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 = 218) 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. Effects of 5-HT_{2C} receptor agonists and antagonists on alarm 208 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

237 spinal transection (Matthews and Varga, 2012).

238

239 2.3.2. Sample size determination

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

254

255 2.3.3. Alarm substance extraction

256 A group of zebrafish was used as donor animals for the extraction of the conspecific
257 alarm substance (CAS). CAS extraction procedure was performed on each animal individually
258 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

282 ## 2.4. Experiment 2: Effects of MK-212 on acute restraint stress-
283 elicited behavior

284 ### 2.4.1. Experimental design

285 In this experiment, we focus on assessing the effects of activation of 5-HT_{2C} receptors on
286 anxiogenic-like effects of acute restraint stress (ARS). In order to do this, we evaluate the
287 behavior of the animals in the novel tank test after being subjected to a 90-min section of
288 restraint stress (Figure 1B). After drug (MK-212 or RS-102221) or vehicle (Cortland's salt
289 solution) injection and anesthesia, each animal was transferred individually to a 2 mL microtube
290 (Eppendorf®) and placed on a plastic microtube rack inside an aquarium with continuous
291 oxygen supply. The microtubes had small holes at both ends to allow free circulation of water
292 inside the tube and to prevent fish from moving around, according to the protocol of Piato et al.
293 (2011). A control group was maintained in a similar tank, but without restraint stress. Animals
294 remained in these conditions for 90 min., sufficient to induce changes in telencephalon
295 neurochemistry (Assad et al., 2020) and marked anxiety-like behavior (Assad et al., 2020;
296 Ghisleni et al., 2012; Piato et al., 2011). After stress, the animals were transferred to the
297 apparatus of the novel tank test, a transparent glass aquarium filled with 5 L of mineral water
298 where the animal can freely explore the space for a period 6 minutes during which their
299 behavior was recorded, following the protocol described in Lima-Maximino et al., (2020). All
300 stages of the experiment were performed under constant white Gaussian noise, producing an
301 average of 58 dB above the tank. Light levels above the tanks were measured using a handheld
302 light meter, and ranged from 254 to 276 lumens (coefficient of variation = 3.401% between
303 subjects). Random allocation was made as described above. In all experiments, experimenters
304 and data analysts were blinded to drugs by using coded vials (with the same code used for

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

2.4.2. Sample size determination

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

2.4.3. Observation apparatus

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

2.5. Behavioral endpoints

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

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

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

2.6. Quality control

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

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

2.7. Statistical analysis

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

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

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).

368 Medium-to-large-sized main effects of treatment ($F_{[1, 114]} = 20.995$, $p = 1.18 \times 10^{-5}$; $\omega^2 =$
369 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
370 small-to-medium interaction effect was also found ($F_{[2, 114]} = 4.085$, $p = 0.0194$; $\omega^2 = 0.037$).
371 Post-hoc tests revealed that CAS increased time on bottom ($p < 0.001$, $d = -0.77$ for the main
372 effect), and the highest MK-212 dose blocked this effect (*1 mg/kg:* $p = 0.39$, $d = -0.64$, non-
373 treated controls vs. treated CAS; $p = 0.38$, $d = 0.6$, non-treated CAS vs. treated CAS; *2 mg/kg:* p
374 $= 0.828$, $d = 0.4$, non-treated controls vs. treated CAS; $p < 0.001$, $d = 1.64$, non-treated CAS vs.
375 treated CAS; Figure 2B).

376 A small-to-medium-sized main effect of treatment ($F_{[1, 114]} = 5.137$, $p = 0.0253$; $\omega^2 =$
377 0.017), but not dose ($F_{[2, 114]} = 2.948$, $p = 0.0565$; $\omega^2 = 0.016$), was found for erratic swimming. A
378 very large interaction effect was also found for this variable ($F_{[2, 114]} = 57.101$, $p < 2 \times 10^{-16}$; $\omega^2 =$
379 0.467). Post-hoc tests showed that CAS greatly increased absolute turn angle in non-treated
380 animals ($p < 0.001$, $d = -2.58$, non-treated controls vs. non-treated CAS), an effect that was
381 blocked by both MK-212 doses (*1 mg/kg*: $p = 1$, $d = -0.04$, non-treated controls vs. treated CAS;
382 $p < 0.001$, $d = 2.53$, non-treated CAS vs. treated CAS; *2 mg/kg*: $p = 0.81$, $d = -0.42$, non-treated
383 controls vs. treated CAS; $p < 0.001$, $d = 2.16$, non-treated CAS vs. treated CAS; Figure 2C);
384 however, MK-212 also increased absolute turn angle in animals which were not exposed to
385 CAS (*1 mg/kg*: $p < 0.001$, $d = -1.95$, non-treated controls vs. treated controls; *2 mg/kg:* $p <$
386 0.001, $d = -1.91$, non-treated controls vs. treated controls).

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

392 No main effects of treatment ($F_{[1, 114]} = 0.005$, $p = 0.9459$; $\omega^2 = -0.008$) nor MK-212 dose

393 ($F_{[2, 114]}$ = 0.211, p = 0.8105; ω^2 = -0.013) were found for swimming speed. While a small-to-
394 medium-sized interaction effect was found ($F_{[2, 114]}$ = 4.652, p = 0.0114; ω^2 = 0.059; f^2 = 0.29),
395 post-hoc tests found no differences across groups (Figure 2E).
396

397 ### 3.1.2. After exposure

398 In the novel tank test after CAS exposure and washout, no main effects of treatment ($F_{[1,}$
399 $_{114]}$ = 0.945, p = 0.333; ω^2 = 0) were found for time on top. However, a medium-sized main
400 effect of MK-212 dose was found ($F_{[2, 114]}$ = 4.892, p = 0.009; ω^2 = 0.06). Interaction effects were
401 also absent ($F_{[2, 114]}$ = 2.393, p = 0.0959; ω^2 = 0.021). Post-hoc tests revealed that MK-212 (1
402 mg/kg) increased time on top in both controls and CAS-exposed animals (*1 mg/kg*: p = 0.019, d
403 = -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
404 vs. non-treated animals; Figure 3A).

405 Medium-to-large-sized main effects of treatment ($F_{[1, 114]}$ = 7.751, p = 0.0063; ω^2 = 0.048)
406 and MK-212 dose ($F_{[2, 114]}$ = 5.385, p = 0.0058) were found for time on bottom. A medium-to-
407 large-sized interaction effect was also found ($F_{[2, 114]}$ = 4.203, p = 0.0173; ω^2 = 0.045). Post-hoc
408 tests suggested that MK-212 (1 mg/kg) decreased time on bottom in control animals (Figure
409 3B), but not in CAS-exposed animals (*1 mg/kg*: p = 0.002, d = 1.31, non-treated controls vs. 1
410 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,
411 non-treated controls vs. 2 mg/kg controls; p = 1, d = 0.01, non-treated CAS vs. 2 mg/kg CAS).

412 No effect of treatment was found for erratic swimming ($F_{[1, 114]}$ = 3.641, p = 0.0589; ω^2 =
413 0.018). A large-sized main effect of drug was found ($F_{[2, 114]}$ = 9.244, p = 0.00019; ω^2 = 0.112),
414 as well as medium-to-large-sized interaction effect ($F_{[2, 114]}$ = 4.951, p = 0.00867; ω^2 = 0.054).
415 Post-hoc tests suggested a synergistic effect between CAS and MK-212 at 2 mg/kg, which

potentiated CAS-elicited increases in erratic swimming (Figure 3C); moreover, 1 mg/kg MK-212 increased erratic swimming in control animals (*1 mg/kg:* $p = 0.026$, $d = -1.05$, non-treated controls vs. 1 mg/kg controls; $p = 1.0$, $d = -0.16$, non-treated CAS vs. 1 mg/kg CAS; *2 mg/kg:* $p = 0.22$, $d = -0.76$, non-treated controls vs. 2 mg/kg controls; $p = 0.002$, $d = -1.21$, non-treated CAS vs. 2 mg/kg CAS) .

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

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

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. A small-to-medium-sized main effect of treatment ($F_{[1, 69]} = 7.404$, $p = 0.00823$, $\omega^2 = 0.08$), but not drug ($F_{[1, 69]} = 1.751$, $p =$

438 0.19011, $\omega^2 = 0.009$), was found for time on top. No interaction effect was found ($F_{[1, 69]} = 1.001$,
439 $p = 0.32067$, $\omega^2 = 0.0$). Post-hoc tests showed that while CAS did not alter time on top ($p =$
440 0.054, $d = 1.0$, non-treated controls vs. non-treated CAS), a synergistic effect was apparent,
441 with WAY-161503-treated animals showing less time on top than controls ($p = 0.381$, $d = 0.618$,
442 non-treated controls vs. WAY-161503 controls; $p = 0.977$, $d = 0.125$, non-treated CAS vs. WAY-
443 161503 CAS; $p = 0.023$, $d = 1.125$, non-treated controls vs. WAY 161-503 CAS; Figure 4A).

