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RESEARCH ARTICLE

Differences in the timing of cardio-respiratory development determine whether marine gastropod embryos survive or die in hypoxia

Tabitha S. Rudin-Bitterli*, John I. Spicer and Simon D. Rundle‡

ABSTRACT

Physiological plasticity of early developmental stages is a key way by which organisms can survive and adapt to environmental change. We investigated developmental plasticity of aspects of the cardio-respiratory physiology of encapsulated embryos of a marine gastropod, *Littorina obtusata*, surviving exposure to moderate hypoxia (P_{O_2}=8 kPa) and compared the development of these survivors with that of individuals that died before hatching. Individuals surviving hypoxia exhibited a slower rate of development and altered ontogeny of cardio-respiratory structure and function compared with normoxic controls (P_{O_2}>20 kPa). The onset and development of the larval and adult hearts were delayed in chronological time in hypoxia, but both organs appeared earlier in developmental time and cardiac activity rates were greater. The velum, a transient, 'larval' organ thought to play a role in gas exchange, was larger in hypoxia but developed more slowly (in chronological time), and velar cilia-driven, rotational activity was lower. Despite these effects of hypoxia, 38% of individuals survived to hatching. Compared with those embryos that died during development, these surviving embryos had advanced expression of adult structures, i.e. a significantly earlier occurrence and greater activity of their adult heart and larger shells. In contrast, embryos that died retained larval cardio-respiratory features (the velum and larval heart) for longer in chronological time. Surviving embryos came from eggs with significantly higher albumen provisioning than those that died, suggesting an energetic component for advanced development of adult traits.

KEY WORDS: Heterokairy, Ecophysiology, Non-adaptive plasticity

INTRODUCTION

Physiological plasticity can enable organisms to survive, and potentially adapt to, stressful environmental conditions unlike those they usually experience (DeWitt and Scheiner, 2004; Ghalambor et al., 2007; Merila and Hendry, 2014; Seebacher et al., 2015). Such plastic responses in physiological systems can be particularly important early in ontogeny, when the maintenance of some level of homeostasis may be necessary to ensure successful development (Woods and Wilson, 2013). Yet, many organisms undergo complex changes to their organ systems and physiological function during early development, which potentially make the task of maintaining a normal developmental trajectory in stressful environments even more challenging.

In aquatic systems there are a number of taxa that have biphasic life cycles, with embryonic and larval stages that possess physiological systems that differ considerably from those in juveniles and adults and that offer the opportunity to study developmental plasticity through complex ontogenetic change. Marine molluscs, for example, have larvae that possess transitory structures such as the velum (Fig. 1), larval heart and larval kidney that carry out important functions before, or at the same time as, adult structures such as the shell and adult heart develop (Page, 2009). While the development of molluscan larval structures is reasonably well documented in terms of when they appear and develop under standardised conditions, the development of their physiological function is not (although see Bitterli et al., 2012). Similarly, while there is considerable information on the effect of changing environmental factors (e.g. temperature, food) on molluscan development (e.g. Przeslawski, 2004), literally nothing is known about the developmental plasticity of physiological function in embryonic and larval molluscs or how such plasticity might affect survival in the face of altered environmental conditions.

One of the forms of physiological plasticity that has received most attention is that in response to hypoxia (Spicer and El-Gamal, 1999; Pelster et al., 2010; Burggren and Reyna, 2011; Spicer, 2016). There is good evidence that vertebrate cardiovascular organs can be remodelled (Crispo and Chapman, 2010; Blank and Burggren, 2014), and cellular and biochemical mechanisms underpinning cardio-respiratory function altered (Eme et al., 2013), when oxygen supply is limited. In vertebrates, such phenotypic alterations have been shown to occur early in development and have been expressed as changes in both the activity and the timing of development of cardio-respiratory function (e.g. Bagatto, 2005; Gamperl and Farrell, 2004; Pelster, 2002). There is also evidence that invertebrates can exhibit hypoxia-induced physiological plasticity early during their development, including alterations to cardio-respiratory structures and function (Harrison, 2015; Callier et al., 2013; Guadagnoli et al., 2011; Centanin et al., 2010; Henry and Harrison, 2004; Reiber and Harper, 2001; Spicer, 2001, 2016; Spicer and El Gamal, 1999).

In most species of marine mollusc, the larva is planktonic and obtains most of its energy for development by feeding in the water column, but some species undergo development within an egg capsule and rely on nutrition provided by the mother. Species with encapsulated development make excellent models to study the developmental biology of embryonic and larval structures, without the complication of culturing and obtaining functional measures from free-living plankton. Such encapsulated embryos may experience highly variable oxygen tensions (P_{O_2}) within egg masses influenced by factors such as embryo density, the

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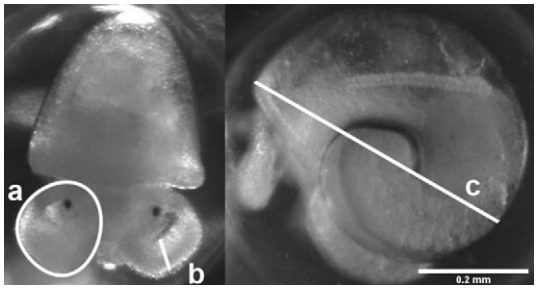

Fig. 1. Morphological measurements of *Littorina obtusata* embryos. The area of one velar lobe is indicated (a), as well as the velar ridge (b) and shell length (c). The scale bar applies to both plates.

presence of algae within the mass as well as external drivers such as temperature (Booth, 1995; Cohen and Strathmann, 1996; Moran and Woods, 2007; Woods and Podolsky, 2007). Those eggs laid in intertidal habitats, such as rock pools, or beneath rocks or boulders will probably experience naturally large diurnal P_{O_2} fluctuations (Morris and Taylor, 1983; Agnew and Taylor, 1986) and these fluctuations are likely to be accentuated by the increased prevalence of eutrophication-driven hypoxia in the world's coastal waters (Diaz, 2001; Rabalais et al., 2002; Diaz and Rosenberg, 2008). Thus, there is a pressing need to study the effects of hypoxia on the early life stages of marine invertebrates, many of which occupy coastal water prone to hypoxia.

