



Multiple-stressor effects of warming and acidification on the embryonic development of an estuarine fiddler crab

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ABSTRACT

Predicted effects of anthropogenic climate change on estuarine and coastal organisms are complex, and early life history stages of calcified ectotherms are amongst the most sensitive groups. Despite the importance of understanding their vulnerability, we lack information on the effects of multiple stressors on the embryonic development of estuarine and burrowing organisms, mainly mangrove-associated species. Here, we determined the combined effects of elevated temperature and decreased pH on the embryonic development of the estuarine fiddler crab *Leptuca thayeri*. Initially, the microhabitat (burrow) of ovigerous (egg-bearing) females was measured for temperature, pH, and salinity, which provided control values in our laboratory experiment. Embryos at the early stage of development were subjected to cross-factored treatments of predicted temperature and pH and evaluated for development rate, survivorship, and volume until their later embryonic stage. Embryo development was faster at early and later stages of development, and survivorship was lower under elevated temperature. Embryos under reduced pH showed advanced embryonic stages at their late development stage. Higher egg volume was observed in a warmer and acidified environment, and lower volume in warmer and non-acidified conditions, indicating that embryo development is synergistically affected by warming and acidification. More than 70% of embryos developed until late stages under the multiple-stressors treatment, giving insights on the effects of a warm and acidified environment on burrowing estuarine organisms and their early stages of development.

1. Introduction

Anthropogenic climate change has been recognised as a significant threat to coastal and marine species (IPCC, 2014; Nagelkerken and Connell, 2015). The changing climate may drive shifts in species distribution and affect the behaviour and physiology of many groups across global environments (Parmesan, 2006; Byrne et al., 2013; Bozinovic and Pörtner, 2015; Clements and Hunt, 2015; Calosi et al., 2017). Coastal and estuarine habitats, in turn, are vulnerable due to their highly restricted areas and complexity of multifaceted environmental factors (Alongi, 2002; Nagelkerken and Connell, 2015; Carstensen and Duarte, 2019; Scanes et al., 2020). While estuarine fauna and flora are well-adapted (physiologically, morphologically and/or behaviourally)

to daily oscillations in abiotic factors (Duke et al., 1998; Madeira et al., 2012), severe and rapid changes in abiotic parameters (e.g., temperature increase and pH decrease) may exceed their physiological thresholds resulting in acute and chronic stress (Crain et al., 2008; Madeira et al., 2012; Byrne and Przeslawski, 2013; Przeslawski et al., 2015; Principe et al., 2018).

The mangrove macrofauna is delimited to a few dominant groups which are generally constituted by crustaceans, molluscs and polychaetes (Nagelkerken et al., 2008). As ectotherms and calcified organisms, temperature and pH-dependence may impose fitness-relevant threats in a changing environment (Madeira et al., 2012; Azra et al., 2020). Nevertheless, infauna organisms are directly affected by environmental changes; species living in the upper sediment layers of

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estuarine habitats are susceptible to warming and changes in sediment carbonate geochemistry, thus affecting their physiological and behavioural processes (Green et al., 2009; Ortega et al., 2016; Clements and Hunt, 2017a, 2017b; Pansch et al., 2018). Several mobile species have mechanisms to avoid adverse abiotic conditions, for instance, building deeper refugia or moving to more suitable areas, but constraints such as acidic and hypoxic deeper layers or dense and highly competitive habitats may affect their fitness (Melzner et al., 2013).

There is an established recognition of the importance of understanding how combined stressors (i.e., multiple stressors) affect organisms (Byrne, 2012; Griffen et al., 2016; Gunderson et al., 2016; Steckbauer et al., 2020). Recent efforts have prioritised the impacts of multiple stressors by incorporating abiotic parameters obtained via downscaling modelling and field measurements (Bozinovic and Pörtner, 2015; Gunderson et al., 2016). The combination of stressors may entail additive, antagonistic and/or synergistic effects, which are, respectively, equal to, lower or greater than the sum of their separate effects (Crain et al., 2008; Griffen et al., 2016; Ong et al., 2017; Steckbauer et al., 2020). The synergistic effect, however, is the most observed impact on early life stages of marine-related species varying amongst ontogeny stages, stressors and biological responses (Przeslawski et al., 2015). Crustacean embryos and larvae, for instance, are potentially susceptible to climate change stressors (Byrne and Przeslawski, 2013; Kroeker et al., 2013; Przeslawski et al., 2015; Pandori and Sorte, 2019). While some species are negatively affected by abiotic variation, others may benefit from or withstand predicted environmental changes. Multiple-stressor experiments combining elevated temperature and reduced pH have shown reduced survival, development and calcification in addition to non-direct impacts for several species (Styf et al., 2013; Przeslawski et al., 2015 and literature within). But despite the susceptibility of estuarine organisms, we still lack information about the vulnerability to multiple stressors in early development stages of many species (Przeslawski et al., 2015; Gunderson et al., 2016).

