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Marine Environmental Research

DOI:
10.1016/j.marenvres.2018.10.001

Published: 01/11/2018

Peer reviewed version

Cyswllt i'r cyhoeddiad / Link to publication

Dyfyniad o'r fersiwn a gyhoeddwyd / Citation for published version (APA):
Ragagnin, M., McCarthy, I., Fernandez, W., Tschiptschin, A., & Turra, A. (2018). Vulnerability of juvenile hermit crabs to reduced seawater pH and shading. *Marine Environmental Research*, *142*, 130-140. https://doi.org/10.1016/j.marenvres.2018.10.001

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22. Nov. 2019

Vulnerability of juvenile hermit crabs to reduced seawater pH and shading

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Abstract

Multiple simultaneous stressors induced by anthropogenic activities may amplify their impacts on marine organisms. The effects of ocean acidification, in combination with other anthropogenic impacts (apart from temperature) are poorly understood, especially in coastal regions. In these areas, shading caused by infrastructure development, such as harbor construction, may potentially interact with CO_2-induced pH reduction and affect invertebrate populations. Here, we evaluated the effects of reduced pH (7.6) and shading (24h in darkness) on mortality, growth, calcification and displacement behavior to live predator (danger signal) and dead gastropod (resource availability signal) odors using juveniles of the hermit crab *Pagurus criniticornis* collected in Araçá Bay (São Paulo state, Southeastern Brazil). After a 98 day experimental period, both stressors had a significant interaction effect on mortality, and an additive effect on total growth. No difference in calcification was recorded among treatments, indicating that individuals were able to maintain calcification under reduced pH conditions. When exposed to odor of live predators, crab responses were only affected by shading. However, an interactive effect between both stressors was observed in response to gastropod odor, leading to reduced displacement behavior. This study shows how local disturbance impacts may enhance the effects of global environmental change on intertidal crustacean populations.

Keywords: Effects, multiple stressors, environmental changes, ocean acidification, photoperiod, *Pagurus criniticornis*.

1. Introduction

Coastal ecosystems are exposed to multiple stressors, of both natural (Elliot and Quintino, 2007; Gamito, 2006) and anthropogenic origin (Dolbeth et al., 2011; Halpern et al., 2007; O'Gorman et al., 2012). Within these ecosystems there is growing evidence that the combined effects of multiple stressors may impact at both the organismal (Blake et al., 2010; Przeslawski et al., 2005; Walther et al., 2010) and community level (Shears and Ross, 2010). However, the combined effects of stressors can vary depending on local environmental conditions (Range et al., 2014) and the biology of the study species (Long et al., 2013). In addition, the complexity of these interactions can

potentially amplify the impacts expected from a single stressor (Cole et al., 2016; Queirós et al., 2015).

The effects of multiple stressors in relation to scenarios of global environmental change have been widely investigated over the past few decades across a range of biomes (e.g. Sala et al., 2000; Schweiger et al., 2010; Tanikawa et al., 2017). Amongst the environmental change stressors acting on marine ecosystems, elevated pCO_2 is one of the major threats, as the global oceans have absorbed about one-third of the anthropogenic carbon dioxide (CO_2) released in atmosphere over the last 200 years (Sabine et al., 2004). This increase in oceanic pCO_2 has already led to a reduction of 0.1 unit from the pre-industrial pH average of about 8.2 (Feely et al., 2009; Orr et al., 2005) and climate models indicate that a further decline of 0.3-0.5 pH units through increased pCO_2 is expected by 2100 (Caldeira and Wicket, 2005). Furthermore, significant impacts on marine organisms have already been noted (e.g., Bressan et al., 2014; Clements and Darrow, 2018; Hendriks et al., 2010; Kroeker et al., 2010; Wang et al., 2017). In this context, calcifying organisms generally demonstrate vulnerability in response to changes in seawater chemistry due to potential impairments in the calcification process and maintenance of their calcified structures (Hoffmann et al., 2010; Iguchi et al., 2012; Zhao et al., 2017).

However, OA may not exclusively be a consequence of absorption of increasing atmospheric CO_2 levels in all marine ecosystems (Provoost et al., 2010). For example, in some coastal regions eutrophication can reduce pH as a result of bacterial degradation of organic matter decreasing oxygen concentration and increasing pCO_2 resulting in acidification (Cai et al., 2011; Melzner, 2013; Wallace et al., 2014). Thus, some coastal waters may be particularly vulnerable to seawater acidification.

The combined impacts of increased pCO_2 and warming on marine invertebrates have been well studied in recent years (e.g., Araújo et al., 2018; Lee et al., 2017; Ong et al., 2017). However, little is known about the effects induced by reduced seawater pH in combination with other local anthropogenic stressors such as light availability (Vogel et al., 2015). In some shallow water habitats, increased pCO_2 (through eutrophication and/or absorption of atmospheric CO_2) and the consequent reduction in seawater pH may occur simultaneously with shading due to the presence of harbors, piers, docks (Roca et al., 2014; Shaffer, 1999), bridges (Struck et al., 2004) or aquaculture systems, such as oyster culture (Bulmer et al., 2012; Forrest et al., 2009). Disturbances in light

availability may drastically influence community structure in coastal zones (Pardal-Souza et al., 2017) with changing irradiance affecting both photosynthetic organisms (Benham et al., 2016) and benthic invertebrates (Glasby 1999; Struck et al., 2004).

Within coastal ecosystems, crustaceans provide several ecological and economic services (LeBlanc, 2007) playing an important role in the structure of food webs (Laidre and Greggor, 2015; Shaffer et al., 1995) and energy flux (Kristensen, 2008; Robertson, 1986). Therefore, the impacts of environmental stressors on crustaceans have been well studied (e.g., hypoxia, Peruzza et al., 2018; hypercapnia, Borges et al., 2018; salinity fluctuations, Joseph and Philip, 2007; and pollutants, Vogt et al., 2018) and sublethal effects are known to cause physiological disruption (LeBlanc, 2007; Moullac and Haffner, 2000), which thereby induce impairments in behavioral performance (Felten et al., 2008; Hebel et al., 1997; Tuomainen and Candolin, 2010).

The interactions among multiple stressors associated with seawater pH reduction resulting from elevated pCO_2 are still poorly understood in crustaceans (Whiteley, 2011) with the existing research focusing on potential synergies with temperature (Dissanayake and Ishimatsu, 2011) and salinity changes (Madeira et al., 2014). Since environmental changes associated increased pCO_2 (pH reduction) and shading are both known to affect coastal benthic biota (e.g., Lorda and Lafferty, 2010; Glaspie et al., 2017), it is likely that in combination these stressors will cause significant and interactive effects on different aspects of performance of benthic organisms in coastal marine habitats, which could include changes to patterns of mortality, growth, calcification and changes in behavioral responses.

