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Divergence in Thermal Physiology Could Contribute to Vertical Segregation in Intertidal Ecotypes of Littorina saxatilis

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Abstract:	Thermal stress is a potentially important selective agent in intertidal marine habitats, but the role that thermal tolerance might play in local adaptation across shore height has been underexplored. Northwest Spain is home to two morphologically distinct ecotypes of the periwinkle Littorina saxatilis , separated by shore height and subject to substantial differences in thermal stress exposure. However, despite other biotic and abiotic drivers of ecotype segregation being well studied, their thermal tolerance has not been previously characterised. We investigated thermal tolerance across multiple life-history stages by employing the Thermal Death Time (TDT) approach, to determine: i) if the two ecotypes differ in thermal tolerance, and: ii) how any differences vary with life history stage. Adults of the two ecotypes differed in their thermal tolerance in line with their shore position: the upper-shore ecotype, which experience more extreme temperatures, exhibited greater endurance of thermal stress compared to the lower shore ecotype. This difference was most pronounced at the highest temperatures tested. The proximate physiological basis for these differences is unknown, but likely due to a multifarious interaction of traits affecting different parts of the TDT curve. Differences in tolerance between ecotypes were less pronounced in early life-history stages, but increased with ontogeny, suggesting partial divergence of this trait during development. Thermal tolerance could potentially play an important role in maintaining population divergence and genetic segregation between the two ecotypes, since the increased thermal sensitivity of the lower-shore ecotype may limit their dispersal onto the upper shore and so restrict gene flow.

Divergence in thermal physiology could contribute to vertical segregation in intertidal ecotypes of *Littorina saxatilis*

Authors: [anonymized]

Key words: Thermal tolerance, ecological speciation, periwinkle, gastropod, TDT curve, CT_{max}, Heat coma

Running page Head: Physiological divergence in ecotypes of *Littorina saxatilis*

What is already known: Thermal adaptation may play a significant role in driving divergence between populations living across shore heights and is predicted to occur across all life-history stages in direct-developing species. Two ecotypes of the direct-developing periwinkle *Littorina saxatilis* are present at different shore heights on the Galician (NW Spanish) coastline and subject to divergent selection along a well-characterised wave-exposure/ predation gradient. Despite being exposed to contrasting temperature regimes, however, differences in their thermal tolerance have not previously been characterized.

What this study adds: The two ecotypes differ in their thermal tolerance in line with their vertical shore height position**:** these differences are more pronounced in adults and magnify during embryonic development. Thermal tolerance differences in the adults may play a hitherto overlooked role in maintaining segregation and divergence between the two ecotypes, potentially reinforcing selection for other habitat-specific traits.

Abstract

Thermal stress is a potentially important selective agent in intertidal marine habitats, but the role that thermal tolerance might play in local adaptation across shore height has been underexplored. Northwest Spain is home to two morphologically distinct ecotypes of the periwinkle *Littorina saxatilis*, separated by shore height and subject to substantial differences in thermal stress exposure. However, despite other biotic and abiotic drivers of ecotype segregation being well studied, their thermal tolerance has not been previously characterised. We investigated thermal tolerance across multiple life-history stages by employing the Thermal Death Time (TDT) approach, to determine: i) if the two ecotypes differ in thermal tolerance, and: ii) how any differences vary with life history stage. Adults of the two ecotypes differed in their thermal tolerance in line with their shore position: the upper-shore ecotype, which experience more extreme temperatures, exhibited greater endurance of thermal stress compared to the lower shore ecotype. This difference was most pronounced at the highest temperatures tested. The proximate physiological basis for these differences is unknown, but likely due to a multifarious interaction of traits affecting different parts of the TDT curve. Differences in tolerance between ecotypes were less pronounced in early life-history stages, but increased with ontogeny, suggesting partial divergence of this trait during development. Thermal tolerance could potentially play an important role in maintaining population divergence and genetic segregation between the two ecotypes, since the increased thermal sensitivity of the lower-shore ecotype may limit their dispersal onto the upper shore and so restrict gene flow.

Introduction

Populations living across environmental gradients may be subject to dramatic variation in levels of biotic and abiotic stress, with the resultant divergent selection regimes driving local adaptation and potentially leading to ecological speciation (Spicer and Gaston 1999; Keller and Seehausen 2012; Nosil 2012). In intertidal marine environments, extreme temperatures represent a significant source of physiological stress, with both intensity and duration of exposure varying in concert with the length of exposure during low tide (McMahon 1990; Helmuth and Hofmann 2001; Tomanek and Helmuth 2002; Little et al. 2009). Notably, intertidal organisms frequently contend with temperatures exceeding the critical limits of aerobic performance, beyond which endurance is time-limited (Sokolova and Pörtner 2003; Pörtner et al. 2017), and so display a variety of mechanisms to prolong survival under these conditions including mobilization of the heat shock response (Tomanek and Somero 1999, 2000; Tomanek and Helmuth 2002), antioxidant defenses (Abele et al. 2007), and anaerobic metabolic pathways (Livingstone 1983; Sokolova and Pörtner 2001). Intertidal organisms have consequently been widely utilized as a model for the study of thermal adaptation. Numerous studies have demonstrated that species living at different shore heights display clinal variation in thermal stress tolerance (Burggren and McMahon 1981; Sanders et al. 1991; Marshall and McQuaid 1992; Stillman and Somero 1996, 2000; Tomanek and Somero 1999, 2000), mirroring patterns seen across latitude (Stillman and Somero 2000; Compton et al. 2007; Somero 2010; Armstrong et al. 2019). However, between-species comparisons are far from ideal for studying the processes leading to thermal adaptation across environmental gradients, because individual species possess unique evolutionary histories which may not represent current patterns of zonation (Garland and Adolph 1994; Keller and Seehausen 2012; Rezende and Diniz-Filho 2012). Intraspecific comparisons, by contrast, reduce the influences of macroevolutionary factors and allow for the potential mechanisms underpinning local population divergence to be elucidated (Coyne and Orr 2004; Keller and Seehausen 2012; Rezende and Diniz-Filho 2012). Intraspecific comparisons have revealed local thermal adaptation leading to population divergence (Kavanagh et al. 2010; Keller and Seehausen

2012; Ohlberger et al. 2013; Gibbons et al. 2016), demonstrating that temperature gradients can promote reproductive isolation and ultimately act as a driver of ecological speciation (Coyne and Orr 2004; Keller and Seehausen 2012; Rezende and Diniz-Filho 2012). Such mechanisms could play a significant role in driving population divergence in the intertidal, where strong patterns of vertical segregation exist in response to thermal stress (Newell 1979; McMahon 1990). In particular, the combination of thermal selection acting in concert with other clinal selective forces present within the intertidal may further enhance divergence *via* multifarious selection (Nosil et al. 2009; Nosil 2012).

The role of early life-history stages in local thermal adaptation is frequently overlooked (Radchuk et al. 2013). Early life-history stages represent a particularly critical period of vulnerability to thermal stress (Przeslawski 2004; Byrne 2012; Radchuk et al. 2013; Lockwood et al. 2018), partly because they may lack physiological and morphological defenses of adults (Andronikov 1975), and because even minor stress-induced alterations in development can have knock-on consequences in later life (Hamdoun and Epel 2007; Nord and Giroud 2020). Variation in the responses of early life-history stages to thermal stress across environmental gradients has been demonstrated in numerous species (Kuo and Sanford 2009; Zippay and Hofmann 2010; Tangwancharoen and Burton 2014), indicating the potential for these stages to play a key role in local thermal adaptation. Despite this vulnerability, early life-history stages have been shown to have both reduced (Zippay and Hofmann 2010; Lockwood et al. 2018) and enhanced (Sewell and Young 1999; Miller et al. 2013; Tangwancharoen and Burton 2014) thermal tolerance compared to adults. It has therefore been suggested that physiological responses may differ across developmental stages, representing an adaptive response to the distinct thermal environments they inhabit (Miller et al. 2013; Tangwancharoen and Burton 2014; Lockwood et al. 2018; Truebano et al. 2018). Although several studies examining within-species thermal tolerance variations across intertidal gradients have incorporated early life-history stages (Bingham et al. 1997; Diederich and Pechenik 2013), these typically utilize species with planktonic larvae that experience different thermal regimes to adults. Highly dispersive, planktonic larvae also increase population connectivity (Sanford and Kelly 2011) reducing the likelihood of local genetic adaptation (Hollander 2008). By contrast, direct developers with poor dispersal, where early life-history stages are exposed to the same environment as their

parents, may show greater capacity for local genetic adaptation of thermal tolerance with shore height, because traits which resist thermal selection during early development are also likely to promote survival in later stages.