Here, we determined the impacts of elevated temperature and decreased pH on the embryonic development of an estuarine crustacean species, the Atlantic mangrove fiddler crab *Leptuca thayeri* Rathbun, 1990. Fiddler crabs are a key estuarine group playing an important role in soil conformation and food webs (Kristensen, 2008; Natálio et al., 2017). Using burrows as refuges, female *L. thayeri* bear their embryos externally, experiencing all daily abiotic environmental changes (Salmon, 1987; Gusmão-Junior et al., 2012), thus making this species a useful model to understand the likely effects of climate change stressors on the embryonic development of a mangrove species. Our experiment consisted of two parts: 1) to characterise the microhabitat of the crab's burrow with respect to temperature, pH and salinity and 2) to determine the combined effect of a predicted temperature increase and pH decrease on their embryonic development using the following response variables: development rate, survivorship, and egg volume. We predicted a synergistic negative effect of temperature and pH on the embryonic development of *L. thayeri*, hypothesising increased development rate, higher mortality and lower volume of eggs under elevated temperature and decreased pH conditions.

2. Material and methods

2.1. Study site and microhabitat characterisation

Field samplings of crabs and abiotic factors (temperature, pH and salinity) were conducted in the Portinho Mangrove at the Ézio Dall'Aqua City Park, Praia Grande - São Paulo/Brazil (23°59'16.74" S 46°24'26.28" W). This mangrove forest is situated in the Mar Pequeno estuarine system (Santos-São Vicente Estuary Complex) and is colonised by black (*Avicennia schaueriana*), red (*Rhizophora mangle*) and white (*Laguncularia racemosa*) mangrove tree species (Checon and Costa, 2017).

We measured temperature (thermometer, Lutron, TM-946, ± 0.01 °C,

Omega K-type thermocouples), pH (pH-meter, mPA2010 MS, Tecnopeon, ± 0.01 , NBS pH Scale) and salinity (Handheld Refractometer ATC, Resolution: 0.2%) of the water within burrows (microhabitat) of *L. thayeri* females bearing eggs at the early stage of development (red/purple egg mass; Christopher et al., 2008) (Table 1). Prior to use, pH-meter calibration was performed with 7.1 and 4.01 National Institute of Standards and Technology (NIST) buffers following the manufacturer's protocol. Burrow water temperature was measured at a 10 cm depth and then water was collected fully with a falcon tube. The time between field water sampling and pH and salinity measurements in the laboratory was less than 30 min. Following temperature collection within each burrow, crabs were measured (carapace width; CW) using standard callipers in the field. In total, 39 burrows of ovigerous females (mean \pm SE CW: 16.14 ± 0.55 mm) were sampled in seven days during summer and spring 2017 (January to March and September to December). We sampled in daylight spring low tides because, despite the recognised underwater activity of some fiddler crabs, ovigerous *L. thayeri* were not observed behaving outside the burrows during high tides in the study conducted by De Grande et al. (2018). Temperature, pH and salinity were obtained in the overlying water column (Table 1) and air temperature was measured after each microhabitat or overlying water column measurement (mean \pm SE: 24.44 ± 0.28 °C).

2.2. Animal collection and maintenance

Ovigerous females with embryos at the early stage of development were manually collected during the summer of 2018 (January to February) by excavating their burrows. The embryonic stage of detached embryos was checked under a stereomicroscope (ZEISS Discovery.V8, Axiocam Erc 5s). Only crabs with embryos at the blastula stage were maintained in the laboratory to average the development stage (classification based on Yamaguchi, 2001 – see Fig. 1 for details). Twelve crabs (mean \pm SE CW: 16.29 ± 0.49 mm) were maintained for one day in tanks containing artificial saltwater (sea salt Blue Treasure Reef Sea dissolved in deionised water at 33, based on microhabitat mean \pm SE value: 33.51 ± 0.56) and PVC pipe connectors (2.5 cm \varnothing) to provide shelter. A 12 h light:dark photoperiod cycle and controlled air and water tank temperature (~ 25 °C) were maintained during acclimation.

2.3. Experimental design

We tested the effects of elevated temperature and acidification on the embryonic development of the fiddler crab *L. thayeri*. Embryos were exposed to cross-factored treatments with two levels of temperature (control = 26 °C, elevated = 30 °C) and pH (control = 6.9, reduced = 6.2 units). Control values for temperature (mean \pm SE: 26.16 ± 0.38) and pH (mean \pm SE: 6.89 ± 0.03) were based on microhabitat characterisation. The elevated temperature value (+4.0 °C) was derived from the IPCC (2014) scenario RCP 8.5 and the lower pH value (−0.7 units) from Caldeira and Wickett (2003). Water temperature was controlled using thermostats (Aquarium heater, H-606 70W, Hopar) and pH values were obtained through CO₂-enriched air injection via a standard regulator and solenoid kits attached to cylinders. Water temperature was continuously monitored with a thermometer (same as used in the microhabitat characterisation), while pH was monitored with a PG 1400 Gehaka pH-meter (electrode 2A09EBI, Analyser, ± 0.01 , NBS pH Scale, calibrated with 7.1 and 4.01 NIST buffers).

