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A multibiomarker approach in the caged neotropical fish to assess the environment health in a river of central Brazilian Cerrado

Douglas dos Santos Silva[a], Bruno Gonçalves[a], Cândido Carvalho Rodrigues[a], Felipe Cirqueira Dias[a], Nicholas Silvestre de Souza Trigueiro[a], Izabella Soares Moreira[a], Daniela de Melo e Silva[c], Simone Maria Teixeira Sabóia-Morais[b], Tânia Gomes[d], Thiago Lopes Rocha[a*]

[a] Laboratory of Environmental Biotechnology and Ecotoxicology, Institute of Tropical Pathology and Public Health, Federal University of Goiás, Goiania, Goiás, Brazil.

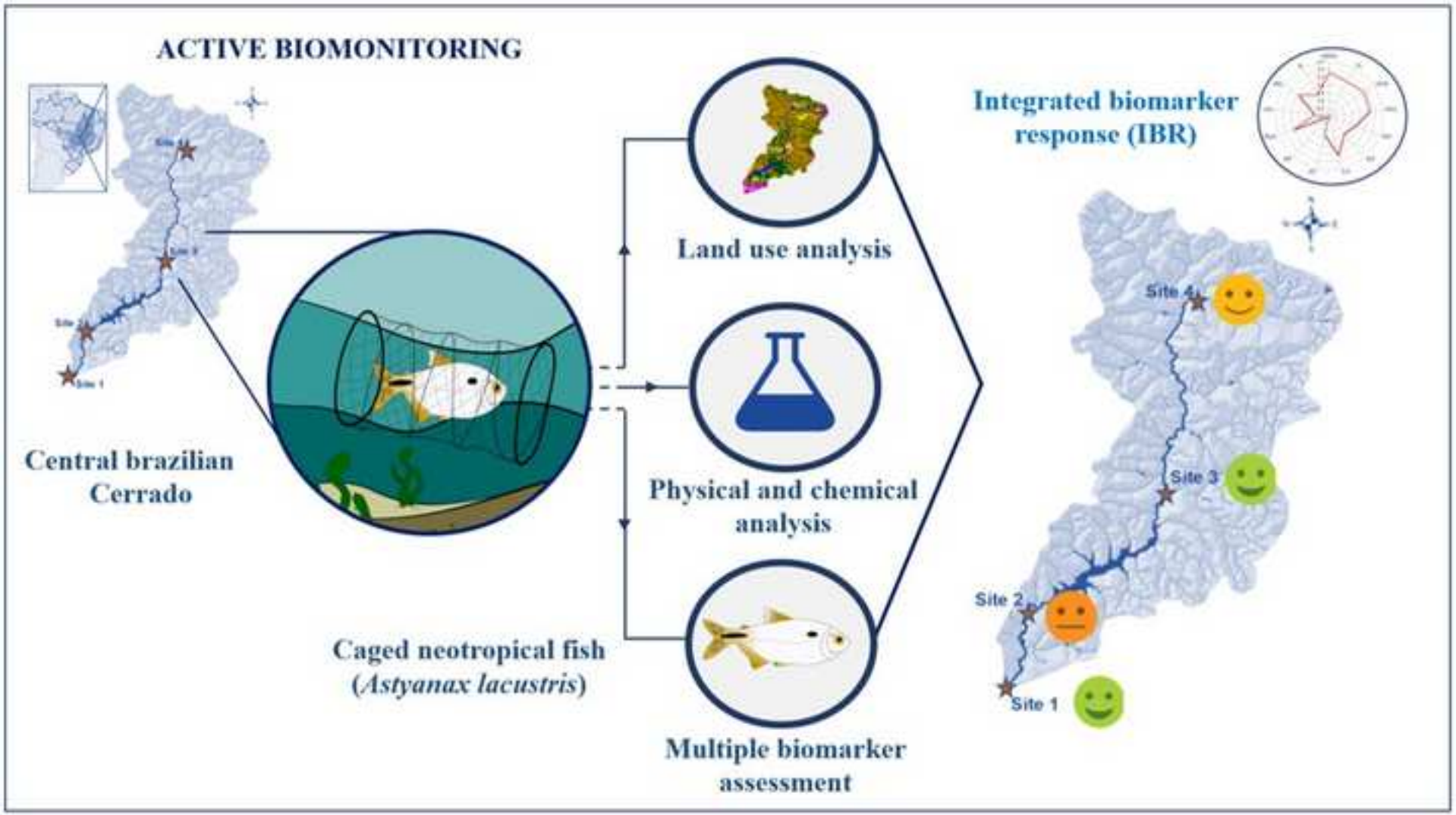
[b] Laboratory of Cellular Behaviour, Department of Morphology, Biological Sciences Institute, Federal University of Goiás, Goiânia, Goiás, Brazil.

[c] Laboratory of Mutagenesis, Biological Sciences Institute, Federal University of Goiás, Goiânia, Goiás, Brazil.

[d] Norwegian Institute for Water Research (NIVA), Section of Ecotoxicology and Risk Assessment, Gaustadalléen 21, N-0349, Oslo, Norway.

[*]Corresponding author: T.L. Rocha, Universidade Federal de Goiás, Instituto de Patologia Tropical e Saúde Pública, Rua 235, Setor Universitário, Goiânia, Goiás, Brasil. CEP: 74605050, Goiânia, Goiás, Brasil. E-mail address: thiagorochabio20@ufg.br

*Graphical Abstract



Highlights

- Multibiomarker approach in neotropical fish to assess the environmental health.
- Somatic, genotoxicity, mutagenicity and tissue-level biomarker in *A. lacustris*.
- DNA damage and leukocyte infiltration in hepatic tissues were sensitive biomarkers.
- *A. lacustris* as suitable biomonitor in active biomonitoring.

Abstract

Water safety is a world-wide concern and several efforts have been made in order to ensure the conservation of aquatic ecosystems. Water quality monitoring must be performed with an integrated approach using biomonitor organisms allied to water parameters. Nonetheless, very few studies have focused on biomarker responses in neotropical fish, especially in the freshwater ecosystem of Brazilian Cerrado savanna. In present study, the active biomonitoring of the João Leite river (central Brazilian Cerrado river) was performed through the evaluation of biomarker responses in caged *Astyanax lacustris* in combination with land use classification and analysis of water parameters. Caged fish were exposed for seven days at four sites along the river and two control groups were kept in a tank under controlled conditions. Results showed that pasture was the predominant land use in the João Leite river basin (54.07 %), followed by natural vegetation (34.92 %) and other kind of land use (11.01 %). Water analyses showed metal concentrations (Mn and Fe) above the maximum allowed by Brazilian regulation, with particularly higher concentrations at Site 2 (near to pasture area). Biomarker responses did not show significant differences for somatic and mutagenic biomarkers between sites. However, the comet assay showed high DNA damage at Sites 2 and 3, indicating genotoxic effects in caged fish at pasture areas. Histopathological analysis showed highest frequency of leukocyte infiltration in liver of fish from Site 2, confirming the ecotoxic effects on *A. lacustris* in streams impacted by grazing activities. DNA damage and leukocyte infiltration in fish hepatic tissues were sensitive biomarkers in the neotropical fish *A. lacustris* to assess the environment health of the Cerrado river. These results showed the importance of using a multibiomarker approach in environmental risk assessment, especially in areas more at risk from anthropogenic pollution.

Keywords**:** ecotoxicology; mutagenicity; genotoxicity; environmental health; biomarker; biomonitoring.

1. Introduction

Water safety is a world-wide concern, due not only to population growth but also to anthropogenic pollution of water sources, which has led to an increasing demand for freshwater resources. In this sense, water quality monitoring networks are invaluable for the assessment, restoration, and protection of water bodies (Ouyang, 2005; Da Silva et al., 2014; Vieira et al., 2017). Although water physicochemical parameters in combination with pollutant concentrations are relevant indicators of environmental quality, the information they provide is not sufficient to describe the bioavailability, ecotoxicity and biological effects induced by pollutants on aquatic organisms (Van der Oost et al., 1996). In this context, water quality monitoring must integrate a wide range of physicochemical and biological parameters (Kannel et al., 2007) using integrated and multidisciplinary approaches (Bebianno et al., 2015). Thus, aquatic environment biomonitoring can be performed through measurable responses on biomonitor organisms, analyzing changes in their normal biological functions. These responses are known as biomarkers, which work as warning indicators of pollutants effects (Nikinmaa, 2014), providing information on alterations on organism health (Moreira et al., 2004).

Besse et al. (2012) proposed two types of biomonitoring methods to address the health condition of biomonitors: passive and active biomonitoring. In passive biomonitoring, biomarker responses on indigenous biomonitor organisms in their natural habitat are normally studied, while in active biomonitoring, standardized

biological material is exposed under pre-established conditions. When comparing both approaches, active biomonitoring can provide some advantages to passive biomonitoring: *i*) it allows to carry out surveys where native fish are not present or found in small numbers in the area of interest; *ii*) it allows for the standardization of phenotypic characteristics of used organisms (i.e., age, sex, size, maturation and linage); *iii*) and it allows for the precise control of variables during exposure (i.e., time of exposure and location of the exposure experiment). On the other hand, this approach can impair the biomonitoring accuracy, as it excludes adaptive factors commonly found on indigenous biomonitor organisms in their natural habitat (Wepener, 2013). Accordingly, the use of caged fish has been widely used on biomonitoring programs to evaluate contaminant effects on aquatic biota (De la Torre et al., 2002; Klobucar et al., 2010; Vieira et al., 2014, 2016, 2017).

