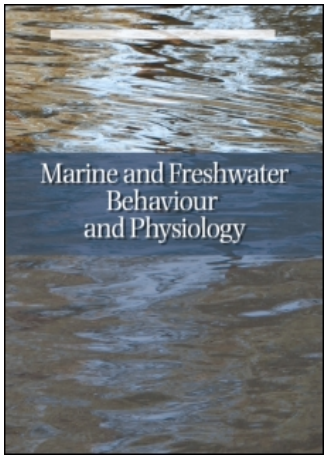

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Do benthic and planktonic diatoms produce equivalent effects in crustaceans?

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Abstract

Hippolyte inermis Leach 1814 is a benthic shrimp characterized by a peculiar mechanism of sex reversal influenced by diatom foods. In fact, the appearance of primary females in spring is due to an apoptotic early disruption of the androgenic gland and of the male gonad, triggered by still unknown compounds present in diatoms of the genus *Cocconeis*. The influence of diatoms on the reproductive ecology and life cycle of planktonic crustaceans has been demonstrated previously: some planktonic diatoms produce aldehydes inducing apoptosis in the embryos and in the larvae of marine copepods, reducing their viability. Both benthic and planktonic diatoms therefore produce compounds having an apoptotic effect on some tissues of target crustaceans, although the ecological significance of the two processes is different: deleterious for copepod populations, regulative for shrimps associated with *Posidonia oceanica*. In the present article we experimentally administered specific planktonic diatoms, their fractions and compounds known to induce apoptosis in planktonic copepods, to *H. inermis* postlarvae, to check whether the apoptotic effect is due to an identical family of diatom compounds, and to establish whether the processes observed in the plankton and in the benthos, respectively, are analogous or homologous, from an ecological point of view. Our results indicated that diatom compounds acting in the two systems are different, since both planktonic diatoms and their aldehydes had negligible effects on the sex ratios of cultured shrimps.

Keywords: Hippolyte inermis, *shrimp, sex reversal, diatom, apoptosis*

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Introduction

Hippolyte inermis Leach is a benthic shrimp (Zariquiey Alvarez 1968; d'Udekem d'Acoz 1996) well-known for its mimicry (Bedini et al. 1997) and for its peculiar strategy of sex reversal. The species is characterized by two yearly periods of reproduction, in spring and fall (Buia et al. 2000). Individuals born in fall develop as males and, after about 1 year, they shift their sex to female (Veillet et al. 1963), as observed in several other decapods (Yaldwin 1966; Adyodi and Adyodi 1970; Bauer 2000). Individuals born in spring, in contrast, develop both as males and females (Zupo 1994). They grow fast and mate the following September, to prime the fall reproductive burst. The different fate of populations born in spring and fall is apparently due to the diatom food available in the field (Mazzella and Buia 1989). Individuals born in spring were observed to ingest high biomasses of diatoms of the genus *Cocconeis*, while these microalgae are almost absent in the diet of the individuals born in fall (Zupo 2001). Laboratory experiments confirmed this field-based evidence (Zupo 2000). It was demonstrated that individuals born of the same females develop mainly as males or females according to the absence or presence of *Cocconeis neothumensis* in their diet, respectively (De Stefano et al. 2000).

Reverberi (1950) observed a peculiar mechanism of sex reversal in this species. He demonstrated that the female gonad is produced from undifferentiated tissues only after the complete disruption of the testes. The absence of an ovotestis was recently confirmed also by Cobos et al. (2005). The sex change mechanism is therefore different from that observed in other decapod crustaceans (Charniaux-Cotton 1960; Charniaux-Cotton and Payen 1988; Calado et al. 2005), in which an ovotestis is normally observed and the development of an ovary follows the classical steps of a hermaphroditic sex change (both simultaneous or not; Bauer 2000). For this reason Reverberi (1950) stated that *H. inermis* reverts its sex, but it cannot be considered hermaphroditic, because there is never contemporaneous presence of gonads or even of gonadic buds (Ginsburger-Vogel and Charniaux-Cotton 1982). This led to confusion in recent times because Cobos et al. (2005) discounted the possibility of observing sex reversal in the absence of an ovotestis, and the question is still under debate. However, Zupo and Messina (2007) demonstrated that the early disruption of the male gonad, preceding the development of an ovary, is due to apoptosis, i.e., programmed cell death (Raff 1998; Evan and Littlewood 1998; Vaux and Korsmeyer 1999) triggered by compounds present, in various concentrations, in different benthic diatoms. Zupo and Messina (2007) also demonstrated that the disruption of the androgenic gland and of the testis takes place in a very early postlarval stage: apoptosis of these organs was observed as early as 2 days after settlement, when active diatoms were administered in the diet.

