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[1], Jéssica Souza Pinheiro[1], 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 (potential threat) exposure, and treated with the 5-HT_{2C} receptor agonists MK-212 or WAY-161503 or with the antagonist RS-102221. The agonists blocked CAS-elicited defensive behavior (distal threat), but not post-exposure increases in defensive behavior (potential threat), suggesting inhibition of responses to distal threat. MK-212 blocked changes in freezing elicited by acute restraint stress, a model of proximal threat, while RS-102221 blocked changes in geotaxis elicited this stressor. We also found that RS-102221, a 5-HT_{2C} receptor antagonist, produced small effect on behavior during and after exposure to CAS.

Preprint: https://www.biorxiv.org/content/10.1101/2020.10.04.324202; **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

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

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

5-HT has been implicated in fish behavioral responses both during and after exposure to

66 CAS. In zebrafish, extracellular 5-HT levels were increased after CAS exposure (Maximino et
67 al., 2014), an effect that can be related to decreased 5-HT uptake (Maximino et al., 2014) and/or
68 decreased monoamine oxidase activity (Lima-Maximino et al., 2020; Quadros et al., 2018). In
69 Crucian carp (*Carassius carassius*), exposure to CAS elicits increases in serotonergic activity in
70 the brainstem and optic tectum (Höglund et al., 2005), structures which have been involved in
71 responses to distal and proximal threat (do Carmo Silva et al., 2018a). However, this only
72 happened when hiding material was unavailable in the tank. In Nile tilapia (*Oreochromis*
73 *niloticus*), CAS did not increase serotonergic activity in the dorsomedial and dorsolateral
74 telencephali (Silva et al., 2015), homologues of the frontotemporal amygdaloid nuclei and
75 hippocampus, respectively (do Carmo Silva et al., 2018a). These results suggest that, during or
76 after exposure, CAS increases serotonergic activity in regions associated with "quick-and-dirty"
77 behavioral responses to distal threat (a "fight/flight/freeze" or "fear" system), but not in the
78 telencephalic areas associated with cautious exploration/risk assessment (a "behavioral
79 inhibition" or "anxiety" system).

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

90 The role of serotonin receptors from the 5-HT_2 family in CAS-elicited behavioral

91 adjustments has also been investigated. Zebrafish has been shown to possess two copies of
92 the 5-HT_{2A} receptor, one copy of the 5-HT_{2B} receptor, and two copies of the 5-HT_{2C} receptor
93 (Sourbron et al., 2016). In Nile tilapia, mianserin, a 5-HT_{2A} and 5-HT_{2C} receptor antagonist that
94 also blocks α_2-adrenoceptors, blocked active components of the alarm reaction (dashing,
95 bristling of dorsal fin spines), but not freezing, during exposure (Barreto, 2012). In zebrafish,
96 methysergide (an antagonist at 5-HT_{2A}, 2B, and 2C receptors, and a 5-HT_{1A} receptor agonist)
97 increased freezing and bottom-dwelling during and after exposure at a high dose (92.79 mg/kg),
98 but not at lower doses (Nathan et al., 2015). These results point to an inhibitory role of the 5-
99 HT_{2A} and 5-HT_{2C} receptors on CAS-elicited fear-like responses. However, since Nathan et al.
100 (2015) observed these effects consistently during a long session, in which behavior is expected
101 to change as CAS concentrations decrease (Mathuru et al., 2012)(i.e., similar to the shift from
102 erratic swimming to freezing that is observed when animals are observed after exposure in a
103 CAS-free context; Lima-Maximino et al., 2020), and since serotonergic drugs can produce
104 opposite effects on behavior during and after CAS exposure (Lima-Maximino et al., 2020), from
105 these results it is not possible to understand whether 5-HT_2 receptors participate in both
106 responses. In rodents, for example, 5-HT_2 receptors appear to inhibit fear and facilitate anxiety
107 at different levels of the neuroaxis (e.g. Graeff et al., 1986; Coimbra and Brandão, 1997;
108 Castilho and Brandão, 2001; Castilho et al., 2002; Alves et al., 2004; Cornélio and Nunes-de-
109 Souza, 2007; Macedo et al., 2007; Nunes-de-Souza et al., 2008; de Paula and Leite-Panissi,
110 2016), while in zebrafish these receptors appear to inhibit both fear and anxiety, although it is
111 currently unknown which brain regions participate in each effect.

112 In this work, we tested whether 5-HT_{2C} receptors participate in responses to CAS during
113 (distal threat) or after exposure (potential threat) and to acute restraint stress (ARS). ARS has
114 been applied in zebrafish to elicit strong stress responses, including activation of the
115 hypothalamus-pituitary-interrenal (HPI) axis and associated behavioral responses (Assad et al.,

116 2020; Ghisleni et al., 2012; Piato et al., 2011). From the point of view of the predatory
117 imminence continuum theory, ARS represents proximal threat (Perusini and Fanselow, 2015).
118 Thus, if 5-HT_{2C} receptors inhibit aversively motivated behavior in zebrafish regardless of threat
119 distance, its activation would inhibit behavioral responses at these three contexts. If, as in
120 rodents, 5-HT_{2C} receptors act at different levels of threat distance to either activate or inhibit
121 defensive responses, then 5-HT_{2C} agonists will not produce the same effect in each of these
122 contexts.

123 The aim of the present work was to test the hypothesis that activation of 5-HT_{2C}
124 receptors would block CAS-elicited defensive reactions during exposure, but not after exposure;
125 moreover, we proposed that 5-HT_{2C} receptor antagonists would affect post-exposure behavior.
126 We also hypothesized that 5-HT_{2C} agonists would not alter the anxiogenic-like effects of acute
127 restraint stress (ARS), a model for proximal threat. We found that 5-HT_{2C} agonists were able to
128 block elements of the alarm reaction, but did not affect post-exposure behavior, nor the full
129 anxiogenic-like effects of ARS, while a 5-HT_{2C} receptor antagonist partially blocked the alarm
130 reaction and more strongly affected post-exposure behavior. This manuscript is a complete
131 report of all the studies performed to test the effect of 5-HT_{2C} agonists and antagonists on
132 zebrafish defensive behavior to distal threat. We report how the sample size was determined, all
133 data exclusions (if any), all manipulations, and all measures in the study.
134

135 2. Materials and methods

136 2.1. Animals and housing

137 Adult (>4 month-old; standard length = 23.0 ± 3.2 mm) zebrafish (*Danio rerio*) from the

138 longfin phenotype (n = 250) were used in the present experiments. The populations used are
139 expected to better represent the natural populations in the wild, due to its heterogeneous
140 genetic background (Parra et al., 2009; Speedie and Gerlai, 2008). Animals were bought from a
141 commercial vendor (Belém/PA) and collectively maintained in 40 L tanks for at least two weeks
142 before the onset of experiments. The animals were fed daily with fish flakes. The tanks were
143 kept at constant temperature (28 °C), oxygenation, light cycle (14:10 LD photoperiod) and a pH
144 of 7.0-8.0, according to standards of care for zebrafish (Lawrence, 2007). Animals were used for
145 only one experiment to reduce interference from apparatus exposure. Potential suffering of
146 animals was minimized by controlling for the aforementioned environmental variables.
147 Furthermore, in the all experiments the animals used were handled, anesthetized and sacrificed
148 according to the norms of the Brazilian Guideline for the Care and Use of Animals for Scientific
149 and Didactical Purposes (Conselho Nacional de Controle de Experimentação Animal -
150 CONCEA, 2017). Since, in all cases, animals were individually stressed and/or individually
151 treated, experimental unit was a single animal, and therefore all sample sizes refer to number of
152 animals. The experimental protocols were approved by UEPA's IACUC under protocol 06/18.
153

154 2.2. Drugs and treatments

155 The 5-HT_{2C} receptor agonists MK-212 (2-Chloro-6-(1-piperazinyl)pyrazine, CAS #64022-
156 27-1) and WAY-161503 (8,9-dichloro-2,3,4,4a-tetrahydro-1H-pyrazino[1,2-a]quinoxalin-5(6H)-
157 one; CAS #75704-24-4) were bought from Sigma-Aldrich (St Louis, USA) on 2018, and
158 dissolved in Cortland's salt solution (NaCl 124.1 mM, KCl 5.1 mM, Na_2HPO_4 2.9 mM, $MgSO_4$ 1.9
159 mM, $CaCl_2$ 1.4 mM, $NaHCO_3$ 11.9 mM, Polyvinylpyrrolidone 4%, 1,000 USP units Heparin; Wolf,
160 1963) and in 1% dimethyl sulfoxide (DMSO), respectively. The 5-HT_{2C} receptor antagonist RS-

161 102221 (8-[5-(2,4-Dimethoxy-5-(4-trifluoromethylphenylsulphonamido)phenyl -5-oxopentyl]-
162 1,3,8-triazaspiro[4.5]decane-2,4-dione; CAS #185376-97-0) was bought from Sigma-Aldrich (St
163 Louis, USA) in 2018, and dissolved in 1% DMSO. While affinities for zebrafish 5-HT_{2C}-like
164 receptors have not been established, WAY-161503 has been reported to displace DOI from
165 human 5-HT_{2C} receptors with a K_i of 3.3 nM (6-fold selectivity over human 5-HT_{2A} receptors and
166 20-fold over human 5-HT_{2B} receptors)(Rosenzweig-Lipson et al., 2006). MK-212 has been
167 shown to be less selective at recombinant human receptors, with a K_i of 7.01 nM at 5-HT_{2C}
168 receptors (vs. 5.99 nM and 6.21 nM at 5-HT_{2A} and 5-HT_{2B} receptors, respectively)(Knight et al.,
169 2004). Finally, RS-102221 has been shown to displace mesulergine from human 5-HT_{2C}
170 receptor with a *pKi* of 8.4 nM (over 100-fold selectivity over human 5-HT_{2A} and 5-HT_{2B} receptors)
171 (Bonhaus et al., 1997).

172 For Experiment 1, animals were injected intraperitoneally either with MK-212 (1 mg/kg
173 and 2 mg/kg, doses which increase anxiety-like behavior in the rat elevated plus-maze; (de
174 Mello Cruz et al., 2005)) or with the vehicle solution (Cortland's salt solution); WAY-161503 (1
175 mg/kg, a dose which produces anxiogenic-like effects in the rat elevated plus-maze: Gomes et
176 al., 2010) or with the vehicle solution (DMSO); or RS-102221 (2 mg/kg, a dose that reduces
177 anxiety in the mouse light/dark test; (Kuznetsova et al., 2006)). For Experiment 2, animals were
178 injected intraperitoneally with MK-212 (2 mg/kg) or vehicle (Cortland's salt solution). Injections
179 were made according to the protocol proposed by Kinkel et al. (2010); briefly, animals were
180 cold-anesthetized and transferred to a sponge-based surgical bed, in which injection was made.
181 Injections were made using a microsyringe (Hamilton® 701N syringe, needle size 26 gauge at
182 cone tip), with total volumes of injection of 5 μL.
183

2.3. Experiment 1: Effects of 5-HT_{2C} receptor agonists and antagonists on alarm reaction and post-exposure behavior

2.3.1. Experimental design

To verify the effects of phasic activation of 5-HT_{2C} receptors on the zebrafish alarm reaction, animals were pre-treated with either receptor agonists and exposed to CAS in a sequential design, with a “washout” period in between tests (Figure 1A). For the exposure stage, each animal was transferred individually to a container (2 L) where after 3 minutes of acclimatization, it was carefully exposed to 7 ml of alarm substance (CAS), extracted using a standardized protocol (do Carmo Silva et al., 2018b). As negative control, a group with the same amount of animals was exposed to the same volume of distilled water, according to the protocol of Lima-Maximino et al. (2020). The animals remained exposed for 6 minutes during which their behavior was recorded using a video camera positioned in front of the aquarium. Then, to verify the residual effects of exposure to the alarm substance, 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 251 to 280 lumens (coefficient of variation = 3.399% between subjects).

Animals were randomly allocated to groups using a random number generator (http://www.jerrydallal.com/random/random_block_size_r.htm), with each subject randomized to a single treatment using random permuted blocks. For each experiment, animals were treated

207 and tested in the order of allocation (i.e., randomly). In all experiments, experimenters and data
208 analysts were blinded to drugs and treatment by using coded vials (with the same code used for
209 randomization); blinding was removed only after data analysis. Experiments were always run
210 between 08:00AM and 02:00 PM. After experiments, animals were sacrificed by prolonged bath
211 in ice-cold water (< 12 ºC), followed by spinal transection (Matthews and Varga, 2012).
212

213 2.3.2. Sample size determination

214 To determine sample size, we incorporated information from control groups derived from
215 previously published experiments on zebrafish alarm substances observing bottom-dwelling
216 (Lima-Maximino et al., 2020; Quadros et al., 2016; Speedie and Gerlai, 2008) and a series of
217 four small experiments on the effects of CAS on behavior during exposure
218 (https://github.com/lanec-unifesspa/5-HT-CAS/tree/master/data/behavioral/metanalysis),
219 following the RePAIR approach (Bonapersona et al., 2019). Sample sizes, means, and standard
220 deviations for the primary endpoint "Time on bottom" were used to produce a prior distribution
221 on the RePAIR script (https://utrecht-university.shinyapps.io/repair/). Final parameters of the
222 distribution were $\mu = 197.149$, $\sigma^2 = 6100.609$, and weighted $N = 120.10$. The parameters of this
223 distribution were then used to calculate sample size, based on an effect size of $d = 0.5$
224 (equivalent to that used to calculate sample sizes in Lima-Maximino et al., 2020) and *a priori*
225 power of 0.8, with one-tailed tests with error probability $\alpha = 0.05$. With these parameters, the
226 final number of animals was 10 animals in the control group and 21 in each experimental group,
227 reaching a prospective power of 92.34%.
228

229 ## 2.3.3. Alarm substance extraction

230 A group of zebrafish was used as donor animals for the extraction of the conspecific
231 alarm substance (CAS). CAS extraction procedure was performed on each animal individually
232 as described by do Carmo Silva et al. (2018b). First, the donor animal was cold anesthetized
233 and transferred to a Petri dish, where the excess water from its body was removed with a paper
234 towel. Then the animal was decapitated with a surgical scalpel and the excess blood from the
235 sectioned region was removed with a swab. Subsequently, the animal's bodies were transferred
236 to another Petri dish where 15 superficial cuts were made in the epidermis of animals (medial-
237 ventral region) and 10 ml of distilled water were added to wash the cuts. After washing, the
238 animal's bodies were removed from the Petri dish, and with the aid of a Pasteur pipette, the fish
239 scales and other impurities were removed from the solution that was stored in a conical tube
240 and preserved on ice. The same extraction procedure was performed for all donated animals.
241

242 ## 2.3.4. Observation apparatuses

243 To assess the effects of drugs on the alarm reaction, animals were transferred to a 12
244 cm x 12 cm x 14 cm glass tank, filled with 1.5 L tank water, and allowed to swim freely for a 3
245 min acclimation period. After this period, 7 mL CAS was added to the tank, from above, and
246 filming started. The video camera (Sony® DCR-DVD610) was positioned in the front of the tank,
247 therefore allowing observation and tracking of vertical distribution. Animals were allowed to
248 freely explore this tank for 6 min before being transferred to a “washout” tank, which contained
249 500 mL tank water. Animals were kept in this tank for 1 min, removing any residues of CAS
250 before transference to the second apparatus. This second apparatus was a 25 cm x 24 cm x 20
251 cm glass tank, filled with 5 L tank water; animals were allowed free exploration of this tank for 6

252 min, during which behavior was filmed from the front of the tank. Tanks for both stages were
253 differently shaped to increase the novelty of the second environment, a variable that is important
254 to induce an anxiety-like “diving” response in animals not exposed to CAS (Bencan et al., 2009).
255

256 2.4. Experiment 2: Effects of MK-212 or RS-102221 on acute 257 restraint stress-elicited behavior

