

1           **An insight into neurotoxic and toxicity of spike fragments SARS-CoV-2 by**  
2                           **exposure environment: A threat to aquatic health?**

3  
4  
5 Ives Charlie-Silva<sup>1\*</sup>, Amanda P. C. Araújo<sup>2,3</sup>, Abraão T. B. Guimarães<sup>2,3</sup>, Flávio P Veras<sup>4</sup>, Helyson L.  
6 B. Braz<sup>5</sup>, Letícia G. de Pontes<sup>6</sup>, Roberta J. B. Jorge<sup>7</sup>; Marco A. A. Belo<sup>8,9</sup>, Bianca H V. Fernandes<sup>10</sup>,  
7 Rafael H. Nóbrega<sup>11</sup>, Giovane Galdino<sup>12</sup>, Antônio Condino-Neto<sup>6</sup>, Jorge Galindo-Villegas<sup>13</sup>, Glauca  
8 M. Machado-Santelli<sup>14</sup>, Paulo R. S. Sanches<sup>15</sup>, Rafael M. Rezende<sup>16</sup>, Eduardo M. Cilli<sup>15</sup>, Guilherme  
9 Malafaia<sup>2,3\*</sup>

10  
11  
12 <sup>1</sup>Department of Pharmacology, Institute of Biomedical Sciences, University of Sao Paulo (SP, Brazil).

13 <sup>2</sup>Post-graduation Program in Biotechnology and Biodiversity, Goiano Federal Institution and Federal  
14 University of Goiás (GO, Brazil).

15 <sup>3</sup>Biological Research Laboratory, Post-graduation Program in Conservation of Cerrado Natural  
16 Resources, Goiano Federal Institute – Urata Campus (GO, Brazil).

17 <sup>4</sup>Center of Research in Inflammatory Diseases, Ribeirão Preto Medical School,  
18 University of São Paulo, Ribeirão Preto, São Paulo, Brazil.

19 <sup>5</sup>Postgraduate Program in Morphological Science, Department of Morphology, School of Medicine,  
20 Federal University of Ceara, Delmiro de Farias St., 60.430-170, Fortaleza-CE, Brazil.

21 <sup>6</sup>Department of Immunology, Institute of Biomedical Sciences. University of Sao Paulo, Sao Paulo,  
22 SP, Brazil (SP, Brazil).

23 <sup>7</sup>Drug Research and Development Center, Federal University of Ceara, Coronel Nunes de Melo St.,  
24 1000, 60.430-275, Fortaleza-CE, Brazil; Department of Physiology and Pharmacology, School of  
25 Medicine, Federal University of Ceara, Coronel Nunes de Melo St., 1127, 60.430-275, Fortaleza-  
26 CE, Brazil.

27 <sup>8</sup>Laboratory of Animal Pharmacology and Toxicology, Brazil University, Descalvado, SP, Brazil.

28 <sup>9</sup>Department of Preventive Veterinary Medicine, São Paulo State University (UNESP),  
29 Jaboticabal, SP, Brazil.

30 <sup>10</sup>Laboratório de Controle Genético e Sanitário, Diretoria Técnica de Apoio ao Ensino e  
31 Pesquisa, Faculdade de Medicina da Universidade de São Paulo.

32 <sup>11</sup>Reproductive and Molecular Biology Group, Institute of Biosciences, São Paulo State University,  
33 Botucatu, São Paulo, Brazil.

34 <sup>12</sup> Institute of Motricity Sciences, Federal University of Alfenas, Alfenas, MG, Brazil.

35 <sup>13</sup>Faculty of Biosciences and Aquaculture, Nord University, 8049 Bodø, Norway

36 <sup>14</sup>Department of Cell Biology, Institute of Biomedical Sciences, University of São Paulo, São Paulo,  
37 Brazil.

38 <sup>15</sup>Institute of Chemistry, São Paulo State University (UNESP), Araraquara -SP, Brazil.

39 <sup>16</sup>Brigham and Women's Hospital, Harvard Medical School, 75 Francis St, Boston, United States.

40

41

42

43

44

45

46 **\*Corresponding author:**

47 **\*Ives Charlie-Silva PhD.** e-mail: [charliesilva4@hotmail.com](mailto:charliesilva4@hotmail.com)

48 Department of Pharmacology, Institute of Biomedical Sciences, University of Sao Paulo (SP, Brazil)

49

50 **\*Guilherme Malafaia. PhD.** e-mail: [guilhermeifgoiano@gmail.com](mailto:guilhermeifgoiano@gmail.com)

51 Biological Research Laboratory, Goiano Federal Institution – Urutaí Campus, Rodovia Geraldo Silva

52 Nascimento, 2,5 km, Zona Rural, Urutaí, Brazil.

53

54

55

56

57

58

59 **HIGHLIGHTS**

60

- 61 • SARS-CoV-2 spike protein peptides (PSDP) were synthesized, purified, and characterized by
- 62 solid phase peptide synthesis.
- 63 • PSDP peptides promoted REDOX imbalance and acute neurotoxicity in tadpoles
- 64 (*Physalaemus cuvieri*)
- 65 • *In silico studies* have shown interactions between peptides and acetylcholinesterase and
- 66 antioxidant enzymes
- 67 • Aquatic particle contamination of SARS-CoV-2 can constitute additional environmental
- 68 damage

69

70

71 **ABSTRACT**

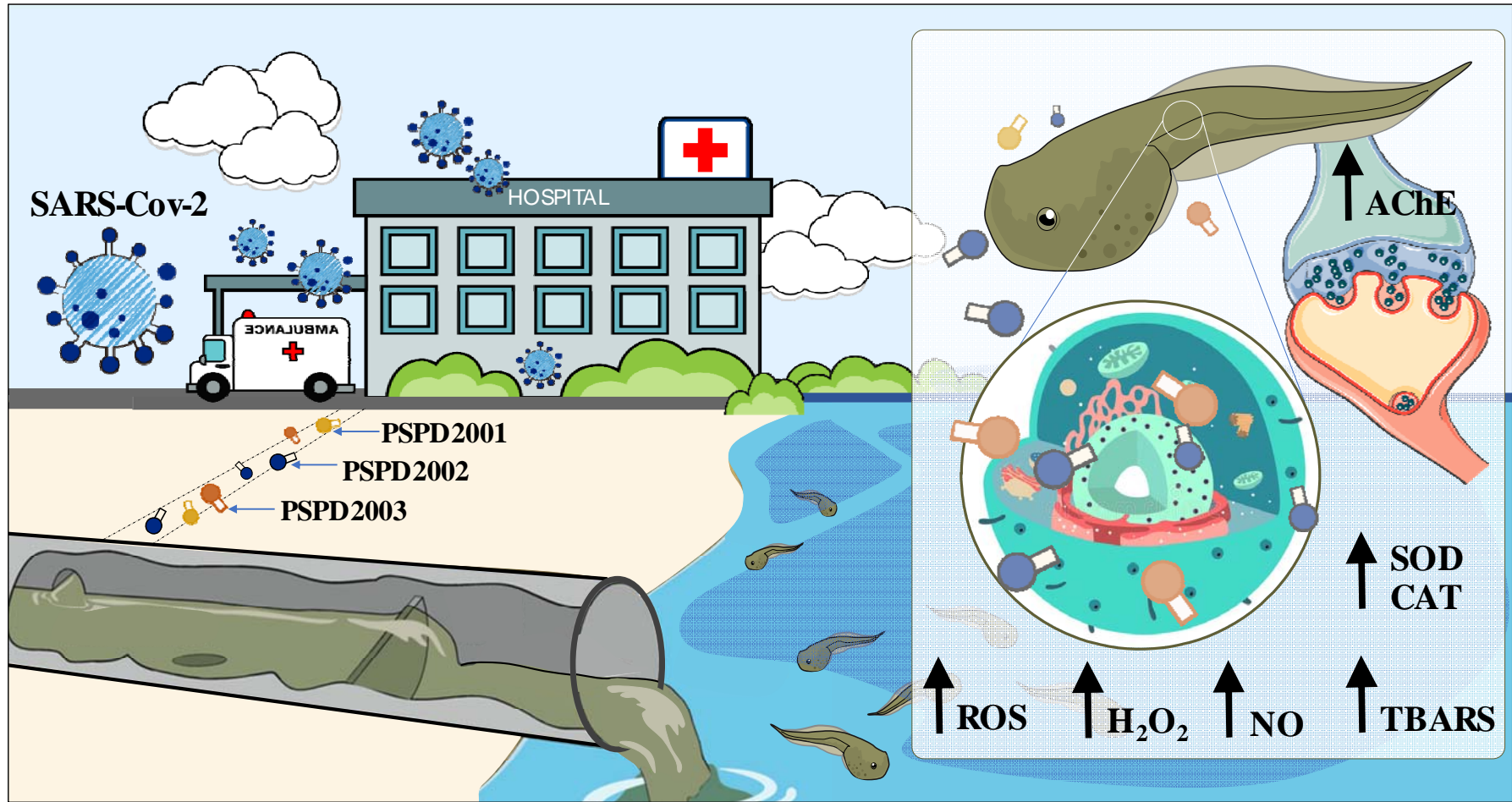
72 The Spike protein (S protein) is a critical component in the infection of the new coronavirus  
73 (SARS-CoV-2). The objective of this work was to evaluate whether peptides from S protein could  
74 cause negative impact in the aquatic animals. The aquatic toxicity of SARS-CoV-2 spike protein  
75 peptides derivatives has been evaluated in tadpoles (n = 50 tadpoles / 5 replicates of 10 animals)  
76 from species *Physalaemus cuvieri* (Leptodactylidae). After synthesis, purification, and  
77 characterization of peptides (PSDP2001, PSDP2002, PSDP2003) an aquatic contamination has  
78 been simulated with these peptides during 24 hours of exposure in two concentrations (100 and 500  
79 ng/mL). The control group ("C") was composed of tadpoles kept in polyethylene containers  
80 containing de-chlorinated water. Oxidative stress, antioxidant biomarkers and neurotoxicity activity  
81 were assessed. In both concentrations, PSDP2002 and PSDP2003 increased catalase and superoxide  
82 dismutase antioxidant enzymes activities, as well as oxidative stress (nitrite levels, hydrogen  
83 peroxide and reactive oxygen species). All three peptides also increased acetylcholinesterase activity  
84 in the highest concentration. These peptides showed molecular interactions in silico with  
85 acetylcholinesterase and antioxidant enzymes. Aquatic particle contamination of SARS-CoV-2 has  
86 neurotoxic effects in *P. cuvieri* tadpoles. These findings indicate that the COVID-19 can constitute  
87 environmental impact or biological damage potential.

88

89

90 **Key words:** oxidative stress; coronavirus; amphibians; acetylcholinesterase, SARS-Cov-2

## GRAPHICAL ABSTRACT



## 93 1. INTRODUCTION

94 Coronavirus Disease-2019 (COVID-19) pandemic, caused by SARS-CoV-2 (Severe acute  
95 respiratory syndrome coronavirus 2), an emergent beta-coronavirus threatening human  
96 health, has led to a dramatic worldwide crisis and presents unprecedented global challenges  
97 on everybody's daily life, social aspects, political affairs, and health measures (Chakraborty &  
98 Prasenjit, 2020)

99 Remarkably, the poor and the most vulnerable people are at critical high risk, and  
100 Oxfam, an international confederation of 20 NGO's already calculates that nearly 500 million  
101 people worldwide may succumb to poverty resulting from the same (OI, 2020). By Dec. 27,  
102 over 79.2 million cases and over 1.7 million deaths have been reported since the start of the  
103 pandemic (FAO, 2020). Resulting from the same, in only 12 months, we have learned a lot  
104 about SARS-CoV-2. Our ability to test for and manage COVID-19 has improved, but ongoing  
105 debate remains about how SARS-CoV-2 is transmitted (Editorial Lancet, 2020).

106 The most recurrent forms of SARS-CoV-2 transmission are through direct contact with  
107 an infected person (Meyerowitz et al., 2020), inhaling respiratory droplets containing the  
108 virus (Harrison et al., 2020), or accessing a contaminated environment where suspended  
109 particles are present over longer distances and time than droplet transmission (Graham et al.  
110 2020).

111 However, by reviewing the environmental threats of the virus reported so far, it is  
112 concluded that the virus can survive on inanimate surfaces such as metal, glass, or plastic for  
113 up to 9 days if any effective disinfection procedure with ozone, ethanol, hydrogen peroxide,  
114 UV light, chlorine or its derivatives as sodium hypochlorite is not conducted in between  
115 (Kampf et al. 2020).

116 Although the direct contact described above concerns meaningful, a different  
117 environmental transmission source with the virus now recognized is the indirect contact  
118 through the infected people's stool and urine (Chen et al., 2020; Xiao et al., 2020; Jones et al.,  
119 2020). Unconventional studies support this notion by reporting positive SARS-CoV-2 viral  
120 titers in domestic sewages (Pandey et al., 2020; Elsamadony et al., 2020; Polo et al., 2020).

121 So far, Sars-Cov-2 has been detected in several countries wastewaters of the American,  
122 European, and Asian continents, suggesting as mandatory the monitoring of the secondary  
123 transmission of the new coronavirus via wastewater (Liu et al. 2020). On a more compelling  
124 perspective, strong evidence suggests that surveillance of primarily settled solids in  
125 wastewater through one-step ddPCR is a solid strategy to track the spread of Covid-19 disease  
126 transmission before the clinical cases break out in a particular location (Graham, 2020).

127 Moreover, following this trend can shed light on the characteristics of infection that are  
128 difficult to capture in clinical investigations, such as the dynamics of infection and early viral  
129 elimination (Wu et al., 2020).

130 The increase in the generation of household waste (Sharma et al., 2020; Zand& Heir,  
131 2020; Urban et al., 2021), hospitals (Abu-Qdais et al., 2020; Sangkham, 2020; Yang et al.,  
132 2020), and notable civil buildings (Carvalho et al., 2020; Abu-Rayash et al., 2020; Santiago et  
133 al., 2020) constitute some of the environmental grounds where no information on the  
134 ecotoxicological effects of SARS-CoV-2 proteic or genetic structural components impact on  
135 freshwater vertebrates exists.

136 Therefore, this lack of knowledge requires urgent attention by developing studies to  
137 assess how COVID-19 impacts the aquatic populations in the close vicinity of the  
138 anthropogenic activities described above. Such studies may focus on supporting actions or  
139 strategies on the remediation or at least mitigation of impacts in favor of conserving non-  
140 target species at the edge of any Sars-Cov-2 variant.

141 The Spike (S) protein is a critical component of the new Sars-Cov-2 coronavirus found  
142 on the surface of the SARS-Cov-2 virus, giving it a "crown" appearance. The S protein is a  
143 granule-shaped structural protein with a length of about 1200 aa, which helps the virus bind  
144 to cell receptors and mediates viral infection and pathogenesis (Coughlan, 2020). The S  
145 protein plays a key role in the receptor recognition and cell membrane fusion process with  
146 ACE-2 (angiotensin-converting enzyme 2) (Huang et al., 2020). Therefore, it is not surprising  
147 this ligand-receptor interaction of the S protein is the primary target to produce vaccines  
148 against COVID-19, as reported in different studies (Bangaru et al., 2020; Samrat et al., 2020;  
149 Keech et al., 2020; Yang et al., 2020; Qi et al., 2020; Ravichandran et al., 2020).

