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Life on the edge: incubation behaviour and physiological performance of squat lobsters in oxygen-minimum conditions

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ABSTRACT: Squat lobsters frequently inhabit low-oxygen waters, facing particular physiological challenges. *Pleuroncodes monodon* inhabits one of the most extreme oxygen minimum zones in the world's oceans at low temperatures, but avoids high temperature hypoxic waters. The present study aimed to determine whether the maternally dependent reproductive outcome is compromised under realistic oxygen/temperature conditions (normoxia and 0.7 mg l^{-1} at 11 and 15°C) and to evaluate some potential metabolic bases. Females incubated for a significantly longer time at low as compared to high temperatures, but reproductive success was only compromised under hypoxic conditions. Brood viability and synchrony were affected by temperature and its interaction with oxygen concentration (especially under hypoxic conditions and 15°C). Non-viable larvae were hatched at hypoxia-15°C, and larvae hatched in hypoxia-11°C did not survive until moulting. Under normoxic conditions, ventilation of the brood mass decreased with advancing embryo development, but remained high or increased under hypoxic conditions, especially at high temperatures. After releasing their broods, females from all treatments had developing oocytes in their ovaries, but the proportion of oocytes in secondary vitellogenesis was larger at 15°C. The diameter of oocytes in secondary vitellogenesis was significantly smaller in hypoxia treatments. Oxygen consumption of ovigerous *P. monodon* was generally higher at 15°C, especially at normoxia, and their critical point was significantly larger at 15°C. Under hypoxic conditions, ovigerous females compensated their energetic requirements using anaerobic pathways (increase of pyruvate kinase:citrate synthase ratio and lactate). This suggests that this and other species living in hypoxic waters might suffer severe challenges in a warming ocean.

KEY WORDS: *Pleuroncodes monodon* · Hypoxia · Temperature · Incubation behaviour · Physiological performance · Reproductive potential

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1. INTRODUCTION

The oxygen concentration in ocean waters is normally above 8 mg O_2 l^{-1} (Talley et al. 2011), but in certain areas of fjords, basins, marginal seas and oxygen minimum zones (OMZs) sensu stricto, oxygen concentrations can drop down to 1.4 mg O_2 l^{-1} and to completely anoxic conditions. Oxygen depletion represents a restriction for most animals (Levin 2003, Vaquer-Sunyer & Duarte 2011, Gilly et al. 2013), because it might limit oxygen uptake rates, and therefore, limit the aerobic energy production rates that allow the organism to meet the basal energy demands for structural integrity and survival.

Uptake and internal oxygen delivery are dependent on concentration gradients (Willmer et al. 2005), so organisms that permanently inhabit OMZs are challenged to enhance energy provision under low oxygen conditions. A common characteristic of organisms in OMZs is their capacity to regulate oxygen uptake rates, so they remain largely independent from environmental oxygen concentrations, down to very low levels (Seibel 2011). Ectothermic organisms that regulate their oxygen uptake rates are known as oxyregulators (Pörtner & Grieshaber 1993). Different adaptations contribute to that end (e.g. high ventilation abilities and/or large gill area to organism volume, among others; Seibel 2011). Nevertheless, as environmental oxygen drops, there is a concentration, known as critical oxygen pressure (P_{crit}), below which basal uptake rates are no longer attainable leading to the onset of anaerobic metabolism (Grieshaber et al. 1994). In general, while the onset or upregulation of anaerobic metabolic pathways below critical oxygen tensions is another common feature among OMZ organisms, its contribution to meet total energetic demands is modest, allowing bursts of activity or temporal exposure to extreme conditions rather than long-term endurance (Seibel et al. 2018). Finally, the reversible metabolic depression, that is, the reduction of total basal metabolic demand, is another common feature of organisms temporarily exposed to extreme hypoxia (Seibel & Childress 2013, Seibel et al. 2018).

Oxygen requirements of marine animals increase with higher temperatures, and hypoxia tolerance of marine ectotherms narrows when the temperature increases beyond the optimum of aerobic performance (Pörtner & Farrell 2008). Therefore, the P_{crit} is variable and depends on both biological conditions (species, sex, reproductive status and ontogeny) and environmental conditions (Whiteley & Taylor 2015). Interactive effects between hypoxia and temperature influence the reproductive success of marine ectotherm invertebrates and consequently their fitness (Newell & Northcroft 1967, Newell & Branch 1980, Grieshaber et al. 1994). Negative effects of hypoxia at high temperatures have been described for gametogenesis, number and quality of sperm and egg, reproductive behaviour, duration of development, hatching (Fernández et al. 2006, Wu 2009) and larval survival (Yannicelli et al. 2012).

Brood incubation is a reproductive strategy with an associated energetic cost that might affect female body condition (Bosch & Slattery 1999, Fernández et al. 2000). For instance, in subtidal brachyuran crabs, oxygen consumption of incubating females displaying active brood care was significantly higher than that of non-brooding females, and it also varied among females carrying embryos at different developmental stages (Baeza & Fernández 2002). In addition, maternal expenditure in movements/behaviours for oxygen supply to the embryo mass showed a positive relationship with temperature and the frequency of events associated with parental care (Brante et al. 2003). For brooding decapod crustaceans, abdominal flapping, pereopod probing and pleopod movements are common behaviours related to oxygen supply to egg masses (Fernández et al. 2002, Fernández & Brante 2003, Baeza et al. 2016). Oxygen consumption increases as eggs develop and, in several crustacean species that inhabit normoxic and/or temporarily hypoxic environments, they oxyconform (decrease oxygen consumption as environmental oxygen decreases). Hypoxia can occur in the centre of the brood mass, with strong gradients in normoxic environments (Fernandez & Brante 2003). Nevertheless, there is a lack of information about incubation in crustacean species that live in the OMZ (permanent natural hypoxia) and how temperature/oxygen interaction affects gametogenesis, embryo development and metabolic rates, active brood care and metabolism (aerobic and anaerobic) and general condition of incubating females.

Some of the most common crustacean species reported from low-oxygen waters around the world are squat lobsters from the taxa Galatheoidea and Chirostyloidea (Lovrich & Thiel 2011). Amongst them, red squat lobsters *Pleuroncodes monodon* (Galatheoidea: Munididae) from the continental shelf of the Humboldt Current System can inhabit the most extreme conditions (Gallardo et al. 2004). This species has historically sustained a valuable fishery in central Chile (30–37° S) (Palma 1994). It is distributed in the eastern Pacific from Mexico to Chile (Franco-Meléndez 2012) and has a wide phenotypic plasticity with a pelagic

(6–25° S) and a benthic adult form (25–37° S) (Haye et al. 2010). Benthic populations are often associated with water temperatures from 11–12°C and oxygen concentrations below 3.00 and down to 0.16 ml l^{-1} (Gallardo et al. 2017). Ovigerous females (OFs) of benthic populations inhabit more oxygenated waters, and the peak reproductive activity coincides with the seasonal weakening of the OMZ influence over the continental shelf (Gallardo et al. 2017). The reproductive period extends over several months, and females produce new broods within a few days after larval hatching under normoxic conditions in laboratory environments (Thiel et al. 2012). To date, 2 contrasting hypotheses have been proposed to explain the shallower distribution of benthic OFs during the reproductive season: coastward migration to favour larval survival after hatching in richer, more oxygenated and protected areas (Palma & Arana 1997), and the potential benefit of brood and (parallel) gonad development under more oxygenated conditions (Gallardo et al. 2017). Meanwhile, pelagic populations inhabit mainly waters above the oxycline, at latitudes where low oxygen water temperatures normally reach 15–16°C (Gallardo 2017). In cold waters, *P. monodon* survives extreme hypoxia for a few hours (Kiko et al. 2015), and the oxygen tension corresponding to the P_{crit} of routine metabolic rates of adults of the close relative *P. planipes* doubles as temperatures rise from 11 to 20°C (Quetin & Childress 1976). This suggests that under high temperatures/hypoxia, process of high energy-demand could be limited during the maternal phase. However, this has never been assessed at temperature/oxygen conditions relevant and realistic for *P. monodon*.

In the present study, we aimed to determine whether female reproduction is compromised under any realistic oxygen/temperature condition and to evaluate some potential metabolic bases. Theoretical considerations and organism distribution suggest that hypoxia, especially at high temperatures, could exert a significant effect (1) on the current reproductive success of OFs through its effect on embryo development until hatching and larval viability, and (2) on future reproduction, through its modulation of female post-hatching condition and gametogenesis. Viable populations do not develop at oxygen tensions that remain permanently below the P_{crit} (routine metabolism), so if our hypothesis is true, only oxygen uptake by brooding females (but not by males or non-brooding females) should show a decrease at natural hypoxia values, and oxygen uptake rate by the egg mass should be efficient even below that level.

2. MATERIALS AND METHODS

In order to accomplish our objective, we conducted both long- and short-term incubation experiments. We maintained reproductive females throughout the incubation period in hypoxic/normoxic waters (0.7 and 8.0 mg O_2 l^{-1}, respectively) at different temperatures (11 and 15°C) (in a fully factorial experimental design) to describe embryo developmental success together with brooding behaviour, final condition of both female and egg mass, gonad development and metabolic potential of *Pleuroncodes monodon* females after larval hatching (long-term incubation experiment). This experiment served to compare brooding behaviour, embryo and gonadal development as well as final female and embryo physiological and proximate composition conditions among treatments. In a short-term experiment, we evaluated the rate of oxygen consumption and metabolic potential of OFs, brood masses, non-reproductive females and males (each individually) at different oxygen concentrations and temperatures. The duration of this experiment depended on the change of slope in respiration curves and served to determine P_{crit} of each sex/stage in each experimental condition.

