Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

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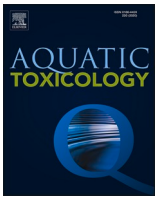

Toxicological impact of SARS-CoV-2 on the health of the neotropical fish, *Poecilia reticulata*

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ABSTRACT

There have been significant impacts of the current COVID-19 pandemic on society including high health and economic costs. However, little is known about the potential ecological risks of this virus despite its presence in freshwater systems. In this study, we aimed to evaluate the exposure of *Poecilia reticulata* juveniles to two peptides derived from Spike protein of SARS-CoV-2, which was synthesized in the laboratory (named PSPD-2002 and PSPD-2003). For this, the animals were exposed for 35 days to the peptides at a concentration of 40 μg/L and different toxicity biomarkers were assessed. Our data indicated that the peptides were able to induce anxiety-like behavior in the open field test and increased acetylcholinesterase (AChE) activity. The biometric evaluation also revealed that the animals exposed to the peptides displayed alterations in the pattern of growth/development. Furthermore, the increased activity of superoxide dismutase (SOD) and catalase (CAT) enzymes were accompanied by increased levels of malondialdehyde (MDA), reactive oxygen species (ROS) and hydrogen peroxide (H_2O_2), which suggests a redox imbalance induced by SARS-CoV-2 spike protein peptides. Moreover, molecular docking analysis suggested a strong interaction of the peptides with the enzymes AChE, SOD and CAT, allowing us to infer that the observed effects are related to the direct action of the peptides on the functionality of these enzymes. Consequently, our study provided evidence that the presence of SARS-CoV-2 viral particles in the freshwater ecosystems offer a health risk to fish and other aquatic organisms.

1. Introduction

Clinically, COVID-19 is described as an acute respiratory disease that affects the upper and lower respiratory systems, resulting in severe respiratory distress syndrome (Huang et al., 2020; World Health Organization (WHO), 2021b). In addition to thousands of deaths (28 January 2022, 5631,457 deaths) (Sarkodie and Owusu, 2021; McKibbin and Fernando, 2021) and social (Saladino et al., 2020; De-Figueiredo et al., 2021) impacts have been directly associated with severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) in different

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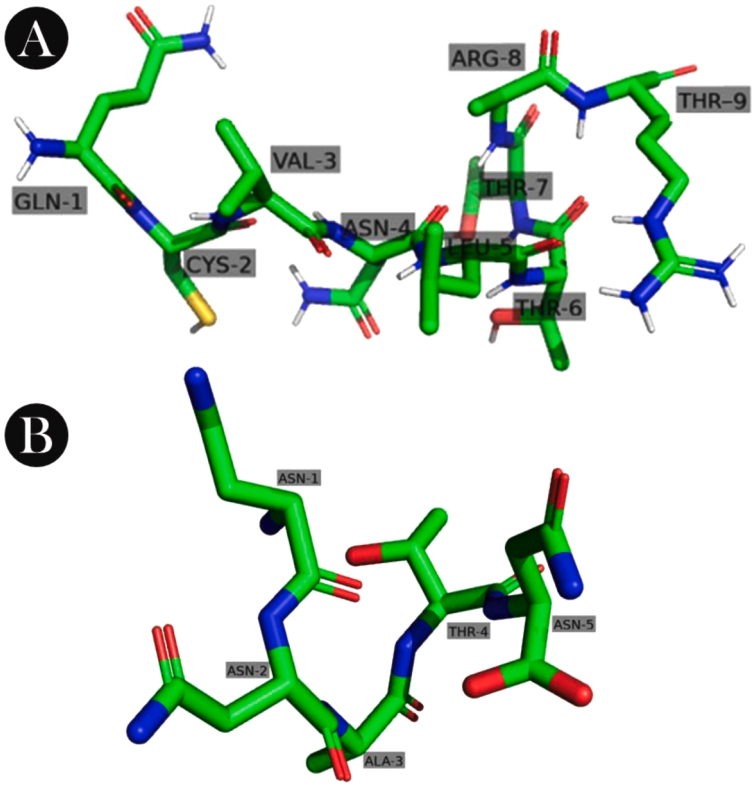

Fig. 1. Molecular models of the active conformations of peptides (A) PSPD2002 and (B) PSPD2003 that were synthesized in the present study.

countries, especially in the poorest ones, where access to health care is precarious and/or in those where government policies have neglected to initially contain the spread of the pandemic (Walker et al., 2020; United Nations (UN), 2021). On the other hand, studies have shown that the consequences of the COVID-19 pandemic go beyond these damages. Increased production of household waste (Yousefi et al., 2021; Amicarelli and Bux, 2021; Leal-Filho et al., 2021), healthcare solid waste (Das et al., 2021; Mekonnen et al., 2021), hospital wastes (Hossain et al., 2020; Kalantary et al., 2021), plastic products (Silva et al., 2020; Vanapalli et al., 2021), medicines/substances (Rolland et al., 2020; Bufquin et al., 2021; McKnight-Eily et al., 2021) and electricity consumption (Bielecki et al., 2021; Beyer et al., 2021; Rouleau and Gosselin, 2021), are some of the aspects that can intensify environmental impacts on natural ecosystems (directly or indirectly). Additionally, the detection of SARS-CoV-2 RNA in wastewater have raised concerns about the environmental consequences of entry of viral particles into aquatic ecosystems via organic waste from infected patients or citizens, especially feces and urine (Jones et al., 2020; Sherchan et al., 2020) (mean 4.96×10^3 copies/L), (Zhou et al., 2021) (cut-off cycle of threshold (Ct) value = 38.96), (Hasan et al., 2021) (viral loads in positive samples ranging between 2.86×10^2 and over 2.90×10^4 gene copies/L); and (Zhao et al., 2022) (one of the three influent samples from Waste Water Treatment Plant tested positive and quantified as 7.4×10^3 copies/L, and two samples from Jinyintan Hospital wastewater system influents (3.8×10^3 and 9.3×10^3 copies/L) were determined as SARS-CoV-2 RNA positive)]. Even though these findings are still incipient for comprehensive conclusions about a possible new viral transmission pathway for COVID-19, recent studies have reported that peptide fragments of SARS-CoV-2 can affect the health of non-target organisms (Charlie-Silva et al., 2021) (at 100 and 500 ng/mL); (Mendonça-Gomes et al., 2021) (at 40 μg/L), which reinforces recent concerns about the risk of COVID-19 to the functioning and biodiversity of aquatic ecosystems.

In this regard, we recently observed that the exposure of *Physalaemus cuvieri* (Leptodactylidae) to synthetic fragments of the Spike protein of SARS-CoV-2 (to 0.1 and 0.5 μg/mL), for only 24 h, was sufficient to induce an increase in oxidative stress, as well as a cholinesterasic effect in animals (Charlie-Silva et al., 2021). In another study, we found that short-term exposure (48 h) to a low concentration (40 μg/L) of the synthesized peptides induced changes in the locomotor and the olfactory-driven behavior of *Culex quinquefascitus* mosquito larvae (Culicidae), which were associated with increased production of reactive oxygen species (ROS) and acetylcholinesterase (AChE) activity (Mendonça-Gomes et al., 2021). In both studies, the tested peptide fragments were generated after a phagolysosomal proteolysis memorization pattern using the virtual proteolytic cleavage tool by Fernandes et al. (2020). This *in silico* method mimics possible degradation of the Spike protein in the environment.

Interestingly, knowledge of the magnitude of the impact of SARS-CoV-2 protein fragments on animals is still very incipient and, therefore, depends on conducting further investigations. It is questioned, for example, what are the effects of prolonged exposure to these fragments, their impacts on the development and growth of animals, as well as whether these fragments can induce physiological changes in other animal groups (in addition to amphibians Charlie-Silva et al., 2021 and insects Mendonça-Gomes et al., 2021). Thus, aiming to expand our knowledge about the effects induced by the presence of SARS-CoV-2 Spike protein peptides in river environments, *Poecilia reticulata* juveniles (Poeciliidae) were exposed to two previously synthesized viral fragments (PSPD-2002 and PSPD-2003). *P. reticulata* is distributed worldwide (Deacon et al., 2011) and widely used in ecotoxicological studies (e.g.: Aich et al., 2015; De-Lima Faria et al., 2021; De-Souza--Trigueiro et al., 2021), which characterizes them as good experimental models. We tested the hypothesis that after 35 days of exposure (at 40 μg/L) these peptides impact the development/growth of animals, induce metabolic changes predictive of redox imbalance, in addition to possibly causing behavioral changes associated with changes in AChE activity. Furthermore, a molecular docking analysis was performed to assess the affinity of these peptide to key protein binding sites. We believe that studies such as ours are important to expand knowledge about impacts

Table 1
Summary of biochemical biomarkers evaluated in *Poecilia reticulata* juveniles exposed or not to PSPD-2002 and PSPD-2003 peptides.

