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

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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 (proximal 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 a phasic inhibition of responses to distal threat. MK-212 did not block changes in behavior elicited by acute restraint stress, a model of proximal threat, suggesting that the phasic role of the 5-HT_{2C} receptor is specific to distal threat. We also found that RS-102221, a 5-HT_{2C} receptor antagonist, did not change behavior during exposure, but it produced a small effect on behavior after exposure to CAS, suggesting a tonic facilitation of responses to potential threat.

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1. Introduction

Serotonergic mechanisms have been implicated in defensive behavior to distal, proximal

45 and potential threat (Graeff, 2004). In current interpretations of the role of serotonin (5-HT) on
46 neurobehavioral responses to threat, the neurotransmitter is released when threat is potential,
47 increasing defensive behavior at this level (i.e., risk assessment/anxiety-like behavior), but
48 inhibiting (non-adaptive) responses to distal and proximal threat (i.e., fight-flight-freeze/fear-like
49 behavior)(Graeff, 2004). These effects are mediated by different structures of the neuroaxis,
50 with more rostral structures (e.g., limbic forebrain) mediating responses to potential threat, and
51 more caudal structures (e.g., periaqueductal gray/griseum centrale) mediating responses to
52 distal and proximal threat (Graeff, 2004).

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

65 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.

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

effect.

These differences could be due to species differences; to effects at different receptors (e.g., 5-HT_{2A} or 5-HT_{2C}); or to difficulties in the protocol used by Nathan et al. (2015), which does not differentiate between responses to proximal, distal, or potential threat. In this work, we tested whether 5-HT_{2C} receptors participate in responses to CAS during (distal threat) or after exposure (potential threat) and to acute restraint stress (ARS). ARS has been applied in zebrafish to elicit strong stress responses, including activation of the hypothalamus-pituitary-interrenal (HPI) axis and associated behavioral responses (Assad et al., 2020; Ghisleni et al., 2012; Piato et al., 2011). From the point of view of the predatory imminence continuum theory, ARS represents proximal threat (Perusini and Fanselow, 2015). Thus, if 5-HT_{2C} receptors inhibit aversively motivated behavior in zebrafish regardless of threat distance, its activation would inhibit behavioral responses at these three contexts. If, as in rodents, 5-HT_{2C} receptors act at different levels of threat distance to either activate or inhibit defensive responses, then 5-HT_{2C} agonists will not produce the same effect in each of these contexts.

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

145 Interestingly, work with rodents clearly suggests phasic facilitation or inhibition of defensive
146 responses by 5-HT_{2C} receptors, since agonists, but not antagonists, produce these effects.
147 The aim of the present work was to test the hypothesis that phasic activation of 5-HT_{2C}
148 receptors would block CAS-elicited defensive reactions during exposure, but not after exposure;
149 moreover, we proposed a tonic facilitation of post-exposure behavior. We also hypothesized
150 that 5-HT_{2C} agonists would not alter the anxiogenic-like effects of acute restraint stress (ARS), a
151 model for proximal threat. We found that 5-HT_{2C} agonists were able to block elements of the
152 alarm reaction, but did not affect post-exposure behavior, nor the anxiogenic-like effects of ARS.
153 This manuscript is a complete report of all the studies performed to test the effect of 5-HT_{2C}
154 agonists and antagonists on zebrafish defensive behavior to distal threat. We report how the
155 sample size was determined, all data exclusions (if any), all manipulations, and all measures in
156 the study.
157

158 2. Materials and methods

159 2.1. Animals and housing

160 Adult (>4 month-old; standard length = 23.0 ± 3.2 mm) zebrafish (*Danio rerio*) from the
161 longfin phenotype (n = 250) were used in the present experiments. The populations used are
162 expected to better represent the natural populations in the wild, due to its heterogeneous
163 genetic background (Parra et al., 2009; Speedie and Gerlai, 2008). Animals were bought from a
164 commercial vendor (Belém/PA) and collectively maintained in 40 L tanks for at least two weeks
165 before the onset of experiments. The animals were fed daily with fish flakes. The tanks were
166 kept at constant temperature (28 °C), oxygenation, light cycle (14:10 LD photoperiod) and a pH

167 of 7.0-8.0, according to standards of care for zebrafish (Lawrence, 2007). Animals were used for
168 only one experiment to reduce interference from apparatus exposure. Potential suffering of
169 animals was minimized by controlling for the aforementioned environmental variables.
170 Furthermore, in the all experiments the animals used were handled, anesthetized and sacrificed
171 according to the norms of the Brazilian Guideline for the Care and Use of Animals for Scientific
172 and Didactical Purposes (Conselho Nacional de Controle de Experimentação Animal -
173 CONCEA, 2017). Since, in all cases, animals were individually stressed and/or individually
174 treated, experimental unit was a single animal, and therefore all sample sizes refer to number of
175 animals. The experimental protocols were approved by UEPA's IACUC under protocol 06/18.
176

177 2.2. Drugs and treatments

178 The 5-HT_{2C} receptor agonists MK-2212 (2-Chloro-6-(1-piperazinyl)pyrazine, CAS
179 #64022-27-1) and WAY-161503 (8,9-dichloro-2,3,4,4a-tetrahydro-1H-pyrazino[1,2-a]quinoxalin-
180 5(6H)-one; CAS #75704-24-4) were bought from Sigma-Aldrich (St Louis, USA) on 2018, and
181 dissolved in Cortland's salt solution (NaCl 124.1 mM, KCl 5.1 mM, Na_2HPO_4 2.9 mM, $MgSO_4$ 1.9
182 mM, $CaCl_2$ 1.4 mM, $NaHCO_3$ 11.9 mM, Polyvinylpyrrolidone 4%, 1,000 USP units Heparin; Wolf,
183 1963) and in 1% dimethyl sulfoxide (DMSO), respectively. The 5-HT_{2C} receptor antagonist RS-
184 102221 (8-[5-(2,4-Dimethoxy-5-(4-trifluoromethylphenylsulphonamido)phenyl -5-oxopentyl]-
185 1,3,8-triazaspiro[4.5]decane-2,4-dione; CAS #185376-97-0) was bought from Sigma-Aldrich (St
186 Louis, USA) in 2018, and dissolved in 1% DMSO. While affinities for zebrafish 5-HT_{2C}-like
187 receptors have not been established, WAY-161503 has been reported to displace DOI from
188 human 5-HT_{2C} receptors with a K_i of 3.3 nM (6-fold selectivity over human 5-HT_{2A} receptors and
189 20-fold over human 5-HT_{2B} receptors)(Rosenzweig-Lipson et al., 2006). MK-212 has been

190 shown to be less selective at recombinant human receptors, with a K_i of 7.01 nM at 5-HT_{2C}
191 receptors (vs. 5.99 nM and 6.21 nM at 5-HT_{2A} and 5-HT_{2B} receptors, respectively)(Knight et al.,
192 2004). Finally, RS-102221 has been shown to displace mesulergine from human 5-HT_{2C}
193 receptor with a *pKi* of 8.4 nM (over 100-fold selectivity over human 5-HT_{2A} and 5-HT_{2B} receptors)
194 (Bonhaus et al., 1997).

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

207 2.3. Experiment 1: Effects of 5-HT_{2C} receptor agonists and
208 antagonists on alarm reaction and post-exposure behavior

209 2.3.1. Experimental design

210 To verify the effects of phasic activation of 5-HT_{2C} receptors on the zebrafish alarm
211 reaction, animals were pre-treated with either receptor agonists and exposed to CAS in a

212 sequential design, with a “washout” period in between tests (Figure 1A). For the exposure
213 stage, each animal was transferred individually to a container (2 L) where after 3 minutes of
214 acclimatization, it was carefully exposed to 7 ml of alarm substance (CAS), extracted using a
215 standardized protocol (do Carmo Silva et al., 2018b). As negative control, a group with the
216 same amount of animals was exposed to the same volume of distilled water, according to the
217 protocol of Lima-Maximino et al. (2020). The animals remained exposed for 6 minutes during
218 which their behavior was recorded using a video camera positioned in front of the aquarium.
219 Then, to verify the residual effects of exposure to the alarm substance, the animals were
220 transferred to the apparatus of the novel tank test, a transparent glass aquarium filled with 5 L of
221 mineral water where the animal can freely explore the space for a period 6 minutes during which
222 their behavior was recorded, following the protocol described in Lima-Maximino et al., (2020).
223 All stages of the experiment were performed under constant white Gaussian noise, producing
224 an average of 58 dB above the tank. Light levels above the tanks were measured using a
225 handheld light meter, and ranged from 251 to 280 lumens (coefficient of variation = 3.399%
226 between subjects).

227 Animals were randomly allocated to groups using a random number 185 generator
228 (http://www.jerrydallal.com/random/random_block_size_r.htm), with each subject randomized to
229 a single treatment using random permuted blocks. One PI attributed a random letter to
230 treatment (e.g., “A” for CTRL, “B” for CAS) and a random integer for drug dose (e.g., “1” for 1
231 mg/kg, “2” for 0 mg/kg [vehicle]), and combinations for letters and integers were randomized.
232 For each experiment, animals were treated and tested in the order of allocation (i.e., randomly).
233 In all experiments, experimenters and data analysts were blinded to drugs and treatment by
234 using coded vials (with the same code used for randomization); blinding was removed only after
235 data analysis. Experiments were always run between 08:00AM and 02:00 PM. After
236 experiments, animals were sacrificed by prolonged bath in ice-cold water (< 12 ºC), followed by

spinal transection (Matthews and Varga, 2012).

2.3.2. Sample size determination

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

2.3.3. Alarm substance extraction

A group of zebrafish was used as donor animals for the extraction of the conspecific alarm substance (CAS). CAS extraction procedure was performed on each animal individually as described by do Carmo Silva et al. (2018b). First, the donor animal was cold anesthetized

259 and transferred to a Petri dish, where the excess water from its body was removed with a paper
260 towel. Then the animal was decapitated with a surgical scalpel and the excess blood from the
261 sectioned region was removed with a swab. Subsequently, the animal's bodies were transferred
262 to another Petri dish where 15 superficial cuts were made in the epidermis of animals (medial-
263 ventral region) and 10 ml of distilled water were added to wash the cuts. After washing, the
264 animal's bodies were removed from the Petri dish, and with the aid of a Pasteur pipette, the fish
265 scales and other impurities were removed from the solution that was stored in a conical tube
266 and preserved on ice. The same extraction procedure was performed for all donated animals.
267

268 ## 2.3.4. Observation apparatuses

269 To assess the effects of drugs on the alarm reaction, animals were transferred to a 12
270 cm x 12 cm x 14 cm glass tank, filled with 1.5 L tank water, and allowed to swim freely for a 3
271 min acclimation period. After this period, 7 mL CAS was added to the tank, from above, and
272 filming started. The video camera (Sony® DCR-DVD610) was positioned in the front of the tank,
273 therefore allowing observation and tracking of vertical distribution. Animals were allowed to
274 freely explore this tank for 6 min before being transferred to a “washout” tank, which contained
275 500 mL tank water. Animals were kept in this tank for 1 min, removing any residues of CAS
276 before transference to the second apparatus. This second apparatus was a 25 cm x 24 cm x 20
277 cm glass tank, filled with 5 L tank water; animals were allowed free exploration of this tank for 6
278 min, during which behavior was filmed from the front of the tank. Tanks for both stages were
279 differently shaped to increase the novelty of the second environment, a variable that is important
280 to induce an anxiety-like “diving” response in animals not exposed to CAS (Bencan et al., 2009).
281