Table 1

Mean (\pm SE) of temperature, pH and salinity values in burrows (microhabitat) of *Leptuca thayeri* ovigerous females and overlying water column.

Abiotic factors	Burrow (microhabitat)	Overlying water column
Temperature (°C)	26.16 ± 0.38	28.42 ± 0.36
pH	6.89 ± 0.03	7.75 ± 0.04
Salinity	33.51 ± 0.56	25.33 ± 0.65

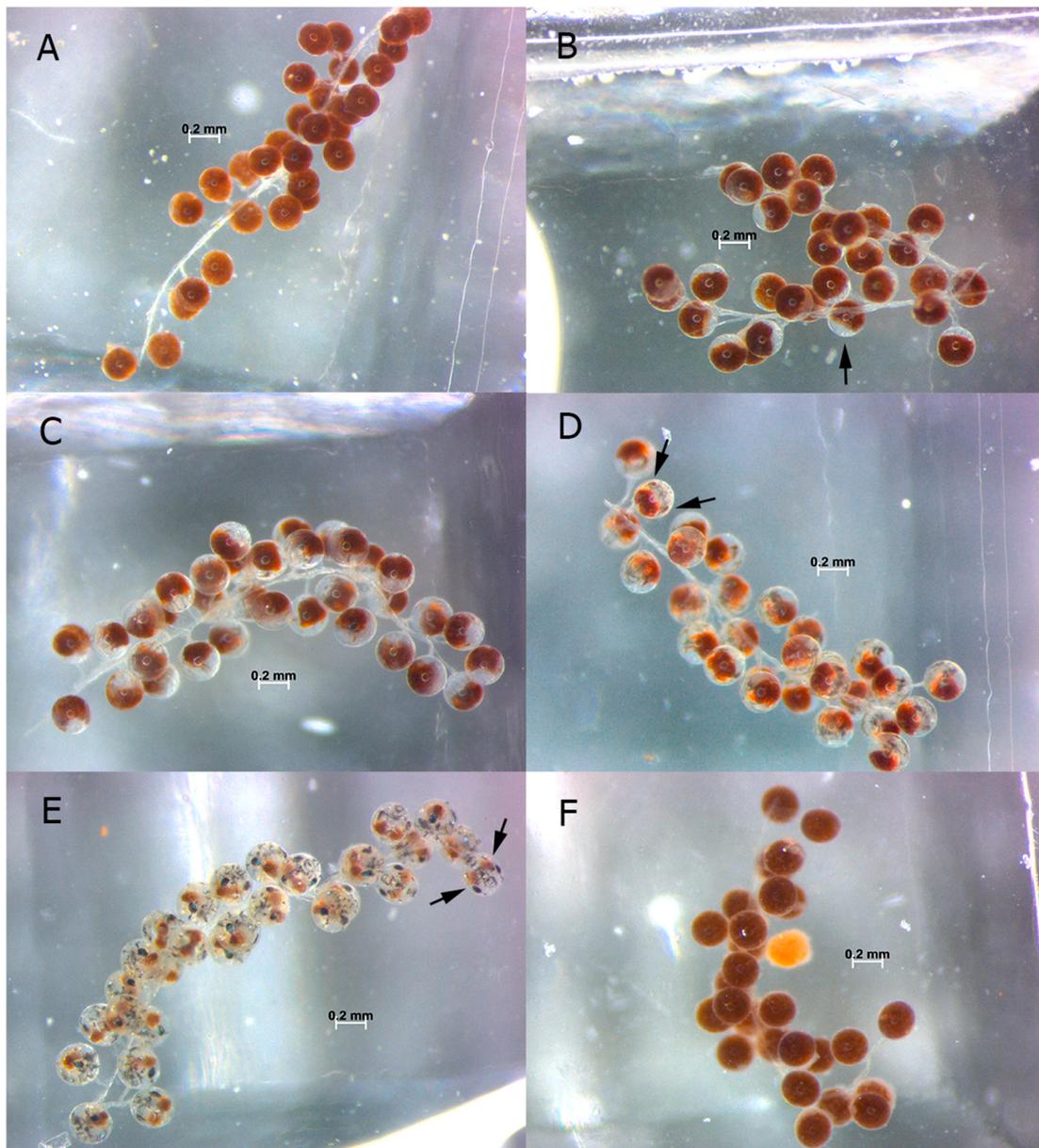


Fig. 1. Classification scheme of the development of *Leptuca thayeri* embryos based on yolk content, eye presence and heartbeat: (A) stage I (egg capsule with almost undistinguished individual cells 24–48 h after ovulation), (B) stage II (small yolk-free portion with a formed germinal disc (indicated by the arrow)), (C) stage III (yolk-free portion increased), (D) stage IV (differentiation of limb buds, decreasing yolk mass, development of eye placodes and eyes (indicated by the arrow) and faint heartbeat) and (E) stage V (completed eye (indicated by the arrow), yolk in four lobes (indicated by the arrow) and strong heartbeat). The description was based on Yamaguchi (2001) for the embryonic development of the fiddler crab *Austruca lactea*. (F) Additional example of degenerated or relatively underdeveloped eggs.

