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ENDOKRINOLOGISCHE ASPEKTEN VAN DE REGULATIE VAN DE VITELLOGENESE BIJ HET PEKELKREEFTJE, *ARTEMIA SP.* (CRUSTACEA ; ANOSTRACA).

Endocrinological aspects of vitellogenesis in the brine shrimp *Artemia sp.* (Crustacea ; Anostraca).

Promotor : Prof. Dr. A. DE LOOF

Proefschrift voorgedragen
tot het behalen van de graad van
Doctor in de Wetenschappen
door

Eddy VAN BEEK

1988

Voor het
IZWO-
sekretariaat

Eddy

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DANKWOORD.

Een woordje van dank, iets dat eigenlijk aan het einde van een werk zoals dit hoort, verdient hier zeker de eerste bladzijde. Een doktoraat schrijven, laat staan maken, doe je immers niet alleen. Verschillende mensen hebben dan ook bijgedragen tot deze studie; soms op een indirekte wijze.

Allereerst wil ik Prof. A. De Loof danken voor de jarenlange begeleiding van mijn werk. Het korps van professoren van het Zoölogisch Instituut, die instonden voor mijn didaktische vorming, wil ik niet vergeten. Aan Prof. F. Ollevier betuig ik verder mijn dank voor het verlenen van materiaal en de mogelijkheid voor de uitbouw van een kweek van pekelkreeftjes. Prof. F. Vandesande dank ik voor het gebruik van apparatuur en het ter beschikking stellen van allerhande antisera voor immunocytochemische doeleinden.

In het kader van het F.K.F.O.-projekt heb ik de kans gekregen samen te werken met diverse personen uit laboratoria van verschillende universiteiten. Luk Moens (UIA, Antwerpen) dank ik voor de gift van het ruwe lipovitelline extract. Mijn dank gaat ook uit naar het Artemia Reference Center (RUG, Gent), met in de eerste plaats Dr. Patrick Sorgeloos die daar het Artemia-projekt coördineerde. Verder nog Patrick Lavens, Wim Tackaert en Ronny Blust (RUCA, Antwerpen) en al de mensen van de Artemia-groep die meehielpen aan de bevoorrading van proefdieren. Lieve Criel dank ik bijzonder voor de talrijke keren dat zij meehielp om pekelkreeftjes te sexen en te determineren. Hilde Walgraeve (RUG, Gent), voor de steun en participatie in het ecdysteroïdenonderzoek.

Ook niet te vergeten zijn de kollega's die samen met mij de lange onderzoekstijd hebben doorgemaakt: Johan Cardoen, Danny De Clerck, Johan Geysen, Roger Huybrechts, Herman Van Mellaert. Ze hebben mijn hoogtes en laagtes gekend. Mijn oprechte dank.
Speciaal dank ik de kollega's van de steroïdgroep voor het scheppen van een aktieve werksfeer: Frans Novak, Guido Paesen en Luc Swevers.

Ik wens ook speciale aandacht te geven aan de personeelsleden die me al die jaren gekend en gesteund hebben: Johny Gijbels, Armand Guisson, Julie Puttemans, René Smisdom, Maria Van Der Eeken en Hendrik Van Den Bergh.
Sommigen van hen hebben me enkel maar een korte periode kunnen helpen; toch wil ik hen niet vergeten te danken: Hilde Bruffaerts, John Evans, Jos Mertens, Christel Sente, Armand Stiers, Jos Vervoort, Roger Vranckx.

De talrijke biologen-vrienden die me in de loop der jaren gesteund hebben mag ik niet vergeten, want zij zijn het die mijn gezaag en geweeklaag, wanneer het niet zo goed ging, hebben moeten aanhoren. Verder nog de talrijke andere kollega's van het labo die door hun aanwezigheid de sfeer erin hielden. De rest van de mensen, en er zijn er te veel om op te noemen; maar om enkele van hen te vermelden: de kuisers Roger en Willy, de nachtwakers, de vrienden van de avonden met gezelschapsspelen, GC,...

Johan en Frank Minne wil ik bedanken voor hun inzet bij het verwerken van de tekst. Hendrik Van Den Bergh ben ik dankbaar voor het nauwgezet nalezen en Julie Puttemans voor het maken van tekeningen.
Buiten al deze mensen die in het onderzoeks- en wetenschapsleven zitten, mag ik zeker mijn ouders niet vergeten. Zij hebben het mogelijk gemaakt dat ik de richting van de universiteit kon inslaan en beëindigen. Evenals mijn schoonouders volgden ze mijn werk steeds met belangstelling. Ook mijn zusters, broers, schoonzusters, schoonbroers en vrienden van de volksdansvereniging dank ik voor de interesse die ze hebben getoond gedurende al die jaren.

Als laatste en enigste die me kontinu geleid heeft, verdragen heeft in de moeilijke tijden, me verschillende avonden moeten missen heeft, de drijvende kracht geweest is om mijn doktoraat te beëindigen, dank ik mijn vrouwtje BEA. Aan haar en onze zoon Jonathan wil ik dan ook mijn werk opdragen dat een einde betekent van vervelende momenten vol spanning, gejaagdheid en zenuwen.

PROBLEEMSTELLING.

Artemia, het pekelkreeftje, leeft in zout water waarvan de saliniteit tot tien maal hoger mag zijn dan de koncentratie van gewoon zeewater. Het is over heel de wereld verspreid.

Vooral de larven of naupliï van Artemia bleken een uitermate proteïnerijke voedselbron te zijn voor talrijke vissen en kreeftachtigen. Het aanwenden van pekelkreeftlarfjes als biomassa voor de kweek van talrijke larven van zee- en zoetwaterdieren is dan ook een praktijk die steeds meer toegepast wordt.

Na verschillende vervellingen bereiken de naupliï het adulte stadium. Typische kenmerken van dit stadium zijn de 11 paar zwempoten of thoracopoden. In bisexuele rassen verschillen de volwassen mannetjes van de wijfjes o.a. door de aanwezigheid van lange antennen. Volwassen Artemia wijfjes produceren, in optimale kweekomstandigheden, per week 20 à 100 eieren. Tijdens de eivorming worden reservestoffen, die als voeding dienen in de eerste levensuren, opgestapeld in de oöcyten. De rijpe eieren in de broedzak kunnen gemakkelijk doorheen de transparante lichaamswand waargenomen worden.

Er wordt sedert een paar jaar gedacht om naast de naupliï ook adulte Artemia's, die men op industriële schaal zou kweken, als biomassa in de aquakultuur aan te wenden. Om dit te kunnen realiseren is het nodig om op een gekontroleerde manier de cyclus van Artemia in een artificiële kweek volledig rond te krijgen. Volwassen pekelkreeftwijfjes produceren onder welbepaalde externe omstandigheden ingekapselde eieren, die cysten genoemd worden. In gedroogde toestand kunnen zij gedurende jaren leefbaar blijven. Terug in kontakt met water gebracht, ontwikkelen zij tot jonge naupliï. Naast deze ovipare vorm van voortplanting is er ook een vorm van ovoviviparitеit, waarbij de eieren niet omgeven worden door een schaal en onmiddellijk levende naupliï worden afgezet in het water. Onder dezelfde strikt omschreven uitwendige kondities kunnen de twee reproduktiepatronen elkaar afwisselen. Het is nog niet gekend welke faktor nu precies bepaalt welke voortplantingsvorm, de ovipare of de ovovivipare, gekozen wordt.

Wil men komen tot kontinue industriële kweek van Artemia, dan is het wenselijk inzicht te hebben in het hormonaal systeem dat de reproduktie bij dit merkwaardig schaaldiertje stuurt. Een vijftal jaren geleden waren de enige aanduidingen voor een eventuele hormonale regulatie van de reproduktie, de aanwezigheid van enkele neurosekretorische cellen in de hersenen. Het doel van ons onderzoek was meer inzicht te verwerven in het proces van de dooiervorming en zijn hormonale regulatie.

Essentieel in de reproduktie is de opstapeling van dooierstoffen in de eieren. Bij Artemia is sedert 1964 bekend dat er endogene synthese van dooiermateriaal is in de jonge oöcyt. Bij insekten staat het onderzoek naar het verloop van vitellogenese veel verder. Bij deze dieren synthetiseert het vetlichaam de grote massa van dooierprecursoren en is de endogene synthese in het ovarium gering. Het leek ons aangewezen om, in eerste instantie na te gaan of er eventueel ook bij Artemia een exogene syntheseplaats van dooierprecursoren is. Dit werd onderzocht met immunocytochemische technieken met behulp van een antiserum gericht tegen een subeenheid van het lipovitellinekomplex.

Aanwijzingen voor de regulatie van het dooierrijpingsproces werden verkregen met in vitro experimenten waarbij thoracopoden geïnkubeerd werden in aanwezigheid van radioaktief gemerkt methionine en hormoonoplossingen.

Algemeen is bij vertebraten en invertebraten de regulatie van de vitellogenese gebaseerd op komplexe interakties van een aantal hormonen. Deze reproduktie gebeurt op een welbepaald tijdstip in de ontwikkeling. Bij hoger geëvolueerde Crustacea zoals bijvoorbeeld bij sommige soorten kreeften en krabben, gebeurt de eiafleg tussen twee vervellingen van de adulte dieren. Bij elke vervelling wordt de oude cuticula afgeworpen en door een nieuwe vervangen. Het vervellingshormoon speelt hierbij een sleutelrol maar dit hormoon is ook in de perioden tussen twee vervellingen aanwezig, zij het in lage koncentratie. Ecdysteroïden spelen mogelijkerwijze een rol bij de regulatie van de dooiervorming. Daarom bestudeerden wij de veranderingen van de ecdysteroïdkoncentraties tijdens de reproduktie- en vervellingscyclus bij Artemia.

Recent zijn bij een aantal ongewervelde diersoorten steroïden aangetoond die bij de gewervelden tussenkomen in de hormonale regulatie van de reproduktie. Ook de koncentratieveranderingen van deze "typische" vertebraat (niet-ecdysteroïd) steroïden werden met radioimmunologische methodes bepaald gedurende de vitellogenese. De chemische identiteit van sommige onder hen werd onomstotelijk vastgesteld door gaschromatografie gekoppeld aan massa-spektroskopie (GC-MS).

Er zijn een aantal gegevens gepubliceerd over het voorkomen van neurohormonen die een invloed op de eiafleg hebben bij invertebraten. Geen enkel van deze hormonen is tot nog toe volledig gekarakteriseerd. Bij vertebraten reguleren een aantal neurohormonen bepaalde aspekten van de reproduktie en deze zijn wel goed gekarakteriseerd. Bij insekten werd de laatste jaren het endokrinologisch onderzoek toegespitst op het lokaliseren en detekteren van "typische" vertebraat-neuropeptiden in de hersenen en de ventrale zenuwstreng met behulp van immunocytochemie. Het neuroëndokrien systeem van Artemia was tot aan ons onderzoek enkel onderzocht met klassieke histologische kleurings-methoden. Wij hebben immunocytochemisch nagegaan of een aantal van die neuropeptide-achtige stoffen, eventueel aanwezig zijn in bepaalde neurosekretorische cellen van het pekelkreeftje.

GENERAL INTRODUCTION.

The brine shrimp Artemia, can survive in saltwater, of which the salinity may exceed ten times the concentration of seawater. This primitive crustacean has a worldwide distribution.

Especially the larvae or nauplii of Artemia appear to constitute an excellent and proteinaceous source of food for a lot of fish and crustacean larvae. Therefore, the brine shrimp is used more and more nowadays as biomass for the propagation of numerous larvae of sea- and freshwater animals.

Nauplii reach the adult stage after several moultings. Typical at that stage are the 11 pairs of swimmerets or thoracopods. In races in which both sexes occur, males differ from females by e.g. the presence of large 'claspers'. In optimal conditions, adult female brine shrimps can produce 20-100 eggs per week. During oogenesis, yolky compounds, which serve as food reserve during the first hours of development, are accumulated in the oocytes. The ripe eggs in the broodsac can easily be observed, due to the transparency of the body wall.

In recent years, attempts are made to use also adult Artemia as biomass in aquaculture. These should then be cultured in high density raceways. In order to make this practicable, it would be useful to obtain the completion of the lifecycle of Artemia from artificial propagation stocks in well controlled conditions. Adult female brine shrimps can produce encysted gastrulae (also called cysts), under certain external conditions. These can be kept for years in a dry state. They will develop into nauplii immediately after contact with water. Besides this oviparous way of reproduction, also ovoviviparity does exist in Artemia. In reproduction after this fashion, eggs are not surrounded by an egg-shell and free-swimming larvae will be released into the water. Sometimes both modes of reproduction can alternate under seemingly identical external conditions. So far, it is not known which factor exactly determines the way of reproduction, oviparity or ovoviviparity, which is adopted.

In order to make it possible to propagate the brine shrimp continuously on an industrial scale, it would be useful to know the hormonal system regulating reproduction in this extraordinary animal. When we started on this research, only some neurosecretory cells that might play a role in reproduction had been described. The goal of our study was to gain more information about the process of yolk formation and its hormonal regulation.

Important in vitellogenesis is the accumulation of yolk proteins in the eggs. Since 1964, it was known that in Artemia there exists endogenous synthesis of yolk in the young oocyte. For insects, many more data and results are available concerning vitellogenesis. In these animals the fat body synthesizes a great part of the yolk precursors and the amount of endogenous synthesis in the ovaries is small. At first instance, we thought it would be better to look for a possible extra-ovarian site of synthesis of yolk precursors. This was done by means of immunocytochemical techniques and with the help of an antiserum raised against a subunit of the lipovitellin complex, we investigated this possibility.

Indications for the regulation of lipovitellin synthesis were provided by in vitro experiments whereby thoracopods were incubated in the presence of sulphur radiolabeled methionine and hormone solutions.

In general, regulation of vitellogenesis in vertebrates and invertebrates is based on complex interactions between several hormones. Reproduction in this manner takes place at a well-defined moment in development. In higher evolved Crustacea, such as some species of crabs and lobsters, eggs are deposited in a period between two moultings of the adults. The moulting hormone plays a key-role in this process, but it is also present at lower concentrations in the interval between two moultings. Ecdysteroids might play a role in the regulation of yolk formation. Therefore, we studied the changes of the concentrations of ecdysteroids during cycles of reproduction and moulting.

Recently, the presence of steroids was demonstrated in a number of invertebrate animals. These compounds are known to intervene in the hormonal regulation of reproduction in vertebrates. So, we quantified these 'typical' vertebrate (non-ecdysteroid) steroids by radioimmunological methods during vitellogenesis. The chemical identity of some of the molecules has been undeniably demonstrated by gas chromatography/mass spectrometry (GC-MS).

Numerous published data are available on the presence of neurohormones that influence egg-laying in invertebrates. None of these hormones is completely characterized as yet. In vertebrates, however, a few neurohormones regulate certain aspects of the reproduction and these are well characterized. We have looked for a few of these 'typical' vertebrate-like neuropeptides possibly present in particular neurosecretory cells of the brine shrimp.

TABLE OF CONTENTS.

LIST OF ABBREVIATIONS.

AS : antiserum
BSA : bovine serum albumin
CHH : crustacean hyperglycemic hormone
DAB : diaminobenzidine
DHT : 5α-dihydrotestosterone
E_1 : estrone
E_2 : estradiol
FSH : follicle stimulating hormone
GAR : goat anti-rabbit
GC-MS : gas chromatography-mass spectrometry
GIH : gonad inhibiting hormone
GSL : Great Salt Lake
HCG : human chorionic gonadotrophin
HPLC : high performance liquid chromatography
IR : immunoreactive
JH : juvenile hormone
LH : luteinizing hormone
LH-RH : luteinizing hormone releasing hormone
LV : lipovitellin
MH : moulting hormone
MIH : moulting inhibiting hormone
NPY : neuropeptide tyrosine (Y)
OIH : ovary inhibiting hormone
Ov : ovary
PAP : peroxidase-anti-peroxidase
PBS : phoshate buffered saline
P_4 : progesterone
P_5 : pregnenolone
PIG : pre-immune goat serum
PIR : pre-immune rabbit serum
RIA : radioimmunoassay
SDS-PAGE : sodium dodecyl sulphate polyacrylamide gel electrophoresis
SG : sinus gland
SOG : suboesophageal ganglion
T : testosterone
Ut : uterus
VG : vitellogenin
VIH : vitellogenesis inhibiting hormone
VSOH : vitellogenin stimulating ovarian hormone
XO : X-organ

LIST OF PUBLICATIONS.

CHAPTER 3:

Van Beek E., Van Brussel M., Criel G. and De Loof A., 1987. A possible extra-ovarian site for synthesis of lipovitellin during vitellogenesis in Artemia sp. (Crustacea; Anostraca). Int. J. Invert. Reprod. Dev. 12, 227-240.

CHAPTER 5:

Van Beek E., Criel G., Walgraeve H. and De Loof A., 1987. Moulting hormone activity in adult Artemia. p. 173-179. In: Artemia Research and its Applications. Vol.2. Physiology, Biochemistry, Molecular biology. Decleir W., Moens L., Slegers H., Jaspers E. and Sorgeloos P. (eds.). Universa Press, Wetteren, Belgium. pp. 562.

CHAPTER 6:

Van Beek E. and De Loof A., 1988. Radioimmunological determinations of concentrations of six C_{21}, C_{19} and C_{18} steroids during the reproductive cycle of female Artemia sp. (Crustacea: Anostraca). Comp. Biochem. Physiol. 89A, 595-599.

APPENDIX VIII:

Walgraeve H., Van Beek E., Criel G., Van Brussel K. and De Leenheer A., 1986. Comparison of three separation systems for the radioimmunoassay of ecdysteroids in Artemia (Crustacea: Branchiopoda). Insect Biochem. 16, 41-44.

CHAPTER 1: TAXONOMICAL CLASSIFICATION, LIFE CYCLE AND CULTURE METHODS OF ARTEMIA SP..

In this chapter the taxonomical classification of the brine shrimp Artemia is sketched. A short description of the life cycle is given. Attention is paid to vitellogenesis and an outline is given of the problems encountered in the cultivation of this salt water animal.

1. Classification of the brine shrimp in animal taxonomy.

After Kükenthal and Krumbach (1927), and Parker and Haswell (1972), Artemia can be classified as follows:

Phylum	Arthropoda
Subphylum	Mandibulata
Classis	Crustacea
Subclassis	Entomostraca
Ordo	Branchiopoda, Phyllopoda
Subordo	Euphyllopoda
Tribus	Anostraca
Familia	Branchinectidae
Genus	Artemia
Species	Artemia franciscana*

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* There has been a lot of controversy about the exact name of the species. In the past, the name Artemia salina was routinely used. But in fact, this name should be strictly reserved to the brine shrimps collected at Lymington in England (A. salina Leach).
According to an international agreement, Barigozzi (1980) proposed that it was better to use the term 'sibling species' (see also Bowen et al., 1980). This name covers a group of different populations which are reproductively isolated in nature, but which seem to be morphologically identical or at least very similar. In this way our experimental animal, which is cultured from cysts of a population of the Great Salt Lake strain (GSL), belongs to the sibling species A. franciscana. A reference number and/or the name of a local harvesting place give further information about the place of origin and year of collection of the cysts. A complete reference of our experimental animals would thus be Artemia sp., GSL 375. This means that the sample of cysts we used is registered as being collected from the northern branch of Great Salt Lake, harvested in the autumn of 1983 and is distributed by the San Franciso Bay Brand Company.

2. Life cycle of the brine shrimp.

Artemia is cosmopolitan. In all places where salt concentrations are much higher than in the oceans, it becomes impossible for most animals to survive. This is not the case for the brine shrimp. Like its name says, it is possible for this small crustacean to maintain itself in salt-lakes with 250 g of salt per litre , which is almost ten times more than the salinity of normal seawater. As a matter of fact, brine shrimps are the predominant life-form in salt lakes, salt pans or salines.

Besides this adaptation the brine shrimp has another important characteristic: it has both an ovoviviparous and an oviparous way of reproduction. Encysted gastrulae or cysts can be stored for years in dry conditions. Back into seawater free-swimming larvae or nauplii emerge. The larvae grow and pass through the stages of metanauplius (I-IV), juvenile or preadult and finally reach the adult phase after 14 moults. These stages can easily be determined with a handlens (Provasoli and Shiraishi, 1959; Provasoli and D'Agostino, 1969). From the tenth moult onwards, sexual differentiation becomes apparent. In males, a paired penis can be observed and the antennae develop into muscular claspers which have a frontal knob at their inner side, while in females the broodsac (brood pouch or uterus) becomes an easily distinguishable characteristic. Adult brine shrimps or reproductively active animals reach a length of 10 to 15 mm, have 11 pairs of functional thoracopods, stalked lateral eyes, sensorial antennulae, a linear digestive tract and paired gonads on both sides of the alimentary canal. Adults are often seen in the so-called 'riding position'. The male grasps the female with his claspers between the last pair of thoracopods and the uterus. These couples are in a pre-copulatory condition and can remain attached to each other for long periods at a time. Copulation itself however, is very rapid: the male abdomen is bent forwards and one penis is introduced into the uterus aperture. Fertilized eggs can develop into free-swimming nauplii (ovoviviparous reproductive mode) or the embryos can be surrounded in the gastrula stage by a thick shell and become cysts that are in diapause (oviparous reproductive mode). For an overview of the life cycle see also Sorgeloos (1977).

3. Successive stages in vitellogenesis.

Due to the transparency of the body wall, we can follow vitellogenesis externally. An arbitrary division - largely based on observation with the naked eye - of the successive stages is depicted in Table 1.1.

Table 1.1. Successive stages in the first vitellogenic cycle.

Stage Ov-	: In this stage no vitellogenesis occurs, the ovaries are fully transparent.
Stage Ov±	: A few oocytes show yolk accumulation.
Stage Ov+	: The ovary is filled at full length with yolk accumulating oocytes.
Stage Ov++	: Oocytes are opaque due to the presence of lipovitellin. They are arranged in a double white strand.
Stage L.S.	: Ripe oocytes have descended into the curled oviducts which now appear as lateral sacs.
Stage Ut+	: Oocytes have descended from the lateral sacs into the uterus or broodsac where they can be fertilized. The ovaries are back in stage Ov-.

The ripe eggs stay in the broodsac until the second vitellogenic cycle has reached stage Ov++. Then, eggs are first expelled from the broodsac and a moult follows (Metalli en Ballardin, 1972). From now on, the ripe oocytes from the second cycle can descend into the oviducts (lateral sac stage). In optimal conditions these stages are species dependent, their duration is fixed and can at times be very short (Bowen, 1962).

Normally in a mass culture, the first cycle is more or less synchronous, but after the second and third vitellogenic cycle a heterogenous distribution of vitellogenic stages and a mixture of different vitellogenic cycles are observed.

4. Culture of actively reproducing brine shrimps.

A first prerequisite for physiological experiments is a continuous supply of the experimental animals. In general, the amount of 500-1000 adult individuals per batch makes it possible to collect all the vitellogenic stages necessary for one experiment.

The strain from Great Salt Lake was chosen for the following reasons:

1) it is a bisexual race
2) the adult stage is fairly big (max. 1.5 cm)
3) the vitellogenic cycle is short
4) the culture from cyst to preadult stage requires only a short period

Preadults were raised in high density cultures in the Artemia Reference Center (ARC) at Ghent (Bossuyt and Sorgeloos, 1980). Specially for this purpose, tanks of 1 m^3 were installed with an air-water-lift device for aeration, a system for semi-automatic food distribution, a plate separator for waste removal and an indirect heat exchanger. Several waste products of major agricultural crops or from bio-industries have been found to be very suitable food sources for Artemia: e.g. rice bran, soybean meal and whey powder (Dobbeleir et al., 1980).

In our institute preadult brine shrimps were further reared in funnel-shaped glass fiber culture tanks of 30 litres (diameter upper part 32 cm, height 40 cm) with a tap at the bottom. One time a day faeces and food remains are tapped off and discarded.

Artificial seawater was made with sea salt enriched with several bioelements (Wiegandt GMBH & Co, Krefeld, West-Germany). Salinity was regularly checked with a density-meter and kept at 35 g per litre. Before use, seawater was filtered through a sieve (mesh width of 50 µm).

In our low density culture, animals were fed daily with a freshly made yeast suspension (Saccharomyces cerevisiae) having a concentration of 0.2 g per 10 litre brine shrimp culture with a density of 100-200 animals per litre. Regularly, the diet was varied by 1 litre suspension of green algae having a concentration of 4.10^6 cells per ml (for culture methods, see further). This was necessary in order to obtain reproductive animals of good quality and to keep them in optimal condition. Turbidity was used as a parameter for the amount of food. It was roughly estimated with the help of a home-made Secchi disc (Bossuyt and Sorgeloos, 1980).

Food particles were kept in suspension by continuous aeration and the temperature was regulated at 25-28°C by a plunging thermostate (220 Volt/300 Watt, RENA). The pH varied between 7.5 and 8.5. It was checked with paper strips

from Merck (Specialindikator pH 6.5-10.0). Brine shrimps need an alkaline medium and do not grow in seawater with pH below 6.8 (Provasoli and D'Agostino, 1969).

Very often our Artemia culture was contaminated with rotifers (Brachionus plicatilis) which appeared to be harmless to the brine shrimps. On the contrary, they probably enzymatically destroy the external protection layer of the yeast cells and thus make the contents available to Artemia (Coutteau, 1987). Devauchelle and Girin already reported in 1974 the possibility of raising mixed cultures of the copepod Trisbe furcata with Brachionus plicatilis based on a diet of living algae.

4.1. Culture of green algae.

To mimic natural food conditions, suspensions of green algae (Tetraselmis suecica obtained from IFREMER, Deva-Sud station at Palavas-les-Flots, France) were supplied. The marine micro algae T. suecica have already been used for the mass culture of rotifers (Trotta, 1980) and for the culture of copepods (Devauchelle and Girin, 1974). The use of algae as a food source implies the continuous presence of a stock of these algae. A starter culture was raised first in small glass tubes, afterwards in small erlenmeyers and later on in big jars (3 or 5 litres). The number of cells in the culture medium was systematically raised until the concentration of 4.10^6 per ml was reached.

These culture tanks were continuously aerated with a mixture of air and 2 percent CO_2 (0.2 bar). Continuous illumination was assured by a screen of 8 fluorescent lamps (Philips, Osram L58W/20, Cool White). Using this setup, a light intensity of 2200 lux (with luxmeter from Gossens) was measured at a distance of 20 cm.

Algal suspensions were filtered every two days through a sieve (mesh width of 50 µm) in order to remove precipitates and aggregations of algae and to brake the development of ciliates that seem to originate in a possible contamination during the culture procedure.

4.2. Artificial seawater and medium for algae.

The culture medium was a mixture of artificial seawater (see above) and 10 ml ES-medium of Provasoli (D'Agostino and Provasoli, 1968) and 5 ml medium of Walne (Gross, 1937; Walne, 1956) per litre. Pure analytical grade products from Merck Company (or from the suppliers specified between brackets) were used to make up this culture medium. The final composition was modified after Mollo and Tudesq, 1985.

A. ES-medium of Provasoli

1 litre distilled water:

3500 mg	$NaNO_3$
500 mg	Na-glycerophosphate
0.1 mg	Vitamin B_{12} (Serva)
5.0 mg	Thiamin (Serva)
0.05 mg	Biotin (Serva)
5000 mg	Tris-buffer
250 ml	Metal-mix A
25 ml	Fe-solution B

Metal-mix A: 1 litre distilled water

1140 mg	H_3BO_3 (Carlo Erba)
49 mg	$FeCl_3.6H_2O$
164 mg	$MnSO_4.4H_2O$ (UCB)
22 mg	$ZnSO_4.7H_2O$
4.8 mg	$CoSO_4.7H_2O$
1000 mg	Na-EDTA

Fe-solution B: 500ml distilled water

351 mg	$Fe(NH_4)_2(SO_4)_2.6H_2O$
330 mg	Na_2-EDTA (UCB)

The medium is brought to pH=7.8 by adding concentrated HCl.

B. Medium of Walne

1 litre distilled water:

0.278 g	$FeSO_4.7H_2O$
4 g	$NaH_2PO_4.2H_2O$ (569 mg P/litre)
30 g	$NaNO_3$
0.47 g	$MnCl_2.4H_2O$

CHAPTER 2: VITELLOGENESIS.

1. Introduction.

In crustaceans, the major constituent of the yolk proteins in eggs is a carotenoid lipo-glycoprotein, called lipovitellin. There has been some controversy about the site of its synthesis. Some investigators assume that lipovitellin is synthesized within the developing oocytes (endogenous synthesis). Others postulate that the yolk proteins are mainly synthesized outside the ovaries, released into the haemolymph and then taken up by the oocytes through pinocytosis. The latter is the case in most insects and vertebrates.

Gradually more results became available which favour the second possibility. For Artemia, this raises the questions about the site of exogenous lipovitellin synthesis and about its possible control by hormones. Other concomitant questions are: Is oviparity and ovoviviparity regulated by hormones? Can vitellogenesis be induced in male brine shrimps?

2. Vitellogenesis in Crustacea (literature survey).

2.1. Vitellogenesis and yolk proteins.

Vitellogenesis is the process of yolk protein synthesis and accumulation. The major constituent of the yolk proteins is vitellogenin. After synthesis, vitellogenin is released into the haemolymph and selectively taken up by the growing oocytes. Following uptake, vitellogenins undergo some changes and are then called vitellins.

In vertebrates, vitellogenin is synthetized by the liver (Wallace and Jared, 1969; Wallace et al., 1972). Vertebrate vitellogenin, once incorporated into the oocyte, undergoes a macromolecular restructuring into lipovitellin and phosvitin. Phosvitin contains almost all the protein-bound phosphate and sugars while nearly all lipids are associated with lipovitellin (Bergink and Wallace, 1974). In insects, the fat body - considered to be analogous to the vertebrate liver - is the site of synthesis of the vitellogenins or yolk polypeptides (see review of Wyatt and Pan, 1978).

In the haemolymph of several crustacean species a female specific lipoglycoprotein* is present (Gilchrist and Lee, 1972; Fyffe and O'Connor, 1974; Croisille et al., 1974; Saiag et al., 1979). Electrophoretically and immunologically this lipoprotein complex is almost identical with lipovitellin** (Kerr, 1969; Pan and Wallace, 1974; Junéra et al., 1977a).

The lipovitellins are carotenoproteins with an average molecular weight of 350,000 dalton. In several crustacean species they have been partially characterized (Wallace et al., 1967). After denaturation with SDS, most yolk proteins split into several polypeptides (Fyffe and O'Connor, 1974). The polypeptide pattern of crustacean lipovitellin is much more complex than that of vertebrates and insects (Bergink et al., 1974; Gellissen et al., 1976; Srdic et al., 1978).

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* The term female specific protein (FSP) refers to any blood-borne protein restricted to females and not immunochemically demonstrable in males (Fyffe and O'Connor, 1974). Usually, it is a lipoglycoprotein transported via the haemolymph to the ovaries and is generally called vitellogenin (VG) (Pan et al., 1969).

**Lipovitellin (LV) is defined as the major high density lipoprotein found in developing oocytes and for which vitellogenin is the precursor (Wallace et al., 1967). It is in fact a lipoprotein complex in which the yolk protein is tightly associated with lipids, sugars and carotenoids. Sometimes, the term vitellins is used for the yolk polypeptides of the oocytes. In flies the yolk protein can be divided in subpolypeptides. We use the term lipovitellin for the female specific protein of the brine shrimp (see 3.3.).

The development of crustacean oocytes comprises two stages. The early growth, called previtellogenesis occurs both during the period of sexual activity (during courtship and mating) and that of sexual rest. This phase can be called autosynthetic while it is mainly characterized by endogenous syntheses such as accumulation of ribosomes, polyribosomes and of glycoprotein granules. Charniaux-Cotton (1980) even divides the period of first growth into previtellogenesis and primary vitellogenesis. According to the terminology used by Dhainaut and de Leersnyder (1976), the second period of oocyte growth is called secondary vitellogenesis and only occurs during sexual activity. In crustaceans, this phase is mainly heterosynthetic.

Several ultrastructural changes occur during the growth of the oocyte. Numerous microvilli appear in the oolemma and pinocytotic vesicles appear as a result of the endocytotic activity. After their fusion, lipovitellin (LV) containing yolk bodies are formed. The number of lipid globules also increases during vitellogenesis (Meusy, 1980).

2.2. Site of lipovitellin synthesis.

During recent decades there were controversies about the site of synthesis of yolk polypeptides in Crustacea. The idea that there had to be an extra-ovarian source of lipovitellin was not accepted by all investigators.

Ganion and Kessel (1972) showed by means of high resolution radioautography, that in the oocytes of the crayfish Orconectes immunis, there is a continuous intracellular synthesis of yolk proteins during vitellogenesis. Hinsch and Cone (1969) also observed by electron microscopy that in the oocytes of the spider crab Libinia emarginata, the granular endoplasmic reticulum appeared active in the synthesis of intracisternal granules which aggregate to produce yolk bodies.

In vitro labeling of the isolated ovary of the crayfish Procambarus and the crab Pachygrapsus crassipes suggested that this organ was capable of synthesizing LV (Lui et al., 1974;1976;1977).

The active role of the ovary in LV synthesis was also demonstrated in the fiddler crab Uca pugilator by Eastman-Reks and Fingerman (1985).

Recently in the kuruma prawn Penaeus japonicus, the protein synthesized by the ovary in vitro was recognized by an anti-vitellin-antiserum. The protein synthesized by the hepatopancreas in vitro was not. Furthermore, immunofluorescence revealed that the follicle cells from early developing oocytes could be responsible for LV synthesis within the ovary (Yano and Chinzei, 1987).

Although the mode of yolk body formation favoured endogenous synthesis, the possibility of exogenous synthesis could not be excluded. Hinsch and Cone already in 1969 suggested the extra-oocytic source for yolk materials. Junéra et al. (1977b) showed in Orchestia gammarella that vitellogenin (VG) was synthetized outside the ovaries while synthesis stopped completely only 5 to 8 days after bilateral ovariectomy. But, Wolin and his co-workers (Wolin and Laufer, 1971; Wolin et al.,1973) undeniably demonstrated that in the eggs of Uca pugilator, Cambarus clarkii and Libinia emarginata, the yolk spheres developed primarily through micropinocytotic uptake of VG from the haemolymph. They observed that fluorescein-conjugated female haemolymph components entered the oocytes only during the period of yolk accumulation. They also proved that in vitellogenic females, extracts of the hepatopancreas contained a protein immunologically identical to LV.

Specific cells in the hepatopancreas of female decapods which synthesize VG (Carcinus maenas and Libinia emarginata) were called vitellogenocytes. These cells were responsible for the extra-ovarian VG synthesis (Paulus and Laufer, 1987).

However, not in all crustaceans is the hepatopancreas the only possible site of VG synthesis.

In the lobster Homarus americanus, Dehn et al. (1981) already suggested an exogenous source for LV. Kerr (1968) pointed out the possibility that hemocytes (cells circulating in the haemolymph) synthesized the serum lipoprotein (vitellogenin) in the blue crab Callinectes sapidus. In Orchestia gammarella (Amphipoda) results of experiments involving ovariectomy suggested that the sub-epidermal adipose tissue of females in secondary vitellogenesis, was the site of VG synthesis (Junéra and Croisille (1980), Croisille and Junéra (1980), see also review in Meusy (1980)). More recently, this fact was supported by electron microscopical observations of the

subepidermal fat-body after ovariectomy by Zerbib and Meusy (1983).

In Parapenaeus longirostris VG was shown to be present in the subepidermal adipose tissue (Tom et al., 1987). Also in Porcellio dilatatus (Isopoda) Picaud (1980) detected VG synthesis in the fat body of ovariectomized females.

Gilchrist and Zagalsky (1983) isolated a lipoprotein, the structure of which was electrophoretically related to LV, from the connective tissue storage cells of female Branchinecta packardi.

In the anomuran crab Clibanarius clibanarius, Varadarajan and Subramoniam (1980) postulated an initial endogenous synthesis that is later overtaken by the exogenous synthesis. Zerbib (1979) divided vitellogenesis in the crayfish Astacus astacus and A. leptodactylus in two phases: the first one was characterized by the ergastoplasmatic activity of the oocyte, and the second was characterized by the accumulation of exogenous yolk materials in the oocyte.

3. Vitellogenesis in Artemia.

3.1. The anatomy of the ovary.

The genital apparatus is depicted in Fig. 2.1. More information about the anatomy can be found in Criel (1980). Egg-development in the brine shrimp was described by Fautrez and Fautrez-Firlefyn (1977).

The two ovaries are thin opaque white strips located on each side of the gut in the posterior part of the body. The germ cells which are located on the ventral side of the ovaries, mature when they are migrating dorsally and medially. The oogonia are located near the ventral side. More dorsally, bands of young oocytes form a curved ribbon. Electron microscopic observations revealed the presence of cytoplasmic bridges which interconnect adjacent oocytes of the ribbon. They could be remnants of incomplete cytokinesis (Anteunis et al., 1966a). On the dorsal side of the ovarian strip, certain cells will become rounder, terminate their previtellogenesis with the formation of a germinal vesicle and then enter vitellogenesis. They phagocytize a large number of nurse cells* (Anteunis et al., 1966b).

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* Nurse cells in Artemia are morphologically identical with young oocytes. The growing oocytes will absorb by phagocytosis all the surrounding nurse cells (Fautrez-Firlefyn, 1951).

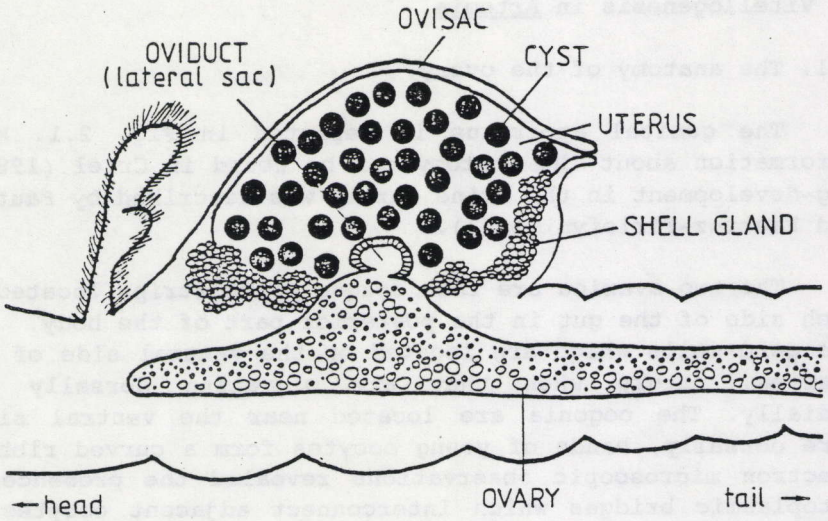

Fig. 2.1. Schematic view on the reproductive system of female Artemia sp.

3.2. Vitellogenesis in Artemia: auto- and heterosynthesis.

The eggs of the brine shrimp are centrolecithal and the yolk is mainly composed of lipid droplets and membrane bound protein granules. Fautrez and Fautrez-Firlefyn (1964) studied vitellogenesis in the brine shrimp by morphological and cytochemical methods. In previtellogenic oocytes they described a structure composed of microgranules. This body is in fact a yolk nucleus and cytochemical reactions have shown its pronounced acid phosphatase activity (Fautrez-Firlefyn et al., 1963). These authors could follow the presence and localization of the yolk nucleus in toto up to the first cleavages. It was possible to observe this body up to the onset of gastrulation. Right at that moment the 'yolk nucleus' was situated in one of the first two cells that invaginate. These two cells are the initial germinal cells (Fautrez-Firlefyn, 1951). The ultrastructure of this yolk nucleus was described by Anteunis et al. (1964). The nucleus is composed of multivesicular bodies, dense bodies, free microvesicles, ribosomes and it is surrounded by Golgi elements and numerous mitochondria. In previtellogenesis 'pre-yolk granules' are split off from the yolk nucleus and migrate towards the cortical plasm. Meanwhile their volume is increasing. Also lipid globules can already be observed at that moment (de Chaffoy de Courcelles, 1979).

de Chaffoy et al. (1980) demonstrated the presence of female specific immunoreactive material when testing it with rabbit anti-lipovitellin antiserum. Furthermore, these authors stated that: 'Since the haemolymph contains not only the apoproteins (LV-α1 and LV-ϵ), but also its hydrolytic products (LV-α2 to LV-α10), it seems likely that these lipovitellin proteins are a result of leakage from the ovary into the haemolymph rather than transport to the ovary through the haemolymph route.'

The fact that in early vitellogenesis autosynthetic yolk protein synthesis is predominant, does not exclude that during late vitellogenesis lipovitellin from an extra-ovarian source is incorporated. Micropinocytotic activity, indicative of this process, was demonstrated by Criel (1984) who followed in vitro the uptake of horse radish peroxidase by ovaries.

When we started our experiments on this subject, the site of extra-ovarian lipovitellin synthesis was largely unknown.

3.3. Lipovitellin in the brine shrimp.

In Artemia, the lipovitellin (LV) complex is confined within small oval granules (2.5-3.2 μm wide and 3.1-5.2 μm long) together with certain enzymes, nucleic acids and diguanosine nucleotides. Each embryo contains about 5.68 x 10^4 yolk granules (Warner et al., 1972). These proportions are in the same range as those for the freshwater crustacean Branchipus stagnalis. In this species LV is packed in small membrane-limited yolk platelets (Zagalsky and Gilchrist, 1976).

