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-10221, 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.

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

1. Introduction

Serotonergic mechanisms have been implicated in defensive behavior to distal, proximal and potential threat (Graeff, 2004). In current interpretations of the role of serotonin (5-HT) on neurobehavioral responses to threat, the neurotransmitter is released when threat is potential,

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

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

63 5-HT has been implicated in fish behavioral responses both during and after exposure to
64 CAS. In zebrafish, extracellular 5-HT levels were increased after CAS exposure (Maximino et
65 al., 2014), an effect that can be related to decreased 5-HT uptake (Maximino et al., 2014) and/or
66 decreased monoamine oxidase activity (Lima-Maximino et al., 2020; Quadros et al., 2018). In
67 Crucian carp (*Carassius carassius*), exposure to CAS elicits increases in serotonergic activity in
68 the brainstem and optic tectum (Höglund et al., 2005), structures which have been involved in
69 responses to distal and proximal threat (do Carmo Silva et al., 2018a). However, this only

70 happened when hiding material was unavailable in the tank. In Nile tilapia (*Oreochromis*
71 *niloticus*), CAS did not increase serotonergic activity in the dorsomedial and dorsolateral
72 telencephali (Silva et al., 2015), homologues of the frontotemporal amygdaloid nuclei and
73 hippocampus, respectively (do Carmo Silva et al., 2018a). These results suggest that, during or
74 after exposure, CAS increases serotonergic activity in regions associated with "quick-and-dirty"
75 behavioral responses to distal threat (a "fight/flight/freeze" or "fear" system), but not in the
76 telencephalic areas associated with cautious exploration/risk assessment (a "behavioral
77 inhibition" or "anxiety" system).

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

88 The role of serotonin receptors from the 5-HT_2 family in CAS-elicited behavioral
89 adjustments has also been investigated. Zebrafish has been shown to possess two copies of
90 the 5-HT_{2A} receptor, one copy of the 5-HT_{2B} receptor, and two copies of the 5-HT_{2C} receptor
91 (Sourbron et al., 2016). In Nile tilapia, mianserin, a 5-HT_{2A} and 5-HT_{2C} receptor antagonist that
92 also blocks α_2-adrenoceptors, blocked active components of the alarm reaction (dashing,
93 bristling of dorsal fin spines), but not freezing, during exposure (Barreto, 2012). In zebrafish,
94 methysergide (an antagonist at 5-HT_{2A}, 2B, and 2C receptors, and a 5-HT_{1A} receptor agonist)

increased freezing and bottom-dwelling during and after exposure at a high dose (92.79 mg/kg), but not at lower doses (Nathan et al., 2015). These results point to an inhibitory role of the 5-HT_{2A} and 5-HT_{2C} receptors on CAS-elicited fear-like responses. However, since Nathan et al. (2015) observed these effects consistently during a long session, in which behavior is expected to change as CAS concentrations decrease (Mathuru et al., 2012)(i.e., similar to the shift from erratic swimming to freezing that is observed when animals are observed after exposure in a CAS-free context; Lima-Maximino et al., 2020), and since serotonergic drugs can produce opposite effects on behavior during and after CAS exposure (Lima-Maximino et al., 2020), from these results it is not possible to understand whether 5-HT_2 receptors participate in both responses.

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

These differences could be due to species differences; to effects at different receptors

120 (e.g., 5-HT_{2A} or 5-HT_{2C}); or to difficulties in the protocol used by Nathan et al. (2015), which does
121 not differentiate between responses to proximal, distal, or potential threat. In this work, we
122 tested whether 5-HT_{2C} receptors participate in responses to CAS during (distal threat) or after
123 exposure (potential threat) and to acute restraint stress (ARS). ARS has been applied in
124 zebrafish to elicit strong stress responses, including activation of the hypothalamus-pituitary-
125 interrenal (HPI) axis and associated behavioral responses (Assad et al., 2020; Ghisleni et al.,
126 2012; Piato et al., 2011). From the point of view of the predatory imminence continuum theory,
127 ARS represents proximal threat (Perusini and Fanselow, 2015). Thus, if 5-HT_{2C} receptors inhibit
128 aversively motivated behavior in zebrafish regardless of threat distance, its activation would
129 inhibit behavioral responses at these three contexts. If, as in rodents, 5-HT_{2C} receptors act at
130 different levels of threat distance to either activate or inhibit defensive responses, then 5-HT_{2C}
131 agonists will not produce the same effect in each of these contexts.

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

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

156 2. Materials and methods

157 2.1. Animals and housing

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

167 animals was minimized by controlling for the aforementioned environmental variables.
168 Furthermore, in the all experiments the animals used were handled, anesthetized and sacrificed
169 according to the norms of the Brazilian Guideline for the Care and Use of Animals for Scientific
170 and Didactical Purposes (Conselho Nacional de Controle de Experimentação Animal -
171 CONCEA, 2017). The experimental protocols were approved by UEPA's IACUC under protocol
172 06/18.
173

174 2.2. Drugs and treatments

175 The 5-HT_{2C} receptor agonists MK-2212 (2-Chloro-6-(1-piperazinyl)pyrazine, CAS
176 #64022-27-1) and WAY-161503 (8,9-dichloro-2,3,4,4a-tetrahydro-1H-pyrazino[1,2-a]quinoxalin-
177 5(6H)-one; CAS #75704-24-4) were bought from Sigma-Aldrich (St Louis, USA) on 2018, and
178 dissolved in Cortland's salt solution (NaCl 124.1 mM, KCl 5.1 mM, Na_2HPO_4 2.9 mM, $MgSO_4$ 1.9
179 mM, $CaCl_2$ 1.4 mM, $NaHCO_3$ 11.9 mM, Polyvinylpyrrolidone 4%, 1,000 USP units Heparin; Wolf,
180 1963) and in 1% dimethyl sulfoxide (DMSO), respectively. The 5-HT_{2C} receptor antagonist RS-
181 102221 (8-[5-(2,4-Dimethoxy-5-(4-trifluoromethylphenylsulphonamido)phenyl -5-oxopentyl]-
182 1,3,8-triazaspiro[4.5]decane-2,4-dione; CAS #185376-97-0) was bought from Sigma-Aldrich (St
183 Louis, USA) in 2018, and dissolved in 1% DMSO. While affinities for zebrafish 5-HT_{2C}-like
184 receptors have not been established, WAY-161503 has been reported to displace DOI from
185 human 5-HT_{2C} receptors with a K_i of 3.3 nM (6-fold selectivity over human 5-HT_{2A} receptors and
186 20-fold over human 5-HT_{2B} receptors)(Rosenzweig-Lipson et al., 2006). MK-212 has been
187 shown to be less selective at recombinant human receptors, with a K_i of 7.01 nM at 5-HT_{2C}
188 receptors (vs. 5.99 nM and 6.21 nM at 5-HT_{2A} and 5-HT_{2B} receptors, respectively)(Knight et al.,
189 2004). Finally, RS-102221 has been shown to displace mesulergine from human 5-HT_{2C}

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

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

204 2.3. Effects of 5-HT_{2C} receptor agonists and antagonists on alarm 205 reaction and post-exposure behavior