The action of diatoms delivered with the diet is very specific and time limited. It takes place from the second to the 12th day of postlarval development and is targeted only against the androgenic gland (AG; Sagi 1988) and the male gonad (Zupo and Messina 2007). It is therefore a very fast and specific process and it leads to the complete disruption of the AG in the first days of postlarval growth and to the appearance of the female sex within a single moult cycle. In fact, the presence of active females (presence of an ovary) with external male appendages was observed before the moult (Reverberi 1950). Active males (presence of mature testis) with external female secondary characters are observed as well, when the moult precedes the disruption of the testis (Katakura 1989). Based on these interesting observations, an EU research project named Pharmapox was started in 2005. It is aimed at isolating, purifying, and characterizing the apoptotic compounds present

in benthic diatoms and to determine their activity for biotechnological purposes (Hannun 1997).

The aim of the present study was to attempt a first extraction of benthic diatom compounds and compare their activity to that of planktonic diatoms. Miralto et al. (1999) demonstrated that diatoms may have an insidious effect on copepod reproduction. These authors also observed that the compounds present in some planktonic diatoms produce apoptosis both in the embryo and in the first larval stages, thus impacting both the survival and the viability of recruits (Romano et al. 2003; Ianora et al. 2004). This was supposed to be, however, a mechanism of defense useful for the diatoms, to reduce the impact of grazers during their plankton blooms (Miralto et al. 1996; Ianora et al. 2004). In contrast, the role of apoptosis in the benthic decapod *H. inermis* appears regulative for the species, since it leads to a higher fitness and guarantees a stable sex ratio in the population. The compounds produced by planktonic diatoms that are detrimental to the development of copepods are mainly aldehydes (Miralto et al. 1999). The compounds present in benthic diatoms, regulating the shrimp populations, are still unknown.

It is an important issue as to whether the same compounds induce apoptosis (with contrasting ecological effects) in benthos and plankton. In such a case, the activity of diatoms on crustacean populations in the two systems could be considered homologous from an ecological point of view. In contrast, the activity of diatoms in the two systems should be considered analogous if the apoptotic compounds they deliver to crustacean populations are different. We therefore performed extractions on both planktonic and benthic diatoms and administered dried diatoms and their extracts to postlarvae of *H. inermis*, to determine whether they produced similar effects on the proportions of females matured in each treatment.

Material and methods

We cultivated both planktonic and benthic diatoms. The planktonic diatoms (*Skeletonema costatum*) were cultivated in glass bowls (4 L) in F2 medium (Sigma-Aldrich), under controlled conditions (18°C, 12/12 h photoperiod in a thermostatic chamber), and the biomass produced after 10 days was filtered on glass fiber filters (GFF) and freeze-dried. To produce benthic diatoms (*Cocconeis* spp., *Navicula* sp., and *Diploneis* sp.), monoclonal cultures were cultivated in sterilized Petri dishes (14 cm diameter), each containing 100 mL of F2 medium. After 15 days of growth in a thermostatic chamber (18°C, 12/12 h photoperiod) Petri dishes were opened, the culture medium was drained, and the dishes were washed twice with distilled water, then freeze-dried and scraped with a metal blade, to collect the biomass produced. Part of the dried materials produced by both planktonic and benthic diatoms was included in composed foods, according to the methods reported below. Another part was used for extraction of active compounds.

