



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

EXPOSURE OF ADULT ZEBRAFISH (*Danio rerio*) TO SARS-CoV-2 AT PREDICTED ENVIRONMENTALLY RELEVANT CONCENTRATIONS: OUTSPREADING WARNS ABOUT ECOTOXICOLOGICAL RISKS TO FRESHWATER FISH

THIARLEN MARINHO DA LUZ

Orientador(a): Prof. Dr. Guilherme Malafaia

Urutaí, março de 2023



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THIARLEN MARINHO DA LUZ

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WARNS ABOUT ECOTOXICOLOGICAL RISKS TO
FRESHWATER FISH**

Orientador(a)

Prof. Dr. Guilherme Malafaia

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Aos trinta dias do mês de março do ano de dois mil e vinte e três, 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 **Thiarlen Marinho da Luz**, discente do **Programa de Pós-Graduação em Conservação de Recursos Naturais do Cerrado do Instituto Federal Goiano - Campus Urutaí**, com trabalho intitulado "**Exposure of adult zebrafish (*Danio rerio*) to SARS-CoV-2: outspreading warns about ecotoxicological risks to freshwater fish**". A sessão foi aberta pelo presidente da banca examinadora, **Prof. Dr. Guilherme Malafaia Pinto**, que fez a apresentação formal dos membros da banca. A palavra, a seguir, foi concedida ao autor da dissertação para, em 40 minutos, proceder à apresentação de seu trabalho. Terminada a apresentação, cada membro da banca arguiu a mestranda, 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 **APROVADO**, 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 do artigo científico oriundo dessa dissertação em periódico 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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“Quem lhe deu a verdade absoluta? Não há nada absoluto. Tudo se transforma, tudo se move, tudo revoluciona, tudo voa e vai.”

(Frida Kahlo)

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SUMÁRIO

LISTA DE FIGURAS	10
RESUMO	11
ABSTRACT	12
1. INTRODUCTION	13
2. MATERIAL AND METHODS	15
2.1. SARS-CoV-2.....	15
2.2. Animals and experimental design.....	15
2.3. Toxicity biomarkers.....	16
2.3.1. Behavioral biomarkers	17
2.3.1.1. Locomotion and anxiety-like behavior	17
2.3.1.2. Habituation learning.....	17
2.3.1.3. Social aggregation test in response to a potential aquatic predator.....	17
2.3.2. Biochemical biomarkers.....	18
2.3.2.1. Sample preparation.....	18
2.3.2.2 Redox imbalance biomarkers.....	19
2.3.2.3. Pro- and anti-inflammatory responses biomarkers.....	19
2.3.2.4. Determination of the total protein level.....	20
2.3.3. Mutagenicity biomarkers.....	20
2.4. Data analysis.....	21
3. RESULTS	21
4. DISCUSSION	29
5. CONCLUSION	33
6. ACKNOWLEDGMENTS	34
7. ETHICAL ASPECTS	34
9. REFERENCES	34
ANEXO I	44
ANEXO II	45

LISTA DE FIGURAS, QUADROS E TABELAS

Figura 1- (A-B) Total crossings at different times of the open field test of adult zebrafish (<i>Danio rerio</i>)	22
Figura 2- Cluster index of shoals of zebrafish (<i>Danio rerio</i>)	23
Figura 3- (A-B) Reactive oxygen species (ROS) production, (C-D) hydrogen peroxide levels (H ₂ O ₂), (E-F) superoxide dismutase, and (G-H) catalase activity	24
Figura 4- Acetylcholinesterase activity in adult zebrafish (<i>Danio rerio</i>)	25
Figura 5- (A-B) Nitrite production, (C) interleukin 10 (IL-10), and (D) interferon γ (IFN- γ) levels in adult zebrafish (<i>Danio rerio</i>).	26
Figura 6- (A-B) Total nuclear abnormalities in erythrocytes and (C) frequency of different types of abnormalities evidenced in adult zebrafish (<i>Danio rerio</i>).	27
Figura 7- Loadings plot of the investigated variables, (B) PC score plot, and (C) PCA biplot of the first two principal components (PCs)	28
Figura 8- Integrated Biomarker Response” index (IBRv2) calculated for groups composed of adult zebrafish (<i>Danio rerio</i>)	29

EXPOSIÇÃO DE ADUTOS DE ZEBRAFISH (*Danio rerio*) AO SARS-CoV-2 EM CONCENTRAÇÕES AMBIENTALMENTE RELEVANTES: DIVULGAÇÃO DOS RISCOS ECOTOXICOLÓGICOS PARA PEIXES DE ÁGUA DOCE

RESUMO

Embora os impactos sociais, econômicos e de saúde pública multifacetados associados à pandemia de COVID-19 sejam conhecidos, pouco se sabe sobre seus efeitos em ecossistemas e organismos aquáticos não-alvo. Assim, objetivamos avaliar a potencial ecotoxicidade da proteína lisada SARS-CoV-2 (SARS.CoV2/SP02.2020.HIAE.Br) em adultos de zebra fish (*Danio rerio*) em concentrações ambientalmente relevantes previstas (0,742 e 2,226 pg/L), durante 30 dias. Embora nossos dados não mostrem alterações locomotoras ou comportamento do tipo ansioso e/ou ansiolítico, notamos que a exposição ao SARS-CoV-2 afetou negativamente a memória de habituação e a agregação social dos animais em resposta a um potencial predador aquático (*Geophagus brasiliensis*). Uma frequência aumentada de anormalidades nucleares nos eritrócitos também foi observada em animais expostos ao SARS-CoV-2. Além disso, nossos dados sugerem que tais alterações foram associadas a um desequilíbrio redox (\uparrow ROS, \uparrow H₂O₂, \downarrow SOD e \downarrow CAT), efeito colinesterásico (atividade \uparrow AChE), bem como a indução de uma resposta imune inflamatória (\uparrow NO, \uparrow IFN-gama e \downarrow IL-10). Para alguns biomarcadores, notamos que a resposta dos animais aos tratamentos não era dependente da concentração. No entanto, a análise de componentes principais (PCA) e o índice “Integrated Biomarker Response” (IBRv2) indicaram uma ecotoxicidade mais proeminente do SARS-CoV-2 em 2.226 pg/L. Portanto, nosso estudo avança no conhecimento sobre o potencial ecotoxicológico do SARS-CoV-2 e reforça a presunção de que a pandemia de COVID-19 tem implicações negativas além de seus impactos econômicos, sociais e de saúde pública.

Palavras-chave: ecotoxicidade, novo coronavírus, *Danio rerio*, biomarcadores, toxicologia ambiental

EXPOSURE OF ADULT ZEBRAFISH (*Danio rerio*) TO SARS-CoV-2 AT PREDICTED ENVIRONMENTALLY RELEVANT CONCENTRATIONS: OUTSPREADING WARNS ABOUT ECOTOXICOLOGICAL RISKS TO FRESHWATER FISH

ABSTRACT

While the multifaceted social, economic, and public health impacts associated with the COVID-19 pandemic are known, little is known about its effects on non-target aquatic ecosystems and organisms. Thus, we aimed to evaluate the potential ecotoxicity of SARS-CoV-2 lysate protein (SARS.CoV2/SP02.2020.HIAE.Br) in adult zebrafish (*Danio rerio*) at predicted environmentally relevant concentrations (0.742 and 2.226 pg/L), by 30 days. Although our data did not show locomotor alterations or anxiety-like or/and anxiolytic-like behavior, we noticed that exposure to SARS-CoV-2 negatively affected habituation memory and social aggregation of animals in response to a potential aquatic predator (*Geophagus brasiliensis*). An increased frequency of erythrocyte nuclear abnormalities was also observed in animals exposed to SARS-CoV-2. Furthermore, our data suggest that such changes were associated with a redox imbalance (↑ROS, ↑H₂O₂, ↓SOD, and ↓CAT), cholinesterasic effect (↑AChE activity), as well as the induction of an inflammatory immune response (↑NO, ↑IFN-gamma, and ↓IL-10). For some biomarkers, we noticed that the response of the animals to the treatments was not concentration-dependent. However, principal component analysis (PCA) and the “Integrated Biomarker Response” index (IBRv2) indicated a more prominent ecotoxicity of SARS-CoV-2 at 2,226 pg/L. Therefore, our study advances knowledge about the ecotoxicological potential of SARS-CoV-2 and reinforces the presumption that the COVID-19 pandemic has negative implications beyond its economic, social, and public health impacts.

Keywords: ecotoxicity, new coronavirus, *Danio rerio*, biomarkers, environmental toxicology.

1. INTRODUCTION

Since the World Health Organization (WHO) has declared the novel coronavirus (COVID-19) outbreak a global pandemic (Cucinotta & Vanelli, 2020), society in general, governments, and institutions linked to public health have registered dramatic numbers of cases and deaths caused by SARS-CoV-2. Recent data from the WHO (as of 28 February 2023) confirm that more than 755 million people have been infected with the novel coronavirus, and 6,859,093 people have died (WHO, 2023). Therefore, these alarming numbers, associated with the social and economic losses observed in several countries because of COVID-19, have motivated scientists worldwide to develop vaccines and therapeutic options to control the disease. However, the ecotoxicological impacts on wild biota associated with the COVID-19 pandemic have yet to receive equal attention (Charlie-Silva & Malafaia, 2022). Although recent reviews suggest that the mismanagement of personal protective equipment during the COVID-19 pandemic, along with the increased use of single-use plastic grocery bags and another food packing, is resulting in widespread environmental pollution (Andeobu et al. al., 2022; Kitz et al., 2022; Hassan et al., 2022; Gautam et al., 2022; Amuah et al., 2022; Hashemi et al., 2023; Miah et al., 2023), few studies have evaluated the potential effects of SARS-CoV-2 on non-target organisms of infection.

Does the presence of SARS-CoV-2 in aquatic environments threaten biota? It is not assumed that the contact of aquatic wild animals with SARS-CoV-2 induces an infectious picture like the acute respiratory syndrome observed in humans, especially considering the differences in several physiological aspects between humans and non-human animals. However, the interaction between SARS-CoV-2 structures and non-target organisms can, by itself, elicit negative biological responses, and our knowledge is restricted to a few studies which focused on the toxicity of only a few small peptide fragments of the new coronavirus. Recently, it was demonstrated that *Physalaemus cuvieri* exposed (for 24 h) to SARS-CoV-2 peptides (named PSPD-2001, PSPD-2002, and PSPD-2003) showed redox imbalance and cholinesterasic alterations (measured by acetylcholinesterase activity) (Charlie-Silva et al., 2021). Later, locomotor and olfactory alterations were reported in *Culex quinquefasciatus* larvae exposed to PSPD-2002 and PSPD-2003 peptides, which were associated with an increase in the production of reactive oxygen species (ROS) and a cholinesterasic effect (Mendonça-Gomes et al., 2021). Furthermore, redox imbalance, behavioral changes, impaired growth/development (Malafaia et al., 2022a), genomic instability, and DNA damage (Gonçalves et al., 2022) in *Poecilia reticulata* exposed to peptides also show, as well as histopathologic changes (Fernandes et al., 2022) and olfactory dysfunction in *Danio rerio* adults

(Kraus et al., 2022). Furthermore, recent studies by Freitas et al. (2022) and Freitas et al. (2023), when evaluating the possible combined effect of different SARS-CoV-2 peptides with a mix of pollutants, reported negative impacts on the health of mayfly larvae (*Cloeon dipterum*) and zebrafish, respectively.