The rough periwinkle, *Littorina saxatilis* (Olivi), is an ideal model organism for the study of the physiological mechanisms driving intraspecific population divergence, due to its presence across intertidal gradients, low dispersal ability, and direct development (Reid 1996; Galindo and Grahame 2014; Rolán-Alvarez et al. 2015; Johannesson 2016). *L. saxatilis* is the only ovoviviparous species within the genus *Littorina*, the eggs developing in a brood pouch inside the female and being subsequently released as shelled crawling juveniles (Reid 1996). Populations of *L. saxatilis* found across many NE Atlantic shores are characterised by the presence of two distinct ecotypes adapted to habitats with contrasting selective forces: wave action (Wave ecotype), and crab predation (Crab ecotype). These ecotypes represent a well-characterised model for parallel divergence and speciation in the face of gene flow (Rolán-Alvarez 2007; Butlin et al. 2014; Rolán-Alvarez et al. 2015; Johannesson 2016; Fernández-Meirama et al. 2017). Strikingly divergent examples of the two ecotypes are found on exposed rocky shores in Galicia, NW Spain (Reid 1996). Here, the local form of the Wave ecotype (referred to henceforth as SU, "smooth unbanded") is present in mussel beds on the lower shore, while the Crab ecotype (referred to henceforth as RB, "ridged banded") is found on and above the barnacle zone on the mid and upper shore. The two ecotypes display divergent morphologies in line with their habitats: a thin shell and large, high-tenacity foot in the SU vs. a reinforced, crab-resistant shell in the RB (reviewed in Rolán-Alvarez 2007). Gene flow between the two ecotypes is restricted by size assortative mating (Johannesson et al. 1995; Rolán-Alvarez et al. 1999; Cruz et al. 2004), differential micro-habitat choice (Otero-Schmitt et al. 1997), and the respective vulnerabilities of the RB and SU to high wave action on the lower shore (Rolán-Alvarez et al. 1997), and high predation on the upper (Boulding et al. 2017).

Crucially, segregation of the two Galician ecotypes by shore height means they are subject to differing windows of exposure to both desiccation and thermal stress at low tide (Rolán-Alvarez et al. 1997). Notably, the RB ecotype experiences more frequent and protracted periods of extremes temperature during the summer than the SU (Fig. 1A-B). Previous studies have suggested that the two

ecotypes may differ in their thermal tolerance as an adaptive response to the distinct thermal conditions of their respective habitats (Rolán-Alvarez et al. 1997; Cruz et al. 2004) with the implication that these differences may act to further reduce gene flow between the two ecotypes (Nosil et al. 2005; Keller and Seehausen 2012). However, despite evidence that the two ecotypes differ in their respiratory physiology (Martínez-Fernández et al. 2008, 2010) and desiccation tolerance (Rolán-Alvarez et al. 1997), the prediction that the two ecotypes differ in thermal tolerance has never been tested in these populations. Accordingly, the aim of our study was to investigate differences in thermal tolerance between the RB and SU ecotypes. We investigated thermal tolerance across life-history stages using the Thermal Death Time (TDT) approach (Rezende et al. 2014), firstly to test the prediction that the RB ecotype should display increased thermal tolerance compared to the SU, and, secondly, to investigate whether such differences originate in early life-history. The TDT approach, which allows for the relative effects of intensity *versus* duration of thermal stress to be distinguished, has been applied to both marine (Rezende et al. 2014; Semsar-kazerouni and Verberk 2018; Truebano et al. 2018; Burton and Einum 2020) and terrestrial taxa (Rezende et al. 2014; Castañeda et al. 2015; Jørgensen et al. 2019) and to comparisons across multiple life-history stages (Truebano et al. 2018), demonstrating its broad applicability across different environmental conditions. The TDT methodology has also been used to identify a trade-off across latitude in organisms' abilities to resist acute *versus* chronic thermal stress (Urban 1994; Rezende et al. 2014; Castañeda et al. 2015; but see Jørgensen et al. 2019). Based upon similarities in patterns of physiological adaptation between intertidal and latitudinal gradients (Stillman 2004; Compton et al. 2007), we anticipated that a comparable trade-off may also be apparent across the height-segregated Galician *L. saxatilis* ecotypes. Since embryos experience the same thermal environment as adults inside the brood pouch, we predicted that thermal tolerance differences between embryos of the two ecotypes would mirror those observed between adults.

Methodology

151 *Animal collection and husbandry*

152 Adult *L. saxatilis* (Fig. 1C) were collected from Silleiro Cape in Galicia, Spain (+42° 6’ 4’’, −8° 53’
153 58’’) from December 2018 to February 2019. The SU ecotype was sampled from mussel beds on the
154 lower shore while the RB ecotype was sampled from the upper shore above the barnacle zone and
155 close to the splash zone (following Boulding et al. 2017; Fig. 1A). These habitats exhibit contrasting
156 thermal regimes which are most pronounced in the summer (Fig. 1A-B). Within 24 h of collection,
157 snails were sent via express airmail to [author’s institution] in plastic boxes containing dampened
158 cardboard inside sealed polystyrene boxes. Upon arrival, they were transferred to 5 L aquaria
159 containing aerated seawater (salinity = 35 ± 1) and held at 15°C, a temperature which approximates
160 field sea temperatures in Galicia at the time of collection. Snails from both ecotypes were held under
161 common laboratory conditions for at least one week prior to experiments. Water changes were
162 conducted weekly, and snails were fed *ad libitum* on *Ulva lactuca*.

163

164 *Acquisition of field temperature data*

165 Field temperature recordings (Fig. 1A-B) were obtained at Silleiro Cape over a 17-day period (8^{th}-25^{th}
166 August 2009) using individual robolimpet data loggers (Lima and Wethey 2009) positioned in
167 microhabitats corresponding to the locations of each ecotype; directly exposed to the sun on the lower
168 shore (corresponding to the position of the SU ecotype during low tide), and in a more protected
169 location (shaded by rock crevicing) on the upper shore. This reflects the location of the RB ecotype
170 within shaded refugia at low tide, a phenomenon not seen in the SU (Author 6, *pers. obs.*). Because of
171 this, it should be noted that the disparity in temperatures between the two dataloggers are likely less
172 extreme than they would be had both been placed in sun- exposed locations at both shore heights.
173 Sensors recorded air temperatures at low tide and water temperatures upon immersion at high tide.
174 Temperature data was recorded at 15-minute increments to the nearest °C.

175

176 *Assessment of thermal tolerance*

To assess thermal tolerance across all life-history stages, we utilized the static approach (Lutterschmidt and Hutchison 1997). We constructed Thermal Death Time (TDT) curves, which allow for the effects of intensity and duration of thermal stress to be distinguished (Rezende et al. 2014). Under the TDT approach, log-transformed endpoint times (lethal or sublethal) derived from static assays are plotted across a range of temperatures, and the resulting regression slopes used to calculate CT_{max} (the extrapolated temperature at which endpoint would occur after a period of 1 minute) and z (the temperature change required for a tenfold change in survival time to occur (Rezende et al. 2014).

Thermal tolerance in embryos

Two developmental stages - mid veliger (characterised by the presence of a larval, but not adult, heart) and late veliger (characterised by the presence of both a larval and adult heart, but still retaining a velum) - were identified and isolated under low power microscopy (Fig. 1C). Although all developmental stages can be found together in *L. saxatilis* brood pouches (Reid 1996), we selected these stages due to their high prevalence in the individuals we dissected, likely due to the relatively long developmental window occupied by these stages relative to earlier stages (Pelseneer 1911; Author 1, *pers. obs.*). In addition, despite the apparent morphological similarity between mid and late veligers (Fig. 1C), significant changes in physiological sensitivity have been demonstrated between these stages in the closely related species *Littorina obtusata* (Bitterli et al. 2012), which are linked to the timing of cardiovascular development.

Thermal assays in embryos from both ecotypes were performed using a custom-built bioimaging system (Tills et al. 2013; Truebano et al. 2018). At the start of each thermal assay, embryos were dissected out of the brood pouch from live females. These were individually added using a paintbrush to separate wells of a 48-well plate (Falcon Scientific, UK) containing 1.25 mL of filtered, autoclaved seawater preheated to one of five assay temperatures (37, 38, 39, 40 and 41°C) and held within an incubation chamber mounted on an XY motorized stage above an inverted camera

and optics (Tills et al. 2013). These temperatures were selected because they represent maximal temperatures experienced by these populations (Fig. 1A-B), and resulted in survival timeframes (1 min – 20 h) suitable for TDT analysis (Rezende et al. 2014; Jørgensen et al. 2019). Temperatures within the chamber were controlled using an UNO Combined controller (OKOLAB, Italy) and desktop PC running OKOLAB's UNO-TS software. Temperature fluctuated by ± 0.3°C around the assay temperature in the first hour following the addition of embryos, after which it stabilized to ± 0.2°C. Salinity was kept at 35 ± 1 by maintaining high humidity within the chamber and by occasional topping up of water during the trials. Oxygen concentrations levels within the wells remained at normoxic levels (>90% air saturation). Due to the high density of embryos carried within the brood pouches of females (Reid 1996), it is possible that oxygen concentrations experienced by embryos in the field would actually be lower than those experienced during our trials. However, we chose to use normoxic conditions to facilitate cross-comparison between ecotypes and temperatures. It should be noted that *L. saxatilis* embryos can be raised successfully in seawater (Pelseneer 1911; Author 1, *pers. obs.*), demonstrating that exposure to normoxic conditions does not inhibit long-term survival.