2.1. Long-term experiment

2.1.1. Collection and maintenance

Adult specimens of *P. monodon* were collected in northern-central Chile at 30° S, by modified Agassiz trawl at depths ranging from 125 to 150 m. Squat lobsters were immediately placed in a plastic container for transportation to the laboratory at the Oceanolab, Universidad Católica del Norte, Coquimbo, Chile. Adults were collected in September 2015 for the treatments at 11°C and in January 2016 for the treatments at 15°C. In order to avoid problems associated with previous breeding/environmental influence on initial female condition, non-brooding females were acclimated (high oxygen concentrations, at 13–15°C, and food ad libitum for 15–30 d). Males were then introduced into a common female aquarium (3 m^3) to mate, and approximately 48 h later, once the brood had been extruded, 32 healthy females with all their appendages and of similar size range (Table 1) were placed in individual containers (20 × 20 × 20 cm; 8 l) to begin long-term incubations. Experimental conditions were (Table 1) hypoxia (H: 0.7 ± 0.1 mg O_2 l^{-1}) and normoxia (N: 8.0 ± 1.0 mg O_2 l^{-1}) at 2 near constant temperatures (11 ± 0.5°C and 15 ± 0.8°C). Here-

Table 1. Number of initial and final replicates. Mean ± SD size (CL: carapace length) of ovigerous female squat lobsters by treatment, and temperature and oxygen conditions in the long-term experiment. H: hypoxia; N: Normoxia

Treatment	n		CL (mm)	Temp. (°C)	Oxygen conc. (mg O_2 l^{-1})
	Initial	Final			
H11	8	8	27.9 ± 3.2	11.1 ± 0.4	0.7 ± 0.4
N11	8	7	29.7 ± 2.5	10.8 ± 0.3	7.9 ± 1.7
H15	8	4	34.3 ± 1.8	15.4 ± 0.9	0.8 ± 0.6
N15	8	8	34.4 ± 1.1	15.2 ± 0.9	6.8 ± 1.5

after, treatments will be referred as H11, H15, N11 and N15.

2.1.2. Experimental design and set-up

Carapace length (CL, mm) and initial mass (g) were measured just before the animals were placed in individual aquaria under controlled conditions. Females were maintained in the experiment until the larvae hatched, the brood was lost, or the female died. Near constant conditions were kept in the sealed individual aquaria using a flow-through system. Seawater was filtered (<0.5 µm and UV-sterilized) and brought to stock tanks in the controlled temperature room where it acquired the experimental temperature. Normoxic water was gently flushed to the individual aquaria directly from the stock tanks. Hypoxic water was prepared at 0.7 mg O_2 l^{-1} with a range between 0.5 and 1 mg O_2 l^{-1}, which was achieved by bubbling 8 holding tanks (50 l each) with a carbon dioxide–nitrogen mix (0.5–999 ppm) manufactured by INDURA (www.indura.cl). This mixture was used to maintain the pH similar between oxygen treatments and avoid possible bias of contrasting pH values in the experimental treatments. The final mix was estimated based on preliminary trials and on the literature (Torres et al. 2013). Once the desired oxygen concentration was reached, water from the hypoxic tanks was isolated from the surrounding air with a floating lid. Water was injected into each aquarium with a pump system at a flow rate of 0.0512 l min^{-1} for 15°C and 0.0466 l min^{-1} for 11°C. Eight OFs maintained in individual aquaria were used for each treatment (H11, N11, H15 and N15). OFs were fed every second day with a paste prepared with flake food for fishes and water; during each feeding, each female received a portion of about 0.125 g (wet mass). Aquarium water change and removal of food leftovers was done the day after feeding.

Each aquarium had a chemical optical (non-invasive) oxygen sensor spot, specific for each oxygen treatment. The O_2 sensor used for the hypoxia treatment was a PSt6 sensor (PreSens) with a measurement range between 0 and 1.8 mg O_2 l^{-1} (±0.010 µmol), and the one used for the normoxia treatment was a PSt3 sensor (PreSens) with a measurement range between 0 and 45 mg O_2 l^{-1} (±0.14 µmol). Oxygen concentration and temperature in each aquarium were monitored using a Fibox 4 (PreSens) every 2 h (between 13:00 and 21:00 h). Fibox 4 uses a fibre optic compatible with the oxygen sensor spot (Fig. 1).

2.1.3. Embryo development time and index of brooding success

Embryo samples (15–25 embryos) were taken randomly from every brood every 4 d to determine the development stages of the embryos following Palma (1994), who defined 5 embryo stages, although in this study, stage V (pre-zoea) was not considered. Stage I is characterized by yolk evenly distributed throughout the embryo. In stage II, cell differentiation is initiated and the yolk is yellowish and grainy. Stage III is defined by the appearance and pigmentation of the ocular structures. In stage IV, the eyes appear completely pigmented and positioned obliquely in the anterior region of the embryo with developed structures. Stage IV is also characterized by the presence of red chromatophores in the dorsal portion of the embryo. We also identified a non-developing embryo category, which is an egg that had arrested development, and appeared completely white (Fig. S1 in the Supplement at www.int-res.com/articles/suppl/m623p051_supp.pdf).

The total time of development was estimated as that from the beginning of incubation until we observed the first hatched larvae. Assumptions of normal distribution and homogeneity of variances were checked using QQ plots and Levene's test (library 'car' 2.1-6; https://r-forge.r-project.org/projects/car/v2.1-6), respectively. A 2-way ANOVA was applied to evaluate the effect of temperature and oxygen conditions, and their interaction, on the total time of embryo development; statistical significance was set at $\alpha = 0.05$. Females that died during the experiment were excluded from this statistical analysis.

In order to determine the brooding success of incubating females, we registered 12 observations and tabulated 0/1 binary variables (Table 2) for each female. For the assigned binary value, 1 was considered

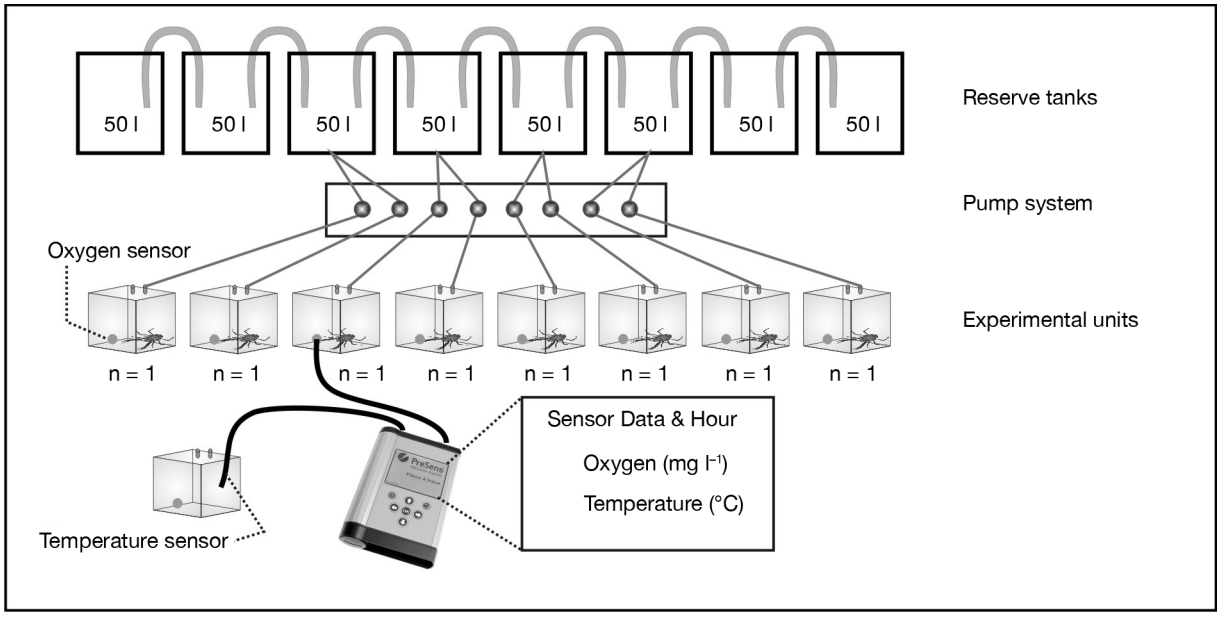

Fig. 1. Set up for the long-term experiment to describe embryo developmental success together with brooding behaviour, final condition of both female and egg mass, gonad development and metabolic potential of *Pleuroncodes monodon* females after larval hatching

as favourable and 0 as not favourable to reproductive success. All females were included to construct an index of brooding success (Table S1 in the Supplement), and females that died before larval hatching received a value of 0 for the related variables. A generalized linear mixed model (GLMM) test was done to estimate reproductive success for each treatment. After backward elimination, the best model was chosen using Akaike's information criterion (AIC). The explained variance (D^2 or pseudo R^2) was calculated following the formula proposed by Zuur et al. (2009). This analysis was done using R statistical software (R Core Team 2015).

2.1.4. Behavioural analysis of brooding females

In order to determine the behaviour of incubating females, these were video-recorded every 4 d for approximately 30 min using a GoPro Hero video

Table 2. Criteria used to build the index of brooding success in squat lobsters. The responses of each criterion are binary: favourable (1) and not favourable (0)

Criterion	Favourable (1)	Not favourable (0)
Non-developing embryos in the embryo mass at any time of development	Absence	Presence
Total number of developmental stages present at the last embryo mass observation	If 1 or 2 stages	If ≥3 stages
Most frequent stage in embryo mass prior to (2 or 3 d before) larval release	If most frequent is >stage III	If most frequent is <stage III
Presence or absence of infections in embryo mass at any time	Absence	Presence
Percentage of embryo stages I–III in embryo mass at the observation prior to (2 or 3 d before) larval release	<50%	>50%
Most frequent stage in embryo mass at larval release	Otherwise	If most frequent is <IV
Presence or absence of incompletely hatched larvae in embryo mass	Absence	Presence
Presence or absence of swimming larvae	Presence	Absence
Presence or absence of dead larvae on the second day after hatching	Absence	Presence
Presence or absence of dead larvae before the first moult	Absence	Presence
Female survival before larval release	Alive	Dead
Female survival after hatching	Alive	Dead

camera placed in front of each aquarium. Videos were taken only at night to avoid disturbance in the laboratory. Based on preliminary observations, 3 common behaviours were identified: (1) pleopod movements, (2) abdominal flapping and (3) pereopod probing (Fig. S2 in the Supplement). These behaviours were previously described for brachyuran crabs (Baeza & Fernández 2002) and the Caribbean spiny lobster (Baeza et al. 2016).

