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1 **Thermal strategies vary with life history stage.**

2

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14 Key words:, TDT curves, thermal tolerance, CT_{max}, thermal sensitivity, trade-off.

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35 **Abstract**

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With both global surface temperatures and the incidence and intensity of extreme temperature events projected to increase, the assessment of species' sensitivities to chronic and acute changes in temperature has become crucial. Sensitivity predictions are based predominantly on adult responses, despite the fact that early life stages may be more vulnerable to thermal challenge. Here, we compared the sensitivity of different life history stages of the intertidal gastropod *Littorina obtusata* using thermal death time curves, which incorporate the intensity and duration of heat stress, and used these to calculate upper critical thermal limits (CT_{max}) and sensitivity to temperature change (z). Early (larval) life stages had both a lower CT_{max} and z than adults, suggesting they are less good at withstanding short term extreme thermal challenges, but better able to survive moderate temperatures in the long term. This result supports the predicted trade-off between acute and chronic tolerance to thermal stress, and is consistent with the different thermal challenges that these stages encounter in the intertidal zone. We conclude that different life history stages employ different thermal strategies that may be adaptive. Our findings caution against the use of predictions of the impact of global warming that are based on only adult responses and, hence, which may underestimate vulnerability.

66 **Introduction**

67

68 The Earth's climate is changing rapidly, with both global surface temperatures and
69 the incidence and intensity of extreme temperature events projected to increase
70 (IPCC, 2014). Within this context, the assessment of species' sensitivities to
71 elevated temperatures over different timescales is a crucial tool for modelling the
72 effects of altered thermal conditions and developing mitigation strategies. These
73 assessments are almost exclusively generated from adult data, despite the fact that
74 responses can differ between life stages (Radchuk et al., 2013), with early life stages
75 often described as more sensitive to altered environmental conditions than later
76 stages (Delorme and Sewell, 2013; Schiffer et al., 2014; Zippay and Hofmann,
77 2010).

78

79 Critical thermal maxima (CT_{max}), which define the upper limit of the thermal tolerance
80 range of organisms, have been used as proxies for predictions of the vulnerability of
81 populations to climate change (Huey et al., 2012). CT_{max} is typically measured as the
82 temperature at which an organism dies upon exposure to steadily increasing
83 temperature (Lutterschmidt and Hutchison, 1997). However, when calculated in this
84 way, its use may be intrinsically limited, as the effect of temperature is not dependent
85 upon just the intensity of thermal challenge, but also the duration of the exposure. As
86 both the organism's physiology and its probability to survive a thermal challenge vary
87 with time, it has been argued that such endpoint temperatures should not be equated
88 to CT_{max} , because these estimates do not control for the duration of the exposure
89 (Castañeda et al., 2015; Santos et al., 2011; Santos et al., 2012; Wang et al., 2007).
90 Thermal death time (TDT) curves address this limitation by providing an approach
91 that incorporates both the intensity and duration of thermal stress, and consequently
92 can generate a more robust method of predicting such responses (Rezende et al.,
93 2014).

94

95 The ability to tolerate extreme temperatures cannot typically be sustained for
96 prolonged periods and species with high CT_{max} tend to be more sensitive to longer
97 term exposure. This trade-off between acute and chronic tolerance to thermal stress

98 (Rezende et al., 2014) may be particularly relevant for marine invertebrates with
99 complex life cycles. Because different life stages often inhabit different environments
100 and thermal regimes, we predict that natural selection should favour different thermal
101 strategies across the life cycle. Here, we test this prediction in the marine intertidal
102 gastropod *Littorina obtusata* (Linnaeus, 1758). Adult *L. obtusata* inhabit the mid to
103 low intertidal where females lay egg masses (100-200 egg capsules) on seaweed
104 (Goodwin, 1979; Williams, 1990). Embryos and larvae undergo direct development,
105 hatching as juvenile snails. Employing TDT curves, we tested whether different life
106 stages (early veliger larva, mid veliger larva and adult) exhibit different sensitivities to
107 elevated temperatures, and if a trade-off exists between tolerance to acute versus
108 chronic thermal challenges across life stages.

