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Morphophysiological traits of an amphibian exposed to historical industrial pollution in a Brazilian biodiversity hotspot

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Abstract. Industrial pollution is a significant global threat to biodiversity, but its consequences on rainforest ecosystems remain poorly understood. Amphibians are especially susceptible to pollutants released on natural environments due to their aquatic-terrestrial life cycle. Here, we explored the association of severe historical air, water, and soil pollution with physiological and morphological alterations on *Rhinella ornata* individuals, an endemic toad species of Atlantic Forest, a world biodiversity hotspot. We hypothesized that individuals sampled in sites closer to the pollution source will present worse indicators of health. As predicted, toads at decreasing distances from the pollution source presented enlargement of organs related to detoxification function (liver and kidneys) and compensatory immunological function (spleen). Contrary to our predictions, however, we do not found significant effects of proximity to the pollution source on individuals' body condition index, on the indicative of fertility (testicles masses) or on macroparasite infection's response (eosinophil counts). Surprisingly, proximity to the pollution source was associated with lower chronic stress levels (neutrophil/lymphocyte ratio) on individuals. We discuss which processes could promote the alterations found on the toads. We also discuss the possible acquirement of local resistance to contamination on toads populations closer from pollution source, giving the more than 60 years of exposure to chemical contaminants in the area.

Keywords: Anura, Bufonidae, conservation, contamination, Cubatão, fertility, immunocompetence, Neotropical species.

Introduction

Since the mid-nineteenth century, industrial pollution grew to be one of the most important environmental issues threatening human and environmental health (Karl and Trenberth, 2003). Industrial activities worldwide contaminated air, water and soil, with adverse effects on organismal physiological performance (Calow, 1991), declines and extinctions of biological populations (Newman, 1979; Carey and Bryant, 1995), and changes in community composition, structure, and vegetation type (Newman, 1979; Carey and Bryant, 1995; Mayer et al., 2000; Szabo et al., 2003). In this scenario, researches aiming to assess wildlife health are essential, especially when focused in vulnerable taxonomic groups.

Amphibians compose a group of animals considered especially vulnerable to environmental contamination. Their cutaneous respiration and biphasic life cycle expose amphibians to sources of contamination in air, water and soil (Carey and Bryant, 1995; Bancroft, Baker, and Blaustein, 2008; Hayes et al., 2010; Kerby et al., 2010). Amphibians exposed to contaminants can present chronic stress and lowered immunocompetence, which increases their susceptibility to parasite infection (Hayes et al., 2006; Rohr et al., 2008). Exposed individuals can also present reduced rate of food ingestion, body condition, and size of energy reserves (Brodeur et al., 2011, 2012). The presence of contaminants can also retard larval growth and metamorphosis (Horne and Dunson, 1995), cause feminization of males (Hayes et al., 2002) and increase masses of organs associated to detoxification, such as liver and kidneys (Arrieta et al., 2004). Finally, contaminants can also cause malformations and impair locomotion, with a consequent reduction in the capacity

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to avoid predators, compete for resources and breed (Carey and Bryant, 1995).

As a consequence of this particularly high vulnerability, the contamination of natural environments has been hypothesized to be one of the main causes of worldwide amphibian population declines, both in pristine and degraded areas (Heyer et al., 1988; Verdade, Rodrigues and Pavan, 2009; Verdade et al., 2011, 2012). Yet, there is a serious mismatch between the geographical distributions of ecotoxicological knowledge – strongly biased towards widely distributed, generalist species from the northern hemisphere – and that of declining amphibian populations (Schiesari, Grillitsch and Grillitsch, 2007; Whitfield, Lips and Donnelly, 2016; Sańchez-Domene et al., 2018). Such geographical biases place especial value in studies relating environmental contamination and amphibian health in biodiversity-rich tropical areas.

The Atlantic Forest is one of the world's top five biodiversity hotspots with respect to vertebrates (Myers et al., 2000), with ∼500 amphibian species or 7% of the world total (Haddad et al., 2013). The Atlantic Forest is, in addition, among the five hotspots with higher rates of endemism (Myers et al., 2000; Paglia et al., 2004; Haddad and Prado, 2005; Haddad, Toledo and Prado, 2008); in fact, 40% of vertebrate species in the biome are endemic (Haddad, Toledo and Prado, 2008). Despite the extreme importance of Atlantic forest to global biodiversity, only 11% of the original Atlantic Forest cover remains, most of which is made up of small fragments surrounded by human occupations (Ribeiro et al., 2009) or adjacent to industrial centers (Verdade et al., 2011). Hence, studies addressing the health of animal populations in remaining tracts of Atlantic Forest may help to diagnose impacts, which in turn can aid in conservation decisions. However, despite previous studies describing declines and extinctions of amphibians in an Atlantic Forest area surrounded by industrial and urban centres (Verdade et al., 2011, 2012), no study has to date empirically assessed detrimental effects of pollution on amphibians.

