5-HT2C agonists and antagonists block different components of behavioral responses to potential, distal, and proximal threat in zebrafish

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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.

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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

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

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

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

93 adjustments has also been investigated. Zebrafish has been shown to possess two copies of
94 the 5-HT_{2A} receptor, one copy of the 5-HT_{2B} receptor, and two copies of the 5-HT_{2C} receptor
95 (Sourbron et al., 2016). In Nile tilapia, mianserin, a 5-HT_{2A} and 5-HT_{2C} receptor antagonist that
96 also blocks α_2-adrenoceptors, blocked active components of the alarm reaction (dashing,
97 bristling of dorsal fin spines), but not freezing, during exposure (Barreto, 2012). In zebrafish,
98 methysergide (an antagonist at 5-HT_{2A}, 2B, and 2C receptors, and a 5-HT_{1A} receptor agonist)
99 increased freezing and bottom-dwelling during and after exposure at a high dose (92.79 mg/kg),
100 but not at lower doses (Nathan et al., 2015). These results point to an inhibitory role of the 5-
101 HT_{2A} and 5-HT_{2C} receptors on CAS-elicited fear-like responses. However, since Nathan et al.
102 (2015) observed these effects consistently during a long session, in which behavior is expected
103 to change as CAS concentrations decrease (Mathuru et al., 2012)(i.e., similar to the shift from
104 erratic swimming to freezing that is observed when animals are observed after exposure in a
105 CAS-free context; Lima-Maximino et al., 2020), and since serotonergic drugs can produce
106 opposite effects on behavior during and after CAS exposure (Lima-Maximino et al., 2020), from
107 these results it is not possible to understand whether 5-HT_2 receptors participate in both
108 responses. In rodents, for example, 5-HT_2 receptors appear to inhibit fear and facilitate anxiety
109 at different levels of the neuroaxis (e.g. Graeff et al., 1986; Coimbra and Brandão, 1997;
110 Castilho and Brandão, 2001; Castilho et al., 2002; Alves et al., 2004; Cornélio and Nunes-de-
111 Souza, 2007; Macedo et al., 2007; Nunes-de-Souza et al., 2008; de Paula and Leite-Panissi,
112 2016), while in zebrafish these receptors appear to inhibit both fear and anxiety, although it is
113 currently unknown which brain regions participate in each effect.

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

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

125 The aim of the present work was to test the hypothesis that activation of 5-HT_{2C}
126 receptors would block CAS-elicited defensive reactions during exposure, but not after exposure;
127 moreover, we proposed that 5-HT_{2C} receptor antagonists would affect post-exposure behavior.
128 We also hypothesized that 5-HT_{2C} agonists would not alter the anxiogenic-like effects of acute
129 restraint stress (ARS), a model for proximal threat. We found that 5-HT_{2C} agonists were able to
130 block elements of the alarm reaction, but did not affect post-exposure behavior, nor the full
131 anxiogenic-like effects of ARS, while a 5-HT_{2C} receptor antagonist partially blocked the alarm
132 reaction and more strongly affected post-exposure behavior. This manuscript is a complete
133 report of all the studies performed to test the effect of 5-HT_{2C} agonists and antagonists on
134 zebrafish defensive behavior to distal threat. We report how the sample size was determined, all
135 data exclusions (if any), all manipulations, and all measures in the study.
136

137 2. Materials and methods

138 2.1. Animals and housing

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

140 longfin phenotype (n = 250) were used in the present experiments. The populations used are
141 expected to better represent the natural populations in the wild, due to its heterogeneous
142 genetic background (Parra et al., 2009; Speedie and Gerlai, 2008). Animals were bought from a
143 commercial vendor (Belém/PA) and collectively maintained in 40 L tanks for at least two weeks
144 before the onset of experiments. The animals were fed daily with fish flakes. The tanks were
145 kept at constant temperature (28 °C), oxygenation, light cycle (14:10 LD photoperiod) and a pH
146 of 7.0-8.0, according to standards of care for zebrafish (Lawrence, 2007). Animals were used for
147 only one experiment to reduce interference from apparatus exposure. Potential suffering of
148 animals was minimized by controlling for the aforementioned environmental variables.
149 Furthermore, in the all experiments the animals used were handled, anesthetized and sacrificed
150 according to the norms of the Brazilian Guideline for the Care and Use of Animals for Scientific
151 and Didactical Purposes (Conselho Nacional de Controle de Experimentação Animal -
152 CONCEA, 2017). Since, in all cases, animals were individually stressed and/or individually
153 treated, experimental unit was a single animal, and therefore all sample sizes refer to number of
154 animals. The experimental protocols were approved by UEPA's IACUC under protocol 06/18.
155

156 2.2. Drugs and treatments

157 The 5-HT_{2C} receptor agonists MK-212 (2-Chloro-6-(1-piperazinyl)pyrazine, CAS #64022-
158 27-1) and WAY-161503 (8,9-dichloro-2,3,4,4a-tetrahydro-1H-pyrazino[1,2-a]quinoxalin-5(6H)-
159 one; CAS #75704-24-4) were bought from Sigma-Aldrich (St Louis, USA) on 2018, and
160 dissolved in Cortland's salt solution (NaCl 124.1 mM, KCl 5.1 mM, Na_2HPO_4 2.9 mM, $MgSO_4$ 1.9
161 mM, $CaCl_2$ 1.4 mM, $NaHCO_3$ 11.9 mM, Polyvinylpyrrolidone 4%, 1,000 USP units Heparin; Wolf,
162 1963) and in 1% dimethyl sulfoxide (DMSO), respectively. The 5-HT_{2C} receptor antagonist RS-

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

174 For Experiment 1, animals were injected intraperitoneally either with MK-212 (1 mg/kg
175 and 2 mg/kg, doses which increase anxiety-like behavior in the rat elevated plus-maze; (de
176 Mello Cruz et al., 2005)) or with the vehicle solution (Cortland's salt solution); WAY-161503 (1
177 mg/kg, a dose which produces anxiogenic-like effects in the rat elevated plus-maze: Gomes et
178 al., 2010) or with the vehicle solution (DMSO); or RS-102221 (2 mg/kg, a dose that reduces
179 anxiety in the mouse light/dark test; (Kuznetsova et al., 2006)). For Experiment 2, animals were
180 injected intraperitoneally with MK-212 (2 mg/kg) or vehicle (Cortland's salt solution). Injections
181 were made according to the protocol proposed by Kinkel et al. (2010); briefly, animals were
182 cold-anesthetized and transferred to a sponge-based surgical bed, in which injection was made.
183 Injections were made using a microsyringe (Hamilton® 701N syringe, needle size 26 gauge at
184 cone tip), with total volumes of injection of 5 μL.
185

186 ## 2.3. Experiment 1: Effects of 5-HT_{2C} receptor agonists and
187 antagonists on alarm reaction and post-exposure behavior

188 ### 2.3.1. Experimental design

189 To verify the effects of phasic activation of 5-HT_{2C} receptors on the zebrafish alarm
190 reaction, animals were pre-treated with either receptor agonists and exposed to CAS in a
191 sequential design, with a "washout" period in between tests (Figure 1A). For the exposure
192 stage, each animal was transferred individually to a container (2 L) where after 3 minutes of
193 acclimatization, it was carefully exposed to 7 ml of alarm substance (CAS), extracted using a
194 standardized protocol (do Carmo Silva et al., 2018b). As negative control, a group with the
195 same amount of animals was exposed to the same volume of distilled water, according to the
196 protocol of Lima-Maximino et al. (2020). The animals remained exposed for 6 minutes during
197 which their behavior was recorded using a video camera positioned in front of the aquarium.
198 Then, to verify the residual effects of exposure to the alarm substance, the animals were
199 transferred to the apparatus of the novel tank test, a transparent glass aquarium filled with 5 L of
200 mineral water where the animal can freely explore the space for a period 6 minutes during which
201 their behavior was recorded, following the protocol described in Lima-Maximino et al., (2020).
202 All stages of the experiment were performed under constant white Gaussian noise, producing
203 an average of 58 dB above the tank. Light levels above the tanks were measured using a
204 handheld light meter, and ranged from 251 to 280 lumens (coefficient of variation = 3.399%
205 between subjects).