Oxidative stress biomarkers	Volume of supernatant used	Reagents and volumes used	Wavelength of sample reading[1]	Base reference for carrying out evaluations
Reactive oxygen species (ROS)	20 μL	• 200 μL PBS • 8.3 μL solution of dichlorofluorescein-diacetate (10 mg/mL)	492 nm	Zhao et al. (2013)
Hydrogen peroxide (H_2O_2) [(mmol/L)/g protein]	10 μL	• 100 μL buffer solution (PBS) • 100 μL solution of ammonium molybdate (0.5% w/v)	405 nm	Elnemma et al. (2004)
Malondialdehyde (MDA) (nmol MDA/g protein)	100 μL	• 150 μL trichloroacetic acid solution (1.6% w/v) + thiobarbituric acid (0.4% w/v). • Standard: solution of 1,1,3,3-etraethoxypropane (2000 nmol/L)	492 nm	Sachett et al. (2020)
Antioxidant biomarkers				
Catalase (CAT)[2] (mmol/g protein)	8 μL	• 200 μL (0.153 g PBS, 7 mL of glacial acetic acid, 14 mL of potassium dichromate (5%))		Sinha et al. (1972)
Superoxide dismutase (SOD)[2] (units/mg protein)		• Phosphate buffered saline. • Piragalol (15 mM) • Bromide of (3-[4,5-*dimetiltiazol*-2H] -2,5-difeniltetrazolium) (1.25 mM) • Dimethylsulfoxide P.A.	630 nm	Del-Maestro & McDonald (1985)
Nutritional status biomarkers				
Total protein (g/dL)	2 μL	• 200 μL of reagent 1[4] • Standard: bovine albumin (5 g/dL)	492 nm	Commercial kit[3] (CAS number: BT1000900), according to Gornall et al. (1949).
Triglycerides (mg/dL)	2 μL	• 200 μL of reagent 1[5] • Standard: glycerol (200 mg/dL)	492 nm	Commercial kit[3] (CAS number: BT1001000), according to Bucolo & David (1973).
Total cholesterol	2 μL	• 200 μL reagente of 1[6] • Standard: cholesterol *200 mg/dL)	492 nm	Commercial kit[3] (CAS number: BT1000400), according to Meiattini et al. (1978).
Cholinesterase effect biomarker				
Acetylcholinesterase (AChE) [(mmol/min/mL)/g protein]	50 μL	• 100 μL acetylcholine solution (0.75 mg/mL) • 100 μL solution of DTNB[7] (0.13 mg/mL))	405 nm	Ellman et al. (1961) and Estrela et al. (2021)

[1]The absorbance measurements of the samples were performed in an ELISA reader.
[2] These molecules are considered first-line antioxidants defenses that are important for preventing physiological oxidative stress.
[3] Commercial kits were obtained from Bioténica Ind. Com. LTD, Varginha, MG, Brazil.
[4] Composition of reagent 1 supplied by commercial kit: copper sulfate ≥ 5 mmol/L, potassium sodium tartrate ≥ 20 mmol/L, potassium iodide ≥ 10 mmol/L, sodium hydroxide ≥ 0.1 mol/L, detergent.
[5] Chemical composition of reagent 1 supplied by commercial kit: Pipes buffer ≥ 20 mmol/L, 4-chlorophenol ≥ 1 mmol/L; 4 - aminoantipyrine ≥ 0.1 mmol/L; ATP - adenosine triphosphate ≥ 0.5 mmol/L; glycerol kinase ≥ 500 U/L; peroxidase ≥ 1000 U/L; lipoprotein lipase ≥ 1000 U/L; glycerol-3-phosphate oxidase ≥ 1000 U/L; activators, detergents, stabilizers, and preservatives.
[6] Chemical composition of reagent 1 supplied by commercial kit: Pipes buffer ≥ 20 mmol/L; 4-aminoantipyrine ≥ 0.1 mmol/L; Phenol ≥ 1.0 mmol/L; peroxidase ≥ 1000 U/L; cholesterol oxidase ≥ 200 U/L; cholesterol esterase ≥ 200 U/L; lipoprotein lipase ≥ 200 U/L; activators; detergents; stabilizers; preservative.
[7] DTNB: 5,5′-Dithiobis-(2-Nitrobenzoic Acid).

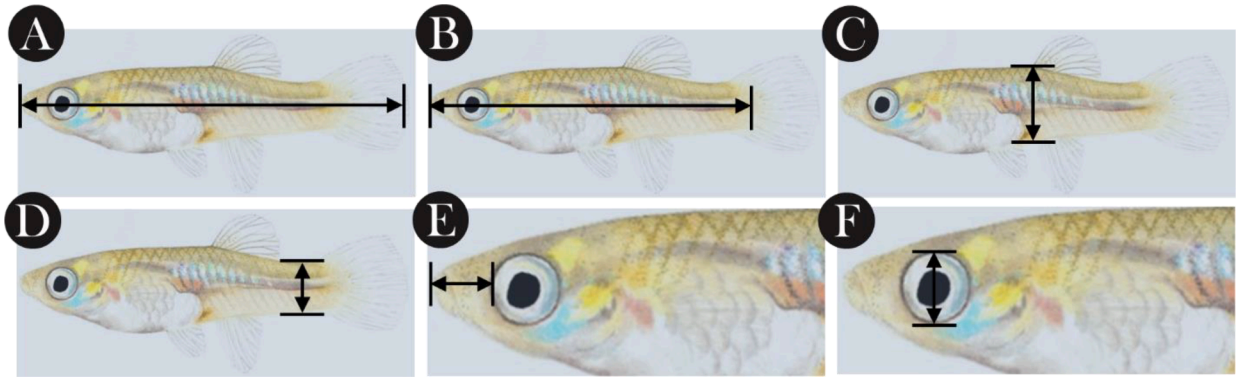

Fig. 2. Schematic drawing of biometric biomarkers (in cm) evaluated in *Poecilia reticulata* juveniles exposed or not to PSPD-2002 and PSPD-2003 peptides. (A) Total length, (B) standard length, (C) body depth, (D) peduncle depth, (E) postorbital length, and (F) eye diameter.

of COVID-19 on aquatic biodiversity; as well as to predict the environmental impacts of the recent pandemic on the populations of neotropical freshwater fishes, which already suffer strong anthropic pressure.

