Environmental stressors induced strong small-scale phenotypic differentiation in a wide-dispersing marine snail

Nicolás Bonel[1,2,3*], Jean-Pierre Pointier[4] & Pilar Alda[1,2]

[1] Centro de Recursos Naturales Renovables de la Zona Semiárida (CERZOS—CCT—CONICET Bahía Blanca), Camino de la Carrindanga km 7, Bahía Blanca 8000, Argentina.

[2] Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Argentina.

[3] Centre d'Écologie Fonctionnelle et Évolutive, UMR 5175, CNRS—Université de Montpellier, Université Paul-Valéry Montpellier—École Pratique des Hautes Études—IRD, 34293 Montpellier Cedex 05, France.

[4] PSL Research University, USR 3278 CNRS–EPHE, CRIOBE Université de Perpignan, Perpignan, France.

*** To whom correspondence should be addressed:** nicobonel@gmail.com (N. Bonel)

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ABSTRACT

Heterogeneous environments pose a particular challenge for organisms because the same phenotype is unlikely to perform best regardless of the types of stress it encounters. To understand how species meet this challenge, we investigated the extent to which contrasting selection pressures induced ecological and phenotypic responses in a natural population of a wide-dispersing marine snail. We analyzed several traits of *Heleobia australis* (Rissooidea: Cochliopidae) collected from heterogeneous habitats from the intertidal area of the Bahía Blanca estuary, Argentina. We also conducted molecular analyses by amplifying the COI gene in individuals sampled from each habitat. We found that subpopulations of *H. australis*, inhabiting close to each other and without physical barriers, exhibited a strong phenotypic differentiation in shell characters and body weight in response to environmental stress conditions, crab predation, and parasites. We proved that this differentiation occurred even early in life as most of the characters observed in juveniles mirrored those found in adults. We also found a clear divergence in penis size in snails collected from each habitat and raised in common garden laboratory conditions. The molecular analyses confirmed that the individuals studied constituted a single species despite the strong phenotypic difference among subpopulations. Altogether, the small-scale phenotypic differentiation in this planktotrophic snail can be interpreted as adaptive plasticity responses to directional selection imposed by strong gradients of abiotic and biotic stressors. These findings might contribute to a more complete understanding of the spatial scale at which adaptive differentiation can occur, which has historically been understudied in intertidal species.

INTRODUCTION

If organisms have difficulties in adapting to human-induced global change and fail to track projected environmental changes, populations become vulnerable to decline and extinction (Hill et al. 2011, Hoffmann & Sgrò 2011). Phenotypic plasticity, a common feature in nature across many taxa, is the ability of a single genotype to express different phenotypes in dissimilar biotic or abiotic environments (Travis 1994, West-Eberhard 2003). Many studies indicate a role of plasticity in shaping phenotypic responses as an effective mechanism that can greatly improve the conditions for persistence of populations facing abrupt environmental shift by facilitating rapid adaptation to the new selection pressures (Price et al. 2003, Ghalambor et al. 2007, Chevin & Lande 2009). Adaptive plasticity that places populations close enough to the new favored phenotypic optimum for directional selection to act provides the first step in adaptation to new environments on ecological time scales (see review in Ghalambor et al. 2007). In other words, such plasticity—when operates jointly with directional selection—can facilitate the process of adaptive evolution in a new environment (De Jong 2005, Fusco & Minelli 2010, Merilä & Hendry 2014) by which non-heritable environmentally induced variation leads to adaptive heritable variation, the so-called Baldwin Effect, or more commonly known as 'genetic assimilation' (reviewed in Waddington 1953, West-Eberhard 1989, Pigliucci et al. 2006; but see West-Eberhard 2003). In this sense, adaptive phenotypic plasticity is crucial for the long-term persistence of populations facing new environmental stress brought about by human-induced global change (Hill et al. 2011).

Temperate marine habitats, in particular the intertidal zone, exhibit great variability in environmental factors mainly driven by the fall and rise of the tides, creating areas on the shore that are alternately immersed and exposed (Denny & Paine 1998, Bourdeau et al. 2015). The transitional nature of the intertidal habitat from marine to terrestrial conditions strongly influences the physiology and the ecology of intertidal organisms due to the increase in environmental harshness (e.g. desiccation, extremes in temperature and salinity) along the vertical zonation of the intertidal area (Vermeij 1972, Denny & Paine 1998, Mouritsen et al. 2018). From this perspective, intertidal organisms, such as aquatic gastropods, are likely to exhibit increased adaptive phenotypic plasticity in response to such environmental selective pressures (Berrigan &

Scheiner 2004). Despite the intertidal area spans a strong selective gradient, small-scale phenotypic divergence and adaption of intertidal organisms has been underestimated (Hays 2007; see also review by Sanford & Kelly 2011).

Phenotypic variation in shell traits is particularly widespread in gastropods (Bourdeau et al. 2015) and their shells offer an easily measured and permanent record of how the organism responded to local abiotic (e.g. water chemistry, temperature) and biotic (e.g. predation, parasitism) agents (Dillon et al. 2013). Major environmental factors as prolonged submersion times, desiccation, high temperature, and extreme salinity across the vertical zonation create contrasting selective pressures that give rise to opposite shell trait responses (Struhsaker 1968, Johannesson & Johannesson 1996). For instance, prolonged submersion times enhance foraging times and the absorption of calcium carbonate from water leading to higher shell growth rates, which result in larger and narrower shells and increased body mass (Vermeij 1973, review by Chapman 1995). By contrast, snails from upper intertidal habitats that are periodically exposed to hot and dry conditions and have only a limited time each day to feed exhibit the opposite shell and body mass responses and are characterized by having a smaller aperture size, which has been correlated to an increase in resistance to high desiccation in upper intertidal habitats (Machin 1967, Vermeij 1973, Chapman 1995, Melatunan et al. 2013, Schweizer et al. 2019).

Predators are one component of the heterogeneity across the intertidal zone because they are often distributed patchily in time and space creating a steep selection gradient in predation risk, which, in turns, can promote an adaptive evolutionary shell response (Boulding & Hay 1993, Bourdeau 2011). Predator-induced plasticity primarily linked to shell defenses are not limited to shell thickening (more resistant to crushing) as snails are capable of plastically altering different aspects of their shell shape (i.e. increased globosity; lower shell length to width ratio) or aperture (i.e. more elongated; higher aperture length to width ratio), which prevents the crab from pulling the soft parts out of the shell (Johannesson 2003, Dillon et al. 2013, Bourdeau et al. 2015). However, not all phenotypic plasticity triggered by environmental conditions is adaptive (Ghalambor et al. 2007). Some plastic trait responses, usually those imposed by the biochemistry and physiology of the organism, can be reversed over short time scales, which is not the

case for developmental plasticity as it tends to be irreversible or takes longer to be reversed (Pigliucci et al., 2006). Parasites, for instance, can induce morphological, behavioral, and physiological change of individual snail hosts thereby influencing several aspects of host life history that can significantly alter size structure, demography, resource use, and intra and interspecific interactions of host population (Fredensborg et al. 2005, Miura et al. 2006, Mouritsen et al. 2018). They have been also reported to induce changes in microhabitat choice (Curtis 1987) and in body size in snails (Mouritsen & Jensen 1994, Probst & Kube 1999, Levri et al. 2005, Miura et al. 2006, Alda et al. 2010). Such pressure exerted by parasites also differs along the intertidal zone (Smith 2001, Alda et al. 2010, 2019) and can cause important shifts in the expression of host phenotypic traits, creating pronounced phenotypic differences between infected and uninfected hosts (Poulin & Thomas 1999, Fredensborg et al. 2006).

These contrasting conditions along the vertical distribution pose a particular challenge for intertidal organisms: the same phenotype is unlikely to perform best regardless of the types of stress it encounters. We can then have as a result (i) the same phenotype everywhere (probably with maladaptation), (ii) a phenotypic plasticity promoting different phenotypes under different conditions, or (iii) a local genetic differentiation, contributing to the phenotypic variation between habitats, favored by restricted gene flow through habitat selection (and probably partial genetic isolation leading to sympatric speciation or cryptic species). Limited gene flow can occur when habitat conditions disrupt dispersal of planktotrophic larvae thereby increasing localized recruitment whereat individuals would be subjected to local (a)biotic selective pressures (Struhsaker 1968, Johannesson & Johannesson 1996, Levin et al. 2006), namely ‘phenotype-environment mismatch’ (reviewed in Marshall et al. 2010). Hence, to understand how species meet this challenge, the first step is to observe phenotypic variation and its association in the field with microhabitats characterized by different sources of stress. If we observe different phenotypes associated with different conditions, the next step is to explore whether such phenotypic variation results from a direct impact of stress (e.g. trait shift induced by parasites) or is it because we are dealing with a cryptic or incipient sympatric species that find alternative niches (e.g. different microhabitats) preventing competition.

Here we tested how strong selection imposed by thermal, saline and dehydration stress, crab predation risk, and parasitism across the intertidal gradient affected snail density and induced different phenotypic responses among individuals from each subpopulation of a wide-dispersing intertidal snail. To do this, we collected and measured shell traits, weighted, and dissected the mud snail *Heleobia australis* (Rissooidea: Cochliopidae) over the four seasons throughout a year from three distinct habitats from the intertidal area of the Bahía Blanca estuary, Southwestern Atlantic, Argentina. Then, we collected juveniles from each habitat and raised them in common garden laboratory conditions until they were adults to investigate if genital morphology varied. Finally, we explored whether individuals living in these contrasting habitats belong to the same species, and not to a species complex, by amplifying the cytochrome oxidase subunit 1 (COI) gene. Altogether, we found clear evidence of small-scale phenotypic differentiation in all traits likely as a result of adaptive plastic responses to strong selection pressures across the vertical gradient.

