INSTITUTO FEDERAL GOIANO, CAMPUS URUTAÍ - GO LICENCIATURA EM CIÊNCIAS BIOLÓGICAS

STEEL WOOLS MICROFIBERS CAUSES IRON OVERLOAD AND INDUCES BIOCHEMICAL CHANGES IN *Gallus gallus domesticus* CHICKS (GALLIFORMES: PHASIANIDAE)

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

Urutaí, GO Março de 2022

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“Por mais difícil que a vida possa parecer, existe sempre algo que você pode fazer e alcançar.”
Stephen Hawking

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ABSTRACT

Steel wool (SW) has a broad-spectrum of applicability, particularly as abrasives, cleaning household utensils and surfaces in general. However, when present in the natural environment, they can be ingested by animals, such as birds, and may represent a risk to the survival of individuals. Accordingly, in this study, we attempted the hypothesis that the ingestion of SW microfibers (SWMs) by *Gallus gallus domesticus* chicks (model system used) alters growth/development, induces redox imbalance and cholinesterasic effect, as well as promotes iron overload in different organs. For this, the animals received SWMs twice (within a 24-hour interval) in an amount corresponding to 12% of their total stomach volume. At the end of the experiment, we observed less weight gain and less head growth, increased production of hydrogen peroxide (in the brain, liver, crop, and gizzard), nitrite (liver, crop, proventriculus and gizzard), malondialdehyde (brain, liver, muscle, proventriculus, and gizzard), along with increased superoxide dismutase activity in the liver, muscle and crop of animals exposed to SWMs. Such results were associated with iron overload observed in different organs, especially in liver, crop, and gizzard. Furthermore, we evidenced an anti-cholinesterasic effect in birds that ingested the SWMs, marked by a reduction in the acetylcholinesterase activity (in brain). Thus, our study sheds light on the (eco)toxicological potential of SWMs in avifauna, conceding us to associate their ingestion (despite ephemeral and occasional) with damage to the health of individuals, requiring a greater attention spotted to disposal of these materials in ecosystems.

Keywords: biochemical toxicity, birds, pollution, fibrous materials, steel, toxicity biomarkers.

1. INTRODUCTION

Among the synthetic materials widely used all over the world, steel wools (SWs) stand out, also known as iron wool, wire wool, steel wire or wire sponge. Such materials were described as a new product in the late 19th century (Iron and Steel Institute, 1896) and have been used as abrasives in finishing and repair work for polishing wood or metal objects, cleaning household utensils, glass, porcelain, windows, and surfaces generally. Commercial SWs are available in a variety of grades that represent roughness or thickness, ranging from coarse to extra-fine, which further expands their applicability in different areas/sectors (Mitra et al., 2014). The SW production process involves the use of low-carbon steel in a process like broaching, in which a heavy steel wire is pulled through a toothed matrix that removes fine, sharp wire shavings (Kogel, 2006). Recently, several studies have investigated the use of SWs to increase the strength and durability of cementitious composite matrix (Begich et al., 2020; Amer et al., 2021; Rmdan-Amer et al., 2021; Saleem et al., 2021) and asphalt material (García et al., 2013; Dinh et al., 2018; Karimi et al., 2020; Hosseinian et al., 2020; Xu et al., 2021a; Fu et al., 2022), as well as for remediation of pollutants (Özer et al., 1997; Mitra et al., 2014; Santos-Juanes et al., 2017; Ike et al., 2018; Hildebrant et al., 2020), increased thermal conductivity of friction composites (Bijwe & Kumar, 2007) and optimization of tribological properties of materials (Vijay et al., 2013). Therefore, the use of SWs in the coming years tends to be greater and more diversified.

However, an unexplored field refers to the (eco)toxicological potential of SWs. During the use of these materials, the breakage of the wires and the release of small pieces that characterize the SW microfibers (SWMs) can occur. In the natural environment, SWMs can result from SW breakage caused by exposure to different environmental factors (including solar radiation, humidity, oxidation, among others), similarly, to processes that result in the formation of microparticles or microfibers of non-metallic materials, such as plastics (Xu et al., 2021b; Naik et al., 2021). Associated with this, steel sponges discarded in household waste, for example, can potentially be ingested by animals, with unknown toxicological consequences. Previous studies have shown that birds can ingest different types of materials, including small metallic fragments or fibers (Holland et al., 2016; Seif et al., 2018; Thaysen et al., 2020; Morkūnas et al., 2021; Vanstreels et al., 2021).

Thus, we can question whether the ingestion (accidental or voluntary) of these materials by birds induces negative effects on the health of these animals. What are the physiological consequences of this ingestion and to what extent does the disposal of SW in natural environments and the presence of SWMs in food or water constitute a risk to the survival of individuals? Aiming to contribute to the resolution of these issues, we evaluated for the first time the possible effects of SWMs ingestion by *Gallus gallus domesticus* chicks (used as a model system), assuming that the short exposure to these materials is sufficient to induce oxidative stress, cholinesterasic effect and change the

development/growth of animals. Furthermore, we evaluated the possible iron overload in different organs/tissues (proportioned by the ingestion of SWMs), correlating it with the different biomarkers evaluated. According to Whelan et al. (2008) and Michel et al. (2020), birds have important roles in ecosystems, being widely distributed in different environmental compartments, and susceptible, therefore, to contact with pollutants from numerous emitting sources. In polluted areas, birds are often exposed to a variety of pollutants through their diet, and via water and atmospheric deposition (Eeva et al., 2020; Celik et al., 2021; Bodziach et al., 2021). Furthermore, birds are often at higher trophic levels and therefore are subject to greater accumulation of pollutants compared to species that occupy lower trophic levels (Hosseini et al., 2013; Ahmadpour et al., 2016). Therefore, evaluating the possible effects of SWMs on these animals implies important subsidies for the conservation of the avifauna. From our study, pioneering evidence on the toxicity of SWMs is provided and, thus, it sheds light on the (eco)toxicological potential of these materials in birds, which together with other pollutants may be contributing to the population decline of several species in recent decades.

2. MATERIAL AND METHODS

2.1. Steel wool microfibers

SWs obtained in commercial establishments (Proeza® - JA PARENTI, Curitiba, PR, Brazil) were used, which are commonly used for household cleaning. To obtain the microfibers, the SW were perforated with scissors and subsequently sieved in a stainless-steel mesh (mesh: 150 μm). Then, the SWMs were stored in a dry container until use. The length and thickness of the SWMs (n=100) were measured using images obtained under a stereoscopic microscope, which were later analyzed via ImageJ software, similarly to the procedures described in Araújo et al. (2020).

2.2. Animals and experimental design

We used *Gallus gallus domesticus* (or *Gallus domesticus*) chicks (Galliformes, Phasianidae) autosexed commercial hybrid chicks "Embrapa-021" (a local variety derived from a White Cornish x White Plymouth Rock cross) obtained from a commercial incubator when they were only seven days old. This species is the most common domestic animals worldwide (Eda, 2021), whose estimated global population in 2017 was greater than >22 billion (FAO, 2021). Furthermore, scientifically, *G. gallus domesticus* chicks have been considered good experimental models in (eco)toxicological studies (Mesak et al., 2018; Vieira et al., 2019; Scalisi et al., 2020; Arcain et al., 2021).

After seven days of acclimation to the laboratory, the chicks (14 days old – body biomass: 128.60 g ± 7.09 g – mean ± SEM) were distributed into two experimental groups. The "control" group was composed of birds that were not exposed to SWMs and in the "SWM" group, the chicks received (orally) microfibers in an amount corresponding to 12% of the weight of the stomach contents of the

birds (i.e.: 0.9246 g ± 0.21 g - mean ± SEM), previously measured in our laboratory. Therefore, each animal in the "SWM" group received daily 0.1041 g ± 0.0005 g (mean ± SEM) of SWMs, which were forcibly introduced into the animals' esophagus, mixed in a small piece of wheat flour dough (0.98 g ± 0.08 g – mean ± SEM).

Considering the lack of studies involving the identification of SWMs in environmental compartments or food, the amount of microfiber offered to birds was based on the study by Seif et al. (2017). Such authors the authors observed that approximately 12% of the total stomach content of three gull species feeding in an urban landfill environment was composed of metallic fragments. Thus, we consider that the amount of SWMs introduced into chicks is environmentally relevant, as it does not overestimate the amount of metallic materials potentially ingested by wild birds. We emphasize that the SWMs were offered twice to the animals, once a day, at an interval of 24 h, simulating the occasional encounter of birds with these materials.

Each experimental group consisted of 10 chicks, which were kept during the experiment in steel cages (70 cm length x 50 cm width x 25 cm height), similarly to Mesak et al. (2018). The rearing cages were illuminated by a 250-W infrared bulb, and an artificial cycle of 12 h light and 12 h dark was provided. Food and water were supplied *ad libitum* and wire-mesh lids prevented the birds from jumping out. At the end of the experiment, the animals were submitted to different evaluations, as described below.

2.3. Toxicity Biomarkers

2.3.1. Biometry

The body condition of the birds (initial and final) was evaluated from the body biomass (BB), wing length (WL), head length (HL) and tarsus length (TL), as well as the BB/WL and BB/TL indices, according to previous studies (Chappell & Titman, 1983; DeVault et al., 2003; Schamber et al., 2009).

2.3.2. Biochemical biomarkers (oxidative stress and antioxidant activity)

Aiming to associate the possible ingestion of SWMs to the induction of a redox imbalance, different biochemical biomarkers were evaluated. For this, the animals were euthanized (via decapitation) to collect brain, liver, muscle (*pectoralis major*), crop, proventriculus, gizzard and intestine fragments. Such fragments were washed in phosphate buffered saline (PBS, pH 7.2), macerated in 1 mL of PBS and then centrifuged (13,000 rpm, 5 min, 4°C) to collect the supernatant, which was subsequently used. Before maceration, the food content of the crop, proventriculus, gizzard and intestine was carefully removed.

Malondialdehyde (MDA), one of the most known secondary products of lipid peroxidation (LPO) (Yaman & Ayhanci, 2021), was used as an oxidative stress biomarker, similarly to other studies

(Tan et al., 2019; Patel et al., 2021; Issac et al., 2021; Rangasamy et al., 2022; Cunha et al., 2022). For this, we adopted the procedures described in detail in the study by Sachett et al. (2020). To measure nitric oxide (NO), we used the Griess colorimetric reaction (Grisham et al., 1996), which consisted of detecting nitrite, resulting from the oxidation of NO, similarly to Ajjuri & O'Donnell (2013) and Estrela et al. (2021). Furthermore, we evaluated the superoxide dismutase (SOD) [according to Del-Maestro & McDonald (1987)] and catalase (CAT) activity [as proposed by Sinha et al. (1972)], considered first line defense antioxidant enzymes (Ighodaro et al., 2018). The acetylcholinesterase (AChE) activity – whose action is crucial in the propagation of nerve impulses (Dunant, 2021) – was evaluated in different organs, assuming a possible cholinesterasic effect induced by the ingestion of SWMs. For this, we adopted the procedures proposed by Ellman et al. (1961), with minor modifications described in Cunha et al. (2022). The results of the analysis of all biomarkers were expressed proportionally to the concentration of total proteins of each organ/tissue, evaluated according to the instructions of the commercial kit used [Commercial kit (Reference number: BT1000900)].

2.4. Determination of iron levels

Iron levels were evaluated in feces and in the fragments of different collected organs/tissues [brain, liver, muscle, crop, proventriculus, gizzard and intestine]. For the quantification of iron in feces, we adopted the protocol proposed by Goswami & Kalita (1988), with some modifications. Briefly, feces were weighed (0.10 g ± 0.0004 g – mean ± SEM) and introduced into beakers containing 5 mL of nitric acid 65% (HNO_3). Afterwards, the samples were placed on a hot plate for 2 h at 100°C. After the samples had been digested, the clock glass was removed, and the heating process continued until the samples had completely dried. Then, HNO_3 (at 5%, v/v) was added to dilute the residue of the samples, bringing the final volume to 25 mL. Posteriorly, 190 µL of the samples were pipetted in a 96-well microplate and mixed with 20 µL of potassium thiocyanate solution (at 40% wt/v). The absorbance values obtained were used to determine the concentration of Fe III in the samples, using the equation obtained from the standard curve (R^2 = 0.9997; Equation: y = 0.0112x + 0.0588) made from different concentrations of ammoniacal ferric sulfate [$NH_4Fe(SO_4)_2$].

In the assessment of iron levels in organs/tissues, we used the procedures described in Oliveira & Naozuka (2021) (with some adaptations), in which the labile fraction of the element was quantified in the supernatant used in the biochemical analyses, as described in item "2.3.2". Aliquots (190 µL) of the supernatant were pipetted in a 96-well microplate and mixed with 20 µL of potassium thiocyanate solution (at 40% wt/v). According to Cabantchik et al. (2014), the labile iron denotes the combined redox properties of iron and its amenability to exchange between ligands, including chelators. Furthermore, labile iron represents a component of non-transferrin-bound iron (NTBI), capable of permeating into organs and inducing tissue iron overload (Cabantchik et al., 2005).

2.5. Visual inspection of organs

We also evaluated the possible adherence of SWMs to the mucosal epithelium of the crop, proventriculus, gizzard and intestine. For this, after collection, the organs had their contents completely removed and were washed 5 times with PBS (pH 7.2). In a petri dish, the organs were analyzed under a stereoscopic microscope and to confirm the presence of identified microfibers, we use a small magnet. In this case, when close to the organ, the magnetic field produced by the magnet promoted a slight movement of the suspected SWMs, which was not observed in structures that were only like the SWMs in shape, color, and size.

2.6. Data analysis

All data were evaluated considering the assumptions for using parametric models. In addition, we used the Shapiro-Wilk test to assess the distribution of residual data and the Bartlett test was used to assess the homogeneity of variances. Student's t-test was used for normally distributed and homogeneous data and Mann-Whitney U-test was used for data with non-normal distribution and when homogeneity of variances was not achieved. Data related to iron levels in feces were submitted to two-way ANOVA, with the "treatments" (two levels: control and SWMs) and "time" (two levels: 24 and 48 h) factors. Multiple comparisons were performed using the Sidak test. Furthermore, correlations were performed using Pearson's (for parametric data) or Spearman's (for non-parametric data) correlation coefficients, as well as linear regression analysis. For all analyses, we considered a significance level of 95% ($p \leq 0.05$), using the Prism 9.0 (GraphPad Software, Inc., CA, USA).

3. RESULTS

3.1. Characterization of steel wool microfibers

The morphological and morphometric parameters of the SWMs, confirmed their heterogeneity, whose dimensions were different – there was a mix of major and minor SWMs (Figure 1A and 1E). SWMs mean length was 1002.07 µm ± 58.54 µm (mean ± SEM) (minimum: 131.0 µm; maximum: 2339.0 µm) and 49% of the microfibers had a length between 400 µm and 1000 µm (Figure 1A). Regarding the thickness of the SWMs, we observed an even greater heterogeneity (Figure 1B). The mean recorded was 77.91 µm ± 1.19 µm (mean ± SEM), with 29% of the SWMs having a thickness between 60 and 65 µm, 19% between 70 and 80 µm and in 42% of the SWMs, the thickness varied from 85 to 90 µm. Regarding the format, some SWMs were straighter (Figure 1F), with different levels of curvature (Figures 1G-J), different spiral shapes (Figures 1K-M) and others with multiple folds (Figure 1N).