'Pleopod movements' involve moving the embryo mass up and down or side to side using the pleopods. These movements are visible as individual movements of the entire embryo mass, and so each coordinated beat of the pleopods corresponds to 1 pleopod movement, i.e. 1 event. In order to analyse pleopod movements, each 30 min recording was divided into non-effective time and effective time of video (ETV). The ETV was the time that allowed for the adequate observation of this female behaviour (i.e. female facing the camera). The number of pleopod movements min^{-1} of ETV was calculated on videos with ETVs longer than 10 min (33 videos out of 107).

'Abdominal flapping' involves moving the abdomen up and down, either entirely or partially, when females are standing on their pereopods, elevated above the substratum. Each abdominal flap, which can occur once or several times in sequence, is counted individually as an event.

In the state 'pereopod probing', females use the 5^{th} pair of pereopods to probe and slightly shake the embryo mass. This state begins when the female starts to touch the embryo mass with its 5^{th} pair of pereopods, and ends when the female pulls the pereopods back out of the embryo mass. In this case, the duration of pereopod probing was measured over each observation period. Abdominal flaps and pereopod probing were visible throughout the observation period, but pleopod movements were only visible when the females were facing the camera. To analyse abdominal flapping, the number of events min^{-1} of video was counted. In case of pereopod probing, the duration of the state was quantified as the percentage of time during the 30 min of observation.

Behaviour was evaluated throughout embryo mass development (Table S2 in the Supplement). As development time changed with temperature and oxygen concentration, comparisons among treatments must rely on comparison of similar developmental stages of the brood mass. Stage I corresponds to the first observation (Day 4) of incubating females, stage II and stage III were considered when >50% of the brood mass was in stage II or III, respectively, and stage IV when >50% of the embryo mass was in stage IV or V.

Statistical analyses of incubating behaviour were made excluding dead females: 2 OFs from treatment H15 and 1 from N11. In order to evaluate the effects of extrinsic (oxygen and temperature) and intrinsic (embryo developmental stage) factors, and their interaction, on the presence/absence of a specific incubating behaviour at any time of each video recording (pleopod movements, abdominal flapping, pereopod probing) among OFs, a GLMM analysis with a binomial link function was conducted. All variables (extrinsic and intrinsic) were integrated in an additive and additive-multiplicative model to determine the significance level for each one; AIC and D^2 were also calculated.

The frequency of the pleopod movements, and their relationship with experimental factors (categorical variables), was analysed using a GLMM with gamma distribution link function. Models with additive and additive-multiplicative terms were tested to define the best model, and the explained variance was calculated. The frequency of abdominal flaps, and their relationship with experimental factors, was analysed using a general linear model (GLM), where the number of flaps was a continuous variable (gamma distribution link function). After backward elimination, the best model was chosen using the stepwise AIC, and the D^2 was calculated.

The percentage of pereopod probing (total time of state in 30 min of observation) was analysed following the suggestions proposed by Zar (2010) for populations with distributions that strongly differ from normal and with different distributions and variances, which consists of reporting the mean and variance for each treatment. All analyses were done using the R statistical software (R Core Team 2015).

2.1.5. Biochemical analyses

Biochemical traits of females were measured on the abdominal muscle at the end of the incubation period. They consisted of the determination of proximate composition (total proteins, carbohydrates and lipids), apparent specific activity of the key enzymes citrate synthase (CS) and pyruvate kinase (PK), respectively associated with aerobic and anaerobic metabolism, and the metabolic end product lactate. Additionally, as an indicator of potential cell stress, levels of the 70 kD heat-shock protein (HSP70) were determined in female gills.

To quantify total carbohydrates and lipids, female abdominal muscles were dried to a constant mass at

60°C. Dry tissues were then pulverized in a mortar and homogenized with deionized water at a proportion (w/v) of 1:1 for carbohydrates and 4:1 for lipids and proteins. The phenol-sulphuric acid method described by Dubois et al. (1956) was used for total carbohydrate determinations. Its concentration was determined in a spectrophotometer (Variant Cary UV) at 490 nm using a solution of glycogen in deionized water at a concentration of 50 µg ml^{-1} as a standard. The procedure used by Mann & Gallager (1985) was followed for lipid determinations. A double extraction with chloroform-methanol and further purification with NaCl was performed. The sample was read in a spectrophotometer at 520 nm using a solution of cholesterol in chloroform-methanol (1:2) at a concentration of 0.8 mg ml^{-1} as a standard.

Total protein was quantified in 0.03 g (wet weight) of abdominal muscle and gill (for HSP70 determinations) samples, following Brokordt et al. (2015). Tissues were homogenized in 150 µl of homogenization buffer (32 mM Tris-HCl at pH 7.5, 2% SDS, 1 mM EDTA, 1 mM Pefabloc and 1 mM protease inhibitor cocktail; Sigma). The homogenate was incubated for 5 min at 100°C, then re-suspended in 100 µl of homogenization buffer and re-incubated at 100°C for 5 min. The homogenate was centrifuged at 10 600 × *g* for 20 min. Total protein was quantified in an aliquot of the supernatant with a Micro-BCA kit using a microplate spectrophotometer EPOCH (BioTek).

HSP70 was measured in the gill tissue of females by ELISA following Brokordt et al. (2015), and validated in previous tests by comparing ELISA results with immunoprobing of Western blots. Western-blot analyses (using the same antibodies described later) showed only 1 band at the level of HSP70. Total protein (30 µg ml^{-1}) was diluted in 0.05 M carbonate-bicarbonate buffer at pH 9.6, and 50 µl of sample per well were incubated in an ELISA plate overnight at 4°C with 3 blanks (containing buffer only) and various concentrations of cognate HSP70 (H8285, Sigma) to generate a standard curve. The plate was washed twice with phosphate-buffered saline (PBS; 200 µl $well^{-1}$). Next, 200 µl of blocking buffer (PBS + 5% skim milk) were added to each well and incubated for 2 h. The wells were washed again with PBS. Subsequently, 100 µl of the primary antibody (polyclonal mono-specific anti-epitope) that recognizes the inducible and constitutive forms of HSP70, developed in immunized mice with a synthetic peptide epitope (Group of Immunological Markers on Aquatic Organisms, Catholic University of Valparaiso), diluted 1:400 in blocking buffer + 0.05% Tween-20, were added to each well, and the plate was incubated overnight at 4°C. The plate was then washed 4 times with PBS, incubated with goat anti-mouse IgG (Thermo Scientific) secondary antibody, diluted in blocking buffer + 0.05% Tween-20 for 2 h at 25°C and washed again 4 times with PBS. Next, 100 µl of substrate solution (10 mg *o*-phenylenediamine dihydrochloride in 25 ml of 0.05 M citrate phosphate buffer) were added, followed by incubation of the plate for 30 min at 25°C. Finally, the plate was read at 450 nm in a microplate spectrophotometer (EPOCH, BioTek). The absorbance of the sample was corrected by the mean absorbance of the blanks and divided by a conversion factor that was estimated from a linear regression curve of cognate HSP70. The calculated result was the concentration of HSP70 in µg mg^{-1} total protein.

The PK:CS activity ratio per female was calculated in order to evaluate the relative predominance of aerobic or anaerobic pathways, considering that CS represents the potential for aerobic metabolic pathways, while PK activity represents the potential for fermentative pathways. For CS and PK determination, samples of abdominal muscle were homogenized on ice in 10 volumes of homogenizing buffer (50 mM imidazole-HCl, 2 mM EDTA-Na_2, 5 mM EGTA, 1 mM dithiothreitol, 0.1% Triton X-100, pH 6.6 or 7.2, respectively for PK and CS). The homogenates were centrifuged at 4°C for 15 min at 600 × *g*. Conditions for enzyme assays were adapted from those used by Brokordt et al. (2000) as follows (all concentrations in mM) for CS: Tris-HCl 75, oxaloacetate 0.3 (omitted for the control), DTNB 0.1, acetyl CoA 0.2, pH 8.0; and for PK: imidazole–HCl 50, $MgSO_4$ 13, KCl 100, phosphoenolpyruvate 5 (omitted for the control), ADP 5, NADH 0.2, excess lactate dehydrogenase, pH 6.6. Enzyme activities were measured at controlled room temperature (20°C) using a microplate spectrophotometer (EPOCH, BioTek) to follow the absorbance changes at 412 nm to detect the transfer of sulphydryl groups from CoASH to DTNB for CS, and that of NAD(P)H at 340 nm for PK. The molar extinction coefficients used for DTNB and NAD(P)H were 13.6 and 6.22, respectively. All assays were run in duplicate, and the specific activities are expressed in international units (IU, µmol of substrate converted to product per min) per gram of abdominal muscle mass. Finally, quantitative determination of lactate in samples of abdominal muscle was achieved with an enzymatic colorimetric kit (Spinreact) following the manufacturer's instructions.

