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Larval development of the barnacle *Amphibalanus improvisus* responds variably but robustly to near-future ocean acidification

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Increasing atmospheric CO_2 decreases seawater pH in a phenomenon known as ocean acidification. In two separate experiments we found that larval development of the barnacle *Amphibalanus (Balanus) improvisus* was not significantly affected by the level of reduced pH that has been projected for the next 150 years. After 3 and 6 days of incubation, we found no consistent effects of reduced pH on developmental speed or larval size at pH 7.8 compared with the control pH of 8.1. After 10 days of incubation, there were no net changes in survival or overall development of larvae raised at pH 7.8 or 7.6 compared with the control pH of 8.0. In all cases, however, there was significant variation in responses between replicate batches (parental genotypes) of larvae, with some batches responding positively to reduced pH. Our results suggest that the non-calcifying larval stages of *A. improvisus* are generally tolerant to near-future levels of ocean acidification. This result is in line with findings for other barnacle species and suggests that barnacles do not show the greater sensitivity to ocean acidification in early life history reported for other invertebrate species. Substantial genetic variability in response to low pH may confer adaptive benefits under future ocean acidification.

Keywords: *Amphibalanus (Balanus) improvisus*, barnacle, CO_2, cyprids, early life history, larvae, development, nauplii, ocean acidification, pH.

Introduction

Increasing atmospheric CO_2 reduces the pH of seawater (Caldeira and Wickett, 2005). This "ocean acidification" (OA) affects marine species in various ways (Kroeker *et al.*, 2010, 2013; Harvey *et al.*, 2013) and has the potential to fundamentally alter the structure of marine ecosystems (e.g. Hale *et al.*, 2011).

Early life-history stages of marine organisms have been reported to be particularly sensitive to environmental changes (Dupont *et al.*, 2008; Kurihara, 2008). In addition, conditions experienced during embryonic and larval development may substantially affect performance in subsequent life-history stages—so-called "carry-over effects" (Pechenik *et al.*, 1998; Thiyagarajan *et al.*, 2002). Consequently the effects of OA on early life-history stages—especially in calcifying species such as barnacles—may have pervasive consequences for juvenile and adult stages.

Investigations of the effects of OA on early life-history stages of barnacles are, however, rare. The only published studies report that larval stages of the barnacle *Amphibalanus amphitrite* were unaffected by even severe OA (pH 7.4; McDonald *et al.*, 2009), whereas embryonic development of *Semibalanus balanoides* was negatively impaired by OA (pH 7.7; Findlay *et al.*, 2009). Interestingly, Pansch *et al.* (2012a) found that larval development time was prolonged under OA (pH 7.4) but only at high and low temperatures; at intermediate temperatures representative of summer conditions, OA had no effect on larval development.

Intraspecific variation is the raw material of natural selection, and different populations of organisms have been shown to respond differently to OA (Walther *et al.*, 2010, pers. obs.; Parker *et al.*, 2011). The magnitude and importance of intrapopulation variation has, in contrast, rarely been addressed in experimental OA studies. This omission is surprising, as such studies provide invaluable insights on the potential of species to adapt to climate change in the coming decades (Sunday *et al.*, 2011; Schlegel *et al.*, 2012).

Here we report the impacts of decreased pH on survival and larval development of multiple batches of the bay barnacle *Amphibalanus improvisus* over two successive years.

Material and methods

Sampling and experiments

The bay barnacle *Amphibalanus improvisus* (also referred to as *Balanus improvisus*; Pitombo, 2004) is a prominent filter-feeder in many fouling communities (Strathmann, 1987; Andersson *et al.*, 1999, 2009) and is globally widespread, occurring in shallow, tidal areas in both salty and brackish waters. It is common throughout the Baltic Sea system, extending from near-full-salinity waters of the Skagerrak to nearly fresh (3‰) waters of the Bay of Bothnia. Eggs hatch internally and are released as free-swimming Stage-I nauplius larvae that develop to Stage-II nauplii within 3–4 h (Thiyagarajan *et al.*, 2003). Stage-II nauplii start feeding and go through another five moults before they develop into the non-feeding cypris stage, which settles and metamorphoses into a juvenile barnacle (post-larva; Jones and Crisp, 1954).