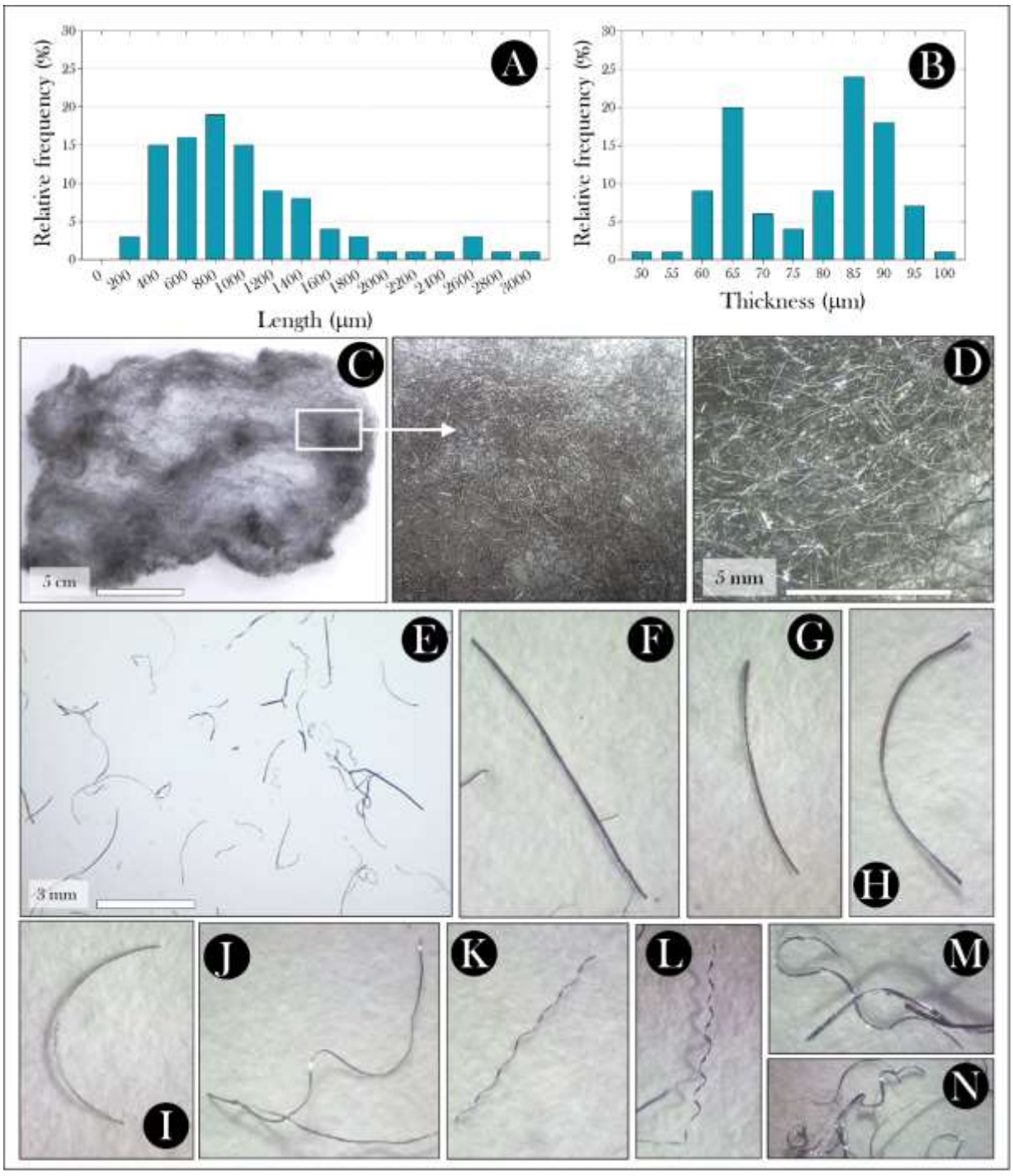

Figure 1. Histograms of distribution of (A) length and (B) thickness of steel wool microfibers (SWMs) (n=100 microfibers) and (C-N) representative photomicrographs of SWMs used in our study, with emphasis on the morphological variety and size.

3.2. Toxicity biomarkers

Assuming that the animals' exposure to SWMs could affect their body condition, different biometric parameters were evaluated. We did not evidence significant differences between the experimental groups in terms of feed and water consumption (graphics not shown), body biomass (Figure 2A), tail, head, and wings lengths (Figures 2B-D, respectively) and calculated indices (Figures

2E-F). However, we observed that at the end of the experiment, the unexposed animals ("control" group) increased body biomass by 12%, while in those exposed to SWMs this increase was only 3.9% (Figure 2A). For head length, we observed an increase of 7.8% in the "control" group and in animals exposed to SWMs the increase was 2.6%, which means a threefold difference.

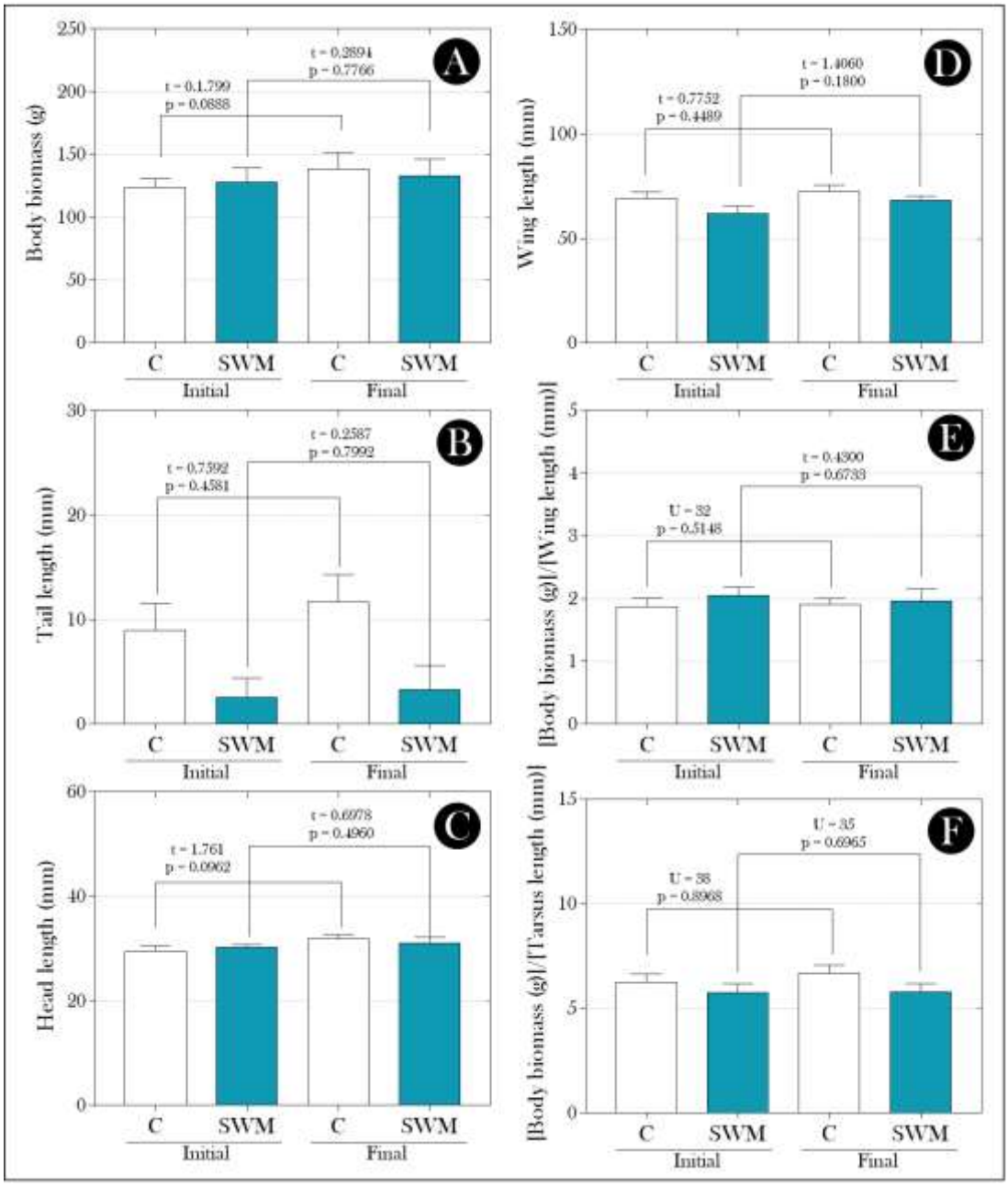

Figure 2. Initial and final biometric parameters of *Gallus gallus domesticus* chicks exposed or not to steel wool microfibers (SWMs). (A) Body biomass, (B) tail length, (C) head length, (D) wing length, (E) [body biomass (g)]/[wing length (mm)] and (F) [body biomass (g)]/[tarsus length (mm)]. The bars indicate the means + SD. Student's t-test was used for normally distributed and homogeneous data and Mann-Whitney U-test was used for data with non-normal distribution and when homogeneity of variances was not achieved (see statistical summary at the top of the graphs). C: group of animals that

did not receive SWMs (control group); SWM: group of chicks that received (orally) SWMs. n=10 animals/group.

On the other hand, we observed increase of the biomarkers of oxidative stress in the birds that ingested SWMs. While the production of H_2O_2 was significantly increased in the brain, liver, crop, and gizzard (Figure 3A) of these animals; MDA levels were high in all organs evaluated, except for the crop and intestine (Figure 3B). The increase in SOD activity (observed in liver, muscle, and crop) (Figure 4A), associated with non-observance of differences in CAT activity (Figure 4B), seem not to have been sufficient to counterbalance the production of H_2O_2, either the increase in LPO (inferred by MDA levels) in animals exposed to SWMs. In liver and crop, we observed a strong (positive) correlation between H_2O_2 levels and SOD activity (Figure 5A-B, respectively) and in brain, MDA and H_2O_2 levels were significantly correlated (Figure 5C). Furthermore, our data suggest the occurrence of a nitrosative stress in liver, crop, proventriculus, and gizzard in animals that ingested the SWMs [marked by increased nitrite levels (Figure 3C)], as well as an anti-cholinesterasic effect in the brain. As seen in Figure 6, cerebral AChE activity in birds exposed to SWMs was 61.9% lower than that observed in the "control" group.

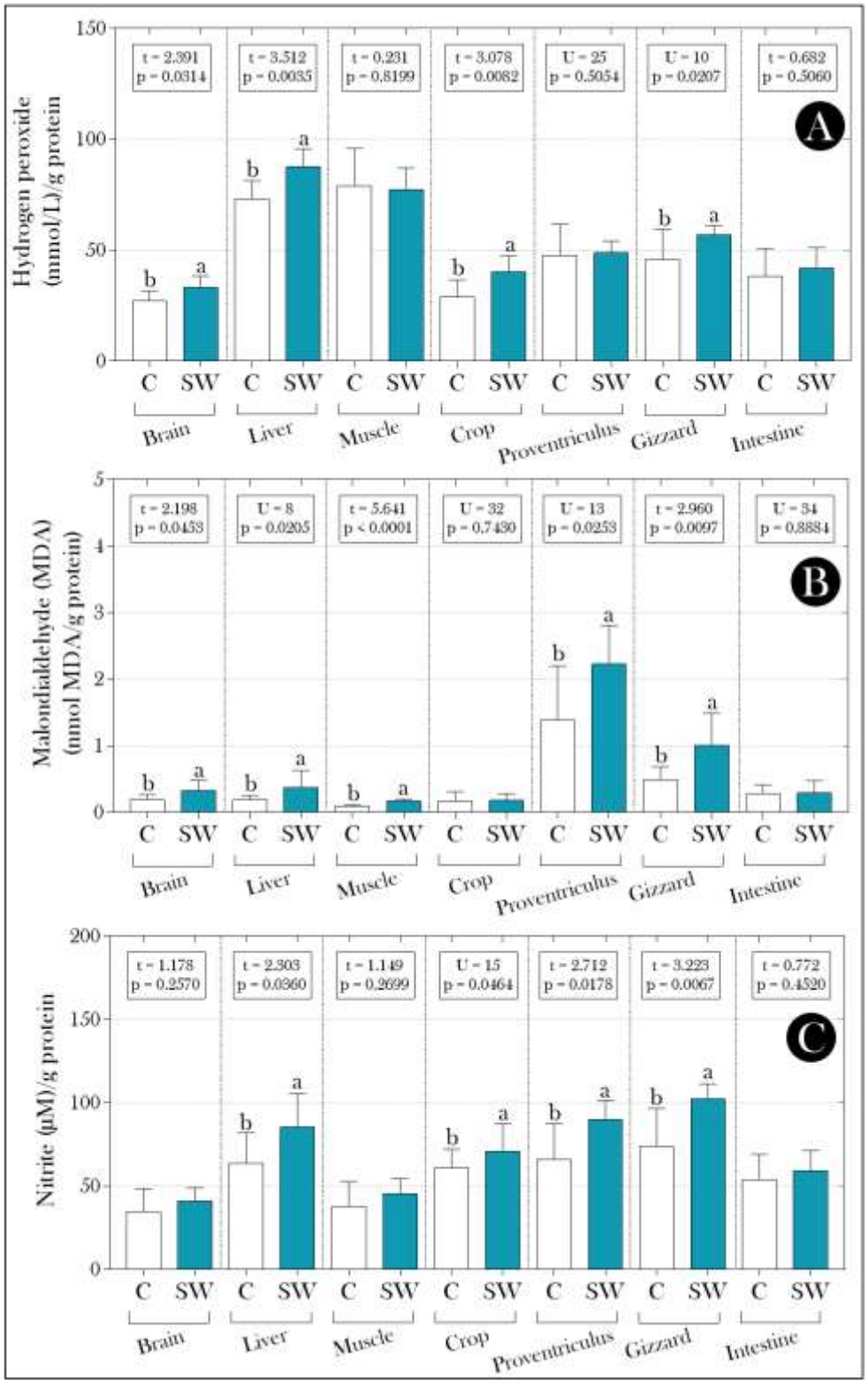

Figure 3. Hydrogen peroxide (H_2O_2) (A), malondialdehyde (MDA) (B) and nitrite (C) levels evaluated in different organs/tissue of *Gallus gallus domesticus* chicks exposed or not to steel wool microfibers (SWMs). The bars indicate the means + SD. Student's t-test was used for normally distributed and homogeneous data and Mann-Whitney U-test was used for data with non-normal distribution and when homogeneity of variances was not achieved (see statistical summary at the top

of the graphs). C: group of animals that did not receive SWMs (control group); SW: group of chicks that received (orally) SWMs. n=10 animals/group.

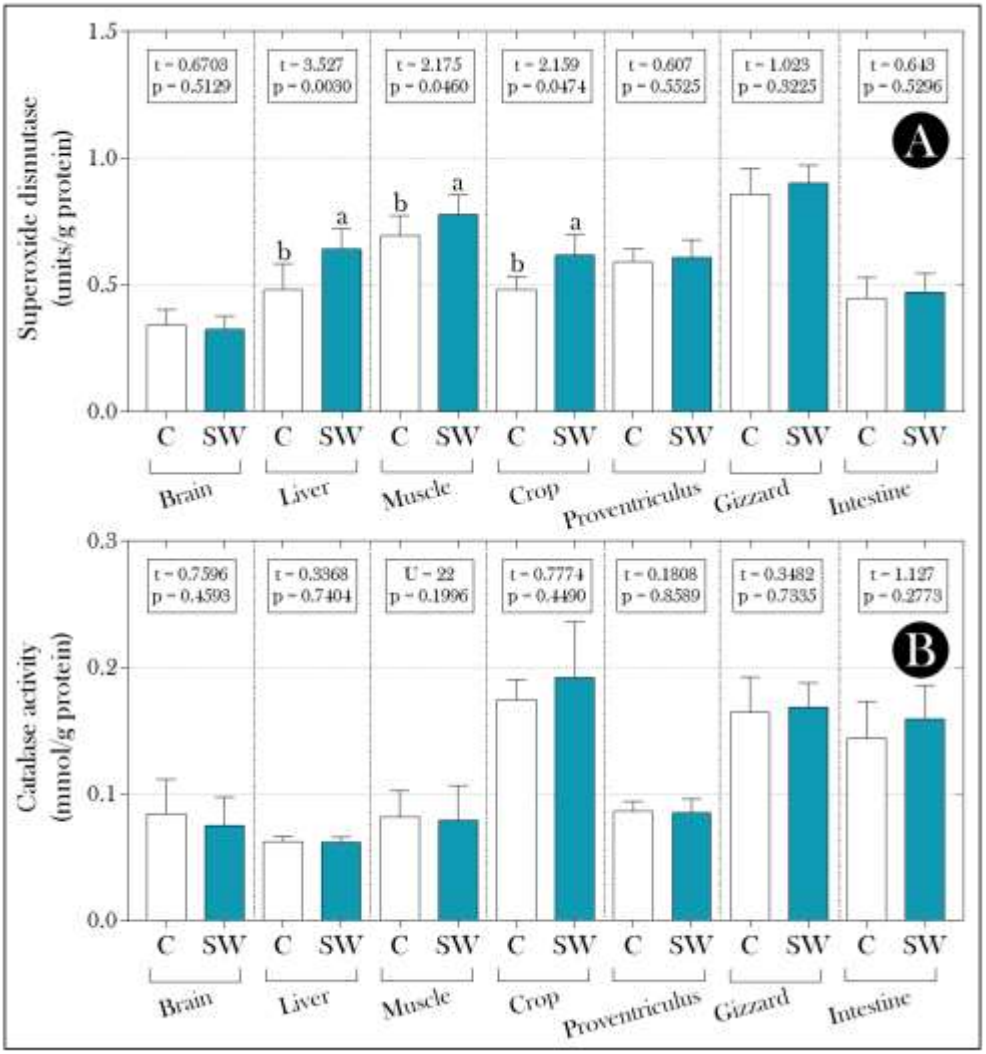

Figure 4. (A) Superoxide dismutase (SOD) and (B) catalase (CAT) activities evaluated in different organs of *Gallus gallus domesticus* chicks exposed or not to steel wool microfibers (SWMs). The bars indicate the means + SD. Student's t-test was used for normally distributed and homogeneous data and Mann-Whitney U-test was used for data with non-normal distribution and when homogeneity of variances was not achieved (see statistical summary at the top of the graphs). C: group of animals that did not receive SWMs (control group); SW: group of chicks that received (orally) SWMs. n=10 animals/group.