The toad *Rhinella ornata* (Amphibia: Anura: Bufonidae) is a terrestrial amphibian found in both open and forested areas of the Atlantic Forest Domain in Southeastern Brazil. As most amphibian species, individuals of *R. ornata* reproduce in temporary and permanent ponds and streams, where adults lay eggs and larvae feed and develop. Adults of *R. ornata*, on the other hand, are mainly terrestrial and occur on the ground in open and forested areas (Baldissera Júnior, Caramaschi and Haddad, 2004; Haddad, Toledo and Prado, 2008). Given its ecological traits, *R. ornata* individuals can be exposed as larvae to water pollution via cutaneous absorption, gill absorption or food and sediment ingestion, and as juveniles and adults to air and soil pollution via cutaneous absorption and contaminated prey consumption. Evidently, environmental compartments are interconnected and airborne contaminants can eventually become water and soil pollution via wet or dry deposition (Furlan, Salatino and Domingos, 1999). Importantly, all species from the *R. ornata* species group are common and widely distributed in Neotropical America and present very similar biologies. Thus, exploring the correlates of contaminated habitat and body condition in *R. ornata* may contribute to make species a suitable group model to assess pollution effects on natural environments.

To evaluate *R. ornata* individuals' health, we assessed morphological and physiological traits generally used as indicators of exposure to contaminants: individual body condition index, proportional organ masses and white blood cell counts. Body condition index, which ultimately expresses the mass-to-length relationship, is widely used as an indicator of individual nutritional condition and has been shown to be positively correlated with fecundity (Reading and Clarke, 1995), individual fitness (Jakob, Marshall and Uetz, 1996) and habitat quality (Janin, Léna, and Joly, 2011), while negatively associated with chronic stress response (Titon et al., 2017). In turn, proportional increases in liver and kidney masses have been shown to reflect increased detoxification function (Arrieta et al., 2004), whereas proportional decreases in gonad mass has been shown to indicate reduced fertility (Rie et al., 2005). Along the same lines, increased spleen masses have been shown to be a response to physiological stress in individuals exposed to contaminants and diseases (McFarland et al., 2012). Furthermore, white blood cell counts indicating high neutrophil counts (neutrophilia), low lymphocyte percentages (lymphopenia) and high neutrophil-to-lymphocyte ratios are commonly used to assay increasing in chronical physiological stress response on anurans (Davis and Maerz, 2008; Shutler and Marcogliese, 2011; Garcia Neto et al., 2020). Finally, eosinophil percentages are commonly related to immunological response to macroparasites infestation (Abbas and Lichtman, 2003; Koprivnikar et al., 2019), wich can be reduced by individuals exposure to contaminants (Kiesecker, 2002).

Based on the arguments above, we hypothesize that *R. ornata* individuals from Atlantic Forest sites located near to a pollution source will present phenotypic traits related to chronic physiological stress, lower body condition, immunosuppression, overcharged detoxification function and reduced fertility. We predict that *R. ornata* individuals will present relatively larger livers, kidneys and spleen, relatively smaller gonads, and higher neutrophil/lymphocyte ratios as closer to a pollution source. Moreover, given that eosinophil counts predictably increase in response to an increase number of macroparasites in the body, and physiological stress caused by contaminant exposure can reduce individual immunocompetence, we also predict that proximity to pollution source will be associated with a reduction in eosinophil number in response to parasite infection in *R. ornata* individuals.

Material and methods

Study site

This study was conducted in Atlantic Forest areas near the industrial complex of Cubatão, a city located in the São Paulo State coast, Southeast of Brazil. The Cubatão Industrial Complex was built in 1950 and contains more than 20 industries, including chemical and petrochemical, fertilizer, cement and cellulose factories (Alonso and Godinho, 1992; Verdade et al., 2011). Heavy industrial activity of the Cubatão Industrial Complex, associated with inappropriate emission control, led to dramatic pollution during the 1970s and 1980s, when the area was called ‘the most polluted forest ecosystem with respect to sulfur, nitrogen, and fluorine’ (Mayer et al., 2000), as also considered ‘the most heavily polluted area in the world’ by Alonso and Godinho (1992). Up to 78 tons of sulphur dioxide, 90 tons of hydrocarbons, and 316 tons of particulate matter, not to mention metals, phosphates, fluorides, aldehydes, and acidic gases and vapors, were released daily in the atmosphere during 20 years, which caused acid rain, soil contamination and the death of local forest vegetation (CETESB, 1991; Alonso and Godinho, 1992; Leitão Filho et al., 1993; Mayer et al., 2000). Additionally, Cubatão is located along a narrow alluvial plain between the slopes of Serra do Mar Mountain Range in the north, northeast and west, and the coast in south and southeast. The local topography (at sea level, but bordered by the steep and locally U-shaped, 1200 m-high Serra do Mar’s peaks) aggravated the contaminant loading originated by Cubatão Industrial Complex and still acts as a natural barrier to the sea-to-land breezes that blow during daytime (Abbas et al., 1993), which favoured local deposition rather than long-range dispersal of atmospheric pollutants (fig. 1).

