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REVIEW

Reproductive regulators in decapod crustaceans: an overview

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Summary

Control of reproductive development in crustaceans requires neuropeptides, ecdysone and methyl farnesoate (MF). A major source of neuropeptides is the X-organ–sinus gland (XO–SG) complex located in the eyestalk ganglia of crustaceans. The other regulatory factors (either peptides or neuromodulators) are produced in the brain and thoracic ganglia (TG). Two other regulatory non-peptide compounds, the steroid ecdysone and the sesquiterpene MF, are produced by the Y-organs and the mandibular organs, respectively. In the current review, I have tried to recapitulate recent studies on the role of gonadal regulatory factors in regulating crustacean reproduction.

Key words: crustaceans, reproduction, gonads, X-organ–sinus gland, peptides.

Introduction

The regulation of reproduction in crustaceans is highly diverse and most species maintain separate sexes (Chang and Sagi, 2008; Parnes et al., 2008). The reproductive biology of crustaceans is crucial for the crustacean industry. The decline in commercial crustacean fisheries around the world is widely known. Major factors contributing to the steady decline in crustacean population number include inadequate legislation providing protection for these species, increases in the harvest rate, decreases in the size of the crustaceans and increases in worldwide consumption. One way to maintain sustainable crustacean populations for consumption is by manipulating the crustacean's endocrine system in order to speed up reproductive development and thus reduce the overall maturation time.

One common method for stimulating gonadal maturation and spawning in crustaceans is eyestalk ablation (ESA), most probably because of the removal of endogenous gonad inhibiting hormones (Brown and Jones, 1949), but eggs frequently do not develop properly following ESA (Anilkumar and Adiyodi, 1985). In the shrimp *Penaeus canaliculatus*, ESA females spawn more frequently than intact females, but the number of eggs formed and the hatching accomplishment are greater in intact animals (Choy, 1987). For the past two and a half decades many scientists and hatchery operators have focused on endocrinological manipulation to induce reproduction without ESA. Alternative techniques have been attempted to stimulate ovarian development, such as administration of gonad inhibitory hormone (GIH) antibody, hormonal level changes by environmental factors such as temperature, salinity and photoperiod, precise functionality of neurotransmitters and double-stranded RNA interference (dsRNAi) to reduce gonad inhibitory peptide transcript. The results from these experiments have revealed the potential (Chang et al., 2001; Fanjul-Moles, 2006; Mazurová et al., 2008; Nagaraju, 2007; Nagaraju and Borst, 2008; Treerattrakool et al., 2008) to stimulate gonad maturity. So, in this context, I will discuss up-to-date research on gonad regulatory factors and their role in reproductive development.

The following topics will be considered in relation to crustacean reproduction: (1) the reproductive system in crustaceans, (2) the role of neuropeptides in reproduction, (3) the role of androgenic hormone in male crustacean reproduction, (4) the role of neurotransmitters in reproduction, (5) the role of opioid peptides in reproduction, (6) the role of steroids and methyl farnesoate (MF) in reproduction, (7) non-neuroendocrine regulation of reproduction in microcrustaceans and (8) the effects of environmental factors on hormone levels and reproduction.

Reproductive system in crustaceans

Most crustaceans have separate sexes, and these can be distinguished by appendages on the abdomen called pleopods (Fig. 1A,C). The first (sometimes the second) pair of pleopods are larger on the male than on the female. These abdominal pleopods have become modified into copulatory organs that transfer the spermatophores from the male penises to the female sexual openings. Female crustaceans store the spermatophores for long periods, and the eggs do not have to be laid immediately after mating. Sperm in the spermatophores fertilizes the eggs as they are laid. The fertilized eggs are attached to the female abdominal pleopods by long sticky threads secreted by the female. Anomurans lack spermatotheca and so cannot store sperm.

The male reproductive system

The reproductive system of male crustaceans consists of the paired testes, vas deferens, seminal vesicles and a genital aperture (Fig. 1B). The testes are white, elongated structures located near the pericardial area. The testicular morphology is similar to the ovarian morphology in that the testis also maintains an H-like appearance. From each testis emerges a vas deferens that links it to the exterior by the gonophores, located on the base of the fifth pair of pereiopods. The vas deferens has three microscopically distinct regions that contain spermatophores in different stages of maturation.

Spermatogenesis is mostly characterized by the differentiation of sperm cells, and their maintenance before fertilization. In

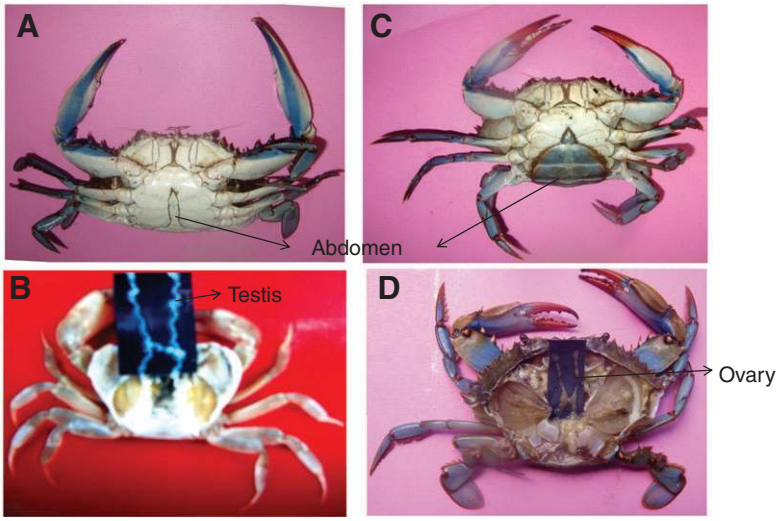

Fig. 1. Male and female crab morphology and reproductive system. (A) Ventral view of the male blue crab *Callinectes sapidus*. The tip of the abdomen is narrow. (B) Reproductive system of the male crab *Oziotelphusa senex senex* (obtained from my PhD thesis). (C) Ventral view of the female blue crab *C. sapidus*. The tip of the abdomen is broad. (D) Reproductive system of the female crab *C. sapidus*.

crustaceans, the testis contains 10–15 lobes each composed of many seminiferous tubules, whose shape changes according to the stage of spermatogenesis. Spermatogenesis starts in the undifferentiated seminiferous tubules (proacini) at the top of each lobe. Meiotic activity occurs in the acini that develop proacini somewhat proximally from the top of each lobe. In this stage, the lobules contain mostly primary spermatocytes. Differentiation of secondary spermatocytes and spermatids takes place in the seminiferous tubules, which lie in the center of each lobe. All these seminiferous tubules are filled with spermatocytes and spermatids surrounded by a layer of otheocytes of mesodermal origin. The hormonal control of spermatogenesis is not completely understood in crustaceans. There is information on MF as a reproductive hormone in males.

The female reproductive system

The reproductive system of female crustaceans consists of the paired ovaries, oviducts, gonophores and an external sperm reception area. The ovarian lobes are connected by a central bridge of ovarian tissue (Fig. 1D). The ovarian lobes are symmetrically arranged and lie in the cephalothorax on the top of the stomach and hepatopancreas (HP). An oviduct arises laterally from each ovary at a point just beside the position of the heart. It extends ventrally and opens through a gonophore in the abdomen. In sexually receptive females, each pore is equipped with a large tuft of long setae, which apparently serve as a tube for the passage of ova. In the fully mature state, the shapes of ovarian limbs can be distinguished only with difficulty as the ovary fills the thoracic region of the body cavity completely.

In the majority of female Malacostraca, oogonial proliferation and ovarian differentiation take place when the ovary is translucent to opaque white (previtellogenic ovary). During vitellogenesis the color of the ovary changes from pale yellow (vitellogenic stage I) to orange (vitellogenic stage II) and it then becomes brown (vitellogenic stage III) to dark brown prior to spawning. Maturation of the ovary also includes an increase in the size of the ovary as the oocytes proliferate and increase in diameter, due to yolk deposition.

Role of neuropeptides in reproduction

The hormonal control of reproduction has been studied in many crustacean species, including crayfish, shrimp, crab, lobsters, etc. A number of hormones from neuroendocrine organs play an essential role in controlling gonad maturation (Chang et al., 2001; Fingerman, 1997a; Laufer et al., 1993a; Mazurová et al., 2008; Nagaraju, 2007; Raviv et al., 2008). Gonad maturation in crustaceans appears to be regulated by two antagonistic neuropeptides: GIH (also called vitellogenesis inhibiting hormone, VIH, in females) synthesized and secreted from the X-organ–sinus gland (XO–SG) complex of the eyestalk (Fig. 2A and Table 1), and gonad stimulating factor (GSF), thought to be produced by the brain and thoracic ganglion (Fig. 2B,C,F, Table 1) (Eastman-Reks and Fingerman, 1984; Otsu, 1963).