109

110 **Methods**

111 **Collection and husbandry of *Littorina obtusata***

112 Adult *Littorina obtusata* were collected from the intertidal zone at Mount Batten,
113 Plymouth, Devon, UK (50°21'25.23" N, 4°07'37.21" W) during low tide. Upon
114 collection, snails were placed in large plastic bags containing damp *Fucus serratus*
115 (Linnaeus, 1753), preventing desiccation and damage during transportation.
116 Individuals were transported to the aquarium facilities at the Marine Biology and
117 Ecology Research Centre at Plymouth University within 2 h of collection, and
118 acclimated to laboratory conditions in 5 L aquaria (n=20 per aquarium) for at least
119 one week. Each aquarium was supplied with aerated sea water (temperature = 16.5
120 ± 0.5°C, salinity = 35 ± 1, PO₂ = 80 ± 10% air saturation, 12h:12h L:D cycle). Water
121 changes were made weekly, and snails were fed *ad libitum* on *F. serratus*.

122

123 **Physiological tolerance in adults**

124 Adult *L. obtusata* were exposed to a static thermal challenge, whereby temperature
125 was kept constant until mortality occurred. Adult snails were placed into individual
126 beakers containing aerated sea water at each of the four treatment temperatures of
127 36 (n=16), 38 (n=18), 40 (n=16) and 42°C (n=15). Sea water temperature was
128 maintained constant by submersion of the beakers in a temperature controlled water

129 bath. Mortality was determined by the absence of foot retraction upon disturbance
130 with a blunt needle, manifested by the lack of movement of the operculum, at which
131 point survival time was recorded (Sandison, 1967). Immediately after, snails were
132 placed in sea water under control conditions for 90 min, to exclude the possibility that
133 the animal had reached sublethal heat coma (McMahon, 1976). Individuals that
134 exhibited retraction of the foot during the recovery period were disregarded from
135 analysis. Individual snails were then frozen and later thawed to facilitate the
136 extraction of soft tissue from the shell. Shells were cracked open, soft tissue was
137 removed, rinsed with deionised water, blotted dry and weighed.

138

139 **Physiological tolerance in embryos**

140 Thermal tolerance tests were carried out on two larval stages: early veliger (i.e. at
141 the start of velar lobe development, approximately 4 days after the first cell division,
142 when reared at 15°C); and mid veliger (i.e. when the larval heart starts to beat,
143 approximately 13 days after the first cell division at 15°C) (Bitterli et al., 2012).
144 Larvae in their individual egg capsules at the target developmental stage were
145 imaged with high temporal and spatial resolution at a range of temperatures for 24 h
146 using an automated, custom-built bioimaging system for time-lapse study of aquatic
147 larvae (Tills et al., 2013). This system comprised a machine vision camera (Pike
148 421B, Allied Vision Technology, Stadtroda, Germany) connected to a zooming lens
149 (VHZ20R, Keyence, Milton Keynes, Buckinghamshire, UK), with dark-field cold
150 illumination provided by an LED light (CD100, Keyence, Milton Keynes,
151 Buckinghamshire, UK). The camera and lens were inverted beneath an XY
152 motorised stage (Scan, Märzhäuser Wetzlar GmbH & Co., Wetzlar, Germany)
153 controlled by a Tango Desktop control unit (Märzhäuser Wetzlar GmbH & Co.,
154 Wetzlar, Germany). A Mac Mini running MICROMANAGER v. 1.4.22 was used to
155 control and synchronise the motorised stage and camera. For a schematic of the
156 system see (Tills et al., 2013). The incubation chamber containing the embryos was
157 mounted on the XY motorised stage above the inverted camera and optics. Embryos
158 were recorded by recording an image sequence (150 images, 1024 x 768 pixels, 7.5
159 frames s⁻¹) of each embryo every 5 min during the period of an experiment. Embryos
160 at the early veliger stage were exposed to four temperature treatments 36 (n=14), 38
161 (n=12), 40 (n=13) and 42°C (n=14) and embryos at the mid veliger stage were