Between six and 16 individuals drawn from each developmental stage were used per thermal assay, with fewer animals per assay used at higher temperatures to reduce setup times. Embryos obtained from a different female were used for each assay, and in the case of the SU ecotype, embryos from multiple females were used within each assay due to the small number of embryos at this developmental stage carried by each parent (Conde-Padín et al. 2007). A minimum of three assays per ecotype were performed for each temperature. At 37-40°C, videos (600x600 pixels, 30x magnification, 10 frames s^{-1}) were recorded onto hard drives (6 TB Barracuda Pro, Seagate, USA) mounted within a hard drive array (eBox TeSU, DatOptic, USA). To provide higher temporal resolution at higher temperatures (where survival times were much shorter), different acquisition frequencies were used at different temperatures (15-20 s at 10-, 5-, or 2-min intervals). Recorded video footage was analyzed using FiJi (Schindelin et al. 2012). A range of criteria were used to define mortality, including the loss of cardiovascular function, loss of gut peristalsis, and cessation of muscular twitching in the head and mantle. Survival times were recorded based upon the last video

sequence in which one of these traits could be observed. Because of the rapid cessation of activity at 41°C, video acquisition was not used, and instead embryos were added to the wells sequentially and endpoints determined through visual assessment using the live camera feed. Total sample sizes were as follows: RB mid veligers (37°C- n = 20, 38°C- n = 20, 39°C- n = 15, 40°C- n = 12, 41°C- n = 14), SU mid veligers (37°C- n = 19, 38°C- n = 17, 39°C- n = 13, 40°C- n = 15, 41°C- n = 14), RB late veligers (37°C- n = 20, 38°C- n = 22, 39°C- n = 16, 40°C- n = 15, 41°C- n = 14) and SU late veligers (37°C- n = 18, 38°C- n = 15, 39°C- n = 13, 40°C- n = 14, 41°C- n = 11). During video analysis, shell and egg sizes were measured using still images obtained from each embryo, with shell height and width measurements taken as described in Reid (1996) using FiJi.

Thermal tolerance in adults

Thermal tolerance in adults of the two ecotypes was assessed using time taken to enter heat coma as a sublethal proxy for survival (henceforth referred to as heat coma time), due to methodological difficulties in determining mortality in adult gastropods using a static approach. Heat coma in Gastropods is characterised by the inability of the nervous and muscular system to respond to external stimuli (Sandison 1967; Truebano et al. 2018) and represents a point of physiological compromise beyond which the animal is unable to react to external threats, such as predation (Cowles and Bogert 1944; Lutterschmidt and Hutchison 1997). Only males were used to remove potential sex-specific differences in thermal tolerance, which also ensured that the use of only reproductively mature individuals (as females cannot be positively distinguished from immature specimens without dissection). The snails were sexed under light microscopy and males positively identified by the presence of a penis behind the right eye (Reid 1996). Prior to the trials, all experimental individuals were fitted with a small (4-6 cm) piece of fine thread attached to the center of the operculum using Super glue (Loctite). Snails were then left in seawater overnight to recover from the addition of the thread, and only individuals which subsequently displayed normal crawling behavior (> 95 % of individuals) were used.

During the trials, snails were held in individual 350 mL glass jars containing seawater preheated to one of the five test temperatures (36, 37, 38, 39 and 40°C) in a Sub Aqua Pro water bath (Grant Instruments, UK). The RB ecotype was tested at all five temperatures (36°C, n = 10; 37°C, n = 12; 38°C, n = 14; 39°C, n = 15; 40°C, n = 15) while the SU was only tested at the four lower temperatures (36°C, n = 13; 37°C, n = 13; 38°C, n = 10; 39°C, n = 13) as exposure at 40°C resulted in heat coma after a period of less than 1 min. Jars were left unsealed ensuring thermal tolerance was assessed under normoxia (> 95 % a.s.). Oxygen levels were measured using a calibrated oxygen sensor (Microx-4, PreSens, Germany) during preliminary trials.

At the start of each trial, snails were added to the preheated jars, and the foot tissue was gently prodded at regular intervals throughout the trial to determine heat coma as per Sandison (1967). At the point when no response to this stimulus was registered within a few seconds, snails were regarded as having reached heat coma, and time elapsed was recorded. The addition of thread to the operculum was necessary because snails sometimes retracted fully into the shell as a response to prodding, preventing subsequent determination of heat coma in these individuals. In these cases, the thread attached to the operculum was gently pulled, and this was sufficient to re-extrude the foot tissue immediately after prodding. Immediately upon reaching heat coma, snails were removed to 200 mL jars containing sea water and held at 15°C for a minimum of 24 h. Snails which did not display full recovery after this time period (manifested by lack of an ability of the foot to attach to the jar sides) were excluded from the analysis. Following recovery assessment, snails were frozen at -20°C. Later, shells were measured (columella height and shell width across the widest part of the shell, perpendicular to the columella) using Vernier calipers, and cracked open to dissect the tissue, which was rinsed, blotted dry and weighed (± 0.01 mg) using a Cubis Semi-Micro Balance (Sartorius, Germany).

Statistical analysis

Statistical analysis was conducted using R version 3.5.3 (R Core Team 2019). The use of different endpoints in adults and embryos prevented direct comparison, thus data from adults was analyzed separately from data for mid and late veliger stage embryos. Differences in survival times between the four different embryonic treatment combinations (mid *vs.* late veliger, RB *vs*. SU) were analyzed independently at each of the five assessed temperatures (37, 38, 39, 40, and 41°C) using two-way ANOVA (with ecotype and developmental stage as factors). Data were log-transformed prior to analysis to meet assumption of homogeneity of variance. Similarly, differences at log-transformed heat coma times in adults from the two ecotypes were tested independently at each of the four temperatures at which both ecotypes were assessed (36, 37, 38, and 39°C) using one-way ANOVA with ecotype as the single factor.

To generate TDT curves, we regressed log-transformed endpoint times against treatment temperature (Rezende et al. 2014). This generated slopes, intercepts, and r-squared values, with CT_{max} generated using the equation CT_{max} = - intercept/slope and z generated using z = 1/slope. To statistically compare between regression slopes, ANCOVA with type 3 sum of squares was used. Differences between embryos were assessed in a two-way ANCOVA including both ecotype and developmental stage (mid- vs late-veliger) as factors, and temperature as a covariate. Adults were assessed using a one-way ANCOVA with ecotype as the single factor and the temperature covariate.

To determine whether there was a significant effect of size of individuals on endpoint times, we conducted MANCOVA tests for each ecotype-stage combination, regressing survival times (in embryos) or heat coma times (in adults) against both treatment temperature and size measurements (shell height in embryos, wet weight and shell height in adults).

Results

Embryos

Comparison of survival times at each individual temperature (Fig. 2A; Fig. 3A-D) revealed significant differences between ecotype: developmental stage combinations at three of the temperatures tested (37, 38 and 41°C), with significantly higher survival times in the RB late veligers relative to other groups at the two lowest temperatures (38 and 37°C). At 38°C, a significant effect of both stage and ecotype was observed (Stage- $F_{1/70} = 20.867$, $p < 0.001$; Ecotype- $F_{1/70} = 17.647$, $p < 0.001$), with RB late veligers displaying survival times 2-3 times longer than the other three treatment groups ($p < 0.001$). Similarly, at 37°C, a significant effect of both stage ($F_{1/73} = 53.801$, $p < 0.001$) and ecotype ($F_{1/73} = 6.788$, $p = 0.011$) was observed, with survival times of RB late veligers being significantly longer than both RB and SU mid veligers (both $p < 0.05$), but not SU late veligers ($p = 0.159$). At 41°C, a significant effect of stage ($F_{1/49} = 6.153$, $p = 0.017$), but not ecotype ($F_{1/49} = 1.907$, $p = 0.174$), was observed, with no significant post-hoc Tukey comparisons (all $p > 0.05$).

Regression lines of semi-log transformed TDT curves generated from survival times demonstrated high goodness-of-fit for all treatments (Fig. 2B). Furthermore, r-squared values were improved through using only mean survival times (all r-squared > 0.96), which is an appropriate method for reducing residual variation by assuming 50 % mortality at each temperature (Rezende et al. 2014; Truebano et al. 2018). Linear regression (ANCOVA) analysis of these TDT curves indicated a significant difference in survival between embryonic stages, irrespective of ecotype (significant interaction between temperature and developmental stage; $F_{1/309} = 4.823$, $p = 0.029$), but no significant effect of ecotype ($F_{1/309} = 0.902$, $p = 0.343$; Fig. 2B). CT_{max} values derived from TDT curves were similar across all ecotype-developmental stage combinations, ranging between 42.00 - 42.25°C, while only slight differences in z-value were apparent between developmental stages (Table 1). It is notable that differences in survival times that were seen at 37- 38°C when analyzed separately at each temperature were not reflected by differences in CT_{max} and z-values in the TDT approach, likely due

to being masked by its logarithmic nature. This is evident in a visual comparison of Fig. 2A (raw survival times) with Fig. 2B (log-transformed survival times).