444 Large main effects of treatment ($F_{[1, 69]} = 10.842$, $p = 0.00157$, $\omega^2 = 0.103$) and drug ($F_{[1,}$
445 $_{69]} = 10.138$, $p = 0.00218$, $\omega^2 = 0.096$) were found for time on bottom. A medium-to-large-sized
446 interaction effect was found ($F_{[1, 69]} = 4.614$, $p = 0.03523$, $\omega^2 = 0.038$). Post-hoc tests revealed
447 that CAS increased time on bottom ($p = 0.007$, $d = -1.287$, non-treated controls vs. non-treated
448 CAS), and WAY-161503 blocked this effect ($p = 0.989$, $d = 0.123$, non-treated controls vs.
449 WAY-161503 controls; $p = 0.002$, $d = 1.181$, non-treated CAS vs. WAY-161503 CAS; Figure
450 4B).

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

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

463 p = 0.544, d = 0.412, non-treated CAS vs. WAY-161503 CAS; Figure 4D).

464 Finally, no effects of treatment ($F_{[1, 69]}$ = 2.303, p = 0.134, ω² = 0.018), drug ($F_{[1, 69]}$ =
465 0.103, p = 0.749, ω² = -0.012), or treatment:drug interaction ($F_{[1, 69]}$ = 0.527, p = 0.471, ω² = -
466 0.006) were found for swimming speed (Figure 4E).

467

468 ### 3.2.2. After exposure

469 No main effects of treatment ($F_{[1, 69]}$ = 3.221, p = 0.0771, ω² = 0.026) were found for time
470 on top, but a small-to-medium effect of drug was found ($F_{[1, 69]}$ = 4.254, p = 0.0429, ω² = 0.039).
471 A medium-sized interaction was also found ($F_{[1, 69]}$ = 6.352, p = 0.014, ω² = 0.064). Post-hoc
472 effects revealed a synergistic effect, with WAY-161503 increasing time on top in animals
473 exposed to CAS (p = 0.922, d = 0.242, non-treated controls vs. non-treated CAS; p = 0.907,
474 0.259, non-treated controls vs. WAY-161503 controls; p = 0.011, d = 0.9833, non-treated CAS
475 vs. WAY-161503 CAS; Figure 5A).

476 No main effects of treatment ($F_{[1, 69]}$ = 1.95, p = 0.1671, ω² = 0.012) were found for time
477 on bottom, but a small-to-medium effect of drug was found ($F_{[1, 69]}$ = 4.107, p = 0.0466, ω² =
478 0.039). No significant interaction was observed ($F_{[1, 69]}$ = 3.267, p = 0.0751, ω² = 0.029). Post-
479 hoc tests suggested a synergistic effect, with WAY-161503 decreasing time on bottom in
480 animals exposed to CAS (p = 0.988, d = -0.1243, non-treated controls vs. non-treated CAS; p =
481 0.999, d = -0.0538, non-treated controls vs. WAY-161503 controls; p = 0.041, d = 0.8369, non-
482 treated CAS vs. WAY-161503 CAS; Figure 5B).

483 A large-sized main effect of treatment ($F_{[1, 69]}$ = 16.119, p = 0.000149, ω² = 0.174) was
484 found for erratic swimming, but no main effect of drug ($F_{[1, 69]}$ = 0.568, p = 0.454, ω² = -0.005)
485 nor an interaction effect (F[1, 69] = 0.042, p = 0.838, ω² = -0.011) were found. However, post-

hoc tests did not find an increase in erratic swimming with CAS ($p = 0.125$, $d = -0.857$), nor a synergistic effect of WAY-161503 ($p = 0.922$, $d = 0.243$, non-treated controls vs. WAY-161503 controls; $p = 0.968$, $d = 0.142$, non-treated CAS vs. WAY-161503 CAS; Figure 5C).

A medium-sized main effect of treatment ($F_{[1, 69]} = 4.726$, $p = 0.0331$, $\omega^2 = 0.43$) was found for freezing, and a medium-sized main effect of drug was also found ($F_{[1, 69]} = 4.17$, $p = 0.04497$, $\omega^2 = 0.037$). A medium-to-large interaction effect was observed for freezing as well ($F_{[1, 69]} = 7.663$, $p = 0.00723$, $\omega^2 = 0.077$). Post-hoc tests revealed that CAS increased freezing ($p = 0.009$, $d = -1.257$, non-treated controls vs. non-treated CAS), while WAY-161503 blocked this effect ($p = 0.816$, $d = -0.338$, non-treated controls vs. WAY-161503 controls; $p = 0.008$, $d = 1.026$, non-treated CAS vs. WAY-161-503 CAS; Figure 5D).

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

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

3.3.1. During exposure

No outliers were detected in any groups. A large effect of treatment ($F_{[1, 68]} = 9.68$, $p = 0.003$, $\omega^2 = 0.011$) was found for time on top, but no main effect of drug ($F_{[1, 68]} = 0.231$, $p = 0.632$, $\omega^2 = -0.01$) nor an interaction effect ($F_{[1, 68]} = 0.096$, $p = 0.757$, $\omega^2 = -0.012$) were found for this variable. Post-hoc tests suggested that CAS decreased time on top ($p = 0.004$, $d = 0.74$ for the main effect); RS-102221 was not able to change this effect ($p = 0.353$, $d = 0.64$, non-

508 treated controls vs. treated CAS; $p = 0.941$, $d = -0.18$, non-treated CAS vs. treated CAS; Figure
509 6A).

510 A very large main effect of treatment ($F_{[1, 68]} = 85.978$, $p = 1.09 \times 10^{-13}$, $\omega^2 = 0.53$), but
511 not dose ($F_{[1, 68]} = 0.352$, $p = 0.555$, $\omega^2 = -0.009$), nor an interaction effect ($F_{[1, 68]} = 0.517$, $p =$
512 0.474, $\omega^2 = -0.006$), were found for time on bottom (Figure 6B). Post-hoc tests suggested that
513 CAS increased time on bottom ($p < 0.001$, $d = -2.22$ for the main effect), and RS-102221 was
514 not able to change this effect ($p < 0.001$, $d = -2.12$, non-treated controls vs. treated CAS; $p =$
515 0.8, $d = 0.29$, non-treated CAS vs. treated CAS).

516 Large main effects of treatment ($F_{[1, 68]} = 25.74$, $p < 0.0001$, $\omega^2 = 0.26$) and drug ($F_{[1, 68]} =$
517 21.06, $p < 0.0001$, $\omega^2 = 0.22$) were found for erratic swimming. An interaction effect was also
518 found ($F_{[1, 68]} = 15.47$, $p < 0.0001$, $\omega^2 = 0.17$). Post-hoc tests suggested that CAS increased
519 erratic swimming ($p < 0.01$, $d = 1.24$for the main effect), an effect that was blocked by RS-
520 102221 ($p =0.829$, $d = -0.33$, non-treated controls vs. treated CAS; $p <0.001$, $d = 1.89$, non-
521 treated CAS vs. treated CAS; Figure 6C).

522 Large main effects of treatment ($F_{[1, 68]} = 21.973$, $p < 0.0001$, $\omega^2 = 0.23$) and drug ($F_{[1, 68]} =$
523 7.06, $p = 0.0098$, $\omega^2 = 0.08$) were found for freezing; no interaction effect was found ($F_{[1, 68]} =$
524 2.98, $p = 0.089$, $\omega^2 = 0.03$). Post-hoc tests revealed that CAS increased freezing ($p < 0.001$, $d =$
525 1.10 for the main effect), and RS-102221 partially blocked this effect ($p = 0.488$, $d = -0.548$,
526 non-treated controls vs. treated CAS; $p = 0.013$, $d = 0.985$, non-treated CAS vs. treated CAS;
527 Figure 6D).

528 Finally, no main effects of treatment ($F_{[1, 68]} = 1.84$, $p = 0.179$, $\omega^2 = 0.01$) nor drug ($F_{[1, 68]}$
529 $= 3.156$, $p = 0.08$, $\omega^2 = 0.03$) were found for swimming speed (Figure 6E). No interaction effects
530 were present ($F_{[1, 68]} = 2.981$, $p = 0.089$, $\omega^2 = 0.03$).

531

532 ### 3.3.2. After exposure

533 No main effects of treatment ($F_{[1, 68]} = 2.449$, $p = 0.122$, $\omega^2 = 0.02$) or drug ($F_{[1, 68]} =$
534 0.002, $p = 0.969$, $\omega^2 = -0.01$), were found for time on top (Figure 7A); an interaction effect was
535 also absent ($F_{[1, 68]} = 1.152$, $p = 0.287$, $\omega^2 = 0.002$).

536 A medium-sized main effect of treatment ($F_{[1, 68]} = 6.322$, $p = 0.0143$, $\omega^2 = 0.07$), as well
537 as a large effect of drug ($F_{[1, 68]} = 22.796$, $p < 0.0001$, $\omega^2 = 0.23$), were found for time on bottom
538 (Figure 7B). A medium-sized interaction effect was also found ($F_{[1, 68]} = 4.08$, $p = 0.047$, $\omega^2 =$
539 0.04). Post-hoc tests suggested that CAS increased time on bottom after exposure ($p = 0.05$, d
540 $= 1.02$, non-treated controls vs. non-treated CAS), an effect that was blocked by RS-102221 (p
541 $= 0.506$, $d = 0.54$, non-treated controls vs. treated CAS; $p < 0.001$, $d = 1.55$, non-treated CAS
542 vs. treated CAS).