Statistical analyses of all biochemical traits were made only for females that completed their brooding period until hatching. QQ plots and Levene's test (library 'car' 2.1-6) were used to check for normal

distribution and homogeneity of variances. A 2-way ANOVA was done in order to determine if oxygen and temperature conditions, or their interactions, explained the variance of proteins, lipids, carbohydrates, PK, CS, PK:CS ratio and lactate in abdominal muscle, and HSP70 levels in gills of incubating females. Tukey's HSD test was used in each case to find specific differences among treatments.

2.1.6. Ovary development

Differences in gonad development between females reared in the different treatments were evaluated by comparing the relative frequency of oocytes in primary and secondary vitellogenesis in each gonad, and also by comparing the turning size of oocytes from primary to secondary vitellogenesis. After larval hatching, female ovaries were fixed in Davidson's solution for 24 h and transferred to 70% ethanol, dehydrated and stained with haematoxylin-eosin. A 5 µm slice of each gonad (through its centre) was prepared for optical microscope analysis. First, oocytes were identified as being in primary or secondary vitellogenesis following the descriptions of Kronenberger et al. (2004) (for *Galathea intermedia*) and Moreno et al. (2012), where oocytes in secondary vitellogenesis differ from those in primary vitellogenesis by the presence of granular yolk (Kronenberger et al. 2004) (Fig. S3 in the Supplement). The number of oocytes in each stage was counted across the histological preparation. The diameters of oocytes that presented visible nuclei were then measured to the nearest 0.01 µm at 50× magnification, after categorizing them into primary or secondary vitellogenesis. A *G*-test was conducted to compare if the proportion of oocytes in primary or secondary vitellogenesis was homogeneous between treatments (Sokal & Rohlf 1995), and afterwards a pairwise *G*-test was applied using the R statistical software (R Core Team 2015). As a measure of oocyte development between treatments, we compared the size at which 50 and 95% of oocytes had reached the secondary vitellogenesis stage. First, the size frequency distribution of oocytes in primary and secondary vitellogenesis was built for each treatment. Following a multi-model comparison approach, the minimal size of secondary oocytes was estimated as the size at which 50 and 95% of observed oocytes had developed the granulated yolk. Hypoxia vs. normoxia treatments were compared for each temperature independently. The difference in the AIC (Zuur et al. 2009) resulting from adjusting a single binomial model to all data at a temperature (with 2 parameters) vs. 2 models at each temperature (1 for each oxygen level, 4 parameters total) was used to choose the best model (Johnson & Omland 2004).

2.2. Short-term experiment

Short-term incubations were conducted to evaluate whether the oxygen consumption rates of adults in different reproductive conditions responded similarly to environmental conditions. Ovigerous females (OFs, carrying their brood), females after brood removal (FABRs), embryo mass (or brood), non-reproductive females (or non-OFs) and males were tested. Organisms were collected in October 2016 and maintained as in the long-term experiment. Nine individuals of each adult type and 9 embryo masses were evaluated under each condition by placing them in individual closed chambers (8 l aquaria). Before the experiment, adults were placed in individual aquaria at the experimental temperature in the controlled temperature room and starved for 24 h. Since in closed respirometry, the time to reach hypoxia might influence the rate of oxygen consumption even in starved organisms, 2 starting oxygen conditions were considered for the 24 h acclimation and experimental conditions: 8.0 and 2.5 mg O_2 l^{-1}. Hypoxic tensions were accomplished as described for the long-term experiment. During the acclimation period, water flow was kept through the aquaria with the corresponding initial temperature and oxygen conditions. Measurements started when the flow was shut down and the first oxygen determination was made. Non-intrusive measurements of oxygen concentration in the sealed aquaria were conducted with Fibox 4 (PreSens) every hour until oxygen concentrations of 3.0 mg O_2 l^{-1} (for normoxia treatment) and 0.05 mg O_2 l^{-1} (for hypoxia treatment) were reached in the chambers.

Oxygen sensors and spots for the hypoxia/normoxia treatments were the same as in the long-term experiment. Wet masses of individuals or broods were measured after the experiment. The embryo masses were removed from the OFs by using a delicate paintbrush, and placed inside 100 ml Winkler bottles. Measurements were done with non-invasive respirometry using spots previously calibrated on each bottle without a period of acclimation, since the Winkler bottles were filled with the same water the embryos had been exposed to when attached to their mothers. After brood removal, females were subject to the same procedure of acclimation and measurements as they had previously experienced as OFs. Data were analysed independently for each stage/sex. Two control chambers with only water with

identical initial conditions as those of the experimental treatments were run in parallel with each stage/sex trial.

Respiration rates were expressed as mg O_2 h^{-1} $ind.^{-1}$ and mg O_2 h^{-1} g^{-1} (wet weight). The slope of the oxygen concentration over time was used for the estimation and referred to the mean oxygen concentration over the period of constant consumption (linear decrease). The average consumption from the control chamber was subtracted. In all cases, consumption was fairly constant except at very low oxygen levels. Since mass-specific consumption rate is dependent on individual mass, an ANCOVA was applied on adult rates to evaluate the effect of oxygen concentration (continuous variable) and temperature (categorical variable) with individual mass as a co-variable. Previously, the significance of individual mass as the explanatory variable was checked as well as the differences between treatment-mass slopes with a homogeneity of slopes models. In the case of significant interactions between the co-variable with any other factor, a separate slope model was conducted instead of an ANCOVA. To evaluate brood consumption rate dependency on oxygen concentration (continuous variable) and temperature (categorical variable), a GLM was applied.

For OFs (no change in slope was observed for the other experimental groups), the P_{crit} for each temperature was estimated by adjusting a polynomial regression to oxygen consumption as a function of oxygen concentration. P_{crit} corresponds to a change of slope in the response variable as a function of the independent variable. The regression was fitted with library 'ggplot2' (version 2.2.1; http://cran.r-project.org/web/packages/ggplot2/), function 'geom_smooth' and method 'LOESS' (local polynomial regression fitting). This allowed us to determine the breakpoint (P_{crit}), represented by a change of slope in the response variable as a function of the independent variable.

Further biochemical determinations of CS, PK and lactate were conducted for muscle tissue of FABRs, OFs, non-reproductive females and males and HSP70 in FABR gills. Tissues were frozen at −80°C immediately after the experiment. The enzymatic analyses (CS, PK and PK:CS ratio) and lactate concentration were performed following the same methodology described for the long-term experiment.

3. RESULTS

3.1. Long-term experiment: reproductive traits

3.1.1. Embryo development time and index of brooding success

Embryo development time was only affected significantly by temperature ($p < 0.0001$, Table 3). Development time was longer at 11°C (mean ± SD = 41.9 ± 3.2 d) than at 15°C (mean = 24.4 ± 2.8 d) (Fig. 2A) regardless of oxygen conditions. The index of brooding success was also significantly affected

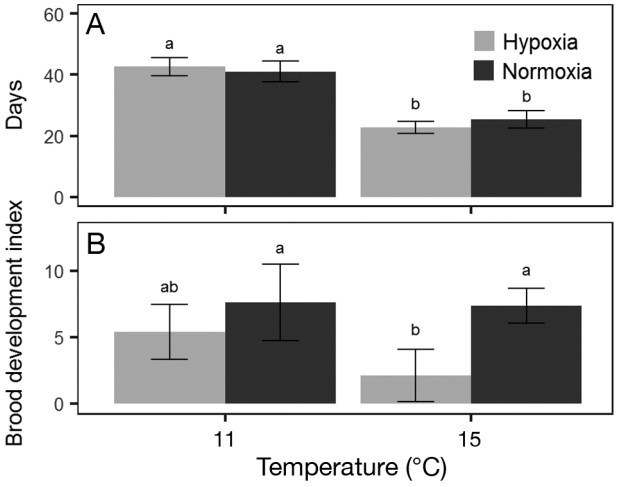

Fig. 2. Mean and SD of (A) embryo development time and (B) index of brooding success of *Pleuroncodes monodon* under different conditions of oxygen and temperature. Index of brooding success is a composite variable based on characteristics describing brood development: the higher the index, the better embryo development (for details, see Table 2 and Table S1). Different letters indicate significant differences at $p < 0.05$

Table 3. Results of the 2-way ANOVA and generalized linear model conducted to test the effect of temperature and oxygen on squat lobster embryo development time and index of brooding success, respectively. Significant values ($p < 0.05$) are highlighted in **bold**

Embryo development time	df	MS	*F*	p
Temperature	1	2074.7	247.826	**<0.0001**
Oxygen	1	0.9	0.102	0.753
Oxygen × Temperature	1	28.6	3.417	0.077
Index of brooding success	Estimate	SE	*Z*	p
(Best model: AIC = 49.5, D^2 = 0.54, residual deviance = 39.5, df = 27)				
Model	1.77071	0.14586	12.139	**<0.0001**
Oxygen	−0.06596	0.20977	−0.314	0.753
Temperature	−121109	0.30448	−3.978	**<0.0001**
Oxygen × Temperature	0.95326	0.38058	2.505	**0.012**

by temperature and decreased from 11 to 15°C (Table 3). A significant interaction between temperature and oxygen conditions was also evident; the index was significantly lower in hypoxia than in normoxia at 15°C but not at 11°C (Table 3, significant interaction term; Fig. 2B). The presence of undeveloped embryos in the brood mass was more frequent at high temperature, especially under hypoxic conditions (Table S1). Asynchrony of brood mass development as indicated by the presence of early stages at, or immediately before, the beginning of hatching, was more frequent at 15 than at 11°C. Three or more developmental stages were more frequently observed at the time of hatching in brood masses in hypoxia at high temperature. The larvae of the hypoxia treatments were not viable: at 11°C, larvae did not reach the first moult because they died within a few days after hatching, while at 15°C, hatching was incomplete and swimming larvae were absent (Table S1).