These studies represent preliminary and incipient findings on the potential effects of SARS-CoV-2 on non-target organisms but undoubtedly reinforce the (eco)toxicological potential of peptide fragments of SARS-CoV-2 in biota. However, it is possible that the interaction of aquatic animals with SARS-CoV-2 triggers more severe effects than those induced by some of its few small protein fragments. To date, the potential impacts of exposure aquatic organisms to SARS-CoV-2, per se, are entirely unknown. On an individual level, exposure of non-target organisms to SARS-CoV-2 can compromise their growth, development, and reproduction and even kill them. On a more holistic level, depending on the scope of the effects observed in individuals, SARS-CoV-2 can dramatically impact the dynamics and distribution of natural populations of organisms not targeted by infection. Therefore, assessing whether the spread of SARS-CoV-2 affects wild aquatic biota is an imminent need for greater attention from scientists and environmental agencies worldwide.

The input of viruses to aquatic environments via domestic/hospital sewage containing feces and urine from infected patients has been reported for SARS-CoV-2 and other related viruses [see review by La-Rosa et al. (2020)] and is a current reality in several countries. In the study by Rimoldi et al. (2020), for example, the authors detected SARS-CoV-2 viral RNA in downstream rivers of the Milan metropolitan region (Italy) during the peak of the COVID-19 disease, the results of which were directly associated with untreated wastewater discharges, or sewage overflows. Similarly, in Brazil, Fongaro et al. (2021) detected SARS-CoV-2 RNA in samples from rivers from different regions of the State of Minas Gerais (Brazil), as well as other similar studies in different freshwater environments from different countries (Rocha et al., 2022; Zhang et al., 2022; Zarza et al., 2022; Maidana-Kulesza et al., 2022; Schilman et al., 2023; Sabzchi-Dehkharghani et al., 2023; Amin et al., 2023). Therefore, aquatic organisms, especially those living in environments receiving domestic and hospital sewage, encounter SARS-CoV-2. Furthermore, considering that SARS-CoV-2 can persist in aquatic environments even after the end of the COVID-19 pandemic (Yang et al., 2022), the dramatic scenario of the possible impact caused to these aquatic organisms seems to be even more “dark”.

Thus, using adults of *D. rerio* (zebrafish) – regarded as one of the most used fish species in the world for aquatic ecotoxicology studies (Verma et al., 2021) – we aimed to evaluate the possible effects induced by exposure to SARS-CoV-2 lysate protein at predicted environmentally

relevant concentrations. More specifically, we assessed whether exposure for 30 days could cause behavioral changes, biochemical alterations, mutagenic effect, and induction of an inflammatory immune response. To the best of our knowledge, our study is a pioneer in this area and therefore advances knowledge about the magnitude of the ecological impact caused by the COVID-19 pandemic.

2. MATERIAL AND METHODS

2.1. SARS-CoV-2

In this study, we used the inactivated form of SARS-CoV-2 (lysate SARS.CoV2/SP02.2020.HIAE.Br), whose procedures for obtaining, isolating, and viral inactivation are detailed in Araújo et al. (2020a). Briefly, the isolated virus was recovered from nasopharyngeal swab samples obtained from symptomatic patients who had acquired COVID-19 during travels to the northwest of Italy (Lombardia region) and returned to São Paulo city in late February (2020). After that, the virus was propagated in Vero cells (E6, CCL-81, and hSLAM), with clear cytopathic effects, having been characterized by whole genome sequencing, electron microscopy, and *in vitro* replication properties. The viral stock solution comprised a supernatant of cultured Vero E6 in Dulbecco minimal essential medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) diluted 1:5 in NucliSENS® Lysis Buffer.

2.2. Animals and experimental design

This study was conducted at the Laboratory of Toxicology Applied to the Environment of the Goiano Federal Institute - Urutaí Campus Urutaí (GO, Brazil), using mixed-sex adults zebrafish (*D. rerio* - wild strain) [age: 6-8 months, body weight: 0.65 ± 0.04 g (mean \pm SEM); total length: 3.08 ± 0.22 cm (media \pm SEM)]. A commercial farm provided fish specimens (Goiânia, GO, Brazil) and, upon arrival at the laboratory, were acclimatized for 20 days. During the acclimatization period, the animals were kept collectively in aquariums (dimensions: 85 cm long x 40 cm wide x 40 cm high) filled with 120 L of naturally dechlorinated water, with constant aeration, light/dark photoperiod of 12 hours:12h and temperature of $26 \pm 1^\circ\text{C}$. The animals were fed twice a day, with commercial fish feed, corresponding to $\approx 2.5\%$ of their live body biomass, according to Lawrence et al. (2012). Aquarium waters were wholly replaced every three days.

After the acclimatization period, 60 healthy zebrafish (male: female ratio 1:1) (i.e., presenting normal swimming behavior and no morphological deformities or apparent lesions) of similar size and body weight were distributed into three experimental groups [each group was composed of four replicates (n=5 animals/replica)]. In the “SARS-I” and “SARS-II” groups,

SARS-CoV-2 lysate was added to the exposure water at 0.742 and 2.226 SARS-CoV-2 lysate protein pg/L, respectively. The “control” group consisted of zebrafish kept in dechlorinated water free of lysate SARS.CoV2/SP02.2020.HIAE.Br. However, this group received the same volume of supernatant of uninfected cultured Vero E6 in DMEM+10% FBS diluted 1:5 in NucliSENS® Lysis Buffer.

To define the SARS-CoV-2 lysate concentrations, we initially measured the total protein content in the viral stock solution using the biuret method (Gornall et al., 1949), using a commercial kit (Biotécnica Ind. Com. Ltda, Varginha, MG, Brazil, PCode: 90.019.00). Furthermore, the total proteins level of the supernatant of uninfected Vero cells incubated in DMEM+10% FBS was measured to determine the levels of viral proteins only. The confirmation of positivity for SARS-CoV-2 of the viral stock solution was performed by chromatographic immunoassay for the qualitative detection of the nucleocapsid protein of the new coronavirus, using a commercial kit (Wondfo Biotech Co. Ltd, Hefei, Anhui Province, China, PCode: W196P0005).

Next, we based on the study by Tampe et al. (2021), in which the maximum SARS-CoV-2 nucleocapsid protein detected in the urine of patients with acute kidney injury COVID-19 was 1.484 pg/mL. From this, considering that approximately only 1% of wastewater is composed of urine (EAWAG, 2008) and assuming a 5% dilution of sewage at a point close to disposal in a watercourse, we arrive at a total concentration of 0.742 pg protein SARS-CoV-2/L for “SARS-I” group. Such concentration is considered predictive and environmentally relevant, having been determined based on the total protein content of the virus, because the studies to date mostly express the concentrations of SARS-CoV-2 in aquatic environments from equivalent viral RNA (genome copies/mL). In the “SARS-II” group, the concentration added to the exposure water was 2.226 pg SARS-CoV-2 lysate protein/L, i.e., three times higher than that defined for the “SARS-I” group, to simulate a pessimistic scenario of high discharge of domestic or hospital sewage into an aquatic environment and therefore a more significant input of SARS-CoV-2. The experimental groups were exposed to the previously described conditions in glass aquariums [containing 1.8 L of dechlorinated water with (“SARS-I” and “SARS-II” groups) or without the SARS-CoV-2 lysate protein (“control” group)]. The animals remained exposed to the SARS-CoV-2 for 30 days in a semi-static condition, i.e., with the complete renewal of the exposure water every 72 h.

2.3. Toxicity biomarkers

2.3.1. Behavioral biomarkers

2.3.1.1. Locomotion and anxiety-like behavior

To evaluate the possible effect of exposure to SARS-CoV-2 lysate protein on the behavior of the animals, at the end of the 30 days of exposure, ten animals from each group were submitted to the open field test (OFT), according to Chagas et al. (2021), with some modifications. Briefly, the test consisted of introducing each animal individually into a rectangular arena with opaque walls (dimensions: 40.5 cm long, 31 cm wide x 15 cm high) containing 5 L dechlorinated water without any pollutant (at $26 \pm 1^\circ\text{C}$).

The behavior of each animal was filmed for 5 min from a camera (coupled to a computer external to the test room) installed 1.5 m above the arena. The test room had acoustic insulation, luminosity, and temperature ($26 \pm 1^\circ\text{C}$) controlled. From the footage, the time the animal spent in proximity to the wall of the experimental arena was quantified and used to calculate the anxiety index (Equation 1), aiming to evaluate the possible SARS-CoV-2 lysate protein-induced anxiogenic or anxiolytic effects. The total number of quadrants ($26.15 \text{ cm}^2/\text{each}$) crossings virtually traced on the computer screen was recorded as locomotor/exploratory activity of the animals. PlusMZ software was used for behavior recording, like Freitas et al. (2023).

$$\text{Anxiety index} = \left[\frac{\left(\frac{\text{Time spent peripheral zone (s)}}{\text{Total test time (300s)}} \right) \times 100}{\text{Total crossing}} \right] \times 100 \quad \text{Eq. 1}$$

2.3.1.2. Habituation learning

To evaluate the possible influence of SARS-CoV-2 on “habituation learning”, we adopted the procedures described in Malafaia et al. (2022a). Habituation learning is one of the forms of non-associative learning, characterized by the reduction of an animal's behavioral response during a behavioral test (Ahmad and Richardson, 2013). Thus, we recorded the locomotor activity of the animals during each minute of the OFT and compared the locomotor activity recorded in the first block of 1 min and the last block (5 min).

2.3.1.3. Social aggregation test in response to a potential aquatic predator

To investigate the possible influence of SARS-CoV-2 on the ability of zebrafish to recognize a potential predator, on the 31st experimental day, the animals were submitted to the social aggregation test, like Chagas et al. (2021) and Freitas et al. (2023), with some modifications. In this test, zebrafish were directly confronted with a predatory stimulus, represented by

Geophagus brasiliensis (Cichlidae family, popularly known as “*acaraá*”), like Araújo et al. (2020b). *G. brasiliensis* presents morphological similarity to the sympatric predator of zebrafish in natural environments [i.e., *Nandus nandus* – Gerlai et al. (2009)]. According to Bastos et al. (2011), *G. brasiliensis* is an omnivorous species with opportunistic eating habits, commonly preying on fry or small fish in natural environments. Briefly, the test started with the introduction of zebrafish (in a shoal of five animals/group) into a polypropylene box (dimensions: 40.5 cm long, 31 cm wide x 15 cm high) containing 7 L of dechlorinated water (free of treatments, temperature: 26±1°C) and filming them for 5 min (habituation session).

