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RESEARCH ARTICLE

How do dietary diatoms cause the sex reversal of the shrimp *Hippolyte inermis* Leach (Crustacea, Decapoda)

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Abstract *Hippolyte inermis* Leach 1915 is a protandric shrimp largely distributed in *Posidonia oceanica* meadows and other Mediterranean seagrasses. Previous studies demonstrated several physiological peculiarities, such as absence of female gonadic buds in adult males (the new female gonad is produced starting from few undifferentiated cells), the consequent absence of an ovotestis, 2 yearly periods of reproduction with different population structures (a spring outburst producing both males and primary females, and a fall reproduction producing mainly males), and a process of sex reversal influenced by the diatom food ingested. We performed several laboratory analyses to compare the effects of various species of benthic diatoms, in order to test the effect of different diatoms and provide information on the mechanism of action of the ingested compounds. In addition, we performed molecular tests (TUNEL) and TEM observations, to check the hypothesis that the effect of benthic diatoms may be mediated by a process of apoptosis acting on the male gonad. The results obtained allowed for a ranking of a series of benthic diatoms according to their effects on sex reversal, and a confirmation of the striking effect of *Cocconeis* sp. diatoms, which are able to trigger the appearance of primary females. We also demonstrated the presence of apoptosis both in the male gonad and in the androgenic glands of postlarvae. The effect is species specific, strictly localized to the male gonad and androgenic gland, and limited to a very short period of time, from the 5th to the 12th day of postlarval development.

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Introduction

The shrimp *Hippolyte inermis* Leach, 1815 lives in shallow waters of the Mediterranean Sea and along the Atlantic coast of Spain (Zariquiei Alvarez 1968). It forms stable populations in seagrass meadows (Gambi et al. 1992), mainly in *Posidonia oceanica* and *Cymodocea nodosa* (Guillen Nieto 1990). Most individuals exhibit a green mimic colour (Bedini et al. 1997). Investigations by Reverberi (1950) and Veillet et al. (1963) demonstrated individuals experiencing a male stage prior to switching to female (i.e. protandric sex reversal; Gherardi and Calloni 1993). We also know (Le Roux 1963; Regnault 1969) that juvenile diet shifts from zooplankton (larvae) to microalgae and microzoobenthos (settled postlarvae). Sex differentiation occurs at a size of 5–7 mm (Veillet et al. 1963); sex reversal was observed in individuals of 10–13 mm, corresponding to an age of 7–12 months (Zupo 1994). Not all individuals exhibit sex reversal. In fact, young females of 5–6 mm length are present in natural populations. They are smaller than any male and are produced by direct differentiation. Large females, originating from sexual inversion, were designated as *alpha* females, while small females, directly developed, were designated as *beta* females (Zupo 1994). Two main periods of recruitment, spring and fall, were detected in the life cycle of *H. inermis*. Individuals born in spring grow quickly and develop as either females or

males, while individuals born in fall grow slowly and develop as males, changing sex in the next spring. The spring period of maximum abundance of *beta* females in natural populations corresponds to a massive epiphytic production in the leaf stratum of *P. oceanica* (Mazzella and Buia 1989; Zupo 1994).

Hippolyte inermis is characterized by a singular feature (Reverberi 1950): female gonads are not produced starting from buds (as in other sex-reverting invertebrates; Charniaux-Cotton 1960) and an ovotestis is never observed (as it occurs in other decapods). They are built up from undifferentiated cells, and the male gonad cannot be influenced by hormones produced by an ovary during its development (Reverberi 1950; Katakura 1989). The absence of an ovotestis was recently observed also by Cobos et al. (2005). Based on this observation these authors concluded that the species should be gonocoric. Actually, the absence of an ovotestis (yet observed by Reverberi in 1950) is not sufficient to negate sex reversal, well demonstrated by previous investigations by Veillet et al. (1963) and Zupo (1994, 2000), since it is known (Reverberi 1950) that this shrimp is protandric, not hermaphroditic (i.e. shift of sex from male to female proceeds in the absence of an ovotestis). Moreover, our present histological investigations demonstrate that the disruption of testis and androgenic gland, quickly followed by the production of an ovary, is a very rapid process, lasting about 1 week and concluded within a single ecdysial cycle.

According to the previous observations, the effect of compounds contained in dietary diatoms (*Cocconeis* sp., according to Zupo 2000) should be directed towards the male gonad, destroying it during its development (Zupo 1994). We hypothesized (Zupo 2000) that the process of sex reversal may proceed through apoptosis (programmed cell death; Raff 1998) of the male gonad. In fact, due to the absence of gonadic buds producing hormonal substances and depressing the male gonad physiology (as it occurs in other protandric crustaceans; Charniaux-Cotton and Payen 1988) the ingested diatoms seemingly contain compounds that selectively destroy cell populations, inducing their suicide. The action of the apoptotic compounds should be species-specific and extremely selective for the male gonad. It should trigger the quick death of cells naturally programmed to die about 12 months after hatching. The target tissues should be those of the male gonad and, probably, the androgenic gland (AG).

