Physiological condition of amphibians 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 remains poorly understood. Amphibians are especially susceptible to pollutants released on natural environments due to their aquatic-terrestrial life cycle. Here, we explored the effects of severe historical air, water and soil industrial pollution of Cubatão Industrial Complex (São Paulo state, Brazil) on the physiological condition of *Rhinella ornate* individuals, an endemic toad species of Atlantic Forest, a world biodiversity hotspot. We hypothesized that individuals sampled at localities closer to the pollution source will present worse indicators of physiological health. As predicted, toads at decreasing distances from the pollution source presented enlargement of organs related with detoxification function (liver and kidneys) and with compensatory immunological function (spleen). Contrary to our predictions, however, we found only a weak negative effect of proximity to the pollution source on individuals' body condition index, and no effects on fertility (testicles masses) or macroparasite infection (eosinophil counts). Surprisingly, proximity to the pollution source was associated with lower chronical stress levels (neutrophil/lymphocyte ratio) on individuals. We discuss which physiological process could promote the alterations found on the toads. We also discuss the possible evolution of a local resistance to contamination on toads from populations closer from pollution source, giving the more than 60 years of exposure to chemical contaminants in the area.

Keywords: Bufonidae, physiological indicator, condition index, chronical stress, organ enlargement, reduced fertility, contamination, local adaptation.

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 phytophysiognomy (Newman, 1979; Carey and Bryant, 1995; Mayer et al., 2000; Szabo et al., 2003). In this scenario, research 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 lower 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 to avoid predators, compete for resources and breed (Carey and Bryant, 1995).

As a consequence of amphibian particular 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 distribution of ecotoxicological knowledge – strongly biased towards widely distributed, generalist species from the northern hemisphere – and that of declining amphibian populations. Such geographical biases place especial value in studies relating environmental contamination and amphibian health in biodiversity rich tropical areas (Schiesari, Grillitsch, and Grillitsch, 2007).

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 endemism rates (Myers et al., 2000; Paglia et al., 2004; Haddad and Prado, 2005; Haddad, Toledo, and Prado, 2008), in which about 40% of vertebrate species are endemic (Haddad, Toledo, and Prado, 2008). Despite the extreme importance of Atlantic forest to 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 found in Atlantic Forest remaining areas may help to diagnose damages, which in turn can aid in conservation decisions. However, despite previous studies describing declines and extinctions of amphibians in an uphill Atlantic Forest area surrounded by industrial and urban centers (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 open or forested areas of Atlantic Forest Domain in Southeast of 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 are exposed to both water pollution as larvae and atmospheric pollution as adults, and also can absorb pollutants deposited by acid rains or through the ingestion of contaminated insect prey eaten when adults. Additionally, all species from *R. ornata* group are commonly found, widely distributed in Neotropical America and present very similar biologies. Thus, explore the impact of pollutants on *R. ornata* may contribute to make the species of this group suitable models to assess pollution effects on natural environments.

To evaluate *R. ornata* individuals' health, we assessed morphological and physiological traits generally used as indicators of physiological condition and exposure to contaminants: individuals' body condition, proportional organ masses and white blood cell counts. Body condition, 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), and negatively associated with chronic stress response (Titon et al., 2017). In turn, proportional increases in liver and kidneys masses have been shown to reflect increased detoxification function (*e. g.* Arrieta et al., 2004), whereas proportional decrease in gonads has been shown to indicate reduced fertility (*e. g.* Rie et al., 2005). Also, increased spleen masses have been registered as a response to physiological stress in individuals exposed to contaminants and diseases (*e. g.* McFarland et al., 2012). Additionally, white blood cell counts indicating high number of neutrophil (neutrophilia), low lymphocyte (lymphopenia) percentages or high neutrophil/lymphocyte ratio can be related to increased circulating levels of glucocorticoids, hormones that modulate physiological stress response (Davis, Maney, and Maerz, 2008). Further, high eosinophil percentages can be related to immunological infection response, particularly against macroparasites (Abbas and Lichtman, 2003).