206 2.3.1. Experimental design

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

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

224 Animals were randomly allocated to groups using a random number 185 generator
225 (http://www.jerrydallal.com/random/random_block_size_r.htm), with each subject randomized to
226 a single treatment using random permuted blocks. One PI attributed a random letter to
227 treatment (e.g., “A” for CTRL, “B” for CAS) and a random integer for drug dose (e.g., “1” for 1
228 mg/kg, “2” for 0 mg/kg [vehicle]), and combinations for letters and integers were randomized.
229 For each experiment, animals were treated and tested in the order of allocation (i.e., randomly).
230 In all experiments, experimenters and data analysts were blinded to drugs and treatment by
231 using coded vials (with the same code used for randomization); blinding was removed only after
232 data analysis. Experiments were always run between 08:00AM and 02:00 PM. After
233 experiments, animals were sacrificed by prolonged bath in ice-cold water (< 12 ºC), followed by
234 spinal transection (Matthews and Varga, 2012).
235

236 ### 2.3.2. Sample size determination

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

252 ### 2.3.3. Alarm substance extraction

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

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

265 ### 2.3.4. Observation apparatuses

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

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

2.4.1. Experimental design

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

302 after data analysis. Experimenters were not blinded to treatment, but data analysts were blinded
303 to both treatment and drug. Experiments were always run between 08:00AM and 02:00 PM.
304 After experiments, animals were sacrificed by prolonged bath in ice-cold water (< 12 ºC),
305 followed by spinal transection (Matthews and Varga, 2012).
306

307 2.4.2. Sample size determination

308 Sample sizes were based on previous experiments with the behavioral effects of ARS
309 (Assad et al., 2020).
310

311 2.4.3. Observation apparatus

312 To analyze the behavioral effects of treatment (ARS exposure) and drug, the same
313 apparatus used in the second stage of the previous experiment (5-L transparent glass tank) was
314 used.
315

316 2.5. Behavioral endpoints

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

320 - Time spent on bottom third of the tank (s)[Primary outcome]
321 - Time spent on top third of the tank (s)[Secondary outcome]
322 - 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: With the exception of outlier exclusion (see 2.7, “Statistical analysis”, below), no exclusion criteria were predetermined.

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

345 on bottom as the main endpoint; values were removed when they were higher or lower than 3
346 MADs around the median (Leys et al., 2013), and the number of outliers was reported in the
347 results.
348

349 3. Results

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

352 3.1.1. During exposure

353 One animal from the control + 0 mg/kg group was detected as outlier and removed.
354 Small-to-medium-sized main effects of treatment ($F_{[1, 114]} = 6.337$, $p = 0.0132$; $\omega^2 = 0.039$) and
355 dose ($F_{[2, 114]} = 3.665$, $p = 0.0287$; $\omega^2 = 0.039$) were found for time on top. A small-to-medium
356 interaction effect was also found ($F_{[2, 114]} = 4.602$, $p = 0.012$; $\omega^2 = 0.052$). Post-hoc tests found
357 that CAS did not alter time on top ($p = 0.94$, $d = 0.3$, non-treated controls vs. non-treated CAS),
358 but MK-212 (1 mg/kg) increased it in non-exposed animals (*1 mg/kg:* $p = 0.024$, $d = -1.05$, non-
359 treated controls vs. 1 mg/kg controls; *2 mg/kg:* $p = 0.994$, $d = -0.18$, non-treated controls vs. 2
360 mg/kg controls; Figure 2A).
361 Medium-to-large-sized main effects of treatment ($F_{[1, 114]} = 20.995$, $p = 1.18 \times 10^{-5}$; $\omega^2 =$
362 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
363 small-to-medium interaction effect was also found ($F_{[2, 114]} = 4.085$, $p = 0.0194$; $\omega^2 = 0.037$).
364 Post-hoc tests revealed that CAS increased time on bottom ($p < 0.001$, $d = -0.77$ for the main

365 effect), and the highest MK-212 dose blocked this effect (*1 mg/kg:* $p = 0.39$, $d = -0.64$, non-
366 treated controls vs. treated CAS; $p = 0.38$, $d = 0.6$, non-treated CAS vs. treated CAS; *2 mg/kg:* p
367 $= 0.828$, $d = 0.4$, non-treated controls vs. treated CAS; $p < 0.001$, $d = 1.64$, non-treated CAS vs.
368 treated CAS; Figure 2B).

369 A small-to-medium-sized main effect of treatment ($F_{[1, 114]} = 5.137$, $p = 0.0253$; $\omega^2 =$
370 0.017), but not dose ($F_{[2, 114]} = 2.948$, $p = 0.0565$; $\omega^2 = 0.016$), was found for erratic swimming. A
371 very large interaction effect was also found for this variable ($F_{[2, 114]} = 57.101$, $p < 2 \times 10^{-16}$; $\omega^2 =$
372 0.467). Post-hoc tests showed that CAS greatly increased absolute turn angle in non-treated
373 animals ($p < 0.001$, $d = -2.58$, non-treated controls vs. non-treated CAS), an effect that was
374 blocked by both MK-212 doses (*1 mg/kg*: $p = 1$, $d = -0.04$, non-treated controls vs. treated CAS;
375 $p < 0.001$, $d = 2.53$, non-treated CAS vs. treated CAS; *2 mg/kg*: $p = 0.81$, $d = -0.42$, non-treated
376 controls vs. treated CAS; $p < 0.001$, $d = 2.16$, non-treated CAS vs. treated CAS; Figure 2C);
377 however, MK-212 also increased absolute turn angle in animals which were not exposed to
378 CAS (*1 mg/kg*: $p < 0.001$, $d = -1.95$, non-treated controls vs. treated controls; *2 mg/kg:* $p <$
379 0.001, $d = -1.91$, non-treated controls vs. treated controls).

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

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

389

3.1.2. After exposure

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

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

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

414 A small-to-medium main effect of treatment ($F_{[1, 114]} = 4.658$, $p = 0.033$; $\omega^2 = 0.025$) and
415 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
416 $= 0.143$). No interaction effects were found ($F_{[2, 114]} = 2.777$, $p = 0.0664$; $\omega^2 = 0.024$). Post-hoc
417 tests revealed that CAS increased freezing ($p = 0.05$, $d = -0.364$ for the main effect), an effect
418 that was blocked by all MK-212 doses (*1 mg/kg:* $p = 0.74$, $d = 0.47$, non-treated controls vs. 1
419 mg/kg controls; $p < 0.001$, $d = 1.5$, non-treated CAS vs. 1 mg/kg CAS; *2 mg/kg:* $p = 0.85$, $d =$
420 0.4, non-treated controls vs. 2 mg/kg controls; $p = 0.002$, $d = 1.22$, non-treated CAS vs. 2 mg/kg
421 CAS; Figure 3D).
422 No main effects of treatment ($F_{[1, 114]} = 0.111$, $p = 0.74$; $\omega^2 = -0.007$) or dose ($F_{[2, 114]} =$
423 1.339, $p = 0.266$; $\omega^2 = 0.006$), nor an interaction effect ($F_{[2, 114]} = 0.981$, $p = 0.378$; $\omega^2 = 0.0$),
424 were found for swimming speed (Figure 3E).
425

426 3.2. Effects of WAY-161503 on alarm reaction and post-exposure
427 behavior

428 3.2.1. During exposure

429 No outliers were detected from any group in this experiment. A small-to-medium-sized
430 main effect of treatment ($F_{[1, 69]} = 7.404$, $p = 0.00823$, $\omega^2 = 0.08$), but not drug ($F_{[1, 69]} = 1.751$, $p =$
431 0.19011, $\omega^2 = 0.009$), was found for time on top. No interaction effect was found ($F_{[1, 69]} = 1.001$,
432 $p = 0.32067$, $\omega^2 = 0.0$). Post-hoc tests showed that while CAS did not alter time on top ($p =$
433 0.054, $d = 1.0$, non-treated controls vs. non-treated CAS), a synergistic effect was apparent,
434 with WAY-161503-treated animals showing less time on top than controls ($p = 0.381$, $d = 0.618$,
435 non-treated controls vs. WAY-161503 controls; $p = 0.977$, $d = 0.125$, non-treated CAS vs. WAY-

436 161503 CAS; $p = 0.023$, $d = 1.125$, non-treated controls vs. WAY 161-503 CAS; Figure 4A).

