

RESEARCH ARTICLE

Both maternal and embryonic exposure to mild hypoxia influence embryonic development of the intertidal gastropod *Littorina littorea*

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ABSTRACT

There is growing evidence that maternal exposure to environmental stressors can alter offspring phenotype and increase fitness. Here, we investigate the relative and combined effects of maternal and developmental exposure to mild hypoxia (65 and 74% air saturation, respectively) on the growth and development of embryos of the marine gastropod *Littorina littorea*. Differences in embryo morphological traits were driven by the developmental environment, whereas the maternal environment and interactive effects of maternal and developmental environment were the main driver of differences in the timing of developmental events. While developmental exposure to mild hypoxia significantly increased the area of an important respiratory organ, the velum, it significantly delayed hatching of veliger larvae and reduced their size at hatching and overall survival. Maternal exposure had a significant effect on these traits, and interacted with developmental exposure to influence the time of appearance of morphological characters, suggesting that both are important in affecting developmental trajectories. A comparison between embryos that successfully hatched and those that died in mild hypoxia revealed that survivors exhibited hypertrophy in the velum and associated pre-oral cilia, suggesting that these traits are linked with survival in low-oxygen environments. We conclude that both maternal and developmental environments shape offspring phenotype in a species with a complex developmental life history, and that plasticity in embryo morphology arising from exposure to even small reductions in oxygen tensions affects the hatching success of these embryos.

KEY WORDS: Developmental plasticity, Gastropod, Maternal effects, Planktotroph

INTRODUCTION

Phenotypically plastic responses in morphology, physiology and behaviour may enable organisms to persist under unfavourable environmental conditions (Ghalambor et al., 2007; Chevin et al., 2010; Merila and Hendry, 2014; Seebacher et al., 2015). Such responses may provide a mechanism by which species can adapt over relatively short time scales, and facilitate the evolution of novel life history traits when exposed to a rapidly changing environment (Price et al., 2003; Wund, 2013). Also, the environment perceived by previous generations is known to be significant in shaping offspring phenotype through transgenerational epigenetic inheritance (transgenerational plasticity, TGP) (Munday et al., 2013). As a


subset of TGP (Rossiter, 1996), maternal effects are well documented (Bernardo, 1996; Mousseau and Fox, 1998; Marshall and Uller, 2007) and are potentially important drivers of plastic responses to new environments (Mousseau and Fox, 1998; Price et al., 2003). Mechanistically, maternal effects can manifest as: the transmission of somatic factors including beneficial proteins and hormones; the transmission of epigenetic marks that influence gene expression in offspring (Munday, 2014); and alterations in the levels of maternal investment, in the form of differential levels of nutrient provisioning (Moran and McAlister, 2009). Increases in levels of nutrient provisioning will influence early developmental stages of offspring and may buffer them against the potentially damaging effects of the environment perceived by the mother (Guisande and Harris, 1995; Räsänen et al., 2005; Allen et al., 2006; Marshall and Bolton, 2007; Burgess and Marshall, 2014).

Whilst many studies have focused on phenotypic plasticity in adult life stages, there is growing interest in earlier stages of development (Pechenik, 2006; Hassel et al., 2008; Hettinger et al., 2012; Truebano et al., 2018). In marine systems, a large number of taxa exhibit complex biphasic lifestyles. Marine invertebrates typically have a larval stage, during which physiological systems and morphological structures vary significantly from that of the adult (Strathmann, 1993; Strathmann, 2007). The transition from larval to adult stages often co-occurs with a transition in environment; for example, benthic adult stages are more liable to encounter variable environmental conditions, such as levels of dissolved oxygen, relative to their larvae suspended in the water column (Bertram and Strathmann, 1998). The ability for mothers to provision their young for environmental conditions they have not themselves experienced remains an important question in characterising the responses of species to perturbations in environmental conditions. Consequently, investigating species with biphasic lifestyles provides an excellent opportunity to study the effects of maternal and developmental exposure to environmental stress.

Phenotypically plastic responses to hypoxia are relatively well documented (Wu et al., 2003; Hassel et al., 2008; Leung et al., 2013; Segura et al., 2014). Instances of ‘mild’ hypoxia occur naturally in aquatic environments via seasonal fluctuations, or restricted water movements, although these occurrences may be exacerbated by future climate change scenarios (Mahaffey et al., 2020). Exposure to sublethal hypoxia during sensitive periods of early development can result in differences to overall development time, and the size and timing of appearance of aspects of embryonic and larval morphology of aquatic animals (Travis, 1994; Rundle and Spicer, 2016), and marine animals in particular (Chan et al., 2008; Hassel et al., 2008; Funch et al., 2016). Furthermore, exposure to relatively mild reductions in oxygen tensions are also known to influence the embryonic and larval development of marine species. Many aquatic species exhibit plasticity in the architecture and sizes of structures implicated in respiratory gas exchange following exposure to reduced oxygen tensions, resulting in more efficient oxygen uptake and

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removal of carbon dioxide (Bond, 1960; McDonald and McMahon, 1977; Burggren and Mwalukoma, 1983; Sollid et al., 2003; Chapman, 2007; Chan et al., 2008). For example, larvae of a tropical freshwater teleost *Trichopodus trichopterus* cultured in hypoxia exhibited significantly larger lamellae, and accelerated growth of these respiratory structures during embryonic development (Blank and Burggren, 2014). Such developmental differences generally may be causally associated with variations in fitness in new environments (Antonovics et al., 1988). Plasticity in the timing of appearance and size of these structures could therefore provide useful quantitative measures of performance under reduced oxygen tensions.

Understanding how maternal and developmental environments influence the phenotype will be crucial in assessing the vulnerability of populations to future climate change scenarios (Bernardo, 1996; Pechenik, 2006; Doney et al., 2012; Munday et al., 2013). Early life stages of marine species possessing planktotrophic stages of development are vulnerable to reductions in oxygen arising from seasonal fluctuations in oxygen levels, and the expansion of areas of coastal hypoxia (Díaz, 2001; Díaz and Rosenberg, 2008; Mahaffey et al., 2020). Characterising the effects of environmental hypoxia on the early development of planktotrophic species will be of importance in predicting how these populations respond to environmental change, as these early life stages are likely to experience markedly different conditions from their mostly benthic adult life stages (Bertram and Strathmann, 1998; Díaz, 2001; Díaz and Rosenberg, 2008). Recent studies have shown that developmental exposure to reduced oxygen tensions can result in significant alterations to the timings of appearance of embryonic traits, and the size of morphological characters in developing embryos of intertidal marine gastropods (Chan et al., 2008; Rudin-Bitterli et al., 2016), and that these effects persist into later life history stages (Pechenik, 2006; Li and Chiu, 2013; Segura et al., 2014). However, the relative influences of both maternal and developmental exposure to sublethal, mild environmental hypoxia on early development of intertidal marine gastropods is not currently known.

Consequently, the main aim of this study was to investigate the effect of chronic maternal and developmental exposure to reduced oxygen on the embryonic development of an invertebrate with a biphasic lifestyle. Possessing both an encapsulated, lecithotrophic embryonic development and a planktotrophic veliger stage, the intertidal marine gastropod *Littorina littorea* (Linnaeus 1758) provides a tractable model species (Lebour, 1937). Pre-hatch embryos were exposed to mild hypoxia (74% air saturation), and a number of key morphological and life history traits were quantified. Specifically the growth of the shell and the size of the multi-function (respiratory gas exchange, feeding and locomotion) transitory structure, the velum, and its associated pre-oral cilia were measured, as were the time of onset of key developmental events. Relationships between levels of nutrient provisioning and embryo morphology were also investigated: *Littorina* eggs contain a distinct albuminous layer surrounding the developing embryo (Lebour, 1937; Moran, 1999), and consequently the amount of yolk is easily quantifiable, and provides a proxy for the level of nutrient provisioning in each egg. Finally, a comparison of morphological traits in embryos that died under mild hypoxia with those that survived was made in order to allow inferences to be made over the selective advantages of particular morphological traits.