In fact, the regulation of the male reproductive system of decapod crustaceans is controlled by the androgenic gland (AG; Sagi et al. 1997b; Sagi and Khalaila 2001). The initiation, completion and intensity of spermatogenic activity are regulated by circulating AG hormone (Charniaux-Cotton and Payen 1988). The AG hormone was purified in Isopoda (Ohira et al. 2003) but has not yet been isolated and characterized in decapod crustaceans. Spermatogenesis starts only when the AGs are fully developed in certain decapod species (Payen 1973; Taketomi et al. 1996). On the other hand, in the male prawn *Macrobrachium rosenbergii* (Nagamine et al. 1980) and in intersex individuals of the Australian red claw crayfish *Cherax quadricarinatus*, removal of the AG leads to cessation or regression of spermatogenesis (Khalaila et al. 1999) and to development of female primary and secondary sex characters (Sagi et al. 1997a, 2002). The expression of the vitellogenin gene was detected by RT-PCR in the hepatopancreas of the AG ablated intersex of various crustaceans (Sagi et al. 2002). There is an ongoing effort to find chemical agents responsible for the regulation of the above shifts from maleness to femaleness in order to control sexual plastic processes in commercially important crustaceans and to determine the sex of offspring groups.

The influence of diatoms on the reproductive ecology and life cycle of other crustaceans (Miralto et al. 1995; Ianora et al. 1995), mainly copepods (Poulet et al. 1994) has been demonstrated. The production of diatom compounds, detrimental to the development and survival of grazers, has major impacts on secondary production (Miralto et al. 1996, 1999). Other effects are hypothesized, however, in *H. inermis* (Adiyodi and Adiyodi 1970; Zupo 1994), according to co-evolutionary processes: it is largely adapted to the life in *P. oceanica* (d'Udekem d'Acoz 1996) and the toxic effect of diatoms is translated into a spring signal for the development of *beta* females, whose presence is a crucial factor for maintaining a constant sex ratio, (Zupo 1994; Buia et al. 2000).

Resuming the previous points, it has been shown that: (a) the ingestion of *Cocconeis* sp. diatoms induces the development of primary (*beta*) females (Zupo 1994); (b) the same mechanism of action occurs both in the laboratory and in the field (Zupo 2001); (c) the process takes place in postlarvae, presumably in the first phases of sex maturation (Zupo 2000); (d) it could hardly be mediated by female hormones (Reverberi 1950; Katakura 1989; Khalaila et al. 2002). Therefore, the objective of this study was to determine the mechanism by which benthic diatoms influence sex determination in *Hippolyte inermis*. We cultured various species of benthic diatoms and included them in the diets of *H. inermis* postlarvae. We studied how organs involved in sex determination are affected by different diets using an in situ cell death detection kit based on TdT-mediated dUTP Nick End Labelling technique

(TUNEL) provided by Roche Diagnostics GmbH. In addition, the presence of apoptosis in specific organs of the treated shrimps was checked by Transmission Electron Microscopy (TEM) and light microscopy observations on testes and AG thin sections.

Material and methods

Diatom collection and culture

Diatom collections were performed by displacing metal panels, covered with a silicon polymer, close to a *Posidonia* meadow, at 1.5 m depth. The low surface tension of silicon coatings facilitates the selection of diatoms of the genus *Cocconeis*, which are characterized by higher adhesive power. The special coating allowed for sampling sufficient amounts of intact microalgae for the next phases. The surface of the panels was sampled weekly in areas of 2 cm^2, by means of a cover slide gently scraped on the wet surface. Each sample was quickly transported to the laboratory, dispersed in 5 ml clean seawater and analysed under an inverse optical microscope. Diatoms of the genus *Cocconeis* were collected by micropipettes, with the aid of a Leica micromanipulator, and individually transferred in sterilized Petri dishes, containing Guillard's "f2" with silicates (Sigma-Aldrich Biochemicals) as a culture medium. After several transfers and selections were conducted, using a micromanipulator at 5-day intervals, monoclonal cultures of diatoms were obtained. Samples of these cultures were collected, filtered, fixed on microscope stubs and gold-sputtered for Scanning Electron Microscope (SEM) examination and species identification (De Stefano et al. 2000). These techniques allowed for isolating live strains of the main species of *Cocconeis* living in *Posidonia* meadows, i.e. *C. scutellum scutellum*, *C. scutellum parva*, *C. neothumensis*, *C. dirupta*. In addition, monoclonal cultures of *Navicula* sp., *Amphora* sp. and *Diploneis* sp. were obtained and used as a control of the bioactivity, by means of bioassays on living shrimps. The obtained monoclonal cultures were maintained in thermostatic chambers at 18°C, under constant irradiance (140 μE), with a 12/12 h photoperiod, and transferred at 15-day intervals.