In this context, hermit crabs may be considered as good biological models to investigate pH-induced behavioral impairment through changes to their behaviors associated with the exploration and selection of new shells and with predator avoidance (Briffa et al., 2008; Gorman et al., 2018). These behavior patterns occur as responses to environmental stimuli (e.g., chemical or visual cues), which determine the decision-making after the perception of dead gastropods or shells (Chiussi et al., 2001; Díaz et al., 1994; Gherardi and Atema, 2005) or predators (Kuhlmann, 1992; Rittschof and Hazlett, 1997). Such behavioral patterns are sensitive to changes in the physical environment associated to elevated pCO_2 (de la Haye et al., 2011, 2012; Kim et al., 2016) and light availability (Díaz et al., 1994, 1995 a, 1995b) and decision-making may be drastically affected thereby compromising individual survival and population maintenance (Briffa et al., 2012).

Here, the hermit crab *Pagurus criniticornis* (Dana, 1852) was used as a biological model to investigate the potential combined effects of two stressors, changes in pH levels and light availability, on populations of crustaceans in coastal regions. This species was chosen as model due to the easy sampling and maintenance in controlled experiments (Turra and Leite, 2003) and its potential to demonstrate changes in behavior patterns following environmental disturbance (Turra and Gorman, 2014). *P. criniticornis* inhabits intertidal and subtidal areas of tropical environments (Melo, 1999) with juveniles commonly occupying shells of the gastropod *Olivella minuta* (Link, 1807), while adults commonly occupy *Cerithium atratum* (Born, 1778) shells (Leite et al., 1998; Turra and Leite, 2003). This species is abundant in the muddy substrate of Araçá Bay (Northern coast of São Paulo State, SE Brazil), an area with potential future shading impacts, due to the expansion project of the adjacent Port of São Sebastião. The initial port expansion proposal would have meant the construction of a suspended concrete slab, which would cover 75% of Araçá Bay area (PIPC, 2011). Despite the changes in the project during the environmental licensing phase, which reduced the covered area to 34% of the bay (CDSS, 2013), the license was suspended due to the lack of studies related to cumulative or synergistic effects of the harbor expansion, combined with the impacts of other coastal enterprises. Thus, the situation in Araçá Bay may be used as an example to evaluate how impacts derived from a continuous, increasing environmental pressure (i.e., reduced seawater pH), on top of which infrastructure-derived disturbances, may potentially exert stronger interaction effects.

The aim of this study was to investigate the combined effects of both reduced seawater pH through increased pCO_2 and shading on ecological and physiological aspects of juveniles of the hermit crab *P. criniticornis*. For this, we tested the null hypothesis that mortality, total growth, calcification and behavioral responses to live predator (danger signal) and dead gastropod (a signal of the availability of empty shells) odors would not vary between organisms in the medium-term (98 days) reared under different treatments from the combination of two different pH and two photoperiod levels

2. Methods

2.1 Experimental design

A fully factorial design experiment was adopted, using two levels of pH (control: pH = 8.1 and reduced: pH = 7.6) and photoperiod (control: 12:12h and darkness: 0:24h). The pH of the "reduced" treatment was derived from predictions of a 0.5 pH unit decrease in seawater by 2100 (Caldeira and Wicket, 2005), while the "shading" treatment light regime was selected as representative of the situation of total darkness expected for areas of the Araçá Bay under the suspended concrete slab as a result of the proposed São Sebastião port expansion project.

Separate recirculating water systems using artificial seawater (Instant Ocean, Blacksburg, USA) were used for each pH condition and reduced pH was maintained by adding CO_2 through a solenoid valve connected to a pH controller system (Aqua Medic, Germany; adapted from Suckling et al. 2014; Widdicombe and Needham, 2007). Each system, with a total water volume around 239 L, consisted of a seawater reservoir (~100 L), a tank for biological filtration (~63 L) and eight experimental tanks used to house hermit crabs exposed to 12:12h and 0:24h conditions (~9.5 L each). Light was excluded from tanks in the shaded treatment by covering the walls and lids with black plastic adhesive sheet. To reduce tank effects, aquaria corresponding to the different photoperiod conditions were randomly placed within the two pH systems. The two recirculating systems did not differ in temperature (Student's *T test*: $t_{162}=0.83$, $p=0.41$) and salinity ($t_{81}=0.45$, $p=0.65$), with overall average values of 25.6°C and 31.9 psu respectively (Table 1). The average pH values for the control and reduced pH treatments were 7.63 and 8.08 (Table 1), which hereafter are referred to as 7.6 and 8.1 respectively. Temperature and salinity were maintained at target levels (25–26ºC and 32 psu respectively) using aquarium heaters (H-606, Hopar, Guangdong, China) and a system consisting of a water level sensor connected to a tank of distilled water was used to prevent salinity changes caused by evaporation. Each recirculating system was connected to a tank containing natural rocky substrate, nitrifying bacteria colony (ATM Colony Marine, Las Vegas, USA) and the algae *Chaetomorpha sp.* for biological filtration. Ammonia, nitrite and nitrate concentrations were measured each month by colorimetric tests (Red Sea kits, Houston, USA) (Table 1). Tanks were cleaned every week by siphoning feces and remaining food, while water volume was replaced by the water level sensor system and salinity was manually corrected by adding extra salt. The experiment was conducted for 98 days.

Table 1: Average values (Mean±SE) and Confidence Interval (CI 95%) for the controlled abiotic parameters (pH, salinity and temperature) during the 98 day exposure of juvenile *Pagurus criniticornis* to different pH and photoperiod treatment conditions.

	Reduced pH		Control pH	
	Mean±SE	CI 95%	Mean±SE	CI 95%
pH	7.63±0.007	7.61 – 7.64	8.06±0.004	8.05 – 8.07
Salinity (psu)	32.05±0.11	31.83 – 32.26	31.74±0.13	31.5 – 31.99
TºC	25.67±0.18	25.31 – 26.02	25.45±0.19	25.07 – 25.83
Ammonia (ppm)	<0.02	nd	0	nd
Nitrate (ppm)	<10	nd	<10	nd
Nitrite (ppm)	<0.5	nd	<0.5	nd

nd – below detection level

2.2 Maintenance and sampling of hermit crabs

Juveniles of the hermit crab *P. criniticornis* were collected by hand from the intertidal zone, during the low tide period in Araçá Bay (Figure 1) in October 2015. Only individuals inhabiting shells of the gastropod *Olivella minuta* were collected, in order to control the individuals' initial size and previous experience of shell occupancy, since juveniles are most commonly found inhabiting shells of this species.