2. Material and methods

2.1. Peptide fragments of the SARS-CoV-2 spike protein

The synthesis, cleavage, purification, and characterization of the

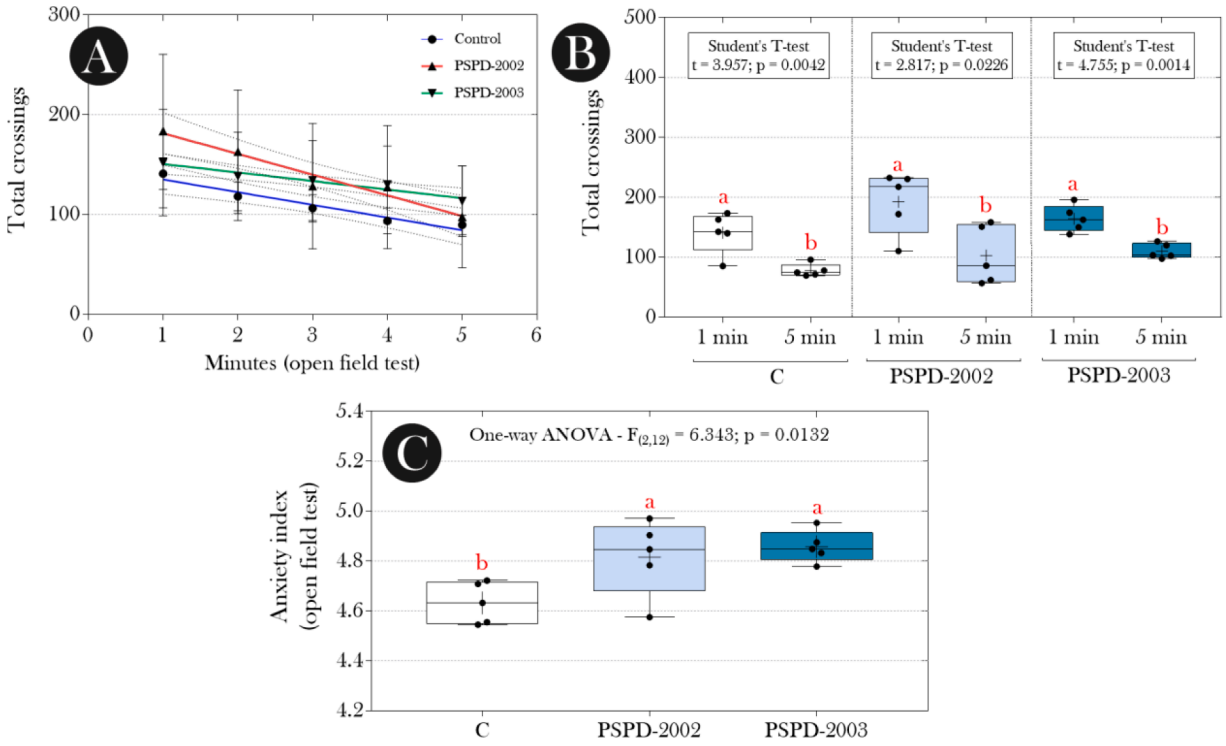

Fig. 3. Locomotor activity of *Poecilia reticulata* juveniles exposed or not to PSPD-2002 and PSPD-2003 peptides, inferred by (A) total quadrant crossings in the open field arena recorded throughout the test and (B) in the first and last minute of the evaluation. (C) Anxiety index recorded in the open field test. In "A", the symbols represent the mean ± SD of the total number of crossings in each minute of the open field test, whose data were submitted to linear regression analysis. In "B" and "C", a boxplot (min to max) is shown, showing all points, whose statistical summary is presented at the top of the graphs. "C": control group, "PSPD-2″: group exposed to SARS-CoV-2 Spike protein peptide 2002 and "PSPD-3″: group exposed to SARS-CoV-2 Spike protein peptide 2003. Each experimental group consisted of five replicate tanks, containing five animals/each. For the behavioral assessment, 2,3 animals/replica were randomly selected and subjected to behavioral test, totaling 12 animals/group. All statistical comparisons were based on the averages of each individual replicate tank (i.e.: $n = 5$ replicate tanks).

peptides from the SARS-CoV-2 Spike protein used in our study were performed according to methods described in detail by Charlie-Silva et al. (2021). Briefly, the synthesis of the Spike S protein was conducted using the solid phase peptide synthesis method (SPPS) following the Fmoc strategy (Raibaut et al., 2014; Behrendt et al., 2016). The resins used in this process were Fmoc-Thr-Wang and Fmoc-Asn-Wang for the PSPD-2002 (sequence: Gln-Cys-Val-Asn-Leu-Thr-Thr-Arg-Thr-COOH; MW: 1035.18 g/mol) and PSPD-2003 (sequence: Asn-Asn-Ala-Thr-Asn-COOH; MW: 532.51 g/mol), respectively. At the end of the synthesis, these resins made it possible to obtain peptides with a carboxylated C-terminal end. After coupling all the amino acid residues of the peptide sequences, the chains were removed from the solid support by means of acid cleavage using trifluoroacetic acid (TFA), similarly to Guy and Fields (1997). The crude compounds were purified by high performance liquid chromatography (HPLC) with a reverse phase column using different purification methods according to the retention time obtained in a gradient program of 5 to 95% in 30 min (exploration gradient) in analytical HPLC [similarly to Klaassen et al., 2019]. Only compounds with purity equal to or greater than 95% were considered for *in vivo* evaluation, following the rules determined by the National Health Surveillance Agency (ANVISA/Brazil) and Food and Drug Administration (FDA/USA). The similarities between the peptides PSPD-2002 and PSPD-2003 was evaluated using the CLUSTAL W software version 1.83 [Higgins et al., 1996; Pais et al., 2014 - http://www.ebi.ac.uk/clustalw/] (Fig. 1).

2.2. Animals and experimental setup

In this study, we used *Poecilia reticulata* (Cyprinodontiformes: Poeciliidae) (wild strain), commonly known as 'guppy', which is native to northwestern South America (Bisazza, 1993). This species was chosen because of its wide distribution in neotropical regions (CABI, 2021), in which it can inhabit strongly impacted aquatic environments where few other species can occur (Araújo et al., 2009), as well as its previous use in different ecotoxicological studies (Aich et al., 2015; De-Lima Faria et al., 2021; De-Souza-Trigueiro et al., 2021).

Fifteen days after the birth of the fingerlings, seventy-five *P. reticulata* juveniles (obtained from matrices of the bioterium of aquatic organisms at the Biological Research Laboratory, IF Goiano – Campus Urutaí, GO, Brazil) were separated and distributed into three experimental groups (five replicates/group). The groups "PSPD-2002″ and "PSPD-2003″ were composed of juveniles of *P. reticulata* exposed (for 35 days) to the respective peptides at a concentration of 40 μg/L, respectively, diluted in water. Such concentration simulates the presence of viral particles in a predicted environmental concentration. The control group consisted of fish kept in dechlorinated water free of viral peptides. Each replica contained five animals (mixed sex) kept in cylindrical aquariums with 2.2L of dechlorinated water (under constant oxygenation), without using filters or substrates. The temperature (25,26 °C) and luminosity (12-12h light:dark cycle) conditions were properly controlled. Every three days there was a complete renewal of the exposure waters, and, at the end of the experiment, the animals were submitted to different evaluations, as described below.

2.3. Behavioral assessment

Assuming that exposure to PSPD-2002 and PSPD-2003 peptides could interfere with the functioning of the nervous system, locomotor aspects and animal emotionality were evaluated. Each animal was submitted to the open field test, considered a good test of boldness and exploratory behavior and for the evaluation of anxiety-like behavior (Burns, 2008). Such a test simulates the involuntary arrival of fish in an