Our experiments were conducted in 2008 and 2009 at the Sven Lovén Centre for Marine Sciences in Tjärnö, Sweden. Barnacle (*A. improvisus*) broodstock comprised a mixed population of adults from Idefjorden (30 km north of Tjärnö; 59°06.2′N 11°21.1′E) and the bay outside the research station (58°52.5′N 11°08.1′E). Broodstock barnacles were held in constant conditions with flow-through seawater at 20°C and fed daily with brine shrimp (*Artemia salina*) *ad libitum*. Freshly released barnacle larvae (Stage-II nauplii) were collected overnight from the broodstock tanks.

We conducted two different experiments to assess the sensitivity of *A. improvisus* larvae in response to decreased seawater pH. In the first experiment, larval survival and development were assessed by rearing nauplius larvae in 6-well plates over 10 days in response to three different pH treatments. In the second experiment, larval stage and size were assessed by rearing nauplius larvae in 5-l glass bottles over 6 days in response to two different pH treatments.

Multiple larval batches of the barnacle *Amphibalanus improvisus* were investigated in repeated experiments. The individual larval batches represent replicate subsamples from the pool of reproducing adults in the studied population of barnacles. Because different adults spawn on different days, genotypic diversity varies between batches, contributing biologically meaningful variation to the observed responses.

Larval survival and development

Filtered seawater (0.2 μm filter) was treated in 2-l glass bottles by adding pure gaseous CO_2 using computerized pH controllers (NBS scale, resolution: 0.01 units, Aqua Medic GmbH, Germany). An 8 ml volume of filtered seawater at each of three pH levels (mean $pH_{NBS} \pm SD = 8.02 \pm 0.06$; 7.80 ± 0.04; 7.57 ± 0.08, Beckman Coulter 400 Series pH meter, NBS scale; total alkalinity = 2133 μmol kg^{-1}) was added to each well of different 6-well plates (Greiner bio-one, Smurfit Kappa GmbH, Neuburg-Germany). 20 nauplii were distributed to each of the wells. The 6-well plates were then placed in seawater–air CO_2 incubators (for details see Egilsdottir *et al.*, 2009; Pansch *et al.*, 2012a). These comprised sealed 11-l plastic aquaria, which were half-filled with filtered seawater (0.2 μm filter) aerated with a mixture of fresh filtered air and pure gaseous CO_2. CO_2 concentrations were controlled using computerized pH controllers as described above to provide mean pH_{NBS} ($\pm$ SD) of 8.03 ± 0.04, 7.86 ± 0.02 and 7.59 ± 0.02. Larvae were reared over 10 days at 25 $\pm$ 1°C and provided with a daily diet of marine diatoms (50:50 mixture of *Chaetoceros calcitrans* and *Skeletonema costatum*) at a concentration of $1–1.5 \times 10^5$ cells ml^{-1} (Strathmann, 1987) under continuous light (Thiyagarajan *et al.*, 2003). The water in the wells was exchanged every second day with filtered seawater at the respective pH.

Larval survival and the number of nauplii that had metamorphosed into cyprids were assessed at the end of the experiment (10 days) using a dissecting microscope (Olympus SZX12). Each treatment was replicated six times within each experiment, and the whole experiment was repeated four times, each time using a different batch of larvae. Treatments were randomly assigned to incubators for each batch.

