# Pronounced phenotypic differentiation in a wide-dispersing marine snail in response to contrasting selection pressures at a local scale

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**Running head:** Pronounced phenotypic differentiation in a wide dispersing snail

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## Abstract

1. 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. The grain size theory predicts that species with high dispersal potential experience a more heterogeneous, fine-grained environment where phenotypic plasticity may evolve to cope with habitat heterogeneity.
2. 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.
3. We collected, measured external and internal characters, weighted, and dissected individuals of *Heleobia australis* (Rissooidea: Cochliopidae) 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.
4. 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 conditions (thermal, saline, and dehydration stress), 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 variation in penis size in snails collected from each habitat and raised in common garden laboratory conditions. The COI gene analysis confirmed that the individuals studied constituted a single species despite the strong phenotypic difference among subpopulations.
5. The pronounced phenotypic differentiation in *H. australis* is all the more remarkable because it occurred at a very small geographical scale, which is rarely documented for a wide-dispersing species. Our findings provide a reasonable ground for advocating that *H. australis* experienced a fine-grained environment and, thus, benefited from the combined effect of directional selection and plasticity to evolve locally adapted phenotypes to contrasting habitat conditions at a local scale.

## 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, Griffiths, & Thomas, 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 (e.g. Chevin & Lande, 2009; Ghalambor, McKay, Carroll, & Reznick, 2007; Price, Qvarnström, & Irwin, 2003). An adaptive evolutionary response to environmentally induced non-heritable variation occurs when phenotypic plasticity operates jointly with directional selection causing the expression of potentially beneficial traits (De Jong, 2005; Fusco & Minelli, 2010; Merilä & Hendry, 2014) and allowing the population to reach the fitness optimum (i.e. 'genetic assimilation'; Pigliucci, 2006; Waddington, 1953; West-Eberhard, 1989). In this sense, 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).

Strategies to cope with environmental variation will depend upon the organism's dispersal ability since it sets the environmental grain and plays a crucial role in determining levels of gene flow among populations, and thus, in the evolution of phenotypic traits within those populations (De Jong, 1999, 2005; Levins, 1968; Mather, 1955; Scheiner, 1998; Via & Lande, 1985). According to the grain-size model, the evolution of traits will follow different pathways in species with contrasting abilities to disperse. For instance, aquatic snails display a wide range of reproductive modes and larval development types, ranging from species with immobile larvae to those with planktotrophic (free-floating) larvae (Janson, 1987; Johannesson, 2003; Parsons, 1997, 1998; Yamada, 1987). Specifically, snails with restricted dispersal (absence of planktotrophic larvae) may experience a coarse-grained environment, as they tend to stay in the same habitat all their life, and their offspring are likely to remain in that habitat. In such conditions, 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 giving rise to different ecotypes. By contrast, wide-dispersing species will experience greater gene flow and encounter different types of habitats more or less at random, thus experiencing a fine-grained environment and exhibiting greater genetic homogeneity than in relatively immobile species. This corresponds to a generalist strategy where phenotypic plasticity may evolve to cope with heterogeneous environments (Bourdeau et al., 2015; Chapman, 1995; Conde-Padín, Grahame, & Rolán-Alvarez, 2007; Hollander, Collyer, Adams, & Johannesson, 2006; Johannesson, 2003; Levins, 1968; Reid, 1996; Vermeij, 1982; Yamada, 1987).

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 (Bourdeau et al., 2015; Denny & Paine, 1998). 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 (Denny & Paine, 1998; Mouritsen, Sørensen, Poulin, & Fredensborg, 2018; Vermeij, 1972). From this perspective, intertidal organisms, such as aquatic gastropods, are likely to exhibit increased phenotypic plasticity in response to such environmental selective pressures (Berrigan & Scheiner, 2004).

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, Jacquemin, & Pyron, 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 (Johannesson & Johannesson, 1996; Struhsaker, 1968). 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 (Chapman, 1995; Machin, 1967; Melatunan, Calosi, Rundle, Widdicombe, & Moody, 2013; Schweizer, Triebskorn, & Köhler, 2019; Vermeij, 1973).