Based in all information compiled above, we hypothesized that *R. ornata* individuals from Atlantic Forest areas adjacent to the industrial region of Cubatão Industrial Complex – São Paulo state, Brazil, considered ‘the most heavily polluted area in the world’ by Alonso and Godinho, 1992 – will present phenotypic traits related to chronic physiological stress, lower body condition, immunosuppression, overcharged detoxification function and reduced fertility. As indicators of these conditions, we expect to find relatively larger livers, kidneys and spleen, relatively smaller gonads and higher neutrophil/lymphocyte ratio in populations of *R. ornata* that are closer to the pollution source. Moreover, given that eosinophil counts increase in response to macroparasite infection, and physiological stress caused by contaminant exposure can reduce individual immunocompetence, we also predicted 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 industries, fertilizer, cement and cellulose factories (Alonso and Godinho, 1992; Verdade et al., 2011). Heavy industrial activity of 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). 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).

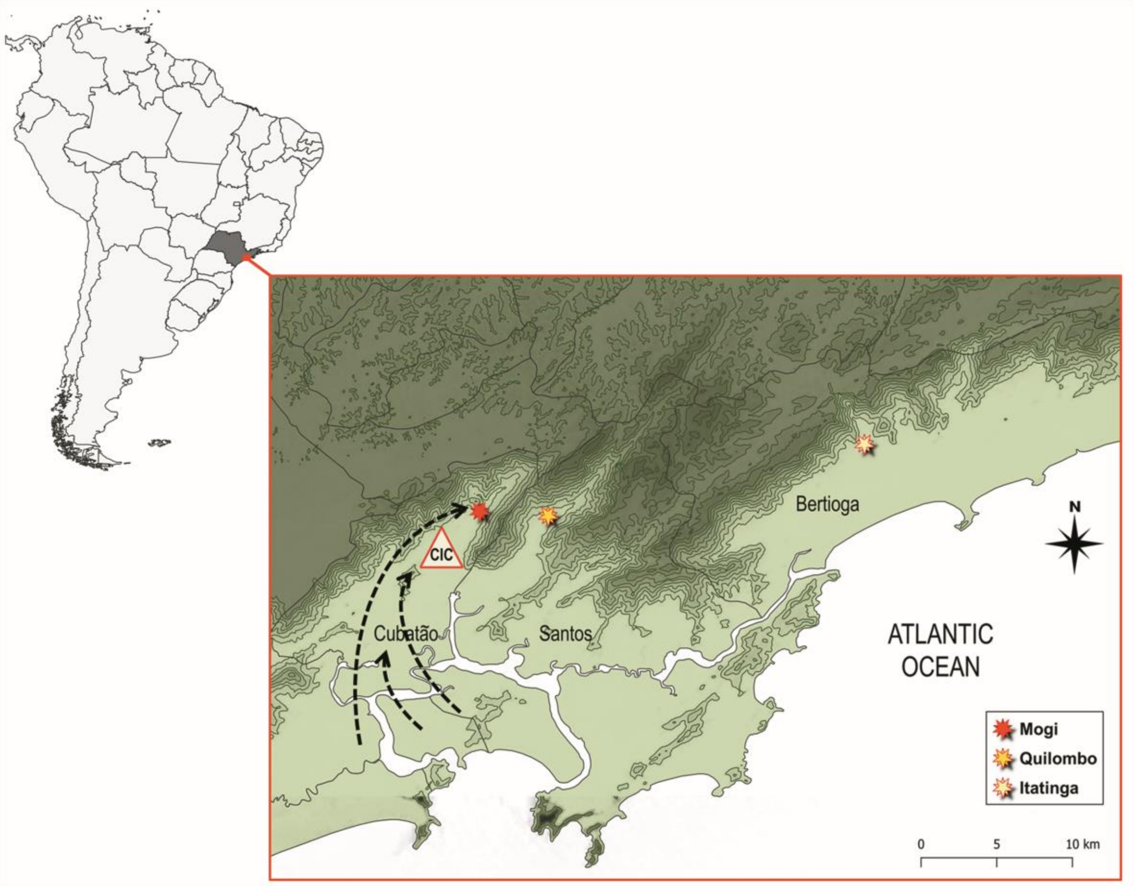

Figure 1. Map with sampled municipalities (Cubatão, Santos and Bertioga) and localities along the São Paulo State coast, Brazil. The arrows show the general movement of air masses in the region, dominated by Southeastern trade winds with a sea-to-land breeze blowing during daytime that comes from the south and is forced towards the North-Northeast as it encounters the slopes of the Serra do Mar (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 (Mogi, test site) and secondarily through the parallel Quilombo River Valley (Quilombo, moderately-polluted site). Due to a barrier effect of Serra do Morrão, a ridge formed by hills of approximately 800 m of altitude, Itatinga Village (Itatinga, control site) does not seem to be affected by pollution from Cubatão Industrial Complex.