We sampled three Atlantic Forest sites at increasing distances from the Cubatão Industrial Complex, representing a hypothesized gradient of decreasing atmospheric deposition of industrial contaminants (fig. 1). We selected the Mogi River Valley (23°48′S, 46°21′W; 54 m a.s.l.; Cubatão city, São Paulo State; 3.2 km aerial distance from the Cubatão Industrial Complex) as our heaviest polluted site, since it was adjacent to the Cubatão Industrial Complex, received the largest amount of pollution and presented death of forest vegetation and consequent landslides (e.g., Klumpp et al., 1996, 1998, 2000, 2002; Furlan et al., 1999; Gonçalves et al., 2000; Mayer et al., 2000a, b; Moraes et al., 2002; Szabo et al., 2003; Schoenlein-crusius et al., 2006) (fig. 1, ‘Mogi’, henceforth ‘polluted site’). Our moderately polluted site was the Quilombo River Valley (23°49′S, 46°18′W; 37 m a.s.l.; 6.3 km aerial distance from the Cubatão Industrial Complex) located in the continental area of the city of Santos (São Paulo State) and separated from polluted site by Serra do Morrão, a ridge formed by hills of approximately 800 m of altitude (Hasui and Sadowski, 1976), which act as a barrier for most part of pollution emitted by Cubatão (fig. 1, ‘Quilombo’, henceforth ‘intermediate site’). Finally, our non-polluted site was the surroundings of Itatinga Village (23°46′36″S, 46°6′43″W; 17 m a.s.l; Bertioga city, São Paulo State; 27 km east-northeast of the Cubatão Industrial

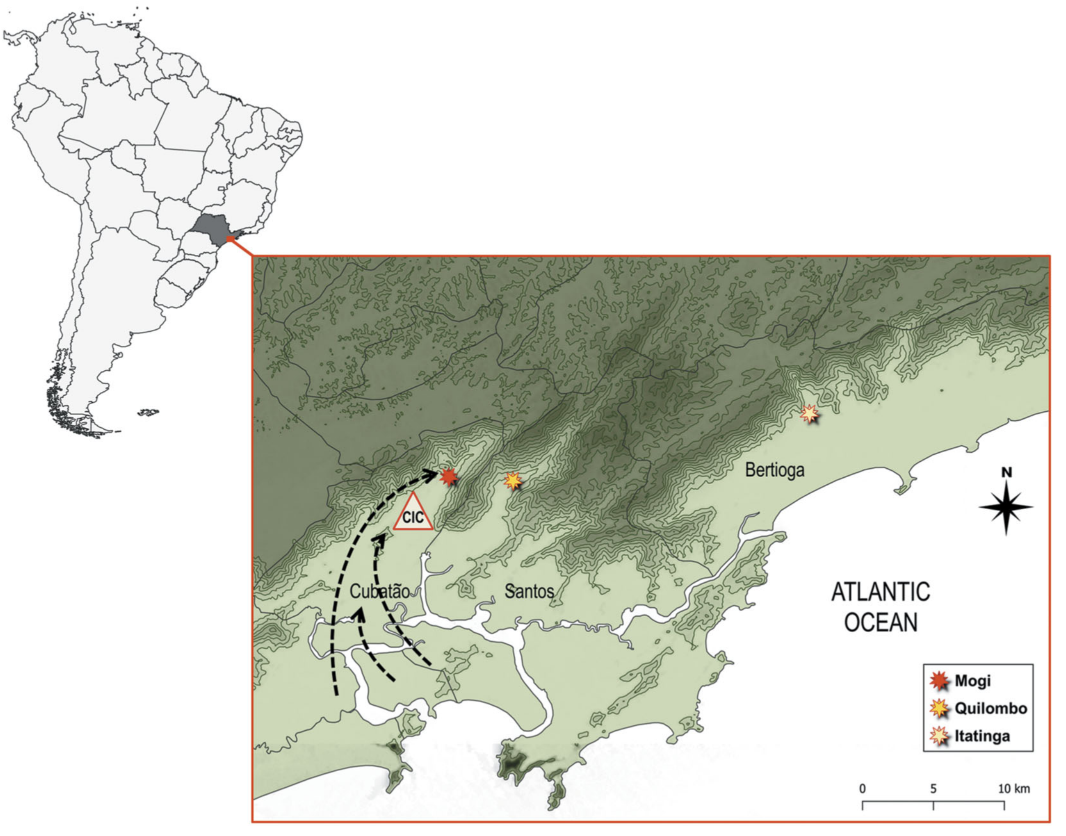

Figure 1. Map with the three sampled sites in the cities of Cubatão, Santos and Bertioga along the São Paulo State coast, Brazil. Arrows show the general daytime movement of air masses in the region according to Abbas et al. (1993). After flowing through the Cubatão Industrial Complex (CIC), winds are forced upwards by the slope of the Serra do Mar predominantly through the Mogi River Valley (polluted site) and secondarily through the parallel Quilombo River Valley (intermediate site). Due to the distance and a barrier effect of Serra do Morrão – a ridge formed by hills of approximately 800 m of altitude – Itatinga Village (reference site) is unlikely to be affected by air pollution from Cubatão Industrial Complex.

Complex), for being comparatively distant and not upwind of the Cubatão Industrial Complex, for the absence of air pollution sources in the area and for showing integrity of the surrounding forests (fig. 1, 'Itatinga', henceforth 'reference site').

Data collection

We collected 43 individuals of *R. ornata* (18 individuals at polluted site, 14 individuals at intermediate site, and 11 at reference site) between September and November 2012. Individuals were collected in Atlantic Forest areas located between 0 and 100 m of altitude, at the base of the Serra do Mar, where the vegetation is composed predominantly by dense ombrophilous forest. Since toad males and females present different behaviours, habits and physiology (Wells, 2007), and given the higher density of males in sampled areas, we collected only adult males.