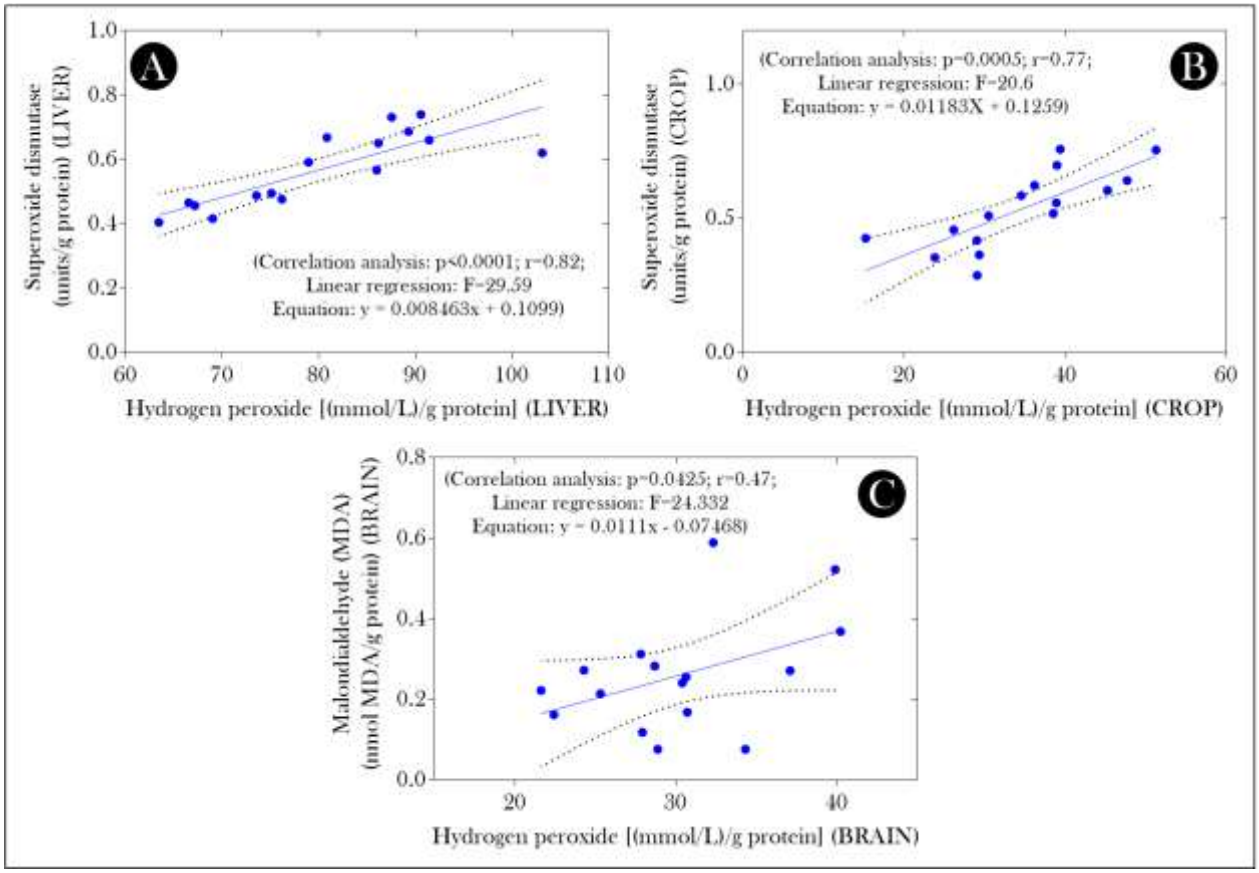

Figure 5. Correlation analysis and linear regression between hydrogen peroxide (H_2O_2) levels and superoxide dismutase (SOD) activity in liver and crop (A-B, respectively) and between H_2O_2 production and malondialdehyde (MDA) levels in brain (C) of *Gallus gallus domesticus* chicks exposed or not to steel wool microfibers (SWMs).

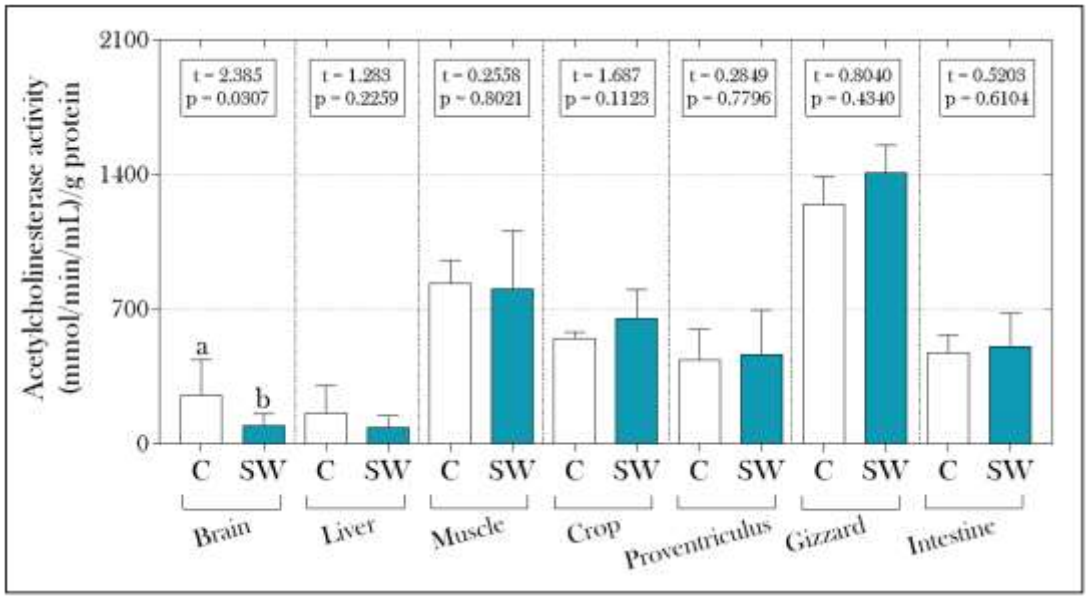

Figure 6. Acetylcholinesterase (AChE) activity evaluated in different organs/tissues of *Gallus gallus domesticus* chicks exposed or not to steel wool microfibers (SWMs). The bars indicate the means +

SD. Student's t-test was used for normally distributed and homogeneous data and Mann-Whitney U-test was used for data with non-normal distribution and when homogeneity of variances was not achieved (see statistical summary at the top of the graphs). C: group of animals that did not receive SWMs (control group); SW: group of chicks that received (orally) SWMs. n=10 animals/group.

3.3. Iron bioaccumulation

Considering the animals' response to SWMs (see summary in Table 1), we evaluated the relationship between the redox imbalance observed in different organs/tissues and the possible iron overload. We observe that although much of the iron content (probably from the SWMs) was excreted via feces (Figure 7A), the labile iron levels in the liver, crop, and gizzard of animals in the "SWMs" group were higher than those identified in the "control" group (Figure 7B). In liver and gizzard, our analyzes revealed a significant correlation between increased H_2O_2 production and SOD activity with labile iron levels in these organs (Figure 8A-B, respectively). In crop, the nitrite levels were proportional to the labile iron levels (Figure 8C). Additionally, the visual inspection performed on the organs revealed the presence of SWMs adhered to the mucosal epithelial tissue of the crop (Figures 9A-C), proventriculus (Figures 9D-F) and gizzard (Figures 9G-H).

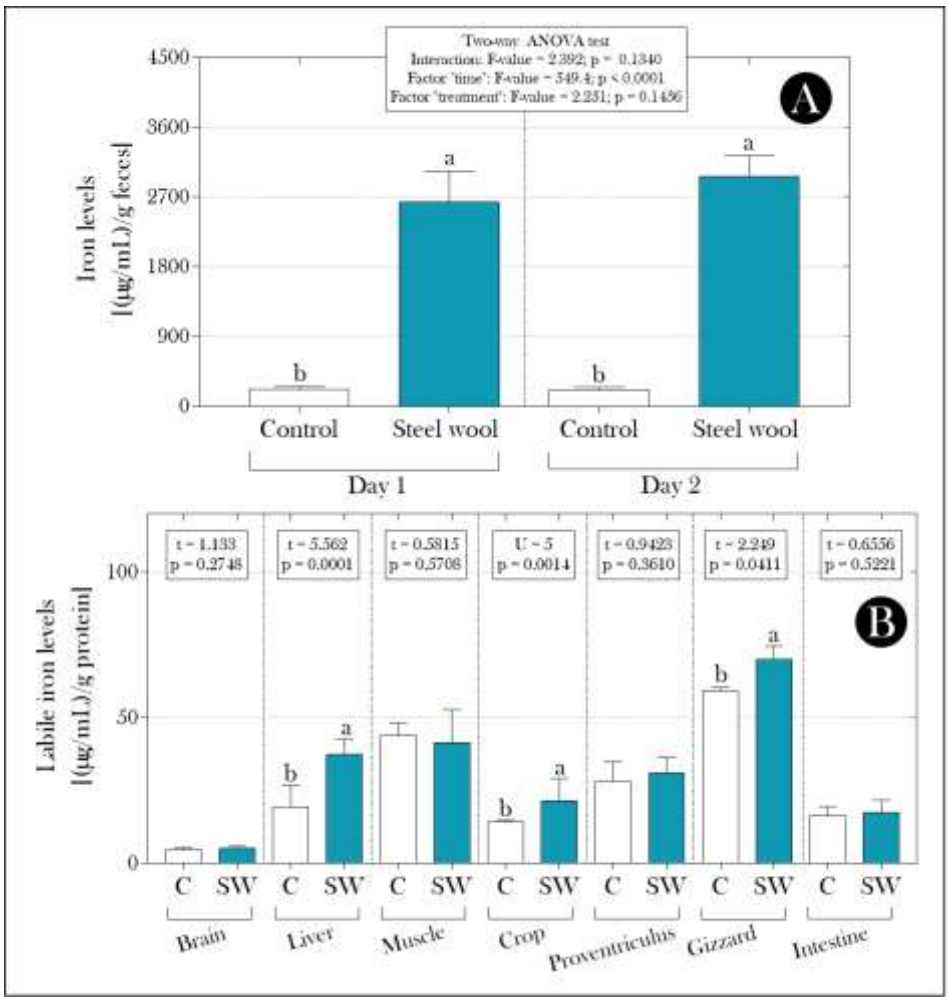

Figure 7. Iron levels in (A) feces and in (B) different organs/tissues of *Gallus gallus domesticus* chicks exposed or not to steel wool microfibers (SWMs). Bars indicate means + SD. In "A", the data were submitted to two-way ANOVA, with Sidak's post-test, at 5% probability. In "B", Student's t-test was used for normally distributed and homogeneous data and Mann-Whitney U-test was used for data with non-normal distribution and when homogeneity of variances was not achieved. C: group of animals that did not receive SWMs (control group); SW: group of chicks that received (orally) SWMs. n=10 animals/group.

Table 1. Synthesis of results obtained in the present study, with identification of increase (↑) decrease (↓) or no change (-) in the parameters evaluated in *Gallus gallus domesticus* chicks exposed or not to steel wool microfibers (SWMs).

Biometry	Experimental groups	
	Control	SWMs
Body biomass	-	-
Tail length	-	-
Head length	-	-
Wing length	-	-
Body condition index #1	-	-
Body condition index #2	-	-

Oxidative stress biomarkers	Brain		Liver		Muscle		Crop		Proventriculus		Gizzard		Intestine	
	C	SW	C	SW	C	SW	C	SW	C	SW	C	SW	C	SW
Hydrogen peroxide	↓	↑	↓	↑	-	-	↓	↑	-	-	↓	↑	-	-
Malondialdehyde	↓	↑	↓	↑	↓	↑	-	-	↓	↑	↓	↑	-	-
Nitrite	-	-	↓	↑	-	-	↓	↑	↓	↑	↓	↑	-	-
Antioxidant activity biomarkers	**C**	**SW**	**C**	**SW**	**C**	**SW**	**C**	**SW**	**C**	**SW**	**C**	**SW**	**C**	**SW**
Superoxide dismutase activity	-	-	↓	↑	↓	↑	↓	↑	↓	↑	↓	↑	-	-
Catalase activity	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Cholinesterasic effect biomarker	**C**	**SW**	**C**	**SW**	**C**	**SW**	**C**	**SW**	**C**	**SW**	**C**	**SW**	**C**	**SW**
Acetylcholinesterase activity	↑	↓	-	-	-	-	-	-	-	-	-	-	-	-

Legend: C: control group; SW: group submitted to ingestion of steel wool microfibers (SWMs); Body condition index #1: [Body biomass (g)]/[Wing length (mm)]; Body condition index #2: [Body biomass (g)]/[Tarsus length (mm)].

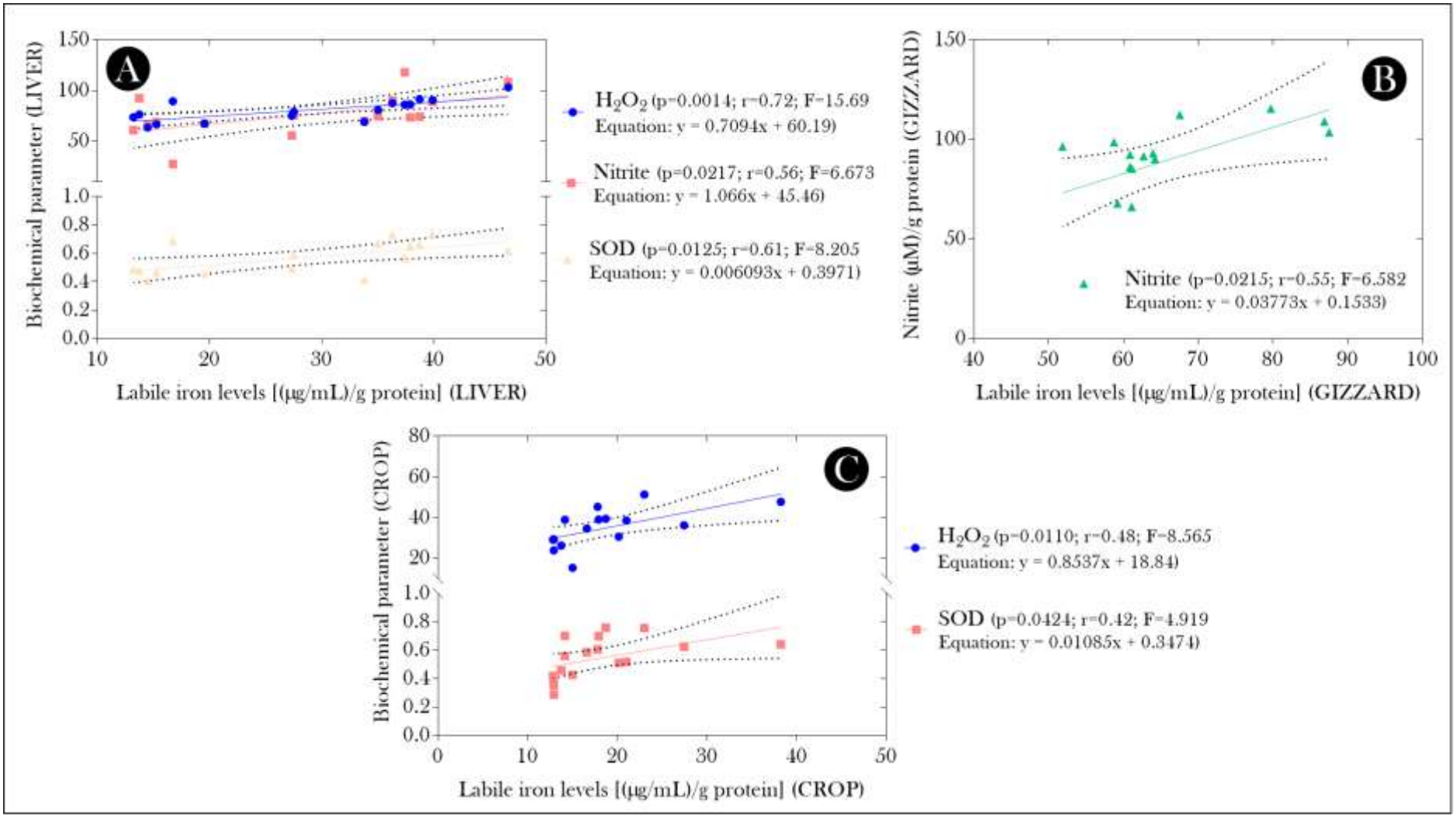

Figure 8. Correlation analysis and linear regression between different biochemical biomarkers and iron labile levels in (A) liver, (B) gizzard and (C) crop of *Gallus gallus domesticus* chicks exposed or not to steel wool microfibers (SWMs).