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 (henceforth ´Mogi´; 23°48'S, 46°21'W; 54 m a.s.l.; Cubatão; 3.2 km aerial distance from the Cubatão Industrial Complex) as our heavily polluted test site, since Mogi 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, 2000b; Moraes et al. 2002; Szabo et al. 2003; Schoenlein-crusius et al. 2006) (Fig. 1, 'Mogi'). Our a moderately polluted test site was the Quilombo River Valley (henceforth ´Quilombo´; 23°49'S, 46°18'W; 37 m a.s.l.; Santos; 6.3 km aerial distance from the Cubatão Industrial Complex) located in the continental area of the city of Santos and separated from Mogi 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'). Finally, our non-polluted site was the surroundings of Itatinga Village (henceforth ´Itatinga´; 23°46'36"S, 46°6'43"W; 17 m a.s.l; Bertioga; 27 km east-northeast of the Cubatão Industrial Complex), for being comparatively distant and not upwind of the Cubatão Industrial Complex, and for the absence of air pollution sources in the area and shows integrity of the surrounding forests (Fig. 1, 'Itatinga').

Data collection

We collected 43 individuals of *R. ornata* (18 individuals at Mogi, 14 individuals at Quilombo, and 11 at Itatinga) 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 males.

We collected blood samples of *R. ornata* through cardiac puncture immediately after capture, to avoid that alterations in individual blood traits caused by manipulation stress (Davis, Maney, and Maerz, 2008). We maintained the blood samples in ice 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 (Mogi n =

12; Quilombo n = 10; Itatinga n = 10). Individual slides with blood smears were preserved in 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 (400x magnification) (Campbell, 2007).

We euthanized all individuals collected with Benzotop® (200 mg/g benzocaine) anesthetic ointment applied on ventral skin, and then measured their snout-vent length. We measured the total individuals' 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 and carcasses were stored in a freezer for later chemical analyses.

To assess the presence of metals on *R. ornata* bodies, we used all livers from collected individuals to compose tissue samples for analysis. We dried the individuals' livers in 1.5 mL tubes using a vacuum centrifuge (Concentrator Eppendorf Plus and 5301 model) at 60°C. Then we put the livers from all *R. ornata* individuals collected in each locality together and macerated these samples. The three composite liver samples (one per locality) 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; see more details in Supplementary material S1) for the presence of cadmium (Cd), lead (Pb), manganese (Mn), iron (Fe) and aluminium (Al) (Supplementary material S2). 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 are tracers for activities of smelting complexes – which is a major historical contributor to airborne metal pollution in the Cubatão Industrial Complex. .

We also assessed the presence of organic contamination on *R. ornata* individuals' bodies. After dissecting the individuals, we removed all organs and sorted one to three of the remaining carcasses of individuals from each locality to compose one composite carcass sample per locality. These three samples were sent to the Laboratory of Environmental Chemistry, Chemistry Institute, University of São Paulo (LEC-IQSC-USP), were they were assayed for 52 organic contaminants by quantification of purgeable organic compounds in water by capillary column gas chromatography (details in Supplementary material

S1). The organic contaminants analysed were those listed in the Resolution 357 of the National Council for the Environment (CONAMA, 2007), an agency of the Brazilian Ministry of the Environment. All material used to manipulate and store samples sent to metal and organic compounds analyses were decontaminated by washing with soap and water and soaking in 10% nitric acid overnight.

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. We used the square root of the residuals as the individuals' body condition index (Băncilă et al., 2010). We did an analysis of deviance in order to evaluate if body condition indices of *R. ornata* individuals were significantly different between localities.

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 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 (φ), while body condition index (BCI) and the locality of origin was used as independent variables (predictors). We compared the following linear models:

$\varphi \sim 1$ → null model;
$\varphi \sim$ locality → φ varies according to locality, independently of BCI variation;
$\varphi \sim$ BCI → φ varies according to BCI, independently of locality;
$\varphi \sim$ BCI + locality → there is an additive effect of locality and BCI on φ;
$\varphi \sim$ BCI * locality → there is an interactive effect of locality and BCI on φ.

