Role of 5-HT2C receptors in zebrafish alarm reactions and post-exposure behavior

Rhayra Xavier do Carmo Silva[1,2], Bianca Gomes do Nascimento[2], Gabriela Cristini Vidal Gomes[2], Nadyme Assad Holanda da Silva[3], Jéssica Souza Pinheiro[3], Suianny Nayara da Silva Chaves[2], Ana Flávia Nogueira Pimentel[2], Bruna Patrícia Dutra Costa[2], Anderson Manoel Herculano[1], Monica Lima-Maximino[3], Caio Maximino[2*]

[1] Laboratório de Neurofarmacologia Experimental – LNE, Universidade Federal do Pará, Belém/PA, Brazil

[2] Laboratório de Neurociências e Comportamento "Frederico Guilherme Graeff" – LaNeC, Universidade Federal do Sul e Sudeste do Pará, Marabá/PA, Brazil

[3] Laboratório de Neurofarmacologia e Biofísica – LaNeF, Universidade do Estado do Pará, Marabá/PA, Brazil

* Corresponding author

Caio Maximino

Laboratório de Neurociências e Comportamento "Frederico Guilherme Graeff" - LaNeC

Faculdade de Psicologia, Instituto de Estudos em Saúde e Biológicas

Universidade Federal do Sul e Sudeste do Pará, Campus Marabá, Unidade III

Av. dos Ipês, S/N – 68500-000

Marabá/PA, Brazil

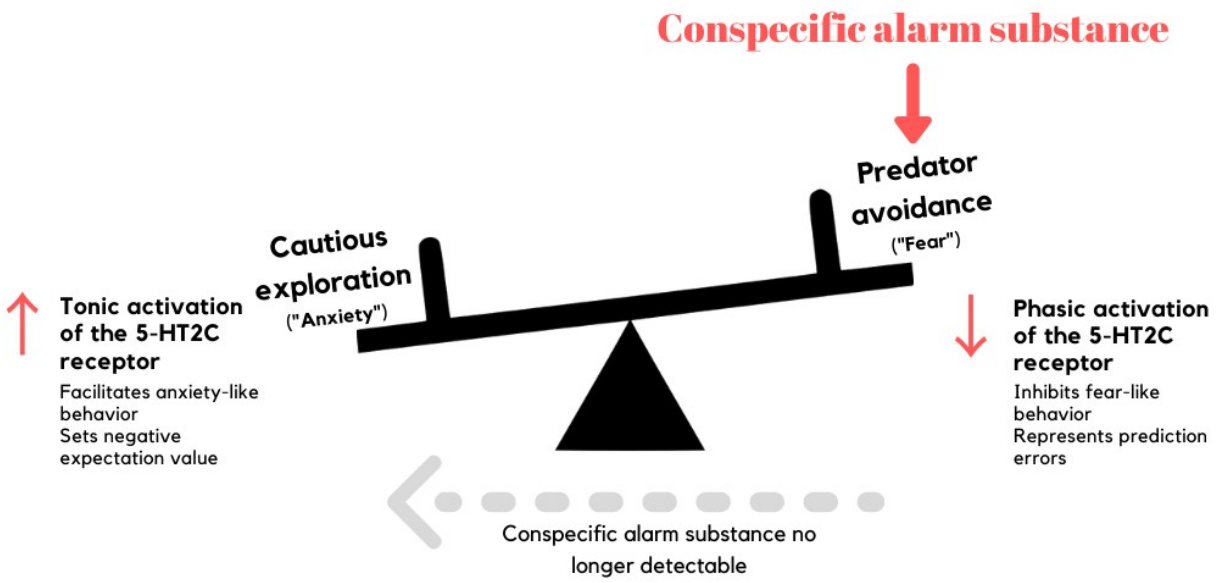
cmaximino@unifesspa.edu.br

Abstract

Serotonin (5-HT) receptors have been implicated in responses to aversive stimuli in mammals and fish, but its precise role is still unknown. Moreover, since at least seven families of 5-HT receptors exist in vertebrates, the role of specific receptors is still debated. Aversive stimuli can be classified as indicators of proximal, distal, or potential threat, initiating responses that are appropriate for each of these threat levels. Responses to potential threat usually involve cautious exploration and increased alertness, while responses to distal and proximal threat involve a fight-flight-freeze reaction. We exposed adult zebrafish to a conspecific alarm substance (CAS) and observed behavior during (distal threat) and after (proximal threat) exposure, and treated with the 5-HT_{2C} receptor agonists MK-212 or WAY-161503 or with the antagonist RS-102221. MK-212 and WAY-161503 blocked CAS-elicited defensive behavior (distal threat), but not post-exposure increases in defensive behavior (potential threat), suggesting a phasic inhibition of responses to distal threat. MK-212 did not block changes in behavior elicited by acute restraint stress, a model of proximal threat, suggesting that the phasic role of the 5-HT_{2C} receptor is specific to distal threat. We also found that RS-102221, a 5-HT_{2C} receptor antagonist, did not change behavior during exposure, but it produced a small effect on behavior after exposure to CAS, suggesting a tonic facilitation of responses to potential threat. The antagonist RS-102221 also did not block changes in behavior elicited by acute restraint stress, suggesting that the tonic role of the 5-HT_{2C} receptor is also specific to distal threat.

Preprint: https://www.biorxiv.org/content/10.1101/2020.10.04.324202v2; **Data and scripts:** https://github.com/lanec-unifesspa/5-HT-CAS/tree/master/data/5HT2C

Keywords: 5-HT2C receptors; Anxiety; Fear; Responses to threat; Zebrafish



Graphical abstract: In zebrafish, activation of the 5-HT_{2C} receptors was able to inhibit behavioral adjustments to a conspecific alarm substance, which elicits fear-like behavior (distal threat), but not adjustments in behavior after the substance was no longer present (potential threat). On the other hand, blocking 5-HT_{2C} receptors inhibited adjustments in behavior after the substance was no longer present, but not when it was present. No effects were observed when behavioral changes were elicited by acute restraint stress (proximal threat). Phasic serotonin signals at the 5-HT_{2C} receptor represent prediction errors on a tonic “background” that sets aversive expectation values.

1. Introduction

Serotonergic mechanisms have been implicated in defensive behavior to distal, proximal and potential threat (Graeff, 2004). In current interpretations of the role of serotonin (5-HT) on neurobehavioral responses to threat, the neurotransmitter is released when threat is potential, increasing defensive behavior at this level (i.e., risk assessment/anxiety-like behavior), but

58 inhibiting (non-adaptive) responses to distal and proximal threat (i.e., fight-flight-freeze/fear-like
59 behavior)(Graeff, 2004). These effects are mediated by different structures of the neuroaxis,
60 with more rostral structures (e.g., limbic forebrain) mediating responses to potential threat, and
61 more caudal structures (e.g., periaqueductal gray/griseum centrale) mediating responses to
62 distal and proximal threat (Graeff, 2004).

63 In zebrafish (*Danio rerio* Hamilton 1822), the alarm reaction has been proposed as a
64 model to study defenses to distal threat (Maximino et al., 2019). The alarm reaction is a
65 behavioral response to the release of an alarm substance ("Schreckstoff") from epitelial club
66 cells after damage (von Frisch, 1938), and involves strategies to avoid potential predators.
67 Since the most likely situation for club cells to be damaged in the wild is predator attack,
68 conspecific alarm substance (CAS) communicates to shoal-mates the potential presence of a
69 predator, eliciting responses that avoid predator attack (Maximino et al., 2019). During CAS
70 exposure, zebrafish display erratic swimming and bottom-dwelling, while after CAS exposure
71 freezing is prominent (Lima-Maximino et al., 2020; Nathan et al., 2015). We suggested that the
72 first component involves defenses to distal threat ("fear-like behavior"), while the second
73 component involves a "return to normal" that is related to potential threat ("anxiety-like
74 behavior")(Lima-Maximino et al., 2020; Maximino et al., 2019).

75 5-HT has been implicated in fish behavioral responses both during and after exposure to
76 CAS. In zebrafish, extracellular 5-HT levels were increased after CAS exposure (Maximino et
77 al., 2014), an effect that can be related to decreased 5-HT uptake (Maximino et al., 2014) and/or
78 decreased monoamine oxidase activity (Lima-Maximino et al., 2020; Quadros et al., 2018). In
79 Crucian carp (*Carassius carassius*), exposure to CAS elicits increases in serotonergic activity in
80 the brainstem and optic tectum (Höglund et al., 2005), structures which have been involved in
81 responses to distal and proximal threat (do Carmo Silva et al., 2018a). However, this only
82 happened when hiding material was unavailable in the tank. In Nile tilapia (*Oreochromis*

83 *niloticus*), CAS did not increase serotonergic activity in the dorsomedial and dorsolateral
84 telencephali (Silva et al., 2015), homologues of the frontotemporal amygdaloid nuclei and
85 hippocampus, respectively (do Carmo Silva et al., 2018a). These results suggest that, during or
86 after exposure, CAS increases serotonergic activity in regions associated with "quick-and-dirty"
87 behavioral responses to distal threat (a "fight/flight/freeze" or "fear" system), but not in the
88 telencephalic areas associated with cautious exploration/risk assessment (a "behavioral
89 inhibition" or "anxiety" system).

90 Manipulations of the serotonergic system impact behavioral and neurovegetative
91 responses to CAS. Treating zebrafish with acute fluoxetine, therefore increasing serotonergic
92 activity, dose-dependently decreased behavior during exposure, but increased post-exposure
93 freezing (Lima-Maximino et al., 2020). Blocking 5-HT receptors with metergoline, or depleting 5-
94 HT with *para*-chlorophenylalanine, had no effect on behavior during exposure, but blocked the
95 effects of CAS on post-exposure behavior. While we suggested that the serotonergic system is
96 recruited *after* CAS exposure to inhibit fear-like responses and promote a cautious "return to
97 normal" (Lima-Maximino et al., 2020), results from Crucian carp (Höglund et al., 2005) open the
98 possibility that inescapability is the variable that is involved in this activation of the serotonergic
99 system.

100 The role of serotonin receptors from the 5-HT_2 family in CAS-elicited behavioral
101 adjustments has also been investigated. Zebrafish has been shown to possess two copies of
102 the 5-HT_{2A} receptor, one copy of the 5-HT_{2B} receptor, and two copies of the 5-HT_{2C} receptor
103 (Sourbron et al., 2016). In Nile tilapia, mianserin, a 5-HT_{2A} and 5-HT_{2C} receptor antagonist that
104 also blocks α_2-adrenoceptors, blocked active components of the alarm reaction (dashing,
105 bristling of dorsal fin spines), but not freezing, during exposure (Barreto, 2012). In zebrafish,
106 methysergide (an antagonist at 5-HT_{2A}, 2B, and 2C receptors, and a 5-HT_{1A} receptor agonist)
107 increased freezing and bottom-dwelling during and after exposure at a high dose (92.79 mg/kg),

but not at lower doses (Nathan et al., 2015). These results point to an inhibitory role of the 5-HT_{2A} and 5-HT_{2C} receptors on CAS-elicited fear-like responses. However, since Nathan et al. (2015) observed these effects consistently during a long session, in which behavior is expected to change as CAS concentrations decrease (Mathuru et al., 2012)(i.e., similar to the shift from erratic swimming to freezing that is observed when animals are observed after exposure in a CAS-free context; Lima-Maximino et al., 2020), and since serotonergic drugs can produce opposite effects on behavior during and after CAS exposure (Lima-Maximino et al., 2020), from these results it is not possible to understand whether 5-HT_2 receptors participate in both responses.

These results are also complicated by results from rodent work. 5-HT_2 receptors have been implicated in the mechanisms of defensive behavior organized by the central gray (PAG/GC) and amygdala in rats. At different amygdalar subnuclei, 5-HT_2 receptors appear to either facilitate (de Paula and Leite-Panissi, 2016) or block (Macedo et al., 2007) unconditioned fear, while at the midbrain 5-HT_2 receptors inhibit defensive responses to distal and proximal threat (Castilho et al., 2002; Castilho and Brandão, 2001; Coimbra and Brandão, 1997; Graeff et al., 1986; Oliveira et al., 2007). These receptors have also been implicated in anxiety-like behavior (defense to potential threat), facilitating these responses in the amygdala (Cornélio and Nunes-de-Souza, 2007), hippocampus (Alves et al., 2004), and GC/PAG (Nunes-de-Souza et al., 2008) of rodents. In general, the 5-HT_{2C} receptor appears to mediate these effects on anxiety-like behavior. Thus, in rodents 5-HT_2 receptors appear to inhibit fear and facilitate anxiety at different levels of the neuroaxis, while in zebrafish these receptors appear to inhibit both fear and anxiety, although it is currently unknown which brain regions participate in each effect.

These differences could be due to species differences; to effects at different receptors (e.g., 5-HT_{2A} or 5-HT_{2C}); or to difficulties in the protocol used by Nathan et al. (2015), which does

133 not differentiate between responses to proximal, distal, or potential threat. In this work, we
134 tested whether 5-HT_{2C} receptors participate in responses to CAS during (distal threat) or after
135 exposure (potential threat) and to acute restraint stress (ARS). ARS has been applied in
136 zebrafish to elicit strong stress responses, including activation of the hypothalamus-pituitary-
137 interrenal (HPI) axis and associated behavioral responses (Assad et al., 2020; Ghisleni et al.,
138 2012; Piato et al., 2011). From the point of view of the predatory imminence continuum theory,
139 ARS represents proximal threat (Perusini and Fanselow, 2015). Thus, if 5-HT_{2C} receptors inhibit
140 aversively motivated behavior in zebrafish regardless of threat distance, its activation would
141 inhibit behavioral responses at these three contexts. If, as in rodents, 5-HT_{2C} receptors act at
142 different levels of threat distance to either activate or inhibit defensive responses, then 5-HT_{2C}
143 agonists will not produce the same effect in each of these contexts.

144 A related question is whether 5-HT_{2C} receptors possess a “tonic” role in defensive
145 behavior in zebrafish. Differently from phasic responses, which are temporally and spatially
146 coupled to neurotransmitter release, tonic responses result from low-level, persistent, and
147 extrasynaptic activation of receptors (Daw et al., 2002). There is some evidence for a
148 serotonergic tone in zebrafish. Tonic optogenetic activation of a habenulo-raphe pathway in
149 zebrafish is aversive, inducing avoidance conditioning, and presentation of a conditioned
150 stimulus consistently produces this tonic activation (Amo et al., 2014); this tonic activity has
151 been proposed to represent a negative expectation value, with phasic signals representing
152 prediction error (Amo et al., 2014; Daw et al., 2002). We have shown that there is evidence for a
153 tonic facilitation of defensive behavior after CAS exposure, but not during exposure, as
154 metergoline and pCPA blocked the first but not the latter (Lima-Maximino et al., 2020).
155 Interestingly, work with rodents clearly suggests phasic facilitation or inhibition of defensive
156 responses by 5-HT_{2C} receptors, since agonists, but not antagonists, produce these effects.

157 The aim of the present work was to test the hypothesis that phasic activation of 5-HT_{2C}

158 receptors would block CAS-elicited defensive reactions during exposure, but not after exposure;
159 moreover, we proposed a tonic facilitation of post-exposure behavior. We also hypothesized
160 that 5-HT_{2C} agonists would not alter the anxiogenic-like effects of acute restraint stress (ARS), a
161 model for proximal threat. We found that 5-HT_{2C} agonists were able to block elements of the
162 alarm reaction, but did not affect post-exposure behavior, nor the anxiogenic-like effects of ARS.
163 This manuscript is a complete report of all the studies performed to test the effect of 5-HT_{2C}
164 agonists and antagonists on zebrafish defensive behavior to distal threat. We report how the
165 sample size was determined, all data exclusions (if any), all manipulations, and all measures in
166 the study.
167

168 2. Materials and methods

169 2.1. Animals and housing

170 Adult (>4 month-old; standard length = 23.0 ± 3.2 mm) zebrafish (*Danio rerio*) from the
171 longfin phenotype (n = 218) were used in the present experiments. The populations used are
172 expected to better represent the natural populations in the wild, due to its heterogeneous
173 genetic background (Parra et al., 2009; Speedie and Gerlai, 2008). Animals were bought from a
174 commercial vendor (Belém/PA) and collectively maintained in 40 L tanks for at least two weeks
175 before the onset of experiments. The animals were fed daily with fish flakes. The tanks were
176 kept at constant temperature (28 °C), oxygenation, light cycle (14:10 LD photoperiod) and a pH
177 of 7.0-8.0, according to standards of care for zebrafish (Lawrence, 2007). Animals were used for
178 only one experiment to reduce interference from apparatus exposure. Potential suffering of
179 animals was minimized by controlling for the aforementioned environmental variables.

