



Marine pollution effects on the southern surf crab *Ovalipes trimaculatus* (Crustacea: Brachyura: Polybiidae) in Patagonia Argentina



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ABSTRACT

We compared the carapace shape and thickness as well as the energy density of *Ovalipes trimaculatus* inhabiting areas comprising a gradient of marine pollution: high, moderate and undetected, in the Nuevo gulf (Patagonia Argentina). The carapace shape was evaluated by means of individual asymmetry scores (=fluctuating asymmetry) whereas the carapace thickness was assessed by measuring the carapace dry weight. The energy density was analyzed through its negative relationship with water content in muscle tissue. The individual asymmetry scores as well as the percentage of water content in muscle tissue were proportional to the marine pollution gradient, whereas the carapaces thickness did not differ among sampling sites. Our results are consistent with previous findings and demonstrate the direct effect of marine pollution on other taxa different from gastropods, cephalopods and polyplacophora and add to long-standing concerns about detrimental effects caused by marine pollution on the benthic community of the Nuevo gulf.

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1. Introduction

Marine pollution comprises a range of threats including oil spills (Neuparth et al., 2012), untreated sewage (Azizullah et al., 2011), nutrient enrichment (Isbell et al., 2013) and persistent organic pollutants (Harrad, 2010), among others. Contaminants at large polluted sites often share critical properties such as toxicity, high environmental persistence, high mobility leading to pollution of groundwater, and high lipophilicity, resulting in bioaccumulation in food webs (Fent, 2004). Organotins belong to the most toxic pollutants known so far to aquatic life (de Mora, 1996). Among them, tributyltin, an anti-fouling paint compound, is one of the most toxic chemicals commonly found in areas with high marine traffic. This compound is introduced in marine waters by ship traffic and scrapping activities as well as sewage disposal, and persists within the sedimentary column for years (Hoch, 2001). Molluscs species are the most sensitive group; among the widespread toxic threats induced by tributyltin, shell malformations and thickness, as well as the appearance of male characters in females (imposex) are the most common (Bryan and Gibbs, 1991).

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Marine shorelines occupy 4725 km of the coast of Argentina and the state of pollution and its effects on marine fauna has been scarcely studied (Barragán et al., 2003). After the study of Penchaszadeh et al. (2001), who related the imposex incidence to tributyltin concentrations in Mar del Plata sediments, other authors have used this alteration in the reproductive systems of gastropods species to assess the state of marine pollution (e.g. Cledón et al., 2006; Bigatti et al., 2009; Arrighetti and Penchaszadeh, 2010). In the Nuevo gulf (Patagonia Argentina), Bigatti and Penchaszadeh (2005) concluded that marine pollution decreases from the Puerto Madryn piers (high tributyltin pollution, 100% imposex) towards the north (50% of imposex), whereas those beaches located south are non-polluted sites with low marine traffic and null imposex. By considering this reported differences in marine pollution in the west coast of the Nuevo gulf, Márquez et al. (2012) reported body weight loss and shell weight loss in the volutid *Odontocymbiola magellanica* inhabiting the polluted area. Malformations of the reproductive system (pseudohermaphroditism) in other taxa were also shown in earlier studies and were associated with marine pollution in the Nuevo gulf (Ortiz and Ré, 2006; Scarano and Ituarte, 2009). Apart from these few studies, the effects produced by marine pollution on members of the benthic community of the Nuevo gulf, have been scarcely researched.

Marine environments biomonitoring programs are usually focused on identifying the best biomarker and bioindicator acting as a prognostic tool for marine pollution levels (Tosti and Gallo,