MATERIALS AND METHODS

Animal collection and maintenance

Adult *Littorina littorea* snails ($N=100$) were collected by hand from the mid-intertidal zone at Mount Batten, Plymouth (50°21'25.3"N,

4°7'33.1"W) in early March 2018 and transferred to the laboratory. Upon arrival, snails were kept in a number of plastic aquaria (volume=8.5 litres) filled with natural seawater ($S=35$, pH 8.1) continually aerated, via a diffuser coupled to an air pump (Mistral 4000, Aqua Medic, Bissendorf, Germany; flow rate 6 l min⁻¹) and maintained at a temperature (T) of 15°C. Water changes took place every 48 h. Snails were exposed to a 12 h:12 h light:dark cycle and fed seaweed (*Fucus serratus*) from the collection site *ad libitum* for 7 days before use in any experiments.

Experimental design

Maternal and embryonic exposures to reduced oxygen were applied using a reciprocal transplant design, which allowed their single and combined effects to be elucidated. Treatment codes were as follows where the first letter denotes maternal exposure and the second embryonic exposure: N-H, H-H, H-N, N-N [where N (normoxia) denotes 100% air saturation and H (hypoxia) denotes 65% air saturation (maternal) or 74% air saturation (embryonic)]. We term the level of oxygen used in this study 'mild hypoxia' as it is double the value of the operational definition of aquatic hypoxia (2.8 mg O₂ l⁻¹ or approximately 35% air saturation) (UNEP, 2011). Furthermore, intertidal gastropod molluscs and their embryonic and/or larval stages may experience considerably greater levels of hypoxic stress in the wild. Adult females were also exposed to a lower oxygen level than developing embryos, as adult life stages occupying intertidal areas are likely to experience more pronounced reductions in oxygen levels than egg capsules that are dispersed into coastal waters (Morris and Taylor, 1983; Mahaffey et al., 2020).

Following laboratory acclimation, six female snails were transferred to each of three airtight containers (18.0×11.5×7.5 cm) per treatment filled with filtered (20 µm) seawater ($T=14^{\circ}\text{C}$, $S=35$, pH 8.1) under a 12 h:12 h light:dark cycle. Female snails were then exposed to either normoxia or mild hypoxia (see below) for 14 days. During the exposure period water was changed every 48 h from 4 litre stock containers and snails were fed *F. serratus ad libitum*.

Production of eggs was continuous through this maternal exposure period; however, embryos for use in experiments were collected after 14 days to ensure maximum exposure time of the mothers. Following this, water containing eggs was filtered (20 µm) from each of the three airtight containers per treatment (normoxia or mild hypoxia) and combined into a single volume. From this volume eggs containing embryos at the four-cell division stage ($N=80$) were selected haphazardly under low power magnification (×10–40) using a micropipette (Sigma-Aldrich). By combining water containing embryos from each of the replicate aquaria into a single volume, we aimed to maximise representativeness from the female population. Embryos were then distributed evenly across treatments ($N=20$ per treatment) in individual wells of two microtitre plates per treatment (Nunc, Microwell, 96 wells, 350 µl per well), containing filtered (20 µm) seawater ($T=14^{\circ}\text{C}$, $S=35$, pH 8.1) and sealed with gas-permeable film (Aeroseal, Excel Scientific, Sigma-Aldrich) to prevent evaporation. Plates were then placed into an airtight container (18.0×11.5×7.5 cm).

Manipulation of seawater P_{O_2}

Embryos were exposed to either normoxia or mild hypoxia for the duration of their embryonic development, as follows.

Normoxic seawater in aquaria containing female snails was produced by aspirating air, supplied by an air pump (Mistral 4000; flow rate ~2 l min⁻¹), through the seawater within each of the airtight containers. Mildly hypoxic seawater within female rearing aquaria was produced by combining bottled N₂ gas with air supplied

from an air pump (Mistral 4000; air flow rate $\sim 1.4 \text{ l min}^{-1}$, N_2 flow rate $\sim 0.8 \text{ l min}^{-1}$). Rates of flow of bottled N_2 and air were adjusted using variable area flow meters (FR2000, Key Instruments). Normoxia or mild hypoxia was achieved within aquaria by supplying air or N_2 -enriched air directly into them through an opening within their lids. All air supplied was scrubbed using a 3 mol l^{-1} solution of KOH to remove CO_2 and standardise between gas mixtures with respect to CO_2 .

Within each airtight container a small 25 ml container filled with filtered ($20 \mu\text{m}$) seawater and sealed with gas-permeable film (Aeraseal) was used for measurements of percent air saturation, salinity, temperature and pH every 24 h, using a handheld dissolved oxygen meter (YSI Pro2030). This container was also used to replenish water within each microtitre well (90%) every 24 h of the embryonic exposure period, using a micropipette (Sigma-Aldrich).

Embryo staging and measurement

Microtitre plates containing developing embryos were removed from their respective airtight containers every 24 h for observations and measurements. An image sequence of individuals was acquired daily (30 s at $2.5 \text{ frames s}^{-1}$) using a QImaging Retiga R6 digital camera attached to a light microscope ($\times 160$ magnification; 1392×1040 pixels, Leica M205 C, Leica Microsystems) controlled using Ocular Scientific Image Acquisition Software (QImaging, 2018; <https://www.photometrics.com/products/ocular>). Water in each well was replenished (90%) with water from the relevant treatment container, before microtitre plates were returned to their respective airtight containers.

Individual images from each image sequence were used to measure pre-oral cilia length (μm), velum area (μm^2) and shell length (μm) every 24 h. Measurements were taken at three time points: (i) T_1 – within 24 h of appearance – the first point at which

measurements were possible; (ii) T_2 – between appearance and hatching – 50% of that individual's total development time; (iii) and T_3 – within 24 h of hatching – the last measurement made before hatching. Velum area was measured using the freehand selection tool in ImageJ (Schindelin et al., 2012) for a single frame in which both velar lobes were fully presented (i.e. parallel to the field of view); a single lobe in this frame was measured and this value doubled (Fig. 1D). A standardised measure of velum size was also calculated as a function of shell length (velum area/shell length), to determine whether differences in velum size were explained by increases in overall body size. Pre-oral cilia length and shell length were quantified using the segmented line tool (Fig. 1B,C) and presented as the mean of 10 haphazardly selected pre-oral cilia. Albumen diameter was also measured within 24 h of fertilisation using ImageJ (Fig. 1A).

Embryo survival and hatching

Embryo mortality and hatching were recorded every 24 h from the image sequences acquired. Mortality was defined as absence of activity (cilia beating, rotational behaviour and gut peristalsis) between one video and the next (24 h later). Shell length at T_3 was used as a proxy for size at hatching.

