Zoological Studies **63:** 8 (2023)

Lethal Consequences and Embryo Shell Shape Alterations in the Marine Gastropod *Trophon geversianus* Due to Elevated Temperatures

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(Received 7 July 2023 / Accepted 27 December 2023 / Published -- 2024)
Communicated by Ka Hou Chu

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Environmental temperature is increasing while natural populations are forced to develop their life cycle under new conditions, resulting in the expression of new phenotypic traits. Still, the links between these new environmental conditions and the subsequent phenotypic expressions are not fully explored. Here, we conducted manipulative experiments with the marine gastropod *Trophon geversianus* embryos to assess the effects of warmer temperatures in shell form. We observed lethal effects together with alterations in the shell form (size + shape) of embryos exposed to 18°C compared to the control temperature (13°C). Our results reveal that *T. geversianus* from

Patagonian coasts growing under warm temperatures will change their phenotype by selecting smaller and more elongated shells in the ontogeny, as well as an expansion of the shell aperture, increasing their predation vulnerability. Therefore, we considered that the embryonic shell shape change could be a good biomarker of thermal stress produced at early developmental stages in marine gastropods.

Key words: Direct development, Atlantic Patagonia, 2D geometric morphometrics, Early development stages, Thermal stress.

Citation: Nieto Vilela RA, Giulianelli S, Zabala S, Bigatti G, Márquez F. 2024. Lethal consequences and embryo shell shape alterations in the marine gastropod *Trophon geversianus* due to elevated temperatures. Zool Stud **63:**08.

BACKGROUND

The Earth's climate is a dynamic system, varying naturally on different time scale ranges: multi-millennial scale changes such as glacier and inter-glacial transitions, inter-decadal cycles such as North Atlantic oscillations and Pacific Decadal, interannual patterns such as ENSO (El Niño, Southern Oscillation), and also in seasonal cycles (Harley et al. 2006). Nevertheless, human activities have become an important additional component of the climate system (IPCC 2022 2021a; Wolff et al. 2020), where greenhouse gas emissions -mostly because of CO_2- increase the temperature (Feely et al. 2004; IPCC 2022). The increase in CO_2 and the consequent increases in global temperature are expected to cause a cascade of physical and chemical changes in marine ecosystems (Arias et al. 2021; Harley et al. 2006). In the marine environment, thermal increase was first detected in the sea surface temperature, then in the durations and frequency of marine heatwaves (Androulidakis and Krestenitis 2022). Nevertheless, the thermal increase impacts the shallow and deep-water environments (Meinen et al. 2020), producing, morphological (Almada-Villela et al. 1982; Hethke et al. 2021) and physiological effects on ectothermic organisms (Pöhlmann et al. 2011; Díaz et al. 2021). Morphologically, high temperatures demand plastic responses in the communities (Hethke et al. 2021; Melatunan et al. 2013; Pigliucci 2001). Exposure to heightened temperatures during development can impact the morphology and dimensions of organisms possessing calcareous structures (Hethke et al. 2021). Furthermore, temperature is a key environmental variable in calcareous shells' biomineralization and growth processes (Gazeau et al. 2013; Mackenzie et al. 2014). Since the shell is the anatomical structure that protects most molluscs against waves,

predators, desiccation, or overheating, its modification has great consequences (Byrne and Fitzer 2019; Melatunan et al. 2013). For example, Olson et al. (2012) showed that temperature can alter the microstructure of the shell, changing the angle and thickness of the aragonite layers. In turn, Almada-Villela et al. (1982) found a relationship between increases in shell growth as a function of temperature in bivalves, but also a decrease in hardness, making them more vulnerable to predation pressure (Mackenzie et al. 2014). Recently, negative effects in calcareous structures due to the acidification by temperature increase were described as a direct consequence of global warming (Bednaršek et al. 2021; Bednaršek et al. 2020). Any modification in the calcareous structure of invertebrates can alter their strategy of shutting themselves up tightly in their shells to survive low tide periods (Pöhlmann et al. 2011). The IPCC, under scenario RCP8.5 predicts an ocean temperature increase from 0.8 ± 0.4°C in the first scenario to 3.1 ± 0.6°C in the fifth (Collins et al. 2013; IPCC 2001a 2021b 2022). Therefore, under IPCC predictions the thermal increase in the following years could have far-reaching effects on the development of organisms with carbonate shells.

