Male Reproductive Hormones

E.S. Chang\textsuperscript{1} and A. Sagi\textsuperscript{2}

\textsuperscript{1}Bodega Marine Laboratory, University of California-Davis, PO Box 247, Bodega Bay, CA 94923, USA, E-mail: eschang@ucdavis.edu

\textsuperscript{2}Department of Life Sciences and the National Institute for Biotechnology in the Negev, Ben Gurion University, PO Box 653, Beer Sheva 84105, Israel. E-mail: sagia@bgu.ac.il

INTRODUCTION

Sex differentiation and reproduction in male vertebrates are regulated by several different peptide and steroid hormones from various endocrine organs. In contrast, insects are thought not to have sex hormones at all (Maas and Dorn, 2005). In the closely related crustaceans, the androgenic gland (AG) appears to be unique, since this single gland regulates both male sex differentiation and male reproductive physiology and since, unlike in vertebrates, the endocrine and gametogenic functions are clearly separated into distinct organs, the androgenic gland and the testis, respectively. As to the other invertebrate phyla, very little is known about the role (or even the existence) of hormones that regulate sex differentiation or male reproductive physiology.

This review is not intended to be comprehensive. It gives a brief historical overview of hormonal regulation of reproduction in male crustaceans, including the androgenic gland hormone (AGH), and then describes some of the more recent work on its isolation, characterization, and mode of action. We then summarize evidence for the role of other chemical mediators in the regulation of male reproduction (ecdysteroids,
vertebrate-like steroids, and methyl farnesoate). A brief discussion of pheromones is included, since they are chemical mediators that influence reproduction and are mechanistically similar to hormones.

The reader is directed to several comprehensive reviews that discuss some aspects of the material covered in this review (Ginsburger-Vogel and Charniaux-Cotton, 1982; Adiyodi, 1985; Charniaux-Cotton, 1985; Charniaux-Cotton and Payen, 1988; Sagi, 1988; Sagi et al., 1997; Sagi and Khalaila, 2001).

THE CRUSTACEAN ANDROGENIC GLAND

Sex Differentiation and the Androgenic Gland

The regulation of reproduction in crustaceans is highly diverse; most species are dioecious (separate sexes), but there are also many hermaphroditic species. In the Malacostraca (e.g., isopods, amphipods, decapods), experiments have demonstrated that individuals of most species possess the genetic information for the development of both males and females. In genetic males, the AG develops and begins to secrete AGH. In the absence of a developed, active AG (i.e., in females), there is an absence of AGH and female structures develop.

The AG was initially discovered by Cronin (1947) in the blue crab (*Callinectes sapidus*) and later suggested by Charniaux-Cotton (1954a) to be the regulator of spermatogenesis and male differentiation in the amphipod *Orchestia gammarella*. The AG is located at the distal portion of the sperm duct. Charniaux-Cotton (1955) observed that the AG is the only male tissue that can mediate the transformation of the ovaries of an immature female into testes. An ovary transplanted into an andrectomized male remained an ovary (Charniaux-Cotton, 1954b, 1955, 1957). Following removal of the AG from a male, spermatogenesis waned and in some cases oocytes appeared (Charniaux-Cotton, 1964). These observations were confirmed in the isopod *Armadillidium vulgare* (Legrand, 1955). More recent experiments have been conducted in the giant freshwater prawn, *Macrobrachium rosenbergii*. Nagamine et al. (1980a) demonstrated complete sex reversal following the removal of the AGs at an early immature stage. This operation resulted in complete female differentiation, complete with ovaries and oviducts including reproductive behavior, successful mating and production of offspring (Sagi and Cohen, 1990; Sagi et al., 1990, 1997). Implantation of AGs into females resulted in the development of male copulatory organs with reported cases of functional sex reversal and progeny obtained when fertile sex-reversed animals were crossed with normal prawns (Nagamine et al., 1980a, b; Malecha et al., 1992).
Macrobrachium rosenbergii males progress through a succession of male morphotypes beginning with small males to orange-claw males to the dominant blue-claw males. Sagi et al. (1990) demonstrated that the AG was necessary for this morphotypic progression. Histological and biochemical supporting evidence for the relationship between morphotypic differentiation and the structure of the AG were presented by Sun et al. (2000). The necessity of the AG for complete maleness in decapods was demonstrated in other species, including the crayfish Procambarus clarkii (Nagamine and Knight, 1987; Taketomi and Nishikawa, 1996) and Cherax destructor (Fowler and Leonard, 1999). The wide array of effects for which this gland is responsible was demonstrated in Cherax quadricarinatus (Khalaila et al., 1999, 2001; Sagi et al., 2002; Manor et al., 2004), including the development of male secondary characteristics and inhibition of female secondary characteristics and vitellogenesis. It was, moreover, shown that the AG induces male-like reproductive and aggressive behavior (Barki et al., 2003; Karplus et al., 2003).