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 & Anderson, 2002). If models with the variable 'locality' showed dAIC smaller than 2.0 we established pairwise comparisons between localities, using a multiple comparison analysis for parametric models.

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 (N/L) (following Davis, Maney, and Maerz, 2008). We elaborated generalized linear statistical models (GLM), in which N/L was the response variable with a Negative Binomial distribution of errors, and compared them according the best fit:

N/L ~1 → null model;

N/L ~ locality → N/L varies according to locality, independently of BCI variation;

N/L ~ BCI → N/L varies according to BCI, independently of locality;

N/L ~ BCI + locality → there is an additive effect of locality and BCI on N/L;

N/L ~ BCI * locality → there is an interactive effect of locality 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 BCI (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), locality, and BCI as predictor variables. For this statistical test, we elaborated the following models:

E ~1 → null model;

E ~ locality → E varies according to locality, independently of P or BCI variation;

E ~ P → E varies according to P, independently of locality or BCI variation;

E ~ BCI → E varies according to BCI, independently of locality or P variation;

E ~ P + locality → there is an additive effect of P and locality on E;

E ~ P * locality → there is an interactive effect of P and locality on E;

E ~ BCI + locality → there is an additive effect of BCI and locality on E;

E ~ BCI * locality → there is an interactive effect of BCI and locality on E.

To deal with data overdispersion, we compared models with N/L as dependent variable with the Quasi Akaike's Information Criterion for small samples (qAICc). Models with E as a dependent variable were compared using the delta Akaike's Information Criterion for small samples (AICc). As above mentioned, if models with variable 'locality' showed criteria values smaller than 2.0, they were considered plausible models to explain dependent variables. If the plausible models contained 'locality' as an independent variable, we evaluated differences between pairwise localities based on coefficient values from the simplest model using a multiple comparison analysis.

All models were implemented and compared with the *lme4* package of the software R version 3.4.3 (R Development Core Team 2017). In all model comparisons, when two or more nested models met this criterion, we considered the simplest one to be the best explanation for the observed data and dismissed the models with additional uninformative parameters (following Arnold, 2010; Burnham & Anderson, 2002).

Results

Animal physiological condition

First, we found a slight, non-significant tendency of improvement in individuals’ body condition index (BCI) with increasing distance of the pollution source (Mogi 0.752 ± 1.489, Quilombo 0.390 ± 0.422, Itatinga 1.376 ± 0.976, F = 2.438, $p = 0.100$, Fig. 2a). When we removed three outliers, however, we found higher BCI values of individuals from Itatinga population, the farthest population from pollution source and our control site (Mogi 0.294 ± 0.383, Quilombo 0.390 ± 0.422, Itatinga 1.145 ± 0.638, F = 11.18; $p < 0.001$, Fig 2b).

