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

Rhayra Xavier do Carmo Silva[1,2], Bianca Gomes do Nascimento[2], Suianny Nayara da Silva Chaves[2], Anderson Manoel Herculano[1], Monica Lima-Maximino[3], Caio Maximino[2*]

[1] Laboratório de Neurofarmacologia Experimental – LNE, Universidade Federal do Pará, Belém/PA, Brazil

[2] Laboratório de Neurociências e Comportamento "Frederico Guilherme Graeff" – LaNeC, Universidade Federal do Sul e Sudeste do Pará, Marabá/PA, Brazil

[3] Laboratório de Neurofarmacologia e Biofísica – LaNeF, Universidade do Estado do Pará, Marabá/PA, Brazil

* Corresponding author

Caio Maximino

Laboratório de Neurociências e Comportamento "Frederico Guilherme Graeff" - LaNeC

Faculdade de Psicologia, Instituto de Estudos em Saúde e Biológicas

Universidade Federal do Sul e Sudeste do Pará, Campus Marabá, Unidade III

Av. dos Ipês, S/N – 68500-000

Marabá/PA, Brazil

cmaximino@unifesspa.edu.br

Abstract

Serotonin (5-HT) receptors have been implicated in responses to aversive stimuli in mammals and fish, but its precise role is still unknown. Moreover, since at least seven families of 5-HT receptors exist in vertebrates, the role of specific receptors is still debated. Aversive stimuli can be classified as indicators of proximal, distal, or potential threat, initiating responses that are appropriate for each of these threat levels. Responses to potential threat usually involve cautious exploration and increased alertness, while responses to distal and proximal threat involve a fight-flight-freeze reaction. We exposed adult zebrafish to a conspecific alarm substance (CAS) and observed behavior during (distal threat) and after (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,

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

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

61 5-HT has been implicated in fish behavioral responses both during and after exposure to
62 CAS. In zebrafish, extracellular 5-HT levels were increased after CAS exposure (Maximino et
63 al., 2014), an effect that can be related to decreased 5-HT uptake (Maximino et al., 2014) and/or
64 decreased monoamine oxidase activity (Lima-Maximino et al., 2020; Quadros et al., 2018). In
65 Crucian carp (*Carassius carassius*), exposure to CAS elicits increases in serotonergic activity in
66 the brainstem and optic tectum (Höglund et al., 2005), structures which have been involved in
67 responses to distal and proximal threat (do Carmo Silva et al., 2018a). However, this only

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

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

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

93 increased freezing and bottom-dwelling during and after exposure at a high dose (92.79 mg/kg),
94 but not at lower doses (Nathan et al., 2015). These results point to an inhibitory role of the 5-
95 HT_{2A} and 5-HT_{2C} receptors on CAS-elicited fear-like responses. However, since Nathan et al.
96 (2015) observed these effects consistently during a long session, in which behavior is expected
97 to change as CAS concentrations decrease (Mathuru et al., 2012)(i.e., similar to the shift from
98 erratic swimming to freezing that is observed when animals are observed after exposure in a
99 CAS-free context; Lima-Maximino et al., 2020), and since serotonergic drugs can produce
100 opposite effects on behavior during and after CAS exposure (Lima-Maximino et al., 2020), from
101 these results it is not possible to understand whether 5-HT_2 receptors participate in both
102 responses.

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

117 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). Interestingly, work with rodents clearly suggests phasic facilitation or inhibition of defensive responses by 5-HT_{2C} receptors, since agonists, but not antagonists, produce these effects.