162 exposed to three 38 (n=12), 40 (n=12) and 42°C (n=14) as the extended survival at
163 36°C led to parasitic infection before mortality occurred. The infection appears to be
164 fungal, but we have yet identified it. We have observed eggs masses infected under
165 field conditions but, as yet, have not investigated how embryos might be impaired by
166 the infection. For experimental purposes, however, the movement of the parasite did
167 not allow accurate estimation of lethal times, and therefore data were excluded.
168 Treatment temperatures fluctuated around the target temperature by <0.5°C for up to
169 60 min, after which period temperature fluctuations were <0.3°C for the next 23 h. A
170 maximum of ten individuals were analysed during each experiment. At 42°C
171 embryos were imaged every 90 s due to greater sensitivity at this temperature.
172 Mortality was assessed by visual inspection of the image sequences using the open-
173 source image analysis software Fiji (Schindelin et al., 2012). A range of body
174 movements were observed including muscle flexing, embryo rotation, velum cilia
175 signals and heartbeat. Mortality was defined by the recession of movement in all of
176 these traits.

177

178 **Data analysis**

179

180 Lethal time was recorded for every individual, and the median lethal time (LT50) at
181 each assay temperature was calculated. Survival probability plots for each stage
182 were generated using the lethal endpoint times of organisms within each treatment.
183 Thermal death time (TDT) curves were modelled from lethal times at three to four
184 static thermal challenges for each life cycle stage. Upper thermal limits and thermal
185 sensitivities were estimated using the following equation (Rezende et al., 2014):

$$186 \quad \log_{10} t = \frac{(CT_{\max} - T)}{z}$$

187 (Eqn1)

188 where t corresponds to lethal time (min), CT_{\max} is the upper critical thermal limit (°C)
189 at 1 min ($\log_{10} t = 0$), T is the assay temperature (°C) and z is the temperature
190 change (°C) required for a 10-fold change in survival times, and therefore quantifies
191 the sensitivity to temperature change. Here, we controlled T, and measured t as the
192 dependent variable, so we estimated CT_{\max} and z for each developmental stage by

193 regressing \log_{10} transformed lethal times against three or four temperature
194 treatments, as $CT_{max} = -\text{intercept/slope}$ and $z = 1/\text{slope}$ (Eq1). A linear model was
195 used to determine the goodness-of-fit. Because average survival times in each
196 treatment closely resembled exposure times required for 50% mortality interpolated
197 from the survival curves ($n = 11$, Pearson $r = 0.999$, $P = 5.7 \times 10^{-13}$), here we report
198 mean \pm SD estimates for simplicity. Data analysis was performed in R (R
199 Development Core Team, 2017).

200

201 Results

202 Mortality curves

203

204 Survival times (8-904 min) differed significantly between life history stages (Figure
205 1). Average survival times at 38°C were 160.3 ± 26.4 min, 522.4 ± 133.9 min and
206 336.8 ± 37.1 min for early veliger, mid veliger and adult respectively (mean \pm SD;
207 $F_{2,39} = 67.2$, $P = 2 \times 10^{-13}$). These decreased to 12.5 ± 5.1 min, 24.3 ± 19.2 min, and
208 51.7 ± 15.5 min, respectively, at 42°C ($F_{2,40} = 27.7$, $P = 2.8 \times 10^{-8}$). Adults exhibited
209 higher survival times in response to more acute thermal challenges and reduced
210 survival times at less extreme temperatures, which was most evident at 36°C when
211 comparing adults against early veliger ($F_{1,28} = 12.2$, $P = 0.0016$). Although there
212 were no data for the mid veliger stage at 36°C, given that survival times in adults at
213 this temperature were statistically indistinguishable from estimates at 38°C for mid
214 veliger ($F_{1,26} = 0.15$, $P = 0.720$), this result should hold in a complete dataset.

