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**Instituto Federal de Educação, Ciência e Tecnologia Goiano – Campus Urutaí**
Programa de Pós-Graduação em Conservação de
Recursos Naturais do Cerrado

# FROM CARRION-EATERS TO PLASTIC MATERIAL PLUNDERERS: TOXICOLOGICAL IMPACTS OF PLASTIC INGESTION ON BLACK VULTURES, *Coragyps atratus* (CATHARTIFORMES: CATHARTIDAE)

WALLACE ALVES CUNHA

**Orientador:** Prof. Dr. Guilherme Malafaia

Urutaí, Agosto de 2022

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*Orientador*
Prof. Dr. Guilherme Malafaia

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# BANCA EXAMINADORA DE DEFESA DE DISSERTAÇÃO

Aos trinta e um dias do mês de agosto do ano de dois mil e vinte e dois, às treze horas, reuniram-se os componentes da banca examinadora em sessão pública realizada por videoconferência, para procederem à avaliação da defesa de dissertação em nível de mestrado, de autoria de **Wallace Alves Cunha,** discente do **Programa de Pós-Graduação em Conservação de Recursos Naturais do Cerrado do Instituto Federal Goiano - Campus Urutaí,** com trabalho intitulado **"FROM CARRION-EATERS TO PLASTIC MATERIAL PLUNDERERS: TOXICOLOGICAL IMPACTS OF PLASTIC INGESTION ON BLACK VULTURES, *Coragyps atratus* (CATHARTIFORMES: CATHARTIDAE)".** A sessão foi aberta pelo presidente da banca examinadora, **Prof. Dr. Guilherme Malafaia Pinto**, que fez a apresentação formal dos membros da banca. A palavra, a seguir, foi concedida ao autor da dissertação para, em 30 minutos, proceder à apresentação de seu trabalho. Terminada a apresentação, cada membro da banca arguiu ao examinado, tendo-se adotado o sistema de diálogo sequencial. Terminada a fase de arguição, procedeu-se à avaliação da defesa. Tendo-se em vista as normas que regulamentam o Programa de Pós-Graduação em Conservação de Recursos Naturais do Cerrado, a dissertação foi **APROVADA**, considerando-se integralmente cumprido este requisito para fins de obtenção do título de **MESTRE EM CONSERVAÇÃO DE RECURSOS NATURAIS DO CERRADO,** na área de concentração em **Ciências Ambientais**, pelo Instituto Federal Goiano – Campus Urutaí. A conclusão do curso dar-se-á quando da entrega na secretaria do Programa de Pós-Graduação em Conservação de Recursos Naturais do Cerrado da versão definitiva da dissertação, com as devidas correções. Assim sendo, a defesa perderá a validade se não cumprida essa condição, em até **60 (sessenta) dias** da sua ocorrência. A banca examinadora recomendou a publicação dos artigos científicos oriundos dessa dissertação em periódicos após procedida as modificações sugeridas. Cumpridas as formalidades da pauta, a presidência da mesa encerrou esta sessão de defesa de dissertação de mestrado, e para constar, foi lavrada a presente Ata, que, após lida e achada conforme, será assinada eletronicamente pelos membros da banca examinadora.

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# FICHA DE APROVAÇÃO DA DISSERTAÇÃO

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| Título da dissertação: | **DE CARNICEIROS À SAQUEADORES DE MATERIAL PLÁSTICO: IMPACTOS TOXICOLÓGICOS DA INGESTÃO PLÁSTICA EM URUBUS-DA-CABEÇA-PRETA C*oragyps atratus* (CATHARTIFORMES, CATHARTIDAE)** |
| Orientador(a): | **Prof. Dr. Guilherme Malafaia** |
| Autor(a): | Wallace Alves Cunha |

Dissertação de Mestrado **APROVADO** em 31 de AGOSTO de 2022, como parte das exigências para obtenção do Título de **MESTRE EM CONSERVAÇÃO DE RECURSOS NATURAIS DO CERRADO**, pela Banca Examinadora especificada a seguir.

Prof. Dr. **Guilherme Malafaia**
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Dra. **Letícia Paiva Matos**
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## ABSTRACT

Despite plastic ingestion has already been reported in several bird species, its physiological impacts have been little inspected, especially in representatives of the Cathartidae family. Thus, in this study, we aimed to identify, characterize, and evaluate the effects arising from the ingestion of plastic materials by *Coragyps atratus* adults, that captured in landfill areas. Herein, a total of 51 individuals were captured, the frequency of plastic intake being higher than 40%. The plastic materials consisted mainly of low-density polyethylene and film-type polystyrene, as well as presenting irregular shapes and diameters between 10 and 30 mm. Biochemically, we observed in animals that contained plastics in the stomach ("plastic" group) high production of reactive oxygen species (ROS), hydrogen peroxide (H2O2) and malondialdehyde (MDA) - especially in the intestine, muscle and brain - whose activity of catalase (CAT) and superoxide dismutase (SOD) was not sufficient to counteract the oxidative stress. Moreover, in the liver of these same animals, we observed high production of nitrite and nitrate, suggesting a hepatic nitrosative stress. Plus, we observed a cholinesterase effect in animals from the "plastic" group, marked by increased activity of butyrylcholinesterase (BChE) (in the brain) and muscle and cerebral acetylcholinesterase (AChE). On the other hand, the biochemical changes perceived were not significantly correlated with the identified plastic material concentrations (2.808 ± 0.598 items/g of stomach content and 0.276 ± 0.070 items/g of stomach – fresh weight), body condition of the animals, size, and shape of the identified plastic materials. Hence, our study sheds the light on the toxicity of plastics deposited in landfills and their ingestion by *C. atratus*, which reinforces the hypothesis that these materials are harming the health of these birds and, consequently, the dynamics of their populations.

**Keywords:** Birds, Synthetic polymer, Plastic pollution, Landfills, Biochemistry, Biometry.

# 1. INTRODUCTION

One of the most emblematic birds of the Cathartidae family (order Cathartiformes) and of great ecological importance are the vultures. As discussed by Blanco et al. (2021) and Van-den-Heever et al. (2021), these animals play a remarkable role in the functioning of ecosystems, especially by foraging for dead animals, promoting clean-up of the environments where they live and preventing the spread of diseases between different trophic levels. Despite this, this finding has not been sufficient for the adequate conservation of these animals. According to the survey carried out by Buechley & Şekercioğlu (2016), vultures, the only obligatory vertebrate scavengers, have experienced the fastest decline in conservation status of any avian group in the last decade, in addition to being the most threatened functional guild in the world, whose causes are especially related to current anthropogenic activities.

There is a close association between declining vulture populations and exposure to different pesticides (Plaza et al., 2019; Odino & Ogada, 2021), polycyclic aromatic hydrocarbons (Gómara et al., 2004; Dhananjayan et al., 2011; Dhananjayan et al., 2013), heavy metals (Borges-Ramírez et al., 2021; Stamenov et al., 2021), pharmacological waste (human and veterinary) (Nambirajan et al., 2018; Moreno-Opo et al., 2021; Jimenez-Lopez et al., 2021; Herrero-Villar et al., 2021) has been reported, among others. The African and Asian continents are good examples of how and how much human activities have threatened vulture species (Bowden, 2017, Oppel et al., 2021). Estimates published by Ogada et al. (2016), in Africa, for instance, are alarming. According to the authors, populations of eight African species assessed decreased by an average of 62% and seven decreased at a rate of approximately 80% (over 30 years). Among these, at least six (*Gyps rueppelli, G. coprotheres, G. africanus, Necrosyrtes monachus, Trigonoceps occipitalis,* and *Torgos tracheliotos*) qualify as "endangered" or "critically endangered" according to the IUCN Red List of Threatened Species.

Importantly, a field of investigation that is still little explored refers to the contributions of plastic pollution to the decline of vulture species. In 2019 alone, the global production of plastics reached 368 million tons (PlasticsEurope, 2020), with a large scale being discarded directly into the natural environment, providing opportunities for the contact (direct or indirect) of these animals with such materials. In the study by Torres-Mura et al. (2015), in the Atacama Desert (Chile), the authors reported that more than 70% of fecal samples from *Cathartes aura* contained plastic material, probably originating from waste disposed along roads and beaches in the studied area. In Mexico, Borges-Ramírez et al. (2021) observed that microplastics (MPs) can be vectors for the exposure of *Coragyps atratus* to different pollutants and Ballejo et al. (2021) demonstrated that *Vultur gryphus, C. atratus* and *C. aura*, when feeding on organic waste, together with plastic waste disposed in dumps, can unintentionally transfer these materials to natural areas where they roost (Northwest Patagonia). Additionally, microtrash ingestion by *G. coprotheres* pups in South Africa (Pfeiffer et al., 2017) and plastic pellets by *C. aura* adults living on a remote and sparsely populated South Atlantic Island have been documented (Augé, 2017). Consequently, these studies provide clear evidence that the ingestion of plastics by different species of vultures is a reality in different locations.

In this interim, we do not know what the effects of this ingestion are on the physiology of these animals, and how much they can impact their health. Different studies have already shown, for example, that plastic ingestion induces changes in REDOX homeostasis in different animal models (Hu & Palić, 2020), such as in invertebrates (Malafaia et al., 2020; Chagas et al., 2021; Muhammad et al., 2021; Muhammad et al., al., 2021; Silva et al., 2021) and vertebrates (Deng et al., 2017; Qiao et al., 2019; Bhagat et al., 2020; Xie et al., 2020; Guimarães et al., 2021; Xu et al., 2021; Meng et al., 2021). Furthermore, the cholinesterase effect has also been previously reported in different animal groups, with exposure to MPs being mostly associated with reduced acetylcholinesterase (AChE) activity (Barboza et al., 2018; Yin et al., 2021). Therefore, it is plausible to assume that similar changes can be observed as a result of the ingestion of plastic material in birds, including vultures. Thus, we pioneered a study focusing on the identification and characterization of plastic materials in the stomach contents of adults of *C. atratus* captured in different landfills, to test the hypothesis of the existence of a close relationship between plastic ingestion and possible biochemical changes in different organs. In addition, the relationships between the frequency of plastic ingestion, body condition, plastic size and animal sex were evaluated. *C. atratus* is a species distributed in tropical and temperate areas along the American continent, being widely represented not only in South and Central America, but also in southern North America (BirdLife International, 2016). Along with that, different authors have reported its occurrence in highly urbanized and impacted areas, as well as urban waste dumps (Rpbbins et al., 1989; Blackwell et al., 2007; Novaes & Cintra, 2013; Campbell, 2014; Araújo et al., 2018; Hill et al., 2021). Therefore, individuals of *C. atratus* constitute good translational models for evaluating the toxicity of plastics in different species of the Cathartidae family. As far as we know, this is the first report that associates plastic ingestion by *C. atratus* with biochemical alterations predictive of oxidative stress, REDOX imbalance, and cholinesterase effect. We strongly believe that studies like ours provide support for the proposition of mitigation/remediation measures for plastic pollution, as well as for the conservation of vulture species.

## 2. MATERIAL AND METHODS

### 2.1. Areas of study and capture of animals

Fifty-one adults of *C. atratus* (of both sexes – see biometrics in Table S1) were captured in landfills in the municipalities of Pires do Rio (-17.260733, -48,285367) and Urutaí (-17.430811, -48.200189) (both located in the State of Goiás, Brazil), having been used a trap of the "pit type", like the one used by Barbara et al. (2017). Such trap was installed on the margins of the final disposal site of solid waste from the respective landfills and to attract the birds, chicken meat baits were placed inside the trap. The authorization to capture the animals was granted by the Biodiversity Information and Authorization System (SISBIO)/Chico Mendes Institute for Biodiversity Conservation (Brazil).

### 2.2. Identification and characterization of plastics

After capture, the animals were taken to the Biological Research Laboratory of the *Instituto Federal Goiano* (Urutaí, GO, Brazil) and were euthanized by decapitation. Then, a laparotomy was performed to extract the stomach and collect fragments of the liver, muscle (pectoral) and small intestine (duodenal region just below the stomach – fragments of approximately 2 cm). In addition, a craniotomy was performed to extract brain fragments from the animals. Then, the samples were properly identified and stored in a freezer at -80oC until the biochemical analyzes were performed (see item "2.2.2").

Afterwards, the stomach contents were washed (with purified water via reverse osmosis) and sieved through a steel mesh (0.075 mm) with running water and placed in a glass petri dish for further analysis under a stereoscopic microscope. The identified plastic material [according to Rosas-Luis (2016)] was collected, washed with 70% alcohol, photographed, and stored individually for further analysis via micro-Raman spectroscopy to determine its polymeric chemical compositions. The spectra obtained were compared with those available in the PublicSpectra © 2019 database (https://publicspectra.com/), whose similarity was greater than 75%. Besides, the size (area and diameter) and shape (circularity) of the plastics were evaluated using the ImageJ software, similarly to the procedure adopted by Araújo et al. (2020). All plastic materials were categorized according to type and color, according to similar procedures adopted by Marti et al. (2020). The frequency of plastic ingestion (FI%) was calculated according to the following equation: FI(%) = (Number of vultures that contained plastics)/(Total number of vultures examined) x 100. The number of plastics in animals was expressed proportionally to dry weight of stomach contents and fresh stomach biomass of animals.

## 2.3. Toxicity biomarkers

### 2.3.1. Biometry

To assess the relationship between plastic material ingestion and possible changes in animal health, different toxicity biomarkers were determined. The body condition of the animals was evaluated from the body mass (BM), body length (BL), tail (TL), wing (WL) and tarsus (T). These parameters were used to determine different biometric indices [BM/T, BM/(BL + WL), BM/BL, BM/WL and BM/TL], which were calculated from previous studies (Chappell & Titman, 1983; DeVault et al., 2003; Schamber et al., 2009). In accordance with Labocha & Hayes (2012), the body condition of birds has been related to the survival, reproduction, and behavior of animals, in addition to being a topic of considerable interest in studies that assess the impacts of human activities on avifauna.

### 2.3.2. Biochemical analysis

Presuming that plastics ingestion can cause biochemical changes, we evaluated in liver, intestine, brain, and muscle fragments the production of hydrogen peroxide (H2O2), reactive oxygen species (ROS), nitric oxide (via nitrite/nitrate production), malondialdehyde (MDA), as well as the activity of the enzymes superoxide dismutase (SOD) and catalase (CAT). In addition, the activity of the enzymes acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) was evaluated assuming the cholinesterase effect of the ingestion of plastic material. For this, the organ fragments were weighed, macerated in 1 mL of phosphate buffered saline (PBS) (pH 7.2) (with semi-automatic macerator) and, subsequently, the samples were centrifuged (10000 rpm, 10 min, 4°C) for the collection of supernatants, which were used to assess the biomarkers summarized in Table 1. Twelve random animals that did not contain plastic in their stomach contents constituted the "control" group and 19 vultures, in which we identified any plastic material, formed the "plastic" group. It is noteworthy that we assume that animals named as "control" do not necessarily constitute animals exempt from ingestion of plastic material (including additives and MPs) hours before the capture. As demonstrated by Ballejo et al. (2021), Torres-Mura et al. (2021) and Platt et al. (2021, regurgitation is quite common in vultures, both in females, to feed their young, and males/females to fend off their attacks and allow easier flights. Therefore, our focus was on the momentary effects of the presence of plastic material in the gut contents of animals and, therefore, we disregard the history of exposure of animals to plastic materials.

**Table 1.** Summary Summary of biochemical biomarkers evaluated in adults of *Coragyps atratus* captured in landfills in the municipalities of Pires do Rio and Urutaí (GO, Brazil).

| **Oxidative stress biomarkers[4]** | | | | |
|---|---|---|---|---|
| | **Volume of supernatant used** | **Reagents and volumes used** | **Wavelength of sample reading[1]** | **Base reference for conducting assessments** |
| Nitrite and nitrate [indirect measurement of nitric oxide (NO)] | | 150 µL of Griess reagent | 492 nm | Bryan & Grisham (2007) |
| Hydrogen peroxide ($H_2O_2$) | 10 µL | 100 µL of phosphate buffered saline (PBS, pH 7.2)<br>100 µL of ammonium molybdate solution (0.5% w/v) | 405 nm | Elnemma (2004) |
| Reactive oxygen species (ROS) | 20 µL | 200 µL of phosphate buffered saline (PBS, pH 7.2)<br>8.3 µL of dichlorofluorescein-diacetate (10 mg/mL) | 492 nm | Maharajan et al. (2018) |
| **Biomarcadores antioxidantes[4]** | | | | |
| Catalase (CAT)[2] | 8 µL | 240 µL of reaction solution [glacial acetic acid P.A. + potassium dichromate (5%)] | 630 nm | Sinha (1972) |

| | | | | |
|---|---|---|---|---|
| Superoxide dismutase (SOD)[2] | 30 µL | 99 µL of phosphate buffered saline (PBS, pH 7.2)<br>15 µL of piragalol (15 mM)<br>6 µL of 3-[4,5-Dimethylthiazol-2H]-2,5-diphenyltetrazolium bromide (1.25 mM)<br>150 µL of dimethylsulfoxide P.A. | 630 nm | Del-Maestro & McDonald (1985) |
| **Cholinesterase effect biomarkers** [4] | | | | |
| Acetylcholinesterase (AChE) e Butyrylcholinesterase (BChE) | 50 µL | 100 µL of acetylcholine or butyrylcholine solution (0.75 mg/mL)<br>100 µL of DTNB[3] solution (0.13 mg/mL)) | 405 nm | Ellman et al. (1961) |

[1] The readings were performed in an ELISA reader.
[2] These molecules are considered first-line antioxidants defenses that are important for preventing physiological oxidative stress.
[3] DTNB: 5,5'-Dithiobis-(2-Nitrobenzoic Acid).
[4] The results of the analyzes of all biomarkers were expressed proportionally to the concentration of total proteins in evaluated organs. Such biomarker was evaluated according to the instructions of the commercial kit used [Commercial kit (CAS number: BT1000900).

## 2.4. Statistical analysis

Initially, the residual normality of all data was evaluated by the Shapiro-Wilk test and the homogeneity of variances by the Bartlett test. The possible difference between the FI(%) of plastics by *C. atratus* females and males was evaluated by the Chi-square test. The means of the different biochemical biomarkers obtained in animals with and without plastics were compared using the Mann-Whitney test (non-parametric) or Student's t test (parametric). The two-way ANOVA (with Tukey's post-test) was used to assess the individual effect or the interaction between the factor’s "contamination" (two levels: presence or absence of plastics in the stomach of animals) and "sex" (two levels: male or female). Furthermore, Pearson (parametric) or Spearman (non-parametric) correlation analyzes were performed, seeking to associate different evaluated biomarkers. In the case of significant correlations, linear regression analysis was performed. For all analyses, p-value < 0.05 was considered as statistically significant. The GraphPad Prism (v. 7.0 version) statistical software was used to perform the statistical analysis and graph construction

## 3. RESULTS

Basically, our analyzes revealed the presence of plastic material in the stomach of almost half of the investigated animals (43.95%), with an equal percentage of females (≈43%) and males (≈44%) in which plastics were identified (Figure 1A). A total of 73 plastic particles were identified, with the mean of items/g of stomach content (dry weight) equal to 2.808 ± 0.598 (mean ± SEM) and 0.276 ± 0.070 items/g of stomach (fresh weight), not having been the factor associated with this variable (Figure 1B). Regarding size, most (84.9%) of the plastic items had a diameter greater than 5 mm (Figure 1C), and 56.2% had a diameter between 10 and 30 mm (Figure 1D). The types of plastics identified (via micro-RAMAN analysis) were, in decreasing order of abundance, low-density polyethylene > polystyrene > nylon (polyamide)/poly (methyl methacrylate)/high-density polyethylene > polyethylene terephthalate/polyvinyl chloride/polybutadiene (Figure 1E).

In this regard, using a classification system from previous studies, plastic materials were divided into four categories according to their shapes: fiber, fragment, pellet, and film. Briefly, a long, thin line with a slender shape was classified as fiber; fragments are hard piece of debris from a broken plastic item; debris with a thin layer was called film; and pellets are microplastics with spherical and cylindrical shapes. As can be seen in Figure 1F, most plastic materials identified were of the “film” type (76.8%), with fibers being less frequent (3.6%). Pellets and fragments represented 8.9% and 10.7% of the identified plastic materials, respectively. As for coloration, most plastics identified were classified as “white” and “transparent” (75.4%) (Figure 2). Images representative of the types, shapes (circularity: 0.20 ± 0.01; mean ± SEM) and colors of the identified plastics are presented in Figures 3 and 4. Although we have not thoroughly characterized the gut contents of animals (in terms of non-plastic material), the presence of plant material (Figure 4I-K), insect larvae (Figure 4L), ticks (Ixodida order) (Figure 4M), animal hair possibly present on carcasses (Figure 4N), eggshells (Figure 4O) and bones (Figure 4P) were commonly found items. On the other hand, our visual evaluation did not evidence the presence of metallic material, pieces of glass or porcelain.

From these results, we evaluated the possible influence of plastic intake on animal health from different biochemical biomarkers. Initially, we observed an increase in the production of

ROS (gut, muscle, and brain), H2O2 (gut, muscle, and brain) and MDA (muscle and brain) of *C. atratus* from the "plastic" group (Figure 5A-C, respectively), as well as of nitric oxide in the liver of these animals (inferred from the levels of nitrite and nitrate) (Figure 5D-E, respectively). As for the activity of the enzymes evaluated, we observed an increase in the activity of CAT in the gut, muscle and brain (Figure 6A), as well as SOD in the gut of animals in the "plastic" group (Figure 6B). On the other hand, changes in BChE activity were observed in the gut and brain ("control" vs. "plastic"; Figure 6C) and an increase in AChE was observed in the muscle and brain (Figure 6D) of these same animals. However, we did not find significant correlations between most of the analyzed biochemical parameters and the plastic material concentrations identified in the animals (Figures S1-2). Furthermore, the size (area in cm2) and shape (inferred from the circularity) of the plastics did not influence the biochemical response of the animals (Figures S3-4).