The Androgenic Gland and Biotechnology

Most economically important cultured crustaceans show sexually bimodal growth patterns in which males grow faster than females or vice versa. Monosex culture has been recently suggested as one of the most promising ways to improve production efficiencies of crustacean aquaculture. Nair et al. (2006) demonstrated in M. rosenbergii that profits from monosex culture could be higher by as much as 60% over a mixed population. An example of the application of endocrine research in crustaceans is the potential for the production of all male monosex M. rosenbergii. This latter example would be the result of AG removal from immature males, which results in sex reversal with complete and functional female differentiation (neo-female). In the prawn M. rosenbergii, neo-female animals are capable of mating with normal specimens to produce all-male offspring. The reason for the occurrence of all-male progeny in such a crossing is the homogametic (ZZ) nature of the males (Sagi and Aflalo, 2005; Aflalo et al., 2006).

Androgenic Gland Hormone

Much effort has been devoted to the purification and characterization of the active AGH belonging to the insulin-like super family. From the isopod A. vulgare, Hasegawa et al. (1987) purified two proteins, AGH1 and AGHII, consisting of 157 and 166 amino acids, respectively. Molecular masses of 17.0 and 18.3 kDa were estimated. Independent
work by Martin et al. (1990) on the same species led to similar results, though there were slight discrepancies in the amino acid compositional data. Bioassays involved masculinization of young female isopods. Protein blotting experiments with polyclonal antisera raised against AGH indicated the presence of a larger, biologically inactive protein. This indicates that AGH may be translated as a prohormone (Martin et al., 1990; Hasegawa et al., 1991). Recent work has resulted in the determination of the structure of the AGH and the gene that codes for it (Martin et al., 1999; Okuno et al., 1999) (Fig. 1). Recombinant isopod AGH has been produced (Okuno et al., 2002).

<table>
<thead>
<tr>
<th>Signal Peptide</th>
<th>B Chain</th>
<th>KR</th>
<th>C Peptide</th>
<th>KR</th>
<th>A Chain</th>
</tr>
</thead>
<tbody>
<tr>
<td>(21)</td>
<td>(44)</td>
<td></td>
<td>(46)</td>
<td></td>
<td>(29)</td>
</tr>
</tbody>
</table>

Fig. 1 Schematic structure of the androgenic gland hormone (AGH) from Armadillidium vulgare. As shown, Okuno et al. (1999) reported an additional signal peptide of 21 amino acid residues adjacent to the B-chain in the prohormone form. Loss of the signal peptide results in the AGH prohormone. Martin et al. (1999) reported a glycan moiety (not shown) attached to one amino acid of the A-chain. The numbers in parentheses indicate the number of amino acid residues in each domain of AGH. The possible cleavage sites are indicated by KR (Lys-Arg). Removal of the C-peptide and formation of disulfide bonds between the A- and B-chains results in the formation of the mature hormone.

In a decapod, a subtractive cDNA library from the AG of the red-claw crayfish C. quadricarinatus has been established revealing an AG-specific gene, expressed exclusively in males, even at early stages of maturation. This gene is termed Cq-IAG (insulin-like AG factor from C. quadricarinatus). In situ hybridization of Cq-IAG confirmed the exclusive localization of its expression in the AG. Following cloning and complete sequencing of the gene, its cDNA was found to contain 1,445 nucleotides encoding a deduced translation product of 176 amino acids. The proposed protein sequence encompasses Cys residue and putative cleaved peptide patterns whose linear and 3D organization are similar to those of members of the insulin/insulin-like growth factor/relaxin family and their receptor recognition surface (Fig. 2). The peptide and its activity remain to be elucidated (Manor et al., 2007).