After that, a species of *G. brasiliensis* was introduced into the box, and the animals were filmed for another 5 min (test session). To avoid biases induced by differences between the biometry of potential predators in zebrafish behavior, the body weight and size of the potential predator were similar (total length: 14.90 ± 0.71 cm, body weight: 36.30 ± 1.33 g - mean ± SEM). In addition, potential predators (n=6) were introduced into the aquariums alternately between the replicas of the same group and between the experimental groups to reduce the influence of the behavior and personality of the predators on the behavior of the prey (i.e., zebrafish). It is also worth noting that the potential predators were captured in a permanent preservation area (Goiano Federal Institute – Urutaí Campus, GO, Brazil) and immediately brought to the laboratory, where they were acclimatized for 90 days, having received a daily diet consisting of specimens of zebrafish adults. Furthermore, we clarify that to avoid a possible predatory attack during the tests, the *G. brasiliensis* were fed moments before being used.

After the test, the videos were analyzed to record the social aggregation behavior against a potential predator. For this, we used a pre-established cluster scores system (Collins et al., 2011; Parker et al., 2014). After the demarcation of 30 quadrants (41.85 cm²/each) in virtual images displayed on the researcher's computer screen, the cluster scores are generated by dividing the maximum number of zebrafish positioned in a given quadrant by the number of quadrants occupied by all the animals (total). Scores were provided at 3 s intervals every 3 s in a 5-min (300 s) test, like Freitas et al. (2023).

2.3.2. Biochemical biomarkers

2.3.2.1. Sample preparation

After exposure, eight animals/group were macerated in 1 mL of phosphate-buffered saline (PBS) (pH 7.2), using cell and tissue disruptor (4000 rpm, 60 s, with two cycles of 20 s interval) (Loccus, L-Deader-6). Next, the homogenate was centrifuged at 13,000 rpm for 10 min at 4°C. After centrifugation, the supernatant was stored at -80°C until use. The whole bodies of the

animals were used in the analyses, considering the limited precision of the isolation of tissues/organs from small animals (Lusher et al., 2017) and the possible “contamination” of the organs by organic matter and/or other particles consumed by zebrafish (Guimarães et al., 2021). Both factors could be a bias in the biochemical analysis applied to the organs.

2.3.2.2. Redox imbalance biomarkers

Predicting some association between exposure to SARS-CoV-2 and the induction of a redox imbalance, the production of ROS, hydrogen peroxide (H_2O_2), and nitric oxide (NO) was evaluated well as the activity of superoxide dismutase and catalase. The ROS production was measured using dichlorofluorescein-diacetate (DCFH-DA) [according to Zhao et al. (2013)], and H_2O_2 levels were determined spectrophotometrically using the protocol proposed by Elnemma (2004). MDA levels were based on Esterbauer & Cheeseman (1990), with previous modifications (Freitas et al., 2022) and superoxide dismutase (SOD) and catalase (CAT) activities were assessed according to Deawati et al. (2017) and Hadwan and Abed (2016), respectively. The potential cholinesterasic effect was inferred by acetylcholinesterase (AChE) activity, which was evaluated according to Ellman's spectrophotometric method (Ellman et al., 1961).

2.3.2.3. Pro- and anti-inflammatory responses biomarkers

To assess the pro- and anti-inflammatory response of zebrafish exposed to SARS-CoV-2, the IL-10 and IFN- γ levels were evaluated. While IL-10 is an anti-inflammatory cytokine that acts as a suppressor and exerts a conserved role in dampening inflammatory responses, IFN- γ belongs to the family of cytokines that mediate immune and inflammatory responses (Zou & Secombes, 2016; Sakai et al., 2021). The levels of these cytokines were measured in the supernatant obtained from the maceration of 10 fish/group (see procedural description in section 2.3.2.1) using the enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer's instructions. The kits were purchased from PeproTech (São Paulo, Brazil). Furthermore, nitric oxide production was evaluated since its levels are associated with several pathophysiological conditions, such as inflammation (Leite et al., 2012). For this, we used the method proposed by Grisham et al. (1998) [modified by Araújo et al. (2022)], which is based on the Griess colorimetric reaction.

2.3.2.4. Determination of the total protein level

The results of all biochemical biomarkers were relativized with the level of total proteins of the analyzed samples, which was determined based on the Bradford (1976) method. This method is based on the electrostatic interaction between the dye (Coomassie Brilliant Blue), basic amino acids (arginine, lysine, histidine amino acid residues, and NH₂ - terminal amino group), and aromatic amino acids residues.

2.3.3. Mutagenicity biomarkers

The possible SARS-CoV-2-induced mutagenic effect was evaluated by recording the frequency of micronucleus and other nuclear abnormalities in the erythrocytes of zebrafish, according to procedures detailed in Gonçalves et al. (2022). In an optical microscope at 100 × magnification, 1000 cells/slide [according to Bolognesi & Hayashi (2011)] totaling 10000 erythrocytes/group were analyzed (n=10 animals/group) based on the criteria reported by Fenech (2007). In addition, the presence of micronuclei and other erythrocyte nuclear abnormalities were recorded, according to the nomenclatures adopted by Braham et al. (2017).

2.4. Data analysis

The comparison of the means of the results obtained between the experimental groups was performed using the one-way ANOVA test (with Tukey post-test) or Kruskal-Wallis test (with Dunn's post-test), defined based on the distribution of residual data (assessed using the Shapiro-Wilk test) and homogeneity of variances (assessed using the Bartlett test). Furthermore, correlation analyses and linear regression were performed. In the social aggregation test in response to a potential aquatic predator, we used the non-parametric Mann-Whitney U test (a paired test) to compare the cluster index of the experimental groups in the sessions with and without the predatory stimulus because the animals in the first and second sessions were the same. Significance levels were set at Type I error (p) values lower than 0.05, and GraphPad Prism software Version 9.0 was used to perform the statistical analyzes.

Biomarker data were also explored based on principal component analysis (PCA) using GraphPad Prism software Version 9.0, like the procedures described in Freitas et al. (2023). Such an analysis was essential to assess whether certain combinations of key variables account for differences between, in our case, experimental groups. In all PCA analyses in this work, the outliers' values (identified via the Grubbs test) were excluded from the original data and sequentially logarithmized before PCA analysis. Furthermore, the results of all biomarkers were applied to the second-generation "Integrated Biomarker Response" index (IBRv2) to transform

the different responses of animals to treatments from the different biomarkers evaluated into a single value, allowing them to analyze their sensitivities to each treatment comparatively. For this, we used the method adopted by Malafaia et al. (2022b), based on Sanchez et al. (2013).

3. RESULTS

Initially, no animal deaths were observed throughout the experimental period in the different experimental groups, which confirms that exposure to SARS-CoV-2 did not induce a lethal effect on zebrafish. Furthermore, the locomotor activity of animals exposed to SARS-CoV-2 did not differ from that recorded in unexposed zebrafish (Figure S1A), and the anxiety indices did not differ between experimental groups (Figure S1B). In Figure 1A, it is possible to notice a decrease in locomotion throughout the OFT in the animals of all groups, following a simple linear regression model), with the slope of the straight lines of each group not statistically different (F-value = 2.349; p-value = 0.1510). However, the locomotor activity of the animals of the “SARS-II” group in the 5th last minute of the OFT did not differ from the activity recorded in the 1st min (Figure 1B), which suggests that exposure to the highest concentration of SARS-CoV-2 influenced the “habituation learning” of these animals. Furthermore, we evidenced that exposure to SARS-CoV-2 induced a different reaction to a potential predator. In the “control” group, the cluster score in the social aggregation test increased when *G. brasiliensis* was introduced into the aquarium, suggesting greater shoal cohesion and responsiveness to potential predators. On the other hand, in zebrafish exposed to SARS-CoV-2, the cluster scores recorded in the presence or absence of predatory stimuli did not differ, denoting the inability of these animals to respond to the predator (Figure 2).