Rearing of larvae and bioassays on *H. inermis* postlarvae

Ovigerous females of *H. inermis* were collected in the field (*Posidonia oceanica* meadow in Lacco Ameno d'Ischia) using the technique described by Zupo (2000) and reared in the laboratory, individually, in aerated 2 l bowls, until larvae were released. Larvae were collected on 60 μm nets and subjected to the standard procedure (Zupo 2000) for production of postlarvae, in approximately 20 days. The technique consists of the culture of larvae in 1 l vessels (containing 800 ml of filtered and UV sterilized seawater) at a density of 1 larva per 10 ml of culture solution, containing three nauplii of *Artemia salina* (450 μm length) and two *Brachionus plicatilis* per ml. The culture vessels, aerated by the use of an air pump, were maintained in a thermostatic chamber at a constant temperature of 18°C, with a 12/12 photoperiod. The culture media was renewed at 2-day intervals, after filtering of larvae on a 60 μm net and counting survivors.

Postlarvae were transferred to 12 cm Petri dishes (filled with 400 ml of filtered seawater) in stocks of 25 individuals. Several (from 2 to 9) replicate Petri dishes (25 ind. each) were used for each treatment, according to the availability of fresh diatoms for each diet (see Table 1). The postlarvae of each treatment were fed on fresh diatoms grown on 12 cm Petri dishes. Postlarvae produced in different vessels and from different females were pooled prior to the start of the bioassays, to randomize any difference due to maternal influences. Prior to starting the bioassay experiments on shrimps, diatoms of each selected species were transferred into individual Petri dishes of 12 cm containing 70 ml of culture media (Guillard's f2). An almost continuous layer of diatoms covered the bottom of the dishes after 15 days. At this time, the culture media was drained, and each Petri was filled with 400 ml of filtered seawater, in order to host postlarvae, which were allowed to feed on the algae adherent to the bottom. A composed dry food (Tetra AZ 300 artificial plankton for shrimps) was used as a diet integrator, since it is known that these shrimps need both animal and plant feeding items (Zupo 2001). Moreover, green macroalgae (*Enteromorpha* sp., cultured in the laboratory) were tested as an additional control. Eight

Table 1 List of the diets considered for the bioassays and respective number of replicates performed

Diet	Content	Nr of replicates
F	Dry food	4
M	Dry food and *Enteromorpha* sp.	7
CSP	*Cocconeis scutellum parva* and dry food	9
CSS	*Cocconeis scutellum scutellum* and dry food	9
CN	*Cocconeis neothumensis* and dry food	2
N	*Navicula* sp. and dry food	9
A	*Amphora* sp. and dry food	6
D	*Diploneis sp.* and dry food	9

different diets were tested, to evaluate the effect of six species of diatoms, compared to the effect of negative controls (macroalgae and dry food) as reported in Table 1.

One individual was sampled from each replicate of each treatment and frozen (−80°C) at 4-day intervals (alternated among the replicates of each treatment, to obtain 2-day lagged data). These individuals were then subjected to TUNEL and TEM analyses. The growth rates of larvae in each treatment were followed by measuring a subset of 20 individuals and by observing the sex maturation (shape of the first two pleopods) in narcotized postlarvae (30 s immersion in a 80 ppm solution of chloroform in seawater, obtained by solving 16 µl chloroform in 200 ml of filtered seawater).

Analysis of sex

Mature postlarvae (in this species, the appearance of secondary sexual characters corresponds to sexual maturity: Zupo 2000) were frozen after 25 days and examined under a dissecting microscope, to record their total length and to collect the second pleopod. The examination of the pleopod II under an optical microscope allowed us for the determination of their sex (presence or absence of the appendix *masculina*). When complete data sets were obtained, statistical analyses (ANOVA on biometric data and Z-test on proportions of males and females) were performed, to evaluate the significance of differences between tests and controls, assess the efficacy of each diatom species and rank their biological activity.

Detection of apoptosis

At the end of the experiment, individuals frozen at various developmental stages (previously collected at 4-day intervals, as above mentioned) were subjected to the TUNEL analysis (Roche Molecular Biochemicals Kit), for the rapid detection of apoptosis, according to the standard procedure (Romano et al. 2003). This molecular technique identifies DNA strand breaks by labelling 3′-OH termini with modified (fluorescein labelled) nucleotides in an enzymatic reaction. Therefore, the technique was here applied to establish whether the abundance of females in the treatments subjected to diatom foods was due to an early apoptosis (programmed cell death) of the testis tissues.

Each individual to be tested was fixed in 4% paraformaldehyde and its abdomen was ablated, to permit a better penetration of the reagents in the carapace (where the gonads and the AGs are located). After 1 day, each individual was rinsed in phosphate buffered saline (PBS) 1x solution, to wash out the fixative, then transferred in citrate buffer (pH 6) and in chitinase, to digest the carapace chitin; finally it was rinsed again in PBS and immersed in Triton-X 100 overnight, to permeabilize its tissues and allow for a better penetration of dyes (see Romano et al. 2003 for a detailed description of the technique).