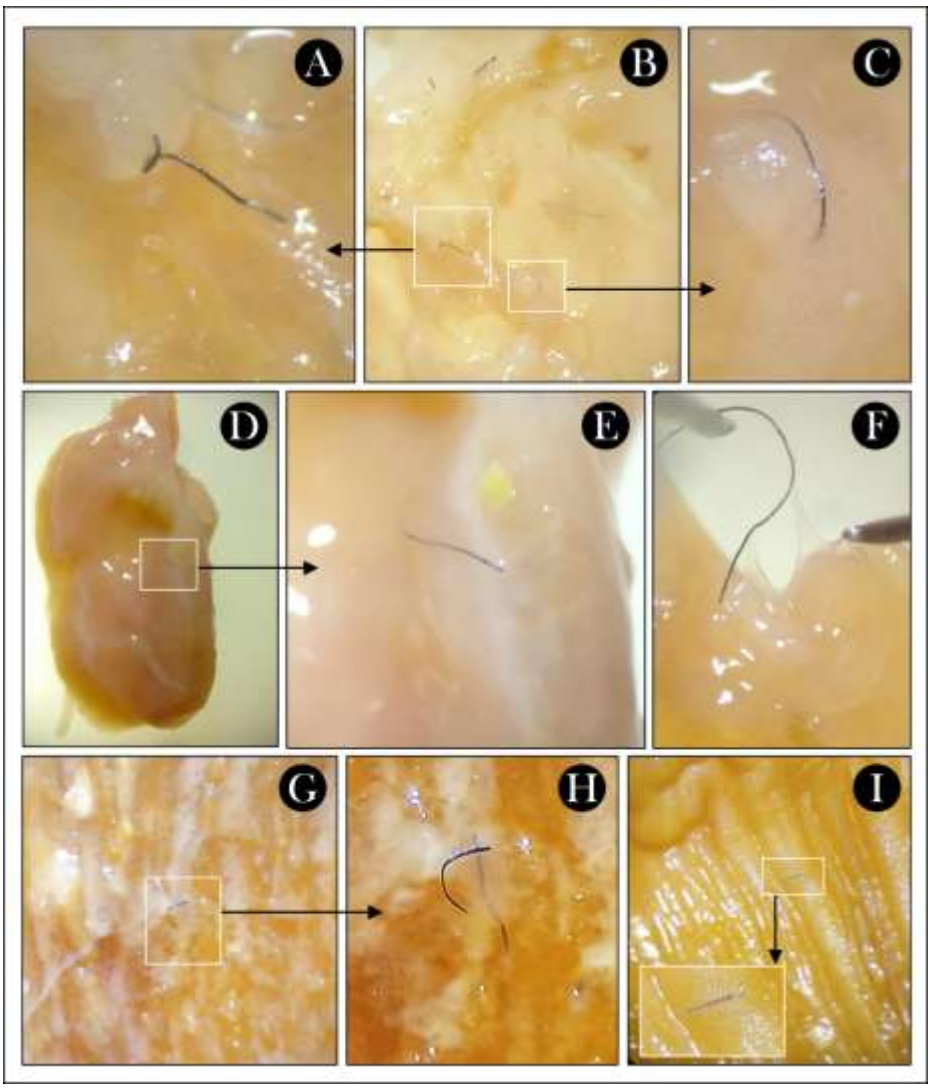

Figure 9. Representative photomicrographs of (A-C) crop, (D-F) proventriculus, and (G-H) gizzard of *Gallus gallus domesticus* chicks exposed to steel wool microfibers (SWMs), with emphasis on the adherence of microfibers to the mucosal epithelial tissue of these organs.

4. DISCUSSION

Generally, it is undeniable that the development and increased production of synthetic materials imply direct benefits to human beings. The use of these materials in different areas and sectors undoubtedly improves the quality of the products, especially regarding their durability and strength. However, the impact that these materials (when present in the natural environment) can have on the biota, needs to be considered, especially in relation to those for which there are no treatment processes before their disposal. This is the case of SWMs which, as shown in this study, if ingested by birds, can represent a risk to the survival of individuals.

We initially evidenced that the short period of exposure to SWMs showed potential to affect the growth/development of *G. gallus domesticus* chicks. The reduced percentage of body biomass gain and head growth of animals in the "SWMs" group (compared to the "control" group) (Figure 2A and

C, respectively) suggest that, in the long term, the ingestion of SWMs may negatively interfere in the growth/development of birds. According to Felter et al (2015), the early life stage is an important stage not only for the growth of animals, but also for the development and maturation of different physiological systems. Thus, changes in development can have drastic consequences in the adult life of animals. Although studies like ours have not been developed so far, our results are like those in which the exposure of juvenile birds to metallic pollutants negatively affected the growth/development of the animals (Huff et al., 1996; Spahn & Sherry, 1999; Abduljaleel et al., 2013). Spahn & Sherry (1999) evidenced that *Egretta caerulea* clicks exposed to Cd had significantly slower growth rates than nonexposed chicks, and exposure to Pb was correlated with increased nestling mortality. In Abduljaleel et al. (2013), the exposure of *G. gallus domesticus* chicks to Pb significantly decreased body biomass of animals after dietary treatment, similarly to the results reported by Huff et al. (1996), involving exposure to aluminum sulfate.

However, reports on the impacts of exposure of chicks to iron (the main constituent of SWMs) on the growth/development of animals are inconclusive, as well as the mechanisms that may explain the trend observed in our study. In Farrow et al. (1983), for example, in the significant effect on growth performance with 2500 mg/kg supplementary iron in White Rock chicks was observed, similarly to the study by Lin et al. (2020), when yellow-feathered broiler chicks received (also from the diet) different iron levels. In this latter study, the results showed that different levels of dietary iron (50 to 150 mg/kg) had no significant impact on the growth performance of yellow broiler chicks during 1 to 21, 22 to 42, or 43 to 63 days, indicating that the iron content in the basic diet was sufficient for the growth of these birds. Ma (2014) reported that the level of dietary iron (47–147 mg/kg) had no effect on the growth performance of AA broilers chicks. Cao et al. (1996) showed that the addition of high doses of iron to the diet (400, 600 and 800 mg/kg) significantly increased body biomass and average daily feed intake of Ross broilers chicks. Therefore, these studies indicate that different chicken breeds had different tolerances and requirements for iron.

On the other hand, it is known that the increase in oxidative stress processes implies greater energy expenditure by the antioxidant system, aiming to counterbalance the production of potentially harmful free radicals to cells. In this case, even though preliminary, we can assume that the lower weight gain of animals exposed to SWMs (three times smaller than the "control" group) is related to an energy reallocation mechanism, aimed at achieving the imbalance between cellular pro-oxidant levels and antioxidant capacity in favor of the somatic homeostasis. However, the increased H_2O_2 production (in the brain, liver, crop, and gizzard – Figure 3A) associated with the increase in SOD activity (in the liver, muscle, and crop – Figure 4A), characterizes a typical redox imbalance observed in animals exposed to SWMs. Thus, it is plausible to assume that the absence of increased catalase activity (which breaks down H_2O_2 into water and molecular oxygen) (Figure 4B) and a possible reduction in glutathione

peroxidase (GPx) activity (which reduces H_2O_2 to water and lipid peroxides to their corresponding alcohol) may explain the high H_2O_2 levels observed in different organs/tissues of animals exposed to SWMs. Lee et al. (1981) reinforces this hypothesis by demonstrating that total GPx activity was decreased in liver of male Sprague-Dawley rats, with high dietary iron. At the same time, we cannot neglect the hypothesis of increased cellular energy expenditure to control or mitigate the processes of LPO (Figure 3B) have influenced the animals' growth. According to Ayala et al. (2014), under subtoxic conditions, cells stimulate their constitutive antioxidant defense systems (which are not restricted to the action of the enzymes evaluated in our study, SOD and CAT) or activate signaling pathways that positively regulate antioxidant proteins, resulting in a response adaptive to stress. All these mechanisms demand energy that could be used in the growth/development of animals.

Another interesting data observed in our study refers to the significant increase in iron levels in the crop, gizzard, and liver (Figure 7B), which indicates the SWMs as a supplementary source of this metal. In crop and gizzard, the iron levels would be related to the deterioration of SWMs, due to corrosion, after encountering water, oxygen, and internal fluids. Although *ad libitum* feeding in chicks reduces the physical use of the crop [since animals adjust the frequency of feed intake to the rate of passage of the digesta (Svihus et al., 2013; Kierończyk et al., 2016)], the adhesion of SWMs in the mucosal layer of this organ (Figure 9A-C) could, by itself, provide opportunities for the availability of additional iron levels. The high labile iron levels in the liver, on the other hand, would be related to iron translocation from the intestine to the circulation hepatic portal, via the absorptive action of the proximal intestinal villus epithelial cells. Although a previous study indicates that chicks can down-regulate or up-regulate uptake of iron (which normally do not develop overload) (Mete et al., 2003), iron overload from SWMs may have caused disturbances in these regulation mechanisms, the which would explain the high absorption of dietary iron relative to body iron needs.

Consequently, it is plausible to suppose that the increase in labile iron levels in the different organs evaluated in our study would be related to the increase in oxidative stress in birds of the "SWM" group. It is agreed that iron is an essential transition metal for organisms, being part of several metalloproteins (Liu et al., 2014), in addition to being involved in different vital biochemical processes, such as those involved in the transport of oxygen in tissues (Gupta , 2014; Abbaspour et al., 2014), electron transfer reactions during mitochondrial respiration (Paul et al., 2017), DNA synthesis and repair (Puig et al., 2017) and in the metabolism of xenobiotics (Crichton, 2016). However, when present in excess in cells and tissues, iron can interrupt redox homeostasis and catalyze the propagation of ROS, leading to oxidative stress. As demonstrated by Merkofer et al. (2006), the presence of unshielded redox-active iron favors Fenton chemistry; what involves the reaction between H_2O_2 and Fe^{2+}, yielding extremely reactive HO• radical dot via ferryl/perferryl intermediates. In spite this radical is short-lived, its high reactivity can oxidize any chemical group that is close to its formation (Galaris et al., 2019). For

example, iron bound to membrane phospholipids catalyzes the initiation of LPO chain reactions, which would explain the increased MDA levels in most organs evaluated in birds exposed to SWMs (Figure 3B). The fact that the labile iron is redox-active, chelatable (i.e.: it can be sequestered by chelating compounds) and exchangeable (i.e.: it can be easily transferred among natural ligands and between cell compartments) (Kakhlon & Cabantchik, 2002) reinforces the hypothesis of excess iron in birds ("SWM" group) is directly associated to the induction of oxidative stress. Several studies that have associated exposure of animal models to high iron concentrations/doses with increased oxidative processes also support this hypothesis (Reardon & Allen, 2009; Sampaio et al., 2014; Handa et al., 2016; Tarifeño-Saldivia et al., 2016; Tarifeño-Saldivia et al., 2016; al., 2018; Jiang et al., 2019; Sönmez-Aydın et al., 2021; Li et al., 2021).

Further result observed in our study refers to the increased nitrite levels in liver, crop, proventriculus and gizzard of chicks from the "SWM" group (Figure 3C), which suggests the induction of a nitrosative stress induced by the ingestion of microfibers. In this case, it is likely that the increase in NO production (inferred by nitrite levels) is associated with the increase in nitric oxide synthase (iNOS) expression by extracellular signal-regulated kinase (ERK1/2) and NF-kappaB activation (induced by iron overload), as also suggested by Cornejo et al. (2005), in a study involving rats fed diet-enriched with carbonyl-iron (to 3% wt/wt). Thus, investigating the hypothesis that iron overload observed in the liver, crop and gizzard of animals exposed to SWMs upregulates the expression of iNOS and NOS activity in conditions of oxidative stress, constituted a fertile future investigative perspective. On the other hand, we cannot neglect the hypothesis that the increase in NO in birds is related to an immune stimulation in response to SWMs adhered to the epithelial layer of the crop (Figure 9A-C) and gizzard (Figure 9G-I). In this case, possible microlesions caused by SWMs would be sufficient to trigger an inflammatory response in animals, with consequent recruitment of phagocytic cells such as monocytes, neutrophils, and macrophages, whose mechanisms of action include the production of NO (Bogdan, 2001; Wink et al., 2011). Alternatively, it is possible that the nitrosative stress observed in birds exposed to SWMs constitutes an adaptive response related to the antioxidant properties of NO. Contrary to the deleterious effects of the reactive nitrogen oxide species formed from either NO/O_2 and NO/O_2^-, it has been pointed out that NO shows antioxidant properties (Rubbo et al., 1994; Rubbo & Radi, 2001; Galleano et al., 2004). Since the biological chemistry of these molecules is dominated by free-radical reactions, the interaction of NO with other free-radical species could lead to either inhibition or potentiation of oxidative damage effect (Beckman et al., 1990). This hypothesis is supported especially by the study by Hummel et al. (2006), in which the authors suggest that the continuous production of NO would yield a steady-state concentration of only 10–20 nM is capable, in human leukemia cells, of inhibiting Fe^{2+}-induced lipidic peroxidation. However, any one of these hypotheses needs to be further investigated in further studies.

Interestingly, we also noticed an anticholinesterasic effect (marked by the reduction of cerebral AChE activity) in animals exposed to SWMs (Figure 6), which agrees with the findings of Perez et al. (2010), involving Wistar rats. Such authors observed reduced AChE activity in animals that received Fe^{2+} at 10 mg/kg of body weight, which was associated with cognitive deficits induced by iron overload. Although the precise mechanisms involved in the reduction of striatal AChE activity in adult rats (Perez et al., 2010) and in *G. gallus domesticus* chicks (in our study) have not been investigated, it is tempting to speculate that in both studies, the iron accumulation would have triggered a cascade of events that impaired cholinergic transmission or induced terminal degeneration. On the other hand, a study involving adults wild-type zebrafish (*Danio rerio*) demonstrated that 24 h of exposure to iron (to 15 mg/L) was sufficient to increase the AChE activity in both, brain (62%) and liver (70%), in relation to unexposed animals (Sant'Anna et al., 2011). Therefore, it is possible that the cholinesterasic response to iron overload is dependent on the studied species, as well as on the iron concentrations and evaluated exposure periods.

Regardless of the biological mechanisms intrinsic to the effects observed in our study, it is evident that the ingestion of SWMs by *G. gallus domesticus* chicks induces alterations that harm the animals' health. The accumulation of iron and, consequently, iron overload can induce, for example, oxidative stress (as noted), as well as causing behavioral changes (associated with a deficiency in cholinergic transmission), damage to the genetic material caused by the increase in free radicals and the association from iron to DNA (including mutations or single and double strand breaks), as well as enzymatic, hormonal and signal transduction dysfunctions that regulate various physiological functions, caused by the binding of the chemical element to different proteins. Thus, taken together, these effects represent a risk to the animals' survival (reducing their fitness), with the potential to change the dynamics of bird populations that ingest SWMs discarded in natural environments.

Based on the above, it is noteworthy that due to the pioneering nature of our study in evaluating the possible toxicity of SWMs in birds, several investigative perspectives that can be explored in the future. It is questioned, for example, whether the effects reported in the present study would be more accentuated in longer exposures and whether similar responses would be observed in adults (males and females). How much the effects evidenced in our study can affect the development/growth of animals (in the long term) and whether the observed changes will impact the adult life of the animals, also constitute a fertile field for further investigation. At the individual level, assessments using predictive biomarkers of gene dysregulation, histopathological, behavioral, and hormonal damages will be essential for a better understanding of how much the ingestion of SWMs (although ephemeral and occasional) can affect the survival of animals. The regulation of ferritin (the main intracellular iron storage protein – Ponka et al., 1998) in the context of SWMs ingestion should also be investigated in the future. At the population level, it is questioned, for example, to what extent the exposure of animals

to these microfibers can affect their social interactions, reproduction, and the dynamics of their populations in natural environments.

