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CRUSTACEAN NEUROENDOCRINOLOGY: PERSPECTIVES FOR THE CONTROL OF REPRODUCTION IN AQUACULTURAL SYSTEMS

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F. Van Herp and G.G. Payen (1991) Crustacean neuroendocrinology: Perspectives for the control of reproduction in aquacultural systems. *Bull. Inst. Zool., Academia Sinica, Monograph* **16**: 513-539. This paper reviews essentially the neuroendocrinological aspects of reproduction in decapod Crustacea obtained during the last few years. After a historical review of the most important data, detailed information is given about the morphology and cytology of the neuroendocrine system, the isolation and characterization of the neuropeptides involved in the reproductive control and their mode of action. The information is concentrated on those regulatory factors which presence and physiological effect are demonstrated with great certitude. Modern techniques such as immunochemistry, microanalytical chemistry and molecular biology are introduced by several research teams. Their contribution in the elucidation of the complexity of the control mechanisms is evaluated and reflections are made to possible spin-offs and application in aquaculture. Ideas about the topics which should be investigated "in priority" in the near future are proposed.

Key words: Crustacea, Neuroendocrinology, Reproduction, Aquaculture.

In the late 1920's and early 1930's E. Scharrer formulated for the first time the phenomenon of neurosecretion based on his studies on the hypothalamic-hypophyseal system of Vertebrates. In that

period, Hanström (1931) extended this type of observations to the invertebrates by the the description of neurosecretory cells in the eyestalk of several decapod crustaceans. Since those pioneer studies, more than 20 candidate neurohormonal factors have been described in Crustacea. They are all synthesized by various regions of the nervous system and have inhibitory and/or stimulatory effects on a variety of behavioral and physiological processes.

A neuroendocrine control mechanism for reproduction in crustaceans was demonstrated about 10 years after the first description on neurosecretion in Crustacea. In 1943 Panouse found an ovarian inhibiting factor in the eyestalk as he observed that eyestalk removal from females of the prawn *Palaemon serratus* during the period of genital rest leads to a rapid increase in ovarian growth. Since that period the presence of a so-called Gonad Inhibiting Hormone (GIH) in the X-organ sinus gland complex of the eyestalk has been confirmed in a number of crustaceans. Nowadays, Panouse's approach is still applicable in aquaculture as unilateral eyestalk ablation is usually practised to trigger vitellogenesis in some cultured species.

Up to now most of the studies on the control of reproduction in crustaceans were dealing with the regulation of female reproduction. From detailed histophysiological studies on oogenesis and vitellogenesis, it was concluded that GIH inhibits vitellogenesis. Therefore the name Vitellogenesis Inhibiting Hormone (VIH) was introduced by Charniaux-Cotton around the eighties. Various investigations are also in favor of the existence of a stimulating neurohormonal control of vitellogenesis. In 1960, Otsu gave the first indications for the presence of such a factor in the thoracic ganglia of the crab *Potamon dehaani*. Later studies postulated that such a factor may act directly on the ovary on an intermediate organ or on both. Recently a stimulatory effect on the growth of oocytes could be associated with one of the isoforms of the Crustacean Hyperglycemic Hormone (CHH) in the lobster *Homarus americanus* (Tensen *et al.*, 1989), but it is still unknown if this is a direct effect. Furthermore, there are indications that the testis or the vas deferens of the male shrimp *Paratya compressa* would secrete an ovary stimulating pheromone which accelerates ovarian

development (Takayanagi *et al.*, 1986). As in vertebrates, completion and maintenance of gametogenesis and related reproductive processes in Crustacea require interactions between factors deriving not only from the nervous system but also from endocrine glands. The Y-organs produce and secrete α-ecdysone which is hydroxylated in the hemolymph to the active hormone β- or 20-OH-ecdysone. The Y-organ has its main function in the stimulation of molting and counteracts in this process with the Molt Inhibiting Hormone (MIH), a neuropeptide also produced in the eyestalks. In addition it has been demonstrated that ecdysteroids in the hemolymph may also play a stimulatory role on ovarian protein synthesis and production of vitellogenin. They seem to intervene also in vitellogenin uptake by the oocyte. The existence of a Vitellogenin Stimulating Ovarian Hormone (VSOH) produced by the follicle cells to stimulate vitellogenin synthesis was proved only in Amphipoda. Finally, the mandibular organs are responsible for the secretion of juvenoid compound factors which seem to play a role in vitellogenesis (Laufer *et al.*, 1986, 1987).

So far not much attention has been paid to the (neuro)hormonal control of the male reproductive system. Since a few years it is known that spermatogonial differentiation requires the presence of the Androgenic Hormone (AH) which is produced by the androgenic gland developing only in males. Completion, maintenance and intensity of spermatogenic activity are generally regulated both by AH and neurohormones. However, most of the studies are still speculative by lacking of suitable bioassays.

Based on reviews from Legrand *et al.* (1982), Payen (1986), Meusy and Payen (1988) and Charniaux-Cotton and Payen (1988), we have summarized the control of reproduction in female and male decapod Crustacea in a schematic representation of "real" and "hypothetical" pathways intervening respectively in vitellogenesis (Fig. 1) and spermatogensis (Fig. 2). Details on the specific references can found in the aforementioned review articles. From both schemes it is clear that the neuroendocrine system, especially the X-organ sinus gland complex in the eyestalk plays an integratory role in the regulation of reproduction. It is responsible for tuning the endocrine and reproductive organs as Y-organ, mandibular

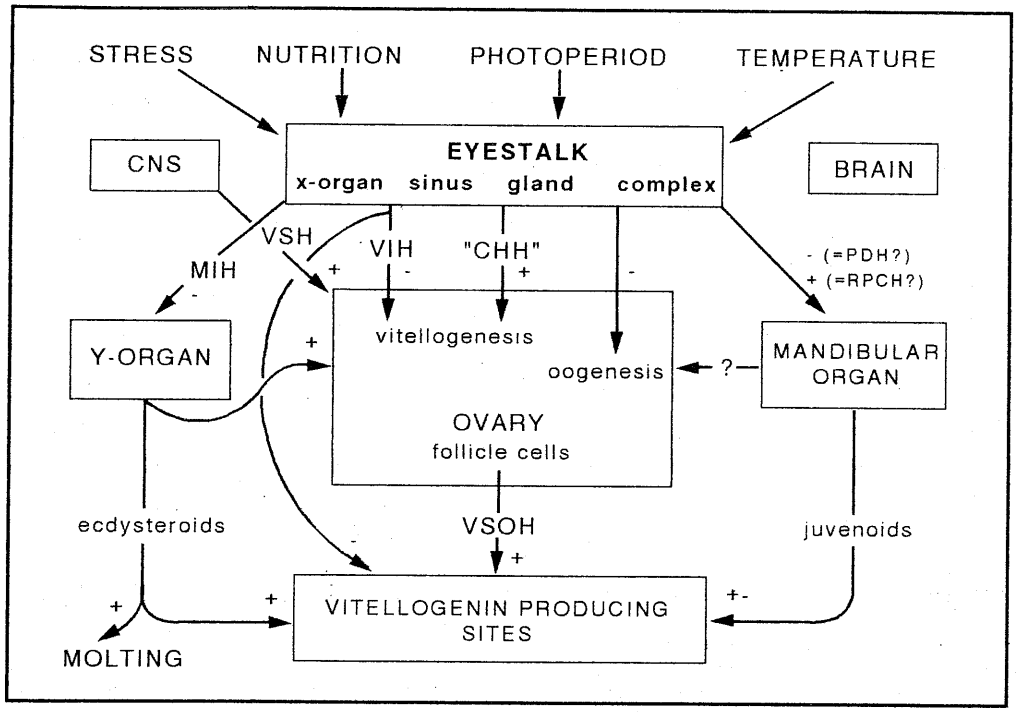


Fig. 1. Control of reproduction in female decapod Crustacea: a schematic representation of real and hypothetical pathways in vitellogenesis.