Other biologically active factors have been isolated from the green crab (Carcinus maenas) AG. The terpenes farnesylacetone and hexahydroxifarnesylacetone were isolated and inhibited the incorporation of radiolabeled leucine by ovaries in vitro (Ferezou et al., 1977). Farnesylacetone was also able to inhibit radiolabeled uridine incorporation, indicating that it inhibits transcription (Berreur-
Fig. 2 Identification and characterization of Cq-IAG (insulin-like AG factor from *Cherax quadricarinatus*), an AG-specific expressed gene. A. RT-PCR (reverse transcriptase-polymerase chain reaction) showing expression of Cq-IAG only in the AG. hAG: hypertrophied androgenic gland; P. glands: peripheral glands; Hepato.: hepatopancreas; EFT-2: control for equal sample loading. B. RT-PCR showing expression of Cq-IAG in juvenile males (M) (8 and 22 days post-release from the mother) and in the base of the fifth walking leg (containing the AG) of a mature male but not in females (F). In the lane marked RNA, RNA was added as a template to the PCR in order to rule out genomic contamination. C. Localization of the expression of Cq-IAG was performed by RNA in situ hybridization. A strong, specific signal in the AG was detected by the antisense probe only. SD: sperm duct. D. Multiple sequence alignment of the putative mature Cq-IAG peptide with representative members of the insulin/insulin-like growth factor/relinxin family (based on SMART results and done by Clustal X; modified from Manor et al., 2007, with permission from Elsevier).

Bonnenfant and Lawrence, 1984). The roles of these terpene AG factors remain to be elucidated, though it is most likely that the decapod AGH is also a peptide as in the isopods (for review, see Sagi and Khalaila, 2001). Secretions from the eyestalk X-organ/sinus gland complex may regulate the AG (Khalaila et al., 2002).

**STEROID HORMONES**

**Ecdysteroids**

A number of studies indicate that the arthropod molting hormones (ecdysteroids) are important regulators of female reproduction in
crustaceans (Subramoniam, 2000; Chang and Kaufman, 2005, for reviews). Much less is known about the involvement of ecdysteroids in male reproduction. While injections of ecdysteroids into crustaceans induce many physiological changes, it is not clear whether the hormone is directly responsible for the associated morphological and biochemical changes (for review see Adiyodi, 1985).

In vitro studies on the effects of ecdysteroids on crustaceans have demonstrated some direct effects of the hormone on male-related physiology. For example, DNA synthesis increased after 2-3 days in sheath cells of cultured isopod (Idotea wosnesenskii) testes incubated with 20-hydroxyecdysone (Fig. 3) (Matlock and Dornfeld, 1982). Exposure of lobster (Homarus americanus) testicular primary cell cultures to 20-hydroxyecdysone caused spermatogonia to proliferate (Brody and Chang, 1989). Addition of 20-hydroxyecdysone resulted in a significant increase in the incorporation of radiolabeled thymidine in cultured testes from M. rosenbergii (Sagi et al., 1991).

![Fig. 3 Structure of 20-hydroxyecdysone.](image)

Several reports circumstantially implicate ecdysteroids as being involved in male reproduction. In shore crabs (Pachygrapsus crassipes), the testes had the highest hydroxylating enzyme activity in converting the prohormone ecdysone to the active molting hormone 20-hydroxyecdysone (Chang et al., 1976).

Expression of cytochrome P<sub>450</sub> genes that code for this enzyme activity were also high in the testes of C. maenas (Styrishave et al., 2004). Recently, Parnes et al. (2006) discovered a molt-dependent mechanism by which old sperm is periodically removed from the reproductive system of male Litopenaeus vannamei shrimp. It was shown that male shrimp go through reproductive cycles that are strictly associated with their molt cycles, which, in turn, are hormonally
regulated. Intact intermolt spermatophores disappeared about 12 h premolt, and a new pair of spermatophores appeared in the ampullae the day after the males had molted.

OTHER STEROIDS

There are a few reports of male vertebrate steroids (such as testosterone) being present (Burns et al., 1984) or capable of being metabolized in various species of male crustaceans (Gilgan and Idler, 1967; Teshima and Kanazawa, 1971; James and Shiverick; 1984, Baldwin and LeBlanc, 1994; LeBlanc and McLachlan, 2000; Verslycke et al., 2002). Histochemical evidence for possible steroidogenic activity through the distribution of lipids 3-alpha and 3-beta-hydroxysteroid dehydrogenase in the AG was reported in *M. rosenbergii* (Veith and Malecha, 1983). Nagabhushanam and Kulkarni (1981) suggested that exogenous testosterone affects the AG of a marine penaeid prawn, *Parapenaeopsis hardwickii*. Recent studies suggest that vertebrate steroid androgens may alter crustacean sex ratio causing the appearance of more males in prawn populations (Baghel et al., 2004; Ohs et al., 2006). The mere presence, exogenous effect and/or metabolism of a steroid, however, do not necessarily mean that the molecule has a physiological role in normal development. Whether testosterone has a physiological role in male reproduction in crustaceans remains to be seen.