Larval stage and size

Filtered seawater (0.2 μm filter) was treated in 5-l glass bottles by adding pure gaseous CO_2 using computerized pH controllers as described above. A fresh batch of nauplii was evenly distributed among the glass bottles (~1 larva ml^{-1}) at each of two pH levels (mean $pH_{NBS} \pm SD = 8.09 \pm 0.07$; 7.80 ± 0.04; total alkalinity = 2134 μmol kg^{-1}). A small subsample of larvae from each bottle was fixed in 10% seawater-buffered formalin at the beginning of the experiment. Larvae were reared over 6 days at 25 $\pm$ 1°C. Larvae were fed with a diet of marine diatoms (*Skeletonema costatum*) at a concentration of $1–1.5 \times 10^5$ cells ml^{-1} (Strathmann, 1987) on Day 1 and Day 3. Larvae were held under a 12:12 h light:dark cycle. A full water exchange was performed after 3 days to remove detritus and faeces as well as dead larvae. Subsamples of nauplii were taken after 3 and 6 days of cultivation and fixed in 10% seawater-buffered formalin. Within each experiment, treatments were replicated three times, and the whole experiment was repeated five times, each time using a different batch of larvae. Incubation bottles were randomized between different batches.

Developmental stages of *A. improvisus* (nauplius Stages I–VI, cyprid = C, juvenile = J) were determined according to Jones and Crisp (1954) using an inverted microscope (Olympus IX71). Larval stage percentages were determined from direct counts of each larval stage in the subsample. Total length (overall length) and carapace length of larvae were assessed according to West and Costlow (West and Costlow, 1987).

Statistical analysis

Although statistical testing of a null hypothesis is almost universally used to analyse the results of biological experiments, this approach conflates statistical significance with biological significance. In climate change research, understanding the magnitude (and variance) of a given effect may be more important than showing a statistically significant difference from "no response". In these circumstances standardized measures of effect size and confidence intervals around them are more informative than traditional null-hypothesis testing (e.g. Nakagawa and Cuthill, 2007; Havenhand *et al.*, 2010). We used this approach to assess responses of *A. improvisus* larvae to the treatments. Specifically, we measured effect size using the mean $\pm$ 95% CI log response ratio (LnRR = log[response in treatment]/log[response in control]; Hedges *et al.*, 1999; Frommel *et al.*, 2012; Schlegel *et al.*, 2012). We chose this metric because of its robust statistical properties and ease of biological interpretation (Hedges *et al.*, 1999; Nakagawa and Cuthill, 2007; Kroeker *et al.*, 2010). A LnRR of zero indicates no effect on the response variable, while positive and negative values indicate positive and negative responses to the treatments, respectively. Overlap of the 95% CI with a given value indicates the effect size is not significantly different from that value ($\alpha = 0.05$).

We additionally performed factorial analysis of variance (ANOVA). All data were tested for normality using the Shapiro–Wilk's W-test

and for homogeneity of variances using Levene's test. Percentage data were arc-sine square-root transformed prior to parametric statistical analyses. When normality or homogeneity of variances was not achieved after transformation we used parametric tests but reduced the level of significance to 0.01 in order to minimize Type 1 errors (Underwood, 1997; Wakefield and Murray, 1998). Larval survival and overall development were tested with a factorial ANOVA with the factors batch (four levels) and pH (three levels). Larval stage at Day 3 and at Day 6 were tested with a factorial ANOVA with the factors batch (five levels) and pH (two levels) using the percentage of larvae developed to Stage V+ (Day 3) or to Stage C+ (Day 6). Total length and carapace length were tested with a factorial ANOVA with the factors batch (five levels) and pH (two levels) for the dominant larval stages (total length: Stage IV, V and V; carapace length: Stage IV, V, VI and C). In all cases the factor "batch" was analysed as a random factor as this was treated as a level of replication. All statistical analyses were done using STATISTICA 8.0 (Stat-Soft, Inc., USA).

Experiments were conducted according to relevant national and international animal welfare laws and no permissions were required for the use of the investigated species.