Here, we investigated plasticity in the cardio-respiratory physiology of embryos of a marine intertidal gastropod, *Littorina obtusata* (Linnaeus 1758), in response to chronic but moderate hypoxia (P_{O_2}=8 kPa). This oxygen tension was considered moderate as eggs of this species can encounter considerably lower P_{O_2} in intertidal pools where the species is found (Morris and Taylor, 1983), and is not as severe as the level that some ecologists would designate as a threshold for hypoxia (Vaquer-Sunyer and Duarte, 2008). In a recent paper, we described the structures associated with cardio-respiratory physiology in *L. obtusata* embryos (Bitterli et al., 2012), namely: the bi-lobed, ciliated velum, which is used early in development in locomotion, feeding and gas exchange (Chaparro et al., 2002; Strathmann and Leise, 1979; Fretter, 1967; Fioroni, 1966); the larval heart, a thin-walled, ectodermal vesicle connected to the foot and velum (Werner, 1955); and the adult heart. These structures are part of four phases in development of cardio-respiratory function: (i) velar-associated, cilial rotation, which we proposed enhanced gas exchange; (ii) circulation by the larval heart; (iii) circulation by both the larval and adult hearts; and (iv) a final phase when circulation is carried out only by the adult heart (Bitterli et al., 2012). Here, we were particularly interested in investigating whether this species exhibited plasticity in either the timing or activity (or both) of these phases and their associated organs; we also wanted to investigate whether certain developmental itineraries were more likely to lead to individuals surviving hypoxic conditions.

MATERIALS AND METHODS

Animal collection and maintenance

Adult *L. obtusata* (N=90) were collected from in and around low water neaps in the intertidal zone at Mount Batten, Plymouth, Devon, UK (50°21.34′N, 04°29.78′W), during January 2012 and transported to the laboratory in buckets containing the sea weed they were collected on (*Fucus serratus*). In the laboratory, they were maintained at a temperature of 15±0.5°C in six Plexiglas aquaria (6 l, 15 individuals per aquarium) containing filtered, aerated seawater (salinity, S=34) and fed *ad libitium* with *F. serratus*. Seawater was fully replaced weekly.

Egg masses were subsequently laid on algal fronds and were carefully removed, using forceps, within 24 h of deposition. They were sampled haphazardly from across all six tanks to minimise the chance of just a few adults contributing to the stock of experimental embryos and were then examined under high-power magnification (×40) and egg masses containing embryos in the 2-cell division stage selected. From each egg mass a single egg was extracted with a razor blade such that each egg was still covered with a thin layer of jelly matrix. A total of 48 embryos were obtained in this way. They were placed into individual wells of microtitre plates (Sterilin Microtiter Plate, 6 wells, 0.4 ml per well) containing filtered treatment seawater (see below). Eight microtitre plates were used and the eggs (6 per plate) were allocated randomly amongst them; the plates were then placed into individual airtight rearing aquaria (1.4 l) maintained at 15±0.5°C under a 12 h light:12 h dark cycle for the duration of the experiment (81 days, i.e. the time taken for all surviving embryos to hatch). Seawater in each well was replaced (90%) daily.

Manipulating P_{O_2} of seawater

Littorina obtusata embryos were exposed to one of two P_{O_2} values within the rearing aquaria throughout their development: normoxia, nominal P_{O_2} 20 kPa; and hypoxia, nominal P_{O_2} 8 kPa.

Normoxia was maintained by bubbling four of the airtight rearing aquaria continuously with compressed air through an opening in the lid of the aquarium, supplied by an air pump (Mistral 4000, Aqua Medic, Bissendorf, Germany; flow rate 17 ml min^{-1}). Hypoxia was produced by continuously ventilating the remaining four rearing aquaria with N_2-enriched air (air flow rate ∼6.8 ml min^{-1}; N_2 flow rate ∼10.2 ml min^{-1}). Air, or N_2-enriched air, left the rearing aquaria through a separate outlet located on the lid of the aquaria. A tube was connected to each outlet and directed the outflow gases into small containers (Starplex Scientific, ON, Canada; 120 ml, one per rearing aquaria) each filled with 80 ml of tapwater to ensure that no air could enter the rearing aquaria through the outlets. All air used was first scrubbed (3 mol l^{-1} KOH) to remove CO_2 and standardise between normoxic and hypoxic gas mixtures.

A small, lidless container (Starplex Scientific; 90 ml) was filled with filtered natural seawater (S=34±1, filter size 0.2 µm) and placed into each rearing aquarium. This reservoir was used for measuring the dissolved O_2 and pH of water in each rearing tank every 24 h, using a hand-held dissolved O_2 meter (YSI Pro2030, Hampshire, UK). Furthermore, reservoir treatment seawater was used to replace the water within the microtitre wells daily (90%). In tanks with normoxic water, the measured P_{O_2} was 20.42±0.04 kPa (mean±s.d.; 98.8±1.9%) and in the tanks with hypoxic water the measured P_{O_2} was 8.03±0.05 kPa (38.8±2.5%). There were no significant differences in water P_{O_2} between the four rearing tanks of the normoxic and hypoxic treatments (repeated measures ANOVA, $F_{1,3}\leq1.8$, $P\geq0.147$ in each case). The P_{O_2} of water within the rearing aquaria did not change significantly throughout the duration of the experiment (normoxia $F_{1,41}$=0.87, P=0.693; hypoxia $F_{1,84}$=0.91; $P\geq0.693$ in each case). The mean pH of the seawater (8.08±0.03) within the rearing aquaria did not differ significantly between treatments (repeated measures ANOVA, $F_{1,3}\leq1.4$, $P\geq0.164$).