MATERIAL AND METHODS

Study species

The intertidal mud snail *Heleobia australis* has a wide geographic range inhabiting marine and estuarine ecosystems from tropical to temperate regions (from Brazil: 22° 54’ S, to Argentina: 40° 84’ S; De Francesco & Isla, 2003) and is the most common benthic macrofaunal species in the estuaries and coastal lagoons. It is a gonochoristic species with internal fertilization (Neves et al. 2010). Mature females lay egg capsules, containing one fertilized egg (Neves et al., 2010), preferably on shells of conspecific individuals, but they may also be laid on shells of other species, on sand grains, or algae, which later develop into a veliger larva that exhibits a short pelagic larval life (Neves et al. 2010), which is, on average, 10±3 days in standard laboratory conditions (12:12 photoperiod, 25 ºC, water salinity 30 PSU, and *ad libitum* food in the form of boiled ground lettuce). Adult snails attain a maximum shell size ranging from 7 to 8 mm and individuals have an averaged shell length of 4.7 mm (1.1 SD) with a lifespan of 2.9 years in a temperate climate and two well-defined recruitment episodes (Carcedo & Fiori 2012). However, in tropical and subtropical areas breeding and recruitment occur year-round (Neves et al. 2010) suggesting that it is likely that *H.*

australis have shorter generation times. This species is capable of creating an air bubble inside its pallial cavity, allowing juveniles and adults to temporarily float in the water column, whereat they are carried along by the tide and wind driven current (Echeverría et al. 2010), increasing its dispersion potential. Moreover, *H. australis* is an obligate intermediate host of a large diversity of parasites that infect several other species hosts. For instance, *H. australis* has been reported to host 18 larval trematode species and 16 of these were from the Bahía Blanca estuary (Argentina), being all but one parasitic castrators, with an individual worm eventually filling up the digestive gland and gonad of the snail host (Alda & Martorelli 2014).

Study area and (a)biotically heterogeneous habitats

This study was conducted in the Villa del Mar saltmarsh-mudflat located in the middle reaches of the Bahía Blanca estuary, Argentina (38° 51’ S – 62° 07’ W). The intertidal mudflat extends for more than 1 km across the tidal gradient. Its topography is characterized by a very gently sloping ramp on the seaward side (Pratolongo et al. 2009) and affected by strong semidiurnal tides and high seasonal variation (Perillo et al. 2001). Close to the mean high tide level, marshes covered by cordgrass (*Spartina alterniflora*) form a narrow, 150 m wide strip of vegetation followed by a mudflat area with no vegetation (Pratolongo et al. 2010). During low tide, these upper areas of the intertidal zone are subjected to high desiccation and temperature and salinity fluctuations as the tide takes *ca*. 10 hours to cover them (P. Pratolongo, *personal communication*; 26 February 2019) whereas low areas located close the seaward edge remain covered by water during low tide.

The existence of a tidal cycle exposes *H. australis* to extreme, but predictable, changes in abiotic conditions (at least twice every day) likely determining their vertical distribution (zonation patterns). Snails in the upper intertidal zone must remain quiescent for long periods of time during daylight hours or during low tide, being exposed to aerial conditions for longer periods than individuals in the lower intertidal zone, which remain cover by water during low tide. The intertidal zone can therefore be characterized in three distinct habitats: flats, marshes, and pans. Flats and marshes are located in the upper zone and they drain at low tide, though flats are free of vegetation and marshes are covered by cordgrass. Pans are free of vegetation but remain covered

by water during low tide and are located close the seaward edge. Thermal, saline, and dehydration stress are strong selective forces occurring mainly in the upper intertidal area (flats and marshes; Denny & Paine 1998, Bourdeau et al. 2015) whereas these inducing agents are weaker in pans, which exhibit low environmental stress condition (Fig. 1).

Biotic agents also vary along the vertical distribution of the intertidal zone (Fig. 1). On the one hand, the grapsid crab *Neohelice granulata* is one of the most abundant macroinvertebrates of intertidal areas of the SW Atlantic estuaries where it commonly inhabits the upper vegetated area (marshes) but uses the entire intertidal zone (Spivak et al. 1994, Alvarez et al. 2013, Angeletti et al. 2014, Angeletti & Cervellini 2015). This burrowing crab could exert a strong pressure on *Heleobia australis* since it can drastically reduce snail density in vegetated areas due to an intense bioturbation activity (Spivak et al. 1994, Alvarez et al. 2013, Angeletti et al. 2014, Angeletti & Cervellini 2015) and/or through snail predation (D'Incao et al. 1990, Barutot et al. 2011). On the other hand, parasite pressure also differs along the intertidal zone. Trematode infection not only inevitably leads to snail castration reducing its fitness to zero (Alda et al. 2019) but also gives rise to smaller shell-sized morphs for infected snails (Alda et al. 2010). The prevalence (percentage of individuals infected) of trematodes is higher in the lower area of the intertidal zone, where parasite infection is predominately caused by one extremely prevalent trematode, *Microphallus simillimus.* Such pressure is stronger in pans than in flats and marshes because prolonged submersion times allow snails to increase time spent foraging and thus ingesting parasite eggs (Alda et al. 2019).

Field sampling and laboratory procedure

We sampled individuals of the intertidal mud snail *H. australis* in summer (March 6), autumn (July 15), winter (September 20), and spring (December 7) of 2012 in a one-hectare plot. We randomly took nine samples from each habitat (flats, marshes, and pans) with 10 cm diameter and 2 cm deep circular samplers (Area = 78.5 cm^2). Snails were sieved from the sediment through a 1 mm-mesh, then transported alive to the lab, kept in aquaria, and fed *ad libitum* with flake fish food.

All 10,367 snails were used to estimate snail density and were also photographed using

a camera attached to a dissecting microscope to analyze shell and aperture morphometric (see below). A random subset of snails (n = 6,250) was used to account for infected and uninfected snails (hereafter infection status) in each habitat. The remaining uncrushed snails were used to estimate snail shell and body weight (n = 3,057; see below) and parasite biomass (n = 1,060).

Snail density and infection status

To test how contrasting habitat conditions affected snail density, we counted all sampled individuals per habitat and sampling date to analyze spatial and temporal variation of snail density. Then, to determine infection status, snails from each habitat were crushed using a mortar and a pestle, tissue was examined under a dissecting microscope, and trematodes were identified under a compound microscope following Alda & Martorelli (2014). Previous studies show that the most prevalent parasite in the study area is *Microphallus simillimus* (Microphallidae), which makes up 87% of the overall prevalence (Alda & Martorelli 2014, Alda et al. 2019). This parasite has life history traits such as abbreviated life cycle (the same snail host can serve as the first- and second-intermediate host), meaning that once snails ingest parasite eggs, the metacercariae encyst within the sporocyst in the infected snail, thereby maximizing transmission success (Alda & Martorelli 2014). One possible approach to the contribution of parasites to the phenotypic variance in host populations is to compare phenotypic responses of uninfected and infected hosts maintained under identical conditions (Poulin & Thomas 1999). Thus, to test for this, we only considered snails infected by the most prevalent trematode *M. simillimus* and also from pans (the habitat with the highest prevalence, Alda et al. 2019), which implies that both infected and uninfected snails grew in the same environmental conditions. By doing so, we can ensure that differences in phenotypic responses can only be attributed to *M. simillimus* effect and not caused by other trematodes or habitat stress conditions.

As older hosts have greater cumulative risk of infection than do young hosts, each sample should be standardized by age, collected at the same time from an area in which hosts are likely to mingle and where they have experienced relatively uniform risks of infection (Lafferty et al. 1994). We therefore identified age cohorts by means of the length-frequency distributions in each sampling date and habitat and removed those

individuals outside the lower 95% confidence limit of the cohort with larger individuals (see Appendix S1 in Supporting Information, '*Snail length-frequency distribution and cohort identification*' for details; Fig. S1).

Shell and aperture morphometric

To analyze variation in shell morphometric across habitat conditions and infection status, we measured four linear variables from each photograph using ImageJ software: shell length (SL), shell width (SW), aperture length (AL), and aperture width (AW). We followed this approach rather than the geometric morphometric analysis because it allowed us for measuring a larger number of individuals (i.e. 10,367 snails). Similar to some other caenogastropod species (e.g. *Littoridina* sp.), the shell of *H. australis* is generally small and conical and the axis of coiling lying at an angle of about 45º above the plane of the elliptical aperture (Vermeij 1973, Hershler & Thompson 1992). We thus used SL and SW to calculate the shell size by calculating the volume of a cone:

$$\text{Shell size: Volume of a cone} = 1/3\ \pi\ (SW/2)^2\ SL$$

To analyze the shell shape, we estimated the SL to SW ratio. Likewise, we analyzed aperture shape by calculating the AL to AW ratio. We used AL and AW to calculate aperture size by calculating the area of an ellipse:

$$\text{Aperture size: Area of ellipse} = (AL/2)\ (AW/2)\ \pi$$

Shell thickness and body mass

To test whether snails subjected to a stronger predation pressure exhibit thicker shells, (considered to be a shell defense trait against predators as it is more resistant to crushing; (Johannesson 2003, Dillon et al. 2013, Bourdeau et al. 2015) and whether contrasting environmental stress conditions affected body mass, we estimated and compared shell weight and ash-free dry-weight among habitats. We used a subsample of individuals (n = 70), representative of the population shell length-frequency distributions obtained previously for all individuals sampled on each date and habitat. We removed sediment and epibiota with a scouring pad before weighing and measuring the organic content of the snail as ash-free dry weight (AFDW, calculated as the

difference between the dry weight and the weight of the incombustible component of the shell), which was considered as body mass. To achieve this, we dried snails individually in porcelain crucibles for 48 h at 60 °C, weighed them with a digital scale (precision 0.1 mg), ashed the snails for 5 h in a muffle furnace at 500 °C and then reweighed them (Bonel & Lorda 2015). The ash shell weight (the incombustible component of the shell) was considered to be an equivalent of shell thickness and used to estimate and compare its variation among individuals along the vertical gradient of the intertidal area.

Variation in penis size

To test for differences in genital morphology, we analyzed variation in the penis size and morphology of individuals from contrasting habitats conditions living in sympatry but raised in common garden laboratory conditions. We sampled snails of *H. australis* from the three distinct habitats of the intertidal area of the Villa del Mar saltmarsh-mudflat in November (Spring) of 2017. Snails were transported to the lab and they were split into adults and juveniles based on their shell-length distribution (as mentioned above). Adults were preserved in alcohol 96% and used to conduct molecular analysis. Juveniles were kept them in three different aquaria (one for each habitat) in common garden laboratory conditions (12:12 photoperiod, 25 ºC, water salinity 30 PSU, and *ad libitum* food in the form of boiled ground lettuce) for 21 months, which ensured that all individuals considered in the analysis were adults. Prior to dissection, we collected, on average, 30 snails from each aquaria and preserved them in the Railliet–Henry's solution (930 ml distilled water, 6 g NaCl [0.85%], 50 ml formaldehyde [37%], 20 ml glacial acetic acid). Then, they were all dissected under the dissecting microscope and the reproductive system of *ca*. 15 males per aquarium was drawn using a camera lucida attachment (Pointier et al. 2004). These were scanned and the penis surface area was calculated using the ImageJ software. Shells were photographed and analyzed as mentioned above to estimate shell size and shape.