3.1.2. Incubating behaviour

The number of females that displayed pleopod movements (Table S2) depended on the developmental stage of embryos and its interaction with temperature. At 15°C, the number of females that bore eggs in stage III and performed the pleopod movements was significantly higher in hypoxic conditions (Table 4, Table S2). This behaviour was sometimes displayed by females simultaneously with pereopod probing. In females with advanced developmental stages of embryos, the frequency of pleopod movements was higher in hypoxia than in normoxia at both temperatures ($p = 0.037$ for stage IV; Table 5, Fig. 3).

Abdominal flapping behaviour occurred among females independently from the studied factors (Table 4). Nevertheless, for females that displayed this type of behaviour, abdominal flaps were more frequent at 15°C than at 11°C. At 11°C, the frequency of abdominal flapping was constant throughout incubation and independent of oxygen concentration. Abdominal flapping became less frequent with advancing embryo development in N15, while in hypoxia it remained constant. In females with stage IV embryos, abdominal flapping per minute was an order of magnitude higher in H15 than in any other condition (Table 5, Fig. 3).

The occurrence of pereopod probing decreased as egg mass developed (Fig. 3). However, due to the interaction between developmental stage and temperature, at advanced stages (especially stage III), pereopod probing was more frequent among females at 15°C than among those at 11°C (Table 4). In the early stages of incubation, there was no particular pattern in the duration of this activity among OFs.

3.1.3. Biochemical analyses

Proximate composition of female muscle as well as embryos were largely unaffected by the experimental conditions. Nevertheless, carbohydrate content in

Table 4. Occurrence of different behaviours in ovigerous females of *Pleuroncodes monodon* kept under hypoxia (H) and normoxia (N) at 11 and 15°C, until hatching of larvae. Number of ovigerous females that showed each observed behaviour is given in Table S2 in the Supplement. Significant values ($p < 0.05$) are highlighted in **bold**

	Estimate	SE	*Z*	p
Pleopod movement				
(Best model: AIC = 111, $D^2 = 0.13$, residual deviance = 93.2, df = 97)				
Model	26.992	10.898	2.477	**0.013**
Stage II	−20.517	11.318	−1.813	0.069
Stage III	−28.730	11.963	−2.401	**0.016**
Stage IV	−16.637	11.806	−1.409	0.159
Temperature 15°C	−0.1352	13.755	−0.098	0.9227
Stage II × Temperature 15°C	26.072	18.323	1.423	0.153
Stage III × Temperature 15°C	37.941	18.685	2.030	**0.042**
Stage IV × Temperature 15°C	22.164	18.605	1.191	0.234
Abdominal flapping				
(Best model: AIC = 150, $D^2 = 0.27$, residual deviance = 136, df = 99)				
Model	0.7049	0.5354	1.316	0.188
Oxygen N	−0.1052	0.4687	−0.224	0.822
Stage II	−0.6586	0.6037	−1.091	0.275
Stage III	−0.9848	0.5926	−1.662	0.097
Stage IV	−10.512	0.6301	−1.668	0.095
Temperature 15°C	0.8466	0.4780	1.771	0.077
Pereopod probing				
(Best model: AIC = 13092, $D^2 = 0.42$, residual deviance = 925, df = 97)				
Model	2.115	1.167	1.813	0.069
Stage II	−2.804	1.251	−2.240	**0.025**
Stage III	−3.433	1.356	−2.531	**0.011**
Stage IV	−4.276	1.644	−2.600	**0.009**
Temperature 15°C	−0.392	1.575	−0.249	0.803
Stage II × Temperature 15°C	1.355	1.672	0.810	0.417
Stage III × Temperature 15°C	5.017	1.983	2.530	**0.011**
Stage IV × Temperature 15°C	6.716	2.580	2.603	**0.009**

Table 5. Frequency of different behaviours in ovigerous females of *Pleuroncodes monodon* kept under hypoxia (H) and normoxia (N) at 11 and 15°C, until hatching of larvae. Number of ovigerous females that showed each observed behavior is given in Table S2. Significant values ($p < 0.05$) are highlighted in **bold**

	Estimate	SE	*Z*	p
Pleopod movement				
(Best model: AIC = 71.5, $D^2 = 0.30$)				
Model	0.038686	0.012903	2.998	**0.003**
Normoxia	−0.005477	0.016333	−0.335	0.737
Stage II	−0.009779	0.015814	−0.618	0.536
Stage III	−0.021789	0.013967	−1.560	0.119
Stage IV	−0.021188	0.014161	−1.496	0.135
Normoxia × Stage II	0.017013	0.023555	0.722	0.470
Normoxia × Stage III	0.040189	0.025997	1.546	0.122
Normoxia × Stage IV	0.084291	0.040323	2.090	**0.037**
Abdominal flapping				
(Best model: AIC = 44.5, $D^2 = 0.47$)				
Model	40.184	0.8320	4.830	**<0.0001**
Temperature 15°C	−26.666	0.6949	−3.837	**0.0003**
Normoxia	−0.8597	0.5930	−1.450	0.153
Stage II	−0.0543	0.8332	−0.065	0.948
Stage III	−0.4789	0.6678	−0.717	0.476
Stage IV	−0.2064	0.8766	−0.235	0.815
Normoxia × Stage II	10.121	11.041	0.917	0.363
Normoxia × Stage III	18.883	10.038	1.881	0.065
Normoxia × Stage IV	96.981	42.570	2.278	**0.027**

the abdominal muscle of incubating females of *Pleuroncodes monodon* increased with temperature ($p < 0.0001$; Table S3 in the Supplement, Fig. 4). In the abdominal muscle of incubating females, neither PK, CS nor their ratio depended on experimental conditions (Fig. 5). Lactate concentration in the abdominal muscle of incubating females of *P. monodon* increased only at H15 (Fig. 5). Statistical differences were found between temperature treatments in HSP70 levels in gills of incubating females. HSP70 levels were greater at 11°C than at 15°C ($p < 0.02$; Table S3), but post hoc analysis did not show differences among treatments (Fig. 5).

3.1.4. Ovary development

The frequency of oocytes in secondary vitellogenesis was significantly different among treatments ($G = 42.1$;

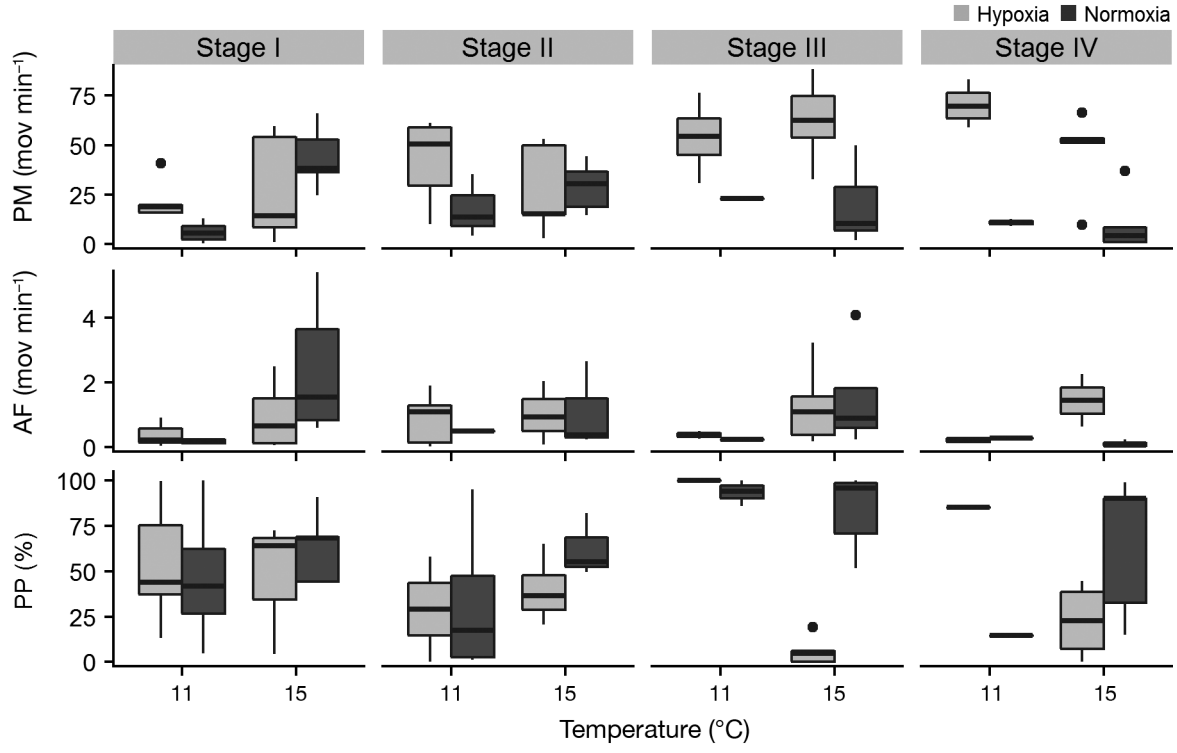

Fig. 3. Frequency of pleopod movements (PM) and abdominal flaps (AF) counted in effective time of video (ETV) during 30 min of observation of ovigerous female *Pleuroncodes monodon*. Pereopod probing (PP) was quantified as the percentage of time that this behaviour occurred during the 30 min of observation. Bar: median; box: interquartile range (IQR): whiskers: min./max. values ≤1.5 × IQR below/above box respectively; dots: outliers