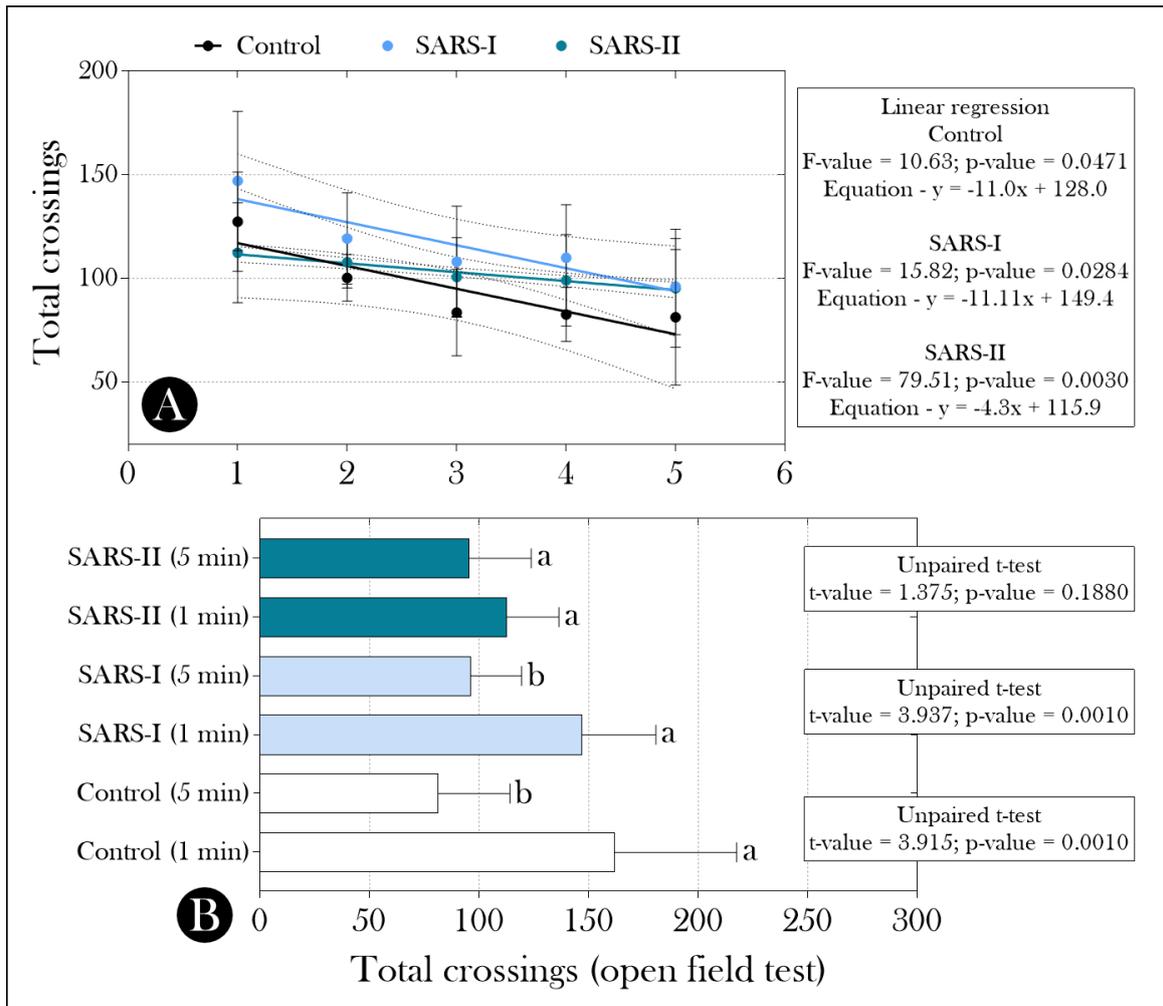


Figure 1. (A-B) Total crossings at different times of the open field test of adult zebrafish (*Danio rerio*) unexposed or exposed to SARS-CoV-2 at different concentrations. In “A”, the correlation and linear regression analyses' statistical summaries are presented next to the graph. In “B”, non-parametric data are displayed by the median and interquartile range, and statistical summaries are shown alongside the graph. Distinct lowercase letters indicate significant differences. SARS-I and SARS-II: composite groups of zebrafish exposed to SARS-CoV-2 lysate protein at 0.742 and 2.226 pg/L, respectively.

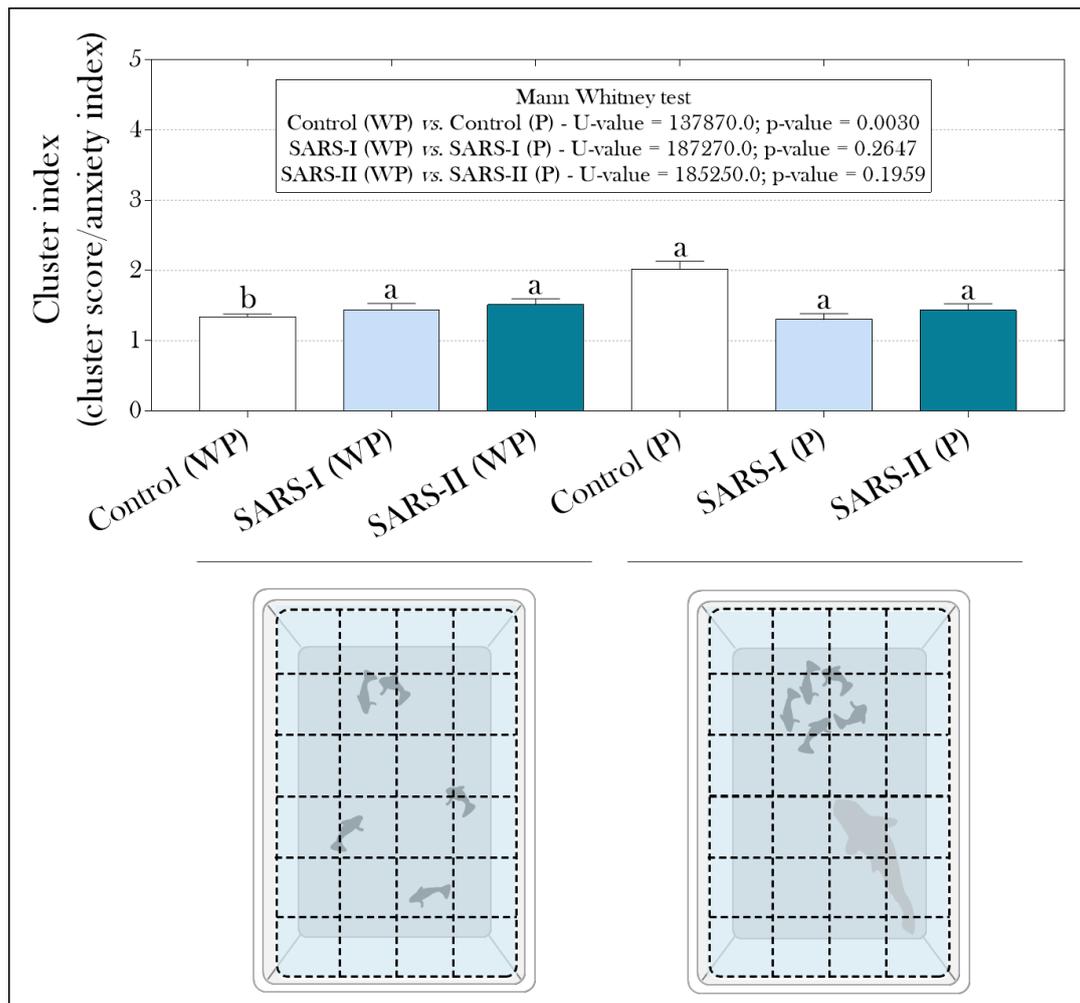


Figure 2. Cluster index of shoals of zebrafish (*Danio rerio*) unexposed or exposed to SARS-CoV-2 at different concentrations in the social aggregation test in response to a potential aquatic predator (*Geophagus brasiliensis*). Non-parametric data are displayed by the median and interquartile range, and statistical summaries are displayed at the top of the graph. Distinct lowercase letters indicate significant differences. SARS-I and SARS-II: composite groups of zebrafish exposed to SARS-CoV-2 lysate protein at 0.742 and 2.226 pg/L, respectively.

Our data also demonstrated that exposure to SARS-CoV-2 induced a concentration-dependent redox imbalance, marked by increased ROS and H_2O_2 production and reduced SOD and CAT activity in animals from the “SARS-II” group (Figure 3). Similarly, we observed a SARS-CoV-2-induced cholinesterasic effect (Figure 4) and nitrosative stress (Figure 5A) also concentration-dependent, with exposure to SARS-CoV-2 at a higher concentration (i.e., 2.226 pg/L) a most prominent influence. On the other hand, in the “SARS-I” and “SARS-II” groups, we observed that exposure to SARS-CoV-2 induced a similar inflammatory immune response, marked by suppression of IL-10 (Figure 5B) and an increase in IFN- γ levels (Figure 5C).

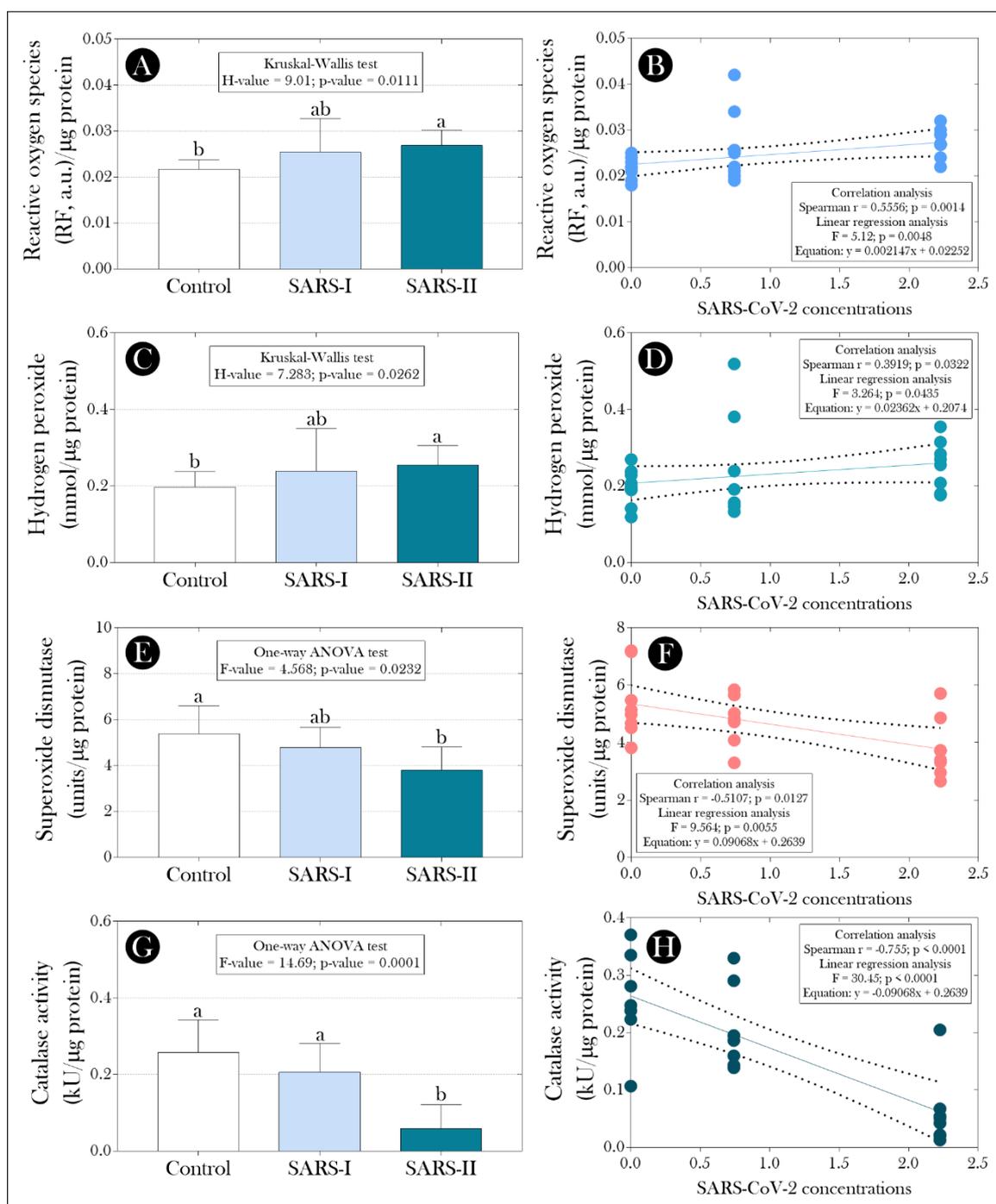


Figure 3. (A-B) Reactive oxygen species (ROS) production, (C-D) hydrogen peroxide levels (H_2O_2), (E-F) superoxide dismutase, and (G-H) catalase activity in adult zebrafish (*Danio rerio*) unexposed or exposed to SARS-CoV-2 at different concentrations. In “A”, “C”, “E”, and “G”, parametric data are presented by the mean + standard deviation, whereas non-parametric data are presented by the median and interquartile range. Distinct lowercase letters indicate significant differences. Statistical summaries are shown next to the graphs. SARS-I and SARS-II

II: composite groups of zebrafish exposed to SARS-CoV-2 lysate protein at 0.742 and 2.226 pg/L, respectively.