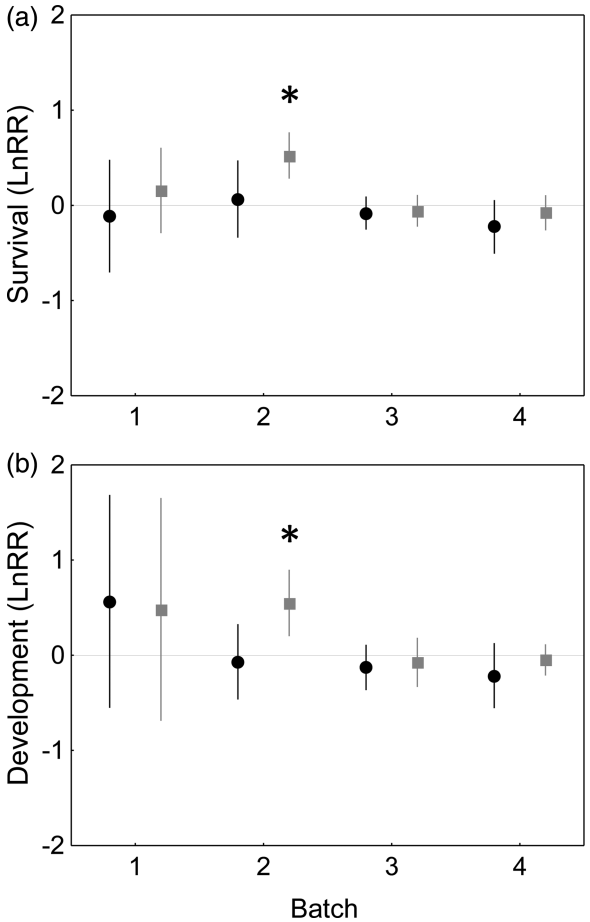

Figure 1. Effects of ocean acidification on the survival (**a**) and development to cyprids or juveniles (**b**) of *Amphibalanus improvisus* after 10 days in pH 7.8 vs. pH 8.0 (black bars) and pH 7.6 vs. pH 8.0 (grey bars) in four different batches of larvae (six replicates per batch). Mean effect size (Log Response Ratio, LnRR) is significant when the 95% confidence interval does not overlap zero (*).

Results

Larval survival and overall development

We observed substantial variability in survival and development between the different batches of larvae (Figure 1, Table 1). Overall survival was highest in batch 3 (73%), and lowest in batch 2 (30%; means over all pH treatments). Overall development was most rapid in batch 3, with 65% of nauplii reaching the cypris stage or metamorphosing to juveniles by the end of the experiment, whereas batch 2 showed the slowest overall development (15%; means over the pH treatments).

The effects of pH on survival and overall development also varied substantially between batches, being positive in some batches and negative in others. OA only had statistically significant effects on survival and overall development of nauplii of the second batch under very low pH conditions: survival was greater and development faster in pH 7.6 water compared with control water of pH 8.0 (Figure 1).

ANOVA revealed that survival and overall development of larvae differed significantly between batches, but there was no significant interaction between the effects of batch and pH, or of pH alone (Table 1).

Larval stage and size

After 3 days, approximately 68% of larvae had developed to Stage V and 25% to Stage VI (means over all pH treatments and batches; Figure 2a). After 6 days, approximately 78% of larvae had developed to the cypris stage and 3% to the juvenile stage (means over all pH treatments and batches; Figure 2b). Consequently, numbers of larvae developing to these stages or further (V+ and C + , respectively) were chosen for analysis of the effects of pH and batch on larval development.

Again, the effects of pH on larval development varied between batches, being positive in some and negative in others. After 3 days a significantly higher proportion of nauplii had developed to Stage V or further (Stage V +) under pH 7.8 compared with pH 8.1 in the first batch (Figure 3a), however this effect was not seen after 6 days of development (Figure 3b).

The effects of pH on total length and carapace length of larvae also varied between batches. After 3 days the total length of Stage

Table 1. Factorial ANOVA on the effects of pH and batch on larval survival and overall development of *Amphibalanus improvises*.