For this purpose, 60 mg of freeze-dried diatoms were added to 2 mL of methanol (MeOH) and homogenized (20°C) using a 2.5 mL potter. The extraction was repeated 3 times and the crude extracts obtained were pooled prior to partitioning between hexane and 10% aqueous MeOH solution. The MeOH phase was then diluted to 40% aq. MeOH (Jüttner et al. 2000) and extracted with CH_2Cl_2. Evaporation of the solvents (Büchi rotavapor) and lyophilization of the aqueous solution permitted us to obtain hygroscopic solids that were stored in a cold (−20°C) and dry environment. The solid residue (mainly empty frustules of diatoms) obtained after the extraction was centrifuged (10 min at 3000 rpm), dried, and included in foods, to check for the presence of any residual activity.

Finally, aldehydes previously identified (Romano et al. 2003; Ianora et al. 2004) in planktonic diatoms (2-*trans*-4-*trans*-decadienal; 2-*trans*-4-*trans*-octadienal) were obtained (Sigma-Aldrich) and incorporated into the shrimp food. Bioassays of diatoms, of their fractions, and of the aldehydes obtained as reported above, indicated where apoptotic activity was principally located.

The materials to be tested (whole diatoms, their extracts, and the diatom aldehydes) were mixed into a basic composite food, containing 40% dried algae (*Enteromorpha* sp.), 40% of dried *Artemia salina* enriched with fatty acids (PUFA, commercially sold as ''SHG Enriched Artemia''), and 20% of enriched *Spirulina* flakes (manufactured by SHG inc., www.superhigroup.com). Small pellets (5 mg each) containing known amounts of the diatoms and their extracts were prepared according to the experimental plan reported in Table I. Each of the organic fractions extracted from 60 mg of dried diatoms (i.e., MeOH fraction, CH_2Cl_2 fraction, hexane fraction, and solid residue) was mixed with 300 mg of dry food, then partitioned into 5 mg pellets. Additionally, 60 mg of dried diatoms for each species of benthic and planktonic microalgae were included into 300 mg dry food and partitioned in 5 mg pellets. Finally, 3 μL of a 2 mg mL^{-1} methanol solution of the two reference aldehydes were added to 300 mg dry food and partitioned into 5 mg pellets.

Mature *H. inermis* females were sampled in the *Posidonia oceanica* meadow off Lacco Ameno d'Ischia (Gulf of Naples, Italy), transported to the laboratory, and cultured until

Table I. Experimental plan consisting of a control and 27 treatments, each with 3 replicates and 25 individuals.

Control/treatment	Materials added to 300 mg basic food
Control	none
Cocconeis neothumensis	60 mg dry diatoms
Cocconeis scutellum parva	60 mg dry diatoms
Diploneis sp.	60 mg dry diatoms
Navicula sp.	60 mg dry diatoms
Skeletonema costatum	60 mg dry diatoms
2-*trans*-4-*trans*-decadienal	6 μg of the *S. costatum* aldehyde
2-*trans*-4-*trans*-octadienal	6 μg of the *S. costatum* aldehyde
C. neothumensis MeOH fraction	Methanolic fraction from 60 mg diatoms
C. neothumensis hexane fraction	Hexane fraction from 60 mg diatoms
C. neothumensis CH_2Cl_2 fraction	CH_2Cl_2 fraction from 60 mg diatoms
C. neothumensis residue	Solid residue collected after the extraction of 60 mg diatoms
C. scutellum parva MeOH fraction	MeOH fraction from 60 mg diatoms
C. scutellum parva hexane fraction	Hexane fraction from 60 mg diatoms
C. scutellum parva CH_2Cl_2 fraction	CH_2Cl_2 fraction from 60 mg diatoms
Cocconeis scutellum parva residue	Solid residue collected after the extraction of 60 mg diatoms
Diploneis sp. MeOH fraction	MeOH fraction from 60 mg diatoms
Diploneis sp. hexane fraction	Hexane fraction from 60 mg diatoms
Diploneis sp. CH_2Cl_2 fraction	CH_2Cl_2 fraction from 60 mg diatoms
Diploneis sp. residue	Solid residue collected after the extraction of 60 mg diatoms
Navicula sp. MeOH fraction	MeOH fraction from 60 mg diatoms
Navicula sp. hexane fraction	Hexane fraction from 60 mg diatoms
Navicula sp. CH_2Cl_2 fraction	CH_2Cl_2 fraction from 60 mg diatoms
Navicula sp. residue	Solid residue collected after the extraction of 60 mg diatoms
S. costatum MeOH fraction	MeOH fraction from 60 mg diatoms
S. costatum hexane fraction	Hexane fraction from 60 mg diatoms
S. costatum CH_2Cl_2 fraction	CH_2Cl_2 fraction from 60 mg diatoms
S. costatum residue	Solid residue collected after the extraction of 60 mg diatoms