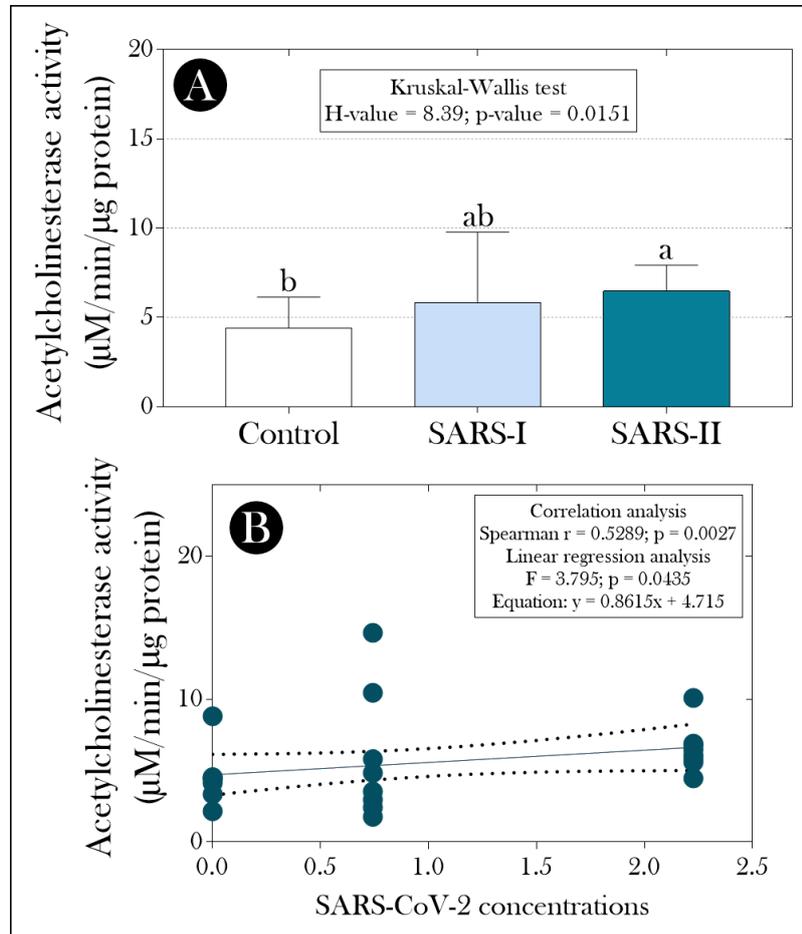


Figure 4. (A-B) Acetylcholinesterase activity in adult zebrafish (*Danio rerio*) unexposed or exposed to SARS-CoV-2 at different concentrations. In “A”, non-parametric data are presented by the median and interquartile range. Distinct lowercase letters indicate significant differences. Statistical summaries are shown next to the graphs. SARS-I and SARS-II: composite groups of zebrafish exposed to SARS-CoV-2 lysate protein at 0.742 and 2.226 pg/L, respectively.

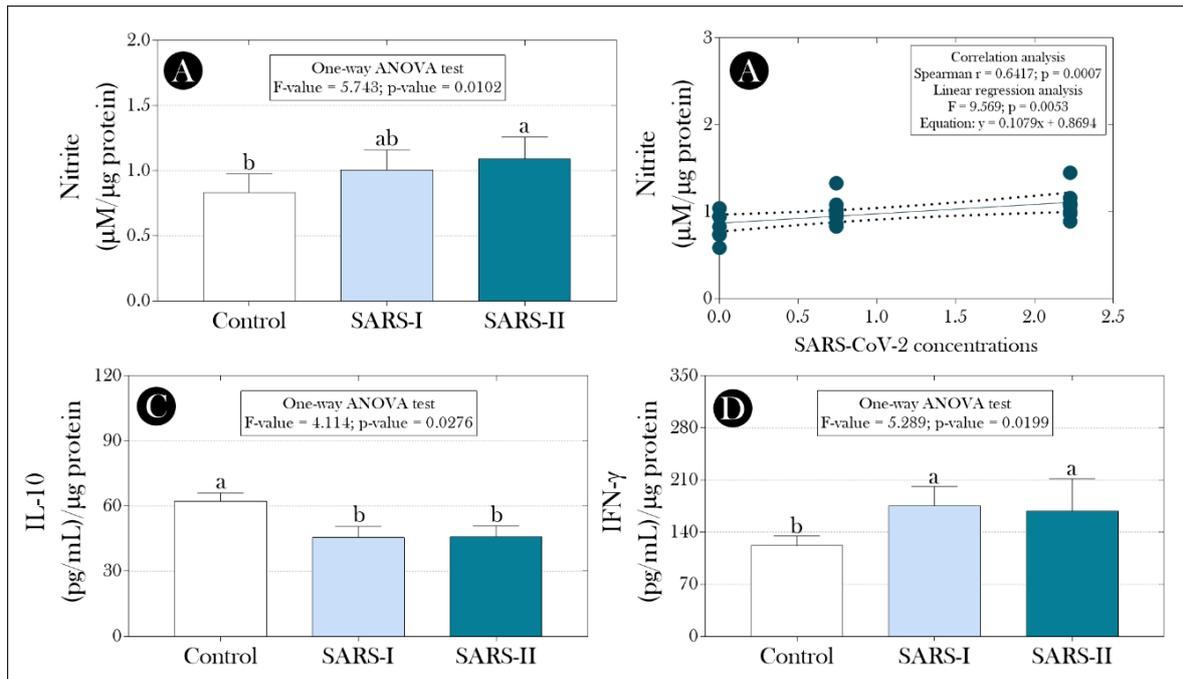


Figure 5. (A-B) Nitrite production, (C) interleukin 10 (IL-10), and (D) interferon γ (IFN- γ) levels in adult zebrafish (*Danio rerio*) unexposed or exposed to SARS-CoV-2 at different concentrations. In “A”, “C” and “D”, parametric data are presented by the mean + standard deviation. Distinct lowercase letters indicate significant differences. Statistical summaries are shown next to the graphs. SARS-I and SARS-II: composite groups of zebrafish exposed to SARS-CoV-2 lysate protein at 0.742 and 2.226 $\mu\text{g/L}$, respectively.

Regarding the micronucleus test results and other erythrocyte nuclear abnormalities, we also evidenced a SARS-CoV-2-induced mutagenic effect, as shown in Figure 6. Throughout the analyses, we recorded the presence of different types of abnormalities, including blebbed nuclei (Figure S2A), notched nuclei (Figure S2B), kidney-shaped nuclei (Figure S2C), nuclear constriction (Figure S2D), micronuclei (Figure S2E-F), and moved nuclei (Figure S2F-H), whose data pointed to a more prominent frequency in animals from the “SARS-II” group (Figure 6A-B).

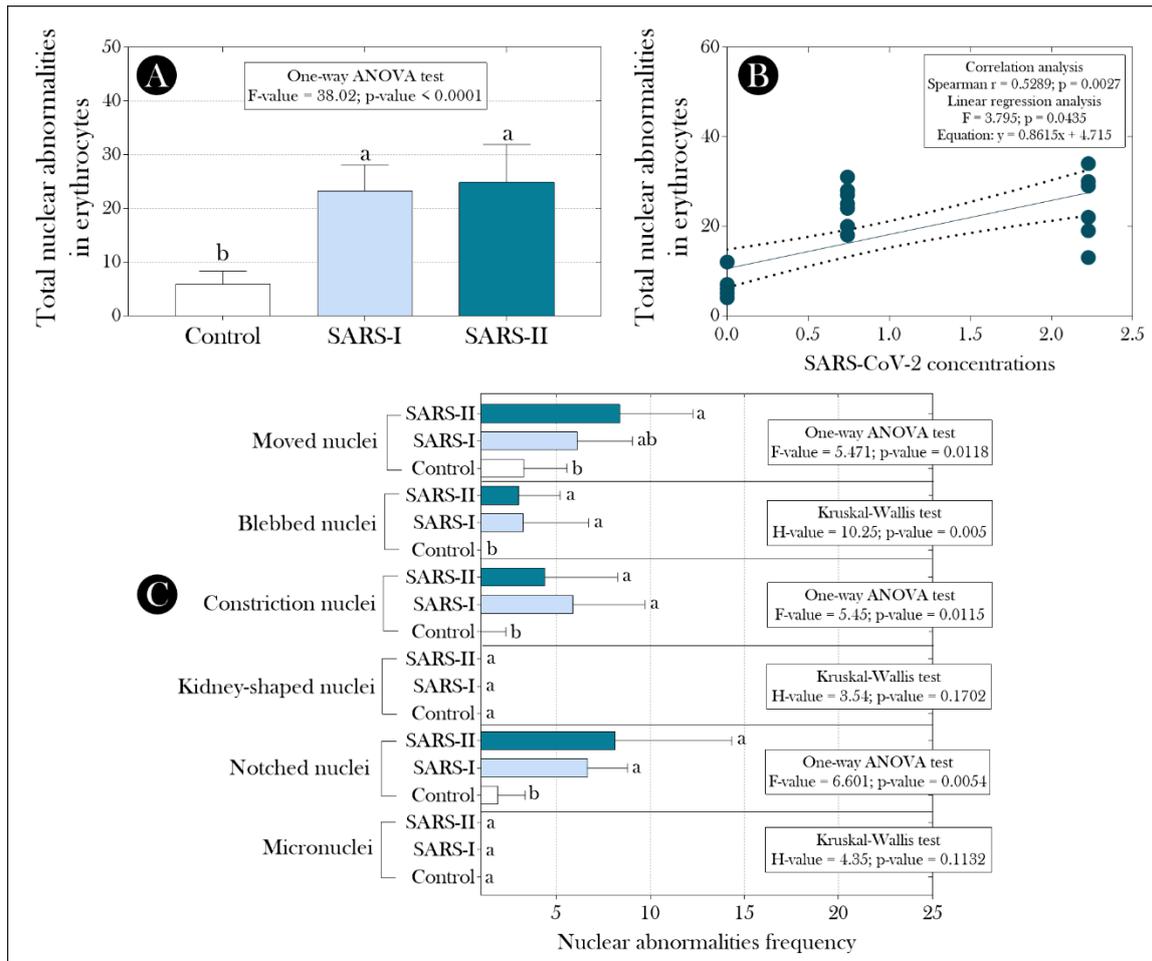


Figure 6. (A-B) Total nuclear abnormalities in erythrocytes and (C) frequency of different types of abnormalities evidenced in adult zebrafish (*Danio rerio*) unexposed or exposed to SARS-CoV-2 at different concentrations. In “A” and “C”, parametric data are presented by the mean + standard deviation, whereas non-parametric data are presented by the median and interquartile range. Distinct lowercase letters indicate significant differences. Statistical summaries are shown next to the graphs. SARS-I and SARS-II: composite groups of zebrafish exposed to SARS-CoV-2 lysate protein at 0.742 and 2.226 pg/L, respectively.

