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

TOXICITY INDUCED BY INGESTION OF NATURALLY-AGED MICROPLASTICS BY A SMALL-SIZED TERRESTRIAL BIRD AND ITS POTENTIAL ROLE AS VECTORS FOR THE DISPERSION OF THESE POLLUTANTS

SINDOVAL SILVA DE SOUZA

Orientador(a): Prof. Dr. Guilherme Malafaia Pinto

Urutaí, 15 de março de 2022

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Orientador(a)
Prof. Dr. Guilherme Malafaia Pinto

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BANCA EXAMINADORA DE DEFESA DE DISSERTAÇÃO

Aos vinte três dias do mês de março 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 **Sindoval Silva de Souza,** discente do **Programa de Pós-Graduação em Conservação de Recursos Naturais do Cerrado do Instituto Federal Goiano - Campus Urutaí,** com trabalho intitulado **"Toxicity induced via ingestion of naturally-aged polystyrene microplastics by a small-sized terrestrial bird and its potential role as vectors for the dispersion of these pollutants".** A sessão foi aberta pelo presidente da banca examinadora, **Prof. Dr. Guilherme Malafaia**, 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 (x) **APROVADA ( ) REPROVADA**, 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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Título da dissertação: Toxicity induced via ingestion of naturally-aged polystyrene microplastics by a small-sized terrestrial bird and its potential role as vectors for the dispersion of these pollutants.

Orientador: Prof. Dr. Guilherme Malafaia Pinto

Autor: Sindoval Silva de Souza

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“A ciência nunca resolve um problema
sem criar pelo menos outros dez.”
(George Bernard Shaw)

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À Deus por ter me concedido esperança, sabedoria, saúde, paz, por ter colocado pessoas tão importes e especiais no meu caminho e por ter me dado forças para tornar tudo isto possível.

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SUMÁRIO

LIST OF FIGURES AND TABLES

TOXICITY INDUCED BY INGESTION OF NATURALLY-AGED MICROPLASTICS BY A SMALL-SIZED TERRESTRIAL BIRD AND ITS POTENTIAL ROLE AS VECTORS FOR THE DISPERSION OF THESE POLLUTANTS

ABSTRACT

In recent years, there has been a growing number of studies on the impact of microplastics (MPs) on biota. However, its effects on bird health are poorly understood. Thus, we aimed to evaluate the possible effects of ingestion of naturally-aged MPs by *Coturnix coturnix japonica* (11 and 22 MP particles/day/bird, once a day, for 9 days), from different toxicity biomarkers. At the end of the experiment, our data showed that birds that ingested MPs showed a significant reduction in body biomass. We also observed an increase in malondialdehyde production in the liver, brain, intestine, and gizzard of the animals, as well as a suppressive effect on hepatic nitric oxide production and superoxide dismutase activity in the liver and intestine. Cerebral catalase activity was reduced in birds exposed to MPs and a cholinesterasic effect (marked by increased acetylcholinesterase activity) was observed in the muscle and brain of these animals. Despite these differences, through the main component analysis, hierarchical clustering analysis, and integrated biomarker response assessment, we observed that, in general, the toxicological effect in birds exposed to different amounts of MPs was similar. We also noticed that the size of MPs was reduced, and their shape was altered as they transited through the gastrointestinal system, which probably explains their accumulation in the liver of birds. In addition, we noticed that expressive amounts of MPs are released by the feces of the animals throughout the experiment. As far as we know, this is the first report that associates MPs ingestion by small-sized terrestrial birds with biochemical alterations predictive of oxidative stress, redox imbalance, and cholinesterase effect, in addition to shedding light on the potential role of these birds as vectors for dispersal of MPs in natural environments.

Keywords: Micropollutants, Ecotoxicology, Avifauna, Biochemistry, Bioaccumulation, Environmental impact.

1. INTRODUCTION

Microplastic pollution has been considered a problem of global magnitude and increasing concern in recent decades (Shruti et al., 2021), especially considering the high production and consequent disposal of plastic products in the environment. In 2017, for example, 8.3 billion metric tons of virgin plastic were produced, and 12 billion tons of plastic wastes are expected to be found in the natural environment by 2050 (Geyer et al. 2017). Obviously, in more urbanized environments, MPs have been identified in high concentrations (Garcia et al., 2020; Piehl et al., 2021; Dacewicz et al., 2022). However, recent studies have shown that MPs can reach remote and pristine areas in which there are very few local sources of plastic, via atmospheric transport (Stefánsson et al., 2021; Zhang et al., 2022; Aves et al., 2022; Liang et al., 2022; Yang et al., 2022). Therefore, the atmospheric long-range transport and surface runoff represent potential pathways to carry MPs from elsewhere to the remote areas and may impact ecosystems in ways that are still poorly understood.

In animals, previous studies have shown that many of the harmful effects of MPs come from dietary exposure, either through the ingestion of plastic particles dispersed in the environment or through the ingestion of prey (via trophic transfer) previously exposed to the pollutants. The accumulation of MPs in several invertebrate species [see reviews by Foley et al. (2018) and Trestrail et al. (2020)] and vertebrates (López-Martínez et al., 2021; Araújo et al., 2021; Wootton et al., 2021; Wang et al., 2021) has already been reported in previous studies, which reinforces the potential of these micropollutants affect the faunal biodiversity of different ecosystems. However, several questions have not yet been elucidated, especially about the effects induced in some groups of animals, which have various ecological functions in the food web.

This is the case of the Aves group, which comprises endotherms organisms that are widely distributed in various habitats worldwide (from the equator to polar areas, and from oceans and freshwater to high plateaus) and that exhibit flight-related morphological and physiological traits that enable them to occupy different habitats and become important members of many ecosystems (Orme et al., 2006). Therefore, these characteristics make birds highly sensitive and vulnerable to external conditions and can be used to monitor environmental changes and in studies to assess the negative effects of pollution (Lovett, 2012; Souto et al., 2018; Carral-Murrieta et al., 2018; Carral-Murrieta et al. al. 2020; Silveira et al., 2021). Birds, in many situations, can confuse plastic (in various compositions, shapes, morphologies, and textures) for prey, which explains the identification of these materials in the gastrointestinal

tracts, feces, and even in feathers and other tissues or organs of several hundred avian species from freshwater (Holland et al., 2016; Reynolds & Ryan, 2018), terrestrial (Zhao et al., 2016; Carlin et al., 2020; Ballejo et al., 2021; Cunha et al., 2022), and marine (Basto et al., 2019; Nam et al., 2021; Morkūnas et al., 2021; Nania & Shugart, 2021) ecosystems.

Despite this, the consequences of MPs ingestion by small-sized terrestrial birds have not been extensively investigated (Wang et al. 2021). As discussed by Yu et al. (2014), these birds are highly diversified and widely distributed relative to raptors. However, only Ryan (1988) and Roman et al. (2019) to date have evaluated the direct consequences of MPs ingestion in small-sized terrestrial birds under laboratory conditions. In Ryan (1988), ingestion of polyethylene pellets by domestic chickens *Gallus gallus domesticus* reduces meal size and thus food consumption when plastic reduces the storage volume of the stomach. On the other hand, Roman et al. (2019) demonstrated that the ingestion of polypropylene (PP) MPs (irregular round shape and diameter: 3–4.5 mm) by Japanese Quail (*Coturnix coturnix japonica*) do not appear to be significant animal health. Although ingestion of plastic caused growth and sexual maturity, the authors reported no evidence of lasting toxicological effects on mortality, adult body weight, organ histology, hormone levels, fertility, hatch rates, and eggshell strength in birds experimentally fed plastic. Furthermore, exposure to MPs did not affect survival or population outcomes for Japanese quail throughout several generations.

Therefore, there is a huge gap in knowledge about the possible effects of MPs on the health of small-sized terrestrial birds. Although the study by Roman et al. (2019) point to the fact that the toxicological implications of MPs ingestion may not be as severe as commonly assumed, in general, it is still very preliminary to consider any optimistic scenario for birds that ingest plastic in general. In the recent study by Cunha et al. (2022), for example, the ingestion of plastic fragments by *Coragyps atratus* adults was associated with redox imbalance in several evaluated organs, hepatic nitrosative stress, and cholinesterase effect observed in the muscle and brain of the animals. Although this study, in particular, did not evaluate small-sized birds, its findings shebd light on the toxicological potential arising from plastic ingestion by these birds.

Thus, aiming to expand our knowledge about the possible effects of MPs, we exposed adult female Japanese Quail (*C. coturnix japonica*) to the ingestion of naturally-aged MPs, assuming the induction of negative biochemical effects in different organs/tissues after nine days of exposure. In addition, we evaluated the possible absorption, translocation of plastic particles to the liver (via the hepatic portal system), as well as the release of MPs through the

feces. As far as we know, this is the first report that associates MPs ingestion by small-sized terrestrial birds with biochemical alterations predictive of oxidative stress, redox imbalance, and cholinesterase effect, in addition to shedding light on the potential role of these birds as vectors for dispersal of MPs in natural environments.

2. MATERIAL AND METHODS

2.1. Microplastics

To carry out this study, we used polystyrene (PS) MPs obtained from large plastic fragments of artificial plants kept in an open urban environment for five years, whose detailed chemical characterization and procedures for obtaining the MPs are presented in a previous study by our research group [see Guimarães et al. (2021)]. We chose to expose animals to this type of plastic due to the extensive use of plastic plants in indoor gardens, for home decoration, and in commercial buildings (Andersen, 2017), which characterizes them as potential sources of MPs for the environment. Furthermore, by using naturally-aged MPs, we brought our experimental design closer to a more realistic context of pollution by these micromaterials.

After obtaining the MPs of different sizes and colors, we selected the green-colored particles (from the leaves of plastic plants) (Figure 1A), which had a diameter of 3293.45 µm ± 60.34 µm (mean ± SEM) [coefficient of variation (CV) = 18.05%], and 80.2% had a diameter between 2400 and 3800 µm (Figure 1B). Furthermore, the MPs had an irregular shape (Figure 1A), and low circularity values were obtained (0.637 ± 0.010, mean ± SEM, CV: 16.16%) for most of the MPs used. So, this reflects the weathering suffered by plastics over years of exposure to natural environmental conditions and the initial crushing of larger particles.

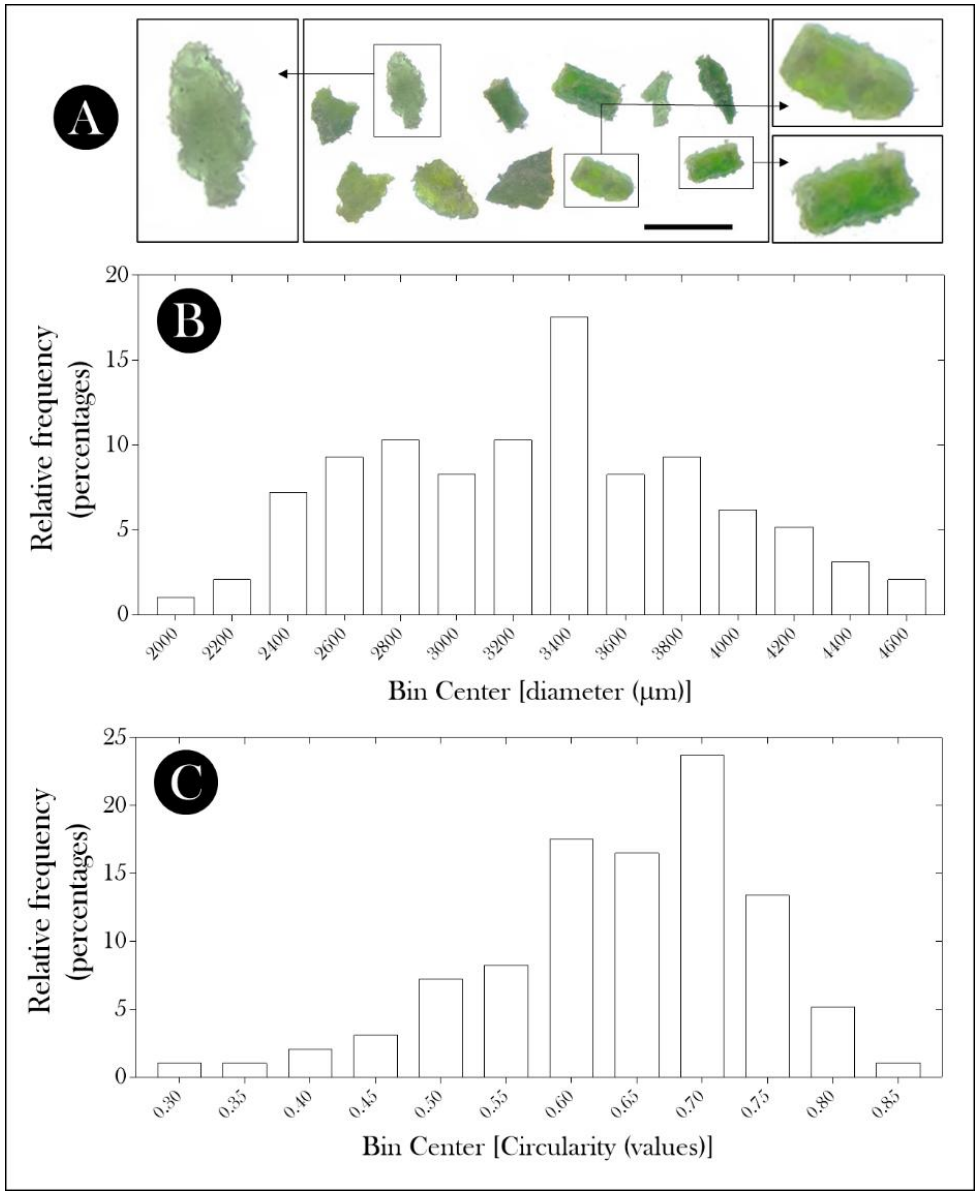

Figure 1. (A) Representative photomicrographs of the naturally-aged polystyrene (OS) microplastics (MPs) used in our study and distribution histograms of the (B) diameter ("µm") and (C) shape (circularity) of the particles. In "A", scale bar = 5000 µm (5 mm).