larvae were released (Le Roux 1963). An experimental larval culture process was devised according to Zupo and Messina (2007) to obtain a sufficient number of postlarvae. Postlarvae obtained from all females were pooled and divided in groups of 25 individuals in Petri dishes to obtain 27 treatments (Table I), each consisting of three replicates. The experimental control was the basic composite food. At the end of the experiment (25 days) all mature postlarvae were sacrificed, fixed, and examined under the dissecting microscope, to record the total length and to collect the second pleopod, in order to determine sex ratios. The ratio 'number of females/total number of mature individuals' (*F*/*tot*) was calculated for each treatment, as well as the mortality rates and the mean size reached by adult shrimp at the end of the experiment. Results were statistically analyzed to check if the planktonic diatoms may have an effect comparable to that of benthic diatoms and also to identify the fractions exhibiting the highest activity. Two-way ANOVA with Bonferroni post-test was performed using Prism software (version 4.00 for Mac, GraphPad Software, San Diego California USA) to evaluate significant differences among treatments. A One-way ANOVA with multiple comparison test was used to check the differences between the control and each treatment. *F*/*tot* ratios between control and treatments were compared by means of a *z*-test on proportions.

Results

The larval growth lasted on average 30 days, while the postlarval growth lasted 25 days. At this age most individuals were mature and they were sacrificed. Low mortality rates for postlarvae were observed for most treatments with freeze-dried diatoms, with the exception of *S. costatum* (10.0%) and *C. scutellum parva* (11.7%; Figure 1). All the remaining treatments with whole diatoms, as well as the control, exhibited mortality rates lower than 10%. The highest *F*/*tot* ratio (number of females on the total number of individuals), indicating high efficiency in the production of primary females, was exhibited by *C. neothumensis* (61.1%; Figure 1). A high *F*/*tot* ratio, significantly different with respect to the control, was also exhibited by the treatment with *C. scutellum parva*. This is in accordance with the results of Zupo and Messina (2007). In contrast, the *F*/*tot* ratios obtained with the treatments *Diploneis* sp. (46.1%), *Navicula* sp. (39.5%) and *S. costatum* (31.3%) were not significantly different in respect to the control (31%; Figure 1).

The size reached at the end of the experiment (Figure 2) adds some insights to the previous information. Two-way ANOVA indicated significant differences among both diatoms and extracts ($p < 0.0001$), with significant statistical interactions between factors. The greatest sizes were reached by *H. inermis* under the treatments with *Diploneis* sp. (7.0 mm) and *Navicula* sp. (6.9 mm). The average size observed in control individuals was 6.4 mm, which is not significantly different from the size obtained with the benthic diatoms *C. scutellum parva* and *C. neothumensis*. A significant difference, with respect to the controls, was observed only for the treatment with *S. costatum* (5.9 mm). Very complex patterns were shown by the size reached under various treatments with solvents (Table II), but the greatest size was reached in individuals under the treatment with fresh and the solid residue of *Diploneis* sp. (7.4 and 7.2 mm, respectively) while the smallest size was reached in individuals under the treatment with the freeze-dried *S. costatum*. The mortalities observed in the treatments with aldehydes were low as well (less than 10.0%) but in this case no significant differences were observed between the *F*/*tot* ratios in the two treatments and in