543 A large main effect of treatment ($F_{[1, 68]} = 36.19$, $p < 0.0001$, $\omega^2 = 0.33$), but not drug ($F_{[1,}$
544 $_{68]} = 2.957$, $p = 0.09$, $\omega^2 = 0.03$), were found for erratic swimming (Figure 7C). No interaction
545 effect was found ($F_{[1, 68]} = 0.002$, $p = 0.965$, $\omega^2 = -0.01$). Post-hoc tests suggested that CAS
546 increased erratic swimming after exposure ($p = 0.001$, $d = 1.514$, non-treated controls vs. non-
547 treated CAS), an effect that was not altered by RS-102221 ($p < 0.001$, $d = -1.92$, non-treated
548 controls vs. treated CAS; $p = 0.56$, $d = -0.41$, non-treated CAS vs. treated CAS).

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

555 Finally, no main effects of treatment ($F_{[1, 68]} = 1.014$, $p = 0.317$, $\omega^2 = 0.0002$) or drug ($F_{[1,}$
556 $_{68]} = 1.83$, $p = 0.181$, $\omega^2 = 0.01$) were found for swimming speed, nor was there an interaction

557 effect for this variable ($F_{[1, 68]}$ = 0.475, p = 0.493, ω² = -0.007. Figure 7E).

558

559

560 3.4. Restraint stress-elicited behavioral changes

561 3.4.1. Effects of MK-212 on restraint stress-elicited behavioral changes

562 No main effects of treatment ($F_{[1, 23]}$ = 0.167, p = 0.687, ω² = -0.026) nor drug ($F_{[1, 23]}$ =
563 3.884, p = 0.061, ω² = 0.089), were found for time on top (Figure 8A). No interaction effects
564 were found as well ($F_{[1, 23]}$ = 4.215, p = 0.052, ω² = 0.1).

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

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

576 No main effects of treatment ($F_{[1, 23]}$ = 0.384, p = 0.541, ω² = -0.016) nor drug ($F_{[1, 23]}$ =
577 0.618, p = 0.44, ω² = -0.01) were found for freezing (Figure 8D). A large interaction effect was
578 found ($F_{[1, 23]}$ = 12.339, p = 0.002, ω² = 0.304). Post-hoc tests suggested that animals injected
579 with MK-212 and subjected to ARS showed decreased freezing in relation to other groups (p =

580 0.033, d = -1.6, non-treated controls vs. treated ARS; p = 0.031, d = 1.6, non-treated ARS vs.
581 treated ARS).

582 Finally, no main effects of treatment ($F_{[1, 23]}$ = 1.0005, p = 0.328, ω² = 0.0) nor drug ($F_{[1, 23]}$
583 = 0.0283, p = 0.868, ω² = -0.038) were found for swimming speed (Figure 8E). No interaction
584 effect was found for this variable ($F_{[1, 23]}$ = 0.5437, p = 0.468, ω² = -0.018).

585

586 3.4.2. Effects of RS-102221 on restraint stress-elicited behavioral changes

587 A main effect of treatment ($F_{[1, 28]}$ = 422.613, p < 0.0001, ω² = 0.93), but not drug ($F_{[1, 28]}$ =
588 0.728, p = 0.401, ω² = -0.009), was found for time on top (Figure 9A). No interaction effect was
589 found ($F_{[1, 28]}$ = 0.416, p = 0.524, ω² = -0.02). ARS increased time on top (p < 0.0001, d = 7.78 for
590 the main effect), and RS-102221 was not able to block this effect.

591 No main effects of treatment ($F_{[1, 28]}$ = 3.051, p = 0.0916, ω² = 0.06) nor drug ($F_{[1, 28]}$ =
592 2.208, p = 0.1485, ω² = 0.04) were found for time on bottom (Figure 9B). However, a significant
593 interaction effect was found ($F_{[1, 28]}$ = 10.832, p = 0.0027, ω² = 0.24). Post-hoc tests revealed
594 that ARS increased time on bottom (p = 0.007 vs. non-treated controls), an effect that was
595 partially blocked by RS-102221 (p = 0.997, non-treated controls vs. treated ARS; p = 0.019,
596 non-treated ARS vs. treated ARS).

597 No main effects of treatment ($F_{[1, 28]}$ = 0.014, p = 0.905, ω² = -0.03) nor drug ($F_{[1, 28]}$ =
598 0.224, p = 0.639, ω² = -0.02), were found for erratic swimming (Figure 9C). An interaction effect
599 was also absent ($F_{[1, 28]}$ = 2.916, p = 0.098, ω² = 0.06).

600 A main effect of treatment ($F_{[1, 28]}$ = 13.564, p = 0.001, ω² = 0.28) was found for freezing
601 (Figure 9D), but no main effect of drug was found ($F_{[1, 28]}$ = 0.122, p = 0.729, ω² = -0.03).
602 Moreover, an interaction effect was also absent ($F_{[1, 28]}$ = 0.022, p = 0.882, ω² = -0.03). ARS

603 increased freezing ($p = 0.001$, $d = 1.4$ for the main effect), an effect that was not blocked by RS-
604 102221 ($p = 0.052$, non-treated controls vs. treated ARS; $p = 0.998$, non-treated ARS vs.
605 treated ARS).

606 Finally, no main effects of treatment ($F_{[1, 28]} = 1.833$, $p = 0.187$, $\omega^2 = 0.03$) nor drug ($F_{[1, 28]}$
607 $= 0.003$, $p = 0.955$, $\omega^2 = -0.03$), neither an interaction effect ($F_{[1, 28]} = 0.051$, $p = 0.823$, $\omega^2 = -$
608 0.03), were found for swimming speed (Figure 9E).

609 4. Discussion

610 The present work tested the hypothesis that phasic activation of the 5-HT_{2C} receptor is
611 involved in behavioral adjustments to distal, but not proximal, threat in zebrafish, and that these
612 receptors exerts a tonic facilitation of defensive behavior to potential threat in zebrafish. We
613 found that 5-HT_{2C} agonists blocked CAS-elicited defensive behavior, but not post-exposure
614 increases in defensive behavior, nor ARS-elicited anxiogenic-like effects. We also found that
615 RS-102221, a 5-HT_{2C} receptor antagonist, did not change behavior during exposure, but it
616 produced a small effect on behavior after exposure to CAS. No effects of RS-102221 were
617 found for ARS-elicited anxiogenic-like effects.
618

619 4.1. Effects of stressors on zebrafish behavior

620 One of the aims of this paper was to confirm the behavioral effects of CAS during and
621 after exposure (Lima-Maximino et al., 2020), given the considerable variation in the literature
622 (Maximino et al., 2019), and to compare the effects after exposure with those of ARS -
623 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 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

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

We have previously found that serotonin participates in responses to CAS, acutely and phasically inhibiting responses to proximal threat but phasically facilitating responses to potential threat (Lima-Maximino et al., 2020; Maximino et al., 2014). We suggested that phasic and tonic serotonin encode an aversive expectation value, switching behavior toward cautious exploration/risk assessment/anxiety when the aversive stimulus is no longer present. In the experimental design that was used in this experiment, behavior during exposure represents proximal threat, as CAS acts as a partial predator stimulus that elicits behavior that decreases the possibility of a predator attack or detection by the predator (Smith, 1992). However, if CAS is no longer present (as in the post-exposure stage of our design), that would signal a decrease in threat levels to potential threat, a situation in which trying to flee or hide is non-adaptive, but resuming normal behavior is also non-adaptive - and therefore cautious, alert exploration is warranted (Maximino et al., 2019). Using Fanselow's taxonomy, the situation in which CAS is present is more akin to "fear", while the situation in which it is no longer present is more akin to "anxiety" (Perusini and Fanselow, 2015). The rodent literature suggests that 5-HT_2 receptors

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

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

691 We have previously shown that, after exposure, serotonin levels are increased in the
692 extracellular fluid of the zebrafish brain (Maximino et al., 2014), an effect that was mediated by
693 both serotonin transporters (Maximino et al., 2014) and monoamine oxidase activity (Maximino
694 et al., 2019; Quadros et al., 2018). This increase in serotonergic activity could activate 5-HT_{2C}
695 and other serotonergic receptors to inhibit ongoing defensive responses to proximal threat and
696 initiate programs of alertness and cautious exploration/risk assessment. This phasic signal

697 represents prediction errors (Amo et al., 2014; Lima-Maximino et al., 2020), while the tonic
698 activity, which facilitates responses to potential threat, has been proposed to represent a
699 negative expectation value (Amo et al., 2014; Lima-Maximino et al., 2020). However, at least for
700 the case of the 5-HT_{2C} receptor, the phasic signals do not appear to represent all aversive
701 values, as no effect of MK-212 was found for ARS-elicited responses. Our results suggest that
702 the 5-HT_{2C} receptor is at least partially responsible for both effects, acting as a "switch" between
703 the two behavioral modes.
704