To evaluate the general response of the zebrafish, considering all the biomarkers being assessed, the data obtained in the “control”, “SARS-I”, and “SARS-II” groups were submitted to the PCA. As shown in Figure 7, the first two principal components cumulatively explained 100% of the total variation (PC1: 88.08% and PC2: 11.92%). The PC-eigenvalues were greater > than 2.0 (PC1: 15.854 and PC2: 2.145), and most of the analyzed variables were positively associated with PC1 and PC2 (Figure 7A and Table S1). Furthermore, the experimental groups were clearly separated into subgroups by PC2. While the “control” group occupied an intermediate position on the PC axis (PC2 score: -0.242), the “SARS” groups were positioned in opposite quadrants

on PC2. The “SARS-I” group was placed in the positive quadrant of PC2 (PC2 Score: 1.570), and the “SARS-II” group in the negative quadrant of the same PC (PC2 Score: -1.329) (Figure 7B-C). Therefore, this suggests a differentiated response of animals to SARS-CoV-2, which was confirmed by the different values reported by IBRV2 (Figure 8), demonstrating that exposure to SARS-CoV-2 lysate protein at 2,226 pg/L, taking the set of animal responses, induced greater toxicity. Through Figures 10B-C, it is possible to notice that this greater toxicity was determined especially by the antioxidant activity of the evaluated enzymes (SOD and CAT), by biomarkers of oxidative (ROS and H₂O₂) and nitrosative (NO) stress, by the cholinesterasic effect (AChE activity), as well as the frequency of moved nuclei, notched nuclei, and micronuclei.

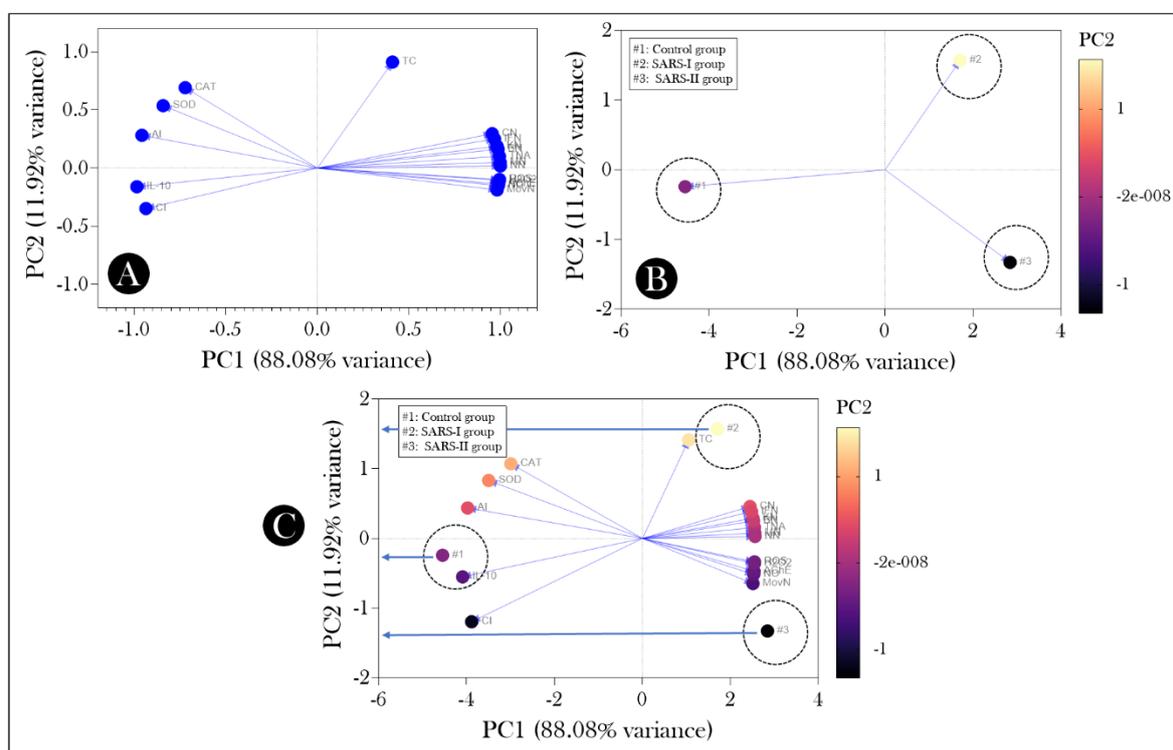


Figure 7. (A) Loadings plot of the investigated variables, (B) PC score plot, and (C) PCA biplot of the first two principal components (PCs) that simultaneously shows scores of experimental groups. See the meanings of the acronyms in Table S1. In “C”, the blue arrows point to the PC2 Scores of the experimental groups. SARS-I and SARS-II: composite groups of zebrafish exposed to SARS-CoV-2 lysate protein at 0,742 and 2.226 pg/L, respectively.

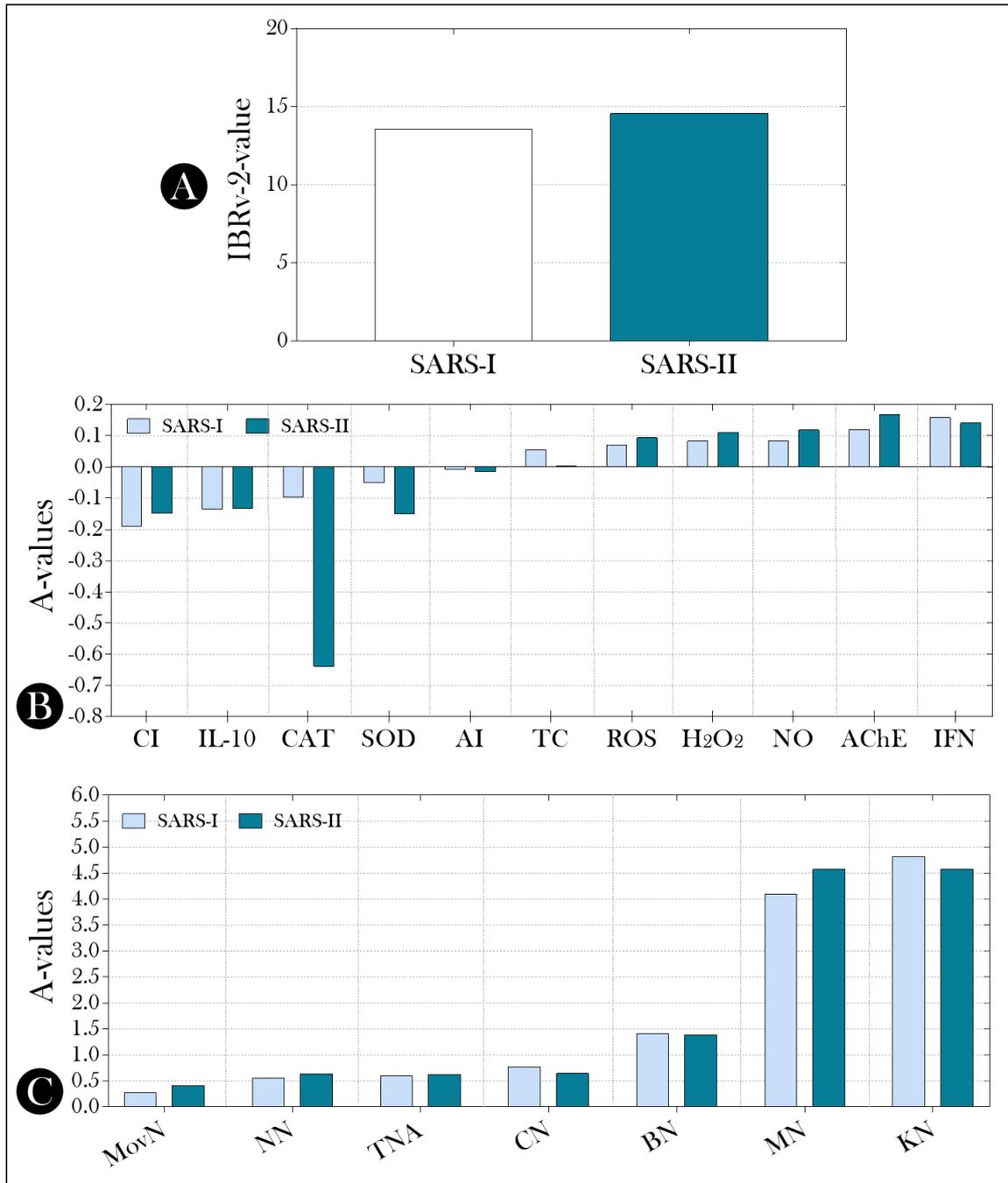


Figure 8. (A) “Integrated Biomarker Response” index (IBRv2) calculated for groups composed of adult zebrafish (*Danio rerio*) unexposed or exposed to SARS-CoV-2 at different concentrations. (B and C): A-values derived from the IBRv2 obtained for the respective groups exposed to the SARS-CoV-2. SARS-I and SARS-II: composite groups of zebrafish exposed to SARS-CoV-2 lysate protein at 0.742 and 2.226 pg/L, respectively.

4. DISCUSSION

It is known that studies to identify and characterize impacts caused by pollutants/contaminants on biota are steps that precede and guide the planning and adoption of

remediation or mitigation measures. As discussed by Mareddy et al. (2017), the absence of these studies makes it difficult to adopt early, efficient, and targeted actions or strategies to combat current pollution. In ecotoxicology, most studies have been dedicated to studying the potentially harmful effects of elements, substances, or chemical compounds on organisms, with the toxicity of non-chemical toxic agents evaluated in a few studies, with rare exceptions for mycotoxins and cyanotoxins (Bucheli et al., 2008; Falfushynska et al., 2023).

In this sense, our study advances knowledge in the area as it sheds light on the ecotoxicity of SARS-CoV-2, reinforcing the hypothesis that the impacts caused by the COVID-19 pandemic are not restricted to economic, social, and human health. Although we did not report behavioral changes predictive of locomotor alterations or the induction of anxiety-like behavior in zebrafish (Figures 1A-B), we observed that exposure to SARS-CoV-2 led to changes in the “habituation memory” of the animals. Despite the locomotion of zebrafish (of all groups) having reduced throughout the OFT (Figure 1A), in the “SARS-II” group, we did not observe differences in the locomotor activity evaluated in the 1st and 5th min of evaluation (Figure 1B), which suggests behavioral impairment. As discussed by different authors, this type of memory is one of the forms of non-associative learning, characterized by the diminution of exploratory activity as a function of repeated exposure to the same environment (Cerbone & Sadile, 1994; Gómez-Laplaza et al., 2010; Ahmad & Richardson, 2013). Therefore, alterations of this nature suggest a negative effect of SARS-CoV-2 on the exploratory abilities of animals that may affect their fitness or even their survival.