5. CONCLUSION

In conclusion, our data confirmed the toxicological potential of SWMs in *G. gallus domesticus* chicks, demonstrating that their ingestion, although ephemeral and occasional, causes iron overload in different organs, redox unbalance, as well as the reduction of cerebral AChE activity in birds exposed to SWMs. However, it must be recognized that our study is not exhaustive and, therefore, constitutes only the "tip of the iceberg" that represents the (eco)toxicological effects associated to SWMs in ecosystems. Thus, we strongly recommend that further studies continue the assessment of the potential toxicity of these materials not only to birds but also to other groups of animals that inhabit areas where the presence of SW and/or SWMs is real.

6. ACKNOWLEDGMENTS

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7. DECLARATION OF COMPETING INTEREST

We confirm that there are no known conflicts of interest associated with this work and there has been no significant financial support for this work that could have influenced its outcome. We confirmed that the manuscript has been read and approved by all named authors and that there are no other persons who satisfied the criteria for authorship but are not listed. We further confirm that the order of authors listed in the manuscript has been approved by all of us. Due care has been taken to ensure the integrity of the work.

8. ETHICAL ASPECTS

All experimental procedures were performed in accordance with the ethical standards for animal experimentation and meticulous efforts were made to ensure that the animals suffered as little as possible and to reduce external sources of stress, pain, and discomfort. The current study has not exceeded the number of animals needed to produce reliable scientific data. This article does not refer to any study with human participants performed by any of the authors.

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10. SUPPLEMENTARY MATERIAL

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Steel wools microfibers causes iron overload and induces biochemical changes in *Gallus gallus domesticus* chicks (Galliformes: Phasianidae)

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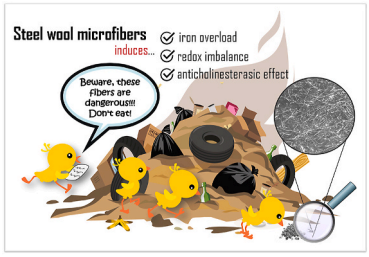
HIGHLIGHTS

- Ingestion of SW microfibers by *G. domesticus* chicks induces iron overload.
- Redox unbalance is observed after ingestion of SWMs in different organs/tissues.
- Anticholinesterasic effect suggests SWM-induced neurotoxicity in chicks.

GRAPHICAL ABSTRACT



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ABSTRACT

Steel wool (SW) has a broad-spectrum of applicability, particularly as abrasives, cleaning household utensils and surfaces in general. However, when present in the natural environment, they can be ingested by animals, such as birds, and may represent a risk to the survival of individuals. Accordingly, in this study, we attempted the hypothesis that the ingestion of SW microfibers (SWMs) by *Gallus gallus domesticus* chicks (model system used) alters growth/development, induces redox imbalance and cholinesterasic effect, as well as promotes iron overload in different organs. For this, the animals received SWMs twice (within a 24-h interval) in an amount corresponding to 12% of their total stomach volume. At the end of the experiment, we observed less weight gain and less head growth, increased production of hydrogen peroxide (in the brain, liver, crop, and gizzard), nitrite (liver, crop, proventriculus and gizzard), malondialdehyde (brain, liver, muscle, proventriculus, and gizzard), along with increased superoxide dismutase activity in the liver, muscle and crop of animals exposed to SWMs. Such results were associated with iron overload observed in different organs, especially in liver, crop, and gizzard. Furthermore, we evidenced an anti-cholinesterasic effect in birds that ingested the SWMs, marked by a reduction in the acetylcholinesterase activity (in brain). Thus, our study sheds light on the (eco)toxicological potential of

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SWMs in avifauna, conceding us to associate their ingestion (despite ephemeral and occasional) with damage to the health of individuals, requiring a greater attention spotted to disposal of these materials in ecosystems.

1. Introduction

Among the synthetic materials widely used all over the world, steel wools (SWs) stand out, also known as iron wool, wire wool, steel wire or wire sponge. Such materials were described as a new product in the late 19th century (Iron and Steel Institute, 1896) and have been used as abrasives in finishing and repair work for polishing wood or metal objects, cleaning household utensils, glass, porcelain, windows, and surfaces generally. Commercial SWs are available in a variety of grades that represent roughness or thickness, ranging from coarse to extra-fine, which further expands their applicability in different areas/sectors (Mitra et al., 2014). The SW production process involves the use of low-carbon steel in a process like broaching, in which a heavy steel wire is pulled through a toothed matrix that removes fine, sharp wire shavings (Kogel et al., 2006). Recently, several studies have investigated the use of SWs to increase the strength and durability of cementitious composite matrix (Begich et al., 2020; Amer et al., 2021; Rmdan-Amer et al., 2021; Saleem et al., 2021) and asphalt material (García et al., 2013; Dinh et al., 2018; Karimi et al., 2020; Hosseinian et al., 2020; Xu et al., 2021a; Fu et al., 2022), as well as for remediation of pollutants (Özer et al., 1997; Mitra et al., 2014; Santos-Juanes et al., 2017; Ike et al., 2018; Hildebrant et al., 2020), increased thermal conductivity of friction composites (Bijwe & Kumar, 2007) and optimization of tribological properties of materials (Vijay et al., 2013). Therefore, the use of SWs in the coming years tends to be greater and more diversified.

However, an unexplored field refers to the (eco)toxicological potential of SWs. During the use of these materials, the breakage of the wires and the release of small pieces that characterize the SW microfibers (SWMs) can occur. In the natural environment, SWMs can result from SW breakage caused by exposure to different environmental factors (including solar radiation, humidity, oxidation, among others), similarly, to processes that result in the formation of microparticles or microfibers of non-metallic materials, such as plastics (Xu et al., 2021b; Naik et al., 2020). Associated with this, steel sponges discarded in household waste, for example, can potentially be ingested by animals, with unknown toxicological consequences. Previous studies have shown that birds can ingest different types of materials, including small metallic fragments or fibers (Holland et al., 2016; Seif et al., 2018; Thaysen et al., 2020; Morkūnas et al., 2021; Vanstreels et al., 2021).

Thus, we can question whether the ingestion (accidental or voluntary) of these materials by birds induces negative effects on the health of these animals. What are the physiological consequences of this ingestion and to what extent does the disposal of SW in natural environments and the presence of SWMs in food or water constitute a risk to the survival of individuals? Aiming to contribute to the resolution of these issues, we evaluated for the first time the possible effects of SWMs ingestion by *Gallus gallus domesticus* chicks (used as a model system), assuming that the short exposure to these materials is sufficient to induce oxidative stress, cholinesterasic effect and change the development/growth of animals. Furthermore, we evaluated the possible iron overload in different organs/tissues (proportioned by the ingestion of SWMs), correlating it with the different biomarkers evaluated. According to Whelan et al. (2008) and Michel et al. (2020), birds have important roles in ecosystems, being widely distributed in different environmental compartments, and susceptible, therefore, to contact with pollutants from numerous emitting sources. In polluted areas, birds are often exposed to a variety of pollutants through their diet, and via water and atmospheric deposition (Eeva et al., 2020; Celik et al., 2021; Bodziach et al., 2021). Furthermore, birds are often at higher trophic levels and therefore are subject to greater accumulation of pollutants compared to species that occupy lower trophic levels (Hosseini et al., 2013; Ahmadpour et al., 2016). Therefore, evaluating the possible effects of SWMs on these animals implies important subsidies for the conservation of the avifauna. From our study, pioneering evidence on the toxicity of SWMs is provided and, thus, it sheds light on the (eco)toxicological potential of these materials in birds, which together with other pollutants may be contributing to the population decline of several species in recent decades.

2. Material and methods

2.1. Steel wool microfibers

SWs obtained in commercial establishments (Proeza® - JA PARENTI, Curitiba, PR, Brazil) were used, which are commonly used for household cleaning. To obtain the microfibers, the SW were perforated with scissors and subsequently sieved in a stainless-steel mesh (mesh: 150 μm). Then, the SWMs were stored in a dry container until use. The length and thickness of the SWMs (n = 100) were measured using images obtained under a stereoscopic microscope, which were later analyzed via ImageJ software, similarly to the procedures described in Araújo et al. (2020).

2.2. Animals and experimental design

We used *Gallus gallus domesticus* (or *Gallus domesticus*) chicks (Galliformes, Phasianidae) autosexed commercial hybrid chicks "Embrapa-021" (a local variety derived from a White Cornish x White Plymouth Rock cross) obtained from a commercial incubator when they were only seven days old. This species is the most common domestic animals worldwide (Eda, 2021), whose estimated global population in 2017 was greater than >22 billion (FAO, 2021). Furthermore, scientifically, *G. gallus domesticus* chicks have been considered good experimental models in (eco)toxicological studies (Mesak et al., 2018; Vieira et al., 2019; Scalisi et al., 2020; Arcain et al., 2021).

After seven days of acclimation to the laboratory, the chicks (14 days old – body biomass: 128.60 g ± 7.09 g – mean ± SEM) were distributed into two experimental groups. The "control" group was composed of birds that were not exposed to SWMs and in the "SWM" group, the chicks received (orally) microfibers in an amount corresponding to 12% of the weight of the stomach contents of the birds (i.e.: 0.9246 g ± 0.21 g - mean ± SEM), previously measured in our laboratory. Therefore, each animal in the "SWM" group received daily 0.1041 g ± 0.0005 g (mean ± SEM) of SWMs, which were forcibly introduced into the animals' esophagus, mixed in a small piece of wheat flour dough (0.98 g ± 0.08 g – mean ± SEM).

Considering the lack of studies involving the identification of SWMs in environmental compartments or food, the amount of microfiber offered to birds was based on the study by Seif et al. (2018). Such authors the authors observed that approximately 12% of the total stomach content of three gull species feeding in an urban landfill environment was composed of metallic fragments. Thus, we consider that the amount of SWMs introduced into chicks is environmentally relevant, as it does not overestimate the amount of metallic materials potentially ingested by wild birds. We emphasize that the SWMs were offered twice to the animals, once a day, at an interval of 24 h, simulating the occasional encounter of birds with these materials.

Each experimental group consisted of 10 chicks, which were kept during the experiment in steel cages (70 cm length x 50 cm width x 25 cm height), similarly to Mesak et al. (2018). The rearing cages were illuminated by a 250-W infrared bulb, and an artificial cycle of 12 h light and 12 h dark was provided. Food and water were supplied *ad libitum* and wire-mesh lids prevented the birds from jumping out. At the end of

the experiment, the animals were submitted to different evaluations, as described below.

2.3. Toxicity biomarkers

2.3.1. Biometry

The body condition of the birds (initial and final) was evaluated from the body biomass (BB), wing length (WL), head length (HL) and tarsus length (TL), as well as the BB/WL and BB/TL indices, according to previous studies (Chappell and Titman, 1983; DeVault et al., 2003; Schamber et al., 2009).

2.3.2. Biochemical biomarkers (oxidative stress and antioxidant activity)

Aiming to associate the possible ingestion of SWMs to the induction of a redox imbalance, different biochemical biomarkers were evaluated. For this, the animals were euthanized (via decapitation) to collect brain, liver, muscle (*pectoralis major*), crop, proventriculus, gizzard and intestine fragments. Such fragments were washed in phosphate buffered saline (PBS, pH 7.2), macerated in 1 mL of PBS and then centrifuged (13,000 rpm, 5 min, 4 °C) to collect the supernatant, which was subsequently used. Before maceration, the food content of the crop, proventriculus, gizzard and intestine was carefully removed.

Malondialdehyde (MDA), one of the most known secondary products of lipid peroxidation (LPO) (Yaman and Ayhanci, 2021), was used as an oxidative stress biomarker, similarly to other studies (Tan et al., 2019; Patel et al., 2021; Issac et al., 2021; Rangasamy et al., 2022; Cunha et al., 2022). For this, we adopted the procedures described in detail in the study by Sachett et al. (2020). To measure nitric oxide (NO), we used the Griess colorimetric reaction (Grisham et al., 1996), which consisted of detecting nitrite, resulting from the oxidation of NO, similarly to Ajjuri and O'Donnell (2013) and Estrela et al. (2021). Furthermore, we evaluated the superoxide dismutase (SOD) [according to Del-Maestro and McDonald (1987)] and catalase (CAT) activity [as proposed by Sinha (1972)], considered first line defense antioxidant enzymes (Ighodaro and Akinloye, 2018). The acetylcholinesterase (AChE) activity – whose action is crucial in the propagation of nerve impulses (Dunant, 2021) – was evaluated in different organs, assuming a possible cholinesterasic effect induced by the ingestion of SWMs. For this, we adopted the procedures proposed by Ellman et al. (1961), with minor modifications described in Cunha et al. (2022). The results of the analysis of all biomarkers were expressed proportionally to the concentration of total proteins of each organ/tissue, evaluated according to the instructions of the commercial kit used [Commercial kit (Reference number: BT1000900)].

2.4. Determination of iron levels

Iron levels were evaluated in feces and in the fragments of different collected organs/tissues [brain, liver, muscle, crop, proventriculus, gizzard and intestine]. For the quantification of iron in feces, we adopted the protocol proposed by Goswami and Kalita (1988), with some modifications. Briefly, feces were weighed (0.10 g ± 0.0004 g – mean ± SEM) and introduced into beakers containing 5 mL of nitric acid 65% (HNO_3). Afterwards, the samples were placed on a hot plate for 2 h at 100 °C. After the samples had been digested, the clock glass was removed, and the heating process continued until the samples had completely dried. Then, HNO_3 (at 5%, v/v) was added to dilute the residue of the samples, bringing the final volume to 25 mL. Posteriorly, 190 μL of the samples were pipetted in a 96-well microplate and mixed with 20 μL of potassium thiocyanate solution (at 40% wt/v). The absorbance values obtained were used to determine the concentration of Fe III in the samples, using the equation obtained from the standard curve ($R^2 = 0.9997$; Equation: $y = 0.0112x + 0.0588$) made from different concentrations of ammoniacal ferric sulfate [$NH_4Fe(SO_4)_2$].

In the assessment of iron levels in organs/tissues, we used the procedures described in Oliveira and Naozuka (2021) (with some adaptations), in which the labile fraction of the element was quantified in the supernatant used in the biochemical analyses, as described in item "2.3.2". Aliquots (190 μL) of the supernatant were pipetted in a 96-well microplate and mixed with 20 μL of potassium thiocyanate solution (at 40% wt/v). According to Cabantchik (2014), the labile iron denotes the combined redox properties of iron and its amenability to exchange between ligands, including chelators. Furthermore, labile iron represents a component of non-transferrin-bound iron (NTBI), capable of permeating into organs and inducing tissue iron overload (Cabantchik et al., 2005).

2.5. Visual inspection of organs

We also evaluated the possible adherence of SWMs to the mucosal epithelium of the crop, proventriculus, gizzard and intestine. For this, after collection, the organs had their contents completely removed and were washed 5 times with PBS (pH 7.2). In a Petri dish, the organs were analyzed under a stereoscopic microscope and to confirm the presence of identified microfibers, we use a small magnet. In this case, when close to the organ, the magnetic field produced by the magnet promoted a slight movement of the suspected SWMs, which was not observed in structures that were only like the SWMs in shape, color, and size.

2.6. Data analysis

All data were evaluated considering the assumptions for using parametric models. In addition, we used the Shapiro-Wilk test to assess the distribution of residual data and the Bartlett test was used to assess the homogeneity of variances. Student's t-test was used for normally distributed and homogeneous data and Mann-Whitney *U* test was used for data with non-normal distribution and when homogeneity of variances was not achieved. Data related to iron levels in feces were submitted to two-way ANOVA, with the "treatments" (two levels: control and SWMs) and "time" (two levels: 24 and 48 h) factors. Multiple comparisons were performed using the Sidak test. Furthermore, correlations were performed using Pearson's (for parametric data) or Spearman's (for non-parametric data) correlation coefficients, as well as linear regression analysis. For all analyses, we considered a significance level of 95% ($p \leq 0.05$), using the Prism 9.0 (GraphPad Software, Inc., CA, USA).

3. Results

3.1. Characterization of steel wool microfibers

The morphological and morphometric parameters of the SWMs, confirmed their heterogeneity, whose dimensions were different – there was a mix of major and minor SWMs (Fig. 1A and E). SWMs mean length was 1002.07 μm ± 58.54 μm (mean ± SEM) (minimum: 131.0 μm; maximum: 2339.0 μm) and 49% of the microfibers had a length between 400 μm and 1000 μm (Fig. 1A). Regarding the thickness of the SWMs, we observed an even greater heterogeneity (Fig. 1B). The mean recorded was 77.91 μm ± 1.19 μm (mean ± SEM), with 29% of the SWMs having a thickness between 60 and 65 μm, 19% between 70 and 80 μm and in 42% of the SWMs, the thickness varied from 85 to 90 μm. Regarding the format, some SWMs were straighter (Fig. 1F), with different levels of curvature (Fig. 1G–J), different spiral shapes (Fig. 1K-M) and others with multiple folds (Fig. 1N).