Most inhabiting organisms along the intertidal and subtidal ecosystems have an external calcareous structure to face the inconstant ambient conditions (Pöhlmann et al. 2011; Raffaelli and Hawkins 2012). They can exhibit plastic responses in different development stages (Melatunan et al. 2013; Pigliucci 2001). Shell shape alteration was described as an early stress indicator for pollution (Primost et al. 2016 2021), thermal stress (Morley et al. 2010; Boomer et al. 2003; Kontrovitz et al. 1987), predation (Stafford et al. 2015; Dietl et al. 2010; Bourdeau 2009) or invasive species detection (Kistner and Dybdahl 2014). In Atlantic North Patagonia, the marine gastropod *Trophon geversianus* (Pallas, 1776) presents a complex phenotype pattern exhibiting a site dependence on shape variation (Nieto-Vilela et al. 2021) and two ecomorphotypes related to physical and biological pressures in the intertidal and subtidal environment (Márquez et al. 2015). This specialist predator (Palomo et al. 2019) is distributed from the province of Buenos Aires to the Burdwood bank in the Namuncurá Reserve in the Southern Atlantic Ocean (Castellanos and Landoni 1993; Griffin and Pastorino 2005; Pastorino 2005) and up to 42º latitude south in the Pacific Ocean. The adult individuals of the subtidal ecomorphotype present a slender and narrower shell shape due to exposure to a less physical stress environment and high predator pressures (Márquez et al. 2015). *Trophon geversianus* is a gonochoristic species with internal fertilization, despite being dioecious does not present external sexual dimorphism, whereas the female snails differ internally by the presence of the albumin and capsule gland (Cumplido et al. 2010). Females produce egg capsules, where the embryos develop until hatching as crawling juveniles surrounded by numerous nurse eggs used as nutritional resources (Zaixso 1973). Each female

can produce 18 egg capsules arranged in clusters, spending 25 or 10 h in attaching each one (Cumplido et al. 2010). Experiments made with intertidal egg capsules revealed an average maturation time of 120 days, with average hatching of 4 embryos of size 2.79 + 0.03 mm (Cumplido et al. 2011).

The increase in thermal stress in the oceans will produce organisms' plastic responses. A strategy for early detection of environmental disturbances in marine populations is to select specific responses to buffer environmental stressors (Edge et al. 2012; Bucheli and Fent 1955). Therefore, we analyzed the shell form, and survival rate, of embryos of the marine gastropod *T. geversianus* under temperatures predicted by the IPCC. We hypothesized that those subtidal embryos that usually develop in a more stable environment than intertidal ones, will modify their shell shape under higher temperatures.

MATERIALS AND METHODS

Sampling design

We carried out a single sampling event in September 2018 in Atlantic Patagonia, the selected area was the shallow subtidal (10 m of deep) of Golfo Nuevo. A total of 150 capsules were collected from 50 different clusters, corresponding to 50 females, by scuba diving, ensuring that the encapsulated individuals were in the first cellular cleavage stage (see fig. 1A in Cumplido et al. 2011). The capsules were transferred to the laboratory and separated under a stereoscopic microscope according to the eggs' stage of maturity (Cumplido 2008; Cumplido et al. 2011). We selected three initial maturity stages considering the presence of nurse eggs as an indicator of the first week of the maturation stage: the *initial*, where the egg contents were not disaggregated; the *intermediate* stage where the mass was condensed into small circles and an *advanced* stage where the eggs began to divide forming irregularly shaped embryos. After that, for acclimatization, the capsules were transferred to the aquarium for two weeks at 13°C. The capsules were placed in a controlled temperature chamber and individually placed inside histological cassettes in 200 ml jars with filtered seawater.

Temperature experiments

After the acclimatization period, we randomly selected 50 egg capsules from the total egg capsules collected (150) and assigned them to three different temperature treatments. The

temperature chambers were monitored by a central control unit that switched on or off the heaters in each container rack according to the sensor readings, -the sensors had a sensitivity of ± 0.1ºC. In addition, data loggers were placed in each rack to keep more records of the temperatures. To assign the capsules to the treatments, we carefully considered that all maturity stages were represented in all three treatments and that egg capsules laid by the same female were assigned to different treatments. The three temperature treatments were defined based on loggers placed in the cost (Nieto-Vilela et al. 2022). The annual temperature average of Golfo Nuevo (13° C) was defined as the control temperature (T13), the naturally high-temperature treatment with the summer mean in the area of 18°C (T18) and the high projected temperature with the mean summer temperature expected with the increase of 4°C expected 22°C (T22) (Collins et al. 2013).

The experiment was placed in six racks containing 30 L of water with 25 independent jars (Supplementary data 1). The individual jars with each egg capsule remained immersed in the water with a gentle airflow (approximately one bubble per second) and continued until the juveniles hatched. Every two days we recorded the pH, salinity, dissolved oxygen, and temperature of each treatment to maintain the water quality (Milwaukee pH 55, multiparametric sonde Hanna). Also, weekly we changed the jar of water and externally cleaned the egg capsules with a brush to evaluate the embryo state. The jars were randomly rotated around the entire rack in each cleaning event. At the end of the experiment, we registered the hatching time for each treatment and the number of hatched embryos from each egg capsule, and randomly select three of them for shell shape analysis. Also, until the end of the experiment, 90 days later, we registered the number of viable and non-viable capsules as well as the number of alive and dead embryos per treatment.