Crustacean hyperglycemic hormone (CHH) from the eyestalk of the shrimp *Marsupenaeus japonicus* has been shown to inhibit protein and mRNA synthesis *in vitro* in ovarian fragments of *Penaeus semisulcatus* (Khayat et al., 1998). This demonstrates the pleiotropic activities of crustacean hormones, given the fact that CHH family peptides can also influence ovarian physiology in these animals (Fanjul-Moles, 2006). Webster (Webster, 1993) reported the occurrence of CHH receptors in various tissues, including oocyte membranes in the crabs *Carcinus maenas* and *Cancer pagurus*, indicating that CHH isomorphs may have specific activities in these tissues. Recent studies indicating that CHH-A and -B mRNAs are present in brain, and TG other than the optic ganglia, suggesting that CHH may have an extra role in the control of molting and reproduction (De Kleijn et al., 1995). The levels of molt inhibiting hormone (MIH)-related neuropeptide mRNA transcript in the eyestalk decrease in the initial (previtellogenic) phase of gonad maturation and increase towards the end of maturation (vitellogenic stage III) in the shrimp *Metapenaeus ensis* (Gu et al., 2002). Likewise, CHH also can stimulate vitellogenesis at early ovarian stages in the female blue crab, *Callinectes sapidus* (Zmora et al., 2009). These results indicate that MIH is a key endocrine regulator in the coordination of molting and reproduction in crustaceans, which simultaneously inhibits molt and induces ovarian maturation.

Role of VIH in female crustacean reproduction

The classic experiment of Panouse (Panouse, 1944) with the shrimp *Leander serratus* (*Palaemon serratus*) demonstrated that removal of the eyestalk during sexual inactivity led to rapid increase in ovarian size and precocious egg deposition, apparently because of the removal of GIH. These observations were well supported by several investigators; for example, Alikunhi and colleagues (Alikunhi et al., 1975) observed successful spawning after bilateral

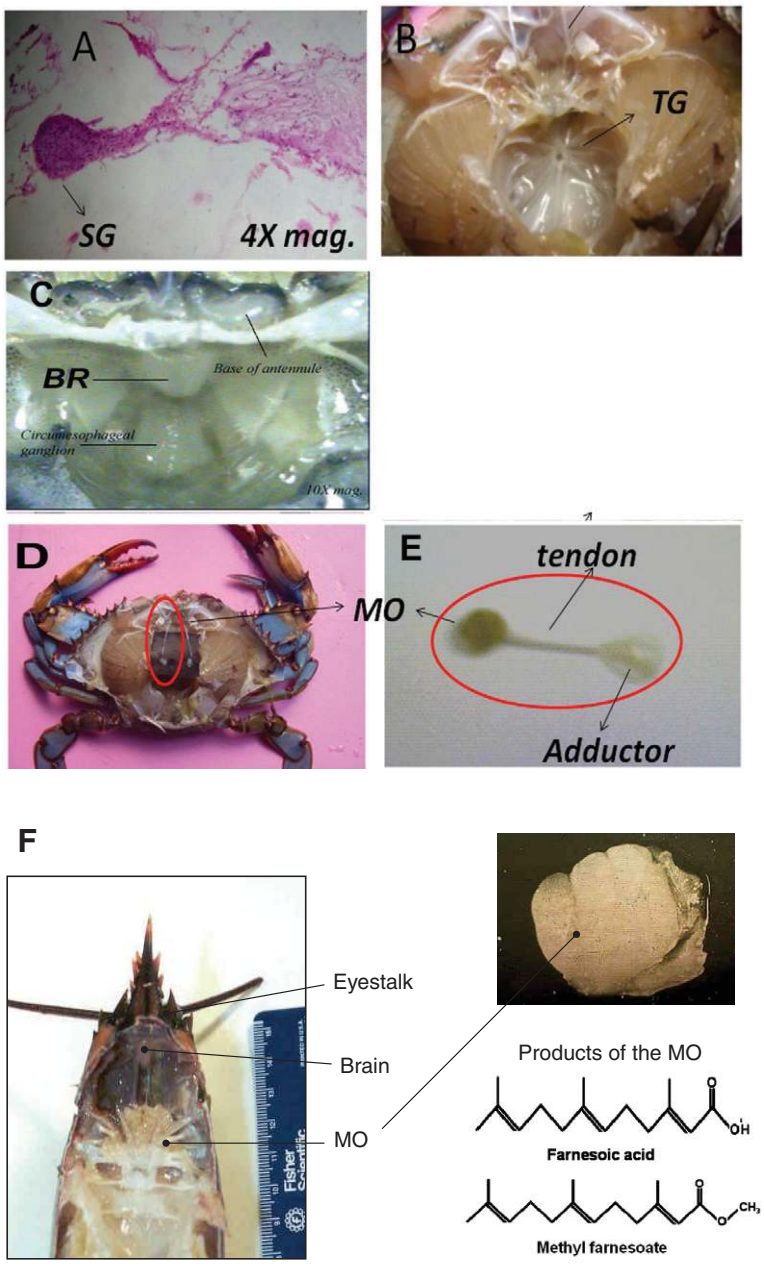

Fig. 2. Neuroendocrine and non-neuroendocrine glands in crustaceans. (A) Sinus gland (SG) in the eyestalk of the crab *O. senex senex* (obtained from my PhD thesis). (B) Thoracic ganglia (TG) location in blue crab *C. sapidus*. (C) Brain (BR) location in blue crab *C. sapidus*. (D) Mandibular organ (MO) location in blue crab *C. sapidus*. (E) Clear view of the mandibular organ. (F) Brain and mandibular organ location in the lobster *Homarus americanus* and MO products such as farnesoic acid and methyl farnesoate.

ESA in *Penaeus merquiensis* and *Penaeus monodon*. Arnstein and Beard (Arnstein and Beard, 1975) reported that maximum ovarian development and survival were observed after the removal of a single eyestalk (unilateral ESA) in *Penaeus orientalis and P. monodon*. In shrimps, VIH appears to be responsible for genital rest by inhibiting secondary vitellogenesis (Charniaux-Cotton and Payen, 1988). Paulus and Laufer (Paulus and Laufer, 1987) have suggested that the targets of VIH are the ovaries and HP. Using a heterologous bioassay (the presence of another VIH that is more potent), Soyez and colleagues suggested that VIH may be concerned with inhibition of the onset of vitellogenesis in lobster (Soyez et al., 2005). Hemolymph VIH levels were high in the previtellogenic (immature) stage of the American lobster, *Homarus americanus* (De Kleijn et al., 1998). Double-stranded RNA, related to the mature Pem-VIH sequence, can elicit a decrease in *Penaeus monodon*–VIH (Pem-VIH) transcript levels in both eyestalk ganglia and abdominal nerve cord explants culture and in female *P. monodon* broodstock. The prominent increase in vitellogenin (Vg) transcript level in the ovary of VIH-knockdown shrimp suggests a negative influence of Pem-VIH on Vg gene expression, and thus implies its role as a VIH (Treerattrakool et al., 2008).