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 effects of activation of 5-HT_{2C} receptors on anxiogenic-like effects of acute restraint stress (ARS). In order to do this, we evaluate the behavior of the animals in the novel tank test after being subjected to a 90-min section of restraint stress (Figure 1B). After drug (MK-212 or 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). After stress, the animals were transferred to the apparatus of the novel tank test, a transparent glass aquarium filled with 5 L of mineral water where the animal can freely explore the space for a period 6 minutes during which their behavior was recorded, following the protocol described in Lima-Maximino et al., (2020). All stages of the experiment were performed under constant white Gaussian noise, producing an average of 58 dB above the tank. Light levels above the tanks were measured using a handheld light meter, and ranged from 254 to 276 lumens (coefficient of variation = 3.401% between subjects). Random allocation was made as described above. In all experiments, experimenters and data analysts were blinded to drugs by using coded vials (with the same code used for

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

2.4.2. Sample size determination

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

2.4.3. Observation apparatus

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

2.5. Behavioral endpoints

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

- Time spent on bottom third of the tank (s)[Primary outcome]
- Time spent on top third of the tank (s)[Secondary outcome]

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

2.6. Quality control

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

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

2.7. Statistical analysis

Data were analyzed using two-way analysis of variance (drug x exposure to stressor)

348 with sequential sum of squares (type I), followed by Tukey's post-tests when $p < 0.05$. Data
349 analysis, table organization, and result graphs were performed using R version 3.6.3 (2020-02-
350 29). Effect sizes for ANOVA effects are shown as ω^2; effect sizes for post-hoc tests were shown
351 as Cohen's d. Outliers were removed based on median absolute differences (MADs), using time
352 on bottom as the main endpoint; values were removed when they were higher or lower than 3
353 MADs around the median (Leys et al., 2013), and the number of outliers was reported in the
354 results.
355

356 2.8. Sequence alignment

357 Zebrafish have two copies of the 5-HT2C receptor gene, *htr2cl1*
358 [ENSDARG00000018228] and *htr2cl2* [ENSDARG00000013210] (Sourbron et al., 2016);
359 currently, only the expression of *htr2cl1* is known, and studies were made in larvae (Schneider
360 et al., 2012). Moreover, receptor binding and drug affinity is currently unknown. Mutation and *in*
361 *silico* studies suggest that the 5-HT_{2C} receptor residues D3.32, S3.36, and Y7.43 are crucial to
362 ligand binding (Canal et al., 2011; Córdova-Sintjago et al., 2012). We looked for the
363 conservation of these residues across zebrafish 5-HT_{2C}-like receptors by aligning protein
364 sequences from zebrafish receptors (XP_017212271.1 and XP_001339040.4) with the murine
365 (NP_032338.3) and human (NP_001243689.2) 5-HT_{2C} receptors in Clustal Omega
366 (https://www.ebi.ac.uk/Tools/msa/clustalo/). Resulting alignments were visually inspected for
367 conserved residues at the positions that are related to ligand affinity.
368

3. Results

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

3.1.1. During exposure

One animal from the control + 0 mg/kg group was detected as outlier and removed. Small-to-medium-sized main effects of treatment ($F_{[1, 114]} = 6.337$, $p = 0.0132$; $\omega^2 = 0.039$) and dose ($F_{[2, 114]} = 3.665$, $p = 0.0287$; $\omega^2 = 0.039$) were found for time on top. A small-to-medium interaction effect was also found ($F_{[2, 114]} = 4.602$, $p = 0.012$; $\omega^2 = 0.052$). Post-hoc tests found that CAS did not alter time on top ($p = 0.94$, $d = 0.3$, non-treated controls vs. non-treated CAS), but MK-212 (1 mg/kg) increased it in non-exposed animals (*1 mg/kg:* $p = 0.024$, $d = -1.05$, non-treated controls vs. 1 mg/kg controls; *2 mg/kg:* $p = 0.994$, $d = -0.18$, non-treated controls vs. 2 mg/kg controls; Figure 2A).

Medium-to-large-sized main effects of treatment ($F_{[1, 114]} = 20.995$, $p = 1.18 \times 10^{-5}$; $\omega^2 = 0.12$) and dose ($F_{[2, 114]} = 11.455$, $p = 2.93 \times 10^{-5}$; $\omega^2 = 0.125$) were found for time on bottom. A small-to-medium interaction effect was also found ($F_{[2, 114]} = 4.085$, $p = 0.0194$; $\omega^2 = 0.037$). Post-hoc tests revealed that CAS increased time on bottom ($p < 0.001$, $d = -0.77$ for the main effect), and the highest MK-212 dose blocked this effect (*1 mg/kg:* $p = 0.39$, $d = -0.64$, non-treated controls vs. treated CAS; $p = 0.38$, $d = 0.6$, non-treated CAS vs. treated CAS; *2 mg/kg:* $p = 0.828$, $d = 0.4$, non-treated controls vs. treated CAS; $p < 0.001$, $d = 1.64$, non-treated CAS vs. treated CAS; Figure 2B).

A small-to-medium-sized main effect of treatment ($F_{[1, 114]} = 5.137$, $p = 0.0253$; $\omega^2 =$

390 0.017), but not dose ($F_{[2, 114]} = 2.948$, $p = 0.0565$; $\omega^2 = 0.016$), was found for erratic swimming. A
391 very large interaction effect was also found for this variable ($F_{[2, 114]} = 57.101$, $p < 2 \times 10^{-16}$; $\omega^2 =$
392 0.467). Post-hoc tests showed that CAS greatly increased absolute turn angle in non-treated
393 animals ($p < 0.001$, $d = -2.58$, non-treated controls vs. non-treated CAS), an effect that was
394 blocked by both MK-212 doses (*1 mg/kg*: $p = 1$, $d = -0.04$, non-treated controls vs. treated CAS;
395 $p < 0.001$, $d = 2.53$, non-treated CAS vs. treated CAS; *2 mg/kg*: $p = 0.81$, $d = -0.42$, non-treated
396 controls vs. treated CAS; $p < 0.001$, $d = 2.16$, non-treated CAS vs. treated CAS; Figure 2C);
397 however, MK-212 also increased absolute turn angle in animals which were not exposed to
398 CAS (*1 mg/kg*: $p < 0.001$, $d = -1.95$, non-treated controls vs. treated controls; *2 mg/kg:* $p <$
399 0.001, $d = -1.91$, non-treated controls vs. treated controls).

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

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

409

410 3.1.2. After exposure

411 In the novel tank test after CAS exposure and washout, no main effects of treatment ($F_{[1,$
412 $_{114]} = 0.945$, $p = 0.333$; $\omega^2 = 0$) were found for time on top. However, a medium-sized main

413 effect of MK-212 dose was found ($F_{[2, 114]} = 4.892$, $p = 0.009$; $\omega^2 = 0.06$). Interaction effects were
414 also absent ($F_{[2, 114]} = 2.393$, $p = 0.0959$; $\omega^2 = 0.021$). Post-hoc tests revealed that MK-212 (1
415 mg/kg) increased time on top in both controls and CAS-exposed animals (*1 mg/kg*: p = 0.019, d
416 = -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
417 vs. non-treated animals; Figure 3A).

418 Medium-to-large-sized main effects of treatment ($F_{[1, 114]} = 7.751$, $p = 0.0063$; $\omega^2 = 0.048$)
419 and MK-212 dose ($F_{[2, 114]} = 5.385$, $p = 0.0058$) were found for time on bottom. A medium-to-
420 large-sized interaction effect was also found ($F_{[2, 114]} = 4.203$, $p = 0.0173$; $\omega^2 = 0.045$). Post-hoc
421 tests suggested that MK-212 (1 mg/kg) decreased time on bottom in control animals (Figure
422 3B), but not in CAS-exposed animals (*1 mg/kg*: p = 0.002, d = 1.31, non-treated controls vs. 1
423 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,
424 non-treated controls vs. 2 mg/kg controls; p = 1, d = 0.01, non-treated CAS vs. 2 mg/kg CAS).

425 No effect of treatment was found for erratic swimming ($F_{[1, 114]} = 3.641$, $p = 0.0589$; $\omega^2 =$
426 0.018). A large-sized main effect of drug was found ($F_{[2, 114]} = 9.244$, $p = 0.00019$; $\omega^2 = 0.112$),
427 as well as medium-to-large-sized interaction effect ($F_{[2, 114]} = 4.951$, $p = 0.00867$; $\omega^2 = 0.054$).
428 Post-hoc tests suggested a synergistic effect between CAS and MK-212 at 2 mg/kg, which
429 potentiated CAS-elicited increases in erratic swimming (Figure 3C); moreover, 1 mg/kg MK-212
430 increased erratic swimming in control animals (*1 mg/kg:* p = 0.026, d= -1.05, non-treated
431 controls vs. 1 mg/kg controls; p = 1.0, d = -0.16, non-treated CAS vs. 1 mg/kg CAS; *2 mg/kg:* p
432 = 0.22, d = -0.76, non-treated controls vs. 2 mg/kg controls; p = 0.002, d = -1.21, non-treated
433 CAS vs. 2 mg/kg CAS) .

434 A small-to-medium main effect of treatment ($F_{[1, 114]} = 4.658$, $p = 0.033$; $\omega^2 = 0.025$) and
435 a large main effect of MK-212 dose were found for freezing ($F_{[2, 114]} = 11.617$, $p = 2.56 \times 10^{-5}$; ω^2
436 $= 0.143$). No interaction effects were found ($F_{[2, 114]} = 2.777$, $p = 0.0664$; $\omega^2 = 0.024$). Post-hoc
437 tests revealed that CAS increased freezing (p = 0.05, d = -0.364 for the main effect), an effect

438 that was blocked by all MK-212 doses (*1 mg/kg:* $p = 0.74$, $d = 0.47$, non-treated controls vs. 1
439 mg/kg controls; $p < 0.001$, $d = 1.5$, non-treated CAS vs. 1 mg/kg CAS; *2 mg/kg:* $p = 0.85$, $d =$
440 0.4, non-treated controls vs. 2 mg/kg controls; $p = 0.002$, $d = 1.22$, non-treated CAS vs. 2 mg/kg
441 CAS; Figure 3D).

442 No main effects of treatment ($F_{[1, 114]} = 0.111$, $p = 0.74$; $\omega^2 = -0.007$) or dose ($F_{[2, 114]} =$
443 1.339, $p = 0.266$; $\omega^2 = 0.006$), nor an interaction effect ($F_{[2, 114]} = 0.981$, $p = 0.378$; $\omega^2 = 0.0$),
444 were found for swimming speed (Figure 3E).