Partial biochemical characterization of lipovitellin (LV) of the brine shrimp has been carried out by de Chaffoy de Courcelles (1979) and his co-workers (de Chaffoy de Courcelles and Kondo, 1980; de Chaffoy et al., 1979,1980). The Artemia lipovitellin complex consists of approximately 3.3 percent carbohydrate, 8.6 percent lipid and 88 percent apoprotein. The apoprotein fraction is constituted of LV-α1 (MW=190,000) and LV-ϵ (MW=67,000) which are present in an equimolar ratio. The native lipovitellin molecule has an estimated molecular weight of 6-700,000 dalton and it

probably represents a dimeric form of a polypeptide with the calculated molecular weight of 290,000. The red-orange colour of lipovitellin is due to carotenoid pigments. Canthaxanthine is the major carotenoid present (97 percent).

de Chaffoy (1979) also described the presence of a specific protease in the yolk granules which is most active in an alkaline environment. This protease is responsible for the limited hydrolysis of lipovitellin. Within the yolk granule the enzyme is associated with lipovitellin, either in an isolated state, or in an integrated state (de Chaffoy et al., 1980). During embryonic development this lipovitellin-specific protease is responsible for the hydrolysis of the LV-α1 while the smallest apoprotein was found to be completely resistant to this proteolytic activity. Gradually LV-α1 is degraded into multiple low molecular weight proteins (from LV-α2 to LV-α10, LV-β, LV-τ). LV-δ (MW=78,000), although it is not a hydrolysis product of LV-α1, is also present in degraded LV molecules. According to these authors, it might play a role in lipid transport.

The proteolysis described above, appears to be the first step in the yolk degradation by the developing embryo of Artemia. The increase in the proportion of lipid globules was found to parallel the decrease in number of the yolk granules during the post-gastrula development.

4. Hormonal control of vitellogenesis in Crustacea (literature survey).

The following paragraphs will give an idea of the diversity of vitellogenesis controlling systems present in crustaceans. The hormones involved may vary according to the species and the physiological state of the animal.

4.1. Correlation between vitellogenin synthesis and the moult cycle.

Evolution of VG synthesis during the moult cycle is illustrated in Fig. 2.2. We will take the situation in Orchestia gammarella as an example and describe the interactions of the moulting hormone with vitellogenesis. In this amphipod, secondary vitellogenesis occurs in parallel with the increase in moulting hormone concentrations.

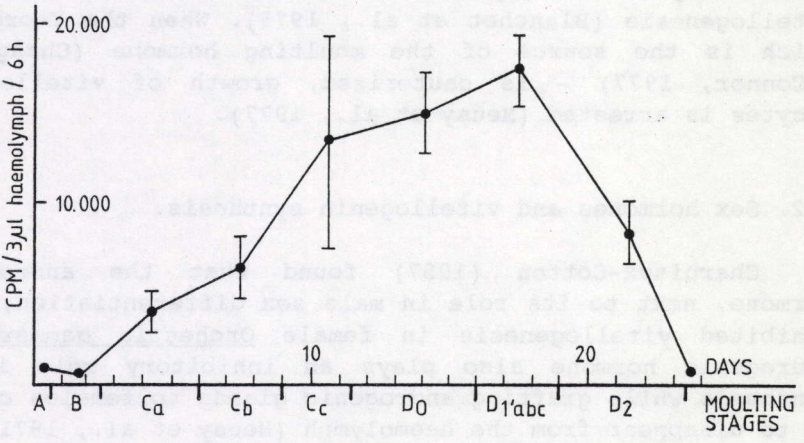

Fig. 2.2. Vitellogenin synthesis in Orchestia gammarella during the moulting cycle. Animals were injected with 2.5 µCi of ^{3}H-leucine. After six hr haemolymph was collected and radioactivity present in vitellogenin was determined after separation of the serum proteins by disc-electrophoresis. Mean values ± standard error are shown. (after Meusy and Charniaux-Cotton, 1984)

In Orchestia gammarella, uptake of VG by the oocytes starts at stage C of the moulting cycle and from then on its synthesis increases quickly. At the end of secondary vitellogenesis, just before egg-laying, VG synthesis drastically decreases. The moulting cycle is then in stage D_2 (From Blanchet-Tournier et al., 1980).

Charniaux-Cotton (1985) has already suggested that the low titer of ecdysteroids at the beginning of the intermoult period could be responsible for the onset of secondary vitellogenesis and that moulting hormone is probably necessary for VG synthesis. Furthermore, in this species the presence of a vitellogenin synthesis-stimulating ovarian hormone (VSOH) which stimulates the subepidermal adipose tissue to produce vitellogenin has been demonstrated. Both VSOH and ecdysteroids are indispensable for normal VG synthesis (Junéra et al., 1977b; Blanchet-Tournier, 1982).

Injection however, of 20-OH-ecdysone into a female immediately after ecdysis inhibits the onset of secondary vitellogenesis (Blanchet et al., 1975). When the Y-organ - which is the source of the moulting hormone (Chang and O'Connor, 1977) - is cauterized, growth of vitellogenic oocytes is arrested (Meusy et al., 1977).

4.2. Sex hormones and vitellogenin synthesis.

Charniaux-Cotton (1957) found that the androgenic hormone, next to its role in male sex differentiation, also inhibited vitellogenesis in female Orchestia gammarella. Androgenic hormone also plays an inhibitory role in VG synthesis while grafting androgenic glands to females caused VG to disappear from the haemolymph (Meusy et al., 1971). In this species, VSOH is demonstrated (see above), a hormone that like the estrogens in vertebrates is of ovarian origin and controls VG synthesis. However, in the isopod Porcellio dilatatus, ovariectomy shows that VG synthesis is not ovarian hormone dependent (Picaud and Souty, 1981).

Typical vertebrate sex-specific molecules can also influence the activity of ovarian cells in crustaceans. In the sand shrimp Crangon crangon, injection of Human Chorionic Gonadotrophin (HCG) accelerates formation of yolk in growing ocytes (Bomirski and Klek-Kawinska, 1976). HCG also stimulates the rate of VG synthesis and its release by the fat body in Idotea balthica basteri (Souty and Picaud, 1984).

Steroids such as progesterone and 17α-hydroxy-progesterone, had a stimulatory effect on vitellogenesis in shrimps and prawns (Yano 1985, 1987) (see also Chapter 6).

4.3. Neurohormonal control of vitellogenesis.

4.3.1. Neuroendocrinology of Crustacea.

4.3.1.1. Crustacean neuroendocrine system.

There is evidence that in crustacean neuroendocrine centers substances are produced which affect osmotic and ionic regulation (Mantel, 1985). Next to the neuropeptides that regulate the concentration of pigments, an hyperglycemic hormone (CCH) has been isolated and characterized from the neurosecretory structures of the crustacean eyestalk (Keller et al., 1985).

The neurosecretory apparatus of Crustacea is complex and elements of it are found throughout the central nervous system. Hormones produced in the neurosecretory cells are transported along axon fibres to release sites. Three main areas of release can be distinguished. The optic ganglia which contain the X-organ-sinus gland complex, are located in the eyestalks. The neurohaemal pericardial organ that receives fibres from centrally and peripherally located somata is more variable in its position, but it is always found in the wall of the pericardium. The post commissural organs are situated immediately after the oesophagus. Each of these release centers is the junction of many fibres which originate elsewhere. The X-organ-sinus gland system is the most important neuroendocrine center in Crustacea and its activity shows a circadian rhythmicity (Aréchiga et al., 1985). The secretion of neurohormones from the crustacean X-organ-sinus gland system is controlled by environmental stimuli, light being the most conspicuous one.

Fig. 2.3 gives an overview of the neuroendocrine structures that are present in the eyestalk of the shrimp Lysmata seticaudata. The main features of this system are common to all crustaceans, but details differ among species and among orders.

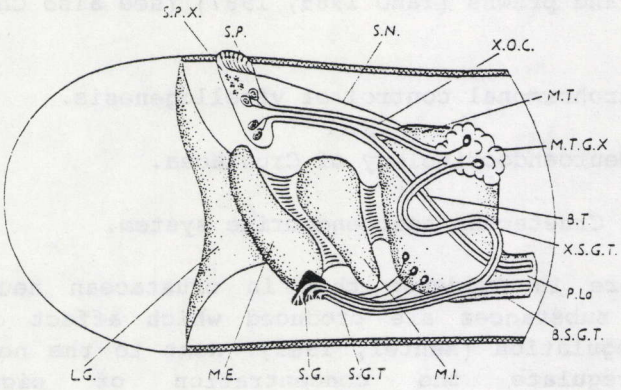

Fig. 2.3. The main nervous and endocrine structures are shown in the anterior diagrammatical view of a dissection of a left eyestalk from the natantian Lysmata seticaudata. B.S.G.T., brain-sinus gland tract; B.T., brain-X organ tract; L.G., lamina ganglionaris; M.E., medulla externa; M.I., medulla interna; M.T., medulla terminalis (sometimes called lobus terminalis of the protocerebrum); M.T.G.X., medulla terminalis ganglionic X organ; P.Lo, peduncle of the optic lobe; S.G., sinus gland; S.G.T., combined sinus gland tract; S.N., sensory nerve; S.P., sensory pore; S.P.X., sensory pore X organ; X.O.C., X organ connective; X.S.G.T., X organ-sinus gland tract. (from Carlisle and Knowles, 1959)

The endocrine regulation of nearly all basic physiological processes studied so far has been traced to the X-organ-sinus gland system (Kleinholz, 1942; Hanstrom, 1947; Bliss et al., 1954; Knowles and Carlisle, 1956; Carlisle and Knowles, 1959; Passano, 1960; Adiyodi and Adiyodi, 1970; Kleinholz and Keller, 1979; Legrand et al., 1982).

The sinus gland acts as the neurohaemal organ for a number of different endocrine neurons situated in the first, second, third and fourth optic ganglia (the lamina ganglionaris, the medulla externa, the medulla interna and the medulla terminalis respectively), and the cerebral ganglia (see van Deijnen, 1986).

The sinus gland functions as a storage organ while the structure of the X-organ resembles that of secretory tissue

in the eyestalk of Crustacea. The term X-organ was given earlier to denote an organ of unknown function found in the eyestalk. Originally the X-organ represents the transformed sensory cells of a rudimentary eye papilla or sensory pore. Since the typical X-organ described by Hanström is found associated with a sensory pore or papilla wherever these are present, Knowles and Carlisle (1956) suggested that Hanström's X-organ should be termed the Sensory Pore X organ or Sensory Papilla X organ (SPX). Later on, structures distinct from Hanström's X-organ, have been found. Depending on the species, the place of these neurosecretory cells varied and other names showed up. Pars distalis X organ (PDX) was proposed for Hanström's X-organ close to the sensory papilla or sensory pore. The term 'Pars ganglionaris X organ (PGX)' was applied to the X-organ situated in the medulla terminalis. There were, however, several neurosecretory cell groups supplying the sinus gland which in essence were not different from each other. Therefore Knowles and Carlisle (1956) suggested that neurosecretory cell groups within the eyestalk should be termed ganglionic X organs and that a prefix denote where they lie. Following this nomenclature the PGX becomes the medulla terminalis ganglionic X organ (MTGX) and the term 'sensory papilla X-organ' is used for SPX, PDX and Hanström's X-organ.

According to Hanström (1947) the brain of all Malacostraca is composed of three regions. The protocerebrum innervates the compound eyes, the nauplius eye, the median frontal organ, the lateral frontal organs (including the X-organ), the sinus gland and muscles of the head. The deutocerebrum innervates the antennulae by both sensory and motor fibres. The tritocerebrum innervates the antennae (by sensory and motor fibres) and possesses a suboesophageal commissure and connections with the stomatogastric system.

The published information about the neurosecretory cells in these regions does not form a coherent whole. Best known are the median zone of the protocerebrum and the thoracic ganglia. The first releases a gonad-inhibiting hormone in isopods (Legrand et al., 1982) and a secondary folliculogenesis-stimulating hormone in Orchestia gammarella (Blanchet-Tournier, 1982). Results obtained by implantation of thoracic ganglion into immature female decapods supports the existence of a gonad-stimulating hormone in this ganglion (Adiyodi and Adiyodi, 1970).

4.3.1.2. Neuroendocrine system of the brine shrimp.

Neurosecretion in Artemia was thus far only investigated by light and E.M. microscopy. Lochhead and Resner (1958) identified 35 to 40 neurosecretory cells in the protocerebrum of Artemia salina. Hentschel (1963, 1965) found the same type of neurosecretory cells in Artemia salina as in Chirocephalus. They are situated in the cerebrum and metamerically spread over the ganglia of the ventral nervous cord. Each ganglion generally contains 2 pairs of neurosecretory cells. Baid and Ramaswami (1965) described 2 types of neurosecretory cells present in the cerebrum and one type in the eyestalk of A. salina. According to these authors, the latter type forms an X-organ.

These data were obtained with the classical histological staining methods. At present nothing is known about the nature of the neurosecretory material of these cells.

We noticed numerous differences when we compared the organisation of the cerebrum and the eyestalks of this species to the system of higher evolved crustaceans (see above). Although in published literature, neurosecretory centers in the brine shrimp, analogous to those in more evolved crustaceans are found. Ultrastructural observations of Elofsson and Lake (1971) and of Rasmussen (1971) showed the presence of a frontal organ which could be the anologue of the (Hanström's) X-organ. Ultrastructural studies have also been conducted on the nauplius eye and adjacent organs of adult Artemia (Anadon and Anadon, 1980). Hentschel (1965) demonstrated a neurohaemal organ but its function as storage organ, similar to the sinus gland, remains to be proven. Two neurosecretory centers close to the nauplius eye were described by Van den Bosch de Aguilar (1974, 1976, 1977), namely the lateral and median neurosecretory cells ('L' and 'M' cells). Secretory activity of the lateral cells increases when reproduction progresses while the product released by the median cells favours salt retention. Now that straightforward immunological methods have become available, the search for neuropeptides in the brine shrimp becomes possible.

For the diagram (see Fig. 2.4) we used data of Cassel (1937), Hentschel (1965) and Van den Bosch de Aguilar (1974).

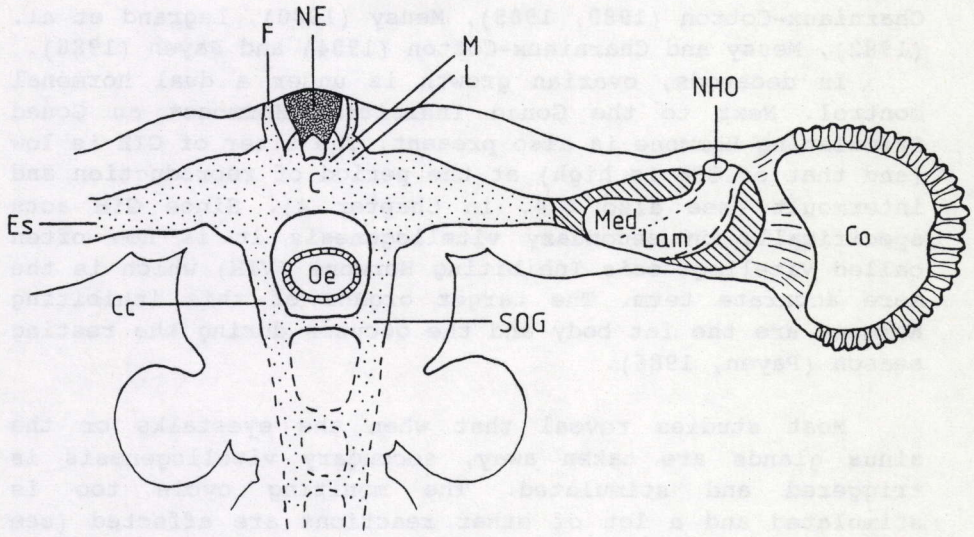

Fig. 2.4. Diagramatic frontal section of the head region and eyestalk of Artemia.
C, cerebrum; Cc, circumoesophageal connective; Co, complex eye; Es, eyestalk; F, frontal organ; L, lateral neurosecretory cells; Lam, lamina; M, median neurosecretory cells; Med, medulla; NE, nauplius eye; NHO, neurohaemal organ; Oe, oesophagus; SOG, sub-oesophageal ganglion*.

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* In literature the term 'post-oesophageal ganglion (POG)' is commonly used.

4.3.2. Neurohormonal control of vitellogenesis.

Endocrine control of vitellogenesis has been extensively studied in malacostracans and was reviewed by Charniaux-Cotton (1980, 1985), Meusy (1980), Legrand et al. (1982), Meusy and Charniaux-Cotton (1984) and Payen (1986).

In decapods, ovarian growth is under a dual hormonal control. Next to the Gonad Inhibiting Hormone* an Gonad Stimulating Hormone is also present. The titer of GIH is low (and that of GSH is high) at the period of reproduction and intermoult (see also 4.4. in Chapter 4). Since GIH acts specifically on secondary vitellogenesis it is now often called Vitellogenesis Inhibiting Hormone (VIH) which is the more accurate term. The target organs of this inhibiting hormone are the fat body and the oocytes during the resting season (Payen, 1986).

Most studies reveal that when the eyestalks or the sinus glands are taken away, secondary vitellogenesis is triggered and stimulated. The moulting cycle too is stimulated and a lot of other reactions are affected (see Chapter 4). The two eyestalk hormones GIH and MIH are different from each other (Meusy and Charniaux-Cotton, 1984).

In the shrimp Palaemon serratus, GIH secretion might be controlled by the X-organ of the medulla externa (MEX) (see above). Secondary vitellogenesis stops after removal of the medulla externa (Faure et al., 1981).

Measurements of Ovary-Inhibiting Hormone (OIH) activity in the eyestalks of female Crangon crangon revealed that the highest activity occurs after the breeding season when the ovaries are in resting condition (Klek-Kawinska and Bomirski, 1975). In the crab Paratelphusia hydrodromous the period at which eyestalk ablation is performed, is also important for a successful completion of vitellogenesis (Anilkumar and Adiyodi, 1985).

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* Gonad Inhibiting Hormone (GIH) is also called Ovary Inhibiting Hormone (OIH) and Vitellogenesis Inhibiting Hormone (VIH). Analogous to GIH, Gonad Stimulating Hormone (GSH) is called OSH and VSH. GIH is partially characterized from eyestalks of male Cancer magister (Bomirski et al., 1981).

The response to eyestalk removal varies with the species, age, season, culture conditions, time of intervention. Often the ablation favours the process in progress (moulting or vitellogenesis) (Legrand et al., 1982). For example in Paratelphusa hydrodromous multiple limb autotomy (MA), long known to stimulate somatic growth, did so only if the animal was physiologically in the somatic phase (this phase lasts until sexual maturity is reached). Vitellogenesis was accelerated if this crab was in the reproductive phase at the time of limb autotomy (Kurup and Adiyodi, 1984).

In species without eyestalks, synthesis of neurohormones is mainly situated at the level of the protocerebrum. Brain neurohormones are involved in the transmission of external stimuli such as temperature and photoperiod which determine the annual period of sexual rest in many crustacean species (Nelson et al., 1983).

In isopods, it seems well established that GIH appears to be released by the median zone of the protocerebrum since removal of this zone triggers vitellogenesis (Legrand et al., 1982). The situation is different in the amphipod Orchestia gammarella in which secondary vitellogenesis does not start when the median part of the protocerebrum is destroyed (Blanchet-Tournier et al., 1980). Nevertheless, in the brain of this species a factor resembling VIH has recently been demonstrated (Blanchet-Tournier, 1987).

In decapods, ovarian enlargement induced by the implantation of a thoracic ganglion into immature females of the crabs Potamon and Paratelphusa, and of the spider crab Libinia, supports the existence of a gonad-stimulating hormone in this ganglion (Adiyodi and Adiyodi, 1970). The thoracic ganglia of Uca pugilator are the source of a Vitellogenesis Stimulating- or Activating- neurohormone (VSH or VAH) (Eastman-Reks and Fingerman, 1984). However, in vitro experiments with the ovary of Paratya compressa revealed the greater effectiveness of extracts of brain over those from thoracic ganglia (Takayanagi et al., 1986). It seems that the ovary-stimulating factor acts directly on the oocytes.

4.4. New perspectives.

In insects, Juvenile Hormone (JH) and JH analogues (JHA)* can influence moulting. Besides ecdysteroids, JH has also been detected in crustaceans (Schneiderman and Gilbert, 1958). The presence of JH in crabs is confirmed by GC/MS analysis (Laufer et al., 1985, 1986a, 1987). JH Analogues were applied by some authors in order to influence moulting and/or reproduction in crustaceans.

Gomez et al. (1973) found that precocious metamorphosis without settlement in the acorn barnacle Balanus galeatus was induced when cyprid larvae were reared in the presence of the synthetic JH analogue ZR-512.

Fig. 2.5. Comparative chemical structures of the acids of the three juvenile hormones known in insects (JH-I, JH-II, JH-III) and one analogue (ZR-512), methoprene (Altosid or ZR-515), and one analogue known in crustaceans, methyl farnesoate. (JHA ZR-515 is isopropyl 11-methoxy-3,7,11-trimethyl-2,4-dodecadienoate).

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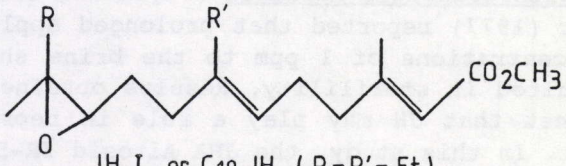
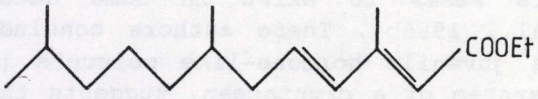
* Juvenile Hormone (JH) analogues are substances of natural or synthetic origin which act in the same way as endogenous JH, the endocrine secretion of the insect corpora allata. JH mimics are known to disrupt embryonic and larval insect development and to induce sterility in adults. The structure of these molecules is shown in Fig. 2.5. (from Payen and Costlow, 1977)

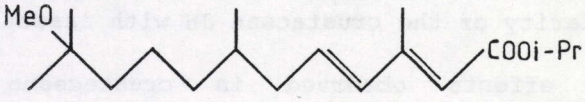


JH I or C_{18} JH (R = R' = Et)
JH II or C_{17} JH (R = Et, R' = Me)
JH III or C_{16} JH (R = R' = Me)

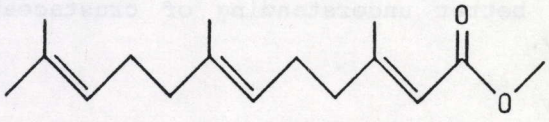
JUVENILE HORMONE



ZR-512



ALTOSID or ZR-515



METHYL (2E, 6E)- FARNESOATE

In Orchestia gammarella (Charniaux-Cotton, 1974), a synthetic JH-I (busulfan or Misulban: 1,4-dimethylsulphonyloxybuthane) inhibits vitellogenesis only when it is injected during the early intermoult period. Methoprene appears to inhibit vitellogenesis also in the mud-crab Rhithropanopeus harrisii (Payen and Costlow, 1977).

Laufer (1977) reported that prolonged application of a JHA at concentrations of 1 ppm to the brine shrimp Artemia salina resulted in sterilility. Results obtained on Daphnia magna suggest that JH may play a role in reproduction and development. In this study, the JHA Altosid ZR-515 was used. The early stages of embryonic development were most sensitive at concentrations of 1.6×10^{-6} M (Templeton and Laufer, 1983).

A correlation between the rate at which methyl farnesoate (MF) (see Fig. 2.5) is secreted, and vitellogenesis seems to exist in some decapod species (Laufer et al., 1986b). These authors concluded that the finding of a juvenile hormone-like molecule (MF), in the circulatory system of a crustacean, suggests that Crustacea may regulate growth and reproduction in a manner similar to that in Insecta. Methyl farnesoate is secreted by the mandibular organs (MO) of decapods. The MO is a structure that resembles the corpora allata (CA) of insects. Laufer et al. (1986b) observed an increase of MF secretion during vitellogenesis in Crustacea. This is another indication of the similarity of the crustacean JH with insect JH.

The effects observed in crustaceans after the administration of compounds which are known to have JH activity in insects, may be caused by the fact that these compounds act in the same or in a similar manner as an endogenous JH. Sometimes doses used in the experiments exceeded physiological doses. Nevertheless, the effects of these various synthetic and natural insect hormones can provide a better understanding of crustacean reproductive physiology.

5. Lipovitellin in the brine shrimp: experimental results.

5.1. Preparation of anti-lipovitellin antiserum.

5.1.1. Sodium Dodecyl Sulphate gradient polyacrylamide gel electrophoresis (SDS-PAGE).

SDS-PAGE is described in Appendix III. After electrophoresis the gels were, depending on the type of experiment, either stained, prepared for blotting or prepared for fluorography (see Appendix VII).

5.1.2. Preparation of antiserum against LV in rabbits.

Lipovitellin was extracted from yolk platelets of the cyst stage. It was roughly purified and contained 12.5 mg protein per ml Tris-HCl buffer (pH=9) (a gift from Dr. L. Moens, Universitaire Instelling Antwerpen). After SDS-PAGE, LV was separated into different protein bands. Gels were stained for 1 hr in Coomassie Blue solution. The band with the lowest mobility was the apoprotein lipovitellin-α1 (MW=190,000). Gels were shortly destained and this band was cut out and rinsed in distilled water. Eight bands were homogenized (both by Elvejhem Potter and by ultrasonics) in 0.9 percent NaCl:Freund's complete adjuvant (Difco), 1:1 (v/v), and injected into a rabbit. Per injection about 60 µg protein was given. Primary injection was made intradermally. Subsequent injections were made subdermally. Blood was taken from an ear vein 1 week after each booster injection. The antibody titer was measured by immunospotting (see further). Serum was frozen in liquid nitrogen and stored at -20°C. Part of the antiserum stock was lyophilized and stored under vacuum at -20°C.

5.2. Screening for the antibody characteristics.

Female specificity of the antiserum was tested by immunospotting on nitrocellulose sheet, immuno-electrophoresis in agarose gels, double immunodiffusion in 1 percent agarose gels.

5.2.1. Immunospotting.

See Appendix IV.1.

Result: The AS can easily be diluted to 1:8,000 (see Fig. 2.6) for its use in immunocytochemistry (see next chapter).

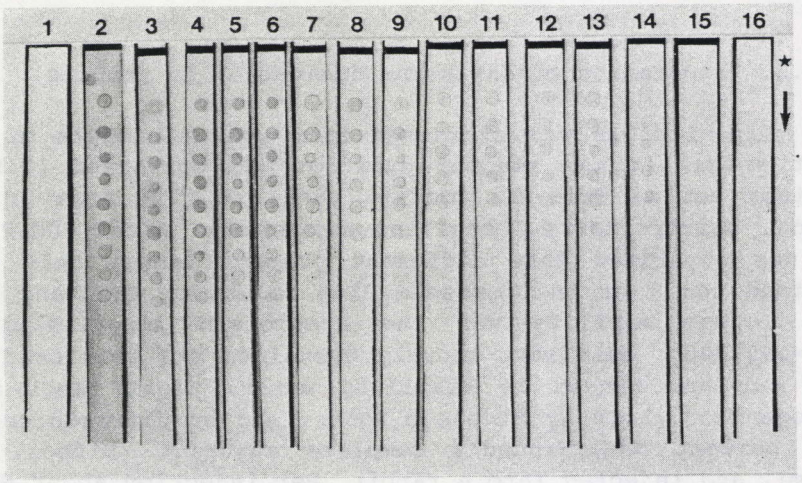


Fig. 2.6. Immunospotting of lipovitellin antigen on nitrocellulose sheet (0.45 µm). Antigen is diluted starting from 1:1, 1:2... up to 1:4096; *=Tris saline. Anti-lipovitellin-α1 antiserum is serially diluted in lane 2-16 up to 100, 200, 400, 800, 1000, 1600, 2000, 3200, 4000, 4500, 5000, 6400, 8000, 9000 and 10,000 times, respectively. In lane 1 pre-immune rabbit (PIR) serum was diluted 1:1000.

5.2.2. Immuno-electrophoresis.

See Appendix IV.2.

Result: Our lipovitellin antiserum reacted against the haemolymph of females of all vitellogenic stages. In male haemolymph no precipitation arcs were seen (see Fig. 2.7).

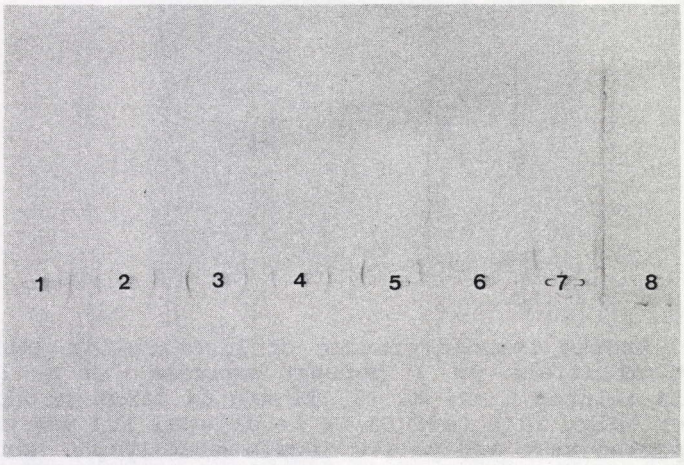


Fig. 2.7. Immuno-electrophoresis of male haemolymph (lane 8) and haemolymph samples from females during vitellogenesis: lane 1=Ov-, Ut+; lane 2=Ov±, Ut+; lane 3=Ov+, Ut+; lane 4=Ov++, Ut+; lane 5=Ov++, Ut-; lane 6=L.S. A lipovitellin sample (12.5 µg/µl) is applied to lane 7. Anti-lipovitellin-α1 antiserum is concentrated 5 times (see 5.2.3.).

5.2.3. Double immunodiffusion.

See Appendix IV.3.

Result: The anti-lipovitellin-α1 antiserum is female specific and gives the best result in immunodiffusion reactions when it is concentrated 5 times (see Fig. 2.8 and also in next chapter).

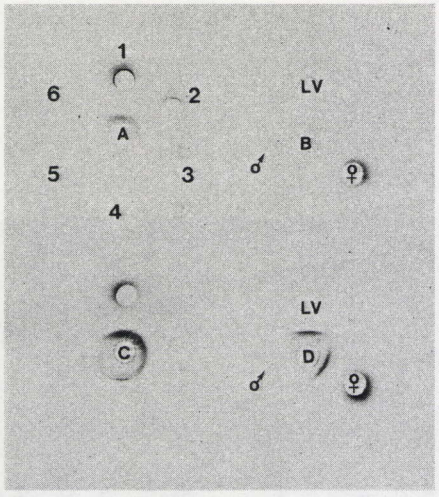


Fig. 2.8. Double immunodiffusion of lipovitellin antigen in different dilutions in 1 percent agarose. In A (and C) antigen is diluted 2, 4, 8, 16, 32 and 64 times in number 1-6, respectively. In B (and D) LV is diluted 1:8 and compared to 5 µl pooled male and pooled female haemolymph. Anti-LV-α1 antiserum is undiluted in A and B, and concentrated 5 times in C and D.

6. Concluding remarks.

The antiserum we raised against LV-α1 seemed to be female specific. The best results are obtained when it is concentrated 5 times. Further characteristics can be given by immunoblotting and immunogold staining.

A dilution of 1:8000 appears to be appropriate for its use in immunocytochemistry. By means of the PAP-staining method we will use this AS in order to find an exogenous source of the lipovitellin synthesis (see next chapter).

CHAPTER 3: A POSSIBLE EXTRA-OVARIAN SITE OF LIPOVITELLIN SYNTHESIS DURING VITELLOGENESIS IN ARTEMIA SP..

1. Introduction.

Whether all yolk proteins are synthesized in the ovarian follicles themselves or whether there is also extra-ovarian synthesis in Crustacea is still a controversial point (see 2.2. in Chapter 2).

In Artemia salina, intra-ovarian yolk protein synthesis occurs in previtellogenic follicles (Anteunis et al., 1964). At a later stage, micropinocytosis takes place which suggests that some yolk precursor proteins at least are synthesized outside the ovary (Criel, 1984) (see 3.2. in Chapter 2). However, neither hepatopancreas nor fat body as a distinct organ can be detected in this primitive crustacean. In this chapter, we describe an extra-ovarian site of synthesis of lipovitellin in the thoracopods.

2. Materials and methods.

2.1. Animals.

Selection and collection of the animals was carried out according to methods described in Appendix I.

2.2 Preparation of the tissues.

Frontal sections of whole adult Artemia were prepared as described in Appendix V.

2.3. Electrophoresis.

SDS-polyacrylamide gradient electrophoresis was carried out on 5-15 percent slab gels according to methods described in Appendix III.

2.4. Preparation of antiserum.

See 5.1. in Chapter 2. Specificity of the antiserum was tested by double immunodiffusion in 1 percent agarose gels, by immunoblotting on nitrocellulose sheets of 0.45 µm pore size and by immunogold staining.

2.5. Immunohistochemistry.

The presence of lipovitellin-like substances in tissue sections was rendered visible by the peroxidase-anti-peroxidase method as described by Vandesande (1983a) (see Appendix V). Method specificity was checked by (1) omitting the primary antiserum; (2) replacing the primary antiserum by a non-immune rabbit antiserum. Antiserum specificity was tested by means of solid phase adsorption. For this purpose, haemolymph proteins of males and vitellogenic females were covalently coupled to beads of CNBr-activated Sepharose 4B (method described by Pharmacia (1979); see also Appendix VI). The anti-lipovitellin antiserum was then applied to the protein-Sepharose conjugate.

2.6. <u>In vitro</u> synthesis of lipovitellin and fluorography.

Thoracopods of 10 vitellogenic females were incubated in filtered seawater with 2 µCi (^{35}S)-labelled methionine (Amersham, >1000Ci/mmol) for 2 hr at room temperature in a moist chamber. They were washed and homogenized with a Potter-Elvehjem homogenizer. The homogenate was then centrifuged for 5 min at 3,000 rpm (Labofuge 6000, Heraeus). The supernatant was incubated overnight with anti-LV-α1. After centrifugation (5 min at 14,000 rpm in an Eppendorf Centrifuge 5415) the pellet was washed, resolved in SDS-sample buffer and subjected to gradient electrophoresis. Gels were prepared for fluorography by the sodium salicylate method of Chamberlain (1979) (see Appendix VII).

3. Results.

3.1. Double immunodiffusion.

Double immunodiffusion test was carried out according to method described in Appendix IV.3. The antiserum appeared to be female specific (Fig. 3.1).

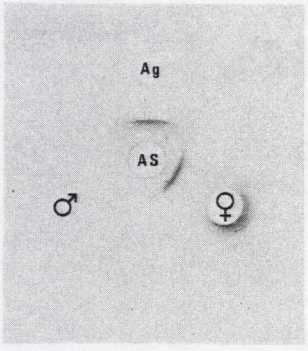


Fig. 3.1. Double immunodiffusion in 1 percent agarose gels. Samples are dissolved in barbital buffer (pH = 8.6). AS: Antiserum of lipovitellin-α1 is concentrated 5 times. Male: 5 µl of pooled male haemolymph. Female: 5 µl of pooled haemolymph of vitellogenic females. Ag: Diluted lipovitellin sample used as antigen, equivalent of 7.8 µg.

3.2. Immunoblotting.

Protocol:

1. Protein transfer.
   - Samples were subjected to SDS-PAGE (see Appendix III).
   - The separated proteins were transferred from the gel onto nitrocellulose sheet (0.45 µm) by vacuumblotting according to Peferoen et al. (1982).
   - For this purpose a blotting buffer was used: 3.025 g Tris, 11.260 g glycine, 200 ml ethanol, to 1 l with distilled H_2O.

2. Immunoblotting.
   - The proteins were visualized using the PAP-immunostaining procedure for blotted antigens according to Peferoen et al. (1982) and Geysen et al. (1984).

Result: Next to the high molecular weight protein LV-α1, a number of low molecular weight proteins can be detected. These are degradation products from the lipovitellin complex that might have the same antigenic determinant as recognized by the antiserum (see Fig. 3.2).

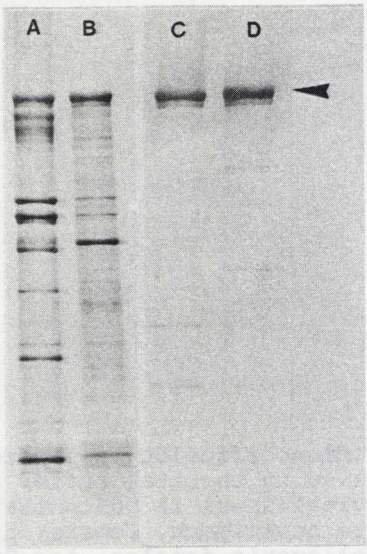


Fig. 3.2. SDS-PAGE of 5-15 percent gradient gel, constant current of 30 mA/gel. Lane A: lipovitellin sample. Lane B: extract of vitellogenic ovaries. Immunoblotting on nitrocellulose of the lipovitellin sample (lane C) and the ovarian extract (lane D) after SDS-PAGE (arrow-head=LV-α1: lipovitellin alpha-1).

3.3. Immunogold staining.

Immunogold staining (IGS) was carried out by Dr. G. Criel (State University of Ghent). Protein A was bound to the Fc-fragment of the female specific lipovitellin-α1 antiserum. These complexes were coupled to IgG gold particles of 15 nm. The electron microscopy was performed according to the method of Roth et al. (1978).

Result: Positive reaction is noticed only in the yolk globules and not in the cytoplasm (Fig. 3.3).

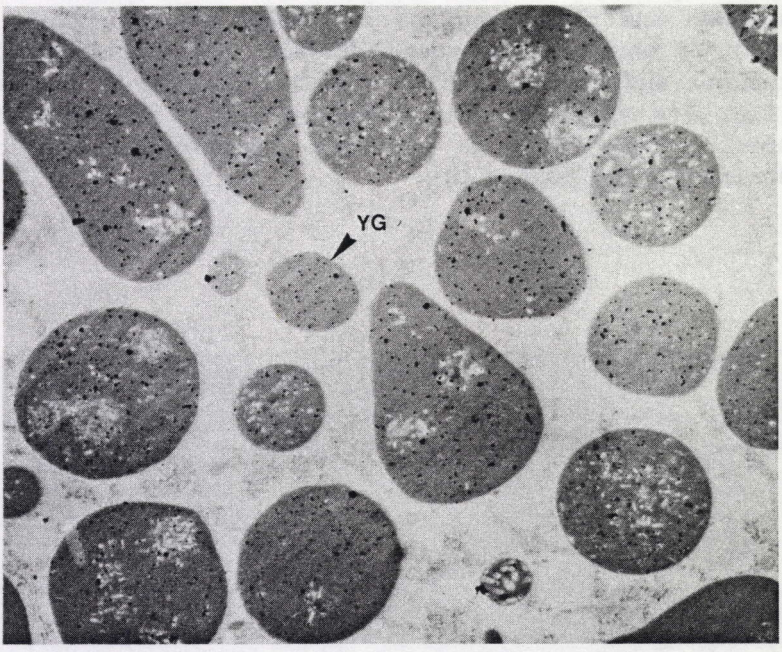


Fig. 3.3. Electron micrograph of immunogold staining of the yolk globules (YG). The tiny dots are the immunogold particles. x9,600.

3.4. Immunocytochemistry.

We studied the presence of lipovitellin during vitellogenesis. In overviews of frontal sections through the abdomen and trunk regions of female adults (Figs. 3.4 and 3.5) we observed in young oocytes the presence of a yolk nucleus and immunopositive granules located in the periphery (Fig. 3.4/A). As the vitellogenic cycles progress, more and more oocytes show a positive immunoreaction after PAP-staining (Fig. 3.4/B,C) .

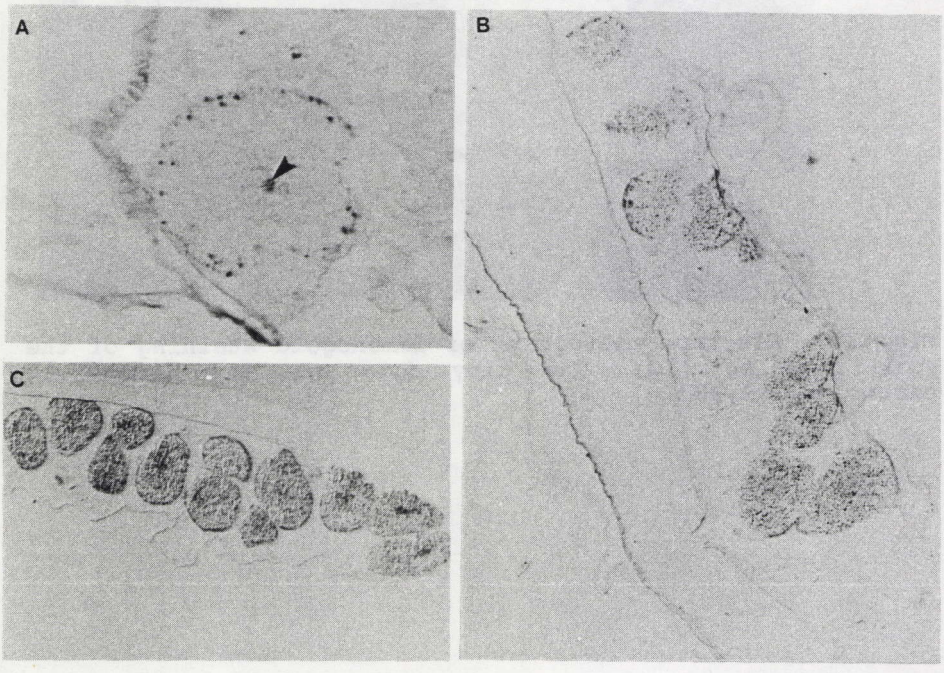


Fig. 3.4. Immunoperoxidase-treated frontal sections through the ovaries of females during a vitellogenic cycle. (A) Young oocyte with positive lipovitellin immunostaining in the yolk nucleus (see arrow-head). x1,000. Tiny dots in the periphery of the oocyte point to the precocious endocytosis of exogenous lipovitellin-like material. (B) Female mid-vitellogenesis (x248) and (C) female towards the end of vitellogenesis showing an increase of immunoreactive material. x180.

