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The effect of ocean acidification on the intertidal hermit crab *Pagurus criniticornis* is not modulated by cheliped amputation and sex

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Highlights

- First study to assess long-term combined effects of OA, autotomy and sex on crustaceans.
- Crabs exposed to OA exhibited reduced survivorship, molting frequency and lipid content.
- Males showed increased molting frequency and higher regeneration rate than females.
- Interactive effects of pH, autotomy and sex were evidenced only for calcium content.
- There are no evident synergy of autotomy and sex on the effects of OA on hermit crabs.

ABSTRACT

Impacts of the interactive effects of ocean acidification (OA) with other anthropogenic environmental stressors on marine biodiversity are receiving increasing attention in recent years. However, little is known about how organismal responses to OA may be influenced by common phenomena such as autotomy and sexual dimorphism. This study evaluated the long-term (120 days) combined effects of OA (pH 7.7), experimental cheliped amputation and sex on physiological stress (mortality, growth, number of molts, cheliped regeneration and startle response) and energy budget (lipid and calcium contents) in the intertidal sexually-dimorphic hermit crab *Pagurus criniticornis*. Crabs exposed to OA reduced survivorship (46%), molting frequency (36%) and lipid content (42%). Autotomised crabs and males molted more frequently (39% and 32%,

respectively). Males presented higher regeneration (33%) and lower lipid content (24%). The few synergistic effects recorded did not indicate any clear pattern among treatments however, (1) a stronger reduction in lipid content was recorded in non-autotomised crabs exposed to low pH; (2) calcium content was higher in males than females only for autotomised crabs under control pH; and (3) autotomised females showed a proportionally slower activity recovery than autotomised males. Although our results suggest an effect of long-term exposure to low pH on the physiological stress and energy budget of *Pagurus criniticornis*, the physiological repertoire and plasticity associated with limb regeneration and the maintenance of dimorphism in secondary sexual characters may provide resilience to long-term exposure to OA.

Keywords: global change; environmental impact; water chemistry; seawater pH; physiological stress; energy budget; limb loss; sexual dimorphism.

1. Introduction

Carbon dioxide concentrations in the atmosphere are increasing, driven by anthropogenic activities, however, approximately one-third of the CO_2 released in the Industrial Age has been absorbed by the oceans modifying seawater chemistry in a process that has been termed ocean acidification (OA) (Sabine et al., 2004; Zeebe, 2012). To date, average seawater pH has declined by 0.1 units compared to pre-industrial values (*i.e.*, from *ca.* 8.2 to *ca.* 8.1) and future 'business-as-usual' IPCC scenario predictions estimate a further reduction of up to 0.4 units by 2100 (IPCC, 2014).

The impacts of OA on marine taxa and biodiversity have been widely discussed in recent reviews (Hofmann et al., 2010; Kelley and Lunden, 2017; Kroeker et al., 2010; Wittmann and Pörtner, 2013). OA can synergistically interact with other human-induced environmental changes (Mostofa et al., 2015), especially ocean warming (Byrne and Przeslawski, 2013; Przeslawski et al., 2015) and it is becoming clear that future OA research should focus on multi-stressor impacts as these may vary among taxa/species and are dependent on habitat, metabolic characteristics, activity patterns and life-cycle (Hofmann and Todgham, 2010; Pörtner et al., 2004; Widdicombe and Spicer, 2008).

Investigations on the effects of OA have generally focused on vulnerable calcifying groups (*e.g.*, corals, Gómez et al., 2015; molluscs, Parker et al., 2013;

echinoderms, Dupont et al., 2010), however, the impacts of OA on crustaceans, have been recognised (Whiteley, 2011) and research on this animal group is gaining momentum (*e.g.*, Borges et al., 2018; Coffey et al., 2017; Lim and Harley, 2018; Ragagnin et al., 2018; Whiteley et al., 2018). Although crustaceans are likely to be more tolerant to changes in seawater pH (Long et al., 2017; Taylor et al., 2015), mainly due to their acid-base regulation ability (Small et al., 2010; Wheatly and Henry, 1992), there is increasing evidence that important ecological aspects may be affected by OA, such as behavioral patterns (Dodd et al., 2015; Roggatz et al., 2016) and reproductive success (Borges et al., 2018). Furthermore, crustacean vulnerability to reduced seawater pH may increase when concurrently exposed to other anthropogenic stressors (Dissanayake and Ishimatsu, 2011; Ragagnin et al., 2018), although this is an issue that still is poorly understood.

Several invertebrate taxa are able to autotomise body parts as a defense response to increase chances of survival and decapod crustaceans represent the most widely-studied group exhibiting this phenomenon (Fleming et al., 2007). However, despite the prevalence of autotomy among crustacean species, the interactive multi-stressor effects of OA and autotomy in crustaceans have not been studied, although data are available for polychaetes (Pires et al., 2015), starfish (McCarthy et al., under review; Schram et al., 2011), sea urchins (Emerson et al., 2017) and brittle stars (Christensen et al., 2017; Hu et al., 2014; Wood et al., 2008, 2010).

Crustaceans may undergo limb loss (self-amputation or autotomy), usually chelipeds, during intra- and interspecific interactions and especially as a strategy to escape from predators (Mace and Curran, 2011; Maginnis, 2006). Although advantageous for survival, energy budgets can be affected with body resources being reallocated to regeneration, potentially at the expense of growth, mating success and ability to compete for resources (Juanes and Smith, 1995; Maginnis, 2006; Mariappan et al., 2000). Thus, the stress/cost of limb regeneration may increase the vulnerability of the organism to other stressors or threats (Mariappan et al., 2000) such as OA.

In addition, the synergistic effects of OA and autotomy may also be sex-dependent, a factor that has been largely neglected in the literature (Ellis et al., 2017). The few studies conducted to date on crustaceans are equivocal with either some (Kurihara et al., 2008), or no (Donohue et al., 2012) evidence of any sex-related effects of OA. The potential for an interactive effect of sex and OA may be especially relevant in sexually-dimorphic taxa where differences in gonad size, body size and secondary sexual characters are observed (Ellis et al., 2017; Shine, 1989). Crustaceans, especially

decapods, exhibit sexual dimorphism in relation to body size (males normally larger than females; Subramoniam, 2017) and in some species cheliped asymmetry is also observed (Salmon, 1987). Cheliped asymmetry is observed in some hermit crabs, *e.g.* in *Pagurus* spp. the right cheliped is larger (Nucci and Melo, 2011) and in *Calcinus* spp. the left cheliped is larger (Nucci and Melo, 2015). In the genus *Pagurus*, males may possess a relatively larger right cheliped than females (Matsuo et al., 2015). Besides the fundamental role in defense, feeding and mating behaviors (Yasuda et al., 2011, 2014), the major cheliped has a particular function for hermit crabs as a weapon during contests for gastropod shells (Arnott and Elwood, 2007; Elwood et al., 2006) and, in the case of males, for mates (Turra, 2005). Therefore, any impairment of claw integrity or regeneration due to reduced pH may potentially impact on fitness at the individual or population level.

