

## Experimental evidence of a sex reversal process in the shrimp *Hippolyte inermis*

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### Summary

*Hippolyte inermis* Leach 1815 is a shrimp which forms stable populations in seagrass meadows of the Mediterranean Sea and along the Atlantic coast of Spain. Investigations from the last century have demonstrated specimens experiencing a male stage prior to switching to females (i.e., protandric sex reversal). Further studies have demonstrated that not all females are derived from sex reversal: young females apparently deriving from direct differentiation are present in natural populations. In recent years some authors have claimed that the species is simply gonochoristic, mainly based on the absence of ovotestis development. In order to establish if the species is a peculiar hermaphrodite or a simple gonochoristic, *H. inermis* postlarvae were individually cultured in Petri dishes in a semi-closed system. Their exuviae were regularly collected, fixed and stained, to monitor the sex and the size of each individual over time. In addition, histological sections were examined and we observed an individual who lost its *appendix masculina* and developed an active ovary. In contrast, specimens that retained their *appendices masculinae* exhibited a male reproductive system. Fourteen such individuals who lost their *appendices masculinae* were observed throughout the experiment, suggesting a mechanism of sex reversal in decapod crustaceans in which an ovotestis may be absent during the transition to the opposite sex.

**Key words:** Crustacea, Decapoda, *Hippolyte inermis*, sex reversal, histology, development, maturation

### Introduction

Male sexual differentiation is thought to be mediated in crustaceans by hormones secreted by the androgenic gland (AG) (Charniaux-Cotton, 1967). All previous investigations on decapod crustaceans have demonstrated that sexual differentiation depends exclusively

on the presence/absence of AG hormones (Sagi et al., 1997). The germinative zone in some species is largely determined as testis tissue due to the presence of the androgenic hormone. A reduction in the hemolymph levels of the hormone can prevent the male determination at given periods during the life of an

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individual, which may lead to intersex individuals and to sex reversal due to an auto-differentiation of the ovary (Sagi and Aflalo, 2005; Baeza, 2006). Therefore, the gametogenesis in hermaphrodite and gonochoristic crustaceans appears to be controlled by the same physiological mechanism, i.e., the presence or absence of the androgenic hormone (Weeks et al., 2006).

As a matter of fact, sex reversal is common among decapod crustaceans (Bauer, 2000). In the past, generalization over genera and families has led to some confusions and errors: in some cases hermaphrodite species were considered gonochoristic and vice-versa (Bauer and VanHoy, 1996; King and Moffit, 1984; Yaldwyn, 1966). For example, in the absence of direct observations, several Hippolytidae shrimps are still considered hermaphrodites due to their similarity to clear hermaphroditic species (d'Udekem d'Acoz, 2002).

Within the multivariate and complex sexual strategy adopted by decapod crustaceans (Yaldwyn, 1966), which includes gonochorism, sequential and simultaneous hermaphroditism and even parthenogenesis (Vogt et al., 2004), *Hippolyte inermis* Leach 1815 is a puzzling case. This shrimp, living in *Posidonia oceanica* and other seagrass meadows (d'Udekem d'Acoz, 1996), was first studied by Reverberi (1950), who discovered a peculiar mechanism of sex reversal. Reverberi (1950) demonstrated that *H. inermis* is sex-reversed only after the complete disruption of the male gonad, and the ovary is developed from embryonic undifferentiated cells. This suggested mechanism contradicts data from all other known decapods, in which an ovotestis is produced by the same germinal tissues as the testis and, subsequently, the regression of testes must occur to obtain a functional female (Bauer and Holt, 1998).