Shape analysis

The three selected embryos were cleaned and orientated over a thin plastic modeling material (plasticine) layer, to minimize the effects of rolling or pivoting. After that, we captured their photos under a Carl Zeiss binocular magnifying glass equipped with AxioVision Rel.4.5 software (©Carl Zeiss Imaging Solutions). Over each picture, we described a configuration of 8 landmarks on the aperture face and 27 semi-landmarks to analyze the shell shape (Fig. 1). Then, the picture digitization was carried out using TPS (Thin Plate Spline; Rohlf 2010) software. First, we used the TpsRelw program (Rohlf and Marcus 1993) to calculate an iterative process of sliding semilandmarks along the contour minimizing the bending energy of the TPS function (Bookstein 1991). After sliding the semilandmarks, the total landmark

configuration was superimposed by a Generalized Procrustes Analysis (Rohlf and Slice 1990); to rotate, and translate the landmark configuration to a common origin and scale them to a unit of the centroid size. Finally, with the Procrustes coordinates of the obtained aligned individuals, we studied the shell shape in the MorphoJ software (Klingenberg 2011). As a proxy for shell size, we used the centroid size of the embryos, defined as the square root of the sum of the squared deviations of landmarks from the centroid (Bookstein 1991; Zelditch et al. 2004).

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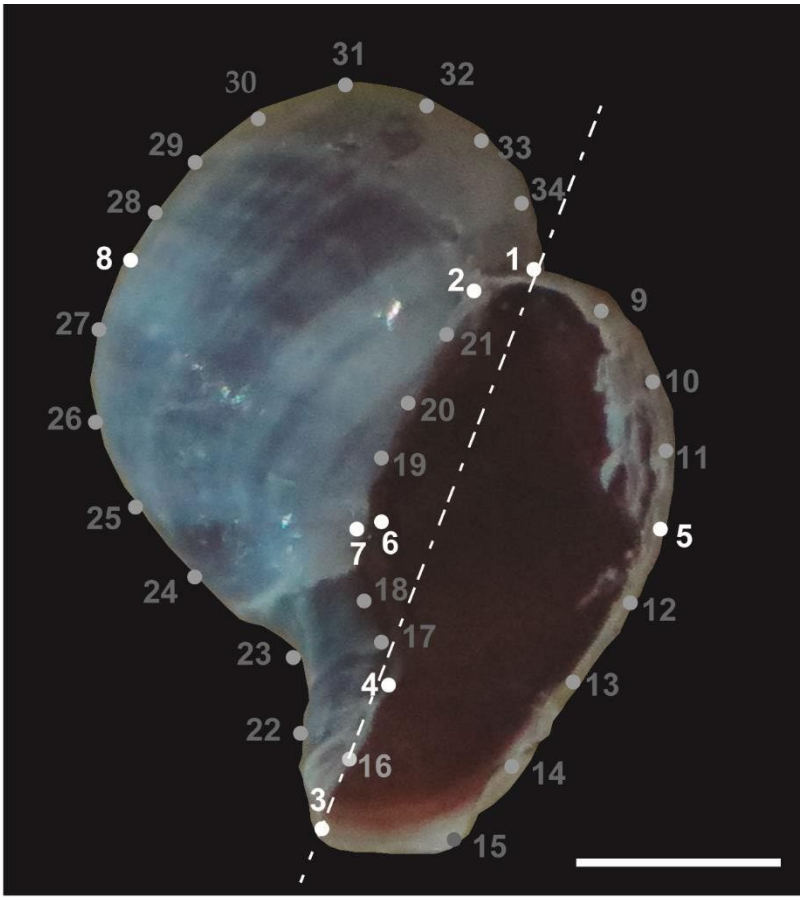

Fig. 1. Embryonic shell of *Trophon geversianus*. Landmarks (LM; white) and semilandmarks (grey) configurations are used to capture the embryo's shell shape. These include: 1- the aperture extreme of the starting point of the last whorl, 2- the union of the aperture suture with the first whorl, 3- maximum depression of columella, 4- maximum protuberance of umbilicus shell, 5- outermost tangent to the dashed line point of the lip, 6- maximum internal concavity of the aperture zone tangent with the dashed line, 7- maximum external concavity of aperture zone tangent with the dashed line and 8- maximum upper curvature of the first whorl tangent with to the dashed line, 9-11- semilandmarks slipped between LM 1 and 5, 12-15- semilandmarks slipped between LM 5 and 3, 16- semilandmark slipped between LM 3 and 4, 17-18- slipped semilandmarks between LM 4 and 7, 19-21- slipped semilandmarks between LM 7 and 2, 22-27- slipped semilandmarks between LM 3 and 8, 28-34- slipped semilandmarks between LM 8 and 1. Scale bar = 1 mm.

