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Patterns of oxygen supply in embryo masses of brachyuran crabs throughout development: the effect of oxygen availability and chemical cues in determining female brooding behavior

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ABSTRACT: Different patterns of variation in oxygen availability throughout development have been observed in embryo masses of brooding species of marine invertebrates, and this variation seems to be related to the strategy to solve the oxygen limitation problem of the broods. As yet, little is known about patterns of oxygen availability and female brooding behavior (abdominal flapping) throughout development in brachyuran crabs, and about which factors trigger abdominal flapping. These issues were experimentally studied in 2 crab species of similar body size (*Cancer setosus* and *Homalaspis plana*). In addition, oxygen consumption of crab embryos and 2 potential factors that could trigger changes in female brooding behavior were studied (oxygen partial pressure and non-identified chemical cues produced by the embryos). Optic fibers were used to monitor oxygen partial pressure (pO_2) in the embryo mass as female behavior was videotaped; optic fibers do not affect female behavior. Microchambers were used to determine oxygen consumption of the embryos. Females carrying early stage embryos connected to containers with water under different treatments were used to evaluate the effect of pO_2 and chemical cues on female behavior. A cyclic pattern in pO_2 was detected in masses of early stage embryos and constant high pO_2 for late stages. As changes in pO_2 in the embryo mass occurred, an increase in oxygen demand by the embryos and an increase in abdominal flapping frequency were detected in both species. Abdominal flapping seems to be affected by low pO_2 in the embryo mass and also by the presence of late stage embryos. These results support previous findings suggesting that oxygen provision to embryos seems to be a critical factor determining parental investment across taxa of marine invertebrates.

KEY WORDS: Brachyuran crabs · Brooding · Marine invertebrates · Oxygen

INTRODUCTION

Brooding in marine systems is restricted to species with small body size (Strathmann & Strathmann 1982), probably because of constraints in oxygen supply to embryo masses (Strathmann & Chaffee 1984, Strathmann & Strathmann 1989). Supporting this hypothesis, strong oxygen gradients have been found in gelatinous egg masses (Cohen & Strathmann 1996). Even in taxa that show an exception to the association between brooding and small body size, such as brachyuran crabs, oxygen availability in the center of the embryo mass is lower than at the periphery (Naylor et al. 1999a, Fernández et al. 2000). The physical constraints of brooding affect the degree of parental investment

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and care provided to the offspring. Among the species that brood, investment in parental care could be in the form of gel (which helps oxygen diffusion into the egg mass; Chaffee & Strathmann 1984, Strathmann & Strathmann 1995, Cohen & Strathmann 1996) or active ventilation (which increases oxygen availability; Baeza & Fernández 2002). In contrast, broadcasting appears to be the simplest and cheapest way to ventilate large numbers of eggs (Strathmann & Chaffee 1984). The physical constraints of brooding can also have important life history consequences among marine invertebrates (Strathmann & Strathmann 1982) as well as implications on management and conservation plans, since differences in early development affect potential for dispersal, population dynamics, and extinction and speciation rates (Valentine & Jablonski 1983).

Oxygen availability in the center of egg masses of marine invertebrates varies over time, but in a different fashion depending on the brooding strategies. Flow regime and light affect daily variations in oxygen availability in gelatinous egg masses (Cohen & Strathmann 1996), while the increase in oxygen demand by the embryos throughout development generates a dramatic decrease in oxygen availability in the center of the egg mass from early to late development (Chaffee & Strathmann 1984, Booth 1995, Cohen & Strathmann 1996). Temporal variation in oxygen availability in the center of the embryo mass of brachyuran crabs shows a different pattern. First, oxygen availability in the center of embryo masses varies over smaller temporal scales (minutes), at least during early development of the embryos (Fernández et al. 2000). Secondly, oxygen availability in the center of the embryo mass increases throughout development, in spite of the increase in oxygen demand of the embryos, at least in the 2 species of *Cancer* that have been studied to date (Naylor et al. 1999a, Baeza & Fernández 2002). The main cause of the differences in temporal variations in oxygen availability between crabs and gelatinous egg masses seems to be the ventilation of the embryos (abdominal flapping) by female crabs, which increases exchange of oxygen rich water (Baeza & Fernández 2002). In *Cancer* species, the frequency of abdominal flapping changes throughout embryo development (Baeza & Fernández 2002). It is not yet clear how consistent this pattern of oxygen provision throughout development is among brachyuran crabs, nor which factors trigger abdominal flapping. To evaluate the consistency of the findings reported for *Cancer* species, patterns of oxygen availability, frequency of abdominal flapping and oxygen consumption by embryos throughout development were studied in 2 crab species of similar body size (*Cancer setosus* and *Homalaspis plana*). Two potential factors that could trigger changes in frequency of abdominal flapping, oxygen partial pressure and non-identified chemical (water-borne) cues produced by the embryos, were also analyzed in 1 of the 2 species (*H. plana*). Here, the broad term brooding behavior was used to refer to abdominal flapping or female ventilation of the embryos, and low or high oxygen availability was used to refer to low or high oxygen partial pressure, which correlates with the concentration of unbound dissolved oxygen.