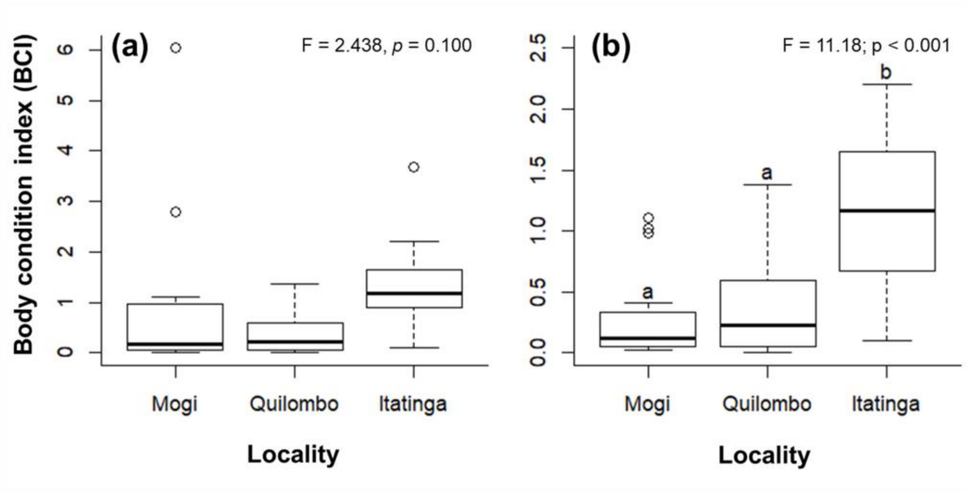

Figure 2. Body condition index (BCI) of *Rhinella ornata* populations at increasing distances from the Cubatão Industrial Complex. Results when considered a) all individuals collected (Mogi n = 18; Quilombo n = 14; Itatinga n = 11) and b) without outliers (Mogi n = 16; Quilombo n = 14; Itatinga n = 10). Letters show statistically significant differences among localities resulting from multiple comparative analyses for parametric models: (a) Mogi-Quilombo: t = -0.914, $p = 0.634$; Mogi-Itatinga: t = 1.464, $p = 0.318$; Quilombo-Itatinga: t = 2.199, $p = 0.084$; (b) Mogi-Quilombo: t = 0.559, $p = 0.842$; Mogi-Itatinga: t = 4.490, $p < 0.001$; Quilombo-Itatinga: t = 3.878, $p = 0.001$. 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.

Three organ-somatic analyses (liver, kidneys and spleen) contained 'locality' as a predictor variable in models with dAIC < 2.0 (Table 1). Moreover, pairwise comparisons between localities using the simplest model ($\varphi \sim$ locality) showed significant differences between relative masses of liver (Mogi $2.13 \times 10^{-2} \pm 0.50 \times 10^{-2}$, Quilombo $1.72 \times 10^{-2} \pm 0.31 \times 10^{-2}$, Itatinga $1.55 \times 10^{-2} \pm 0.11 \times 10^{-2}$), kidneys (Mogi $4.37 \times 10^{-3} \pm 0.70 \times 10^{-3}$, Quilombo $3.61 \times 10^{-3} \pm 0.66 \times 10^{-3}$, Itatinga $0.36 \times 10^{-3} \pm 0.44 \times 10^{-3}$) and spleen (Mogi $9.71 \times 10^{-4} \pm 5.10 \times 10^{-4}$, Quilombo $7.11 \times 10^{-4} \pm 1.86 \times 10^{-4}$, Itatinga $6.25 \times 10^{-4} \pm 2.19 \times 10^{-4}$). Individuals from test site Mogi always presented higher relative masses of liver, kidneys and spleen than individuals from our control site Itatinga. Gonadosomatic index values did not differ among *R. ornata* populations sampled (Mogi $2.76 \times 10^{-3} \pm 1.08 \times 10^{-3}$, Quilombo $2.53 \times 10^{-3} \pm 0.98 \times 10^{-3}$, Itatinga $2.37 \times 10^{-3} \pm 0.82 \times 10^{-3}$), since 'locality' was not present in plausible models to explain this variable (Table 1) (Fig 3).

Table 1. Model selection procedure assessing the effect of body condition index (BCI) and locality on 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
φ ~ 1	-340.1	12.5	2	<0.001	-492.8	13.9	2	<0.001	-550.4	3.2	2	0.113	**-470.9**	**0**	**2**	**0.536**
φ ~ locality	**-352.7**	**0**	**4**	**0.47**	**-506.7**	**0**	**4**	**0.56**	**-553.6**	**0**	**4**	**0.564**	-468.1	2.8	4	0.134
φ ~ BCI	-338.5	14.2	3	<0.001	-492.3	14.4	3	<0.001	-548.4	5.2	3	0.042	**-469.0**	**1.8**	**3**	**0.213**
φ ~ BCI + locality	**-352.4**	**0.2**	**5**	**0.42**	**-505.9**	**0.8**	**5**	**0.38**	**-551.7**	**1.9**	**5**	**0.222**	-466.5	4.4	5	0.059
φ ~ BCI * locality	-349.9	2.8	7	0.12	-502.3	4.5	7	0.06	-549.1	4.5	7	0.059	-466.4	4.5	7	0.058