445

446 3.2. Effects of WAY-161503 on alarm reaction and post-exposure
447 behavior

448 3.2.1. During exposure

449 No outliers were detected from any group in this experiment. A small-to-medium-sized
450 main effect of treatment ($F_{[1, 69]} = 7.404$, $p = 0.00823$, $\omega^2 = 0.08$), but not drug ($F_{[1, 69]} = 1.751$, $p =$
451 0.19011, $\omega^2 = 0.009$), was found for time on top. No interaction effect was found ($F_{[1, 69]} = 1.001$,
452 $p = 0.32067$, $\omega^2 = 0.0$). Post-hoc tests showed that while CAS did not alter time on top ($p =$
453 0.054, $d = 1.0$, non-treated controls vs. non-treated CAS), a synergistic effect was apparent,
454 with WAY-161503-treated animals showing less time on top than controls ($p = 0.381$, $d = 0.618$,
455 non-treated controls vs. WAY-161503 controls; $p = 0.977$, $d = 0.125$, non-treated CAS vs. WAY-
456 161503 CAS; $p = 0.023$, $d = 1.125$, non-treated controls vs. WAY 161-503 CAS; Figure 4A).

457 Large main effects of treatment ($F_{[1, 69]} = 10.842$, $p = 0.00157$, $\omega^2 = 0.103$) and drug ($F_{[1,}$
458 $_{69]} = 10.138$, $p = 0.00218$, $\omega^2 = 0.096$) were found for time on bottom. A medium-to-large-sized
459 interaction effect was found ($F_{[1, 69]} = 4.614$, $p = 0.03523$, $\omega^2 = 0.038$). Post-hoc tests revealed

460 that CAS increased time on bottom ($p = 0.007$, $d = -1.287$, non-treated controls vs. non-treated
461 CAS), and WAY-161503 blocked this effect ($p = 0.989$, $d = 0.123$, non-treated controls vs.
462 WAY-161503 controls; $p = 0.002$, $d = 1.181$, non-treated CAS vs. WAY-161503 CAS; Figure
463 4B).

464 Very large main effects of treatment ($F_{[1, 69]} = 108.37$, $p = 8.66 \times 10^{-16}$, $\omega^2 = 0.311$) and
465 drug ($F_{[1, 69]} = 122.99$, $p = 2 \times 10^{-16}$, $\omega^2 = 0.353$) were found for erratic swimming. A very large
466 interaction effect was also found ($F_{[1, 69]} = 44.24$, $p = 5.67 \times 10^{-9}$, $\omega^2 = 0.125$). Again, post-hoc
467 tests suggested that CAS increased erratic swimming ($p < 0.001$, $d = -3.984$, non-treated
468 controls vs. non-treated CAS), while WAY-161503 blocked this effect ($p = 0.302$, $d = 0.6759$,
469 non-treated controls vs. WAY-161503 controls; $p < 0.001$, $d = 3.9537$, non-treated CAS vs.
470 WAY-161503 CAS; Figure 4C).

471 A large main effect of treatment ($F_{[1, 69]} = 8.582$, $p = 0.0046$, $\omega^2 = 0.094$), but not of drug
472 ($F_{[1, 69]} = 0.564$, $p = 0.4552$, $\omega^2 = -0.005$), was found for freezing duration; no interaction effect
473 was found ($F_{[1, 69]} = 1.434$, $p = 0.2352$, $\omega^2 = 0.005$). Post-hoc tests revealed that CAS increased
474 freezing ($p = 0.047$, $d = -1.02$, non-treated controls vs. non-treated CAS), but this effect was not
475 blocked by WAY-161503 ($p = 0.967$, $d = -0.178$, non-treated controls vs. WAY-161503 controls;
476 $p = 0.544$, $d = 0.412$, non-treated CAS vs. WAY-161503 CAS; Figure 4D).

477 Finally, no effects of treatment ($F_{[1, 69]} = 2.303$, $p = 0.134$, $\omega^2 = 0.018$), drug ($F_{[1, 69]} =$
478 0.103, $p = 0.749$, $\omega^2 = -0.012$), or treatment:drug interaction ($F_{[1, 69]} = 0.527$, $p = 0.471$, $\omega^2 = -$
479 0.006) were found for swimming speed (Figure 4E).

480

481 ### 3.2.2. After exposure

482 No main effects of treatment ($F_{[1, 69]} = 3.221$, $p = 0.0771$, $\omega^2 = 0.026$) were found for time

483 on top, but a small-to-medium effect of drug was found ($F_{[1, 69]}$ = 4.254, p = 0.0429, ω^2 = 0.039).
484 A medium-sized interaction was also found ($F_{[1, 69]}$ = 6.352, p = 0.014, ω^2 = 0.064). Post-hoc
485 effects revealed a synergistic effect, with WAY-161503 increasing time on top in animals
486 exposed to CAS (p = 0.922, d = 0.242, non-treated controls vs. non-treated CAS; p = 0.907,
487 0.259, non-treated controls vs. WAY-161503 controls; p = 0.011, d = 0.9833, non-treated CAS
488 vs. WAY-161503 CAS; Figure 5A).

489 No main effects of treatment ($F_{[1, 69]}$ = 1.95, p = 0.1671, ω^2 = 0.012) were found for time
490 on bottom, but a small-to-medium effect of drug was found ($F_{[1, 69]}$ = 4.107, p = 0.0466, ω^2 =
491 0.039). No significant interaction was observed ($F_{[1, 69]}$ = 3.267, p = 0.0751, ω^2 = 0.029). Post-
492 hoc tests suggested a synergistic effect, with WAY-161503 decreasing time on bottom in
493 animals exposed to CAS (p = 0.988, d = -0.1243, non-treated controls vs. non-treated CAS; p =
494 0.999, d = -0.0538, non-treated controls vs. WAY-161503 controls; p = 0.041, d = 0.8369, non-
495 treated CAS vs. WAY-161503 CAS; Figure 5B).

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

502 A medium-sized main effect of treatment ($F_{[1, 69]}$ = 4.726, p = 0.0331, ω^2 = 0.43) was
503 found for freezing, and a medium-sized main effect of drug was also found ($F_{[1, 69]}$ = 4.17, p =
504 0.04497, ω^2 = 0.037). A medium-to-large interaction effect was observed for freezing as well
505 ($F_{[1, 69]}$ = 7.663, p = 0.00723, ω^2 = 0.077). Post-hoc tests revealed that CAS increased freezing
506 (p = 0.009, d = -1.257, non-treated controls vs. non-treated CAS), while WAY-161503 blocked
507 this effect (p = 0.816, d = -0.338, non-treated controls vs. WAY-161503 controls; p = 0.008, d =

508 1.026, non-treated CAS vs. WAY-161-503 CAS; Figure 5D).

509 Finally, no main effects of treatment ($F_{[1, 69]}$ = 3.85, p = 0.0538, ω² = 0.038) or drug ($F_{[1, 69]}$
510 = 0.416, p = 0.5212, ω² = -0.008) were found for swimming speed, and an interaction effect was
511 also absent (F[1, 69] = 0.062, p = 0.8037, ω² = -0.013)(Figure 5E).

512

513 3.3. Effects of RS-102221 on alarm reaction and post-exposure 514 behavior

515 3.3.1. During exposure

516 No outliers were detected in any groups. A large effect of treatment ($F_{[1, 68]}$ = 9.68, p =
517 0.003, ω² = 0.011) was found for time on top, but no main effect of drug ($F_{[1, 68]}$ = 0.231, p =
518 0.632, ω² = -0.01) nor an interaction effect ($F_{[1, 68]}$ = 0.096, p = 0.757, ω² = -0.012) were found
519 for this variable. Post-hoc tests suggested that CAS decreased time on top (p = 0.004, d = 0.74
520 for the main effect); RS-102221 was not able to change this effect (p = 0.353, d = 0.64, non-
521 treated controls vs. treated CAS; p = 0.941, d = -0.18, non-treated CAS vs. treated CAS; Figure
522 6A).

523 A very large main effect of treatment ($F_{[1, 68]}$ = 85.978, p = 1.09 x 10^{-13}, ω² = 0.53), but
524 not dose ($F_{[1, 68]}$ = 0.352, p = 0.555, ω² = -0.009), nor an interaction effect ($F_{[1, 68]}$ = 0.517, p =
525 0.474, ω² = -0.006), were found for time on bottom (Figure 6B). Post-hoc tests suggested that
526 CAS increased time on bottom (p < 0.001, d = -2.22 for the main effect), and RS-102221 was
527 not able to change this effect (p < 0.001, d = -2.12, non-treated controls vs. treated CAS; p =
528 0.8, d = 0.29, non-treated CAS vs. treated CAS).

529 Large main effects of treatment ($F_{[1, 68]}$ = 25.74, p < 0.0001, ω² = 0.26) and drug ($F_{[1, 68]}$ =

530 21.06, $p < 0.0001$, $\omega^2 = 0.22$) were found for erratic swimming. An interaction effect was also
531 found ($F_{[1, 68]} = 15.47$, $p < 0.0001$, $\omega^2 = 0.17$). Post-hoc tests suggested that CAS increased
532 erratic swimming ($p < 0.01$, $d = 1.24$for the main effect), an effect that was blocked by RS-
533 102221 ($p = 0.829$, $d = -0.33$, non-treated controls vs. treated CAS; $p < 0.001$, $d = 1.89$, non-
534 treated CAS vs. treated CAS; Figure 6C).

535 Large main effects of treatment ($F_{[1, 68]} = 21.973$, $p < 0.0001$, $\omega^2 = 0.23$) and drug ($F_{[1, 68]} =$
536 7.06, $p = 0.0098$, $\omega^2 = 0.08$) were found for freezing; no interaction effect was found ($F_{[1, 68]} =$
537 2.98, $p = 0.089$, $\omega^2 = 0.03$). Post-hoc tests revealed that CAS increased freezing ($p < 0.001$, $d =$
538 1.10 for the main effect), and RS-102221 partially blocked this effect ($p = 0.488$, $d = -0.548$,
539 non-treated controls vs. treated CAS; $p = 0.013$, $d = 0.985$, non-treated CAS vs. treated CAS;
540 Figure 6D).

541 Finally, no main effects of treatment ($F_{[1, 68]} = 1.84$, $p = 0.179$, $\omega^2 = 0.01$) nor drug ($F_{[1, 68]}$
542 $= 3.156$, $p = 0.08$, $\omega^2 = 0.03$) were found for swimming speed (Figure 6E). No interaction effects
543 were present ($F_{[1, 68]} = 2.981$, $p = 0.089$, $\omega^2 = 0.03$).

544

545 ### 3.3.2. After exposure

546 No main effects of treatment ($F_{[1, 68]} = 2.449$, $p = 0.122$, $\omega^2 = 0.02$) or drug ($F_{[1, 68]} =$
547 0.002, $p = 0.969$, $\omega^2 = -0.01$), were found for time on top (Figure 7A); an interaction effect was
548 also absent ($F_{[1, 68]} = 1.152$, $p = 0.287$, $\omega^2 = 0.002$).

549 A medium-sized main effect of treatment ($F_{[1, 68]} = 6.322$, $p = 0.0143$, $\omega^2 = 0.07$), as well
550 as a large effect of drug ($F_{[1, 68]} = 22.796$, $p < 0.0001$, $\omega^2 = 0.23$), were found for time on bottom
551 (Figure 7B). A medium-sized interaction effect was also found ($F_{[1, 68]} = 4.08$, $p = 0.047$, $\omega^2 =$
552 0.04). Post-hoc tests suggested that CAS increased time on bottom after exposure ($p = 0.05$, d
553 $= 1.02$, non-treated controls vs. non-treated CAS), an effect that was blocked by RS-102221 (p
554 $= 0.506$, $d = 0.54$, non-treated controls vs. treated CAS; $p < 0.001$, $d = 1.55$, non-treated CAS

vs. treated CAS).