Recording development

Every 24 h, the microtitre plates were removed from the rearing aquaria and embryos were visualised using a zooming lens system (Zoom 70 XL, Optem, Luxembourg) at ×50 magnification. The system was connected to a Pike F-210C 2 Megapixel colour camera

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Table 1. Results of one-way ANOVA testing for the effect of reduced P_{O_2} on the timing of various developmental events in *Littorina obtusata* embryos

	Time (days)					
Event	Normoxia	Hypoxia	d.f.	*F*	*P*	R^2 (%)
Velum						
Appearance	2.6±0.2	3.0±0.2	1,29	3.0	0.296	6.52
Disappearance	18.8±0.4	38.6±2.2	1,29	158.5	**<0.001**	84.99
Max. area	8.3±0.4	14.2±0.7	1,29	58.7	**<0.001**	66.60
Max. relative area	5.9±0.2	5.7±0.4	1,29	0.2	0.670	2.90
Larval heart						
Appearance	9.2±0.2	10.2±0.2	1,29	5.0	**0.034**	15.06
Disappearance	22.2±0.4	42.0±2.7	1,29	116.8	**<0.001**	80.66
Max. activity	11.6±0.5	17.7±0.8	1,29	44.5	**<0.001**	61.40
Adult heart						
Appearance	14.4±0.3	19.9±0.6	1,29	134.0	**<0.001**	82.72
Shell						
Appearance	6.1±0.9	6.3±0.7	1,29	0.9	0.365	2.94
Hatching	26.0±0.5	57.2±2.5	1,29	309.5	**<0.001**	91.70
Rotational behaviour						
First activity	4.8±1.4	9.3±2.2	1,25	3.1	0.095	12.2
Last activity	10.8±1.9	14.0±2.9	1,25	0.9	0.359	3.80

Values are means±1 s.e.m. (normoxia, *N*=22; hypoxia, *N*=9). Significant probabilities are given in bold.

(Allied Vision Technology, Stadtroda, Germany), which operated with 1280×960 pixels at 15 Hz. The camera was controlled by AVT SmartView 1.11 software. After an acclimatisation period of 3 min, each embryo was filmed for 60 s (equivalent to 900 frames). The embryos were then returned to their rearing aquaria and well water was replaced (90%) with fresh treatment seawater.

Mortality and hatching success

Embryo mortality and hatching success were determined every 24 h from recorded image sequences. An embryo was considered dead if no activity (e.g. spinning, crawling, adult and larval heart beats, radula activity) was observed for 60 s and was confirmed by observations on the following 3 days. Hatching was defined as when the embryos had completely removed themselves from the egg capsule. On the day of hatching, hatchlings were first recorded for 60 s within the wells and then removed from the microtitre plates.

Morphological measurements

Key aspects of the morphology of the velum (area) and shell (length) were quantified every 24 h for each embryo. The area of one velum lobe was measured using the freehand selection tool in ImageJ on single frames extracted from videos in which the entire velum was visible, i.e. where both velar lobes were parallel to the field of view (see Fig. 1). Velum area was calculated as twice the measurement of this lobe. A standardised measure of velum size (relative velum area) was calculated by expressing velum area as a function of shell length.

Physiological and behavioural measurements

The larval and adult hearts were distinguished by their position within the larva, with the larval heart lying on the floor of the mantle cavity and the adult heart located beneath the right posterior dorsal surface of the shell (see Bitterli et al., 2012, for details). Heart rate was measured manually every 24 h from the 60 s-long recordings and expressed as beats min^{-1}. Rotational activity was expressed as the percentage of the 60 s interval spent rotating within the capsule.

Data analysis

We investigated the effects of hypoxia on developmental traits in both real time (referred to here as chronological time in days) and relative to the overall rate of development. For this second measure (referred to as developmental time), chronological time for each individual was first normalised by dividing the developmental day of the observation by the total development time for that individual (i.e. time to hatching).

All statistical analyses were performed using IBM SPSS 20.0 (IBM Corp., released 2011). One-way ANOVA was used to test for

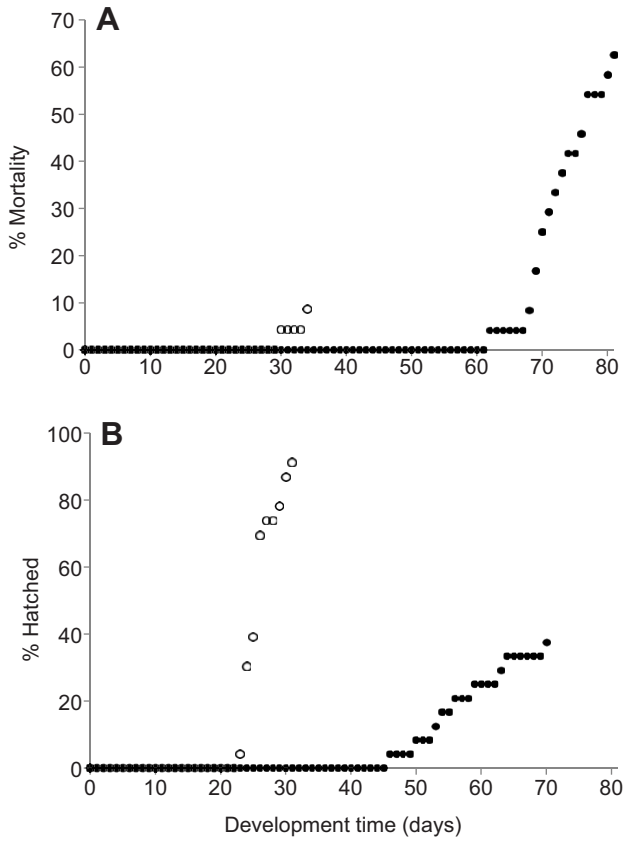

Fig. 2. Effect of hypoxia on survival. (A) Percentage mortality and (B) hatching success of *L. obtusata* embryos reared in normoxic (open circles, *N*=24) and hypoxic (filled circles, *N*=24) conditions. All values are means (±1 s.e.m.).

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the effects of hypoxic culture on morphological and physiological characters measured at a single time point in chronological time. Repeated measures ANOVA were also used to test for differences in traits between successful (individuals that hatched from the egg and lived) and unsuccessful (individuals that died before hatching) embryos through chronological time. For developmental time data, general linear model (GLM) ANOVA were used to test for differences between treatments and time blocks and the interaction between treatment and time blocks, with time and individual (nested within treatment) as random factors.

RESULTS

Survival and development rate

Hatching success was reduced and mortality increased in embryos reared under hypoxic conditions (Fig. 2), with only 38% of individuals hatching in hypoxia compared with 92% in normoxia. All embryos that hatched survived and grew well as hatchlings (T.R.-B., unpublished observations). There was also a clear effect of hypoxia on development time, with the time from egg laying to hatching in hypoxia being twice as long as that in normoxic conditions (Fig. 2B, Table 1). Given this differential mortality, we could separate our developmental trait data from embryos in the hypoxia treatment according to whether they survived or not and so made *ad hoc* formal comparisons of trait values in 'successful' and 'unsuccessful' individuals. First, however, we present an analysis based only on the individuals that hatched under normoxic (N=22) and hypoxic (N=9) conditions.