To date, very few studies have used multiple biomarker responses on neotropical fish species as tools to assess water quality and environmental health. Among the most commonly used neotropical biomonitors, the genus *Astyanax* comprises of more than 107 recognized species of freshwater fish (Eschmeyer, 2007). The tetra fish *A. lacustris* (Lütken, 1875), now including the formerly known *A. altiparanae, A. asuncionensis* and *A. jacuhiensis* (Lucena and Soares, 2016), is a native and abundant species along the Brazilian Cerrado rivers, such as João Leite river (Santana et al., 2007), which has been used in ecotoxicological studies both *in situ* (Vieira et al., 2014; Pereira et al., 2017; Vieira et al., 2017) and in laboratory studies (Martinez & Souza, 2002; Pereira et al., 2014; Bettim et al., 2016; Kida et al., 2016; Galvan et al., 2016; Lopes et al., 2017) due to its biomarker responses sensitivity to a range of pollutants. Accordingly, the aim of this study was to evaluate the water quality of a Cerrado river (João Leite river) in the Center-West Brazil by using a multibiomarker approach in caged *A. lacustris*. This

approach included the assessment of fish biometric parameters, mutagenicity, genotoxicity and hepatic tissue-level biomarkers, in combination with physico-chemical parameters of water and land use analysis. Thus, the hypothesis that the water quality of Brazilian savanna rivers can be evaluated by multiple biomarker response in caged *A. lacustris* was tested. This is the first study using an integrated multibiomarker approach in caged neotropical fish in a Brazilian Cerrado river.

2. Material and Methods

2.1. Study area

The exposure and water sampling were carried out at four sites along the João Leite river (Fig. 1). The sampling sites were chosen to represent different types of land use along of the João Leite river. Site 1 (S1) (16°38'32.91"S; 49°15'1.97"W) is located inside the water catchment station of the Sanitation Company of Goiás, within the urban area of the city of Goiânia (SANEAGO). Site 2 (S2) (16°34'30.54"S; 49°13'55.02"W) is located in a farm area in the city of Goiânia, about 4 km downstream from the João Leite reservoir dam and the Altamiro de Moura Pacheco Ecological Park. Site 3 (S3) (16°28'25.05"S; 49°6'43.87"W) is located in the rural zone of the city of Terezópolis de Goiás, and it is surrounded by crop farms and cattle ranches. Site 4 (S4) (16°18'16.98"S; 49°5'43.98"O) is located in a strait river section inside the SANEAGO water catchment station, within the rural zone of the municipality of Campo Limpo de Goiás. Crop farms and cattle ranches surround this area. Photographic records of each sample site are also available in the supplementary material (Fig. S1).

2.2. Land use analysis

The analysis on drainage area, land cover and land use in the João Leite river basin was performed using the software ArcGis 10.1. Land cover (natural vegetation and water bodies), land use (pasture, urban area, annual agriculture, perennial agriculture, forestry, mining, mosaic of occupation, exposed ground) and not observed (surfaces where observation wasn't possible) were classified according to TerraClass Cerrado Project 2013 (BRASIL, 2015). In order to evaluate possible impacts of different land uses on water quality, we have defined two methods for delimiting areas able to affect water quality at each sample site. The first took into account a local disturbance within a circular buffer of 1 km radius which was defined around each sample site, while the second method is a whole catchment approach which consider the entire drainage area to each sample site based on the João Leite river basin landform and its tributaries streams (Silva & Williams, 2001; Viana et al. 2018; Mwaijengo et al., 2020). As sample sites are located along the same river, the drainage areas are cumulative from S4 to S1. The values for land cover and use at each sample site are corresponding to its surface area (ha) and its percentage (%) related to drainage area and 1 km buffer zone area referring to each sample site. These values were measured by TerraClass shapefile analysis using ArcGis. This data was used to account all kind of land use present across the basin and its association to biomarkers response in the caged fish.

2.3. Experimental design

Specimens of *A. lacustris* were collected from the pisciculture at the SANEAGO (16°38'11.00"S 49°15'37.40"O) in the city of Goiânia – Goiás – Brazil, transported to the experimental fish farm at Federal University of Goiás and acclimated for 40 days in a 1000 L tank in a constant flow-through system with water under constant temperature

125 (21 ± 2.0) and pH (7.0 ± 0.3). Fish were fed daily with commercial fish food (Cardume®
126 42 % protein, VB Alimentos, Brazil). The animal handling and experimental procedures
127 were approved by Human and Animal Research Ethics Committee of the UFG (protocol
128 no. 094/17).

129 Fish (total weight: 8.33 ± 2.92 g; standard length: 66.95 ± 6.88 mm) were
130 separated into three groups, 2 control groups and 1 exposed group. The first group,
131 called initial control group (n=10 fish), comprised of individuals taken for analysis to
132 reflect the condition of fish at the beginning of the caging exposure (T0). The second
133 group was named exposure group (n=120 fish) and included the fish used for the caging
134 experiment within the João Leite river. This group of fish was transported to the four
135 exposure sites in plastic bags with aeration and placed in the João Leite river inside
136 cylindrical cages (nylon mesh 0.5 mm and steel structure). Cages were submerged (60 ±
137 10 cm) at every site for 7 days (14^{th} to 21^{st} of September 2018 – dry season), with two
138 cages *per* sample site, each containing 15 fish. During the exposure period, the fish
139 were kept in the water column without contact with the underlaying sediment
140 compartment. The third group (final control group) included the fish (n=30 fish) kept in
141 the acclimatization tank in two replicate cages (similar as those used for the exposure
142 group) during the exposure period (7 days) to measure any possible stress responses
143 resulting from the caging process.

144 At the beginning of the exposure (initial control group) and after 7 days of
145 exposure (exposed group and final control group), cages were retrieved and fish from
146 each site transported to laboratory, sensitized with hypothermia and euthanized by
147 decapitation. For all groups, fish were kept within the plastic bags with aeration (1
148 fish/L) for a total of 90 min in order to standardize the time spent in the bags during the
149 transport and avoid artifacts in biomarker responses. For each fish, 20 μL of blood from

150 the tail artery was immediately collected and diluted in PBS buffer solution (500 µL, pH
151 7.2), while the liver was dissected and immediately fixed by immersion in Karnovsky's
152 solution (4 % paraformaldehyde and 2.5 % glutaraldehyde in 0.1 M PBS buffer at pH
153 7.2).
154
155 **2.4. Water analysis**
156 Several environmental parameters were measured in the field (i.e., environment
157 temperature, water temperature, pH and conductivity) using a multiparametric probe,
158 while others were determined in the laboratory at the begin and end of exposure. Thus,
159 water samples (5 L) were collected from all sampling sites, and sent to the Water
160 Laboratory of the SANEAGO for analysis of physical and chemical parameters
161 (turbidity, true color, total alkalinity, total hardness, organic matter, chlorides, total iron,
162 total dissolved solids, total phosphorus, nitrate, nitrite, total ammoniacal nitrogen,
163 manganese, dissolved oxygen, BOD 5 days – 20 °C), total coliform index and
164 *Escherichia coli* index. Metal levels (Ag, Al, As, Ba, Co, Cr, Cu, Fe, Mn, Ni, P, Pb, Sb,
165 Se, U, V and Zn) and organic pollutants concentrations in the water were also
166 determined using optical emission spectrophotometer with inductively coupled plasma
167 (ICP-OES) (VARIAN® 710-ES 230.S/N: IP0801M049), gas chromatograph with mass
168 detector (GC/MS) with Combi-pal injector (VARIAN® - 3900;S/N: 102288), mass
169 detector (VARIAN® - 2100T;S/N: 06362) and automatic sampler (CTC Analitics® -
170 Combi-pal; S/N: 128422). Water sampling was performed according to the procedures
171 established by Brazilian regulation (IT07.0101, IT07.0743 and IT07.0613 – Brazil,
172 2005). Water samples were analyzed using the methods set by the Standard Methods for
173 the Examination of Water and Wastewater (SMEWW) and the United States
174 Environmental Protection Agency (USEPA). Parameter values were analyzed according

175 to the Brazilian environmental regulation (Resolution 357/2005 of the National Council
176 for Environment - CONAMA).
177
178 **2.5. Biomarker analysis**
179 **2.5.1. Somatic indexes**
180 The total length (cm), standard length (cm), total weight (g) and liver (g) and
181 gonad (g) weights were determined in all sampled fish and the following somatic
182 biomarkers obtained: Condition Factor (Fulton's K) = Total weight/(Standard Length)^3
183 (Vazzoler, 1982); Hepatosomatic Index (HSI) = (liver weight/body weight) *x* 100;
184 Gonadosomatic Index (GSI) = (gonad weight/body weight) *x* 100 (Lamas and Godinho,
185 1996).
186
187 **2.5.2. Genotoxicity**
188 The alkaline comet assay was performed according to Singh et al. (1988) with
189 modifications. Peripheral blood samples of *A. lacustris* (n = 10 fish per group) were
190 obtained from the tail artery (20 µL) and diluted in 500 µL of 0.1 M PBS buffer solution
191 at pH 7.2. This solution (15 µL) was mixed with 120 µL of 0.5 % low melting point
192 agarose (LMPA) at 37 °C, and spread onto two replicate microscope slides, pre-coated
193 with 1.5 % normal melting point agarose. Coverslips were added to the slides and after
194 LMPA solidification, the coverslips were removed and the slides were immersed in cold
195 lysing solution (1 % Triton X-100, 10 % DMSO, 2.5 M NaCl, 100 mM EDTA, 10 mM
196 Tris, 1 % N-lauroylsarcosine sodium salt, pH 10) for 12 h at 4 ºC, in the dark. Slides
197 were placed in an electrophoresis buffer (300 mM NaOH, 1 mM EDTA, pH > 13) for
198 30 min, and the electrophoretic run was conducted for 30 min at 25 V and 250 mA.
199 After electrophoresis, neutralization was performed with 0.4 M Tris buffer solution (pH

7.5) for three times (5 min each), fixed in 99.8 % ethanol for 10 min and dried at room temperature overnight. DNA was stained with SYBR® Green (S9430 Sigma-Aldrich) and examined under an Axio Imager fluorescence microscope (Carl Zeiss, Jena, Germany). A total of 100 nucleoids were randomly analyzed for each fish (n = 1000 *per* experimental condition) using the Comet Imager® (metasystem, version 2.2, GmbH) analysis system. Three parameters related to DNA damage were scored: tail length (TL), DNA percentage in the tail (% DNA), and Olive tail moment (OTM). Furthermore, the score of % DNA in the tail was ranked according to Almeida et al. (2011): zero or minimal 10 % DNA, low damage >10 – 25 %, mild damage >25 – 50 %, high damage >50 – 75 %, and extreme damage > 75 %.