Statistical analysis

Analyses were carried out using R (version 1.0.136; <https://www.r-project.org/>). A Shapiro–Wilk test for normality and Levene's test for homogeneity of variance were used to assess normality and equality of variances. Differences in temperature, salinity, pH and percent air saturation between treatments during maternal, embryonic and veliger exposures were analysed using a repeated-measures ANOVA. The effects of maternal and developmental environments and their interaction on embryonic traits were analysed using a two-

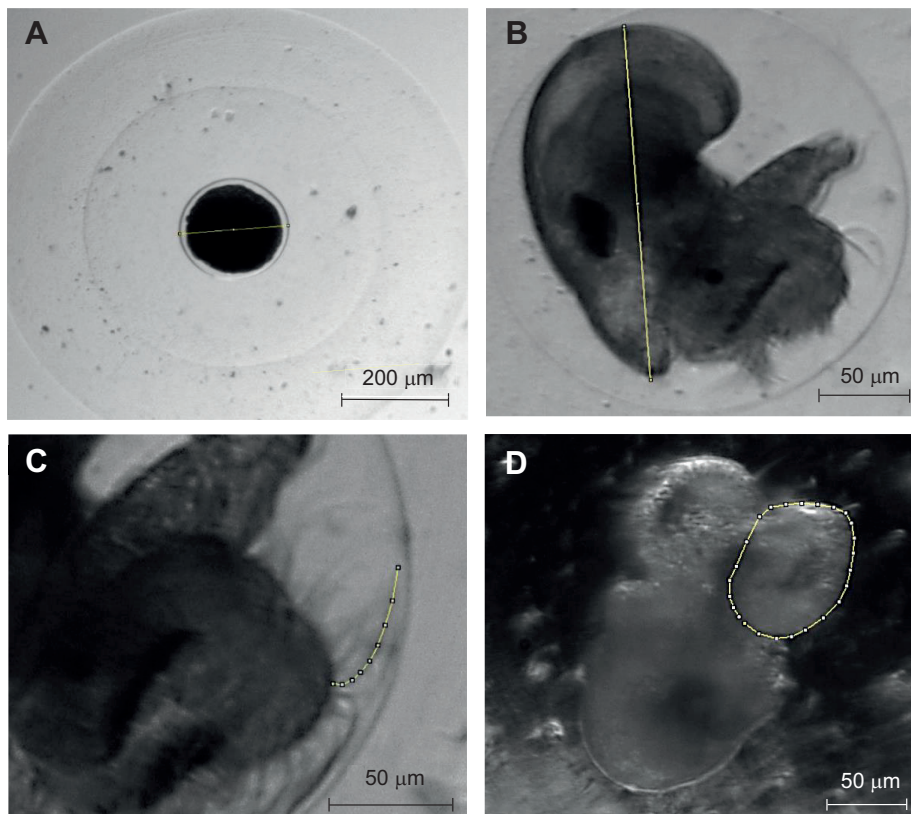


Fig. 1. Morphological measurements of *Littorina littorea* embryos. Measurements were made using the image analysis software ImageJ (Schindelin et al., 2012). (A) Albumen diameter. (B) Shell length. (C) Pre-oral cilia length. (D) Velum area.

Table 1. Summary of physical measurements between treatments of maternal and embryonic exposures

	Measurement	Normoxia (100%)	Mild hypoxia (70%)	d.f.	F	P
Maternal exposure	Temperature (°C)	13.64±0.04	13.67±0.03	1	0.193	0.108
	Salinity	34.97±0.23	34.42±0.23	1	2.785	0.664
	pH	8.09±0.02	8.10±0.03	1	0.012	0.92
	Air saturation (%)	94.67±1.04	65.26±2.24	27	0.89	0.607
Embryonic exposure	Temperature (°C)	14.06±0.037	14.09±0.039	1	0.161	0.69
	Salinity	34.68±0.11	34.34±0.119	1	2.712	0.107
	pH	8.09±0.03	8.09±0.04	1	3.502	0.0679
	Air saturation (%)	102.27±0.367	73.94±1.55	27	1.09	0.401

Temperature, salinity and pH: one-way ANOVA. Air saturation (%): repeated measures ANOVA, $P < 0.05$.

way ANOVA. Trait values in hatched veligers were analysed using a three-way ANOVA to test for effects of maternal and developmental environments as fixed factors and status (hatched or died) nested within treatments. For significant interactions, Tukey's honest significant difference (HSD) tests were used to test for significant pairwise differences between treatments. Linear regressions were used to test for relationships between albumen diameter (μm) and morphological traits of developing embryos.

RESULTS

Physico-chemical measurements

Mean values of temperature, salinity and pH in culture conditions did not vary significantly between treatments for either the maternal or embryonic exposure periods (maternal: temperature, $F_{1,44}=0.193$, $P=0.108$; salinity, $F_{1,44}=2.785$, $P=0.664$; pH, $F_{1,44}=0.012$, $P=0.92$; embryonic: temperature, $F_{1,44}=0.161$, $P=0.69$; salinity, $F_{1,44}=2.712$; $P=0.107$; pH, $F_{1,44}=3.502$, $P=0.0679$). Mean percent air saturation for control and mild hypoxia treatments did not vary significantly for the duration of either maternal or embryonic

exposure (repeated measures ANOVA: maternal normoxia, $F_{27,26}=0.89$; $P=0.607$; mild hypoxia, $F_{27,26}=0.98$, $P=0.523$; embryonic normoxia, $F_{26,25}=1.09$, $P=0.401$; mild hypoxia, $F_{26,25}=0.92$, $P=0.622$). Within mildly hypoxic maternal exposure tanks, percent air saturation was $65.26 \pm 2.24\%$, and the percent air saturation within microplate wells containing mildly hypoxic embryos was $73.94 \pm 1.55\%$ (Table 1).

Embryo survival and hatching

Mortality was higher among embryos reared under mild hypoxia (N-H: 30%) compared with those under normoxia (N-N: 20%) (Fig. 2A), and hatching success was reduced under mild hypoxia (N-H: 60%) compared with those reared under normoxia (N-N: 70%) (Fig. 2B). Maternal exposure to mild hypoxia increased mortality of embryos under normoxic (H-N: 25%; N-N: 20%) and mildly hypoxic (H-H: 55%; N-H: 30%) conditions, and reduced hatching success in those reared under normoxic (H-N: 60%; N-N: 70%) and mildly hypoxic (N-H: 60%; H-H: 45%) conditions.

Hatching time and size at hatching were only significantly influenced by embryonic environment. Embryos hatched significantly later under mild hypoxia, taking approximately 4 days longer to hatch than those reared under normoxia (N-H: 13.1 ± 1.3 days; H-H: $13. \pm 2.3$ days compared with H-N: 8.9 ± 1.1 days; N-N: 8.8 ± 0.6 days) ($F_{1,43}=11.26$, $P=0.0016$) (Fig. 3B). Hatchlings reared under normoxia were approximately 47% larger than those reared under mild hypoxia ($F_{1,33}=26.383$, $P < 0.0001$).

Timings of appearance of embryonic traits

There was a significant interaction between maternal and embryonic environment on pre-oral cilia appearance ($F_{1,64}=6.212$, $P=0.015$), which was significantly delayed in embryos reared under mild hypoxia whose mothers had been maintained under normoxia (N-H: 2.6 ± 0.1 days) compared with all other treatments (H-H: 2.2 ± 0.1 days; H-N: 2.3 ± 0.1 days; N-N: 2.2 ± 0.1 days) ($F_{3,64}=3.403$, $P=0.0221$) (Fig. 3A).

