Subtle but significant effects of CO$_2$ acidified seawater on embryos of the intertidal snail, *Littorina obtusata*

Robert P. Ellis*, Jess Bersey, Simon D. Rundle, Jason M. Hall-Spencer, John I. Spicer

Marine Biology and Ecology Research Centre, University of Plymouth, Plymouth PL4 8AA, UK

ABSTRACT: Our understanding of the effects of ocean acidification on whole organism function is growing, but most current information is for adult stages of development. Here, we show the effects of reduced pH seawater (pH 7.6) on aspects of the development, physiology and behaviour of encapsulated embryos of the marine intertidal gastropod *Littorina obtusata*. We found reduced viability and increased development times under reduced pH conditions, and the embryos had significantly altered behaviours and physiologies. In acidified seawater, embryos spent more time stationary, had slower rotation rates, spent less time crawling, but increased their movement periodicity compared with those maintained under control conditions. Larval and adult heart rates were significantly lower in acidified seawater, and hatchling snails had an altered shell morphology (lateral length and spiral shell length) compared to control snails. Our findings show that ocean acidification may have multiple, subtle effects during the early development of marine animals that may have implications for their survival beyond those predicted using later life stages.

KEY WORDS: Ocean acidification · Climate change · Seawater pH · Embryonic development · Marine gastropod · *Littorina obtusata*

INTRODUCTION

By the end of this century the pH of surface seawater is predicted to decrease by 0.5 units, due to the increase of carbonic acid formed when CO$_2$ is absorbed by the ocean (Caldeira & Wicket 2003, Feely et al. 2004). Over the next few centuries this pH decline could reach 0.8 to 1.4 units (Royal Society 2005), causing the oceans to become less alkaline than they have been for the last 55 million years (Zachos et al. 2005).

Early indications of the potential impact that ocean acidification may have on marine ecosystems suggest that effects could be complex, with organisms responding directly (e.g. Przeslawski et al. 2005) and indirectly (e.g. Bibby et al. 2007) to the chemical change in their environment. Different organismal groups may demonstrate differing levels of vulnerability to the change in oceanic chemistry (Hall-Spencer et al. 2008, Widdicombe & Spicer 2008). For

example, effects may be disproportionately large for calcifying organisms, as in many cases calcification rates decrease linearly with increasing pCO$_2$ (Gazeau et al. 2007), leading to reduced shell mineralization (e.g. Kurihara et al. 2007 although cf. Wood et al. 2008). Evidence is also growing of the diverse effects of CO$_2$-induced seawater acidification on whole organism function (Pörtner et al. 2004, Fabry et al. 2008, Widdicombe & Spicer 2008), ranging from impaired immunological function (e.g. Bibby et al. 2008) and extracellular acid–base disruption (e.g. Michaelidis et al. 2005, Miles et al. 2007, Spicer et al. 2007) through to growth, reproduction (e.g. Michaelidis et al. 2005, Berge et al. 2006, Mayor et al. 2007) and ecosystem effects (Hall-Spencer et al. 2008).

Whilst our understanding of the effects of ocean acidification on marine organisms is growing, most of this information is for late (i.e. postembryonic, predominantly sexually mature) stages of development

and little is known about effects early in ontogeny. Recent findings, however, suggest that earlier developmental stages of marine fish may be more sensitive to CO_2-induced seawater acidification than those later in development (Ishimatsu et al. 2004). A similar pattern was found for the copepod *Calanus finmarchicus*, in which adult survival and egg production were unaffected by reduced seawater pH but egg development was reduced dramatically (Mayor et al. 2007). Other invertebrates also show a disruption of early development: fertilization, cleavage and overall development rates were all slower, and the pluteus larval stage was smaller in the sea urchins *Hemicentrotus pulcherrimus* and *Echinometra methaei* grown under elevated CO_2 conditions (Kurihara et al. 2004, Kurihara & Shirayama 2004); additionally the morphology and shell mineralization of larvae of the oyster *Crassostrea gigas* were disrupted at pH 7.4 (Kurihara et al. 2007). The study of Kurihara et al. (2007) is the first invertebrate study that, by concentrating on the process of mineralization, begins to focus on a mechanistic developmental process that might be affected by reduced pH. Such processes are often key to understanding how the environment affects organism fitness (e.g. Spicer & El-Gamal 1999).