		SS	d.f.	MS	F	p
Survival	batch	1.851	3	0.617	38.636	* < 0.001
	pH	0.097	2	0.049	2.722	0.144
	batch × pH	0.111	6	0.018	1.156	0.342
Overall development	batch	3.268	3	1.089	40.454	* < 0.001
	pH	0.035	2	0.018	0.487	0.637
	batch × pH	0.220	6	0.037	1.363	0.244

Significant effects are indicated with asterisks.

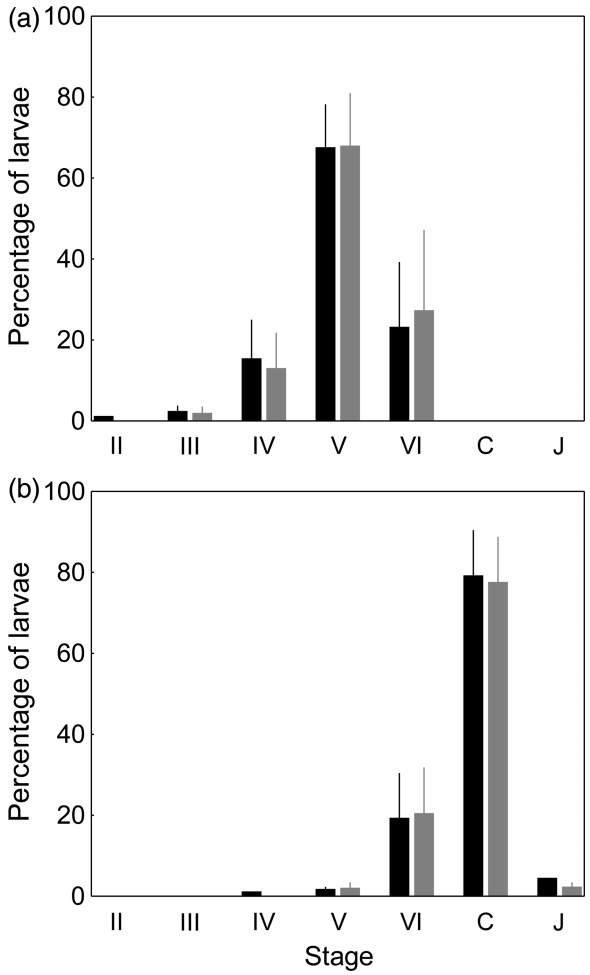

Figure 2. Percentage of larvae of *Amphibalanus improvisus* in different developmental stages (nauplius larvae = II–VI, cypris = C, and juveniles = J) on Day 3 (**a**) and Day 6 (**b**) in pH 8.1 (black bars) and pH 7.8 (dark grey bars; means over all five batches ± 95% CI; three replicates per batch).