Yaldwin (1966) and Veillet et al. (1963) confirmed that the shrimp reversed its sex about 1 year after hatching. Zupo (1994) confirmed again the presence of sex reversed females, based on a population dynamic investigation, but for the first time demonstrated the presence of primary females or, at least, very small females developing simultaneously with the male spring cohort. These were named "beta" females, in contrast to the larger "alpha" females deriving from sex reversal at the age of 1 year. Diatoms (*Cocconeis* sp.) were found in the gut contents of specimens sampled in the period of the appearance of beta females (Zupo, 2001). The feeding of *Cocconeis neothumensis* by *H. inermis* post-larvae has been shown to trigger the production of large amounts of beta females, while individuals fed on control foods (without diatoms) produced mainly males (Zupo, 2000). Recently, Zupo and Messina (2007) also suggested that the diatoms of the genus *Cocconeis* induce apoptosis of AG in *H. inermis*, followed by the

complete disruption of the testis. This remarkable process leads to the production of beta females and supports the peculiar mechanism suggested by Reverberi (1950). However, Cobos et al. (2005) suggested that *H. inermis* is a simple gonochoristic species, based mainly on two observations: (a) the absence of any ovotestis in their histological sections and (b) not detecting reduction in the size of the *appendix masculina*, as observed in other sex-reversing decapods.

The present research was conducted in order to compare the conflicting conclusions of studies by Reverberi (1950) vs. Cobos et al. (2005) regarding the process of sex differentiation in *H. inermis*. In particular, starting from the well demonstrated absence of an ovotestis (all previous studies are in agreement about this point) we aim at understanding if the lack of an intersex stage means, as concluded by Cobos et al. (2005) that the shrimp is gonochoristic, or it means that the shrimp undergoes a peculiar process of sex reversal, as stated by Reverberi (1950). Direct observation of growing individuals in the laboratory was performed following morphological changes in their exuvia and reproductive system. In addition, we conducted histological investigations in order to provide support that the external sex characteristics observed (presence/absence of *appendix masculina*) were consistent with the internal reproductive organs.

## Material and Methods

All experimental specimens were derived from laboratory hatching of eggs laid by gravid females of *H. inermis*. Twenty-eight gravid females were collected in Lacco Ameno d'Ischia (Gulf of Naples, Italy) by a plankton trawl that scraped the surface of *Posidonia oceanica* leaves. After the collection, females were placed individually in 2 l aerated vessels containing 1.5 l of filtered and UV sterilized seawater, reared in a thermostatic chamber (18°C) with a 12:12 h photoperiod. Most females spawned 1–3 days after the collection and were then released into the field. Larvae produced by each female were divided into groups of 80 individuals. Each group was placed in a 1 l aerated vessel (containing 800 ml of filtered seawater) in the same thermostatic chamber. They were fed with *Brachionus plicatilis* (4 ind/ml) and *Artemia salina* nauplia (4 ind/ml) enriched for 12 h with a plant integrator (Algamac). Every 2 days larvae were collected using a 400 µm mesh filter and the culture media was renewed. After 25 days all larvae settled. Postlarvae deriving from different mothers were pooled, to exclude any maternal influence on sex, and divided into replicates of 20 individuals. Nine replicates were cultured in 500 ml dishes containing 400 ml filtered

seawater and a dry food (BDF), composed of 33% (by weight) of dry *Artemia salina* enriched with PUFA (Super-Hi Food Corp.), 33% of pure dried *Spirulina* and 33% of “AZ” shrimp food (Tetra Corp.). This basic food was pressed into small (5 mg DW) pellets. One pellet was administered every day to each dish. Every 2 days postlarvae were collected using a plastic pipette; the dish bottom was washed and water was replaced.

The food administered allowed for rapid growth of the larvae and postlarvae, with postlarval mortality as low as 13.9% on the 39th day after settlement. Most postlarvae reached sexual maturity after 39 days. In total, 155 postlarvae were individually transferred into small dishes (6 cm diameter) covered with a net (mesh size 0.2 mm) and clustered into an 80 l glass tank filled with filtered seawater. The tank was aerated with air-stones and an external filter (Eheim Classic 2215) filled with perlon wool and activated charcoal. The water flowing out from the filter was sterilized by UV (15 W) lamp prior to returning into the tank. Every 2 days the net covering each dish was removed, exuviae produced were collected and fixed in a solution of ethanol and rose Bengal (200 mg/100 ml 70% alcohol), and another small pellet of dry food was added to the bottom prior to restoring the dish in the tank. Fifty percent of the water in the tank was replaced every 2 days with clean seawater. The experiment lasted 279 days and it was concluded when only eight individuals were still alive. During this period the highest mortalities (20% and 40%) were observed during the 4th and 7th month of growth, respectively, in correspondence with two technical failures of the filtering system, producing temporary deterioration of the water quality.