2.5.3. Mutagenicity

The mutagenicity was analyzed by the Micronucleus test (MN) and Erythrocyte Nuclear Abnormalities (ENA) according to Carrasco et al. (1990) and Fenech et al. (2003), with modifications. Peripheral blood was obtained from the tail artery of fish (n = 10 fish per group), diluted in 500 μL of 0.1 M PBS buffer at pH 7.2, extended in microscopic slides and stained with hematological stain (Panótico Rápido – Newprov®). The ENA was measured by determining the frequency of cells with nuclear abnormalities, such as binucleated nucleus (BN), kidney-shaped nucleus (KN), lobed nucleus (LN), micronucleus (MN) and segmented nucleus (SN), as previously described by Carrasco et al. (1990), Fenech et al. (2003) and Vignardi et al. (2015). The total ENA frequency was determined by the sum of all nuclear abnormalities: BN + KN + LN + MN + SN.

2.5.4. Histopathological analysis

225 After fixation, livers of *A. lacustris* from all groups (n = 10 *per* group) were
226 dehydrated in ethanol (70 to 95 %) and embedded in glycol-methacrylate resin (Leica
227 Historesin, Germany), according to the manufacturer's instructions. Liver sections with
228 3 µm thickness were cut using a Leica Ultramicrotome (Leica UTC Ultracut) and 30
229 sections were used per specimen (n = 10 sections *per* slide, 3 slides *per* fish, 10 fish *per*
230 group). Slides were stained with 1 % Toluidine blue at pH 8.5. Histopathological
231 assessments were performed using a light microscope (Leica DMLB) associated with a
232 Moticam 2300 camera and digital images were recorded using the Motic Image PLUS
233 2.0® software.
234
235 **2.6. Integrated biomarker response (IBR)**
236 The biomarkers determined in *A. lacustris* were combined into a stress index
237 termed "integrated biomarker response" (IBR) described by Beliaeff and Burgeot
238 (2002) and modified by Guerlet et al. (2010). This index was calculated for each group
239 as follows: individual areas A_i connecting the *ith* and the $(i + 1)th$ radius coordinates of
240 the star plot were obtained in a simpler way, according to the formula:

$$A_i = \frac{1}{2} \sin\left(\frac{2\pi}{n}\right) S_i S_{i+1}$$

241 where Si and Si+1 represent the individual biomarker scores (calculated from
242 standardized data) and their successive star plot radius coordinates and n represent the
243 number of radii corresponding to the biomarkers used in the survey. The considerations
244 described by Cravo et al. (2013) regarding the selection of biomarkers in terms of type
245 of pollution response and biological hierarchy were taken into consideration, as to avoid
246 over emphasizing the final index value. In accordance, biomarkers used for the IBR
247 calculation were ranged clockwise according to their hierarchy of biological
248 organization, from subcellular to individual level.

249

250 **2.7. Statistical analysis**

251 The one-way ANOVA and/or the non-parametric Kruskall-Wallis test were used
252 to compare the results from the different sites and control groups, accordingly the
253 distribution of data and homogeneity of variances (Shapiro-Wilk and Levene's tests).
254 Multiple comparisons were performed using the Tukey test with Bonferroni corrections.
255 Correlation matrixes were used to evaluate the relationship between biomarker
256 responses, pollutant concentrations and land use. Water parameters are expressed as the
257 mean values recorded from samples collected on Day 1 and Day 7. Statistical analyses
258 were performed using the software Statistica® 7.0 (Statsoft Inc., 2005, Tulsa, OK, USA)
259 and R Studio®. Statistical significance was set at $p < 0.05$.

260

261 **3. Results and discussion**

262 **3.1. Land use**

263 The João Leite river was analyzed in terms of drainage (Fig. 1A) and land use
264 (Fig. 1B; Table 1). The pasture is the predominant type of land use in this area (54 %),
265 followed by natural vegetation (35 %), that in total cover about 89 % of the João Leite
266 river basin area. On the other hand, agriculture only corresponds to 2 % of the total area
267 (mainly corn and soybeans), while the remaining types of occupation represent 11 % of
268 the total basin area (Table 1). The altitude quotas across the João Leite river basin area
269 ranged from 733 m to 1022 m above sea level, being that S4 is located in the upper João
270 Leite river basin (922 m above sea level) and S1 in the lower part of the basin (690 m
271 above sea level).

272 The drainage area in S1 corresponds to the total area of the João Leite river
273 basin, as it potentially receives water from all parts of the basin. Accordingly, the land

use values in the drainage area for this site are the same as those showed for the total basin area. However, when the additional 1 km radius circular buffer was considered, S1 had different land use values when compared to the drainage analysis, being the highest area covered by urban areas (58.29 %), followed by natural vegetation (39.53 %) and water bodies (2.18 %). S1 is located in the urban area of the city of Goiania, which is a very populous metropolis with more than 1.000.000 inhabitants. When considering the 1 km radius circular buffer, S1 was not affected by pasture, mining or agriculture activities.

In S2, large areas covered by natural vegetation were found within the area delimited by the 1 km radius (64.80 % of the area), in addition to pasture areas (26.86 %) and small urban areas (8.25 %). In contrast, agriculture, forestry and mining activities were not found within the 1 km radius circular buffer. When taking into account the whole drainage area within S2, pasture represents the largest area covered (55.88 %), followed by natural vegetation (35.27 %), forestry activity (2.17 %), urban areas (2.10 %), water bodies (1.95 %), annual agriculture (1.52 %), perennial agriculture (0.62 %), mosaic of occupation (0.21 %), exposed ground (0.15 %), as well as mining (0.02 %). In accordance, S2 has the highest areas covered by natural vegetation between all sample sites, for both drainage area and circular buffer radius of 1 km.

S3 is the sampling site most affected by pasture areas, both in the case of drainage area (59.97 %) and 1 km radius circular buffer (57.80 %) analyses. Natural vegetation was identified as the second largest area both in the drainage area (31.12 %) and the 1 km radius circular buffer (33.14 %) analyzed. Similar to S1 and S2, the additional analysis of the 1 km radius circular buffer did not identify the potential effect of agriculture activities in this site, even though the drainage area analysis showed the presence of agriculture areas (1.88 % annual agriculture and 0.78 % perennial

299 agriculture). The presence of these agriculture areas has a potential to affect water
300 quality at S3, given that this site is the closest to agriculture areas. Furthermore, the 1
301 km radius buffer analysis showed the presence of the smallest urban area (0.27 %) at
302 this site when compared to the remaining sites (Table 1).

303 For S4, pasture was the predominant type of land use (49.07 % drainage and
304 57.13 % buffer), followed by natural vegetation (32.44 % - drainage and 24.11 % -
305 buffer), urban areas (7.48 % - drainage and 11.39 % - buffer), forestry activity (3.97 % -
306 drainage and 4.69 % - buffer) and water bodies (0.37 % - drainage and 2. 69 % - buffer).
307 Agriculture activities areas were found only by the drainage area analysis (3.89 %
308 annual agriculture and 1.53 % perennial agriculture), while mining activities were not
309 found by either analysis (Table 1).

310 The 1 km radius circular buffer analysis was not able to identify effects from
311 agricultural and mining activities, suggesting that the residues from these activities are
312 not responsible for the deterioration of water quality at any of the sampling locations. In
313 the other hand, the drainage area analysis was successful in showing changes in land use
314 within the João Leite river basin, which can affect water quality at the sampling sites.
315 The João Leite river has faced intense land use changes within its hydrographic basin
316 area showing several problems related to environmental degradation by anthropogenic
317 activities. This increased anthropogenic pressure can lead to the modification of the
318 chemical composition of sediments, and consequently compromise the quality of the
319 surrounding water and organisms living therein (Rios et al., 2013).

320

321 **3.2. Water analysis**

322 The physicochemical parameters of collected water samples were similar
323 between the sampling sites, except for total K and Mn, which were highest at S2 (see

supplementary material Table S1). From the analyzed metals, only Mn and Fe showed values above the limit established by Brazilian environmental regulation (Resolution 357/2005 of the National Council for Environment - CONAMA) (Table S2). Of the selected organic compounds (insecticides, herbicides, pesticides, fungicides, solvents and others), the majority were either not detected or detected in very low levels in all sampling sites (Table S3).

