al.*, 1980, 1981 ; Seidel *et al.*, 1982 ; Lèger and Sorgeloos, 1984). Furthermore, various investigators (Wickins, 1972, Watanabe *et al.*, 1980) have shown that the nutritional quality of *Artemia* nauplii can be vastly improved by maintaining them on an algal or yeast diets high in 20:5ω3. Freshwater fishes can elongate 18:3ω3 to 20:5ω3 but marine fishes fail to have this ability and must be supplied with sufficient quantities of 20:5ω3 for reproduction and survival (Kanazawa *et al.*, 1979). Circumstantial evidence suggests that *Artemia* can metabolize 18:3ω3 to 20:5ω3 (Kayama *et al.*, 1963 ; Jezyk and Penicnak, 1966) and recently Schauer and Simpson (1985) showed conclusively that Australian *Artemia* could elongate 18:3ω3 to 20:5ω3.

Watanabe *et al.* (1978, 1980) and Fujita *et al.* (1980) have indicated that much less than 5 % of 20:5ω3 brings about a reduction in fish size and reproductive success. Presumably, the same quantity of 18:3ω3 is required by freshwater organisms (Watanabe *et al.*, 1978 ; 1980 ; Fujita *et al.*, 1980 ; Schauer *et al.*, 1980 ; Seidel *et al.*, 1982).

The purpose of the present investigation was to examine *Artemia* cysts from California-USA, Brazil, and Colombia, the three major sources available to American users, and, within each of these regions, to examine the variations in concentrations of eighteen essential elements and polyunsaturated fatty acids, chiefly the ω3s.

Materials and methods

Sources of *Artemia* cysts

Artemia cysts were purchased from San Francisco Bay Brand, Inc., in Newark, California-USA, (Lot No. 503-48), Aquarium Products in Glen Burnie, Maryland, USA (no lot number), and for comparison Reference *Artemia* Cysts II (RAC II) were donated by the US EPA Narragansett Laboratory in Rhode Island. Lot number 503-48 arrived in an assortment of six cans all with the same lot number. It was decided to treat this lot of six as though they were six different samples. There is no statement with lot number 503-48 that verifies its geographical origin. The origin of cysts purchased from Aquarium Products is Galera Zamba, Colombia, the source of cysts from RAC II is Sosal-Salmac, Guanabara, Brazil, and those from California represent unknown origins.

Sampling procedure

All samples were analyzed in triplicate. Thus a total of 24 aliquots were analyzed : 18 from California, three from each of Brazil and Colombia. Before each aliquot was taken, samples were thoroughly mixed by shaking the container 25 times top to bottom followed by 25 times from side to side.

Physical analyses

All samples were dried at 60 °C to a constant weight which was achieved in 72 h. It is of interest to note that lipid extraction from wet *Artemia* cysts is incomplete. Complete extraction

can only be achieved with dry cysts, because the amount of water present is critical. If more water is present than is known to the analyst the extraction is incomplete (Bligh and Dyer, 1959).

Cyst diameter variation was measured microscopically on 100 cysts collected randomly from each vessel. These cysts were non-decapsulated or untreated.

Cyst viability variation was estimated microscopically on three aliquots of 100 cysts each from each of the eight samples. Cysts were divided into unbroken (viable) and broken categories.

Inorganic chemistry

Twenty-four aliquots of *Artemia* cysts were analyzed for the presence of sodium, potassium, copper, magnesium, calcium, zinc, phosphorus, molydenum, iron, manganese, cobalt, nickel, vanadium, and chromium. These were acid digested with H_2SO_4 and HNO_3. The residue was taken up in 10 ml of 6N HCl and made up to volume with double distilled water. Solid samples were analyzed for selenium by digesting the material with H_3PO_4 and HNO_3 according to the method of Reamer and Veillon (1981).

Selenium and arsenic were detected by hydride generation-atomic absorption spectrometry in all samples. Chlorine was determined by neutron activation. Calcium, magnesium, sodium, and potassium were measured using flame atomic absorption spectrometry. Sulfur quantities were determined by Leco combustion and ion chromatography (Small *et al.*, 1975 ; Stevens and Turkelson, 1977 ; Rawa, 1979). All other elements were analyzed by use of inductively coupled plasma optical emission spectroscopy.

The error of determination was ±2 % in all cases. Percent recovery studies were carried out for all elements and found to be acceptable (95-102 %). Sodium and arsenic concentrations were confirmed by neutron activation analysis.

Two aliquots, one from sublot A and the other from sublot B, of the San Francisco Bay collection had solid residues remaining after acid digestion with H_3PO_4 and HNO_3. These were examined for crystalline substances by X-ray powder diffraction analysis. The elemental composition of the residues was qualitatively examined by X-ray fluorescence.

Mineralogical analysis was carried out by high resolution X-ray diffraction utilizing the Guinier-Hagg camera. Each sample was exposed to CrK_α, radiation for 8 h in vacuum. Data sets were collected on CEA reflex film.

Nondestructive chemical analysis of the solid residue in the two samples was carried out using an energy dispersive Kevex 0700 fluorescence unit with a Ge target.

Lipid extraction and analysis

For the extraction of total lipids, a modified procedure of Bligh and Dyer (1959) was employed. Cysts were homogenized in 2:1 (v/v) chloroform:methanol. The resulting mixture was filtered and the filtrate was extracted twice with 3:48:47 chloroform:methanol:water centrifuged at 2 000 rpm and the upper phase discarded each time. Concentration was carried out with purified N_2. The lipid fraction was finally dried to constant weight at 60 °C. Total lipid weight was determined gravimetrically and reported on a dry weight basis as percent.

Weighed aliquots of the dried lipid samples were dissolved in benzene. The fatty acid analysis was carried out by employing a sodium methoxide transesterification method previously described by Cowgill *et al.* (1984). To determine the identities of the methyl esters of the major components in the transesterified samples, three sample solutions and one standard solution were

studied using positive ion chemical ionization mass spectrometry with methane as the reagent gas.

Identities of the major methyl esters in the three sample solutions were established by comparing their quasi molecular (M + 1) ions and GC retention times against the quasi molecular ions and GC retention times of the corresponding methyl esters in a standard solution. Where no standards were available, identities were suggested on the basis of quasi molecular ions. It was not possible to determine the isomeric structures of the fatty acid methyl esters (FAME) using chemical ionization mass spectrometry.

The gas chromatograph used in this study was a Hewlett Packard 5890 GC equipped with a flame ionization detector and a SUPELCOWAX 10 fused silica capillary column, 15 m × 0.32 mm I.D., 0.25 μm d_f. The temperature was programmed at 80 °C for 2 min and then increased at a rate of 3 °C/min up to 225 °C. A sample size of 1 μl was used throughout the study. Samples dissolved in isooctane containing a small amount of sodium sulfate were injected at 250 °C employing a splitless injector. Fig. 1 and 2 show chromatograms obtained under these conditions of a FAME mixture and an extract of RAC II : Brazil. For illustrative purposes a smaller sample size was used.

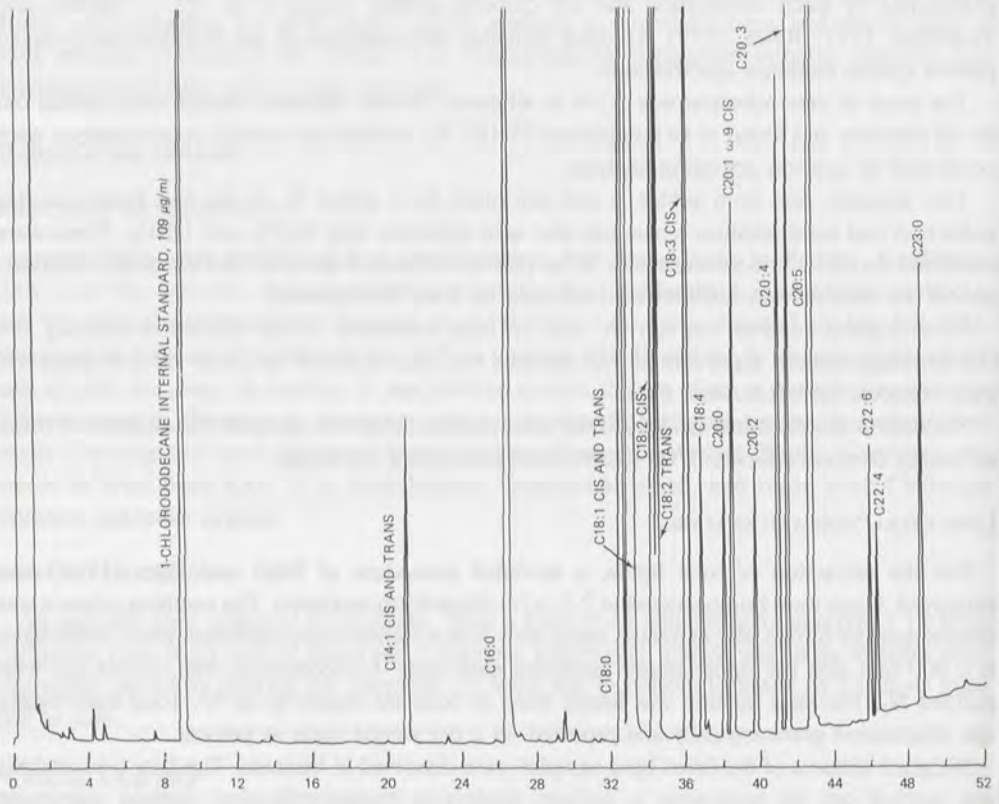

FIG. 1. Chromatogram of fatty acid methyl esters obtained from standard mixture 724-18.1 with SUPELCOWAX 10 Column.

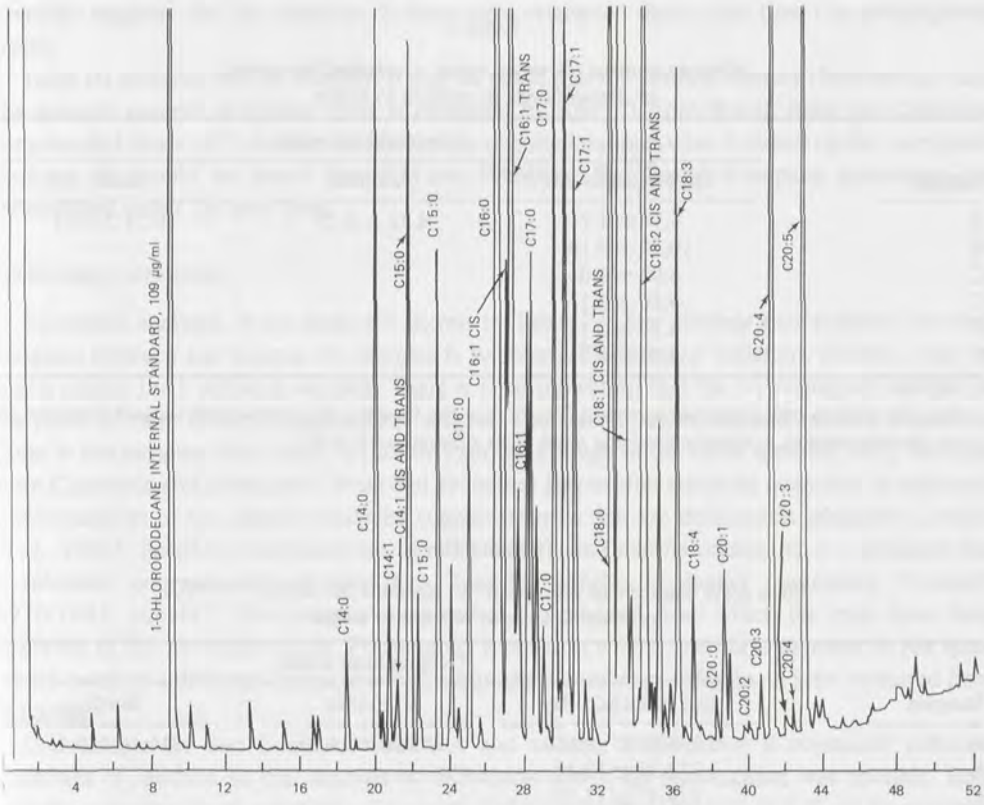

FIG. 2. Chromatogram of fatty acid methyl esters obtained from a lipid extract (RAC II : Brazil) with SUPELCOWAX 10 Column.

Results were quantified using 1-chlorododecane in benzene as an internal standard. Studies with triglycerides of fatty acids suggest the percent recovery of the transesterification procedure around 82 %. The results are presented as percent fatty acid methyl ester (FAME) of the dry weight samples. All fatty acid methyl esters reported here had their identity confirmed by mass spectrometry in the chemical ionization mode.

Results

PHYSICAL ANALYSES

Table I shows the results (mean ± standard deviation) of moisture analyses performed on six aliquots of each of the eight samples under study. The mean moisture content of all the California samples was 5.70 ± 2.39 % while those of Brazil and Colombia exhibited much less variability among the aliquots analyzed. The range for California samples was 4.22-10.70 %.

Table II shows the mean diameter (µm) of 100 cysts removed from each of eight samples. The grand mean for the California samples was 237.4 ± 14.6 µm with a range of 212.4 to 255.8. The variation exhibited by these samples is in excess of those noted for Colombia and Brazil, and

TABLE I

Moisture content (% water, mean ± standard deviation) of *Artemia* cysts in relation to origin

Samples	Geographical origin		
	San Francisco Bay	Colombia	Brazil
A	5.14 ± 1.71	4.32 ± 0.29	10.51 ± 0.21
B	10.70 ± 0.14		
C	5.08 ± 0.05		
D	4.80 ± 0.17		
E	4.22 ± 0.12		
F	4.27 ± 0.25		

Only one lot from each region was purchased. The lot from San Francisco Bay arrived in six separate containers and it was decided therefore to examine this lot in terms of six subsamples (A to F).

TABLE II

Sizes (cyst diameter in μm, mean ± standard deviation ; N = 100) of *Artemia* cysts in relation to origin

Samples	Geographical origin		
	San Francisco Bay	Colombia	Brazil
A	238.05 ± 9.62	241.26 ± 11.06	216.16 ± 8.45
B	212.38 ± 12.25		
C	233.37 ± 15.57		
D	238.24 ± 13.29		
E	255.75 ± 14.86		
F	246.86 ± 15.41		

Note as in Table I.

TABLE III

Viability of *Artemia* cysts in relation to origin (N = 300)

Samples	Geographical origin					
	San Francisco Bay		Colombia		Brazil	
	% Viable	% Broken	% Viable	% Broken	% Viable	% Broken
A	81.0 ± 4.0	19.0 ± 4.0	82.0 ± 2.6	18.0 ± 2.6	77.7 ± 2.9	22.3 ± 2.9
B	82.3 ± 3.1	17.7 ± 3.1				
C	84.3 ± 2.1	15.7 ± 2.1				
D	81.7 ± 3.2	18.3 ± 3.2				
E	84.7 ± 2.1	15.3 ± 2.1				
F	81.0 ± 4.4	19.0 ± 4.4				

Note as in Table I.

certainly suggests that the majority of these cysts originated from more than one geographical locale.

Table III presents data on viability. It may be noted that the poorest viability (hatchability) and the greatest percent of broken cysts is exhibited by RAC II from Brazil. Both the California samples and those of Colombia do not have a viability less than what is stated on the purchased package. It should be noted that the San Francisco Bay Brand Company guarantees the hatchability noted on their label.

Inorganic chemistry

Elemental analyses of the cysts are shown in Table IV. The element that exhibits the most variation between and among the samples is selenium. The greatest variation, however, may be noted among the California samples. There is clear indication that the six containers labelled as the same lot have different geographical origins. The arsenic, selenium, and calcium concentrations in the samples from Brazil are lower than the average of the other samples. Only the cysts from Colombia and container C from San Francisco Bay exhibit adequate quantities of selenium. The remainder of the samples analyzed suggest *Artemia* that are deficient in selenium (Cowgill *et al.*, 1986). Based on many analyses of many aquatic and marine organisms, it is assumed that a selenium concentration of less than 2 mg/kg signifies a dietary inadequacy (Cowgill, 1970-1981, unpubl.). This situation may reflect the position from which the cysts have been collected in the particular salina. Presumably, a position where *Artemia* is exposed to sea spray would result in a different chemical composition than *Artemia* growing in an area protected from sea spray.

One sample for San Francisco sublot A and another from sublot B contained sufficient quantities of residues so that analysis by diffraction and X-ray fluorescence was possible. Both samples contained large quantities of α-quartz (SiO_2) and albite ($NaAlSi_3O_8$). X-ray fluorescence showed that the crystalline structures were composed of silicon, potassium, aluminium, and iron. Substitution of potassium for sodium in albite is common and probably accounts for the detection of potassium in the minerals. Sublot B also contained sulfur. No sulfur-containing nor iron-containing phase was identified. A large amount of amorphous scattering was noted in both samples suggesting the presence of unidentified material.

Lipid analysis

Table V shows the percent fat extracted from the cysts on a dry weight basis. Samples from Brazil and Colombia contain comparable quantities of fat while those taken from the six containers from California exhibit unacceptable degrees of variation indicating a variety of geographical origins.

Tables VI, VII and VIII present the fatty acid composition of whole dried cysts from Brazil, Colombia, and California. Of the saturated fatty acids (Table VI) palmitic (16:0) is the most predominant. Since this appears to be the case for many strains of *Artemia* (Schauer *et al.*, 1980), presumably is inherently characteristic of the organism. Among the monoenoic unsaturated fatty acids the most prevalent are, in order of decreasing quantity, oleic (18:1ω9), elaidic (trans 18:1ω9) – many columns used on gas chromatographs fail to separate these two compounds, and finally palmitoleic (16:1). Linoleic (18:2ω6) is the most predominant among the polyenoic unsaturated fatty acids.

TABLE IV

Inorganic chemical composition of *Artemia* cysts in relation to geographical origin (mg/kg dry weight)

Source		Na	K	Cu	Mg	Ca	Zn	P	S	Se	As	V	Mo	Cr	Cl	Fe	Mn	Ni	Co
Brazil	m̄	1 446.7	7240.0	8.0	2490.0	946.3	74.0	7153.3	6090.0	0.62	9.7	6.0	6.0	9.0	3600.0	1210.0	18.7	2.7	2.0
	SD	15.3	303.5	0	60.8	42.2	7.8	87.4	50.0	0.34	0.6	1.0	0	1.0	65.6	199.2	0.6	0.6	0
Colombia	m̄	1 716.7	6493.3	13.6	2366.7	1810.0	82.7	8030.0	4860.0	1.7	15.7	4.7	5.0	7.3	4186.7	553.3	17.0	1.7	1.0
	SD	23.1	171.6	1.5	89.6	151.0	7.0	293.1	177.8	1.4	0.6	0.6	1.0	0.6	28.9	104.1	1.0	0.6	0
SFB A-	m̄	3 240.0	6893.0	7.3	2366.6	3683.0	101.3	8257.0	4783.0	1.2	18.2	7.0	6.3	9.3	6653.0	1470.0	107.3	3.7	2.0
	SD	121.0	346.0	1.2	70.2	210.8	7.6	241.0	455.0	0.38	0.3	1.0	0.6	0.6	329.6	346.0	3.1	0.6	0
B-	m̄	3 977.0	6287.0	6.0	2790.0	1860.0	65.7	7667.0	5366.7	0.48	11.0	6.3	7.0	9.7	8087.0	1323.0	280.0	5.0	2.3
	SD	582.0	346.0	1.7	226.1	132.3	1.2	773.0	1307.0	0.63	1.0	0.6	0	0.6	977.5	283.0	26.5	0	0.6
C-	m̄	4 397.0	7030.0	7.7	2586.6	2543.0	104.7	8083.0	4560.0	1.96	19.5	5.3	5.7	7.7	9170.0	1033.3	85.3	3.3	1.7
	SD	510.8	293.0	1.5	121.0	255.0	13.3	81.0	900.7	1.61	0.5	0.6	0.6	0.6	1040.0	133.0	11.5	0.6	0.6
D-	m̄	4 057.0	6893.0	7.0	2336.7	2557.0	98.7	7867.0	4483.0	0.19	19.0	5.3	6.0	7.7	8380.0	1096.0	80.3	4.7	2.0
	SD	281.5	275.0	1.0	115.9	235.0	10.5	588.0	600.0	0.17	1.0	0.6	1.0	0.6	589.5	322.0	8.5	2.9	0
E-	m̄	2 750.0	6724.0	6.3	2363.3	3220.0	92.3	7433.0	4653.0	0.43	18.5	6.0	6.3	8.3	5840.0	1160.0	92.3	3.3	1.7
	SD	278.7	709.0	1.5	240.1	360.0	5.5	605.0	728.0	0.27	0.5	1.0	1.2	1.2	547.0	34.6	9.0	0.6	0.6
F-	m̄	2 630.0	7640.0	6.7	2310.0	3283.0	140.0	7583.0	4700.0	0.90	18.7	6.0	6.0	7.7	5513.0	1226.6	98.3	10.3	2.0
	SD	79.4	1051.0	1.5	407.3	430.0	56.7	141.5	811.7	1.22	1.2	1.0	1.7	1.2	211.0	259.0	17.8	5.1	1.0

m̄ = mean, SD = standard deviation, SFB = San Francisco Bay.

TABLE V

Lipid content (% dry weight) of *Artemia* cysts in relation to geographical origin

Samples	Geographical origin		
	San Francisco Bay	Colombia	Brazil
A	9.60 ± 0.88	11.75 ± 0.09	11.10 ± 0.27
B	11.26 ± 1.11		
C	8.63 ± 0.98		
D	9.92 ± 0.59		
E	10.53 ± 0.18		
F	9.58 ± 0.99		

Note as in Table I.

TABLE VI

Quantities of saturated fatty acid methyl esters (FAME) on a dry weight basis (% of total FAME ; mean ± standard deviation, triplicate analyses) in relation to source

FAME	Brazil	Colombia	San Francisco Bay					
			A	B	C	D	E	F
14:0	1.20± 0.23	0.68± 0.06	1.37± 0.21	0.73± 0.10	1.15± 0.15	1.06± 0.06	1.16± 0.09	1.25± 0.14
15:0	0.51± 0.11	0.18± 0.03	0.50± 0.04	0.34± 0.03	0.39± 0.03	0.34± 0.03	0.40± 0.05	0.37± 0.03
16:0	8.97± 0.42	7.63± 0.70	9.00± 0.61	7.31± 1.02	6.80± 0.49	6.93± 0.70	7.99± 1.57	8.16± 2.30
17:0	1.31± 0.26	0.41± 0.08	0.69± 0.06	0.48± 0.01	0.53± 0.02	0.45± 0.03	0.57± 0.09	0.58± 0.11
18:0	1.81± 0.18	1.77± 0.36	2.39± 0.25	1.39± 0.23	1.82± 0.20	1.95± 0.36	2.12± 0.65	2.23± 0.66
20:0	ND-<0.05	ND-<0.05	ND-0.05	<0.04-<0.05	<0.04-<0.05	ND-<0.05	ND-<0.05	ND-0.07

ND = not detected at or below 0.03 %.
Note as in Table I.

TABLE VII

Quantities of unsaturated fatty acid methyl esters (FAME) on a dry weight basis (% of total FAME ; mean ± standard deviation, triplicate analyses) in relation to source

			San Francisco Bay					
FAME	Brazil	Colombia	A	B	C	D	E	F
14:1	0.14± 0.02	0.09± 0.01	0.22± 0.03	0.08± 0.006	0.19± 0.02	0.14± 0.02	0.19± 0.05	0.22± 0.04
16:1 *cis/trans*	9.07± 1.18	3.14± 0.63	12.65± 0.53	4.60± 0.44	8.62± 0.62	9.17± 0.99	11.47± 1.90	11.99± 3.37
17:1	1.85± 0.34	0.75± 0.24	1.38± 0.17	0.97± 0.08	1.22± 0.16	0.99± 0.04	1.25± 0.29	1.27± 0.20
18:1 *cis/trans*	17.53± 0.08	15.64± 0.52	20.04± 2.04	14.58± 0.36	15.05± 1.49	16.18± 0.97	18.44± 5.74	20.15± 5.14
18:2ω6	5.08± 0.43	3.17± 0.67	2.28± 0.81	3.52± 0.84	2.79± 0.39	2.50± 0.64	2.87± 0.56	3.25± 0.33
18:3ω3	0.72± 0.19	11.62± 3.45	1.76± 1.19	8.35± 3.18	4.04± 1.08	2.34± 0.87	2.57± 0.65	2.92± 0.52
18:4ω6	0.12± 0.03	1.37± 0.69	0.30± 0.33	1.26± 0.63	0.72± 0.35	0.47± 0.27	0.43± 0.02	0.61± 0.10
20:1	0.18± 0.03	0.20± 0.02	0.26± 0.02	0.21± 0.03	0.22± 0.04	0.22± 0.04	0.26± 0.07	0.31± 0.05
20:2	ND	<0.05-0.06	ND-<0.05	0.08± 0.03	ND-<0.05	ND-<0.05	ND-<0.05	ND-0.02
20:3ω6	ND-<0.05	ND-<0.05	ND-<0.05	ND-0.07	ND-<0.05	ND-0.06	0.09± 0.04	0.09± 0.02
20:3ω3	ND	0.14± 0.05	ND-<0.05	0.11± 0.4	<0.05-0.11	ND-<0.05	ND-<0.05	ND-<0.05
20:4ω6	1.87± 0.46	0.29± 0.16	0.60± 0.62	0.49± 0.24	0.77± 0.32	0.82± 0.47	1.27± 0.40	1.49± 0.15
20:5ω3	2.41± 0.70	1.20± 0.58	3.98± 4.42	0.48± 0.37	3.88± 2.27	4.21± 2.62	6.71± 2.63	7.94± 1.25
24:1	ND-0.29	ND-0.20	ND-0.57	ND-0.37	ND-0.12	ND-0.20	ND	ND-0.05

ND = not detected at or below 0.03 %.
Note as in Table I.

TABLE VIII

Quantities of some monobranched saturated fatty acid methyl esters (FAME) some monoenoic unsaturated and polyenoic unsaturated FAME, incompletely identified, on a dry weight basis (% of total FAME ; mean ± standard deviation, triplicate analyses) in relation to source Retention time (RT) is in parenthesis below FAME or in the case of unknows noted as RT

			San Francisco Bay					
FAME	Brazil	Colombia	A	B	C	D	E	F
14:0 (18.53)	0.10± 0.01	0.11± 0.02	0.12± 0.03	0.09± 0.01	0.13± 0.02	0.09± 0.02	0.11± 0.005	0.11± 0.005
15:0 (21.82)	0.71± 0.14	0.62± 0.05	0.90± 0.09	0.80± 0.09	0.97± 0.12	0.71± 0.006	0.79± 0.12	0.87± 0.12
15:0 (22.26)	0.16± 0.03	0.37± 0.05	0.44± 0.06	0.21± 0	0.41± 0.04	0.31± 0.02	0.40± 0.06	0.43± 0.07
16:0 (25.03)	0.38± 0.06	0.62± 0.12	0.35± 0.05	0.32± 0.02	0.47± 0.03	0.29± 0.02	0.33± 0.05	0.29± 0.04
16:1 (27.64)	0.16± 0.04	ND-<0.05	<0.04-0.10	ND-<0.05	ND-0.10	0.10± 0.006	0.12± 0.03	0.17± 0.07
16:1 (28.16)	0.48± 0.09	0.34± 0.06	0.49± 0.03	0.35± 0.006	0.57± 0.03	0.46± 0.10	0.48± 0.08	0.51± 0.12
16:0 (28.59)	0.26± 0.05	0.73± 0.14	0.47± 0.03	0.36± 0.01	0.52± 0.03	0.37± 0.01	0.41± 0.06	0.43± 0.07
16:0 (28.98)	ND-0.08	ND-0.06	0.31± 0.14	ND-<0.05	0.37± 0.05	ND-0.27	0.34± 0.07	0.38± 0.07
18:1 (29.68)	0.65± 0.16	ND	0.29± 0.04	<0.05-1.27	0.21± 0.03	ND-0.19	0.25± 0.06	0.26± 0.04
18:1 (30.14)	ND	ND-0.39	ND-<0.05	<0.05-0.24	ND-0.10	ND-0.07	ND-0.08	0.08± 0.02
18:0 (30.66)	ND	ND-0.10	ND-<0.05	ND-0.10	ND-<0.05	ND-0.04	ND-0.10	ND-0.10
RT 34.95	ND	ND	ND-0.10	ND	ND-<0.05	ND-0.09	ND	0.10± 0.00
RT 35.14	0.29± 0.09	<0.05-0.10	ND-0.48	0.31± 0.11	0.25± 0.05	0.23± 0.11	0.34± 0.07	0.39± 0.04
RT 35.72	ND	ND-0.05	ND-0.18	ND-<0.05	ND-<0.05	ND-0.13	ND-0.16	0.17± 0.02
20:5 (41.28)	ND	0.13± 0.07	<0.05-0.10	0.22± 0.11	0.18± 0.09	<0.05-0.15	0.09± 0.05	0.13± 0.02
22:6 (41.85)	ND	ND	ND-0.29	ND	ND-0.10	ND	ND	ND-0.05
RT 28.34	0.51± 0.12	<0.05-0.28	ND	ND-<0.23	ND	ND-0.18	ND-0.23	ND-0.25
RT 29.95	ND	ND	ND-0.28	ND-<0.05	0.13± 0.09	0.13± 0.02	0.19± 0.08	0.20± 0.03
RT 44.87	ND	ND	ND	ND	ND	ND	ND	ND-0.04

TABLE VIII. Continued.

FAME	Brazil	Colombia	San Francisco Bay A	B	C	D	E	F
RT=46.41	ND	ND-0.27	ND	ND	ND	ND-0.71	ND	ND-0.34
RT=47.34	ND	ND-0.15	ND-0.22	ND	ND	ND-0.23	ND	ND-0.26
RT=51.36	ND	ND	ND	ND	ND-0.10	ND-0.23	ND	ND
RT=52.07	ND	ND-0.41	ND-0.13	ND	ND-0.10	ND-0.42	ND	ND-0.41
Grand Total	3.70	2.92	3.66	2.62	4.21	2.70	3.80	4.52

Beyond 22:1 there are several longer chained fatty acids but the total amount in all cases is less than 0.6 %.

A comparison of our results for RAC II cysts and the results of Bengtson *et al.* (1985) for RAC II nauplii (not based on dry weight) are presented in Table IX. Both sets of data have been normalized to 100 for comparison but it should be noted that our data are based on dry cysts that have been dried to constant weight at 60 °C. Part of the discrepancy noted in Table IX is due to the lack of analyzing a substance of known constant weight and another part is due to the differences in analytical technique. The results of Bengtson *et al.* (1985) were developed using the methodology described by Schauer and Simpson (1978). Their data were not confirmed by mass spectrometry and the columns used failed to separate the various fatty acids to the extent made possible by recently developed columns. It is interesting that the data are as similar as they are given the fact that the results of Bengtson *et al.* (1985) represent nauplii and those of this paper represent cysts.

TABLE IX

Comparison of fatty acid profiles (each fatty acid methyl ester quantity expressed as % of total FAME) for RAC II nauplii (EPA results ; Bengtson *et al.*, 1985) and cysts (this paper)

FAME	EPA results	This paper
14:0	1.93	2.44 ± 0.47
15:0	0.50	1.04 ± 0.22
15:1	0.42	Not detected (<0.07)
16:0	14.81	18.24 ± 0.85
16:1	16.82	18.45 ± 2.40
16:2ω4	4.12	Not detected (<0.07)
18:0	4.30	3.68 ± 0.37
18:1	34.82	35.65 (*cis/trans*) ± 0.16
18:2ω6	11.80	10.33 (*cis/trans*) ± 0.87
18:3ω3	0.30	1.46 ± 0.39
20:0	0.23	Not detected (<0.07) - 0.10
20:4ω6	4.86	3.80 ± 0.94
20:5ω3	5.14	4.90 ± 1.42

Discussion

There are many laboratories presently testing the effects of effluents and chemicals utilizing both freshwater and marine organisms. It is customary to feed newly-hatched *Artemia* nauplii to both freshwater and marine fish and marine mysids. As mentioned earlier, one is unlikely to encounter consistently *Artemia* that will contain sufficient quantities of 18:3ω3 and 20:5ω3 to maintain organisms from both ambient media. In addition, most laboratories fail to have either the available facilities or the personnel to check every batch of purchased *Artemia* cysts for the presence of sufficient amounts of the two essential fatty acids. The following discussion provides the rationale for the alternative of algal supplementation.

Recently, Cowgill *et al.* (1984) published the variation in fatty acid composition of *Daphnia magna* Straus 1820 fed a variety of algal diets. The algae were cultured in a revised Bold's medium (Cowgill *et al.*, 1986) and a revised medium originally developed by Provasoli and Pintner (1953). *Selenastrum capricornutum* Printz cultured in either of these two media contained 18:3ω3 in their tissues (Cowgill *et al.*, 1984). *S. capricornutum* cultured in the revised Bold's medium (Cowgill *et al.*, 1986) contained 17.5 % 18:3ω3 while that reared in the organic medium of Provasoli and Pintner (1953) contained only 5.20 %. The advantage of using *S. capricornutum* cultured in revised Bold's medium is that the alga so reared contains sufficient selenium (Cowgill *et al.*, 1986) in its tissues to fulfill the needs of freshwater fish. Thus, a solution to the predicament of purchasing chemically inconsistent *Artemia* is to feed freshwater fish the nauplii and *S. capricornutum* reared on the revised Bold's medium (Cowgill *et al.*, 1986). It should be noted that most laboratories testing with freshwater and marine organisms also maintain cultures of *S. capricornutum*. This green alga can easily be cultured in the revised medium, thus solving the nutritional problems encountered in rearing freshwater fishes. It should be noted that malnourished fishes used for testing will respond to toxicants in a more sensitive manner than properly nourished fishes (Bengtson *et al.*, 1984), thus it is even more important to ensure that the nutritional state of test organisms is adequate.

The chemical inconsistency encountered among frequently purchased lots of *Artemia* cysts may be responsible for population declines so commonly noted by mysid culturists. In this instance the lack of sufficient amounts of 20:5ω3 as well as low levels of selenium may be responsible. It is of interest to note that none of the freshwater algae grown in the two media described above (Cowgill *et al.*, 1984, 1986) contained detectable quantities of 20:5ω3 but the *Daphnia* consuming these diets do and apparently are able to elongate 18:3ω3 to 20:5ω3. Watanabe *et al.* (1983) solved the problem by feeding marine copepods or enriching the food with chemical supplements high in ω3 fatty acids. It is clear that mysids cannot be maintained on *Artemia* nauplii alone so long as it is not possible to procure *Artemia* that have a certified chemical composition. Acceptable supplements may be selected marine algae or emulsified enrichment diets (review in Léger *et al.*, 1986). Since these *Artemia* might lack some essential elements, it is suggested that tin, vanadium, iodine, and selenium be added at rates previously described (Cowgill *et al.*, 1986).

Another item of interest that needs to be addressed is the matter of expressing data on some constant basis such that figures obtained can be compared on an equal basis. Different workers have analyzed *Artemia* cysts and reported the results on a wet basis (Watanabe *et al.*, 1978, 1980, 1983 ; Fujita *et al.*, 1980) and on an "as arrived" basis (Schauer and Simpson, 1978, 1985 ; Schauer *et al.*, 1980 ; Seidel *et al.*, 1982 ; Bengtson *et al.*, 1984, 1985). Sometimes cysts are

dried in an oven at an uncontrolled or unknown temperature. The present work was based on constant weight obtained at 60 °C in an oven. The amount of time needed to achieve constant weight of a 2 g sample was 72 h. This treatment may reduce the quantity of polyunsaturated fats that have carbon chains longer than 14. A more successful approach is to use a vacuum desiccator with fresh sulfuric acid placed in the bottom. Cysts spread out on petri dishes will achieve constant weight in 24 hr.

It should be noted that the data presented here for RAC II cysts is not concordant with the EPA results (Bengtson *et al.*, 1985) for nauplii. The latter presents the results of only two fatty acid analyses from one sample taken from the 250 kg of RAC II cysts. The results for RAC II presented in this paper are either higher than those published by Bengtson *et al.* (1985) or within the range of acceptable statistical error. Given this situation it is quite important to address the matter of reproducibility. Table X shows the results of three aliquots taken from lipid extracted from San Francisco Bay – container F. It may be noted that no coefficient of variation exceeds 7 %. However, an examination of Tables VI, VII, and VIII clearly shows that randomly selected aliquots extracted from tins of *Artemia* cysts often exceed this coefficient of variation.

TABLE X

Reproducibility obtained when aliquots of the same lipid sample (SFB-F) are studied in triplicate. Samples were analyzed by gas chromatography and confirmed by mass spectrometry

FAME	Percent composition (mean ± S.D.)	Coefficient of variation (%)
14:0	1.21 ± 0.06	4.98
14:1	0.20 ± 0.01	5.00
15:0	0.43 ± 0.02	4.80
16:0	5.98 ± 0.17	2.85
16:1	8.94 ± 0.31	3.52
17.0	0.55 ± 0.02	2.78
17:1	1.24 ± 0.04	3.26
18:0	1.54 ± 0.03	1.87
18:1	16.40 ± 0.09	0.55
18:2	3.00 ± 0.09	2.83
18:3	2.80 ± 0.095	3.39
20:1	0.25 ± 0.006	2.34
20:2	<0.05 ± 0	0
20:3ω6	0.083 ± 0.006	6.93
20:4ω6	1.52 ± 0.036	2.37
20:5ω3	7.71 ± 0.14	1.82

Conclusions

A study has been described that addresses the problem of the chemical inconsistency of *Artemia* cysts purchased from various areas of the world. The following conclusions were drawn:

1. The material purchased from San Francisco Bay Brand Company, lot (503-48) which arrived in six containers originated from more than one geographical locale.

2. Of the distribution of eighteen elements examined in aliquots from Brazil, Colombia, and California, selenium and manganese proved to be the most variable. Material from Brazil and four containers of the six obtained from California have insufficient selenium to maintain young fish or mysids.
3. It is clear from the fatty acid data that one source of *Artemia* will not supply the needs of both freshwater and marine organisms. Since it is not possible to purchase *Artemia* cysts that have a chemical certification of the contents and since natural variability appears to be great, it is concluded that diets using *Artemia* nauplii should be supplemented with selected freshwater or marine algae, or fatty acid enrichment diets depending upon the nutritional needs of the species being maintained.

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Biochemical evaluation of the brine shrimp *Artemia* from the Kuyalnitsky liman (USSR)

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Abstract

The Kuyalnitsky liman reservoir in the northwest part of the Black Sea is a potential water body to grow and collect *Artemia*.

The *Artemia* have been analyzed for their protein, lipid, and sterol contents. The amount of protein was determined on lipid-free dry matter, while lipids and sterols were determined following thin-layer chromatography.

The protein and lipid contents averaged respectively 9.40 % and 2.86 % of the wet weight. Of the sterols, cholesterol, methostenol and quick-acting sterols (total) averaged respectively 9.96, 0.85, and 0.77 % of the unsaponifiable matter.

Introduction

Artemia is an important and unique source of live food for growing fish fry and Decapoda in aquaculture in the USSR (Oleinikova and Pleskachevskaya, 1975 ; Chaga, 1976 ; Polikarpov, 1984). Numerous investigations carried out in this aspect give evidence for the value of using *Artemia* as fish food (Voskresensky and Khaidarov, 1967 ; Voronov, 1973, 1977), including works on *Artemia* from the Sivash as food for sturgeons (Voskresensky, 1960 ; Nechaev, 1961 ; Gunko and Pleskachevskaya, 1962 ; Gunko, 1967). In the USSR, the brine shrimp from Crimean brackish water bodies has been studied in detail (Voronov, 1977). Literature on the brine shrimp of the Kuyalnitsky liman is limited to the end of the last century.

The Kuyalnitsky liman with a marked variation in salinity is included in a series of limans which are enclosed in the northwestern Black Sea coast. According to investigations from 1878 to 1968 salinity fluctuated from 29 to 269 ‰. The lowest salinity was observed when the liman was connected with the sea. Natural cycles of changes in salinity take up to 11-15 years. Our observations from 1971-1979 refer to the period of lowest mineralization when the salinity of water in the liman fluctuated in the range of 70-105 ‰ depending on the season. The Kuyalnitsky liman is shallow and temperatures of 25-30 °C warm the water from June to September.

Materials and methods

Samples of *Artemia* were taken at a permanent sampling site in different seasons from 1971-1979 two or four times a month. The cysts and adult forms of brine shrimp from the

sampling site were studied biochemically. Lipids were extracted with a chloroform-ethanol solvent mixture by the modification of Bligh and Dyer's (1959) method. The extract was dried in a vacuum oven at room temperature. Besides total lipid content, the dry matter after lipid extraction was weighed and expressed as percentage wet weight. The lipid fractions were determined by thin-layer chromatography (Shthal, 1965) using densitometry. Chromatograms were developed with 5 % phosphoric-molybdenic acid. Sterols were determined by thin-layer chromatography (Lisboa, 1969), colorimetric (Moore and Baumann, 1952), and spectrophotometric (Yakhimovich *et al.*, 1974) methods. Chromatograms were developed with $SnCl_5$.

Results and discussion

The results of our investigations (Table I) have shown that the lipid content of *Artemia* was 1.99 % in the summer, increasing to 2.24 % wet weight in the autumn. The percentage of dry matter declined from 10.74 % to 8.16 % correspondingly, while the percentage of all lipid fractions, excluding phospholipids, increased. The amount of cholesterol esters predominated over sterols.

The typically provitamin D, 7-dehydrocholesterol, is absent in *Artemia*. Cholesterol, the main sterol in *Artemia*, methostenol and quick-acting sterols (total) have been registered in *Artemia*, 9.96, 0.85 and 0.77 % insaponifiable matter correspondingly. As cholesterol has been found in *Artemia* in the absence of 7-dehydrocholesterol, then cholesterol synthesis occurs through desmosterol which was not determined experimentally.