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

2. Materials and methods

2.1. Animals and housing

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

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

172 2.2. Drugs and treatments

173 The 5-HT_{2C} receptor agonists MK-2212 (2-Chloro-6-(1-piperazinyl)pyrazine, CAS
174 #64022-27-1) and WAY-161503 (8,9-dichloro-2,3,4,4a-tetrahydro-1H-pyrazino[1,2-a]quinoxalin-
175 5(6H)-one; CAS #75704-24-4) were bought from Sigma-Aldrich (St Louis, USA) on 2018, and
176 dissolved in Cortland's salt solution (NaCl 124.1 mM, KCl 5.1 mM, Na_2HPO_4 2.9 mM, $MgSO_4$ 1.9
177 mM, $CaCl_2$ 1.4 mM, $NaHCO_3$ 11.9 mM, Polyvinylpyrrolidone 4%, 1,000 USP units Heparin; Wolf,
178 1963) and in 1% dimethyl sulfoxide (DMSO), respectively. The 5-HT_{2C} receptor antagonist RS-
179 102221 (8-[5-(2,4-Dimethoxy-5-(4-trifluoromethylphenylsulphonamido)phenyl -5-oxopentyl]-
180 1,3,8-triazaspiro[4.5]decane-2,4-dione; CAS #185376-97-0) was bought from Sigma-Aldrich (St
181 Louis, USA) in 2018, and dissolved in 1% DMSO. While affinities for zebrafish 5-HT_{2C}-like
182 receptors have not been established, WAY-161503 has been reported to displace DOI from
183 human 5-HT_{2C} receptors with a K_i of 3.3 nM (6-fold selectivity over human 5-HT_{2A} receptors and
184 20-fold over human 5-HT_{2B} receptors)(Rosenzweig-Lipson et al., 2006). MK-212 has been
185 shown to be less selective at recombinant human receptors, with a K_i of 7.01 nM at 5-HT_{2C}
186 receptors (vs. 5.99 nM and 6.21 nM at 5-HT_{2A} and 5-HT_{2B} receptors, respectively)(Knight et al.,
187 2004). Finally, RS-102221 has been shown to displace mesulergine from human 5-HT_{2C}

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

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

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

2.3.1. Experimental design

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

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

Animals were randomly allocated to groups using a random number 185 generator (http://www.jerrydallal.com/random/random_block_size_r.htm), with each subject randomized to a single treatment using random permuted blocks. One PI attributed a random letter to treatment (e.g., “A” for CTRL, “B” for CAS) and a random integer for drug dose (e.g., “1” for 1 mg/kg, “2” for 0 mg/kg [vehicle]), and combinations for letters and integers were randomized. For each experiment, animals were treated and tested in the order of allocation (i.e., randomly). In all experiments, experimenters and data analysts were blinded to drugs and treatment by using coded vials (with the same code used for randomization); blinding was removed only after data analysis. 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).

2.3.2. Sample size determination

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

2.3.3. Alarm substance extraction

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

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

2.3.4. Observation apparatuses

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

2.4. Experiment 2: Effects of MK-212 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

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: 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

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; ω^2 = 0.039) and dose ($F_{[2, 114]}$ = 3.665, p = 0.0287; ω^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; ω^2 = 0.052). Post-hoc tests found that CAS did not alter time on top (p = 0.94, d = 0.3, non-treated controls vs. non-treated CAS), but MK-212 (1 mg/kg) increased it in non-exposed animals (*1 mg/kg:* p = 0.024, d = -1.05, non-treated controls vs. 1 mg/kg controls; *2 mg/kg:* p = 0.994, d = -0.18, non-treated controls vs. 2 mg/kg controls; Figure 2A).