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 (Bourdeau et al., 2015; Dillon et al., 2013; Johannesson, 2003). However, not all phenotypic plasticity triggered by environmental conditions is adaptive. 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, Mouritsen, & Poulin, 2005; Miura, Kuris, Torchin, Hechinger, & Chiba, 2006; Mouritsen et al., 2018). They have been also reported to induce changes in microhabitat choice (Curtis, 1987) and in body size in snails (Alda, Bonel, Cazzaniga, & Martorelli, 2010; Levri, Dillard, & Martin, 2005; Miura et al., 2006; Mouritsen & Jensen, 1994; Probst & Kube, 1999). Such pressure exerted by parasites also differs along the intertidal zone (Alda et al., 2010; Alda, Bonel, Cazzaniga, Martorelli, & Lafferty, 2019; Smith, 2001) and can cause important shifts in the expression of host phenotypic traits, creating pronounced phenotypic differences between infected and uninfected hosts (Fredensborg, Mouritsen, & Poulin, 2006; Poulin & Thomas, 1999).

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 (Johnson, Watts, & Black, 1994) whereat individuals would be subjected to local (a)biotic selective pressures (Johannesson & Johannesson, 1996; Struhsaker, 1968). 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 morphs 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 investigated how different selective pressures induced ecological and phenotypic responses in an intertidal mud snail with planktotrophic larval dispersal. For our study, we collected and measured external and internal characters, weighted, and dissected the 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. The (a)biotically contrasting habitat conditions of the study area create the potential for niche differences that could affect snail density and induce different phenotypic responses among individuals from each subpopulation. Finding strong evidence of phenotypic differentiation in external characters (shell traits and body mass), we then investigated whether internal characters as the genital morphology varied among snails from each subpopulation. We found that juveniles collected from each habitat, and raised in common garden laboratory conditions until they were adults, showed a remarkable difference in penis size. We also

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. We confirmed that the individuals here studied constitute a single *Heleobia* species. Collectively, our findings clearly advocate that the strong phenotypic differentiation in *H. australis* might have resulted from the combined effect of directional selection and plasticity that led to the evolution of locally adapted phenotypes in response to contrasting selection pressures at a local scale.

## 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, Valentin, & Figueiredo, 2010). Mature females lay egg capsules, containing one fertilized egg (N. Bonel, *personal observation*, but see 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; N. Bonel *personal observation*). 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, Neves, Pessoa, & Paiva, 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, Kirby, Plater, & Brinson, 2009) and affected by strong semidiurnal tides and high seasonal variation (Perillo, Piccolo, Parodi, & Freije, 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, Perillo, & Piccolo, 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; Bourdeau et al., 2015; Denny & Paine, 1998) whereas these inducing agents are weaker in pans, which exhibit low environmental stress condition (Fig. S1).

Biotic agents also vary along the vertical distribution of the intertidal zone (Fig. S1). 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 (Alvarez et al., 2013; Angeletti & Cervellini, 2015; Angeletti, Lescano, & Cervellini, 2014; Spivak, Anger, Luppi, Bas, & Ismael, 1994). 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 (Alvarez et al., 2013; Angeletti & Cervellini, 2015; Angeletti et al., 2014; Spivak et al., 1994) and/or through snail predation (Barutot, D'Incao, & Fonseca, 2011; D'Incao, Silva, Ruffino, & Braga, 1990). 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; N. Bonel and P. Alda, *unpublished data*).

**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 et al., 2019; Alda & Martorelli, 2014). 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, Sammond, & Kuris, 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. S2).

**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 (Hershler & Thompson, 1992; Vermeij, 1973). 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; Bourdeau et al., 2015; Dillon et al., 2013; Johannesson, 2003) 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, Noya, Amarista, & Théron, 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,

Lambert, Brouillet, & Achaz, 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 infection status or habitat). 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, Mächler, Bolker, & Walker, 2014), *nlme* (Pinheiro, Bates, DebRoy, Sarkar, & R Core Team, 2017), *car* (Fox et al., 2011), *effects* (Fox, 2003), *MASS* (Ripley et al., 2013), *plyr* (Wickham, 2011), *dplyr* (Wickham, François, Henry, & Müller, 2019), *devtools* (Wickham, Hester, & Chang, 2019), *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. 1). 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). This increase across seasons was mainly driven by juvenile recruitment (Table S1; Fig. S3). 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. S3).