Hermit crabs are considered appropriate biological models to both understand the consequences of limb autotomy and the importance of the regeneration process for individual fitness and population maintenance (Yasuda et al., 2014) and to evaluate behavioral effects of ocean acidification, since they demonstrate clear impaired responses under environmental changes and stimuli (Briffa et al., 2012). Studies on the effects of OA on hermit crabs have shown decreases in growth (Ragagnin et al., 2018), "antennular flicking" (de la Haye et al., 2011; 2012; Kim et al., 2016), shell exchange (de la Haye et al., 2011), locomotory activity (de la Haye et al., 2012), the ability to approach food (Newman and Dubuque, 2013) and to identify signals of shell availability (Ragagnin et al., 2018), as well as increased mortality (Ragagnin et al., 2018) and an increase in the time needed to select a new shell (de la Haye et al., 2011). Some of these effects are related to changes in the olfactory function and represent significant impacts on information gathering in hermit crabs (Kim et al., 2016). However, the studies evaluating chemosensory capacity and behavioral responses of hermit crabs have been under short-term exposure to reduced pH as a single stressor (de la Haye et al., 2011; 2012; Newman and Dubuque, 2013), and little is known about the potential effects of long-term exposure to OA. Therefore, considering the potential impacts on hermit crabs caused by changes in seawater chemistry, especially when it occurs simultaneously with other stressors (Ragagnin et al., 2018), we predict that the long-term effects of reduced pH may be amplified by the physiological repertoire that has evolved in hermit crabs regarding limb regeneration and the development of secondary sexual characters.

In this context, this study evaluated the potential long-term (120 day exposure) synergistic effects of ocean acidification expected by the end of the 21st century (pH 7.7; RCP8.5 pCO_2 levels predicted for 2100; IPCC, 2014) with experimental right cheliped amputation and sexual dimorphism on physiological stress (mortality, growth, number of molts, cheliped regeneration, and startle response) and energy budget (lipid and calcium contents) of the intertidal and shallow subtidal tropical hermit crab *Pagurus criniticornis* (Dana, 1852) (Decapoda, Anomura).

2. Materials and methods

2.1. Sampling and experimental design

Individuals of *Pagurus criniticornis* (Dana, 1852) were collected by hand in August 2016 at Araçá Bay, located on the northern coast of São Paulo State (23°48'47''S, 45°24'30''W). Immediately after collection, individuals were placed in thermal boxes containing seawater aerated using battery-powered air pumps and with empty gastropod shells to minimize agonistic interactions, and transported to the Oceanographic Institute at the University of São Paulo. In the laboratory, crabs were acclimatized for two weeks in seawater aquaria (salinity 31; Light:dark photoperiod of 12:12 hours; temperature ca. 23 ºC, pH 8.1) with constant aeration and biological and mechanical filtration and fed *ad libitum* with pelleted food for crustaceans (JBL, NovoPrawn, Germany).

After the acclimation period, the hermit crabs were removed from their gastropod shells to identify sex and, after allowing each crab to return to its shell, 160 individuals (80 males and 80 females; shield length: 4.45 ± 0.51 mm and 4.35 ± 0.58 mm, respectively; although males are on average larger than females, crab size was controlled in the experiment.) were individually allocated to plastic containers with drilled walls to ensure good water circulation. Eight combinations of treatments (n=20 crabs each) were set up in an orthogonal design and maintained for 120 days (25th August to 22nd December, 2016). The experimental treatments consisted of two levels of pH [pH 8.25 (Control) and pH 7.7 (reduced pH)], cheliped amputation (autotomised and non-autotomised individuals), and sex (male and female).

For the treatments with autotomised crabs (i.e., experimentally amputated), 80 individuals (40 males and 40 females) were individually anesthetized with 7.5% magnesium chloride ($MgCl_2$) and their right cheliped (*i.e.* larger) was sectioned with a pair of scissors between the ischium and the merus.

Two independent recirculating water systems were used, one for each pH treatment, using artificial seawater (HW Marinemix Reefer, HW Wiegandt, Germany). Each system (*ca.* 440 L of total seawater volume) consisted of a seawater reservoir (310 L), 10 tanks used to house the hermit crabs (9.5 L each) and a tank for biological filtration (35 L). The control pH treatment was initially planned at pH 8.1 to represent an average actual pH in the ocean. However, the system stabilised at a value of 8.25 approximately 3 weeks after the experiment had started due to the biological filtering system. We decided to not correct the control pH by modifying the biological filtering system (the same in the low pH treatment) or introducing other substances in the aquaria to avoid additional influences and oscillations in the system. Since *Pagurus criniticornis* is an intertidal species commonly occupying tide pools (Turra and Denadai, 2003; Nucci and Melo, 2011), where pH tend to be more alkaline (Truchot and Duhamel-Jouve, 1980; Wolfe et al., 2013), this relatively high "control" pH represents a natural condition for this species and can be used to be contrasted to the experimental (low) pH. In this way, the experimental treatments are robust to allow unequivocal considerations about the effect of reduced pH on the tested species of hermit crab. In addition, we note that the mortality rates for *P. criniticornis* exposed to control pH values of 8.25 for 120 days in the current study (see results), were comparable to those recorded for control *P. criniticornis* reared at pH 8.1 for 98 days (Ragagnin et al., 2018).

The reduced pH treatment was based on the expected reduction of 0.3-0.5 pH units by the end of the 21st century (the 'business-as-usual' IPCC scenario; IPCC, 2014). Crabs were introduced into this system and the pH from the experimental system was slowly reduced over 48 hours to reach 7.7 units in order to avoid any effect on survivorship related to a sudden pH decrease. The reduced pH recirculating system was maintained at pH 7.7 by bubbling CO_2 in the seawater reservoir through a solenoid valve linked to a pH controller system (accuracy of ±0.01 unit; Aqua Medic, Germany).

Eight separately-housed individuals were allocated per tank, two individuals of each sex and amputation treatments. All crabs were fed *ad libitum* with pelleted food for crustaceans (JBL, NovoPrawn, Germany) during the experimental period and three empty gastropod shells were provided for each separately-housed individual to avoid growth limitation. Salinity, pH and temperature were measured daily. Dissolved inorganic carbon (DIC) and total alkalinity (TA) samples were taken monthly, and analyzed in triplicate by infrared detection (LICOR-AIRICA, Marianda, Belgium) and potentiometric titration

(Titrino, Metrohm, Brazil), respectively (Dickson et al., 2003). Data were corrected using certified reference materials (Scripps Institution of Oceanography, USA).

2.2. Physiological stress

2.2.1. Mortality

Any dead individuals were recorded daily during the 120-day experimental period and immediately removed from the tanks, in order to minimize changes in water quality. Mortality patterns were evaluated from the cumulative mortality (*i.e.*, cumulative number of deaths) per 30-day experimental period (*i.e.*, after 30, 60, 90, and 120 days respectively) and compared among treatments after 120 days.