Medium-to-large-sized main effects of treatment ($F_{[1, 114]}$ = 20.995, p = 1.18 x 10^{-5}; ω^2 = 0.12) and dose ($F_{[2, 114]}$ = 11.455, p = 2.93 x 10^{-5}; ω^2 = 0.125) were found for time on bottom. A small-to-medium interaction effect was also found ($F_{[2, 114]}$ = 4.085, p = 0.0194; ω^2 = 0.037). Post-hoc tests revealed that CAS increased time on bottom (p < 0.001, d = -0.77 for the main

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

367 A small-to-medium-sized main effect of treatment ($F_{[1, 114]}$ = 5.137, p = 0.0253; ω^2 =
368 0.017), but not dose ($F_{[2, 114]}$ = 2.948, p = 0.0565; ω^2 = 0.016), was found for erratic swimming. A
369 very large interaction effect was also found for this variable ($F_{[2, 114]}$ = 57.101, p < 2 x 10^{-16}; ω^2 =
370 0.467). Post-hoc tests showed that CAS greatly increased absolute turn angle in non-treated
371 animals (p < 0.001, d = -2.58, non-treated controls vs. non-treated CAS), an effect that was
372 blocked by both MK-212 doses (*1 mg/kg*: p = 1, d = -0.04, non-treated controls vs. treated CAS;
373 p < 0.001, d = 2.53, non-treated CAS vs. treated CAS; *2 mg/kg*: p = 0.81, d = -0.42, non-treated
374 controls vs. treated CAS; p < 0.001, d = 2.16, non-treated CAS vs. treated CAS; Figure 2C);
375 however, MK-212 also increased absolute turn angle in animals which were not exposed to
376 CAS (*1 mg/kg*: p < 0.001, d = -1.95, non-treated controls vs. treated controls; *2 mg/kg:* p <
377 0.001, d = -1.91, non-treated controls vs. treated controls).

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

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

387

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) .

412 A small-to-medium main effect of treatment ($F_{[1, 114]}$ = 4.658, p = 0.033; ω^2 = 0.025) and
413 a large main effect of MK-212 dose were found for freezing ($F_{[2, 114]}$ = 11.617, p = 2.56 x 10^{-5}; ω^2
414 = 0.143). No interaction effects were found ($F_{[2, 114]}$ = 2.777, p = 0.0664; ω^2 = 0.024). Post-hoc
415 tests revealed that CAS increased freezing (p = 0.05, d = -0.364 for the main effect), an effect
416 that was blocked by all MK-212 doses (*1 mg/kg:* p = 0.74, d = 0.47, non-treated controls vs. 1
417 mg/kg controls; p < 0.001, d = 1.5, non-treated CAS vs. 1 mg/kg CAS; *2 mg/kg:* p = 0.85, d =
418 0.4, non-treated controls vs. 2 mg/kg controls; p = 0.002, d = 1.22, non-treated CAS vs. 2 mg/kg
419 CAS; Figure 3D).
420 No main effects of treatment ($F_{[1, 114]}$ = 0.111, p = 0.74; ω^2 = -0.007) or dose ($F_{[2, 114]}$ =
421 1.339, p = 0.266; ω^2 = 0.006), nor an interaction effect ($F_{[2, 114]}$ = 0.981, p = 0.378; ω^2 = 0.0),
422 were found for swimming speed (Figure 3E).
423

424 3.2. Effects of WAY-161503 on alarm reaction and post-exposure
425 behavior

426 3.2.1. During exposure

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

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

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

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

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

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

3.2.2. After exposure

No main effects of treatment ($F_{[1, 69]}$ = 3.221, p = 0.0771, ω^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, ω^2 = 0.039). A medium-sized interaction was also found ($F_{[1, 69]}$ = 6.352, p = 0.014, ω^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, ω^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, ω^2 = 0.039). No significant interaction was observed ($F_{[1, 69]}$ = 3.267, p = 0.0751, ω^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, ω^2 = 0.174) was found for erratic swimming, but no main effect of drug ($F_{[1, 69]}$ = 0.568, p = 0.454, ω^2 = -0.005) nor an interaction effect (F[1, 69] = 0.042, p = 0.838, ω^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, ω^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, ω^2 = 0.037). A medium-to-large interaction effect was observed for freezing as well