2012). The use of invertebrate populations as biomonitors of environmental quality has been extensively reported (Clarke, 1993 and references therein) and typically assessed by means of conventional life history or physico-chemical parameters (Giblock and Crain, 2013; Pereira et al., 2006), but also throughout the use of morphological indicators (Frontalini and Coccioni, 2011; Nuñez et al., 2012). Because the proportion of morphological abnormalities increases with pollution, it is sometimes considered as an indicator of the exposition of an organism to pollution (Le Cadre and Debenay, 2006). Since the pioneer work of Ludwig (1932), asymmetry in otherwise normally symmetrical traits has been used as a measure of morphological abnormalities. Specifically, among the three forms of biological asymmetries (see Palmer and Strobeck, 2003 for examples) fluctuating asymmetry, which refers to the small and completely random departures from bilateral symmetry observed in individuals (Klingenberg and McIntyre, 1998), was widely used as an overall indicator of stress in organisms inhabiting polluted environments (Parsons, 1992). It should be mentioned, however, that controversial results regarding the association between environmental stress and fluctuating asymmetry are found in the literature (for examples, see Beasley et al., 2013). Nevertheless and although some recognized disagreements (for a review, see also Leung et al., 2003), the utility of fluctuating asymmetry for measuring slight morphological alterations has been proven (Savriama and Klingenberg, 2011).

The southern surf crab, *Ovalipes trimaculatus* (De Haan 1833) (Crustacea: Polybiidae) is adapted to live in high-latitude environments (Stephenson and Rees, 1968). According to present interpretation of its taxonomic status, this species is found on the margins of the southwest and southeast Atlantic, south and southeast Pacific and Indian Oceans (Schoeman and Cockcroft, 1993) on sandy substrates (Fenucci and Boschi, 1975), from the low intertidal to about 100-m deep (Boschi et al., 1992). On the coast of South America it is reported in the western coast of the Atlantic Ocean from Brazil (Melo, 1996) to Argentina (25°–45° S; Boschi et al., 1992) and in the eastern coast of the Pacific Ocean from Peru to Chile (14°–41° S; Retamal, 1994). It is an edible resource and as such, it must be studied regarding its general biology and the effects that marine pollution could cause on it.

Because of its intertidal habitat and its close association with sediments, in addition to the reported effect of marine pollution on the aquatic fauna from the west coast of the Nuevo gulf (e.g. Bigatti and Penchaszadeh, 2005; Ortiz and Ré, 2006; Scarano and Iturarte, 2009; Bigatti et al. 2009; Márquez et al., 2012), we hypothesized that the southern surf crab inhabiting this area might be harmed by marine pollution. Therefore we predict that crabs exposed to marine pollution will exhibit higher levels of morphological alterations, carapace thickness loss and low energy density in contrast to those from not exposed sites. The main objective of this study is to assess if the levels of morphological alterations in the carapace (shape and structure) and the energy density of *O. trimaculatus* differ among sites exposed to different degrees of marine pollution in the Nuevo gulf. A close association between the marine pollution gradient and morphological/physiological effects might be considered as evidence of the fact that the southern surf crab is acting as a prognostic tool for marine pollution levels in the Nuevo gulf.

2. Materials and methods

Study area and sample collection: Sampling sites comprised three sandy beaches separated by 30 km of coastline in the west coast of the Nuevo gulf: El Doradillo (ED; 42.37° S; 64.56° W), Puerto Madryn (PM; 42.45° S; 65.02° W) and Cerro Avanzado (CA; 42.50° S; 64.52° W) (Fig. 1). The sampling sites are similar

in terms of its sediment characteristics (Ferrando et al., 2010) as well as wave energy and topographic conditions (Monti and Bayarsky, 1996), but different regarding marine pollution, particularly by tributyltin. PM and CA were previously assessed by Bigatti et al. (2009) and while PM has tributyltin 1.7 ng Sn/g sediment dry weight and 100% of imposex, CA has 0% of imposex. ED, situated 15 km north of PM, presents 50% of imposex (Bigatti et al., 2009). Based on these results, which highlight that at least the tributyltin concentration varies gradually in the west coast of the Nuevo gulf, PM, ED and CA were considered as high, moderate and undetected marine polluted sites respectively.