437 Large main effects of treatment ($F_{[1, 69]} = 10.842$, $p = 0.00157$, $\omega^2 = 0.103$) and drug ($F_{[1,$
438 $_{69]} = 10.138$, $p = 0.00218$, $\omega^2 = 0.096$) were found for time on bottom. A medium-to-large-sized
439 interaction effect was found ($F_{[1, 69]} = 4.614$, $p = 0.03523$, $\omega^2 = 0.038$). Post-hoc tests revealed
440 that CAS increased time on bottom ($p = 0.007$, $d = -1.287$, non-treated controls vs. non-treated
441 CAS), and WAY-161503 blocked this effect ($p = 0.989$, $d = 0.123$, non-treated controls vs.
442 WAY-161503 controls; $p = 0.002$, $d = 1.181$, non-treated CAS vs. WAY-161503 CAS; Figure
443 4B).

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

451 A large main effect of treatment ($F_{[1, 69]} = 8.582$, $p = 0.0046$, $\omega^2 = 0.094$), but not of drug
452 ($F_{[1, 69]} = 0.564$, $p = 0.4552$, $\omega^2 = -0.005$), was found for freezing duration; no interaction effect
453 was found ($F_{[1, 69]} = 1.434$, $p = 0.2352$, $\omega^2 = 0.005$). Post-hoc tests revealed that CAS increased
454 freezing ($p = 0.047$, $d = -1.02$, non-treated controls vs. non-treated CAS), but this effect was not
455 blocked by WAY-161503 ($p = 0.967$, $d = -0.178$, non-treated controls vs. WAY-161503 controls;
456 $p = 0.544$, $d = 0.412$, non-treated CAS vs. WAY-161503 CAS; Figure 4D).

457 Finally, no effects of treatment ($F_{[1, 69]} = 2.303$, $p = 0.134$, $\omega^2 = 0.018$), drug ($F_{[1, 69]} =$
458 0.103, $p = 0.749$, $\omega^2 = -0.012$), or treatment:drug interaction ($F_{[1, 69]} = 0.527$, $p = 0.471$, $\omega^2 = -$
459 0.006) were found for swimming speed (Figure 4E).

460

3.2.2. After exposure

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

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

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

A 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, ω² = 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, ω² = 0.038) or drug ($F_{[1, 69]}$ = 0.416, p = 0.5212, ω² = -0.008) were found for swimming speed, and an interaction effect was also absent (F[1, 69] = 0.062, p = 0.8037, ω² = -0.013)(Figure 5E).

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

3.3.1. During exposure

One outlier was detected in the group exposed to CAS and injected with vehicle. A large effect of treatment ($F_{[1, 68]}$ = 8.89, p = 0.004, ω² = 0.101) was found for time on top, but no main effect of drug ($F_{[1, 68]}$ = 0.166, p = 0.685, ω² = -0.011) nor an interaction effect ($F_{[1, 68]}$ = 0.096, p = 0.757, ω² = -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-10221 was not able to change this effect (p = 0.353, d = 0.64, non-treated controls vs. treated CAS; p = 0.941, d = -0.18, non-treated CAS vs. treated CAS; Figure 6A).

A very large main effect of treatment ($F_{[1, 68]}$ = 80.6, p = 1.09 x 10^{-13}, ω² = 0.53), but not dose ($F_{[1, 68]}$ = 0.189, p = 0.665, ω² = -0.005), nor an interaction effect ($F_{[1, 68]}$ = 0.517, p = 0.474, ω² = -0.003), were found for time on bottom (Figure 6B). Post-hoc tests suggested that CAS increased time on bottom (p < 0.001, d = -2.22 for the main effect), and RS-10221 was not able

507 to change this effect ($p < 0.001$, $d = -2.12$, non-treated controls vs. treated CAS; $p = 0.8$, $d =$
508 0.29, non-treated CAS vs. treated CAS).

509 A large main effect of treatment ($F_{[1, 68]} = 36.85$, $p = 7.98 \times 10^{-8}$, $\omega^2 = 0.33$) was found for
510 erratic swimming, but no main effect of drug was found ($F_{[1, 68]} = 2.86$, $p = 0.095$, $\omega^2 = 0.017$),
511 nor was an interaction effect found ($F_{[1, 68]} = 0.002$, $p = 0.965$, $\omega^2 = -0.009$). Post-hoc tests
512 suggested that CAS increased erratic swimming ($p < 0.01$, $d = -1.5$ for the main effect), an effect
513 that was not blocked or increased by RS-10221 ($p < 0.001$, $d = -1.92$, non-treated controls vs.
514 treated CAS; $p = 0.56$, $d = -0.41$, non-treated CAS vs. treated CAS; Figure 6C).

515 A large main effect of treatment ($F_{[1, 68]} = 37.61$, $p = 2.46 \times 10^{-8}$, $\omega^2 = 0.325$), but not drug
516 ($F_{[1, 68]} = 2.65$, $p = 0.108$, $\omega^2 = 0.015$), was found for freezing; no interaction effects were found
517 ($F_{[1, 68]} = 3.23$, $p = 0.077$, $\omega^2 = 0.020$). Post-hoc tests revealed that CAS increased freezing ($p <$
518 0.001, $d = -1.52$ for the main effect), and RS-10221 partially blocked this effect ($p = 0.025$, $d = -$
519 1.12, non-treated controls vs. treated CAS; $p = 0.041$, $d = 0.85$, non-treated CAS vs. treated
520 CAS; Figure 6D).

521 Finally, no main effects of treatment ($F_{[1, 68]} = 1.80$, $p = 0.184$, $\omega^2 = 0.011$) nor drug ($F_{[1, 68]}$
522 $= 2.146$, $p = 0.148$, $\omega^2 = 0.016$) were found for swimming speed (Figure 6E). Interaction effects
523 were also absent ($F_{[1, 68]} = 0.475$, $p = 0.493$, $\omega^2 = -0.007$).

524

525 ### 3.3.2. After exposure

526 No main effects of treatment ($F_{[1, 68]} = 1.615$, $p = 0.21$, $\omega^2 = 0.009$) or drug ($F_{[1, 68]} =$
527 0.032, $p = 0.858$, $\omega^2 = -0.013$), were found for time on top (Figure 7A); an interaction effect was
528 also absent ($F_{[1, 68]} = 1.152$, $p = 0.287$, $\omega^2 = 0.002$).

529 A medium-sized main effect of treatment ($F_{[1, 68]} = 4.39$, $p = 0.04$, $\omega^2 = 0.035$), as well as
530 a large effect of drug ($F_{[1, 68]} = 22.7964$, $p = 9.96 \times 10^{-6}$, $\omega^2 = 0.18$), were found for time on
531 bottom (Figure 7B). A medium-sized interaction effect was also found ($F_{[1, 68]} = 4.08$, $p = 0.047$,

532 $\omega^2 = 0.032$). Post-hoc tests suggested that CAS increased time on bottom after exposure ($p =$
533 0.05, $d = 1.02$, non-treated controls vs. non-treated CAS), an effect that was blocked by RS-
534 10221 ($p = 0.506$, $d = 0.54$, non-treated controls vs. treated CAS; $p < 0.001$, $d = 1.55$, non-
535 treated CAS vs. treated CAS).

