

# EFFECTS OF NATURAL COMPOUNDS IN THE SEX DETERMINATION OF DECAPOD CRUSTACEANS AND POSSIBLE AQUACULTURE APPLICATIONS: A REVIEW

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## Summary

Some natural substances, especially those produced by aquatic plants, can have different biological effects on decapod crustaceans. Several studies demonstrated the activity of wound-activated compounds on the physiology of planktonic and benthic crustaceans. Many of these compounds are produced at the time of the rupture of the cell wall, in order to reduce the pressure of grazing. They may have toxic or teratogenic effects and have been identified mainly in the plankton (reduction of grazing by planktonic copepods on some diatoms). Other compounds had probably a deterrent mean, but they are used as seasonal signals by some decapods and they are able to stabilize natural populations. This is the case of the substances that trigger a rapid apoptosis (programmed cell death) of androgenic gland in postlarvae of the protandric shrimp *Hippolyte inermis*, thus leading to their sex reversal and the production of young females. Extracts of benthic diatoms containing these bioactive compounds have been tested on various benthic and planktonic organisms, in order to define their mechanism of action and their chemical nature. Bioassays aimed at the elucidation of the molecular structure of the active compound are still ongoing. Our aim was initially to establish if it was possible to obtain pseudo-females of commercially interesting species (e.g. *Cherax* spp.) to be crossed with natural males, in order to obtain an all-male generation. This result might, in fact, produce clear benefits to the environment (non-native species could never be introduced in natural environments in an appropriate gender proportion) and the economy (fastest growing of conspicuous males). First results have failed to obtain a change of sex by simply administrating diatom crude extracts, but the future explication of molecular mechanisms that control the specificity of apoptogenic activity will enable the development of biotechnologies to help the control of sex in decapod crustaceans.

## Introduction

Shrimp aquaculture in the world has largely developed in the last decades. However, new technological advances in hormonal manipulation of shrimp reproduction are increasingly requested for effective stock enhancement and to maximize the yields with minimum efforts (Rungsin *et al.*, 2006). This claims for striking progresses in shrimp endocrinology, because we need to develop newer hormonal manipulation techniques. At date, eyestalk hormones, ecdysteroids, and vertebrate-type steroid hormones are known and applied to female reproduction. Eyestalk ablation induces ovarian development, indicating the role of the eyestalk hormones and vitellogenesis-inhibiting hormone (Sroyraya *et al.*, 2010). Hemolymph levels of ecdysteroids and vertebrate-type steroid hormones are not involved in the regulation of ovarian development (Okumura and Hara, 2004). For male reproduction, the Androgenic Gland (AG) showed distinct structural changes in relation to male reproductive activity, indicating that the hormone has a key role in the regulation of male reproductive activity as well in the male maturation of sex (Sagi and Aflalo, 2005; Fowler and Leonard, 1999).

At present, no hormones are technically available for controlling shrimp reproduction, besides the hormone changes produced by eyestalk ablation, that is the only technique routinely used. In conclusion, endocrinology of shrimp reproduction is not yet sufficiently understood (Okumura, 2004). Only a few hormones have been detailed so far, and many hormones are still unidentified. Another important field of research involving hormonal regulation of decapod physiology is the control of sex (Fingerman, 1997). Some species are characterized by striking differences between sexes (in terms of size, growth rate, colour, etc) and the possibility to obtain populations of a single sex could provide unprecedented opportunities for dramatic improvements of productions (Rungsin, 2006). The techniques in this field are still in their infancy, since we still apply surgical ablation or chemical destruction of gonads in broodstocks, with enormous mortality and high costs (Schechter *et al.*,

2005). Recent investigations on the effect of natural products on the AG of selected decapods (Zupo, 2000), however, open some interesting perspectives of research to obtain elegant and low-cost results.

