

# 1 Phasic and tonic serotonin modulate alarm reactions 2 and post-exposure behavior in zebrafish

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## 26 List of abbreviations

27	4-OH quinoline	4-hydroxyquinoline
28	5-HT	Serotonin
29	5,7-DHT	5,7-dihydroxytryptamine
30	ANOVA	Analysis of variance
31	CAS	Conspecific alarm substance
32	CONCEA	Conselho Nacional de Controle de Experimentação Animal
33	CTRL	Control groups
34	IACUC	Institutional Animal Care and Use Committee
35	Ibama	Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis
36	IV	Independent variable
37	MAD	Median absolute difference
38	pCPA	<i>para</i> -chlorophenylalanine
39	PI	Principal Investigator
40	ppm	Parts per million
41	UEPA	Universidade do Estado do Pará
42	WAY 100,635	<i>N</i> -[2-[4-(2-Methoxyphenyl)-1-piperazinyl]ethyl]- <i>N</i> -(2-pyridyl)cyclohexanecarboxamide
43		
44	zMAO	Zebrafish monoamine oxidase

## 45 **Abstract**

46 Current theories on the role of serotonin (5-HT) in vertebrate defensive behavior suggest that this  
47 monoamine increases anxiety but decreases fear, by acting at different levels of the neuroaxis. This  
48 paradoxical, dual role of 5-HT suggests that a serotonergic tone inhibits fear responses, while an  
49 acute increase in 5-HT would produce anxiety-like behavior. However, so far no evidence for a  
50 serotonergic tone has been found. Using zebrafish alarm responses, we investigate the participation  
51 of phasic and tonic 5-HT levels in fear-like behavior, as well as in behavior after stimulation.  
52 Conspecific alarm substance (CAS) increased bottom-dwelling and erratic swimming, and animals  
53 transferred to a novel environment after CAS exposure (post-exposure behavior) showed increased  
54 bottom-dwelling and freezing. Clonazepam blocked CAS effects during and after exposure. Acute  
55 fluoxetine dose-dependently decreased fear-like behavior, but increased post-exposure freezing.  
56 Metergoline had no effect on fear-like behavior, but blocked the effects of CAS on post-exposure  
57 behavior; similar effects were observed with pCPA. Finally, CAS was shown to decrease the  
58 activity of monoamine oxidase in the zebrafish brain after exposure. These results suggest that  
59 phasic and tonic serotonin encode an aversive expectation value, switching behavior towards  
60 cautious exploration/risk assessment/anxiety when the aversive stimulus is no longer present.

61 *Keywords:* Serotonin; Fear; Alarm substance; Zebrafish; Panic

## 62 **1. Introduction**

63       The neurocircuitry of defensive reactions involves regulation by a plethora of  
64 neuromodulators, including monoamines and peptides (Maximino 2012). In vertebrates, the  
65 monoamine serotonin (5-HT) is produced in specific brain nuclei, including the raphe, and is  
66 thought to inhibit fear/escape responses to proximate threat by acting on more caudal structures of  
67 the aversive brain system (Paul *et al.* 2014; Deakin and Graeff 1991; Maximino 2012). This  
68 response appears to be dependent on the specific brain region in which serotonin acts, as well as on  
69 the receptor that is activated. For example, in the rodent periaqueductal gray, the activation of 5-  
70 HT<sub>1A</sub> and 5-HT<sub>2</sub>-type receptors inhibit fear responses, while in amygdaloid nuclei the activation of  
71 5-HT<sub>2</sub>- and 5-HT<sub>3</sub>-type receptors increase anxiety-like responses (Guimarães *et al.* 2008; Paul *et al.*  
72 2014; Hale and Lowry 2011). There is also evidence for a serotonergic “tone” inhibiting anxiety,  
73 since antagonists usually inhibit anxiety-like responses in animal models; however, antagonists do  
74 not appear to modify fear-like responses, suggesting that phasic, not tonic, serotonin is involved in  
75 fear. For example, 5-HT levels do not change in the basolateral amygdala or in the dorsal  
76 periaqueductal gray during chemical stimulation of this latter structure in rats (Zanoveli *et al.* 2009),  
77 a manipulation that induces panic-like responses (Brandão *et al.* 2008). This suggests that the  
78 inhibitory role of serotonin in fear functions as a “switching” signal: as the threatening stimulus  
79 ceases, serotonin is released, inhibiting the fear reactions that are now non-adaptive, and initiating  
80 careful exploration and risk assessment responses to ensure that the threat is actually over.

81       In non-mammalian vertebrates, including teleost fish, 5-HT is produced in additional brain  
82 regions, including pretectal and hypothalamic populations (Herculano and Maximino 2014). There  
83 is some evidence that 5-HTergic neurons innervate areas of the teleostean brain which participate in  
84 defensive behavior, including prosencephalic and mesencephalic regions (do Carmo Silva *et al.*  
85 2018a). A role for 5-HT in modulating fish defensive behavior has been demonstrated before: in  
86 zebrafish, 5-HT<sub>1A</sub> and 5-HT<sub>1B</sub> receptor antagonists decrease anxiety-like behavior (Maximino *et al.*  
87 2013; Nowicki *et al.* 2014; Herculano *et al.* 2015; Maximino *et al.* 2015), while 5-HT<sub>2</sub>- and 5-HT<sub>3</sub>-

88 type antagonists increase it (Nowicki *et al.* 2014). Little is known, however, of the modulation of  
89 fear-like responses. Microinjection of the serotonergic neurotoxin 5,7-dihydroxytryptamine (5,7-  
90 DHT) in the telencephalon of zebrafish, destroying most serotonergic innervation in regions  
91 associated with aversive learning, impairs the acquisition of active avoidance (Amo *et al.* 2014),  
92 suggesting that serotonin encodes an aversive expectation value.

93 In zebrafish and other Actinopterygian fish, specialized club cells in the skin produce a  
94 substance (conspecific alarm substance, CAS) that, when the skin is damaged, is dispersed in the  
95 water, signaling to conspecifics a potential threat (von Frisch 1941; Hüttel 1941; von Frisch 1938;  
96 Døving and Lastein 2009; Maximino *et al.* 2019). CAS induces defensive behavior in zebrafish,  
97 including increased bottom-dwelling, erratic swimming, and freezing (Maximino *et al.* 2019; Egan  
98 *et al.* 2009; Speedie and Gerlai 2008; Maximino *et al.* 2014). These responses have been exploited  
99 as a model system to study fear in more basal vertebrates (Maximino *et al.* 2019; Jesuthasan and  
100 Mathuru 2008).

101 The serotonergic system has also been implicated in some of these behavioral functions.  
102 CAS increases extracellular serotonin levels (Maximino *et al.* 2014) and inhibits monoamine  
103 oxidase activity (Quadros *et al.* 2018) in the zebrafish brain after exposure. Zebrafish exposed to  
104 CAS show increased anxiety-like behavior in the light/dark test after exposure (i.e., when the  
105 substance is no longer present), an effect that is blocked by fluoxetine but not by the 5-HT<sub>1A</sub>  
106 receptor antagonist WAY 100,635 (Maximino *et al.* 2014). Interestingly, WAY 100,635 blocked the  
107 analgesic effects of CAS in zebrafish (Maximino *et al.* 2014), suggesting that this receptor  
108 participates in some, but not all, neurobehavioral responses to threatening stimuli. While WAY  
109 100,635 was not able to alter anxiety-like behavior *after* exposure, the drug blocked the increased  
110 geotaxis *during* CAS exposure, both in the first minutes of exposure and in the last minutes (Nathan  
111 *et al.* 2015). Blocking 5-HT<sub>2</sub>-type receptors with methysergide did not affect these responses,  
112 except at a sedative dose (Nathan *et al.* 2015). These results are difficult to interpret, but suggest

113 that CAS increases serotonergic activity after exposure, and that a serotonergic tone on the 5-HT<sub>1A</sub>  
114 receptor is involved in behavioral switching after exposure – that is, when the threat is no longer  
115 present, and risk assessment begins; whether this is true for behavioral responses *during* exposure –  
116 that is, when the threat is present – is unknown.

117 The present paper investigated whether phasic and tonic serotonin participates in the alarm  
118 response in zebrafish during and after exposure. Our results reinforce the idea that behavior during  
119 CAS exposure is qualitatively different from behavior after CAS exposure. We also show that  
120 behavior in both contexts are differentially sensitive to clonazepam, a high potency benzodiazepine  
121 commonly used in the clinical management of panic disorder (Caldirola *et al.* 2016). Increasing  
122 serotonin levels by treating zebrafish with acute fluoxetine blocked the effects of CAS during and  
123 after exposure, but blocking serotonin receptors with metergoline, or blocking serotonin synthesis  
124 with *pCPA*, produced an effect only after exposure. Finally, we show that CAS inhibited  
125 monoamine oxidase activity in the brain. Results are discussed in terms of the putative role of  
126 serotonin in an homeostatic “neurobehavioral switch” in the absence of threat after predatory risk.

127

## 128 **2. Methods**

### 129 **2.1. Animals, housing, and baseline conditions**

130 435 zebrafish (*Danio rerio*) from the longfin phenotype were used in the present  
131 experiments; details for sample size calculations can be found on each experimental section, below  
132 (Figure 1). Outbred populations were used due to their increased genetic variability, decreasing the  
133 effects of random genetic drift that could lead to the development of uniquely heritable traits (Parra  
134 *et al.* 2009; Speedie and Gerlai 2008). The populations used in the present experiments are expected  
135 to better represent the natural populations in the wild. Animals were bought from a commercial  
136 vendor (Fernando Peixes, Belém/PA) and arrived in the laboratory with an approximate age of 3  
137 months (standard length =  $13.2 \pm 1.4$  mm), and were quarantined for two weeks; the experiment

138 began when animals had an approximate age of 4 months (standard length =  $23.0 \pm 3.2$  mm).  
139 Animals were kept in mixed-sex tanks during acclimation, with an approximate ratio of 50-50  
140 males to females (confirmed by body morphology). The breeder was licensed for aquaculture under  
141 Ibama's (Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis) Resolution  
142 95/1993. Animals were group-housed in 40 L tanks, with a maximum density of 25 fish per tank,  
143 for at least 2 weeks before experiments begun. Tanks were filled with non-chlorinated water at  
144 room temperature (28 °C) and a pH of 7.0-8.0. Lighting was provided by fluorescent lamps in a  
145 cycle of 14-10 hours (LD), according to standards of care for zebrafish (Lawrence, 2007). Water  
146 quality parameters were as follows: pH 7.0-8.0; hardness 100-150 mg/L CaCO<sub>3</sub>; dissolved oxygen  
147 7.5-8.0 mg/L; ammonia and nitrite < 0.001 ppm. Potential suffering of animals was minimized by  
148 controlling for the aforementioned environmental variables and scoring humane endpoints (clinical  
149 signs, behavioral changes, bacteriological status), following Brazilian legislation (Conselho  
150 Nacional de Controle de Experimentação Animal - CONCEA 2017). Animals were used for only  
151 one experiment and in a single behavioral test, to reduce interference from apparatus exposure.  
152 Experiments were approved by UEPA's IACUC under protocol 06/18.

153

## 154 **2.2. Alarm substance extraction**

155 CAS was extracted at a ratio of 1 donor fish for 10 ml distilled water. A detailed protocol for  
156 extraction can be found at protocols.io (Silva, Rocha, Lima-Maximino, & Maximino, 2018;  
157 <https://dx.doi.org/10.17504/protocols.io.tr3em8n>). Briefly, a donor fish was cold-anesthetized and  
158 euthanized, and 15 shallow cuts were made on the side of its trunk to lesion club cells. The cuts  
159 were washed with 10 mL distilled water, and 7 mL of the eluate was reserved as 1 unit CAS.

160

## 161 **2.3. General experimental design**

162           After the onset of drug effects (see details below for each drug), animals were individually  
163 transferred to a 1.5 L tank (12 cm X 12 cm x C cm, w X l X h), filled with system water, and left to  
164 acclimate for 3 min. Filming was started, and animals were exposed to either 7 mL distilled water  
165 (CTRL groups) or 7 mL (1 unit) alarm substance (CAS groups). Exposure was made by slowly  
166 pouring the substance on the water from the top. Animals were then left undisturbed as filming  
167 continued for 6 min; this was termed “alarm reaction”. The animal was then transferred to a tank  
168 with 500 mL mineral water for a 1 min “washout” period, to remove potential residues from the  
169 alarm substance. After this period, the animal was transferred to a 5 L tank (A cm X 24 cm X 22  
170 cm, w X l X h) and freely explored for 6 min, during which its behavior was recorded; this was  
171 termed “post-exposure behavior” (Figure 2A). Tanks for both stages were differently shaped to  
172 increase the novelty of the second environment, a variable that is important to induce an anxiety-  
173 like “diving” response in animals not exposed to CAS (Bencan *et al.* 2009). Light levels above the  
174 tanks were measured using a handheld light meter, and ranged from 251 to 280 lumens (coefficient  
175 of variation = 3.399% between subjects) In all experiments, the following variables were recorded:

- 176           • Time spent on the bottom third of the tank (s) [Primary outcome]
- 177           • Time spent on the top third of the tank (s) [Secondary outcome]
- 178           • Absolute turn angle (equivalent to erratic swimming) [Secondary outcome]
- 179           • Freezing: duration of complete movement cessation, defined as speed lower than 0.5 cm/s.  
180           [Secondary outcome]
- 181           • Swimming speed (cm/s) [Secondary outcome]

182           Variables were extracted by automated video tracking, using the software  
183 TheRealFishTracker v. 0.4.0 (<http://www.dgp.toronto.edu/~mccrae/projects/FishTracker/>), running  
184 on a Windows platform. Animals were randomly allocated to groups using a random number  
185 generator ([http://www.jerrydallal.com/random/random\\_block\\_size\\_r.htm](http://www.jerrydallal.com/random/random_block_size_r.htm)), with each subject



186 randomized to a single treatment using random permuted blocks. One PI attributed a random letter  
187 to treatment (e.g., “A” for CTRL, “B” for CAS) and a random integer for drug dose (e.g., “1” for 1  
188 mg/kg, “2” for 0 mg/kg [vehicle]), and combinations for letters and integers were randomized. For  
189 each experiment, animals were treated and tested in the order of allocation (i.e., randomly). In all  
190 experiments, experimenters and data analysts were blinded to drugs and treatment by using coded  
191 vials (with the same code used for randomization); blinding was removed only after data analysis.  
192 Experiments were always run between 08:00AM and 02:00 PM. After experiments, animals were  
193 sacrificed by prolonged bath in ice-cold water (< 12 °C), followed by spinal transection (Matthews  
194 and Varga 2011).