We collected blood samples of *R. ornata* through cardiac puncture with 1 ml syringes containing heparin (anticoagulant). To avoid alterations in individual blood traits caused by handling stress, we conducted the blood collection procedure within 1 to 10 min after capture and without anaesthetics (Davis, Maney and Maerz, 2008). We maintained the blood samples in ice for up to 5 hours until the arrival at the laboratory. We then prepared slide smears with the blood from individuals that provided enough amount of blood (polluted site n = 12; intermediate site n = 10; reference site n = 10). Individual slides with blood smears were preserved via methanol immersion for 3 min, air-dried and colored with 5% Giemsa stain solution for 20 min. We counted 100 leukocytes per *R. ornata* individual sample and classified them as neutrophils, lymphocytes, eosinophils, basophils and monocytes in an optical microscope (400× magnification) (Campbell, 2007).

All individuals were euthanized with Benzotop® anesthetic ointment (200 mg/g benzocaine) applied on ventral skin, and have their snout-vent length measured. We also measured individual body mass, dissected them and recorded the wet masses of liver, kidneys, spleen and gonads (testicles) with an analytical balance (0.001 g). We dissected intestines and lungs, and counted the approximate number of visible helminth parasites under a stereoscopic microscope. The incidence of helminth parasite loads influences eosinophil counts and was used in this study as a covariate for interpreting blood cell counts. Livers were stored in a −20°C freezer for later chemical analyses.

To assess the presence of metals in *R. ornata* bodies, we combined all dissected livers from each site as a composite tissue sample for chemical analysis. We dried the individuals' livers in 1.5 ml tubes using a vacuum centrifuge (Concentrator Eppendorf Plus 5301 model) at 60°C. Then we put the livers from all *R. ornata* individuals collected in each site together and macerated these samples. The three composite liver samples (one per site) were sent to the Analytical Center of the Chemistry Institute, at University of São Paulo, where they were analysed by Inductively Coupled Plasma – Atomic Emission Spectrometry (ICP-AES) for the presence of cadmium (Cd), lead (Pb), manganese (Mn), iron (Fe) and aluminium (Al). We chose these metals because they are present in high abundance in previous studies of environmental contamination in areas exposed to the Cubatão Industrial Complex's pollution (Furlan, Salatino, and Domingos, 1999) and because they are tracers for activities of smelting complexes – which is a major historical contributor to airborne metal pollution in the Cubatão Industrial Complex.

Statistical analysis

Body condition index. We performed a linear regression between individual snout-vent length (mm) and body mass (g), with body mass as a dependent variable, and used the squared residuals multiplied by two as the individuals' body condition index (Băncilă et al., 2010). To explore if individual's body condition indeces were significantly different between sites, we compared two linear models: null model and a model with site as a predictor variable. We used the Akaike Information Criterion (AIC) to compare the models and considered the model with delta AIC (dAIC) smaller than 2.0 equally plausible to explain the observed data (Burnham and Anderson, 2002).

Because body condition index can affect individual responses to other stressful factors, body condition index values were also used as a predictor variable in other analyses (see below). In these cases, we used standardized values of body condition index (Schielzeth, 2010), calculated using the function *scale* of the software R version 3.4.3 (R Development Core Team, 2017).

Organ-somatic indices. We used organ and body wet masses to calculate relative organ masses (organ-somatic indices, i.e., organ mass/body mass × 100). We used organ-somatic indices (OSI) of liver, kidneys, spleen and gonads (testicles) to elaborate four sets of statistical linear models, one for each organ. In these models, each organ-somatic index is used as a dependent variable with Gaussian distributions of errors, while standardized values of body condition index (BCI) and the site of origin was used as independent variables (predictors). We compared the following models: null model, OSI varies only with site, OSI varies only with BCI; additive effect of site and BCI on OSI; interactive effect of site and BCI on OSI.

We compared these models using the Akaike Information Criterion (AIC) and considered models with delta AIC (dAIC) smaller than 2.0 equally plausible to explain the observed data (Burnham and Anderson, 2002). When more the one model dAIC $\leqslant 2.0$, we conducted a model averaging analysis to assess if the predictor 'site' presented a significant effect on OSI (see confidence intervals of full model average tables in supplementary material). We also used the best fitted model to conduct pairwise comparisons between sites (see below).

Haematological indicators. As an indicator of chronic stress, we calculated an individual stress index by the ratio between total number of neutrophils and total number of lymphocytes found in differential blood cell counted for each *R. ornata* individual (following Davis, Maney and Maerz, 2008). We elaborated generalized linear statistical models (GLM), in which the response variable N was modelled as having a binomial probability distribution (proportion of N found in the total N + L possibilities, N/L). We compared the following models: null model; N/L varies only with site; N/L varies only with BCI; additive effect of site and BCI on N/L interactive effect of site and BCI on N/L.

As a surrogate of immunological response to parasitism, we used the total number of eosinophil counted (E). Since E can increase as an immunological response to macroparasites, and such immunological response can depend of the individual body condition index (Alonso-Alvarez and Tella, 2001), we used E as a dependent variable with a Poisson distribution of errors and the total number of helminth parasites found in lungs and intestine (P), site, and BCI as predictor variables. For this statistical test, we elaborated the following models: null model; E varies only with site; E varies only with P; E varies only with BCI; additive effect of P and site on E; interactive effect of P and site on E; additive effect of BCI and site on E interactive effect of BCI and site on E.