180 Furthermore, in the all experiments the animals used were handled, anesthetized and sacrificed
181 according to the norms of the Brazilian Guideline for the Care and Use of Animals for Scientific
182 and Didactical Purposes (Conselho Nacional de Controle de Experimentação Animal -
183 CONCEA, 2017). Since, in all cases, animals were individually stressed and/or individually
184 treated, experimental unit was a single animal, and therefore all sample sizes refer to number of
185 animals. The experimental protocols were approved by UEPA's IACUC under protocol 06/18.
186

187 2.2. Drugs and treatments

188 The 5-HT_{2C} receptor agonists MK-212 (2-Chloro-6-(1-piperazinyl)pyrazine, CAS #64022-
189 27-1) and WAY-161503 (8,9-dichloro-2,3,4,4a-tetrahydro-1H-pyrazino[1,2-a]quinoxalin-5(6H)-
190 one; CAS #75704-24-4) were bought from Sigma-Aldrich (St Louis, USA) on 2018, and
191 dissolved in Cortland's salt solution (NaCl 124.1 mM, KCl 5.1 mM, Na_2HPO_4 2.9 mM, $MgSO_4$ 1.9
192 mM, $CaCl_2$ 1.4 mM, $NaHCO_3$ 11.9 mM, Polyvinylpyrrolidone 4%, 1,000 USP units Heparin; Wolf,
193 1963) and in 1% dimethyl sulfoxide (DMSO), respectively. The 5-HT_{2C} receptor antagonist RS-
194 102221 (8-[5-(2,4-Dimethoxy-5-(4-trifluoromethylphenylsulphonamido)phenyl -5-oxopentyl]-
195 1,3,8-triazaspiro[4.5]decane-2,4-dione; CAS #185376-97-0) was bought from Sigma-Aldrich (St
196 Louis, USA) in 2018, and dissolved in 1% DMSO. While affinities for zebrafish 5-HT_{2C}-like
197 receptors have not been established, WAY-161503 has been reported to displace DOI from
198 human 5-HT_{2C} receptors with a K_i of 3.3 nM (6-fold selectivity over human 5-HT_{2A} receptors and
199 20-fold over human 5-HT_{2B} receptors)(Rosenzweig-Lipson et al., 2006). MK-212 has been
200 shown to be less selective at recombinant human receptors, with a K_i of 7.01 nM at 5-HT_{2C}
201 receptors (vs. 5.99 nM and 6.21 nM at 5-HT_{2A} and 5-HT_{2B} receptors, respectively)(Knight et al.,
202 2004). Finally, RS-102221 has been shown to displace mesulergine from human 5-HT_{2C}

203 receptor with a *pKi* of 8.4 nM (over 100-fold selectivity over human 5-HT_{2A} and 5-HT_{2B} receptors)
204 (Bonhaus et al., 1997).

205 For Experiment 1, animals were injected intraperitoneally either with MK-212 (1 mg/kg
206 and 2 mg/kg, doses which increase anxiety-like behavior in the rat elevated plus-maze; (de
207 Mello Cruz et al., 2005)) or with the vehicle solution (Cortland's salt solution); WAY-161503 (1
208 mg/kg, a dose which produces anxiogenic-like effects in the rat elevated plus-maze: Gomes et
209 al., 2010) or with the vehicle solution (DMSO); or RS-102221 (2 mg/kg, a dose that reduces
210 anxiety in the mouse light/dark test; (Kuznetsova et al., 2006)). For Experiment 2, animals were
211 injected intraperitoneally with MK-212 (2 mg/kg) or vehicle (Cortland's salt solution). Injections
212 were made according to the protocol proposed by Kinkel et al. (2010); briefly, animals were
213 cold-anesthetized and transferred to a sponge-based surgical bed, in which injection was made.
214 Injections were made using a microsyringe (Hamilton® 701N syringe, needle size 26 gauge at
215 cone tip), with total volumes of injection of 5 µL.
216

217 2.3. Effects of 5-HT_{2C} receptor agonists and antagonists on alarm 218 reaction and post-exposure behavior

219 2.3.1. Experimental design

220 To verify the effects of phasic activation of 5-HT_{2C} receptors on the zebrafish alarm
221 reaction, animals were pre-treated with either receptor agonists and exposed to CAS in a
222 sequential design, with a "washout" period in between tests (Figure 1A). For the exposure
223 stage, each animal was transferred individually to a container (2 L) where after 3 minutes of
224 acclimatization, it was carefully exposed to 7 ml of alarm substance (CAS), extracted using a

225 standardized protocol (do Carmo Silva et al., 2018b). As negative control, a group with the
226 same amount of animals was exposed to the same volume of distilled water, according to the
227 protocol of Lima-Maximino et al. (2020). The animals remained exposed for 6 minutes during
228 which their behavior was recorded using a video camera positioned in front of the aquarium.
229 Then, to verify the residual effects of exposure to the alarm substance, the animals were
230 transferred to the apparatus of the novel tank test, a transparent glass aquarium filled with 5 L of
231 mineral water where the animal can freely explore the space for a period 6 minutes during which
232 their behavior was recorded, following the protocol described in Lima-Maximino et al., (2020).
233 All stages of the experiment were performed under constant white Gaussian noise, producing
234 an average of 58 dB above the tank. Light levels above the tanks were measured using a
235 handheld light meter, and ranged from 251 to 280 lumens (coefficient of variation = 3.399%
236 between subjects).

237 Animals were randomly allocated to groups using a random number generator
238 (http://www.jerrydallal.com/random/random_block_size_r.htm), with each subject randomized to
239 a single treatment using random permuted blocks. One PI attributed a random letter to
240 treatment (e.g., “A” for CTRL, “B” for CAS) and a random integer for drug dose (e.g., “1” for 1
241 mg/kg, “2” for 0 mg/kg [vehicle]), and combinations for letters and integers were randomized.
242 For each experiment, animals were treated and tested in the order of allocation (i.e., randomly).
243 In all experiments, experimenters and data analysts were blinded to drugs and treatment by
244 using coded vials (with the same code used for randomization); blinding was removed only after
245 data analysis. Experiments were always run between 08:00AM and 02:00 PM. After
246 experiments, animals were sacrificed by prolonged bath in ice-cold water (< 12 ºC), followed by
247 spinal transection (Matthews and Varga, 2012).
248

249 ## 2.3.2. Sample size determination

250 To determine sample size, we incorporated information from control groups derived from
251 previously published experiments on zebrafish alarm substances observing bottom-dwelling
252 (Lima-Maximino et al., 2020; Quadros et al., 2016; Speedie and Gerlai, 2008) and a series of
253 four small experiments on the effects of CAS on behavior during exposure
254 (https://github.com/lanec-unifesspa/5-HT-CAS/tree/master/data/behavioral/metanalysis),
255 following the RePAIR approach (Bonapersona et al., 2019). Sample sizes, means, and standard
256 deviations for the primary endpoint "Time on bottom" were used to produce a prior distribution
257 on the RePAIR script (https://utrecht-university.shinyapps.io/repair/). Final parameters of the
258 distribution were $\mu = 197.149$, $\sigma^2 = 6100.609$, and weighted $N = 120.10$. The parameters of this
259 distribution were then used to calculate sample size, based on an effect size of $d = 0.5$
260 (equivalent to that used to calculate sample sizes in Lima-Maximino et al., 2020) and *a priori*
261 power of 0.8, with one-tailed tests with error probability $\alpha = 0.05$. With these parameters, the
262 final number of animals was 10 animals in the control group and 21 in each experimental group,
263 reaching a prospective power of 92.34%.
264

265 ## 2.3.3. Alarm substance extraction

266 A group of zebrafish was used as donor animals for the extraction of the conspecific
267 alarm substance (CAS). CAS extraction procedure was performed on each animal individually
268 as described by do Carmo Silva et al. (2018b). First, the donor animal was cold anesthetized
269 and transferred to a Petri dish, where the excess water from its body was removed with a paper
270 towel. Then the animal was decapitated with a surgical scalpel and the excess blood from the
271 sectioned region was removed with a swab. Subsequently, the animal's bodies were transferred

272 to another Petri dish where 15 superficial cuts were made in the epidermis of animals (medial-
273 ventral region) and 10 ml of distilled water were added to wash the cuts. After washing, the
274 animal's bodies were removed from the Petri dish, and with the aid of a Pasteur pipette, the fish
275 scales and other impurities were removed from the solution that was stored in a conical tube
276 and preserved on ice. The same extraction procedure was performed for all donated animals.
277

278 ## 2.3.4. Observation apparatuses

279 To assess the effects of drugs on the alarm reaction, animals were transferred to a 12
280 cm x 12 cm x 14 cm glass tank, filled with 1.5 L tank water, and allowed to swim freely for a 3
281 min acclimation period. After this period, 7 mL CAS was added to the tank, from above, and
282 filming started. The video camera (Sony® DCR-DVD610) was positioned in the front of the tank,
283 therefore allowing observation and tracking of vertical distribution. Animals were allowed to
284 freely explore this tank for 6 min before being transferred to a “washout” tank, which contained
285 500 mL tank water. Animals were kept in this tank for 1 min, removing any residues of CAS
286 before transference to the second apparatus. This second apparatus was a 25 cm x 24 cm x 20
287 cm glass tank, filled with 5 L tank water; animals were allowed free exploration of this tank for 6
288 min, during which behavior was filmed from the front of the tank. Tanks for both stages were
289 differently shaped to increase the novelty of the second environment, a variable that is important
290 to induce an anxiety-like “diving” response in animals not exposed to CAS (Bencan et al., 2009).
291

2.4. Experiment 2: Effects of MK-212 on acute restraint stress-elicited behavior

2.4.1. Experimental design

In this experiment, we focus on assessing the effects of activation of 5-HT_{2C} receptors on anxiogenic-like effects of acute restraint stress (ARS). In order to do this, we evaluate the behavior of the animals in the novel tank test after being subjected to a 90-min section of restraint stress (Figure 1B). After drug (MK-212) or vehicle (Cortland's salt solution) injection and anesthesia, each animal was transferred individually to a 2 mL microtube (Eppendorf®) and placed on a plastic microtube rack inside an aquarium with continuous oxygen supply. The microtubes had small holes at both ends to allow free circulation of water inside the tube and to prevent fish from moving around, according to the protocol of Piato et al. (2011). A control group was maintained in a similar tank, but without restraint stress. Animals remained in these conditions for 90 min., sufficient to induce changes in telencephalon neurochemistry (Assad et al., 2020) and marked anxiety-like behavior (Assad et al., 2020; Ghisleni et al., 2012; Piato et al., 2011). After stress, the animals were transferred to the apparatus of the novel tank test, a transparent glass aquarium filled with 5 L of mineral water where the animal can freely explore the space for a period 6 minutes during which their behavior was recorded, following the protocol described in Lima-Maximino et al., (2020). All stages of the experiment were performed under constant white Gaussian noise, producing an average of 58 dB above the tank. Light levels above the tanks were measured using a handheld light meter, and ranged from 254 to 276 lumens (coefficient of variation = 3.401% between subjects). Random allocation was made as described above. In all experiments, experimenters and data analysts were blinded to drugs by using coded vials (with the same code used for randomization); blinding was removed only

after data analysis. Experimenters were not blinded to treatment, but data analysts were blinded to both treatment and drug. Experiments were always run between 08:00AM and 02:00 PM. After experiments, animals were sacrificed by prolonged bath in ice-cold water (< 12 ºC), followed by spinal transection (Matthews and Varga, 2012).

2.4.2. Sample size determination

Sample sizes were based on previous experiments with the behavioral effects of ARS (Assad et al., 2020).

2.4.3. Observation apparatus

To analyze the behavioral effects of treatment (ARS exposure) and drug, the same apparatus used in the second stage of the previous experiment (5-L transparent glass tank) was used.

2.5. Behavioral endpoints

Video files for each experiment were stored and later analyzed using automated video tracking (TheRealFishTracker; http://www.dgp.toronto.edu/~mccrae/projects/FishTracker/). The following variables were extracted:

- Time spent on bottom third of the tank (s)[Primary outcome]
- Time spent on top third of the tank (s)[Secondary outcome]
- Erratic swimming, measured as absolute turn angle [Secondary outcome]

- Freezing (s), measured as time spent in a speed lower than 0.5 cm/s [Secondary outcome]
- Swimming speed (cm/s) [Secondary outcome]

2.6. Quality control

Exclusion criteria: Animals were to be excluded if showing signs of poor health or distress before beginning experiments, using the Zebrafish Health and Welfare Glossary (https://wiki.zfin.org/display/ZHWG/Zebrafish+Health+and+Welfare+Glossary+Home; Goodwin et al., 2016). Moreover, animals could be excluded from analysis based on statistical detection as outlier (see 2.7, “Statistical analysis”, below).

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

2.7. Statistical analysis

Data were analyzed using two-way analysis of variance (drug x exposure to stressor) with sequential sum of squares (type I), followed by Tukey's post-tests when $p < 0.05$. Data

342 analysis, table organization, and result graphs were performed using R version 3.6.3 (2020-02-
343 29). Effect sizes for ANOVA effects are shown as ω^2; effect sizes for post-hoc tests were shown
344 as Cohen's d. Outliers were removed based on median absolute differences (MADs), using time
345 on bottom as the main endpoint; values were removed when they were higher or lower than 3
346 MADs around the median (Leys et al., 2013), and the number of outliers was reported in the
347 results.
348

349 3. Results

350 3.1. Effects of MK-212 on alarm reaction and post-exposure 351 behavior

352 3.1.1. During exposure

353 One animal from the control + 0 mg/kg group was detected as outlier and removed. Main
354 effects of treatment ($F_{[1, 114]} = 6.337$, $p = 0.0132$; $\omega^2 = 0.039$) and dose ($F_{[2, 114]} = 3.665$, $p =$
355 0.0287; $\omega^2 = 0.039$) were found for time on top. An interaction effect was also found ($F_{[2, 114]} =$
356 4.602, $p = 0.012$; $\omega^2 = 0.052$). Post-hoc tests found that CAS did not alter time on top ($p = 0.94$,
357 $d = 0.3$, non-treated controls vs. non-treated CAS), but MK-212 (1 mg/kg) increased it in non-
358 exposed animals (*1 mg/kg:* $p = 0.024$, $d = -1.05$, non-treated controls vs. 1 mg/kg controls; *2*
359 *mg/kg:* $p = 0.994$, $d = -0.18$, non-treated controls vs. 2 mg/kg controls; Figure 2A).
360 Main effects of treatment ($F_{[1, 114]} = 20.995$, $p = 1.18 \times 10^{-5}$; $\omega^2 = 0.12$) and dose ($F_{[2, 114]} =$
361 11.455, $p = 2.93 \times 10^{-5}$; $\omega^2 = 0.125$) were found for time on bottom. An interaction effect was

362 also found ($F_{[2, 114]} = 4.085$, $p = 0.0194$; $\omega^2 = 0.037$). Post-hoc tests revealed that CAS increased
363 time on bottom ($p < 0.001$, $d = -0.77$ for the main effect), and the highest MK-212 dose blocked
364 this effect (*1 mg/kg:* $p = 0.39$, $d = -0.64$, non-treated controls vs. treated CAS; $p = 0.38$, $d = 0.6$,
365 non-treated CAS vs. treated CAS; *2 mg/kg:* $p = 0.828$, $d = 0.4$, non-treated controls vs. treated
366 CAS; $p < 0.001$, $d = 1.64$, non-treated CAS vs. treated CAS; Figure 2B).

367 A main effect of treatment ($F_{[1, 114]} = 5.137$, $p = 0.0253$; $\omega^2 = 0.017$), but not dose ($F_{[2, 114]} =$
368 2.948, $p = 0.0565$; $\omega^2 = 0.016$), was found for erratic swimming. An interaction effect was also
369 found for this variable ($F_{[2, 114]} = 57.101$, $p < 2 \times 10^{-16}$; $\omega^2 = 0.467$). Post-hoc tests showed that
370 CAS greatly increased absolute turn angle in non-treated animals ($p < 0.001$, $d = -2.58$, non-
371 treated controls vs. non-treated CAS), an effect that was blocked by both MK-212 doses (*1*
372 *mg/kg*: $p = 1$, $d = -0.04$, non-treated controls vs. treated CAS; $p < 0.001$, $d = 2.53$, non-treated
373 CAS vs. treated CAS; *2 mg/kg*: $p = 0.81$, $d = -0.42$, non-treated controls vs. treated CAS; $p <$
374 0.001, $d = 2.16$, non-treated CAS vs. treated CAS; Figure 2C); however, MK-212 also increased
375 absolute turn angle in animals which were not exposed to CAS (*1 mg/kg*: $p < 0.001$, $d = -1.95$,
376 non-treated controls vs. treated controls; *2 mg/kg:* $p < 0.001$, $d = -1.91$, non-treated controls vs.
377 treated controls).

378 A main effect of treatment ($F_{[1, 114]} = 15.219$, $p = 0.000162$; $\omega^2 = 0.105$), but no effect of
379 MK-212 dose ($F_{[2, 114]} = 1.037$, $p = 0.358$; $\omega^2 = 0.001$). No interaction effects were found ($F_{[2, 114]} =$
380 1.662, $p = 0.194$; $\omega^2 = 0.01$). Post-hoc tests suggested that CAS increased freezing at the
381 lowest MK-212 dose and controls ($p < 0.001$, $d = -0.72$ for the main effect of CAS; Figure 2D).