The lipid content in *Artemia* cysts was slightly lower than in adult forms, 1.63 % and 1.99 % correspondingly. However, in the autumn the lipid content in cysts increased up to 4.12 %. Besides an increase of lipid content in cysts toward autumn, the amount of dry matter also sharply rises up to 30.32 % wet weight.

The percentage of lipid fractions in cysts in contrast to adult forms declines from summer to autumn excluding phospholipids, the content of which increases to 30.79 % of the total amount of lipids (Table I).

The total amount of provitamins D, methostenol and cholesterol is also lowered from summer to autumn. In comparison to adult forms, the amount of provitamins in cysts is higher both in the summer and autumn. The amount of cholesterol in the autumn is markedly lower in cysts as it is gradually used up for the development of nauplii (Table I).

In spite of the high food value and widespread distribution of *Artemia* on the territory of the USSR, up to date it has not been fully used in Soviet aquaculture.

When comparing the Kuyalnitsky liman with the Crimean salt lakes (Voronov, 1977), which have been extensively studied, it can be noted that the distribution and reproduction time are similar in both water bodies. However, the number of *Artemia* in the Kuyalnitsky liman is greater than in the Crimean salt lakes. Thus, the Kuyalnitsky liman can be placed on the same scale with the most productive water bodies for collecting *Artemia* cysts used in aquaculture. The number of cysts differs in a seasonal aspect which can be explained because of the reproductive characteristics of *Artemia*. Thus, in samples obtained from May to August inclusively, only a small number of females have brood sacs filled with cysts, their number rarely exceeding 18-32. In the summer, ovoviviparity predominates. Hatching of nauplii in experimental conditions from cysts collected in nearshore strands in summer does not exceed 4 %. As a rule, the cysts contain a large mixture of empty shells which occur as a result of nauplii hatching.

TABLE I

Biochemical composition of cysts and adults of *Artemia*

	Cysts		Adults	
	Summer	Autumn	Summer	Autumn
Dry matter (in % of wet weight)	13.38	30.32	10.74	8.16
Total lipids (in % of wet weight)	1.63	4.12	1.99	2.24
Lipid fractions (in % of total lipids)				
– phospholipids	25.15	31.79	33.01	21.20
– sterols	19.55	17.80	17.80	19.48
– triglycerides	22.01	15.79	19.81	24.13
– sterol esters	29.38	28.60	19.66	35.17
Insaponifiable fraction IF (in g)	0.0176	0.0141	0.0359	–[1]
Total provitamin D :				
in % of IF	1.16	1.07	0.77	–
in % of wet weight	0.0041	0.0030	0.0056	–
Methostenol :				
in % of IF	1.17	1.45	0.85	–
in % of wet weight	0.0042	0.0026	0.0061	–
Cholesterol :				
in % of IF	10.78	3.33	9.96	–
in % of wet weight	0.0394	0.0094	0.0715	–

[1] Not determined.

In the second half of September and October, there is a marked decrease in temperature of ir and water (up to 9-11 °C) and an increase in salinity, more than 100 ‰, as a result of summer vaporations, and the quantity of undamaged cysts in strands reaches 90 %. Under experimental onditions, 40-50 % of the cysts hatch into nauplii. Toward the end of August and September he quantity of cysts increases in the water body as well as in beach strands. Individual fertility f brine shrimp is enhanced. There are about 46-79 cysts in the brood sacs.

Thus, in the Kuyalnitsky liman it is best to collect cysts towards the end of summer in the first alf of autumn. Cysts collected in this period hatch into the greatest number of nauplii.

According to the data obtained on the Kuyalnitsky liman, *Artemia* which concentrate mainly the near-shore region in the summer and autumn months attain quite high numbers, and have high biochemical content characterizing their nutritional value. This brings us to the conclusion n the significance of *Artemia* for commercial aquaculture.

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Laboratory culture and nutritional assessment of *Artemia* from Didwana Salt Lake (India)

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Abstract

Studies on laboratory culture, nutritionally important biochemical constituents, and caloric value have been made on *Artemia* of Didwana Salt Lake (India). Maximum hatching (73 ± 2 %) was recorded at a salinity of 35 ‰ and a water temperature of 28 ± 2 °C after 28 h of incubation. Best survival and growth, under laboratory conditions, were observed at a salinity of 55 ‰. Protein, lipid contents, and caloric value showed decreasing trends during development, while carbohydrate exhibited no specific trend.

Introduction

Artemia is very widely used as a live food in the culture of fish. Gross biochemical estimates and caloric values have been reported for *Artemia* of the Great Salt Lake (Paffenhofer, 1967 ; Von Hentig, 1971) and San Francisco Bay strain (Dutrieu, 1960 ; Von Hentig, 1971 ; Helfrich, 1973 ; Benijts *et al.*, 1975 ; Gallagher and Brown, 1975) from the USA and Tuticorin from India (Royan, 1980ab). Enzler *et al.* (1974), Fujita *et al.* (1980), and Schauer *et al.* (1980) studied the lipid contents and fatty acids of different strains of *Artemia*. Seidel *et al.* (1980) reported the amino acid composition of *Artemia* from a few geographical locations. Tobias *et al.* (1980) and Jines *et al.* (1980) provided data on post-instar I stages of *Artemia*. The present paper embodies the first study on the laboratory culture with *Artemia* cysts of Didwana Salt Lake (India) origin and nutritional assessment of its various developmental stages.

Materials and methods

Floating *Artemia* cysts were collected from Didwana Salt Lake (27°3'N, 74°5'E) during March-April 1984. These were cleaned, dried, and kept in airtight plastic containers. Cysts were decapsulated following the method of Sorgeloos and Kulasekarapandian (1984) and hatched under laboratory conditions (temperature 28 ± 2 °C; light intensity 1 000 lux) in conical polyethylene bags of 2 l capacity each, filled with water of 35 ‰ salinity prepared from brine of the same lake. Aeration was maintained from the bottom of the hatching bags.

Newly hatched nauplii were transferred to media of different salinities (35, 55, 75 and 95 ‰) in small plastic pools supplied with aeration. The pools were manured with a combination of pig

manure slurry and ground nut oil cake, as used by Dwivedi *et al.* (1980). Survival and growth observation were made each day.

For the assessment of the nutritional value, freshly hatched nauplii, 24 and 48 h old larvae and 15 days old adults were separately harvested from the culture, dried, powdered, and used for biochemical and caloric estimates. Assessments were also made for untreated and decapsulated eggs of *Artemia*. Total protein was measured with a Kjeltec II apparatus (Swiss made), carbohydrate by the anthrone method (Scott and Meluin, 1953) and lipids according to the Bligh and Dyer (1959). Caloric values were estimated with the Gallenkamp Ballistic Bomb Calorimeter (UK).

Results and discussion

Laboratory culture

A maximum of 73 ± 2 % hatching was obtained at a salinity of 35 ‰ and a water temperature of 28 ± 2 °C. Although hatching began 18 h after incubation, maximum hatching was attained at 28 h.

Table I provides data on the hatching time for cysts of Indian origin. In their study on hatching rate of *Artemia* cysts of 16 geographical strains, at 25 °C, Vanhaecke and Sorgeloos (1982) have found the time until appearance of the first nauplius (T_0) to vary between 13.9 and 34.0 h. The longest T_0 reported is of the Tuticorin (India) strain, *i.e.* 34.0 h which is about double that of the Didwana strain (present study). Dwivedi *et al.* (1980) reported T_0 of 12 h so far shortest one known, at a temperature of 26 °C for *Artemia* cysts collected from Bombay (India). The period for maximum hatching (T_{max}) of Didwana cysts is also much shorter (28 h) in comparison to that of the Tuticorin strain (48.3 h) (Vanhaecke and Sorgeloos, 1982). Earlier, Royan (1976) recorded the time taken for maximum hatching (50 %) of the Tuticorin strain cysts as 41 h. Salinity level remained more or less the same during all these studies, but the temperature varied somewhat.

Table I

A comparison of time until appearance of the first nauplius (T_0) and maximum hatching (T_{max}) for *Artemia* cysts of different geographical areas in India

Source of cysts	Temperature (°C)	T_o (h)	T_{max} (h)
Didwana Salt Lake (Present study)	28 ± 2	18.0	28.0
Tuticorin (Vanhaecke and Sorgeloos, 1982)	25	24.0	48.3
Bombay (Dwivedi *et al.*, 1980)	26	12.0	–

Cysts used without decapsulation, in the present study, began to hatch at 24 h and showed maximum hatching (61 %) at 31 h. At higher water temperatures (35-45 °C) no significant hatching was recorded in untreated as well as decapsulated eggs. Sorgeloos (1980) observed that

an increase of the water temperature within the range of about 33 to 40 °C causes interruption of the cyst metabolism during hatching.

In culture experiments with instar I nauplii in four sets of different salinity (a-35 ‰, b-55 ‰, c-75 ‰, d-95 ‰) maintained at 30 ± 2 °C temperature, a sharp decline in population in set a, c, and d was observed and surprisingly not a single larva survived on the 3rd day after inoculation. Salinity 55 ‰ (set b) was found to be acceptable for the propagation and growth of the brine shrimp despite a 30 % mortality. Dwivedi *et al.* (1980) found a salinity range of 45-75 ‰ most favourable.

The brine shrimp attained the adult stage in 15 days after emerging from the cyst. The population consisted of all females, being the strain parthenogenetic. The size of the adults varied between 10 and 14.5 mm. The egg sac began to develop on the 20th day and the *Artemia* attained maturity by the 34th day. Breeding commenced on the 48th day.

Biochemical and caloric evaluation

Table II provides data on the nutritional assessment in terms of major biochemical constituents and caloric value of various stages of *Artemia* of Didwana Salt Lake and Tuticorin.

Table II

Biochemical constitution of various stages of *Artemia* of Didwana Salt Lake and Tuticorin

Place of origin and stage	Individual dry wt (µg)	Protein (% dry wt)	Lipids (% dry wt)	Carbohydrate (% dry wt)	kcal/ g dry wt	Reference
Didwana Salt Lake, India						Present study
Untreated cysts		48.31	15.2	11.92	4.148	
Decapsulated cysts		50.05	19.5	14.87	5.227	
Nauplii freshly hatched	2.9	47.60	29.1	4.52	6.076	
Nauplii 24 h old	4.8	45.21	21.3	8.34	4.839	
Larvae 48 h old	6.3	43.31	11.1	7.47	3.514	
Adults	520	41.38	9.6	5.64	3.301	
Tuticorin, India						Royan, 1980ab
Egg		58.00	25.64	7.76	4.924	
Decapsulated cysts		61.23	23.12	9.13	5.146	
Nauplii freshly hatched		59.03	20.75	8.88	4.818	
Juveniles		57.96	18.15	7.21	4.456	
Adults		59.22	16.02	6.69	4.284	

The data indicate that freshly-hatched nauplii have a very high individual dry weight. The only record of instar I nauplii of *Artemia* weighing more than that of Didwana (2.9 µg) is of the Margarita di Savoia, Italy, strain (3.33 µg) (Vanhaecke and Sorgeloos, 1980). Vanhaecke and Sorgeloos (1982) found very large differences among the larval dry weights of different biogeographical strains. Comparative information on the dry weight of advanced stages of different

strains is not available. Reeve (1963), however, found adult *Artemia* to weigh 500 times more than freshly-hatched nauplii, whereas this increase was about 180 times during the present study.

Maximum protein content (50.05 % dry wt) was found in decapsulated cysts. The protein content gradually decreased after hatching and during development, reaching 41.38 % in adults. The highest lipid content (29.15 % dry wt) was recorded in freshly-hatched nauplii and, like the protein content, it decreased during development, being only 9.6 % in adults. The carbohydrate content was found to reach a maximum (8.34 % dry wt) in 24 h larvae. However, it showed no fixed trend with development.

The caloric value of freshly-hatched nauplii was found to be the richest (6.076 kcal/g dry wt). This value is comparable to nauplii of the San Francisco Bay strain (6.6 kcal/g dry wt) as recorded by Dutrieu (1960) but higher than that of the similar stage in most of the other strains. The energy content, as revealed by the present study, declined with the development, by about half from the naupliar to the adult stage (3.301 kcal/g). However, Paffenhofer (1967) found that 7 day-old *Artemia* (fed) had an energy content of 5.584 kcal/g and Gabaudan *et al.* (1980) found that commercially available frozen adults had about 5.1 kcal/g.

Von Hentig (1971) and Helfrich (1973) reported that during growth, the fat content decreases from about 20 % to less than 10 % of the dry wt and the protein content increases from about 42 % to 60 %. Our results, however, show a decrease in protein as well as fat contents during development. Royan (1980a) noted a decline in all the three major constituents from decapsulated cysts through the adult stage. Sorgeloos (1980) stated that upon molting from instar I to II, 27 % of the caloric content and an equal amount of the lipid content are lost. Sillero *et al.* (1980) observed a significantly higher level of proteolytic activity during development in the parthenogenetic populations than in the bisexual one.

From our data, it is clear that the decapsulated cysts are the richest in protein and carbohydrate content while the freshly-hatched nauplii are the richest in lipid and energy content. Overall nutritional value of freshly-hatched nauplii, in view of biochemical composition and energy content, is found to be high. Lipid content has been identified as a very important determinant of the overall nutritional value and a major source of the diet's metabolizable energy directly linked to the growth of the consumer organisms (Pandian, 1975 ; Schauer *et al.*, 1980).

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Characterization of *Artemia* from different localities in Tunisia with regard to their use in local aquaculture

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Abstract

Artemia cysts have been collected from different saltworks and natural salt lakes in Tunisia. The cyst material was processed and used for the following characterization analyses : cyst and naupliar biometrics, cyst hatching characteristics, fatty acid pattern of the nauplii, nutritional value of the nauplii for mysid shrimp, naupliar growth rate and temperature resistance, mode of reproduction, and characterization of sibling species. The cross-breeding tests and cyst biometrics reveal that only *Artemia tunisiana* occurs in Tunisia. Although quality improvements may be expected through improved harvesting, Tunisian *Artemia* have acceptable hatching characteristics and are a good food for mysid shrimp. Small variations in hatching quality observed between cyst samples harvested from the same locality, might indicate fluctuations in the environmental conditions prior to the cyst harvest. Naupliar growth rate is high in most strains but naupliar resistance to high temperatures is limited.

Introduction

In future years coastal aquaculture in Tunisia is expected to expand rapidly with the production of sea bass *Dicentrarchus labrax*, sea bream *Sparus aurata*, and *Penaeus* sp. Since Tunisian fish and shrimp hatcheries will need substantial quantities of *Artemia*, and since several natural *Artemia* habitats are known in Tunisia (Persoone and Sorgeloos, 1980 ; Vanhaecke *et al.*, 1987), a survey was set up to sample brine shrimp in order to characterize their potential value in marine fish and shrimp farming. Cysts were processed and submitted to standard laboratory tests to define the taxonomic classification of Tunisian *Artemia*, their suitability for local culture, and the economical and nutritional quality of their cysts.

Material and methods

Cyst samples

Cysts were collected from three salt lakes, Sebkret el Kourzia, Sebkret Sidi el Hani, and Sebkret mta Moknine, and from the solar saltworks near Sfax, Bekalta, and Mégrine (Fig. 1). Sebkret el Kourzia and Sebkret Sidi el Hani, both inland lakes, were dry at the time of sampling but cysts were found embedded in the salt crusts. At Sebkret mta Moknine, *Artemia* cysts were collected along the shore of the lake. In the Mégrine and Bekalta salterns, cysts were sampled from the shore of evaporation ponds with a salinity of 152 and 200-230 ‰ respectively. Two samples were taken in Sfax, *i.e.* from the shore of a 117 ‰ S evaporation pond (Sfax 117) and scooped from the water in a 218 ‰ S brine collector pond (Sfax 218).

All samples were processed at the Artemia Reference Center. Cysts were cleaned by means of the bi-flotation technique as described by Sorgeloos *et al.* (1978) and, exception made for the Sfax samples, dried in a fluidized bed dryer at a temperature of 35 °C. The Sfax material was dried on a 120 µm screen in a temperature-controlled room (35 °C) under continuous ventilation. The lake samples, containing considerable amounts of salt and impurities, produced only limited amounts of full cysts, especially the Kourzia sample, restricting the characterization of the latter to the analysis of the reproductive mode.

Fig. 1. Location of the Tunisian *Artemia* sources studied.

ARTEMIA CULTURE TESTS

All *Artemia* culture tests were carried out in 35 ‰ S artificial seawater (formula in Sorgeloos *et al.*, 1983). Once a day the larvae were fed *Dunaliella viridis* cells according to optimal feeding regimes as determined by Vanhaecke (1983).

For the analysis of the reproduction mode *Artemia* larvae were cultured at 25 °C in cylindro-conical tubes each containing 150 freshly-hatched nauplii in 300 ml seawater. When sexual differentiation was clearly pronounced, survival and sex ratio were determined and the animals transferred to test tubes, each holding 10 females in 15 ml seawater or 10 couples in 25 ml seawater. Both couple-cultures and all-female cultures, were regularly checked for viable offspring.

Artemia sibling species were defined for Sfax and Mégrine brine shrimp in cross-breeding tests performed following procedures outlined by Tackaert *et al.* (1987) with *Artemia* from San Francisco Bay (California-USA) and Larnaca Lake (Cyprus) as reference material for respectively *A. franciscana* (Bowen *et al.*, 1978) and *A. tunisiana* (Vanhaecke *et al.*, 1987).

Larval growth and survival of Mégrine, Bekalta, Sfax, Moknine, and Sidi el Hani *Artemia* were determined in a standard culture test as outlined by Vanhaecke and Sorgeloos (1980a). Growth of the nauplii was expressed as percent of the growth recorded for the San Francisco Bay strain 288-2596 which was included as an internal standard.

The response of Tunisian *Artemia* to high temperatures was studied following the test procedure of Vanhaecke *et al.* (1984), but omitting the different salinity regimes. Mégrine, Bekalta, and Sfax *Artemia* were cultured at a salinity of 35 ‰ and temperatures of 30 °C and 34 °C ; *Artemia* from Moknine was cultured at 30 °C. *Artemia* from Great Salt Lake (1977), known to have a high temperature tolerance (Vanhaecke *et al.*, 1984), was selected as a reference.

HATCHING TESTS

Hatching tests were carried out in 35 ‰ S artificial seawater (formula of Dietrich and Kalle, 1957) at an incubation temperature of 25 °C ± 0.5 °C under continuous illumination of 1 000 lux. The hatching vessels, were cylindro-conical glass tubes and cysts were kept in suspension by gentle air bubling from the cone of the tube. Hatching percentage, hatching efficiency, and hatching rate (and synchrony) were analyzed as outlined by Bruggeman *et al.* (1979), Sorgeloos *et al.* (1978), and Vanhaecke and Sorgeloos (1982) respectively. Hatching efficiency is expressed as the number of nauplii hatched out of 1 g dry cyst product after 48 h incubation ; hatching rate is expressed as number of hours of incubation needed to reach the time of first appearance of nauplii and 50 % and 90 % of the maximal hatching value.

CYSTS AND NAUPLII BIOMETRICS

Diameter, volume, and chorion thickness of the cysts and length of the nauplii were analyzed for the Mégrine, Bekalta, and Sfax 218 samples, while cysts from Moknine and Sfax 117 were analyzed respectively for the first two and first three biometrical parameters above. Size analysis of the cysts was performed on both untreated and decapsulated (Bruggeman *et al.*, 1980) fully-hydrated cysts using Coulter Counter equipment (Vanhaecke *et al.*, 1980). Analysis of the naupliar length was performed according to the method of Vanhaecke and Sorgeloos (1980b) and Vanhaecke (1983). Individual dry weight of the nauplii was calculated from the equation based on cyst volume (Vanhaecke, 1983).

Fatty acid analysis

The fatty acid profile was determined for freshly-hatched nauplii from the Mègrine and Sfax 218 samples. After homogenization with an ultrasonic homogenizer, lipid extraction, saponification, and esterification were done according to the procedure described by Schauer and Simpson (1978). Fatty acid methyl esters were injected on a capillary column (25 m fused silica, 0.32 mm ID ; liquid phase : Silar 10 C ; film thickness, 0.3 μm) installed on a Carlo Erba Fractovap 2330 gas chromatograph. Operating conditions were as follows : solid injector ; hydrogen as carrier gas at a flow rate of 1.9 ml/min ; FID detection ; oven temperature program : 154 °C-200 °C at 1.5 °C/min. Peak identification and quantification was done with a calibrated plotter-integrator (Hewlett Packard 3390A). The internal standard procedure with 20:2ω6 was used for quantitative analysis.

Culture test with mysid shrimp

The culture test with the marine mysid *Mysidopsis bahia* was performed as outlined by Léger *et al.* (1987). Mysid juveniles were fed daily with freshly-hatched nauplii. Survival was registered daily and at the end of the experiment growth and reproductive characteristics of the mysids were examined. Nauplii hatched from Reference *Artemia* Cysts and Great Salt Lake North Arm cysts were included in the culture experiment as positive and negative controls, respectively.

Data analysis

Data from the standard *Artemia* growth test, the temperature resistance experiments, and the bioassay with mysid shrimp were treated statistically in a one-way analysis of variance (Sokal and Rohlf, 1969). Duncan's multiple range test was used to determine significant differences among means (Goodnight, 1979) and the survival data were normalized through an arcsin $\sqrt{\%}$ transformation prior to analysis (Snedecor and Cochran, 1967).

Results and discussion

All Tunisian *Artemia* tested so far are bisexual (Table I) : sex ratios are high and females cultured in the absence of males produced non-viable eggs only. Since the crossbreeding tests between either Sfax or Mègrine *Artemia* with Larnaca (Cyprus) brine shrimp yielded viable F1 offspring, whereas Sfax and San Francisco Bay matings were sterile, Sfax and Mègrine *Artemia* can be classified within the *Artemia tunisiana* sibling species complex.

Table I

Sex-ratio and reproductive mode in *Artemia* from Tunisia

	Mègrine	Bekalta	Sfax 218	Moknine	Sidi el Hani	Kourzia	Ariana[1]
Sex ratio (male/female)	1.38	1.5	1.0	1.0	0.9	–[2]	–
Reproductive mode	B[3]	B	B	B	B	B	B

[1] After Clark and Bowen (1976).

[2] No data available.

[3] Bisexual.

With the exception of the strain from Sfax, the cyst diameters of the Tunisian populations (Table II) resemble those of the other Old World bisexual populations (Léger *et al.*, 1986) ; *i.e.* significantly larger than the mean cyst diameter of *Artemia franciscana* strains, thus providing further evidence for their designation as *Artemia tunisiana*. Despite being a representative of the *Artemia tunisiana* sibling species as proven by their reproductive compatibility with the *Artemia tunisiana* strain from Larnaca, the biometrical characteristics of Sfax *Artemia* are aberrant since their cyst diameter is significantly smaller than the mean value found for the other *Artemia tunisiana* strains, while their chorion thickness lies between the mean values obtained for *Artemia tunisiana* and *Artemia franciscana*.

TABLE II

Diameter and chorion thickness (in µm) of *Artemia* cysts form Tunisia

	Mégrine	Bekalta	Sfax 218	Sfax 117	Moknine
Diameter of untreated cysts	258.8	251.6	235.4	239.7	252.6
standard deviation	(14.9)	(13.6)	(15.2)	(14.4)	–[1]
Diameter of decapsulated cysts	234.1	228.0	215.1	218.9	–
standard deviation	(11.7)	(12.2)	(12.9)	(11.9)	–
Chorion thickness	12.4	11.8	10.2	10.4	–

[1] No data available.

Table III shows high hatching percentages for the samples from solar saltworks. Maybe that the cysts collected from beaches of the salt lakes have been exposed to suboptimal conditions, *e.g.* repeated hydration-dehydration cycles or too long exposure to sunlight (Sorgeloos *et al.*, 1976 ; Vanhaecke and Sorgeloos, 1982) which can result in mortality of a part of the embryos.

TABLE III

Hatching characteristics of *Artemia* cysts form Tunisia

	Mégrine	Bekalta	Sfax 218	Sfax 117	Moknine	Sidi el Hani
Hatching percentage	60.5	83.2	84.8	76.0	14.2	43.1
Hatching efficiency (in nauplii/g product)	169 173	229 920	305 120	187 413	23 040	–
Hatching rate						
T_0	15.2	16.0	14.0	–	–	–
T_{50}	24.3	25.8	22.6	–	–	–
T_{90}	39.8	36.2	32.8	–	–	–
T_s	22.0	15.8	16.2	–	–	–

T_0 : time until appearance of first nauplii.
T_{50} : time until 50 % hatching is attained.
T_{90} = time until 90 % hatching is attained.
$T_s = T_{90} - T_{10}$. T_s is a measure for hatching synchrony.

A good illustration of the importance of pre-harvest conditions can be given for the cysts collected from the Sfax salterns : *i.e.* the hatching percent of the Sfax 218 sample, harvested from the brine, exceeds the value of the Sfax 117 cysts which were collected from the shore, by almost 10 %. The even larger difference in hatching efficiency reflects the risks of higher amounts of impurities, *e.g.* sand, when collecting the cysts from the shore. Despite a similar hatching percent value for the Bekalta and Sfax samples, the hatching efficiency of the latter is significantly higher. This can be attributed to the smaller size of the Sfax cysts and the purity of the sample.

The slow hatching rate and poor hatching synchrony observed in all samples (Table III, Fig. 2) is a drawback for their optimal use in aquaculture hatcheries. At the time of harvest (T_{90}) already part of the nauplii will have molted into the instar II and III stage which have lost a considerable amount of energy in comparison with the instar I stage (Benijts *et al.*, 1976 ; Vanhaecke *et al.*, 1983). As a consequence good yields of instar I nauplii can only be obtained when applying a two-step harvest, re-incubating the unhatched cysts after a first harvest of nauplii for example at T_{50}.

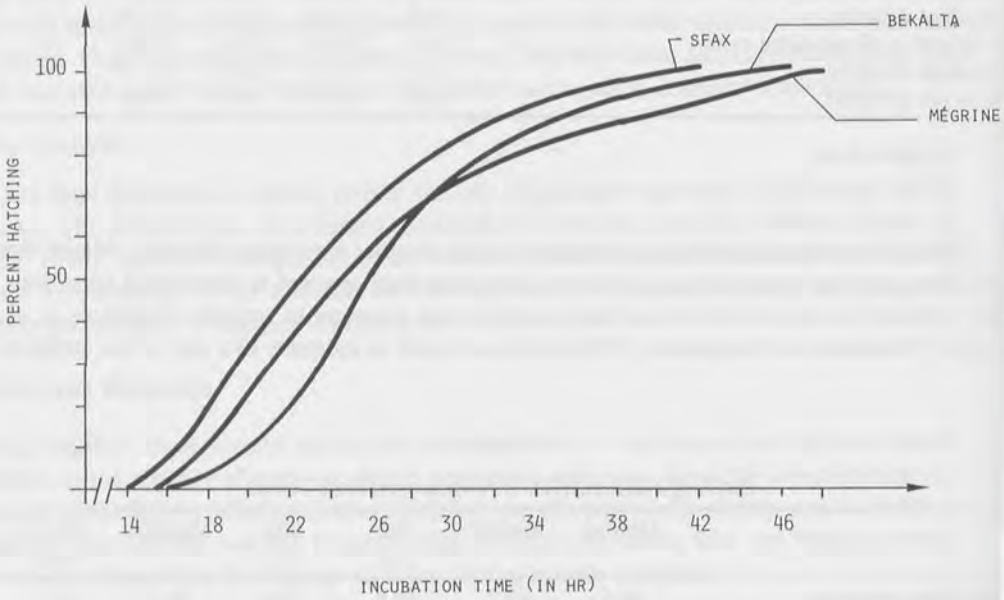

FIG. 2. Hatching curves for different *Artemia* cyst sources from Tunisia.

With the exception of the Sfax nauplii, which approximate the size range of San Francisco Bay nauplii (Vanhaecke and Sorgeloos, 1980b), the Tunisian *Artemia* produce rather large nauplii with a correspondent high dry weight (Fig. 3). Nevertheless sizes are still significantly smaller than the values obtained for parthenogenetic *Artemia*. They approximate the value for Great Salt Lake nauplii (Vanhaecke and Sorgeloos, 1980b) which means that only the smallest fish larvae might have ingestion problems with Tunisian *Artemia* (Beck and Bengtson, 1981).

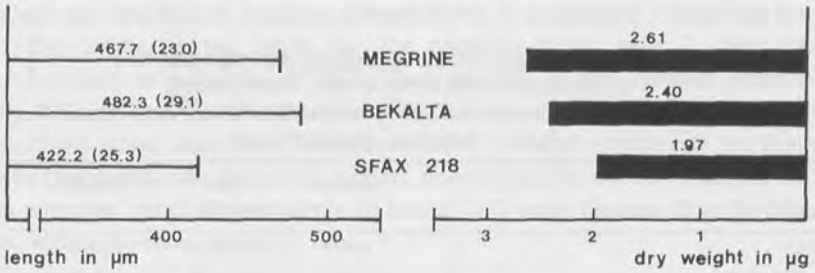

FIG. 3. Length and dry weight of instar I *Artemia* nauplii from various locations in Tunisia (standard deviations in parentheses).

Whereas Mégrine *Artemia* contain high levels of the HUFA 20:5ω3 (Table IV), Sfax nauplii contain only 3.1 mg/g which is marginally acceptable for a good *Artemia* diet for marine predator larvae (Léger *et al.*, 1986). As in most other *Artemia* strains no 22:6ω3 was detected in Tunisian *Artemia* (Léger *et al.*, 1986). The nutritional experiments with mysid shrimp reveal that the two Tunisian *Artemia* sources are an adequate diet (Table V ; Fig. 4). Survival, biomass production and reproduction activity of the shrimp fed the Tunisian *Artemia* are similar to the data obtained with nutritionally good Reference *Artemia*, and are significantly higher than the data obtained for Great Salt Lake, North Arm *Artemia*, known to be of inferior quality (Léger *et al.*, 1986). The good results with the Sfax strain, although low in 20:5ω3, is not surprising. The HUFA profile in Sfax *Artemia* is comparable to that found for Great Salt Lake South Arm cysts (Schauer *et al.*, 1980) which ensured good results with mysids (Johns *et al.*, 1981) but caused total mortality with brachyuran crab larvae (Johns *et al.*, 1980) and poor growth and survival in *Penaeus vannamei* and *P. stylirostris* (Léger *et al.*, 1986). As indicated by the latter authors, a 20:5ω3 content between 3 % and 4 % of total fatty acid methyl esters (*i.e.* 3 to 4 mg/g in instar I nauplii) seems to represent a marginal value of which the acceptability by different predators is not yet fully understood. It would therefore be incautious to designate the Sfax sample as a good quality product without additional culture data for other marine predators.

TABLE IV

Fatty acid methyl esters in freshly-hatched *Artemia* ;
expressed as mg fatty acid methyl ester per g *Artemia* dry weight
(GSLNA = Great Salt Lake, North Arm ; RAC = Reference *Artemia* cysts)

	Mégrine	Sfax 218	GSLNA	RAC
8:2ω6	6.3	5.3	6.0	6.2
8:3ω6	0.5	0.5	0.5	0.6
8:3ω3	15.6	17.4	18.0	3.0
0:4ω3	1.0	0.4	0.5	–[1]
0:5ω3	7.3	3.1	0.2	10.6
2:6ω3	–	–	–	–

[1] Undetectable.

TABLE V

Survival, dry weight, length and reproductive characteristics of *Mysidopsis bahia* cultured with *Artemia* nauplii from different cyst sources (GSLNA = Great Salt Lake, North Arm ; RAC = Reference *Artemia* Cysts)

	Mégrine	Sfax 218	GSLNA	RAC
Survival (%)	89.7[a]	94.7[a]	56.7[b]	93.2[a]
Standard deviation	(12.0)	(12.3)	(4.3)	(12.0)
Dry weight (µg)	319.3[ab]	330.1[a]	214.8[c]	280.7[ab]
Standard deviation	(48.0)	(20.0)	(34.9)	(40.9)
Length (µm)	5 209[a]	5 287[a]	4 321[c]	4 832[b]
Standard deviation	(105)	(219)	(273)	(169)
Reproductive characteristics[1] :				
sexually differentiated animals	100.0	98.1	89.3	100.0
$♀_i$	–	–	75.0	–
$♀_+$	82.4	74.8	25.0	80.0
$♀_m$	17.6	25.9	–	20.0

[abc] Means with different superscript are significantly different ($\alpha = 0.05$).

[1] Data in percent.

$♀_i$ = immature females.

$♀_+$ = females with eggs in ovaria.

$♀_m$ = females with eggs in marsupium.

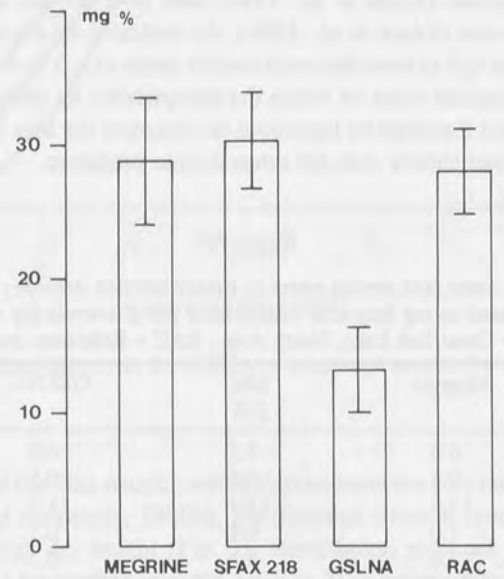

FIG. 4. Biomass production (expressed as mg %) of *Mysidopsis bahia* larvae cultured with *Artemia* nauplii from different cyst sources (GSLNA = Great Salt Lake, North Arm ; RAC = Reference *Artemia* Cysts).

Growth and survival data of Tunisian *Artemia* larvae in a standard culture test are given in Table VI. The average larval length for the reference strain after 7 days culture was 3.44 mm ± 0.18 mm at a survival of 90 %. Survival rates of the Tunisian *Artemia* are not significantly different from the reference strain. *Artemia* from Bekalta and Sfax grow significantly faster, respectively slower than the SFB reference strain. As larval growth rate is a strain specific characteristic (Vanhaecke and Sorgeloos, 1980a), Bekalta *Artemia* should be selected for biomass production purposes, yielding respectively 13 % and 21 % more biomass than the Mégrine and Sfax strains within the same period of time.

TABLE VI

Survival and growth of different *Artemia* strains from Tunisia in a standard culture test

	Mégrine	Bekalta	Sfax 218	Moknine	Sidi el Hani
Survival at day 7 (%)	85	88 86[2]	95	85	88
Growth[1]	100[b3]	115[a] 112[2]	85[c]	97[b]	103[b]

[1] Expressed as % of growth recorded for San Francisco Bay 288-2596.
[2] Result of replicate test in time.
[3] Means with different superscript are significantly different ($\alpha = 0.05$).

Fig. 5 reveals the poor tolerance to high temperature of Tunisian *Artemia*. This confirms the findings of Thoeye *et al.* (1987) who submitted Tunisian *Artemia* to diurnal temperature cycles. Since the GSL reference strain in our tests only yields 78 % and 32 % survival at respectively 30 °C and 34 °C, while in previous tests survival data of 90 % and 70 % respectively were recorded (Vanhaecke *et al.*, 1984), we suspect that our culture conditions, maybe the quality of the *Dunaliella*, were not optimal. Nevertheless the results show that the three indigenous strains already suffer considerable mortalities at a temperature of 30 °C.

This experiment provides interesting confirmation of limited temperature resistance of Tunisian *Artemia* of which the natural populations disappear during early summer when water temperatures exceed 30 °C. It should be mentioned that besides low temperature resistance of local strains, the production potential of *Artemia* in several saltworks is further limited by the presence of fish (*Aphanius* sp.) predating on *Artemia* at salinities as high as 150 ‰.

Conclusions

In conclusion it may be recognized that, although open for improvement, the overall quality of Tunisian *Artemia* is good, both in terms of hatching characteristics and nutritional effectiveness. Consequently, they can be used as an acceptable food source in aquaculture hatcheries. Furthermore, in view of their good growth rate, local strains can be used for intensive biomass production. Extensive culture might be limited, because of the poor resistance of local strains to high temperatures. Additional work is needed to assess the potential of the region for extensive cyst and biomass production.

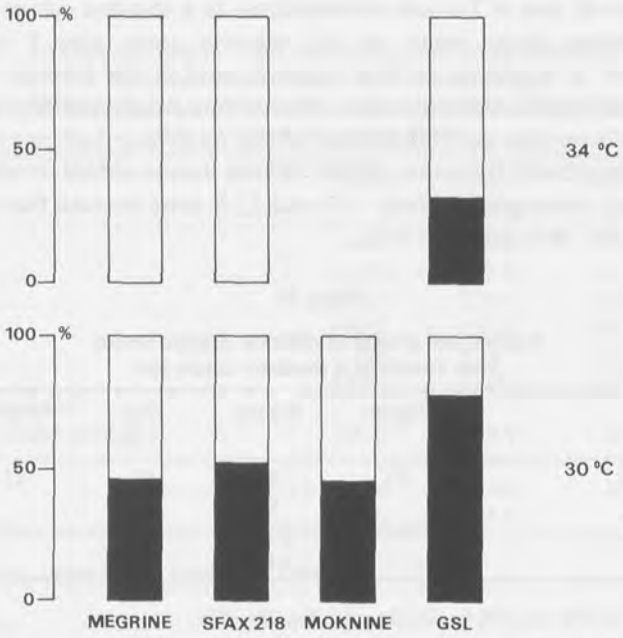

FIG. 5. Percent survival (black bars) of *Artemia* larvae from various origin reared at 30 °C and 34 °C in a standardized culture test (GSL = Great Salt Lake batch 1977).

Acknowledgements

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Characterization of Portuguese *Artemia* strains

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Abstract

Artemia cysts have been collected from various salinas along the Portuguese coast. Data on biometrical and hatching characteristics of the cysts as well as information on the reproduction type of various *Artemia* strains are provided.

The fatty acid composition and caloric content of cysts and nauplii was determined to evaluate the nutritive value of these *Artemia* to be used in local aquaculture operations.

Introduction

As part of the project "Study of the Portuguese *Artemia*" carried out by the Instituto Nacional de Investigação das Pescas (INIP), *Artemia* cysts have been harvested from naturally occurring populations in salinas along the Portuguese coast.

In this study various *Artemia* strains are characterized with regard to their type of reproduction as well as to biometrical characteristics of cysts and instar I nauplii, hatching efficiency, caloric content and fatty acid composition. Based on this information, we will be able to select the appropriate strain among the Portuguese brine shrimp for mariculture or other specific purposes.

Materials and methods

Artemia cysts from 17 different locations of the Portuguese coast were collected for characterization. The geographical distribution of the source of cysts is given in Fig. 1.

The cysts were processed according to Sorgeloos *et al.* (1978).

A Reichert microscope equipped with a micrometric ocular lens was used for doing all the measurements.

Measurements were performed on cysts harvested from three salinas at Aveiro, where exclusively adult parthenogenetic females were found on the sampling day. Measurements were also performed on cysts harvested from cultures of wild females collected from these locations and kept under standard laboratory conditions (*Platymonas suecica* food, 18±1 °C temperature and seawater 30-32 ‰ salinity).

For the analysis of the biometrical characteristics of the nauplii, cysts were incubated in autoclaved seawater 30 ‰ salinity at 18±1 °C temperature and continuous artificial light. Nauplii were collected within 2 h after hatching, immediately preserved in formalin in order to obtain a homogeneous population of instar I nauplii for all the strains.

Caloric content was measured in a Gallenkamp Ballistic Bomb Calorimeter CB-370 and the results are given as kcal/g ash free dry weight. Sample incineration was done in a muffle furnace

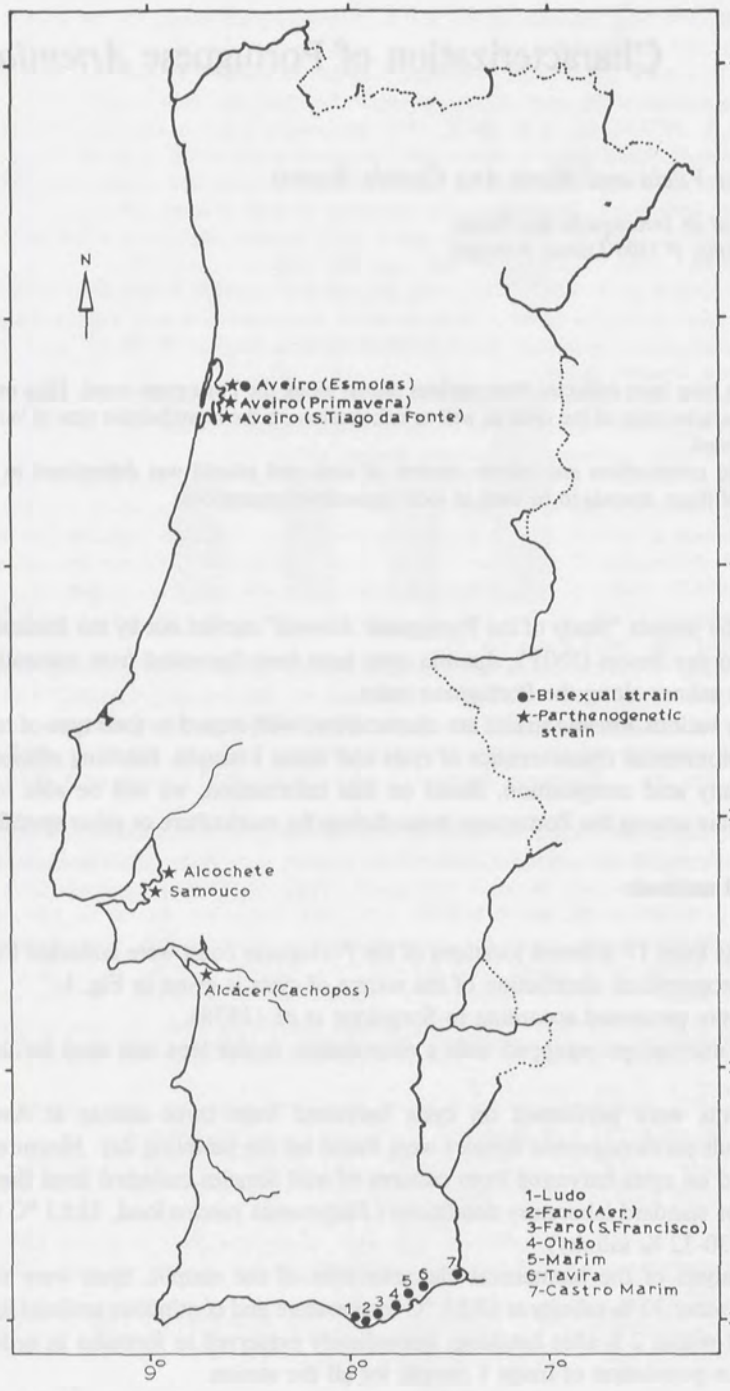

FIG. 1. Geographical distribution of *Artemia* strains in Portugal.

at 520 °C for about 5 h. The ash values were determined and subtracted from the sample dry weight in order to obtain the estimation of the absolute caloric values (kcal/g ash free dry weight).