S2 showed the highest metal concentrations in water, even though this site is surrounded by the largest natural vegetation areas compared to other sites. The levels of dissolved Fe (1.37 – 1.66) and total Mn (0.28 - 0.33) detected at this site were three times higher than the maximum allowed by Brazilian law (Resolution CONAMA 357/2005). When it comes to Mn levels in water bodies, concentrations higher than 0.05 mg L^{-1} can cause negative effects in organisms (USEPA 2009), such as change in ion- and osmoregulation, and lead to metabolic disorders (Partridge and Lymbery 2009), lipid peroxidation, and oxidative stress in fish (Vieira et al. 2012). Recently, several metals, as Al, Fe, Mn and Hg, were detected in the water samples at Meia Ponte River (Goiânia, GO) above the recommended by Brazilian law, indicating embryotoxic effects on zebrafish (*Danio rerio*) (Ribeiro et al., 2020). Similar results were obtained in the present study for the caged neotropical fish *A. lacustris*.

3.3. Multibiomarker responses

3.3.1. Somatic biomarkers

After the 7 days exposure period, the condition factor (Fulton's K) decreased for all caged fish, as well as the final control group when compared to initial control group ($F(5, 50)=10.503$; p-value < 0.0001) (Fig. 2A). However, no significant differences were found between the exposed groups and the final control group, except for the

Fulton's K -values with differences only between S3 and S4 (p-value = 0.03). Similarly, no significant differences were detected for HSI (H(5, N= 56)=5.722807; p-value = 0.3341), as well as for GSI (H(5, N= 56) =9,406266; p-value = 0.0939) between the exposed and control groups (Fig. 2B-C).

The Fulton's K is a quantitative measure that makes possible to evaluate the health of fish and provides a relation between fish body condition and/or physiological state and the surrounding environment (Angelescu et al., 1958). Thus, the Fulton's K can be used as an indicator of environmental disturbances associated to aquatic pollution (Barrilli et al., 2015). Lizama and Ambrosio (2002) found lowest Fulton's K-values in *A. altiparanae* from upper Paraná basin in September and highest values in April. In this study, fish exposure was carried out in September, but no differences were found for Fulton's K-values between the exposed groups and the final control group. However, the exposed groups and the final control group have showed a decrease on Fulton's K-values when compared to initial control group, probably because they were isolated by cages, making feeding difficult.

3.3.2. Genotoxicity

The genotoxicity of contaminants present in the exposure sites is expressed as % DNA in the tail (Fig. 3), as this endpoint has been shown to be the most meaningful to assess genotoxicity (Kumaravel and Jha, 2006). The comet assay showed significant differences in DNA damage between the sampled sites and control group (F(4, 41)=28.186, p-value < 0.0001), being that fish from S2 and S3 exhibited higher DNA damage (25.33 ± 4.29 % and 23.38 ± 3.20 %, respectively) compared to the other experimental groups (Fig. 3). On the other hand, similar DNA damage was observed in fish from S1, S4 and control group (p-value > 0.05; Fig. 3). Results of the OTM and TL

374 are in supplementary material (Fig. S2), which further confirmed the genotoxic effects
375 observed in the caged fish from S2 and S3.

376 The score of % DNA in the tail was ranked as zero or minimal 10 % DNA (Fig.
377 3 B), low damage >10 – 25 % (Fig. 3C), mid damage >25 – 50 % (Fig. 3D), high
378 damage >50 – 75 % (Fig. 3E) and extreme damage > 75 % (not found). Minimal DNA
379 damage was only found on fish from S1 (16.67 %), while low DNA damage was found
380 on fish from all groups [control (100 %), S1 (83 %), S2 (40 %), S3 (70 %) and S4 (90
381 %)]. Mild DNA damage showed highest frequency at S2 (60 %) followed by S3 (30 %)
382 and S4 (10 %). However, mid DNA damage was not found for S1 and the control
383 group, while high and extreme DNA damage was not found for any group (See
384 supplementary material Table S4).

385 Results obtained for the caged *A. lacustris* showed highest scores of DNA
386 damage in fish exposed at S2, where the highest concentrations of metals (Fe and Mn)
387 were found in the water. Heavy metals, as well as other pollutants, have been associated
388 to oxidative stress in aquatic animals, including fish (Valavanidis et al., 2006;
389 Sevcikova et al., 2011; Qu et al., 2014). Thus, exposure to heavy metals can induce the
390 generation of reactive oxygen species (ROS) leading to cellular injuries due to
391 alterations in DNA, proteins and membranes (Leonard et al., 2004). In a study
392 performed by Vieira et al. (2017), the increased occurrence of LPO and DNA damage in
393 *A. altiparanae* suggested an exhaustion phase in individuals chronically exposed to
394 chemical substances. These authors suggested that after reaching the compensation
395 limits, a significant increase in the response of biochemical biomarkers (as
396 biotransformation metabolism and oxidative stress) led to energy reserves depletion and
397 resulted in degenerative events such as LPO, DNA damage, and cell necrosis and lysis.

In the present study, the exposure to metals, even at low concentrations, can also induce DNA damage in the neotropical fish *A. lacustris*, indicating interactive effects.

In addition to heavy metals, the anthropogenic pressure present in the sampling locations could also account for the increased DNA damage in exposed fish. Ghisi et al. (2017) found more DNA damage on *Astyanax* collected downstream from an urban zone compared to fish sampled from agriculture areas and a reserve. These responses were likely due a synergic interaction between agriculture and industrial chemical effluents, as well as ineffective sewage treatments. However, results obtained in this study did not show a direct influence of land use on the degree of DNA damage assessed on *A. lacustris.* Another study by De Andrade et al. (2004) observed a positive correlation between DNA damage in fish and the size of the city located near the sampling site. The same cannot be seen in this study, as higher DNA damage was recorded in fish from S4 compared to S1. S1 is located in the urban area in the city of Goiânia, which is a city far larger than Campo Limpo, where S4 is located. Other authors state that additional cumulative factors such as agriculture and industrial residues (Clements et al., 1997; Alberto et al., 2005; Wirzinger et al. 2007; Ghisi et al., 2014; 2017), as well as domestic sewage, can have a significant impact on DNA damage observed in freshwater fish (Wirzinger et al., 2007; Grisolia et al., 2009; Rocco et al., 2012; Ghisi et al., 2014). However, of the selected organic compounds quantified in the water collected, the majority were either not detected or detected in very low levels in all sampling sites, ruling out their possible effect in the damage seen in exposed fish. Although this is the first study in the selected area, a more comprehensive chemical analysis of water sampled at these sites should be performed in future studies, as well as the underlying sediment, as to get a better sense of which compounds can be responsible for the damage seen in fish and consequently the decrease in water quality.

Overall, the assessment of DNA damage showed to be a sensitive biomarker in caged *A. lacustris* and indicated that the environmental conditions in S2 and S3 induced more genotoxic effects.

3.3.3. Mutagenicity

The ENA frequency observed in caged *A. lacustris* exposed in the João Leite river and control groups are in Fig. 4. The total ENA observed on *A. lacustris* erythrocytes is a sum of different individual endpoints, namely MN, KN, LN, BN and SN (Fig. 4). A similar ENA total frequency was detected on fish from control groups and those exposed in the different sites ($H(5, N = 55) = 9.458623$; p-value = 0.0921). The presence of MN was only observed on fish exposed at S2, S3 and S4, with no significant differences between sites (Fig. 4B). No differences between both controls and exposed groups were seen for KN and LN (Fig. 4C-D), even though S2 had a higher frequency of LN (Fig. 4D). The presence of BN and SN were only recorded in fish from the initial control and S4 and S2, respectively, but with no differences between groups (Fig. 4E-F).

Several studies showed an increase on ENA frequency in fish of the genus *Astyanax* from sites under anthropic pressure (de Lemos et al., 2008; de Moraes Pantaleão et al., 2006; Vieira et al., 2014; Vieira et al., 2017; Viana et al., 2018). However, in the study performed by Vieira et al. (2017) on freshwater biomonitoring using *A. altiparanae*, the highest ENA frequency was found on feral fish, while caged fish exposed in the same sites did not show differences compared to the control group. These authors concluded that feral fish are more vulnerable to bioavailability and bioaccumulation processes in comparison to caged fish, which due to limited dislocation routes are less influenced by chemical exposure. In addition, Carrasco et al.

448 (1990) did not found a consistent correlation between variations in nuclear morphology
449 in wild fish and levels of contaminants in fish tissues and sediments. The authors
450 therefore state that piscine micronucleus test is a highly questionable method as an *in*
451 *situ* indicator of biological effects in wild fish exposed to chemical contaminants. In the
452 present study, even though no differences were detected between the control and
453 exposed groups for any of the mutagenic related endpoints, fish exposed at S2 had
454 higher frequencies of total ENA and LN, suggesting the presence of mutagenic
455 compounds at this site.
456
457 **3.3.4. Histopathological analysis**
458 The liver is an important organ that plays vital functions, such as protein
459 synthesis and glycogen storage, as well as detoxification of contaminants (Heath, 1987).
460 There is a great similarity between *Astyanax* spp. liver structure and the structure
461 described for other teleosts, with hepatocytes arranged in cords with pancreatic tissue
462 distributed throughout the liver tissue (Marcon et al., 2015). In this study, images taken
463 from fish livers showed that the hepatic tissue of *A. lacustris* is formed by a parenchyma
464 with hepatocytes arranged in a tubular pattern, shaping a cord structure along the
465 sinusoid vessels (Fig. 5 A-B). Image analysis showed that most frequent hepatic
466 alterations found on caged fish livers were leukocyte infiltrations in different stages
467 (Fig. 5), in addition to other changes, such as cytoplasmic vacuolation or steatosis.
468 Leukocyte infiltration at stage I was found in fish from all sampling sites, as well as for
469 the control groups, with S2 presenting the highest frequency at stage I (80 %) followed
470 by S3 (76.67 %), S1 (50 %), S4 (36.67 %), final control (23.33 %) and initial control
471 (10 %) (Fig. 6). Leukocyte infiltration at stage II was recorded in all groups in lower
472 frequency than the observed at stage I (S3 > S2 > S1 > S4 > final control), except for

473 the initial control where no stage II was detected. Finally, leukocyte infiltration at stage
474 III (6.67 %) was only observed on hepatic tissue from fish exposed at S2 (Fig. 6). These
475 findings are corroborated by results found by Freire et al. (2015) and Nimet et al.
476 (2018), which showed increased leukocyte infiltration on hepatic tissue of *Astyanax*
477 exposed to environmental contaminants.