The timing of velum appearance was only influenced by maternal environment, with mildly hypoxic maternal exposure corresponding to a significantly delayed appearance of the velum (H-H: 4.5 ± 0.2 days; H-N: 4.8 ± 0.3 days) relative to embryos from mothers maintained under normoxia (N-H: 4.1 ± 0.1 days; N-N: 4.4 ± 0.2 days) ($F_{1,59}=5.496$, $P=0.022$) (Fig. 3A).

In contrast, only embryonic environment significantly influenced the timing of appearance of the shell. The shell started to form later in embryos reared under mild hypoxia (N-H: 5.5 ± 0.3 days; H-H: 5.6 ± 0.3 days) relative to those reared under normoxia (H-N: 5.1 ± 0.1 days; N-N: 4.9 ± 0.1 days) ($F_{3,53}=2.809$, $P=0.0466$) (Fig. 3A). There was no significant interaction between maternal and embryonic environment ($F_{1,53}=8.243$, $P=0.0056$), or main effect of maternal environment ($F_{1,53}=0.178$, $P=0.675$) on the timing of shell formation (Table 2).

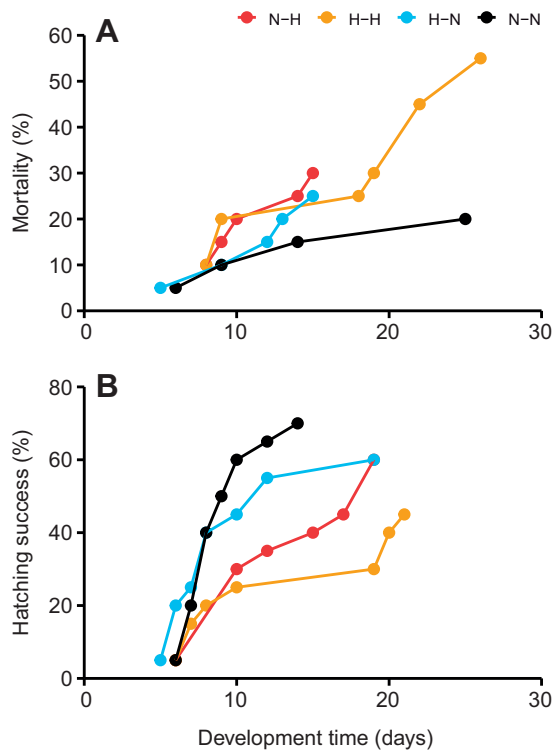


Fig. 2. Survival of embryos across treatments. (A) Percentage mortality of *L. littorea* embryos across treatments N-H ($N=19$), H-H ($N=17$), H-N ($N=20$) and N-N ($N=17$). (B) Percentage of successfully hatched *L. littorea* embryos across treatments N-H ($N=19$), H-H ($N=17$), H-N ($N=20$) and N-N ($N=17$).

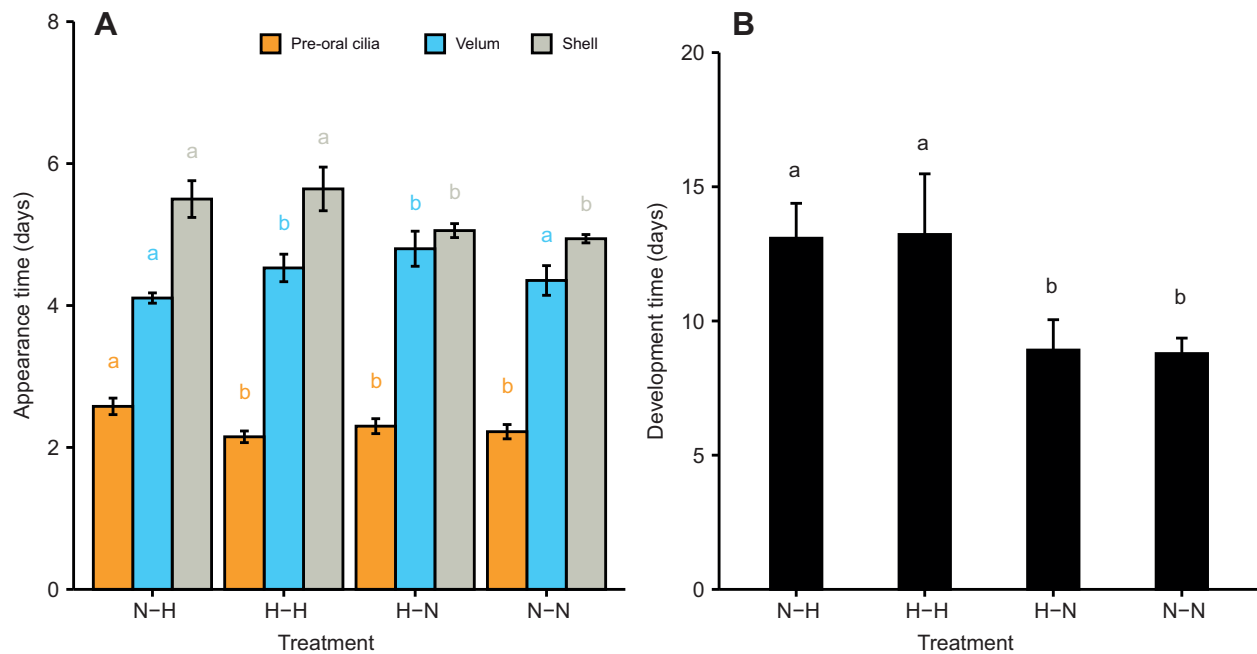


Fig. 3. Development time and appearance timings of aspects of embryo morphology between treatments. Treatments were N-H ($N=19$), H-H ($N=17$), H-N ($N=20$) and N-N ($N=17$) (means \pm s.e.m.). (A) Appearance time (days) of pre-oral cilia, velum and shell; pairwise significant differences (two-way ANOVA, $P<0.05$) between the appearance times of individual traits are denoted by lower case letters above treatments. (B) Overall development time (days); pairwise significant differences (two-way ANOVA, $P<0.05$) are denoted by lower case letters above treatments.

Embryo morphological traits

Pre-oral cilia length

Treatment effects on pre-oral cilia length were only detected early in T_1 individuals. There was a significant interaction between maternal and embryonic environment on T_1 pre-oral cilia length (μm) ($F_{1,60}=4.881$, $P=0.032$). Embryos reared under mild hypoxia whose mothers were maintained under mild hypoxia (H-H) had significantly longer pre-oral cilia compared with all other treatments ($F_{3,60}=4.665$, $P=0.0069$). Neither maternal nor embryonic environment was found to significantly influence the length of T_2 (maternal: $F_{1,54}=1.002$, $P=0.323$; embryonic: $F_{1,54}=2.747$, $P=1.05$) or T_3 pre-oral cilia (maternal: $F_{1,56}=0.212$, $P=0.648$; embryonic: $F_{1,56}=3.882$, $P=0.069$) (Fig. 4A, Tables 2 and 3).

Velum size

Only embryonic environment was found to significantly influence T_1 velum size ($F_{1,36}=22.813$, $P<0.0001$) with embryos reared under mild hypoxia having a significantly larger velum compared with those reared under normoxia ($F_{3,36}=20.33$, $P=0.0016$). This significant effect of developmental environment on velum size was also apparent at T_2 and T_3 time points (T_2 : $F_{3,32}=13.95$, $P<0.0001$; T_3 : $F_{3,32}=41.358$, $P<0.0001$) (Fig. 4B, Tables 2 and 3).