Lipid extraction was done by the method of Bligh and Dyer (1959). For saponification and esterification the technique adopted at the Artemia Reference Center, Ghent, was used. Fatty acid methyl esters (FAME) were injected into a Perkin Elmer F_{11} Gas Chromatograph unit equipped with a flame ionization detector and operated isothermally at 230 °C. The chromatograph had two diethylene glycol succinate columns on Chromosorb W HMDS 80-100 mesh, with 2 m length and 3.2 mm outer diameter.

Identification and quantification of the FAME were made with an electronic integrator Hewlett Packard.

Results and discussion

Important differences in cyst size among parthenogenetic and bisexual strains were observed (Table I). The parthenogenetic strains present, in general, a larger cyst size. Nevertheless the strain from Alcochete (Vau) has, among the parthenogenetic strains, the smallest cyst diameter (241.2 µm) and the bisexual strain harvested in Aveiro and described by Vieira and Teles (1984) presents the largest (268.0 µm). The other strains from Aveiro are parthenogenetic and the measurements concern laboratory produced cysts.

TABLE I

Biometrical characteristics of hydrated cysts and instar I nauplii of various Portuguese *Artemia* strains. For all determinations, N=50

	Cysts		Nauplii
	Diameter (µm) ($\bar{X} \pm$ SD)	Mean volume ($\times 10^6$ µm^3)	Length (µm) ($\bar{X} \pm$ SD)
veiro (Esmolas) (n)[1]	268.0 ± 15	10.08	401.0 ± 3.1
veiro (Esmolas) (c)	262.6 ± 14.1	9.48	–
veiro (Primavera) (c)	253.8 ± 8.7	8.56	–
veiro (S. Tiago Fonte) (c)	252.8 ± 8.8	8.46	–
lcochete (Vau) (n)	241.2 ± 10.2	7.35	429.1 ± 29.1
amouco (n)	264.1 ± 15.4	9.65	503.5 ± 25.6
lcácer (Cachopos) (n)	251.3 ± 16.8	8.31	484.7 ± 20.3
udo (n)	237.7 ± 8.0	7.01	446.5 ± 26.0
aro (Má vontade) (n)	246.5 ± 10.2	7.84	422.3 ± 18.6
aro (Aeroporto) (n)	234.7 ± 13.3	6.77	450.3 ± 22.5
aro (S. Francisco) (n)	230.4 ± 11.1	6.40	445.4 ± 17.9
lhão (Afincão) (n)	247.3 ± 9.6	7.92	446.6 ± 22.2
lhão (Farisol) (n)	241.0 ± 8.5	7.33	458.2 ± 21.6
lhão (Belamandil) (n)	239.4 ± 9.2	7.18	452.5 ± 18.5
arim (n)	231.4 ± 10.2	6.49	446.8 ± 19.9
vira (n)	247.5 ± 10.6	7.94	448.9 ±20.0
astro Marim (n)	231.2 ± 8.0	6.47	441.6 ± 27.9
M. (Compasal) (n)	242.2 ± 8.7	7.44	458.9 ± 27.1

(n) = collected from nature. (c) = collected from cultures.

[1] Data from Vieira and Teles (1984).

Comparison of the cyst size data and naupliar length data (Table I) indicate that in general there is a correlation between the biometrical characteristics of the cysts and their respective nauplii. However, this proportionality is sometimes not verified, *e.g.*, the Aveiro bisexual strain, which has a relatively large cyst volume (probably due to the large chorion thickness), the instar I nauplii length is rather small (Vieira and Teles, 1984).

The *Artemia* strains from Faro, Olhão, Marim, and Tavira revealed a good hatching efficiency (Table II) and, because of the similarity of the results within 24 and 48 h period they apparently have a high hatching synchrony.

TABLE II

Hatching efficiency of *Artemia* cysts collected from various Portuguese salinas and information on their reproduction type

	24 h	48 h	
	Number of nauplii per g cysts	Number of nauplii per g cysts	Reproduction type[3]
Aveiro[1]	–	–	PB
Alcochete (Vau)[2]	19 312	58 864	P
Samouco[2]	18 512	40 224	P
Alcacer (Cachopos)[2]	41 824	67 312	P
Ludo	60 480	77 760	B
Faro (Má vontade)	38 400	85 800	B
Faro (Aeroporto)[2]	101 936	127 360	B
Faro (S. Francisco)[2]	166 736	177 376	B
Olhão (Afincão)	131 200	132 000	B
Olhão (Farisol)	38 400	62 171	B
Olhão (Belamandil)	117 440	143 200	B
Marim	159 680	164 160	B
Tavira	109 440	129 712	B
Castro Marim[2]	68 912	79 312	B
C.M. (Compasal)	49 120	89 280	B

[1] After Vieira and Teles (1984).
[2] After Narciso (1982).
[3] P = parthenogenetic ; B = bisexual.

With regard to the type of reproduction of *Artemia*, it appears that, in general, the parthenogenetic strains are from salinas situated in the north (Ria de Aveiro) and central part of the country (Tejo and Sado Estuaries) and the bisexual strains are native to salinas along the Portuguese south coast. However, in Aveiro (Esmolas) both adult parthenogenetic females and cysts of a bisexual strain were found and harvested. The cysts were collected and described by Vieira and Teles (1984). The possibility of co-existence of mixed populations of bisexual and parthenogenetic *Artemia* at some locations has been discussed previously (Amat, 1980).

Considerable differences exist in caloric content between cysts and newly hatched nauplii (Table III). The mean caloric value varies from 6.5 to 7.0 kcal/g ash-free dry wt in eggs and from 7.0 to 8.2 kcal/g ash-free dry wt in nauplii. Comparing the caloric values between the cysts and

the newly hatched nauplii of each *Artemia* strain, the difference varies from 0.8 kcal/g ash-free dry wt in Faro (Aeroporto) strain to 1.4 kcal/g ash-free dry wt in Alcochete (Vau) strain. Of the geographical strains analysed the nauplii of Alcochete (Vau) strain present the highest caloric value.

TABLE III

Caloric values of *Artemia* cysts and newly hatched nauplii from various Portuguese salinas. N = number of analyses

	Sample size (mg)	N	Ash content (% dry weight)	kcal/ash free g ($\bar{X} \pm$ S.D.)
Cysts				
Alcochete (Vau)	700	3	3.03	6.812 ± 0.184
Samouco	700	3	4.37	6.989 ± 0.533
Alcácer (Cachopos)	700	3	3.60	6.653 ± 0.106
Faro (Aeroporto	700	3	4.52	6.882 ± 0.211
Faro (S. Francisco)	700	3	4.95	6.706 ± 0.310
Castro Marim	700	3	3.65	6.506 ± 0.263
Nauplii				
Alcochete (Vau)	700	3	6.90	8.218 ± 0.326
Alcácer (Cachopos)	700	3	5.78	7.865 ± 0.290
Faro (Aeroporto)	672-700	4	7.42	7.635 ± 0.996
Faro (S. Francisco)	700-735	3	7.30	7.858 ± 0.245

In aquatic animals the range of intraspecies variation of calorific value is a considerable one (1-2 kcal) and it can be said that the same organisms can accumulate energy at a different quantity, depending on age, developmental stage, physiological stage or food conditions (Prus, 1970).

The major fatty acids in the *Artemia* strains tested (Table IV) were 16:0, 16:1 (except in Castro Marim cysts and Samouco nauplii which presented a significant amount of 18:0) and 18:1 (except in Samouco nauplii). In addition substantial levels of 18:3, 18:4 and/or 20:5 fatty acids were detected in some strains.

Watanabe *et al.* (1978) classified the *Artemia* strains into two types by their fatty acid composition ; one containing a high amount of 18:3ω3 which is the essential fatty acid (EFA) for freshwater fish, and the other with a high content of 20:5ω3, which, along with 22:6ω3, is an EFA for marine fish.

Although our values should be considered as preliminary information and a more detailed study is needed, it appears from the data that Samouco, Alcácer (Cachopos) and Faro (Aeroporto) strains contain significant amounts of 18:3 or 18:4 and 20:5 fatty acids and therefore they can be included in either of the two types.

In the Faro (S. Francisco) strain no trace of 20:5ω3 was detected suggesting a poor food value for marine fish.

Castro Marim strain revealed a high level of 18:4 fatty acid and although no trace of 20:5 was found, it contained a high amount of 22:5 (?) fatty acid whose importance as food value for fish is not well defined.

TABLE IV

Fatty acid composition of cysts and newly hatched *Artemia* nauplii of some Portuguese strains. Results are expressed as area % fatty acid methyl ester of total fatty acid methyl esters

	Cysts		Newly-hatched nauplii			
	Samouco	Castro Marim	Samouco	Alcácer (Cach.)	Faro (Aerop.)	Faro (S.F.)
14:0	2.3	–[1]	–	2.0	1.0	1.1
14:1	2.3	–	–	2.4	2.2	2.5
15:0	0.7	–	0.1	0.8	0.4	0.4
15:1	0.7	–	1.7	0.9	1.3	1.5
16:0	16.0	3.3	2.3	12.7	13.3	12.5
16:1	22.0	2.8	0.6	16.4	8.8	8.9
17:0	0.2	1.5	0.7	0.8	–	1.5
17:1	1.7	0.4	14.3	2.4	1.7	2.1
18:0	3.5	16.1	19.9	3.2	5.7	5.7
18:1	28.7	14.4	0.6	29.2	32.7	33.0
18:2	3.7	1.0	1.2	4.3	8.8	9.8
20:0 (?)	–	1.7	4.6	0.3	–	–
18:3+20:1	7.1	2.9	–	4.2	16.3	–
18:3	–	–	28.8	–	–	0.3
20:1 (?)	–	–	–	–	–	17.3
18:4 (?)	0.2	28.8	2.6	–	3.8	3.4
22:0	–	5.0	8.6	–	–	–
22:1	1.5	–	0.7	2.5	–	–
20:5 (?)	9.4	–	1.6	7.7	3.8	–
24:0 (?)	–	4.9	–	–	–	–
22:4	–	–	11.1	10.2	–	–
22:5 (?)	–	17.2	–	–	–	–

[1] Not detected.

The only *Artemia* strain analysed for fatty acids in both cysts and nauplii was the one from Samouco. The data revealed a quite different profile of fatty acids before and after hatching. This appears to indicate that the chorion of this strain contains a significant amount of fatty acids which is contrary to the findings of Schauer *et al.* (1980).

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Biometrics of *Artemia* from Milos (Greece)

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Summary

Parthenogenetic *Artemia* cysts and adults were collected from Milos saltworks during the months of July 1982 and July 1983. Water salinities ranged from 150 to 250 ‰, temperature from 27 to 30 °C and pH from 7.0 to 8.0.

The allometrical data for nine morphological characteristics are given in Table I and appear to be in accordance with those found in Spanish parthenogenetic *Artemia* (Amat Domenech, 1980).

FIG. 1. Adult *Artemia* from Milos saltworks.

TABLE I

Allometrical data for *Artemia* from Milos (Greece)
(n = 40 ; average ± standard deviation of the mean)

Total length	9.740 mm ± 0.037 mm
Length of abdomen	5.158 mm ± 0.052 mm
Maximal width of brood pouch	1.335 mm ± 0.087 mm
Width of third abdominal segment	0.471 mm ± 0.059 mm
Length of furca	0.260 mm ± 0.112 mm
Width of head	0.792 mm ± 0.070 mm
Length of first antenna	1.170 mm ± 0.038 mm
Maximal diameter of complex eye	0.3492 mm ± 0.0219 mm
Distance between complex eyes	1.454 mm ± 0.038 mm

Cysts cleaned following the methods outlined in Sorgeloos *et al.* (1978) yielded a hatching percentage of 72 %. Upon decapsulation (Sorgeloos *et al.*, 1977) the hatching percentage increased up to 82.8 %.

Hydrated untreated cysts measured 263.1 µm in diameter (n = 215 ; standard deviation of the mean = 12.1 µm), hydrated decapsulated cysts 251.89 µm (n = 213 ; s.d. = 10.8 µm). Thickness of the chorion = 5.61 µm.

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Preliminary characterization of four populations of *Artemia* from the Dominican Republic

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Abstract

Bisexual populations of the brine shrimp (*Artemia*) were found at four solar saltworks in the Dominican Republic, *i.e.*, Monte Cristi and Isla Cabra on the Atlantic coast, and at Las Calderas and Puerto Alejandro on the Caribbean.

Population studies revealed that the highest *Artemia* densities occur at the Caribbean sites with up to 70 and 300 ind./l at, respectively, Puerto Alejandro and Las Calderas, in the samples taken near the shore.

The following parameters were recorded for the cysts harvested from the four sites : cyst diameter, nauplius length, hatching-efficiency.

Introduction

The first data on *Artemia* strains found in Las Calderas, Dominican Republic, was reported by Jakowska *et al.* (1980). The populations of *Artemia* found in other parts of the country present the possibility that these strains could be useful in national and/or international aquaculture.

The characterization of these newly found strains necessary for their future exploitation (nutrition, reproductive ability, etc.) is now being conducted by the Centro de Investigaciones de Biologia Marina (CIBIMA). The four populations of brine shrimp *Artemia* were found in four sites associated with salt production, an important industry of the Dominican Republic which could be complemented with the commercial production of *Artemia*.

The Dominican Republic provides numerous coastal lagoons as well as lake Enriquillo, the largest tropical hypersaline lake of the western hemisphere, which could provide a favorable area for *Artemia* development (Lysenko, 1984).

The salt farms where brine shrimp were found are located on the southern coast (Las Calderas, Puerto Alejandro) and on the northern coast (Monte Cristi, Isla Cabra) of the Dominican Republic (Fig. 1). These salt farms include reservoir ponds which have subterranean or surface communication to the sea. Specific data on the four habitats is presented in Table I.

When a certain degree of salinity is reached the brine is carried away to the concentration and evaporation ponds by means of canals and tubing. During different times of the year the period of crystallization varies from 7 to 30 days.

For analytical purposes we consider these four *Artemia* populations as different strains (Persoone and Sorgeloos, 1980).

FIG. 1. Locations of the principal salt farms in the Dominican Republic where *Artemia* populations were collected for this study.

TABLE I

Data on four habitats of *Artemia* in the Dominican Republic

Habitats (salt farms)	Total area of salt ponds (ha)	Reservoir			
		Area (ha)	Salinity range (‰)	Temperature range (°C)	Highest density *Artemia*/l
Las Calderas	30	15	120-200	26-3.4	366
Puerto Alejandro	15	10	160-200	28-32	102
Monte Cristi	50	8	90	25-29	216
Isla Cabra	2	0.5	160	29	–

Materials and methods

Morphometrical analysis was performed on live animals collected from their natural environments. Only adult individuals were selected according to their sexual characteristics. Biometrics were taken following the methods used by Amat (1980ab). Measurements were made with a Bausch & Lomb binocular microscope equipped with an ocular micrometer. Thirty individuals of both sexes were measured.

The cysts collected in reservoir ponds in Las Calderas (X-83 and VIII-84) and Puerto Alejandro (V-83) were cleaned of debris and dried. The diameter of 200 dried cysts were measured with a microscope equipped with an ocular micrometer.

Hatching efficiency of the cysts mentioned above was performed in standard conditions (Sorgeloos *et al.*, 1978). The length of nauplii was determined under microscope within 24 and 48 h after hatching. In each case 150 nauplii were measured.

Results and discussion

All four analyzed strains were bisexual.

Differences in body lengths were observed within and among the four Dominican strains. Within-strain differences were due to sexual dimorphism, but among-strain differences may be related to variations in environmental conditions, especially salinity. Among all the Dominican strains, Las Calderas showed the greatest uniformity in length of the two sexes, *i.e.*, females ranged from 10 to 12 mm and males from 8 to 10 mm. In the Monte Cristi strain, some females reached 13-14 mm in length, but the males remained smaller (7-11 mm). Adults from Isla Cabra seemed to be the smallest.

Preliminary morphometric analysis (not pictured here) indicated that abdomen length, ovisac width, head width and interorbital distance were all strongly related to total length for all four strains ; however, abdomen width, furca length, antennulae length and eye diameter were much less strongly related to total length.

FIG. 2. Morphology of ovisac in four Dominican *Artemia* strains sampled from their natural habitats.

Morphological differences among the four strains are visible in the ovisac (Fig. 2), furca (Fig. 3), and head (Fig. 4).

Cyst diameter and naupliar length at 24 h and 48 h after hatching (Table II) suggest that the Dominican strains may be larger than those strains analyzed by Amat (1983).

The data we have obtained so far do not permit us to make any conclusions about differences among the four Dominican strains. Further evaluations will be carried out under standard culture conditions.

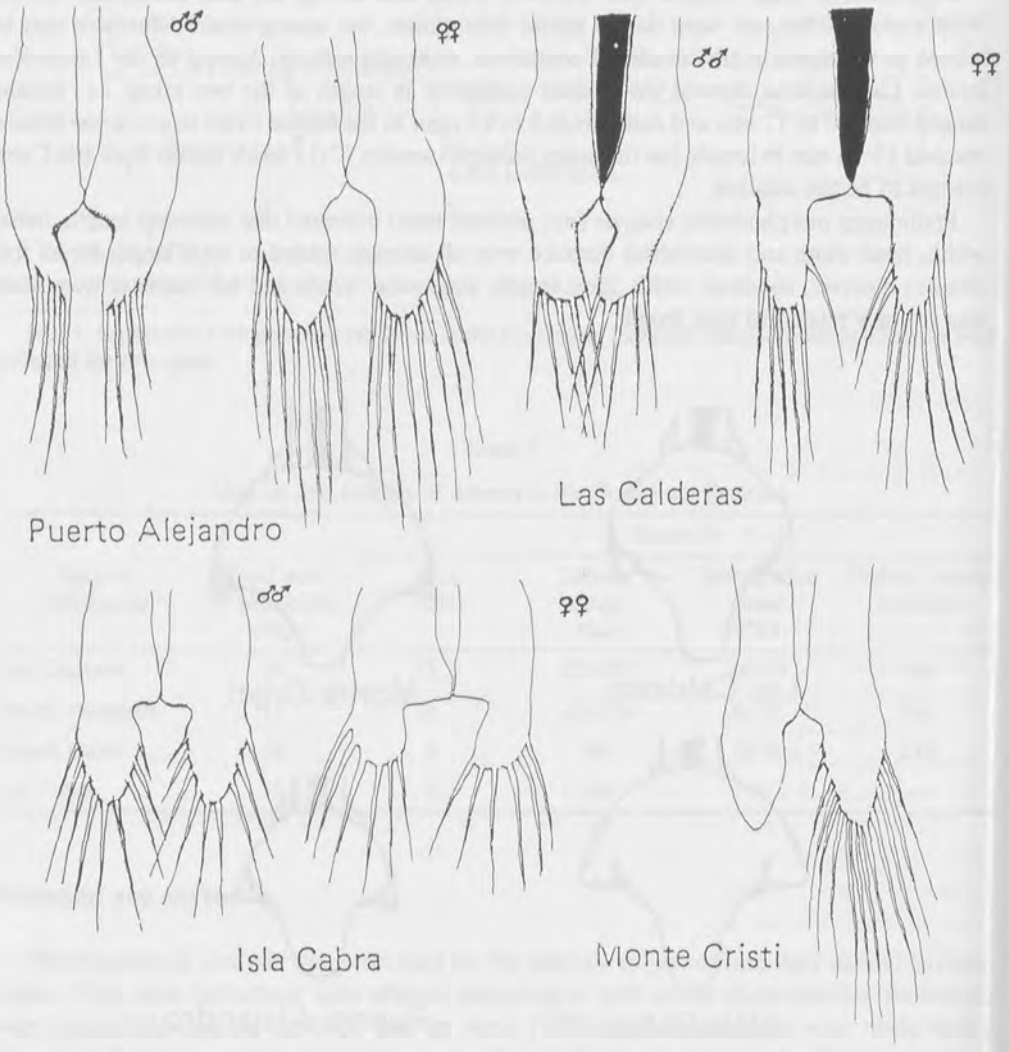

FIG. 3. Morphology of the furca in four Dominican *Artemia* strains sampled from their natural habitats.

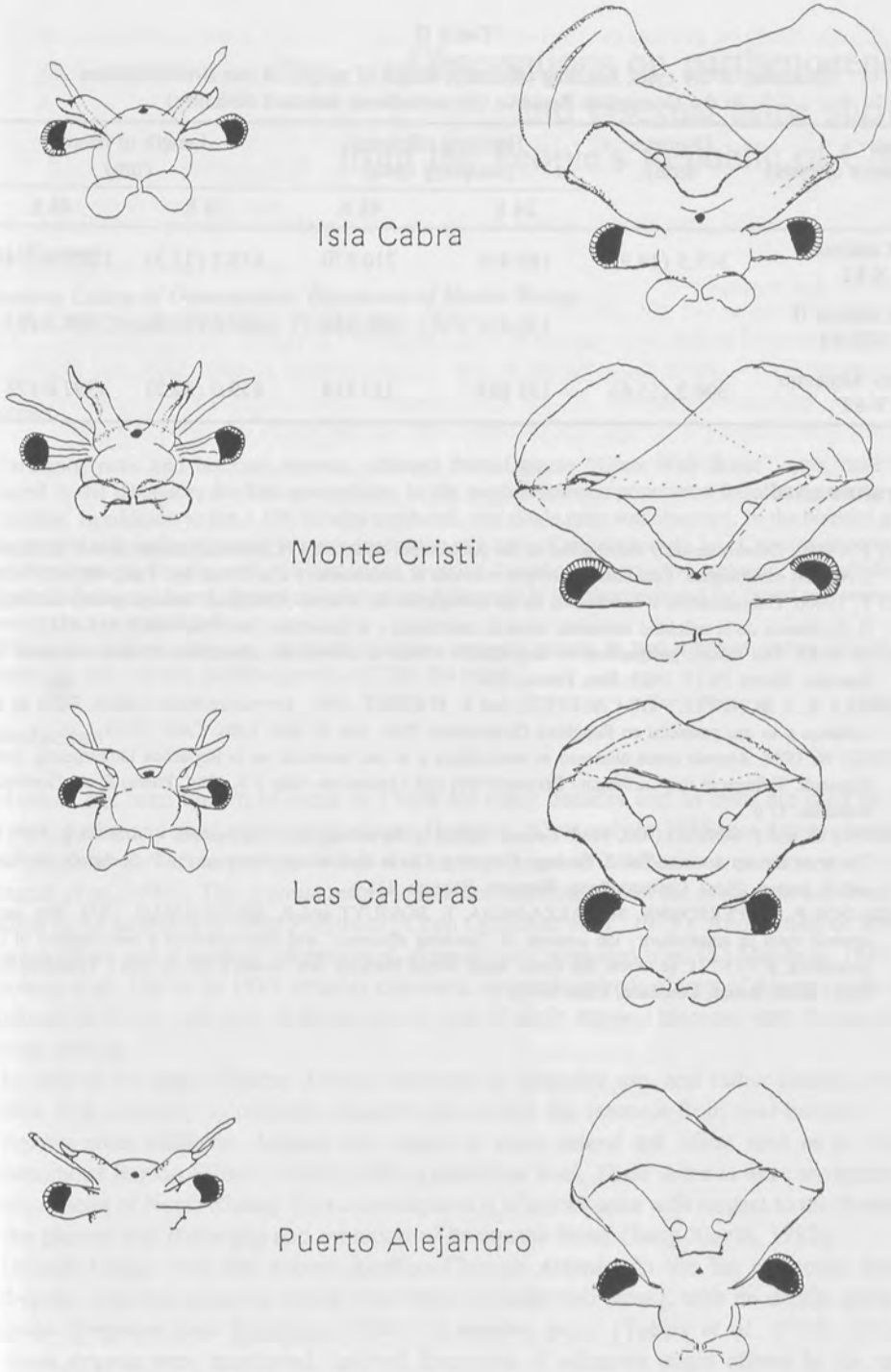

FIG. 4. Morphology of the head in four Dominican *Artemia* strains sampled from their natural habitats.

TABLE II

Diameter of dry cysts, hatching efficiency, length of nauplii of two *Artemia* strains in the Dominican Republic (in parentheses standard deviation)

Strains (source of cysts)	Diameter (µm)	Hatching efficiency (nauplii/g cysts)		Length of nauplii (µm)	
		24 h	48 h	24 h	48 h
Las Calderas I X-83	319.5 (14.9)	186 916	210 970	674.7 (77.3)	1 007.0 (74.9)
Las Calderas II VIII-84		136 054	186 916	607.0 (35.0)	939.3 (93.5)
Puerto Alejandro V-83	306.2 (15.6)	135 685	127 714	659.0 (82.2)	997.0 (75.6)

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Observations on parthenogenetic and bisexual brine shrimp from the People's Republic of China

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Abstract

Parthenogenetic and bisexual *Artemia*, obtained from Chinese "Great Wall Brand" cysts, have been cultured in the laboratory for four generations. In the parthenogenetic population females appear in every generation. In addition to the 1 898 females produced, one single male was observed. In the bisexual group both females and males appeared in every generation at a ratio of approximately 1:1. Crossing experiments of parthenogenetic females with bisexual males revealed complete reproductive isolation, *i.e.* only female offspring is being produced. Several morphological differences in the abdomen and the brood sac were noted between the two populations.

These observations allow to conclude that these *Artemia* belong to two different sibling species, *i.e.* *Artemia* sp. and *Artemia parthenogenetica* of Tian Jin origin.

Introduction

Artemia has been known to occur in China for many decades and its cysts are used as food for pigs, ducks, and chickens in many places. However, it was only in 1958 that *Artemia* was used as a live food for larvae and fry of fish and shrimp larvae in hatcheries and nurseries (Huang Jingxia *et al.*, 1980). The *Artemia* resource and the hatching rate of the cysts were explored and studied in 13 saltfields of three Provinces (Chen Qingchao *et al.*, 1975). Application of *Artemia* in aquaculture and the use of decapsulated *Artemia* cysts were also tried (Li Maotang, 1980 ; Li Maotang *et al.*, 1982). In 1984, roughly estimated, several hundreds of tons of *Artemia* cysts were produced in China and tens of thousands of tons of adult *Artemia* biomass were harvested for shrimp rearing.

In view of the huge Chinese *Artemia* resource, its extensive use, and rather limited *Artemia* studies, it is necessary to reinforce research and protect the resource from over-harvest.

Besides solar saltfields, *Artemia* also occurs in many inland salt lakes, such as in Xizang Autonomous Region (Tibet), 4 000-5 000 m above sea level. These *Artemia* were recognized as a relic species of North Xizang. This consideration is of significance with respect to the formation of the plateau and the origin and evolution of its aquatic fauna (Jiang Xiezhi, 1982).

Outside China, very few papers mention Chinese *Artemia*. In the list of recent *Artemia* field-spots, only two places in China were listed (actually two cities), with no details about the *Artemia* (Persoone and Sorgeloos, 1980). In another paper (Tobias *et al.*, 1980), Chinese bisexual *Artemia* were mentioned, hatched from cysts of unknown origin offered by the representative of China at the FAO in Rome (Sorgeloos, pers. commun.). In a paper of the

International Study on *Artemia* the biometrics of a Chinese bisexual *Artemia* strain were studied, but again its origin is unknown (Vanhaecke and Sorgeloos, 1980). The nutritional quality of an *Artemia* strain (high in 20:5ω3 concentration, low in 18:3ω3 from a salt pan in China near Tian Jin, was reported by Fujita *et al.* (1980). There was still another paper of the International Study on *Artemia* on combined effects of temperature and salinity to its survival (Vanhaecke *et al.*, 1984).

The purpose of this paper was to initiate studies on *Artemia* species and populations in China.

Materials and methods

The *Artemia* used in this study originated from the cysts of a commercial product called "Great Wall *Artemia* Cysts" which is distributed by the "China National Cereals, Oils, and Foodstuffs Import and Export Corporation" in Tian Jin (Tientsin). Cysts sold under this tradename were collected from different solar saltfields such as Tang Gu, Han Gu, Yang Kou, and others, in the coastal regions near Tian Jin.

Artemia were cultured in natural seawater (30 ‰ salinity) without aeration in glass culture vessels of 15 cm height and 20 cm in diameter. The salinity of the medium increased gradually through evaporation. The *Artemia* were mostly fed with *Phaeodactylum tricornutum*. Periodically faecal pellets and waste material were siphoned out of the vessels, in order to allow the addition of new algal medium. The room temperature ranged between 20 and 27 °C throughout the entire experiment.

The appearance of several males in one vessel allowed further culture of this batch and thus increased the number of males. Finally about 30 couples were selected for further culturing through four generations. The other culture series was set up with one parthenogenetic female taken from the vessel which had no male during the first generation and was cultured further through four generations.

Results

Bisexual *Artemia*

In all four generations, there were about equal numbers of males and females. In the second generation, a sample was fixed for examination, which showed 78 males, 108 females, and some individuals too small to be sexed. The remainder of the second generation was cultured to produce the third generation. From this third generation two samples were taken, the first one comprised 83 males, 145 females, and some individuals too small to be sexed ; in the second one 20 males, 30 females, and 151 individuals in the larval stage occurred. The remainder of the third generation was cultivated to produce the fourth generation. This generation also had about equal numbers of males and females but they gradually died off, for the culturing condition deteriorated.

Parthenogenetic *Artemia*

Four generations of the cultivated parthenogenetic *Artemia* were all females except for one male. The details were as follows : in the second generation there were 36 females, in the third generation 248 females and one male, in the fourth generation 1 650 females ; or in total 1 934 females and one male.

CROSSING TESTS

Males of bisexual *Artemia* were crossed with females of parthenogenetic *Artemia.* First, young males of bisexual *Artemia* and females of parthenogenetic *Artemia* were selected and cultured separately. After maturation, 10 mixed couples were reared in a vessel for crossing experiments. They showed grasping behavior, but all of the 81 offspring cultured to near maturity and fixed for examination were females.

MORPHOMETRIC OBSERVATIONS

With regard to external morphological features of females, the two *Artemia* species were obviously different (Fig. 1 and 2). The abdomen of the parthenogenetic *Artemia* is long and slender (mean length = 5.6 mm). Whereas the bisexual one is short and thick (mean length = 3.9 mm). The total length of the bisexual *Artemia* was also shorter (mean = 8.8 mm *versus* 10.5 mm for the parthenogenetic one). The ratio of abdominal length to width and the ratio of total length to abdominal length (Table I) were both highly significantly different for the two species (t-test ; $P < 0.01$). Compared with females of San Francisco Bay *Artemia*, the ratio of abdominal length to total length given by Amat Domenech (1980) was somewhat different (Fig. 3).

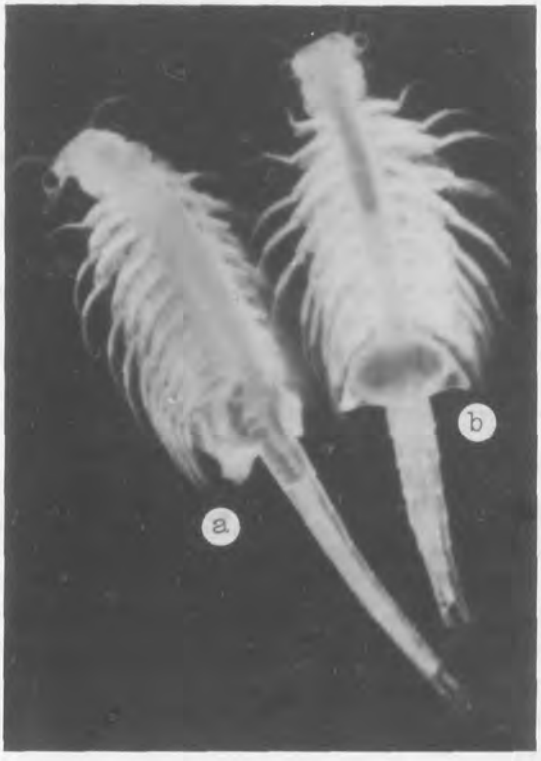

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FIG. 1. a. Parthenogenetic female *Artemia.* b. bisexual female *Artemia.*
FIG. 2. Mating in the bisexual *Artemia.*

TABLE I

Comparison of morphometric data
for bisexual *versus* parthenogenetic *Artemia* females
from Tian Jin, China. Data are given as mean ± standard deviation (N = 25)

	Bisexual	Parthenogenetic
Abdominal width/ abdominal length	0.19 ± 0.02	0.11 ± 0.02
Abdominal length/ total length	0.45 ± 0.04	0.53 ± 0.02

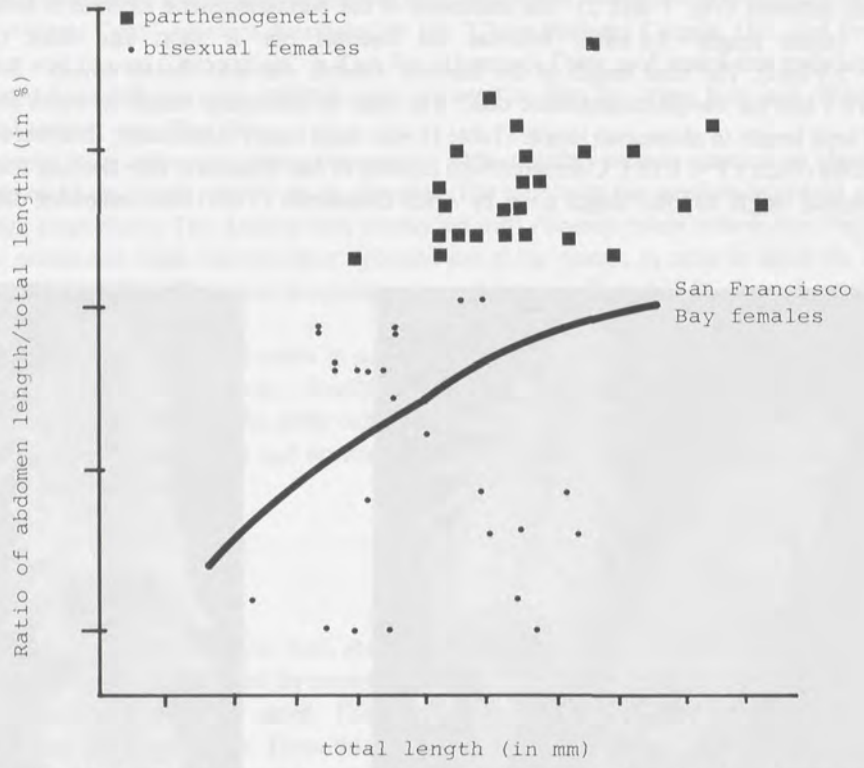
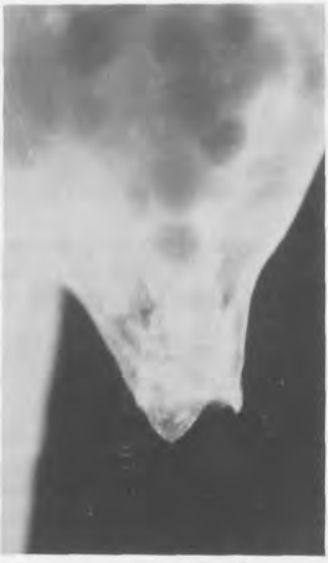


FIG. 3. Ratios of abdominal length to total length for females of the bisexual (●) and parthenogenetic (■) *Artemia* from the Chinese strain (N = 25), and for the San Francisco Bay *Artemia* strain (curve after Amat Domenech, 1980).

Furthermore, the edges of the ovisac openings of these two species were also different. The parthenogenetic one had a sharp angle with the upper lip and two sharp angles with the lower lip, so that they coincided when they closed (Fig. 4). The bisexual one was smooth along its upper and lower lips (Fig. 5).

According to the above external morphological differences it was easy to distinguish the two species.



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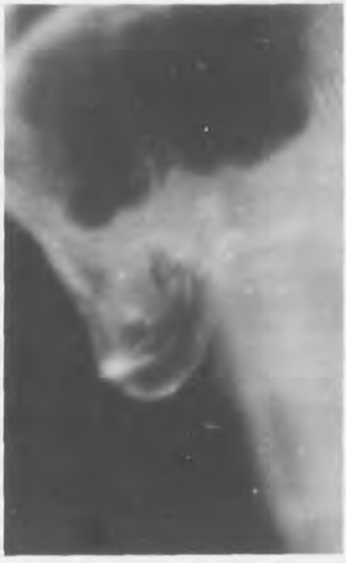

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FIG. 4. Ovisac opening of the parthenogenetic *Artemia.*
FIG. 5. Ovisac opening of the bisexual *Artemia.*

Discussion

From these results it may be concluded that we are dealing with two different *Artemia* species, i.e. *Artemia parthenogenetica* from Tian Jin and *Artemia* sp. Crossing experiments should be performed with other bisexual *Artemia, i.e.* with the strain (of unknown origin in China) as used by Tobias *et al.* (1980) and by Vanhaecke and Sorgeloos (1980) and with bisexual *Artemia* belonging to well-known sibling species (Bowen *et al.*, 1978). Only then can we know if this bisexual strain belongs to one of the existing species or if it should be designated as a new *Artemia* sibling species.

Since the Tian Jin "Great Wall *Artemia* Cysts" are collected from different solar saltfields in the very extensive Tang Gu area, only detailed survey work can reveal whether the bisexual and parthenogenetic *Artemia* populations are found in different natural habitats or co-occur in the same ponds. Co-occurrence has been reported in Spain (Amat Domenech, 1980). In such mixed populations the sex ratios should differ greatly from 1. It may be that sex ratios of 3 and 5 found in *Artemia* populations in Taiwan (Qiu Jiajin, 1979) and India (Lal Mohan, 1980) are the result of co-existence of parthenogenetic and bisexual *Artemia.*

Acknowledgements

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The effect of diurnal temperature cycles on survival of *Artemia* from different geographical origin

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Abstract

The temperature in a natural *Artemia* habitat usually shows diurnal cyclic variations. It is important for inoculation and culturing purposes to know the survival under such conditions.

The survival of four *Artemia* strains, Great Salt Lake and San Francisco Bay (USA), and Sfax and Mègrine (Tunisia) was studied over a period of 7 days, in different conditions of constant or diurnal fluctuating temperatures at two salinities.

For the Great Salt Lake strain, the temperature fluctuation had no effect on the survival, as compared to constant temperature regimes (at 35 ‰). For the other strains, temperature fluctuations had either no influence, or a beneficial influence on survival.

In general, the tolerance of *Artemia* to high temperatures was higher under conditions of alternating temperatures than of constant ones.

Introduction

When using constant experimental temperatures, one is always dealing with animals that are fully acclimated to the temperature. In nature, this is usually not the case. From this point of view, rearing at constant temperatures might be unbiological, in the sense that it does not allow any assertions to be made about the environmental requirements of a species (Laudien, 1973). Growing in conditions of diurnal temperature fluctuations could represent an important prerequisite for the wellbeing and the normal completion of an animal's life cycle (Kinne, 1970). Somero and Hochachka (1976) raise the question whether a regular (predictable) change in habitat temperature can offer favourable potentials for a marine organism.

For *Artemia*, to date no studies have been made on the impact of daily fluctuating temperatures on metabolism or survival. In a first group of experiments, we studied survival as a criterion of tolerance. We chose some biologically important temperature levels, and temperature fluctuations with sharp transmissions from one temperature to another.

Material and methods

The four *Artemia* strains tested were :

1) Great Salt Lake (GSL), Utah, USA (batch 375)

2) San Francisco Bay (SFB), California, USA (batch 2149)

Both belong to the *franciscana* sibling species (Bowen and Sterling, 1978 ; Abreu-Grobois and Beardmore, 1980).

3) Sfax (SFX), Tunisia (batch 502)

4) Mégrine (MEG), Tunisia (batch 361 p).

Both belong to the *tunisiana* sibling species (Van Ballaer *et al.*, 1987).

The *Artemia* cysts were hatched under optimal conditions (25 °C, salinity 35 ‰, 1 000 lux (Sorgeloos, 1980). Instar I nauplii were transfered to 3 ml-plastic containers with 2 ml aerated, filtered (0.22 µ) artificial seawater (HW-Wimex), with a density of 20 organisms/2 ml medium. Twelve replicates were used for each experiment. The fluctuating and constant temperature regimes were obtained by using a precision incubator (Memmert), with cooling set and program controller, enabling to change the incubation temperature every 15 min. In the case of fluctuating temperatures, the transition of one temperature to another was accomplished in 40 min, for increases as well as decreases. When starting experiments with fluctuating temperatures, the organisms were always transferred from the hatching temperature (25 °C) to the lowest temperature in the temperature cycle (*i.e.* either 20 °C or 25 °C), and kept at this level for at least 8 hours before the temperature was raised for the first time.

