

Fertilisation and larval development in an Antarctic bivalve, *Laternula elliptica*, under reduced pH and elevated temperatures

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ABSTRACT: Elevated temperatures associated with ocean warming and acidification can influence development and, ultimately, success of larval molluscs. The effect of projected oceanic changes on fertilisation and larval development in an Antarctic bivalve, *Laternula elliptica*, was investigated through successive larval stages at ambient temperature and pH conditions (−1.6°C and pH 7.98) and conditions representative of projections through to 2100 (−0.5°C to +0.4°C and pH 7.80 to pH 7.65). Where significant effects were detected, increased temperature had a consistently positive influence on larval development, regardless of pH level, while effects of reduced pH varied with larval stage and incubation temperature. Fertilisation was high and largely independent of stressors, with no loss of gamete viability. Mortality was unaffected at all development stages under experimental conditions. Elevated temperatures reduced occurrences of abnormalities in D-larvae and accelerated larval development through late veliger and D-larval stages, with D-larvae occurring 5 d sooner at 0.4°C compared to ambient temperature. Reduced pH did not affect occurrences of abnormalities in larvae, but it slowed the development of calcifying stages. More work is required to investigate the effects of developmental delays of the magnitude seen here in order to better determine the ecological relevance of these changes on longer term larval and juvenile success.

KEY WORDS: Ocean acidification · Early life history · D-larvae · Invertebrate · Abnormal development · Climate change · CO₂ · Antarctica · Fertilisation

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INTRODUCTION

Since the industrial revolution, a 40% increase in atmospheric CO₂ (>400 ppm) has resulted in large-scale changes in global climate (IPCC 2013, Tans & Keeling 2014). Even under best-case mitigation scenarios further increases in atmospheric CO₂ are projected (up to 450 ppm by 2100; Calvin et al. 2009, Fishedick et al. 2011). Radiative forcing due to increased greenhouse gases results in an increased energy uptake in global climate systems, and subsequently, atmospheric temperatures are predicted to increase. Over 90% of the total energy increase observed in the climate system is stored in the oceans

(60% in the upper 700 m), leading to warmer oceans (IPCC 2013). Additionally, increased CO₂ concentrations affect ocean chemistry through hydrolysis with seawater, which results in increased hydrogen ion (H⁺) concentrations and a subsequent drop in pH (Seibel & Walsh 2003, Orr et al. 2005). Since measurements began, surface pH levels have reduced by approximately 0.1 units with up to a further drop of 0.3 units projected by 2100 (IPCC 2013). The increased [H⁺] is buffered by carbonate ions through the formation of bicarbonate, reducing the carbonate saturation state (Ω). As Ω decreases to undersaturation ($\Omega < 1$), dissolution of calcium carbonate (CaCO₃) occurs. As CO₂ dissolves more readily in cold water,

the effects of ocean acidification will be felt first in polar and/or deep-sea regions (IPCC 2013). Organisms using aragonite (a less stable form of CaCO_3) in shell formation may soon experience undersaturation in winter months in the Antarctic (McNeil & Matear 2008).

Early life stages of invertebrates are important in the context of environmental stressors as sensitivity may affect species persistence, success and adaptation (Byrne 2011). Due to the high percentage of cells exposed to ocean conditions, invertebrate larvae may be more vulnerable to changes in ocean chemistry compared to adults (Pechenik 1999, Melzner et al. 2009). As oceanic $p\text{CO}_2$ increases, diffusion of CO_2 out of the cells becomes more difficult. In adults, and some juveniles, pH gradient can be maintained either by increasing the metabolic rate or by switching to anaerobic metabolism. However, larvae have a limited capacity to regulate internal pH and ion transport systems (Melzner et al. 2009, Waldbusser et al. 2013).

Responses to temperature stress in invertebrate larvae are diverse and may include altered growth rates, increases in abnormal development and mortalities, as well as mitigation of pH effects (Byrne et al. 2011, 2013b, Davis et al. 2013, Pecorino et al. 2014). The impacts of changes in pH and temperature on fertilisation vary in marine organisms, with many species being resistant to all but the most extreme conditions and others only showing negative impacts when stressors are combined (Byrne 2011, Ericson et al. 2012, Gonzalez-Bernat et al. 2013). Furthermore, the observed effects of temperature and pH stressors on fertilisation may be dependent on sperm concentrations, population or experimental design (Reuter et al. 2011, Ho et al. 2013, Sewell et al. 2014). Other responses to temperature and pH stress include smaller larvae, reduced lipid content, reduced calcification and increased abnormal development (Talmage & Gobler 2011, Nguyen et al. 2012, Andersen et al. 2013, Byrne et al. 2013a). Reduced pH can cause down-regulation of genes involved in skeletogenesis and metabolism in sea urchin larvae (O'Donnell et al. 2010), while reduced survivorship, particularly in later development stages, has been observed in mollusc larvae (Talmage & Gobler 2009, Van Colen et al. 2012).

In bivalves, changes in shell development have occurred in response to ocean acidification and warming. These include reduced calcification, weakened shells, increased pitting, and changes in CaCO_3 crystal structure (Watson et al. 2009, Beniash et al. 2010, Gaylord et al. 2011, Gobler & Talmage 2013).

Additionally, deformities in shell hinges and valve edges have occurred, which may significantly reduce larval survival by impairing feeding behaviour and mobility (Talmage & Gobler 2010, Andersen et al. 2013, Gazeau et al. 2013).

The Antarctic geoduck, *Laternula elliptica*, is a deep burrowing bivalve with a circumpolar distribution. It occurs at depths from 0 to 360 m, but is common between 5 and 30 m where densities of up to 170 ind. m^{-2} have been recorded (Powell 1965, Hendler 1982, Cummings et al. 2011). Adult *L. elliptica* are temperature sensitive, with animals from the Antarctic Peninsula region having a reduced capacity for activities such as reburying at 2.5°C and exhibiting a complete loss of reburying capability at 5°C (Urban & Silva 1998, Peck et al. 2002, 2004). Above 6°C, increased oxygen consumption followed by a switch to anaerobic metabolism occurs, and prolonged exposure to temperatures exceeding 9°C is lethal (Heise et al. 2003, Peck et al. 2004). *L. elliptica* may be able to withstand small elevations in temperature by entering into low or anaerobic metabolic states allowing for the conservation of energy until conditions become more favourable (Morley et al. 2007). Longer-term exposure to elevated temperatures suggests that adults have little or no capacity for acclimation, although their responses may be dependent on other factors such as season and food supply (Morley et al. 2012a,b).

The responses of *L. elliptica* to reductions in pH are less well studied. When exposed to low pH seawater, empty shells are prone to rapid dissolution (McClintock et al. 2009). Living *L. elliptica* adults exposed to low pH increased expression of the heat shock protein HSP70 and chitin synthase, an enzyme involved in shell formation (Cummings et al. 2011). Oxygen consumption also increased with decreased pH, indicating a metabolic effect. Despite these changes in protein expression and metabolism, exposure to reduced pH did not result in mortality after 120 d (Cummings et al. 2011). Although the adults appear resilient (at least in the short term) to lowered pH and elevated temperatures on the order predicted for 2100, effects on early life history of this key bivalve are as yet unknown.