COI analysis

To confirm that individuals living in these contrasting habitats belong to the same species and not to a species complex, we conducted molecular analyses by amplifying the cytochrome oxidase subunit 1 (COI) gene in 10 individuals sampled from each of

the three sites. Then, we built a gene tree and a haplotype network to verify if individuals gather in clusters depending on the habitat they come from. We also applied a species-delimitation method: Automatic Barcode Gap Detection (ABGD, Puillandre et al. 2012). ABGD detects barcode gaps, which can be observed whenever the divergence among organisms belonging to the same species is smaller than divergence among organisms from different species (see Supporting Information for specific details on these procedures).

Statistical analyses

To test for spatial and temporal variation of snail density, we performed a two-way ANOVA including habitats (flats, marshes, and pans) and seasons (summer, autumn, winter, spring) as fixed effect, and their interaction. We transformed density (natural-log) data to meet assumptions of normality and homoscedasticity.

To test for phenotypic variation in shell characters, we defined three categories (cat.) of snails that comprised: (*i*) uninfected adults, (*ii*) infected adults, and (*iii*) uninfected juveniles. These three categories (cat. *i*–*iii*) were considered to test whether contrasting (a)biotic stress conditions (environmental stress and crab predation) induced variation in shell traits (morphometric and thickness) and body mass. To evaluate the effect exerted by the trematode *Microphallus simillimus* on shell morphometry of *Heleobia australis* from pans (the habitat with the highest prevalence), we created a fourth category that included uninfected and infected adult snails (cat. *iv*). We performed four Principal Components Analyses (PCAs) to visualize the main components of the morphological shell variation for each snail category. Then, we tested for differences in shell morphology (size and shape of the shell and aperture) by fitting independent linear mixed models (LMMs), with a Gaussian error distribution, for the above-mentioned four categories (cat. *i*–*iv*).

To test the effect of habitat conditions on shell traits (cat. *i*–*iii*), we considered habitat as a fixed effect. To test for morphometric differences between infected and uninfected (cat. *iv*), we included infection status (infected and uninfected) as a fixed effect as we only considered individuals from one habitat (i.e. same habitat conditions for infected and uninfected snails). For all the four categories (cat. *i*–*iv*), we included sex (male and

female) as a fixed factor (and its interaction with habitat or infection status). We added sampling season as a random factor in all models as it can explain a significant portion of the variance in measured variables. Likewise, we also considered shell size as a covariate when testing for shell and aperture shape, but the models were all ran with and without this effect to check the extent to which the effects were mediated by shell size. We also incorporated shell size as a covariate when testing for shell thickness and body weight (by means of LMMs) and estimates were statistically corrected for variation that could be explained by the covariate. Statistical significance of the fixed effects was obtained from model comparisons using likelihood-ratio tests. Random effects were separately tested using chi-square likelihood-ratio tests with the corrections indicated by Zuur (2009). To test for differences in penis size (area of the penis), we performed a linear model with habitat as a factor and we added shell size (volume) as a covariate, which was used to statistically correct estimates for variation explained by the covariate. All individuals analyzed were free of parasites, meaning that the differences found are not due to parasite effect.

Post-hoc tests were performed when the effects were significant (P <0.05), using Holm-Bonferroni correction for multiple testing to compare their effects. Values are given as means ± 1 SE unless otherwise stated. All analyses and figures were performed with R v.3.3.3 packages *lme4* (Bates et al. 2014), *nlme* (Pinheiro et al. 2017), *car* (Fox et al. 2011), *effects* (Fox 2003), *MASS* (Ripley et al. 2013), *plyr* (Wickham 2011), *dplyr* (Wickham et al. 2019a), *devtools* (Wickham et al. 2019b), *ggbiplot* (Vu 2011), *psych* (Revelle 2018), *ggplot2* (Wickham & Chang 2008), and *outliers* (Komsta 2011).

RESULTS

Overview

Overall, the mud snail *Heleobia australis* showed a strong variation in snail density and a remarkable variation in several traits linked to contrasting (a)biotic selective pressures (environmental stress, predation, or parasite infection) occurring along the vertical zonation of the intertidal area. Tables 1 to 3 summarize descriptive statistics on density and shell traits measured for the first and fourth snail categories (cat. *i* and *iv*). In the appendix, we reported results of the snail density split by age classes (juvenile and adult; Table S1), principal components analyses (Table S2), descriptive statistics and

the linear mixed models for the four snail categories on shell traits (Tables S3–S8) and for shell weight and body mass (Table S9).

Strong habitat and seasonal effect on snail density

We found spatial differences in total density (including juveniles and adult snails; Table 1; Fig. 2). We observed no significant interaction between habitats and seasons ($F_{(6, 106)} = 1.56$, $P = 0.167$). Marshes showed the lowest density whereas flats and pans showed a marginal non-significant difference (Table 1). We found significant differences between seasons (Table 1). Density was higher in spring than in autumn, winter, and summer (Table 1). Snail density in autumn did not differ from that of summer or winter, these latter two being not different either (Table 1), but it largely increased from autumn to spring (particularly in pans as compared to flats and marshes; Fig. 2). This increase across seasons was mainly driven by juvenile recruitment (Table S1; Fig. S2). By contrast, the effect of habitat conditions and seasons on adult density showed a significant interaction because density was higher in pans from autumn to winter whereas it decreased in marshes for the same period, and in flats it decreased from summer to autumn and the than remained fairly constant (Table S1; Fig. S2).

Main components of the morphological shell variation within each snail group

For each of the snail categories (cat. *i*–*iv*), principal component analyses decomposed the shell morphometric measures (shell and apertures size and shape) into four principal components of which the first two explained, overall, more than 73% of the variance in the original data (Table S2; Fig. S3). The component loadings showed that the shell and aperture size are highly correlated one another and with PC1, whereas PC2 highly contrast shell shape with aperture shape. In other words, the first component was strongly correlated with size indices, and the second component with shape (further details in Table S2). The distribution of individual data points from flats and marshes highly overlapped, and this was consistent in all the three snail categories (cat. *i*–*iii*). This constituted one group that clearly differed from the other group of data points that was composed by individual observations from pans (Fig. S2). Likewise, the scatter of the data showed two groups when decomposing the variation of morphometric measures between infected and uninfected adult snails (cat. *iv*).

Strong habitat effect on shell morphometric

The morphometric responses to habitat conditions were similar in all the three snail categories analyzed (cat. *i*–*iii*). For simplicity, we present results and figures only for uninfected adult snails (cat. *i*) in the main text and those for infected adults (cat. *ii*) and juvenile snails (cat. *iii*) are shown in Supporting Information (Tables S4-S7; Figs. S4-S5).

Shell size

We found a significant interaction between fixed effects (habitat and sex; $X^2_2 = 8.42$, $P = 0.015$). This was mainly driven by differences in this trait between sexes in pans, whereas such difference between males and females was not detected in snails from flats and marshes. In other words, males exhibited a larger shell size (mm^3) compared to females in pans, meaning that the sex effect was much stronger in creating size differences between sexes compared to flats and marshes where the sex effect was weaker resulting in no difference in size between sexes. By contrast, the effect of habitat conditions was stronger in both sexes. Both male and female snails from flats and marshes showed a smaller shell size (a decrease of 12 and 22 % in shell size; respectively) relative to individuals from pans (Tables 2 and S3; Fig. 2).

Shell shape

The interaction between fixed effects was significant ($X^2_2 = 8.20$, $P = 0.017$), but when we statistically corrected for shell size, it became marginally non-significant ($X^2_2 = 5.69$, $P = 0.058$). Snails from pans (sex pooled) exhibited the most elongated shells relative to individuals from marshes and flats. At the sex level (habitats pooled), males had a more elongated shell shape than females (Table 2 and S3; Fig. 2).

Aperture size

We found a strong habitat and sex effect on aperture size; the interaction between these variables was not significant ($X^2_2 = 3.05$, $P = 0.217$). Across habitats (sex pooled), snails from pans showed the largest aperture size (mm^2) whereas in flats and marshes it was 9 to 12% smaller, respectively. Moreover, we observed a significant difference in aperture size between sexes (habitats pooled), males had bigger apertures than females and this difference was consistent across habitats (Table 2 and S3; Fig. 2).

Aperture shape

We found no significant difference in aperture shape between sexes (X^2_1 = 0.65, P = 0.724), and this pattern was similar even after correcting for shell size (X^2_2 = 2.20, P = 0.333). However, individuals from pans (sex pooled) showed the most rounded shape relative to those from flats and marshes, which showed a more elongated aperture shape (Table 2 and S3; Fig. 2).

Pronounced trematode effect on shell morphometric

Infection caused by the trematode *M. simillimus* strongly shifted the mean value of most phenotypic traits of *H. australis* to lower values when comparing infected and uninfected snails from pans.

Shell size

Shell size of infected snails (sex pooled) decreased 10% relative to uninfected ones, whereas females (infection status pooled) were 5% smaller than males (Table 3; Fig. 4). We found no significant interaction between the fixed effects (status and sex; X^2_1 = 0.70, P = 0.402).

Shell shape

We found a significant interaction between status and sex (X^2_1 = 15.86, P <0.001). This was because, at the status level, uninfected males were more elongated (higher SL to SW ratio) than uninfected females, but infected males and females showed no difference in shell shape. We found the same pattern at the sex level; that is, infected male and female snails have a more elongated shell relative to uninfected male and female individuals (Table 3; Fig. 4).

Aperture size

We observed a strong effect of infection status and sex in aperture size. Infected snails (sex pooled) and females (status pooled) had a smaller aperture than uninfected and male snails (15 and 3% respectively; Table 3; Fig. 4). We found no significant interaction between fixed effects (X^2_1 = 2e-04, P = 0.990).