Here we investigate how ocean acidification affects developing embryos, using the intertidal gastropod *Littorina obtusata*. A direct developing gastropod, this species is ideal for observing development, including traits such as spinning activity and heart rates (S. Rundle et al. unpubl. data). We focussed on behavioural (percentage spinning/crawling time, movement rate and periodicity) and physiological (larval and adult heart activity) effects, but also measured total development time, hatching success, hatchling morphology and heart rate in order to infer potential links between egg viability, hatchling condition and the embryonic traits measured.

MATERIALS AND METHODS

Collection and maintenance of adult snails. Adult *Littorina obtusata* (N = 90) were collected by hand from the intertidal zone at Mount Batten, Plymouth, Devon, UK (50° 21.34′ N, 04° 07.45′ W) and Talland Bay, West Looe, Cornwall, UK (50° 20.21′ N, 04° 29.78′ W) during September 2007. Snails were transported to the laboratory within 1 h of harvest and immediately distributed between 6 aquaria (volume = 9 l, stocking density = 15 ind. aquarium^{-1}) each containing aerated seawater (salinity = 35 PSU, temperature = 15 ± 0.5°C, filtered using a 10 carbon block filter [10 µm Matrikx]). All aquaria were subject to a 16 h light:8 h dark regime. Snails were fed ad libitum on *Fucus serratus*. Snails

were housed in collection batches, which were sorted by date, to ensure no effect of disturbance through addition of subsequently collected animals and to ensure all individual snails had equal acclimation time in the laboratory.

Egg laying occurred on algal fronds and was initiated by exposing snails to 'dirty water' followed by a recovery period (e.g. Boyle & Yoshino 2000): after 5 daily water changes and constant aeration, aerated aquarium water was left unchanged and snails were left unfed for 72 h, followed by 36 h with no aeration; after this time water was renewed and aerated, and snails were given fresh *Fucus serratus*. Daily water changes were resumed until sufficient egg batches had been laid. This experimental method was demonstrated to induce oviposition in snails, which, when left to occur 'spontaneously', does so at a rate that is unsuitable for large experimental manipulations (Boyle & Yoshino 2000).

Egg batch maintenance and seawater acidification. Fronds containing egg masses were removed from aquaria within 24 h of laying. Each mass (N = 9) was detached from the frond using a scalpel and divided into 2 equal halves (from now on these halves are referred to as batches). Each batch was then transferred to an experimental chamber (volume = 100 ml, polypropylene) with an aspirator supplying either air (untreated seawater) or air enriched with CO_2 (acidified seawater), with a batch from each egg mass being allocated to 1 of each of the 2 treatments. Air treatments were prepared as follows: ambient air scrubbed with NaOH (2 mol l^{-1}) was mixed with CO_2 using adjustable airline gang valves (Algarde) to produce a nominal CO_2 concentration of 1100 ppm (measured mean = 1093 ± 181 ppm). The resultant gas mixture was dried, and the CO_2 content was measured using a CO_2 analyzer (LI-700, Li-Cor) and then supplied to either (1) each of the experimental chambers, via an airline connected to a glass pipette inserted through the top of the experimental chamber, or (2) a reservoir tank (volume = 9 l) containing seawater, producing seawater at pH 7.6, which was used to replenish water in experimental chambers every 24 h. This procedure was replicated for the control treatment, except that the air was not scrubbed and CO_2 was not added, which resulted in water through which this gas mixture was aspirated having a nominal pH of 8.1. The pH of experimental water was measured every 24 h (just prior to a complete change of seawater in experimental chambers) using a pH electrode (pH 209 meter, Hanna Instruments).

Embryo viability and hatching success. Every 24 h each egg batch was removed from its chamber, placed onto a scaled graticule slide (1 mm), immersed in chamber seawater, and imaged using a digital camera

(Nikon Coolpix 4500) mounted on an optical light microscope (Leica MZ12; Mazurek optical series, mag. ×8–50). Three images were taken of each egg batch daily, containing a minimum of 3, typically 10 to 20, haphazardly selected eggs. Egg viability was subsequently calculated, with imaged eggs being recorded as viable or non-viable, non-viable being those that were either deformed or twin (i.e. 2 egg capsule^{-1}), as no embryo from either of these classes was observed to develop successfully. Viability was analysed up until the first hatching event, as, after this point in development, successful hatching would alter the visible numbers of viable eggs.