After three weeks of acclimatization, crabs were individually allocated to 80 mL plastic containers to prevent agonistic interactions and facilitate the collection of exuviae. Containers were labeled and perforated to allow water exchange and a total of 48 crabs were used in each treatment group, divided between four tanks (n=12 per tank).

At the beginning of the experiment, three small shells of varied sizes of *C. atratum* were provided to the crabs, to avoid growth limitation. After the first molt from the *C. atratum* shell initially selected, each hermit crab was offered three larger *C. atratum* shells of different sizes that were left in the plastic container until the end of the experiment. Crabs were fed with commercial pellet food specific for crustaceans (two pellets per individual, three times per week; JBL, NovoPrawn, Germany). Molts and dead individuals were collected daily and stored in 70% ethanol for subsequent measurements of shield length and elemental analysis (see below).

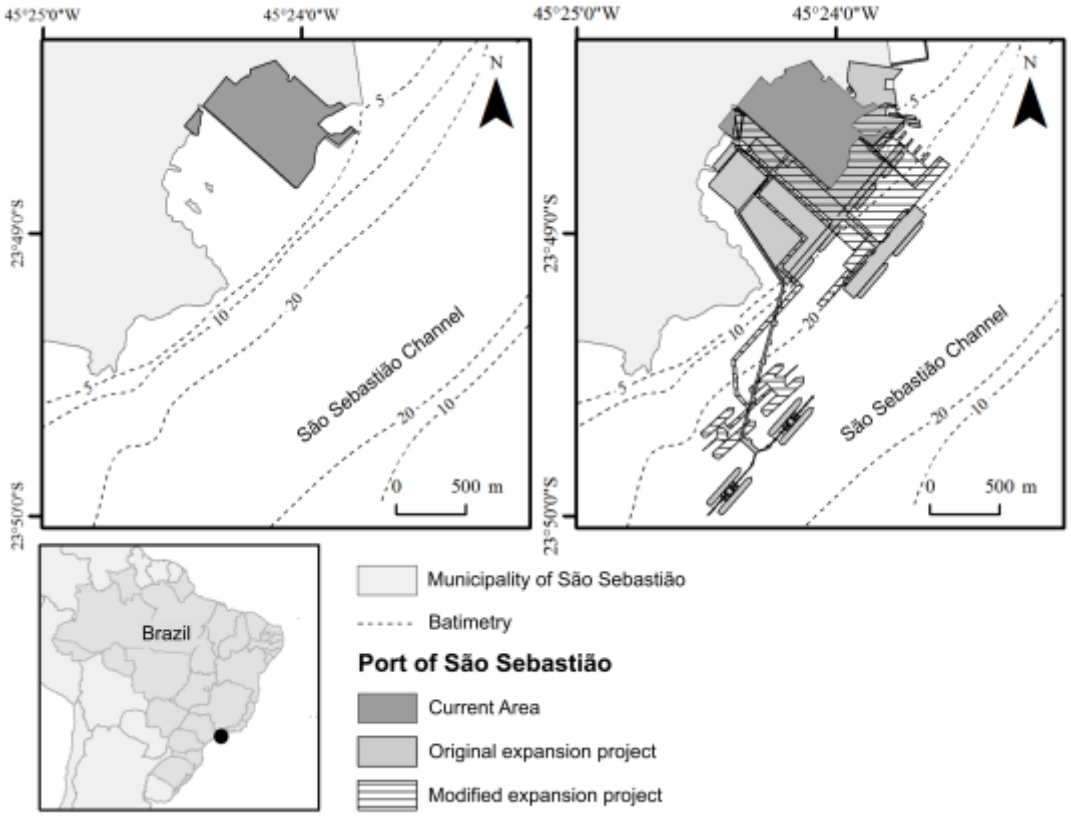

Figure 1: Araçá Bay location within the municipality of São Sebastião (Northern coast of São Paulo State, Brazil). The emphasized infrastructures correspond to the present-day harbor area, the initial project with 75% of expansion area and the current modified project with 34% of expansion area proposed.

2.3 Mortality

Average cumulative mortality was analyzed as a function of time (days), between treatment conditions, using a two-way Analysis of Covariance (ANCOVA), considering time as covariate.

2.4 Growth

Cephalothorax shield length (mm) from the first molt and for each live crab at the end of the experiment was measured, using *ImageJ* software. Since no difference in initial shield size was recorded among treatments (One-way ANOVA: $F_{3,64,}$=2.07; *DF*=3; *P*=0.11), total growth was calculated by the difference between the final and initial shield length. Damaged carapaces were excluded from this analysis (n=16), and only living individuals at the end of the experiment period were considered.

All the surviving individuals from 7.6/0:24h treatment were used for the growth analysis (n=17) and in order to balance the sample sizes for statistical analysis, 17

hermit crabs were randomly selected from the other three treatments. Data were tested for normality (Shapiro-Wilk test) and homogeneity of variances (Cochran's test) and differences in total growth were analyzed by two-way Analysis of Variance (ANOVA, pH x photoperiod), followed by Tukey's HSD test.

2.5 Calcification

The last molt of the crabs was used to compare the variation in calcification among experimental conditions (n=6 per treatment). Molt samples were carbon sputter-coated (Balzers, BAL-TEC SCD 050, Germany), and subsequently analyzed by scanning electron microscopy with energy dispersive X-ray spectrometry (SEM-EDS; FEI, Inspect F50/EDAX, Netherlands). SEM-EDS was used to estimate calcium and magnesium content by weight percentage (%w) using a semi-quantitative approach, similarly to Taylor et al. (2015). The accelerating voltage was 20 kV, the working distance 10 mm and the tilt angle 0^{o}. Monte Carlo modeling of the maximum range of X-ray penetration depth in the molt resulted in a droplet 3.0 µm wide and 1.8 µm in height.