3.2. Toxicity biomarkers

Assuming that the animals' exposure to SWMs could affect their body condition, different biometric parameters were evaluated. We did not evidence significant differences between the experimental groups in terms of feed and water consumption (graphics not shown), body biomass (Fig. 2A), tail, head, and wings lengths (Fig. 2B–D, respectively) and calculated indices (Fig. 2E–F). However, we observed that at the end

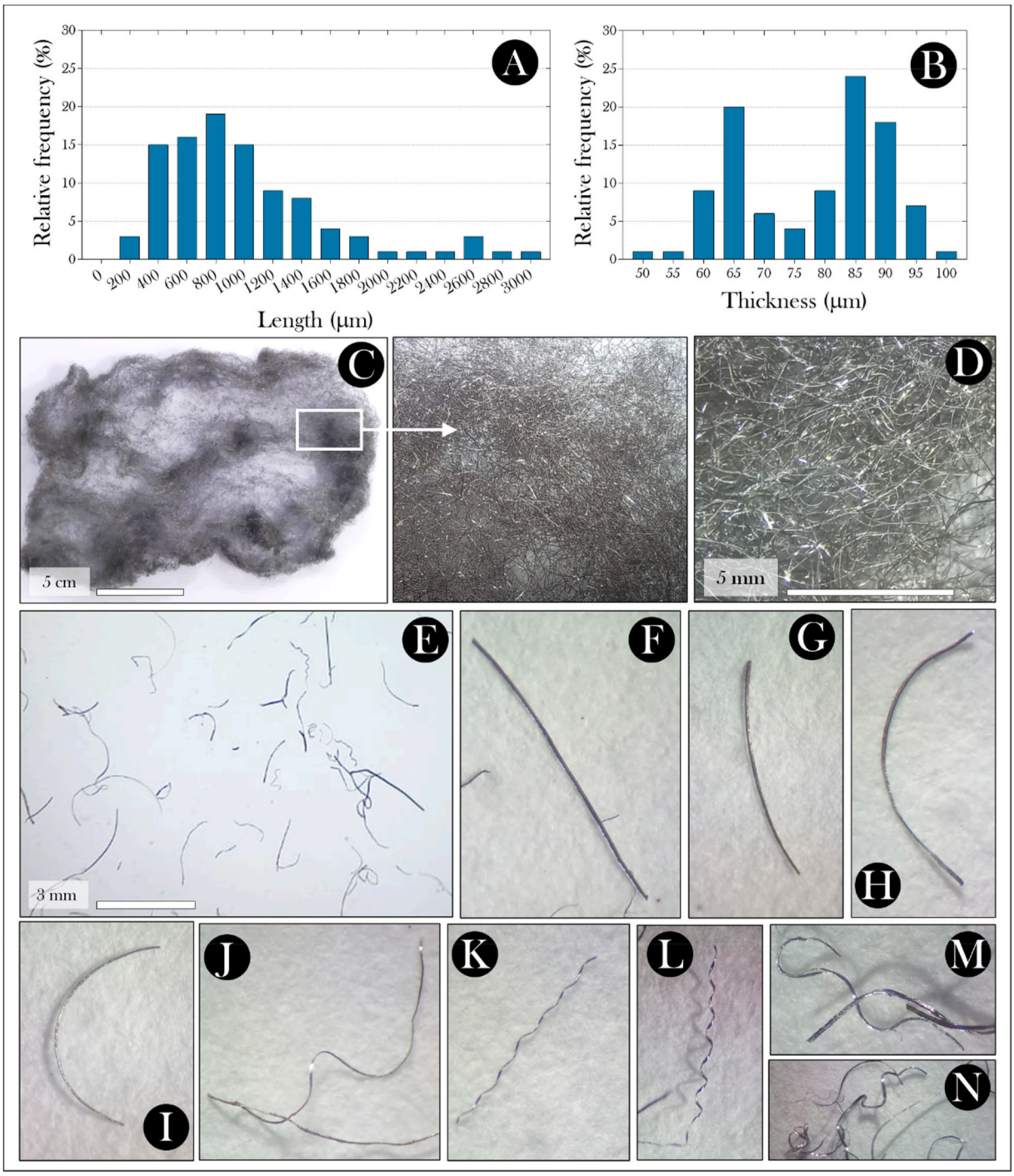

Fig. 1. Histograms of distribution of (A) length and (B) thickness of steel wool microfibers (SWMs) (n = 100 microfibers) and (C–N) representative photomicrographs of SWMs used in our study, with emphasis on the morphological variety and size.

of the experiment, the unexposed animals ("control" group) increased body biomass by 12%, while in those exposed to SWMs this increase was only 3.9% (Fig. 2A). For head length, we observed an increase of 7.8% in the "control" group and in animals exposed to SWMs the increase was 2.6%, which means a threefold difference.

On the other hand, we observed increase of the biomarkers of oxidative stress in the birds that ingested SWMs. While the production of H_2O_2 was significantly increased in the brain, liver, crop, and gizzard (Fig. 3A) of these animals; MDA levels were high in all organs evaluated, except for the crop and intestine (Fig. 3B). The increase in SOD activity (observed in liver, muscle, and crop) (Fig. 4A), associated with non-observance of differences in CAT activity (Fig. 4B), seem not to have been sufficient to counterbalance the production of H_2O_2, either the increase in LPO (inferred by MDA levels) in animals exposed to SWMs. In liver and crop, we observed a strong (positive) correlation between H_2O_2 levels and SOD activity (Fig. 5A–B, respectively) and in brain, MDA and H_2O_2 levels were significantly correlated (Fig. 5C). Furthermore, our data suggest the occurrence of a nitrosative stress in liver, crop, proventriculus, and gizzard in animals that ingested the SWMs [marked by increased nitrite levels (Fig. 3C)], as well as an anti-cholinesterasic effect in the brain. As seen in Fig. 6, cerebral AChE activity in birds exposed to SWMs was 61.9% lower than that observed in the "control" group.

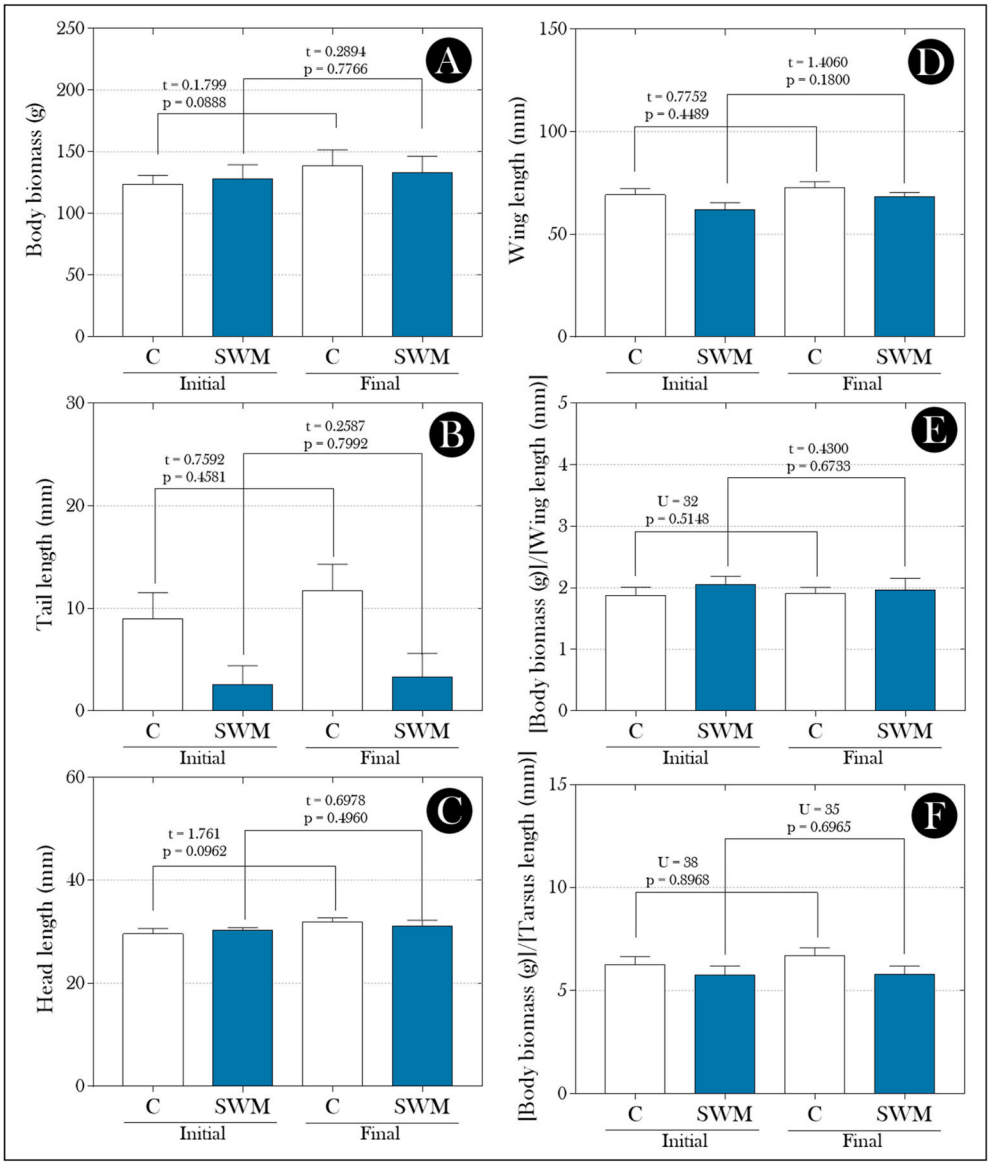

Fig. 2. Initial and final biometric parameters of *Gallus gallus domesticus* chicks exposed or not to steel wool microfibers (SWMs). (A) Body biomass, (B) tail length, (C) head length, (D) wing length, (E) [body biomass (g)]/[wing length (mm)] and (F) [body biomass (g)]/[tarsus length (mm)]. The bars indicate the means + SD. Student's t-test was used for normally distributed and homogeneous data and Mann-Whitney *U* test was used for data with non-normal distribution and when homogeneity of variances was not achieved (see statistical summary at the top of the graphs). C: group of animals that did not receive SWMs (control group); SWM: group of chicks that received (orally) SWMs. n = 10 animals/group.

3.3. Iron bioaccumulation

Considering the animals' response to SWMs (see summary in Table 1), we evaluated the relationship between the redox imbalance observed in different organs/tissues and the possible iron overload. We observe that although much of the iron content (probably from the SWMs) was excreted via feces (Fig. 7A), the labile iron levels in the liver, crop, and gizzard of animals in the "SWMs" group were higher than those identified in the "control" group (Fig. 7B). In liver and gizzard, our analyzes revealed a significant correlation between increased H_2O_2 production and SOD activity with labile iron levels in these organs (Fig. 8A–B, respectively). In crop, the nitrite levels were proportional to the labile iron levels (Fig. 8C). Additionally, the visual inspection performed on the organs revealed the presence of SWMs adhered to the mucosal epithelial tissue of the crop (Fig. 9A–C), proventriculus (Fig. 9D–F) and gizzard (Fig. 9G–H).

4. Discussion

Generally, it is undeniable that the development and increased production of synthetic materials imply direct benefits to human beings. The use of these materials in different areas and sectors undoubtedly improves the quality of the products, especially regarding their durability and strength. However, the impact that these materials (when present in the natural environment) can have on the biota, needs to be considered, especially in relation to those for which there are no treatment processes before their disposal. This is the case of SWMs which, as shown in this study, if ingested by birds, can represent a risk to the survival of individuals.

We initially evidenced that the short period of exposure to SWMs showed potential to affect the growth/development of *G. gallus domesticus* chicks. The reduced percentage of body biomass gain and head growth of animals in the "SWMs" group (compared to the "control" group) (Fig. 2A and C, respectively) suggest that, in the long term, the ingestion of SWMs may negatively interfere in the growth/development of birds. According to Felter et al. (2015), the early life stage is an important stage not only for the growth of animals, but also for the development and maturation of different physiological systems. Thus, changes in development can have drastic consequences in the adult life of animals. Although studies like ours have not been developed so far, our results are like those in which the exposure of juvenile birds to metallic pollutants negatively affected the growth/development of the

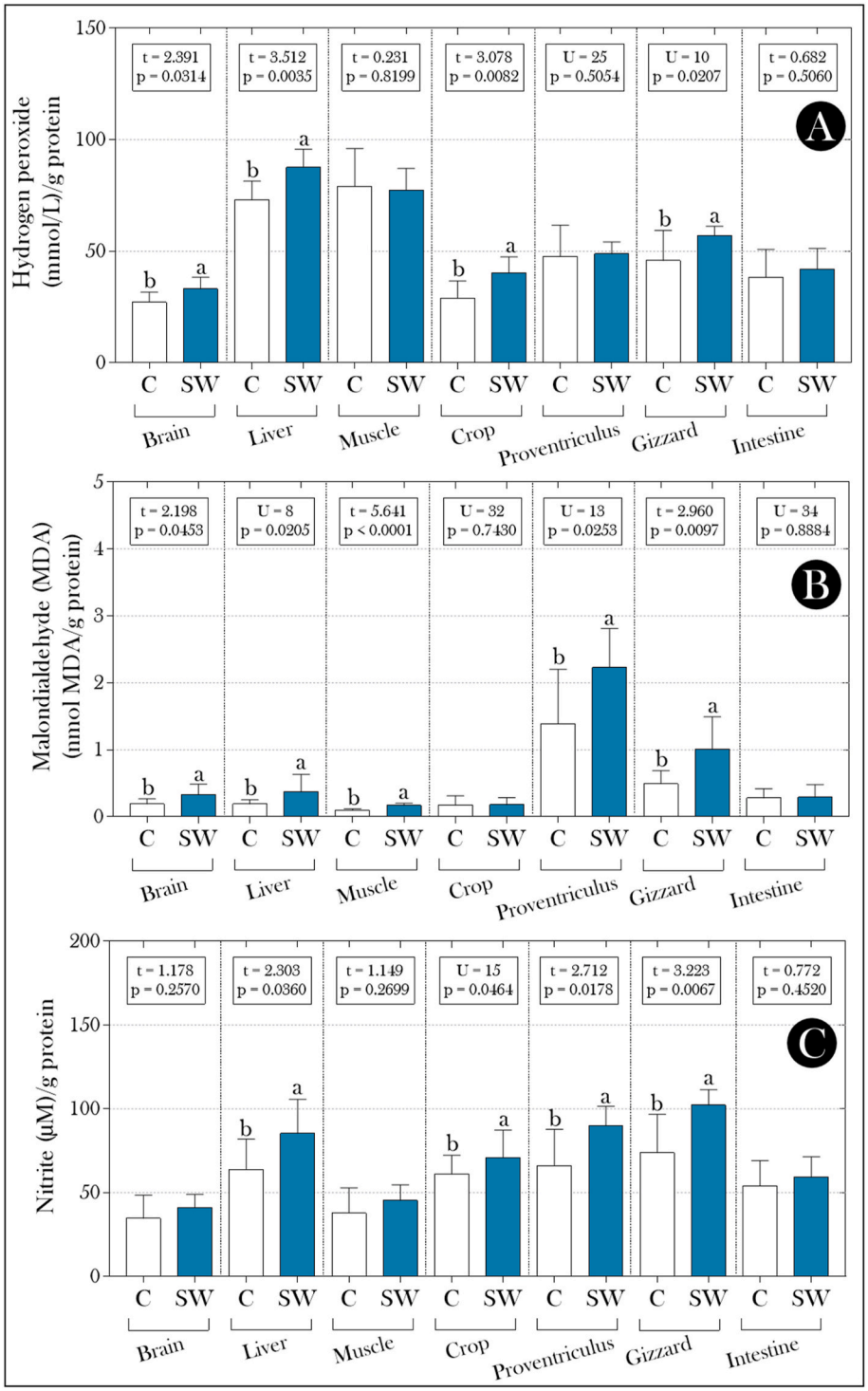

Fig. 3. Hydrogen peroxide (H_2O_2) (A), malondialdehyde (MDA) (B) and nitrite (C) levels evaluated in different organs/tissue of *Gallus gallus domesticus* chicks exposed or not to steel wool microfibers (SWMs). The bars indicate the means + SD. Student's t-test was used for normally distributed and homogeneous data and Mann-Whitney *U* test was used for data with non-normal distribution and when homogeneity of variances was not achieved (see statistical summary at the top of the graphs). C: group of animals that did not receive SWMs (control group); SW: group of chicks that received (orally) SWMs. n = 10 animals/group.

animals (Huff et al., 1996; Spahn and Sherry, 1999; Abduljaleel and Shuhaimi-Othman, 2013). Spahn and Sherry (1999) evidenced that *Egretta caerulea* clicks exposed to Cd had significantly slower growth rates than nonexposed chicks, and exposure to Pb was correlated with increased nestling mortality. In Abduljaleel and Shuhaimi-Othman (2013), the exposure of *G. gallus domesticus* chicks to Pb significantly decreased body biomass of animals after dietary treatment, similarly to the results reported by Huff et al. (1996), involving exposure to aluminum sulfate.