Aperture shape

Uninfected snails exhibited a more elongate aperture shape (higher AL to AW ratio) than infected individuals whereas we found no sex effect on this shell trait (Table 3; Fig. 4). We found no significant interaction between fixed effects and no sex effect on aperture shape, even after correcting by shell size (X^2_1 = 0.33, P = 0.564).

Thicker shells and lower body mass in habitats with high (a)biotic stress

We found that snails differed in shell weight (~ thickness) across habitats (X^2_2 = 48.93, P <0.001). Snails from pans showed the thinnest shells (11.53±0.23 mg) compared to snails from marshes (12.54±0.15 mg; $P_{\text{Pans<Marshes}}$ <0.001) and flats (12.00±0.15 mg; $P_{\text{Pans<Flats}}$ = 0.001). By contrast, in marshes individuals showed the heaviest/thickest shells ($P_{\text{Marshes>Flats}}$ <0.001; Fig. 4). Body mass also differed across habitats (X^2_2 = 19.67, P <0.001). Snails from pans exhibited the heaviest body mass (0.50±0.05 mg) relative to individuals from marshes (0.32±0.03 mg) and flats (0.33±0.04 mg) ($P_{\text{Pans>Marshes}}$ <0.001; $P_{\text{Pans>Flats}}$ <0.001), which showed no differences in body weight ($P_{\text{Marshes<Flats}}$ = 0.390; Fig. 5). Further details on observations, corrected and uncorrected mean values, and statistics are indicated in the appendix (Table S9; Fig. S6).

Variation in penis size

The penis shape confirmed that individuals from each habitat of the Bahía Blanca estuary belong to the same species (Fig. S8; Gaillard & Castellanos, 1976). We found, however, a clear difference in penis size ($F_{(2, 39)}$ = 8.42, P <0.001). Individuals from marshes showed the largest size relative to flats ($P_{\text{flats<marshes}}$ <0.001) and pans ($P_{\text{pans<marshes}}$ = 0.034); snails collected from these two habitats showed a marginal non-significant difference ($P_{\text{flats<pans}}$=0.090; Fig. 6). We found no significant effect of shell size, meaning that differences in penis size were not due differences in individual's shell size ($F_{(1, 39)}$ = 0.27, P=0.608). Further, we found no differences in shell shape, even after controlling by shell size ($F_{(2, 43)}$ = 0.851, P = 0.435).

Molecular analysis of the cytochrome oxidase subunit 1 (COI) gene

The species-delimitation analysis implemented in ABGD found only one partition (prior maximal distance, P=0.001) that confirmed that the individuals here studied constitute a single *Heleobia* species, which is in agreement with the similar penis shape found

among habitats. In fact, we did not observe any type of structure among individuals. The gene tree and haplotype network showed that individuals did not gather in clusters depending on the habitat they come from (Figs. S8 and S9).

DISCUSSION

This study provides clear evidence of ecological and phenotypic trait variation in response to contrasting biotic and abiotic conditions at a small scale. Snail density and most of mean values of phenotypic traits measured shifted to lower values in habitats with high physical stress conditions (flats and marshes), crab predation (marshes), and parasite (pans) pressure (summarized in Fig. 7). These findings support the standpoint that *Heleobia australis* might have experienced a fine-grained environment where the combined effect of directional selection and adaptive plasticity led to phenotypic divergence in response to contrasting selection pressures across the vertical gradient. In the following, we discuss these main findings with emphasis on the abiotic and biotic stressors that have shaped adaptive phenotypes among subpopulations of this intertidal snail.

Reduced snail density under high environmental and predatory stress

Density of *Heleobia australis* was the lowest in marshes (vegetated area that drains at low tide) relative to flats (unvegetated area that drains at low tide) and pans (covered by water at low tide) whereas flats and pans showed no difference. However, when analyzed by age classes, adult density was higher in pans during winter but remarkably low in marshes, even compared to flats (both habitats from the upper area). This is consistent with the idea that prolonged submersion times could increase the survival rate of snails during harsh climatic conditions (Fig. S2). As for juvenile snails, their density increased from autumn to winter in all the three habitats and kept increasing until spring time where it was 1.6 and 2.4 times higher in pans relative to flats and marshes (Table S1; Fig. S2). We found little support for the idea that intense environmental stress, combined with harsh climatic conditions, could strongly decrease their survival rate affecting their abundance, especially, in the upper area. We found, instead, that conditions at pans might have favored juvenile recruitment, which could explain the differences observed in spring.

It is puzzling, however, that total density in marshes was low relative to flats because *H. australis* is positively associated to marsh plants (the smooth cordgrass *Spartina alterniflora*, which dominates the lower marsh), as they buffer physical stress factors (thermal and dehydration stress) relative to uncovered areas, promoting snail aggregation (Canepuccia et al. 2007 and references therein). One possible explanation for the low density in marshes, relative to flats, is if this resulted from a negative interaction between *H. australis* and the grapsid crab *Neohelice granulata*, whose aggregation is also facilitated by marsh plants (Spivak et al. 1994, Alvarez et al. 2013, Angeletti et al. 2014, Angeletti & Cervellini 2015) where it attains density peaks in late spring and early summer but it is absent throughout the year in unvegetated areas (flats or pans; (Angeletti & Cervellini 2015). It is therefore likely that the low density of *H. australis* in vegetated crab-rich habitats resulted from an increased bioturbation activity caused by *N. granulata*, particularly during that period of time (Alvarez et al. 2013). Another mutually non-exclusive hypothesis is that this difference between marshes and flats could result from a higher predation pressure caused by this crab (D'Incao et al. 1990, Barutot et al. 2011), in which case it might have induced predatory shell defenses as shell thickening or narrower apertures (Boulding & Hay 1993, Johannesson & Johannesson 1996, Rolán-Alvarez 2007) review by (Bourdeau et al. 2015). Accordingly, snails from marshes exhibited a strong variation in shell characters that support this hypothesis, which we discuss later in this section.

Snails exhibited larger, narrower, and thinner shells and heavier body mass in habitats with low stress conditions

Snails subjected to different levels of stress showed a strong phenotypic variation. In all categories analyzed (cat. *i–iii*), individuals showed a clear shift in their phenotype likely in response to different selective pressures. Overall, the distribution of trait values tended to represent two distinct groups, one for flats and marshes and another for pans. Such responses seem to be primarily linked to the level of exposure to physical stress (temperature and salinity fluctuation and desiccation) of individuals during low tide. Prolonged submersion times enhance foraging times and the absorption of calcium carbonate from water increasing shell growth rate, which, in turn, gives rise to larger but more elongated shells (review by Chapman, 1995). As there is a maximal rate at which calcium carbonate can be absorbed from the water, rapidly growing individuals produce

thinner shells for the same amount of body weight than slower growing individuals. This results in a larger internal volume allowing for accommodating a higher body mass, which is related to higher growth rate favored by longer foraging times (Palmer 1981, Kemp & Bertness 1984, Chapman 1995, Trussell 2000a). Accordingly, snails that remain covered by water during low tide (pans) showed larger, narrower, and thinner shells and heavier body mass relative to individuals from habitats with high environmental stress conditions (flats and marshes). As higher body weight is directly linked to higher fecundity (i.e. egg production, Fredensborg et al. 2006), it is therefore likely that the heaviest body weight of individuals from pans is related to a higher recruitment, which would explain the increased juvenile abundance compared to flats and marshes (Fig. S2).

Juvenile and adult snails from flats and marshes showed a smaller aperture size (smaller aperture surface area) with respect to individuals from pans. It could be that higher environmental stress conditions favored the expression of a smaller aperture size as a potentially beneficial trait increasing resistance to high desiccation in these habitats that drain at low tide. This is consistent with other studies showing that the size of the shell aperture in gastropods is smaller in response to hot and dry conditions (Machin 1967, Vermeij 1973, Chapman 1995, Melatunan et al. 2013, Schweizer et al. 2019). Moreover, individuals from flats and marshes showed thicker shells and lower body mass. Shell thickening could be due to a higher deposition of calcium carbonate at a slow grow rate (likely in response to unfavorable conditions in more exposed habitats from the upper intertidal) that traded off against investment in body mass.

Crab predation pressure would favor the expression of shell defenses in *Heleobia australis*

Snails from marshes showed a more rounded and thicker shell, reduced body mass, and a narrow aperture shape for three snail categories analyzed (cat. *i–iii*). Such characteristics are consistent with shell defenses probably induced by the high presence of the predatory crab *Neohelice granulata* in that habitat in particular (e.g. Appleton & Palmer 1988, Palmer 1990, Bourdeau et al. 2015). Considering that crushing predators exert a strong selective pressure driving the evolution of behavioral, chemical, and morphological defense traits (Appleton & Palmer 1988, Palmer 1990, Trussell 2000b,

Johannesson 2003, Bourdeau et al. 2015), our results, combined with its remarkably low density, support the hypothesis that predation pressure is stronger in marshes than in flats and pans. Yet, specific information on whether crabs actively predates on *H. australis* is limited or inexistent. Earlier studies reported that *N. granulata* consumes mollusks but in a low frequency (D'Incao et al. 1990, Barutot et al. 2011). These studies, however, have used visual examination of gut/stomach contents. This procedure may have under-estimated true predation levels as crabs only eat the soft tissue after crushing/peeling the shell whereby the remains found in guts or stomach become unidentifiable due to maceration and digestion. Thus, further studies using molecular-based tools are needed for detecting the presence of *Heleobia*'s tissue in crab's stomachs as an alternative or complementary approach to visual identification (e.g. Albaina et al. 2010, Collier et al. 2014). By doing so, we would be able to establish the trophic link between these two species that is highly likely to exist. Evidence found in this study clearly reveals that *H. australis* phenotypically responded to the presence of predatory crabs, even early in life as juveniles from marshes also showed a narrow aperture shape and a more rounded shell. Our results therefore warrant further investigation to understand the adaptive value of the plastic shell responses of gastropods induced by predators.