MATERIALS AND METHODS

Samples. Two crab species belonging to different families were selected: Cancridae: *Cancer setosus* (Molina 1782), and Xanthidae: *Homalaspis plana* (Milne-Edwards 1834). Both species exhibit similar ranges of distribution (Retamal 1981) and body size, brood during winter and early spring, and support commercial fisheries (Fernández & Castilla 1997). Crabs were collected by SCUBA in El Quisco (33° 23′ S, 71° 42′ W), Las Cruces (33° 29′ S, 71° 38′ W) and Coquimbo (29° 58′ S, 71° 22′ W), Chile. After collection, females were measured (carapace width; CW) and maintained in holding tanks (3 m in diameter, 0.5 m deep) with circulating seawater at 14°C and constant aeration. In the holding tanks, crabs were fed *ad libitum* on fresh mussels (*Choromytilus chorus*). Size of brooding females ranged between 97.5 and 136.7 mm for *C. setosus* ($\bar{x}$ = 124.1 mm ± 10.1) and between 80.3 and 129.5 mm for *H. plana* ($\bar{x}$ = 100.9 ± 13.7). Size of control (non-brooding) females ranged between 94.5 and 125.3 mm for *C. setosus*, and between 88.9 and 129.1 mm for *H. plana*. Mean diameter of the embryo mass was 56.14 mm ± 7.69 (n = 66) in *H. plana* and 59.7 mm ± 7.99 (n = 66) in *C. setosus*. The developmental stage of the embryos was determined in each female used in the experiments (Wear 1974). Three stages were determined: Stage I: gastrulation (some cells without vitellium of the animal pole); Stage II: nauplius (first appendages just appearing); and Stage III: metanauplius (heart pumping, ocular pigment and chromatophores present). All experiments and calibrations of oxygen consumption experiments were conducted at constant, average temperature in the study area (14°C).

Oxygen availability in the center of the embryo mass of brooding females. To determine patterns of oxygen availability in the center of the embryo mass of brooding females carrying embryos in Stages I (early), II (intermediate) and III (late), laboratory experiments were conducted. Optic fibers (Microoptodes Presens) with a tip diameter between 20 and 50 µm were used to continuously monitor oxygen partial pressure in the center of the embryo mass (Microx I; Holst et al. 1997, Klimant et al. 1997). A small hole (<1 mm) was drilled

into the abdomen ($^1/_2$ of the VI segment) of brooding females and a plastic tube (of variable length depending on embryo mass diameter) was inserted and fixed to the abdomen. The microoptode was inserted into the tube after calibration (0% air saturation: solution saturated with Na_2SO_3; 100% air saturation: aerated water from the tank where the experiment was conducted). Both the tube and the microoptode were fixed to the carapace using cyanoacrylate glue and dental wax. The tip of the optic fiber reached 2 mm outside the catheter and was in contact with the embryos. After the microoptode was fixed, females were placed in the experimental tank (25 × 25 × 25 cm) filled with a 5 cm layer of shell hash and rocks, and unfiltered seawater. Constant aeration was maintained during the whole experimental period. After 1 h, oxygen availability (mm Hg) in the center of the embryo mass was monitored continuously for 24 h and recorded in a computer every 5 s. Using the 24 h records, the percentage of time that the embryos from the center of the mass were exposed to 2 levels of oxygen partial pressure (pO_2; low <39.7 and high >79.4 mm Hg) was calculated for each female. These 2 categories of pO_2 were selected considering: (1) the level of pO_2 below which embryo oxygen consumption is always affected (<39.7 mm Hg); and (2) the level of pO_2 above which oxygen consumption is not affected (>79.4 mm Hg; Naylor et al. 1999a, Fernández et al. 2000, Baeza & Fernández 2002). Four replicates were conducted for each species and each developmental stage. Two-way ANOVAs were used to test for differences in the mean proportion of time exposure of the embryos to each category of pO_2 between species and stage. Data were transformed in 1 case (>79.4 mm Hg) in order to meet the assumptions of the ANOVA model. A SNK test was used for post hoc comparisons (Zar 1996).

In addition, autocorrelation analyses were conducted to detect the presence of cycles in pO_2. When cycles were detected (all females carrying embryos in Stages I and II but not in Stage III, see 'Results'), the data were fitted to Fourier series (minimum-square method) and a periodogram was obtained. Stationary time series were also obtained. Spectral analyses were conducted to determine the dominant frequency in each time series (Chatfield 1989). In each replicate, the 5 largest periodogram peaks (frequency domain) were recorded, transformed to period and expressed in minutes needed to complete 1 full cycle (Chatfield 1989). A 2-way ANOVA (fixed factors) was used to test for differences in the mean time needed to complete a pO_2 cycle between species and stages (only females carrying embryos in Stages I and II showed cycles and were compared).

