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Potential Use of Distinct Biomarkers (Trace Metals, Micronuclei and Nuclear Abnormalities) in a Heterogeneous Sample of Birds in Southern Brazil.

JOANA TOMAZELLI (joanatomazelli1@gmail.com)
FEEVALE University: Universidade FEEVALE https://orcid.org/0000-0001-6060-5579

Gabriela Zimmermann Prado Rodrigues
FEEVALE University: Universidade FEEVALE

Danielle Franco
Federal University of Rio Grande do Sul: Universidade Federal do Rio Grande do Sul

Mateus Santos de Souza
Federal University of Rio Grande do Sul: Universidade Federal do Rio Grande do Sul

Jorge Henrique Burghausen
FEEVALE University: Universidade FEEVALE

Jenifer Panizzon
FEEVALE University: Universidade FEEVALE

Juliana Machado Kayser
FEEVALE University: Universidade FEEVALE

Márcia Regina Loiko
FEEVALE University: Universidade FEEVALE

Anelise Schneider
FEEVALE University: Universidade FEEVALE

Rafael Linden
FEEVALE University: Universidade FEEVALE

Gunther Gehlen
FEEVALE University: Universidade FEEVALE

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Abstract

The analysis of metal concentrations in feathers and the micronuclei (MN) and other nuclear abnormalities (NA) test in birds are tools used for evaluating the impacts that anthropogenic actions have been causing to the environment and organisms. Here we used these biomarkers to investigate the response of birds to disturbances observed in three areas with different environmental characteristics (natural, agricultural and urban) in southern Brazil. We obtained a diverse sample (108 individuals from 25 species and 17 families) without significant differences in metal concentrations, frequency of MN and AN between sampling points. The concentrations of zinc (Zn) and copper (Cu) were significantly different between trophic guilds (Zn: p = 0.0006, Cu: p = 0.04) and age classes (Zn: p = 0.01, Cu: p = 0.03). Omnivore birds contributed to the increase in the number of MN (ΔAICc: 0.00; w = 0.40) and NA, which was also influenced by age classes and body condition index (BCI) (ΔAICc: 0.00; w = 0.34; ΔAICc: 0.89; w = 0.22; ΔAICc: 1.15; w = 0.19; ΔAICc: 1.33; w = 0.18). We showed that in a diverse sample of birds, the variables analyzed affected each biomarker in distinct ways.

1. Introduction

Environmental discharges of many kinds of toxic compounds that result from anthropogenic activities such as pesticides, herbicides and industrial and vehicular atmospheric emissions, are associated with the degradation of natural habitats (Olayemi e Jagun 2014; Baesse et al. 2019). These compounds release metals into the environment and in high concentrations they can affect the health of various animal species, including birds (Pandiyan et al. 2020). The effects of metals on birds are related to growth, reproduction and, consequently, survival. Among the most important effects are decrease of testicular mass, spermatogenesis failure, decrease in egg production and egg mass, alterations in the eggshell, increase in embryo mortality and reduction of the incubation success (Burger e Gochfeld 1995). Besides that, these chemical compounds have mutagenic and carcinogenic potential, that can lead to genotoxicity, i.e., alterations in the levels of genetic compositions, increasing predisposition to chromosome-related disorders (Alimba and Bakare 2016; de Souza et al. 2017; Baesse et al. 2019).

In this context, quantification of metals and genotoxicity evaluation are frequently used tools of environmental monitoring in studies with birds. Birds are in a global level considered bioindicators of environmental quality (Solgi et al. 2020) because they quickly reflect environmental changes (Baesse et al. 2015), are easily found, live in many different habitats and occupy distinct trophic levels (Becker 2003; Abbasi et al. 2015a; Solgi et al. 2020). Moreover, the collection of biological samples, like feathers and blood, is considered a non-destructive procedure (Burger and Gochfeld 2000; Braga et al. 2010). Some authors have made efforts to evaluate the potential of birds as bioindicators by capturing a great number of species, but they rarely succeeded in identifying a good bioindicator species for genotoxic damage (Baesse et al. 2015) or in determining a species more sensitive to metal contamination (Abbasi et al. 2015a; Grúz et al. 2018). Other authors have restricted their attention to only one or a few species (Tsarpali et al. 2020; Mukhtar et al. 2020) and were able to achieve more robust conclusions, which is mainly attributed to known biological characteristics of the target species (Frixione and Rodríguez-Estrella 2020).

Birds are exposed to contaminants through their interactions with the environment, by breathing, by food and water ingestion (Dauwe et al. 2000; Mukhtar et al. 2020) and by their contact with the ground (Leonzio et al. 2009; Souto et al. 2018). Feathers are widely used in environmental studies because of their capability of metal accumulation (Solgi et al. 2020) externally, via deposition and internally, through the bloodstream (Burger e Gochfeld 2000), reflecting the reflect long-term damage (Dauwe et al. 2000). Furthermore, the collection is considered a non-destructive technique (Burger and Gochfeld 1992).

The micronucleus (MN) test (Baesse et al. 2015) and the nuclear abnormalities (NA) test (Quero et al. 2016) are used for genotoxicity evaluation. MN and NA are formed when organisms are exposed to genotoxic agents (Angeletti e Carere, 2014). MN are small chromatin bodies outside the nucleus, generated by chromosomal break and spindle or centromere dysfunction during cell division (Bonisoli-Alquati 2014). NA are nuclear malformations attributable to errors during the development of erythrocytes (De Mas et al. 2015). These alterations represent impacts over DNA (Baesse et al. 2015) and are indicatives of recent exposure to contaminants (Santos et al. 2017).