705 4.3. Limitations and translational potential

706 While the present work presents results on zebrafish behavioral pharmacology inspired
707 on results from mammals, which can potentially underscore conserved mechanisms for
708 responses to threat across vertebrates (cf. Gerlai, 2014, for a discussion on how using fish as
709 well as mammals can decrease uncertainty regarding translational relevance and evolutionary
710 conservation of mechanisms), there are limitations in using zebrafish as experimental models in
711 neuroscience. One of them is that, due to the teleost genome duplication event, zebrafish
712 present two copies of the gene that codes the 5-HT_{2C} receptor (Schneider et al., 2012), and
713 therefore it is currently not possible to know which protein (HTR2CL1 or HTR2Cl2) was targeted
714 by the drugs used in the present experiments. Moreover, while we propose that alarm reactions
715 and post-exposure behavior can be exploited to better understand panic disorder (Silva et al.,
716 2020), the paradigm that was used represents the normal, adaptive behavior of zebrafish under
717 threatening circumstances, and therefore cannot yet be used to generate novel hypotheses
718 about a pathological organism (cf. Maximino and van der Staay, 2019, for a discussion on the
719 limitations of using behavioral assays in experimental psychopathology).
720 Another important limitation of our results is that sex differences were not considered

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

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

737

738

739 Significance statement

740 While the neurotransmitter serotonin has been associated with mental disorders, we currently
741 do not know the mechanisms through which serotonin influences responses to threat. This is
742 relevant to future studies on anxiety disorders, for example. Here we report that, in zebrafish,
743 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 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

998 differences at the $p < 0.05$ level; similar letters indicate lack of statistically significant
999 differences. Data are presented as individual data points (dots) superimposed over the
1000 median ± interquartile ranges. Dashed lines on panels A and B represent change levels.
1001 Dots connected by lines represent group means. CTRL = controls (water-exposed
1002 animals); CAS = conspecific alarm substance. Final sample sizes: CTRL + VEH: n = 10
1003 animals; CTRL + 1 mg/kg WAY-161503: n = 21 animals; CAS + VEH: n = 21 animals;
1004 CAS + 1 mg/kg WAY-161503: n = 21 animals.

1005 Figure 5 – Effects of WAY-161503 on behavior <u>after</u> CAS exposure. (A) Time spent on top third
1006 of the tank. (B) Time spent on bottom third of the tank. (C) Erratic swimming. (D) Total
1007 time spent freezing. (E) Swimming speed. Different letters represent statistical
1008 differences at the $p < 0.05$ level; similar letters indicate lack of statistically significant
1009 differences. Data are presented as individual data points (dots) superimposed over the
1010 median ± interquartile ranges. Dashed lines on panels A and B represent change levels.
1011 Dots connected by lines represent group means. CTRL = controls (water-exposed
1012 animals); CAS = conspecific alarm substance. Final sample sizes: CTRL + VEH: n = 10
1013 animals; CTRL + 1 mg/kg WAY-161503: n = 21 animals; CAS + VEH: n = 21 animals;
1014 CAS + 1 mg/kg WAY-161503: n = 21 animals.

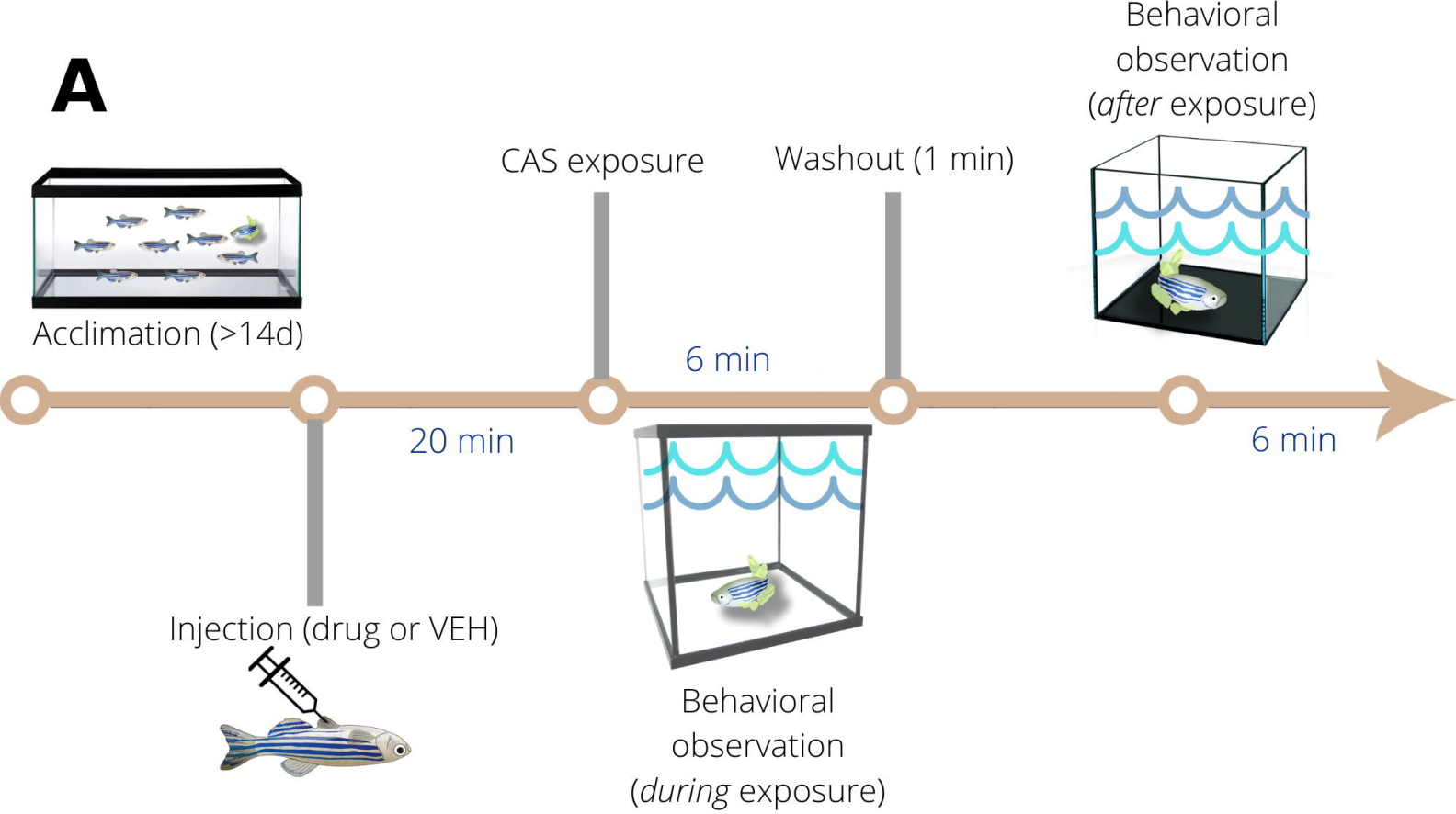
1015 Figure 6 – Effects of RS-102221 on behavior <u>during</u> CAS exposure. (A) Time spent on top third
1016 of the tank. (B) Time spent on bottom third of the tank. (C) Erratic swimming. (D) Total
1017 time spent freezing. (E) Swimming speed. Different letters represent statistical
1018 differences at the $p < 0.05$ level; similar letters indicate lack of statistically significant
1019 differences. Data are presented as individual data points (dots) superimposed over the
1020 median ± interquartile ranges. Dashed lines on panels A and B represent change levels.
1021 Dots connected by lines represent group means. CTRL = controls (water-exposed
1022 animals); CAS = conspecific alarm substance. Final sample sizes: CTRL + VEH: n = 10
1023 animals; CTRL + 2 mg/kg RS-102221: n = 21 animals; CAS + VEH: n = 20 animals; CAS
1024 + 2 mg/kg RS-102221: n = 21 animals.