478 One of the organs most affected by contaminants present in water is the liver,
479 mainly due to its role in the detoxification and biotransformation processes of
480 xenobiotics (Van der Oost et al., 2003). Accordingly, several studies have reported
481 accumulation of contaminants in fish liver (Vinodhini & Narayanan, 2008; Malik et al.,
482 2010; Weber et al., 2013), as well as damage on hepatic tissue (Zeitoun et al., 2014;
483 Cupertino et al., 2015). In the present study, high concentrations of Fe and Mn were
484 found in water samples collected at S2, which was the site where histopathological
485 analysis indicated the highest frequency of leukocyte infiltration on *A. lacustris* liver
486 tissue. These results suggest that the presence of metals in water might be responsible
487 for the occurrence of leukocyte infiltration on fish liver, as previously described by
488 other authors (Younis et al., 2013; Santana et al., 2018; Neves et al., 2018), probably as
489 a part of the inflammation process.

490 Recently, innate immunity has provided a wide set of biomarkers for
491 immunotoxicity against multiple xenobiotics (Slaninova et al., 2009; Jovanović & Palić,
492 2012; Cordero et al., 2016), such as leukocytes dynamics, phagocytic activity, lysozyme
493 production, production of antimicrobial peptides, cytokines expression, and ROS
494 production (Rehberger et al., 2017). Thus, early kinetics of innate parameters induction
495 associated with the sensitivity to detect additive or synergistic effects of contaminants
496 makes them an effective tool for ecotoxicological studies (Torrealba et al., 2019).
497 Immune cells in particular play an important role in organisms defense, mainly in

depuration of pathogens or other foreign elements (Gustafson et al., 2015), such as metals and organic compounds. The inflammation process in response to toxicants is considered the final component of internal innate immunity occurring when tissues are injured or when phagocytosis alone fail to prevent infection, and normally involves inflammatory responses as vasodilatation, increased vascular permeability, activation of blood clotting and infiltration of phagocytic cells (leukocytes) into injured tissues (Bols et al., 2001).

3.4. Integration of the biological effects response (IBR)

Integration of the biological effects determined in *A. lacustris* tissues was performed using the IBR (Fig. 7). The positioning of the biomarkers around the star plot can influence the output of the IBR, therefore care was taken to position the biomarkers based on their similarity in either cellular and/or physiological function as recommended (Cravo et al., 2013). Results obtained for the IBR showed that the 4 selected sites with the João Leite river are differently impacted (Fig. 7), with contaminants, environmental factors and different anthropogenic pressures influencing the spatial biomarker responses. IBR values within the exposed groups ranged from 2.69 to 10.44, while within the control groups the IBR values were within 1.33 and 1.92. As suspected, the highest IBR response was clearly identified in S2, with main contributions from % of DNA, ENA and the histopathological index. S4 was the second highest value calculated, with main contributions from GSI, KN and TL. The least impacted site within the João Leite river was S1, with small contributions from DNA damage biomarkers, GSI and condition factor. The lowest IBR was recorded at the final control, with the star plot revealing small contributions from the condition factor and % DNA damage.

The use of indices like the IBR, that combine only biomarker responses, can provide a general idea of the water quality of a chosen aquatic environment and have been shown to be an appropriate and valuable decision support tool for environmental managers (Beliaeff and Burgeot, 2002; Guerlet et al., 2010; Cravo et al., 2013). However, it is crucial to be aware that the magnitude and interaction between contaminants, mixed anthropogenic sources, as well as environmental and physiological stress, can have an influence and even mask the response of some biomarkers, and thus influence the result of the applied IBR. For this reason, this type of indices needs to be used with caution and should be interpreted along with chemical data, environmental parameters and if possible, consider the reproductive cycle or other parameters reflecting physiological stress, as to avoid misinterpretation of data.

4. Conclusion

The present study is the first one to apply an integrated multibiomarker approach to caged neotropical fish in a Brazilian Cerrado river, combined with results from physico-chemical characterization of water and land use analysis. Based on results, the hypothesis that the water quality of Brazilian savanna rivers can be evaluated by multiple biomarker response in caged *A. lacustris* was confirmed. Land use analysis was successful in showing differences between the sampling sites within the João Leite river basin, which can potentially affect water quality. Chemical characterization of the water from João Leite river (S2 and S3) showed metal concentrations (Mn and Fe) above limits allowed by Brazilian regulation. These results indicated that the presence and interactive effect of these metals, even at low concentrations, can affect fish health. As for biomarker responses, although the MN test was not able to detect mutagenic effects on *A. lacustris* during the exposure period, the comet assay was an effective

548 biomarker to identify DNA damage on caged fish exposed in the João Leite river. In
549 addition, hepatic leukocyte infiltration was also an important tissue-level biomarker,
550 highlighting S2 as the sampling site causing the higher impact on caged fish. The IBR
551 calculation was also able to integrate the biological responses of caged fish and showed
552 a clear distinction between sampling sites, with fish exposed at S2 showing higher
553 biological responses. Overall, the results obtained in this study highlighted the need to
554 implement multiple biomarker approaches covering a wider range of biological
555 responses in caged neotropical fish *A. lacustris* in future biomonitoring programs. In
556 addition, the observed results showed that a more comprehensive chemical analysis of
557 water and sediment sampled at these sites should also be performed in future studies, as
558 to get a better sense of which compounds can be responsible for the type of damage
559 seen in fish and consequently the decrease in water quality.
560
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571
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879

880 **Figure legends**

881

882 **Figure 1.** Sampling sites along the João Leite river basin area in relation to drainage
883 and land use. (A) Represents the drainage of each sample site and (B) represents
884 different types of land use across João Leite river basin. Site 1 is located in the urban
885 area of Goiânia, Site 2 is located in the rural area of the city of Goiânia, Site 3 is in the
886 rural zone of the municipality of Terezópolis and Site 4 is located in the rural zone of
887 the municipality of Campo Limpo.

888

889 **Figure 2**. Somatic biomarker responses of *A. lacustris* exposed in the João Leite river
890 and control groups. A) Condition Factor (Fulton's K) of *A.* lacustris. (B) Hepatosomatic
891 index (HIS) of *A. lacustris*. (C) Gonadosomatic index (GSI) of *A. lacustris*.

892

893 **Figure 3.** DNA damage in erythrocytes of *A. lacustris* from final control group and
894 exposed in the João Leite river for 7 days, obtained using the Comet Assay (A). The
895 black bars represent the DNA damage score expressed as % DNA in the tail (mean ±
896 SD). Letters indicate statistical differences, in which equal letter show no statistically
897 significant differences. (B-E). Representative image of nucleoids obtained from *A.*
898 *lacustris* taken with a fluorescence microscope using a 40*x* magnification after staining
899 with Syber Green. (B) Integral nucleus. (C) Nucleus with low DNA damage. (D)
900 Nucleus with moderate DNA damage. (E) Nucleus with severe DNA damage.

901

902 **Figure 4.** Erythrocyte nuclear abnormalities (ENA) frequency of controls and *A.*
903 *lacustris* exposed in the João Leite river for 7 days. (A) Total ENA, (B) cells with the

presence of micronucleus, (C) cells with kidney-shaped nucleus, (D) cells with lobbed nucleus, (E) number of binucleated cells and (F) cells with segmented nucleus. Similar letters show no statistically significant differences.

Figure 5. Histopathological changes in the liver of *A. lacustris* exposed in the João Leite river for 7 days and control groups. (A - B) Normal hepatic tissue, (C - D) Leukocyte infiltration at stage I, (E - F) Leukocyte infiltration at stage II, (G - H) Leukocyte infiltration at stage III. A, C, E and G: 20*x* magnification. B, D, F and H: 40 *x* magnification.

Figure 6. Frequency of leukocyte infiltration on hepatic tissues of *A. lacustris* exposed in the João Leite river.

Figure 7. Integrated biological response index (IBR) of *A. lacustris* exposed in the João Leite river for 7 days and control groups. Star plots represent the integration of biomarkers used for the calculation of the IBR for each site. IC – Initial control, S1 – Site 1, S2 – Site 2, S3 – Site 3, S4 – Site 4, FC – Final control. TL – Tail length, OTM – Olive tail moment, ENA – Erythrocyte nuclear abnormalities, MN – Micronucleus, KN – Kidney-shaped nucleus; LN – Lobed nucleus; SN – Segmented nucleus; BN – Binucleated nucleus, Histo – Histopathological index, GSI – Gonadosomatic index, HSI – Hepatosomatic index, K – Condition factor.