150 Several in vivo platforms to dissect the cellular and molecular programs governing  
151 Sars-Cov-2 viral dissemination on vertebrates are available. However, the number of aquatic  
152 model animals that may support trials and provide reliable information is almost inexistent.  
153 Among them, the zebrafish model represents an attractive model to explore the desired  
154 effects on the context of a full vertebrate (Galindo-Villega, 2020). Unfortunately, the zebrafish  
155 has not been vigorously infected in vivo trials so far by the causative agent of Covid-19  
156 (Gaudin & Goetz, 2021).

157 Following a synthetic approach in previous research, we have developed three  
158 peptides of the full-length SARS-CoV-2 Spike protein (PSPD2001, PSPD2002, and PSPD 2003)  
159 after a pattern memorization phagolysosomal proteolysis (Fernandes et al. 2020). To attempt  
160 to elucidate whether and how the Sars-Cov-2 influence the aquatic animals, in this study, we

161 investigate the same by adding the three produced synthetic peptides to mimic the resulting  
162 Covid-19 aquatic contamination in wastewater. The tadpole *Physalaemus cuvieri*, is a prevalent  
163 amphibian species found in many freshwater habitats throughout Brazil and South America  
164 ((Miranda et al., 2019; Herek et al., 2020; Araújo et al., 2020ab; Rutkoski et al., 2020). Its  
165 population stability and abundance in the areas that occur (Frost 2017), good adaptability in the  
166 laboratory, and early biological response justify the species' choice (Herek et al., 2020; Araújo et  
167 al., 2020ab; Rutkoski et al., 2020). Previous studies using this species report the effects of water  
168 pollution caused by wastewater runoff (Wrubleswski et al. 2018). Therefore, in this study, we  
169 selected *P. cuvieri* as our choice of a translational model vertebrate.

170 From different biomarkers indicative of an imbalance in oxidation-reduction (REDOX)  
171 and neurotoxicity processes, we aimed to test the hypothesis that nanometric concentrations  
172 of the SARS-CoV-2 Spike protein fragments in water may affect the health of amphibians. We  
173 believe that studies like ours are needed not only to expand our knowledge about the impacts  
174 of COVID-19 on aquatic biodiversity; but also to predict the environmental impacts of the  
175 recent pandemic on the populations of neotropical amphibians, which have already, over the  
176 years, shown a drastic population decline (Pechmann et al., 1991; Blaustein et al., 2002;  
177 Ranvestel et al., 2004; Grant et al., 2020).

178

179

## 180 2. MATERIAL AND METHODS

### 181 2.1. Synthesis, purification, and characterization of peptides

#### 182 2.1.1. Synthesis of SARS-CoV-2 Spike protein peptides

183 The peptides were obtained manually using the solid phase peptide synthesis method (SPFS)  
184 using the Fmoc strategy (Raibaut et al., 2014; Behrendt et al., 2016). The couplings were carried  
185 out by activating the carboxyl groups of the Fmoc-amino acids with a solution of  
186 diisopropylcarbodiimide and hydroxybenzotriazole (HOBT), for 2 h. In this step, a 2-fold excess of  
187 Fmoc-amino acids and coupling agents in relation to the number of reactive sites in the resin was  
188 used. Deprotection of the amino group after coupling, i.e., removal of the base labile Fmoc group  
189 was carried out by reaction with a 20% solution of 4-methyl-piperidine in dimethylformamide  
190 (DMF) following the exit of the protective group through the colorimetric test ninhydrin (Luna et  
191 al., 2016), which identifies free amine groups converting the yellow solution to violet-blue after  
192 incubation at 110°C for 3 min. The resins used for synthesis were Fmoc-Cys (Trt)-Wang, Fmoc-Thr  
193 (TBu)-Wang, and Fmoc-Asn (Trt)-Wang for peptides Arg-Val-Tyr-Ser-Ser-Ala-Asn-Asn-Cys-

194 COOH (PSPD2001); Gln-Cys-Val-Asn-Leu-Thr-Thr-Arg-Thr-COOH (PSPD2002) and Asn-Asn-  
195 Ala-Thr-Asn-COOH (PSPD2003), respectively

#### 196 2.1.2. Cleavage of SARS-CoV-2 Spike protein peptides

197 After coupling all the amino acid residues from the peptide sequences, the chains were  
198 removed from the solid support by acid cleavage using trifluoroacetic acid (TFA) for 2 h [similarly  
199 to Guy & Fields (1997)]. In addition to TFA, reaction suppressors were added according to the  
200 sequence of each peptide. After cleavage, the peptides were precipitated with cold ether and later  
201 extracted with 0.045% TFA solution in purified water. The solutions were lyophilized to obtain solid  
202 crude material.

203

#### 204 2.1.3. Purification of SARS-CoV-2 Spike protein peptides

205 The crude compounds were purified by high-performance liquid chromatography (HPLC)  
206 with a reverse-phase column using different purification methods according to the retention time  
207 obtained in a gradient program of 5 to 95% in 30 min (exploration gradient) in Analytical HPLC  
208 (Klaassen et al.,2019). Table S1 (see “Supplementary Material”) presents a summary of the  
209 purification methods adopted in our study. The purification solvents were water containing 0.045%  
210 TFA (solvent A) and acetonitrile containing 0.036% TFA (solvent B).

211 After collecting, lyophilizing, and weighing the pure material fractions, its yield was  
212 calculated, obtaining 13.0% for PSPD2001, 21.4% for PSPD2002, and 18.2% for PSPD2003. The  
213 pure and solid material was subjected to chromatographic analysis to determine the purity of the  
214 final product. Only compounds with purity equal to or greater than 95% were considered for  
215 biological analysis,

216

#### 217 2.1.4. Characterization of SARS-CoV-2 Spike protein peptides

218 The analysis of the synthesized peptides' identity was carried out in a mass spectrometer  
219 (Metzger et al., 1994) Thermo LCQ-fleet, with ESI-IT-MS configuration. For this, the sample  
220 solutions were directly infused at a concentration of approximately 10 mg/L in acetonitrile/water  
221 containing 0.1% v/v formic acid. The infusion rate was adjusted to 5.0  $\mu$ L/min, and the electrospray  
222 source was operated in a positive mode, applying 4.5 kV to the electrospray capillary.

223

#### 224 2.2. Alignment of SARS-CoV-2 Spike protein peptides

225 The similarities between the PSPD2001, PSPD2002 and PSPD2003 peptides synthesized in  
226 the present study were tested using the CLUSTAL W version 1.83 software [Higgins et al. (1996),  
227 Pais et al. (2014) - <http://www.ebi.ac.uk/clustalw/>]. The peptides were aligned with proteins



228 deposited in the NCBI/BLAST (Basic Local Alignment Search Tool), consisting of a set of programs  
229 that look for similarities between different sequences. The investigated sequences' alignment was  
230 carried out with the nucleic acid and/or protein database (<http://www.ncbi.nlm.nih.gov/blast>).  
231 Within BLAST, the search was carried out in the “Protein blast” using as a database the “Swissprot  
232 protein sequence (swissprot)”, algorithm - blastp (protein BLAST) and the search was restricted to  
233 *Physalaemus cuvieri* (taxid:218685). The database UniProtKBSwissProt (<http://www.uniprot.org/>)  
234 was used to obtain detailed information on the protein aligning with the selected peptides revealed.

235

### 236 2.3. Model system and experimental design

237 To evaluate the synthesized peptides' aquatic toxicity, we used tadpoles of the species  
238 *Physalaemus cuvieri* (Leptodactylidae) as a model system. All tadpoles used came from an ovigerous  
239 mass (containing approximately 1500 eggs), according to Pupin et al. (2010). They were collected in  
240 a lentic environment (Urutaí, GO, Brazil) surrounded by native vegetation from the Cerrado biome,  
241 under license no. 73339-1 of the Biodiversity Authorization and Information System  
242 (SISBIO/MMA/ICMBio) in Brazil.

243 Upon arrival at the laboratory, the eggs were kept in an aquarium (40.1 x 45.3 x 63.5 cm)  
244 containing 80 L of naturally dechlorinated water (for at least 24 h), under controlled light  
245 conditions (light-dark cycle, 12:12 h), temperature ( $26\text{ }^{\circ}\text{C} \pm 1\text{ }^{\circ}\text{C}$  - similar to the natural  
246 environment) and constant aeration (maintained by air compressors), being fed once a day (ad  
247 libitum) with commercial fish food (guarantee levels: 45% crude protein, 14% ether extract, 5%  
248 crude fiber, 14% mineral matter and 87% dry matter). After the eggs hatched, the tadpoles  
249 remained in these conditions until they reached stage 27G, according to Gosner (1960) (body  
250 biomass:  $70\text{ mg} \pm 4.1\text{ mg}$  and total length:  $20.1\text{ mm} \pm 0.7\text{ mm}$  - mean  $\pm$  SEM). The healthy  
251 tadpoles (i.e., with normal swimming movements and without morphological deformities or  
252 apparent lesions) were divided into seven experimental groups ( $n = 50$  tadpoles / each - 5 replicates  
253 composed of 10 animals/each. The control group (“C”) was composed of tadpoles kept in  
254 polyethylene containers containing 50 ml of de-chlorinated water, free of any peptide. The animals  
255 kept in water containing the peptides comprised the groups “PSPD2001”, “PSPD2002”, and  
256 “PSPD2003”. Two concentrations were tested for each peptide (100 and 500 ng/mL, defined based  
257 on reports that SARS-CoV-2 in sweet environments occurs in minimal concentrations (Shutler et  
258 al., 2020; Guerrero-Latorre et al., 2020; Tran et al., 2020). The exposure period (24 h; in the static  
259 system) was defined considering the low persistence of SARS-CoV-2 in the aquatic environment  
260 after being released with human feces (Bivins et al., 2020). We emphasize that, throughout the  
261 exposure period, different physical-chemical parameters of the quality of the exposure waters were

262 monitored (every 6 hours), keeping them equitable between treatments (temperature:  $23^{\circ}\text{C} \pm 1.14$ ;  
263 atmospheric pressure (atm) :  $0.91 \pm 0.0001$ ; electrical resistivity ( $\Omega\text{m}$ ):  $0.01 \pm 0.0001$ ; electrical  
264 conductivity ( $\mu\text{S}/\text{cm}^2$ ):  $96.2 \pm 1.83$ ; total dissolved solids (mg/L):  $48, 2 \pm 0.83$ ; salinity:  $0.04 \pm$   
265  $0.004$ ; oxidation-reduction potential (ORP):  $130.21 \pm 6.17$ ; dissolved oxygen (mg/L):  $7.72 \pm 0.78$   
266 and pH:  $7, 2 \pm 0.38$ ).

267

## 268 **2.4. Toxicity biomarkers**

269 We evaluated peptide-induced toxicity from predictive biomarkers of REDOX imbalance  
270 and neurotoxicity after exposure, considered classic and essential parameters in ecotoxicological  
271 studies (Valavanidis et al., 2016). For this, pools of four animals/each composed the samples to be  
272 analyzed. Such animals were weighed and later macerated in 1 mL of phosphate-buffered saline  
273 (PBS), centrifuged at 13.000 rpm for 5 min (at  $4^{\circ}\text{C}$ ). The supernatant was separated into aliquots to  
274 be used in different biochemical evaluations. Entire bodies were used in the experiment due to the  
275 hard time isolating specific organs from small animals. Organ-specific biochemical assessment in  
276 tadpole requires highly accurate dissection due to their small size, making it difficult to process large  
277 sample numbers under a time constraint (Khan et al. 2015). Organ “contamination” by organic  
278 matter and/or by other particles consumed by tadpole can be biased at biochemical analysis applied  
279 to organs at dissection time (Lusher et al. 2017; Guimarães et al., 2021).

280

### 281 **2.4.1. REDOX state**

#### 282 **2.4.1.1. Oxidative stress biomarkers**

283 The effects of exposure to peptides on oxidative stress reactions were evaluated based on  
284 indirect nitric oxide (NO) determination on REDOX regulated processes via nitrite measurement  
285 (Soneja et al. 2005); thiobarbituric acid reactive species (TBARS), a predictive of lipid peroxidation  
286 (De-Leon & Borges, 2020); production of reactive oxygen species (ROS) and on hydrogen peroxide  
287 ( $\text{H}_2\text{O}_2$ ), which plays an essential role in responses to oxidative stress in different cell types (Sies,  
288 2017). The Griess colorimetric reaction was used to measure NO (Grisham et al., 1996). This  
289 reaction consisted of detecting nitrite resulting from NO oxidation. TBARS levels were determined  
290 based on procedures described by Ohkawa et al. (1979) and modified by Sachett et al. (2020), with  
291 adaptations for conduction in microtubes and ELISA microplate reading. The reagent of 1,1,3,3-  
292 tetra-ethoxy-propane was used as a standard solution in the reaction with thiobarbituric acid (TBA)  
293 reactive substance, according Ro et al. (2020). In brief, this method's principle depends on the  
294 Determination of the pink color produced by TBA interaction with malondialdehyde (MDA). The

295 production of hydrogen peroxide and ROS was evaluated using the methodologies proposed by  
296 Elnemma et al. (2004) and Maharajan et al. (2018).

297

298

299

#### 300 **2.4.1.2. Antioxidant response biomarkers**

301 The activation or suppression of antioxidant activity by peptides was assessed by determining  
302 catalase and superoxide dismutase (SOD) enzyme activities, which are considered critical first-line  
303 antioxidants for defense strategies against oxidative stress (Ighodaro&Akinloye, 2018; Jing et al.,  
304 2020). While catalase activity was assessed based on Sinha et al. (1972) andMontalvão et al.  
305 (2021). The SOD was determined according to the method described by Del-Maestro & McDonald  
306 (1985) (initially) and adapted by Estrela et al. (2021). To assess the balance between the synthesis  
307 of hydrogen peroxide by SOD and its decomposition by catalase, the SOD/CAT ratio was calculated  
308 and recorded, as Liu et al. (2017).

309

#### 310 **2.4.2. Neurotoxicity**

311 Peptide neurotoxicity was assessed by quantifying acetylcholinesterase (AChE) activity,  
312 based on the method by Ellman et al. (1961), with minor detailed modifications in Estrela et al.  
313 (2021). AChE activity is used as a cholinergic function marker since it regulates the acetylcholine  
314 (ACh) amount interacting with its receptors (Tougu, 2001).

315

#### 316 **2.4.3. Determination of the protein level**

317 All results of the biochemical analyzes were expressed by the “g of proteins” of the samples.  
318 In this case, the protein level was determined with a commercial kit (Biotécnica Ind. Com. LTD,  
319 Varginha, MG, Brasil. CAS number: 10.009.00) based on biuret reaction (Gornall et al., 1949;  
320 Henry et al., 1957). In general,  $\text{Cu}^{2+}$  ions, in an alkaline medium, react with the peptide bonds of  
321 proteins forming the blue complex specifically with protein, and the intensity of color, measured by  
322 an ELISA reader at a wavelength of 492 nm, is proportional to the protein concentration.