Shell length

Across all time points, only embryonic environment was found to be significant in influencing shell length (μm) (T_1 : $F_{1,60}=47.977$, $P<0.0001$; T_2 : $F_{1,40}=47.294$, $P<0.0001$). Embryos reared under mild hypoxia showed significantly reduced shell lengths relative to those reared under normoxia throughout their development ($F_{3,60}=25.2962$, $P<0.0001$; $F_{3,60}=17.66$, $P<0.0001$, respectively) (Fig. 4C, Tables 2 and 3).

Albumen diameter correlations

Albumen diameter did not change significantly during the course of development in any treatment ($R^2=0.003$, $P=0.719$). There were no

significant relationships detected between albumen diameter and pre-oral length in any treatment, across all time points. However, embryos reared under mild hypoxia after maternal exposure to normoxia (N-H) showed a significant negative correlation between T_1 albumen diameter (μm) and T_3 velum area (μm^2) ($R^2=0.5319$, $P=0.04006$) (Fig. 5B). In those embryos reared under mild hypoxia (N-H), significant positive correlations between T_1 albumen diameter and shell length were also detected at time points T_2 and T_3 ($R^2=0.4874$, $P=0.01689$; $R^2=0.3556$, $P=0.04909$, respectively) (Fig. 5A, Table 4).

Comparisons of embryos that hatched and those that died

There were pre-hatch mortalities in all treatments (N-H: 6; H-H: 10; H-N: 5; N-N: 4), and so the opportunity was taken to compare embryos that successfully hatched with those that died to identify traits linked with successful hatching (see also Rudin-Bitterli et al., 2016). By presenting data for comparisons of those that successfully hatched and those that died, inferences can also be made over the selective advantages of morphological traits under mild hypoxia. Measurements for those that died were taken using image sequences <24 h preceding death.

Successfully hatched individuals reared under mild hypoxia had larger T_1 albumen diameters (N-H: $F_{9,19}=12.08$, $P=0.0027$) (Fig. 6A), and longer T_3 pre-oral cilia (N-H: $F_{1,13}=4.007$, $P=0.0455$; H-H: $F_{1,9}=2.216$, $P=0.013$) (Fig. 6B) and T_3 velum areas (N-H: $F_{1,10}=5.511$, $P=0.0387$) (Fig. 6C) relative to those that died. Shell length was comparable between embryos that survived and those that died under mild hypoxia (Fig. 6D) ($F_{1,23}=3.630$, $P=0.0630$).

DISCUSSION

Maternal or embryonic exposure?

This study aimed to investigate the extent to which chronic maternal and developmental exposure to mild hypoxia (65 and 74% air saturation, respectively) influenced the embryonic development of the intertidal gastropod *L. littorea*. Our findings suggest that plasticity in the size of morphological characters and overall development time

Table 2. Relative influences of maternal and embryonic exposure to mild hypoxia on timing and sizes of T_3 embryo morphological traits

Trait	d.f.	Sum of squares	Mean square	F	P
Development time					
Maternal	1	0.000	0.000	0.000	0.995
Embryonic	1	214.70	214.70	11.260	0.002
Maternal×Embryonic	1	0.000	0.000	0.000	0.998
Residuals	43	819.70	19.060		
Pre-oral cilia appearance					
Maternal	1	0.626	0.626	3.151	0.080
Embryonic	1	0.168	0.168	0.846	0.000
Maternal×Embryonic	1	1.233	1.233	6.212	0.061
Residuals	73	14.493	0.199		
Velum appearance					
Maternal	1	3.750	3.750	5.496	0.022
Embryonic	1	1.220	1.220	1.788	0.186
Maternal×Embryonic	1	0.000	0.000	0.003	0.953
Residuals	69	47.110	0.683		
Shell appearance					
Maternal	1	0.120	0.120	0.178	0.675
Embryonic	1	5.440	5.440	8.243	0.006
Maternal×Embryonic	1	0.000	0.000	0.005	0.944
Residuals	63	41.600	0.660		
Pre-oral cilia length					
Maternal	1	427	427	0.212	0.648
Embryonic	1	12,548	12,548	3.882	0.069
Maternal×Embryonic	1	1361	1361	0.673	0.417
Residuals	36	72,735	2020		
Velum size					
Maternal	1	231,557	231,557	1.684	0.206
Embryonic	1	5,688,420	5,688,420	41.358	0.000
Maternal×Embryonic	1	528,755	528,755	3.844	0.061
Residuals	26	3,576,099	137,542		
Shell length					
Maternal	1	7518	7518	0.080	0.779
Embryonic	1	2,465,581	2,465,581	36.740	0.000
Maternal×Embryonic	1	362,767	362,767	3.882	0.0573
Residuals	26	3,083,951	93,453		

Two-way ANOVA, $P < 0.05$.

is driven primarily by exposure during embryonic development, whereas the timings of developmental events are shaped mainly by the maternal environment and its interaction with the embryonic environment. Plasticity arising from embryonic exposure to mild hypoxia included significant increases in velum size and development time, delays to the appearance of the shell, and significant reductions in shell length, whereas timings of appearance of morphological characters were found to be driven by interactive

effects between maternal and developmental environment (pre-oral cilia), and only the maternal environment (velum).

Overall development and hatching success

Embryonic exposure to mild hypoxia significantly increased development time and saw higher mortality relative to embryos that developed under normoxia. Increases in development time and reductions in growth rate of developing embryos in response to

Table 3. Sizes of aspects of embryo morphology and associated sample sizes across all treatments and time points

	T_1		T_2		T_3	
	N	Mean±s.e.m.	N	Mean±s.e.m.	N	Mean±s.e.m.
Pre-oral cilia length (μm)						
N–H	11	34.61±0.99	12	59.75±1.39	10	61.81±1.38
H–H	10	40.74±0.94	9	57.95±1.59	7	62.44±1.47
H–N	11	36.43±1.55	12	58.85±1.73	9	57.42±1.36
N–N	13	35.54±1.04	15	54.24±1.51	14	59.19±1.35
Velum size (μm)						
N–H	10	179.37±19.69	9	207.93±20.48	8	203.91±14.94
H–H	7	120.31±21.03	7	168.83±23.96	7	156.61±26.55
H–N	10	95.84±6.27	10	91.41±2.94	10	97.45±7.14
N–N	14	90.33±2.81	12	83.06±4.23	11	91.03±5.74
Shell length (μm)						
N–H	12	128.75±8.55	11	129.56±10.25	9	137.29±8.54
H–H	9	135.89±8.29	9	143.76±12.59	7	155.38±13.69
H–N	11	169.58±6.69	11	186.92±8.69	10	185.59±10.32
N–N	13	186.34±3.43	14	215.70±7.88	11	207.67±8.60

N is the number of individual embryos.