Diet (Burger and Gochfeld 2000; De Mas et al. 2015; Abbasi et al. 2015a; Solgi et al. 2020), behavioral and feeding strategies (Costa et al., 2011; Tsarpali et al. 2020; Tasneem et al. 2020) and food resources availability (Fritsch et al. 2012; Quero et al. 2016) are some of the factors responsible for metal contamination and alterations of the numbers of MN and NA in birds. Birds in higher trophic levels present higher concentrations of metals (Zolfaghari et al. 2007; Lodenius and Solonen 2013; Abbasi et al. 2015) due to the biomagnification process (Burger and Gochfeld 1995; Tasneem et al. 2020). Food preferences might influence the levels of MN (Quirós et al. 2008) and the formation of NA (Quero et al. 2016). Quirós et al. (2008) found significant differences between heron nestlings species that feed in aquatic and terrestrial habitats, reporting higher levels of MN to terrestrial species, which usually feed on insects. However, there is limited information about the relation between MN and NA levels and diet in the literature.

Studies relating the presence of metals, MN, NA and the nutritional status of birds are scarce. High concentrations of metals were found in the liver and kidney of owls with low reserve of lipids (Esselink et al. 1995), however, in structures like feathers, this relation was investigated recently (Innangi et al. 2019) and more studies on the subject are needed. MN and NA were not correlated to the body condition (Tsarpali et al. 2020; Frixione and Rodríguez-Estrella 2020), but inferior health and/or nutritional status might increase the amount of damage to the cell nucleus (Santos et al. 2017).

Regarding wildlife exposure to chemical compounds, age is an important factor to be considered (Squadrone et al. 2016). Studies indicate that metal concentration in several tissues are frequently higher in adult birds (Burger and Gochfeld 1995; Leonzio et al. 2009; López-Perea et al. 2019; Innangi et al. 2019; Ackerman et al. 2019) because of long-term exposure to contaminated environments and consequent bioaccumulation (Grúz et al. 2018; Innangi et al. 2019). As the feathers of young individuals are recently formed and are less exposed to atmospheric conditions, they have smaller concentrations of metals (Dauwe et al. 2000). Age is also an important factor for MN an NA. Different generations might present different levels of these alterations (Santos et al. 2017; Tsarpali et al. 2020). MN and NA increased with the reduction of the age classes in aquatic birds (Santos et al. 2017), however, few studies tried to establish this relation.

The Sinos River Hydrographic Basin (SRHB) had its vegetation cover altered by human activities initiated after the arrival of European immigrants and this process was intensified since 1940's (Franz et al. 2010). Deforestation to open areas for agriculture and cattle grazing is the main reason for the reduction of the vegetation cover in the basin to 10% of its original area. Besides that, these activities also contributed to the reduction of water quality (Figueiredo et al. 2010). In the metropolitan area, the high population and industrial density with its associated environmental problems (atmospheric emissions, industrial wastewater, lack of basic sanitation and intense vehicle traffic) contribute to the environmental degradation observed in this basin (Figueiredo et al. 2010). Previous studies with different bioindicators and biomarkers have shown an important environmental disturbance in the SRHB, caused by multiple factors. In fish, in addition to morphological alterations, high levels of metals have been reported (Dalzochio et al. 2018). In *Tradescantia pallida* var. *purpurea*, genotoxic damages influenced by low air quality were observed (Cassanego et al. 2015).

In this context, it is important to analyze the response of birds to the environmental impacts that occur in this region. The aim of this study was to evaluate the effect of anthropogenic disturbance in wild birds that inhabit areas with different environmental characteristics along the SRHB through the analysis of concentration of trace metals (chromium, manganese, zinc and copper) in feathers and the frequency of MN and NA in peripheral blood. Besides that, we evaluated whether there was variation of metals concentrations, MN and NA frequencies between sampling point, trophic guild, species, body condition index (BCI) and age classes. We hypothesized that birds inhabiting regions with less environmental impact would present lower concentrations of metals and lower frequencies of MN and NA in comparison to birds inhabiting more impacted regions. We also tested whether species respond in different ways to environmental disturbance, as well as whether adult birds and birds with more lipidic reserves would present higher metal concentration and whether young birds would present lower concentrations of metals and higher frequencies of MN and NA.

2. Methodology

2.1 Study area

The study was conducted in two cities, Taquara and Novo Hamburgo, which are part of the Sinos River Hydrographic Basin. Three sampling sites were defined, each one located in a distinct environmental zone (natural, rural and urban zones) (Fig. 1). The climate, according to the Köppen classification, is Cfa, with no defined dry season and the average air temperature higher than 22°C in the hottest month (Peel et al., 2007). The areas are predominantly in the Semideciduous Seasonal Forest domain (Ibge 2012).

2.1.1 Ilha River (Taquara city)

The sampling site 1 (S1) is located near the source of the Ilha River (29º32'51.62"S 50º37'34.14"W) and its vegetation is typical of the highest altitude areas in the basin, i.e., it has a dense vegetation cover with the occurrence of exotic invader species, like the japanese raisin tree (*Hovenia dulcis*) (Fontanella et al., 2009). Some elements of Ombrophilous Forest are observed in this region, as it is in an ecotone between this plant formation and the Semideciduous Seasonal Forest (Comitesinos, 2016). This place is also characterized by surrounding areas with plantations of exotic species like *Pinus* sp. and *Eucalyptus* sp., There are also small farms and low population density.

The sampling site 2 (S2) is situated near the mouth of the Ilha River (29º40'41.81"S 50º44'25.30"W). There are more houses and rural properties in this point than in S1, and activities like cattle raising are carried out in the region. The vegetation has been altered due to greater anthropogenic action (Fontanella et al. 2009). Native vegetation at this sampling site is restricted to riparian vegetation, which is surrounded by rice plantations.