2.2.2. Growth and number of molts

Shield length (± 0.001 mm) was measured from the first molt and from the live crabs at the end of the experiment. These measurements were taken from digital images of the molts (or crabs) obtained using a stereomicroscope coupled with a camera and analyzed using the ImageJ 1.51d software (Abramoff et al., 2004) through the "Measure" function after scale setting ("Set Scale" function). Molts with damaged shields were excluded from the analysis and only individuals that survived until the end of the experiment were included in the statistical analysis. For these individuals, the total number of molts was recorded at the end of the experiment.

2.2.3. Cheliped regeneration

The cheliped regenerative capacity was evaluated by comparison to the length of the right cheliped amputated at the beginning of the experiment. These measurements (to the nearest mm, using digital images collected as described above) were taken at the end of the 120-day experiment for all surviving experimentally-autotomised males and females.

2.2.4. Startle Response

To verify changes in behavioral responses due to the experimental conditions, the startle response (Briffa et al., 2008; White and Briffa, 2016) of all surviving hermit crabs was analysed at the end of the 120 days of experiment. Each individual was removed from the experimental system, placed in a nine-liter aquarium with the same physico-

chemical parameters as their treatment water and allowed to acclimate for 10 minutes. Then, the hermit crab shells were turned, using forceps, so the shell aperture was uppermost, and the time taken (seconds) by each individual to turn the shell with the locomotory appendages back to the substrate was recorded (Briffa et al., 2008; White and Briffa, 2016) using a stopwatch (accuracy ± 0.01s).

2.3. Energy budget

2.3.1. Lipid content

The total lipid content was measured in all individuals that survived to the end of the experiment using the extraction method of Folch et al. (1957) and quantified following Frings et al (1972). For this analysis, the whole body of each crab was used, except the left cheliped, which was used for calcium content analysis (see below). Each individual was weighed (g) and homogenized in a 2:1 solution of chloroform and methanol (according to Folch et al. 1957) using an ultrasonic processor. Then, 0.5 ml distilled water was added, the solution was centrifuged for 5 minutes at 1,000 rpm and dried in liquid nitrogen for 45 minutes. Following this procedure, the total lipid content was determined according to Frings et al. (1972). Briefly, 100 µl of the sample was pipetted into 50 ml falcon tubes in triplicate, 1 ml of chloroform was added, the solution mixed, samples were evaporated for 15 min in an oven at 60 °C. Then, 200 µl of concentrated sulfuric acid was added and the solution was heated for 10 minutes in boiling water on a heating plate at 100 °C. The solution was then cooled in an ice bath for 5 minutes, 5 ml of phospovaniline was added, and the samples were reheated on heating plate for 15 minutes at 37 °C. Finally, absorbance at 540 nm was measured by spectrophotometry and related to a calibration curve prepared with cod liver oil (Sigma – Cod liver oil fatty acid methyl esters – C5650 10g) as a standard.

2.3.2. Calcium content

The calcium content in the left cheliped was measured in all surviving individuals. The samples were dried in an oven at 60 °C for 48 hours, weighed (g), digested in solution of nitric acid (HNO_3) and hydrogen peroxide (H_2O_2), maintained in boiling water in a heating plate at 100 °C for one hour, and 10 ml of deionized water was added. Calcium concentration was then analyzed by flame atomic absorption spectrometry [AAS vario 6 model with automatic sampler AS52 (AnalytikJenaAG, Jena, Germany) and hollow cathode lamp (Narva, Germany)].

2.4 Statistical analyses

All experimental data were first tested for normality (Shapiro-Wilk test) and homogeneity of variance (Levene's test) and met the assumptions for parametric statistical analysis (Zar, 2010) except for the startle response data that was transformed as $Log_{10}(SR+1)$ prior to analysis. Measures of physiological stress (mortality, growth, number of molts and startle response) and energy budget (lipid and calcium content) after 120 days were each analysed using a three-way ANOVA (pH, sex and autotomy plus two-way and three-way interactions) followed by Tukey HSD *post-hoc* tests (Zar, 2010). Crab growth was evaluated by the difference between the initial and final shield length (mm). Differences in cheliped regeneration between treatments after 120 days were analyzed using a two-way ANOVA (pH and sex), followed by Tukey HSD post-hoc tests (Zar, 2010).

3. Results

During the 120-day experimental period, the physico-chemical parameters of the seawater (pH, salinity, temperature, dissolved inorganic carbon, and total alkalinity) exhibited little variation over time (Table 1). The exception being the pH of the control treatment that increased from 8.1 to 8.25 during the first 3 weeks of the study but then stabilised for the remainder of the experiment.

Cumulative mortality increased over time in all treatments (Fig. 1A) with a total of 63 crabs (39%) dying by the end of the experiment. The accumulated mortality over the 120 days showed that autotomised males reared in reduced pH tended to present a higher number of deaths from the beginning of the experiment compared to all other treatments (15 crabs *cf.* 3 to 11 crabs in the other treatments; Fig. 1A). However, ANOVA analysis on total mortality after 120 days revealed that only pH as a single factor affected crab survival with no interactions among treatments (Table 2). Crabs maintained at low pH represented 73% (n = 46) of the total mortality, 2.7 times higher than the mortalities of crabs maintained at control pH (n = 17) (Table 2; Fig. 1B). Alternatively, survival rate was 46% lower in crabs maintained at low pH (34 out of 80; 43%) in comparison to control pH (63 out of 80; 79%).

Crab growth during the 120-day experiment, expressed as the difference between the final and initial shield length, was not influenced by pH, amputation or sex, with no interactions observed between any of these three treatments (Table 2; Fig. 2). A total of

21 crabs did not molt during the experimental period: 3 males and 7 females from the reduced pH condition and 3 males and 8 females from the control pH. Hermit crabs performed a maximum of five molts with the average number of molts during the 120-day experiment of 1.5 ± 1.1. Amputated males reared in control pH performed the highest average number of molts (2.3 ± 1.8) with non-amputated females maintained in reduced pH performing the lowest average of number of molts (0.6 ± 0.7). Although crab growth was not affected by treatments, the average number of molts was influenced by pH, amputation and sex, with no interactive effects observed between treatments (Fig. 3, Table 2). In general, males showed a higher average number of molts (32% higher) than females (1.7 ± 0.6 and 1.2 ± 0.5, respectively; Fig. 3A). In addition, the average number of molts of autotomised crabs was 39% higher than non-autotomised (1.8 ± 0.5 and 1.1 ± 0.6, respectively; Fig. 3B), while it was 36% lower for individuals reared in reduced pH compared to those maintained in control pH (1.8 ± 0.5 and 1.1 ± 0.5, respectively; Fig. 3C).