382 No main effects of treatment ($F_{[1, 114]} = 0.005$, $p = 0.9459$; $\omega^2 = -0.008$) nor MK-212 dose
383 ($F_{[2, 114]} = 0.211$, $p = 0.8105$; $\omega^2 = -0.013$) were found for swimming speed. While an interaction
384 effect was found ($F_{[2, 114]} = 4.652$, $p = 0.0114$; $\omega^2 = 0.059$; $f^2 = 0.29$), post-hoc tests found no
385 differences across groups (Figure 2E).

386

3.1.2. After exposure

In the novel tank test after CAS exposure and washout, no main effects of treatment ($F_{[1, 114]} = 0.945$, $p = 0.333$; $\omega^2 = 0$) were found for time on top. However, a main effect of MK-212 dose was found ($F_{[2, 114]} = 4.892$, $p = 0.009$; $\omega^2 = 0.06$). Interaction effects were also absent ($F_{[2, 114]} = 2.393$, $p = 0.0959$; $\omega^2 = 0.021$). Post-hoc tests revealed that MK-212 (1 mg/kg) increased time on top in both controls and CAS-exposed animals (*1 mg/kg*: p = 0.019, d = -0.63 vs. non-treated animals; p = 0.038, d = 0.54 vs. 2 mg/kg; *2 mg/kg:* p = 0.92, d = -0.09 vs. non-treated animals; Figure 3A).

Main effects of treatment ($F_{[1, 114]} = 7.751$, $p = 0.0063$; $\omega^2 = 0.048$) and MK-212 dose ($F_{[2, 114]} = 5.385$, $p = 0.0058$) were found for time on bottom. An interaction effect was also found ($F_{[2, 114]} = 4.203$, $p = 0.0173$; $\omega^2 = 0.045$). Post-hoc tests suggested that MK-212 (1 mg/kg) decreased time on bottom in control animals (Figure 3B), but not in CAS-exposed animals (*1 mg/kg*: p = 0.002, d = 1.31, non-treated controls vs. 1 mg/kg controls; p = 1.0, d = 0.1, non-treated CAS vs. 1 mg/kg CAS; *2 mg/kg:* p = 0.99, d = 0.21, non-treated controls vs. 2 mg/kg controls; p = 1, d = 0.01, non-treated CAS vs. 2 mg/kg CAS).

No effect of treatment was found for erratic swimming ($F_{[1, 114]} = 3.641$, $p = 0.0589$; $\omega^2 = 0.018$). A main effect of drug was found ($F_{[2, 114]} = 9.244$, $p = 0.00019$; $\omega^2 = 0.112$), as well as an interaction effect ($F_{[2, 114]} = 4.951$, $p = 0.00867$; $\omega^2 = 0.054$). Post-hoc tests suggested a synergistic effect between CAS and MK-212 at 2 mg/kg, which potentiated CAS-elicited increases in erratic swimming (Figure 3C); moreover, 1 mg/kg MK-212 increased erratic swimming in control animals (*1 mg/kg:* p = 0.026, d= -1.05, non-treated controls vs. 1 mg/kg controls; p = 1.0, d = -0.16, non-treated CAS vs. 1 mg/kg CAS; *2 mg/kg:* p = 0.22, d = -0.76, non-treated controls vs. 2 mg/kg controls; p = 0.002, d = -1.21, non-treated CAS vs. 2 mg/kg CAS) .

411 A main effect of treatment ($F_{[1, 114]} = 4.658$, $p = 0.033$; $\omega^2 = 0.025$) and a main effect of
412 MK-212 dose were found for freezing ($F_{[2, 114]} = 11.617$, $p = 2.56 \times 10^{-5}$; $\omega^2 = 0.143$). No
413 interaction effects were found ($F_{[2, 114]} = 2.777$, $p = 0.0664$; $\omega^2 = 0.024$). Post-hoc tests revealed
414 that CAS increased freezing ($p = 0.05$, $d = -0.364$ for the main effect), an effect that was blocked
415 by all MK-212 doses (*1 mg/kg:* $p = 0.74$, $d = 0.47$, non-treated controls vs. 1 mg/kg controls; $p <$
416 0.001, $d = 1.5$, non-treated CAS vs. 1 mg/kg CAS; *2 mg/kg:* $p = 0.85$, $d = 0.4$, non-treated
417 controls vs. 2 mg/kg controls; $p = 0.002$, $d = 1.22$, non-treated CAS vs. 2 mg/kg CAS; Figure
418 3D).

419 No main effects of treatment ($F_{[1, 114]} = 0.111$, $p = 0.74$; $\omega^2 = -0.007$) or dose ($F_{[2, 114]} =$
420 1.339, $p = 0.266$; $\omega^2 = 0.006$), nor an interaction effect ($F_{[2, 114]} = 0.981$, $p = 0.378$; $\omega^2 = 0.0$),
421 were found for swimming speed (Figure 3E).

422

423 3.2. Effects of WAY-161503 on alarm reaction and post-exposure
424 behavior

425 3.2.1. During exposure

426 No outliers were detected from any group in this experiment. A main effect of treatment
427 ($F_{[1, 69]} = 7.404$, $p = 0.00823$, $\omega^2 = 0.08$), but not drug ($F_{[1, 69]} = 1.751$, $p = 0.19011$, $\omega^2 = 0.009$),
428 was found for time on top. No interaction effect was found ($F_{[1, 69]} = 1.001$, $p = 0.32067$, $\omega^2 =$
429 0.0). Post-hoc tests showed that while CAS did not alter time on top ($p = 0.054$, $d = 1.0$, non-
430 treated controls vs. non-treated CAS), a synergistic effect was apparent, with WAY-161503-
431 treated animals showing less time on top than controls ($p = 0.381$, $d = 0.618$, non-treated
432 controls vs. WAY-161503 controls; $p = 0.977$, $d = 0.125$, non-treated CAS vs. WAY-161503

433 CAS; $p = 0.023$, $d = 1.125$, non-treated controls vs. WAY 161-503 CAS; Figure 4A).

434 Main effects of treatment ($F_{[1, 69]} = 10.842$, $p = 0.00157$, $\omega^2 = 0.103$) and drug ($F_{[1, 69]} =$
435 10.138, $p = 0.00218$, $\omega^2 = 0.096$) were found for time on bottom. An interaction effect was
436 found ($F_{[1, 69]} = 4.614$, $p = 0.03523$, $\omega^2 = 0.038$). Post-hoc tests revealed that CAS increased
437 time on bottom ($p = 0.007$, $d = -1.287$, non-treated controls vs. non-treated CAS), and WAY-
438 161503 blocked this effect ($p = 0.989$, $d = 0.123$, non-treated controls vs. WAY-161503 controls;
439 $p = 0.002$, $d = 1.181$, non-treated CAS vs. WAY-161503 CAS; Figure 4B).

440 Main effects of treatment ($F_{[1, 69]} = 108.37$, $p = 8.66 \times 10^{-16}$, $\omega^2 = 0.311$) and drug ($F_{[1, 69]} =$
441 122.99, $p = 2 \times 10^{-16}$, $\omega^2 = 0.353$) were found for erratic swimming. An interaction effect was also
442 found ($F_{[1, 69]} = 44.24$, $p = 5.67 \times 10^{-9}$, $\omega^2 = 0.125$). Again, post-hoc tests suggested that CAS
443 increased erratic swimming ($p < 0.001$, $d = -3.984$, non-treated controls vs. non-treated CAS),
444 while WAY-161503 blocked this effect ($p = 0.302$, $d = 0.6759$, non-treated controls vs. WAY-
445 161503 controls; $p < 0.001$, $d = 3.9537$, non-treated CAS vs. WAY-161503 CAS; Figure 4C).

446 A main effect of treatment ($F_{[1, 69]} = 8.582$, $p = 0.0046$, $\omega^2 = 0.094$), but not of drug ($F_{[1, 69]}$
447 $= 0.564$, $p = 0.4552$, $\omega^2 = -0.005$), was found for freezing duration; no interaction effect was
448 found ($F_{[1, 69]} = 1.434$, $p = 0.2352$, $\omega^2 = 0.005$). Post-hoc tests revealed that CAS increased
449 freezing ($p = 0.047$, $d = -1.02$, non-treated controls vs. non-treated CAS), but this effect was not
450 blocked by WAY-161503 ($p = 0.967$, $d = -0.178$, non-treated controls vs. WAY-161503 controls;
451 $p = 0.544$, $d = 0.412$, non-treated CAS vs. WAY-161503 CAS; Figure 4D).

452 Finally, no effects of treatment ($F_{[1, 69]} = 2.303$, $p = 0.134$, $\omega^2 = 0.018$), drug ($F_{[1, 69]} =$
453 0.103, $p = 0.749$, $\omega^2 = -0.012$), or treatment:drug interaction ($F_{[1, 69]} = 0.527$, $p = 0.471$, $\omega^2 = -$
454 0.006) were found for swimming speed (Figure 4E).

455

3.2.2. After exposure

No main effects of treatment ($F_{[1, 69]} = 3.221$, $p = 0.0771$, $\omega^2 = 0.026$) were found for time on top, but a main effect of drug was found ($F_{[1, 69]} = 4.254$, $p = 0.0429$, $\omega^2 = 0.039$). An interaction was also found ($F_{[1, 69]} = 6.352$, $p = 0.014$, $\omega^2 = 0.064$). Post-hoc effects revealed a synergistic effect, with WAY-161503 increasing time on top in animals exposed to CAS (p = 0.922, d = 0.242, non-treated controls vs. non-treated CAS; p = 0.907, 0.259, non-treated controls vs. WAY-161503 controls; p = 0.011, d = 0.9833, non-treated CAS vs. WAY-161503 CAS; Figure 5A).

No main effects of treatment ($F_{[1, 69]} = 1.95$, $p = 0.1671$, $\omega^2 = 0.012$) were found for time on bottom, but a main effect of drug was found ($F_{[1, 69]} = 4.107$, $p = 0.0466$, $\omega^2 = 0.039$). No significant interaction was observed ($F_{[1, 69]} = 3.267$, $p = 0.0751$, $\omega^2 = 0.029$). Post-hoc tests suggested a synergistic effect, with WAY-161503 decreasing time on bottom in animals exposed to CAS (p = 0.988, d = -0.1243, non-treated controls vs. non-treated CAS; p = 0.999, d = -0.0538, non-treated controls vs. WAY-161503 controls; p = 0.041, d = 0.8369, non-treated CAS vs. WAY-161503 CAS; Figure 5B).

A main effect of treatment ($F_{[1, 69]} = 16.119$, $p = 0.000149$, $\omega^2 = 0.174$) was found for erratic swimming, but no main effect of drug ($F_{[1, 69]} = 0.568$, $p = 0.454$, $\omega^2 = -0.005$) nor an interaction effect ($F[1, 69] = 0.042$, $p = 0.838$, $\omega^2 = -0.011$) were found. However, post-hoc tests did not find an increase in erratic swimming with CAS (p = 0.125, d = -0.857), nor a synergistic effect of WAY-161503 (p = 0.922, d = 0.243, non-treated controls vs. WAY-161503 controls; p = 0.968, d = 0.142, non-treated CAS vs. WAY-161503 CAS; Figure 5C).

A main effect of treatment ($F_{[1, 69]} = 4.726$, $p = 0.0331$, $\omega^2 = 0.43$) was found for freezing, and a medium-sized main effect of drug was also found ($F_{[1, 69]} = 4.17$, $p = 0.04497$, $\omega^2 = 0.037$). An interaction effect was observed for freezing as well ($F_{[1, 69]} = 7.663$, $p = 0.00723$, $\omega^2 = 0.077$).

Post-hoc tests revealed that CAS increased freezing ($p = 0.009$, $d = -1.257$, non-treated controls vs. non-treated CAS), while WAY-161503 blocked this effect ($p = 0.816$, $d = -0.338$, non-treated controls vs. WAY-161503 controls; $p = 0.008$, $d = 1.026$, non-treated CAS vs. WAY-161-503 CAS; Figure 5D).

Finally, no main effects of treatment ($F_{[1, 69]} = 3.85$, $p = 0.0538$, $\omega^2 = 0.038$) or drug ($F_{[1, 69]} = 0.416$, $p = 0.5212$, $\omega^2 = -0.008$) were found for swimming speed, and an interaction effect was also absent ($F[1, 69] = 0.062$, $p = 0.8037$, $\omega^2 = -0.013$)(Figure 5E).

3.3. Effects of RS-102221 on alarm reaction and post-exposure behavior

3.3.1. During exposure

One outlier was detected in the group exposed to CAS and injected with vehicle. A main effect of treatment ($F_{[1, 68]} = 8.89$, $p = 0.004$, $\omega^2 = 0.101$) was found for time on top, but no main effect of drug ($F_{[1, 68]} = 0.166$, $p = 0.685$, $\omega^2 = -0.011$) nor an interaction effect ($F_{[1, 68]} = 0.096$, $p = 0.757$, $\omega^2 = -0.012$) were found for this variable. Post-hoc tests suggested that CAS decreased time on top ($p = 0.004$, $d = 0.74$ for the main effect); RS-102221 was not able to change this effect ($p = 0.353$, $d = 0.64$, non-treated controls vs. treated CAS; $p = 0.941$, $d = -0.18$, non-treated CAS vs. treated CAS; Figure 6A).

A main effect of treatment ($F_{[1, 68]} = 80.6$, $p = 1.09 \times 10^{-13}$, $\omega^2 = 0.53$), but not dose ($F_{[1, 68]} = 0.189$, $p = 0.665$, $\omega^2 = -0.005$), nor an interaction effect ($F_{[1, 68]} = 0.517$, $p = 0.474$, $\omega^2 = -0.003$), were found for time on bottom (Figure 6B). Post-hoc tests suggested that CAS increased time on bottom ($p < 0.001$, $d = -2.22$ for the main effect), and RS-102221 was not

502 able to change this effect ($p < 0.001$, $d = -2.12$, non-treated controls vs. treated CAS; $p = 0.8$, d
503 $= 0.29$, non-treated CAS vs. treated CAS).

504 A main effect of treatment ($F_{[1, 68]} = 36.85$, $p < 0.0001$, $\omega^2 = 0.33$) was found for erratic
505 swimming, but no main effect of drug was found ($F_{[1, 68]} = 2.86$, $p = 0.095$, $\omega^2 = 0.017$), nor was
506 an interaction effect found ($F_{[1, 68]} = 0.002$, $p = 0.965$, $\omega^2 = -0.009$). Post-hoc tests suggested
507 that CAS increased erratic swimming ($p < 0.01$, $d = -1.5$ for the main effect), an effect that was
508 not blocked or increased by RS-102221 ($p < 0.001$, $d = -1.92$, non-treated controls vs. treated
509 CAS; $p = 0.56$, $d = -0.41$, non-treated CAS vs. treated CAS; Figure 6C).

510 A main effect of treatment ($F_{[1, 68]} = 37.61$, $p < 0.0001$, $\omega^2 = 0.325$), but not drug ($F_{[1, 68]} =$
511 2.65, $p = 0.108$, $\omega^2 = 0.015$), was found for freezing; no interaction effects were found ($F_{[1, 68]} =$
512 3.23, $p = 0.077$, $\omega^2 = 0.020$). Post-hoc tests revealed that CAS increased freezing ($p < 0.001$, d
513 $= -1.52$ for the main effect), and RS-102221 partially blocked this effect ($p = 0.025$, $d = -1.12$,
514 non-treated controls vs. treated CAS; $p = 0.041$, $d = 0.85$, non-treated CAS vs. treated CAS;
515 Figure 6D).

516 Finally, no main effects of treatment ($F_{[1, 68]} = 1.80$, $p = 0.184$, $\omega^2 = 0.011$) nor drug ($F_{[1, 68]}$
517 $= 2.146$, $p = 0.148$, $\omega^2 = 0.016$) were found for swimming speed (Figure 6E). Interaction effects
518 were also absent ($F_{[1, 68]} = 0.475$, $p = 0.493$, $\omega^2 = -0.007$).

519

520 3.3.2. After exposure

521 No main effects of treatment ($F_{[1, 68]} = 1.615$, $p = 0.21$, $\omega^2 = 0.009$) or drug ($F_{[1, 68]} =$
522 0.032, $p = 0.858$, $\omega^2 = -0.013$), were found for time on top (Figure 7A); an interaction effect was
523 also absent ($F_{[1, 68]} = 1.152$, $p = 0.287$, $\omega^2 = 0.002$).

524 A main effect of treatment ($F_{[1, 68]} = 4.39$, $p = 0.04$, $\omega^2 = 0.035$), as well as a large effect
525 of drug ($F_{[1, 68]} = 22.7964$, $p < 0.0001$, $\omega^2 = 0.18$), were found for time on bottom (Figure 7B). An
526 interaction effect was also found ($F_{[1, 68]} = 4.08$, $p = 0.047$, $\omega^2 = 0.032$). Post-hoc tests suggested

527 that CAS increased time on bottom after exposure ($p = 0.05$, $d = 1.02$, non-treated controls vs.
528 non-treated CAS), an effect that was blocked by RS-102221 ($p = 0.506$, $d = 0.54$, non-treated
529 controls vs. treated CAS; $p < 0.001$, $d = 1.55$, non-treated CAS vs. treated CAS).