Morphological responses to hypoxia

The velum appeared after ∼3 days in both hypoxia and normoxia (Table 1, Fig. 3). After this time, however, there was a difference in velum ontogeny in chronological (i.e. development time in days) time between treatments: in normoxia, velum size reached a maximum in 8 day old embryos, whereas maximum velum size occurred at 14 days in hypoxic embryos (Fig. 3). However, maximum velum area was significantly (∼12%) larger in hypoxia (47,619±1018 μm^2) than in normoxia (42,666±540 μm^2; one-way ANOVA $F_{1,40}$=4.6, P=0.038), as was overall velum size through development (repeated measures ANOVA $F_{1,70}$=419.5, P<0.001) and relative velum area, which was 34% higher in hypoxic compared with normoxic embryos (repeated measures ANOVA $F_{1,70}$=603.8, P<0.001; Fig. 3).

In developmental time (i.e. percentage of total development time), there was a significant interaction between experimental treatment and time (interaction term $F_{1,9}$=3.1, P<0.001), with the velum showing a relatively rapid increase in size early in development in hypoxia, followed by a more rapid decrease in size (Fig. 4). This apparent advance in velum development in relative terms in hypoxia was even more pronounced for relative velum area (Fig. 4; interaction term $F_{1,28}$=10.6, P<0.001).

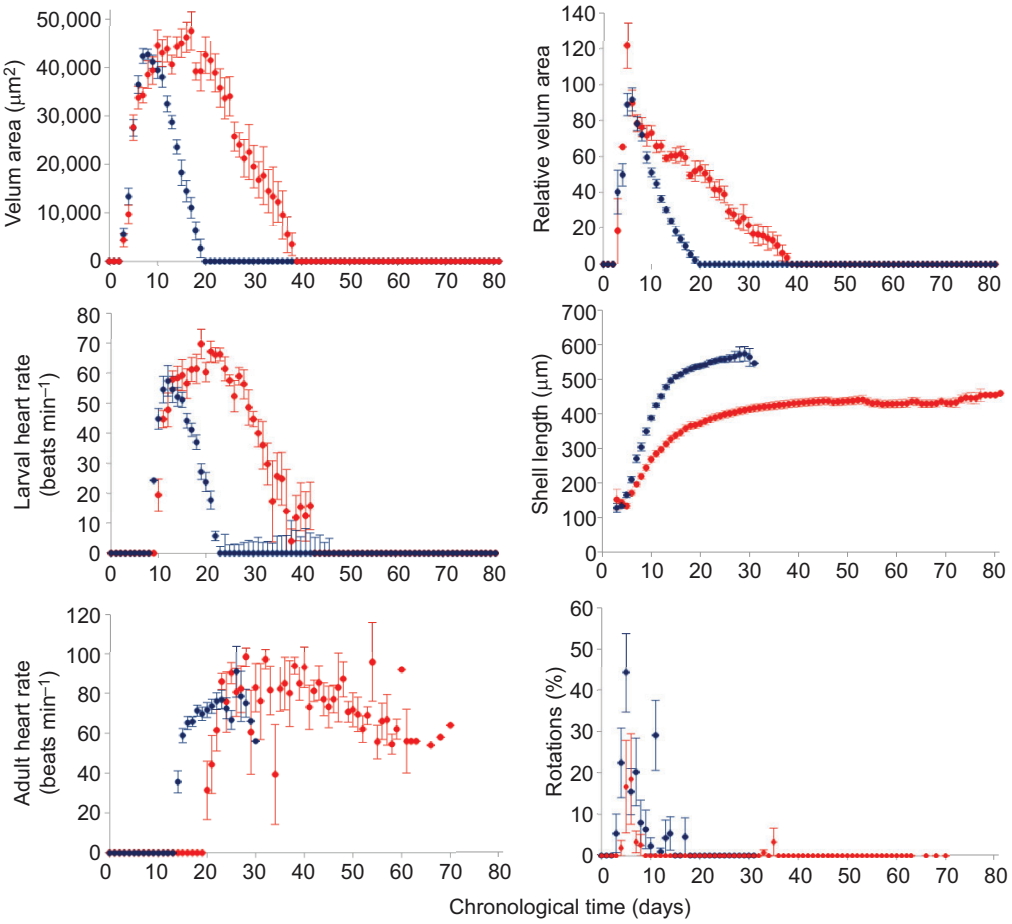

Fig. 3. Developmental traits of *L. obtusata* embryos cultured in normoxia or hypoxia through chronological time. Blue circles, normoxia (N=22); red circles, hypoxia (N=9). All values are means (±1 s.e.m.).

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Physiological responses to chronic hypoxia

In chronological time, the larval heart appeared significantly later in hypoxia than in normoxia (Table 1). The maximum beat rate of the larval heart also occurred significantly (6 days) later in hypoxia (Fig. 3, Table 1), but was significantly (22%) greater in hypoxia ($F_{1,25}$=6.20, P=0.020). The overall beat rate of the larval heart was also significantly higher in hypoxia through development (repeated measures ANOVA $F_{1,70}$=180.4, P<0.001) (Fig. 3). In developmental time, the larval heart had a significantly higher beat rate early on in hypoxia compared with that in normoxia, followed by a significantly lower beat rate in hypoxia later in development, reflected in a significant interaction between treatment and time (interaction term $F_{1,9}$=38.0, P<0.001; Fig. 4).

In chronological time, the adult heart began to beat, on average, 5 days earlier in normoxia (14 days) than in hypoxia (Fig. 3, Table 1). Its activity then increased in both treatments, but was significantly higher and more variable in hypoxia (repeated measures ANOVA $F_{1,66}$=81.4, P<0.001). In developmental time, however, both the appearance and activity of the adult heart were advanced significantly by hypoxia (Fig. 4; interaction term $F_{1,9}$=21.5, P<0.001).

Overall rotational activity through development was significantly higher in normoxic compared with hypoxic conditions in both chronological (repeated measures ANOVA $F_{1,66}$=15.1, P<0.001; Fig. 3) and developmental time (Fig. 4; interaction term $F_{1,9}$=4.3, P<0.001), but there was no significant difference as a result of hypoxia in the timing of this trait in chronological or developmental time (Table 1).