Abdominal flapping. To assess patterns of abdominal flapping in relation to embryo developmental stages, females were videotaped as pO_2 in the center of the embryo mass was being measured (both instruments were set to the same time). Thus, the same number of replicates as above were used for this experiment. A 12:12 h day:night cycle was simulated with white and red lights, respectively, using an automatic switch system. Female behavior was videotaped using a Sony time-lapse video (SVT-3000) and a Pelco vigilance camera. Recording of female behavior began 1 h after the microoptode was introduced into the embryo mass. Two video blocks (2 h each) were randomly analyzed for each experimental female, one during the day and another during the night. Day and night time blocks were included in order to account for differences in female brooding behavior during a 24 h cycle as part of the variance in the data. Abdominal flapping and resting time (defined as having no apparent activity) were recorded. Non-brooding females were also videotaped and used as a control group in order to assess if the total time that females spent flapping the abdomen or resting was related to the presence of the embryo mass. The percentage of the total time spent flapping the abdomen (or resting) was estimated and compared between species and developmental stages using a 2-way ANOVA. Data were transformed when the assumptions of the model were not met (Table 1). SNK post hoc comparisons were conducted to determine differences among levels in each factor of the ANOVA.

To assess the effect of the optic fiber on the behavior of brooding females, the percentage of time spent performing 2 behaviors, i.e. abdominal flapping by brooding females and walking by brooding and non-

Table 1. Results of the 2-way ANOVAs conducted to test for differences in the mean percentage of time allocated to resting and abdominal flapping between species (*Cancer setosus* and *Homalaspis plana*) and among female condition (non-brooding females and brooding females carrying embryos at Stages I, II and III). Transformations are indicated between parentheses. Mean values (and SD) are reported in Fig. 2C,D. **Statistical significant differences were detected

Variable	Factor: species			Factor: developmental stage			Interaction term		
	F	df	p	*F*	df	p	*F*	df	p
Resting (1/*x*)	1.49	1, 24	0.23	6.45	3, 24	0.002**	2.49	3, 24	0.085
Abdomen flapping (ln)	0.002	1, 24	0.97	17.16	3, 24	<0.00001**	1.082	3, 24	0.37

Table 2. Results of the 2-way ANOVAs conducted to test for the effect of the optic fiber on female behavior (abdominal flapping and walking) between developmental stages (early and late embryos) for the 2 experimental conditions used (with and without optic fiber). The analysis was conducted for both species (*Cancer setosus* and *Homalaspis plana*). **Statistical significant differences were detected

Factor	*F*	df	p
(A) *Cancer setosus*: abdominal flapping (natural log transformation)			
Stage of development of the embryos	13.69	1	0.0018**
Experimental condition	2.67	1	0.1207
Interaction	0.193	1	0.671
Error		17	
(B) *Cancer setosus*: walking (data were not transformed)			
Stage of development of the embryos	0.97	1	0.347
Experimental condition	0.004	1	0.948
Interaction	1.75	1	0.203
Error		17	
(C) *Homalaspis plana*: abdominal flapping (natural log transformation)			
Stage of development of the embryos	29.6	1	0.0001**
Experimental condition	3.98	1	0.063
Interaction	0.31	1	0.59
Error		16	
(D) *Homalaspis plana*: walking (data were not transformed)			
Stage of development of the embryos	1.098	1	0.3113
Experimental condition	0.001	1	0.971
Interaction	0.17	1	0.691
Error		16	

brooding females, was compared. The experiment was conducted using females carrying early (Stage I) and late (Stage III) embryos (n = 4), because of the contrast in their behavior (Baeza & Fernández 2002). Female behavior was recorded using the same experimental set-up described above. A 2-way ANOVA was used to compare the mean percentage of time spent performing a behavior (abdominal flapping and walking) between females with or without the optic fiber and between developmental stages (Zar 1996). Abdominal flapping data were transformed to meet the assumptions of the model (Table 2).

Oxygen consumption of crab embryos. Laboratory experiments were conducted to determine oxygen consumption of embryos of both species at 2 pO_2 (<39.7 and >79.4 mm Hg) and 3 stages of development (I, II and III). Oxygen consumption of the embryos was recorded under constant temperature, using a double walled, closed microchamber filled with 2 ml of filtered (0.2 µm) seawater (with added antibiotic). After calibration, a blank (seawater without embryos) was used in order to determine the presence of bacteria that could affect oxygen consumption estimates. Another blank was repeated at the end of the experiment. Oxygen consumption of the embryos was measured only when oxygen consumption of the blank was 0. A small number of embryos were removed from the periphery of the embryo mass of brooding females and placed on a fine grid in the microchamber. Water was stirred inside the chamber using a stir bar to avoid oxygen depletion near the embryos when unstirred (Naylor et al. 1997); the grid avoided direct contact between the stir bar and the embryos. Oxygen depletion was monitored continuously with oxygen electrodes (Eschweiler M200; measure range: 0 to 760 mm Hg; resolution 0.1 mm Hg) for variable times depending on the developmental stage of the embryos until oxygen was depleted to 30 mm Hg. At the end of the experimental period, the embryos were weighed (wet weight). A minimum of 5 measurements was conducted for each stage and pO_2 level. Oxygen consumption per unit of time and weight was estimated for each developmental stage, species and pO_2, and compared using a 3-way ANOVA. Data were transformed (natural log) in order to meet the assumptions of the model.