The third day, all individuals were rinsed twice in PBS and incubated in the TUNEL reagents, to label the DNA fragments. One positive control was previously treated with DNAse (to check the effectiveness of TUNEL treatment), while a negative control was immersed in the TUNEL label solution, without immersion in the terminal deoxynucleotidyl transferase enzyme (TdT), catalysing the polymerization of nucleotides at the 3′-OH end of the DNA fragments (to check for the absence of fluorescence). After 90 min of incubation at 37°C and three rinses in PBS, to wash out the fluorescent reagent, all samples were ready for observations. The treated individuals were examined under a UV complanar microscope (Leica Z16 APO) or under a laser confocal microscope (Zeiss 410 He/Ne laser 543 nm), to obtain 3-D images and record any fluorescence due to apoptosis. The results of molecular analyses were confirmed by observations at the TEM of thin slices of the same individuals, after post-fixation with 1% osmium tetroxide and embedding in Epon 812 resin.

In addition, histological sections (5 µm) of several male and female shrimps were obtained (after fixing in Carnoy and embedding in paraffin) and stained according to the ematoxilin-eosin technique, to map the shrimp morphology and check the position and shape of the fluorescent structures detected by TUNEL analyses.

Results

In total, 37 adult females of *Hippolyte inermis* were sampled and each produced a mean of 75 larvae. Pooling the productions of all females, 2,784 larvae were obtained and subjected to growing experiments. During larval growth (lasting meanly 24 days; Fig. 1) 20.54% mortality was recorded, while during the postlarval growth (lasting meanly 25 days), 15.11% (±10.28) mortality was observed. The mean size at the reaching of postlarval stage was 3.4 ± 0.4 mm (Fig. 1). All individuals were subjected, during the larval phase, to the same treatment and cultured contemporaneously in the same thermostatic chamber. In contrast, different growth rates were recorded among treatments

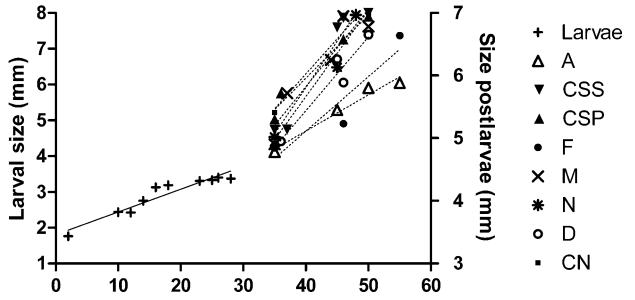

Fig. 1 Larval (first 25 days after hatching, left plot) and postlarval (next 20 days, right plots) growth rates expressed as size of larvae (left axis) and postlarvae (right axis) in mm. A single curve expresses larval growth, because all larvae were cultured in identical conditions. Several *dotted lines* express postlarval growth, according to individual treatments. The names of treatments refer to the acronyms reported in Table 1

performed on postlarvae (Fig. 1; $R^2 > 0.9$), with *Cocconeis scutellum scutellum* and *Navicula* sp. exhibiting the highest slopes of the growth curves. Most treatments exhibited a sex maturation period of 25 days from the production of postlarvae (24 days after hatching) to the appearance of secondary sexual characters (49 days after hatching), but the treatments with *Amphora* sp. and dry food lasted 30 days.

The postlarvae sacrificed at the end of each treatment had a total mean length of 6.72 mm (±0.37), but differences were observed among diets (Fig. 2). *Amphora* sp. exhibited the smallest size (5.88 mm), while *Navicula* sp, *Cocconeis scutellum scutellum* and *Cocconeis neothumensis* exhibited the largest sizes (7.01, 7.00 and 6.97 mm, respectively). Differences among slopes of growth curves were significant (ANOVA, $P < 0.01$) only for *Amphora* sp. and dry food. The average percentage of sexually undetermined (and sexually immature) postlarvae at the end of the experiments was 37.6% (±25.4), but differences were observed among replicates (Fig. 2). Moreover, the treatments "Dry food", *Cocconeis neothumensis* and *Cocconeis scutellum scutellum* exhibited the lowest percentage of sexually undetermined postlarvae (5.75, 10.00 and 24.97%, respectively), while the treatments *Amphora* sp., *Diploneis* sp. and *Cocconeis scutellum parva* displayed the highest percentages of sexually undetermined postlarvae (63.74, 45.56 and 37.69%, respectively). Also the mortality during postlarval growth was low, on average, but differences were observed among treatments (Fig. 2), with *Cocconeis neothumensis* (35.75%), *Amphora* sp. (27.60%) and macroalgae (14.88%), respectively, producing the highest mortalities.

The female/matures ratio, indicating the efficacy of each species of diatoms for triggering the development of *beta* females, exhibited large variations among the replicates as well (Fig. 3). Significant differences, however, were observed between several treatments. Considering dry food (F) and macroalgae (M) as the negative controls, *Navicula* sp. (N) and *Amphora* sp. (A) shared similar percentages of females on the total of mature individuals, which indicate absence of activity on the sex reversal process. In contrast, other species of diatoms exhibited a significant effect, triggering the production of larger percentages of *beta* females (Fig. 3). In particular, *Diploneis* sp., *Cocconeis neothumensis* and *C. scutellum parva* exhibited the highest