530 A main effect of treatment ($F_{[1, 68]} = 36.1896$, $p < 0.0001$, $\omega^2 = 0.325$), but not drug ($F_{[1, 68]}$
531 $= 2.9573$, $p = 0.09$, $\omega^2 = 0.018$), were found for erratic swimming (Figure 7C). No interaction
532 effect was found ($F_{[1, 68]} = 0.0019$, $p = 0.965$, $\omega^2 = -0.009$). Post-hoc tests suggested that CAS
533 increased erratic swimming after exposure ($p = 0.001$, $d = 1.514$, non-treated controls vs. non-
534 treated CAS), an effect that was not altered by RS-102221 ($p < 0.001$, $d = -1.92$, non-treated
535 controls vs. treated CAS; $p = 0.56$, $d = -0.41$, non-treated CAS vs. treated CAS).

536 A main effect of treatment ($F_{[1, 68]} = 39.7692$, $p < 0.0001$, $\omega^2 = 0.334$) and a main effect of
537 drug ($F_{[1, 68]} = 4.16$, $p = 0.045$, $\omega^2 = 0.027$), were found for freezing; no interaction effects were
538 found ($F_{[1, 68]} = 3.23$, $p = 0.077$, $\omega^2 = 0.019$). Post-hoc tests suggested that CAS increased
539 freezing after exposure ($p < 0.001$, $d = 1.96$, non-treated controls vs. non-treated CAS), an
540 effect that was partially blocked by RS-102221 ($p = 0.025$, $d = -1.12$, non-treated controls vs.
541 treated CAS; $p = 0.041$, $d = 0.85$; Figure 7D).

542 Finally, no main effects of treatment ($F_{[1, 68]} = 1.014$, $p = 0.317$, $\omega^2 = 0.0$) or drug ($F_{[1, 68]} =$
543 1.83, $p = 0.181$, $\omega^2 = 0.011$) were found for swimming speed, nor was there an interaction effect
544 for this variable ($F_{[1, 68]} = 0.475$, $p = 0.493$, $\omega^2 = -0.007$. Figure 7E).

545

546 3.4. Restraint stress-elicited behavioral changes

547 3.4.1. Effects of MK-212 on restraint stress-elicited behavioral changes

548 No main effects of treatment ($F_{[1, 23]} = 0.167$, $p = 0.687$, $\omega^2 = -0.026$) nor drug ($F_{[1, 23]} =$
549 3.884, $p = 0.061$, $\omega^2 = 0.089$), were found for time on top (Figure 8A). No interaction effects

550 were found as well ($F_{[1, 23]}$ = 4.215, p = 0.052, ω² = 0.1).

551 A main effect of treatment ($F_{[1, 23]}$ = 6.3864, p = 0.019, ω² = 0.099), but not drug ($F_{[1, 23]}$ =
552 0.0879, p = 0.77, ω² = -0.017), was found for time on bottom (Figure 8B). A large
553 treatment:drug interaction effect was found ($F_{[1, 23]}$ = 8.5023, p = 0.008, ω² = 0.22). Post-hoc
554 tests showed that ARS increased time on bottom (*p* = 0.038, *d* = -1.566 vs. non-treated
555 controls), and MK-212 (2 mg/kg) blocked this effect (*p* = 0.757, *d* = -0.495 non-treated controls
556 vs. treated ARS).

557 A main effect of treatment ($F_{[1, 23]}$ = 17.9732, p = 0.00031, ω² = 0.398), but not drug ($F_{1, 23]}$
558 = 0.446, p = 0.511, ω² = -0.013), was found for erratic swimming (Figure 8C). No interaction
559 effects were found ($F_{[1, 23]}$ = 0.193, p = 0.664, ω² = -0.019). Post-hoc tests suggested that a ARS
560 increased erratic swimming (p < 0.001, d = -1.57 for the main effect), an effect that was not
561 blocked by MK-212 (*p* = 0.006, d = -1.85, non-treated controls vs. treated ARS).

562 No main effects of treatment ($F_{[1, 23]}$ = 0.384, p = 0.541, ω² = -0.016) nor drug ($F_{[1, 23]}$ =
563 0.618, p = 0.44, ω² = -0.01) were found for freezing (Figure 8D). An interaction effect was found
564 ($F_{[1, 23]}$ = 12.339, p = 0.002, ω² = 0.304). Post-hoc tests suggested that animals injected with MK-
565 212 and subjected to ARS showed decreased freezing in relation to other groups (p = 0.033, d =
566 -1.6, non-treated controls vs. treated ARS; p = 0.031, d = 1.6, non-treated ARS vs. treated
567 ARS).

568 Finally, no main effects of treatment ($F_{[1, 23]}$ = 1.0005, p = 0.328, ω² = 0.0) nor drug ($F_{[1, 23]}$
569 = 0.0283, p = 0.868, ω² = -0.038) were found for swimming speed (Figure 8E). No interaction
570 effect was found for this variable ($F_{[1, 23]}$ = 0.5437, p = 0.468, ω² = -0.018).

571

572 ### 3.4.2. Effects of RS-102221 on restraint stress-elicited behavioral changes

573 A main effect of treatment ($F_{[1, 28]}$ = 422.613, $p < 0.0001$, ω^2 = 0.93), but not drug ($F_{[1, 28]}$ =
574 0.728, p = 0.401, ω^2 = -0.009), was found for time on top (Figure 9A). No interaction effect was
575 found ($F_{[1, 28]}$ = 0.416, p = 0.524, ω^2 = -0.02). ARS increased time on top ($p < 0.0001$, d = 7.78 for
576 the main effect), and RS-102221 was not able to block this effect.

577 No main effects of treatment ($F_{[1, 28]}$ = 3.051, p = 0.0916, ω^2 = 0.06) nor drug ($F_{[1, 28]}$ =
578 2.208, p = 0.1485, ω^2 = 0.04) were found for time on bottom (Figure 9B). However, a significant
579 interaction effect was found ($F_{[1, 28]}$ = 10.832, p = 0.0027, ω^2 = 0.24). Post-hoc tests revealed
580 that ARS increased time on bottom (p = 0.007 vs. non-treated controls), an effect that was
581 partially blocked by RS-102221 (p = 0.997, non-treated controls vs. treated ARS; p = 0.019,
582 non-treated ARS vs. treated ARS).

583 No main effects of treatment ($F_{[1, 28]}$ = 0.014, p = 0.905, ω^2 = -0.03) nor drug ($F_{[1, 28]}$ =
584 0.224, p = 0.639, ω^2 = -0.02), were found for erratic swimming (Figure 9C). An interaction effect
585 was also absent ($F_{[1, 28]}$ = 2.916, p = 0.098, ω^2 = 0.06).

586 A main effect of treatment ($F_{[1, 28]}$ = 13.564, p = 0.001, ω^2 = 0.28) was found for freezing
587 (Figure 9D), but no main effect of drug was found ($F_{[1, 28]}$ = 0.122, p = 0.729, ω^2 = -0.03).
588 Moreover, an interaction effect was also absent ($F_{[1, 28]}$ = 0.022, p = 0.882, ω^2 = -0.03). ARS
589 increased freezing (p = 0.001, d = 1.4 for the main effect), an effect that was not blocked by RS-
590 102221 (p = 0.052, non-treated controls vs. treated ARS; p = 0.998, non-treated ARS vs.
591 treated ARS).

592 Finally, no main effects of treatment ($F_{[1, 28]}$ = 1.833, p = 0.187, ω^2 = 0.03) nor drug ($F_{[1, 28]}$
593 = 0.003, p = 0.955, ω^2 = -0.03), neither an interaction effect ($F_{[1, 28]}$ = 0.051, p = 0.823, ω^2 = -
594 0.03), were found for swimming speed (Figure 9E).

4. Discussion

The present work tested the hypothesis that phasic activation of the 5-HT_{2C} receptor is involved in behavioral adjustments to distal, but not proximal, threat in zebrafish, and that these receptors exerts a tonic facilitation of defensive behavior to potential threat in zebrafish. We found that the 5-HT_{2C} agonist MK-212 blocked CAS-elicited defensive behavior (bottom-dwelling, erratic swimiming, freezing) at the highest dose (2 mg/kg), with limited effects at the lowest dose (1 mg/kg); MK-212 did not block post-exposure increases in defensive behavior. The participation of the 5-HT_{2C} receptor on this effect was confirmed by treating animals with another agonist, WAY-161503, which had similar effects. MK-212 (2 mg/kg) did not change ARS-elicited anxiogenic-like effects. We also found that RS-102221, a 5-HT_{2C} receptor antagonist, did not change behavior during exposure, but it produced a small effect on behavior after exposure to CAS. No effects of RS-102221 were found for ARS-elicited anxiogenic-like effects.

4.1. Effects of stressors on zebrafish behavior

One of the aims of this paper was to confirm the behavioral effects of CAS during and after exposure (Lima-Maximino et al., 2020), given the considerable variation in the literature (Maximino et al., 2019), and to compare the effects after exposure with those of ARS - considering that, while both CAS and ARS are anxiogenic stressors, from an ecological point of view both should affect different behavioral endpoints.

In the present experiments, CAS consistently increased bottom-dwelling and erratic swimming during exposure, with a smaller component of freezing. After exposure, a strong

617 component of freezing was present, while erratic swimming contributed less to the overall
618 behavioral pattern. These results are consistent with what was observed both with a washout
619 period (Lima-Maximino et al., 2020), and in the absence of a washout, but with an extended
620 observation interval (Mathuru et al., 2012; Nathan et al., 2015). These effects suggest that, as
621 threat levels change from distal (i.e., CAS is present) to potential (i.e., CAS is no longer
622 present), a “residual” effect emerges that is marked by different behavioral components. This is
623 similar to what is observed with electrical stimulation of the PAG/GR in rats (Brandão et al.,
624 2008). We have previously shown that serotonin differentially mediates these behaviors,
625 phasically inhibiting responses during exposure and tonically facilitating responses after
626 exposure (Lima-Maximino et al., 2020).
627 ARS-elicited behavioral effects have been explored in the literature with mixed results.
628 While Ghisleni et al. (2012) found that a 90-min restraint protocol did not change bottom-
629 dwelling after stress when animals were tested individually, a similar protocol showed marked
630 increases in this variable (Assad et al., 2020) - albeit control animals in the latter experiment
631 spent most of the session in the upper half of the tank instead of in the lower half. In the present
632 experiments, ARS increased bottom-dwelling as well. Moreover, we observed a small
633 component of erratic swimming, consistent with what is observed by Ghisleni et al. (2012).
634 While increased bottom-dwelling was also observed as a pattern of post-exposure behavior
635 after CAS in the present experiments and in Lima-Maximino et al. (2020), freezing was a major
636 component of post-exposure behavior, suggesting that stressors do not produce similar
637 behavioral effects.
638

4.2. Role of the 5-HT_{2C} receptor in CAS-elicited behavioral adjustments

Both 5-HT_{2C} receptor agonists were able to block CAS-elicited behavioral adjustments *during* exposure, but the effects on behavior after exposure were less impressive, with agonists blocking the increased geotaxis. While RS-102221, a 5-HT_{2C} receptor antagonist, did not change behavior during exposure, it blocked some of the effects on post-exposure behavior. These results suggest that the 5-HT_{2C} receptor has opposite roles in both stages, phasically inhibiting defensive responses to proximal threat and tonically facilitating responses to potential threat.

We have previously found that serotonin participates in responses to CAS, acutely and phasically inhibiting responses to proximal threat but phasically facilitating responses to potential threat (Lima-Maximino et al., 2020; Maximino et al., 2014). We suggested that phasic and tonic serotonin encode an aversive expectation value, switching behavior toward cautious exploration/risk assessment/anxiety when the aversive stimulus is no longer present. This model is an expansion of the Deakin/Graeff theory of the serotonergic modulation of defensive behavior (e.g., Deakin & Graeff, 1991), which was adopted in previous work from our group (e.g., Maximino et al., 2013, 2014). In its simplest version, this theory states that serotonin facilitates behavior elicited by potential threat (i.e., anxiety-like behavior) by acting in forebrain structures such as the amygdala and the septo-hippocampal system, and inhibits behavior elicited by distal and proximal threat (i.e., fear-like behavior) by acting in midbrain structures such as the periaqueductal gray area. In the experimental design that was used in this experiment, behavior during exposure represents proximal threat, as CAS acts as a partial predator stimulus that elicits behavior that decreases the possibility of a predator attack or

662 detection by the predator (Smith, 1992). However, if CAS is no longer present (as in the post-
663 exposure stage of our design), that would signal a decrease in threat levels to potential threat, a
664 situation in which trying to flee or hide is non-adaptive, but resuming normal behavior is also
665 non-adaptive - and therefore cautious, alert exploration is warranted (Maximino et al., 2019).
666 Using Fanselow's taxonomy, the situation in which CAS is present is more akin to "fear", while
667 the situation in which it is no longer present is more akin to "anxiety" (Perusini and Fanselow,
668 2015).

669 The rodent literature suggests that 5-HT_2 receptors participate in fear and anxiety.
670 Salchner and Singewald (2006) have shown that MK-212 potentiates escape responses to an
671 airjet in rats, and the antagonist SB-242084 has been shown to produce anxiolytic-like effects in
672 rats (Kennett et al., 1997; Martin et al., 2002). In the elevated T-maze, an apparatus which tests
673 anxiety-like behavior (inhibitory avoidance) and fear-like behavior (one-way escape), 5-HT_{2C}
674 agonists facilitate, and antagonists impair, inhibitory avoidance, but only agonists facilitate one-
675 way escape (Mora et al., 1997), suggesting tonic facilitation of responses to potential threat and
676 phasic inhibition of responses to distal or proximal threat. Thus, a conserved role for this
677 receptor appears to be related to phasic and tonic modulation of defensive responses.

678 A role for 5-HT_2 receptors has also been suggested by pharmacological experiments in
679 fish as well. In Nile tilapia the 5-$HT_{2A/2C}$ and α_2-adrenoceptor antagonist mianserin blocked
680 active components of the alarm reaction (dashing, bristling of dorsal fin spines), but not freezing,
681 during exposure (Barreto, 2012). In zebrafish, the 5-$HT_{2A/2B/2C}$ receptor antagonist/5-HT1A
682 receptor agonist methysergide increased freezing and bottom-dwelling during and after
683 exposure at a high dose (92.79 mg/kg), but not at lower doses (Nathan et al., 2015). Our results
684 are consistent with a major component of 5-HT_{2C} receptors in these responses, although we did
685 not find an effect of the antagonist during exposure. Thus, our results point to a specific role of
686 the 5-HT2C receptor in phasic (inhibitory) control of defensive responses to proximal threat and

687 tonic (stimulatory) control of responses to potential threat.

688 We have previously shown that, after exposure to CAS, serotonin levels are increased in
689 the extracellular fluid of the zebrafish brain (Maximino et al., 2014), an effect that was mediated
690 by both serotonin transporters (Maximino et al., 2014) and monoamine oxidase activity
691 (Maximino et al., 2019; Quadros et al., 2018). This increase in serotonergic activity could
692 activate 5-HT_{2C} and other serotonergic receptors to inhibit ongoing defensive responses to
693 proximal threat and initiate programs of alertness and cautious exploration/risk assessment.
694 This phasic signal represents prediction errors (Amo et al., 2014; Lima-Maximino et al., 2020),
695 while the tonic activity, which facilitates responses to potential threat, has been proposed to
696 represent a negative expectation value (Amo et al., 2014; Lima-Maximino et al., 2020).
697 However, at least for the case of the 5-HT_{2C} receptor, the phasic signals do not appear to
698 represent all aversive values, as no effect of MK-212 was found for ARS-elicited responses. Our
699 results suggest that the 5-HT_{2C} receptor is at least partially responsible for both effects, acting
700 as a “switch” between the two behavioral modes.
701

702 4.3. Limitations and translational potential

703 While the present work presents results on zebrafish behavioral pharmacology inspired
704 on results from mammals, which can potentially underscore conserved mechanisms for
705 responses to threat across vertebrates (cf. Gerlai, 2014, for a discussion on how using fish as
706 well as mammals can decrease uncertainty regarding translational relevance and evolutionary
707 conservation of mechanisms), there are limitations in using zebrafish as experimental models in
708 neuroscience. One of them is that, due to the teleost genome duplication event, zebrafish
709 present two copies of the gene that codes the 5-HT_{2C} receptor (Schneider et al., 2012), and
710 therefore it is currently not possible to know which protein (HTR2CL1 or HTR2Cl2) was targeted

711 by the drugs used in the present experiments. Moreover, while we propose that alarm reactions
712 and post-exposure behavior can be exploited to better understand panic disorder (Silva et al.,
713 2020), the paradigm that was used represents the normal, adaptive behavior of zebrafish under
714 threatening circumstances, and therefore cannot yet be used to generate novel hypotheses
715 about a pathological organism (cf. Maximino and van der Staay, 2019, for a discussion on the
716 limitations of using behavioral assays in experimental psychopathology).