The presence of VIH in embryos and larvae may be an indication of its gonad inhibitory role before adolescence (Rotllant et al., 1995; Rotllant et al., 1993). De Kleijn and colleagues (De Kleijn et al., 1998) observed the expression, storage and release of VIH and CHH during the reproductive cycle in female *H. americanus*. Wongsawang and colleagues (Wongsawang et al., 2005) reported that eyestalk extract contains both gonad inhibitory and gonad stimulatory peptides such as VIH and GSF. These results indicate that each eyestalk peptide may actively participate in ovarian development depending on the animal's physiological condition. For example, crustaceans are reproductively active when the titers of VIH and ecdysteroid are low and those of GSF and MF are high (Chang et al., 2001; Nagaraju, 2007). Receptor binding studies in

Table 1. Effect of neuroendocrine and non-neuroendocrine hormones on target tissues and their physiological action in crustaceans

Hormone	Site of production	Target	Physiological action
Crustacean hyperglycemic hormone	X-organ–sinus gland of eyestalk	Many organs	Regulates glucose level Regulates reproduction Regulates growth
Gonad (vitellogenic) inhibiting hormone	X-organ–sinus gland of eyestalk	Gonads and HP	Inhibits gonad maturation
Molt inhibiting hormone	X-organ–sinus gland of eyestalk	Y-organ	Inhibits growth Stimulates vitellogenesis
Gonad stimulating factor	Brain, thoracic ganglia	Gonads and HP	Stimulates gonad development
Neurotransmitters: 5-HT, DA and OA	X-organ–sinus gland of eyestalk, brain, TG	Gonads, HP, brain, TG, etc.	Influences gonad development, growth and metabolism
Methyl farnesoate	Mandibular organ	Gonads, HP, Y-organ, brain and TG	Stimulates gonad development Stimulates ecdysteroid production
Farnesoic acid	Mandibular organ	Gonads and HP	Stimulates gonad development
Ecdysteroid	Y-organ	Eyestalk, gonads and HP	Stimulates growth Stimulates gonad development
Opioid peptides	Eyestalk	Brain, TG, ovary and HP	May inhibit or stimulate gonad maturation May stimulate molt
Prostaglandins		X-organ–sinus gland, brain, TG, ovary and HP	May inhibit or stimulate gonad maturation May stimulate molt
FSH, LH, HCG		Ovaries	Stimulates ovarian maturation
Estrogens, progesterone		Hemolymph and ovaries	May stimulate ovaries
Androgenic hormone	Androgenic hormone	Testis, HP, brain and TG	Masculine characteristics, spermatogenesis in the testis, secondary male characteristics

5-HT, serotonin; DA, dopamine; OA, octopamine; HP, hepatopancreas; TG, thoracic ganglia; FSH, follicle stimulating hormone; LH, luteinizing hormone; HCG, human chorionic gonadotrophin.

the crab *C. maenas* and the crayfish *Orconectes limosus* indicate that CHH may have different targets and maybe also a positive influence on reproduction (Webster, 1993).

To date, VIH has only been found in a few crustacean species (Fig. 3) such as *Homarus gammarus* (Ollivaux et al., 2006), *H. americanus* (De Kleijn et al., 1995), the Norway lobster *Nephrops norvegicus* (Edomi et al., 2002), the giant river prawn *Macrobrachium rosenbergii* (Yang and Rao, 2001), the shrimp *Rimicaris kairei* (Qian et al., 2009) and the woodlouse *Armadillidium vulgare* (Greve et al., 1999). Molecular analysis of VIHs isolated from females of a few crustacean species shows that they consist of signal peptides (20–31 amino acid residues) and mature peptides (77–83 amino acid residues; Fig. 3). They also show a considerable degree of sequence similarity with MIH, including the preservation of six cysteine residues at the same relative locations (Demeusy, 1953; Leung-Trujillo and Lawrence, 2009; Nagaraju and Borst, 2008; Rotllant et al., 1993; Wongsawang et al., 2005; Yano et al., 1988) forming three intramolecular disulfide bonds (1–5; 2–4; 3–6 fashion); these are major chemical forces, which help to maintain the VIH tertiary structure (Fig. 3) (Soyez, 2005; Udomkit, 2000; Nagaraju et al., 2009). Reverse phase high performance liquid chromatography (RP-HPLC) analysis revealed that the neuropeptide in *H. americanus* has two enantiomeric isoforms with the fourth residue being either *l*-tryptophan or *d*-tryptophan. The two VIH isoforms had the same sequence (77 amino acid residues), molecular mass (9.135 kDa) and isoelectric point, but they varied in hydrophobicity due to a C-terminal amidation (Soyez et al., 1991). An 8.4 kDa peptide fraction was purified from the crayfish *Procambarus bouvieri* by Aguilar and colleagues (Aguilar et al., 1992), which had an inhibitory activity on vitellogenesis in the ovary of the white shrimp, *Litopenaeus vannamei*. A partial amino acid sequence showed that this peptide is a member of the CHH family. De Kleijn and colleagues (De Kleijn et al., 1992) found that the structures of GIH and crab MIH (prepro hormones, as well as mature peptide) showed a high degree of amino acid identity. They also proposed that GIH may be a central modulator of the production or release of hormones involved in molting as well as reproduction. Ollivaux and colleagues (Ollivaux et al., 2006) purified VIHs from the lobster *H. gammarus* and *H. americanus* by RP-HPLC, and characterized these peptides by Fourier transform ion cyclotron resonance mass spectroscopy (MS). The existence of an extra small peptide CHH precursor related peptide, between the signal and the mature peptide, distinguished CHH from MIH and VIH. VIH had similar retention times and molecular masses in the two species, hence suggesting that the VIH peptide sequence was highly conserved between the two species. The functional consequence of such structural isoforms is far from obvious. In contrast to CHH, VIH has an unblocked N-terminus and an amidated (more peptides) C-terminus, as well as many uneven residue numbers in the C-terminus (Bocking et al., 2001). Immunocytochemistry and *in situ* hybridization studies indicate that there are no remarkable differences in the number of VIH/GIH neuroendocrine cells of the XO of female and male *H. americanus*, suggesting an endocrine role for this hormone in male reproduction as well (De Kleijn et al., 1992).

The physiological pathway of VIH in inhibiting secondary vitellogenesis requires closer examination. Among the possibilities open to investigation are the following: (i) VIH may act directly on oocytes by inhibiting uptake of Vg or synthesis of yolk protein; this might be studied by *in vitro* culture of oocytes (Jugan and Soyez, 1985) with Vg or vitellein (Derelle et al., 1986) and a range of concentrations of pure VIH; (ii) determination of GIH/VIH titer in hemolymph and mRNA levels during different reproductive stages. In a relationship like this between the MIH from the SG and the Y-organ, GIH/VIH may inhibit the release of a GSH from TG or brain of the central nervous system or mandibular organ (MO; Fig. 2D,E,F); and (iii) VIH may either bind to Vg, preventing its binding to the receptor, or bind to the receptor to block the Vg binding site (Charniaux-Cotton and Payen, 1988; Van Herp, 1993).

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Homga_VIH       MVTRVASGFSVQRVWLLLVIVVVLCGSVTQQASAWFTND-ECPGVMGNRDLYEKVAWVCND
Homam_VIH       MVTRVGSGFSVQRVWLLLVIVVVLCGSVTQQASAWFTND-ECPGVMGNRDLYEKVAWVCND
Nepno_VIH       MVTRVASGFSVQRVWLLLVIVVVLCGSVTQQASAWFTND-ECPGVMGNRDLYEKVAWVCND
Rimka_VIH       MVGQVNHDISVQRVLRLAALVISLLLITGTSARNLYDLDTECPGVMGNRDLYEKVVRVCDD
Penmo_VIH       -----------MKTWL------LLATLVVGASLANILDS-KCRGAMGNRDMYNKVERVCED
Meten_VIH       -----------MRTWLTFVAVMVWASLLVDESSAFSIDY-TCTGAMGNRDIYNKVSRVCDD
Macro_VIH       MASRLNQAFTLKKLTYVAIMMAVFGILLVDQTSARFLDD-ECRGVMGNRDLYEYVVRICDD
Armvu_VIH       ----------------------YNIPLGWGRRDMPG---CLGVLGNRDLYDDVSRICSD
                                                .   * *.:****:*: *   :*.*

Homga_VIH       CANIFRNNDVGVMCKKDCFHTMDFLWCVYATERHGEIDQFRKWVSILRAGRK- 100%
Homam_VIH       CANIFRNNDVGVMCKKDCFHTMDFLWCVYATERHGEIDQFRKWVSILRAGRK- 99.1%
Nepno_VIH       CANIFRINDVGVKCKKDCFHNMDFLWCVYATERHGEIDQFRKWISILRAGRK- 96.4%
Rimka_VIH       CSNIFRENDVGTRCRKECFFNVDFLWCVYATERHGDVEQLNRWMSILRAGRK- 59.0%
Penmo_VIH       CTNIYRLPQLDGLCRNRCFNNQWFLMCLHSAKREAELEHFRLWISILNAGRPW 38.2%
Meten_VIH       CANIYRLPGLDGMCRNRCFNNFWFMICLRAAKREDEIDKFRVWISILNPGGAW 43.1%
Macro_VIH       CENLFRKSNVGSRCKKNCFYNEDFMWCVRATERTDELEHLNRAMSIIRVGRK- 45.5%
Armvu_VIH       CQNVFRDKNVESKCRSDCFSTSYFETCIMALDLAEKISDYKLHASILKE---- 34.1%
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Fig. 3. Vitellogenesis inhibiting hormone (VIH) amino acid sequence comparison between crustacean species. *Homarus gammarus* (*Homga_VIH*; DQ181793), *Homarus americanus* (*Homam_VIH*, X87192), *Nephrops norvegicus* (*Nepno_VIH*; AF163771), *Rimicaris kairei* (*Rimka_VIH*; FJ447500), *Penaeus monodon* (*Penmo_VIH*, ABG33898), *Metapenaeus ensis* (*Meten_VIH*; AF294648), *Macrobrachium rosenbergii* (*Macro_VIH*; AF432347) and *Armadillidium vulgare* (*Armvu_VIH*; P83627). Asterisks indicate a single, fully conserved residue, colons indicate conservation of strong groups, stops indicate conservation of weak groups and dashes indicate no consensus.