During October and November 2012, 50 crabs (25 non-ovigerous females and 25 males; carapace width range in mm = 52.18 to 102.37 and 53.66 to 122.37 for females and males respectively) were manually collected from the sea bottom by SCUBA diving close inshore at 1–6 m deep in each sampling site concurrently. Non-parasitized and intact crabs (e.g. without any noticeable damage or additional epifauna) were selected and then transferred alive to the laboratory where they were sorted by sex based on the morphology of the pleon and dissected. In order to avoid any bias due to differences in ontogeny and/or molt stage, only mature crabs in the final stage of the intermolt period were included. Maturity was established according to P. Barón (personal communication) and the molt stage was determined according to Alvarez et al. (2009). All groups included crabs with comparable size distribution.

Data acquisition: Cephalothoraxes (henceforth carapaces) were photographed using a calibrated digital camera (Sony Cyber Shot DSC-W200, 12.1 Megapixels) according to the standard procedure reported by Lezcano et al. (2012). The dorsal view of each carapace was photographed considering the scaling procedure and the carapace orientation, thus avoiding distortions. Images were compiled, scaled and digitized in the TpsUtil and TpsDig programs (Rohlf, 2010a,b) and the carapace shapes were assessed by means of 2D geometric morphometric analysis (Bookstein, 1991). Given that the carapace has an internal plane of symmetry (Klingenberg et al., 2002) we based our analysis on object symmetry. In order to achieve a good representation of the carapace shape, twice the same observer (AHL) digitized 22 landmarks (henceforth landmarks configuration) in two separate sessions. A total of 2 midline landmarks and 10 pairs of landmarks on the left and right side of the carapace were digitized (Fig. 2). Before setting the landmarks

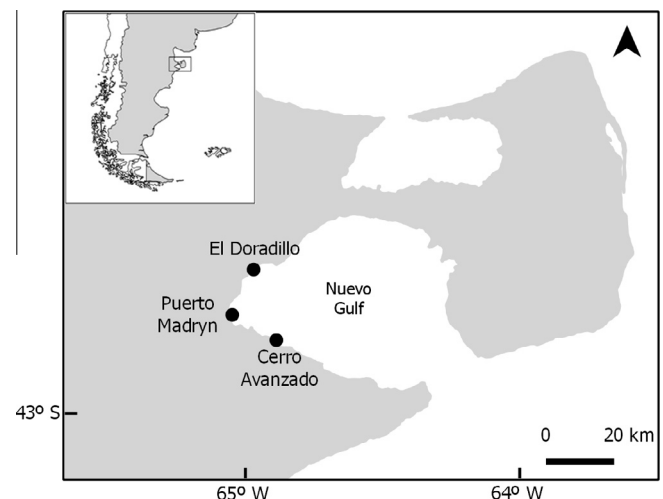


Fig. 1. Sampling sites in the west coast of the Nuevo gulf. El Doradillo (ED), located 15 km north of Puerto Madryn (PM), is the moderate marine polluted site. PM is the high marine polluted site and Cerro Avanzado (CA), which is located 17 km south of PM, is the non-detected marine pollution site.

configuration, the homologous landmark falling on the sagittal symmetry axis was defined as indicated in Idaszkin et al. (2013). Briefly, 9 semi-landmarks were digitized along the posterior side of the carapace contour and slid according to a sliding algorithm that minimizes the bending energy (Gunz et al., 2005) in the tps-Relw software (Rohlf, 2010c). After the detection of the sagittal axis and the consequent definition of landmark 2 (which corresponds to the middle of the configuration originated from 9 semi-landmarks), the additional 8 semi-landmarks were discarded (see also Heuzé et al., 2012). Then, the landmarks configuration of 22 homologous landmarks were used directly for shape analysis using 2D geometric morphometric analysis, which divides both sides of the carapace by considering the median axis (landmarks 1 and 2; Fig. 2) as the symmetry axis (Mardia et al., 2000; Klingenberg et al., 2002). The landmarks configurations were superimposed using a Generalized Procrustes Analysis (Rohlf and Slice, 1990; Goodall, 1991) which removes the effects of translation, rotation, and scaling (Dryden and Mardia, 1998). After superimposition, pure shape information is preserved in the specimens aligned landmarks, and variation around the mean shape in the sample (consensus) is decomposed into a component of symmetric variation and a component of asymmetry (Klingenberg and McIntyre, 1998; Klingenberg et al., 2002). In order to control and exclude any specimen strongly deviated from the average, both the total sample and each sex-specific group were inspected for outliers according to the standard procedure reported in MorphoJ program (Klingenberg, 2011). Due to the fact that the preliminary screening for outliers (results not shown) showed a good correspondence between the observed data and shape distances expected under a multivariate normal distribution model (no long tails in the distribution and no evident outliers), we did not exclude any specimens from the analysis. Given that the interest lies on asymmetry, the components of symmetric variation were disregarded. The levels of fluctuating asymmetry were computed by means of the individual asymmetry scores, which quantify the individual asymmetries of shape (as deviations from the mean asymmetry) by using Mahalanobis distances, and they are scaled in relation to the variation of the asymmetry in the sample (Klingenberg and Monteiro, 2005). Individual asymmetry scores were used to assess shape differences among sampling sites.