A large main effect of treatment ($F_{[1, 68]} = 36.19$, $p < 0.0001$, $\omega^2 = 0.33$), but not drug ($F_{[1, 68]} = 2.957$, $p = 0.09$, $\omega^2 = 0.03$), were found for erratic swimming (Figure 7C). No interaction effect was found ($F_{[1, 68]} = 0.002$, $p = 0.965$, $\omega^2 = -0.01$). Post-hoc tests suggested that CAS increased erratic swimming after exposure ($p = 0.001$, $d = 1.514$, non-treated controls vs. non-treated CAS), an effect that was not altered by RS-102221 ($p < 0.001$, $d = -1.92$, non-treated controls vs. treated CAS; $p = 0.56$, $d = -0.41$, non-treated CAS vs. treated CAS).

A large main effect of treatment ($F_{[1, 68]} = 39.763$, $p < 0.0001$, $\omega^2 = 0.35$) and a small main effect of drug ($F_{[1, 68]} = 4.16$, $p = 0.045$, $\omega^2 = 0.04$), were found for freezing; no interaction effects were found ($F_{[1, 68]} = 3.23$, $p = 0.077$, $\omega^2 = 0.03$). Post-hoc tests suggested that CAS increased freezing after exposure ($p < 0.001$, $d = 1.96$, non-treated controls vs. non-treated CAS), an effect that was partially blocked by RS-102221 ($p = 0.025$, $d = -1.12$, non-treated controls vs. treated CAS; $p = 0.041$, $d = 0.85$; Figure 7D).

Finally, no main effects of treatment ($F_{[1, 68]} = 1.014$, $p = 0.317$, $\omega^2 = 0.0002$) or drug ($F_{[1, 68]} = 1.83$, $p = 0.181$, $\omega^2 = 0.01$) were found for swimming speed, nor was there an interaction effect for this variable ($F_{[1, 68]} = 0.475$, $p = 0.493$, $\omega^2 = -0.007$. Figure 7E).

3.4. Restraint stress-elicited behavioral changes

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

No main effects of treatment ($F_{[1, 23]} = 0.167$, $p = 0.687$, $\omega^2 = -0.026$) nor drug ($F_{[1, 23]} = 3.884$, $p = 0.061$, $\omega^2 = 0.089$), were found for time on top (Figure 8A). No interaction effects were found as well ($F_{[1, 23]} = 4.215$, $p = 0.052$, $\omega^2 = 0.1$).

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

584 A large main effect of treatment ($F_{[1, 23]}$ = 17.9732, p = 0.00031, ω^2 = 0.398), but not drug
585 ($F_{1, 23]}$ = 0.446, p = 0.511, ω^2 = -0.013), was found for erratic swimming (Figure 8C). No
586 interaction effects were found ($F_{[1, 23]}$ = 0.193, p = 0.664, ω^2 = -0.019). Post-hoc tests suggested
587 that a ARS increased erratic swimming (p < 0.001, d = -1.57 for the main effect), an effect that
588 was not blocked by MK-212 (*p* = 0.006, d = -1.85, non-treated controls vs. treated ARS).

589 No main effects of treatment ($F_{[1, 23]}$ = 0.384, p = 0.541, ω^2 = -0.016) nor drug ($F_{[1, 23]}$ =
590 0.618, p = 0.44, ω^2 = -0.01) were found for freezing (Figure 8D). A large interaction effect was
591 found ($F_{[1, 23]}$ = 12.339, p = 0.002, ω^2 = 0.304). Post-hoc tests suggested that animals injected
592 with MK-212 and subjected to ARS showed decreased freezing in relation to other groups (p =
593 0.033, d = -1.6, non-treated controls vs. treated ARS; p = 0.031, d = 1.6, non-treated ARS vs.
594 treated ARS).

595 Finally, no main effects of treatment ($F_{[1, 23]}$ = 1.0005, p = 0.328, ω^2 = 0.0) nor drug ($F_{[1, 23]}$
596 = 0.0283, p = 0.868, ω^2 = -0.038) were found for swimming speed (Figure 8E). No interaction
597 effect was found for this variable ($F_{[1, 23]}$ = 0.5437, p = 0.468, ω^2 = -0.018).

598

599 3.4.2. Effects of RS-102221 on restraint stress-elicited behavioral changes

600 A main effect of treatment ($F_{[1, 28]}$ = 422.613, p < 0.0001, ω^2 = 0.93), but not drug ($F_{[1, 28]}$ =

601 0.728, $p = 0.401$, $\omega^2 = -0.009$), was found for time on top (Figure 9A). No interaction effect was
602 found ($F_{[1, 28]} = 0.416$, $p = 0.524$, $\omega^2 = -0.02$). ARS increased time on top ($p < 0.0001$, $d = 7.78$ for
603 the main effect), and RS-102221 was not able to block this effect.

604 No main effects of treatment ($F_{[1, 28]} = 3.051$, $p = 0.0916$, $\omega^2 = 0.06$) nor drug ($F_{[1, 28]} =$
605 2.208, $p = 0.1485$, $\omega^2 = 0.04$) were found for time on bottom (Figure 9B). However, a significant
606 interaction effect was found ($F_{[1, 28]} = 10.832$, $p = 0.0027$, $\omega^2 = 0.24$). Post-hoc tests revealed
607 that ARS increased time on bottom ($p = 0.007$ vs. non-treated controls), an effect that was
608 partially blocked by RS-102221 ($p = 0.997$, non-treated controls vs. treated ARS; $p = 0.019$,
609 non-treated ARS vs. treated ARS).

610 No main effects of treatment ($F_{[1, 28]} = 0.014$, $p = 0.905$, $\omega^2 = -0.03$) nor drug ($F_{[1, 28]} =$
611 0.224, $p = 0.639$, $\omega^2 = -0.02$), were found for erratic swimming (Figure 9C). An interaction effect
612 was also absent ($F_{[1, 28]} = 2.916$, $p = 0.098$, $\omega^2 = 0.06$).

613 A main effect of treatment ($F_{[1, 28]} = 13.564$, $p = 0.001$, $\omega^2 = 0.28$) was found for freezing
614 (Figure 9D), but no main effect of drug was found ($F_{[1, 28]} = 0.122$, $p = 0.729$, $\omega^2 = -0.03$).
615 Moreover, an interaction effect was also absent ($F_{[1, 28]} = 0.022$, $p = 0.882$, $\omega^2 = -0.03$). ARS
616 increased freezing ($p = 0.001$, $d = 1.4$ for the main effect), an effect that was not blocked by RS-
617 102221 ($p = 0.052$, non-treated controls vs. treated ARS; $p = 0.998$, non-treated ARS vs.
618 treated ARS).

619 Finally, no main effects of treatment ($F_{[1, 28]} = 1.833$, $p = 0.187$, $\omega^2 = 0.03$) nor drug ($F_{[1, 28]}$
620 $= 0.003$, $p = 0.955$, $\omega^2 = -0.03$), neither an interaction effect ($F_{[1, 28]} = 0.051$, $p = 0.823$, $\omega^2 = -$
621 0.03), were found for swimming speed (Figure 9E).

622

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

The sequences of *Danio rerio* 5-HT_{2C}-like receptors were aligned to the sequence of the human and murine receptors using Clustal Omega. Sequence alignment showing the transmembrane domains is shown in Figure 10. While target residues were conserved across all sequences, differences in residues close to D3.32 the 5-HT_{2C}-like receptors zebrafish receptor (S3.30Y in 5-HT_{2CL1} and S3.30F in 5-HT_{2CL2}), as well as close to Y7.43 (V7.44I in5-HT_{2CL2}) were found.

4. Discussion

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

4.1. Effects of stressors on zebrafish behavior

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

In the present experiments, CAS consistently increased bottom-dwelling and erratic swimming during exposure, with a smaller component of freezing. After exposure, a strong component of freezing was present, while erratic swimming contributed less to the overall behavioral pattern. These results are consistent with what was observed both with a washout period (Lima-Maximino et al., 2020), and in the absence of a washout, but with an extended observation interval (Mathuru et al., 2012; Nathan et al., 2015). These effects suggest that, as threat levels change from distal (i.e., CAS is present) to potential (i.e., CAS is no longer present), a “residual” effect emerges that is marked by different behavioral components. This is similar to what is observed with electrical stimulation of the PAG/GR in rats (Brandão et al., 2008). We have previously shown that serotonin differentially mediates these behaviors, phasically inhibiting responses during exposure and tonically facilitating responses after exposure (Lima-Maximino et al., 2020).

ARS-elicited behavioral effects have been explored in the literature with mixed results. While Ghisleni et al. (2012) found that a 90-min restraint protocol did not change bottom-dwelling after stress when animals were tested individually, a similar protocol showed marked 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. In the present experiments, ARS increased bottom-dwelling as well. Moreover, we observed a small

668 component of erratic swimming, consistent with what is observed by Ghisleni et al. (2012).
669 While increased bottom-dwelling was also observed as a pattern of post-exposure behavior
670 after CAS in the present experiments and in Lima-Maximino et al. (2020), freezing was a major
671 component of post-exposure behavior, suggesting that stressors do not produce similar
672 behavioral effects.
673

674 4.2. Role of the 5-HT_{2C} receptor in CAS-elicited behavioral 675 adjustments

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

683 We have previously found that serotonin participates in responses to CAS, acutely and
684 phasically inhibiting responses to proximal threat but phasically facilitating responses to
685 potential threat (Lima-Maximino et al., 2020; Maximino et al., 2014). We suggested that phasic
686 and tonic serotonin encode an aversive expectation value, switching behavior toward cautious
687 exploration/risk assessment/anxiety when the aversive stimulus is no longer present. In the
688 experimental design that was used in this experiment, behavior during exposure represents
689 proximal threat, as CAS acts as a partial predator stimulus that elicits behavior that decreases
690 the possibility of a predator attack or detection by the predator (Smith, 1992). However, if CAS

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

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

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

729 One potential limitation of behavioral pharmacological work in zebrafish is the relative
730 divergence between 5-HT_{2C}-like receptors in teleosts and mammals, which makes it difficult to
731 ascertain whether drugs which act as agonists and antagonists at mammalian 5-HT_{2C} receptors
732 will target zebrafish receptors. Using sequence alignment, it was found that residues that, in
733 humans, are crucial for ligand binding (and selectivity with the 5-HT_{2A} receptor, for example;
734 Canal et al., 2011; Córdova-Sintjago et al., 2012) are conserved in both zebrafish proteins. This
735 suggests that ligand affinity is conserved for these proteins, which strengthens the hypothesis
736 that MK-212, WAY-161503, and RS-102221 targeted 5-HT_{2C} receptors in the behavioral
737 experiments. Differences were found in residues near the conserved binding sites, however;
738 even in mammalian receptors, though, the role of these residues in ligand affinity and/or efficacy
739 is unknown.