258 2.4.1. Experimental design

259 In this experiment, we focus on assessing the roles of 5-HT_{2C} receptors on anxiogenic-
260 like effects of acute restraint stress (ARS). In order to do this, we evaluate the behavior of the
261 animals in the novel tank test after being subjected to a 90-min section of restraint stress
262 (Figure 1B). After drug (MK-212 or RS-102221) or vehicle (Cortland’s salt solution) injection and
263 anesthesia, each animal was transferred individually to a 2 mL microtube (Eppendorf®) and
264 placed on a plastic microtube rack inside an aquarium with continuous oxygen supply. The
265 microtubes had small holes at both ends to allow free circulation of water inside the tube and to
266 prevent fish from moving around, according to the protocol of Piato et al. (2011). A control group
267 was maintained in a similar tank, but without restraint stress. Animals remained in these
268 conditions for 90 min., sufficient to induce changes in telencephalon neurochemistry (Assad et
269 al., 2020) and marked anxiety-like behavior (Assad et al., 2020; Ghisleni et al., 2012; Piato et
270 al., 2011). Animals in the control groups were subjected to all manipulations with the exception
271 of restraint. After stress, the animals were transferred to the apparatus of the novel tank test, a
272 transparent glass aquarium filled with 5 L of mineral water where the animal can freely explore
273 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), using the same parameters as the CAS sample size determination, with the exception of effect size (which was defined as d = 4.56263, based on 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

317 are publicly available. Behavioral records were reviewed by at least one PI for
318 administration/scoring accuracy, in order to ensure adherence to protocols and consistency
319 across tests.
320

321 2.7. Statistical analysis

322 Data were analyzed using two-way analysis of variance (drug x exposure to stressor)
323 with sequential sum of squares (type I), followed by Tukey's post-tests when $p < 0.05$; since
324 multiple comparisons were made in post-tests, p-values were calculated using Tukey's method.
325 Data analysis, table organization, and result graphs were performed using R version 3.6.3
326 (2020-02-29). Effect sizes for ANOVA effects are shown as ω^2; effect sizes for post-hoc tests
327 were shown as Cohen's d. Outliers were removed based on median absolute differences
328 (MADs), using time on bottom as the main endpoint; values were removed when they were
329 higher or lower than 3 MADs around the median (Leys et al., 2013), and the number of outliers
330 was reported in the results.
331

332 2.8. Sequence alignment

333 Zebrafish have two copies of the 5-HT2C receptor gene, *htr2cl1*
334 [ENSDARG00000018228] and *htr2cl2* [ENSDARG00000013210] (Sourbron et al., 2016);
335 currently, only the expression of *htr2cl1* is known, and studies were made in larvae (Schneider
336 et al., 2012). Moreover, receptor binding and drug affinity is currently unknown. Mutation and *in*
337 *silico* studies suggest that the 5-HT_{2C} receptor residues D3.32, S3.36, and Y7.43 are crucial to
338 ligand binding (Canal et al., 2011; Córdova-Sintjago et al., 2012). We looked for the

339 conservation of these residues across zebrafish 5-HT_{2C}-like receptors by aligning protein
340 sequences from zebrafish receptors (XP_017212271.1 and XP_001339040.4) with the murine
341 (NP_032338.3) and human (NP_001243689.2) 5-HT_{2C} receptors in Clustal Omega
342 (https://www.ebi.ac.uk/Tools/msa/clustalo/). Resulting alignments were visually inspected for
343 conserved residues at the positions that are related to ligand affinity.
344

345 2.9 Open science practices

346 The studies presented here were not formally pre-registered. A preprint version was
347 posted to bioRxiv **(**https://www.biorxiv.org/content/10.1101/2020.10.04.324202v2. Data and
348 analysis scripts can be found on a Github repository (https://github.com/lanec-unifesspa/5-HT-
349 CAS/tree/master/data/5HT2C).
350

351 3. Results

352 3.1. Effects of MK-212 on alarm reaction and post-exposure 353 behavior

354 3.1.1. During exposure

355 One animal from the control + 0 mg/kg group was detected as outlier and removed. Full
356 results for ANOVAs can be found on Table 1. Post-hoc tests found that CAS did not alter time
357 on top (p = 0.94, d = 0.3, non-treated controls vs. non-treated CAS), but MK-212 (1 mg/kg)
358 increased it in non-exposed animals (*1 mg/kg:* p = 0.024, d = -1.05, non-treated controls vs. 1

359 mg/kg controls; *2 mg/kg:* $p = 0.994$, $d = -0.18$, non-treated controls vs. 2 mg/kg controls; Figure
360 2A).

361 Post-hoc tests revealed that CAS increased time on bottom ($p < 0.001$, $d = -0.77$ for the
362 main effect), and the highest MK-212 dose blocked this effect (*1 mg/kg:* $p = 0.39$, $d = -0.64$,
363 non-treated controls vs. treated CAS; $p = 0.38$, $d = 0.6$, non-treated CAS vs. treated CAS; *2*
364 *mg/kg:* $p = 0.828$, $d = 0.4$, non-treated controls vs. treated CAS; $p < 0.001$, $d = 1.64$, non-treated
365 CAS vs. treated CAS; Figure 2B).

366 Post-hoc tests showed that CAS greatly increased erratic swimming (absolute turn
367 angle) in non-treated animals ($p < 0.001$, $d = -2.58$, non-treated controls vs. non-treated CAS),
368 an effect that was blocked by both MK-212 doses (*1 mg/kg*: $p = 1$, $d = -0.04$, non-treated
369 controls vs. treated CAS; $p < 0.001$, $d = 2.53$, non-treated CAS vs. treated CAS; *2 mg/kg*: $p =$
370 0.81, $d = -0.42$, non-treated controls vs. treated CAS; $p < 0.001$, $d = 2.16$, non-treated CAS vs.
371 treated CAS; Figure 2C); however, MK-212 also increased absolute turn angle in animals which
372 were not exposed to CAS (*1 mg/kg*: $p < 0.001$, $d = -1.95$, non-treated controls vs. treated
373 controls; *2 mg/kg:* $p < 0.001$, $d = -1.91$, non-treated controls vs. treated controls).

374 Post-hoc tests suggested that CAS increased freezing at all MK-212 doses and controls
375 ($p < 0.001$, $d = -0.72$ for the main effect of CAS; Figure 2D).

376 While a small-to-medium-sized interaction effect was found for swimming speed (Table
377 1), post-hoc tests found no differences across groups (Figure 2E).

378

379 3.1.2. After exposure

380 Table 2 presents full ANOVA results for effects in the novel tank test after CAS exposure
381 and washout. 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).

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).

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) .

Post-hoc tests revealed that CAS increased freezing ($p = 0.05$, $d = -0.364$ for the main effect), an effect that was blocked by all MK-212 doses (*1 mg/kg:* $p = 0.74$, $d = 0.47$, non-treated controls vs. 1 mg/kg controls; $p < 0.001$, $d = 1.5$, non-treated CAS vs. 1 mg/kg CAS; *2 mg/kg:* $p = 0.85$, $d = 0.4$, non-treated controls vs. 2 mg/kg controls; $p = 0.002$, $d = 1.22$, non-treated CAS vs. 2 mg/kg CAS; Figure 3D).

No effects were found for swimming speed (Figure 3E).

402 ## 3.2. Effects of WAY-161503 on alarm reaction and post-exposure
403 behavior

404 ### 3.2.1. During exposure

405 No outliers were detected from any group in this experiment. Table 3 presents full
406 ANOVA results for this experiment. Post-hoc tests showed that while CAS did not alter time on
407 top ($p = 0.054$, $d = 1.0$, non-treated controls vs. non-treated CAS), a synergistic effect was
408 apparent, with WAY-161503-treated animals showing less time on top than controls ($p = 0.381$,
409 $d = 0.618$, non-treated controls vs. WAY-161503 controls; $p = 0.977$, $d = 0.125$, non-treated
410 CAS vs. WAY-161503 CAS; $p = 0.023$, $d = 1.125$, non-treated controls vs. WAY 161-503 CAS;
411 Figure 4A).

412 Post-hoc tests revealed that CAS increased time on bottom ($p = 0.007$, $d = -1.287$, non-
413 treated controls vs. non-treated CAS), and WAY-161503 blocked this effect ($p = 0.989$, $d =$
414 0.123, non-treated controls vs. WAY-161503 controls; $p = 0.002$, $d = 1.181$, non-treated CAS
415 vs. WAY-161503 CAS; Figure 4B).

416 Again, post-hoc tests suggested that CAS increased erratic swimming ($p < 0.001$, $d = -$
417 3.984, non-treated controls vs. non-treated CAS), while WAY-161503 blocked this effect ($p =$
418 0.302, $d = 0.6759$, non-treated controls vs. WAY-161503 controls; $p < 0.001$, $d = 3.9537$, non-
419 treated CAS vs. WAY-161503 CAS; Figure 4C).

420 Post-hoc tests revealed that CAS increased freezing ($p = 0.047$, $d = -1.02$, non-treated
421 controls vs. non-treated CAS), but this effect was not blocked by WAY-161503 ($p = 0.967$, $d = -$
422 0.178, non-treated controls vs. WAY-161503 controls; $p = 0.544$, $d = 0.412$, non-treated CAS
423 vs. WAY-161503 CAS; Figure 4D).

424 Finally, no effects were found for swimming speed (Figure 4E).

425

426 ### 3.2.2. After exposure

427 Full ANOVA results for this experiment can be found on Table 4. Post-hoc effects
428 revealed a synergistic effect for time on top, with WAY-161503 increasing time on top in animals
429 exposed to CAS ($p = 0.922$, $d = 0.242$, non-treated controls vs. non-treated CAS; $p = 0.907$,
430 0.259, non-treated controls vs. WAY-161503 controls; $p = 0.011$, $d = 0.9833$, non-treated CAS
431 vs. WAY-161503 CAS; Figure 5A).

432 Post-hoc tests suggested a synergistic effect also on time on bottom, with WAY-161503
433 decreasing time on bottom in animals exposed to CAS ($p = 0.988$, $d = -0.1243$, non-treated
434 controls vs. non-treated CAS; $p = 0.999$, $d = -0.0538$, non-treated controls vs. WAY-161503
435 controls; $p = 0.041$, $d = 0.8369$, non-treated CAS vs. WAY-161503 CAS; Figure 5B).

436 While ANOVA found effects of treatment on erratic swimming, post-hoc tests did not
437 find an increase in erratic swimming with CAS ($p = 0.125$, $d = -0.857$), nor a synergistic effect of
438 WAY-161503 ($p = 0.922$, $d = 0.243$, non-treated controls vs. WAY-161503 controls; $p = 0.968$, d
439 $= 0.142$, non-treated CAS vs. WAY-161503 CAS; Figure 5C).

440 Post-hoc tests revealed that CAS increased freezing ($p = 0.009$, $d = -1.257$, non-treated
441 controls vs. non-treated CAS), while WAY-161503 blocked this effect ($p = 0.816$, $d = -0.338$,
442 non-treated controls vs. WAY-161503 controls; $p = 0.008$, $d = 1.026$, non-treated CAS vs. WAY-
443 161-503 CAS; Figure 5D).

444 Again, no effects were found for swimming speed (Figure 5E).

445

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

3.3.1. During exposure

No outliers were detected in any groups. Full results for ANOVAs can be found on Table 5. 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).

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

Post-hoc tests suggested that CAS increased erratic swimming ($p < 0.01$, $d = 1.24$for the main effect), an effect that was blocked by RS-102221 ($p =0.829$, $d = -0.33$, non-treated controls vs. treated CAS; $p <0.001$, $d = 1.89$, non-treated CAS vs. treated CAS; Figure 6C).

Post-hoc tests revealed that CAS increased freezing ($p < 0.001$, $d = 1.10$ for the main effect), and RS-102221 partially blocked this effect ($p = 0.488$, $d = -0.548$, non-treated controls vs. treated CAS; $p = 0.013$, $d = 0.985$, non-treated CAS vs. treated CAS; Figure 6D).

Finally, no effects were found for swimming speed (Figure 6E).

3.3.2. After exposure

Full results for ANOVAs for this experiment can be found on Table 6. No effects were found for time on top (Figure 7A);

Post-hoc tests suggested that CAS increased time on bottom after exposure ($p = 0.05$, $d = 1.02$, non-treated controls vs. non-treated CAS), an effect that was blocked by RS-102221 (p

469 = 0.506, d = 0.54, non-treated controls vs. treated CAS; p < 0.001, d = 1.55, non-treated CAS
470 vs. treated CAS)(Figure 7B).
471 Post-hoc tests suggested that CAS increased erratic swimming after exposure (p =
472 0.001, d = 1.514, non-treated controls vs. non-treated CAS), an effect that was not altered by
473 RS-102221 (p < 0.001, d = -1.92, non-treated controls vs. treated CAS; p = 0.56, d = -0.41, non-
474 treated CAS vs. treated CAS)(Figure 7C).
475 Post-hoc tests suggested that CAS increased freezing after exposure (p < 0.001, d =
476 1.96, non-treated controls vs. non-treated CAS), an effect that was partially blocked by RS-
477 102221 (p = 0.025, d = -1.12, non-treated controls vs. treated CAS; p = 0.041, d = 0.85; Figure
478 7D).
479 Finally, no effects were found for swimming speed (Figure 7E).
480

481 ## 3.4. Restraint stress-elicited behavioral changes

482 ### 3.4.1. Effects of MK-212 on restraint stress-elicited behavioral changes

483 Full results for ANOVAs for this experiment can be found on Table 7. No effects were
484 found for time on top (Figure 8A).
485 Post-hoc tests showed that ARS increased time on bottom (*p* = 0.038, *d* = -1.566 vs.
486 non-treated controls), and MK-212 (2 mg/kg) blocked this effect (*p* = 0.757, *d* = -0.495 non-
487 treated controls vs. treated ARS; Figure 8B
488 Post-hoc tests suggested that a ARS increased erratic swimming (p < 0.001, d = -1.57
489 for the main effect), an effect that was not blocked by MK-212 (*p* = 0.006, d = -1.85, non-treated
490 controls vs. treated ARS; Figure 8C).
491 Post-hoc tests suggested that animals injected with MK-212 and subjected to ARS

492 showed decreased freezing in relation to other groups ($p = 0.033$, $d = -1.6$, non-treated controls
493 vs. treated ARS; $p = 0.031$, $d = 1.6$, non-treated ARS vs. treated ARS; Figure 8D).
494 Finally, no effects were found for swimming speed (Figure 8E).
495

496 3.4.2. Effects of RS-102221 on restraint stress-elicited behavioral changes

497 Full results for ANOVAs for this experiment can be found in Table 8. ARS increased time
498 on top ($p < 0.0001$, $d = 7.78$ for the main effect), and RS-102221 was not able to block this
499 effect (Figure 9A).
500 Post-hoc tests revealed that ARS increased time on bottom ($p = 0.007$ vs. non-treated
501 controls), an effect that was partially blocked by RS-102221 ($p = 0.997$, non-treated controls vs.
502 treated ARS; $p = 0.019$, non-treated ARS vs. treated ARS; Figure 9B).
503 No effects were found for erratic swimming (Figure 9C).
504 ARS increased freezing ($p = 0.001$, $d = 1.4$ for the main effect), an effect that was not
505 blocked by RS-102221 ($p = 0.052$, non-treated controls vs. treated ARS; $p = 0.998$, non-treated
506 ARS vs. treated ARS; Figure 9D).
507 Finally, no effects were found for swimming speed (Figure 9E).
508

509 3.5. Conservation of residues related to ligand binding in 510 zebrafish 5-HT_{2C} receptors

511 The sequences of *Danio rerio* 5-HT_{2C}-like receptors were aligned to the sequence of the
512 human and murine receptors using Clustal Omega. Sequence alignment showing the
513 transmembrane domains is shown in Figure 10. While target residues were conserved across

514 all sequences, differences in residues close to D3.32 the 5-HT_{2C}-like receptors zebrafish
515 receptor (S3.30Y in 5-HT_{2CL1} and S3.30F in 5-HT_{2CL2}), as well as close to Y7.43 (V7.44I in5-
516 HT_{2CL2}) were found.
517

518 4. Discussion

519 The present work tested the hypothesis that phasic activation of the 5-HT_{2C} receptor is
520 involved in behavioral adjustments to distal, but not proximal, threat in zebrafish, and that these
521 receptors exerts a tonic facilitation of defensive behavior to potential threat in zebrafish. We
522 found that 5-HT_{2C} agonists blocked CAS-elicited defensive behavior, but not post-exposure
523 increases in defensive behavior (with the exception of freezing), nor ARS-elicited anxiogenic-
524 like effects. We also found that RS-102221, a 5-HT_{2C} receptor antagonist, did not change
525 behavior during exposure, but it produced a small effect on behavior after exposure to CAS. No
526 effects of RS-102221 were found for ARS-elicited anxiogenic-like effects. Residues related to
527 ligand binding in the human 5-HT_{2C} receptors were conserved in both zebrafish isoforms, but
528 differences exist in residues close to these sites.
529

530 4.1. Effects of stressors on zebrafish behavior

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

535 view both should affect different behavioral endpoints.