For the above mentioned %wCa and %wMg analyses, both chelipeds of each crab were used due to their crucial role in hermit crab behavior associated with feeding (Schembri, 1982), shell fighting (Briffa and Dallaway, 2007), burying (Rebach, 1974), reproduction (Goshima et al., 1998; Turra, 2005) and investigation in response to chemical stimuli (Rittschof et al., 1992). Moreover, chelae are rigid structures, usually with a high calcium concentration due to its function (Greenaway, 1985), and are more likely to remain intact after molting.

Four different areas (300x400µm) were selected, one each from the carpus and propodus segments of both chelipeds. Analyzing the %wCa and %wMg from the targeted areas, calcification of the cheliped segments was compared among treatments. Cheliped morphology prevents exact replication of measurements in each segment, since its structure is not flat, and the precision of quantification depends on the angle in which the X-rays of the EDS system are projected onto the sample. Thus, the %wCa and %wMg data from the four measured areas were arcsine-square-root transformed and a repeated measures ANOVA was performed (pH x photoperiod) to determine whether calcification differed among treatment conditions.

2.6 Behavioral responses

At the end of the experiment, crabs were randomly selected to evaluate the behavioral responses to odor stimuli, which mimicked the presence of either live predators (n=19) or dead gastropods (n=18).

The blue crab *Callinectes danae* Smith, 1869 was used to provide the predator odor (danger signal) in this experiment, as it is a natural predator of *P. criniticornis* in Araçá Bay (Turra et al., 2005). Three specimens of *C. danae* were kept in 10 L of seawater for two hours, after which a 1 mL aliquot of that water was used as odor cue for each hermit crab. For dead gastropod assays, 30 specimens of *C. atratum* were frozen, macerated and mixed with 1 L of seawater and 1 mL from the supernatant was used as odor cue for each hermit crab assay. The dead gastropod odor was used to indicate the possibility of resource availability (i.e., new shells for exchange).

The behavioral odor response assessment was adapted from Hazlett (1996) and Rittschof and Hazlett (1997), using each individual's displacement as the response parameter to the cues. Prior to odor exposure, experimental crabs were kept without access to food for 48 hours. In each assay, hermit crabs were held in an aquarium filled with 500 mL of seawater from the appropriate treatment condition, with a 1x1 cm grid drawn on the bottom. Both odor experiments were run in the dark, using red light to avoid any influence of (white) light on crabs' behavior responses (Hazlett, 1966; Turra and Denadai, 2003). After cue insertion, the displacement of each crab was filmed for 3 minutes and then subsequently analyzed in *Kinovea* software, by counting of lines crossed.

Differences in displacement were analyzed by two-way ANOVA, followed by Tukey's HSD test, as above for growth and calcification. Displacement data were log(x+1)-transformed, due to the heterogeneity of variances and non-normal distribution. In addition, responses of all crabs experiencing both odor experiments were used to compare behavioral patterns between the environmental conditions using linear correlation with non-transformed data.

3. Results

3.1 Mortality

Mortality increased over time and was influenced by pH, resulting in a higher general mortality for pH 7.6 treatments compared to 8.1 treatments (Table 2; Figure 2). The effect of reduced pH over time was demonstrated by the progressively increasing mortality in the low-pH treatment (7.6/12:12h), becoming significantly different from the control in the fourteenth week (>8.1/12:12h, Figure 2). Dead individuals in this treatment (7.6/12:12h) represented 23.9% of the total mortalities during the experiment. Photoperiod alone did not affect mortality, with no difference observed between shaded (8.1/0:24h) and control treatments, during the experimental period. Both treatments represented 6.5% (8.1/0:24h) and 2.2% (8.1/12:12h) of total deaths, respectively.

Nevertheless, photoperiod may present an additional effect to acidification condition over time, as demonstrated by the significant interaction observed between the three factors (pH*photoperiod*time) (Table 2). This effect resulted in a higher mortality of hermit crabs being observed in the combined acidified/darkness treatment (7.6/0:24h; Table 3), 56 days earlier compared to the other treatments (Figure 2). Of all individual hermit crabs that died during the experiment, 67% were kept in the combined acidified/darkness treatment (7.6/0:24h), thus providing evidence for synergistic effects. At the end of the experiment, a total of 46 dead individuals (i.e. 24%) were recorded.

Table 2: Analysis of Covariance (ANCOVA) of the average cumulative mortality of juvenile *Pagurus criniticornis* reared under different conditions of pH (8.1 and 7.6) and photoperiod (12:12h e 0:24h), using time (98 days), as covariate.

	DF	*MS*	*F*	*p*
Intercept	1	7.86	13.17	<0.001
pH	1	5.92	9.92	<0.001
Photoperiod	1	0.34	0.57	0.45
Time	1	165.86	277.99	<0.001
pH*Photoperiod	1	0.36	0.61	0.44
Photoperiod*Time	1	39.25	65.79	<0.001
pH*Time	1	112.18	188.01	<0.001
pH* Photoperiod*Time	1	31.02	51.98	<0.001
Error	216	0.60		

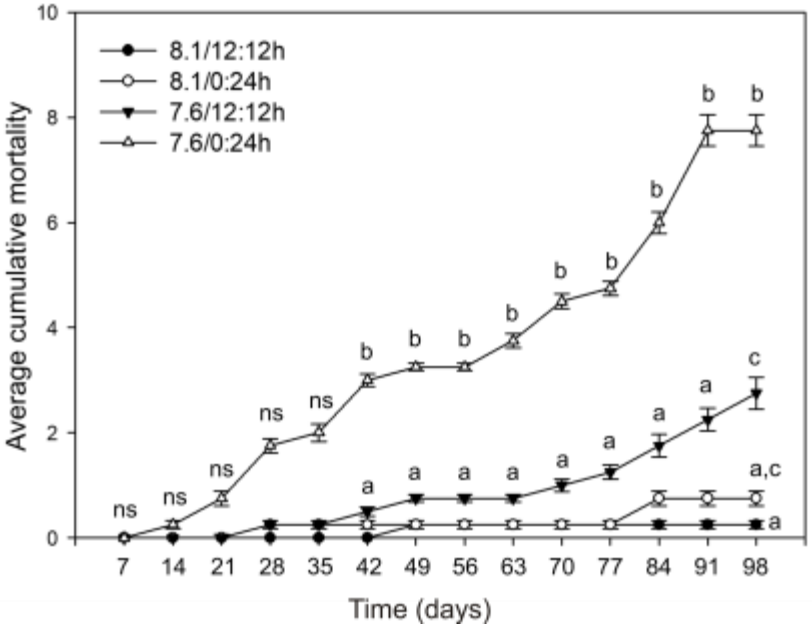

Figure 2: Mortality of juvenile *Pagurus criniticornis*, expressed by the average cumulative mortality (Mean±SE) during 98 days of exposure, to different treatments of pH (8.1 and 7.6) and photoperiod (12:12h and 0:24h). Different letters represent significant differences by Tukey test among treatments at each time interval (7 days); ns= not significant.