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

209 and tested in the order of allocation (i.e., randomly). In all experiments, experimenters and data
210 analysts were blinded to drugs and treatment by using coded vials (with the same code used for
211 randomization); blinding was removed only after data analysis. Experiments were always run
212 between 08:00AM and 02:00 PM. After experiments, animals were sacrificed by prolonged bath
213 in ice-cold water (< 12 ºC), followed by spinal transection (Matthews and Varga, 2012).
214

215 2.3.2. Sample size determination

216 To determine sample size, we incorporated information from control groups derived from
217 previously published experiments on zebrafish alarm substances observing bottom-dwelling
218 (Lima-Maximino et al., 2020; Quadros et al., 2016; Speedie and Gerlai, 2008) and a series of
219 four small experiments on the effects of CAS on behavior during exposure
220 (https://github.com/lanec-unifesspa/5-HT-CAS/tree/master/data/behavioral/metanalysis),
221 following the RePAIR approach (Bonapersona et al., 2019). Sample sizes, means, and standard
222 deviations for the primary endpoint "Time on bottom" were used to produce a prior distribution
223 on the RePAIR script (https://utrecht-university.shinyapps.io/repair/). Final parameters of the
224 distribution were $\mu = 197.149$, $\sigma^2 = 6100.609$, and weighted $N = 120.10$. The parameters of this
225 distribution were then used to calculate sample size, based on an effect size of $d = 0.5$
226 (equivalent to that used to calculate sample sizes in Lima-Maximino et al., 2020) and *a priori*
227 power of 0.8, with one-tailed tests with error probability $\alpha = 0.05$. With these parameters, the
228 final number of animals was 10 animals in the control group and 21 in each experimental group,
229 reaching a prospective power of 92.34%.
230

231 ## 2.3.3. Alarm substance extraction

232 A group of zebrafish was used as donor animals for the extraction of the conspecific
233 alarm substance (CAS). CAS extraction procedure was performed on each animal individually
234 as described by do Carmo Silva et al. (2018b). First, the donor animal was cold anesthetized
235 and transferred to a Petri dish, where the excess water from its body was removed with a paper
236 towel. Then the animal was decapitated with a surgical scalpel and the excess blood from the
237 sectioned region was removed with a swab. Subsequently, the animal's bodies were transferred
238 to another Petri dish where 15 superficial cuts were made in the epidermis of animals (medial-
239 ventral region) and 10 ml of distilled water were added to wash the cuts. After washing, the
240 animal's bodies were removed from the Petri dish, and with the aid of a Pasteur pipette, the fish
241 scales and other impurities were removed from the solution that was stored in a conical tube
242 and preserved on ice. The same extraction procedure was performed for all donated animals.
243

244 ## 2.3.4. Observation apparatuses

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

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

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

2.4.1. Experimental design

In this experiment, we focus on assessing the roles of 5-HT_{2C} receptors on anxiogenic-like effects of acute restraint stress (ARS). In order to do this, we evaluate the behavior of the animals in the novel tank test after being subjected to a 90-min section of restraint stress (Figure 1B). After drug (MK-212 or RS-102221) or vehicle (Cortland’s salt solution) injection and anesthesia, each animal was transferred individually to a 2 mL microtube (Eppendorf®) and placed on a plastic microtube rack inside an aquarium with continuous oxygen supply. The microtubes had small holes at both ends to allow free circulation of water inside the tube and to prevent fish from moving around, according to the protocol of Piato et al. (2011). A control group was maintained in a similar tank, but without restraint stress. Animals remained in these conditions for 90 min., sufficient to induce changes in telencephalon neurochemistry (Assad et al., 2020) and marked anxiety-like behavior (Assad et al., 2020; Ghisleni et al., 2012; Piato et al., 2011). Animals in the control groups were subjected to all manipulations with the exception of restraint. After stress, the animals were transferred to the apparatus of the novel tank test, a transparent glass aquarium filled with 5 L of mineral water where the animal can freely explore the space for a period 6 minutes during which their behavior was recorded, following the

protocol described in Lima-Maximino et al., (2020). All stages of the experiment were performed under constant white Gaussian noise, producing an average of 58 dB above the tank. Light levels above the tanks were measured using a handheld light meter, and ranged from 254 to 276 lumens (coefficient of variation = 3.401% between subjects). Random allocation was made as described above. In all experiments, experimenters and data analysts were blinded to drugs by using coded vials (with the same code used for randomization); blinding was removed only after data analysis. Experimenters were not blinded to treatment, but data analysts were blinded to both treatment and drug. Experiments were always run between 08:00AM and 02:00 PM. After experiments, animals were sacrificed by prolonged bath in ice-cold water (< 12 ºC), followed by spinal transection (Matthews and Varga, 2012).

2.4.2. Sample size determination

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

319 are publicly available. Behavioral records were reviewed by at least one PI for
320 administration/scoring accuracy, in order to ensure adherence to protocols and consistency
321 across tests.
322

323 2.7. Statistical analysis

324 Data were analyzed using two-way analysis of variance (drug x exposure to stressor)
325 with sequential sum of squares (type I), followed by Tukey's post-tests when $p < 0.05$; since
326 multiple comparisons were made in post-tests, p-values were calculated using Tukey's method.
327 Data analysis, table organization, and result graphs were performed using R version 3.6.3
328 (2020-02-29). Effect sizes for ANOVA effects are shown as ω^2; effect sizes for post-hoc tests
329 were shown as Cohen's d. Outliers were removed based on median absolute differences
330 (MADs), using time on bottom as the main endpoint; values were removed when they were
331 higher or lower than 3 MADs around the median (Leys et al., 2013), and the number of outliers
332 was reported in the results.
333

334 2.8. Sequence alignment

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

341 conservation of these residues across zebrafish 5-HT_{2C}-like receptors by aligning protein
342 sequences from zebrafish receptors (XP_017212271.1 and XP_001339040.4) with the murine
343 (NP_032338.3) and human (NP_001243689.2) 5-HT_{2C} receptors in Clustal Omega
344 (https://www.ebi.ac.uk/Tools/msa/clustalo/). Resulting alignments were visually inspected for
345 conserved residues at the positions that are related to ligand affinity.
346

347 2.9 Open science practices

348 The studies presented here were not formally pre-registered. A preprint version was
349 posted to bioRxiv **(**https://www.biorxiv.org/content/10.1101/2020.10.04.324202v2. Data and
350 analysis scripts can be found on a Github repository (https://github.com/lanec-unifesspa/5-HT-
351 CAS/tree/master/data/5HT2C).
352

353 3. Results

354 3.1. Effects of MK-212 on alarm reaction and post-exposure 355 behavior

356 3.1.1. During exposure

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

361 mg/kg controls; *2 mg/kg:* $p = 0.994$, $d = -0.18$, non-treated controls vs. 2 mg/kg controls; Figure
362 2A).

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

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

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

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

380

381 3.1.2. After exposure

382 Table 2 presents full ANOVA results for effects in the novel tank test after CAS exposure
383 and washout. Post-hoc tests revealed that MK-212 (1 mg/kg) increased time on top in both

384 controls and CAS-exposed animals (*1 mg/kg*: p = 0.019, d = -0.63 vs. non-treated animals; p =
385 0.038, d = 0.54 vs. 2 mg/kg; *2 mg/kg:* p = 0.92, d = -0.09 vs. non-treated animals; Figure 3A).
386 Post-hoc tests suggested that MK-212 (1 mg/kg) decreased time on bottom in control
387 animals (Figure 3B), but not in CAS-exposed animals (*1 mg/kg*: p = 0.002, d = 1.31, non-treated
388 controls vs. 1 mg/kg controls; p = 1.0, d = 0.1, non-treated CAS vs. 1 mg/kg CAS; *2 mg/kg:* p =
389 0.99, d = 0.21, non-treated controls vs. 2 mg/kg controls; p = 1, d = 0.01, non-treated CAS vs. 2
390 mg/kg CAS).
391 Post-hoc tests suggested a synergistic effect between CAS and MK-212 at 2 mg/kg,
392 which potentiated CAS-elicited increases in erratic swimming (Figure 3C); moreover, 1 mg/kg
393 MK-212 increased erratic swimming in control animals (*1 mg/kg:* p = 0.026, d= -1.05, non-
394 treated controls vs. 1 mg/kg controls; p = 1.0, d = -0.16, non-treated CAS vs. 1 mg/kg CAS; *2*
395 *mg/kg:* p = 0.22, d = -0.76, non-treated controls vs. 2 mg/kg controls; p = 0.002, d = -1.21, non-
396 treated CAS vs. 2 mg/kg CAS) .
397 Post-hoc tests revealed that CAS increased freezing (p = 0.05, d = -0.364 for the main
398 effect), an effect that was blocked by all MK-212 doses (*1 mg/kg:* p = 0.74, d = 0.47, non-treated
399 controls vs. 1 mg/kg controls; p < 0.001, d = 1.5, non-treated CAS vs. 1 mg/kg CAS; *2 mg/kg:* p
400 = 0.85, d = 0.4, non-treated controls vs. 2 mg/kg controls; p = 0.002, d = 1.22, non-treated CAS
401 vs. 2 mg/kg CAS; Figure 3D).
402 No effects were found for swimming speed (Figure 3E).
403

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

3.2.1. During exposure

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

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

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

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

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

427

428 ### 3.2.2. After exposure

429 Full ANOVA results for this experiment can be found on Table 4. Post-hoc effects
430 revealed a synergistic effect for time on top, with WAY-161503 increasing time on top in animals
431 exposed to CAS ($p = 0.922$, $d = 0.242$, non-treated controls vs. non-treated CAS; $p = 0.907$,
432 0.259, non-treated controls vs. WAY-161503 controls; $p = 0.011$, $d = 0.9833$, non-treated CAS
433 vs. WAY-161503 CAS; Figure 5A).

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

438 While ANOVA found effects of treatment on erratic swimming, post-hoc tests did not
439 find an increase in erratic swimming with CAS ($p = 0.125$, $d = -0.857$), nor a synergistic effect of
440 WAY-161503 ($p = 0.922$, $d = 0.243$, non-treated controls vs. WAY-161503 controls; $p = 0.968$, d
441 $= 0.142$, non-treated CAS vs. WAY-161503 CAS; Figure 5C).

442 Post-hoc tests revealed that CAS increased freezing ($p = 0.009$, $d = -1.257$, non-treated
443 controls vs. non-treated CAS), while WAY-161503 blocked this effect ($p = 0.816$, $d = -0.338$,
444 non-treated controls vs. WAY-161503 controls; $p = 0.008$, $d = 1.026$, non-treated CAS vs. WAY-
445 161-503 CAS; Figure 5D).