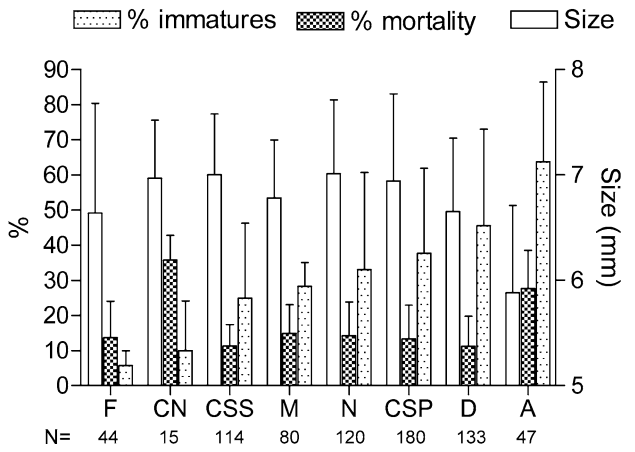

Fig. 2 Percentage of immatures (sexually undetermined, as explained in the text), mortality and size (mm) reached by shrimps at the end of the experiment, according to each treatment (the names on the x-axis refer to the acronyms reported in Table 1). The standard deviations among replicates are reported for each diet. The size of each treatment group (N = total number of individuals at the end of the experiment) is reported on the bottom

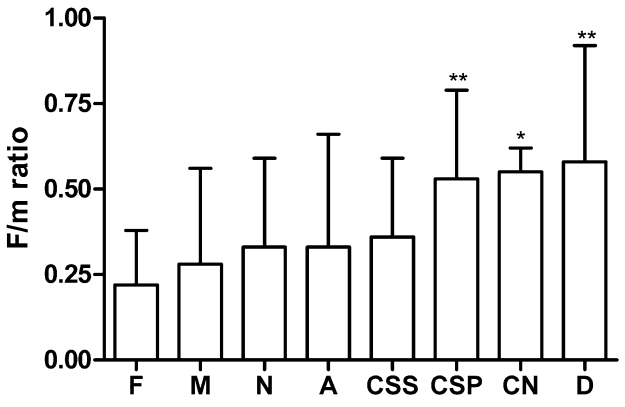

Fig. 3 Females/mature ratios obtained for each diet, ranked according to effectiveness. The names of the treatments are reported on the x-axis according to the acronyms contained in Table 1. The standard deviations among replicates of the same treatment are reported. *Asterisks* on the bars indicate the significance of differences against the controls (*$P < 0.05$; **$P < 0.01$) determined by means of Z-test. The sizes of treatment groups (N) are reported in Fig. 2

female/mature ratios (0.62, 0.55 and 0.53, respectively). Z-tests performed to compare the female/mature ratios obtained in controls and tests demonstrated significant differences (rejection of the null hypothesis, absence of differences, at $P < 0.05$) for *C. neothumensis* and highly significant differences ($P < 0.01$) for *C. scutellum parva*, and *Diploneis* sp.

The TUNEL technique, applied to test and control shrimps sampled during the culture of postlarvae, indicated presence of apoptosis in various organs of shrimps feeding on *C. neothumensis*, *C. scutellum parva* and *Diploneis* sp. (Fig. 4). In particular, evidence of apoptosis was detected in the AG glands (Fig. 4b); moreover, in some individuals, testes under apoptosis were reduced to very small masses (Fig. 4c) in respect to the control shrimps (Fig. 4a, d). Clear signals of apoptosis in the *vasa deferentia* and in the AG's of various individuals were detected by TUNEL under a UV complanar microscope (Fig. 5c). The acinous shape and the position of the AG gland yielded by the fluorescent dye was in accordance with the morphology of the gland observed in histological sections (Fig. 5d). The results of TUNEL tests (Fig. 5) were confirmed by TEM analyses (Fig. 6): thin sections showed DNA fragmentation and apoptosomes (dark vesicles containing nuclear materials and organelles) both in the male gonad (Fig. 6b) and in the androgenic glands. After pooling the results of all experimental sets, apoptosis was detected in male gonads within the treatments with *C. scutellum scutellum*, *C. scutellum parva*, *Diploneis* sp. and *C. neothumensis*, from the 5th to the 12th day of experiment (Table 2). In addition, we observed apoptosis of the AG in *Diploneis* sp. treatments, at the 7th day of experiment. Individuals sampled prior the 5th day and after the 12th day of experiment did not show any TUNEL fluorescence. Controls, as well, did not exhibit any TUNEL positive structure, from the 2nd to the 24th day of experiment.