536 In the present experiments, CAS consistently increased bottom-dwelling and erratic
537 swimming during exposure, with a smaller component of freezing. After exposure, a strong
538 component of freezing was present, while erratic swimming contributed less to the overall
539 behavioral pattern. These results are consistent with what was observed both with a washout
540 period (Lima-Maximino et al., 2020), and in the absence of a washout, but with an extended
541 observation interval (Mathuru et al., 2012; Nathan et al., 2015). These effects suggest that, as
542 threat levels change from distal (i.e., CAS is present) to potential (i.e., CAS is no longer
543 present), a “residual” effect emerges that is marked by different behavioral components. While
544 the meanings of freezing and erratic swimming are likely context-dependent, and much up to
545 debate, it could be argued that they represent “passive” or “risk assessment” responses in the
546 case of freezing, and “active” or “escape” responses in the case of erratic swimming. Thus, as
547 CAS is no longer present, the animal transitions from a “risky” to a “safety” behavioral state, as
548 the absence of predators or partial predator stimuli are used as a positive prediction error. This
549 is similar to what is observed with electrical stimulation of the PAG/GR in rats (Brandão et al.,
550 2008). We have previously shown that serotonin differentially mediates these behaviors,
551 phasically inhibiting responses during exposure and tonically facilitating responses after
552 exposure, and suggested that it is the phasic activation of serotonin receptors after exposure
553 that represent prediction errors (Lima-Maximino et al., 2020). The continued absence of these
554 threat signals sets negative expectation values, proposed by Amo et al. (2014) to be encoded
555 by raphe serotonergic neurons.

556 ARS-elicited behavioral effects have been explored in the literature with mixed results.
557 While Ghisleni et al. (2012) found that a 90-min restraint protocol did not change bottom-
558 dwelling after stress when animals were tested individually, a similar protocol showed marked
559 increases in this variable (Assad et al., 2020) – albeit control animals in the latter experiment

spent most of the session in the upper half of the tank instead of in the lower half. It is difficult to find other measures which are differentially affected by CAS vs. ARS, as physiological endpoints such as cortisol are increased by both (Ghisleni et al., 2012; Abreu et al., 2017). In the present experiments, ARS increased bottom-dwelling as well. Moreover, we observed a small component of erratic swimming, consistent with what is observed by Ghisleni et al. (2012). While increased bottom-dwelling was also observed as a pattern of post-exposure behavior after CAS in the present experiments and in Lima-Maximino et al. (2020), freezing was a major component of post-exposure behavior, suggesting that stressors do not produce similar behavioral effects.

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

The pattern of drug effects across behavioral experiments are complex, and summarized on Table 9. 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 freezing. RS-102221, a 5-HT_{2C} receptor antagonist, blocked changes in erratic swimming and freezing during exposure, and blocked increases in geotaxis and freezing after exposure. 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.

In the experimental design that was used in this experiment, behavior during exposure represents distal threat, as CAS acts as a partial predator stimulus that elicits behavior that decreases the possibility of a predator attack or detection by the predator (Smith, 1992).

583 However, if CAS is no longer present (as in the post-exposure stage of our design), that would
584 signal a decrease in threat levels to potential threat, a situation in which trying to flee or hide is
585 non-adaptive, but resuming normal behavior is also non-adaptive -- and therefore cautious, alert
586 exploration is warranted (Maximino et al., 2019). Using Fanselow's taxonomy, the situation in
587 which CAS is present is more akin to "fear", while the situation in which it is no longer present is
588 more akin to "anxiety" (Perusini and Fanselow, 2015). The rodent literature suggests that 5-HT_2
589 receptors participate in both fear and anxiety. Salchner and Singewald (2006) have shown that
590 MK-212 potentiates escape responses to an airjet in rats, and the antagonist SB-242084 has
591 been shown to produce anxiolytic-like effects in rats (Kennett et al., 1997; Martin et al., 2002). In
592 the elevated T-maze, an apparatus which tests anxiety-like behavior (inhibitory avoidance) and
593 fear-like behavior (one-way escape) in rodents, 5-HT_{2C} agonists facilitate, and antagonists
594 impair, inhibitory avoidance, but only agonists facilitate one-way escape (Mora et al., 1997),
595 suggesting tonic facilitation of responses to potential threat and phasic inhibition of responses to
596 distal or proximal threat. Thus, a conserved role for this receptor appears to be related to phasic
597 and tonic modulation of defensive responses.

598 We have previously found that serotonin participates in responses to CAS, acutely and
599 phasically inhibiting responses to distal threat but phasically facilitating responses to potential
600 threat (Lima-Maximino et al., 2020; Maximino et al., 2014). While it is not known whether
601 serotonin levels vary during exposure (i.e., whether CAS elicits physiological, adaptive changes
602 in serotonin levels), pharmacological experiments (Lima-Maximino et al., 2020) show that acute
603 (phasic) increases in serotonin during exposure block behavioral effects of CAS, while
604 decreasing serotonin levels with *para*-chlorophenylalanine did not produce effects during
605 exposure. In the present experiments, 5-HT_{2C} receptor agonists blocked the effects of CAS
606 during exposure, suggesting that during exposure phasic activation of the 5-HT_{2C} receptor is
607 inhibitory. However, in the present experiments the antagonist RS-102221 also blocked effects

608 on erratic swimming and (partially) freezing during exposure. In Nile tilapia, the 5-$HT_{2A/2C}$ and α_2-
609 adrenoceptor antagonist mianserin blocked active components of the alarm reaction (dashing,
610 bristling of dorsal fin spines), but not freezing, during exposure (Barreto, 2012). If erratic
611 swimming and geotaxis are indeed the main components of the alarm reaction during exposure,
612 the 5-HT_{2C} receptor could inhibit concurrent risk assessment (freezing) responses while
613 facilitating active escape/avoidance responses (erratic swimming).

614 We have found that, after exposure, CAS increases serotonin levels in the extracellular
615 fluid of the zebrafish brain (Maximino et al., 2014), an effect that can be mediated by serotonin
616 transporters (Maximino et al., 2014) and/or inhibition of monoamine oxidase activity (Lima-
617 Maximino et al., 2020). Regardless of the mechanism, increased serotonin levels *after* exposure
618 are suggested to encode an aversive expectation value, switching behavior toward cautious
619 exploration/risk assessment/anxiety when the aversive stimulus is no longer present (Lima-
620 Maximino et al., 2020). With increased serotonin levels, receptor antagonists are expected to
621 produce effects at this stage. This increase in serotonergic activity could activate 5-HT_{2C} and
622 other serotonergic receptors to inhibit ongoing defensive responses to proximal threat (e.g.,
623 freezing) and initiate programs of alertness and cautious exploration/risk assessment, including
624 geotaxis and erratic swimming (which are main components of the response during exposure).
625 However, while in the present experiments RS-102221, a 5-HT_{2C} receptor antagonist, blocked
626 increases in erratic swimming and geotaxis observed after exposure, 5-HT_{2C} receptor agonists
627 blocked freezing observed after exposure, contrary to what would be expected if that was true.
628 Moreover, 5-HT_{2C} receptors have been shown to inhibit 5-HT neuronal discharge in
629 anesthetized rats (Queree et al., 2009) through a regulatory loop mediated by GABAergic
630 neurons in the raphe, habenula, and/or frontal cortex (Sharp et al., 2007). Thus, the effects of
631 receptor agonists in this stage could represent the over-activation of this regulatory loop in
632 conditions of elevated serotonin levels. Moreover, other receptors can be implicated; indeed, in

633 zebrafish, both the 5-HT_2 receptor antagonist methysergide and the 5-HT1A receptor antagonist
634 WAY-100635 increased freezing and bottom-dwelling during CAS exposure and during a
635 recovery period (Nathan et al., 2015), suggesting that other receptors from the 5-HT_2 family or
636 the 5-HT_{1A} receptor (but see Maximino et al., 2014) control freezing behavior after exposure.
637 Thus, serotonergic signals emerging after exposure, representing negative expectation values
638 (Lima-Maximino et al., 2020), would modulate behavior through the 5-HT_{2C} receptor (inhibiting
639 active responses that would emerge in distal threat) and other receptors (facilitating freezing/risk
640 assessment responses that emerge in potential threat).

641 However, at least for the case of the 5-HT_{2C} receptor, the phasic signals do not appear to
642 represent all aversive values, as no effect of MK-212 was found for most ARS-elicited
643 responses, with the exception of freezing. Moreover, RS-102221 blocked the effects of ARS on
644 geotaxis. In rats, acute restraint stress increases serotonin release in the central amygdala, an
645 effect that is mediated by corticotropin-releasing factor receptors (Mo et al., 2008). In mce,
646 restraint stress for 45 min. increased serotonin turnover in the hippocampus, an effect blocked
647 by the 5-$HT2_{B/2C}$ receptor agonist RO 60-0175 (Mongeau et al, 2010). In Nile tilapia, acute
648 restraint stress (20 min) increased serotonin turnover in the dorsolateral and dorsomedial
649 telencephali (Silva et al., 2015), homologous to the hippocampus and frontotemporal amygdala
650 complex, respectively. Interestingly, the 5-HT_{2C} receptor antagonist SB 242084 blocked the
651 inhibitory effect of restraint stress on social interactions in mice (Mongeau et al., 2010). Thus,
652 the effects of RS-102221 on geotaxis could be mediated by blockade of 5-HT_{2C} receptors in the
653 forebrain, while the effects of MK-212 would be more likely to involve activation of the regulatory
654 loop. Our results suggest that the 5-HT_{2C} receptor is at least partially responsible for both
655 effects, acting as a “switch” between behavioral modes.

656 One potential limitation of behavioral pharmacological work in zebrafish is the relative
657 divergence between 5-HT_{2C}-like receptors in teleosts and mammals, which makes it difficult to

658 ascertain whether drugs which act as agonists and antagonists at mammalian 5-HT_{2C} receptors
659 will target zebrafish receptors. Using sequence alignment, it was found that residues that, in
660 human receptors, are crucial for ligand binding (and selectivity with the 5-HT_{2A} receptor, for
661 example; Canal et al., 2011; Córdova-Sintjago et al., 2012) are conserved in both zebrafish
662 proteins (HTR2Cl1 and HTR2Cl2). This suggests that ligand affinity is conserved for these
663 proteins, which strengthens the hypothesis that MK-212, WAY-161503, and RS-102221
664 targeted 5-HT_{2C} receptors in the behavioral experiments. Differences were found in residues
665 near the conserved binding sites, however; even in mammalian receptors, the role of these
666 residues in ligand affinity and/or efficacy is unknown.
667

668 4.3. Limitations and translational potential

669 While the present work presents results on zebrafish behavioral pharmacology inspired
670 on results from mammals, which can potentially underscore conserved mechanisms for
671 responses to threat across vertebrates (cf. Gerlai, 2014, for a discussion on how using fish as
672 well as mammals can decrease uncertainty regarding translational relevance and evolutionary
673 conservation of mechanisms), there are limitations in using zebrafish as experimental models in
674 neuroscience. One of them is that, due to the teleost genome duplication event, zebrafish
675 present two copies of the gene that codes the 5-HT_{2C} receptor (Schneider et al., 2012), and
676 therefore it is currently not possible to know which protein (HTR2CL1 or HTR2Cl2) was targeted
677 by the drugs used in the present experiments. Importantly, both proteins had conserved
678 residues at sites known to affect ligand affinity in the human 5-HT_{2C} receptor, and therefore both
679 are likely to have produced the effects. Moreover, while we propose that alarm reactions and
680 post-exposure behavior can be exploited to better understand panic disorder (Silva et al., 2020),
681 the paradigm that was used represents the normal, adaptive behavior of zebrafish under

threatening circumstances, and therefore cannot yet be used to generate novel hypotheses about a pathological organism (cf. Maximino and van der Staay, 2019, for a discussion on the limitations of using behavioral assays in experimental psychopathology).

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

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

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

Ballesteros, J. A., Jensen, A. D., Liapakis, G., Rasmussen, S. G., Shi, L., Gether, U., Javitch, J. A., 2001. Activation of the beta 2-adrenergic receptor involves disruption of an ionic lock between the cytoplasmic ends of transmembrane segments 3 and 6. J. Biol. Chem. 276, 29171-29177. https://doi.org/10.1074/jbc.M103747200

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

Canal, C. E., Cordova-Sintjago, T. C., Villa, N. Y., Fang, L.-J., & Booth, R. G. (2011). Drug discovery targeting human 5-HT2C receptors: Residues S3.36 and Y7.43 impact ligand —Binding pocket structure via hydrogen bond formation. Eur. J. Pharmacol., 673, 1–12. https://doi.org/10.1016/j.ejphar.2011.10.006

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.

Córdova-Sintjago, T., Sakhuja, R., Kondabolu, K., Canal, C. E., Booth, R. G., 2012. Molecular determinants of ligand binding at serotonin 5-HT_{2A} and 5-HT_{2C} GPCRs: Experimental affinity results analyzed by molecular modeling and ligand docking studies. Int. J. Quantum Chemistry, 112, 3807-3814. https://doi.org/Córdova-Sintjago, T., Sakhuja, R., Kondabolu, K., Canal, C. E., & Booth, R. G. (2012). Molecular determinants for ligand binding at serotonin 5-HT2Aand 5-HT2CGPCRs: Experimental affinity results analyzed by molecular modeling and ligand docking studies. International Journal of Quantum Chemistry, 112(24), 3807–3814. doi:10.1002/qua.24237

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

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

812 do Carmo Silva, R.X., Lima-Maximino, M.G., Maximino, C., 2018a. The aversive brain system of
813 teleosts: Implications for neuroscience and biological psychiatry. Neurosci. Biobehav.
814 Rev. 95, 123–135. https://doi.org/10.1016/j.neubiorev.2018.10.001
815 do Carmo Silva, R.X., Rocha, S.P., Lima-Maximino, M.G., Maximino, C., 2018b. Extraction of
816 alarm substance in zebrafish. protocols.io. https://doi.org/10.17504/protocols.io.tr3em8n
817 Genario, R., Abreu, M. S., Giacomini, A. C. V. V., Demin, K. A., Kalueff, A. V. Sex differences in
818 behavior and neuropharmacology of zebrafish. Eur. J. Neurosci. 51, 2586– 2603.
819 https://doi.org/10.1111/ejn.14438
820 Gerlai. R., 2014. Fish in behavior research: Unique tools with a great promise! J. Neurosci.
821 Methods 234, 54-58. https://doi.or/g10.1016/j.jneumeth.2014.04.015
822 Ghisleni, G., Capiotti, K.M., Da Silva, R.S., Oses, J.P., Piato, Â.L., Soares, V., Bogo, M.R.,
823 Bonan, C.D., 2012. The role of CRH in behavioral responses to acute restraint stress in
824 zebrafish. Prog. Neuropsychopharmacol. Biol. Psychiatry 36, 176–182.
825 https://doi.org/10.1016/j.pnpbp.2011.08.016
826 Gomes, F., Greidinger, M., Salviano, M., Couto, K.C., Scaperlli, G.F., Alves, S.H. de S., Cruz,
827 A.P. de M., 2010. Antidepressant- and anxiogenic-like effects of acute 5-HT2C receptor
828 activation in rats exposed to the forced swim test and elevated plus maze. Psychol. &
829 Neurosci. 3, 245–249. https://doi.org/10.3922/j.psns.2010.2.014
830 Goodwin, N., Karp, N. A., Blackledge, S., Clark, B., Keeble, R., Kovacs, C., Murray, K. N., Price,
831 M., Thompson, P., Bussell J., 2016. Standardized welfare terms for the zebrafish
832 community. Zebrafish 13, Suppl. 1, S164-S168. https://doi.org/10.1089/zeb.2016.1248
833 Graeff, F.G., 2004. Serotonin, the periaqueductal gray and panic. Neurosci. Biobehav. Rev. 28,
834 239–259. https://doi.org/10.1016/j.neubiorev.2003.12.004
835 Graeff, F.G., Brandão, M.L., Audi, E.A., Schütz, M.T.B., 1986. Modulation of the brain aversive
836 system by GABAergic and serotonergic mechanisms. Behav. Brain Res. 22, 173–180.
837 https://doi.org/10.1016/0166-4328(86)90038-0
838 Herculano, A.M., Maximino, C., 2014. Serotonergic modulation of zebrafish behavior: Towards a
839 paradox. Prog. Neuropsychopharmacol. Biol. Psychiatry, Special Issue: Zebrafish
840 models of brain disorders 55, 50–66. https://doi.org/10.1016/j.pnpbp.2014.03.008
841 Höglund, E., Weltzien, F.-A., Schjolden, J., Winberg, S., Ursin, H., Døving, K.B., 2005.
842 Avoidance behavior and brain monoamines in fish. Brain Res. 1032, 104–110.
843 https://doi.org/10.1016/j.brainres.2004.10.050
844 Kennett, G.A., Wood, M.D., Bright, F., Trail, B., Riley, G., Holland, V., Avenell, K.Y., Stean, T.,