2.1.2 Parque Henrique Luís Roessler (Novo Hamburgo city)

The sampling site 3 (S3) corresponds to the Parque Henrique Luís Roessler, a municipal conservation unit (Novo Hamburgo 2009), located in the urban area of the city of Novo Hamburgo (29°40'53.67"S 51° 6'33.38"W). The unit is surrounded by streets, houses and residential and commercial buildings. The native vegetation is characterized as early stage of succession. Some exotic species are present, as the *Pinus elliotti* (Cappelatti and Schmitt 2009). There are three springs within the unit, all of them contaminated by illegal discharge of domestic wastewater (Leuck, 2010).

2.2 Capture of birds and data collection

Bird were captured between November 2019 and May 2020, by mist-nets (9 m x 3 m, 15 mm mesh size). Each sampling site was sampled four times during this period, for approximately 5 hours in the morning. In order to avoid animal stress, the nets were checked every 15–30 minutes during the sampling period. Species identification followed Sigrist (2014). All individuals were marked with metallic rings supplied by CEMAVE-ICMBio and grouped by taxa according to the Brazilian Ornithological Records Committee (Comitê Brasileiro de Registros Ornitológicos) (de Piacentini et al. 2015).

Age classes determination of birds was based on information from feathers condition and other attributes from the plumage (molt and molt limits, shape of flight feathers, presence or absence of growing bars) (Ralph et al. 1996; Howell et al. 2003). Additional characteristics, like skull ossification, iris and bill color and commissure of the bill were observed (Ralph et al. 1996). In order to categorize the age of the birds, the system of classification based on the molting cycle – Wolfe-Ryder-Pyle (WRP) (Wolfe et al. 2010; Johnson et al. 2011; Johnson and Wolfe 2017) was used. Specimens were classified as juveniles when they were on, or presented signs of, the first cycle, i.e., they had juvenile plumage – acquired after leaving the nest – or formative plumage – acquired before reaching sexual maturity. Specimens that had finished the first cycle were classified as adults and a third category, undetermined age, was defined to include those animals that were not confidently classified as juveniles or adults.

Birds were grouped in four trophic guilds, following the classification proposed by Willman et al. (2014). This database defines trophic guilds based on the proportion of different food items consumed by each species. The guilds used in this study were: (1) insectivore, (2) herbivore/granivore and (3) omnivore.

Body condition can be estimated by dividing body mass by any linear measure of body size (Labocha and Hayes 2012). Therefore, the BCI (body condition index) was defined as the body mass divided by the tarsus length. Individuals with a high index value are heavier in relation to the skeleton size, and probably have better lipidic reserves (Gaiotti et al. 2020). The length of the tarsus (in mm) was measured by a caliper rule and the mass was inferred with a digital weighing scale (precision of 0.1 g).

A sample of blood was taken from the ulnar vein using a sterile needle (0.45 x 13 mm – subcutaneous standard). Blood smears (two for individuals) were immediately made on glass slides (Braga et al. 2010), which were dried at environmental temperature and fixed in methanol for 10 minutes.

For the analysis of metals, some contour feathers were taken from both sides of the chest of each bird, to reduce the possibility of harm (Burger and Gochfeld 2000). The feathers were placed in zipped plastic bags (Abbasi et al., 2015b), identified with a label containing the ring number of each bird and stored at room temperature.

2.3 Washing, digestion and detection of metals in feathers

In order to remove any contaminants deposited on its surface, the feathers were washed alternately with deionized water (1 minute) and PA acetone (1 minute) in decontaminated falcon tubes, which were shaken by hand (Veerle et al., 2004; Abdullah et al. 2015). This procedure was repeated three times. After washing, the feathers were dried at 80°C in a oven for 1 hour (Abbasi et al. 2015a).

The procedure for digestion of feathers followed adaptations of the methodology proposed by Reglero et al. (2008). The feathers (average of 0.03g) were initially immersed in a solution of HNO_3 (65%). After that they were digested in a Microwave Accelerated Reaction System (MARS6 – CEM). Briefly, the feathers were calcined for 20 minutes until reaching the temperature of 180°C, maintained at 180°C for more 15 minutes and then refrigerated for 15 minutes. Digested samples were diluted in a solution of 1520 µl of HNO_3 (65%) (Merck, Darmstadt, Germany) and 106 µl of Triton (Baker Analyzed®) and had the volume adjusted to 100 ml with Milli-Q H_2O.
A graphite furnace atomic absorption spectrometer (GFAAS - Perkin Elmer Analyst 600) was used for detection of metals. To provide quality control data, solutions used to dilute processed samples in each batch of analysis were taken as blank. The concentration of metals in blank solutions was always bellow detection limits. Detection limits for metals chromium (Cr), manganese, copper (Cu) and zinc (Zn) were 0.006, 0.01, 0.01, and 0.3 µg/g, respectively.

2.4 Preparation and analysis of slides

In laboratory, the slides were stained with Giemsa solution (5%) for 10–15 minutes. After that, they were codified and analyzed by a single person (Alimba and Barake 2016) in an optical microscope with the highest magnification (1000x). For every bird, 3000 erythrocytes were analyzed (Hussain et al. 2012).

Criterions for identification of MN and NA were adapted from Quero et al. (2016). Thus, MN were identified as oval or circular structures with 1/3 to 1/16 of the nucleus size, with the same focal plane, color and texture as the nucleus, without bridges or chromatin overlaps (Tolbert et al. 1992) (Online Resource 1).

Among the NA, nuclear buds had the same morphology as the MN, but remained connected to the nucleus, without formation of chromatin bridges or a constriction at one extremity and had between 1/4 and 1/3 of the main nucleus size (Thomas et al. 2009). Binucleated cells presented two nuclei about the same size and color, with or without contact between them (Thomas et al. 2009; Jindal and Verma 2015). Nucleus that had a progressive narrowing and elongation at one extremity, were considered nuclear tails (Kursa and Bezrukov 2008). Nucleoplasmic bridges were considered as two nuclear structures of the same color, having equal or different sizes, connected by a chromatin bridge (Tolberd et al. 1992). A nucleus was considered notched when it had a well-defined notch that extended to a considerable depth and was limited by the nuclear envelope (Carrasco et al. 1990; Alimba and Bakare 2016) (Online Resource 1). Cases in which the morphology of the nucleus did not meet any of the preestablished criterions and/or raised doubts during the analysis were not included in the data.