($F_{[1, 69]}$ = 7.663, p = 0.00723, ω^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, ω^2 = 0.038) or drug ($F_{[1, 69]}$ = 0.416, p = 0.5212, ω^2 = -0.008) were found for swimming speed, and an interaction effect was also absent (F[1, 69] = 0.062, p = 0.8037, ω^2 = -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, ω^2 = 0.101) was found for time on top, but no main effect of drug ($F_{[1, 68]}$ = 0.166, p = 0.685, ω^2 = -0.011) nor an interaction effect ($F_{[1, 68]}$ = 0.096, p = 0.757, ω^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-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}, ω^2 = 0.53), but not dose ($F_{[1, 68]}$ = 0.189, p = 0.665, ω^2 = -0.005), nor an interaction effect ($F_{[1, 68]}$ = 0.517, p = 0.474, ω^2 = -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

505 to change this effect ($p < 0.001$, $d = -2.12$, non-treated controls vs. treated CAS; $p = 0.8$, $d =$
506 0.29, non-treated CAS vs. treated CAS).

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

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

519 Finally, no main effects of treatment ($F_{[1, 68]} = 1.80$, $p = 0.184$, $\omega^2 = 0.011$) nor drug ($F_{[1, 68]}$
520 $= 2.146$, $p = 0.148$, $\omega^2 = 0.016$) were found for swimming speed (Figure 6E). Interaction effects
521 were also absent ($F_{[1, 68]} = 0.475$, $p = 0.493$, $\omega^2 = -0.007$).

522

523 ### 3.3.2. After exposure

524 No main effects of treatment ($F_{[1, 68]} = 1.615$, $p = 0.21$, $\omega^2 = 0.009$) or drug ($F_{[1, 68]} =$
525 0.032, $p = 0.858$, $\omega^2 = -0.013$), were found for time on top (Figure 7A); an interaction effect was
526 also absent ($F_{[1, 68]} = 1.152$, $p = 0.287$, $\omega^2 = 0.002$).

527 A medium-sized main effect of treatment ($F_{[1, 68]} = 4.39$, $p = 0.04$, $\omega^2 = 0.035$), as well as
528 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
529 bottom (Figure 7B). A medium-sized interaction effect was also found ($F_{[1, 68]} = 4.08$, $p = 0.047$,

530 $\omega^2 = 0.032$). Post-hoc tests suggested that CAS increased time on bottom after exposure ($p =$
531 0.05, $d = 1.02$, non-treated controls vs. non-treated CAS), an effect that was blocked by RS-
532 10221 ($p = 0.506$, $d = 0.54$, non-treated controls vs. treated CAS; $p < 0.001$, $d = 1.55$, non-
533 treated CAS vs. treated CAS).

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

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

546 Finally, no main effects of treatment ($F_{[1, 68]} = 1.014$, $p = 0.317$, $\omega^2 = 0.0$) or drug ($F_{[1, 68]} =$
547 1.83, $p = 0.181$, $\omega^2 = 0.011$) were found for swimming speed, nor was there an interaction effect
548 for this variable ($F_{[1, 68]} = 0.475$, $p = 0.493$, $\omega^2 = -0.007$. Figure 7E).

549

550

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

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

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

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

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

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

574 = 0.0283, p = 0.868, ω^2 = -0.038) were found for swimming speed (Figure 8E). No interaction
575 effect was found for this variable ($F_{[1, 23]}$ = 0.5437, p = 0.468, ω^2 = -0.018).
576

577 4. Discussion

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

586 4.1. Effects of stressors on zebrafish behavior

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

595 behavioral pattern. These results are consistent with what was observed both with a washout
596 period (Lima-Maximino et al., 2020), and in the absence of a washout, but with an extended
597 observation interval (Mathuru et al., 2012; Nathan et al., 2015). These effects suggest that, as
598 threat levels change from distal (i.e., CAS is present) to potential (i.e., CAS is no longer
599 present), a “residual” effect emerges that is marked by different behavioral components. This is
600 similar to what is observed with electrical stimulation of the PAG/GR in rats (Brandão et al.,
601 2008). We have previously shown that serotonin differentially mediates these behaviors,
602 phasically inhibiting responses during exposure and tonically facilitating responses after
603 exposure (Lima-Maximino et al., 2020).