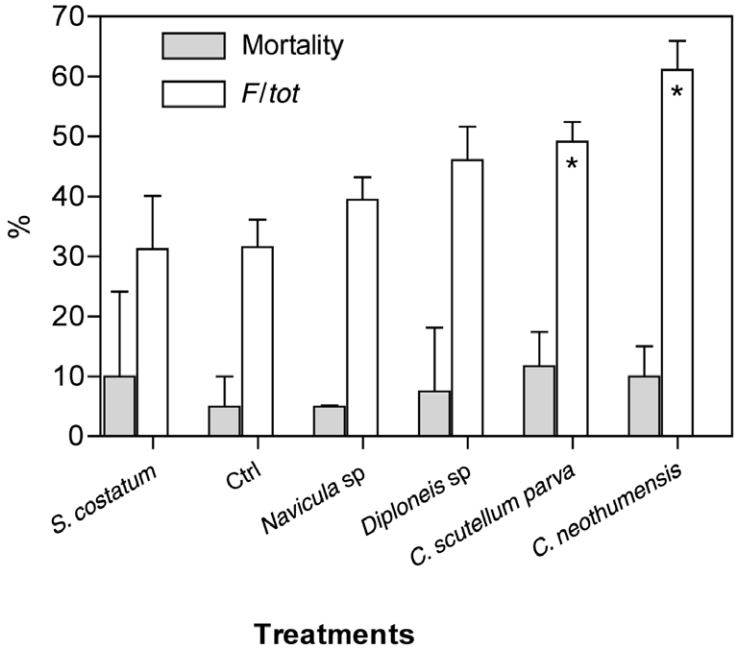

Figure 1. Mean mortality (% of dead individuals at the end of the 25-day experiment) and percent of females on the total number of individuals (*F/tot*) of *Hippolyte inermis* in three replicates, ordered according to the efficacy of treatments. Only treatments with freeze-dried diatoms are considered here. An asterisk denotes the treatments significantly different from the controls.

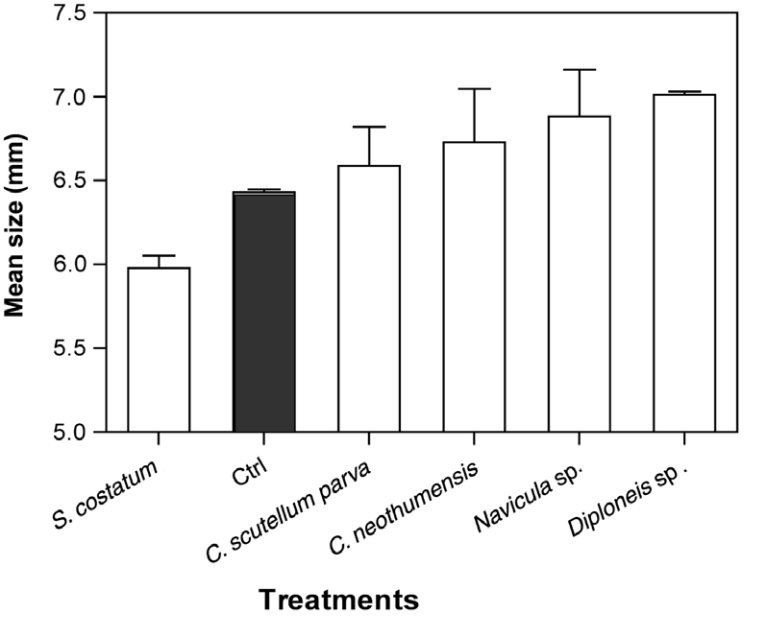

Figure 2. Mean size (mm) + 1 SD ($n = 3$) reached by *Hippolyte inermis* postlarvae at the end of the 25-day experiment. A black bar denotes the control.

Table II. Mean size (mm) reached in each treatment and for each solvent, followed by standard deviations (SD) and number of individuals (N). N (75 at the start of experiment) varies according to the mortality observed in each treatment. All fractions were compared to the same control. CTRL = control; Fresh = whole diatoms; F-D = freeze-dried diatoms; Met = methanol fraction; Hex = Hexane fraction; CH_2CL_2 = CH_2Cl_2 fraction; Res = solid residue.