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

447

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

471 = 0.506, d = 0.54, non-treated controls vs. treated CAS; p < 0.001, d = 1.55, non-treated CAS
472 vs. treated CAS)(Figure 7B).
473 Post-hoc tests suggested that CAS increased erratic swimming after exposure (p =
474 0.001, d = 1.514, non-treated controls vs. non-treated CAS), an effect that was not altered by
475 RS-102221 (p < 0.001, d = -1.92, non-treated controls vs. treated CAS; p = 0.56, d = -0.41, non-
476 treated CAS vs. treated CAS)(Figure 7C).
477 Post-hoc tests suggested that CAS increased freezing after exposure (p < 0.001, d =
478 1.96, non-treated controls vs. non-treated CAS), an effect that was partially blocked by RS-
479 102221 (p = 0.025, d = -1.12, non-treated controls vs. treated CAS; p = 0.041, d = 0.85; Figure
480 7D).
481 Finally, no effects were found for swimming speed (Figure 7E).
482

483 3.4. Restraint stress-elicited behavioral changes

484 3.4.1. Effects of MK-212 on restraint stress-elicited behavioral changes

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

494 showed decreased freezing in relation to other groups ($p = 0.033$, $d = -1.6$, non-treated controls
495 vs. treated ARS; $p = 0.031$, $d = 1.6$, non-treated ARS vs. treated ARS; Figure 8D).
496 Finally, no effects were found for swimming speed (Figure 8E).
497

498 3.4.2. Effects of RS-102221 on restraint stress-elicited behavioral changes

499 Full results for ANOVAs for this experiment can be found in Table 8. ARS increased time
500 on top ($p < 0.0001$, $d = 7.78$ for the main effect), and RS-102221 was not able to block this
501 effect (Figure 9A).
502 Post-hoc tests revealed that ARS increased time on bottom ($p = 0.007$ vs. non-treated
503 controls), an effect that was partially blocked by RS-102221 ($p = 0.997$, non-treated controls vs.
504 treated ARS; $p = 0.019$, non-treated ARS vs. treated ARS; Figure 9B).
505 No effects were found for erratic swimming (Figure 9C).
506 ARS increased freezing ($p = 0.001$, $d = 1.4$ for the main effect), an effect that was not
507 blocked by RS-102221 ($p = 0.052$, non-treated controls vs. treated ARS; $p = 0.998$, non-treated
508 ARS vs. treated ARS; Figure 9D).
509 Finally, no effects were found for swimming speed (Figure 9E).
510

511 3.5. Conservation of residues related to ligand binding in 512 zebrafish 5-HT_{2C} receptors

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

516 all sequences, differences in residues close to D3.32 the 5-HT_{2C}-like receptors zebrafish
517 receptor (S3.30Y in 5-HT_{2CL1} and S3.30F in 5-HT_{2CL2}), as well as close to Y7.43 (V7.44I in5-
518 HT_{2CL2}) were found.
519

520 4. Discussion

521 The present work tested the hypothesis that phasic activation of the 5-HT_{2C} receptor is
522 involved in behavioral adjustments to distal, but not proximal, threat in zebrafish, and that these
523 receptors exerts a tonic facilitation of defensive behavior to potential threat in zebrafish. We
524 found that 5-HT_{2C} agonists blocked CAS-elicited defensive behavior, but not post-exposure
525 increases in defensive behavior (with the exception of freezing), nor ARS-elicited anxiogenic-
526 like effects. We also found that RS-102221, a 5-HT_{2C} receptor antagonist, did not change
527 behavior during exposure, but it produced a small effect on behavior after exposure to CAS. No
528 effects of RS-102221 were found for ARS-elicited anxiogenic-like effects. Residues related to
529 ligand binding in the human 5-HT_{2C} receptors were conserved in both zebrafish isoforms, but
530 differences exist in residues close to these sites.
531

532 4.1. Effects of stressors on zebrafish behavior

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

537 view both should affect different behavioral endpoints.

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

558 ARS-elicited behavioral effects have been explored in the literature with mixed results.
559 While Ghisleni et al. (2012) found that a 90-min restraint protocol did not change bottom-
560 dwelling after stress when animals were tested individually, a similar protocol showed marked
561 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).

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

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

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

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

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

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

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

660 ascertain whether drugs which act as agonists and antagonists at mammalian 5-HT_{2C} receptors
661 will target zebrafish receptors. Using sequence alignment, it was found that residues that, in
662 human receptors, are crucial for ligand binding (and selectivity with the 5-HT_{2A} receptor, for
663 example; Canal et al., 2011; Córdova-Sintjago et al., 2012) are conserved in both zebrafish
664 proteins (HTR2Cl1 and HTR2Cl2). This suggests that ligand affinity is conserved for these
665 proteins, which strengthens the hypothesis that MK-212, WAY-161503, and RS-102221
666 targeted 5-HT_{2C} receptors in the behavioral experiments. Differences were found in residues
667 near the conserved binding sites, however; even in mammalian receptors, the role of these
668 residues in ligand affinity and/or efficacy is unknown.
669

670 4.3. Limitations and translational potential

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

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

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

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

704

705

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").

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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).

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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

990 differences at the $p < 0.05$ level; similar letters indicate lack of statistically significant
991 differences. Data are presented as individual data points (dots) superimposed over the
992 median ± interquartile ranges. Dashed lines on panels A and B represent change levels.
993 Dots connected by lines represent group means. CTRL = controls (water-exposed
994 animals); CAS = conspecific alarm substance. Final sample sizes: CTRL + VEH: n = 10
995 animals; CTRL + 1 mg/kg WAY-161503: n = 21 animals; CAS + VEH: n = 21 animals;
996 CAS + 1 mg/kg WAY-161503: n = 21 animals.
997 Figure 5 – Effects of WAY-161503 on behavior <u>after</u> CAS exposure. (A) Time spent on top third
998 of the tank. (B) Time spent on bottom third of the tank. (C) Erratic swimming. (D) Total
999 time spent freezing. (E) Swimming speed. Different letters represent statistical
1000 differences at the $p < 0.05$ level; similar letters indicate lack of statistically significant
1001 differences. Data are presented as individual data points (dots) superimposed over the
1002 median ± interquartile ranges. Dashed lines on panels A and B represent change levels.
1003 Dots connected by lines represent group means. CTRL = controls (water-exposed
1004 animals); CAS = conspecific alarm substance. Final sample sizes: CTRL + VEH: n = 10
1005 animals; CTRL + 1 mg/kg WAY-161503: n = 21 animals; CAS + VEH: n = 21 animals;
1006 CAS + 1 mg/kg WAY-161503: n = 21 animals.
1007 Figure 6 – Effects of RS-102221 on behavior <u>during</u> CAS exposure. (A) Time spent on top third
1008 of the tank. (B) Time spent on bottom third of the tank. (C) Erratic swimming. (D) Total
1009 time spent freezing. (E) Swimming speed. Different letters represent statistical
1010 differences at the $p < 0.05$ level; similar letters indicate lack of statistically significant
1011 differences. Data are presented as individual data points (dots) superimposed over the
1012 median ± interquartile ranges. Dashed lines on panels A and B represent change levels.
1013 Dots connected by lines represent group means. CTRL = controls (water-exposed
1014 animals); CAS = conspecific alarm substance. Final sample sizes: CTRL + VEH: n = 10
1015 animals; CTRL + 2 mg/kg RS-102221: n = 21 animals; CAS + VEH: n = 20 animals; CAS
1016 + 2 mg/kg RS-102221: n = 21 animals.
1017 Figure 7 – Effects of RS-102221 on behavior <u>after</u> CAS exposure. (A) Time spent on top third of
1018 the tank. (B) Time spent on bottom third of the tank. (C) Erratic swimming. (D) Total time
1019 spent freezing. (E) Swimming speed. Different letters represent statistical differences at
1020 the $p < 0.05$ level; similar letters indicate lack of statistically significant differences. Data
1021 are presented as individual data points (dots) superimposed over the median ±
1022 interquartile ranges. Dashed lines on panels A and B represent change levels. Dots

1023 connected by lines represent group means. CTRL = controls (water-exposed animals);
1024 CAS = conspecific alarm substance. Final sample sizes: CTRL + VEH: n = 10 animals;
1025 CTRL + 2 mg/kg RS-102221: n = 21 animals; CAS + VEH: n = 20 animals; CAS + 2
1026 mg/kg RS-102221: n = 21 animals.
1027 Figure 8 – Effects of MK-212 on behavior after acute restraint stress (ARS). (A) Time spent on
1028 top third of the tank. (B) Time spent on bottom third of the tank. (C) Erratic swimming.
1029 (D) Total time spent freezing. (E) Swimming speed. Different letters represent statistical
1030 differences at the $p < 0.05$ level; similar letters indicate lack of statistically significant
1031 differences. Data are presented as individual data points (dots) superimposed over the
1032 median ± interquartile ranges. Dashed lines on panels A and B represent change levels.
1033 Dots connected by lines represent group means. CTRL = controls (water-exposed
1034 animals); ARS = acute restraint stress. Final sample sizes: CTRL + VEH: n = 8 animals;
1035 CTRL + 2 mg/kg MK-212: n = 5 animals; ARS + VEH: n = 6 animals; ARS + 2 mg/kg
1036 MK-212: n = 8 animals.
1037 Figure 9 – Effects of RS-102221 on behavior after acute restraint stress (ARS). (A) Time spent
1038 on top third of the tank. (B) Time spent on bottom third of the tank. (C) Erratic swimming.
1039 (D) Total time spent freezing. (E) Swimming speed. Different letters represent statistical
1040 differences at the $p < 0.05$ level; similar letters indicate lack of statistically significant
1041 differences. Data are presented as individual data points (dots) superimposed over the
1042 median ± interquartile ranges. Dashed lines on panels A and B represent change levels.
1043 Dots connected by lines represent group means. CTRL = controls (water-exposed
1044 animals); ARS = acute restraint stress. Final sample sizes: CTRL + VEH: n = 8 animals;
1045 CTRL + 2 mg/kg RS-102221: n = 8 animals; ARS + VEH: n = 8 animals; ARS + 2 mg/kg
1046 RS-102221: n = 8 animals.
1047 Figure 10 – Amino acid sequences for zebrafish 5-HT_{2C}RL1 and 5-HT_{2C}RL2 aligned with
1048 sequences of mice (*Mus*) and humans (*Homo*). Residues associated with the binding
1049 site are labeled above the sequences (D3.32, S3.36, Y7.43), according to the
1050 Ballesteros et al. (2001) nomenclature. Colors code physicochemical properties: red,
1051 small amino acids+hydrophobic amino acids (including aromatic amino acids except
1052 tyrosine [Y]); blue, acidic amino acids; magenta, basic amino acids except histidine [H];
1053 green, hydroxyl amino acids+sulfhydryl amino acids+amine+glycine. Asteriks (*) show
1054 residues which are conserved across all four sequences, while colons (:) indicate
1055 conservation between groups scoring > 0.5 in the Gonnet PAM 250 matrix, and periods