Role of eyestalk extracts (inhibitory hormones) in crustacean male reproduction

Development of the crustacean male reproductive system has been studied by several investigators. The crustacean eyestalk is known to control testicular function (Figs 4 and 5). For example, eyestalk removal in non-breeding adult males induces precocious spermatogenesis, and hypertrophy in the androgenic gland (Demeusy, 1953; Gomez and Nayar, 1965; Otsu, 1963). ESA of male white shrimp, *L. vannamei*, increased testicular size and doubled mating success (Chamberlain and Lawrence, 2009; Sreekumar and Adiyodi, 1983) suggested that ablation stimulates spermatogenesis and that inhibitory eyestalk principles (possibly GIH or MIH) play a role in the synchronized regulatory processes of reproduction and molting in *Macrobrachium idella*. Eyestalk removal in male *Penaeus vannamei* also enhanced the testicular index, spermatophore weight and total number of sperm but did not affect sperm viability (Leung-Trujillo and Lawrence, 2009). Unilaterally, ESA male *P. monodon* showed a significantly higher sperm count, larger sperm head diameter and longer spikes in comparison to unablated ones, but no observable changes were noted in testicular index, spermatophore weight and sperm viability (Gomes and Honculada-Primavera, 1993). We also found that the ESA of *C. maenas* caused testicular index to increase to 2-fold higher than that of intact green crabs (Nagaraju and Borst, 2008). These and other research studies have led to the proposal that the eyestalk contains an inhibitory factor called GIH (Fingerman, 1997a; Haihui et al., 2006; Kulkarni et al., 1984), but the structural information and molecular and cellular mechanisms remain to be elucidated.

Role of GSFs in crustacean reproduction

The effects of ESA and implantation of TG or brain indicated the presence of a GSF (Aiken and Waddy, 1980; Gomez and Nayar, 1965; Otsu, 1963), but whether these factors are peptides or non-peptides is still uncertain. Clearly, a more complete characterization of GSFs is desirable. In the prawns *Paratya compressa* and *Parapenaeopsis hardwickii*, extracts of cerebral (brain) tissue as well as TG stimulated ovarian growth *in vivo* and *in vitro*; but brain extracts were more effective than TG in *P. compressa* (Kulkarni et al., 1981; Takayanagi et al., 2005). Brain and TG extract could induce ovarian maturation and the development of secondary oocytes in *Uca pugilator* (Chang, 1985). Joshi and Khanna (Joshi and Khanna, 1984) injected TG extracts into male *Potamon koolooense* during spermatogonial quiescence, which produced hypertrophy of the androgenic glands (AGs) along with the onset of spermatogenesis, an increase in the gonad index, and enlarged testicular tubules and vas deferens. In the crab *U. pugilator*, GSF production fluctuates depending on the stage of the annual reproductive cycle (Eastman-Reks and Fingerman, 1984). Yano and Wyban (Yano and Wyban, 1992) reported that, in a small number of cases, implants of the TG of *H. americanus* into non-reproductive *L. vannamei* resulted in the stimulation of ovarian maturation. Injections of aqueous extracts prepared from TG and brain as well as *in vitro* incubation experiments have shown that vitellogenesis can be stimulated by TG extract (Yano and Wyban, 1992). Evidence for GIH seems well established through classical endocrine procedures, but sufficiency studies with the GSF from TG of the central nervous system are lacking, probably because of the difficulty with surgery. These results also suggest that the GSF is not species specific.

Nevertheless, several other compounds play a vital role in regulating reproduction, for example, androgenic gland hormone, MF, steroids, opioid peptides and neurotransmitters (Fig. 5). These compounds are also known to have several metabolic functions in crustacean species, including carbohydrate metabolism, molt and reproduction, etc.

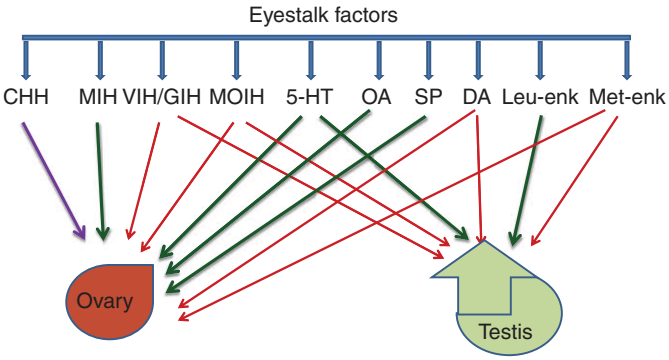

Fig. 4. The effect of eyestalk factors on reproduction in crustaceans. CHH, crustacean hyperglycemic hormone; MIH, molting inhibiting hormone; VIH, vitellogenesis inhibiting hormone; GIH, gonad inhibiting hormone; MOIH, mandibular organ inhibiting hormone; 5-HT, 5-hydroxytryptamine; OA, octopamine; SP, spiperone; DA, dopamine; Leu-enk, leucine-enkephalin; Met-enk, methionine-enkephalin. Green arrows indicate positive influence; red arrows indicate negative regulation; purple arrow indicates either positive or negative regulation.

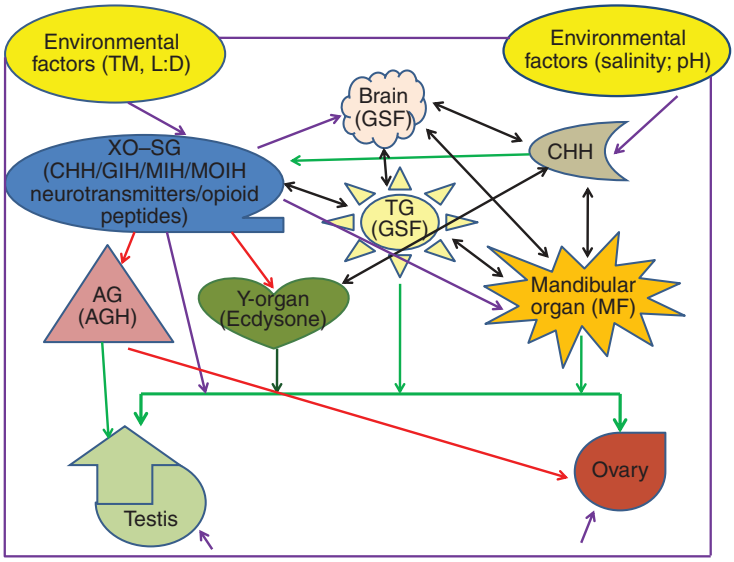

Fig. 5. The effect of environmental factors and neuroendocrine and non-neuroendocrine hormones on reproduction in crustaceans. T, temperature; L:D, light and dark; GSF, gonad stimulating factor; TG, thoracic ganglia; CHH, crustacean hyperglycemic hormone; GIH, gonad inhibiting hormone; MIH, molting inhibiting hormone; MOIH, mandibular organ inhibiting hormone; MF, methyl farnesoate; AG, androgenic gland; AGH, androgenic gland hormone. Green arrows indicate positive influence; red arrows indicate negative regulation; purple arrows indicate either positive or negative regulation; black arrows indicate unknown regulation.

Role of androgenic hormone in male crustacean reproduction

The AG is specific to male malacostracan crustaceans, a group that includes decapods and isopods. The AG is found alongside the distal area of the male gamete ducts. Within male crustaceans, in addition to GIH and GSF, the androgenic gland hormone (AGH) has a key role in the regulation of spermatogenesis (Fig. 5). The purpose of the AG in regulating expansion and maturation of the crustacean male reproductive system and secondary sexual characteristics was first explained by Charniaux-Cotton (Charniaux-Cotton, 1954). Spermatogenesis is inhibited when the AGs are ablated in male crustaceans (Charniaux-Cotton, 1964; Nagamine et al., 1980). ESA, thereby eliminating the preliminary place of GIH, results in hypertrophy of the AGs and a rapid increase in spermatogenesis (Payen et al., 1971). Hence, the eyestalk peptide GIH seems to affect the testes indirectly, by inhibiting the AGs. Further GSF is essential to trigger AG spermatogenesis (Juchault and Legrand, 1965). Gupta (Gupta, 1989) found from their studies of the crab *Purutelphusa hydrodromus* that the inactive stage of the testis is due to an increase in the hemolymph titer of GIH with correlated decreases in the titers of GSF and AGH.