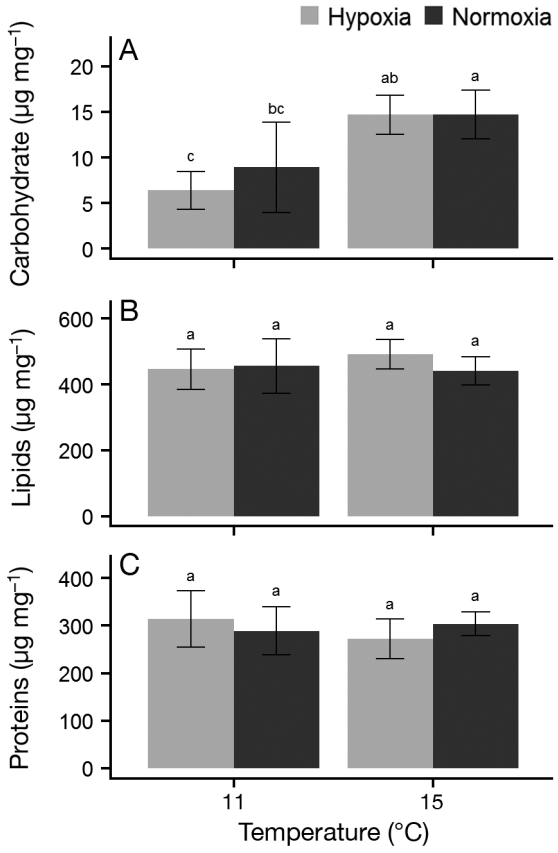

Fig. 4. Contents (µg mg^{-1} of abdominal muscle) of total (A) carbohydrates, (B) lipids and (C) proteins in incubating ovigerous females of *Pleuroncodes monodon* were maintained for the duration of embryo mass development at different conditions of temperature and oxygen (hypoxia: 0.7 ± 0.1 mg O_2 l^{-1}; and normoxia: 8.0 ± 1.0 mg O_2 l^{-1}). Data are means ± SE, different letters indicate statistical differences at $p < 0.05$

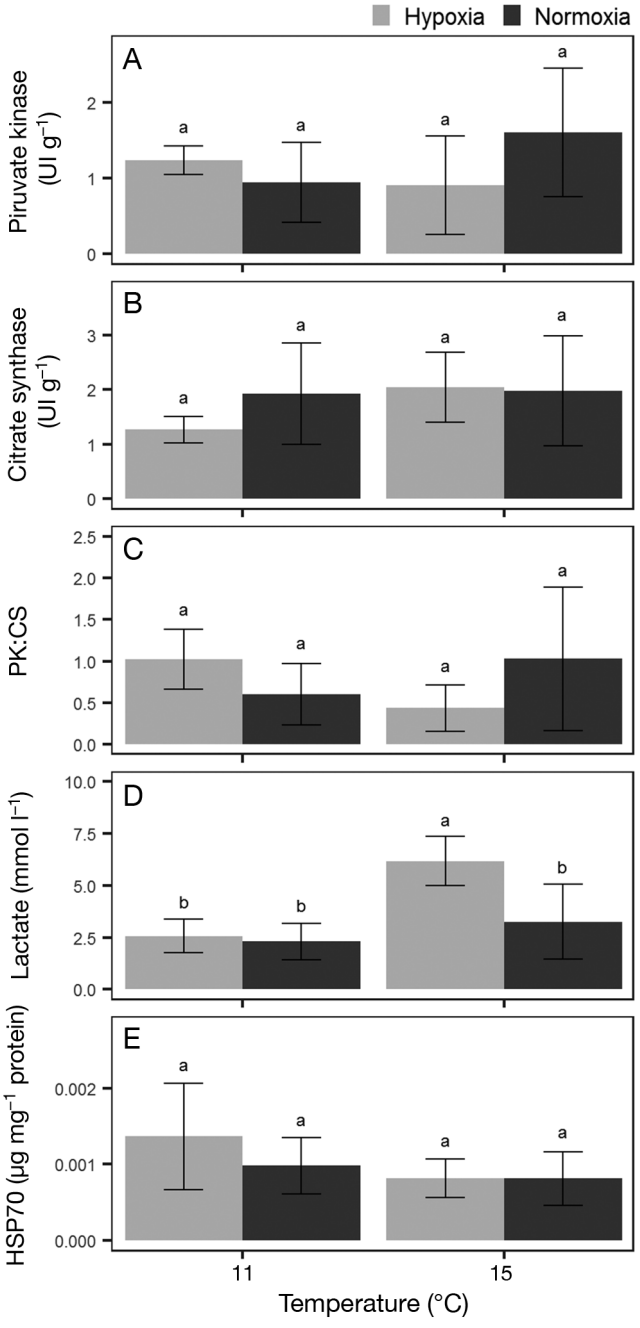

Fig. 5. Mean ± SD (A) pyruvate kinase (PK) apparent specific activity per g, (B) citrate synthase (CS) apparent specific activity per g, and (C) PK:CS ratio in abdominal muscle; and mean ± SD (D) lactate concentration and (E) heat-shock protein 70 (HSP70) levels in gill tissue of incubating females of *Pleuroncodes monodon* at different conditions of temperature and oxygen

df = 3; $p < 0.0001$), with the highest proportion attained at N15 (Table 6B). The size at which 50 and 95% of oocytes I turned into oocytes II showed an important difference in AIC (larger than that corresponding to $p < 0.05$) between normoxia and hypoxia at both temperatures (Table 6C; Fig. S4 in the Supplement). Vitellogenesis II was reached at a diameter 1/5 smaller in hypoxia than in normoxia.

3.2. Short-term experiment: rate of oxygen consumption

Considering individual mass as a co-variable, oxygen consumption rates depended on oxygen concentration and temperature for most adult categories considered (Fig. 6, Table 7). Consumption rates of adults increased with temperature. OFs showed the highest mean respiration rate at 15°C (as compared with other treatments). Data dispersion allowed us to calculate the P_{crit} only for OFs, which was higher at 15°C than at 11°C (~2 and 0.9 mg O_2 l^{-1}, respectively;

Table 6. Ovary development in *Pleuroncodes monodon* females in the long-term experiment. (A) Percentage of oocytes in secondary vitellogenesis (VII). (B) Analysis of frequency *G*-test; values in **bold** indicate significant differences between treatments. Treatments: H11 (H15): hypoxia at 11°C (15°C); N11 (N15): normoxia at 11°C (15°C). (C) Model fitting for comparison of sizes at which 50% and 95% of oocytes I turned into oocyte II

(A)

Treatment	Percentage of secondary oocytes
H11	26.9
N11	30.8
H15	22.5
N15	33.6

(B)

G = 42. 1 df = 3 p < 0.0001

	H11	N11	H15
N11	**0.00425**	–	–
H15	**0.03554**	**2.20×10^{-5}**	–
N15	**0.00033**	**2.20×10^{-5}**	0.521

(C)

Treatment		Model	Size (µm)	Parameter	logL	AIC	AICc
Hypoxia 11°C vs. Normoxia 11°C	1 model	50%	235	2	−1152	2308	2307
		95%	287				
	2 models	50% H	201	4	−843	1695	1690
		95% H	228				
		50% N	248				
		95% N	288				
Hypoxia 15°C vs Normoxia 15°C	1 model						
		50%	245	2	−342.5	693	688.3
		95%	280				
	2 models	50% H	205	4	−222	453.9	449.2
		95% H	224				
		50% N	249				
		95% N	270				

Fig. 6). This P_{crit} observed from respiration rates was accompanied by a higher concentration of lactate at 15°C observed for OFs (Fig. 7D). In the case of females (non-OF and FABR), the effect of oxygen on respiration rates was marginally significant, and for males, the effect of oxygen was observed only at 15°C (Fig. 6, Table 7).

Broods, on the other hand, are oxyconformers, as they responded linearly to oxygen concentration and independently from temperature (Fig. 6). Broods were in stage II of development, so a large part of the eggs had energetic reserves rather than larval tissue. Because the brood mass was <10% of the carrying female biomass, and oxygen consumption rates per brood are similar to those of OF (female+brood), brood mass respiration should comprise less than 5% of such measurements. Enzymatic activity, but especially the PK:CS ratio (the higher the ratio, the greater the relative capacity for anaerobic activity) responses to short-term exposure to low oxygen concentration at the 2 temperatures contrasted with the lack of differences in the long-term experiment, although the range of values was consistent. In OFs, CS increased with oxygen at 15°C (Fig. 7). At low oxygen levels, PK measurements of females at 15°C were concentrated in the higher range of values. Nevertheless, the dispersion throughout higher oxygen levels was large. PK:CS ratios showed a decreasing trend from low to high oxygen concentrations in both ovigerous and non-ovigerous females (Fig. 7C,G); males did not show any trend for enzyme activities individually or for their rate (Fig. 7K). PK and CS showed the lowest values in OFs (Fig. 7). In contrast, the largest concentration of lactate was observed for OFs at 15°C and mainly at low oxygen, as compared with the other adult categories, where it was similar at both temperatures (Fig. 7D,H,L). Lactate was independent of temperature and oxygen for the other adults.