845 Upton, N., Bromidge, S., Forbes, I.T., Brown, A.M., Middlemiss, D.N., Blackburn, T.P.,
846 1997. SB 242084, a Selective and Brain Penetrant 5-HT2C Receptor Antagonist.
847 Neuropharmacology 36, 609–620. https://doi.org/10.1016/S0028-3908(97)00038-5
848 Kinkel, M.D., Eames, S.C., Philipson, L.H., Prince, V.E., 2010. Intraperitoneal Injection into
849 Adult Zebrafish. JoVE J. Vis. Exp. e2126. https://doi.org/10.3791/2126
850 Knight, A.R., Misra, A., Quirk, K., Benwell, K., Revell, D., Kennett, G., Bickerdike, M., 2004.
851 Pharmacological characterisation of the agonist radioligand binding site of 5-HT2A, 5-
852 HT2B and 5-HT2C receptors. Naunyn. Schmiedebergs Arch. Pharmacol. 370, 114–123.
853 https://doi.org/10.1007/s00210-004-0951-4
854 Kuznetsova, E.G., Amstislavskaya, T.G., Shefer, E.A., Popova, N.K., 2006. Effect of 5-HT2C
855 receptor antagonist RS 102221 on mouse behavior. Bull. Exp. Biol. Med. 142, 76–79.
856 https://doi.org/10.1007/s10517-006-0296-8
857 Lima-Maximino, M., Pyterson, M.P., Silva, R.X. do C., Gomes, G.C.V., Rocha, S.P., Herculano,
858 A.M., Rosemberg, D.B., Maximino, C., 2020. Phasic and tonic serotonin modulate alarm
859 reactions and post-exposure behavior in zebrafish. J. Neurochem. 153, 495–509.
860 https://doi.org/10.1111/jnc.14978
861 Macedo, C.E., Martinez, R.C.R., Albrechet-Souza, L., Molina, V.A., Brandão, M.L., 2007. 5-
862 HT2- and D1-mechanisms of the basolateral nucleus of the amygdala enhance
863 conditioned fear and impair unconditioned fear. Behav. Brain Res. 177, 100–108.
864 https://doi.org/10.1016/j.bbr.2006.10.031
865 Martin, J.R., Ballard, T.M., Higgins, G.A., 2002. Influence of the 5-HT2C receptor antagonist,
866 SB-242084, in tests of anxiety. Pharmacol. Biochem. Behav., Serotonin: Pharmacology,
867 Biochemistry & Behaviour 71, 615–625. https://doi.org/10.1016/S0091-3057(01)00713-4
868 Mathuru, A.S., Kibat, C., Cheong, W.F., Shui, G., Wenk, M.R., Friedrich, R.W., Jesuthasan, S.,
869 2012. Chondroitin Fragments Are Odorants that Trigger Fear Behavior in Fish. Curr.
870 Biol. 22, 538–544. https://doi.org/10.1016/j.cub.2012.01.061
871 Matthews, M., Varga, Z.M., 2012. Anesthesia and Euthanasia in Zebrafish. ILAR J. 53, 192–
872 204. https://doi.org/10.1093/ilar.53.2.192
873 Maximino, C., van der Staay, J., 2019. Behavioral models in psychopathology: Epistemic and
874 semantic considerations. Behav. Brain Funct. 15, 1. https://doi.org/10.1186/s12993-019-
875 0152-4
876 Maximino, C., do Carmo Silva, R.X., Campos, K. dos S., Oliveira, J.S. de, Rocha, S.P.,
877 Pyterson, M.P., Souza, D.P. dos S., Feitosa, L.M., Ikeda, S.R., Pimentel, A.F.N., Ramos,

878 P.N.F., Costa, B.P.D., Herculano, A.M., Rosemberg, D.B., Siqueira-Silva, D.H., Lima-
879 Maximino, M., 2019. Sensory ecology of ostariophysan alarm substances. J. Fish Biol.
880 95, 274–286. https://doi.org/10.1111/jfb.13844
881 Maximino, C., Lima, M.G., Costa, C.C., Guedes, I.M.L., Herculano, A.M., 2014. Fluoxetine and
882 WAY 100,635 dissociate increases in scototaxis and analgesia induced by conspecific
883 alarm substance in zebrafish (*Danio rerio* Hamilton 1822). Pharmacol. Biochem. Behav.
884 124, 425–433. https://doi.org/10.1016/j.pbb.2014.07.003
885 Mo, B., Feng, N., Renner, K., Forster, G., 2008. Mo, B., Feng, N., Renner, K., & Forster, G.
886 (2008). Restraint stress increases serotonin release in the central nucleus of the
887 amygdala via activation of corticotropin-releasing factor receptors. Brain Res. Bul. 76,
888 493–498. https://doi.org/10.1016/j.brainresbull.2008.02.011
889 Mora, P.O., Netto, C.F., Graeff, F.G., 1997. Role of 5-HT2A and 5-HT2C Receptor Subtypes in
890 the Two Types of Fear Generated by the Elevated T-Maze. Pharmacol. Biochem.
891 Behav. 58, 1051–1057. https://doi.org/10.1016/S0091-3057(97)00057-9
892 Nathan, F.M., Ogawa, S., Parhar, I.S., 2015. Kisspeptin1 modulates odorant-evoked fear
893 response via two serotonin receptor subtypes (5-HT1A and 5-HT2) in zebrafish. J.
894 Neurochem. 133, 870–878. https://doi.org/10.1111/jnc.13105
895 Nunes-de-Souza, V., Nunes-de-Souza, R.L., Rodgers, R.J., Canto-de-Souza, A., 2008. 5-HT2
896 receptor activation in the midbrain periaqueductal grey (PAG) reduces anxiety-like
897 behaviour in mice. Behav. Brain Res. 187, 72–79.
898 https://doi.org/10.1016/j.bbr.2007.08.030
899 Oliveira, L.C., Broiz, A.C., de Macedo, C.E., Landeira-Fernandez, J., Brandão, M.L., 2007. 5-
900 HT2 receptor mechanisms of the dorsal periaqueductal gray in the conditioned and
901 unconditioned fear in rats. Psychopharmacology (Berl.) 191, 253–262.
902 https://doi.org/10.1007/s00213-006-0653-3
903 Parra, K.V., Adrian, J.C., Gerlai, R., 2009. The synthetic substance hypoxanthine 3-N-oxide
904 elicits alarm reactions in zebrafish (Danio rerio). Behav. Brain Res. 205, 336–341.
905 https://doi.org/10.1016/j.bbr.2009.06.037
906 Perusini, J.N., Fanselow, M.S., 2015. Neurobehavioral perspectives on the distinction between
907 fear and anxiety. Learn. Mem. 22, 417–425. https://doi.org/10.1101/lm.039180.115
908 Petrovich, G. D., Lougee, M. A., 2011. Sex differences in fear-induced feeding cessation:
909 Prolonged effect in female rats. Physiol. Behav. 104, 996-1101.
910 https://doi.org/10.1016/j.physbeh.2011.06.020

911 Piato, A.L., Rosemberg, D.B., Capiotti, K.M., Siebel, A.M., Herrmann, A.P., Ghisleni, G., Vianna,
912 M.R., Bogo, M.R., Lara, D.R., Bonan, C.D., 2011. Acute Restraint Stress in Zebrafish:
913 Behavioral Parameters and Purinergic Signaling. Neurochem. Res. 36, 1876.
914 https://doi.org/10.1007/s11064-011-0509-z
915 Quadros, V.A., Costa, F.V., Canzian, J., Nogueira, C.W., Rosemberg, D.B., 2018. Modulatory
916 role of conspecific alarm substance on aggression and brain monoamine oxidase activity
917 in two zebrafish populations. Prog. Neuropsychopharmacol. Biol. Psychiatry 86, 322–
918 330. https://doi.org/10.1016/j.pnpbp.2018.03.018
919 Quadros, V.A., Silveira, A., Giuliani, G.S., Didonet, F., Silveira, A.S., Nunes, M.E., Silva, T.O.,
920 Loro, V.L., Rosemberg, D.B., 2016. Strain- and context-dependent behavioural
921 responses of acute alarm substance exposure in zebrafish. Behav. Processes 122, 1–
922 11. https://doi.org/10.1016/j.beproc.2015.10.014
923 Queree, P., Peters, S., Sharp, T., 2009. Further pharmacological characterization of 5-HT(2C)
924 receptor agonist-induced inhibition of 5-HT neuronal activity in the dorsal raphe nucleus
925 in vivo. Br. J. Pharmacol., 158, 1477–1485. https://doi.org/10.1111/j.1476-
926 5381.2009.00406.x
927 Rosenzweig-Lipson, S., Zhang, J., Mazandarani, H., Harrison, B.L., Sabb, A., Sabalski, J.,
928 Stack, G., Welmaker, G., Barrett, J.E., Dunlop, J., 2006. Antiobesity-like effects of the 5-
929 HT2C receptor agonist WAY-161503. Brain Res. 1073–1074, 240–251.
930 https://doi.org/10.1016/j.brainres.2005.12.052
931 Salchner, P., Singewald, N., 2006. 5-HT receptor subtypes involved in the anxiogenic-like action
932 and associated Fos response of acute fluoxetine treatment in rats. Psychopharmacology
933 (Berl.) 185, 282–288. https://doi.org/10.1007/s00213-005-0247-5
934 Schneider, H., Fritzky, L., Williams, J., Heumann, C., Yochum, M., Pattar, K. Noppert, G., Mock,
935 V., Hawley, E., 2012. Cloning and expression of a zebrafish 5-HT_{2C} receptor gene. Gene
936 502, 108-117. https://doi.org/10.1016/j.gene.2012.03.070
937 Sharp, T., Boothman, L., Raley, J., Queree, P., 2007. Important messages in the 'post': Recent
938 discoveries in 5-HT neurone feedback control. Trends Pharmacol. Sci., 28, 629-635.
939 https://doi.org/10.1016/j.tips.2007.10.009
940 Silva, P.I.M., Martins, C.I.M., Khan, U.W., Gjøen, H.M., Øverli, Ø., Höglund, E., 2015. Stress
941 and fear responses in the teleost pallium. Physiol. Behav. 141, 17–22.
942 https://doi.org/10.1016/j.physbeh.2014.12.020
943 Silva, R.X. do C., Rocha, S.P., Herculano, A.M., Lima-Maximino, M.G., Maximino, C., 2020.

Animal models for panic disorder. Psychol. Neurosci. 13, 1–18. https://doi.org/10.1037/pne0000177

Smith, R.J.F., 1992. Alarm signals in fishes. Rev. Fish Biol. Fish. 2, 33–63. https://doi.org/10.1007/BF00042916

Sourbron, J., Schneider, H., Kecskés, A., Liu, Y., Buening, E.M., Lagae, L., Smolders, I., de Witte, P., 2016. Serotonergic Modulation as Effective Treatment for Dravet Syndrome in a Zebrafish Mutant Model. ACS Chem. Neurosci. 7, 588–598. https://doi.org/10.1021/acschemneuro.5b00342

Speedie, N., Gerlai, R., 2008. Alarm substance induced behavioral responses in zebrafish (Danio rerio). Behav. Brain Res. 188, 168–177. https://doi.org/10.1016/j.bbr.2007.10.031

von Frisch, K., 1938. Zur Psychologie des Fisch-Schwarmes. Naturwissenschaften 26, 601–606. https://doi.org/10.1007/BF01590598

Wolf, K., 1963. Physiological Salines for Fresh-Water Teleosts. Progress. Fish-Cult. 25, 135–140. 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 [distal threat] and post-exposure behavior [potential threat]) and (B) experiment 2 (ARS-elicited behavioral responses [proximal threat]). *Abbreviations:* ARS – acute restraint stress; CAS – conspecific alarm substance; VEH – vehicle

Figure 2 – Effects of MK-212 on behavior <u>during</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; 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 change 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 <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; 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 change 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 <u>during</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

988 differences at the $p < 0.05$ level; similar letters indicate lack of statistically significant
989 differences. Data are presented as individual data points (dots) superimposed over the
990 median ± interquartile ranges. Dashed lines on panels A and B represent change levels.
991 Dots connected by lines represent group means. CTRL = controls (water-exposed
992 animals); CAS = conspecific alarm substance. Final sample sizes: CTRL + VEH: n = 10
993 animals; CTRL + 1 mg/kg WAY-161503: n = 21 animals; CAS + VEH: n = 21 animals;
994 CAS + 1 mg/kg WAY-161503: n = 21 animals.
995 Figure 5 – Effects of WAY-161503 on behavior <u>after</u> CAS exposure. (A) Time spent on top third
996 of the tank. (B) Time spent on bottom third of the tank. (C) Erratic swimming. (D) Total
997 time spent freezing. (E) Swimming speed. Different letters represent statistical
998 differences at the $p < 0.05$ level; similar letters indicate lack of statistically significant
999 differences. Data are presented as individual data points (dots) superimposed over the
1000 median ± interquartile ranges. Dashed lines on panels A and B represent change levels.
1001 Dots connected by lines represent group means. CTRL = controls (water-exposed
1002 animals); CAS = conspecific alarm substance. Final sample sizes: CTRL + VEH: n = 10
1003 animals; CTRL + 1 mg/kg WAY-161503: n = 21 animals; CAS + VEH: n = 21 animals;
1004 CAS + 1 mg/kg WAY-161503: n = 21 animals.
1005 Figure 6 – Effects of RS-102221 on behavior <u>during</u> CAS exposure. (A) Time spent on top third
1006 of the tank. (B) Time spent on bottom third of the tank. (C) Erratic swimming. (D) Total
1007 time spent freezing. (E) Swimming speed. Different letters represent statistical
1008 differences at the $p < 0.05$ level; similar letters indicate lack of statistically significant
1009 differences. Data are presented as individual data points (dots) superimposed over the
1010 median ± interquartile ranges. Dashed lines on panels A and B represent change levels.
1011 Dots connected by lines represent group means. CTRL = controls (water-exposed
1012 animals); CAS = conspecific alarm substance. Final sample sizes: CTRL + VEH: n = 10
1013 animals; CTRL + 2 mg/kg RS-102221: n = 21 animals; CAS + VEH: n = 20 animals; CAS
1014 + 2 mg/kg RS-102221: n = 21 animals.
1015 Figure 7 – Effects of RS-102221 on behavior <u>after</u> CAS exposure. (A) Time spent on top third of
1016 the tank. (B) Time spent on bottom third of the tank. (C) Erratic swimming. (D) Total time
1017 spent freezing. (E) Swimming speed. Different letters represent statistical differences at
1018 the $p < 0.05$ level; similar letters indicate lack of statistically significant differences. Data
1019 are presented as individual data points (dots) superimposed over the median ±
1020 interquartile ranges. Dashed lines on panels A and B represent change levels. Dots