2.2. Animal model and experimental design

For the accomplishment of this study, adult female Japanese Quail adults (*C. coturnix japonica*) (16 weeks of age) were used, considered a species of great adaptability in captivity, being classified by the International Union for Conservation of Nature's Red List (IUCN), as Least Concern (BirdLife International, 2018). We chose to work with females, similarly to Faria et al. (2018) and Sampaio et al. (2019), to minimize possible negative effects of the naturally aggressive behavior of males throughout the experimental period, which could interfere with the results. In addition, *C. coturnix japonica* has been recommended as a model small-sized

terrestrial bird species to clarify the potential regulatory mechanisms associated with physiology, behavior, and neuroendocrinology upon exposure to different sizes of MPs (Wang et al., 2021). All individuals were acquired in commercial breeding facilities (Pires do Rio, GO, Brazil) and kept under recommended sanitary conditions for the species in the vivarium of the Laboratory of Toxicology Applied to the Environment, Goiano Federal Institute – Campus Urutaí (GO, Brazil).

After 15 days of acclimatization to laboratory conditions, the animals were divided into three experimental groups (n=10 animals), with equitable body biomass between them (one-way ANOVA test; $F = 0.0498$; $p = 0.9515$). The "MP-I" and "MP-II" groups were composed of quails that received, orally, 11 and 22 MP particles/bird, respectively, once a day, for nine consecutive days. The control group was composed of animals that did not receive any type of plastic particle. The animals were kept in steel cages (dimensions: 37.5 cm long x 34 cm wide x 22 cm high) and housed in an animal facility room with controlled temperature (24 to 25 °C) and light (12 h light cycle), according to Hussain et al. (2014) and Faria et al. (2018). In addition, the quails received water and standard commercial feed ad libitum (all bowls were stainless steel). Similar to Roman et al. (2019), each group was housed separate from other treatment groups but within the same animal room facility to reduce between-room environmental variation.

2.2.1. Quantity of microplastics and exposure procedures

The amounts of plastic particles used in the exposure of quails were defined based on studies by Zhao et al. (2016) and Carlin et al. (2020), in which the authors (together) identified plastic material in the gastrointestinal contents of 20 species of terrestrial birds of different feeding habits (carnivores, herbivores, and omnivores) captured in Shanghai (China) and Florida (USA), respectively. Zhao et al. (2016) observed that mean density by number in all specimens was 10.6 particles (plastic fibers and fragments)/bird and, in Carlin et al. (2020), the overall mean number of MPs for species of bird was 11.9. Therefore, the average number of MPs identified in the gastrointestinal/bird content in these studies is equivalent to the number of particles offered to the quails of the "MP-I" group (n=11 MPs particles/quail/day) which represents a theoretical environmentally relevant plastic loading in terrestrial birds. On the other hand, the amount of particles offered to the animals of the "MP-II" group (i.e., twice the amount ingested by the quails of the "MP-I" group) simulates a pessimistic scenario of microplastic contamination in small-sized terrestrial bird species.

Daily, the experimental birds were force-fed plastic, like Ryan (1988). For this, the pre-defined total number of MPs (in both groups) was introduced into the animals mixed with a small pellet of moistened wheat flour (0.80 g ± 0.08 g – mean ± SEM) to avoid additional stress by the repeated introduction of particles daily. The wheat flour pellet was carefully introduced on the back of the tongue of the animals (always in the morning), using metallic forceps previously sterilized and sanitized with ethanol (up to 70%, v/v). The animals in the "control" group received the wheat flour pellets free of any plastic particles.

2.3. Toxicity biomarkers

2.3.1. Body biomass assessment

Considering that the body biomass constitutes a general indicator of the health status of the animals (Depoorter et al., 2015), including the Japanese quail (Faria et al., 2018), at the end of the experiment the individuals were weighed, using electronic scales to the nearest 0.1 g.

2.3.2. Biochemical analyzes

2.3.2.1. Sample preparation

Assuming that exposure to MPs could induce predictive alterations of oxidative and nitrosative stress, a deficit of antioxidant response, and cholinesterasic effect in different organs, at the end of the experiment the birds were euthanized (via decapitation) and dissected to collect the crop, proventriculus, gizzard, and small intestine, muscle (pectoral), brain and liver fragments, according to standard methods (Van Franeker, 2004). After that, the muscle, brain, and liver fragments were immediately stored in a -80^{o}C freezer for further analysis. Crop, proventriculus, gizzard, and intestinal fragments had their food contents completely removed and washed with phosphate-buffered saline (PBS) (pH 7.2 at 4^{o}C) (in abundance) before being stored in a freezer at -80^{o}C for analysis later. Furthermore, the food contents extracted from the gizzard and intestine were dried in an oven (at 40^{o}C, until constant weight) and later stored in containers previously sanitized with ethanol (at 70%, v/v).

For the biochemical evaluation, the collected fragments were processed according to Cunha et al. (2022), initially consisting of weighing the respective fragments and subsequent maceration in 1 mL of PBS (with a semi-automatic macerator). Subsequently, the samples were

centrifuged (10,000 rpm, for 10 min, at 4°C) for the collection of supernatants, which were used to assess the biomarkers.

2.3.2.2. Reactive species production

Assuming that the treatments could induce an increase in the production of reactive species, we evaluated the levels of hydrogen peroxide (H_2O_2), total reactive oxygen species (ROS), and nitric oxide (NO) in the different collected organs/tissues. For the measurement of H_2O_2, we adopted the procedures described in Elnemma et al. (2004), with some modifications. Briefly, 100 µL of PBS was added to 96-well microplates and preheated to 37ºC. Subsequently, 10 µL of supernatant and 100 µL of ammonium molybdate solution (at 0.5%, w/v in purified water) were added, mixed, and after 5 min the samples were ELISA reader reading, at 405 nm. H_2O_2 concentrations were obtained from the standard curve previously obtained ($y = 0.0047x + 0.0126$, $R2 = 0.9984$). The total ROS levels were determined from the protocol adapted from Maharajan et al. (2018). In this case, 20 µL of the supernatant was mixed (96-well microplate) with 200 µL of PBS and 8.3 µL of dichlorofluorescein-diacetate (to 10 mg/mL). Subsequently, the samples were incubated for 5 min (at room temperature) and read in an ELISA reader at 492 nm, according to Cunha et al. (2022). For the determination of NO production (via nitrite production), we used the method proposed by Bryan & Grisham et al. (2007), with some modifications. Initially, 30 µL of the supernatant was mixed with 150 µL of Griess reagent in a 96-well microplate. Griess reagent was prepared by mixing equal volumes of a solution of N-(1-naphthyl) ethylenediamine dihydro (1 mg/mL) and a sulfanilic acid (10 mg/mL) solution in 5% phosphoric acid. Subsequently, the sample was incubated for 5 min, at room temperature, and read at 540 nm. Nitrite concentrations were obtained via a previously made standard curve ($y = 0.0019x - 0.003$; $R2 = 0.9996$).

Furthermore, the malondialdehyde (MDA) levels were useful to infer the possible consequences of oxidative stress induced by treatments, considering that MDA is an indicator of lipid peroxidation produced by reactive species (Grotto et al., 2009; Ayala et al., 2014). For this, we used the procedures reported by Malafaia et al. (2022), by which MDA levels were determined using trichloroacetic acid (at 16%, w/v) and thiobarbituric acid (at 0.4%, w/v), with the standard curve established with different concentrations of 1,1,3,3-tetraethoxypropane (the precursor MDA), like Nasr et al. (2022).

2.3.2.3. Antioxidant response

The possible stimulatory or suppressive action of MPs on the antioxidant activity of individuals was evaluated through the activity of superoxide dismutase (SOD) and catalase (CAT), taken as enzymes that make up the organisms' first line of antioxidant defense, acting mainly in suppressing or preventing the formation of free radicals or reactive species in cells (Ighodaro & Akinloye, 2018). The SOD activity in each analyzed organ was determined from the protocol established by Dieterich et al. (2000), with some modifications. Measurements were based on SOD's ability to scavenge superoxide radical anion, which decreases the overall pyrogallol autoxidation rate. One SOD activity unit was defined as the amount of enzymes inhibiting the pyrogallol autoxidation rate by 50%, which was determined at 630 nm. On the other hand, catalase activity was determined from the protocol adapted from Sinha et al. (1972). In this case, 16 µL of the supernatant was mixed (in a 96-well microplate) with 240 µL of the reaction solution [PBS + glacial acetic acid P.A. + potassium dichromate (at 5%, w/v)] and incubated for 20 min at 37^{o}C. Subsequently, the samples were read in ELISA reading at 630 nm. Catalase activity was obtained via a standard curve previously made ($y = 14.204x – 2.4088$; $R2 = 0.9902$).

2.3.2.4. Acetilcolinesterase activity

To assess the association between MP ingestion and a possible cholinesterase or anticholinesterase effect, acetylcholinesterase (AChE) activity in muscle and brain was evaluated. As highlighted by Chen et al. (2011), AChE is a cholinergic enzyme responsible for the hydrolysis of the neurotransmitter, acetylcholine, in the nervous system and the neuromuscular junctions. In both organs, AChE activity was evaluated according to the spectrometric method proposed by Ellman (1961), using iodized acetylcholine substrate (750 µg/mL) and 5,5′-dithiobis (2-nitrobenzoic acid) (130 µg/mL).

2.3.2.5. Determination of the protein level

All results of biochemical analyzes were expressed in unit/g protein in the samples. Thus, we used a commercial kit (Bioténica Ind. Com. LTD, Varginha, MG, Brazil, code #10.009.00), whose total protein levels were determined based on the colorimetric reaction resulting from the reaction of Cu^{2+} ions and peptide bonds of proteins, giving rise to a blue color detected in an ELISA reader at 492 nm.

2.4. Microplastics quantification

2.4.1. Microplastics in feces and stomach (gizzard) and intestinal contentes

From the visual inspection of the feces (via stereoscopic microscope) and, later identification of microplastic particles, we quantified MPs after 12 h of the first exposure. Thereafter, assessments were performed every 24 h after each subsequent exposure. For this, we adopted the modified procedures by Yan et al. (2020). Briefly, 0.1 g of feces (previously dried in an oven at 40^{o}C) were introduced into glass beakers and mixed with 50 mL of hydrogen peroxide solution (at 30%, v/v) and 10 mL of iron catalyst solution [$FeSO_4$ at 15 g/L in purified water, with pH adjusted to 3, using concentrated sulfuric acid (at 95%, v/v)]. The iron catalyst solution (Fenton's reagent) was used to accelerate the reaction of hydrogen peroxide on the organic material of the samples. Then, the samples were incubated at 65^{o}C for 75 min and, later, the samples were centrifuged (at 1000 rpm, for 10 min, at room temperature) and the supernatant was filtered through a nitrocellulose membrane (pore: 0.45 µm), using a vacuum pump. Then, the filters were placed in a Petri dish with a cover, dried at room temperature, and later analyzed via stereoscopic microscopy to record the total number of MPs in the digested samples. In each evaluation, 10 replicates of feces samples from each group (including samples from the "control" group) were digested and evaluated.

The quantification of MPs in gizzard content (0.1 g) and intestinal content (small intestine) (0.1 g) was performed using the same procedures described above. However, the volumes of hydrogen peroxide and iron catalyst solution used were 25 mL and 5 mL, respectively. The MPs identified in the samples were photographed and later analyzed in the ImageJ software to evaluate the parameters related to the size (diameter) and shape (circularity) of the plastic particles (n=100 particles were analyzed for each type of sample), like Araújo et al. (2020). We emphasize that these organs were chosen because of their important contributions to the digestive process of birds. The gizzard acts as a storage organ for food, in addition to being specially modified for grinding food (Svihus, 2011). The small intestine is the primary site of absorption of most nutrients (Duke, 1997), whose functionality is similar in both birds and mammals (Lavin et al., 2008).