717 Another important limitation of our results is that sex differences were not considered
718 throughout the experiment. The field of sex differences in zebrafish research is barely emerging
719 (Genario et al., 2020), due to difficulties in ascertaining sex only through external morphology in
720 common (commercial) zebrafish phenotypes; as a result, we did not separate animals per sex in
721 our experiments. Future experiments will consider the role of sex in these responses,
722 considering that sex differences were observed in fear-like behavior in rodents (e.g., Petrovich
723 and Lougee, 2011).

724 Despite these limitations, the work presented has translational potential in understanding
725 fear, anxiety, and panic states (cf. Silva et al., 2020, for a discussion on the use of alarm
726 reactions to study fear in zebrafish). In general, our results are the first to determine a specific
727 role of 5-HT_{2C} receptors in zebrafish behavior, and add to the small literature on the role of this
728 receptor in mammals as well. Further work is needed to understand whether this receptor
729 interacts with other serotonin receptors known to be involved in defensive behavior in this
730 species (Herculano and Maximino, 2014) and if the effects during exposure are related to
731 changes in serotonin levels. Moreover, given the importance of these phenotypes to
732 understanding fear and panic states (Silva et al., 2020), further work will clarify the usefulness of
733 this pharmacological profile in modeling panic disorder and anxiety.

734

735

Significance statement

While the neurotransmitter serotonin has been associated with mental disorders, we currently do not know the mechanisms through which serotonin influences responses to threat. This is relevant to future studies on anxiety disorders, for example. Here we report that, in zebrafish, the 5-HT_{2C} receptor “pushes the break” on (fear-like) responses to threat that is actually present, and keeps on facilitating (anxiety-like) responses to threat that is only implied. Thus, this receptor appears to act as a “switch” between two modes that facilitate escape (“fear”) or cautious exploration (“anxiety”).

Acknowledgments

This work was supported by a Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) grant (#400726/2016-5). CM is the recipient of a CNPq productivity grant (PQ2, #302998/2019-5). The funders had no influence on the study design, the collection, analysis and interpretation of data, as well as writing and submission of this manuscript.

Conflict of interests statement

The authors declare no conflict of interest.

Authors' contributions

Rhayra Xavier do Carmo Silva: Conceptualization, Methodology, Investigation, Data curation, Formal analysis, Visualization, Writing – original draft, Writing – review & editing; *Bianca Gomes do Nascimento:* Investigation, Data curation, Formal analysis; *Gabriela Cristini Vidal Gomes:* Investigation, Data curation, Formal analysis; *Nadyme Assad Holanda da Silva:* Investigation, Data curation; *Jéssica Souza Pinheiro:* Investigation, Data curation; *Suianny Nayara da Silva Chaves:* Investigation, Data curation, Formal analysis, Visualization; *Ana Flávia Nogueira Pimentel:* Investigation, Data curation, Formal analysis; *Bruna Patrícia Dutra Costa:* Investigation, Data curation, Formal analysis; *Anderson Manoel Herculano:* Conceptualization, Supervision, Resources, Validation; *Monica Lima-Maximino:* Conceptualization, Validation, Writing – original draft; *Caio Maximino:* Conceptualization, Formal analysis, Funding acquisition, Methodology, Project Administration, Resources, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing.

Data accessibility

Data and analysis scripts for this work can be found at a GitHub repository (https://github.com/lanec-unifesspa/5-HT-CAS/tree/master/data/5HT2C/; doi: 10.5281/zenodo.4437275).

References

Alves, S.H., Pinheiro, G., Motta, V., Landeira-Fernandez, J., Cruz, A.P.M., 2004. Anxiogenic effects in the rat elevated plus-maze of 5-HT2C agonists into ventral but not dorsal hippocampus. Behav. Pharmacol. 15, 37–43.

Amo, R., Fredes, F., Kinoshita, M., Aoki, R., Aizawa, H., Agetsuma, M., Aoki, T., Shiraki, T., Kakinuma, H., Matsuda, M., Yamazaki, M., Takahoko, M., Tsuboi, T., Higashijima, S., Miyasaka, N., Koide, T., Yabuki, Y., Yoshihara, Y., Fukai, T., Okamoto, H., 2014. The

Habenulo-Raphe Serotonergic Circuit Encodes an Aversive Expectation Value Essential for Adaptive Active Avoidance of Danger. Neuron 84, 1034–1048. https://doi.org/10.1016/j.neuron.2014.10.035

Assad, N., Luz, W.L., Santos-Silva, M., Carvalho, T., Moraes, S., Picanço-Diniz, D.L.W., Bahia, C.P., Oliveira Batista, E. de J., da Conceição Passos, A., Oliveira, K.R.H.M., Herculano, A.M., 2020. Acute Restraint Stress Evokes Anxiety-Like Behavior Mediated by Telencephalic Inactivation and GabAergic Dysfunction in Zebrafish Brains. Sci. Rep. 10, 5551. https://doi.org/10.1038/s41598-020-62077-w

Barreto, R.E., 2012. Mianserin affects alarm reaction to conspecific chemical alarm cues in Nile tilapia. Fish Physiol. Biochem. 43, 193–201. https://doi.org/10.1007/s10695-016-0279-2

Bencan, Z., Sledge, D., Levin, E.D., 2009. Buspirone, chlordiazepoxide and diazepam effects in a zebrafish model of anxiety. Pharmacol. Biochem. Behav. 94, 75–80. https://doi.org/10.1016/j.pbb.2009.07.009

Bonapersona, V., Hoijtink, H., RELACS, Sarabdjitsingh, R.A., Joëls, M., 2019. RePAIR: a power solution to animal experimentation. bioRxiv 864652. https://doi.org/10.1101/864652

Bonhaus, D.W., Weinhardt, K.K., Taylor, M., DeSouza, A., McNeeley, P.M., Szczepanski, K., Fontana, D.J., Trinh, J., Rocha, C.L., Dawson, M.W., Flippin, L.A., Eglen, R.M., 1997. RS-102221: a novel high affinity and selective, 5-HT2C receptor antagonist. Neuropharmacology 36, 621–629. https://doi.org/10.1016/s0028-3908(97)00049-x

Brandão, M.L., Zanoveli, J.M., Ruiz-Martinez, R.C., Oliveira, L.C., Landeira-Fernandez, J., 2008. Different patterns of freezing behavior organized in the periaqueductal gray of rats: Association with different types of anxiety. Behav. Brain Res. 188, 1–13. https://doi.org/10.1016/j.bbr.2007.10.018

Castilho, V., Macedo, C., Brandão, M., 2002. Role of benzodiazepine and serotonergic mechanisms in conditioned freezing and antinociception using electrical stimulation of the dorsal periaqueductal gray as unconditioned stimulus in rats. Psychopharmacology (Berl.) 165, 77–85. https://doi.org/10.1007/s00213-002-1246-4

Castilho, V.M., Brandão, M.L., 2001. Conditioned antinociception and freezing using electrical stimulation of the dorsal periaqueductal gray or inferior colliculus as unconditioned stimulus are differentially regulated by 5-HT2A receptors in rats. Psychopharmacology (Berl.) 155, 154–162. https://doi.org/10.1007/s002130100697

Coimbra, N.C., Brandão, M.L., 1997. Effects of 5-HT2 receptors blockade on fear-induced analgesia elicited by electrical stimulation of the deep layers of the superior colliculus

and dorsal periaqueductal gray. Behav. Brain Res. 87, 97–103. https://doi.org/10.1016/S0166-4328(96)02267-X

Conselho Nacional de Controle de Experimentação Animal - CONCEA, 2017. Diretriz brasileira para o cuidado e a utilização de animais para fins científicos e didáticos - DBCA. Anexo I. Peixes mantidos em instalações de instituições de ensino ou pesquisa científica, Resolução Normativa CONCEA nº 34.

Cornélio, A.M., Nunes-de-Souza, R.L., 2007. Anxiogenic-like effects of mCPP microinfusions into the amygdala (but not dorsal or ventral hippocampus) in mice exposed to elevated plus-maze. Behav. Brain Res. 178, 82–89. https://doi.org/10.1016/j.bbr.2006.12.003

Daw, N.D., Kakade, S., Dayan, P., 2002. Opponent interactions between serotonin and dopamine. Neural Netw. 15, 603–616. https://doi.org/10.1016/S0893-6080(02)00052-7

Deakin, J. F., Graeff, F. G. (1991). 5-HT and mechanisms of defence. J Psychopharmacol 5, 305-315. https://doi.org/10.1177/026988119100500414

de Mello Cruz, A.P., Pinheiro, G., Alves, S.H., Ferreira, G., Mendes, M., Faria, L., Macedo, C.E., Landeira-Fernandez, J., 2005. Behavioral effects of systemically administered MK-212 are prevented by ritanserin microinfusion into the basolateral amygdala of rats exposed to the elevated plus-maze. Psychopharmacology (Berl.) 182, 345–354. https://doi.org/10.1007/s00213-005-0108-2

de Paula, B.B., Leite-Panissi, C.R.A., 2016. Distinct effect of 5-HT1A and 5-HT2A receptors in the medial nucleus of the amygdala on tonic immobility behavior. Brain Res. 1643, 152–158. https://doi.org/10.1016/j.brainres.2016.04.073

do Carmo Silva, R.X., Lima-Maximino, M.G., Maximino, C., 2018a. The aversive brain system of teleosts: Implications for neuroscience and biological psychiatry. Neurosci. Biobehav. Rev. 95, 123–135. https://doi.org/10.1016/j.neubiorev.2018.10.001

do Carmo Silva, R.X., Rocha, S.P., Lima-Maximino, M.G., Maximino, C., 2018b. Extraction of alarm substance in zebrafish. protocols.io. https://doi.org/10.17504/protocols.io.tr3em8n

Genario, R., Abreu, M. S., Giacomini, A. C. V. V., Demin, K. A., Kalueff, A. V. Sex differences in behavior and neuropharmacology of zebrafish. Eur. J. Neurosci. 51, 2586– 2603. https://doi.org/10.1111/ejn.14438

Gerlai. R., 2014. Fish in behavior research: Unique tools with a great promise! J. Neurosci. Methods 234, 54-58. https://doi.or/g10.1016/j.jneumeth.2014.04.015

Ghisleni, G., Capiotti, K.M., Da Silva, R.S., Oses, J.P., Piato, Â.L., Soares, V., Bogo, M.R., Bonan, C.D., 2012. The role of CRH in behavioral responses to acute restraint stress in

844 zebrafish. Prog. Neuropsychopharmacol. Biol. Psychiatry 36, 176–182.
845 https://doi.org/10.1016/j.pnpbp.2011.08.016
846 Gomes, F., Greidinger, M., Salviano, M., Couto, K.C., Scaperlli, G.F., Alves, S.H. de S., Cruz,
847 A.P. de M., 2010. Antidepressant- and anxiogenic-like effects of acute 5-HT2C receptor
848 activation in rats exposed to the forced swim test and elevated plus maze. Psychol. &
849 Neurosci. 3, 245–249. https://doi.org/10.3922/j.psns.2010.2.014
850 Goodwin, N., Karp, N. A., Blackledge, S., Clark, B., Keeble, R., Kovacs, C., Murray, K. N., Price,
851 M., Thompson, P., Bussell J., 2016. Standardized welfare terms for the zebrafish
852 community. Zebrafish 13, Suppl. 1, S164-S168. https://doi.org/10.1089/zeb.2016.1248
853 Graeff, F.G., 2004. Serotonin, the periaqueductal gray and panic. Neurosci. Biobehav. Rev. 28,
854 239–259. https://doi.org/10.1016/j.neubiorev.2003.12.004
855 Graeff, F.G., Brandão, M.L., Audi, E.A., Schütz, M.T.B., 1986. Modulation of the brain aversive
856 system by GABAergic and serotonergic mechanisms. Behav. Brain Res. 22, 173–180.
857 https://doi.org/10.1016/0166-4328(86)90038-0
858 Herculano, A.M., Maximino, C., 2014. Serotonergic modulation of zebrafish behavior: Towards a
859 paradox. Prog. Neuropsychopharmacol. Biol. Psychiatry, Special Issue: Zebrafish
860 models of brain disorders 55, 50–66. https://doi.org/10.1016/j.pnpbp.2014.03.008
861 Höglund, E., Weltzien, F.-A., Schjolden, J., Winberg, S., Ursin, H., Døving, K.B., 2005.
862 Avoidance behavior and brain monoamines in fish. Brain Res. 1032, 104–110.
863 https://doi.org/10.1016/j.brainres.2004.10.050
864 Kennett, G.A., Wood, M.D., Bright, F., Trail, B., Riley, G., Holland, V., Avenell, K.Y., Stean, T.,
865 Upton, N., Bromidge, S., Forbes, I.T., Brown, A.M., Middlemiss, D.N., Blackburn, T.P.,
866 1997. SB 242084, a Selective and Brain Penetrant 5-HT2C Receptor Antagonist.
867 Neuropharmacology 36, 609–620. https://doi.org/10.1016/S0028-3908(97)00038-5
868 Kinkel, M.D., Eames, S.C., Philipson, L.H., Prince, V.E., 2010. Intraperitoneal Injection into
869 Adult Zebrafish. JoVE J. Vis. Exp. e2126. https://doi.org/10.3791/2126
870 Knight, A.R., Misra, A., Quirk, K., Benwell, K., Revell, D., Kennett, G., Bickerdike, M., 2004.
871 Pharmacological characterisation of the agonist radioligand binding site of 5-HT2A, 5-
872 HT2B and 5-HT2C receptors. Naunyn. Schmiedebergs Arch. Pharmacol. 370, 114–123.
873 https://doi.org/10.1007/s00210-004-0951-4
874 Kuznetsova, E.G., Amstislavskaya, T.G., Shefer, E.A., Popova, N.K., 2006. Effect of 5-HT2C
875 receptor antagonist RS 102221 on mouse behavior. Bull. Exp. Biol. Med. 142, 76–79.
876 https://doi.org/10.1007/s10517-006-0296-8