Statistical analysis

To compare the survival percentage among temperatures, we performed a Kaplan-Meier test with a Log-rank test (Kaplan and Meier 1958). Also, we evaluated hatching time by analysis of Variance (ANOVA) and finally compare the mean number of alive embryos per treatment with a Student's *t*-test. In both cases, the assumptions of homoscedasticity and normality were tested with a Levene and Shapiro-Wilk test, respectively. The statistical analyses were performed in GraphPad Prism 5 (CA, EE.UU.). It should be noted that to determine the survival percentage among temperatures along the experiment, the egg capsule was first evaluated qualitatively. Where those that presented an epibiotic increase, discoloration, and loss of turgor were marked as in poor condition - following the description of Cumplido 2008- recording the status and date. At the end of the experiment, its status was verified by opening and viewing its content under a magnifying glass.

To evaluate and correct the putative allometric effect we performed a multivariate regression of shape on centroid size variables (change in the shape associated with size increment) (Bookstein 1991; Monteiro 1999) for treatment. To assess the independence between the shape and size variables, we performed a permutation test with 10000 rounds (Bookstein 1991; Zelditch et al. 2004). Centroid size was examined using a one-way ANOVA. Principal component analysis was carried out to arrange the individuals on maximum shape variation axes to describe this variation. Finally, we performed a discriminant analysis to find the shell shape components that maximize the separation between temperature treatments. The difference between means was tested by the T2 Hotelling test with 1000 permutations.

RESULTS

Within the 50 egg capsules placed in the control and both treatments, we found differences in survival numbers (Figure 2). Forty-seven capsules from T13 were viable (95%), whereas for T18, 37 egg capsules were viable (75%). Finally, the T22 was not taken into account for the shape analysis, since after 60 days of the experiment, no embryos were recorded hatching from the egg capsule. Hatching times were not different between T13 and T18 (ANOVA, $F_{1,83} = 0.01$, $p = 0.92$) during the length of the experiment (90 days). The first egg capsule hatched from T18 after 71 days after starting the experiment, and the last one at 90 days was from T13 and T18. The number of embryos per egg capsule was variable in each case, registering a maximum of 35 in T18 (Table 1). Although comparing the number of live embryos between T13 and T18, the total was lower in T18, the survival differences between

them were statistically different (Mantel-Cox test $p = 0.005$). The lowest survival rate was recorded in T22, 50% after 25 days of the experiment starting and 0% at the end (Fig. 2).

Table 1. Total embryos in the 13°C and 18°C treatments, showing the maximums (Max.) and minimums (Min.) found in each egg capsule

Treatments	Total embryos	Max	Min	Alive embryos
13°C	7.6±3.2	17	2	7.4±3.3
18°C	8.4±5.3	35	3	7.5±5.4

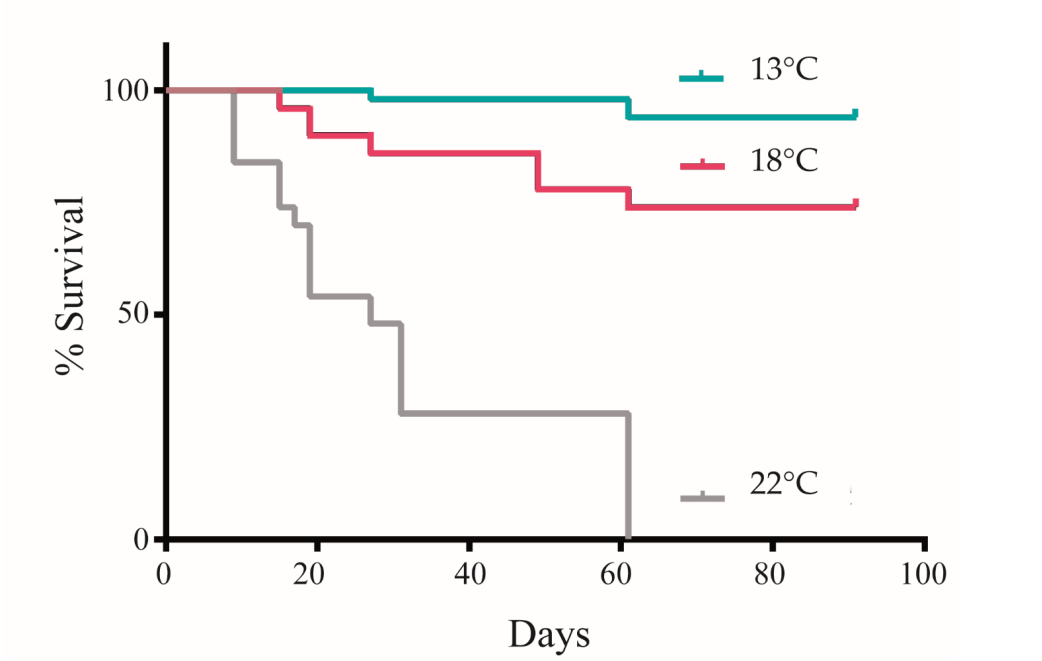

Fig. 2. Kaplan-Meier survival curve for *Trophon geversianus* embryos. Temperature treatments were 13°C, 18°C and 22°C versus the experiment duration, 90 days (N = 150, 50 egg capsules for each experimental temperature). Log-rank test: 13°C vs 18°C $p < 0.0001$; 13°C vs 22°C $p < 0.0001$; 18°C vs 22°C $p < 0.0001$.