Each chamber was inspected every 24 h for hatchlings, which were then removed using a small brush. After appropriate physiological measurements had been made (see following subsection), hatchlings were collected using a pipette, counted, transferred to a glass vial and stored at −20°C for subsequent morphological analysis (see following subsection).

Measuring physiological and behavioural traits of embryos. We measured embryo activity and selected aspects of physiological function throughout development in a subsample of 3 egg batches from each treatment. Embryo activity (as defined below) was quantified every 24 h from the fourth day post hatching (i.e. when spinning commenced, as prior to this point embryos remained stationary) using 5 min videos of 3 randomly selected eggs from within each of the 3 egg batches. These batches were placed on a slide immersed in seawater taken from their chambers and videoed for 5 min d^{-1}. Digital video imaging was produced using a motion camera (Pixera Corporation) mounted upon a light microscope coupled to a PC (Pixera Motion v.2.1). Videos were recorded at a magnification of ×40 to 100 depending on the stage of development and were analysed to determine embryo behaviour. Several behavioural measures were employed based on the cilia-driven rotation, spinning, movements observed in early stage gastropod embryos (e.g. Diefenbach et al. 1991) and the crawling activity exhibited later in development (e.g. Smirthwaite et al. 2007), including: (1) the percentage of time embryos spent stationary, spinning, or crawling; (2) the spinning rate of embryos, recorded as rotations per minute (RPM); and (3) the periodicity of embryo movement, being the total number of both stationary (inactive) and motile (active) periods, highlighting how often embryos switched from stationary to active motion, or active to stationary, during a recording period. Spinning activity is of particular interest due to its proposed function in mixing of cellular fluids increasing embryo environmental oxygen delivery (Goldberg et al. 2008); embryo rotational behaviour is also linked to early neural development (Diefenbach et al. 1991), and, hence, its

disruption could indicate effects on the embryonic nervous system.

As well as measuring the activity of the adult heart (beats min^{-1}), which appears and begins to function midway through embryonic development, we also quantified the activity of the 'larval heart'. This extracardiac structure, which is linked with perfusion of the velum and has a presumed circulatory function, appears before the adult heart, disappearing once the adult heart has been established (Fretter & Graham 1962). Larval and adult heart activities were quantified every 24 h throughout development, from the day that each respective organ began to function. In this case direct counts were made from the same 3 egg batches from each treatment used for behavioural videos. Both larval and adult heart activities were measured separately in 3 randomly selected individuals from each egg batch every day for 1 min. In the case of the larval heart only, days where this structure remained fully functional in all recorded replicates, up to Day 21, were included in this analysis. Adult heart activity analysis only included days on which the adult heartbeat was established in all individuals (i.e. from 17 d). The area of the velum was also measured throughout development using imageJ (http://rsb.info.nih.gov/ij/).

Hatchling morphology and heart beat. Aspects of the physiology and morphology of post-hatch juvenile snails were quantified to determine whether the embryonic developmental environment might have affected this later stage of development. Firstly, the adult heart activity (the larval heart was not present at this stage) was measured manually for 60 s for the first 5 hatchling individuals from 3 egg batches in each treatment.

Hatchling morphology was measured in 5 individuals from each of the last 3 egg batches to be laid in each treatment (N = 30), photographed in the same way as were embryos. Eight measures were obtained: lateral shell length, ventral shell length, ventral shell width (spire height), aperture length, aperture width (widest), lateral shell mid-length, spiral length and aperture area (Fig. 1).