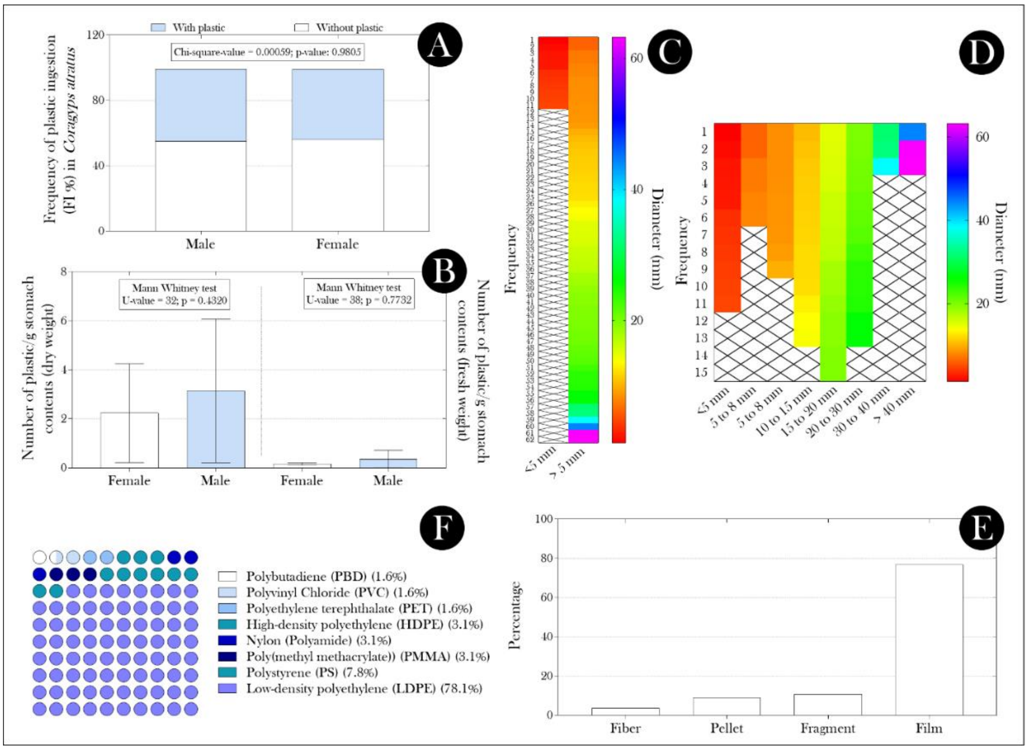


**Figure 1.** (A) Frequency of plastic ingestion, (B) plastic concentrations identified in animals (by "g" of stomach content (dry weight) and by "g" of stomach (fresh weight); (C) distribution of frequency of plastic material with diameter smaller and larger than 5 mm; (D) frequency distribution of plastic material according to the categories of intervals of diameters (mm) measured; (E) percentage distribution of polymer types and (F) percentage of types of plastic classified according to form (fibers, pellets, fragments or film) identified in the stomach contents of adults of *Coragyps atratus* (females and males) captured in landfills in two municipalities in the State of Goiás (Brazil). In "B", bars represent mean ± SD.

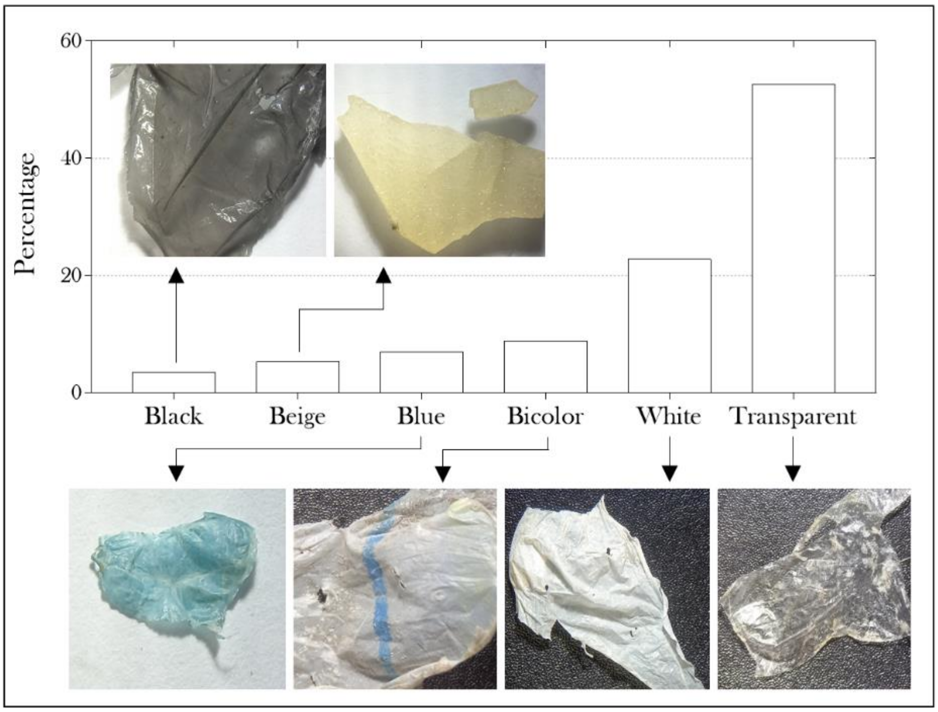
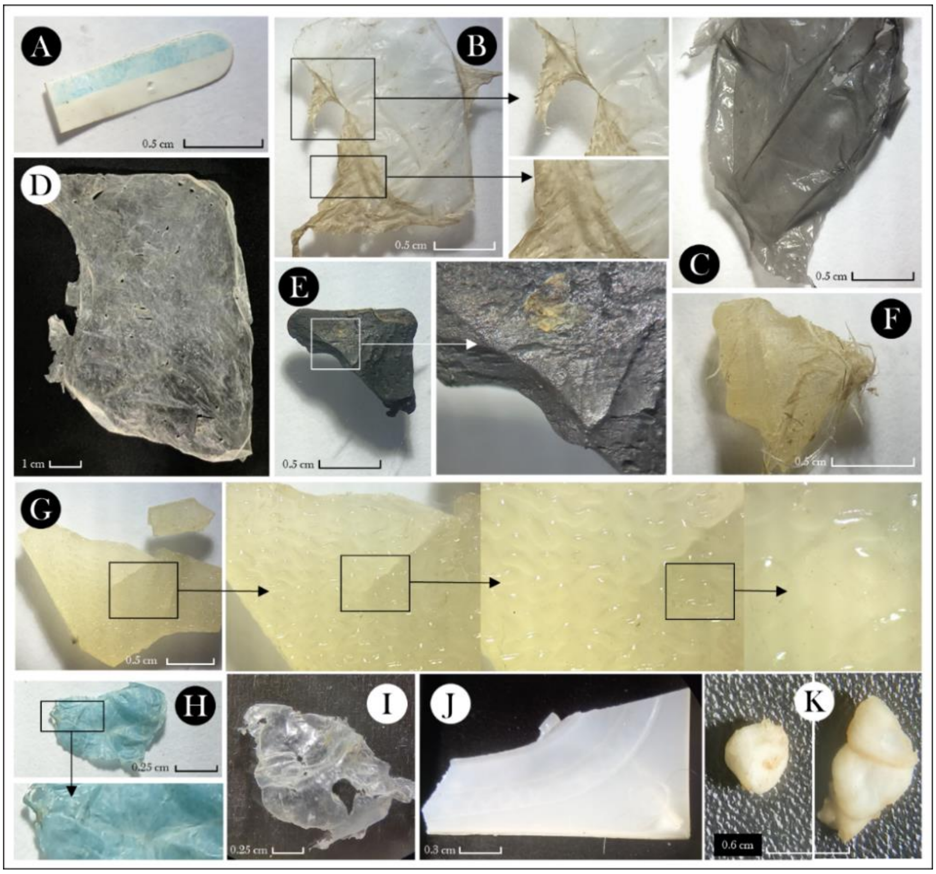


**Figure 2.** Percentage of the color of plastic materials identified in the stomach content of *Coragyps atratus* (female and male) adults captured in landfills (State of Goiás, Brazil).



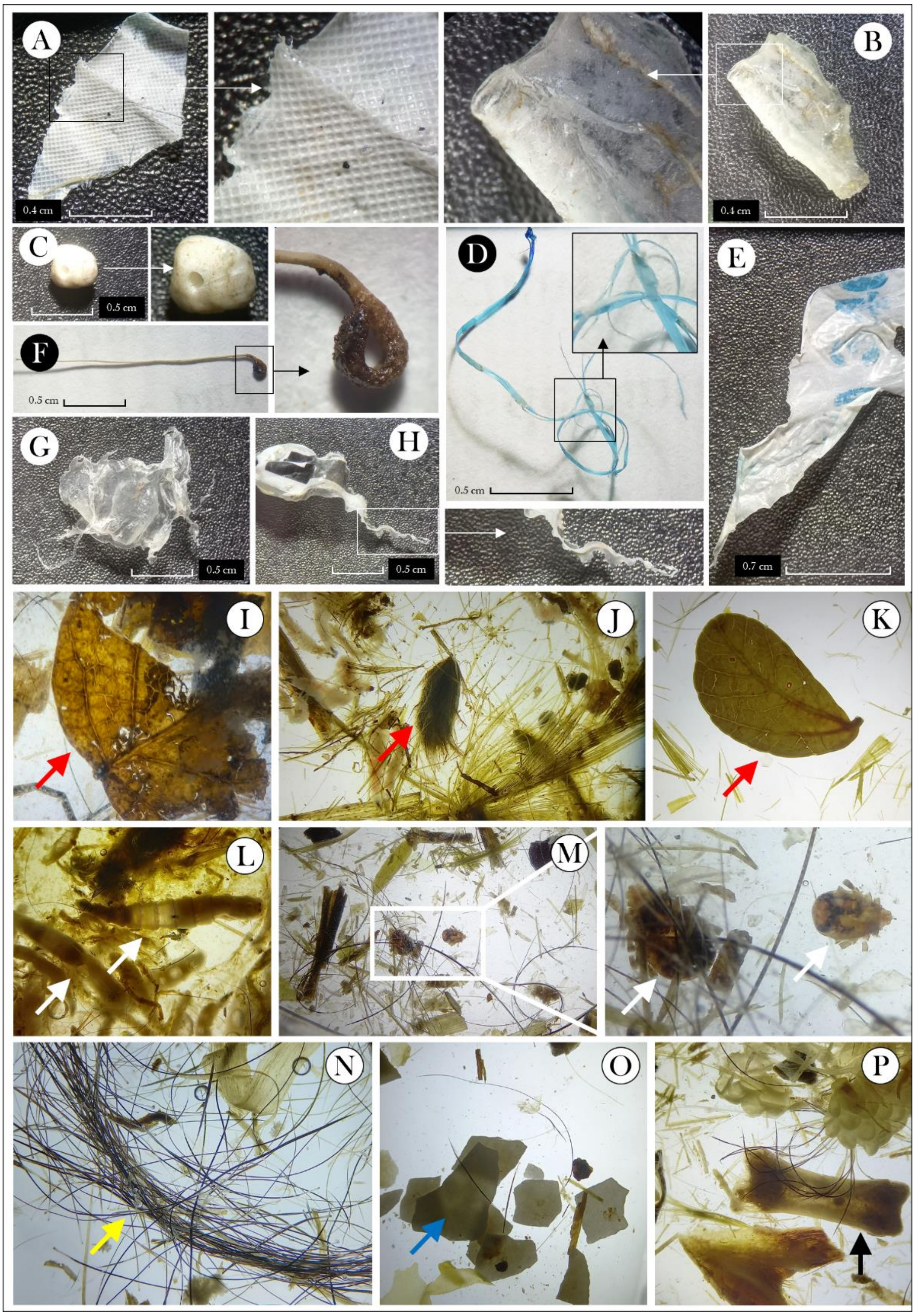


**Figure 3.** Images representing the types, shapes and colors of plastic materials identified in the gut contents of *Coragyps atratus* adults (female and male) captured in landfills (State of Goiás, Brazil). (A, E, G, and H): film; (B): fragment; (C) pellet and (D and F) fiber. Other materials (non-plastic) identified in the gut contents of animals - **(I-K)**: plant material (red arrows); **(L-M)**: insect larvae and tick (white arrows); (N): hair (yellow arrow); (O) eggshell (blue arrow) and **(P)** bone pieces (black arrow). 241

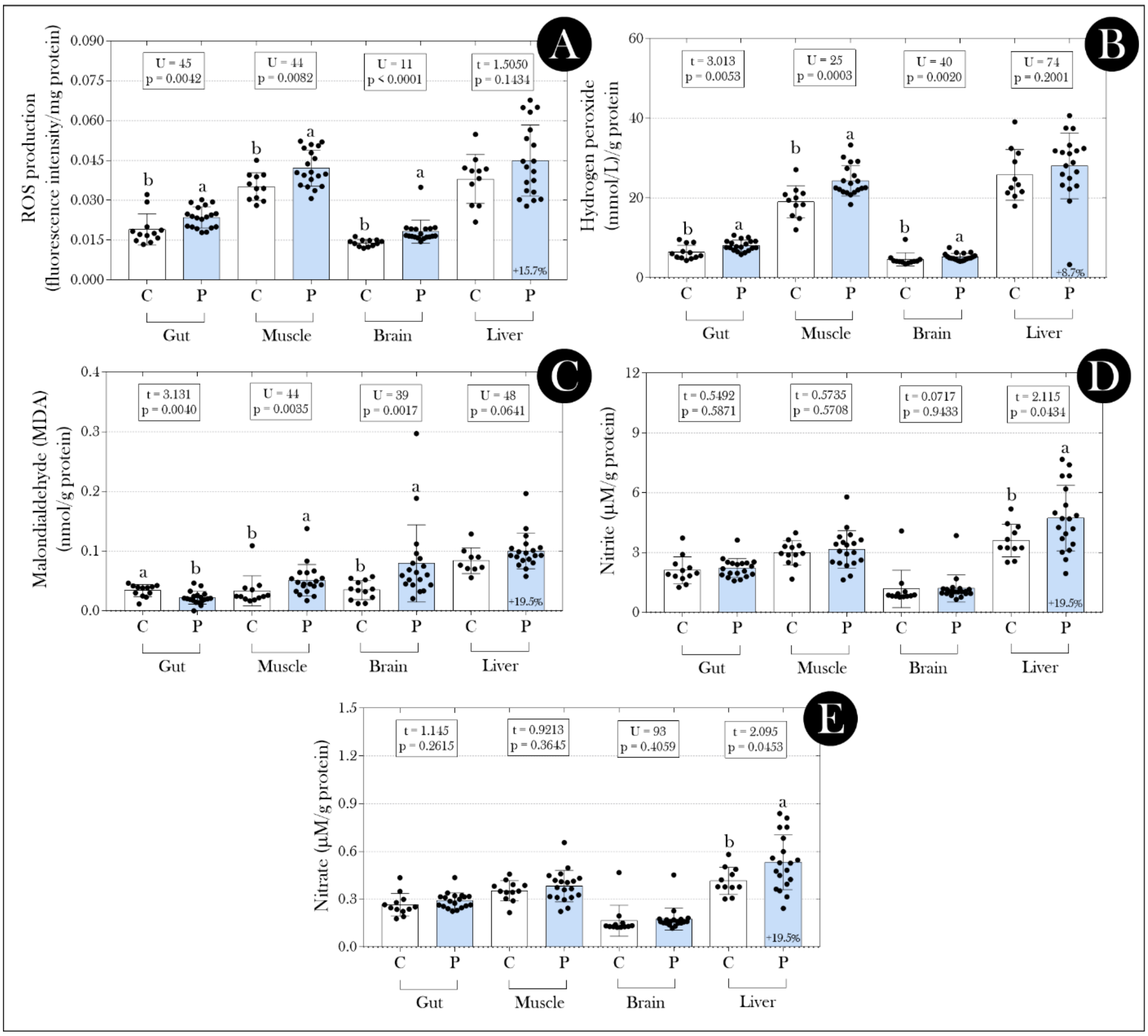


**Figure 4.** Scatter dot plot of production of (A) reactive oxygen species (ROS), (B) hydrogen peroxide (H2O2); (C) malondialdehyde (MDA); (D) nitrite and (E) nitrate in different organs of *Coragyps atratus* (female and male) adults captured in landfills (State of Goiás, Brazil). (n=12, control, and n=19, plastic). The "control" group (C) refers to animals in which no plastic material was identified in the stomach contents and "plastic" group (P) includes animals that have ingested some plastic material. Distinct lowercase letters indicate statistical differences between "C" and "P" groups.

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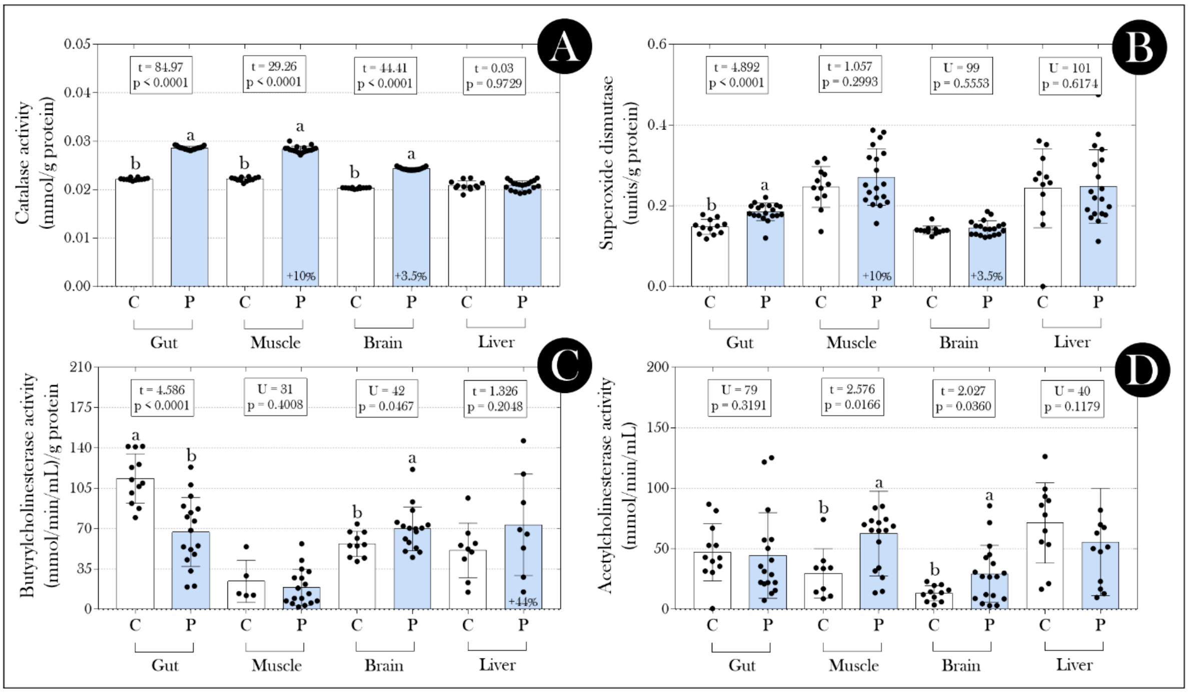

**Figure 5.** Scatter dot plot of (A) catalase (CAT), (B) superoxide dismutase (SOD); (C) butyrylcholinesterase (BChE) and (D) acetylcholinesterase (AChE) activity in different organs of *Coragyps atratus* (female and male) adults captured in landfills (State of Goiás, Brazil). (n=12, control, and n=19, plastic). The "control" group (C) refers to animals in which no plastic material was identified in the stomach contents and "plastic" group (P) includes animals that have ingested some plastic material.

Moreover, we quantified whether sex and the biometric indices assessed were correlated with the deemed biochemical changes. In this case, we noticed that the production of ROS in the gut of females in the "plastic" group was greater (56.5%) than in those in the "control" group (Figure 7A), although in the brain and liver we noticed increases of 31% and 26.3%, respectively (Figure 7B-C). In males, the production of ROS in the brain was higher than that observed in females and males in the "control" group ($p<0.05$) (Figure 7B). The production of H2O2 was higher in the gut ($p<0.05$) of females in the "plastic" group, compared to their respective "control" group ($p<0.05$) (Figure 7E) and in males "with plastics" the production of this biomarker was exceeding that recorded in females "without plastics" (Figure 7H).

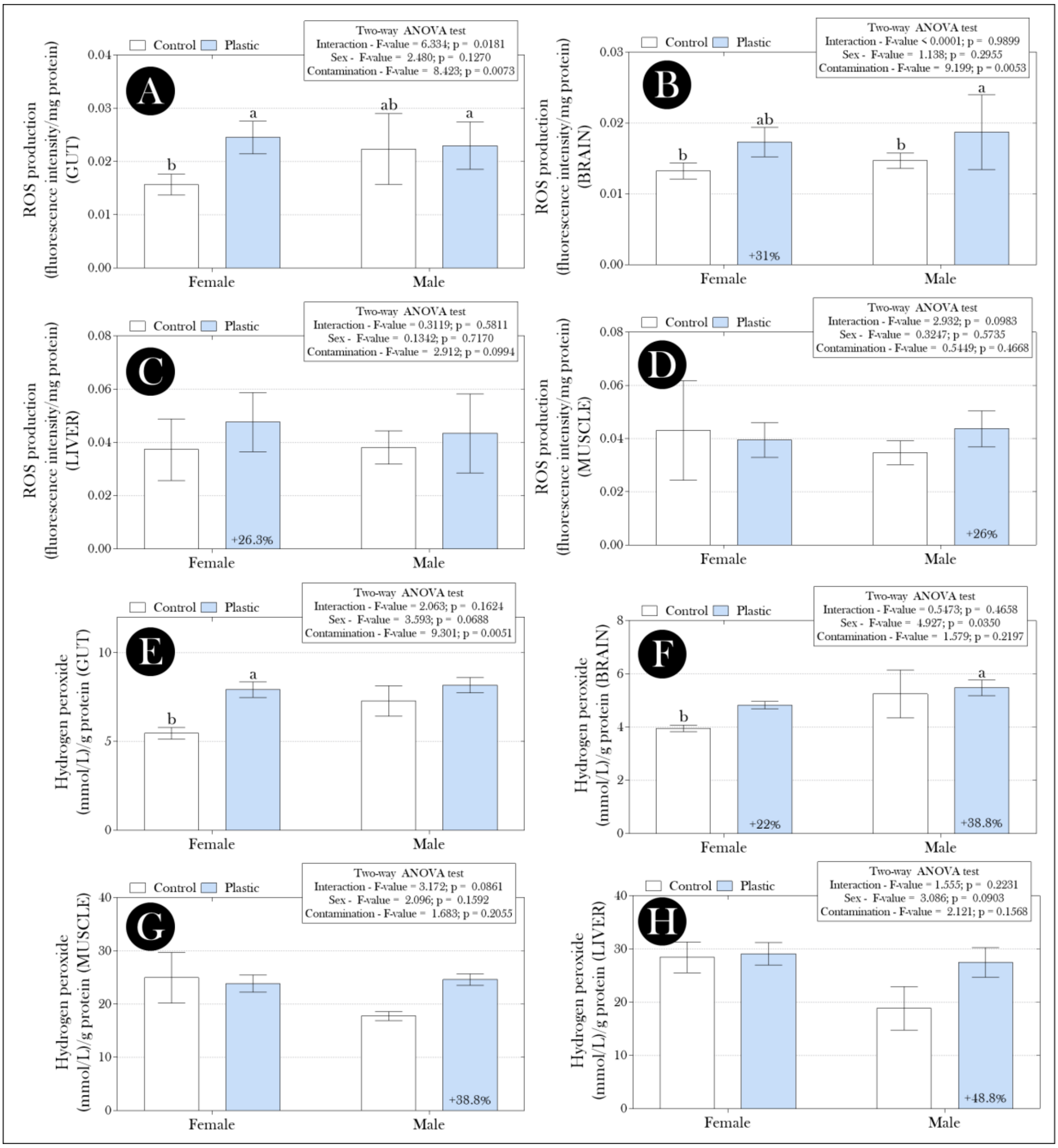

**Figure 6.** Production of reactive oxygen species (ROS) [(A) in gut, (B) brain, (C) liver and (D) muscle] and hydrogen peroxide (H2O2) [(E) in gut, (F) brain, (G) muscle and (H) liver] in *Coragyps atratus* (female and male) adults captured in landfills (State of Goiás, Brazil). The bars represent the mean ± SEM (n=12, control, and n=19, plastic). The "control" group (C) refers to animals in which no plastic material was identified in the stomach contents and "plastic" group (P) includes animals that have ingested some plastic material. Distinct lowercase letters indicate statistical differences between groups.