2.4 Data analysis

In order to verify how much of the response variables (MN and NA) were explained by the predictor variables (sampling site, trophic guilds, species, BCI and age), we used Generalized Linear Models (GLM). The Poisson distribution was used in the MN models, while in the NA models the negative binomial distribution was used, to correct for data overdispersion. The link function used was the logarithm (link = log).

We built 27 candidate models for each response variable (n = 54), including null models (no effect). Model selection was performed according to the Akaike information criterion for small sample size (AICc) (Burnham and Anderson 2002), using the value of ΔAICc < 2 (difference of AIC between given model and the best model) and the weight (w - weight of the evidence in favor of each model) for comparisons. The significance of each factor and the percentage of variation explained by the best models was calculated by applying the ANOVA test on the models residuals and subsequently applying the formula proposed by Ye et al. (2001), represented by the deviance of the variable divided by model residual deviance multiplied by 100.

For the statistical analysis of the metals data, we tested normality using the Shapiro-Wilk test and applied the non-parametric Kruskal-Wallis test followed by the post-hoc Dunn's test for compare medians between points, species, trophic guilds and age classes. We used the Spearman correlation (r) to verify the existence of correlation between metals and BCI. The analyses were performed using the packages "vegan", "AICcmodavg" and "MASS" in the software "R" (R Core Team 2020), with significance level of $\alpha \leq$ 0,05. The graphs were made in GraphPad Prism 8.0.1.

3. Results

Overall, 108 individuals (= 216 slides) from 25 species and 17 families were captured (Online Resource 2). Age classes and trophic guilds presented varied composition between sampling points (Online Resource 3). Adult Individuals were more frequently captured (57.4%), followed by juveniles (35.8%) and the undetermined age class (7.4%). Insectivore (55.5%) was the best represented trophic guild, followed by omnivore (40.7%) and herbivore/granivore (3.8%). The highest BCI was recorded in S3 (1.7), followed by P2 (1.56) and P1 (1.18).

3.1 Detection of metals

The concentrations of metals analyzed were not significantly different between sampling points (Online Resource 4). Significant differences were observed between some species, trophic guilds, age classes and

BCI. Concentrations of Zn were higher in *Myiothlypis leucoblephara* compared with *Turdus albicollis* (p = 0.01) and *Turdus rufiventris* (p = 0.02). Insectivores presented higher concentrations of Zn (p = 0.02) and Cu (p = 0.03) than omnivores. The undetermined age class presented higher concentrations of Zn when compared with adult individuals (p = 0.001), and juveniles individuals presented higher concentrations of Cu than adults (p = 0.004) (Online Resource 5). Only Zn and Cu were significantly correlated with the BCI, both negative and weak correlations (Zn: r = − 0.33, p = 0.0008, Cr: r = − 0.22, p = 0.02).

3.2 Micronuclei and nuclear abnormalities

A total of 76 MN and 3,267 NA were recorded in 46.8% and 99% of the birds, respectively. Considering the number of captured individuals, the overall mean frequency of MN was 0.70 / 3,000 (or 2.33 / 10,000) and the overall mean frequency of NA was 5 / 3,000 (or 16.68 / 10,000). The most frequent NA were notched nuclei (95,4 %), binucleated cells (82,4%), nuclear buds (62,0%), nucleoplasmic bridges (49,1 %) and nuclear tails (26,8%). While MN presented varied frequencies, NA presented similar number between sampling points (Online Resource 3).

3.3 Generalized Linear Models (GLMs)

Despite distinctive vegetation characteristics and anthropogenic pressure observed in the region of each sampling site, they did not influence the GLM (MN = ΔAICc: 5.53; w = 0.03; NA = ΔAICc: 26.17; w = 0.00).

The best supported GLM Poisson model showed that the omnivore guild contributed significantly and positively to data variation, being the factor responsible for the increase in the number of MN (Table 1). In all negative binomial GLM, the variable BCI significantly and positively influenced on the number of NA, showing that birds with better body condition have more NA. The presence of the herbivore/granivore guild decreased, while the omnivore guild increased the NA in some models. The adult and undetermined age classes reduced significantly the NA number in the second model, indicating that juveniles have more NA than other age classes (Table 1).

Table 1

Results of the best GLM models selected for MN (Poisson) and NA (negative binomial) with parameters estimated under each model, standard error (SE), p value (*p*), AICc, ΔAICc and the weight of the model (*w*). The intercept values represent the insectivore guild and the juvenile age classes.

Models	**Variables**	**Estimates**	**SE**	***z-value***	***p***	**AICc**	**ΔAICc**	***w***
MN ~ trophic guild	Intercept	− 0.6604	0.1796	-3.677	< 0.001	279.36	0.00	0.40
	Herbivore/granivore	− 0.7259	1.0160	-0.715	0.474			
	Omnivore	0.6604	0.2345	2.816	< 0.01			
NA ~ trophic guild + BCI	Intercept	1.9945	0.2427	8.219	< 0.001	927.20	0.00	0.34
	Herbivore/granivore	-1.7553	0.6122	-2.867	< 0.01			
	Omnivore	0.6877	0.1997	3.444	< 0.001			
	BCI	0.6809	0.1662	4.097	< 0.001			
NA ~ age classes + trophic guild * BCI	Intercept	2.2168	0.3725	5.951	< 0.001	928.09	0.89	0.22
	Adult	-0.3517	0.2078	-1.693	0.09			
	Undetermined age	-0.7753	0.3887	-1.995	< 0.01			
	Herbivore/granivore	-0.9359	0.9711	-0.964	0.33			
	Omnivore	-0.6173	0.8695	-0.710	0.47			
	BCI	0.6519	0.2673	2.439	0.01			
	Herbivore/granivore:BCI	-0.2619	0.3622	-0.723	0.47			
	Omnivore:BCI	0.8340	0.5222	1.597	0.11			
NA ~ trophic guild * BCI	Intercept	1.7164	0.3210	5.347	< 0.001	928.35	1.15	0.19
	Herbivore/granivore	-0.6152	0.9678	-0.636	0.52			
	Omnivore	-0.2575	0.8787	-0.293	0.76			
	BCI	0.8992	0.2350	3.827	< 0.001			
	Herbivore/granivore:BCI	-0.5466	0.3468	-1.576	0.11			