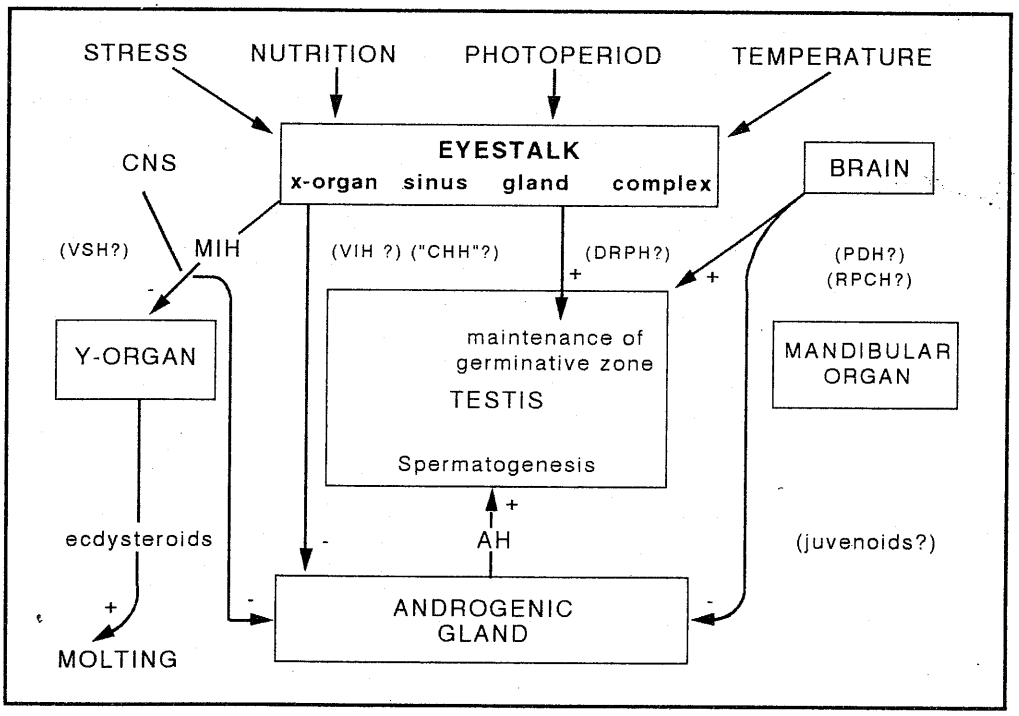


Fig. 2. Control of reproduction in male decapod Crustacea: a schematic representation of real and hypothetical pathways in spermatogenesis.

organ, androgenic gland and testis or ovary to the variations in the environment. Among the external factors photoperiodism and temperature seem to be the most important. However, in studies carried out in recent years on aquacultural species to improve the production of farming, it is shown that a significant effect of nutrition and stress on growth (molting) and reproduction may not be excluded.

In this paper we have reviewed recent data on the neuroendocrine component involved in the regulation of crustacean reproduction obtained by the application of microanalytical chemistry, immunocytochemistry and molecular biology. We have evaluated the contribution of these methods in the elucidation of the complexity of the control mechanisms. With reflections to the possible spin-offs and application in aquaculture, we have concentrated our contribution on those regulatory factors which presence and physiological effect is demonstrated with great certitudes.

MORPHOLOGY AND CYTOLOGY OF THE NEUROENDOCRINE SYSTEMS INVOLVED IN REPRODUCTIVE CONTROL

The neuroendocrine system of Crustacea is complex and components are found throughout the central nervous system. Neurons with a putative neuroendocrine function in reproduction occur in the optic ganglia of the eyestalk as well as in the cerebral and thoracic ganglia, but the X-organ sinus gland complex in the eyestalk shows a remarkable clustering of neuroendocrine cell groups important for the synthesis of neuropeptides controlling reproduction. Iontophoretic and retrograde cobalt injection methods revealed that the sinus gland acts as a neurohemal region for neuroendocrine products arising from the cells in the Medulla Terminalis Ganglionic X-organ (MTGX) and in a lower extent from those of the Medulla Externa X-organ (MEX) (Andrew *et al.*, 1978; Jaros, 1978). By those techniques it was also possible to identify connections with the cerebral ganglia. A light microscopical picture of a longitudinal section of an eyestalk is shown in Fig. 3. Immunocytochemical studies made it possible to demonstrate a more extensive peptidergic system than formerly recognized (Van Deijnen *et al.*, 1985; Van Herp, 1988). The morphology of this system suggests that one part has a neurohormonal character and that the other part has a