2.4.2. Microplastics in the liver

Assuming that the passage of MPs through the gastrointestinal tract could cause the fragmentation of plastic particles (culminating in the reduction of their sizes), we evaluated the possible absorption, translocation of MPs, and subsequent accumulation in the liver of the animals. For this, after extracting the liver and removing the fragment used in the biochemical analyses, the rest of the organ was macerated in a porcelain crucible and, later, 0.1 g of the sample was weighed and submitted to alkaline digestion, according to the procedures described above, the volumes of hydrogen peroxide and iron catalyst solutions were also adjusted to 25 mL and 5 mL, respectively.

After the steps of centrifugation and filtration of the supernatant (see item “2.4.1”), the membrane filters were processed and analyzed according to the methodology proposed by Malafaia et al. (2022a), with minor modifications. Briefly, the filtered membranes were introduced into glass beakers containing 5 mL of Nile red dye solution (up to 100 µg/mL, in acetone PA) and 3 mL of acetonitrile (PA), aiming at the complete dissolution of the membranes and release of the MPs withheld. After complete membrane dissolution, aliquots of each sample were introduced into the Neubauer chambers for MP counting. For each animal, six Neubauer chambers were analyzed (i.e., 54 quadrants/animal), totaling 540 quadrants/group. The MPs identified in the samples (via fluorescence microscopy) were photographed and later also had their diameters and circularity values measured (by ImageJ software) (n=25 particles were analyzed). The concentrations of MPs in the liver of the animals were expressed in “number of MPs/mg liver”. It is worth mentioning that the adoption of a differentiated methodology for the identification and counting of MPs in liver samples (compared to the one described in item “2.4.1”) is justified due to the reduced size of the micromaterials possibly accumulated in the organ. The use of Nile red dye helped in the identification of MPs in a fluorescence microscope.

2.5. Integrated Biomarker Response Index

To evidence the toxicity of the MPs, the results of all biomarkers evaluated (in all organs/tissues) were applied into the "Integrated Biomarker Response Index" (IBRv2), similarly to the procedures described in detail in Malafaia et al. (2022b), which were based on Sanchez et al. (2013). In summary, the deviation between biomarkers measured in Japanese quail exposed to MPs was compared to those measured in birds not exposed to micropollutants ("control" group). The biomarker response scores were plotted as radar graphs. The area above 0 reflects biomarker induction, and the area below 0 indicates biomarker inhibition.

2.6. Statistical analyses

Initially, all data obtained were evaluated regarding the assumptions for using parametric models. For this, 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. The data that met the assumptions for the use of parametric models were analyzed via one-way ANOVA test (with Tukey post-test) and the non-parametric data were compared via Kruskal-Wallis test (with Dunn's post-test). The average concentrations of MPs in the different organs of the animals of the "MP-I" and "MP-II" groups were compared by Student's t-test (if parametric) or Mann-Whitney test (if non-parametric). Data related to the number of MPs/g feces were submitted to two-way ANOVA (with Tukey post-test), considering the factors “treatment” (MP-I and MP-II groups) and “time” (nine evaluations along the of the trial period). The principal component analysis (PCA) was performed to explore correlations between treatments, based on the average value of each biomarker evaluated. In this regard, the number of principal components (PC) was selected based on scree plots (Peres-Neto et al., 2004). Outliers' values [identified via ROUT method (Q = 1%)] were excluded from all analyzes and, before the multivariate analysis, the data were logarithmized. Additionally, we performed correlation and linear regression analyses, as well as hierarchical clustering analysis, based on Ward's method (Eszergár-Kiss & Caesar, 2017). Significance levels were set at Type I error (p) values lower than 0.05. GraphPad Prism Software Version 9.0 and PAST (PALaeontology STatistic) software were used to perform the statistical analyses.

3. RESULTS

Initially, we observed that the quail survival rate at the end of the experimental period was similar among the experimental groups (all 100%). However, at the end of the experiment, the quails that ingested MPs had lower body biomass than the animals in the “control” group (Figure 2). Although we did not show an increase in H_2O_2 induced by exposure to MPs (in any organ/tissue evaluated) (Figure 3A), total ROS levels were higher in muscle and liver of quail exposed to MPs (“MP-I” and “MP -II” groups), with an average increase of 17.4% and 23.2%, respectively, in relation to the “control” group (Figure 3B). In addition, we observed that animals exposed to the highest number of MPs also had higher production of MDA in the liver, brain, intestine, and gizzard (Figure 4A). However, hepatic nitrite levels of birds exposed to MPs were lower than those reported in the “control” group (Figure 4B).

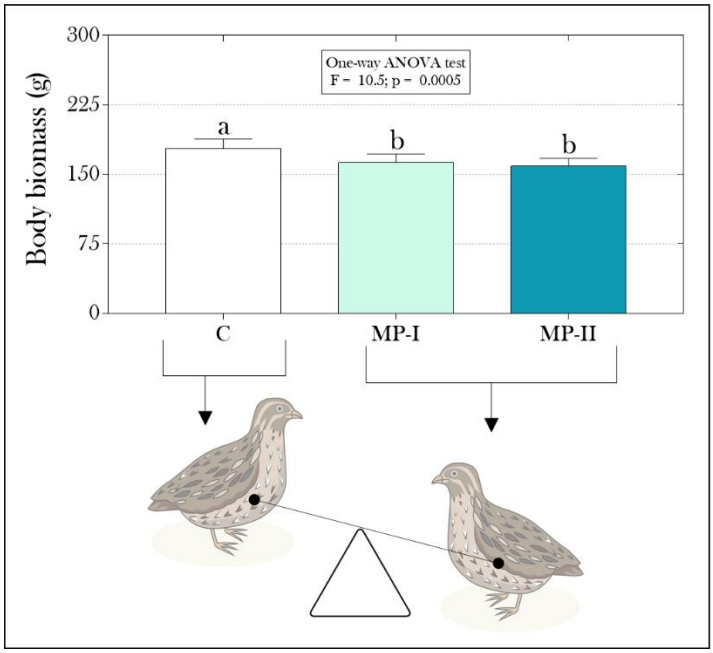

Figure 2. Body biomass of adult female Japanese quail (*Coturnix coturnix japonica*) exposed and unexposed to naturally-aged polystyrene microplastics (MPs). The bars indicate the mean + SD and the statistical summary is displayed at the top of the graph. Distinct lowercase letters indicate significant differences. C: group composed of quails not exposed to MPs ("control") and the "MP-I" and "MP-II" groups were composed of quails that received 11 and 22 MP particles, respectively, once a day, for nine consecutive days. n=10 animals/group.

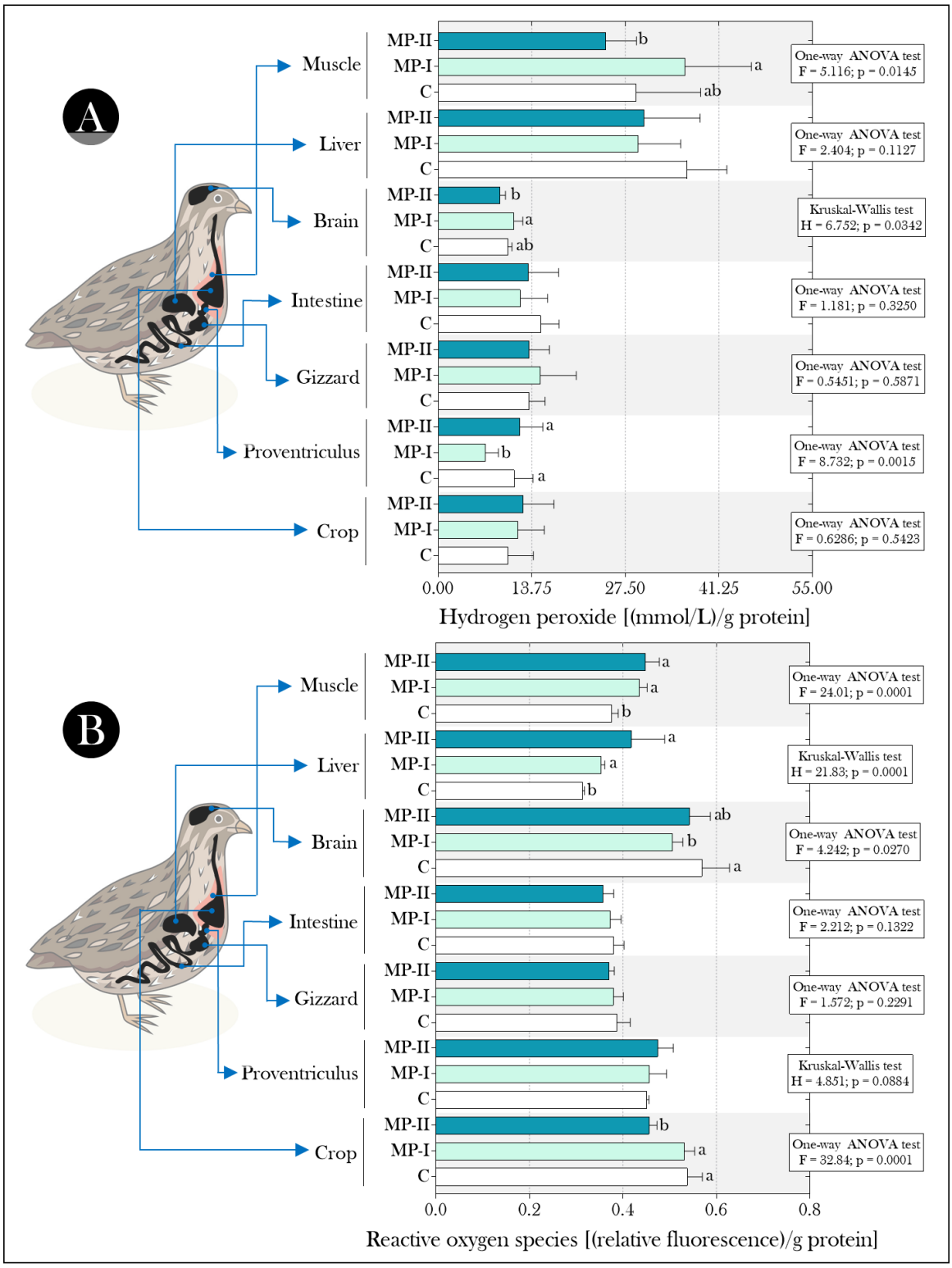

Figure 3. (A) Hydrogen peroxide (H_2O_2) and (B) total reactive oxygen species (ROS) production in different organs/tissues of adult female Japanese quail (*Coturnix coturnix japonica*) exposed and unexposed to naturally-aged polystyrene microplastics (MPs). Parametric data are presented by the mean ± SD whereas non-parametric are presented by the median and interquartile range. Summaries of statistical analyzes are displayed next to the bars. C: group composed of quails not exposed to MPs ("control") and the "MP-I" and "MP-II" groups were

composed of quails that received 11 and 22 MP particles, respectively, once a day, for nine consecutive days. n=10 animals/group.