Statistical analysis. All data were analysed using MINITAB 15. First, data were transformed when necessary, including $\log_{10}(x + 1)$ (hatchling duration, hatchling number, movement periodicity and rate, larval, adult and hatchling heartbeat) and arcsine (percentage of time spent stationary, spinning or crawling) transformation. Two-way analyses of variance (ANOVAs), using the general linear model, were then used to investigate the effects of developmental time, pH and their interaction on all traits observed during development. On finding a significant interaction, post hoc Tukey tests were used to assess pairwise differences between the treatments on specific
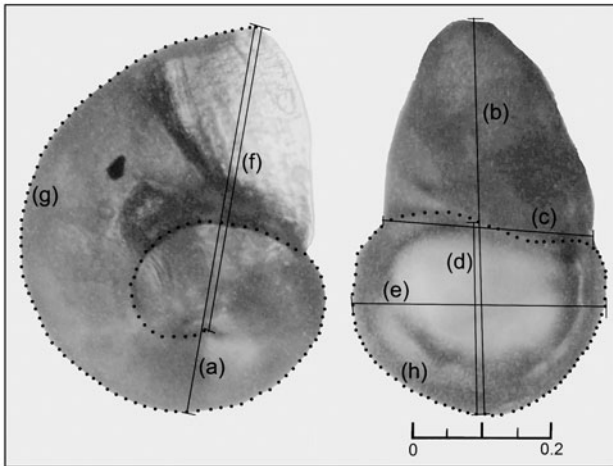
Fig. 1. *Littorina obtusata*. Hatchling morphological measures: (a) lateral shell length, (b) ventral shell length, (c) ventral shell width (spire height), (d) aperture length, (e) aperture width (widest), (f) lateral shell mid-length, (g) spiral length and (h) aperture area. Scale bar = 0.2 mm
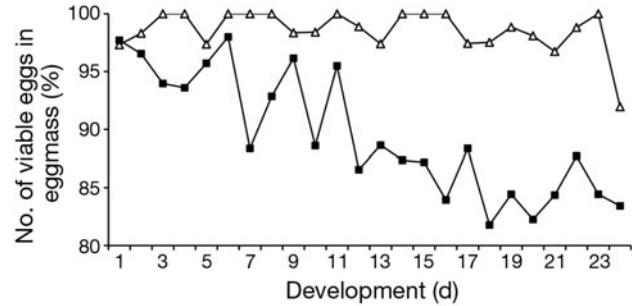

Fig. 2. *Littorina obtusata*. Percentage egg batch viability, throughout development, of egg batches maintained in untreated (control) and reduced pH (treatment) seawater. Values are total numbers of viable eggs from within each treatment, on each day, as a percentage of total numbers of eggs observed. △: control treatment; ■: acidified treatment

days throughout development, indicating at which point during development the treatment has a significant effect. Differences in hatchling morphology, hatchling heart rate and development duration between the control and low pH treatment were investigated using 1-way ANOVA.

RESULTS

Embryo viability and hatching success

There was a clear effect of acidified seawater on the viability of *Littorina obtusata* embryos. During development, percentage egg viability in untreated seawater remained >96% until the first hatching event on Day 24. In acidified seawater, however, percentage viability fell to <94% after Day 6 and declined further to 88% by Day 7, remaining between 81 and 88% from Day 12 through 24 (Fig. 2). The cumulative number of hatchlings was lower in acidified seawater (N = 449) compared with the number in the control (N = 575) (Fig. 3), and mean duration of development was significantly longer in the acidified treatment group (mean ± SE: 27.5 ± 0.1 d) compared with the duration in the control (26.9 ± 0.05 d) (ANOVA, $F_{1,1022} = 56.29$, p > 0.001).

Embryo activity

Embryos spent a significantly greater proportion of their time stationary in reduced pH compared with the control seawater (Fig. 4a, Table 1), which was due to

reduced crawling (Fig. 4c, Table 1). There was also a significant interaction between development time and pH for the percentage of time spent crawling (Fig. 4c, Table 1); post hoc analysis indicated a significant difference between treatments 22 d into development. In contrast, there was no effect on the percentage of time spent spinning (Table 1), which occurred earlier in development than crawling (Days 7 to 20) (Fig. 4b). Spinning rate was also affected by pH, with embryos in acidified seawater rotating at a significantly reduced rate (Fig. 5, Table 2). Periodisation of embryo movement was greater in the acidified treatment (Fig. 6, Table 2).