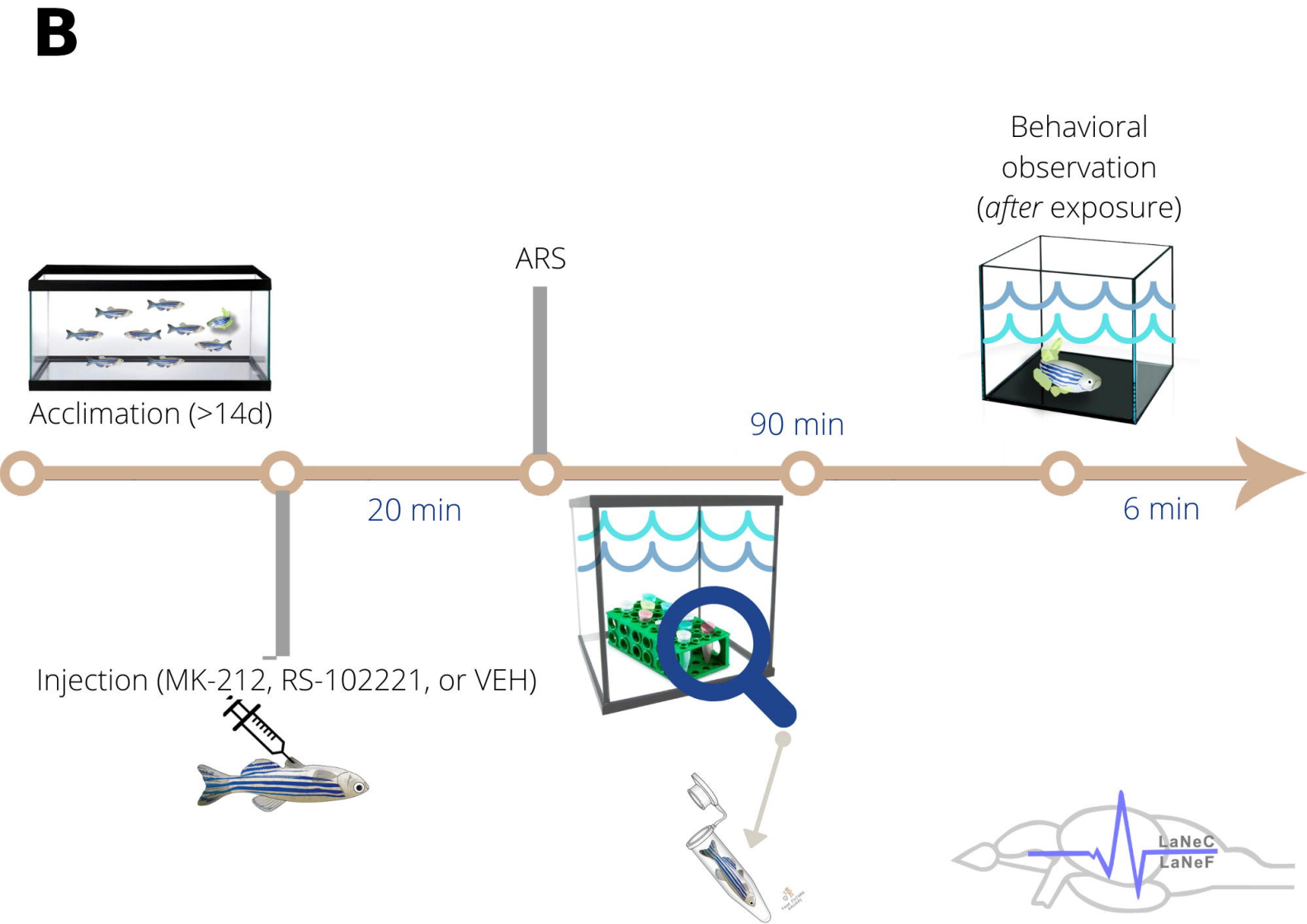
1025 Figure 7 – Effects of RS-102221 on behavior <u>after</u> CAS exposure. (A) Time spent on top third of
1026 the tank. (B) Time spent on bottom third of the tank. (C) Erratic swimming. (D) Total time
1027 spent freezing. (E) Swimming speed. Different letters represent statistical differences at
1028 the $p < 0.05$ level; similar letters indicate lack of statistically significant differences. Data
1029 are presented as individual data points (dots) superimposed over the median ±
1030 interquartile ranges. Dashed lines on panels A and B represent change levels. Dots

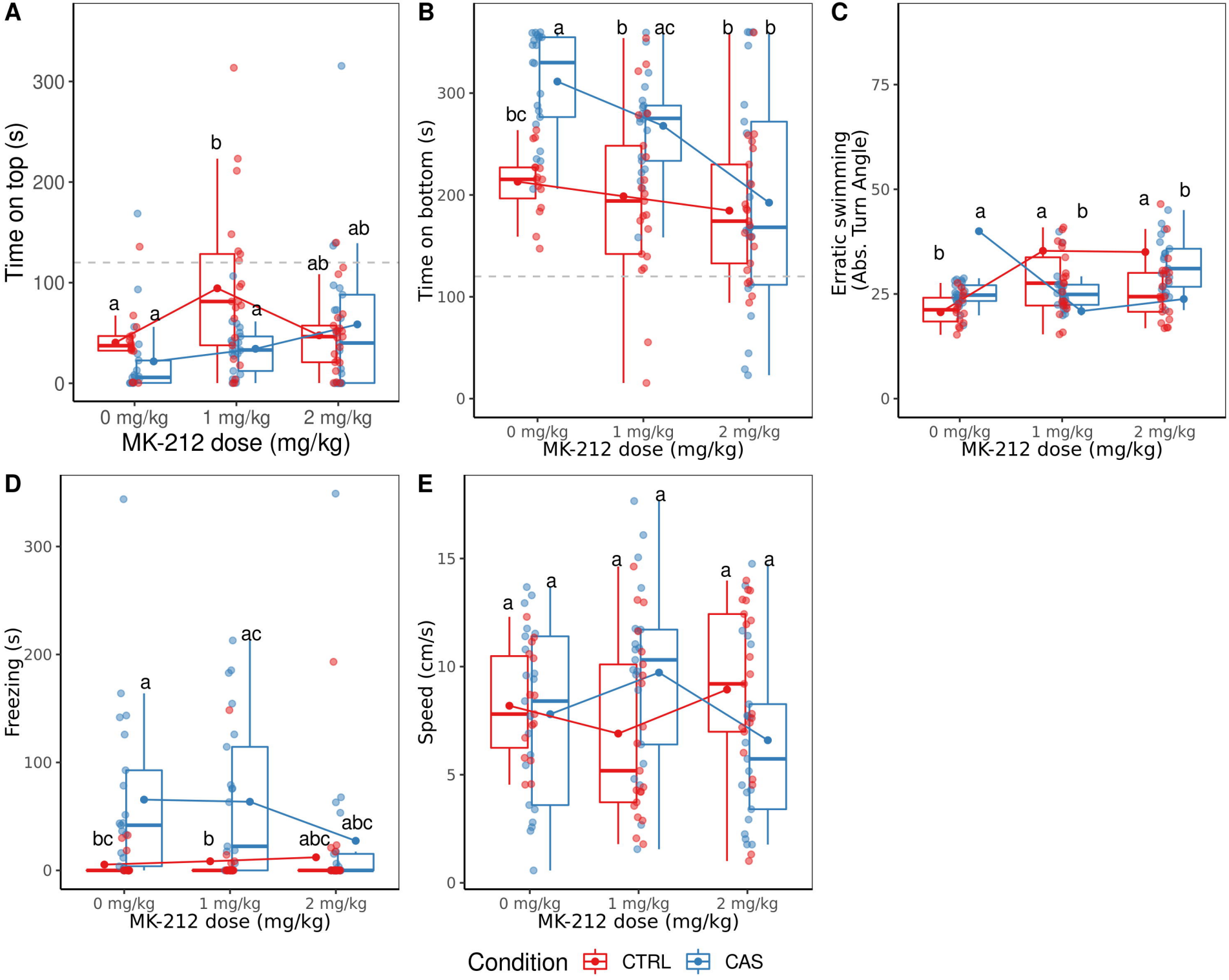
connected by lines represent group means. CTRL = controls (water-exposed animals); CAS = conspecific alarm substance. Final sample sizes: CTRL + VEH: n = 10 animals; CTRL + 2 mg/kg RS-102221: n = 21 animals; CAS + VEH: n = 20 animals; CAS + 2 mg/kg RS-102221: n = 21 animals.