1056 (.) 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

1060

1061 Table 2 – Full ANOVA results for effects of MK-212 on behavioral endpoints after CAS

1062 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

1063

1064

1065 Table 3 – Full ANOVA results for effects of WAY-161503 on behavioral endpoints during CAS
1066 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

1067

1068 Table 4 – Full ANOVA results for effects of WAY-161503 on behavioral endpoints after CAS
1069 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

1070

1071 Table 5 – Full ANOVA results for effects of RS-102221 on behavioral endpoints during CAS
1072 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

1073

1074 Table 6 – Full ANOVA results for effects of RS-102221 on behavioral endpoints after CAS
1075 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

1076

1077 Table 7 – Full ANOVA results for effects of MK-212 on behavioral endpoints after restraint
1078 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

1079

1080 Table 8 – Full ANOVA results for effects of RS-102221 on behavioral endpoints after restraint
1081 stress.
1082

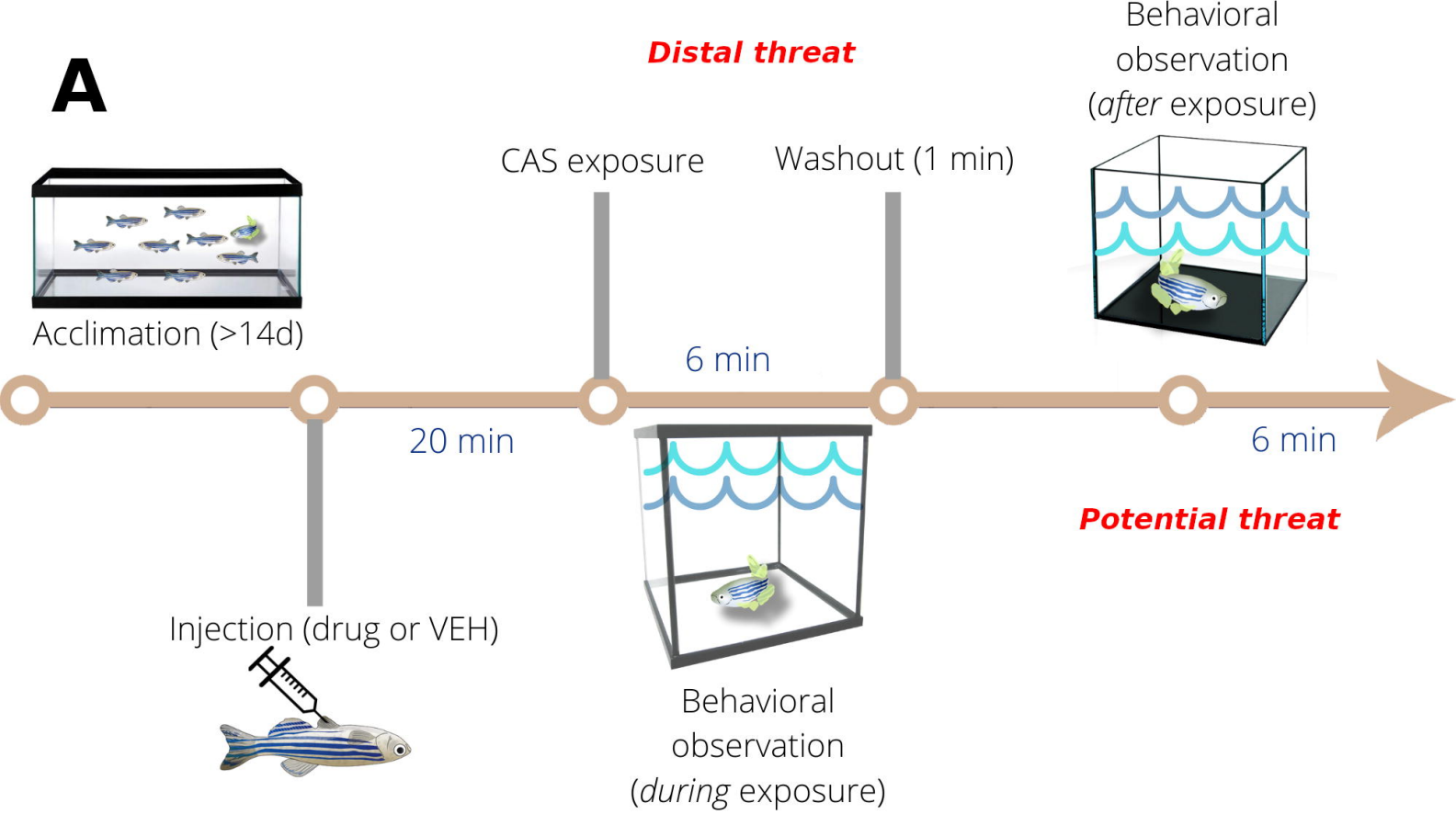
Context	**Drug**	**Endpoint**	**Effect** **[df1, df2]**	**F**	**P-value**	**Effect size** **(ω^2)**
After restraint stress	RS-102221 (5-HT_{2C}R antagonist)	Time on top (s)	Treatment [1, 23]	422.613	< 0.0001	0.93
			Drug [1, 23]	0.728	0.401	-0.009
			Interaction [1, 23]	0.416	0.524	-0.02
		Time on bottom (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