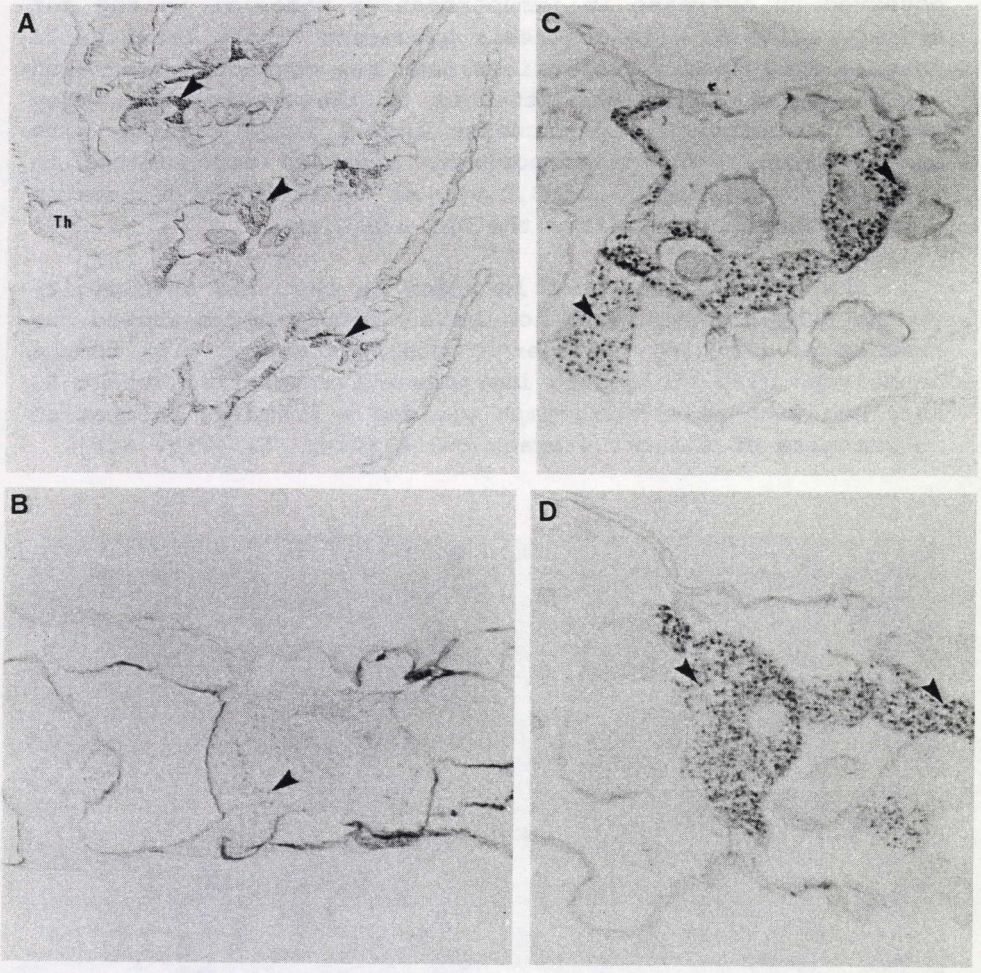


Fig. 3.5. Frontal sections through (A) the thoracopods (Th) of female adult Artemia during vitellogenesis showing lipovitellin immunopositive cells (see arrow-heads). x180. Detail of the thoracopods showing fat cells or fat storage cells containing lipovitellin immunoreactive granules (see arrow-heads) in a female at the onset of vitellogenesis (B) (x720), mid-vitellogenesis (C) (x720) and in a female at the end of yolk accumulation (D). x800.

In appendages of the thorax (Fig. 3.5), we also observed an increase in immunoreactive material in the fat storage cells as vitellogenesis proceeded. These large cells possess cytoplasmic protrusions that are connected with each other and are sometimes attached to the exoskeleton. They contain numerous lipid vacuoles and a large nucleus with many nucleoli. The immunopositive granules are located in the cytoplasm (Figs. 3.5/B,C,D), as verified by a hemalum staining immediately after the PAP-staining.

The control treatment in order to test the specificity of the PAP-procedure with non-immune rabbit serum showed the absence of staining. Antiserum totally absorbed with female haemolymph also yielded an immunonegative reaction, while AS absorbed with male haemolymph yielded a brownish coloration in granules of the fat storage cells after PAP (Fig. 3.6).

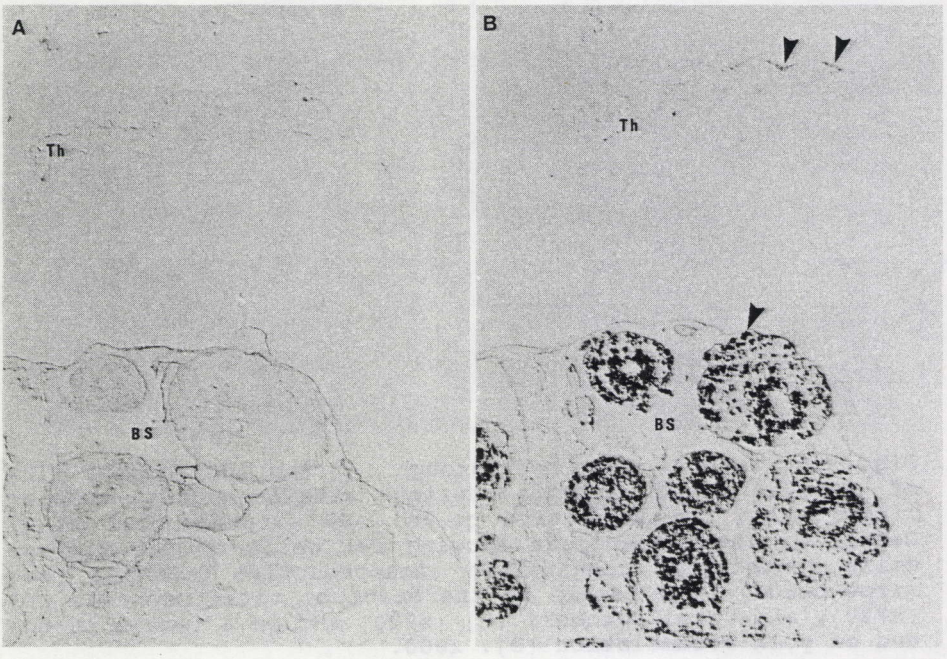


Fig. 3.6. Frontal sections through the broodsac region of a female in mid-vitellogenesis, after PAP-staining using LV-α1-antiserum absorbed with (A) female and (B) male haemolymph. Arrow-heads: immunopositive reaction in fat storage cells of the thoracopod (Th) of the posterior pair and in the encysted gastrulae of the broodsac (BS). x248.

Remark: Either female haemolymph proteins or a sample of lipovitellin extract could be coupled to the CNBr-activated Sepharose 4B beads. The total loss of positive reaction in the PAP-staining procedure was obtained with both.

When the peroxidase-antiperoxidase unlabeled antibody enzyme (PAP) staining method was performed on male tissue no trace of positive reaction with the lipovitellin antiserum could be observed.

Fluorography after SDS-PAGE of incubated thoracopods of vitellogenic females shows the synthesis of several proteins (Fig. 3.7, lane 2). Even in the incubation medium a detectable amount of newly synthesized proteins could be visualized (lane 3). After immunoprecipitation (see Appendix VII) with anti-LV- 1 (lane 1), we could clearly observe that lipovitellin-like substances are present in the homogenate of the thoracopods, probably originating in the fat storage cells.

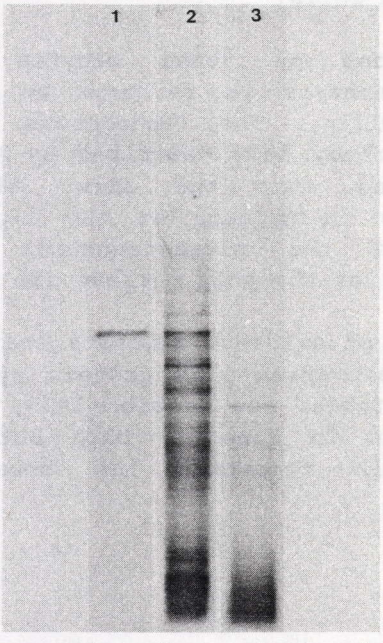


Fig. 3.7. Fluorography of an electrophoretic separation of polypeptides synthetized by female thoracopods in the presence of (^{35}S)-labelled methionine. Lane 1: polypeptides immunoprecipitated by anti-lipovitellin-α1 antibodies. Lane 2: homogenate of the incubated thoracopods. Lane 3: proteins present in the incubation medium.

4. Discussion.

The antiserum we obtained against lipovitellin-α1 was female-specific. Ultrastructurally, positive immunoreaction was visible only in the yolk globules. However, in immunoblotting the antiserum showed positive reaction against several lower molecular weight proteins. These proteins were probably degradation products and had some common antigenic determinants. de Chaffoy et al. (1980a,b) described the proteolytic breakdown of the largest apoprotein molecule of the lipovitellin complex during the embryonic development (see Chapter 2).

Fat storing cells were first described in Artemia by Leydig (1851) and Claus (1886). Bruntz (1905), who injected various substances in the body, called these cells 'phagocytotic nephrocytes'. Lochhead and Lochhead (1941) defined these cells as phagocytic storage cells taking into account the two major functions observed: phagocytosis of old blood cells and storage of fat and glycogen. We prefer the term fat storage cells.

The observations on young oocytes suggest that endogenous yolk synthesis is followed by endocytosis of exogenous lipovitellin. The endogenous synthesis of lipovitellin has already been described by Anteunis et al. (1964), but is now for the first time visualized immunocytochemically in Artemia at the light microscopic level. Studies on the ultrastructural level (using immunogold staining) of the yolk nucleus are in progress.

A growing body of evidence suggests that a combination of intra- and extra-ovarian yolk protein synthesis is not exceptional in Crustacea: see Zerbib (1979) and Zerbib and Mustel (1984). Even in annelids both autosynthetic and heterosynthetic yolk formation has been demonstrated (Eckelbarger, 1979).

In the present study, the demonstration of lipovitellin-containing cells, the micropinocytotic activity in the oocyte and the ability of thoracopods to release newly synthesized lipoproteins *in vitro*, also favour the dual mechanism of yolk protein synthesis. Our findings about the fat storage cells as being an extra-ovarian site of LV synthesis are comparable with other data from literature. Meusy et al. (1983) found a well developed RER in the adipocytes of vitellogenic females of *Orchestia gammarella* and demonstrated the presence of vitellogenin in the dense bodies of these cells. In female *Branchinecta packardi*, Gilchrist and Zagalsky (1983), isolated a lipoprotein, structurally related to lipovitellin, from connective tissue storage cells and which might be a precursor to oocyte lipovitellin. The electron microscopic studies of the storage cells suggested they were active in biosynthesis.

Our *in vitro* experiments demonstrate that thoracopods are able to synthesize lipovitellin-like proteins. Whether these are produced by the fat storage cells only, needs to be investigated further.
In the following chapters the endocrinology of vitellogenesis in crustaceans and *Artemia* will be discussed. More evidence for the site of lipovitellin synthesis and its possible control is given in Chapter 8.

CHAPTER 4: MOULTING, VITELLOGENESIS AND ECDYSTEROIDS.

1. Introduction.

Crustaceans, like other arthropods are surrounded by an exoskeleton that is regularly shed and replaced by a new one. Increase in volume has to be realised in the short period before the newly formed cuticle hardens. This happens through an active uptake of water and a passive stretching of the epidermal epithelium. The changing of the animals 'outfit' is regulated by moulting hormones (MH's) or ecdysteroids. These C_{27}-molecules are polyhydroxysterols with highly hydrophilic characteristics. Their concentrations can be measured by radioimmunoassay (RIA).

Female brine shrimps moult after every vitellogenic cycle. Male individuals also moult at regular intervals. To discover a relationship between moulting and the reproductive cycle, we first need to measure the titer and concentrations of ecdysteroids at various stages of vitellogenesis.

2. Moulting.

2.1. Moulting in Crustacea.

A new cuticle is secreted by the underlying epidermal layer and deposited under the old one. Hereby a great part of the old exoskeleton is reabsorbed. Between these two layers an exuvial space is formed. Frequently the term 'apolysis' is used for the removal of the epidermal cells from the old cuticle (Jenkin and Hinton, 1966; see also Fig. 4.1). Moulting or ecdysis means then the event of shedding the old cuticle or exuvium. To give the newly formed cuticle its rigidity, calcium carbonate is incorporated in the form of calcite. This mineralisation of the exoskeleton is a postecdysial event. The change of the epidermal cells and the formation of organules (such as mechano- and thermoreceptors, dermal glands) in the exocuticle of arthropods during an intermoult/moult cycle has been described by Gnatzy and Romer (1984).

A moulting cycle can be divided in several stages according to the progress of hardening of the tegumental skeleton. Also the changing of the matrix of the hairs (or

setae) can be taken into account. In this way, Drach (1939) made the following division: metecdysis or postmoult (stage A+B), intermoult (stage C)*, proecdysis or premoult (stage D) and ecdysis or moult or exuviation (stage E). Each stage can be subdivided according to particular morphological and physiological events, such as body weight, water content, calcification,... (Lockwood, 1968). Later on, the description of intermoult periods based on the morphogenesis of setae was generalized by Drach and Tchernigovtzeff (1967).

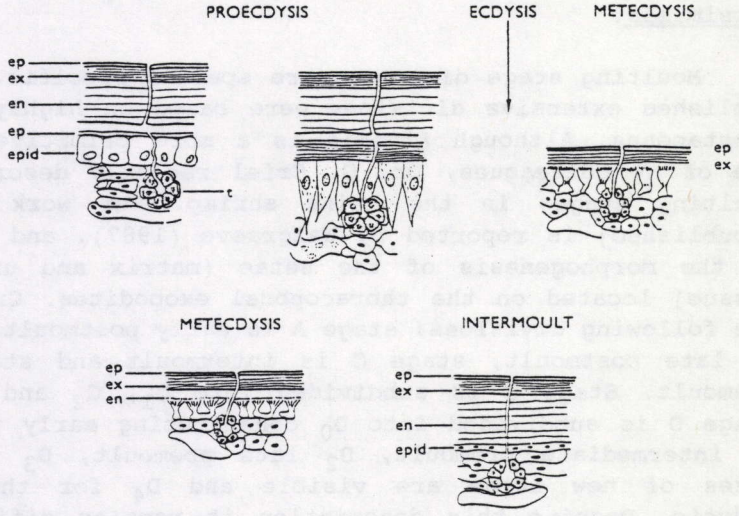


Fig. 4.1. Structure of the integument of the land crab, Gecarcinus lateralis, at each stage of the moult cycle. During early proecdysis the epidermal cells enlarge, separate from the old cuticle (apolysis), and secrete a new epicuticle. By late proecdysis the epidermal cells are enlarged still further and secretion of the new exocuticle has begun. After ecdysis, exocuticle secretion is complete and endocuticle production begins, to be completed during metecdysis. The epidermal cells then decrease in size and remain small during the intermoult period. Epicuticle (ep), exocuticle (ex), endocuticle (en), epidermal cells (epid), tegumental gland (t). (from Highnam and Hill, 1977)

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* In decapods intermoult can be of two types according to Knowles and Carlisle (1956).
Anecdysis is a long period of rest between the end of one metecdysis and the beginning of the next proecdysis. This is found in animals which moult seasonally.
Diecdysis is a short period during which a metecdysis passes imperceptibly into the succeeding proecdysis. This is found in animals which moult all the year round.

2.2. Moulting in Artemia.

The appearance of new appendages after moulting is a sure indication of the life stage for the larval or preadult animals. When the adult stage is reached this criterion can no longer be used. Observations on individual animals demonstrated that moulting does occur in adults at regular intervals as has been mentioned already by Bowen (1962) for the Utah race of Great Salt Lake and California race of San Francisco Bay. This moulting pattern has been described more in detail by Metalli and Ballardin (1972) in Artemia persimilis.

Moulting stage divisions are species specific. All the published extensive divisions were based on highly evolved crustaceans. Although Artemia is a more primitive animal, one of my colleagues, Dr. G. Criel recently described the moulting stages in the brine shrimp. Her work (as yet unpublished) is reported by Walgraeve (1987), and is based on the morphogenesis of the setae (matrix and underlying tissue) located on the thoracopodal exopodites. Criel used the following divisions: stage A is early postmoult, stage B is late postmoult, stage C is intermoult and stage D is premoult. Stage C is subdivided into C_1, C_2 and C_3, and stage D is subdivided into D_0 constituting early premoult, D_1 intermediate premoult, D_2 late premoult, D_3 when the bases of new setae are visible and D_4 for the actual ecdysis. Despite this description it remains difficult to use a microscopic procedure and to collect large numbers of animals for ecdysteroid measurements. Therefore we chose a moulting stage determination based on the external characteristics of the vitellogenic stages (see Table 1.1).

3. Control of moulting.

There are several reviews on control of moulting in crustaceans (Kleinholz, 1942; Knowles and Carlisle, 1956; Passano, 1960, 1961; Highnam and Hill (1977), Kleinholz and Keller, 1979; Spindler et al., 1980; Chang, 1985). From the available data it is clear that the sinus gland-X-organ complex* in the eyestalks controls the production of

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* The sinus gland is a neurohaemal organ located in the eyestalks which stores up and releases the neurohormones coming from the pericaryons of the Hanström organ (medulla terminalis ganglionaris-X-organ or MTGX, see Chapter 2).

ecdysone by the Y organ (see also Watson and Spaziani, 1985). There are inhibiting and stimulating factors and feedback systems (Mattson and Spaziani, 1986). The model proposed by these authors (Fig. 4.2.) clearly shows that the release of the peptide hormone MIH (Moult Inhibiting Hormone) from neurosecretory cells of the X-organ (XO)-sinus gland (SG) complex is stimulated by activated serotonergic eyestalk neurons (through the mediation of environmental stimuli, e.g. stress, Mattson and Spaziani, 1985). MIH in haemolymph binds to putative Y-organ cell surface receptors (R) resulting in activation of adenylate cyclase (AC) and generation of cAMP.

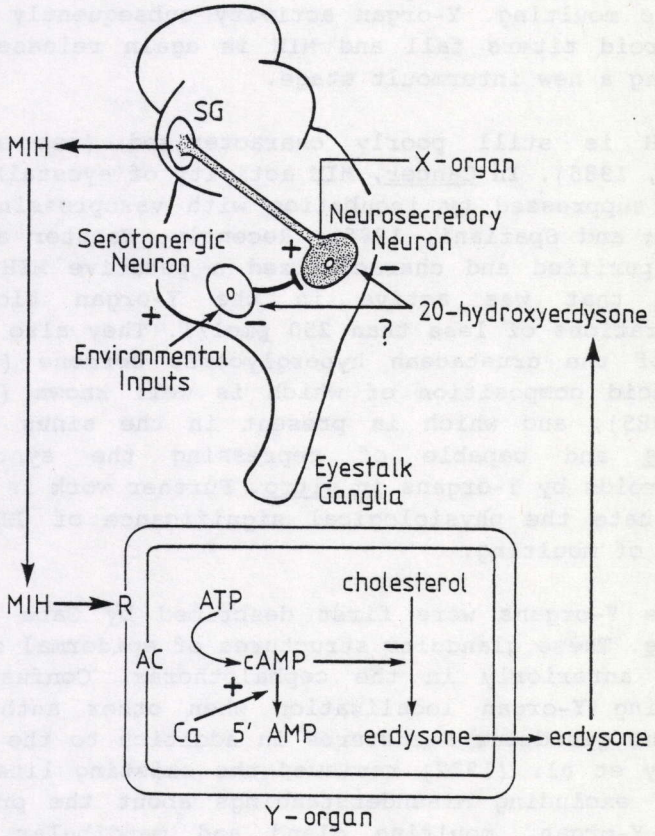


Fig. 4.2. Model of neuroendocrine regulatory interactions of the X-organ sinus gland Y-organ system. See text for further explanation. (from Mattson and Spaziani, 1986)

Ecdysone production from cholesterol is suppressed by cAMP and this effect is antagonized by calcium which activates a calcium-calmodulin-sensitive cAMP-phosphodiesterase. Calcium becomes available when it is reabsorbed from the old cuticle just before the start of new cuticulogenesis. Thus as long as MIH is released, the ecdysone titer remains low and the intermoult state is maintained. On the other hand when MIH release decreases (e.g. due to a transient increase in haemolymph ecdysteroid levels or to reduced peripheral neural input) ecdysone production is increased. Ecdysone is converted in peripheral tissues to 20-hydroxyecdysone which eventually exerts a negative feedback on the release of MIH from XO-SG cells, resulting in a very considerable rise of ecdysteroid titers prior to moulting. Y-organ activity subsequently declines, ecdysteroid titers fall and MIH is again released thereby instating a new intermoult stage.

MIH is still poorly characterised (see review by Skinner, 1985). In Cancer, MIH activity of eyestalk extracts can be suppressed by incubation with vasopressin antisera (Mattson and Spaziani, 1985). Recently, Webster and Keller (1986) purified and characterized a putative MIH, a novel peptide that was active in the Y-organ bioassay at concentrations of less than 250 pmol/l. They also found two forms of the crustacean hyperglycemic hormone (CHH), the amino acid composition of which is well known (Keller et al., 1985), and which is present in the sinus glands of Carcinus and capable of repressing the synthesis of ecdysteroids by Y-organs in vitro. Further work is necessary to evaluate the physiological significance of CHH for the control of moulting.

The Y-organs were first described by Gabe (1953) in Carcinus. These glandular structures of epidermal origin are located anteriorly in the cephalothorax. Confusion arose concerning Y-organ localization when other authors found different glandular structures in addition to the Y-organs. Sochasky et al. (1972) reviewed the existing literature in view of excluding misunderstandings about the presence of Gabe's Y-organ, moulting gland and mandibular organ in decapod Crustacea. Now, all malacostracan Y-organs are considered to be maxillary and to be homologous with the 'Y-organ of Carcinus'. The term Y-organ is now synonymous with moulting gland in the Crustacea (Sochasky and Aiken, 1974).

In certain crustaceans, Y-organs are not the only sources of moulting hormones (Spindler et al., 1980). A 'cephalic gland' in Orconectes and in Astacus secretes ecdysteroids in vitro (Gersch, 1979; Gersch and Birkenbeil, 1979; Gersch et al., 1979), and the ovary is the site of active ecdysteroid synthesis in Carcinus (Lachaise and Hoffmann, 1977).

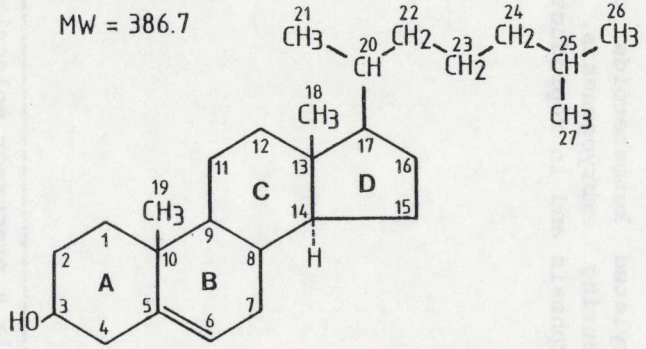
4. Ecdysteroids.

4.1. Structure of moulting hormones.

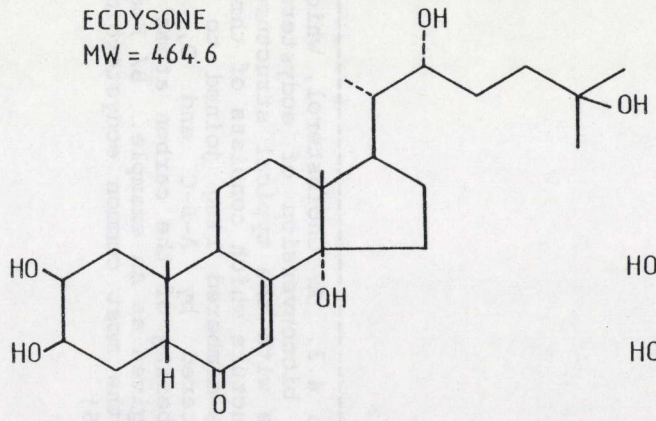
Ecdysteroids are polyhydroxylated ketosteroids that play major regulatory roles during embryogenesis, in postembryonic development, metamorphosis and in reproduction of arthropods.

Fig. 4.3. A/ Cholesterol, which is a precursor molecule in the bioconversion of ecdysteroids and steroids, is shown here with the typical structure of a steroid nucleus (ring structure which consists of three six-membered rings and one five-membered ring joined to each other by common sides lettered by A-B-C and D, respectively). The typical numbering of the carbon atoms in this C_{27}-steroid molecule is given as an example. B/ Ecdysone and C/ 20-OH-ecdysone as the most common ecdysteroids. (after Schulster et al., 1976)

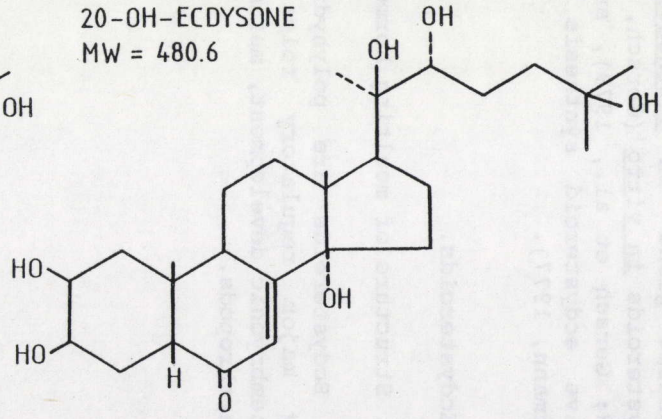
CHOLESTEROL
MW = 386.7



ECDYSONE
MW = 464.6



20-OH-ECDYSONE
MW = 480.6



These molecules differ from the known vertebrate-type steroids by the presence of a side-chain. Ecdysone (α-ecdysone or 2ß,3ß,14α,22R,25-pentahydroxy-5ß-cholest-7-en-6-one*) and 20-hydroxy-ecdysone (ß-ecdysone, ecdysterone, crustecdysone or 2ß,3ß,14α,20R,22R,25-hexahydroxy-5ß-cholest-7-en-6-one) are the most predominant arthropod moulting hormones or the so-called ecdysteroids (Goodwin et al., 1978). The structural formulae of these three molecules are depicted in Fig. 4.3 together with the numbering and nomenclature of such molecules.

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* From Schulster, Burstein and Cooke, 1976:

*1. An atom or group attached to a ring is termed alpha (α) if it lies below the plane of the paper (=plane of the rings) or beta (ß) if it lies above the plane of the paper. In formulae, bonds to atoms or groups lying below the plane of the paper are shown as broken (---) lines and bonds to atoms or groups lying above the plane of the paper are shown as solid lines (-).

*2. The Ingold system or Sequence Rule procedure for nomenclature of substituents in the C_{17} side-chain relates to the three-dimensional position of each substituent on any particular carbon atom. The symbols R for right (rectus) and S for left (sinister) are used. When there is no substituent on C_{17} or C_{21}, 20α- is equivalent to (20S)-, and 20ß- to (20R)-. These relationships may be reversed when additional substituents are present.

*3. Unless implied or stated to the contrary, use of a steroid name implies that atoms or groups attached at the ring-junction position 8, 9, 10, 13 and 14 are oriented as (8ß, 9α, 10ß, 13ß, 14α) and a carbon chain attached at position 17 is assumed to be ß-oriented.

4.2. Presence of ecdysteroids in Crustacea.

Ecdysone was first isolated by Butenandt and Karlson (1954) from the pupae of the silkworm Bombyx mori. Y-organ ablation experiments of Echalier (1954) in Carcinus maenas demonstrated the presence of an endocrine organ that would probably be the source of a moulting hormone. The fact that crustacean extracts showed positive results in the Calliphora bioassay suggested that moulting in crustaceans would prove to be regulated by ecdysone and/or 20-OH-ecdysone (Carlisle, 1965). Firm evidence showed up in 1966 when Horn et al. demonstrated moulting hormones of insects and crustaceans to be identical.

During recent decades a lot of ecdysteroid concentrations have been measured in several species. 20-hydroxyecdysone has been found to be the active form of arthropod moulting hormone, both in extracts of whole animals and in the haemolymph (Spindler et al., 1980). Ecdysone has been identified as the secretory product of the crustacean moulting gland, the Y-organ (Chang and O'Connor, 1977). It is converted to 20-OH-ecdysone in several organs by a C_{20}-hydroxylase, both in vivo (King and Siddall, 1969) and in vitro (Chang et al., 1976; Lachaise and Feyereisen, 1976). There are also a number of polar and apolar metabolites present in crustaceans; some of them are distributed in a tissue-specific pattern (Gorell et al., 1972a,b; Lachaise et al., 1976; Kuppert et al., 1978), but their structure and functions remain unknown.

So far, only a few authors (outside our research team) have reported the presence of ecdysteroids in Artemia. A German research team demonstrated ecdysone and 20-OH-ecdysone with RIA, GC and HPLC in Artemia salina (the unpublished results of Spindler and Radi reported in Spindler et al. (1980)). According to this group, the presence of free and conjugated ecdysteroids together with ponasterone A in the early developmental stages of this species might be an indication for a probable involvement of ecdysteroids in the secretion of embryonic envelopes and in embryonic moulting (Spindler et al., 1984). Funke and Spindler demonstrated in 1987 the coincidence of a high level of free ecdysteroids during emergence and hatching, with the high chitinolytic activity at these stages. This

would indicate that the investigated chitinolytic enzymes may act as hatching enzymes during these developmental stages*.

4.3. Ecdysteroids and moulting.

During moulting a well-defined pattern of ecdysteroid concentrations can be shown. When the MIH level decreases - through internal and external stimuli (e.g. by changes in photoperiod, Quackenbush and Herrnkind, 1983a) ecdysone production by the Y-organs is increased (Chang and O'Connor, 1978). The circulating ecdysone is metabolized to hydroxylated derivatives in the target tissues. Generally, ecdysteroid concentrations stay low in the intermoult stage. They slowly increase during early premoult (D_0,D_1) followed by a steep rise at D_2. After this maximum a sharp decline occurs just prior to ecdysis. A minimum is reached at stages A+B (postmoult).

This pattern of circulating ecdysteroids is typical during a normal moulting cycle. When moulting is induced by eyestalk ablation, similar patterns of circulating haemolymph ecdysteroids are present during proecdysis (Hopkins, 1983).

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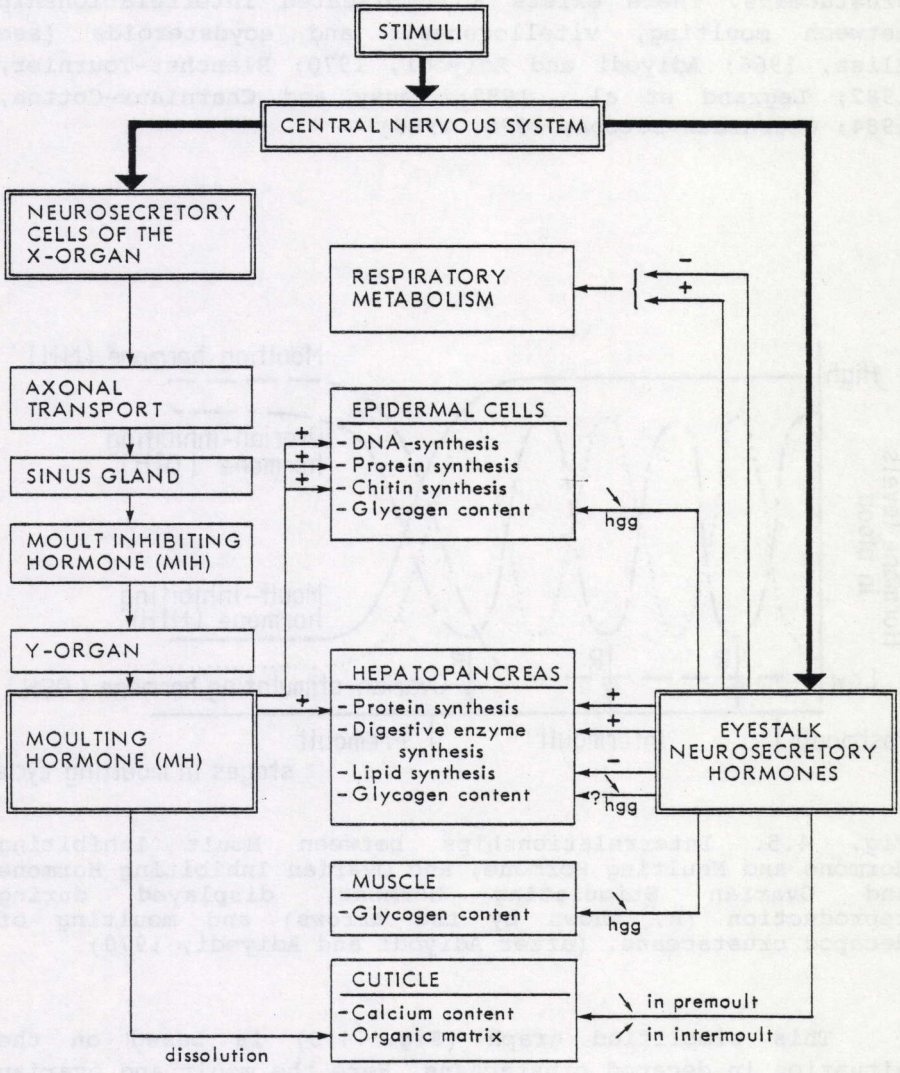
* Chitin-degrading enzymes play an important role in the moulting cycle of arthropods. Together with proteolytic enzymes they are involved in the apolysis of the exoskeleton. After reabsorption, the end products of hydrolysis can be reused for the formation of a new exoskeleton. Since there is a positive correlation between the activity of chitinolytic enzymes and the ecdysteroids (Spindler-Barth et al., 1986), emergence and hatching and also the enzymatic activities may be under hormonal control (see Funke and Spindler, 1987).

In endocrinological research on Crustacea, many attempts have been made to induce moulting or correlated morphogenetic changes by administration of ecdysone or 20-OH-ecdysone as exogenous ecdysteroids. Whether these experiments resulted in complete ecdyses or not, depended on moulting stage, on concentrations and the number of applications (Spindler et al., 1980). Besides the numerous effects of ecdysteroids on moult induction, also several specific biochemical events related to the moulting process are induced by 20-OH-ecdysone (see Fig. 4.4).

The effect in all these reactions is different according to the moulting stage of the animals and the endogenous concentration of ecdysteroids.

Fig. 4.4. Overview of the actions of moulting hormone and the eyestalk neurosecretory hormones during the crustacean moult cycle. Moulting in crustaceans is controlled by a two-step hormonal sequence (left side of the figure). The moulting hormone is secreted by an epithelial endocrine gland (Y-organ) the activity of which is inhibited by a neurosecretory hormone. This Moult Inhibiting Hormone is produced in the X-organ and released from the sinus gland. MH is produced when secretion of the MIH ceases. (after Highnam and Hill, 1977)


STIMULI
CENTRAL NERVOUS SYSTEM
NEUROSECRETORY CELLS OF THE X-ORGAN
AXONAL TRANSPORT
SINUS GLAND
MOULT INHIBITING HORMONE (MIH)
Y-ORGAN
MOULTING HORMONE (MH)
RESPIRATORY METABOLISM
EPIDERMAL CELLS
- DNA-synthesis
- Protein synthesis
- Chitin synthesis
- Glycogen content
hgg
HEPATOPANCREAS
- Protein synthesis
- Digestive enzyme synthesis
- Lipid synthesis
- Glycogen content
?hgg
EYESTALK NEUROSECRETORY HORMONES
MUSCLE
- Glycogen content
hgg
CUTICLE
- Calcium content
- Organic matrix
in premoult
in intermoult
dissolution

4.4. Ecdysteroids, moulting and vitellogenesis.

The overwhelming majority of studies undertaken so far deal with effects of ecdysteroids on vitellogenesis and reproductive behaviour, specially in the higher evolved crustaceans. There exists a complicated interrelationship between moulting, vitellogenesis and ecydsteroids (see Bliss, 1966; Adiyodi and Adiyodi, 1970; Blanchet-Tournier, 1982; Legrand et al., 1982; Meusy and Charniaux-Cotton, 1984; Charniaux-Cotton, 1975, 1985).

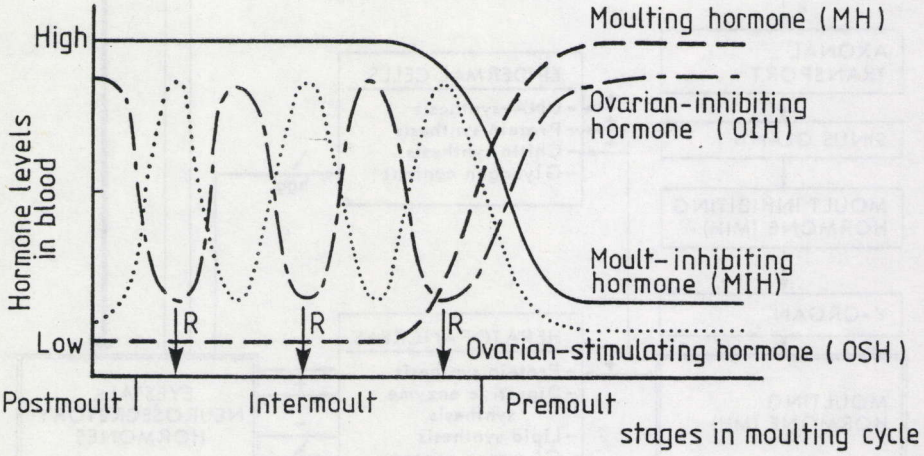


Fig. 4.5. Interrelationships between Moult Inhibiting Hormone and Moulting Hormone, and Ovarian Inhibiting Hormone and Ovarian Stimulating Hormone, displayed during reproduction (R, shown by the arrows) and moulting of decapod crustaceans. (after Adiyodi and Adiyodi, 1970)

This simplified graph (Fig. 4.5) is based on the situation in decapod crustaceans. Here the moult and ovarian inhibiting hormones act synergistically during postmoult stages, but antagonistically during intermoult stages when reproductive development occurs. Sometimes there is more than one vitellogenic cycle during the intermoult phase. The moulting pattern thus imposes lifelong, seasonal or annual reproductive cycles according to the species.

A strict correlation between ecdysteroids and vitellogenesis does not exist in Crustacea and species dependent differences are a general rule. For example, a terminal ecdysis is present in the spider crab with subsequent cycles of vitellogenesis and embryonic development. However, in Orchestia (Amphipoda) the ovarian activity is cyclic and closely synchronized with the moulting cycle: vitellogenesis occurs during an intermoult and ecdysis is followed by egg laying.

In many malacostracans spawning or oviposition is obligatorily preceded by a moult. Especially in those animals that carry their brood in a broodchamber on the ventral side of the abdomen (e.g. Macrobrachium rosenbergii) or where the eggs are attached to the abdominal appendages (e.g. Astacus sp.). In the former animal, successful mating can only take place between hard-shelled males and ripe females which have just completed their pre-mating moult and are soft-shelled (New and Singholka, 1982).

Moulting can be induced by eyestalk ablation or administration of ecdysteroids. This means that moulting somehow requires a high level of ecdysteroid concentrations. Vitellogenesis however, appears to be independent of high levels of moulting hormone. The drop of ecdysteroid titer would permit cell movements in the course of folliculogenesis to occur. After folliculogenesis, the follicle cells become endocrinologically active: they trigger synthesis and the release of a large amount of female-specific protein (vitellogenin) which enters the oocyte (Charniaux-Cotton, 1975). This phenomenon is only true in species where secondary vitellogenesis* and moult cycle are synchronous (e.g. O. gammarella and some isopods). The low ecdysteroid titer at the beginning of the intermoult stage is presumably responsible for the onset of secondary vitellogenesis (Charniaux-Cotton, 1985).

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* The term secondary vitellogenesis covers the massive entrance of vitellogenin in the oocytes through micropinocytosis, while primary vitellogenesis deals with the endogenous synthesis of yolk proteins (see Chapter 2).

In Orchestia gammarella and in Isopoda the presence of MH is necessary both for vitellogenin synthesis and for secondary vitellogenesis (Blanchet-Tournier, 1982). In this species, also the simultaneous presence of both ovarian and moult hormone is necessary for vitellogenin synthesis. The secondary follicle cells are probably the source of this vitellogenin-stimulating ovarian hormone (VSOH) (Charniaux-Cotton, 1985).

CHAPTER 5: MOULTING HORMONE CONCENTRATIONS IN CYSTS, DURING EARLY DEVELOPMENT AND IN ADULT BRINE SHRIMP ARTEMIA SP..

1. Identification and quantification of ecdysteroids.

Considerable progress has been made in the detection of moulting hormones and moulting activity. It started with the development of a bioassay system for MH based on sclerotization of the dipteran puparium and stemmed from the work of Fraenkel (1935). The so-called Calliphora-bioassay depends on the induction of sclerotization in the abdominal cuticle of a dipteran larva when the posterior section of the body is isolated by ligation from the brain-ring gland complex, and thus is deprived from the influence of ecdysone secretion (Thomson, 1974).