METHYL FARNESOATE

Methyl farnesoate (MF; Fig. 4) is a sesquiterpenoid that was identified in crustacean hemolymph using gas chromatography/mass spectrometry with selected ion monitoring (Borst et al., 1987; Laufer et al., 1987). It is produced by the paired mandibular organs (Fig. 5). Methyl farnesoate is very similar in structure to insect juvenile hormone III, differing only by the absence of an epoxide group in MF. In insects, the juvenile hormones regulate metamorphosis and reproduction (Goodman and Granger, 2005). Crustaceans have many morphological, developmental, and physiological features in common with insects. Because of the striking similarities between these two groups of arthropods, biologists have speculated about the existence of crustacean juvenile hormone-like

![Fig. 4 Structure of methyl farnesoate.](image-url)
molecules for at least 50 years (Chaudonneret, 1956). Support for this hypothesis was presented shortly thereafter (Schneiderman and Gilbert, 1958). A number of different effects on crustacean development and reproduction have been attributed to MF (for review, see Homola and Chang, 1997). The function of MF in crustaceans is still ripe for investigation. It is probable that MF has roles similar to those of juvenile hormones in insects, but it is also possible that it has novel functions. In many species of insects, juvenile hormones play several distinct roles in female reproduction (Raikhel et al., 2005, for review). Primarily, they stimulate the synthesis of the yolk protein precursor vitellogenin in the female. In some species of insects, juvenile hormones maintain the functional state of the male accessory sex organs. One effect of MF is on the duration of the crustacean molt interval (Yudin et al., 1980; Borst et al., 1987; Tamone and Chang, 1993). Methyl farnesoate may also be involved in mediating various stress responses (Lovett et al., 1997, 2001). Several
lines of evidence indicate that it may have a permissive or stimulatory effect upon reproduction in both female and male crustaceans (see other chapters in this volume for discussions on the role of MF in female reproduction).

**Methyl Farnesoate as a Gonadotropin**

A number of studies implicate MF as a gonadotropin—a stimulator of gonadal development. Spider crabs (*Libinia emarginata*) can be divided into various morphotypes on the basis of overall size, claw size, and shell condition (abraded or unabraded). Homola et al. (1991) observed that the group of males with the largest reproductive index comprised the large, unabraded males with large claws. These male crabs had the highest rates of MF production when their mandibular organs were cultured in vitro. They also had the greatest reproductive activity (Sagi et al., 1994).

Injection of MF into crabs (*Oziotelphusa senex senex*) resulted in significant increases in the size of individual testicular follicles and the overall testicular index (Kalavathy et al., 1999; Reddy et al., 2004).

**Methyl Farnesoate and the Regulation of Morphotypic Development**

As mentioned in the previous section, differences in MF status are correlated with reproduction in male *L. emarginata* (Homola et al., 1991). Adult males of this species have alternative morphotypes, or distinct external features corresponding to differences in reproductive development and behavior (Laufer et al., 1994). In *L. emarginata*, male morphotypes can be distinguished by the relative size of the claws and the condition of the epicuticle. Males with large claws and worn epicuticles display mating behavior and have high MF titers (39-67 ng/ml) in the hemolymph. Males with small claws and intact epicuticles do not display mating behavior. They have hemolymph with significantly lower MF titers (3-30 ng/ml) (Sagi et al., 1993, 1994). This crab species offers the opportunity to predict differences in MF titer using external morphological markers. Male morphotypes of *L. emarginata* could be an important model for testing the reproductive function of MF when a technique for manipulating MF titer becomes available. Sex-specific differences in MF titer occur in several species of crustaceans. Male *L. emarginata* and *H. americanus* have higher levels of MF in the hemolymph than females (Borst et al., 1987; Laufer et al., 1987). In *H. americanus*, the relative size of the mandibular organ increases in males after sexual maturity, but not in females, suggesting that the mandibular organ may function differently in males and females (Waddy et al., 1995). Methyl
farnesolate titers in females may be lower than in males because the mandibular organs are smaller or less active, or because MF is sequestered by the gonads.

Development of different morphotypes depends upon the differential growth of various body parts (allometric growth). Laufer et al. (2002) were able to modify ecdysteroid levels via eyestalk ablation. This operation removes the X-organ/sinus gland complex resulting in the disinhibition of ecdysteroid secretion by the molting gland. These authors were also able to modify MF levels via injections. They concluded that ecdysteroids in the presence of low MF concentrations promoted allometric claw growth, while ecdysteroids with relatively high concentrations of MF inhibited it (Laufer et al., 1997).