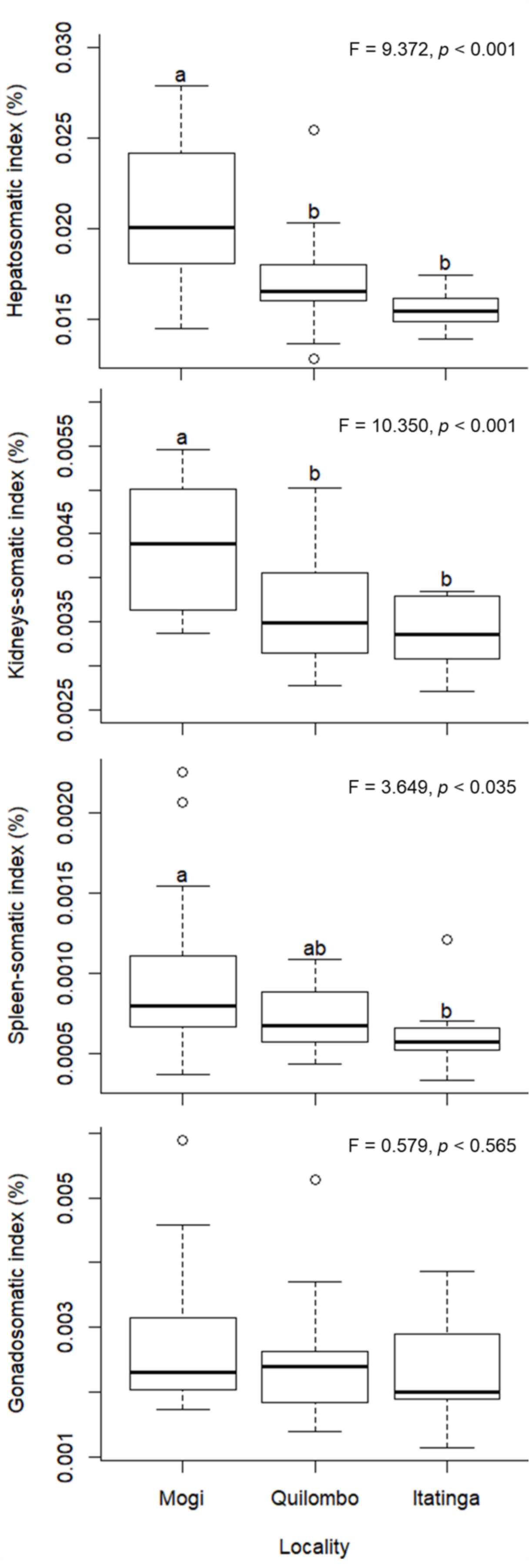
Hepatosomatic index (%)
0.015
0.020
0.025
0.030
F = 9.372, p < 0.001
a
b
b
Kidneys-somatic index (%)
0.0025
0.0035
0.0045
0.0055
F = 10.350, p < 0.001
a
b
b
Spleen-somatic index (%)
0.0005
0.0010
0.0015
0.0020
F = 3.649, p < 0.035
a
ab
b
Gonadosomatic index (%)
0.001
0.003
0.005
F = 0.579, p < 0.565
Mogi
Quilombo
Itatinga
Locality

Figure 3. Organ-somatic indices of *Rhinella ornata* populations at increasing distances from the Cubatão Industrial Complex (Mogi n=18, Quilombo n=14, Itatinga n=11). Letters show statistically significant differences among localities resulting from multiple comparative analyses for parametric models: Hepatosomatic index - Mogi-Quilombo: t = -3.085, p = 0.009; Mogi-Itatinga: t = -4.031, p < 0.001; Quilombo-Itatinga: t = -1.100, p = 0.519; Kidneys-somatic index - Mogi-Quilombo: t = -3.36, p = 0.005; Mogi-Itatinga: t = -4.17, p < 0.001; Quilombo-Itatinga: t = -0.99, p = 0.587; Spleen-somatic index - Mogi-Quilombo: t = -1.998, p = 0.125; Mogi-Itatinga: t = -2.475, p = 0.046; Quilombo-Itatinga: t = -0.583, p = 0.829. 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.

Haematological indicators

Individual stress index, as indicated by neutrophil/lymphocyte ratios (N/L), was only predicted by the model containing ‘locality’ as the only explanatory variable (Table 2). Lower N/L values were found in individuals from test site Mogi when compared with individuals from Quilombo and Itatinga, which did not differ from each other (Fig 4a).