The experiment was conducted for 10 days in February 2018. After 10 days of development, fiddler crab embryos are expected to present completed eyes, yolk in four lobes and strong heartbeats, representing an advanced stage of embryonic development (Yamaguchi, 2001). We used detached embryos at the blastula stage from six ovigerous females to exclude the maternal effect on egg development. Roggatz et al. (2016) claimed that ovigerous female crabs, which provide oxygen to embryos during their development, had their clutch ventilation and egg mass care behaviour affected by seawater acidification. While females are usually in charge of controlling the hatching time (De Vries and Forward, 1989), detached embryos can develop when separated from their clutch (e.g., Miller et al., 2016). Thus, we eliminated the female crab influence to verify the effects of elevated temperature and reduced pH on the embryos. A portion of the clutches was detached with a forceps only from the central region of the egg mass because inner and outer embryos may

have different oxygen availability during early development (Fernández et al., 2003). Embryo aggregations (EAs) were gently detached under the stereomicroscope with thin hypodermic needles. Each aggregation consisted of 20–40 embryos at the blastula stage; most of the embryo filaments detached from the clutch had 20–40 eggs each and to ensure embryo integrity we maintained the EAs with their natural egg disposition. Embryo aggregations had their eggs counted, transferred using pipets to a well (2 ml vol. each) in a 24-well cell culture plate and then relocated to the microcosms with water temperature and pH set to the control treatment values ($n = 24$ EAs per tank/288 EAs for all treatments). Transfer pipets and needles were constantly cleaned during the detaching process with distilled water to avoid contamination.

2.4. Water chemistry

Water samples were taken at the beginning and end of the experiment and then measured for total alkalinity (A_T , $\mu\text{mol kg}^{-1}$) (T50 automatic titrator, Mettler Toledo) following Dickson et al. (2007). Partial pressure of carbon dioxide, bicarbonate, carbonate, aragonite and calcite saturation state (Ω) were calculated using CO2SYS (Robbins et al., 2010) from obtained pH (NBS), temperature, salinity and A_T values with carbonate dissociation constants K_1 and K_2 from Mehrbach et al. (1973) refitted by Dickson and Millero (1987), K_{SO4} from Dickson (1990) and total boron from Lee et al. (2010) (see Supplementary Table 1 for water chemistry details).

2.5. Experimental apparatus

Microcosms ($n = 3$ per treatment) consisted of tanks ($39 \times 9 \times 14$ cm) filled with 4.91 l of artificial saltwater (salinity level, 33). Tank effect was reduced by randomly interspersed treatment levels (Havenhand et al., 2010). Each tank had a 24-well cell culture plate attached with Velcro to the tank's borders. The plastic bottom of each well was removed and substituted by a nylon mesh (180μ), allowing water circulation. Each tank had two connected compartments: one for the culture plate and other for controlling abiotic factors and apparatus setup including the thermostat, plastic tubes providing CO_2 , and oxygen supply by air pumps with air stones bubbling at a similar intensity (see Supplementary Figure 1 for details). Water level was refilled in our microcosms, when necessary, with distilled water at similar pH and temperature values to maintain controlled salinity values. Photoperiod regime was set in a 12 h light:dark cycle. Experimental set-points for elevated temperature and reduced pH values were gradually reached over 1 day (~ 0.05 pH unit decrease and ~ 0.25 °C temperature increase every 2 h).

2.6. Response variables: stage of development, survivorship, and egg volume

Every other day, one EA per microcosm was systematically chosen to assure female brood independence throughout the experiment. Embryos were photographed at the same magnification with the stereomicroscope mentioned above (scale: 0.2 mm) and scored as dead or alive. Embryos with pale yellow/white coloration and degenerated underdeveloped eggs were considered dead (Förster and Baeza, 2001; Przeslawski et al., 2005) (see Fig. 1 for details). Embryonic stages of all living embryos were based on the detailed classification by Yamaguchi et al. (2001) and EAs were classified as: stage I (egg capsule with almost undistinguished individual cells 24–48 h after ovulation), stage II (small yolk-free portion with a formed germinal disc), stage III (yolk-free portion increased), stage IV (differentiation of limb buds, decreasing yolk mass, development of eye placodes and eyes and faint heartbeat) and stage V (completed eye, yolk in four lobes, strong heartbeat) (see Fig. 1 for all embryonic stages used as reference images). Embryo aggregations did not return to the microcosms after manipulation.