323

#### 324 **2.5. Bioinformatics *in silico* analysis**

325 Seeking to predict the binding mode and affinity of the bonds between the peptides  
326 synthesized in our study and the protein structures of the enzymes AChE, catalase, and SOD, we  
327 performed docking and chemoinformatic screens (Kolb et al., 2009). For this, we obtained the  
328 peptide ligand PSPD2002 and PSPD2003 in three dimensions through the web server PEP-FOLD3

329 (<https://bioserv.rpbs.univ-paris-diderot.fr/services/PEP-FOLD3/>). Protein structures and sequences  
330 of the *P.cuivieri* (i.e.:Leptodactylidae) taxonomic family were not found in the biological structure  
331 databases. Therefore, we use as target structures those from the Xenopodinae family, a family  
332 phylogenetically close to the group of Leptodactylidae (Jetz& Pylon, 2018). The AChE and catalase  
333 enzymes' structures were obtained using the homology construction technique with values of  
334 similarity 65.48% and 87.14% to structures (targets) used for comparative modeling on the server  
335 SWISS-MODEL (<https://swissmodel.expasy.org/>), respectively. On the other hand, the structure of  
336 the SOD was obtained by Research Collaboratory for Structural Bioinformatics protein databank  
337 (<https://www.rcsb.org>) PDB code: 1XSO with 1.49Å resolution, obtained by X-ray diffraction of  
338 Xenopodinae origin. For molecular docking simulations, AutoDock tools (ADT) v4.2 (Morris et al.,  
339 2009) and AutoDock Vina 1.1.2 (Trott & Olson, 2010) were used. The procedure was carried out  
340 by removing water molecules and other residues present in the target structures. A polar hydrogen  
341 group was added to establish hydrogen bonds between the macromolecule and the ligand tested.  
342 The grid box was chosen based on the native ligand of the macromolecules (targets). The binding  
343 potency ( $\Delta G$  affinity) was used to determine better molecular interactions. The results were  
344 visualized using ADT and UCSF Chimera X (Pettersen et al., 2021).

345

## 346 2.6. Data analysis

347 GraphPad Prism Software Version 8.0 (San Diego, CA, USA) was used to perform the  
348 statistical analysis. Initially, data were checked for deviations from the normality of variance and  
349 homogeneity of variance before analysis. Normality of data was assessed using the Shapiro-Wilks  
350 test, and homogeneity of variance by the Bartlett's test. Multiple comparisons were performed using  
351 a one-way ANOVA and Tukey's posthoc analysis (for parametric data) or the Kruskal-Wallis test,  
352 with Dunn's posthoc (for non-parametric data). Correlation analysis was performed through  
353 Pearson's (parametric data) or Spearman's method (non-parametric data). Besides, the regression  
354 analysis was performed when significant differences were detected between different treatments.  
355 Levels of significance were set at (p) less than 0.05, 0.01, or 0.001.

356

## 357 3. RESULTS AND DISCUSSION

### 358 3.1. Synthesis and characterization of SARS-CoV-2 Spike peptides

359 Our study's first stage was to synthesize and characterize the SARS-CoV-2 Spike peptides  
360 arbitrary named PSPD2001, PSPD2002, and PSPD2003. During the peptides' cleavage, we  
361 performed the addition of different reaction suppressors to avoid the return of the side chain  
362 protectors present in some amino acids with a reactive side chain. The results obtained in this step

363 are shown in Table 1. Regarding the mass spectrometry analysis, the spectra obtained indicated the  
 364 molecular mass/charge ratio (m/z) of the identified compounds, allowing us to confirm the  
 365 deprecated molecules' achievement. The spectra can be seen in Figure S1 (see "Supplementary  
 366 Material"), and Table 2 summarizes the results obtained in this step. Figure 1 also shows the  
 367 structural models of the PSPD2001, PSPD2002, and PSPD2003.

368

369

370

371 **Table 1.** General information on the synthesis of the peptides used in the present study.

Sequences	Codes	M.W.	Resins	Cleavage Solution
Arg-Val-Tyr-Ser-Ser-Ala-Asn-Asn-Cys-COOH	PSPD2001	1013,09	Fmoc-Cys-Wang SG*: 0,55 mmol/g	94% TFA 2,5% H <sub>2</sub> O 2,5% DODT* 1% TIS*
Gln-Cys-Val-Asn-Leu-Thr-Thr-Arg-Thr-COOH	PSPD2002	1035,18	Fmoc-Thr-Wang SG: 0,55 mmol/g	94% TFA 2,5% H <sub>2</sub> O 2,5% DODT 1% TIS
Asn-Asn-Ala-Thr-Asn-COOH	PSPD2003	532,51	Fmoc-Asn-Wang SG: 0,51 mmol/g	95% TFA 2,5% H <sub>2</sub> O 2,5% TIS

372 M.W.: molecular weight; SG: substitution grade– Number of active sites available for the growth of the peptide chain.

373 DODT: 2,2'-(Ethylendioxy) diethanol; TIS: triisopropylsilane; TFA: trifluoroacetic acid.

374

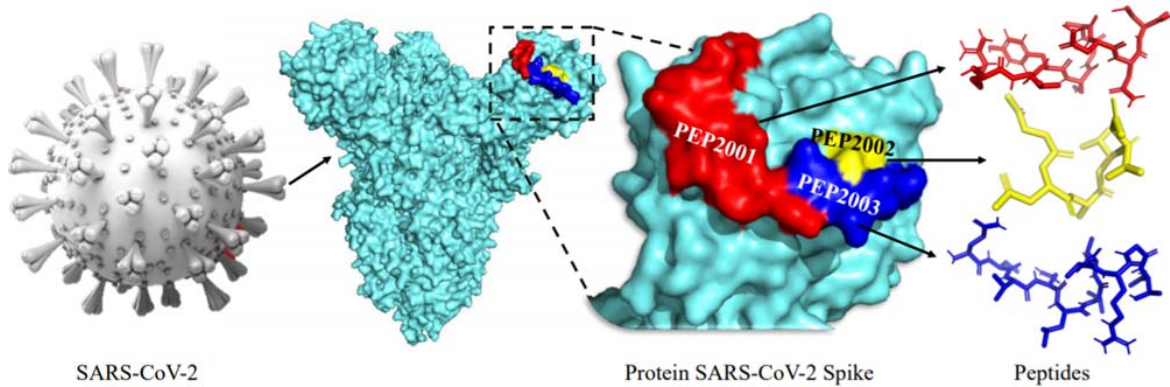
375 **Table 2.** Mass spectrometry analysis after peptide purification

	PSPD2001	PSPD2002	PSPD2003
M.W.	1013,09 g/mol	1035,18 g/mol	532,51 g/mol
m/z calculated	1014,09; 507,54	1036,18;518,59	533,51
m/z obtained	1013,41; 507,52	1035,41;518,96	533,04;1064,86

376 M.W.: molecular weight; PSPD2001: Arg-Val-Tyr-Ser-Ser-Ala-Asn-Asn-Cys-COOH; PSPD2002: Gln-Cys-Val-Asn-Leu-Thr-Thr-  
 377 Arg-Thr-COOH; PSPD2003: Asn-Asn-Ala-Thr-Asn-COOH.

378

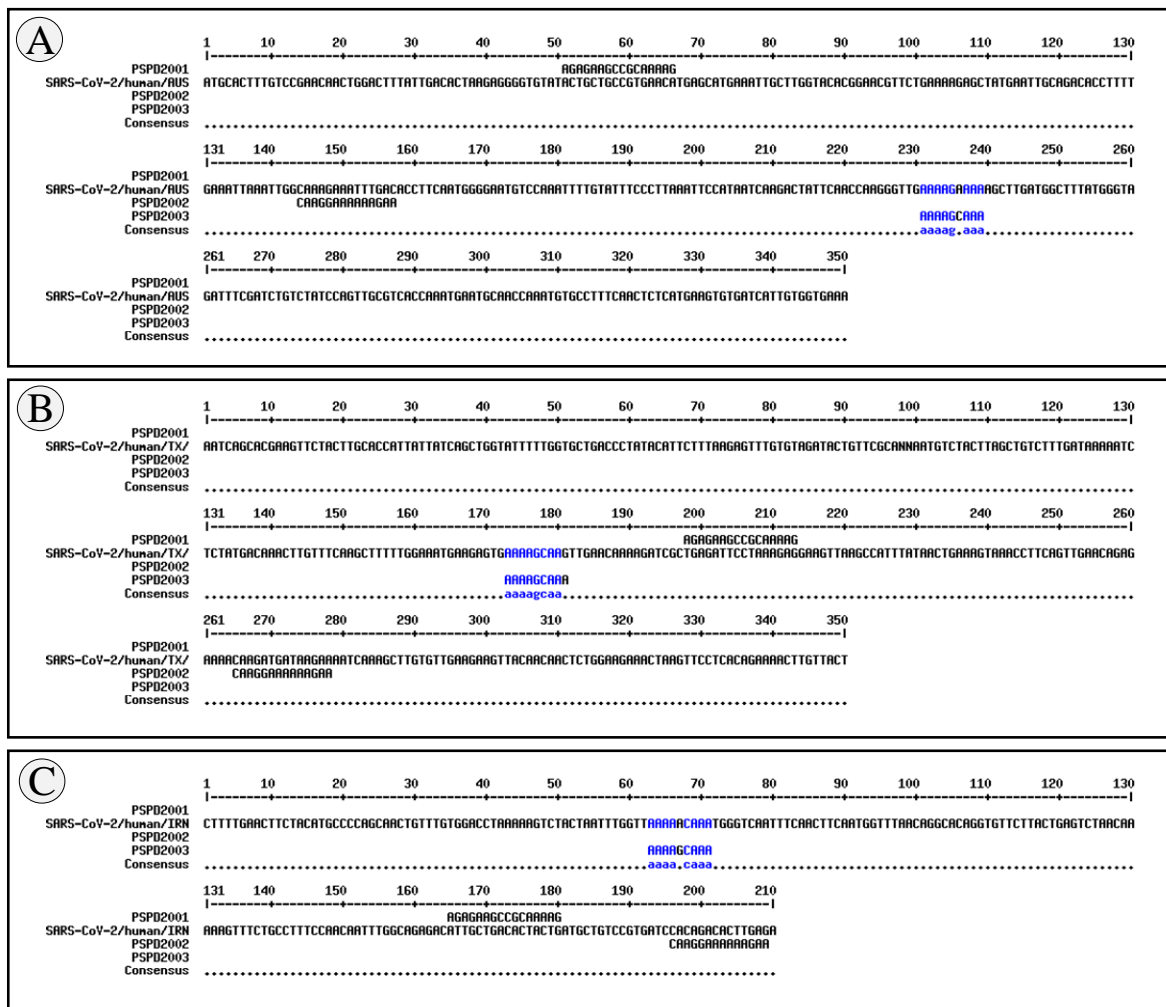
379



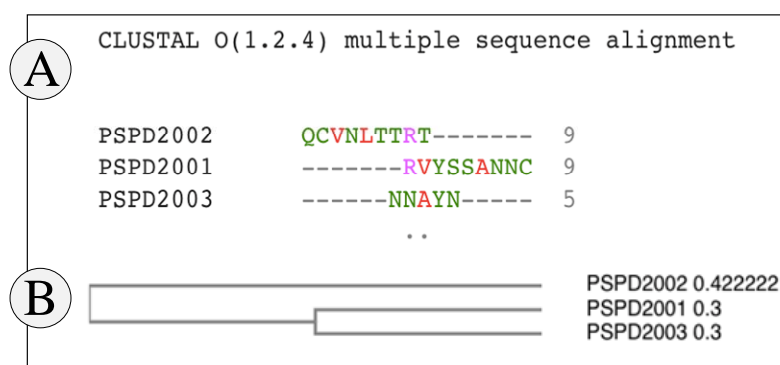
**Figure 1.** Structural models of peptides (A) PSPD2001, (B) PSPD2002, and (C) PSPD2003 that were synthesized in the present study.

380

381           Regarding the alignment of the obtained sequences, our analyzes revealed the existence of  
382 similarities between the nucleotide sequences of the synthesized peptides and different regions  
383 conserved in SARS-Cov-2, whose comparisons were made from three datasets COVID, originated  
384 from Texas (USA), Iran, and Australia (Figure 2). These data demonstrated that the peptides  
385 PSPD2001, PSPD2002, and PSPD2003 are, in fact, part of the protein structure of the etiological  
386 agent of COVID-19. However, the sequences obtained for *P. cwieri* (taxid: 218685) showed two  
387 main agreements belonging to five of the total of nine peptides found. The identification of possible  
388 linear epitopes was performed by BLAST with the Swissprot protein sequence database restricted to  
389 *P. cwieri*. All peptides obtained in the sequencing were evaluated. However, only the main results  
390 found for five peptides are being presented in Table S1, two in the form SARS-CoV-2, in addition  
391 to the analysis made for the consensus obtained in alignment with Clustal W.



**Figure 2.** Alignment of the nucleotide sequence encoding PSPD2001, PSPD2002 and PSPD2003 with SARS-CoV-2 obtained from the (A) COVID dataset from Australia (SARS-Cov-2 / human / AUS), (B) Texas, USA (SARS-Cov-2 / human / TX) and (C) Iran (SARS-Cov-2 / human / IRN). The blue markings refer to similar nucleotides between the synthesized peptides and those present in SARS-CoV-2.



**Figure 3.** (A) Peptide alignment and (B) Guide Tree Phylogram. Both for the *Physalaemus cuvieri* form (taxid: 218685) by the Clustal W program. The green regions highlight 50% of the conserved region and, in red, 50% to 85% of the conserved region.