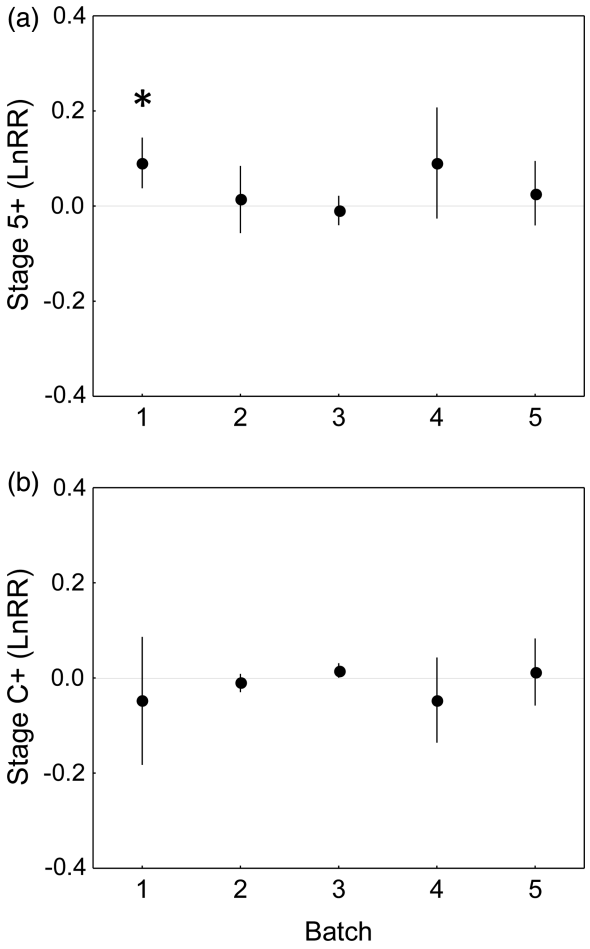

Figure 3. Effects of ocean acidification on the percentage of larvae of *Amphibalanus improvisus* developed to Stage V and further on Day 3 (**a**) and to Stage C and further on Day 6 (**b**) in pH 7.8 vs. pH 8.1 in five different batches of larvae (three replicates per batch). Mean effect size (Log Response Ratio, LnRR) is significant when the 95% confidence interval does not overlap zero (*).

IV larvae was lower at pH 7.8 compared with pH 8.1 in the second and the third batches (Figure 4a). Once again, however, this effect was not seen for Stage V larvae after 3 days or for Stage VI larvae after 6 days of development in the same batch (Figure 4b and c). After 6 days the carapace length of Stage C larvae was significantly increased under pH 7.8 compared with that under pH 8.1 in the fourth batch (Figure 5).

ANOVA revealed that, as for the larval survival and development data, there was a significant effect of batch (in most cases) but there were no significant interactions between the effects of batch and pH on development, total length and carapace length of larvae, and there was no significant effect of pH alone (Table 2).

Discussion

Our results do not support the hypothesis that early life-history stages of *Amphibalanus improvisus* will be negatively affected by near-future levels of OA. Even relatively severe pH treatments (down to pH_{NBS} 7.6, equivalent to scenario estimates for the year 2250; Caldeira and Wickett, 2005) did not cause substantial changes in survival and development rate of larvae. We also found no significant effects of OA on larval development rate or size, with most larvae reaching the cypris stage after 6 days, matching development rates recorded elsewhere for this species (Semmler *et al.*, 2009; Pansch *et al.*, 2012a). We conclude, therefore, that early life-history stages of this species might be tolerant to near-future OA.

The substantial interbatch difference in larval development in both experiments is a common characteristic of cultures of this species (pers. obs.) and also of related species such as *Semibalanus improvisus* (Jarrett and Pechenik, 1997). This most likely results from heterogeneous parentage (and hence genetic composition) of larval batches. Although differences may also be attributed to variability in the quality of the microalgal food supplied to the larvae (Nasrolahi *et al.*, 2007), these conditions were standardized in our experiments. Interestingly, even though responses of different larval batches—and thus genetic composition—varied, we found no significant interaction effects between batch and pH. This indicates that responses to OA did not differ between batches, strengthening our conclusion that near-future levels of OA (pH 7.8 to 7.6) are unlikely to affect larval development of *A. improvisus*.

More generally, it has been suggested that early life-history stages of marine invertebrates are highly susceptible to OA (Kurihara,

2008). Larval development of the oyster *Crassostrea gigas* and the mussel *Mytilus galloprovincialis* have been shown to be negatively impacted by pH 7.4, probably triggered by acidification impacts on calcification processes (Kurihara *et al.*, 2007, 2009), and even small pH changes (a pH reduction by 0.2 units) have been shown to induce 100% larval mortality in the brittlestar *Ophiothrix fragilis* (Dupont *et al.*, 2008). Similar results have been found in other species (reviewed in Ross *et al.*, 2011; Byrne, 2012; Kroeker *et al.*,