Since not all EAs were alive on the 10th day, 26 living EAs for each treatment were randomly chosen from the microcosms using a random sequence generator. We avoided female brood repetition and only EAs at stage V were used to standardise the embryonic stage. Five embryos of each EA were also arbitrarily selected, photographed (same as above) and measured for smaller and larger diameter with ImageJ. We verified the volume of each embryo by applying the formula for oblate spheroids: $V = 1/6 (\pi \cdot d^2 \cdot D)$ (d = smaller diameter, D = larger diameter) and estimated mean volume of EA (Simoni et al., 2011).

2.7. Statistical analysis

Data were checked for normality and homoscedasticity using Shapiro-Wilk's and Levene's test, respectively. Stages of development

(categorical data) were analysed as present (1) or absent (0) for each mentioned stage (five stages) and compared with a Type III SS three-way Permutational Analysis of Variance (PERMANOVA) (Anderson, 2001). The PERMANOVA is considered a robust test for non-normal distributions and univariate analysis when analysed in a Euclidean distance (Anderson et al., 2008). Analyses were undertaken on the Euclidean matrices of untransformed data using 9999 permutations with time (fixed, five levels: 2nd, 4th, 6th, 8th and 10th day), temperature (fixed, two levels: control and elevated) and pH (fixed, two levels: control and reduced). Post-hoc pairwise tests were performed for multiple comparisons amongst significant factors.

Survivorship data were organised according to percentage of living embryos per EA (= unit of replication) (log-transformed prior to the analysis). We applied a three-way Analysis of Variance (ANOVA) with temperature (two levels: control and elevated) and pH (two levels: control and reduced) as fixed factors. Egg volume on the last day of the experiment (10th day) was compared amongst treatments via a two-way ANOVA with temperature (two levels: control and elevated) and pH (two levels: control and reduced) as fixed factors.

Models were performed with egg number as a covariate; since there were non-significant interactions between factors and egg number, the covariate was dropped from all models. Significant factors were checked by Tukey's *post hoc* with Bonferroni correction for pairwise comparisons. The critical level (α) was set at a 95% confidence interval ($\alpha = 0.05$) for all analyses. Values throughout the text are reported as mean and standardised error. Analyses were conducted in the SPSS 25.0 (SPSS Inc., Armonk, NY, U.S.A.) and PRIMER v.7 (PERMANOVA+, Anderson et al., 2008).

3. Results

3.1. Stage of development

Time, and its interaction with temperature and pH, influenced the embryonic development stages (Table 2, Fig. 2). Under elevated temperature, embryos developed faster from the 2nd to 4th day (PERMANOVA: $t = 5.567$, $p = 0.003$) and 8th to 10th day (PERMANOVA: $t = 5.136$, $p = 0.006$) and were at advanced stages on the 10th day (PERMANOVA: $t = 4.654$, $p = 0.002$) when compared to the control temperature group. Embryos under reduced pH were also at more advanced stages on the 10th day when compared to the 8th day (PERMANOVA: $t = 4.935$, $p = 0.004$), but not under controlled pH (PERMANOVA: $t = 1.940$, $p = 0.107$).

3.2. Survivorship

Survivorship was influenced by the interaction between time and temperature with a higher number of living embryos on the 4th day and

Table 2

Summary results of three-way PERMANOVA test for comparison of stages of embryonic development under elevated temperature (control = 26 °C, elevated = 30 °C) and reduced pH (control = 6.9, reduced = 6.2 units) over time. Significant effects are represented in bold ($N = 3$ embryo aggregations (EAs) per time/treatment).

Factor	df	MS	Pseudo-F	P (perm)
Stage of development				
Time	4	22.275	19.37	0.001
Temperature	1	3.1	2.695	0.082
pH	1	4.433	3.855	0.036
Time \times Temperature	4	6.3917	5.558	0.001
Time \times pH	4	3.225	2.804	0.004
Temperature \times pH	1	0.366	0.318	0.739
Time \times Temperature \times pH	4	0.575	0.5	0.859
Residual	40	1.15		

df: degrees of freedom, MS: mean square, permutation P-value: P (perm).