Effect of pO_2 and chemical cues on female brooding behavior. A laboratory experiment was conducted in order to evaluate possible factors that could trigger female brooding behavior. As stated above, brooding behavior was used to refer to abdominal flapping or female ventilation of the embryos (Baeza & Fernández 2002). A second, small hole (<1 mm) drilled into the abdomen was used to insert a plastic tube (of variable length depending on embryo mass diameter), which was used to direct water maintained under the different treatments into the embryo mass of brooding

females using a peristaltic pump. Treatments resulted from the combination of the different levels of the 2 factors analyzed: embryo stage and pO_2. Water containing early (Stage I) and late (Stage III) embryos or no embryos was used as levels for the embryo stage factor. For oxygen partial pressure, 2 levels were used: seawater maintained at high (158.8 mm Hg) and low (15.9 mm Hg) pO_2; these were used because of the patterns of oxygen availability observed in the center of the embryo mass (Fernández et al. 2000, Baeza & Fernández 2002). Thus, treatments were: (1) low oxygen-no embryos; (2) low oxygen-early embryos; (3) low oxygen-late embryos; (4) high oxygen-no embryos; (5) high oxygen-early embryos; and (6) high oxygen-late embryos. Water was pumped into the embryo mass of brooding females (carrying early stage embryos) at a flow rate of 20 ml min^{-1} from 1 l containers filled with seawater under the different treatments. In the treatments where embryos were added, 20 g of embryos were used per container. The experiment was started at 20:00 h. During the experimental period, pO_2 and female behavior were monitored simultaneously (as explained above), beginning 1 h before treatments were applied. Treatments were randomly applied to each crab for 1 h each, after which no further water was pumped to experimental females, but pO_2 and brooding behavior continued to be monitored (control). To incorporate the control (no water pumped into the embryo mass) into the analysis while maintaining the factorial experimental design, the difference between each treatment and the control for each female was estimated. Thus, the response variable used in the 2-way ANOVAs was the difference between the treatment and the control. Data were not transformed since the assumptions of the model were met.

RESULTS

Oxygen availability in the center of the embryo mass of brooding females

pO_2 in the center of the embryo mass showed a distinctive pattern for each developmental stage and this pattern was consistent in both species (Fig. 1). A cyclic pattern of pO_2 was detected in Stages I and II, and relatively constant oxygen availability was found for Stage III. The results shown in Fig. 1 are for 1 female in each case and stage (different females were used for each stage), but the patterns were very consistent in all females studied (Fig. 2A,B). In *Cancer setosus*, the peaks appeared shaved off on the left side and asymmetric compared with the symmetric peaks of *Homalaspis plana*. The mean time used to complete a full cycle of pO_2 in the center of the embryo mass was not significantly different between species (ANOVA: $F = 0.13$, df = 1,11, $p = 0.73$), but varied with embryo developmental stage (ANOVA: $F = 17.92$, df = 1,11, $p = 0.001$; Fig. 2A). The interaction term was not significant (ANOVA: $F = 0.17$, df = 1,11, $p = 0.69$). The mean frequency of pO_2 cycles was significantly higher in females carrying embryos in Stage II than I ($p < 0.05$); females carrying embryos in Stage III were not included in the analysis because no cycle in pO_2 was found (Fig. 2A). Oxygen availability never reached low pO_2 in masses of Stage III embryos.