Adults

In contrast to embryos, comparisons at each temperature in adults revealed consistently higher thermal tolerance in the RB compared to the SU ecotypes across all temperatures ($p < 0.01$; Fig. 2C; Fig. 3E-F). Unlike in embryos, these differences were most pronounced at the highest temperatures. For instance, at 39°C, almost a fourfold difference in survival time was observed between RB and SU adults ($F_{1,26} = 77.5$, $p < 0.001$). At 36°C, the lowest temperature measured, differences in survival times were less pronounced but still significant ($F_{1, 21} = 19.5$, $p < 0.001$). Mirroring this, analysis of TDT curves (Fig. 2D) indicated that RB snails had substantially higher z and CT_{max} values than SU snails (Table 1). This is indicative of greater acute thermal tolerance at the highest temperature extremes in the RB ecotype, indicated by a significant interaction between ecotype and temperature ($F_{1, 111} = 40.484$, $p < 0.010$) in ANCOVA. This is apparent when heat coma times are plotted logarithmically (Fig. 2D) as opposed to as raw values (Fig. 2C). It is important to note that the lower CT_{max} values we observed in adults compared to embryos likely reflect the lower, sublethal endpoint threshold used and thus do not equate to reduced thermal tolerance in absolute terms in the adults.

Controlling for size differences between ecotypes

Shell height ($t = 46.631$, $p < 0.001$) and wet weight ($t = 21.784$, $p < 0.001$) differed between adults of the two ecotypes. RB snails were larger, with mean shell length of 9.86 ± 0.96mm, whereas SU adults had a mean shell length of 3.44 ± 0.47mm. We found no significant effect of either shell height or wet weight ($p > 0.05$) on time taken to enter heat coma within adults of either ecotype, suggesting size differences are not responsible for the higher thermal tolerance observed in RB compared to SU snails. In embryos, there was no significant difference in shell length between late veligers of the two ecotypes, while RB mid veligers were significantly larger than SU mid veligers

(0.401 ± 0.038 mm *vs*. 0.380 ± 0.045 mm, respectively; $t = 3.278$ $p < 0.001$). As with adults, there was no significant relationship in embryos between shell height and survival times within each treatment group ($p > 0.05$).

Discussion

In this study we compared the thermal sensitivities across different life stages of two ecotypes of *L. saxatilis* from Galician populations (NW Spain) which are associated with different height levels of the intertidal zone and therefore exposed to substantially different thermal environments. We found that adults of the upper shore RB ecotype, which experience higher *in situ* habitat temperatures, had greater tolerance times across the entire range of temperatures tested. This difference was most pronounced at the highest temperatures and corresponded with a shift in CT_{max} and z-values between the two ecotypes. By contrast, differences in responses between embryos were more subtle than those in adults, with increased survival times in the RB being only apparent at the lowest temperatures measured and not associated with a shift in CT_{max} and z-values. Our results support the prediction that the two ecotypes, adapted to contrasting selective forces, also differ in their thermal tolerance in line with the environmental conditions of their microhabitats (Rolán-Alvarez et al. 1997; Rolán-Alvarez 2007). We propose that the increased vulnerability of the SU ecotype to thermal stress may inhibit their small-scale dispersal into the RB habitat on the upper shore. Such limited dispersal would act a partial barrier to gene flow between the two ecotypes, reinforcing selection for other adaptive traits and contributing to the maintenance of two distinct ecotypes and the evolution of greater divergence and reproductive barriers in the Galician population.

Our employment of the TDT approach (Rezende et al. 2014) provided us with the ability to distinguish between the effects of intensity and duration of thermal stress, which is significant given the substantial variation in intensity and duration of heat exposure corresponding with shore height position (Newell 1979; McMahon 1990; Helmuth and Hofmann 2001; Tomanek and Helmuth 2002). Recent latitudinal comparisons (Rezende et al. 2014; Castañeda et al. 2015) have indicated a trade-off

between CT_{max} and z across latitude (Jørgensen et al. 2019), with populations from colder, higher latitudes possessing greater acute tolerance at the highest temperatures but increased sensitivity at less extreme temperatures. Our prediction that the RB ecotype, being exposed to higher environmental temperatures, would accordingly possess greater tolerance of prolonged exposure than the SU, but reduced acute tolerance, was not met. Instead, the RB ecotype had higher CT_{max} and z values than the SU, indicative of increased acute tolerance at the very highest temperatures without a reduction in chronic tolerance at lower temperatures, as they displayed greater thermal tolerance across the entire temperature range analyzed (36-39°C). It should be noted that if the TDT curves for the two ecotypes were extrapolated to lower temperatures, then the curves would intersect at a temperature of 35.23°C (at a survival time of 19 h 38 min) below which tolerance times in the SU ecotype would theoretically become greater than those in the RB ecotype. While this could indicate the presence of a trade-off, we do not consider differences at this end of the TDT curve to be of ecological consequence, because such long exposure times required at these temperatures would never be reached in the intertidal zone due to tidal and day/night cycles. Thus, the positive shift in both z and CT_{max} observed in the RB relative to the SU ecotypes is apparently not associated with detrimental consequences for longer term survival, as is seen in other study systems (Rezende et al. 2014; Castañeda et al. 2015).

The proximate physiological basis for greater acute tolerance in the RB is unknown and beyond the scope of this study, and could be due to a multifarious interaction of traits affecting different parts of the TDT curve (e.g., Figure S2 in Rezende et al. 2014). Metabolic demands of tissues may differ substantially between the two ecotypes, especially due to the foot muscle of the SU ecotype being 1.4 times larger than that of the RB (Martínez-Fernández et al. 2008). Oxygen limitation plays an important role in determining thermal performance and tolerance limits in many marine ectotherms, as the capacity of circulatory systems to meet increasing oxygen demands beyond a certain temperature threshold becomes limited, leading to a reduction in aerobic scope (Pörtner 2001, 2010; Pörtner et al. 2017). The high test temperatures used in our study, which exceed threshold temperature for the onset of anaerobiosis in water for this species (Sokolova and Pörtner 2003), may suggest that the responses we observed were driven chiefly by passive tolerance mechanisms

activated above the critical limits for aerobic scope, beyond which survival is time-limited (Rezende et al. 2014; Pörtner et al. 2017). Anaerobic metabolic pathways play a crucial role in the passive thermal tolerance of many intertidal species (Burggren and McMahon 1981; Sokolova and Pörtner 2003; Monaco et al. 2017; Pörtner et al. 2017), and it is possible that anaerobic energy production via the succinate pathway is enhanced in the RB similarly to other upper-shore *L. saxatilis* populations (Sokolova and Pörtner 2001). However, there is also evidence of upregulation of proteins associated with rapid anaerobic production of ATP in the SU, potentially linked to increased muscle activity required to resist high wave action on the lower shore (Martínez-Fernández et al. 2008). Greater acute tolerance in the RB adults could also be linked to a more rapid ability to mobilize heat shock proteins in response to acute thermal stress relative to the SU (Rezende et al. 2014), as has been observed in upper *versus* lower shore congeners of the genus *Tegula* (Tomanek and Somero 2000). The underlying mechanisms for the observed differences in thermal tolerance between the ecotypes warrant further investigation.

Thermal sensitivity through development might be predicted to reflect environmental conditions experienced by each ontogenetic stage (Lockwood et al. 2018; Truebano et al. 2018). A previous study on the mid-shore flat periwinkle *Littorina obtusata* demonstrated that z-values were lower in embryos compared to adult snails (Truebano et al. 2018), a difference that was attributed to the fact that *L. obtusata* embryos develop within egg masses fixed to the substrate, thus experiencing a substantially different thermal environment to adults, which are mobile and so able to escape thermal stress. Accordingly, we predicted that differences in thermal sensitivity between embryos of the two ecotypes of the brooding *L. saxatalis* would correspond closely to those observed in the adults. However, differences in CT_{max} and z values between adults of the two ecotypes were not reflected in the embryos. There are several potential explanations for this apparent mismatch. Firstly, this may be an artefact of our use of a sub-lethal endpoint in adults *vs.* a lethal endpoint in embryos, given that different levels of biological organization may be independently affected by thermal stress (Rezende et al. 2014; Rezende and Bozinovic 2019; Bozinovic et al. 2020). Secondly, limitations to thermal tolerance may be imposed by the need to resist other stressors associated with living in the

intertidal, which are only experienced by adults. As noted, adults of the SU ecotype must cope with the physiological demands of increased wave exposure on the lower shore (Rolán-Alvarez et al. 1997); demands that are not experienced by their embryos. Also, we cannot exclude the possibility that phenotypic plasticity in the form of irreversible thermal acclimatization (Garland and Adolph 1991; Bourdeau et al. 2015) may have contributed to differences between adults, particularly as adults may have experienced exposure to extreme temperatures during summers prior to collection. However, between-ecotype differences in this species have been found to be largely invariant of environmental effects (Conde-Padín et al. 2007; Hollander and Butlin 2010; Galindo et al. 2019), including, notably, in proteome expression (Martínez-Fernández et al. 2010). In addition, the fact we observed an increase in sensitivity differences through development (i.e., between mid and late veliger stages) further suggests that the differences we observed between ecotypes are likely to have a substantial genetic (or at least heritable) component.