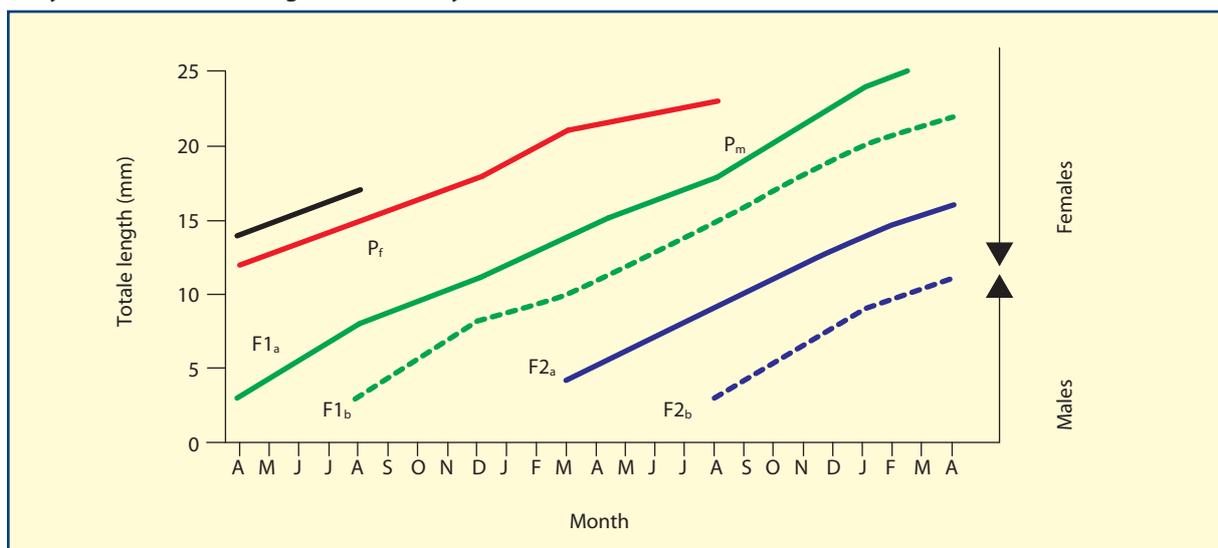
**Key Words:** Natural Compounds, Sex Determination, Decapod, Androgenic Gland

### Control of sex in decapod crustaceans

Any newborn organism must take a first fundamental decision: male or female? In most of higher organisms this decision implies the realization of a very complex hormonal network, assuring stability during the development. In crustacean decapods this process is relatively simple, since the sex is determined by the activity of a single organ, the Androgenic Gland (AG). This is a small endocrine organ, often covered by a layer of connective tissue, attached to the distal part of spermatic ducts and ejaculatory bulbs (Huberman, 2000). The control by the androgenic gland (AG) of male sex differentiation, in crustaceans, was first described in the amphipod *Orchestia gammarella* (Charniaux-Cotton, 1954). In decapod crustaceans, removal of the AG from a male produces regression of male characteristics, while implantation of AG into juvenile females results in the inhibition of vitellogenesis and development of male sexual features (Nagamine and Knight, 1987; Sagi *et al.*, 1990; Lee *et al.*, 1993). As a result, decapod crustaceans have a natural tendency to develop as females, but the simple presence (or implant) of an AG induce the masculinization of that individual. In addition, a direct inhibition by the sinus gland (in shrimp's eyestalks) on the AG was demonstrated (Khalaila *et al.*, 2002; Srorya *et al.*, 2010), and this suggests an endocrine axis-like relationship between the sinus gland, the AG, and the male reproductive system. Therefore, the "simple" hormonal regulation of decapod sex is under the control of the "complex" eyestalk gland system.

To date, attempts to identify active AG factors in decapods through either classical purification techniques or sequence similarity with isopod AG hormones have proven unsuccessful. Manor *et al.* (2007) identified an insulin-like proteinaceous compound, produced in the AG and composed of 176 amino acids, able to control the sex in the crayfish *Cherax quadricarinatus*. Presently, cDNA libraries have been constructed for various species of decapods, in order to test the hypothesis that this same compound is the main hormone produced by the AG of decapod crustaceans (Sagi and Khalaila, 2001).