In addition, the carapace width was also measured as the straight line between landmarks 21 and 22 (Fig. 2) using the Tmorph Gen6 program (from Integrated Morphometrics Package series; <http://www.canisius.edu/~sheets/morphsoft.html>). Given that carapace width is a well-known measure in morphological

studies, it was used as a proxy for overall carapace size in the comparison of the carapace thickness (see below).

Also, the carapace thickness and the energy density were calculated on a subset of twenty crabs (10 males and 10 females) per sampling site. Assuming that, for equally sized carapaces, the thinner are also the lighter, the carapace thickness was measured as the carapace dry weight. Carapaces were dried until achieving constant weight (96 h at 60 °C). The energy density was assessed through its negative relationship with the percentage of water content in muscle tissue (for references see Ciancio et al., 2013). Wet and dry weights were recorded by weighting two samples of muscle tissue per crab (~2 gr) before and after drying until achieving constant weight (96 h at 60 °C), and its mean value used in subsequent analysis. Weights were recorded with a precision of 0.01 gr.

2.1. Data analysis

Because subtle asymmetries are often so small, it is important to distinguish fluctuating asymmetry from other types of asymmetries (e.g. directional asymmetry and antisymmetry; Palmer and Strobeck, 2003; Graham et al., 2010) and from measurement error, which is another factor that inflates the asymmetry estimates (Leamy, 1984; Merilä and Björklund, 1995). In order to calculate the measurement error and to quantify directional asymmetry, the data were evaluated by means of Procrustes Anova (Klingenberg and McIntyre, 1998). To test for potential antisymmetry, the histogram obtained from left-right distances among paired landmarks was inspected for signs of bimodality using the Kolmogorov–Smirnov test of normality (Palmer, 1994).

The individual asymmetry scores and the percentage of water content in muscle tissue were tested for normality with the Shapiro–Wilks test and for homogeneity of variance with the Levene test. Sexual dimorphism in the aforementioned estimates (individual asymmetry scores and the percentage of water content) was tested prior to data analysis using one-way ANOVAs to decide whether to consider the sex of specimens as an independent factor in the analysis. Given that neither the variable sex (individual asymmetry scores: $F = 0.3345$; $df = 1$; percentage of water content in muscle tissue: $F = 0.869$; $df = 1$) nor the interaction term (individual asymmetry scores: $F = 0.1565$; $df = 2$; percentage of water content in muscle tissue: $F = 0.5205$; $df = 2$) were significant ($p > 0.05$), sexes were pooled and the variables were analyzed by means of one-way ANOVAs with sampling sites as the main factor. Tukey honest significant difference test was applied for paired