Table 2. Model selection procedure assessing the effect of body condition index (BCI) and locality on individual stress index (neutrophil/lymphocyte ratio, N/L) of *Rhinella ornata* (see text for details). The values of the Quasi-likelihood delta AIC (dqAIC), 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	dqAIC	df	Weight
N/L ~ 1	9.5	1	0.006
N/L ~ locality	**0**	**3**	**0.744**
N/L ~ BCI	9.8	2	0.006
N/L ~ BCI+ locality	2.3	4	0.233
N/L ~ BCI* locality	8.5	6	0.011

Two models were equally plausible to explain the observed variation in the proportion of eosinophil count (E) and both had ‘locality’ as a dependent variable (Table 3). However, despite the best model of E analysis included ‘locality’ as a predictor variable, the multiple comparison analyses for non-parametric models did not find statistically significant differences among localities (Fig 4b).

Table 3. Model selection approach assessing the effect of body condition index (BCI), total parasite load (P) and locality on the proportion of eosinophils (E) of *Rhinella ornata*. The values of the Quasi Akaike’s Information Criterion for small samples (qAICc), delta qAICc, 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	qAICc	dqAICc	df	Weight
E ~ 1	181.9	2.1	1	0.132
E ~ Locality	**179.9**	**0**	**3**	**0.371**
E ~ P	182.1	2.3	2	0.120
E ~ BCI	183.3	3.4	2	0.068
E ~ P + Locality	**181.8**	**1.9**	**4**	**0.144**
E ~ BCI + Locality	182.4	2.5	4	0.105
E ~ P * Locality	184.2	4.3	6	0.043
E ~ BCI * Locality	186.0	6.2	6	0.017

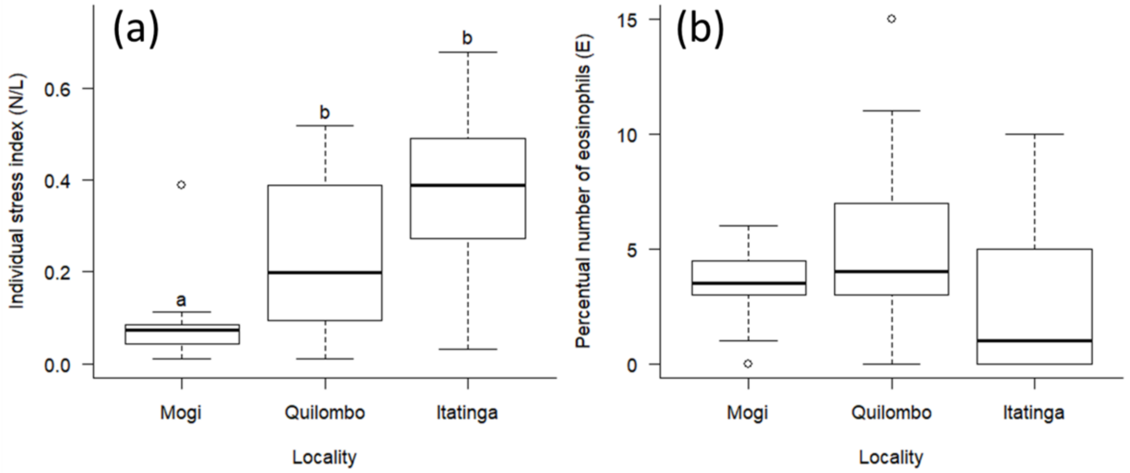

Figure 4. Individual stress index (neutrophil/lymphocyte ratio, N/L) of *Rhinella ornata* from populations at increasing distances from the Cubatão Industrial Complex (Mogi n = 13; Quilombo n = 10; Itatinga n = 10). As probabilistic distributions of these models are not Gaussian, the model analysis does not provide F and *p* values. Letters show statistically significant differences among localities resulting from multiple comparative analyses for non-parametric models: Individual stress index (N/L) - Mogi-Quilombo: z = 2.521, $p = 0.031$; Mogi-Itatinga: z = 3.589, $p < 0.001$; Quilombo-Itatinga: z = 1.237, $p = 0.427$; Percentual number of eosinophils (E) - Mogi-Quilombo: z = 2.210, $p = 0.069$; Mogi-Itatinga: z = -0.149, $p = 0.988$; Quilombo-Itatinga: z = -2.229, $p = 0.066$. 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.

Patterns of current contamination

Liver samples of the three localities had detectable concentrations of iron (Fe) and aluminium (Al), but not of manganese (Mn), cadmium (Cd) and lead (Pb). Surprisingly, *R. ornata* from Itatinga had the highest amounts of Fe and Al in their livers (Table 4). All organic contaminants in the composite toad carcass samples were below quantification limits (Supplementary material S2).