536 A large main effect of treatment ($F_{[1, 68]} = 36.1896$, $p = 7.98 \times 10^{-8}$, $\omega^2 = 0.325$), but not
537 drug ($F_{[1, 68]} = 2.9573$, $p = 0.09$, $\omega^2 = 0.018$), were found for erratic swimming (Figure 7C). No
538 interaction effect was found ($F_{[1, 68]} = 0.0019$, $p = 0.965$, $\omega^2 = -0.009$). Post-hoc tests suggested
539 that CAS increased erratic swimming after exposure ($p = 0.001$, $d = 1.514$, non-treated controls
540 vs. non-treated CAS), an effect that was not altered by RS-10221 ($p < 0.001$, $d = -1.92$, non-
541 treated controls vs. treated CAS; $p = 0.56$, $d = -0.41$, non-treated CAS vs. treated CAS).

542 A large main effect of treatment ($F_{[1, 68]} = 39.7692$, p = 2.46 x 10-8, $\omega^2 = 0.334$) and a
543 small main effect of drug ($F_{[1, 68]} = 4.16$, $p = 0.045$, $\omega^2 = 0.027$), were found for freezing; no
544 interaction effects were found ($F_{[1, 68]} = 3.23$, $p = 0.077$, $\omega^2 = 0.019$). Post-hoc tests suggested
545 that CAS increased freezing after exposure ($p < 0.001$, $d = 1.96$, non-treated controls vs. non-
546 treated CAS), an effect that was partially blocked by RS-10221 ($p = 0.025$, $d = -1.12$, non-
547 treated controls vs. treated CAS; $p = 0.041$, $d = 0.85$; Figure 7D).

548 Finally, no main effects of treatment ($F_{[1, 68]} = 1.014$, $p = 0.317$, $\omega^2 = 0.0$) or drug ($F_{[1, 68]} =$
549 1.83, $p = 0.181$, $\omega^2 = 0.011$) were found for swimming speed, nor was there an interaction effect
550 for this variable ($F_{[1, 68]} = 0.475$, $p = 0.493$, $\omega^2 = -0.007$. Figure 7E).

551

552

553 ## 3.4. Effects of MK-212 on restraint stress-elicited behavioral

554 changes

555 No main effects of treatment ($F_{[1, 23]}$ = 0.167, p = 0.687, ω^2 = -0.026) nor drug ($F_{[1, 23]}$ =
556 3.884, p = 0.061, ω^2 = 0.089), were found for time on top (Figure 8A). No interaction effects
557 were found as well ($F_{[1, 23]}$ = 4.215, p = 0.052, ω^2 = 0.1).

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

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

569 No main effects of treatment ($F_{[1, 23]}$ = 0.384, p = 0.541, ω^2 = -0.016) nor drug ($F_{[1, 23]}$ =
570 0.618, p = 0.44, ω^2 = -0.01) were found for freezing (Figure 8D). A large interaction effect was
571 found ($F_{[1, 23]}$ = 12.339, p = 0.002, ω^2 = 0.304). Post-hoc tests suggested that animals injected
572 with MK-212 and subjected to ASR showed decreased freezing in relation to other groups (p =
573 0.033, d = -1.6, non-treated controls vs. treated ASR; p = 0.031, d = 1.6, non-treated ASR vs.
574 treated ASR).

575 Finally, no main effects of treatment ($F_{[1, 23]}$ = 1.0005, p = 0.328, ω^2 = 0.0) nor drug ($F_{[1, 23]}$

576 = 0.0283, p = 0.868, ω² = -0.038) were found for swimming speed (Figure 8E). No interaction
577 effect was found for this variable ($F_{[1, 23]}$ = 0.5437, p = 0.468, ω² = -0.018).
578

579 4. Discussion

580 The present work tested the hypothesis that phasic activation of the 5-HT_{2C} receptor is
581 involved in behavioral adjustments to distal, but not proximal, threat in zebrafish, and that these
582 receptors exerts a tonic facilitation of defensive behavior to potential threat in zebrafish. We
583 found that 5-HT_{2C} agonists blocked CAS-elicited defensive behavior, but not post-exposure
584 increases in defensive behavior, nor ARS-elicited anxiogenic-like effects. We also found that
585 RS-10221, a 5-HT_{2C} receptor antagonist, did not change behavior during exposure, but it
586 produced a small effect on behavior after exposure to CAS.
587

588 4.1. Effects of stressors on zebrafish behavior

589 One of the aims of this paper was to confirm the behavioral effects of CAS during and
590 after exposure (Lima-Maximino et al., 2020), given the considerable variation in the literature
591 (Maximino et al., 2019), and to compare the effects after exposure with those of ARS -
592 considering that, while both CAS and ARS are anxiogenic stressors, from an ecological point of
593 view both should affect different behavioral endpoints.
594 In the present experiments, CAS consistently increased bottom-dwelling and erratic
595 swimming during exposure, with a smaller component of freezing. After exposure, a strong
596 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-10221, 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 participate in fear and anxiety. Salchner and Singewald (2006) have shown that MK-212

641 potentiates escape responses to an airjet in rats, and the antagonist SB-242084 has been
642 shown to produce anxiolytic-like effects in rats (Kennett et al., 1997; Martin et al., 2002). In the
643 elevated T-maze, an apparatus which tests anxiety-like behavior (inhibitory avoidance) and fear-
644 like behavior (one-way escape), 5-HT_{2C} agonists facilitate, and antagonists impair, inhibitory
645 avoidance, but only agonists facilitate one-way escape (Mora et al., 1997), suggesting tonic
646 facilitation of responses to potential threat and phasic inhibition of responses to distal or
647 proximal threat. Thus, a conserved role for this receptor appears to be related to phasic and
648 tonic modulation of defensive responses.

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

659 We have previously shown that, after exposure, serotonin levels are increased in the
660 extracellular fluid of the zebrafish brain (Maximino et al., 2014), an effect that was mediated by
661 both serotonin transporters (Maximino et al., 2014) and monoamine oxidase activity (Maximino
662 et al., 2019; Quadros et al., 2018). This increase in serotonergic activity could activate 5-HT_{2C}
663 and other serotonergic receptors to inhibit ongoing defensive responses to proximal threat and
664 initiate programs of alertness and cautious exploration/risk assessment. This phasic signal
665 represents prediction errors (Amo et al., 2014; Lima-Maximino et al., 2020), while the tonic

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

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

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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

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 = 10 animals; CTRL + 1 mg/kg WAY-161503: n = 21 animals; CAS + VEH: n = 21 animals; CAS + 1 mg/kg WAY-161503: n = 21 animals.