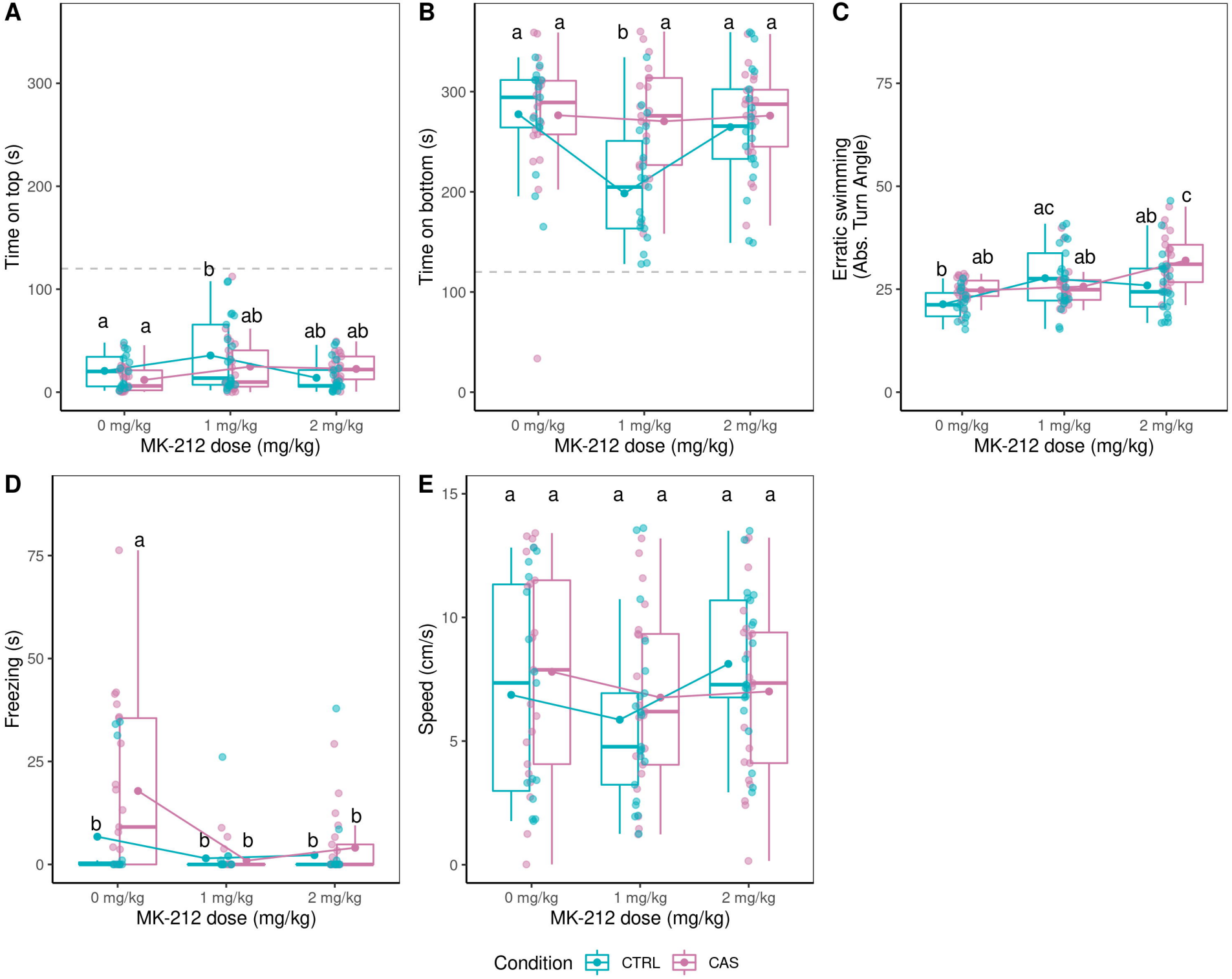
Figure 8 – Effects of MK-212 on behavior after acute restraint stress (ARS). (A) Time spent on top third of the tank. (B) Time spent on bottom third of the tank. (C) Erratic swimming. (D) Total time spent freezing. (E) Swimming speed. Different letters represent statistical differences at the $p < 0.05$ 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.

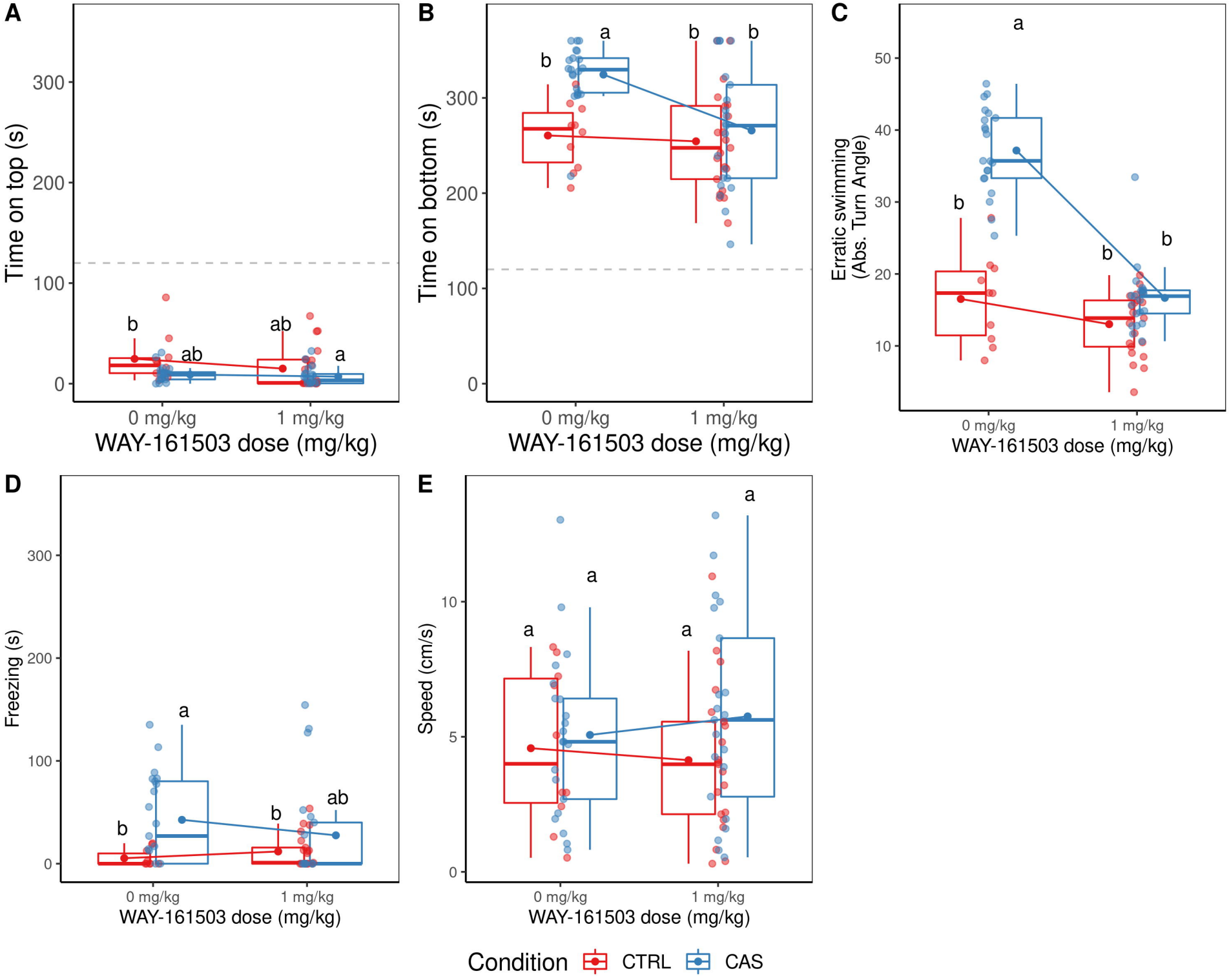
Figure 9 – Effects of RS-102221 on behavior after acute restraint stress (ARS). (A) Time spent on top third of the tank. (B) Time spent on bottom third of the tank. (C) Erratic swimming. (D) Total time spent freezing. (E) Swimming speed. Different letters represent statistical differences at the $p < 0.05$ 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.

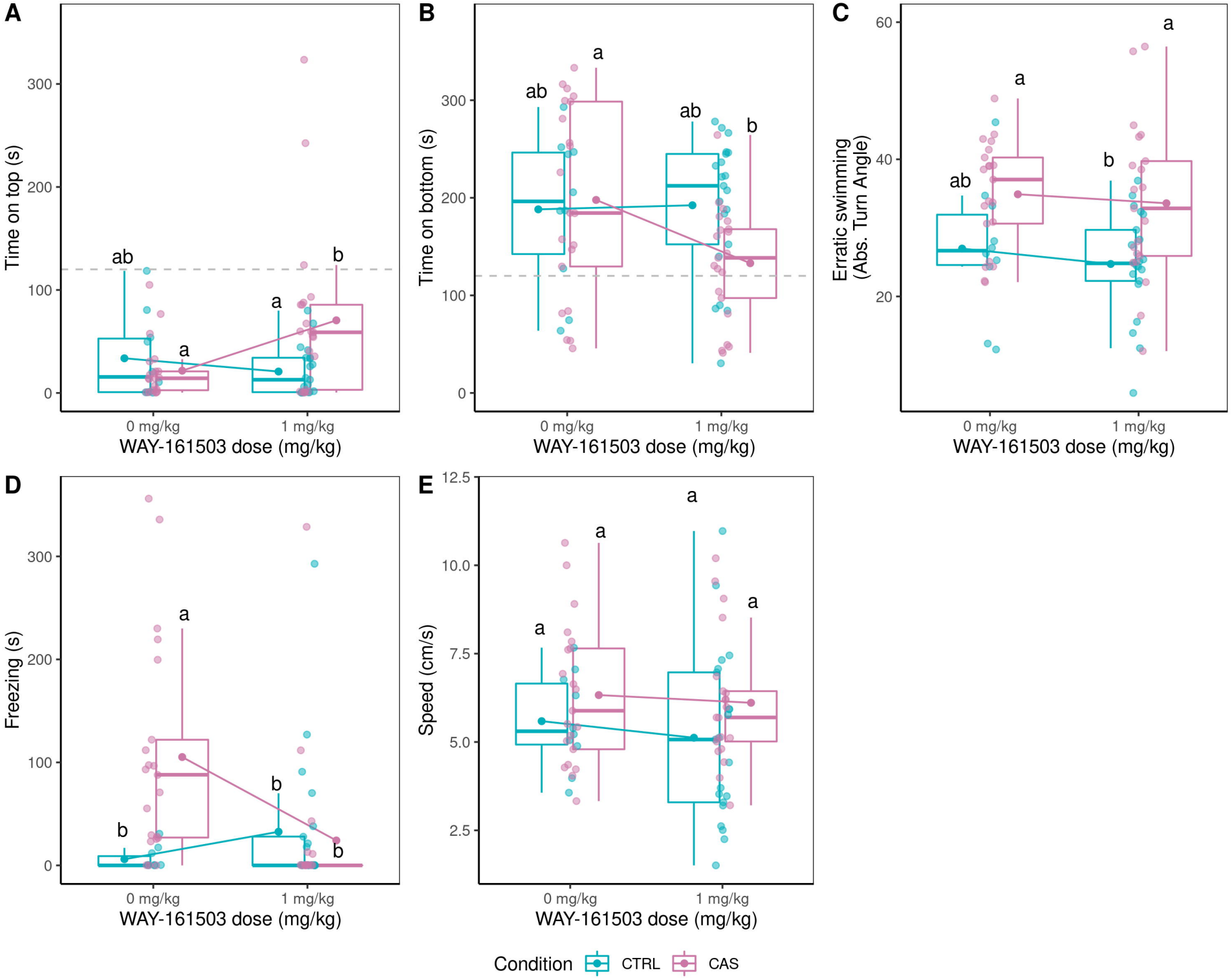

A
Acclimation (>14d)
CAS exposure
Washout (1 min)
Behavioral observation (*after* exposure)
6 min
20 min
6 min
Injection (drug or VEH)
Behavioral observation (*during* exposure)