Table legends

928 **Table 1**. Different kinds of land use in the João Leite river basin classified *per* size area
929 and percent from the basin total area.
930
931 **Supplementary material captions**
932
933 **Table S1.** Physicochemical analysis of the water collected from the João Leite river.
934 (M.A.V) = maximum allowed value by CONAMA for water bodies classified into class
935 II. (*) represents values above limit allowed by CONAMA 357. (NR)= Not Relevant.
936 (ND) = Not Detected.
937
938 **Table S2.** Metal concentration (mg L^{-1}) in the water from the João Leite river. (M.A.V)
939 = maximum allowed value by CONAMA for water bodies classified into class II, (*)
940 indicates values above limit established by CONAMA 357, (NR) = not relevant, (ND) =
941 not detected, (-) = not performed.
942
943 **Table S3**. Organic compounds and organochlorine concentrations (μg L^{-1}) found in the
944 water collected from the João Leite river. (M.A.V) = maximum allowed value by
945 CONAMA for water bodies classified into class II, (*) represents values above limit
946 established by Brazilian regulation (CONAMA 357), (NR) = not relevant, (ND) = not
947 detected.
948
949 **Table S4.** Frequency of % DNA in the tail ranked into 5 categories of DNA damage.
950
951 **Figure S1.** Sampling sites along the João Leite river basin area. (A) Site 1 is located in
952 the urban area of Goiânia. (B) Site 2 is located in the rural area of the city of Goiânia.

953 (C) Site 3 is in the rural zone of the municipality of Terezópolis. (D) Site 4 is located in
954 the rural zone of the municipality of Campo Limpo.
955

956 **Figure S2**. Tail length (A) and Olive Tail Moment (OTM) in erythrocytes of *A.*
957 *lacustris* from final control group and exposed in the João Leite river for 7 days. Letters
958 indicate statistical differences, in which equal letter show no statistically significant
959 differences.
960

Figure 1
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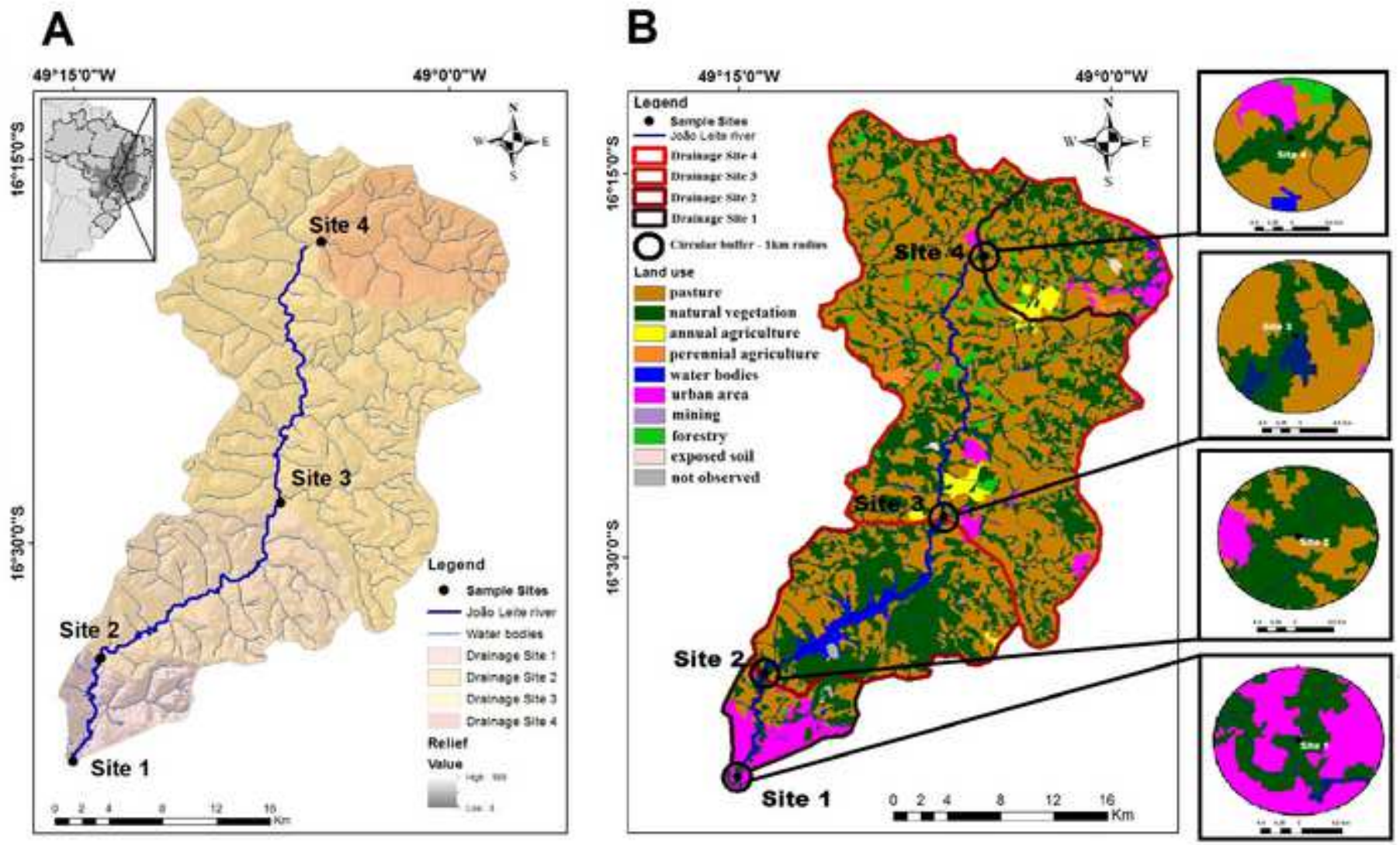

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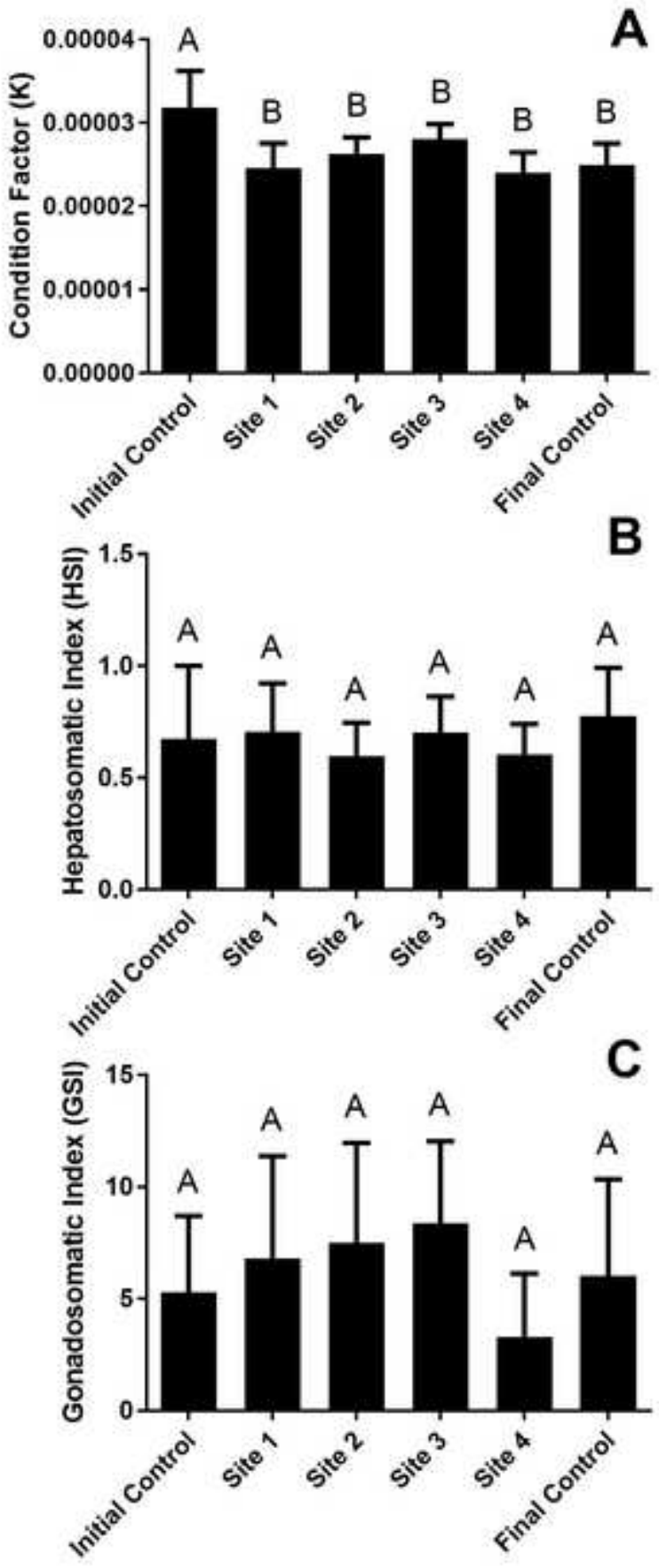