It should be noted that, while the differences we identified between ecotypes and developmental stages appear subtle, even comparatively small differences in the shape of TDT curves may have substantial consequences for the survival of populations, especially when cumulative mortality over multiple successive days of thermal stress is considered (Rezende et al. 2014, 2020). For example, while mean survival times in embryos apparently exceeded maximal upper-shore habitat temperatures recorded by our temperature loggers, the high levels of variation we observed in survival times of embryos across all temperatures suggests that partial brood mortality is already likely to occur in the field during the summer, especially in the RB ecotype. This is in spite of our temperature data from the upper shore having been recorded from a shaded location, suggesting that the apparent behavioral preference of the RB ecotype for shaded refugia at low tide (as noted in our methodology) may not be completely sufficient to avoid embryo mortality. A potential strategy to further mitigate against this would be for females to breed preferentially at other times of year. However, *L. saxatilis* populations typically brood continuously year-round (Reid 1996), and while there is some evidence from Galician populations that numbers of recently hatched juveniles are highest in the spring and autumn (Carballo et al. 2005), this could equally be explained by seasonal changes in embryo

462 viability, or juvenile survival, rather than female fecundity. Substantially larger broods are produced
463 by RB females compared to SU females (Saura et al. 2011), which, as well as mitigating against
464 attrition from predation, could potentially serve to offset against partial losses of broods due to
465 thermal stress in the summer.

466 We note that there are risks associated with directly inferring physiological adaptation from
467 two-population/species comparisons, including a lack of statistical replication and the potential for
468 confounding phylogenetic factors (Garland and Adolph 1994). However, the thermal tolerance
469 differences we observe in this study fall within a wider context of adaptive morphological and
470 physiological differentiation across shore height between closely related ecotypes (Rolán-Alvarez et
471 al. 1997; Rolán-Alvarez 2007) and align closely with patterns of thermal tolerance across vertical
472 shore gradients seen in many taxa (Newell 1979; Burggren and McMahon 1981; Sanders et al. 1991;
473 Tomanek and Somero 1999; Stillman and Somero 2000) including in other populations of *L. saxatilis*
474 where divergent ecotypes are not present across shore height (Sokolova et al. 2000; Sokolova and
475 Pörtner 2001). Regardless of whether the thermal tolerance differences we describe are a direct
476 adaptive response, or partially environmentally mediated, the presence of such differences may be
477 significant in maintaining the vertical segregation of the two ecotypes and reinforcing selection for
478 other traits. In particular, the increased susceptibility of the SU ecotype to thermal stress identified in
479 our study may prevent their dispersal onto the upper shore (e.g., immigrant inviability; Nosil et al.
480 2005), thereby acting as an additional prezygotic isolation barrier and maintaining a selection gradient
481 for habitat-specific traits (Keller and Seehausen 2012; Nosil 2012). It is also possible that thermal
482 stress works in tandem with the increased susceptibility of the SU to crab predation (Boulding et al.
483 2017), as SU adults weakened by the effects of heat exposure may become more vulnerable to
484 predators through their inability to escape (Lutterschmidt and Hutchison 1997). The combination of
485 both thermal physiological stress and the predation / wave exposure gradient may therefore lead to
486 further enhancement of reproductive barriers (via multifarious selection; Nosil et al. 2009; Nosil
487 2012) in the Galician RB and SU, potentially contributing to the greater genetic divergence between

488 the Galician ecotypes in comparison to other Wave and Crab ecotype pairs from localities where
489 thermal stress is likely to be less significant (such as the UK; Butlin et al. 2014).

490 In conclusion, our study has demonstrated, for the first time, differences in thermal tolerance
491 between the RB and SU ecotypes of *L. saxatilis* from Galician (NW Spain) populations, indicating
492 that they may act as an important and hitherto overlooked factor in maintaining ecotype segregation
493 and promoting the evolution of barriers to gene flow. Further research into the proximate causes of
494 these differences may invite their use as a model for how interspecific differences in physiological
495 traits, such as thermal tolerance, evolve along fine-scale environmental gradients. However, divergent
496 responses in embryos and adults of the two ecotypes also suggest that thermal stress may have
497 differing fitness and selection consequences across life-history stages, and highlight that further work
498 is needed to explore to what extent these traits are genetic *versus* environmentally mediated in adults.
499 Our research further highlights the importance of including multiple life-history stages in assessments
500 of thermal tolerance and susceptibility to future climate change.

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Table legends

Table 1: Z-values and CTmax values for different ecotype: stage combinations, generated by regressing log-transformed endpoint times against treatment temperature (see methods). Estimates for embryos were derived from survival times, while estimates for adults were derived from heat coma time, a sublethal marker of physiological compromise.

720

721 **Figure legends**

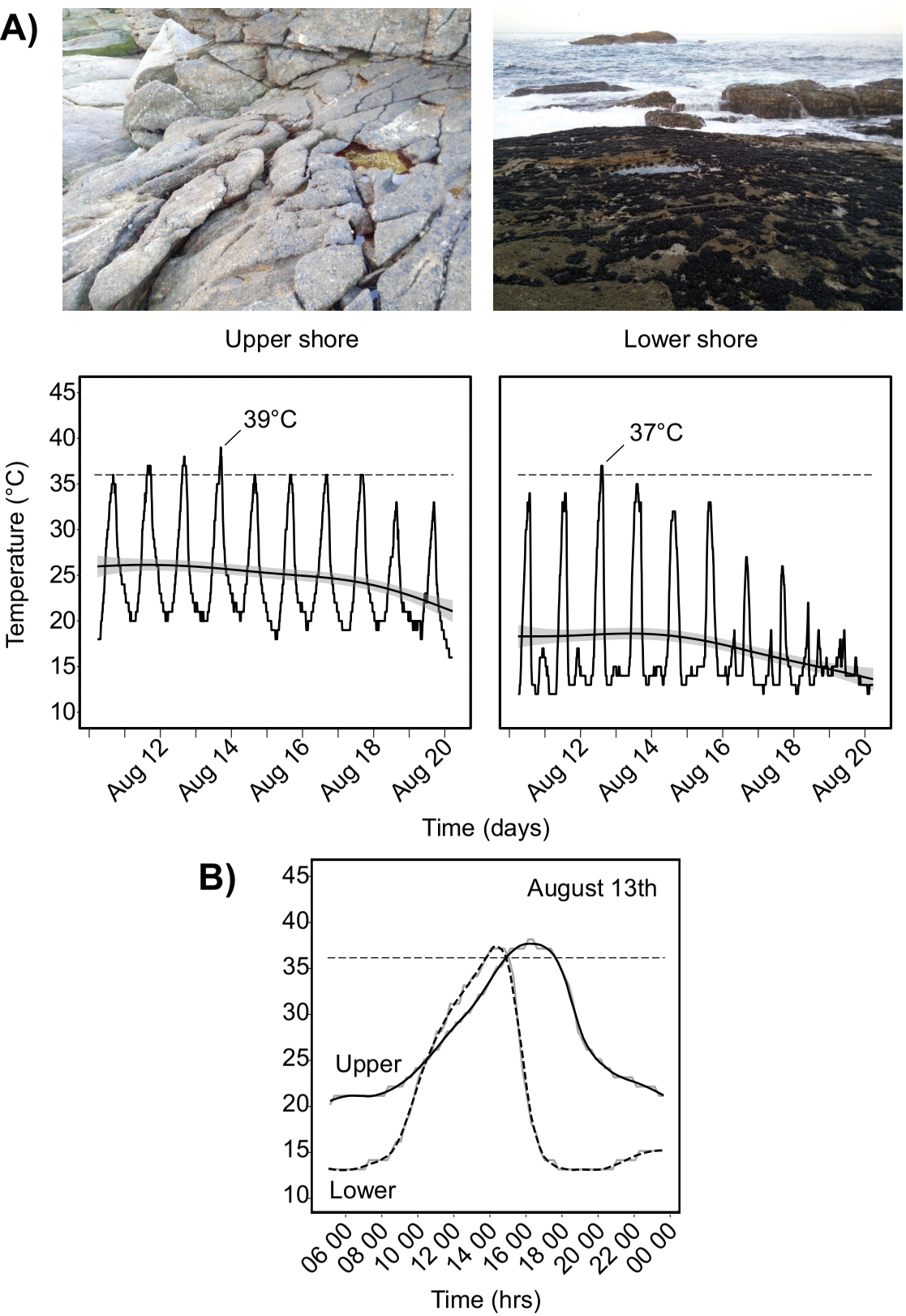
722 Figure 1: Field temperature data and life-history stages. (A) Examples of typical habitats on the upper
723 and lower shores, Silleiro Cape, Galicia (NW Spain), accompanied by habitat temperatures recorded
724 using robolimpet data loggers over a ten-day period from 11- 20 August 2009. Maximum
725 temperatures experienced during the recorded period are annotated, while solid shaded lines represent
726 average temperatures. Upper and lower shore habitats respectively correspond to the microhabitat
727 temperature regimes experienced by the RB and SU ecotypes of *Littorina saxatilis* (see methods).
728 Dashed line represents 36°C, the lowest assay temperature used in our study. Temperatures were
729 recorded at 15-minute increments to the nearest °C. (B) Temperatures over the course of a single day,
730 representing the second warmest and warmest days during the ten-day period, respectively, for the
731 upper and lower shore. (C) Individuals of each *Littorina saxatilis* ecotype – developmental stage
732 combination used in this study. Separate scale bars for adults and embryonic stages are depicted.
733 Images of embryos are stills derived from experimental video data captured early in static thermal
734 tolerance trials before lethal endpoints were reached.