We compared haematological indicators models (N/L and E) with the Akaike's Information Criterion for small samples (AICc). As above mentioned, when more than one model presented dAIC < 2.0, we conducted a model averaging analysis to assess if the predictor 'site' presented a significant effect on both haematological indicators (see confidence intervals in the full model average tables in supplementary material). We also used the best fitted model to conduct pairwise comparisons between sites (see below).

All models were implemented and compared with the *lme4* package of the software R version 3.4.3 (R Development Core Team, 2017). Model averaging analyses are conducted by using *model.avg* function (*MuMIn* package, R Development Core Team version 1.43.17). All model comparisons were made with the *ICtab* function (*bbmle* package, R Development Core Team version 1.0.23.1). To conduct models pairwise comparisons, we used the *emmeans* function to access marginal means estimated for the best fitted model in model selections and the function *pairs* to conduce post-hoc pairwise contrasts, both functions from *emmeans* package (Lenth et al., 2021; R Development Core Team version 1.5.4).

Results

Body condition indices did not present differences according original site of *R. ornata* individuals (polluted site 0.256 ± 1.765, intermediate site 0.238 ± 1.272, and reference site −0.722 ± 2.341). The null model presented the lowest AIC between the two concurrent models, but both models presented dAICc <2.0 (null model: dAIC = −175.7, df = 2, weight = 0.68; model with site as predictor: dAIC = 1.5, df = 4, weight = 0.32).

In three organ-somatic model selection analyses (OSI = liver, kidneys and spleen) models with dAIC < 2.0 contained 'site' as a predictor variable (table 1). In the model average analyses made with models that presented with dAIC < 2.0 for these three OSI variables, the estimated effect of 'site' was significant (confidence intervals did not include zero, see supplementary material). Additionally, pairwise comparisons between sites using best fitted models showed significant differences between relative masses of liver (polluted site $2.13 \times 10^{-2} \pm 0.50 \times 10^{-2}$, intermediate site $1.72 \times 10^{-2} \pm 0.31 \times 10^{-2}$, reference site $1.55 \times 10^{-2} \pm 0.11 \times 10^{-2}$), kidneys (polluted site $4.37 \times 10^{-3} \pm 0.70 \times 10^{-3}$, intermediate site $3.61 \times 10^{-3} \pm 0.66 \times 10^{-3}$, reference site $0.36 \times 10^{-3} \pm 0.44 \times 10^{-3}$) and spleen (polluted site $9.71 \times 10^{-4} \pm 5.10 \times 10^{-4}$, intermediate site $7.11 \times 10^{-4} \pm 1.86 \times 10^{-4}$, reference site $6.25 \times 10^{-4} \pm 2.19 \times 10^{-4}$) (see supplementary material). Individuals from the polluted site consistently presented higher relative masses of liver, kidneys and spleen than individuals from intermediate and reference sites (fig. 2). Gonadosomatic index values did not differ among *R. ornata* populations sampled (polluted site $2.76 \times 10^{-3} \pm 1.08 \times 10^{-3}$, intermediate site $2.53 \times 10^{-3} \pm 0.98 \times 10^{-3}$, reference site $2.37 \times 10^{-3} \pm 0.82 \times 10^{-3}$), since null model of this index presented dAIC < 2.0 (table 1, fig. 2).

Individual stress index, indicated by neutrophil/lymphocyte ratios (N/L) varied according site, since the confidence intervals of 'site' predictor from model average analysis with all models with dAIC <2.0 (table 2) did not include zero (see supplementary material). In this case, however, lower N/L values were found in individuals from heaviest polluted site when compared with individuals from intermediate and reference site (fig. 3, supplementary table S11).

Only one model was plausible to explain the observed variation in the proportion of eosinophil count (E) and had 'site' as a dependent variable (supplementary table S12). However, as we noted an outlier in our data (see highlighted point in fig. 3), we conducted a new model selection analysis without this individual (id 39). In this new model selection, tree models presented dAICc < 2.0 and two of them did not included 'site' as a predictor variable (supplementary table S13).

Liver samples of the three sites had detectable concentrations of iron (Fe) and aluminium (Al), but not of manganese (Mn), cadmium (Cd) and

Table 1. Model selection procedure assessing the effect of body condition index (BCI) and site on organ-somatic indices of liver, kidneys, spleen and gonads of *Rhinella ornata* (see text for details). The AIC, likelihood delta AIC (dAIC), the degrees of freedom (df) and the weight of evidence (Weight) are presented. Bold numbers are used to highlight the supported models.