604 ARS-elicited behavioral effects have been explored in the literature with mixed results.
605 While Ghisleni et al. (2012) found that a 90-min restraint protocol did not change bottom-
606 dwelling after stress when animals were tested individually, a similar protocol showed marked
607 increases in this variable (Assad et al., 2020) - albeit control animals in the latter experiment
608 spent most of the session in the upper half of the tank instead of in the lower half. In the present
609 experiments, ARS increased bottom-dwelling as well. Moreover, we observed a small
610 component of erratic swimming, consistent with what is observed by Ghisleni et al. (2012).
611 While increased bottom-dwelling was also observed as a pattern of post-exposure behavior
612 after CAS in the present experiments and in Lima-Maximino et al. (2020), freezing was a major
613 component of post-exposure behavior, suggesting that stressors do not produce similar
614 behavioral effects.
615

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

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

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

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

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

670 In general, our results are the first to determine a specific role of 5-HT_{2C} receptors in
671 zebrafish behavior, and add to the small literature on the role of this receptor in mammals as
672 well. Further work is needed to understand whether this receptor interacts with other serotonin
673 receptors known to be involved in defensive behavior in this species (Herculano and Maximino,
674 2014) and if the effects during exposure are related to changes in serotonin levels. Moreover,
675 given the importance of these phenotypes to understanding fear and panic states (Silva et al.,
676 2020), further work will clarify the usefulness of this pharmacological profile in modelling panic
677 disorder and anxiety.
678

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682 #302998/2019-5).

References

Alves, S.H., Pinheiro, G., Motta, V., Landeira-Fernandez, J., Cruz, A.P.M., 2004. Anxiogenic effects in the rat elevated plus-maze of 5-HT2C agonists into ventral but not dorsal hippocampus. Behav. Pharmacol. 15, 37–43.

Amo, R., Fredes, F., Kinoshita, M., Aoki, R., Aizawa, H., Agetsuma, M., Aoki, T., Shiraki, T., Kakinuma, H., Matsuda, M., Yamazaki, M., Takahoko, M., Tsuboi, T., Higashijima, S., Miyasaka, N., Koide, T., Yabuki, Y., Yoshihara, Y., Fukai, T., Okamoto, H., 2014. The Habenulo-Raphe Serotonergic Circuit Encodes an Aversive Expectation Value Essential for Adaptive Active Avoidance of Danger. Neuron 84, 1034–1048. https://doi.org/10.1016/j.neuron.2014.10.035

Assad, N., Luz, W.L., Santos-Silva, M., Carvalho, T., Moraes, S., Picanço-Diniz, D.L.W., Bahia, C.P., Oliveira Batista, E. de J., da Conceição Passos, A., Oliveira, K.R.H.M., Herculano, A.M., 2020. Acute Restraint Stress Evokes Anxiety-Like Behavior Mediated by Telencephalic Inactivation and GabAergic Dysfunction in Zebrafish Brains. Sci. Rep. 10, 5551. https://doi.org/10.1038/s41598-020-62077-w

Barreto, R.E., 2012. Mianserin affects alarm reaction to conspecific chemical alarm cues in Nile tilapia. Fish Physiol. Biochem. 43, 193–201. https://doi.org/10.1007/s10695-016-0279-2

Bencan, Z., Sledge, D., Levin, E.D., 2009. Buspirone, chlordiazepoxide and diazepam effects in a zebrafish model of anxiety. Pharmacol. Biochem. Behav. 94, 75–80. https://doi.org/10.1016/j.pbb.2009.07.009