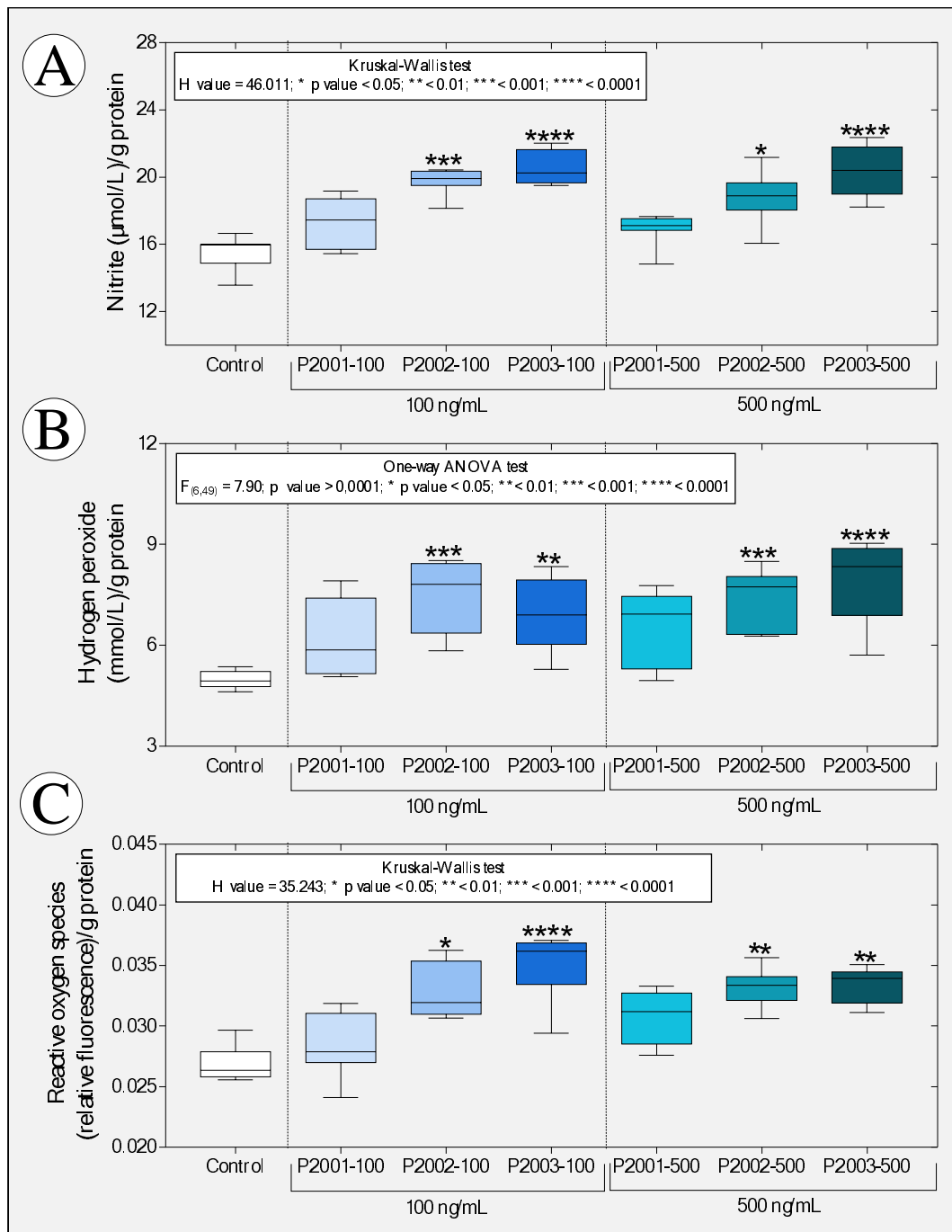
392

393           The *in vivo* experiments revealed that short exposure to SARS-CoV-2 Spike peptides was  
394 able to induce significant biochemical changes in *P. cuvieri* tadpoles. After 24 h of exposure, we  
395 observed that the peptides PSPD2002 and PSPD2003 (100 and 500 ng/mL) induced a significant  
396 increase in nitrite production (the indirect measurement of NO (Soneja et al. 2005) and hydrogen  
397 peroxide (Figure 4A-B, respectively), which in association with the higher levels of ROS (Figure  
398 4D), suggest an increase in oxidative stress processes in the animals. The PSPD2003 peptide, in  
399 particular, demonstrated an even more significant effect on NO production, exceeding a 30%  
400 increase, to the control group, in both tested concentrations (100 and 500 ng/ml); almost 60%  
401 increase in hydrogen peroxide levels in the group exposed to 500 ng/mL, and 29% ROS in the  
402 animals treated with 100 ng/mL. However, we did not observe significant differences between the  
403 groups regarding MDA levels (Figure S2-A), suggesting that the treatments did not intensify the  
404 lipid peroxidation processes in the tadpoles.

405

406





**Figure 4.** Boxplot of data obtained from predictive oxidative stress biomarkers [(A) nitrite levels, (B) hydrogen peroxide and (C) reactive oxygen species] in tadpoles of *P. cwieri* (phase 27G) exposed or not to peptides PSPD 2001, 2002 and 2003 of the SARS-CoV-2 Spike protein. The summaries of the statistical analyzes are shown in the upper left corner of the graphs. Asterisks indicate significant differences between the respective groups and the control group. (n = 50 animals/group). PSPD2001: Arg-Val-Tyr-Ser-Ser-Ala-Asn-Asn-Cys- COOH; PSPD2002: Gln-Cys-Val-Asn-Leu-Thr-Thr-Arg-Thr-COOH; PSPD2003: Asn-Asn-Ala-Thr-Asn-COOH.

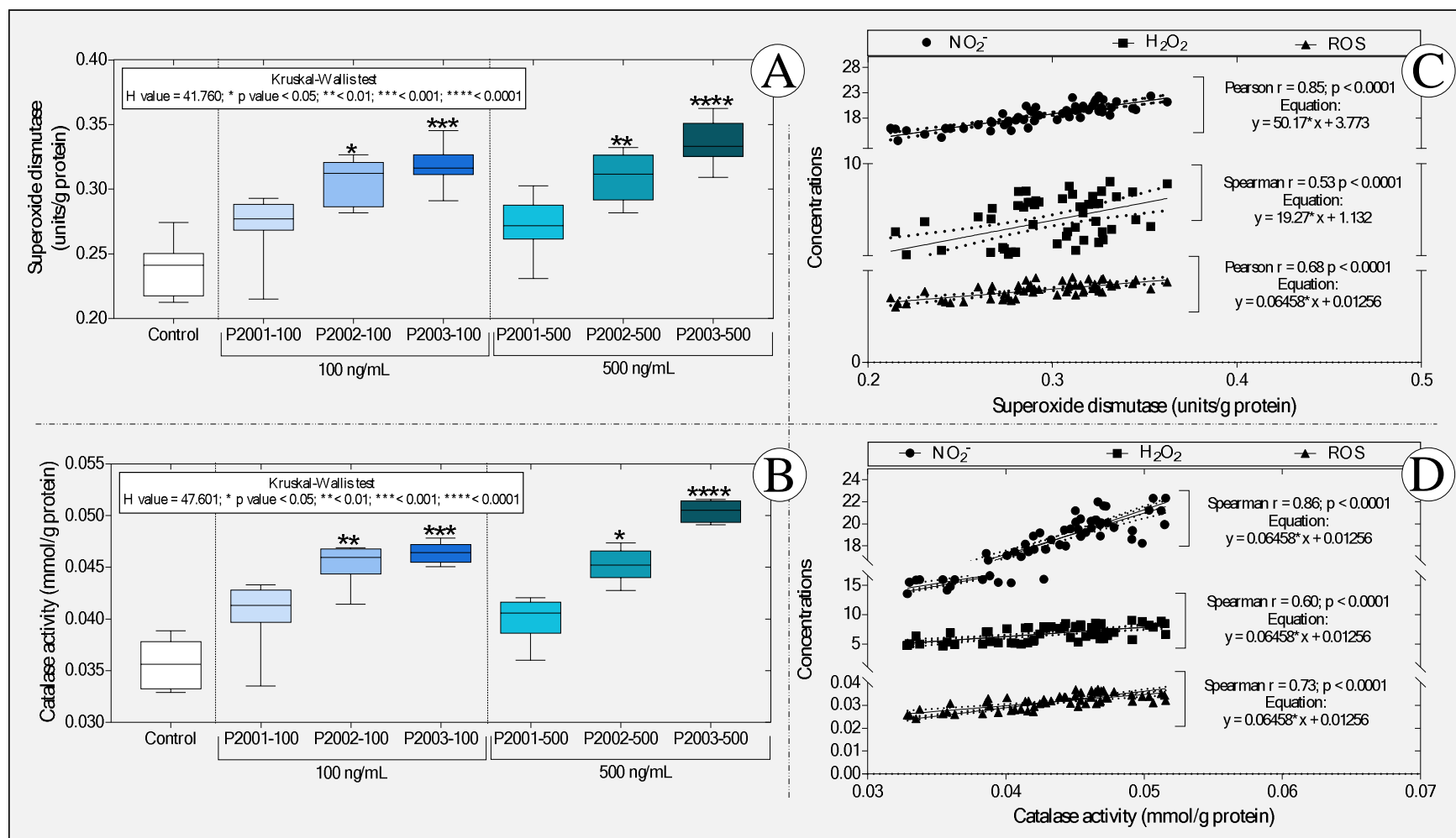
407 Similar to the previous findings, we observed that the animals exposed to PSPD2002 and  
408 PSPD2003 showed an increase, in a concentration-dependent manner, of the activity of the  
409 enzymes SOD and catalase (Figure 5A-B), with these data being positively and significantly  
410 correlated with the increase in the levels of nitrite, peroxide hydrogen and ROS (Figure 5C-D). We  
411 also observed that PSPD2003, once again, induced more intense effects on the antioxidant activity;  
412 there was an increase above 36% as compared to the control group for the two concentrations  
413 tested (100 and 500 ng/mL). The levels of SOD and catalase in the tadpoles exposed to PSPD2002  
414 fragments were 28.9% higher than those reported in the control group. However, the SOD/catalase  
415 ratio was unaffected or decreased which indicates the relative balance between hydrogen peroxide  
416 synthesis by SOD and its decomposition by catalase (Figure 2S-B).

417 These data are exciting since they corroborate previous studies that describe the critical role  
418 the SARS-CoV-2 Spike protein in inducing oxidative stress in COVID-19 infection [see the review  
419 of Suhail et al. (2020)] while demonstrating that the peptides evaluated, even in a non-host  
420 organism, can cause metabolic disorders related to the increase in reactive species. On the other  
421 hand, the impairment of antioxidant defenses observed in several viral infections (Fraternale et al.,  
422 2006), including COVID-19 (Baradaran et al., 2020; Polonikov, 2020; Bayindir & Bayindir, 2020;  
423 Abouhashem et al., 2020), was not evident in the studied organism. These data also reinforce the  
424 hypothesis that the responses to the peptide fragments tested may be different between hosts and  
425 non-hosts of SARS-CoV-2; they also confirm the ability of peptides PSPD2002 and PSPD2003 to  
426 induce metabolic changes that alter REDOX homeostasis towards oxidative stress in tadpoles.

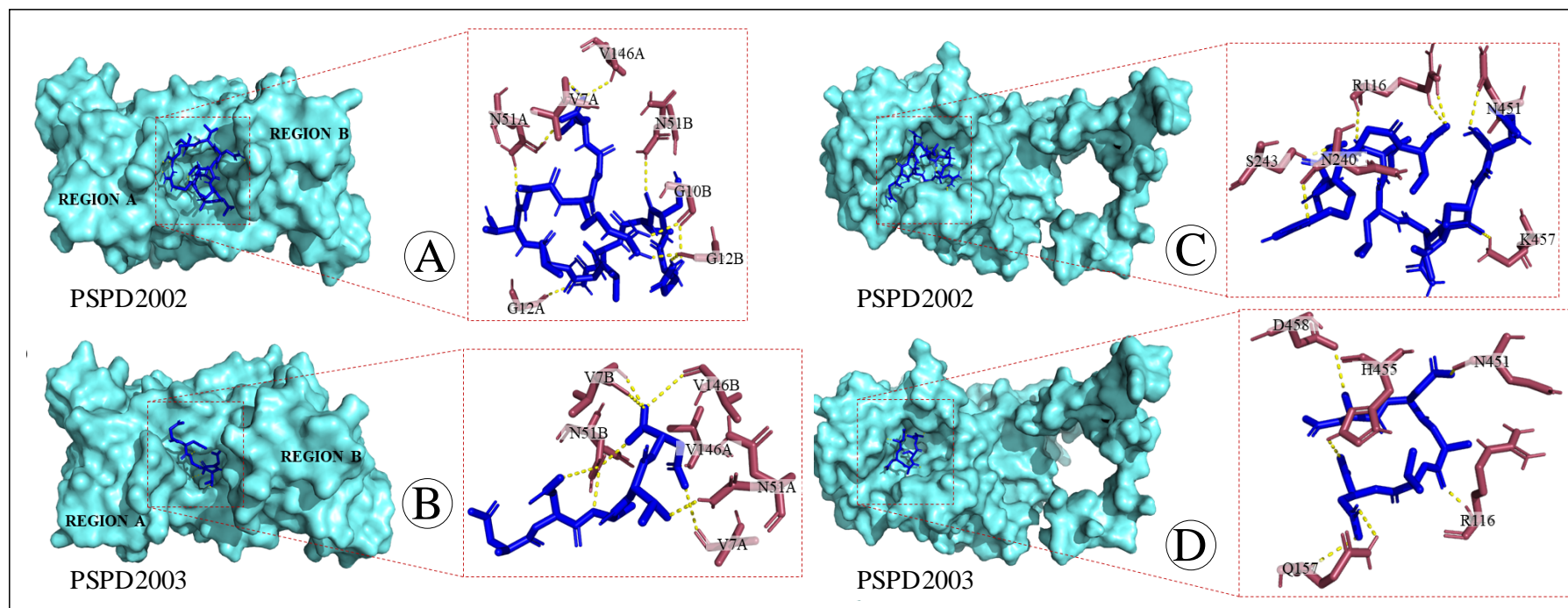
427 The proportional increase in oxidative stress biomarkers and the activity of SOD and  
428 catalase enzymes [two essential and indispensable molecules in cellular antioxidant defense  
429 strategies -Nishikawa et al. (2009), Hu & Tirelli (2012) and Ighodaro & Akinloye (2018)]  
430 reinforces our hypothesis, showing that the increase in antioxidant defenses does not seem to have  
431 been sufficient to reduce oxidative stress. The proposition of an action mechanism explaining the  
432 increase in these enzymes' activity is very incipient, either due to our study's pioneering nature or  
433 the need to deepen biochemical assessments in future studies. However, it is tempting to speculate  
434 that the interactions between PSPD2002 and PSPD2003 peptides and antioxidant enzymes  
435 evaluated in tadpoles (confirmed by molecular docking) have induced functional changes in SOD  
436 and catalase, similarly to what was observed by Jing et al. (2020) by exposing hepatocytes isolated  
437 from C57BL6 mice to different concentrations of naphthalene. The data obtained from the  
438 molecular docking reinforces our hypothesis by confirming the affinity between the PSPD2002 and  
439 PSPD2003 peptides and the referred enzymes and the existence of interactions with residues from  
440 all tested moorings (Figure 6). In the interactions with PSPD2002, it was possible to verify several

441 hydrogen bonds in the threonine mixture (T9), revealing the potencies of the binding affinities and  
442 the central region of interaction in the active sites of the tested targets. In contrast, PSPD2003  
443 interactions showed  $\geq 20$  hydrogen interactions in all the tested couplings, with the structures of  
444 valine (V2) and serine (S4) (central of the peptide) considered to have the best affinity region of the  
445 ligand.

446 An increase in NO production (inferred by high levels of nitrite) in tadpoles exposed to  
447 PSPD2002 and PSPD2003 (Figure 4A) suggests that the production of this free radical gas  
448 constitutes a standard response to the constituents of the SARS-CoV protein -2 Spike of the new  
449 coronavirus, both in the evaluated non-host organism and in humans infected with SARS-CoV-2.  
450 The ability of NO (both endogenous and exogenous) to inhibit the replication cycle of other viruses  
451 in the Coronaviridae family, affecting their proteins and reducing viral RNA (Chen et al., 2004;  
452 Keyaerts et al., 2004; Åkerström et al., 2005; Jung et al., 2010), has even motivated studies, whose  
453 preliminary results point to its potential therapeutic use in patients infected with SARS-CoV-2  
454 (Alvarez et al., 2020). Alternatively, we cannot neglect the hypothesis of increased NO in tadpoles  
455 due to the innate immune response modulated by the peptides, with a consequent increase in the  
456 production of inflammatory cytokines. In this case, studies reporting a positive correlation between  
457 NO production and increased pro-inflammatory cytokine levels (TNF- $\alpha$ , IL-6, IL-17, IL-12, and  
458 interferon- $\gamma$ ) in patients with COVID-19 reinforce our hypothesis (Karki et al., 2020; Del Valle et  
459 al., 2020; Costela-Ruiz et al., 2020).