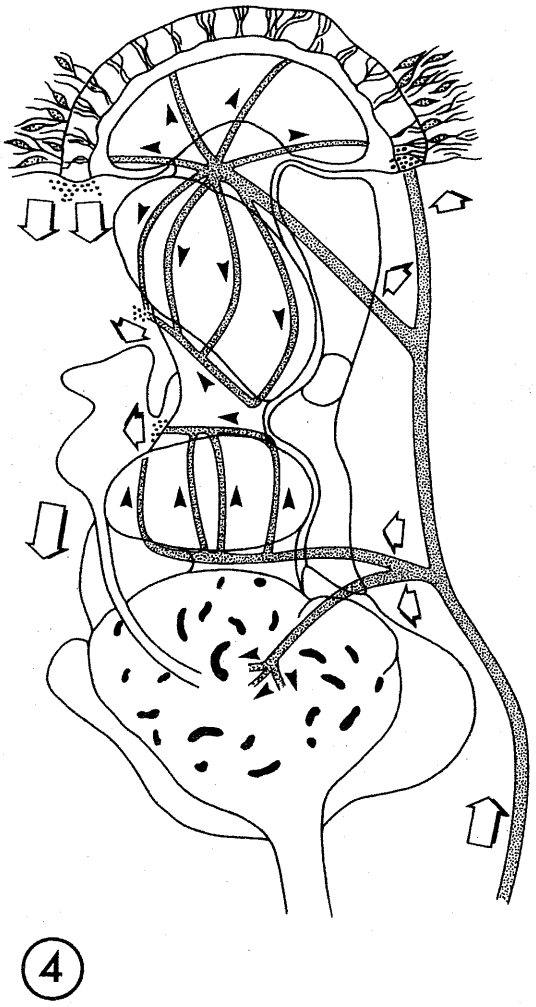


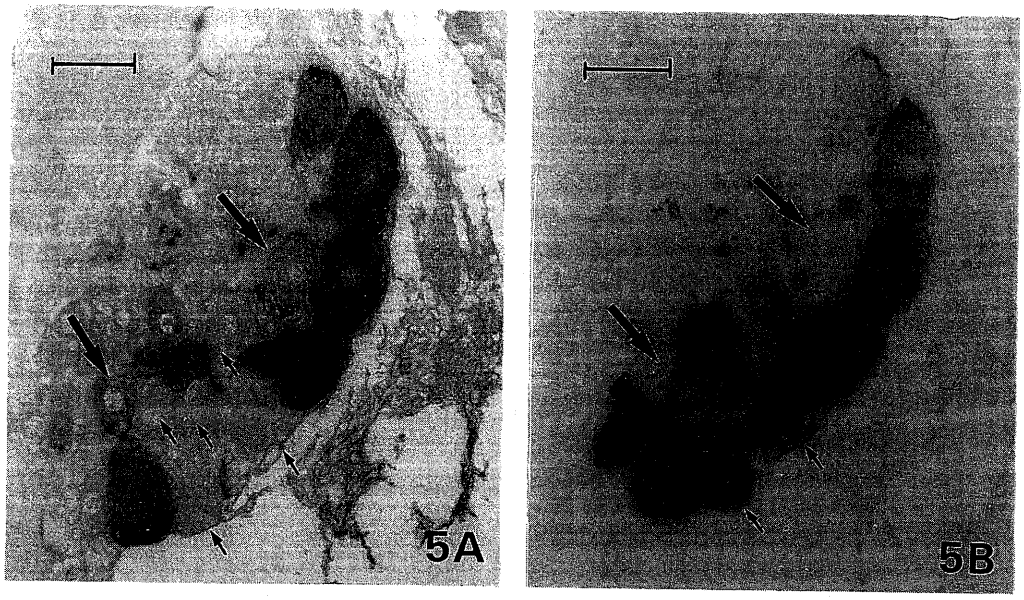
Fig. 3. Longitudinal section of the eyestalk of the crayfish *Astacus leptodactylus* showing the optic ganglia and neuroendocrine centers. (H: hemolymph; LG: lamina ganglionaris; ME: medulla interna; MT: medulla terminalis; MTGX: medulla terminalis X-organ; O: ommatidia; SG: sinus gland; arrows indicate neuroendocrine cells and material in the neurohemal region. Bar represents 100 μm).

Fig. 4. Schematic representation of the vascular system in the eyestalk of the crayfish *Astacus leptodactylus*. The flow of the hemolymph is indicated by arrows and arrow heads. Modified from Van Deijnen, 1986.

neurotransmitter and/or neuromodulatory nature. Perfusion experiments of the vascular system in the optic ganglia showed that the hemolymph is highly compartimentalized in the eyestalk (Sandeman, 1967, 1982). This may have a significant consequence for the regulatory role upon the sinus gland mediated by the hemolymph. As illustrated in Fig. 4 the efferent blood vessels from the optic ganglia, with exclusion of the medulla terminalis, discharge into the hemocoel through the sinus of this neurohemal organ. This arrangement implies that the peptidergic axon terminals from the three upper optic ganglia are connected to the sinus gland by the vascular system. This opens the possibility that the secreted factors control the release of the neurohormones deriving from the X-organs and accumulating in the sinus gland.

In our laboratories polyclonal antibodies against purified *Homarus americanus* VIH were used for the identification of the VIH producing cell system in the eyestalk of the lobster (Meusy *et al.*, 1987; Kallen and Meusy, 1989). An immunopositive reaction was found in neuroendocrine cell somata belonging to the cluster of the MTGX, in the tractus to the sinus gland and in this neurohemal region itself. Comparing the localization of the CHH producing cells in the MTGX, which were visualized by a polyclonal serum against CHH of the crayfish, revealed a frequent but not complete co-location of VIH and CHH in a variable number of the same group of perikarya. Furthermore, a small number of cells only showed an immuno-response with anti-VIH serum. Electron microscopical observations of the sinus gland demonstrated that both neuropeptides are accumulated in distinct axonal endings and are related to different granule types. Figs. 5A-5F and 6A-6B illustrate the immunocytochemical localizatin of VIH and CHH in the MTGX of the lobster. Taking these observations into account the following postulations can be made: 1) CHH and VIH might be synthesized in the same perikarya *via* separate biosynthetic pathways; 2) CHH and VIH might be encoded by one primary RNA transcript; 3) both CHH and VIH may originate from a common precursor.

By means of immunocytochemical and morphological studies at the light and electron microscopical level, it was possible to understand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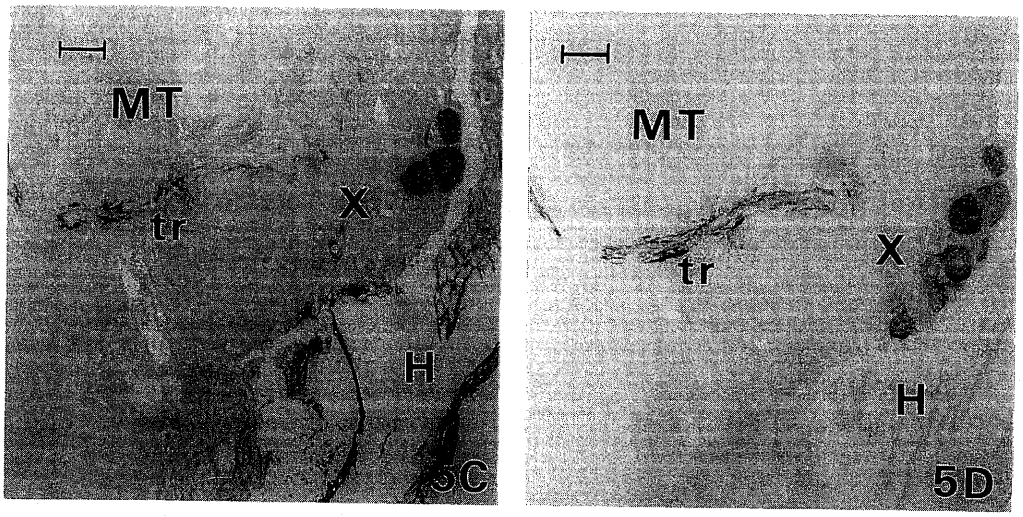
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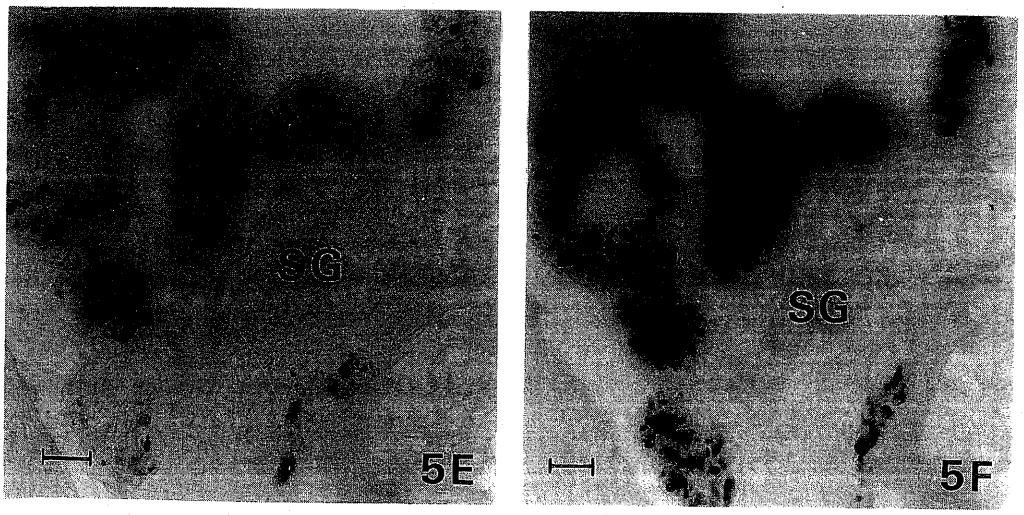
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the secretory dynamics of CHH producing cell system in several crayfish species (see review Van Herp and Kallen, 1991). This knowledge is certainly useful for further detailed studies on the dynamics of the VIH producing cell system. Concerning the CHH system we know that: 1) the secretory dynamics show an endogenous circadian rhythmicity entrained by the light/dark schedule and may be controlled by a photoreceptor located in the optic ganglia of the eyestalk rather than in the compound eyes (Gorgels-Kallen and Voorter, 1985; Kallen *et al.*, 1988); 2) the secretory activity of the system is firmly reduced during the molting period and at that time the nocturnal activity peak is completely absent (Kallen, 1985); 3) the system develops immediately after hatching and is already active during the larval and postlarval life cycle (Gorgels-Kallen and May, 1985); 4) CHH axon branches have a serotoninergic synaptic input and also dopamine and met-enkephalin are good candidates for the neuromodulatory regulation of the system (Van Herp and Kallen, 1991; Kallen *et al.*, in preparation).