195

## 196 **2.4. Quality control**

197 **Exclusion criteria:** With the exception of outlier exclusion (described in 2.5.3), no  
198 exclusion criteria were predetermined.

199 **Behavioral data:** Quality control of samples was maintained by periodic assessment of  
200 water quality and health parameters. All experimenters were trained in the behavioral methods  
201 before experiments; training included observation of all experiments by a PI (CM or MGL) on at  
202 least two occasions. After these observations, each trainee performed two mock experiments, on a  
203 single subject each, while being observed by the PI. All protocols were reviewed by all PIs, and are  
204 publicly available. Behavioral records were reviewed by at least one PI for administration/scoring  
205 accuracy, in order to ensure adherence to protocols and consistency across tests.

206 **Biochemical data:** All experimenters were trained in the analytical method before  
207 experiments. Quality control was achieved periodically using Levey-Jennings charts for known  
208 concentrations of kynuramine, adopting a  $1_{2S}$  rule.

209

## 210 **2.5. Experiments 1-4: Effects of clonazepam and serotonergic drugs on** 211 **alarm reaction and post-exposure behavior**

### 212 **2.5.1. Sample size calculations**

213 Sample size calculations were based on a power analysis for a 2-way ANOVA with  
214 interaction effects, with  $\alpha = 0.05$ ,  $\beta = 0.8$ , and expected effect size  $f = 0.25$  for each independent  
215 variable (IV); Effect sizes used for estimating sample sizes were based on the range of effects  
216 observed after pharmacological manipulations on zebrafish anxiety-like behavior in a metanalysis  
217 (Kysil et al., 2017). Sample size of 15 animals/group was established. Thus, a total of 270 animals  
218 were used for experiments, and another 135 animals were used to produce CAS. The distribution of  
219 samples through groups can be found in Figure 1.

220

### 221 **2.5.2. Drugs and treatments**

222 Clonazepam (CAS #1622-61-3) was bought from Roche on 2018, and dissolved in  
223 Cortland's salt solution (NaCl 124.1 mM, KCl 5.1 mM, Na<sub>2</sub>HPO<sub>4</sub> 2.9 mM, MgSO<sub>4</sub> 1.9 mM, CaCl<sub>2</sub>  
224 1.4 mM, NaHCO<sub>3</sub> 11.9 mM, Polyvinylpyrrolidone 4%, 1,000 USP units Heparin; Wolf 1963).  
225 Clonazepam, as a high-potency benzodiazepine widely used in treating panic disorder (Cloos 2005;  
226 Caldirola *et al.* 2017), is expected to decrease fear-like responses to CAS, and therefore used as a  
227 positive control. Fluoxetine is expected to acutely increase serotonin levels in the synapse (Figure  
228 2B). Fluoxetine hydrochloride (CAS #54910-89-3) was bought from Libbs on 2017, and dissolved  
229 in Cortland's salt solution. Metergoline is expected to block 5-HT receptors from the 5-HT<sub>1</sub>, 5-HT<sub>2</sub>,  
230 and 5-HT<sub>7</sub> families (Figure 2C). Metergoline (CAS #17692-51-2) was bought from Virbac on 2017,  
231 and dissolved in Cortland's salt solution. pCPA is expected block 5-HT synthesis, therefore greatly  
232 reducing serotonergic tone on all receptors (Figure 2D). 4-chloro-DL-phenylalanine (pCPA; CAS  
233 #7424-00-2) was bought from Sigma-Aldrich (C6506) on 2018, and dissolved in 10% DMSO. For  
234 Experiment 1, animals were injected intraperitoneally with either vehicle (Cortland's salt solution)  
235 or clonazepam (0.05 mg/kg; Maximino, Silva, Gouveia Jr., & Herculano, 2011). For Experiment 2,

236 animals were injected intraperitoneally with vehicle (Cortland's salt solution) or fluoxetine (2.5 or  
237 25 /kg; Maximino et al. 2014). For Experiment 3, animals were injected intraperitoneally with  
238 vehicle (Cortland's salt solution) or metergoline (1 mg/kg; Pimentel et al. 2019). For Experiment 4,  
239 animals were injected intraperitoneally with either vehicle (DMSO) or pCPA (one injection of 150  
240 mg/kg/day for 2 days, followed by 24 h without treatment; Curzon et al. 1978). Injections were  
241 made according to the protocol proposed by Kinkel et al. (2010); briefly, animals were cold-  
242 anesthetized and transferred to a sponge-based surgical bed, in which injection was made. Injections  
243 were made using a microsyringe (Hamilton® 701N syringe, needle size 26 gauge at cone tip), with  
244 total volumes of injection ranging from 4.81 to 5.05  $\mu$ L. Cold-anesthesia has been shown to  
245 produce satisfactory results in zebrafish, with faster recovery and less animal loss than commonly  
246 used anesthetics such as MS-222 (Matthews and Varga 2011). The sponge allowed gill perfusion to  
247 be kept, minimizing suffering. 20 min after recovery, animals were subjected to CAS or water.

248

### 249 **2.5.3. Statistical analysis**

250 Outliers were removed based on median absolute differences (MADs), using time on bottom  
251 as main endpoint; values were removed when they were higher or lower than 3 MADs around the  
252 median (Leys *et al.* 2013), and the number of outliers was reported in the results. Differences  
253 between groups were analyzed using two-way analyses of variance (ANOVAs) with robust  
254 estimators on Huber's M-estimators, using the R package 'rcompanion' (Mangiafico 2017;  
255 <https://cran.r-project.org/package=rcompanion>). Normality was not assumed, and thus no specific  
256 test for normality was performed; however, this type of analysis is resistant to deviations from the  
257 assumptions of the traditional ordinary-least-squares ANOVA, and are robust to outliers, thus being  
258 insensitive to distributional assumptions (such as normality)(Huber 1981). Behavioral variables  
259 were included as outcomes, with treatment and drug used as independent variables; interaction

260 between IVs was assessed as the most important predictor. P-values were adjusted for the false  
261 discovery rate.

262

## 263 **2.6. Experiment 5: Effects of CAS on monoamine oxidase activity**

### 264 **2.6.1. Sample size**

265 Based on a power analysis for two-sample unpaired t-test.  $\alpha = 0.05$ , power = 0.8, and  
266 expected effect size  $d = 1.5$ , a sample size of 10 animals/group was established. Thus, a total of 20  
267 animals were used for experiments, and 10 more used to produce CAS. The distribution of samples  
268 through groups can be found in Figure 1.

269

### 270 **2.6.2. Methods**

271 z-MAO activity was determined as reported previously (Quadros *et al.* 2018). Two zebrafish  
272 brains were pooled per sample and homogenized in 0.5 mL of buffer solution containing 16.8 mM  
273  $\text{Na}_2\text{HPO}_4$  and 10.6 mM  $\text{KH}_2\text{PO}_4$ , pH 7.4, isotonized with sucrose. Samples ( $n = 10$  per group) were  
274 centrifuged at  $1.000 \times g$  for 5 min, and the supernatants were kept on ice for the experiments.  
275 Protein samples (approximately 100  $\mu\text{g}$ ) were mixed with 460  $\mu\text{L}$  of assay buffer (168 mM  
276  $\text{Na}_2\text{HPO}_4$  and 10.6 mM  $\text{KH}_2\text{PO}_4$ , pH 7.4, isotonized with KCl) and preincubated at  $37^\circ\text{C}$  for 5 min.  
277 The reaction started by adding 110  $\mu\text{M}$  kynuramine hydrobromide in a final volume of 700  $\mu\text{L}$ , and  
278 was stopped 30 min later with 300  $\mu\text{L}$  10% trichloroacetic acid. Reaction products were further  
279 centrifuged at  $16.000 \times g$  for 5 min and supernatants (800  $\mu\text{L}$ ) were mixed with 1M NaOH (1 mL).  
280 Fluorescence was measured using excitation at 315 nm and emission at 380 nm. Product formation  
281 (4-hydroxyquinoline) was estimated and enzyme activity was expressed as expressed as nmol 4-OH  
282 quinoline/min/mg protein.

283

### 284 **2.6.3. Statistical analysis**

285 Data were analyzed with an asymptotic general independence test, using the R package  
286 ‘coin’ (Hothorn et al. 2006; <https://cran.r-project.org/package=coin>).

287

## 288 **2.7. Open science practices**

289 Experiments were formally preregistered at Open Science Framework  
290 (<https://doi.org/10.17605/OSF.IO/QM3PX>). Data packages and analysis scripts for all experiments  
291 can be found at a GitHub repository (<https://github.com/lanec-unifesspa/5-HT-CAS>). Preprints for  
292 the manuscript can be found at bioRxiv (<https://doi.org/10.1101/827824>).

293

### 294 **2.7.1. Changes from pre-registration**

295 During pre-registration, we proposed to use manual recording of behavioral variables.  
296 During experiments, we decided to use automated tracking, due to the higher availability of open  
297 source software; automated tracking allows for a better reproducibility and precision in measures  
298 but, as a trade-off, some measurements were not possible. In the present experiments, the software  
299 TheRealFishTracker (v. 0.4.0, for Windows;  
300 <http://www.dgp.toronto.edu/~mccrae/projects/FishTracker/>) was used. Moreover, during pre-  
301 registration we proposed to also analyze melanophore responses to CAS; this data will appear in a  
302 separate paper, on behavioral and physiological aspects of the alarm reaction, and the data is  
303 available at a GitHub repository ([https://github.com/lanec-unifesspa/5-HT-  
304 CAS/tree/master/data/melanophore](https://github.com/lanec-unifesspa/5-HT-CAS/tree/master/data/melanophore)). Finally, the following doses were changed from pre-  
305 registration: clonazepam was reduced to 0.05 mg/kg, to avoid unwanted sedation; a second dose of  
306 fluoxetine was added to approach the range in which fluoxetine blocks fear conditioning at a high  
307 shock intensity (Santos *et al.* 2006). Due to a problem with solubility, the pCPA dose was reduced  
308 to 150 mg/kg, a dose that has been shown to reduce serotonin levels in the rat brain by about 50%,  
309 and to block the release of 5-HT elicited by electrical stimulation of the raphe (Curzon *et al.* 1978).

310

### 311 **3. Results**

#### 312 **3.1. Experiment 1**

313 One outlier was removed from the group in which animals were exposed to CAS and  
314 injected with vehicle; and one outlier was removed from the group in which were exposed to water  
315 and injected with vehicle in Experiment 1. During CAS exposure, significant effects of treatment ( $p$   
316 = 0.0006), dose ( $p = 0.0044$ , and interaction ( $p = 0.0006$ ) were found for time on top (Figure 3A);  
317 post-hoc tests suggested that CAS decreased time on top (adjusted  $p = 3.91 \cdot 10^{-5}$ ), and CLZ blocked  
318 this effect. Significant effects of treatment ( $p = 0.0466$ ), dose ( $p = 0.0002$ ), and interaction ( $p <$   
319  $0.0001$ ) were found for time on bottom (Figure 3B); CAS increased time on bottom (adjusted  $p$   
320 =  $9.44 \cdot 10^{-6}$ ), and CLZ blocked this effect. Main effects of treatment ( $p = 0.0004$ ) and dose ( $p =$   
321  $0.00038$ ), as well as an interaction effect ( $p = 0.00042$ ), were found for absolute turn angle (Figure  
322 3C); CAS increased absolute turn angle (adjusted  $p = 0.00033$ ), and CLZ blocked this effect. Main  
323 effects of treatment ( $p = 0.00028$ ) and dose ( $p = 0.0031$ ), as well as an interaction effect ( $p =$   
324  $0.0021$ ), were found for freezing (Figure 3D); again, CAS increased freezing (adjusted  $p = 1.26 \cdot 10^{-6}$ ),  
325 and CLZ blocked this effect. Main effects were found for treatment ( $p = 0.04$ ) and dose ( $p =$   
326  $0.02$ ) for speed; however, post-hoc tests failed to uncover differences between groups (Figure 3E).