The exuviae collected and stained were examined using optical microscopy. A picture of each exuvia was obtained by a Leica Z16-APO photomicroscope, equipped with a computerized system of image analysis permitting the measurement of total length (TL, from the tip of the rostrum to the posterior medial notch of the telson). The second pleopods on the exuviae were separated and photographed in order to record any change of secondary sex characters (*appendix masculina*) and to track biometry during the culture of individual shrimps.

Finally, some individuals were collected before or after the sex change for histological preparation and observation, to confirm the presence of primary sexual characters in shrimps whose sex was determined based on the presence/absence of the *appendix masculina*. For this purpose, shrimps were fixed in a modified Carnoy solution, dehydrated and embedded in paraffin. Serial sections (5  $\mu$ m each) were stained in haematoxylin and eosin for optical microscopy observations. These preparations, as explained above, were not aimed at the

detection of intersex individuals, since the absence of ovotestes was demonstrated in *H. inermis* by previous authors (Reverberi, 1950; Cobos et al., 2005). Plots were obtained to check the relationships between the size of *appendix masculina* and the total length of shrimps (King and Moffit, 1984).

## Results

Fourteen individuals exhibiting an *appendix masculina* at the start of the experiment changed their sex throughout the test period, transforming into females (Fig. 1). A good correlation ( $R^2 = 0.82$ ) was found between the number of these alpha (sex reverted) females and the average size of the shrimps at each time (Fig. 1). In contrast, the *appendix masculina* did not change its relative shape during shrimp growth and its length exhibited a linear correlation ( $R^2 = 0.47$ ) to shrimp total length (Fig. 2). The collection and staining of each exuvia produced by cultured shrimps (Fig. 3) allowed for easy monitoring of the total length and

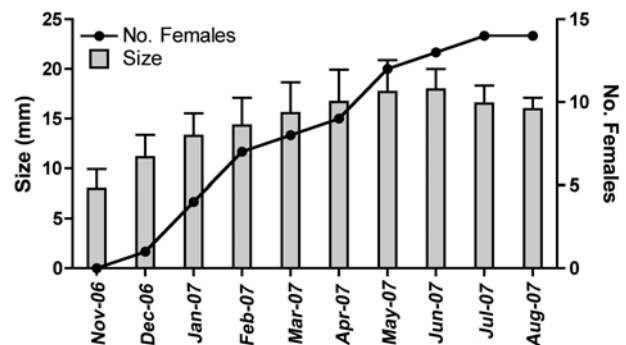


Fig. 1. Average size of the cultured *Hippolyte inermis* (vertical bars) and cumulative number of individuals that have reversed their sex from male to female (line).

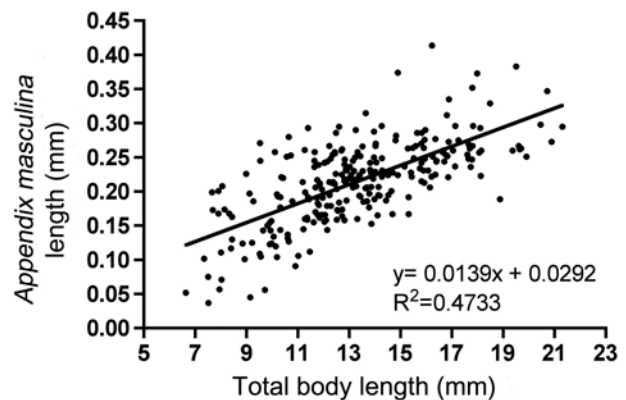


Fig. 2. Relationship of size of *appendix masculina* (mm) to total length of the shrimp (mm) in exuviae collected throughout the experiment.