As in many decapod crustacean species genital and spawning activity are related to molting, one may not exclude a possible relationship between the control of molting and reproduction. Therefore, it is worthwhile to notice that the MIH is also synthesized in the MTGX. Using a polyclonal antiserum against crab MIH, it was possible to identify the MIH producing cells (Dircksen *et al.*, 1988). The MIH cells are intermingled with the CHH cells, their axons follow the same tractus to the sinus gland and their axon terminals are distributed among the

Fig. 5. Serial sections (5 μm) demonstrating the immunocytochemical localization of VIH and CHH in the X-organ sinus gland complex of the lobster *Homarus americanus*. A-B: co-localization of the VIH cells (A) and CHH cells (B) in the MTGX. Arrows indicate the cells that are stained only with anti-VIH (large arrows) or with anti-CHH (small arrows). Bar represents 50 μm. C-D: view of the VIH cells and their axons (C) and the CHH cells and their axons (D) in the medulla terminalis. (H: hemolymph; MT: medulla terminalis; tr: tractus from the X-organ to the sinus gland; X: X-organ. Bar represents 50 μ). E-F: immunopositive reaction for anti-VIH (E) and anti-CHH (F). (H: hemolymph; SG: sinus gland. Bar represents 30 μm). Results according to Kallen and Meusy, 1989.

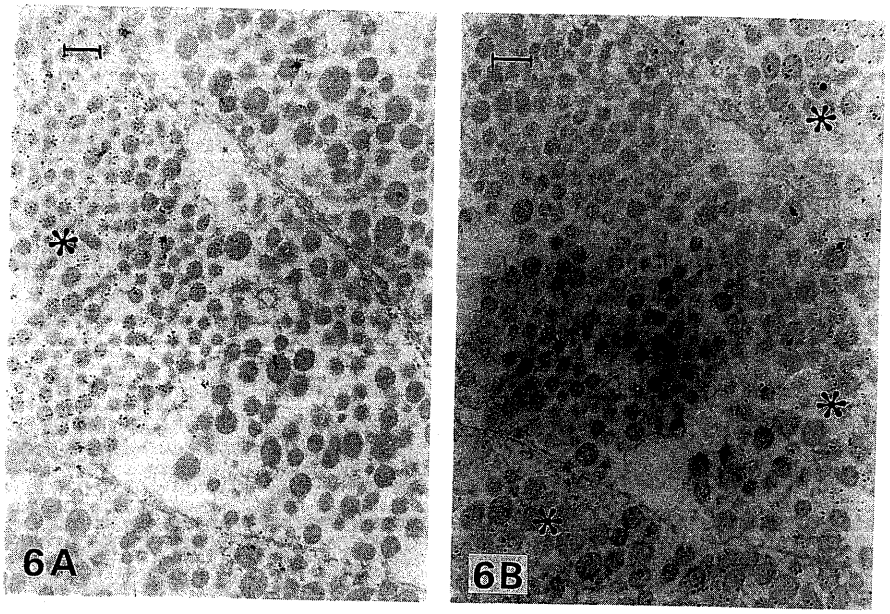

Fig. 6. Consecutive ultrathin sections of the sinus gland treated with anti-VIH (A) and anti-CHH (B) showing that these two neurohormones are located in different granule types (see *). Glutaraldehyde fixation; Epon embedding and Protein-A 16 nm immunogold labelling. Bar represents 200 μm. Results according to Kallen and Meusy, 1989.

numerous CHH axon terminals. Despite the similarity in the morphology of the systems no co-localization was observed.

Furthermore, antibodies raised against synthetic derivatives from Red Pigment Concentrating Hormone (RPCH) and Pigment Dispersing Hormone (PDH) revealed that RPCH is also produced by neuroendocrine cells belonging to the MTGX and that the axon terminals both neuropeptides end in the sinus gland (Bellon-Humbert *et al.*, 1986; Mangerich *et al.*, 1986, 1987; Schooneveld *et al.*, 1989). In recent studies indications are made on the stimulation by RPCH and on the inhibition by β-PDH of the secretion of methyl farnesoate by the mandibular organs (Landau *et al.*, 1989).

In conclusion, as the aforementioned native crustacean neuropeptides playing a role in the regulation of reproduction are, for the greater part, grouped in the same regions of the X-organ sinus gland complex, research carried out on their synthesis, storage and release, in relation with seasonal, diurnal and other variations, has good prospects in the near future.

ISOLATION AND CHARACTERIZATION OF (NEURO) PEPTIDES INVOLVED IN REPRODUCTIVE CONTROL

Inhibiting factors

Several attempts to purify VIH have been reported among decapod crustaceans.

A factor with VIH activity has been isolated from eyestalks of male crab *Cancer magister* and tested in female shrimp *Crangon crangon* (Bomirsky *et al.*, 1981). The neurohormone is neither sex nor species specific, it is thermostable and its MW is about 2 kDa.

VIH activity from eyestalks of the spiny lobster *Panulirus argus* has been bioassayed in female crab *Uca pugilator* (Quackenbush and Herrnkind, 1983). The hormone has been partially characterized and the MW was determined at 5 kDa.

An inhibibitory from eyestalks of the shrimp *Penaeus vannamei* assayed *in vitro* on protein synthesis by both the hepatopancreas and the ovary was estimated to be 3,300 daltons (Quackenbush, 1989). This result is in agreement with previous data obtained from *P. setiferus* eyestalks (Quackenbush and Keeley, 1988) where the partially purified material inhibited *in vitro* production of vitellogenin by ovarian tissue of the crab *Uca pugilator*.