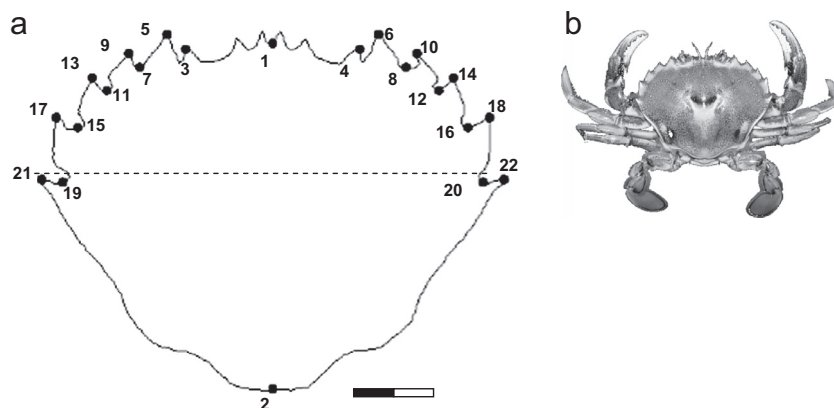


Fig. 2. (a) Landmarks configuration on the carapace of *O. trimaculatus*. Landmarks 1 and 2 lie on the midline of the carapace and define the internal plane of symmetry; they indicate the anterior and posterior edge of the carapace respectively. The remaining 20 landmarks have natural pairings through their corresponding positions on the left and right sides of the carapace. Landmarks 3 to 22 are located on the external and internal border of the rostral and anterolateral teeth. Dotted line is for the carapace width and the scale bar = 10 mm. (b) *Ovalipes trimaculatus* whole crab (CW = 105.7 mm).

comparison in cases where a significant site effect was observed in the ANOVA table.

Since the carapace dry weight varies exponentially with carapace width (Hartnoll, 1982; du Preez and McLachlan, 1984), we fitted exponential models for each site and compared them by likelihood ratio tests (LRT). In order to test if each sampling site showed a unique relationship and therefore differential carapace thickness, we fitted and compared 2 alternative models with different complexity (number of parameters). The general or simpler model (Model 1) consisted in a unique model for all three populations (carapace dry weight = $N_0 * e^{(r * \text{carapace width})}$) and the complex model (Model 2) that considered a site specific curve with varying values of r parameter for each sampling site (carapace dry weight = $N_0 * e^{(r_i * \text{carapace width})}$, where i takes values of 1, 2 or 3 for CA, ED and PM respectively). The two models were compared with LRT, which allows statistical consideration of whether the data support rejecting the simpler model in favor of the more complex one (Kimura, 1980). Prior to this analysis and following the same LRT routine, we evaluated the sexual dimorphism by comparing models with (complex model) and without sex (simpler model) as a factor. For the three populations we could not reject the simpler model (without effect of sex) ($P > 0.05$), therefore sexes were pooled for the population comparison.

For all statistical analysis, we employed the “R” language and environment for statistical computing version 3.0.1 for Windows (R Development Core Team, 2013), and the bbmle library (Bolker and R Development Core Team, 2012) for likelihood ratio tests. 2D geometric morphometric analysis were carried out with MorphoJ v1.05a (Klingenberg, 2011).

3. Results

Results of the Procrustes Anova indicated a significant effect of both directional and fluctuating asymmetry, but the latter being the most important source of left-right differences. Measurement error was negligible in relation to side \times individual effect, and since a normal, rather than a bimodal distribution of differences was obtained, antisymmetry was discarded as an important source of asymmetries (results not shown).

The individual asymmetry scores and the percentage of water content in muscle tissue were normally distributed with homogeneous variance. The differences among sampling sites for both variables were significant (one-way Anova in individual asymmetry scores: $F = 58.95$; $df = 2$; $p < 0.0001$, and in the percentage of water content in muscle tissue: $F = 39.97$; $df = 2$; $p < 0.0001$; Fig 3). The Tukey honest significant difference test indicated that all sampling sites were significantly different for both, the individual asymmetry scores and the percentage of water content in muscle tissue ($p < 0.05$; Fig 3). Both, the levels of individual asymmetry scores and the values of percentage of water content in muscle tissue were proportional to the gradient of marine pollution: crabs inhabiting the high polluted site (PM) were those showing the highest individual asymmetry scores (mean = 4.54; confidence interval, CI = 0.17) and the highest percentage of water content in muscle tissue (mean = 39.77; CI = 0.64). The crabs from ED (moderate polluted site) displayed intermediate values of individual asymmetry scores (mean = 3.62; CI = 0.15) and percentage of water content in muscle tissue (mean = 35.25; CI = 1.21), whereas the crabs from the undetected marine pollution site (CA) showed the lowest individual asymmetry scores (mean = 3.30; CI = 0.16) and the lowest percentage of water content in muscle tissue (mean = 33.12; CI = 1.18) (Fig. 3).