Concerning nitrite and nitrate, its production was higher in the liver of animals (p<0.05)) that ingested the plastic materials (both females and males) (Figures 8D-H, respectively) and, in the gut, increases greater than 30% were observed in females in the “plastic” group compared to those in the “control” group (Figure 8A-E). On the other hand, MDA production in muscle and brain of females “with plastics” (compared to those “without plastics”) was greater than 65% (Figure 9A-B, respectively). In addition, although not statistically significant, muscle and brain MDA levels in females in the “plastic” group were 30.2% and 82.1% higher than those recorded in males in that same group (Figures 9A-B). As for the enzymes evaluated, we did not observe any effect of the factor’s “sex” and “contamination” on the SOD activity in the evaluated organs (Figure 10A-D); but, except for the liver, the CAT activity was higher in animals in the “plastic” group (both females and males) in relation to their respective “control” groups (Figure 10E-H). On the other hand, we noticed in animals that ingested plastics a significant reduction in BChE activity in the gut (Figure 11A) and an increase in muscle and brain AChE (Figures 11F-G, respectively), regardless of sex. Plus, we observed that plastic ingestion was not correlated with any change in the animals' body condition [inferred by the different calculated biometric indices (Figure S3)] and that size (area) (Figures S4-5) and shape (circularity) (Figures S6-7) of plastic materials were not correlated with most biochemical biomarkers assessed.

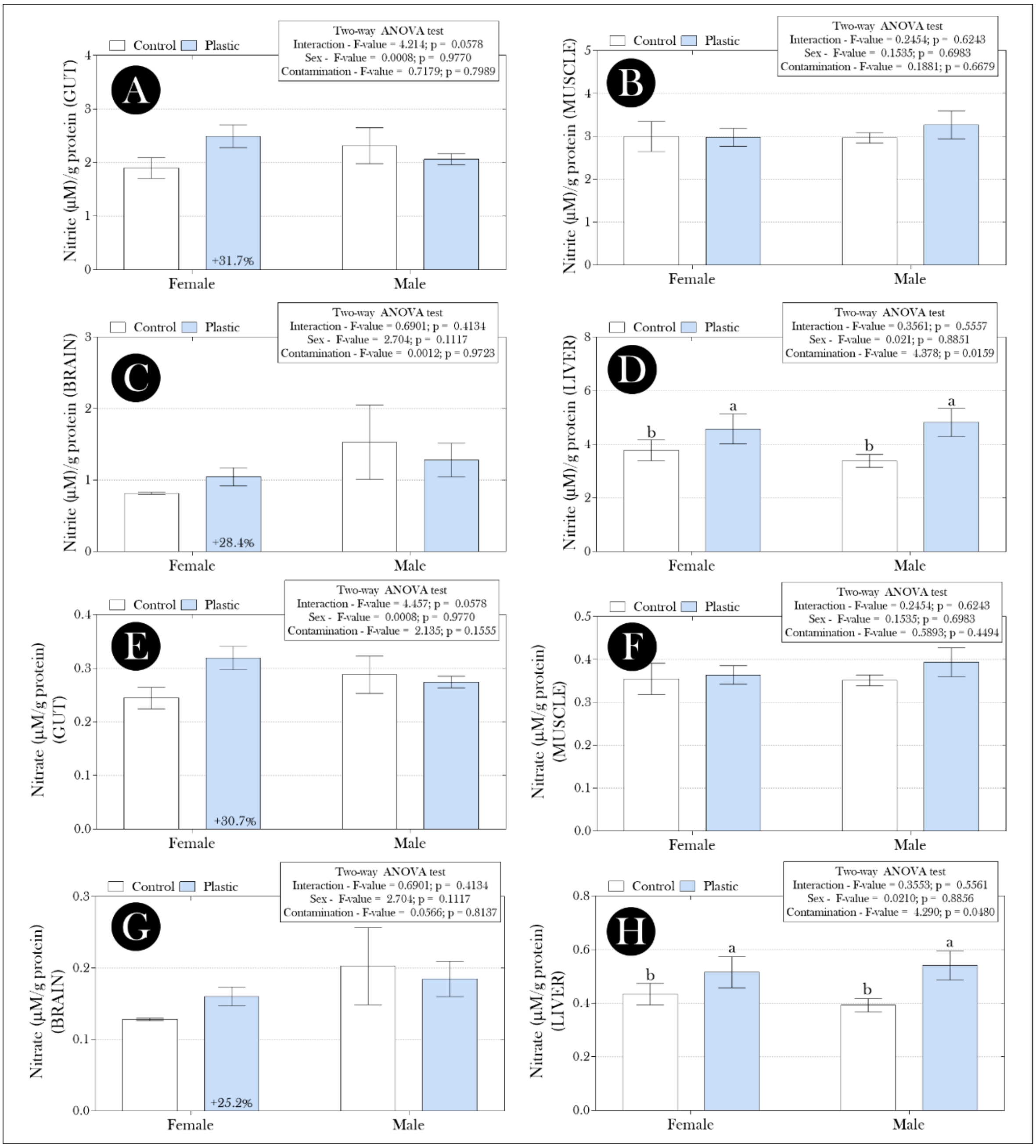

**Figure 7.** Production of nitrite [(A) in gut, (B) muscle, (C) brain and (D) liver] and nitrate [(E) in gut, (F) muscle, (G) brain and (H) liver] in *Coragyps atratus* (female and male) adults captured in landfills (State of Goiás, Brazil). The bars represent the mean ± SEM (n=12, control; and n=19, plastic). The “control” group (C) refers to animals in which no plastic material was identified in the stomach contents and “plastic” (P) includes animals that have ingested some plastic material. Distinct lowercase letters indicate statistical differences between groups.

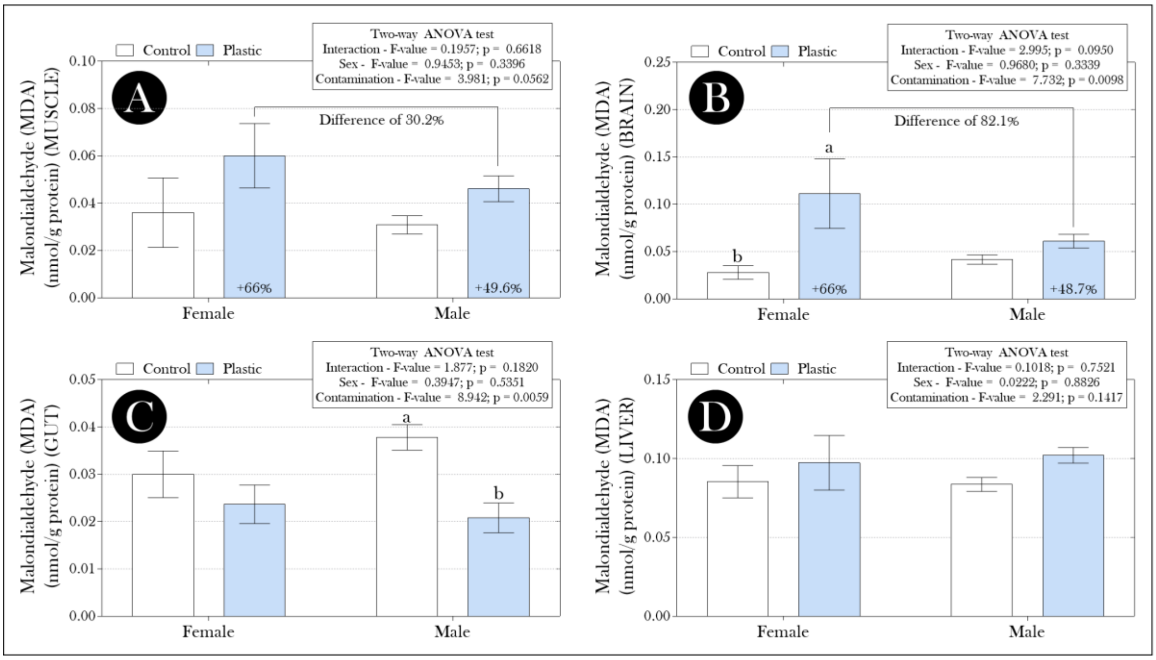

**Figure 8.** Malondialdehyde (MDA) production [(A) in muscle, (B) brain, (C) gut and (D) liver] in *Coragyps atratus* (female and male) adults captured in landfills (State of Goiás, Brazil). The bars represent the mean ± SEM (n=12, control; and n=19, plastic). The "control" group refers to animals in which no plastic material was identified in the stomach contents and "plastic" includes animals that have ingested some plastic material. Distinct lowercase letters indicate statistical differences between groups.

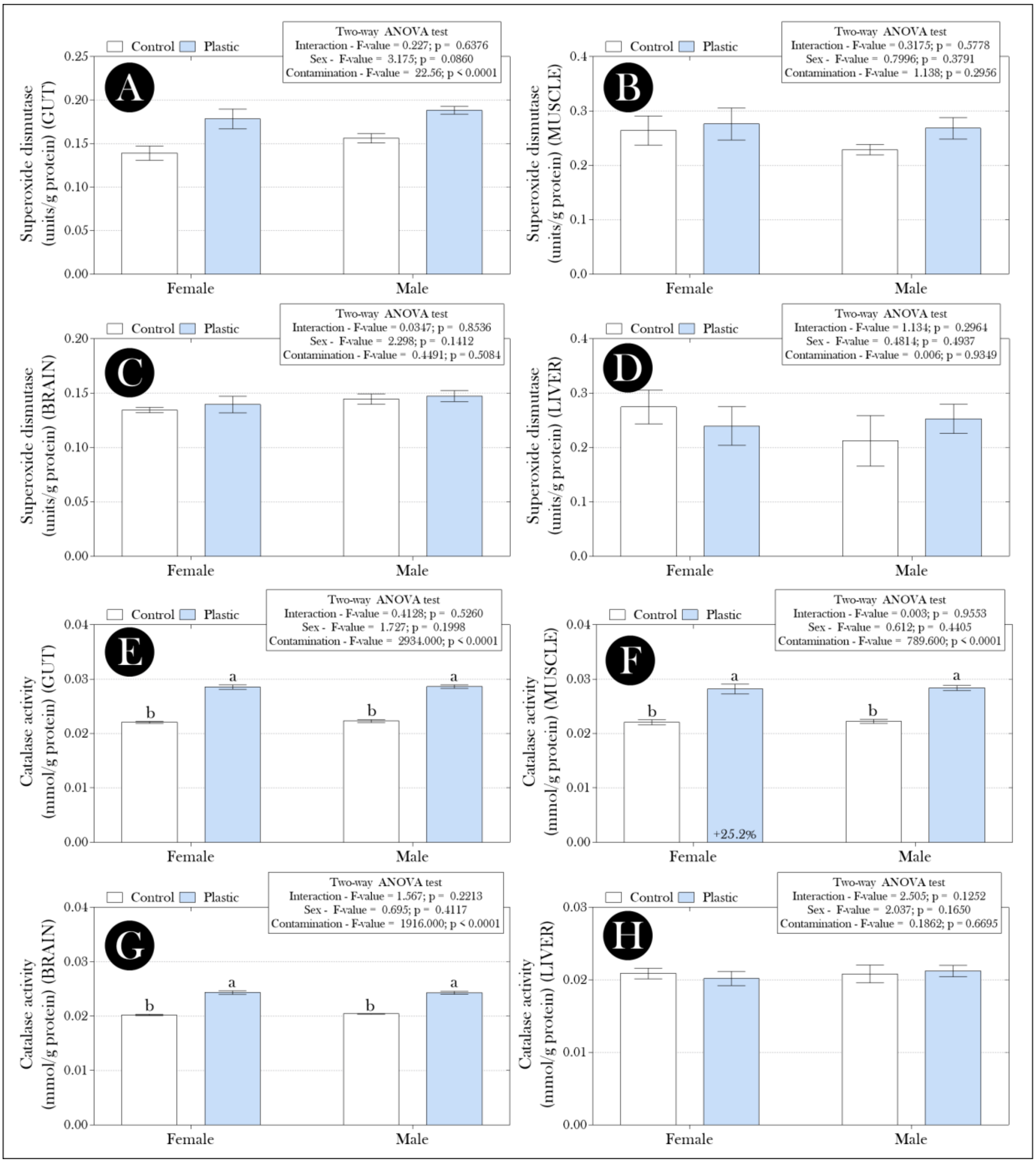

**Figure 9.** Superoxide dismutase (SOD) enzymes [(A) in gut, (B) muscle, (C) brain and (D) liver] and catalase (CAT) activity [(E) in gut, (F) muscle, (G) brain and (H) liver] in *Coragyps atratus* (female and male) adults captured in landfills (State of Goiás, Brazil). The bars represent the mean ± SEM (n=12, control; and n=19, plastic). The "control" group refers to animals in which no plastic material was identified in the stomach contents and "plastic" includes animals that have ingested some plastic material. Distinct lowercase letters indicate statistical differences between groups.

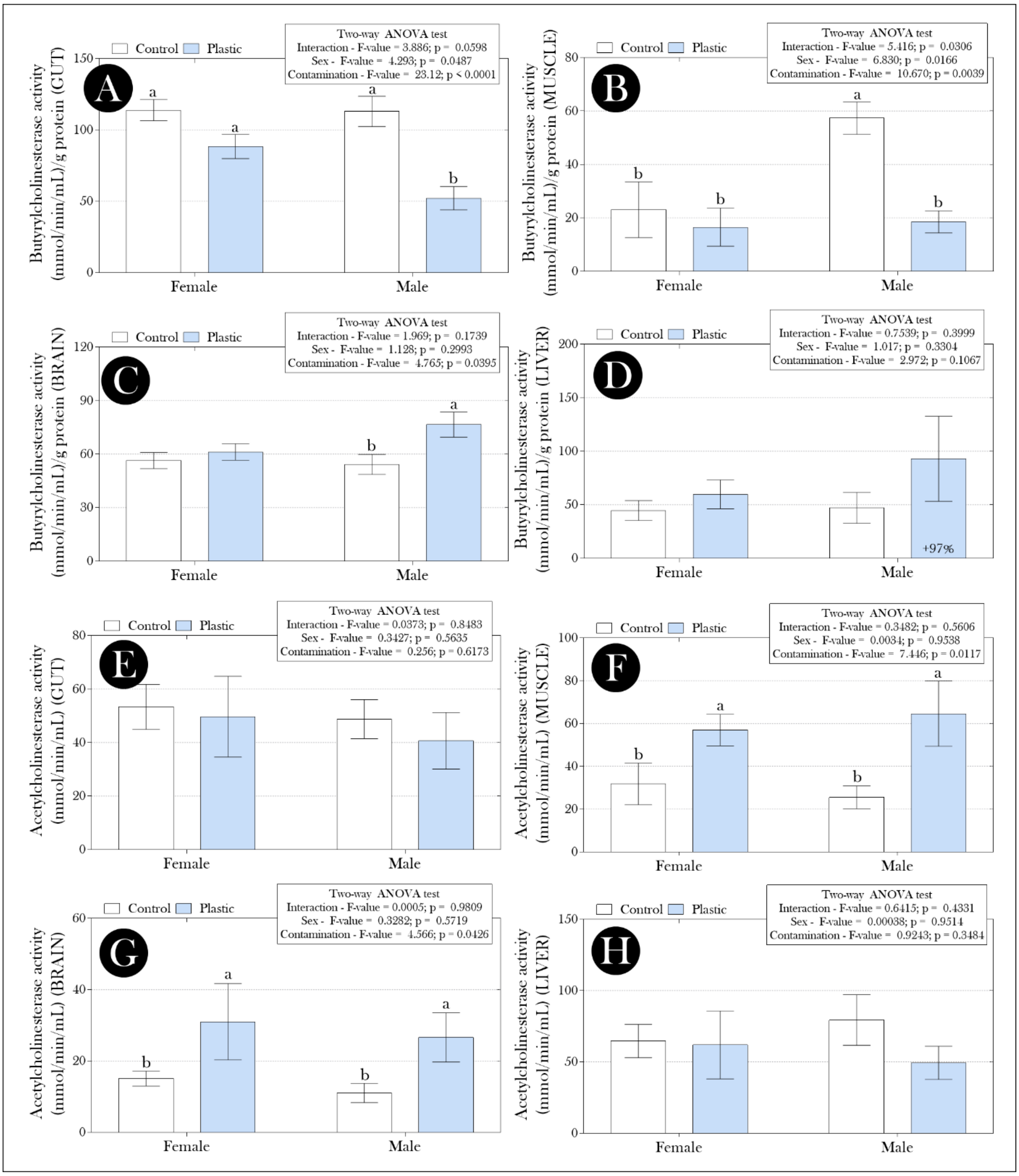

**Figure 10.** Butyrylcholinesterase (BChE) enzymes [(A) in gut, (B) muscle, (C) brain and (D) liver] and acetylcholinesterase (AChE) activity [(E) in gut, (F) muscle, (G) brain and (H) liver] in *Coragyps atratus* (female and male) adults captured in landfills in two municipalities in the State of Goiás (Brazil). The bars represent the mean ± SEM (n=12, control; and n=19, plastic). The "control" group refers to animals in which no plastic material was identified in the stomach contents and "plastic" includes animals that have ingested some plastic material. Distinct lowercase letters indicate statistical differences between groups.

## 4. DISCUSSION

It is readily apparent that the identification, characterization, and evaluation of the impacts of plastic ingestion by birds constitute an important opportunity to predict the ecotoxicological impacts of these materials on avifauna. Based on studies like ours, subsidies are acquirable for planning actions related not only to pollution remediation, but also to species conservation. Focusing on vultures (*C. atratus species*) we confirm that these individuals are subject to involuntary ingestion of plastic material disposed in the investigated areas (landfills). From the visual inspection and polymeric chemical characterization of the identified materials, we note that plastic bags (Figures 4E, 5B and 5D) and probably food packaging (Figures 3I and 4G) (mainly made of low-density polyethylene - Figure 1F) constitute the main sources of material ingested by animals, which are commonly present in waste disposed of in landfills (Quaghebeur et al., 2013; Osra et al., 2021).

Substitutable results have been documented in studies involving other species of the Cathartidae family, such as in the works by Kelly et al. (2007) (*Coragyps atratus* and *Cathartes aura*), Ballejo & De-Santis (2013) and Borges-Ramírez et al. (2021) (*Coragyps atratus*), Torres-Murra et al. (2015) and Augé (2017) (*Cathartes aura*) and Ballejo et al. (2021) (*Vultur gryphus, Coragyps atratus* and *Cathartes aura*). In most of these studies, like what we observed, the plastic materials identified (especially in animal feces) had an appearance compatible with pieces of plastic bags, as well as small pieces of plastic with sharp or pointed edges. On the other hand, the identification of polystyrene polymers (styrofoam pellets) (Figures 3K and 4B), polybutadiene fragment (tire constituent) (Figure 3E) and poly (methyl methacrylate) (common constituent of acrylic material used in construction civil – Figures 3G and 3J) demonstrate how variable the type of material ingested by adults of *C. atratus* can be. In this case, it is possible that this is associated not only with the animals' capture sites, but also with their eating habits and tolerance to highly urbanized sites.

However, an unexplored field in the aforementioned studies refers to the impacts of plastic material ingestion on the health of these animals, which demonstrates that plastic ingestion by species of the Cathartidae family has not received much attention, compared to other important threats, such as poisoning by agricultural pesticides (Plaza et al., 2019) and contamination by heavy metals such as lead (Plaza & Lambertucci, 2018), aluminum (Borges-Ramírez et al., 2021), cadmium, copper and zinc (López-Berenguer et al., 2021). This is especially disturbing, since the ingestion of plastic material by vultures may be inducing silent harmful effects on their populations which, added to the impacts caused by other pollutants, increase the threat of anthropogenic activities to the survival of the species. In our study we observed that although the plastic materials identified in the stomach of *C. atratus* were not correlated with changes in the biometric indices (ie: body conditions) of the animals (Figure S3), biochemical differences were observed between the "control" and "plastic" groups" denounce for the first time some of the physiological consequences arising from these materials on the health of the individuals analyzed. The high production of ROS (Figure 5A), H2O2 (Figure 5B) and MDA (Figure 5C) especially in the gut, muscle, and brain of animals in the "plastic" group suggest the induction of oxidative stress possibly motivated by the ingestion of plastic materials, whose antioxidant activity (measured by CAT and SOD enzymes – Figures 6A-B, respectively) seems not to have been sufficient to counteract cellular oxidative processes. Similar results have already been reported in the context of exposure of different animal groups to plastic materials [see review by Hu & Palić, (2020)]. In the liver, the increased production of nitrite and nitrate (Figures 5D-E, respectively) also suggests hepatic alterations that may be related both to the activation of the immune response mediated especially by Kupffe cells, and to a nitrosative stress from the oxide isoform inducible nitric synthase (iNOS).

Obviously, the assumption of any mechanism of action responsible for inducing the biochemical alterations observed in our study is preliminary. However, it is tempting to speculate that in the gut, particularly, the high production of ROS and H2O2 (Figures 5A-B, respectively), as well as of CAT and SOD (Figures 6A-B, respectively) may be related to physical and chemical impacts induced by plastic materials, especially by those classified as "fragments". Physical changes (most obvious) include injuries and perforation of the intestinal epithelium [caused by sharp and sharp plastic fragments – e.g.: Figure 3G and 3J – already reported in seabirds, by Roman et al. (2019)], inflammatory processes (Pirsaheb et al., 2020), as well as digestive obstruction [caused by the accumulation of pieces of bags (Figure 3D) or plastic fibers (Figure 4D), e.g.: – also reported in *Puffinus gravis* and *Morus bassanus*, by Pierce et al. (2004)]. On the other hand, we cannot neglect the hypothesis that these changes are related (direct or indirect) with the release of additives/chemical compounds used in the manufacture of plastics, particularly during the digestive action in the stomach of birds, as well as the presence of MPs/NPs (non-targets of our study). Regardless of the mechanism responsible for the changes, developments in oxi-reduction processes can lead to intestinal dysfunctions, crucial for the maintenance of the animals' energy homeostasis, with negative physiological consequences for the animals. Intestinal alterations can, for example, totally compromise, in some cases, the physiological functions of the intestines, directly affecting the digestive and absorptive processes (Peda et al., 2016).