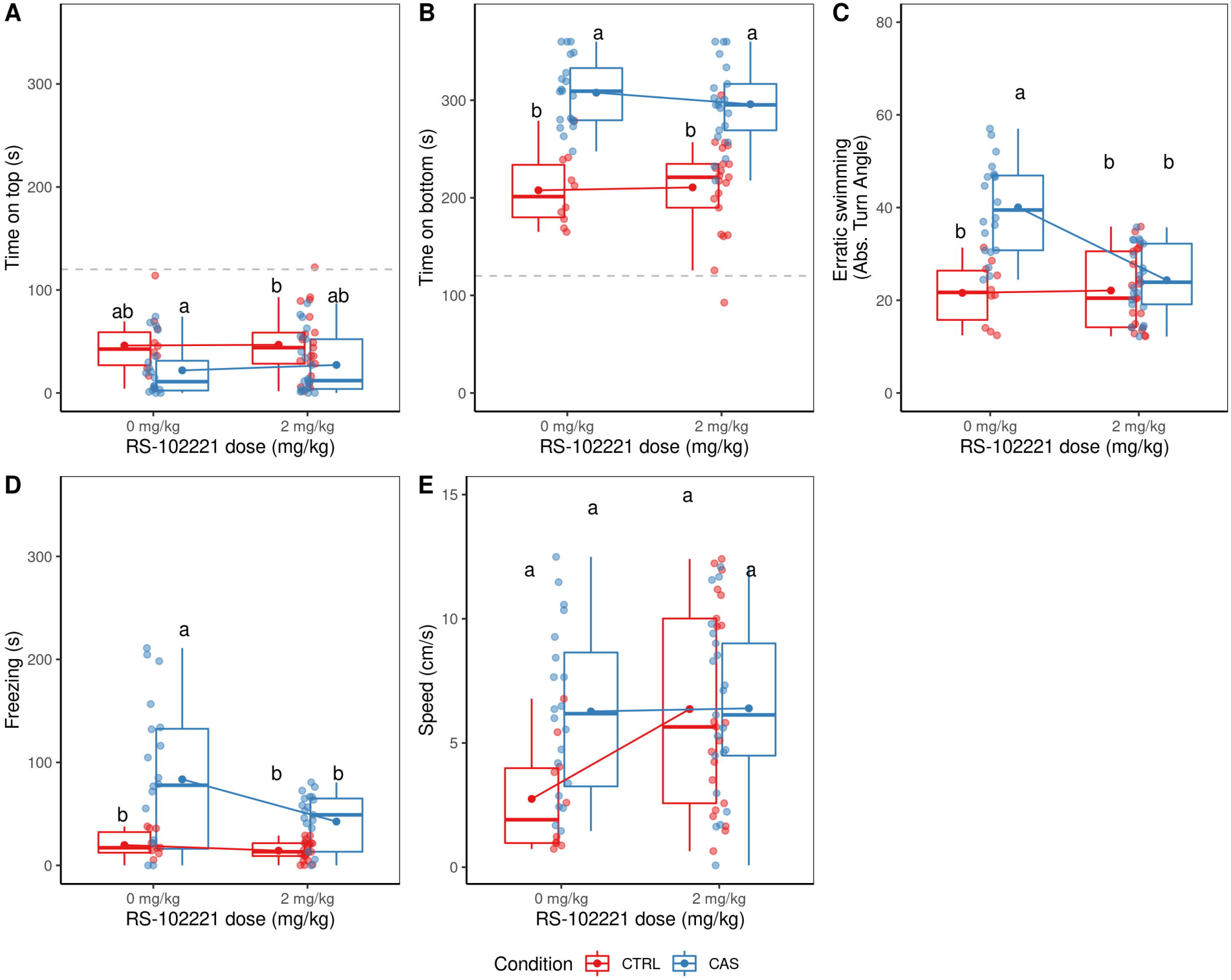

B
Acclimation (>14d)
ARS
Behavioral observation (*after* exposure)
90 min
20 min
6 min
Injection (MK-212, RS-102221, or VEH)
LaNeC
LaNeF

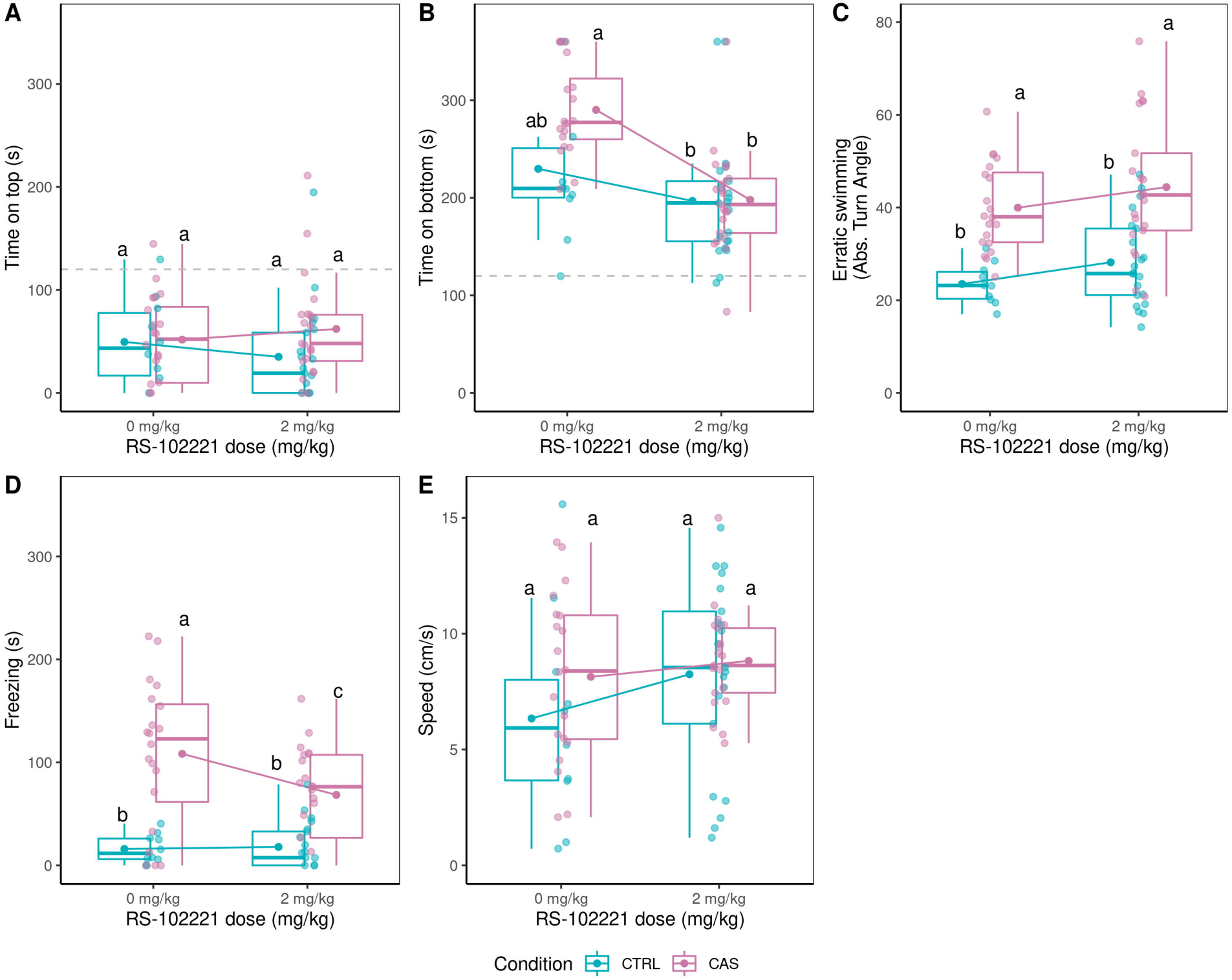

A
Time on top (s)
300
200
100
0
a
a
b
a
ab
ab
0 mg/kg
1 mg/kg
2 mg/kg
MK-212 dose (mg/kg)
B
Time on bottom (s)
300
200
100
0
bc
a
b
ac
b
b
0 mg/kg
1 mg/kg
2 mg/kg
MK-212 dose (mg/kg)
C
Erratic swimming
(Abs. Turn Angle)
75
50
25
0
b
a
a
b
a
b
0 mg/kg
1 mg/kg
2 mg/kg
MK-212 dose (mg/kg)
D
Freezing (s)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