* significant *p* value ($p \leq 0.05$)

Models	Variables	Estimates	SE	*z-value*	*p*	AICc	ΔAICc	*w*
	Omnivore:BCI	0.4922	0.5280	0.932	0.35			
NA ~ trophic guild + BCI * age classes	Intercept	1.4283	0.5516	2.589	< 0.001	928.53	1.33	0.18
	Herbivore/granivore	-1.3864	0.6066	-2.286	< 0.05			
	Omnivore	0.8309	0.2077	4.000	< 0.001			
	BCI	1.2284	0.4094	3.000	< 0.01			
	Adult	0.7567	0.6847	1.105	0.26			
	Undetermined age	1.130	1.4966	0.760	0.44			
	BCI:Adult	-0.7653	0.4584	-1.670	0.09			
	BCI:Undetermined age	-2.3940	2.1153	-1.132	0.25			
* significant *p* value ($p \leq 0.05$)								

The results of the ANOVA test of the MN model showed that the variable trophic guild was significant and explained 7.64% of the model (Table 2). In the NA GLM, the ANOVA test showed that the variables trophic guild and BCI were significant and explanatory in all models. The age classes also had significant contribution to the explanation of the second and fourth best models (Table 2). All models explained 119.03% of data variation.

Table 2
ANOVA results of the Generalized Linear Models for values of MN (Poisson) and NA (negative binomial), number of parameters (Df).

Models	Df	Deviance	Residuals Df	Residual deviance	*p*	% explained
Null model			107	126.91		
MN ~ trophic guild						
Guild	2	9.6977	105	117.22	< 0.01	7.64
Null model			107	163.41		
NA ~ trophic guild + BCI						
Guild	2	31.810	105	131.60	< 0.001	19.47
BCI	1	11.998	104	119.61	< 0.001	7.34
					Total	26.81
Null model			107	173.85		
NA ~ age classes + trophic guild * BCI						
Age	2	18.6850	105	155.17	< 0.001	10.75
Guild	2	25.6706	103	129.50	< 0.001	14.76
BCI	1	8.1230	102	121.37	< 0.01	4.67
Guild:BCI	2	2.8403	100	118.53	0.24	1.63
					Total	31.81
Null model			107	167.28		
NA ~ trophic guild * BCI						
Guild	2	32.594	105	134.69	< 0.001	19.48
BCI	1	12.283	104	122.40	< 0.001	7.34
Guild:BCI	2	3.415	102	118.99	0.28	2.04
					Total	28.86
Null model			107	173.06		
NA ~ trophic guild + BCI * age						
Guild	2	33.768	105	139.29	< 0.001	19.51
BCI	1	12.710	104	126.58	< 0.001	7.34
Age	2	5.755	102	120.83	0.05	3.32

* significant *p* value ($p \leq 0.05$)

Models	Df	Deviance	Residuals Df	Residual deviance	*p*	% explained
BCl:age	2	2.391	100	118.44	0.30	1.38
					Total	31.55
* significant *p* value ($p \leq 0.05$)						

4. Discussion

4.1 Trace metals

The overall mean concentration of Zn in feathers recorded in this study (437.77 ± 241.25 µg/g) is similar to those recorded by Abdullah et al. (2015) in feathers of herons from polluted aquatic environments in Pakistan (226 to 529 µg/g). However, the species *M. leucoblephara* presented mean concentration of 762,82 ± 309,06 µg/g, which is significantly higher when compared with *T.rufiventris* and *T. albicollis* (352.97 ± 132.00 and 336.18 ± 93.54 µg/g, respectively). These values are higher than concentrations found in feathers of a passerine bird from a DDT contaminated area in Africa (207.45 a 291.51µg/g) (Baker et al. 2017). According to Tasneem et al. (2020), high concentrations of this metal in feathers might be related to deposition from exogenous sources, but Abdullah et al. (2015) argue that when there is a high concentration of Zn in the organism, it can be deposited in feathers, as a way of excretion.

The metal Mn presented an overall mean concentration of 29.63 ± 16.74 µg/g, which is comparable to the value found in a passerine species living in a gradient of industrial pollution (means from 17.4 to 43.8 µg/g) (Janssens et al. 2001) and in an aquatic bird species (16 to 21.9 µg/g) (Abdullah et al. 2015). In *Passer domesticus*, however, Baker et al. (2017) found concentrations ranging from 15.73 to 78.38 µg/g. Concentrations observed in the present study are high when compared with values reported in the literature but are not high enough to indicate manganese contamination. Manganese is an essential metal that participates in a series of biochemical reactions in the organisms (Abdullah et al. 2015), but exogenous contamination might occur through vehicular pollution, contaminated dust and food intake (Burger and Gochfeld; 1995; Abdullah et al. 2015).

The metal Cr presented an overall mean concentration of 2.41 ± 1.84 µg/g. This metal might have neurotoxic effects on birds (Burger and Gochfeld 2000) and these authors assert that concentrations higher than 2.80 µg/g in feathers indicate contamination. The overall mean concentration of Cu found in the present study (7.84 ± 4.55 µg/g) is low when compared to other studies in polluted areas (Manjula et al. 2015; Baker et al. 2017). According to Baker et al. (2017) exogenous contamination by this metal is related to anthropogenic sources, like emissions from fossil fuels and industrial activities. Our results, however, are similar to those recorded in passerine birds that inhabit an urban area in Pakistan (Cr, 1.11 ± 0.70 to 1.95 ± 0.16, Cu, 2.19 ± 0.81 to 4.14 ± 0.20) (Abbasi et al. 2015b).