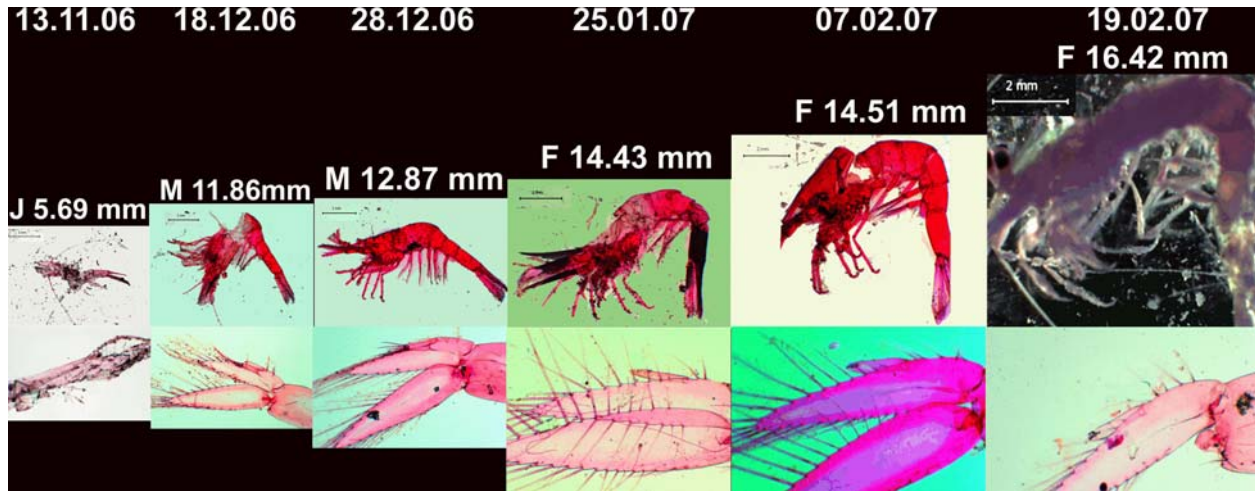


Fig. 3. Images of stained exuviae and pleopods from *H. inermis* during the development of a representative individual. Loss of *appendix masculina* was observed at the fourth recorded moult, third month of culture, at a size of 14.43 mm as presented in the enlarged pictures of the second pleopod (reported below). Exuvies were collected in the dates reported on top of the figure. The second row indicates sex and size (total length expressed in mm) J, juvenile; M, male; F, female.

presence of *appendix masculina*. This technique, devised ad hoc, was sufficient for sketching the growth of individual shrimps and detecting processes of sex reversal (see in Fig. 3a representative individual which reverted its sex at total length of 14.43 mm, for example). Histological investigations were performed on three males and one sex reverted female (sacrificed immediately after the observation of the loss of *appendix masculina*), confirming the correspondence of external morphology with the anatomical features. Individuals bearing an *appendix masculina* (classified as males) exhibited mature testis (Fig. 4A–D) and very large and convoluted *vasa deferentia* containing mature spermatozoa (Fig. 4C, D). Suspected androgenic gland tissue was detected close to the base of the fifth walking leg (Fig. 4F). In contrast, a representative sex reverted female (similar to the one shown in Fig. 3) exhibited a mature ovary containing follicles and oocytes (Fig. 5A, B), oviducts and a gonopore close to the base of the third walking leg (Fig. 5C–E).

### Discussion

The present study and laboratory culture demonstrated that 14 males lost their *appendix masculina* during the experimental period and developed the external sexual character typical of females. We have also demonstrated that males, externally identified by means of their *appendix masculina*, contained mature testes and that a female, identified after the loss of the *appendix masculina*, contained a mature ovary. These observations lead to the conclusion that the species is

capable of sex reversal from male to female (Veillet et al., 1963; Zupo, 2000).