Physiological measures from embryo

A functional larval heart appeared 12 d into development in both the control and reduced pH treatments, although the larval heart rate was significantly lower in acidified seawater (Fig. 7, Table 3). Adult heart activity was also significantly lower in acidified seawater (Fig. 7, Table 3).
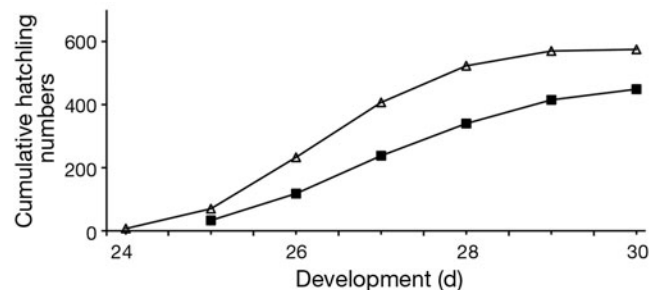

Fig. 3. *Littorina obtusata*. Cumulative hatchling numbers, from egg batches maintained in untreated (control) and reduced pH (treatment) seawater from the first hatching event until the termination of the experiment. △: control treatment; ■: acidified treatment
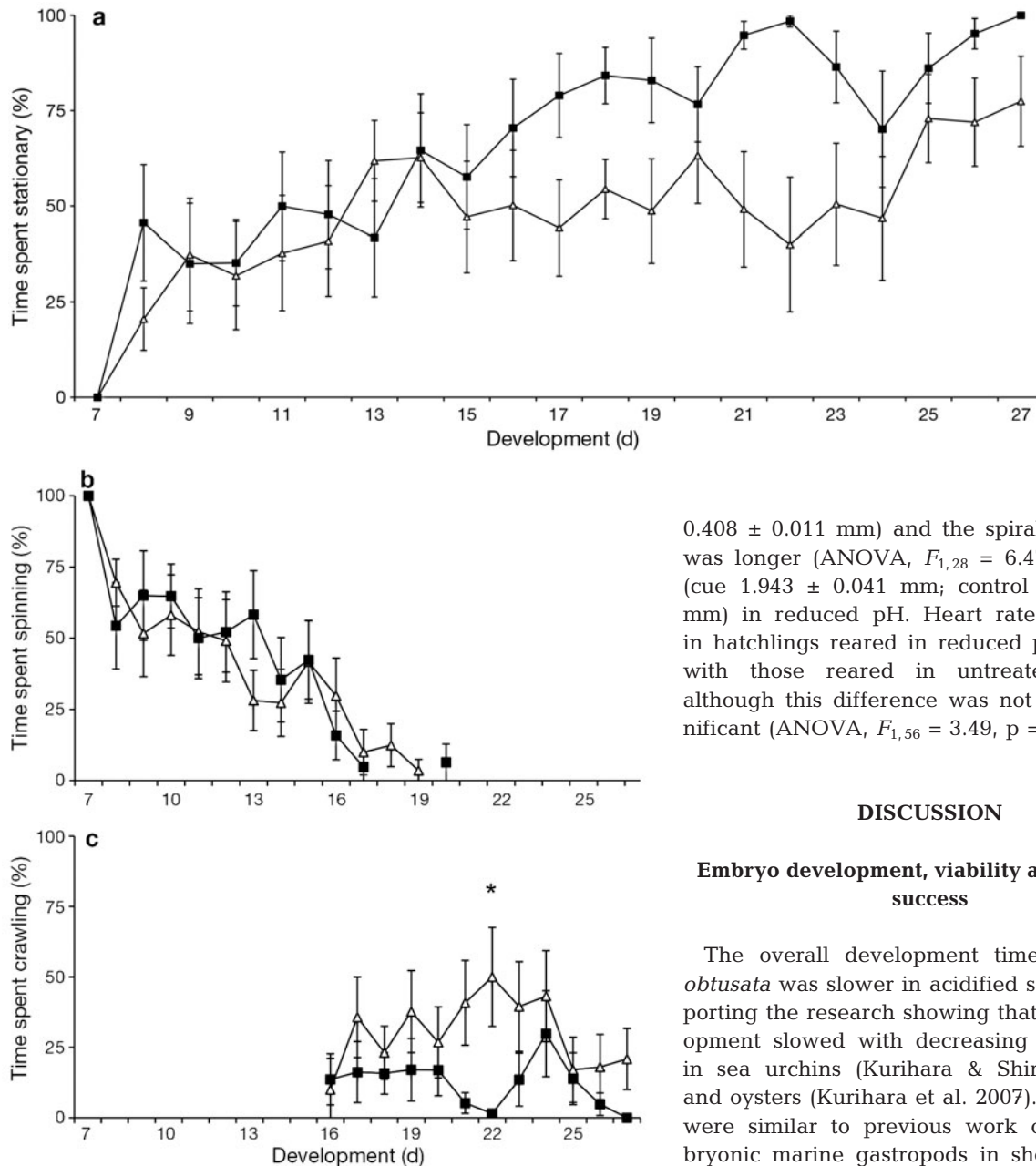
Fig. 4. *Littorina obtusata*. Percentage of time spent (a) stationary, (b) spinning and (c) crawling, throughout development, in embryos developed in untreated (control) and reduced pH (treatment) seawater (mean ± SE); asterisk indicates a significant difference between treatment group means as tested in post hoc analyses. △: control treatment; ■: acidified treatment