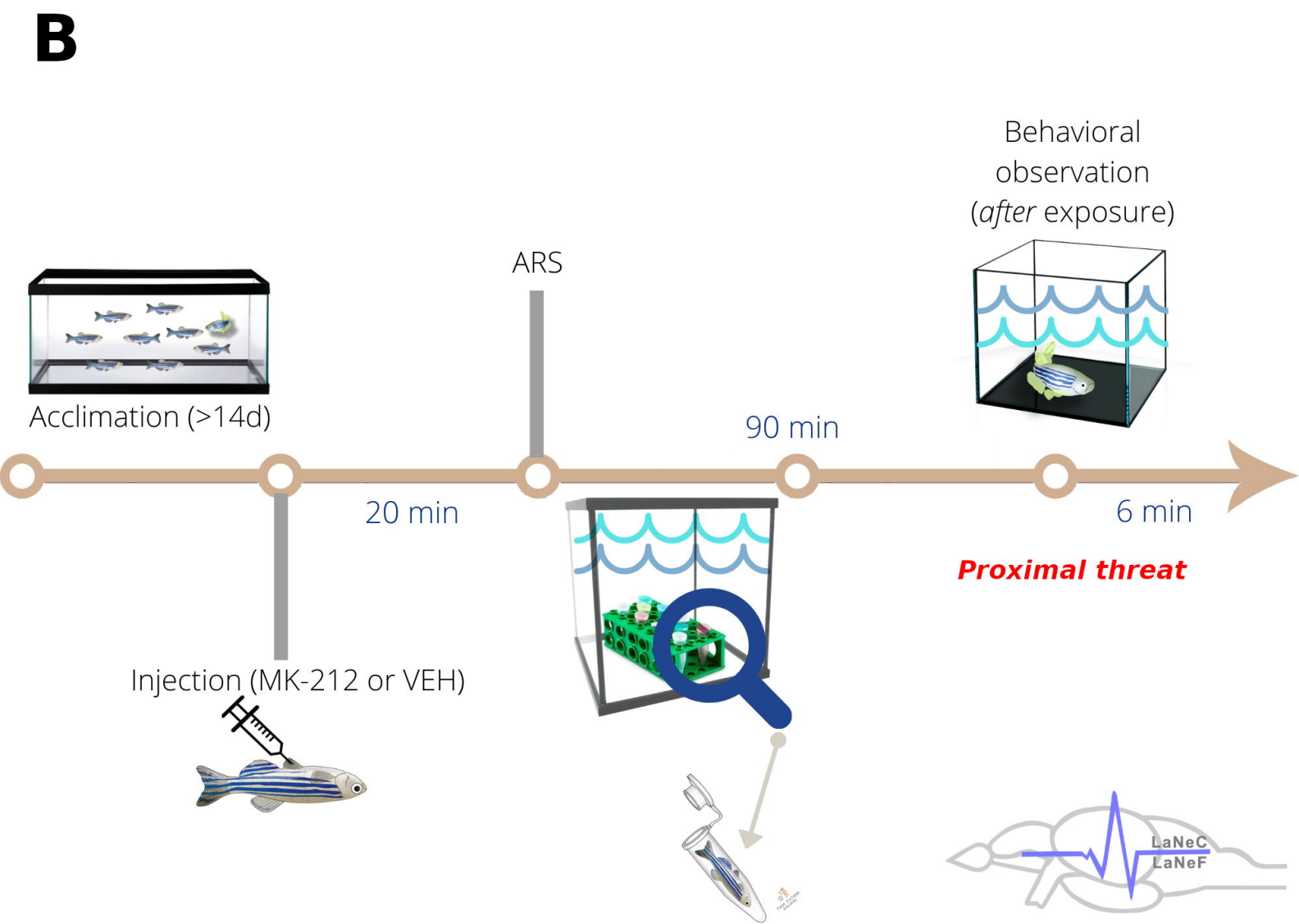
1083

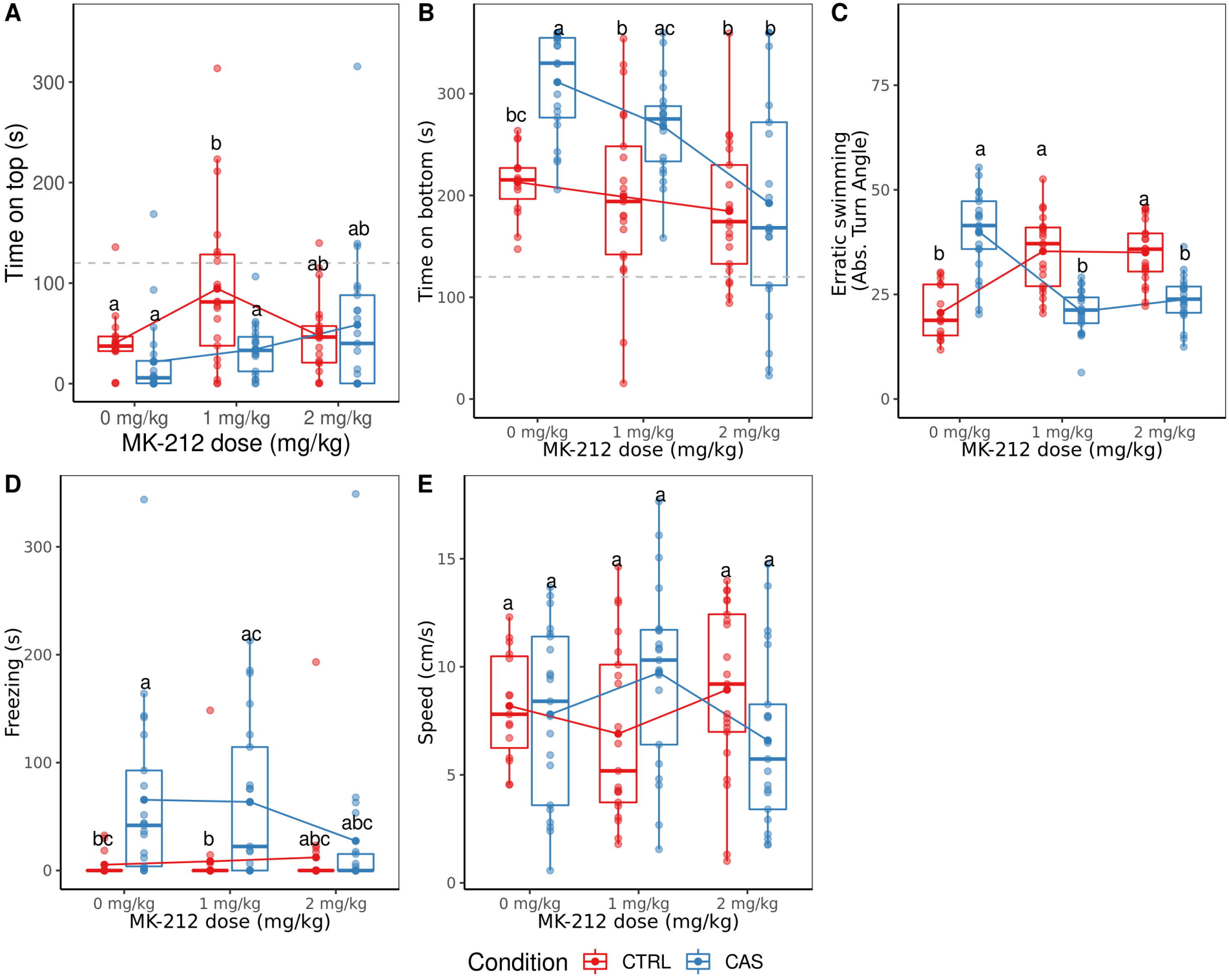
1084 Table 9 – Behavioral effects of 5-HT2C receptor agonists (MK-212 and WAY-161503) and
1085 antagonists (RS-102221) on behavior during and after CAS exposure, and after ARS in adult
1086 zebrafish.

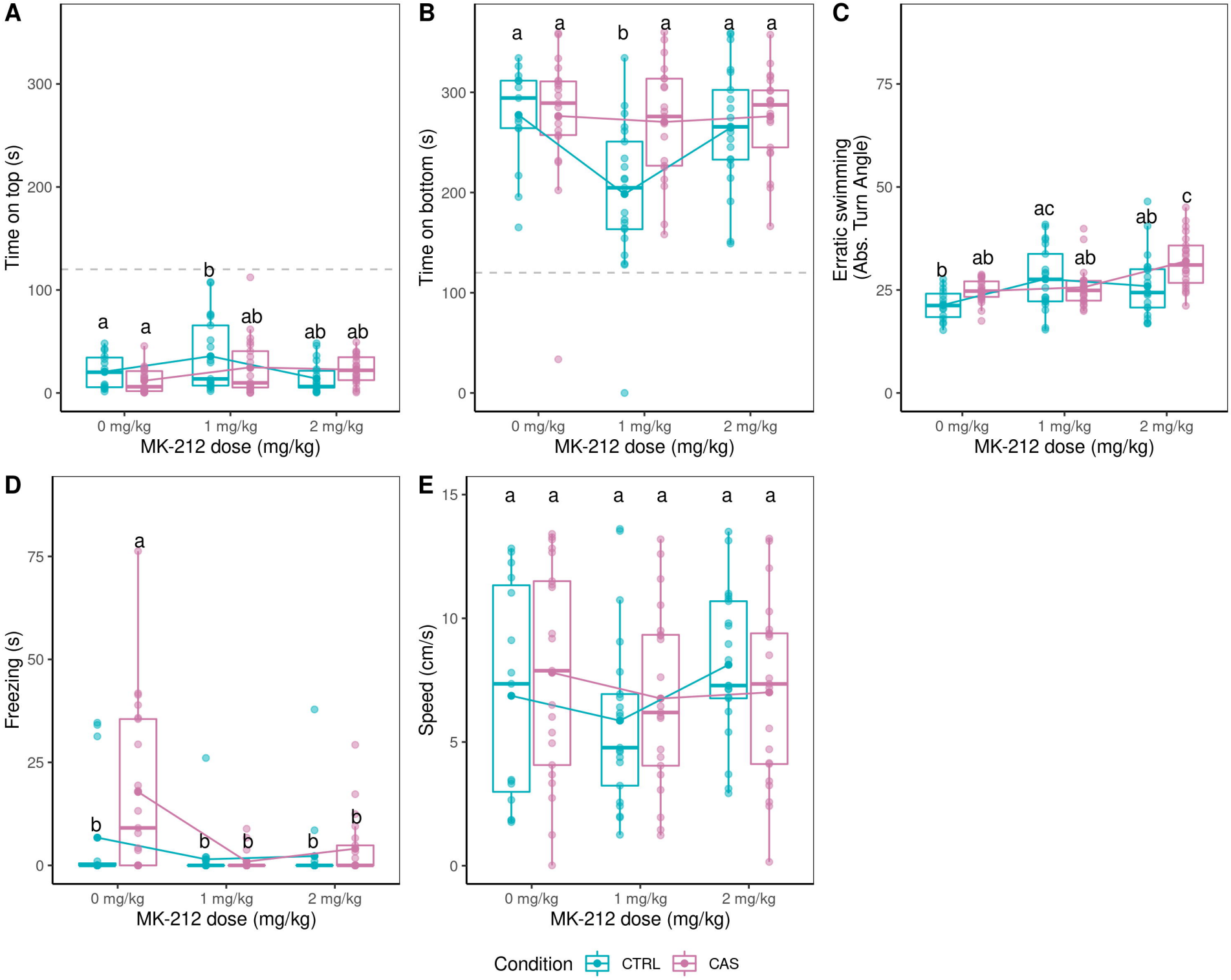
Experiment		**Behavior**		
		Geotaxis	**Erratic swimming**	**Freezing**
During CAS exposure	CAS effect	↑↑	↑↑	↑
	Agonist effect	Block	Partial block	Block
	Antagonist effect	--	Block	Partial block
After CAS exposure	CAS effect	↑	↑	↑↑
	Agonist effect	--	--	Partial block
	Antagonist effect	Block	--	Partial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