However, the presence of beta females (apparently primary females) in the spring cohort was demonstrated by Zupo (2004). The case of these small females, which appear to be derived from direct differentiation (Wenner, 1972), remains to be explained in order to find out whether these females are also the result of an early sex reversal process (Zupo and Messina, 2007). If these are primary females, it may support the hypothesis that this species represents a case of partial protandry, as observed in other decapod crustaceans (Rudolph et al., 2007). However, it is known that other factors (e.g., stress and growth conditions; Rider et al., 2005) may influence the sex maturation of decapod crustaceans.

The sex reversal process in protandric decapods is supposed to be accompanied by a size reduction of the *appendix masculina* when individuals approach the transitional period (King and Moffit, 1984; Schatte and Saborowski, 2006). Contrary to this, the biometric data found in the present study indicated that the *appendix masculina* increases its size in correlation with the total length of individuals and seems to be lost in a single moult when the sex reversal is recorded. This is in contrast with the observations made on most protandric decapods, but is in agreement with the data presented by Reverberi (1950). He suggested that the process is very fast and could lead to unparallel development of primary and secondary characters. This process is supported by his report on cases, in natural populations, of apparent males (individuals bearing a normal *appendix masculina*) containing an ovary while the male appendix is still present. Additionally, a small percentage of

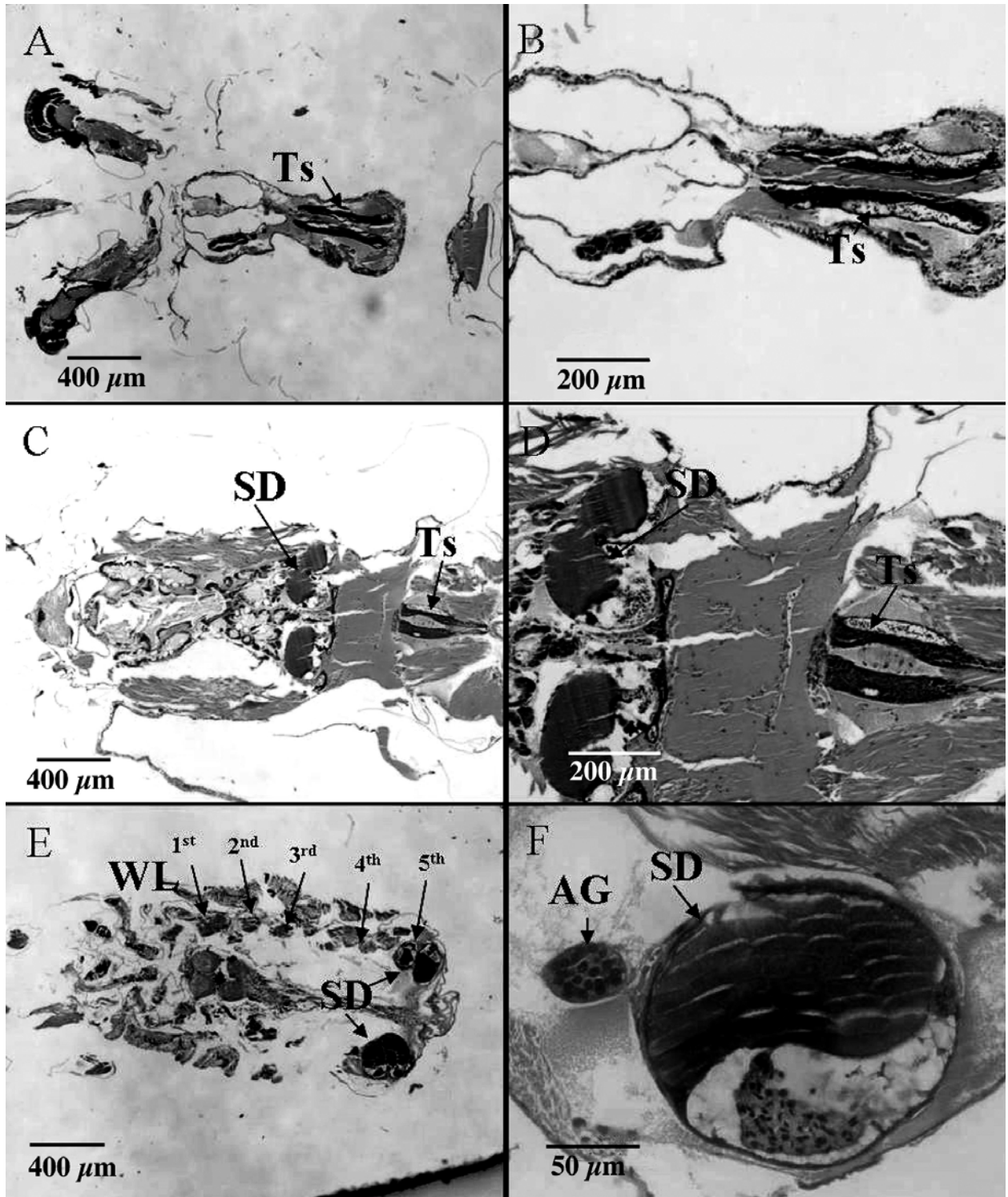


Fig. 4. Dorsoventral sections of a representative male *H. inermis* showing the major features of its reproductive system. A,B, Dorsal section view. C,D, Mid-body section view. E, Ventral section view. F, High magnification of the terminal sperm duct showing the suspected androgenic gland tissue. Ts, testis; SD, vas deferens; AG, androgenic gland; WL, walking leg bases (indicated from the 1st to the 5th).