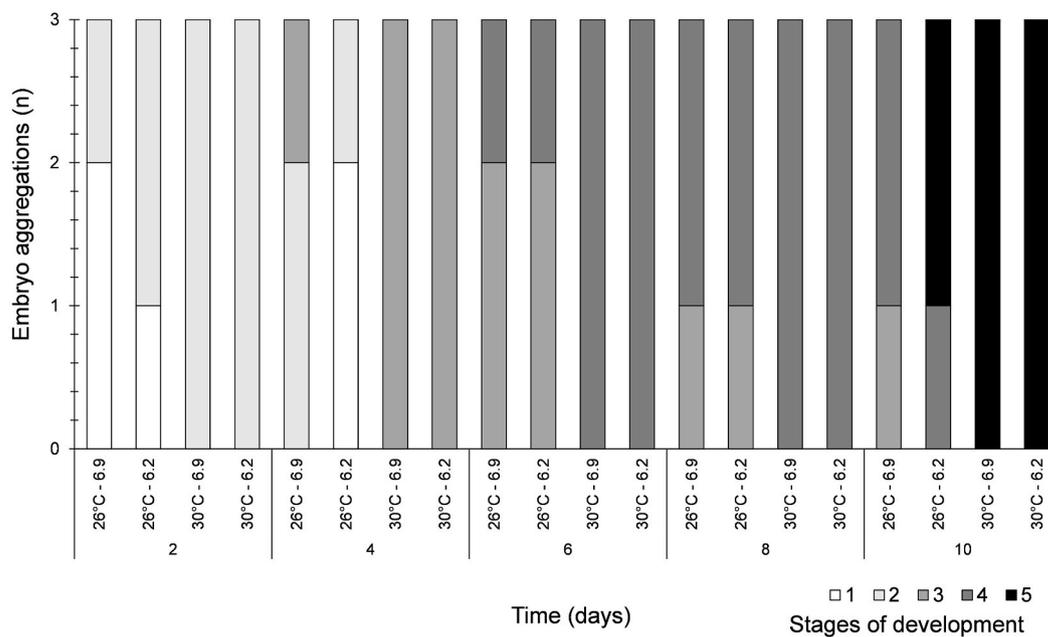


Fig. 2. Stages of development (1–5) of embryo aggregations (EAs) of *Leptuca thayeri* over time under elevated temperature (control = 26 °C, elevated = 30 °C) and reduced pH (control = 6.9, reduced = 6.2 units) (N = 3 EAs per treatment/time).

lower on the 10th day under elevated temperature (Tukey’s test: $p = 0.05$ and $p = 0.018$, respectively) (Table 3, Fig. 3).

3.3. Egg volume

There was a significant temperature \times pH interaction whereby embryos that developed under elevated temperature had lower volume under control pH conditions and higher volume under reduced pH (Tukey’s test: $p < 0.001$) (Table 4, Fig. 4).

4. Discussion

We investigated the effects of predicted warming and acidification on the embryonic development of the fiddler crab *L. thayeri*. Our hypothesis was partially supported, where elevated temperature and reduced pH affected embryos, but several embryos were alive until late stages of development. Elevated temperature accelerated their development at early and late development stages but also increased

mortality. Embryos under reduced pH were also at more advanced stages in their late development. Higher egg volume was observed in a warmer and acidified environment, and lower volume in warmer and non-acidified conditions.

Embryonic development of ectotherms is strictly temperature-dependent. The success of developing embryos was assessed in twenty-one species of British crustaceans, and temperature was the main factor affecting their early stages (Wear, 1974 and literature within). In the present study, embryos at early (2nd to 4th day) and late (8th to 10th day) stages developed faster under elevated temperature conditions supporting that temperature is indeed one of the main drivers of embryonic development (Wear, 1974; Anger, 2001; Przeslawski et al., 2015; Azra et al., 2020); however, faster development does not mean better development. Larval viability, fitness and survivorship rate are usually negatively affected when embryos develop with temperatures ranging outside usual variations (Wear, 1974; Kroeker et al., 2013; Harvey et al., 2013; Perez-Miguel et al., 2020). As we observed, *L. thayeri* embryos exposed to elevated temperature showed a lower survivorship rate at late stages of development (10th day of experiment). In a robust meta-analysis, Harvey et al. (2013) evidenced that survivorship of early life history stages is negatively and synergistically affected by temperature and pH. Thermal stress and low pH have been recognised as an important threat to oxygen supply, calcification, growth, reproduction, and survival (Pörtner and Knust 2007; Bozinovic and Pörtner, 2015; Griffen et al., 2016), and survivorship is amongst the most evaluated response variable to those stressors (Crain et al., 2008; Przeslawski et al., 2015). A low pH environment, however, also seems to affect embryos at late stages of development. Some broods are indeed more tolerant to acidified environments (Gravinese et al., 2018) and examples of tolerance to low pH conditions are observed across taxa where growth, development and survivorship of estuarine and marine early life stages were not affected/partially affected (Pansch et al., 2012; Range et al., 2012; Miller et al., 2016; Jarrold and Munday, 2018), or even positively affected (Ries et al., 2009; McMahon et al., 2020). Coastal and estuarine habitats already experience similar or wider pH

Table 3

Summary results of an ANOVA for comparisons of survivorship (% of living *Leptuca thayeri* embryos) under elevated temperature (control = 26 °C, elevated = 30 °C) and reduced pH (control = 6.9, reduced = 6.2 units) over time. Significant effects are represented in bold (N = 3 embryo aggregations (EAs) per time/treatment).