In the last few years, our two laboratories studied the neuropeptides of the lobster *Homarus americanus* extensively. A VIH factor has been characterized from extracts of sinus gland using an *in vivo* bioassay developed in the shrimp *Palaemonetes varians* measuring the changes in growth of the oocytes in secondary vitellogenesis (Soyez *et al.*, 1987). After a two-step purification by reversed phase HPLC, the bioactivity was recovered in a symmetrical peak. SDS-urea-PAGE revealed the presence of one band corresponding to a MW of 7-8 kDa. The used HPLC protocol allowed an 85-fold enrichment of the active material and the recovery was about 20-30 ng/10 μg of starting material, which is approximately 0.2% of the total extractable protein content of the sinus gland. This lobster VIH seems to be specific in its physiological actions and it displays no hyperglycemic activity. In subsequent studies the neuropeptide content from lyophilized sinus glands was analysed by HPLC and special attention was given to the peptides with polarity close to that of VIH. After separation by cation-exchange HPLC, a vitellogenesis inhibiting activity is

present in two distinct fractions (Tensen *et al.*, 1989), while only one peak (peptide 3, recently named Hoa-VIH I) displayed a significant bioactivity after fractionation of lobster sinus gland extract by RP-HPLC (Soyez *et al.*, 1987). Preliminary biochemical analysis (Soyez *et al.*, 1990) as well as immunochemical studies (Meusy and Soyez, 1991) indicate that a structurally related isoform (peptide 4, Hoa-VIH II) is present in the sinus gland. This isoform was found to be inactive in the *Palaemonetes* assay. Amino acid sequence both isoforms was recently established by gas phase microsequencing and FAB/MS analysis of native peptide and of fragments derived from enzymatic cleavage experiments (Soyez *et al.*, 1991). The two peptides are neutral (pI 6.8), have a free N-terminus, contain 77 amino acid residues among which 6 cysteins. The MW deduced from the sequence fully agrees with the value determined by FAB/MS (9,135 Da). In conclusion, structurally related to lobster CHH (Tensen *et al.*, 1991c) and MIH (Chang *et al.*, 1990), VIH of *Homarus americanus* clearly appears as an original member of the CHH family. Based on the amino acid sequence of VIH, a set of two cDNA probes were synthesized and hybridized in a Northern blot analysis of total RNA extracted from lobster X-organs. A positive was found in a zone corresponding to a size of approximately 2.5 kb (Laverdure *et al.*, in preparation). Recently we used the polymerase chain reaction (PCR) in combination with a set of selected degenerated oligonucleotides for specific amplification of a partial cDNA. The PCR reaction yielded an expected product of 160 bps. Sequence analysis of recombinants, containing the 160 bps product, revealed the presence of a cDNA encoding a partial sequence of VIH. This deduced amino acid sequence fully agrees with the appropriate amino acid sequence determined by the chemical approach (Da Kleijn *et al.*, in preparation). Immunochemical investigation, using a dot immunobinding assay (DIA) and an enzyme linked immunosorbent assay (ELISA), revealed information about species specificity based on immunochemical criteria (Meusy *et al.*, 1987). Antisera raised against VIH of *Homarus americanus* cross-react with sinus gland extracts of *Palaemon varians*, *Palaemon serratus*, *Macrobrachium rosenbergii*, *Carcinus maenas* and *Porcellio dilatatus* and not with extracts from *Penaeus vannamei*, *Penaeus monodon*, *Astacus*

leptodactylus, *Orconectes limosus* and *Jasus paulensis*.

Recent studies on the isolation and characterization of neuropeptides of the sinus gland from the terrestrial isopod, *Armadillidium vulgare* revealed that the amino acid sequence of isopod VIH and CHH are very similar (Martin *et al.*, personal communication).

Stimulatory factors

Boiled aqueous extracts of thoracic ganglia from *Uca pugilator* stimulate vitellogenesis in both intact and destalked crabs indicating the presence of perhaps a neuropeptide with a small ME (Eastman Reks and Fingerman, 1984). Takayanagi *et al.*, (1986) demonstrated that aqueous extracts not only from thoracic ganglia but also from brain have a stimulating effect on vitellogenesis in the shrimp *Paratya compressa*. From those studies the existence of an aqueous soluble VSH, secreted by nervous cells seems to be established but no details about further purification and characterization are published until now.

During our studies on the isolation, characterization and physiological specificity of neuropeptides from the sinus gland of the lobster *Homarus americanus*, we demonstrated that lobster CHH is polymorphic and that hyperglycemia is elicited by closely related peptides with similar molecular weights and amino acid sequences (Tensen *et al.*, 1988, 1991b, 1991c). A hyperglycemic response is provoked by two 8,577 Da peptides and one of the 8,633Da components. In addition, a stimulatory effect on the oocyte growth in *Palaemon varians* with non-vitellogenic ovaries is associated with the elution time of both 8,633Da peptides (Tensen *et al.*, 1989). So far, it is still unknown if this stimulatory effect is a direct effect or if it is provoked by a higher rate in carbohydrate metabolism after injection of these 8,633Da isoforms. It is striking to observe that injection of the smaller isoforms of CHH have no effect on oocyte growth. Anyhow, the existence of stimulatory effect in relation with the CHH peptides is promising for further detailed investigations on the interactions of hyperglycemic neuropeptides in reproduction.

Concerning the characterization of AH produced by the androgenic glands in male crustaceans, for some time it was not clear whether a protein or a lipoidal factor was involved. Evidence for the protein nature of AH is obvious in the

isopod *Armadillidium vulgare* (Hasegawa *et el.*, 1987; Martin *et al.*, 1990). In the last study AH was purified from hypertrophied androgenic glands of intersexed animals. Two isohormones labelled AH1 and AH2 with similar molecular weights in the range of 17,000-18,000 Da were isolated as in normal *A. vulgare*. Likewise, amino acid composition analysis performed by the two groups of authors, demonstrated the absence of cystein in both forms, but the physiological significance of the polymorphism is still unknown. Up to now no detailed studies have been published on the characterization of VSOH in Amphipods. As it seems to play a similar role as oestradiol-17β in egg laying vertebrates, one may not exclude that VSOH also belongs to the steroid hormones.

Other factors

Partial characterization of a neuroendocrine factor involved in the development of the male genital apparatus in decapod Crustacea indicated the presence of a thermostable molecule eluted in the same zone as the Distal Retinal Pigment Hormone (DRPH), which MW is around 2 kDa (Payen *et al.*, 1983).

As shown in Figs. 1 and 2, several reflections have been made about the neuromodulatory functions of neuropeptides with primary chromatophorotropic activities, such as RPCH, PDH and DRPH. RPCH from *Pandalus borealis* was the first invertebrate neurohormone that has been isolated and fully characterized by determing the amino acid sequence: PGlu-Leu-Asn-Phe-Ser-Pro-Gly-Trp-NH_2 (Fernlund and Josefsson, 1972). It is also the first invertebrate neuropeptide that was chemically synthesized (Fernlund, 1976) and commercially available. The first structural elucidation of a peptide with pigment dispersing activities, DRPH, was performed by Fernlund in 1976 from eyestalks of *Pandalus borealis*. In the meantime at least 4 different PDHs have been isolated and sequenced, for example β-PDH from *Uca pugilator* (Rao and Riehm, 1989) and *Cancer magister* (Kleinholz *et al.*, 1986). The amino acid sequence of DRPH (α-PDH) from *Pandalus borealis* was determined as: Asn-Ser-Gly-Met-Ile-Asn-Ser-Ile-Leu-Gly-Ile-Pro-Arg-Val-Met-Thr-Glu-Ala-NH_2, the other PDHs consist also of 18 amino acids, have an amidated C-terminal and share identical amino acids on positions 1, 2, 5, 6, 7, 9, 10, 12 and 18.