Cheliped regeneration was influenced by crab sex but not pH (Table 3), with cheliped length on average 33% greater in males than in females after 120 days (18.40 mm and 13.79 mm, respectively; Fig. 4). No significant interaction between sex and pH was recorded (Table 3).

The startle response on day 120 did not show any clear patterns among treatments (Table 2; Fig. 5), with a significant interaction between amputation and sex. Autotomised females showed a proportionally slower response time than autotomised males. Autotomised males showed the highest SR values, *i.e.* responded faster compared to autotomised females (average response times: 76.5 seconds and 21.5 seconds, respectively) (Fig. 5).

Lipid content after 120 days was affected by pH, sex and an interaction between pH and amputation (Table 4) with a greater reduction in lipid content recorded in non-autotomised crabs exposed to low pH (Fig. 6A). In general, OA reduced average lipid content by 42% in comparison to that recorded in crabs reared at the control pH (0.85 mg/g and 1.45 mg/g, respectively; Fig. 6A). In addition, average lipid content in males was 24% lower than the lipid content in female crabs (0.99 mg/g and 1.30 mg/g, respectively; Fig. 6A). The calcium content of the left cheliped was influenced by the interaction between amputation and sex (Fig. 6B; Table 4), with higher values in males than females only for autotomised crabs under control pH conditions. In general, calcium content was 6 % higher in males (176.75 mg/g) than females (166.13 mg/g).

4. Discussion

The present study has highlighted some potential effects of long-term exposure to the reduced surface seawater pH predicted for the end of this century (pH 7.7), combined with autotomy events (as represented by experimental cheliped amputation) and sexual dimorphism, on the performance of the intertidal, sexually-dimorphic hermit crab *Pagurus criniticornis*. No synergistic effects of pH, amputation and sex were demonstrated on mortality, growth, and cheliped regeneration, although single treatment effects of pH (mortality) and sex (cheliped regeneration) were observed. The few interactive effects recorded were: (1) between sex and amputation for startle response, with amputated females responding more slowly than amputated males; (2) between pH and amputation for lipid content, with a significant reduction in lipid content in non-amputated crabs exposed to reduced pH; and (3) among pH, sex and autotomy for calcium content - the only interactive effect between the three conditions - with higher calcium concentrations in males than females, but only for amputated crabs under control pH condition. Thus, no clear patterns of synergistic effects were recorded in this study.

Ocean acidification has been reported to significantly increase mortality in marine crustaceans (Dissanayake et al., 2010; Dissanayake and Ishimatsu, 2011; Findlay et al., 2010; Kurihara et al., 2008; Long et al., 2013; Ragagnin et al., 2018). Our results indicated a similar pattern of mortality, with long-term exposure to reduced pH resulting in higher mortality rates than control pH conditions. One aim of our study was to determine any interaction between sex and pH on mortality, however, no sex-related effects were observed. Since *Pagurus criniticornis* is a sexually dimorphic species, with larger cheliped asymmetry in males than in females, we expected that amputation of the right (larger) cheliped would amplify the potential cumulative role of sex on the effects of OA. However, this effect was not supported by the data gathered. Few studies have investigated how sex may modulate the effects of OA on crustaceans, with the available data indicating that sex-related effects may vary among species. For example, in the Pacific grass shrimp *Palaemon pacificus*, males exhibited higher survival rates (but not growth) than females when exposed to reduced seawater pH values of 7.89 or 7.64 compared to control seawater at pH 8.16 for 30 weeks (Kurihara et al., 2008). In contrast, no evidence of sex-related effects were seen in the burrowing shrimp *Upogebia deltaura* exposed to reduced seawater pH values of 7.64, 7.35 or 6.71 compared to control seawater at pH 7.99 for 35 days (Donohue et al., 2012). Since sensitivities to OA can vary among

taxa, species and populations (Kroeker et al., 2013; Przeslawski et al., 2015; Whiteley, 2011; Wittman and Pörtner, 2013), more research is needed to determine whether there are any consistent effect of sex on mortality rates in crustaceans.

It was expected that amputation would increase the effect of reduced pH on mortality, however, no differences were observed between autotomised and non-autotomised individuals. Autotomy is a natural defense mechanism and despite the immediate survival benefit, the regrowth of an appendage may be physiologically expensive, especially in cases of the loss of the major cheliped (Maginnis et al., 2014). Although autotomy itself may not increase mortality risk *per se*, crabs may become more vulnerable as a result of the limb loss, since it may impair feeding ability (Flynn et al., 2015) and/or increase susceptibility to predators and competitors (Darnell et al., 2018; Maginnis et al., 2014). However, mortality associated with limb loss in crustaceans may depend on its function in the behavior and ecology of a species. For example, stone crabs *Menippe* spp. display increased mortality in the natural environment following forced claw removal during fishing activities since amputated crabs have limited ability to crush their bivalve prey (Duermit et al., 2015, 2017). In addition, autotomised hermit crabs change shells less frequently (Matsuo et al., 2014), which may prevent crabs from finding and using more adequate shells within which they can withdraw and protect themselves from predators. Crabs in low adequacy shells (*i.e.*, shells relatively smaller than crabs) are more susceptible to predation than crabs in adequate shells (Vance, 1972). Since chelipeds are also relevant to hermit crabs for feeding (Turra and Denadai, 2003), burrowing (Rebach, 1974) and mating (Yasuda et al., 2011, 2014; Turra, 2005), autotomy may have chronic effects at both the individual and population level. These effects may be intensified under OA, which was demonstrated here to reduce survivorship and lipid content (*e.g.*, energy for reproduction). Nevertheless, further studies on the ecological responses under these natural situations in the context of OA would be necessary to understand species-specific vulnerability.

In the present study, neither sex, pH or autotomy affected the overall growth of *P. criniticornis* but individually these factors all influenced the number of molts. Although no interaction was observed, molting rate was reduced at low pH (i.e. molting frequency was 36% lower than crabs exposed to control pH), as has already been demonstrated for other crustaceans (Findlay et al., 2010; Kurihara et al., 2008; Long et al., 2013; Zheng et al., 2015). Previously, we have shown that juvenile *P. criniticornis* exhibited reduced growth at pH 7.6 (Ragagnin et al., 2018), indicating that different responses may be

observed among life stages (Byrne, 2011; Byrne and Przeslawski, 2013). Reduced growth will be associated with energetic trade-offs, where the costs to physiological maintenance may impair processes related to growth and reproduction (Kurihara et al., 2013; Pörtner et al., 2004; Wood et al., 2008). Thus, it is likely that hermit crabs exposed to reduced pH presented fewer molt cycles due to the higher energetic costs incurred due to OA (Whiteley, 2011).