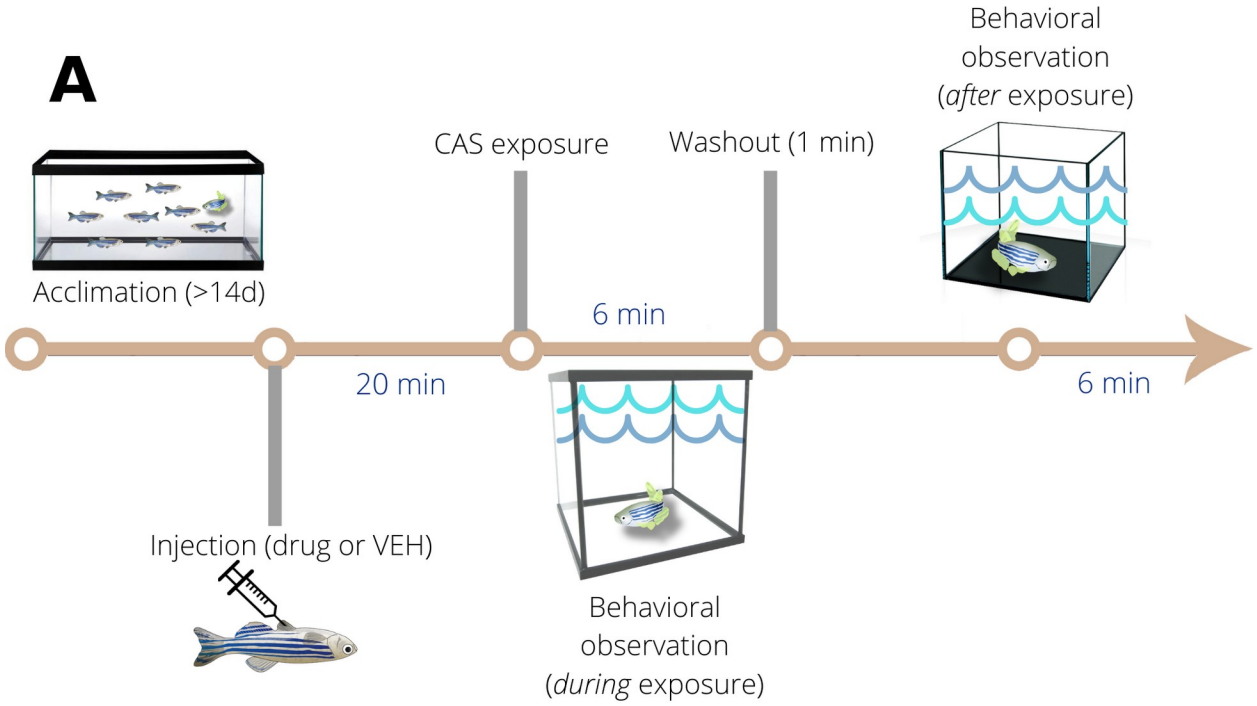
877 Lima-Maximino, M., Pyterson, M.P., Silva, R.X. do C., Gomes, G.C.V., Rocha, S.P., Herculano,
878 A.M., Rosemberg, D.B., Maximino, C., 2020. Phasic and tonic serotonin modulate alarm
879 reactions and post-exposure behavior in zebrafish. J. Neurochem. 153, 495–509. https://
880 doi.org/10.1111/jnc.14978
881 Macedo, C.E., Martinez, R.C.R., Albrechet-Souza, L., Molina, V.A., Brandão, M.L., 2007. 5-
882 HT2- and D1-mechanisms of the basolateral nucleus of the amygdala enhance
883 conditioned fear and impair unconditioned fear. Behav. Brain Res. 177, 100–108. https://
884 doi.org/10.1016/j.bbr.2006.10.031
885 Martin, J.R., Ballard, T.M., Higgins, G.A., 2002. Influence of the 5-HT2C receptor antagonist,
886 SB-242084, in tests of anxiety. Pharmacol. Biochem. Behav., Serotonin: Pharmacology,
887 Biochemistry & Behaviour 71, 615–625. https://doi.org/10.1016/S0091-3057(01)00713-4
888 Mathuru, A.S., Kibat, C., Cheong, W.F., Shui, G., Wenk, M.R., Friedrich, R.W., Jesuthasan, S.,
889 2012. Chondroitin Fragments Are Odorants that Trigger Fear Behavior in Fish. Curr.
890 Biol. 22, 538–544. https://doi.org/10.1016/j.cub.2012.01.061
891 Matthews, M., Varga, Z.M., 2012. Anesthesia and Euthanasia in Zebrafish. ILAR J. 53, 192–
892 204. https://doi.org/10.1093/ilar.53.2.192
893 Maximino, C., van der Staay, J., 2019. Behavioral models in psychopathology: Epistemic and
894 semantic considerations. Behav. Brain Funct. 15, 1. https://doi.org/10.1186/s12993-019-
895 0152-4
896 Maximino, C., do Carmo Silva, R.X., Campos, K. dos S., Oliveira, J.S. de, Rocha, S.P.,
897 Pyterson, M.P., Souza, D.P. dos S., Feitosa, L.M., Ikeda, S.R., Pimentel, A.F.N., Ramos,
898 P.N.F., Costa, B.P.D., Herculano, A.M., Rosemberg, D.B., Siqueira-Silva, D.H., Lima-
899 Maximino, M., 2019. Sensory ecology of ostariophysan alarm substances. J. Fish Biol.
900 95, 274–286. https://doi.org/10.1111/jfb.13844
901 Maximino, C., Lima, M.G., Costa, C.C., Guedes, I.M.L., Herculano, A.M., 2014. Fluoxetine and
902 WAY 100,635 dissociate increases in scototaxis and analgesia induced by conspecific
903 alarm substance in zebrafish (*Danio rerio* Hamilton 1822). Pharmacol. Biochem. Behav.
904 124, 425–433. https://doi.org/10.1016/j.pbb.2014.07.003
905 Maximino, C., Puty, B., Benzecry, R., Araújo, J., Lima, M. G., Batista, E. J. O., Oliveira, K. R.
906 M., Crespo-Lopez, M. E., Herculano, A. M., 2013. Role of serotonin in zebrafish (*Danio*
907 *rerio*) anxiety: relationship with serotonin levels and effect of buspirone, WAY 100635,
908 SB 224289, fluoxetine and para-chlorophenylalanine (pCPA) in two behavioral models.
909 Neuropharmacology 71, 83-97. https://doi.org/10.1016/j.neuropharm.2013.03.006

910 Mora, P.O., Netto, C.F., Graeff, F.G., 1997. Role of 5-HT2A and 5-HT2C Receptor Subtypes in
911 the Two Types of Fear Generated by the Elevated T-Maze. Pharmacol. Biochem.
912 Behav. 58, 1051–1057. https://doi.org/10.1016/S0091-3057(97)00057-9
913 Nathan, F.M., Ogawa, S., Parhar, I.S., 2015. Kisspeptin1 modulates odorant-evoked fear
914 response via two serotonin receptor subtypes (5-HT1A and 5-HT2) in zebrafish. J.
915 Neurochem. 133, 870–878. https://doi.org/10.1111/jnc.13105
916 Nunes-de-Souza, V., Nunes-de-Souza, R.L., Rodgers, R.J., Canto-de-Souza, A., 2008. 5-HT2
917 receptor activation in the midbrain periaqueductal grey (PAG) reduces anxiety-like
918 behaviour in mice. Behav. Brain Res. 187, 72–79.
919 https://doi.org/10.1016/j.bbr.2007.08.030
920 Oliveira, L.C., Broiz, A.C., de Macedo, C.E., Landeira-Fernandez, J., Brandão, M.L., 2007. 5-
921 HT2 receptor mechanisms of the dorsal periaqueductal gray in the conditioned and
922 unconditioned fear in rats. Psychopharmacology (Berl.) 191, 253–262.
923 https://doi.org/10.1007/s00213-006-0653-3
924 Parra, K.V., Adrian, J.C., Gerlai, R., 2009. The synthetic substance hypoxanthine 3-N-oxide
925 elicits alarm reactions in zebrafish (Danio rerio). Behav. Brain Res. 205, 336–341.
926 https://doi.org/10.1016/j.bbr.2009.06.037
927 Perusini, J.N., Fanselow, M.S., 2015. Neurobehavioral perspectives on the distinction between
928 fear and anxiety. Learn. Mem. 22, 417–425. https://doi.org/10.1101/lm.039180.115
929 Petrovich, G. D., Lougee, M. A., 2011. Sex differences in fear-induced feeding cessation:
930 Prolonged effect in female rats. Physiol. Behav. 104, 996-1101.
931 https://doi.org/10.1016/j.physbeh.2011.06.020
932 Piato, A.L., Rosemberg, D.B., Capiotti, K.M., Siebel, A.M., Herrmann, A.P., Ghisleni, G., Vianna,
933 M.R., Bogo, M.R., Lara, D.R., Bonan, C.D., 2011. Acute Restraint Stress in Zebrafish:
934 Behavioral Parameters and Purinergic Signaling. Neurochem. Res. 36, 1876.
935 https://doi.org/10.1007/s11064-011-0509-z
936 Quadros, V.A., Costa, F.V., Canzian, J., Nogueira, C.W., Rosemberg, D.B., 2018. Modulatory
937 role of conspecific alarm substance on aggression and brain monoamine oxidase activity
938 in two zebrafish populations. Prog. Neuropsychopharmacol. Biol. Psychiatry 86, 322–
939 330. https://doi.org/10.1016/j.pnpbp.2018.03.018
940 Quadros, V.A., Silveira, A., Giuliani, G.S., Didonet, F., Silveira, A.S., Nunes, M.E., Silva, T.O.,
941 Loro, V.L., Rosemberg, D.B., 2016. Strain- and context-dependent behavioural
942 responses of acute alarm substance exposure in zebrafish. Behav. Processes 122, 1–

943 11. https://doi.org/10.1016/j.beproc.2015.10.014
944 Rosenzweig-Lipson, S., Zhang, J., Mazandarani, H., Harrison, B.L., Sabb, A., Sabalski, J.,
945 Stack, G., Welmaker, G., Barrett, J.E., Dunlop, J., 2006. Antiobesity-like effects of the 5-
946 HT2C receptor agonist WAY-161503. Brain Res. 1073–1074, 240–251.
947 https://doi.org/10.1016/j.brainres.2005.12.052
948 Salchner, P., Singewald, N., 2006. 5-HT receptor subtypes involved in the anxiogenic-like action
949 and associated Fos response of acute fluoxetine treatment in rats. Psychopharmacology
950 (Berl.) 185, 282–288. https://doi.org/10.1007/s00213-005-0247-5
951 Schneider, H., Fritzky, L., Williams, J., Heumann, C., Yochum, M., Pattar, K. Noppert, G., Mock,
952 V., Hawley, E., 2012. Cloning and expression of a zebrafish 5-HT_{2C} receptor gene. Gene
953 502, 108-117. https://doi.org/10.1016/j.gene.2012.03.070
954 Silva, P.I.M., Martins, C.I.M., Khan, U.W., Gjøen, H.M., Øverli, Ø., Höglund, E., 2015. Stress
955 and fear responses in the teleost pallium. Physiol. Behav. 141, 17–22.
956 https://doi.org/10.1016/j.physbeh.2014.12.020
957 Silva, R.X. do C., Rocha, S.P., Herculano, A.M., Lima-Maximino, M.G., Maximino, C., 2020.
958 Animal models for panic disorder. Psychol. Neurosci. 13, 1–18.
959 https://doi.org/10.1037/pne0000177
960 Smith, R.J.F., 1992. Alarm signals in fishes. Rev. Fish Biol. Fish. 2, 33–63.
961 https://doi.org/10.1007/BF00042916
962 Sourbron, J., Schneider, H., Kecskés, A., Liu, Y., Buening, E.M., Lagae, L., Smolders, I., de
963 Witte, P., 2016. Serotonergic Modulation as Effective Treatment for Dravet Syndrome in
964 a Zebrafish Mutant Model. ACS Chem. Neurosci. 7, 588–598.
965 https://doi.org/10.1021/acschemneuro.5b00342
966 Speedie, N., Gerlai, R., 2008. Alarm substance induced behavioral responses in zebrafish
967 (Danio rerio). Behav. Brain Res. 188, 168–177. https://doi.org/10.1016/j.bbr.2007.10.031
968 von Frisch, K., 1938. Zur Psychologie des Fisch-Schwarmes. Naturwissenschaften 26, 601–
969 606. https://doi.org/10.1007/BF01590598
970 Wolf, K., 1963. Physiological Salines for Fresh-Water Teleosts. Progress. Fish-Cult. 25, 135–
971 140. https://doi.org/10.1577/1548-8659(1963)25[135:PSFFT]2.0.CO;2

Figure captions

Figure 1 – Experimental designs for (A) experiment 1 (CAS-elicited behavioral responses and post-exposure behavior) and (B) experiment 2 (ARS-elicited behavioral responses). *Abbreviations:* ARS – acute restraint stress; CAS – conspecific alarm substance; VEH – vehicle


A
Acclimation (>14d)
CAS exposure
Washout (1 min)
Behavioral observation (*after* exposure)
6 min
20 min
6 min
Injection (drug or VEH)
Behavioral observation (*during* exposure)

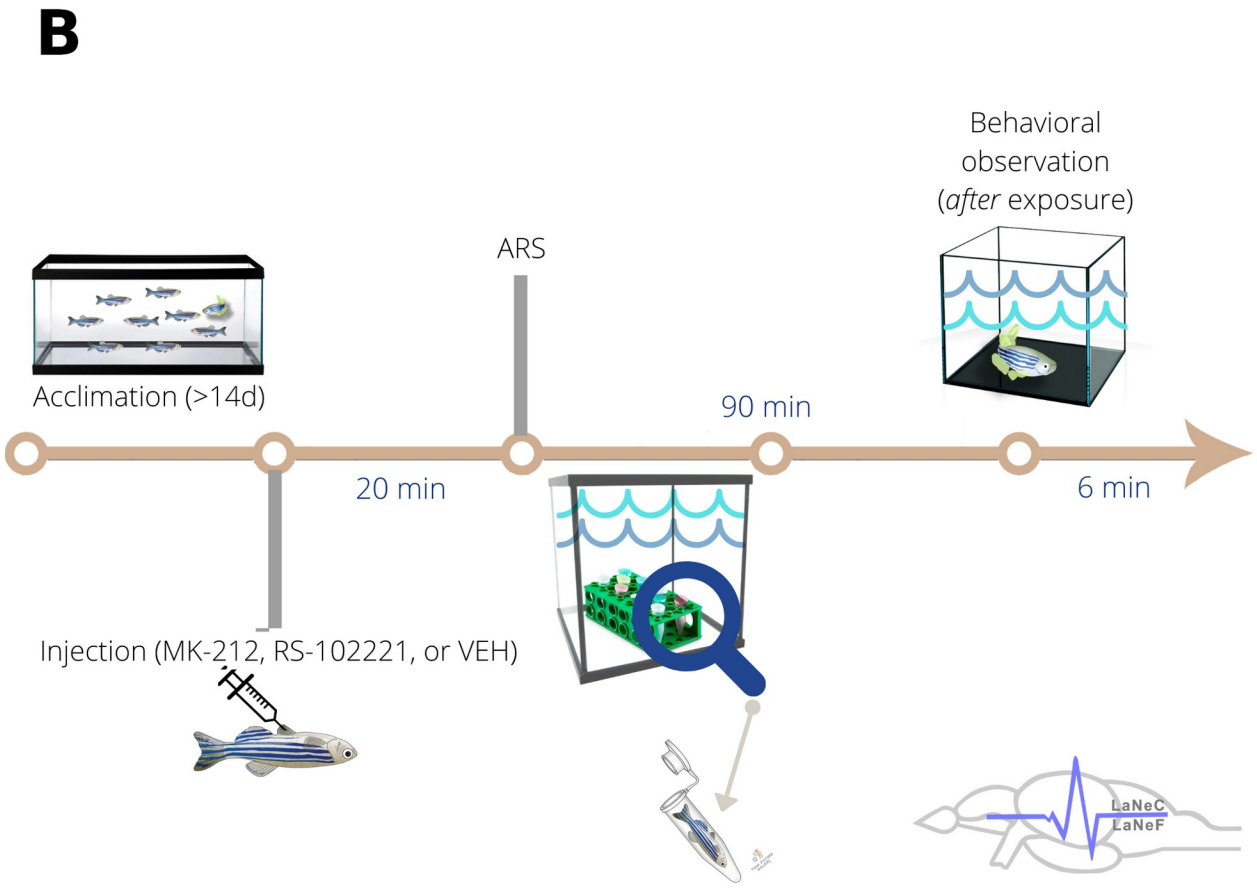

B
Acclimation (>14d)
ARS
Behavioral observation (*after* exposure)
90 min
20 min
6 min
Injection (MK-212, RS-102221, or VEH)
LaNeC
LaNeF

Figure 2 – Effects of MK-212 on behavior during CAS exposure. (A) Time spent on top third of the tank. (B) Time spent on bottom third of the tank. (C) Erratic swimming. (D) Total time spent freezing. (E) Swimming speed. Different letters represent statistical differences at the $p < 0.05$ level in pairwise comparisons with all other groups; similar letters indicate lack of statistically significant differences. Data are presented as individual data points (dots) superimposed over the median ± interquartile ranges. Dashed lines on panels A and B represent chance levels. Dots connected by lines represent group means. CTRL = controls (water-exposed animals); CAS = conspecific alarm substance. Final sample sizes: CTRL + VEH: n = 15 animals; CTRL + 1 mg/kg MK-212: n = 21 animals; CTRL + 2 mg/kg MK-212: n = 21 animals; CAS + VEH: n = 21 animals; CAS + 1 mg/kg MK-212: n = 21 animals; CAS + 2 mg/kg MK-212: n = 21 animals.