Bonapersona, V., Hoijtink, H., RELACS, Sarabdjitsingh, R.A., Joëls, M., 2019. RePAIR: a power solution to animal experimentation. bioRxiv 864652. https://doi.org/10.1101/864652

Bonhaus, D.W., Weinhardt, K.K., Taylor, M., DeSouza, A., McNeeley, P.M., Szczepanski, K., Fontana, D.J., Trinh, J., Rocha, C.L., Dawson, M.W., Flippin, L.A., Eglen, R.M., 1997. RS-102221: a novel high affinity and selective, 5-HT2C receptor antagonist. Neuropharmacology 36, 621–629. https://doi.org/10.1016/s0028-3908(97)00049-x

Brandão, M.L., Zanoveli, J.M., Ruiz-Martinez, R.C., Oliveira, L.C., Landeira-Fernandez, J., 2008. Different patterns of freezing behavior organized in the periaqueductal gray of rats: Association with different types of anxiety. Behav. Brain Res. 188, 1–13.

https://doi.org/10.1016/j.bbr.2007.10.018

Castilho, V., Macedo, C., Brandão, M., 2002. Role of benzodiazepine and serotonergic mechanisms in conditioned freezing and antinociception using electrical stimulation of the dorsal periaqueductal gray as unconditioned stimulus in rats. Psychopharmacology (Berl.) 165, 77–85. https://doi.org/10.1007/s00213-002-1246-4

Castilho, V.M., Brandão, M.L., 2001. Conditioned antinociception and freezing using electrical stimulation of the dorsal periaqueductal gray or inferior colliculus as unconditioned stimulus are differentially regulated by 5-HT2A receptors in rats. Psychopharmacology (Berl.) 155, 154–162. https://doi.org/10.1007/s002130100697

Coimbra, N.C., Brandão, M.L., 1997. Effects of 5-HT2 receptors blockade on fear-induced analgesia elicited by electrical stimulation of the deep layers of the superior colliculus and dorsal periaqueductal gray. Behav. Brain Res. 87, 97–103. https://doi.org/10.1016/S0166-4328(96)02267-X

Conselho Nacional de Controle de Experimentação Animal - CONCEA, 2017. Diretriz brasileira para o cuidado e a utilização de animais para fins científicos e didáticos - DBCA. Anexo I. Peixes mantidos em instalações de instituições de ensino ou pesquisa científica, Resolução Normativa CONCEA nº 34.

Cornélio, A.M., Nunes-de-Souza, R.L., 2007. Anxiogenic-like effects of mCPP microinfusions into the amygdala (but not dorsal or ventral hippocampus) in mice exposed to elevated plus-maze. Behav. Brain Res. 178, 82–89. https://doi.org/10.1016/j.bbr.2006.12.003

Daw, N.D., Kakade, S., Dayan, P., 2002. Opponent interactions between serotonin and dopamine. Neural Netw. 15, 603–616. https://doi.org/10.1016/S0893-6080(02)00052-7

de Mello Cruz, A.P., Pinheiro, G., Alves, S.H., Ferreira, G., Mendes, M., Faria, L., Macedo, C.E., Landeira-Fernandez, J., 2005. Behavioral effects of systemically administered MK-212 are prevented by ritanserin microinfusion into the basolateral amygdala of rats exposed to the elevated plus-maze. Psychopharmacology (Berl.) 182, 345–354. https://doi.org/10.1007/s00213-005-0108-2

de Paula, B.B., Leite-Panissi, C.R.A., 2016. Distinct effect of 5-HT1A and 5-HT2A receptors in the medial nucleus of the amygdala on tonic immobility behavior. Brain Res. 1643, 152–158. https://doi.org/10.1016/j.brainres.2016.04.073

do Carmo Silva, R.X., Lima-Maximino, M.G., Maximino, C., 2018a. The aversive brain system of teleosts: Implications for neuroscience and biological psychiatry. Neurosci. Biobehav. Rev. 95, 123–135. https://doi.org/10.1016/j.neubiorev.2018.10.001