740

741 ## 4.3. Limitations and translational potential

742 While the present work presents results on zebrafish behavioral pharmacology inspired
743 on results from mammals, which can potentially underscore conserved mechanisms for
744 responses to threat across vertebrates (cf. Gerlai, 2014, for a discussion on how using fish as
745 well as mammals can decrease uncertainty regarding translational relevance and evolutionary
746 conservation of mechanisms), there are limitations in using zebrafish as experimental models in
747 neuroscience. One of them is that, due to the teleost genome duplication event, zebrafish
748 present two copies of the gene that codes the 5-HT_{2C} receptor (Schneider et al., 2012), and
749 therefore it is currently not possible to know which protein (HTR2CL1 or HTR2Cl2) was targeted
750 by the drugs used in the present experiments. Importantly, both proteins had conserved
751 residues at sites known to affect ligand affinity in the human 5-HT_{2C} receptor, and therefore both
752 are likely to have produced the effects. Moreover, while we propose that alarm reactions and
753 post-exposure behavior can be exploited to better understand panic disorder (Silva et al., 2020),
754 the paradigm that was used represents the normal, adaptive behavior of zebrafish under
755 threatening circumstances, and therefore cannot yet be used to generate novel hypotheses
756 about a pathological organism (cf. Maximino and van der Staay, 2019, for a discussion on the
757 limitations of using behavioral assays in experimental psychopathology).

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

765 Despite these limitations, the work presented has translational potential in understanding

766 fear, anxiety, and panic states (cf. Silva et al., 2020, for a discussion on the use of alarm
767 reactions to study fear in zebrafish). In general, our results are the first to determine a specific
768 role of 5-HT_{2C} receptors in zebrafish behavior, and add to the small literature on the role of this
769 receptor in mammals as well. Further work is needed to understand whether this receptor
770 interacts with other serotonin receptors known to be involved in defensive behavior in this
771 species (Herculano and Maximino, 2014) and if the effects during exposure are related to
772 changes in serotonin levels. Moreover, given the importance of these phenotypes to
773 understanding fear and panic states (Silva et al., 2020), further work will clarify the usefulness of
774 this pharmacological profile in modeling panic disorder and anxiety.
775
776

777 Significance statement

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

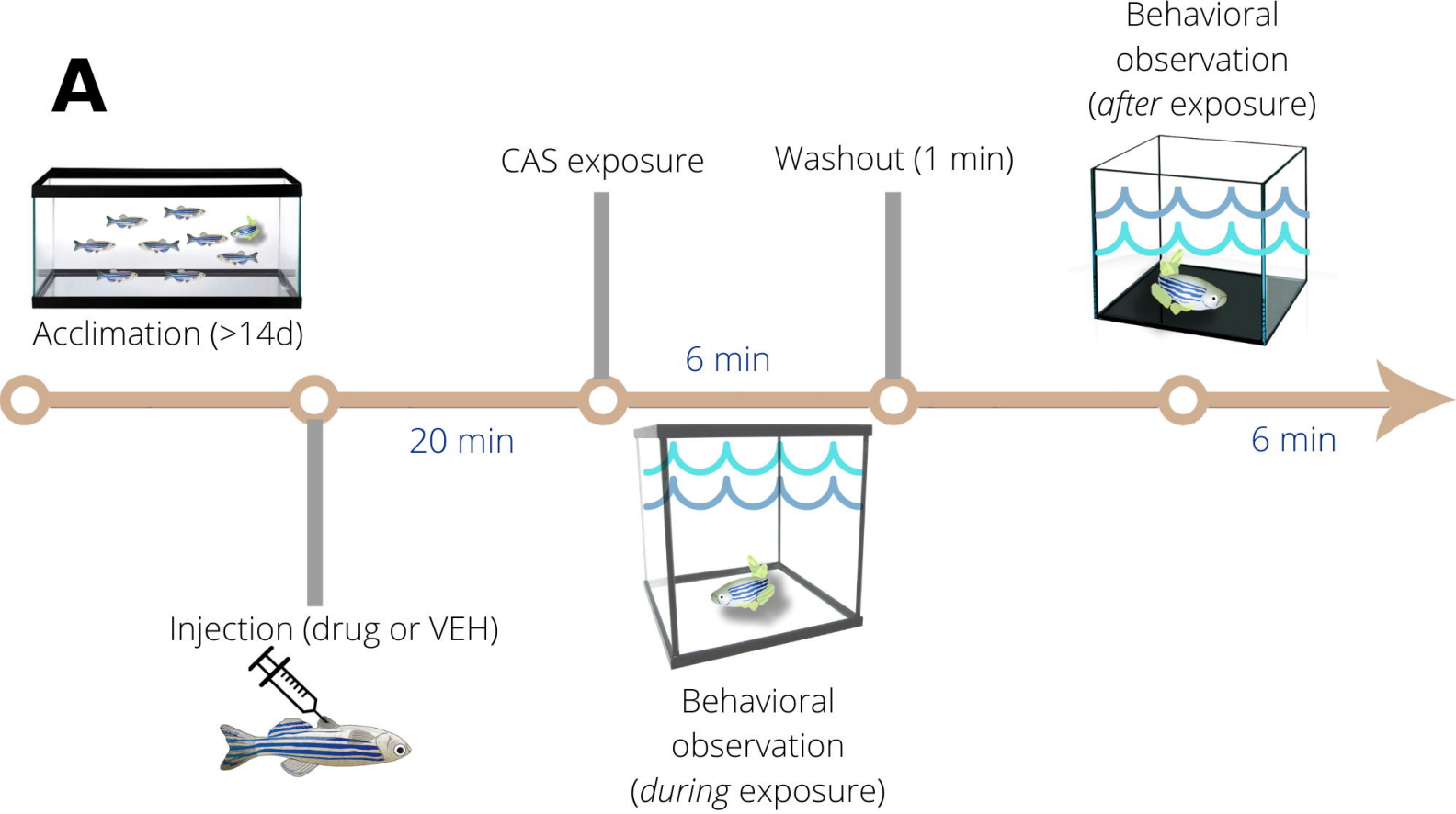
Figure 4 – Effects of WAY-161503 on behavior <u>during</u> CAS exposure. (A) Time spent on top third of the tank. (B) Time spent on bottom third of the tank. (C) Erratic swimming. (D) Total time spent freezing. (E) Swimming speed. Different letters represent statistical

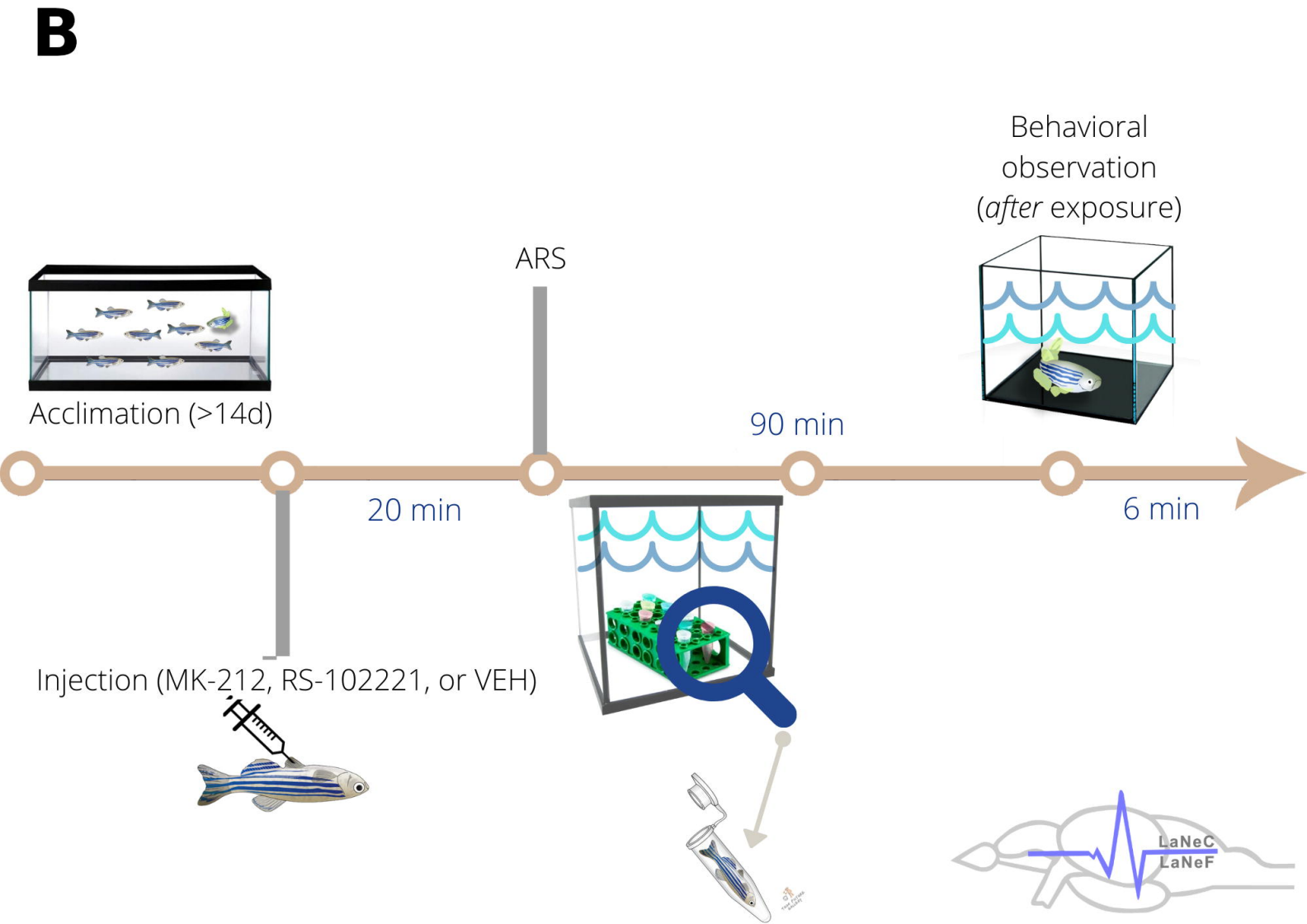
1048 differences at the $p < 0.05$ level; similar letters indicate lack of statistically significant
1049 differences. Data are presented as individual data points (dots) superimposed over the
1050 median ± interquartile ranges. Dashed lines on panels A and B represent change levels.
1051 Dots connected by lines represent group means. CTRL = controls (water-exposed
1052 animals); CAS = conspecific alarm substance. Final sample sizes: CTRL + VEH: n = 10
1053 animals; CTRL + 1 mg/kg WAY-161503: n = 21 animals; CAS + VEH: n = 21 animals;
1054 CAS + 1 mg/kg WAY-161503: n = 21 animals.
1055 Figure 5 – Effects of WAY-161503 on behavior after CAS exposure. (A) Time spent on top third
1056 of the tank. (B) Time spent on bottom third of the tank. (C) Erratic swimming. (D) Total
1057 time spent freezing. (E) Swimming speed. Different letters represent statistical
1058 differences at the $p < 0.05$ level; similar letters indicate lack of statistically significant
1059 differences. Data are presented as individual data points (dots) superimposed over the
1060 median ± interquartile ranges. Dashed lines on panels A and B represent change levels.
1061 Dots connected by lines represent group means. CTRL = controls (water-exposed
1062 animals); CAS = conspecific alarm substance. Final sample sizes: CTRL + VEH: n = 10
1063 animals; CTRL + 1 mg/kg WAY-161503: n = 21 animals; CAS + VEH: n = 21 animals;
1064 CAS + 1 mg/kg WAY-161503: n = 21 animals.
1065 Figure 6 – Effects of RS-102221 on behavior during CAS exposure. (A) Time spent on top third
1066 of the tank. (B) Time spent on bottom third of the tank. (C) Erratic swimming. (D) Total
1067 time spent freezing. (E) Swimming speed. Different letters represent statistical
1068 differences at the $p < 0.05$ level; similar letters indicate lack of statistically significant
1069 differences. Data are presented as individual data points (dots) superimposed over the
1070 median ± interquartile ranges. Dashed lines on panels A and B represent change levels.
1071 Dots connected by lines represent group means. CTRL = controls (water-exposed
1072 animals); CAS = conspecific alarm substance. Final sample sizes: CTRL + VEH: n = 10
1073 animals; CTRL + 2 mg/kg RS-102221: n = 21 animals; CAS + VEH: n = 20 animals; CAS
1074 + 2 mg/kg RS-102221: n = 21 animals.
1075 Figure 7 – Effects of RS-102221 on behavior after CAS exposure. (A) Time spent on top third of
1076 the tank. (B) Time spent on bottom third of the tank. (C) Erratic swimming. (D) Total time
1077 spent freezing. (E) Swimming speed. Different letters represent statistical differences at
1078 the $p < 0.05$ level; similar letters indicate lack of statistically significant differences. Data
1079 are presented as individual data points (dots) superimposed over the median ±
1080 interquartile ranges. Dashed lines on panels A and B represent change levels. Dots