The presence of allometry in the individuals from T13 and T18 was proved ($p < 0.0001$). Although it explained a low percentage of association between shape and size (4.48%), the subsequent statistical analyzes used the residuals of that regression as new allometry-free shape variables. The principal component (PC) 1 was related to the opening and contour of the last whorl (Fig. 3). Individuals in the negative extreme of PC1, which was associated with a globose general shape, presented constriction of both the apex and the area of the siphonal canal, with an expansion of the opening and in the last whorl. At the positive extreme, the opposite trend was recorded, resulting in a more fusiform shape.

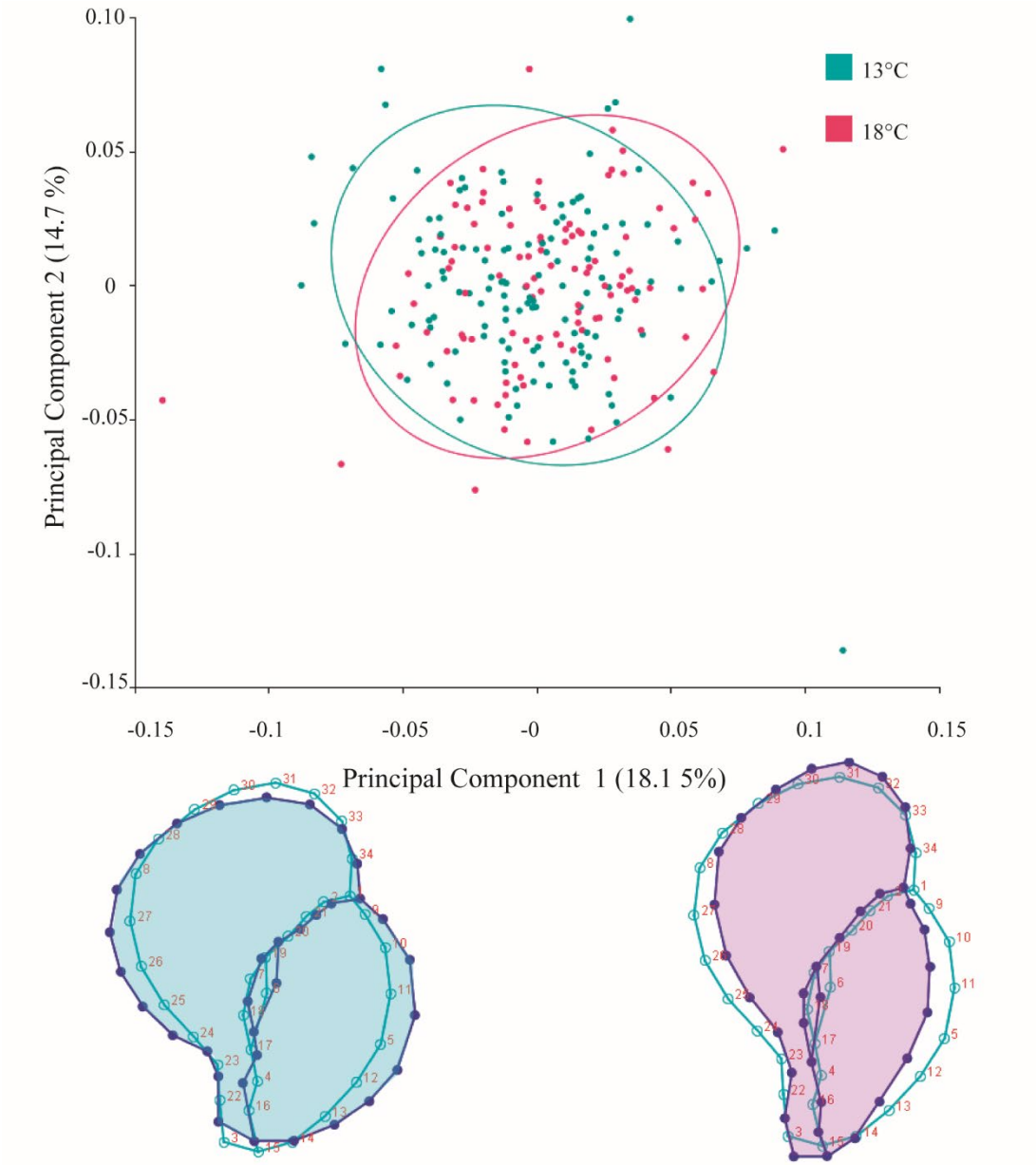

Fig. 3. Dispersion plot between the first two principal components (PC1 vs PC2) with the percentage of the variation explained by each axis. The ellipses represent 90% confidence around the mean shape of the treatments. The lower graphs represent the extremes of variation with a scale factor of -0.15 and 0.15 respectively. In both cases, the deformation is shown in color compared to the consensus form of light blue polygon.