Survivorship	df	MS	F	p value
Intercept	1	21064.197	1.848	<0.0001
Time	4	620.056	96.123	0.037
Temperature	1	1.295	2.830	0.939
pH	1	0.289	0.006	0.971
Time \times Temperature	4	583.405	2.662	0.046
Time \times pH	4	534.896	2.441	0.062
Temperature \times pH	1	0.000	0.000	0.999
Time \times Temperature \times pH	4	184.694	0.843	0.506
Residual	40	219.138		

df: degrees of freedom, MS: mean square.

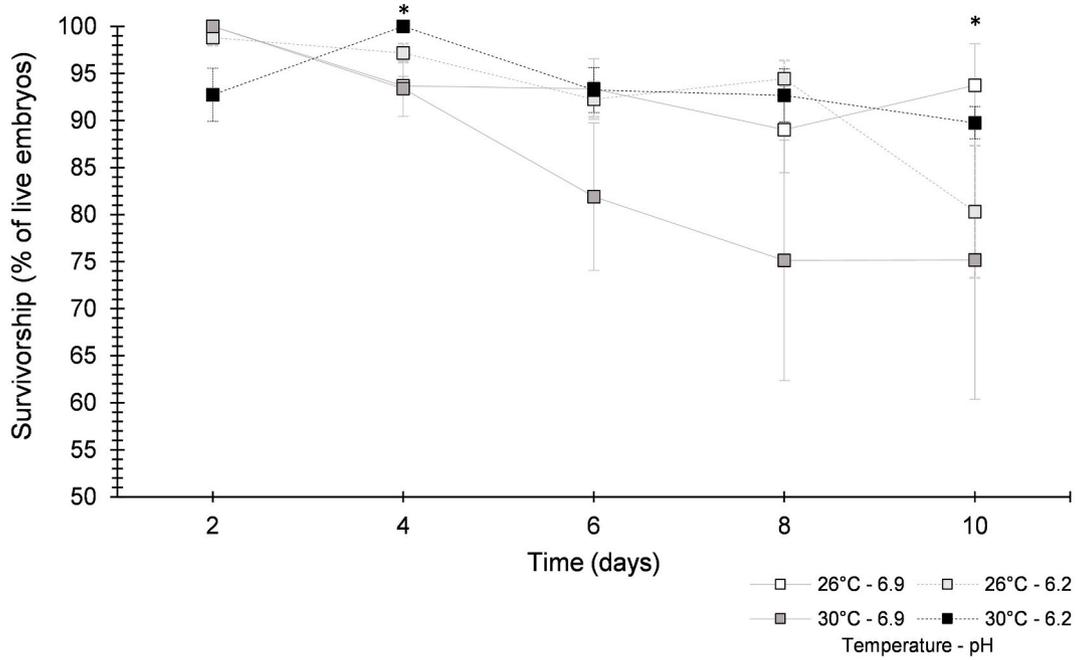


Fig. 3. Mean survivorship (% of living embryos) (\pm SE) of *Leptuca thayeri* embryo aggregations (EAs) over time under elevated temperature (control = 26 °C, elevated = 30 °C) and reduced pH (control = 6.9, reduced = 6.2 units) (N = 3 EAs per treatment/time). Asterisk indicates statistical difference within time between temperature treatments (ANOVA, $p < 0.05$).

Table 4

Deviance analysis for ANOVA fitted to identify the effects of elevated temperature and reduced pH on egg volume of late-developed *Leptuca thayeri* embryos. Significant effects are represented in bold (N = 26 embryo aggregations (EAs) per treatment).

Source	df	MS	F	P
Intercept	1	0.0002	331.058	<0.001
Temperature	1	<0.0001	2.775	0.099
pH	1	<0.0001	24.894	<0.001
Temperature \times pH	1	<0.0001	34.956	<0.001
Residue	99	<0.0001		

variation than predictions for the late 21st century (Dai et al., 2009; Duarte et al., 2013; Carstensen et al., 2018; Carstensen and Duarte, 2019) where several species present biological mechanisms to deal with low pH conditions, such as intracellular regulation (Hendriks et al., 2015).

Infaunal organisms are constantly exposed to temperature and pH oscillations (Przeslawski et al., 2009). Thus, sediment geochemistry variations may cause acute to severe effects in organism fitness depending on the temporal scale and species (Green et al., 2009; Bauer and Miller, 2010; Clements and Hunt, 2017a, 2017b), especially in potentially sensitive embryonic stages (Przelawski et al., 2015). Low pH conditions experienced by several infaunal organisms (e.g., bivalves) in shallow-water marine sediments, for instance, are primarily driven by