In muscle, the REDOX imbalance (marked by increased production of ROS, H2O2 and CAT activity – Figures 5A-B and 6A, respectively) observed in animals from the "plastic" group may be associated with the accumulation of MPs (diameter not identified in our study), and indirect impacts caused by macroplastics. In this case, physiological disorders in the birds' pectoral muscles can cause changes in their locomotor abilities, especially those related to flight, considering that such muscles are substantial to produce the aerodynamic force necessary to support the animal's weight in the air and to overcome drag (Cao & Jin, 2020). On the other hand, increased oxidative processes in the brain of these animals [marked by the increased production of ROS (Figure 5A), H2O2 (Figure 5B), MDA (Figure 5C) and CAT (Figure 6A)] may also be directly or indirectly associated with the ingestion of macroplastics or by MPs/NPs accumulated in the central nervous system, which can impact various physiological processes in animals. This includes everything from the vital functions of different organs to the modulation of their behavior. Furthermore, it is important to emphasize that REDOX imbalance can also be related to inflammatory and immune responses. Both the nitrosative stress observed in the animals' livers (Figures 5D-E) and other previous studies [see review by Hu & Palić (2020)] reinforce this hypothesis.

On the other hand, the increased activity of BChE (in the brain) and AChE (in the muscle and brain) of the "plastic" group vultures (Figures 6C-D respectively), suggests a stimulatory effect on the animals' cholinergic system. In this case, previous studies support the hypothesis that these increases are associated with the oxidative stress observed in these same organs. Schallreuter et al. (2004) and Garcimartín et al. (2017) reported, in vitro, that the high concentration of H2O2, for example, stimulated cholinesterase activity. On the other hand, the increase in cerebral BChE activity may be related to a dysfunction in the AChE activity, whose increase observed in the muscle and brain of these animals (Figure 6D) would constitute a compensatory physiological mechanism. As discussed by Mesulam et al. (2002), BChE plays an important role in supporting AChE in the regulation of cholinergic transmission, especially in the absence or inefficiency of this enzyme.

On the other side, it is possible that the oxidative stress observed in the brain of these animals and the increase in lipid peroxidation processes (inferred by the high levels of MDA – Figure 5C) may have contributed to greater release of acetylcholine and butyrylcholine in the cholinergic synaptic clefts and to the overstimulation of postsynaptic receptors, culminating in the consequent increase in BChE and AChE activity. This hypothesis, in particular, is supported by Barboza et al. (2020) who, at the time, associated the increase in lipid peroxidation and AChE levels to the accumulation of MPs in the muscle, gills and brain of *Dicentrarchus labrax*, *Trachurus trachurus* and *Scomber colonies*. On the other hand, it is known that the increase in AChE and BChE has also been associated with the presence of inflammatory processes, compared to healthy individuals (De-Oliveira et al., 2012; Santarpia et al., 2013), which also may explain its increased levels in animals of the "plastic" group. Regardless of the mechanisms involved, the increased activity of AChE and BChE in the brain can be considered a predictive response of neurological changes, whose consequences are likely to affect the fitness of individuals, increase energy demand, induce discoordination, behavioral changes, among others. On the other hand, the increased activity of these enzymes in muscles can induce nicotinic effects, which are the result of sympathetic hyperactivity and neuromuscular dysfunction.

Obviously, knowledge of the mechanisms underlying these different responses requires further studies focusing on more specific biomarkers correlated with the sex of *C. atratus*. However, it has been shown in studies involving other animal models that the response of males and females exposed to different pollutants, in fact, can be differentiated (Bao et al., 2020; Gade et al., 2021; Vega et al., 2021; Kochi et al., 2021; Gokulan et al., 2021). Overall, these studies have hypothesized that sex-based differential susceptibility is related to sexual dimorphisms in anatomy, gray matter distribution, hormones, and/or epigenetic and metabolic factors. In *Coturnix coturnix japonica*, for example, differences between sexes were recorded in the response of microbiota to trichlorfon (organophosphate insecticide) (Crisol-Martínez et al., 2016). Erikstad et al. (2013) demonstrated that *Larus hyperboreus* females contaminated with organochlorines are more sensitive to the negative effects of these pesticides, when compared to males. In *Ficedula hypoleuca* adults that live in areas contaminated by heavy metals, Eeva et al. (2006) predicted lower local survival of males in polluted areas compared to females. On the other hand, we observed that the chronic ingestion of water containing tannery effluent by *Melopsittacus undulates* (female and males) adults induced a similar mutagenic effect in both sexes (Souza et al., 2017). Therefore, it is noted that the sex-dependent response to pollutants is influenced not only by the pollutant and its accumulation levels, but also by the avifauna species and its physiological characteristics, which should be better studied in the future.

Eventually, it is noteworthy that our data shed light on the toxic effects of plastic materials ingested by *C. atratus* adults, which had not been previously studied. Therefore, evidence is provided to reinforce the hypothesis that plastic materials may be harming the health of these animals, with consequences that are still poorly understood. The absence of a concentration-dependent, size and shape (of plastics)-dependent effect observed in our study emerges as an additional concern, since the simple presence of plastic material in the gastrointestinal system of these birds (regardless of number, size, and shape) can trigger harmful physiological changes. Obviously, any robust conclusion about the extent to which these materials have contributed to the population decline of the studied species and other representatives of the Cathartidae family is incipient. However, it is known that any change in REDOX homeostasis or cholinesterase effect represents a risk to the health of these birds, with negative consequences for the fitness of individuals, including their reproductive performance, eating habits, behaviors, as well as their adaptive plasticity. Thus, investigations focusing on the impacts of plastic ingestion on these aspects emerge as investigative perspectives that will contribute to a better understanding of how plastic pollution may be enhancing the negative effect of other pollutants on the health of vultures

## 5. CONCLUSION

To sum up, our study confirms the hypothesis that plastic material ingestion by *C. atratus* adults causes sex- and organ-dependent biochemical alterations predictive of REDOX imbalance and cholinesterase effect without necessarily affecting the body conditions of these animals. We also revealed that the small amount of ingested plastic seems to be enough to trigger negative physiological responses in animals. Holistically, this may not only be an issue for vulture populations but also for a range of bird species that inhabit highly polluted areas (such as landfills) that may ingest or be affected by anthropogenic debris, in particular plastic bags. Thus, we defend the idea that continued monitoring of the impact of potential contaminants including plastics on vultures is necessary for the conservation of these animals, whose ecological importance goes far beyond their low popularity among humans.

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# SUPPLEMENTARY MATERIAL

**Table S1.** Biometric biomarkers evaluated in Coragyps atratus (female and male) adults captured in landfills (State of Goiás, Brazil).

| **Female** | | | | | **Statistical summary**<br>**Female *vs.* male** |
|---|---|---|---|---|---|
| **Parameters** | **Mean** | **Std. Deviation** | **Std. Error of Mean** | **Coefficient of variation (%)** | |
| Body mass (kg) | 2.225 | 0.234 | 0.058 | 10.26 | Body mass (kg): t = 0.9046; p = 0.3702 |
| Body length (cm) | 58.140 | 5.761 | 1.440 | 9,91 | Body length (cm): U = 271.5; p = 0.9959 |
| Wing length (cm) | 40.800 | 9.257 | 2.314 | 23.95 | Wing length (cm): U = 196.5; p = 0.1518 |
| Beak length (cm) | 5.100 | 0.406 | 0.1016 | 7.99 | Beak length (cm): t = 1.114; p = 0.2709 |
| Head length (cm) | 6.000 | 0.539 | 0.134 | 8.93 | Head length (cm): t = 0.749; p = 0.4573 |
| Wingspan (cm) | 128.900 | 6.355 | 1.589 | 4.93 | Wingspan (cm): U = 268.5; p = 0.9466 |
| Tarsus length (cm) | 8.625 | 0.366 | 0.091 | 4.24 | Tarsus length (cm): t = 0.299; p = 0.7656 |
| **Male** | | | | | |
| Body mass (kg) | 2.187 | 0.391 | 0.067 | 17.92 | |
| Body length (cm) | 57.710 | 9.699 | 1.663 | 16.81 | |
| Wing length (cm) | 41.220 | 1.441 | 0.250 | 3.50 | |
| Beak length (cm) | 5.253 | 0.523 | 0.089 | 9.97 | |
| Head length (cm) | 5.921 | 0.503 | 0.086 | 8.50 | |
| Wingspan (cm) | 128.2 | 9.278 | 1.591 | 7.24 | |
| Tarsus length (cm) | 8.700 | 0.603 | 0.105 | 7.03 | |

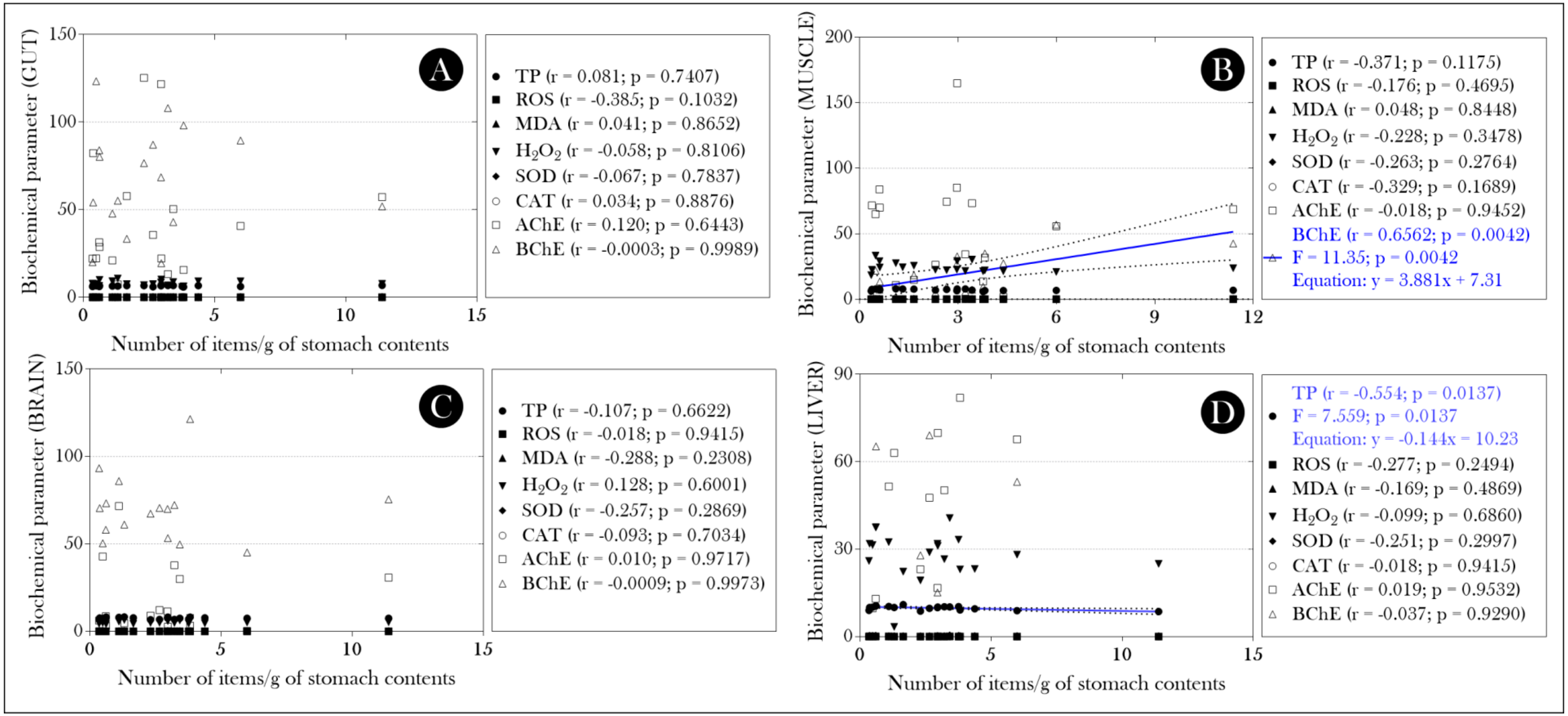

**Figure S1.** Correlation analysis between plastic material concentrations (in number of plastic items/g of stomach contents - dry weight) and different biochemical biomarkers evaluated in (A) gut, (B) muscle, (C) brain and (D) liver of *Coragyps atratus* (female and male) adults captured in landfills (State of Goiás, Brazil). TP: total proteins, ROS: reactive oxygen species, MDA: malondialdehyde, H2O2: hydrogen peroxide, SOD: superoxide dismutase, CAT: catalase, AChE: acetylcholinesterase and BChE: butyrylcholinesterase.

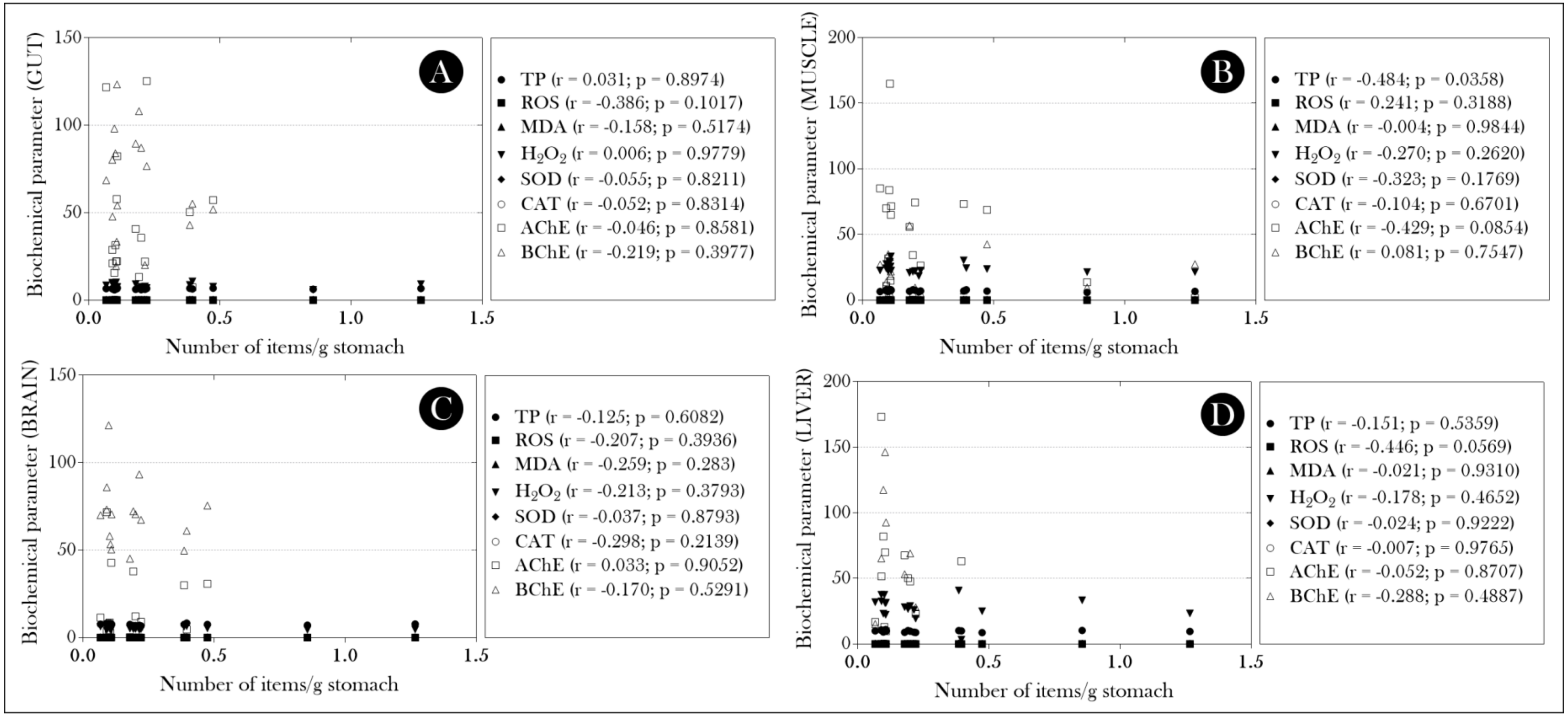

**Figure S2.** Correlation analysis between plastic material concentrations (in number of plastic items/g of stomach – fresh weight) and different biochemical biomarkers evaluated in (A) gut, (B) muscle, (C) brain and (D) liver of *Coragyps atratus* (female and male) adults captured in landfills (State of Goiás, Brazil). TP: total proteins, ROS: reactive oxygen species, MDA: malondialdehyde, H2O2: hydrogen peroxide, SOD: superoxide dismutase, CAT: catalase, AChE: acetylcholinesterase and BChE: butyrylcholinesterase.

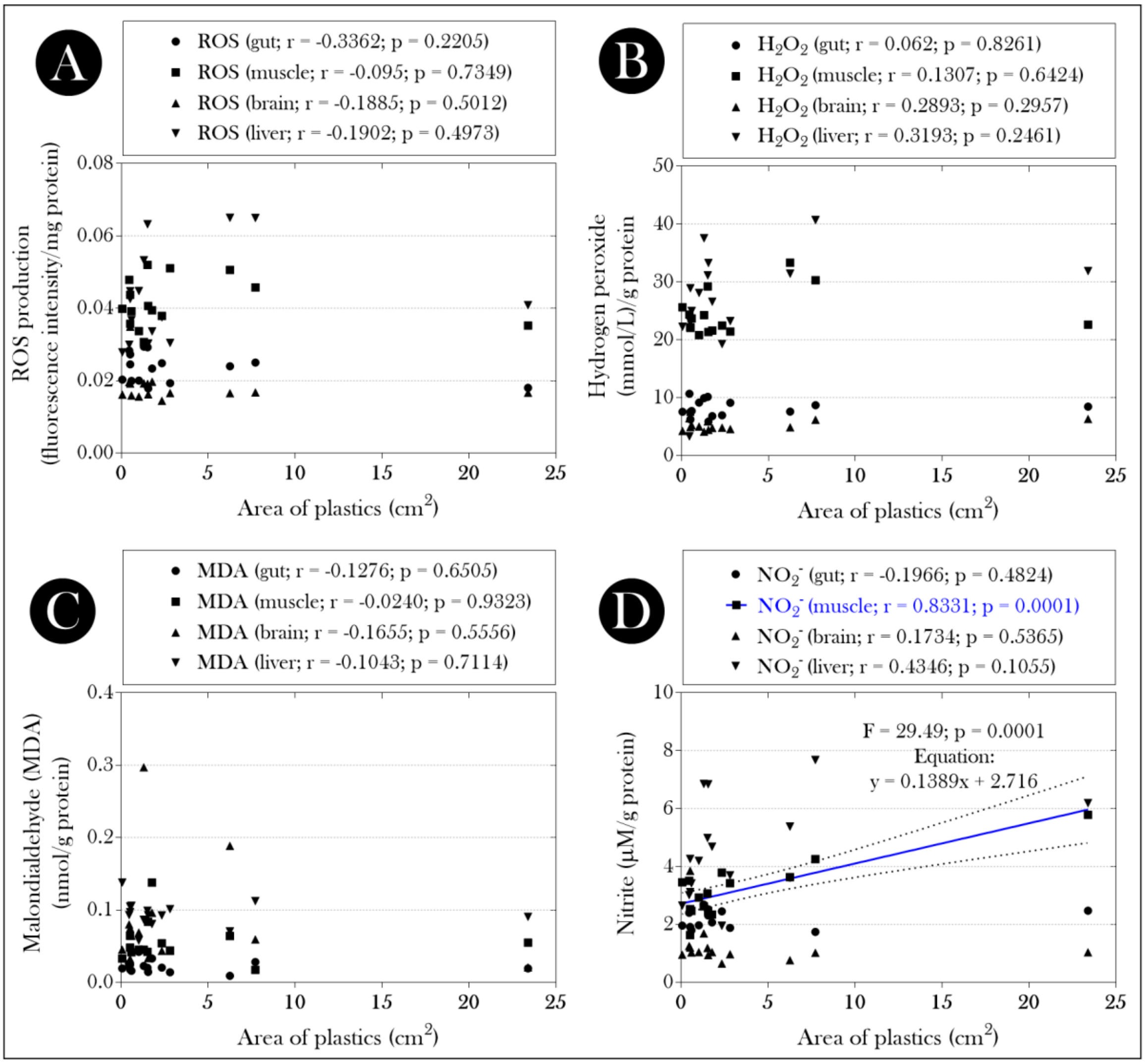

**Figure S3.** Correlation analysis between the size of plastic materials (area – cm2) and the levels of (A) reactive oxygen species (ROS), hydrogen peroxide (H2O2), (C) malondialdehyde (MDA) and (D) nitrite in different organs of *Coragyps atratus* (female and male) adults captured in landfills (State of Goiás, Brazil).