Models	Hepatosomatic index				Kidney-somatic index				Spleen-somatic index				Gonadosomatic index			
	AIC	dAIC	df	Weight	AIC	dAIC	df	Weight	AIC	dAIC	df	Weight	AIC	dAIC	df	Weight
Null	−340.1	17.6	2	<0.001	−492.8	13.9	2	<0.001	−550.4	13.1	2	0.001	**−354.3**	**0**	**2**	**0.391**
Site	−352.7	5.1	4	0.045	**−506.7**	**0**	**4**	**0.56**	−553.6	9.9	4	0.006	**−353.8**	**0.5**	**4**	**0.309**
BCI	−346.0	11.8	3	0.002	−490.9	15.8	3	<0.001	−552.4	11.1	3	0.003	−352.3	2.0	3	0.144
BCI + site	**−357.8**	**0.0**	**5**	**0.577**	**−504.9**	**1.8**	**5**	**0.22**	−559.3	4.1	5	0.111	−351.8	2.5	5	0.114
BCI ∗ site	**−356.9**	**0.9**	**7**	**0.377**	**−504.9**	**1.8**	**7**	**0.22**	**−563.5**	**0.0**	**7**	**0.879**	−349.9	4.4	7	0.043

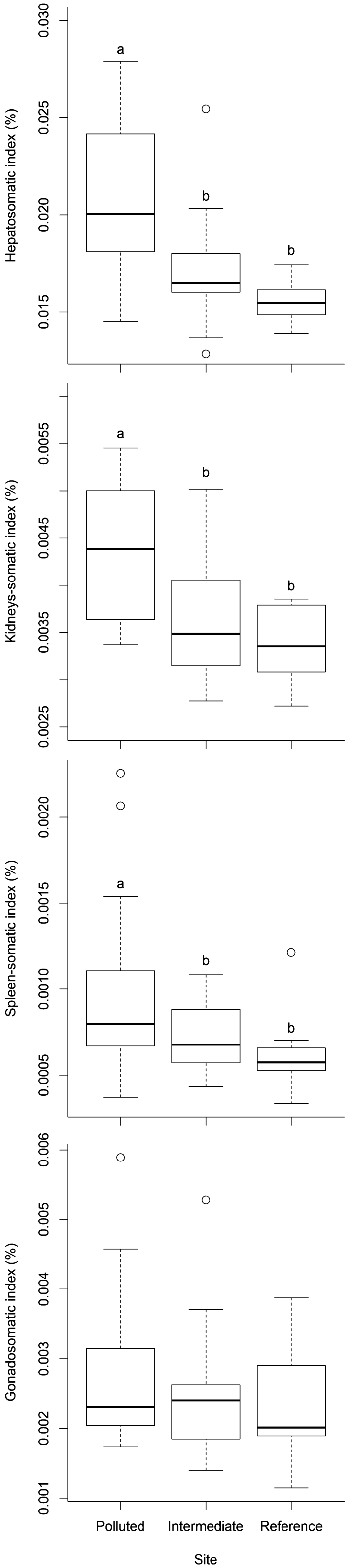

Table 2. Model selection procedure assessing the effect of body condition index (BCI) and site on individuals' stress index (neutrophil/lymphocyte ratio, N/L) of *Rhinella ornata* (see text for details). The values of the Akaike's Information Criterion (AIC), the likelihood delta AIC (dAIC), the degrees of freedom (df) and the weight of evidence (Weight) of the proposed models are presented. Bold numbers are used to highlight the supported models.

Models	AIC	dAIC	df	Weight
N/L ∼ 1	434.1	101.8	1	<0.001
N/L ∼ site	**333.6**	**1.3**	**3**	**0.220**
N/L ∼ BCI	425.6	93.3	2	<0.001
N/L ∼ BCI + site	**332.3**	**0.0**	**4**	**0.410**
N/L ∼ BCI ∗ site	**332.5**	**0.2**	**6**	**0.370**

lead (Pb). Surprisingly, *R. ornata* from reference site had the highest amounts of Fe and Al in their livers (table 3).

Discussion

Our assessment of the *Rhinella ornata* morphophysiological traits provided a range of responses. As hypothesized, proximity to the pollution source (Cubatão Industrial Complex)

Figure 2. Organ-somatic indices of *Rhinella ornata* populations at increasing distances from the Cubatão Industrial Complex (polluted site n = 18, intermediate site n = 14, reference site n = 11). Letters show statistically significant differences among sites based on confidence intervals of pairwise analyses made with the best fitted linear statistical model for each dependent variable (see text for details and values in the supplementary material). Hepatosomatic index (39 degrees of freedom) – polluted site-intermediate site: standard error = 0,0013, t-ratio = 3.2970, $p = 0.0058$; polluted site-reference site: standard error = 0,0014, t-ratio = 3.6300, $p = 0.0023$; intermediate site-reference site: standard error = 0,0015, t-ratio = 0.6110, $p = 0.8152$. Kidneys-somatic index (40 degrees of freedom) – polluted site-intermediate site: standard error = 0,0002, t-ratio = 3.3600, $p = 0.0048$; polluted site-reference site: standard error = 0,0002, t-ratio = 4.1700, $p = 0.0005$; intermediate site-reference site: standard error = 0.0003, t-ratio = 0.9900, $p = 0.5876$. Spleen-somatic index (37 degrees of freedom) – polluted site-intermediate site: standard error = 0.0001, t-ratio = 2.7090, $p = 0.0268$; polluted site-reference site: standard error = 0.0001, t-ratio = 3.3280, $p = 0.0055$; intermediate site-reference site: standard error = 0.0001, t-ratio = 0.8120, $p = 0.6982$. Boxes indicate the interval between first and third quartiles, central lines indicate the median, and whiskers indicate 1.5 times the value of the quartiles.