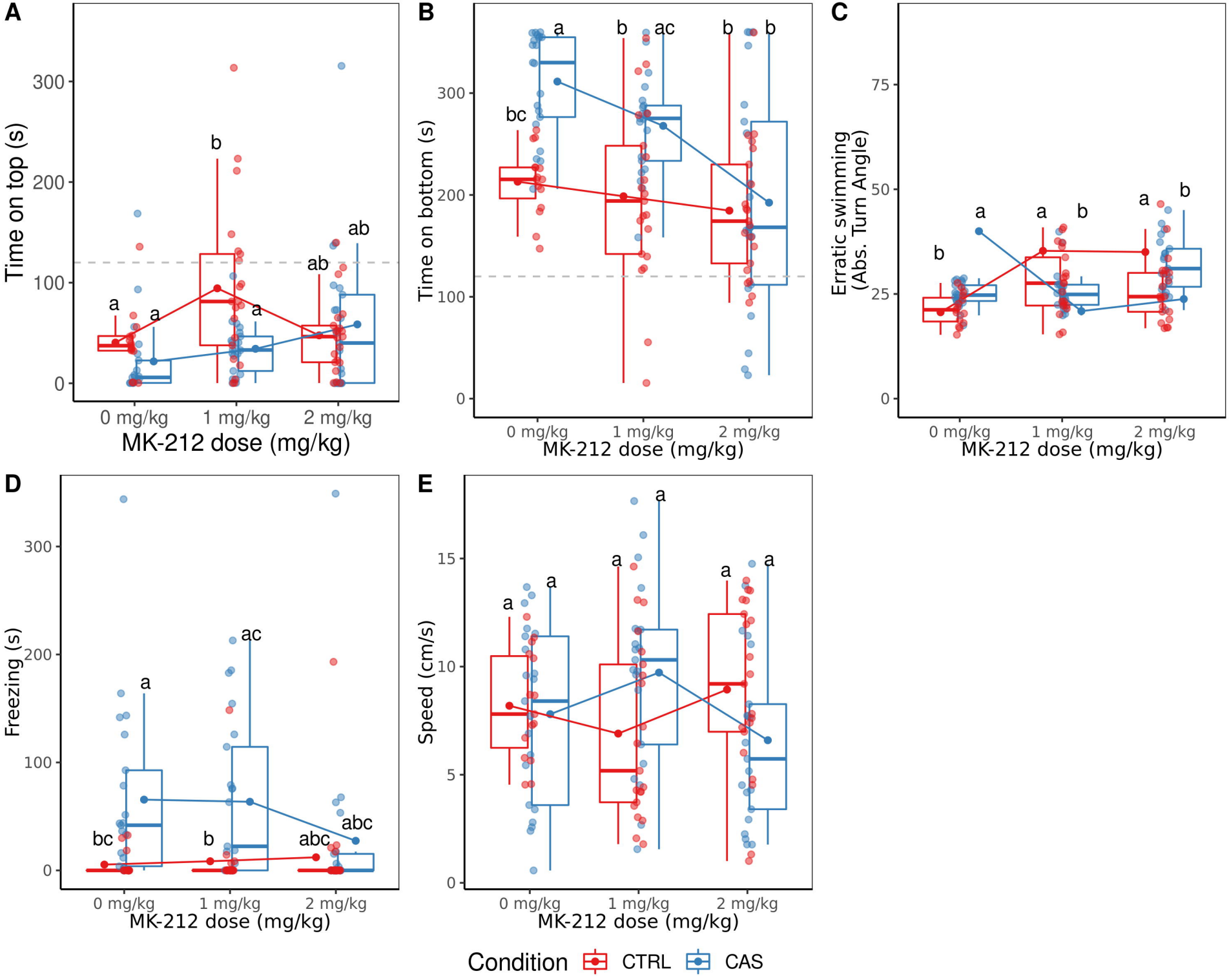
connected by lines represent group means. CTRL = controls (water-exposed animals); CAS = conspecific alarm substance. Final sample sizes: CTRL + VEH: n = 10 animals; CTRL + 2 mg/kg RS-102221: n = 21 animals; CAS + VEH: n = 20 animals; CAS + 2 mg/kg RS-102221: n = 21 animals.

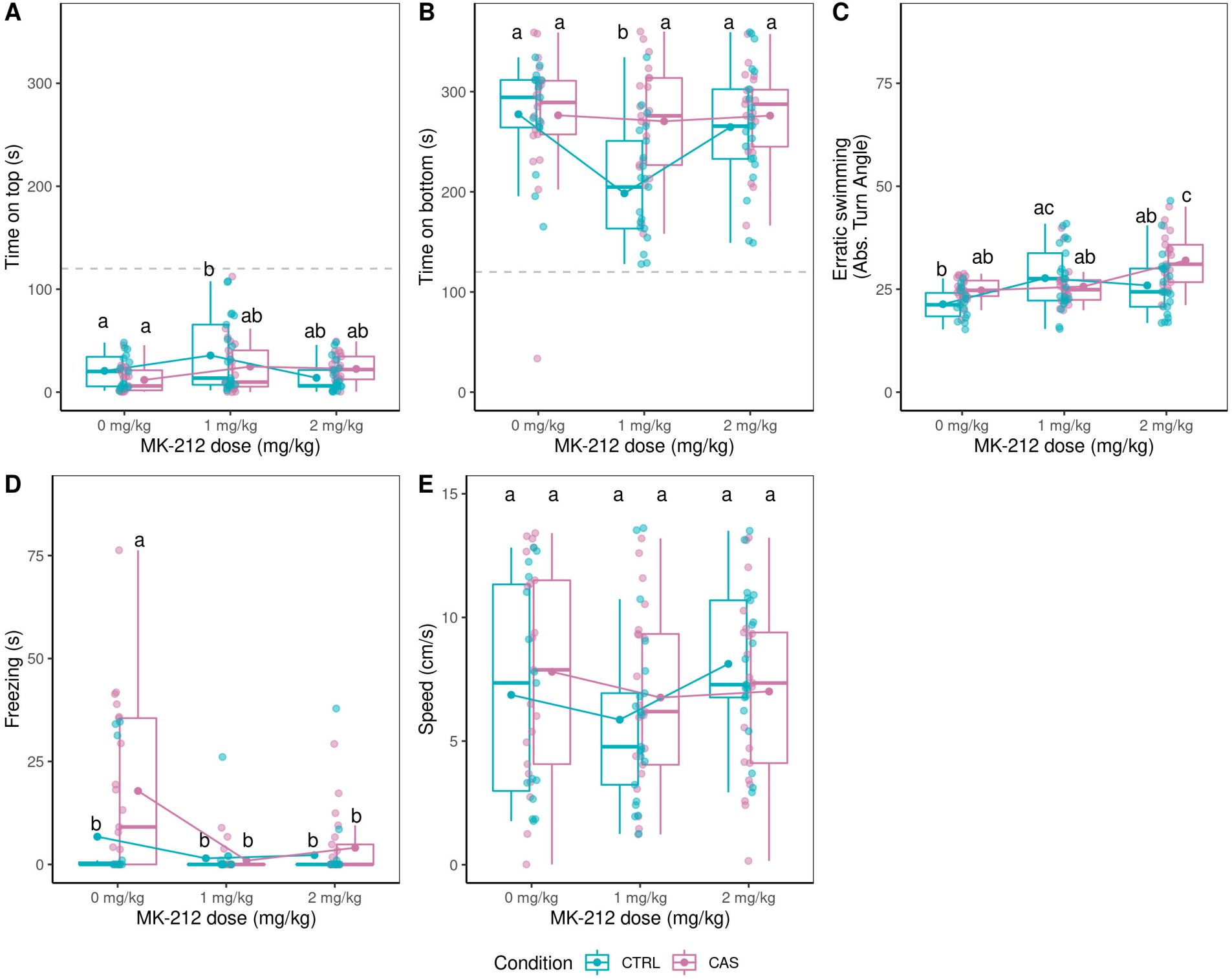
Figure 8 – Effects of MK-212 on behavior after acute restraint stress (ARS). (A) Time spent on top third of the tank. (B) Time spent on bottom third of the tank. (C) Erratic swimming. (D) Total time spent freezing. (E) Swimming speed. Different letters represent statistical differences at the $p < 0.05$ level; 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); ARS = acute restraint stress. Final sample sizes: CTRL + VEH: n = 8 animals; CTRL + 2 mg/kg MK-212: n = 5 animals; ARS + VEH: n = 6 animals; ARS + 2 mg/kg MK-212: n = 8 animals.