L. elliptica is a simultaneous hermaphrodite with seasonally dependent growth and gonad development (Ahn et al. 2003). Larvae are large (220 μm) and lecithotrophic (Pearse et al. 1985, 1986). Spawning times vary with significant interannual variation (Ahn et al. 2003). Peak spawning occurs from late February to mid-May in McMurdo Sound (Ross Sea), and in late December through February on the

Antarctic Peninsula, with juveniles settling in the sediment in the following months (Pearse et al. 1985, 1986, Ahn et al. 2000). The sensitivity of *L. elliptica* fertilisation and larval development to warming and acidification is addressed in this study. Fertilisation success and subsequent progression of development, abnormality and mortality are examined in the context of ecologically relevant elevated temperature and reduced pH.

MATERIALS AND METHODS

Collection

Adult *Laternula elliptica* (4.2–8.9 cm shell length) were collected from the McMurdo Station intake jetty (77° 51.093' S, 166° 39.931' E), Ross Sea, Antarctica, in November 2012. They were transported to New Zealand and held in flow-through tanks with filtered (0.1 µm) seawater chilled to –1.6°C at pH 7.98 (ambient conditions in the Ross Sea at the time of collection) until March 2013. They were fed Shellfish Diet 1800 (Reed Aquaculture), a liquid algal mix that included Antarctic species.

Experiment setup

Combinations of 3 pH and 3 temperature treatments were chosen to determine the effects of acidification and/or warming on fertilisation and subsequent embryonic and larval development of *L. elliptica*. The treatments included temperature and pH controls (target settings: –1.6°C and pH 7.98, respectively). Projections of changes in sea surface temperature and pH through to 2100 were represented by 2 elevated temperatures (–0.5 and +0.4°C

and 2 reduced pHs (pH 7.80 and 7.65) (Orr et al. 2005, IPCC 2013) (Table 1). A maximum of 8 treatments were possible due to system logistical constraints, therefore the combination of 0.4°C and pH 7.80 was not included.

Temperature and pH manipulation and measurement

Temperature and pH manipulations were performed in 8 separate header tanks, which supplied the 48 treatment tanks through insulated lines. The pH in each header tank was manipulated through the diffusion of food grade CO₂ and controlled using Omega pH controllers (Model PHCN-37-AI-230-03). Temperature was manipulated using 2000 W submersible heater elements controlled by Omega CN740 controllers connected to precision PT100 probes, which also logged temperature (Table 1). Temperatures and pH (on the total hydrogen scale) were monitored for each header tank 8 times per day using LabView® software. This automated system, detailed in McGraw et al. (2010), allows precise control of pH by measuring the pH spectrophotometrically and correcting for aberrations from target pHs (McGraw et al. 2010, Cummings et al. 2011) (Table 1). Flow was maintained at 200 ml min⁻¹. At 6, 16 and 41 d, water samples were taken from each of the 8 header tanks and preserved with HgCl₂ for analysis of dissolved inorganic carbon (DIC) and alkalinity (A_T) (Table 1). Aragonite (Ω_{Ar}) and carbonate (Ω_{Ca}) saturation states and partial pressure of CO₂ (pCO₂) at experimental temperatures and salinity were calculated from measured pH and A_T using refitted equilibrium constants (Mehrbach et al. 1973, Dickson & Millero 1987). Analytical methods follow those detailed in Cummings et al. (2011).

Table 1. Seawater conditions for all experimental treatments. Average temperature (°C; n = 360); pH (measured on the total hydrogen scale; n = 360); total alkalinity (A_T; n = 3); dissolved inorganic carbon (DIC, n = 3); partial pressure of CO₂ (pCO₂) is calculated from A_T and pH, carbonate saturation states Ω_{Ar} and Ω_{Ca} are calculated from A_T and pH. Values presented are mean ± SE, except pH where mean and range are shown. Salinity was 34.2 ppt

Temp (°C)	pH	A _T (µmol kg ⁻¹)	DIC (µmol kg ⁻¹)	pCO ₂ (ppm)	Ω _{Ar}	Ω _{Ca}
–1.6 ± 0.01	7.968 (7.829–7.996)	2263.3 ± 8.2	2183.8 ± 8.1	350.1 ± 1.3	1.37 ± 0.01	2.18 ± 0.01
	7.787 (7.754–7.826)	2260.3 ± 8.9	2233.0 ± 4.0	554.6 ± 2.2	0.92 ± 0.01	1.47 ± 0.01
	7.630 (7.571–7.686)	2266.0 ± 5.8	2271.2 ± 9.4	823.3 ± 2.1	0.65 ± 0.01	1.04 ± 0.01
–0.5 ± 0.01	7.969 (7.871–8.111)	2265.0 ± 8.3	2174.4 ± 3.8	367.2 ± 1.4	1.37 ± 0.01	2.19 ± 0.01
	7.797 (7.729–7.863)	2264.8 ± 6.2	2254.0 ± 22.4	569.0 ± 1.6	0.95 ± 0.01	1.52 ± 0.01
	7.643 (7.593–7.680)	2264.0 ± 7.2	2270.8 ± 15.0	835.6 ± 2.7	0.68 ± 0.01	1.08 ± 0.01
0.4 ± 0.01	7.969 (7.810–8.003)	2260.0 ± 8.4	2173.8 ± 5.8	383.6 ± 1.4	1.38 ± 0.01	2.19 ± 0.01
	7.632 (7.586–7.670)	2260.5 ± 9.2	2260.0 ± 4.2	893.0 ± 3.7	0.66 ± 0.01	1.06 ± 0.01

Gamete collection and fertilisation

Eggs and sperm were collected from separate individuals to avoid self-fertilisation. The eggs were collected from 22 individuals by piercing the female portion of the gonad and collecting the eggs that flowed out in a Pasteur pipette. The eggs were pooled and the volume made up to 1.5 l with -1.6°C seawater. Thirty ml of the egg solution (~ 7000 eggs) was added to a 400 ml fertilisation container suspended in a 4 l insulated tank. Seawater flowed through the 4 l tanks at the experimental temperature, maintaining experimental conditions in the fertilisation containers. Eggs were acclimatised to experimental conditions by adding seawater at the respective experimental temperature and pH to make up the volume within the fertilisation containers to 200 ml.

Sperm was collected 24 h later from 10 different individuals by cutting into the male portion of the gonad until sperm flowed out. A small sample of sperm collected from each bivalve was examined to determine motility. Sperm was pooled in a 1 l glass beaker, the volume topped to 500 ml using -1.6°C seawater and 10 ml of sperm solution was added to each fertilisation container with eggs. Sperm concentration in the fertilisation containers was $\sim 3 \times 10^7$ sperm ml^{-1} . Once an hour, the water was gently agitated with a Pasteur pipette to ensure mixing. A sample of ~ 50 eggs was removed from each container using a Pasteur pipette 4 h after the addition of the sperm. The sample was preserved in Carriker's solution for observations of fertilisation success as described below (Carriker 1950). Following this sampling, the contents of the fertilisation containers were carefully emptied and rinsed into the 4 l tanks, through which seawater flowed at experimental conditions. The eggs were negatively buoyant and remained on the bottom of the tanks.