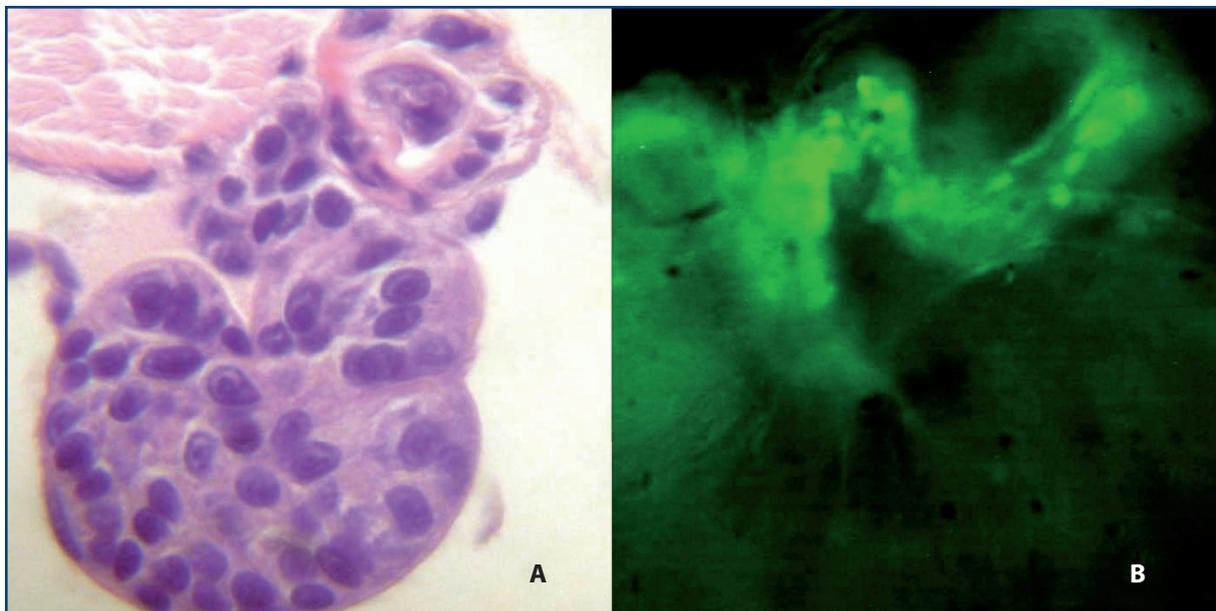
**Figure 1. Life Cycle of *H. inermis* (From Zupo, 1994, modified). Pf, Pm, Parental female generation and Parental male generations. F1, F2, filial generations. a, April generation (both males and small females); b, September generation (only males, that will change sex the next year).**



However, the "simple" mechanism of sex determination, based on a single gland and, probably, on a single insulin-like hormone, makes the sex of decapods very plastic. Several species are hermaphroditic, both sequential or contemporaneous, and sex may change due to environmental cues (Zupo, 2000) or due to parasitic influences (Reverberi, 1943; Reverberi, 1952), stress (Rider *et al.*, 2005) and other factors.