4. DISCUSSION

4.1. Brooding success

The results of the long-term experiment showed that the reproductive process and reproductive success of the species is modulated by the interaction of environmental oxygen concentration and temperature. The range of conditions considered are realistic

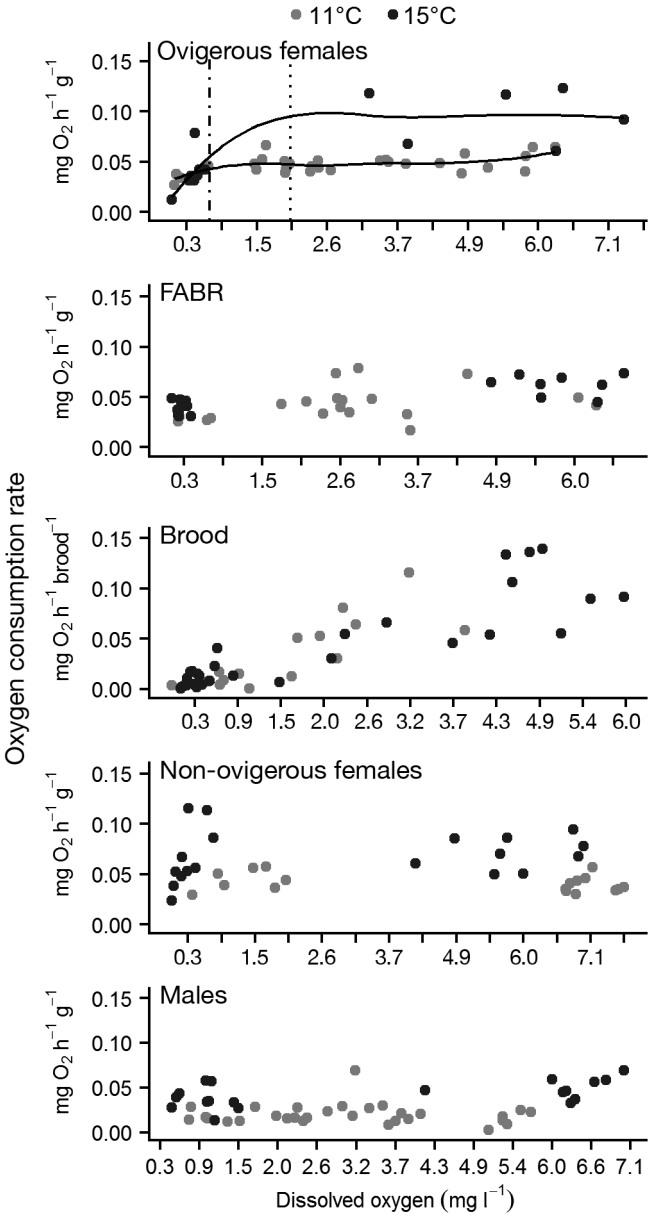

Fig. 6. Rates of oxygen consumption in the short-term experiment: ovigerous females, females after brood removal (FABR), broods, non-ovigerous females and males of *Pleuroncodes monodon* exposed to different oxygen concentration and 2 temperatures (11°C in grey and 15°C in black). In ovigerous females, the curves were constructed by the 'LOESS' (local polynomial regression fitting) method. The curves were used to detect the breakpoint (P_{crit}), represented by a change of slope in the response variable as a function of the independent variable. P_{crit} was higher at 15°C than at 11°C (~2 vs. 0.7 mg O_2 l^{-1}, dotted and dash-dotted lines, respectively)

Table 7. Separate slope models analysing the oxygen consumption of *Pleuroncodes monodon* in the short-term experiment, in different components of the population. DO: dissolved oxygen. Significant values ($p < 0.05$) are highlighted in **bold**

	Estimate	SE	WaldStat	p
Ovigerous females				
Intercept	−2.152	0.351	37.662	**0.000**
Temperature	−0.044	0.015	8.514	**0.003**
log (Weight)	0.057	0.024	5.376	**0.020**
Oxygen	−0.108	0.052	4.315	**0.037**
Females after brood removal				
Intercept	−3.060	0.429	50.858	**<<0.001**
11°C × Weight	−0.062	0.026	5.561	**0.018**
15°C × Weight	−0.009	0.019	0.232	0.630
11°C × DO	0.105	0.040	6.815	**0.009**
15°C × DO	0.073	0.028	6.615	**0.010**
Broods				
Intercept	−4.152	0.208	398.659	**<<0.001**
Oxygen	0.386	0.053	52.580	**<<0.001**
Temperature	0.146	0.104	1.979	0.159
Non-ovigerous females				
Intercept	−2.369	0.180	173.907	**0.000**
Temperature	−0.032	0.008	14.687	**0.000**
log (Weight)	0.024	0.011	4.538	**0.033**
Oxygen	−0.100	0.052	3.780	0.051
Males				
Intercept	−2.729	0.324	70.750	**0.000**
Temperature	−0.035	0.011	10.088	**0.001**
log (Weight)	0.059	0.023	6.434	**0.011**
Oxygen	−0.364	0.071	25.985	**0.000**

across the latitudinal and depth distribution of *Pleuroncodes monodon* (Gallardo 2017), since this is a bentho-pelagic species, as are many other galatheids. Successful populations might occupy benthic, pelagic or bentho-pelagic habitats, so both the actual range of temperature/dissolved oxygen values experienced by the populations as well as those clearly constraining their habitat use (vertical distribution) are relevant. Hypoxia under low temperature is characteristic of southern benthic populations (Gallardo et al. 2017), while high temperature normoxia characterizes Peruvian pelagic habitats, and hypoxia/high temperatures characterize the vertical distribution limit of the species (Gutiérrez et al. 2008). Continuous hypoxic conditions and high temperature during brood incubation were detrimental for both the female and brood hatching success. Even if females survived chronic exposure to warm temperatures and hypoxia until the end of brood carrying, they did not produce viable larvae. OFs that encounter hypoxia for short periods of time at high temperatures (15°C) may supplement their metabolic costs using anaerobic pathways, but during long brood incubation, this capacity apparently decreases.

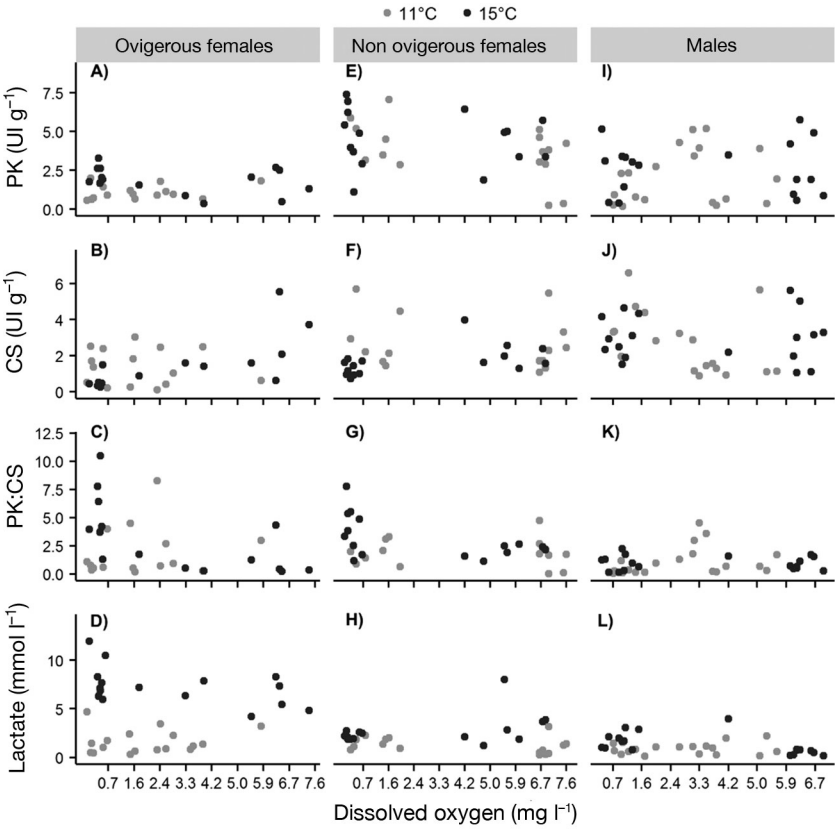

Fig. 7. Apparent specific activity of enzyme and lactate concentration in muscle of ovigerous females, non-ovigerous females and males of *Pleuroncodes monodon* at different conditions of temperature and oxygen. (A,E,I) Pyruvate kinase (PK) apparent specific activity per g. (B,F,J) Citrate synthase (CS) apparent specific activity per g. (C,G,K) PK:CS ratio. (D,H,L) Lactate concentration per g

Embryo development time shortened by almost 50% at 15°C compared to 11°C, but brood development synchrony and brood success were also lower. Developmental times coincided with those previously reported by Thiel et al. (2012) for the same species at ~11°C, and fit well within expected values for the squat lobster group. The duration of incubation has an exponential negative relationship with temperature for squat lobsters between 9 and 21°C (Thiel & Lovrich 2011), and lengthening of development is steepest for the range of temperatures studied herein. Populations of *P. monodon* are found in a temperature range from 11–21°C, so we evaluated the development response to temperature over the lower half of the range found throughout its latitudinal distribution. Nonetheless, in natural conditions, temperatures above 18°C do not coincide with hypoxia, and there are no previous observations on the effect of combined temperature–oxygen conditions on development time for squat lobsters or other crustaceans that permanently inhabit low oxygen zones. Our results show that oxygen concentration did not have an effect on development time to first hatching. The non-significant effect of oxygen on development time of *P. monodon* broods confirms that embryonic development of *P. monodon* is substantially shorter than that previously estimated from field surveys (90–120 d, Palma & Arana 1997) for populations inhabiting low temperature hypoxic waters.

Interestingly, oxygen concentrations did have an effect on the index of brooding success at hypoxia 15°C. Changes in asynchrony and success of development caused by temperature at levels close to the upper limit of the environmental temperature range have been found in broods or capsules of other marine organisms that keep embryos packed during embryo development (Fernández et al. 2006). Nevertheless, in other studies, hypoxic conditions did show a strong effect on development synchrony and the proportion of undeveloped embryos across temperatures (Steer et al. 2002). Besides external oxygen concentration, developmental asynchrony is related to oxygen gradients within crustacean embryo masses (Fernández et al. 2002) that might be overcome by active brood ventilation by the female (Fernández et al. 2003). When asynchrony is observed, respiration rates of embryos are significantly affected by oxygen concentrations (Fernández & Brante 2003), and something similar occurs in the centre of the brood under hypoxic conditions. The internal oxygen gradient within the brood develops as a balance between embryo consumption and diffusion rates in the centre of the embryo mass (Fernández et al. 2000). As the response of oxygen consumption rates to environmental oxygen concentration at low temperature is narrower at 11°C than at 15°C, embryos would be less affected at low temperature hypoxia, especially during the last developmental stages.