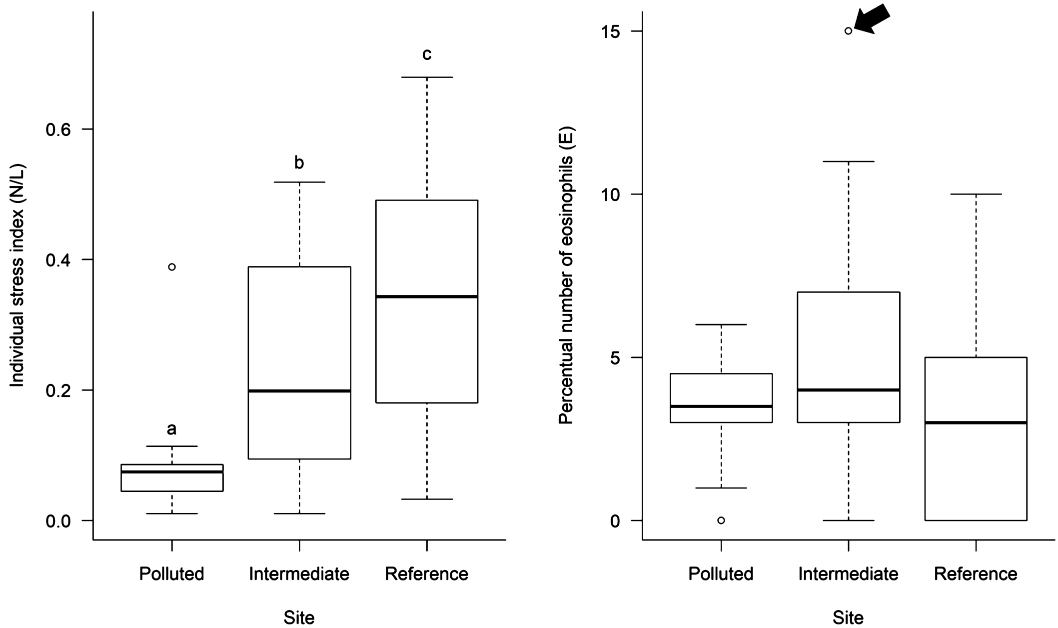

Figure 3. Individual stress index (neutrophil/lymphocyte ratio, N/L) of *Rhinella ornata* from populations at increasing distances from the Cubatão Industrial Complex (polluted site n = 13, intermediate site n = 10, reference site n = 10. Letters show statistically significant differences among sites based on confidence intervals of pairwise analyses made with the best fitted generalized linear statistical model (see text for details and values in the supplementary material). Individual stress index (N/L) – polluted site-intermediate site: standard error = 0,144, z-ratio = −6.651, $p < 0.001$; polluted site-reference site: standard error = 0,139, z-ratio = −9.113, $p < 0.001$; intermediate site-reference site: standard error = 0,124, z-ratio = −2.505, $p = 0.0328$, residual deviance 199.96 on 29 degrees of freedom). Percentual number of eosinophils (E): polluted site n = 12, mean = 3.4167, SD = 1.6765; intermediate site n = 10, mean = 5.4, SD = 4.5995; reference site n = 10, mean = 3.3, SD = 3.4009.

Table 3. Concentrations of metals (ppm, dry mass) found in the livers of *Rhinella ornata* from polluted site, intermediate site and reference site.

Metals	Polluted site	Intermediate site	Reference site
Al	<0.01	6.27	21.94
Cd	<0.005	<0.005	<0.005
Fe	703.7	642.22	2250.36
Mn	<0.001	<0.001	<0.001
Pb	<0.009	<0.009	<0.009

was associated with a clear increase in liver, kidneys and spleen masses. However, proximity to the Cubatão Industrial Complex was associated with a decrease in individuals' stress index (N/L), as also no significant effects on body condition index (BCI) or relative gonad masses. Additionally, we did not achieve a robust indicative effect of the proximity to the pollution source on proportional eosinophils. For most organ-somatic indices there was a monotonic gradient in response, with toads from the polluted site standing out as significantly different from those from the intermediate site and the reference site which, in turn, did not differ from each other. Indeed, when using several indicators of organismal exposure to contaminants and/or their effects, it is not uncommon to observe a mosaic of positive, neutral, and negative responses even in heaviest contaminated sites or in controlled laboratory studies (see, for example, Tables 12.5 and 12.6 in Grillitsch and Schiesari, 2010, for a summary of ∼50 studies of the effects of metals on reptiles).

Among the morphological indicators analysed in this study, the enlargements of livers, kidneys and spleen are the strongest signal of exposure of *R. ornata* to environmental contamination in the polluted site. Exposure to contaminants is known to alter liver and kidney cell metabolism, leading to the development of fibrosis and granulomas (Williams and Iatropoulos, 2002; Linzey et al., 2003; Boncompagni et al., 2004; Păunescu et al., 2010), ultimately increasing organ masses and negatively affecting individual health (Linzey et al., 2003). Increased liver and kidney masses are also interpreted as an organismal effort for increased detoxification in face of exposure to contaminants (Vogiatzis and Loumbourdis, 1998; Arrieta et al., 2004; Stolyar et al., 2008). Spleen, on the other hand, has a role in the production of cells involved in vertebrate immune response (John, 1994), housing one-quarter of all lymphocytes in the body (Li et al., 2006), and tends to enlarge concurrent to intensified immunological responses and/or infections (John, 1994; Forbes, McRuer and Shutler, 2006).