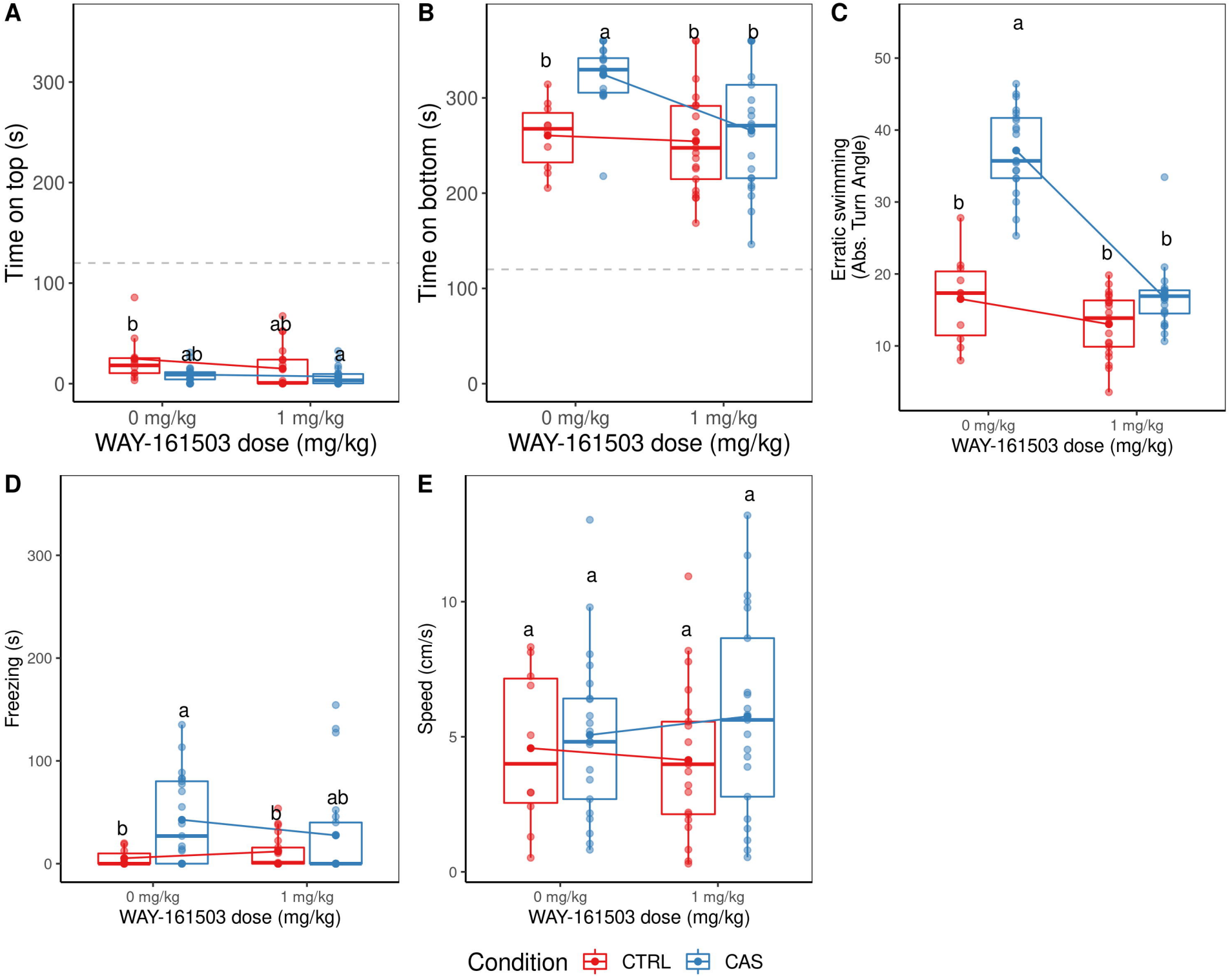
1087
1088

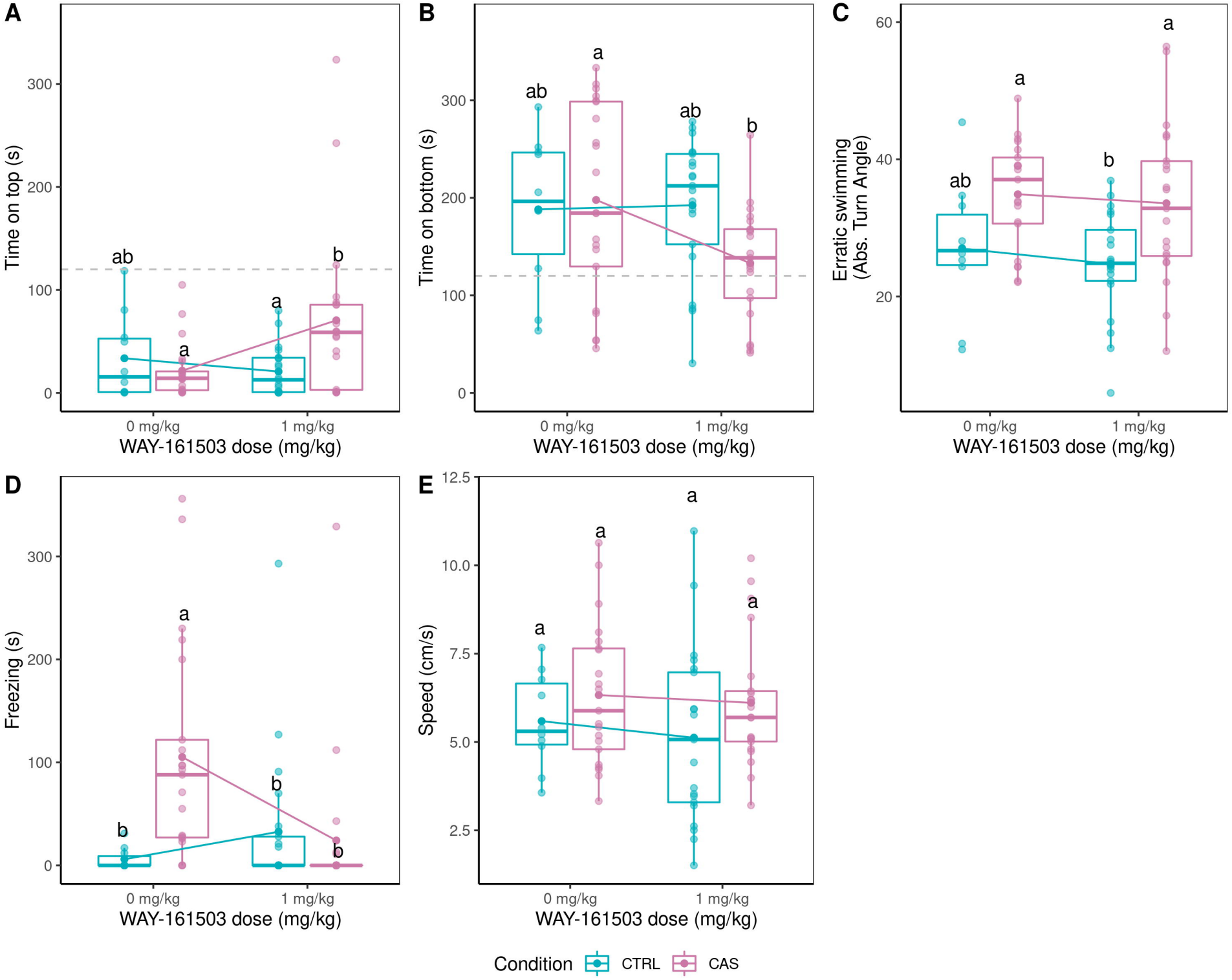

A
Distal threat
Behavioral observation (*after* exposure)
CAS exposure
Washout (1 min)
Acclimation (>14d)
6 min
20 min
6 min
Potential threat
Injection (drug or VEH)
Behavioral observation (*during* exposure)