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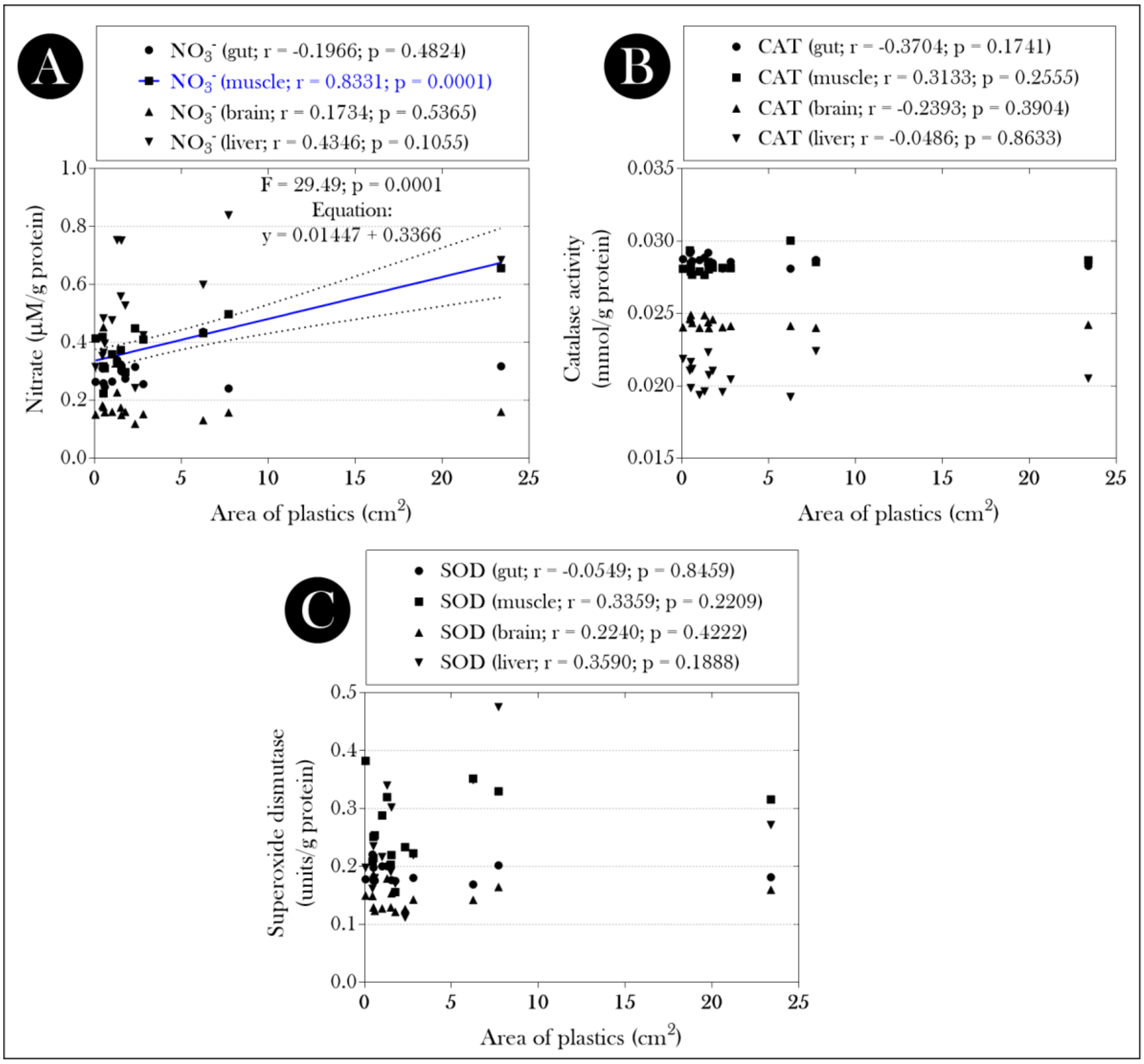

**Figure S4.** Correlation analysis between the size of plastic materials (area - cm2) and the levels of (A) nitrate, (B) catalase (CAT) and (C) superoxide dismutase (SOD) in different organs of *Coragyps atratus* (females and males) adults captured in landfills (State of Goiás, Brazil).

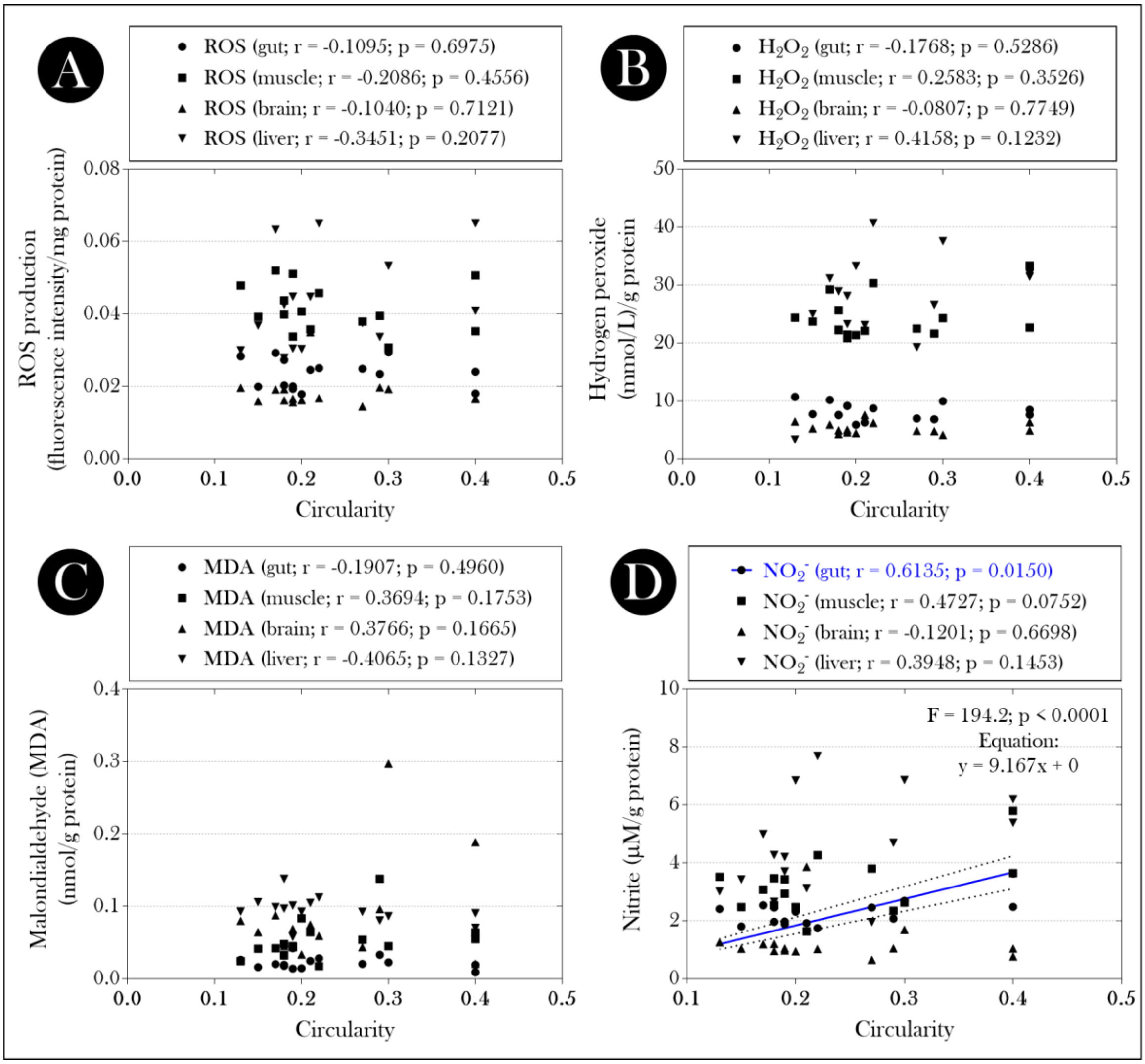

**Figure S5.** Correlation analysis between the shape of plastic materials (circularity) and the levels of (A) reactive oxygen species (ROS), (B) hydrogen peroxide (H2O2), (C) malondialdehyde (MDA) and (D) nitrite in distinct organs of *Coragyps atratus* (female and male) adults captured in landfills (State of Goiás, Brazil).

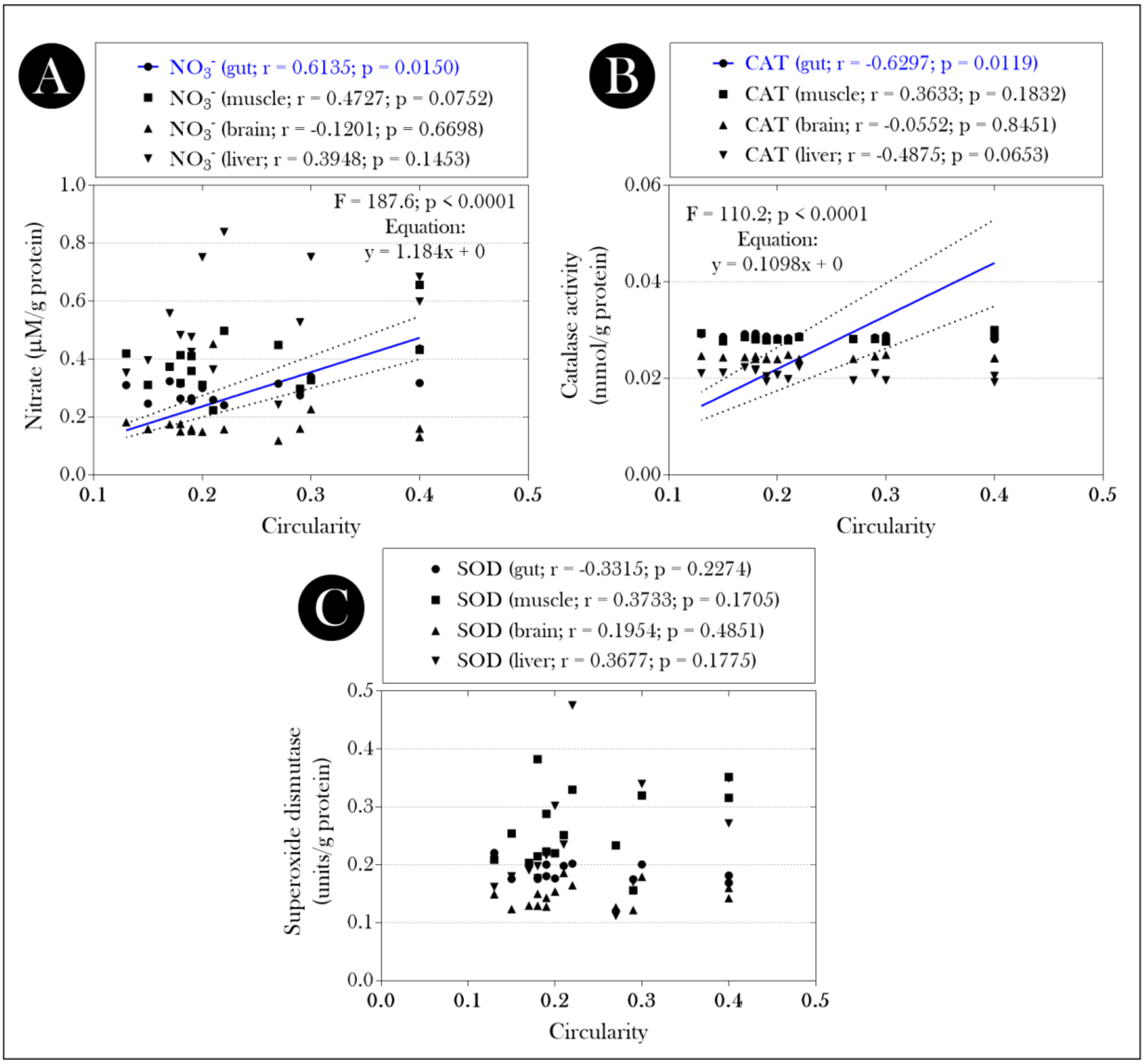

**Figure S6.** Correlation analysis between the shape of plastic materials (circularity) and the levels of (A) nitrate, (B) catalase (CAT) and (C) superoxide dismutase (SOD) in different organs of *Coragyps atratus* (female and male) adults captured in landfills (State of Goiás, Brazil).

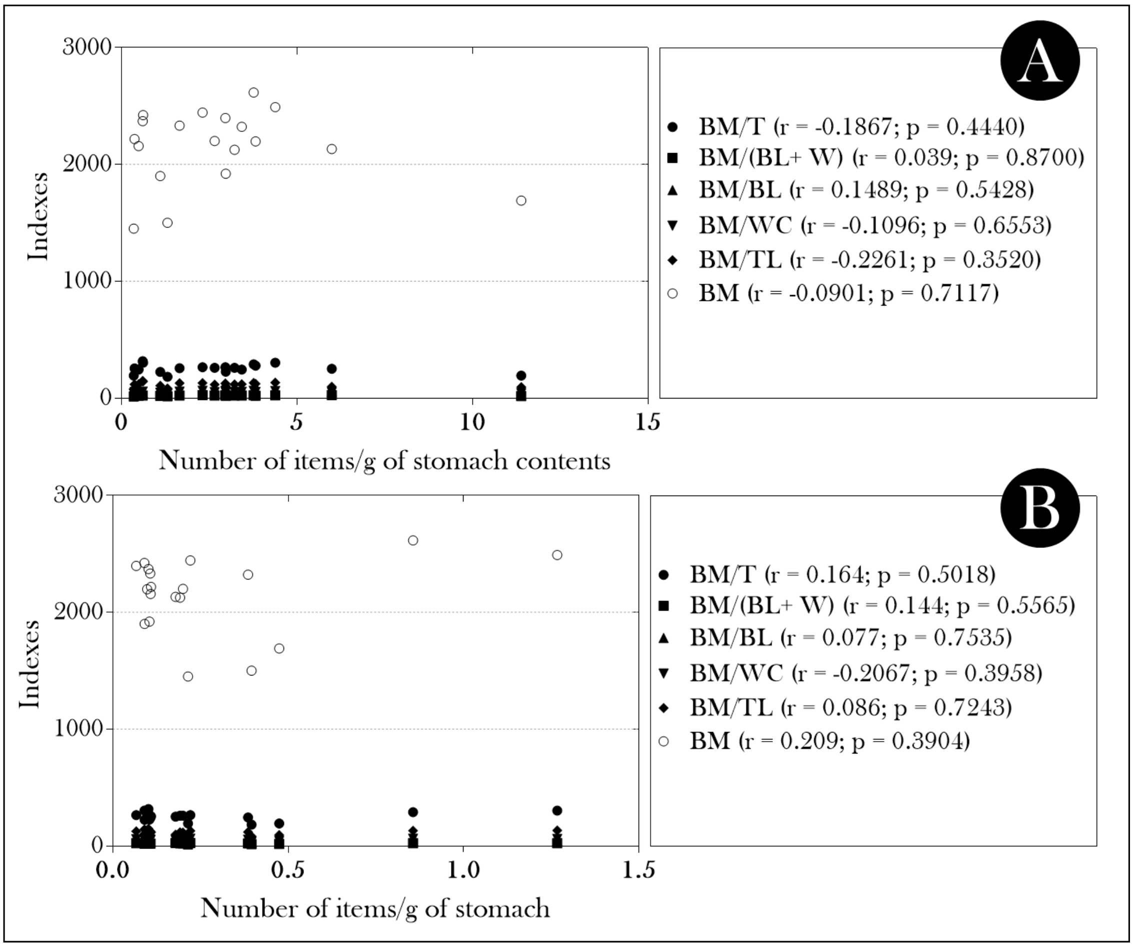

**Figure S7.** Correlation analysis between plastic material concentrations in (A) number of plastic items/g of stomach contents (dry weight) and (B) number of plastic items/g of stomach (fresh weight) and different biometric indices calculated for *Coragyps atratus* (female and male) adults captured in landfills (State of Goiás, Brazil). BM: body mass, T: tarsus, BL: body length, WC: wing length and TL: tail length.

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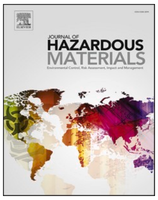

# From carrion-eaters to plastic material plunderers: Toxicological impacts of plastic ingestion on black vultures, *Coragyps atratus* (Cathartiformes: Cathartidae)

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## ABSTRACT

Despite plastic ingestion has already been reported in several bird species, its physiological impacts have been little inspected, especially in representatives of the Cathartidae family. Thus, in this study, we aimed to identify, characterize, and evaluate the effects arising from the ingestion of plastic materials by *Coragyps atratus* adults, that captured in landfill areas. Herein, a total of 51 individuals were captured, the frequency of plastic intake being higher than 40%. The plastic materials consisted mainly of low-density polyethylene and film-type polystyrene, as well as presenting irregular shapes and diameters between 10 and 30 mm. Biochemically, we observed in animals that contained plastics in the stomach ("plastic" group) high production of reactive oxygen species (ROS), hydrogen peroxide ($H_2O_2$) and malondialdehyde (MDA) - especially in the intestine, muscle and brain - whose activity of catalase (CAT) and superoxide dismutase (SOD) was not sufficient to counteract the oxidative stress. Moreover, in the liver of these same animals, we observed high production of nitrite and nitrate, suggesting a hepatic nitrosative stress. Plus, we observed a cholinesterase effect in animals from the "plastic" group, marked by increased activity of butyrylcholinesterase (BChE) (in the brain) and muscle and cerebral acetylcholinesterase (AChE). On the other hand, the biochemical changes perceived were not significantly correlated with the identified plastic material concentrations ($2.808 \pm 0.598$ items/g of stomach content and $0.276 \pm 0.070$ items/g of stomach – fresh weight), body condition of the animals, size, and shape of the identified plastic materials. Hence, our study sheds the light on the toxicity of plastics deposited in landfills and their ingestion by *C. atratus*, which reinforces the hypothesis that these materials are harming the health of these birds and, consequently, the dynamics of their populations.

## 1. Introduction

One of the most emblematic birds of the Cathartidae family (order Cathartiformes) and of great ecological importance are the vultures. As discussed by Blanco and de Tuesta (2021) and Van-den-Heever et al. (2021), these animals play a remarkable role in the functioning of ecosystems, especially by foraging for dead animals, promoting clean-up of the environments where they live and preventing the spread of diseases between different trophic levels. Despite this, this finding has not been sufficient for the adequate conservation of these animals. According to

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**Table 1.**
Summary of biochemical biomarkers evaluated in adults of Coragyps atratus captured in landfills in the municipalities of Pires do Rio and Urutaí (GO, Brazil).

| **Oxidative stress biomarkers**[4] | | | | |
|---|---|---|---|---|
| | **Volume of supernatant used** | **Reagents and volumes used** | **Wavelength of sample reading**[1] | **Base reference for conducting assessments** |
| Nitrite and nitrate [indirect measurement of nitric oxide (NO)] | | 150 μL of Griess reagent | 492 nm | Bryan and Grisham (2007) |
| Hydrogen peroxide ($H_2O_2$) | 10 μL | 100 μL of phosphate buffered saline (PBS, pH 7.2)<br>100 μL of ammonium molybdate solution (0.5% w/v) | 405 nm | Elnemma (2004) |
| Reactive oxygen species (ROS) | 20 μL | 200 μL of phosphate buffered saline (PBS, pH 7.2)<br>8.3 μL of dichlorofluorescein-diacetate (10 mg/mL) | 492 nm | Maharajan et al. (2018) |
| **Biomarcadores antioxidantes**[4] | | | | |
| Catalase (CAT)[2] | 8 μL | 240 μL of reaction solution [glacial acetic acid P.A. + potassium dichromate (5%)] | 630 nm | Sinha (1972) |
| Superoxide dismutase (SOD)[2] | 30 μL | 99 μL of phosphate buffered saline (PBS, pH 7.2)<br>15 μL of piragalol (15 mM)<br>6 μL of 3-[4,5-Dimethylthiazol-2 H]− 2,5-diphenyltetrazolium bromide (1.25 mM)<br>150 μL of dimethylsulfoxide P.A. | 630 nm | Del-Maestro and McDonald (1985) |
| **Cholinesterase effect biomarkers**[4] | | | | |
| Acetylcholinesterase (AChE) e Butyrylcholinesterase (BChE) | 50 μL | 100 μL of acetylcholine or butyrylcholine solution (0.75 mg/mL)<br>100 μL of DTNB[3] solution (0.13 mg/mL)) | 405 nm | Ellman et al. (1961) |

[1] The readings were performed in an ELISA reader.

[2] These molecules are considered first-line antioxidants defenses that are important for preventing physiological oxidative stress.

[3] DTNB: 5,5′-Dithiobis-(2-Nitrobenzoic Acid).

[4] The results of the analyzes of all biomarkers were expressed proportionally to the concentration of total proteins in evaluated organs. Such biomarker was evaluated according to the instructions of the commercial kit used [Commercial kit (CAS number: BT1000900).

the survey carried out by Buechley and Şekercioğlu (2016), vultures, the only obligatory vertebrate scavengers, have experienced the fastest decline in conservation status of any avian group in the last decade, in addition to being the most threatened functional guild in the world, whose causes are especially related to current anthropogenic activities.

There is a close association between declining vulture populations and exposure to different pesticides (Plaza et al., 2019; Odino and Ogada, 2021), polycyclic aromatic hydrocarbons (Gómara et al., 2004; Dhananjayan et al., 2011; Dhananjayan et al., 2013), heavy metals (Borges-Ramírez et al., 2021a,b; Stamenov et al., 2021), pharmacological waste (human and veterinary) (Nambirajan et al., 2018; Moreno-Opo et al., 2021; Jimenez-Lopez et al., 2021; Herrero-Villar et al., 2021) has been reported, among others. The African and Asian continents are good examples of how and how much human activities have threatened vulture species (Bowden, 2017; Oppel et al., 2021). Estimates published by Ogada et al. (2016), in Africa, for instance, are alarming. According to the authors, populations of eight African species assessed decreased by an average of 62% and seven decreased at a rate of approximately 80% (over 30 years). Among these, at least six (*Gyps rueppelli*, *G. coprotheres*, *G. africanus*, *Necrosyrtes monachus*, *Trigonoceps occipitalis*, and *Torgos tracheliotos*) qualify as “endangered” or “critically endangered” according to the IUCN Red List of Threatened Species.

Importantly, a field of investigation that is still little explored refers to the contributions of plastic pollution to the decline of vulture species. In 2019 alone, the global production of plastics reached 368 million tons (PlasticsEurope, 2020), with a large scale being discarded directly into the natural environment, providing opportunities for the contact (direct or indirect) of these animals with such materials. In the study by Torres-Mura et al. (2015), in the Atacama Desert (Chile), the authors reported that more than 70% of fecal samples from *Cathartes aura* contained plastic material, probably originating from waste disposed along roads and beaches in the studied area. In Mexico, Borges-Ramírez et al. (2021a,b) observed that microplastics (MPs) can be vectors for the exposure of *Coragyps atratus* to different pollutants and Ballejo et al. (2021a,b) demonstrated that *Vultur gryphus*, *C. atratus* and *C. aura*, when feeding on organic waste, together with plastic waste disposed in dumps, can unintentionally transfer these materials to natural areas where they roost (Northwest Patagonia). Additionally, microtrash ingestion by *G. coprotheres* pups in South Africa (Pfeiffer et al., 2017) and plastic pellets by *C. aura* adults living on a remote and sparsely populated South Atlantic Island have been documented (Augé, 2017). Consequently, these studies provide clear evidence that the ingestion of plastics by different species of vultures is a reality in different locations.