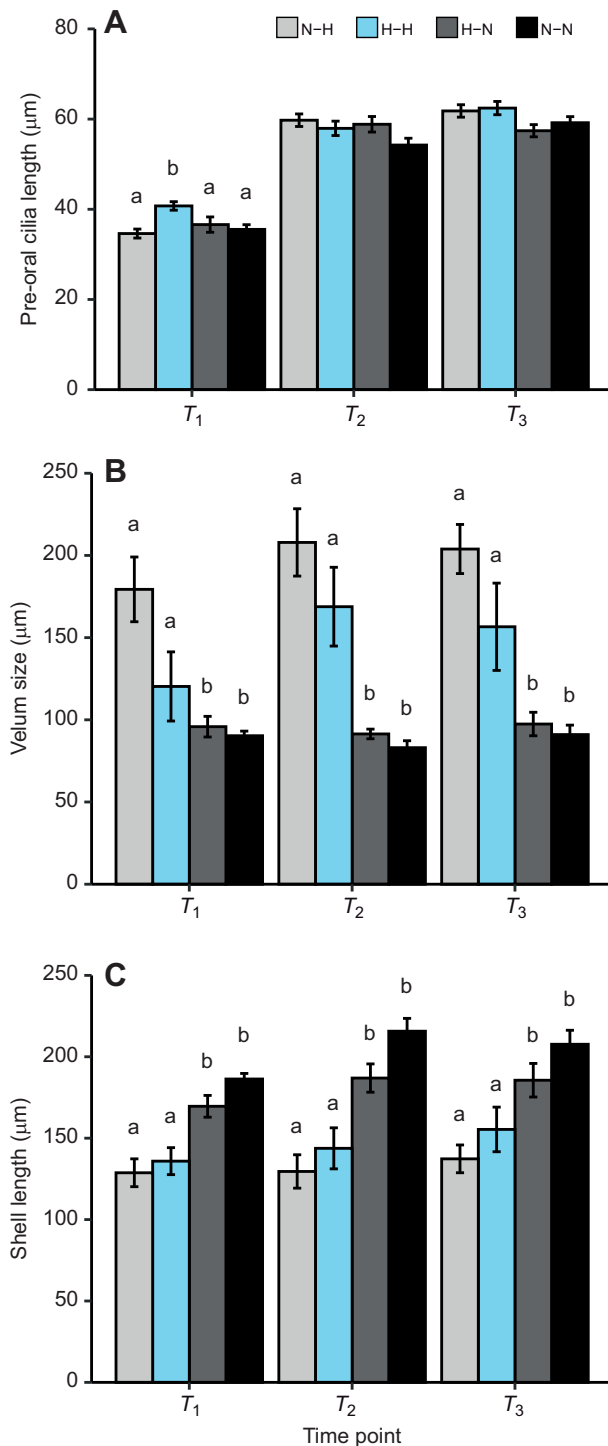


Fig. 4. Morphological traits of developing F1 embryos between treatments at three time points. Values are means \pm s.e.m. for time points T_1 , T_2 and T_3 . (A) Pre-oral cilia length (μm). (B) Velum size (μm) [calculated by dividing velum area (μm^2) by shell length (μm)]. (C) Shell length (μm). Pairwise significant differences (two-way ANOVA, $P < 0.05$) within time points are denoted by lower case letters above treatments.

hypoxia have been observed in multiple taxa (Morrison, 1971; Widdows et al., 1989; Wang and Widdows, 1991; Shang and Wu, 2004). For example, when reared at a dissolved oxygen concentration of 3 mg l^{-1} ($\sim 37.5\%$ air saturation), embryonic development of the gastropod *Reticunassa* (as *Nassarius*) *festivus* was significantly delayed relative to those reared under normoxia (Chan et al., 2008).

Closer to the percentage saturation of oxygen used in the current study, Cancino et al. (2003) showed that when developmentally exposed to moderate hypoxia (57.5% air saturation), the muricid gastropod *Chorus giganteus* had a significantly delayed embryonic development and reduced hatching success. It is important to note that the oxygen saturation used in these experiments was considerably lower than that of the current study. This may indicate that intertidal gastropods possessing planktotrophic stages of development are more sensitive to reductions in oxygen than previous data would suggest. Extended development time under reduced oxygen may arise from reductions in aerobic performance, thereby limiting the amount of energy available for undisturbed growth (Calosi et al., 2013). In addition to the direct mortality resulting from development of *L. littorea* in mild hypoxia, retarded development may have consequences at later life history stages on the survival of these individuals. Predation pressure on pelagic veliger larvae is high (Thorson, 1950; Cowden et al., 1984) and an extended time spent within the plankton will probably increase mortality arising from predation. Those that metamorphose faster are exposed to predators for less time; therefore (according to the stage duration hypothesis: Leggett and Deblois, 1994), their chances of survival are greatly increased.

The observed increased mortality and reduced hatching success under mild hypoxia is in general agreement with previous studies (Huntington and Miller, 1989; Brante et al., 2008; Vasquez, 2013). In gastropod molluscs, the encapsulated larvae are released by three main mechanisms including: increases in osmotic pressure within the capsule by uptake of water (Kennedy and Keegan, 1992); chemical dissolution of the capsule by the production of hatching enzymes (Vaughn, 1953); and mechanical action on the capsule by the larvae (Pechenik, 1975). Mild hypoxia may act to impair these mechanisms by, for example, impairing movement as a result of reduced aerobic performance, associated with the onset of anaerobiosis (Pörtner, 2001; 2010).

Relative influences of maternal and developmental environments

The maternal environment

The timing of appearance of pre-oral cilia was driven by interactive effects between maternal and embryonic environment. Appearance was significantly delayed in those embryos reared under mild hypoxia, although maternal exposure to mild hypoxia resulted in a recovery of appearance time similar to control individuals. Additionally, appearance of the velum was found only to be influenced by maternal environment. Given that albumen diameter was not different across treatments and was not significantly correlated with development time of embryos, these differences in the timing of developmental events are unlikely to have resulted from differences in the quantity of nutrient provisioned. Instead, differences in developmental trajectories in this species may arise from some form of epigenetic mechanism (Jablonka and Raz, 2009; Munday, 2014), or through alterations to the nutritional quality, rather than quantity, of albumen supplied within the egg capsule (Fukazawa et al., 2005; Moran and McAlister, 2009). The recovery in appearance timing of pre-oral cilia arising from maternal pre-exposure may also indicate a form of ‘anticipatory maternal effect’, where the mother adjusts her phenotype in response to the environment she perceives (Simonini and Prevedelli, 2003; Marshall and Uller, 2007; Untersee and Pechenik, 2007; Parker et al., 2012). This accelerated appearance of pre-oral cilia in H–H individuals also probably explains the increase in T_1 pre-oral cilia length H–H individuals, as this trend fails to persist into other measured time points, notably into the T_3 timepoint.

Table 4. Statistically significant results of linear regression analyses of albumen diameter and aspects of embryo morphology

Correlation	Treatment	Time point	d.f., error d.f.	Slope	Intercept	R ²	P
AD×SL	N–H	T ₂	1, 9	705	1932	0.4874	0.01689
AD×SL	N–H	T ₃	1, 7	946	1888	3.556	0.04909
AD×VA	N–H	T ₃	1, 6	-7.4×10 ⁻⁵	2196	0.5319	0.04006

AD, albumen diameter (μm); SL, shell length (μm); VA, velum area (μm²). Statistical significance at $P < 0.05$.

Whilst no significant differences in albumen diameter were observed between mothers exposed to normoxia and mild hypoxia, albumen diameter was found to be linked with the sizes of embryonic traits. Albumen diameter was negatively correlated with velum area in embryos reared under mild hypoxia (N–H), and significantly positively correlated with shell length. Organic content is known to significantly influence offspring performance and aspects of morphology (Guisande and Harris, 1995), and there is good evidence to suggest that higher levels of maternal investment can act to recover aspects of morphology or physiology under environmental stressors, similar to levels of control individuals (Räsänen et al., 2005; Allen et al., 2006; Marshall and Bolton, 2007). Albumen diameter was also found to be significantly positively correlated with shell length at hatching. Increases in body size and sizes of specific morphological characters arising from greater levels of nutrient provisioning can be found in marine invertebrates with planktotrophic modes of development (Bertram and Strathmann, 1998; Emler et al., 1987; Allen et al., 2006). Greater levels of nutrient provisioning in *L. littorea* reared under mild hypoxia may act to shift this trade-off, towards a greater shell size, at the expense of respiratory surface area, potentially reducing their vulnerability to predation in the water column once hatched (Bertram, 1993).