Furthermore, we noticed in the no social aggregation test in response to the potential aquatic predator that animals exposed to SARS-CoV-2 did not respond to *G. brasiliensis* similarly to unexposed zebrafish (Figure 2). The increase of the cluster index in the “control” group (in the presence of the predator) suggests a greater cohesion among the individuals of the shoal, interpreted as a typical antipredatory response, which was not observed in the zebrafish of the “SARS-I” in the “SARS- II” groups (Figure 2). Although few studies have evaluated the antipredatory response of shoals of fish in direct confrontation with a potential predator after exposure to pollutants [e.g., Chagas et al., 2021], Gerlai (2014) points out that the formation of shoaling benefits individuals in different ways. Descriptive investigations on zebrafish biology reveal that the formation of shoaling in *D. rerio* [reported both in the natural environment (Engeszer et al., 2007) and in the laboratory (Saverino & Gerlai, 2008; Buske & Gerlai, 2011)] constitutes one of the strongest consistent features of this species. In addition, it has been demonstrated that the formation of shoals also optimizes foraging, facilitates access to reproductive partners, and provides greater protection to individuals (Litvak, 1993; Dlugos &

Rabin, 2003; Gerlai, 2014). Furthermore, Krause & Ruxton (2002) point out that forming groups has been shown to reduce the risk of predation, either by confusing the predator (via movement of several individuals) (Landeau & Terborgh, 1986) or by increasing the efficiency of early detection of predators (Pitcher & Parrish, 1993). Therefore, our data suggest that exposure to SARS-CoV-2 negatively influenced the animals' response to the predatory stimulus provided by *G. brasiliensis*.

We also noticed in our study a mutagenic effect in animals exposed to SARS-CoV-2 (Figure 6) like those reported by Gonçalves et al. (2022). At the time, these authors showed that exposure of *P. reticulata* fish to the SARS-CoV-2 peptide PSPD-2002 for ten days (at 40 µg/L) induced an increase in the frequency of erythrocyte nuclear abnormalities, as well as DNA damage, which reinforces the potential of viral particles of the new coronavirus to cause genomic instability in freshwater fish. As discussed by Canedo et al. (2021), changes of this nature may have multifaceted consequences for the health of animals that, in association with other changes (e.g., behavioral), affect the survival, reproduction, or genetic heritage of animals. While the presence of micronuclei may indicate aneugenic (i.e., during the abnormal anaphase) or clastogenic effects (chromosome breakdown), the increased frequency of other nuclear abnormalities [e.g., blebbed, kidney-shaped, and notched nuclei (Figure S2)] have been associated with changes in the cell cycle and events that precede the formation of micronuclei (Shimizu et al., 2000; Lindberg et al., 2007; Kalsbeek & Golsteyn, 2017; Hintzsche et al., 2017), which can induce cell death, cause genomic instability and/or induce cancer development (Alimba & Bakare, 2016; Souza et al., 2017).

Obviously, the alterations reported in our study can be associated with several causes, including the biochemical responses of the animals to the treatments. In our study, it is possible that the redox imbalance (Figure 3), the cholinesterasic effect (Figure 4), nitrosative stress, and the inflammatory response (Figure 5) induced by exposure to SARS-CoV-2 were predominant. It is known that the increased production of reactive species affects different physiological mechanisms capable of eliciting altered behavioral responses. Habituation memory, social behavior, and antipredatory response in zebrafish are regulated by underlying neural networks that, under conditions of disturbances in biochemical homeostasis, can be potentially affected. Previous studies have already associated different types of behavioral changes in zebrafish with increased oxidative stress processes (Müller et al., 2017; Strungaru et al., 2018; Gonçalves et al., 2020; Attaran et al., 2020; Chagas et al., 2021; Salahinejad et al., 2021; Salahinejad et al., 2022), which reinforces our hypothesis. In addition, changes in AChE activity - a key enzyme in biological neurotransmission, which can degrade acetylcholine (ACh) in cholinergic synapses and

ensure the normal transmission of nerve signals in organisms (Dvir et al., 2010; Cavalcante et al., 2020) – have already been considered the cause of behavioral changes not only in zebrafish (Raja et al., 2019; Pullaguri et al., 2020; Rosales-Pérez et al., 2022) but also in other animal models (Almeida et al. al., 2010; Zugno et al., 2014; Bartos et al., 2022).

In recent studies by Charlie-Silva et al. (2021), Malafaia et al. (2022), and Mendonça-Gomes et al. (2021), the authors reported that exposure of *P. cuvieri* tadpole, *P. reticulata* fish, and *C. quinquefasciatus* larvae (respectively) to peptide fragments of SARS-CoV-2 induced an increase in AChE activity, which coincides with our findings. At the time, it was suggested that this increase could be one of the consequences of the increase in lipid peroxidation processes – with a consequent increase in the release of ACh from synaptic vesicles – of the direct interaction between the enzyme and the peptide fragments of SARS-CoV-2, or the release of ACh in the synaptic clefts via activation of the cholinergic anti-inflammatory pathway (CAP). Therefore, we cannot rule out the hypothesis that similar mechanisms induced by SARS-CoV-2 occurred in the zebrafish evaluated in our study. On the other hand, different results were evidenced when mice were exposed to peptide fragments PSPD-2002 from SARS-CoV-2. At the time, Luz et al. (2022) recorded a memory deficit in mice exposed to peptides, which was associated with cholinesterasic suppression, marked by a significant reduction in AChE activity in the brain of Swiss and C57Bl/6J mice. Therefore, these studies demonstrate that the behavioral/biochemical response of non-target organisms to SARS-CoV-2 is dependent on the species, as well as on the concentrations and routes of exposure, and this field should be more comprehensively explored in the future.

In biochemical terms, studies involving the exposure of non-target organisms to SARS-CoV-2 peptides have pointed to oxidative stress as one of the mechanisms responsible for the reported changes, whether in fish (Malafaia et al., 2022), amphibians (Charlie-Silva et al., 2021), insects (Mendonça-Gomes et al., 2021) or mammals (Luz et al., 2022). Charlie-Silva et al. (2021) and Malafaia et al. (2022), for example, proposed that the redox imbalance caused by the exposure of non-target organisms evaluated to SARS-CoV-2 (*P. cuvieri* tadpole and *P. reticulata*, respectively) is related to functional changes in the antioxidant activity induced by the interaction between viral fragments and important enzymes in the regulation of redox homeostasis. Therefore, it is possible that a similar phenomenon occurred in our study, especially considering the diversity of viral fragments to which zebrafish were exposed. On the other hand, our data also suggest that the redox imbalance caused in animals is associated with the induction of a predominant Th1-specific immune response in zebrafish exposed to SARS-CoV2. In animals from the “SARS-I” and “SARS-II” groups, we noticed an increase in IFN- γ levels (Figure 5D),

which coincided with the increase in NO production (Figure 5B) and with the suppression of IL-10 levels (Figure 5C), which can be explained by the functionality of each cytokine evaluated, as well as by the co-operative action of these “immunosuppressive” and “immunostimulatory” cytokines. This response would be related to several aspects of immunity, including stimulation of antigen presentation and activation of macrophages and T helper (Th) lymphocytes, among others, which should be better investigated in the future. However, the stimulatory effects of IFN- γ on fish monocytes/macrophages are well documented in teleost fish, with increased production of NO and ROS being a consequence of increased levels of this cytokine (Pereira et al., 2019; Gan et al., 2020), which reinforces our hypothesis. On the other hand, the suppressive action of IFN- γ on IL-10 expression (Hu et al., 2006) may explain the low levels of IL-10 in animals exposed to SARS-CoV-2. According to Yanagawa et al. (2009), the IL-10 is responsible for the limitation and eventual termination of inflammatory responses, which seems not to have occurred in animals exposed to SARS-CoV-2, considering that the animals' contact with the viral fragments was continuous during the exposure period.

Obviously, the mechanisms that explain the induction of a Th1 polarized immune response in fish analyzed in our study are still unknown, although we know that in humans, innate immunity mediated by pattern recognition receptors and the renin-angiotensin-aldosterone system (RAAS) has a central role in immunopathology involving SARS-CoV-2 infection (Laghi et al., 2022). However, it is necessary to consider that although zebrafish have an immune system like that of humans (Bailone et al., 2020), including orthologs of classic cytokines (Zou & Secombes, 2016), some peculiarities prevent us from proposing the exact mechanisms described in humans. Ladies et al. (2020) point out, for example, that although the zebrafish genome contains a unique and unequivocal ortholog of the gene that encodes angiotensin-converting enzyme 2 (ACE2), the SARS-CoV-2 receptor, modest conservation of amino acids at the binding interface makes it unlikely that zebrafish ACE2 proteins bind to bind the spike virus efficiently. Furthermore, since the beginning of the COVID-19 pandemic, many other receptors and co-receptors for SARS-CoV-2 in humans have been identified, many of which are not expressed in fish (Zamorano Cuervo & Grandvaux, 2020). Therefore, future studies will be necessary to understand the mechanisms intrinsic more comprehensively to the adverse effects of exposure of non-target aquatic organisms to SARS-CoV-2.

5. CONCLUSION

In conclusion, our study confirms the initial hypothesis by demonstrating that exposure of zebrafish (a non-target organism) to SARS-CoV-2 lysate at predicted environmentally relevant

induces changes in habituation memory behavior and antipredatory response in shoals, an mutagenic effect, biochemical changes predictive of redox imbalance, cholinesterasic effect, nitrosative stress, as well as a predominant Th1 specific immune response. Although the animals' response was not concentration-dependent for some biomarkers evaluated, PCA and IBRv2 indicated greater toxicity of SARS-CoV-2 at 2.226 pg/L. However, it is necessary to consider that the ecotoxicity of SARS-CoV-2 in non-target aquatic organisms is still an embryonic field that should be better explored in future studies. The identification and characterization of the impacts of SARS-CoV-2 on wild biota are undoubtedly helpful for us to understand the magnitude of the effects caused by the COVID-19 pandemic on ecosystems and biota, as well as to think about ways or strategies that aim at the conservation of the aquatic biodiversity in similar pandemic contexts.

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7. 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 authors.