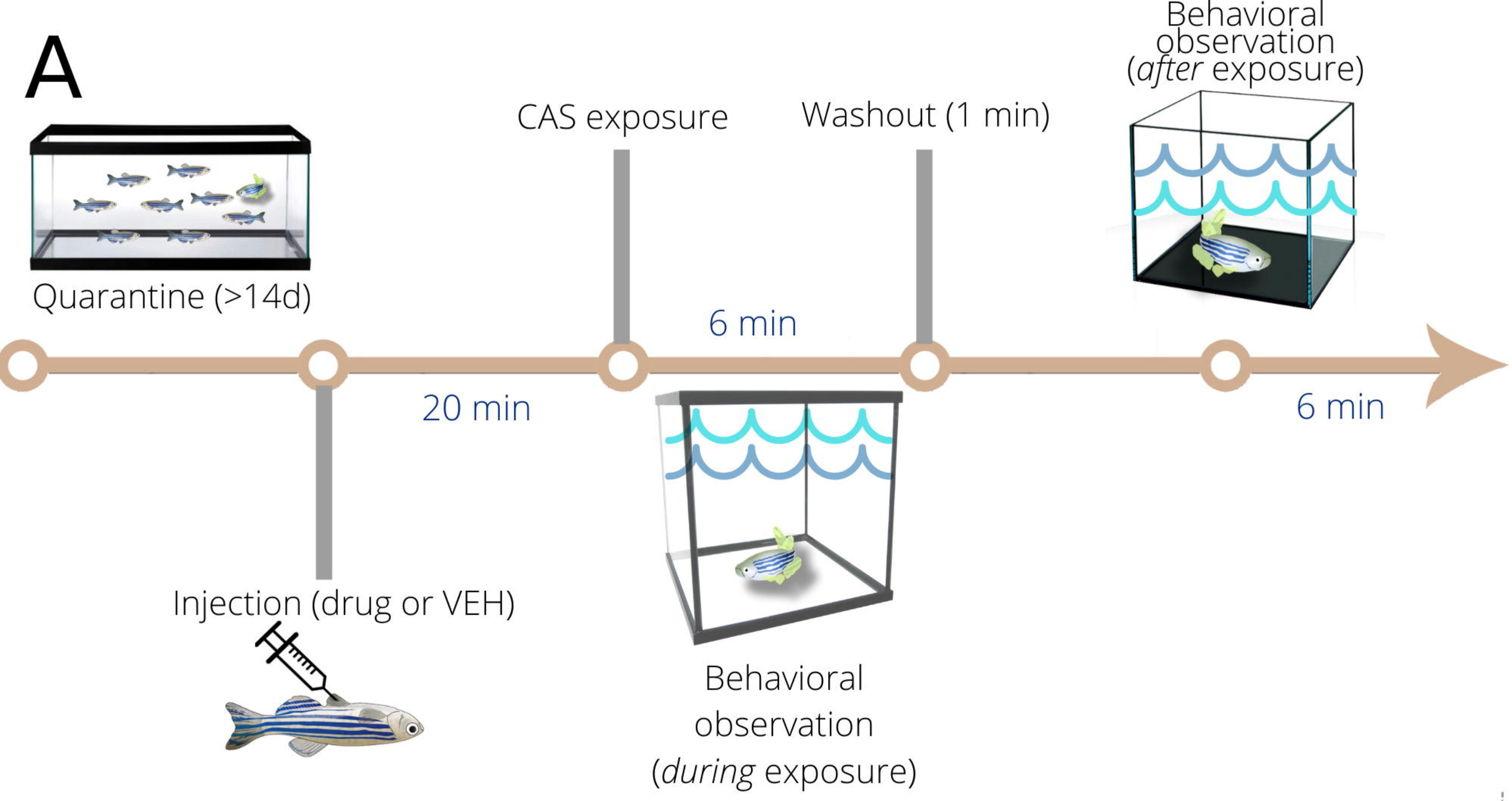
Figure 5 – Effects of WAY-161503 on behavior <u>after</u> CAS exposure. (A) Time spent on top third of the tank. (B) Time spent on bottom third of the tank. (C) Erratic swimming. (D) Total time spent freezing. (E) Swimming speed. Different letters represent statistical differences at the $p < 0.05$ level; 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 = 10 animals; CTRL + 1 mg/kg WAY-161503: n = 21 animals; CAS + VEH: n = 21 animals; CAS + 1 mg/kg WAY-161503: n = 21 animals.

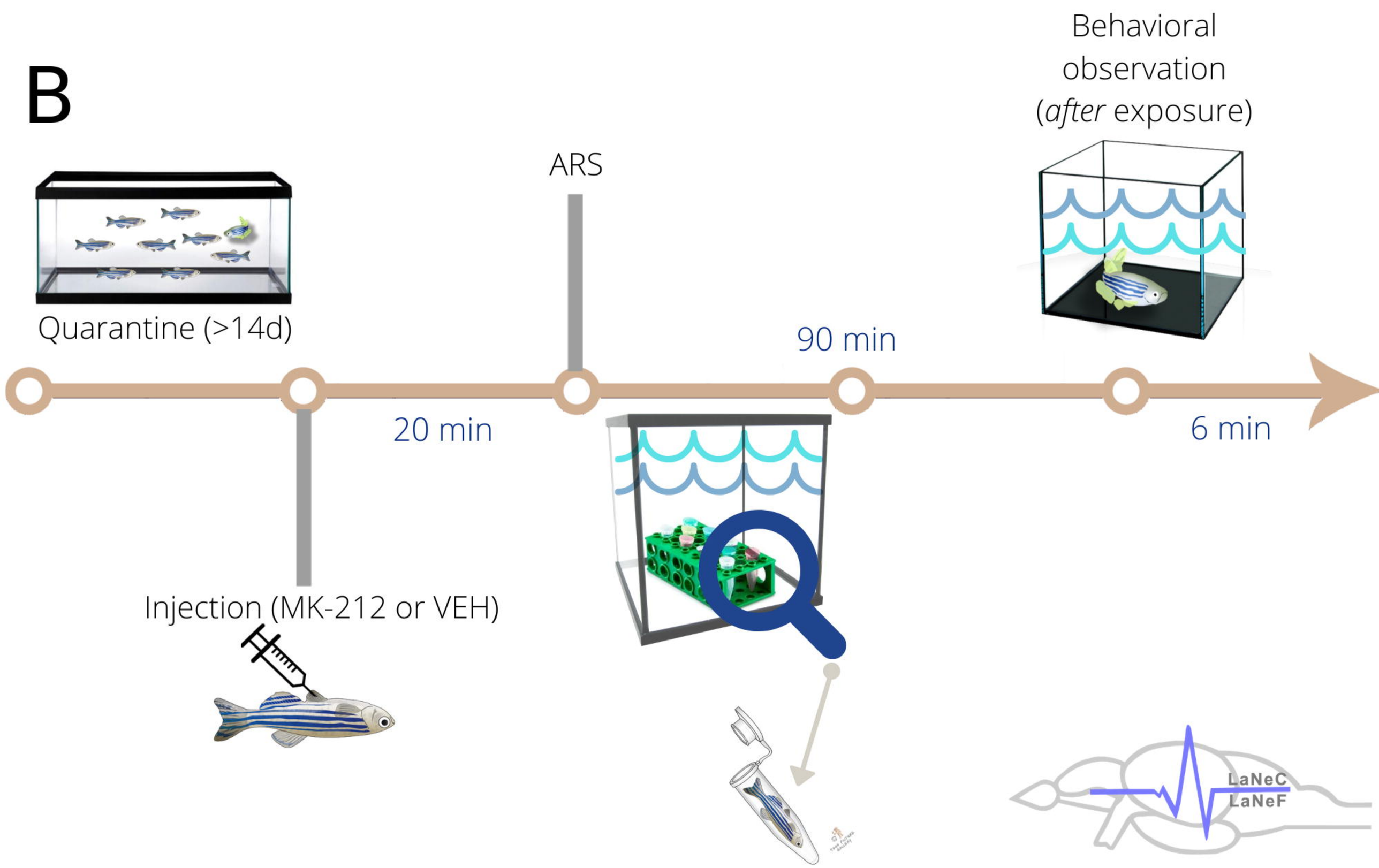
Figure 6 – Effects of RS-10221 on behavior <u>during</u> CAS exposure. (A) Time spent on top third of the tank. (B) Time spent on bottom third of the tank. (C) Erratic swimming. (D) Total time spent freezing. (E) Swimming speed. Different letters represent statistical differences at the $p < 0.05$ level; 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 = 10 animals; CTRL + 2 mg/kg RS-10221: n = 21 animals; CAS + VEH: n = 20 animals; CAS + 2 mg/kg RS-10221: n = 21 animals.