The developmental environment

Whilst maternal environment was found to significantly influence the timings of appearance of the velum and its associated pre-oral cilia, only the developmental environment significantly influenced timing of appearance of the shell, and the growth of embryonic morphological traits.

The appearance of the shell was significantly delayed in those reared under mild hypoxia. Differences in the timing of developmental events driven by environmental perturbations, termed ‘heterokairy’ (Spicer and Burggren, 2003), may constitute a significant form of phenotypic plasticity, and have been proposed to act as a mechanism by which differences in developmental sequences between a species and its ancestor, known as ‘heterochrony’, may evolve (Spicer and Rundle, 2007).

The velum is thought to act as the main surface for gas exchange in planktotrophic veligers (Fioroni, 1966). Here, the velum was significantly larger in embryos developed under mild hypoxia. This hypertrophic response in respiratory surface area probably reflects an increased effort to enhance rates of oxygen uptake, and can be observed across multiple taxa, with the majority focusing on fish and amphibians (Bond, 1960; McDonald and McMahon, 1977; Burggren and Mwalukoma, 1983; Sollid et al., 2003; Chapman, 2007; Chan et al., 2008). Few studies focus on embryonic stages of gastropod molluscs (Chan et al., 2008; Rudin-Bitterli et al., 2016), and of these two studies, only one focuses on a species with a planktotrophic mode of development (*Nassarius festivus*). Whilst lower than the O₂ saturation used in the present study, Chan et al. (2008) reported a reduction in velum area under moderate hypoxia (57.5% air saturation) in planktotrophic *Reticunassa* (as *Nassarius*) *festivus*, contrasting with our results. Rudin-Bitterli et al. (2016) proposed that enhanced plasticity in encapsulated developers, relative to those with planktotrophic modes of development, could arise from greater fluctuations in P_{O₂} in the intertidal environment (Morris and Taylor, 1983). Here, planktotrophic *L. littorea* embryos, the focus of the present study, are shown to respond to relatively small reductions in dissolved oxygen, despite the supposed less extreme fluctuations these embryos are likely to experience in the water column (Agnew and Taylor, 1986).

The larger velum in individuals that developed under mild hypoxia was accompanied by a smaller relative shell size. Gastropod molluscs are known to invest relatively large amounts of energy into construction of the shell (Chow, 1987; Cheung et al., 2008). As aerobic performance declines under low oxygen, energy production becomes limited (De Zwaan et al., 1991; Roman et al., 2019). As a result, investment of energy into growth may become constrained, ultimately leading to the reductions in shell length observed in the present study. Additionally, the observed increases in velum size may result in a further reduction in energy allocated towards production of the shell, whereby energy is invested into structures that will assist survival under conditions larvae immediately find themselves in, at the expense of functionally unrelated structures (Schaack and Chapman, 2003). Stressor-induced changes in morphogenesis can be found in multiple marine species (Andraso, 1997; Bayne, 2004; Bagatto, 2005). For example, when reared

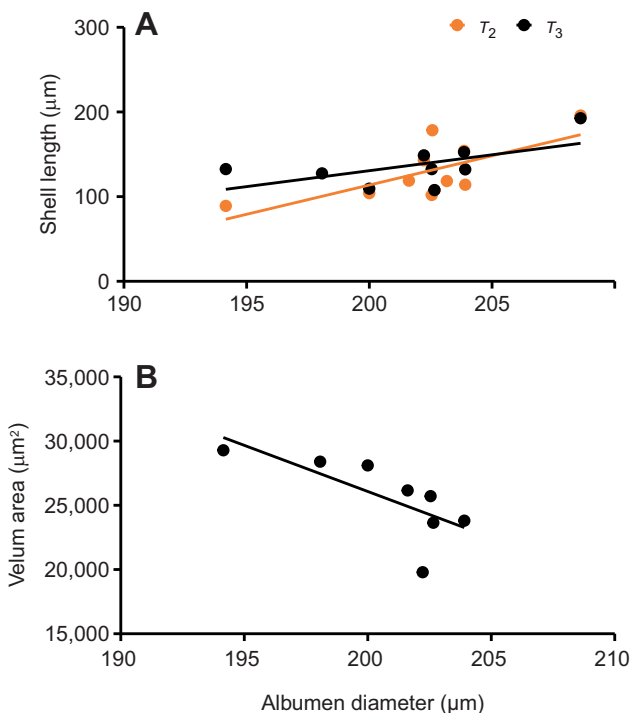


Fig. 5. Statistically significant correlations between albumen diameter and morphological traits in *L. littorea* embryos. Values are means±s.e.m.; statistical significance at $P < 0.05$. (A) T₂ and T₃ shell length (μm) and albumen diameter (μm). (B) T₃ velum area (μm²) and albumen diameter (μm) in N–H individuals.

under reduced food availability, larvae of the urchins *Strongylocentrotus purpuratus* and *S. franciscanus* exhibited a trade-off by increasing the size of their feeding apparatus to enhance rates of feeding, whilst reducing their stomach size (Miner, 2005). These reductions in shell length in those reared under mild hypoxia are likely to have consequences on the success of these individuals in the wild, given that greater larval size has been shown to significantly reduce mortality arising from predation (Webb, 1981; Bertram, 1993). For example, Gliwicz and Umana (1994) showed that increases in body size of the cladocerans *Daphnia* spp. and *Ceriodaphnia reticulata* resulted in significantly reduced predation by the freshwater copepod *Acanthocyclops robustus*. Whilst a greater velum area of *L. littorea* probably enhances the rates of gas exchange under mild hypoxia, survivability in the plankton may ultimately be compromised as a result of reduced body sizes.

Comparison of embryos that hatched or died under mild hypoxia

By comparing embryos that successfully hatched and died under mild hypoxia, inferences can be drawn on which morphological characters

are linked with survival under mild hypoxia. Increased levels of nutrient provisioning in the form of a greater albumen diameter were linked with successful hatching under mild hypoxia. Greater levels of nutrient provisioning are known to increase survival (Bernardo, 1996; Moran and McAlister, 2009; Rudin-Bitterli et al., 2016), and are of particular importance for species with planktotrophic modes of development, given the relatively small amount of nutrients provided to the offspring by the mother (Gimenez and Anger, 2001). In the current study we also show that the sizes of aspects of embryo morphology are linked with survival under reduced oxygen. Those that successfully hatched possessed a significantly larger velum, suggesting that an increased surface area for gas exchange is linked with survival under mild hypoxia, and may confer a selective advantage under such conditions. Furthermore, we found that an increased length of pre-oral cilia was associated with those that successfully hatched. Cilia of molluscan larvae exert pressure on the egg capsule during hatching, probably weakened by the action of hatching enzymes (Boletsky, 1979; Pilkington, 1974; Boletsky, 1989; *Littorina littorea*: S. D. Rundle and O. Tills, unpublished observations), and are also involved in mixing of fluid within the egg