4.2 Micronuclei and nuclear abnormalities

The overall mean frequency of MN found in the present study (0.70 / 3,000 or 2.33 / 10,000) is similar to the data in the literature. Baesse et al. (2019) found a mean of 1.04MN/10,000 and Baesse et al. (2015) reported a mean of 1.30 MN /5,000 (ou 2.6 MN/10,000) in different sized forest fragments close to urban areas. In coffee farms with different sizes and different productive capacities, the mean was 3 MN/10,000 (Souto et al. 2018), which is higher than the values reported in other studies, but the evaluated environments and the chosen species might have contributed to this value.

The NA most frequently found in this study (notched nucleus and binucleated cells) were also the most representative abnormalities in a community of birds from a desert environment (Quero et al. 2016) and in a falcon species from an island environment (Tsarpali et al. 2020). Notched nucleus was the second most frequent NA found in a falcon species in an agricultural area (Frixione and Rodríguez-Estrella 2020), but the causes and the mechanism behind the formation of this abnormality are unknown (Quero et al. 2016). The presence of a great number of binucleated cells in birds have been related to exposure to particular contaminants, like in Japanese quails (*Coturnix japonica*) exposed to landfill leachate (Alimba and Bakare 2016) and atrazine (Hussain et al. 2012) and in Australian parakeets (*Melopsittacus undulates*) exposed to tannery effluents (de Souza et al. 2017). These chemical substances might inhibit the cytokinesis during cell division (Alimba and Bakare 2016), affecting its final stages (de Faria et al. 2018).

4.3 Influence of the sampling points

Although our sampling points are located in regions with different environmental characteristics, which are apparently impacted by different levels of anthropogenic pressure, our results did not indicate different levels of contamination between these regions. None of the evaluated metals or the frequency of MN and NA presented significant differences between sampling points. Moreover, all metals presented the highest concentrations on S1, and this sampling point had the second highest frequency of MN, followed by S2. Thus, this sampling point, located in a region that apparently has a lower degree of impacts, might be being contaminated by a wide range of pollutants that could enter even preserved areas by the movement of atmospheric air (Abbasi et al. 2015), as already reported by Alves et al. (2018) in the SRBH.

Even though we did not find any significant relation, the impact on birds that inhabit urban areas was evidenced by a higher frequency of MN in S3. Stocker (2019) reports higher levels of MN in birds that live near the airport area in Porto Alegre, Brazil, when compared with captive animals. Baesse et al. (2019) found a higher number of MN in forest fragments close to urbanization, suggesting that the greater the vehicles traffic near the fragment, the greater the number of MN in birds. Sasamori et al. (2012) assessing the genotoxic potential of the air using *Tradescantia Pallida* as a bioindicator in S3, found a higher frequency of MN in plants in this place when compared with a control group, suggesting that pollution from motor vehicles might be the source of the genotoxic agent in this region. However, based on our results, there is no clear distinction between potential genotoxicity and metal contamination between sampling points.

4.4 Influence of trophic guilds

In this study, trophic guilds had a relevant contribution to the results, but were related in different ways to the concentrations of metals and genotoxicity. Insectivores presented concentrations of Zn and Cu significantly higher than omnivores. GLM results indicated that a generalist diet (omnivore) is related to the increase in the number of MN and NA.

Our results are in agreement with studies that found higher levels of metals in insectivore birds in comparison to omnivore birds (Leonzio et al. 2009; Gong et al. 2012; Abbasi et al. 2015b; Ackerman et al. 2019). Besides that, we observed that a particular insectivore species presented concentration of Zn significantly higher than omnivores birds. However, food intake is not the primary source of Zn contamination in birds (Philpot et al. 2019), because the transference of this metal through the food chain is modified by homeostatic mechanisms to maintain adequate physiological levels (Gong et al. 2012). The concentration of Zn found in this guild (465.61 ± 221.38 μg/g) is higher than in insectivore passerines in Pakistan (41.58 to 51.04 μg/g) (Abbasi et al. 2015b) and in other insectivore birds from the same country (75.25 μg/g) (Abbasi et al. 2015a).

The concentration of Cu in the insectivore guild was not high (9.20 ± 5.99 μg/g), but it was higher than values reported in insectivore passerines (1.04 to 1.58 μg/g) and in other insectivore birds from Pakistan (2.88 μg/g) (Abbasi et al. 2015a; b). The detection of high concentrations of Cu is related to exogenous contamination (Leonzio et al. 2009). A recent study with samples from the food chain and levels of metals in feathers, muscle and blood, indicates that there is no trophic transference of Cu between terrestrial species of birds (Tasneem et al. 2020).

Due to the nature of the metals analyzed, it is not possible to affirm that the significance of the results is related to contamination through the diet. Environment characteristics (Fritsch et al. 2012) and the interaction of birds with it might influence the food items that will be consumed and consequently influence the availability of metals (Abbasi et al. 2015a; Berglund 2018), because species have distinct foraging characteristics and behavior, and can explore different areas when searching for food (Fritsch et al. 2012; Berglund 2018).

Omnivore birds are able to change their diet when exposed to adverse environmental conditions (Willis 1979). In forests, omnivores can forage near its edges, being favored by plant heterogeneity found in these areas (Bispo e Scherer-Neto 2010). Besides that, due to feeding plasticity, omnivore populations tend to stabilize or to grow in fragmented environments (Anjos et al. 2004). Therefore, animals belonging to this guild are more able to adapt to different environments and explore a wide range of food resources in different strata, what exposes them to many contaminants.