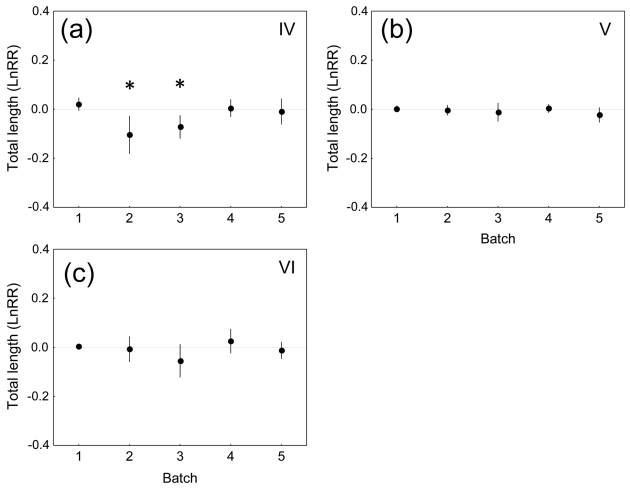

Figure 4. Effects of ocean acidification on the total length of nauplius larvae of *Amphibalanus improvisus* at Stage IV (**a**) and Stage V (**b**) on Day 3, and Stage VI (**c**) on Day 6 in pH 7.8 vs. pH 8.1 in five different batches of larvae (three replicates per batch). Mean effect size (Log Response Ratio, LnRR) is significant when the 95% confidence interval does not overlap zero (*).

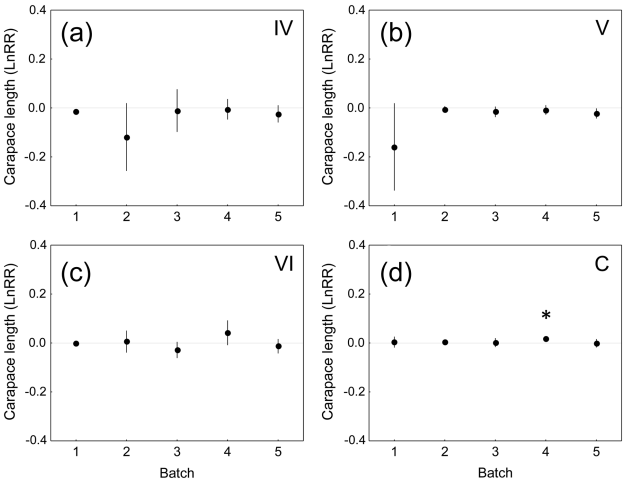

Figure 5. Effects of ocean acidification on the carapace length of nauplius larvae of *Amphibalanus improvisus* at Stage IV (**a**) and Stage V (**b**) on Day 3, and of Stage VI (**c**) and cyprids (**d**) on Day 6 in pH 7.8 vs. pH 8.1 in five different batches of larvae (three replicates per batch). Mean effect size (Log Response Ratio, LnRR) is significant when the 95% confidence interval does not overlap zero (*).

Table 2. Factorial ANOVA on the effects of pH and batch on the percentage of larvae of *Amphibalanus improvisus* developed to Stage V and further (Stage V + VI + C + P = V+) on Day 3, and to Stage C and further (Stage C + P = C+) at the end of the experiment on Day 6, as well as factorial ANOVA on the effects of pH and batch on the total length and the carapace length of nauplius (Stage IV, V and VI) and cypris larvae (Stage C).