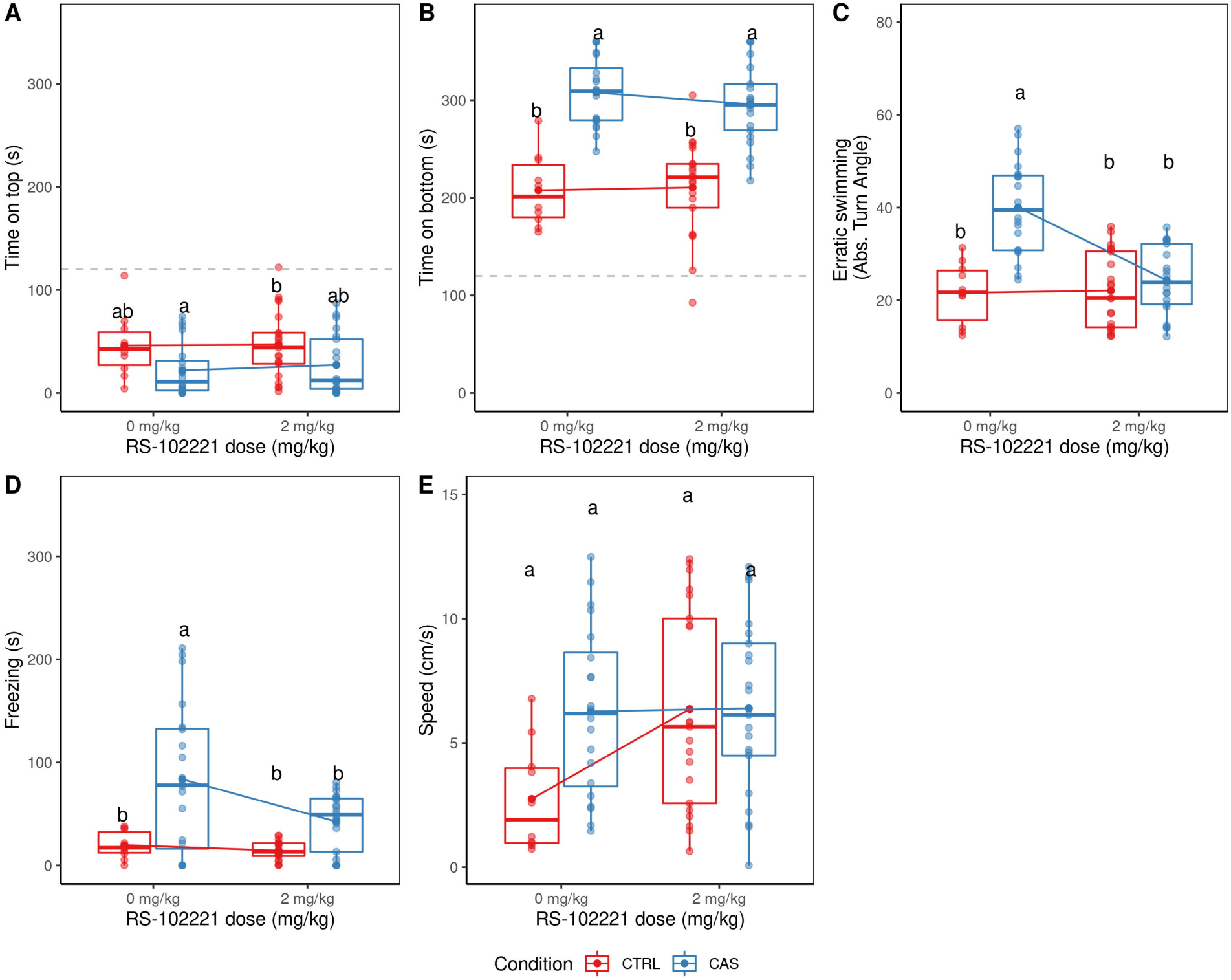

B
Behavioral observation (*after* exposure)
ARS
Acclimation (>14d)
90 min
20 min
6 min
Proximal threat
Injection (MK-212 or VEH)
LaNeC
LaNeF

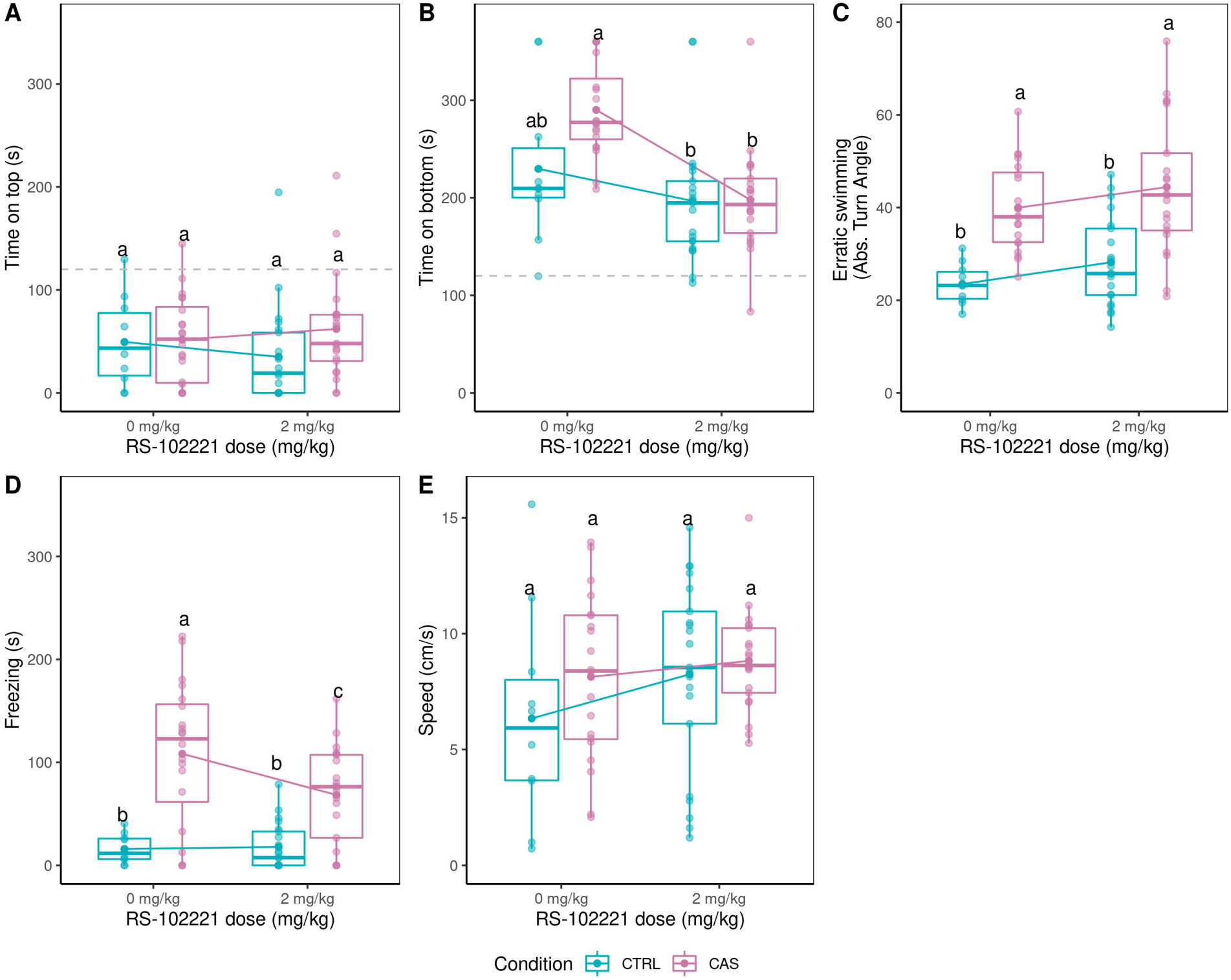

A
Time on top (s)
300
200
100
0
a
a
b
a
ab
ab
0 mg/kg
1 mg/kg
2 mg/kg
MK-212 dose (mg/kg)
B
Time on bottom (s)
300
200
100
0
bc
a
b
ac
b
b
0 mg/kg
1 mg/kg
2 mg/kg
MK-212 dose (mg/kg)
C
Erratic swimming
(Abs. Turn Angle)
75
50
25
0
b
a
a
b
a
b
0 mg/kg
1 mg/kg
2 mg/kg
MK-212 dose (mg/kg)
D
Freezing (s)
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

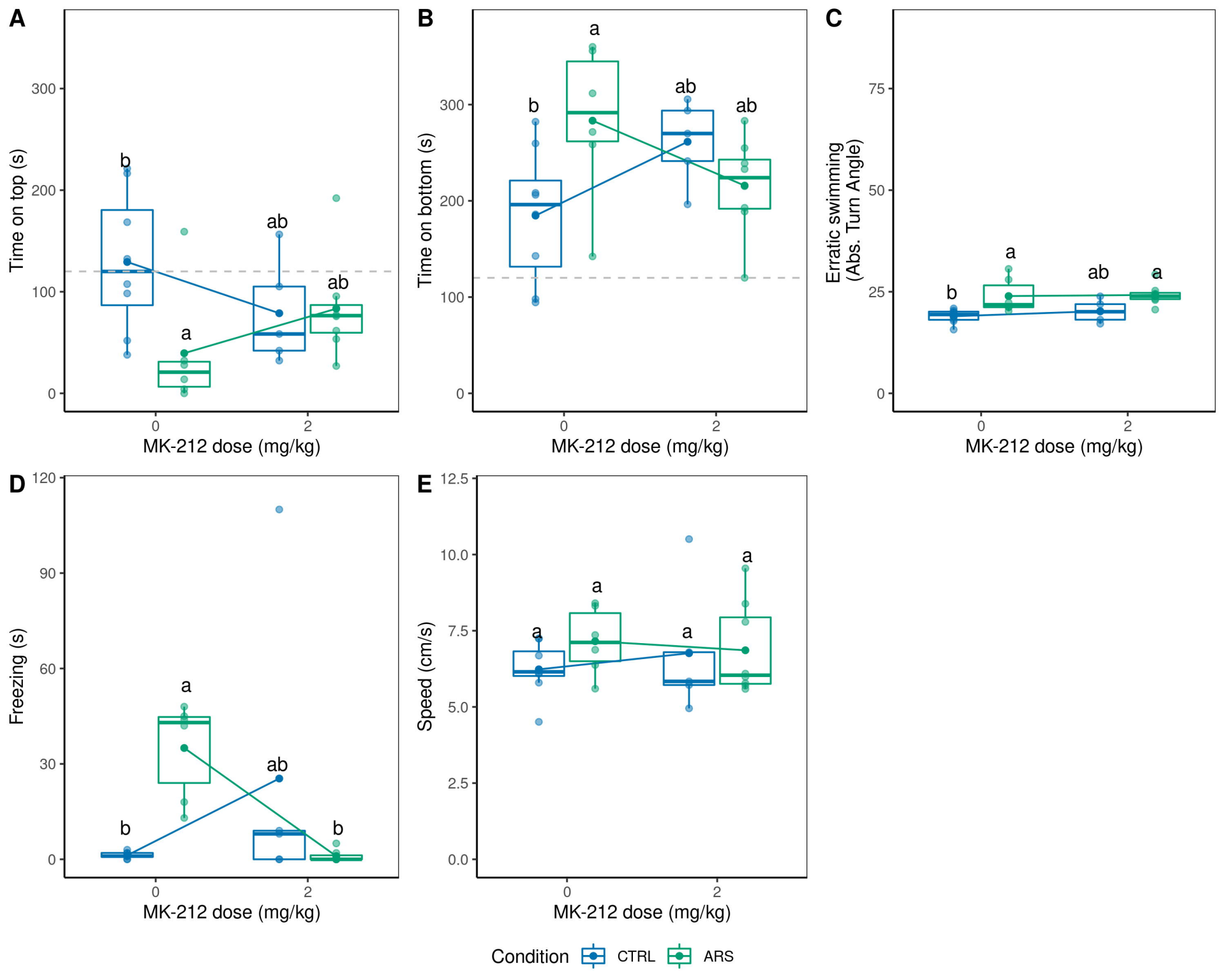

A
Time on top (s)
300
200
100
0
a
a
b
ab
ab
ab
0 mg/kg
1 mg/kg
2 mg/kg
MK-212 dose (mg/kg)
B
Time on bottom (s)
300
200
100
0
a
a
b
a
a
a
0 mg/kg
1 mg/kg
2 mg/kg
MK-212 dose (mg/kg)
C
Erratic swimming
(Abs. Turn Angle)
75
50
25
0
b
ab
ac
ab
ab
c
0 mg/kg
1 mg/kg
2 mg/kg
MK-212 dose (mg/kg)
D
Freezing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