Table 2. Projected first observations of individual *Laternula elliptica* larval stages

Sample point	Stage (first observation)
4 h	2-cell embryo
24 h	8-cell embryo
48 h	16-cell embryo
4 d	Blastula
10 d	Trochophore
17 d	Veliger
20 d	D-larvae

Assessing fertilisation and larval development

Larval development of *L. elliptica* was tracked at varying intervals (hours to days), chosen to target particular embryonic and larval stages. Samples were collected at 4, 6, 24 and 48 h post-insemination and every 1 to 2 d thereafter for 41 d. All larval data presented in this study from here onwards refer to times and data collected post-insemination. Sample points were chosen to reflect previous observations in ambient conditions of the first occurrence of each development stage in *L. elliptica* (C. H. Bylenga pers. obs.) (Table 2). The larvae are negatively buoyant and remain encapsulated through to the D-larval stage (C. H. Bylenga pers. obs., Ansell & Harvey 1997). On each occasion, a sample of ≥ 50 eggs, embryos or larvae was removed from each replicate tank by running a Pasteur pipette along the bottom of the tank to ensure haphazard selection. Fertilisation success was determined after 4, 6, 24 and 48 h. At these and the following sample points, embryos and larvae were classified into 7 developmental stages using a stereomicroscope in order to assess the progression of larval development (Fig. 1). The percentages of fertilised larvae dead or abnormally devel-

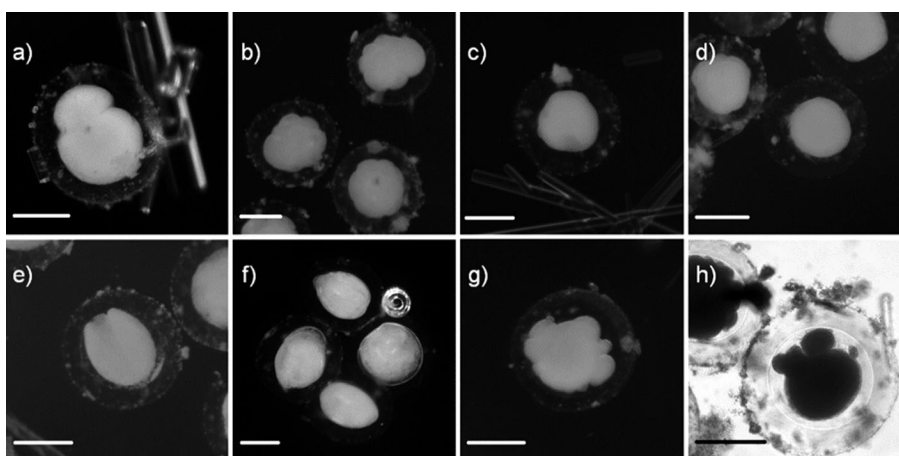


Fig. 1. Examples of embryos and larvae of *Laternula elliptica* at (a) 2-cell embryo (6 h post-insemination), (b) 16-cell embryo (3 d), (c) blastula (11 d), (d) trochophore (with cilia, 16 d), (e) veliger (25 d) and (f) D-larvae (25 d) developmental stages. (g,h) Examples of abnormal development observed during the blastula stage (11 d). Imaged larvae were raised at ambient conditions (-1.6°C and pH 7.98). Scale bars = 100 μm

oped were also determined. Larvae were considered abnormal if they displayed unusual cell development (e.g. Fig. 1g,h).

Statistical analysis

All statistical analysis was performed using SPSS ver. 20. Normality of the data was verified using Shapiro-Wilk's test and equality of variance was confirmed using Levene's test. The relation of the developmental responses to pH and temperature was examined by fitting the data to a general linear model. Percent fertilisation, the individual developmental stages and percent abnormalities were used as dependent variables while pH and temperature, and Ω_{Ar} at the later stages, were fixed factors, and a pH \times temperature interaction term was used. Where interactive effects were significant, or trended towards significance ($p < 0.100$), individual between group *t*-tests were performed for temperature at each experimental pH and for pH at each experimental temperature. In order to reduce Type I errors, a Bonferroni correction was used to account for multiple measures ($\alpha = 0.017$): only *p*-values $< \alpha$ were considered significant. If the general linear model indicated overall individual statistical significance ($p < 0.050$) of either temperature or pH, a post-hoc Bonferroni multiple comparison test was performed to determine effects of pH averaged over temperature and

temperature averaged over pH on larval stage percentages and abnormalities at each time point.

Progression of development through the trochophore and D-larval stages was analysed using a repeated measures general linear model with temperature and pH (trochophore stage), or Ω_{Ar} (D-larval stage), as fixed factors.

RESULTS

Temperature and pH data are detailed in Table 1. Ω_{Ar} was undersaturated at pH 7.80 and 7.65, while Ω_{Ca} remained above but close to undersaturation at pH 7.65 (Table 1). All adults dissected had fully mature and ripe gonads with large numbers of eggs and sperm. Eggs ranged in size from 180 to 230 μm and were encapsulated in a thick (30 μm), slightly sticky membrane. Sperm were highly motile ($\sim 99\%$) when activated with fresh seawater. Fertilised eggs often clumped and the sticky membranes collected algae and other debris. Abnormal development of larvae was observed at all stages in all treatments.

Fertilisation success and early embryonic stages

Fertilisation success reached a maximum of $\sim 85\%$ at 48 h (Fig. 2). A significant interactive effect of pH and temperature was observed in the first 4 to 6 h

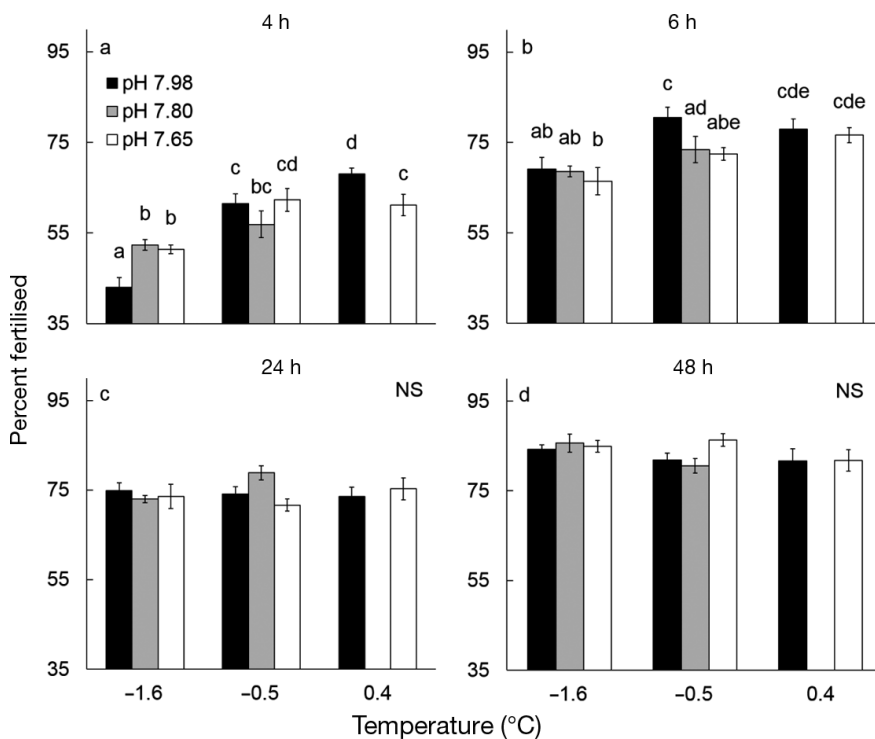


Fig. 2. Percentage (\pm SE) of *Laternula elliptica* eggs fertilised at experimental temperatures ($^{\circ}\text{C}$) and pH after (a) 4 h (b) 6 h (c) 24 h and (d) 48 h post-insemination. Letters indicate significance among treatments where $p < \alpha$ (0.017); treatments sharing the same letter are not significantly different. The temperature/pH combination of 0.4°C /pH 7.80 was not used. NS: not significant

after insemination (Table 3), with the impact of reduced pH on fertilisation success dependent on the incubation temperature. In the ambient temperature