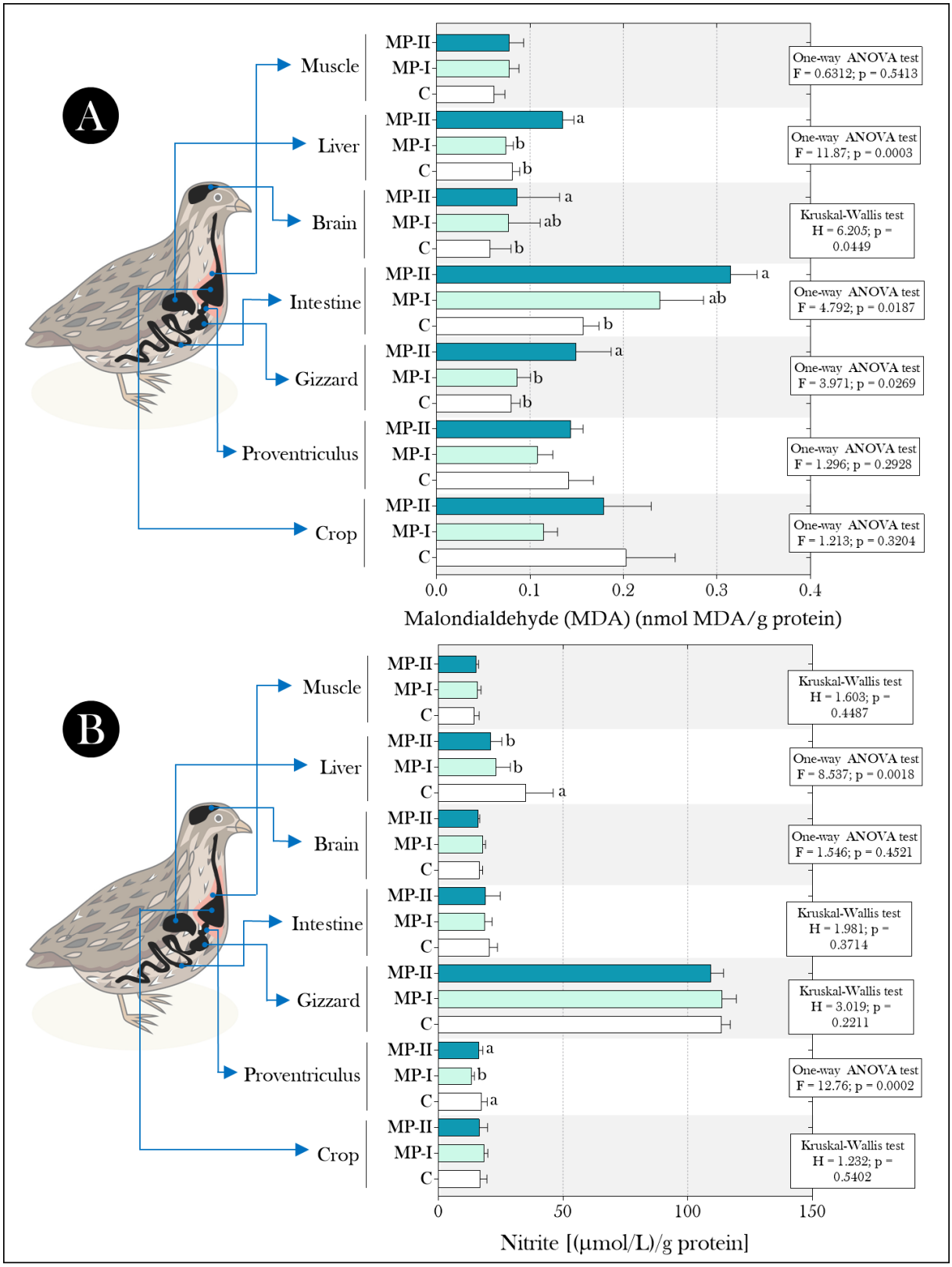

Figure 4. (A) Malondialdehyde (MDA) and (B) nitrite (NO_{2}-) production in different organs/tissues of adult female Japanese quail (*Coturnix coturnix japonica*) exposed and unexposed to naturally-aged polystyrene microplastics (MPs). Parametric data are presented by the mean ± SD whereas non-parametric are presented by the median and interquartile range. Summaries of statistical analyzes are displayed next to the bars. C: group composed of quails not exposed to MPs ("control") and the "MP-I" and "MP-II" groups were composed of quails that received 11 and 22 MP particles, respectively, once a day, for nine consecutive days. n=10 animals/group.

Regarding the antioxidant response of the animals, we observed that SOD activity was reduced in the liver and intestine of quails exposed to MPs, whose average reduction was 22.5 and 20.1%, respectively, in relation to the control group. In addition, we observed a lower activity of this enzyme in the crop of the animals of the "MP-II" group (Figure 5A). In the other organs, SOD activity did not differ between the experimental groups. However, we did not observe a well-defined pattern regarding the effect of exposure to MPs on catalase activity in the different organs/tissues evaluated (Figure 5B). While in muscle, catalase activity was higher in quails from groups exposed to pollutants (average of 11.3%); in the intestine, a suppressive effect was observed in these same animals (Figure 5B). Furthermore, the activity of this enzyme in the liver of the animals in the "MP-II" group was increased by 20.8% in relation to the "control" group; however, in the brain, a reduction of close to 10% was noticed in these same animals. On the other hand, in the crop of the animals of the "MP-I" group, we observed an increase in catalase activity and, in the gizzard and proventriculus of these same animals, a suppressive effect was evidenced (Figure 5B). As for AChE activity, although we did not observe statistical differences between the experimental groups, we noticed a trend towards the increased activity of this enzyme in the muscle and brain of animals exposed to MPs (Figure 6). Such trends represented mean increases >22% and >7.5% in the respective tissues.

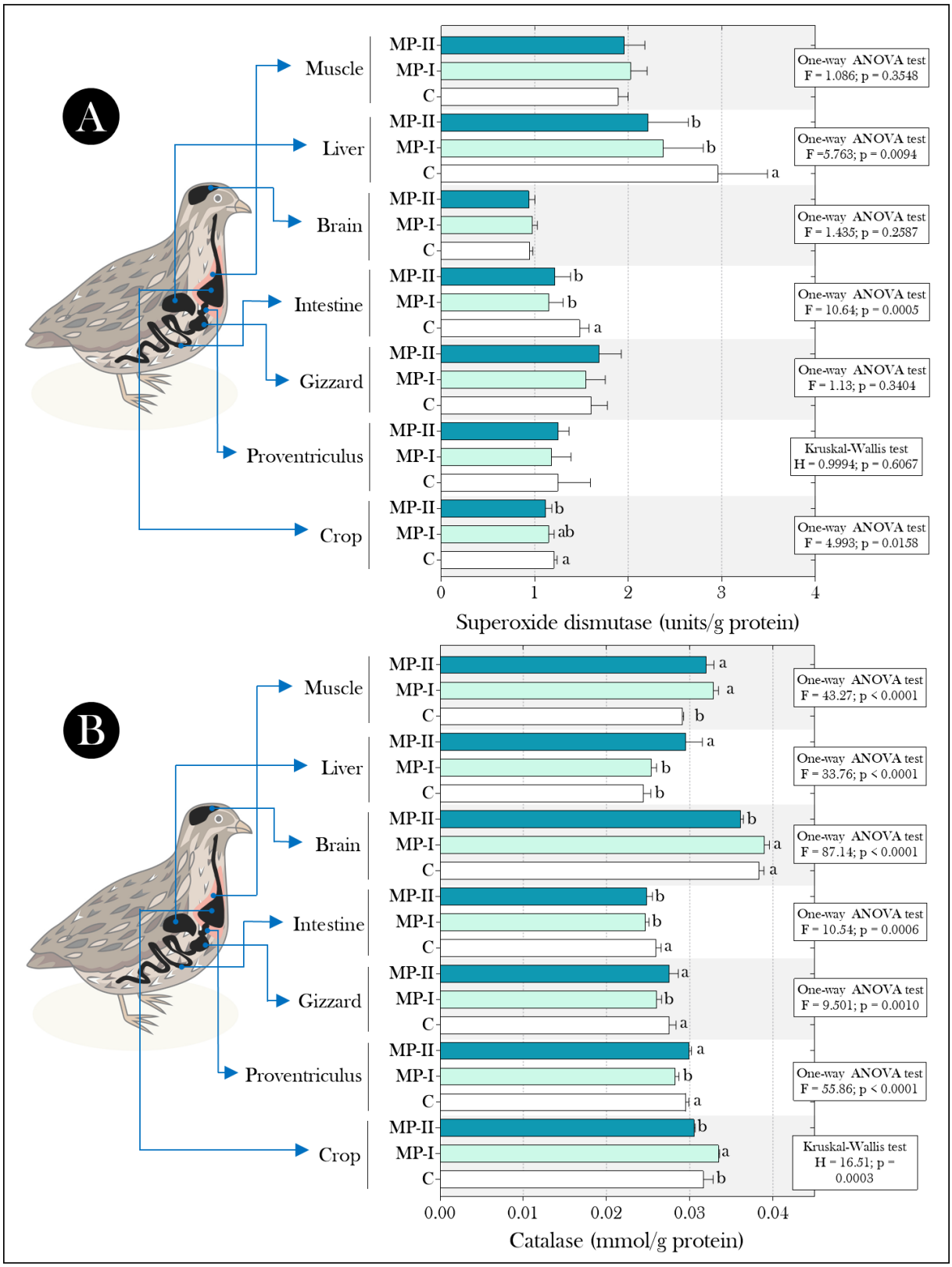

Figure 5. (A) Superoxide dismutase (SOD) and (B) catalase activity in different organs/tissues of adult female Japanese quail (*Coturnix coturnix japonica*) exposed and unexposed to naturally-aged polystyrene microplastics (MPs). Parametric data are presented by the mean ± SD whereas non-parametric are presented by the median and

interquartile range. Summaries of statistical analyzes are displayed next to the bars. C: group composed of quails not exposed to MPs ("control") and the "MP-I" and "MP-II" groups were composed of quails that received 11 and 22 MP particles, respectively, once a day, for nine consecutive days. n=10 animals/group.

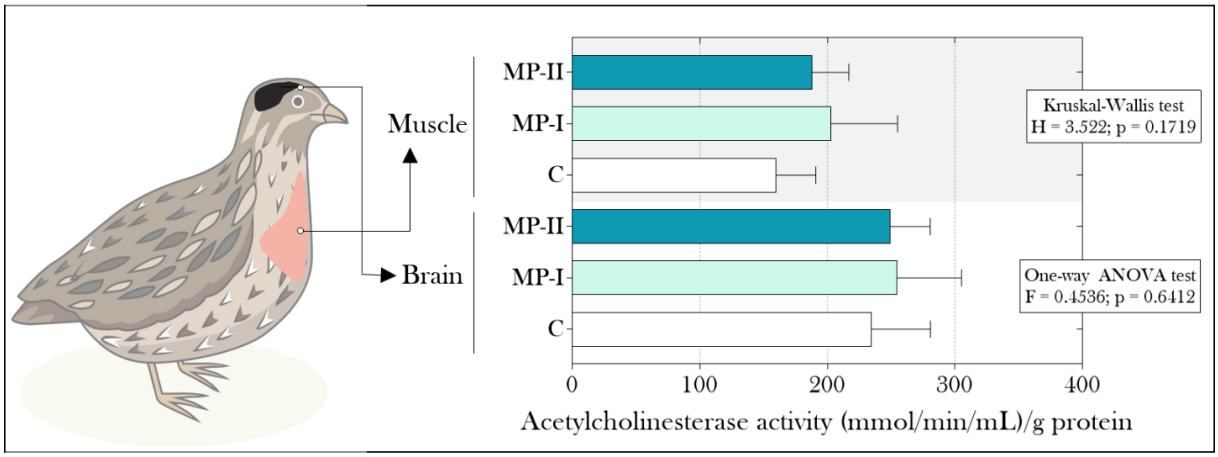

Figure 6. Acetylcholinesterase (AChE) activity in muscle and brain of adult female Japanese quail (*Coturnix coturnix japonica*) exposed and unexposed to naturally-aged polystyrene microplastics (MPs). Parametric data are presented by the mean ± SD whereas non-parametric are presented by the median and interquartile range. Summaries of statistical analyzes are displayed next to the bars. C: group composed of quails not exposed to MPs ("control") and the "MP-I" and "MP-II" groups were composed of quails that received 11 and 22 MP particles, respectively, once a day, for nine consecutive days. n=10 animals/group.

Regarding the quantification of MPs in the gizzard and intestine, our data confirm the visual analysis of their contents, in which the evident presence of plastic particles was noticed (Figure 7B-C) mixed with food content. As expected, we noticed a higher concentration of MPs in the gizzard and intestine of the quails of the "MP-II" group, compared to the birds that received a lower number of MPs (Figure 7A). On the other hand, the number of plastic particles in the intestinal contents (from both groups exposed to MPs) was on average 113.3% higher than that observed in the gizzard. Furthermore, the presence of MPs in the analyzed liver samples suggests that part of the ingested MPs was absorbed by intestinal cells and passed through the hepatic portal system. We showed that the concentration of MPs in the liver of the quails of the "MP-II" group was 29.1% higher than that identified in the quails of the "MP-I" group (Figure 7D-E). Furthermore, our data point to significant correlations between the accumulation of MPs in these organs and different biomarkers evaluated (Figure 8). On the other hand, we noticed the elimination of plastic particles in the feces shortly after 12 h of the first exposure (Figure 9A-C). Additionally, we observed that the number of particles/g of fecal

content remained constant throughout subsequent evaluations. As expected, a greater number of MPs was observed in the feces of birds in the "MP-II" group (Figure 9D).