4.2. Parental care behaviour

Brooding success in squat lobsters is related to parental care, improving offspring growth and sur-

vival (Thiel & Lovrich 2011). Frequency of parental care behaviours may be a response to oxygen demand of embryos (Baeza & Fernández 2002), since oxygen consumption varies within the brood mass, not only through development of embryos, but also with oxygen distribution (partial pressure) within the brood. Also, high temperatures are associated with more intense incubating behaviours, and this is further intensified during embryo development (Brante et al. 2003).

The 3 common incubating behaviours described for *P. monodon* in the present study are pleopod movements, abdominal flapping, and fifth-pair pereopod probing, similar to what has been shown for anomuran (Pohle 1989) and brachyuran crabs (Fernández et al. 2000, Baeza & Fernández 2002, Fernández & Brante 2003). Pleopod movements are used to ventilate the embryo mass, and are thought to provide oxygen to the embryo mass (Fernández et al. 2000, Baeza et al. 2016). The frequency of pleopod fanning is greater in females carrying late-stage embryos, which commonly have higher oxygen requirements (Baeza et al. 2016). Indeed, for *P. monodon* under hypoxic conditions, pleopod movements were more frequent than under normoxia, and probably represent the attempts of females to better ventilate the brood mass and provide oxygen to the developing embryos. Nonetheless, there was no link between pleopod movements and embryo developmental stage, but there was an interaction between oxygen and temperature, indicating that females are sensitive to oxygen concentrations in the brood mass.

Abdominal flapping is an energy-expensive behaviour, which could imply a higher effort for OFs under hypoxic conditions (Fernández & Brante 2003), even more so when temperatures rise. This might mean that there were strong costs associated with oxygen provisioning to the brood, especially in OFs of *P. monodon* under hypoxia at 15°C. Abdominal flapping commonly results in increased oxygen availability in the centre of the embryo mass (Baeza & Fernández 2002). Abdominal flapping has also been described for *Munida gregaria* (Dellatorre & Barón 2008) and *Panulirus argus* (Baeza et al. 2016). Baeza & Fernández (2002) affirmed that the frequency of abdominal flaps was significantly higher in females carrying late-stage embryos than in females carrying early-stage embryos. Females of *Pleuroncodes monodon* exhibit frequent abdominal flapping throughout embryonic development, maintaining high levels in hypoxic conditions at 15°C in later embryonic stages. Possibly, under this particular condition, OFs spend more energy because embryos require more oxygen. Our measurements of brood oxygen consumption were undertaken for eggs in stage II, when embryos comprise a small fraction of overall egg mass/volume; oxyconformity might contribute to diminish intra-brood oxygen gradients. Nevertheless, as development proceeds oxygen consumption increases in crustacean eggs, and it is necessary for development. This is congruent with the higher incidence of maternal care behaviours at later developmental stages. Pereopod probing is used for grooming and removal of dead embryos from the embryo mass (Förster & Baeza 2001) and does not affect oxygen availability in the centre of the brood (Fernández & Brante 2003, Dellatorre & Barón 2008). Baeza et al. (2016) found that pereopod probing frequency increased in later embryo stages, but in the present study, pereopod probing diminished for N11 and H15 in later-stage embryos.

Female condition is affected by the level of parental care investment, especially at high temperatures at low oxygen concentrations. Increase in lactate in the abdominal muscle of females by the end of the incubation period indicates their reliance on anaerobic metabolism to sustain abdominal flapping for ventilation and is consistent with the lowest carbohydrate levels among the 4 treatments. It has been suggested that additional effort in incubating behaviours under stressful conditions could cause the reduction of the female's fitness (Brante et al. 2003). In this study, cellular stress, as indicated by HSP70 levels, were similar among females incubating under different conditions of temperature and oxygen. However, lowest carbohydrate levels found in abdominal muscle of incubating females maintained at hypoxia at low temperature can be attributed to direct costs of parental care, since carbohydrates are the main macromolecule that provides energy for muscle contraction activity (Jimenez & Kinsey 2015). Among treatments, higher routine metabolic rates with increasing temperature might lead to a reduced aerobic scope of muscle activity, especially in hypoxia. Observed levels of lactate and carbohydrate concentrations at the end of brooding suggest that female mortality, growth and future broods could be compromised in this environmental regime.

4.3. Potential for producing a subsequent brood

Temperature has an important effect on ovary development as in all other developmental rates due to increased metabolism. The mean size of oocytes in secondary vitellogenesis is similar in females that

completed egg maturation at 11 and 15°C, even though developmental time at 11°C is almost twice as long (~42 d) than at 15°C (~25 d). In normoxia, females start ovary maturation immediately after egg extrusion, and gonad maturation advances in parallel to egg mass development. The sooner the ovary completes secondary vitellogenesis after completing brood incubation, the sooner females can produce a new brood. Successive carrying events with few days in between have been observed in the laboratory, where some female *P. monodon* produced up to 5 broods (in normoxic conditions) during the annual reproductive season (Thiel et al. 2012). Continuous exposure to cyclic hypoxia appears to suppress the number of broods a female grass shrimp is capable of producing (Brown-Peterson et al. 2008). In our case, the diameter of oocytes turning into secondary vitellogenesis differed among treatments, and the larger ones appeared in normoxia at both 11 and 15°C. On the other hand, the frequency of oocytes in vitellogenesis II was lowest in the 2 hypoxic treatments, which implies that exposure to hypoxia during early gonad development might affect the size, thus the egg energetic reserves, and number of eggs of the future brood. This is also consistent with the increased demands of energy for maternal care and lower upper limit of energy acquisition in this condition. Consistently, the largest effect was observed on oocyte II proportions at 15°C, and females in hypoxia at 15°C had the lowest aerobic metabolic potential as measured by CS activity.

In nature, OFs are distributed according to oxygen conditions in a range from 0.7–1.42 mg O_2 l^{-1} at 11°C (Gallardo et al. 2017). The observed field threshold coincides with our experimental results, indicating that embryonic development starts to be affected at and below those oxygen levels. Hypoxic conditions at higher temperatures are detrimental to reproductive success and female survival, and therefore tend to be avoided by incubating females. In the field, at latitudes where the OMZ is associated with higher temperatures, *P. monodon* is found in its pelagic form above the oxycline. Late first maturity (2 to 3 yr old) in typical cold-water populations, together with larger embryo masses at first extrusion (number of embryos proportional to size), would be a disadvantage at high temperatures, where internal dissolved oxygen gradients could be less marked in smaller embryo masses typical of smaller (younger, pelagic) forms. Reproduction onset at smaller (younger) size, small broods and higher brooding frequency (as is known for pelagic populations at high temperatures; Gutiérrez et al. 2008) could be advantageous above 15°C in normoxic conditions, while hypoxia at that temperature should be avoided due to the high associated incubation costs and the risk of reproduction failure.

Overall, our results show that sub-thermocline conditions observed in northern Chile and southern Perú (hypoxia 15°C) can severely suppress the reproductive potential of *P. monodon*. In fact, at those latitudes (10–22° S), OFs remain above the oxycline, in normoxic warm waters. The pelagic adults attain smaller sizes than the ones in benthic cold hypoxic waters (Franco-Meléndez 2012), so the restrictive effects of 15°C hypoxia observed in our experiment could be overcome. The pelagic population is in fact a very productive one, as indicated by the very high observed biomasses (Gutiérrez et al. 2008). In central-Chile (30–37° S), the reproductive period tends to coincide with more oxygenated bottom waters, and females are found preferentially above 0.5 ml l^{-1} (Gallardo et al. 2017). Nevertheless fast (hours), intense (over 50% change in oxygen saturation) and persistent deoxygenation events over scales of days might occur during the season, and the short- vs. long-term differences in enzymatic activity would be more pronounced during those periods. Non-OFs could tolerate these episodic events, although if the escape response of OFs is not entrained fast enough, they might survive, but conditions are detrimental for egg and ovarian development. Video recordings of benthic populations at about 30° S have shown OFs in extremely hypoxic conditions lying on their backs, reducing all other activity except abdominal flapping (J. Sellanes unpubl. data).

4.4. Implication for population dynamics

The observed effects (here on reproductive females) can influence not only their reproductive potential but also their growth. Differences in female and male growth rates have been repeatedly reported after annual stock assessments (Roa & Tapia 1998). Lower growth rates in females are expected due to energy allocation to gonad vitellogenesis (Bascur et al. 2018) (extruded egg mass might represent up to 5% of female body mass), especially during years when environmental conditions allow for an extended reproduction period, and eventually more carrying events per individual females. In addition, if important (extended and/or intense) hypoxic events occur during incubation, growth would be further compromised since other costs of maternal care are

enhanced under such conditions together with a lower maximum energy processing capacity, due to hypoxic limits on oxygen consumption above routine metabolic rate.

Our results point to a new way to re-evaluate population assessments in view of environmental variability (dissolved oxygen, temperature), causing interannual variability in female mortality, length of the reproductive period and fecundity, among others. Temperature and oxygen concentration thus significantly influence the reproductive success of *P. monodon* in its natural environment. These effects could possibly explain large density-independent recruitment failures among the squat lobster populations.

The definition of hypoxic conditions is related to species-specific abilities to withstand low dissolved oxygen levels, and similarly, oxygen concentration might impose 'hypoxic' restrictions at high temperatures. Hypoxia thresholds might also vary between different ontogenetic stages and reproductive conditions. We conclude that environmental oxygen cycles in southern-central Chile and episodic temperature oscillations might affect brood development, as well as long-term survival of OFs, and are the missing factor to explore the relationship between these conditions and interannual recruitment success.

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