Table 3 General linear model results for (a) fertilisation success of *Laternula elliptica*, (b) larval development progression and (c) abnormal larval development by time post-insemination. Temperature, pH and a temperature × pH interactive term were fixed factors. Bold p-values: $p < 0.050$

	Temperature		pH		Temp. × pH	
	F _{2,40}	p	F _{2,40}	p	F _{3,40}	p
(a) Fertilisation						
4 h	31.823	<0.001	0.097	0.908	5.992	0.002
6 h	9.478	<0.001	2.114	0.134	0.964	0.419
24 h	0.317	0.730	0.977	0.385	1.67	0.189
48 h	1.858	0.169	0.808	0.453	1.027	0.391
(b) Developmental progression						
2-cell embryo						
4 h	128.883	<0.001	0.811	0.452	2.234	0.099
6 h	20.327	<0.001	3.651	0.035	0.752	0.527
8-cell embryo						
24 h	8.222	0.001	3.971	0.027	2.599	0.065
16-cell embryo						
48 h	16.164	<0.001	20.465	<0.001	9.146	<0.001
Trochophore						
15 d	30.019	<0.001	1.485	0.239	0.959	0.959
Veliger						
17 d	352.473	<0.001	12.186	<0.001	6.412	0.001
20 d	470.43	<0.001	0.752	0.478	2.314	0.090
D-larvae						
17 d	152.312	<0.001	3.374	0.044	3.944	0.015
18 d	82.969	<0.001	11.38	<0.001	1.88	<0.001
20 d	75.513	<0.001	7.087	0.002	8.416	<0.001
22 d	83.985	<0.001	7.116	0.002	4.437	0.009
24 d	122.428	<0.001	32.121	<0.001	5.025	0.005
27 d	22.803	<0.001	11.029	<0.001	3.511	0.024
29 d	16.293	<0.001	6.912	0.003	0.459	0.712
(c) Abnormal development						
4 h	2.298	0.114	0.543	0.585	5.511	0.003
6 h	2.910	0.066	2.103	0.135	7.472	<0.001
48 h	2.582	0.088	1.624	0.210	0.593	0.623
3 d	0.07	0.933	0.508	0.605	0.354	0.786
4 d	1.207	0.310	0.352	0.705	3.030	0.040
6 d	0.489	0.619	1.24	0.301	6.295	0.001
8 d	19.455	<0.001	1.749	0.187	3.855	0.016
10 d	4.027	0.026	1.647	0.205	0.247	0.863
11 d	7.656	0.002	0.183	0.834	1.439	0.246
13 d	3.719	0.033	0.160	0.853	0.151	0.929
15 d	3.841	0.030	1.611	0.212	0.672	0.574
17 d	2.39	0.105	0.478	0.624	0.280	0.840
18 d	2.276	0.116	0.422	0.659	0.450	0.750
20 d	1.948	0.156	1.605	0.214	0.133	0.940
22 d	0.620	0.543	0.636	0.535	0.562	0.643
24 d	4.460	0.018	1.393	0.260	0.552	0.650
27 d	3.709	0.033	0.331	0.720	0.158	0.924
29 d	0.784	0.463	0.375	0.689	0.377	0.770
35 d	3.597	0.037	0.449	0.641	0.161	0.922
41 d	5.568	0.007	2.341	0.109	0.097	0.961

treatments, fertilisation was significantly lower at ambient pH than at either of the 2 reduced pH levels (pH 7.80: $t_{10} = 4.09$, $p = 0.001$ and pH 7.65: $t_{10} = 3.68$, $p = 0.002$; Fig. 2a). Conversely, at 0.4°C, fertilisation was lower at pH 7.65 compared to the ambient pH ($t_{10} = 2.98$, $p = 0.006$; Fig. 2a).

Irrespective of pH, temperature had an effect on the fertilisation success that was dependent on time. Elevated temperature treatments had a higher fertilisation success than the ambient temperature treatment at 4 and 6 h, regardless of pH treatment (Fig. 2a,b, Table 3). However by 24 and 48 h, there were no individual or interactive effects of temperature treatment on the fertilisation success (Fig. 2c,d, Table 3).

Progression of embryonic and larval development through each stage was impacted by both elevated temperature and reduced pH (Tables 3 & 4). Temperature strongly influenced the development to the 2-cell stage at both 4 and 6 h. Post-hoc tests show significantly fewer 2-cell-stage embryos were found at ambient temperature compared to both elevated temperatures ($p < 0.002$; Fig. 3b, Table 3). In the intermediate temperature treatment (−0.5°C; Fig. 3a), 4 h post-insemination, fewer 2-cell-stage embryos were observed with reduced pH ($t_{10} = 3.88$, $p = 0.002$). At 6 h, there were fewer 2-cell stage embryos at ambient pH compared to reduced pH (Fig. 3b, Table 3). Interactive effects were non-significant at 6 h post-insemination (Table 3).

By 24 h, over 50 % of the embryos were at the 8-cell development stage in all treatments (Table 4, Fig. 3c). While there was no interactive effect of pH and temperature, both variables individually influenced development to the 8-cell stage (Table 3). Post-hoc tests revealed that percentages of normally developed embryos in the 8-cell stage were significantly higher at both elevated temperatures than at ambient temperatures (−0.5°C: $p = 0.012$; 0.4°C: $p = 0.006$; Fig. 3c). Individuals raised at reduced pH had significantly higher percentages of normally developed larvae in the 8-cell stage compared to ambient pH ($t_{10} = 2.52$, $p < 0.015$; Fig. 3c).

By 48 h, embryonic development had progressed to the 16-cell stage (Table 4, Fig. 3d). An effect of pH was observed only in the 2 elevated temperature treatments, where percentages of normally developed embryos at the 16-cell stage were significantly higher at reduced pH (Fig. 3d, Table 3).