As food for the *Artemia*, 150 µg/replicate/day of dried *Spirulina* powder was provided (being the optimal dose as determined in preliminary experiments). Every day, the dead animals were counted and removed.

The experimental temperature-salinity conditions are presented in Table I.

TABLE I

Experimental temperature-salinity conditions

Temperature (°C)	Salinity (‰)
25 constant	35
25 constant	120
30 constant	35
30 constant	120
25-35 cycle	35
20-30 cycle	35
20-30 cycle	120
20-35 cycle	35

The 120 ‰ salinity experiments were incorporated for their similarity with inoculation conditions. Survival rates were compared using Student's t-test, $P < 0.05$. The survival data were transformed by means of an arc sin $\sqrt{p_i}$ transformation with p_i = the percentage survival (Sokal and Rohlf, 1981).

Results

The time-% survival data from the four *Artemia* strains and for each experimental condition are presented in the Fig. 1 to 8.

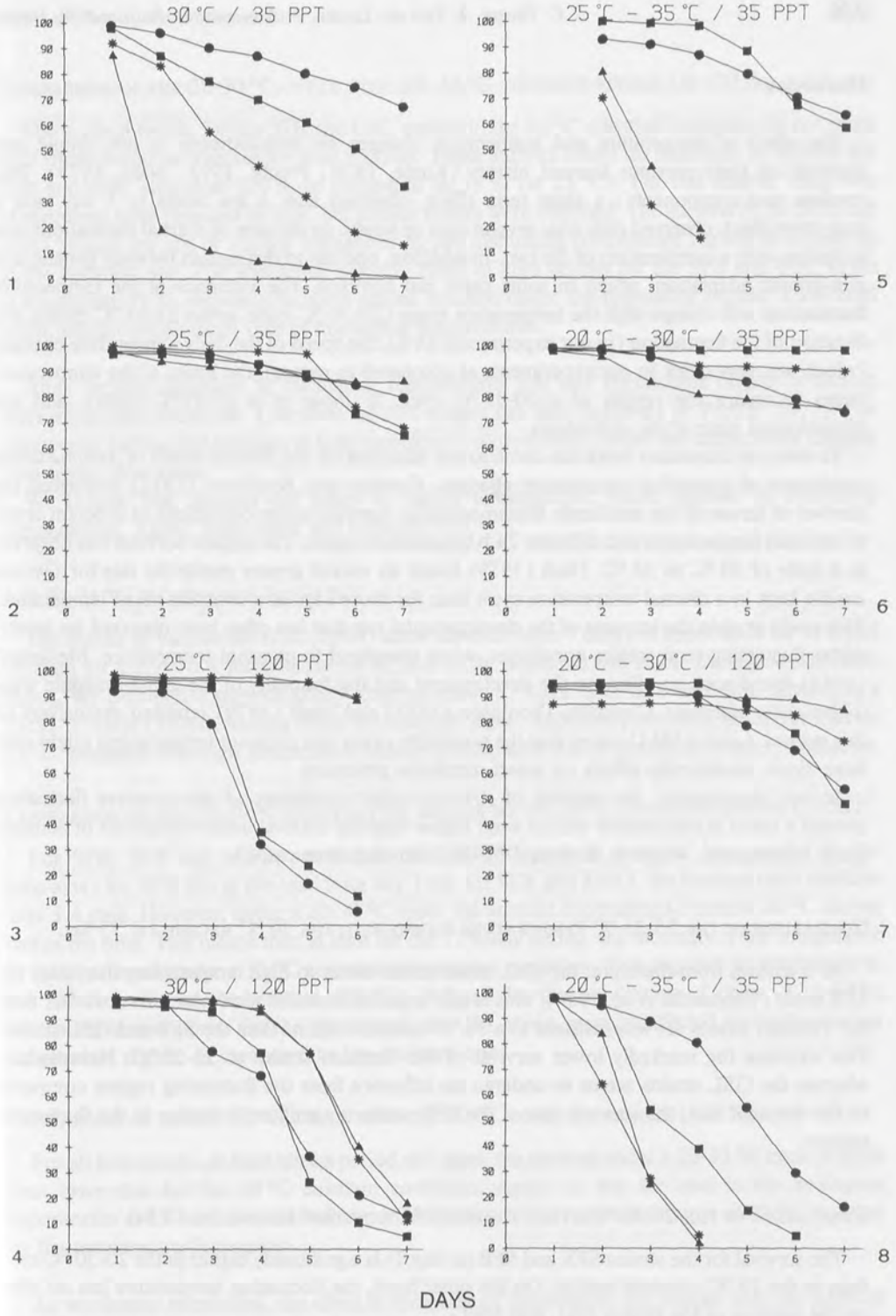

FIG. 1 to 8. Survival as a function of time during 7-days experiments under various temperature-salinity conditions ; (●) GSL ; (■) SFB ; (★) SFX ; (▲) MEG).

Discussion

The effect of temperature and temperature changes on poikilotherms is not simple and depends on their previous thermal history (Kinne, 1970 ; Precht, 1973 ; Nash, 1979). This involves two components : a short term effect, observed after a few hours to 1 day, and a long-term effect, observed only after several days or weeks. In the case of diurnal fluctuations one is dealing with a combination of the two. In addition, one has to distinguish between genetic and non-genetic adaptation, which in some cases can combine. The influence of the temperature fluctuations will change with the temperature range (20-30 °C cycle *versus* 25-35 °C cycle), the duration of the fluctuation (in our experiments 24 h), the speed of the daily temperature changes (which was very quick in our experiments as compared to nature), the limits of the temperature range (compare the results of a 20-35 °C cycle to those of a 25-35 °C cycle), and the physiological state of the individuals.

To date, no laboratory work has come to our attention on the thermal death of *Artemia* under conditions of controlled temperature changes. Costlow and Bookhout (1971) compared the survival of larvae of the mud-crab *Rhithropanopeus harrissii* under conditions of different levels of constant temperatures and different 24 h temperature cycles. The highest survival was observed in a cycle of 30 °C to 35 °C. Nash (1979) found an overall greater metabolic rate for *Oniscus asellus* kept in a diurnal temperature cycle than for those kept at a constant mean temperature. This could explain the increase of the developmental rate that has often been observed for insects under fluctuating temperature conditions, when compared to constant temperature. Messenger (1964) found such an effect on the development and the fecundity of the aphid-braconic wasp *Therioaphis maculata*. Cloudsley-Thompson (1953) and Nash (1979) reported extensively on this subject. Lamb (1961) stated that the possibility exists that constant temperatures might even have direct, undesirable effects on insect metabolic processes.

In our experiments, the survival of *Artemia* under conditions of temperatures fluctuating around a mean is comparable with or even higher than the survival under conditions of constant mean temperature, which is illustrated by the following three cases.

Comparison of the 25-35 °C temperature regime with the 30 °C regime (at 35 ‰)

As is known from literature, the GSL strain resists better to high temperatures than does the SFB strain (Vanhaecke *et al.*, 1984). This is also apparent from the survival results at 30 °C. Both the Tunisian strains are less resistant to a 30 °C constant regime than the SFB and GSL strains. This explains the markedly lower survival of the Tunisian strains at 25-35 °C. Nevertheless, whereas the GSL strains seems to undergo no influence from the fluctuating regime compared to the constant one, the survival rate of the SFB strain is significantly higher in the fluctuating regime.

Comparison of the 20-30 °C cycle to the 25 °C constant regime (at 35 ‰)

The survival for the strains SFX and SFB (at day 7) is significantly higher in the 20-30 °C cycle than in the 25 °C constant regime. On the other hand, the fluctuating temperature has no effect on the survival of the strains GSL and MEG.

COMPARISON OF THE 20-30 °C CYCLE WITH THE 25 °C CONSTANT REGIME (at 120 ‰)

The survival results for the SFB and GSL strains in the 25 °C constant condition do not agree with those found by Vanhaecke *et al.* (1984). These authors found no reduction in survival for SFB and GSL strains at 120 ‰ as compared to 35 ‰ (at 25 °C). For this reason, these two experiments were repeated in time, but similar results were obtained. The survival of the SFX and MEG strains are not or slightly influenced by the fluctuating-temperature regime as compared to the constant one. The apparently negative effect of high salinity for the SFB and GSL strain at a constant 25 °C regime is to some extent lessened under the fluctuating regime. This could again indicate a beneficial role of fluctuating temperatures.

After variable exposure to fluctuating temperatures fish and crustaceans display a distinct increase in heat resistance. Literature on this subject has been reviewed by Precht (1973). For *Gammarus salinus* this increase in heat resistance is more distinct under fast temperature changes than under slow ones.

We believe that *Artemia* can adapt to higher temperatures, when exposed to fluctuating temperatures. Again, three cases will illustrate this.

THE 25-35 °C FLUCTUATION (at 35 ‰)

The results of Vanhaecke *et al.* (1984) show survival (after 7 days) of about 10 % for SFB and between 90 % and 70 % for GSL, at 34 °C and 35 ‰, when fed live algae, *Dunaliella tertiolecta* Butch. We obtained a survival after 7 days of about 65 % for both SFB and GSL strains, at a 25-35 °C cycle (35 ‰), feeding dried *Spirulina* sp. algae. Apparently, the SFB strain can tolerate 35 °C whenever this high temperature regime is alternated with a period of lower temperature.

COMPARISON OF THE 20-30 °C TEMPERATURE REGIME TO THE 30 °C CONSTANT REGIME (35 ‰)

For SFB, SFX and MEG, survival in the 20-30 °C cycle is more or less parallel to the time-axis ; for SFB this is the case from day 1 on, for SFX and MEG, the survival rates stabilize after 3-4 days. However, under a 20-30 °C cycle, the animals experience a constant 30 °C during half of the time. This means that, at least for the 12 h/day period, the mortality is not comparable to the one found under a 30 °C constant temperature condition. This suggests an adaptation to the relatively higher temperature (30 °C), induced by the conditions of daily (12 h-12 h) fluctuating temperatures. On the other hand, the GSL strain seems unaffected by the fluctuating temperature regime as compared to the constant one.

COMPARISON OF THE 20-30 °C CYCLE WITH THE 30 °C CONSTANT REGIME (120 ‰)

For all four strains, at least after a period of 7 days, the survival under a 20-30 °C cycle is more than twice that for the 30 °C constant condition. Again, as was the case in the analogous experiments at 35 ‰, this could indicate an adaptation to the high temperature of 30 °C, induced by the temperature fluctuation.

As we already mentioned, the effect of temperature fluctuations will change with the limits of the temperature range. This is obvious when we compare the results of a 20-35 °C cycle with

those of a 25-35 °C cycle. For the four strains, survival under a 25-35 °C cycle is significantly higher than the survival under a 20-35 °C cycle. The capability of SFB to increase its resistance to higher temperature, which was the case under the 25-35 °C cycle, is no longer present under the 20-35 °C cycle.

The body temperature of aquatic invertebrates closely follows that of the environment. This means that, in conditions of temperature fluctuations, an organism has to be able to maintain its physiological functions in spite of changes in body temperature. The main mechanism to compensate for these changes in temperature is metabolic adaptation. Four groups of control of the many different enzymatic reactions can be distinguished : 1) change in the enzyme concentration with changing temperature ; 2) change in substrate and cofactor concentration ; 3) modulation of enzyme activities ; and 4) formation of enzymes with different catalytic properties (Somero and Hochachka, 1976). Two mechanisms seem most likely to be responsible for the adaptive capacity of an organism to the diurnal changing temperatures : the system of producing different isoenzymes with a different temperature optimum, and the temperature-dependent interconversion between different conformational states of the same enzyme (Kinne, 1970 ; Precht, 1973 ; Gilles, 1975 ; Addink and Zandee, 1978).

Conclusions

1. The survival of SFX, MEG and SFB under conditions of temperatures fluctuating around a mean is comparable with or even higher than the survival in the conditions of the constant mean temperatures.
2. *Artemia* can tolerate higher temperatures when exposed to conditions of alternating temperatures, as compared to conditions of constant temperatures.
3. This increase in tolerance diminishes when the difference between the two temperatures is too large.
4. At least for 35 ‰, the GSL strain seems to undergo no influence from temperature fluctuations around a mean, as compared with constant mean temperature conditions.

Acknowledgements

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Preliminary data on the heritability of some quantitative characteristics in *Artemia*

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Abstract

A standard method has been developed to produce F_1-generation cysts from different zygogenetic strains and their cross-breds. The F_1 cysts were used to determine the quantitative heritability of the following characteristics : cyst hatchability, cyst diameter, larval growth, and temperature resistance of the freshly-hatched nauplii.

Data are given for cysts produced from nine strains and seven cross-breds. Important differences for all characteristics studied were noted between the cross-breds and the parental strains.

Introduction

The worldwide distribution of the brine shrimp *Artemia* in a variety of isolated habitats, characterized by climatological and physico-chemical conditions has resulted in the existence of numerous geographical strains. A recent list of *Artemia* sites compiled by Vanhaecke *et al.* (1987) extends to over 350 localities. Among these strains a high degree of genetic variability (review by Abreu-Grobois, 1987) as well as unique diversity in various quantitative characteristics (Vanhaecke, 1983) have been observed. Some of these characteristics (*e.g.* the nutritional value of the freshly-hatched nauplii) are phenotypical (Léger *et al.*, 1986) and change from year to year or season to season. Others, however, (*e.g.* cyst diameter, growth rate, resistance to high temperature) are strain specific and remain constant (Vanhaecke and Sorgeloos, 1980ab ; Vanhaecke *et al.*, 1984), *i.e.* they have become genotypical as a result of long-term adaptations of the strain to local conditions. While the nutritional value can be manipulated (review by Léger *et al.*, 1986), changes in strain specific characteristics to better suit their use for aquacultural purposes will require selection of strains and/or their cross-breds. For a large number of strains Vanhaecke (1983) determined quantitative strain-specific characteristics providing a basis for selection of the most suitable strain for specific aquacultural purposes. The production of cross-breds combining well-defined desirable characteristics selected from parental strains into new genotypes (eventually exploiting heterotic effects) could provide further improvements for *Artemia* use in aquaculture.

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For the present study, cyst offspring was produced from various pure-bred strains and some of their mutual crosses all cultured under standard conditions. The cysts were used to verify strain specificity (genetic contribution) and to determine the heritability of the following characteristics into the cross-breds : cyst hatchability, cyst diameter, larval growth, and temperature resistance of the freshly-hatched nauplii.

Materials and methods

Geographical origin, sibling species, and abbreviations of the strains used in this study are given in Table I. Prior to forming the pure-bred and cross-bred mating populations, *Artemia* strains were separately grown from freshly-hatched nauplii to sexually distinct preadults. These 9-day cultures were carried out in 800 ml cylindroconical tubes filled with 35 or 70 ‰ artificial seawater (Lavens *et al.*, 1985) at a temperature of 25 °C. Each cone was inoculated at a density of 2 nauplii/ml. The *Artemia* larvae were fed daily with a defined number of *Dunaliella tertiolecta* cells (see feeding scheme in Table II, adapted from Vanhaecke and Cooreman, 1979) which sustained good growth and survival in all strains. On day 10, sexually-distinct preadult males and females from the zygogenetic strains were separated and further cultured under the same conditions as above.

TABLE I

List of *Artemia* strains used in this study

Geographical origin	Sibling species	Abbreviation
San Francisco Bay, USA	*A. franciscana*	SFB
Great Salt Lake, USA	*A. franciscana*	GSL
Chaplin Lake, Canada	*A. franciscana*	ChL
Macau, Brazil	*A. franciscana*	Mac
Manaure, Colombia	*A. franciscana*	Man
Galera Zamba, Colombia	*A. franciscana*	GZ
Shark Bay, Australia	*A. franciscana*	SB
Buenos Aires, Argentina	*A. persimilis*	BA
Tientsin, PR China	*A. parthenogenetica*	Ti

After 1 week of separate culture, 250 ♂♂ and ♀♀ from the same bisexual strain (500 ♀♀ for the parthenogenetic strain from Ti) and from different bisexual strains were brought together in a 5 l aquarium to form respectively pure-bred controls and various combinations of cross-bred populations. Normal culture conditions were 25 °C, 90 ‰ seawater, and optimal feeding regime (Table II), although some populations were also kept at a salinity of 35 ‰ and under suboptimal feeding conditions (lower number of cells).

The following routine manipulations were performed weekly : change of culture medium, collection and storage (in saturated brine) of produced cysts, removal of eventual ovoviviparous offspring ; adjustment of feeding quantity and culture volume (1 animal/10 ml of culture medium) determined from survival of animals.

TABLE II

Daily amounts of *Dunaliella* cells (in 10^6) per 100 *Artemia*

Age of *Artemia* (in days)	Daily food regime (in 10^6 cells)
1	15
2	30
5,6	45
7	60
8	75
9	112
10,11	114
12,13	180
14,15	216
16,17	252
18,19	306
20,21	360
22,23	396
24,25	450
All following days	450

Cysts stored in brine were processed using the biphase flotation method as described in Sorgeloos *et al.* (1986), oven dried at 35 °C during 24 h and preserved under vacuum at ambient temperature for up to a few months prior to characterization. Hatchability of cysts was evaluated by determining the hatching percentage under optimal conditions (35 ‰ seawater, 25 °C, continuous illumination with an intensity of 1 000 lux (Sorgeloos *et al.*, 1986). Analysis of the cyst diameter was performed by microscopical measurement of 100 fully hydrated cysts (conditioned according to Vanhaecke *et al.*, 1980) under a microscope equipped with a calibrated micrometer eyepiece. Cyst batch SFB 288-2596 used by Vanhaecke for his characterisation studies was used here as a control. The larval growth of *Artemia* was determined by the standard culture test of Vanhaecke and Sorgeloos (1980b). Growth results are expressed as percentage of the growth recorded for the reference strain (SFB 288-2596). Resistance of *Artemia* to high temperatures was studied in a 9-day culture test at 35 ‰ and 34 °C following the same procedure as used by Vanhaecke *et al.*, (1984) but with omission of the other combinations of temperature and salinity. The GSL strain, batch 1977, which is known for its high temperature resistance, was included as an inner control.

Results and discussion

Table III summarizes the hatching results of cysts produced from various pure-breds and cross-breds kept under identical as well as different conditions of salinity and feeding regime. Very important differences are noted among pure-bred strains cultured under identical conditions. The highest values are obtained for SFB pure-breds, while GSL and ChL pure-breds record lowest values. Only the Mac pure-bred strain approximates the results obtained with SFB pure-breds. The differences in hatching results found between pure-bred strains and their mutual cross-breds suggest the existence of genetic control on hatchability, *e.g.* the values obtained for

the cross-breds are mostly intermediate to these of both parental pure-breds. The observed differences in hatching percentage might have been masked by a different regulation of diapause inhibition, which also appears to be strain specific and most probably under genetical control (Lavens and Sorgeloos, 1987). Application of specific diapause deactivation techniques (*e.g.* freezing or peroxide treatment of the cysts) can significantly influence the hatching characteristics of dormant cysts in at least some strains (Lavens *et al.*, 1986) but none of these were applied in the present experiment. This strain specific character of diapause deactivation sensivity is also hypothesized by Lavens *et al.*, (1986) and Lavens and Sorgeloos (1987), who explained it as originating from a long-term genetic adaptation of the strain to the specific climatic conditions of their habitat. Our hatching results furthermore indicate that besides genetic influences, cyst hatchability is also determined by the environmental conditions in which the reproducing populations are kept, *i.e.* hatchability is higher in cysts produced from populations kept under optimal feeding conditions and high salinity (90 ‰) than those produced under suboptimal feeding regime and low salinity (35 ‰). A similar interaction between hatchability of cysts and culture conditions was demonstrated by Lavens *et al.* (1986), who could significantly improve the hatchability of laboratory-produced cysts from the Lavalduc and SFB strain by increasing the food levels in the parental cultures (review by Lavens and Sorgeloos, 1987).

TABLE III

Hatching percentage of cysts produced under different culture conditions from pure-bred and cross-bred *Artemia*

Strain combination	Salinity of culture medium	
	35 ‰	90 ‰
GSL × GSL	11.1	35.1
SFB	–[a]	84.4
Ti × Ti	51.8 (30.1)[b]	60.2
ChL × ChL	14.5 (7.1)	19.2
Mac × Mac	–	74.4
BA × BA	–	55.4
ShB × ShB	61.7 (53.4)	–
GZ × GZ	–	32.8
Man × Man	–	62.8
GSL ♀ × SFB ♂	66.1 (44.3)	73.1
GSL ♂ × SFB ♀	45.9	51.0
GZ ♀ × SFB ♂	–	42.1
GZ ♂ × SFB ♀	–	43.0
Man ♀ × SFB ♂	–	71.1
Man ♂ × SFB ♀	–	73.1

[a] No data available.

[b] In parentheses the hatching percentage of cysts produced under suboptimal feeding conditions is given.

Diameters of cysts produced from some pure-breds and cross-breds are given in Table IV. Significant differences in cyst diameter found among pure-breds produced under identical conditions confirm the hypothesis of Vanhaecke and Sorgeloos (1980a) that cyst diameter is a

genotypical strain characteristic. However, most cysts produced from pure-breds are smaller than their parental material (Vanhaecke and Sorgeloos, 1980a ; Vanhaecke, 1983). Moreover, different culture conditions (feeding regime and salinity) seem also to affect the diameter of the cysts produced. Vanhaecke and Sorgeloos (1980a) reported but a few cases of significant differences among cyst batches of the same strain, *i.e.* lab produced *versus* their original material, cysts collected from populations transplanted into different countries *versus* their parental stock (seeding) material. These observations, together with our results allow to suggest that aside from genetic factors, the cyst diameter is also controlled by environmental factors. From the values obtained for the cross-breds it appears that cyst diameter is not equally inherited from both parental strains but rather from the mother shrimp. Instead of being intermediate between both parentals, the cyst diameters of the cross-breds approximate those of their respective female parentals, irrespective of the male population crossed with. This suggests, that the genetic factors determining cyst diameter are sex linked.

TABLE IV

Diameter of cysts (in µm) produced under different culture conditions from pure-bred and cross-bred *Artemia*

Strain combination	Salinity of culture medium	
	35 ‰	90 ‰
Ti × Ti	265.2 (256.8)[a]	247.5
GSL × GSL	237.2	233.3
SFB × SFB	–[b]	219.8
BA × BA	–	254.6
Man × Man	–	218.0
GZ × GZ	–	231.7
GSL♀ × SFB♂	231.2 (222.1)	230.7
GSL♂ × SFB♀	–	221.6
GZ♀ × SFB♂	–	231.0
GZ♂ × SFB♀	–	222.3
Man♀ × GSL♂	–	223.5
Man♂ × GSL♀	–	237.3
GZ♀ × GSL♂	–	232.6
GZ♂ × GSL♀	–	233.9
Man♀ × SFB♂	–	219.6
Man♂ × SFB♀	–	216.7

[a] In parentheses the diameter of cysts produced under suboptimal feeding conditions is given.

[b] No data available.

Data on survival at high temperature (34 °C) of the F_1-generation nauplii produced from some pure-breds and cross-breds are given in Table V. The pure-breds display similar tolerances towards high temperature as reported earlier by Vanhaecke *et al.* (1984) for the parental material they used, *i.e.* moderately low temperature resistance of SFB and Ti parental material and high temperature tolerance of GSL, GZ, and Man parental material, is reflected in the respective pure-breds produced in our experiments. This suggests that temperature resistance is (at least

partly) under genetic control. Since our tests were limited to pure-breds produced under identical conditions, a possible interaction of environmental factors (different culture conditions) on temperature resistance of produced offspring, could not be quantified. Crosses between the least temperature resistant strain (SFB) and different high temperature resistant strains (GSL, GZ, and Man) yield values which always are closest to the high temperature resistant parental one. This indicates that high temperature resistance is dominant over low temperature resistance. Crosses between two strains both showing high temperature resistance, do not seem to give notably better temperature resistance, suggesting that for these characteristics heterosis cannot be exploited by cross-breeding.

TABLE V

Survival of pure-bred and cross-bred *Artemia* F1 offspring reared at 34 °C and 35 ‰

Strain combinations	Survival (%)
SFB × SFB	12
Ti × Ti	17
GSL × GSL	63
Man × Man	57
GZ × GZ	76
GSL♂ × SFB♀	46
GSL♀ × SFB♂	45
GZ♂ × SFB♀	65
GZ♀ × SFB♂	67
Man♂ × SFB♀	50
Man♀ × SFB♂	67
Man♂ × GSL♀	69
Man♀ × GSL♂	75
GZ♂ × GSL♀	74

TABLE VI

Growth and survival of pure-bred and cross-bred *Artemia* F1 offspring after 7 days culturing

Strain combinations	Produced at 35 ‰		Produced at 90 ‰	
	Survival (%)	Growth[a]	Survival (%)	Growth
GSL × GSL	72	115	92	115
SFB × SFB	–[b]	–	72	57
GZ × GZ	–	–	90	123
GSL♀ × SFB♂	94	100	–	–
GSL♂ × SFB♀	90	100	–	–
GZ♀ × SFB♂	–	–	83	103
GZ♂ × SFB♀	–	–	76	97
GZ♀ × GSL♂	–	–	90	122
GZ♂ × GSL♀	–	–	84	117

[a] In % of the figure obtained with the reference strain SFB 288-2596.

[b] No date available.

Growth and survival data of F_1 larvae from some pure-breds and cross-breds are given in Table VI. Our results seem to confirm the findings of Vanhaecke and Sorgeloos (1980b) that growth is a strain specific characteristic ; *i.e.* the pure-breds produced in our experiment exhibit growth performances comparable to their respective parental strains. Similar growth rates for GSL pure-breds produced at 35 and 90 ‰ furthermore suggest that environmental factors (at least salinity) do not interact. The few data obtained for cross-breds seem to indicate that high growth rate cannot be cross-bred. Crosses between SFB and strains with higher growth rate (GSL and GZ) yield values close to the lower growth rate of SFB.

Conclusions

This study provides extra evidence on the contribution of genetic and environmental factors in the expression of various quantitative characteristics, and on the differential inheritance of these characteristics in cross-breds of *Artemia*. Although no superior characteristics could be obtained through cross-breeding, our results indicate that cross-breeding could yield new *Artemia* strains with combined desirable characteristics which are normally only found in separate strains. In this regard cross-breeding might become an interesting tool to complement artificially selected traits with antagonistic desirable characteristics in order to create a strain with overall superior performance. Prior to consider the use of new cross-breds in inoculation/transplantation work it is essential to further study the inheritance characteristics of these new cross-bred lines over many generations.

Acknowledgements

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Reproduction and genetics of Mexican *Artemia*

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Abstract

The purpose of this paper is the characterization of four Mexican *Artemia* populations. Reproductive isolation was determined through interbreeding in both reciprocal ways. It was found that all of the interbreeding was fertile. Reproduction patterns were also determined ; it was found that the *Artemia* were bisexual. From all the observations, only in one bottle with three females from San Crisanto strain were nauplii produced parthenogenetically. In relation to chromosome number we could observe only some metaphases which showed 42 chromosomes.

Introduction

The present work is one of a large program on *Artemia*, which is being carried out at the Universidad Autónoma Metropolitana-Xochimilco. The objective of this program is a better knowledge of this crustacean for use as food to develop aquaculture in México.

The purpose of this paper is the characterization of four *Artemia* populations from the following sites : Bahia de Ceuta, Sinaloa ; Yavaros, Sonora ; San Crisanto, Yucatán, and Ecatepec, State of México, the latter being introduced from San Francisco Bay, California, USA, in December, 1975.

Previous to this work, only a few aspects of the characterization of the Mexican *Artemia* populations have been studied (Bowen, 1964 ; Abreu-Grobois and Beardmore, 1980).

The criteria employed to characterize the four populations were reproduction patterns and reproductive isolation. In addition, observations were made of some chromosome preparations. These characteristics have been studied in *Artemia* populations in other parts of the world and there have been indications of the existence of isolation among populations (Barigozzi, 1974, 1980).

Bowen (1964) in her hybridization work made several crosses with the purpose of investigating reproductive isolation among the populations studied. She mentioned that the only two studies in this field had been done by Barigozzi and Tosi (1959) and Gilchrist (1960).

Materials and methods

The chromosome observations were made on nauplii immediately after hatching. The four populations were handled according to the Barigozzi technique (Barigozzi, pers. commun.), which consists in putting the nauplii in a hypotonic solution of 0.5 % sodium citrate for 40 min.

Later the nauplii were transferred into a 1:1 acetic acid-methanol solution for 3 min, followed by 60 % acetic acid for 30 s at 40 °C. Each nauplius is macerated to disintegrate the cells and promote the dispersion of chromosomes. The preparations were stained with natural 2 % orcein and dehydrated with 95 % alcohol, finally mounted in Balsam.

To obtain an average of 30 metaphases for each population, 8 to 15 preparations were observed (three nauplii in each one).

For the studies of reproductive isolation and reproduction patterns we hatched 0.2 g of cysts in 500 ml of seawater (32 ‰ S) in inverted bottles. After 27 h the nauplii were transferred to aquaria of 4 l capacity filled with seawater. After 15-20 days, the *Artemia* reached sexual maturity and could be sexed without any difficulty. Males and females were separated, transferred to 250 ml bottles and observed for 1 week to make sure that the females were not fertilized.

During the time that the nauplii and adults remained in the aquarium or in the bottles, they were fed *Chlamydomonas* sp. each 3rd day and maintained under equal conditions of illumination, aeration, salinity, and temperature. After 1 week of observations two males and one female were put in a 250 ml bottle with artificial seawater and fed also with *Chlamydomonas* sp.

The (F_1) nauplii of each interbreeding were grown until they reached sexual maturity and were then interbred to obtain F_2 nauplii. Control crosses within each strain were made.

At the same time, in each test, three females were put in a bottle to observe the behavior and investigate possible parthenogenetic reproduction.

Results and discussion

The ploidy level was difficult to establish, however, 42 chromosomes could be counted in some preparations (Fig. 1-3). On the other hand, most of the crosses were fertile, which suggests they have the same ploidy level.

All crosses produced F_1 nauplii, which eventually proved to be fertile and produced F_2 nauplii, so there appears to be no reproductive isolation (Table I).

TABLE I

Results of reciprocal crosses of four wild *Artemia* populations from Mexico

Parental cross Male – Female	Fertile pairs/ total matings	Hybrids production	F_2 production
♀ Ceuta-♂ Texcoco	5/11	Yes	Yes
♂ Ceuta-♀ Texcoco	6/7	"	"
♀ Ceuta-♂ Yavaros	3/11	"	"
♂ Ceuta-♀ Yavaros	3/7	"	"
♀ Texcoco-♂ Yavaros	3/11	"	"
♂ Texcoco-♀ Yavaros	2/7	"	"
♀ Texcoco-♂ San Crisanto	7/7	"	"
♂ Texcoco-♀ San Crisanto	9/11	"	"
♀ Yavaros-♂ San Crisanto	4/7	"	"
♂ Yavaros-♀ San Crisanto	5/11	"	"
♂ Ceuta-♀ Ceuta	4/15	"	"
♂ Texcoco-♀ Texcoco	8/11	"	"
♂ Yavaros-♀ Yavaros	6/15	"	"
♂ San Crisanto-♀ San Crisanto	10/15	"	"

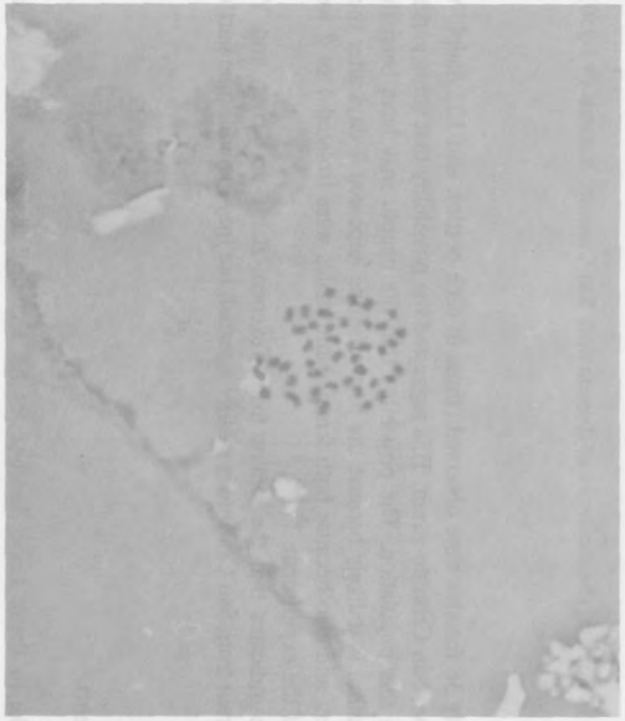

FIG. 1. Somatic chromosomes of *Artemia* from Bahia de Ceuta, Sinaloa (× 800).

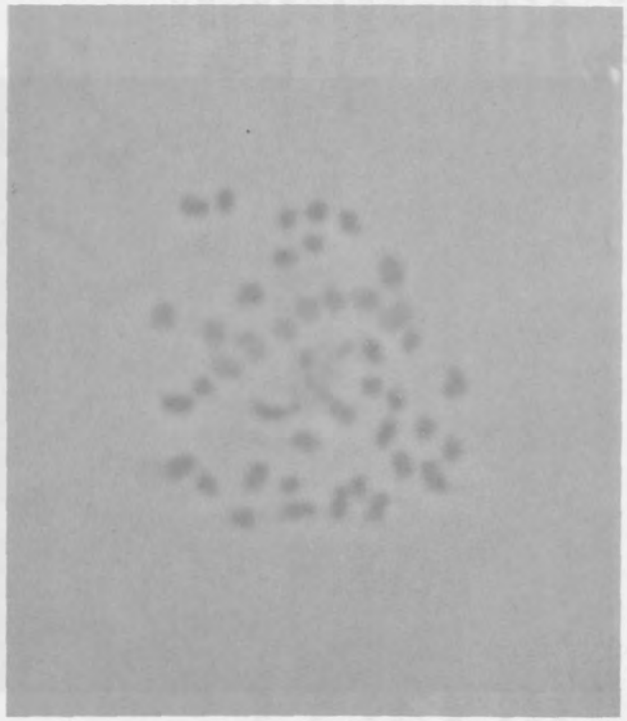

FIG. 2. Somatic chromosomes of *Artemia* from Yavaros, Sonora (× 1 250).

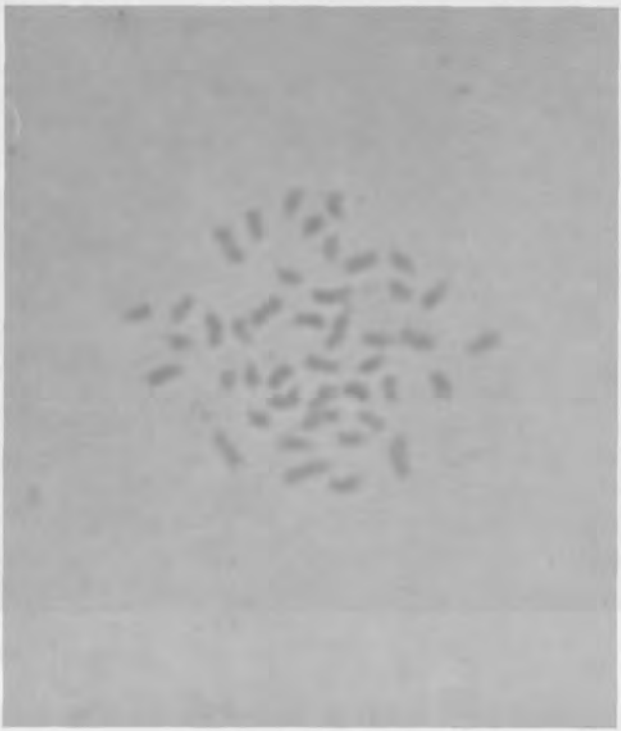

FIG. 3. Somatic chromosomes of *Artemia* from San Crisanto, Yucatan (× 1 250).

Simultaneously 36 females were observed (three in each bottle), and 132 nauplii were found in one bottle from San Crisanto strain. This suggested the possibility that some parthenogenetic females exist in this population. The possibility that the nauplii may have been the result of previous fertilization is unlikely because the first offspring appeared 7 days after the beginning of the experiment and also because both females and males were isolated 1 or 2 weeks before the beginning of the experiment.

The evidence indicates that the strains from Texcoco, Bahia de Ceuta, and Yavaros are bisexual. The strain from San Crisanto is mainly bisexual, but may include some parthenogenetic animals.

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BOWEN S. T. 1964. The genetics of *Artemia salina*. IV. Hybridization of wild populations with mutant stocks. Biol. Bull. 126(3):333-344.

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

Toxicology

This section contains material from two subject areas, ecotoxicology and radiobiology, each of which is reviewed in the initial papers (1-2). The abstract of Radchenko (3) represents the only experimental paper in radiobiology presented at this symposium. The subsequent experimental papers on ecotoxicology cover lethal effects on *Artemia* of inorganic chemicals (4-5), reference toxicants under different environmental conditions (6), and organic chemicals (7-8) as well as sublethal responses at the physiological (9) or behavioral (10) level.

The workshop report (11), clearly states the present paradox of *Artemia* usage in ecotoxicology, *i.e.* the toxicity test is standardized and convenient, but the applicability to the natural environment has yet to be demonstrated.

(1) G. Persoone and P. G. Wells.
Artemia in aquatic toxicology : a review

(2) R. P. McCourt.
The radiobiology of *Artemia* : a review.

(3) L. A. Radchenko.
The combined effect of temperature, salinity, and gamma-radiation on biological characteristics of *Artemia.*

(4) R. Blust, F. Bernaerts, A. Van der Linden, and C. Thoeye.
The influence of aqueous copper chemistry on the uptake and toxicity of copper in *Artemia.*

(5) B. L. Freeman, R. L. Bernstein, and S. T. Bowen.
Selenium toxicity in two populations of *Artemia franciscana.*

(6) G. Persoone and M. Van Steertegem.
The influence of temperature and salinity on the sensitivity of *Artemia* nauplii to chemical compounds.

(7) J. Castritsi-Catharios.
Short-term toxicity tests with oil dispersant mixtures using two *Artemia* strains.

(8) M. Moraitou-Apostolopoulou and G. Verriopoulos.
Effects of pre-exposure on the tolerance of *Artemia* to oil and oil dispersants.

(9) J. L. Littlepage and A. Guevara.
Effect of salinity and sodium lauryl sulphate on adenosine triphosphate levels in *Artemia* nauplii.

10) N. M. Trieff, J. P. Saunders, E. E. Kalmaz, and T. Uchida.
Measurement of photoattraction of *Artemia* nauplii : effect of mercuric ion.

11) A. D. Beck, M. D. Johns, and M. Van Steertegem.
Workshop report : Ecotoxicology.

Artemia *Research and its Applications. 1987. Vol. 1. Morphology, Genetics, Strain characterization, Toxicology*
P. Sorgeloos, D. A. Bengtson, W. Decleir, and E. Jaspers (Eds). Universa Press, Wetteren, Belgium. 380 p.

Artemia in aquatic toxicology : a review

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Abstract

Due to the commercial availability of dried cysts from which live test material can be hatched at will, *Artemia* is used extensively in research and applied toxicology.

Despite the extensive literature on dose-effect relationships of chemicals on brine shrimp, it was not until 1980 that an experimental protocol was developed for a simple acute toxicity test with *Artemia* nauplii, meeting the prerequisites for standardization.

The reliability and accuracy of this short-term test were determined during an intercalibration exercise involving 80 laboratories and were found to be quite satisfactory. Consequently, the so-called ARC test, which is one of the very few standardized marine toxicity tests, is now used routinely at the international level.

Recent research on the use of *Artemia* in ecotoxicology has focused on the development of testing procedures and screening bioassays with sublethal responses. The medical, drug, and food sectors seem to use *Artemia* assays as frequently as laboratories investigating environmental concerns.

Toxicity tests with brine shrimp have a significant potential in QSAR research because of their simplicity, rapidity, and cost-effectiveness. *Artemia* tests also have a good predictive potential as alternatives for other crustacean test species.

This review postulates the future role of *Artemia* tests in aquatic toxicology to be that of a reference or quality control in rapid screening tests, as much as that of a predictor of chemical effects on species in marine environments.

ntroduction

Artemia continues to be used extensively in research and applied toxicology laboratories orldwide. Uses include the investigation of sources of toxicity in chemical mixtures and nvironmental samples, the acute screening of chemicals, the detection of natural toxins in odstuffs and in pharmaceuticals, the study of models of toxic action of substances, and the study f the trophic transfer of pollutants. *Artemia* is proving to be a versatile and valuable organism single-species toxicity tests, particularly if studied with other endemic species. This brief review escribes recent studies, programs, and developments within this wide range of applications and iscusses *Artemia*'s future role in basic and applied aquatic toxicology.

Hazard assessment

The hazard resulting from the release of anthropogenic chemicals into aquatic environments is a function of the probability and intensity of the exposure of biological systems to the chemicals, and of the potential of chemicals to harm biological systems, which in turn depends upon the chemical's physico-chemical properties and the unique characteristics of the exposed biota.

Hence, hazard assessment strategies always include two components :

1) the exposure analysis to determine the concentration of the pollutant at a particular time and place ;
2) the effects analysis to determine the negative effects which the chemical may exert on biota living at the site of concern.

Such strategies have been described in many recent documents (*e.g.* Bergman *et al.*, 1986).