Hatchling morphology and heart activity

Two morphological characteristics of the hatchling shell were significantly affected by reduced pH: the lateral shell length was shorter (ANOVA, $F_{1,28} = 7.49$, p = 0.011) (mean ± SE: cue 0.369 ± 0.008 mm; control 0.408 ± 0.011 mm) and the spiral shell length was longer (ANOVA, $F_{1,28} = 6.47$, p = 0.015) (cue 1.943 ± 0.041 mm; control 1.811 ± 0.03 mm) in reduced pH. Heart rate was greater in hatchlings reared in reduced pH compared with those reared in untreated seawater, although this difference was not formally significant (ANOVA, $F_{1,56} = 3.49$, p = 0.067).

DISCUSSION

Embryo development, viability and hatching success

The overall development time of *Littorina obtusata* was slower in acidified seawater, supporting the research showing that larval development slowed with decreasing seawater pH in sea urchins (Kurihara & Shirayama 2004) and oysters (Kurihara et al. 2007). Our findings were similar to previous work on other embryonic marine gastropods in showing slower development due to adverse environmental conditions, with previous studies demonstrating a significant impact of temperature, salinity, air exposure and solar radiation on rate of development (e.g. Pechenik et al. 2003, Przeslawski 2005, Przeslawski et al. 2005). However, our results and those for the sea urchins and oyster contrast with studies on *Calanus finmarchicus*, in which there was a CO_2-related reduction in hatching rate but no effect on growth or egg production (Mayor et al. 2007), and on another copepod *Acartia tsuensis*, in which there was no effect of CO_2 (2380 ppm) on developmental rate, survival, or body size (Kurihara & Ishimatsu 2008).

Table 1. 2-way ANOVA assessing differences in behavioural traits (expressed as percentage time spent in that activity) of *Littorina obtusata* embryos in untreated and reduced pH (Treatment) seawater through time (Day). ***p < 0.001; *p < 0.05; –: p > 0.05, significance levels

Factor	df	SS	MS	*F*	p
Stationary					
Day	20	28.6221	1.4311	3.96	<0.001***
Treatment	1	5.2286	5.2286	14.48	<0.001***
Day × Treatment	20	6.4853	0.3243	0.90	0.590 –
Error	317	114.4790	0.3611		
Total	358				
Spinning					
Day	20	52.8176	2.6409	15.4	<0.001***
Treatment	1	0.0193	0.0193	0.11	0.737 –
Day × Treatment	20	2.3197	0.116	0.68	0.849 –
Error	317	54.3503	0.1715		
Total	358				
Crawling					
Day	20	11.5701	0.5785	4.14	<0.001***
Treatment	1	2.5651	2.5651	18.36	<0.001***
Day × Treatment	20	4.7229	0.2361	1.69	0.034*
Error	317	44.2804	0.1397		
Total	358				

Table 2. 2-way ANOVA assessing differences in movement parameters of *Littorina obtusata* embryos in untreated and reduced pH (Treatment) seawater through time (Day). ***p < 0.001; *p < 0.05; –: p > 0.05, significance levels

Factor	df	SS	MS	*F*	p
Rate of spinning (RPM)					
Day	20	6.7956	0.3398	11.84	<0.001***
Treatment	1	0.1169	0.1169	4.07	0.044*
Day × Treatment	20	0.4491	0.0225	0.78	0.735 –
Error	317	9.1002	0.0287		
Total	358				
Total periodisation of movement					
Day	20	1.3371	0.0669	2.60	<0.001***
Treatment	1	0.2830	0.2830	10.99	0.001***
Day × Treatment	20	0.4992	0.0250	0.97	0.499 –
Error	317	8.1600	0.0257		
Total	358				