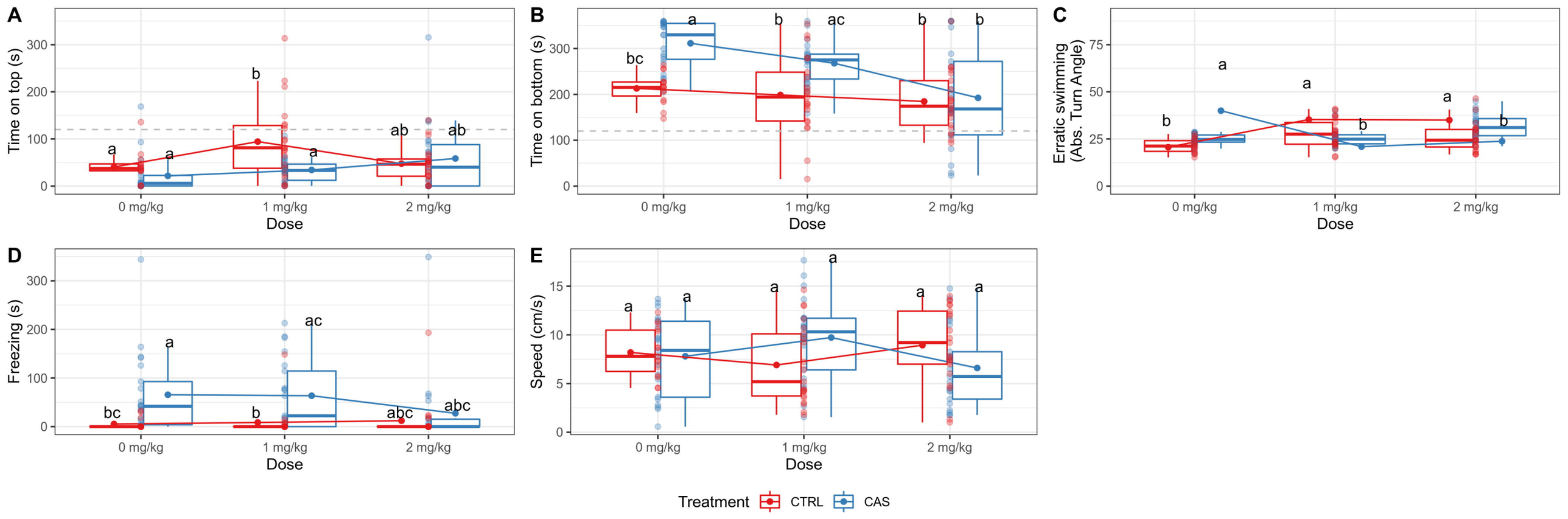
Figure 7 – Effects of RS-10221 on behavior <u>after</u> CAS exposure. (A) Time spent on top third of the tank. (B) Time spent on bottom third of the tank. (C) Erratic swimming. (D) Total time spent freezing. (E) Swimming speed. Different letters represent statistical differences at the $p < 0.05$ level; 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

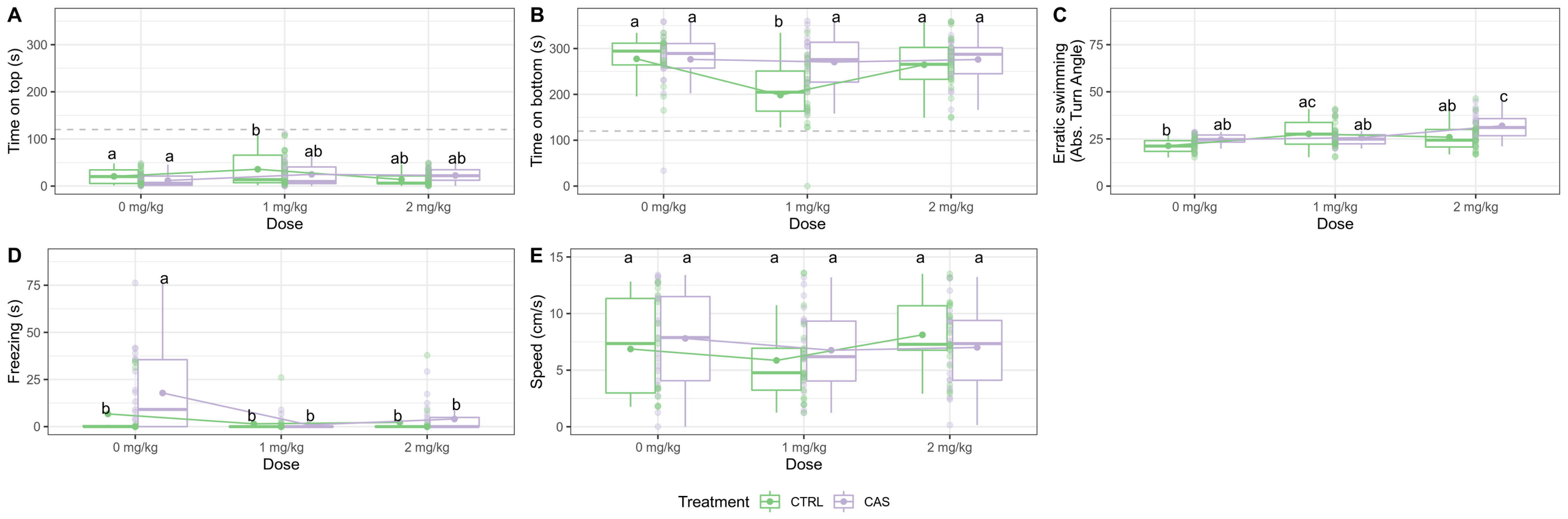
connected by lines represent group means. CTRL = controls (water-exposed animals); CAS = conspecific alarm substance. Final sample sizes: CTRL + VEH: n = 10 animals; CTRL + 2 mg/kg RS-10221: n = 21 animals; CAS + VEH: n = 20 animals; CAS + 2 mg/kg RS-10221: n = 21 animals.