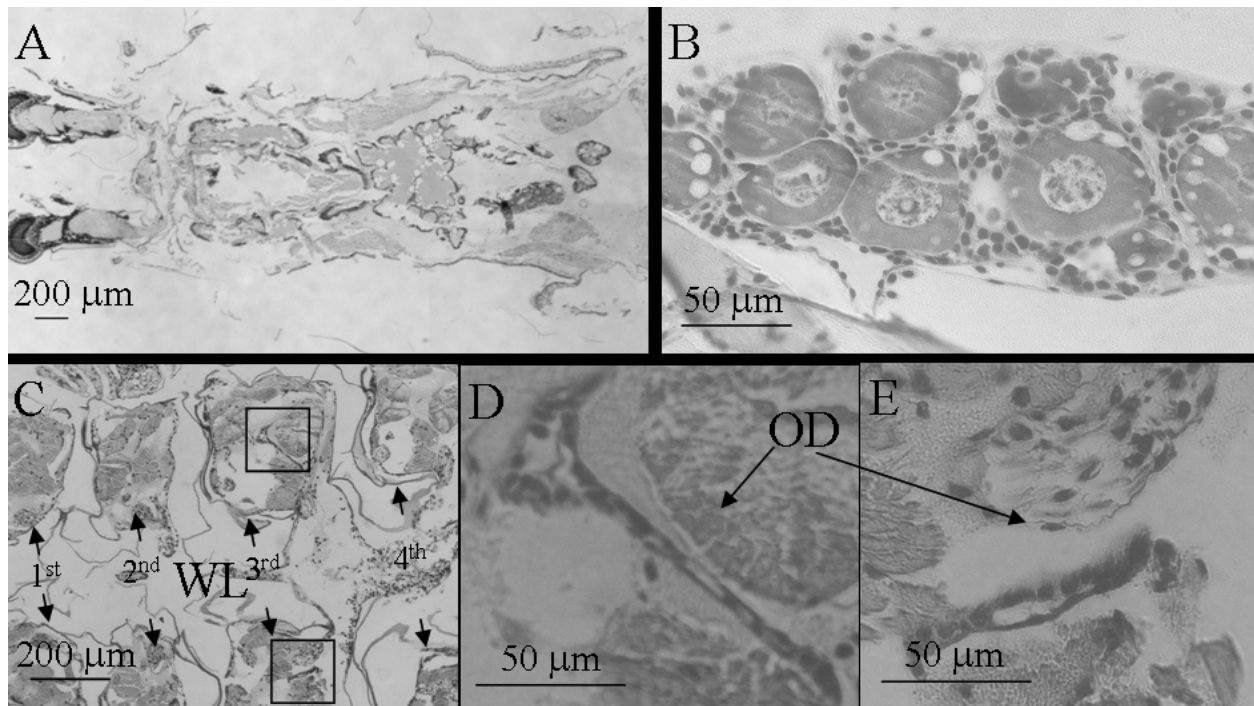


Fig. 5. Dorsoventral sections of a sex-reverted female *H. inermis* showing the major features of its reproductive system. A, Dorsal section view. B, Higher magnification showing the ovary. C, Ventral section view. D, E, Higher magnification of the 3rd walking legs showing the oviducts. Ov, ovary; OD, oviduct; WL, walking leg bases (indicated from the 1st to the 4th).

apparent females (individuals not bearing an *appendix masculina*) may be found, still containing a functional testis (Reverberi, 1950). Support to this notion comes from one such transitional male, found during the present research (Zupo, personal observation; data not shown).

The rapid sex change observed in these shrimps might also be explained according to the sex allocation theory (Charnov, 1982). The shrimp has only two narrow periods of reproduction every year, in April and September (Zupo, 1994), so it may be beneficial to the species when sex changes as quickly as possible, rather than progressing through several intermediate stages. This strategy could also explain the absence of an ovotestis, in terms of best allocation of resources (Speakman, 2005; Charnov et al., 2007). Moreover, the two periods of reproduction, in spring and fall, correspond to very different environmental conditions in terms of leaf density (exposure to predators), meadow spatial complexity (Zupo et al., 2006) and food availability. It is also known that sex ratios are differently controlled in a spatially variable environment (Charnov et al., 1981). Finally, we know that sex allocation may be size-dependent, with smaller hermaphrodites allocating more resources to male reproduction than larger ones (Baeza, 2007). The peculiar life strategy of *H. inermis* (double period of reproduction with different

sexual strategy applied by young individuals and a variable proportion of resources invested in large and small females, respectively) may be viewed as a useful approach to increase mating opportunities and adjust sex allocations seasonally in order to improve its fitness to a high predation pressure (Zupo, 1994).