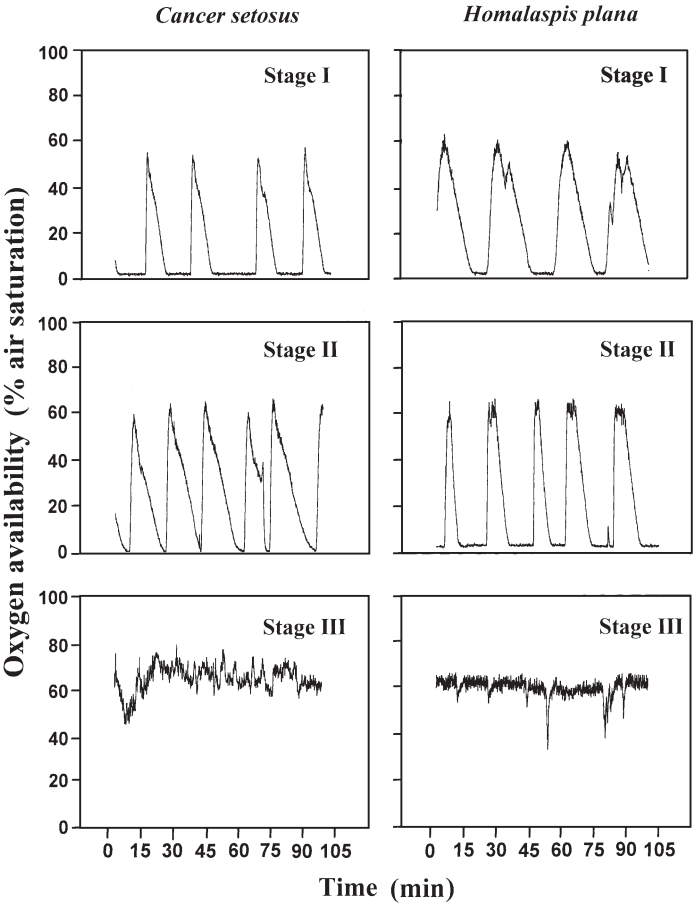

Fig. 1. Patterns of oxygen partial pressure in the center of the embryo mass of *Cancer setosus* (left) and *Homalaspis plana* (right) for early (I), intermediate (II) and late (III) stages of embryo development. The patterns shown here are for 1 female of each species and embryo developmental stage; however, these results were consistent across replicates and the summary of the information for all females analyzed is shown in Fig. 2. Fractions of the 24 h recording (105 min) were randomly selected to depict the pattern of pO_2 of both species and the 3 stages of development

Table 3. Results of the 2-way ANOVAs conducted to test for differences in the mean percentage of time that embryos were exposed to each level of pO_2, between species (*Cancer setosus* and *Homalaspis plana*) and among the 3 developmental stages (brooding females carrying embryos at Stages I, II, and III). Independent ANOVAs were conducted for the 2 percentages of time that embryos were exposed to each range of pO_2 (<39.7 and >79.4 mm Hg). Mean values (and SD) are shown in Fig. 2B. In both cases, significant differences among developmental stages were found and all levels were significantly different (I < II < III; $p < 0.05$). **Statistical significant differences were detected

Variable	Factor: species			Factor: developmental stage			Interaction term		
	F	df	p	*F*	df	p	*F*	df	p
% time spent at pO_2 < 39.7 mm Hg (sq rt)	8.34	1,18	0.0098**	160.86	2,18	<0.00001**	2.65	2,18	0.10
% time spent at pO_2 > 79.4 mm Hg	0.40	1,18	0.54	102.67	2,18	<0.00001**	1.99	2,18	0.16

Early embryos (Stage I) were exposed to low pO_2 (<39.7 mm Hg) for significantly longer time periods than Stages II and III embryos ($p < 0.05$; Fig. 2B). As a consequence, late embryos (III) were exposed most of the time to high pO_2 (>79.4 mm Hg; $p < 0.05$; Fig. 2B). No differences between species were found at high pO_2, but embryos of *Homalaspis plana* were exposed to pO_2 < 39.7 mm Hg a higher percentage of time than embryos of *Cancer setosus* (Table 3, Fig. 2B). The interaction term was not significant in both cases (Table 3).

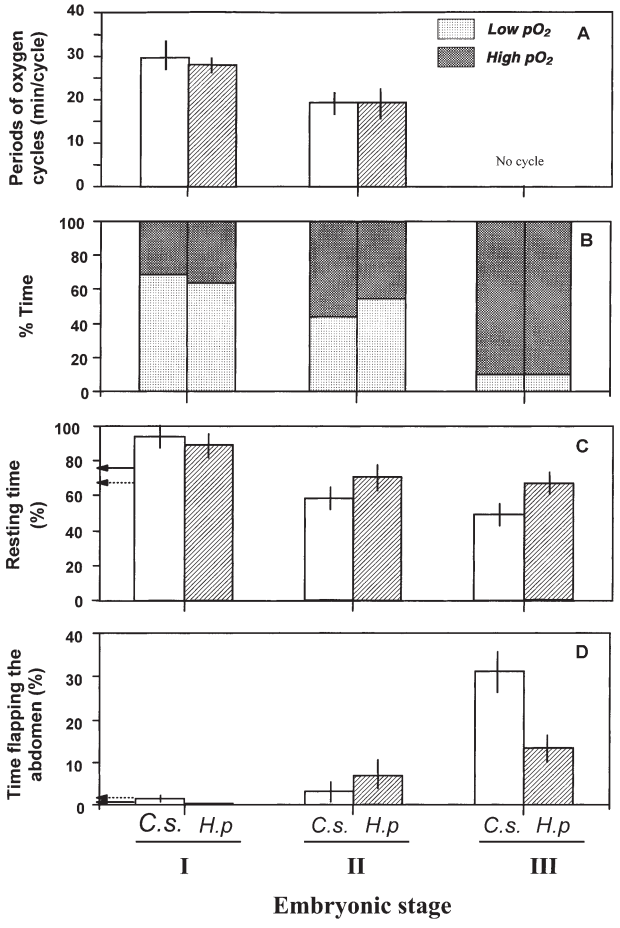

Fig. 2. (A) Periods of oxygen partial pressure (mm Hg) cycles as detected by the microoptodes in the center of the embryo mass of ovigerous females of *Cancer setosus* (*C.s.*, left) and *Homalaspis plana* (*H.p.*, right) for early (I), intermediate (II) and late (III) stages of embryo development. The same pattern for the bars and symbols are used for each species in the rest of the panels; (B) mean percentage of the total time (24 h) that low (<39.7 mm Hg) and high (>79.4 mm Hg) oxygen partial pressures were measured in the center of the embryo masses of *C. setosus* and *H. plana* females carrying embryos at the 3 developmental stages (Stages I, II and III); (C) percentage of time (of the total time monitored) that females adopted a resting position at each stage of embryo development. The arrows indicate the mean percentage of time that control females (no embryos) were inactive (solid line: *C. setosus*; broken line: *H. plana*). (D) Percentage of time that females spent flapping the abdomen at each stage of embryo development. The arrows indicate the mean percentage of time that control females (no embryos) flapped the abdomen. Horizontal bars always indicate SE