**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. S4). 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. S5-S6).

***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. 3). 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 (or exhibited 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. 3).

### *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. 3). 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. 3). 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. 4). Further details on observations, corrected and uncorrected mean values, and statistics are indicated in the appendix (Table S9; Fig. S7).

**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. 5). 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. S9-S10).

## Discussion

This study provides clear evidence of ecological and phenotypic trait variation in response to contrasting biotic and abiotic conditions at a local 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. 6). The pronounced phenotypic differentiation in *Heleobia australis* is all the more remarkable because it occurred at a very small geographical scale, which is rarely documented for a wide-dispersing species. These findings support the standpoint that this species might have experienced a fine-grained environment where the combined effect of directional selection and plasticity led to the evolution of locally adapted phenotypes in response to contrasting selection pressures.

### 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. S3). As for juvenile snails, their density increased during winter season in all the three habitats (Fig. S3). This was somehow unexpected as we hypothesized that intense environmental stress in the upper area of the intertidal zone, combined with harsh climatic conditions, could strongly decrease their survival rate affecting their abundance. It is puzzling, however, that 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 (Alvarez et al., 2013; Angeletti & Cervellini, 2015; Angeletti et al., 2014; Spivak et al., 1994) 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 (Barutot et al., 2011; D'Incao et al., 1990), 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, snail 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 (Chapman, 1995; Kemp & Bertness, 1984; Palmer, 1981; 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. S3).

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 (Chapman, 1995; Machin, 1967; Melatunan et al., 2013; Schweizer et al., 2019; Vermeij, 1973). 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; Bourdeau et al., 2015; Palmer, 1990). Considering that crushing predators exert a strong selective pressure driving the evolution of behavioral, chemical, and morphological defense traits (Appleton & Palmer, 1988; Bourdeau et al., 2015; Johannesson, 2003; Palmer, 1990; Trussell, 2000b), 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 (Barutot et al., 2011; D'Incao et al., 1990). 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, Fitzgerald, Hice, Frisk, & McElroy, 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, likely 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 (Fredensborg et al., 2006; Lafferty, 1993). 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 (Agnew, Bedhomme, Haussy, & Michalakis, 1999; Lafferty, 1993), 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, which is all the more remarkable as 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. However, we found no differences in shell shape, even after controlling by shell size, among male individuals reared in laboratory conditions. This result suggests that the differences in penis size might not be related to differences in growth rate. Future studies are therefore needed to exclude other processes (e.g. genetic differences) 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 (Hollander, Smadja, Butlin, & Reid, 2013; Kameda, Kawakita, & Kato, 2009). 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 protein-coding COI gene confirmed that specimens considered in this study constitute a single species.

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

The intertidal mud snail *H. australis* exhibited a strong phenotypic variation in shell morphology and other characteristics associated to contrasting biotic (parasitism and predation) and abiotic (high temperature, salinity, and desiccation stress) factors among habitats that are separated just a few meters away from each other within the intertidal area. Such phenotypic differentiation at a very small geographical scale has been rarely documented for a natural population of a wide-dispersing species. It does seem reasonable to interpret the observed variation as an adaptive phenotypic response to contrasting selection pressures occurring along the vertical gradient of the intertidal area (Levins, 1968; Pigliucci, 2001). Environmentally-induced phenotypic change has been reported for a large number of snail species (review by Bourdeau et al., 2015). The appearance of an environmentally induced novel phenotype relies on plasticity unique ability to generate an immediate phenotypic response to the surrounding habitat conditions (West-Eberhard, 2003). If genetic variation in plasticity exists (or arises), then an environmentally induced novel phenotype can be refined by selection on the expression of such phenotype through processes such as genetic accommodation or assimilation (see Pigliucci et al., 2006; Waddington, 1953; West-Eberhard, 1989, 2003, 2005 for further details). Both processes are potentially relevant in fostering divergent phenotypes within populations and subsequently driving diversification (e.g. Pfennig et al., 2010 and references therein; Suzuki & Nijhout, 2006; West-Eberhard, 2003, 2005). Multilocus or genomic analyses are therefore needed to unveil whether the phenotypic differentiation of external and internal characters might have become genetically assimilated, likely due to by local disruption to dispersal caused by environmental stress conditions thereby increasing localized recruitment (e.g. Parsons, 1997; Struhsaker, 1968). If so, the individuals studied herein would be part of a species complex that have recently diverged, which would explain why individuals showed, for instance, differences in the genital morphology but did not cluster when analyzing the conserved COI gene (Janzen et al., 2017; Matos□Maraví, Wahlberg, Antonelli, & Penz, 2019).