New analytical methods became involved in the search for ecdysteroids in biological extracts (see Morgan and Wilson, 1980; Hoffmann and Hetru, 1983). Spectroscopic methods such as ultraviolet (UV) absorption, infrared (IR) absorption, nuclear magnetic resonance spectroscopy (NMR), mass spectrometry (MS) and fluorescence were useful in the determination of the molecular structure of ecdysteroids. Chromatographic techniques such as thin layer chromatography (TLC), high performance liquid chromatography (HPLC) and gas chromatography (GC) were mostly used for examination of ecdysteroids in biological material. Radioimmunoassay (RIA) is however the most widely used method for ecdysteroid determination (Hirn and Delaage, 1980).

The basic principle of this method is the competition reaction between radiolabeled antigen (Ag^x) and the unknown Ag (present in a biological extract) for the specific antibody (Ab). When there is more unlabeled Ag, less Ag^x can be bound and the final concentration of the (Ag^xAb) complex decreases. In fact, in this assay the immunological and not the biological activity is measured. Also some standard solutions are incubated with a known amount of Ab and Ag^x. After equilibration free and bound antigens are separated and radioactivity is measured. Dose-response curves are established based on the percentages of binding (on Y-axis) and the respective standard concentration (on X-axis). From this standard curve the concentration of the unknown antigen

can be read directly on the X-axis that corresponds with the percentage of binding of the sample.

RIA has become an interesting analytical tool for the measurement of circulating ecdysteroids in individual crustaceans. Also the ecdysteroids secreted by tissues (such as the moulting gland) in vitro can be identified (O'Connor, 1985).

Our results of measurements of ecdysteroid concentrations in Artemia sp. are obtained by RIA, after the free and bound ligands are separated by a double antibody precipitation method (Walgraeve et al., 1986; Walgraeve, 1987). In this method gammaglobulins of the first AS function as antigens for the binding with the second antibody. More specifically in this case: first antibodies, raised against an ecdysteroid in rabbits, are precipitated with Goat-anti-rabbit-gamma-globulins (GARGG).

2. Extraction of ecdysteroids from biological material.

The initial step in the procedure of MH determination consists certainly in answering the question 'How to get all the ecdysteroids out of my material'? In 1981, I already described the extraction procedure of ecdysteroids from whole adult flies. However, the nature and volume of solvent depend on the properties of the biological sample. According to Morgan and Poole (1976) mostly acetonitrile, methanol, aqueous ethanol (96 percent) and methanol:aceton (1:1, v/v) are used as extraction solvents. We always homogenized our Artemia samples in methanol:acetone. Proteins are precipitated but the crude extract still contains many impurities such as lipids, carotenes and polar products that often interfere in immunological and/or chromatographic assays. To get a selective purification we then used solvent:solvent partitioning. We tried different solvent:solvent systems taking into account that the polar ecdysteroids normally would be present in the more polar phase:

1) Benzene:water resulted in a good separation of the two layers but benzene is highly toxic.
2) Chloroform:water often resulted in formation of a gel.
3) Dichloromethane:water resulted also in two clearly separate layers but the organic phase stayed underneath and this caused problems in collecting the aqueous phase.
4) Butanol:water also resulted in a nice bilayer but the evaporation of butanol proved difficult.
5) Diethylether:water (6:1, v/v) is a system also used for the extraction of steroids. We got a stable bilayer and the phases could easily be separated by freezing the glass tubes. The organic phase could then be discarded. A recovery test after addition of radiolabeled ecdysteroids revealed that about 10 percent of the initial radioacitvity was present in the organic phase.
6) n-Hexane:water (6:1, v/v) showed a clear dividing line. After freezing, the two phases could be separated in the same way as diethylether:water. However, ecdysteroids are recovered in the aqueous phase for 99.9 percent. The aqueous phase was then further purified by column-chromatography. We used short columns. Mostly they were filled with silica*. In one run it was easy to separate the free ecdysteroids, from apolar and polar conjugates. Finally, we chose for Sep-pak C_{18} cartridges (Waters Associates) as they were easy to handle and extremely useful for the isolation of ecdysteroids from crustacean tissues (Watson and Spaziani, 1982). In our study, the recovery of ecdysteroids obtained with this type of column was 98 percent.

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* Short columns were home-made starting from Pasteur pipettes (long type). These were first plugged with a small ball of glass wool or a tiny glass sphere and then the solid phase e.g. Sephadex LH-20 (Pharmacia) was placed on top in order to purify an ecdysteroid extract of Artemia cysts. A 6 cm column was thus made and afterwards eluted with benzene:dichloromethane:methanol (60:35:5, v/v). After application of the sample to the column an orange band (due to the carotenoids present in the extract) first eluted from the column. Then methanol (100 percent) was used as solvent. Ecdysteroids now passed out of the column. The recovery of an external radiolabeled ecdysteroid standard was 82.1 percent. The only disadvantage was that the elution time for ecdysteroids proved rather long (in the range of 20 min).

We successfully used the mixture n-hexane:water (4:1, v/v and even 1:1, v/v) (Van Beek et al., 1986; Van Beek et al., 1987). Finally we preferred the system n-hexane:water (4:1, v/v) which also gave good results in the extraction of steroids (Van Beek and De Loof, 1988). This procedure enabled us to collect the aqueous phase for the ecdysteroid analysis and the organic phase for the measurement of steroid concentrations (see Appendix II).

3. Preparation of an anti-ecdysteroid antibody.

Ecdysteroids are small molecules ('haptens') and they need to be coupled chemically to a carrier protein in order to be rendered immunogenic (Hirn and Delaage, 1980). The procedures followed for preparing a specific antiserum against ecdysteroids can be found elsewhere (Briers, 1981 and Walgraeve, 1987).

Since 20-OH-ecdysone is the most active MH, it would be best to obtain an antiserum against this molecule. We tried to raise an AS more specific for 20-OH-ecdysone than for ecdysone. We followed the method of Porcheron et al. (1976) for the derivatizing of the ecdysteroid molecule and the coupling on bovine serum albumin (BSA) according to Skowski and Fisher (1972). However, the titer was too low to be suitable for RIA.

In our preliminary experiments we used an AS prepared by Dr. T. Briers and which was 10 times more specific for 20-OH-ecdysone than for ecdysone. Later on, we used an AS which was a gift from Dr. J. Koolman (Marburg, FRG). It was more specific for ecdysone. Specificity of the latter was characterized in Walgraeve et al. (1986) (see Appendix VIII).

4. 20-OH-ecdysone as tracer.

Problems were encountered when the tritiated 20-OH-ecdysone tracer was no longer commercially available. The production of ^{3}H-20-OH-ecdysone was only carried out in some research laboratories. We tried it ourselves following the method of Feyereisen et al. (1976) who described the Malpighian tubules as a primary site of ecdysone-20-hydroxylation in Locusta migratoria migratorioides. We couldn't complete this work as it was too time consuming.

Meanwhile we used doubly tritium labeled ecdysone as tracer in our RIA. It was obtained from New England Nuclear (U.S.A.).

5. Separation of free and bound ecdysteroids and scintillation counting.

Although the fraction of unbound label can be absorbed by dextran-coated charcoal, a method which is widely used for other steroids (see Chapter 6), we preferred the method of precipitating the immuno-complexes. At first we tried to precipitate the complexes with saturated ammoniumsulphate in the presence of immunoglobulins [(2 percent BSA in PBS (=Phosphate buffered saline, see Appendix II)], but the use of polyethylene glycol (PEG 6000 from UCB, 25 percent in PBS) according to a modified procedure of Desbuquois and Aurbach (1971) proved to be the more efficient. PEG withdraws water from the complex and the bound ecdysteroids precipitate. Free ecdysteroids in the supernatants are discarded.

The PEG-precipitate is dissolved in 100 µl 0.5 N NaOH. Distilled water (200 µl) and scintillation cocktail (2.5 ml Rialuma PEG from Lumac, The Netherlands) are added and radioactivity is counted in a liquid scintillation spectrophotometer (Beckman LS 9000) with automatic quench correction mode.

Some of our results of ecdysteroid concentrations measured during a vitellogenic cycle were described by Walgraeve et al. (1986). The RIA method for separation of bound and free antigens in this paper was based on the principle of double antibody precipitation. The best results were obtained with the pre-precipitated second antibody procedure (see Appendix VIII).

6. Concentrations in cysts and during early development.

In aquaculture, decapsulation is performed to facilitate emergence and to get a better hatching ratio. Decapsulated cysts have lost their chorion layer or tertiary envelope but still possess a permeability barrier (see Funke and Spindler, 1987). The idea that emergence and hatching may be under hormonal control and the fact that physiological amounts of ecdysteroids directly influence the cuticle, inspired us to look into ecdysteroid concentrations of decapsulated cysts.

Results were obtained on extracts of cyst material and early developmental stages of Artemia sp. Decapsulation of the cysts was carried out for 10-15 min in the presence of sodium hypochlorite solution (2 g dry cysts per gram active product*) according to the method of Bruggeman et al. (1979). Pulverizing the dry cysts in a mortar with a pestle is apparently the best homogenization method.

Extracts were purified on Sephadex columns (see note in 2.). RIA was carried out according to the procedure already described (Van Beek, 1981). The antiserum was more specific for 20-OH-edysone and tritiated 20-OH-ecdysone was used as a tracer.

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* The concentration (in g/l) of the active product (Y) of a fresh NaOCl solution can be calculated from the equation: $Y = 3000 X - 4003$; with X = the refractive index which can be measured with a refractometer. For a complete decapsulation 2 g of dry cysts per gram active product are used at a density of 15 g dry cysts per 200 ml decapsulation solution (= seawater and NaOCl). The process can be optimized by adding 2.5 ml of a 40 percent NaOH solution per 100 ml decapsulation solution (from Bruggeman et al., 1979). Afterwards cysts are washed for 30 sec in 0.1 N HCl and thoroughly rinsed under tapwater.

Results of 1 g untreated cysts and 1 g decapsulated cysts from different strains were compared (see Table 5.1). Ecdysteroid concentrations in extracts from untreated cysts varied between 10 and 150 ng/g dry weight. The lowest value was found in the strain from Chaplin Lake and peak values were measured in the San Francisco Bay strain. It was difficult to compare these results with the those obtained from decapsulated cysts. No relationship could be demonstrated while data were so incoherent. Vanhaecke and Sorgeloos (1980) already had described the difference in volume and chorion volume for cysts of different Artemia strains. We couldn't make a comparison between these volume differences and the ecdysteroid concentrations in extracts from decapsulated and untreated cysts.

Table 5.1. Total ecdysteroid activity, expressed in ng ecdysteroid activity per gram dry cysts, in A/ untreated cysts and B/ decapsulated cysts of different Artemia populations.

	A	B
1. San Francisco Bay Brand (1976)	107.98	42.44
2. San Francisco Bay Brand (243)	130.51	57.31
3. Macau (791030-1980)	14.84	100.17
4. Macau (1981)	34.45	53.95
5. Great Salt Lake (1977)	34.51	63.15
6. Great Salt Lake (217-1977)	46.26	26.49
7. Tientsin (1980)	11.22	124.10
8. Tientsin (242)	51.67	37.47
9. Shark Bay (1977)	51.67	229.59
10. Buones Aires (223-1980)	23.54	303.54
11. Margherita di Savoia (214-1977)	12.01	72.57
12. Chaplin Lake (241-1980)	10.75	28.45
13. Lavalduc (256-1978)	31.75	184.42
14. Lavalduc (1980)	63.53	295.93
15. Larnaca (305)	50.67	15.38

We also measured ecdysteroid concentrations in the course of the decapsulation procedure (see Fig. 5.1). From our previous orientating data we concluded that decapsulation showed no clear influence on ecdysteroid concentrations. However, when we followed cyst development more accurately, we observed a slight increase in concentration at 5-6 min. This ecdysteroid peak could coincide with the first moulting which takes place within the cyst. Although these observations are not complete, we think that these data are promising for future research.

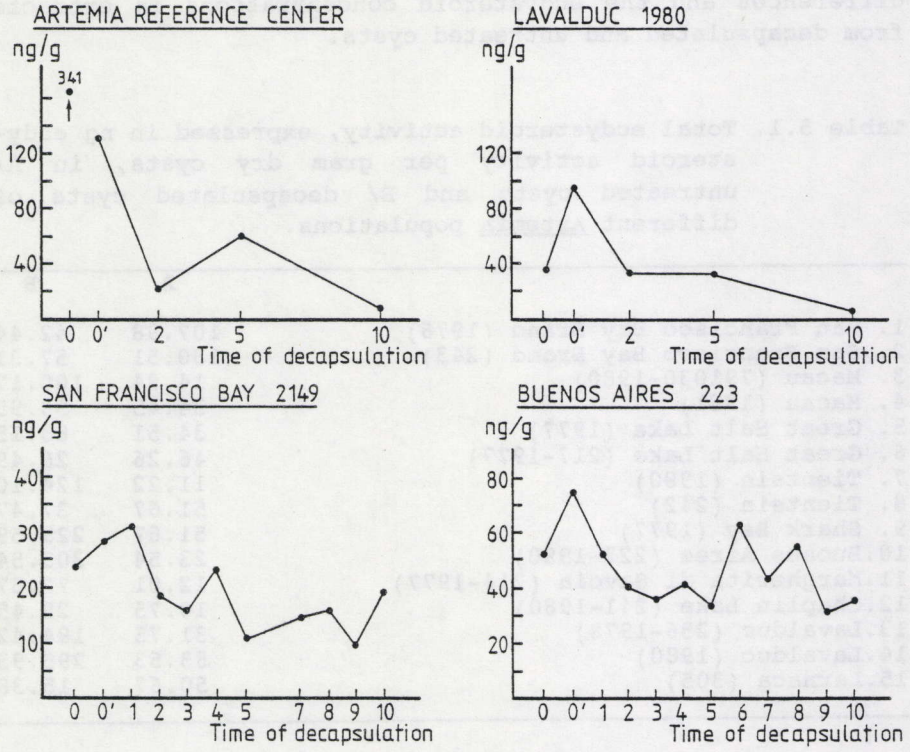


Fig. 5.1. Total ecdysteroid activity expressed in ng 20-OH-ecdysone/g dry weight for four different Artemia strains during decapsulation: A/ Macao, B/ Lavalduc (1980), C/ San Francisco Bay (2149) and Buenos Aires (223). Time is expressed in min and o=untreated condition, o'=after 2 hr hydratation in seawater.

Different stages in the lifecycle of animals from the Great Salt Lake strain were also used for the measurement of ecdysteroids. These stages were: 15.5 hr, 23.5 hr, 31 hr, 48 hr, 4, 11, 14, 18 and 22 days after contact with seawater (see Fig. 5.2).

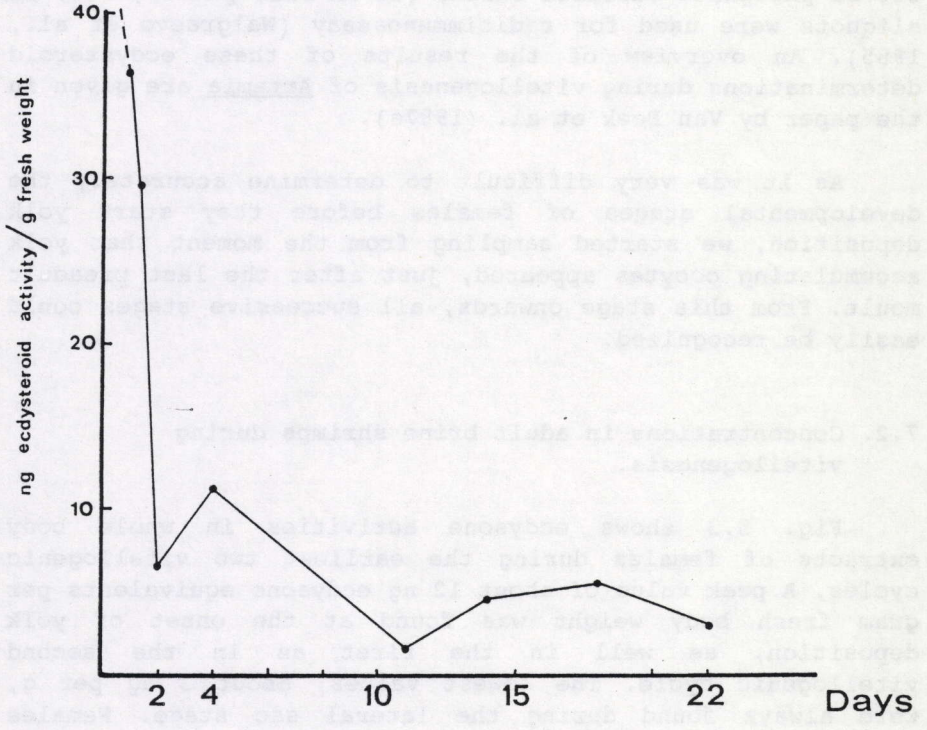


Fig. 5.2. Concentration of ecdysteroids expressed in ng 20-OH-ecdysone equivalents/g fresh weight during the lifecycle of brine shrimps from the Great Salt Lake strain.

In this preliminary study it could not be demonstrated that each moult is proceeded or accompanied by an ecdysteroid peak. Despite the fact that the stages we used were not timed with sufficient precision, we clearly observe that the ecdysteroid pattern varies during larval development. Collection of very precisely staged animals might reveal pronounced peaks.

7. Ecdysteroid measurement during vitellogenesis.

7.1. Estimation of ecdysteroid levels.

Further RIA measurements were carried out in collaboration with our colleague Dr. H. Walgraeve. Total body extracts (and haemolymph extracts) were dissolved in 500 µl phosphate buffered saline (10 mM PBS, pH=7.4). 100 µl aliquots were used for radioimmunoassay (Walgraeve et al., 1985). An overview of the results of these ecdysteroid determinations during vitellogenesis of Artemia are given in the paper by Van Beek et al. (1987a).

As it was very difficult to determine accurately the developmental stages of females before they start yolk deposition, we started sampling from the moment that yolk accumulating oocytes appeared, just after the last preadult moult. From this stage onwards, all successive stages could easily be recognized.

7.2. Concentrations in adult brine shrimps during vitellogenesis.

Fig. 5.3 shows ecdysone activities in whole body extracts of females during the earliest two vitellogenic cycles. A peak value of about 12 ng ecdysone equivalents per gram fresh body weight was found at the onset of yolk deposition, as well in the first as in the second vitellogenic cycle. The lowest values, about 3 ng per g, were always found during the lateral sac stage. Females moulted just prior to this stage.

A similar pattern was observed in data obtained from haemolymph extracts: the peak value (13.1 ng/ml) was measured at the period when the most intensive yolk accumulation by the oocytes was taking place.

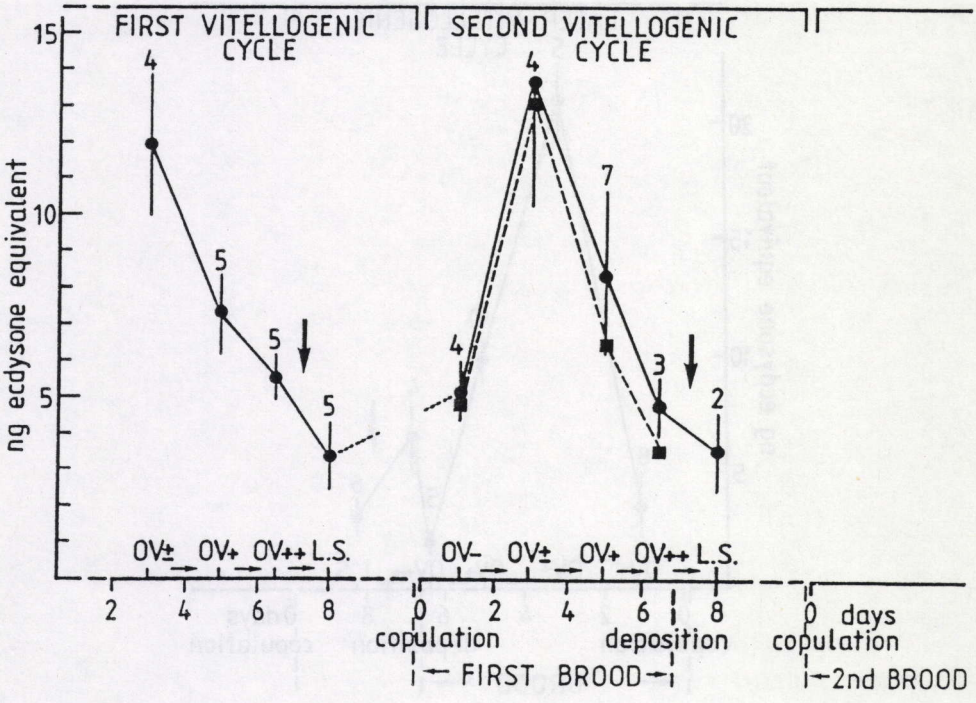


Fig. 5.3. Ecdysteroid activity in adult females of Artemia (Great Salt Lake strain) during the earliest stages of the vitellogenic cycle. Concentrations are expressed in ng ecdysone equivalents per g body weight (●—●, with S.D.) and per ml haemolymph (■--■). Arrows (/) indicate moults, and data correspond to sets of 20 individuals. An approximate time scale is given.

In subsequent vitellogenic cycles (Fig. 5.4) the same general pattern was found for moulting hormone levels (peak value of 21 ng/g during vitellogenesis, lowest value of 1.8 ng/g when oocytes were loaded with lipovitellin). However, a minor peak (6.6 ng/g) was observed after egg-deposition and before moulting. The value of 3.1 ng/g at the lateral sac stage could be compared with the data for the corresponding stage in the first two cycles. No haemolymph samples were collected here.

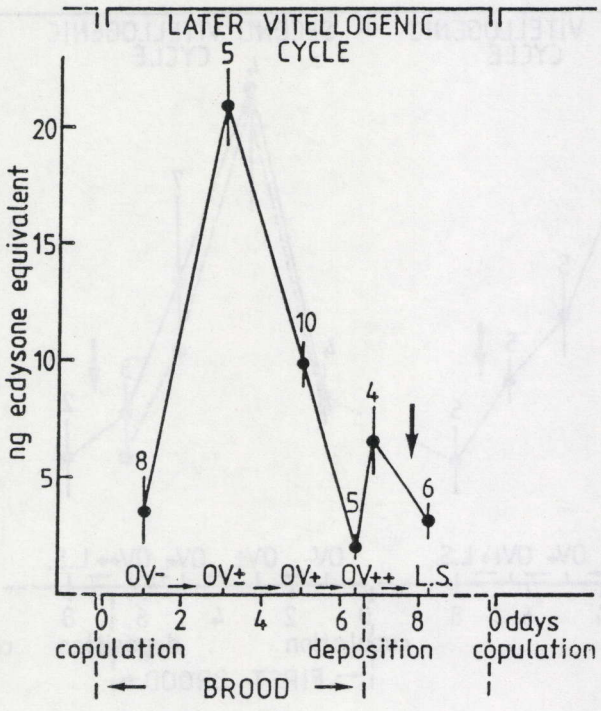


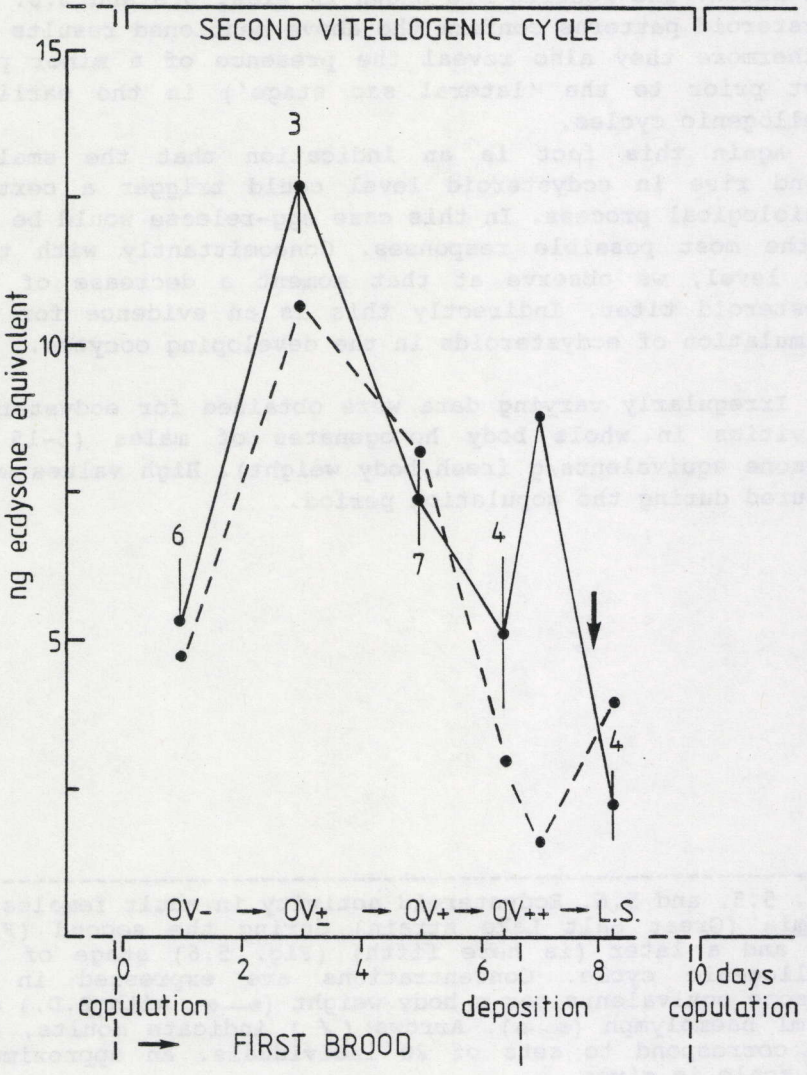
Fig. 5.4. Ecdysteroid activity in adult females of Artemia (Great Salt Lake strain) during a later stage of the vitellogenic cycle. Concentrations are expressed in ng ecdysone equivalents per g body weight (●—● , with S.D.). Arrows (/) indicate moults, and data correspond to sets of 20 individuals. An approximate time scale is given.

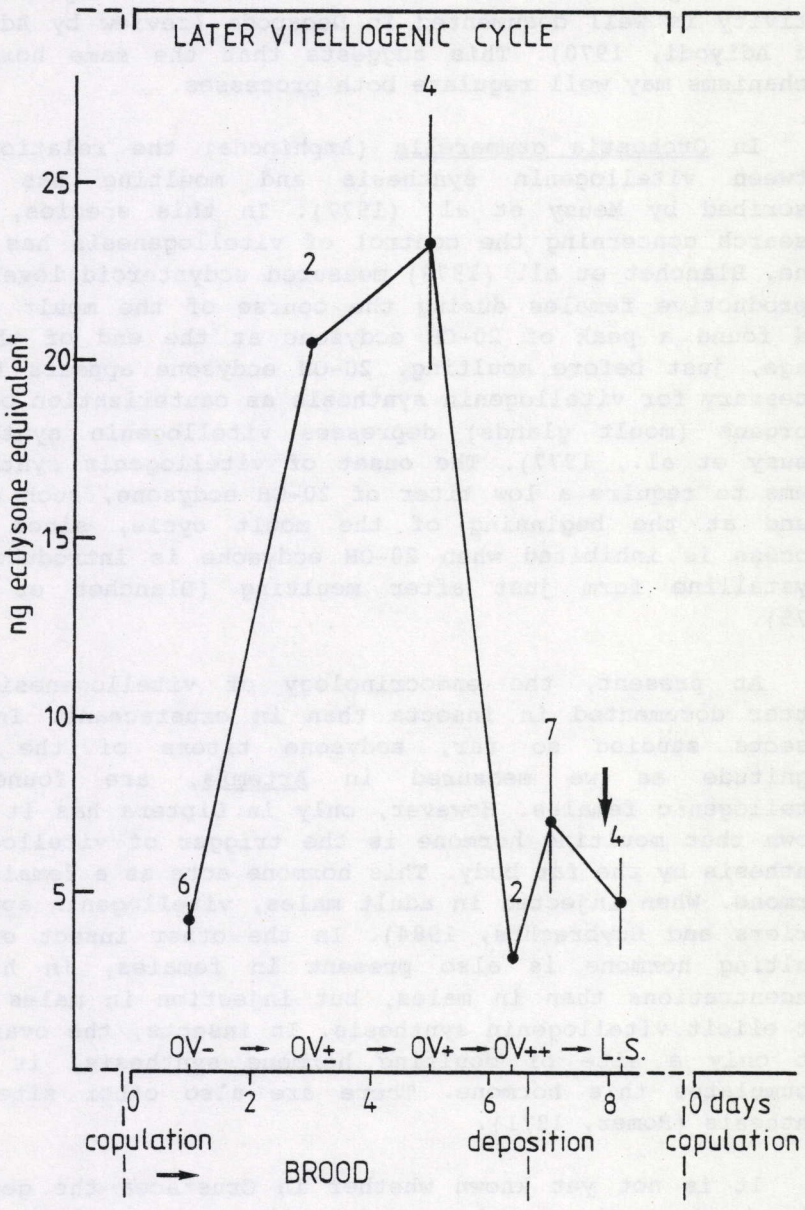
Following a more accurate sampling technique we investigated the ecdysteroid pattern during the second and the fifth vitellogenic cycle in animals derived from the same batch. The results are shown in Figs. 5.5 and 5.6. The ecdysteroid patterns confirm the above mentioned results and furthermore they also reveal the presence of a minor peak (just prior to the 'lateral sac stage') in the earliest vitellogenic cycles.

Again this fact is an indication that the smaller second rise in ecdysteroid level could trigger a certain physiological process. In this case egg-release would be one of the most possible responses. Concomittantly with this peak level, we observe at that moment a decrease of the ecdysteroid titer. Indirectly this is an evidence for the accumulation of ecdysteroids in the developing oocytes.

Irregularly varying data were obtained for ecdysteroid activities in whole body homogenates of males (5-15 ng ecdysone equivalents/g fresh body weight). High values were measured during the copulation period.

Figs. 5.5. and 5.6. Ecdysteroid activity in adult females of Artemia (Great Salt Lake strain) during the second (Fig. 5.5) and a later (is here fifth) (Fig. 5.6) stage of the vitellogenic cycle. Concentrations are expressed in ng ecdysone equivalents per g body weight (●—●, with S.D.) and per ml haemolymph (●--●). Arrows (/) indicate moults, and data correspond to sets of 20 individuals. An approximate time scale is given.


SECOND VITELLOGENIC CYCLE
15
10
5
ng ecdysone equivalent
3
6
7
4
4
OV- → OV± → OV+ → OV++ → L.S.
0
2
4
6
8
0 days
copulation
deposition
copulation
FIRST BROOD


LATER VITELLOGENIC CYCLE
ng ecdysone equivalent
25
20
15
10
5
4
2
6
2
7
4
OV-
OV±
OV+
OV++
L.S.
0
2
4
6
8
0 days
copulation
deposition
copulation
BROOD

7.3. Discussion.

The synchronization between moulting and reproductive activity is well documented in Decapoda (review by Adiyodi and Adiyodi, 1970). This suggests that the same hormonal mechanisms may well regulate both processes.

In Orchestia gammarella (Amphipoda) the relationship between vitellogenin synthesis and moulting has been described by Meusy et al. (1977). In this species, much research concerning the control of vitellogenesis has been done. Blanchet et al. (1979) measured ecdysteroid levels in reproductive females during the course of the moult cycle and found a peak of 20-OH ecdysone at the end of the D_2 stage, just before moulting. 20-OH ecdysone appears to be necessary for vitellogenin synthesis as cauterization of the Y-organs (moult glands) depresses vitellogenin synthesis (Meusy et al., 1977). The onset of vitellogenin synthesis seems to require a low titer of 20-OH ecdysone, such as is found at the beginning of the moult cycle, since this process is inhibited when 20-OH ecdysone is introduced in crystalline form just after moulting (Blanchet et al., 1975).

At present, the endocrinology of vitellogenesis is better documented in insects than in crustaceans. In all insects studied so far, ecdysone titers of the same magnitude as we measured in Artemia, are found in vitellogenic females. However, only in Diptera has it been shown that moulting hormone is the trigger of vitellogenin synthesis by the fat body. This hormone acts as a female sex hormone. When injected in adult males, vitellogenin appears (Briers and Huybrechts, 1984). In the other insect orders moulting hormone is also present in females, in higher concentrations than in males, but injection in males does not elicit vitellogenin synthesis. In insects, the ovary is not only a site of moulting hormone synthesis, it also accumulates this hormone. There are also other sites of synthesis (Romer, 1971).

It is not yet known whether in Crustacea the general rule is that the ovaries are a major source of moulting hormone. Since the highest activities and titers are found during the period of active yolk deposition, the situation might be similar to that in insects.

Junéra et al. (1977) proved in Orchestia gammarella (Amphipoda) that vitellogenin synthesis is controlled by an ovarian hormone called 'vitellogenin stimulating ovarian hormone' or VSOH (see also Meusy, 1980; Meusy and Charniaux-Cotton, 1984). This VSOH stimulates the sub-epidermal adipose tissue to produce vitellogenin (Charniaux-Cotton, 1980; Blanchet-Tournier, 1982).

In Artemia, according to Anteunis et al. (1964) and Criel (1980a) yolk formation starts in the center of an oocyte where an atypical vitellin nucleus is found that probably forms proteinaceous vitellin granules (see Chapter 2). Criel (1984) also obtained evidence that exogenous vitellogenin synthesis takes place in Artemia. Preliminary experiments, using immunocytochemical localisation of lipovitellin, demonstrated the presence of this protein in the 'fat storage cells' (Lochhead and Lochhead, 1941) in the limbs (see Chapter 3). In Branchinecta packardi (Anostraca), Gilchrist and Zagalsky (1983) found 'connective tissue storage cells' which appear to be the extra-ovarian site of biosynthesis of yolk protein precursors. The situation could well be similar to that known in insects where the bulk of vitellogenin is synthesized by the fat body and only trace amounts by the ovarian follicles themselves. Further research will be needed to find out whether 20-OH ecdysone controls vitellogenin synthesis.

Ultrastructural observations on the oviduct of Artemia (Californian strain), showed the existence of a secretory cycle (Criel, 1980b). During previtellogenesis the glandular cells produce a scarce merocrine secretion. The typical apocrine secretion starts at the period when the highest ecdysone level is reached, at the onset of vitellogenesis, and lasts until ovulation. It remains to be demonstrated that ecdysone controls the activity of these glandular cells.

We have no exact data on hormonal activity in reproducing males. In this sex, moulting occurs at regular time intervals and always after copulation. We measured high ecdysone activities during the copulation period. When an accurate sampling technique for male Artemia stages will have been worked out, the role of ecdysteroids in reproduction in males can be studied.

Our next goal is the elucidation of the role of this and other hormones on reproduction. We expect that moulting hormone will only be one out of a whole series of hormonal factors controlling reproduction.

8. Concluding remarks.

Ecdysteroid concentrations in the brine shrimp increase during oogenesis and prior to moulting. The appearance of the second peak during a later vitellogenic cycle could be important for egg-release and a more accurate timing of the samples at this period could well confirm this idea. However, the rapid decrease in ecdysteroid level after egg-release seems to be in favour of the maternal origin of the embryonic ecdysteroids. Since a lot of ecdysteroids are present in cysts this finding could point to a production and/or an accumulation of ecdysteroids by the ovaries. In insects, ovarian ecdysteroids possibly play a role in the control of vitellogenin synthesis and in the early events of embryogenesis (Hoffmann, 1986).

When we look at the complicated network of inhibiting and/or stimulating factors in the eyestalks of crustaceans, it appears possible that apart from the presence of ecdysteroids other factors also will prove to be responsible for the regulation of vitellogenesis in the brine shrimp. With this in mind we investigated the presence of vertebrate-type steroids in Artemia (see Chapter 6).

CHAPTER 6: NON-ECDYSTEROID STEROIDS AND THEIR POSSIBLE ROLE.

1. Introduction.

In vertebrate endocrinology steroids have an important function in reproduction. During the recent decade, however, it has become evident that the so called Vertebrate-type steroids are also present in a variety of invertebrate species belonging to different phyla (see review Sandor and Mehdi, 1979; Sandor, 1980; De Clerck et al., 1984; Voogt et al., 1984; Mechoulam et al., 1984; De Loof and De Clerck, 1986). In some invertebrates, concentrations are comparable with those present in vertebrates. Some data suggest that C_{21}, C_{19} and C_{18} steroids might exert an hormonal function in arthropods, be it that evidence is still rather limited (Rothschild and Ford, 1966; Lehoux and Sandor, 1970).

In addition, since a few years highly sensitive analytical methods are used for the identification of non-ecdysteroid steroids in arthropods. Afte such steroids had been found in an insect species (De Clerck et al., 1984), extracts of haemolymph of the crustacean Astacus leptodactylus were subjected to derivatization followed by gas chromatography/mass spectrometry. The following steroids could be identified: pregnenolone, 17α-hydroxypregnenolone, testosterone, cholesterol and 6ß-hydroxyprogesterone. The latter could only be demonstrated in haemolymph of females (Ollevier et al., 1986).

Thus it seemed interesting to look for such steroids (C_{21}-steroids or progestagens, C_{19}-steroids or androgens and C_{18}-steroids or estrogens) in the brine shrimp Artemia and this again in relation to vitellogenesis.

In this chapter we demonstrate the presence of pregnenolone in the brine shrimp by GC-MS (see 4.). We also present the results of RIA determinations on changes in concentrations of six non-ecdysteroid steroids in purified total body extracts of this primitive crustacean (see 5.), this as a first attempt to obtain information about their possible functions.

2. Literature survey.

2.1. Occurrence of non-ecdysteroid steroids in arthropods.

In vertebrates, some steroids play an important role in sexual development. However, the existence of sex specific hormones in arthropods was not so evident. Injection of 20-OH-ecdysone in male flies induced the synthesis of the female specific yolk protein vitellogenin (Huybrechts and De Loof, 1977, 1981). All data gathered showed that 20-OH-ecdysone, at least in Diptera, might be the equivalent of estrogens in egg-laying vertebrates (De Loof et al., 1980). Naisse (1966a,b,c) demonstrated the existence of a male specific sex-hormone in insects. She found an androgenic substance in Lampyris noctiluca which is synthetized by the testes under the influence of a brain hormone. In crustaceans an androgenic hormone produced by the androgenic gland is present in Orchestia gammarella (Charniaux-Cotton, 1957, 1962). Some scientists supposed it to be a peptide (Juchault and Legrand, 1978) while others found some evidence for its steroidal nature (Sarojini, 1964). In vertebrates also some peptides function as hormone releasing factors and probably both kind of molecules have to be kept in mind.

Since steroids seem to be highly conserved molecules during evolution, it is not exaggerated to state that steroids - as universal biomolecules - might be potential sex hormones in invertebrates (Sandor and Mehdi, 1979; Barrington, 1986).

Steroids are present in a very wide variety of living organisms (Lehoux and Sandor, 1970; Sandor and Mehdi, 1979; Sandor, 1980).

Already in 1940, Donahue reported the estrogenic effect of extracts of lobster ovaries when applied in mammalian bioassays. Further proofs for the presence of estrogens in lobsters were obtained by chromatographic techniques and fluorimetry (Donahue, 1948, 1957; Lisk, 1961). Testosterone was isolated and identified in Homarus americanus from the haemolymph and testes (Burns et al., 1984b). Estradiol-17ß and progesterone were quantified in tissues of this lobster by radioimmunoassay (Couch et al., 1987). The presence of estrogen was demonstrated by gas chromatography in the ovary of the shrimp Parapenaeus fissurus (Jeng et al., 1978). Gas chromatography coupled to mass spectrometry (GC-MS) allowed the identification of nonecdysteroid steroids in haemolymph

of both male and female Astacus leptodactylus (Ollevier et al., 1986).

So far as we know, our RIA-report is the first study about the presence of C_{21}, C_{19} and C_{18}-steroids in the brine shrimp (see 5.). We also demonstrated the presence of pregnenolone in extracts of adult Artemia by means of GC-MS (see 4.).

2.2. Metabolization.

At first sight, it could be supposed that the presence of these steroids is due to the uptake through the food. Arthropods, specially insects, need cholesterol or phytoecdysteroids in their diet as a precursor for a lot of sterols. Also some marine crustaceans have been found to lack the ability to synthetize sterols starting from acetate and/or mevalonate (Teshima and Kanazawa, 1971a,d). On the other hand, it was demonstrated that the Alaskan king crab Paralithodes camtschatica contained a variety of sterols in addition to cholesterol (Idler and Wiseman, 1968). It has been generally assumed that some of them originate from the diet.

In Artemia, dietary cholesterol is required and 0.8 mg percent (w/v) is found to be sufficient (Provasoli and D'Agostino, 1969; D'Agostino, 1980). The need for cholesterol is in agreement with the report of Kanazawa et al. (1971) who found no evidence of sterol synthesis in Artemia. In the brine shrimp dietary cholesterol can be substituted in part by ergosterol, 24-methylcholesterol, stigmasterol and ß-sitosterol as these compounds can be converted to cholesterol (Teshima, 1971; Teshima and Kanazawa, 1971b,d). We can conclude that this phytophagous animal probably modifies phyto-sterols, present in algae, into cholesterol. Cholesterol is, besides the small percentage of cholestanol, the primary component of the sterol fraction in Artemia salina from Mono Lake (Payne and Kuwahara, 1972).