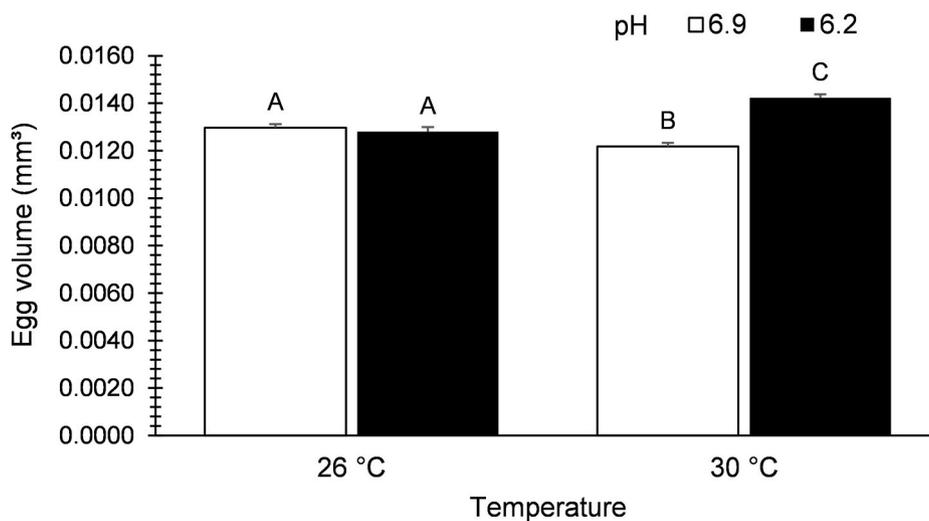


Fig. 4. Mean (\pm SE) egg volume of embryo aggregations (EAs) of *Leptuca thayeri* at final embryonic development stages exposed to elevated temperature (control = 26 °C, elevated = 30 °C) and reduced pH (control = 6.9, reduced = 6.2 units) (N = 26 EAs per treatment). Different letters indicate statistical difference within and between treatments (ANOVA, Tukey's test: $p < 0.001$).

high rates of decomposition of organic matter and microbial aerobic activity (Clements and Hunt, 2017b and literature within). For burrowing mangrove crabs, within-burrow respiration in addition to microbial activity may drive even lower pH conditions in the microhabitat, which may also explain the tolerance to acidification by the embryos. As mentioned, fiddler crab embryos are bore in the female's clutch, thereby restricted to soil surface and burrow conditions until reaching their larvae stages. Thus, although crab burrows are indeed hypercapnic environments (as observed in our field measurements) and likely experience changes in water pCO₂ concentrations (Pinder et al., 1993), embryos seem resilient to predicted acidified conditions throughout embryonic development, despite showing potential impairments to next life stages due to higher volume under the multiple-stressor treatment.

Egg volume may entail a complementary interpretation of both elevated temperature and reduced pH effects. Embryos in the elevated temperature and control pH treatment may have had their metabolic rate exceeding the physiological threshold, which explains a lower volume at the last stage of embryonic development. Otherwise, a higher volume of embryos in our experiment may be linked to the lack of osmoregulation capacity and impairment of ion and gas exchanges due to high pCO₂ concentrations (Simoni et al., 2011; Gravinese, 2018), which are likely due to the combined high temperature and low pH conditions, and resultant extreme pCO₂ values. Thus, our sub-lethal response variables support a synergistic and negative effect of pH and temperature throughout the embryonic development of *L. thayeri*. Summing up the effects, elevated temperature increases development rate, and pH may disrupt homeostasis, affecting exchanges with the surrounding environment.

Warming and acidification have been extensively discussed as important stressors in marine and coastal habitats (Duarte et al., 2013; Nagelkerken and Connell, 2015; Gunderson et al., 2016). In a climate change context, abiotic stressors drive directional selection and define winners and losers at different scales (Somero, 2010). Physiological plasticity plays an important role in how species deal with climate change (Seebacher et al., 2015), and the relevance of individual genetic variability may trigger species acclimation and define their adaptation by natural selection (Pistevos et al., 2011). This is especially relevant when those responses are observed in an estuarine, calcified ectothermic organism with an r-selected reproductive strategy. In our study, a mean of more than 70% of EAs in all treatments developed well until their late stage of development. Although we have used EAs as our unit of replication, eggs in the same tank and clutch have occasionally showed varied responses to stressors, highlighting the individuality of each embryo. Thus, it would be important to understand the potential adaptation of fiddler crabs at the next life stages using varied performance response variables such as ontogeny, populations, and generations (e.g., Stillman et al., 2020). Complementary field manipulative experiments and more realistic approaches would be relevant to complement our results. To the extent of our knowledge, this is the first study exploring the embryonic development of a mangrove crab species under a multiple-stressor and climate change-related predicted scenario. Our results also highlight the importance of applying varied morphological response variables when dealing with multiple-stressor experiments. Multiple-response variables provide a broader and more realistic perspective of the performance of a given species under predicted warming and acidified environments.

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Author contributions statement

JCFP was responsible for the conception, design, and execution of the experiments, for the data analysis and manuscript drafting; TMC was responsible for the conception, design and revision of the manuscript. Both authors approved the final version for publication.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ecss.2021.107296>.

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