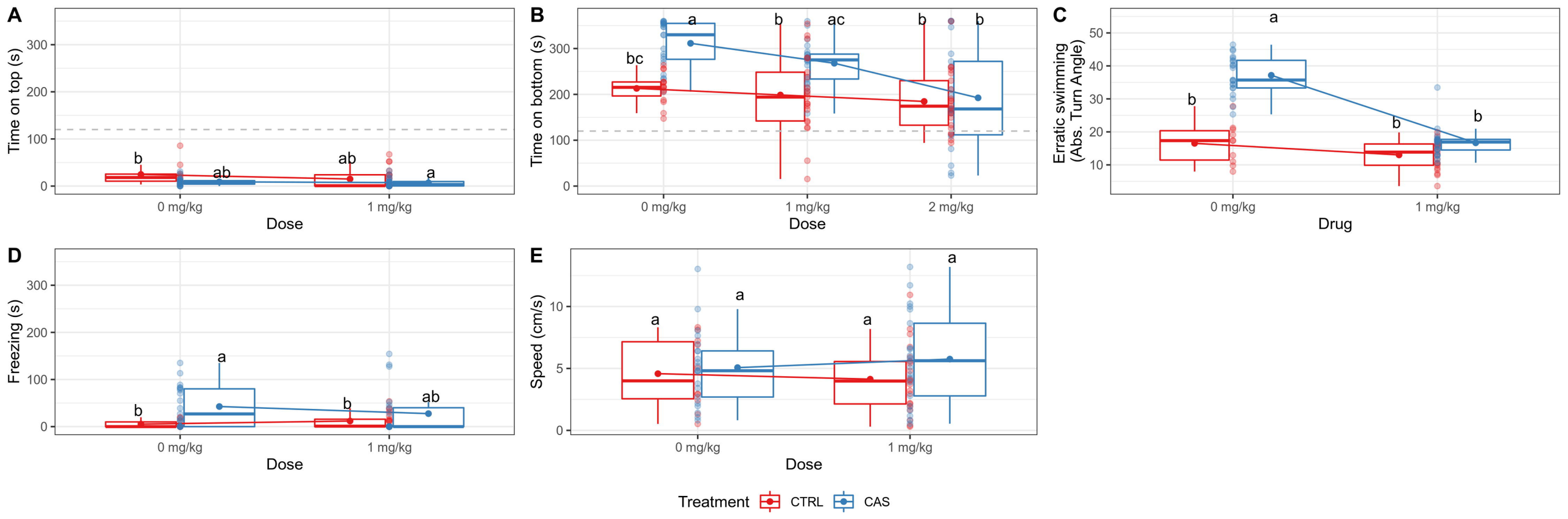
Figure 8 – Effects of MK-212 on behavior after acute restraint stress (ARS). (A) Time spent on top third of the tank. (B) Time spent on bottom third of the tank. (C) Erratic swimming. (D) Total time spent freezing. (E) Swimming speed. Different letters represent statistical differences at the $p < 0.05$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

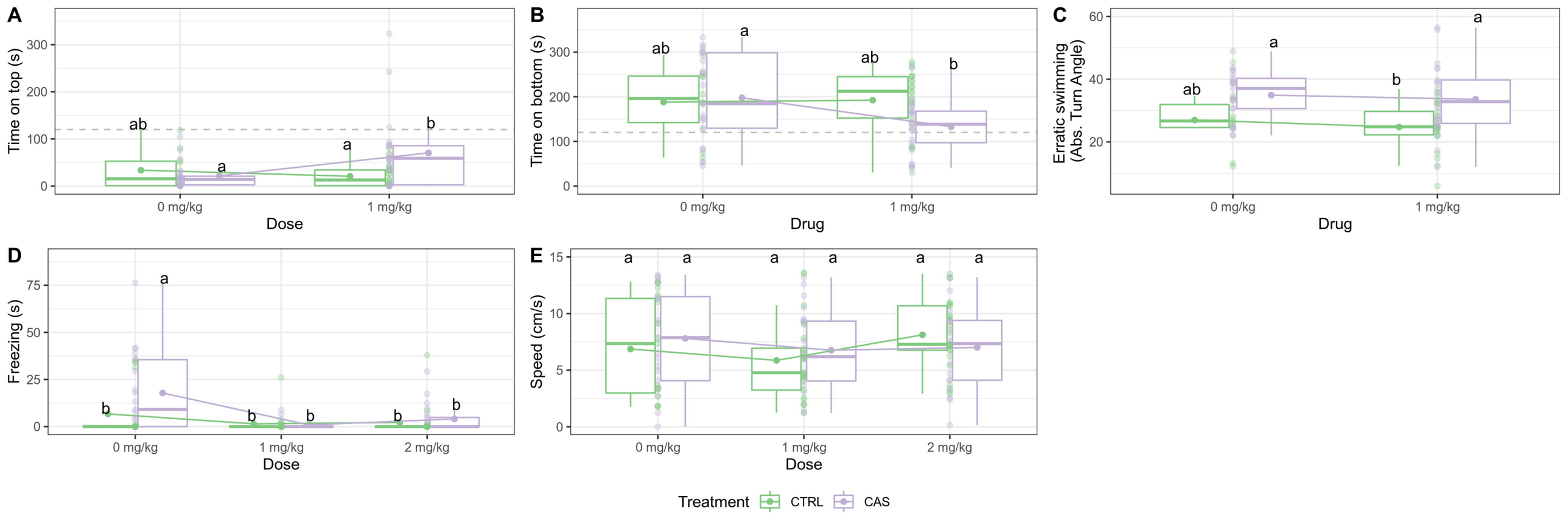

A
Quarantine (>14d)
CAS exposure
Washout (1 min)
Behavioral observation (*after* exposure)
6 min
20 min
6 min
Injection (drug or VEH)
Behavioral observation (*during* exposure)

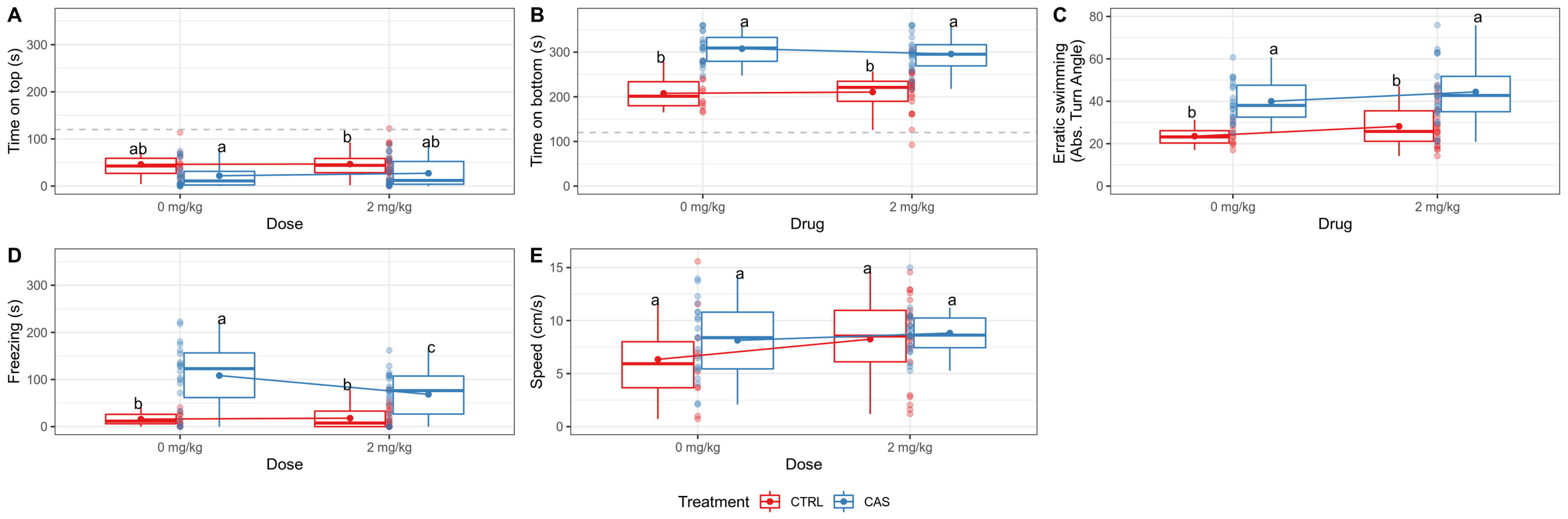

B
Quarantine (>14d)
ARS
Behavioral observation (*after* exposure)
90 min
20 min
6 min
Injection (MK-212 or VEH)
LaNeC
LaNeF