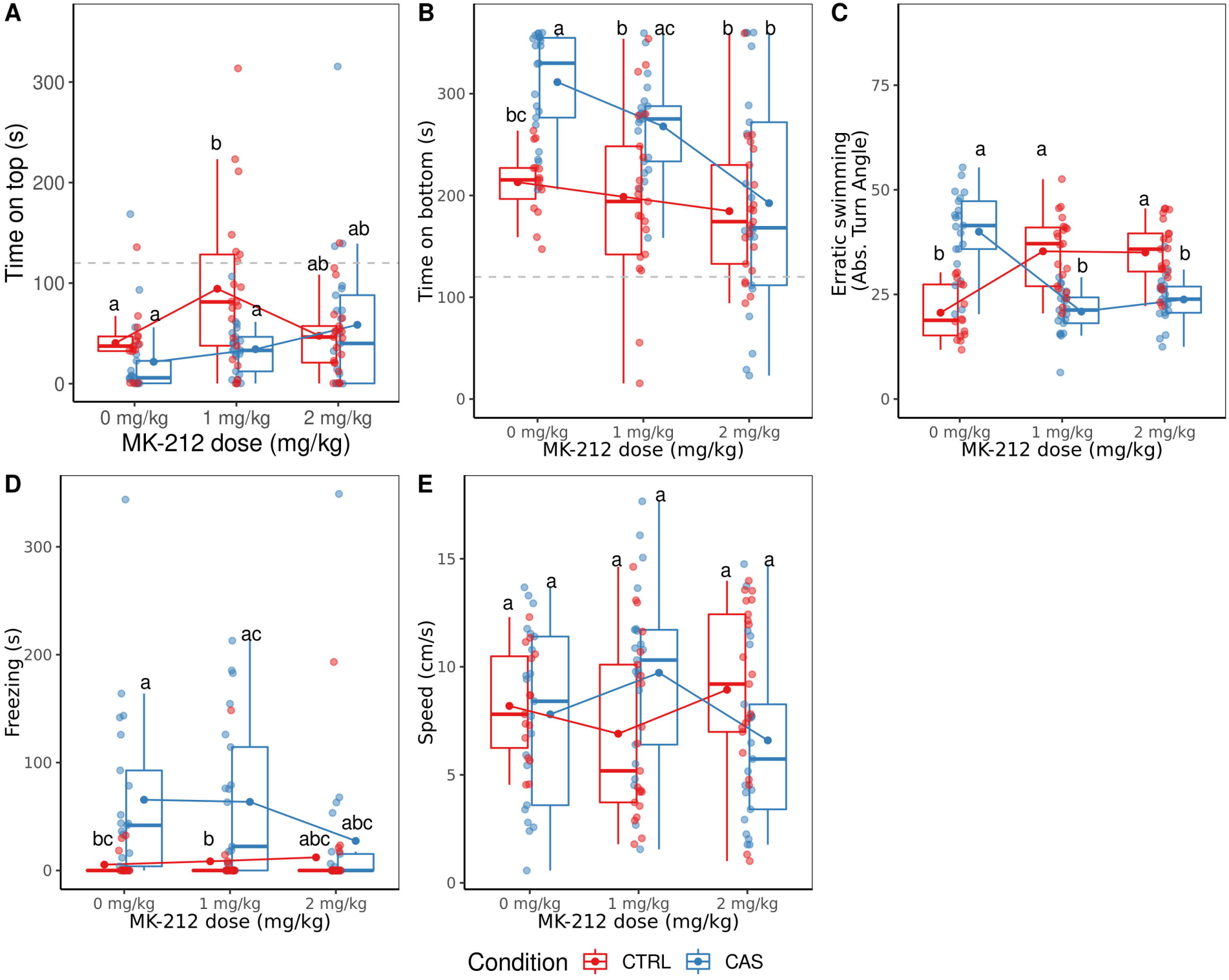
Figure 9 – Effects of RS-102221 on behavior after acute restraint stress (ARS). (A) Time spent on top third of the tank. (B) Time spent on bottom third of the tank. (C) Erratic swimming. (D) Total time spent freezing. (E) Swimming speed. Different letters represent statistical differences at the $p < 0.05$ level; 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); ARS = acute restraint stress. Final sample sizes: CTRL + VEH: n = 8 animals; CTRL + 2 mg/kg RS-102221: n = 8 animals; ARS + VEH: n = 8 animals; ARS + 2 mg/kg RS-102221: n = 8 animals.

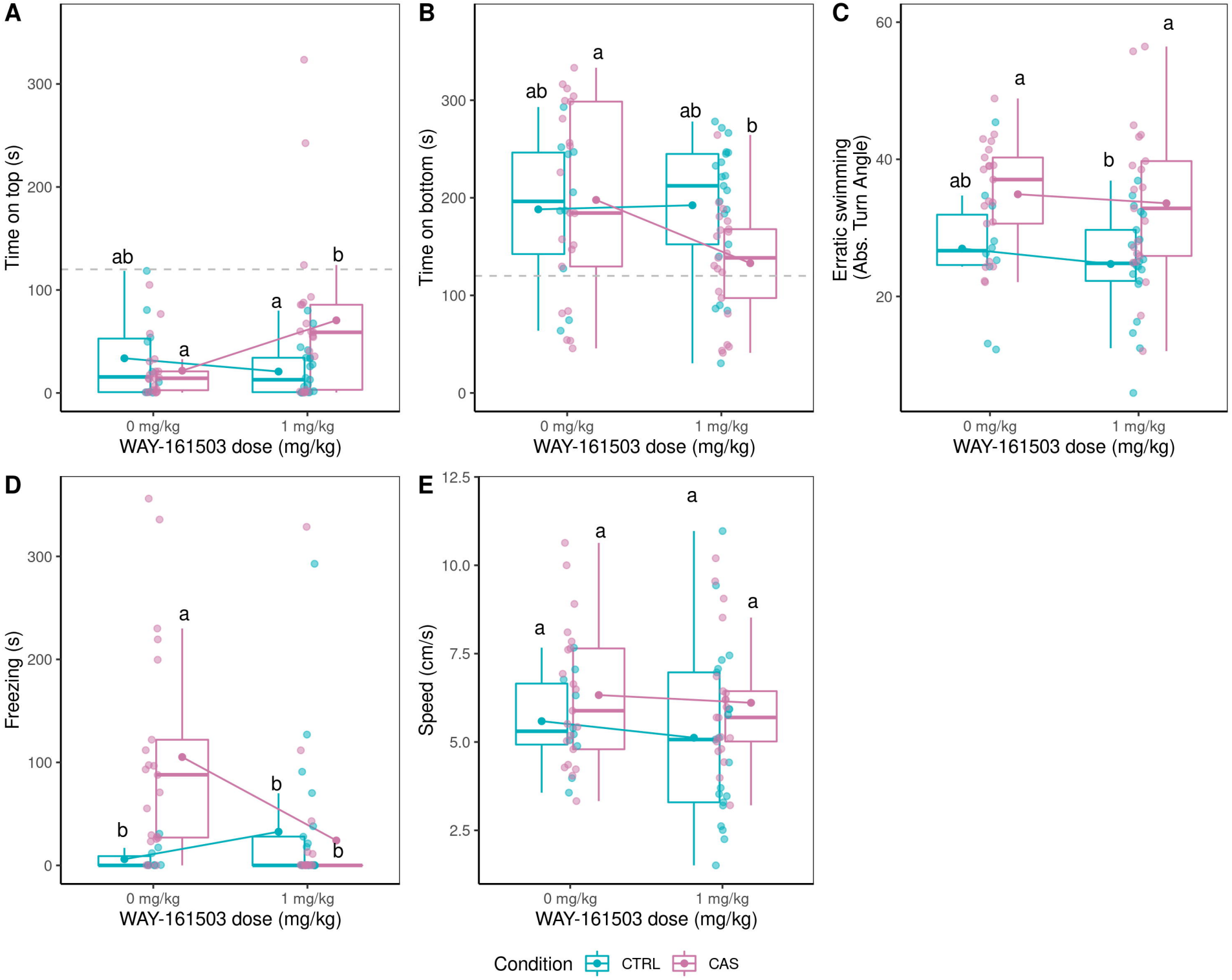

A
Acclimation (>14d)
CAS exposure
Washout (1 min)
Behavioral observation (*after* exposure)
6 min
20 min
6 min
Injection (drug or VEH)
Behavioral observation (*during* exposure)

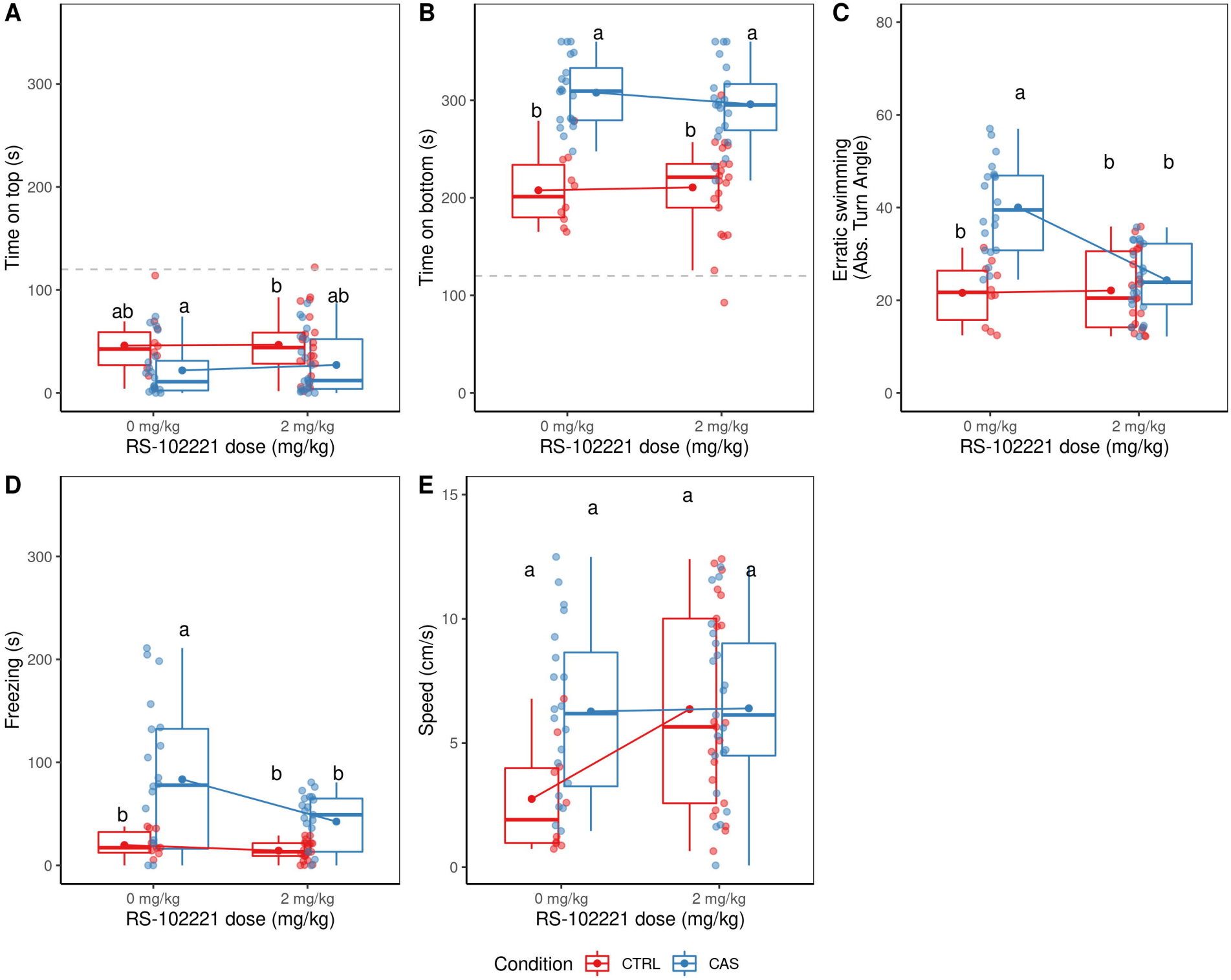

B
Acclimation (>14d)
ARS
Behavioral observation (*after* exposure)
90 min
20 min
6 min
Injection (MK-212, RS-102221, or VEH)
LaNeC
LaNeF