**Methyl Farnesoate and Sexual Differentiation**

Methyl farnesoate also acts as a sex determinant. In *Daphnia magna*, application of physiological amounts of exogenous MF (400 nM) to egg-maturing females resulted in all-male broods. There was a correlation with lower MF concentrations and increasing proportions of males in the broods (Olmstead and LeBlanc, 2002). Similar effects were seen in four other species of cladocerans (Kim et al., 2006). The production of male offspring following MF induction will be a powerful tool for the elucidation of the hormone’s action at the molecular level (Rider et al., 2005).

**PHEROMONES**

For decapod crustaceans, pheromones may play an important role in both soft-shelled mating crab families (Eales, 1974) and hard-shelled mating crab families (Warner, 1977; Hardege et al., 2002). It has been suspected that during the days leading up to the actual molt, the female of some species releases a pheromone into the environment to attract a male (Ryan, 1966; Snow and Neilson, 1966; Dunham, 1978; Ekerholm and Hallberg, 2005). Males in the vicinity of the premolt female begin a search behavior that is distinct from the behavior displayed upon the detection of either food or other males (Atema and Engstrom, 1971; Eales, 1974; Kamio et al., 2002). Upon location of the female, the male may initiate a courtship display. It may then attempt to grasp the female and carry her upside-down with their ventral surfaces in contact until she molts. The male protects the female from predators and other males. After the female molts, the male copulates and then maintains a post-mating embrace until her exoskeleton has partially hardened.
The source of these attractant pheromones is likely urine. There have been a number of studies focused on isolating and identifying the urinary pheromone, but with limited success (Dunham, 1978). Hormones such as the ecdysteroids have been proposed to be pheromones (Kittredge et al., 1971; Seifert, 1982; Gleeson et al., 1984). Those results have been inconclusive. It may be that a suite of compounds acts as a multi-component signal rather than a solitary chemical messenger (Warner, 1977; Wyatt, 2003). The attractant pheromone of the female crab *Erimacrus isenbeckii* has been identified as a novel ceramide (Asai et al., 2000).

Other research indicates that alternative sources of pheromones may exist. This was demonstrated by showing that post-molt female lobsters were still attractive to males, even though the female’s urine was collected by catheters and not released into the environment (Snyder et al., 1993). In *C. maenas*, the pheromone may be contact mediated and not released in the urine (Ekerholm and Hallberg, 2005).

Males also likely produce pheromones. In *H. americanus* and crayfish (*Astacus leptodactylus*), release of urine is timed with agonistic interactions between males (Breithaupt and Atema, 2000; Breithaupt and Eger, 2002) and, along with visual cues, likely provides information about the status of the males. Establishment of dominance results in more successful mating by the winner of the contest.

**CONCLUSIONS**

This is an exciting time for research on crustacean endocrinology. There is increased interest in the topic due to aquaculture applications and the focus on crustaceans as keystone species in aquatic environments. Some key areas of future research on the hormonal control of male reproduction include the following:

1. The identification of the AGH in decapod crustaceans will enable a better understanding of the regulation of sexual differentiation and masculine reproductive physiology. In case the AG active factor is found to be a member of the insulin-like super family, it will support the notion that insulin may have evolved in the context of regulating sexual differentiation.

2. Except for the ecdysteroid receptor, research on crustacean hormone receptors has been minimal. The characterization of MF and AGH receptors will yield insight into the molecular action of these important regulatory molecules.

3. New functions are being attributed to members of the crustacean hyperglycemic hormone (CHH) peptide family. Mandibular organ-inhibiting hormone is a member of this family (see Chang
and Kaufman, 2005 for review). Structure-function studies of the peptides comprising this family will yield information about key amino acid positions and their resulting biological activities.

4. Further research on the biological activities and mode of action of MF will be of comparative interest in relation to juvenile hormone studies in insects. Both the developmental and reproductive aspects of MF need to be addressed.

5. There are indications of other chemical mediators involved in male reproduction. These factors include neurotransmitters and peptides such as gonad-inhibiting hormone (also known as vitellogenesis-inhibiting hormone). Most of the work on these factors has been focused on the female. More research needs to be conducted to determine whether they have a role in male reproduction.

6. There is growing interest in the field of invertebrate endocrine disrupters (deFur et al., 1999). Because of the biological importance of the Crustacea, there will probably be much more research devoted to the determination of the effects of exogenous chemicals upon various physiological processes, including male reproduction. Crustaceans will likely be useful indicator species for environmental contamination.

7. The isolation and characterization of crustacean pheromones will be important for basic research, development of novel methods of harvesting commercially important fisheries species, and the control of nuisance and alien crustaceans.

Acknowledgments

We thank Sharon A. Chang for editorial assistance. E.S.C. is grateful to Prof. Howard A. Bern for introducing him to the crab androgenic gland over three decades ago.

References