Marine crustaceans such as the prawn Penaeus japonicus and the brine shrimp Artemia possess the enzyme systems for the dealkylation at C-24 of C_{28}- and C_{29}-sterols (Teshima, 1971). In the spiny lobster Panulirus japonica, Kanazawa and Teshima (1971) reported enzyme systems capable of catalyzing the conversion of (4-^{14}C)-cholesterol to the steroid hormones progesterone, 17α-hydroxyprogesterone, androstenedione and testosterone. Crustacean gonads also contain

several enzymes that are capable of converting progesterone into steroid metabolites (Tcholakian and Eik-Nes, 1971; Teshima and Kanazawa, 1971c; Burns et al., 1984a). Among the molluscs, the gonads of the cuttlefish (Cephalopoda) are known to possess several enzyme systems that intervene in the biosynthesis of androgens (Carreau and Drosdowsky, 1977). In insect gonads, pregnenolone can be transformed to progesterone (Lehoux et al., 1970). Besides the conversion of pregnenolone to progesterone in Sarcophaga bullata larvae, numerous unidentified metabolites are demonstrated in in vivo metabolization experiments (De Clerck et al., 1987). Furthermore, this study revealed no formation of C21 or C19 steroids out of cholesterol. It was only recently that in the insect Manduca sexta, for the first time, the bioconversion of cholesterol to a C_{21}-steroid conjugate was shown (Thompson et al., 1986). In this way, endogenous steroid biosynthesis was demonstrated indirectly.

As enzyme systems for steroidogenesis are present, attention has to be paid to the possible role of these steroids in invertebrates.

2.3. Possible functions.

A specific role is as yet not obvious except for the steroids found in the defensive secretions of some water beetles (Schildknecht, 1970; Chadha et al., 1970). Certainly, a number of possible effects have been reported in the recent decade but a real hormonal function for steroids in invertebrates never was demonstrated.

Experimentally administered nandralone phenyl propionate is highly active as an insect growth regulator in Anopheles stephensi (Sita et al., 1986). Both injections of testosterone-propionate and androgenic gland extracts in young female crabs resulted in masculinisation (Sarojini, 1963). Topical application of testosterone gave rise to hyperplasia and hypertrophy of the androgenic gland of the prawn Parapenaeopsis hardwickii (Nagabhushanam and Kulkarni, 1981). Progesterone however, stimulated oogonia to undergo maturation and accelerated growth of oocytes at the initiation of ovarian vitellogenesis in this species (Kulkarni et al., 1979). Induced ovarian maturation by progesterone injection was also reported in Metapenaeus ensis (Yano, 1985). Yano demonstrated in 1987 that injection of 17α-hydroxy-progesterone in early vitellogenic stages of

the prawn Penaeus japonicus resulted in an increase of haemolymph vitellogenin. Since progesterone is converted into 17α-hydroxy-progesterone in the ovaries of the crab Portunus trituberculatus (Teshima and Kanazawa, 1971c), progesterone may serve during ovarian vitellogenesis as a precursor of 17α-hydroxy-progesterone, which stimulates vitellogenin synthesis and/or release into the haemolymph in female prawns.

We can only make some preliminary assumptions about the possible functions of the steroids in the brine shrimp (see 5.).

3. Structure of the steroids investigated.

We have restricted our work to the quantification of six representative steroids: pregnenolone and progesterone (as C_{21}-steroids or progestagens), testosterone and 5α-dihydrotestosterone (as C_{19}-steroids or androgens), estrone and estradiol (as C_{18}-steroids or estrogens).

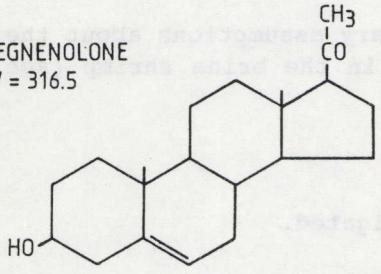
The trivial names normally used in steroid nomenclature are described in Table 6.1 and Fig. 6.1 gives the structural formulae of these molecules (after the work of Schulster et al. (1976), see also note in 4.1. in Chapter 4).

Table 6.1. The trivial names of the six steroids investigated.

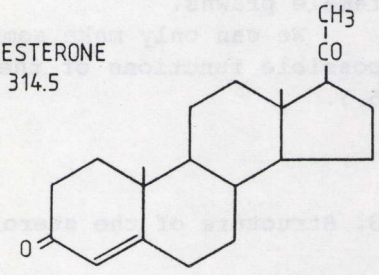
Pregnenolone (P_5): 5-Pregnene-3ß-ol-20-one
Progesterone (P_4): 4-Pregnene-3,20-dione
Testosterone (T): 17ß-Hydroxy-4-androsten-3-one
5α-Dihydrotestosterone (DHT): 17ß-Hydroxy-5α-androstan-3-one
Estrone (E1): 3-Hydroxy-1,3,5,(10)-estratrien-17-one
Estradiol (E2): 1,3,5,(10)-estratriene-3,17ß-diol

Fig. 6.1. Structural formulae of the six steroids investigated.

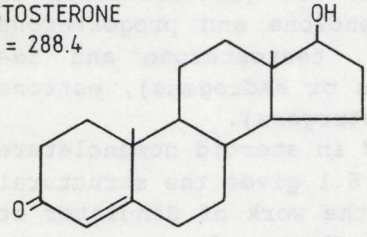
PREGNENOLONE
MW = 316.5



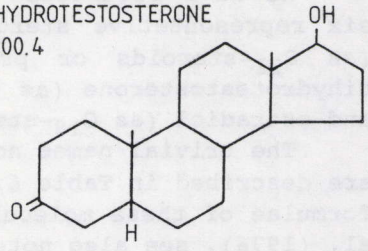
PROGESTERONE
MW = 314.5



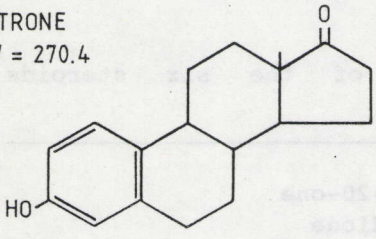
TESTOSTERONE
MW = 288.4



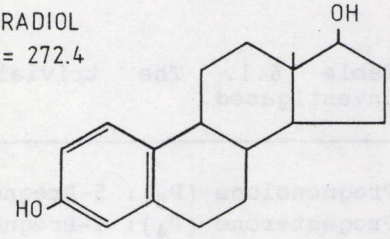
5α-DIHYDROTESTOSTERONE
MW = 300.4



ESTRONE
MW = 270.4



ESTRADIOL
MW = 272.4



4. Identification of C_{21}, C_{19} and C_{18} steroids in adult brine shrimp Artemia sp. by gas chromatography-mass spectrometry.

4.1. Extraction of the steroids.

For this purpose we used a mixture of male and female adults. Fifteen samples each weighing about 1 g of frozen animals were homogenized as is described in Appendix II. Supernatants were pooled and evaporated with a Büchi rotavapor. Next, solvent partition was carried out with the solvent system n-hexane:water (10:1, v/v). After equilibrium the vial was cooled for 2 hr at -20°C. The organic phase was decanted and the aqueous phase was washed once more with 100 ml n-hexane. Afterwards the organic phases were evaporated and samples were dissolved in dichloromethane and stored at 4°C until use.

4.2. Treatment of the extracts.

4.2.1. Purification.

A first purification was achieved by chromatography on a silica column. Then, the six steroids Pregnenolone (P_5), Progesterone (P_4), Testosterone (T), 5α-dihydrotestosterone (DHT), estrone (E_1) and estradiol (E_2) were separated by celite chromatography (see Appendix II) and further purified by Sep-pak chromatography*. The different fractions were evaporated under liquid nitrogen and dissolved in dichloromethane.

---

* The Sep-pak C_{18} cartridges are first prewet with pure ethanol and then with 80 percent ethanol. Steroid samples are dissolved in 80 percent ethanol and applied to the column. The pure steroid fraction is immediately eluted while impurities remain on the column.

4.2.2. Derivatization.

The method described by Schoonen and Lambert (1988) was followed.

Trimethylsilyl (TMS) and oxime-trimethylsilyl derivatives were prepared in the presence of hydroxylammonium chloride in pyridine. In this initial step, ketone functions of the steroids were converted in hydroxyl groups. To obtain the the TMS-ether derivatives of the steroids, a mixture of the derivatization reagents trimethyl-chlorosilane (TMCS) and N,O-bis-(trimethylsilyl)-acetamide (BSA) (1:9, v/v, both from Fluka) was added. Finally, the steroid derivative fraction was dissolved in hexane and an aliquot of 2 µl was subjected to GC-MS.

4.2.3. Capillary gas chromatography/mass spectrometry.

A Hewlett-Packard 5992 B gas chromatograph/mass spectrometer with a Hewlett-Packard fused silica capillary column (Ultra 1, cross linked methyl silicone, film thickness, 0.17 µm, 25 m x 0.31 mm i.d.) was used with helium as carrier gas at a flow rate of 2 ml/min.

After elution from the column, molecules were fractionated by electron bombardment and the positive fragment ions were counted by an electron-multiplier. The obtained mass spectra were non-normalized and for total ion monitoring the scan range of 200-600 m/e was used.
Steroids from the biological samples could be identified by comparing the obtained spectra with the spectra of standards at their expected GC retention time.

As internal standards 5α-androstane-17ß-ol-TMS and 5-pregnene-3ß,17α,20ß-tri-TMS (obtained from Steraloids Inc.) were used. The characteristic ions for the first were the molecular ion m/e 348.4 and the mass fragments m/e 334.4, m/e 243 and m/e 258; for the second the molecular ion was m/e 550.3 and the mass fragments were m/e 434, m/e 343 and m/e 253.

4.3. Results.

The lower detection limit in our system was: 5 ng for P_5; >100 ng for P_4; 6 ng for T; 6 ng for DHT; 1-2 ng for E_1 and 1 ng for E_2.

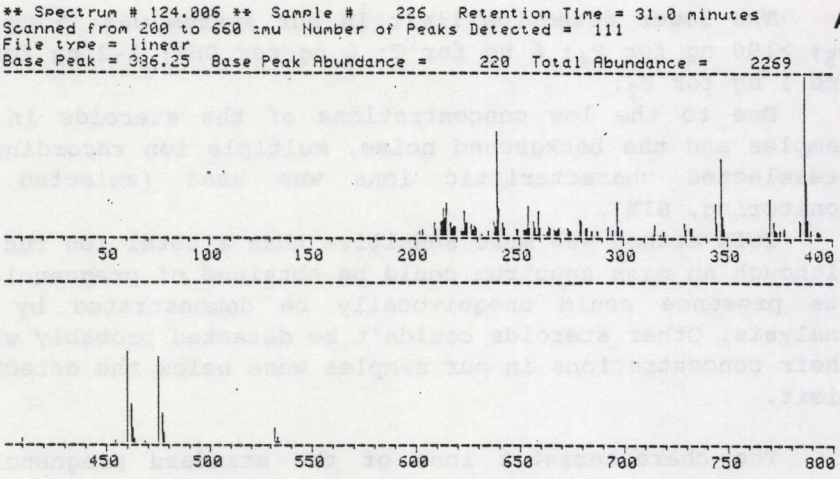
Due to the low concentrations of the steroids in our samples and the background noise, multiple ion recording of preselected characteristic ions was used (selected ion monitoring, SIM).

This method was more sensitive than a total ion run and although no mass spectrum could be obtained of pregnenolone, its presence could unequivocally be demonstrated by SIM analysis. Other steroids couldn't be detected probably while their concentrations in our samples were below the detection limit.

The characteristic ions of the standard pregnenolone derivative were present at retention time of 31.0 min. Its molecular ion was m/e 475.2 (M^+) and its characterictic mass fragments were m/e 460.2 (M^+-CH_3), m/e 386.3 (M^+-OTMS), m/e 346.2 and m/e 239.0. The relative abundance for the ions 386.3, 239.0, 460.2, 475.2 and 346.2 was 100:65:57:54:48 (see Fig. 6.2).

Fig. 6.2.
A/. Mass-spectra (non-normalized) of pregnenolone-oxim-diTMS standard. The characteristic ions are the molecular ion m/e 475.2 and the mass fragments m/e 460.2, m/e 386.3, m/e 346.2 and m/e 239.0.
B/. Table of the relative abundance of all the detected fragment ions.
C/. Structural formula of the standard pregnenolone derivative with the characteristic fragment ions.

A



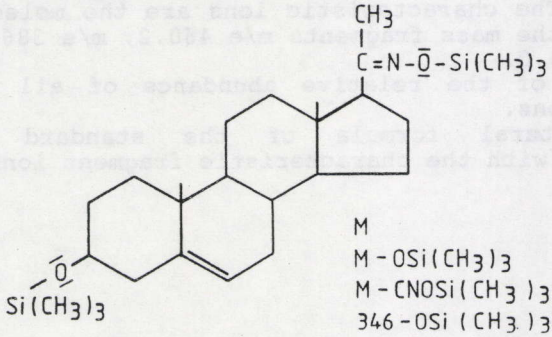
B

Lower Abundance Cutoff Level = 5.0%

MASS	ABUNDANCE (%)	MASS	ABUNDANCE (%)	MASS	ABUNDANCE (%)
208.95	7.7	240.00	17.3	344.20	20.0
211.05	8.6	241.00	8.2	345.20	7.7
211.95	9.5	253.10	5.5	346.20	47.7
213.05	16.8	254.10	18.2	347.30	11.8
214.05	10.9	255.00	5.0	370.30	23.2
215.05	19.5	256.10	8.6	371.20	9.1
216.05	7.7	257.00	5.0	384.15	10.0
217.05	5.0	259.00	14.5	385.25	9.1
217.85	6.4	264.10	5.9	386.25	100.0
218.90	6.4	265.10	5.0	387.25	33.6
223.00	15.0	279.05	9.5	388.25	10.0
224.00	8.2	280.05	9.1	460.20	57.3
224.90	8.2	281.05	5.0	461.20	25.5
226.00	7.3	281.95	5.5	462.00	10.0
227.90	5.9	292.15	6.4	475.20	54.1
228.90	5.0	296.15	21.4	476.30	18.6
237.00	9.1	297.25	6.4	477.30	6.8
238.00	5.9	315.05	7.3	531.15	10.5
239.00	64.5	329.10	8.2		

C

PREGNENOLON-OXIM-diTMS



M = 475.2

M - OSi(CH3)3 = 386.3

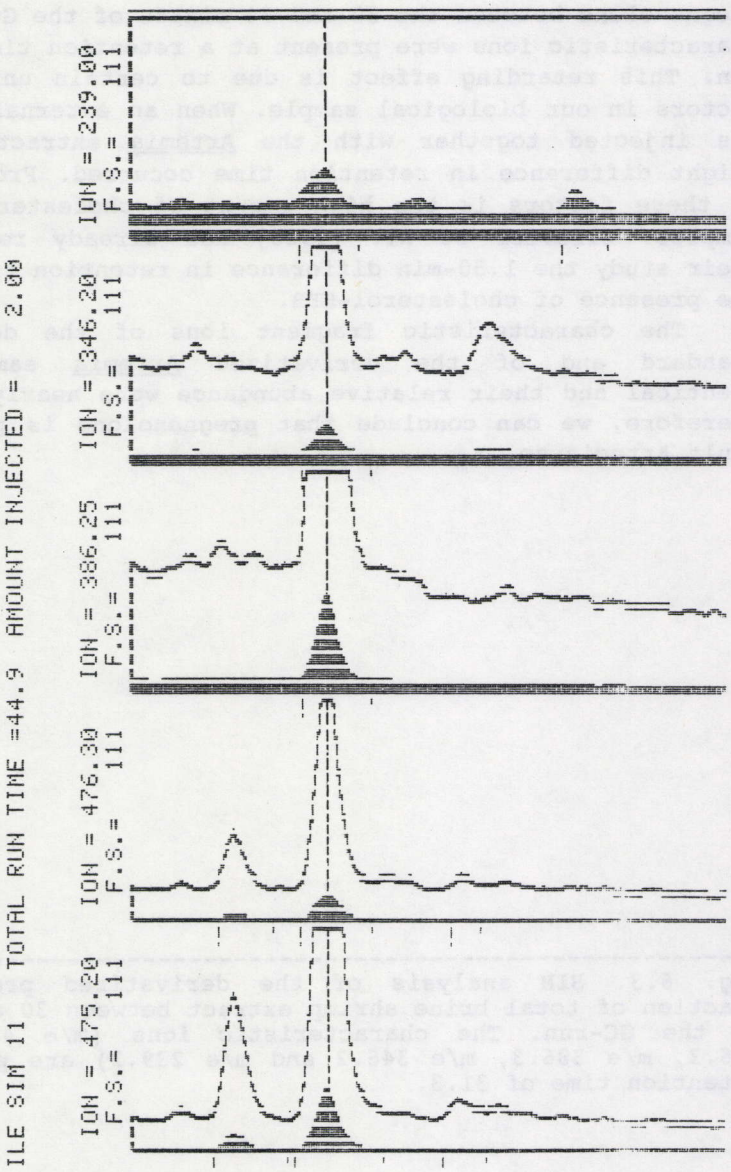
M - CNOSi(CH3)3CH3 = 346.2

346 - OSi(CH3)3-CH3 = 239.0

Multiple Ion Search of the pregnenolone derivative of the brine shrimp extract is shown in Fig. 6.3. We looked very closely for the fragment ions of the steroid derivative pregnenolone between the 30 and 34 minute of the GC-run. The characteristic ions were present at a retention time of 31.3 min. This retarding effect is due to certain unidentified factors in our biological sample. When an external standard was injected together with the Artemia extract, also a slight difference in retention time occurred. Probably one of these factors is the high amount of cholesterol in our samples. Ollevier et al. (1986) had already reported in their study the 1.50-min difference in retention time due to the presence of cholesterol-HFB.

The characteristic fragment ions of the derivatized standard and of the derivatized Artemia sample were identical and their relative abundance were nearly similar. Therefore, we can conclude that pregnenolone is present in adult Artemia sp.

Fig. 6.3. SIM analysis of the derivatized pregnenolone fraction of total brine shrimp extract between 30 and 34 min of the GC-run. The characteristic ions (m/e 475.2, m/e 476.3, m/e 386.3, m/e 346.2 and m/e 239.2) are present at retention time of 31.3.


ILE SIM 11 TOTAL RUN TIME =44.9 AMOUNT INJECTED = 2.00
ION = 475.20
F.S.= 111
ION = 476.30
F.S.= 111
ION = 386.25
F.S.= 111
ION = 346.20
F.S.= 111
ION = 239.00
F.S.= 111

4.4. Discussion.

These results of GC-MS analysis are the first data about the presence of a C_{21} vertebrate-type steroid in Artemia sp. Despite the fact that only pregnenolone is clearly identified in the brine shrimp extract, it is not unlikely that other steroids such as estradiol will also be identified by GC-MS in the near future.

In the next chapter, we report on data obtained with RIA. The results of that report confirm the GC-MS analysis of pregnenolone and further demonstrate the presence of progesterone, testosterone, 5α-dihydrotestosterone, estrone and estradiol in female brine shrimp during vitellogenesis.

Recently a tentative scheme of the biochemical reactions for the synthesis of vertebrate-type steroids in insects was proposed (De Clerck et al., 1987). According to these authors, pregnenolone might be a precursor for further steroid synthesis in invertebrates, as it is the case in vertebrates. Moreover, in vitro incubation of testis from the insects Gromphodorina portentosa and Byrsotria furnigata showed the conversion of pregnenolone into progesterone (Lehoux et al., 1970). The identification of pregnenolone in our preliminary results of GC-MS together with its high concentrations during vitellogenesis (see 5.) are two facts in agreement with the above mentioned bioconversion pattern.

5. Quantification of steroids.

In this research domain also a lot of progress has been made. Originally all methods were based on what was known from vertebrate endocrinology but gradually protocols adapted to specific invertebrates have been developed. In vertebrates, the basic material for steroid investigations was mostly serum. However, in small invertebrates even extracts of whole animals were commonly used. This needed an extended extraction and purification procedure.

5.1. Extraction and purification procedure.

The same methods are followed as for the extraction of ecdysteroids. Samples are further purified by celite chromatography (see Appendix II).

Remarks:

- Haemolymph is treated in the same way but for steroid analysis in the brine shrimp it was extremely difficult to get sufficiently large amounts.
- By the celite chromatography procedure, the different steroids are separated in such a way that cross reaction in the radioimmunoassay did not exceed 0.5 percent (Novak et al., 1987).
- Results of the recoveries of the whole purification method with brine shrimp extracts are described by Van Beek and De Loof (1988).

5.2. Radioimmunological determinations of concentrations of six C_{21}, C_{19} and C_{18} steoids during the reproductive cycle of female Artemia sp..

5.2.1. Radioimmunological detection.

The assay protocol as described by bioMérieux was followed. The RIA procedure is similar to the assay of ecdysteroids except that the free steroids are precipitated with dextran coated charcoal (2.686 g charcoal and 0.269 g dextran T_{70} per litre buffer). Afterwards the antibody bound steroids, present in 500 µl aliquots of the supernatant, are counted.

The antisera used for radioimmunoassay were obtained from Radioassay Systems Laboratories, Inc. (AHS, Belgium). The properties of the antisera have been described by Novak et al. (1987).

Remarks:

- All incubations and the centrifugation need to be carried out at 4°C.
- The charcoal suspension has to be stirred continuously.
- A rigorous timing is absolutely essential.

5.2.2. Materials and methods.

Female Artemia were selected according to an arbitrary chosen vitellogenic stage (see Table 6.2). Batches of 50-120 individuals were collected (see Appendix I). Samples were homogenized and purified according to methods described in Appendix II.

Table 6.2.: Stages in an arbitrary reproductive cycle of Artemia after the first vitellogenic cycle.

1. Ov-,Ut+	: Ripe oocytes in the broodsac. No yolk formation in the fully transparent ovaries.
2. Ov±,Ut+	: A few oocytes are opaque as the result of presence of beginning yolk deposition. Eggs in broodsac.
3. Ov+,Ut+	: More yolk containing oocytes become visible. They form an extended cord in each ovary. Eggs in broodsac.
4. Ov++,Ut+	: A double strand of ripe oocytes is present, while the broodsac is still full with the previous brood.
5. Ov++,Ut-	: A double strand of ripe oocytes is present in the ovaries. The (fertilized) eggs are released from the broodsac.
6. L.S.	: The new ripe oocytes have descended into the lateral sacs or extended oviducts.

By addition of tritium labelled steroids to the samples, it was shown that recoveries of progesterone, pregnenolone, 5α-dihydrotestosterone, testosterone, estrone and estradiol were 94, 89, 93, 85, 95 and 89 percent, respectively.

5.2.3. Results.

Due to the transparency of the body wall, yolk accumulation can easily be followed microscopically. Each vitellogenic cycle ends with the descent of the ripe oocytes through the oviducts or lateral sacs into the uterus or broodsac. The brood stays there while the subsequent cycle proceeds. Just before this descent the previous brood is released into the water. When we take the development of ovaries (Ov) and uterus (Ut) into account as main external features, the vitellogenic cycle can be divided into six successive stages as described in Table 6.2.

The results of RIA measurements are shown in Figs. 6.4-6.9. All diagrams show a vitellogenic cycle subdivided into six stages according to Table 6.2. Per stage, 3 to 5 different batches were analysed.

The concentration of all hormones decreases after egg-release (stage 5). At the onset of vitellogenesis (stage 2) concentrations are also low, with an exception for estrone.

The amount of progesterone (P_4) is relatively low during vitellogenesis (mean values between 2 and 6 ng/g). High amounts are found in the beginning and at the end of a vitellogenic cycle (mean values are 18.2 ng/g and 22.5 ng/g, extreme values are 86 ng/g and 85.5 ng/g respectively) (Fig. 6.4).

Concentrations of pregnenolone (P_5) are 2-3 times higher than those of progesterone and more than 10 times higher than those of the other steroids investigated. Relatively low concentrations are measured during vitellogenesis (about 23 ng/g), while at the beginning and at the end of a vitellogenic cycle high values could be detected (95 ng/g and 58.6 ng/g respectively) (Fig. 6.5).

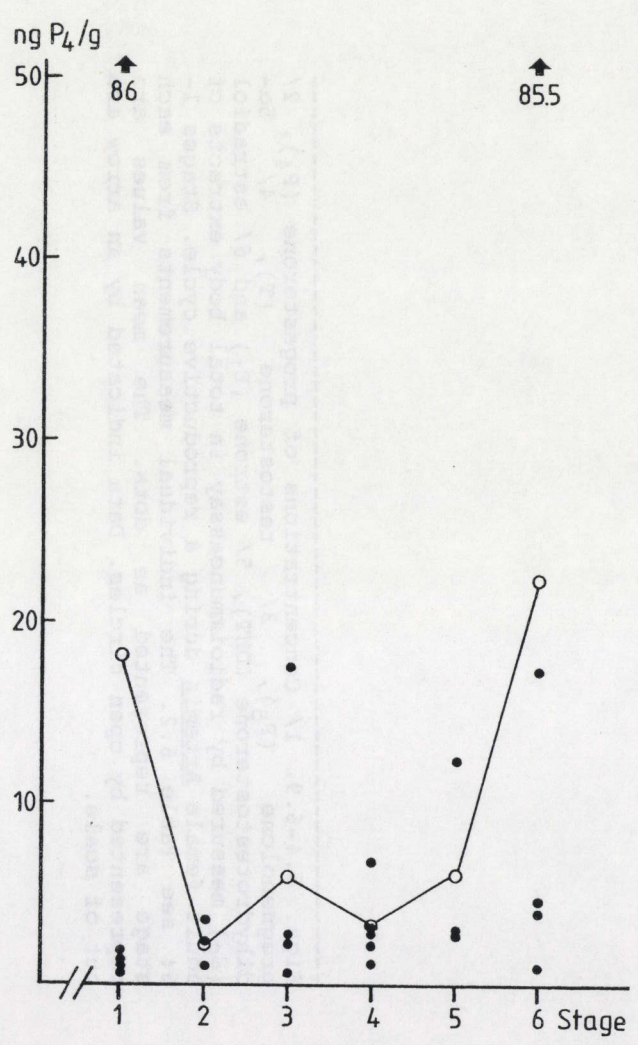
The amount of 5α-dihydrotestosterone (DHT) is very low at the onset of vitellogenesis (mean value of 539 pg/g). At the end of vitellogenesis a peak value (mean value of 1,190 pg/g) is detected. Like the progestagen concentrations, that of DHT is high between vitellogenic cycles (Fig. 6.6).

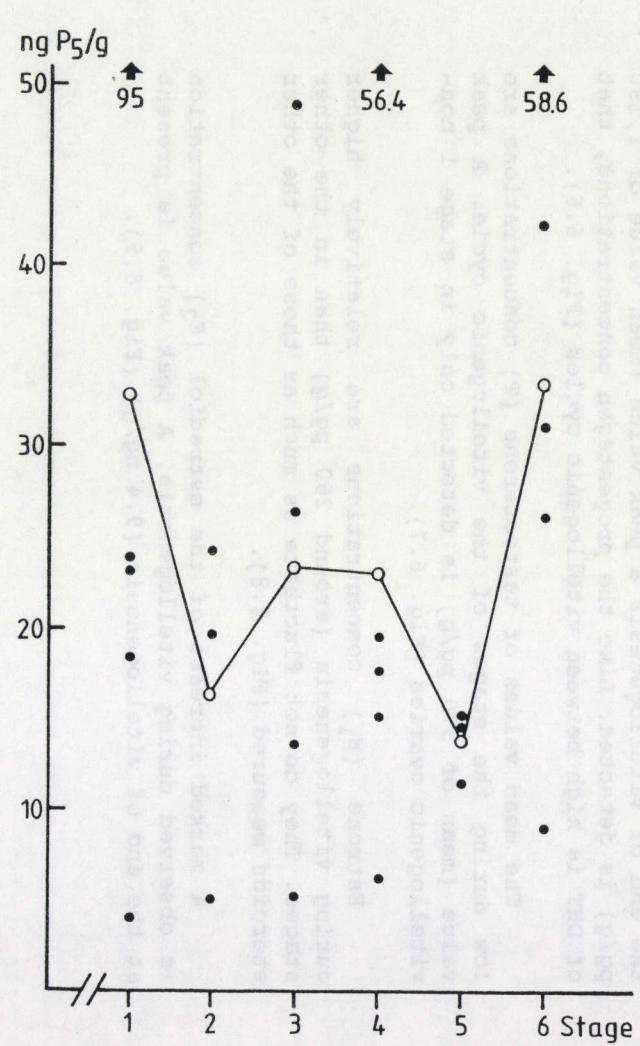
The mean values of testosterone (T) concentrations are low during the stages of the vitellogenic cycle. A peak value (mean of 370 pg/g) is detected only in stage 1 non-vitellogenic ovaries (Fig. 6.7).

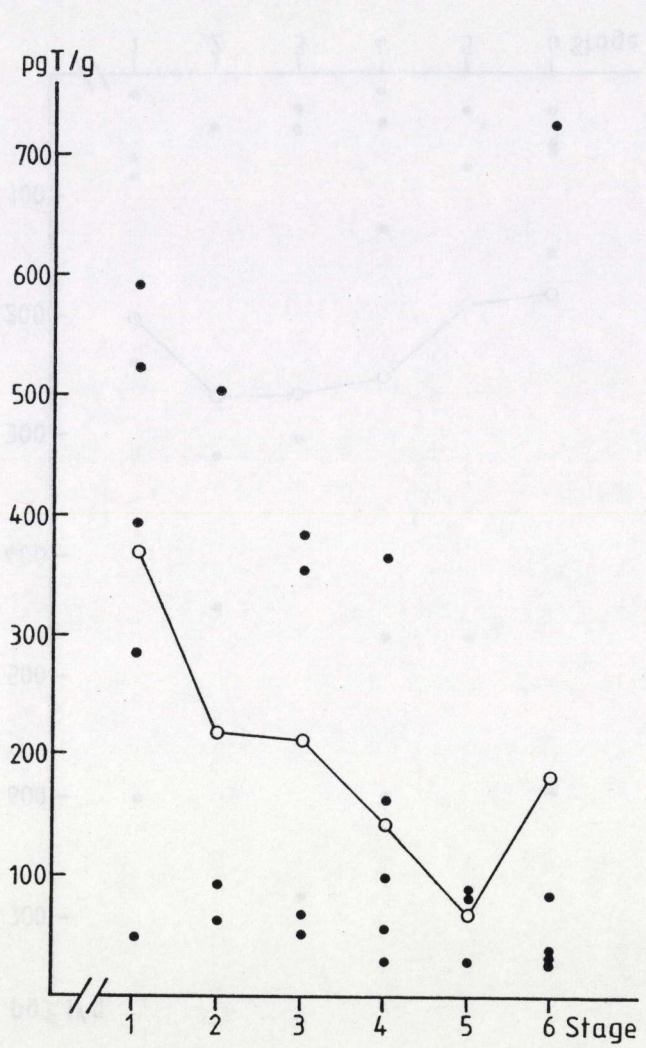
Estrone (E_1) concentrations are relatively higher during vitellogenesis (around 260 pg/g) than in the other stages. They do not fluctuate as much as those of the other steroids measured (Fig. 6.8).

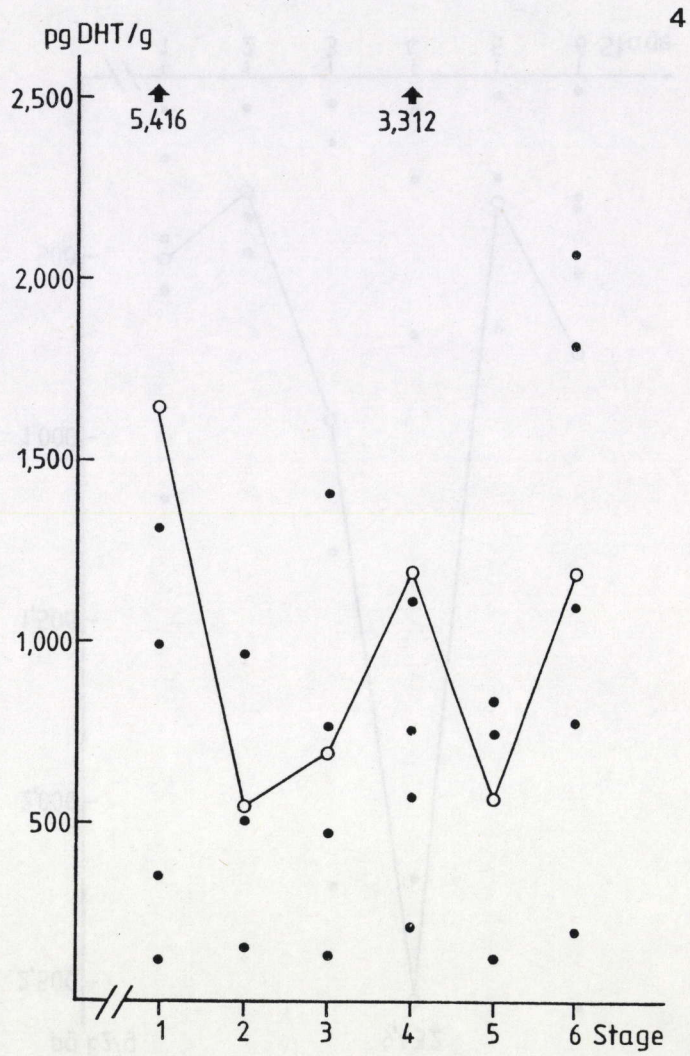
A marked increase of the estradiol (E_2) concentration is observed during vitellogenesis. A peak value is present at the end of vitellogenesis (9.4 ng/g) (Fig. 6.9).

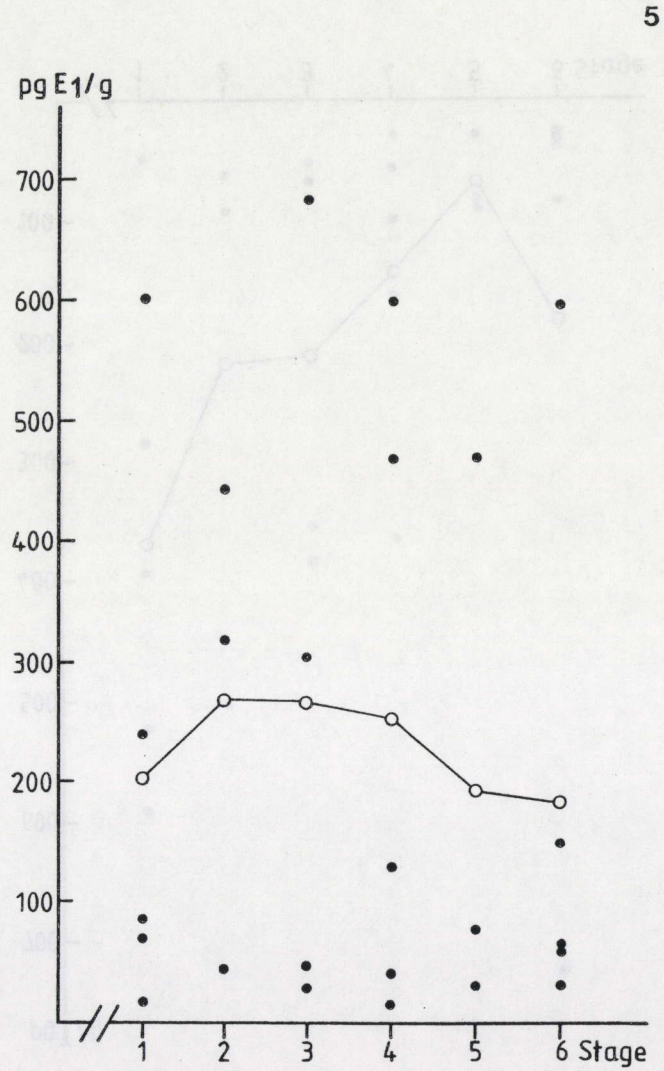
Figs. 6.4-6.9. 1/ Concentrations of progesterone (P_4), 2/ pregnenolone (P_5), 3/ testosterone (T), 4/ 5α-dihydrotestosterone (DHT), 5/ estrone (E_1) and 6/ estradiol (E_2) measured by radioimmunoassay in total body extracts of adult female Artemia during a reproductive cycle. Stages 1-6: see Table 6.2. The individual measurements from each stage are represented as dots. The mean values are represented by open circles. Data indicated by an arrow are out of scale.

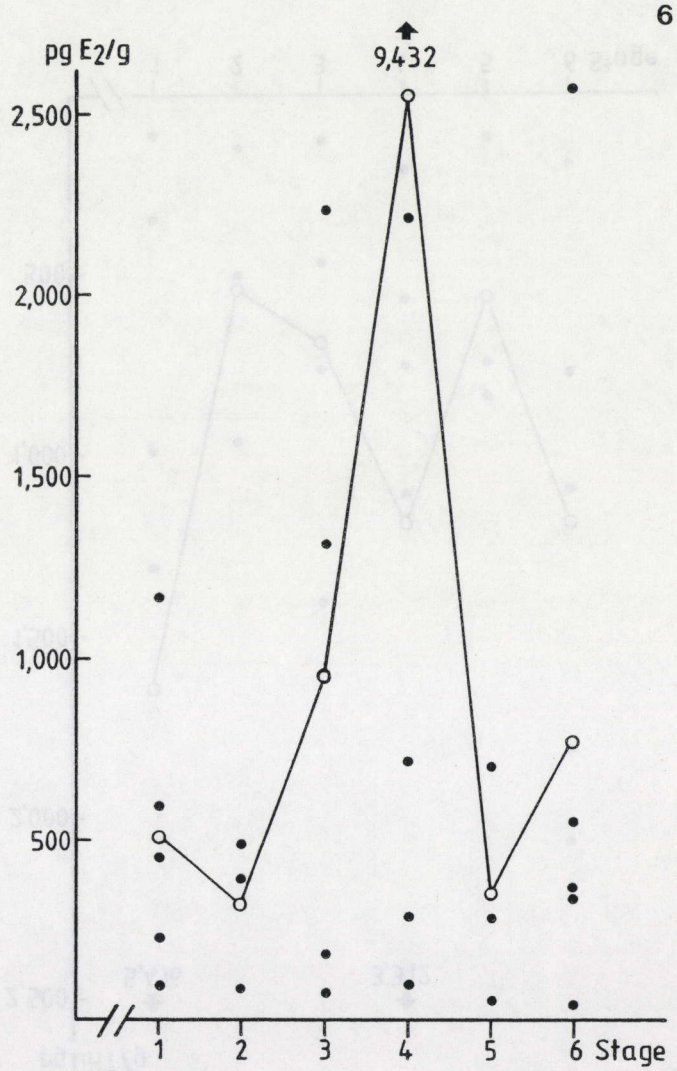

1
ng P_4/g
50
40
30
20
10
86
85.5
1
2
3
4
5
6 Stage


2
ng P_5/g
50
40
30
20
10
95
56.4
58.6
1
2
3
4
5
6 Stage


3
pg T / g
700
600
500
400
300
200
100
1
2
3
4
5
6 Stage


4
pg DHT / g
2,500
2,000
1,500
1,000
500
5,416
3,312
1
2
3
4
5
6 Stage


5
pg E1/g
700
600
500
400
300
200
100
1
2
3
4
5
6 Stage


6
pg E2/g
9,432
2,500
2,000
1,500
1,000
500
1
2
3
4
5
6 Stage

5.2.4. Discussion.

A first observation on the steroid patterns is the presence of low concentrations of progestagens during vitellogenesis, followed by a sharp increase at stage 5 and a decrease in stage 2. However, in preliminary experiments high values were measured at the onset of the first vitellogenic cycle when preadults change into the adult phase. Voogt et al. (1984) found that in starfish a decrease in the progesterone level seemed to be essential for the onset of vitellogenesis.

Estrogen levels, on the contrary, reached peak values during vitellogenesis. In Artemia, a progesterone-estradiol antagonism may be present instead of progesterone-estrone as in starfish (Voogt et al., 1984). We found that in Artemia, estradiol concentrations especially were high just before the egg-release from the uterus (stage 5). According to Takeda (1979), estrogens stimulate egg-laying in Deroceras reticulatus and Limax flavus (gastropods). Concentrations of all hormones investigated were low at the moment of egg-release. Preliminary experiments showed that cysts or encysted gastrulae of Artemia accumulate ecdysteroids as well as other steroids. RIA measurements revealed that an extract of 1g of cysts contained 193 pg P_4, 2,290 pg P_5, 265 pg DHT, 91 pg T, 2,131 pg E_1 and 134 pg E_2. This shows that some steroids are present in the eggs in a non-conjugated form. The measurements may partially explain the decrease of steroid concentrations at egg release.

The variability of the several steroid concentrations is rather high. As far as we could check, it is not due to inperfections in our extraction or RIA procedures. It may be that peak values of short duration occur, which are difficult to detect in different batches. Some stage overlapping is possible, especially between stage 6 and 1 or between 3 and 4. Indeed some vitellogenic stages take 2 hr, others last for days (Bowen, 1962). Measurements in extracts of haemolymph might be more reliable, but the small size of Artemia makes sampling of sufficient amounts of haemolymph extremely difficult.

Artemia is capable of synthesizing cholesterol from precursor molecules such as ergosterol and 24-methylcholesterol (Teshima, 1971; Teshima and Kanazawa, 1971). The steroid metabolic pathways in Artemia have not yet been studied. The relatively low concentrations of testosterone and estrone (average value of 200 pg/g) suggest a rapid conversion into dihydrotestosterone and estradiol respectively. Pregnenolone*, present in high amounts (average value of 20 ng/g), could be a precursor for steroidogenesis, as is suggested for insects by De Loof and De Clerck (1986). In future research, experiments with radiolabeled steroid precursors will be necessary to elucidate the biosynthetic pathway.