AGH was isolated and structurally characterized in *A. vulgare*. AGH is composed of glycosylated dimeric peptides (8.7 kDa) joined by two disulfide bridges. This structure is highly conserved among isopods (Okuno et al., 1999). AGH is responsible for the development and continuation of male primary and secondary sexual characteristics, inhibition of Vg synthesis and stimulation of spermatogenesis (Chang and Sagi, 2008). When AGH is defective, intersex characteristics arise where normal male secondary sex characteristics increase but the animal contains both oviduct and testes (LeBlanc, 2007). Several reports are available on AG ablation and implantation, and AGH administration has been investigated in a few crustacean species. Suzuki and Yamasaki (Suzuki and Yamasaki, 1998) found that when AG extracts were injected into female *A. vulgare* at different developmental stages there was sex reversal after gonadal differentiation. These results suggest that it is a sex differentiating factor and not a sex determining factor. Likewise, AG implanted into female crab *Scylla paramamosain* inhibited further progress of the ovaries (Cui et al., 2005). Deterioration of the ovaries was seen 7–26 days after AG treatment. Cui and colleagues (Cui et al., 2005) also incubated sections of ovarian tissue *in vitro* with AG extracts and found that uptake of amino acids by ovary tissues were reduced by 50%. These results suggest that AGH seems to function as a sex differentiating factor and may induce male sex characteristics and inhibit vitellogenesis. Barki and colleagues (Barki et al., 2006) removed AG from juvenile intersex crayfish and observed that in the adult stage they did not fight with other males and did not initiate mating behaviors with females. In addition, AG removal stimulated vitellogenesis. These results suggest that the AG is not only responsible for male sex characteristics but may also be involved in male-like behavior in the crayfish.

Role of neurotransmitters in reproduction

The role of neurotransmitters in reproduction has been studied in several crustacean species (Figs 4 and 5). Beltz (Beltz, 1988) found that the biogenic amines 5-hydroxytryptamine (5-HT) and octopamine (OA) play a significant role in determining mating behavior in the lobster *H. americanus*. The level of 5-HT in the central nervous system and ovary of crayfish *Procambarus clarkii* and *M. rosenbergii* was quantified by HPLC (Kulkarni and Fingerman, 1992; Tinikul et al., 2008). Other studies reported that 5-HT and dopamine (DA) were widely distributed in the CNS of *O. limosus*, *P. clarkii* and *M. rosenbergii* (Elekes et al., 1988; Fong et al., 2005; Mercier et al., 1991; Tinikul et al., 2009a), the crab *C. maenas* (Kerkut et al., 1966) and the lobster *H. gammarus* (Barthe et al., 1989) eyestalk (Martínez, 1991), brain (Braley, 1985), thoracic, esophageal and abdominal ganglia (Matsutani, 1990). Biogenic amines act as neurotransmitters in several thoracic animals such as insects and crustaceans (Fingerman et al., 1985; Werman, 1966) and are involved in the release of neurohormones in crustaceans (Fingerman, 1997b; Sainath and Reddy, 2010). This hypothesis is supported by evidence that administration of 5-HT activates the release of GSF from TG in fiddler crab *U. pugilator* (Richardson et al., 1991; Sarojini et al., 1993) and in *P. clarkii* (Kulkarni et al., 1991; Sarojini et al., 1994). The eyestalk extract from *P. clarkii* inhibited ^{14}C leucine incorporation into the ovary, but the extract from the brain, TG and subesophageal ganglia increased ^{14}C leucine incorporation (Kulkarni et al., 1991). *In vivo* but not *in vitro* treatment with 5-HT raised ^{14}C leucine incorporation into ovarian proteins in *P. clarkii* (Sarojini et al., 1995a). The ^{14}C leucine incorporation method has been used to quantify newly formed proteins and determine their origin and deposition. Further, DA has been found to antagonize the gonad initiating action of 5-HT in *P. clarkii* females and *U. pugilator*

males (Sarojini et al., 1995a; Sarojini et al., 1995b), whereas OA increased ovarian growth in a dose-dependent manner in *H. americanus* (Howard and Talbot, 2005). 5-HT injection induced ovarian development in *L. vannamei* (Vaca and Alfaro, 2000), but at lower rates than unilateral ESA.

Alfaro and colleagues (Alfaro et al., 2004) stimulated ovarian development and spawning in shrimp *L. vannamei* and *Liptopenaeus stylirostris* by treatment with the 5-HT and DA antagonist spiperone. Administration of 5-HT plus spiperone as well as spiperone alone stimulated ovarian maturation and embryonic development, and increased ovarian index and oocyte diameters in *M. rosenbergii* (Tinikul et al., 2009b). *In vivo* spiperone treatment has also been reported to stimulate ovarian maturation in crayfish *Cherax quadricarinatus* and subsequently produced an augmented percentage of spawning females (Cahansky et al., 2008). Cahansky and colleagues also reported that gonadosomatic index (GSI) in *Aegla platensis* was stimulated by food containing spiperone (Cahansky et al., 2008). Similarly, lipid and cholesterol levels of both ovaries and HP from *Aegla uruguayana schmitt* were substantially increased in response to spiperone (Castiglioni et al., 2009). They found that higher GSI was also correlated with a higher lipid content of gonads and/or HP, suggesting an increased energetic demand in accordance with an active investment in reproduction. In further studies, administration of 5-HT raised hemolymph Vg concentration 15–26 times from the control levels within 16 days in ESA brood stock *M. rosenbergii* prawns (Chen et al., 2003). Recently, Meeratana and colleagues (Meeratana et al., 2006) reported that 5-HT-primed TG medium stimulates ovarian maturation and oocyte growth in *M. rosenbergii*. These studies indicate that 5-HT indirectly acts on ovary. It appears that 5-HT plays a significant role in gonad development and spawning processes of crustaceans. With respect to females, spiperone produced an increase of the GSI in *P. clarkii*, when injected during early vitellogenesis (Rodríguez et al., 2001).

In addition to the effect of 5-HT on female crustaceans, it also has a role in the reproductive system of male crustaceans (Figs 4 and 5; Table 1). Injection of 5-HT into male lobsters stimulated the dominant attitude of male reproductive behavior (Beltz, 1988). In the fiddler crab, injection of 5-HT stimulates testicular development (Sarojini et al., 1993). Injection of 5-HT antagonist has no inducing effect on testes and AGs. From these observations, Sarojini and colleagues hypothesized that 5-HT indirectly stimulates the release of GSF, which in turn stimulates the AGs to synthesize and release AGH, resulting in the activation of testicular development (Sarojini et al., 1994).

Role of opioid peptides in crustacean reproduction

Opioid peptides are short sequences of amino acids that bind to specific receptors in the brain; opiates and opioids mimic the effect of these peptides. Opioid peptides may also be involved in the control of reproduction in decapods (Fig. 4, Table 1). One of the first reports of a reproductive role for endogenous opioid peptide in crustaceans was published by Fingerman and colleagues (Fingerman et al., 1985). They observed inhibition of ovarian maturation in *U. pugilator* after the administration of synthetic methionine-enkephalin. Subsequently, Sarojini and colleagues (Sarojini et al., 1995a) found that testicular growth in *U. pugilator* decreased after methionine-enkephalin injection. Naloxone, an antagonist of enkephalinergic receptors, can stimulate ovarian growth in both *U. pugilator* and *P. clarkii* (Sarojini et al., 1995a). Cahansky and colleagues (Cahansky et al., 2008) reported that ovarian maturation in *A. platensis* was stimulated by food containing naloxone. Kishori and Reddy (Kishori and Reddy, 2003) found an antagonistic action of opioid peptides in the regulation of ovarian maturation in the crab *Oziotelphusa senex senex*. Reddy (Reddy, 2000) also observed stimulation of ovarian development in the prawn *Penaeus indicus* after leucine-enkephalin injection. Injection of leucine-enkephalin stimulated molting and also enhanced ovarian maturation and vitellogenesis (Kishori and Reddy, 2003). The mode of action of opioid peptides in reproduction in crustaceans is unclear. The isolation and identification of opioid receptors and their levels in crustaceans is essential in order to clarify several issues related to the physiology of crustaceans.