Strong parasite effect on shell characters: a trade-off between growth and early reproduction

Infected and uninfected snails from pans showed a clear phenotypic differentiation in shell traits. Trematode infection inevitably leads to snail host castration drastically reducing host fitness, which, in evolutionary terms, is equivalent to death of the host (Lafferty 1993, Fredensborg et al. 2006). In this sense, being castrated by a parasite is similar as being eaten by a predator (Kuris 1974). This means that parasitic castrators can exert a strong selective force that could favor the expression of behavioral, physiological, and morphological adaptations to minimize the negative impact of parasitism on host fitness (Lafferty 1993). Early maturation, which results from fast growing individuals, is an effective strategy that increases current reproductive effort over future reproduction (Cole 1954, Lewontin 1965, Roff 1992) which equals to 0 in castrated hosts. In *Heleobia*, castration caused by *Microphallus* can have a profound

impact on snail fitness as this parasite shows an extensive host exploitation occupying the entire gonad and most of digestive gland (see Fig. S2 in Alda et al., 2019).

Our results showed that infected snails had a more elongated shell shape but a smaller size compared to uninfected snails. Despite infected snails were smaller, they exhibited the same number of whorls (six) as uninfected snails, which indicates that individuals analyzed were adults (Gaillard & Castellanos 1976). It could therefore be that the elongated shape resulted from an initial higher growth rate (review by Chapman, 1995) likely followed by an energy reallocation from growth to early reproduction (Lafferty 1993, Agnew et al. 1999), which could explain the smaller shell size compared to uninfected snails (Alda et al. 2010, 2019) this study). Infected snails also showed a smaller aperture size but a more rounded shape relative to uninfected individuals. This change in aperture size and shape could be a side effect of selection for high rate of shell growth (Boulding & Hay 1993) induced by parasites. Together, these findings support the idea that infection by trematodes exerted a strong selective pressure on the snail host causing important shifts in the expression of shell traits towards lower mean values, creating a pronounced phenotypic difference between infected and uninfected snails (Poulin & Thomas 1999).

Difference in penis size in a single *Heleobia* species

Interestingly, individuals from each habitat showed a clear variation in penis size in spite of they were raised in common garden laboratory conditions (no habitat effect) and, when dissected, we observed that none of them was infected by parasites (no parasite effect). It could be that this variation resulted from different growth rates as well as the differences in shell shape observed for wild snails. We found, however, no differences in shell shape among male individuals reared in laboratory conditions, even after controlling by shell size. One interpretation of this divergence in penis size is not related to dissimilar rates of growth but to genetic differences, suggesting that this is likely to be a non-plastic trait. Despite the difference in size, its shape indicates that individuals from each subpopulation belong to the same species, more specifically to *Heleobia australis australis* (see Gaillard & Castellanos, 1976 for further detail on taxonomy). Accordingly, the COI gene confirmed that specimens considered in this study constitute a single species.

Future experimental (laboratory breeding) and non-experimental approaches (multilocus or genomic analyses) are needed to exclude other processes that may have contributed to this difference in penis size and to understand whether this genital divergence could be an indicative of pre-zygotic reproductive isolation among subpopulations of *Heleobia australis* living in sympatry (Kameda et al. 2009, Hollander et al. 2013). If this is true, it could be that individuals studied herein would be part of a species complex that have recently diverged, which would be in line with the lack of clusters when analyzing the conserved COI gene (Janzen et al. 2017, Matos-Maraví et al. 2019).

Does the small-scale phenotypic differentiation in *Heleobia australis* reflect an adaptive evolutionary response to local selection pressures?

Theory predicts that differentiation via adaptive phenotypic plasticity is expected to be more common than genetic adaptation in wide-dispersing species relative to species with restricted dispersal because of greater gene flow (Mather 1955, Levins 1968, Via & Lande 1985, Scheiner 1998, De Jong 1999, 2005), which homogenizes genotype frequencies across space thereby countering the effect of natural selection (review by Lenormand 2002). However, an increasing number of experimental studies indicates that local genetic adaptation may be more common than predicted by theory in marine species with planktonic dispersal, especially in systems characterized by strong selection over relatively fine spatial scale (reviewed in Sanford & Kelly 2011). The pronounced phenotypic divergence in *Heleobia australis* provides a reasonable ground for advocating that local adaptation might be possible in spite this phenomenon has been historically understudied in wide-dispersing species (Hays 2007, Marshall et al. 2010, Sanford & Kelly 2011). Below, we discuss whether there is scope for adaptive evolution in the intertidal mud snail *H. australis*.

One possible explanation for the evolution of locally adapted phenotypes in species with planktonic dispersal is linked to pre- and post-colonization barriers to connectivity (i.e. phenotype-environment mismatch) that increase localized recruitment (Levin et al. 2006, Marshall et al. 2010). That is, local adaptation may be ensured by genetic polymorphism because immigration is rare and the intrapopulation gene pool is highly reliant upon local recruitment whereat individuals are exposed to local selection

pressures. The appearance of an environmentally induced novel phenotype relies on plasticity unique ability to generate an immediate phenotypic response to the surrounding habitat conditions, which can be refined by selection on the expression of such phenotype through processes such as genetic accommodation or assimilation (see Waddington 1953, West-Eberhard 1989, 2003, 2005, Pigliucci et al. 2006 for further details). Both processes are potentially relevant in fostering divergent phenotypes within populations and subsequently driving diversification (e.g. West-Eberhard 2003, 2005, Suzuki & Nijhout 2006, Pfennig et al. 2010). In this sense, it is therefore possible that *H. australis* benefited from the combined effect of directional selection and plasticity to evolve locally adapted phenotypes to contrasting habitat conditions if strong selection pressures caused a local disruption to dispersal of planktotrophic larvae leading to phenotype-environment mismatch and increasing localized recruitment (e.g. Struhsaker 1968, Parsons 1997). This study was not designed to test for an adaptive basis to phenotypic variation of external and internal characters. The next step is therefore to perform experimental approaches (including common garden designs and reciprocal transplants) to unveil whether the phenotypic differentiation of such traits is genetically assimilated.

CONCLUSION

Our results show that individuals of *H. australis* are likely to be constituted by a mosaic of interacting plastic and non-plastic traits that are subjected to directional selection imposed by strong gradients of abiotic and biotic stressors. It does seem reasonable to interpret that most of the variation in phenotypic traits are adaptive plasticity responses, meaning that the shift in the mean trait values is in the same direction favored by the prevailing selection pressure at each habitat. Overall, this study provides compelling evidence on small-scale phenotypic differentiation in an intertidal snail with planktonic dispersal. These findings might contribute to a more complete understanding of the spatial scale at which adaptive differentiation can occur in marine systems. Studies providing this information are critical for improving prediction of the biological impacts of climate change and for effective conservation management approaches of marine populations.

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AUTHORS' CONTRIBUTIONS

NB and PA conceived the ideas and designed methodology; NB, JPP, and PA collected the data; NB analyzed the data and led the writing of the manuscript. All authors contributed critically to the drafts and gave final approval for publication.

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Tables

Table 1. Summary of means (±SE) and statistical significance of the two-way ANOVA testing for habitat and seasonal effect on total snail density (ind./per sample; one sample being 78.5 cm^{-2}) of the intertidal mud snail *Heleobia australis* from the Bahía Blanca estuary, Argentina. Number of samples are indicated between parentheses. Value in bold indicates overall mean. We reported raw estimates whereas the model fits for Ln-transformed density. See Methods section for details.

	Seasons	Habitats			Mean by Season	Habitat effect	Habitat comparison	Effect size	Season effect	Season comparison	Effect size
		Flats (F)	Marshes (M)	Pans (P)							
Total density	Summer (Su)	132±17 (9)	52±14 (9)	128±20 (9)	104±12 (27)	$F_{(2, 106)}$ = 15.48***	P *vs.* M	5.49***	$F_{(3, 106)}$ = 6.22***	Au *vs.* Sp	4.26***
	Autumn (Au)	65±13 (9)	87±42 (9)	70±14 (9)	74±15 (27)		M *vs* . F	-3.54***		Wi *vs.* Sp	2.69*
	Winter (Wi)	92±20 (8)	70±24 (9)	206±30 (9)	124±18 (26)		P *vs.* F	1.91		Su *vs.* Sp	2.57*
	Spring (Sp)	187±36 (9)	114±36 (9)	317±34 (9)	206±26 (27)					Su *vs.* Au	1.68
										Au *vs.* Wi	1.53
	Mean by Habitat	120±14 (35)	81±15 (36)	180±20 (36)	**127±10 (107)**					Su *vs.* Wi	0.14

∗ $P < 0.05$; ∗∗ $P < 0.01$; ∗∗∗ $P < 0.001$. We applied the Holm-Bonferroni correction for multiple testing when comparing effect sizes.

Table 2. Uninfected adult snails. Summary of means (±SE) and statistical significance of habitat and sex effect on shell and aperture morphometry of uninfected adult individuals of the mud snail *Heleobia australis* from the intertidal area of the Bahía Blanca estuary, Argentina. Variables are the same as indicated in table 2. Number of observations are indicated between parentheses, which are only shown for shell size but are the same for other traits measured.

Traits measured	Habitat type	Sex: Males (♂)	Sex: Females (♀)	*Sex pooled*	Habitat effect	Habitat comparison	Effect size	Sex effect	Sex comparison	Effect size
Shell size Φ	Flats (F)	6.19±0.18 (388)	6.17±0.18 (396)	6.17±0.16 (784)	*Males*	P *vs.* F	17.91***	*Flats*		
	Marshes (M)	6.78±0.13 (266)	6.64±0.11 (245)	6.71±0.11 (511)	X_2^2 = 346.22***	P *vs.* M	11.01***	X_1^2 =0.20; n.s.	♂ *vs.* ♀	0.50
	Pans (P)	7.97±0.36 (600)	7.55±0.40 (738)	7.74±0.40 (1338)		M *vs* . F	4.08***	*Marshes*		
	Habitats pooled	7.14±0.22 (1254)	6.95±0.25 (1379)		*Females*	P *vs.* F	15.20***	X_1^2 =1.57; n.s.	♂ *vs.* ♀	1.25
					X_2^2 = 249.33***	P *vs.* M	9.01***	*Pans*		
						M *vs* . F	3.62***	X_1^2 =24.30***	♂ *vs.* ♀	4.93***
Shell shape †	Flats	2.27±0.02	2.26±0.02	2.27±0.02	X_2^2 = 101.02***	P *vs.* F	10.13***	X_1^2 = 29.02***	♂ *vs.* ♀	5.39***
	Marshes	2.32±0.01	2.29±0.01	2.31±0.01		P *vs.* M	5.31***			
	Pans	2.35±0.01	2.31±0.01	2.33±0.01		M *vs* . F	3.61***			
	Habitats pooled	2.32±0.01	2.29±0.01							
Aperture size	Flats	1.82±0.05	1.83±0.06	1.82±0.05	X_2^2 = 362.62***	P *vs.* F	17.10***	X_1^2 = 8.54**	♂ *vs.* ♀	2.93**
	Marshes	1.90±0.04	1.86±0.03	1.88±0.03		P *vs.* M	12.79***			
	Pans	2.10±0.09	2.05±0.10	2.07±0.09		M *vs* . F	1.63			
	Habitats pooled	1.97±0.06	1.94±0.07							
Aperture shape	Flats	1.73±0.03	1.73±0.03	1.73±0.03	X_2^2 = 535.73***	P *vs.* F	-22.91***	X_1^2 =0.04; n.s.	♂ *vs.* ♀	-0.20
	Marshes	1.70±0.02	1.71±0.01	1.70±0.02		P *vs.* M	-15.35***			
	Pans	1.60±0.02	1.60±0.02	1.60±0.02		M *vs* . F	-3.86***			
	Habitats pooled	1.66±0.02	1.66±0.03							