Larval and adult heart function

The effect of reduced pH on both the larval and adult hearts of *Littorina obtusata* supports the idea that reduced pH may affect the metabolism of developing embryos. The fact that these activities were reduced under low pH conditions is at odds with any hypothesis that could be advanced in which metabolism is increased and oxygen levels decreased under low pH. To our knowledge the effect of environmental cues on larval heart function is unexplored, rendering comparisons with our work premature.
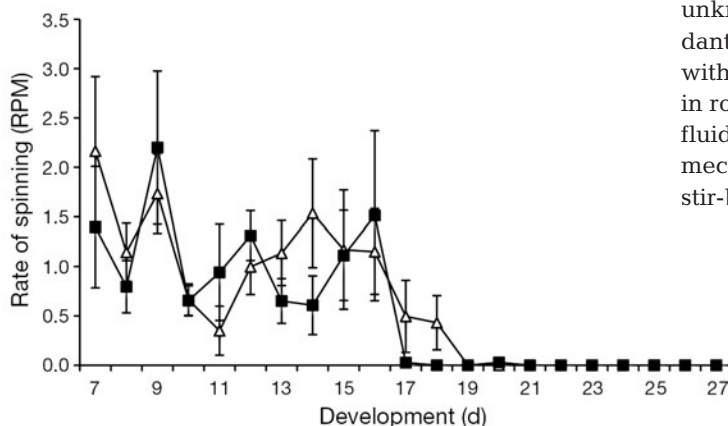

Fig. 5. *Littorina obtusata*. Rate of spinning (RPM, rotations per minute) in embryos developed in untreated (control) and reduced pH (treatment) seawater (mean ± SE). △: control treatment; ■: acidified treatment

Embryo activity

During development, snails in acidified seawater spent less time crawling and, despite no reduction in the time spent spinning, their spinning rate was significantly reduced. Under acidified conditions the snails had short bursts of active motility interspersed with frequent stationary periods as opposed to prolonged active periods. All of the behavioural alterations we observed have been noted previously in embryonic snails (Moran 1999, Chaparro et al. 2002, Uyan & Aral 2003), but there was no previous information on the effects of CO_2-induced low pH. The effect of CO_2 on sexually mature stages usually led to a reduction in activity; for example, Batten & Bamber (1996) found a reduction in burrowing activity in adult polychaetes *Nereis virens* exposed to pH 7.5, although Widdicombe & Needham's (2007) study on the same species reported no CO_2-related change in activity.

Why embryonic movement is affected by CO_2 is unknown. Kuang et al. (2002) showed a dose-dependant and reversible increase in embryonic rotation rate with O_2 concentration, with a hypoxia-related increase in rotation rate facilitating the mixing of intra-capsular fluid, facilitating O_2 diffusion to the embryo. This mechanism has recently been termed the 'embryo stir-bar hypothesis' (Goldberg et al. 2008), and it is possible that the alterations to activity we observed reflect a link between reduced pH and O_2 in experimental animals, potentially through an indirect influence on metabolism.

Hatchling morphology

The shape of hatchling shells was altered by culture in elevated CO_2, with the lateral shell