In addition, the higher number of molts in autotomised compared to non-autotomised individuals, and in males compared to females (39% and 32%, respectively) may be associated with the important role the major cheliped plays, especially in males crabs, in mating behavior and male-male contests (Yasuda et al., 2014). Previous work has shown that autotomy accelerates molting cycles, possibly as an adaptive response to recover the major claw considering its functional role (Darnell et al., 2018). Males may regenerate their major cheliped at the first molt in some species (Yasuda et al., 2014). Indeed, our results showed that males had a significantly higher regeneration rate of the right cheliped compared to females (*ca.* 33%), with no influence of reduced pH exposure. These results highlight the importance of energy allocation to a fast growth of the major claw in males of *Pagurus criniticornis* due to the potential ecological costs related to this condition (*e.g.*, disadvantage in male-male contests for females and shells and less protection against predators), as demonstrated for other *Pagurus* species (Yasuda et al., 2011, 2014).

Regarding behavioral responses, autotomised females demonstrated a clearly slower startle response (*i.e.*, activity recovery) than autotomised males, irrespective of pH treatment. Sex-dependent differences in startle response between males and females have been rarely explored in behavioral studies. To the best of our knowledge, the only studies considering the effect of sex on startle response are by Briffa et al. (2008) and White and Briffa (2016), who showed no influence of sex in *Pagurus bernhardus* in the field and in the laboratory in the absence/presence of predator cues (Briffa et al., 2008) or exposed to high concentrations of copper White and Briffa (2016). Sex also had no effect on shell abandoning response by the hermit crab *Pagurus criniticornis* when exposed to experimental entrapment of the shell (Gorman et al., 2015). In the present study, the faster response of males may be related to increased aggressiveness expected in autotomised males compared to autotomised females, considering the importance of aggression in the frequent male-male contests observed in hermit crabs (Suzuki et al., 2012; Yasuda and Koga, 2016). Unexpectedly, reduced pH did not affect behavioral activity of. *P.*

criniticornis, contradicting some studies reporting changes in startle response in *Pagurus spp.* exposed to other environmental stressors (temperature; Briffa et al. 2013; copper, White and Briffa, 2016) as well as reduction in displacement behavior in *P. criniticornis* exposed to combined effect of AO and shadow in response to gastropod odor (Ragagnin et al., 2018).

In our study, pH-dependent differences in lipid concentrations were observed, with lower mean lipid concentrations recorded in individuals maintained in the acidified treatment, thus providing evidence of higher energy expenditure under OA (Carter et al., 2013). There is also evidence that lipids reserves were significantly reduced under increased temperature (25.2 ± 0.6 °C) and pCO_2 (763.0 ± 104.6 ppm) on the whelk *Dicathais orbita* (Valles-Regino et al., 2015). Further, lipid reserves decreased in crustaceans exposed to other stressful environmental conditions, for example cadmium (freshwater crab *Sinopotamon henanense*; Yang et al., 2013) and crude oil (blue crab, *Callinectes sapidus*; Wang and Stickle, 1988), showing that lipid content of marine invertebrates can be affected by adverse environmental conditions. In addition, it is still unclear how these effects may induce consequences on the community scale, but there is evidence that impacts on fatty acids composition of lower trophic levels organisms may impair growth and reproduction of consumers (Rossol et al., 2012).

In the present study, an interaction among the three treatments (pH, autotomy and sex) was observed for calcium content. In general, males presented a higher calcification than females, feature that was maintained in autotomised males compared to autotomised females reared in control pH, but not under low pH. Although it is well known that several species of crustaceans may demonstrate both maintenance or increased rates of calcification when specimens are subjected to long-term exposure to ocean acidification conditions (Long et al., 2013; McDonald et al., 2009; Ries et al., 2009; Ragagnin et al., 2018; Small et al., 2010; Taylor et al., 2014), in the present study, exposure to low pH eliminated the difference in cheliped calcification between autotomised males and females. Based on the arguments presented above on the importance of chelipeds for males, pH may have an additional effect on male fitness through cheliped weakening.

In conclusion, this is the first study to evaluate the potential interactive effects of ocean acidification with intrinsic physiological characteristics and behaviors, *i.e.* autotomy combined with startle responses, and to consider the influence of sexual dimorphism on such effects in crustaceans. In addition, few studies have investigated the synergistic effects of OA with other environmental stressors under a long-term exposure

perspective. In general, our results highlight the negative effects of long-term exposure to reduced pH, through reducing survival, molt frequency and lipid content. Although our results did not provide evidence for clear patterns of synergistic impacts of the tested factors, the few interactive effects recorded highlight the different responses of these organisms to a scenario of reduced pH exposure and cheliped loss. The results indicate that males may invest more energy into faster regeneration, resulting in lower concentrations of lipids and greater number of molts, processes not governed by low pH itself *per se*. Such a response may be a consequence of evolutionary processes and osmoregulatory adaptations of intertidal species, as *Pagurus criniticornis*, as already suggested by Whiteley et al. (2018) for sea urchins. Additional evidence for the synergistic effect of amputation and sexual dimorphism with low pH might be provided by studies on subtidal hermit crabs species that exhibit cheliped asymmetry. In addition, the already existing adaptations regarding limb regeneration and the maintenance of dimorphism on the secondary sexual characters overcame the effect of OA, potentially alleviating some additional effects due to alterations in behavioral intereactions. However, other impacts of exposure to low pH (reduced survival, number of molts and lipid content) may reveal the potential chronic effects that can be up-scaled to higher levels of organization.