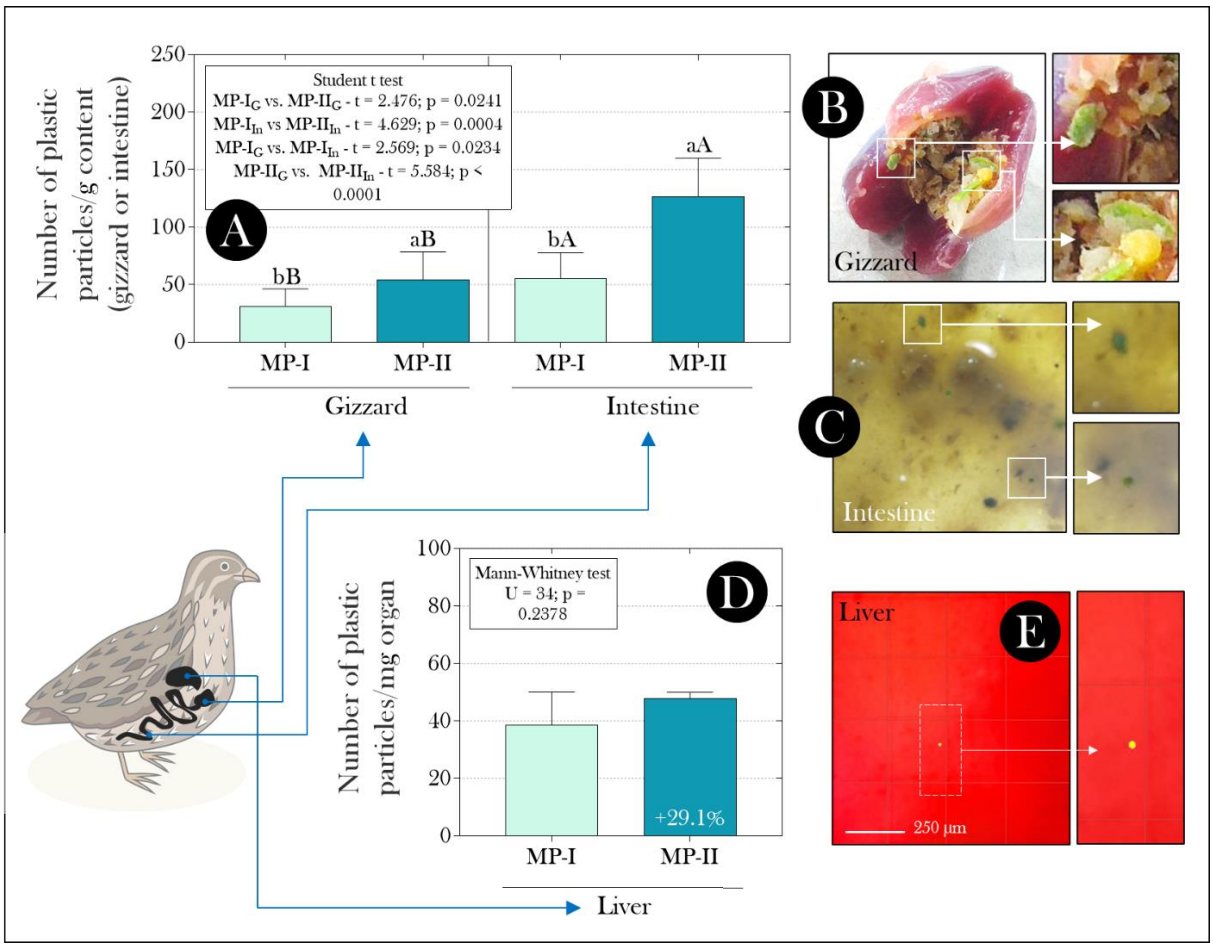

Figure 7. The number of plastic particles identified in (A-C) gizzard and small intestine and (D-E) liver of adult female Japanese quail (*Coturnix coturnix japonica*) exposed to naturally-aged polystyrene microplastics (MPs). In "A and D", parametric data are presented by the mean ± SD whereas non-parametric are presented by the median and interquartile range. Summaries of statistical analyzes are displayed at the top of the graphs. In "A", distinct lowercase letters indicate significant differences between the experimental groups within each organ evaluated. The distinct capital letters indicate significant differences between the number of plastic particles of Organs evaluated organs. In "B and C" we highlight the presence of MPs (green particles) in the contents of gizzard (B) and intestine (C) of quails exposed to pollutants. In "E", a representative image of the presence of PM in the Neubauer chamber is presented during the analysis of liver samples from quail exposed to the pollutant. "MP-I" and "MP-II" groups were composed of quails that received 11 and 22 plastic particles, respectively, once a day for nine consecutive days. n=10 animals/group.

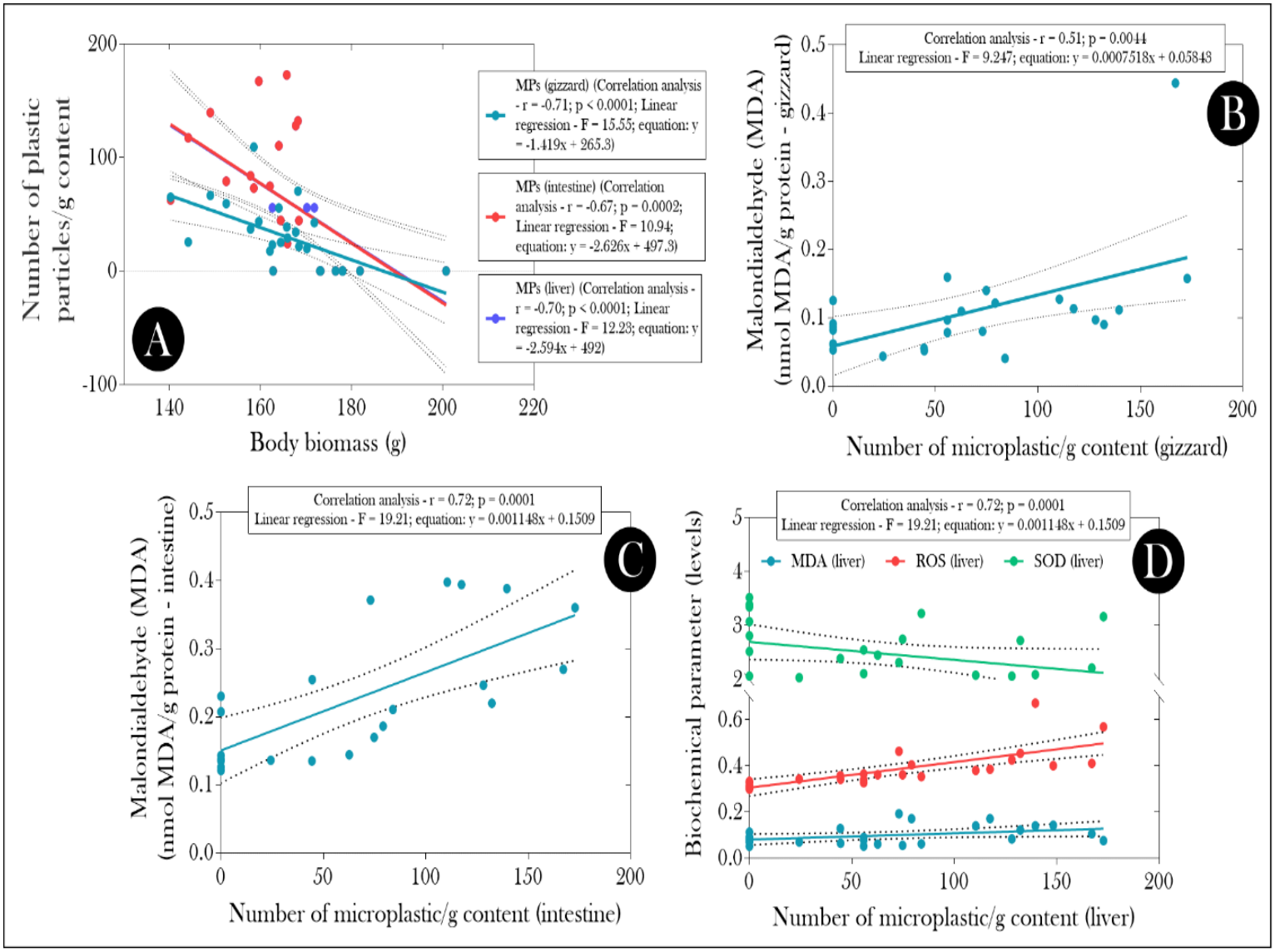

Figure 8. Correlation and linear regression analyses between (A) "accumulation of naturally-aged polystyrene microplastics (MPs) in the gizzard, small intestine, and liver" vs. "body biomass"; (B-C) "number of MPs in gizzard and intestine" vs. "MDA levels" in the respective organs, and (D) "MPs accumulation in the liver" vs. "MDA and ROS levels and SOD activity" in quail liver. The graphics show the 95% confidence bands of the best-fit line in linear regression analysis. MPs: naturally-aged polystyrene microplastics (number of plastic particle/g content); MDA: malondialdehyde (nmol/g protein); ROS: total reactive oxygen species (relative fluorescence/g protein); SOD: superoxide dismutase activity (units/g protein).

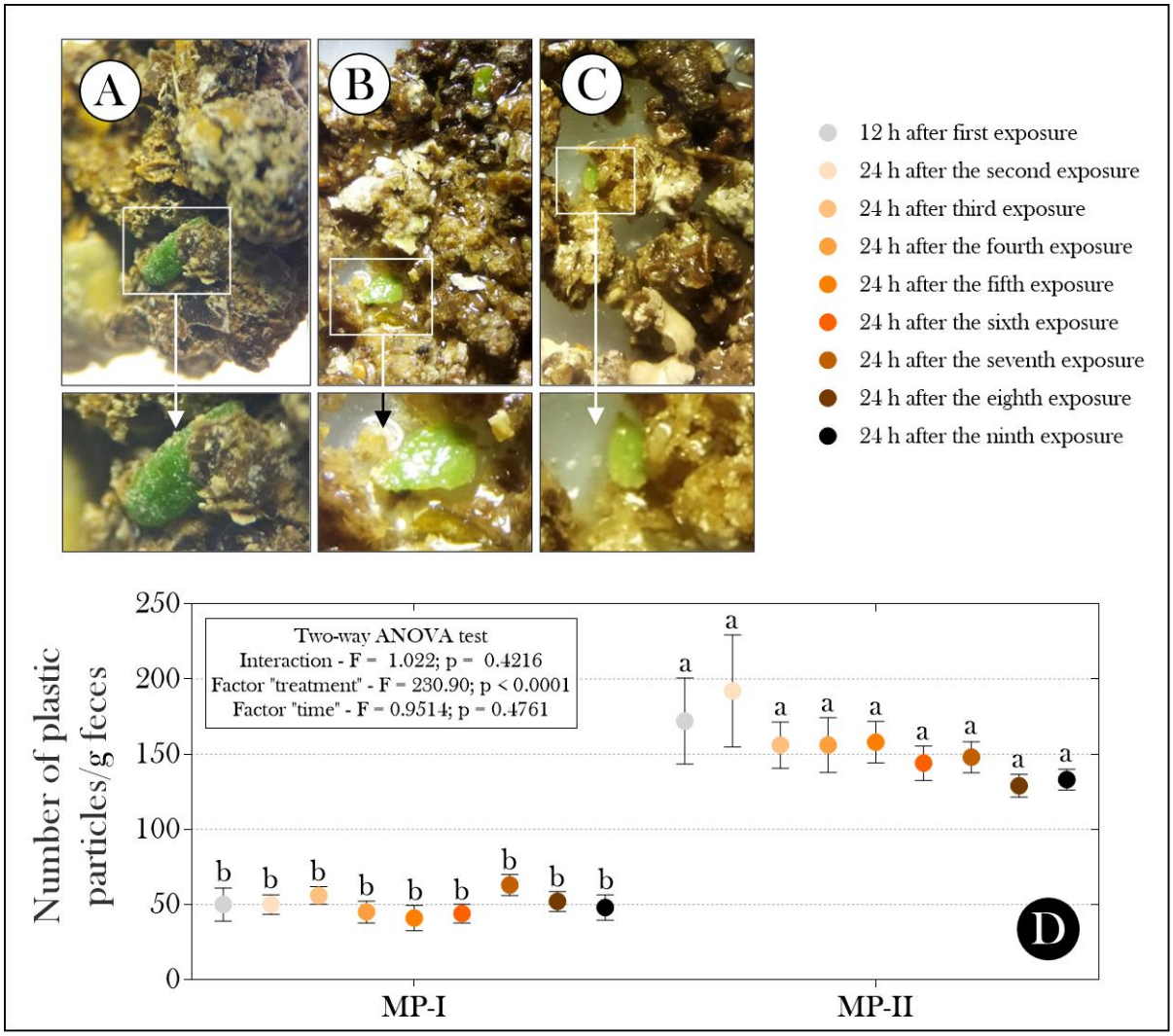

Figure 9. (A-C) Representative photomicrographs of fecal samples from adult female Japanese quail (*Coturnix coturnix japonica*) exposed to naturally-aged polystyrene microplastics (MPs), shortly after 12 h of first exposure. (D) The number of particle plastic/g feces identified during the daily assessments carried out. In "D", the circles indicate the mean ± SD, and the summary of the statistical analysis is presented at the top of the graph. Distinct lowercase letters indicate significant differences. The "MP-I" and "MP-II" groups were composed of quails that received 11 and 22 plastic particles, respectively, once a day for nine consecutive days. n=10 animals/group.

We also observed that the size and shape of ingested MPs were modified as they transited through the animals gastrointestinal tract. As shown in Figure 10A, the diameter of the MPs was organ-dependent. In the gizzard content, we observed, on average, a reduction of 46.8% in the diameter of the MPs in relation to the plastic particles initially ingested, and a more accentuated reduction was observed in the particles identified in the intestinal content (average reduction of 80.2%). In the liver, the identified MPs had a diameter of 9.18 µm ± 0.89 µm (mean ± SEM), which represents a size 364.8 times smaller than that recorded in the

particles initially ingested by the animals (Figure 10A). Furthermore, we observed that the diameter of the MPs identified in the feces was like that identified in the intestinal content (626.90 µm ± 40.77 µm; general mean ± SEM) and, in terms of shape, we did not observe differences between the circularity values of the MPs identified in gizzard, intestine, and feces (Figure 10B). On the other hand, in the liver, MPs showed higher circularity-values in relation to the particles initially ingested by the animals and those identified in the gizzard and intestine (Figure 10B). As shown in Figure 10E, 88.8% of MPs in the liver had values ≥ 0.9, which indicates more spheroidal shapes (Figure 10C-D).