1021 connected by lines represent group means. CTRL = controls (water-exposed animals);
1022 CAS = conspecific alarm substance. Final sample sizes: CTRL + VEH: n = 10 animals;
1023 CTRL + 2 mg/kg RS-102221: n = 21 animals; CAS + VEH: n = 20 animals; CAS + 2
1024 mg/kg RS-102221: n = 21 animals.
1025 Figure 8 – Effects of MK-212 on behavior after acute restraint stress (ARS). (A) Time spent on
1026 top third of the tank. (B) Time spent on bottom third of the tank. (C) Erratic swimming.
1027 (D) Total time spent freezing. (E) Swimming speed. Different letters represent statistical
1028 differences at the $p < 0.05$ level; similar letters indicate lack of statistically significant
1029 differences. Data are presented as individual data points (dots) superimposed over the
1030 median ± interquartile ranges. Dashed lines on panels A and B represent change levels.
1031 Dots connected by lines represent group means. CTRL = controls (water-exposed
1032 animals); ARS = acute restraint stress. Final sample sizes: CTRL + VEH: n = 8 animals;
1033 CTRL + 2 mg/kg MK-212: n = 5 animals; ARS + VEH: n = 6 animals; ARS + 2 mg/kg
1034 MK-212: n = 8 animals.
1035 Figure 9 – Effects of RS-102221 on behavior after acute restraint stress (ARS). (A) Time spent
1036 on top third of the tank. (B) Time spent on bottom third of the tank. (C) Erratic swimming.
1037 (D) Total time spent freezing. (E) Swimming speed. Different letters represent statistical
1038 differences at the $p < 0.05$ level; similar letters indicate lack of statistically significant
1039 differences. Data are presented as individual data points (dots) superimposed over the
1040 median ± interquartile ranges. Dashed lines on panels A and B represent change levels.
1041 Dots connected by lines represent group means. CTRL = controls (water-exposed
1042 animals); ARS = acute restraint stress. Final sample sizes: CTRL + VEH: n = 8 animals;
1043 CTRL + 2 mg/kg RS-102221: n = 8 animals; ARS + VEH: n = 8 animals; ARS + 2 mg/kg
1044 RS-102221: n = 8 animals.
1045 Figure 10 – Amino acid sequences for zebrafish 5-HT_{2C}RL1 and 5-HT_{2C}RL2 aligned with
1046 sequences of mice (*Mus*) and humans (*Homo*). Residues associated with the binding
1047 site are labeled above the sequences (D3.32, S3.36, Y7.43), according to the
1048 Ballesteros et al. (2001) nomenclature. Colors code physicochemical properties: red,
1049 small amino acids+hydrophobic amino acids (including aromatic amino acids except
1050 tyrosine [Y]); blue, acidic amino acids; magenta, basic amino acids except histidine [H];
1051 green, hydroxyl amino acids+sulfhydryl amino acids+amine+glycine. Asteriks (*) show
1052 residues which are conserved across all four sequences, while colons (:) indicate
1053 conservation between groups scoring > 0.5 in the Gonnet PAM 250 matrix, and periods

1054 (.) indicates conservation between groups scoring < 0.5 in the Gonnet PAM 250 matrix.

Tables and table captions

Table 1 – Full ANOVA results for effects of MK-212 on behavioral endpoints during CAS exposure.

Context	**Drug**	**Endpoint**	**Effect** [df, df2]	**F**	**p-value**	**Effect size (ω^2)**
During exposure	MK-212 (5-HT_{2C}R agonist)	Time on top (s)	Treatment [1, 114]	6.337	0.0132	0.039
			Dose [2, 114]	3.665	0.0287	0.039
			Interaction [2, 114]	4.602	0.012	0.052
		Time on bottom (s)	Treatment [1,114]	20.995	1.18×10^{-5}	0.12
			Dose [2, 114]	11.455	2.93×10^{-5}	0.125
			Interaction [2, 114]	4.085	0.0194	0.037
		Erratic swiming (abs. turn angle)	Treatment [1,114]	5.137	0.0253	$\omega^2 = 0.017$
			Dose [2, 114]	2.948	0.0565	0.016
			Interaction [2, 114]	57.101	$< 2 \times 10^{-16}$	0.467
		Freezing (s)	Treatment [1,114]	15.219	0.000162	0.105
			Dose [2, 114]	1.037	0.358	0.001
			Interaction [2, 114]	1.662	0.194	0.01
		Swimming speed (cm/s)	Treatment [1,114]	0.005	0.9459	-0.008
			Dose [2, 114]	0.211	0.8105	0.013
			Interaction [2, 114]	4.652	0.0114	0.059

1058

1059 Table 2 – Full ANOVA results for effects of MK-212 on behavioral endpoints after CAS

1060 exposure.

Context	**Drug**	**Endpoint**	**Effect** [df, df2]	**F**	**p-value**	**Effect size (ω^2)**
After exposure	MK-212 (5-HT_{2C}R agonist)	Time on top (s)	Treatment [1, 114]	0.945	0.333	0.0
			Dose [2, 114]	4.892	0.009	0.06
			Interaction [2, 114]	2.393	0.0959	0.021
		Time on bottom (s)	Treatment [1,114]	7.751	0.0063	0.048
			Dose [2, 114]	5.385	0.0058	
			Interaction [2, 114]	4.203	0.0173	0.045
		Erratic swiming (abs. turn angle)	Treatment [1,114]	3.641	0.0589	0.018
			Dose [2, 114]	9.244	0.00019	0.112
			Interaction [2, 114]	4.951	0.00867	0.054
		Freezing (s)	Treatment [1,114]	4.658	0.033	0.025
			Dose [2, 114]	11.617	2.56×10^{-5}	0.143
			Interaction [2, 114]	2.777	0.0664	0.024
		Swimming speed (cm/s)	Treatment [1,114]	0.111	0.74	-0.007
			Dose [2, 114]	1.339	0.266	0.006
			Interaction [2, 114]	0.981	0.378	0.0

1061

1062

1063 Table 3 – Full ANOVA results for effects of WAY-161503 on behavioral endpoints during CAS
1064 exposure.

Context	**Drug**	**Endpoint**	**Effect** **[df1, df2]**	**F**	**P-value**	**Effect size** **(ω^2)**
During exposure	WAY-161503 (5-HT_{2C}R agonist)	Time on top (s)	Treatment [1, 69]	7.404	0.00823	0.08
			Drug [1, 69]	1.751	0.19011	0.009
			Interaction [1, 69]	1.001	0.32067	0.0
		Time on bottom (s)	Treatment [1, 69]	10.842	0.00157	0.103
			Drug [1, 69]	10.138	0.00218	0.096
			Interaction [1, 69]	4.614	0.03523	0.038
		Erratic swiming (abs. turn angle)	Treatment [1, 69]	108.37	< 0.0001	0.311
			Drug [1, 69]	122.99	< 0.0001	0.353
			Interaction [1, 69]	44.24	< 0.0001	0.125
		Freezing (s)	Treatment [1, 69]	8.582	0.0046	0.094
			Drug [1, 69]	0.564	0.4552	-0.005
			Interaction [1, 69]	1.434	0.2352	0.005
		Swimming speed (cm/s)	Treatment [1, 69]	2.303	0.134	0.018
			Drug [1, 69]	0.103	0.749	-0.012
			Interaction [1, 69]	0.527	0.471	-0.006

1065

1066 Table 4 – Full ANOVA results for effects of WAY-161503 on behavioral endpoints after CAS
1067 exposure.

Context	**Drug**	**Endpoint**	**Effect** **[df1, df2]**	**F**	**P-value**	**Effect size** **(ω^2)**
After exposure	WAY-161503 (5-HT_{2C}R agonist)	Time on top (s)	Treatment [1, 69]	3.221	0.0771	0.026
			Drug [1, 69]	4.254	0.0429	0.039
			Interaction [1, 69]	0.039 6.352	0.014	0.064
		Time on bottom (s)	Treatment [1, 69]	1.95	0.1671	0.012
			Drug [1, 69]	4.107	0.0466	0.039
			Interaction [1, 69]	3.267	0.0751	0.029
		Erratic swiming (abs. turn angle)	Treatment [1, 69]	16.119	0.000149	0.174
			Drug [1, 69]	0.568	0.454	-0.005
			Interaction [1, 69]	0.042	0.838	-0.011
		Freezing (s)	Treatment [1, 69]	4.726	0.0331	0.43
			Drug [1, 69]	4.17	0.04497	0.037
			Interaction [1, 69]	7.663	0.00723	0.077
		Swimming speed (cm/s)	Treatment [1, 69]	3.85	0.0538	0.038
			Drug [1, 69]	0.416	0.5212	-0.008
			Interaction [1, 69]	0.062	0.8037	-0.013

1068

1069 Table 5 – Full ANOVA results for effects of RS-102221 on behavioral endpoints during CAS
1070 exposure.

Context	**Drug**	**Endpoint**	**Effect** **[df1, df2]**	**F**	**P-value**	**Effect size** **(ω^2)**
During exposure	RS-102221 (5-HT_{2C}R antagonist)	Time on top (s)	Treatment [1, 68]	9.68	0.003	0.011
			Drug [1, 68]	0.231	0.632	-0.01
			Interaction [1, 68]	0.096	0.757	-0.012
		Time on bottom (s)	Treatment [1, 68]	85.978	< 0.0001	0.53
			Drug [1, 68]	0.352	0.555	-0.009
			Interaction [1, 68]	0.517	0.474	-0.006
		Erratic swiming (abs. turn angle)	Treatment [1, 68]	25.74	< 0.0001	0.26
			Drug [1, 68]	21.06	< 0.0001	0.22
			Interaction [1, 68]	15.47	< 0.0001	0.17
		Freezing (s)	Treatment [1, 68]	21.973	< 0.0001	0.23
			Drug [1, 68]	7.06	0.0098	0.08
			Interaction [1, 68]	2.98	0.089	0.03
		Swimming speed (cm/s)	Treatment [1, 68]	1.84	0.179	0.01
			Drug [1, 68]	3.156	0.08	0.03
			Interaction [1, 68]	2.981	0.089	0.03

1071

1072 Table 6 – Full ANOVA results for effects of RS-102221 on behavioral endpoints after CAS
1073 exposure.

Context	**Drug**	**Endpoint**	**Effect** **[df1, df2]**	**F**	**P-value**	**Effect size** **(ω^2)**
After exposure	RS-102221 (5-HT_{2C}R antagonist)	Time on top (s)	Treatment [1, 68]	2.449	0.122	0.02
			Drug [1, 68]	0.003	0.969	-0.01
			Interaction [1, 68]	1.152	0.287	0.002
		Time on bottom (s)	Treatment [1, 68]	6.322	0.0143	0.07
			Drug [1, 68]	22.796	< 0.0001	0.23
			Interaction [1, 68]	4.08	0.047	0.04
		Erratic swiming (abs. turn angle)	Treatment [1, 68]	36.19	< 0.0001	0.33
			Drug [1, 68]	2.957	0.09	0.03
			Interaction [1, 68]	0.002	0.965	-0.01
		Freezing (s)	Treatment [1, 68]	39.763	< 0.0001	0.35
			Drug [1, 68]	4.16	0.045	0.04
			Interaction [1, 68]	3.23	0.077	0.03
		Swimming speed (cm/s)	Treatment [1, 68]	1.014	0.317	0.0002
			Drug [1, 68]	1.83	0.181	0.01
			Interaction [1, 68]	0.475	0.493	-0.008

1074

1075 Table 7 – Full ANOVA results for effects of MK-212 on behavioral endpoints after restraint
1076 stress.

Context	**Drug**	**Endpoint**	**Effect** **[df1, df2]**	**F**	**P-value**	**Effect size** **(ω^2)**
After restraint stress	MK-212 (5-HT_{2C}R agonist)	Time on top (s)	Treatment [1, 23]	0.167	0.687	-0.026
			Drug [1, 23]	3.884	0.061	0.089
			Interaction [1, 23]	4.215	0.052	0.1
		Time on bottom (s)	Treatment [1, 23]	6.3864	0.019	0.099
			Drug [1, 23]	0.0879	0.77	-0.017
			Interaction [1, 23]	8.5023	0.008	0.22
		Erratic swiming (abs. turn angle)	Treatment [1, 23]	17.9732	0.00031	0.398
			Drug [1, 23]	0.446	0.511	0.013
			Interaction [1, 23]	0.193	0.664	-0.019
		Freezing (s)	Treatment [1, 23]	0.384	0.541	-0.016
			Drug [1, 23]	0.618	0.44	-0.01
			Interaction [1, 23]	12.339	0.002	0.304
		Swimming speed (cm/s)	Treatment [1, 23]	1.0005	0.328	0.0
			Drug [1, 23]	0.0283	0.868	-0.038
			Interaction [1, 23]	0.5437	0.468	-0.018

1077

1078 Table 8 – Full ANOVA results for effects of RS-102221 on behavioral endpoints after restraint
1079 stress.
1080

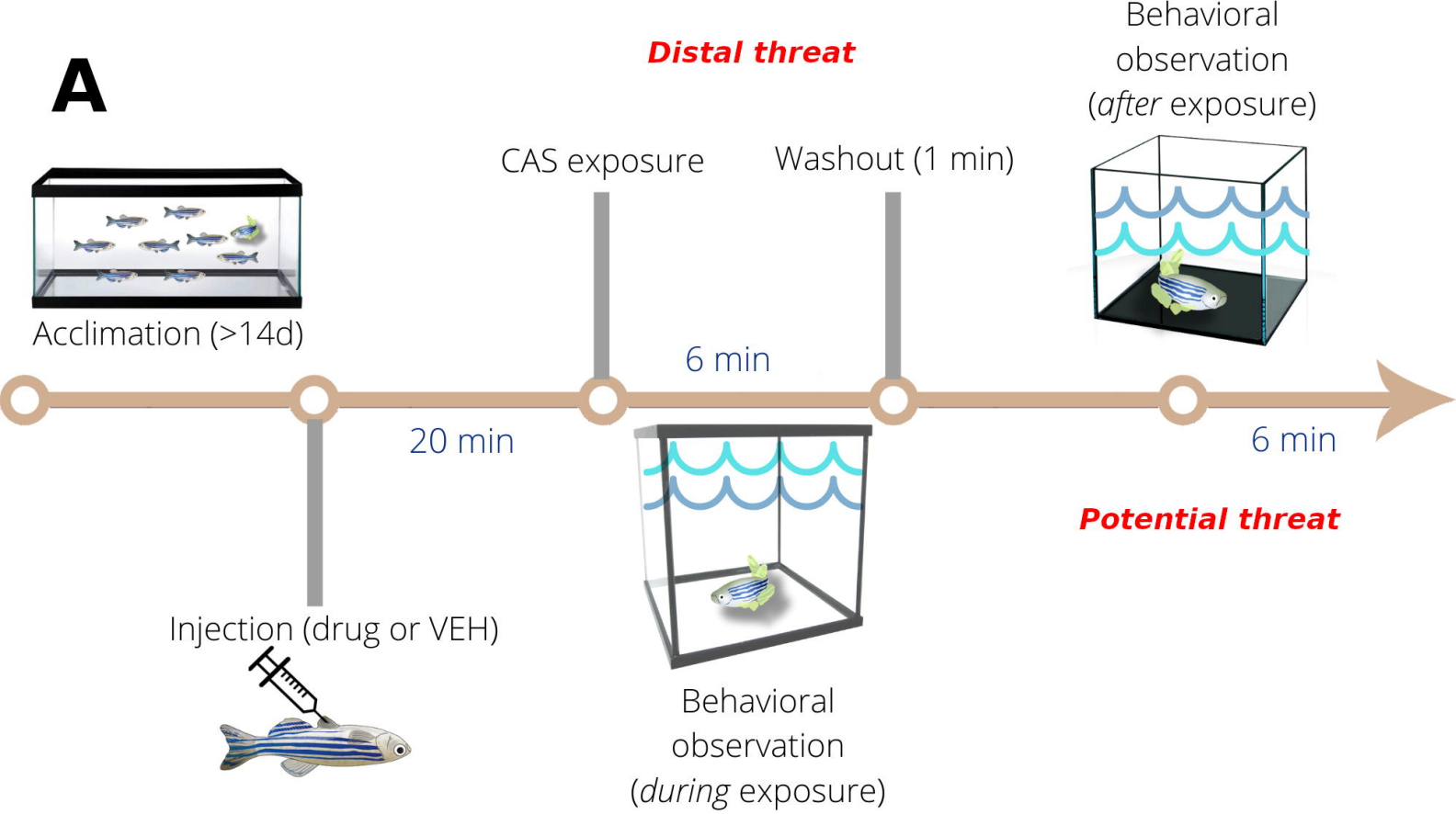
Context	**Drug**	**Endpoint**	**Effect** **[df1, df2]**	**F**	**P-value**	**Effect size** **(ω^2)**
After restraint stress	RS-102221 (5-HT_{2C}R antagonist)	Time on top (s)	Treatment [1, 23]	422.613	< 0.0001	0.93
			Drug [1, 23]	0.728	0.401	-0.009
			Interaction [1, 23]	0.416	0.524	-0.02
		Time on bottom (s)	Treatment [1, 23]	3.051	0.0916	0.06
			Drug [1, 23]	2.208	0.1485	0.04
			Interaction [1, 23]	10.832	0.0027	0.24
		Erratic swiming (abs. turn angle)	Treatment [1, 23]	0.014	0.905	-0.03
			Drug [1, 23]	0.224	0.639	-0.02
			Interaction [1, 23]	2.916	0.098	0.06
		Freezing (s)	Treatment [1, 23]	13.564	0.001	0.28
			Drug [1, 23]	0.122	0.729	-0.03
			Interaction [1, 23]	0.022	0.882	-0.03
		Swimming speed (cm/s)	Treatment [1, 23]	1.833	0.187	0.03
			Drug [1, 23]	0.003	0.955	-0.03
			Interaction [1, 23]	0.051	0.823	-0.03