215

216 Differences in thermal tolerance profiles between stages

217 Differences in survival times across temperatures were adequately encapsulated by
218 our semi-log model (Figure 2). Curves exhibited high goodness-of-fit at all stages
219 (adult, $R^2_{adj} = 0.89$, $F_{1,63} = 519.2$, $P < 0.001$; mid veliger, $R^2_{adj} = 0.86$, $F_{1,36} = 233.6$, P
220 < 0.001 ; early veliger, $R^2_{adj} = 0.96$, $F_{1,51} = 1294$, $P < 0.001$). Importantly, R^2 estimates
221 were even higher when only mean estimates of survival times per temperature were
222 considered, which is an appropriate strategy for removing variance in survival within
223 temperatures, due to the probabilistic nature of survival curves (Santos et al., 2011).
224 These TDT curves revealed contrasting differences in thermal tolerance between

225 stages, with adults exhibiting a higher CT_{max} (52.8°C) than embryos (45.6°C for both
226 stages) and lower sensitivity to temperature change, i.e. z values suggested that a
227 10-fold change in survival times resulted from a 6.01°C change in temperature for
228 adults compared with 3.47°C and 2.85°C in early and mid veligers, respectively. A
229 trade-off between CT_{max} and z is apparent because adults tend to survive for longer
230 at high temperatures, whereas early life stages survive for longer at less extreme
231 temperatures (Figure 2). For adults only, there was a significant effect of size on
232 survival ($t_{62} = 2.646$, $P = 0.01$).

233

234 **Discussion**

235

236 The relative thermal sensitivities of three life history stages of the intertidal gastropod
237 *L. obtusata* were assessed using survival plots and TDT curves. Early life stages had
238 both a lower CT_{max} and a lower z than adults, which suggests that they are less good
239 at withstanding short term extreme thermal challenges, but better able to survive
240 moderate temperatures in the long term.

241

242 Given the putative trade-off between acute tolerance and long term survival,
243 adaptive strategies are expected to differ between organisms experiencing different
244 thermal regimes. Rezende et al (Rezende et al., 2014) indicate that low z values
245 should be beneficial in thermally stable environments, at the expense of a high
246 CT_{max} , whereas highly variable environments would favour a high CT_{max} at the
247 expense of low z values. The intertidal zone is characterised by large thermal
248 variability both in space and time, with rapid and severe fluctuations in temperature
249 associated with the tidal cycle (Helmuth and Hofmann, 2001). The intensity and time
250 that intertidal individuals experience thermal stress will depend on the shore height
251 and microhabitat they inhabit. *Littorina obtusata* adults typically inhabit a range of
252 microhabitat types on the shore, grazing epiphytes from furoid algae and epilithic
253 algae from rocks (Kemppainen et al., 2005). Hence, they are likely to experience
254 greater thermal variation than embryos, which are contained within egg masses
255 (Woods and DeSilets, 1997) glued to algal fronds or rocks and, hence, in a fixed
256 position on the shore (Goodwin, 1979). The low acute tolerance in embryos may be
257 associated with the protection conferred by the characteristics and positioning of the
258 egg mass, which potentially buffers environmental insult (Woods and DeSilets,

259 1997). Our results support the predicted trade-off between CT_{max} and z , by which
260 embryos, which develop in more constant environments, display lower acute thermal
261 tolerance, but are able to survive longer exposure to less extreme temperatures than
262 adults, which experience more variable environments.