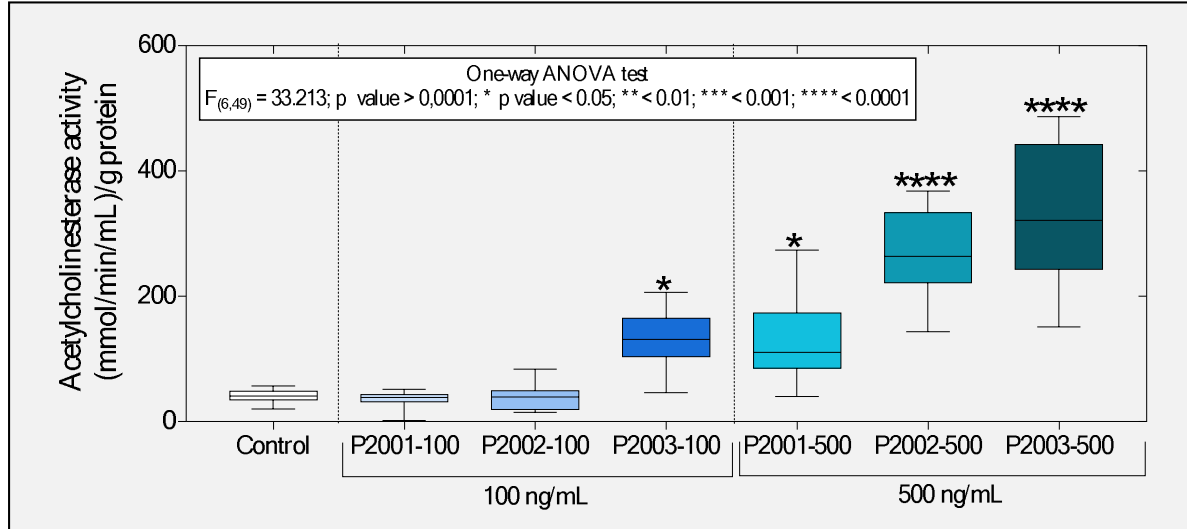


**Figure 5.** Boxplot of the activity of the enzymes (A) superoxide dismutase and (B) catalase, as well as correlations between the levels of (C) superoxide dismutase and (D) catalase and the different predictive biomarkers of oxidative stress. NO<sub>2</sub><sup>-</sup>: nitrite; H<sub>2</sub>O<sub>2</sub>: hydrogen peroxide and ROS: reactive oxygen species. In "A" and "B," the statistical analyses' summaries are shown in the graphs' upper left corner. Asterisks indicate significant differences between the respective groups and the control group. (n = 50 animals / group). PSPD2001: Arg-Val-Tyr-Ser-Ser-Ala-Asn-Asn-Cys-COOH; PSPD2002: Gln-Cys-Val-Asn-Leu-Thr-Thr-Arg-Thr-COOH; PSPD2003: Asn-Asn-Ala-Thr-Asn-COOH.



**Figure 6.** Three-dimensional surface-ligand coupling of interactions between peptides PSPD2002 and PSPD-2003 and the enzyme (A-B) superoxide dismutase (SOD) and (C-D) catalase (C-D), all in surface mode and highlighted active site. In “B and D”, we also observe regions A and B of the homo-dimer structure. Interaction residues in “A” (SOD-PSPD2002): G12A; N51A \*; V7A; V146B; G10B \*; G12B \*; N51B (affinity (kcal / mol) = -8.3). In “B” (SOD-PSPD2002): N51A; V7A; V146A; N51B \*\*, V7B; V146B \* (affinity (kcal / mol) = -8.6). In “C” (Catalase-PSPD2002): K457; N240; N451; R116 \*\*, S243 (affinity (kcal / mol) = -9.3). In “D” (Catalase-PSPD2003): D458; H455; N451; Q157 \*; R116 (affinity (kcal / mol) = -6.8). An "asterisk" indicates two interactions in the same residue. Two "asterisks" indicate the existence of three interactions in the same residue.

464 We also evaluated the peptides' possible neurotoxicity in tadpoles exposed to the peptide  
465 fragments of the SARS-CoV-2 Spike protein. Interestingly, we observed that 100 ng/mL PSPD2003  
466 induced an increase greater than 220% concerning the control group. However, at a concentration  
467 of 500 ng/mL, all the peptides evaluated exerted an effect in the cholinergic system, causing an  
468 increase in the activity of AChE (Figure 7). While the peptides PSPD2001 and PSPD2002 induced  
469 increases of 219 and 553.8% in relation to AChE activity in the control group's animals,  
470 respectively; the PSPD2003 peptide impressively induced an even more significant increase  
471 (697.3%). Therefore, these data confirmed the initial hypothesis that the SARS-CoV-2 Spike  
472 fragments induce neurotoxic effects, inferred by the stimulatory effect of the cholinergic system of  
473 the animals evaluated, especially in those exposed to the highest concentration (500 ng/mL) of the  
474 peptides.  
475



**Figure 7.** Boxplot of the enzyme acetylcholinesterase activity evaluated in tadpoles of *P. cuvieri* exposed or not to the peptides PSPD 2001, 2002, and 2003 of the SARS-CoV-2 Spike protein. The summaries of the statistical analyzes are shown in the upper left corner of the graphs. Asterisks indicate significant differences between the respective groups and the control group. (n = 50 animals / group). PSPD2001: Arg-Val-Tyr-Ser-Ser-Ala-Asn-Asn-Cys- COOH; PSPD2002: Gln-Cys-Val-Asn-Leu-Thr-Thr-Arg-Thr-COOH; PSPD2003: Asn-Asn-Ala-Thr-Asn-COOH.

476

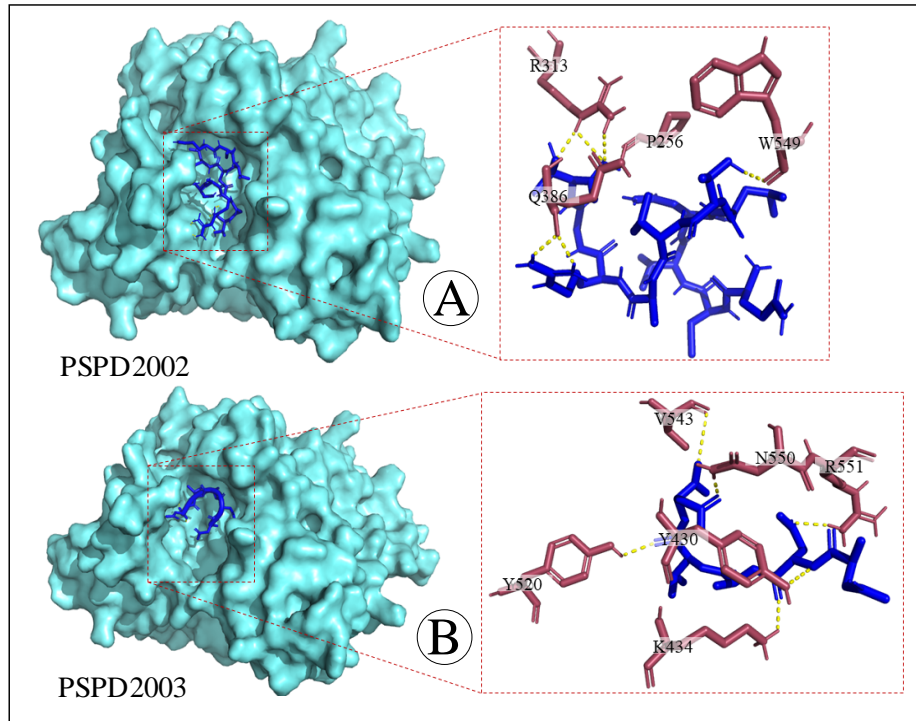
477 Interestingly, these data differ from other studies that report suppression in AChE induced  
478 by increased cellular oxidative stress (Flora et al., 2013; Kayode et al., 2016; Bali et al., 2019;  
479 Ezeoyili et al., 2019; Pala et al., 2019; Ibrahim et al., 2020). In general, such studies argue that this  
480 can occur due to the deterioration of neurotransmission and oxidative damage. Additionally, AChE

481 inhibition impairs oxidative phosphorylation and is followed by neuronal  $\text{Ca}^{2+}$  influx and activation  
482 of nNOS, associated with the neurons' oxidative and nitrosative injury (Milatovic et al., 2006).  
483 However, the increased AChE activity observed in tadpoles exposed to the peptides may be related  
484 to the activation of the cholinergic anti-inflammatory pathway (CAP), which has been found  
485 beneficial in preventing inflammatory conditions such as sepsis and acute respiratory distress  
486 syndrome in animal models [see the review of Liu et al. (2020)]. As discussed by Osman (2020),  
487 CAP constitutes a neural mechanism that modulates inflammation through the release of  
488 acetylcholine (ACh), that have led to increased AChE synthesis to decompose higher levels of this  
489 neurotransmitter [see details in Tracey (2007)]. This mechanism has been reported in different  
490 studies involving patients infected with the new coronavirus (Bonaz et al., 2020; Mazloom et al.,  
491 2020; Pomara et al., 2020), strengthening the presumption that this mechanism may constitute  
492 another similar physiological response between SARS-CoV-2 non-host and host organisms. Besides,  
493 it is plausible to assume not only that the peptide composition of the SARS-CoV-2 Spike protein  
494 participates in the CAP activation (both in humans and in the evaluated tadpoles) but also that the  
495 neuroimmune system of the tadpoles has an essential role in responding to exposure of peptides  
496 PSPD2001, PSPD2002 and PSPD2003.

497 Alternatively, the tadpoles' cholinergic system's stimulation may also be explained by the  
498 direct interactions between the tested peptides and AChE, whose affinity was demonstrated in the  
499 molecular docking analysis (Figure 8). In this case, future studies will be useful to understand if  
500 these interactions induced a significant change in the association and catalysis mechanism or  
501 expansion of the enzyme efficiency with an increase of the substrate affinity to the active site  
502 (increasing the catalytic constant was increased and decreasing the Michaelis constant). In both  
503 situations, a significant increase in AChE activity can occur, either as part of a compensatory  
504 mechanism that will aim to compensate for the enzyme's catalytic deficit or as a more efficient  
505 response to the increased release of acetylcholine in synaptic clefts via CAP activation. The  
506 hypothesis that increased AChE activity in these animals was associated with positive AChE gene  
507 regulation due to Spike protein peptides' inhibitory effect needs to be tested in future studies.

508 Finally, it is essential to emphasize that although our study gathers clear and pioneering  
509 evidence on the negative impact of the SARS-CoV-2 Spike fragments (especially PSPD2002 and  
510 PSPD2003) on the biochemical parameters evaluated in *P. cuvieri* tadpoles, many questions about  
511 the consequences of the presence of these fragments in the aquatic environment remains obscure.  
512 The evaluation of the effects of prolonged exposure to the tested peptides (in higher and lower  
513 concentrations), the use of other experimental models (expanding the environmental  
514 representativeness), and the use of multiple toxicity biomarkers are some future investigative

515 perspectives. Equally important will be to deepen the mechanisms of action of the peptides of the  
516 SARS-CoV-2 Spike protein when in direct contact with non-host organisms of the new coronavirus.  
517 Approaches of this nature can significantly expand our knowledge of the impact of COVID-19 on  
518 the environment and the functioning of ecosystems, and support the proposal for strategies to  
519 remedy or mitigate aquatic contamination by SARS-Cov2 particles.  
520



**Figure 8.** Three-dimensional surface-ligand coupling of interactions between peptides (A) PSPD2002 and (B) PSPD-2003 and the enzyme acetylcholinesterase, all in surface mode and highlighted active site. Interaction residues in "A" (AChE-PSPD2002): P256; Q386\*; R313\*\*;; W549 (affinity (kcal / mol) = -9.4). In "B" (AChE-PSPD2003): K434; N550; R551; V543; Y430; Y520 (affinity (kcal / mol) = -8.4). An "asterisk" indicates two interactions in the same residue. Two "asterics" indicate the existence of three interactions in the same residue.

521

#### 522 4. CONCLUSIONS

523 From a systemic approach that included the synthesis, cleavage, purification, and alignment  
524 of peptides to *P. cuvieri* tadpoles' exposure to peptide fragments of Spike protein, we gathered  
525 evidence that confirms the toxicity of viral constituents in the evaluated animal model. The  
526 increase in predictive biomarkers of REDOX imbalance and neurotoxic action is, therefore, an  
527 insight into how aquatic particle contamination of SARS-CoV-2 can constitute additional  
528 environmental damage to the COVID-19 pandemic. In this sense, we strongly suggest conducting



529 further studies necessary to understand the real magnitude of the biological/environmental impact  
530 of COVID-19.

531

## 532 5. ACKNOWLEDGMENT

533 This work was supported by São Paulo Research Foundation (FAPESP 2020/05761-3),  
534 Brazilian National Research Council (CNPq) (426531/2018-3) and *Instituto Federal Goiano* for the  
535 financial support (23219.001309.2020-19). Malafaia G. holds productivity scholarship from CNPq  
536 (307743/2018-7).

537

## 538 6. REFERENCES

539 Abouhashem, A. S., Singh, K., Azzazy, H. M., & Sen, C. K. (2020). Is Low Alveolar Type II Cell  
540 SOD3 in theLungsofElderlyLinkedtotheObservedSeverity of COVID-19?.*Antioxidants&*  
541 *Redox Signaling*.

542 Abu-Qdais, H. A., Al-Ghazo, M. A., & Al-Ghazo, E. M. (2020). Statistical analysis and  
543 characteristics of hospital medical waste under novel Coronavirus outbreak. *Global Journal*  
544 *of Environmental Science and Management*, 6(Special Issue (Covid-19)), 21-30.

545 Abu-Rayash, A., &Dincer, I. (2020). Analysis of the electricity demand trends amidst the COVID-  
546 19 coronavirus pandemic. *Energy Research & Social Science*, 68, 101682.

547 AdrieliSachett, Matheus Gallas-Lopes, Greicy M MConterato, RadharaniBenvenuti , Ana P  
548 Herrmann, Angelo Piato 2020. Quantification of thiobarbituric acid reactive species  
549 (TBARS) optimized for zebrafish brain tissue. *protocols.io*.  
550 <https://dx.doi.org/10.17504/protocols.io.bjp8kmrw>

551 Åkerström, S., Gunalan, V., Keng, C. T., Tan, Y. J., &Mirazimi, A. (2009). Dual effectofnitric oxide  
552 on SARS-CoVreplication: viral RNA production and palmitoylationofthe S protein are  
553 affected. *Virology*, 395(1), 1-9.