By 3 d, embryonic development was entering the blastula stage and by 4 d all normally developing embryos had progressed to the blastula stage (Table 4). Development progression and abnormalities through

Table 4. Development timing by treatment showing when at least 50% of the normally developing *Laternula elliptica* larvae were at a particular stage. Time in h or d after insemination

Stage	Time of approximate 50% abundance							
	-1.6		-0.5		0.4		0.4	
Temp. (°C):	-1.6	-1.6	-1.6	-0.5	-0.5	-0.5	0.4	0.4
pH:	7.98	7.80	7.65	7.98	7.80	7.65	7.98	7.65
Fertilised	4 h	4 h	4 h	4 h	4 h	4 h	4 h	4 h
2-cell embryo	6 h	6 h	6 h	4 h	4 h	6 h	4 h	4 h
8-cell embryo	24 h	24 h	24 h	24 h	24 h	24 h	24 h	24 h
16-cell embryo	48 h	48 h	48 h	48 h	48 h	48 h	48 h	48 h
Blastula	4 d	4 d	4 d	4 d	4 d	4 d	4 d	4 d
Trochophore	15 d	15 d	15 d	15 d	16 d	15 d	15 d	15 d
Veliger	22 d	22 d	22 d	17 d	17 d	18 d	17 d	17 d
D-larvae	24 d	25 d	26 d	22 d	22 d	25 d	18 d	22 d

the later blastula stage were not significantly impacted by temperature and/or pH.

Trochophore and veliger stage development

Larval development time to the trochophore stage was significantly affected by elevated temperature (Fig. 4, Table 3), with no individual or interactive effect of pH. Trochophore larvae appeared first in the 0.4°C treatment at 11 d and after 13 d at -0.5°C. At ambient temperature, only a very small percentage of trochophore larvae were noted 13 d post-insemination, after which trochophore development quickly

progressed. By 17 d, there were no significant differences among treatments.

Timing of the veliger stage was significantly influenced by temperature and pH and an interaction term was detected (Table 3). The first of the veliger larvae appeared 15 d after insemination in the elevated temperatures compared to 17 d at ambient temperature and pH and 20 d at ambient temperature and pH 7.65. By 17 d, only 9% of the developing larvae at ambient temperature/pH were at the veliger stage compared to over 50%

at -0.5°C ($t_{10} = 14.13$, $p < 0.001$) and 80% at 0.4°C ($t_{10} = 23.40$, $p < 0.001$; Table 4, Fig. 5a). After 20 d, this pattern of temperature dependence was still very evident as the percentage of veliger stage larvae in both elevated temperature treatments was >90%, significantly higher than levels at ambient temperature (<25%) (Fig. 5b, Tables 3 & 4).

The effect of reduced pH was dependent on the incubation temperature. At -0.5°C, 17 d, significantly fewer larvae were observed in the reduced pH treatment (pH 7.65) compared to ambient pH ($t_{10} = 7.65$, $p < 0.001$) and pH 7.80 ($t_{10} = 7.60$, $p < 0.001$; Fig. 5a). Conversely, 20 d at -1.6°C, significantly more veligers were observed in the lowest pH treatment than

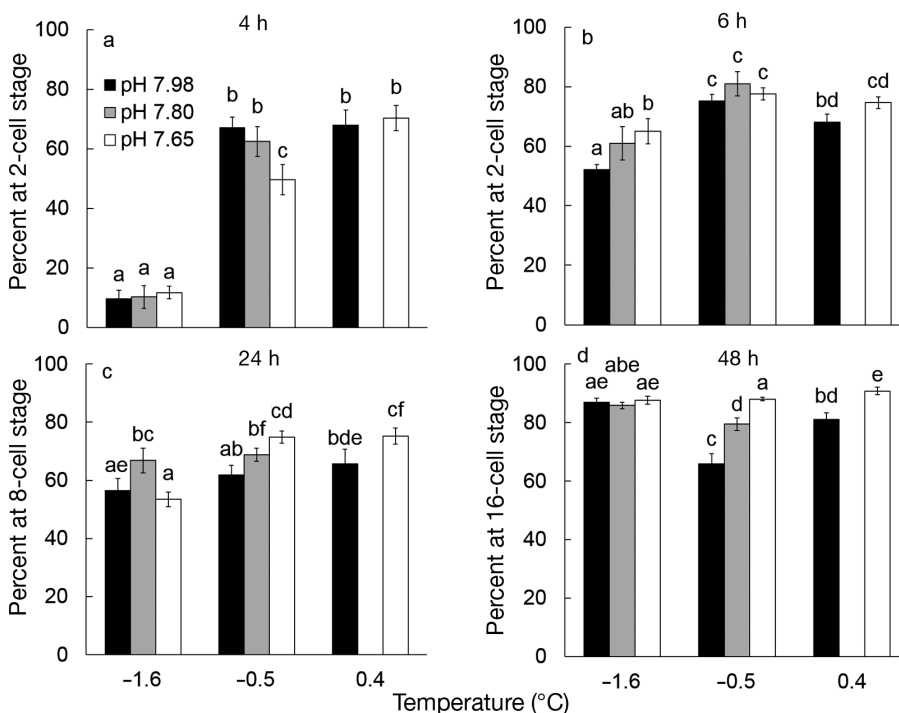


Fig. 3. Percentage (\pm SE) of normally developing *Laternula elliptica* embryos at the (a) 2-cell developmental stage at 4 and (b) 6 h post-insemination, (c) the 8-cell stage at 24 h and (d) the 16-cell stage at 48 h at experimental temperatures (°C) and pH. Letters indicate significance as described in Fig. 2. The temperature/pH combination of 0.4°C/pH 7.80 was not used

in the ambient pH treatment ($t_{10} = 3.87$, $p = 0.002$; Fig. 5b). There was no significant effect of pH at the highest temperature on either day (17 d: $t_{10} = 1.34$, $p = 0.105$; 20 d: $t_{10} = 1.19$, $p = 0.130$; Fig. 5a,b).

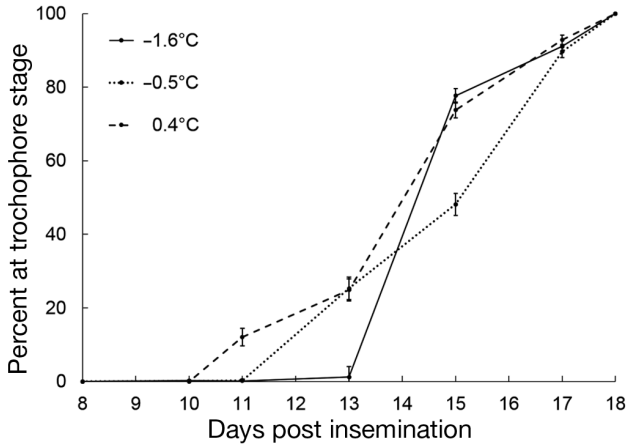


Fig. 4. Progression of normal *Laternula elliptica* larval development through the trochophore stage (% ± SE) at each temperature (°C), averaged over experimental pHs