In this interim, we do not know what the effects of this ingestion are on the physiology of these animals, and how much they can impact their health. Different studies have already shown, for example, that plastic ingestion induces changes in REDOX homeostasis in different animal models (Hu and Palić, 2020), such as in invertebrates (Malafaia et al., 2020; Chagas et al., 2021; Muhammad et al., 2021; Muhammad, al. et al., 2021; Silva et al., 2021) and vertebrates (Deng et al., 2017; Qiao et al., 2019; Bhagat et al., 2020; Xie et al., 2020; Guimarães et al., 2021; Xu et al., 2021; Meng et al., 2021). Furthermore, the cholinesterase effect has also been previously reported in different animal groups, with exposure to MPs being mostly associated with reduced acetylcholinesterase (AChE) activity (Barboza et al., 2018; Yin et al., 2021). Therefore, it is plausible to assume that similar changes can be observed as a result of the ingestion of plastic material in birds, including vultures. Thus, we pioneered a study focusing on the identification and characterization of plastic materials in the stomach contents of adults of *C. atratus* captured in different landfills, to test the hypothesis of the existence of a close relationship between plastic ingestion and possible biochemical changes in different organs. In addition, the relationships between the frequency of plastic ingestion, body condition, plastic size and animal sex were evaluated. *C. atratus* is a species distributed in tropical and temperate areas along the American continent, being widely represented not only in South and Central America, but also in southern North America (BirdLife International, 2016). Along with that, different authors have reported its occurrence in highly urbanized and impacted areas, as well as urban waste dumps (Robbins et al., 1989; Blackwell et al., 2007; Novaes and Cintra, 2013; Campbell, 2014; Araújo et al., 2018; Hill et al.,

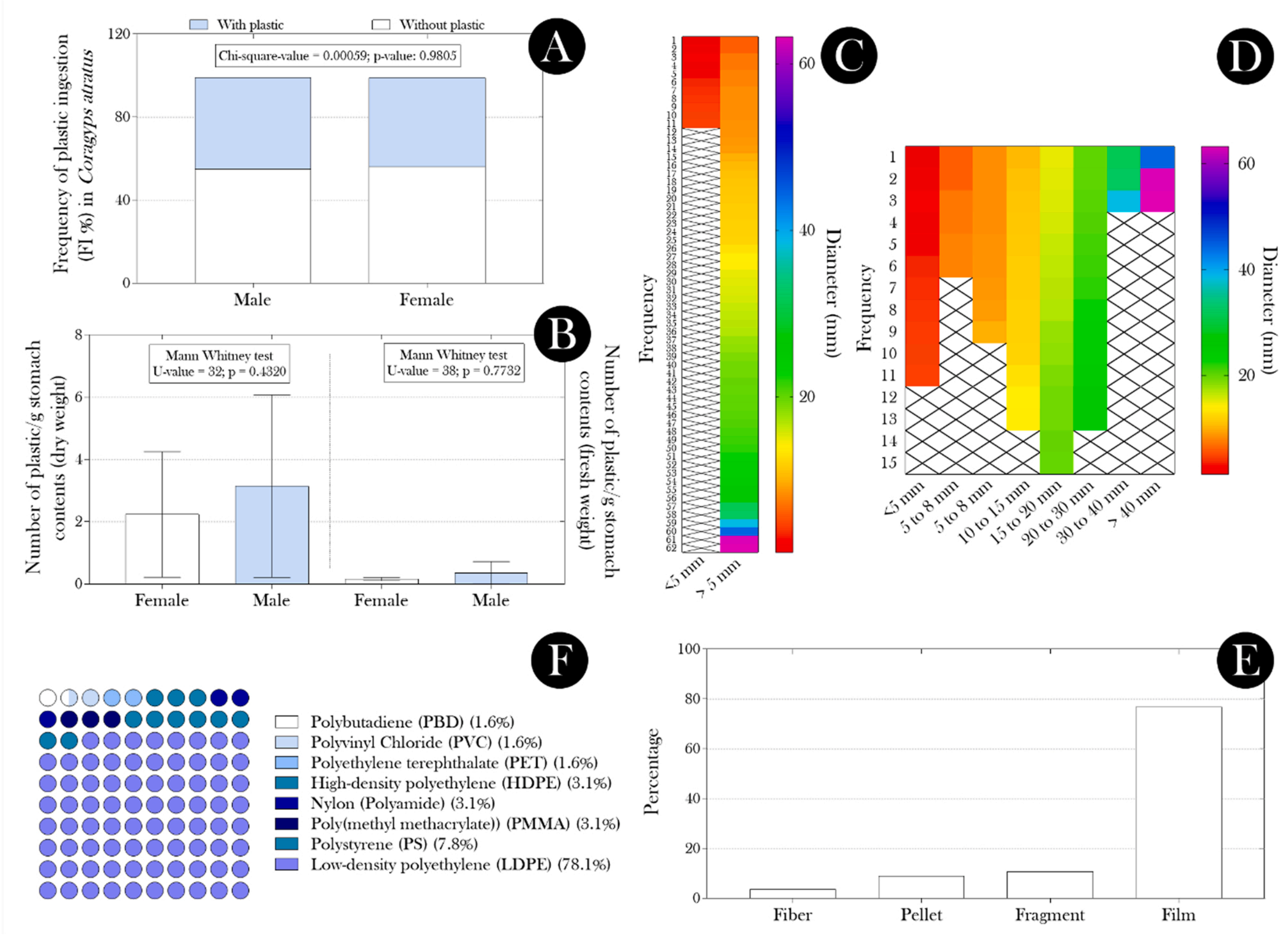

**Fig. 1.** (A) Frequency of plastic ingestion, (B) plastic concentrations identified in animals (by "g" of stomach content (dry weight) and by "g" of stomach (fresh weight); (C) distribution of frequency of plastic material with diameter smaller and larger than 5 mm; (D) frequency distribution of plastic material according to the categories of intervals of diameters (mm) measured; (E) percentage distribution of polymer types and (F) percentage of types of plastic classified according to form (fibers, pellets, fragments or film) identified in the stomach contents of adults of *Coragyps atratus* (females and males) captured in landfills in two municipalities in the State of Goiás (Brazil). In "B", bars represent mean ± SD.

2021). Therefore, individuals of *C. atratus* constitute good translational models for evaluating the toxicity of plastics in different species of the Cathartidae family. As far as we know, this is the first report that associates plastic ingestion by *C. atratus* with biochemical alterations predictive of oxidative stress, REDOX imbalance, and cholinesterase effect. We strongly believe that studies like ours provide support for the proposition of mitigation/remediation measures for plastic pollution, as well as for the conservation of vulture species.

## 2. Material and methods

### *2.1. Areas of study and capture of animals*

Fifty-one adults of *C. atratus* (of both sexes – see biometrics in Table S1) were captured in landfills in the municipalities of Pires do Rio (−17.260733, −48,285367) and Urutaí (−17.430811, −48.200189) (both located in the State of Goiás, Brazil), having been used a trap of the "pit type", like the one used by Barbara et al. (2017). Such trap was installed on the margins of the final disposal site of solid waste from the respective landfills and to attract the birds, chicken meat baits were placed inside the trap. The authorization to capture the animals was granted by the Biodiversity Information and Authorization System (SISBIO)/Chico Mendes Institute for Biodiversity Conservation (Brazil).

### *2.2. Identification and characterization of plastics*

After capture, the animals were taken to the Biological Research Laboratory of the *Instituto Federal Goiano* (Urutaí, GO, Brazil) and were euthanized by decapitation. Then, a laparotomy was performed to extract the stomach and collect fragments of the liver, muscle (pectoral) and small intestine (duodenal region just below the stomach – fragments of approximately 2 cm). In addition, a craniotomy was performed to extract brain fragments from the animals. Then, the samples were properly identified and stored in a freezer at −80 °C until the biochemical analyzes were performed (see item "2.2.2").

Afterwards, the stomach contents were washed (with purified water via reverse osmosis) and sieved through a steel mesh (0.075 mm) with running water and placed in a glass petri dish for further analysis under a stereoscopic microscope. The identified plastic material [according to Rosas-Luis (2016)] was collected, washed with 70% alcohol, photographed, and stored individually for further analysis via micro-Raman spectroscopy to determine its polymeric chemical compositions. The spectra obtained were compared with those available in the PublicSpectra © 2019 database (https://publicspectra.com/), whose similarity was greater than 75%. Besides, the size (area and diameter) and shape (circularity) of the plastics were evaluated using the ImageJ software, similarly to the procedure adopted by Araújo et al. (2020). All plastic materials were categorized according to type and color, according to similar procedures adopted by Marti et al. (2020). The frequency

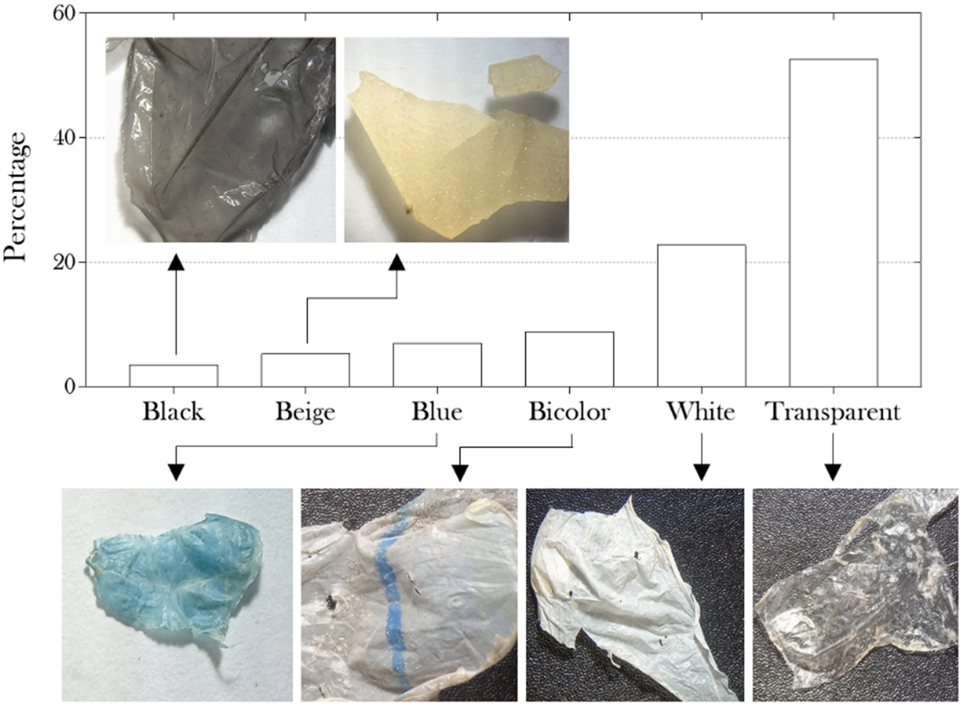

**Fig. 2.** Percentage of the color of plastic materials identified in the stomach content of *Coragyps atratus* (female and male) adults captured in landfills (State of Goiás, Brazil).

of plastic ingestion (FI%) was calculated according to the following equation: FI(%) = (Number of vultures that contained plastics)/(Total number of vultures examined) x 100. The number of plastics in animals was expressed proportionally to dry weight of stomach contents and fresh stomach biomass of animals.

### 2.3. Toxicity biomarkers

#### 2.3.1. Biometry

To assess the relationship between plastic material ingestion and possible changes in animal health, different toxicity biomarkers were determined. The body condition of the animals was evaluated from the body mass (BM), body length (BL), tail (TL), wing (WL) and tarsus (T). These parameters were used to determine different biometric indices [BM/T, BM/(BL + WL), BM/BL, BM/WL and BM/TL], which were calculated from previous studies (Chappell and Titman, 1983; DeVault et al., 2003; Schamber et al., 2009). In accordance with Labocha and Hayes (2012), the body condition of birds has been related to the survival, reproduction, and behavior of animals, in addition to being a topic of considerable interest in studies that assess the impacts of human activities on avifauna.

#### 2.3.2. Biochemical analysis

Presuming that plastics ingestion can cause biochemical changes, we evaluated in liver, intestine, brain, and muscle fragments the production of hydrogen peroxide ($H_2O_2$), reactive oxygen species (ROS), nitric oxide (via nitrite/nitrate production), malondialdehyde (MDA), as well as the activity of the enzymes superoxide dismutase (SOD) and catalase (CAT). In addition, the activity of the enzymes acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) was evaluated assuming the cholinesterase effect of the ingestion of plastic material. For this, the organ fragments were weighed, macerated in 1 mL of phosphate buffered saline (PBS) (pH 7.2) (with semi-automatic macerator) and, subsequently, the samples were centrifuged (10,000 rpm, 10 min, 4 °C) for the collection of supernatants, which were used to assess the biomarkers summarized in Table 1. Twelve random animals that did not contain plastic in their stomach contents constituted the “control” group and 19 vultures, in which we identified any plastic material, formed the “plastic” group. It is noteworthy that we assume that animals named as “control” do not necessarily constitute animals exempt from ingestion of plastic material (including additives and MPs) hours before the capture. As demonstrated by Ballejo et al. (2021a,b), Torres-Mura et al. (2015) and Platt et al. (2021), regurgitation is quite common in vultures, both in females, to feed their young, and males/females to fend off their attacks and allow easier flights. Therefore, our focus was on the momentary effects of the presence of plastic material in the gut contents of animals and, therefore, we disregard the history of exposure of animals to plastic materials.

### 2.4. Statistical analysis

Initially, the residual normality of all data was evaluated by the Shapiro-Wilk test and the homogeneity of variances by the Bartlett test. The possible difference between the FI(%) of plastics by *C. atratus* females and males was evaluated by the Chi-square test. The means of the different biochemical biomarkers obtained in animals with and without plastics were compared using the Mann-Whitney test (non-parametric) or Student’s *t*-test (parametric). The two-way ANOVA (with Tukey’s post-test) was used to assess the individual effect or the interaction between the factor’s "contamination" (two levels: presence or absence of plastics in the stomach of animals) and "sex" (two levels: male or female). Furthermore, Pearson (parametric) or Spearman (non-parametric) correlation analyzes were performed, seeking to associate different evaluated biomarkers. In the case of significant correlations, linear regression analysis was performed. For all analyses, p-value $<$ 0.05 was considered as statistically significant. The GraphPad Prism (v. 7.0 version) statistical software was used to perform the statistical analysis and graph construction.

## 3. Results

Basically, our analyzes revealed the presence of plastic material in

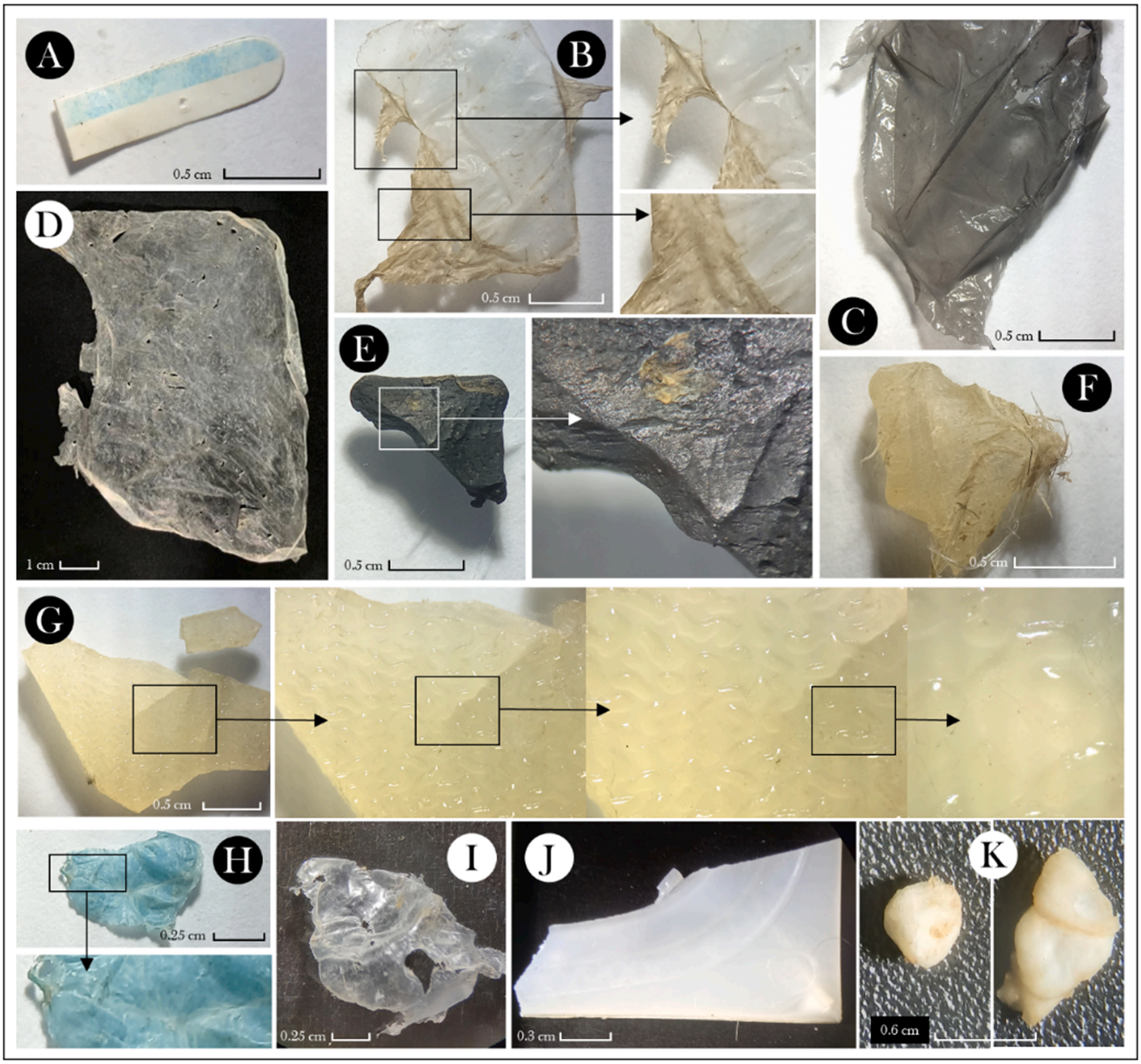

**Fig. 3.** Images representing the types, shapes and colors of plastic materials identified in the stomach contents of *Coragyps atratus* adults (female and male) captured in landfills (State of Goiás, Brazil). (A, E, F, G and J): fragments; (B, C, D, H, and I): film and (K): pellet.

the stomach of almost half of the investigated animals (43.95%), with an equal percentage of females (≈43%) and males (≈44%) in which plastics were identified (Fig. 1A). A total of 73 plastic particles were identified, with the mean of items/g of stomach content (dry weight) equal to 2.808 ± 0.598 (mean ± SEM) and 0.276 ± 0.070 items/g of stomach (fresh weight), not having been the factor associated with this variable (Fig. 1B). Regarding size, most (84.9%) of the plastic items had a diameter greater than 5 mm (Fig. 1 C), and 56.2% had a diameter between 10 and 30 mm (Fig. 1D). The types of plastics identified (via micro-RAMAN analysis) were, in decreasing order of abundance, low-density polyethylene > polystyrene > nylon (polyamide)/poly (methyl methacrylate)/high-density polyethylene > polyethylene terephthalate/polyvinyl chloride/polybutadiene (Fig. 1E).

In this regard, using a classification system from previous studies, plastic materials were divided into four categories according to their shapes: fiber, fragment, pellet, and film. Briefly, a long, thin line with a slender shape was classified as fiber; fragments are hard piece of debris from a broken plastic item; debris with a thin layer was called film; and pellets are microplastics with spherical and cylindrical shapes. As can be seen in Fig. 1F, most plastic materials identified were of the "film" type (76.8%), with fibers being less frequent (3.6%). Pellets and fragments represented 8.9% and 10.7% of the identified plastic materials, respectively. As for coloration, most plastics identified were classified as "white" and "transparent" (75.4%) (Fig. 2). Images representative of the types, shapes (circularity: 0.20 ± 0.01; mean ± SEM) and colors of the identified plastics are presented in Figs. 3 and 4. Although we have not thoroughly characterized the gut contents of animals (in terms of non-plastic material), the presence of plant material (Fig. 4I-K), insect larvae (Fig. 4L), ticks (Ixodida order) (Fig. 4M), animal hair possibly present on carcasses (Fig. 4N), eggshells (Fig. 4O) and bones (Fig. 4P) were commonly found items. On the other hand, our visual evaluation did not evidence the presence of metallic material, pieces of glass or porcelain.