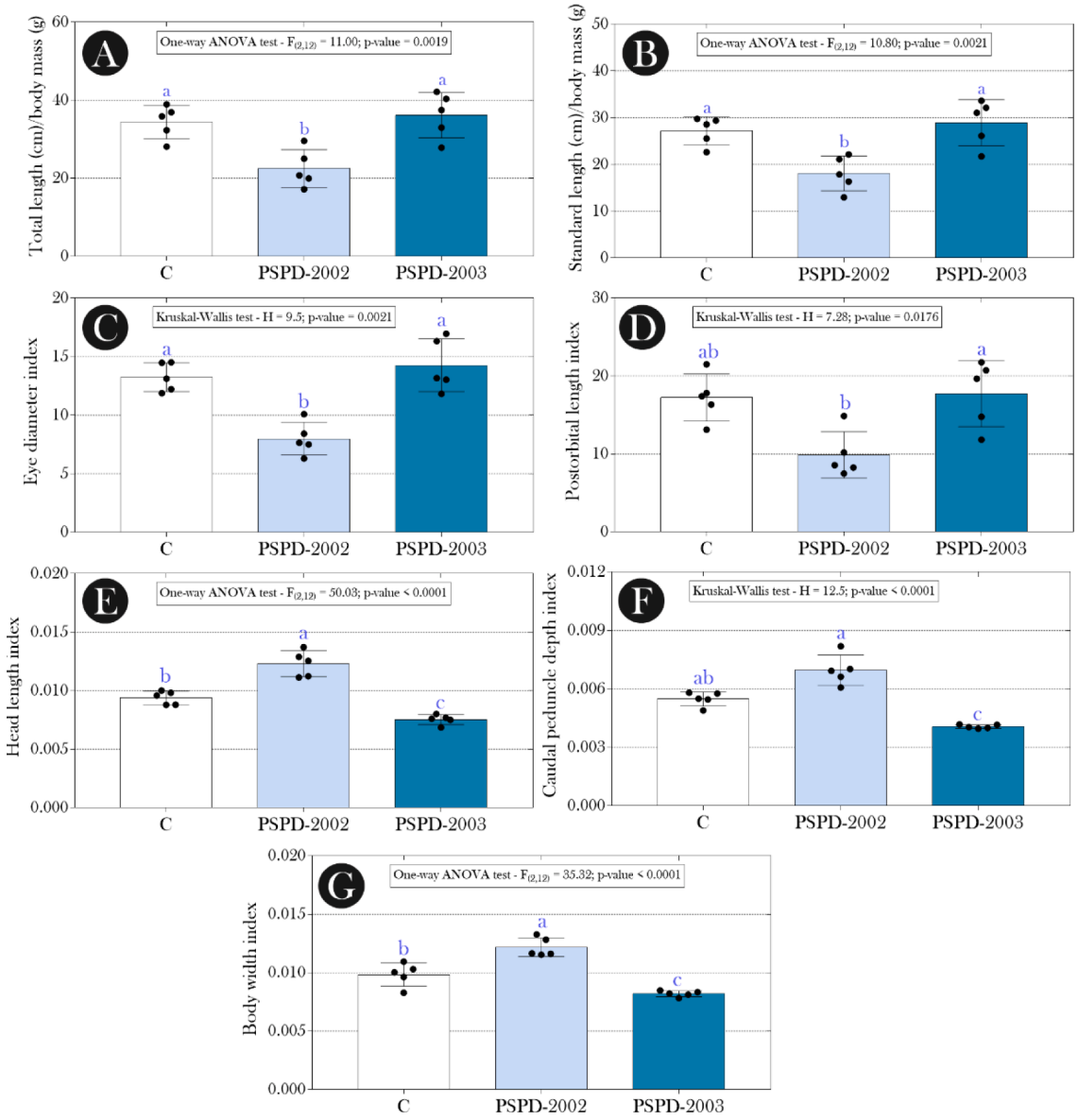

Fig. 4. Biometric parameters evaluated in juveniles *Poecilia reticulata* exposed or not to SARS-CoV-2 Spike protein peptides. (A) Total length/body mass, (B) standard length/body mass (C) eye diameter index, (D) posterbital length index, (E) head length index, (F) caudal peduncle depth and (G) body width. It is presented in boxplot graphs (min to max) showing all points, whose statistical summary is presented at the top of the graphs. "C": control group, "PSPD-2″: group exposed to SARS-CoV-2 Spike protein peptide 2002 and "PSPD-3″: group exposed to SARS-CoV-2 Spike protein peptide 2003. Each experimental group consisted of five replicate tanks, containing five animals/each. For the biometric assessment, 2,3 animals/replica were randomly selected and evaluated, totaling 13 animals/group. All statistical comparisons were based on the averages of each individual replicate tank (i.e.: $n = 5$ replicate tanks).

unknown environment in response to the exposure with a stressor such as presence of a predator (Magurran, 2005). To conduct the test, we adopted the procedures described by Burns (2008), with some modifications. Briefly, the test consisted of introducing the animals (individually) into the center of a cylindrical arena (Ø: 14 cm), containing 500 mL of water (previously dechlorinated and free of any peptide) and filming them for 5 min. The walls of the arenas were lined with brown paper to prevent external signals from interfering with the animals' behavioral response. A circular zone ($Ø = 9.5$ cm) was also delineated in the center and served as the starting point. Between one session and another, the water in the arenas was replaced by previously dechlorinated fresh water, also free of pollutants. The tests were carried out in a specific room that had sound insulation, artificial lighting, controlled temperature (25,26 °C) and two cameras coupled to a computer located externally. Automated video recording of behavior started upon entry in the test apparatus and the "chronology" function of the PlusMZ software was used to record and analyze behavior.

During testing, the experimenter was outside the view of the *P. reticulata* juveniles to avoid disturbance of behavioral responses. After performing the tests, we evaluated the "*general locomotor activity*" of the animals [inferred by recording the total distance covered (in cm) throughout the test] and the "*anxiety-like behavior*". The latter was calculated as the ratio between animal movement in the peripheral zone and total locomotion in the test arena, being considered a biomarker of anxiety-like behavior as it assesses the animals' normal preference for the periphery in favor of the central area of the arena (Godwin et al., 2012). In addition, we evaluated the possible influence of treatments on the "*habituation learning*" of animals, considered one of the forms of non-associative learning, characterized by the reduction of an animal's behavioral response during a test (Gómez-Laplaza and Gerlai, 2010; Ahmad and Richardson, 2013). In our study, this endpoint was evaluated by recording the locomotor activity of the animals during each

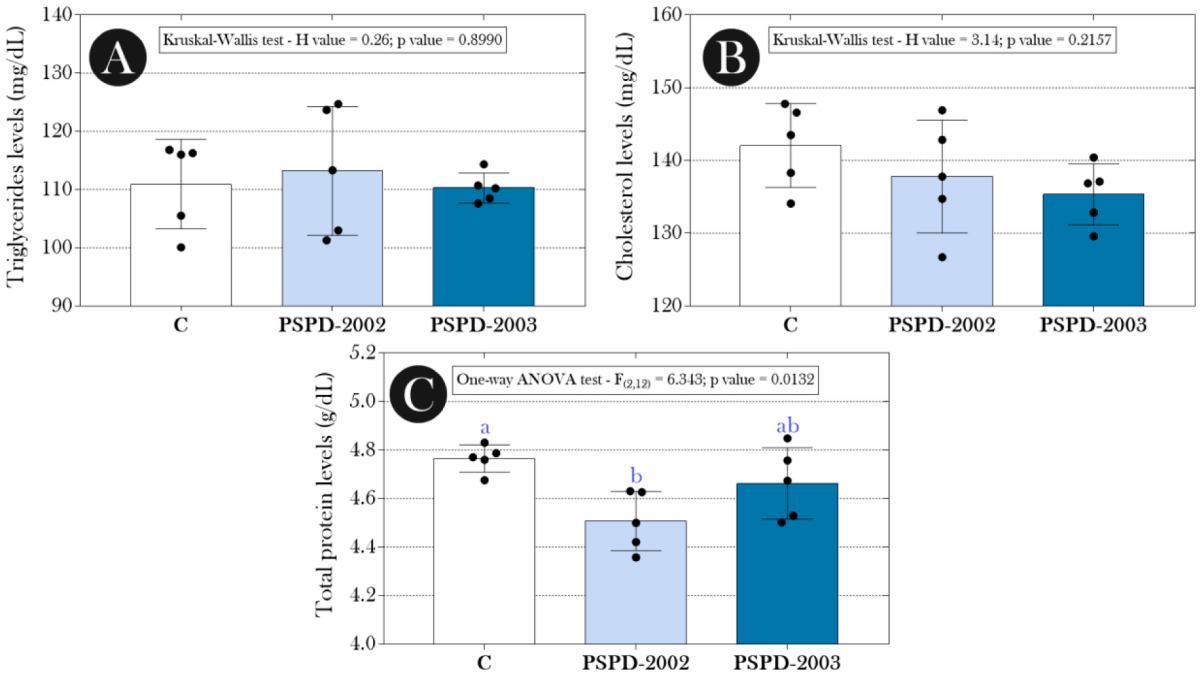

Fig. 5. (A) Triglyceride, (B) total cholesterol and (C) total proteins levels of *Poecilia reticulata* juveniles exposed or not to SARS-CoV-2 Spike protein peptides. Boxplot graphs (min to max) are presented showing all points, whose statistical summary is presented at the top of the graphs. "C": control group, "PSPD-2": group exposed to SARS-CoV-2 Spike protein peptide 2002 and "PSPD-3": group exposed to SARS-CoV-2 Spike protein peptide 2003. Each experimental group consisted of five replicate tanks, containing five animals/each. For evaluation of biochemical biomarkers, 2 animals/replica were randomly selected and evaluated, totaling 10 animals/group. All statistical comparisons were based on the averages of each individual replicate tank (i.e.: $n = 5$ replicate tanks).

minute of the test and comparing the locomotor activity recorded in the first block of 1 min and in the last block (also of 1 min). Between 2 and 3 animals per replicate, totaling 12 animals/group were randomly selected and subjected to behavioral test.

2.4. Biochemical assessments

In order to associate the behavior of animals exhibited in the test described above with possible biochemical dysfunctions, different antioxidant, oxidative stress and cholinesterase effect biomarkers were evaluated. For this, after the behavioral tests, the animals were euthanized (on ice), weighed (5.2 mg ± 0.97 mg – mean ± SEM) and macerated in 1 mL of phosphate buffered saline (PBS) (pH 7.2). Then, the samples were centrifuged (3000 g, 5 min, 4 °C) and the supernatants were used for the evaluation of the biomarkers summarized in Table 1. Between 2 and 3 animals per replicate, a total of 13 animals/group were randomly selected and evaluated at this stage.