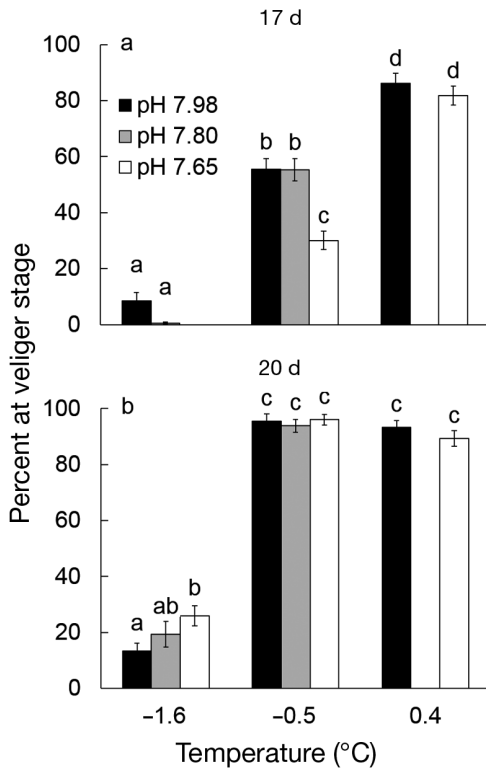


Fig. 5. Percentage (± SE) of developing *Laternula elliptica* larvae at the veliger stage at (a) 17 and (b) 20 d post-insemination. Letters indicate significance as described in Fig. 2. The temperature/pH combination of 0.4°C/pH 7.80 was not used

D-larval stage development

D-larvae first appeared at 17 d in the +0.4°C and -0.5°C treatments, and 5 d later in the ambient temperature treatments (Fig. 6). A significant interactive effect of pH and temperature on the progression of D-larvae development was observed from 17 to 27 d. Development to the D-larval stage was slowest in the reduced pH treatments at all temperatures, with the longest delays occurring at elevated temperatures (Table 3). At each experimental temperature, pH had no effect on the first observance of D-larvae; however, the time needed for 50% of the normally developing larvae to reach the D-larval stage was increased at reduced pH, an effect that was amplified at elevated temperatures (Table 4, Fig. 6). At the lowest pH, larvae in the ambient temperature treatments were delayed 1 to 2 d ($t_{10} = 4.83$, $p < 0.001$; Table 4, Fig. 6a), compared to 3 and 4 d at the mid-range ($t_{10} = 10.73$, $p < 0.001$; Table 4, Fig. 6b) and highest temperatures ($t_{10} = 11.28$, $p < 0.001$; Table 4, Fig. 6c), respectively. At 29 d, a pH effect independent of temperature was

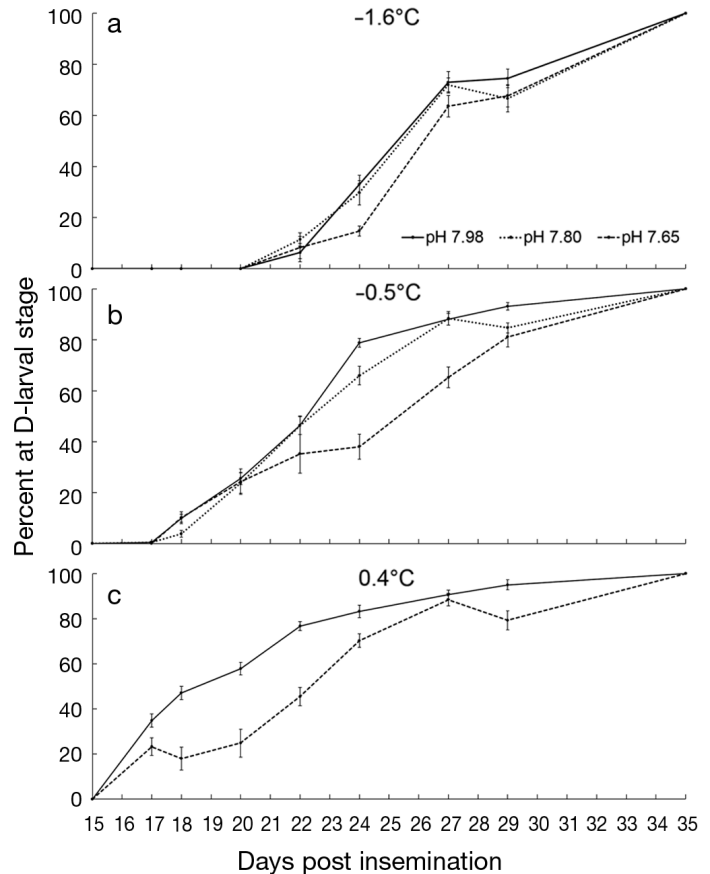


Fig. 6. Percentage (± SE) of normally developing *Laternula elliptica* D-larvae raised at (a) -1.6, (b) -0.5 and (c) 0.4°C

still evident, with D-larvae development being significantly higher in all ambient pH treatments, compared to both reduced pHs (Fig. 6, Table 3).

Elevated temperatures accelerated development to the D-larval stage. This trend was apparent regardless of pH treatment (Fig. 6). At 17 d, less than 1% of the normally developing larvae in the mid-range temperature of -0.5°C were D-larvae compared to 23 and 35% at 0.4°C (Fig. 6b,c, Table 3). All normally developed larvae had reached the D-larval stage in all treatments by between 29 and 35 d.

Abnormal development

Instances of abnormal development were influenced by both temperature and pH (Fig. 7). However, the effect of temperature and pH on abnormal-

ities was largely dependent on larval stage. At 4 h, 2–8% of the embryos, on average, were abnormally developed, and by 6 h post-insemination this number had increased slightly to 5–16% (Fig. 7a,b). Despite these low occurrences, a significant interactive effect was observed between pH and temperature at both time points (Table 3). By 4 h, at ambient temperature, significantly fewer abnormalities were observed in the ambient pH treatment compared to the reduced pH treatment (pH 7.65: $t_{10} = 2.61$, $p = 0.013$; Fig. 7a).

By 6 h, abnormal development in response to elevated temperature and reduced pH was variable (Fig. 7b). At ambient temperature, the reduced pH (pH 7.65) caused increased abnormal development compared to the mid-range pH (pH 7.80; $t_{10} = 3.07$, $p = 0.006$). The highest occurrence of abnormality at -0.5°C was observed within the mid-range pH treatment ($t_{10} = 6.15$, $p < 0.001$; Fig. 7b).