746 do Carmo Silva, R.X., Rocha, S.P., Lima-Maximino, M.G., Maximino, C., 2018b. Extraction of
747 alarm substance in zebrafish. protocols.io. https://doi.org/10.17504/protocols.io.tr3em8n
748 Ghisleni, G., Capiotti, K.M., Da Silva, R.S., Oses, J.P., Piato, Â.L., Soares, V., Bogo, M.R.,
749 Bonan, C.D., 2012. The role of CRH in behavioral responses to acute restraint stress in
750 zebrafish. Prog. Neuropsychopharmacol. Biol. Psychiatry 36, 176–182.
751 https://doi.org/10.1016/j.pnpbp.2011.08.016
752 Gomes, F., Greidinger, M., Salviano, M., Couto, K.C., Scaperlli, G.F., Alves, S.H. de S., Cruz,
753 A.P. de M., 2010. Antidepressant- and anxiogenic-like effects of acute 5-HT2C receptor
754 activation in rats exposed to the forced swim test and elevated plus maze. Psychol. &
755 Neurosci. 3, 245–249. https://doi.org/10.3922/j.psns.2010.2.014
756 Graeff, F.G., 2004. Serotonin, the periaqueductal gray and panic. Neurosci. Biobehav. Rev. 28,
757 239–259. https://doi.org/10.1016/j.neubiorev.2003.12.004
758 Graeff, F.G., Brandão, M.L., Audi, E.A., Schütz, M.T.B., 1986. Modulation of the brain aversive
759 system by GABAergic and serotonergic mechanisms. Behav. Brain Res. 22, 173–180.
760 https://doi.org/10.1016/0166-4328(86)90038-0
761 Herculano, A.M., Maximino, C., 2014. Serotonergic modulation of zebrafish behavior: Towards a
762 paradox. Prog. Neuropsychopharmacol. Biol. Psychiatry, Special Issue: Zebrafish
763 models of brain disorders 55, 50–66. https://doi.org/10.1016/j.pnpbp.2014.03.008
764 Höglund, E., Weltzien, F.-A., Schjolden, J., Winberg, S., Ursin, H., Døving, K.B., 2005.
765 Avoidance behavior and brain monoamines in fish. Brain Res. 1032, 104–110.
766 https://doi.org/10.1016/j.brainres.2004.10.050
767 Kennett, G.A., Wood, M.D., Bright, F., Trail, B., Riley, G., Holland, V., Avenell, K.Y., Stean, T.,
768 Upton, N., Bromidge, S., Forbes, I.T., Brown, A.M., Middlemiss, D.N., Blackburn, T.P.,
769 1997. SB 242084, a Selective and Brain Penetrant 5-HT2C Receptor Antagonist.
770 Neuropharmacology 36, 609–620. https://doi.org/10.1016/S0028-3908(97)00038-5
771 Kinkel, M.D., Eames, S.C., Philipson, L.H., Prince, V.E., 2010. Intraperitoneal Injection into
772 Adult Zebrafish. JoVE J. Vis. Exp. e2126. https://doi.org/10.3791/2126
773 Knight, A.R., Misra, A., Quirk, K., Benwell, K., Revell, D., Kennett, G., Bickerdike, M., 2004.
774 Pharmacological characterisation of the agonist radioligand binding site of 5-HT2A, 5-
775 HT2B and 5-HT2C receptors. Naunyn. Schmiedebergs Arch. Pharmacol. 370, 114–123.
776 https://doi.org/10.1007/s00210-004-0951-4
777 Kuznetsova, E.G., Amstislavskaya, T.G., Shefer, E.A., Popova, N.K., 2006. Effect of 5-HT2C
778 receptor antagonist RS 102221 on mouse behavior. Bull. Exp. Biol. Med. 142, 76–79.