From these results, we evaluated the possible influence of plastic intake on animal health from different biochemical biomarkers. Initially, we observed an increase in the production of ROS (gut, muscle, and brain), $H_2O_2$ (gut, muscle, and brain) and MDA (muscle and brain) of *C. atratus* from the "plastic" group (Fig. 5A-C, respectively), as well as of nitric oxide in the liver of these animals (inferred from the levels of nitrite and nitrate) (Fig. 5D-E, respectively). As for the activity of the enzymes evaluated, we observed an increase in the activity of CAT in the gut, muscle and brain (Fig. 6A), as well as SOD in the gut of animals in

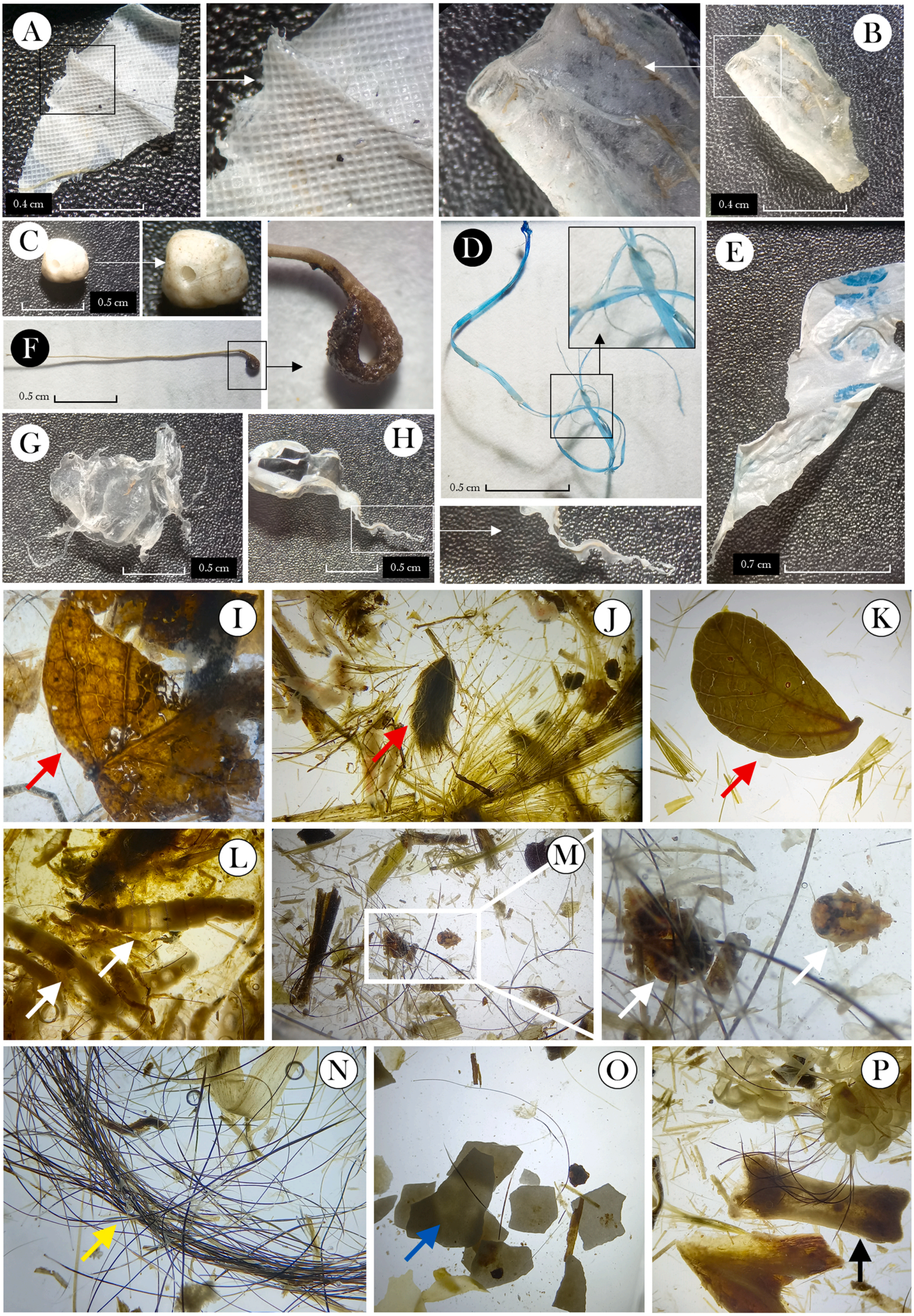

**Fig. 4.** Images representing the types, shapes and colors of plastic materials identified in the gut contents of *Coragyps atratus* adults (female and male) captured in landfills (State of Goiás, Brazil). (A, E, G, and H): film; (B): fragment; (C) pellet and (D and F) fiber. Other materials (non-plastic) identified in the gut contents of animals - (I-K): plant material (red arrows); (L-M): insect larvae and tick (white arrows); (N): hair (yellow arrow); (O) eggshell (blue arrow) and (P) bone pieces (black arrow). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

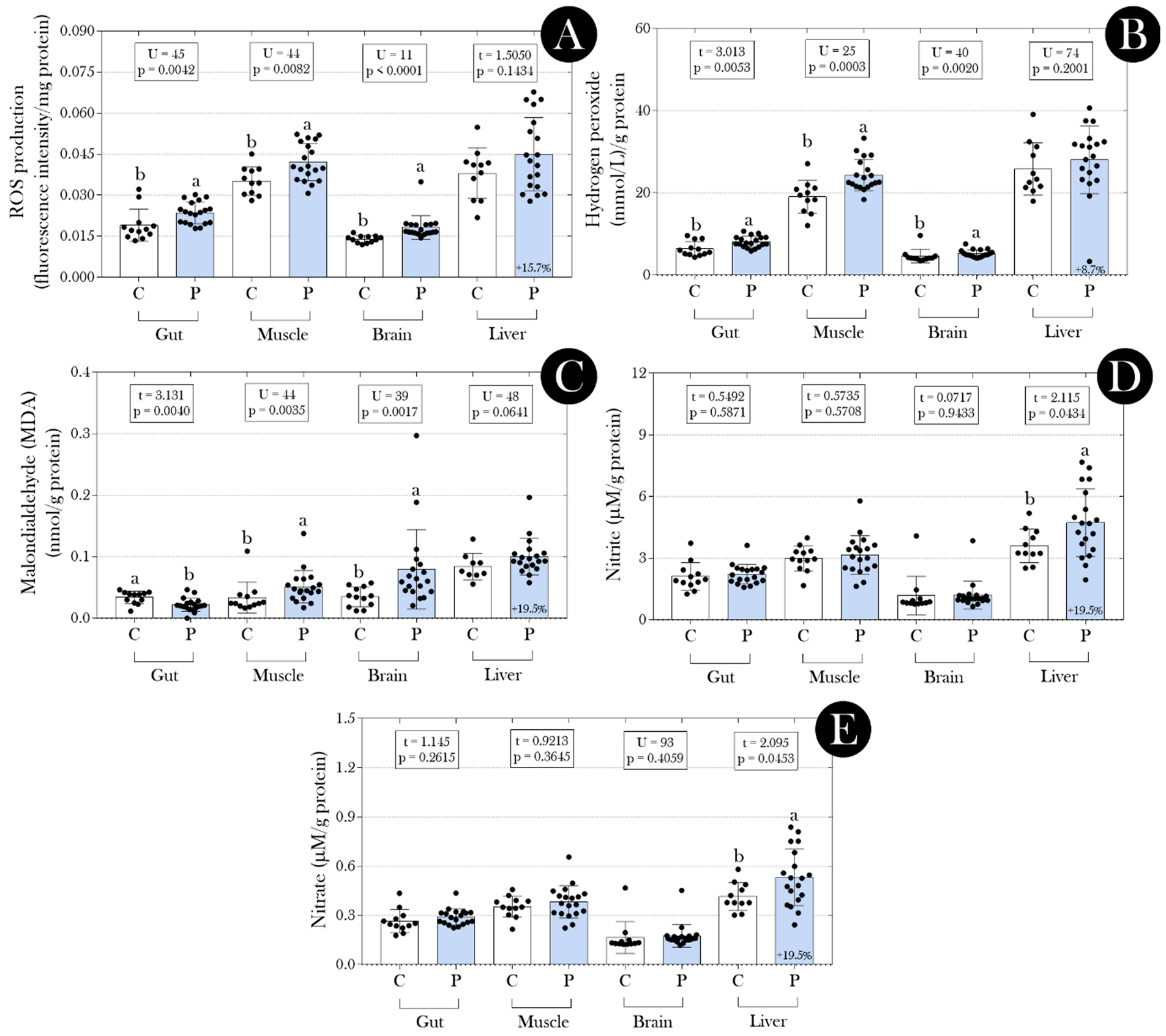

**Fig. 5.** Scatter dot plot of production of (A) reactive oxygen species (ROS), (B) hydrogen peroxide ($H_2O_2$); (C) malondialdehyde (MDA); (D) nitrite and (E) nitrate in different organs of Coragyps atratus (female and male) adults captured in landfills (State of Goiás, Brazil). (n = 12, control, and n = 19, plastic). The "control" group (C) refers to animals in which no plastic material was identified in the stomach contents and "plastic" group (P) includes animals that have ingested some plastic material. Distinct lowercase letters indicate statistical differences between "C" and "P" groups.

the "plastic" group (Fig. 6B). On the other hand, changes in BChE activity were observed in the gut and brain ("control" vs. "plastic"; Fig. 6C) and an increase in AChE was observed in the muscle and brain (Fig. 6D) of these same animals. However, we did not find significant correlations between most of the analyzed biochemical parameters and the plastic material concentrations identified in the animals (Figs. S1–2). Furthermore, the size (area in $cm^2$) and shape (inferred from the circularity) of the plastics did not influence the biochemical response of the animals (Figs. S3–4).

Moreover, we quantified whether sex and the biometric indices assessed were correlated with the deemed biochemical changes. In this case, we noticed that the production of ROS in the gut of females in the "plastic" group was greater (56.5%) than in those in the "control" group (Fig. 7A), although in the brain and liver we noticed increases of 31% and 26.3%, respectively (Fig. 7B-C). In males, the production of ROS in the brain was higher than that observed in females and males in the "control" group ($p < 0.05$) (Fig. 7B). The production of $H_2O_2$ was higher in the gut ($p < 0.05$) of females in the "plastic" group, compared to their respective "control" group ($p < 0.05$) (Fig. 7E) and in males "with plastics" the production of this biomarker was exceeding that recorded in females "without plastics" (Fig. 7H).

Concerning nitrite and nitrate, its production was higher in the liver of animals ($p < 0.05$)) that ingested the plastic materials (both females and males) (Fig. 8D-H, respectively) and, in the gut, increases greater than 30% were observed in females in the "plastic" group compared to those in the "control" group (Fig. 8A-E). On the other hand, MDA production in muscle and brain of females "with plastics" (compared to those "without plastics") was greater than 65% (Fig. 9A-B, respectively). In addition, although not statistically significant, muscle and brain MDA levels in females in the "plastic" group were 30.2% and 82.1% higher than those recorded in males in that same group (Fig. 9A-B). As for the enzymes evaluated, we did not observe any effect of the factor's "sex" and "contamination" on the SOD activity in the evaluated organs (Fig. 10A-D); but, except for the liver, the CAT activity was higher in animals in the "plastic" group (both females and males) in relation to their respective "control" groups (Fig. 10E-H). On the other hand, we noticed in animals that ingested plastics a significant reduction in BChE activity in the gut (Fig. 11 A) and an increase in muscle and brain AChE (Fig. 11F-G, respectively), regardless of sex. Plus, we observed that plastic ingestion was not correlated with any change in the animals' body condition [inferred by the different calculated biometric indices (Fig. S3)] and that size (area) (Figs. S4–5) and shape (circularity)

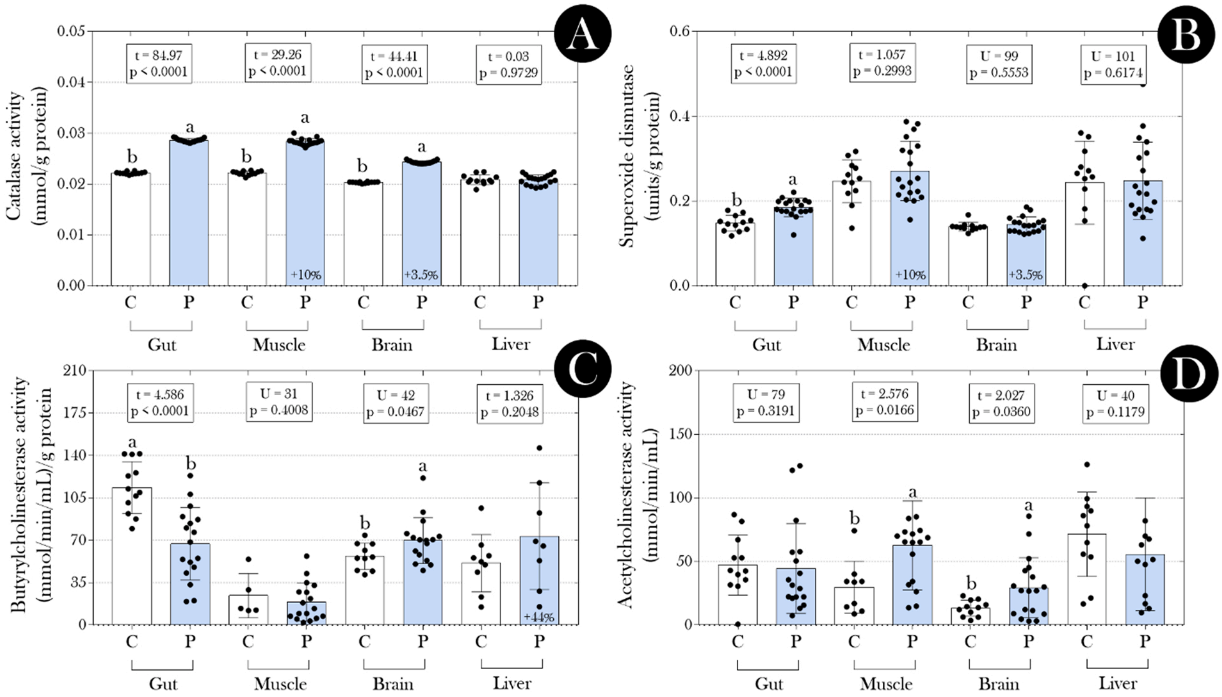

**Fig. 6.** Scatter dot plot of (A) catalase (CAT), (B) superoxide dismutase (SOD); (C) butyrylcholinesterase (BChE) and (D) acetylcholinesterase (AChE) activity in different organs of *Coragyps atratus* (female and male) adults captured in landfills (State of Goiás, Brazil). (n = 12, control, and n = 19, plastic). The "control" group (C) refers to animals in which no plastic material was identified in the stomach contents and "plastic" group (P) includes animals that have ingested some plastic material.

(Figs. S6–7) of plastic materials were not correlated with most biochemical biomarkers assessed.

## 4. Discussion

It is readily apparent that the identification, characterization, and evaluation of the impacts of plastic ingestion by birds constitute an important opportunity to predict the ecotoxicological impacts of these materials on avifauna. Based on studies like ours, subsidies are acquirable for planning actions related not only to pollution remediation, but also to species conservation. Focusing on vultures (*C. atratus species*) we confirm that these individuals are subject to involuntary ingestion of plastic material disposed in the investigated areas (landfills). From the visual inspection and polymeric chemical characterization of the identified materials, we note that plastic bags (Figs. 4E, 5B and D) and probably food packaging (Figs. 3I and 4G) (mainly made of low-density polyethylene - Fig. 1F) constitute the main sources of material ingested by animals, which are commonly present in waste disposed of in landfills (Quaghebeur et al., 2013; Osra et al., 2021).

Substitutable results have been documented in studies involving other species of the Cathartidae family, such as in the works by Kelly et al. (2007) (*Coragyps atratus* and *Cathartes aura*), Ballejo and de Santis (2013) and Borges-Ramírez et al. (2021a,b) (*Coragyps atratus*), Torres-Murra et al. (2015) and Augé (2017) (*Cathartes aura*) and Ballejo et al. (2021a,b) (*Vultur gryphus*, *Coragyps atratus* and *Cathartes aura*). In most of these studies, like what we observed, the plastic materials identified (especially in animal feces) had an appearance compatible with pieces of plastic bags, as well as small pieces of plastic with sharp or pointed edges. On the other hand, the identification of polystyrene polymers (styrofoam pellets) (Figs. 3K and 4B), polybutadiene fragment (tire constituent) (Fig. 3E) and poly (methyl methacrylate) (common constituent of acrylic material used in construction civil – Fig. 3G and J) demonstrate how variable the type of material ingested by adults of *C. atratus* can be. In this case, it is possible that this is associated not only with the animals' capture sites, but also with their eating habits and tolerance to highly urbanized sites.

However, an unexplored field in the aforementioned studies refers to the impacts of plastic material ingestion on the health of these animals, which demonstrates that plastic ingestion by species of the Cathartidae family has not received much attention, compared to other important threats, such as poisoning by agricultural pesticides (Plaza et al., 2019) and contamination by heavy metals such as lead (Plaza and Lambertucci, 2018), aluminum (Borges-Ramírez et al., 2021a,b), cadmium, copper and zinc (López-Berenguer et al., 2021). This is especially disturbing, since the ingestion of plastic material by vultures may be inducing silent harmful effects on their populations which, added to the impacts caused by other pollutants, increase the threat of anthropogenic activities to the survival of the species. In our study we observed that although the plastic materials identified in the stomach of *C. atratus* were not correlated with changes in the biometric indices (ie: body conditions) of the animals (Fig. S3), biochemical differences were observed between the "control" and "plastic" groups" denounce for the first time some of the physiological consequences arising from these materials on the health of the individuals analyzed. The high production of ROS (Fig. 5 A), H2O2 (Fig. 5B) and MDA (Fig. 5 C) especially in the gut, muscle, and brain of animals in the "plastic" group suggest the induction of oxidative stress possibly motivated by the ingestion of plastic materials, whose antioxidant activity (measured by CAT and SOD enzymes – Fig. 6A-B, respectively) seems not to have been sufficient to counteract cellular oxidative processes. Similar results have already been reported in the context of exposure of different animal groups to plastic materials [see review by Hu and Palić (2020)]. In the liver, the increased production of nitrite and nitrate (Fig. 5D-E, respectively) also suggests hepatic alterations that may be related both to the activation of the immune response mediated especially by Kupffe cells, and to a nitrosative stress from the oxide isoform inducible nitric synthase (iNOS).

Obviously, the assumption of any mechanism of action responsible for inducing the biochemical alterations observed in our study is preliminary. However, it is tempting to speculate that in the gut,

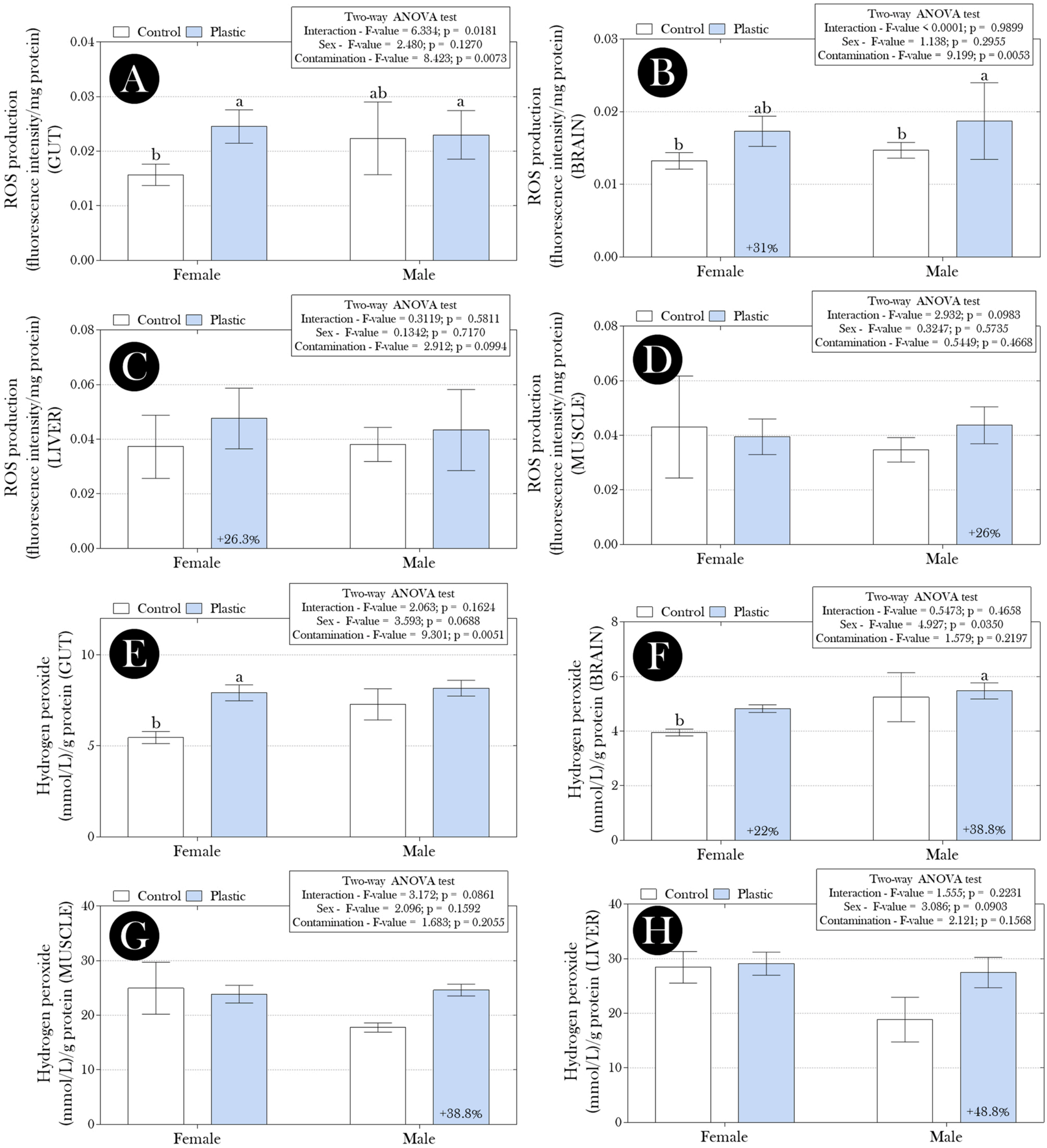

**Fig. 7.** Production of reactive oxygen species (ROS) [(A) in gut, (B) brain, (C) liver and (D) muscle] and hydrogen peroxide ($H_2O_2$) [(E) in gut, (F) brain, (G) muscle and (H) liver] in *Coragyps atratus* (female and male) adults captured in landfills (State of Goiás, Brazil). The bars represent the mean ± SEM (n = 12, control, and n = 19, plastic). The "control" group (C) refers to animals in which no plastic material was identified in the stomach contents and "plastic" group (P) includes animals that have ingested some plastic material. Distinct lowercase letters indicate statistical differences between groups.

particularly, the high production of ROS and $H_2O_2$ (Fig. 5A-B, respectively), as well as of CAT and SOD (Fig. 6A-B, respectively) may be related to physical and chemical impacts induced by plastic materials, especially by those classified as "fragments". Physical changes (most obvious) include injuries and perforation of the intestinal epithelium [caused by sharp and sharp plastic fragments – e.g.: Fig. 3 G and 3 J – already reported in seabirds, by Roman et al. (2019)], inflammatory processes (Pirsaheb et al., 2020), as well as digestive obstruction [caused by the accumulation of pieces of bags (Fig. 3D) or plastic fibers (Fig. 4D), e.g.: – also reported in *Puffinus gravis* and *Morus bassanus*, by Pierce et al. (2004)]. On the other hand, we cannot neglect the hypothesis that these changes are related (direct or indirect) with the release of additives/chemical compounds used in the manufacture of plastics, particularly during the digestive action in the stomach of birds, as well as the presence of MPs/NPs (non-targets of our study). Regardless of the mechanism responsible for the changes, developments in oxi-reduction processes can lead to intestinal dysfunctions, crucial for the maintenance of the animals' energy homeostasis, with negative physiological

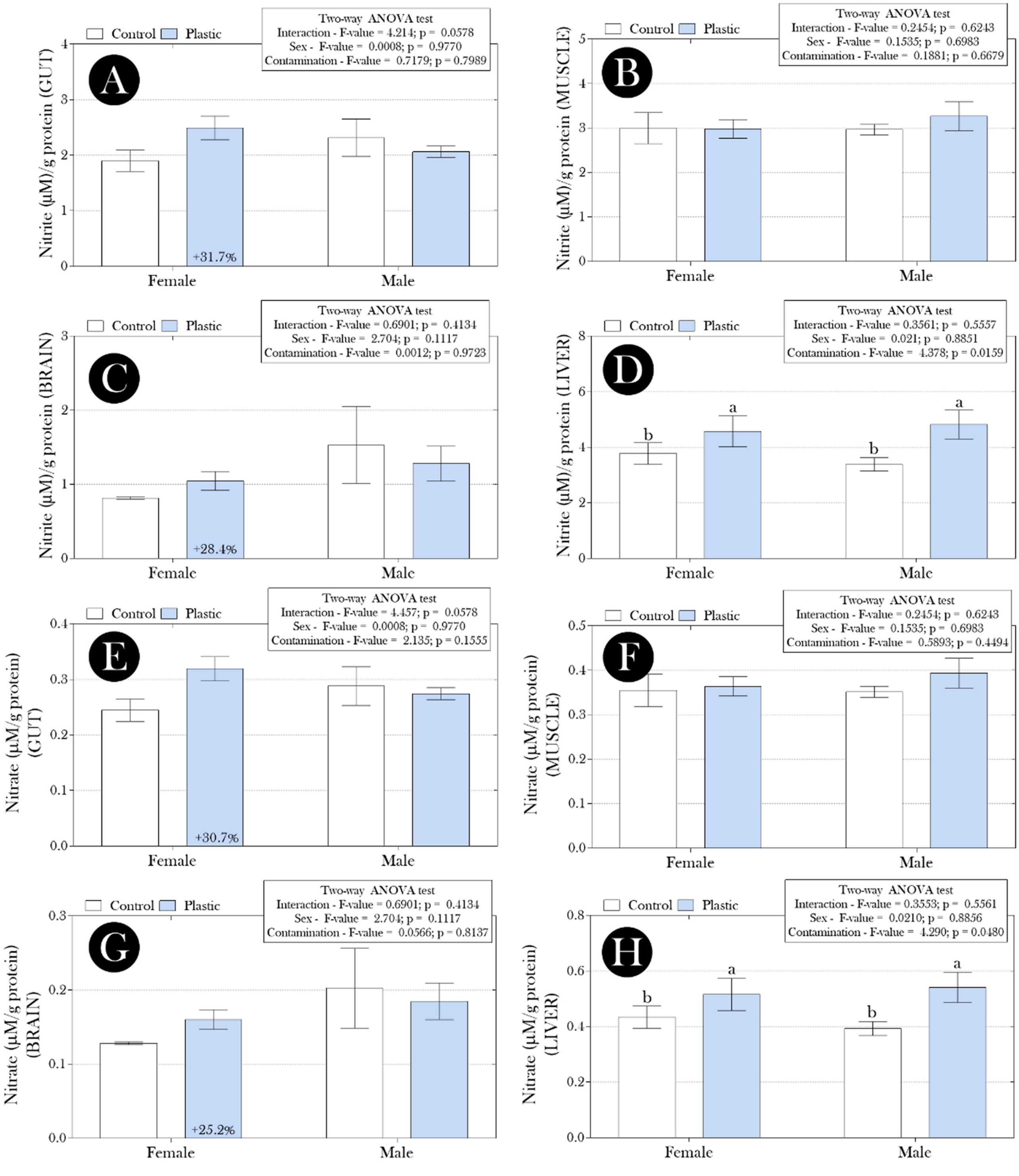

**Fig. 8.** Production of nitrite [(A) in gut, (B) muscle, (C) brain and (D) liver] and nitrate [(E) in gut, (F) muscle, (G) brain and (H) liver] in *Coragyps atratus* (female and male) adults captured in landfills (State of Goiás, Brazil). The bars represent the mean ± SEM (n = 12, control; and n = 19, plastic). The "control" group (C) refers to animals in which no plastic material was identified in the stomach contents and "plastic" (P) includes animals that have ingested some plastic material. Distinct lowercase letters indicate statistical differences between groups.

consequences for the animals. Intestinal alterations can, for example, totally compromise, in some cases, the physiological functions of the intestines, directly affecting the digestive and absorptive processes (Peda et al., 2016).