---

* Pregnenolone was already identified by GC-MS and its presence is now confirmed by this study. In order to succeed in further GC-MS analysis, we have to deal with the following remarks:

1. When we look to the RIA measurements (Van Beek and De Loof, 1988) pregnenolone (out of the six investigated steroids) is present in the highest concentration (mean value of 20 ng/g fresh weight). For the identification of the other steroids probably more than 15 g of frozen animals is needed (see 4.).
2. Steroid concentration varies during vitellogenesis. Therefore, animals must be collected more accurately according to their reproductive stage and the investigated steroid. Although it is easy to collect a bulk mass of material, it is difficult to determine the moment when concentrations have reached peak levels.
3. Our purification procedure appears to be satisfactory both for RIA quantification and GC-MS analysis.

The possible functions of these steroids remain to be elucidated. For insects, where research in this domain is more advanced, some suggestions have been made. Injected estradiol-17ß into whole pupae and isolated pupal abdomens of Bombyx mori inhibited the rate of oviposition when admittedly extremely high doses were used (Ogiso and Ohnishi, 1986) (see also 2.3.). In Artemia, the high amounts of estradiol may be of some importance in egg-release.

Vitellogenesis and moulting are closely connected in crustaceans. In Artemia, after the release of the brood from the uterus (stage 5), a moult occurs. Then the next batch of eggs descends from the ovaries into the oviducts (stage 6). Van Beek et al. (1987a) described the relationship between vitellogenesis and moulting in Artemia. Vitellogenic cycles alternate with the moult cycles and ecdysone concentrations reach peak values when vitellogenesis is most intensive. It remains to be demonstrated whether or not there exists an interaction between ecdysteroid-steroids and the other steroids during vitellogenic cycles.

Reviews of Adiyodi and Adiyodi (1970) and Kleinholz and Keller (1979) describe the complex system of eyestalk stimulating and inhibitory hormones, brain factors and gonadal hormones, controlling reproduction in crustaceans. Neurosecretory control of egg-laying was also described in snails (Kupfermann, 1972; Geraerts and Bohlken, 1976; Takeda, 1977; Rothman et al., 1983; Scheller and Axel, 1984). In insects, neuropeptides trigger ecdysone synthesis in the ovary (Hagedorn et al., 1979; Charlet et al., 1979). Neurosecretion in Artemia was already described by Van den Bosch de Aguilar (1976, 1977) (see Chapter 2) but until now it is not known whether in some of the diverse neurosecretory cells a gonadotrophic factor inducing egg-laying is present as is the case in gastropods e.g.(Takeda, 1979).

As at least some non-ecdysteroid steroids show up in all invertebrate species, when they are looked for, it is probable that they are universal biomolecules as suggested by Sandor (1980). The elucidation of their roles will require additional research.

CHAPTER 7: IMMUNOCYTOCHEMICAL LOCALISATION OF A FEW NEUROPEPTIDES IN ARTEMIA SP..

1. Introduction.

Organisms perceive all kinds of stimuli coming from the external environment. They have to adjust their physiology accordingly. Two systems ensure internal regulation: the nervous system and the endocrine system.

There are also neurons in the central nervous system with axons that are not ending at an effector cell but close to or in the circulatory system. These cells synthesize and secrete into the body fluid, material of a proteinaceous nature and with an endocrine function. Therefore these neurons are called neuroendocrine cells (van Deijnen, 1986).

The study of neuroendocrine cell systems reveals that neurology and endocrinology are two closely related fields. Twenty years ago, neurosecretory cells that release their biologically active peptides into the circulation or at 'neurosecretomotor junctions' were denoted as 'peptidergic neurons' (see review by Scharrer, 1978). Peptides that are known to have an hormonal function, can also act as neurotransmitters or they can be recognized as neuromodulators.

Abbreviations used in this chapter:
PYY: peptide tyrosine tyrosine, MSH: melanocyte stimulating hormone, ACTH: adrenocorticotropic hormone, LPH: lipotropic hormone, CRF: corticotropin releasing factor, GRF: growth releasing factor, VIP: vaso-intestinal peptide, CCK: cholecystokinin, TRH: thyrotropin releasing hormone.

From numerous recent data in comparative endocrinology, it has become clear that there is much more similarity between the vertebrate and invertebrate endocrine system than previously thought. Furthermore the nervous system in invertebrates now also appears to be much more complex than thought earlier. Recently, a lot of 'typical' vertebrate-type neuropeptides have been found in insects (Verhaert, 1987), in crustaceans (van Deijnen, 1986), in coelenterates (George and Dubois, 1984), in annelids (Rzasa et al., 1984) and in molluscs (Martin et al., 1984).

Neurosecretory cells in Artemia salina were described by various authors (see Chapter 2). The identity of the neurosecretory materials in those cells is still unknown. In this chapter, we have looked in Artemia, for neuropeptides playing a role in reproduction of vertebrates, namely gonadotropins. We also investigated the presence of other neuropeptides with the PAP-method. The antisera which used in this study had previously been tested on insect nervous tissue.

With this immunocytochemical technique we have been able to demonstrate neurosecretory cells not yet observed earlier.

2. Role of neuropeptides.

A number of data are available about the presence of vertebrate-type neuropeptides in invertebrates, but their role is still unclear.

Immunocytochemical observations by van Deijnen et al. (1985) in the crayfish Astacus leptodactylus reveal a more extensive peptidergic system than was recognized previously. They distinguish 30 cell groups in the nervous system of the eyestalk. Their results support the view that substances immunologically and structurally related to peptides with a hormonal function are ubiquitous in invertebrates.

Enkephalin-like immunoreactivity in the retina cells of the spiny lobster Panulirus interruptus suggests a possible neurotransmitter function of enkephalin or of a closely related peptide in the primary photoreceptors (Mancillas et al., 1981). Met-enkephalin stimulates the release of pigment concentrating hormones in the fiddler crab Uca pugilator (Quackenbush and Fingerman, 1984). The integumentary chromatophores are important for protection against predation and UV radiation, for temperature regulation of moulting, and perhaps as an indicator of reproductive readiness (Quackenbush and Herrnkind, 1983a). Fingerman et al. (1985) reported the presence of substance P-like, Leu- and Met-enkephalin-like, and FMRFamide-like immunoreactivity in the eyestalk of U. pugilator. They suggested that at least part of the material responsible for SP-like and Leu-enkephalin-like immunoreactivity, was derived from material that was axonally transported into the eyestalk. All the peptides located were apparently present in non-neurosecretory neuronal components and this suggests that they function as neuroregulators (neuromodulators/neuro-transmitters). Moreover, the presence of SP-like and enkephalin-like immunoreactivity in the sinus gland would suggest that they have a role in the modulation of the release of neurohormones. On the other hand, they could possibly have sequences in common with some of the peptide neurohormones released from this neurohaemal organ.

Results of experiments with gonadotropins in Idotea balthica basteri (Souty and Picaud, 1984) and in Crangon crangon (Bomirski and Clegg, 1976) suggest the presence of a hormonal system, which regulates reproduction and which is analogous to vertebrates. In these crustaceans, HCG (Human Chorionic Gonadotropin) stimulates vitellogenin synthesis. Cooremans (1984) already immunocytochemically demonstrated the presence of a gonadotropin-like substance in Crangon crangon. Future research will elucidate the relation of these gonadotropins with Gonad Stimulating Hormone (GSH).

3. Immunocytochemical localisation of gonadotropins, FMRF-amide, Neuropeptide Y, Methionine- and Leucine-enkephalin in the brain and suboesophageal ganglion of adult brine shrimp Artemia sp..

3.1. Immunocytochemistry.

Preparations for immunocytochemistry are described in Appendix V.

3.2. Primary antisera.

FMRFamide antiserum (code 544), a gift from C.J.P. Grimmelikhuijzen (University of Heidelberg, FRG), was raised in Guinea pig (GP). This antiserum is selected for its inability to react with bovine and avian pancreatic polypeptide (BPP and APP). Also its unique staining properties in colchicine-treated and untreated Guinea pig brain have revealed that it does not cross-react with any of the other known brain peptides (Triepel and Grimmelikhuijzen, 1984).

The Neuropeptide tyrosine (NPY) or Neuropeptide Y antiserum was radioimmunologically characterised and only weak cross reactions were observed with synthetic APP (0.1 percent) and PYY (0.4 percent). Anti-NPY did not cross-react with α-MSH, 1-39 ACTH, ß-LPH, CRF, ß-endorphin, Leu-enkephalin, Met-enkephalin, GRF, VIP, CCK, somatostatin, TRH and neurotensin (Danger et al., 1985). Also its specificity has been checked by liquid-phase adsorptions with the homologous antigen (NPY) on sections of insect brain (Schoofs et al., 1988a) and with various heterologous antigens such as BPP, PYY, FMRFamide and τ_3-MSH on sections of frog (Danger et al., 1986) and feline (Léger et al., 1987) brain. The immunoreaction was totally abolished after preincubation with NPY, but was not affected by preincubation with the heterologous antigens.

The Methionine- and Leucine-enkephalin antisera were raised in rabbits. Emulsions consisting of equal volumes of Freund's complete adjuvant and the conjugate were injected subcutaneously. For this purpose, the two pentapeptides (both from UCB Bioproducts, Belgium) were linked with bovine thyroglobulin (Sigma) by carbodiimide as described by Skowsky and Fisher (1972). Serum specificity was examined earlier by means of solid and liquid phase adsorption. The preabsorbed enkephalin antisera were applied to microscopic

sections of several insect species (Verhaert and De Loof, 1985; Schols et al., 1987; Schoofs et al., 1988c). Antigen spot tests, where antigens were immobilized by dotting on nitrocellulose (NC), revealed that Leu-Enk antiserum showed no affinity to Met-Enk, and that Met-Enk antiserum only weakly recognized NC dotted Leu-Enk (Schols et al., 1987).

In this study we also used antisera against human Luteinizing Hormone (hLH, from UCB), Luteinizing Hormone Releasing Hormone (antiserum from synthetic LHRH) and human Follicle Stimulating Hormone (hFSH, from UCB).

A working dilution of 1:2,000 for NPY and LH-RH, and 1:1,000 for the other antisera, appeared to be suitable during 16 hr of incubation. All dilutions were made in Tris-saline (0.01 M Tris/HCl buffer, pH=7.6, containing 0.9 percent NaCl). Afterwards, in the case of FMRFamide, an anti-GP-antiserum conjugated to horseradish peroxidase (Nordic, Tilburg, The Netherlands) was used. Routinely, goat anti-rabbit globulin (GAR) was used as secondary antiserum, followed by rabbit peroxidase-anti-peroxidase complex. GAR and PAP antisera were kindly put at our disposal by Prof. Dr. F. Vandesande. The diaminobenzidine reaction, described by Graham and Karnovsky (1966), was used to visualize the peroxidase attached in the PAP complex. After staining the sections were dehydrated and mounted in DePeX mounting medium (British Drug House, Poole, U.K.).

3.3. Specificity tests.

Method specificity was checked by the application of non-immune rabbit serum (diluted 1:1,000) and also by a series of PAP-stainings in which each reagent was in turn omitted from the regular staining sequence. Serum specificity was also verified by liquid phase adsorption. To this end, diluted antisera and antigen solutions (10^{-6} M) were preincubated for 24 hr at 4°C. After centrifugation (4 min, at 3,000 rpm in a Beckman Microfuge B) the supernatants were used in the immunocytochemical stainings. Double PAP-staining was performed according to Vandesande (1983b) (see Appendix V.3.). Of the purified antigens: enkephalins were from UCB, FMRFamide from Bachem (Switzerland) and synthetic NPY was a generous gift of Dr. S. Saint-Pierre (Sherbrooke University).

Some of the sections were subsequently counterstained for 1 min in Mayer's hemalum solution to trace the site of staining in the cytoplasm.

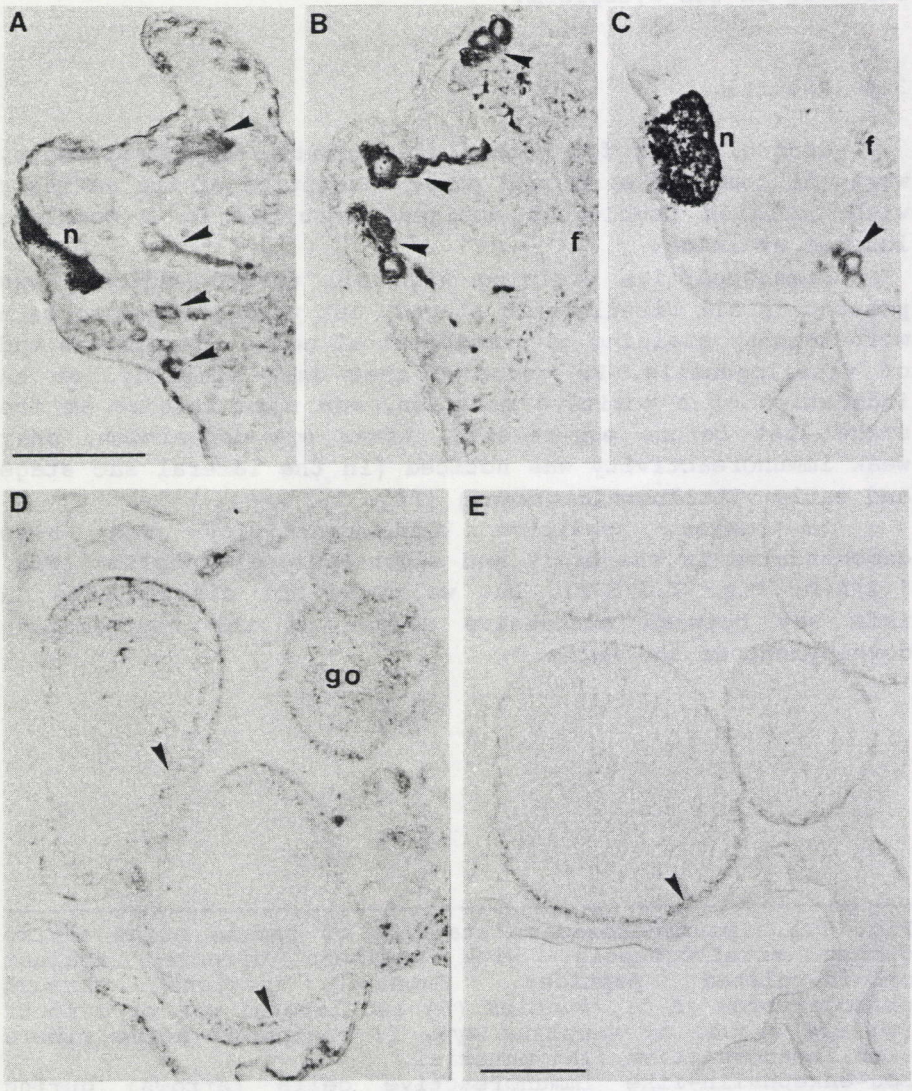
3.4. Results.

Control tests for method specificity, mentioned above, were all conclusive. Liquid phase adsorption of the antisera with purified homologous antigens resulted in a complete loss of staining.

Immunopositive staining with all tested antisera was present in all vitellogenic stages, but there seemed to be a more intense staining of protocerebral cells towards the end of vitellogenesis. We observed that dark staining, as an indication of a positive reaction, was more intense at the stage just before egg-release. After egg deposition, only weak immunoreactivity was noticed (in the lateral sac stage and early vitellogenic stages) (Fig. 7.1/A-C).

In males, positive immunoreactivity was also demonstrated in the brain and suboesophageal ganglion (Fig. 7.2/A-D, Fig. 7.3/E-F), but we could not discriminate in this way between successive stages of the reproductive development of the males.

Fig. 7.1. Immunoperoxidase staining of female brine shrimp during vitellogenesis, with antisera directed against opioid-related peptides. Frontal sections through protocerebrum (A-C), ovaries (D) and lateral sac or oviduct (E) are shown. n: nauplius eye, f: region of nerve fibers with immunoreactive (IR) material.
Leu-enkephalin-like immunoreactive cells (arrows) during early vitellogenesis (A), during late vitellogenesis (B) and during the lateral sac stage (C). Note the most sensitive immunoreaction is shown in the late vitellogenesis stage and the presence of axons of the median neurosecretory cells.
Met-enkephalin-like IR material is evenly distributed in growing oocytes (go) but is more concentrated at the periphery of vitellogenic oocytes and ripe oocytes (arrows).
Bar A-D and E, 10 μm.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C
n
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D
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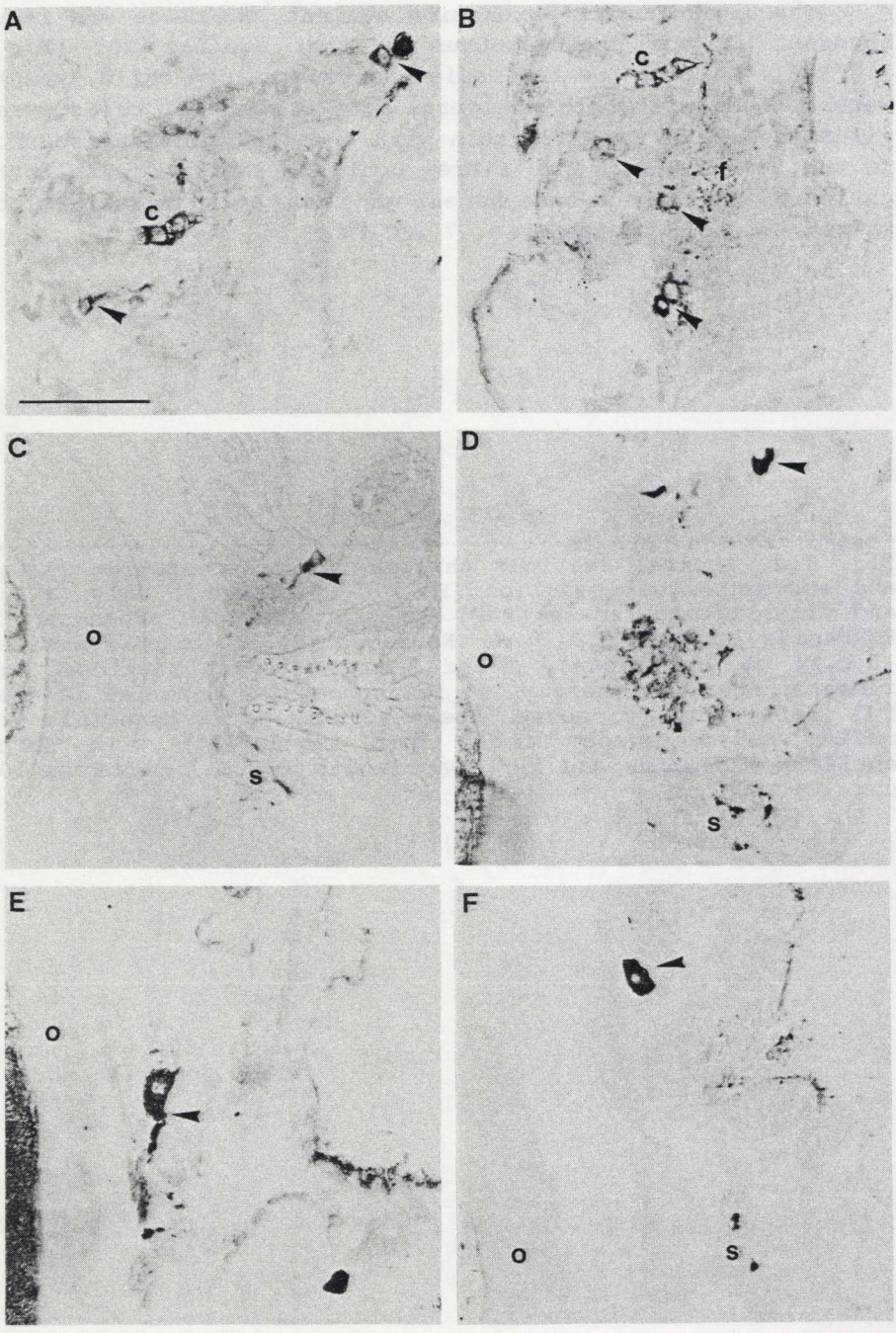
E

3.4.1. FMRFamide and Neuropeptide Y.

The immunopositive reaction against FMRFamide was less abundant in the protocerebrum as that against NPY (Fig. 7.2/A-B). Besides the few median neurosecretory cells, which were stained with both antisera, many more small cells were visualised with the NPY- than with the FMRFamide-antiserum. In the same areas, nerve fibres could be labelled with both antisera. In the sub-oesophageal ganglion both antisera also marked two cell bodies (Fig. 7.2/C-F).

Fig. 7.2. Frontal sections through the protocerebrum (A-B) and suboesophageal ganglion (SOG; C-F) of adult male (A-D) and vitellogenic female (E-F) Artemia, show the presence of FMRFamide-like (A,C,E) and Neuropeptide Y immunoreactive (B,D,F) cells (arrows). A and B are adjacent sections, o: suboesophageal epithelium, s: immunoreactive material in the SOG (left part), f: nerve fibers containing IR material, c: median neurosecretory cells that immunoreact with both antisera FMRFamide and NPY. Bar length for all parts is 10 µm.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3.4.2. Leu- and Met-enkephalins.

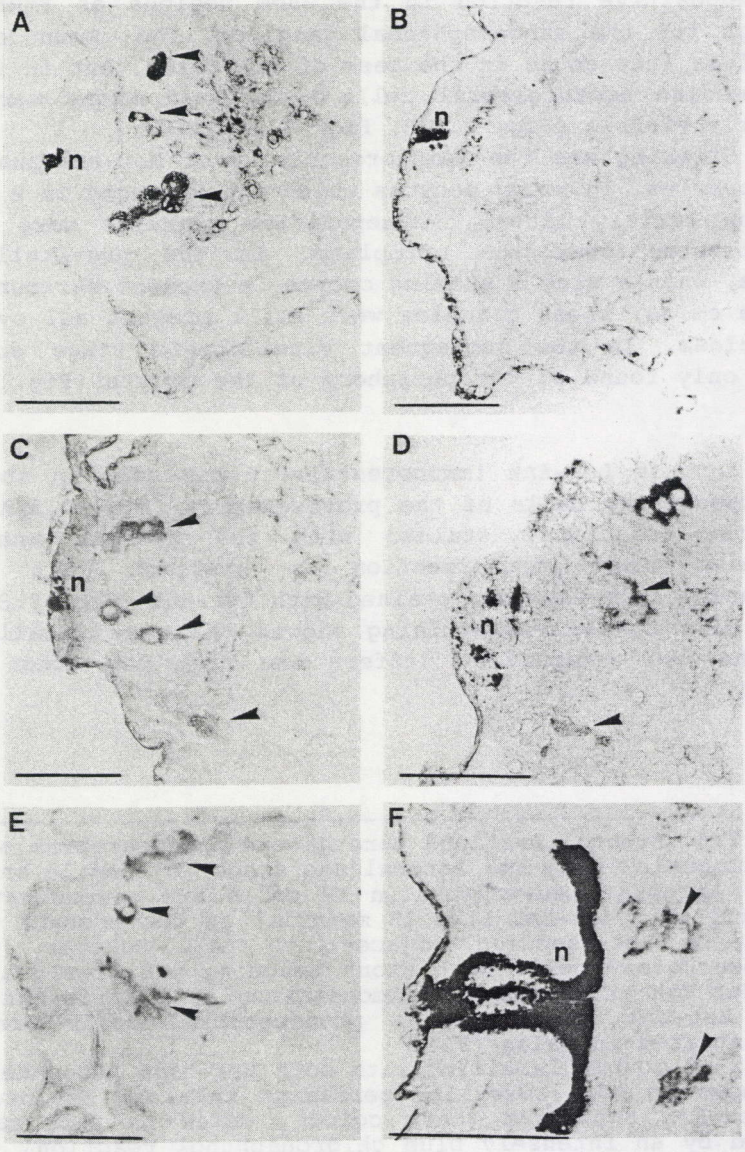
The antisera against these peptides showed immunopositive reaction in the same regions as FMRFamide, except for the suboesophageal ganglion. The immunoreaction was less intense as in the case of FMRFamide, but in females the median protocerebral cells with their axons were very clearly visible (Fig. 7.1/B, Fig. 7.3/C).

Striking was the immunoreactivity of Met-enkephalin in the ovaries. In young oocytes which are arranged in a curved ribbon (Criel, 1980), immunopositive granules were evenly distributed over the cytoplasm. In the previtellogenic stage, when a single growing oocyte is present surrounded by nurse cells, these granules were still present all over the cytoplasm. In the subsequent vitellogenic stage granules were only found at the periphèry of the oocyte (Fig. 7.1/D-E).

Intense Leu-Enk immunoreactivity was seen in about 10 neurosecretory cells of the protocerebrum (Fig. 7.3/A). The adjacent sections, stained with the Met-Enk antiserum revealed weak immunoreaction in distinct cell bodies different from the ones stained with Leu-Enk (Fig. 7.3/B-C).

Also double PAP-staining showed that the localisation of the two enkephalins differs one from the other (Fig. 7.3/D).

Fig. 7.3. Frontal sections through the protocerebrum of mid-vitellogenic (A-C) and lateral sac stage (D) female and male (E-F) Artemia. Leu-enkephalin IR cells are demonstrated in (A) and (E). Met-Enk-like IR material is not present in (B) which is the section adjacent to (A). However, median neurosecretory cells with axons reacting positively against the Met-Enk antiserum are demonstrated at a different level (C). Met-Enk immunoreactive protocerebral cells are also demonstrated in males (F).
Result of double labelling with both Met- and Leu-enkephalin is shown in (D), revealing perikarya that are stained only by Leu-Enk antiserum (dark colour, which in this case is caused by an intensely blue chloronaphthol reaction) in the lateral part of the protocerebrum. The median neurosecretory cell bodies stained by Met-Enk are indicated by arrows (lighter colour, brown diaminobenzidine staining). n: dark staining due to the pigment of the nauplius eye. All bars, 10 μm.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3.4.3. LH, LHRH and FSH.

No immunoreactivity could be found in the protocerebrum or in the SOG region when using LH-RH and FSH antisera, neither in males nor in females. With the LH antiserum only weak immunoreaction was demonstrated in the lateral parts of the protocerebrum of male and female shrimps. Two or three small neurosecretory cells reacted positively (Fig. 7.4).

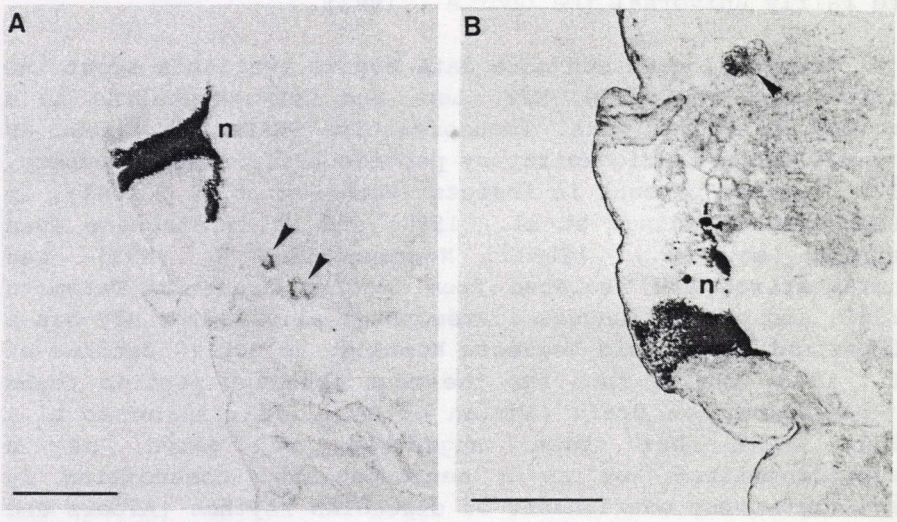

Fig. 7.4. Frontal sections through the protocerebrum of male (A) and mid-vitellogenic female (B) Artemia. Two weak immunoreactive perikarya containing LH-like material are shown in the lateral part of the protocerebrum. n: dark staining of the pigment granules of the nauplius eye. Bars, 10 µm.

3.5. Discussion.

In vertebrates, neuropeptides are biologically active compounds acting as neurotransmitters, neuronal modulators, and coordinators of growth, maturation and muscular activities. Greenberg and Price (1983) reviewed the invertebrate peptides and made a distinction between two groups of neuropeptides, namely the 'native' and the 'naturalised' types. The latter type is identified primarily by its binding to antisera raised against known vertebrate peptides. In our study, we demonstrated the presence of four neuropeptides of this type in male and female adult Artemia. There is recently a tendency to omit this classification and to think rather in terms of endocrinological principles that are fairly universal (De Loof A., 1987).

Recently, more and more data became available about the presence of FMRFamide, NPY, Leu- and Met-enkephalins in a variety of animal taxa. Immunoreactive FMRFamide, known as the molluscan cardioexcitatory peptide (Price and Greenberg, 1977) is also present in insects (Verhaert et al., 1985), in nemerteans (Jennings et al., 1987) and in crustaceans (van Deijnen et al., 1985). Neuropeptide Y (NPY) was characterized and isolated from porcine brain by Tatemoto (1982) and his colleagues (Tatemoto et al., 1982). NPY has a 50 percent amino acid sequence homology to BPP (O'Donohue et al., 1985) and is thus far the most abundant peptide found in rat and human brain (Adrian et al., 1983, Allen et al., 1983). NPY has been suggested to serve as a neurotransmitter, or as a neuromodulator controlling in vertebrates the entrainment of circadian rhythms (Albers and Ferris, 1984), the food and water intake (Levine and Morley, 1984) and the secretion of hormones such as Luteinizing Hormone (LH) and Growth Hormone (GH) (McDonald et al., 1985). Its presence in frog brain was demonstrated by Danger et al. (1985). Our immunocytochemical investigation, together with the study of Schoofs et al. (1988a), who found NPY immunoreactive cells in the brain of insects, are the first reports on the presence of immunoreactive NPY in invertebrates.

Enkephalins, first discovered in higher vertebrates by Hughes (1975), are members of the family of endogenous opioids that have been shown to affect systems involved in the perception of pain (Scharrer, 1978). Molecules immunologically related to opioid peptides are also present in insects (Verhaert and De Loof, 1985; De Loof, 1987; Schoofs et al., 1988c), crustaceans (Mancillas et al., 1981; Jaros and Keller, 1984; Jaros et al., 1985), and molluscs (Martin et al., 1984). Leung and Stefano (1984) even isolated and identified enkephalins in Mytilus edulis (Mollusca).

Fingerman et al. (1985) reported the presence of substance P-like, Leu- and Met-enkephalin-like, and FMRFamide-like immunoreactivity in the eyestalk of Uca pugilator. They suggested that at least part of the material responsible for substance P-like and Leu-enkephaline-like immunoreactivity, was derived from material that was axonally transported into the eyestalk. Earlier immunohistochemical observations in the laboratory of A. De Loof on brain sections of Crangon crangon demonstrated the presence of FMRFamide, Leu- and Met-enkephalin in immunoreacting cerebral neurosecretory cells (Cooremans, 1984), and are in agreement with the present study. We ignore if the primary structures of the demonstrated peptides are identical to the typical vertebrate molecules. However the widespread distribution throughout the animal kingdom of these vertebrate resembling peptides, suggests that they are of an evolutionary conserved nature.

In Artemia, only a few immunoreactive cells (see Fig. 7.2/A,B) are stained by both NPY and FMRFamide antisera in the protocerebrum. The cross-reaction of both types of antisera is not so surprising. The common chemical feature of the tetra-peptide FMRFamide (Phe-Met-Arg-Phe-NH_2) and NPY (a 36 amino acid peptide) is the C-terminal amide structure: both have an arginine and an amidated aromatic amino acid in similar positions (RFamide and RYamide, respectively). The FMRFamide antiserum chosen does not cross-react with BPP or APP. Since NPY is a member of the PP family cross reaction is not to be expected. Nevertheless we clearly observed a few cells immunoreacting with both antisera. Further research will reveal whether this is due to the recognition of FMRFamide immunoreactive cells by the NPY antiserum, or vice versa. Many immunopositive fibers are also present

throughout the neuropile region of the brain, suggesting that FMRFamide and NPY-like peptides might function as neuromodulators or as neurotransmitters. This is suggested for FMRFamide in molluscs by Cottrell et al. (1983a) . In decapod crustaceans, Hooper and Marder (1984) suggest that the presence of an FMRFamide-like substance in fibers of the stomatogastric nerve (STN), is correlated with the pyloric system of the stomatogastric ganglion (STG). These fibers originate in the paired commissural ganglia (CG) and the single oesophageal ganglion (OG) and modulate the neural action of the STG.

Although Leu- and Met-enkephalin differ only by one amino acid (Leu-Enk : Tyr-Gly-Gly-Phe-Leu and Met-Enk : Tyr-Gly-Gly-Phe-Met), the localization of these two pentapeptides in the protocerebral cells of the brine shrimp is distinct. A similar phenomenon was also observed in earlier studies on insects (see 3.2.).

In vertebrates, Rossier et al. (1986) demonstrated pro-enkephalin, a precursor molecule of Met-enkephalin. Stern et al. (1979) isolated Met-enkephalin (Arg^6-Phe^7) from bovine adrenal medullary granules and striatum. The amidated form of this opioid heptapeptide (Tyr-Gly-Gly-Phe-Met-Arg-Phe-NH_2 or YGGFMRFamide) has been shown to have effects similar to FMRFamide in the invertebrate Helix (Cottrell et al., 1983b). These concurrent data not withstanding in Artemia, FMRFamide and enkephalin immunoreactivity certainly show distinct localizations. Beside immunoreactivity in the brain region, FMRFamide is present in the SOG, whereas Met-Enk is present in the ovaries.

The appearance of Met-enkephalin in the ovaries and its changes in distribution pattern during vitellogenesis suggest that Met-Enk-like peptide might play a role in reproductive physiology. These findings can be compared to the results of Schoofs et al. (1988c), who described the same phenomenon in the gonads of the insect species Locusta migratoria and Sarcophaga bullata. Georges and Dubois (1984) earlier observed Met-enkephalin immunoreactivity in the ovary of the protochordate Ciona intestinalis. The presence of Met-enkephalin-like material in gonads of several animal orders indicates that we are dealing with a biological process of a general nature although its physiological

role remains to be elucidated. The possibility exists that a larger molecule than Met-enkephalin is recognized in the ovaries by the antiserum. This molecule can be quite unrelated to Met-enkephalin but it shares at least one epitope as is demonstrated by preadsorption experiments.

An intensive immunoreaction (for all of the 4 peptides) is observed during the second half of the female reproductive cycle, especially when the ovaries are full of ripe oocytes. Also many immunoreactive nerve fibres are found. Van den Bosch de Aguilar (1974) had already suggested that the secretory activity by the lateral protocerebral cells is correlated to reproduction. We have no evidence as yet that the immunopositive cells we demonstrated are indeed directly involved in control of reproduction.

Neuropeptides, such as LHRH and FSH, are known to play a role in reproduction in vertebrates. Although these hormones are present in adult American cockroaches (Verhaert and De Loof, 1986), they could not be detected in Artemia. Only weak immunoreaction can be observed with the LH antiserum. Although androgenic steroids (C_{19}-steroids) are present in Artemia (Van Beek and De Loof, 1988), the presence of gonadotropins need to be further investigated to obtain more data on a possible interrelationship between peptides and steroids.

This study is the first one dealing with the presence of vertebrate neuropeptide-like material in a primitive crustacean. Further research will be necessary on the neuro-endocrinology of Artemia in order to draw up a fruitful comparison between this endocrinological system and that described in higher Crustacea where inhibiting and stimulating factors derived from the X-organ-sinus gland complex regulate several physiological functions (Legrand et al., 1982).

4. Concluding remarks.

It seems that in invertebrates, Artemia included, all of a sudden a whole range of substances are showing up with potential hormonal functions. They are similar or closely related to substances which in vertebrates function as hormones, active peptides or neuropeptides.

That the demonstrated lateral protocerebral cells possess neurohormones that might influence the secretion of lipovitellin material from the thoracopods into the haemolymph needs further investigation. According to Van den Bosch (1974), the activity of the neurosecretory cells of the median protocerebral region varies in the brine shrimp in relation to salinity changes. It could well be possible that these cells also intervene in the ecdysteroid synthesis while the activity of these cells increases during vitellogenesis. This occurs just at the period when the ecdysteroid concentration reaches its peak level (see Chapter 5).

In the insect Rhodnius prolixus, release of the prothoracotropic hormone (PTTH) from the brain (probably the median neurosecretory cells of the pars intercerebralis) stimulates the prothoracic glands which in turn produce ecdysteroids (Steel et al., 1982). Also in Drosophila, two or more neural factors interact to stimulate ecdysteroid synthesis by the larval ring gland (Henrich et al., 1987).

Future research can comprise further characterization of the localized neuropeptides by HPLC and RIA (e.g. Jaros et al., 1985). Application of extracts from nervous tissue to bioassays can also give some information about the structure and activity of the neuropeptides that are immunocytochemically demonstrated (e.g. Schoofs et al., 1988b).

CHAPTER 8: IN VITRO SYNTHESIS OF LIPOVITELLIN.

1. Evidence for the site of lipovitellin synthesis by in vitro experiments.

By means of UV-spectrophotometry, we had earlier measured the total protein concentration in haemolymph during vitellogenesis (see Fig. 8.1). The increase in total protein content was proportional with the gradual increase of lipovitellin components present in haemolymph samples during vitellogenesis. Haemolymph proteins were separated on SDS-PAGE and the specificity was checked by immunoblotting. These results were the first indications that there was an exogenous source of lipovitellin in the brine shrimp.

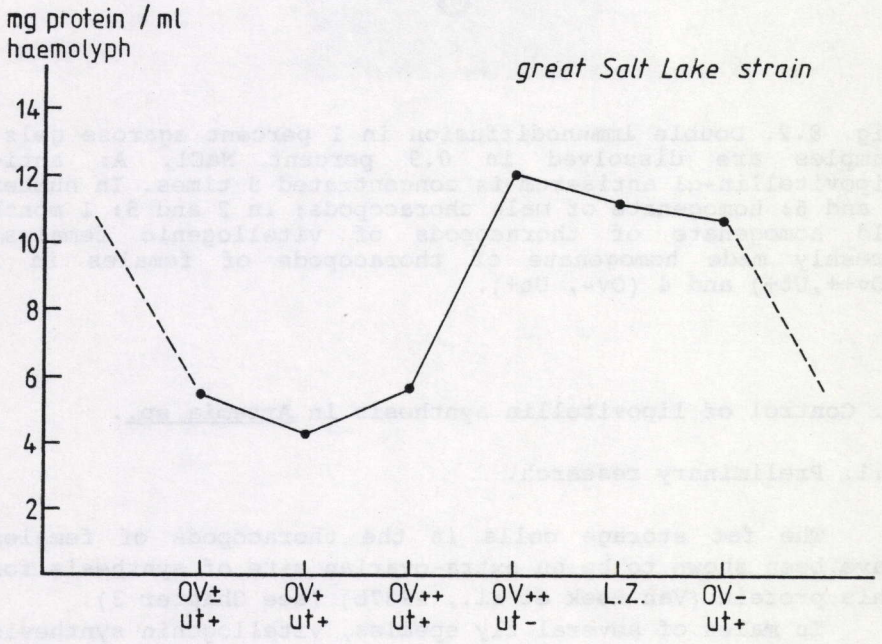

Fig. 8.1. Total protein concentration in haemolymph during vitellogenesis.

Microscopical investigations (see Chapter 3) revealed a possible site for lipovitellin synthesis. This observation was confirmed when we subjected an extract of female and male thoracopods to double immunodiffusion: whereas female thoracopod extract gave a clear precipitation arc, male extracts did not (see Fig. 8.2). To further document this; we incubated female thoracopods in the presence of sulphur radiolabelled methionine. Afterwards, immunoprecipitation has been applied to the incubation medium in order to identify the present proteins. We succeeded in confirming our results with <u>in vitro</u> RNA translation experiments.

The <u>in vitro</u> incubation of thoracopods, RNA-extraction and <u>in vitro</u> translation are described in Appendix VII.

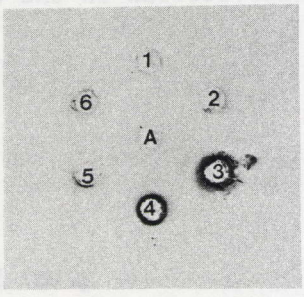

Fig. 8.2. Double immunodiffusion in 1 percent agarose gels. Samples are dissolved in 0.9 percent NaCl. A: anti-lipovitellin-α1 antiserum is concentrated 5 times. In number 1 and 6: homogenate of male thoracopods; in 2 and 5: 1 month old homogenate of thoracopods of vitellogenic females; freshly made homogenate of thoracopods of females in 3 (Ov++,Ut+) and 4 (Ov-, Ut+).

2. Control of lipovitellin synthesis in <u>Artemia sp.</u>.

2.1. Preliminary research.

The fat storage cells in the thoracopods of females have been shown to be an extra-ovarian site of synthesis for this protein (Van Beek et al., 1987b) (see Chapter 3).

In males of several fly species, vitellogenin synthesis can be induced by 20-OH-ecdysone. According to Cardoen et al. (1986) the mRNA for vitellogenin is not present in untreated male flies.