327 After CAS exposure, significant main effects of treatment ( $p = 0.0041$ ) and dose ( $p =$   
328  $0.0023$ ), as well as an interaction effect ( $p = 0.0021$ ), were found for time on top (Figure 4A); CAS  
329 decreased time on top (adjusted  $p = 1.07 \cdot 10^{-5}$ ), and CLZ partially blocked this effect. Main effects  
330 of treatment ( $p = 2 \cdot 10^{-4}$ ) and dose ( $p = 2.1 \cdot 10^{-4}$ ), as well as an interaction effect ( $p = 0.0044$ ), were  
331 found for time on bottom (Figure 4B), and post-hoc tests suggested that CAS increased time on  
332 bottom ( $p = 0.0009$ ) while CLZ blocked this effect. No main effects ( $p > 0.2$ ), nor an interaction  
333 effect ( $p = 0.4$ ) were found for absolute turn angle (Figure 4C). A main effect of treatment ( $p =$   
334  $2.1 \cdot 10^{-4}$ ) and drug ( $p = 2.1 \cdot 10^{-4}$ ), as well as an interaction effect ( $p = 2.3 \cdot 10^{-4}$ ), were found for

335 freezing (Figure 4D); CAS increased freezing (adjusted  $p = 0.0009$ ), and CLZ blocked this effect.  
336 No main effects were found for swimming speed ( $p > 0.08$ ), nor were interaction effects found ( $p >$   
337  $0.3$ ) (Figure 4E).

338

### 339 **3.2. Experiment 2**

340 One outlier was removed from the group exposed to CAS and treated with 2.5 mg/kg  
341 fluoxetine. During CAS exposure, significant effects of treatment ( $p = 0.0458$ ), dose ( $p < 0.0001$ ),  
342 and interaction ( $p < 0.0001$ ) were found for time on top. Fluoxetine alone increased time on top at  
343 2.5 mg/kg (adjusted  $p = 3.218 \cdot 10^{-5}$  vs. control), CAS decreased it (adjusted  $p = 0.01132$ ), and  
344 fluoxetine blocked the effect of CAS at both doses (Figure 5A). Main effects of treatment ( $p =$   
345  $0.004$ ) and dose ( $p < 0.0001$ ), but no interaction ( $p = 0.6152$ ), were found for time on bottom (Figure  
346 5B); CAS increased time on bottom (adjusted  $p < 0.0236$ ), fluoxetine decreased it at both doses  
347 (adjusted  $p < 0.01$ ), and fluoxetine blocked the effect of CAS at the highest dose. A main effect of  
348 treatment ( $p = 0.0436$ ), but not dose ( $p = 0.1102$ ) nor interaction ( $p = 0.1148$ ), was found for  
349 absolute turn angle (Figure 5C); post-hoc tests suggested that CAS increased absolute turn angle  
350 (adjusted  $p = 0.0061$  vs. control), and fluoxetine partially (2.5 mg/kg) or fully (25 mg/kg) blocked  
351 this effect. A main effect of treatment ( $p < 0.0001$ ) and dose ( $p = 0.0004$ ), as well as an interaction  
352 effect ( $p = 0.00002$ ), were found for freezing (Figure 5D); CAS increased freezing (adjusted  $p =$   
353  $0.0058$ ), and both doses partially blocked this effect. Finally, no effect was found on swimming  
354 speed ( $p > 0.39$ ; Figure 5E).

355 Significant effects of treatment ( $p = 0.0072$ ) and interaction ( $p = 0.0196$ ) were found for  
356 time on top after exposure (Figure 6A). Post-hoc pairwise permutation tests found a difference  
357 between control and CAS-exposed animals (adjusted  $p = 1.194 \cdot 10^{-5}$ ), an effect that was not  
358 blocked by fluoxetine. Similarly, main effects of treatment ( $p = 0.0002$ ), but not a drug ( $p = 0.2914$ )  
359 nor an interaction effect ( $p = 0.5878$ ) were found for time on bottom (Figure 6B), with a significant

360 increase in CAS-exposed animals (all adjusted  $p < 0.001$ ). No effects were found for erratic  
361 swimming ( $p > 0.7$ ; Figure 6C). Significant treatment ( $p < 0.0001$ ), dose ( $p = 0.025$ ), and  
362 interaction effects ( $p = 0.0014$ ), were found for freezing (Figure 6D), with CAS increasing freezing  
363 at all drug treatments, and the highest fluoxetine dose potentiating this effect. No effects were found  
364 for swimming speed ( $p > 0.24$ ; Figure 6E).

365

### 366 **3.3 Experiment 3**

367 One outlier was removed from the group exposed to water and treated with vehicle. During  
368 CAS exposure, significant effects of treatment ( $p < 0.0001$ ), but not metergoline ( $p = 0.6682$ ) or  
369 interaction ( $p = 0.5162$ ), were found for time on top (Figure 7A); post-hoc comparisons suggested  
370 that CAS decreased time on top (adjusted  $p = 1.977 \cdot 10^{-6}$ ), but metergoline did not block effect.  
371 Significant effects of treatment ( $p = 0.0432$ ), but not metergoline ( $p = 0.9518$ ) nor interaction ( $p =$   
372  $0.4174$ ) were found for time on bottom (Figure 7B), with CAS increasing time on bottom (adjusted  
373  $p = 0.01472$ ) and no effect of metergoline. Significant effects of treatment ( $p = 0.0002$ ), but not  
374 metergoline ( $p = 0.6496$ ) nor interaction ( $p = 0.1814$ ), were found for absolute turn angle (Figure  
375 7C), with CAS increasing absolute turn angle (adjusted  $p = 0.02627$ ) and metergoline having no  
376 effect. Significant effects of treatment ( $p < 0.0001$ ), but not metergoline ( $p = 0.462$ ) nor interaction  
377 ( $p = 0.1922$ ), were found for freezing (Figure 7D); post-hoc tests found significant differences  
378 between CAS-exposed animals and controls treated with vehicle (adjusted  $p = 0.02073$ ), but  
379 metergoline did not block the effects of CAS on freezing. No effects of treatment ( $p =$ , metergoline,  
380 and interaction (all  $p > 0.1$ ) were found for swimming speed (Figure 7E).

381 After CAS exposure, no main nor interaction effects were found for time on top (all  $p >$   
382  $0.13$ ; Figure 8A). A dose ( $p = 0.0156$ ) and an interaction ( $p = 0.047$ ) effects were found for time on  
383 bottom, but a treatment effect was not found ( $p = 0.827$ ); CAS increased time on bottom (adjusted  $p$   
384  $= 0.0292$ ), an effect that was decreased by metergoline (Figure 8B). No main nor interaction effects



385 were found for absolute turn angle ( $p > 0.33$ ; Figure 8C). A main effect of treatment ( $p = 0.0036$ ),  
386 but not drug ( $p = 0.4182$ ), nor interaction ( $p = 0.1508$ ), was found for freezing (Figure 8D); CAS  
387 increased freezing (adjusted  $p = 0.0075$ ), and metergoline partially blocked this effect. No main  
388 effects were found for swimming speed ( $p > 0.27$ ), but an interaction effect was found ( $p = 0.026$ );  
389 however, post-hoc tests failed to find significant differences between groups (Figure 8E).

390

### 391 **3.4. Experiment 4**

392 Three outliers were removed from the group exposed to water and treated with vehicle, two  
393 from the group exposed to water and treated with pCPA, one from the group exposed to CAS and  
394 treated with vehicle, and two from the group exposed to CAS and treated with pCPA. During CAS  
395 exposure, a main effect of treatment ( $p < 0.0001$ ), but no drug ( $p = 0.801$ ) nor interaction effects ( $p$   
396  $= 0.5386$ ) were found for time on top (Figure 9A); CAS decreased time on top on both vehicle- and  
397 pCPA-injected animals (both adjusted  $p > 0.0025$ ). Likewise, a main effect of treatment ( $p = 0.0022$ ),  
398 but no drug ( $p = 0.6496$ ) nor an interaction effects ( $p = 0.1166$ ), were found for time on bottom  
399 (Figure 9B); CAS increased time on bottom on both vehicle- and pCPA-injected animals (both  
400 adjusted  $p = 0.05$ ). A main effect of treatment ( $p = 0.0002$ ), but not an effect of drug ( $p = 0.8162$ )  
401 nor interaction ( $p = 0.5612$ ), was found for absolute turn angle (Figure 9C); CAS increased absolute  
402 turn angle on both vehicle- and pCPA-injected animals (both adjusted  $p < 0.002$ ). A main effect of  
403 treatment ( $p < 0.0001$ ), but not an effect of drug ( $p = 0.6822$ ) nor interaction ( $p = 0.4156$ ), was  
404 found for freezing (Figure 9D); CAS increased freezing on both vehicle- and pCPA-injected  
405 animals (both adjusted  $p < 0.018$ ). No main or interaction effects were found for speed (all  $p > 0.11$ ;  
406 Figure 9E).

407 After CAS exposure, no main (all  $p > 0.08$ ) or interaction ( $p = 0.1472$ ) effects were found  
408 for time on top (Figure 10A). A main effect of drug ( $p < 0.0001$ ), but not of treatment ( $p = 0.4514$ ),  
409 nor an interaction effect ( $p = 0.5436$ ), was found for time on bottom (Figure 10B); pCPA decreased

410 time on bottom at both controls and CAS-exposed animals (all adjusted  $p < 0.001$ ). No main (all  $p$   
411  $> 0.3$ ) nor interaction ( $p = 0.6958$ ) effects were found for absolute turn angle (Figure 10C). A main  
412 effect of treatment ( $p = 0.0108$ ), but not drug ( $p = 0.1892$ ) nor interaction ( $p = 0.2576$ ), was found  
413 for freezing (Figure 10D); CAS increased freezing (adjusted  $p = 0.02278$ ), an effect that was  
414 blocked by pCPA (adjusted  $p = 0.01453$ ). No main (all  $p > 0.19$ ) nor interaction ( $p = 0.0954$ ) effects  
415 were found for swimming speed (Figure 10E).

416

### 417 **3.5. Experiment 5**

418 No outliers were removed. After CAS exposure, zMAO activity was reduced in the brain ( $Z$   
419  $= 3.205$ ,  $p = 0.00135$ ; Figure 11).

420

## 421 **4. Discussion**

422 The present work attempted to clarify the role of phasic and tonic serotonin in the alarm  
423 reaction of zebrafish during (fear-like behavior) and after (recovery) exposure. We found that  
424 clonazepam decreased fear-like behavior, as well as post-exposure behavior, suggesting a good  
425 predictive validity of the assay. Moreover, acute fluoxetine decreased fear-like behavior at the  
426 highest dose, but increased freezing post-exposure. Metergoline had no effect on fear-like behavior,  
427 but blocked the effects of conspecific alarm substance (CAS) on post-exposure behavior; similar  
428 effects were observed with pCPA. Finally, CAS was shown to decrease the activity of monoamine  
429 oxidase in the zebrafish brain after exposure.

430

### 431 **4.1. Behavior during and after CAS exposure**

432 In zebrafish, reported behavioral effects of CAS vary widely as a function of timing,  
433 extraction method, and whether animals are tested alone or in groups (Maximino *et al.* 2019). When

434 animals are exposed and/or tested alone, as in the present experiments, bottom-dwelling, freezing,  
435 and erratic swimming consistently increases *during* exposure (Eachus *et al.* 2017; Nathan *et al.*  
436 2015; Ogawa *et al.* 2014; Maximino *et al.* 2014), but effects *after* exposure are less clear (Quadros  
437 *et al.* 2016; Schirmer *et al.* 2013; Nathan *et al.* 2015; Egan *et al.* 2009). In the present experiments,  
438 CAS consistently increased bottom-dwelling and erratic swimming during exposure, while after  
439 exposure bottom-dwelling and freezing were increased. The first effects were blocked by treatment  
440 with the panicolytic drug clonazepam, which nonetheless had a very mild effect on post-exposure  
441 behavior. Thus, two components can be elicited by CAS: the first, dominated by erratic swimming,  
442 occurs when the substance is present, and the second, dominated by freezing, occurs when the  
443 concentrations of CAS decrease.

444 Observing the context in which defensive behavior, and not only the response topography, is  
445 important to understand the function of a specific response. The context in which CAS elicits alarm  
446 reactions is akin to a circa-strike defensive situation (Maximino *et al.* 2019), therefore producing  
447 freezing and escape reactions that are fear-like; when CAS signals decrease, however (i.e., *after*  
448 *exposure* in the present experiments), the context is akin to a post-encounter defensive situation,  
449 eliciting avoidance and freezing behavior (see Perusini and Fanselow 2015 for a discussion on  
450 predatory imminence, defensive reactions, and fear vs. anxiety). In these contexts, increases in  
451 freezing, for example, can be interpreted as representing two different functions: to escape detection  
452 by predators in the first case, and to allow careful vigilance, in the second case.

453 The differences in behavior during and after exposure are reminiscent of the different types  
454 of freezing elicited during and after electrical stimulation of the periaqueductal gray in rodents  
455 (Brandão *et al.* 2008). The increased erratic swimming observed during CAS exposure suggest  
456 escape and/or avoidance attempts, while the increased freezing observed after CAS exposure  
457 suggest a role in risk assessment. The effects of clonazepam also suggest different neurobehavioral  
458 states: this drug usually decreases panic attacks, but has small effects on generalized anxiety in

459 human clinical settings (Caldirola *et al.* 2016; Cloos 2005). These results imply good predictive  
460 validity, suggesting that behavior during and after CAS can be used to study fear- vs. anxiety-like  
461 effects.

462

## 463 **4.2. Role of phasic serotonin on CAS effects**

464 Fluoxetine, at the highest dose, blocked the alarm reaction (fear-like behavior during CAS  
465 exposure) and post-exposure behavior in zebrafish. The results from the lower dose (2.5 mg/kg) are  
466 harder to interpret, as they could represent not (partial) blocking, but an additive effect, at least on  
467 bottom-dwelling. These results are similar to what was previously observed in the light/dark test, in  
468 which fluoxetine (2.5 mg/kg) blocked post-exposure effects on scototaxis, freezing, and erratic  
469 swimming (Maximino *et al.* 2014). While the role of phasic increases in serotonergic signaling on  
470 acute fear-like responses in zebrafish has not been previously investigated, a higher dose (10  
471 mg/kg) blocked the alarm reaction (i.e., during exposure) in the piauçu *Leporinus macrocephalus*  
472 (Barbosa *et al.* 2012). This phasic role of serotonin is likely highly conserved, as serotonin has been  
473 shown to decrease responses to aversive odors in *Caenorhabditis elegans* (Li *et al.* 2012; Harris *et*  
474 *al.* 2009). The effects of fluoxetine strongly suggest that serotonin phasically inhibits fear-like  
475 behavior in zebrafish, acting as a switch towards risk assessment.