Discussion

The number of larvae produced by each female and the growth rates obtained are in agreement with previous studies on *Hippolyte inermis* (Regnault 1969; Zupo 2000). The mortality rates were low enough to suggest absence of stress in the cultured shrimps. This point, in fact, may be critical for avoiding biases in the sex ratio due to stress (Austin and Meewan 1999), because recent studies indicated that stress may be one of the

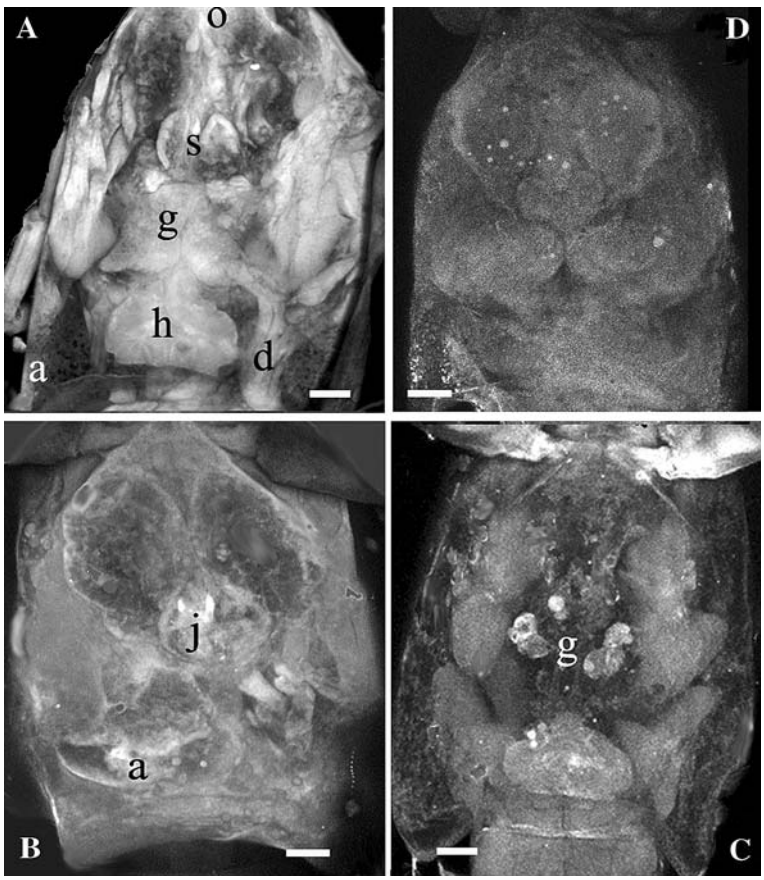

Fig. 4 Confocal microscopy analyses (bars: 150 μm). **a** Positive control individual stained with 1.1-dicoctadecyl-3,3,3′,3-tetrametyl-indocarbocyanine die, to show the shape, the position and the normal size of the main organs; **b** *Cocconeis scutellum parva* treatment at the 5th day of experiment. Male individual stained by the TUNEL technique; **c** *Diploneis* sp. treatment at the 7th day of experiment. Male individual stained by the TUNEL technique. **d** Negative control male individual fed on diet "M" stained by the TUNEL technique to show the absence of fluorescence. *o* optical chain, *s* gut, *g* gonad, *h* Heart, *d* vas deferens, *a* Androgenic Gland, *j* gastrolites

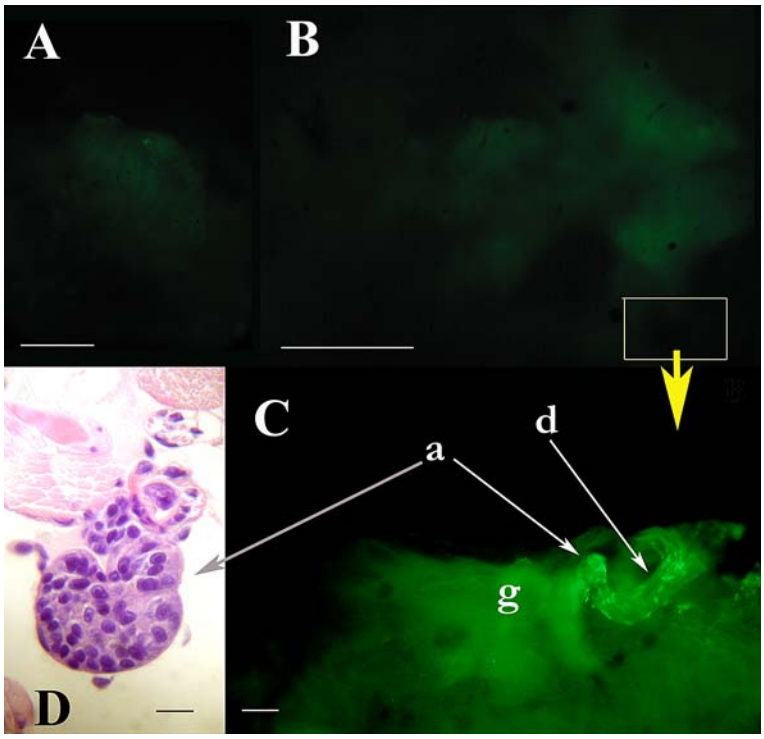

Fig. 5 **a** TUNEL detection of apoptosis in a 6-day-old male subjected to control treatment (diet "M") observed under a UV complanar microscope (14×; bar: 500 μm) to show absence of apoptosis (i.e. absence of fluorescence). **b** 6-day old male under *Cocconeis neothumensis* treatment observed under a UV complanar microscope (25×; bar: 500 μm) to show absence of fluorescence (pre-treatment control). The *rectangle* on the bottom roughly corresponds to the area shown in (**c**), after the TUNEL treatment. **c** TUNEL detection of apoptosis in a 6-day-old male (the same shown in (**b**), prior to be treated) under *Cocconeis neothumensis* treatment, observed under a UV complanar microscope (230×; bar: 100 μm). The *green* fluorescence of tissues indicates the presence of apoptosis. **d** AG of a young male in a section stained by hematoxilin-eosin, observed under an optical microscope (400×; bar: 25 μm). *a* Androgenic Gland, *d* deferens vasa, *g* testis