Abdominal flapping

Significant differences in the mean percentage of time that females used for abdominal flapping and resting were detected between stages of development (Table 1). No differences between species were found (Table 1). Females carrying early embryos (I) and control females spent most of the time resting; in both species the mean percentage of time assigned to resting decreased as embryos developed (Stages II and III, $p < 0.05$; Fig. 2C). The opposite pattern was found for the mean percentage of time spent flapping the abdomen (Table 1, Fig. 2D); again no differences were found between control and brooding females carrying early embryos. The mean percentage of time spent flapping the abdomen increased for females carrying embryos in Stage II, and was highest for females carrying Stage III embryos (Table 1, Fig. 2D; $p < 0.05$). Abdominal flapping occurred when females were in a standing position; one or more flapping events (movement backwards and forwards of the abdomen) may occur each time that females adopt the standing position. During flapping, the entire embryo mass can be shaken using the pleopods.

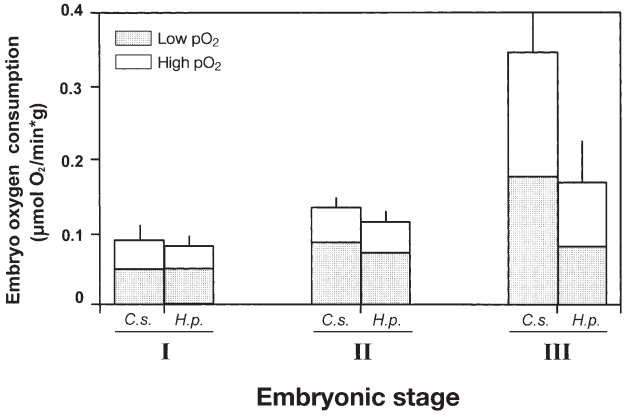

Fig. 3. Mean oxygen consumption of embryos (per g wet weight) of *Cancer setosus* (*C.s.*, left) and *Homalaspis plana* (*H.p.*, right) at each developmental stage and level of oxygen partial pressure. Vertical lines indicate SE

The experimental condition (use of the optic fiber) did not affect female behavior (Table 2). The mean frequency of abdominal flapping increased with embryo development, as shown above; however, the mean time spent performing this behavior was not affected but the presence of the optic fiber was (Table 2). The non-brooding behavior analyzed (walking) was not affected by the optic fiber either, and did not change throughout development (Table 2).

Oxygen consumption of crab embryos

Mean oxygen consumption of crab embryos was significantly different among embryonic developmental stages (ANOVA: F = 10.79, df = 2,52, p = 0.0001), between species (ANOVA: F = 9.14, df = 1,52, p = 0.0039) and between pO_2 levels (ANOVA: F = 11.17, df = 1,52, p = 0.0015; Fig. 3). None of the interaction terms were significant (p > 0.061). Oxygen consumption was higher for embryos of *Cancer setosus* than *Homalaspis plana*, increased significantly from Stage I to II, and from Stage II to III, and was higher at pO_2 > 79.4 mm Hg than at pO_2 < 39.7 mm Hg (Fig. 3).

Effect of pO_2 and chemical cues on female brooding behavior

The mean difference in pO_2 between treatments and control was significantly higher at high pO_2 treatments (ANOVA: F = 66.67, df = 1,34, p < 0.00001; Fig. 4A). No differences in pO_2 in the embryo mass among embryo stage treatments were found (ANOVA: F = 0.871, df = 2,34, p = 0.871; Fig. 4A). The interaction term was not significant (ANOVA: F = 0.275, df = 2,34, p = 0.761). The main effects could not be tested for the second variable (mean difference in the frequency of abdominal flapping) since the interaction term was significant (ANOVA: F = 4.46, df = 1,30, p = 0.02; Fig. 4B). This was due to the dramatic increase in the mean difference in frequency of abdominal flapping when the effects of high pO_2 and late stage embryos were combined (Fig. 4B). Abdominal flapping frequency was lower when seawater containing no embryos or early embryos at high pO_2, was pumped into the embryo mass (Fig. 4B). No differences in abdominal flapping frequency were detected among embryo stage treatments maintained at low pO_2 and late embryos maintained at high pO_2 (Fig. 4B). Mean pO_2 and abdominal flapping frequency per treatment are reported in Table 4.