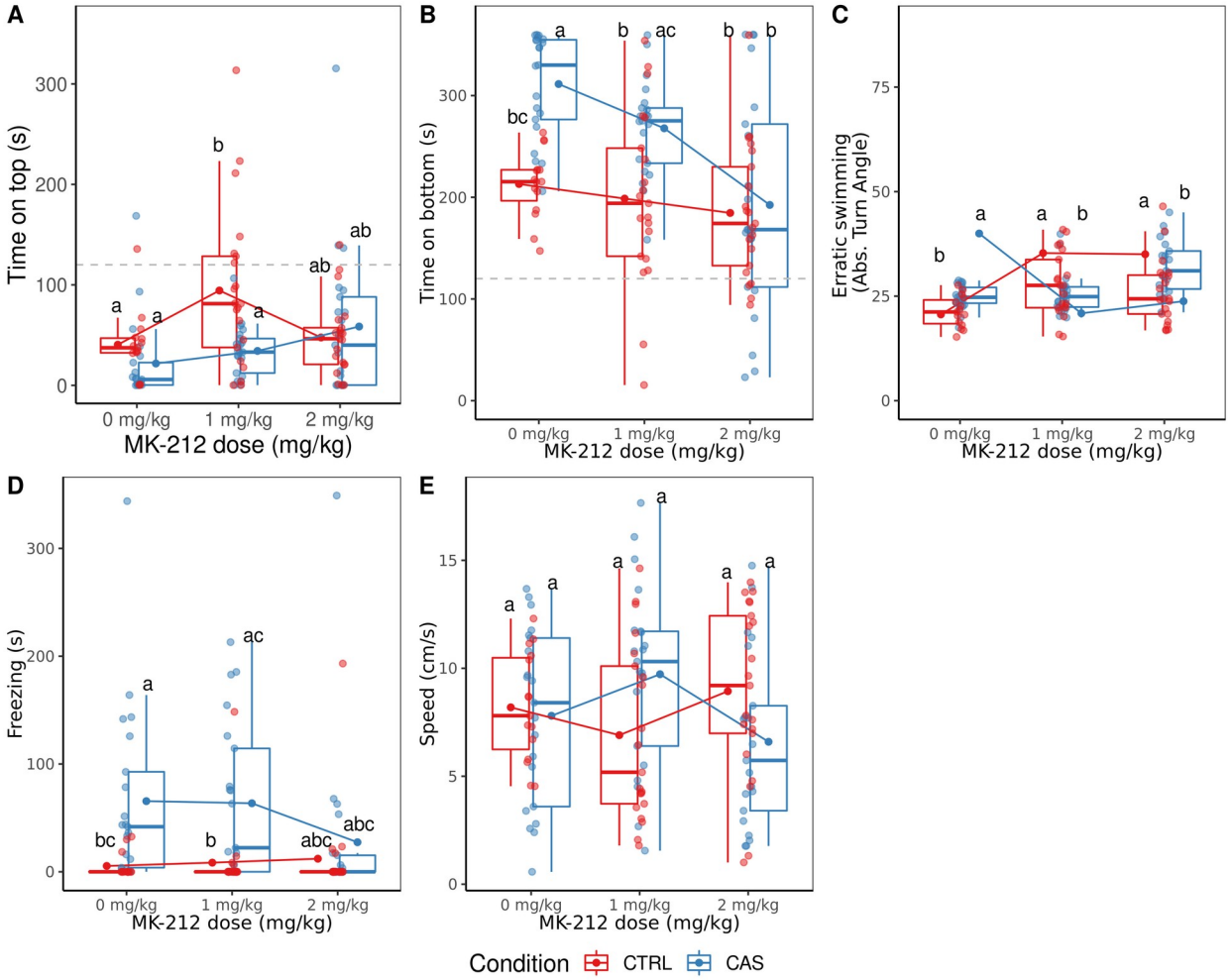


Figure 3 – Effects of MK-212 on behavior after CAS exposure. (A) Time spent on top third of the tank. (B) Time spent on bottom third of the tank. (C) Erratic swimming. (D) Total time spent freezing. (E) Swimming speed. Different letters represent statistical differences at the $p < 0.05$ level in pairwise comparisons with all other groups; similar letters indicate lack of statistically significant differences. Data are presented as individual data points (dots) superimposed over the median ± interquartile ranges. Dashed lines on panels A and B represent chance levels. Dots connected by lines represent group means. CTRL = controls (water-exposed animals); CAS = conspecific alarm substance. Final sample sizes: CTRL + VEH: n = 15 animals; CTRL + 1 mg/kg MK-212: n = 21 animals; CTRL + 2 mg/kg MK-212: n = 21 animals; CAS + VEH: n = 21 animals; CAS + 1 mg/kg MK-212: n = 21 animals; CAS + 2 mg/kg MK-212: n = 21 animals.

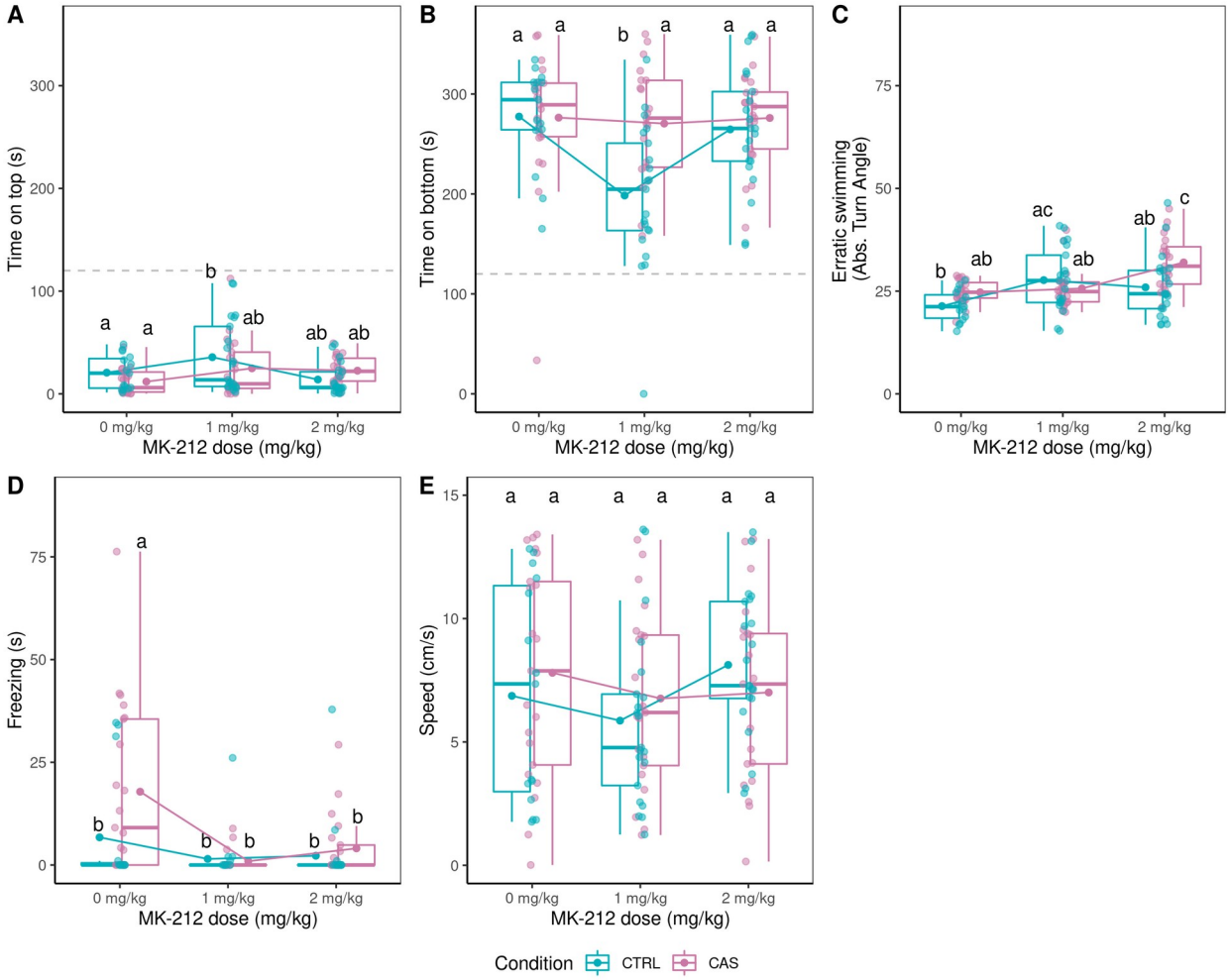


Figure 4 – Effects of WAY-161503 on behavior during CAS exposure. (A) Time spent on top third of the tank. (B) Time spent on bottom third of the tank. (C) Erratic swimming. (D) Total time spent freezing. (E) Swimming speed. Different letters represent statistical differences at the $p < 0.05$ level; in pairwise comparisons with all other groups similar letters indicate lack of statistically significant differences. Data are presented as individual data points (dots) superimposed over the median ± interquartile ranges. Dashed lines on panels A and B represent chance levels. Dots connected by lines represent group means. CTRL = controls (water-exposed animals); CAS = conspecific alarm substance. Final sample sizes: CTRL + VEH: n = 10 animals; CTRL + 1 mg/kg WAY-161503: n = 21 animals; CAS + VEH: n = 21 animals; CAS + 1 mg/kg WAY-161503: n = 21 animals.

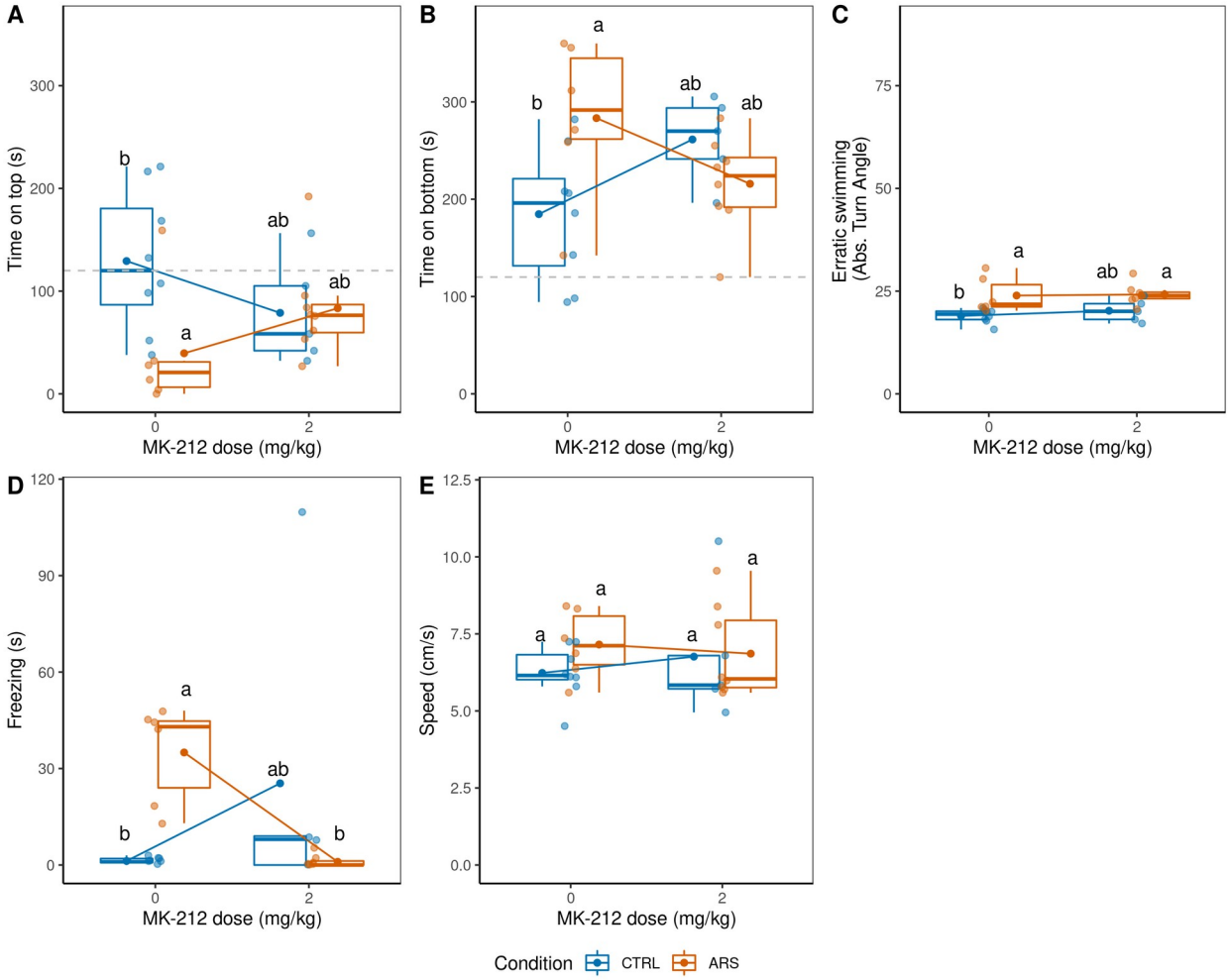


Figure 5 – Effects of WAY-161503 on behavior <u>after</u> CAS exposure. (A) Time spent on top third of the tank. (B) Time spent on bottom third of the tank. (C) Erratic swimming. (D) Total time spent freezing. (E) Swimming speed. Different letters represent statistical differences at the $p < 0.05$ level in pairwise comparisons with all other groups; similar letters indicate lack of statistically significant differences. Data are presented as individual data points (dots) superimposed over the median ± interquartile ranges. Dashed lines on panels A and B represent chance levels. Dots connected by lines represent group means. CTRL = controls (water-exposed animals); CAS = conspecific alarm substance. Final sample sizes: CTRL + VEH: n = 10 animals; CTRL + 1 mg/kg WAY-161503: n = 21 animals; CAS + VEH: n = 21 animals; CAS + 1 mg/kg WAY-161503: n = 21 animals.