263

264 The mechanisms employed under these contrasting thermal strategies are likely to
265 differ. The fact that early life stages had low CT_{max} values compared to adults,
266 suggests that high upper thermal limits develop during ontogeny. It is possible that
267 energy allocation to cellular division and rearrangements during development lead to
268 greater susceptibility to acute stress in developing embryos compared to adults
269 (Hammond and Hofmann, 2010). Acute thermal tolerance in adults is conferred by
270 mechanisms involved in the heat shock response, which may manifest at a lower
271 capacity in early embryo (Brown et al., 2004; Sconzo et al., 1995, but see Hammond
272 and Hofmann, 2010) perhaps because overexpression in early embryos inhibits
273 development (Krebs and Feder, 1998). While embryos are equipped with defenses
274 that ensure developmental stability under different environmental conditions
275 (Hamdoun and Epel, 2007), adaptive strategies and acute changes may defeat such
276 defenses, leading to disruptions in development and subsequent mortality. In adults,
277 acute tolerance is higher, but cannot be sustained for long periods of exposure to
278 even less extreme conditions. The thermal tolerance of an organism is proportional
279 to the magnitude of temperature variation it experiences (Deutsch et al., 2008).

280 Ectotherms inhabiting the intertidal can experience large daily and seasonal
281 temperature fluctuations, thus leading to high upper thermal limits (Stillman, 2002).
282 The higher sensitivity to longer term exposure to less extreme temperatures could
283 reflect, to some extent, the ability to avoid such conditions through regulating body
284 temperature by minimising heat exposure. Behavioural plasticity of habitat use is an
285 essential thermoregulatory strategy in ectotherms (Kearney et al., 2009; Sunday et
286 al., 2014). Thermoregulatory behaviours have been described in intertidal
287 gastropods (Iacarella and Helmuth, 2012; Miller and Denny, 2011; Ng et al., 2017),
288 and can potentially ameliorate the impacts of warming temperatures (Marshall et al.,
289 2015).

290

291 Our results also highlight the inherent complexity involved in predicting the potential
292 impact of warming temperatures on intertidal organisms with multiple life stages.

293 Climate change scenarios predict both a gradual increase in surface temperatures,
294 and an increase in temperature extremes (IPCC, 2014). While it is tempting to focus
295 on the impact of temperature anomalies in adult mortality because adult snails are
296 generally exposed to more pronounced fluctuations, relatively moderate changes in
297 water temperature could have major consequences in larval mortality should the
298 observed differences in z between life stages hold across species. For instance, the
299 10-fold decrease in survival times expected with a $z = 3.47^{\circ}\text{C}$ observed in early
300 veliger implies a drop of 6.4% in survival times for every 0.1°C increase in
301 temperature (i.e., t at 36.1°C corresponds to $0.936 \times t$ at 36°C , Eqn1). A shift of this
302 magnitude in the whole survival curve (Figure 1) would increase the mortality from
303 50% at a given temperature to roughly 84% at 0.1°C higher if exposure times are
304 held constant (with 95% confidence intervals corresponding to 81.8% and 85.6%
305 based on the intervals estimated for z of 3.29 and 3.67°C). In contrast, a similar
306 calculation with $z = 6.01^{\circ}\text{C}$ of adult snails results in a drop of 3.7% in survival times
307 per 0.1°C , or an increase in mortality from 50% to 59% (95% CI between 58.9% and
308 59.8% given the intervals for z of 5.53 and 6.58°C) everything else being equal. A
309 formal implementation of these calculations to estimate the impact of different
310 thermal regimes on mortality rates constitutes work in progress (E.L.R. unpublished
311 results).

312

313 The complexity of responses in intertidal habitats is increased by the fact that
314 organismal physiology differs during immersion and emersion (Bjelde and Todgham,
315 2013; Truchot, 1990). In our experiments, measurements of thermal tolerance were
316 made in water for both adults and early life stages to ensure that the experimental
317 environment was standardized. It is highly likely, however, that sensitivity predictions
318 would have been different if experiments had been performed in air. Comparisons of
319 thermal responses between immersed and emersed adult gastropods show that
320 thermal limits are higher in air (Bjelde and Todgham, 2013; Drake et al., 2017),
321 which may reflect adaptations of an intertidal lifestyle that confer thermal tolerance
322 when emersed. Increased oxygen availability in air versus water (Truchot, 1990),
323 may mean that adult intertidal animals are able to meet oxygen demands more
324 easily when emersed (Pörtner, 2001) and are also less reliant on anaerobic
325 metabolism. Intertidal adults may also be able to upregulate cellular defenses in
326 response to emersion that allow greater tolerance of thermal extremes (Bjelde and

327 Todgham, 2013) or may exhibit circatidal variation in gene expression that underpin
328 increased thermal tolerance during emersion (Gracey et al., 2008). They may also
329 be able to rely partly on evaporative cooling that will be associated with exposure
330 (Cleland et al., 1990; McMahon, 1990).