	Fresh	SD	N	F-D	SD	N	Met	SD	N	Hex	SD	N	CH_2CL_2	SD	N	Res	SD	N
Control	6.43	0.02	57	6.43	0.02	57	6.43	0.40	57	6.43	0.02	57	6.43	0.02	57	6.43	0.02	57
C. neothumensis	6.40	0.25	49	6.73	0.32	54	6.57	0.34	60	6.60	0.16	49	6.59	0.16	52	6.54	0.23	58
C. scutellum parva	7.06	0.16	51	6.59	0.23	53	6.27	0.02	58	6.82	0.45	52	6.77	0.24	56	6.73	0.15	50
Diploneis sp.	7.45	0.67	37	7.01	0.02	37	6.74	0.57	43	6.53	0.17	36	7.10	0.06	37	7.25	0.77	36
Navicula sp.	6.79	0.09	38	6.88	0.28	38	6.64	0.12	37	6.67	0.02	35	6.53	0.04	34	6.96	0.26	37
Skeletonema costatum	6.21	0.20	32	5.98	0.08	36	6.50	0.12	32	6.50	0.25	40	6.19	0.31	29	6.68	0.26	32

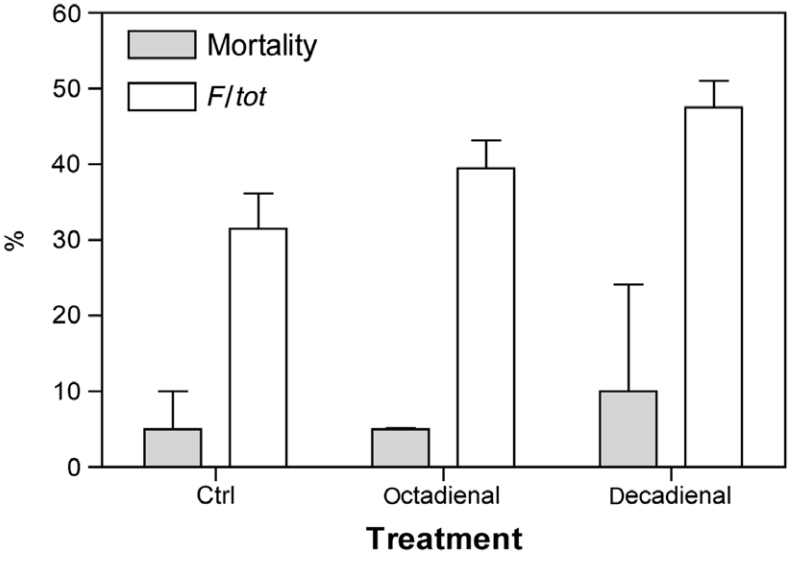

Figure 3. Mean mortality with SD ($n = 3$) (percent of dead individuals at the end of the 25-day experiment) and percent of females on the total individuals of *H. inermis* in three replicates (*F/tot*) ordered according to the efficacy of the treatments. Treatments with aldehydes derived from the benthic diatom *S. costatum* are considered here.

the control (Figure 3). The highest percentage of females was obtained with the 'decadienal' treatment (47.5%).

The results obtained with the diatom extracts showed a complex pattern but, on average, the methanolic fractions appeared to be the most active in most treatments (Figure 4). The solid residues were also active however (large *F/tot* ratios), especially in the treatments with some diatoms (*S. costatum*, *C. scutellum parva*, and *C. neothumensis*). This indicates that the solvents used for the extraction were not efficient. However, both the hexane and dichloromethane fractions exhibited a low efficacy in promoting the development of primary females, and their activities were inversely proportioned to the one of solid residue (Figure 4). It is worth noting that the activity of the methanolic fractions was higher in the treatments with *C. scutellum parva*, *Navicula* sp. and *C. neothumensis*, thus demonstrating the highest efficacies (Figure 4) as a whole. A high *F/tot* ratio was exhibited also by the MeOH fraction of *Diploneis* sp., which was characterized by the lowest activity of the solid residue. In contrast, the efficacy of the methanolic fractions was low in *S. costatum*.