476 Phasic serotonin has been proposed to modulate fear-like behavior in mammals before  
477 (Zangrossi Jr *et al.* 2001; Paul *et al.* 2014; Guimarães *et al.* 2010); the Deakin/Graeff theory  
478 suggests a “panic inhibition system” (Paul *et al.* 2014; Silva *et al.* 2019) that inhibits behavioral  
479 and sympathoexcitatory responses to these stimuli, and is mediated by serotonergic signaling. The  
480 theory proposes a “dual role” for serotonin, increasing anxiety-like responses and inhibiting fear-  
481 like responses. A similar mechanism has been proposed for zebrafish based on data regarding  
482 serotonergic drugs in anxiety-like behavior (Herculano and Maximino 2014). We propose that, at  
483 least in zebrafish, phasic serotonin does not physiologically inhibit fear responses; instead, the

484 inhibitory role of serotonin in fear functions as a “neurobehavioral switch”: as the threatening  
485 stimulus ceases, serotonin is released, inhibiting the fear reactions that are now non-adaptive, and  
486 initiating careful exploration and risk assessment responses to ensure that the threat is actually over  
487 (Figure 11). This is consistent with expectancy value theories, in which serotonin signals represent  
488 the expectation of risk/threatening outcomes (aversive expectation values), from which appropriate  
489 behavioral strategies can be selected (Amo *et al.* 2014; Cools *et al.* 2011).

490 A role for the serotonin transporter has also been proposed for the selection of behavior at  
491 different levels of a threat imminence continuum: animals with lower expression of the transporter  
492 are more cautious and readily show defensive responses under distal threat, while animals with high  
493 expression show more defensive responses under proximal threat (Kroes *et al.* 2019). While the  
494 expression levels of the serotonin transporter are more associated with controlling serotonergic  
495 tone, this protein has been also shown to mediate the increases in serotonin levels after CAS  
496 exposure in zebrafish (Maximino *et al.* 2014), suggesting a participation also in phasic signals.  
497 Whether serotonin transporter expression levels are associated with the alarm reaction and/or post-  
498 exposure behavior in zebrafish is still unknown.

499

### 500 **4.3. Is there a tonic inhibition of fear-like responses in zebrafish?**

501 The hypothesis that serotonin functions as a “neurobehavioral switch” signal in zebrafish  
502 aversive behavior would be strengthened if decreasing the effects of serotonin on its receptors  
503 inhibited post-exposure behavior. Indeed, metergoline, which non-specifically blocks 5-HT<sub>1</sub>, 5-HT<sub>2</sub>,  
504 and 5-HT<sub>7</sub> receptors, had no effect on the alarm reaction, but blocked the post-exposure effects of  
505 CAS on bottom-dwelling and homebase use; no effect was observed during exposure, suggesting  
506 that fear-like responses are not under tonic inhibition. pCPA had similar effects. Nathan *et al.* (2015)  
507 observed that blocking 5-HT<sub>1A</sub> and 5-HT<sub>2</sub> receptors potentiates freezing and bottom-dwelling both  
508 in the initial moments of exposure and in a “recovery period”; however, during the recovery period

509 animals were still exposed to CAS, the doses which produced effect in Nathan et al. (2015) were  
510 higher than reported in other experiments with zebrafish (Maximino *et al.* 2013), and important  
511 controls were lacking, making comparison of results difficult.

512 Further support for this hypothesis is lent by sophisticated experiments made by Amo et al.  
513 (2014) using the serotonergic neurotoxin 5,7-DHT. Injection of this neurotoxin in the telencephalon  
514 destroyed most serotonergic fibers projecting to it, and led to an inability to learn an active  
515 avoidance contingency (Amo *et al.* 2014), suggesting that serotonergic signaling in the  
516 telencephalon represents an aversive expectation value. Amo et al. (2014) demonstrated that this  
517 circuitry is under the control of projections from the ventral habenula which are not necessary for  
518 classical fear conditioning. This suggests that this habenula-raphé-telencephalon pathways do not  
519 simply process a fear response, but instead represents expectations values that can be used to inhibit  
520 fear when threat is no longer present.

521 A caveat of the results from metergoline and pCPA experiments is that these drugs also  
522 affect other neurotransmitter systems. Metergoline also acts as a non-selective dopamine receptor  
523 antagonist (<https://www.ebi.ac.uk/chebi/searchId.do?chebiId=CHEBI:64216>); although its affinity  
524 for 5-HT<sub>2</sub> receptors is ~25 times higher than for the dopamine D<sub>2</sub> receptor, the affinity for 5-HT<sub>1</sub>  
525 receptors is comparable to D<sub>2</sub> receptors (Dukhovich *et al.* 2004). While pCPA has been reported to  
526 produce a selective effect on serotonin levels in zebrafish (Sallinen *et al.* 2009), not altering levels  
527 of catecholamines, at the moment we cannot discard the possibility that the treatment used in the  
528 present article were not due to changes in these systems. We cannot discard, then, the participation  
529 of catecholamines along with serotonin in the effects of these drugs on post-exposure behavior.

530 In addition to the effects of the manipulations of the serotonergic system on post-exposure  
531 behavior, zMAO activity has been shown to be decreased after CAS exposure, which would  
532 increase serotonin levels at this moment. Previously, CAS has been shown to increase 5-HT levels  
533 in the extracellular fluid of the zebrafish brain 20 min after CAS stress (Maximino *et al.* 2014), and

534 repeated (7 day) exposure to CAS decreases the mRNA levels of the serotonergic genes *pet1* and  
535 *slc6a4a* (serotonin transporter)(Ogawa *et al.* 2014). These results suggest that CAS increases  
536 serotonergic activity after the stimulus is no longer present, but it is not known whether CAS does  
537 so during exposure.

538

#### 539 **4.4. Which receptors are involved?**

540 The present work did not investigate specific receptors which are involved in the alarm  
541 reaction in zebrafish. However, a role for 5-HT<sub>1</sub>-, 5-HT<sub>2</sub>-, and 5-HT<sub>7</sub>-like receptors is suggested by  
542 the effects of metergoline. The 5-HT<sub>1A</sub> receptor antagonist WAY 100,635 has been previously  
543 shown to block fear-induced analgesia elicited by CAS, but not the increase in anxiety-like behavior  
544 in the light/dark test (Maximino *et al.* 2014). At higher doses, however, WAY 100,635 potentiated  
545 the effects of CAS both in the early responses (0-8 min) and in the late phase (8-13 min) (Nathan *et*  
546 *al.* 2015). These contradictory results can be explained by differences in exposure methods, as well  
547 as differences in behavioral scoring techniques. Methysergide, which non-selectively blocks 5-  
548 HT<sub>2A</sub>, 5-HT<sub>2B</sub>, and 5-HT<sub>2C</sub> receptors, also potentiates the effects of CAS at both times (Nathan *et al.*  
549 2015). However, 5-HT<sub>1A</sub>, 5-HT<sub>2A</sub>, and 5-HT<sub>2C</sub> receptors in the dorsolateral periaqueductal gray  
550 area have been shown to phasically inhibit escape/fear responses in rats (Soares and Zangrossi Jr  
551 2004). While currently it is unknown whether the griseum centrale, the teleostean homolog of the  
552 periaqueductal gray area, is involved in fear responses in zebrafish or not, its anatomical position  
553 and homology suggests so (Maximino *et al.* 2019; do Carmo Silva *et al.* 2018a). Thus, 5-HT<sub>1A</sub> and  
554 5-HT<sub>2</sub>-like receptors appear to be involved in phasic inhibition of fear-like behavior, but so far  
555 evidence for a tonic inhibition is lacking.

556

## 557 **5. Conclusion**

558           The present experiments evidenced two qualitatively different stages of the alarm reaction in  
559 zebrafish, one in the presence of the alarm substance, and another when it is no longer present, both  
560 sensitive to clonazepam. Results from biochemistry and pharmacological manipulations suggest  
561 that phasic and tonic serotonin acts as a neurobehavioral switch towards cautious exploration/risk  
562 assessment/anxiety when the aversive stimulus is no longer present. These results refine previous  
563 theories on the role of serotonin in anxiety and fear, suggesting new avenues of research.

564

## 565 **Acknowledgments**

566           This work was supported by a Conselho Nacional de Desenvolvimento Científico e  
567 Tecnológico/CNPq grant to C.M. (#400726/2016-5). M.P.P. was the recipient of a CNPq  
568 undergraduate scholarship. D.B.R. is the recipient of a CNPq research productivity grant  
569 (#307595/2015-3). The funders had no influence on the study design, the collection, analysis and  
570 interpretation of data, as well as writing and submission of this manuscript.

571

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721

## 722 **Figure captions**

723 **Figure 1** – Experimental design and sample sizes for each experiment. In the right-most boxes,  
724 “donors” refer to animals which were sacrificed and used to produce alarm substance, and therefore  
725 not used as subjects.

726

727 **Figure 2** – (A) Time-course of the experiments, with behavioral observations during two 6-min  
728 blocks, “CAS exposure” and “Post exposure”, separated with a 1-min washout period. The boxes in  
729 red indicate the moment that the observation is made in each block. (B-D) Representation of the  
730 synaptic effects of the pharmacological manipulations: acute fluoxetine (B) is expected to increase  
731 synaptic and extra-synaptic serotonin levels, while metergoline (C) is expected to block receptors  
732 from the 5-HT<sub>1</sub>, 5-HT<sub>2</sub>, and 5-HT<sub>7</sub> family; pCPA (D) is expected to decrease synaptic and extra-  
733 synaptic serotonin levels.

734

735 **Figure 3 – Clonazepam (0.05 mg/kg) blocks all CAS-elicited increases in defensive behavior**  
736 **during exposure.** (A) Time spent on the top third of the tank; (B) Time spent on the bottom third of  
737 the tank; (C) Absolute turn angle; (D) Freezing; (E) Swimming speed. Different letters represent  
738 statistical differences at the  $p < 0.05$  level; similar letters indicate lack of statistically significant  
739 differences. Data are presented as individual data points (dots) superimposed over the median  $\pm$   
740 interquartile ranges. CTRL = controls (water-exposed animals); CAS = conspecific alarm substance.  
741 Final sample sizes: CTRL + VEH:  $n = 14$  animals; CTRL + CLZ:  $n = 15$  animals; CAS + VEH:  $n =$   
742  $14$  animals; CAS + CLZ:  $n = 15$  animals.

743

744 **Figure 4 – Clonazepam (0.05 mg/kg) blocks only the CAS-elicited increases in bottom-**  
745 **dwelling after exposure.** (A) Time spent on the top third of the tank; (B) Time spent on the bottom  
746 third of the tank; (C) Absolute turn angle; (D) Freezing; (E) Swimming speed. Different letters  
747 represent statistical differences at the  $p < 0.05$  level; similar letters indicate lack of statistically

748 significant differences. Data are presented as individual data points (dots) superimposed over the  
749 median  $\pm$  interquartile ranges. CTRL = controls (water-exposed animals); CAS = conspecific alarm  
750 substance. Final sample sizes: CTRL + VEH: n = 14 animals; CTRL + CLZ: n = 15 animals; CAS +  
751 VEH: n = 14 animals; CAS + CLZ: n = 15 animals.

752

753 **Figure 5 – Acute fluoxetine dose-dependently blocks all the CAS-elicited increases in defensive**  
754 **behavior during exposure.** (A) Time spent on the top third of the tank; (B) Time spent on the  
755 bottom third of the tank; (C) Absolute turn angle; (D) Freezing; (E) Swimming speed. Different  
756 letters represent statistical differences at the  $p < 0.05$  level; similar letters indicate lack of  
757 statistically significant differences. Data are presented as individual data points (dots) superimposed  
758 over the median  $\pm$  interquartile ranges. CTRL = controls (water-exposed animals); CAS =  
759 conspecific alarm substance. Final sample sizes: CTRL + 0 mg/kg: n = 15 animals; CTRL + 2.5  
760 mg/kg: n = 15 animals; CTRL + 25 mg/kg: n = 15 animals; CAS + 0 mg/kg: n = 15 animals; CAS +  
761 2.5 mg/kg: n = 14 animals; CAS + 25 mg/kg: n = 15 animals.

762

763 **Figure 6 – Acute fluoxetine dose-dependently blocks only the CAS-elicited increases in**  
764 **bottom-dwelling after exposure.** (A) Time spent on the top third of the tank; (B) Time spent on the  
765 bottom third of the tank; (C) Absolute turn angle; (D) Freezing; (E) Swimming speed. Different  
766 letters represent statistical differences at the  $p < 0.05$  level; similar letters indicate lack of  
767 statistically significant differences. Data are presented as individual data points (dots) superimposed  
768 over the median  $\pm$  interquartile ranges. CTRL = controls (water-exposed animals); CAS =  
769 conspecific alarm substance. CTRL = controls (water-exposed animals); CAS = conspecific alarm  
770 substance. Final sample sizes: CTRL + 0 mg/kg: n = 15 animals; CTRL + 2.5 mg/kg: n = 15  
771 animals; CTRL + 25 mg/kg: n = 15 animals; CAS + 0 mg/kg: n = 15 animals; CAS + 2.5 mg/kg: n  
772 = 14 animals; CAS + 25 mg/kg: n = 15 animals.