			SS	d.f.	MS	F	p
Development	Stage V+ (Day 3)	batch	0.645	4	0.161	79.694	* < 0.001
		pH	0.006	1	0.006	3.116	0.093
		batch × pH	0.008	4	0.002	0.935	0.464
	Stage C+ (Day 6)	batch	0.936	4	0.234	240.109	* < 0.001
		pH	0.001	1	0.001	1.330	0.312
		batch × pH	0.004	4	0.001	1.079	0.393
Total length	Stage IV	batch	2144.2	4	536.0	3.042	0.041
		pH	1337.3	1	1337.3	1.824	0.248
		batch × pH	2931.0	4	732.8	4.159	0.013
	Stage V	batch	4158.6	4	1039.6	14.417	* < 0.001
		pH	97.7	1	97.7	1.861	0.244
		batch × pH	209.9	4	52.5	0.728	0.583
	Stage VI	batch	625.4	4	156.4	0.433	0.783
		pH	273.1	1	273.1	0.490	0.522
		batch × pH	2229.7	4	557.4	1.543	0.228
Carapace length	Stage IV	batch	1835.1	4	458.8	2.499	0.079
		pH	567.4	1	567.4	2.444	0.193
		batch × pH	930.6	4	232.7	1.268	0.319
	Stage V	batch	10239.6	4	2559.9	9.921	* < 0.001
		pH	1625.5	1	1625.5	2.253	0.208
		batch × pH	2884.3	4	721.1	2.795	0.054
	Stage VI	batch	1077.5	4	269.4	2.214	0.104
		pH	0.8	1	0.8	0.003	0.956
		batch × pH	989.9	4	247.5	2.034	0.128
	Stage C	batch	3843.3	4	960.8	29.065	* < 0.001
		pH	55.7	1	55.7	2.255	0.207
		batch × pH	98.6	4	24.7	0.746	0.573

Significant effects indicated with asterisks.

2013), however, we did not find these patterns in this study for the bay barnacle *A. improvisus*. Lack of sensitivity of early life-history stages to OA has also been shown in a different population of the same species by Pansch *et al.* (2012a).

We are not alone in finding that early life-history stages of barnacles are rather tolerant to expected variations in seawater pH. This conclusion was also reached in recent investigations of the closely related barnacle species *Amphibalanus amphitrite* (McDonald *et al.*, 2009). As barnacle larvae do not develop calcified structures until they settle and metamorphose into the juvenile stage, they may be less susceptible to OA stress compared with other invertebrate larvae (Kurihara *et al.*, 2007, 2009; Kurihara, 2008; Dupont *et al.*, 2008). Moreover, crustaceans generally are known to have high metabolic rates that facilitate control of extracellular pH through active ion transport (Gohad *et al.*, 2009; Whiteley, 2011). Consequently even at the larval stage, they may be capable of tolerating variations in seawater pH (Kurihara *et al.*, 2004; Arnold *et al.*, 2009).

Although survival, size, and development rates of *A. improvisus* larvae were not affected by pH, other processes may have been impacted (Thiyagarajan *et al.*, 2002). Nothing is known about OA impacts on the fertilization process, but slower embryo development was found in *Semibalanus balanoides* at pH 7.7 (Findlay *et al.*, 2009), which could lead to a delayed release of larvae. Barnacle nauplii generate lipid vesicles, which are used as energy reservoirs during the non-feeding cyprid stage, for swimming, settlement and metamorphosis. These reservoirs have a profound importance for growth of juvenile barnacles (Thiyagarajan *et al.*, 2002, 2003). Hypercapnia-induced carry-over effects can thus be fatal for subsequent life-history stages (Beckerman *et al.*, 2002). These issues have rarely been addressed in this species (Pansch *et al.*, 2012a).

A range of biological and abiotic processes can cause the pH of shallow coastal habitats to vary substantially over the year or even on a daily basis (Blackford and Gilbert, 2007; Shim *et al.*, 2007; Salisbury *et al.*, 2008; Wootton *et al.*, 2008; Feely *et al.*, 2010; Thomsen *et al.*, 2010, 2013). Species inhabiting these habitats, such as the barnacle *A. improvisus,* will likely have undergone selection for tolerance to these fluctuating conditions, and may therefore be better able to cope with climate change than organisms from more stable habitats (Miller *et al.*, 2009; Parker *et al.*, 2011; Pansch *et al.*, 2012a, b). It is clear from our results that substantial intraspecific variability in responses to OA exists, indicating that there may be additional substantial additive genetic variability upon which selection can act. Whether, and how, other barnacle populations will cope with near-future levels of ocean acidification remains unclear, as it is difficult to draw general conclusions from the few available studies.

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