The carapace dry weight, in contrast, did not differ among sampling sites. All three populations showed the same carapace dry

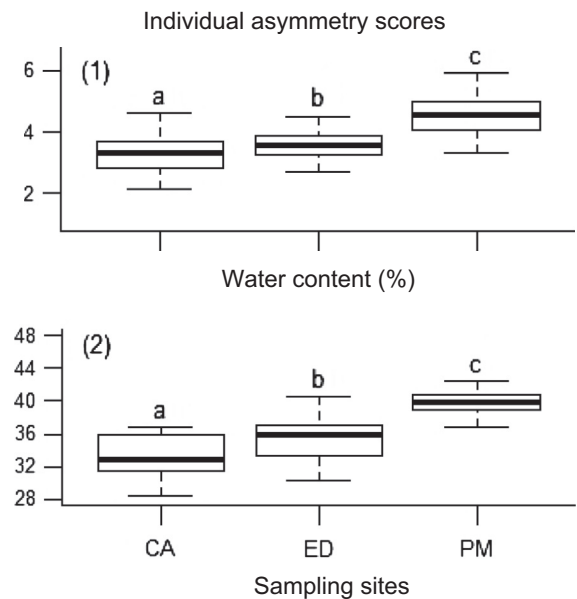


Fig. 3. Box plots of individual asymmetry score (1) and percentage of water content in muscle tissue (2) of *O. trimaculatus* for each sampling site. Boxes bounded by the 75th and 25th percentiles, and whisker lines represent the minima and maxima; black line inside the boxes are mean values and the different letters above whiskers represent significant differences ($p < 0.05$) after a *post hoc* Tukey honest significant difference test. Abbreviations for sampling sites are as in Fig. 1.

weight vs. carapace width relationship (Model 1_{df=2} vs. Model 2_{df=4}, simple model vs. complex; $P > 0.05$).

4. Discussion

This is the first study comparing morphological and physiological aspects of a subtidal keystone species such as *O. trimaculatus*, which inhabits sites with differential influence of marine pollution in the Nuevo gulf (Patagonia Argentina). Our results are in accordance to previous studies and, as predicted, the southern surf crab was overtly harmed by marine pollution. This resulted evident through the elevated fluctuating asymmetry values in the carapace shape as well as the low percentage of water content (indicating low energy density) in crabs captured in Puerto Madryn, the highest polluted site; and the degree of responses ranged in accordance to the marine pollution gradient (PM > ED > CA). The carapace thickness, in contrast, did not evidence any effect.

The molting process (i.e. changes of the exoskeleton) of decapods crustaceans is defined as the set of morphological changes from proecdysis to postecdysis in which rapid growth of already developed tissues occurs (Aiken, 1980). It is expected that the molting process modifies subtle anatomical abnormalities throughout a homeostatic regulatory mechanism (Petriella and Boschi, 1997). However, the exposure to pollutants could alter this normal process (Rodríguez et al., 2007) and growth retardation is a common response to pollution, especially by tributyltin (Weis et al., 1992). Even when the mechanism whereby pollutants alter the normal carapace morphology is still unclear, the high departure of symmetry in crabs from polluted sites suggests that the pollutant disturbs the normal developmental pathway, thus producing developmental noise, which is commonly measured as fluctuating asymmetry (Leamy and Klingenberg, 2005). The close association between the marine pollution gradient and fluctuating asymmetry levels could be considered as an evidence of a direct effect of pollution on the carapace morphology. Nevertheless, fluctuating asymmetry is a non-specific indicator of the stress level

experienced by the organism during its development (Leung et al., 2003) and high levels of fluctuating asymmetry might also be caused by other factors different from marine pollution (e.g. crowding, temperature, population density). Although our sampling design included sites which differ mainly in the levels of marine pollution, the influence of multiple stressors cannot be neglected. It is also worth noting that we did not test the repeatability of our results across populations and/or time (for examples, see Floate and Coghlin, 2010) and in order to validate the current methodology, measurements from other population and/or different seasons would be needed.