773

774 **Figure 7 – Metergoline does not block the CAS-elicited increases in defensive behavior after**  
775 **exposure.** (A) Time spent on the top third of the tank; (B) Time spent on the bottom third of the  
776 tank; (C) Absolute turn angle; (D) Freezing; (E) Swimming speed. Different letters represent  
777 statistical differences at the  $p < 0.05$  level; similar letters indicate lack of statistically significant  
778 differences. Data are presented as individual data points (dots) superimposed over the median  $\pm$   
779 interquartile ranges. CTRL = controls (water-exposed animals); CAS = conspecific alarm substance.  
780 CTRL = controls (water-exposed animals); CAS = conspecific alarm substance. Final sample sizes:  
781 CTRL + VEH:  $n = 14$  animals; CTRL + MET:  $n = 15$  animals; CAS + VEH:  $n = 15$  animals; CAS +  
782 MET:  $n = 15$  animals.

783

784 **Figure 8 – Metergoline blocks the CAS-elicited increases in bottom-dwelling and freezing after**  
785 **exposure.** (A) Time spent on the top third of the tank; (B) Time spent on the bottom third of the  
786 tank; (C) Absolute turn angle; (D) Freezing; (E) Swimming speed. Different letters represent  
787 statistical differences at the  $p < 0.05$  level; similar letters indicate lack of statistically significant  
788 differences. Data are presented as individual data points (dots) superimposed over the median  $\pm$   
789 interquartile ranges. CTRL = controls (water-exposed animals); CAS = conspecific alarm substance.  
790 Final sample sizes: CTRL + VEH:  $n = 14$  animals; CTRL + MET:  $n = 15$  animals; CAS + VEH:  $n =$   
791  $15$  animals; CAS + MET:  $n = 15$  animals.

792

793 **Figure 9 – pCPA does not block the CAS-elicited increases in defensive behavior during**  
794 **exposure.** (A) Time spent on the top third of the tank; (B) Time spent on the bottom third of the  
795 tank; (C) Absolute turn angle; (D) Freezing; (E) Swimming speed. Different letters represent  
796 statistical differences at the  $p < 0.05$  level; similar letters indicate lack of statistically significant  
797 differences. Data are presented as individual data points (dots) superimposed over the median  $\pm$

798 interquartile ranges. CTRL = controls (water-exposed animals); CAS = conspecific alarm substance.  
799 Final sample sizes: CTRL + VEH: n = 12 animals; CTRL + pCPA: n = 13 animals; CAS + VEH: n  
800 = 14 animals; CAS + pCPA: n = 13 animals.

801

802 **Figure 10 – pCPA blocks the CAS-elicited increases in bottom-dwelling and freezing after**  
803 **exposure.** (A) Time spent on the top third of the tank; (B) Time spent on the bottom third of the  
804 tank; (C) Absolute turn angle; (D) Freezing; (E) Swimming speed. Different letters represent  
805 statistical differences at the  $p < 0.05$  level; similar letters indicate lack of statistically significant  
806 differences. Data are presented as individual data points (dots) superimposed over the median  $\pm$   
807 interquartile ranges. VEH = Vehicle (10% DMSO); pCPA = *para*-chlorophenylalanine; CTRL =  
808 controls (water-exposed animals); CAS = conspecific alarm substance. Final sample sizes: CTRL +  
809 VEH: n = 12 animals; CTRL + pCPA: n = 13 animals; CAS + VEH: n = 14 animals; CAS + pCPA:  
810 n = 13 animals.

811

812 **Figure 11 – Conspecific alarm substance (CAS) reduces the activity of monoamine oxidase in**  
813 **the brain after exposure.** Different letters represent statistical differences at the  $p < 0.05$  level.  
814 Data are presented as individual data points (dots) superimposed over the median  $\pm$  interquartile  
815 ranges. CTRL = controls (water-exposed animals); CAS = conspecific alarm substance. Final  
816 sample sizes: CTRL: n = 10 animals; CAS: n = 10 animals.

817

818 **Figure 12 – Hypothetical mechanism of the serotonergic signaling in zebrafish defensive**  
819 **behavior during and after exposure to conspecific alarm substance.** CAS elicits responses  
820 dominated by erratic swimming, which decreases as the substance's concentrations decline. After  
821 CAS exposure, the behavioral response is dominated by freezing. Serotonin shifts responding from  
822 the first to the second (represented by the purple arrow, as well as by the arrows connecting the



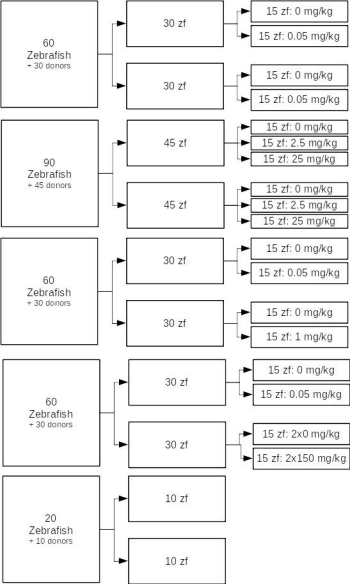
823 raphe to the “switch” green boxes), putatively by switching control from the mesencephalic  
824 aversive circuit (“switch OFF”) to the prosencephalic aversive circuit (“switch ON”).

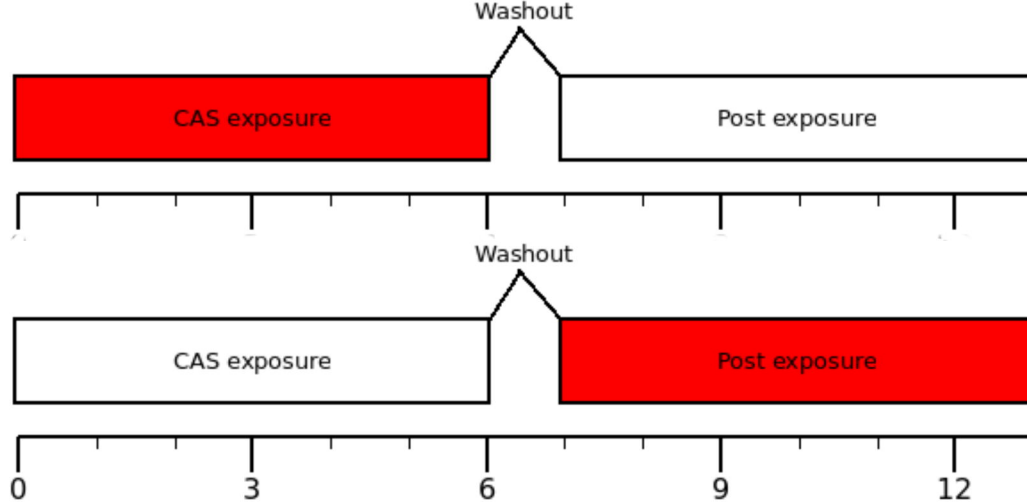
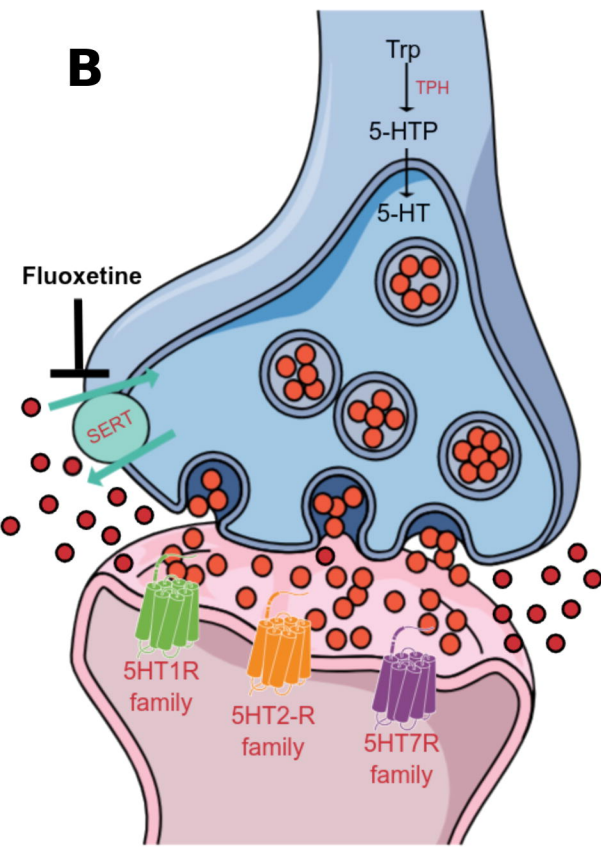
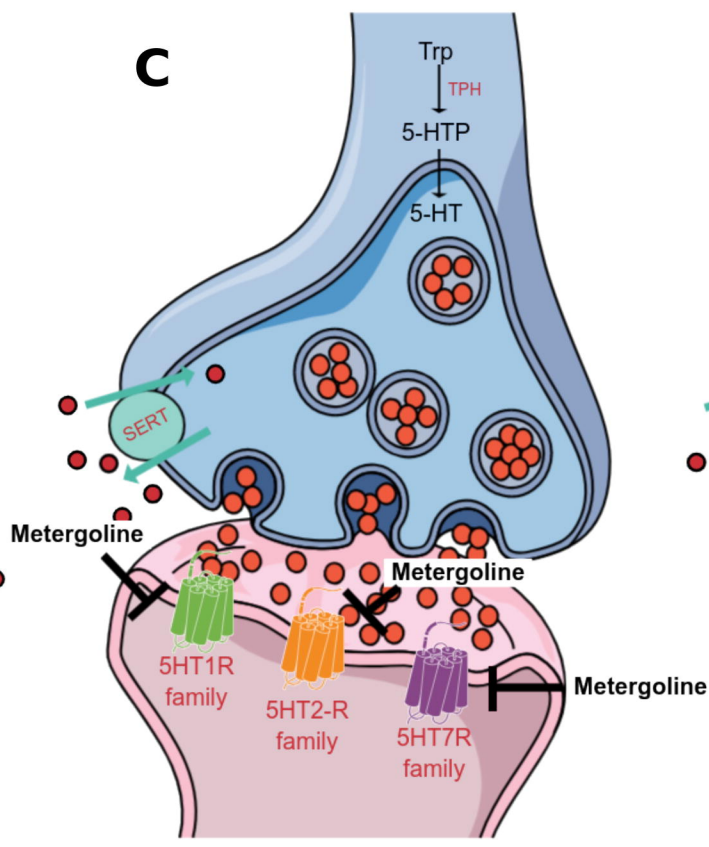
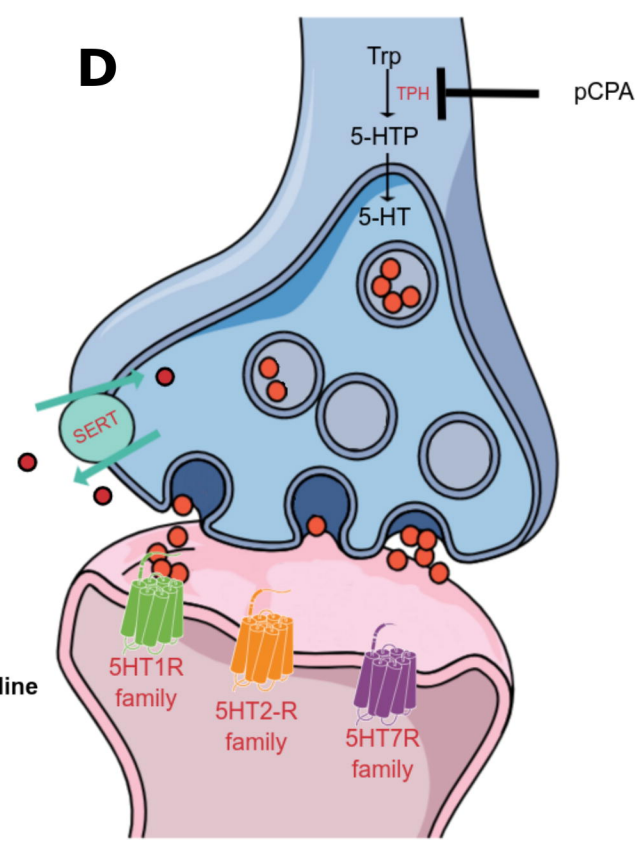
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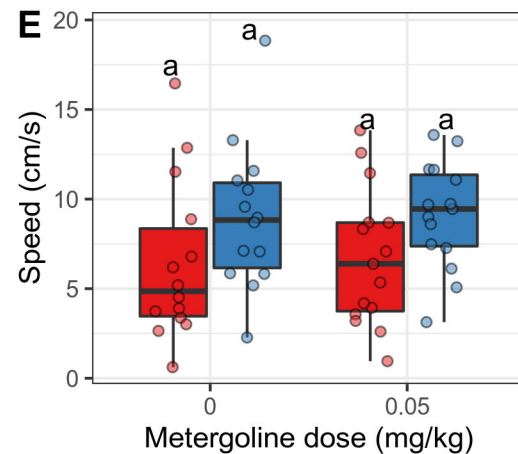
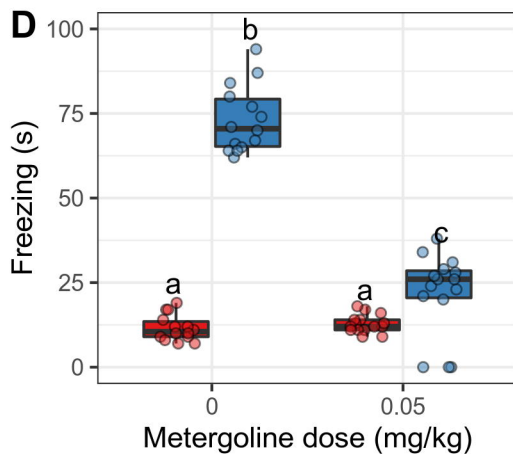
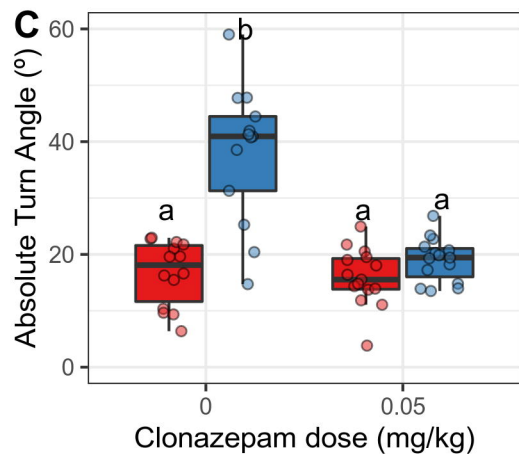
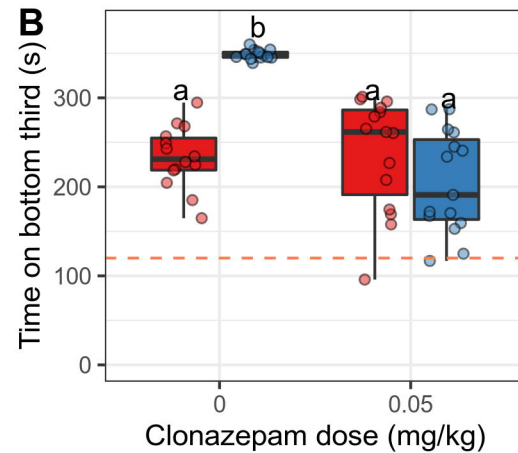
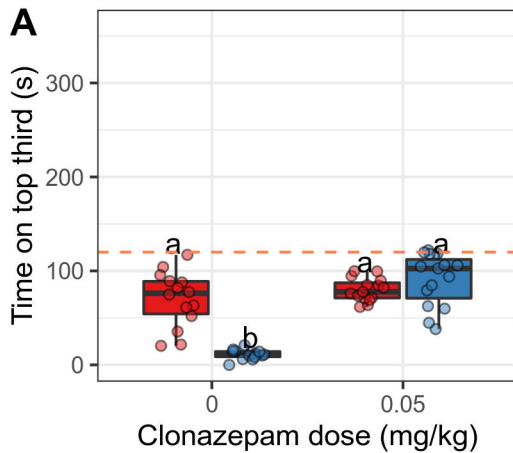
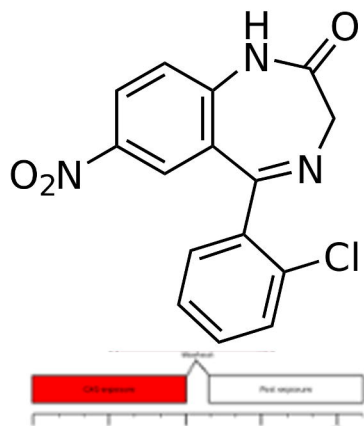
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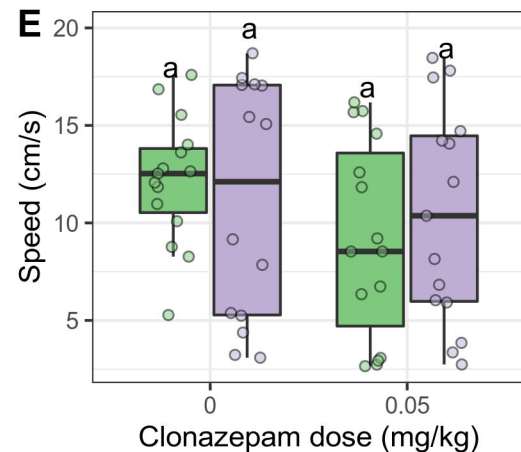
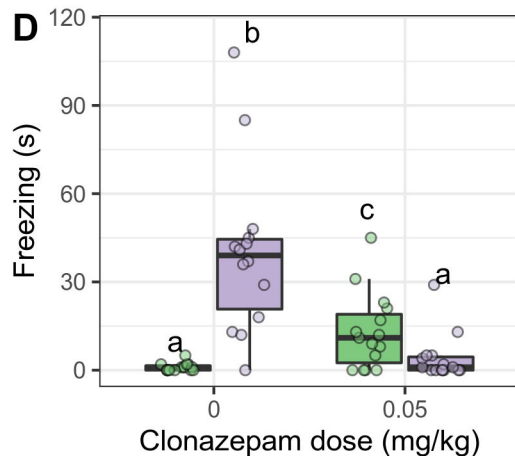
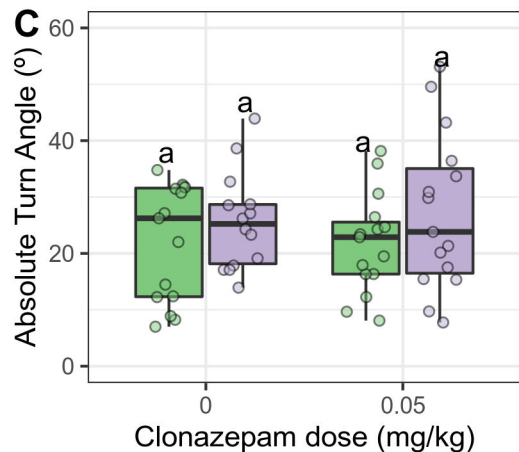
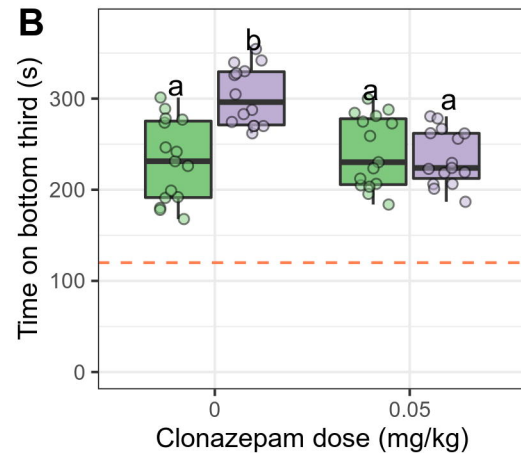
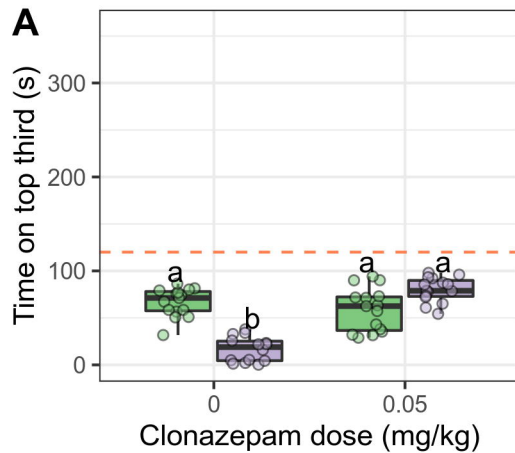
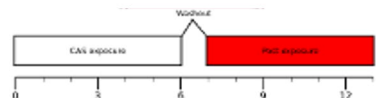
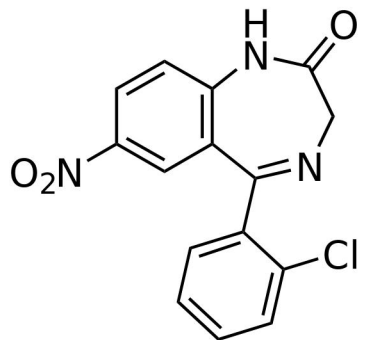
**1****A****B****C****D**