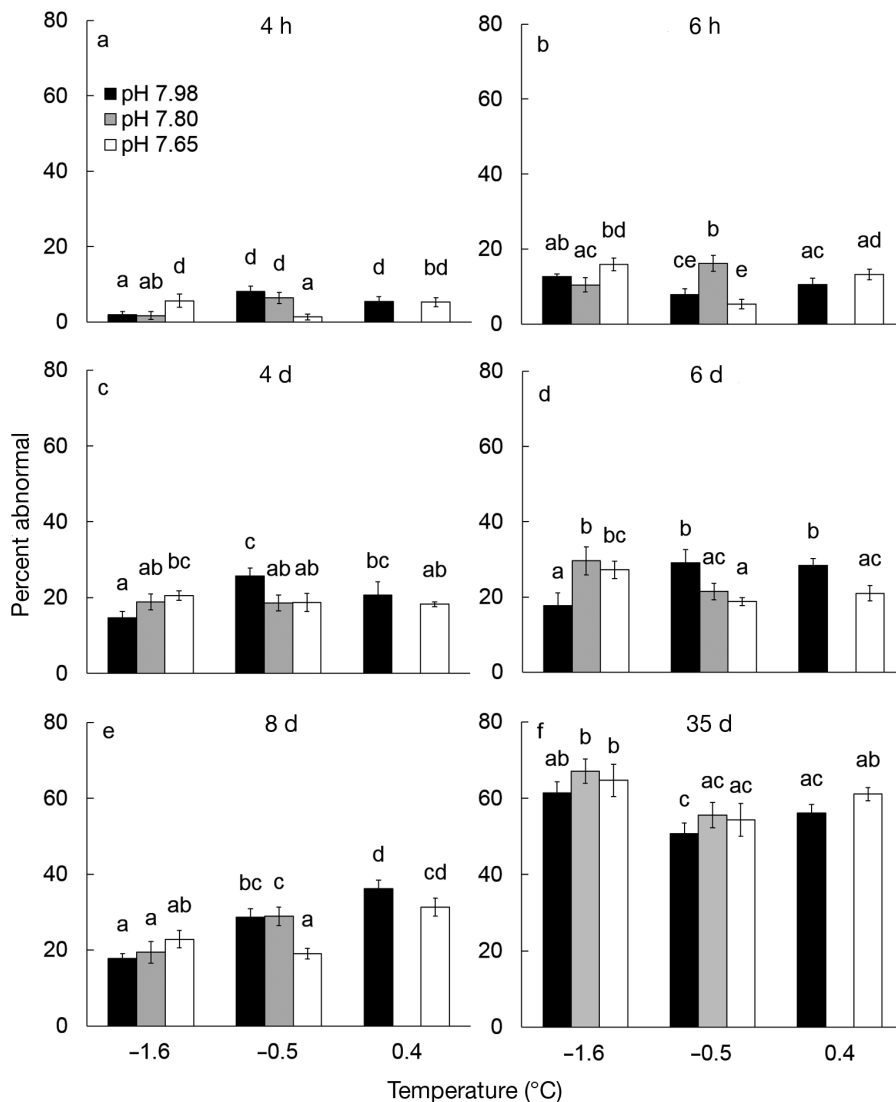


Fig. 7. Percentage (\pm SE) of developing *Laternula elliptica* larvae that were abnormal at experimental temperatures and pH following fertilisation at (a) 4 and (b) 6 h post insemination; during early developmental stages at (c) 4, (d) 6, and (e) 8 d post-insemination; and (f) during D-larvae development 35 d post-insemination. Letters indicate significance as described in Fig. 2. The temperature/pH combination of $0.4^{\circ}\text{C}/\text{pH } 7.80$ was not used

A significant interactive effect of pH and temperature on the percentages of abnormally developing blastulas was observed at 4, 6 and 8 d post-insemination (Fig. 7c–e, Table 3). Percentages of abnormal development were always lowest at ambient temperature and pH (-1.6°C , pH 7.98). Embryos from the ambient temperature treatments exhibited significantly higher abnormality percentages at reduced pH (4 d: $t_{10} = 2.49$, $p = 0.016$; Fig. 7c; 6 d: $t_{10} = 3.49$, $p = 0.003$; Fig. 7d). Although this trend was still evident at 8 d, it was not statistically significant after Bonferroni correction ($t_{10} = 2.09$, $p = 0.031$; Fig. 7e). Conversely, in the mid and high temperature treatments (-0.5°C and $+0.4^{\circ}\text{C}$), the highest abnormality percentages occurred at ambient pH (Fig. 7c,d). At -0.5°C , both reduced pH treatments had fewer abnormally developing individuals at 4 and 6 d post-insemination compared to ambient pH (pH 7.80: $t_{10} = 3.12$, $p = 0.005$ and $t_{10} = 3.01$, $p = 0.007$; pH 7.65: $t_{10} = 2.96$, $p = 0.007$ and $t_{10} = 3.79$, $p = 0.002$, respectively). A similar pattern was noted in the 0.4°C treatment 6 d post-insemination; higher occurrences of abnormality were observed at ambient pH ($t_{10} = 2.71$, $p = 0.011$; Fig. 7d). By 8 d, at -0.5°C , the trend between ambient pH and pH 7.65 was still significant ($t_{10} = 3.98$, $p = 0.001$), but abundances of abnormalities had increased at pH 7.80 to equal percentages at ambient pH (Fig. 7e). At 0.4°C , the differences in abnormal percentages due to pH were no longer significant after Bonferroni correction ($t_{10} = 1.97$, $p = 0.039$; Fig. 7e).

Abnormalities were high in all treatments at the D-larval stage, ranging between 47 and 68% (Fig. 7f). No interactive effects were observed. Post-hoc tests revealed ambient temperature treatments had the highest percentages of abnormal larvae but differences were only significant against mid-range temperature treatments ($p = 0.042$; Fig. 7f). There were no differences in abnormality percentages at -0.5°C and $+0.4^{\circ}\text{C}$ (35 d: $p = 0.108$). At all temperatures there were indications of negative impacts of reduced pH on D-larvae, however these were non-significant (Fig. 7f). Aragonite saturation state had no significant effect on abnormal development in D-larvae ($p = 1.000$).

DISCUSSION

Our data are the first to show that larval development in *Laternula elliptica* is influenced by reduced pH and elevated temperatures. The observed effects varied with both developmental stage and stressor combination. Where significant effects were detected,

increased temperature had a consistently positive influence on development, regardless of pH level, resulting in faster fertilisation and development, and reducing occurrences of abnormalities at later developmental stages. The effects of reduced pH were varied, promoting development during some stages while delaying it in others. Independent from experimental temperatures, reduced pH did not significantly impact occurrences of abnormal development. Interactive effects of pH and temperature on development varied with stage.

Fertilisation was high in all treatments, consistent with the highest rates reported in Powell et al. (2001). Overall, fertilisation in *L. elliptica* was robust against temperature and pH changes projected for 2100, both individually and in combination. However, the sperm concentrations in this experiment were within the optimal range (reported by Powell et al. 2001). Effects of temperature and pH stressors on fertilisation may be more evident at very low sperm concentrations (Reuter et al. 2011, Ho et al. 2013, Sewell et al. 2014) as may be experienced in the wild. We noted higher fertilisation success at 4 and 6 h in the elevated temperature treatments. An initially high fertilisation success is potentially important because as time progresses past spawning, gametes may lose viability and local currents may dilute sperm concentrations. Consequently, the greater fertilisation success at elevated temperature observed for *L. elliptica* 4 and 6 h may help overcome such issues.

The tolerance and faster development through to the D-larval stage at elevated temperatures observed here reflect observations of other Antarctic invertebrates where small increases in temperature have positive or neutral impacts until thermal tolerance thresholds are reached (Stanwell-Smith & Peck 1998, Ericson et al. 2012, Kapsenberg & Hofmann 2014). The temperature tolerance thresholds in *L. elliptica* may be well above existing conditions in the southern Ross Sea (McMurdo Sound). Populations along the Antarctic Peninsula may experience higher summer temperatures (ranging from 0.5 to 1.5°C), which are within and above the projected temperatures for the Ross Sea (Brey et al. 2011, Morley et al. 2012a). In adults, temperature tolerance limits are higher in Antarctic Peninsula compared to McMurdo Sound populations (Morley et al. 2012a). However, limits may be further influenced by animal size, oxygen saturation, exposure to additional stressors, or acclimation to elevated temperatures (Pörtner et al. 2006, Peck et al. 2007, Morley et al. 2012a,b). In our study, the observed positive responses of *L. elliptica* larval development to temperatures up to 2°C above those

currently found in McMurdo Sound also indicated some resilience, although the magnitude of the increase they can tolerate is unknown. Additionally, other stressors could further impact larval responses (e.g. reduced salinity, UV exposure). Exposure and acclimation of adults to adverse conditions can also influence tolerance of their offspring. For example, in the Antarctic sea urchin *Sterechinus neumayeri* long-term (17 mo) exposure of adults to reduced pH and elevated temperature improves larval performance, while 6 mo exposure does not (Suckling et al. 2015). In our experiment, gametes were collected from adults developing in ambient pH and temperature conditions for the Ross Sea.