Discussion

The results obtained with the dry diatom bioassays confirm the activity of *C. neothumensis* and *C. scutellum parva* in promoting the development of primary females (Zupo and Messina 2007). They also demonstrate that the planktonic diatom *S. costatum* exhibits a negligible effect on the sex reversal of the shrimp *H. inermis*. Since previous investigations (Zupo 2000; Zupo and Messina 2007) indicated that the development of primary females is due to a very early disruption by apoptosis of the androgenic gland (AG) of young postlarvae, followed by the death of the testis and the development of an ovary

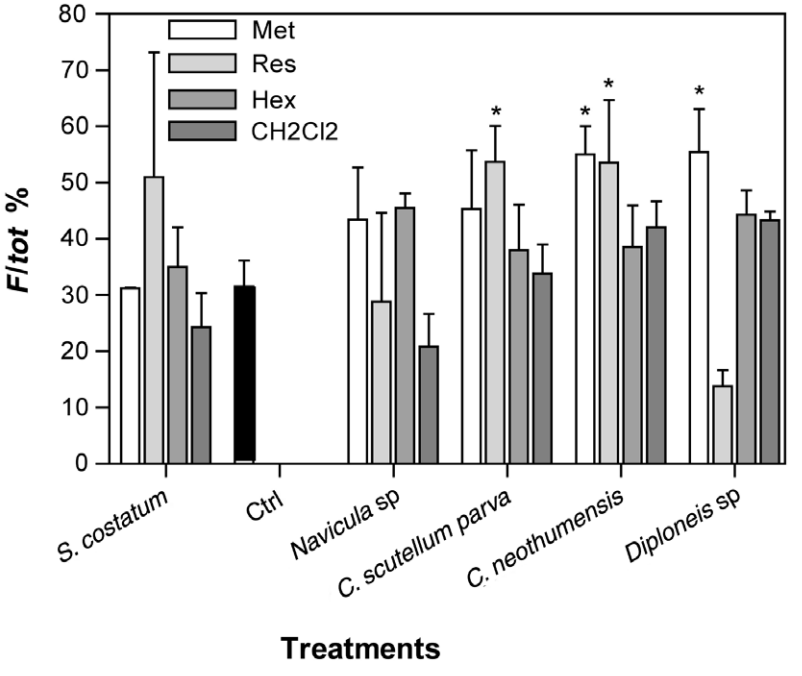

Figure 4. Mean percent of females on the total individuals of *Hippolyte inermis* (*F/tot*) ordered according to the efficacy of treatments. Treatments with extracts are considered here. A black bar denotes the control. An asterisk denotes the treatments significantly different from the control. Met = methanol extract; Res = solid residue after the extraction; Hex = Hexane extract; CH2Cl2 = CH_2Cl_2 extract.

(Charniaux-Cotton 1954; Austin and Meewan 1999; Khalaila et al. 2002), we should infer that *C. neothumensis* and *C. scutellum parva* contain compounds able to specifically trigger the apoptosis of the AG in *H. inermis* postlarvae, while the planktonic diatom *S. costatum*, previously demonstrated to induce apoptosis in embryos and larvae of planktonic copepods (Poulet et al. 1994; Ianora et al. 1995), does not contain the same compound.

In fact it is evident that the disruption of the testis and the formation of an ovary should be preceded by the disappearance of the AG (Payen 1983; Nagamine et al. 1980; Khalaila et al. 1999), since in *H. inermis* intersex individuals were never observed (Sagi et al. 1997, 2002; Cobos et al. 2005). The compounds occurring in *S. costatum* are apparently characterized by a specific toxicity on *H. inermis*, as demonstrated by the low size reached at the end of the experiment with this planktonic diatom. However, the toxic effect was not due to the apoptotic compounds (aldehydes) previously found in this diatom. The mortality recorded in the treatments with octadienal aldehyde was lower than the mortality recorded in the treatment with the whole diatom.

The data on the size reached at the end of the experiment with fractions may add some information to this puzzling question. Shrimps exposed to the CH_2Cl_2 fraction of *S. costatum* exhibited the smallest size and the highest mortality. This indicates that the toxic compound present in the planktonic diatom is selectively extracted by this solvent (Jüttner 2001). Recent studies (d'Ippolito et al. 2004) indicated that other compounds, besides aldehydes, may be responsible for the effects observed in the planktonic copepods and that these compounds could be selectively extracted by CH_2Cl_2.