In this regard, studies relating the presence of MN and/or NA with the diet of birds are scarce. Souto et al. (2018) tested the hypothesis that insectivore birds would present more MN in coffee farms, because there is a great number of insects in these places. However, the omnivore guild presented the highest MN frequency, in opposition to their hypothesis and corroborating our results. Similarly, Oliveira (2020) observed that in two different environment (conserved and agricultural) omnivore species presented the highest frequencies of MN, and no significant NA differences were found.

The herbivore/granivore guild did not show significant influence on the MN number, but when associated with the BCI this guild contributed negatively to the increase in NA in some GLM. Plants have lower concentrations of metals when compared with insects (Gong et al. 2012), therefore herbivores/granivores might be less susceptible to contamination through the food chain. The sample size of this guild, however, was not representative in this study.

4.5 Influence of body condition

Our results reveled a negative association between Zn and Cu with the increase of the BCI, even though it had a small relevance. The negative association of metals like selenium (Se) (López-Perea et al. 2019) and mercury (Hg) (Ackerman et al. 2019) in blood, and cadmium (Ca) and barium (Ba) in feathers (Innangi et al. 2019) with the body condition was also reported in the literature.

Ackerman et al. (2019) suggest two hypotheses to explain these observations. The first one considers that concentrations of metals might be reduced because they are diluted as the body mass increases. The second one considers the opposite relationship, observing that the highest concentrations of metals are found in birds with lower body condition. The last hypothesis corroborates the affirmation of Lodenius and Solonen (2013), that malnutrition might be responsible for physiological stress and worse body condition, increasing the concentration of metals at the expense of body condition. These discussions support our results, as we observed insectivore birds (with the lowest BCI) presenting the highest Zn and Cu concentrations.

Regarding genotoxicity, BCI and trophic guilds contributed to the increase in the number of NA, according to the GLM and the ANOVA test on models' residuals. Our results indicate that omnivore birds (with higher BCI) have more NA. Frixione and Rodríguez-Estrella (2020), studying a falcon species in an area of agricultural production, did not find significant relationship between MN and NA frequencies with the body condition index, but smaller birds presented higher frequencies of NA (Frixione and Rodríguez-Estrella 2020). Souto et al. (2018) showed that the size of a bird influenced the number of MN, explaining 95% of MN frequency.

The variation of body condition might be related to the reproductive period, in which birds expend more energy (Kitaysky et al. 1999), but it is directly related to the diet (Brown and Sherry 2006). Birds with specialized feeding habits (insectivores) might be affected by fluctuations in resource availability, so that the generalist diet is favored, because it has greater flexibility (Teles et al. 2017).

4.6 Age classes influence

The undetermined age class presented higher concentration of Zn than the adults, however, a few individuals were present in this age class. In this study, concentrations of Cu were higher in juveniles when compared with adults (9.18 ± 4.26 and 6.31 ± 3.13 μg/g respectively), in opposition to information available in the literature.

Copper (Cu) is an essential metal, which is required in low concentrations in birds (Grúz et al. 2018). Besides that, it has affinity with keratin, a protein involved in feathers development and growing (Baker et al. 2017). The accumulation of metals in feathers of juvenile birds might occur during growth, when the nestling receives food from its parents, which might be different from the adult diet (Fritsch et al. 2012; Grúz et al. 2018). Berglund et al. (2011) report that nestlings of a passerine species presented higher levels of essential elements (cadmium, nickel and zinc) in their liver when compared to adult females, in a polluted environment. Fritsch et al. (2012) found higher concentrations of metals in juveniles than in adults of a passerine species in a gradient of polluted areas. Among the explanations, the authors highlight the hypothesis of the forage behavior, in which younger and less experienced individuals would not consume the same food items consumed by the adults (Fritsch et al. 2012).

The age classes influenced the number of NA in the second best GLM and the significance of the ANOVA test based on model's residuals. Birds in the undetermined age class and adult birds presented lower numbers of NA when compared to juveniles. Santos et al. (2017) reported that juveniles of white stork (*Ciconia ciconia*) in the process of rehabilitation presented higher frequencies of NA and MN than adults. In a falcon species the MN frequency in juveniles was higher than in adults (Tsarpali et al. 2020). Zúñiga-González et al. (2000) comment that this result is might be attributable to the reticuloendothelial system (involved with the removal of old erythrocytes from the blood), which only matures in older birds, so juveniles would not be able to eliminate damaged and old erythrocytes so efficiently as the adults.

5. Conclusion

We defined the field sampling based on previous studies in which many species of birds were captured and during the analysis of biomarkers, some of them revealed potential to be used as bioindicators of environmental quality (Abbasi et al. 2015a; Baesse et al. 2015; Baesse et al. 2019; Quero et al. 2016; Souto et al. 2018). However, we observed that in our study area, this kind of "random" capture was not effective.

This is partially due to the fact that only a few species had similar sampling sizes in all sampling sites, what restricted comparisons between individuals of the same species from different sites, making it difficult to evaluate their bioindicator potential. Our samples were predominantly composed of a few individuals from many different species with different interspecific biological and ecological characteristics (behavior, diet, availability of food resources, preferences for distinct foraging areas) and also potential physiological differences (absorption, retention and excretion of contaminants) (Burger and Gochfeld 2000; Costa et al., 2011; Quero et al. 2016). Therefore, they might respond in varied ways to exposure to contaminants (Eens et al. 1999; Manjula et al. 2015).

The mainly results in our study were mainly related to the diet. Therefore, in studies using birds as bioindicators, we recommend that researchers focus on one or a few species which have well-known specific characteristics, like the forage area and feeding preferences (Tsarpali et al. 2020; Frixione e Rodríguez-Estrella, 2020).