Role of steroids and MF in reproduction

Reproduction of crustaceans is not only affected by abiotic constraints. For instance, it is known from experiments with the microcrustacean *Daphnia* that a dietary source of polyunsaturated fatty acids (e.g. food quality) significantly improves the production of viable offspring (Wacker and Martin-Creuzburg, 2007). Interestingly, these fatty acids are precursors for prostaglandins and other eicosanoids, which might play a significant role in reproductive processes. In addition to eyestalk peptides, several additional non-neuroendocrine products also play direct or indirect roles in regulating the reproductive process. For example, MF and ecdysteroids stimulate male and female reproduction in crustaceans and are regulated by neuropeptides (Fig. 5) (Chang et al., 2001; Fingerman, 1997a; Nagaraju, 2007).

Ecdysteroids

Though ecdysteroids are primarily considered to be molting hormones, recent studies indicate that they play a major role in regulating vitellogenesis, ovarian maturation and protein synthesis in decapods (Fig. 5, Table 1) (Wongsawang et al., 2005; Young et al., 1993; Subramoniam, 2000; Brown et al., 2009). In crustaceans, the inactive parent compound, ecdysone, and a biologically active metabolite, 20-hydroxyecdysone, are synthesized from dietary sterols in the Y-organ (Chang, 1985). Ecdysteroids have been identified in the ovaries and eggs of *Parapenaeus fissures* (Jeng et al., 1978), *H. americanus* (Couch et al., 1987), *P. serratus* (Spindler et al., 1987), *H. gammarus* (Goudeau et al., 1990), *M. rosenbergii* (Young et al., 1991), *Libinia emarginata* (Laufer et al., 1993a) and *P. monodon* (Laufer et al., 1993a). These findings indicated that 20-hydroxyecdysone is the physiological equivalent of estrogens. Lachaise and colleagues (Lachaise et al., 1981) showed an increase in the levels of ecdysteroids in ovaries of the crab *C. maenas* during ovarian development. A positive correlation between vitellogenesis and hemolymph ecdysteroid titers has been observed in the spider crab *Acanthonyx lunulatus* (Chaix and De Reggi, 1982), *M. nipponense* (Okumura et al., 1992) and *M. rosenbergii* (Laufer et al., 1993a). Hansen and colleagues (Hansen et al., 2008) observed that ecdysteroid concentrations were higher in female *Calanus finmarchicus* with large egg sacs, suggesting that ecdysteroids may be involved in egg maturation and reproduction. 20-Hydroxyecdysone apparently stimulated *M. ensis* Vg1 (MeVg1) gene expression in both HP and ovary explants *in vitro* (Tiu et al., 2006). Therefore, in females a gonadotropin (MF) is likely to stimulate ecdysteroid production by the ovaries. However, Young and colleagues (Young et al., 1993) found decreasing levels of hemolymph ecdysteroids during vitellogenesis in *P. monodon*. On the other hand, it is still uncertain as to whether ecdysteroids directly affect vitellogenesis or whether their levels during vitellogenesis are

merely indicative of the consequent stage of the molt cycle. Exposure to 20-hydroxyecdysone induced spermatogenesis in the lobster (Brody and Chang, 1989). Ecdysteroids have been shown to enhance DNA synthesis in the testes of *M. rosenbergii* (Sagi et al., 1991). Ecdysteroids are also found in the testis sheath of the isopod *Idothea wosneskii* (Matlock and Dornfeld, 1982) and the spider crab, *L. emarginata* (Laufer et al., 1993b; Rotllant et al., 2000). It might be worthwhile to investigate the effect of ecdysteroids on reproduction in a more systematic way than has been done so far.

Vertebrate-type steroids, prostaglandins and mammalian hormones

Crustacean gonads have been shown to possess steroids more usually identified with vertebrates and the enzymatic capacity to synthesize vertebrate sex steroids (Brown et al., 2009; Gunamalai et al., 2006; Lafont et al., 2005). Fairs and colleagues (Fairs et al., 1990) studied changes in steroid titers during vitellogenesis. They found high titers of estrogens during vitellogenic stages, suggesting a possible role in the stimulation of vitellogenesis. Progesterone (17α-hydroxyprogesterone, 20α-hydroxyprogesterone and 6β-hydroxyprogesterone) and estradiol (17β-estradiol, estrone and testosterone) are the vertebrate-type steroids. A positive relationship between Vg levels in hemolymph and circulatory levels of both progesterone and 17β-estradiol has been observed for shrimp *P. monodon* (Quinitio et al., 1994), prawns (Yano et al., 2000) and crabs (Shih, 1997). Fluctuating levels of estradiol and progesterone in the ovary and hemolymph at different vitellogenic stages of the crab *Scylla serrata* were also reported (Warrier et al., 2001). In contrast, a negative relationship between ovarian maturation and hemolymph levels of steroids was found in *M. japonicus* (Okumura and Sakiyama, 2004). Injection of progesterone induced ovarian development in the shrimp *P. hardwickii* (Kulkarni et al., 1979). Progesterone and estradiol apparently stimulated MeVg1 gene expression in both HP and ovary explants of *M. ensis* (Tiu et al., 2006). Administration of 17α-hydroxyprogesterone stimulated ovarian growth and vitellogenesis in the kuruma prawn *M. japonicus* (Yano, 1987). 17β-Estradiol stimulated vitellogenesis by ovary fragments *in vitro* (Yano et al., 2000) and *in vivo* in crayfish (Coccia et al., 2010). Injection of 17α-hydroxyprogesterone induced ovarian maturation in the crab *O. senex senex* (Reddy et al., 2006). Ghosh and Ray (Ghosh and Ray, 1994) observed that estrogen stimulated lipogenic activity in the ovary of the prawn *M. rosenbergii*. Additional studies are necessary to examine the mode and action of steroid hormones. Steroid hormones have to bind to nuclear receptors in order to generate a physiological reaction. Recent studies indicate an immunological role for progesterone and estradiol receptors in the HP and gonad of the freshwater crayfish *Austropotamobius pallipes* (Paolucci et al., 2002). While no genome-wide surveys have been done in crustaceans, a lack of steroid hormone receptors is feasible.

To date, in crustacean reproduction, a number of studies on the effects of prostaglandins have been published (Table 1). The reproductive functions, like discharging of hatching factors in barnacle *Balanus balanoides* and ovarian development (Holland et al., 1985), are known to be under the control of prostaglandins. Spaziani and colleagues (Spaziani et al., 1993) observed the occurrence and gradual rise in ovarian prostaglandin E2 (PGE2) and prostaglandin F2 (PGF2) during vitellogenesis in the crayfish *P. paeninsulanus*. Sagi and colleagues (Sagi et al., 1995) observed that PGE2 significantly stimulates cyclic adenosine monophosphate (cAMP) synthesis in ovarian tissue in the prawn *M. rosenbergii*. The presence and effect of the classical prostaglandins, particularly prostaglandin D2 (PGD2), PGF2 and PGE2, in ovarian tissue and maturation were studied in the prawn *Metapenaeus affinis* (Sarojini et al., 1987) and the crayfish *P. paeninsulanus* (Spaziani and Hinsch, 1997) and *C. quadricarinatus* (Silkovsky et al., 1998). Yano and colleagues reported that the incorporation of prostaglandins into food pellets, stimulated final ovarian maturation in prawns *in vivo* (Yano et al., 2000). Prostaglandin H synthase activity was considerably increased in the ovary during the final vitellogenic stage in comparison to the previtellogenic ovary in the crab *O. senex senex* (Reddy et al., 2004). The authors also observed that injection of PGF2 and PGE2 stimulated ovarian development but PGD2 did not affect ovarian maturation. These findings demonstrate the occurrence of a prostaglandin biosynthetic system in the ovary, and that it plays a vital role in vitellogenesis. However, the mode of prostaglandin biosynthesis in crustaceans and enzymes involved in these pathways have remained uncertain.