In late larval development stages, high percentages of abnormalities were observed in all treatments, although mitigated by elevated temperature. If the high occurrences observed here reflect abnormality rates in the wild, temperatures projected for 2100 may result in larger populations at the settlement stage, due to fewer occurrences of abnormalities, and a reduction in time spent at stages vulnerable to predation. Warmer oceans may also alter temperature cues that initiate spawning or settling and metamorphosis, or may affect food availability during settlement by impacting the timing and magnitude of algal blooms (Clarke 1982, Pechenik 1999, Byrne 2011, Ericson et al. 2012). Additionally, faster settlement could come with a potential trade-off of reduced larval dispersal and thus genetic connectivity (Pechenik 1999). However, species with demersal larvae such as *L. elliptica* may rely on close proximity to other individuals for optimal spawning success, and decreased dispersal may even increase fertilisation success (Pechenik 1999, Byers & Pringle 2006, Byrne 2011).

The effects of reduced pH on larval development were variable and largely dependent on larval stage and incubation temperature. In late-stage larvae, delays from reduced pH were greater at elevated temperatures. However, larvae raised at high temperature and low pH developed faster than those raised in ambient conditions (22 vs. 25 d to 50% abundance at the D-larval stage) due to the overall faster development rates at elevated temperature. Our results reflect observations in other species where the effects of reduced pH on development timing are mitigated by elevated temperature (Shepard-Brennan et al. 2010, Arnberg et al. 2013, Byrne et al. 2013b). In other species, pH effects are amplified by increased temperature (Talmage & Gobler 2011, Pansch et al. 2012) or both effects are fully independent (Parker et al. 2009, 2010).

In single-stressor studies, developmental delays in sea urchin larvae in response to reduced pH are attributed to the allocation of energetic resources away from growth and development in order to maintain cellular function and calcification (Stumpp et al. 2011). Slight delays in development timing may negatively affect larvae by prolonging planktonic stages resulting in transport away from favourable settlement conditions or through prolonged exposure to predation or unfavourable conditions (Dupont et al. 2010, Stumpp et al. 2011).

Lecithotrophic larvae, such as those of *L. elliptica*, are dependent on maternally provided lipid stores during gametogenesis until hatching and settlement (Pearse et al. 1986). Ocean acidification could prolong development, draining energy resources, although this may be mitigated by the faster development observed with elevated temperatures. Due to sufficient energetic resources, other encapsulated invertebrate larvae display an initial capacity for high calcification during early development even under acidified conditions (Timmins-Schiffman et al. 2013). Under ambient conditions, other Antarctic species have lipid stores in excess of what is needed for larval development, allowing for the development of larger juveniles (McClintock & Pearse 1986). However, energetic reserves are limited and developmental delays, as well as environmental stress and subsequent metabolic responses, may increase the use of energy reserves to the point where available energy is insufficient for development (Pörtner 2008, Dupont et al. 2010, Dorey et al. 2013). For example, reduced lipid content coinciding with prolonged development timing was observed in bivalve larvae exposed to elevated $p\text{CO}_2$ (Talmage & Gobler 2011).

Incidences of abnormal development in D-larvae were not influenced by reductions in pH. The constancy of abnormal development, even under reduced pH, may be related to *L. elliptica* larval encapsulation. Larvae are encapsulated in a thick (30 μm) gelatinous egg membrane, which may provide protection by buffering against external conditions, or conversely, create high $p\text{CO}_2$ environments within the eggs (Ansell & Harvey 1997, Pechenik 1999). Encapsulated larvae of the gastropod *Crepidula fornicata* exhibit reduced calcification at reduced pH, but abnormalities and differences in shell sizes indicate a much greater tolerance compared to other non-encapsulated mollusc larvae (Noisette et al. 2014). Egg cases of cuttlefish significantly limit gas diffusion creating a high intercapsular $p\text{CO}_2$ as development progresses, even under ambient pH (Dorey et al. 2013). However, despite the reduced

pH, cuttlefish are able to begin calcification of their aragonite shells and continue calcification after hatching as juveniles (Melzner et al. 2009, Dorey et al. 2013). If a similar limitation of diffusion occurs in *L. elliptica*, larvae could be adapted to calcification under high $p\text{CO}_2$.

The D-larval stage is not only a point of high calcification as the shell is formed, it is at a stage where isolation of calcifying surfaces may be more energetically demanding compared to later developmental stages (Waldbusser et al. 2013). Additionally, shell formation may be influenced by the availability and solubility of CaCO_3 . Aragonite was undersaturated ($\Omega_{\text{Ar}} < 1$) at both reduced pH levels in our experiment (Table 1), indicating that the observed D-larvae developmental delay may be due to difficulties in shell maintenance. Closer examination of the D-larvae is required to assess treatment differences in shell formation and morphology.

Reduced calcification in D-larvae would result in smaller, weaker larval shells, which would be more susceptible to crushing injury and predation upon settlement. The requirements for successful settlement and metamorphosis into juveniles in *L. elliptica* are unknown. In other bivalves, settlement success can be dependent on a number of factors including substrate suitability or sediment saturation state (Snelgrove et al. 1993, Green et al. 2009, 2013). Protracted development times indicate that shell formation in juveniles may occur in winter months in the Ross Sea when aragonite saturation states, at $\Omega_{\text{Ar}} < 1$ (McNeil & Matear 2008), may be unfavourable for shell maintenance in the near future. Impacts of ocean acidification would likely persist past the D-larval stage, potentially impacting settlement cues and reducing survival and development of juveniles.

To conclude, fertilisation in *L. elliptica* was affected by elevated temperature and reduced pH during the first 4 to 6 h, after which fertilisation was high in all treatments and robust to temperature and pH. No loss of gamete viability was observed. The observed influences of temperature and pH on development of the later veliger and D-larval stages suggest that future ocean warming may accelerate development to these stages, but that ocean acidification may limit this increase. Additionally, elevated temperatures reduced occurrences of abnormalities in D-larvae, while reduced pH ultimately had no effect. Despite no change in abnormalities, the slower development at reduced pH noted for this calcifying stage may indicate the diversion of energetic resources away from calcification/shell maintenance. Under continued exposure to these conditions, or in combination

with other stressors, the negative effect of reduced pH on larvae may become significant through reduced shell growth rates, increased energy demands, or increased mortality rates. More work is required to investigate the links between developmental delays, energetic reserves, and shell morphology in order to determine the ecological relevance of these changes in terms of longer-term larval success.

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