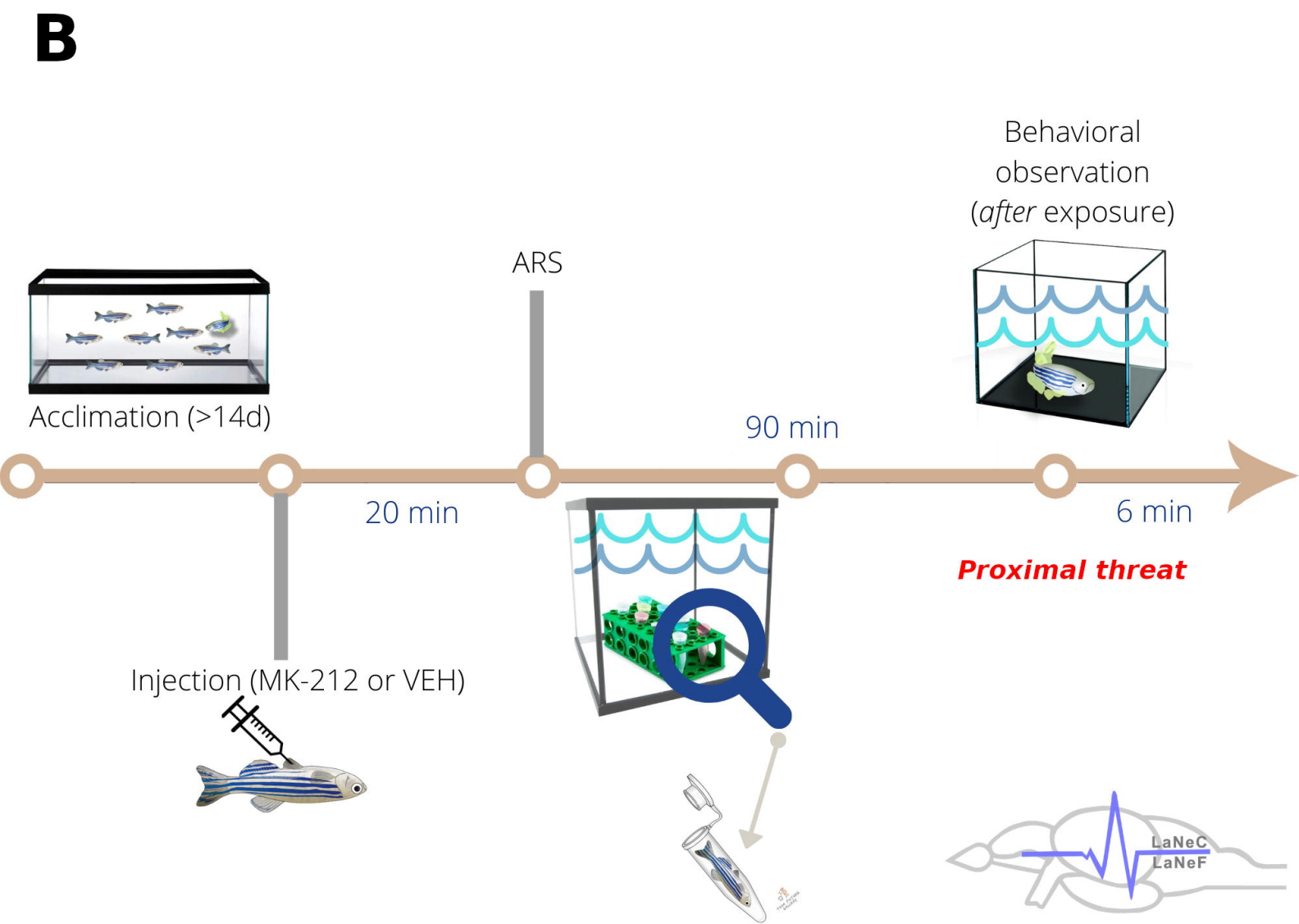
1081

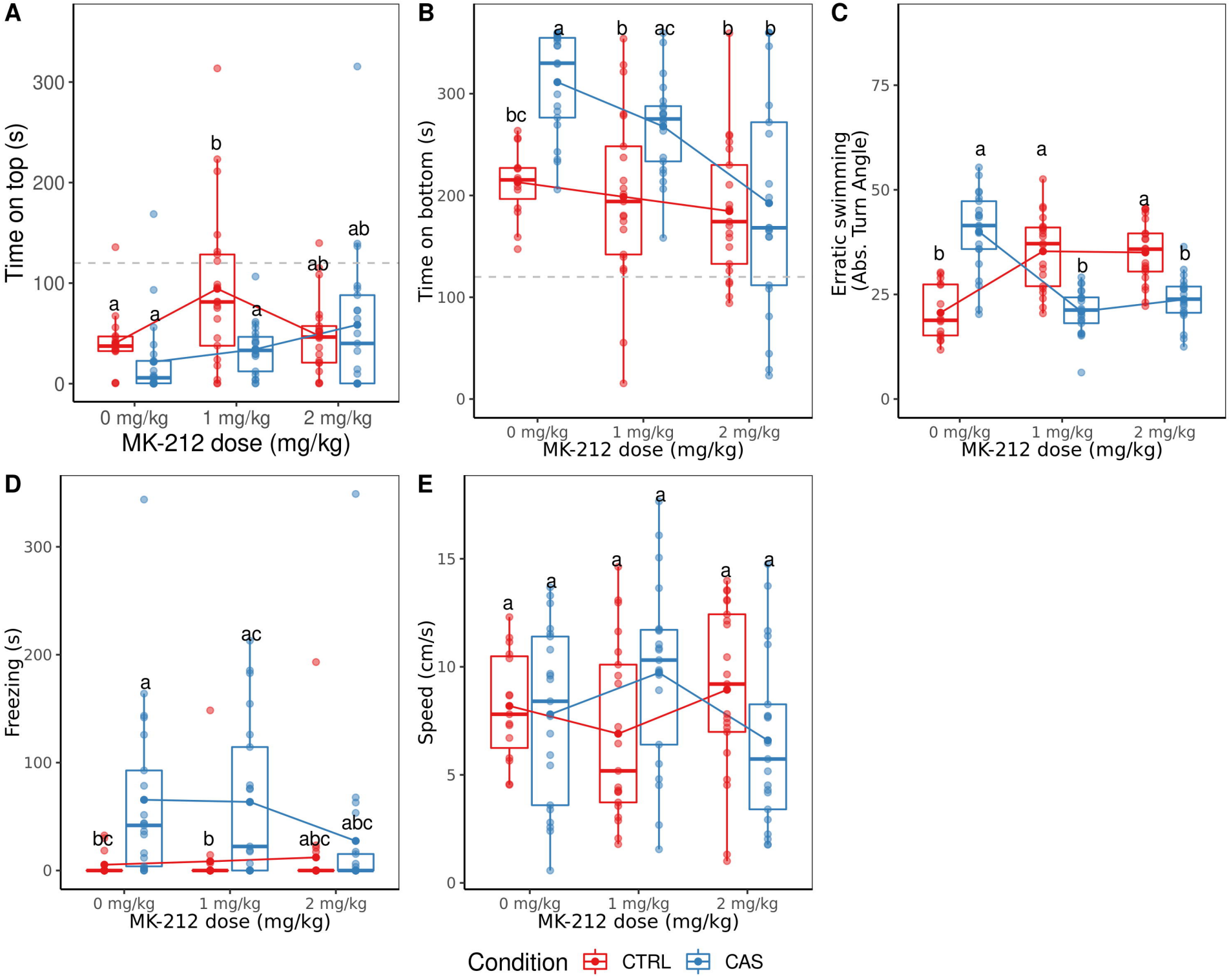
1082 Table 9 – Behavioral effects of 5-HT2C receptor agonists (MK-212 and WAY-161503) and
1083 antagonists (RS-102221) on behavior during and after CAS exposure, and after ARS in adult
1084 zebrafish.

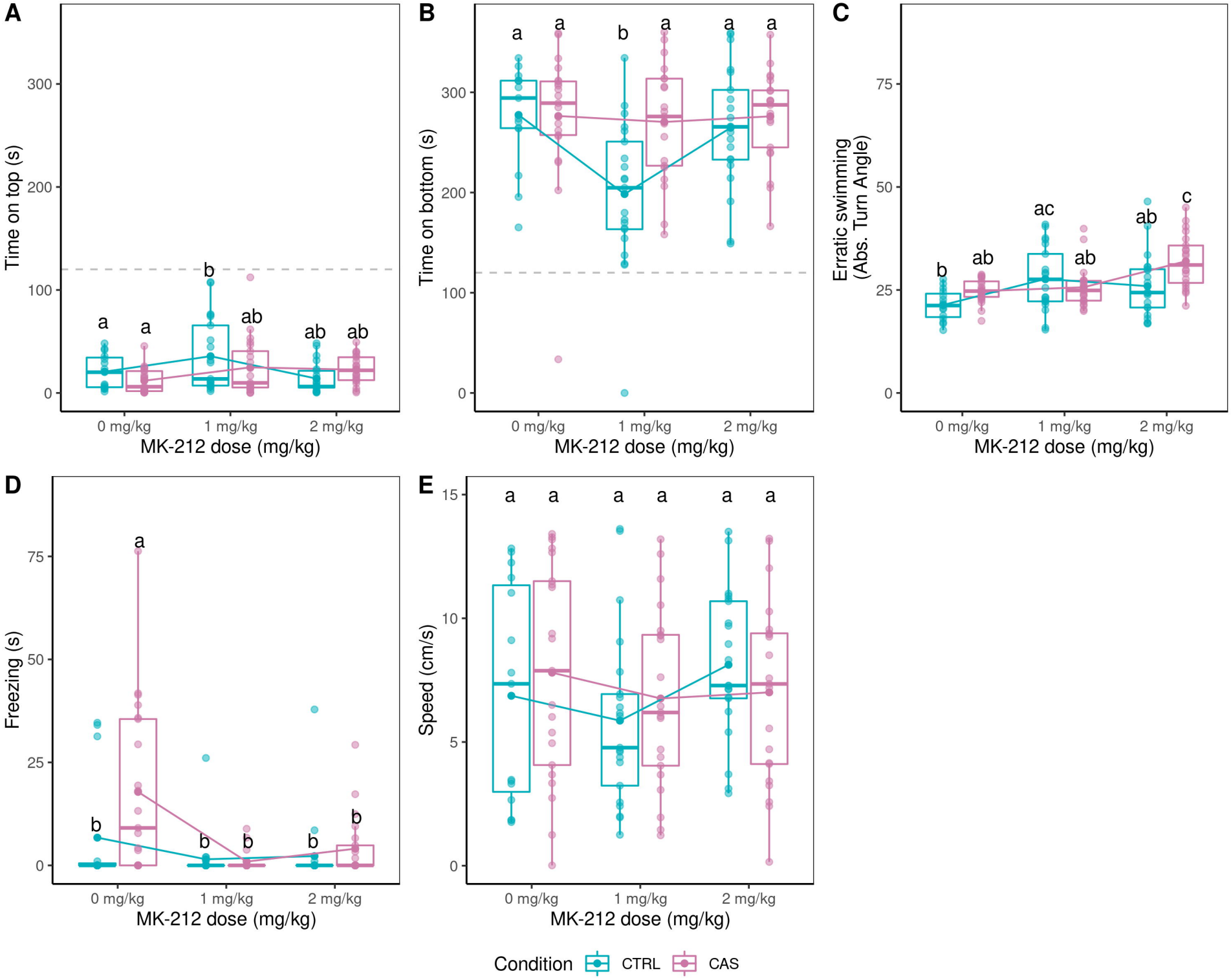
Experiment		**Behavior**		
		Geotaxis	**Erratic swimming**	**Freezing**
During CAS exposure	CAS effect	↑↑	↑↑	↑
	Agonist effect	Block	Partial block	Block
	Antagonist effect	--	Block	Partial block
After CAS exposure	CAS effect	↑	↑	↑↑
	Agonist effect	--	--	Partial block
	Antagonist effect	Block	--	Partial Block
After ARS	ARS effect	↑	↑	↑
	Agonist effect	--	--	Block
	Antagonist effect	Partial block	--	--