Table 4. Concentrations of metals (ppm, dry mass) found in the livers of *Rhinella ornata* from Mogi, Quilombo and Itatinga.

Metals	Mogi	Quilombo	Itatinga
Al	<0.01	6.27	21.94
Cd	<0.005	<0.005	<0.005
Fe	703.7	642.22	2,250.36
Mn	<0.001	<0.001	<0.001
Pb	<0.009	<0.009	<0.009

Discussion

Our assessment of the physiological condition of *Rhinella ornata* provided a range of responses. As hypothesized, proximity to the Cubatão Industrial Complex was associated with a relative increase in liver, kidneys and spleen masses. Contrary to our hypothesis, however, proximity to the Cubatão Industrial Complex was associated with a decrease in individuals' stress index (N/L), weak effects on body condition index, and no significant effects on proportional eosinophils number and relative gonad masses. Irrespective of the direction of response, for most organ-somatic indices there was a linear gradient in response, with toads from the heavily-polluted site (Mogi) standing out as significantly different from those from the moderately-polluted site (Quilombo) and the control site (Itatinga) 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 heavily contaminated sites or in controlled laboratory studies (see, for example, Tables 12.5 and 12.6 in Grillitsch & Schiesari, 2010, for a summary of ~50 studies of the effects of metals on reptiles).

Among the morphological indicators analyzed in this study, the enlargement of livers, kidneys and spleen is the strongest signal of exposure of *R. ornata* to environmental contamination in Mogi. 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 mass and negatively affecting individual health (Linzey et al., 2003). Increased liver and kidney mass is 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 Mogi. 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). Moreover, the injection of polycyclic aromatic hydrocarbons (PAHs) promotes lymphocytosis in spleens of the common carp (Reynaud and Deschaux, 2005). Therefore, lower N/L and higher spleen masses found in individuals of *R. ornata* from Mogi might result from direct detrimental effects of pollution and/or an increase of organisms’ susceptibility to parasitic diseases.

Several stressors including infection by parasites, exposure to radioactivity, pesticides and metals – such as Pb, Cd, Zn, and Cu –, are known to increase glucocorticoid secretion (Davis, Maney, and Maerz, 2008). Increased glucocorticoid secretion usually causes lymphopenia and neutrophilia within a few hours, consequently increasing the neutrophil/lymphocyte (N/L) ratio in circulation (Davis, Maney, and Maerz, 2008). However, we found lower individual stress index (N/L) in Mogi than in Quilombo and Itatinga. Additionally, we found only weak differences in body condition index and no differences in reproductive capacity between the sampled populations. It is possible that selective pressures imposed by local contamination input over 60 years promoted local physiological adaptations in the Mogi population. It is also possible that the populations of *R. ornata* from Quilombo and Itatinga 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 in *R. ornata* from Mogi.

Given the very broad range of contaminants released by the Cubatão Industrial Complex, it is not possible to know which contaminants (and which other environmental factors) caused the changes in physiological condition observed in toads.

None of the 52 organic contaminants nor 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 2,250 ppm), both considered nonpriority pollutants of medium ecotoxicological relevance (Grillitsch and Schiesari, 2010). Even if patterns of aluminium and iron contamination are somewhat contradictory to physiological indicators (see below), 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 & 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 3,300 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.

Nevertheless, the observation that toad livers from control site Itatinga 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 Mogi River Valley (Klumpp et al., 1998; Mayer et al., 2000; Moraes et al., 2002), but given the topography impairing wind directions and land use, it is unlikely that the higher metal concentrations observed in toad livers from Itatinga 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 Itatinga 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.

We found toads with lower body condition index and increased masses of liver, kidneys and spleen in the Mogi population – the nearest locality from Cubatão Industrial Complex. On the other hand, we found no differences in the toads' masses of testicles and we found a reduction in toads individuals' stress index (N/L) of Mogi, probably related with the spleen enlargement presented by them. We concluded that, despite changes caused by exposition to contaminants during the past 60 years, toads form polluted areas are maintaining their reproductive capacity. We suggest that future studies should test the hypothesis that toad populations from the Mogi River Valley have evolved 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 region,, as also a combination of laboratory and field experiments with crossing population origin to environmental conditions.

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