Butler (1978) defined ecotoxicology as "the science concerned with the toxic effects of chemical and physical agents on living organisms, especially on populations and communities within defined ecosystems, including the transfer pathways of those agents and their interactions with the environment". Consequently, testing of the effects of man-made chemicals should in principle always be carried out on multispecies systems, such as micro-ecosystems (*i.e.* microcosms, mesocosms) which simulate natural conditions (National Research Council, 1981 ; Cairns, 1985). Calamari *et al.* (1985), however, portrayed the inverse relationship existing between ecological realism and simplicity of testing in test systems of increasing complexity (Fig. 1). With regard to species and response criteria, Persoone (1980) on the other hand, showed the inverse relationship existing between the ecological realism and the sensitivity and costs of bioassays (Fig. 2). Most of the ecotoxicological knowledge to-date is based on single-species testing, the majority being acute tests for reasons of practicality, reliability, and general application. Bioassays with *Artemia* rank highly as candidates for rapid and cost-effective routine bioassays in hazard assessment schemes incorporating single-species and multiple-species approaches (Hammons, 1981 ; National Research Council, 1981 ; Cairns, 1985).

Development of a short-term *Artemia* test

During the past 30 years, many papers have been published on the effects of chemicals on brine shrimp, using different procedures, response criteria, life stages, and durations of the tests (see updated bibliography on *Artemia* by McCourt and Lavens, 1985). Research on *Artemia* ecotoxicology was initiated in 1975 at the State University of Ghent in Belgium, to evaluate the usefulness and reliability of different published toxicity testing methods with brine shrimp (Vanhaecke *et al.*, 1980). This evaluation and our own experimentation showed that none of the published methods were acceptable for use in a standardized, acute routine test.

Hence, a list of theoretical prerequisites and important parameters was derived for developing a simple and reliable screening test with *Artemia*. Following existing methods, an experimental protocol for a routine toxicity test was developed, called the Artemia Reference Center (ARC) test. Four decisions were made : the type of test was static, the duration was 24 h, the life stages were nauplii, and the response criterion was mortality, expressed as an LC50. After 2 years of research, the accuracy, reliability and reproducibility of the ARC-test were considered to be acceptable. The test was submitted for criticism to a special workshop on *Artemia* toxicity tests

during the First International Symposium on The Brine Shrimp, held at Corpus Christi, Texas, in 1979 (Persoone *et al.*, 1980). The test procedure was considered logical and well-developed. A recommendation was formulated that the reliability, accuracy, and precision of the bioassay in the various laboratories should be determined by a Round Robin (Intercalibration) Exercise (Persoone and d'Agostino, 1980).

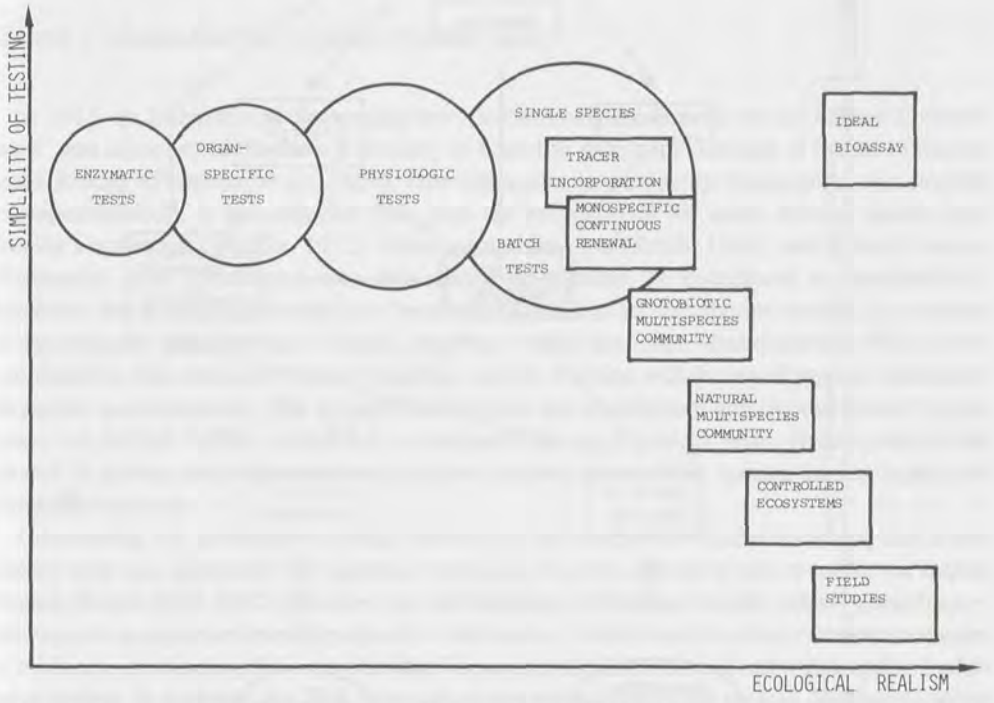


FIG. 1. Inverse relationship between simplicity and ecological realism in test systems of increasing complexity (modified from Calamari *et al.*, 1985).

Intercalibration exercise-ARC test

A call for participation in the Round Robin Exercise was sent to a large number of institutes, laboratories, and companies throughout Europe in late 1980. A similar exercise in North America was launched from the Freshwater Institute, Winnipeg, Canada. Positive replies were received – approximately 100 from Europe and 125 from Canada and the USA. Each laboratory was then provided with materials (cysts, seawater salts, reference chemicals, instructions, reply forms). Sixty European and 20 North American laboratories participated ; the very low response from the American contingency was due to a long postal strike in Canada.

Two points regarding this exercise are important. With 80 replies, this Round Robin on an aquatic toxicity test was the largest study conducted to-date. In addition, for two-thirds of the participating laboratories, the intercalibration exercise was their first experience with *Artemia* as a toxicity test-species. Hence, their personnel had few or no prior skills in hatching cysts, handling nauplii, or making observations during the assays.

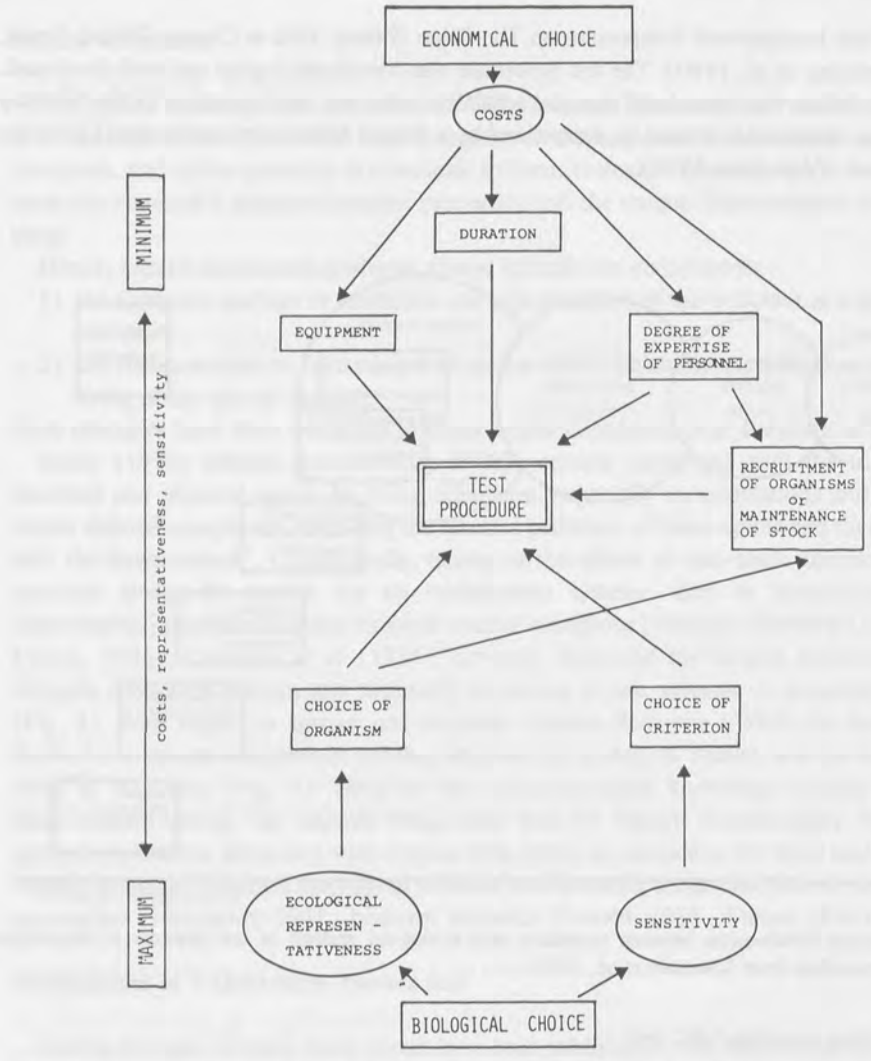


FIG. 2. Interrelationships of the basic factors determining the choice of bioassay test methods (from Persoone, 1980).

Results of the Round Robin were published in a EEC Report (Persoone *et al.*, 1981) and were presented in 1981 at the INSERM Symposium on Acute Aquatic Ecotoxicological Tests in France (Vanhaecke and Persoone, 1981). Most laboratories conducted the prescribed test with relatively few difficulties. Both the intra- and interlaboratory variabilities of the ARC test were satisfactory in comparison to those of other Round Robin tests conducted in Europe for the EEC (*e.g.* the acute *Daphnia* and *Brachydanio* tests, now adopted by the OECD and subsequently endorsed by the EEC). As stated above, two-thirds of the participating laboratories had their first encounter with *Artemia* in this Round Robin, compared to other exercises with *Daphnia* and zebrafish (*Brachydanio rerio*) with which most participants were already familiar. It is likely that with more practice and skill, the repeatability and reproducibility of the ARC test will improve.

The intercalibration exercise was very helpful in identifying weak points in the experimental protocol, resulting in continually improved versions (*e.g.* Wells *et al.*, 1982, 1985). Our collective efforts have led to an acute screening-testing protocol of intermediate sensitivity, satisfactory repeatability and reproducibility, low cost, minimum maintenance of animals, and universal, year-round applicability.

Martox – standardization of marine toxicity tests

In 1983, an International Symposium on "Ecotoxicological Testing for the Marine Environment" was convened at the State University of Ghent to determine the state of the art of marine ecotoxicology (Persoone *et al.*, 1984). One discussion session at the Symposium was devoted to standardization. It was apparent that, with the exception of the acute *Artemia* nauplii test, Woelke's oyster test (Woelke, 1972), Reish's polychaete test (Reish, 1984), and echinoid assays (Kobayashi, pers. commun.) very few marine tests could be considered as standardized. Relatively few Round Robin tests have been carried out thus far with marine species, the number of laboratories participating is small, and the results are often disappointing. With other zooplankton, this situation is now changing, notably for tests with larvae of mysids, copepods, decapods, and echinoids. This is particularly due to the involvement and interest in the United States, of ASTM, APHA, and EPA in standardizing acute toxicity tests. Hence, there soon should be a data base with which to compare *Artemia versus* other species on key aspects of standard protocols.

Considering the advantages of using *Artemia* as a test species for routine bioassays, one would expect wide use, especially for regulatory purposes. In fact, regulatory use at present is largely limited to the 1978 EEC Directive on the dumping of titanium wastes, which prescribes – without giving any experimental protocol – that next to "tests for acute toxicity on certain species of molluscs, crustaceans, fish, and plankton" bioassays should be carried out with larval and adult brine shrimp. In addition, the EPA continues to use *Artemia* for testing oil spill dispersants, along with other crustaceans and fish. Some international conventions, such as the Oslo Convention, have recently excluded *Artemia* as a test organism from their sets of mandatory or recommended bioassays. Consequently the organism and the testing protocol have had a mixed reception.

There are several reasons for opposition to using *Artemia* in regulatory hazard assessments. *Artemia* is not present in the sea, thus it is not a natural or endemic marine organism. However, *Artemia* is highly euryhaline ; it can be cultured at salinities of 5 up to 150 ‰. Since it is not competitive with other zooplankton, it is mainly found in high salinity biotopes, not those of typical estuaries and coastal waters. A second reason is that *Artemia*, because of its specialized tolerance to high salinities, is presumed not to be very sensitive to contaminants. This is usually correct for the mortality criterion, especially compared to other microcrustaceans such as *Pseudocalanus minutus* (see next section). It is debatable however, whether this reason negates the many advantages that *Artemia* offers as a test organism in acute screening assays. This is particularly true when, for some toxicants, the sensitivities of other species are predictable from the *Artemia* data (Wells *et al.*, 1982 ; Abernethy *et al.*, 1986). The third reason for opposition is that some experimenters have had little success with *Artemia*, probably due to incorrect techniques for hatching the cysts and manipulating the nauplii during holding and in experiments ; this reason is particularly invalid for rejecting a valuable reference test organism.

approach, rather than through newer, innovative, multi-species, ecological toxicology. However, the number of reported studies, from many countries, underlines the animals usefulness rather than its limitations.

One area of research for which *Artemia* tests seem to have a significant potential is QSAR (quantitative-structure-activity-relationships). QSAR's have been used extensively and are still used in pharmacology and food science to determine relationships between the structure of related chemicals and their metabolic and toxicological activity within living organisms. The QSAR approach, in use for several decades, has recently been rediscovered and applied by environmental chemists and toxicologists to determine the relationship between selected physico-chemical properties of xenobiotic compounds and their acute lethal and sublethal toxicity (Veith and Konasewitch, 1975 ; Goldberg, 1983 ; Kaiser, 1984). Since QSAR's are in fact based on large series of identically conducted bioassays with many chemicals, in homologous and non-homologous series, it is clear that *Artemia* larvae constitute ideal aquatic test organisms for such research, not the least for cost-effective reasons.

Foster and Tullis (1984) selected the octanol-water partition coefficient as a representative parameter of molecular structure. This factor is used frequently as a rapid predictor of the bioconcentration potential of organic pollutants in water. It also has wide applicability as a predictor of acute toxicity. The acute toxicity to *Artemia* larvae of 11 organic compounds (naphthalene and its derivatives, phenanthrene, pyridine, 1, 2-dichloroethane, chloroform) was determined. A highly positive linear relationship between log P (*i.e.* the octanol-water partition coefficient) and "activity" (log 1/IC50, where IC50 was the median immobilization concentration) was found. The equation (log 1/IC50 = 1.57 + 0.88 × log P) was derived. A general equation for the relationship between *Artemia* naupliar toxicity and the partitioning coefficient of chemicals (log 1/TR + a + b × log P) was developed, in which TR is the measured toxic response.

TABLE

Developments in ecotoxicological research with brine shrimp

Category	Reference	C
1. Reviews	Grozdov *et al.* (1983)	D
	Vanhaecke *et al.* (1981)	D
	Vanhaecke and Persoone (1984)	
	Wells (1984a)	B
		pr
2. Culture for toxicology	Beck and Bengtson (1982)	Ev
		m
	Groat *et al.* (1980)	C
	Sleet and Brendel (1983)	In
		po
	Leonhard (1981)	C
3. Development of bioassays	Amiard-Triquet *et al.* (1981)	D
	Bengtson *et al.* (1984)	D
		th
	Denuit *et al.* (1982)	St
	Kerster and Schaeffer (1983)	D
		ex
	Vanhaecke *et al.* (1980)	D
		ac
	Vanhaecke *et al.* (1981)	Pr
	Vanhaecke and Persoone (1984)	D
		of
4. Screening assays	Adema and Vink (1981)	C
		se
	Amiard-Triquet (1983)	C
	Aubert *et al.* (1983)	To
	Betz and Blogoslawski (1982)	Ev
	Bijl *et al.* (1981)	Ev
		ot
	Bijl *et al.* (1982)	D

We are convinced that, if the standard ARC test was better understood, improved upon by individual investigators, and used actively as one of several marine, single-species screening tests, it would find gradual acceptance as a reference test in the array of toxicity tests and approaches needed for national and international pollution research and control. Interestingly enough, *Artemia* seems to be included more often in manuals describing bioassay procedures for testing chemicals and effluents. The recent US-EPA methods document for testing acute toxicity of industrial effluents (Peltier and Weber, 1985) includes *Artemia* for both food and test organisms. In Canada, Environment Canada (EPS) lists *Artemia* as one of its suggested zooplankton toxicity tests (MacGregor and Wells, 1984). The ARC test is slowly but surely being adopted and used in more laboratories for research, screening, and regulatory purposes.

Developments in ecotoxicological research with brine shrimp since the first *Artemia* symposium, 1979

Table I, which summarizes published work in *Artemia* ecotoxicology since 1979, shows that efforts have been considerable including development of testing procedures, screening bioassays, and lethal and sublethal research assays. The last category represents extensive efforts covering many sublethal responses and environmental samples or suspected toxins and toxicants. The medical, drug- and food sectors use the assays as frequently as those laboratories investigating environmental problems. The brine shrimp is used primarily with the classical aquatic toxicology

since the first International *Artemia* Symposium (1979)

	omments
	escription of bioassays used to assess marine pollution. Includes *Artemia*.
	escription of methodology of short-term standardized test with nauplii.
	ief review of current use and continued development of *Artemia* toxicity ocedures.
	aluation of five strains of *Artemia* used as diet for Atlantic silversides, *Menidia enidia*, used in toxicological studies. Standard strain is recommended.
	ulturing of *Artemia* for toxicological studies with *Aurelia aurita* larvae.
	nprovement of methods for harvesting and counting nauplii from synchronous opulations.
	ulturing technique for *Artemia* used in toxicology.
	velopment of acute toxicity procedures with *Artemia*.
	emonstration of *Artemia* diet quality effects on the results of toxicity tests with ree species of marine organisms.
	udy of the effect of developmental stage on *Artemia* sensitivity to metals.
	evelopment of teratogen test system based on disrupted elongation of nauplii, posed to wide range of contaminants.
	escription of seven factors crucial to acceptable reproducibility of a routine, ute toxicity test with nauplii.
	oposal of a standard procedure for acute toxicity test with nauplii.
	etailed description of a standard acute toxicity test with nauplii and evaluation intra- and interlaboratory variation of results with two chemicals.
	omparison of toxicity of dieldrin to three crustaceans. *Artemia* nauplii most nsitive.
	omparison of sensitivities of several developmental stages of several organisms.
	oxicity of silicon compounds to *Artemia*.
	valuation of toxicity of dinoflagellates using an LD50 (ingestion) shrimp test.
	valuation of mycotoxin toxicity. *Artemia* preferred for simplicity of test to five her species.
	tection of trichothecenes in food with the aid of *Artemia* bioassay.

TABLE I. Continued

Category	Reference	Comments
	Chattopadhyay (1983)	Study of pharmacological activity in isoquinoline-derived alkaloids.
	Cooper *et al.* (1981)	Comparative toxicology of jet fuels to *Artemia* and *Daphnia magna.*
	Eng-Wilmot and Martin (1979)	Toxicity of algal and dinoflagellate cultures to *Artemia.*
	Eng-Wilmot and Martin (1981)	Interactions between algal and dinoflagellate cultures to mitigate toxic effects on *Artemia.*
	Meyer *et al.* (1982)	Use of *Artemia* in simple bioassay of active (*i.e.* toxic) plant constituents.
	Podojil *et al.* (1979)	Use of *Artemia* bioassay to examine human, bacterial and fungal toxins.
	Prior (1979)	Bioassay of mycotoxins in animal feedstuffs with *Artemia* larvae.
	Smolka and Schulz (1980)	Use of *Artemia* bioassay to test isolates of filamentous fungi from apples.
5. Screening assays (QSAR)	Abernethy *et al.* (1986)	Comparative and QSAR-related toxicology of hydrocarbons to *Artemia* nauplii and *Daphnia magna.*
	Foster and Tullis (1984)	Establishment of QSAR relationship between partition coefficients and acute toxicity of naphthalenes and other hydrocarbons, using *Artemia* nauplii.
	Foster and Tullis (1985)	Examination of QSAR relationships in osmotically stressed *Artemia* nauplii exposed to various organic chemicals.
6. Lethal assays (research)	Castritsi-Catharios *et al.* (1980)	Study of effects of several surfactants and one dispersant on hatching and survival of *Artemia.*
	Castritsi-Catharios *et al.* (1982)	Study of toxicity of three surfactants and one dispersant to nauplii-survival and hatching.
	Castritsi-Catharios *et al.* (1984)	Study of toxicity of an oil dispersant on the intestinal epithelium of two strains of *Artemia.*
	Castritsi-Catharios *et al.* (1986)	Comparison of sensitivities of two *Artemia* populations to a dispersant and its mixture with oil.
	El-Zayat *et al.* (1985)	Screening of "biologically active" organic compounds.
	Gaeta *et al.* (1983)	Toxicity of pesticide-mercury mixtures to *Artemia* larvae.
	Jacob *et al.* (1980)	Comparative toxicity of metals, oils, dispersants, mixtures to various species, including *Artemia.*
	Jones (1980)	Acute toxicity tests to nauplii of two drilling mud additives, and comparison to other regulatory toxicity testing protocols.
	Nikonenko and Aivazova (1983)	Toxicity of phenol to several aquatic organisms, including *Artemia.*
	Olney *et al.* (1980)	Analysis of nauplii of *Artemia* from Brazil, Australia, Italy, and USA for chlorinated hydrocarbons. All levels less than 100 ppb on wet weight basis.

TABLE I. Continued

Category	Reference	Comments
	Pankhurst *et al.* (1980)	Fluoride (NaF) inhibited growth of *Artemia* (12 d, 5 ppm), in comparative study with bivalves, krill and sole.
	Persoone *et al.* (1986)	Report on combined effects of temperature and salinity on sensitivity of nauplii to potassium dichromate and sodium lauryl sulphate.
	Suarez *et al.* (1981)	Toxicity screening of fungal strains from starches with nauplii.
	Tanaka *et al.* (1982)	Toxicity study of 17 metallic compounds and their mixtures with mycotoxins.
	Verriopoulos and Moratiou-Apostolopoulou (1983)	Comparison of toxicities of a crude oil, an oil dispersant and its mixture using *Artemia*.
	Weber and Rosenberg (1980)	Examination of toxicity of toxaphene from estuarine sediments to *Artemia*.
	Wells (1984b)	Presentation of acute toxicity data on *Artemia* nauplii and marine copepods exposed to oil spill dispersants.
	Wells *et al.* (1982)	Acute toxicity studies with Corexit 9527 dispersant and mineral oil, on *Artemia* nauplii.
	Wells *et al.* (1985)	Acute toxicity studies with solvent and surfactant components of oil spill dispersants, on *Artemia* nauplii and *Daphnia magna*.
7. Biochemical and physiological assays (research)	Alayse-Danet *et al.* (1979, 1980)	Measurement of variations in enzymes (amylase, trypsin), and growth in *Artemia* exposed to copper and zinc. Enzyme responses were generally more sensitive.
	Austerberry *et al.* (1979)	Study of di-N butyl phthalate hydrolysing enzymes in developing nauplii.
	Castritsi-Catharios *et al.* (1984)	Acute toxicity of four surfactants and an oil spill dispersant.
	Dechev and Matveeva (1978)	Proposal of respiration response as a method for examining toxicity of oils and dispersants.
	Hudson *et al.* (1981)	Study of uptake, metabolism and toxicity of di-N-butyl phthalate to synchronously developing larvae. Extraction of enzymes that may detoxify the phthalate.
	Hudson *et al.* (1982)	Isolation and purification of the hydrolysing enzyme from phthalate exposed larvae.
	Matveeva (1979)	Measurement of respiratory rates of *Artemia* under crude oil and oil products exposures, followed by recoveries in clean water.
	Samain *et al.* (1981)	Measurement of correlations between amylase and trypsin content of *Artemia* (San Francisco strain) and copper toxicity.
	Sleet and Brendel (1982)	Measurement of selective toxicity of model toxicants with different developmental stages.
8. Reproductive and developmental assays (research)	Browne (1980)	Measurement of survival and lifetime reproductive performance in shrimp (five strains) exposed to copper sulphate.

TABLE I. Continued

Category	Reference	Comments
	Kerster and Schaeffer (1983)	Development of teratogen testing system based on disruption of elongation of nauplii, and assay of a wide range of contaminants. Not a very sensitive test.
	Kissa *et al.* (1984)	Estimation of LC50's, and EC50's (hatching rate) of four metals (Cd, Cr, Ni, Co).
	Kuwabara *et al.* (1980)	Development and assessment of hatchability as a test method with approximately 40 contaminants.
	Landau and Rao (1980)	Measurement of effects of precocene II on hatching, survival and activity of nauplii.
	Leonhard and Lawrence (1980)	Application of acute and chronic tests in study of effects of cadmium on reproduction.
	Okasako and Siegel (1980)	Toxicity of sodium chloride, sulphur group (VIa) compounds on hatching of cysts.
	Sleet and Brendel (1983)	Examination of nauplii for potential in teratogen screening tests. Instars I to IV were suitable for indicating developmental effects of inorganics.
9. Food chain assays (research)	Cosson (1979)	Comparison of water versus food routes of contamination by copper, with shrimp, mussels and several fish.
	Komatsu *et al.* (1978)	Food chain experiments with radiation, including phytoplankton, *Artemia*, and several fish.
	Komatsu *et al.* (1981)	Study of accumulation through food chain with diatoms, *Artemia* and Killifish.
	Milner (1982)	Use of *Artemia* in study of zinc accumulation by flatfish.
	Snarski and Olson (1982)	Use of *Artemia* in study of influence of diet on mercury toxicity and bioaccumulation in fathead minnows.
	Wrench *et al.* (1979)	Use of *Artemia* in study of arsenic metabolism in algal-crustacean food chain.
10. Model ecosystem (research)	Higuchi *et al.* (1980)	Assessment of bioaccumulation kinetics and sublethal (growth, fecundity) radiation effects in brine shrimp reared in model ecosystem and exposed to tritium.

Very recently, Mackay and co-workers at the University of Toronto (Abernethy *et al.*, 1986) have used an improved ARC test and the acute *Daphnia* test for QSAR determinations with 37 hydrocarbons and chlorinated hydrocarbons. Good correlations were found between the aqueous solubility of the chemicals and their acute toxicity to *Artemia* and *Daphnia* as expressed by the 24 h LC50 (Fig. 3).

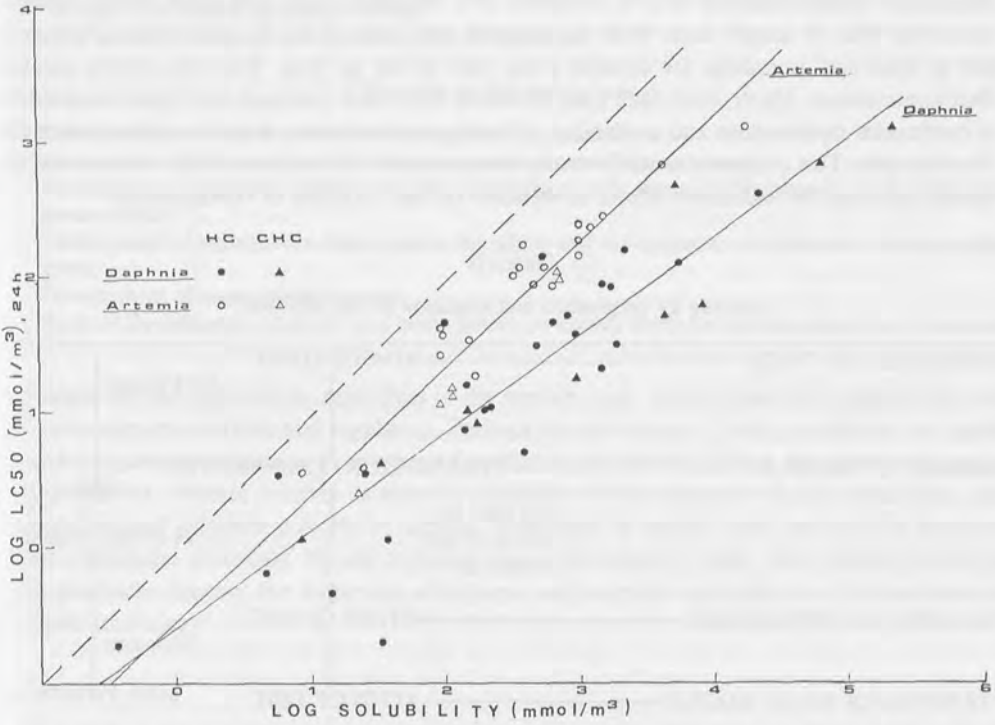


FIG. 3. Correlation between aqueous solubility of hydrocarbons (HC) and chlorinated hydrocarbons (CHC), and their acute toxicity to *Daphnia* and *Artemia* (from Abernethy *et al.*, 1986).

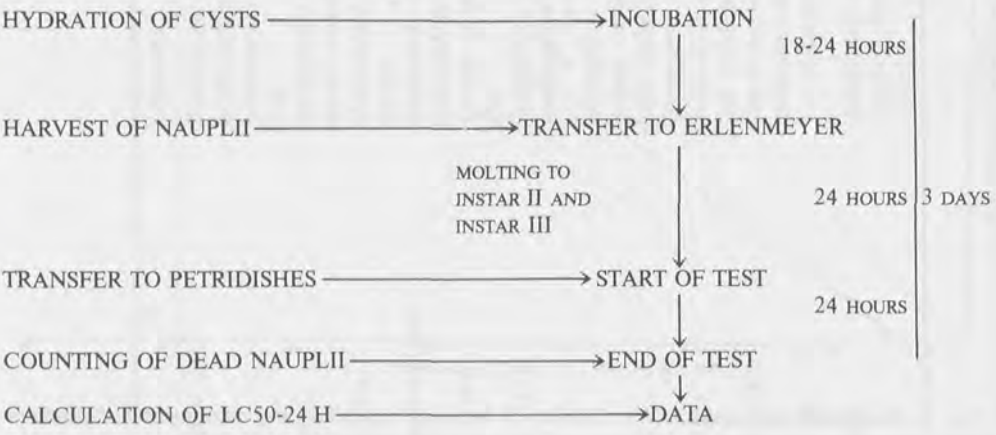
An important conclusion from both studies with *Artemia* and *Daphnia* is that acute toxicities of many organic compounds to crustaceans are largely non-selective. In other words, acute toxicity is not influenced primarily by molecular structure. It is rather correlated with the rate and success of organism-water partitioning of the chemical (Abernethy *et al.*, 1986), which for nonpolar, organic compounds is reflected by aqueous solubility and/or octanol-water partition coefficients. *Artemia's* role in this fundamental research in the QSAR field is underlined here.

Mackay and co-workers in Toronto, Canada, recently also emphasized the predictive potential of *Artemia* tests. During extensive studies with zooplankton including *Artemia*, and oils, oil dispersants and their components, it was discovered that the acute lethal toxicity of a chemical or formulation to *Daphnia magna* and marine copepods was often predictable from the *Artemia* data (Wells *et al.*, 1982, 1985 ; Abernethy *et al.*, 1986).

The advantages of the acute *Artemia* ARC test for routine experimentation in aquatic toxicology have been demonstrated recently at the State University of Ghent (Persoone *et al.*, 1986). Comparative series of acute bioassays (24 h LC50's) have been conducted with three well-known test species – *Daphnia magna, Artemia,* and the brackish water rotifer *Brachionus plicatilis* – to determine the effect which different combinations of environmental variables (temperature, salinity) have on acute toxicities of two chemicals. The *Artemia* part of the comparative study consisted of 150, complete 24 h bioassays, each with eight concentrations, triplicated with 10 nauplii each. With the standard ARC test (Table II), each assay is set up in half an hour and mortalities are counted a day later in half an hour. Both the *Artemia* and the *Brachionus* assays, which could each time be started from inert cysts and were thus independent of continuous maintenance and availability of healthy stock-cultures, were completed before the *Daphnia* tests. This comparative study clearly demonstrated the usefulness of the *Artemia* test for rapidly studying the interactive effects of variables on the toxicities of contaminants.

TABLE II

Schedule for preparation and execution of the ARC-test



The future of *Artemia* ecotoxicology

We have presented the status of the ARC test, current toxicological research with *Artemia*, and promising avenues of ecotoxicological research being explored with *Artemia* in Belgium and Canada. The role of *Artemia* in ecotoxicology, particularly aquatic, is shown in Table III, where the distinction is made between the various applications of the ARC test (*e.g.* screening, comparing, investigating effects of other variables) and the research areas with both standard and unique, continually developing methods (QSAR, teratogenic assays, investigations into modes of toxic action, comparative toxicology, etc.). Although we may have given the impression that *Artemia* is or should be a "key" species in aquatic toxicology, we would like to emphasize that *Artemia*'s usefulness in the hazard assessment of chemicals and environmental samples should be evaluated objectively. No single organism or testing protocol fulfills all criteria to determine the toxicity of materials, and as underlined by Cairns in many papers, there are inherent dangers

TABLE III

Fields of immediate application of the standard ARC-test

- Routine monitoring of ambient waters (freshwater and marine)
- Testing of effluent toxicity prior to release
- Testing of waste toxicity prior to ocean dumping
- Testing of the toxicity of mixtures of chemicals
- Testing of oil and oil dispersant toxicity
- First toxicity ranking of new chemicals and formulations

Research in *Artemia* toxicology

- Determination of QSAR's with various categories of chemicals
- Comparative toxicity studies with other test-species for predictive purposes
- Development of sensitive sublethal bioassay methods (growth, reproduction, physiological and biochemical criteria)
- Development of multispecies tests to study the effects and the dynamics of pollutants between trophic levels
- Development of bioaccumulation tests
- Study of the influences of abiotic and biotic factors on toxicity levels for various categories of chemicals

in single-species approaches, regardless of the species used. *Artemia* has been useful in the past to both research workers and regulators. Perhaps its role is one of being a reference or quality control organism in assays, as much as a predictor of chemical effects on species in marine environments. *Artemia* deserves its place in the battery of test species for aquatic toxicology, and should be used wherever possible to identify, understand or assess, solve, and prevent problems from xenobiotic chemicals. We are confident that in the years to come, more people worldwide will gradually discover the numerous advantages and potential applications of *Artemia* tests in aquatic toxicology.

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The radiobiology of *Artemia*: a review

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Introduction

Reports of studies of the radiobiology of *Artemia* have been published from 1922 until this year with most of them occurring in the 1960's and 1970's. They have dealt with many aspects of brine shrimp biology including studies of the irradiation of cysts, larvae and adults ; population studies ; cytological and genetic effects and studies of the uptake of radioactive materials. Previous review papers that contain more details on certain aspects of *Artemia* radiobiology have been published by Metalli and Ballardin (1962a, 1963) and Metalli (1980). This review will attempt to summarize what has been reported by many of these studies and to suggest directions which radiobiological studies of the brine shrimp might follow in the future.

Historical aspects

The earliest report describing the effects of radiation on *Artemia* was published by Gajewskaya in 1922. She irradiated adult animals with radiations from natural salts of radium. Estimates of the doses are difficult to determine with any degree of accuracy as the roentgen (R) or other units of dose were not used at that time. Bonham and Palumbo (1951) estimated that the doses she administered were probably less than 1 000 R. She was apparently attempting to compare artificial radiation with the natural radiation present in saline bodies of water near Sebastopol (USSR). She reported that several morphological abnormalities were produced including an

altered number of abdominal segments, changes in the length of the heart, eye deficiencies, sterility, and variations in the gills and setae.

Radiobiology of cysts

General effects on emergence, hatching, and survival after hatching

Comparisons of studies of the irradiation of cysts carried out by different investigators (particularly in the earlier reports) are frequently difficult due to unknown variability in hatching methods (salinity, temperature, etc.) and failure to distinguish between emergence, hatching, and post-emergence viability. **Emergence** involves the cracking of the shell and the escape of the embryo from the shell while still surrounded by a membrane. **Hatching** involves the escape of the instar I nauplius from the surrounding membrane into the medium. **Post-emergence viability** can be defined as any movement or coordinated swimming movements in a larva after the nauplius has hatched.

The first report describing the irradiation of cysts was by Bonham and Palumbo (1951). They irradiated San Francisco Bay cysts (both dry and wet) with doses of X-rays from 0.008 KR to 100 KR. The "wet" cysts were soaked in either tap water for 24 h, or seawater for 10, 15 or 24 h prior to irradiation. From a large series of experiments they concluded that the LD_{50} for both the wet and dry cysts, when measured during the 1st or 2nd day after hatching, was approximately the same, ranging from 40 KR to 80 KR. When measured on the 3rd through the 5th day, differences appeared between the "wet" and dry cysts, the LD_{50} being 80 KR for the former and 93 KR for the latter. Beginning on the 6th day and continuing for a week or two the LD_{50} was 20 KR for the wet cysts and 50 KR for the dry cysts. This agrees with similar observations of other biological specimens in the wet and dry state. They also observed that doses greater than 30 KR inhibited growth. After the first day following hatching this growth inhibition became significantly greater in the wet group.

Most of the studies of cysts have been reported by Iwasaki and her coworkers (26 references) from 1958 until 1980. The first paper in the series (Iwasaki, 1964a) described the irradiation of dry cysts with 480 KR and 600 KR of Co-60 gamma radiation. The beginning time of hatching was 12 h in the control, 22 h at 480 KR and 26 h at 600 KR. Thus, there is clearly a delay in hatching time which is greater with increasing doses. The hatching percent (expressed as % of controls) was 70 % at 480 KR and 41 % at 600 KR after 72 h of hydration. The time required for 50 % hatching was 16 h in the control, 29 h at 480 KR and 32 h at 600 KR. In another series of experiments (doses 60 KR to 900 KR) the LD_{50} was estimated to be 500 KR. Other reports not cited elsewhere are Iwasaki (1964d, 1973c), Iwasaki and Nakanishi (1966) and Nakanishi *et al.* (1963).

Radiosensitivity and hydration

Studies of the effects of hydration on radiosensitivity have been carried out by several persons. Rugh and Clugston (1955) irradiated cysts with doses from 100 KR to 600 KR in the dry state and immediately after hydration (no time delay). The hatching percentage of the dry cysts after 24 h at 100 KR was not affected but at higher doses hatching was reduced. At 260 KR 50 % hatched and 1.5 % hatched at 600 KR. After 48 h the values were 100 % at 300 KR and 22 % at 600 KR. In the wet irradiated cysts, all percentages were lower indicating an increased

sensitivity. With respect to motility after hatching, the effect of hydration is less than it was for hatching.

Engel and Fluke (1961, 1962) studied the effects of irradiating cysts with four different doses (150, 300, 450, and 600 KR) of Co-60 gamma rays at varying water contents (0, 1.3, 8.45, 50 % and "wet"). They also studied the effects of post radiation storage by irradiating cysts with 1 MeV electrons (doses approximately the same as the gamma ray doses) followed by storage in air at room temperature. At all doses they found that the hatching percent was moderate in the driest cysts, increasing at intermediate water contents and then becoming lowest in the wet cysts. The effect was greater with increasing doses. They also detected a time delay in the maximum hatching time which increased with increasing doses. They also stated that "... deformities were frequently noted in the nauplii" and "... nearly all nauplii which hatched after large doses were deformed or moribund". They did not describe the nature of these deformities or how frequently they occurred. They found that post radiation storage decreased hatching percentages proportionally to storage time, reaching 0 % at the highest dose (600 KR) after 30 days storage. They suggest that the hydration effect may be explained in terms of physical changes in DNA fibers known to be produced by water. They also mentioned the possible role of free radicals which will be discussed further.

Iwasaki (1959, 1964b, 1965b) and Iwasaki and Nakanishi (1966) examined the effect of varying water content on the radiosensitivity of cysts. Prior to irradiation with 432 KR of Co-60 gamma rays the water content was regulated to values ranging from 5.8 to 54.0 % using different concentrations of H_2SO_4. In general, the hatching percentage decreased as the water content of the cysts increased. However, at one specific level (9.6 %) above the minimum value (5.8 %), the hatching percentage rose slightly before beginning its decline. This had also been noted in an earlier study with X-rays (Iwaski, 1958b). In an attempt to further understand the nature of the hydration effect and its possible reversibility, cysts which had been hydrated to various percentages of water were redesiccated to the original level (5.8 %). This revealed that the hydration effect was reversed as the hatching percentages after redesiccation were returned to values similar to cysts that had not been hydrated originally. This seems to indicate that no irreversible physiological changes were produced by the increased hydration. To further clarify this conclusion, cysts with varying water contents were irradiated at -10 °C and 25 °C after having been stored at 0 °C and 25 °C for uncited time periods. These experiments indicated that the temperature during hydration did not change the hatching percentages but the temperature at the time of irradiation did. The cysts irradiated at -10 °C were only slightly influenced by higher hydration percentages at either storage temperature. The cysts irradiated at 25 °C showed significant reductions in hatching percentages at both storage temperatures. She therefore concluded (although some of the results do not confirm this) that radiosensitivity of dry cysts depends primarily on the physical state of the cyst constituents at the time of irradiation.

Iwasaki (1964c) further investigated the effect of hydration for varying periods of time (1 to 7 h) and at two temperatures (0 ° and 27 °C) prior to irradiation at 100 and 200 KR. Hatching percentages decreased slightly more at the lower hydration temperature at both doses and much more by the higher dose. However, at both doses and hydration temperatures, the percentages only decreased slightly by increasing hydration times and the decreases were not uniform falling to a low point after 3 h (at 100 KR) to 5 h (at 200 KR). This was interpreted by suggesting that there are many sensitive stages during the course of development and the final measured result depends upon whether the radiation is delivered at one of these time specific stages.