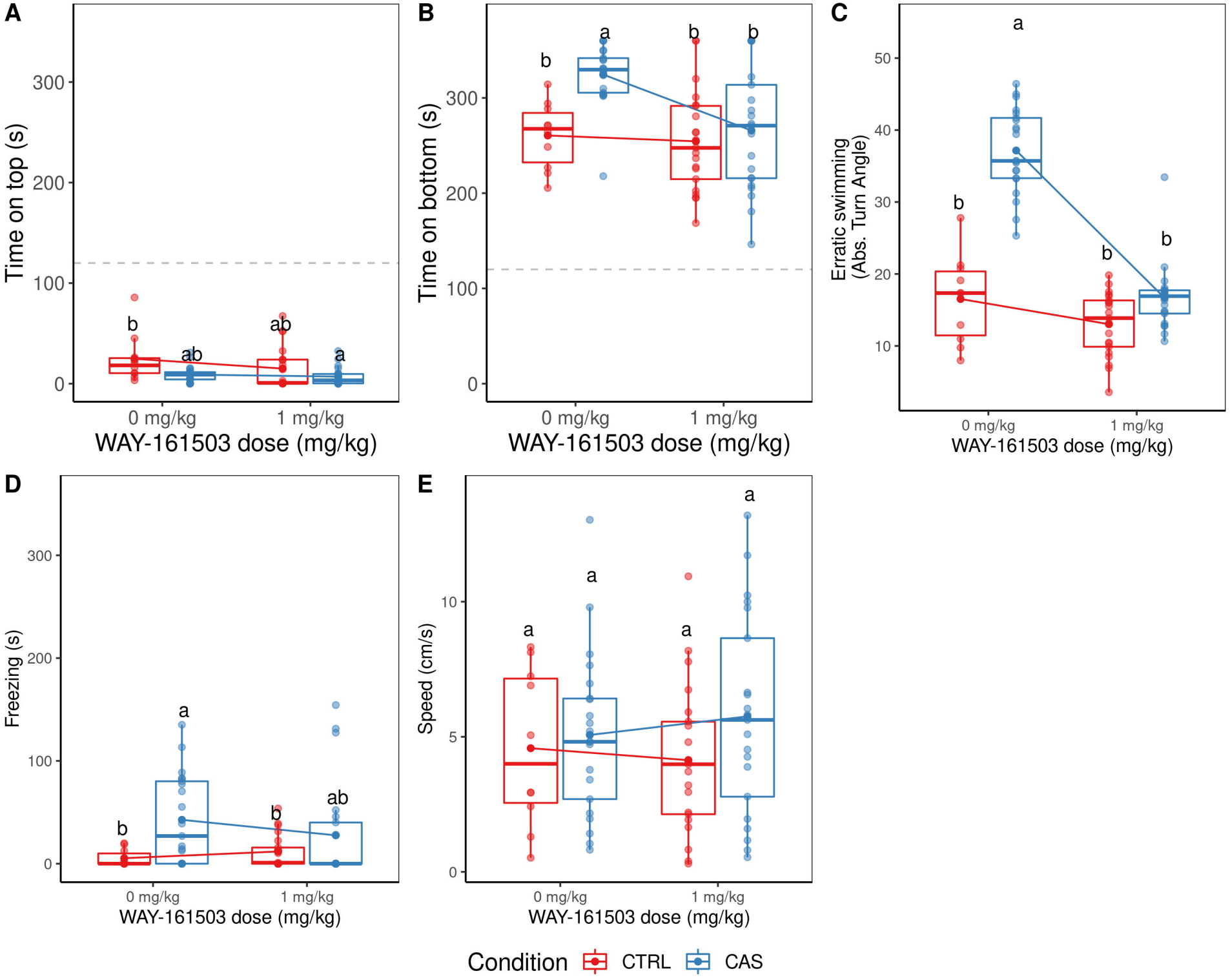
1085

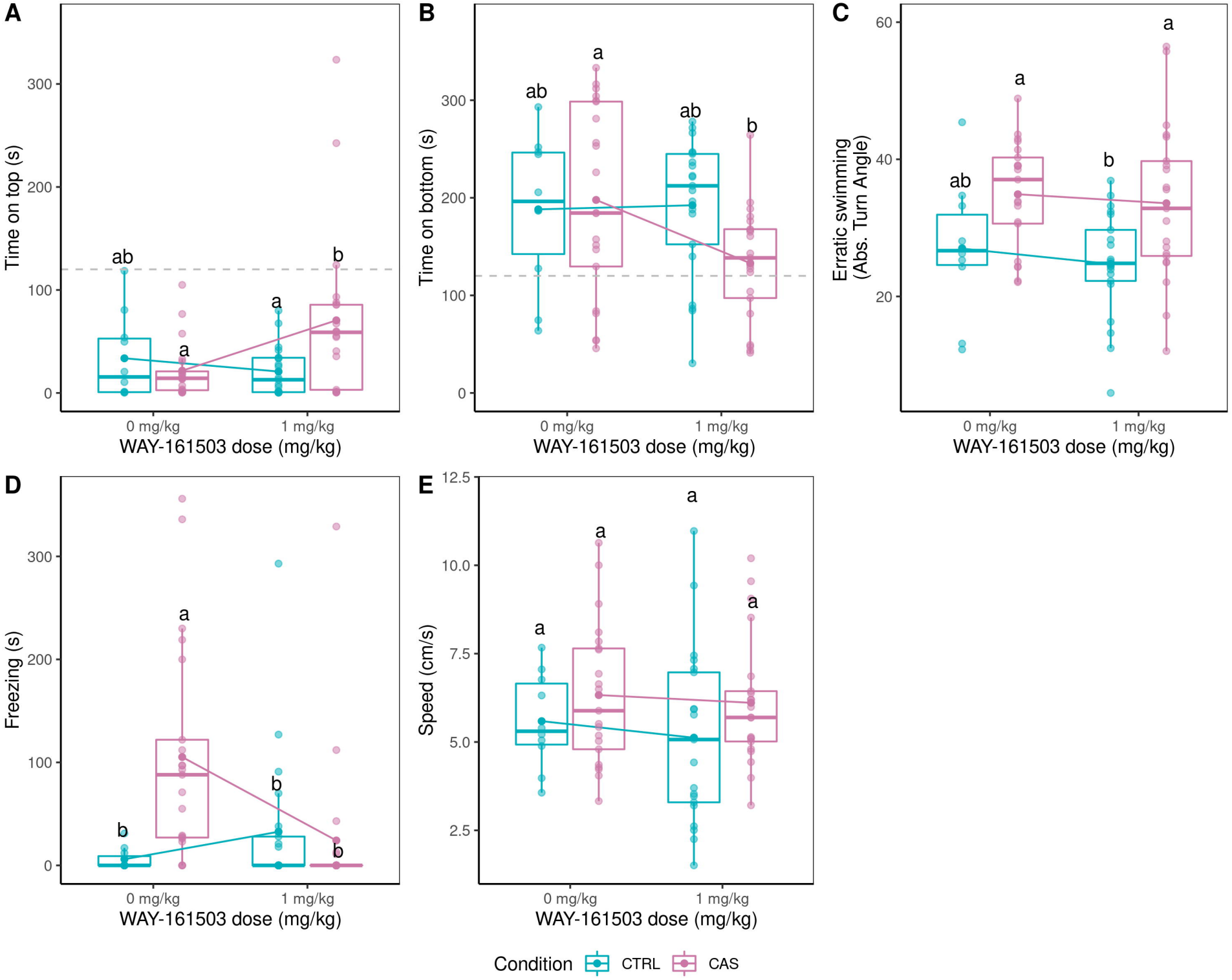

A
Distal threat
Behavioral observation (*after* exposure)
CAS exposure
Washout (1 min)
Acclimation (>14d)
6 min
20 min
6 min
Potential threat
Injection (drug or VEH)
Behavioral observation (*during* exposure)

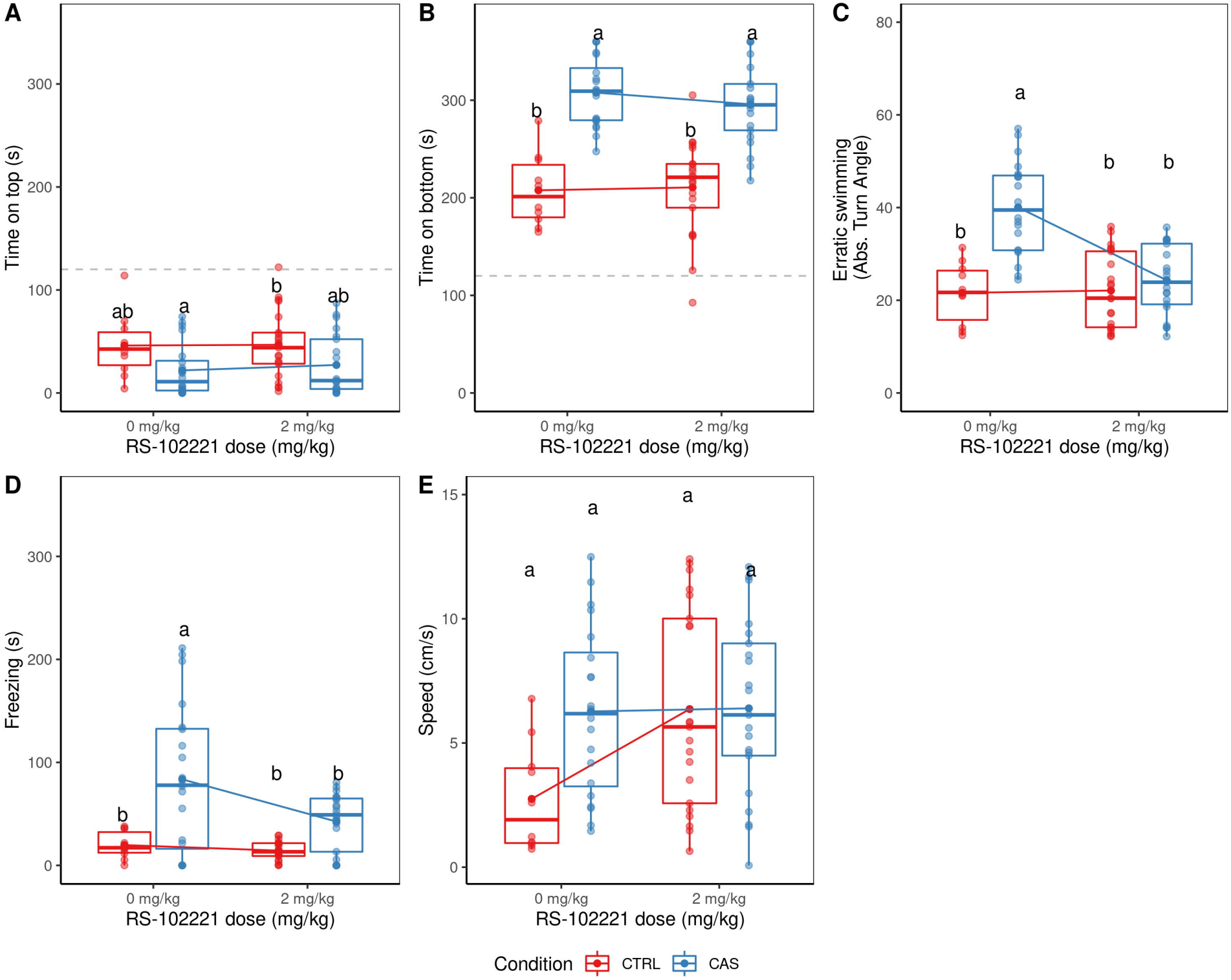

B
Behavioral observation (*after* exposure)
ARS
Acclimation (>14d)
90 min
20 min
6 min
Proximal threat
Injection (MK-212 or VEH)
LaNeC
LaNeF

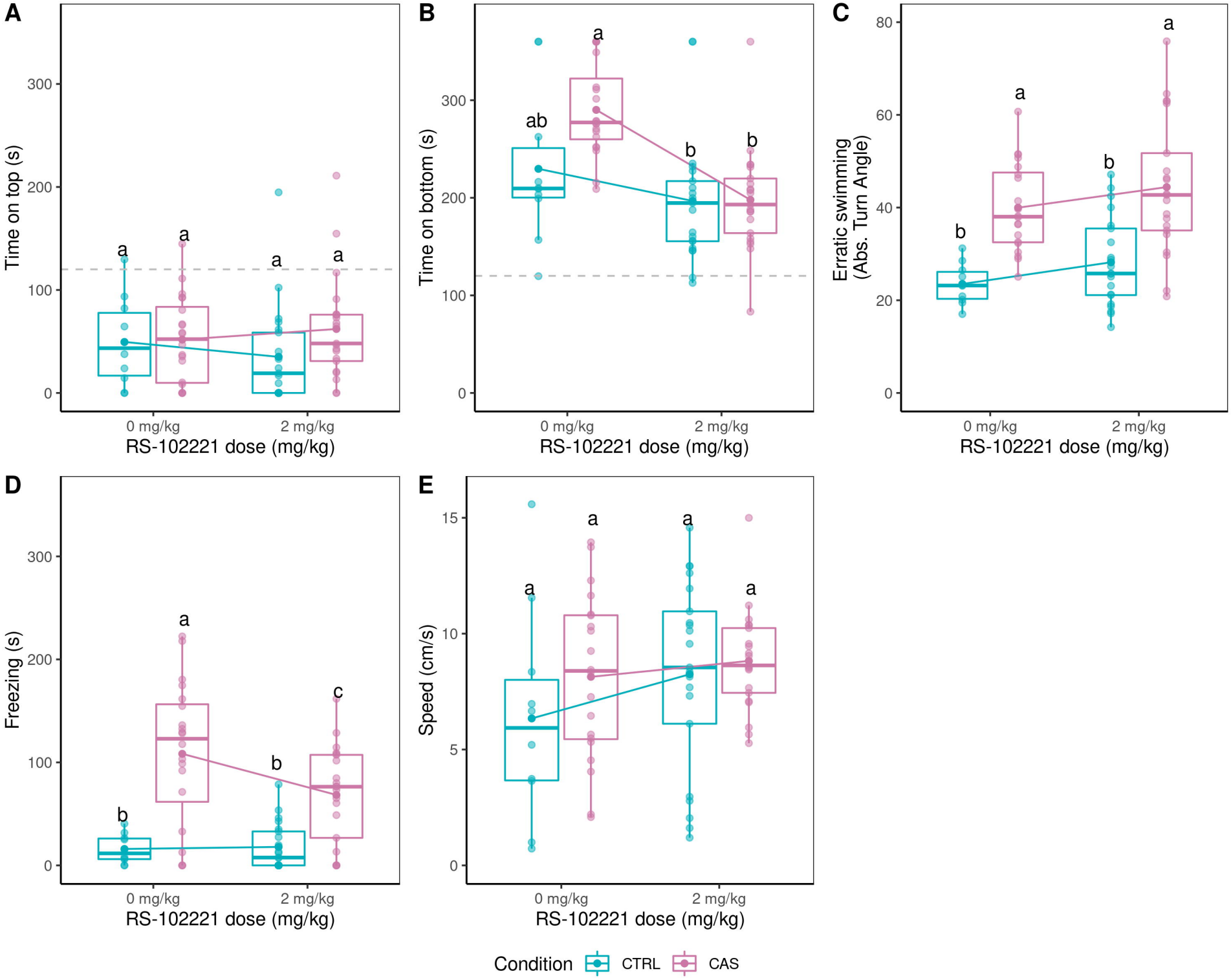

A
Time on top (s)
300
200
100
0
a
a
b
a
ab
ab
0 mg/kg
1 mg/kg
2 mg/kg
MK-212 dose (mg/kg)
B
Time on bottom (s)
300
200
100
0
bc
a
b
ac
b
b
0 mg/kg
1 mg/kg
2 mg/kg
MK-212 dose (mg/kg)
C
Erratic swimming
(Abs. Turn Angle)
75
50
25
0
b
a
a
b
a
b
0 mg/kg
1 mg/kg
2 mg/kg
MK-212 dose (mg/kg)
D
Freezing (s)
300
200
100
0
bc
a
b
ac
abc
abc
0 mg/kg
1 mg/kg
2 mg/kg
MK-212 dose (mg/kg)
E
Speed (cm/s)
15
10
5
0
a
a
a
a
a
a
0 mg/kg
1 mg/kg
2 mg/kg
MK-212 dose (mg/kg)
Condition
CTRL
CAS

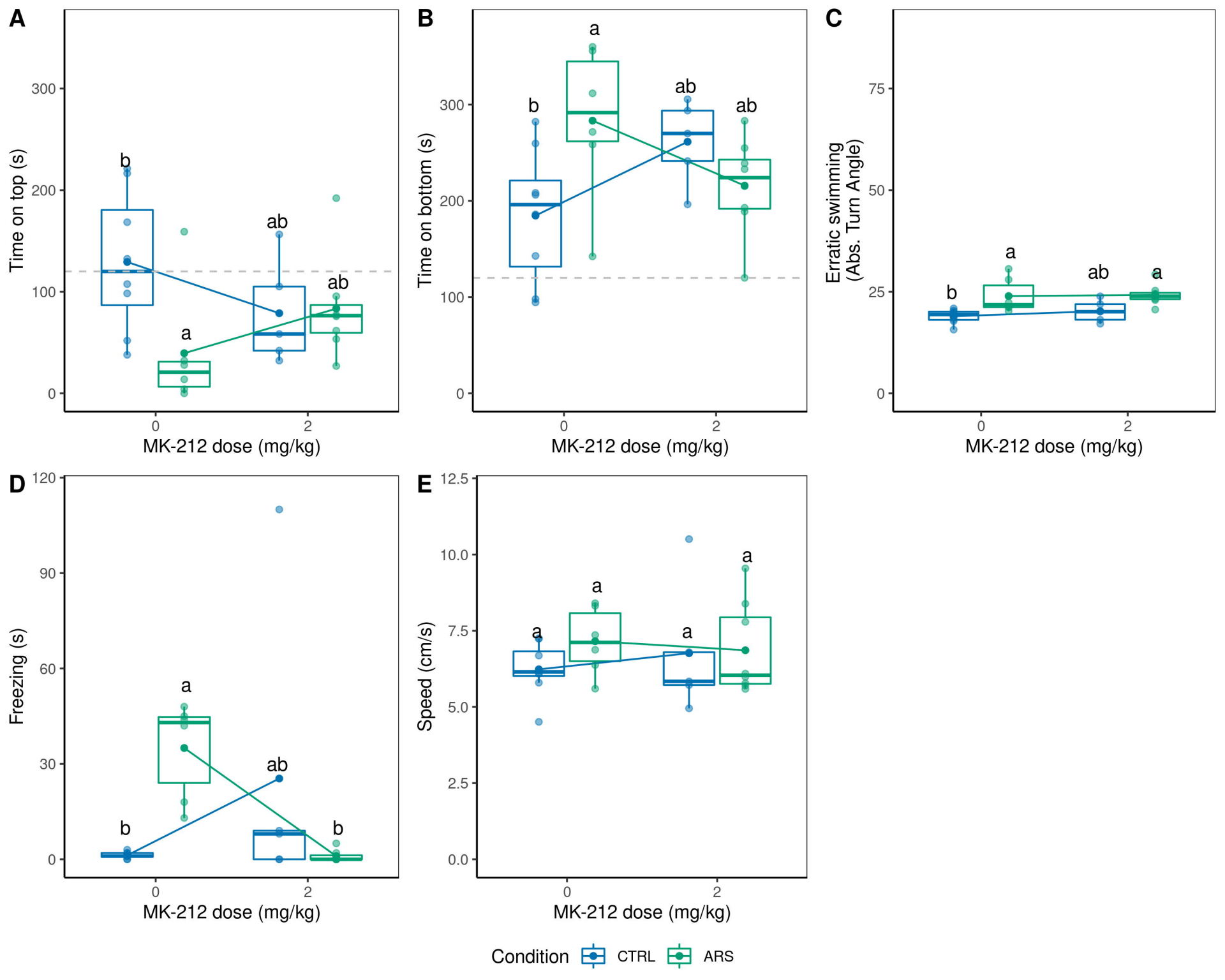

A
Time on top (s)
300
200
100
0
a
a
b
ab
ab
ab
0 mg/kg
1 mg/kg
2 mg/kg
MK-212 dose (mg/kg)
B
Time on bottom (s)
300
200
100
0
a
a
b
a
a
a
0 mg/kg
1 mg/kg
2 mg/kg
MK-212 dose (mg/kg)
C
Erratic swimming
(Abs. Turn Angle)
75
50
25
0
b
ab
ac
ab
ab
c
0 mg/kg
1 mg/kg
2 mg/kg
MK-212 dose (mg/kg)
D
Freezing (s)
75
50
25
0
a
b
b
b
b
b
0 mg/kg
1 mg/kg
2 mg/kg
MK-212 dose (mg/kg)
E
Speed (cm/s)
15
10
5
0
a
a
a
a
a
a
0 mg/kg
1 mg/kg
2 mg/kg
MK-212 dose (mg/kg)
Condition
CTRL
CAS