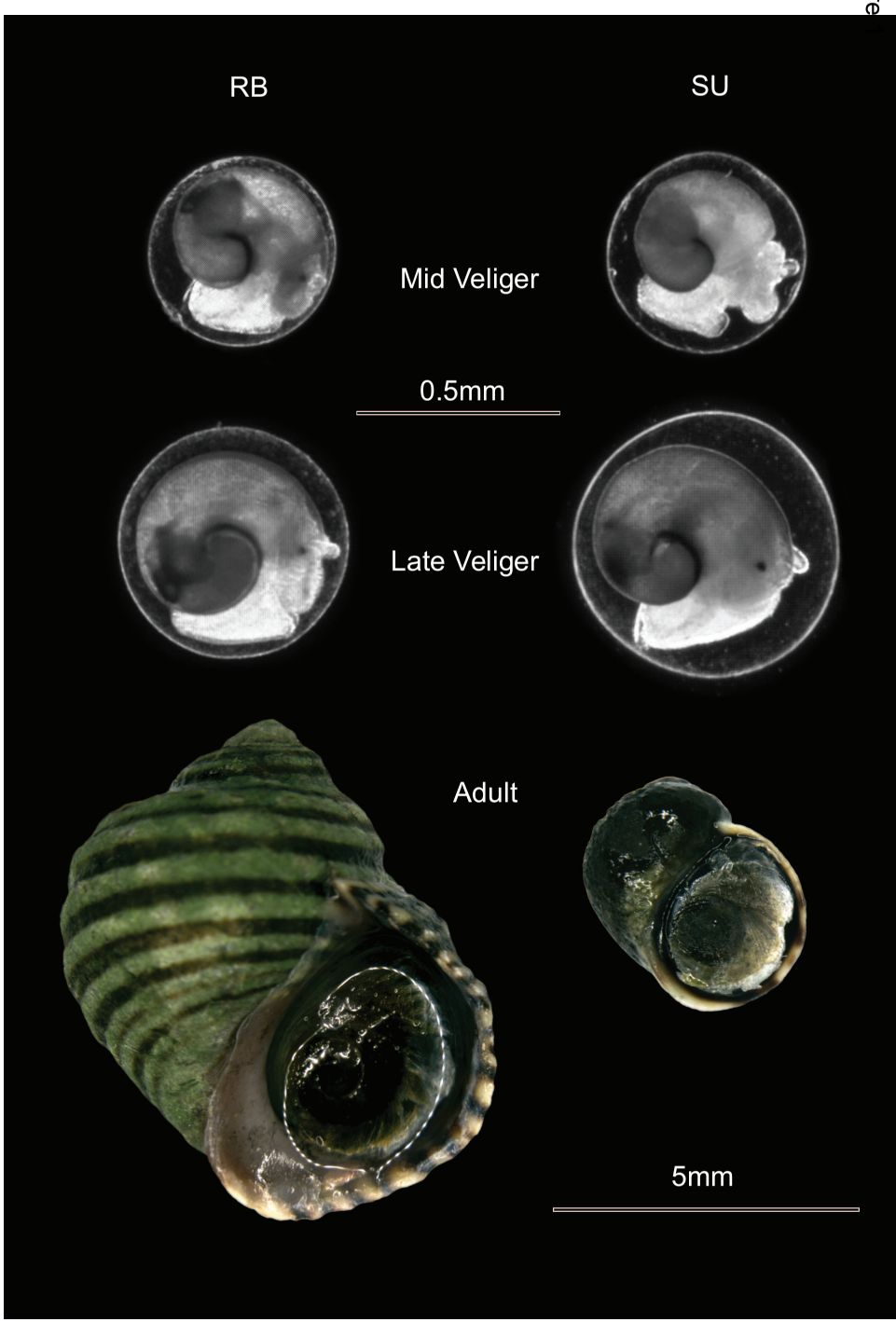
735 Figure 2: Thermal Death Time curves. (A-B) TDT curves for mid and late veliger stage embryos of
736 two Galician *Littorina saxatilis* ecotypes (RB and SU) displaying survival times across five
737 temperatures (37-41°C). (C-D) TDT curves for adults of the two ecotypes, displaying heat coma times
738 across four (SU; 36-39°C) or five temperatures (RB; 36-40°C). Large and small datapoints represent
739 mean values for each temperature, and individual endpoint times, respectively. Left-hand panels (A,
740 C) depict raw data values, with asterisks indicating significant differences between treatment groups
741 at the respective temperature; for details see Results. Right hand panels (B, D) depict the same data
742 log-transformed, illustrating the semilogarithmic relationship between temperature tolerance and
743 exposure time associated with the TDT method. R-squared values for regression lines in (B, D) were
744 as follows: RB Mid Veliger = 0.86; SU Mid Veliger = 0.87; RB Late Veliger = 0.91; SU Late Veliger
745 = 0.90; RB Adult = 0.96; SU Adult = 0.96.

Figure 3: Survival plots. (A-D) probability of survival for embryos under increasing exposure time at 5 temperatures for (A) RB mid veligers, (B) SU mid veligers (C) RB late veligers and (D) SU late veligers. (E-F) probability of remaining uncompromised (not in heat coma) for adults under increasing exposure time at 5 temperatures and 4 temperatures respectively in (E) RB and (F) SU ecotypes. Note that to aid clarity of presentation, several datapoints representing survival times longer than 1000 minutes have been omitted from the survival plots for mid and late veligers; these datapoints are depicted in Fig. 2A.

Table 1

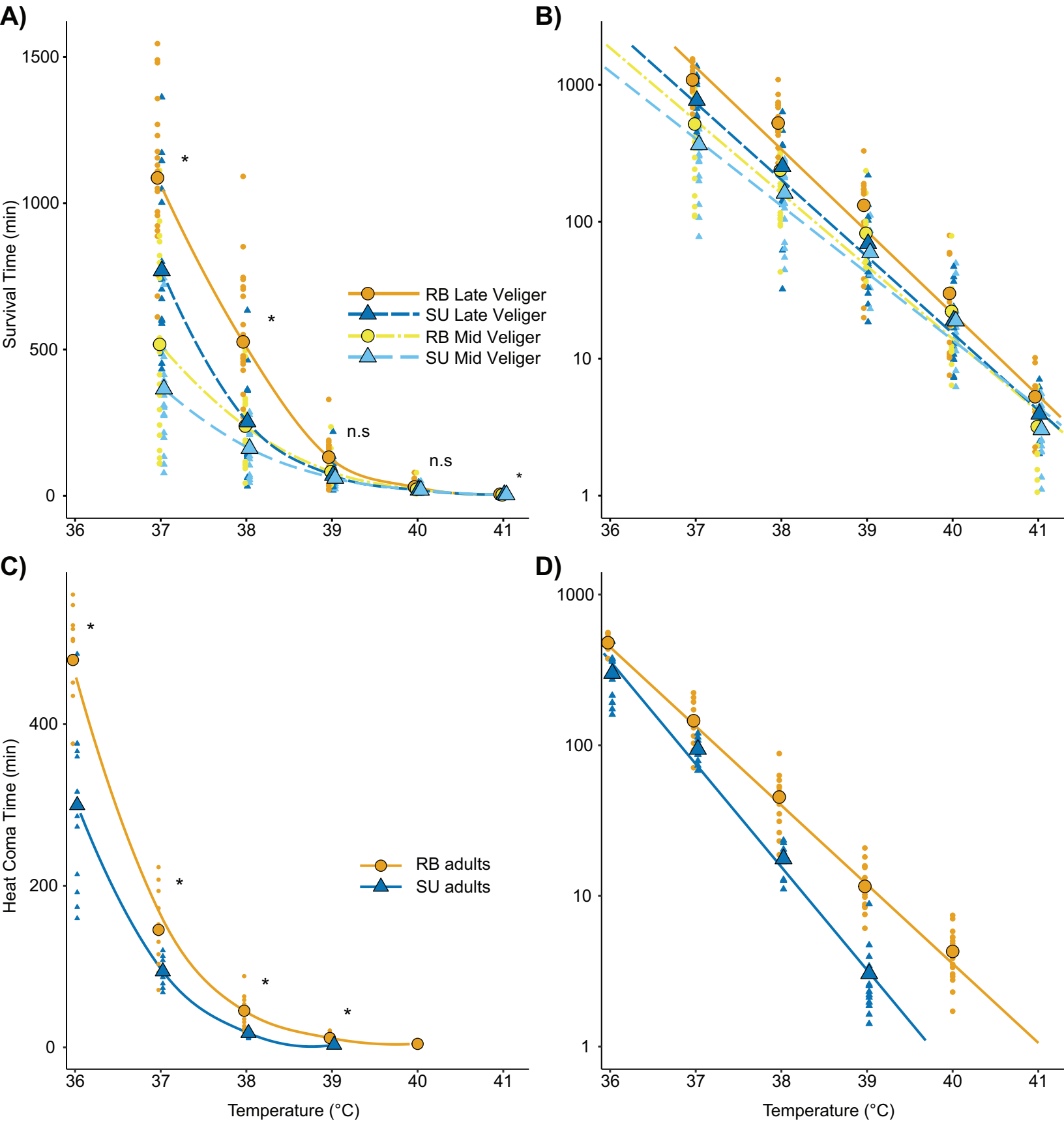
Ecotype: Stage combination	z-value (°C)	CT_{max} (°C)
By endpoint:		
Survival:		
RB Mid Veliger	1.89	42.20
SU Mid Veliger	1.98	42.16
RB Late Veliger	1.67	42.25
SU Late Veliger	1.74	42.00
Heat Coma:		
RB Adult	1.90	41.07
SU Adult	1.46	39.72