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Figure captions

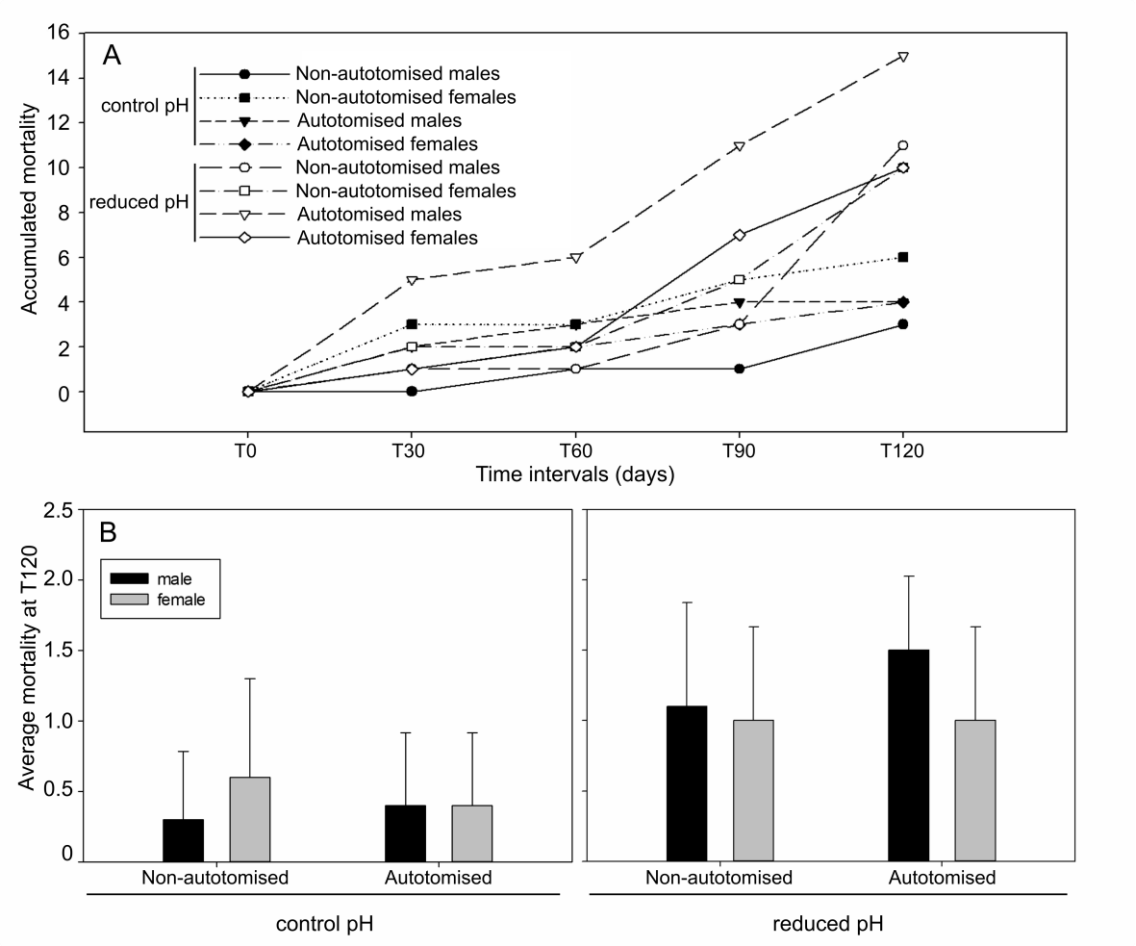

Figure 1. Mortality of males and females of the hermit crab *Pagurus criniticornis* subjected to experimental amputation of the right cheliped (autotomised and non-autotomised) and maintained at different pH treatments (control: pH 8.25; reduced: pH 7.70), during a 120-day experimental period. (A) Accumulated mortality, represented by the accumulated number of dead individuals across the time intervals (30, 60, 90 and 120 days); and (B) Average mortality [number of dead individuals per experimental tank (n=10) per treatment; Mean ± Standard deviation] at the end of the experiment (T120).

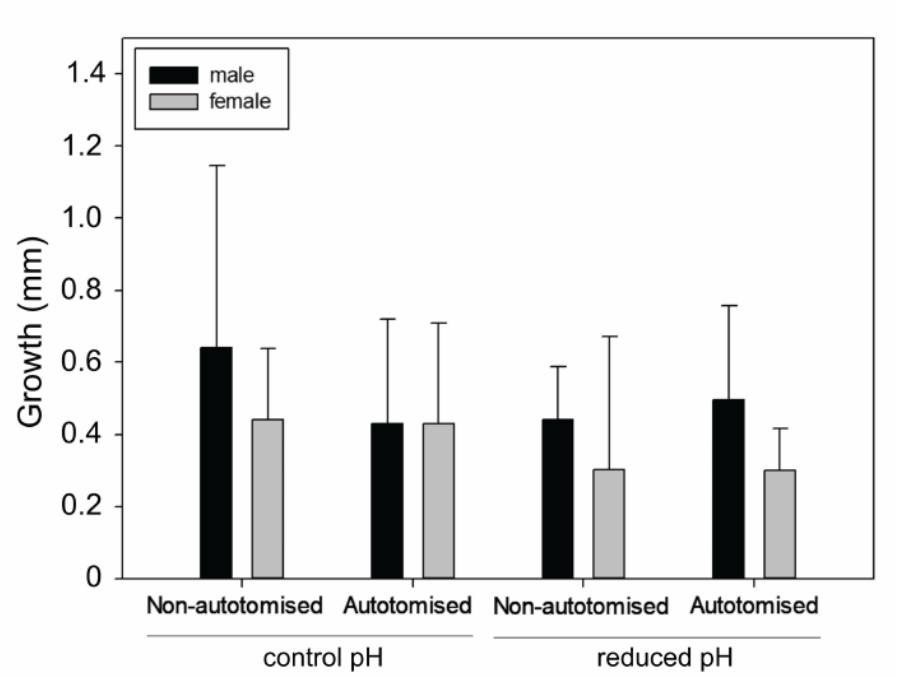

Figure 2. Growth (Mean ± Standard deviation), expressed by the difference between the final and initial shield length (mm), of males and females of the hermit crab *Pagurus criniticornis* subjected to experimental amputation of the right cheliped (autotomised and non-autotomised) and maintained at different pH treatments (control: pH 8.25; reduced: pH 7.70) during a 120-day experimental period.

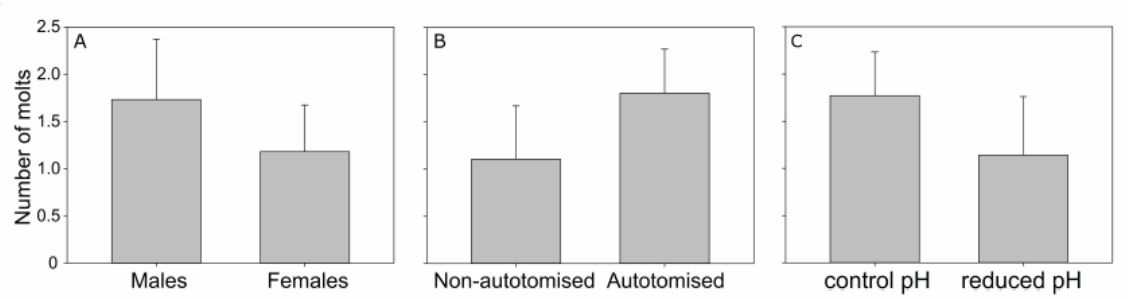

Figure 3. Number of molts (Mean ± Standard deviation) of males and females (A) of the hermit crab *Pagurus criniticornis* subjected to experimental amputation of the right cheliped (autotomised and non-autotomised) (B) and maintained at different pH treatments (control: pH 8.25; reduced: pH 7.70) (C) during a 120-day experimental period.