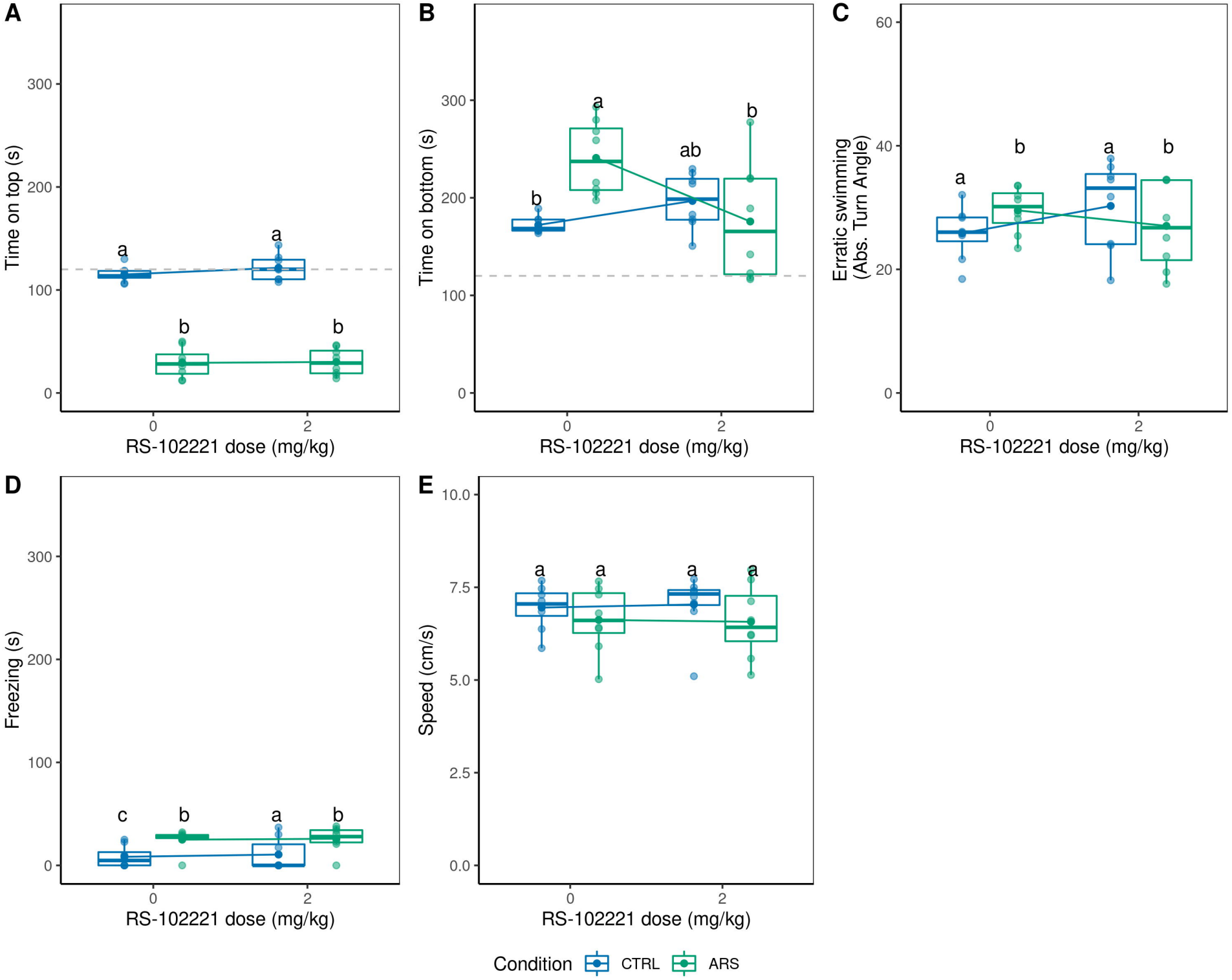

A
Time on top (s)
300
200
100
0
b
ab
ab
a
0 mg/kg
1 mg/kg
WAY-161503 dose (mg/kg)
B
Time on bottom (s)
300
200
100
0
b
a
b
b
0 mg/kg
1 mg/kg
WAY-161503 dose (mg/kg)
C
Erratic swimming
(Abs. Turn Angle)
50
40
30
20
10
a
b
b
b
0 mg/kg
1 mg/kg
WAY-161503 dose (mg/kg)
D
Freezing (s)
300
200
100
0
a
b
b
ab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

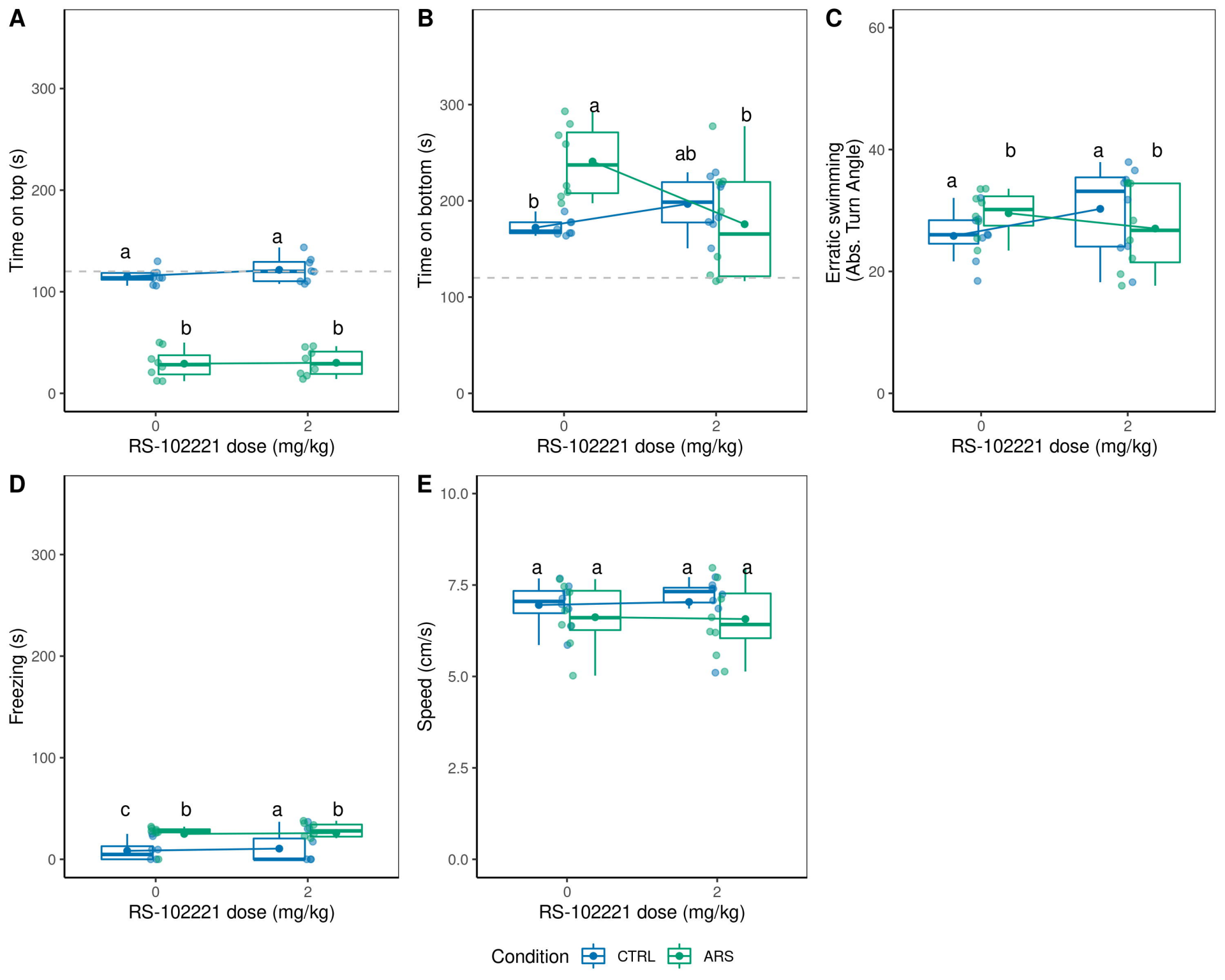

A
Time on top (s)
300
200
100
0
ab
a
b
ab
0 mg/kg
2 mg/kg
RS-102221 dose (mg/kg)
B
Time on bottom (s)
300
200
100
0
b
a
b
a
0 mg/kg
2 mg/kg
RS-102221 dose (mg/kg)
C
Erratic swimming
(Abs. Turn Angle)
80
60
40
20
0
b
a
b
b
0 mg/kg
2 mg/kg
RS-102221 dose (mg/kg)
D
Freezing (s)
300
200
100
0
b
a
b
b
0 mg/kg
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
```


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
a
b
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