2.5. Biometry

To assess the effects of the peptides on the growth of the animals, the total length, standard length, body depth, peduncle depth, postorbital length and eye diameter were measured (Fig. 2), similarly to Sheridan and Pomiankowski (1997). For this, the animals were anesthetized (on ice) and photographed, for further evaluation in the ImageJ software and calculation of different biometric indices (see equations below).

$$\textbf{Total length index} = \frac{Total\ length\ (cm)}{Body\ mass\ (g)} \quad (1)$$

$$\textbf{Standard length index} = \frac{Standard\ length\ (cm)}{Body\ mass\ (g)} \quad (2)$$

$$\textbf{Body width index} = \frac{Body\ width\ (cm)}{Total\ length\ index} \quad (3)$$

$$\textbf{Caudal peduncle depth index} = \frac{Caudal\ peduncle\ depth\ (cm)}{Total\ length\ index} \quad (4)$$

$$\textbf{Head length index} = \frac{Caudal\ peduncle\ depth\ (cm)}{Total\ length\ index} \quad (5)$$

$$\textbf{Eye diameter index} = \frac{Eye\ diameter(cm)}{Head\ length\ index} \quad (6)$$

$$\textbf{Postorbital length index} = \frac{Postorbital\ length\ (cm)}{Head\ length\ index} \quad (7)$$

2.6. Molecular docking

Molecular docking analysis was performed to predict the possibility of binding and affinity between PSPD-2002 and PSPD-2003 peptides and the protein structures of SOD, CAT and AChE enzymes. For this, the structures of the PSPD-2002 and PSPD-2003 peptides were modeled using the PEP-FOLD3 server (https://bioserv.rpbs.univ-paris-diderot.fr/services/PEP-FOLD3/). The sequences of the protein structures (targets) of *P. reticulata* (Taxon: 8081) (SOD, CAT and AChE) were obtained from the European Nucleotide Archive (ENA) repository (https://www.ebi.ac.uk/ena/browser/home) and modeled by the homology construction technique by the SWISS-MODEL server (https://swissmodel.expasy.org/). The validation of the structures took place through the SAVES v.6.0 server (https://saves.mbi.ucla.edu/). For molecular docking simulations, AutoDock tools (ADT) v4.2 was used to prepare the binders and targets [according to Morris et al., 2009] and to perform the calculations the AutoDock Vina 1.1.2 was used (Pettersen et al., 2021).

2.7. Statistical analysis

Initially, all data were checked for deviations from normality (via Shapiro-Wilk test) and homogeneity of variance (using the Bartlett's test) before the analysis. These analyzes were important to define which

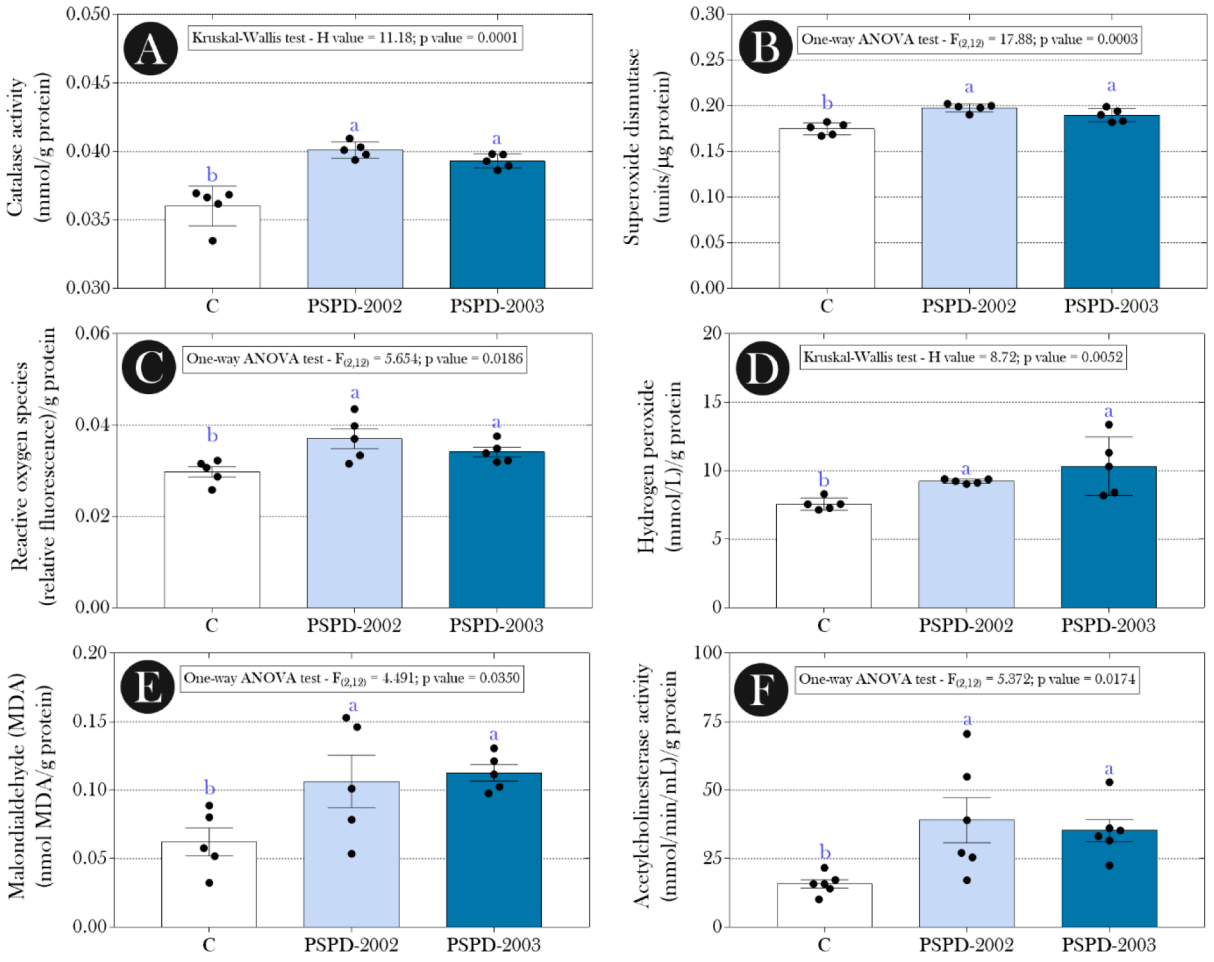

Fig. 6. Biochemical biomarkers evaluated in *Poecilia reticulata* juveniles exposed or not to SARS-CoV-2 Spike protein peptides (PSPD-2002 and PSPD-2003). (A) Catalase (CAT), (B) superoxide dismutase (SOD), (C) reactive oxygen species (ROS), (D) hydrogen peroxide (H_2O_2), (E) malondialdehyde (MDA) and (F) acetylcholinesterase (AChE). Boxplot graphs (min to max) are presented showing all points, whose statistical summary is presented at the top of the graphs. "C": control group, "PSPD-2″: group exposed to SARS-CoV-2 Spike protein peptide 2002 and "PSPD-3″: group exposed to SARS-CoV-2 Spike protein peptide 2003. Each experimental group consisted of five replicate tanks, containing five animals/each. For evaluation of biochemical biomarkers, 2 animals/replica were randomly selected and evaluated, totaling 10 animals/group. All statistical comparisons were based on the averages of each individual replicate tank (i.e.: $n = 5$ replicate tanks).

Table 2
Correlation matrix for antioxidant and oxidative stress biomarkers evaluated in *Poecilia reticulata* juveniles exposed or not to SARS-CoV-2 Spike protein peptides (PSPD-2002 and PSPD-2003).