## Conclusion

Our results show a remarkable shift in the expression of phenotypic traits, even early in life, in response to strong selective pressures exerted by biotic (parasites, predation) and abiotic (temperature, salinity, desiccation) stressors, which also affected ecological processes such as snail density (summarized in Fig. 6) in a natural population at a very small geographical scale. While this study was not designed to test for an adaptive basis to phenotypic variation, these findings provide a reasonable ground for advocating that *H. australis* benefited from the combined effect of directional selection (likely through genetic assimilation) and plasticity to evolve locally adapted phenotypes to contrasting habitat conditions. The demonstration of the adaptive nature of these differences in life history features will be the object of a future study.

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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 indicate 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.** 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 2.** 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 3.** 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 4.** 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 5.** 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 6.** Summary of the responses observed in snail population density, shell and aperture morphometry, 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

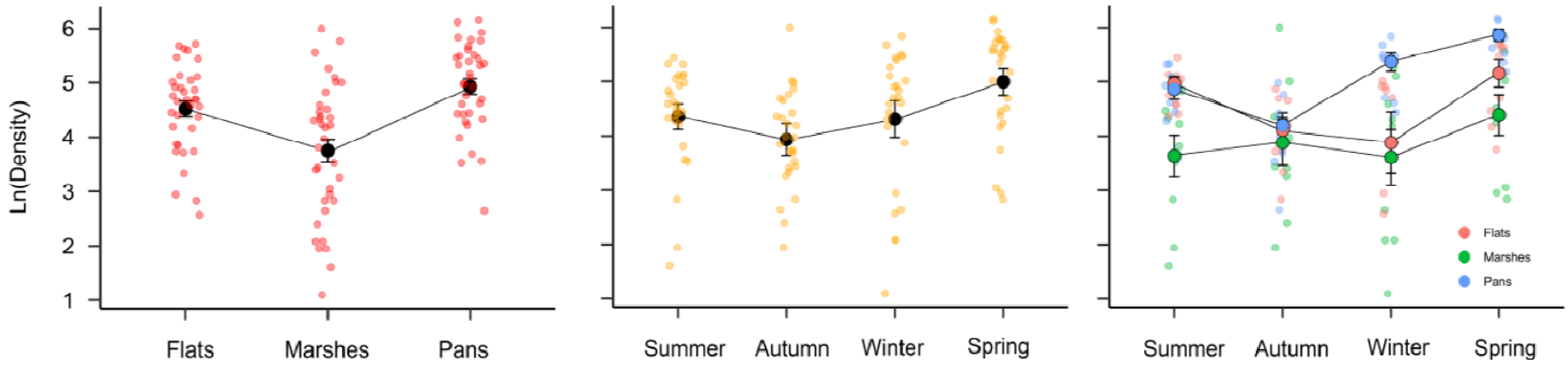


**Figure 1.**

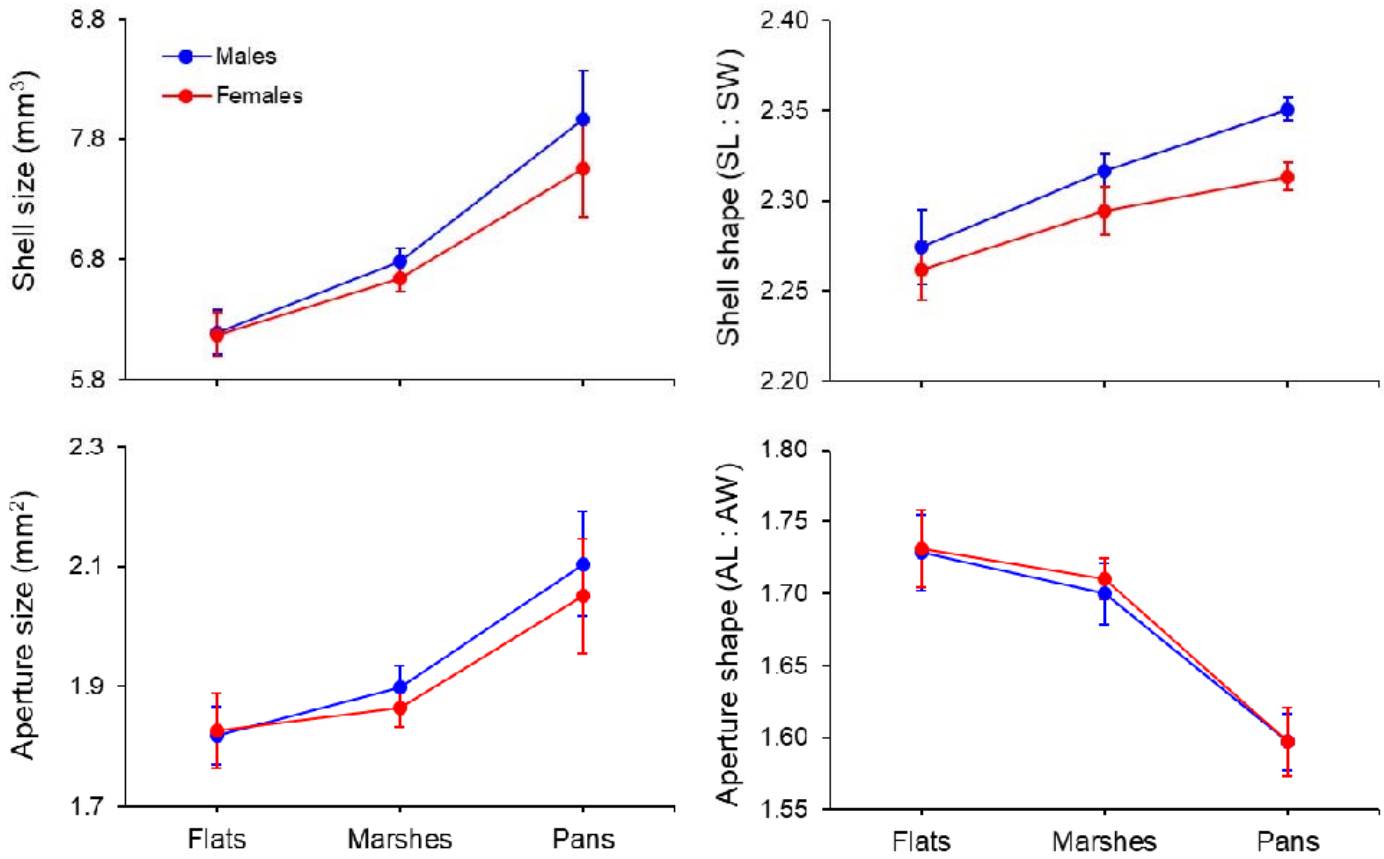
Males
Females
Shell size (mm3)
8.8
7.8
6.8
5.8
Shell shape (SL : SW)
2.40
2.35
2.30
2.25
2.20
Aperture size (mm2)
2.3
2.1
1.9
1.7
Aperture shape (AL : AW)
1.80
1.75
1.70
1.65
1.60
1.55
Flats
Marshes
Pans
Flats
Marshes
Pans