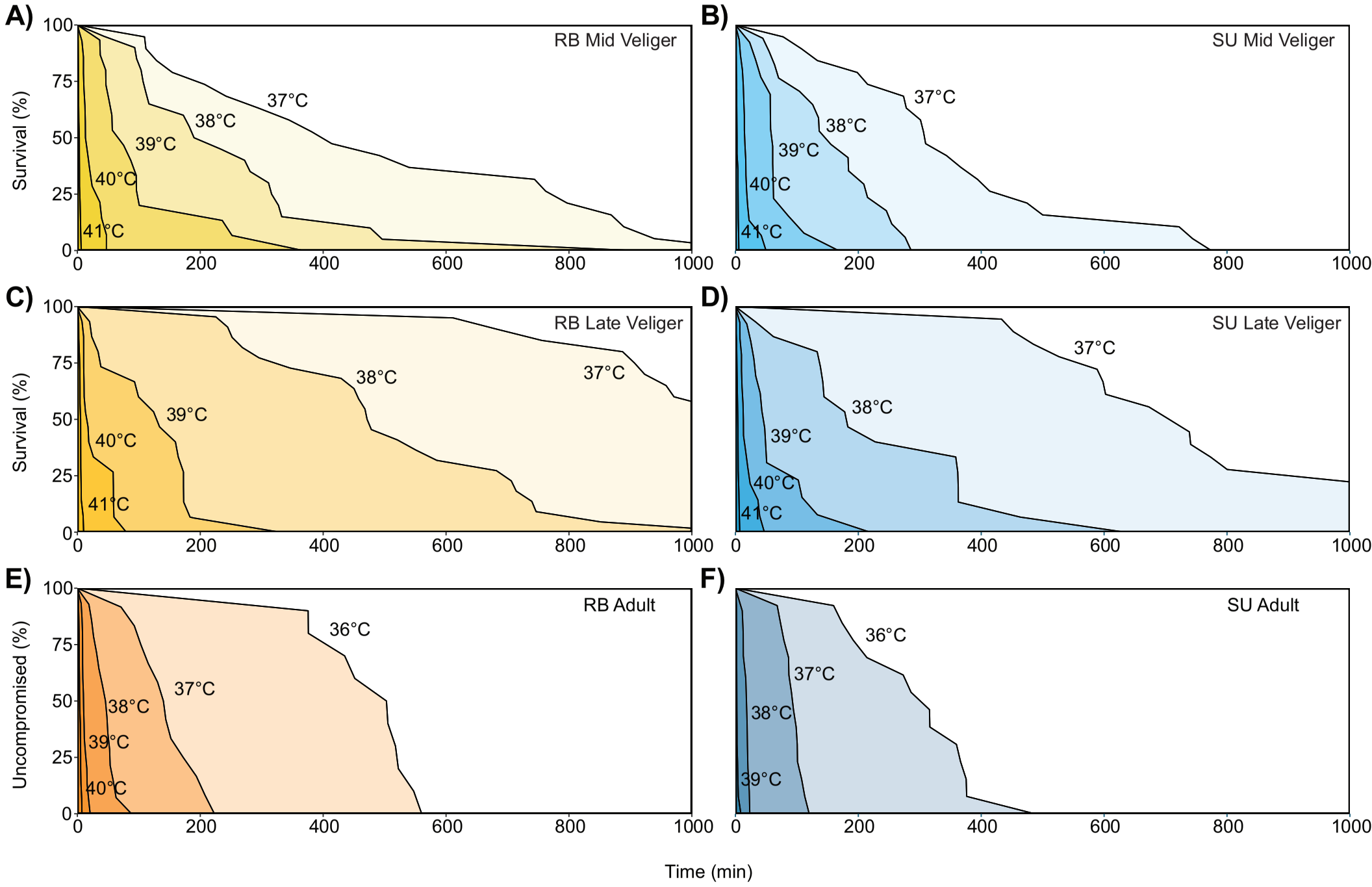

A)
Upper shore
Lower shore
Temperature (°C)
45
40
35
30
25
20
15
10
39°C
37°C
Aug 12
Aug 14
Aug 16
Aug 18
Aug 20
Time (days)
B)
August 13th
Upper
Lower
06 00
08 00
10 00
12 00
14 00
16 00
18 00
20 00
22 00
00 00
Time (hrs)


C)
RB
SU
Mid Veliger
0.5mm
Late Veliger
Adult
5mm

Figure

Figure 2


A)
B)
C)
D)
Survival Time (min)
Heat Coma Time (min)
Temperature (°C)
RB Late Veliger
SU Late Veliger
RB Mid Veliger
SU Mid Veliger
RB adults
SU adults
n.s
n.s
*


A)
RB Mid Veliger
37°C
38°C
39°C
40°C
41°C
B)
SU Mid Veliger
37°C
38°C
39°C
40°C
41°C
C)
RB Late Veliger
37°C
38°C
39°C
40°C
41°C
D)
SU Late Veliger
37°C
38°C
39°C
40°C
41°C
E)
RB Adult
36°C
37°C
38°C
39°C
40°C
F)
SU Adult
36°C
37°C
38°C
39°C
Survival (%)
Uncompromised (%)
0
25
50
75
100
0
200
400
600
800
1000
Time (min)

Reviewer 1 Comments

The paper entitled "Divergence in thermal physiology could contribute to vertical segregation in intertidal ecotypes of Littorina saxatilis" describes the upper thermal limits in the Galician ecotypes of the common rough periwinkle. The upper limits were established via laboratory experiments with adults and larvae stages, and expectedly proved to be higher in the upper shore ecotype.

Though this result was expected (since the upper shore ecotype experiences a heavier thermal stress in the wild) it is important for our understanding of mechanisms underlying divergence of ecotypes in natural populations. Unfortunately, the study does not go beyond analysis of survival under thermal stress and does not concern biochemical mechanisms of how snails of different ecotypes cope with this stress.

REPLY:

We are pleased that the reviewer agrees that our study is important for understanding the mechanisms underlying divergence between the two ecotypes. The reviewer is correct to point out that the aim of our study was to characterize physiological differences between the two ecotypes across multiple life-history stages, using a powerful modelling approach, and that the underlying biochemical mechanisms were beyond the scope of this study. Below, we respond to each comment and make the necessary clarifications herein and within the text.

Two other main flaws are as follows.

(1) The authors test the hypothesis of the trade-off between resistance to acute and chronic thermal stress. When formulating (and discussing) the hypothesis, they make a reference to papers on terrestrial insects (Drosophila, mainly). For some reason, they do not mention and do not discuss that mechanisms underlying thermal limits in marine vs terrestrial ectotherms differ tremendously. In the former group of organisms, these are related to ability to cope with hypoxia, since thermal stress in marine ectotherms is inevitably accompanied with hypoxia and their thermal tolerance limits depend on ability to withstand under hypoxia conditions, see e.g.:

REPLY:

The reviewer raises an important point that thermal tolerance and performance in marine organisms is linked to oxygen limitation. However, the mechanistic basis for the observed differences between the two ecotypes are beyond the scope of our study. When introducing the TDT approach, we focused on early experimental work presenting the method which involved the use of insects. However, the relationship between duration vs. intensity of thermal stress exposure has since been

widely demonstrated in aquatic species as well as terrestrial species. We have clarified this and cited additional references in **lines 134-142**. We have also clarified that the relationship between acute and chronic tolerance is likely to be the result of multiple interacting mechanisms, potentially including oxygen limitation, which all contribute to TDT curves observed at the organismal level (**lines 397-399, also lines 33-34 of abstract**). We also note that *L. saxatilis* have a high anaerobic capacity and are likely to be above the threshold temperatures for anaerobiosis when at the test temperatures used in this study (Sokolova and Pörtner 2003). Therefore, it is likely that in our study, oxygen limitation has already occurred at lower temperatures. Anaerobic capacity is likely to be important in determining tolerance at extreme temperatures such as those used in this study, where tolerance is time-limited. We have extensively incorporated these points into the discussion in **lines 397-420** and addressed the general importance of these mechanisms for intertidal organisms in **lines 49-54** of our Introduction.

(2) The authors do not describe the shell-closing behavior in experimental animals during heat exposure: did the snails withdraw into their shells under heating? How fast? Was this observed in both ecotypes under all tested temperatures for all stages studied? As mentioned, hypoxia is an important player in thermal tolerance limits and closing behavior additionally restricts breathing; moreover, such restrictions are expected to be different in veligers (with soft thin shells) vs adults. This behavior (isolation) should be paid attention to in all the parts of the paper: methods, results and discussion.

REPLY:

The shell-closing behavior reported in adults was an instantaneous response to the prodding methodology used to determine whether the snails entered heat coma and was not a response to high temperatures. In fact, upon immersion in hot water, the foot muscle relaxes causing the operculum to open. The operculum only closes as a temporary response to prodding as the foot muscle retracts. In most cases (especially in individuals close to reaching heat coma), the foot muscle relaxes again within seconds and the shell reopens. In cases where individuals retracted fully into their shells, the thread attached to the operculum was gently pulled, and this was sufficient to re-extrude the foot tissue immediately after prodding. A gentle pull of the thread was sufficient to extrude the foot. These behaviors occurred across all temperatures and in both ecotypes. Retraction into the shell was similarly not observed in veliger embryos at any of the temperatures analyzed. We therefore do not consider restriction of breathing as a result of retraction a factor of concern.