Effects of fractionated radiation

Iwasaki (1962, 1966a) investigated the effects of fractionated radiation on dry cysts. This should produce information regarding fractionation effects in an essentially non-metabolizing system. In the first set of experiments, cysts with a water content of 7.4 % were irradiated at room temperature with gamma rays at a dose rate of 12 KR/min. In all cases the total dose was 480 KR. The doses were delivered as a single dose of 480 KR and as two doses of 240 KR at intervals of 1, 3, and 7 days. Following each irradiation the cysts were stored either at room temperature or dry ice temperature. When the cysts were stored at dry ice temperatures, there was no significant difference in the hatchability of the cysts receiving the radiation as a single dose and those receiving any of the fractionated doses. When the cysts were stored at room temperature, however, the hatchability was greatly reduced and in proportion to the storage time (1 day : 56 to 40 % ; 3 days : 56 to 25 % ; 7 days : 56 to 5 %).

In a second set of experiments (Iwasaki, 1966a), a single dose (480 KR) was delivered and split unequally (120-360 KR ; 240-240 KR ; 360-120 KR). The cysts were then stored either at room temperature or dry ice temperature for 7 days. In those cysts stored at dry ice temperature, there was no difference in hatchability whether the dose was delivered singly or fractionated in any way. In those stored at room temperature, however, there were marked decreases in hatchability, the decreases being much greater when the second dose was the larger (480 KR : 49 % ; 120-360 KR : 17 % ; 240-240 KR : 7 % ; 360-120 KR : 0 %). It is clear from these experiments that there is no indication of recovery or repair of radiation damage during the time periods used.

Effects of radiations with different LET

Investigations of the comparative effects of radiations of different LET (linear energy transfer) were reported by several investigators. LET is a measure of the number of ionizing events occurring during a unit distance travelled by the specific radiation. In general, it is higher for heavier particulate radiations.

Hutchinson and Easter (1960) using dry cysts and measuring both hatching and emergence found that Co-60 gamma rays (low LET) produce a typical sigmoid survival curve with little change in survival with increasing dose at low doses, with an abrupt decrease in survival above a threshold dose. On the other hand, following irradiation with 40 MeV helium ions (LET=240 equivalents/100 Å) the threshold is significantly reduced, while following irradiation with 160 MeV oxygen ions (LET=3800 equivalents/100 Å) the threshold is no longer present. They suggested four possible reasons for this loss of a threshold : 1) The gamma ray curve may indicate that a certain number (20 to 60) or a certain fraction of functioning units must be damaged to prevent development. A single heavy oxygen atom might produce these necessary events. However, a calculation from the dose curves indicates that 24 and 9 oxygen ions/m^2 are necessary to suppress emergence and hatching, respectively, to 37 % of the controls. Thus, the chance that a single oxygen ion will do all the necessary damage is small ; 2) The simultaneous inactivation of several widely separated areas by a heavy ion might be biologically more effective than several consecutive inactivations by gamma rays. This mechanism might be valid for a metabolizing system or at higher doses, but probably does not apply to dry, non-metabolizing cysts ; 3) As the ionizing events would occur very close together in the track of high LET oxygen

atoms, the resulting physical and chemical changes might be different and therefore more effective in producing the resulting damage ; 4) The most likely explanation is that if a certain amount of damage is produced within some specific small volume, the egg will not develop. This damage can be produced by several gamma rays, a smaller number of helium ions, or a single oxygen ion. The dimensions of this volume are probably less than 1 μm and there are probably many of them in each cyst (cell).

Easter and Hutchinson (1961), expanding on their earlier work, used six different types of radiation (Co-60 gamma rays, 1 MeV electrons, and 10 MeV helium, carbon, oxygen, and nitrogen ions). They measured both emergence and hatching following various periods of presoaking (dry, 6 h, 14 h, 24 h) prior to irradiation. In addition to confirming their earlier observations and conclusions, and more precisely defining the point of the change from a threshold, to no threshold, to a point between LET values of 190 and 1700 MeV/g/cm^2, they further demonstrated the effect of hydration on radiosensitivity which was discussed earlier. They found that the effects of increased hydration time were greater with radiations with low LET than with the heavy ions where the difference was minimal. They also reported little change in radiosensitivity between 14 and 24 h of hydration time. They were not yet aware of the precise developmental events occurring during the time period prior to emergence and hatching.

Iwasaki *et al.* (1971) described the effects of Co-60 gamma rays and 2 MeV fast neutrons on post hatching survival times, and the numbers and kinds of mitotic cells in larvae. They reported that larvae irradiated with doses of 10 KR of neutrons or 20 KR of gamma rays reach the "mature stage" (?) with no apparent difference from unirradiated controls. Doses of 200 KR (gamma rays) or 100 KR (neutrons) had no significant effect on hatching, but the viability of the hatched larvae is greatly reduced. They also stated that the fast neutrons were 2.1, 2.8, and 4.8 times more effective in producing mortality of larvae at 1, 2, and 3 days post hatching. Doses greater than 150 KR (neutrons) or 350 KR (gamma rays) do not prevent emergence and hatching but the larvae died shortly afterwards without passing through the first molt.

Iwasaki and Maruyama (1971) reported on the comparative effects of Co-60 gamma rays and 29 MeV electrons on hatching and the number of mitotic cells in larvae immediately after and 10 h after hatching. They found small decreases in hatching percentages with doses of 50 to 250 KR. The percentages decreased only slightly with increasing dose and there were no differences between the gamma rays and electrons. The time of first hatching increased linearly from 15 to 19 h with increasing doses for both types of radiation. The number of mitotic cells in larvae decreased with increasing doses although some of these changes were erratic (decreasing, then increasing and then decreasing again). Also, with increasing dose, the number of cells in metaphase increased from 40 to 80 %, the number of cells in prophase decreased from 40 to 20 % while the number of cells in ana-telophase decreased slightly and then remained the same. Other studies of these effects have been reported by Iwasaki *et al.* (1974, 1977, 1980), Iwasaki and Kumamoto (1974), and Fujikawa *et al.* (1979).

Radiation of cysts and larval viability

Bowen (1963b), in an attempt to determine the X-ray dose which would be most efficient for producing mutations without impairing the viability of the larvae or greatly reducing the fertility of the stocks which would be derived from them, performed investigations on the effects of X-rays upon the survival of larvae hatched from irradiated cysts. She also examined the larvae and four

generations of their progeny for mutations (see below). Dry cysts were irradiated with X-rays (400, 2 000, 10 000, 20 000 R) and stored either in a dark room at room temperature or frozen for time periods of 1, 13, 52 days, 21, 22, and 25 months. Preliminary tests indicated that 400 or 2 000 R did not significantly affect hatchability of the cysts or the survival of the larvae so that later tests were made at only 10 and 50 KR. The results indicated that there was no significant change in viability at either 10 or 50 KR even after the longest storage period. When tests for survival of larvae for at least 3 weeks after hatching were carried out, the results of some individual tests were erratic. However, when all tests were combined, it was revealed that survival of larvae derived from cysts irradiated with 10 KR was not significantly different from those derived from non-irradiated cysts. Also, the larvae in the 10 KR group were normal in size and other obvious characteristics. On the other hand, survival of larvae from cysts irradiated with 50 KR was nearly zero at the end of 3 weeks. Also, throughout the survival tests the larvae in the 50 KR group were smaller than either those in the 10 KR or control group. The two survivors (out of 450 which hatched) in the 50 KR group were one male and one female. Both displayed abnormalities and decreased size, and they survived only a few days after the three week period.

Radiation and oxygen

Iwasaki and Kumamoto (1975) published an abstract describing the effects of oxygen, nitrogen and nitrous oxide on the radiosensitivity of dry cysts. The cysts were exposed to these gases at pressures of 1, 3, and 5 atmosphere and then irradiated with 5 to 600 KR of X-rays. The 50 % hatchability doses were 450, 395, and 375 KR for nitrogen, nitrous oxide and oxygen respectively. Mixtures of oxygen and nitrogen produced results similar to oxygen. The increased radiosensitivity in oxygen was proportional to the oxygen pressure and the curves had two components : a radiosensitive one at lower doses, and a radioresistant one at higher doses. Similar findings were reported by Iwasaki and Kumamoto (1976a). After determining that it took 10 h for the diffusion of oxygen to reach equilibrium, experiments revealed that at the 50 % hatchability level, the OER (oxygen enhancement ratios) were 1.4, 4.5, and 17 at 0.2, 0.6, and 3 atmosphere, respectively. Changes in the slope of the curves at higher oxygen pressures again suggested that there might be two different components to radiation sensitivity to oxygen.

In a more complete report (Iwasaki and Kumamoto, 1976b) irradiation of dry cysts after overnight exposure to oxygen (which insures complete equilibration) resulted in 50 % hatchability with only 24 KR as compared to 335 KR after a short exposure to oxygen (no equilibration). The OER in this case was again found to be 19. They point out that this value is much higher by a factor of two than other OER values reported for other organisms. They again reported a sudden change in the slope of the curve at high doses indicating that there are two components to the oxygen effect. They attribute this change to oxygen depletion due to lack of diffusion during the radiation exposures. Birnbaum (1973) also examined the effect of oxygen levels on hatchability of cysts.

Space flight studies

Cysts have been a favorite passenger on several space flights (particularly Apollo and Cosmos) and 28 publications deal with various aspects of these studies (Eugster, 1955, 1964 ; Von Borstel *et al.*, 1971 ; Bucker *et al.*, 1972, 1973ab, 1974 ; Bucker, 1974, 1975, 1976 ; Bucker and Horneck, 1974, 1975ab ; Planel *et al.*, 1974ab, 1975, 1980 ; Ruther *et al.*, 1974 ; Graul and

Ruther, 1976, 1977 ; Blanquet *et al.*, 1977 ; Heinrich, 1977 ; Schopper *et al.*, 1977 ; Lukassowitz, 1978 ; Gaubin *et al.*, 1979ab, 1980 ; Kovalev *et al.*, 1981).

The material that will be described here is based on the results of the experiments performed on board of Apollo 16 (Planel *et al.*, 1980). The main radiations considered in these experiments were heavy ions (HZE particles). By means of elaborate dosimetric techniques which are described in detail in several of the above references it was possible to determine which cysts had been hit by HZE particles and which had not. In general, cysts "hit" by these particles had reduced **emergence** percentages (10 % as compared to flight non-hit of 30 % as compared to ground controls of 65 %). Even smaller percentages **hatched** from the "hit" cysts (6 %) and even a smaller percentage attained an age of 4.5 days. There was also a delay in reaching each of the stages mentioned and the few larvae that survived beyond 4.5 days were reduced in size and many of them had abnormalities which resulted in their death before reaching maturity. Incidently, there were several effects observed in flight "non-hit" cysts which could not be explained.

Cyst irradiation and free radicals

It has been known for some time that in the production of radiation damage in biological materials one of the initial events following ionization is the production of short and long lived free-radicals. An early publication (Snipes and Gordy, 1963) described some simple experiments studying free radical production but the tentative results were in agreement with later publications, so it will not be described here.

This phenomenon has been investigated much more extensively by Iwasaki (1965b). Using ESR spectrometry the quantity of free radicals in cysts was determined before and 10 min after irradiation doses of 60 to 960 KR with Co-60 gamma rays. She found that the total amount of free radicals increased with increasing dose although the increase was not entirely linear. As mentioned earlier, the hatchability curve declined and was sigmoidal. She also investigated the decay of free radicals during post radiation storage at 25 °C. Decay of the free radicals occurred rapidly during the first 3 days, more slowly for the next 4 days, and then remained at this elevated level for long (?) periods of time. In all cases, all of these levels were higher at higher radiation doses. In another set of experiments it was determined that the free radicals decayed much more slowly at dry ice temperatures.

Iwasaki (1966b) next investigated two post radiation treatments that had been found to modify radiation damage in cysts in relation to free radical changes produced by these same treatments. The two treatments investigated were : 1) heat treatment (100 °C for 5 min) and 2) hydration for 30 min at 4 °C followed by redesiccation. The result showed that both treatments decreased the number of free radicals while improving the hatchability values as compared with untreated cysts. Thus a fairly clear relationship has been established between the number of free radicals present and the damage (decreased hatchability).

Radiation and chemical protection

A good deal of experimental evidence exists to indicate that several SH-containing compounds, such as the mercaptenes, exert a radioprotective effect on biological materials. Whether these materials function by protecting biologically important molecules directly or by assisting in the transfer of energy from the biologically important molecules to other molecules or out of the system is not known with certainty. They very likely may function in any event through free

radical reactions. Iwasaki (1971) investigated this phenomenon using mercaptoethylamine hydrochloride (MEA) as the protective chemical. The results of several groups of experiments can be summarized as follows : 1) the maximum dose reduction factor (DRF) produced by MEA in cysts is 1.2 in both the dry and wet state ; 2) MEA must be present at the time of irradiation to afford protection ; 3) higher concentrations of MEA were required to afford this protection when compared to other organisms (this may be due to cyst penetration problems) ; 4) there is a direct relationship between free radical decreases and protection produced by MEA ; 5) MEA has no effect on post radiation changes in damage. These results would seem to indicate that MEA exerts its effect primarily on short-lived free radicals produced at the time of irradiation.

In another set of experiments Iwasaki (1973a) investigated the same phenomenon using fast neutrons (2 MeV) as the radiation source. In general, the results were essentially the same as with the previous experiments with gamma radiation. Thus the protection by MEA appears to operate the same for neutrons as it does for gamma radiation.

Stimulatory effects of radiation and other agents

Several publications in Russian (Ivanovskii, 1980ab ; Ivanovskii and Kulinich, 1980) report a stimulatory effect of radiation. They report that doses of 1 and 2 KR increase the life span as does treatment with N-methylnitrosourea (1 µg/ml) or 1-4-bis-diazoacetylbutane (0.1 µg/ml). Higher doses of any of these treatments decrease life span. Also, the stimulatory effect is no longer present after the animals reach sexual maturity. Similar effects have been noted by other investigators but they have been unable to demonstrate statistically significant differences at these low dose levels.

Radiobiology of larval stages

Survival and growth

Few reports are available concerning the effects of radiation on developing larvae. One problem mentioned by some investigators involves the difficulty of maintaining reasonable numbers of larvae for long periods of time. Another problem is the difficulty of obtaining reasonably large numbers of larvae at the same stage of development. This becomes increasingly difficult at later stages due to the fact that the larvae do not all grow at the same rate. Thus, selecting a group of larvae at a particular time after hatching does not guarantee that they will all be in the same stage of development. This requires selecting individuals visually and the extra handling decreases their longevity.

In a publication preliminary to studies of radiation and ploidy, Metalli *et al.* (1961) described the results of irradiating newly hatched nauplii and a later stage (metanauplius IV) with X-ray doses of 11.5 to 46 KR. Their primary interest was to compare diploid and tetraploid varieties. They reported percent cumulative mortality of control values for 7 days. Mortality increased exponentially with time and was proportional to the dose. The LD_{50} range was much lower than for dry cysts and they reported that tetraploid forms are more resistant than diploid forms. In a later publication (Metalli and Ballardin, 1962a), they mention that more stages will be tested but they apparently did not do this.

Boswell (1966), in an unpublished Ph.D. thesis, described studies of the irradiation of cysts and three different larval and adult stages (stages 0, 12, and 19 after Weisz, 1946) with doses

of 5 to 200 KR of Co-60 gamma rays. The following are the major results of his study : 1) the larger the dose, the shorter the survival time of all stages ; 2) the cyst was the least sensitive stage ; post hatching stage 0 was the most sensitive, stage 12 next and then finally stage 19 (adult) ; 3) the total length of the developing larvae was reduced in those irradiated in the cyst stage or in stage 0 with the amount of reduction being proportional to the dose ; this was also true of the length-width ratio ; 4) eye abnormalities were noted in these same two groups but were more frequent in those irradiated at stage 0 ; 5) reduction in the size of and cessation of mitotic activity in blood-forming organs was noted following doses of 100 and 175 KR in animals irradiated at stage 19 ; 6) adult (stage 19) females were more sensitive than males ; 7) mating activity in adults was reduced in direct proportion to dose.

During the course of some recent work (Slowik, 1975 ; Lauro, unpubl. ; McCourt, unpubl.), larvae have been irradiated with Cesium-137 gamma rays (doses in the range of 5 to 30 KR) at several stages of larval development from immediately after hatching and emergence to 18 days after hatching. In our initial experiments we used only time after hatching to select experimental animals, but as mentioned earlier, this does not insure that all larvae are in the same developmental stage at the time of irradiation. In future studies, we will also visually select larvae that are all in the same stage of development.

The results of some of our work are summarized in Table I and Fig. 1. The results indicate that newly-hatched larvae are much more radiosensitive than either cysts or adults. Following acute doses of 5.8, 12, 17.5 and 23 KR, no larvae survived beyond 8 days in the 23 KR group or beyond day 14 in the 17 KR group. The time after which 50 % of the larvae had died was 3 days in the 23 KR group, 4.5 days in the 17 KR group, 13.5 days in the 12 KR group, and 15 days in the 5.8 KR group. Some deaths occurred in the control group but 74 % were still alive at the end of 16 days.

TABLE I

Survival rate over a 16 day period of newly hatched larvae exposed to different doses of γ-radiation

Days after irradiation	Dose (in R)				
	0	5800	12000	17500	23000
0	100	100	100	100	100
1	100	98	100	86	70
2	96	98	96	80	58
3	96	94	90	72	50
4	96	90	80	56	40
5	96	80	76	40	34
6	96	74	74	30	20
7	96	74	70	18	6
8	94	72	66	12	0
9	92	72	60	4	0
10	86	72	60	4	0
11	86	70	56	2	0
12	84	64	54	2	0
13	80	60	54	2	0
14	80	54	48	0	0
15	80	50	40	0	0
16	74	40	34	0	0

Similar experiments in which the irradiated stages are older (10 days) are shown in Fig. 2. They produce similar results but the survival times at the same doses are longer with respect to both time of survival to either any larvae or 50 % of the larvae. Although there is a general trend that the older the larvae, the longer the survival time, it appears that there might be several specific time periods during larval development when the larvae vary in sensitivity. Due to the fact that at later larval times, the actual stages irradiated are more varied, makes it more difficult to evaluate this suggested observation with accuracy. Further studies in which larvae irradiated are selected by specific stage rather than by time may produce a more definite answer to this question.

Another part of our study has involved the measurement of growth rate of irradiated larvae. Newly hatched larvae irradiated with the same doses described above have been measured (total length) following irradiation (Fig. 3). Growth rates are reduced with the amount of reduction being greater at higher doses. However, growth rates at the two highest doses (17.5 and 23 KR) are only slightly different (not statistically significant). It may be that beyond a certain dose, growth rate is not further affected. We plan to investigate mitotic activity in irradiated larvae to see if there is a relationship between this and the degree of growth rate reduction. We also plan to measure other size parameters to determine whether changes are produced and whether any of the changes are stage specific.

Major abnormalities have not been noticed in larvae following irradiation at these doses but what appear to be minor defects have appeared from time to time. As time permits, we hope to

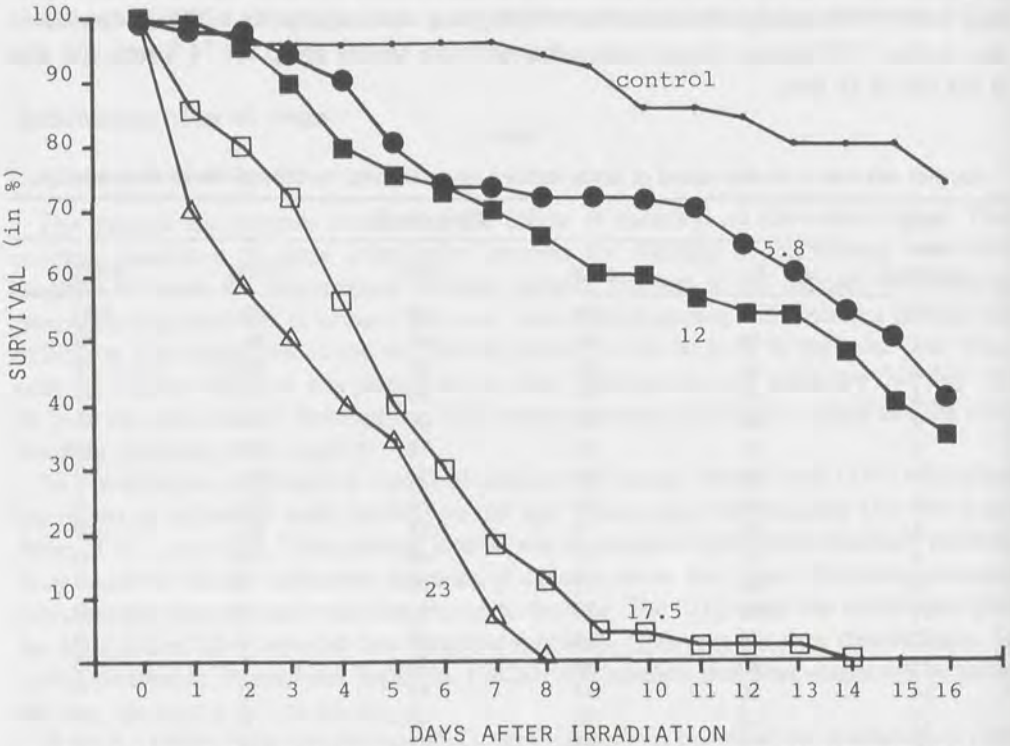

FIG. 1. Survival of 1-day-old nauplii irradiated with acute doses of Cesium-137 γ-rays (in KR).

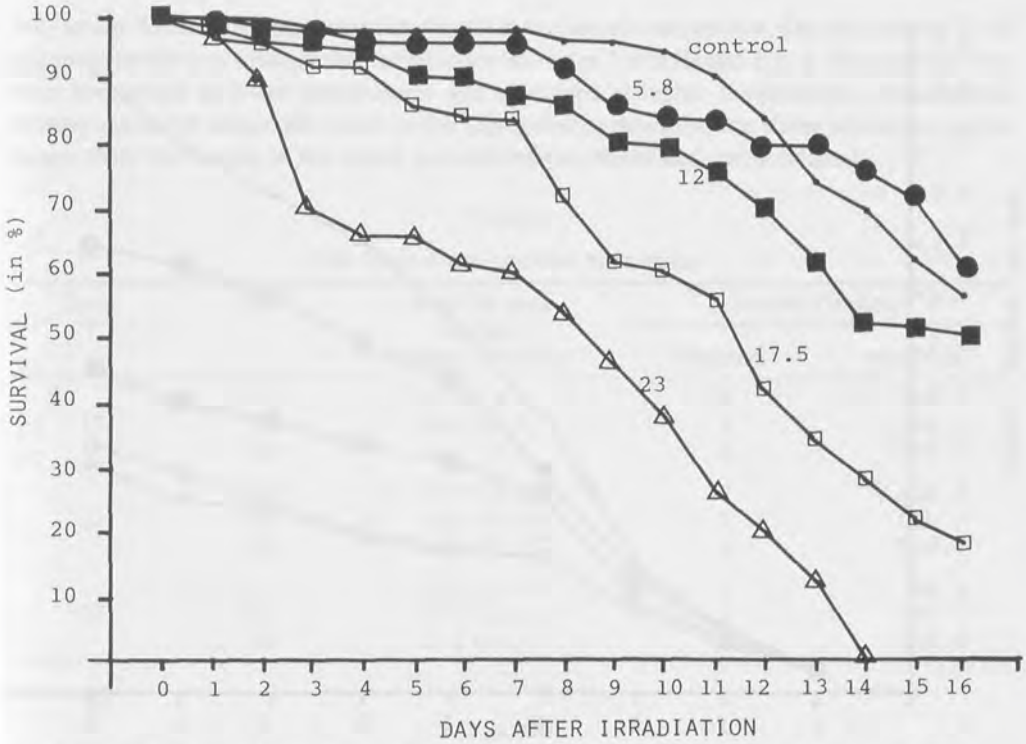

FIG. 2. Survival of 10-days-old larvae irradiated with acute doses of Cesium-137 γ-rays (in KR).

study some of these in greater detail. It is possible that certain abnormalities are more prevalent after irradiation at certain specific time periods. This phenomenon has been noted following irradiation of embryos of vertebrates and if it turns out to be true for *Artemia* it might be possible to relate specific abnormalities to specific developmental events occurring at the time of irradiation. Further, as has been the case in other developmental systems, it may be possible to use radiation as a tool to learn more about developmental mechanisms.

PHYSIOLOGICAL EFFECTS

The only physiological event studied in larvae with respect to the effect of radiation has been respiration. Publications by Angelovic *et al.* (1966, 1969, 1970), Engel *et al.* (1965, 1966) have described investigations of the combined effects of radiation and different salinity levels on respiration in the brine shrimp. Most of this work has been summarized by Engel and Davis (1976).

Angelovic and Engel (1968) studied the interaction of radiation and salinity on the respiratory rate of newly hatched *Artemia* nauplii. They irradiated nauplii which had been cultured for 24 h in solutions with salinities of 5, 50, 100, and 200 ‰ with acute doses of Co-60 gamma radiation (10, 20, 40, and 80 KR). Respiration was measured during the first 4 h at both 20 °C and 30 °C. The results indicated that the respiratory rate of both control and irradiated larvae was higher at 50 than at 5 ‰, and lowest at 200 ‰. The rate in the controls (non-irradiated) declined

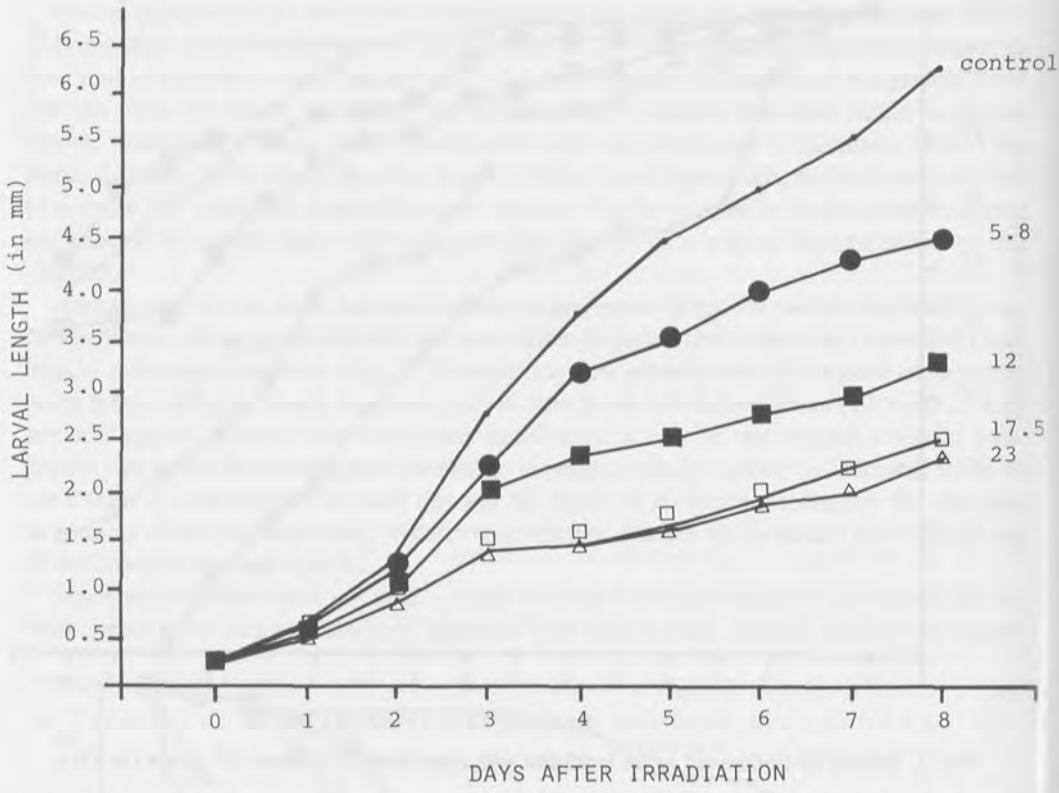

FIG. 3. Larval growth of newly hatched nauplii irradiated with acute doses of Cesium-137 γ-rays (in KR).

continuously between 50 and 200 ‰. The rate in the irradiated groups was nearly constant between 50 and 150 ‰. They mentioned that the 40 and 80 KR groups in 200 ‰ died within 24 h. They speculated that the effects they observed might be due to defects in the "branchiae" which are not present yet in one day old shrimp. They also mentioned that some larvae "appeared to be" under a severe physiological stress and that they "did not survive very long".

Radiobiology of adult stages

LIFE SPAN STUDIES

Decreases in life span following irradiation of adults have been reported by many investigators (Grosch and Erdman, 1955 ; Metalli and Ballardin, 1965a ; Ballardin and Metalli, 1966b ; Squire, 1969 ; Squire and Grosch, 1970 ; Suyama and Iwasaki, 1976). In the most complete study Suyama and Iwasaki (1976) first made a careful determination of normal life spans of males, females, and mixed groups ; then they continued with an analysis of radiation effects (0 to 200 KR) at three different temperatures (15, 25, and 32 °C). Control life spans are shown in Table II. The differences at the 15 and 25 °C levels were significant at the 5 % level. The mixed populations had the longest life spans, followed by the female only groups and finally the male

only group. These differences were not present at the highest temperature. The life spans at 25 °C following irradiation with gamma radiation are shown in Table III and Fig. 4. The survival times were lengthened at lower temperatures and shortened at higher temperatures. Although the females displayed longer life spans in the segregated populations, the males tended to survive longer than the females in the mixed populations (irradiated and non-irradiated).

TABLE II

Life spans of non-irradiated brine shrimp

Temp. (°C)	Sex	Mean life span (in days) (standard deviation)	Survival (in days)	
			Minimum	Maximum
15	F	62.4 (24.9)	26	156
	M	49.8 (26.0)	9	124
	FM	75.2 (30.9)	6	182
25	F	45.5 (28.1)	7	134
	M	39.9 (20.9)	9	122
	FM	52.1 (35.1)	4	204
32	F	23.3 (11.3)	2	59
	M	23.4 (12.4)	5	100
	FM	23.3 (14.5)	1	100

TABLE III

Life spans of brine shrimp following irradiation

Dose (KR)	Sex	Mean life span (in days) (standard deviation)	Survival (in days)	
			Minimum	Maximum
0	MF	52.1 (35.1)	4	204
10	MF	39.1 (23.1)	1	99
20	MF	25.4 (12.3)	6	60
30	MF	16.2 (6.2)	4	26
40	MF	11.6 (4.3)	3	19
50	MF	9.6 (3.2)	2	17
100	MF	3.5 (0.8)	1	5
200	MF	1.2 (0.5)	1	3

Grosch and Erdman (1955) irradiated adults (during their first 3 days of maturity) with doses of X-rays from 0.5 to 358.785 KR. Some males were able to survive for several days at doses below 200 KR while females did not survive at doses exceeding 150 KR. For any dose above 10 KR, the variability in time of survival was not great, being less than 10 days in almost all cases. The range for unirradiated adults ranged from 18 to at least 45 days. If a survival curve were drawn using their data, a typical sigmoid curve would result. They also examined several aspects of reproduction in females. At doses greater than 5.16 KR, there was no reproduction except from those elements that were already formed before the irradiation. There were reductions in total number of broods, average number of broods, total number of gametes, % oviviparous

reproduction, and average number of gametes. The average life span was also reduced. All of these were roughly proportional to the dose received. They state that the female sterility dose is in the range of 2 to 3 KR.

Squire (1969) irradiated adults with 1 to 100 KR of gamma radiation. The mean life span for non-irradiated males and females is 50 and 43 days respectively. Doses up to 5 KR did not significantly alter life span. Higher doses decreased mean life span to 47 days for males (probably not significant) and 26 days for females after 10 KR, to 15 days for males and 10 days for females after 50 KR, and to 6 days for males and 5 days for females after 100 KR. Similar but sometimes less precise results have been reported by others. Since often details are lacking about experimental parameters and strains used, precise comparisons are impossible. It is clear that life span shortening does occur but no clear explanation for its cause is apparent. Ballardin and Metalli (1966b) performed similar studies and found differences between diploid and tetraploid strains (Squire questioned some of the details of their report) and also reported that fractionation of the dose did not change the results.

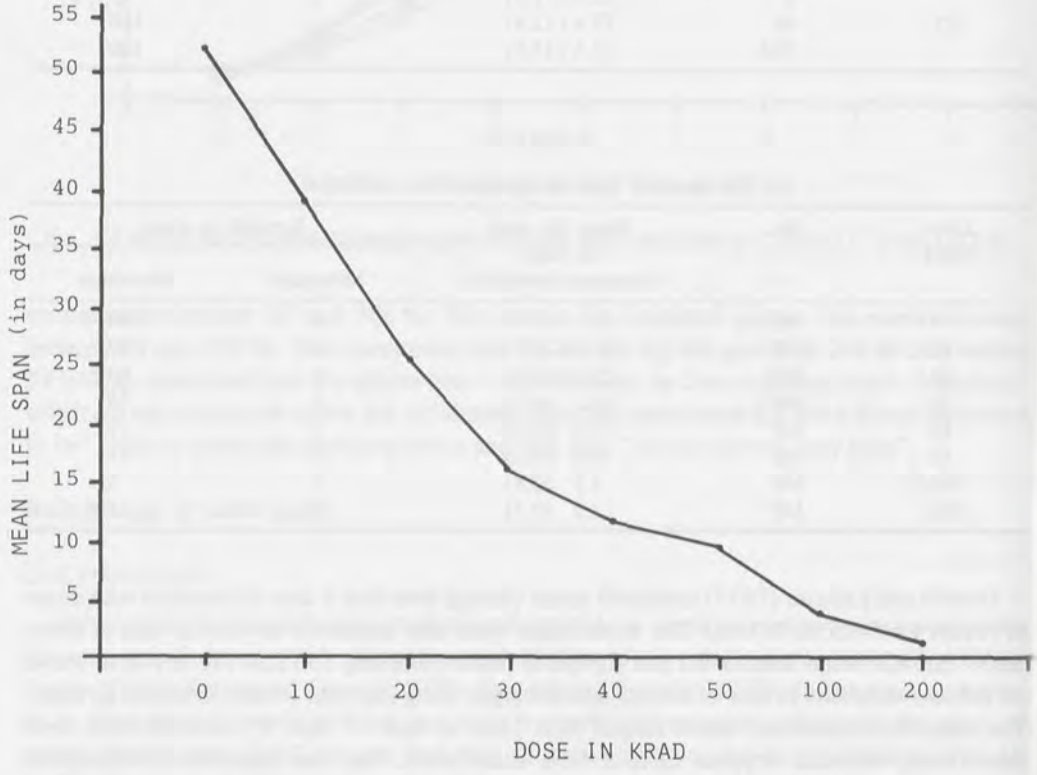

FIG. 4. Mean life span at 25 °C of mixed male and female population irradiated with different doses of γ-radiation.

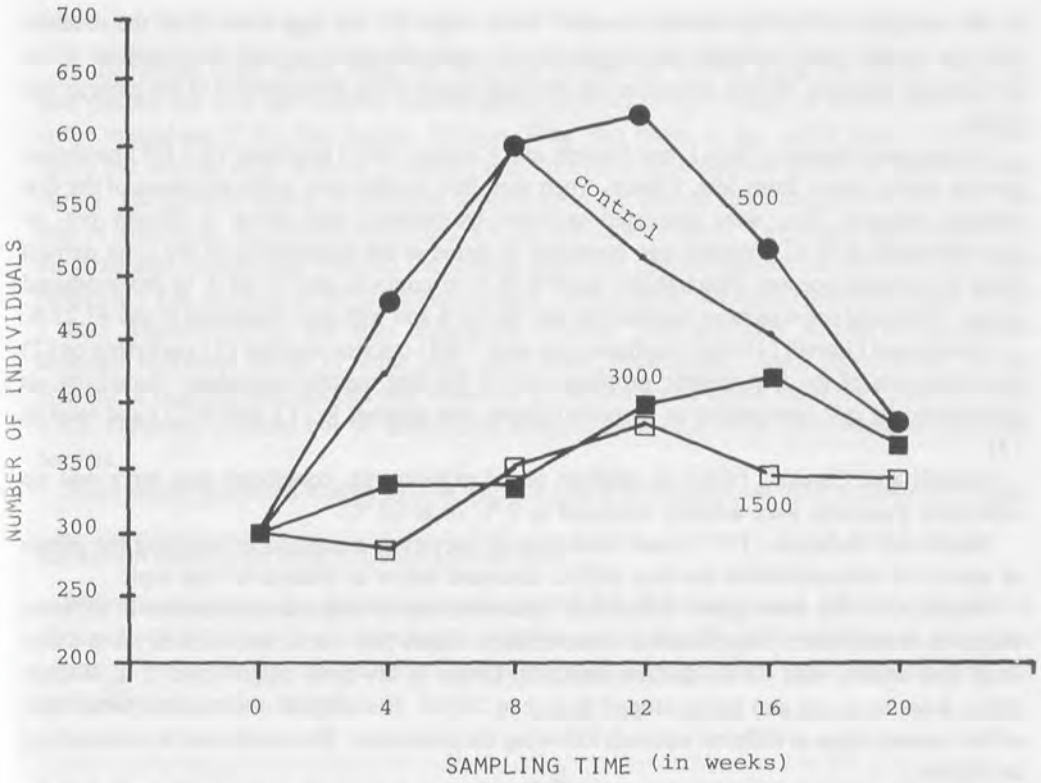

FIG. 5. Change of population size (7th instar and older) at 4 week intervals following irradiation with different doses of γ-radiation (in R).

RADIATION EFFECTS ON REPRODUCTIVE ORGANS

Several publications exist dealing with the effects of radiation on female reproductive tissue. For an extensive review of this topic see Metalli and Ballardin (1972) and Barigozzi (1974). Other publications are Auerbach (1963), Ballardin *et al.* (1963), Ballardin and Metalli (1965ab, 1966a, 1968a), Grosch and Sullivan (1955), Metalli and Ballardin (1965b), and Nakanishi *et al.* (1964). First, a brief review of some of the facts relating to maturation of the female germ cells in *Artemia* will be presented (Metalli and Ballardin, 1962b). It has been determined that all of the oocytes in a specific brood are synchronous in their development, so that by macroscopic observation of adult females it is possible to know in general what is occurring cytologically. The first event (stage B) is the appearance of two rows of small longitudinal dots located along the lateral parts of the two genital and first few abdominal segments. These are new oocytes that are beginning to accumulate yolk materials. The oocytes go through a period of enlargement as yolk accumulates. It has been determined histologically that, during this time (stage C), they are passing through all of the earlier stages of the first prophase (leptotene, zygotene, diplotene and diakinesis) of the first meiotic division. Next (stage D), the oocytes are pushed out of the ovaries into the oviducts (lateral pouches of the ovisacs). The oocytes are now

in the metaphse of the first meiotic division. Next (stage E), the eggs move from the oviducts into the central ovisac at which time maturation is resumed beginning with the anaphase of the first meiotic division. Shortly thereafter the cleavage stages of the development of the embryo will begin.

Cervini and Giavelli (1965a), and Giavelli and Cervini (1965) irradiated (0.5 KR) parthenogenetic adults (strain from Sète, France) when their first broods were in the prophase of the first meiotic division. They were irradiated at room temperature with either a 30 min pre- or post-treatment at 0 °C. Damage was measured in terms of the hatchability of the cysts derived from the treated oocytes. Hatchability was 94.26 % in controls and 37.68 % in the irradiated group. With cold pre-treatment hatchability was 48.03 % and with post-treatment it was 43.22 %.

Cervini and Giavelli (1965b) irradiated (0.5 and 1 KR) oocytes in either (1) pachytene or (2) late diakinesis of the first meiotic prophase or (3) the first meiotic metaphase. Sensitivity, as determined by cyst hatchability as described above, was greatest in (1), less in (2) and least in (3).

Giavelli and Cervini (1966), in another set of experiments, concluded that there was no difference if oocytes were actually irradiated at 0 °C or at 22 °C.

Metalli and Ballardin (1972) used irradiation of oocytes as a method for studying the effects of ploidy on radiosensitivity but they will be discussed below in relation to that topic.

Iwasaki (1973b) investigated differential radiosensitivity of oogonia and oocytes at different stages of development. She irradiated three different stages (dry cysts, immature larvae 6-8 mm long, and adults) with Co-60 gamma radiation. Doses to dry cysts ranged from 2 to 300 KR while doses to young and adults ranged from 2 to 20 KR. Histological observations were made of the ovarian tissue at different intervals following the irradiation. The results can be summarized as follows :

Dry cysts

Larvae from irradiated cysts were examined within 1 day after hatching. The germ cells at this stage normally consist of only 4 to 8 cells. Cells from cysts irradiated with less than 25 KR were intact and normal in appearance. Some cells were pycnotic in larvae from cysts irradiated with more than 25 KR. In larvae from cysts receiving 150 KR or more, normal mitoses were still found but the cells were "loosened" in appearance. The ovaries of adults from cysts receiving 10 KR or less showed no changes. The ovaries of adults from cysts receiving 100 KR contained normal growing oocytes but some pycnotic oocytes were also found.

Immature larvae

The ovary at this stage normally consists of a large population of mitotically active oogonia. Changes in the ovarian tissue were apparent at all radiation levels but more changes were produced at higher doses. In larvae receiving 2 KR, some of the oogonia underwent mitosis but some pycnotic cells appeared within 1 day. During the first 4 days after irradiation oogonia degenerated and the ovary became greatly depleted. Within 10 days to 2 weeks, however, surviving oogonia entered mitosis, "healthy-appearing" oogonia were observed, and the ovary increased in size. After doses of 5, 10, and 20 KR, the effects were similar but more pronounced. However, the larvae at these higher doses usually died before any recovery of ovarian damage had occurred.