This study was a pioneer in using wild birds in the SRHB region and, despite the limitations described, we concluded that trophic guilds, age classes and BCI significantly influenced the accumulation of trace metals and the frequency of MN and NA. It was not possible, however, to determine the exact interaction between the biomarkers used to understand the response of the birds to the environmental pollution in the study area.

Declarations

Ethical approval

All procedures performed in this study were approved by the Ethics Committee on the Use of Animals of the Feevale University (Comitê de Ética no Uso de Animais, CEUA-FEEVALE), project 02.19.075, and by Brazilian environmental agencies (SISBIO authorization 70856-1 and CEMAVE autorization 159/2019).

Consent to participate "Not applicable"

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Availability of data and materials “Not applicable”

Competing interests

The authors declare that they have no conflict of interest

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Author`s contributions

JT was responsible for conceptualizing the project, data analysis, acquisition of financial support, methodology (capture of birds, data and biological samples collection, slide analysis and processing of feathers) and for writing the manuscript. GZPR helped with the methodology (MN and NA analysis, and feather processing), data analysis and manuscript writing. DF helped with the methodology (capture of birds), data analysis and manuscript writing. MSdeS helped with the methodology (capture of birds and collection of biological samples) and revised the language and grammar of the manuscript. JP helped with the methodology (capture of birds and collection of biological samples). JHB did part of data analysis and helped with the methodology (capture of birds and collection of biological samples). JMK helped with the methodology (processing of feathers). MRL helped with the methodology (collection of biological samples). AS helped with the methodology (analysis and detection of metals in the samples). RL helped with the methodology (analysis and detection of metals in the samples). GG helped with the conceptualization of the project, was responsible for the supervision and validation of the project, acquisition of financial support and helped with manuscript writing.

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Figures

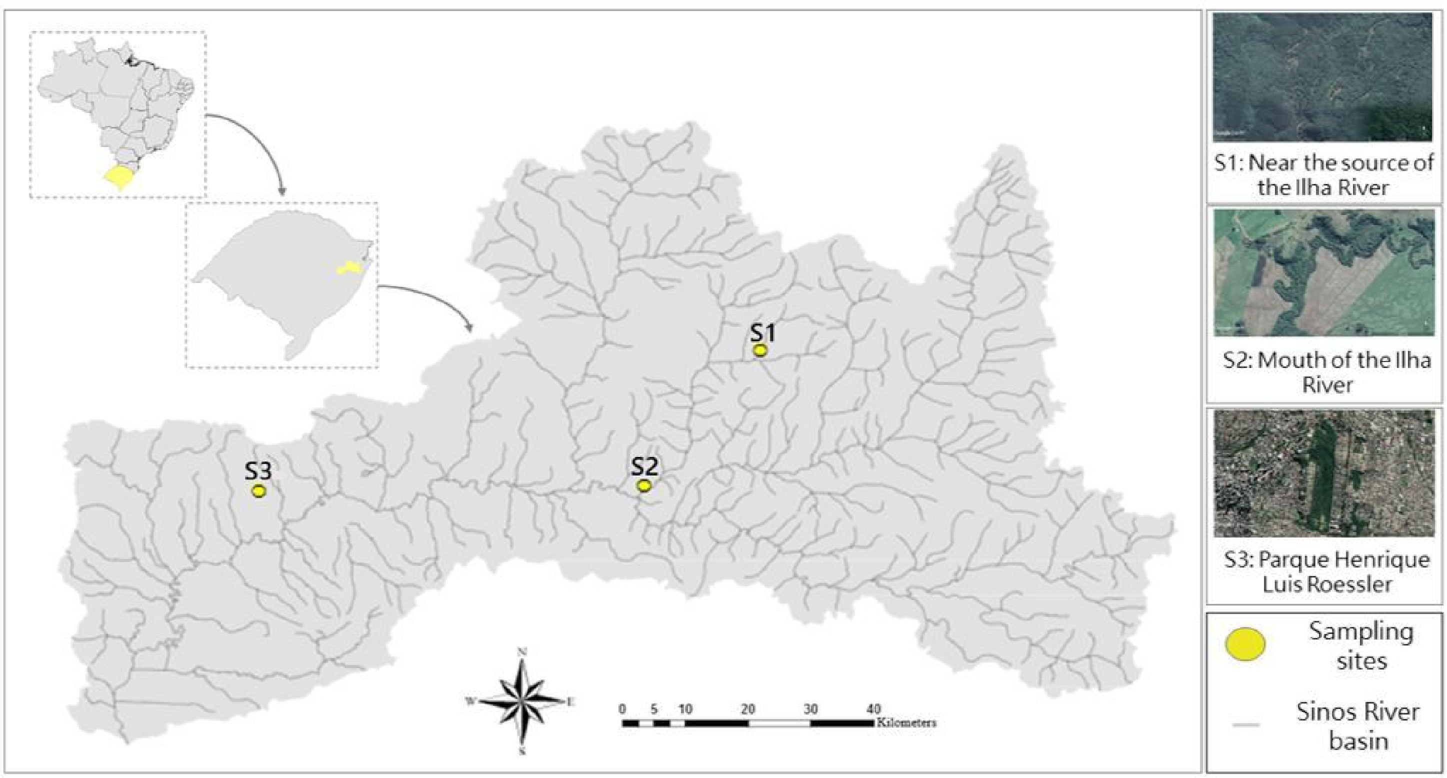

Figure 1

Map with the location of the sampling points in the SRHB, Rio Grande do Sul State, Brazil. Sampling sites 1 (S1 - natural zone) and 2 (S2 - rural zone) are located in Taquara city, while sampling site 3 (S3 – urban zone) is located in Novo Hamburgo city. Note: The designations employed and the presentation of the material on this map do not imply the expression of any opinion whatsoever on the part of Research Square concerning the legal status of any country, territory, city or area or of its authorities, or concerning the delimitation of its frontiers or boundaries. This map has been provided by the authors.

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