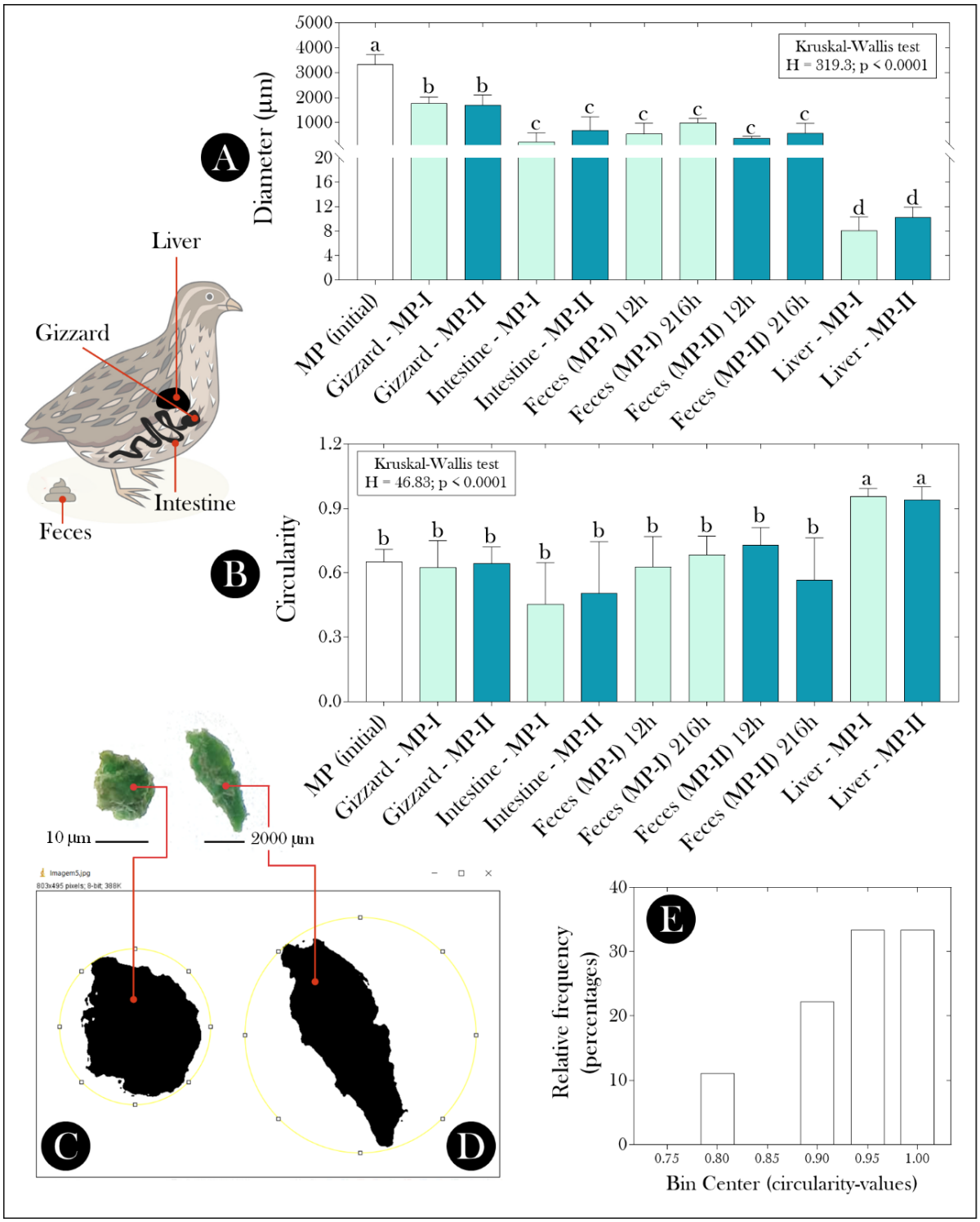

Figure 10. (A) Diameter (µm) and (B) circularity-values of naturally-aged polystyrene microplastics (MPs) before ingestion (initial) and in those identified in gizzard and intestine contents, feces, and liver of adult female Japanese quail (*Coturnix coturnix japonica*). (C-D) Representative images of the shape of the plastic particles observed in the (C) liver and (D) feces of the animals. (E) Histogram of circularity-value distribution of particles accumulated in the quail liver. In "A" and "B", non-parametric data are presented by the median and interquartile range. Statistical summaries are displayed at the top of the graphs and distinct lowercase letters indicate significant

differences. The “MP-I” and “MP-II” groups were composed of quails that received 11 and 22 plastic particles, respectively, once a day for nine consecutive days. n=10 animals/group. 216h: indicates that the evaluation was performed 216h after the first exposure of the animals. Alternatively, this indication can be interpreted as “24 h after the ninth exposure”.

To show an overview of the results and correlations between the groups and the variables evaluated in this study, data from all biomarkers were submitted to a PCA. According to this analysis, the first two principal components (PC1 and PC2) cumulatively explained 100% of the total variation (PC1 = 56.79%; PC2 = 43.21%), with the eigenvalues > 20 (PC1 = 26.69; PC2 = 20.31). The loadings plot (which shows the relationship between the PCs and the original variable – Figure 11A) and Table 1 demonstrate a diversified positioning of the variables in the PCs. On the other hand, in the PCA biplot, we noticed that the groups exposed to MPs were clearly separated from the “control” group by PC1, the latter having a negative score (C: -5.74) and the other groups positive scores (“MP-I ”: 3.00 and “MP-II”: 2.74) (Figure 11B), which confirms the trends observed in the tests presented above (one-way ANOVA or Kruskal-Wallis tests). In addition, the similarity of the PC1 scores of the “MP-I” and “MP-II” groups in PC1 allowed them to be grouped into a single group (Figure 11B), which was also observed in the hierarchical clustering analysis performed according to Ward's method (Figure 11C).

Additionally, to complement the PCA performed, the IBRv2 was applied to provide an overall evaluation of the toxicological effects of MPs on quails. As shown in Figure 12A, the proximity between the scores (absolute value) of biomarker responses of the “MP-I” and “MP-II” groups indicates a similar sensitivity to toxic stress, which also confirms the clustering of these groups in the PCA (Figure 11B). The IBR star plots combining scores of biomarker response in the different organs/tissues evaluated and corresponding IBRv2 values of different treatments are shown in Figure 12B. Of all biomarkers, the scores of MDA response in most organs/tissues evaluated were markedly higher than those of other biomarkers, indicating that lipid peroxidation displayed the highest response sensitivity. Additionally, SOD (muscle) and catalase (brain) activity, as well as ROS (liver and muscle) and H_2O_2 (crop) production were

more sensitive to exposure to MPs, with MDA levels (brain, intestine, and liver) were more sensitive to the ingestion of a higher number of MPs (Figure 12B and 13).

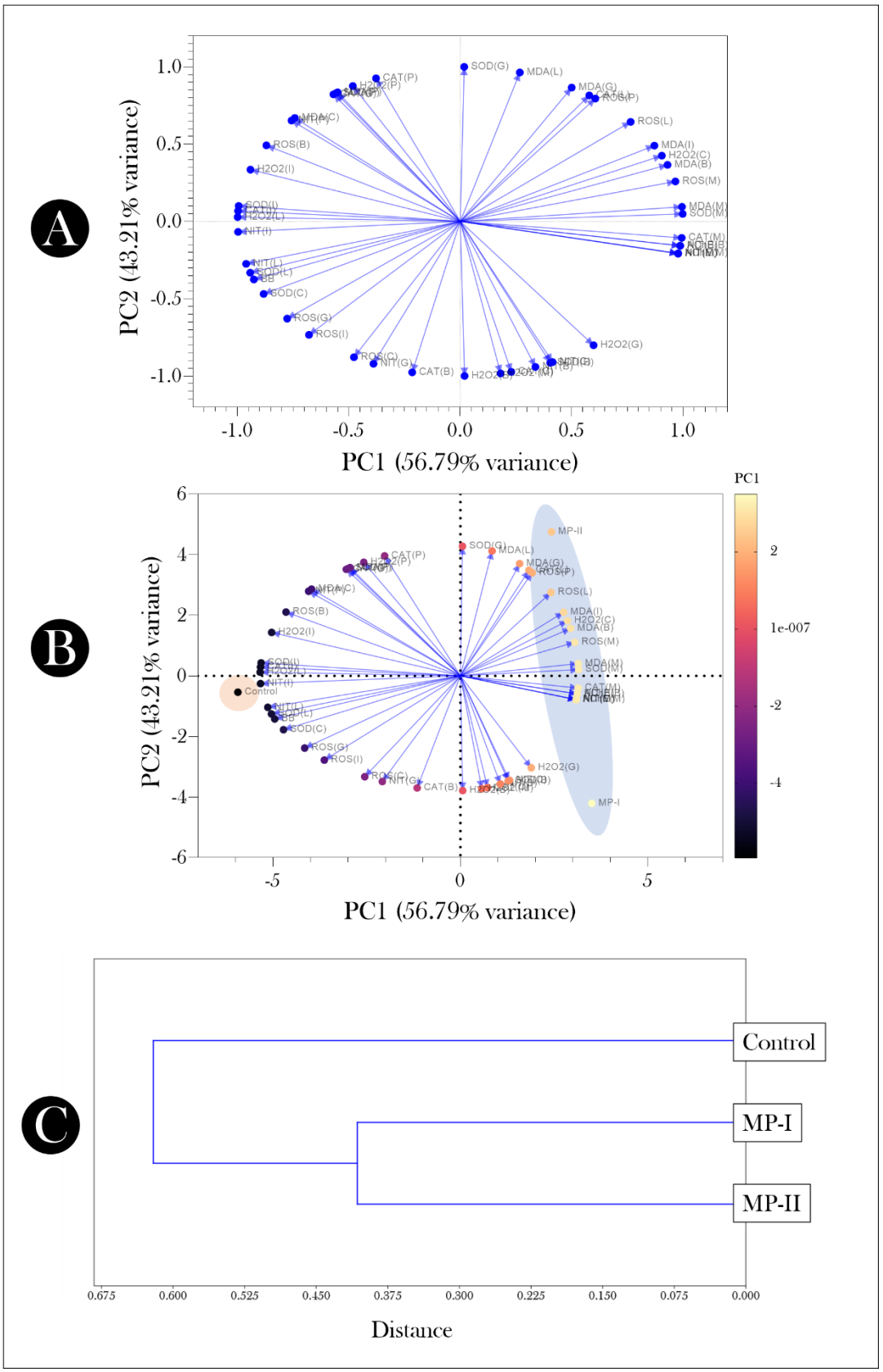

Figure 11. (A) Loadings plot of the investigated variables, (B) PCA biplot of the first two main components that simultaneously shows PC1 scores experimental loadings of explanatory variables (vectors), and (C) cluster analysis dendrogram. C: group composed of quails not exposed to MPs ("control") and the "MP-I" and "MP-II" groups were composed of quails that received 11 and 22 MP particles, respectively, once a day, for nine consecutive days. CAT: catalase activity; SOD: superoxide dismutase activity; H_2O_2: hydrogen peroxide production; ROS: total reactive oxygen species production; MDA: production of malondialdehyde (MDA); NIT: nitrite production (an indirect measure of nitric oxide production); AChE: acetylcholinesterase activity; BB: body biomass. The letters in parentheses refer to the initials of the brain (B), crop (C), liver (L), small intestine (I), muscle (M), gizzard (G), and proventriculus (P).

Table 1. Rotated loading (coefficient) matrix provided by the multivariate analysis to define factors or principal components PC1 and PC2.