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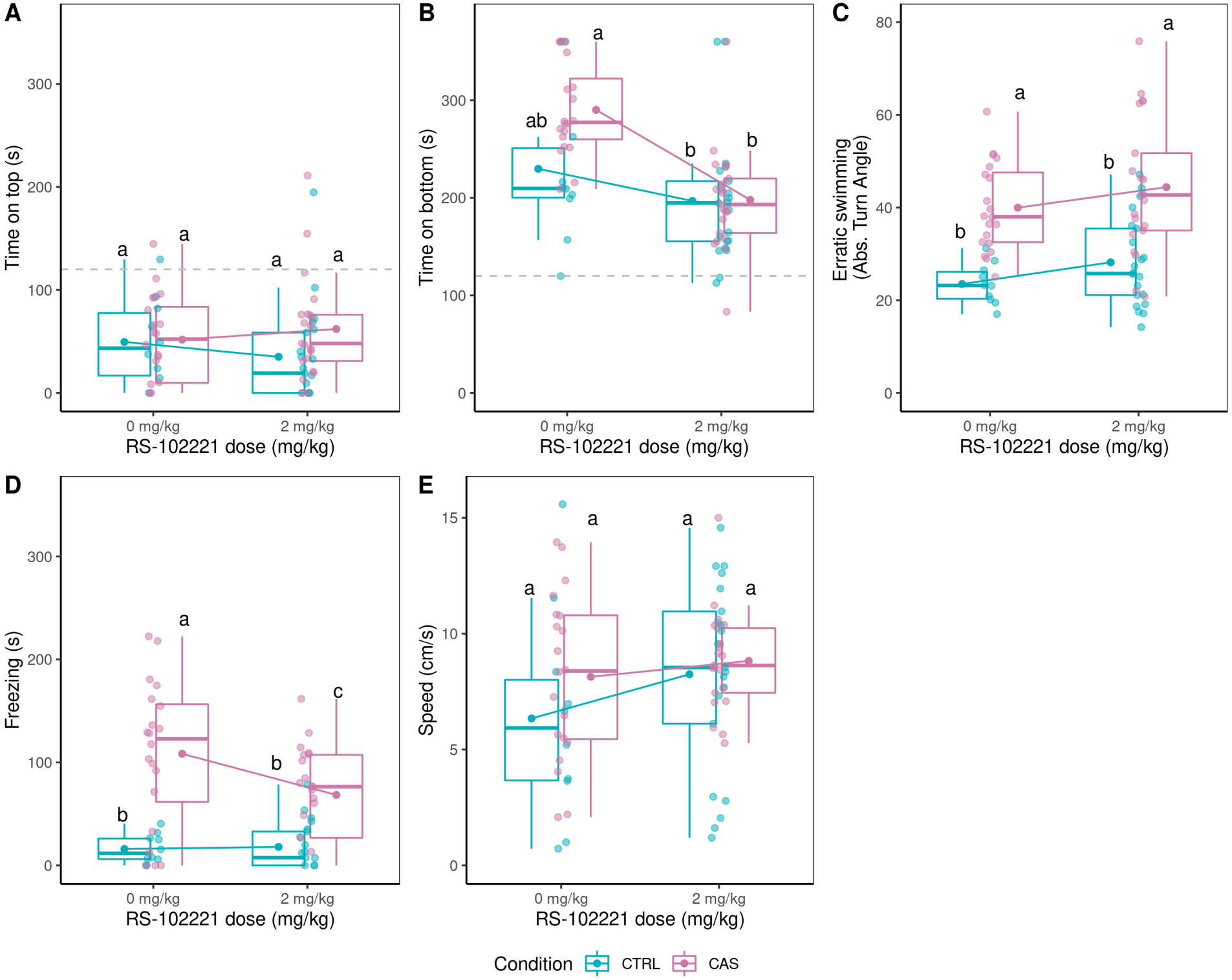
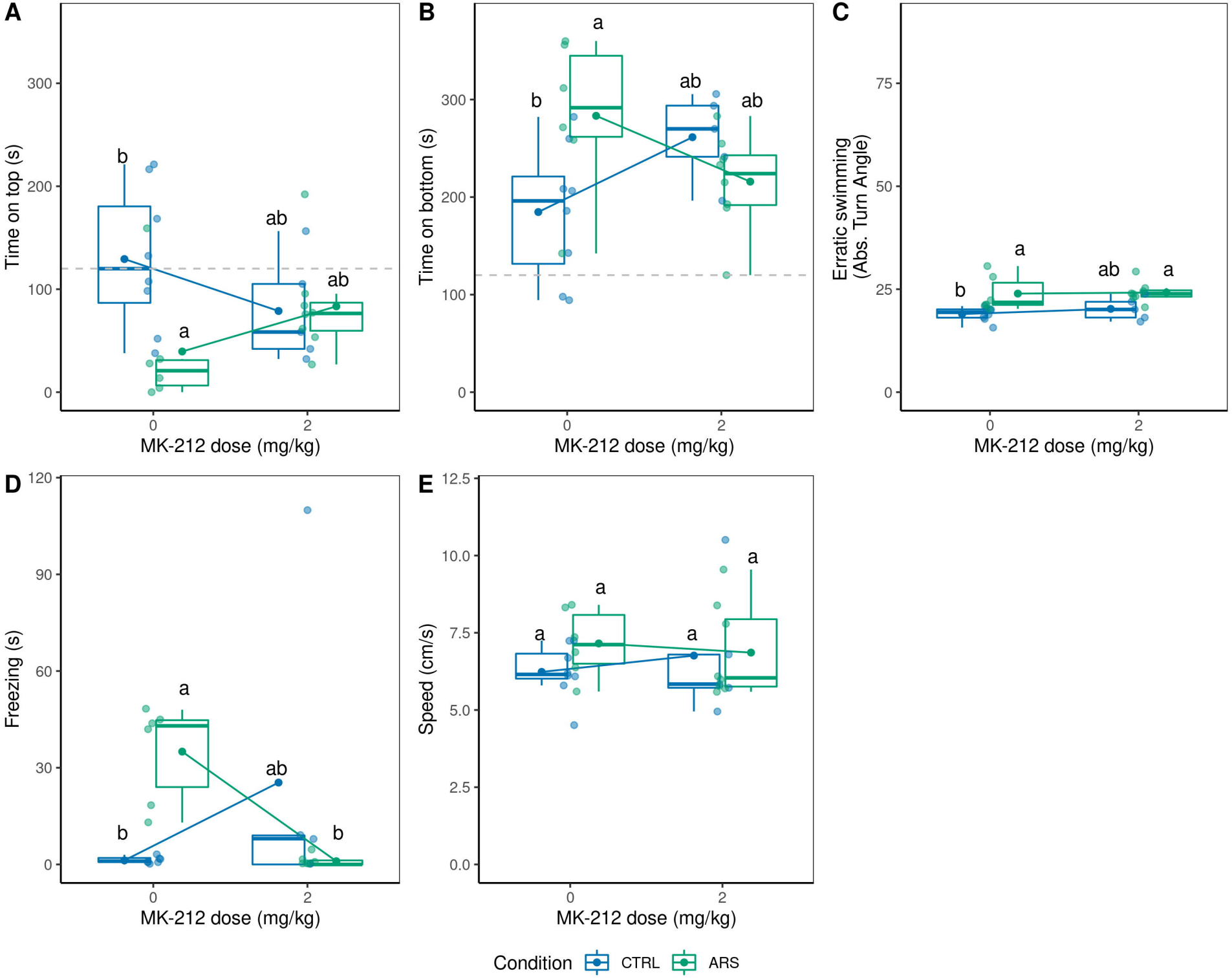
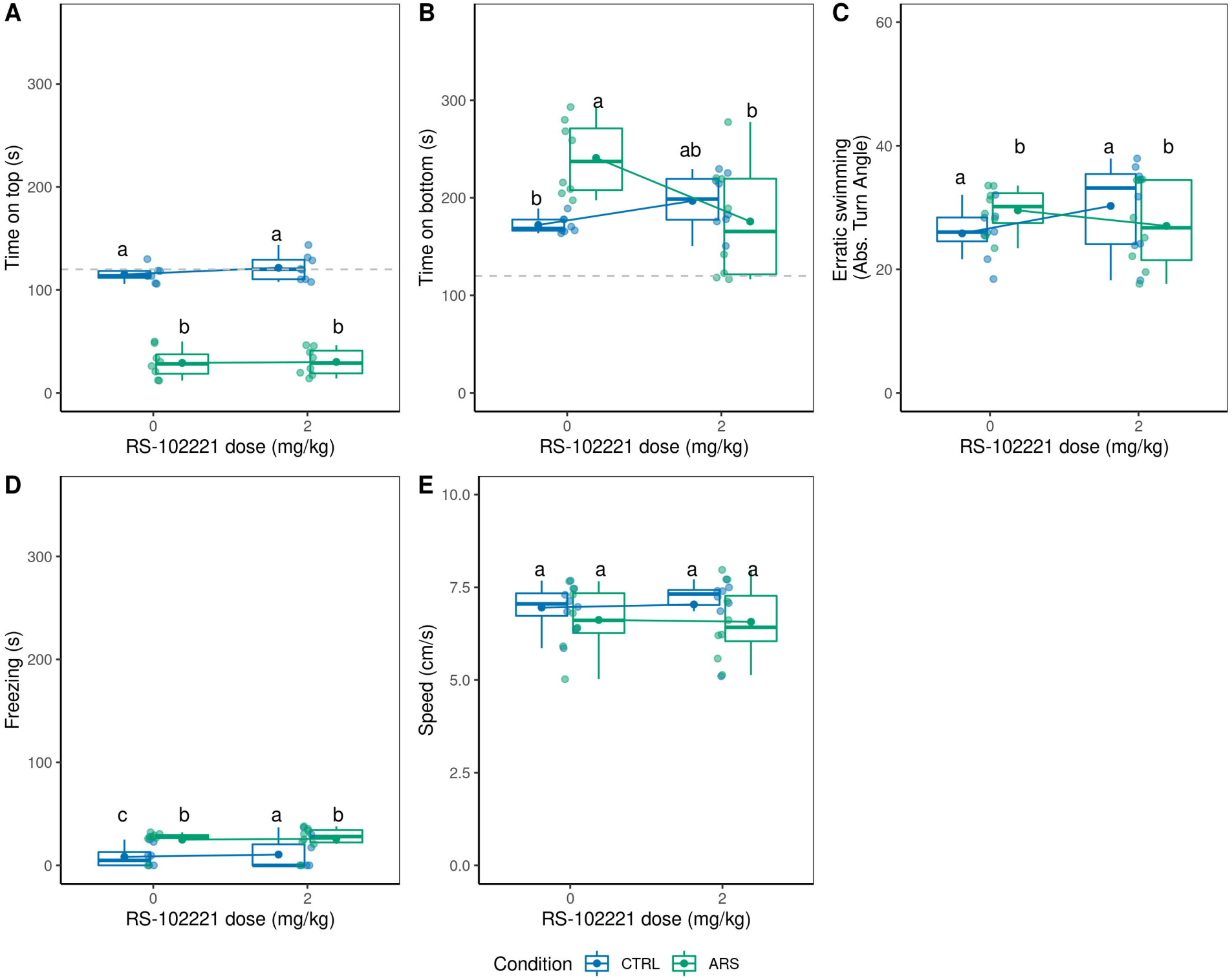
Time on top (s)
300
200
100
0
ab
a
b
ab
0 mg/kg
2 mg/kg
RS-102221 dose (mg/kg)
B
Time on bottom (s)
300
200
100
0
b
a
b
a
0 mg/kg
2 mg/kg
RS-102221 dose (mg/kg)
C
Erratic swimming
(Abs. Turn Angle)
80
60
40
20
0
b
a
b
b
0 mg/kg
2 mg/kg
RS-102221 dose (mg/kg)
D
Freezing (s)
300
200
100
0
b
a
b
b
0 mg/kg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

```
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
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