In muscle, the REDOX imbalance (marked by increased production of ROS, $H_2O_2$ and CAT activity – Figs. 5A-B and 6 A, respectively) observed in animals from the "plastic" group may be associated with the accumulation of MPs (diameter not identified in our study), and indirect impacts caused by macroplastics. In this case, physiological disorders in the birds' pectoral muscles can cause changes in their locomotor abilities, especially those related to flight, considering that such muscles are substantial to produce the aerodynamic force necessary to support the animal's weight in the air and to overcome drag (Cao and Jin, 2020). On the other hand, increased oxidative processes in the brain of these

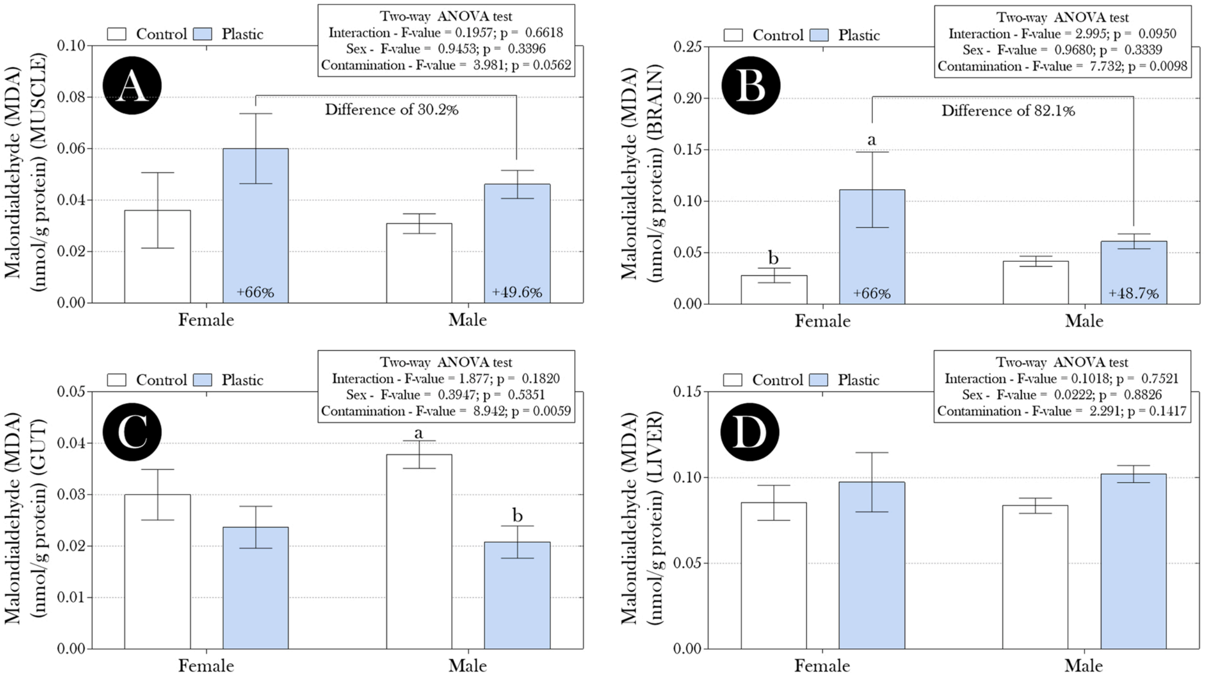

**Fig. 9.** Malondialdehyde (MDA) production [(A) in muscle, (B) brain, (C) gut and (D) liver] in *Coragyps atratus* (female and male) adults captured in landfills (State of Goiás, Brazil). The bars represent the mean ± SEM (n = 12, control; and n = 19, plastic). The "control" group refers to animals in which no plastic material was identified in the stomach contents and "plastic" includes animals that have ingested some plastic material. Distinct lowercase letters indicate statistical differences between groups.

animals [marked by the increased production of ROS (Fig. 5 A), $H_2O_2$ (Fig. 5B), MDA (Fig. 5 C) and CAT (Fig. 6A)] may also be directly or indirectly associated with the ingestion of macroplastics or by MPs/NPs accumulated in the central nervous system, which can impact various physiological processes in animals. This includes everything from the vital functions of different organs to the modulation of their behavior. Furthermore, it is important to emphasize that REDOX imbalance can also be related to inflammatory and immune responses. Both the nitrosative stress observed in the animals' livers (Fig. 5D-E) and other previous studies [see review by Hu and Palić (2020)] reinforce this hypothesis.

On the other hand, the increased activity of BChE (in the brain) and AChE (in the muscle and brain) of the "plastic" group vultures (Fig. 6C-D respectively), suggests a stimulatory effect on the animals' cholinergic system. In this case, previous studies support the hypothesis that these increases are associated with the oxidative stress observed in these same organs. Schallreuter et al. (2004) and Garcimartín et al. (2017) reported, in vitro, that the high concentration of $H_2O_2$, for example, stimulated cholinesterase activity. On the other hand, the increase in cerebral BChE activity may be related to a dysfunction in the AChE activity, whose increase observed in the muscle and brain of these animals (Fig. 6D) would constitute a compensatory physiological mechanism. As discussed by Mesulam et al. (2002), BChE plays an important role in supporting AChE in the regulation of cholinergic transmission, especially in the absence or inefficiency of this enzyme.

On the other side, it is possible that the oxidative stress observed in the brain of these animals and the increase in lipid peroxidation processes (inferred by the high levels of MDA – Fig. 5C) may have contributed to greater release of acetylcholine and butyrylcholine in the cholinergic synaptic clefts and to the overstimulation of postsynaptic receptors, culminating in the consequent increase in BChE and AChE activity. This hypothesis, in particular, is supported by Barboza et al. (2020) who, at the time, associated the increase in lipid peroxidation and AChE levels to the accumulation of MPs in the muscle, gills and brain of *Dicentrarchus labrax*, *Trachurus trachurus* and *Scomber colonies*. On the other hand, it is known that the increase in AChE and BChE has also been associated with the presence of inflammatory processes, compared to healthy individuals (De-Oliveira et al., 2012; Santarpia et al., 2013), which also may explain its increased levels in animals of the "plastic" group. Regardless of the mechanisms involved, the increased activity of AChE and BChE in the brain can be considered a predictive response of neurological changes, whose consequences are likely to affect the fitness of individuals, increase energy demand, induce discoordination, behavioral changes, among others. On the other hand, the increased activity of these enzymes in muscles can induce nicotinic effects, which are the result of sympathetic hyperactivity and neuromuscular dysfunction.

Obviously, knowledge of the mechanisms underlying these different responses requires further studies focusing on more specific biomarkers correlated with the sex of *C. atratus*. However, it has been shown in studies involving other animal models that the response of males and females exposed to different pollutants, in fact, can be differentiated (Bao et al., 2020; Gade et al., 2021; Vega et al., 2021; Kochi et al., 2021; Gokulan et al., 2021). Overall, these studies have hypothesized that sex-based differential susceptibility is related to sexual dimorphisms in anatomy, gray matter distribution, hormones, and/or epigenetic and metabolic factors. In *Coturnix Coturnix japonica*, for example, differences between sexes were recorded in the response of microbiota to trichlorfon (organophosphate insecticide) (Crisol-Martínez et al., 2016). Erikstad et al. (2013) demonstrated that *Larus hyperboreus* females contaminated with organochlorines are more sensitive to the negative effects of these pesticides, when compared to males. In *Ficedula hypoleuca* adults that live in areas contaminated by heavy metals, Eeva et al. (2006) predicted lower local survival of males in polluted areas compared to females. On the other hand, we observed that the chronic ingestion of water containing tannery effluent by *Melopsittacus undulates* (female and males) adults induced a similar mutagenic effect in both sexes (Souza et al., 2017). Therefore, it is noted that the sex-dependent response to pollutants is influenced not only by the pollutant and its accumulation levels, but also by the avifauna species and its physiological characteristics, which should be better studied in the future.

Eventually, it is noteworthy that our data shed light on the toxic

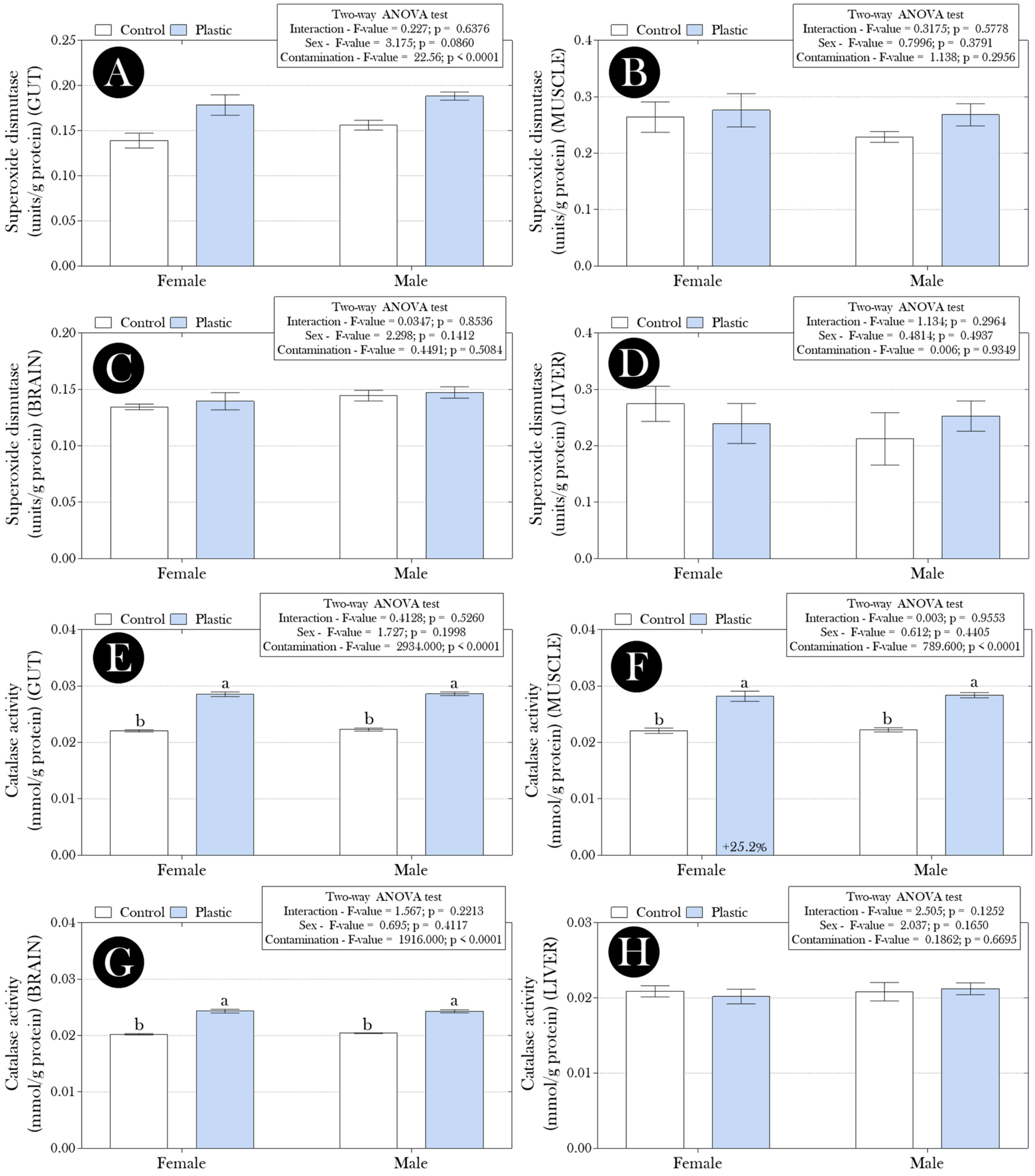

**Fig. 10.** Superoxide dismutase (SOD) enzymes [(A) in gut, (B) muscle, (C) brain and (D) liver] and catalase (CAT) activity [(E) in gut, (F) muscle, (G) brain and (H) liver] in *Coragyps atratus* (female and male) adults captured in landfills (State of Goiás, Brazil). The bars represent the mean ± SEM (n = 12, control; and n = 19, plastic). The "control" group refers to animals in which no plastic material was identified in the stomach contents and "plastic" includes animals that have ingested some plastic material. Distinct lowercase letters indicate statistical differences between groups.

effects of plastic materials ingested by *C. atratus* adults, which had not been previously studied. Therefore, evidence is provided to reinforce the hypothesis that plastic materials may be harming the health of these animals, with consequences that are still poorly understood. The absence of a concentration-dependent, size and shape (of plastics)-dependent effect observed in our study emerges as an additional concern, since the simple presence of plastic material in the gastrointestinal system of these birds (regardless of number, size, and shape) can trigger harmful physiological changes. Obviously, any robust conclusion about the extent to which these materials have contributed to the population decline of the studied species and other representatives of the Cathartidae family is incipient. However, it is known that any change in REDOX homeostasis or cholinesterase effect represents a risk to the health of these birds, with negative consequences for the fitness of individuals, including their reproductive performance, eating habits, behaviors, as well as their adaptive plasticity. Thus, investigations focusing on the impacts of plastic ingestion on these aspects emerge as investigative perspectives that will contribute to a better understanding of how plastic pollution may be enhancing the negative effect of other pollutants on the health of vultures.

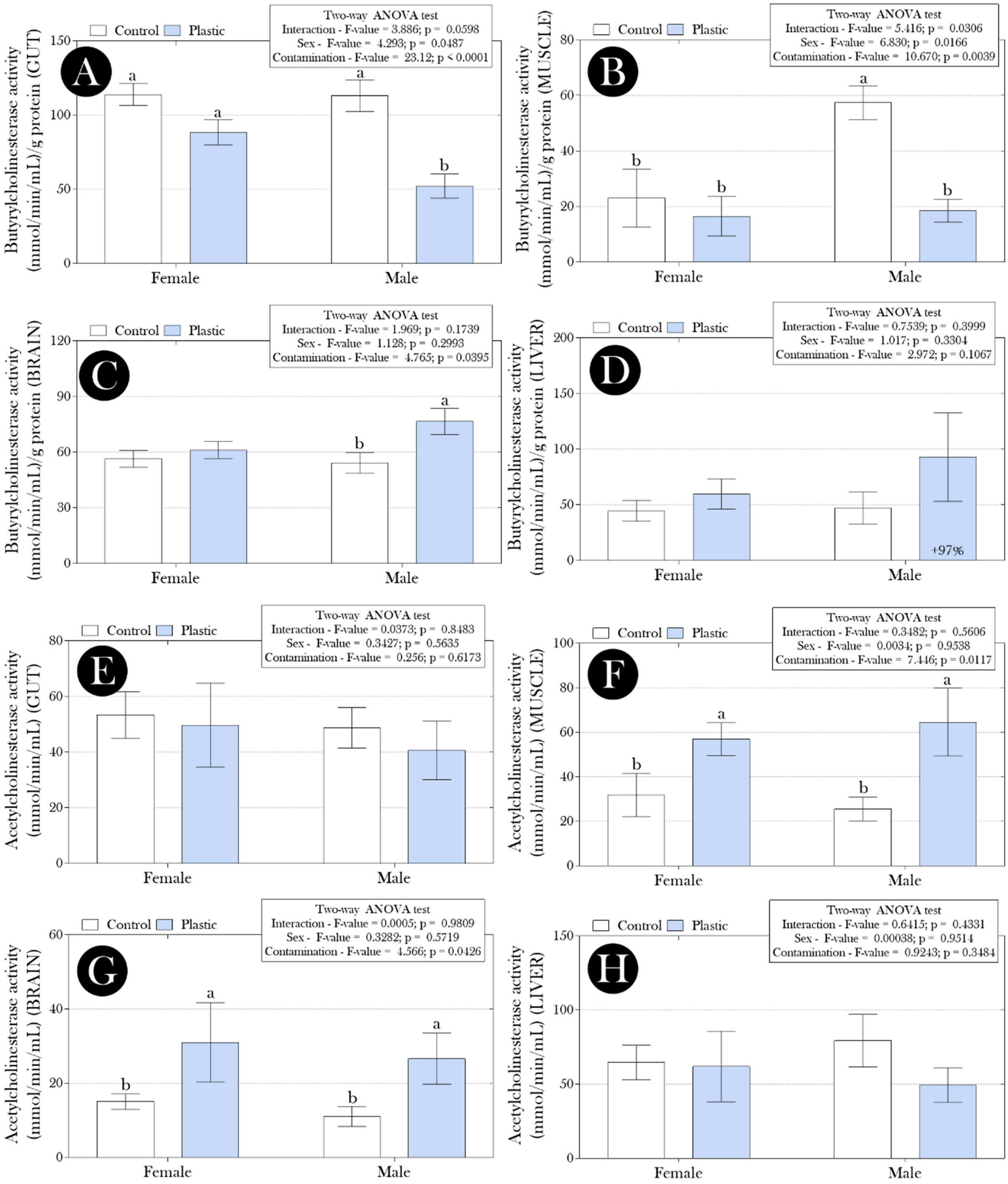

**Fig. 11.** Butyrylcholinesterase (BChE) enzymes [(A) in gut, (B) muscle, (C) brain and (D) liver] and acetylcholinesterase (AChE) activity [(E) in gut, (F) muscle, (G) brain and (H) liver] in *Coragyps atratus* (female and male) adults captured in landfills in two municipalities in the State of Goiás (Brazil). The bars represent the mean ± SEM (n = 12, control; and n = 19, plastic). The "control" group refers to animals in which no plastic material was identified in the stomach contents and "plastic" includes animals that have ingested some plastic material. Distinct lowercase letters indicate statistical differences between groups.

## 5. Conclusion

To sum up, our study confirms the hypothesis that plastic material ingestion by *C. atratus* adults causes sex- and organ-dependent biochemical alterations predictive of REDOX imbalance and cholinesterase effect without necessarily affecting the body conditions of these animals. We also revealed that the small amount of ingested plastic seems to be enough to trigger negative physiological responses in animals. Holistically, this may not only be an issue for vulture populations but also for a range of bird species that inhabit highly polluted areas (such as landfills) that may ingest or be affected by anthropogenic debris, in particular plastic bags. Thus, we defend the idea that continued monitoring of the impact of potential contaminants including plastics on vultures is necessary for the conservation of these animals, whose ecological importance goes far beyond their low popularity among humans.

## CRediT authorship contribution statement

**Wallace Alves Cunha**: study conception and design, data collection, analysis, and interpretation of results, and draft manuscript preparation. **Ítalo Nascimento Freitas**: data collection. **Lux Attiê Santos Gomes:** data collection. **Sandy de Oliveira Gonçalves:** data collection**. Abner Marcelino Silva**: data colletion. **Mateus Flores Montalvão**: data collection. **Mohamed Ahmed Ibrahim Ahmed**: analysis, and interpretation of results, and draft manuscript preparation. **Alex Rodrigues Gomes**: data collection. **Thiarlen Marinho da Luz**: data collection and analysis, and interpretation of results. **Amanda Pereira da Costa Araújo**: conceived of the presented idea; data collection and analysis, and interpretation of results. **Guilherme Malafaia**: conceived of the presented idea, collected the data, provided funding. All authors reviewed the results and approved the final version of the manuscript.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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### *Compliance With Ethical Standards*

### *Ethical approval*

All experimental procedures were carried out in compliance with ethical guidelines on animal experimentation, having been approved by the Ethics Committee on Animal Use (CEUA) of the IF Goiano (number: 2020128747). Furthermore, the authorization for the capture of specimens was obtained from the Biodiversity Information and Authorization System (SISBIO - Brazil), under protocol number 80241–1. Meticulous efforts were made to assure that animals suffered the least possible and to reduce external sources of stress, pain, and discomfort. The current study did not exceed the number of animals necessary to produce trustworthy scientific data. This article does not refer to any study with human participants performed by any of the authors.

## Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jhazmat.2021.127753.

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