In preliminary experiments we tried to induce vitellogenesis in male Artemia by micro-injection of ecdysone, 20-OH-ecdysone, juvenile hormone III, progesterone and estradiol. No traces of lipovitellin in male haemolymph could be detected after injecting any of these substances with the exception of ecdysone. Administration of ecdysone resulted in the appearance of a high molecular weight protein band (MW=120,000) detected by SDS-PAGE and which could be compared with LV-α10. Immunoblotting with anti-LV-α1 antiserum however, showed no positive reaction. Further investigation with micro-injection experiments is needed to find out whether ecdysone is needed for in vivo synthesis of a possible lipovitellin-like protein in males. However, in Artemia injection experiments followed by collection of haemolymph is difficult.

Gohar and Souty (1983) incubated fat body of the male isopod Porcellio dilatatus in the presence of ^{14}C-leucine and deprived of all female hormonal influence. They found the rate of vitellogenin synthesis comparable with that from females.

In order to obtain information about the possible hormonal control of lipovitellin synthesis in Artemia, we decided to use in vitro experiments. These were routinely carried out by incubating male thoracopods in the presence of ^{35}S-methionine.

2.2. In vitro incubation of male thoracopods.

The same in vitro culturing system was used as described in Appendix VII.

Total RNA was extracted also from male thoracopods and its concentration was similar to that of female thoracopods, namely 200-320 µg total RNA per 200 animals. The same translation system was used (see Appendix VII).

2.3. Results.

The electrophoretic patterns of haemolymph and thoracopod extracts in Fig. 8.3 show no pronounced differences between male and female samples. However, immunoblotting reveals that in males, lipovitellin is neither circulating in the haemolymph, nor present in the thoracopods, the site of synthesis in vitellogenic females.

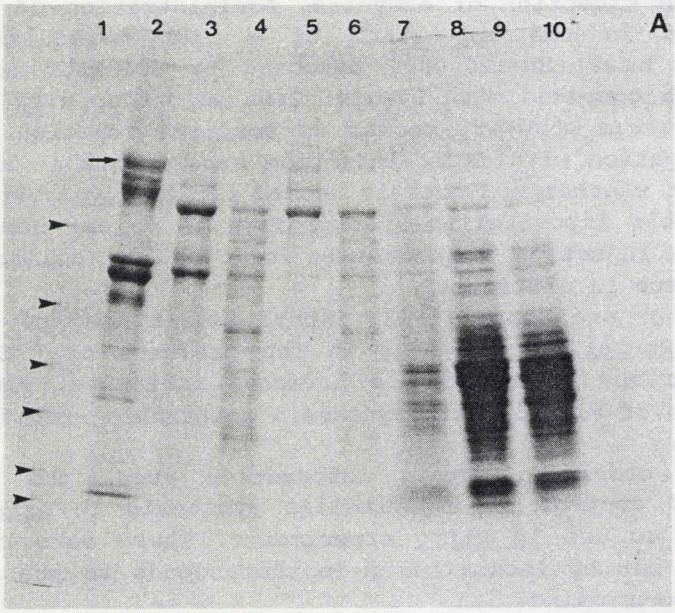

Fig. 8.3./A. Electrophoretic pattern on a 5-15 percent gradient gel after SDS-PAGE, constant current of 30mA/gel. Lipovitellin-α1 with MW=190,000 is indicated by an arrow. Lane 1: low molecular weight proteins from Electrophoresis calibration kit (Pharmacia) used as markers (Phosphorylase b, Bovine Serum Albumin, Ovalbumin, Carbonic Anhydrase, Soybean Trypsin Inhibitor and α-Lactalbumin with respective MW of 94,000, 67,000, 43,000, 30,000, 20,100 and 14,400). Lane 2: lipovitellin sample. Lane 3: male haemolymph. Lane 4: female haemolymph (L.S.). Lane 5: haemolymph from vitellogenic female. Lane 6: female haemolymph (Ov-,Ut+). Lane 7: broodsac homogenate from vitellogenic females. Lane 8: male thoracopod homogenate. Lane 9: thoracopod homogenate from vitellogenic females. Lane 10: sample buffer.

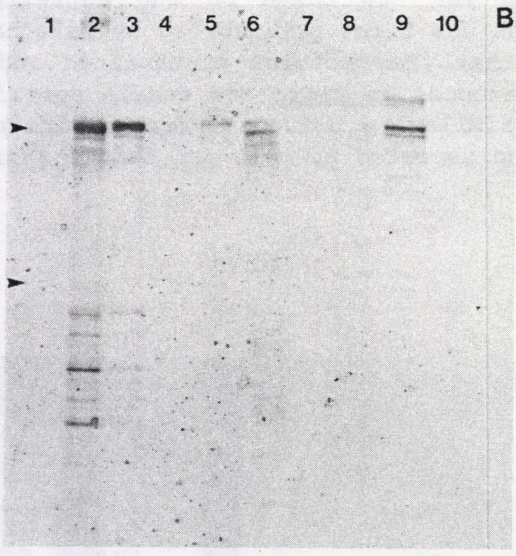

Fig. 8.3./B. Immunoblotting on nitrocellulose sheet (0.45 μm) according to the method of Peferoen et al. (1982). Lipovitellin-α1 (MW=190,000) and lipovitellin-ϵ (MW=67,000) are both indicated by an arrow. Lane 1: homogenate of male animals. Lane 2: lipovitellin sample. Lane 3: homogenate of ovaria. Lane 4: male haemolymph. Lane 5: homogenate of lateral sacs. Lane 6: haemolymph from vitellogenic females. Lane 7: female haemolymph (Ov-,Ut+). Lane 8: broodsac homogenate from vitellogenic females. Lane 9: thoracopod homogenate from vitellogenic females. Lane 10: male thoracopod homogenate.

In Fig. 8.4, the results obtained by immunoprecipitation are based on the anti-lipovitellin-α1 antiserum which was first preadsorbed by male haemolymph. We can conclude that the proteins synthetized and secreted by the male thoracopods in vitro are female specific. There is also a great similarity between immunoprecipitated products synthetised and secreted by male and female thoracopods.

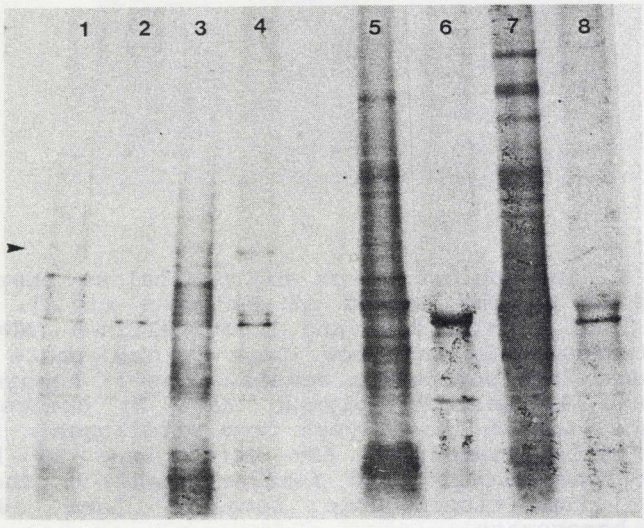

Fig. 8.4. Fluorography of in vitro synthesis by female (lane 1-4) and male (lane 5-8) thoracopods incubated for 4 hr in (^{35}S)-labelled methionine. Secreted (lane 1 and 5) and synthetised (lane 3 and 7) polypeptides are separated by SDS-PAGE. The pattern of the immunoprecipitated proteins from the incubation medium is shown in lane 2 and 6; the pattern of the immunoprecipitated proteins from the thoracopod homogenate is shown in lane 4 and 8. For this purpose lipovitellin antiserum underwent first liquid-liquid phase adsorption with an equal volume of male haemolymph. Method for immunoprecipitation is described in Appendix VII. The place of the marker with MW=67,000 is indicated by an arrow.

Fig. 8.5 gives an idea of the amount of synthetised proteins in the thoracopods (A) and of the secreted ones in the incubation medium (B). Thoracopod extracts show more or less the same pattern of incorporated radioactivity (for counting analysis see Appendix VII) under all conditions studied. An indication for the presence of lipovitellin-like material in the homogenate of thoracopods, is clearly seen in condition 4, namely in the presence of homogenate of heads from females in mid-vitellogenesis.

Secretion of proteins into the medium seems to be influenced by some factors present in the haemolymph when we compare the results from the control with the other experimental ones. Furthermore, this effect seems to be present only when haemolymph is added originating from the same sex as the thoracopods. Possible endogenous factors can play a role in this phenomenon, but when haemolymph is replaced by head extracts (male or female), secretion is stimulated 2-3 fold. There could be a stimulating factor present in the head compartments but when male haemolymph is added too, strong inhibition is observed. Only in the presence of head extracts from mid-vitellogenic and preadult females (condition 10 and 7 respectively) secretion of proteins into the medium reached the same level as when only male haemolymph is added.

Also incubated male heads together with male haemolymph show secretion activity (condition 14). This can be explained by the presence of some fat storage cells in the head region. In females these cells contain immunopositive granules after applying the peroxidase-anti-peroxidase technique using anti-LV-α1-antiserum.

Both the experiments with ecdyson (15) and with estradiol (16) fail to show a positive effect on the secretion activity. It is striking however, that under all conditions where secretion activity is low, relatively more immunopositive proteins are secreted than in the other experiments. Most lipovitellin-like proteins are secreted in experiment 12 (female head-homogenate, lateral sac stage) and in the presence of estradiol (experiment 16).

Fig. 8.5. Histogram of the rate of <u>in vitro</u> protein synthesis of male thoracopods. Incorporation of (^{35}S)-methionine into proteins synthetised by thoracopodal tissue (A) and into proteins secreted into the incubation medium (B). Specific incorporation of the label into synthetised (A) and secreted (B) yolk polypeptides is shown by the shaded parts of the bars. Expression of data, see materials and methods. Conditions used in the <u>in vitro</u> synthesis experiments: 1. Co; 2. Co + MaHae; 3. Co + FeHae; 4. Co + HeHom Fe/Ov+,Ut+; 5. Co + HeHom Ma; 6. Co + MaHae + HeHom Ma; 7. Co + MaHae + HeHom Fe/preadult; 8. Co + MaHae + HeHom Fe/Ov-,Ut+; 9. Co + MaHae + HeHom Fe/Ov±,Ut+; 10. Co + MaHae + HeHom Fe/Ov+,Ut+; 11. Co + MaHae + HeHom Fe/Ov++,Ut+; 12. Co + MaHae + HeHom Fe/L.S.; 13. Co + MaHae; 14. Ma He + MaHae; 15. Co + MaHae + Ecd; 16. Co + MaHae + Est; Co=control, Hae=haemolymph, Fe=female, Ma=male, He=head, Hom=homogenate, Ecd=ecdysone, Est=estradiol.
Equivalents of 3 heads were added to the incubation media except for condition 5 where 6 head equivalents were used.

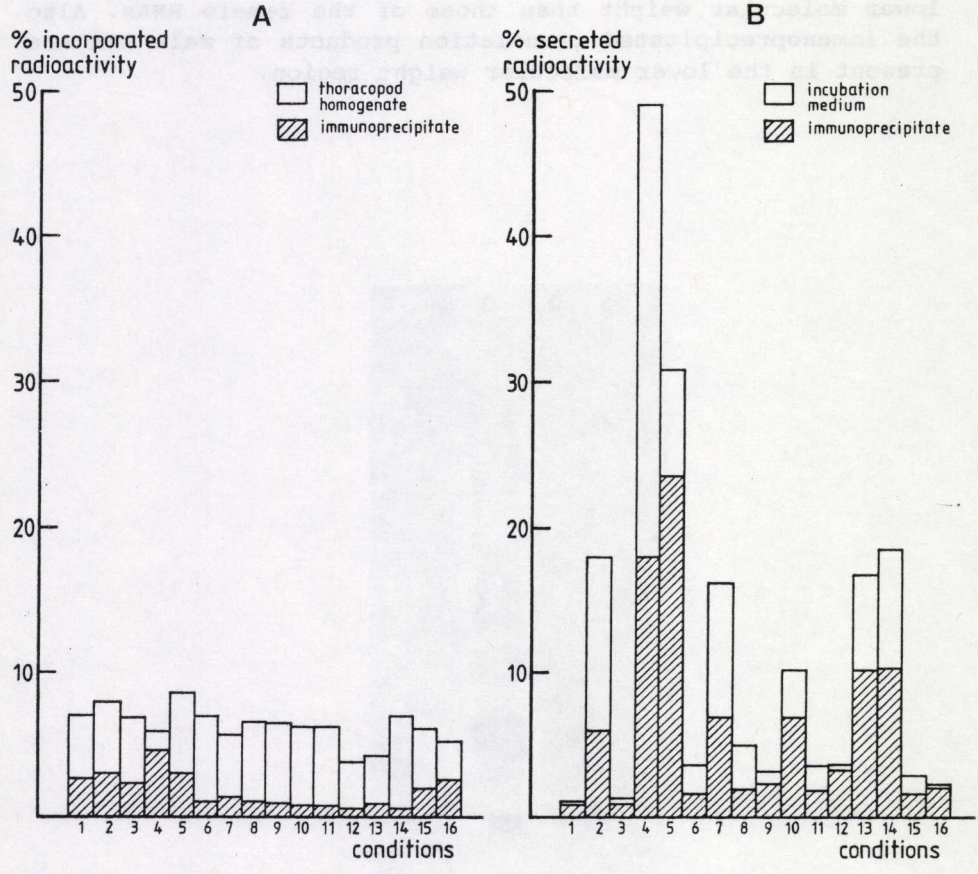
A
% incorporated radioactivity
thoracopod homogenate
immunoprecipitate
50
40
30
20
10
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
conditions
B
% secreted radioactivity
incubation medium
immunoprecipitate
50
40
30
20
10
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
conditions

Fig. 8.6 shows the translation products of total RNA of male and female thoracopods translated in rabbit reticulocyte lysate. The electrophoretic separation revealed that the translation proteins from the male RNAs have a much lower molecular weight than those of the female RNAs. Also the immunoprecipitated translation products of male RNA are present in the lower molecular weight region.

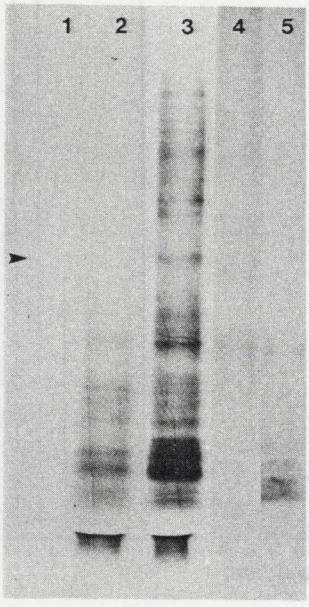

Fig. 8.6. Fluorography of <u>in vitro</u> translation of 4 µg total RNA extracted from male (Lane 2) and female thoracopods (Lane 3) in a rabbit reticulocyte lysate system. Immunoprecipitation with anti-LV-α1 antiserum of the translation products from male (Lane 5) and female thoracopods (Lane 4) translated in reticulocyte lysate. Endogeneous protein synthesis is shown in Lane 1 where no exogeneous RNA is added.
The place of the marker with MW=67,000 is indicated by an arrow.

Female thoracopods were also incubated with extracts of heads and the results are depicted in Fig. 8.7. This figure shows an increase in protein release in the presence of male and female head extracts from vitellogenic females. It confirms the results obtained from the in vitro study with male thoracopods.

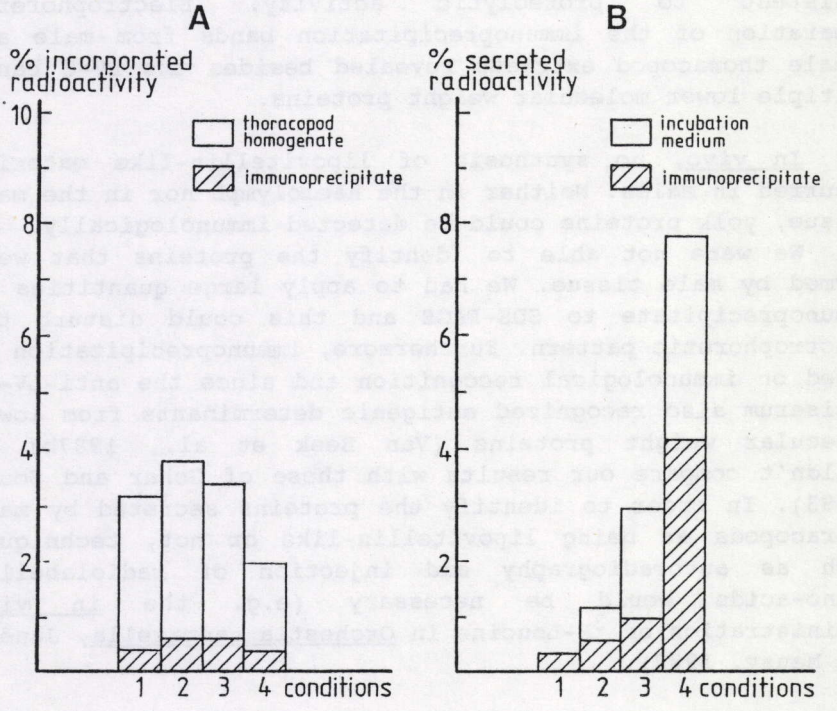

Fig. 8.7. Histogram of the rate of in vitro protein synthesis of female thoracopods (females varied from stage L.S. to Ov-,±). Conditions were: 1. Co + FeHae; 2. Co + FeHae + HeHom Ma; 3. Co + FeHae + HeHom Fe/Ov+,++,Ut+; 4. FeHe + FeHae. Explanation of abbreviations see legend to Fig. 8.5.

3. Discussion.

Working along lines similar to those of Gohar and Souty (1983) for Porcellio, we also tried to induce lipovitellin synthesis in male tissue. We incubated male thoracopods in different experimental conditions. Results revealed that after 4 hr, radioactivity was incorporated and secreted into the medium. Electrophoretic patterns showed that in thoracopod homogenates and in incubation media of all experimental conditions, LV-ϵ was present. We have already mentioned the proteolytic breakdown of the lipovitellin complex: LV-α1 is completely degraded while LV-ϵ was resistent to proteolytic activity. Electrophoretic separation of the immunoprecipitation bands from male and female thoracopod extracts revealed besides the LV-ϵ band, multiple lower molecular weight proteins.

In vivo, no synthesis of lipovitellin-like material occurred in males. Neither in the haemolymph nor in the male tissue, yolk proteins could be detected immunologically.

We were not able to identify the proteins that were formed by male tissue. We had to apply large quantities of immunoprecipitate to SDS-PAGE and this could disturb the electrophoretic pattern. Furthermore, immunoprecipitation is based on immunological recognition and since the anti-LV-α1 antiserum also recognized antigenic determinants from lower molecular weight proteins (Van Beek et al., 1987b) we couldn't compare our results with those of Gohar and Souty (1983). In order to identify the proteins secreted by male thoracopods as being lipovitellin-like or not, techniques such as autoradiography and injection of radiolabelled amino-acids would be necessary (e.g. the in vivo administration of ^{3}H-Leucine in Orchestia gammarella, Junéra and Meusy, 1982).

However, the most important data obtained by in these in vitro experiments were the indications that the protein release from the thoracopodal cells (see Figs. 8.5 and 8.7) could be influenced by some unidentified factors present in the haemolymph and in the head compartments of males and females.

In vitro translation in a rabbit reticulocyte lysate system of total RNA extracted from male thoracopods, showed low molecular weight protein bands after immunoprecipitation. The fluorographic signal after 4 hr of incubation is, however, too weak to conclude that these bands were lipovitellin-like.

It could be possible that vitellogenesis in male milieu is strictly limited to some animal orders, such as is the case with Lepidoptera in insects (review Lamy, 1984). In some instances vitellogenin is only synthetised by the male insect fat body after hormone injection (De Loof et al., 1981). However, in the amphipod *Orchestia gammarella*, administration of 20-OH-ecdysone to females, has no positive effect on the female-specific protein synthesis (Blanchet et al., 1975). Injection of ecdysone and 20-OH-ecdysone in *Artemia* males didn't result in lipovitellin synthesis (see above). In our *in vitro* experiments ecdysone treatment also failed to result in clear stimulation and the result of the use of estradiol only differed by the presence of more lipovitellin-like proteins in the media. Estradiol however, a non-ecdysteroid steroid typical for vertebrates, was demonstrated to be present in *Artemia* (Van Beek and De Loof, 1988) (see Chapter 6). Concentrations showed peak values just before egg-release, the moment when lipovitellin concentration in the haemolymph reached its maximum.

4. Concluding remarks.

Results of *in vitro* incubations of female and male thoracopods revealed that a brain factor seems to be necessary for the secretion of proteins. The concentration of this factor probably varies in the course of vitellogenesis.

Since male thoracopods incubated in the presence of head homogenate show a substantial secretion of female specific proteins, neurohormonal action can be postulated. This factor remains to be specified.

APPENDIX I: COLLECTING ANIMALS, HAEMOLYMPH, THORACOPODS AND HEADS FROM ARTEMIA SP.

1. Selecting individual brine shrimps.

Adult animals were selected according to sex and to vitellogenic stage. They were sucked up with a glass pipette to minimize damage. Males in riding position were collected separately from the free-swimming ones. Female animals were staged according to the development of their gonads as determined by a handlens or a microscope. Each vitellogenic cycle could be divided into successive stages (see Table 1.1), which could be easily followed, owing to the transparency of the body wall.

The selected animals were then rinsed with distilled water, blotted dry on a filter paper and put on a clean glass slide.

2. Sampling animals for total body extraction.

Groups of 20 selected individuals were frozen in liquid nitrogen and stored at -20°C until use.

3. Collecting haemolymph.

To obtain haemolymph, a few legs were removed or the body wall was punctured. Haemolymph was then sucked up with a micropipette (2 or 5 µl, Brand, Wertheim, FRG) and collected in an Eppendorf tube hanging in liquid nitrogen, and subsequently stored at -20°C.

4. Collecting thoracopods.

The gut from selected animals was removed by pinching off the hindpart of the abdomen and pulling out the gut together with the anterior alimentary glands. Head, abdomen and the reproductive organs were cut off with microsurgery scissors. The remainder comprising the thoracopods which

contain the fat storage cells was washed in X_{TH}-medium (9.46 g NaCl, 0.46 g KCl, 0.14 g $MgCl_2$, 0.2 g $CaCl_2$, 0.05 g Na_2PO_4, 2.4 g $NaHCO_3$ per litre respectively, pH=7.6) and blotted dry. They were put in an eppendorf vial and were immediately frozen in liquid nitrogen. Then thoracopods were stored at -20°C until use.

5. Collecting head compartments.

Heads from selected animals were cut off with microsurgery scissors, rinsed in X_{TH}-medium, blotted dry on a filter paper and put in an Eppendorf vial. Frozen heads were stored at -20°C until use.

APPENDIX II: EXTRACTION AND PURIFICATION PROCEDURE FOR ECDYSTEROIDS AND STEROIDS.

Animals are collected according to sex and vitellogenic stage (see Appendix I). The same extraction methods are followed for the extraction of ecdysteroids as for steroids:

- Frozen brine shrimps are weighed and homogenized in a cold analytical grade acetone:methanol mixture (1:1, v/v; 15 ml solvent per gram of material) using an Ultra-turrax mixer (Janke & Kunkel, IKA-Werk, Staufen).
- Homogenates are kept overnight at 4°C.
- Then they are centrifuged for 20 min, at 4°C and at 15,000 rpm (Sorval RC-5B centrifuge, DuPont Instruments).
- Pellets are thoroughly washed with 5 ml acetone:methanol, kept for 4 hr at 4°C and centrifuged.
- All supernatants are evaporated under vacuum with a Büchi rotavapor apparatus, using a waterbath in which temperature gradually is increased from 30°C to 50°C.
- Samples are partitioned in a mixture of water:n-hexane (from Carlo Erba, 1:4, v/v).
- After equilibrium, bilayer is put at -20°C.
- Organic phases are decanted.
- Aqueous phases are washed and kept apart for ecdysteroid quantification.
- Organic phases are evaporated, dissolved in dichloromethane and stored at 4°C.

1. Purification procedure for ecdysteroids.

- The aqueous phase is further purified by column-chromatography. We used Sep-pak C_{18} cartridges (Waters Associates).
- Columns are first prewet with pure methanol and then the aqueous samples are applied by means of a plastic syringe.
- Ecdysteroids are eluted from the column with pure methanol.
- Methanol is evaporated under a gentle stream of nitrogen and the sample is dissolved in PBS (=Phosphate buffered saline: 36.92 g $Na_2HPO_4.2H_20$, 6.86 g $NaH_2PO_4.2H_2O$, 9 g NaCl and 1 g NaN_3 per litre, pH=7.4).

- Extracts are stored at -20°C until use in radioimmunoassay.

2. Purification procedure for steroids.

- Stored organic samples are evaporated and dissolved in isooctane.
- Samples are purified by column chromatography: home-made short columns (in a short type Pasteur pipette, see note in 2. of Chapter 5) of 1.5-2 cm silicagel 60 (purchased from Merck), were used.
- After application, column is washed with 6 ml isooctane.
- Subsequently, the column is eluted with 7 ml acetone.
- Acetone is evaporated and dissolved in 200 µl isooctane.

- Samples are further purified by celite column chromatography (Chromatolithe A, bioMérieux): 5 ml graduated glass pipette is filled rather tightly in two times with the solid phase to the mark of 4 ml; the outlet is first closed with a tiny glass sphere; columns are made just before use.
- Columns are first eluted with 4 ml 100 percent isooctane.
- Samples are applied and washed two times with 200 µl isooctane taking care that columns do not become dry.
- Fraction A is now kept apart; column is further eluted with 500 µl and 3.5 ml 100 percent isooctane; this fraction contains P_4 and P_5.
- Stepwise elution follows with different solvent mixtures using an increasing gradient of ethylacetate in the presence of isooctane.
- Fraction B, which contains P_5 and DHT, is eluted with 7 ml 6 percent ethylacetate.
- Fraction C is eluted with 6 ml 20 percent ethylacetate and contains T and E_1.
- Finally fraction D is eluted with 6 ml 40 percent ethylacetate and contains E_2.
- All solvents are evaporated under a gentle stream of nitrogen and samples are dissolved in phosphate buffer and stored at 4°C until the radioimmunoassay is carried out; the used PBS-BSA buffer: 1.78 g $Na_2HPO_4.2H_20$, 1.56 g $NaH_2PO_4.2H_2O$, 7.3 g NaCl, 0.975 g NaN_3 and 1 g BSA per litre, pH=7.4).

APPENDIX III: SODIUM DODECYL SULPHATE GRADIENT POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE).

SDS-PAGE was performed according to the discontinuous buffer system of Laemmli (1970) with the vertical slab gel apparatus of Bio-Rad (Protean 32 CM). Routinely 5-15 percent gradient gels were used.

Reagents:

1. Acrylamide stock (30 percent, w/v)
   30 g acrylamide (Serva)
   0.8 g N,N-methylenebisacrylamide (Serva)
   to 100 ml with H_2O, add Dowex AG ion exchanger (type 1x8, 20-50 µm particle diameter, from Serva), stir for 1 hr and remove Dowex by filtering before use.

2. Run buffer
   12.1 g Tris (hydroxymethyl)aminomethane (Merck)
   to 50 ml with H_2O
   to pH 8.8 with HCl

3. Stacking buffer
   3.03 g Tris
   to 50 ml with H_2O
   to pH 6.8. with HCl

4. Stacking gel
   5 ml stacking buffer
   2 ml acrylamide stock
   to 20 ml H_2O
   add 60 µl ammoniumpersulphate and 30 µl TMED

5. Electrode buffer
   144 g glycine (Merck)
   30 g Tris
   to 1 l with H_2O

6. Ammoniumpersulphate (APS) 10 percent stock solution
   1 g ammoniumpersulphate (Serva)
   to 10 ml with H_2O

7. TMED (N,N,N',N'-Tetramethylenediamine, Serva)

8. SDS-sample buffer
   5 ml stacking buffer
   0.5 g sodium dodecyl sulphate (SDS) (Serva)
   5 µl ß-mercaptoethanol (Fluka)
   traces of bromophenolblue (Serva)
   to 10 ml with glycerin (Merck)

9. Coomassie Brilliant Blue staining solution
   1.86 g Coomassie Brilliant Blue R-250 (Serva)
   800 ml ethanol
   200 ml acetic acid, glacial
   to 2 l with H_2O

5-15 percent gradient gels were prepared as follows:

5 percent : 2.5 ml acrylamide, 3 ml run buffer (no glycerin)
to 15 ml H_2O

15 percent: 7.5 ml acrylamide, 3 ml run buffer, 4 ml glycerin
to 15 ml H_2O

Gels were polymerized with 30 µl APS and 15 µl TMED. Gradient gels were prepared by means of a 3 way peristaltic pump (Pharmacia) and a mixer in order to obtain a linear gradient. After polymerization stacking gel was applied on top of the separating gel using an injection needle. Subsequently, the appropriate sample spacer was applied. Samples were denaturated by adding 2 volumes of SDS-sample buffer. They were shaken, heated in boiled water for 5 min and then applied to the gel. The lower compartment of the gel apparatus contained 800 ml electrode buffer and 7.2 l distilled water. The upper compartment was filled with upperbuffer (50 ml electrode buffer, 5 ml 10 % SDS, to 500 ml with H_2O). The sample was separated at a constant current of 20 mA/gel; when the frontline had reached the separating gel, current was increased to 30 mA/gel.

After staining overnight the gels were rinsed several times in destaining solution and photographed.

APPENDIX IV: SCREENING FOR THE ANTIBODY CHARACTERISTICS.

1. Immunospotting.

According to this technique the lipovitellin sample used for the preparation of the AS, is serially diluted. Aliquots of maximum 1 µl are spotted on nitrocellulose sheets (pore size 0.45 µm, from Millipore). The control solvent was Tris saline (1.2 g Tris, 9 g NaCl, 1 ml Triton X-100 and 0.04 g Merthiolate (Fluka AG) or NaN_3 in 1 litre distilled H_2O, pH=7.6; all other products were from Merck). The lipovitellin-α1 antiserum was also serially diluted.

Protocol:

1. All strips are rinsed in distilled H_2O and put into a glass tube with 1.2 ml pre-immune goat serum (PIG) + 10.8 ml Tris saline for 30-60 min.
2. Individual strips are placed into glass tubes filled with the respective AS dilution for 1 hr.
3. They are thoroughly washed (5 x 1 min) with Tris saline (200 ml Tris saline + the 10 percent PIG-Tris saline from no. 1).
4. All strips are put together in a glass tube filled with diluted goat-anti-rabbit (GAR) 1:50 (v/v), for 30 min.
5. They are washed as in no. 3.
6. All strips are put in a diluted solution of peroxidase-anti-peroxidase (PAP) 1:500 (v/v), for 30 min.
7. They are washed as in no. 3.
8. Afterwards they are washed (5 x 1 min) with Tris stock (180 ml distilled H_2O + 20 ml Tris stock (60.5 g Tris/litre, pH=7.6) in a petri dish.
9. Staining in diaminobenzidine (DAB) solution: 180 ml distilled H_2O + 20 ml Tris stock + 25 mg DAB is filtered and immediately before use 2 ml of H_2O_2 solution (10 ml distilled H_2O + 100µl H_2O_2) is added. Staining time varied from 2-4 min.
10. Strips are rinsed with distilled H_2O and dried on filter paper.

2. Immuno-electrophoresis.

Universal Electrophoresis Agarose film^{R} was purchased from Corning. 1 µl aliquots of sample were applied to the slots in the gel. The whole film was put in a Corning Aci apparatus and each of the buffer compartments were filled with 95 ml barbital buffer (10.3 g Na-barbiturate; 0.35 g EDTA, both from Merck, in 1 litre distilled H_2O; pH is adjusted at 8.6-8.8 with concentrated HCl). The electrophoresis lasted for 35 min. The lipovitellin antiserum was concentrated 5 times (see results in 5.2.3. of Chapter 2) and dissolved in barbital buffer. Then it was applied to the gel in the small strokes between the electrophoretic lanes. After overnight incubation, the gel was soaked for 30 min in 0.4 M NaCl. The gel was then covered with wet filter paper, several layers of dry filter paper and a heavy weight. This pressing was done 3 x 15 min. The gel was washed in distilled H_2O and again pressed 3 x 15 min. The gel was then completely dried and stained with a filtered amido-black solution (0.5 g amidoblack 10B (Serva), 5 g mercuric chloride (Sigma), 5 ml glacial acetic acid, to 100 ml with H_2O). Destaining was effected in 2 percent glacial acetic acid (v/v). The gel was dried and stored at room temperature.

3. Double immunodiffusion.

Protocol for home-made agarose gels:

1. Pre-coating of glass plates.
   - Glass plates (8 x 8 cm) were coated on one side with a 0.1 percent agarose solution.
   - Agarose was dried overnight and heated for 1 hr at 100°C.
2. Preparation of 1 percent agarose with low melting point.
   - 1 Percent agarose was dissolved in hot water bath in 0.05 M barbital-EDTA buffer, pH=8.6 for at least 30 min.
   - The mixture was stirred continuously and the reservoir was kept closed.
   - A minute quantity of NaN_3 was added as a fungicide and the agarose stock was divided into different fractions of 8 ml which were stored in glass tubes at 4°C.

3. Casting of agarose gel.
   - One pre-coated glass plate was covered with a U-like frame (1.5 mm thick).
   - An uncoated glass was placed against it and the two glass plates were kept tightly together with large paper-clips.
   - The glass tube with agarose 1 percent was heated in a hot water bath and the melted agarose was poured between the two glass plates.
   - The gel was left to cool down and the uncoated glass plate was then removed.
   - The gel was stored at 4°C until use.

4. Application of samples prior to immunodiffusion.
   - Holes were made in the gel with a gel cutter ('puncher') and the tiny gel pieces were sucked up by means of a vacuumpump.
   - 5 µl aliquots of antiserum were applied in the central hole.
   - Antigen dilutions or samples were put in the surrounding holes.
   - Overnight incubation was carried out in a moist chamber.
   - Precipitation lines were observed by strong backlighting.
   - Afterwards the washing procedure of the gel in 0.4 M NaCl and distilled water was followed according to the 'pressing' method described in 2.
   - The gel was stained in filtered Coomassie Brilliant Blue solution (see Appendix III), destained and dried.
   - The gel could then be stored at room temperature.

APPENDIX V: IMMUNOHISTOCHEMISTRY.

1. Imbedding of whole adult *Artemia* and preparation of sections.

1.1. Fixation of the material.
The animals collected (see appendix I) were rinsed in distilled H_2O and then fixed in AFA solution (ethanol:formaldehyde:glacial acetic acid; 20:75:5, v/v/v). After maximum 2 hr they were washed and stored in 70 percent ethanol.

1.2. Imbedding and sectioning.
Dehydratation: 1 hr in 70 percent ethanol
1 hr in 95 percent ethanol
1 hr in 100 percent ethanol 1
1 hr in 100 percent ethanol 2
1 hr in ethanol:xylol, 50:50
2 hr in 100 percent xylol
12 hr in xylol:Paraplast, 50:50
(second time in xylol:Paraplast may take longer)

Embedding: The specimens were placed in Paraplast for 24 hr.
Then the Paraplast was renewed and the whole animals were oriented with the dorsal side upwards.
The medium was cooled on water.

Sectioning: Frontal sections of 4 µm were obtained on a LKB Historange microtome.
Sections were secured to the glass slide with the help of albumin adhesive: a tiny drop of the solution was rubbed on the slide (the freshly made solution of the proteins of a hen's egg:glycerine (1:1, v/v) was filtered and a few cristals of camphor were added for storage).
Sections had to dry for at least 1 week at 45°C.

2. PAP-technique.

2.1. Deparaffination steps.

5 min in xylol 100 percent (I)
5 min in xylol 100 percent (II)
5 min in ethanol 100 percent (I)
5 min in ethanol 100 percent (II)
1 min in distilled water
2 min in lugol (I)
2 min in lugol (II)
2 min in sodium-thiosulphate (10 g per 200 ml)
1 min in distilled water
5 min in Tris saline (may take longer)

2.2. Pre-incubation.

Sections were pre-incubated with PIG (pre-immune goat serum) diluted to 1:5 with Tris saline for 45 min in a moist chamber to reduce background staining caused by non-immunological adsorption of either the primary or secondary antibody.
Sections were always covered with a minimum of 0.5 ml solvent.

2.3. Primary and secondary antibodies.

Sections were rinsed in Tris saline and were incubated for 5 min with Tris saline.
Next they were incubated with primary AS for 18-24 hr.
Then they were washed with Tris saline and were incubated for 5 min with Tris saline.
Goat anti-rabbit (GAR, 1:30) as a second antibody was applied for 25 min and the rinsing procedure was repeated.
Sections were incubated with PAP (1:300) for 25 min.

2.4. Staining procedure.

Sections were washed with Tris saline and placed for 5 min in Tris saline.
Rinsing procedure was repeated with Tris stock.
Staining procedure was carried out according to the precipitation reaction described by Graham and Karnovsky (1966).
The same procedure was followed as in Appendix IV.1. but the reaction was stopped afterwards with Tris stock.

2.5. Dehydratation of the sections and mounting.

1 min in distilled H_2O
5 min in ethanol 100 percent (I)
5 min in ethanol 100 percent (II)
5 min in ethanol 100 percent (III)
5 min in xylol 100 percent (I)
5 min in xylol 100 percent (II)
5 min in xylol 100 percent (III)
Sections were mounted in DePeX (British Drug House, Poole, U.K.) and dried at 45°C.

2.6. Control steps in the PAP-technique.

a. The primary antiserum was omitted in the PAP-procedure and replaced by Tris saline.
b. The primary AS was replaced by pre-immune rabbit serum (PIR).
c. Site of positive reaction was checked by counterstaining for 1 min in Mayer's hemalum solution.
d. The primary antibodies were removed from the AS by liquid or solid phase adsorption. Liquid phase adsorption was carried out as described in Chapter 7, solid phase adsorption according to Appendix VI.

3. Double PAP-staining method.

Information about co-localisation and cross-reaction was obtained by the double PAP-staining method according to Vandesande (1983b). In this procedure a first PAP-staining is carried out and followed by electrophoresis for 2 hr at 20 V/cm in a mixture of 0.2 M Glycine/HCl buffer (pH=2.2, and containing 0.5 M NaCl), dimethylformamide and bidistilled water (1:2:4, v/v). A home-made electrophoresis apparatus is used. All compartments, except the electrophoresis chamber, are filled with buffer and paper bridges are made from Gelman absorbent sheets. In this step, even the antibodies of high affinity are selectively and completely removed. Sections were washed in distilled water and the second PAP-procedure is carried out. Routinely, for the first staining reaction DAB is used while in the second one 4-chloro-1-naphthol is used for the enzymatic precipitation reaction. Afterwards sections were mounted in chrome-glycerine jelly.

APPENDIX VI: SOLID PHASE ADSORPTION.

Protocol after the manual of affinity chromatography from Pharmacia Fine Chemicals (Pharmacia, Uppsala, Sweden, 1979):

- 1 g of dry CNBr-activated Sepharose 4B (Pharmacia AB, Sweden) is weighed and put in a centrifuge tube.
- Then the gel is soaked and washed 4-5 times in acid water (1 mM HCl, UCB, 200 ml/g gel)(1 g dry Sepharose results in 3.5 ml gel after soaking).
- Supernatant is discarded after centrifugation (1000 rpm, 1 min, Beckman Microfuge B).
- Protein is dissolved in coupling buffer (0.1 M $NaHCO_3$ and 0.5 M NaCl, pH=8.3).
- Routinely, 5 mg pure protein (determined with UV-spectrophotometry according to the method of Bradford, 1976) is covalently coupled on 1 g Sepharose beads (1 volume of gel with 2 volumes of coupling buffer).
- The mixture is continuously shaken and incubated for 2 hr and 30 min at room temperature.
- The reaction is stopped with blocking agent (coupling buffer and 0.2 M Glycine (Merck), pH=8.00), for 2 hr at room temperature.
- The gel is rinsed in coupling buffer and afterwards in acetate buffer (0.1 M CH_3COONa (UCB) and 0.5 M NaCl, pH=4).
- The last two steps are repeated 3 times.
- Then gel is washed 2 times in distilled water.
- Gel can be divided in small portions and stored at 4°C.
- When portions are used: gel is washed with Tris buffer (0.1 M Tris and 0.5 M NaCl, pH=8.00) and a diluted AS solution is added.
- The mixture is incubated overnight (end-over-end mixing).
- The supernatant which contains the preadsorbed antiserum is used in the PAP-procedure for checking the serum specificity.

Remarks: All washes used in this technique can be carried out on a sintered glass filter connected to a vacuumpump. The gel can be scraped from the filter instead of having to be centrifuged. It is better to use an excess of gel in order to bind all proteins to it. The progress of the coupling reaction can be followed by measuring the protein concentration at regular intervals.

APPENDIX VII: *IN VITRO* EXPERIMENTS.

1. *In vitro* incubation of thoracopods.

1.1. Tissue preparation.

The collection of haemolymph and the preparation of thoracopods and heads were carried out according to methods described in Appendix I.

1.2. *In vitro* culturing of fat storage cells.

The thoracopods of 10 adult brine shrimps were incubated in an Eppendorf tube with 100 µl filtered X_{TH}-medium that contained 5 µCi (^{35}S)-methionine (> 800 Ci/mmole, Amersham) and a 5 µl aliquot of haemolymph. The whole system was continuously stirred by a gentle stream of air. Routinely, the incubation lasted for 4 hr. Tissues were washed three times and were stored at -20°C until they were used.