# Clonazepam treatment - During exposure



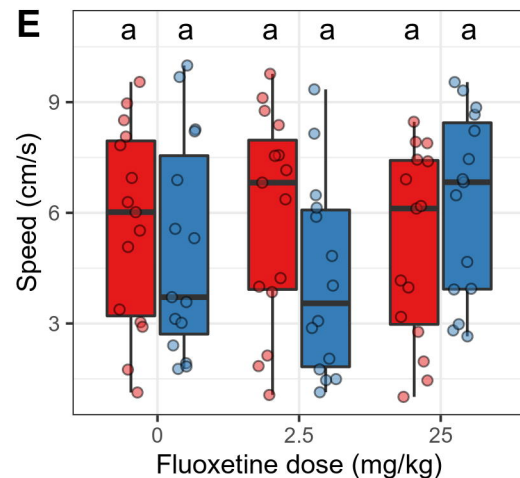
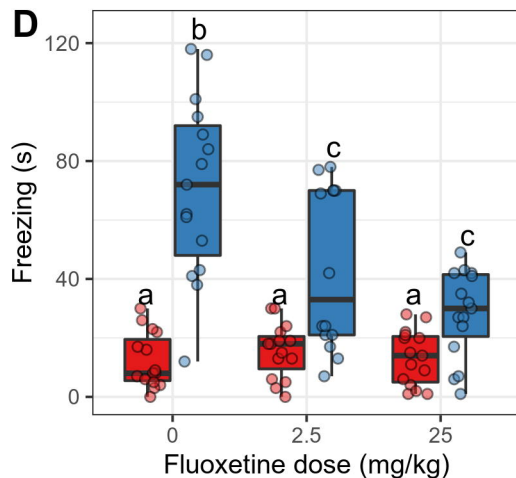
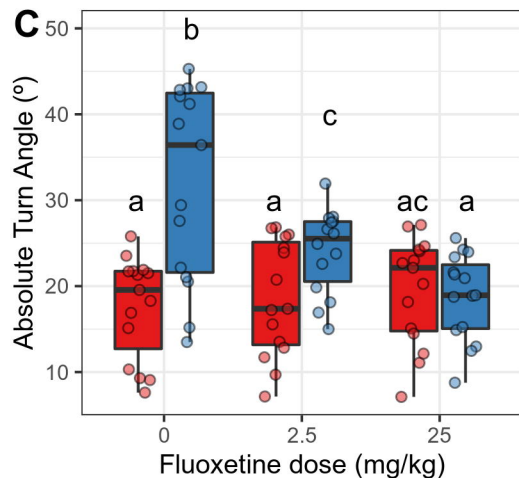
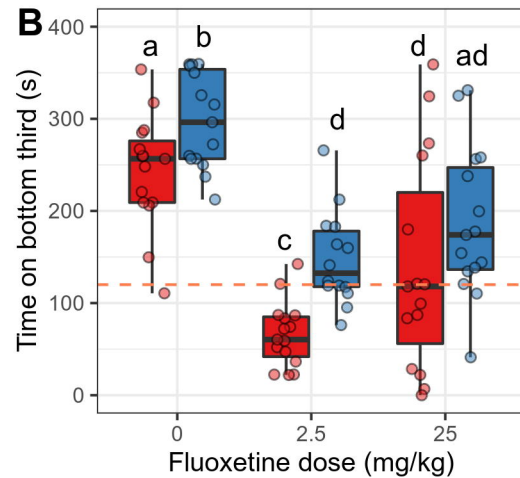
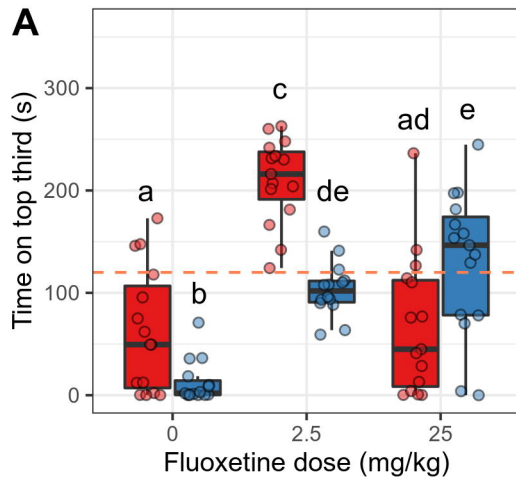
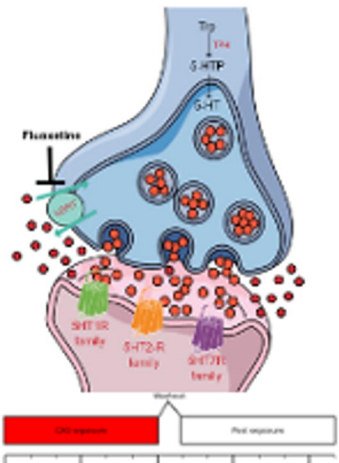
Treatment  CTRL  CAS