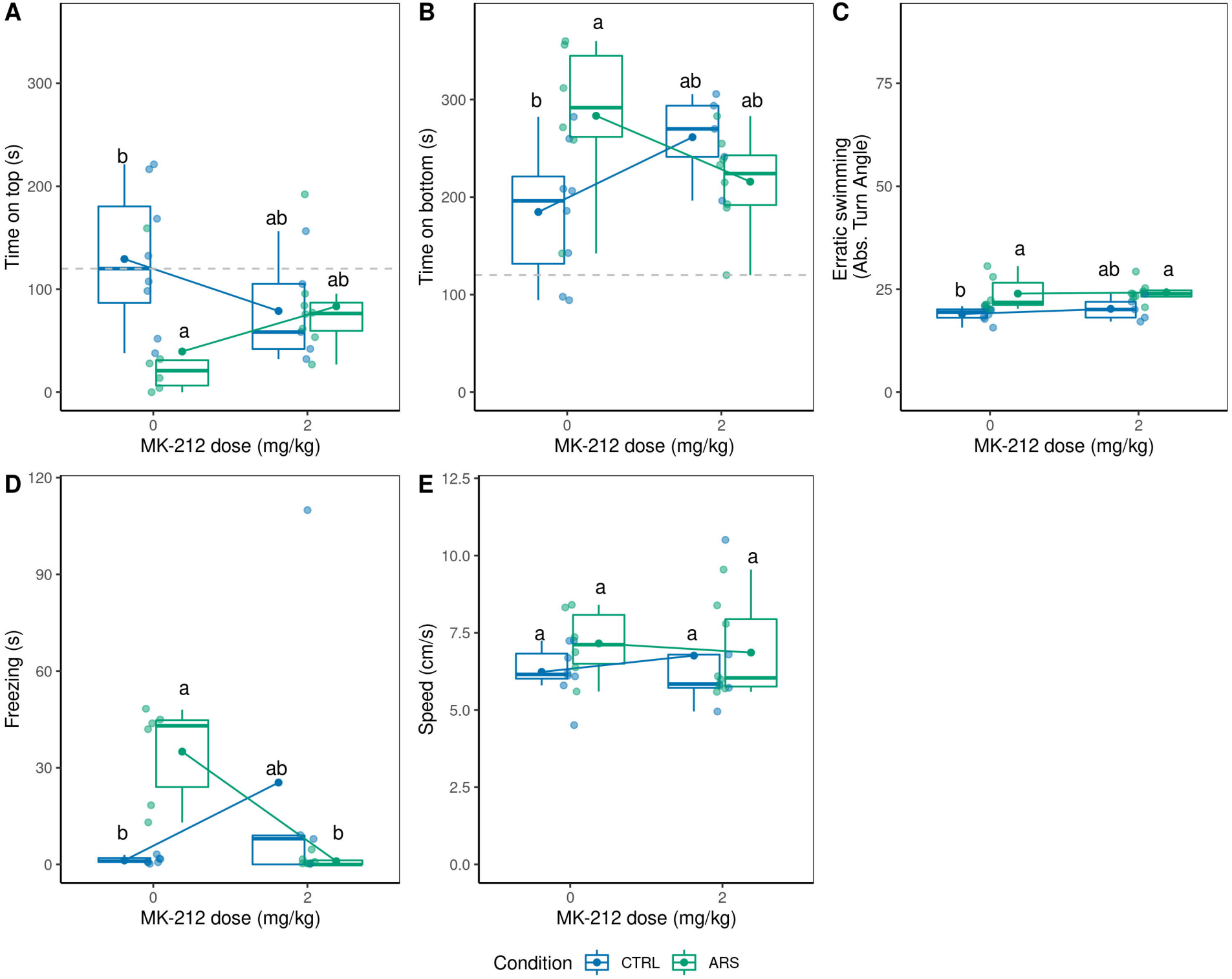

A
Time on top (s)
300
200
100
0
a
a
b
ab
ab
ab
0 mg/kg
1 mg/kg
2 mg/kg
MK-212 dose (mg/kg)
B
Time on bottom (s)
300
200
100
0
a
a
b
a
a
a
0 mg/kg
1 mg/kg
2 mg/kg
MK-212 dose (mg/kg)
C
Erratic swimming
(Abs. Turn Angle)
75
50
25
0
b
ab
ac
ab
ab
c
0 mg/kg
1 mg/kg
2 mg/kg
MK-212 dose (mg/kg)
D
Freezing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

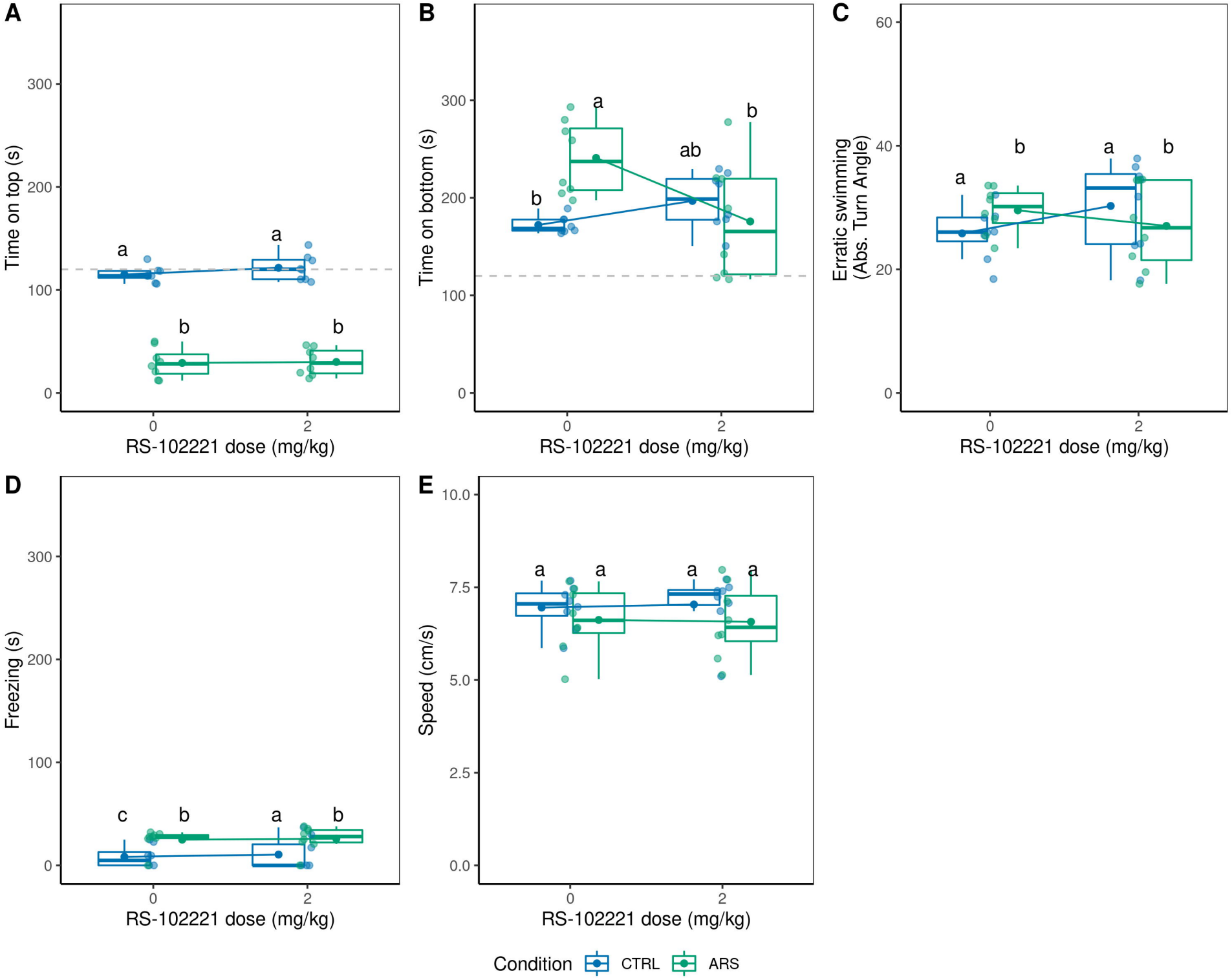

A
Time on top (s)
300
200
100
0
b
ab
ab
a
0 mg/kg
1 mg/kg
WAY-161503 dose (mg/kg)
B
Time on bottom (s)
300
200
100
0
b
a
b
b
0 mg/kg
1 mg/kg
WAY-161503 dose (mg/kg)
C
Erratic swimming
(Abs. Turn Angle)
50
40
30
20
10
a
b
b
b
0 mg/kg
1 mg/kg
WAY-161503 dose (mg/kg)
D
Freezing (s)
300
200
100
0
a
b
b
ab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