The morphometric evidence found, based on direct laboratory observations suggests that *H. inermis* is a protandric species, contradicting the conclusions reached by a previous study on the histomorphology of the female gonads collected from a field population (Cobos et al., 2005). The latter authors concluded that this species is gonochoristic based on: (a) the absence of ovotestis in their samples; (b) the absence of reduction in size of the *appendix masculina*, as expected for other protandric decapods; (c) the presence of small females in the natural populations. As stated above, the absence of an ovotestis in this species was previously observed by Reverberi (1950), whose evidence indicated that the sex reversal process does indeed occur rapidly, during a single moult, and without an intermediate ovotestis phase. This critical stage should be studied more closely.

Bauer and VanHoy (1996) demonstrated that in some protandric species there is a reduction in the size of the *appendix masculina* as the individual approaches its sex reversal event. The absence of a reduction of the

*appendix masculina* prior to the sex reversal found by Cobos et al. (2005) is confirmed in the present study. In fact, in each of the 14 sex reverted individuals it could be seen that sex reversal occurred without any change in the size of *appendix masculina*.

The presence of smaller females, previously observed by Zupo (1994), could be the result of an early protandric sex reversal due to diatom food metabolites (Raniello et al., 2007). In fact, it was demonstrated (Zupo and Messina, 2007) that some diatoms induce quick destruction of the androgenic gland in *H. inermis*, which is the central controlling organ of male sex differentiation in crustaceans (Sagi and Khalaila, 2001; Sagi et al., 1997). The demonstrations of sex reversal reported above allow us to conclude that *H. inermis* is a protandric or, at least, partially protandric shrimp, in common with other decapod crustaceans (Rudolph et al., 2007).

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