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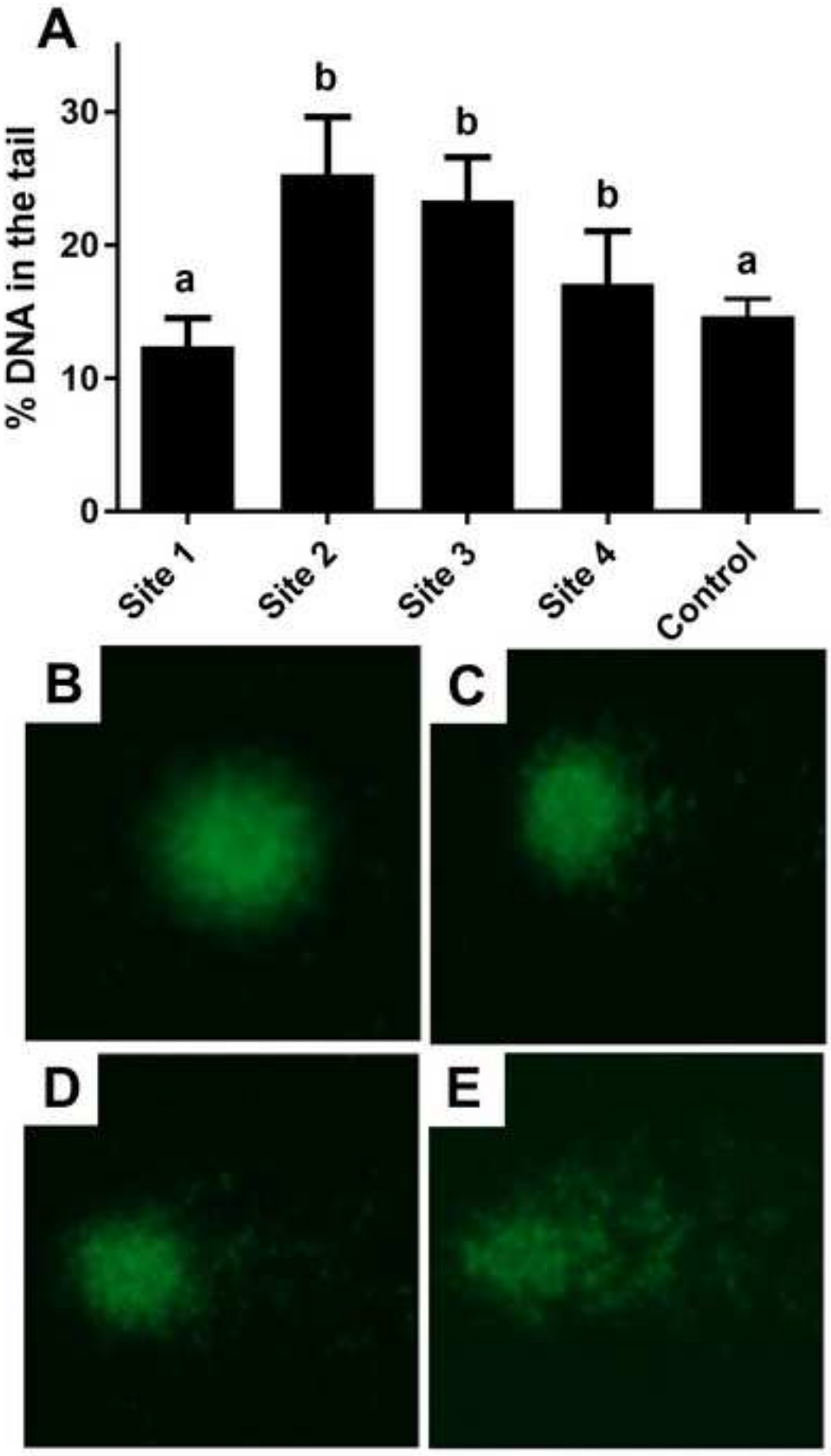

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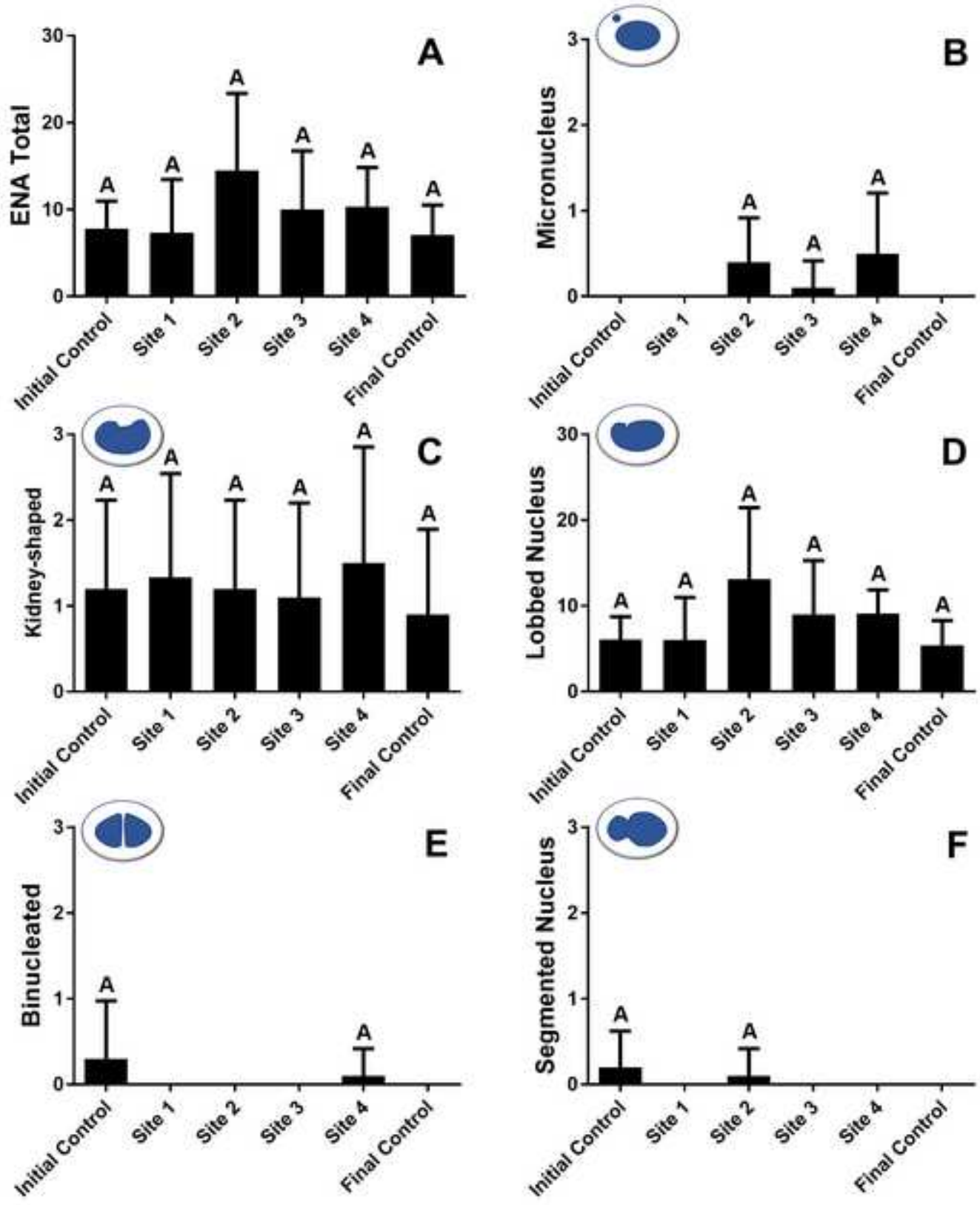

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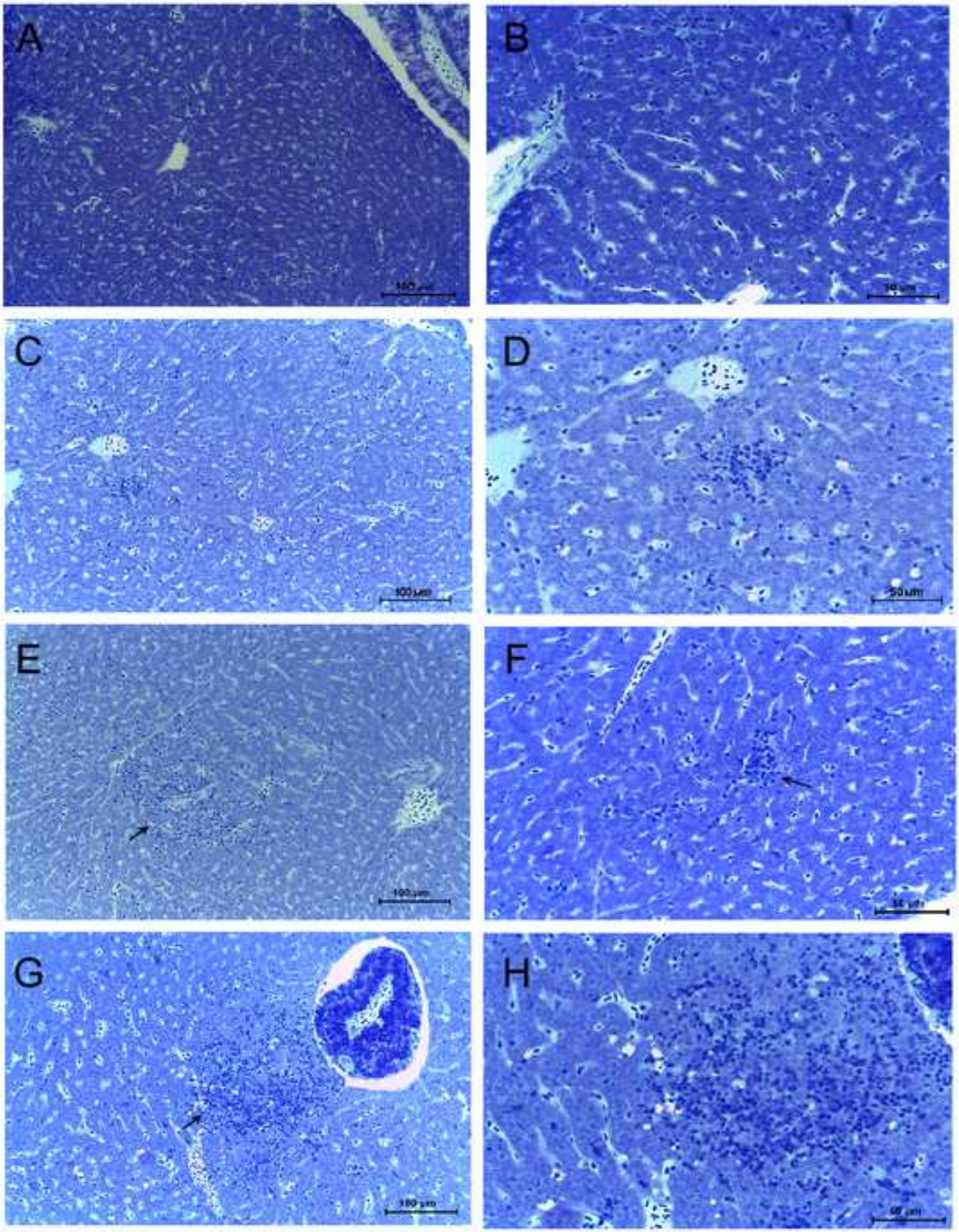