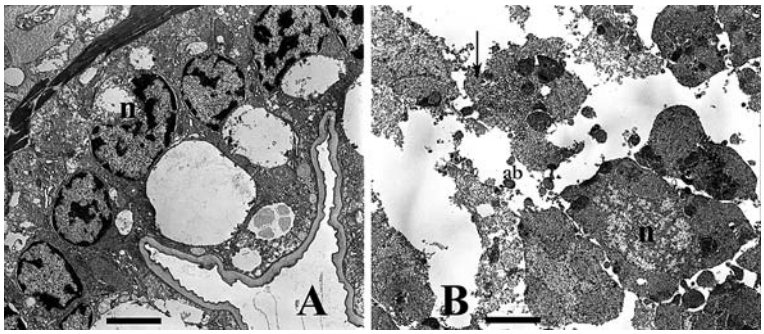

Fig. 6 **a** TEM image (2800×; bar: 5 μm) obtained on a transverse thin section of a testicular lobule from an individual of *H. inermis* subjected to a control diet, to show the absence of apoptotic processes. **b** TEM image (2800×; bar: 5 μm) obtained on a transverse thin section of a testicular lobule from an individual of *H. inermis* subjected to *Cocconeis neothumensis* diet, to confirm apoptotic processes and DNA fragmentation (*arrow*). *Ab* apoptosomes, n nucleus

factors triggering the shift to female sex in protandric decapods (Bauer 2000; Calado et al. 2005).

The size reached by most shrimps at the end of feeding experiments was enough to guarantee a large percentage of mature individuals (Zupo 1994). Larger abundances of immatures, exhibited by the treatments with *Amphora* sp. and other diatoms, may be due to feeding deficiencies: *H. inermis* needs both animal and plant prey during the postlarval growth (Zupo 2001) and experimental diets may lack some feeding items (e.g. vitamins, fatty acids or essential amminoacids) indispensable to promote growth and maturation. However, the female/mature ratios were low, as expected (Zupo 2000), even in control treatments, indicating that sex ratios were not altered by any stress due to feeding deficiencies. Similarly, the slopes of growth

Table 2 TUNEL positive structures detected on various days of treatment

Days	Treatments							
	F	M	N	A	CSS	CSP	CN	D
5						mg		
7					mg			AG
8						mg	mg	
10								mg
12								mg

Days refer to postlarval growth (from 25 days after hatching, i.e. day 1, to 45 days after hatching, i.e. day 20). In most treatment groups, all days of culture were covered by collections, by sampling alternatively in different replicates, but only the days in which positive structure were detected are reported in the table. Empty cells indicate absence of fluorescence in any body region. "mg" indicates apoptosis of the male gonad; "AG" indicates apoptosis of the Androgenic Gland. The first row indicates the treatments, according to the acronyms reported in Table 1

curves exhibited by most treatments were scarcely influenced by the composition of individual diets. In fact, differences among slopes were significant only for *Amphora* sp. and dry food (needing 30 days to reach the postlarval stage), as a confirmation of the lower efficiency of these diets. However, the growth efficiency descriptors (e.g. size, % immatures and % mortality at the end of the experiment) were not correlated in any way to the sex ratios. This evidence indicates that the factors influencing the growth rates are independent from the factors triggering the appearance of primary females in *H. inermis* populations (Calado et al. 2005).

It is worth observing that *Cocconeis neothumensis*, which previously demonstrated to effectively promote significant modifications of the sex ratio in *H. inermis* (large production of *beta* females; Zupo 2000), was one of the most effective diatoms, among the tested species. This study, in addition, demonstrated that *C. neothumensis* is not the unique diatom exhibiting an influence on the sex reversal of this shrimp (Zupo 2000), because other congeneric diatoms (e.g. *C. scutellum parva*) promoted a similar effect, as well as different diatom genera (e.g. *Diploneis* sp.). Not all the considered diatoms triggered the production of primary females, because within the same species, the two subspecies *C. scutellum scutellum* and *C. scutellum parva* exhibited contrasting effects. Finally, such genera of benthic diatoms as *Amphora* sp. demonstrated negligible effects, as well as *Navicula* sp. and dry food (controls). We may conclude, therefore, that the factor produced by benthic diatoms, triggering the appearance of primary females in *H. inermis*, is unevenly distributed among various species and absent in several species of marine microalgae.

Cocconeis sp. are particularly abundant in the field (and in gut contents of *H. inermis*; Zupo 2001) in spring. This explains why the April offspring contains both males and small (primary) females (Zupo 1994). In contrast, these diatoms are scarcely abundant in fall (both in the field and in *H. inermis* gut contents; Zupo 2001). This explains why the fall offspring contains mainly males, which change their sex in the course of the next year (Zupo 1994).