Sequential hermaphrodites in decapod crustaceans have been included as a part of reviews with different purposes by Yaldwyn (1966), Fukuhara (1999), and Chiba, (2007), among the others. Chiba (2007), in particular, listed 57 species of protandric hermaphrodite decapods, including partial protandric or protandric-simultaneous hermaphrodite. No protogynous hermaphrodite decapods have so far been reported. In fact, any factor able to reduce in size or destroy the small AG present in males, is able to trigger a sex change to female. For the same reason, we observe a variety of sexual layouts. Some species (*Marsupenaeus* spp., *Palaemon* spp. etc) conserve their sex for the whole life, because their AG is very stable and persistent, and it is conserved throughout the whole lifespan. Therefore, males never change their sex (Bauer and van Hoy, 1996). Some other species (e.g., *Lysmata* spp.; Bauer, 2002; Bauer, 2006) are simultaneous hermaphrodites, because their AG reduces in size during the development, but it is never totally destroyed. Consequently we observe individuals that are males in the first phases of their adult life, then they loose some male external characters but they conserve the ability to mate as males or females and, of course, they conserve both ovaries and testes. Some species (e.g., *Processa edulis*; Noel, 1973; *Pandalus* spp.; Bergström, 2000) are protandrous hermaphrodites, because their AG is slowly destroyed. In this case, the initial regression of the AG corresponds to a regression of the testes, and the production of a new organ defined "ovotestis". Finally, the destruction of the AG is completed, the regression of the testes continues, and the ovotestis evolves into a functional ovary. Contemporaneously, the external sexual characters change accordingly: the appendix masculina is progressively reduced in size and number of hairs; the male gonopores are closed, while female gonopores appear on the walking legs; eventually, colours and shape of chelipeds may change, if a sexual dimorphism characterizes the considered species. This is the "typical" case of sex reversal in decapods, because it consists of well-known temporal phases, characterizing several species (Chiba, 2007). However, several important exceptions make this process variable and somewhat puzzling. Also stress may trigger the appearance of a higher number of females in some species (Rider *et al.*, 2005). Various environmental factors like temperature (Allen, 1959) and food (Zupo, 1994; Calado *et al.*, 2005) may influence the sex change in some species of decapods.

**Figure 2. Androgenic gland of *Hippolyte inermis*. A, normal physiologic conditions (Hematoxylin-Eosin staining, 400 X). B, during its apoptosis (TUNEL staining for the rapid detection of apoptosis, 200 X).**



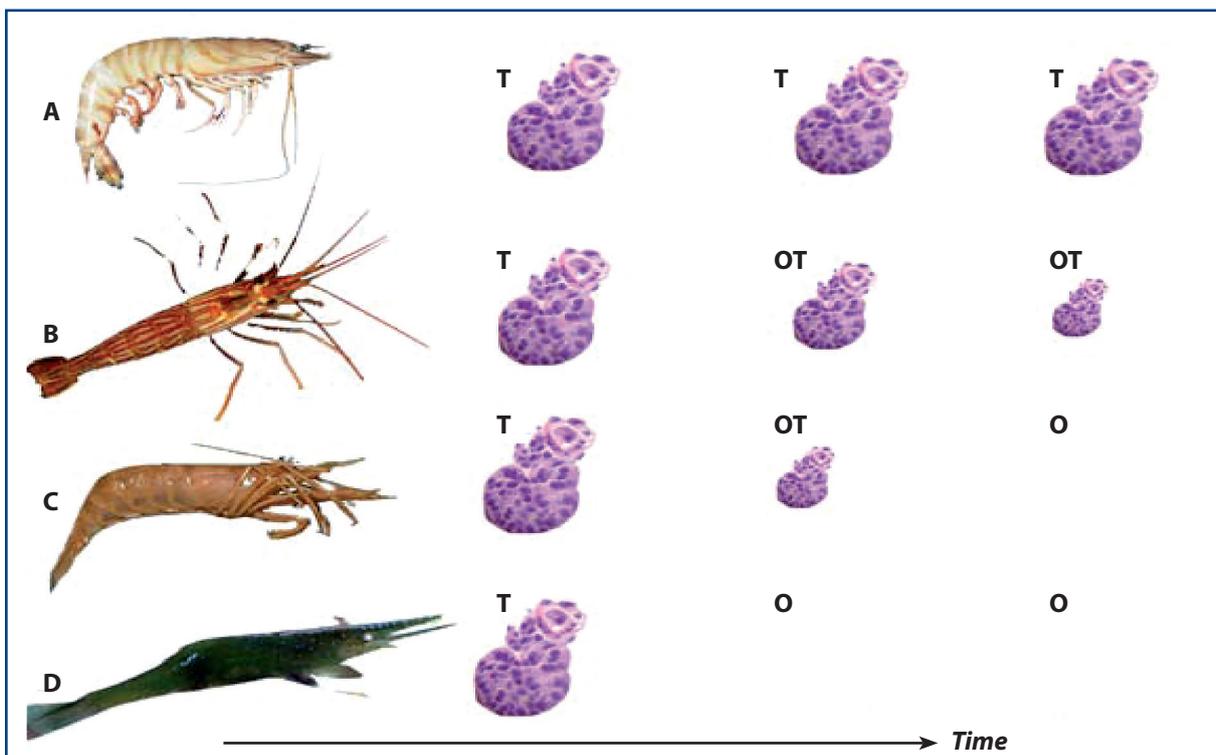
### The peculiar case of *Hippolyte inermis* for comprehension of general trends