Φ As there was a significant interaction between Habitat and Sex when testing shell size, we report effect sizes separately for this trait.
† We report estimates of shell shape corrected for shell size, as the pattern observed was not the same when removing the covariate from the model.
∗ $P < 0.05$; ∗∗ $P < 0.01$; ∗∗∗ $P < 0.001$. We applied the Holm-Bonferroni correction for multiple testing when comparing effect sizes

Table 3. Infected vs. Uninfected adult snails. Summary of means (±SE) and statistical significance of status (infected of uninfected) and sex effect on shell and aperture morphometry of the intertidal mud snail *Heleobia australis* from the Bahía Blanca estuary, Argentina. In these analyses we only considered individuals from pans, which allowed for preventing habitat effect on morphometry. Shell size estimated as the volume of a cone (mm^3), shell shape as the length to width ratio (SL/SW), aperture size as the area of an ellipse (mm^2), and aperture shape as the ratio between aperture length and width (AL/AW). Number of observations are indicated between parentheses, which are only shown for shell size but are the same for other traits measured.

Traits measured	Habitat type	Sex: Male (♂)	Sex: Female (♀)	*Sex pooled*	Status effect	Status comparison	Effect size	Sex Effect	Sex comparison	Effect size
Shell size	Uninfected (U)	7.97±0.36 (600)	7.55±0.40 (738)	7.74±0.38 (1338)	X_1^2 = 149.69***	U *vs.* I	12.42***	X_1^2 = 31.68***	♂ *vs.* ♀	5.65***
	Infected (I)	7.01±0.14 (432)	6.76±0.20 (573)	6.86±0.16 (1005)						
	Status pooled	7.59±0.28 (1032)	7.22±0.32 (1311)							
Shell shape Φ	Uninfected	2.36±0.01	2.32±0.01	2.34±0.01	*Males*			*Uninfected*		
	Infected	2.40±0.01	2.39±0.02	2.40±0.01	X_1^2 = 11.82***	U *vs.* I	3.43***	X_1^2 = 37.51***	♂ *vs.* ♀	6.12***
	Status pooled	2.38±0.01	2.35±0.01							
					Females			*Infected*		
					X_1^2 = 99.50***	U *vs.* I	9.98***	X_1^2 = 0.09; n.s.	♂ *vs.* ♀	-0.30
Aperture size	Uninfected	2.10±0.09	2.05±0.10	2.07±0.09	X_1^2 = 408.65***	U *vs.* I	21.11***	X_1^2 = 14.95***	♂ *vs.* ♀	3.88***
	Infected	1.80±0.04	1.76±0.06	1.77±0.05						
	Status pooled	1.98±0.07	1.92±0.08							
Aperture shape	Uninfected	1.60±0.02	1.60±0.02	1.60±0.02	X_1^2 = 60.57***	U *vs.* I	7.83**	X^2_1 = 1.3e-3; n.s.	♂ *vs.* ♀	0.03
	Infected	1.56±0.02	1.56±0.02	1.56±0.02						
	Status pooled	1.58±0.02	1.58±0.02							

Φ As there was a significant interaction between Status and Sex when testing shell shape, we report effect sizes separately for this trait.
∗ $P < 0.05$; ∗∗ $P < 0.01$; ∗∗∗ $P < 0.001$. We applied the Holm-Bonferroni correction for multiple testing when comparing effect sizes.

Figure Captions

Figure 1. Contrasting habitat conditions at the intertidal zone of the Bahía Blanca estuary (Villa del Mar), Argentina. Three distinct habitats can be characterized: flats, marshes, and pans. Flats and marshes are located in the upper zone and they drain at low tide, though flats are free of vegetation and marshes covered by cordgrass (*Spartina alterniflora*). Pans are free of vegetation but remain covered by water during low tide and are located close the seaward edge. Thermal, saline, and dehydration stress are strong selective forces occurring mainly in the upper intertidal area (flats and marshes) whereas these inducing agents are weaker in pans, which exhibit low environmental stress condition. Biotic agents also vary along the vertical distribution of the intertidal zone. Predation risk is higher in the upper area (marshes) mainly driven by the grapsid burrowing crab *Neohelice granulata*, which is commonly found in high abundances in marshes, though it uses the entire intertidal zone. Parasite pressure also differs along the intertidal zone. The prevalence (percentage of individuals infected) of trematodes is higher in the lower area of the intertidal zone (pans), where parasite infection is predominately caused by one extremely prevalent trematode, *Microphallus simillimus*.

Figure 2. Variation in total density (juveniles and adults pooled) of the planktotrophic snail *Heleobia australis* across habitats and seasons in the intertidal area of the Bahía Blanca estuary, Argentina. Bars represent ± 1 SE.

Figure 3. Phenotypic variation in shell and aperture morphometrics of uninfected adult snails *Heleobia australis* (cat. *i*) in the Bahía Blanca estuary, Argentina. Blue and red dots indicate mean values of each variable and sex in each habitat (flats, marshes, and pans). Mean values of shell shape were statistically corrected for shell size. Bars represent ± 1 SE.

Figure 4. Phenotypic variation in shell and aperture morphometrics of infected and uninfected adult snails *Heleobia australis* (cat. *iv*) in response to a strong parasite pressure from a habitat with low environmental stress conditions but high parasite prevalence (pans) in the Bahía Blanca estuary, Argentina. Blue and red dots indicate mean values of each variable and sex (males and females, respectively). Mean values of aperture size were statistically corrected for shell size. Bars represent ± 1 SE.

Figure 5. Shell weight (as a proxy of thickness) and body mass variation of the intertidal mud snail *Heleobia australis* across habitats with different biotic and abiotic selective pressures in the Bahía Blanca estuary, Argentina. Bars represent ± 1 SE.

Figure 6. Differences in penis size (mm^2) of uninfected adult snails *Heleobia australis* collected from three environmentally distinct habitats from the Bahía Blanca estuary, Argentina and kept in standard laboratory conditions for 21 months, which ensured that individuals analyzed were all adults. Bars represent ± 1 SE.

Figure 7. Summary of the responses observed in snail population density, shell and aperture morphometrics, and shell and body weight of the intertidal mud snail *Heleobia australis* from the Bahía Blanca estuary, Argentina. Such responses were induced by: (i) environmental pressure, whereby snails experiencing prolonged submersion times in habitats that remain covered by water during low tide (pans) exhibited higher density and higher mean values of morphological traits relative to those from more environmental stress conditions (flats and marshes), which showed opposite phenotypic responses; (ii) predation pressure caused by the presence of the burrowing crab *Neohelice granulata* could be linked to the remarkable low density in marshes and could have favored the expression of shell defenses as shell thickening, increased shell globosity (lower SL to SW ratio), narrower apertures (higher AL to AW ratio); and (iii) strong parasite pressure caused by *Microphallus simillimus* where morphological traits of infected (I) snails shifted to lower mean values compared to uninfected (U) ones; note that polymorphisms are only shown for pans, where parasite prevalence was the highest. Responses observed in this study are indicated by color-filled boxes, white-filled boxes indicate possible mechanisms or processes that might explain the observed responses. Green color refers to responses induced by environmental pressure, orange to polymorphism induced by crab predation, and pink by parasite pressure. Upper arrows (↑) indicate increase/higher/longer and down arrows (↓) indicate decrease/lower/shorter.

Figures

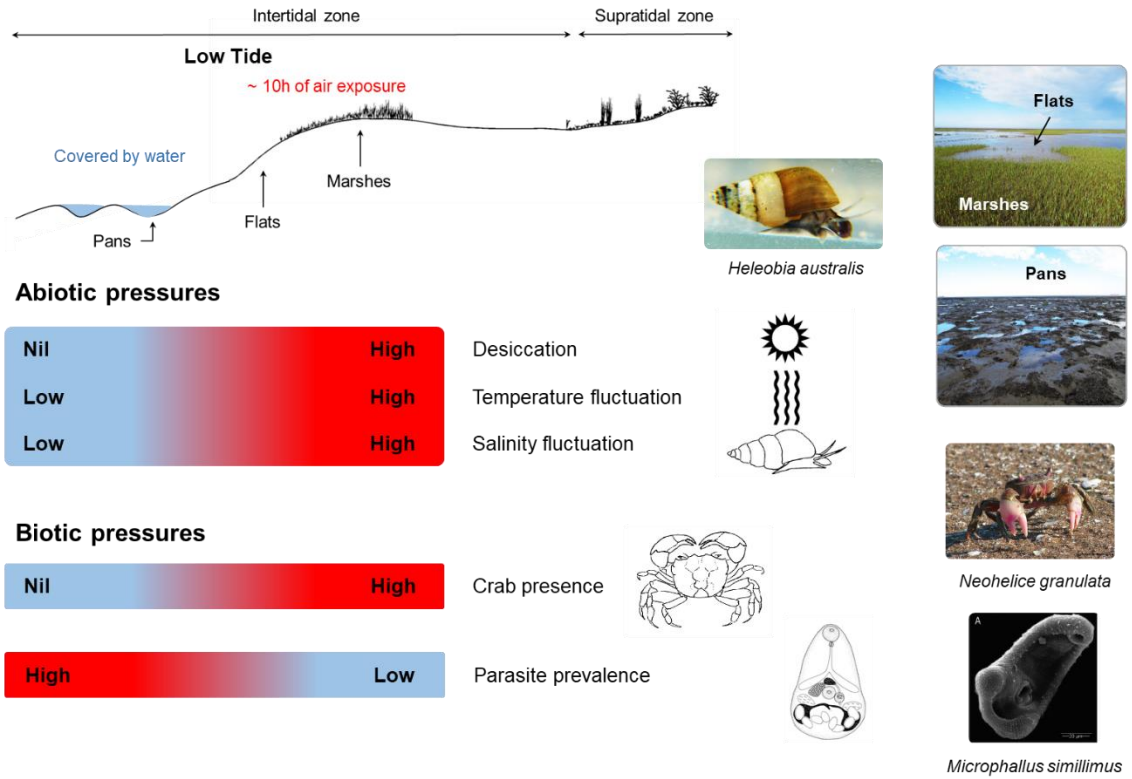

Figure 1.