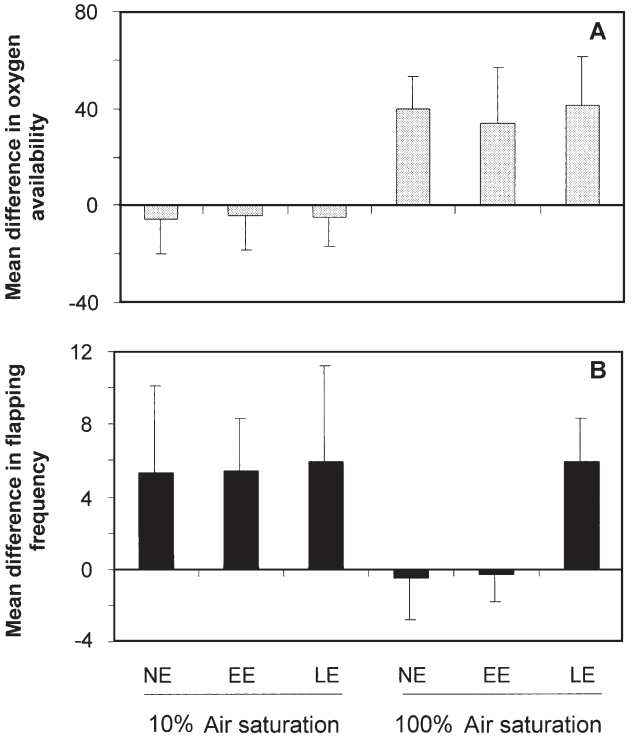

Fig. 4. (A) Mean difference in oxygen partial pressure when water containing no embryos (NE), early stage embryos (EE) or late stage embryos (LE) at low (15.9 mm Hg) or high (158.8 mm Hg) oxygen partial pressure was pumped into the embryo mass of females carrying early stage embryos. (B) Mean difference in abdominal flapping frequency under the same conditions. In both cases, the difference was calculated between the application of a treatment and the control situation (no water pumped into the embryo mass). Vertical lines indicate SE

Table 4. Mean pO_2 and mean frequency of abdominal flapping in the different experimental treatments: control, water containing low (or high) pO_2 and no embryos, early stage embryos or late stage embryos. Water from the experimental containers under the different treatments was pumped into the embryo mass of females carrying early stage embryos. SE is indicated between parenthesis

Treatment	Mean oxygen availability	Mean abdominal flapping
Control	60.60 (6.25)	12.2 (0.60)
High pO_2–no embryos	52.95 (9.00)	11.8 (0.92)
High pO_2–early embryos	54.44 (8.36)	11.7 (0.94)
High pO_2–late embryos	52.91 (7.48)	18.2 (1.17)
Low pO_2–no embryos	105.69 (12.13)	17.6 (1.59)
Low pO_2–early embryos	106.86 (12.51)	17.7 (1.92)
Low pO_2–late embryos	116.87 (12.35)	18.2 (2.15)

DISCUSSION

The main findings of this study can be divided in 2 categories: (1) those related to the concurrent changes in patterns of oxygen availability, female behavior and oxygen demand of the embryos; and (2) those related to the identification of 2 possible factors causing female brooding behavior.

The cyclic pattern in pO_2 in the embryo mass during early development is consistent with previous reports for other crab species (Fernández et al. 2000, Baeza & Fernández 2002). The slightly different shapes of the pO_2 peaks between species could be due to: (1) differences in oxygen consumption of the embryos, i.e. those of *Homalaspis plana* consumed less oxygen than embryos of *Cancer setosus*; (2) differences in the size of the embryos, i.e. those of *H. plana* are larger than embryos of *C. setosus*, so water flow through the embryo mass may vary between species (Strathmann & Chaffee 1984); and (3) differences in alternative mechanisms of ventilation not detected here e.g. internal ventilation (Wheatly 1981, Mantel 1983, Naylor et al. 1999b). What is clear, however, is that the slight differences in the shape of the pO_2 peaks did not have a major effect on the percentage of the time that the embryos were exposed to different pO_2 levels.

The changes in pO_2 throughout development produced differences in the total time that embryos were exposed to different pO_2; early embryos spent most of the time at low pO_2, while late embryos spent most of the time at high pO_2. These 2 extreme ranges in pO_2 could have an effect on embryo development since embryo oxygen demand was negatively affected at the same low, experimental pO_2. It is interesting to note that as embryo oxygen demand increased, brooding females spent less time resting and more time flapping the abdomen. Thus, late stage embryos, which consume oxygen at higher rates, were never exposed to low pO_2. Recently Baeza & Fernández (2002) showed a direct link between abdominal flapping and oxygen availability in *Cancer setosus*, suggesting that abdominal flapping is the main behavior directed towards providing oxygen to the embryos. Results suggest that the same mechanism of oxygen provision may operate in other crab species, given the similarities found in all the species studied so far. Active embryo mass ventilation has already been suggested for *Cancer pagurus* as well as the importance of the standing position, abdominal flapping and pleopods in ventilation of the embryos (Naylor et al. 1999a). However, the results of this study show that brooding females ventilate the embryo mass throughout development, rather than only during late development as has previously been suggested (Naylor et al. 1999a). These observations clearly suggest that: (1) as females became more active and spent more time flapping the abdomen, the cycles in oxygen availability decreased and embryos experienced higher levels of oxygen in the center of the embryo mass; and (2) these changes in female behavior and oxygen availability corresponded to increases in oxygen demand by the embryos.