	CAT	SOD	H_2O_2	MDA	ROS
CAT	–	$r = 0.659$; $p = 0.0002$	$r = 0.629$; $p = 0.0004$	$r = 0.579$; $p = 0.0020$	$r = 0.694$; $p < 0.0001$
SOD	$r = 0.659$; $p = 0.0002$	–	$r = 0.567$; $p = 0.0020$	$r = 0.445$; $p = 0.0200$	$r = 0.531$; $p = 0.0044$
H_2O_2	$r = 0.629$; $p = 0.0004$	$r = 0.567$; $p = 0.0020$	–	$r = 0.471$; $p = 0.0130$	$r = 0.529$; $p = 0.0045$
MDA	$r = 0.579$; $p = 0.0015$	$r = 0.445$; $p = 0.0200$	$r = 0.471$; $p = 0.0131$	–	$r = 0.640$; $p = 0.0003$
ROS	$r = 0.694$; $p = 0.0001$	$r = 0.531$; $p = 0.0044$	$r = 0.529$; $p = 0.0045$	$r = 0.640$; $p < 0.0001$	–

Legend: CAT: Catalase activity (mmol/g protein); SOD: Superoxide dismutase (units/mg protein); H_2O_2: Hydrogen peroxide (mmol/L)/g protein; MDA: Malondialdehyde (MDA) (nmol MDA/g protein); ROS: Reactive oxygen species (relative fluorescence)/g protein. r: Spearman's correlation coeficiente; p: *p*-value, at 5% probability.

statistical models would be used *a posteriori*. Data regarding the locomotor activity of the animals (total crossings) in the first and last minutes of the open field test were compared using Student's *t*-test. Additionally, the correlation between the variable's "time" and "total crossings" of the animals in each experimental group during the open field test was evaluated (based on Pearson's correlation coefficient) and, after confirming the existence of a direct relationship between these variables, the data were submitted to linear regression analysis. The decreasing locomotor behavior (over time) in the open field test is one of the presuppositions to infer "habituation learning". Multiple comparisons between the anxiety index as well as different biometric and biochemical biomarkers were performed based on one-way ANOVA, Tukey's post-hoc analysis (for parametric data) or Kruskal-Wallis's test, with Dunn's post-hoc (for non-parametric data). The relationships between biochemical biomarkers were also assessed using correlation analyzes based on Pearson's or Spearman's correlation coefficients (for parametric and non-parametric data, respectively) and regression analysis was performed when significant correlations between variables (biochemical biomarkers) were identified. Significance levels were set at Type I error (p) values lower than 0.05. GraphPad Prism Software Version 9.0 (San Diego, CA, USA) was used to perform the statistical analyses.

Table 3
Summary of linear regression analyzes regarding correlations between different biochemical biomarkers evaluated in *Poecilia reticulata* juveniles exposed or not to SARS-CoV-2 Spike protein peptides (PSPD-2002 and PSPD-2003).

Biomarker	*F*-value	*p*-value	Equation
ROS *vs.* CAT	22.04	< 0.0001	$y = 0.3048x + 0.0284$
ROS *vs.* SOD	11.29	0.0025	$y = 1.76x + 0.1286$
ROS *vs.* H_2O_2	1.27	0.2705	–
ROS *vs.* MDA	11.53	0.0023	$y = 5.91x - 0.1032$
CAT *vs.* ROS	22.04	< 0.0001	$y = 1.537x + 0.3475$
CAT *vs.* SOD	18.17	0.0003	$y = 4.597x + 0.009949$
CAT *vs.* H_2O_2	3.652	0.0676	–
CAT *vs.* MDA	12.61	0.0016	$y = 13.68x - 0.4337$
SOD *vs.* ROS	11.29	0.0025	$y = 0.1768x + 0.0002209$
SOD *vs.* CAT	18.17	0.0003	$y = 0.09157x + 0.02142$
SOD *vs.* H_2O_2	1.671	0.2079	–
SOD *vs.* MDA	4.965	0.0351	$y = 1.357x - 0.1604$
H_2O_2*vs.* ROS	1.27	0.2705	–
H_2O_2*vs.* CAT	3.652	0.0676	–
H_2O_2*vs.* SOD	1.671	0.2079	–
H_2O_2*vs.* MDA	9.731	0.0045	$y = 0.0106x - 0.002133$
MDA *vs.* ROS	11.53	0.0023	$y = 0.05342x + 0.02832$
MDA *vs.* CAT	12.61	0.0016	$y = 0.02451x + 0.03626$
MDA *vs.* SOD	4.965	0.0351	$y = 0.1221x + 0.1758$
MDA *vs.* H_2O_2	9.731	0.0045	$y = 26.44x + 6.568$

Legend: CAT: Catalase activity (mmol/g protein); SOD: Superoxide dismutase (units/mg protein); H_2O_2: Hydrogen peroxide (mmol/L)/g protein; MDA: Malondialdehyde (nmol MDA/g protein); ROS: Reactive oxygen species (relative fluorescence)/g protein.

3. Results

Initially, we evaluated the possible behavioral effects of animal exposure to PSPD-2002 and PSPD-2003 peptides. All groups showed a decrease in locomotion throughout the test (Fig. 3A), following a simple linear regression model (Table 1S), with the slope of the straight lines of each group were not statistically different (*F* test – *F*-value = 1848; DFn = 2; DFd = 164; *p*= 0.1608). Furthermore, we observed that the locomotor activity of the animals in the 5th last minute of the test was statistically lower than that observed in the 1st minute of evaluation (Fig. 3B). Therefore, these data suggest that "*habituation learning*" was not altered by the peptides. However, our analyses revealed that the Y-intercepts of the straight lines differed significantly among the experimental groups (*F* test – *F*-value = 5394; DFn = 2; DFd = 166; *p*-value = 0.0054), which indicates that at any of the evaluation times, the animals in the control group showed less locomotor activity, inferred by the total number of crossings of the quadrants in the open field area. Furthermore, we observed that the animals exposed to the peptides exhibited anxiety-like behavior, inferred by their longer stay in the peripheral quadrants of the open field arena (Fig. 3C).

Regarding biometric parameters, we also observed that the treatments induced changes in different parameters evaluated. Animals exposed to the PSPD-2002 peptide had a lower proportion between length and body biomass (total and standard – Fig. 4A,B, respectively), as well as reduced eye diameter index (Fig. 4C). On the other hand, both PSPD-2002 and PSPD-2003 peptides induced different changes in head length and body width (Fig. 4E–G, respectively), suggesting a disproportionate growth in relation to total length of animals.

On the other hand, assuming that behavioral and growth changes in animals could be associated with changes in lipid and protein metabolism, we estimated the total levels of triglycerides, cholesterol, and proteins in the animals. However, we did not observe differences in triglyceride and total cholesterol levels among different experimental groups (Fig. 5A,B), but total protein levels were lower in animals exposed to PSPD-2002 peptides, compared to the control group (Fig. 5C).

Importantly, our study also demonstrated that the SARS-CoV-2 Spike protein peptides caused a redox imbalance in *P. reticulata*, marked by an increase in CAT and SOD activity (Fig. 6A,B) and a concomitant increase in the production of ROS, H_2O_2 and MDA (Fig. 6C–E, respectively), which suggests that the increase in antioxidant activity was not able to counteract the oxidative stress induced by the treatments. Statistical analyses showed a positive and significant correlation between antioxidant biomarkers and those predictive of oxidative stress (Table 2), following a linear increase for most of the evaluated correlations (Table 3 and Fig. 7). Furthermore, we observed that both peptides evaluated induced a cholinesterasic effect in animals, marked by a significant increase in AChE activity (Fig. 6F).

Assuming that the observed biochemical effects could be related to the possible binding of SARS-CoV-2 Spike protein peptides to the protein structures of the enzymes SOD, CAT and AChE, we performed molecular docking analysis. In this case, all interactions evaluated showed acceptable affinity data exceeding the low-quality threshold (-6.0 kcal/mol). For the PSPD-2002 peptide the affinity values for AChE was -7.9 kcal/mol, for CAT it was -7.2 kcal/mol and for SOD it was -8.8 kcal/mol. Interactions between PSPD-2002 and AChE involved residues TYR93, TYR144, ASN95, ASN108, MET104, SER304, SER310 and TYR354. Interactions with CAT involved residues ILE343, GLU344, MET339, GLN415, ARG382, ASN385, GLN387 and HIS395; and with SOD, ASN92, GLU76, ALA179, LYS30, MET27, GLY155, ILE140, ARG142 and ALA179. For the PSPD-2003 peptide, the affinity values for AChE, CAT and SOD were -8.1 kcal/mol, -9.9 kcal/mol and -9.1 kcal/mol, respectively, whose interactions involved the residues ASN584, ASP331, ASN255, THR256 and GLN423 (for AChE), THR381, ARG382, VAL383 and ALA384 (for CAT) and GLN42, ASN80, THR81A and THR81B (for SOD). The results of couplings between peptides and enzyme active sites are shown in Fig. 8.