Comparing embryos that survived or died in hypoxia

The most obvious differences between embryos that survived or died in hypoxia were in the development of their shell and adult heart. Those individuals that survived hypoxia developed their adult heart significantly earlier (~8 days) than those that died (Table 2) and had a significantly higher adult heart rate (Fig. 5). The growth of the shell was also significantly greater in surviving embryos (Fig. 5, Table 3).

Differences in the velum and larval heart between surviving and dying embryos were more complex but suggested a significantly greater investment in these larval structures in individuals that died in hypoxia. Although there was no significant difference in the time of appearance of the larval heart between successful and unsuccessful individuals, there was a significant survival×time interaction for its activity. The larval heart rate was significantly higher in those animals that survived compared with those that died early during its development; after reaching its maximum beat rate, the larval heart rate was higher in those embryos that died (Fig. 5, Table 3). There were also significant survival×time interactions for the size of the velum, which reflected a comparatively larger velum size in individuals that died, particularly late in development (Fig. 5,

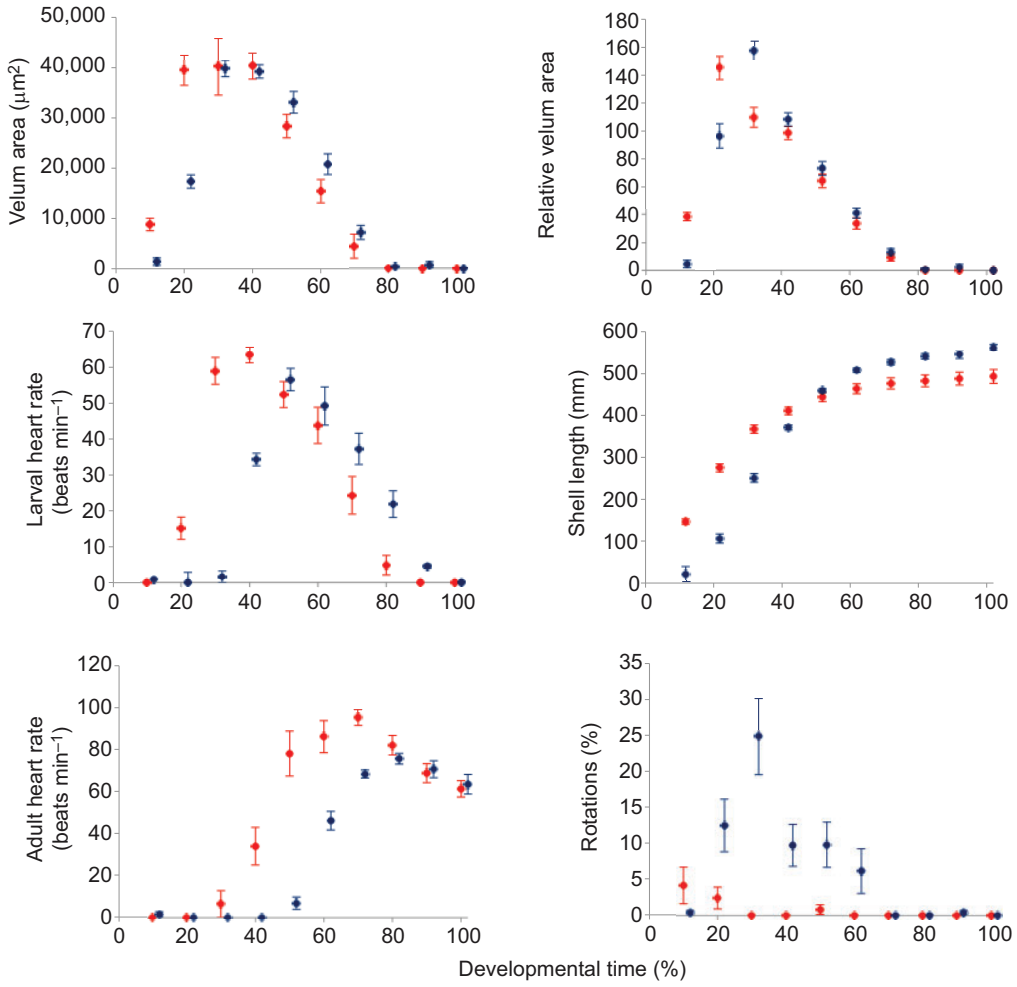

Fig. 4. Developmental traits of *L. obtusata* embryos cultured in normoxia or hypoxia through developmental time. Blue circles, normoxia (N=22); red circles, hypoxia (N=9). Data points are means (±1 s.e.m.) for 10 time blocks (see Materials and methods for further details).

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Table 2. Results of one-way ANOVA testing for timing differences of various developmental events between *L. obtusata* embryos that survived or died in hypoxia

	Time (days)					
Event	Survived	Died	d.f.	*F*	*P*	R^2 (%)
Velum						
Appearance	3.0±0.2	2.5±0.2	1,23	1.5	0.231	6.50
Disappearance	38.6±2.2	44.9±2.3	1,23	2.9	0.105	11.50
Max. area	14.2±0.7	16.1±1.0	1,23	1.4	0.257	5.80
Max. relative area	5.7±0.4	6.0±0.3	1,23	0.4	0.554	1.60
Larval heart						
Appearance	10.2±0.2	10.3±0.2	1,23	0.1	0.686	0.80
Disappearance	42.0±2.7	49.3±2.6	1,23	3.0	0.095	12.10
Max. activity	17.7±0.8	21.2±1.4	1,23	3.1	0.095	8.00
Adult heart						
Appearance	19.9±0.6	28.2±1.9	1,23	11.4	**0.003**	31.20
Shell						
Appearance	6.3±0.7	6.7±0.2	1,23	1.3	0.262	5.70
Rotational behaviour						
First activity	9.3±2.2	8.1±1.9	1,20	0.1	0.767	0.50
Last activity	14.0±2.9	17.1±2.8	1,20	0.3	0.576	1.70

Data (means±1 s.e.m.) are for the mean day on which an event occurred (survived, *N*=9; died, *N*=15). Significant probabilities are given in bold.

Table 3). Finally, there were significant survival×time interactions for rotational activity that reflected a greater rotation rate late during development in those embryos that died (Fig. 5, Table 3).