Table 3: Summary data of sample sizes, crab size (Mean±SE), mortality and carapace mineral content for the different conditions of pH (8.1 and 7.6) and photoperiod (12:12h and 0:24h). N_0 = initial sample size; N_t = final sample size; $CTSL_0$ = initial cephalothorax shield length (mm); $CTSL_t$ = final cephalothorax shield length (mm); wCa% = weight of calcium (%) and wMg% = weight of magnesium (%).

	8.1/12:12	8.1/0:24h	7.6/12:12	7.6/0:24
N_0	48	48	48	48
$CTSL_0$	2.02±0.04	2.07±0.05	2.13±0.03	1.97±0.05
N_t	47	45	37	17
$CTSL_t$	2.69±0.07	2.61±0.09	2.65±0.05	2.34±0.09
% Mortality	2.08	6.25	22.92	64.58
wCa %	25.96	25.91	27.32	27.72
wMg %	2.00	2.03	2.31	2.09

3.2 Growth

Initial and final cephalothorax shield lengths are presented in Table 3. Photoperiod and pH affected growth (Table 4), indicating a general pattern of lower growth rates observed in individuals exposed to reduced pH compared to control pH (i.e., total growth 8.1>7.6) and for individuals exposed to darkness compared to the 12:12h control photoperiod. Therefore, crabs reared at pH 8.1 had an average 27% larger shield length, than those kept under reduced pH 7.6 conditions. Similarly,

individuals from control photoperiod treatments showed an >24% higher growth, compared to those exposed to 24h of darkness.

The combined stressor treatment (7.6/0:24h) demonstrated the lowest growth: 46% slower than the control treatment (pH 8.1/12:12h; Figure 3). Meanwhile, single-stressor treatments showed intermediate (and similar) growth, with shield lengths 22% (7.6/12:12h) and 19% (8.1/0:24h) smaller than the control crabs (Figure 3). Thus, despite the influence of these conditions on growth, the non-interaction between them indicates the additive effect of both stressors (Table 4).

Table 4: Two-way Analysis of Variance (ANOVA) of total growth of juvenile *Pagurus criniticornis* reared under different treatments of pH (8.1 and 7.6) and photoperiod (12:12h and 0:24h) following a 98 day exposure period (n=17 per treatment).

	DF	*MS*	*F*	*p*
Intercept	1	18.63	338.9	<0.001
pH	1	0.46	8.43	0.01
Photoperiod	1	0.34	6.16	0.02
pH*Photoperiod	1	0	0.05	0.82
Error	64	0.05		

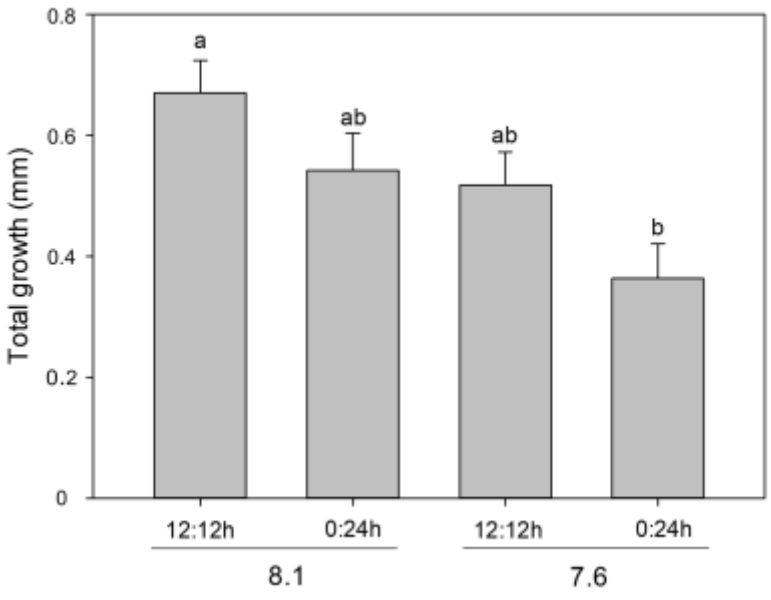

Figure 3: Total growth (Mean+SE) of juvenile *Pagurus criniticornis* (n=17) maintained under different treatments of pH (8.1 and 7.6) and photoperiod (12:12h and 0:24h) during 98 days of exposure. Different letters represent the significant difference among treatments by Tukey test.

3.3 Calcification

Crab carapaces presented an overall average mineral content of 26.7% wCa and 2.1% wMg respectively at the end of the experiment (Table 3). Although calcium and magnesium %w varied between the four measured areas of the chelipeds, the average %w of these minerals were similar between the experimental conditions with no effect of pH or photoperiod on cheliped calcification during the experiment (Table 5).

Table 5: Repeated-measures Analysis of Variance (ANOVA) of calcium and magnesium %w, measured in four measured areas of the chelipeds, for juvenile *Pagurus criniticornis* reared under different treatments of pH (8.1 and 7.6) and photoperiod (12:12h and 0:24h) following a 98 day exposure period (n=6 per treatment).

	F	*DF*	*p*
Repeated Measures ANOVA (%wCa)			
Area	5.34	3	0.002
Area*pH	2.69	1	0.83
Area*photoperiod	0.02	1	0.50
Area*pH*photoperiod	0.05	1	0.66
Repeated Measures ANOVA (%wMg)			
Area	8.45	3	<0.001
Area*pH	2.13	1	0.57
Area*photoperiod	0.56	1	0.58
Area*pH*photoperiod	1.06	1	0.27

3.4 Behavioral responses

Only reduced photoperiod (shaded condition) affected behavioral responses to predator odor exposure in hermit crabs, with no effect of pH (Table 6). Displacement after *C. danae* stimuli was 45% lower in individuals reared in 24h of darkness compared to individuals from 12:12h treatments (Figure 4A). However, effects of photoperiod, pH and an interactive effect between both factors were demonstrated in response to gastropod odor exposure (Table 6; Figure 4B). Thus, the effect of pH was evidenced only in the 0:24h condition, since individuals of 7.6/0:24h moved 70% less, compared to individuals from low pH condition and photoperiod controls (7.6/12:12h). Although the displacement of hermit crabs observed in single-stressor treatments did not significantly differ from control, crabs in shaded conditions moved 34% less compared to individuals under control pH and photoperiod (8.1/12:12h) conditions (Figure 4B).