However, reports on the impacts of exposure of chicks to iron (the main constituent of SWMs) on the growth/development of animals are inconclusive, as well as the mechanisms that may explain the trend observed in our study. In Farrow et al. (1983), for example, in the significant effect on growth performance with 2500 mg/kg supplementary iron in White Rock chicks was observed, similarly to the study by Lin et al. (2020), when yellow-feathered broiler chicks received (also from the diet) different iron levels. In this latter study, the results showed that different levels of dietary iron (50–150 mg/kg) had no significant impact on the growth performance of yellow broiler chicks during 1–21, 22 to 42, or 43–63 days, indicating that the iron content in the basic diet was sufficient for the growth of these birds. Ma (2014) reported that the level of dietary iron (47–147 mg/kg) had no effect on the growth performance

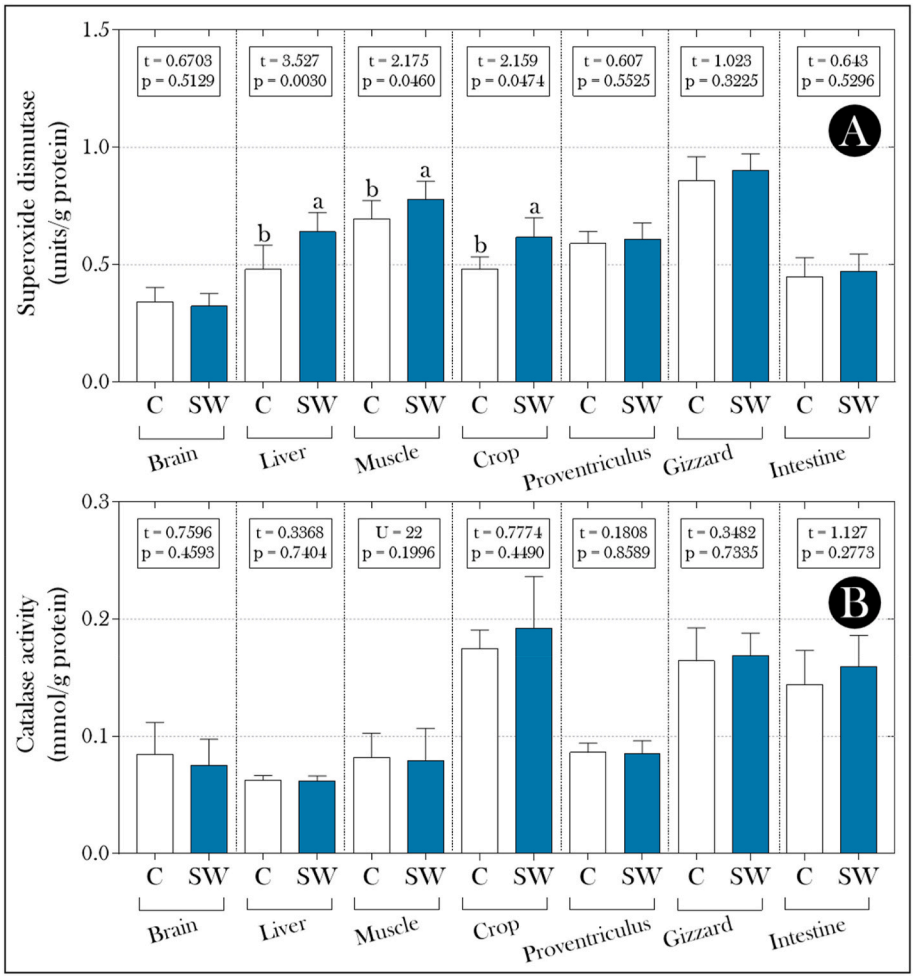

Fig. 4. (A) Superoxide dismutase (SOD) and (B) catalase (CAT) activities evaluated in different organs of *Gallus gallus domesticus* chicks exposed or not to steel wool microfibers (SWMs). The bars indicate the means + SD. Student's t-test was used for normally distributed and homogeneous data and Mann-Whitney *U* test was used for data with non-normal distribution and when homogeneity of variances was not achieved (see statistical summary at the top of the graphs). C: group of animals that did not receive SWMs (control group); SW: group of chicks that received (orally) SWMs. n = 10 animals/group.

of AA broilers chicks. Cao et al. (1996) showed that the addition of high doses of iron to the diet (400, 600 and 800 mg/kg) significantly increased body biomass and average daily feed intake of Ross broilers chicks. Therefore, these studies indicate that different chicken breeds had different tolerances and requirements for iron.

On the other hand, it is known that the increase in oxidative stress processes implies greater energy expenditure by the antioxidant system, aiming to counterbalance the production of potentially harmful free radicals to cells. In this case, even though preliminary, we can assume that the lower weight gain of animals exposed to SWMs (three times smaller than the "control" group) is related to an energy reallocation mechanism, aimed at achieving the imbalance between cellular pro-oxidant levels and antioxidant capacity in favor of the somatic homeostasis. However, the increased H_2O_2 production (in the brain, liver, crop, and gizzard – Fig. 3A) associated with the increase in SOD activity (in the liver, muscle, and crop – Fig. 4A), characterizes a typical redox imbalance observed in animals exposed to SWMs. Thus, it is plausible to assume that the absence of increased catalase activity (which breaks down H_2O_2 into water and molecular oxygen) (Fig. 4B) and a possible reduction in glutathione peroxidase (GPx) activity (which reduces H_2O_2 to water and lipid peroxides to their corresponding alcohol) may explain the high H_2O_2 levels observed in different organs/tissues of animals exposed to SWMs. Lee et al. (1981) reinforces this hypothesis by demonstrating that total GPx activity was decreased in liver of male Sprague-Dawley rats, with high dietary iron. At the same time, we cannot neglect the hypothesis of increased cellular energy expenditure to control or mitigate the processes of LPO (Fig. 3B) have influenced the animals' growth. According to Ayala et al. (2014), under subtoxic conditions, cells stimulate their constitutive antioxidant defense systems (which are not restricted to the action of the enzymes evaluated in our study, SOD and CAT) or activate signaling pathways that positively regulate antioxidant proteins, resulting in a response adaptive to stress. All these mechanisms demand energy that could be used in the growth/development of animals.

Another interesting data observed in our study refers to the significant increase in iron levels in the crop, gizzard, and liver (Fig. 7B), which indicates the SWMs as a supplementary source of this metal. In crop and gizzard, the iron levels would be related to the deterioration of SWMs, due to corrosion, after encountering water, oxygen, and internal fluids. Although *ad libitum* feeding in chicks reduces the physical use of the crop [since animals adjust the frequency of feed intake to the rate of passage of the digesta (Svihus et al., 2013; Kierończyk et al., 2016)], the adhesion of SWMs in the mucosal layer of this organ (Fig. 9A–C) could, by itself, provide opportunities for the availability of additional iron levels. The high labile iron levels in the liver, on the other hand, would be related to iron translocation from the intestine to the circulation hepatic portal, via the absorptive action of the proximal intestinal villus epithelial cells. Although a previous study indicates that chicks can down-regulate or up-regulate uptake of iron (which normally do not develop overload) (Mete et al., 2003), iron overload from SWMs may have caused disturbances in these regulation mechanisms, the which would explain the high absorption of dietary iron relative to body iron needs.

Consequently, it is plausible to suppose that the increase in labile iron levels in the different organs evaluated in our study would be related to the increase in oxidative stress in birds of the "SWM" group. It

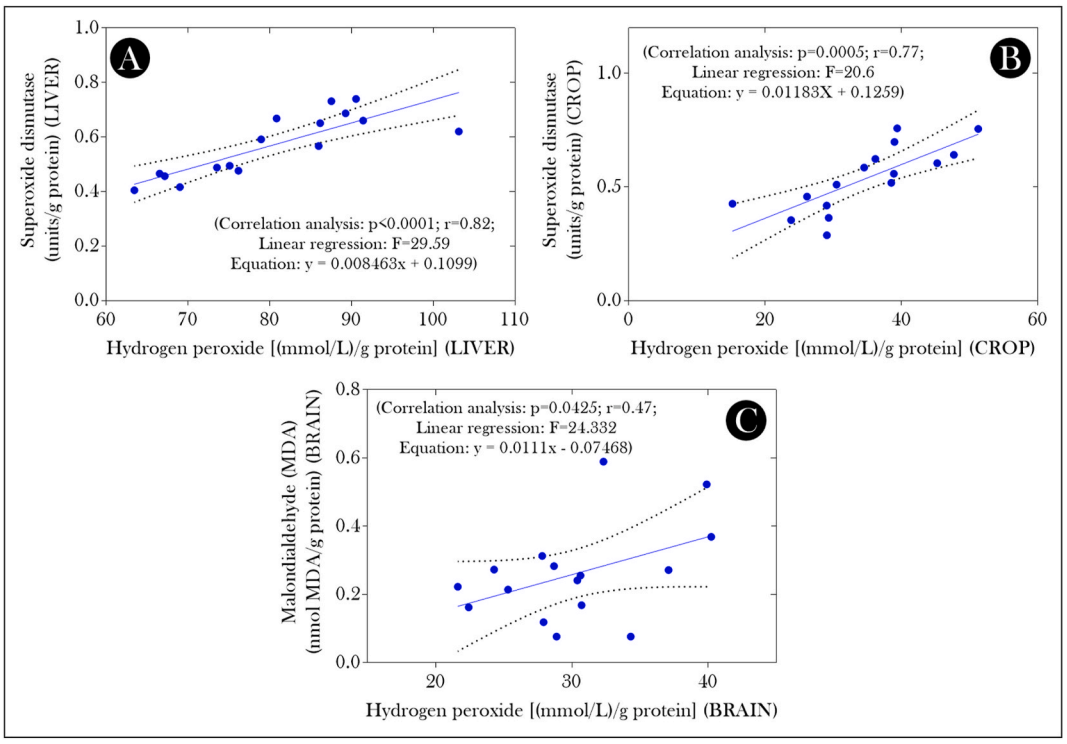

Fig. 5. Correlation analysis and linear regression between hydrogen peroxide (H_2O_2) levels and superoxide dismutase (SOD) activity in liver and crop (A-B, respectively) and between H_2O_2 production and malondialdehyde (MDA) levels in brain (C) of *Gallus gallus domesticus* chicks exposed or not to steel wool microfibers (SWMs).

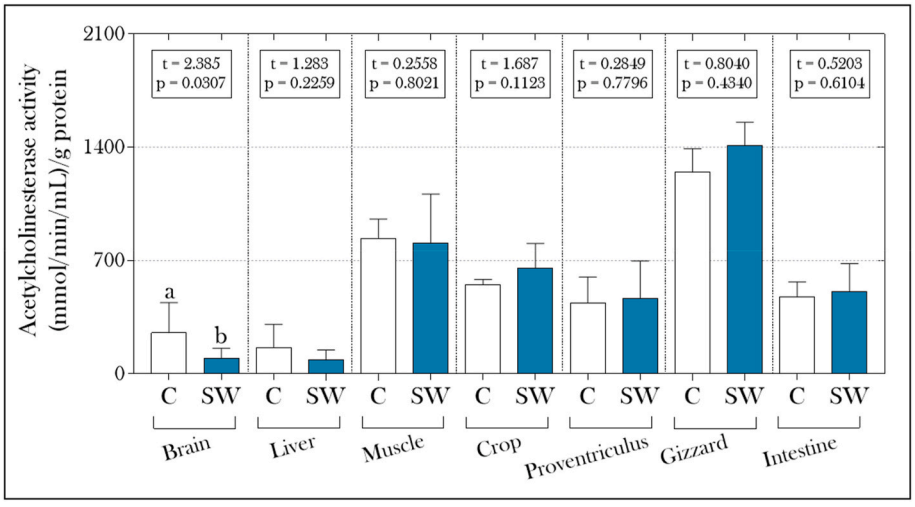

Fig. 6. Acetylcholinesterase (AChE) activity evaluated in different organs/tissues of *Gallus gallus domesticus* chicks exposed or not to steel wool microfibers (SWMs). The bars indicate the means + SD. Student's t-test was used for normally distributed and homogeneous data and Mann-Whitney *U* test was used for data with non-normal distribution and when homogeneity of variances was not achieved (see statistical summary at the top of the graphs). C: group of animals that did not receive SWMs (control group); SW: group of chicks that received (orally) SWMs. n = 10 animals/group.

is agreed that iron is an essential transition metal for organisms, being part of several metalloproteins (Liu et al., 2014), in addition to being involved in different vital biochemical processes, such as those involved in the transport of oxygen in tissues (Gupta, 2014; Abbaspour et al., 2014), electron transfer reactions during mitochondrial respiration (Paul et al., 2017), DNA synthesis and repair (Puig et al., 2017) and in the metabolism of xenobiotics (Crichton, 2016). However, when present in excess in cells and tissues, iron can interrupt redox homeostasis and catalyze the propagation of ROS, leading to oxidative stress. As demonstrated by Merkofer et al. (2006), the presence of unshielded redox-active iron favors Fenton chemistry; what involves the reaction between H_2O_2 and Fe^{2+}, yielding extremely reactive HO• radical dot via ferryl/perferryl intermediates. In spite this radical is short-lived, its high reactivity can oxidize any chemical group that is close to its formation (Galaris et al., 2019). For example, iron bound to membrane phospholipids catalyzes the initiation of LPO chain reactions, which would explain the increased MDA levels in most organs evaluated in birds exposed to SWMs (Fig. 3B). The fact that the labile iron is redox-active, chelatable (i.e.: it can be sequestered by chelating compounds) and exchangeable (i.e.: it can be easily transferred among natural ligands and between cell compartments) (Kakhlon and Cabantchik, 2002) reinforces the hypothesis of excess iron in birds ("SWM" group) is directly associated to the induction of oxidative stress. Several studies that have associated exposure of animal models to high iron concentrations/doses with increased oxidative processes also support this hypothesis (Reardon and Allen, 2009; Sampaio et al., 2014; Handa et al., 2016;

Table 1
Synthesis of results obtained in the present study, with identification of increase (↑) decrease (↓) or no change (–) in the parameters evaluated in *Gallus gallus domesticus* chicks exposed or not to steel wool microfibers (SWMs).

Biometry	Experimental groups	
	Control	SWMs
Body biomass	–	–
Tail length	–	–
Head length	–	–
Wing length	–	–
Body condition index #1	–	–
Body condition index #2	–	–

Oxidative stress biomarkers	**Brain**		**Liver**		**Muscle**		**Crop**		**Proventriculus**		**Gizzard**		**Intestine**	
	C	**SW**	**C**	**SW**	**C**	**SW**	**C**	**SW**	**C**	**SW**	**C**	**SW**	**C**	**SW**
Hydrogen peroxide	↓	↑	↓	↑	–	–	↓	↑	–	–	↓	↑	–	–
Malondialdehyde	↓	↑	↓	↑	↓	↑	–	–	↓	↑	↓	↑	–	–
Nitrite	–	–	↓	↑	–	–	↓	↑	↓	↑	↓	↑	–	–
Antioxidant activity biomarkers	**C**	**SW**	**C**	**SW**	**C**	**SW**	**C**	**SW**	**C**	**SW**	**C**	**SW**	**C**	**SW**
Superoxide dismutase activity	–	–	↓	↑	↓	↑	↓	↑	↓	↑	↓	↑	–	–
Catalase activity	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Cholinesterasic effect biomarker	**C**	**SW**	**C**	**SW**	**C**	**SW**	**C**	**SW**	**C**	**SW**	**C**	**SW**	**C**	**SW**
Acetylcholinesterase activity	↑	↓	–	–	–	–	–	–	–	–	–	–	–	–

Legend: C: control group; SW: group submitted to ingestion of steel wool microfibers (SWMs); Body condition index #1: [Body biomass (g)]/[Wing length (mm)]; Body condition index #2: [Body biomass (g)]/[Tarsus length (mm)].