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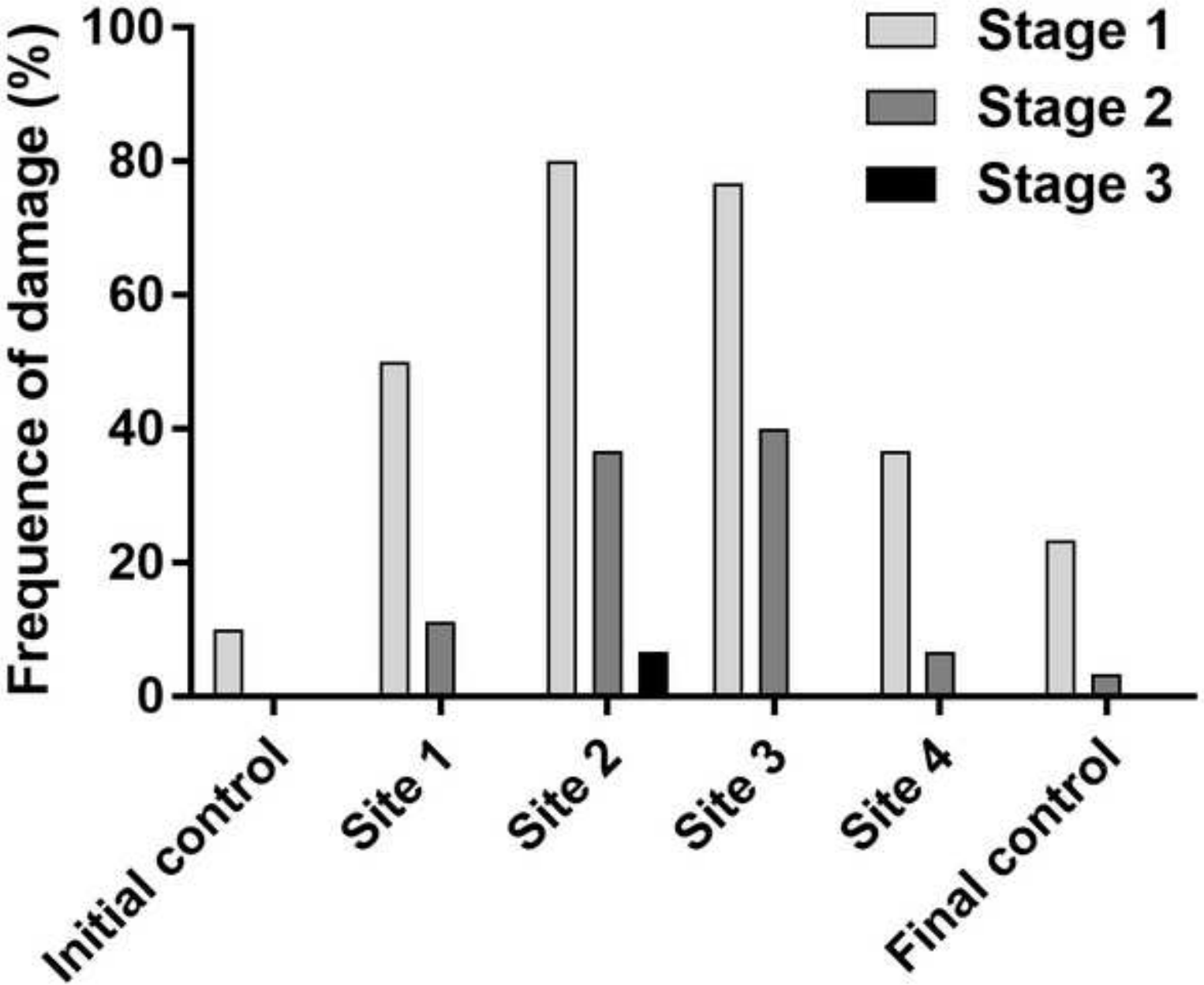

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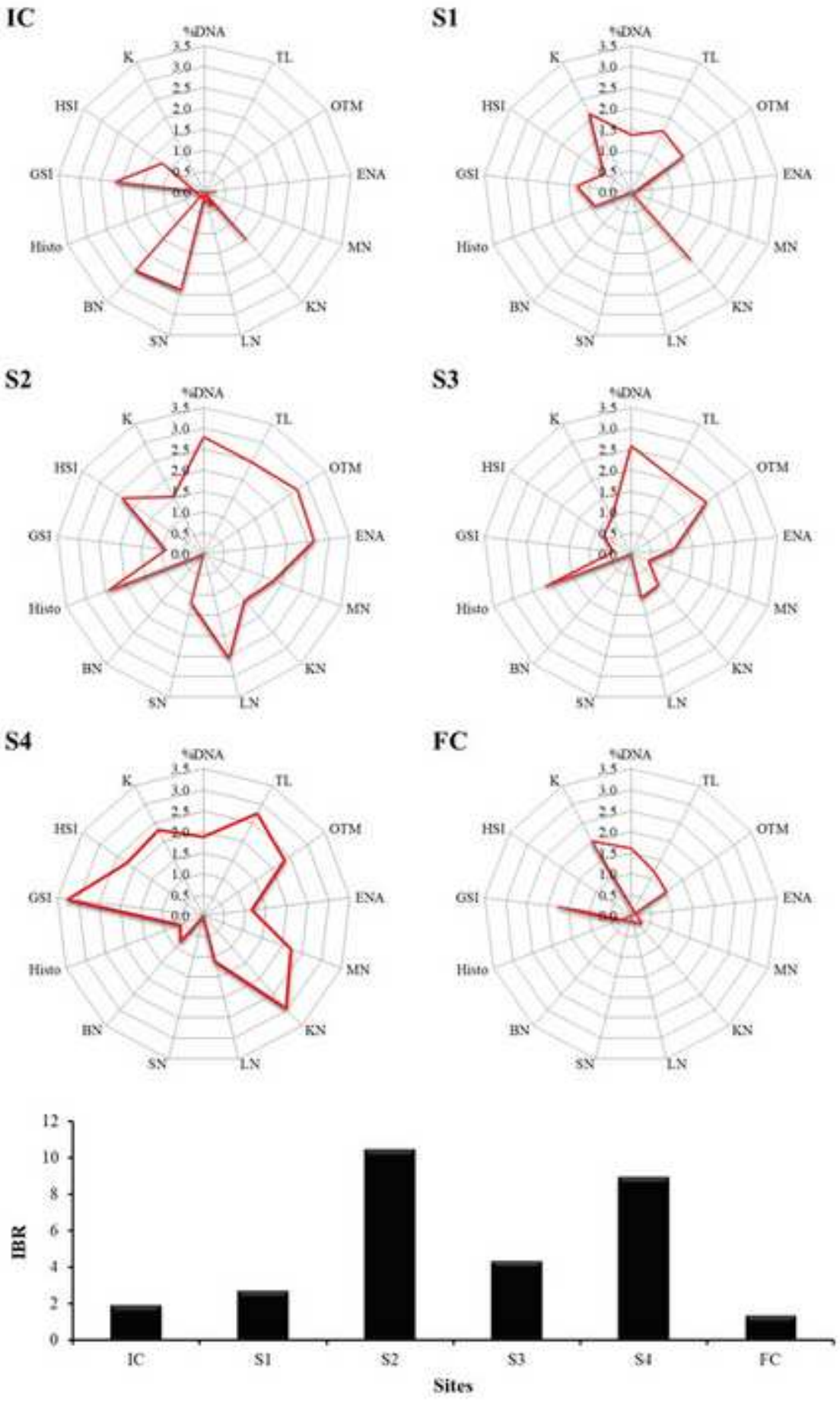

CRediT author statement

Douglas dos Santos Silva: methodology, data curation, writing-reviewing and editing. **Bruno Gonçalves**, **Cândido Carvalho Rodrigues**, **Felipe Cirqueira Dias** and **Izabella Soares Moreira**: methodology and formal analysis. **Nicholas Silvestre de Souza Trigueiro**: methodology, data curation, writing-reviewing and editing. **Daniela de Melo e Silva**, **Simone Maria Teixeira Saboia-Morais** and **Tânia Gomes**: data curation, writing-reviewing and editing. **Thiago Lopes Rocha**: conceptualization, supervision, data curation, writing-reviewing and editing.