The mean shape of the embryos extracted from T13 was statistically different from T18 (Hotelling's T-squared, $p = 0.036$). The cross-validation function indicated that the percentage of individuals correctly assigned to T13 was 77% and 58% in the assignment to T18 (Fig. 4). The general shell shape appearance of the T18 embryos was concerning an elongated shell with an expansion of the anterior aperture, and restriction on the narrowing of the contour opposite to the opening. In the case of T13 embryos, a more globose and compressed shell shape was found.

In the centroid size (CS) analysis, we found a lower CS in the T18 (5.26 mm,) than in T13

(5.7 mm) (ANOVA, $F_{1,238} = 10.3$, $p = 0.0015$). In T18, a group of individuals with sizes below the mean was observed (Fig. 5).

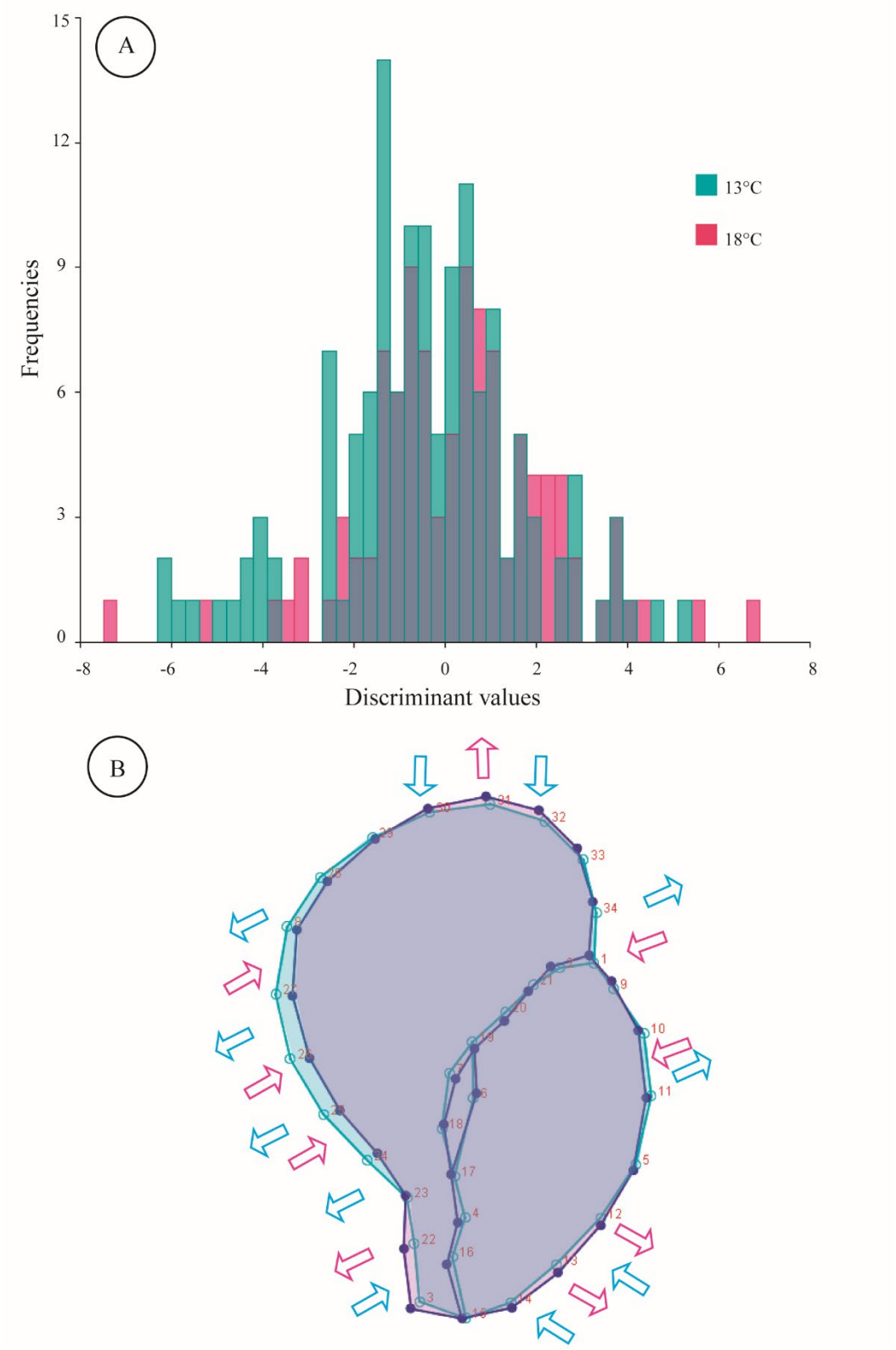

Fig. 4. A-Frequencies of discriminant values predicted by the cross-validation test. B-The image shows the mean of the shape of each treatment overlapped, increasing the variation three times. The 13°C and 18°C shapes are shown in light blue and pink, respectively. Arrows indicate the general direction of the shell shape variation.