# Clonazepam treatment - After exposure

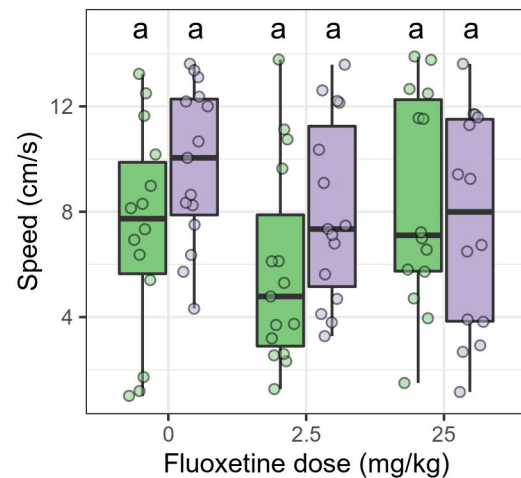
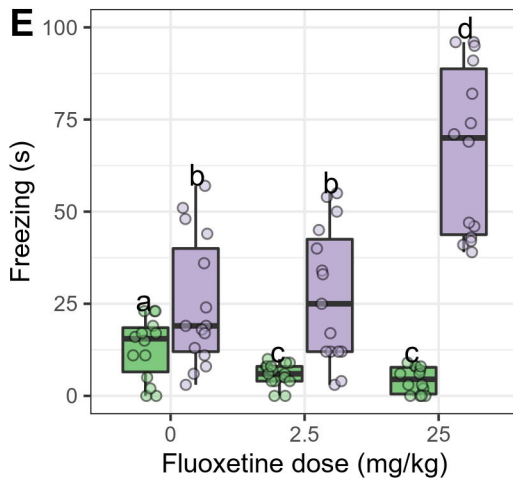
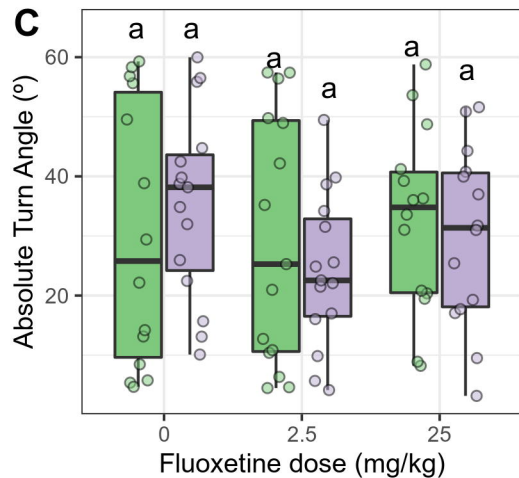
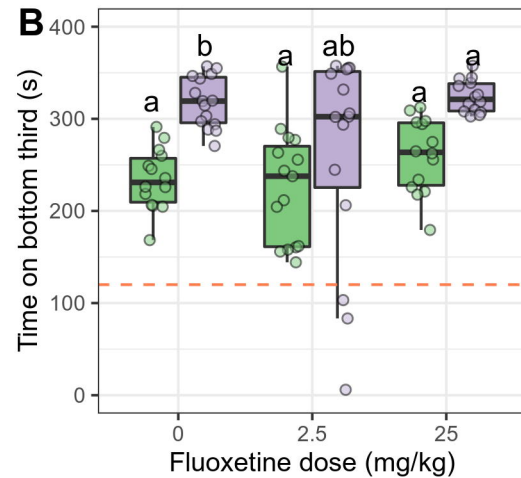
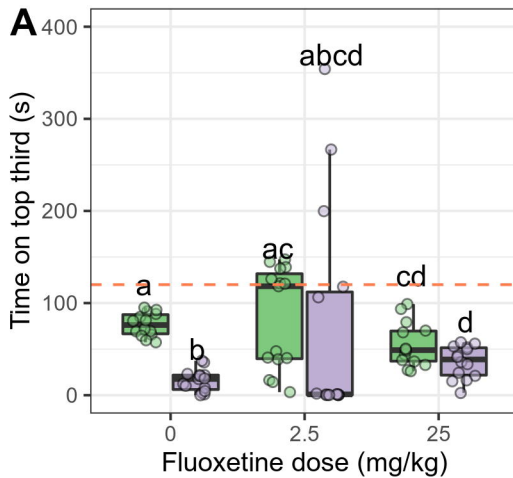
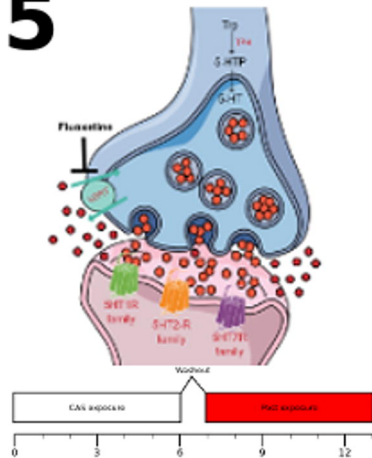


Treatment  CTRL  CAS

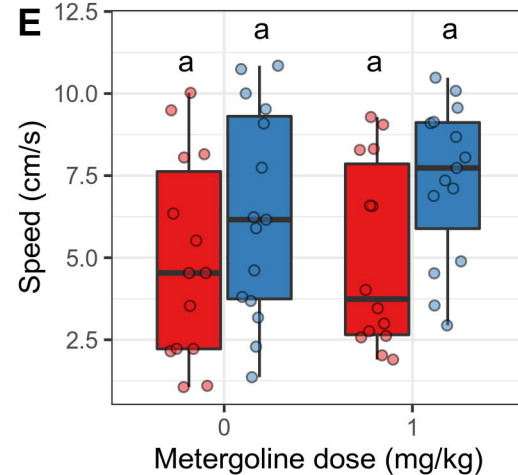
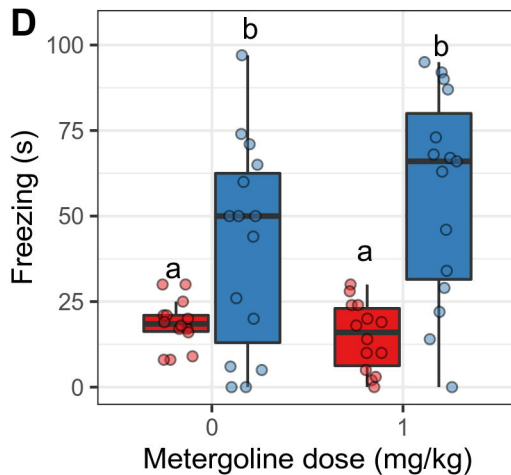
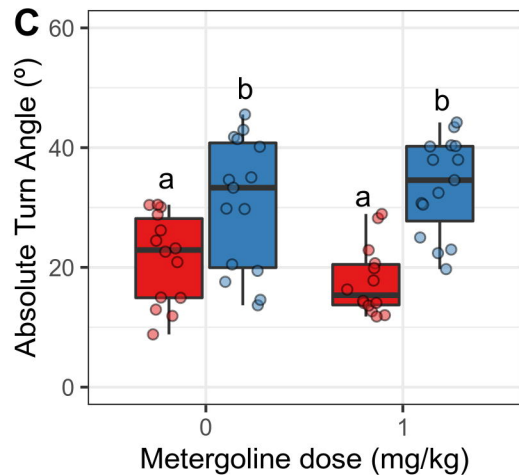
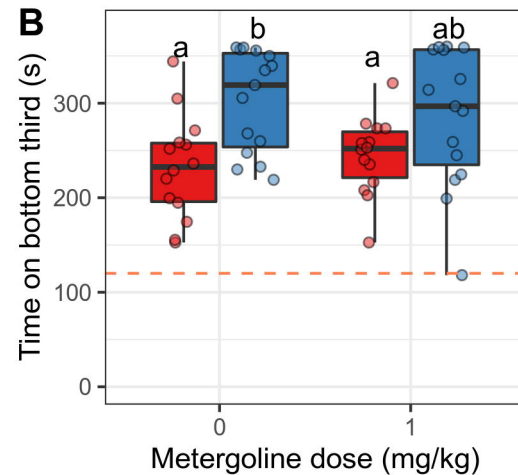
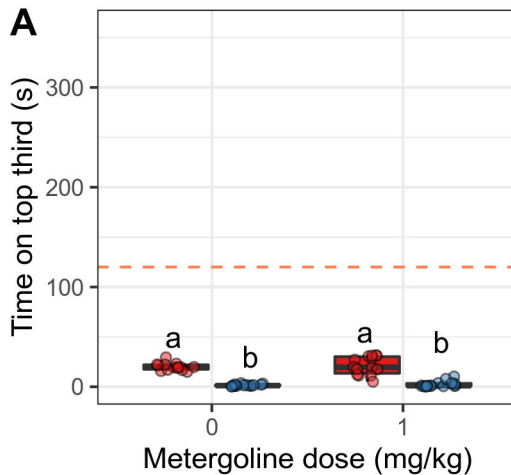
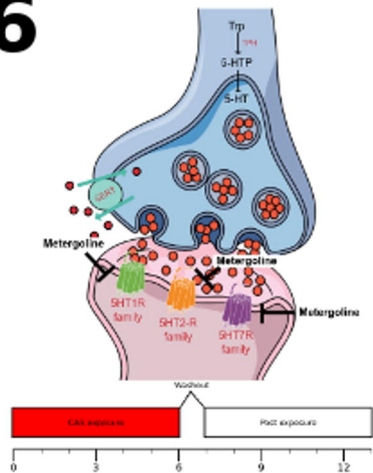
# 4



Treatment  CTRL  CAS

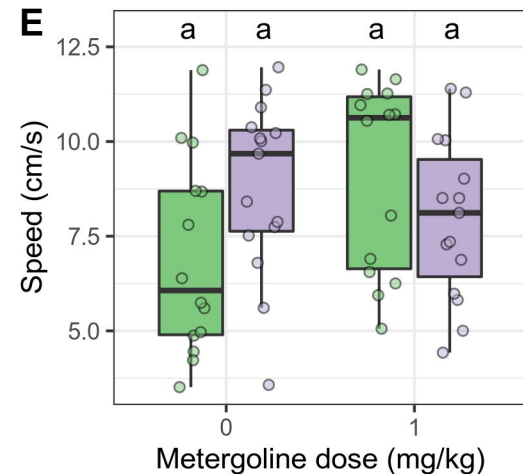
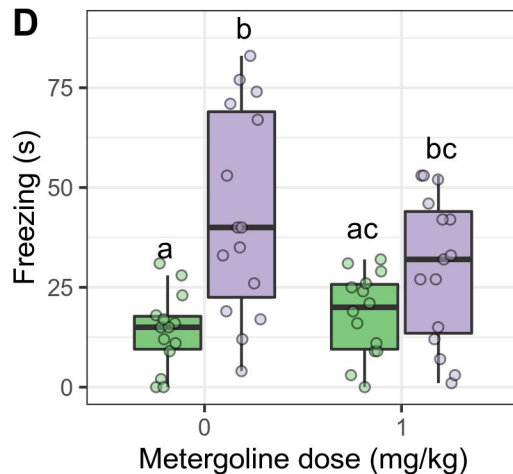
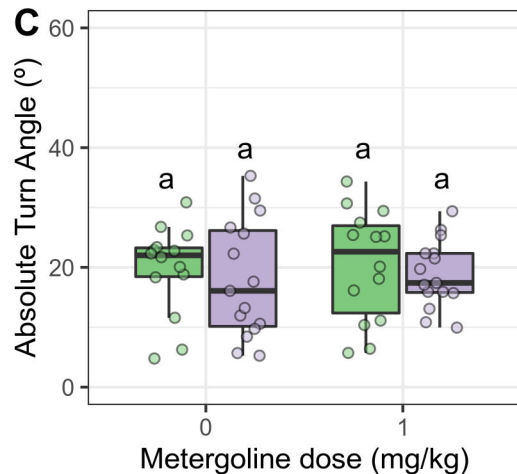
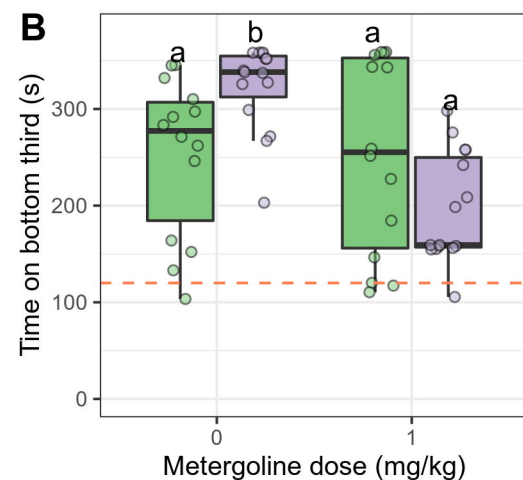
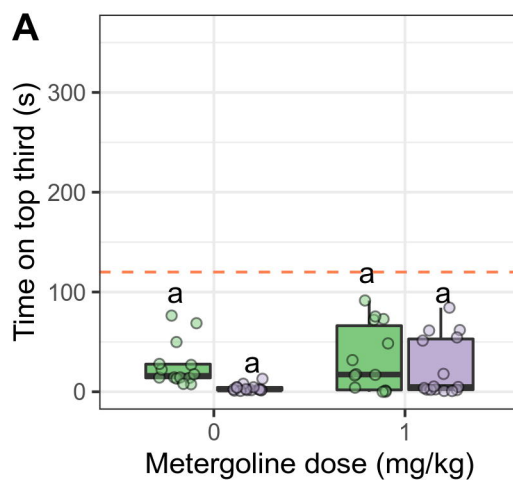
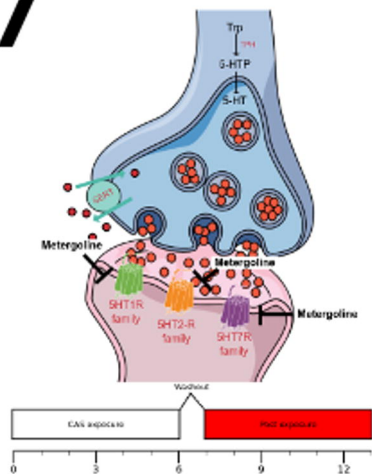
**5**