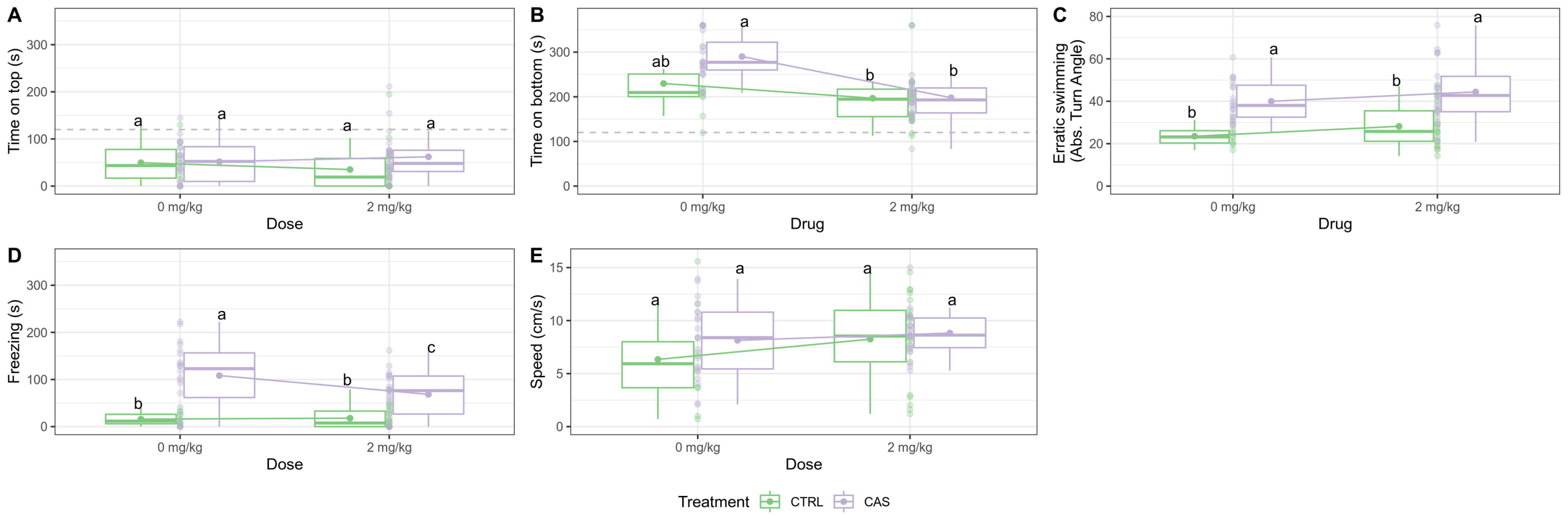

A
Time on top (s)
300
200
100
0
a
a
b
a
ab
ab
0 mg/kg
1 mg/kg
2 mg/kg
Dose
B
Time on bottom (s)
300
200
100
0
bc
a
b
ac
b
b
0 mg/kg
1 mg/kg
2 mg/kg
Dose
C
Erratic swimming
(Abs. Turn Angle)
75
50
25
0
b
a
a
b
a
b
0 mg/kg
1 mg/kg
2 mg/kg
Dose
D
Freezing (s)
300
200
100
0
bc
a
b
ac
abc
abc
0 mg/kg
1 mg/kg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

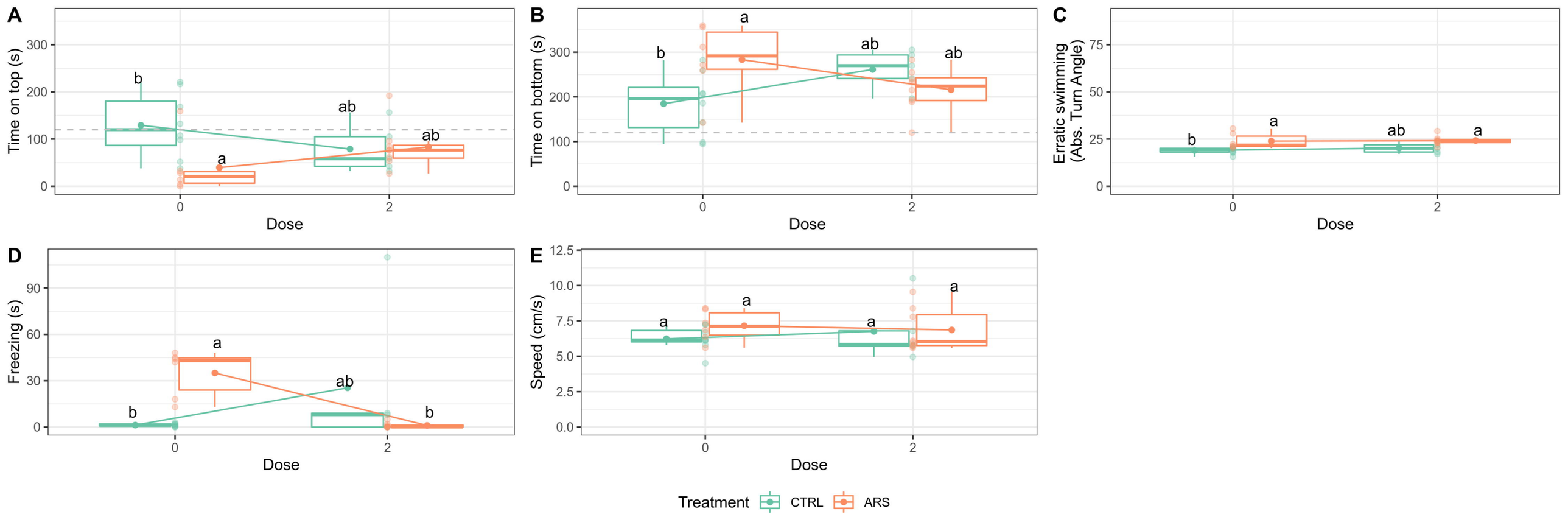

A
Time on top (s)
300
200
100
0
a
a
b
ab
ab
ab
0 mg/kg
1 mg/kg
2 mg/kg
Dose
B
Time on bottom (s)
300
200
100
0
a
a
b
a
a
a
0 mg/kg
1 mg/kg
2 mg/kg
Dose
C
Erratic swimming
(Abs. Turn Angle)
75
50
25
0
b
ab
ac
ab
ab
c
0 mg/kg
1 mg/kg
2 mg/kg
Dose
D
Freezing (s)
75
50
25
0
b
a
b
b
b
b
0 mg/kg
1 mg/kg
2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


A
Time on top (s)
300
200
100
0
b
ab
ab
a
0 mg/kg
1 mg/kg
Dose
B
Time on bottom (s)
300
200
100
0
bc
a
b
ac
b
b
0 mg/kg
1 mg/kg
2 mg/kg
Dose
C
Erratic swimming
(Abs. Turn Angle)
50
40
30
20
10
b
a
b
b
0 mg/kg
1 mg/kg
Drug
D
Freezing (s)
300
200
100
0
b
a
b
ab
0 mg/kg
1 mg/kg
Dose
E
Speed (cm/s)
10
5
0
a
a
a
a
0 mg/kg
1 mg/kg
Dose
Treatment
CTRL
CAS


A
Time on top (s)
300
200
100
0
ab
a
a
b
0 mg/kg
1 mg/kg
Dose
B
Time on bottom (s)
300
200
100
0
ab
a
ab
b
0 mg/kg
1 mg/kg
Drug
C
Erratic swimming
(Abs. Turn Angle)
60
40
20
ab
a
b
a
0 mg/kg
1 mg/kg
Drug
D
Freezing (s)
75
50
25
0
b
a
b
b
b
b
0 mg/kg
1 mg/kg
2 mg/kg
Dose
E
Speed (cm/s)
15
10
5
0
a
a
a
a
a
a
0 mg/kg
1 mg/kg
2 mg/kg
Dose
Treatment
CTRL
CAS


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


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


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