In the experimental conditions thoracopods of 10 females or 10 males were incubated in the presence of 5 µl aliquots of haemolymph and/or 10 µl aliquots of prepared solutions (head extracts or hormone solutions):

- Heads were put in an Eppendorf vial filled with 100 µl X_{TH}-medium. Head extracts were then homogenised twice for 30 seconds by ultrasonic disintegration (Soniprep 150, MSE) (while they were cooled on ice), and centrifugated (3,000 rpm, 4 min, in a Labofuge 6000 centrifuge from Heraeus). Equivalents of 3 heads were added to the incubation media.
- α-Ecdysone (10 µl of a solution of 1 µg/µl 20 percent ethanol) was purchased from Simes (Milano) and ß-estradiol (10 µl of a solution of 0.5 µg/µl 40 percent ethanol) was obtained from Serva.

2. RNA-extraction and <u>in vitro</u> translation.

For RNA isolation, the procedure described by Cardoen et al. (1986) was modified. Thoracopods of reproductively active females or adult males were collected as mentioned in Appendix I and immediately placed in liquid nitrogen. They were homogenized with an Ultra-turrax homogenizer (Janke & Kunkel, IKA-Werk, Staufen) in buffer solution (0.15 M Na acetate, 0.05 M Tris/HCl (pH=9.0), 1 mM EDTA (Titriplex III) and 1 percent SDS). An equal volume of Tris-saturated phenol was added, the mixture was shaken and after 10 min it was centrifugated (10 min, 4,000 rpm, Sorvall RC-5B centrifuge, DuPont Instruments). The upper aqueous phase was extracted three times with an equal volume of phenol and once with phenol:chloroform:isoamyl alcohol (25:24:1). RNA from the aqueous phase was precipitated overnight with 2.5 volumes of ice cold ethanol at -20°C. After centrifugation (10 min, 4,000 rpm) the RNA pellet was rinsed twice with 70 percent ethanol, vacuum dried and resuspended in 1 ml buffer solution (10 mM Tris/HCl (pH=7.8), 1mM EDTA, 0.1 percent SDS). Residual proteins were digested for 1 hr at 37°C with proteinase K (100 µg/ml). The solution was mixed with an equal volume of phenol:chloroform (1:1) and after centrifugation (5 min, 4,000 rpm) the RNA was precipitated with 2.5 volumes of ethanol overnight. The RNA pellet was collected by centrifigation (15 min, 5,000 rpm), vacuum dried and washed once with 70 percent ethanol. It was resuspended in deionised H_2O till a concentration was reached of 0.5 µg/µl (estimated with a Beckman spectrophotometer at 260 nm). Thoracopods from 200 animals yielded 200-320 µg total RNA (for both sexes). The total RNA was translated in a rabbit reticulocyte-lysate system (Amersham, N.90) for 1 hr at 30°C in the presence of (^{35}S)-methionine.

3. Analysis of the samples.

3.1. Homogenization.

Thoracopods were homogenized in 100 µl X_{TH}-medium by applying ultrasonic disintegration (Soniprep 150, MSE). After centrifugation (3000 rpm, 4 min, in a Labofuge 6000 from Heraeus) 20 µl aliquots of these homogenates and of the incubation media (also from the translation experiments) were checked for female specific yolk polypeptides.

3.2. Immunoprecipitation.

Analysis of female specificity was done by immunoprecipitation using an antibody raised against the high molecular weight apoprotein of the lipovitellin complex (Van Beek et al., 1987b). An equal volume of the primary AS was added to 20 µl of the incubation medium or the homogenate of the in vitro experiments. The mixture was incubated for 30 min at 37°C and then for 4 hr at 4°C. Goat-anti-rabbit (GAR) was added as a secondary antibody (4 times the volume of primary AS). Incubation was continued for 30 min at 37°C and then overnight at 4°C. The immunoprecipitate was isolated by centrifugation for 5 min at 14,000 rpm (Eppendorf Centrifuge 5415). The pellet was thoroughly washed (3 times) with a 0.9 percent NaCl solution. Finally, the pellet was dissolved in 0.9 percent NaCl.

All the samples were then mixed with SDS-sample buffer (1:1, v/v) cooked for 10 min and analysed by SDS-gradient (5-15 percent) polyacrylamide gel electrophoresis.

3.3. Fluorography.

After overnight staining and destaining, the gel was rinsed in tapwater until a pH > 6.6 was reached. The gel was then prepared for fluorography by soaking it in 1M sodium salicylate for 30 min (Chamberlain, 1979). Subsequently, the gel was briefly rinsed in distilled H_2O and dried for 2-3 hr on a slab gel dryer (Bio-Rad, Model 1125). The vacuum dried gel was placed against Kodak X-Omat S film at -60°C. Films were developed with Kodak LX 24 developer, rinsed in 1 percent acetic acid for 15 sec and fixed in Kodak AL 4 fixative.

3.4. Trichloroacetic acid precipitation.

The radioactivity incorporated in the thoracopods was counted with a LS 9000 (Beckman) liquid scintillator with automatic quench correction mode. Therefore 5µl samples were placed on 1 cm^2 Whatman 3M filter paper and submitted to trichloroacetic acid (TCA) precipitation (15 min in 10 percent cold TCA, 5 min in 5 percent cold TCA, washed with distilled H_2O, methanol and aceton respectively). The paper squares were then dried and counted in 4 ml scintillation cocktail (Aqualuma, Lumac).

3.5. Counting.

The results are expressed as the percentage incorporated (secreted) radioactivity (= radioactivity in TCA-precipitated proteins) present in the initial volume of thoracopod homogenate (incubation medium) over total radioactivity added to incubation medium. The data of incorporated (secreted) radioactivity into immuno-precipitated lipovitellin-like proteins, are expressed in the same way.

APPENDIX VIII: COMPARISON OF THREE SEPARATION METHODS FOR THE RADIOIMMUNOASSAY OF ECDYSTEROIDS IN ARTEMIA SP..*

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1. Introduction.

Radioimmunoassay (RIA) has become an important analytical tool for the estimation of ecdysteroid levels in many organisms (Hirn and Delaage, 1980). A critical step in the RIA procedure is the separation of bound and free antigen prior to counting the isotope. Double-antibody-based separation techniques are both specific and efficient but require a fairly long second incubation time to achieve adequate precipitation (Lazarovici et al., 1983). The length of the separation time can be reduced by the use of a pre-precipitated second antibody (Ysewijn-Van Brussel et al., 1981), by the addition of reaction accelerating substance such as polyethyleneglycol (Hartmann et al., 1982) or by linking the second antibody to a solid phase (Butt, 1984). These three approaches were tried and compared in the course of developing a new method for RIA of ecdysteroids using the antiserum DUL-2.

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* Published in Insect Biochem. 16, No. 1, 41-44, 1986.

2. Materials and methods.

2.1. Chemicals.

Reagents and solvents were all of analytical grade from Merck (Darmstadt, FRG). Normal rabbit serum, goat anti-rabbit gamma-globulins and Tachisorb^{R}-suspension were from Boehring Calbiochem (San Diego, California, U.S.A.). Polyethyleneglycol 6000 was from Fluka (Buchs, Switzerland). (23,24-^{3}H)ecdysone of specific activity 58 Ci/mmol was obtained from New England Nuclear (Boston, Massachusetts, U.S.A.). Solutions of labeled ecdysone were purified every 2 months by HPLC. Unlabeled ecdysteroids were obtained from Simes (Milan, Italy), Sigma (St. Louis, Missouri, U.S.A.), Dr. R. Lafont (Paris, France) and Dr. M. Thompson (Beltsville, Maryland, U.S.A.; see Table 1). The anti-ecdysteroid antiserum DUL-2 (lyophilised) was a gift from Dr. J. Koolman (Marburg, FRG; Reum et al., 1981). Before use, it was reconstituted at its original concentration with distilled water.

Table 1. Cross-reaction factors for antiserum DUL-2.

Ecdysteroid	Source	Cross-reaction (%)
Ecdysone	Simes (Milan, Italy)	100
20-OH-ecdysone	Simes (Milan, Italy)	2.70
2-Deoxy-ecdysone	Dr R. Lafont (Paris, France)	65.60
2-Deoxy-20-OH-ecdysone	Dr R. Lafont (Paris, France)	1.15
26-OH-ecdysone	Dr M. Thompson (Beltsville, Maryland, U.S.A.)	0.56
20,26-Di-OH-ecdysone	Sigma (St Louis, Missouri, U.S.A.)	0.25
Ponasterone *A*	Dr R. Lafont (Paris, France)	1.10
Polypodine *B*	Sigma (St Louis, Missouri, U.S.A.)	0.28
Makisterone *A*	Simes (Milan, Italy)	0
20-OH-22-acetate	Sigma (St Louis, Missouri, U.S.A.)	0.23

Binding (50%) of ecdysone was observed at a concentration of 1.24 nM.

2.2. Standard curves.

Standard curves are obtained by adding increasing amounts of an ecdysteroid to constant amounts of antibody and (^{3}H)ecdysone. Concentrations of stock solutions were determined by measuring the absorbance at 242 nm (ϵ=12,000 l mol^{-1} cm^{-1}; Karlson, 1966). Results were expressed as bound percentages of radiolabel (cpm bound in the sample/cpm bound at zero concentration: B/B_o x 100) against the concentration of ecdysone. Concentrations were then calculated using the logit-log transformation (Rodbard et al., 1970).

2.3. Radioimmunoassay.

All assays were performed in duplicate in 3 ml polyethylene vials (Milli^R-3-vials) from Lumac, according to a modified procedure of Briers and De Loof (1981): 65 pg (3000 cpm) of radiolabeled ecdysone in 100 µl 10mM phosphate-buffered saline, pH 7.4, was incubated overnight at 4°C together with 100 µl of the sample (either standard or buffer) and 100 µl antiserum at the appropriate dilution (1/2000) binding approximately 50 percent tracer. Following separation (see below) and centrifugation (10 min, 1500 g at 4°C) the supernatant was decanted. The pellet, containing the bound fraction, was resuspended in 100 µl 0.5 N NaOH and 200 µl water. After addition of 2.5 ml scintillation liquid (Rialuma, Lumac) radioactivity was counted in a model 3390 Packard Tricarb Liquid Scintillation Counter (Packard Instrument Co., Downers Grove, Illinois, U.S.A.). All data were corrected for non-specific counts.

2.4. Separation systems.

2.4.1. Pre-precipitated (insoluble) second antibody.

When the second antibody is precipitated with its antigen, before being added to the assay, separation time can be reduced from 16 to 1 hr (Ysewijn-Van Brussel et al., 1981). First the optimum ratio of second antibody to carrier immunoglobulin must be determined. To this end, ten different precipitates were prepared overnight in separate tubes. Ten microlitres of normal rabbit serum (containing 10 mg ml^{-1} immunoglobulins) were precipitated with respectively 25, 50, 75... 225 or 250 µl goat anti-rabbit gamma-globulin

in a total volume of 1 ml phosphate-buffered saline. Zero-dose values (B_o) and non-specific counts were measured in duplicate for each combination. The ratio which yielded the highest zero-dose value was used. To obtain separation of bound and free antigen, 200 µl pre-precipitate was added to the assay vial, followed by a short incubation (60 min, 4°C). Cold phosphate-buffered saline (1ml) was added to minimise unspecific adsorption, and the tubes were centrifuged for 10 min (1500 g, 4°C). The supernatant was decanted and the radioactivity of the pellet was determined as described above.

2.4.2. Accelerated precipitation.

One hundred microlitres of 2 percent normal rabbit serum in buffer was added to the assay vials as a carrier globulin, followed by 100 µl of the appropriate second antibody dilution (goat anti-rabbit gamma globulin, 1:10, v/v, as indicated by the manufacturer). After 30 min incubation at 4°C, 1.0 ml of 5 percent polyethyleneglycol 6000 in phosphate-buffered saline was added to accelerate precipitation. The tubes were centrifuged and the pellet counted after redissolving, as described above.

2.4.3. Protein A-bound second antibody (Tachisorb^R).

Tachisorb^R-suspension is a ready-for-use separation reagent, specific for rabbit gamma-globulins, consisting of second antibody (anti-rabbit gamma-globulins) covalently bound to Staphylococcus aureus cells (Cowan I strain). These bacterial cell walls contain an antigen (protein A) which interacts specifically with the Fc fragment of many immunoglobulins (Kessler, 1975). Due to the binding of gamma-globulins to the solid phase, less second antibody is required in RIA and no carrier protein is needed. According to the manufacturer's manual, the reaction mixture-Tachisorb^R suspension-buffer solution is used in a ratio of 1:1:2 (v/v). To obtain the separation, 300 µl suspension was added to the assay vials. Following an incubation of 30 min at 4°C, 600 µl cold PBS buffer was added. After centrifugation the pellet was redissolved and the radioactivity was counted as described above.

2.5. Animals.

Brine shrimps (Artemia sp.) from a Great Salt Lake strain were reared under optimal conditions (Lavens et al., 1985) at the Laboratory for Mariculture (Artemia Reference Center, Ghent, Belgium). The vitellogenic stage of the adult females was determined by microscopic examination. Selected animals were thoroughly rinsed with distilled water, dried, and stored immediately at -20°C.

2.6. Extraction and purification of ecdysteroids.

For each determination 20 animals (approx. 180 mg) were homogenised with an Ultra-turrax (Janke & Kunkel, IKA-Werk, Staufen) in 2 ml methanol-acetone (1:1, v/v). Homogenates were left standing overnight at 4°C and then centrifuged (20 min, 15,000 g). After evaporation of the supernatant (Büchi rotavapor, Eislingen/Fils, FRG) the residue was partitioned between n-hexane and water (1:1, v/v). The aqueous layer containing the ecdysteroids was adsorbed on a Sep-pak^{R} C_{18} cartridge (Waters Ass., Milford, Maryland, U.S.A.). The column was rinsed with 5 ml water and ecdysteroids were eluted with 5 ml methanol (Lafont et al., 1982). The methanol was evaporated under a stream of nitrogen, and the residue was redissolved in 500 µl phosphate-buffered saline. A 100 µl aliquot of each extract was subjected to RIA.

3. Results.

3.1. Pre-precipitated second antibody.

The use of a pre-precipitated second antibody allows us to reduce the separation time from 16 to <1 hr. Non-specific counts are fewer in number than those that result from the use of non-specific precipitation techniques involving ammonium sulphate (Reum and Koolman, 1979) or 25 percent polyethyleneglycol 6000 (Briers et al., 1981). Using the latter method we found that non-specific binding varied between 8 and 15 percent of the total radioactivity. The reproducibility of this procedure was checked by replicate analysis of variation of the percentage bound (B_o/T) (see Table 2). The minimum detectable dose (B_o - 2 SD) was estimated at 18 pg total ecdysone. Using this technique, we

examined the specific affinity of DUL-2 anti-ecdysteroid antiserum for nine ecdysteroids (see Table 1 and Fig. 1).

Table 2. Reproducibility of the RIA for ecdysteroids using the pre-precipitated second-antibody separation; B_0/T.

Table 2. Reproducibility of the RIA for ecdysteroids using the pre-precipitated second-antibody separation: B_0/T

	Mean (%)	SD (%)	CV (%)	n
Within assay	51.6	3.0	5.7	10
Between assay	59.0	7.8	13.2	5

Radioimmunoassay of ecdysteroids in *Artemia*

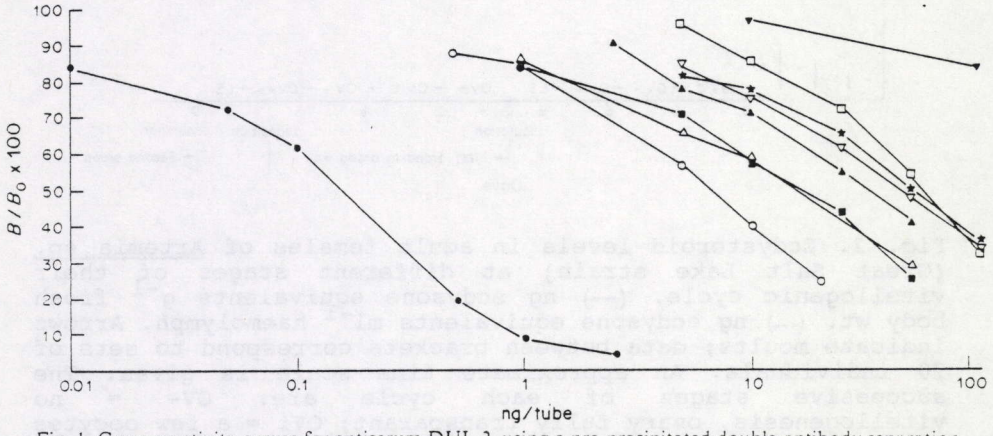

Fig. 1. Cross-reactivity curves for antiserum DUL-2, using a pre-precipitated double-antibody separation. (●) Ecdysone; (○) 20-hydroxyecdysone; (△) 2-deoxy-20-hydroxyecdysone; (▲) 26-hydroxyecdysone; (■) ponasterone *A*; (▽) polypodine *B*; (▼) makisterone *A*; (□) 20-hydroxyecdysone-22-acetate; (✱) 20,26-dihydroxyecdysone.

These results demonstrate the high affinity of the antiserum for ecdysone and 2-deoxyecdysone (data not shown). Other ecdysteroids cross-reacted to a lesser extent (<3 percent). We applied this RIA procedure to whole-body extracts and haemolymph of female brine shrimps during their vitellogenic development. Ecdysone was used as a competitor; and the results are expressed in ng ecdysone equivalents (see Fig. 2). High-performance liquid chromatography is introduced to separate ecdysone from other interfering ecdysteroids (e.g. 2-deoxyecdysone). The immunological response of the various fractions collected subsequent facilitates determination of ecdysone titer and screening for the presence of other ecdysteroids in Artemia sp.

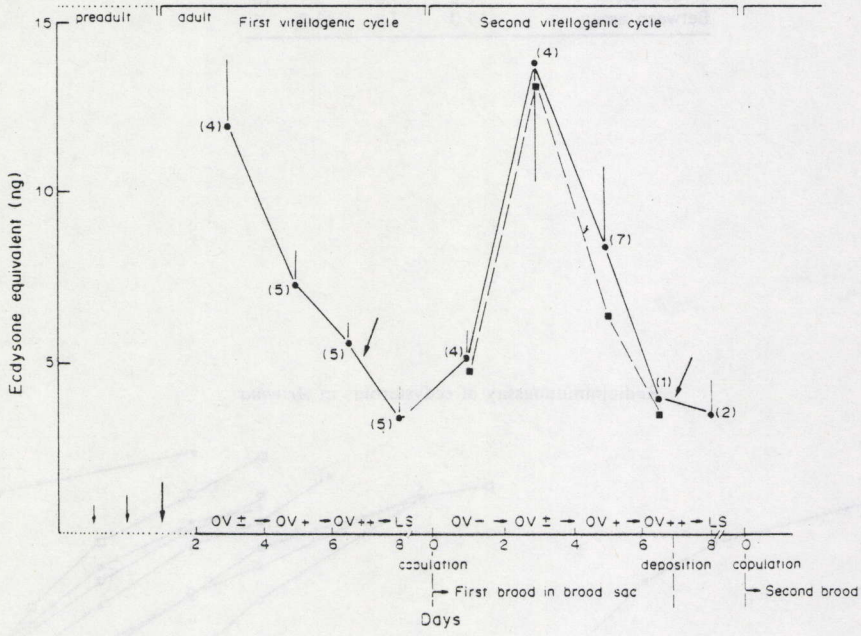

Fig. 2. Ecdysteroid levels in adult females of Artemia sp. (Great Salt Lake strain) at different stages of their vitellogenic cycle. (—) ng ecdysone equivalents g^{-1} fresh body wt. (---) ng ecdysone equivalents ml^{-1} haemolymph. Arrows indicate moults; data between brackets correspond to sets of 20 individuals. An approximate time scale is given. The successive stages of each cycle are: OV- = no vitellogenesis, ovary fully transparant; OV± = a few oocytes have started yolk accumulation,; OV+ = oocytes accumulating yolk are found over the whole length of the ovary; OV++ = oocytes opaque due to the presence of lipovitellin and ordered in a thick white double strand; LS = ripe eggs in lateral sacs.

3.2. Accelerated precipitation.

Polyethyleneglycol has a pronounced effect on the kinetics of the precipitation reactions (Creighton et al., 1973). Following the addition of carrier and second-antibody dilution (1:10, v/v, according to the manufacturer) we incubated the tubes during 0, 15, 30, 60 and 120 min before adding 5 percent polyethyleneglycol 6000 in buffer. For reasons of convenience we chose a 30 min incubation time, but simultaneous addition of goat anti-rabbit gamma-globulin and polyethyleneglycol 6000 gave similar results. No rise in zero-dose values was obtained when addition of polyethyleneglycol was followed by an incubation of up to 60 min. Longer incubation, however, seemed to disrupt the first equilibrium. The polyethyleneglycol-accelerated precipitation approach yielded a coefficient of variation (within a day) of 7.4 percent (n=11) for values of percentage of binding (B_o/T). Non-specific counts were low (2.8 ± 0.4 percent; n=8).

3.3. Protein A-bound second antibody.

For the sake of comparison with the above method a Tachisorb^R suspension was used in the RIA separation step. Because of the binding of anti-rabbit gamma-globulins to the solid phase, less antibody is required and the separation is obtained quickly (<30 min at 4°C). An additional washing of the pellet obtained after centrifugation and decanting of the supernatant reduces non-specific binding to <1 percent.

4. Discussion.

Second-antibody separations have become a standard procedure, comparable in accuracy to other separation methods, but they need to be optimised by titration of the antiserum against the carrier chosen. Since this antiserum (DUL-2) was raised in the rabbit (Reum et al., 1981), goat anti-rabbit gamma-globulins were used as the second antibody and normal rabbit serum was added as a carrier. To eliminate the long second incubation time necessary in double-antibody separation systems, three precipitation methods were compared for their usefulness in the ecdysteroid RIA.

The relative reliability of these methods was determined by comparison of the values found by conventional separation techniques (using ammonium sulphate, or polyethyleneglycol 6000). Correlation between insoluble (i.e. pre-precipitated) and soluble second antibody techniques was good (r=0.9935, n=5) as is shown in Fig. 3. With all three methods non-specific binding was significantly lower than with the non-specific precipitation techniques. For the insoluble second-antibody technique and the polyethyleneglycol-accelerated precipitation method, values of 3.4 ± 1.0 percent (n=8) and 2.8 ± 0.4 percent (n=8), respectively, were obtained. When using 5 percent polyethyleneglycol 6000 to enhance precipitation, the pellet obtained is easier to distinguish, which constitutes an additional practical advantage. The same is true for the application of Tachisorb^{R}.

However, we preferred the pre-precipitated second antibody for several reasons. In our hands this technique yielded the best reproducibility and sensitivity. Manipulations are simple, and simultaneous analysis of a large number of samples can easily be achieved. Moreover, this method consumes less goat anti-rabbit gamma-globulin than does the polyethyleneglycol-accelerated method. The third technique, using Tachisorb^{R}, was also more expensive, and therefore was not considered suitable for application on a routine basis.

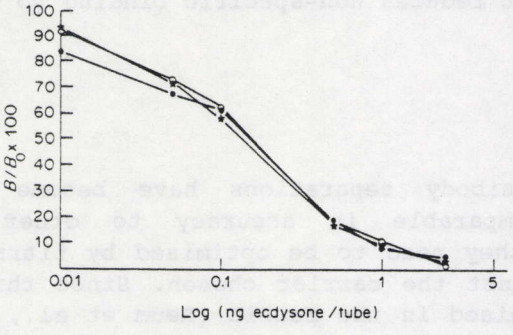

Fig. 3. Comparison of standard curves using three different double-antibody separation techniques. (●) Pre-precipitated second antibody; (★) accelerated double-antibody; (○) protein A double-antibody.

5. Summary.

Three separation techniques based on double antibodies have been investigated in order to constitute a basis for ecdysteroid radioimmunoassay. The best results were obtained with the pre-precipitated second-antibody procedure. Using this technique we studied the specificity of the antiserum DUL-2 (a gift of Dr. J. Koolman) and we determined the ecdysteroid titres in extracts from adult female Artemia sp. (Great Salt Lake strain) during their vitellogenic development.

SAMENVATTING EN SLOTBESCHOUWINGEN.

Het doel van ons onderzoek was meer inzicht te krijgen in de regulatie van de vitellogenese van het pekelkreeftje. Vitellogenese of het dooierrijpingsproces werd tot 1981 alleen morfologisch beschreven. Men had morfologische aanwijzingen dat de dooiervorming endogeen startte vanuit een dooierkern in de oöcyt. Voor het voorkomen van een exogene dooiersynthese had men in het begin van dit onderzoek geen aanwijzingen en men dacht dat dat proces niet plaatsvond bij Artemia.

Extra-ovariële syntheseplaats voor lipovitelline en de hormonale regulatie van de synthese van dit proteïne.

Lipovitelline werd geëxtraheerd uit dooierplaatjes van cysten en gescheiden door SDS-PAGE. Een wijfjesspecifiek antiserum werd aangemaakt in konijnen tegen het hoogmoleculair gewicht apoprotein lipovitelline alfa-1 (LV-α1) van het lipovitelline komplex. Dit anti-LV-α1 werd door middel van de peroxidase-anti-peroxidase kleuringsmethode op frontale paraffine snedes (4 µm) van totale dieren getest. De wijfjes werden bestudeerd gedurende een volledige vitellogene cyclus. De aanwezigheid van exogene dooierprecursoren werd aangetoond in de vetcellen van de zwempoten. De hoeveelheid wijfjesspecifieke dooiereiwitten en het aantal positieve cellen veranderde daarbij doorheen de vitellogene cyclus toe.

Resultaten van in vitro experimenten met ^{35}S-radioaktief gemerkte methionine leverden het bewijs voor de synthese van lipovitelline-achtige substanties door de vetcellen van vitellogene wijfjes.

Het ontworpen in vitro systeem bleek een uitstekende screenings-methode te zijn om de invloed na te gaan van extrakten met potentiële hormoonaktiviteit op de synthese en sekretie van lipovitelline. Inkubaties van mannelijke poten in de aanwezigheid van kopextrakten en hemolymfe, toonden het bestaan aan van een faktor, die de proteïnesekretie stimuleert.

De vetcellen in de thoracopoden van Artemia, die als extra-ovariële syntheseplaats voor de precursoren van dooiereiwitten fungeren, zijn dus te vergelijken met het vetlichaam bij insekten. Bij vertebraten is de lever de plaats van synthese van vitellogenine.

De in vitro sekretieaktiviteit van die vetcellen is te beïnvloeden door een faktor uit de hersenen en/of de hemolymfe. Dit zijn de eerste aanwijzingen voor een mogelijke neurohormonale regulatie van de lipovitelline synthese in wijfjes van Artemia.

Observaties van individuele pekelkreeftjes toonden aan dat in de adulte fase van Artemia, vervellingen optraden met een strikte regelmaat. In de stam van de geleedpotigen zijn de ecdysteroïden of vervellingshormonen verantwoordelijk voor dit proces. De vervellingshormonen spelen in het algemeen een belangrijke rol in de reproduktie bij Crustacea.

Vervellingshormonen gedurende de vitellogenese.

In onze experimenten gebruikten we dieren van een Artemia populatie van Great Salt Lake (GSL 375). Extrakten van hemolymfe en totale adulte pekelkreeftjes werden verder opgezuiverd door vloeistofpartitie- en kolomchromatografie. De koncentraties van vervellingshormonen werden bepaald door middel van radioimmunoassays en uitgedrukt in ng ecdysteroïdaktiviteit/g versgewicht of per ml.

Door een nauwkeurige verzameltechniek te gebruiken, gebaseerd op de reproduktiecyclus van de wijfjes, konden we een diagram van de hormoonaktiviteit opstellen voor de ganse cyclus.

In de wijfjes alterneren de vervellingscycli met de vitellogene cycli. De laagste waarden van het vervellingshormoon ecdyson (3-6 ng ecdyson equivalenten/g lichaamsgewicht of /ml hemolymfe) werden genoteerd tijdens de previtellogenese en gedurende de periode dat de rijpe eieren in de zogenaamde laterale zakjes vertoeven. Maximale waarden (± 20 ng/g) vinden we in iedere vitellogene cyclus terug op het moment dat de dooieropname het sterkst is.

Bij mannetjes is een exacte indeling van de ontwikkeling gedurende de adulte fase veel moeilijker dan bij wijfjes. Ecdysteroïdkoncentraties zijn maximaal (± 10 ng ecdyson equivalenten/g lichaamsgewicht) gedurende de kopulatieperiode.

Wij hebben aangetoond dat de koncentratiestijging van vervellingshormonen gekorreleerd is met het tijdstip van de aanzet van de sekundaire (=exogene) vitellogenese in Artemia. Dit is te vergelijken met de hoger geëvolueerde Crustacea (vb. in Orchestia gammarella), waar de koncentratie van ecysteroïden ook het hoogst is op het moment van de maximale aktiviteit van de vitellogenese. Om te mogen stellen dat deze hormonen in een direkte manier tussenkomen in de vitellogenese is verder onderzoek noodzakelijk.

Het jongste decennium is het endokrinologisch onderzoek in invertebraten een andere richting ingeslagen. Een gevolg was dat nu meer en meer bewijzen voorhanden zijn die aantonen dat C_{21}, C_{19} en C_{18}-steroïden wel degelijk geïdentificeerd kunnen worden in tal van ongewervelde dieren. Deze steroïden of tenminste een aantal van deze, zijn echter "typische" vertebraathormonen die onder andere tussenkomen in de reproduktie van vertebraten.

Naast insekten werden tot nog toe enkel hoger ontwikkelde crustaceeën onderzocht op de aanwezigheid van deze steroïden. Evolutief gezien in het kader van ons onderzoek, was het interessant om deze gegevens verder uit te breiden met metingen bij een meer primitief schaaldiertje.

Kwantifikatie van "C_{21}, C_{19} en C_{18}-steroïden" gedurende de vitellogenese.

In pekelkreeftjes hebben wij niet alleen ecdysteroïden maar ook "typische" vertebraat-type steroïden (C_{21}, C_{19} en C_{18} steroïden) gevonden.

Wijfjes werden in welbepaalde stadia van de reproduktiecyclus verzameld en hun opgezuiverde extrakten werden geanalyseerd door radioimmunoassay. Hierbij werden de koncentraties van progesterone, pregnenolone, 5α-dihydrotestosterone, testosterone, estrone en estradiol bepaald.

Progestagenen (progesterone en pregnenolone) zijn in grote hoeveelheid aanwezig (20-30 ng/g) tussen de opeenvolgende vitellogene cycli terwijl de koncentratie van estrogenen piekwaarden bereikte tijdens de vitellogenese (gemiddelde waarde van 269 pg/g en 2.720 pg/g voor estrone en estradiol respektievelijk).

De koncentraties van 5α-dihydrotestosterone stegen tot 1.190 pg/g op het moment dat de testosterone koncentraties het laagst waren (144 pg/g), nl. in het stadium vóór de eiafleg.

De aanwezigheid van pregnenolone werd niet alleen radioimmunologisch maar ook met behulp van gas chromatografie/massa spektrometrie aangetoond.

Analoog met de regulatie van de vitellogenese bij Echinodermata schijnt bij Artemia, de mogelijkheid te bestaan van een progesterone-estrogenen antagonisme. Meer bepaald zou de koncentratie van estradiol een belangrijke rol kunnen spelen in de eiafleg. Niettegenstaande onze gegevens het feit bevestigen dat steroïden universele biomoleculen zijn, is hun rol en funktie verre van opgelost. Naast hun verdere identifikatie is verder onderzoek naar de fysiologische betekenis zeker aangewezen.

Verschillende fysiologische processen, zoals ovipositie, worden bij vertebraten en invertebraten geregeld door neurohormonen. Sommige van die "typische vertebraat-neuropeptiden" zijn recent in ons laboratorium duidelijk aangetoond in de hersenen en de ventrale zenuwstreng van insekten. Beschouwd vanuit een vergelijkend oogpunt, was het interessant om na te gaan of enkele van die neuropeptiden ook voorkomen in het pekelkreeftje. In de optiek van de resultaten uit het bovenvermelde hormoononderzoek, was het interessant om te kijken naar de aanwezigheid van gonadotrofine-achtige proteïnes.

In de hersenen en de ventrale zenuwstreng waren enkel neurosekretorische cellen beschreven in Artemia met behulp van de klassieke histologische kleuringsmethoden. De identiteit van het sekreet, alsook de mogelijke funkties van die neurosekretorische cellen, waren tot zover nog niet onderzocht.

De aanwezigheid van "vertebraat-type" neuropeptiden.

Met behulp van de peroxidase-anti-peroxidase techniek hebben we de aanwezigheid aangetoond van FMRFamide-, Neuropeptide Y- (NPY), Leucine- and Methionine-enkefaline-achtige peptiden in de neurosekretorische cellen (laterale en mediane) van het protocerebrum van zowel vrouwelijke als mannelijke Artemia. FMRFamide- en NPY- bevattende cellen waren ook aanwezig in het suboesofageaal ganglion (SOG). In de oöcyten kon alleen Met-enkefaline immunoreaktiviteit teruggevonden worden. Bij previtellogene oöcyten waren de immunoreaktieve korrels voornamelijk in het centrum gelokaliseerd terwijl ze gedurende de vitellogenese meer verspreid lagen in de periferie. Algemeen werd waargenomen dat de kleuring van de immunopositieve cellen in het protocerebrum intenser bleek naar het einde van de vitellogenese toe.

Met de antisera tegen Luteïniserend Hormoon Releasing Hormoon (LHRH) en Follikel Stimulerend Hormoon (FSH) werden geen positieve reakties verkregen. Antiserum tegen Luteïniserend Hormoon (LH) leverde een zwakke reaktie op in de hersenen van mannelijke en vrouwelijke pekelkreeftjes.

De aanwezigheid van neurosekretorische cellen in het protocerebrum (de "M" en "L" cellen) van het pekelkreeftje en de veranderingen van hun kleurbaarheid naarmate de vitellogenese vordert, zijn belangrijke aanwijzigingen voor een verband tussen vitellogeneseaktiviteit en neurosekretie.

We kunnen konkluderen dat in het primitieve schaaldiertje Artemia, net zoals bij insekten, er aanwijzingen zijn voor gelijkenissen met het endokrien systeem van vertebraten.

SUMMARY AND CONCLUSIONS.

The regulation of vitellogenesis was investigated in the brine shrimp. Up to 1981, the reports on vitellogenesis or the process of yolk maturation were chiefly concerned with morphological descriptions. Morphological evidence was present about endogenous yolk formation which started in a yolk nucleus of the young oocyte. No indications were known about a possible exogenous site of yolk synthesis in Artemia.

Extra-ovarian site of lipovitellin synthesis and its possible regulation of this synthesis.

Lipovitellin samples, extracted from yolk platelets of cysts, were subjected to SDS-PAGE. A female specific antiserum was raised against the high molecular weight apoprotein lipovitellin alpha-1 (LV-α1) of the lipovitellin complex. This anti-LV-α1 was used in the peroxidase-anti-peroxidase staining method on frontal paraffin sections (of 4 μm) of whole embedded Artemia. Females were studied during a complete vitellogenic cycle. The presence of exogenous yolk precursors in the fat storage cells of the thoracopods of female Artemia was demonstrated. The amount of the female specific yolk polypeptides and the number of positively stained cells changed during the vitellogenic cycle.

In vitro experiments with ^{35}S-radiolabeled methionine showed the synthesis of lipovitellin-like substances by an extra-ovarian source in fat storage cells of vitellogenic females.

The in vitro system we developed, appeared to be an excellent screening-method for extracts with potential hormone activity influencing the synthesis and secretion of lipovitellin. Incubations of male thoracopods in the presence of head extracts and haemolymph, showed the existence of a factor that stimulates the protein secretion.

The fat storage cells in the thoracopods of Artemia, which function as an extra-ovarian site of synthesis for the precursors of yolk proteins, can be compared to the fat body in insects. (In vertebrates, the liver is the site of synthesis of vitellogenins.)

The *in vitro* secretory activity of these fat cells is influenced by an unknown factor from the brain and/or the haemolymph. This is the first indication for a possible neurohormonal control of lipovitellin synthesis in female *Artemia*.

When individual brine shrimps are observed, it is striking that in the adults also moultings occur according to a very regular rhythm. In arthropods, ecdysteroids or moulting hormones are responsible for this process. In general, moulting hormones play an important role in the reproduction of crustaceans.

Moulting hormone pattern during vitellogenesis.

The *Artemia* strain from Great Salt Lake (GSL 375) was used in our experiments. Total body extracts and haemolymph of adult *Artemia* were purified by solvent partition and column chromatography. Moulting hormone concentrations were then determined by a specific radioimmunoassay (RIA). Results are expressed in ng ecdysteroid activity/g fresh weight (or per ml).

Initially, the RIA data on extracts of adults were highly variable. The use of a more accurate sampling technique based on the reproductive cycle of the females allowed us to determine a hormone activity pattern.

In females, moulting cycles alternate with vitellogenic cycles. Lowest values of ecdysone (3-6 ng ecdysone equivalents/g fresh body weight or /ml haemolymph) occur during previtellogenesis and when ripe eggs, contained in the lateral sacs are present. Peak values (± 20 ng/g) are reached during every vitellogenic cycle at periods corresponding to the phase of most intensive yolk accumulation.

Exact timing of the progress of development in the adult stage is much more difficult in males than in females. Ecdysteroid peak values (± 10 ng ecdysone equivalents/g fresh body weight) are measured during the copulation period.

We clearly demonstrated the relationship between the increase of ecdysteroid activity and the onset of secondary (=exogenous) vitellogenesis in Artemia. This phenomenon can be compared to the events in higher evolved crustaceans (e.g. in Orchestia gammarella), in which the concentration of ecdysteroids reaches its peak value at the moment of maximal activity of vitellogenesis. Whether ecdysteroids regulate vitellogenesis in a direct way needs further investigation.

In the course of the recent decade endocrinological research in invertebrates has taken a new direction. The result was that nowadays a lot more evidence is available demonstrating the presence of C_{21}, C_{19} and C_{18} steroids in numerous invertebrate species. These steroids or at least part of them, are 'typical' vertebrate hormones that play a role in the reproduction of vertebrates.

Apart from insects, intensive research on the presence of steroids in arthropods was limited to the higher evolved crustaceans. From an evolutionary point of view, it was interesting to complete these data with measurements in the more primitive brine shrimp.

Quantification of vertebrate-type steroids during vitellogenesis.

Not only did we find ecdysteroids in the brine shrimp, we also demonstrated 'typical' vertebrate-type steroids (C_{21}, C_{19} and C_{18} steroids).

Progesterone, pregnenolone, 5α-dihydrotestosterone, testosterone, estrone and estradiol were measured by radioimmunoassay in purified total body extracts of adult female Artemia during the vitellogenic cycle.

Progestagens were present in large amounts (20-30 ng/g) during intervals between the vitellogenic cycles while estrogen concentrations had peak values during vitellogenesis (mean values of 269 pg/g and 2,720 pg/g for estrone and estradiol respectively).

When testosterone concentrations were lowest (144 pg/g), concentrations of 5α-dihydrotestosterone increased up to 1,190 pg/g.

We were able to demonstrate the presence of pregnenolone by gas chromatography/mass spectrometry.

In Artemia the existence of an progesterone-estrogen antagonism seems possible. The regulation of vitellogenesis would thus present a certain amount of analogy with events known to occur in Echinodermata. More specifically, the concentration of estradiol could play an important role in the process of egg-laying. Although our data confirm the fact that steroids are universal biomolecules, their role and function is far from clear. Further identification and more research is needed to establish the physiological significance of these steroids.

Several physiological processes, such as oviposition, are regulated by neurohormones both in vertebrates and invertebrates. In our laboratory, some of these 'typical vertebrate-like neuropeptides' were recently demonstrated in the brain and the ventral nerve cord of insects. From a comparative point of view, it seemed interesting to look for the presence of some of these neuropeptides in the brine shrimp. With the above mentioned results of hormone measurements in mind, we looked for the presence of gonadotropin-like peptides.

So far, neurosecretory cells in the brain and the ventral nerve cord of Artemia had been demonstrated by classical histological coloration methods. Only the identity of the secretion products and the possible functions of these neurosecretory cells were thus far not studied.

The presence of vertebrate-like neuropeptides.

By means of the peroxidase-anti-peroxidase technique we demonstrated the presence of FMRFamide-, Neuropeptide Y- (NPY), Leucine- and Methionine-enkephalin-like resembling peptides in neurosecretory protocerebral cells (lateral and median) of the brine shrimp Artemia, both females and males. FMRFamide- and NPY-containing cells were also present in the suboesophageal ganglion (SOG). Only Met-enkephalin immunoreactivity was demonstrated in the oocytes. Here, immunoreactive granules were observed mainly in the center of previtellogenic oocytes, whereas during vitellogenesis, they are located more at the periphery. Staining of immunopositive cells in the protocerebrum seemed to be more intense towards the end of vitellogenesis.

No staining was obtained with antisera to Luteinizing Hormone Releasing Hormone (LHRH) or Follicle Stimulating Hormone (FSH). Luteinizing Hormone (LH) antiserum yielded a weak reaction in the brain of male and female shrimps.

The presence of neurosecretory cells in the protocerebrum (the 'M' and 'L' cells) of the brine shrimp and their change in immunoreactivity during the progress of vitellogenesis are important indications for a possible relationship between neurosecretion and vitellogenic activity.

We can conclude that in the primitive crustacean Artemia, as in insects, indications are found which strongly point to similarities with the endocrine system of vertebrates.

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