Biomarkers	Principal components[a]		Biomarkers	Principal components[a]	
	PC1	PC2		PC1	PC2
CAT(M)	0.994	-0.107	ROS(B)	-0.871	0.492
CAT(L)	0.579	0.815	ROS(I)	-0.679	-0.734
CAT(B)	-0.216	-0.976	ROS(G)	-0.777	-0.629
CAT(I)	-0.998	0.068	ROS(P)	0.607	0.795
CAT(G)	-0.570	0.822	ROS(C)	-0.477	-0.879
CAT(P)	-0.378	0.926	MDA(M)	0.996	0.094
CAT(C)	0.229	-0.973	MDA(L)	0.268	0.964
SOD(M)	0.999	0.049	MDA(B)	0.931	0.366
SOD(L)	-0.943	-0.332	MDA(I)	0.872	0.490
SOD(B)	0.404	-0.915	MDA(G)	0.500	0.866
SOD(I)	-0.995	0.100	MDA(P)	-0.550	0.835
SOD(G)	0.018	1.000	MDA(C)	-0.743	0.669
SOD(P)	-0.557	0.830	NIT(M)	0.978	-0.209
SOD(C)	-0.883	-0.469	NIT(L)	-0.962	-0.275
H_2O_2(M)	0.181	-0.983	NIT(B)	0.338	-0.941
H_2O_2(L)	-1.000	0.028	NIT(I)	-0.998	-0.068
H_2O_2(B)	0.019	-1.000	NIT(G)	-0.390	-0.921
H_2O_2(I)	-0.942	0.335	NIT(P)	-0.758	0.653
H_2O_2(G)	0.599	-0.801	NIT(C)	0.414	-0.910
H_2O_2(P)	-0.482	0.876	NIT(M)	0.978	-0.207
H_2O_2(C)	0.905	0.425	NIT(B)	0.988	-0.158
ROS(M)	0.966	0.259	AChE(M)	0.978	-0.207
ROS(L)	0.765	0.644	AChE(B)	0.988	-0.158
			BB	-0.927	-0.376

Legend: CAT: catalase activity; SOD: superoxide dismutase activity; H_2O_2: hydrogen peroxide production; ROS: total reactive oxygen species production; MDA: production of malondialdehyde (MDA); NIT: nitrite production (an indirect measure of nitric oxide production); AChE: acetylcholinesterase activity; BB: body biomass. The letters in parentheses refer to the initials of the brain (B), crop (C), liver (L), small intestine (I), muscle (M), gizzard (G), and proventriculus (P).

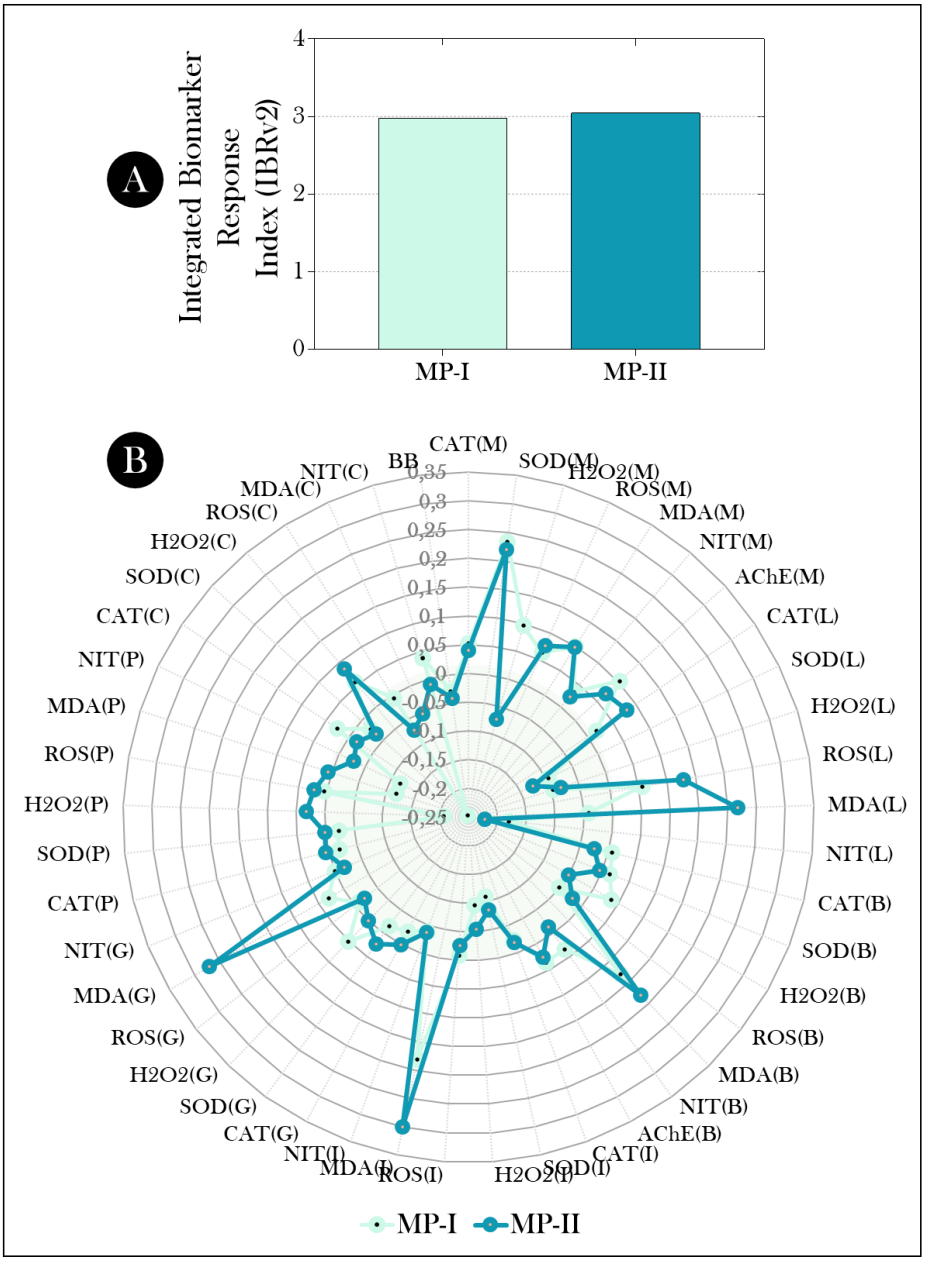

Figure 12. (A) Results of integrated biomarker responses index (IBRv2) calculations and (B) star plot for "MP-I" and "MP-II" groups. In "B", the calculated value for each biomarker (of each organ/tissue) was reported in a star plot in a reference deviation of each investigated biomarker. The area above 0 reflects induction of the biomarker and below 0 indicates a reduction of the biomarker. The "MP-I" and "MP-II" groups were composed of quails that received 11 and 22 MP particles, respectively, once a day for nine consecutive days. CAT: catalase activity; SOD: superoxide dismutase activity; H_2O_2: hydrogen peroxide production; ROS: total reactive oxygen species production; MDA: production of malondialdehyde (MDA); NIT: nitrite production (an indirect measure of nitric

oxide production); AChE: acetylcholinesterase activity; BB: body biomass. The letters in parentheses refer to the initials of the brain (B), crop (C), liver (L), small intestine (I), muscle (M), gizzard (G), and proventriculus (P).

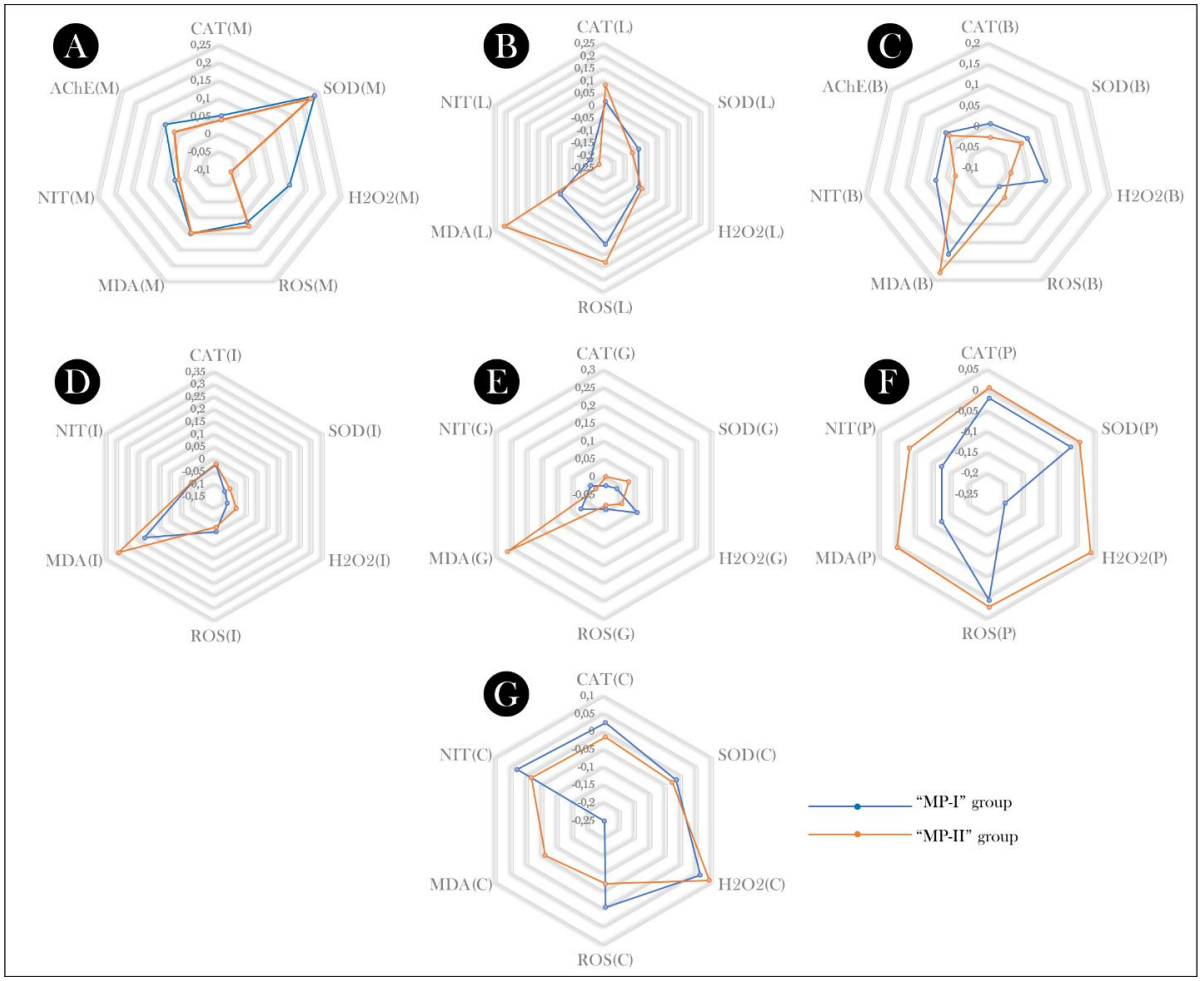

Figure 13. Star plot for the calculated IBRv2 index of the (A) muscle, (B) liver, (C) brain, (D) small intestine, (E) gizzard, (F) proventriculus, and (G) crop of adult female Japanese quail (*Coturnix coturnix japonica*) exposed to naturally-aged polystyrene microplastics (MPs). The "MP-I" and "MP-II" groups were composed of quails that received 11 and 22 MP particles, respectively, once a day for nine consecutive days. CAT: catalase activity; SOD: superoxide dismutase activity; H_2O_2: hydrogen peroxide production; ROS: total reactive oxygen species production; MDA: production of malondialdehyde (MDA); NIT: nitrite production (an indirect measure of nitric oxide production); AChE: acetylcholinesterase activity; BB: body biomass. The letters in parentheses refer to the initials of the brain (B), crop (C), liver (L), small intestine (I), muscle (M), gizzard (G), and proventriculus (P).

4. DISCUSSION

It is agreed that one of the major challenges of (eco)toxicological evaluation studies is to understand how pollutants/contaminants affect different organisms. Especially in small-sized terrestrial birds, the identification and characterization of plastic ingestion are important not only to fill a gap in our knowledge but especially to allow prediction of the impacts of MPs on these important components of avifauna. As highlighted by Yu et al. (2014), these animals are highly diversified and widely distributed in different ecological habitats. However, this important argument has not been enough to leverage current studies on the possible impacts arising from the ingestion of MPs. In the recent review by Wang et al. (2021), the authors point out that very little is known about the relationship between the occurrence of macroplastics and MPs in small-sized terrestrial birds, as well as about their impacts on the health of these animals. Therefore, the evaluation of the possible effects of microplastic ingestion by these birds becomes important to support the planning and mitigation, and conservation actions of these animals.

In our study, using the experimental model *C. cotunix japonica* (a representative of small-sized terrestrial birds), we demonstrated that the ingestion of MPs can induce alterations that put their survival at risk. Initially, it was evidenced that the ingestion of MPs for only nine days was able to affect the body biomass of the animals. At the end of the experiment, the weight of the quails in the "MP-I" and "MP-II" groups was on average 10% lower than that of the animals in the "control" group, which was also reported by Spear et al. (1995), when studying seabirds captured in the tropical Pacific between 1984 to 1991. At the time, the authors hypothesized that the lower weight of birds that contained plastic in the gastrointestinal contents would be due to physical damage or blockage of the digestive tract and/or impairment of digestive efficiency induced by these materials. On the other hand, when subjecting domestic chickens *G. gallus domesticus* to the ingestion of plastic fragments, Ryan (1988) observed a significant reduction in the birds feeding, which was associated with the reduced gizzard volume, considering the occupation of a large part of the stomach cavity by the birds plastic particles. Therefore, these studies provide evidence of what may have caused the reduction in body biomass of quails exposed to MPs in our study.

On the other hand, it is also possible that this weight loss is associated with increased energy expenditure to maintain redox homeostasis in the different organs evaluated. As discussed by Hou et al. (2015), in many circumstances, energy expenditure is proportional to the rate of free radical production, which in turn is proportional to the net oxidative damage.