The molting process in crustaceans is controlled by two hormones: ecdysone, a steroid and MIH, a peptide. Several experiments showed that MIH inhibits ecdysteroid synthesis by the Y-organs and that the presence of 20-OH-ecdysone is necessary not only for the induction of molting but also for the onset and completion of vitellogensis. In this respect one may propagate that MIH may also have an indirect regulatory influence on reproduction. A report on the amino acid sequence of MIH from *Homarus americanus* has been recently published (Chang *et al.*, 1990). The identified peptide has also a significant hyperglycemic activity and therefore it belongs to the family of CHH.

MODE OF ACTION OF NEUROPEPTIDES INVOLVED IN REPRODUCTIVE CONTROL

As in most animals the oocyte of a female crustacean must acquire the capacity to generate the species and therefore it undergoes a sequence of major morphological transformation. The most intensively investigated aspect of oogenesis in crustaceans concerns vitellogenesis, a process that is mainly characterized by extra-oocytic yolk production and accumulation of this material in the growing oocyte. In several publications the term "maturation" is used in relation with vitellogenesis, although maturation corresponds to the stage of breakdown of the germinal vesicle and resumption of meiosis following vitellogenesis. The earlier steps in crustacean oocyte growth are called oogenesis and previtellogenesis. Oogensis is mainly characterized by mitotic and meiotic activities leading to the formation of fully grown previtellogenic oocytes. When endogenous glycoproteins accumulate in the oocytes, the process of "endogenous" vitellogenesis starts. This stage stops when the oocyte reaches a diameter typical for the species and can be retained for a longer period in young females and during genital rest in puberal females. Fig. 7 summarizes the chronological events of oocyte development. The undifferentiated gonad forms the germinative zone of the ovary and the gonia become oogonia. Each of these cells is completely surrounded by mesodermal cells and undergoes oogonial mitosis exclusively in the germinative zone. Some oogonia leave continuously this zone and enter meiotic prophase. During previtellogenesis the oocytes

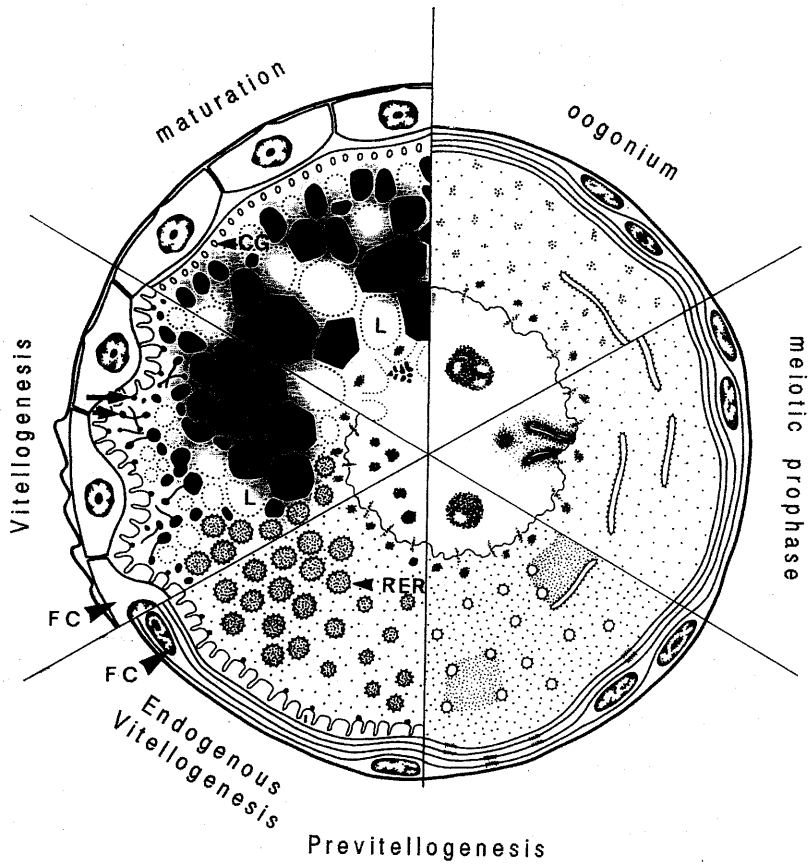

Fig. 7. Diagram summarizing the chronological and cytological events of oocyte development. (CG: cortical granule; FC: follicle cell; L: lipid globule; RER: rough endoplasmic reticulum; YB: yolk body; arrow: endocytotic activity during vitellogenesis). Modified from Charniaux-Cotton, 1980.

accumlate ribosomes and a rough endoplasmic reticulum (RER) develops. When this RER becomes active the oocytes synthesize intra-oocytotic yolk which accumulates in the RER. At the end of the previtellogenic stage the oocytes develop microvilli and the follicle cells also become active. At the season of reproduction, oocytes at the end of previtellogenesis enter vitellogenesis. Each of them is surrounded by a single layer of follicle cells and oocyte microvilli cross the vitellin envelope. Oocytes accumulate yolk proteins which major component is called vitellin. This yolk protein originates from vitellogenin which is taken up from the hemolymph by endocytosis. Synthesis of glycoproteins by the oocyte RER continues during vitellogenesis and carotenoids are associated with vitellin resulting in

bright colored oocytes. After maturation, ovulation occurs when the follicular epithelium retracts at the periphery of the ovary.

Details about the regulation of oogenesis are well documented in the reviews by Charniaux-Cotton (1980), Legrand *et al.* (1982), Meusy and Charniaux-Cotton (1984), Charniaux-Cotton (1985), Adiyodi (1985), Charniaux-Cotton and Payen (1988) and Meusy and Payen (1988). In female crustaceans, the regulation of oogenesis up to the end of previtellogenesis is only partially under the control of neurohormonal factors and detailed cellular mechanisms are still missing. Summarizing: 1) Ovarian differentiation of the gonad rudiment is a process of auto-differentiation, Oogenesis from oogonia to the end of previtellogenesis takes place spontaneously, in the absence of any hormone. 2) Initiation of oogenesis does not appear to be controlled by a neurohormone. No acceleration of the onset of oogenesis has been observed after removal of the eyestalks in larvae and young females. 3) The maintenance of the germinative zone of the ovary does not need the presence of a neurohormone. 4) On the contrary, the increase in number of oogonial mitosis and in number of oocytes entering into meiotic prophase seems to be controlled by a moderating hormone.

Various studies have shown that oocyte growth during vitellogenesis is controlled by neurohormones. 1) A moderating factor seems to interfere with the development of the follicular tissue. 2) VIH plays a predominant role in the regulation of endocytosis of vitellogenin as well as in the regulation of vitellogenin synthesis. 3) From studies in the amphipod *Orchestia*, one can deduce that the follicle cells of the ovary contribute in the stimulation of vitellogenin synthesis by secreting a VSOH. 4) The control of yolk production is also under the control of ecdysteroids and juvenoids. 5) A VIH antagonistic neuropeptide, VSH, is responsible for stimulation of yolk incorporation and has a positive effect on ovarian protein synthesis.