DISCUSSION

Hypoxia-induced developmental plasticity

We found clear evidence for developmental plasticity in cardiovascular function in *L. obtusata* embryos in response to even moderately hypoxic conditions. Hypoxia delayed the development of the larval and adult hearts in chronological time, but the overall rate of development was decreased and both organs appeared earlier in developmental time. The beat rate of both the larval and adult hearts was also greater in hypoxia. Together, these findings suggest that prolonged development time and increased activity of the larval and adult hearts are characteristics that either allow or at least do not compromise larval respiratory gas exchange. However, the fact that just over half of hypoxia-reared embryos died late on in development suggests that the costs of these responses are very high for some individuals.

Other studies have shown negative effects of hypoxia on survival and growth of marine gastropods. Comparisons with previous studies where isolated gastropod eggs or embryos were exposed to chronic hypoxia show a general concordance with the impacts on growth, development and survival that we observed in *L. obtusata* (but see discussion of velum plasticity below). Only around 1 in 10 eggs of the slipper limpet, *Crepidula fornicata*, survived a 3 day exposure to an environmental P_{O_2} of 3.2 kPa (Brante et al., 2008) and no embryos of the nassarid *Nassarius festivus* hatched successfully when cultured in seawater of P_{O_2} of just less than 1 kPa (Chan et al., 2008). It should be noted that both these P_{O_2} values are considerably lower than we used in our study. However, there is also evidence of negative effects even at P_{O_2} values similar to those we used (8 kPa), which may not be classified by ecologists as being environmental hypoxia (Vaquer-Sunyer and Duarte, 2008). While the survival of the encapsulated slipper limpet, *C. coquimbensis*, was not significantly affected by exposure to P_{O_2} of ∼11.5 kPa (Brante et al., 2008), Brante et al. (2009) later found that aerobic metabolism of embryos of this species and the related *C. fornicata* decreased significantly at P_{O_2} <12 kPa. Similarly, development, hatching and shell secretion of the encapsulated muricid snail *Chorus giganteus* were all compromised by culture in seawater of P_{O_2} ∼10 kPa (Cancino et al., 2003, 2011) and embryonic development of *N. festivus* was significantly delayed when cultured at a P_{O_2} of ∼7.6 kPa (Chan et al., 2008).

There are also studies on the effects of hypoxia within egg masses that offer a chance for comparison with our study. Low P_{O_2} is a common feature within egg masses of gastropods and is induced by respiration of embryos (Chaffee and Strathmann, 1984; Brante et al., 2008; Cancino et al., 2011). Levels of hypoxia often vary throughout the mass, with consequences for embryonic development. Eggs of the sand snail *Conuber* (as *Polinices*) *sordidus* lying close to the surface of an egg mass did not experience P_{O_2} <5 kPa, but those more centrally within the mass became more hypoxic over time, experiencing P_{O_2} of >1 kPa and had inhibited oxygen uptake and retarded development (Booth, 1995). The increased realism that studies on development within egg masses offer makes them an important strand of research on the effects of hypoxia. Hence, further work on the developmental physiology of *L. obtusata* embryos within masses, including measurements of intracapsular O_2, would be highly instructive.

The significant hypoxia-induced increase in activity of the larval and adult hearts we observed in *L. obtusata* could be interpreted as a compensatory response to cope with the more limited O_2 supply. Compensatory tachycardia in response to long-term hypoxia exposure is rarely observed in invertebrates (DeFur and Mangum, 1979; McMahon, 1988; Spicer, 2016). At low chronic P_{O_2}, the typical response is an unaltered heart rate or bradycardia, sometimes compensated for by an increase in stroke volume so that cardiac output is sustained or enhanced (Airriess and McMahon, 1994; Guadagnoli et al., 2011; Reiber and McMahon, 1998; Wheatly and Taylor, 1981). Stroke volume has been shown to be invariant in embryonic or larval crustaceans (Harper and Reiber, 2004; J.I.S. and S. P. Eriksson, unpublished observations), but it has not yet been possible to reliably quantify stroke volume in these embryonic gastropods. There is evidence that some vertebrate early-life stages can increase heart rate in response to chronically reduced P_{O_2}. Jacob et al. (2002) reported increased heart rate and stroke volume in zebrafish, *Danio rerio*, larvae raised in a P_{O_2} of 10 kPa (∼50% air saturation) 4 days post-fertilization (dpf). Similarly, zebrafish larvae raised in a P_{O_2} of 3 kPa (∼15% air saturation) from 24 dpf responded

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Table 3. *F*-values from ANOVA investigating differences in developmental traits between *L. obtusata* embryos that survived or died in hypoxia

	Velum	Relative velum size (velum:shell)	Larval heart	Adult heart	Shell	Rotation
Time	$F_{1,44}$=21.6***	$F_{1,44}$=16.1***	$F_{48,1}$=10.1***	$F_{43,1}$=2.5***	$F_{39,1}$=157***	$F_{1,44}$=10.1***
Survival	$F_{1,1}$=31.2***	$F_{1,1}$=46.6***	$F_{1,1}$=0.1	$F_{1,1}$=35.1***	$F_{1,1}$=1281***	$F_{1,1}$=0.1
Time×survival	$F_{1,44}$=2.5***	$F_{1,44}$=2.9***	$F_{48,1}$=5.8***	$F_{43,1}$=3.7***	$F_{39,1}$=0.5	$F_{1,44}$=5.8***

See Materials and methods for details of the type of analysis employed. Asterisks denote levels of significance (*P<0.05, **P<0.01, ***P<0.001).

with a tachycardia and increased stroke volume from 7 dpf to 11 dpf; this pattern was reversed at 4 and 5 dpf, where a bradycardia was observed (Yaqoob and Schwerte, 2010). We tentatively suggest that the developing cardiovascular system of *L. obtusata* is perhaps just as responsive to reduced P_{O_2} as more complex vertebrate systems.

Larval heart responses to chronically reduced P_{O_2} were remarkably similar to those of the adult heart. Chronic hypoxia stimulated larval heart activity, and larval beat frequencies in hypoxia exceeded those of the control. This study lends further credence to an earlier hypothesis that the production of the beat in the two hearts is regulated in similar ways (Bitterli et al., 2012). Unfortunately, we know little of the ontogeny of adult heart regulation in molluscs (McMahon et al., 1997), let alone that of the larval heart, or the effect of environmental perturbation on both.