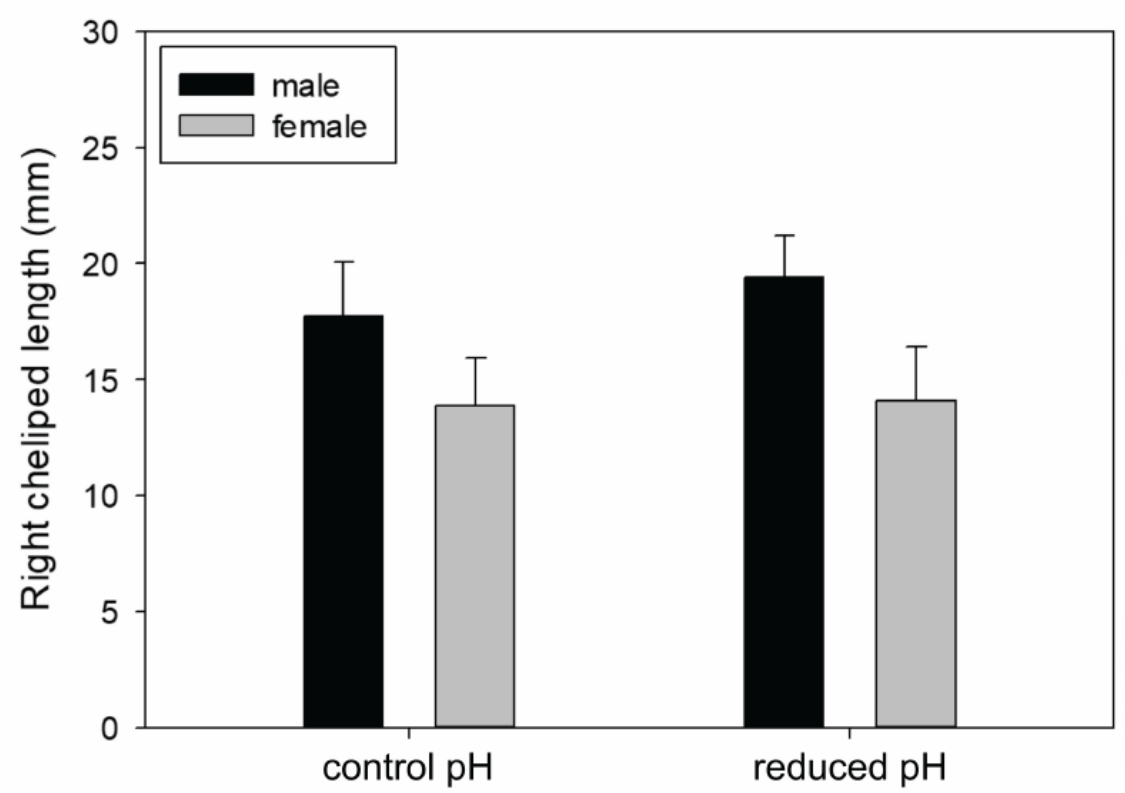

Figure 4. Cheliped regeneration, represented by the right cheliped length (Mean ± Standard deviation; mm) at the end of the experiment, of males and females of the hermit crab *Pagurus criniticornis* subjected to experimental amputation and maintained at different pH treatments (control: pH 8.25; reduced: pH 7.70) during a 120-day experimental period.

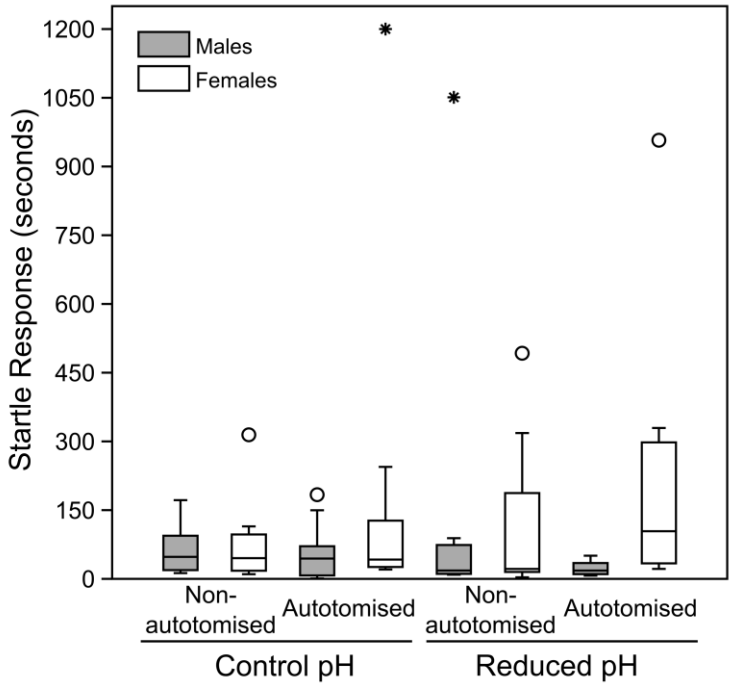

Figure 5. Startle response (Mean ± Standard deviation; s^{-1}) of males and females of the hermit crab *Pagurus criniticornis* subjected to experimental amputation of the right cheliped (autotomised and non-autotomised) and maintained at different pH treatments (control: pH 8.25; reduced: pH 7.70) during a 120-day experimental period.