The changes in female ventilatory behavior and oxygen provision could be related to oxygen demand by the embryos. Similar changes have been found in the ventilatory behavior of female amphipods (Dick et al. 1998) and in caridean shrimps (Pandian 1994), although the changes in behavior were not accompanied by measures of oxygen conditions of the brood. The effect of oxygen limitation during embryonic development on developmental rate, survival and performance of subsequent stages is not yet well understood. Aynchronic development of inner embryos has been related to low oxygen availability in embryo masses (Chaffee & Strathmann 1984, Strathmann & Strathmann 1995). Severe oxygen limitation of embryos incubated in the center of the mass during early development could also be related to the high variability in post-hatching larval viability within a clutch which has been recorded for some decapods (Pandian 1970a,b, Pandian & Katre 1972, Kunisch & Anger 1984). Thus, changes in female behavior corresponding to increases in oxygen consumption rate may help to maintain a synchronic developmental rate within the embryo mass (see Chaffee & Strathmann 1984, Strathmann & Strathmann 1995) or may increase survival probability for later developmental stages.

Considering that ventilation patterns of brooding females seem to follow oxygen demands of the embryos, it can be expected that pO_2 or some cue produced by the embryos (or products of embryo metabolism) could serve as a signal for brooding females to ventilate (see Forward et al. 1987, Rittschof et al. 1989,

Saigusa 1996). Results suggest that in fact, both factors had a significant effect on abdominal flapping frequency. Low pO_2 explained the increase in mean flapping frequency as compared with controls, regardless of the presence or the stage of embryos. The flapping frequency in the control treatment (no water pumped into the embryo mass) was similar to that reported for *Cancer setosus* (Baeza & Fernández 2002), suggesting that the experimental manipulation (holes drilled in the abdomen) did not affect female behavior. However, pO_2 in the embryo mass does not seem to be the only factor affecting female behavior since abdominal flapping frequency was affected by water containing late embryos. The compound that affected abdominal flapping frequency could be a product of embryo metabolism, increasing its concentration as metabolic rate increases during embryo development (Booth 1995, Cohen & Strathmann 1996). A response to pheromones is also possible since chemical responses to pheromones have been detected during the hatching of crab embryos and seem to produce the same behavior (abdominal flapping; Forward et al. 1987, Rittschof et al. 1989, Saigusa 1996). The effect of this additional factor is obvious when water from late stage embryos is pumped into the embryo mass of brooding females carrying early stage embryos. Furthermore, the presence of this unknown compound could also be responsible for the negative effect in flapping frequency, when water containing no embryos or early embryos at high pO_2 was pumped into the embryo mass of brooding females. We hypothesize that this same compound, at higher concentrations, may trigger the behavior adopted by females during larval hatching (Tankersley et al. 2002). The presence of 2 different factors affecting female brooding behavior is also suggested by the natural patterns of oxygen availability and flapping frequencies reported above. If pO_2 was the only factor triggering female behavior, then oxygen availability should show a cyclic pattern (or at least some peaks of low pO_2) also during late development. Biological rhythms may not be a plausible explanation for the changes in female behavioral patterns throughout development since abdominal flapping frequency in females carrying early embryos were modified experimentally.

Finally, it is important to emphasize that the results of this study support previous findings suggesting that oxygen is a limiting factor in the egg masses of several taxa of marine invertebrates. This implies that oxygen provision to the embryos may be an important factor shaping life history patterns and one of the critical factors determining parental investment among marine invertebrates. Parental investment in relation to oxygen provision to embryos may range from no investment in ventilation (broadcasting, as the simplest way to provide oxygen to a large number of embryos) to investment in extraembryonic material (Chaffee & Strathmann 1984, Lee & Strathmann 1998), or in active brooding behaviors that can help to provide oxygen to the embryos (Naylor et al. 1999a). Thus, patterns of oxygen provision and the adjustment of oxygen provision according to embryo demand may vary greatly among taxa. In many taxa of marine invertebrates, investment in extraembryonic material, i.e. gel that helps oxygen diffusion, can be substantial and is assigned during egg deposition (Perron 1981, Defreese & Clark 1983). Taxa that show more active brooding behavior related to oxygen provision (e.g. brachyuran crabs: Fernández et al. 2000; octopuses: Voight & Grehan 2000) may be able to change their energy allocation to ventilation depending on embryo demand and environmental conditions that affect oxygen consumption of the embryos (e.g. temperature; Clarke 1982). Among brachyuran crabs, investment in brooding may be substantial, especially for late stage embryos (a 2-fold increase in oxygen consumption was found in brooding females when compared to non-brooding females; Fernández et al. 2000, Baeza & Fernández 2002). In light of these results, the current approach to studying the investment in reproduction among marine invertebrates, i.e. ratio of reproductive biomass, gonad or egg mass weight to the mass of the parent, regardless of the mode of development of the embryos, seems not to be appropriate and may require urgent revision.

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