4. Discussion

Understanding the environmental/ecological impacts caused by viral particles, especially the new coronavirus (SARS-CoV-2), inevitably permeates studies that assess how much exposure to these particles can damage the biology of various organisms. Thus, our study pioneered that both tested peptides (PSPD-2002 and PSPD-2003) induced negative impacts on the evaluated fish (*P. reticulata* juveniles), which included neurobehavioral alterations, an effect on growth, as well as a redox imbalance. Initially, we observed an anxiety-like behavior in fish exposed to peptides (Fig. 3C), coincided with an cholinesterasic effect observed in these animals, marked by an increase in AChE activity (Fig. 6F). Similar results were recently reported by Charlie-Silva et al. (2021) and Mendonça-Gomes et al. (2021). In these studies, the authors evidenced that the short exposure of *P. cuvieri* tadpoles and *C. quinquefasciatus* larvae (respectively) to PSPD-2002 and PSPD-2003 peptides was able to induce a cholinesterasic effect as observed for *P. reticulata* juveniles in this study (Fig. 6F). However, the effects of PSPD-2002 and PSPD-2003 peptides on the emotionality of animals had not been demonstrated previously. Obviously, it is still incipient to propose any mechanisms that can explain the observed effects, and, in this case, different mechanisms (isolated or concomitant) may be related to the observed anxiogenic effect.

The relationship between the increase in AChE activity in *P. reticulata* exposed to peptides and the induction of anxiety-like behavior needs to be further investigated, as it has been controversially reported that an decrease in AChE in larvae, juveniles and/or adults of fish exposed to different pollutants was considered the cause of induction of an anxiety-like behavior in these animals (Golombieski et al., 2008; Giacomini et al., 2020; Raja et al., 2019; Pullaguri et al., 2020; Duncan et al., 2020). On the other hand, the hypothesis that the stimulation of the cholinergic system of *P. reticulata* is a consequence of direct interactions between the tested peptides and AChE is supported by molecular docking analysis (Fig. 8A and D), similarly to reports by Charlie-Silva et al. (2021) and Mendonça-Gomes et al. (2021). However, future investigations should be conducted to elucidate whether these interactions would be capable

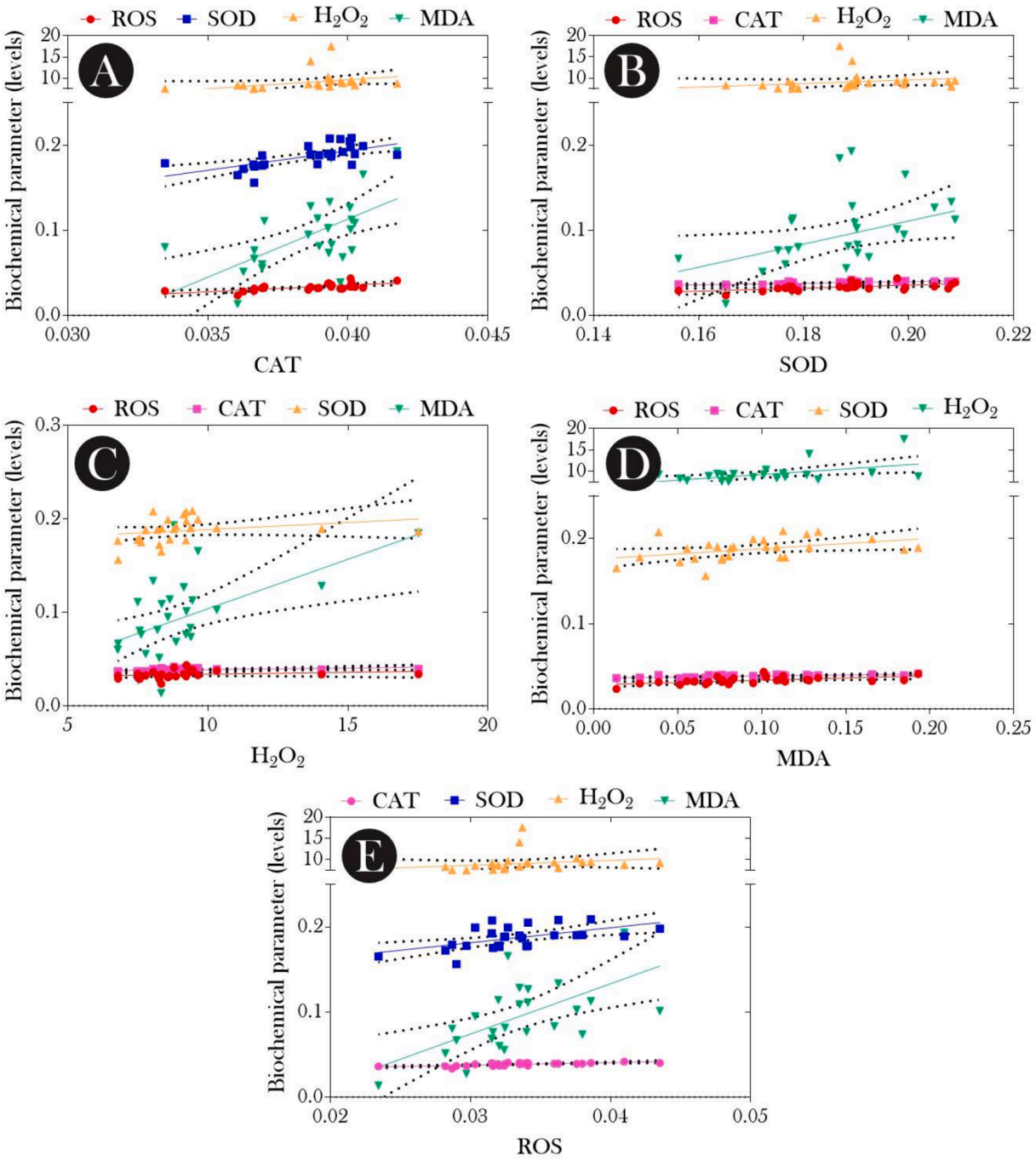

Fig. 7. Graphic representations of linear regression analyze involving antioxidant and oxidative stress biomarkers evaluated in *Poecilia reticulata* juveniles exposed or not to SARS-CoV-2 Spike protein peptides (PSPD-2002 and PSPD-2003). (A) CAT: Catalase activity, (B) SOD: Superoxide dismutase, (C) H_2O_2: Hydrogen peroxide, (D) MDA: Malondialdehyde (MDA) and ROS: Reactive oxygen species. The summary of statistical analyzes is shown in Table 3.

of causing dysfunctions or an increase in the enzymatic efficiency of AChE. In the first situation, the increase in AChE activity in *P. reticulata* would characterize a compensatory mechanism in response to the catalytic deficit induced by the peptides. In this case, it is possible that the peptides would bind AChE instead of the natural ligand and thus reduce catalysis of acetylcholine (ACh). In the second, the increase would be explained by a more efficient response of the enzyme to the increase in the release of ACh in the synaptic clefts via activation of the cholinergic anti-inflammatory pathway (CAP).