Mammalian hormones also have an influence on crustacean reproduction and spawning (Table 1). For example, Zukowska-Arendarczyk observed an inducing effect of hypophysis gonadotropins (follicle stimulating hormone, FSH, and luteinizing hormone, LH) on ovarian maturation in *Crangon crangon* (Zukowska-Arendarczyk, 1981). The response of crustacean ovaries to mammalian FSH and LH indicates that gonadotropins may act on invertebrates. However, to date no structurally similar counterparts of FSH and LH have been isolated from any invertebrate. Injection of human chorionic gonadotrophin (HCG) stimulated maturation and spawning in shrimp and prawn (Jayaprakas and Sambhu, 1998; Yano, 1993). Laufer and Landau (Laufer and Landau, 1991) found similar patterns in ovarian maturation for *P. indicus* given food containing HCG. HCG positively influenced vitellogene synthesis in *Idotea balthica* and the sand shrimp, *C. crangon* (Laufer and Landau, 1991). Akta and Kumlu (Akta and Kumlu, 2005) observed that injection of 5-HT hormones induced maturation and spawning in *P. semisulcatus*, but minimal effects were observed in treatments with HCG and luteinizing hormone releasing hormone. Further studies are required to fully understand the effects of mammalian hormones on fertility and hatching rates of crustaceans.

MF

Recently, the well-known juvenile hormone (JH) family compound MF was shown to play a key role in the regulation of crustacean reproduction (Fig. 5 and Table 1) (for a review, see Nagaraju, 2007). MF is a sesquiterpene discovered in the mandibular organ (MO; Fig. 2D,E,F) of crustaceans about 20 years ago (Borst et al., 1987; Laufer et al., 1987). Since then, it has been found in more than 35 crustacean species. MF is negatively regulated by the mandibular organ inhibiting factor produced by the eyestalk SG (Fig. 5) (Borst et al., 2001; Borst et al., 2002; Nagaraju et al., 2005; Nagaraju et al., 2003; Wainwright et al., 1996). MF is structurally similar to the JHs, only differing in the presence of an epoxide moiety at the terminal end. Juvenile hormone is involved in several aspects of reproduction in female insects, including secondary vitellogenesis and Vg uptake. By analogy, MF may have comparable roles in crustaceans (Borst et al., 1994).

This view is supported by several observations. Histological studies of MOs from *C. maenas* and *L. emarginata* suggest that they are more active during ovarian maturation (Hinsch, 1980; Le Roux, 1968). MOs in female crabs doubled in size during reproduction (Nagaraju et al., 2003). Injection of MO extracts from

males into immature female spider crabs enhanced ovarian maturation (Hinsch, 1980). The MO appears to produce a 'gonad-stimulating factor', most likely MF. *In vitro*, MF production has been associated with ovarian development in *C. maenas*, *L. emarginata* and *U. pugilator* (Borst et al., 1987; Laufer et al., 1987). Similarly, *in vivo* MF levels have been correlated with ovarian maturation and molting in the crab *O. senex senex* (Nagaraju et al., 2006). Administration of MF increased the Vg titer in hemolymph of ESA spider crab (Vogel and Borst, 1989). Unilateral and bilateral ESA enhances MF levels and increases the ovarian index in *L. emarginata* (Laufer et al., 1987). These results are important because ESA also removes another inhibitory peptide, GIH/VIH (Que-Tae et al., 1999). Administration of MF stimulated ovarian maturation and testicular development in several crustacean species (Nagaraju, 2007; Nagaraju and Borst, 2008; Nagaraju et al., 2004; Nagaraju et al., 2003). *In vitro* studies showed that MF stimulated oocyte size and Vg messenger in HP and the ovary (Otsu, 1963). These results indicate MF acts directly on both HP and ovary, whereas in male crustaceans it is not clearly known how MF acts on the testes.

The MO also plays a role in male reproduction and behavior. In several crustacean species, the MO is much larger in adult males. For example, the male lobster (carapace >80 mm; sexually mature) and crab (carapace >30 mm; sexually mature) have larger MOs than females, and MOs from reproductively active males synthesize more MF than those from sexually active females of the same size (Nagaraju et al., 2003; Waddy et al., 1995). Sagi and colleagues (Sagi et al., 1993) found a correlation between hemolymph MF and gonad maturity in *L. emarginata*. High MF levels were also correlated with mating behavior; males with higher MF levels actively courted and coupled with females, while males with lower MF levels did not show this behavior (Sagi et al., 1994). In addition, in the crab *O. senex senex*, the green crab, *C. maenus*, and the prawn *M. malcholumsonii*, injection of MF caused an increase in the testicular index (Kalavathy et al., 1999; Nagaraju and Borst, 2008; Nagaraju et al., 2004). These results suggested that MF stimulates the testes either directly or indirectly by stimulating (GSF or AGH) or inhibiting GIH secretions. In addition to this, MF levels may also change, depending on color, behavior, reproduction, molt and stress (Nagaraju, 2007). It is worth clarifying these issues for better outcomes in crustacean aquaculture.

In addition to MF, farnesoic acid (FA; Fig. 2F) is still a viable candidate for a crustacean hormone synthesized *in vitro* and secreted from the MOs. FA is first metabolized by farnesoic acid *O*-methyltransferase (FA-O-MeT) in the presence of *S*-adenosyl methionine (SAM) and a cytochrome P450 monooxygenase to form MF (Holford et al., 2004). FA-O-MeT transcripts have been found in multiple tissues such as brain (Fig. 2C,F), eyestalk (Fig. 2F), epidermis, MO (Fig. 2D,E,F), muscle, gill, heart, ovary, HP and the gut of the edible crab *C. pagurus*, and in the lobster *H. americanus* (Li et al., 2010; Ruddell et al., 2003). However, the multiple spatial locations of FA-O-MeT gene expression are an open area of interest to all crustacean sesquiterpenoid researchers. Recently, Mak and colleagues (Mak et al., 2005) demonstrated that FA (low concentrations) can also stimulate *Charybdis feriatus* Vg gene expression in the HP. Similarly, FA also consistently stimulated MeVg1 expression by the HP explants, while both FA and 20-hydroxyecdysone stimulated ovarian explants of *M. ensis in vitro* (Tiu et al., 2006). These studies have revealed distinct hormonal effects *in vitro*, in which the stimulatory effect of FA is consistent and more potent than that of MF. As neither MF nor JH-III shows up-regulation of the Vg gene at low concentrations, the apparent stimulatory effect at high concentrations suggests that the metabolism of these sesquiterpenoids could be important for crustacean vitellogenesis.

Non-neuroendocrine regulation of reproduction in microcrustaceans

Microcrustaceans are essential components in freshwater as well as marine water food webs; because of their abundance and their high grazing activity on phytoplankton, they provide a vital link between primary and secondary producers. Ecologically these species have been well investigated, e.g. geographical distribution and migration patterns (Thorisson, 2006). However, neuroendocrine and non-neuroendocrine systems in microcrustaceans have not been adequately investigated. The basic microcrustacean neuroendocrine and non-neuroendocrine system information has to be translated from research on insects and larger crustaceans, such as crabs, lobster, shrimp, crayfish and prawns. Neuroendocrine signaling pathways among the microcrustaceans are mediated by MF and ecdysteroids. These hormones are absent in vertebrates. Microcrustaceans are incapable of *de novo* cholesterol synthesis and, consequently, must obtain these vital nutrients from their diet (Wacker and Martin-Creuzburg, 2007). The conversion of dietary cholesterol into 20-hydroxyecdysone necessitates several enzymatic hydroxylation steps (Lafont et al., 2005). In microcrustaceans these enzyme systems have not been studied.

Very little is known about regulatory systems controlling lipid consumption, ecdysteroid synthesis and initiation of reproduction in microcrustaceans in general. Cholesterol-enriched diet arouses both egg production and hatching rates without changing the cholesterol content of plasma membranes in the copepod *Acartia hudsonica* (Crockett and Hassett, 2005). A decrease in ecdysteroid levels provoked by environmental chemicals has been found to result in developmental abnormalities in *Daphnia magna* embryos, which suggests that ecdysteroids are essential for the embryogenesis of daphnids (Mu and LeBlanc, 2002). Mu and LeBlanc (Mu and LeBlanc, 2004) also reported a progressive decline in ecdysteroid levels during the first stages of embryonic development, followed by a slight increase in subsequent developmental stages. This suggested that daphnid embryos rely on maternally derived ecdysteroids in their early development, and that this pool of ecdysteroids is exhausted and subsequently replenished with endogenously synthesized ecdysteroids in late embryonic development. Nevertheless, evidence has been provided that ovarian ecdysteroids are transferred into the eggs, either as free or as conjugated ecdysteroids, most probably for subsequent use by the developing embryo (Subramoniam, 2000).