The molluscan velum is presumed to be the primary area for respiratory gas exchange in planktonic larvae and encapsulated embryos (Werner, 1955; Fioroni, 1966). The velar ridge (shown in Fig. 1) is well supplied with haemolymph, which is circulated from the velum to the inner body of the animal by the larval heart (Werner, 1955; Kriegstein, 1977; Bitterli et al., 2012). Hence, it is perhaps not surprising that this gas exchange organ was enlarged in *L. obtusata* cultured under hypoxia, which could enable an increase in gas exchange through an increase in the area available for O_2 uptake and CO_2 elimination under hypoxic conditions. Increases in the area of gas exchange surfaces appear to be a general response of both invertebrates and vertebrates (Bábak, 1907; Drastich, 1925; Bond, 1960; Burggren and Mwalukoma, 1983; Loudon, 1989; Hoback and Stanley, 2001; Henry and Harrison, 2004; Chapman, 2007). This plasticity takes the form of hypertrophy – an increase in the volume of an organ due to the enlargement of its cells rather than

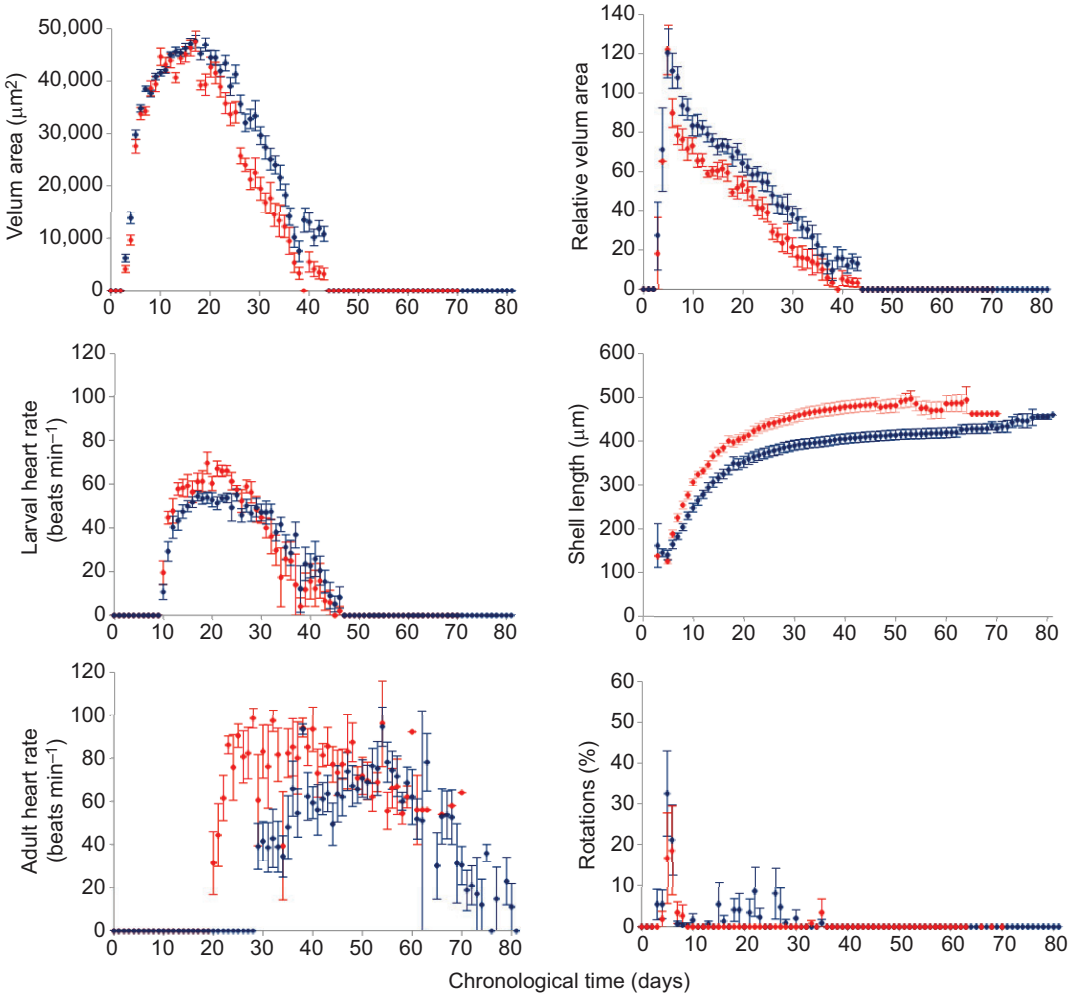

Fig. 5. Developmental traits of *L. obtusata* embryos surviving or dying in hypoxic conditions through chronological time. Red circles, surviving (N=9); blue circles, dying (N=15). All values are means (±1 s.e.m.).

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an increase in cell number. Paradoxically, the only other study investigating the influence of reduced P_{O_2} (~11.5 kPa) on gastropod velum plasticity reported a reduction in velum size in terms of both absolute size (length) and when size was standardised relative to the size of the shell (Chan et al., 2008) in the dogwhelk, *N. festivus*. Swimming behaviour and dispersal velocities were also lower in snails reared in hypoxic compared with normoxic conditions, suggesting that the locomotory function of the velum was impaired by hypoxia.

Whilst it is unwise to infer an adaptive difference between planktonic (e.g. *N. festivus*) and encapsulated (e.g. *L. obtusata*) life history types based on a two-species comparison (Huey, 1987), we propose the hypothesis that the two developmental modes differ in their plastic responses to low P_{O_2}. Egg capsules are often fixed to the substrate (as is the case with *L. obtusata*) and, hence, encapsulated embryos are vulnerable to changes in local environmental conditions. For those egg masses laid in intertidal habitats, this variation may be particularly extreme, including large fluctuations in P_{O_2} (Truchot and Duhamel-Jouve, 1980; Agnew and Taylor, 1986). Furthermore, embryos are often crowded within egg masses and the jelly matrix around the eggs limits O_2 diffusion (Booth, 1995; Strathmann and Strathmann, 1995; Moran and Woods, 2007). Hence, it might be predicted that plasticity in velum size that increased the surface area for gaseous exchange would be more likely to be associated with encapsulated, intertidal embryos than planktonic larvae that are able to disperse in the water column.