The results of molecular and ultrastructural analyses demonstrated that the influence of some diatoms on the sex reversal of shrimps is due to apoptosis (Vaux and Korsmeyer 1999), as hypothesized in previous studies (Zupo 2000). In fact, *H. inermis* lacks gonadic buds (Reverberi 1950) and the female gonad is produced starting from undifferentiated cells, only after the complete disruption of the testis. Hormonal influences of the ovary on the testis (e.g. an AG suppressor; Ginsburger-Vogel and Charniaux-Cotton 1982; Gherardi and Calloni 1993; Martin et al. 1999) may be excluded since an ovotestis was never observed (Reverberi 1950; Cobos et al. 2005). Therefore, the development of an ovary starts only after the suicide of the testis tissues.

The discovery of the apoptotic disruption of the male gonad, selectively triggered by benthic diatoms, is important because it opens new biotechnological frontiers in the application of this fundamental mechanism of cell death (Hannun 1997; Jimeno 2002). Most apoptotic compounds applied to human medicine (Kaufmann and Earnshaw 2000; Frankfurt and Krishan 2001) as well as physical influences inducing cell death (e.g. X-rays) are "generalist effectors": they act on a large variety of cells, in any physiological state. In contrast, the factor present in the considered benthic diatoms is effective only on the male gonad (and the androgenic gland) and exclusively in a short period of their life, i.e. from the 5th to the 12th day of growth.

Noteworthy, various marine natural products induce growth arrest and apoptosis of human neoplastic cells in vitro and in vivo (Schwartsmann et al. 2001; Jimeno 2002; Dirsch et al. 2004) and demonstrated strong activity against cancer and lower toxicity if compared with traditional chemotherapeutic agents. Some examples are aplidin, a marine compound isolated from *Aplidium albicans*, and ecteinascidin-743 derived from *Ecteinascidia turbinata*. (Broggini et al. 2003; Erba et al. 2001; Schwartsmann et al. 2003). Therefore, further studies aimed at elucidating the structure and the mechanism of action of the factors present in benthic diatoms, whose effectiveness was demonstrated by this study, could be crucial to clarify the cellular mecha-

nisms triggering specificity of the apoptotic effect, when directed against selected cell populations (O'Gorman and Cotter. 2001).

Another important finding of this study is the detection of apoptosis in the androgenic glands of shrimps fed on *Diploneis* sp. This finding could help to understand the mechanism of action of the diatom compounds (Nagamine et al. 1980; Martin et al. 1999; Hengartner 2000). The disruption of the AG seemingly precedes the apoptosis of testes. It was not observed in all tested shrimps due to the size of these fundamental glands (few cells), the consequent rapidity of the process (few days) and the intervals (2 days) chosen for sampling the shrimps analysed by the TUNEL technique. The testes, in contrast, are characterized by larger size, may need several days to be destroyed, and are frequently positive to TUNEL in the treatments CSS, CSP, CS and D. Suicide of testis tissues was detected in various phases, starting from the rear portion, close to the heart, and proceeding towards the anterior part, close to the gut (see Payen 1973 for a description of the morphogenesis of these structures in a decapod crustacean). These observations may be explained considering the fact that in crustaceans the androgenic gland is the sole source of hormones responsible for sex differentiation (Payen 1983; Abdu et al. 2002), i.e. the commitment of an embryo to either the female or the male pathway (Charniaux-Cotton 1954; Adiyodi and Adiyodi 1970). In male crustaceans, unlike male vertebrates, the endocrine and gametogenic functions are clearly separated into two distinct organs, the AG and the testis, respectively (Ginsburger-Vogel and Charniaux-Cotton 1982; Charniaux-Cotton and Payen 1988). In fact, sex differentiation can be manipulated through the removal of the AG, without damaging the gonads. In *M. rosenbergii*, for example, AG removal from immature males resulted in sex reversal, with complete female differentiation. Similarly, AG implantations into immature females lead to the development of a male reproductive system (Sagi 1988). Sex-reversed *M. rosenbergii* were capable of mating with natural males producing offspring. Therefore, the early disruption of AG in *H. inermis* feeding on *Cocconeis* sp., followed by the apoptosis of testes in the next 5 days, should be considered as the starting phase of *H. inermis* sex reversal (Zupo 2000).

Further studies will clarify the chemical structure of the apoptotic factor whose action was detected in this study, and they will allow for biotechnological applications of the compound (Bongiorni and Pietra 1996) and for clarification of the factors influencing the specificity of apoptosis in animal cells (Evan and Littlewood 1998). This study, however, demonstrated that the peculiar reproductive strategy of *Hippolyte inermis* observed in the field (Zupo 1994, 2001) is due to apoptotic compounds, present in variable concentrations in several benthic diatoms and all abundant during the spring reproductive period of *H. inermis* (Mazzella and Buia 1989). They are able to trigger the suicide of cells in the AG and in the male gonad of these shrimps in an early phase of the postlarval growth, so permitting the development of a primary female.

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