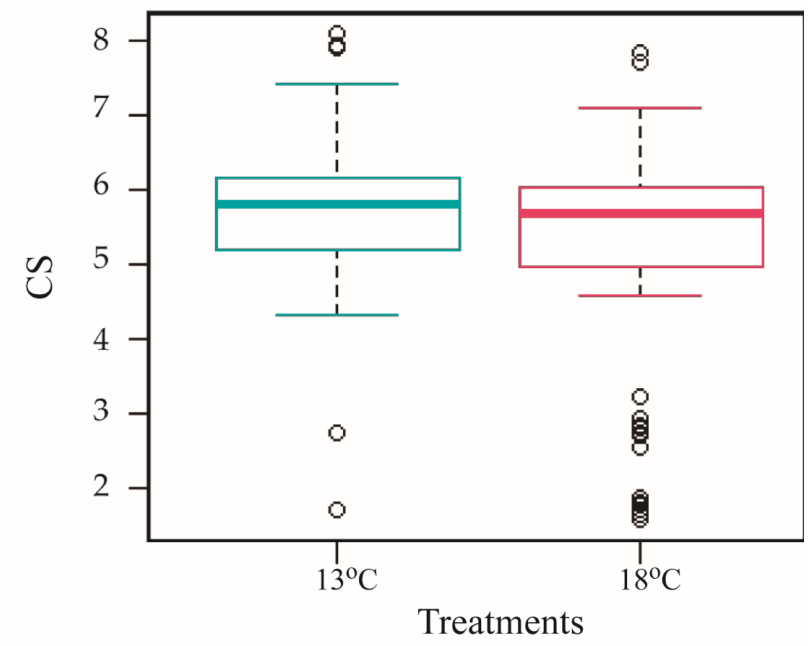

Fig. 5. Centroid size (CS) means between the treatments. The differences were significative p= 0.0015.

DISCUSSION

Water temperature is a critical environmental variable that significantly affects biomineralization processes recorded in many shelled marine molluscs (Gazeau et al. 2013) as well as the survival rate (Rawlings 1999). The present study found that temperature increase modifies *T. geversianus* subtidal embryos' shell form (size + shape) and it also affects the survival rate.

We found that the embryos from subtidal environments were tolerant to temperatures as high as 18ºC, but were not able to survive until hatch at 22ºC. These results were similar to those described in experiments of thermal and salinity tolerance in eggs from the marine snails *Bembicium nanum*, *Siphonaria denticulata,* and *Dolabrifera brazieri*, where it was shown that the increase in temperature and salinity also increased the mortality rate (Deschaseaux et al. 2010). However, they could not find a common mortality pattern, and it was determined as specific to each species (see table 1 in Deschaseaux et al. 2010). Mortality in gastropod *Thais haemastoma canaliculata* capsules was related to oxygen consumption. As the temperature increased, the larvae's oxygen consumption decreased significantly (Roller and Stickle 1989). However, studies performed on adults of *Kelletia kelletii* demonstrated that at temperatures higher than 22°C the oxygen consumption rate increased to 50% (Díaz et al. 2021). In the present work, the subtidal egg capsules of the *T. geversianus* population were

unviable at temperatures of 22°C, 4 degrees higher than the warmest temperature recorded at the studied site. Also, in this population (subtidal population of Golfo Nuevo), a failure in the thermal defense was found in adults exposed to higher temperatures than 18°C (Nieto-Vilela et al. 2022). Hence, for subtidal populations of *T. geversianus* 18°C is a limit in thermal defense for adults, and the embryos are not able to survive until hatching at 22°C. Therefore, if the most extreme projections of the global warming scenario expected for 2100 occur, the summer season will be challenging for this species (RCP 8.5 see Collin et al. 2013).