Furthermore, it is known that activation of cellular damage repair mechanisms (e.g.: removal of peroxidized acyl chains from phospholipids, DNA double-strand break or base excision repair, and methionine sulfoxide repair) can increase the overall metabolic energy pool (Hou et al., 2015) which, in the medium and long term, can result in damage to the organism. Furthermore, we cannot neglect the hypothesis that the immune response of birds to MPs imposed additional energy costs that could have contributed to the weight loss of the animals. The low production of nitrite observed in the liver of birds in the "MP-I" and "MP-II" groups (Figure 4B), for example, is suggestive of a suppressive effect on hepatic NO production and/or isoforms of nitric oxide synthases in different cells that play a crucial role in immune homeostasis in the liver, including liver sinusoidal endothelial cells, hepatocytes, Kupffer cells (liver resident macrophages), hepatic stellate cells, smooth muscle cells, cholangiocytes, and other immune cells (Iwakiri & Kim, 2015). Therefore, prioritizing energy for an antioxidant, damage repair, and immune response could conflict with systems that are necessary for meeting ongoing ecological challenges, such as those involving the maintenance of body biomass and tissue energy reserves. In this case, future evaluations on the impact of MPs ingestion on the metabolic reorganization processes in birds may favor our understanding of the mechanisms that lead to weight reduction in these animals, which was evidenced both in our study and in the work of Spear et al. al. (1995). In addition, assessments of the lipid composition of reserve tissues, of subcellular fractions of other tissues, and their variations as a function of plastic ingestion are strongly recommended.

Another aspect observed in our study refers to the differentiated pro- and antioxidant response in the organs/tissues of quails exposed to MPs. Although the biochemical changes in some organs have not been directly associated with the accumulation of MPs; for others, the cause of the reported changes was proportional to the concentrations of plastic particles in these organs. The presence of MPs in the gizzard and intestine, for example, was positively correlated with the production of MDA in these organs (Figures 8B-C) which, contrary to our expectations, was not associated with the production of ROS (similar between the experimental groups, Figure 3B). In this case, it is plausible to assume that the increase in MDA levels is part of an adaptive response to try to counterbalance the oxidative stress induced by MPs. As discussed by Morales & Munné-Bosch (2019) and more recently by Rangasamy et al. (2022), MDA increases may represent acclimation processes rather than damage, since MDA can exert a positive role by activating regulatory genes involved in animal defense and development and

granting cell protection under oxidative stress conditions. Anyway, this presumption needs to be validated by future studies.

On the other hand, in the liver, accumulated MPs were significantly associated with increased production of ROS and MDA, as well as reduced hepatic SOD activity (Figure 8D). Therefore, these data suggest a typical redox imbalance, characterized by the inability of the hepatic antioxidant system to counterbalance ROS production, with reduced SOD activity indirectly contributing to the increase in lipid peroxidation processes in the liver (inferred by MDA levels). The significant correlations between these biomarkers (Figure 8) reinforce our hypothesis. In any case, these alterations can lead to a series of other hepatic alterations (at a cellular, structural, or molecular level) that compromise the function of this important organ. Although no studies have investigated the consequences of MP accumulation in the liver of birds, several liver changes have already been reported in other animal models, which instigate further investigations involving small-sized terrestrial birds exposed to the pollutant. In *Physalaemus cuvieri* tadpoles, for example, we demonstrated that the bioaccumulation of MPs in the liver was correlated to different histopathological changes (blood vessel dilation, infiltration, congestion, hydropic degeneration, hypertrophy, and hyperplasia), which showed the histopathotoxicity of MPs (Araújo et al. al., 2020b). Similarly, Deng et al. (2017) demonstrated that compared with control mice, inflammation and lipid droplets were observed in the livers of PS-MPs-treated ICR mice. In C57BL/6 male mice, it was observed that PS-MPs induced hepatocyte apoptosis and abnormal glycolytic flux through ROS-driven calcium overload (Li et al., 2021). Furthermore, the transcriptomic analysis indicated that the PS-MPs resulted in hepatic glycolipid disruption metabolism in zebrafish (Danio rerio), which led to liver dysfunction and degeneration eventually (Zhao et al., 2020).

Interestingly, we also observed that the ingestion of MPs by quails induced biochemical changes in organs that are not directly interconnected with the animals' gastrointestinal system, which confirms the systemic toxicity associated with MPs. In the brain, for example, an average increase of 43% in the production of MDA was observed in the animals exposed to MPs (Figure 4A), and in the muscle, the production of total ROS was higher, on average, by 17.5% compared to the control group (Figure 3B). Furthermore, although AChE activity in muscle and brain did not differ significantly between the experimental groups, mean increases of 22.2% and 7.5%, respectively, were observed in quails that ingested MPs (Figure 6). From a biological point of view, these results can be interpreted as a moderate cholinesterase effect induced by MPs, which has also been demonstrated in previous studies involving Danio rerio (Guimarães et al., 2021),

Culex quinquefasciatus larvae (Malafaia et al., 2020), ICR mice (Deng et al., 2017), earthworms (Eisenia foetida) (Chen et al., 2020), and wild fish from North-East Atlantic Ocean (Dicentrachus labrax, Trachurus trachurus, Scomber colias) (Barboza et al., 2020). Future investigations should be conducted to investigate the mechanisms intrinsic to these alterations, which are unknown in birds, but also complex and ambiguous in different animal organisms. However, we cannot neglect the hypothesis that the biochemical changes reported, especially in the brain and muscle, are related to the release of heavy metals adsorbed to the surfaces of naturally-aged MPs used in our study. During the chemical characterization of these materials, previously carried out by Guimarães et al. (2021), we observed weak peaks of Fe, Ca, Cu, Si, Al, Na, and S, which were observed in spectra found through electron microscopy. Therefore, it is plausible to suppose that these chemical elements may have been leached from MPs, absorbed via the gastrointestinal system, and, consequently, have contributed to the occurrence of the reported changes.

In any case, the increase in oxidative stress processes and the increase in AChE in the brain and muscle can lead to harmful consequences for animals. Biochemical changes in the brain induced by micro(nano)plastics have, for example, been associated with neurobehavioral disorders important for the survival of other animal models (e.g.: Mattsson et al., 2015; Mattsson et al., 2017; Zaheer et al., 2022). Elevated AChE activity, in particular, can lead to dramatic effects that include, among others, reduced cholinergic neurotransmission efficiency due to decreased acetylcholine levels in the synaptic cleft (Ferreira et al., 2012, Teodorak et al., 2015), which, in its turn, lead to several changes. As discussed by Guimarães et al. (2021), important neurophysiological processes at the very root of several motor and cognitive functions are linked to cholinergic neurotransmission at the synaptic, pathway, and circuital levels. Furthermore, it is well established that highly reactive molecules have many deleterious effects on the muscle, such as a reduction of force generation and increased muscle atrophy (Steinbacher & Eckl, 2015). Therefore, the changes observed in the brain and muscle of quails exposed to MPs can culminate in comprehensive physiological changes.

On the other hand, although we have reported different effects in the evaluated organs/tissues, taken together, our data suggest that the ingestion of MPs induced similar toxicological effects in the “MP-I” and “MP-II” groups, which was clearly evidenced in the PCA analysis (Figure 11B), hierarchical clustering analysis (Figure 11C), and in the IBRv2-values obtained for both groups (Figure 12a). Such results demonstrate, therefore, that the ingestion of a low number of MPs is sufficient to cause a toxicological effect like that induced

by the ingestion of larger amounts. In this case, even if preliminary, it is tempting to speculate on the possible activation of tolerance mechanisms in the animals of the "MP-II" group, to prevent more extensive damage from occurring due to exposure to a greater amount of MPs. In the pharmacological area, the dose-response curve, which postulates that a change in drug dose will produce a proportionate and predictable change in drug effect, is assumed to provide an adequate description of the dose-effect relation (Peper, 2009). However, Yang et al. (2021) warn that existing conceptualizations of the relationship between drug dose and drug effect display fundamental contradictions. Therefore, the possible mechanisms that modulate the response of organisms (including the birds that are the focus of our study) to the exposure of MPs (in different concentrations/doses) constitute fertile and useful investigative perspectives to be explored in future investigations.

Another important result observed in our study refers to the changes that occurred in the size of MPs when transiting through the digestive tract of birds. As shown in Figure 10A, the MPs identified in the gizzard and intestine showed significantly reduced sizes when compared to the diameter of the plastic particles initially ingested, suggesting, therefore, the occurrence of MPs fragmentation by the direct action of the gizzard's mechanical functions. As described by Svihus (2011), the gizzard of birds of the order Galliformes has as a typical characteristic the adaptation to the grinding of coarse particles, which occurred through the development of a very large mass of strongly smooth muscles posterior to the glandular stomach immediately. Therefore, this may explain the large number of plastic particles identified in the animals' feces. We observed that throughout the experiment, on average, 49.9 ± 2.22 MP particles and 153.80 ± 6.44 MP particles (mean ± SEM) were released daily per "g" of feces produced by quails in the "MP -I" and "MP-II", respectively (Figure 9D). In this case, if the daily production of feces by a quail is equivalent to approximately 7% of its body weight, as already reported for domestic pigeon (Columbia livia) (Walia & Walia, 2021), it is possible to predict that after only 12 h of the first MP ingestion occurred, 588.8 and 1722.24 MP particles/day were released by the quails of the "MP-I" and "MP-II" groups, respectively. This represents an increase of 53.3 and 78.2 times (respectively) in the number of particles ingested by birds initially. Therefore, these data point to the real possibility of small-sized terrestrial birds, acting (unintentionally) as vectors for dispersal of MPs in environments, as already reported for birds of the order Falconiformes (e.g.: Falco tinnunculus, Buteo buteo, and Milvus migrans lineatus (Zhao et al., 2016). In addition to the ingestion of plastic particles inducing changes that are predictive of damage to the health of animals, the release of MPs through feces can greatly increase the dispersion power

of these pollutants in natural environments and, consequently, impact organisms from different trophic levels of the food chain.

5. CONCLUSIONS

In conclusion, our study confirms the initial hypothesis demonstrating that the ingestion of naturally-aged MPs induces biochemical changes in different organs of adult female *C. coturnix japonica*. In addition, we observed that the reduction in size and changes in the shape of the particles, along the digestive tract, contribute to the release (via feces) of large amounts of MPs (which demonstrates the potential of these birds to act as vectors for dispersal of MPs. in natural environments), in addition to providing opportunities for absorption, translocation via the hepatic portal system and consequent accumulation of these particles in the liver of birds. Obviously, our study is not exhaustive, and our data represent only the "tip of an iceberg" that represents the impacts that MPs can have on avifauna. Therefore, it is recommended that similar studies be carried out, expanding the representation of small-sized terrestrial birds, based on the evaluation of other animal models, as well as evaluating other biomarkers of toxicity (e.g.: histopathological, molecular, mutagenic, genotoxic, reproductive) and the potential mechanisms of action (direct or indirect) of MPs. The development of studies such as ours constitutes a unique opportunity to support the planning of strategies to mitigate the impacts of MPs on small-sized terrestrial birds, as well as the conservation of their species.

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MATERIAL SUPPLEMENTARY

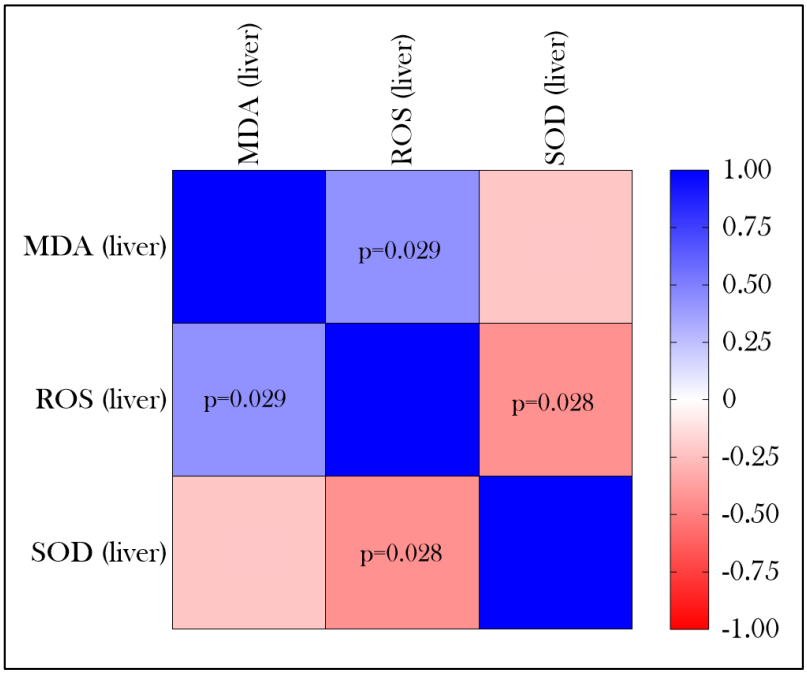

Figure S1. Heat map of correlations between biochemical parameters evaluated in the liver of an adult female Japanese quail (*Coturnix coturnix japonica*). MDA: malondialdehyde; SOD: superoxide dismutase; ROS: reactive oxygen species.

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