Adults

In the normal ovary at this stage, the prophase oocytes are located along the ovaries. They are then pushed out into the oviducts (paired lateral pouches of the ovisacs) at which time they are in the metaphase of the first meiotic division. Then they move to the central part of the ovisac where the second polar body is produced and embryonic development up to the gastrula stage occurs. The specific effects of radiation depend upon the precise stage the oocytes are in at the time of irradiation. In *Artemia* receiving 2 KR, no effects were noted. Two weeks after irradiation, the irradiated oocytes had reached the mature stage and normal maturation occurred. In those receiving 5 KR, oocytes in the pre-meiotic stages were severely damaged (pycnotic nuclei within 1 day). Mature oocytes and eggs undergoing cleavage were generally normal in appearance although abnormal nuclei in some eggs were found occasionally. Also, repopulation by the division of oogonia and oocytes was observed in the ovaries of adults receiving doses of 2 and 5 KR. However, it could not be determined whether these cells went on to become functional oocytes.

There are no published studies dealing with the effects of radiation on male reproductive tissue.

RADIATION EFFECTS ON OTHER TISSUES AND ORGANS

With the exception of a brief examination of the blood-forming tissue following irradiation (Boswell, 1966), there is no information with respect to the effects of radiation on any other organs. It would certainly be desirable to examine blood-forming tissue, digestive tract tissue and perhaps others in this respect.

Population effects, reproductive potential, and fitness

Several publications were concerned with radiation effects as they might affect populations, while others studied environmental radiation in the form of radioisotopes in the surrounding medium and the actual effect on a population.

The first group (Holton, 1968 ; Squire, 1969, 1970, 1973 ; Squire and Grosch, 1970 ; Holton *et al.*, 1971ab) described two independent research projects that were being carried out at least partly during the same time period. Although there are some similarities in the two projects, each of them will be described separately, and then be compared.

The first (Squire, 1969) listed the following goals : 1) to develop the use of *Artemia* as an organism for radiobiological and genetic studies ; 2) to estimate the overall effects of acute gamma radiation on fitness components of amphigonic *Artemia* ; 3) to study reproductive behavior of X_1 radiation heterozygotes. This was to be accomplished by : 1) studying the effect of various acute doses, from 100 R to 100 KR, on the life span and reproductive potential of adult *Artemia* ; 2) studying the reproductive behavior and life span of X_1 animals after their mother's oogonia had received a dose of 1 KR acute gamma radiation ; 3) estimating the type of genetic load in the species by using an inbred line of *Artemia*.

Squire examined several aspects of reproductive behavior in non-irradiated and gamma irradiated females and males such as life span, fecundity, fertility, cyst hatchability, number of broods, survival to adulthood, sex ratios and other characteristics. Using information derived from these experiments, an attempt was made to predict the effects of an acute dose of radiation

on a population of *Artemia*. Long term experiments were designed to determine the effects of acute maternal doses of radiation on the intrinsic natural increase of an *Artemia* population and also the effects of inbreeding.

The following is a brief summary of Squire's results :

Life span and reproductive behavior

- significant reductions of female life span occurred after 10-100 KR ;
- significant reductions of male life span occurred after 50-100 KR ;
- female fecundity was reduced after 1 to 5 KR and became zero after 10 to 100 KR ;
- cyst hatchability showed no dominant lethality after 1 KR but it was evident after 5 to 10 KR ;
- the average number of broods and oviviparity was reduced after 2 to 5 KR ;
- survival to adulthood, adult mortality, and reproductive patterns in the first brood were affected in animals from females receiving 1 to 2 KR ;
- sex ratios were not significantly changed by any doses ;
- males were permanently sterile after 50 to 100 KR and permanently sterile after a 12 day fertile period after 5 to 10 KR. After 2 KR, most males were temporarily sterile following a 12 day fertile period and after 0.1 to 1 KR there was no detectable sterility ;
- animals derived from males after 2 KR were not affected with respect to hatchability but there was 73 % lethality after 10 KR as well as reductions in survival to adulthood, fertility, and life span upon adulthood. Sterility seemed to be greater in the X_1 than in the fathers.

Predicted effects of acute irradiation on a population

- A dose of 2 KR or more would be expected to result in the death or almost complete sterility of all stages except cysts ;
- A dose of 1 KR would be something less than that in 1.

Effects of inbreeding

Rapid and pronounced inbreeding depression occurred in all lines and was manifested as reductions of life span, brood size, cyst hatchability, number of broods, survival to adulthood, net reproductive rate, intrinsic rate of increase, and total sterility in some males. In two of the six lines, fecundity became zero in the third generation. After several generations, the inbred lines were usually totally inviable. These observations should be seriously considered by anyone carrying out long term population experiments not only with radiation but with other materials.

Effects of maternal irradiation on an inbred line

This line had an estimated coefficient of inbreeding of 0.76, but an acute dose of 1 KR to maternal oogonia had no significant effect on the reproductive behavior of the next generation.

The second major population study (Holton, 1968) lists as objectives : 1) to determine what doses of gamma radiation will affect the reproductive ability of *Artemia* ; 2) to determine the dose required to reduce the reproductive ability of a population to zero and thus fix the level at which a laboratory population would go to extinction.

In these experiments, all irradiations were performed on larvae 22 or 23 days of age (10-20 % in stage 10, 80-90 % in stage 11 of Heath, 1924). The doses ranged from 300 to 6 000 R. Twenty

duplicate pair matings were performed at each dose and continued until both parents died (males were replaced if they died first). All offspring were followed until they reached maturity. The results of these experiments are summarized in Table IV.

TABLE IV

Effects of irradiation as determined from pair matings (20 pairs at each dose). (Standard errors omitted)

Dose (R)	Number of broods	Broods/ pair	Total nauplii	Nauplii/ pair	Nauplii/ brood	% survival to adulthood	Total adults	Adults/ pair	Adults/ brood
0	87	4.35	9 692	484.6	111.4	64.9	6 290	314.5	72.3
300	92	4.60	9 916	495.8	107.8	67.8	6 723	336.2	73.1
600	89	4.45	10 305	515.2	115.8	56.4	5 812	290.6	65.3
900	60	3.00	7 005	350.2	116.7	59.3	4 154	207.7	69.2
1 200	53	2.65	8 321	416.0	157.0	50.7	4 219	211.0	79.6
1 500	39	1.95	4 831	241.5	123.9	40.3	1 947	97.4	49.9
1 800	26	1.30	1 894	94.7	72.8	31.1	589	29.4	22.7
2 100	9	0.45	237	11.8	26.3	0	0	0	0
2 400	0	0	0	0	0	0	0	0	0
3 000	0	0	0	0	0	0	0	0	0
4 500	0	0	0	0	0	0	0	0	0
6 000	0	0	0	0	0	0	0	0	0

Examination of the results reveals several important points. The range of doses employed did cover the range of reproductive sensitivity since doses of 2 100 KR and above produced almost complete sterility even though the animals themselves survived essentially (?) as long as the controls. Although the slight increase in reproduction per pair at 300 R was not statistically significant, it should be investigated further, as others have reported stimulatory effects of low doses of radiation (Ivanovskii and Mitrofanov, 1978 ; Ivanovskii, 1980ab ; Ivanovskii and Kulinick, 1980 ; Fedorik, 1983 ; Radchenko, 1984). The number of adults/pair remains relatively constant at doses up to 1 200 R and then decreases rapidly, perhaps indicating some type of threshold. Below that dose, the decrease is due primarily to a reduction in the number of broods produced.

In his second major experiment, Holton (1968) established several populations. At that time, a random sample of the population (including all stages) was removed and irradiated with doses ranging from 500 to 3 000 R. At 4 week intervals for 20 weeks (about five generations), counts of the total number of individuals (age 7th instar or older) in the population were made. The results indicate that, after 8 weeks, the numbers in the control population had nearly doubled. However, a comparison of this increase with the pair mating experiments revealed that the population had only realized 1/80 of its potential for growth. Thus, some environmental factor was limiting the population size. This is further confirmed by the fact that the control population increased much more slowly after the 4th week and actually declined during the final weeks. The 500 R population revealed a similar pattern of growth but with a 4 week time lag. Although the 1 500 and 3 000 R populations increased more slowly, by the end of the experiment all

populations were approaching the same size. This might be interpreted to imply that the irradiated populations had recovered from whatever radiation damage they had incurred (see further).

A third experiment, in which pair matings were performed with individuals taken from the irradiated populations, gave a possible answer to this question of recovery. These experiments revealed that all reproductive parameters were reduced by the radiation by the end of 4 weeks and continued at reduced values for the 20 week length of the experiment. In the 500 R group, these reductions were less pronounced and some of them recovered to near normal values within 20 weeks. However, in the 1 500 and 3 000 R groups, the reductions were not only more pronounced, but only minimal recovery, if any, had occurred by the end of 20 weeks.

Thus, it was concluded that even though the irradiated populations had incurred significant radiation damage to all of the elements producing its reproductive potential, they were still able to maintain near normal population levels within the environmental constraints to which all of the populations were subjected. This clearly indicates that merely measuring total population size will not necessarily give an accurate assessment of damage by radiation and perhaps other injurious agents.

One additional publication can be mentioned. Ballardin and Metalli (1968b) performed similar but perhaps less extensive experiments. They irradiated 14-16 day old diploid, parthenogenetic shrimp with doses of 500 and 1 000 R of X-rays. Each new generation was irradiated at that rate. They measured fecundity (number of eggs produced/20 days), fertility or hatchability (% of eggs that hatch within 24 h), and survival (% of larvae that reach adulthood). They found that no significant changes occurred for seven generations but in the eighth generation there was a sudden reduction in most of the measured components.

Another group of experiments investigating the effects of radiation on populations was carried out by Grosch and Plumb (1959), and Grosch (1962, 1966). Three liter populations of *Artemia* were established and they were treated initially and/or periodically with different quantities of P-32, Zn-65, or by the irradiation of adults with X-rays. Periodically they were tested by performing pair matings. Measurements made were survival of the cultures, adult life span, and various aspects of reproductive fitness (broods/pair, cysts and larvae/pair, cysts and larvae/brood, % of larvae surviving to adulthood, mature adults/pair). Other components measured were hatchability of cysts and sex ratios. Some of the populations were followed over a 4-year period with others for shorter time periods. Some of the major results and conclusions were :

- Populations do not persist if more than 90 μC of P-32 or more than 30 μC of Zn-65 are added. Populations with 30 μC of P-32 do not survive the addition of a second 30 μC. The same is true if more than 2 000 R of X-rays are administered to the ten pairs of individuals used to begin these cultures.
- The treatments used had no effects on the original adults treated. The effects appeared only in subsequent generations.
- A decrease on both zygotes produced and survival to adulthood contributed to the decline in population size.
- With respect to sex ratios there were more females in the controls but more males in the irradiated groups.
- Part way through the experiments the salinity of the media used for the pair mating tests was modified and both the life span and reproductive behavior was improved or increased.

In a separate experiment (Grosch and Plumb, 1959), it was found that the uptake of P-32 was greater in adult females than in males, although the levels in females declined with time until they equaled that in males after about 12-15 days. It was suggested that this occurred as the P-32 was incorporated into the eggs and young that they were producing.

In experiments continuing his earlier work, Grosch (1966) further investigated the effects of periodic additions of more radioactive material. In some cases, adding a second dose to a population that had received an earlier dose can cause extinction even though a dose equal to the sum of the two doses would not have caused extinction initially. Recovery from the effects of the first dose may take 2 to 4 years. The frequency of brood deposit is not affected by the treatments. As opposed to an earlier conclusion, there are now more females than males in all groups. Maintenance of populations of 300 adults/3 l requires only 0.2 % of the reproductive potential in controls. In experimental cultures, 1 % or more of their potential must be utilized to maintain the same numbers.

Cytological and genetic effects

Cytological effects

Except in the context of other types of studies mentioned above, there are few publications dealing with the cytological effects of radiation in *Artemia*. One important study, however, studied the fine structure of the chromosomes in *Artemia* using colchicine treatment and X-ray treatment (Stefani and Cadeddu, 1967). They attempted to determine whether fragments of chromosomes produced by X-ray damage are transmitted to succeeding cells. From their results they concluded that broken pieces of chromosomes behave as if they have their own centromere. Therefore, they concluded that *Artemia* has a diffuse or non-localized centromere. If this is the case, it contributes to an explanation of the higher radioresistance of *Artemia* cells.

Genetic effects

Many of the effects of radiation on all stages of *Artemia* are undoubtedly produced by genetic changes. This is assumed in the case of dominant lethal and other types of mutations produced during developmental stages. However, little specific quantitative information is available describing specific mutations produced by radiation.

Bowen (1963b), in an attempt to find a dose of radiation which would be most efficient for inducing mutations, irradiated cysts with 0.4, 2, and 10 KR and the shrimp derived from these were examined for abnormalities. When one was found it was outcrossed to the control stock and its siblings and parents were inbred to possibly produce more with the mutant phenotype. Most of the abnormalities found (absence of setae on distal lobe of legs, swollen abdomen, bent abdomen, kidney-shaped eyes, bump on seventh abdominal segment) were not sufficiently viable for genetic experiments. However, one shrimp with garnet eye color appeared in the second generation of the 10 KR line. Further study revealed that the trait was inherited as a recessive autosomal gene (see Hanson, 1963 for details).

Other investigators (Bowen, 1962, 1963a ; Bowen and Hanson, 1962 ; Hanson, 1963 ; Cervini, 1965 ; Bowen *et al.*, 1966 ; Squire and Grosch, 1967 ; Barigozzi *et al.*, 1968, 1969) have encountered mutant forms during the course of other research projects, some of which may

have involved the use of radiation. Therefore, some of these mutants may have been produced by radiation but usually no estimates of the doses which may have produced them are possible.

Radiation effects and ploidy

Several publications are dealing with the effects of ploidy on radiation effects in *Artemia*. Most of these are a series of papers by Metalli, Ballardin and others which were summarized by Metalli and Ballardin (1972) and Metalli (1980). The reader is referred to these for a detailed description and discussion of their work. The following lists and discusses some of their general conclusions :

- in *Artemia*, tetraploidy is clearly associated with an increased resistance to ionizing radiation ;
- this increased resistance in the adult is greatest in oocytes of parthenogenetic strains irradiated in early meiotic stages when the chromosomes are condensed ;
- the best hypothesis to explain this difference is probably a greater ability of the tetraploid cells to buffer the lethal radiation damage ;
- the difference does not seem to be related to repair or recovery events nor damage to the recovery systems ;
- the effect is greatest overall for total embryonic lethality in the irradiation of dry cysts (as much as 2.5 times greater) where disturbances of both cell division, and cell and tissue differentiation are involved in the final appearance of the radiation damage ;
- the effects of radiation on life span are apparently independent of the degree of ploidy or other genetic and strain differences. This is associated with a complete absence of cell differentiation and a mitotic rate that is zero in most tissues and extremely low in a few others.

Uptake of radioactive materials

The uptake of radioactive materials by *Artemia* has been widely studied. Although, strictly speaking, most publications do not deal with radiation effects, they will be briefly summarized at this point. Others not specifically described here are Chipman (1972), Mauchline and Templeton (1964), Polikarpov (1966, 1967), and Seymour (1962).

Boroughs *et al.* (1957, 1958) described the uptake of Sr-89/Sr-90, Y-90 by brine shrimp. They found that the amount taken up reached a steady state after 16 h and increased only slightly after 96 h. It was concentrated about 40 fold (concentration ratio 0.13). Chipman (1958ab) showed that *Artemia* concentrates cesium-137 more rapidly and to a higher level than it does with strontium-85.

Rice (1963) demonstrated that the concentration of Zn-65 is much greater in *Artemia* when it is present in the food than when it is only present in the surrounding water.

Hallopeau (1969ab) described the uptake of fission products (2 µCi/l) and cesium-137 (40 µCi/l). Early larvae (24-48 h after hatching) were placed in solutions and their development and reproduction were studied for a period of 5 months. They reported no significant differences between treated animals and controls.

Jennings and Rainbow (1979ab) compared the uptake of cadmium directly from seawater and from ingested food material. The cadmium (0.1, 1 and 10 ppm) was presented in four ways :

1) in solution ; 2) in solution in the presence of latex "food" particles ; 3) in solution with cadmium-rich *Dunaliella* as a food source ; 4) none in solution but with cadmium-rich *Dunaliella* as a food source. The ratios of cadmium accumulated from solution to cadmium accumulated from food was 1:4.9 (0.1 ppm), 1:6.7 (1 ppm), and 1:1.1 (10 ppm). They concluded that the food chain is the major source of cadmium as long as the previous trophic level has the ability to accumulate the metal and make it more available than by direct intake from the water.

Mukade and Higuchi (1974) studied the uptake of tritium (H-3) from seawater and its incorporation into *Artemia*. The seawater tritium concentrations were from 100 μCi to 7.5 mCi/ml. The body content of tritium increased rapidly during the first 8 to 30 h when it became constant and practically the same as the surrounding medium. When returned to tritium-free water, the tritium content of the animals slowly decreased. The highest concentration (7.5 mCi/ml) did not affect the hatchability of cysts. However, animals reared at this concentration "could not mature" and died within 24 days. Concentrations lower than 3.5 mCi/ml had no effect on growth rate. Animals reared in concentrations above 500 μCi/ml showed significant reductions in life span. Rearing in 100 and 500 μCi/ml caused reductions in number of broods/female, reproductive span, and larvae/brood resulting in a decreased total number of larvae/female. No reproduction occurred above 750 μCi/ml. Animals were reared for seven generations with no significant cumulative changes. They suggested that the internal radiation from β-decay seems to result in more severe effects on reproductive capacity than external radiation with respect to total dose.

Higuchi and Mukade (1976), using autoradiographic methods, studied the distribution of tritium taken up by 2 month-old adults who had been grown in tritium solution (0.5 mCi/ml) since they were 2-3 days old. The autoradiographs revealed that although the tritium was taken up by all tissues of the body, the density is much greater in the ovisac and/or eggs. Also, the periphery of the tail in young females, where the ovary localizes, also had a greater density than the rest of the tail. They therefore concluded that the greater effect of tritium radiation noted above is due not only to the larger RBE of β-particles but also due to the fact that it is concentrated in the developing female reproductive cells.

Higuchi *et al.* (1980) attempted to assess the effect of tritium radiation on a model ecosystem with *Artemia* and diatoms (*Chaetoceros*). In one case the tritium was administered directly from the medium and in the other via the diatoms and from the medium. A lyophilizing apparatus was used to separate free and bound tritium which were then measured separately. In order to assess the isotope effect and its effect on metabolism of the organism a specific activity ratio (R value) was defined as the ratio of the specific activity of tritium incorporated into the organism to the specific activity of the external medium. If no isotope effect exists the R value would be one and the greater the isotope effect the greater the deviation of the R value from 1. The R value of bound and free tritium both increased until both reached a maximum after 20-30 h. At that time the R value for bound tritium was half that of the R value of free tritium. Also, the R value for bound tritium was 0.5 without the food chain and 0.75 when the food chain was used. The autoradiographs of females again showed a concentration of tritium in the ovary (eggs) and ovisac. Fecundity as measured by larvae/female, duration of breeding, broods/female and larvae/brood were all reduced with increasing concentrations of tritium from 0.1 μCi/ml to 1 mCi/ml. A rough calculation of dose from 0.1 mCi/ml results in 26.2 R/day from free tritium and 0.6 R/day from bound tritium. Thus the total dose after 15 days would be slightly greater than 400 R. They also concluded that tritium incorporation through the food chain in addition to incorporation

directly from the medium increases the amount of tritium in the bound form and therefore increases the radiation effect by 50 % over that from tritium only from the medium.

Another phenomenon was suggested by Nakazawa and Yasumasu (1963). They state that they confirmed a specific incorporation of P-32 into the RNA of *Artemia* cysts immediately after incubation in 2 % NaCl while incorporation into DNA did not begin until 10 h of incubation. They treated hydrated cysts with 20 μCi/ml of inorganic P-32 during the last minute of the first 4 h of incubation or the entire first 4 h period of incubation. The two were not significantly different in the total radioactivity present in the embryo but the radioactivity of the RNA fraction was 25 times higher in those treated for the entire 4 h. The DNA fraction contained no radioactivity in either group. The 1 minute treatment showed no change in hatchability as compared to the control. The 4 h treatment, however, produced a decreased hatchability as well as a delay in the hatching period beginning with cysts stored 15 days and both changes became more pronounced after 30 days of storage. They suggest that these phenomena are produced not by the β-radiation but by the transmutation of the phosphorus atoms incorporated into the RNA.

Applications of radiobiological studies to aquaculture

Can the information derived from any of these investigations of the radiobiology of *Artemia* be applied to solving practical problems in the use of the brine shrimp in aquaculture? Probably not in any obvious way at the present time. However, a fuller understanding of any facet of an organism's biology frequently leads to applications that one cannot anticipate. Certainly the knowledge derived from population and other ecological investigations may lead to applications in mass culturing. It is theoretically possible to use radiation in the production of mutations which may result in animals with characteristics that would be desirable when using *Artemia* as a food source in aquaculture. However, this first necessitates the identification of those characteristics which are "desirable" from the standpoint of aquaculture and then determining if they are genetically controlled. Then, as the presence of these desirable features can usually not be determined merely by examining a brine shrimp, lengthy cross-breeding and culture procedures must be performed. Only then can it be determined whether a new strain with the desirable feature has been developed.

Summary and discussion

– *Artemia* cysts are highly radioresistant, the LD_{50} for hatching being in the vicinity of 500 KR. The degree of hydration and other environmental characteristics (oxygen, temperature, storage) will modify radiosensitivity. There is probably no recovery or repair of cyst damage. High LET radiations are more effective in producing damage. The effect on larval viability is greater than that on hatching. The amount of damage is related to free radicals produced. Chemical protective agents are effective in cysts.

– Larval stages are much more sensitive to radiation with earlier stages being more sensitive than later stages. Growth rates are reduced in irradiated larvae. Visible abnormalities are not common in surviving larvae. Some physiological changes (*e.g.* respiration rate) are affected by radiation.

– Adult *Artemia* are less sensitive than larvae but much more sensitive than cysts. Decreased life spans are produced by doses beginning at 10 KR and decrease further at higher doses. In the

female, early maturation stages are more sensitive than later stages. Little information is available with respect to other tissues.

– Ecological studies have revealed that many aspects of reproduction (in females) are modified by moderate doses (1000 R) of radiation. However, it has been demonstrated that measurements of total population numbers can lead to incorrect conclusions regarding effects of radiation on "fitness" for survival. Inbreeding depression has been clearly demonstrated. Similar effects have been demonstrated in populations grown in cultures containing various radioisotopes.

– Cytological studies have demonstrated that *Artemia* apparently has diffuse (non-localized) centromeres. This may explain, in part, the high radioresistance of *Artemia*. There are few quantitative studies of mutation induction.

– Tetraploidy clearly imparts an added radioresistance at all stages except in the adult (*e.g.* life span).

– Radiobiological studies of *Artemia* have produced much basic information about many aspects of its life which will ultimately be utilized in practical applications in the field of aquaculture.

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The combined effect of temperature, salinity, and gamma-radiation on biological characteristics of *Artemia*

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Abstract

The effects of γ-irradiation at doses of 2.5, 5.0, 7.5, and 10.0 Gy at salinities of 18, 54, and 108 ‰ and average temperatures of 15, 22, 25, and 27 °C on *Artemia* are reported. The total survival, survival at different developmental stages, average life duration, mortality, and development were measured. The *Artemia* nauplii were γ-irradiated for 1 day.

A relationship was established between the irradiation effect (stimulation or inhibition) and the temperature which is favorable for the development of *Artemia.*

Analysis of the survival rate at different developmental stages showed that greater age directly enhances the effect of temperature on survival and inversely the irradiation effect as compared to the control.

The temperature appeared to influence the dynamics of mortality more than did the irradiation dose in the range used. At 15 and 22 °C the curves had a single peak, at 25 and 27 °C the peak height is less but multiple peaks occur.

The combination of temperature, salinity, and irradiation affected characteristics such as average life duration, larval developmental rate and sexual maturation.

It is suggested that γ-radiation doses of up to 5 Gy may be regarded as low doses for *Artemia.*

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The influence of aqueous copper chemistry on the uptake and toxicity of copper in *Artemia*

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Abstract

The effect of organic complexation, salinity, and pH on the uptake and toxicity of copper in *Artemia* was studied. The results were compared with data on the chemical speciation and lipid solubility of copper in saline solutions.

The uptake experiments took 90 min and the toxicity tests 24 h. A computer model was used to calculate the saline solution equilibrium speciation. The lipid solubility of copper was determined in a series of saline/olive oil partition tests.

Complexation of copper by EDTA reduced the uptake and toxicity of copper. Salinity had no apparent effect on both processes. The results of these uptake and toxicity experiments agreed well with the copper speciation and lipid solubility data. The effect of pH on the uptake and toxicity of copper is more complex. The uptake rate is maximal around pH 7.6 and lowest below 7.0. The toxicity increases towards alkaline conditions. These results do not relate to the speciation and lipid solubility data in a straightforward manner.

Possibly two distinct ligands determine the biological availability of copper in solution. Ionized species interact with the charged ligands present on the external surface of cell membranes (*i.e.* glycoproteins, proteins, polar heads of lipids) and neutral species move across the lipid bilayer by passive diffusion.

Introduction

Many aquatic invertebrates, including the brine shrimp, can accumulate and tolerate very high levels of metals in the tissues. Although the concentration of metals in most saline water systems is rather low, point source discharges such as municipal and industrial wastewater outfalls can lead to high environmental concentrations (Moore and Ramamoorthy, 1984 ; Salomons and Förstner, 1984).

Geochemical and physiological processes are both important in determining the biological availability of metals. A major factor which complicates studies on the accumulation and toxicity of metals is the complexity of their water chemistry. In addition, the nature of the interface between environment and organism has an important effect upon the absorption process. This interface is essentially a lipid bilayer membrane, impregnated with molecules that sequester polar substances and help in their transport. Several studies suggest that metals are taken up through facilitated diffusion of the metal species with the highest free energy, *e.g.* the free cation (Andrew *et al.*, 1977 ; Anderson and Morel, 1978 ; Sunda and Lewis, 1978 ; Canterford and Canterford, 1980 ; Zamuda and Sunda, 1982). Alternatively, it has been suggested that neutral inorganic metal species, which are more lipid soluble than the corresponding cations, may permeate cell membranes (Gutknecht, 1981, 1983 ; Simkiss, 1983, 1984). A poor understanding of the processes that control the biological availability of metals is a major impediment to define, prove or predict metal impacts on nature.

Copper is of special interest because of its known toxicity to aquatic organisms and its presence in industrial effluents. This study was undertaken with two objectives : to determine the influence of inorganic and organic speciation on the uptake and toxicity of copper, and to evaluate the usefulness of metal speciation and lipid solubility data to model the biological availability of copper. A chemically-defined model system was used to study the effects or organic complexation, salinity, and pH on the uptake and toxicity of copper to *Artemia*.

Materials and methods

Culturing of brine shrimp

Brine shrimp cysts (San Francisco Bay, USA) were hatched in 34.5 ‰ artificial seawater (HW-Wimex) at 25 °C under continuous illumination and aeration. The nauplii were reared in 60 l air-water lift operated raceways and fed with a suspension of *Spirulina maxima* (Cyanophycea). Animals reached maturity after 2 to 3 weeks and were used before they were 6 weeks old.

Copper uptake and toxicity

Tests were conducted at 25.0 ± 0.5 °C under continuous illumination from fluorescent tubes. Uptake experiments were performed in aerated 1.5 l PET jars, each containing 100 individuals. Animals were acclimated for 5 days to the experimental conditions and starved for 24 h before the experiment. The metal-containing test solutions were prepared 1 day before use. The composition of the chemically-defined saline solution is given in Table I.

Table I

Composition of the chemically-defined saline solution

Reagent	Concentration	
	g/l	M/l
NaCl	24.407	0.409
Na_2SO_4	4.155	0.0286
KCl	0.727	0.00955
$NaHCO_3$	0.204	0.00238
$MgCl_2.6H_2O$	17.338	0.0835
$CaCl_2.2H_2O$	1.527	0.0102
H_3BO_3	0.0269	0.000426

Each chemical must be completely dissolved before another is added.
Salinity = 34.5 ‰, pH = 8.20, pE = 12.50, I = 0.599.

After equilibration the air-saturated solution had a pH of 8.20 and a salinity of 34.5 ‰. Test solutions with a salinity higher or lower than 34.5 ‰ were prepared by dissolving the required quantity of the five principal salts in deionized water. The added amount of $NaHCO_3$ and H_3BO_3 was the same over the entire salinity range. The pH of the medium was adjusted by adding HCl. Copper was added as sulfate and EDTA as sodium salt.

Each uptake experiment took 90 min and animals were sampled for copper analysis after 30, 60, and 90 min (for EDTA also after 10 and 20 min). The jars were emptied over a 250 µm sieve and the collected specimens rinsed with deionized water before transfer to storage tubes for desiccation at 60 °C for 24 h. The 24 h acute toxicity tests were carried out in exactly the same way as the uptake experiments. The animals were considered dead when they were completely immobile and did not respond to mechanical stimulation. All experiments were repeated five times.

Copper lipid solubility

The lipid solubility of copper, *i.e.* the metal's distribution coefficient between saline and a lipid bulk phase, was determined using an experimental set-up based upon the design described by Simkiss (1983). Extraction ratios for copper were determined between 50 ml chemically-defined saline and 5 ml olive oil (Aldrich Chemie) in screw-capped polystyrene vials. The vials were placed on a shaking table which was placed in a controlled-temperature room (25.0 ± 0.5 °C). Samples of the saline solutions were taken after 24 h.

Metal analysis

Copper determinations were made by atomic absorption using a Perkin-Elmer 703 Spectrophotometer fitted with a Burner Control for flame measurements and a Heated Graphite Atomizer HGA-500 for furnace analysis. Concentrations above 1 mg/l were generally determined by flame, while submilligram analytical work was done with the graphite atomizer, applying stabilized temperature atomization and deuterium arc background correction (Slavin *et al.*, 1983).

HNO_3 acidified seawater-solutions or filtrates were analyzed directly against a matrix matched calibration curve. Initially the biological material was digested with ultrapure nitric acid in a teflon-lined high pressure bomb (Paus, 1972). Later, this method was abandoned and samples were digested in a microwave oven (Blust *et al.*, 1985).

Chemical modeling

Chemical species equilibrium concentrations and activities were calculated using the computer program PHREEQE (Parkhurst *et al.*, 1982). Based on an ion-pairing aqueous model, the program allows the calculation of the composition of solutions in equilibrium with gaseous and solid phases. For our specific applications we compiled a thermodynamic data base, based on the data listed in Martell and Smith (1974, 1982), Dickson and Whitfield (1981), and Turner and Whitfield (in press). Case-specific input includes total concentrations of metals and ligands, pH, pE, temperature, and mineral phases which are maintained at equilibrium with the solution. The program was run on a CDC Cyber 180-825 computer.

Results

Organic complexation

At pH 8.20 $CuCO_3$ is the most important copper species. Cu^{2+} is one of the minor species and accounts for about 5 % of the total copper concentration (Fig. 1). The speciation calculations

indicate that copper is readily complexed by EDTA above a ligand concentration of 0.1 µM/l. At 10 µM/l virtually all copper is bound to the chelator. The partitioning of copper between the saline solution and olive oil is reduced by the addition of the organic ligand (Fig. 2). EDTA significantly reduces the uptake and toxicity of copper (Fig. 3 and 4). The results of all these separate experiments agree very well.

Salinity

Increasing the salinity from 5 ‰ to 125 ‰ has no important effect on the concentration of $CuCO_3$, but the concentrations of the minor species change considerably (Fig. 5). The salinity does not significantly alter the lipid solubility of copper either (Fig. 6). Likewise, the uptake and toxicity of copper do not change with the salinity of the medium (Fig. 7 and 8).

pH

The speciation of copper in saline waters depends largely upon the pH of the medium. With decrease in pH (increasing hydrogen activity) Cu^{2+} and $CuSO_4$ assume more importance, at the expense of $CuCO_3$ (Fig. 9). The extraction of copper in the lipid layer is also influenced by the pH (Fig. 10). Above pH 7.0 the lipid solubility remains constant and seems independent of pH. Below pH 7.0 the lipid solubility drops quickly and almost no copper is extracted. The effect of pH on the uptake and toxicity of copper in brine shrimp is more complex. The accumulation rate is highest around pH 7.6 and lowest below pH 7.0 (Fig. 11). The effect of pH on the toxicity does not show such an optimum, but the response is also minimal below pH 7.0 (Fig. 12). Neither the speciation calculations nor the lipid solubility data seem to relate to the biological availability of copper in a straightforward manner.

Discussion

The synthetic chelator EDTA reduces the uptake and toxicity of copper in *Artemia* (Fig. 3 and 4). There is a considerable amount of literature which indicates that complexation by organic substances reduces the biological availability of copper to aquatic invertebrates and fishes (Sprague, 1968 ; Biesinger *et al.*, 1974 ; Stephenson and Taylor, 1975 ; Knezovich *et al.*, 1981 ; Zamuda and Sunda, 1982). Most of the evidence comes from toxicity studies. It should, however, be noted that toxicity is not a good measure for the biological availability of a metal unless each absorbed species elicits the same response. Nevertheless, the results show that complexation of copper by EDTA affects both uptake and toxicity in the same way. The ability of a chelator to reduce the biological availability of copper is related to its log K, which indicates the strength of the copper complex. Thus, the biological availability of copper does not relate to the total concentration of the metal in solution. Addition of EDTA to the saline solution reduces the lipid solubility by formation of the hydrophilic copper-chelate (Fig. 2) in the same way as it alters the uptake and toxicity of the metal.

Although the effect of complexation by an organic ligand on the biological availability of a metal is often related to a decrease in the cupric ion concentration, the chemical speciation calculations show that complexation by an organic ligand reduces the concentration of all inorganic species (Fig. 1). This means that in an environment with a low organic load the

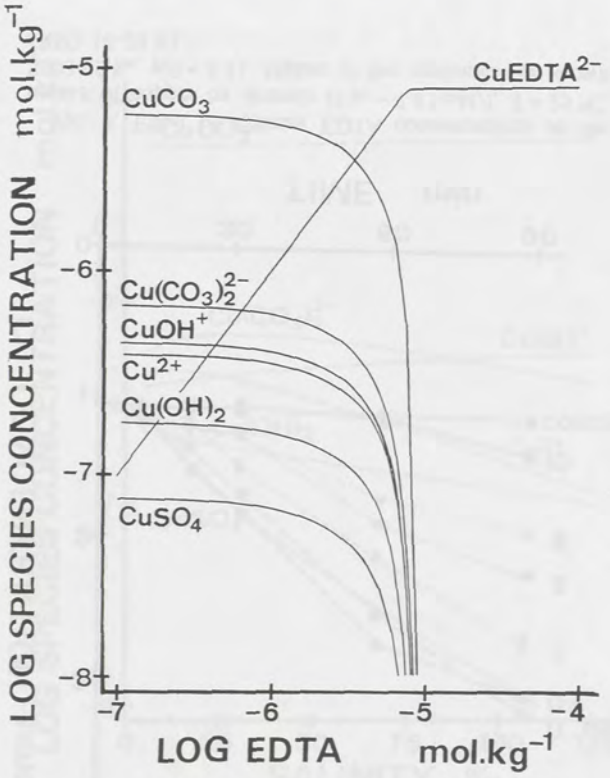

FIG. 1. Effect of EDTA on the calculated speciation of copper in a saline solution (total copper concentration Cu_T = 7.87 µM/l, T = 25 °C, S = 34.5 ‰, pH = 8.2).

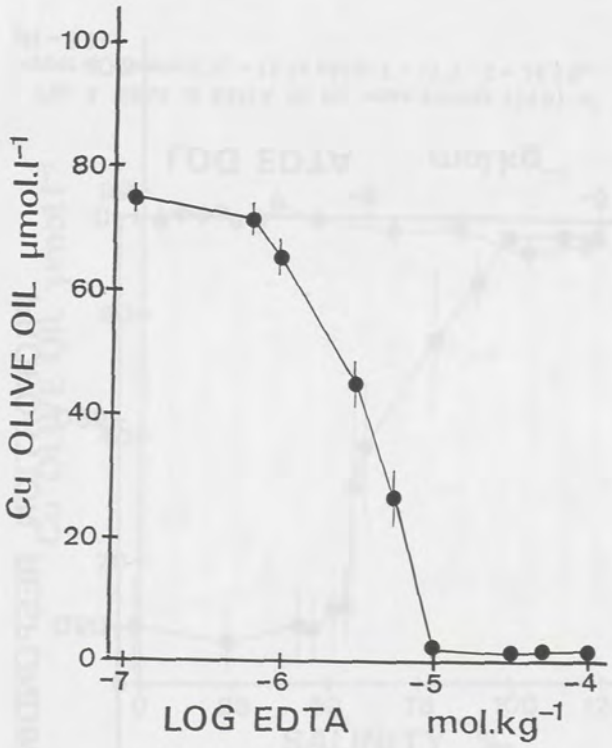

FIG. 2. Effect of EDTA on the lipid solubility of copper in a saline solution (Cu_T = 7.87 µM/l, T = 25 °C, Sal = 34.5 ‰, pH = 8.2).

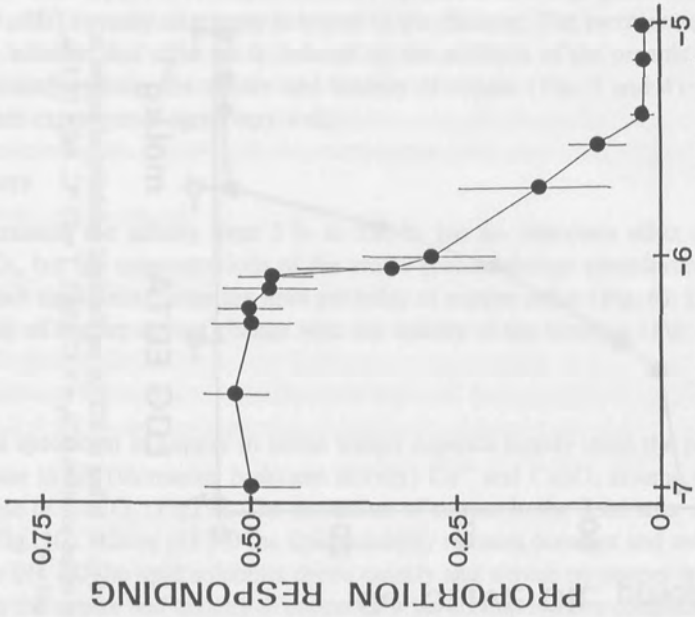

FIG. 4. Effect of EDTA on the acute toxicity (24 h) of copper to *Artemia* (Cu_T = 15.74 µM/l, T = 25 °C, S = 34.5 ‰, pH = 8.2).

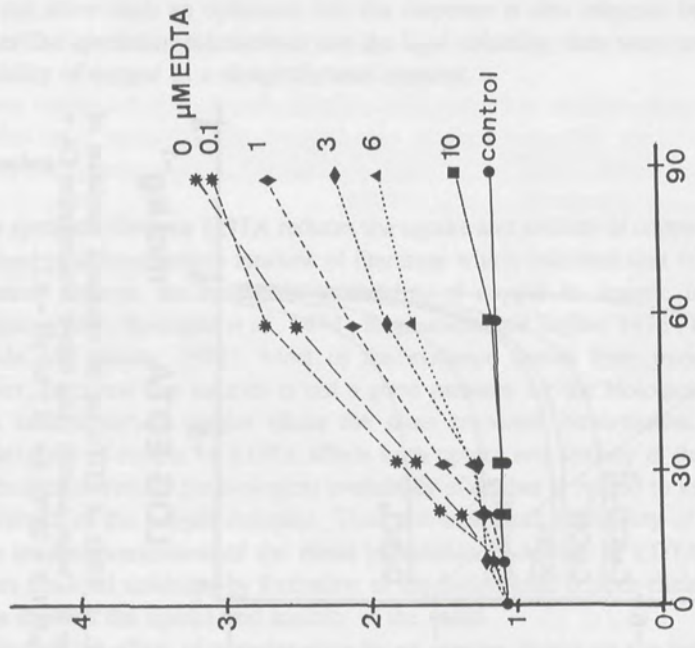

FIG. 3. Effect of different EDTA concentrations on the uptake of copper by *Artemia* (Cu_T = 7.87 µM/l, T = 25 °C, S = 34.5 ‰, pH = 8.2). Means of five replicates are shown (RSD 10-20 %).

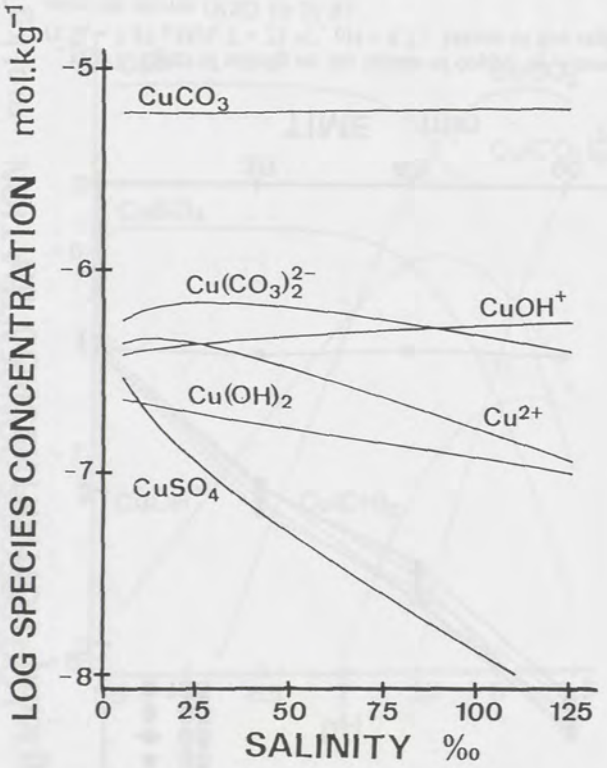

FIG. 5. Effect of salinity on the calculated speciation of copper in a saline solution (Cu_T = 7.87 µM/l, T = 25 °C, pH = 8.2).