The plastic response of the velum to hypoxia in *L. obtusata* was not matched by the rotational activity driven by this organ, with this behaviour decreasing in hypoxia. This rotational activity, which is driven by cilia located on the velar lobes, has been proposed as a mechanism to mix intracapsular fluids and thus enhance O_2 diffusion into molluscan egg capsules (Hunter and Vogel, 1986; Goldberg et al., 2008). In freshwater gastropod embryos, rotational activity increases during short-term hypoxia in a dose-dependent manner (Kuang et al., 2002; Byrne et al., 2009; Shartau et al., 2010). We have also shown that rotational activity is particularly important in early development of *L. obtusata*, when it may play an important regulatory role as P_{O_2} decreases and larval heart activity is weak (Bitterli et al., 2012). Given this suggested potential adaptive value of rotational activity, the comparatively low rotation rate in hypoxia observed in the current study suggests that the relationship between hypoxia supply and demand and the energetics of cilia-driven rotational activity may be both subtle and complex. Whilst some authors suggest that this activity may be costly because of the energy needed for cilial activity (Goldberg et al., 2008; Shartau et al., 2010), there are other studies which propose that rotational activity is a low energy-cost process (Silvester and Sleigh, 1984; Widdows and Hawkins, 1989; Riisgård and Larsen, 2001). The fact that embryos survived hypoxia in the current study despite no apparent increase in their rotation rate suggests that, if rotation is costly, embryos can obtain enough oxygen by performing some base level of rotation.

Which embryos survived hypoxia?

We took the opportunity to use the relatively late death of non-survivors to assess whether there were any alterations in gas exchange and cardiovascular structure or function characteristics of embryos that survived hypoxic conditions. This analysis revealed clear differences, which suggested that survival was associated with the earlier development of adult traits (shell and adult heart). It appeared that those embryos that survived also exhibited a slightly delayed onset, and earlier loss, of the larval heart and velum. In contrast, those embryos that died in hypoxia appeared to retain their velum for longer.

This pattern suggests that individuals that survived hypoxia grew faster and had an advanced adult development, with early investment in larval structures during development being maladaptive. As marine animals develop, they are confronted with the task of maintaining a balance between energy intake and energy consumption (Chaparro and Paschke, 1990; Bayne, 2004). Those embryos and larvae that undergo encapsulated development are dependent on maternal provisioning for their energy supply, which can occur extra-embryonically through additional nurse eggs (Rivest, 1983; Chaparro and Paschke, 1990), cannibalism (adelophagy) (Lesoway et al., 2014; Thomsen et al., 2014) or albumen deposited within the egg capsule by the mother at the time of laying (Moran, 1999). The development rate of marine embryos and larvae has been linked to the maternal provisioning they receive. Brante et al. (2009), for example, showed that supplying additional albumen to embryos of the slipper limpet, *C. fornicata*, enhanced growth rate. Although we did not measure albumen levels in the eggs of *L. obtusata* throughout their development, we did quantify albumen levels (measured as the diameter of albumen within the egg capsule) from images at the start of development. These indicated that embryos surviving hypoxia had significantly higher levels of albumen (Fig. 6; $F_{1,22}$=18.2, P<0.001). Hence, the advanced adult development associated with survivors could have an energetic basis linked to higher provisioning of albumen. This hypothesis would be worth exploring, particularly given the potential importance of this link for this species' chances of surviving hypoxia.

Altered timing of developmental itineraries within species in response to environmental conditions has been proposed as a potential driver of adaptive evolutionary change (Spicer and Rundle, 2007; Spicer et al., 2011). When such intraspecific altered timing occurs as a result of the influence of environmental factors, it is a form of developmental plasticity termed heterokairy (Spicer and Burggren, 2003; Spicer and Rundle, 2007). Heterokairy has been proposed as a potential mechanistic basis for the evolutionary pattern of heterochrony – differences in developmental timing between ancestors and their descendants (Tills et al., 2013; Mueller et al., 2015). At the same time, it may be of ecological importance, allowing species to tolerate and/or survive stressful environmental conditions.

Several examples of heterokairy have been demonstrated in species exposed to hypoxic conditions, such as: advanced adult ability to oxyregulate in brine shrimp, *Artemia franciscana* (Spicer and El-Gamal, 1999) and Norway lobster, *Nephrops norvegicus* (Spicer and Eriksson, 2003); the earlier expression of cardiovascular

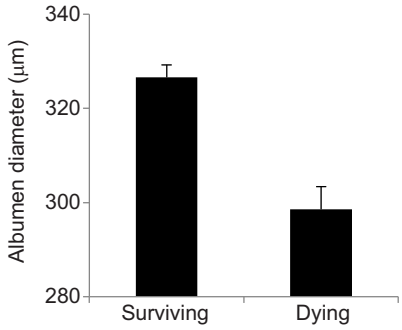

Fig. 6. Albumen diameter in *L. obtusata* embryos surviving (*N*=9) or dying (*N*=15) under hypoxic conditions. All values are means (±1 s.e.m.).

control mechanisms (andronergic response and vasoconstriction) in zebrafish (Bagatto, 2005); and the onset of air breathing in the three spot gourami, *Trichopodus trichopterus*, and Siamese fighting fish, *Betta splendans* (Mendez-Sanchez and Burggren, 2014). The early expression of adult traits during development has also been documented as a form of developmental plasticity in marine invertebrates in response to increased levels of food (Gibson and Gibson, 2004; Strathmann et al., 1992). In our study, we also observed advanced timing of development of the adult heart in animals exposed to hypoxia. Within this broad-scale developmental plasticity there were subtle differences in timing between individuals that appeared to enable them to survive hypoxia. This type of plastic response, where a few individuals within a population are able to survive altered environmental conditions but, in doing so, are only able to produce a suboptimal phenotype (in this case a more protracted development period and smaller size at hatching) has been defined as non-adaptive phenotypic plasticity (Ghalambor et al., 2007). Such non-adaptive plasticity has been suggested to have a potentially important role in the evolution of plasticity, as those individuals that survive the new environmental conditions may be more open to selection (Ghalambor et al., 2007, 2015). Our studies of *L. obtusata* suggest that this species might offer an excellent opportunity to explore how non-adaptive heterokairy might play an important role in the ability of marine species to adapt to hypoxic conditions.

Competing interests

The authors declare no competing or financial interests.

Author contributions

T.S.R.-B. carried out the laboratory experiments and part of the statistical analysis; S.D.R. completed additional statistical analysis and co-ordinated the manuscript preparation; all three authors designed the experiment and contributed equally to the writing of the manuscript.

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