554 Åkerström, S., Mousavi-Jazi, M., Klingström, J., Leijon, M., Lundkvist, Å., &Mirazimi, A. (2005).  
555 Nitric oxide inhibitsthereplicationcycleofsevereacuterespiratorysyndrome coronavirus.  
556 *Journal of virology*, 79(3), 1966-1969.

557 Alvarez RA, Berra L, Gladwin MT. Home nitric oxide therapyfor COVID-19. *Am J*  
558 *RespirCritCareMed*, 202(1): 16-20, 2020.

559 Araújo, A. P. C., de Melo, N. F. S., de Oliveira Junior, A. G., Rodrigues, F. P., Fernandes, T., de  
560 Andrade Vieira, J. E., ... &Malafaia, G. (2020a). How much are microplastics harmful to  
561 the health of amphibians? A study with pristine polyethylene microplastics and  
562 *Physalaemus cuvieri*. *Journal of hazardous materials*, 382, 121066.

- 563 Araújo, A. P. C., Gomes, A. R., & Malafaia, G. (2020b). Hepatotoxicity of pristine polyethylene  
564 microplastics in neotropical physalaemus cuvieri tadpoles (Fitzinger, 1826). *Journal of*  
565 *Hazardous Materials*, 386, 121992.
- 566 Bali, Y. A., Kaikai, N. E., Ba-M'hamed, S., & Bennis, M. (2019). Learning and  
567 memory impairments associated to acetylcholinesterase inhibition and oxidative stress  
568 following glyphosate-based herbicide exposure in mice. *Toxicology*, 415, 18-25.
- 569 Bangaru, S., Ozorowski, G., Turner, H. L., Antanasijevic, A., Huang, D., Wang, X., ... & Patel, N.  
570 (2020). Structural analysis of full-length SARS-CoV-2 spike protein from an advanced  
571 vaccine candidate. *Science*, 370(6520), 1089-1094.
- 572 Baradaran, A., Ebrahimzadeh, M. H., Baradaran, A., & Kachooei, A. R. (2020).  
573 Prevalence of comorbidities in COVID-19 patients: A systematic review and meta-analysis.  
574 *Archives of Bone and Joint Surgery*, 8(Suppl 1), 247.
- 575 Bayindir, M., & Bayindir, E. E. (2020). Synergic viral-bacterial co-infection in catalase-deficient  
576 COVID-19 patients causes suppressed innate immunity and  
577 lung damages due to detrimental elevation of hydrogen peroxide concentration. Available at  
578 SSRN 3648292.
- 579 Behrendt, R., White, P., & Offer, J. (2016). Advances in Fmoc solid-phase peptide synthesis.  
580 *Journal of Peptide Science*, 22(1), 4-27.
- 581 Bivins, A., Greaves, J., Fischer, R., Yinda, K. C., Ahmed, W., Kitajima, M., ... & Bibby, K. (2020).  
582 Persistence of SARS-CoV-2 in water and wastewater. *Environmental Science &*  
583 *Technology Letters*.
- 584 Blaustein, A. R., & Kiesecker, J. M. (2002). Complexity in conservation: lessons from the global  
585 decline of amphibian populations. *Ecology letters*, 5(4), 597-608.
- 586 Bonaz, B., Sinniger, V., & Pellissier, S. (2020). Targeting the cholinergic anti-  
587 inflammatory pathway with vagus nerve stimulation in patients with Covid-19? *Bioelectronic*  
588 *medicine*, 6(1), 1-7.
- 589 Braz HLB, Silveira JAM, Marinho AD, de Moraes MEA, Moraes Filho MO, Monteiro HSA, Jorge  
590 RJB. In silico study of azithromycin, chloroquine and hydroxychloroquine and their  
591 potential mechanisms of action against SARS-CoV-2 infection. *Int J Antimicrob Agents*.  
592 2020 Sep;56(3):106119. doi: 10.1016/j.ijantimicag.2020.106119. Epub 2020 Jul 30. PMID:  
593 32738306; PMCID: PMC7390782.
- 594 Carvalho, M., Bandeira de Mello Delgado, D., de Lima, K. M., de Camargo Cancela, M., dos  
595 Siqueira, C. A., & de Souza, D. L. B. (2020). Effects of the COVID-19 pandemic on the

- 596 Brazilian electricity consumption patterns. *International Journal of Energy Research*,  
597 e5877.
- 598 Chen L, Liu P, Gao H, Sun B, Chao D, et al. (2004) Inhalation of nitric oxide in  
599 the treatment of severe acute respiratory syndrome: a rescue trial in Beijing. *Clin Infect Dis* 39:  
600 1531-1535.
- 601 Chen, Y., Chen, L., Deng, Q., Zhang, G., Wu, K., Ni, L., ... & Yang, J. (2020). The presence of  
602 SARS-CoV-2 RNA in the feces of COVID-19 patients. *Journal of medical virology*.
- 603 Colston, J. T., Chandrasekar, B., & Freeman, G. L. (2002). A novel peroxide-  
604 induced calcium transient regulates interleukin-6 expression in cardiac-derived fibroblasts.  
605 *Journal of Biological Chemistry*, 277(26), 23477-23483.
- 606 Costela-Ruiz, V. J., Illescas-Montes, R., Puerta-Puerta, J. M., Ruiz, C., & Melguizo-Rodríguez, L.  
607 (2020). SARS-CoV-2 infection: the role of cytokines in COVID-19 disease.  
608 *Cytokine & Growth Factor Reviews*.
- 609 Coughlan, L. (2020). Snatching the Crown from SARS-CoV-2. *Cell Host & Microbe*, 28(3), 360-  
610 363.
- 611
- 612 Chakraborty, I & Prasenjit, M. COVID-19 outbreak: Migration, effects on society, global  
613 environment and prevention. *Science of the Total Environment*, 2020, 138882.
- 614
- 615 Del Valle, D. M., Kim-Schulze, S., Hsin-hui, H., Beckmann, N. D., Nirenberg, S., Wang, B., ...  
616 & Marron, T. (2020). An inflammatory cytokine signature helps predict COVID-19 severity  
617 and death. *medRxiv*.
- 618 Del-Maestro, R. F., & McDonald, W. (1985). Oxidative enzymes in tissue homogenates. *Handbook*  
619 *of methods for oxygen radical research*, 291-296.
- 620 Elnemma, E. M. (2004). Spectrophotometric determination of hydrogen peroxide by a  
621 hydroquinone-aniline system catalyzed by molybdate. *Bulletin of the Korean Chemical*  
622 *Society*, 25(1), 127-129.
- 623 Elsamadony, M., Fujii, M., Miura, T., & Watanabe, T. (2020). Possible transmission of viruses from  
624 contaminated human feces and sewage: Implications for SARS-CoV-2. *Science of the*  
625 *Total Environment*, 755, 142575.
- 626 Emmanuel Daanoba Sunkari, Harriet Mateko Korboe, Mahamuda Abu, Tefide Kizildeniz,  
627 Estrela, F. N., Guimarães, A. T. B., Silva, F. G., da Luz, T. M., Silva, A. M., Pereira, P. S., &  
628 Malafaia, G. (2021). Effects of polystyrene nanoplastics on *Ctenopharyngodon idella* (grass

629 carp) after individual and combined exposure with zinc oxide nanoparticles. Journal of  
630 Hazardous Materials, 403, 123879.

631 European Centre for Disease Prevention and Control (ECDC). COVID-19 situation update for the  
632 EU/EEA and the UK, as of week 50 2020. Available in:  
633 <https://www.ecdc.europa.eu/en/cases-2019-ncov-eueea>. Accession: 21 december 2020.  
634

635 Editorial, The Lancet Respiratory Medicine, COVID-19 transmission—up in the air,  
636 The Lancet Respiratory Medicine, Volume 8, Issue 12, 2020, Page 1159, ISSN 2213-2600,  
637 [https://doi.org/10.1016/S2213-2600\(20\)30514-2](https://doi.org/10.1016/S2213-2600(20)30514-2).

638 Ezeoyili, I. C., Mgbenka, B. O., Atama, C. I., Ngwu, G. I., Madu, J. C., & Nwani, C. D. (2019).  
639 Changes in Brain Acetylcholinesterase and Oxidative Stress Biomarkers in  
640 African Catfish Exposed to Carbendazim. Journal of Aquatic Animal Health, 31(4), 371-379.

641 Farsalinos, K., Niaura, R., Le Houezec, J., Barbouni, A., Tsatsakis, A., Kouretas, D., ... & Poulas, K.  
642 (2020). Nicotine and SARS-CoV-2: COVID-19 may be a disease of the nicotinic  
643 cholinergic system. Toxicology Reports.

644 Fernandes, B.H., Feitosa, N. M., Barbosa, A. P., Bomfim, C. G., Garnique, A. M. B., Gomes, F. I. F.,  
645 ... & Charlie-Silva, I. (2020). Zebrafish studies on the vaccine candidate to COVID-19, the  
646 Spike protein: Production of antibody and adverse reaction.  
647 <https://doi.org/10.1101/2020.10.20.346262>

648 Flora, S. J., Mehta, A., Satsangi, K., Kannan, G. M., & Gupta, M. (2003). Aluminum-  
649 induced oxidative stress in rat brain: response to combined administration of citric acid and  
650 HEDTA. Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology,  
651 134(3), 319-328.

652 Fraternali A, Paoletti MF, Casabianca A, Oiry J, Clayette P, Vogel JU, Cinatl J, Jr, Palamara AT,  
653 Sgarbanti R, Garaci E, Millo E, Benatti U, Magnani M. Antiviral and  
654 immunomodulatory properties of new pro-glutathione (GSH) molecules. Curr Med Chem.  
655 2006;13(15):1749–1755. doi: 10.2174/092986706777452542

656 Frost DR. Amphibian Species of the World: an Online Reference. Version 6.0. Available in:  
657 <http://research.amnh.org/vz/herpetology/amphibia/>. Access on: 11 march. 2017.

658 Gaudin, Raphael; Goetz, Jacky G. Tracking Mechanisms of Viral Dissemination In Vivo. Trends in  
659 Cell Biology, 2021.

660

661 Gornall, A.G.; Bardawill, C.J.; David, M. M. Determination of serum proteins by means of the  
662 biuret reaction. J. Biol. Chem. v.177, p.751-766, 1949.

- 663 Gosner KL. A simplified table for staging anuran embryos and larvae with notes on identification.  
664 *Herpetologica*, 16:183–190 1960.
- 665 Grant, E. H. C., Miller, D. A., & Muths, E. (2020). A Synthesis of Evidence of Drivers of Amphibian  
666 Declines. *Herpetologica*.
- 667 Graham, Katherine E., et al. SARS-CoV-2 RNA in Wastewater Settled Solids Is Associated with  
668 COVID-19 Cases in a Large Urban Sewershed. *Environmental science & technology*,  
669 2020.
- 670 Guerrero-Latorre, L., Ballesteros, I., Villacrés-Granda, I., Granda, M. G., Freire-Paspuel, B., & Ríos-  
671 Touma, B. (2020). SARS-CoV-2 in river water: Implications in low sanitation countries.  
672 *Science of the Total environment*, 743, 140832.
- 673 Guimarães, A. T. B., Charlie-Silva, I., & Malafaia, G. (2020). TOXIC EFFECTS OF  
674 NATURALLY-AGED MICROPLASTICS ON ZEBRAFISH JUVENILES: A MORE  
675 REALISTIC APPROACH TO PLASTIC POLLUTION IN FRESHWATER  
676 ECOSYSTEMS. *Journal of Hazardous Materials*, 124833.
- 677 Guimarães, A. T. B., de Lima Rodrigues, A. S., Pereira, P. S., Silva, F. G., & Malafaia, G. (2021).  
678 Toxicity of polystyrene nanoplastics in dragonfly larvae: An insight on how these pollutants  
679 can affect benthic macroinvertebrates. *Science of The Total Environment*, 752, 141936.
- 680 Guy, C. A., & Fields, G. B. (1997). [5] Trifluoroacetic acid cleavage and deprotection of resin-  
681 bound peptides following synthesis by Fmoc chemistry. *Methods in enzymology*, 289, 67-83.
- 682 Harrison, A. G., Lin, T., & Wang, P. (2020). Mechanisms of SARS-CoV-2 transmission and  
683 pathogenesis. *Trends in immunology*.
- 684 HENRY, R. J.; SOBEL, C.; BERKMAN, S. Interferences with biuret methods for serum proteins.  
685 Use of Benedict's qualitative glucose reagent as a biuret reagent. *Anal. Chem.* v.29, p.1491-  
686 1495, 1957.
- 687 Herek, J. S., Vargas, L., Trindade, S. A. R., Rutkoski, C. F., Macagnan, N., Hartmann, P. A., &  
688 Hartmann, M. T. (2020). Can environmental concentrations of glyphosate affect survival  
689 and cause malformation in amphibians? Effects from a glyphosate-based herbicide on  
690 *Physalaemus cuvieri* and *P. gracilis* (Anura: Leptodactylidae). *Environmental Science and*  
691 *Pollution Research*, 1-12.
- 692 Higgins, D. G., Thompson, J. D., & Gibson, T. J. (1996). [22] Using CLUSTAL for multiple  
693 sequence alignments. In *Methods in enzymology* (Vol. 266, pp. 383-402). Academic Press.
- 694 Hu, P., & Tirelli, N. (2012). Scavenging ROS: superoxide dismutase/catalase mimetics by the use  
695 of an oxidation-sensitive nanocarrier/enzyme conjugate. *Bioconjugate chemistry*, 23(3), 438-  
696 449.

- 697 Huang, Y., Yang, C., Xu, X. F., Xu, W., & Liu, S. W. (2020). Structural and functional properties of  
698 SARS-CoV-2 spike protein: potential antivirus drug development for COVID-19. *Acta*  
699 *Pharmacologica Sinica*, 41(9), 1141-1149.
- 700 Ibrahim, K. A. E. M., Abdelrahman, S. M., Elhakim, H. K., & Ragab, E. A. (2020).  
701 Single or combined exposure to chlorpyrifos and cypermethrin provoke oxidative stress and  
702 downregulation in monoamine oxidase and acetylcholinesterase gene  
703 expression of the rat's brain. *Environmental Science and Pollution Research*, 1-12. Pomara, N.,  
704 & Imbimbo, B. P. (2020). Impairment of the cholinergic anti-inflammatory pathway in  
705 older subjects with severe COVID-19. *Medical hypotheses*.
- 706 Ighodaro, O. M., & Akinloye, O. A. (2018). First line defence antioxidants-superoxide dismutase  
707 (SOD), catalase (CAT) and glutathione peroxidase (GPX): Their fundamental role in  
708 the entire antioxidant defence grid. *Alexandria journal of medicine*, 54(4), 287-293.
- 709 Ighodaro, O. M., & Akinloye, O. A. (2018). First line defence antioxidants-superoxide dismutase  
710 (SOD), catalase (CAT) and glutathione peroxidase (GPX): Their fundamental role in the  
711 entire antioxidant defence grid. *Alexandria journal of medicine*, 54(4), 287-293.
- 712 Jetz, W, and R. A. Pyron. 2018. The interplay of past diversification and evolutionary isolation with  
713 present imperilment across the amphibian tree of life. *Nature Ecology & Evolution* 2:850-  
714 858.
- 715 Jing, M., Han, G., Wan, J., Zhang, S., Yang, J., Zong, W., ... & Liu, R. (2020). Catalase and  
716 superoxide dismutase response and the underlying molecular mechanism for naphthalene.  
717 *Science of The Total Environment*, 139567.
- 718 Jones, D. L., Baluja, M. Q., Graham, D. W., Corbishley, A., McDonald, J. E., Malham, S. K., ... &  
719 Wilcox, M. H. (2020). Shedding of SARS-CoV-2 in feces and urine and its potential role  
720 in person-to-person transmission and the environment-based spread of COVID-19. *Science*  
721 *of the Total Environment*, 749, 141364.
- 722 Galindo-Villegas, Jorge. The zebrafish disease and drug screening model: A strong ally against  
723 Covid-19. *Frontiers in Pharmacology*, 2020, 11: 680.
- 724 Jung K, Gurnani A, Renukaradhya GJ, Saif LJ (2010) Nitric oxide is elicited and inhibits viral  
725 replication in pigs infected with porcine respiratory coronavirus but not porcine reproductive  
726 and respiratory syndrome virus. *Vet Immunol Immunopathol* 136 335-339. [Crossref]
- 727 Kampf, G., Todt, D., Pfaender, S., & Steinmann, E. (2020). Persistence of coronaviruses on  
728 inanimate surfaces and their inactivation with biocidal agents. *Journal of Hospital*  
729 *Infection*, 104(3), 246-251.