Spleen mass enlargement (splenomegaly) might be also related with the reduction of individuals' stress index (N/L) found in individuals of *R. ornata* from the polluted site. Splenomegaly associated with the activation of lymphocyte proliferation (lymphocytosis) has been observed in situations of parasitic and bacteriological infection, as well as after tissue damage or cell necrosis caused by exposure to contaminants (Larsson, Haux and Sjöbeck, 1985). Wild wood mice from populations exposed to heavy metal pollution showed higher parasitic infection and splenomegaly, a possible consequence of increased parasitism (Tersago et al., 2004). Stressors as infection by parasites, exposure to radioactivity, pesticides and metals – such as Pb, Cd, Zn, and Cu – are also known to increase glucocorticoid secretion (Davis, Maney and Maerz, 2008), which usually causes lymphopenia and neutrophilia within a few hours, consequently increasing the neutrophil/lymphocyte (N/L) ratio in circulation (Davis, Maney and Maerz, 2008). We found lower individual stress index (N/L) in the polluted site than the intermediate site and the reference site. Lower N/L and higher spleen masses found in individuals of *R. ornata* from the polluted site might be a result from direct detrimental effects of pollution and/or an increase in an organisms' susceptibility to parasitic diseases.

We found no differences in body condition index and testes relative mass (indicative of reproductive capacity, see above) between *R. ornata* sampled populations. It is possible that selective pressures imposed by local contamination input over 60 years promoted responses on anuran bodies of the polluted site population. It is also possible that the populations of *R. ornata* from the intermediate site and the reference site are more sensitive to the smaller pollution input in these areas. Sublethal concentrations of contaminants have been shown to induce local adaptations on amphibians, such as increased contamination tolerance (Hua, Morehouse and Relyea, 2013) and changes in the time or size of metamorphosis (Howe et al., 2004). Moreover, a previous study with an amphibian species (*Lithobates sylvaticus*) found that, in a geographical contamination gradient, populations located nearer to a pollution source evolved higher tolerance to carbaryl, possibly involving genetic assimilation (Hua et al., 2015). Additional studies are necessary to test the hypothesis of local adaptation of *R. ornate* individuals.

Given the very broad range of contaminants released by the Cubatão Industrial Complex, it is not possible to know exactly which contaminants (and which other environmental factors) caused the changes in morphophisilogical traits observed in toads. None of the two metals of ecotoxicological relevance (Cd, Pb) presented detectable quantities in toad livers. The only two contaminants detected in toad livers were aluminium (ranging from <0.01 to 21.94 parts per million – ppm) and iron (642 to 2250 ppm), both considered nonpriority pollutants of medium ecotoxicological relevance (Grillitsch and Schiesari, 2010). Even if patterns of aluminium and iron contamination are contradictory to our predictions, it is useful to discuss whether these elements reached toxic levels to toads. There is no recent systematic compilation of metal contaminant loads in toad tissues. Grillitsch and Schiesari (2010) reviewed all literature available about metal contamination in reptiles until 2010 and found that liver concentrations for iron in terrestrial species ranged from ∼350 to 13 000 ppm (N = 6 studies, dry weight, as in this study); values as high as 3300 ppm were reported as being found in individuals from seemingly unpolluted areas. In turn, liver concentrations for aluminium in terrestrial species ranged from 120 to 500 ppm (N = 2 studies, dry weight). Thus, taking into consideration the limited available information, it seems that metal contamination in toad livers was not particularly high.

The fact that toad livers from the reference site contained the highest concentrations of aluminium and iron is puzzling. There is relatively abundant data documenting heavy pollution in air, water, soil and biota in the polluted site (Klumpp et al., 1998; Mayer et al., 2000; Moraes et al., 2002). However, given the topography impairing wind directions and land use, it is unlikely that the higher metal concentrations observed in toad livers from reference site represent a faithful, long term depiction of exposure to pollutants in the region. No meaningful sources of metals other than the Cubatão Industrial Complex exist in the region. One possible exception is a railroad near our collection sites in reference site that could be a small scale but a local relevant source of metal contamination. A further factor that could contribute to the disparity in expected versus measured liver contamination is the variation in other environmental properties that modulate the bioavailability of metals to toads or their prey, such as water pH or dissolved organic carbon (Freda, 1991). Clearly, broader sampling surveys are necessary to establish a clear environmental assessment of metal contamination in the region.

In conclusion, we found toads with increased masses of liver, kidneys and spleen in the polluted site population – the nearest site from Cubatão Industrial Complex, our pollution source. We found no differences in the toads' body condition index, masses of testicles or eosinophil counts, as also found a reduction in toads' individuals' stress index (N/L) of polluted site, probably related with the observed spleen enlargement. We concluded that, despite toads from population found at the polluted site survive and reproduce without any apparent difficult, the chronic exposure to pollutants probably are promoting important changes in morphophysiological traits of *R. ornata*, which could impair individuals health, reproduction and, ultimately, population viability. These results are robust, even given the small samples size we collected in each site and the absence of profound contamination analysis. Thus, our results allow us to suggest a causal correlation between pollution and morphophysiological traits. However, future studies should test the hypothesis that toad populations from the Mogi River Valley (polluted site) have acquired tolerance to the high levels of contaminants released in Cubatão by the Industrial Complex. To do so, future studies should focus on a detailed assessment of environmental contamination in these regions, and also on a combination of laboratory and field experiments with the crossing of population origins to environmental conditions.

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