**Figure 2.**

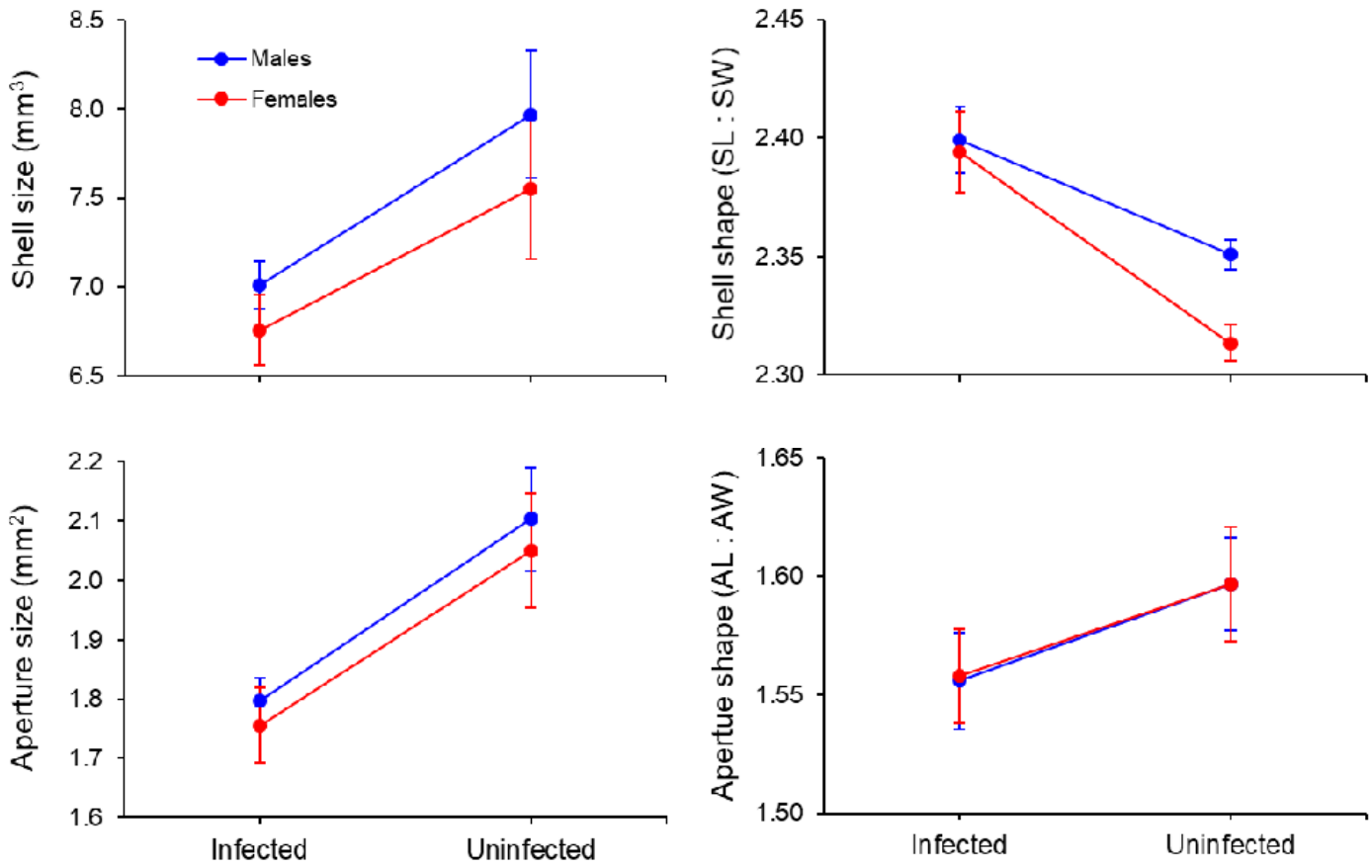
Males
Females
Shell size (mm3)
8.5
8.0
7.5
7.0
6.5
Shell shape (SL : SW)
2.45
2.40
2.35
2.30
Aperture size (mm2)
2.2
2.1
2.0
1.9
1.8
1.7
1.6
Apertue shape (AL : AW)
1.65
1.60
1.55
1.50
Infected
Uninfected
Infected
Uninfected