Table 6: Two-way Analysis of Variance (ANOVA) of displacement activity (number of liens crossed) for juvenile *Pagurus criniticornis* reared under different treatments of pH (8.1 and 7.6) and photoperiod

(12:12h and 0:24) for 98 days, in response to the odors of live predators (n=19) and dead gastropods (n=18). Data were log (x+1) transformed prior to analysis.

	Predator odor				Gastropod odor			
	DF	*MS*	*F*	*p*	*DF*	*MS*	*F*	*p*
Intercept	1	426.75	287.98	<0.001	1	611.17	738.23	<0.001
pH	1	0.02	0.01	0.92	1	3.64	4.39	0.04
Photoperiod	1	19.74	13.32	<0.001	1	20.54	24.81	<0.001
pH*Photoperiod	1	2.06	1.39	0.24	1	5,.35	6.46	0.01
Error	72	1.48			68	0.83		

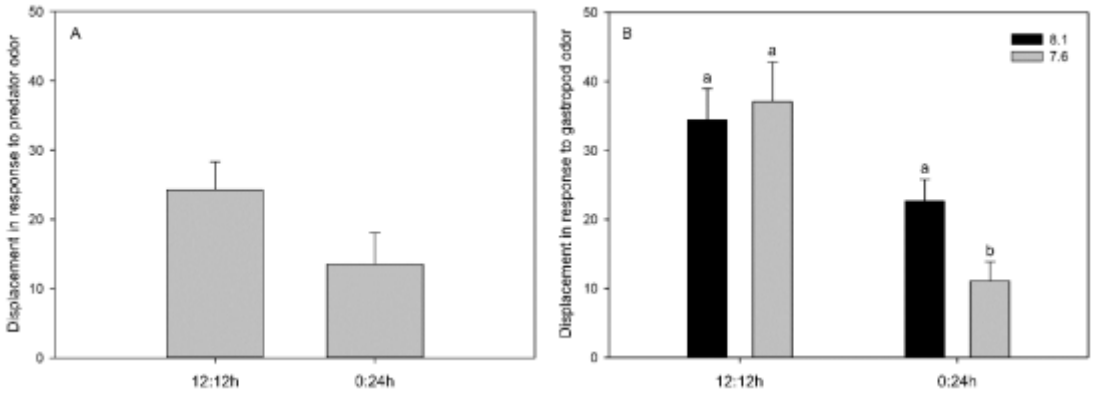

Figure 4: Displacement activity (Mean+SE), expressed by the number of lines crossed, of juvenile *Pagurus criniticornis* reared under different treatments of pH (8.1 and 7.6) and photoperiod (12:12h and 0:24h) during 98 days of exposure: (A) response to live predator odor (*Callinectes danae*) (n=19) and (B) in response to dead gastropod odor (*Cerithium atratum*) (n=18), from non-transformed data. Different letters represent significant difference by Tukey test between experimental conditions of pH and photoperiod, as evidenced by the significant interaction (Table 6).

Displacement patterns were not correlated between both stimuli for individuals reared in control and low-pH conditions (p=0.33 and p=0.23, respectively; Figure 5A and B). Responses of crabs reared in shading condition (8.1/0:24h) showed significant correlation, even though moderate (p=0.01; r=0.55) (Figure 5C). In addition, individuals maintained on combined stressors condition showed strong correlation between predator and gastropod odors and therefore strong tendency to present similar reduced response to both stimuli (p<0.001; r=0.79) (Figure 5D).

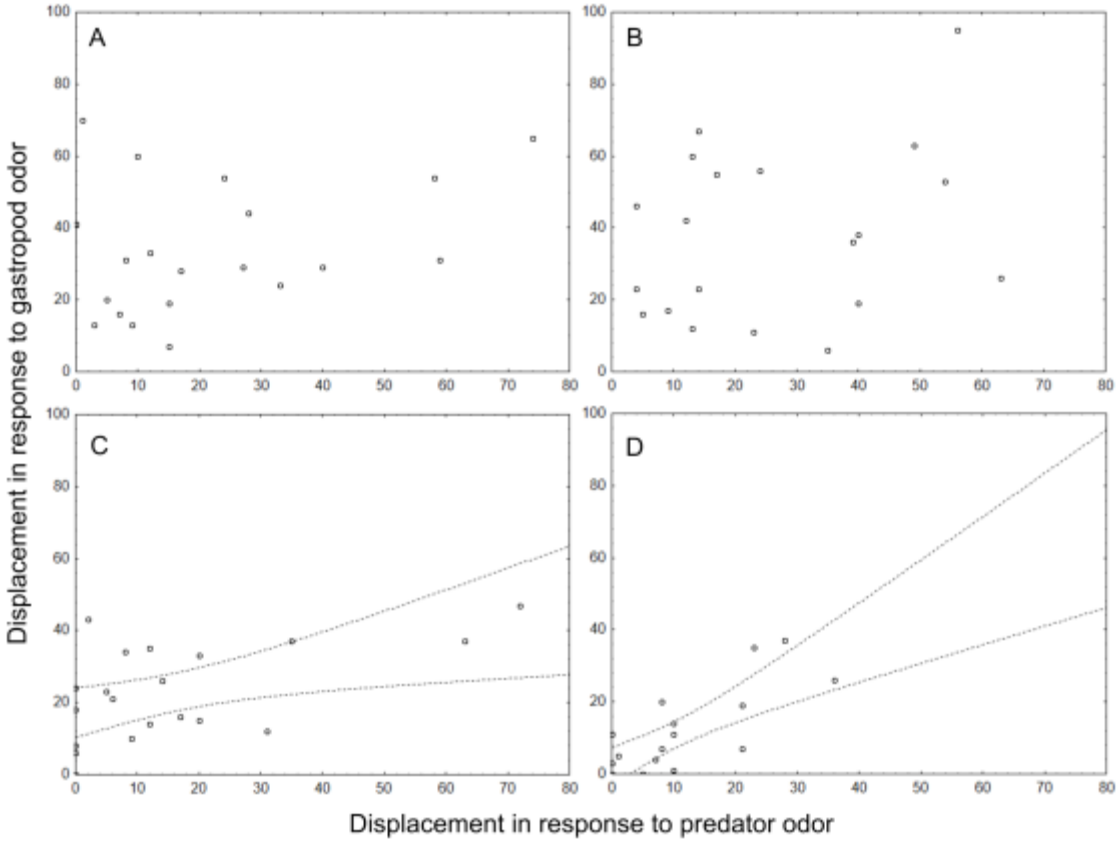

Figure 5: Correlation between displacement activity, expressed by the number of line crossed, in response to predator and gastropod odors for juvenile *Pagurus criniticornis* reared for 98 days under different conditions of pH (8.1 and 7.6) and photoperiod (12:12h and 0:24h), presented non-transformed data. A) 8.1/12:12h, control pH and control photoperiod (p=0.33); B) 7.6/12:12h, reduced pH and control photoperiod condition (p=0.23); C) 8.1/0:24h, control pH and shading (p=0.01; r=0.55); D) 7.6/0:24h, combined condition of reduced pH and shading (p<0.001; r=0.79). Dotted lines represent a 95% confidence interval.