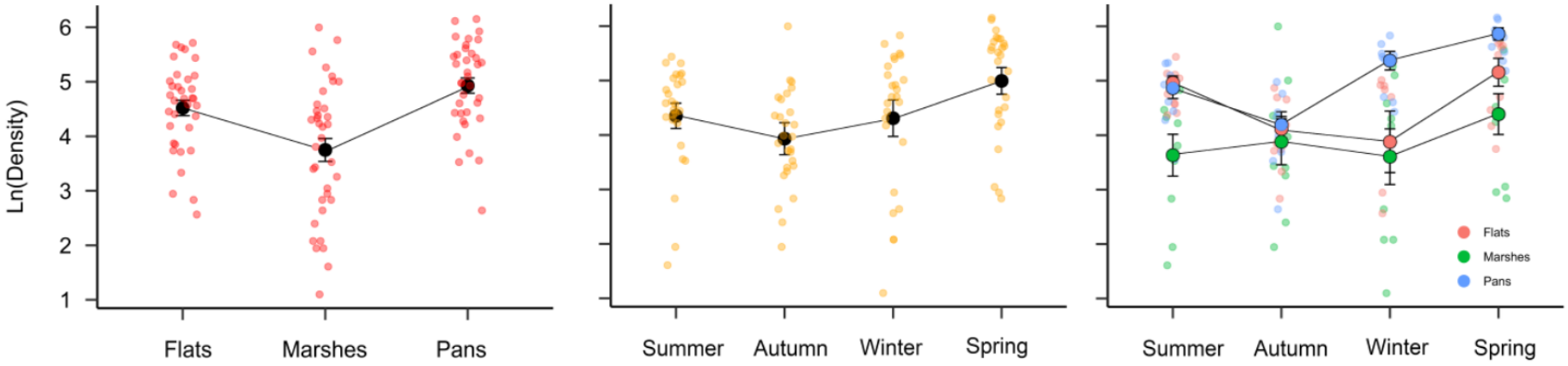
Ln(Density)
6
5
4
3
2
1
Flats
Marshes
Pans
Summer
Autumn
Winter
Spring
Summer
Autumn
Winter
Spring
Flats
Marshes
Pans

Figure 2.

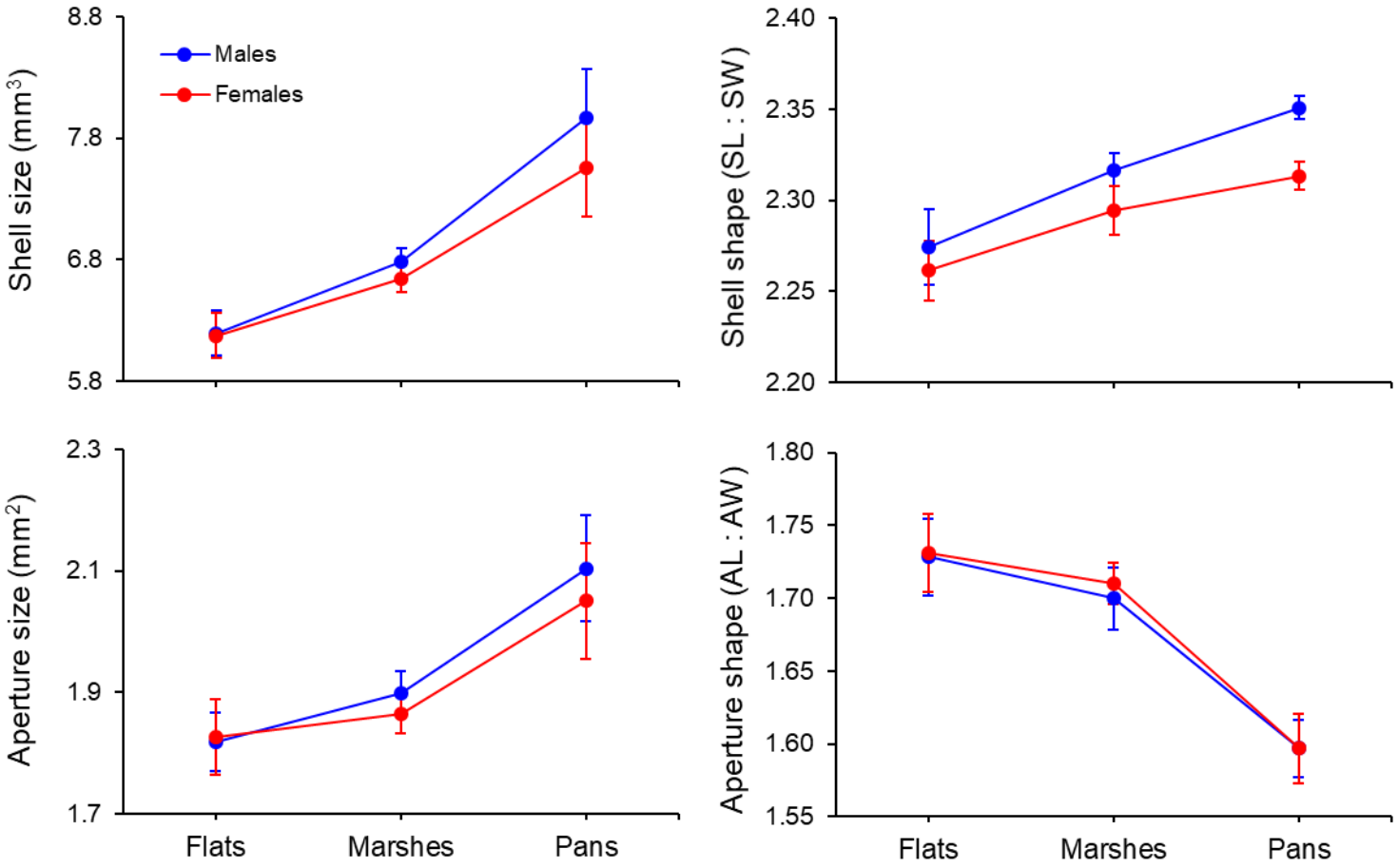
Males
Females
Shell size (mm3)
8.8
7.8
6.8
5.8
Shell shape (SL : SW)
2.40
2.35
2.30
2.25
2.20
Aperture size (mm2)
2.3
2.1
1.9
1.7
Aperture shape (AL : AW)
1.80
1.75
1.70
1.65
1.60
1.55
Flats
Marshes
Pans
Flats
Marshes
Pans

Figure 3.

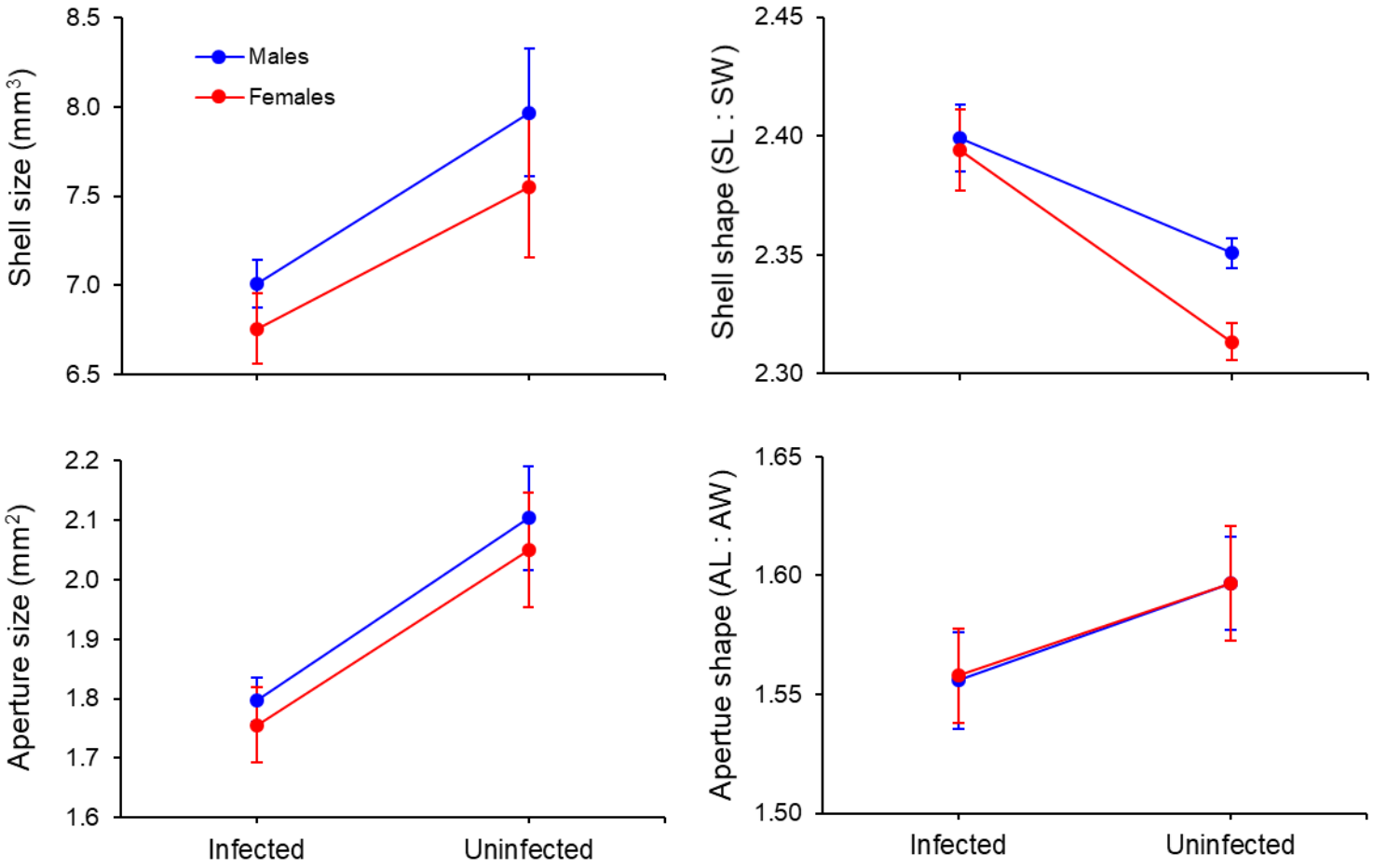
Males
Females
Shell size (mm3)
8.5
8.0
7.5
7.0
6.5
Shell shape (SL : SW)
2.45
2.40
2.35
2.30
Aperture size (mm2)
2.2
2.1
2.0
1.9
1.8
1.7
1.6
Apertue shape (AL : AW)
1.65
1.60
1.55
1.50
Infected
Uninfected
Infected
Uninfected

Figure 4.