**Figure 3.**

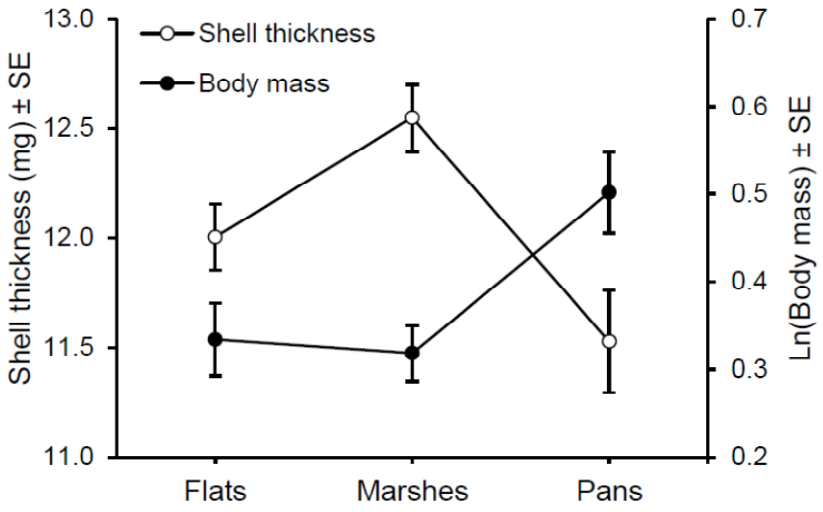
Shell thickness
Body mass
Shell thickness (mg) ± SE
Ln(Body mass) ± SE
13.0
12.5
12.0
11.5
11.0
0.7
0.6
0.5
0.4
0.3
0.2
Flats
Marshes
Pans