4. Discussion

Although there was no evidence for interactive stressor effects for all response variables, our results highlight how combined environmental stressors may compromise several ecological aspects for *Pagurus criniticornis*. The vulnerability of hermit crabs to a combination of global environmental change effects and local quasi-permanent disturbances was demonstrated by high mortality, reduced growth and changes in behavioral responses that are fundamental to survival. In addition, although this study investigated acute effects - since chronic effects should consider several life stages or generations (Dupont et al., 2013) - the medium-term exposure (98 days) allows the understanding of impacts in terms of lethal effects (Kurihara et al., 2008) and impacts on advanced development stages, such as juveniles (Albright et al., 2012; Range et al., 2014), both of which may interfere in maintaining local populations.

Reduced seawater pH is known to increase mortality in several marine invertebrate taxa (Amaral et al., 2011; Donohue et al., 2012; Metzger et al., 2007), especially for the most vulnerable life stages, such as larvae and juveniles (Gosselin and Qian, 1997). In the current study, juvenile hermit crabs reared under reduced pH conditions (7.6/12:12h) did not present an immediate increase in mortality (*i.e.*, significant increase not seen until 14 weeks). This delayed response has also been observed in other crustacean species maintained under OA conditions (Kurihara et al., 2008; Long et al., 2013; Zheng et al., 2015). Although shading did not affect mortality, a synergistic effect was evidenced in the 7.6/0:24h treatment with mortalities occurring earlier, after ~42 days exposure, compared to other treatments clearly showing the additional effect of shading on the tolerance of hermit crabs to hypercapnia.

Long et al. (2013) suggest that crustaceans may be tolerant to hypercapnia and can survive during short- or medium- term exposure due to their acid-base balance regulatory capabilities. However, compensating to maintain acid-base balance implies a high energetic cost that impairs individual performance and survival in long-term exposure (Miles et al., 2007; Pörtner et al., 2004). In addition, internal compensation of external disturbances is associated with energetic costs and thus implies physiological trade-offs (Wood et al., 2008). Such trade-offs may compromise other essential physiological processes (Kurihara et al., 2013; Langenbuch et al., 2006; Wood et al., 2008) and lead to impairment of growth and reproduction (Michaelidis et al., 2005; Pörtner et al., 2004). Thus, continuous long-term exposure to reduced pH may be detrimental, even for organisms tolerant to natural extreme pH variability (e.g., *Petrolisthes cinctipes* exposed to pH 7.58 for ~40 days; Ceballos-Osuna et al., 2013), as observed in intertidal habitats (Morris and Taylor, 1983; Truchot, 1986) and as demonstrated in this study.

Physiological responses to environmental stressors may explain the reduced total growth of *P. criniticornis* reared under the combined stress of reduced pH and darkness (7.6/0:24h) compared to control conditions (8.1/12:12h), thus demonstrating an additive effect of both stressors on the growth of hermit crabs. Further, total growth was similar in the 7.6/12:12h and 8.1/0:24h treatments (*i.e.*, exposure to only one stressor) and both tended to be lower than control. Several studies have clearly showed slower growth rates in crustaceans exposed to low pH (Findlay et al., 2010; Kurihara et al., 2008; Long et al., 2013; Zheng et al., 2015). In addition, similar effects have observed in

crustaceans reared in constant darkness (Andrés et al., 2010; Chittleborough, 1975; Gardner and Maguire, 1998), since photoperiod may influence the molting process (Aiken, 1969) and growth (Hoang et al., 2003). However, photoperiod effects are dependent on both species and life stage (Yue et al., 2009) and opposite effects may occur in organisms with nocturnal activity (Morales and Barba Jr., 2015). Therefore, species vulnerable to reduced pH and/or shading may show intensified responses to the combination of both conditions, resulting in lower growth rates.

In this study, crabs showed the ability to maintain calcification over 98 days, since cheliped %wCa and %wMg did not differ among treatments. Calcium and magnesium are typically used for exoskeletal mineralization of crustaceans and the effects of hypercapnia on mineral content may vary among species. For example, while some crustaceans under hypercapnia may demonstrate increased concentration only for calcium (Taylor et al., 2015) or magnesium (deVries et al., 2016; Small et al., 2010), other species may have their exoskeletal mineral composition unaffected (Lowder et al., 2017), as observed for *P. criniticornis* in the current study, even under the combined stressors treatment. The ability to maintain, or even increase calcification rates and/or compensate dissolution rates when exposed to seawater acidification is well known amongst crustaceans (McDonald et al., 2009; Ries et al., 2009; Wickins, 1984), mollusks and echinoderms (Findlay et al. 2011; Wood et al., 2008). This capacity is associated with the ability of these calcifying organisms to produce $CaCO_3$ from CO_2 and bicarbonate (HCO_3^-), with no dependence on seawater carbonate saturation for calcification (Cameron and Wood, 1985; Roleda et al., 2012). In addition, the carapace of crustaceans is mostly composed of calcite, a less soluble form of $CaCO_3$, which makes the calcification process less vulnerable to seawater acidification (Boßelmann et al., 2007). Furthermore, the strong regulatory ability of crustaceans allows the recovery of acid-base balance after disturbances by the active transport of ions through the gills between the haemolymph and the external environment (Henry and Wheatley, 1992; Pörtner et al., 2004). Under such situations, excretion of H^+ accompanied by Ca^{2+} and HCO_3^- intake may be favorable to the calcification process (Cameron and Wood, 1985). Nevertheless, the ability to modulate calcification rates (i.e. maintain or increase dependent on species/conditions), will have energetic consequences for marine invertebrates (Findlay et al., 2011) and trade-offs thus occur between the maintenance of calcified structures and energy allocation to other biological processes (Findlay et al.,

2011; Small et al., 2010; Wood et al., 2008), such as growth and potential reduced survival, as shown in this study for *P. criniticornis*.