Regarding the biometric evaluation, our data also revealed that morphometric changes were induced by exposure to PSPD-2002 and PSPD-2003 peptides, aspects that had not yet been investigated in previous studies. In this case, it is possible that such changes are reflections of the redox imbalance induced by the evaluated peptides. The increase in antioxidant activity (inferred by SOD and CAT levels) (Fig. 6) was not sufficient to counterbalance the high levels of ROS, H_2O_2 and MDA observed in *P. reticulata* exposed to the peptides, which may have demanded a high energy cost of exposed animals. Considering that the juvenile phase naturally consists of a phase of high energy demand (Geist et al., 2013; Di Pane et al., 2019; Mejri et al., 2021), the reallocation of energy to maintain physiological homeostasis, can compromise the growth and development of animals. Although no other study has evaluated similar effects in fish exposed to SARS-CoV-2 or its components, investigations involving fish larvae or juveniles exposed to other

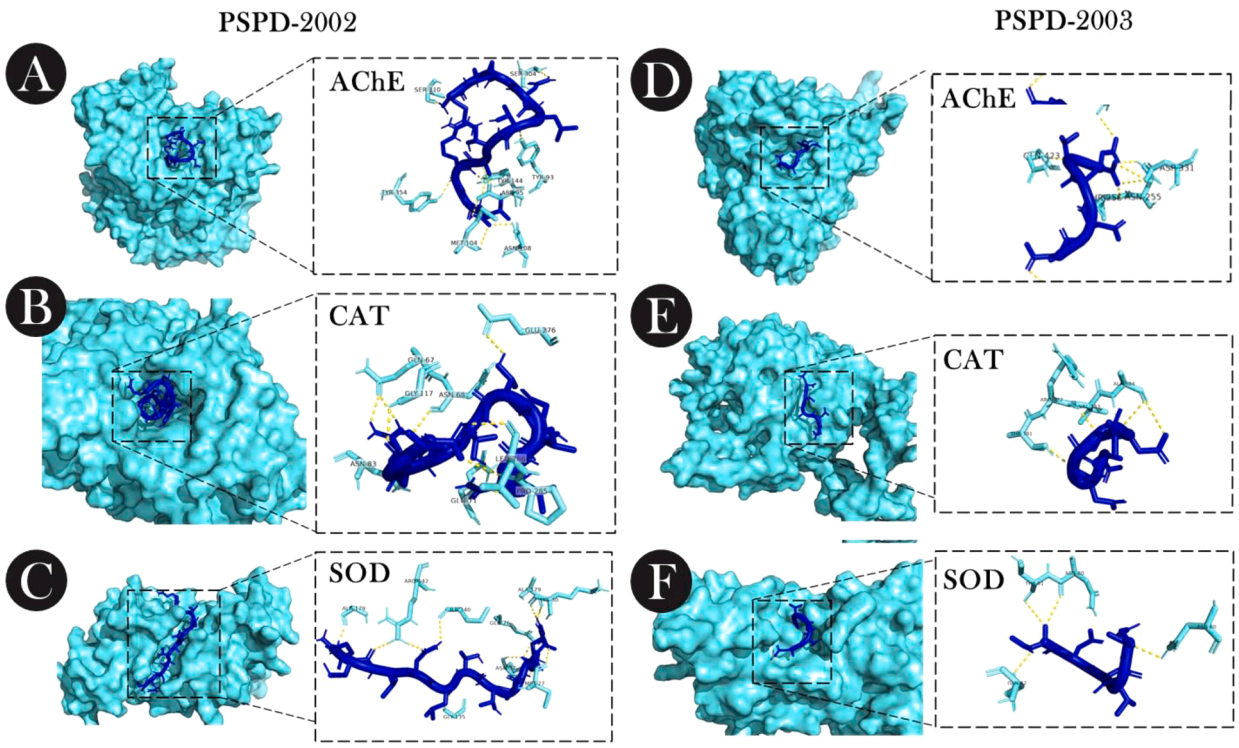

Fig. 8. Three-dimensional surface-ligand coupling of PSPD-2002 (A–C) and PSPD-2003 (E,F) peptide interactions and the active sites of acetylcholinesterase (AChE), catalase (CAT) and superoxide dismutase (SOD) enzymes.

pollutants also relate changes in growth and development to oxidative stress (Zhu et al., 2008; Mao et al., 2020; Chowdhury and Saikia, 2020). As demonstrated by several authors, the physiological impacts of increased oxidative stress are manifold and systemic and, therefore, a number of other toxicological effects have already been reported in different species with elevated ROS, such as (i) DNA damage; (ii) damage to membrane integrity, (iii) functional modifications of proteins; (iv) histopathological damage; (v) changes in the immune response; (vi) endocrine disruption; and (vii) metabolic disturbances, among others (Slaninova et al., 2009; Lushchak, 2016; Birnie-Gauvin et al., 2017; Biller and Takahashi, 2018; Chowdhury and Saikia, 2020). Together, these effects can directly or indirectly affect the growth and development of animals. On the other hand, it is possible that the biometric differences observed in *P. reticulata* constitute an adaptive response to the stress induced by the peptides, rather than a direct proof of the biological impact of protein fragments on animal growth. This hypothesis is supported, for example, by Oleksiak (2008), who reported that bodily changes in *Fundulus heteroclitus* that inhabit polluted areas were associated with alterations in the expression of distinct ontological genes, such as those related to muscle and skeletal development (Oleksiak, 2008; Vidal-Dorsch et al., 2012). Therefore, the high dimensionality of possible physiological responses to exposure to SARS-CoV-2 spike protein peptides demands the development of new studies, aiming to understand their association with changes in fish development patterns.

Moreover, our data corroborate previous studies describing the important role of spike protein constituents in inducing a disproportionate cellular antioxidant-oxidant balance in SARS-Cov-2 infection (Ntyonga-Pono, 2020; Delgado-Roche and Mesta, 2020; Cecchini and Cecchini, 2020; Suhail et al., 2020). In these studies, the high production of ROS has been attributed to mitochondrial dysfunctions caused by the penetration of the virus into cells (Ntyonga-Pono, 2020), accompanied by the release of a "*cytokine storm*", such as IL-2, IL-6, IL-7, TNF-α, etc. (Mehta et al., 2020; Noroozi et al., 2020), as well as hyperinflammation and hyperferritinemia (Camini et al., 2017). Particularly, *P. reticulata* were not directly exposed to SARS-CoV-2, but to synthetic peptide fragments of the Spike protein. Therefore, it is possible that the oxidative stress observed was due to phagocytosis/endocytosis of these peptides by cells known to have a relevant role in the physiological response to foreign agents. Such cells include, for example, neutrophils, which represent the most important cell type in this context, since they produce significant superoxide free radicals and H_2O_2, constituting an important mechanism in the elimination of pathogens (Naumenko et al., 2018), including SARS-CoV-2 (Borges et al., 2020; Reusch et al., 2021; Cavalcante-Silva et al., 2021). Furthermore, it is possible that activation of agranulocytes, such as macrophages, led to a respiratory burst in response to the presence of SARS-CoV-2 Spike protein peptides and may also induce ROS production, similarly to what has been reported in studies focusing on human (Abassi et al., 2020; Boumaza et al., 2020; Zhang et al., 2021) and experimental infection by the new coronavirus (Hoang et al., 2021). On the other hand, it is possible that the oxidative stress observed in *P. reticulata* exposed to PSPD-2002 and PSPD-2003 peptides is related to some enzymatic dysfunction caused by their strong interactions with SOD and CAT, suggested by molecular docking analysis (Fig. 8). In this case, the increased activity of these enzymes may also have resulted from some physiological compensatory mechanism to counterbalance the high production of the evaluated oxidative biomarkers.

Eventually, it is important to emphasize that although our study gathered evidence on the negative effects of SARS-CoV-2 Spike protein peptides (PSPD-2002 and PSPD-2003) on behavior, growth, and redox balance in *P. reticulata* juveniles, many issues still need to be investigated. Assessments of the toxicity of these peptides, as well as other SARS-CoV-2 particles, at different concentrations and exposure periods and at different stages of animal life constitute some future investigative perspectives. Equally important will be to expand the list of biomarkers to be evaluated (e.g.: histopathological, molecular, endocrine, among others), as well as the environmental representativeness of the animal models to be studied, since the sensitivity to viral peptides can be different between non-host organisms of the new coronavirus.

Monitoring the effects observed in the adult life of animals, as well as their consequences at the population level and on their ecological roles, should also be the focus of further studies. We believe that approaches of this nature will be useful for a better understanding of the extent of the environmental/ecological impact of the COVID-19 pandemic, whether in the short, medium, or long term.

5. Conclusion

In conclusion, our study confirms the hypothesis that exposure to PSPD-2002 and PSPD-2003 peptides induces physiological changes in *P. reticulata* juveniles, marked by behavioral changes, growth, cholinesterasic effect, and redox imbalance. Taken together, our data reinforce that the (eco)toxicological risks arising from the presence of SARS-CoV-2 Spike protein peptides in freshwater environments cannot be neglected. Therefore, our study appends the list of recent works that exhibit that aquatic contamination by SARS-Cov-2 particles can constitute a significant environmental concern of the COVID-19 pandemic. Thus, we strongly suggest that further studies must be carried out, in the hope of obtaining subsidies for the proposition of mitigation/remediation measures for the environmental and ecological impacts arising from SARS-CoV-2 impact.

Declaration of Competing Interest

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

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.aquatox.2022.106104.

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