**Figure 4.**

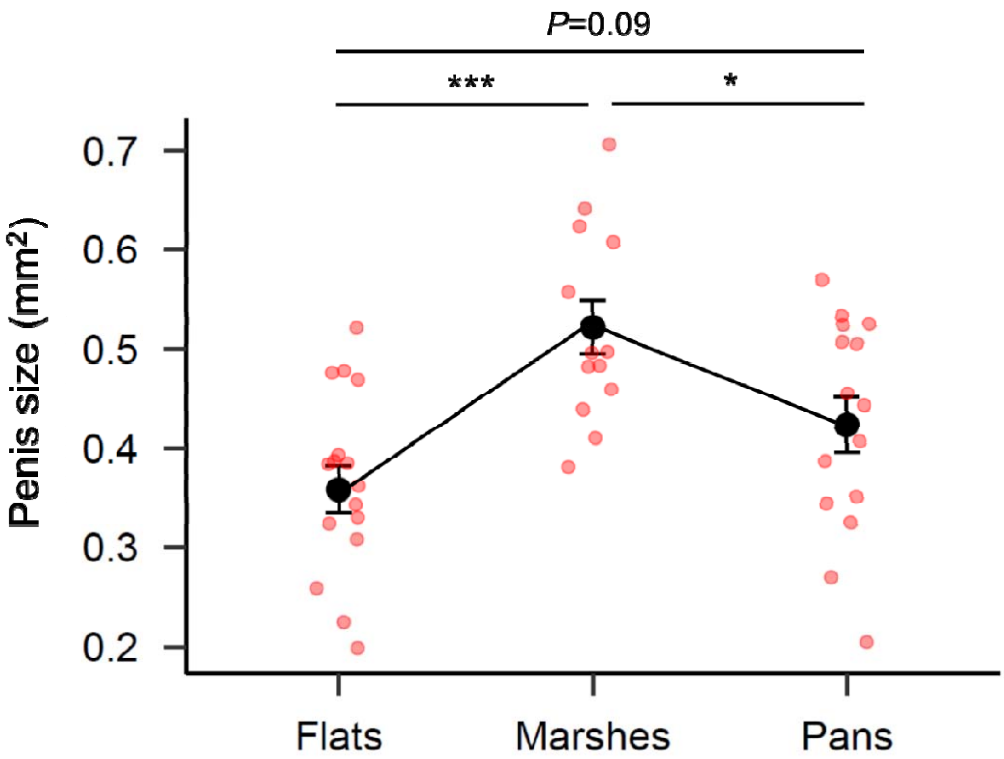


**Figure 5.**

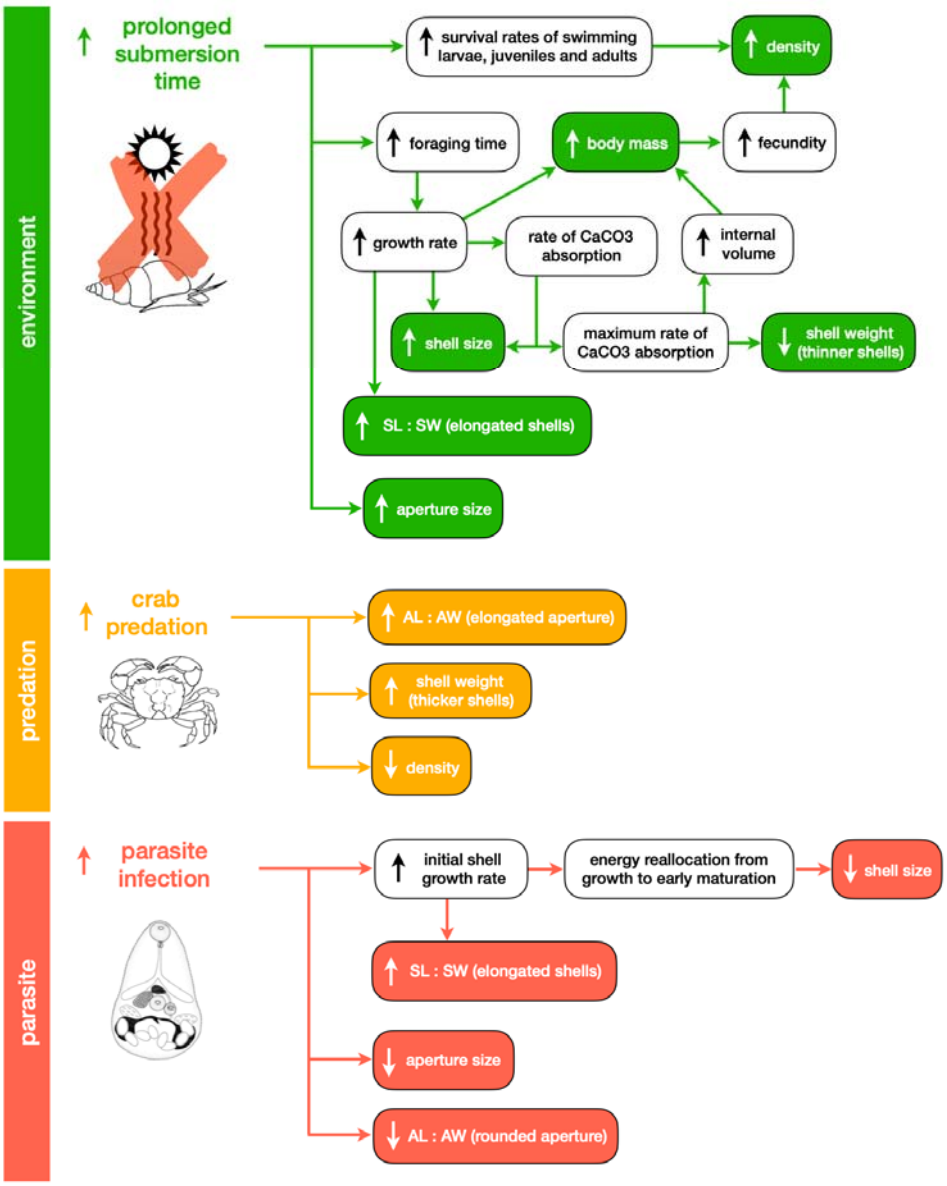
environment
prolonged submersion time
survival rates of swimming larvae, juveniles and adults
density
foraging time
body mass
fecundity
growth rate
rate of CaCO3 absorption
internal volume
shell size
maximum rate of CaCO3 absorption
shell weight (thinner shells)
SL : SW (elongated shells)
aperture size
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 6.**