The shell shape also registered variations associated with the increase in temperature. The embryos that developed in an environment with higher temperatures presented fusiform forms, with elongated shells and wide aperture zones compared to those exposed to a controlled temperature. Aperture zone variations were reported in marine snails concerning different factors and with different consequences. Whereas smaller aperture zones were related to defense against predation in *Littorina saxatilis*, *Nucella lamellose,* and *N. lapillus* (Bourdeau 2009; Carvajal Rodríguez et al. 2005; Guerra-Varela et al. 2009), since a smaller inner surface in the aperture zone leaves less space for a crab attack (Johannesson 1986). Also, shell shape variations were described in early ontogeny in the muricid *Acanthina monodon*, founding thicker shells in embryos from egg capsules exposed to predator treatments, and thinner shells in those exposed to water turbulence (Solas et al. 2015). The elongated shell shape in subtidal embryos of *T. geversianus* exposed to higher temperatures presents a more expansive anterior aperture zone. This increase in the aperture zone could affect the crab's attack defense, so the temperature rise may lead to heightened vulnerability of *T. geversianus*.

An implication that might be seen as beneficial is that a larger apertural facilitate improved adhesion to the substrate (Kitching et al. 1966). Larger aperture zones were identified as adaptations to avoid dislodgments in *Lymnaea peregra* (Lam and Calow 1988) and *Littorina saxatilis* (Conde-Padín et al. 2007). Nevertheless, considering that the studied population inhabits a gulf where wave energy remains relatively low, the advantage of possessing a larger aperture size becomes negligible. Hence, the notably expansive anterior aperture zone observed in subtidal embryos of *T. geversianus*, which were subjected to elevated temperatures as revealed in this study, could suggest a noteworthy escalation in predation vulnerability during upcoming warming scenarios without any benefit associated with the substrate attachment.

Finally, we found differences in size related to temperature increase. The shell size of organisms exposed to higher temperatures was smaller than the control, probably due to investing energy in physiological thermal defense rather than growth, a typical consequence of warmer conditions (Daufresne et al. 2009; Moore and Folt 1993; Pöhlmann et al. 2011).

Research conducted on *L. saxatilis* demonstrated that smaller sizes faced higher predation rates from crabs (Johannesson 1986), implying that under prospective conditions, *T. geversianus* might encounter intensified predation pressures. Whereas the thicker shells found with the higher temperature were described previously in marine snails (Doyle et al. 2010; Melatunan et al. 2013) and associated with increased in calcite and aragonite saturation states with increasing temperature (Dickson 2010). Considering the previous field studies on the species concerning the shell shape variation (Márquez et al. 2015; Nieto-Vilela et al. 2021), and the phenotypic plasticity found in the early development stage provided by the present work, we consider that subtidal populations of *T. geversianus* will change their phenotype under future thermal increases by selecting smaller and more elongated shells in the ontogeny. On the other hand, we identify that embryos cannot survive to hatch at 22ºC. Future inquiries will allow us to determine the consequences resulting from these phenotypic changes. Simultaneously, they will make it easier to recognize potential adjustments in its distribution, helping to improve the knowledge degree regarding of the adaptive responsiveness of this species.

CONCLUSIONS

Under higher water temperatures, *T. geversianus* embryos change their phenotype and diminish the shell size. Also, we found a thermal tolerance limit for embryos in temperatures up to 22°C. In conclusion, the embryonic shell shape change could be used as a sentinel biomarker of thermal stress produced at early developmental stages.

Acknowledgments: We thank Julio Rúa, Nestor Ortiz and Ricardo Vera for field logistics and also the staff of the CCT CONICET-CENPAT aquarium for their support during the experiment development. This work was part of the doctoral thesis of RANV at the Universidad Nacional de la Patagonia San Juan Bosco. RANV wants to thank the BIOS institution for funding training courses on climate warming effects. These courses have not only illuminated their career path but also provided the inspiration behind the current work. Finally, we would like to express my gratitude to the anonymous reviewers for their invaluable comments and suggestions. This work was partially supported by PICT 2017-1750 (SG), PICT 2018-0969 (GB), PICT 2018-04386 (SZ), PICT2018-3197 (FM) and ANERIS project (RANV). RANV is a postdoctoral fellow of CONICET; FM, GB, SZ and SG are members of the Research Career (CONICET). Special thanks are extended to Dr. Benny K.K. Chan for his exceptionally valuable insights. This is publication #189 of the Laboratorio de Reproducción y

Biología Integrativa de Invertebrados Marinos (LARBIM).

Authors' contributions: Rocío Nieto-Vilela: Conceptualization, Data curation, Formal Analysis, Investigation, Writing – original draft, Writing – review and editing. Sebastián Giulianelli: Conceptualization, Funding acquisition, Validation, Writing – review and editing. Soledad Zabala: Conceptualization, Investigation, Validation, Funding acquisition, Writing – review and editing. Gregorio Bigatti: Investigation, Validation, Writing – review and editing. Federico Márquez: Conceptualization, Project administration, Investigation, Validation, Writing – review and editing.

Competing interests: All authors declare that they have no conflict of interest.

Availability of data and materials: The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Consent for publication: Not applicable.

Ethics approval consent to participate: Not applicable.

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Supplementary materials

Supplementary data 1. Experiment diagram. Three temperature treatments in six 30 L water racks with 25 independent jars with egg capsules in histological cassettes. (download)