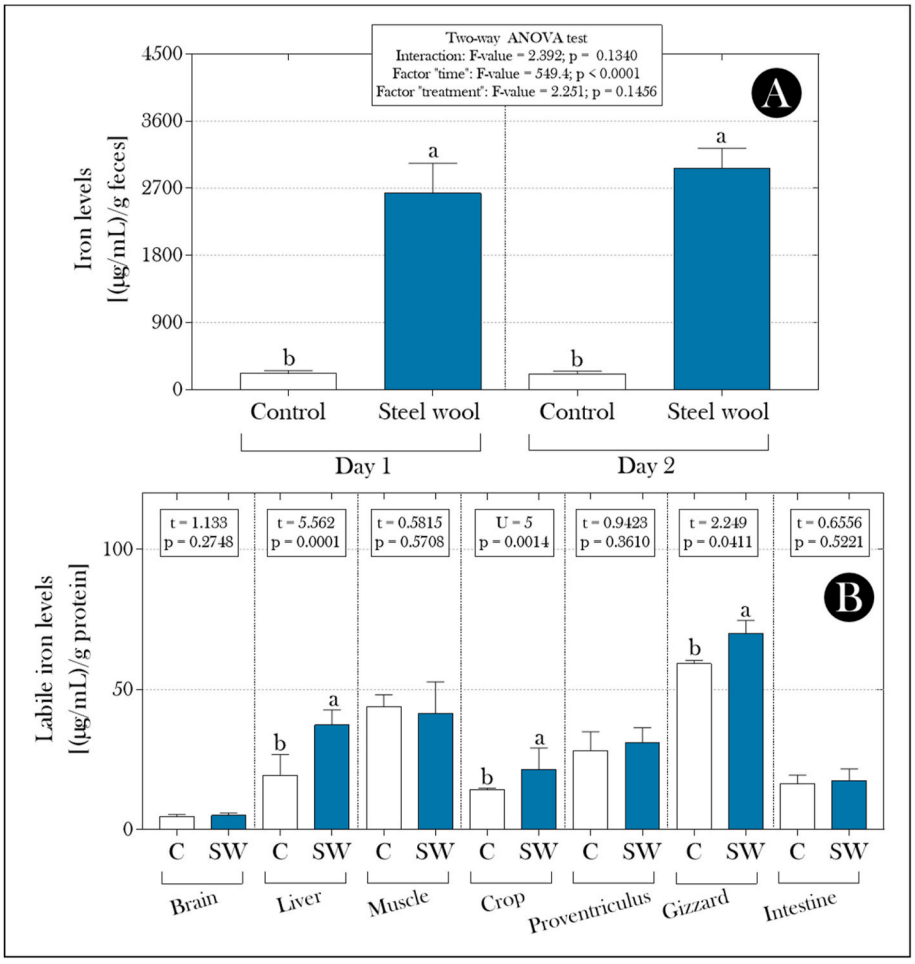

Fig. 7. Iron levels in (A) feces and in (B) different organs/tissues of *Gallus gallus domesticus* chicks exposed or not to steel wool microfibers (SWMs). Bars indicate means + SD. In "A", the data were submitted to two-way ANOVA, with Sidak's post-test, at 5% probability. In "B", Student's t-test was used for normally distributed and homogeneous data and Mann-Whitney *U* test was used for data with non-normal distribution and when homogeneity of variances was not achieved. C: group of animals that did not receive SWMs (control group); SW: group of chicks that received (orally) SWMs. n = 10 animals/group.

Tarifeño-Saldivia et al., 2018; Jiang et al., 2019; Sönmez-Aydın et al., 2021; Li et al., 2021).

Further result observed in our study refers to the increased nitrite levels in liver, crop, proventriculus and gizzard of chicks from the "SWM" group (Fig. 3C), which suggests the induction of a nitrosative stress induced by the ingestion of microfibers. In this case, it is likely that

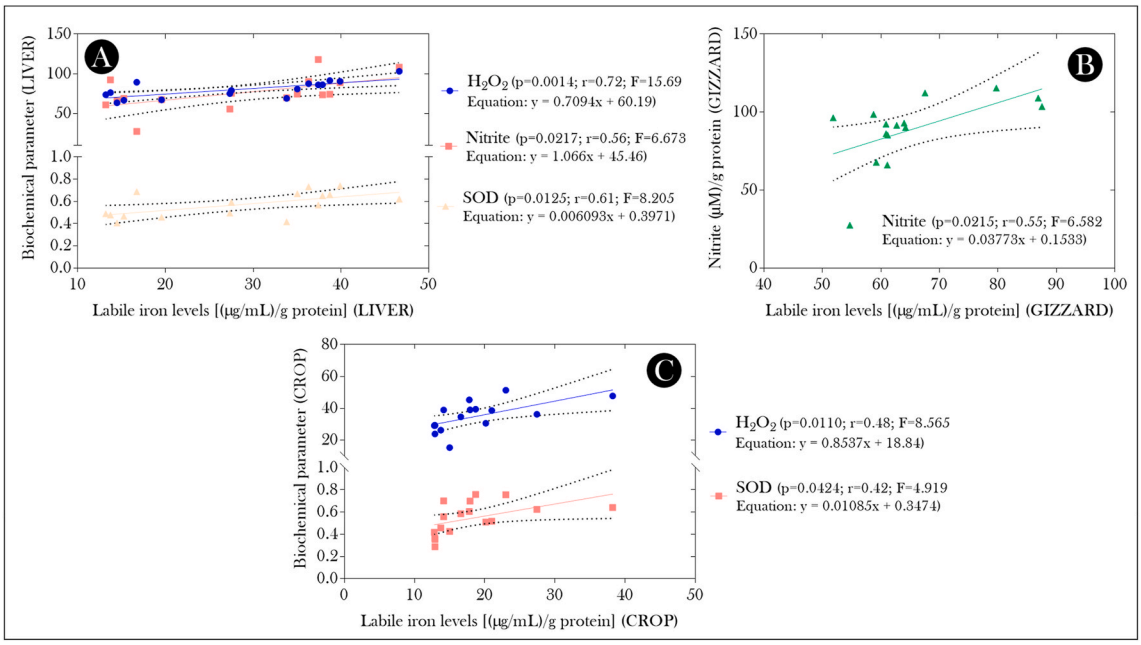

Fig. 8. Correlation analysis and linear regression between different biochemical biomarkers and iron labile levels in (A) liver, (B) gizzard and (C) crop of *Gallus gallus domesticus* chicks exposed or not to steel wool microfibers (SWMs).

the increase in NO production (inferred by nitrite levels) is associated with the increase in nitric oxide synthase (iNOS) expression by extracellular signal-regulated kinase (ERK1/2) and NF-kappaB activation (induced by iron overload), as also suggested by Cornejo et al. (2005), in a study involving rats fed diet-enriched with carbonyl-iron (to 3% wt/wt). Thus, investigating the hypothesis that iron overload observed in the liver, crop and gizzard of animals exposed to SWMs upregulates the expression of iNOS and NOS activity in conditions of oxidative stress, constituted a fertile future investigative perspective. On the other hand, we cannot neglect the hypothesis that the increase in NO in birds is related to an immune stimulation in response to SWMs adhered to the epithelial layer of the crop (Fig. 9A–C) and gizzard (Fig. 9G–I). In this case, possible microlesions caused by SWMs would be sufficient to trigger an inflammatory response in animals, with consequent recruitment of phagocytic cells such as monocytes, neutrophils, and macrophages, whose mechanisms of action include the production of NO (Bogdan, 2001; Wink et al., 2011). Alternatively, it is possible that the nitrosative stress observed in birds exposed to SWMs constitutes an adaptive response related to the antioxidant properties of NO. Contrary to the deleterious effects of the reactive nitrogen oxide species formed from either NO/O_2 and NO/O_2^-, it has been pointed out that NO shows antioxidant properties (Rubbo et al., 1994; Rubbo and Radi, 2001; Gallean et al., 2004). Since the biological chemistry of these molecules is dominated by free-radical reactions, the interaction of NO with other free-radical species could lead to either inhibition or potentiation of oxidative damage effect (Beckman et al., 1990). This hypothesis is supported especially by the study by Hummel et al. (2006), in which the authors suggest that the continuous production of NO would yield a steady-state concentration of only 10–20 nM is capable, in human leukemia cells, of inhibiting Fe^{2+}-induced lipidic peroxidation. However, any one of these hypotheses needs to be further investigated in further studies.

Interestingly, we also noticed an anticholinesterasic effect (marked by the reduction of cerebral AChE activity) in animals exposed to SWMs (Fig. 6), which agrees with the findings of Perez et al. (2010), involving Wistar rats. Such authors observed reduced AChE activity in animals that received Fe^{2+} at 10 mg/kg of body weight, which was associated with cognitive deficits induced by iron overload. Although the precise mechanisms involved in the reduction of striatal AChE activity in adult rats (Perez et al., 2010) and in *G. gallus domesticus* chicks (in our study) have not been investigated, it is tempting to speculate that in both studies, the iron accumulation would have triggered a cascade of events that impaired cholinergic transmission or induced terminal degeneration. On the other hand, a study involving adults wild-type zebrafish (*Danio rerio*) demonstrated that 24 h of exposure to iron (to 15 mg/L) was sufficient to increase the AChE activity in both, brain (62%) and liver (70%), in relation to unexposed animals (Sant'Anna et al., 2011). Therefore, it is possible that the cholinesterasic response to iron overload is dependent on the studied species, as well as on the iron concentrations and evaluated exposure periods.

Regardless of the biological mechanisms intrinsic to the effects observed in our study, it is evident that the ingestion of SWMs by *G. gallus domesticus* chicks induces alterations that harm the animals' health. The accumulation of iron and, consequently, iron overload can induce, for example, oxidative stress (as noted), as well as causing behavioral changes (associated with a deficiency in cholinergic transmission), damage to the genetic material caused by the increase in free radicals and the association from iron to DNA (including mutations or single and double strand breaks), as well as enzymatic, hormonal and signal transduction dysfunctions that regulate various physiological functions, caused by the binding of the chemical element to different proteins. Thus, taken together, these effects represent a risk to the animals' survival (reducing their fitness), with the potential to change the dynamics of bird populations that ingest SWMs discarded in natural environments.

Based on the above, it is noteworthy that due to the pioneering nature of our study in evaluating the possible toxicity of SWMs in birds, several investigative perspectives that can be explored in the future. It is questioned, for example, whether the effects reported in the present study would be more accentuated in longer exposures and whether similar responses would be observed in adults (males and females). How much the effects evidenced in our study can affect the development/growth of animals (in the long term) and whether the observed changes will impact the adult life of the animals, also constitute a fertile field for further investigation. At the individual level, assessments using predictive biomarkers of gene dysregulation, histopathological, behavioral, and hormonal damages will be essential for a better understanding of how much the ingestion of SWMs (although ephemeral and occasional)

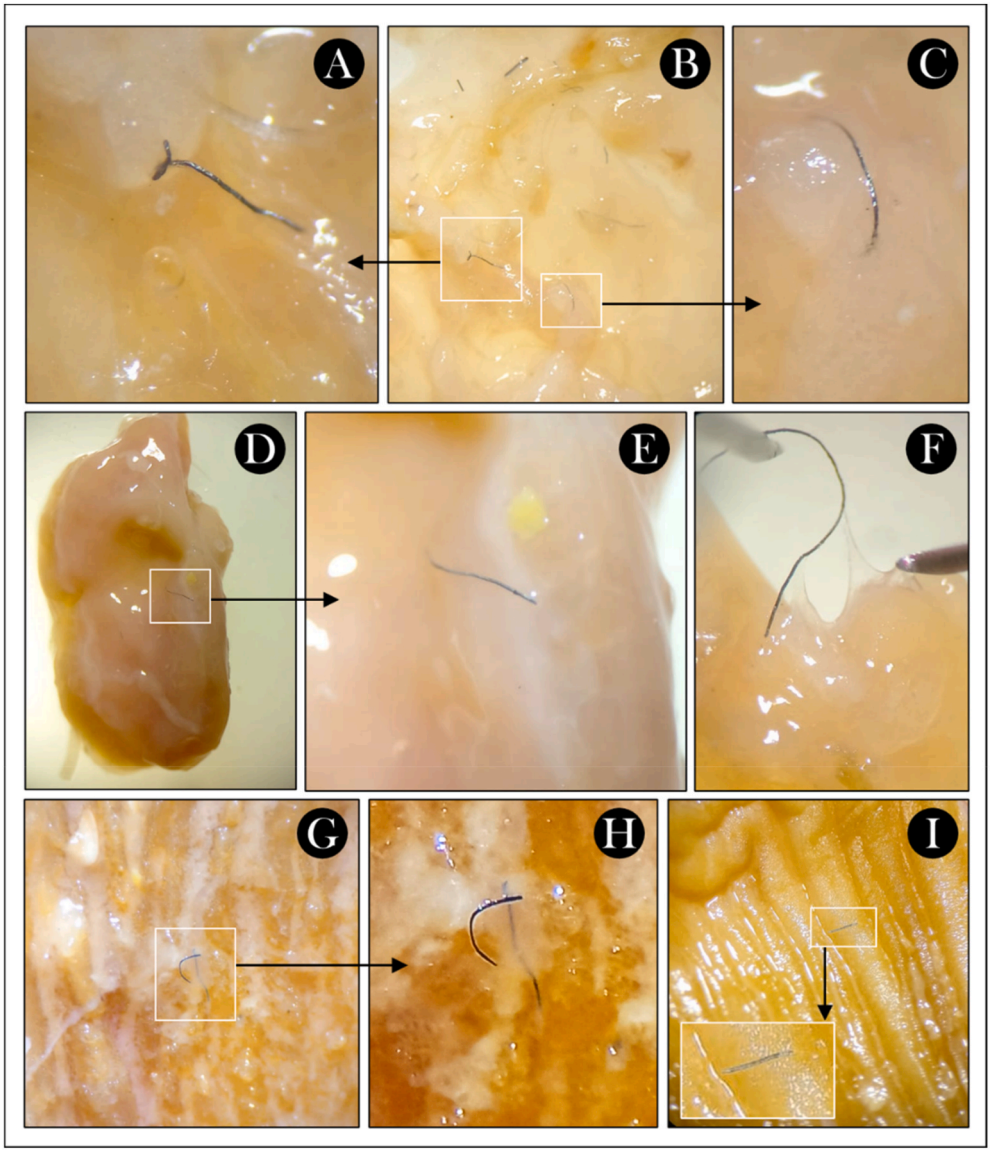

Fig. 9. Representative photomicrographs of (A–C) crop, (D–F) proventriculus, and (G–H) gizzard of *Gallus gallus domesticus* chicks exposed to steel wool microfibers (SWMs), with emphasis on the adherence of microfibers to the mucosal epithelial tissue of these organs.

can affect the survival of animals. The regulation of ferritin (the main intracellular iron storage protein – Ponka et al., 1998) in the context of SWMs ingestion should also be investigated in the future. At the population level, it is questioned, for example, to what extent the exposure of animals to these microfibers can affect their social interactions, reproduction, and the dynamics of their populations in natural environments.

5. Conclusion

In conclusion, our data confirmed the toxicological potential of SWMs in *G. gallus domesticus* chicks, demonstrating that their ingestion, although ephemeral and occasional, causes iron overload in different organs, redox unbalance, as well as the reduction of cerebral AChE activity in birds exposed to SWMs. However, it must be recognized that our study is not exhaustive and, therefore, constitutes only the "tip of the iceberg" that represents the (eco)toxicological effects associated to SWMs in ecosystems. Thus, we strongly recommend that further studies continue the assessment of the potential toxicity of these materials not only to birds, but also to other groups of animals that inhabit areas where the presence of SW and/or SWMs is real.

Author contribution statements

Ítalo Freitas Nascimento: study conception and design, data collection, analysis, and interpretation of results, and draft manuscript preparation. **Sindoval Silva de Souza:** data colletion. **Thiarlen Marinho da Luz:** data colletion. **Lux Attiê Santos Gomes:** data colletion. **Sandy de Oliveira Gonçalves:** data colletion. **Mohamed Ahmed Ibrahim Ahmed:** analysis, and interpretation of results, and draft manuscript preparation. **Abraão Tiago Batista Guimarães:** data colletion, analysis, and interpretation of results, and draft manuscript preparation. **Aline Sueli de Lima Rodrigues:** analysis, and interpretation of results, and draft manuscript preparation. **Guilherme Malafaia:** conceived of the presented idea, collected the data, provided funding, analysis, and interpretation of results, and draft manuscript preparation. All authors reviewed the results and approved the final version of the manuscript.

Ethical aspects

All experimental procedures were performed in accordance with the

ethical standards for animal experimentation and meticulous efforts were made to ensure that the animals suffered as little as possible and to reduce external sources of stress, pain, and discomfort. The current study has not exceeded the number of animals needed to produce reliable scientific data. This article does not refer to any study with human participants performed by any of the authors.

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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