Treatment ■ CTRL ■ CAS

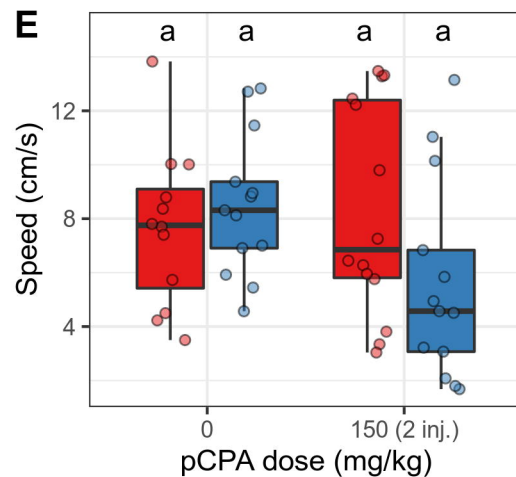
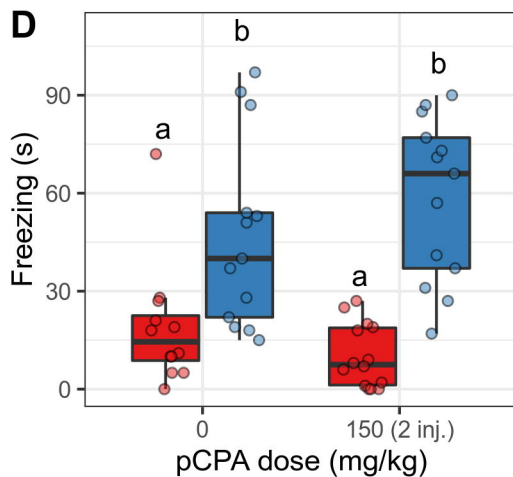
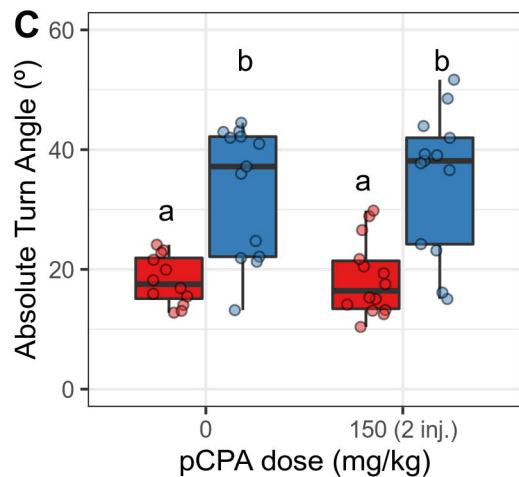
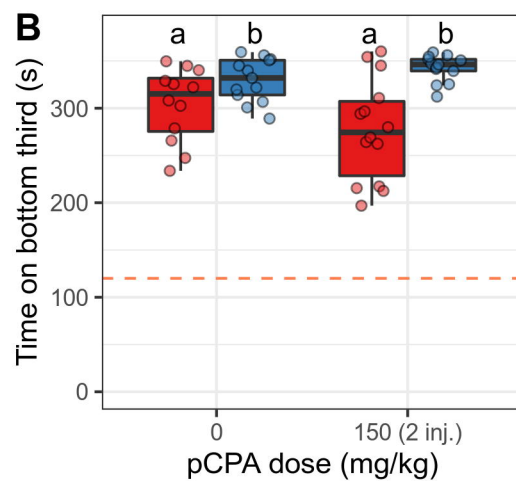
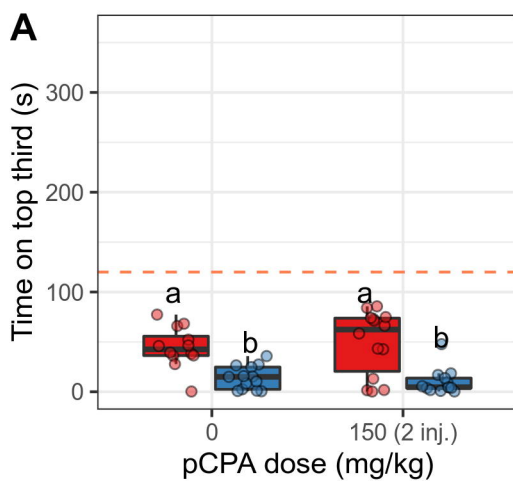
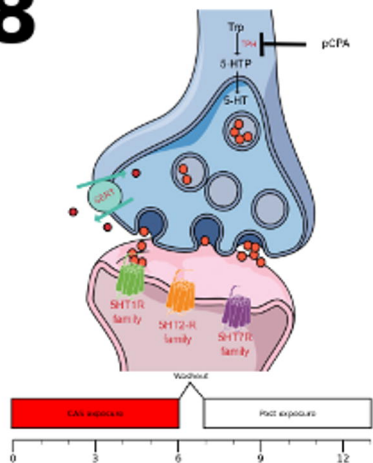
**6**

Treatment: CTRL (red), CAS (blue)

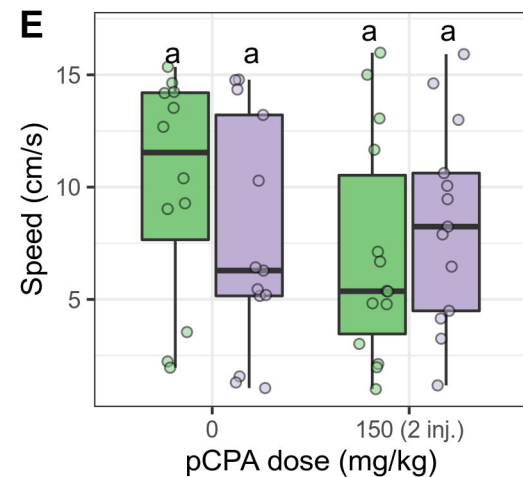
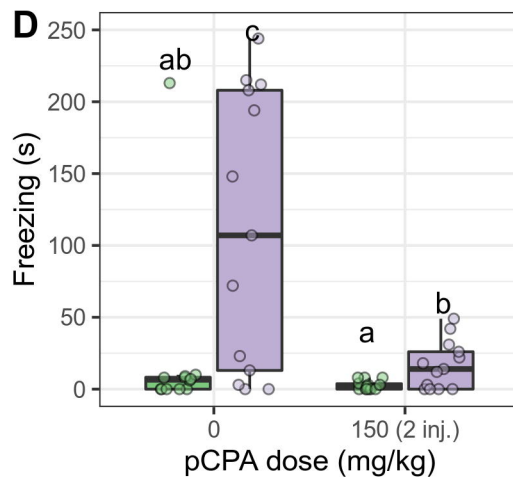
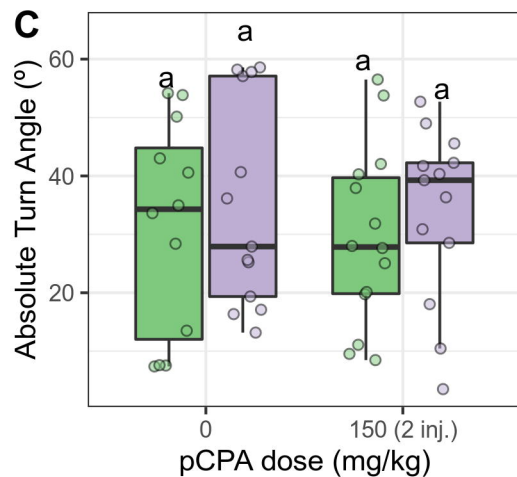
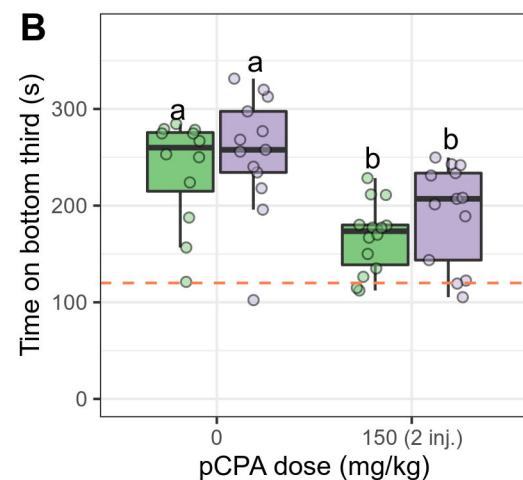
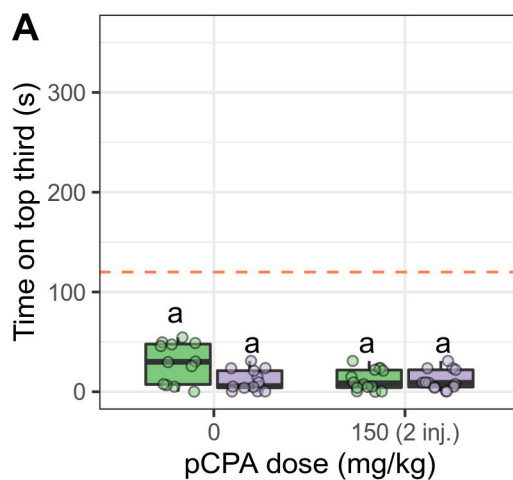
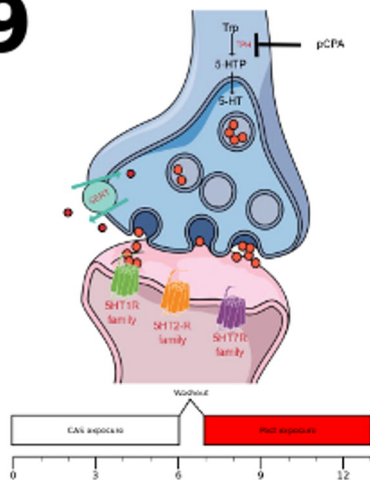


**7**

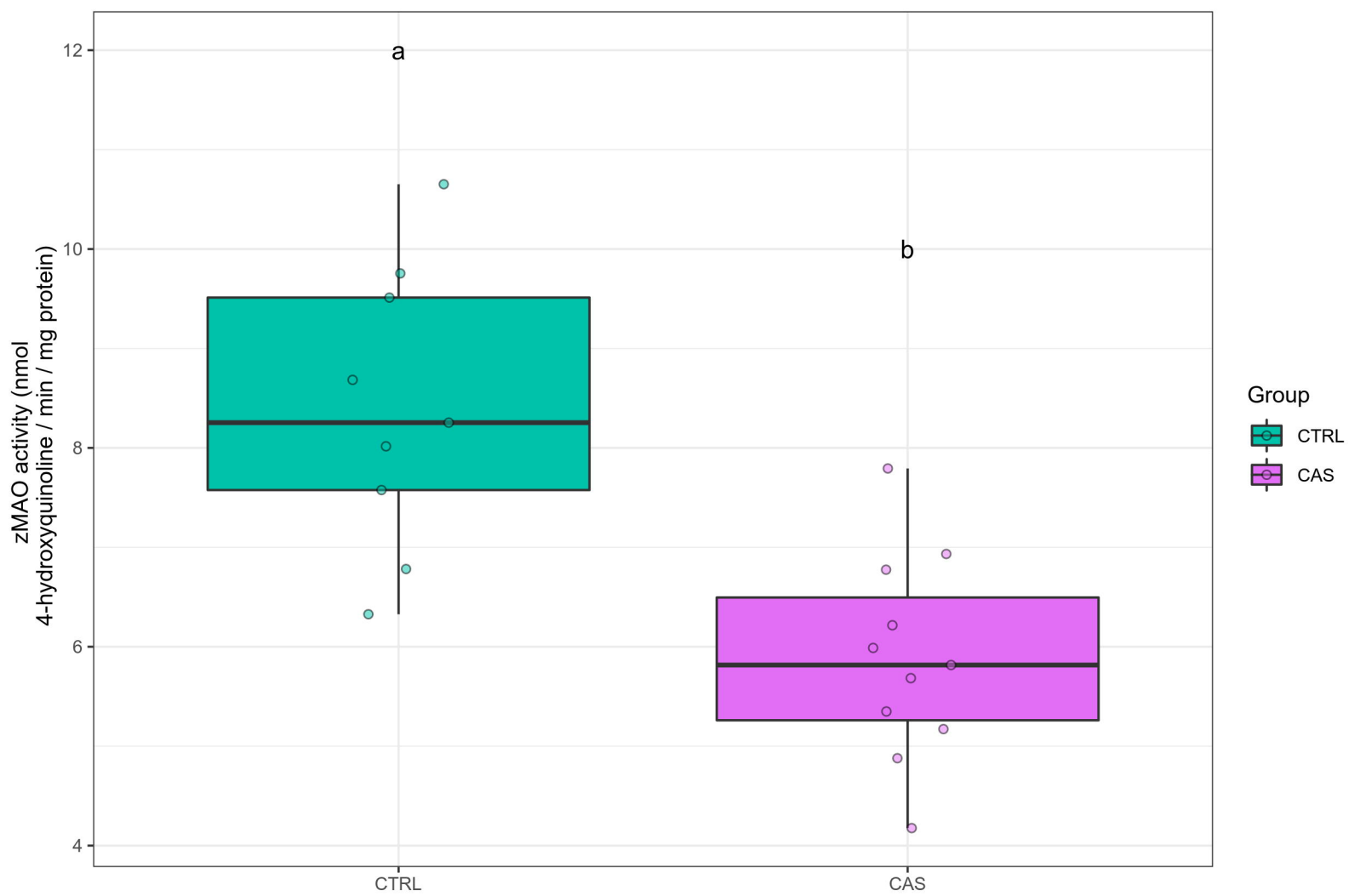
Treatment  CTRL  CAS


**8**

Treatment: CTRL (red), CAS (blue)

**9**

Treatment  CTRL  CAS



**5-HT** 

**Switch ON**

Activation of  
**prosencephalic circuit**

Freezing / Risk  
assessment

**Switch OFF**

Activation of  
**mesencephalic circuit**

Erratic  
Swimming /  
Fear

Hb

POA

GC

Raphe