length being shorter and the spiral shell length longer. Modification of shell shape in *Littorina obtusata* by abiotic factors such as predation have been recorded previously (Brookes & Rochette 2007), but this is the first time it has been documented for hatchlings and for snails cultured in acidified seawater. The extent to which these impacts and the observed decreased viability of embryos can be attributed to the behavioural and physiological responses of embryonic *L. obtusata* is not known. The cost of developing under acidified conditions for organisms at later life cycle stages should be a research priority. Such information would allow us to understand how any trade-offs imposed by a permanently reduced seawater pH will impact an organism's ecology and, ultimately, its fitness (Black 1993).
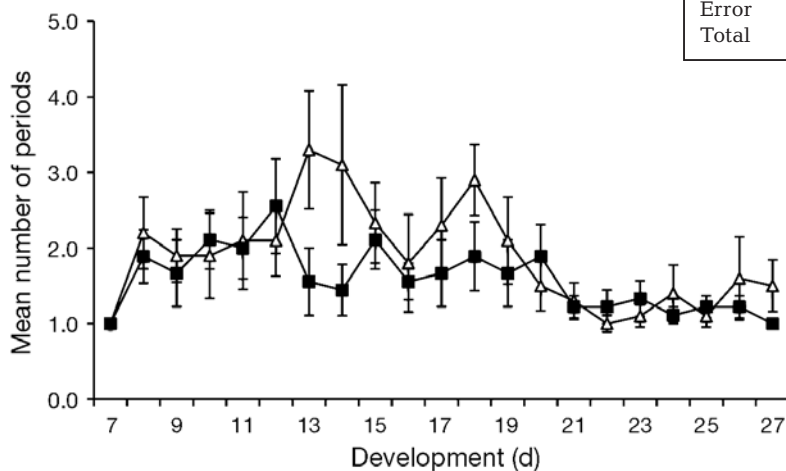
Table 3. Results of 2-way ANOVAs assessing differences in larval and adult heart rates of *Littorina obtusata* embryos in untreated and reduced pH (Treatment) seawater through time (Day). ***p < 0.001; **p < 0.01; *p < 0.05; –: p > 0.05, significance levels

Factor	df	SS	MS	*F*	p
(a) Larval heart rate					
Day	9	1.1062	0.1229	1.52	0.146 –
Treatment	1	0.4444	0.4444	5.50	0.020*
Day × Treatment	9	0.7559	0.0840	1.04	0.412 –
Error	143	11.5577	0.0808		
Total	162				
(b) Adult heart rate					
Day	10	4.1333	0.4133	3.90	<0.001***
Treatment	1	0.9242	0.9242	8.72	0.004**
Day × Treatment	10	1.1816	0.1182	1.11	0.354 –
Error	159	16.8503	0.1060		
Total	180				

Fig. 6. *Littorina obtusata*. Periodisation of embryo movement during the recording period (mean ± SE). △: control treatment; ■: acidified treatment
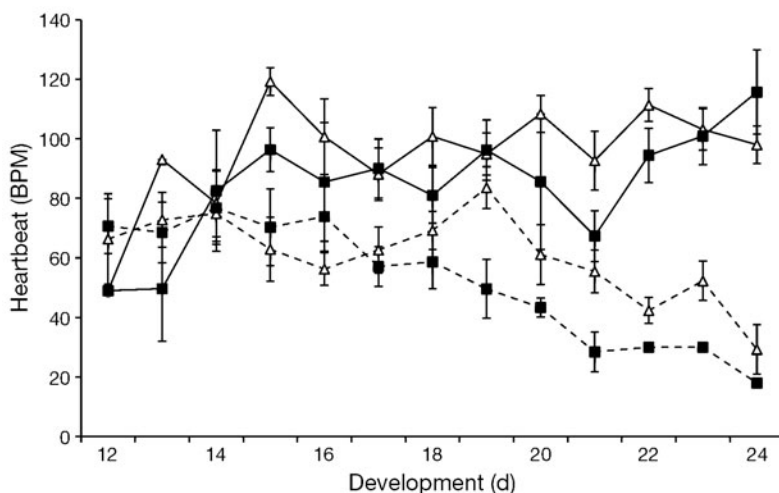

Fig. 7. *Littorina obtusata*. Larval and adult heart rate (BPM, beats per minute), throughout development, of embryos developed in untreated (control) and reduced pH (treatment) seawater (mean ± SE). Dashed lines: larval heart rate; continuous lines: adult heart beat; △: control treatment; ■: acidified treatment

CONCLUSIONS

We demonstrate that ocean acidification may affect embryonic marine organisms in significant but subtle ways. Furthermore, such effects are observed at a level of seawater acidification (pH 7.6) predicted to occur over the next century. It is now of considerable interest to know the long-term costs (if any) of CO_2-related modifications in the development of this important intertidal species. Whether or not there are fitness implications from environmental challenges early in development is a key area of study in ecological and evolutionary biology (Mathis et al. 2008), the findings of which are critical for our understanding of the effect of ocean acidification on marine species and ecological communities. The ultimate goal, however, should be to try and factor the embryonic stage into long-term studies aiming to address whether marine invertebrates such as *Littorina obtusata* are likely to be able to adapt to the predicted changes in ocean chemistry.

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