- 730 Karki, R., Sharma, B. R., Tuladhar, S., Williams, E. P., Zalduondo, L., Samir, P., ... & Schreiner, P.  
731 (2020). Synergism of TNF- $\alpha$  and IFN- $\gamma$  triggers inflammatory cell death, tissue damage, and  
732 mortality in SARS-CoV-2 infection and cytokine shock syndromes. *Cell*.
- 733 Kayode AO, Sulaiman O, Emmanuel AG, Dorcas W. Acetylcholinesterase activity and oxidative  
734 stress indices in cerebellum, cortex and hippocampus of rats exposed to lead and manganese;  
735 *International Journal of Biological Research*, 4(2): 157-164, 2016.
- 736 Keech, C., Albert, G., Cho, I., Robertson, A., Reed, P., Neal, S., ... & Smith, G. (2020). Phase 1–2  
737 trial of a SARS-CoV-2 recombinant spike protein nanoparticle vaccine. *New England*  
738 *Journal of Medicine*.
- 739 Keyaerts E, Vijgen L, Chen L, Maes P, Hedenstierna G (2004) Inhibition of SARS-coronavirus  
740 infection in vitro by S-nitroso-N-acetylpenicillamine, a nitric oxide donor compound. *Int J*  
741 *Infect Dis*. 8: 223-226. [Crossref]
- 742 Khan, F. R., Syberg, K., Shashoua, Y., & Bury, N. R. (2015). Influence of polyethylene microplastic  
743 beads on the uptake and localization of silver in zebrafish (*Danio rerio*). *Environmental*  
744 *pollution*, 206, 73-79.
- 745 Kharazmi, A., Nielsen, H., Rechnitzer, C., & Bendtzen, K. (1989). Interleukin 6 primes human  
746 neutrophil and monocyte oxidative burst response. *Immunology letters*, 21(2), 177-184.
- 747 Klaassen, N., Spicer, V., & Krokhin, O. V. (2019). Universal retention standard for peptide  
748 separations using various modes of high-performance liquid chromatography. *Journal of*  
749 *Chromatography A*, 1588, 163-168.
- 750 Kolb, P., Ferreira, R. S., Irwin, J. J., & Shoichet, B. K. (2009). Docking and chemoinformatic  
751 screens for new ligands and targets. *Current opinion in biotechnology*, 20(4), 429-436.
- 752 Lamiable A, Thévenet P, Rey J, Vavrusa M, Derreumaux P, Tufféry P. PEP-FOLD3: previsão de  
753 estrutura de novo mais rápida para peptídeos lineares em solução e em complexo.  
754 *Nucleic Acids Res*. 8 de julho de 2016; 44 (W1): W449-54.
- 755 Li, Y., Hu, Y., Yu, J., & Ma, T. (2020). Retrospective analysis of laboratory testing in 54  
756 patients with severe or critical-type 2019 novel coronavirus pneumonia.  
757 *Laboratory Investigation*, 1-7.
- 758 Liu, D., Thompson, J. R., Carducci, A., & Bi, X. (2020). Potential secondary transmission of SARS-  
759 CoV-2 via wastewater. *Science of The Total Environment*, 749, 142358.
- 760 Liu, H., Wu, J., Yao, J. Y., Wang, H., & Li, S. T. (2017). The role of oxidative stress in  
761 decreased acetylcholinesterase activity at the neuromuscular junction of the diaphragm during  
762 sepsis. *Oxidative medicine and cellular longevity*, 2017.

- 763 Liu, W., Liu, Z., & Li, Y. C. (2020). COVID-19-related myocarditis and cholinergic anti-  
764 inflammatory pathways. *Hellenic Journal of Cardiology*.
- 765 Luna, O. F., Gomez, J., Cárdenas, C., Albericio, F., Marshall, S. H., & Guzmán, F. (2016).  
766 Deprotection reagents in Fmoc solid phase peptide synthesis: moving away from  
767 piperidine?. *Molecules*, 21(11), 1542.
- 768 Lusher, A. L., Mchugh, M., & Thompson, R. C. (2013). Occurrence of microplastics in the  
769 gastrointestinal tract of pelagic and demersal fish from the English Channel. *Marine  
770 pollution bulletin*, 67(1-2), 94-99.
- 771 Maharajan, K., Muthulakshmi, S., Nataraj, B., Ramesh, M., & Kadirvelu, K. (2018). Toxicity  
772 assessment of pyriproxyfen in vertebrate model zebrafish embryos (*Danio rerio*): a multi  
773 biomarker study. *Aquatic Toxicology*, 196, 132-145.
- 774 Mazloom, R. (2020). Feasibility of Therapeutic Effects of the Cholinergic Anti-Inflammatory Pathway on  
775 COVID-19 Symptoms. *Journal of Neuroimmune Pharmacology*, 1-2.
- 776 Metzger, J. W., Kempter, C., Wiesmuller, K. H., & Jung, G. (1994). Electrospray mass spectrometry  
777 and tandem mass spectrometry of synthetic multicomponent peptide mixtures:  
778 determination of composition and purity. *Analytical biochemistry*, 219(2), 261-277.
- 779 Meyerowitz, E. A., Richterman, A., Gandhi, R. T., & Sax, P. E. (2020). Transmission of SARS-  
780 CoV-2: a review of viral, host, and environmental factors. *Annals of internal medicine*.
- 781 Milatovic, D., Gupta, R. C., & Aschner, M. (2006). Anticholinesterase toxicity and oxidative stress.  
782 *The Scientific World Journal*, 6.
- 783 Miranda NEO, Maciel NM, Ribeiro MSL, Colli GR, Haddad FB, Collevatti RG. Diversification of  
784 the widespread neotropical frog *Physalaemus cuvieri* in response to Neogene-Quaternary  
785 geological events and climate dynamics. *Molecular Phylogenetics and Evolution*, 132: 67-  
786 80, 2019.
- 787 Montalvão, M. F., Guimarães, A. T. B., de Lima Rodrigues, A. S., & Malafaia, G. (2021). Carbon  
788 nanofibers are bioaccumulated in *Aphyllawilliamsoni* (Odonata) larvae and cause REDOX  
789 imbalance and changes of acetylcholinesterase activity. *Science of The Total Environment*,  
790 143991.
- 791 Nishikawa, M., Hashida, M., & Takakura, Y. (2009). Catalase delivery for inhibiting ROS-  
792 mediated tissue injury and tumor metastasis. *Advanced drug delivery reviews*, 61(4), 319-326.
- 793 Ohkawa, H., Ohishi, N., & Yagi, K. (1979). Assay for lipid peroxides in animal tissues by  
794 thiobarbituric acid reaction. *Analytical biochemistry*, 95(2), 351-358.
- 795 Osman, A. H. (2020). COVID-19: Targeting the cytokine storm via cholinergic anti-inflammatory  
796 (Pyridostigmine). *Int. J. Clin. Virol*, 4, 041-046.



- 797 Osman, A. H. (2020). COVID-19: Targeting the cytokine storm via cholinergic anti-inflammatory  
798 (Pyridostigmine). *Int. J. Clin. Virol*, 4, 041-046.
- 799 Pais, F. S. M., de Cássia Ruy, P., Oliveira, G., & Coimbra, R. S. (2014). Assessing the efficiency of  
800 multiple sequence alignment programs. *Algorithms for molecular biology*, 9(1), 4.
- 801 Pala, A. (2019). The effect of a glyphosate-based herbicide on acetylcholinesterase (AChE) activity,  
802 oxidative stress, and antioxidant status in freshwater amphipod: *Gammarus pulex*  
803 (Crustacean). *Environmental Science and Pollution Research*, 26(36), 36869-36877.
- 804 Pandey, D., Verma, S., Verma, P., Mahanty, B., Dutta, K., Daverey, A., & Arunachalam, K. (2020).  
805 SARS-CoV-2 in wastewater: Challenges for developing countries. *International journal of*  
806 *hygiene and environmental health*, 113634.
- 807 Pechmann, J. H., Scott, D. E., Semlitsch, R. D., Caldwell, J. P., Vitt, L. J., & Gibbons, J. W. (1991).  
808 Declining amphibian populations: the problem of separating human impacts from natural  
809 fluctuations. *Science*, 253(5022), 892-895.
- 810 Pettersen, EF, Goddard, TD, Huang, CC, et al. UCSF ChimeraX: Structure visualization for  
811 researchers, educators, and developers. *Protein Science*. 2021; 30: 70– 82.  
812 <https://doi.org/10.1002/pro.3943>
- 813 Polo, D., Quintela-Baluja, M., Corbishley, A., Jones, D. L., Singer, A. C., Graham, D. W.,  
814 & Romalde, J. L. (2020). Making waves: Wastewater-based epidemiology for SARS-CoV-  
815 2—Developing robust approaches for surveillance and prediction is harder than it looks.  
816 *Water Research*.
- 817 Polonikov, A. (2020). Endogenous Deficiency of Glutathione as the Most Likely Cause  
818 of Serious Manifestations and Death in COVID-19 Patients. *ACS Infectious Diseases*.
- 819 Pupin, N. C., Gasparini, J. L., Bastos, R. P., Haddad, C. F., & Prado, C. (2010). Reproductive  
820 biology of an endemic *Physalaemus* of the Brazilian Atlantic forest, and the trade-off between  
821 clutch and egg size in terrestrial breeders of the *P. signifer* group. *The Herpetological Journal*,  
822 20(3), 147-156.
- 823 Qi, X., Ke, B., Feng, Q., Yang, D., Lian, Q., Li, Z., ... & Liao, G. (2020). Construction and  
824 immunogenic studies of a mFc fusion receptor binding domain (RBD) of spike protein as a  
825 subunit vaccine against SARS-CoV-2 infection. *Chemical Communications*, 56(61), 8683-  
826 8686.
- 827 Raibaut, L., El Mahdi, O., & Melnyk, O. (2014). Solid phase protein chemical synthesis. In *Protein*  
828 *Ligation and Total Synthesis II* (pp. 103-154). Springer, Cham.

- 829 Ranvestel, A. W., Lips, K. R., Pringle, C. M., Whiles, M. R., & Bixby, R. J. (2004). Neotropical  
830 tadpoles influence stream benthos: evidence for the ecological consequences of decline in  
831 amphibian populations. *Freshwater Biology*, 49(3), 274-285.
- 832 Ravichandran, S., Coyle, E. M., Klenow, L., Tang, J., Grubbs, G., Liu, S., ... & Khurana, S. (2020).  
833 Antibody signature induced by SARS-CoV-2 spike protein immunogens in rabbits. *Science*  
834 *Translational Medicine*.
- 835 Ro, J. H., Liu, C. C., & Lin, M. C. (2020). Resveratrol Mitigates Cerebral Ischemic Injury by  
836 Altering Levels of Trace Elements, Toxic Metal, Lipid Peroxidation, and Antioxidant  
837 Activity. *Biological Trace Element Research*, 1-10.
- 838 Rutkoski, C. F., Macagnan, N., Folador, A., Skovronski, V. J., do Amaral, A. M., Leitemperger, J.  
839 W., ... & Hartmann, M. T. (2020). Cypermethrin-and fipronil-based insecticides cause  
840 biochemical changes in *Physalaemus gracilis* tadpoles. *Environmental Science and Pollution*  
841 *Research*, 1-11.
- 842 Sachett, A., Bevilaqua, F., Chitolina, R., Garbinato, C., Gasparetto, H., Dal Magro, J., ... & Siebel,  
843 A. M. (2018). Ractopamine hydrochloride induces behavioral alterations and oxidative  
844 status imbalance in zebrafish. *Journal of Toxicology and Environmental Health, Part A*,  
845 81(7), 194-201.
- 846 Samrat, S. K., Tharappel, A. M., Li, Z., & Li, H. (2020). Prospect of SARS-CoV-2 spike protein:  
847 Potential role in vaccine and therapeutic development. *Virus research*, 198141.
- 848 Sangkham, S. (2020). Face mask and medical waste disposal during the novel COVID-19 pandemic  
849 in Asia. *Case Studies in Chemical and Environmental Engineering*, 2, 100052.
- 850 Santiago, I., Moreno-Munoz, A., Quintero-Jiménez, P., Garcia-Torres, F., & Gonzalez-Redondo, M.  
851 J. (2020). Electricity demand during pandemic times: The case of the COVID-19 in Spain.  
852 *Energy policy*, 148, 111964.
- 853 Sharma, H. B., Vanapalli, K. R., Cheela, V. S., Ranjan, V. P., Jaglan, A. K., Dubey, B., ... &  
854 Bhattacharya, J. (2020). Challenges, opportunities, and innovations for effective solid waste  
855 management during and post COVID-19 pandemic. *Resources, conservation and recycling*,  
856 162, 105052.
- 857 Shutler, J., Zaraska, K., Holding, T. M., Machnik, M., Uppuluri, K., Ashton, I., ... & Dahiya, R.  
858 (2020). Risk of SARS-CoV-2 infection from contaminated water systems. *MedRxiv*.
- 859 Silva, F. F. D., Silva, J. M. D., Silva, T. D. J. D., Tenorio, B. M., Tenorio, F. D. C. A. M., Santos, E.  
860 L., ... & Soares, E. C. (2020). Evaluation of Nile tilapia (*Oreochromis niloticus*) fingerlings  
861 exposed to the pesticide pyriproxyfen. *Latin american journal of aquatic research*, 48(5),  
862 826-835.