331

332 Measurements of thermal tolerance have not been made previously for encapsulated
333 embryos and larvae of intertidal species and would require careful design due to the
334 confounding factor of desiccation, which has a large effect on these small life stages.
335 Under this scenario, we speculate that, in contrast to adults, larval stages would
336 exhibit substantially lower heat tolerance in air than estimates in water. Further work
337 on the physiological and molecular mechanisms in early life stages will be needed to
338 unravel the capacity for these stages to tolerate thermal extremes under emersion
339 and immersion. Nonetheless, the main take-home message is clear: in water, early
340 life stages with low z should be more sensitive to temperature changes (see also
341 Castañeda et al., 2015), and therefore relatively small changes in their thermal
342 environments could have important consequences to population dynamics and
343 ultimately resilience to climate change. Thus, while intuition suggests that in highly
344 variable environments, such as the intertidal, tolerance to thermal extremes may be
345 more important for long-term species persistence than changes in surface
346 temperatures, our analyses suggest that the latter might be equally important.

347

348 In summary, the thermal tolerance of *L. obtusata* varies across its life cycle, with
349 early life stages having lower acute tolerance but greater long term tolerance to
350 thermal challenges compared to adults. The trade-off between these two parameters
351 may reflect different adaptive thermal strategies imposed by differential thermal
352 challenges they are likely to encounter in the environment. Our study highlights the
353 importance of considering different life stages if we are to make robust predictions of
354 environmental sensitivity and the impacts of global warming on populations.

355

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358

359 **Competing interests**

360 No competing interests declared.

361

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365

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367

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487 **Figure legends**

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489 **Figure 1. Survival plots.** Probability of survival with increasing exposure time at
490 each test temperature for A) early veliger (n=14, 12, 13 and 14 at 36, 38, 40 and
491 42°C respectively), B) mid veliger (n=12, 12 and 14 at 38, 40 and 42°C respectively)
492 and C) adult snails (n=16, 18, 16 and 15 at 36, 38, 40 and 42°C respectively).

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495 **Figure 2 Thermal death time curves and the trade-off between CT_{max} and z .** A)
496 Thermal death time (TDT) curves for adults (blue, $n=16, 18, 16$ and 15 at $36, 38, 40$
497 and 42°C respectively), early veliger (red, $n=14, 12, 13$ and 14 at $36, 38, 40$ and
498 42°C respectively) and mid veliger (green, $n=12, 12$ and 14 at $38, 40$ and 42°C
499 respectively). The interpolation at the abscissa represents CT_{max} , or the tolerated
500 temperature following 1 min of exposure (Eqn1), whilst z corresponds to the
501 reciprocal of the slope. **Errors bars represent standard deviations.** The R-square
502 obtained for these curves, as well for linear models including mean estimates per
503 temperature, are shown in the inset. Curves exhibited high goodness-of-fit at all
504 stages (adult, $R^2_{adj} = 0.89$, $F_{1, 63} = 519.2$, $P < 0.001$; mid veliger, $R^2_{adj} = 0.86$, $F_{1, 36} =$
505 233.6 , $P < 0.001$; early veliger, $R^2_{adj} = 0.96$, $F_{1, 51} = 1294$, $P < 0.001$). B) Relationship
506 between Ct_{max} and z for early veliger (red) mid veliger (green) and adult (blue) snails.
507 A trade-off between CT_{max} and z is apparent.