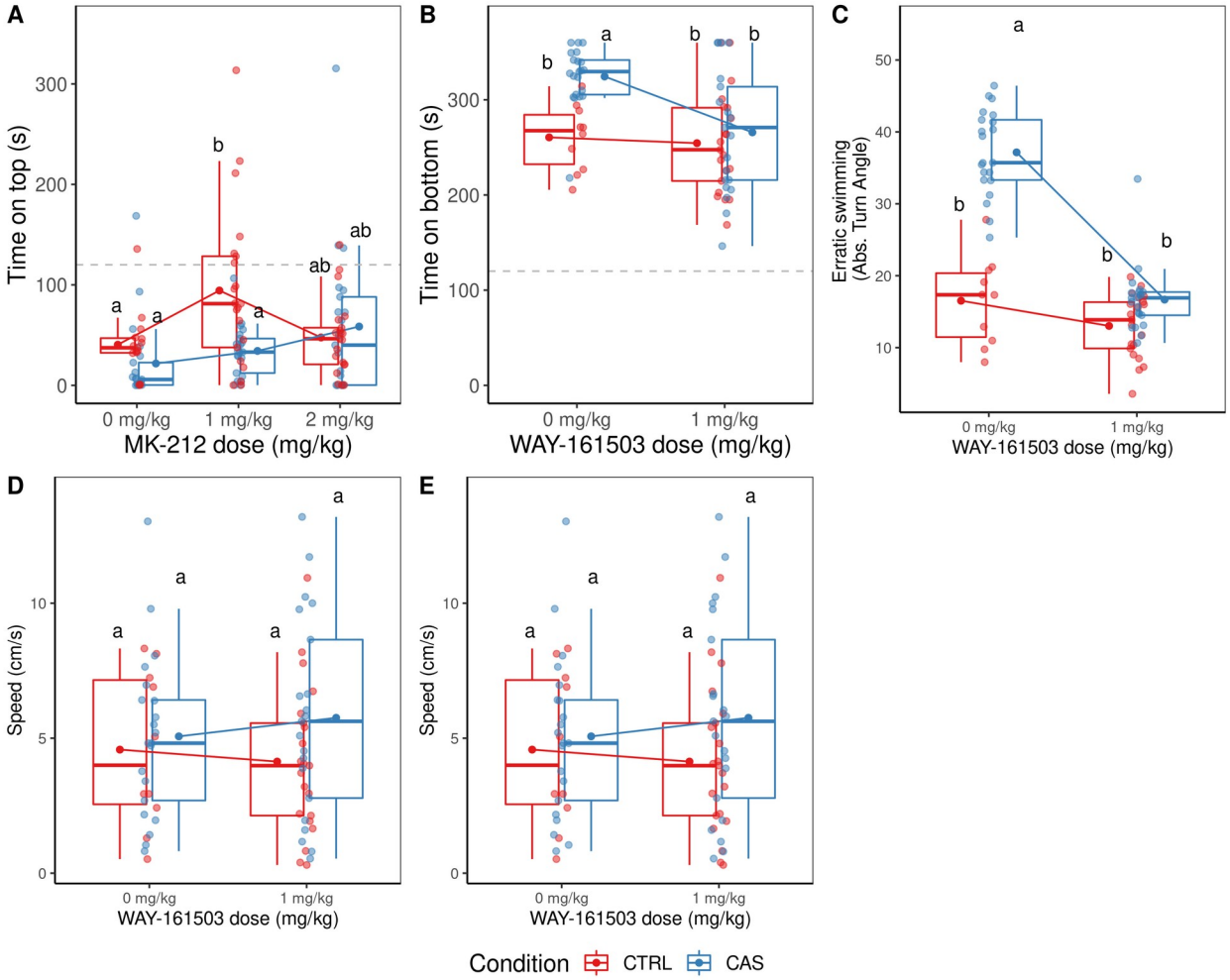
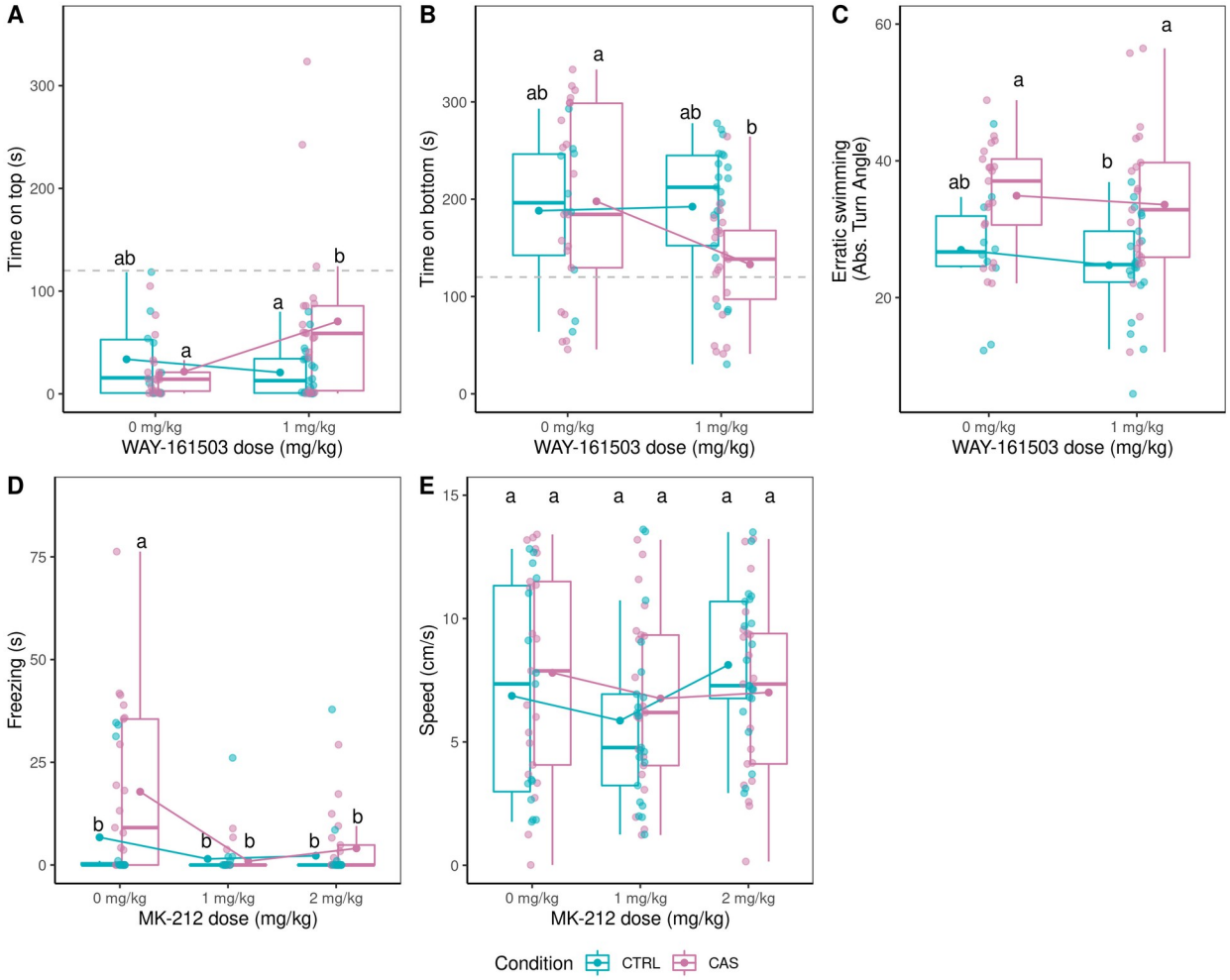
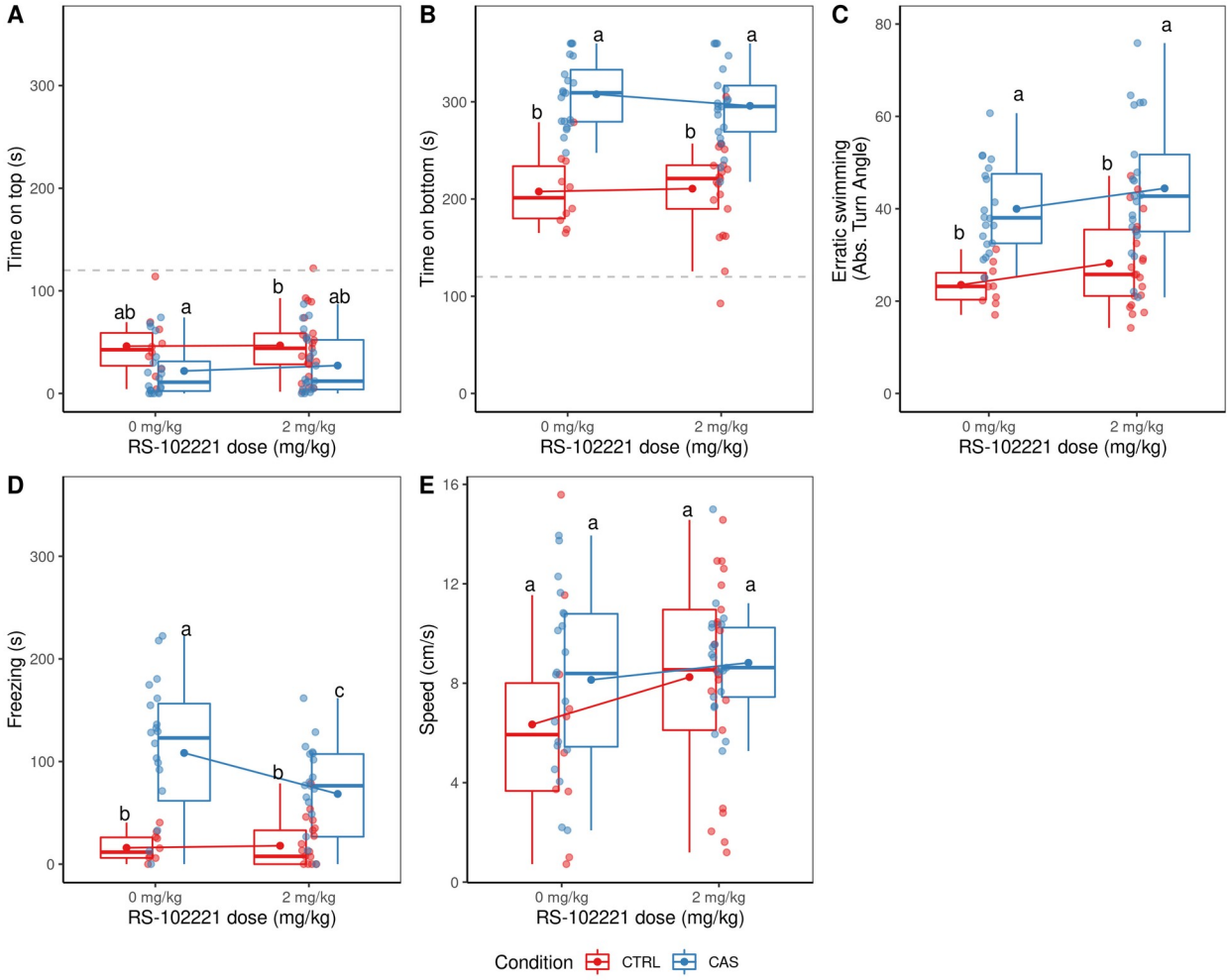


Figure 6 – Effects of RS-102221 on behavior during CAS exposure. (A) Time spent on top third of the tank. (B) Time spent on bottom third of the tank. (C) Erratic swimming. (D) Total time spent freezing. (E) Swimming speed. Different letters represent statistical differences at the $p < 0.05$ level in pairwise comparisons with all other groups; similar letters indicate lack of statistically significant differences. Data are presented as individual data points (dots) superimposed over the median ± interquartile ranges. Dashed lines on panels A and B represent chance levels. Dots connected by lines represent group means. CTRL = controls (water-exposed animals); CAS = conspecific alarm substance. Final sample sizes: CTRL + VEH: n = 10 animals; CTRL + 2 mg/kg RS-102221: n = 21 animals; CAS + VEH: n = 20 animals; CAS + 2 mg/kg RS-102221: n = 21 animals.



1038 Figure 7 – Effects of RS-102221 on behavior after CAS exposure. (A) Time spent on top third of
1039 the tank. (B) Time spent on bottom third of the tank. (C) Erratic swimming. (D) Total time
1040 spent freezing. (E) Swimming speed. Different letters represent statistical differences at
1041 the $p < 0.05$ level in pairwise comparisons with all other groups; similar letters indicate
1042 lack of statistically significant differences. Data are presented as individual data points
1043 (dots) superimposed over the median ± interquartile ranges. Dashed lines on panels A
1044 and B represent chance levels. Dots connected by lines represent group means. CTRL
1045 = controls (water-exposed animals); CAS = conspecific alarm substance. Final sample
1046 sizes: CTRL + VEH: n = 10 animals; CTRL + 2 mg/kg RS-102221: n = 21 animals; CAS
1047 + VEH: n = 20 animals; CAS + 2 mg/kg RS-102221: n = 21 animals.



1049

Figure 8 – Effects of MK-212 on behavior <u>after</u> acute restraint stress (ARS). (A) Time spent on top third of the tank. (B) Time spent on bottom third of the tank. (C) Erratic swimming. (D) Total time spent freezing. (E) Swimming speed. Different letters represent statistical differences at the $p < 0.05$ level in pairwise comparisons with all other groups; similar letters indicate lack of statistically significant differences. Data are presented as individual data points (dots) superimposed over the median ± interquartile ranges. Dashed lines on panels A and B represent chance levels. Dots connected by lines represent group means. CTRL = controls (water-exposed animals); ARS = acute restraint stress. Final sample sizes: CTRL + VEH: n = 8 animals; CTRL + 2 mg/kg MK-212: n = 5 animals; ARS + VEH: n = 6 animals; ARS + 2 mg/kg MK-212: n = 8 animals.

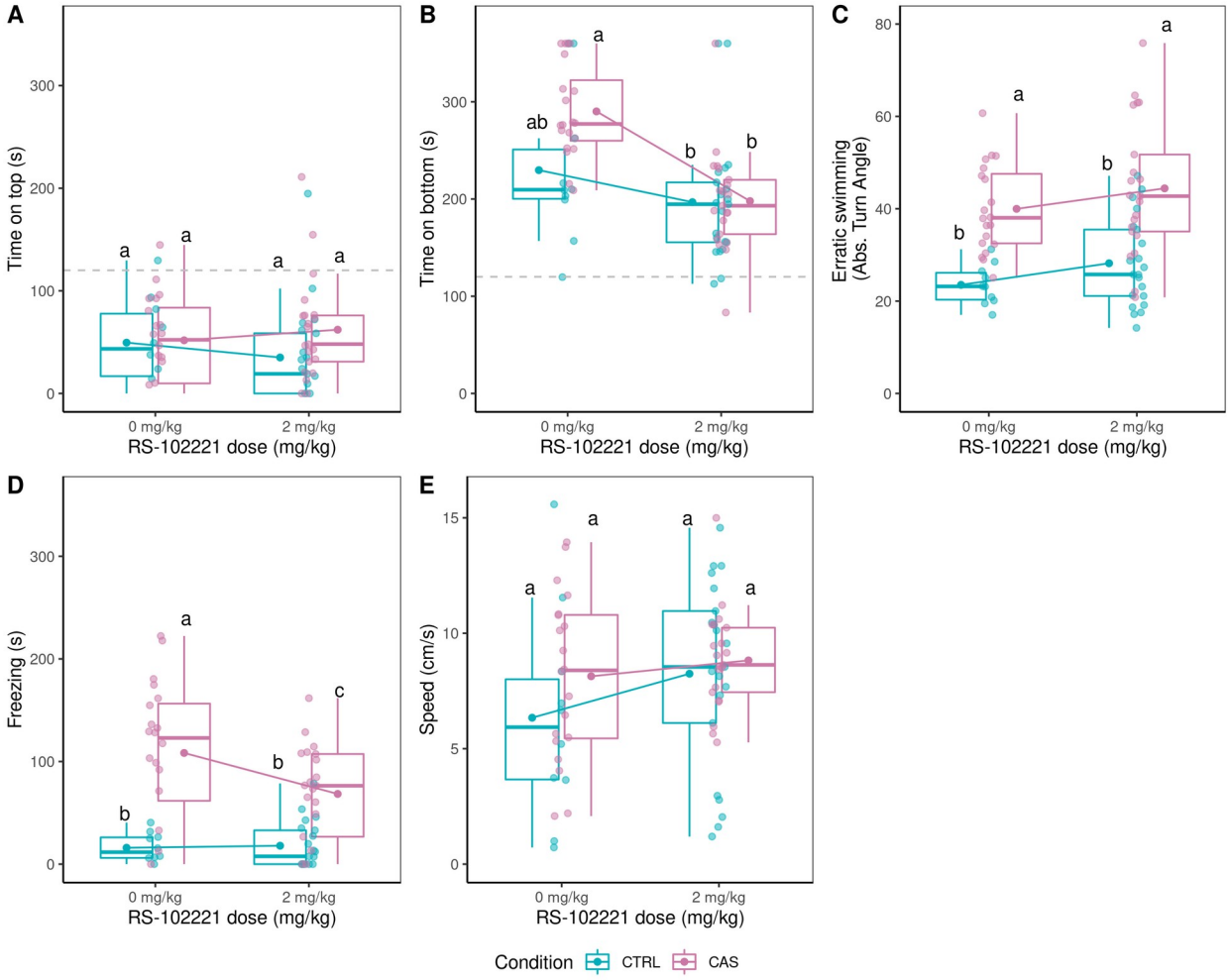
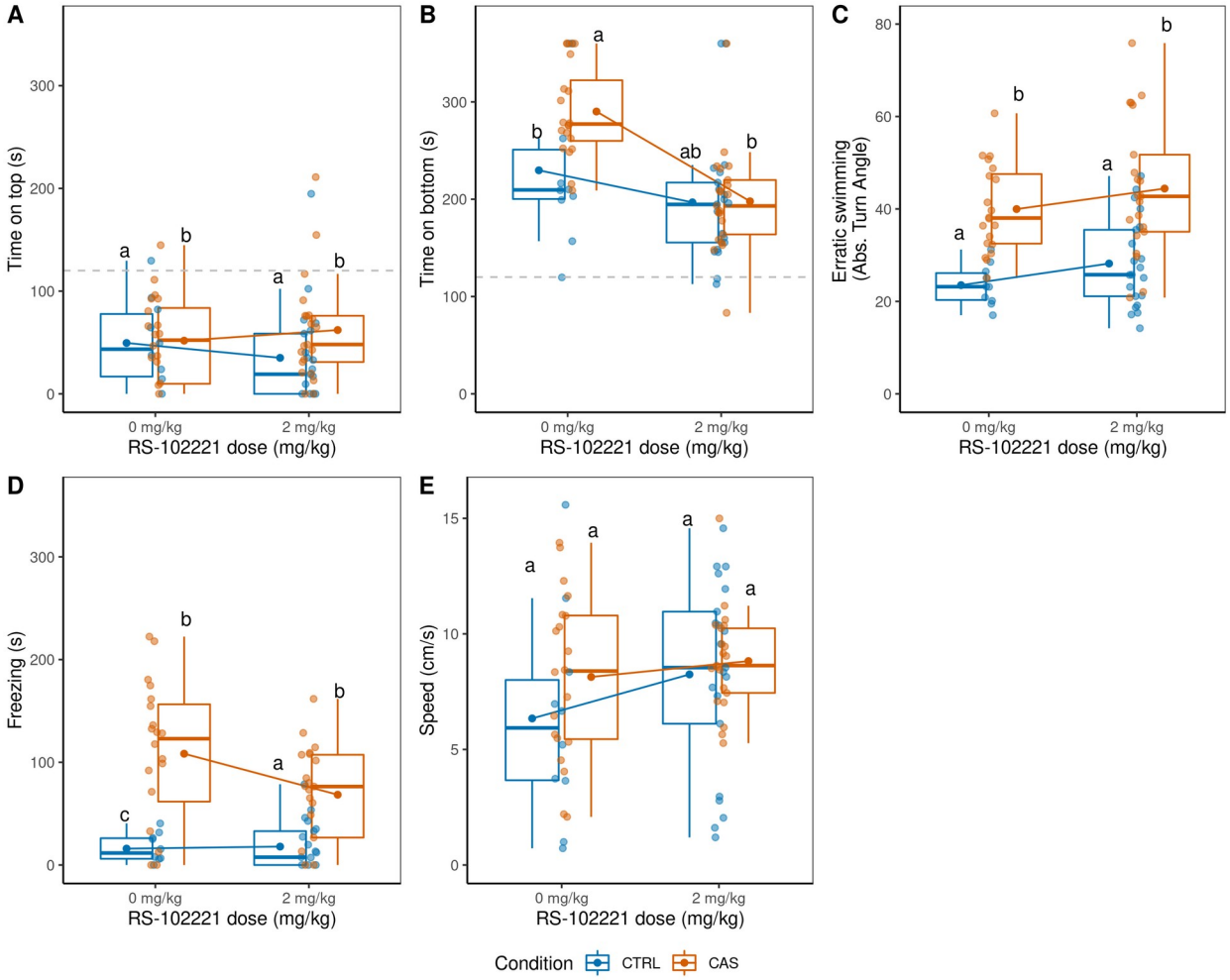



Figure 9 – Effects of RS-102221 on behavior after acute restraint stress (ARS). (A) Time spent on top third of the tank. (B) Time spent on bottom third of the tank. (C) Erratic swimming. (D) Total time spent freezing. (E) Swimming speed. Different letters represent statistical differences at the $p < 0.05$ level in pairwise comparisons with all other groups; similar letters indicate lack of statistically significant differences. Data are presented as individual data points (dots) superimposed over the median ± interquartile ranges. Dashed lines on panels A and B represent chance levels. Dots connected by lines represent group means. CTRL = controls (water-exposed animals); ARS = acute restraint stress. Final sample sizes: CTRL + VEH: n = 8 animals; CTRL + 2 mg/kg RS-102221: n = 8 animals; ARS + VEH: n = 8 animals; ARS + 2 mg/kg RS-102221: n = 8 animals.