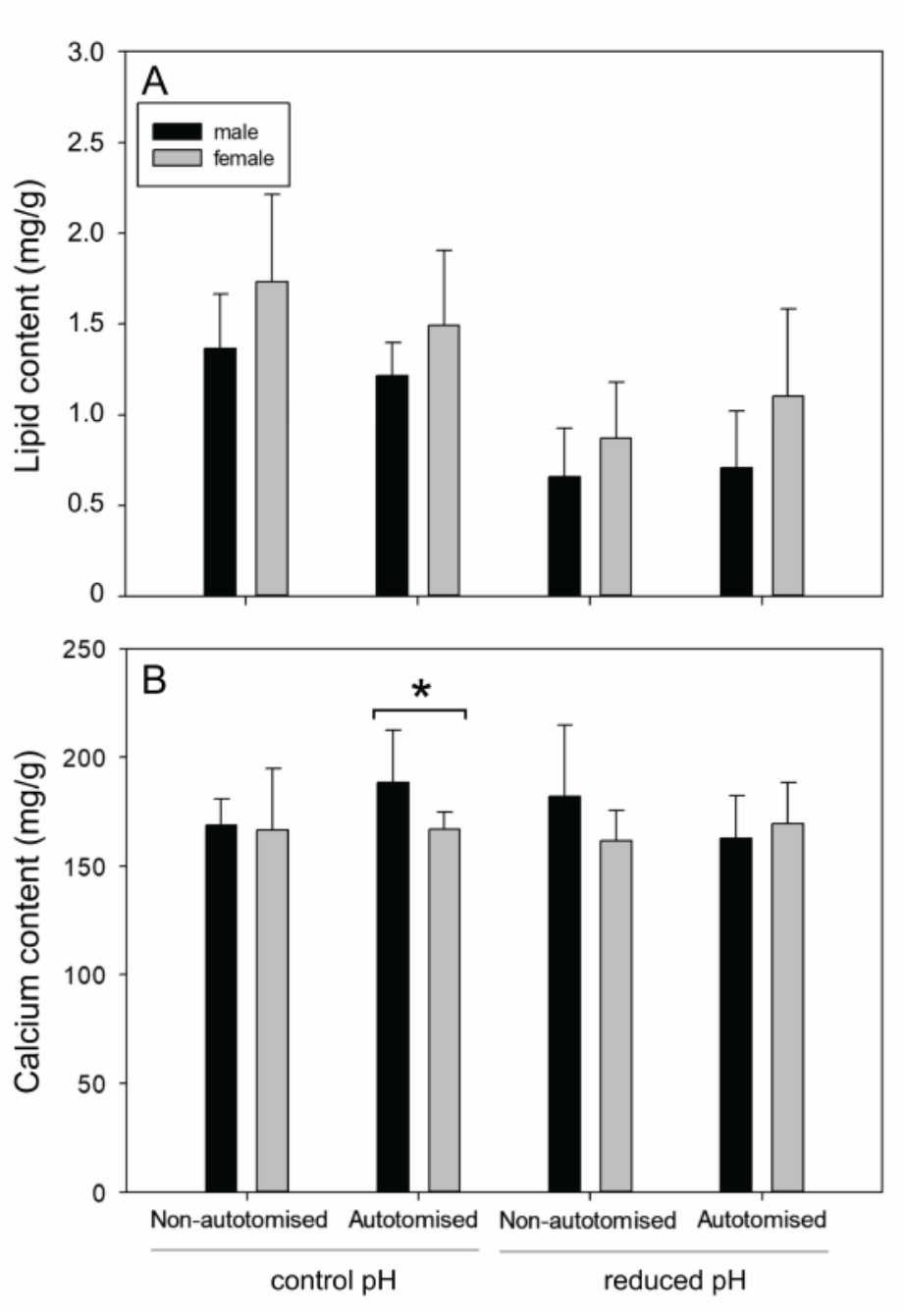

Figure 6. Energy budget, represented by (A) Lipid content (Mean ± Standard deviation; mg/g) and (B) Calcium content of the left cheliped (Mean ± Standard deviation; mg/g), of males and females of the hermit crab *Pagurus criniticornis* subjected to experimental amputation of the right cheliped (autotomised and non-autotomised) and maintained at different pH treatments (control: pH 8.25; reduced: pH 7.70) during a 120-day experimental period. * indicate significant difference in calcium content between males and females within the combination of amputation and pH treatments.

Table caption

Table 1. Mean (± standard deviation; SD) of abiotic seawater parameters measured daily (n=120; pH, salinity, temperature) and monthly (n=4; dissolved inorganic carbon – DIC; total alkalinity – TA) in the control and experimental treatments.

Parameters	Mean (± SD)	
	Control	Experimental
pH	8.25 (±0.11)	7.70 (± 0.11)
Salinity	31.2 (± 0.1)	31.1 (± 0.3)
Temperature (°C)	23.2 (± 0.5)	23.2 (± 0.4)
DIC (µmol/kgSW)	2,573 (± 192)	2,872 (± 70)
TA (µmol/kgSW)	2,366 (± 19.3)	2,287 (± 149)

Table 2. Three-way ANOVA of the physiological stress indicators of males and females of the hermit crab *Pagurus criniticornis* subjected to experimental amputation of the right cheliped (autotomised and non-autotomised) and maintained at different pH treatments (control: pH 8.25; reduced: pH 7.70) during a 120-day experimental period. A) Mortality (average number of dead individuals per experimental tank; n=10); B) Growth (average difference in the shield length between final and initial conditions; mm); C) Number of molts (average number of molts per individual) and D) Startle response (s^{-1}; data transformed as $Log_{10}[SR+1]$ prior to ANOVA).

Effect	A) Mortality				B) Growth				C) Number of molts				D) Startle Response			
	DF	*MS*	*F*	*p*	*DF*	*MS*	*F*	*p*	*DF*	*MS*	*F*	*p*	*DF*	*MS*	*F*	*p*
Intercept	1	49.613	133.787	<0.001	1	6.991	59.418	<0.001	1	183.898	171.618	<0.001	1	203.744	751.403	<0.001
pH	1	10.513	28.348	<0.001	1	0.017	0.141	0.709	1	8.605	8.031	0.006	1	0.009	0.033	0.855
Sex	1	0.113	0.303	0.583	1	0.165	1.400	0.244	1	6.615	6.173	0.015	1	1.822	6.942	0.010
Autotomy	1	0.113	0.303	0.583	1	0.093	0.794	0.378	1	10.645	9.934	0.002	1	0.0004	0.001	0.973
pH*Sex	1	1.01	2.73	0.102	1	0.011	0.096	0.758	1	0.028	0.026	0.872	1	0.145	0.536	0.466
pH*Autotomy	1	0.313	0.842	0.361	1	0.043	0.365	0.549	1	0.720	0.672	0.414	1	0.139	0.514	0.475
Sex*Autotomy	1	0.613	1.651	0.202	1	0.011	0.091	0.765	1	0.149	0.139	0.710	1	1.529	5.640	0.020
pH*Sex*Autotomy	1	0.013	0.033	0.854	1	0.038	0.321	0.574	1	1.185	1.105	0.296	1	0.115	0.423	0.518
Error	72	0.37			40	0.118			92	1.072			81	0.271		

Table 3. Two-way ANOVA of the cheliped regeneration, represented by the right cheliped length (mm) at the end of the experiment, of males and females of the hermit crab *Pagurus criniticornis* subjected to experimental amputation and maintained at different pH treatments (control: pH 8.25; reduced: pH 7.70) during a 120-day experimental period.

Effect	*SS*	*DF*	*MS*	*F*	*p*
Intercept	10583.53	1	10583.53	2210.618	<0.001
Sex	210.59	1	210.59	43.987	<0.001
pH	8.77	1	8.77	1.831	0.183
Sex*pH	5.12	1	5.12	1.07	0.306
Error	201.08	42	4.79		

875 Table 4. Three-way ANOVA of the energy budget (lipid and calcium contents; mg/g) of males and females of the hermit crab *Pagurus criniticornis*
876 subjected to experimental amputation of the right cheliped (autotomised and non-autotomised) and maintained at different pH treatments (control:
877 pH 8.25; reduced: pH 7.70) during a 120-day experimental period.
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Effect	A) Total lipid content				B) Calcium content			
	DF	*MF*	*F*	*p*	*DF*	*MF*	*F*	*p*
Intercept	1	122.546	946.564	<0.001	1	4129006.422	9270.433	<0.001
pH	1	8.9	68.741	<0.001	1	261.128	0.586	0.445
Sex	1	2.302	17.777	<0.001	1	3958.665	8.888	0.003
Autotomy	1	0.021	0.164	0.687	1	357.14	0.802	0.372
pH*Sex	1	0.002	0.017	0.898	1	78.11	0.175	0.676
pH*Autotomy	1	0.66	5.095	0.027	1	1576.124	3.539	0.062
Sex*Autotomy	1	0.012	0.092	0.762	1	22.667	0.051	0.822
pH*Sex*Autotomy	1	0.111	0.856	0.357	1	3917.977	8.797	0.004
Error	86	0.13			133	445.395		

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