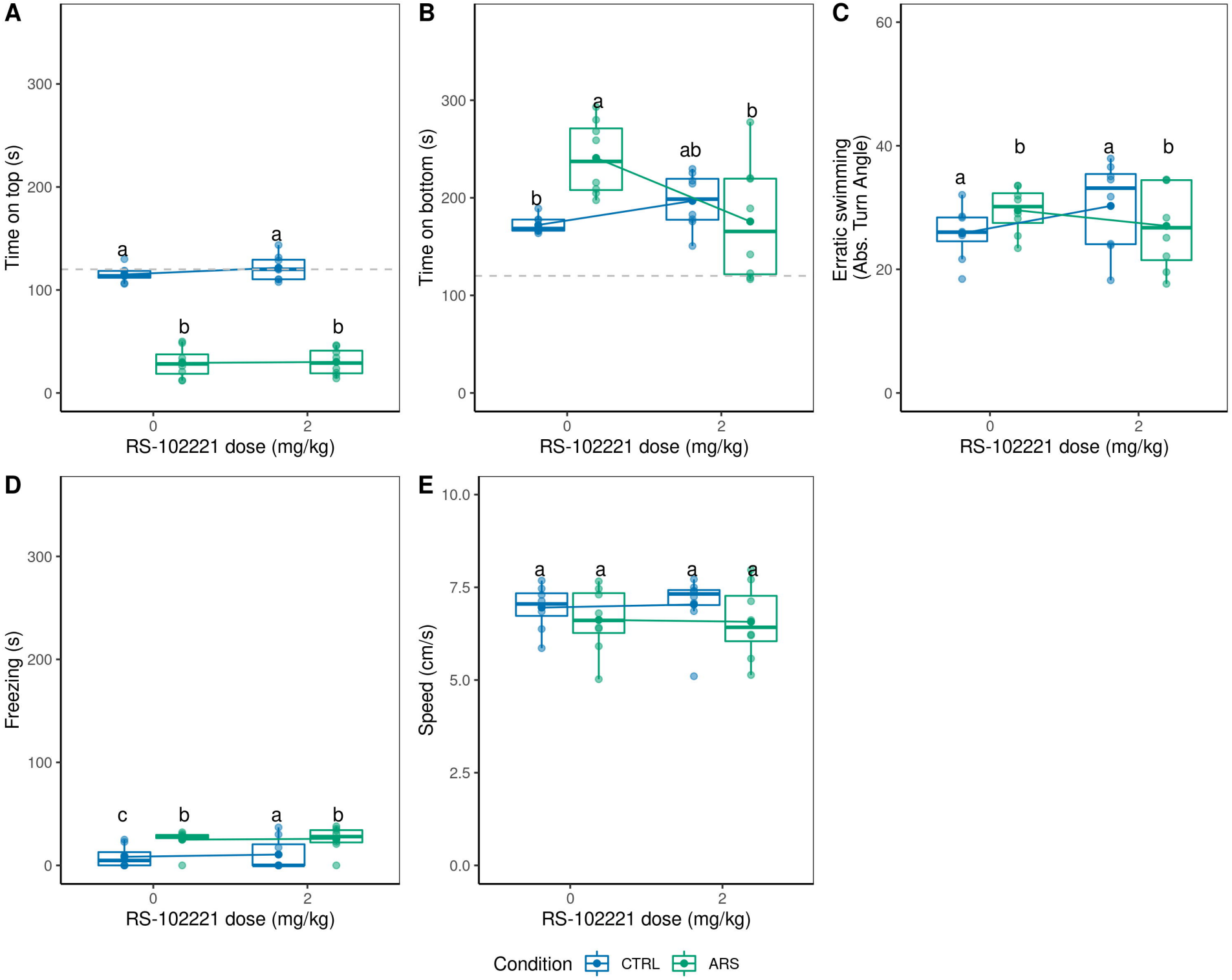

A
Time on top (s)
300
200
100
0
b
ab
ab
a
0 mg/kg
1 mg/kg
WAY-161503 dose (mg/kg)
B
Time on bottom (s)
300
200
100
0
b
a
b
b
0 mg/kg
1 mg/kg
WAY-161503 dose (mg/kg)
C
Erratic swimming
(Abs. Turn Angle)
50
40
30
20
10
a
b
b
b
0 mg/kg
1 mg/kg
WAY-161503 dose (mg/kg)
D
Freezing (s)
300
200
100
0
a
b
b
ab
0 mg/kg
1 mg/kg
WAY-161503 dose (mg/kg)
E
Speed (cm/s)
10
5
0
a
a
a
a
0 mg/kg
1 mg/kg
WAY-161503 dose (mg/kg)
Condition
CTRL
CAS


A
Time on top (s)
300
200
100
0
ab
a
a
b
0 mg/kg
1 mg/kg
WAY-161503 dose (mg/kg)
B
Time on bottom (s)
300
200
100
0
ab
a
ab
b
0 mg/kg
1 mg/kg
WAY-161503 dose (mg/kg)
C
Erratic swimming
(Abs. Turn Angle)
60
40
20
ab
a
b
a
0 mg/kg
1 mg/kg
WAY-161503 dose (mg/kg)
D
Freezing (s)
300
200
100
0
b
a
b
b
0 mg/kg
1 mg/kg
WAY-161503 dose (mg/kg)
E
Speed (cm/s)
12.5
10.0
7.5
5.0
2.5
a
a
a
a
0 mg/kg
1 mg/kg
WAY-161503 dose (mg/kg)
Condition
CTRL
CAS


A
Time on top (s)
300
200
100
0
ab
a
b
ab
0 mg/kg
2 mg/kg
RS-102221 dose (mg/kg)
B
Time on bottom (s)
300
200
100
0
b
a
b
a
0 mg/kg
2 mg/kg
RS-102221 dose (mg/kg)
C
Erratic swimming
(Abs. Turn Angle)
80
60
40
20
0
b
a
b
b
0 mg/kg
2 mg/kg
RS-102221 dose (mg/kg)
D
Freezing (s)
300
200
100
0
b
a
b
b
0 mg/kg
2 mg/kg
RS-102221 dose (mg/kg)
E
Speed (cm/s)
15
10
5
0
a
a
a
a
0 mg/kg
2 mg/kg
RS-102221 dose (mg/kg)
Condition
CTRL
CAS


A
Time on top (s)
300
200
100
0
a
a
a
a
0 mg/kg
2 mg/kg
RS-102221 dose (mg/kg)
B
Time on bottom (s)
300
200
100
0
ab
a
b
b
0 mg/kg
2 mg/kg
RS-102221 dose (mg/kg)
C
Erratic swimming
(Abs. Turn Angle)
80
60
40
20
0
b
a
b
a
0 mg/kg
2 mg/kg
RS-102221 dose (mg/kg)
D
Freezing (s)
300
200
100
0
b
a
b
c
0 mg/kg
2 mg/kg
RS-102221 dose (mg/kg)
E
Speed (cm/s)
15
10
5
0
a
a
a
a
0 mg/kg
2 mg/kg
RS-102221 dose (mg/kg)
Condition
CTRL
CAS


A
Time on top (s)
300
200
100
0
b
a
ab
ab
0
2
MK-212 dose (mg/kg)
B
Time on bottom (s)
300
200
100
0
b
a
ab
ab
0
2
MK-212 dose (mg/kg)
C
Erratic swimming
(Abs. Turn Angle)
75
50
25
0
b
a
ab
a
0
2
MK-212 dose (mg/kg)
D
Freezing (s)
120
90
60
30
0
b
a
ab
b
0
2
MK-212 dose (mg/kg)
E
Speed (cm/s)
12.5
10.0
7.5
5.0
2.5
0.0
a
a
a
a
0
2
MK-212 dose (mg/kg)
Condition
CTRL
ARS


A
Time on top (s)
300
200
100
0
a
a
b
b
0
2
RS-102221 dose (mg/kg)
B
Time on bottom (s)
300
200
100
0
b
a
ab
b
0
2
RS-102221 dose (mg/kg)
C
Erratic swimming (Abs. Turn Angle)
60
40
20
0
a
b
a
b
0
2
RS-102221 dose (mg/kg)
D
Freezing (s)
300
200
100
0
c
b
a
b
0
2
RS-102221 dose (mg/kg)
E
Speed (cm/s)
10.0
7.5
5.0
2.5
0.0
a
a
a
a
0
2
RS-102221 dose (mg/kg)
Condition
CTRL
ARS

```
5HT2CR|Mus        -------MVNLGTAVRSLLVHLIGLLVWQFDISISPVAAIVTDT-------FNSSDGGRL    46
5HT2CR|Homo       -------MVNLRNAVHSFLVHLIGLLVWQSDISVSPVAAIVTDI-------FNTSDGGR-    45
5HT2Cl1R|Danio    MMGAPGGPVLGGFSSTTSSLDLVGWMIWPGNTTVSLNQSFFGTDYSFNLSSSSSSSPHGV    60
5-HT2Cl2R|Danio   ------------------------------------------------MALLNTTASCNV    12
                                                                     .::

5HT2CR|Mus        FQFPDGVQNWPALSIVVIIIMTIGGNILVIMAVSMEKKLHNATNYFLMSLAIADMLVGLL   106
5HT2CR|Homo       FKFPDGVQNWPALSIVIIIIMTIGGNILVIMAVSMEKKLHNATNYFLMSLAIADMLVGLL   105
5HT2Cl1R|Danio    KREEMKEKNWPALLILVIIILTIGGNILVILAVSLEKKLQNATNFFLRSLAVADMLVGIL   120
5-HT2Cl2R|Danio   TLDTDHSYSWPALFILVIIFFTIAGNILVIMAVSIESKLHNPTNFFLCSLAVADMLVGFL    72
                          .**** *:.**:.**.******.***.*.**.* **.** ***.******.*
                                                D3.32
5HT2CR|Mus        VMPLSLLAILYDYVWPLPRYLCPVWISLDVLFSTASIMHLCAISLDRYVAIRNPIEHSRF   166
5HT2CR|Homo       VMPLSLLAILYDYVWPLPRYLCPVWISLDVLFSTASIMHLCAISLDRYVAIRNPIEHSRF   165
5HT2Cl1R|Danio    VMPISLINILYDYAWPLPSALCPIWIYLDVLFSTASIMHLCAISLDRYVAIRNPIEHSRF   180
5-HT2Cl2R|Danio   VMPISLISILYNHTWPLPQLLCPLWIFLDVLFSTASIMHLCAISLDRYIGIRNPFSISHT   132
                  ***.**: ***..:****  ***.** ********************.:****:. *:
                                            S3.36
5HT2CR|Mus        NSRTKAIMKIAIVWAISIGVSVPIPVIGLRDESKVFVNNTTCVLNDPNFVLIGSFVAFFI   226
5HT2CR|Homo       NSRTKAIMKIAIVWAISIGVSVPIPVIGLRDEEKVFVNNTTCVLNDPNFVLIGSFVAFFI   225
5HT2Cl1R|Danio    NSRTKAMLKIAAVWTISIGISMPIPVIGLSNREKVLKN-GICALNEELFILVGSFVAFFI   239
5-HT2Cl2R|Danio   QSSRKAMIKIITVWTISIVLSLPIPVMGLQDEQKVFID-CACVLNEPRFILVGSIVAFFV   191
                  :*  **.:**  **.*** .*.****.** :..**: :   *.**:  *.*.**.****:

5HT2CR|Mus        PLTIMVITYFLTIYVLRRQTLMLLRGHTEEELR--------------------NISLNFL   266
5HT2CR|Homo       PLTIMVITYCLTIYVLRRQALMLLHGHTEEP-P--------------------GLSLDFL   264
5HT2Cl1R|Danio    PLVIMVVTYCLTVQALQRQATVFLYEGKASSQQPLQPPAPPSSQLVPPLAPSRRSSLNCL   299
5-HT2Cl2R|Danio   PLVIMVVCYYLTLHHLQKHSKSFPHDSKSSCMHSS-------------IE----------   228
                  **.***. * **:  *.:::  :    . .

5HT2CR|Mus        KCCCKKGDEEE------------NAPNPN-PDQKPR-RKKKEKRPRGTMQAINNEKKASK   312
5HT2CR|Homo       KCCKRNTAEEE------------NSANPN-QDQNARRRKKKERRPRGTMQAINNERKASK   311
5HT2Cl1R|Danio    RISNSDGNTLGLTVPPDTISIIPSSEAPSQMNSPAGRDPAGAHGRRGMMQAIKNERRASK   359
5-HT2Cl2R|Danio   ---TSDTSFL-------------NSKSSSKMSAFPVIKRSPLKSRQGMNRALENERRASK   272
                       .                .:   .  .          :  :*  :*.:**.:***
                                                          Y7.43
5HT2CR|Mus        VLGIVFFVFLIMWCPFFITNILSVLCGKACNQKLMEKLLNVFVWIGYVCSGINPLVYTLF   372
5HT2CR|Homo       VLGIVFFVFLIMWCPFFITNILSVLCEKSCNQKLMEKLLNVFVWIGYVCSGINPLVYTLF   371
5HT2Cl1R|Danio    VLGVVFFLFLFMWCPFFITNVLYVLCHRTCKETLLTELLNVFVWVGYISSGVNPLVYTLF   419
5-HT2Cl2R|Danio   VLGIVFFLFLIMWCPFFITNVLNAICRDNCSEY-VHKLLDIFVWVGYVSSGVNPLIYTLF   331
                  ***.***.**.*********.* .:*   *.:  : .**.:***.**.:.**.***.****

5HT2CR|Mus        NKIYRRAFSKYLRCDYKPDKKPPVRQIPRVAATALSGREL---NVNIYRHTNER------   423
5HT2CR|Homo       NKIYRRAFSNYLRCNYKVEKKPPVRQIPRVAATALSGREL---NVNIYRHTNEP------   422
5HT2Cl1R|Danio    NKTYRRAFSNYMHCQYRHVGLKPITINVPCPSHAVVTPILMCDKVSIDRNSNCRNGDGNG   479
5-HT2Cl2R|Danio   NKTYRQAFARYLRCKFH-----------------------------------------   348
                  ** **.**:.*.:*.::

5HT2CR|Mus        ---VVRKANDTEPGIEMQVENLELPVNPSNVVSERISSV    459
5HT2CR|Homo       ---VIEKASDNEPGIEMQVENLELPVNPSSVVSERISSV    458
5HT2Cl1R|Danio    IRNLETLDIDTDTGLELKPGISEISISSGHSHTEHTSSV    518
5-HT2Cl2R|Danio   --------------------------------------     348
```