During the last few years our laboratories studied the neurohormonal control of vitellogenin endocytosis by the oocytes is some detail as the cellular mechanism of this process was still unknown. Jugan and Soyez (1985) demonstrated that a sinus gland extract inhibits the binding of colloidal-gold labelled vitellin on the oocyte microvilli (Figs. 8A-8C) and Jugan (1985)

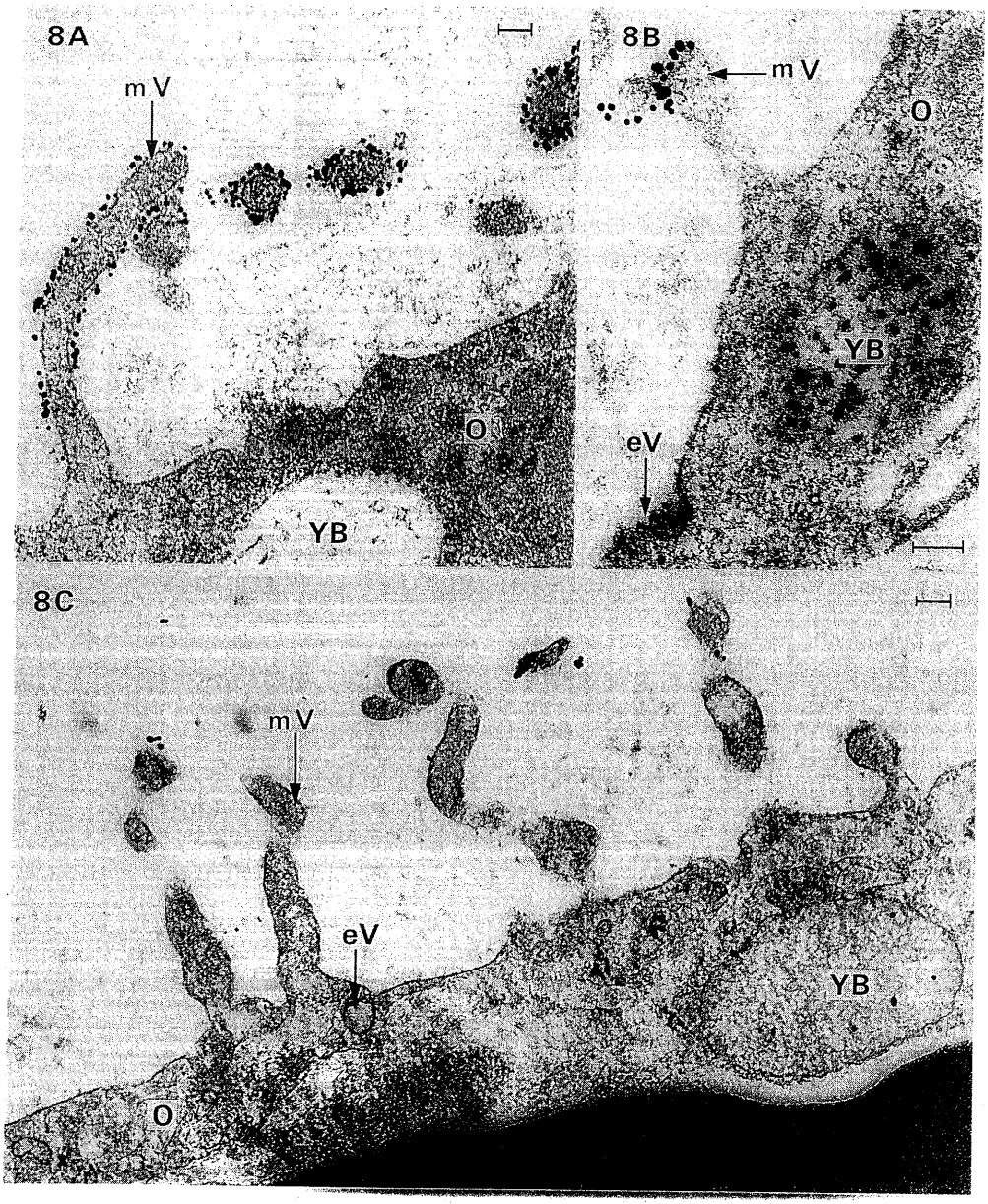

Fig. 8. Binding of colloidal-gold labelled vitellin conjugate on the oocyte microvilli of an oocyte from *Macrobrachium rosenbergii*. A-B: oocyte incubated without addition of lobster sinus gland extract. C: oocyte incubated with addition of lobster sinus gland extract. (eV: endocytotic vesicle; mV: microvilli; o: oocyte; YB: yolk body. Bar represents 0.1 μm). From Jugan, 1985.

reported in a preliminary study, using peroxidase-labelled vitellin, that the affinity of VIH for the receptors to vitellin would be higher than of vitellin itself. Both results give evidence for a direct hormonal control of vitellogenin uptake by the oocytes of the prawn *Macrobrachium*

rosenbergii, In introductory studies we characterized a 28 kDa protein in the oocyte membrane of the crayfish *Orconectes limosus* that specifically binds vitellogenin (Jugan and Van Herp, 1989). Antibodies against this protein should offer the possibility to set up further experiments in order to investigate the cellular interactions of neurohormonal factors controlling vitellogenesis and to study the

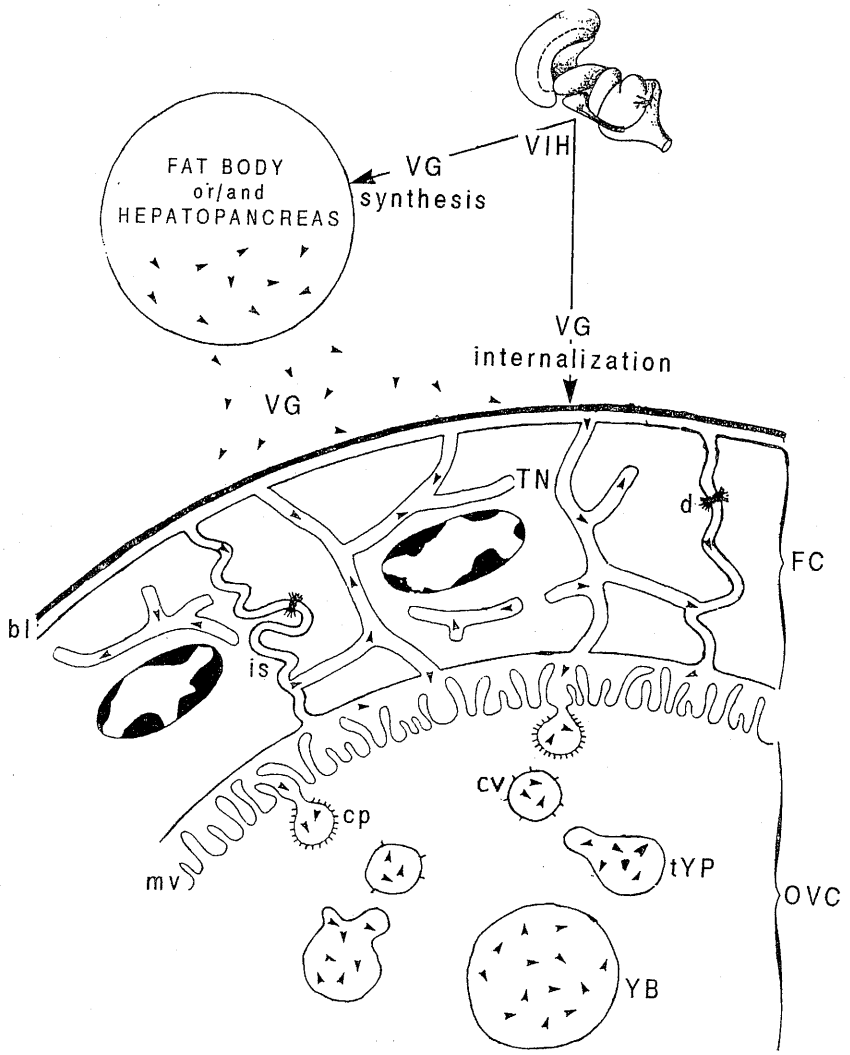

Fig. 9. Schematic representation of vitellogenin accumulation in the crustacean oocyte and the control by VIH from the X-organ sinus gland complex in the eyestalk. (bl: basal lamina; cp: coated pit; cv: coated vesicle; d: desmosome; FC: follicle cell; is: intercellular space; mv: microvilli; ovc: ovocyte; tYP: transitional yolk body; TN: tubular network; VG: vitellogenin; VIH: vitellogenin inhibiting hormone; YB: yolk body). Modified from Charniaux-Cotton and Payen, 1988.