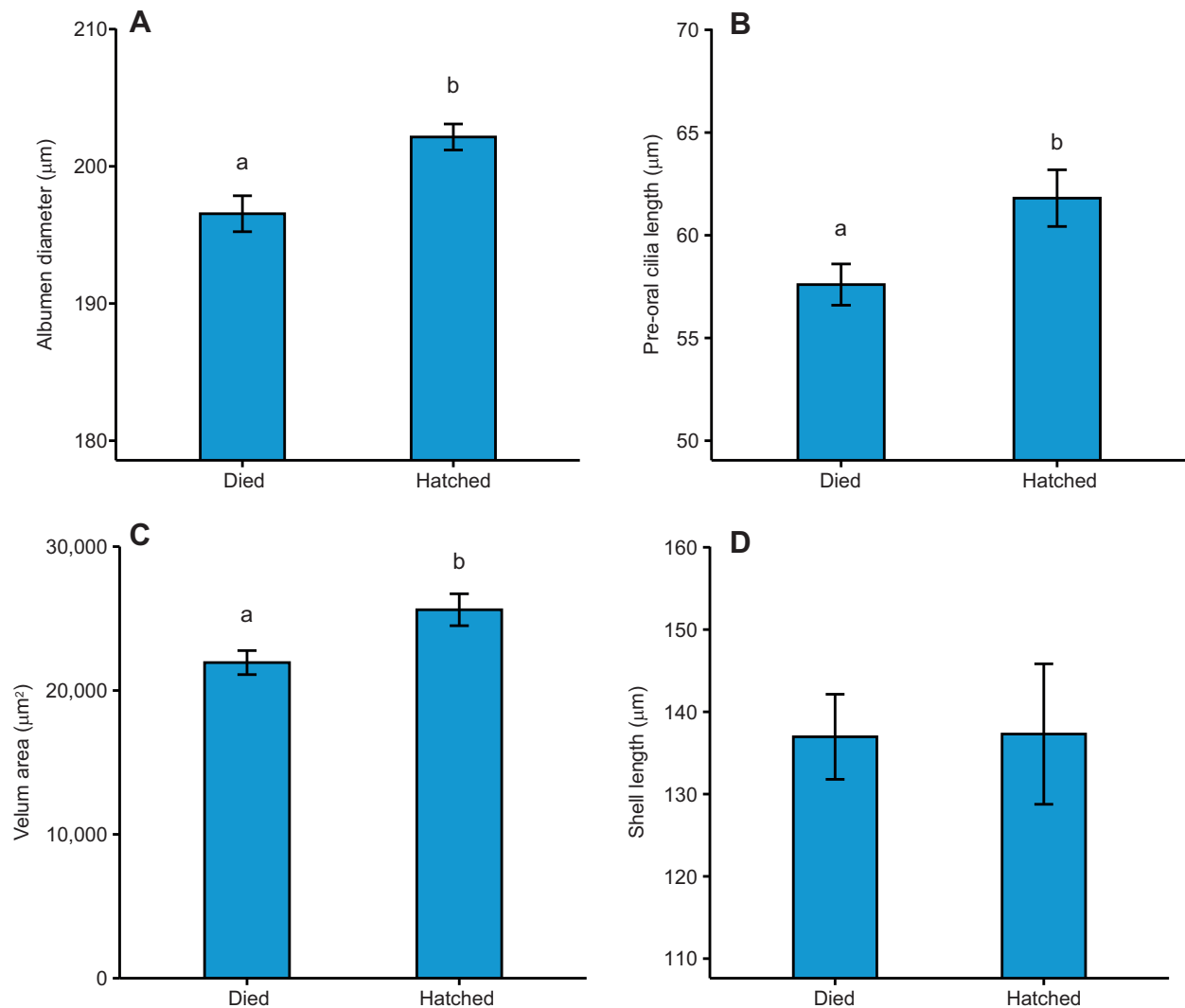


Fig. 6. Comparisons of morphological traits in *L. littorea* embryos that either survived or died within the N-H treatment. (A) Albumen diameter (µm) of successfully hatched ($N=13$) and dead ($N=7$) embryos. (B) Pre-oral cilia length (µm) of successfully hatched ($N=10$) and dead ($N=6$) embryos. (C) Velum area (µm²) of successfully hatched ($N=8$) and dead ($N=6$) embryos. (D) Shell length (µm) of successfully hatched ($N=9$) and dead ($N=5$) embryos. Values are means ± s.e.m. Pairwise significant differences are denoted by lower case letters above treatments (three-way ANOVA, $P < 0.05$).

capsule (Goldberg et al., 2008). The increased pre-oral cilia length in successfully hatched individuals can be interpreted in one of two ways: (1) an increased capacity to disperse normoxic waters within the capsule, thereby enhancing mixing of oxygenated water (Widdows et al., 1989; Goldberg et al., 2008); and/or (2) increased mechanical action on the capsule during hatching by the larger pre-oral cilia.

Given the significantly improved hatching success and survivability of those individuals possessing a greater velum area and pre-oral cilia length, it is not unreasonable to question why these phenotypes are not present in all individuals, including those reared under control conditions. However, given that our study focused on the embryonic development stage of *L. littorea*, it is not possible to make inferences on the selective benefits of these traits in later stages of development. Hypertrophy in the velum and pre-oral cilia, accompanied by reductions in shell length may have consequences on the success of individuals during larval, juvenile and adult stages, despite being shown to be linked with survivability during embryonic development.

Conclusions

Here, both maternal and developmental exposure to mild hypoxia significantly influenced the embryonic development of *L. littorea*; however, the type of effect elicited by these treatments differed. Whilst the maternal environment and its interaction with the developmental environment significantly influenced the timings of appearance of developmental traits, only the developmental environment was found to significantly influence the size of morphological traits. Furthermore, embryos of *L. littorea* responded to reductions in oxygen saturation considerably lower than those of previous studies, indicating that embryos of species possessing planktotrophic stages of development may be more sensitive to reductions in oxygen levels than previously assumed. Hypertrophy in the velum probably reflects an increased effort to enhance rates of oxygen uptake and removal of carbon dioxide under mild hypoxia (Fioroni, 1966; Chan et al., 2008; Rudin-Bitterli et al., 2016). Reductions in shell length under mild hypoxia probably reflect a trade-off towards structures linked with survival under reduced oxygen tensions (Schaack and Chapman, 2003). The greater velum area and pre-oral cilia length in embryos that survived hypoxia compared with those that died, indicate that hypertrophy of these structures may be linked with survival and successful hatching under mild hypoxia. Despite showing no significant differences between treatments, levels of maternal nutrient provisioning in the form of albumen diameter are shown to be important in offspring survival and shaping phenotype under mild hypoxia (Mousseau and Fox, 1998).

We can infer that the consequences of maternal and developmental exposure to mild hypoxia on the embryonic development of *L. littorea* are complex, and interactive in some situations. Future studies should focus on how alterations to key aspects of embryo morphology such as velum size and shell size affect the success of later life stages, such as the larval veliger stage and the subsequent juvenile stage. In doing so, better inferences could be made on any adaptive significances of hypertrophy of structures implicated in respiratory gas exchange and locomotion, and the consequences reduced hatchling sizes of veliger larvae have for this species in areas of reduced oxygen tensions.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: J.C.S.M., O.T., J.I.S., S.D.R.; Methodology: J.C.S.M., O.T., J.I.S., S.D.R.; Formal analysis: J.C.S.M.; Investigation: J.C.S.M.; Writing – original draft: J.C.S.M.; Writing – review & editing: J.C.S.M., O.T., J.I.S., S.D.R.; Visualization: J.C.S.M.; Supervision: O.T., J.I.S., S.D.R.; Project administration: J.C.S.M.

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