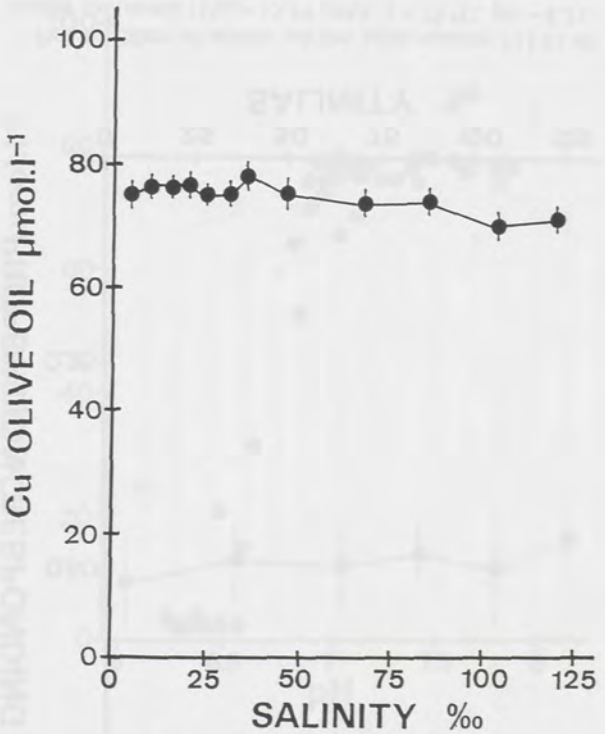

FIG. 6. Effect of salinity on the lipid solubility of copper (Cu_T = 7.87 µM/l, T = 25 °C, pH = 8.2).

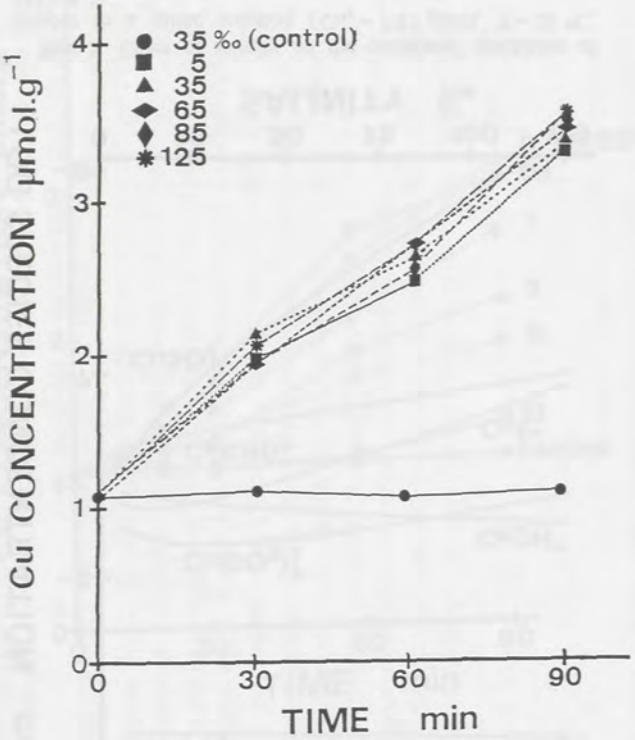

FIG. 7. Effect of salinity on the uptake of copper by *Artemia* (Cu_T = 7.87 µM/l, T = 25 °C, pH = 8.2). Means of five replicates are shown (RSD 10-20 %).

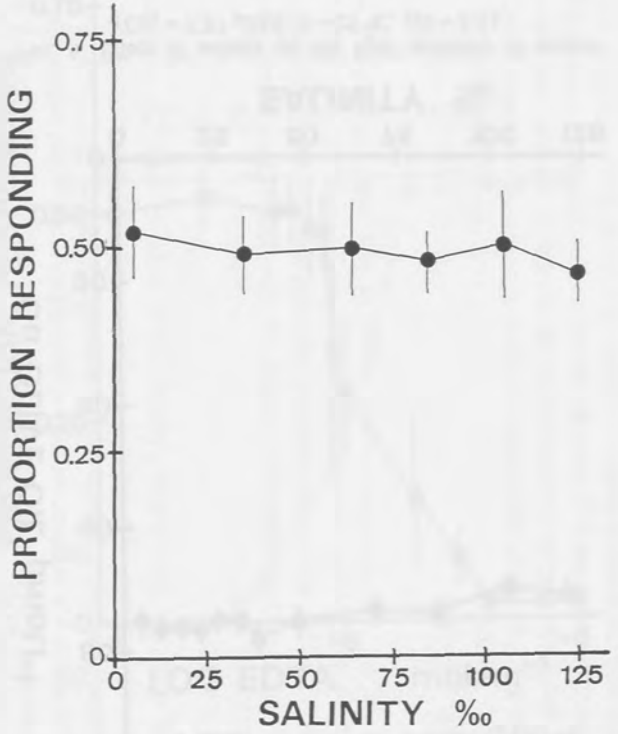

FIG. 8. Effect of salinity on the acute toxicity (24 h) of copper to *Artemia* (Cu_T = 15.74 µM/l, T = 25 °C, pH = 8.2).

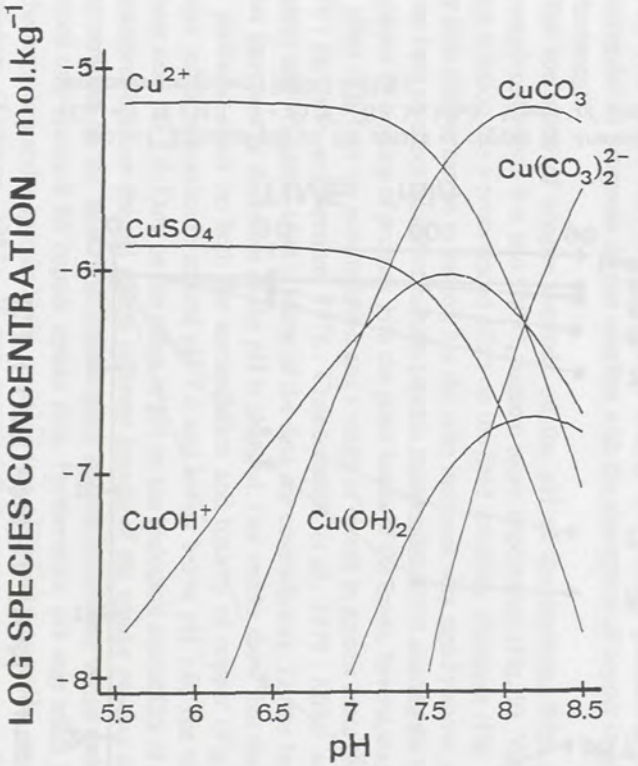

FIG. 9. Effect of pH on the calculated speciation of copper in a saline solution in equilibrium with atmospheric carbon dioxide. (Cu_T = 7.87 µM/l, T = 25 °C, S = 34.5 ‰, p_{CO_2} = $10^{-3.48}$ atm).

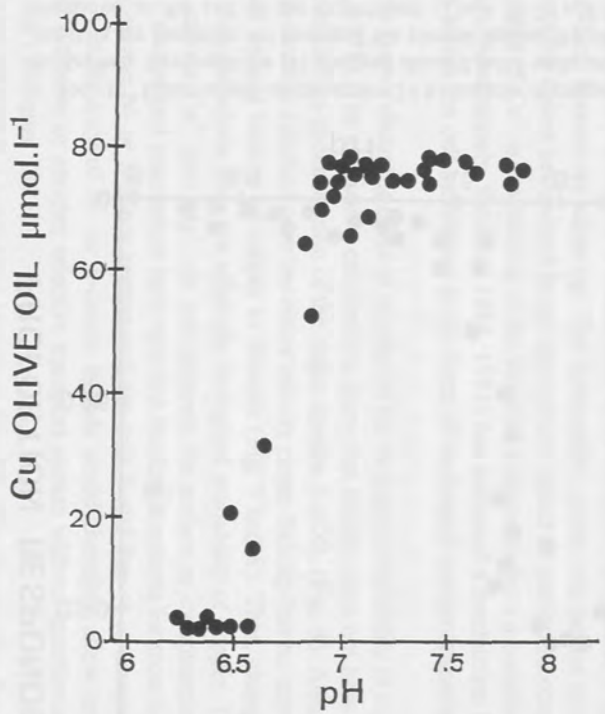

FIG. 10. Effect of pH on the lipid solubility of copper. Overnight the pH changed about 0.1-0.3 units and the data are therefore not averaged but plotted against the pH measured at the end of the experiment (Cu_T = 7.87 µM/l, T = 25 °C, S = 34.5 ‰).

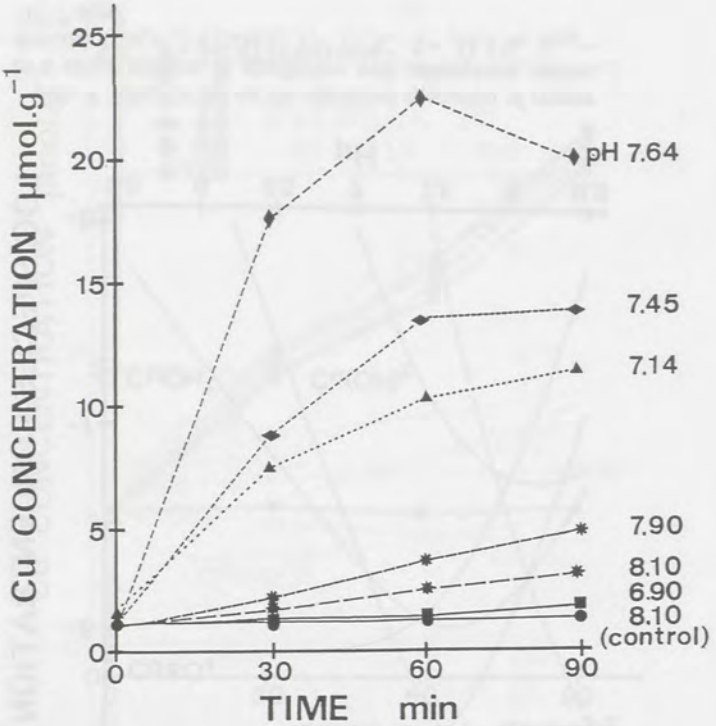

FIG. 11. Effect of pH on the uptake of copper by *Artemia* (Cu_T = 7.78 µM/l, T = 25 °C, S = 34.5 ‰). Means of five replicates are shown (RSD 10-25 %).

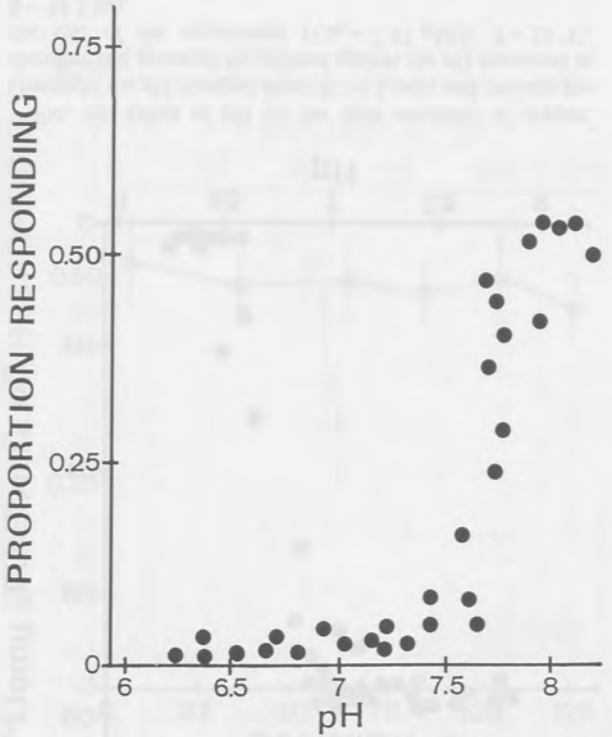

FIG. 12. Effect of pH on the acute (24 h) toxicity of copper to *Artemia*. Overnight the pH changed about 0.1-0.3 units and the data are therefore not averaged but plotted against the pH measured at the end of the experiment (Cu_T = 15.74 µM/l, T = 25 °C, S = 34.5 ‰).

biological availability of copper is determined by its inorganic speciation. It is, however, not clear which of these species are taken up. The hydrophilic cupric ion cannot cross the hydrophobic cell membrane unless neutralized by an appropriate ligand or carrier. Accordingly, the first step in the uptake of Cu^{2+} is the binding of the ion with a ligand to form a complex that can pass into the tissues. Alternatively, Simkiss (1983, 1984) has proposed a mechanism by which metals are taken up across the cell membrane in the form of uncharged inorganic species, *e.g.* $Cu(OH)_2$ or $CuCO_3$.

Little is known about the effect of salinity on the biological availability of copper (Jones, 1975 ; Phillips, 1976). The speciation calculations show that salinity does not have an important effect on the equilibrium concentration of the major species $CuCO_3$ (Fig. 5). Also, the lipid solubility of copper remains constant over the entire salinity range. Salinity has no apparent effect on both the uptake by and toxicity of copper to *Artemia* (Fig. 7 and 8). Thus, changing the Na, K, Ca, and Mg concentrations does not affect the biological availability of copper. This implies that the divalent cations Ca^{2+} and Mg^{2+} do not compete for surface or cytoplasmic binding sites with copper. Calcium and magnesium have very low binding constants for most ligands. They do not bind strongly to N- or S-donor centres and are only found free or in association with O-donor groups. A sufficiency of N- or S-donor ligands could therefore allow uptake of copper by facilitated diffusion or another selective transport system without interference from calcium or magnesium (Williams, 1981).

The brine shrimp is a euryhaline organism capable of a remarkable degree of hypoosmotic regulation in highly concentrated media. The isoosmotic point is at about 9 ‰. Below this level the animal is hyperosmotic in relation to its environment (Croghan, 1958 ; Russler and Mangos, 1978). This means that, as a function of salinity, the osmotic gradient is reversed. Apparently osmoregulatory processes do not interfere with the absorption of copper in high and low salinity environments.

The speciation of copper depends on the pH of the medium. With decreasing pH the uncomplexed cupric ion and $CuSO_4$ assume more importance (Fig. 9). Varying the pH of the saline solution has a pronounced effect on the lipid solubility of copper (Fig. 10). Below pH 7.0 very little copper can be extracted, but the ratio increases very rapidly above pH 7.0. The pH *per se* can have an effect on the extraction process independent from altering the chemical speciation of copper by binding of protons with the polar heads of the lipids. Several studies have dealt with the effect of pH on the accumulation and toxicity of metals in aquatic biota (Sunda and Guillard, 1976 ; Howarth and Sprague, 1978 ; Chakoumakos *et al.*, 1979 ; Miller and Mackay, 1980 ; Sheffrin and Williams, 1984). Many of the data are contradictory, *i.e.* the biological availability either increases or decreases as the pH is changed. Our results show that the pH has a different but profound effect on both the accumulation and toxicity of copper (Fig. 11 and 12). The copper influx is maximum around pH 7.6 and lowest below pH 7.0. The toxicity of copper is highest near pH 8.0. Data on the effect of pH on the biological availability of copper are difficult to interpret because the pH affects different aspects of the uptake process simultaneously. The concentration of the most important copper species cannot be varied independently of pH. Protons may compete for copper uptake sites. Furthermore, pH may affect the metabolic state of the organism and a specific biotic response to a pH-metal interaction may reflect the altered physiology (Knutzen, 1981). If one assumes that copper uptake is a function of copper complexing ligands on the animals' surface, *e.g.* facilitated diffusion, the biological availability is controlled by the equilibrium that exists between these ligands, hydrogen, and copper ions. In

this case uptake and toxicity depend on the acid dissociation and copper stability constants of the ligands located at the membrane. The existence of a pH optimum at which the copper influx is maximal, supports this view. The effect of pH on the uptake of copper cannot be explained by the lipid solubility model. The effect of pH on the toxicity of copper, however, seems to relate somewhat better to the lipid solubility data.

Possibly two distinct processes determine the biological availability of copper in solution. Ionized species interact with the charges present on the external surface of cell membranes (*i.e.* glycoproteins, proteins, polar heads of lipids), while neutral species move across the lipid bilayer by passive diffusion.

Conclusions

The biological availability of copper to *Artemia* is determined by the chemical speciation of the metal in solution. Salinity does not alter the uptake or toxicity of copper. Complexation by an organic ligand reduces the availability by decreasing the concentrations of the inorganic species. Both observations are in agreement with the chemical speciation and lipid solubility data. The effect of pH on the uptake and toxicity of copper is more complex and is not merely function of the altered copper speciation. Thus, information on the chemical speciation and lipid solubility of copper in a particular water system is essential but does not suffice to model the biological availability of copper to *Artemia*.

The influence of abiotic factors such as organic load and pH on the biological availability of metals should be considered in biomonitoring programs and laboratory bioassays.

Acknowledgements

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Selenium toxicity in two populations of *Artemia franciscana*

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Abstract

Selenium inhibits survival of first instar *Artemia* nauplii in two culture media : artificial seawater (TDS 35.2 g/l ; Cl/SO_4 molar ratio of 19.4) and medium #170 (TDS 78.1 g/l ; Cl/SO_4 ratio of 2.5). In the latter, viability of untreated nauplii was increased whereas selenium toxicity was enhanced. In medium #170, viability was slightly but significantly reduced after 7 days in 0.2 ppm selenium (0.0025 mM) and greatly reduced after 3 days at 2 ppm selenium ; the 7-day LC50 is <2 ppm. In both media the Great Salt Lake population is more resistant than the San Francisco Bay race over the concentration range of 0.025-1.0 mM selenium, with LC50 values about twofold greater For San Francisco Bay nauplii in artificial seawater, the LC50-24h of selenite (Na_2SeO_3) is 0.6 mM (47 mg Se/l=46 ppm). Although cadmium also reduces *Artemia* viability, San Francisco Bay nauplii are more tolerant of cadmium (up to 1.0 mM) than of equivalent concentrations of selenium. In combination, selenium and cadmium showed little if any interaction in reducing viability.

Introduction

Selenium is an essential trace element for animals and plants (Lindstrom, 1983). In 100-fold doses above the minimum requirement, however, selenium is toxic (Shamberger, 1983). In many regions of the western United States, high concentrations of selenium in surface waters have resulted in toxic effects on plants and animals. The antagonism of cadmium and synergistic effect of alkalinity and sulfate ionson this toxicity have been recognized for many years (Wilber, 1983). Interest in selenium levels in both fresh water (Burau, 1985) and seawater (Cutter and Bruland, 1984) has revived due to increased agricultural irrigation and proposals to drain the concentrated effluent into reservoirs and estuaries. In the Central Valley of California, some waters have selenium concentrations equivalent to 3-5 ppm, the toxicity threshold for foods in mammals (Burau, 1985). As selenium is concentrated by the food chain, the value may reach >10 ppm in mosquito fish in Central Valley reservoirs (Marshall, 1985). Because of the toxicity of selenium, a sensitive and reproducible bioassay has been sought.

In the present paper, we test the toxicity of selenium on first instar *Artemia* nauplii maintained in two media. Artificial seawater was used to make our studies similar to the 24 h toxicity test on *Artemia* larvae of Vanhaecke *et al.* (1980). A simple synthetic solution, medium #170, was used for two week viability tests because it made the test more sensitive and efficient, permitting detection of the toxic effects of quantities of <2 ppm. We will demonstrate that medium #170 increases the viability of untreated shrimps while enhancing the toxicity of selenium on the

Artemia nauplii. Because cadmium is known to interact with selenium and is often concentrated along with selenium in arid lands, its effect upon selenium toxicity was determined. In western North America, brine shrimp are found in alkaline sulfate lakes in regions where soils are reported to be high in selenium. Therefore, we anticipated that racial differences in tolerance might be found. Two populations of shrimp were compared for response to selenium toxicity in both the seawater and the high sulfate medium (#170).

Materials and methods

Culture media

Artificial seawater was made from Instant Ocean salts with omission of the trace elements. The value of Total Dissolved Solids (TDS) is 35.2 g/l ; Cl/SO_4 molar ratio is 19.4 and density is 1.02. Each liter of medium #170 contains 30.8 Na_2SO_4, 38.5 g NaCl, 14.32 g $MgSO_4.7H_2O$, 1.17 g $CaCl_2$, 0.866 g K_2SO_4, 1 g $Na_2B_4O_7.10H_2O$, 0.125 g Na_2CO_3, and 0.1 g $NaHCO_3$. The value for TDS is 78.1 g/l ; Cl/SO_4 molar ratio is 2.5 and density is 1.06.

Toxicity tests

Hatching and toxicity tests were carried out at 24 °C. Nauplii were hatched from two lots of cysts (collected in different years) from each of the two populations : San Francisco Bay salterns (California, USA) and Great Salt Lake (Utah, USA). Within 3 h of hatching, five nauplii were placed in each glass vial (21 mm diameter × 70 mm) containing 5 ml of culture medium with 0-1 mM sodium selenite (0-345.8 mg Na_2SeO_3/l of medium). In some experiments, divalent cadmium cation was added as $CdCl_2$. The weekly additions of yeast food have been described earlier (Bowen *et al.*, 1985). The numbers of living shrimp were recorded every 24 h until all had died. To facilitate statistical analysis, independent records were kept for each of the 4-30 vials within one treatment. Groups were compared by two-tailed Student's t-test following square root transformation of the mortality data and by the Wilcoxon signed rank paired-sample test. The probability value reported for each conclusion is the larger of the two figures. The LC50 values reported here were calculated by interpolating between the two values of cumulative % mortality which bracketed 50% mortality (Reed and Muench, 1938).

Results

In a comparison of the two culture media (data in Table I), viability of untreated 1st instar *Artemia* nauplii was higher in medium #170 than in artificial seawater ($P < 0.001$). However, in the presence of selenium (0.5-1.0 mM), both races of shrimp had lower viability in #170 than in seawater. The greater effect of selenium in medium #170 is significant for each of the two populations ($P < 0.01$, data not given) and highly significant for the data pooled from both populations ($P \ll 0.001$, Table I).

In medium #170, the 24 h LC50 of selenite was 0.4 mM for San Francisco Bay and 0.8 mM for Great Salt Lake *Artemia* (Table II). After 48 h, the LC50 was reduced to 0.1 mM selenium. In Table III, it is evident that the 7-day LC50 for both populations is <0.025 mM selenite (<2 ppm).

TABLE I

Effect of two culture media on viability (survivors/total) of 1st instar *Artemia* nauplii in presence and absence of selenium.
Data pooled from one experiment on Great Salt Lake nauplii and two independent experiments on San Francisco Bay nauplii, all maintained at 24°C

Selenium mM	Day	Artificial seawater	Medium #170
0	1	116/136 (85 %)	145/150 (97 %)
	2	78/136 (57 %)	141/150 (94 %)
0.25	1	83/105 (79 %)	96/105 (91 %)
	2	25/105 (24 %)	14/105 (13 %)
0.50	1	88/135 (66 %)	64/135 (47 %)
	2	24/135 (18 %)	9/135 (9 %)

TABLE II

Selenium sensitivity of 1st instar nauplii (survivors/total) from two *A. franciscana* populations in medium #170 at 24°C

		Populations	
Selenium mM	Day	San Francisco Bay	Great Salt Lake
0	1	49/50 (98 %)	47/50 (94 %)
	2	49/50 (98 %)	43/50 (86 %)
0.25	1	32/35 (91 %)	33/35 (94 %)
	2	2/35 (6 %)	8/35 (23 %)
0.50	1	15/50 (30 %)	49/50 (98 %)
	2	0/50 (0 %)	9/50 (18 %)
1.00	1	1/20 (5 %)	4/20 (20 %)
	2	0/20 (0 %)	0/20 (0 %)

In artificial seawater, the 24 h LC50 of selenite was 0.6 mM for the San Francisco Bay population and >1.5 mM for the Great Salt Lake population. The LC50 value for San Francisco Bay nauplii was calculated from these 24 h survival values (survivors/total) : 80/89, 49/85, and 7/55 in 0.0, 0.5 and 1.0 mM selenite, respectively.

Great Salt Lake *Artemia* were more resistant than the San Francisco Bay strain over the range of 0.0025-1.0 mM selenite in medium #170 ($P \ll 0.001$, data in Tables II and III). The higher tolerance of the Utah population was also significant ($P < 0.01$) in artificial seawater in the range of 0.25-1.0 mM selenite (data not given). In 0.025 mM Se, San Francisco Bay nauplii showed 84 % mortality after 3 days, whereas the Great Salt Lake larvae still had less than 84 % mortality after 13 days. Control populations in medium #170 without selenium had less than 20 % mortality after 3 weeks, permitting the assay of toxic effects of low selenium over this longer period.

TABLE III

Effect of low concentrations of selenium on the viability of first instar nauplii (survivors/total) maintained in medium #170 at 24°C. Each value based on data recorded from 10 vial cultures

Day	Selenium concentrations		
	0.0 mM	0.0025 mM	0.025 mM
	San Francisco Bay nauplii		
1	50/50 (100 %)	50/50 (100 %)	49/50 (98 %)
2	48/50 (96 %)	44/50 (88 %)	40/50 (80 %)
3	48/50 (96 %)	44/50 (88 %)	8/50 (16 %)
7	41/50 (82 %)	36/50 (72 %)	0/50 (0 %)
10	40/50 (80 %)	35/50 (70 %)	--
14	40/50 (80 %)	34/50 (68 %)	--
	Great Salt Lake nauplii		
1	51/51 (100 %)	50/50 (100 %)	49/49 (100 %)
2	50/51 (98 %)	49/50 (98 %)	49/49 (100 %)
3	50/51 (98 %)	49/50 (98 %)	37/49 (76 %)
7	47/51 (92 %)	44/50 (88 %)	21/49 (43 %)
10	47/51 (92 %)	40-50 (80 %)	12/49 (24 %)
14	46/51 (90 %)	37/50 (74 %)	6/49 (12 %)
17	43/51 (84 %)	34/50 (68 %)	4/49 (8 %)

A comparison of 24 h viabilities of San Francisco Bay nauplii in 1 mM cadmium and in 1 mM selenium (both in medium #170) revealed that viability was higher in cadmium (83 %) than in selenium (5 %). When tested at 1 mM cadmium with and without the presence of selenium (0.25 and 0.5 mM), shrimp viability decreased proportionally to the separate effects of the two elements. Thus, no antagonism or synergism was detected. These statements hold for both artificial seawater and medium #170. However, a white precipitate was immediately evident when both cadmium ion and selenite were added to artificial seawater. This precipitate was resuspended and added to the test vials. Thus, the soluble cadmium and selenium may be less than reported above (in solutions containing both elements). No precipitate was evident in medium #170.

When artificial seawater was made 5 mM in respect to selenite, gray crystals were conspicuous in the bottom of the container after 24 h. They were not seen in medium #170 made 5 mM Se. At selenite concentrations of 0.25-1 mM, a reddish-brown discoloration of the detritus at the bottom of each vial was evident by the 3rd day in seawater but not in medium #170. Although the red color appeared in the absence of nauplii, it was not detected in the absence of yeast. It is probably due to reductive metabolism of Se (+4) to elemental selenium by the living yeast cells in the diet. The color did not appear in media which included 1 mM cadmium.

Discussion

The Great Salt Lake strain is more resistant to selenite than the San Francisco Bay population. The concentrations of selenium in the upper and lower strata of Great Salt Lake today are less than 0.009 ppm (Tayler *et al.*, 1980). Thus, we cannot account for the difference in tolerance between the two strains of *Artemia*. The selenium tolerance of the Utah shrimp may be a

non-adaptive trait or the progenitors of the Great Salt Lake population may have encountered high selenium values in the past. In medium #170, the Great Salt Lake *Artemia* are more resistant than the San Francisco Bay population during a two week period of exposure (Table III). These experiments at lower doses over longer times exclude the possibility that differences in nutrient storage in the encysted blastulae might have accounted for the strain differences in selenium intolerance.

Previous work showed that a simple synthetic culture medium, #70, yields higher viability than seawater for most *A. franciscana* populations in the absence of toxic agents (Bowen *et al.*, 1985). However, medium #70 is made by adding salts to an artificial seawater. Therefore, it is not completely defined and contains substantial amounts of trace elements. Because many heavy metals interact with selenium (Shamberger, 1983), we anticipated that variable results might be obtained with different types of artificial seawater. In the present study, we used medium #170 which is identical to #70 in regard to major ions (Na, K, Ca, Mg, Cl, SO_4, BO_3, and CO_3) but which is made from reagent grade chemicals and lacks the trace elements in seawater. We demonstrate that high sulfate medium #170 yields higher viability of untreated nauplii than does artificial seawater. This fact, along with the enhanced sensitivity of the nauplii to selenium, makes it a desirable culture medium for a sensitive bioassay of selenium. Furthermore, over the range 0.25-5 mM selenite, there was less reduction of Se (+4) to elemental selenium in medium #170 than in seawater.

Trieff (1980) reported LC50 values for divalent heavy metal ions (Cd, Hg, As, Cu, Zn, and Pb) in *Artemia* from Macau, Brazil. The LC50-24 h values were higher than the LC50 values reported for other organisms. For San Francisco Bay nauplii in seawater, we found the LC50-24 h to be 0.6 mM for selenium and about 1.0 mM for cadium (to be contrasted with the 0.6 mM value for cadmium reported by Trieff). Mammalian studies have shown that cadmium and selenium antagonize one another's toxic effects (Shamberger, 1983). In the experimental conditions described here, the two elements are neither antagonistic nor synergistic. Not only are brine shrimp more resistant than mammals to these two elements, but shrimp do not show the antagonistic interaction of selenium and cadmium at the levels which are toxic in short-term tests. Under slightly different culture conditions, the LC50 values might be considerably different. Trieff reported 3- to 50-fold differences in LC50 values for heavy metals in experiments using different "editions" of the commercial preparations of artificial seawater.

For San Francisco Bay nauplii in medium #170, the LC50-24 h is 0.4 mM ; the LC50-48 h is 0.1 mM Se. The LC50 estimate after 7 days of exposure is less than 0.025 mM (<2 ppm Se) for both strains tested in medium #170 (Table III). The LC50 value for the entire pre-reproductive period might be still lower. Furthermore, in extrapolating our results to ecological systems, one must remember that selenium would be accumulated at each step of the food chain (Shamberger, 1983 ; Burau, 1985).

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The influence of temperature and salinity on the sensitivity of *Artemia* nauplii to chemical compounds

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Abstract

The toxicity of chemicals to aquatic organisms is mostly determined in a set of experimental conditions which are close to optimal for the test species.

Because in nature organisms have to cope with fluctuating environmental conditions which ran range during the course of the year from optimal to suboptimal, up to the limits of the tolerance range, the conditions under which routine bioassays are normally performed do, however, not reflect the large range of situations occurring in the field.

Considering the scarcity of information on the effect of fluctuating environmental conditions on the sensitivity of test species to toxicants, a comparative laboratory study has been undertaken to determine the combined influence of environmental variables on the sensitivity of three aquatic invertebrates : the brackish water rotifer *Brachionus plicatilis*, the freshwater crustacean *Daphnia magna*, and the marine crustacean *Artemia*.

This study reports on the combined effects of two important abiotic variables, namely temperature and salinity, on the sensitivity of *Artemia* nauplii for two chemical compounds : potassium dichromate and sodium laurylsulphate.

LC50 24 h have been determined, using the standardized ARC-test in a factorial design with temperatures ranging from 10 to 30 °C in combination with salinities ranging from 5 to 50 ‰.

The results reveal that whereas the LC50 in standard conditions is approximately 22 mg/l for both chemicals, the LC50's ranged from 9 up to 291 mg/l for potassium dichromate and from 7 up to 154 mg/ for sodium laurylsulphate in the various combinations of the factorial experiment.

Consequently, changes in environmental conditions thus influenced the outcome of the test by increasing the sensitivity of the test organism by a factor of 2.5 and 3 respectively, or decreasing it by a factor 7 up to 13, in comparison to the so-called "standard" conditions.

Furthermore, comparing the results with analogous experiments carried out with the two other test species named above, it appeared that the pattern and the magnitude of the variation in toxicity resulting from different environmental conditions is chemical as well as species dependent.

This study thus clearly demonstrates once more the necessity to take environmental factors into consideration for the predictive determination of the hazard of manmade chemicals.

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Effects of pre-exposure on the tolerance of *Artemia* to oil and oil dispersants

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Abstract

This paper tries to contribute to a better understanding of the effects of chronic low-level oil contamination of seawater which, according to some authors, constitutes a major long-term problem for the marine environment.

Are the biological processes of marine organisms affected by low levels of petroleum as some authors claim, or does the continuous input of petroleum have but little or no demonstrable adverse effects on marine populations or their activities ?

Since metal resistant strains of organisms have been collected from contaminated areas and since it has been demonstrated that the tolerance of marine organisms to metals can be increased by pre-exposure to low levels of metals, the question arises in the case of oil pollution, whether one can obtain animals of increased tolerance by pre-exposing them to oil.

Experiments have been conducted in which adult *Artemia* were exposed to various concentrations of oil (Tunisian crude, zaraitine type), two oil dispersants (Finasol OSR-2 and OSR-5), and a mixture of oil and dispersants.

After this pre-exposure the *Artemia* were subjected to the same toxicants whereby two effects were measured :

– acute toxicity (LC50 48 h) ;
– sublethal toxicity (respiration rate).

The results show that adaptation to oil and oil dispersants occurs after pre-exposure as expressed by a higher resistance of the brine shrimp when subsequently submitted to the toxicants.

In the case of pre-exposure to low concentrations of both pollutants, the acquisition of resistance is a slowly-progressing mechanism which is not reversed nor lost after transfer into clean water (detoxification). Pre-exposure to higher concentrations of the chemicals results in a rapid induction of resistance which is, however, partially lost after transfer to clean water conditions.

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Effect of salinity and sodium lauryl sulphate on adenosine triphosphate levels in *Artemia* nauplii

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Abstract

The value of adenosine triphosphate (ATP) as a biological indicator of physiological stress was examined in the brine shrimp (*Artemia*). The ATP content was measured every 12 h, for 72 h, in organisms cultured at different salinities. The results showed that intracellular ATP concentrations decreased as environmental salinity increased from 20 to 150 ‰. This reflects differences in metabolic activity between organisms held at different salinities.

Artemia ATP levels were also measured after 24 h of exposure to sodium lauryl sulphate (SLS), a typical anionic detergent. The ATP concentration showed a significant decrease with increasing concentration of SLS. This reflects stress related alteration of metabolic activity in organisms exposed to different concentrations of a known toxicant.

The ATP concentration in *Artemia* showed significant changes when subjected to physical and chemical perturbation and demonstrates the usefulness of ATP as a measure of physiological stress in the brine shrimp.

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Measurement of photoattraction of *Artemia* nauplii : effect of mercuric ion

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Abstract

Living organisms exhibit a phototactic response which can be altered by certain environmental toxic chemical species. The analysis of photobehavior can help in elucidating environmental factors that influence photomotility reactions of the organisms.

A simple method has been developed that measures the phototactic response of *Artemia* nauplii in synthetic seawater. The phototactic response of *Artemia* nauplii is measured quantitatively by observing the movements of the organisms from a darkened half to a lighted half of an experimental vessel containing synthetic seawater. The number of organisms moving as a function of time is determined by removing aliquots from both the light and dark sides and then plating on agar for counting under a dissecting microscope. These measurements consistently show a significant movement of nauplii to the lighted side within 45 min of the start of the experiments.

Studies on the effects of pollutants such as mercuric ion were also performed. The present investigation demonstrates that at concentrations as low as 0.0010 mg $HgCl_2$/l an enhancement of the phototactic effect on *Artemia* nauplii by mercuric ion occurs as compared to the control. The phototactic response of the *Artemia* nauplii is altered by mercuric ion in a dose-related manner. The hypothesis is put forward that the effect may be related to the formation of a metal-S bond, but no direct experimental evidence has as yet been determined.

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Workshop report : Ecotoxicology

Moderator

Allan D. Beck, US Environmental Protection Agency, Environmental Research Laboratory (Narragansett, Rhode Island, USA)

Rapporteurs

Michael D. Johns, US Environmental Protection Agency, Environmental Research Laboratory (Narragansett, Rhode Island, USA)

Marleen Van Steertegem, Laboratory for Biological Research in Aquatic Pollution, State University of Ghent
(Belgium)

The workshop was opened with a brief presentation by the authors of the posters dealing with this subject. These presentations underlined the suitability of *Artemia* as a test organism in certain types of ecotoxicological tests.

Most of the discussion in this workshop dealt with the question of whether a standardized *Artemia* test would be widely useful in toxicity testing programmes. As one participant suggested, the question of the value of a standardized *Artemia* test concerns both the precision and accuracy of the test. Precision, defined as the ability to repeat a toxicity test with little variability, has been demonstrated for some chemical compounds using a standardized *Artemia* test. Accuracy, defined as the predictive usefulness or relevancy of test data to eventual environmental impacts, however, has not been addressed as yet, and must be done before the full potential of a standardized ecotoxicological test using *Artemia* can be determined.

It was the general feeling of the participants that in certain circumstances, such as development of water quality criteria, an *Artemia* test might not be suitable, while in other applications it might be very useful as a quick screen test. For example, the standardized short-term *Artemia* test seems to be very suitable for toxicological SAR (structure activity relationship) research ; it may also constitute an excellent and very cheap tool for routine testing of effluents.

In conclusion, Prof. Persoone (State University of Ghent, Belgium) asked that researchers interested in ecotoxicological testing with *Artemia* please contact him to discuss the possibility of future group discussions.

Concluding remarks

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Concluding remarks for Symposium Session I: Morphology, Ecotoxicology, Radiobiology, Genetics

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Morphology

Numerically the contributions in this area to the symposium have been few. However, recent work, particularly that based on electron-microscopic investigation, is providing a more accurate picture of differentiation and development in *Artemia.*

For the future, comparative studies on different species would be of considerable value.

Ecotoxicology

It is evident that *Artemia* is an organism admirably suited for bioassay in ecotoxicology though this is perhaps not recognised as widely as it should be.

During the symposium the question of the value of a standardized *Artemia* test has received much discussion. The conclusion appears to be that such a test has high repeatability value but not necessarily a high predictive value in terms of assessing environmental impact. The use of the test could then be seen as very suitable in some circumstances but much less so in others.

Radiobiology

Artemia is very radiation resistant in both somatic and gonadal tissue, and mutant induction by ionizing radiation appears to be difficult. The oxygen effect appears to be more pronounced than in many other life forms. The biological basis for these observations remains poorly known and there is a need for systematic investigation of these and related phenomena.

Genetics and strain characterization

It is evident that there has been a considerable increase in work on topics in this area since the symposium in 1980 as Dr. Abreu-Grobois' review ably documents.

In cytology accurate information on the chromosome numbers, incidence of aneuploidy (surprisingly high in larvae from many strains) and the distribution of chromocenters (heterochromatin) visible in interphase nuclei is now available.

Electrophoretic studies of genetic variation in populations have demonstrated that levels of genetic variation are high in *Artemia* and that strains and species can be characterized accurately. Data of this sort have been used to derive evolutionary pathways of phylogenetic descent which

throw light on a variety of phenomena including the origin of the parthenogenetic mode of reproduction.

Studies of life history traits have also begun and are yielding information likely to be of both fundamental and practical value. One striking example of this is the work by Dr. Browne on reproductive capacity of coexisting clones of diploid parthenogenetic *Artemia*. Ecological aspects of genetic differentiation continue to excite interest. The populations of Mono Lake ($Cl^-/CO_3^{--}/SO_4^{--}$) and Jesse Lake (CO_3^{--}) are of considerable interest in this connection and the possibilities for flow of genes between the normal Cl^- populations of *A. franciscana* and these populations via Fallon Lake are being studied.

Parthenogenesis is widespread in *Artemia*. The levels of fixed heterozygosity in parthenogenetic strains are generally high and correlate positively with ploidy levels. Diploid parthenogens appear to be capable of generating significant amounts of new genetic variation with obvious consequences for evolutionary persistence. Clearly more work is needed on the significance of the parthenogenetic mode of reproduction in *Artemia* and on the extent of genetic variation between clones.

Discussion in the workshop demonstrates that considerable advances could be expected in strain characterization and analysis of genetic variation by the developments in the use of isoelectric focussing, restriction enzyme technology and mitochondrial DNA.

A few words on the role that genetics can play in *Artemia* biology and aquaculture are probably appropriate at this point. To this observer following points appear to be the most important :

- Collaboration of geneticists with workers in other branches of the biology of *Artemia* is likely to be fruitful.
- Analysis of characterisation of geographic population (and species?) from areas poorly known (especially large parts of Asia) is an urgent task.
- Application of a variety of genetic techniques to modification of a number of quantitative characters like larval size, nutritional quality and temperature tolerance is a goal of very great importance for advances in many areas of aquaculture.

The final section of these concluding remarks is devoted to a topic which is becoming increasingly important.

While the deliberate inoculation, by man, of saline waters with *Artemia* frequently ensures social and economic benefits particularly in developing countries, this practice also carries with it certain dangers. Where the waters inoculated (or those close by) contain natural populations of *Artemia*, it is almost always likely to be the case that (at best) some genotypes and (at worst) the entire indigenous and possibly unique population or species will become extinct. Such losses of germplasm are matters for concern to biologists, to those engaged in commercial exploitation of *Artemia* and to geneticists and those engaged in conservation because they represent a significant loss of resources valuable for both practical and scientific reasons.

The following resolution was put to the final session of the symposium and received unanimous acceptance : "The Second International Symposium on *Artemia* meeting in Antwerp (Belgium) in September 1985 resolves that all possible measures be taken to ensure that the genetic resources of natural *Artemia* populations are conserved. Such measures include the establishment of gene-banks (cysts), close monitoring of inoculation policies and where possible the use of indigenous *Artemia* for inoculating *Artemia*-free waters".

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