This point raised by the reviewer clearly reflects a lack of clarity in our original manuscript, and we have therefore revised our description of the protocol accordingly in **lines 251-271**.

Minor things.

Lines 193-196: What were O2\CO2 concentrations? Atmospheric? Can such conditions be

hyperoxic for larvae? I suspect that O2 concentration within the brood pouch is lower than in air.

REPLY:

The reviewer raises a really interesting point regarding the oxygen concentrations in the brood pouch, which we considered extensively throughout our experimental design. We agree with the reviewer that O2 concentrations may be lower in the brood pouch than in experimental conditions in the study. We have observed that embryos are typically packed densely within the brood pouches of both ectoypes, although the numbers and stages of embryos can vary considerably between individuals and therefore it is likely that so to would O2 concentrations. However, as yet we have not been able to measure O2 concentrations in the pouch due to methodological difficulties. As such, we decided to measure all embryos to the same conditions to allow for comparisons. Since we exposed embryos from the two ecotypes to the same conditions during our study, any confounding effects of hyperoxia (it is unclear whether these would be beneficial or negative) on thermal tolerance would have been consistent across treatments. *L. saxatilis* embryos can be reared successfully *in vitro* in seawater, and therefore exposure to normoxic seawater allows for successful development and long term survival. We have addressed this in **lines 210-216**.

Lines 227-228: How sex of snails was determined without dissection?

and

Line 320: Was the age taken into account in any way? (is age possible to be determined, e.g., based: on shell?)

REPLY:

The adult snails used in the study were sexed without dissection under light microscopy and males positively identified by the presence of a penis behind the right eye. Age was not factored into our study; however, our use of only reproductively mature males precluded the use of immature specimens. We have clarified this in **Lines 247-251.**

Lines 385-386: why aerobic demand is stressed? There were differences in abundances of arginine-kinase and bisphosphate-aldolase between ecotypes revealed and both enzymes are not participants of aerobic metabolism.

REPLY:

We thank the reviewer for their comment and have removed this reference from our discussion. We suspect that aerobic demand in the SU ecotype may indeed be higher, due to unpublished observations from our group of higher mitochondria density in the foot tissue of the SU compared to the RB. However, this conclusion is not supported in the currently published literature. We have however retained the more general statement that differences in foot size may be linked to differences in metabolic demand between the two ecotypes, potentially contributing to the observed differences in thermal tolerance, in **Lines 399-401**.

Reviewer 2 Comments

This is a carefully constructed set of experiments about field-relevant conditions testing a hypothesis that thermal tolerance helps segregation in two co-occurring ecotypes of one species. The methods and hypotheses are clear. It is impressive that multiple stages of development and adults were tested for comparisons. The figures and statistics are appropriate.

REPLY:

We are pleased to see that the reviewer finds our study to be carefully constructed and clearly written, and that they are appreciative of the fact we incorporated multiple developmental stages into our comparison. We agree that this is a strength of our approach.

The difference in adult tolerance, though significant, is very small. The difference at the larval stages is even smaller. The authors admit that the adults may be responding to the environment they experience, with plasticity rather than genetic differences (lines 388-390). Because these adults were collected from the field and tested within a short time, their prior experiences are likely to have influenced their acclimation to temperature, even though they were collected in winter.

REPLY:

We recognize and agree that acclimation may partially explain the more pronounced differences between adults compared to embryos, and that a shortcoming of our study is that this cannot be distinguished from heritable genetic differences in the adults, a point addressed in our concluding remarks in the manuscript. However, although we suspect that there is a heritable genetic component to these differences (because differences are also observed between embryos), we contend that even if the differences were entirely due to plasticity, they would still potentially play a role in the vertical segregation of the ecotypes, for example due to immigrant inviability, which we have emphasized in **Line 476.** In addition, shell morphology is highly heritable in the two ecotypes (Galindo et al. 2019. J Exp Mar Bio Ecol 513:27–34) and it is possible that this could be linked to other phenotypic traits related to temperature tolerance.

I am curious to know if the robolimpet gathering the field temperatures in summer was in a crevice or near algal cover, as might be expected for live snails in the heat of the day. If not, then the temperatures recorded would not necessarily capture the actual microhabitats of the snail locations.

REPLY:

The robolimpet data were collected from locations which directly reflect where the snails are found at low tide on hot days: a protected site on the upper shore (shaded by rock crevicing) versus a sun-exposed rockface within mussel beds on the mid shore. The temperatures data therefore closely reflects temperatures experienced by the two ecotypes, as well as accounting for divergent behaviours (the upper shore RB seek shelter in crevices at low tide, while the mid shore SU do not). In fact, because the SU lack this refugia-seeking behavior at low tide (based upon personal

observations), it is possible that any individuals which attempted to colonize the upper shore would be exposed to even higher temperatures than those experienced by the RB. We have provided further clarification of the placement of sensors in **lines 166-172.** We have also elaborated on how the different behaviors of the two ecotypes relate to habitat temperatures in the discussion (**lines 454-457**).

In either case the temperatures measured were within the tolerance of both groups, and the graph of a single day shows extended exposure to a temperature that both could handle.

This leads me to the conclusion that the heat tolerance is probably not contributing much to the segregation of these two species, although perhaps it is contributing something. This might be a case of statistical significance but not much biological significance.

REPLY:
We agree that the observed differences between ecotypes appear small, however recent work has demonstrated that comparatively small changes in the shape of the TDT curve can have major effects upon survival under fluctuating field temperatures (discussed in Rezende et al. 2020, including supplementary information). We have extensively incorporated this into our discussion in **lines 447-454**, as we recognize that the potential biological significance of small differences in the TDT curve may not be immediately apparent to the reader. Work is currently ongoing to address this question further in the populations used in this study.

The lack of distinction in the larval results suggests that the adult females would probably avoid exposure to high temperatures when brooding.

REPLY:

The reviewer raises a very interesting point. Avoidance of high temperatures, via the seeking of shaded refugia, appears to be a general response across both males and females in the RB ecotype, as we have noted in **lines 165-169.** Because females brood year-round (see below) specific behavioural avoidance of high temperatures to protect embryos in the female (but not male) population would potentially inhibit feeding activity and is also inconsistent with the r-type reproductive strategy pursued by the RB ecotype, which maximizes reproductive output at the expense of limited maternal investment and high mortality. We hypothesize that larger brood sizes might actually be an adaptive response to cope with greatly increased embryo mortality in the upper shore due to thermal stress, and we have incorporated this in **lines 462-465** of the discussion. By contrast, our temperature data suggest that the SU ecotype (which pursues a k-type reproductive strategy) is less vulnerable to brood mortality, and we would therefore not anticipate, nor have we observed, increased refugia-seeking behavior in female SU.

Does this species brood over summer, or only in winter when heat stress is less likely?

REPLY:

Previous research has indicated that there is a seasonal pattern in the population density of shelled juvenile snails (which had already left the brood pouch), with densities being highest in the autumn at some localities and in the spring at others (Carballo et al. 2005). However, this result could equally be driven by seasonal changes in fecundity, changes in the viability of embryos, or in the survival rates of newly hatched juveniles. The latter two may, for instance, be adversely influenced by thermal stress in the summer and high levels of precipitation (leading to osmotic stress) in the winter. Additionally, *L. saxatilis* is largely unique (among congeners) in being reproductively active all through the year (Reid 1996). This, as well as our experience in the field, lead us to suspect that there are no substantial seasonal changes in fecundity in these populations. This is an interesting point which we have incorporated into our discussion in **lines 458-462**

Is this why the authors avoid using females in the study? Might one hypothesize that females could be less heat tolerant, or more heat avoidant behaviorally, as well?

REPLY:

We have provided further clarification on our reasons for using only male adults in **lines 247-250.** See also our reply to Reviewer 1's comment on how the snails were sexed. We see no *a priori* reason to assume that female adults would be less heat tolerant than males, although as noted, it is possible that high brood mortality occurs in the RB ecotype (**Lines 451-462**).

The photo does not show the upper shell of the two types of Littorina. Are they similar in color and would insolation affect each one similarly?

REPLY:

The question of how differences in coloration between the two ecotypes relate to insolation is an intriguing one. To our knowledge, this has not been examined in these populations. Research into the effect of shell coloration on thermal tolerance is in general quite limited, and mostly restricted to terrestrial taxa (Seuront et al. 2018. J Molluscan Stud 84:203–232). However, data from other Littorinid species suggests that coloration differences may affect body temperature by 0.2-2.0°C, while behavioral responses such as "shell standing" may affect body temperature by 2-4°C (Miller and Denny 2011. Biol Bull 220:209-223). Due to the methodology, we used, differences in shell colouration would not have influenced our results, therefore we consider this issue to not be of direct relevance to our study and so have not incorporated it into our manuscript.

The color patterns depicted in the photo are representative of patterning across the entirety of the shell, thus we have chosen to depict the shells in the standard conformation used in the literature (e.g., Butlin et al. 2014).