Ecdysteroid analysis may provide a valuable tool for identifying environmental cues associated with diapause induction, thus facilitating the formulation of diapause induction models that can assess the impact of diapause on crustacean species (Hind et al., 2000; Qiu et al., 2007). Ecdysteroids are at low levels during diapause, and therefore they can, at most, indirectly control diapause (Johnson, 2003). Only small amounts of ecdysteroids were found in newly deposited eggs of *D. magna*, which suggests a sparing investment of maternal ecdysteroids in the eggs for early embryogenesis (Martin-Creuzburg et al., 2007). In copepods, gravid females carrying late stage pre-hatch embryos contained significantly more 20-hydroxyecdysone than gravids carrying early embryos (Block et al., 2003). Egg production rates on the supplemented diet are up to 2-fold higher in *A. hudsonica* and up to 3-fold higher in *C. finmarchicus* than in animals on diets not supplemented with cholesterol (Hassett, 2004). It has been shown

that daphnids allocate dietary sterols into their eggs presumably to provide the developing embryo with sufficient amounts of sterols (Wacker and Martin-Creuzburg, 2007). In copepods, the dietary sterol content was found to affect not only egg production rates but also egg viability (Crockett and Hassett, 2005).

Administration of vertebrate-type steroidal androgens (testosterone, androstenedione) to daphnids causes aberrant embryo development (LeBlanc, 2007). Co-administration of 20-hydroxyecdysone protected embryos against this effect (Mu and LeBlanc, 2002). These observations indicate that the activity of testosterone in daphnids is mediated by its ability to interfere with ecdysteroid signaling and not through an androgen signaling pathway.

Less is known about eicosanoids in crustaceans, although during the last three decades considerable evidence has been collected concerning their synthesis and mode of action. Generally, eicosanoids are derived from fatty acids acquired in the diet. They play a very important role in the regulation of reproduction and immune functions. Martin-Creuzburg et al. (Martin-Creuzburg et al., 2009) reported that population growth of *D. magna* is improved more by the addition of sterols and eicosapentaenoic acid (EPA) together than by adding either nutrient separately, which implies a synergistic effect of sterol and EPA supplementation on reproduction. Further, they also found in *D. magna* that somatic growth is primarily constrained by the availability of sterols, and reproduction is primarily constrained by the availability of EPA. Current evidence suggests that the main mode of action of the non-steroidal anti-inflammatory drug ibuprofen in *D. magna* relates to interruption of eicosanoid biosynthesis, which reduces fecundity (Heckmann et al., 2008), where both prostanoids and lipoxygenase products appear to be important agents in oogenesis and embryogenesis (Medeiros et al., 2004).

It was recently reported that the exposure of MF to *Daphnia* oocytes during late ovarian development causes the oocytes to develop into males, whereas only females are produced from control, unexposed animals (Mu and Leblanc, 2004; Ikuno et al., 2008). These results suggest that MF may act as a sex determinant. Further studies indicated that MF, fenoxycarb and pyriproxyfen all disrupted ecdysteroid-regulated aspects of embryo development in daphnids (Mu and LeBlanc, 2004). The data indicate that MF modulates ecdysteroid activity in crustaceans (Tamone and Chang, 1993). Further studies are needed to verify the other physiological roles of MF in microcrustaceans.

Effects of environmental factors on hormone levels and reproduction

Crustacean reproduction is controlled by several hormones, which aid in evaluating environmental conditions to determine the best time to reproduce (Fig. 5). Environmental variables such as salinity, temperature and seasonality play vital roles in regulating crustacean physiology, including reproduction, behavior, molting, morphogenesis and feeding (Charmantier-Daures et al., 1994; Fingerman, 1997a; Mazurová et al., 2008; Spanings-Pierrot et al., 2000). We observed that when red phase crabs were transferred into diluted seawater, the percentage of MF increased to nearly 100% at 11°C and 18°C (Nagaraju and Borst, 2008). These results agree with previously published reports. For example, as the environmental water salinity is diluted to 5 p.p.t., the green crab's MF levels in hemolymph increased up to 5- to 10-fold greater than that of crabs in isosmotic seawater (Lovett et al., 2006). Increased temperature (32°C), anoxia (0.25 p.p.m. O_2) and decreased salinity were followed by a significant increase in the level of hemolymph MF in *C. maenas* (Lovett et al., 1997). Similarly, a stenohaline crab, *L. emarginata,* showed increases in hemolymph MF levels when transferred to low (20 p.p.t.) saline water (G.P.C.N., unpublished). In contrast, exposure of another euryhaline crab, *C. sapidus*, to low salinity sea water (15 p.p.t.) did not increase its hemolymph levels of MF (Henry and Borst, 2005). Similar results were also observed in green phase crabs when they were treated with low saline water at 11°C (Nagaraju and Borst, 2008). Olmstead and LeBlanc (Olmstead and LeBlanc, 2007) found that environmental factors can cause aberrant sex determination *via* perturbations in MF signaling in *D. magna*.

Techniques that are less conventional than the ESA method, such as temperature, salinity and photoperiod manipulations, have been employed in different shrimp species. Cripe (Cripe, 1994) and Akta and colleagues (Akta et al., 2003) studied the effects of temperature variation on stimulated ovarian development and spawning in *Penaeus duorarum* and *P. semisulcatus*, respectively, with a high level of success. Ohtomi and colleagues (Ohtomi et al., 1998) observed that reproduction was stimulated by photoperiod in the deep-water shrimp *Solenocera melantho* and *Plesionika semilaevis* from Kagoshima Bay, where the bottom water temperature tended to be constant throughout the year at 15.8±0.5°C (Noro et al., 1991). However, stability of water temperature and optimal temperature along with salinity were essential to maintaining reproductive captivity in marine crustaceans. Neuroendocrine coordination of the appropriate environmental factor is essential to ensure that annual reproductive and molting events take place in the appropriate temporal sequence. Information about the environmental and endogenous factors that control crustacean molt and reproduction is required to enhance profitable aquaculture programs (Yano, 1987). Photoperiod has been reported to be a strong inducer of reproduction in several crustacean species. Ovarian maturation and Vg synthesis increased significantly in *H. americanus* when a longer photoperiod was set in the laboratory (Quackenbush, 1994). In Penaeidae, a longer photoperiod also caused ovarian maturation in adults (Yano, 1993). Earlier studies showed that high temperature causes stress in some male crustaceans. Pascual and colleagues (Pascual et al., 2007) confirmed that 24–27°C temperatures delay spermatophore deterioration and melanization, and 33°C was shown to be an acute temperature that considerably affected sperm quality in *L. setiferus*. These results are analogous to those of Bray and colleagues (Bray et al., 2009) who found in an earlier experiment that temperatures between 25 and 26°C avoid a decrease in sperm quality during the first 30 days of exposure in *L. setiferus*, leading to satisfactory sperm quality preservation for 60 days in 33% of the shrimps.

Conclusion and future directions

The neuroendocrine and non-neuroendocrine control of reproduction in decapods has been the basis of study and a topic of debate for the past six decades. Structural and functional genomics as well as endocrine manipulations (*via* changing environmental factors) and biochemical studies need to be combined with a reproductive developmental approach. The hormones and neuromodulators involved in the reproductive and non-reproductive response of decapods, and their overlapping functions as well as their diverse behavior at various targets, remain unclear. Interestingly, dsRNAi/microRNAi studies may give new perspectives on the functional significance of GIH or gonad stimulatory peptides and proteins. Integrative comparative aspects of reproductive physiology, such as salinity, temperature, circadian rhythmicity and development are a classical approach that should

be investigated to answer the many exciting questions about eyestalk, brain and TG peptides and proteins.

List of abbreviations

AG	androgenic gland
AGH	androgenic gland hormone
cAMP	cyclic adenosine monophosphate
CHH	crustacean hyperglycemic hormone
DA	dopamine
dsRNAi	double-stranded RNA interference
EPA	eicosapentaenoic acid
ESA	eyestalk ablation
FA	farnesoic acid
FA-O-MeT	farnesoic acid *O*-methyl transferase
FSH	follicle stimulating hormone
GIH	gonad inhibitory hormone
GSF	gonad stimulating factor
HCG	human chorionic gonadotrophin
HP	hepatopancreas
HPLC	high performance liquid chromatography
5-HT	5-hydoxytryptamine
JH	juvenile hormone
LH	luteinizing hormone
MeVg1	*Metapenaeus ensis* Vg1
MF	methyl farnesoate
MIH	molt inhibiting hormone
MO	mandibular organ
MS	mass spectroscopy
OA	octopamine
PG	prostaglandin
SG	sinus gland
SP	spiperone
TG	thoracic ganglia
Vg	vitellogenin
VIH	vitellogenesis inhibiting hormone

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