*Hippolyte inermis* is a small shrimp living in seagrasses of the Mediterranean and it is characterized by a peculiar mechanism of sex reversal (Reverberi, 1950) influenced by the ingestion of diatom foods (Zupo, 2000). In fact, the development of the ovary in this species is preceded by the total destruction of the testes (Reverberi, 1950) and an ovotestis is never observed (Cobos *et al.*, 2005). For this reason, the latter authors hypothesized a gonochoristic life strategy for this species. However, its sex reversal was demonstrated in the laboratory (Zupo

*et al.*, 2007a) although it is very fast, takes place in a single moult, and corresponds to an abrupt disappearance of the appendix masculina. In this case (Figure 1), it was demonstrated that the AG is destroyed normally at an age of 1 year, in individuals of about 10-12 mm (TL). However, there are small females, in natural populations, in which the AG is destroyed after about 1 month from the hatching of eggs. In this case, the AG destruction is due to the ingestion of diatoms of the genus *Cocconeis*, and it leads to the appearance of young females of 6-7 mm TL. The apoptogenic effect (Figure 2) of diatom compounds is fast (3-4 days), limited to a specific temporal window (from the 2nd to the 8th day after settlement of postlarvae), specific for the tissues of the AG (no toxic effects have been demonstrated in other tissues of the shrimps) and dose dependent (Zupo and Messina, 2007). This effect is perfectly natural and it stabilizes natural populations, because it provides a higher number of young females, indispensable to produce a reproductive burst in autumn.

When we observe the variety of sexual layouts above summarized, a very complex situation appears to characterize the decapod determination of sex. However, a canonical comparison of the different strategies indicates that a single factor, represented by the tissue fragility of the decapod's AG, may produce such a range of life strategies (Figure 3). In fact, in some decapods the AG is persistent and strong: in this case we simply observe gonochorism (Cobos *et al.*, 2005). In other species, it is slowly destroyed, but part of it remains alive, and we observe simultaneous hermaphroditism (Bauer and Newman, 2004). In other species, the AG is slowly destroyed, and we observe the typical cases of protandric hermaphroditism (Noel, 1973). In other species, finally, the AG is very weak and it may be destroyed by stress or environmental impacts (temperature, food, etc) and these are the "peculiar" cases that generate many discussions among scientists (Zupo *et al.*, 2007b). In conclusion, the role played by a single gland (the AG) and the tissue persistence of this small structure, generates the variety of sexual adaptations we observe in various species of crustacean decapods. In other words, according to the grade of fragility of an AG (very stable, partially weak, very unstable and easily destroyed by external or physiologic factors), we observe gonochorism, simultaneous hermaphroditism or protandric sex change, respectively. Of course, hermaphroditism and sex change should be seen in the light of Darwin's theory and species applying these strategies evolve when they have selective advantages (Giselin, 1969; Giselin, 2006), but the sex control ruling the physiology of decapod crustaceans makes the choice "simpler".