In contrast, the largest size was reached in the treatment with *Diploneis* sp. and in the treatment with its solid residue. This seems to indicate that the solid residue of diatoms still contains some compounds or feeding principle useful to promote growth of shrimp. The final size reached by shrimps under the treatments with the two species of benthic *Cocconeis* is not, however, significantly different from the size of shrimps under the control diet or fed with other benthic diatoms. This indicates that the process of apoptosis disrupting the AG of young shrimps is not due to a toxic effect, but rather to a specific activity influencing their physiology (Zupo 1994). No differences in food acceptance were observed among treatments and all cultured shrimps produced similar quantities of fecal pellets. These observations mean that size differences cannot be ascribed to the amount of ingested food. In addition, our data demonstrated that the main apoptotic compounds found in planktonic diatoms, i.e., octadienal and decadienal aldehydes, have negligible effects on the sex maturation of *H. inermis* and, therefore, these compounds should be different from those responsible for the effects on the sex ratio produced by benthic diatoms (Zupo et al. 2000).

The results obtained by administering the fractions obtained from various benthic diatoms should be considered indicative, but not conclusive, in starting the characterization of the compounds responsible for the effect on the sex ratio of *H. inermis*. A high residual activity was found in the solid fraction of diatoms after the extraction with different solvents, and this indicates the need for further investigations with different sets of solvents. However, the methanolic fraction was significantly active (in respect to the control) in the case of the effective diatoms *C. neothumensis* and *C. scutellum parva*. The activity of both hexane and dichloromethane fractions, in all diatoms, was low ($F/tot < 46$ and 43%, respectively) and their effects were not significantly different from the control group. It is also evident that the activity of the methanolic fraction of *S. costatum*, whose effect on sex maturation was negated by other experimental manipulations (freeze dried biomasses), was low and comparable to that of the hexane and dichloromethane fractions. This confirms the absence of activity of this planktonic alga and represents a further indication of the ability of methanol to segregate at least part of the active compound (since another important part, apparently, remains in the solid residue). In contrast, *Diploneis* sp. exhibiting slight activity as a whole diatom (Figure 1) demonstrated the highest methanolic activity and the lowest activity of the solid residue ('Res' in Figure 4). This may indicate that the residual activity in the solid residue is linked to some physical properties of diatoms and that the activities of the solid residue and of the methanolic fraction are inversely proportioned, since an effective extraction may move most of the activity from the solid part to the 'MeOH' fraction (Fink et al. 2006).

Taking into account that the effects of *S. costatum* on the sex of our model shrimp were not significant within the planned factorial experiment and that benthic diatoms triggered a significant shift to female sex, we conclude that the apoptotic compounds present in the diatoms examined are different. Moreover, *S. costatum* promoted a significant increase of mortality and a lower growth of postlarvae, thus demonstrating that, in the benthos as well as in the plankton, this diatom is characterized by a high toxicity (d'Ippolito et al. 2004). The results obtained with freeze-dried diatoms were confirmed by the tests with aldehydes, demonstrating high mortality, low growth rates, and non-significant effects on the sex ratio of our test shrimps. The solvent apparently extracting the active compound was methanol, although some activity remained in the solid residue. This observation indicates that, besides a difficulty of extraction, the active factor should be at least a partially polar compound (Blom and Jüttner 2005). Further research, using different sets of solvents, will be necessary to fractionate and further characterize the active compound occurring in some

benthic diatoms, absent in planktonic diatoms. We suggest that the compound is probably useful for biotechnological applications (Schwartsmann et al. 2001, 2003), due to its specific activity (Bongiorni and Pietra 1996) on the androgenic gland of *H. inermis*. The apoptotic activities promoted by planktonic and benthic diatoms, both producing effects on the physiology of various crustaceans (reducing the size of recruitment in planktonic copepods, stabilizing the natural populations in benthic crustaceans) are analogous, because they are based on different chemical compounds.

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