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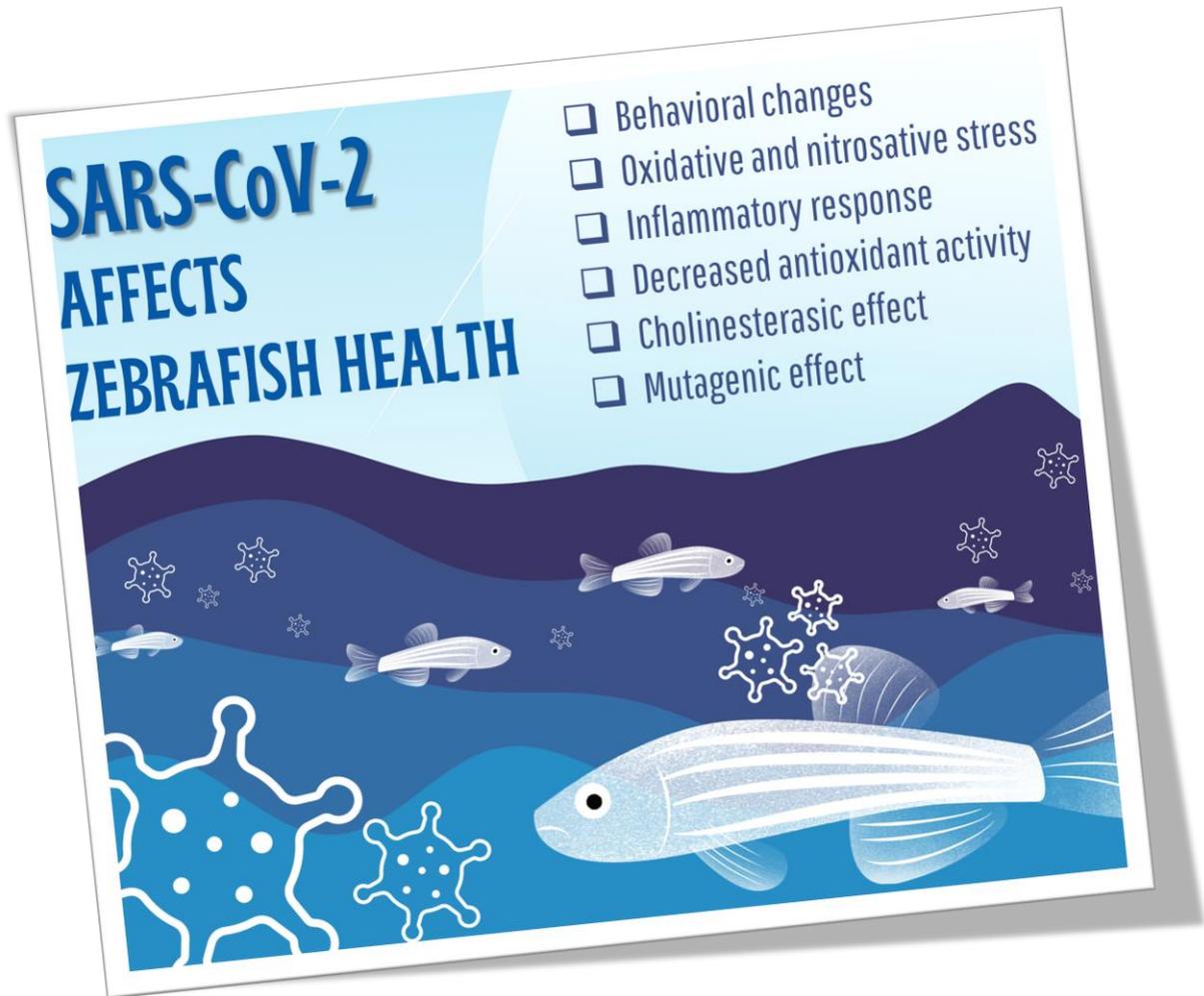
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ANEXO I

GRAPHIC ABSTRACT



ANEXO II

SUPPLEMENTARY MATERIAL

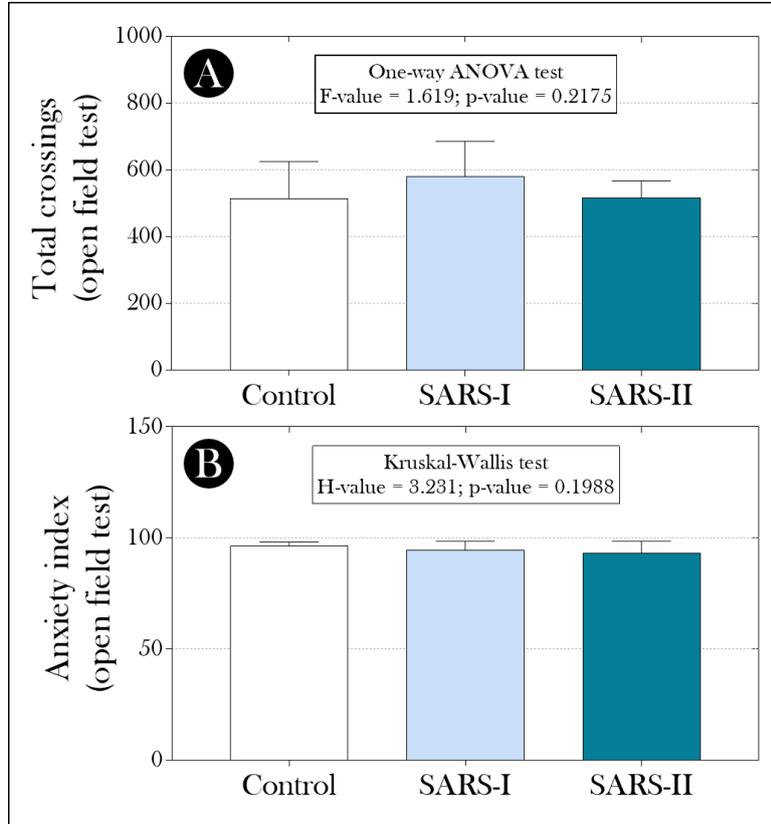


Figure S1. (A) Total crossings and (B) anxiety index in the open field test of adult zebrafish (*Danio rerio*) unexposed or exposed to SARS-CoV-2 at different concentrations. Parametric data are presented by the mean + standard deviation, whereas non-parametric data are presented by the median and interquartile range. Statistical summaries are displayed at the top of the graphs. Distinct lowercase letters indicate significant differences. SARS-I and SARS-II: composite groups of zebrafish exposed to SARS-CoV-2 lysate protein at 0.742 and 2.226 pg/L, respectively.

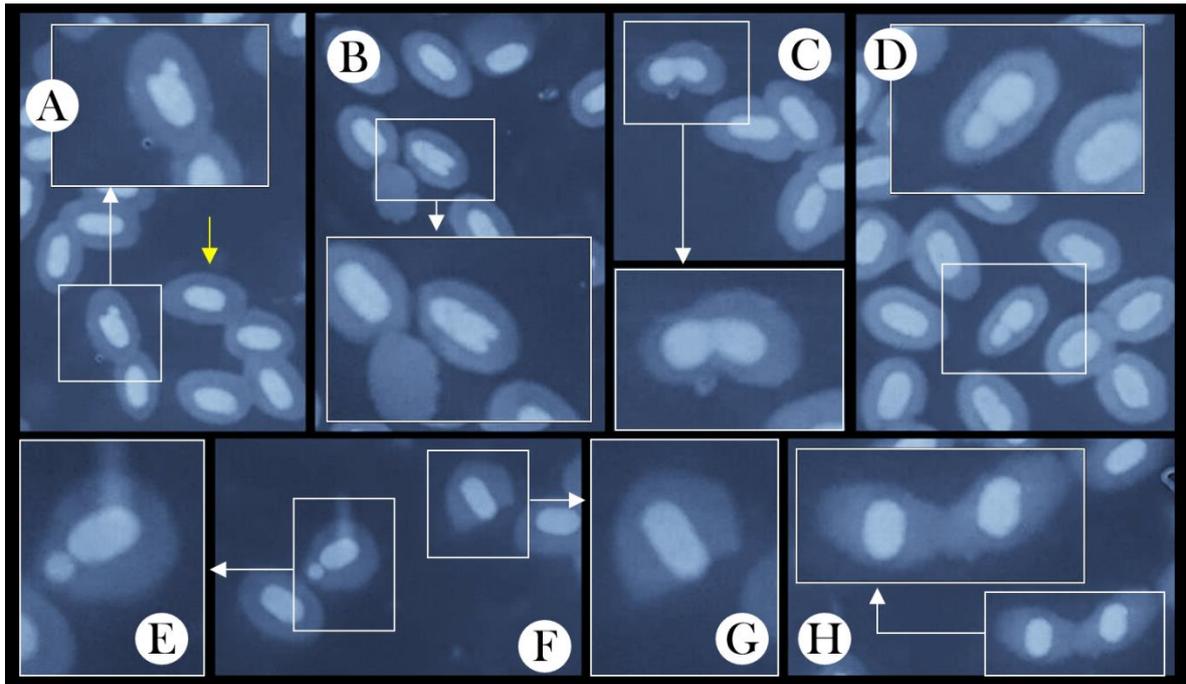


Figure S2. Representative images of erythrocytes identified in adult zebrafish (*Danio rerio*) unexposed or exposed to SARS-CoV-2 at different concentrations [(A) erythrocytes with a blebbed nucleus, (B) notched nucleus, (C) kidney-shaped nucleus, (D) constricted nucleus, (E-F) micronucleus, (F to H) moved nucleus. In “A”, the yellow arrow indicates an erythrocyte with an unchanged nucleus.

Table S1. Loading (coefficient) matrix provided by the multivariate analysis to define factors or principal components (PC1 and PC2).

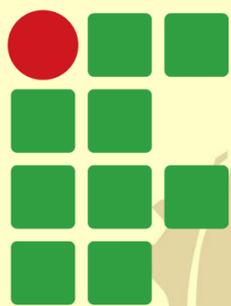
Biomarkers	Abbreviations	Principal components	
		PC1	PC2
Anxiety index	AI	-0,959	0,282
Total crossings	TC	0,410	0,912
Cluster index	CI	-0,937	-0,348
Total nuclear abnormalities	TNA	0,995	0,101
Micronuclei	MN	0,999	0,048
Notched nuclei	NN	1,000	0,018
Kidney-shaped nuclei	KN	0,982	0,188
Constricted nuclei	CN	0,955	0,295
Blebbled nuclei	BN	0,987	0,162
Moved nuclei	MovN	0,982	-0,188
Reactive oxygen species	ROS	0,995	-0,098
Superoxide dismutase activity	SOD	-0,844	0,537
Catalase activity	CAT	-0,723	0,691
Nitric oxide level	NO	0,989	-0,150
Acetylcholinesterase activity	AChE	0,991	-0,137
Hydrogen peroxide level	H ₂ O ₂	0,994	-0,105
Interleukin 10	IL-10	-0,987	-0,161
Interferon γ	IFN	0,968	0,250

ANEXO III

SUPPLEMENTARY MATERIAL

Produto Técnico Tecnológico (PTT)

<https://drive.google.com/file/d/1Aaj9SxFXP3hicyLpH3HX-FUF8HCggobN/view?usp=sharing>



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