- 863 Suhail, S., Zajac, J., Fossum, C., Lowater, H., McCracken, C., Severson, N., ... & Bhattacharyya, S.  
864 (2020). Role of Oxidative Stress on SARS-CoV (SARS) and SARS-CoV-2 (COVID-19)  
865 Infection: A Review. *The protein journal*, 1-13.
- 866 Tougu, V. (2001). Acetylcholinesterase: mechanism of catalysis and inhibition. *Current Medicinal*  
867 *Chemistry-Central Nervous System Agents*, 1(2), 155-170.
- 868 Tracey KJ. Physiology and immunology of the cholinergic anti-inflammatory pathway. *J Clin Invest.*  
869 2007; 117: 289-296.
- 870 Tran, H. N., Le, G. T., Nguyen, D. T., Juang, R. S., Rinklebe, J., Bhatnagar, A., ... & Chao, H. P.  
871 (2020). SARS-CoV-2 coronavirus in water and wastewater: A critical review about  
872 presence and concern. *Environmental Research*, 110265.
- 873 Trott, O; Olson, A.J. AutoDock Vina: improving the speed and accuracy of docking with a new  
874 scoring function, efficient optimization, and multithreading. *J Comput Chem.*  
875 2010;31(2):455-461. doi:10.1002/jcc.21334
- 876 Tsujimoto, M., Yokota, S., Vilček, J., & Weissmann, G. (1986). Tumor necrosis factor  
877 provokes superoxide anion generation from neutrophils. *Biochemical and*  
878 *biophysical research communications*, 137(3), 1094-1100.
- 879 Urban, R. C., & Nakada, L. Y. K. (2021). COVID-19 pandemic: Solid waste and environmental  
880 impacts in Brazil. *Science of the Total Environment*, 755, 142471.
- 881 Valavanidis, A., Vlahogianni, T., Dassenakis, M., & Scoullou, M. (2006). Molecular biomarkers of  
882 oxidative stress in aquatic organisms in relation to toxic environmental pollutants.  
883 *Ecotoxicology and environmental safety*, 64(2), 178-189.
- 884 Wang, X. W., Li, J. S., Jin, M., Zhen, B., Kong, Q. X., Song, N., ... & Si, B. Y. (2005). Study on the  
885 resistance of severe acute respiratory syndrome-associated coronavirus. *Journal of*  
886 *virological methods*, 126(1-2), 171-177.
- 887 Waterhouse, A., Bertoni, M., Bienert, S., Studer, G., Tauriello, G., Gumienny, R., Heer, F.T., de  
888 Beer, T.A.P., Rempfer, C., Bordoli, L., Lepore, R., Schwede, T. SWISS-MODEL: homology  
889 modelling of protein structures and complexes. *Nucleic Acids Res.* 46(W1), W296-W303  
890 (2018).
- 891 Wu, F., Xiao, A., Zhang, J., Moniz, K., Endo, N., Armas, F., ... & Duvallet, C. (2020). SARS-CoV-2  
892 titers in wastewater foreshadow dynamics and clinical presentation of new COVID-19  
893 cases. *Medrxiv*.
- 894 Wrubleswski, J., Reichert, F. W., Galon, L., Hartmann, P. A., & Hartmann, M. T. (2018). Acute  
895 and chronic toxicity of pesticides on tadpoles of *Physalaemus cuvieri* (Anura,  
896 *Leptodactylidae*). *Ecotoxicology*, 27(3), 360-368.

897

898 Xiao, F., Sun, J., Xu, Y., Li, F., Huang, X., Li, H., ... & Zhao, J. (2020). Infectious SARS-CoV-2 in  
899 feces of patient with severe COVID-19. *Emerging infectious diseases*, 26(8), 1920.

900 Yang, J., Wang, W., Chen, Z., Lu, S., Yang, F., Bi, Z., ... & Hong, W. (2020). A vaccine targeting  
901 the RBD of the S protein of SARS-CoV-2 induces protective immunity. *Nature*,  
902 586(7830), 572-577.

903 Yang, L., Yu, X., Wu, X., Wang, J., Yan, X., Jiang, S., & Chen, Z. (2020). Emergency response to  
904 the explosive growth of health care wastes during COVID-19 pandemic in Wuhan, China.  
905 *Resources, Conservation and Recycling*, 164, 105074.

906 Zand, A. D., & Heir, A. V. (2020). Emerging challenges in urban waste management in Tehran,  
907 Iran during the COVID-19 pandemic. *Resources, Conservation, and Recycling*, 162,  
908 105051.

909

## SUPPLEMENTARY MATERIAL

910

911 **Table S1.** Summary information on the methodological procedures adopted in the purification stage  
912 of the present study's compounds.

Compound	Retention time (min)	Purificationmethod
PSPD2001	7,7	Gradient 3 to 43% B in 90 min
PSPD2002	10,0	Gradient 10 to 50% B in 90 min
PSPD2003	2,0 e 13,3	Isocratic 2% to B in 20 min and gradient 2 to 53% B in 90 min

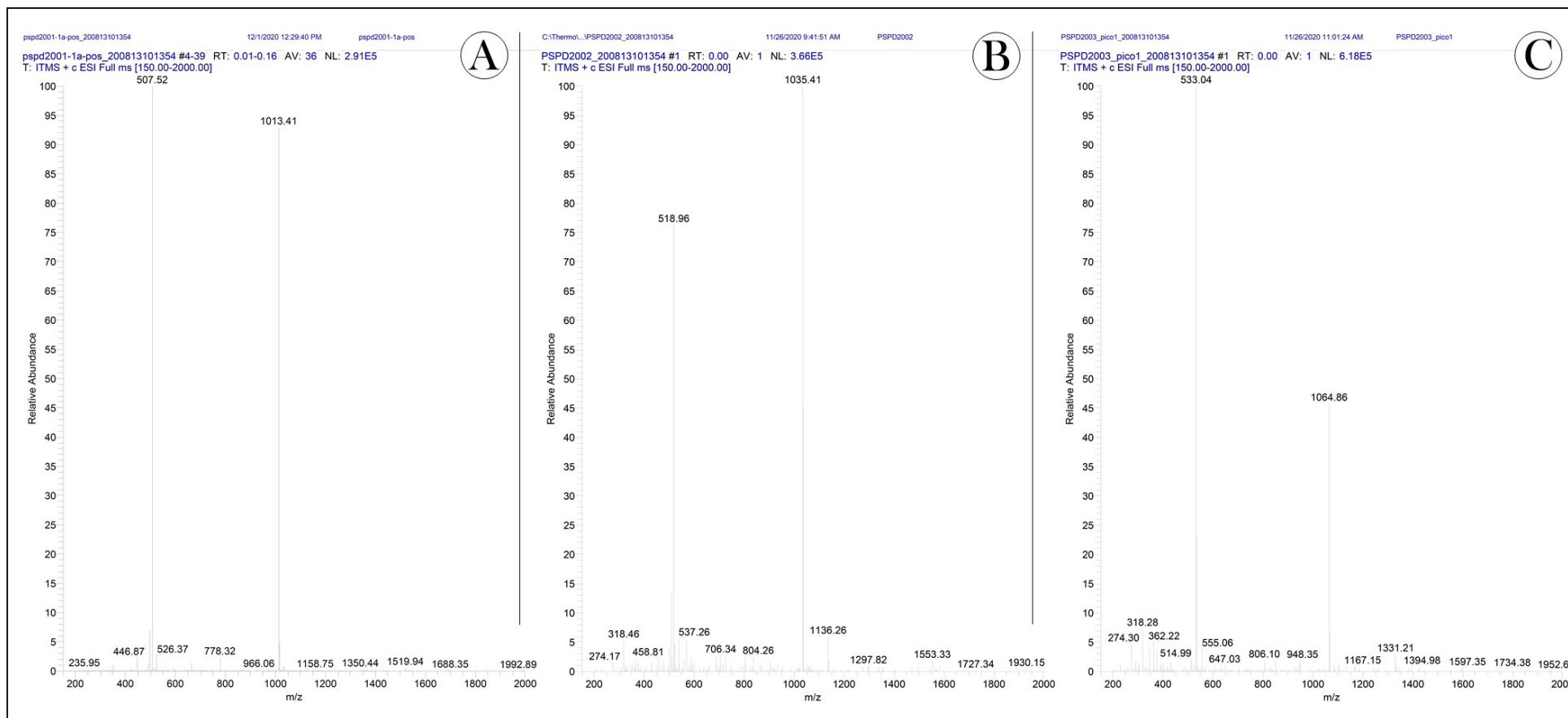
913

PSPD2001: Arg-Val-Tyr-Ser-Ser-Ala-Asn-Asn-Cys- COOH; PSPD2002: Gln-Cys-Val-Asn-Leu-Thr-Thr-Arg-Thr-COOH;

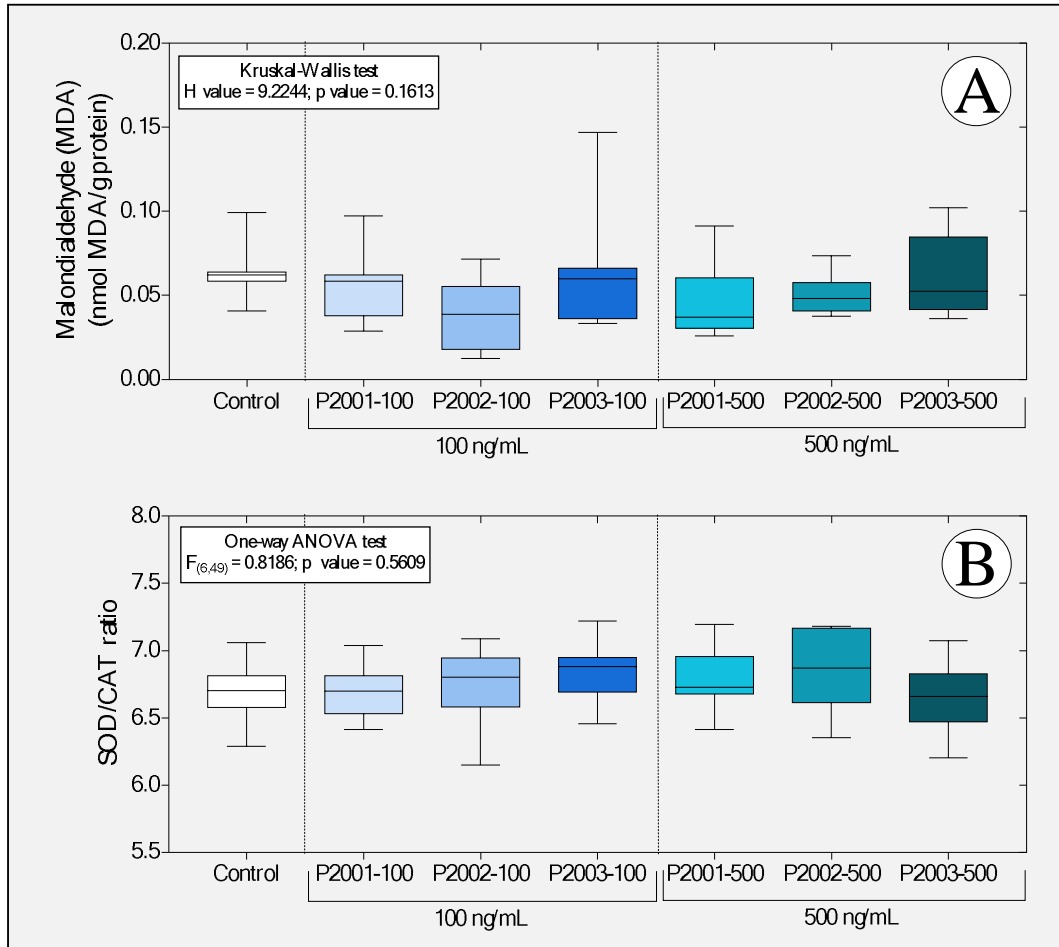
914

PSPD2003: Asn-Asn-Ala-Thr-Asn-COOH.

915

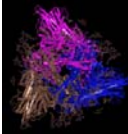
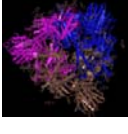
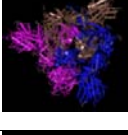
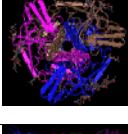
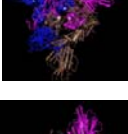
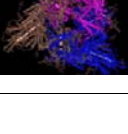


**Figure S1.** Mass spectra obtained for peptides (A) PSPD2001, (B) PSPD2002 and (C) PSPD2003. PSPD2001: Arg-Val-Tyr-Ser-Ser-Ala-Asn-Asn-Cys-COOH; PSPD2002: Gln-Cys-Val-Asn-Leu-Thr-Thr-Arg-Thr-COOH; PSPD2003: Asn-Asn-Ala-Thr-Asn-COOH.



**Figure S2.** Boxplot of (A) concentrations of malondialdehyde and (B) SOD / CAT ratio in tadpoles of *P. cuvieri* (phase 27G) exposed or not to PSPD peptides 2001, 2002, and 2003 of the SARS-CoV-2 Spike protein. Summaries of statistical analyzes are shown in the upper left corner of the graph. (n = 50 animals / group). PSPD2001: Arg-Val-Tyr-Ser-Ser-Ala-Asn-Asn-Cys-COOH; PSPD2002: Gln-Cys-Val-Asn-Leu-Thr-Thr-Arg-Thr-COOH; PSPD2003: Asn-Asn-Ala-Thr-Asn-COOH. SOD: superoxide dismutase; CAT: catalase.

916 **TableS1:** Alignment of the sequences and consensus for PSPD2001, showing the similarities found  
 917 with the proteins noted in the GeneBank database by BLAST, with the Sequence ID, the protein's  
 918 name, the Number of Matches, the alignment and the Protein 3D Structure.

Sequence ID	Protein	Number of Matches	Alignment	Protein 3D Structure
6XR8_A	Distinct conformational states of SARS-CoV-2 spike protein [Severe acute respiratory syndrome coronavirus 2]	1	<b>Query</b> 1    RVYSSANNC 9 <b>Sbjct</b> 158    RVYSSANNC 166 RVYSSANNC 166	
6XKL_A	SARS-CoV-2 HexaPro S One RBD up [Severe acute respiratory syndrome coronavirus 2]	1	<b>Query</b> 1    RVYSSANNC 9 <b>Sbjct</b> 158    RVYSSANNC 166 RVYSSANNC 166	
6VSB_A	Prefusion 2019-nCoV spike glycoprotein with a single receptor-binding domain up [Severe acute respiratory syndrome coronavirus 2]	1	<b>Query</b> 1    RVYSSANNC 9 <b>Sbjct</b> 158    RVYSSANNC 166 RVYSSANNC 166	
6Z43_A	Cryo-EM Structure of SARS-CoV-2 Spike : H11-D4 Nanobody Complex [Severe acute respiratory syndrome coronavirus 2]	1	<b>Query</b> 1    RVYSSANNC 9 <b>Sbjct</b> 158    RVYSSANNC 166 RVYSSANNC 166	
6ZGG_A	Furin Cleaved Spike Protein of SARS-CoV-2 with One RBD Erect [Severe acute respiratory syndrome coronavirus 2]	1	<b>Query</b> 1    RVYSSANNC 9 <b>Sbjct</b> 189    RVYSSANNC 197 RVYSSANNC 197	
6ZGE_A	Uncleavable Spike Protein of SARS-CoV-2 in Closed Conformation [Severe acute respiratory syndrome coronavirus 2]	1	<b>Query</b> 1    RVYSSANNC 9 <b>Sbjct</b> 189    RVYSSANNC 197 RVYSSANNC 197	

919  
 920