In addition to the crabs from the Nuevo gulf, we also evaluated the fluctuating asymmetry in a small sample of 15 crabs (11 females and 4 males) collected in the coast off Mar del Plata. It is the principal fishery port in Argentina and the first record of tributyltin pollution in Atlantic waters was done in this area by Penchaszadeh et al. (2001). Even when it is an extremely polluted site (13–128 ng Sn/g of tributyltin; Bigatti et al., 2009), we found that the values of fluctuating asymmetry were similar to those of Puerto Madryn (mean = 4.33, CI = 0.16). This is a striking result, since it suggests that even when the difference in pollution concentration is elevated (up to a 60-fold difference in tributyltin concentrations between sites) fluctuating asymmetry appears as a common response to marine pollution (specifically by tributyltin) and denotes that the carapace morphology of *O. trimaculatus* is very sensitive to pollution. Given the relatively small sample size ($n = 15$), we decided not to include those crabs in our initial analysis as a fourth treatment (extremely high polluted site). In order to reconcile our findings from the Nuevo gulf with those from Mar del Plata, more research is required; particularly by adding more crabs from Mar del Plata's pier.

Besides the fluctuating asymmetry in the carapace shape, marine pollution also affected the energy density, which varied in relation to marine pollution. The effect of marine pollution could be due to synergic factors; it could cause direct physiological imbalances and a depletion of somatic energy, thus reducing the energy density in the exposed crabs, and/or asymmetric crabs could be less aerodynamic, thus resulting in an increased metabolic cost of swimming. The importance of shape in the energy cost of swimming has been extensively reported since Schmidt-Nielsen (1972); commonly expressed as "streamlining", it is well known from fish and whales though less studied in crabs. The hydrodynamic efficiency related to the carapace shape in crabs was studied by Schäfer (1954), who recognized the importance of symmetry in the swimming ability. According to this author, major energy expenditure for the asymmetric swimming crabs is expected. The low energy density in crabs from Puerto Madryn is in agreement with Márquez et al. (2012), who reported body weight loss of *O. magellanica* at the most polluted site.

Contrary to the carapace shape, the carapace thickness of *O. trimaculatus* did not evidence any effect related to marine pollution. The lack of effect on the carapace thickness suggests that, even when pollution could alter the normal molting process and lead to high levels of fluctuating asymmetry in the carapace shape by means of developmental noise, those molt-related events that encompass the synthesis of new tissues are not affected by pollution. Fragile shells (shells that can be broken by hand) were registered by Márquez et al. (2012) in animals from polluted zones, suggesting that marine pollution affects the shell density in gastropods from the Nuevo gulf. According to our results and contrary to those findings, the metabolic pathway involved in the secretion of new endocuticle and posterior mineralization of exocuticle of the southern surf crab is not affected by marine pollution in the same area.

Although some acknowledge limitations, our results add to the long-standing concerns about detrimental effects caused by

marine pollution on marine biota. It also highlights the need of an integrative approach with observational data from nature, experimental studies of key processes, as well as expanded in situ and chronic toxicity testing, pointed to assert the effect of this type of stress on natural resources. Our approach offers an alternative tool to complex, time consuming, and usually too expensive chemical analysis to assess the presence of pollutants in natural waters, especially because it can be employed as a first step in an environmental monitoring program to identify stressed populations inhabiting polluted zones. Given the relevance of *O. trimaculatus* as a potential fishery resource, our study will serve as a base for futures studies to assess the utility of the southern surf crab as a prognostic tool for marine pollution, particularly by means of the analysis of fluctuating asymmetry because it is a methodology that is possible to apply on living specimens, thus avoiding killing individuals.

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