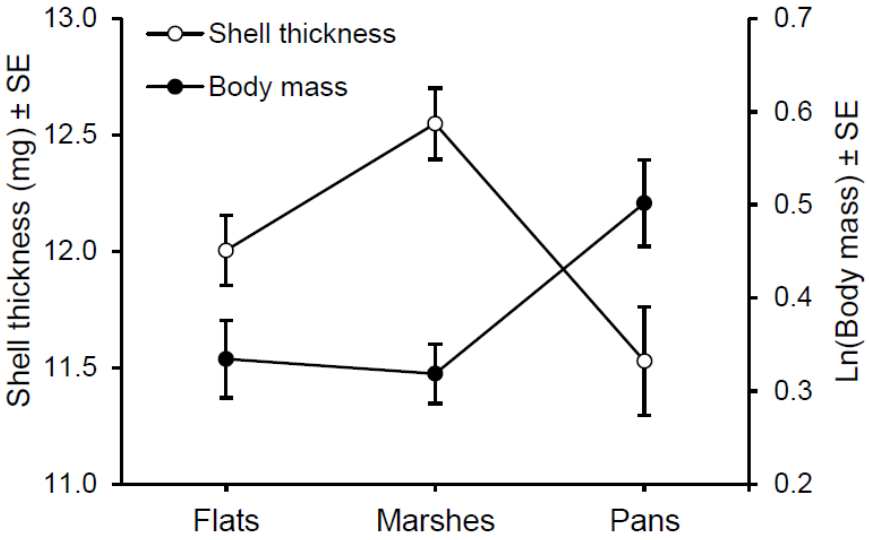
Shell thickness
Body mass
Shell thickness (mg) ± SE
Ln(Body mass) ± SE
13.0
12.5
12.0
11.5
11.0
0.7
0.6
0.5
0.4
0.3
0.2
Flats
Marshes
Pans

Figure 5.

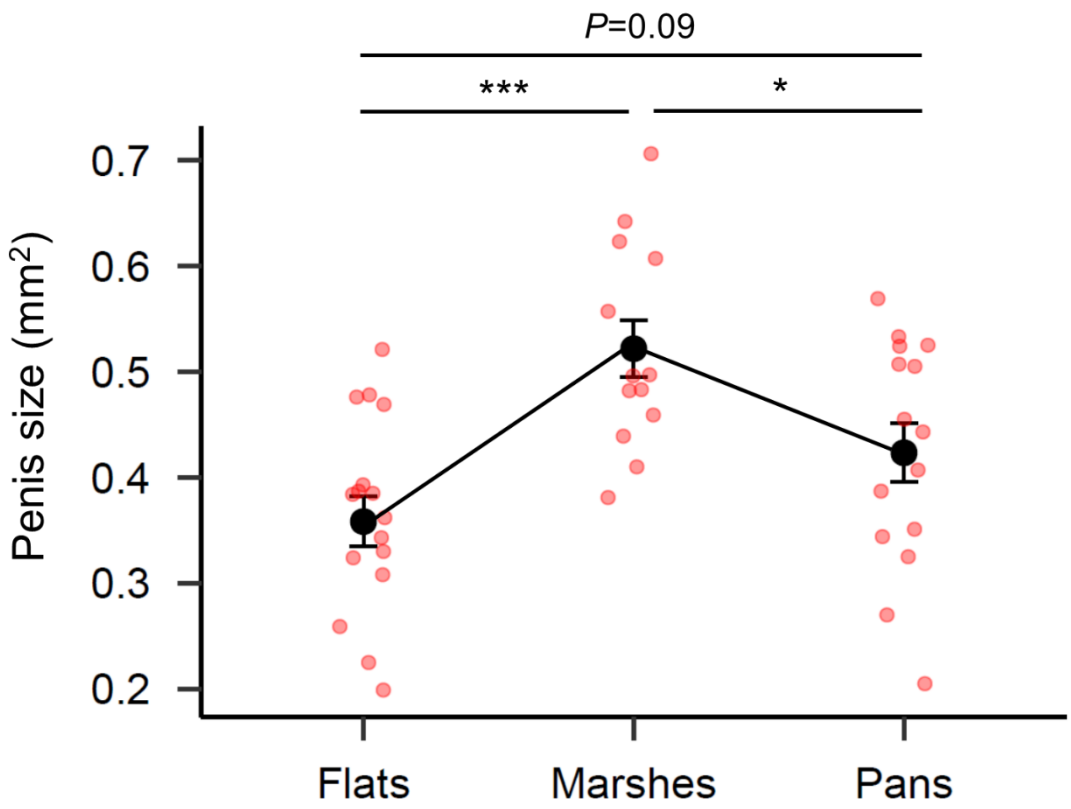

Figure 6.

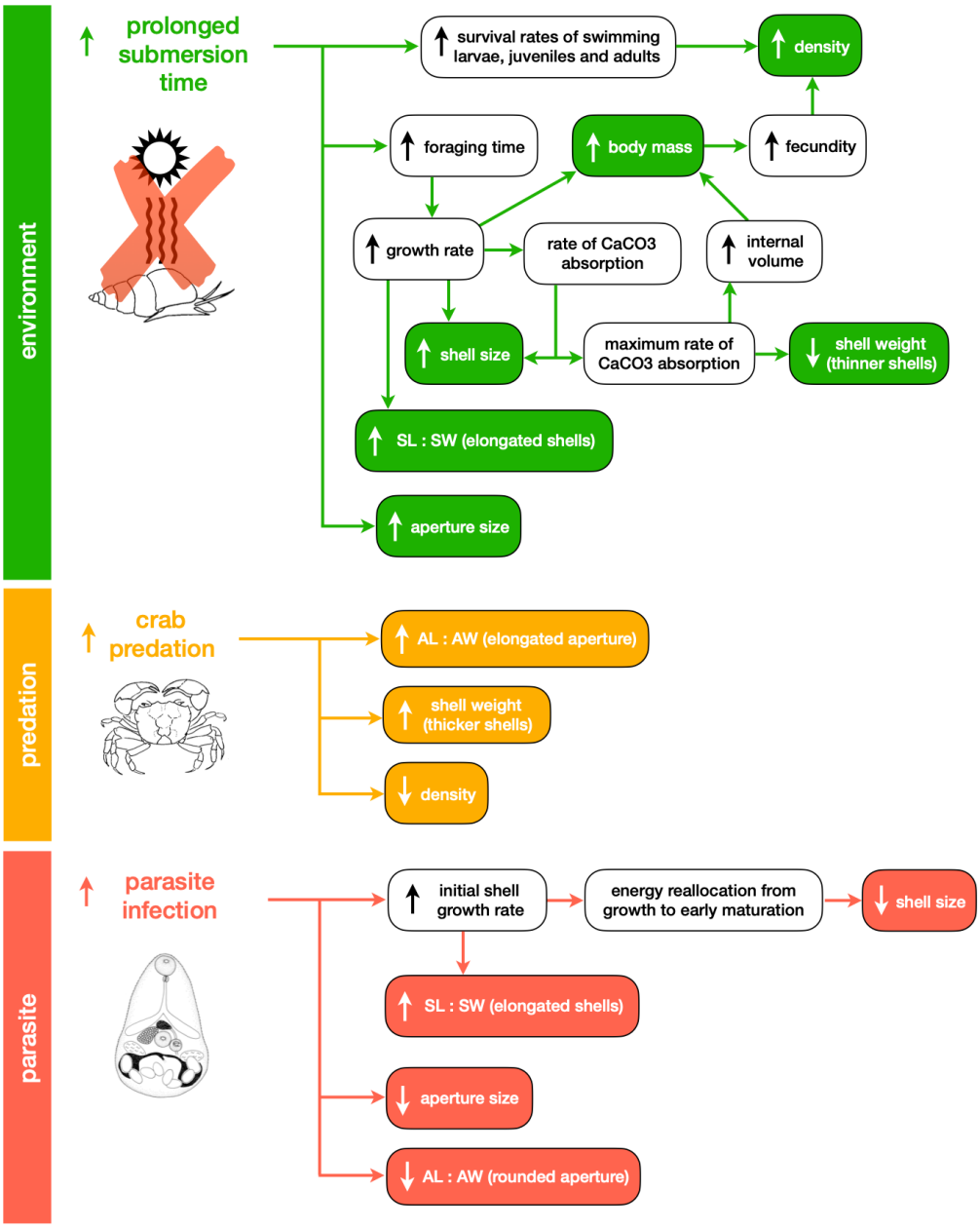
environment
prolonged submersion time
survival rates of swimming larvae, juveniles and adults
density
foraging time
body mass
fecundity
growth rate
rate of CaCO3 absorption
internal volume
shell size
maximum rate of CaCO3 absorption
shell weight (thinner shells)
SL : SW (elongated shells)
aperture size
predation
crab predation
AL : AW (elongated aperture)
shell weight (thicker shells)
density
parasite
parasite infection
initial shell growth rate
energy reallocation from growth to early maturation
shell size
SL : SW (elongated shells)
aperture size
AL : AW (rounded aperture)

Figure 7.