The results of our behavioral experiments suggest an increased vulnerability to predation, for example, in situations which predators detect their prey by chemical cues (Zhou and Rebach, 1999). Hermit crabs are able to visually identify predators, moving in the opposite direction when a predator's silhouette or dark area is associated to odor cues (Orihuela et al., 1992). This behavioral response is also demonstrated in other decapod species (megalopa stage of *Aratus pisonii*, Díaz et al., 1995a; *Uca cumulanta*, Chiussi and Díaz, 2002). In the current study, individuals kept in 24h of darkness were less stimulated to active flight (Figure 4A) suggesting that light availability, which helps to recognize potential predators after chemical cue perception, may be crucial to induce escape responses more efficiently. Furthermore, since predator odor represents a survival risk, even a low concentration may suffice to motivate certain behavioral responses (Hazlett, 1997). For *P. criniticornis*, a potential reduction in odor detection, due to the low pH conditions, was not sufficient to affect crab displacement in response to a predator signal, as occurred in shaded conditions.

Here, the interactive effect between stressors was shown in response to the gastropod odor cue, where reduced displacement was only evident for crabs reared in the 7.6/0:24h treatment (i.e. combined stressors). This result is likely associated to the reduced motivation to search for newly available shells, which highlights the influence of environmental change on decision making and acquisition of vital resources (see de la Haye et al., 2011). Although displacement of *P. criniticornis* exposed to low pH (7.6/12:12h) was not affected in response to dead gastropod stimuli, hermit crabs may show decreased movement in more extreme pH reductions (e.g., *Pagurus bernhardus* reared in pH 6.8; de la Haye et al., 2012). In addition, hermit crabs may be less motivated to investigate new shells and take longer to exchange shells (de la Haye et al., 2011). Although not statistically significant, individuals in shaded conditions (8.1/0:24h) also tended to reduce displacement compared to those under control conditions (8.1/12:12h). Thus, disturbances in behavioral patterns in response to gastropod odor may increase the vulnerability to predators, since decreased motivation to search for suitable shells for protection may increase predation risk. Thus, the results of the current study demonstrate how the effects of such stressors on the behavior of

marine organisms may be underestimated when individually evaluated, in contrast to evaluation of the effects of combined environmental changes.

In addition, the different correlation results across environmental conditions indicate that behavioral patterns may vary depending on the anthropogenic pressures placed on populations. The laboratory study of predicted environmental change allows for investigations on how anthropogenic stressors may affect behavioral syndromes (i.e., consistent individual differences across multiple contexts and/or situations; Sih et al 2004) or plasticity (i.e., behavioral flexibility; Hazlett, 1995) of marine organisms. This is a field of research that has been little studied in crustaceans in general (Gherardi et al., 2012) with the exception of hermit crabs (see Briffa and Bibost, 2009; Briffa et al., 2008; Gorman et al., 2018; Mowles et al., 2012). However, even in hermit crabs the study of OA impacts on behavior has been restricted to the single stressor of pCO_2 (Briffa et al. 2012; de la Haye et al. 2011, 2012) and not under combined environmental changes (i.e. multiple stressors) induced by anthropogenic activities (e.g. reduced pH and shading; this study). Thus, although the present study had not assessed the potential effects of such human-induced environmental changes on behavioral syndromes or plasticity (Sih, 2013) and their role in individual performance and the cascade effects through populations, this area of research should be further investigated.

4.1 Conclusions

This is the first study to evaluate how reduced seawater pH, combined with persistent shading may affect a crustacean species, increasing its vulnerability to environmental stressors induced by human activities. This combination of stressors (i.e. reduced pH and shading) has been little investigated and the few existing studies focus on the effects on reef-building coral species (Suggett et al., 2013; Vogel et al. 2015). Our results provide evidence that calcification in hermit crabs can be maintained at the expense of decreased growth and increased and earlier mortality in populations of crabs exposed to these combined stressors. Such findings emphasize the need for long-term OA experiments in the context of multiple stressors (i.e. reduced pH in combination with other environmental changes), to assess the effects on biological processes, to determine the potential consequences at the population or even ecosystem level. Population impacts may be intensified by changes in behavioral patterns induced by the interactions between multiple stressors, for example, effects on decision-making in

hermit crabs may interfere with selection of new shells, creating optimal conditions for growth (Fotheringham, 1976), fecundity (Elwood et al., 1995) and protection against predators (Reese, 1969). In addition, we suggest that future studies focusing on behavioral responses, associated to environmental changes, consider behavioral syndromes and include repeatability of responses in order to find context-dependent consistencies.

This study suggests that, in addition to photosynthetic and calcifying organisms (Suggett et al., 2013; Vogel et al., 2015), populations of crustacean species inhabiting coastal regions also may be impacted by future scenarios of low pH and reduced light availability. Some species may be able to adapt to, or tolerate future acidification conditions (Kim et al., 2016). However, any adaptation in a multiple stressor context bears costs associated to intensified physiological stress (Rosa and Seibel, 2008), leading to potential disturbances on interspecific interactions (Keppel et al., 2015) and community structure (Menge and Sutherland, 1987). For this reason, future studies need to consider environmental changes scenarios on environmental impact assessment in order to provide a more realistic perspective to management (Heller and Zavaleta, 2009; Mani-Peres et al., 2016; Tompkins and Adger, 2004). In coastal regions, effective strategies for local mitigation of anthropogenic impacts may be employed, aiming at the reduction of potential interactive effects with global environmental changes.

5. Acknowledgements

We are thankful to Dr Nia Whiteley and Dr Coleen Suckling for their help and contributions in the experimental design. Dr. Miguel Mies for his help with water systems set up and to Dr. Francesc Montserrat for his review of the manuscript.

6. Funding: This work was supported by the São Paulo Research Foundation (FAPESP) [grant number 2015/02727-0].

7. Author contributions:

MNR: wrote the main text, set up and maintained the experiment, ran the behavioral responses experiment and data analyses, revised the manuscript.
IDM wrote the main text, contributed in statistical analysis and revised the manuscript.
WF: contributed in statistical analysis and revised the manuscript.
APT: supervised the calcification analysis and revised the text.

AT: supervised the study and revised the manuscript.

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