**Figure 3. Diagrammatic representation of various possible sexual layouts in decapods. A. The case of a typical gonochoristic species, bearing a persistent AG. B. The case of *Lysmata*-type contemporaneous hermaphrodites. C. The case of *Processa*-type protandric species. D. The case of *Hippolyte inermis*. The relative size of the AG during the lifespan of a male individual is sketched. T, presence of testis. OT, presence of an ovotestis. O, presence of an ovary.**



## All male populations for decapod aquaculture

Males of some species of decapods grow faster and reach higher weights at harvest, compared to females. This fact makes the culture of all-male populations desirable. It is the case, for example, of *Macrobrachium rosenbergii* (Aflalo *et al.*, 2006), *Cherax quadricarinatus* (Shechter *et al.*, 2005) and other species of decapod crustaceans, in which a bimodal growth pattern is exhibited by the two sexes and males show superior growth in comparison to females. As a matter of fact, monosex culture strategy has become a common practice in fish-based aquaculture (Beardmore *et al.*, 2001; Devlin and Nagahama, 2002), and attempts have been made to apply these techniques to crustacean culture (Lawrence *et al.*, 2000; Sagi *et al.*, 1997; Siddiqui *et al.*, 1997), since male and female crustaceans may differ in terms of growth rates, behaviour patterns and husbandry needs. Various crustacean species exhibit bimodal growth patterns, and males exhibit superior growth to females or vice-versa (Hartnoll, 1982). According to the data previously reported, sex differentiation can be manipulated in decapod crustaceans through the removal of the AG, without damaging the gonads, and such manipulations can play a key role in producing monosex cultures. Surgical removal of the AG from juvenile *M. rosenbergii* at an early developmental stage resulted in complete sex reversal, leading to the development of functional females capable of mating and producing progeny (Sagi *et al.*, 1997). Crossing of sex reverted males (pseudo-females) with normal males, yields an all male progeny, as forecasted by the homogametic male theory (Katakura, 1989; Sagi and Cohen, 1990). A feasible sex reversal procedure was developed by Aflalo *et al.* (2006) using a two-phase microsurgical AG ablation. Neofemales capable of generating an all-male progeny were produced, and they served as broodstock for monosex culture.

The advantages of such a technique are the higher weights of the male prawns, the shorter time for maturation, the saving of resources that would have been necessary for growing females, and the simplification of reproduction in grow-out ponds. It is obvious that applied significance lies in the understanding of the AG and sexual differentiation processes. However, the surgical ablation of AG is a complex operation, producing high mortality and needing very specialized operations. Therefore, the chemical destruction of AGs using a mechanism parallel to the one observed in males of *Hippolyte inermis* could lead to great simplification, reduction of costs, and virtual absence of mortality. Unfortunately, the compounds contained in *Cocconeis* spp. diatoms are very specific and they act only on the testes of *Hippolyte inermis* (Zupo *et al.*, 2007a). In fact, when we tested crude extracts of the diatoms on the AGs of *Cherax quadricarinatus*, *in vitro*, we obtained no significant effects. However, we aim at elucidating the mechanism of action of these compounds, starting from the peculiar case *Hippolyte-Cocconeis*, in order to develop new biotechnologies effective on selected species of shrimps and prawns.

## Conclusions and future perspectives

We are presently testing diatom fractions obtained from crude extracts of *Cocconeis scutellum parva*, on post-larvae of *Hippolyte inermis*. At date, we demonstrated that the active compound, *i.e.*, the factor able to trigger the appearance of larger number of females, in culture, is contained in a non-polar fraction. Therefore, the active compound having such specific apoptogenic activity on the AGs of the shrimp, could be a fatty acid or a lipoprotein. When the chemical structure of the active compound will be fully elucidated, then we will test its effect *in vitro* using a micro-array technique. This will allow for determining its mechanism of action. Based on these data, we will attempt the development of active compounds effective on AGs of other species of decapod crustaceans.

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