recepor-mediated endocytosis. Based on the concept of receptor-mediated endocytosis used by cells for recognition and uptake of specific molecules (review Goldstein *et al.*, 1979) and the selective uptake of vitellogenin from blood by developing oocytes (Roth *et al.*, 1976), we made a schematic presentation of vitellogenin accumulation in the crustacean oocyte and the mode of action of VIH (Fig. 9).

Studies on the control of the development of the male genital apparatus and of the onset of spermatogenesis proved that AH has several roles: it induces male differentiation of the genital tract and of the gonia; it stimulates the development of the androgenic gland primordia in genetic females; it allows the first initiation of the spermatogenic cycle and regulates its intensity. It is also necessary for the completion of meiotic prophase I and often ensures the onset of new spermatogenic cycles. Furthermore, AH also controls the development of the external characteristics. However, most of these studies need further detailed information and suitable bioassays are necessary. In this respect, the set-up of a bioassay based on the variations of the specific activity of aspartate trans-carbomylase in the male genital apparatus is encouraging for further researh on male genitalotropic neurohormones (Payen *et al.*, 1983).

PERSPECTIVES AND CONCLUSION

At present, the picture of the neuroendorine control of reproduction in Crustacea is still far from complete. However, in recent years modern techniques such as immunocytochemisty for localization at the light and electron microscopic level, microanalytical chemistry for protein sequencing and molecular biological techniques for studying DNA and mRNA, have been introduced by several research groups intersted in the neuroendocrinology of crustaceans. These studies must proceed as much for their fundamental interest, as for their purposes in aquaculture and must be extended, in particular, in the field of the control of crustacean reproduction. Further progress will mostly come from complete characterization of the involved neurohormones in terms of amino acid sequence and the unequivocal determination and comparison of their biological activities.

Great improvements in the biochemical isolation and purification of crustacean neuropeptides were obtained with the introduction of High Performance Liquid Chromatography (HPLC). The polymorphic nature of CHH (Tensen *et al.*, 1989, 1991b, 1991c) and VIH (Soyez *et al.*, 1991; Meusy and Soyez, 1991) has been recently demonstrated and the HPLC purification procedures yield peptides pure enough for further characterization studies.

Amino acid sequence determinations by gasphase microsequencing and precise molecular weight analysis by Fast Bombardment Mass Spectrometry (FAB/MS) and Electro Spray Mass Spectrometry (ES/MS) became powerful tools for the analysis of the primary structure of MIH (Chang *et al.*, 1990), CHH (Tensen *et al.*, 1991c) and VIH (Soyez *et al.*, 1991) in the lobster. Molecular biological techniques such as molecular cloning of cDNA starting from cDNA libraries or generated by the Polymerase Chain Reaction (PRC) using degenerated oligonucleotide primers, are necessary not only to support the microanalytical approach for determination of the amino acid sequences of native neuropeptides (Tensen *et al.* 1991b), but also to obtain detailed information on the preprohormone products *e.g.*, the CHH preprohormone (Weidemann *et al.*, 1989; Tensen, 1991). Such knowledge is, for example, required to unravel the biosynthesis of VIH and CHH and to elucidate the biochemical and functional relationship between both crustacean neuropeptides.

After establishment of the complete primary and/or secondary structures of the neuropeptides controlling reproduction in crustaceans, it will be fruitful to obtain the various neurohormones available in quantities that allow detailed studies of their function. Therefore, it will be necessary to synthesize fragments and/or complete synthetic derivatives of the factors in order to study their interactions with the receptors of the target organs and their cellular mode of action. Selected fluorescent derivatives of the neuropeptides can be prepared in order to utilize them for microscopic visualization and localization of hormone receptors at the level of the target organs.

The synthetic probes can also be used for the preparation of polyclonal and monoclonal antibodies which are applicable in further detailed immunocytochemical studies and for the development of quantification methods for measuring

neurohormonal concentrations. Recently we showed that an Enzyme Linked Immuno Sorbent Assay (ELISA) is a suitable method for determining hormone levels in the hemolymph of crustaceans (Kallen *et al.*, 1990). In that study it is shown that the circadian rhythm of blood CHH levels in the crayfish *Orconectes limosus* has a pattern similar to that found for blood glucose: basal levels during daytime and an increase of blooch CHH content during the first hour of darkness.

The first application of cDNA sequence in studies on the regulation of the gene(s) encoding CHH in lobster and crayfish has recently been introduced. Non-radioactive labelled complementary RNA (cRNA) probes derived from cDNA sequences were applied in "*in situ*" hybridization studies of eyestalks. In combination with immunocytochemical staining results, it could be concluded that these cRNA probes can be used for the visualization of CHH encoding mRNA within the CHH producing cell system (Tensen *et al.*, 1991a). These complementary RNA probes were also useful for the quatitative detection of mRNA encoding CHH in the eyestalk of the crayfish, using a sensitive hybridization method, the RNase protection assay, which allows to quantify changes in levels of CHH encoding mRNA. We demonstrated that this assay is very suitable to determine differences in mRNA concentrations in individual animals. First results on the dynamics of CHH gene expression during a day/night cycle suggest that the increase in CHH protein synthesis is preceded in CHH mRNA transcripts (De Kleijn *et al.*, 1991).

To conclude, in the last few years sophisticated methods have been introduced which permit the development of specific probes/labels for detailed studies on the complexity of the control of crustacean reproduction. In our opinion, the following topics should be investigated "in priority": 1) Cellular dynamics of the neuroendocrine cell systems in the X-organ of the eyestalk producing VIH, CHH, MIH and neuromodulatory factors (for example RPCH). 2) Quantification of the concentration levels of the aforementiond neurohormonal factors in the secreting tissue and in circulation of specific target organs by means of visualization and localization of hormone receptors. 4) Mode of action of the different involved neuropeptides and their isoforms

using synthetic derivatives and adapted bioassays.

Such studies should be undertaken with special reference to environmental and internal stimulatory and inhibitory effectors, and in relation with different biological cycles, especially the reproductive and molting cycle. As species- and systematic group specificity have to be considered, it is worthwhile to select experimental models which may reflect their utility in aquacultural systems. In addition, further investigations would now gain by being carried out on a limited number of species in order to compare identical mechanisms and to facilitate the understanding of the hormonal actions. At that stage of research the development of practically adapted methods to screen the stock of cultured animals, in relation with environmental conditions, should be introduced with the ultimate goal to offer control and manipulation techniques for aquacultural systems. Finally, since it does not appear that most of the neurohormones are sex specific, it is necessary to pay more attention to the control of reproduction in male crustaceans.

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