779 https://doi.org/10.1007/s10517-006-0296-8
780 Lima-Maximino, M., Pyterson, M.P., Silva, R.X. do C., Gomes, G.C.V., Rocha, S.P., Herculano,
781 A.M., Rosemberg, D.B., Maximino, C., 2020. Phasic and tonic serotonin modulate alarm
782 reactions and post-exposure behavior in zebrafish. J. Neurochem. 153, 495–509. https://
783 doi.org/10.1111/jnc.14978
784 Macedo, C.E., Martinez, R.C.R., Albrechet-Souza, L., Molina, V.A., Brandão, M.L., 2007. 5-
785 HT2- and D1-mechanisms of the basolateral nucleus of the amygdala enhance
786 conditioned fear and impair unconditioned fear. Behav. Brain Res. 177, 100–108. https://
787 doi.org/10.1016/j.bbr.2006.10.031
788 Martin, J.R., Ballard, T.M., Higgins, G.A., 2002. Influence of the 5-HT2C receptor antagonist,
789 SB-242084, in tests of anxiety. Pharmacol. Biochem. Behav., Serotonin: Pharmacology,
790 Biochemistry & Behaviour 71, 615–625. https://doi.org/10.1016/S0091-3057(01)00713-4
791 Mathuru, A.S., Kibat, C., Cheong, W.F., Shui, G., Wenk, M.R., Friedrich, R.W., Jesuthasan, S.,
792 2012. Chondroitin Fragments Are Odorants that Trigger Fear Behavior in Fish. Curr.
793 Biol. 22, 538–544. https://doi.org/10.1016/j.cub.2012.01.061
794 Matthews, M., Varga, Z.M., 2012. Anesthesia and Euthanasia in Zebrafish. ILAR J. 53, 192–
795 204. https://doi.org/10.1093/ilar.53.2.192
796 Maximino, C., do Carmo Silva, R.X., Campos, K. dos S., Oliveira, J.S. de, Rocha, S.P.,
797 Pyterson, M.P., Souza, D.P. dos S., Feitosa, L.M., Ikeda, S.R., Pimentel, A.F.N., Ramos,
798 P.N.F., Costa, B.P.D., Herculano, A.M., Rosemberg, D.B., Siqueira□Silva, D.H., Lima-
799 Maximino, M., 2019. Sensory ecology of ostariophysan alarm substances. J. Fish Biol.
800 95, 274–286. https://doi.org/10.1111/jfb.13844
801 Maximino, C., Lima, M.G., Costa, C.C., Guedes, I.M.L., Herculano, A.M., 2014. Fluoxetine and
802 WAY 100,635 dissociate increases in scototaxis and analgesia induced by conspecific
803 alarm substance in zebrafish (Danio rerio Hamilton 1822). Pharmacol. Biochem. Behav.
804 124, 425–433. https://doi.org/10.1016/j.pbb.2014.07.003
805 Mora, P.O., Netto, C.F., Graeff, F.G., 1997. Role of 5-HT2A and 5-HT2C Receptor Subtypes in
806 the Two Types of Fear Generated by the Elevated T-Maze. Pharmacol. Biochem.
807 Behav. 58, 1051–1057. https://doi.org/10.1016/S0091-3057(97)00057-9
808 Nathan, F.M., Ogawa, S., Parhar, I.S., 2015. Kisspeptin1 modulates odorant-evoked fear
809 response via two serotonin receptor subtypes (5-HT1A and 5-HT2) in zebrafish. J.
810 Neurochem. 133, 870–878. https://doi.org/10.1111/jnc.13105
811 Nunes-de-Souza, V., Nunes-de-Souza, R.L., Rodgers, R.J., Canto-de-Souza, A., 2008. 5-HT2

receptor activation in the midbrain periaqueductal grey (PAG) reduces anxiety-like behaviour in mice. Behav. Brain Res. 187, 72–79. https://doi.org/10.1016/j.bbr.2007.08.030

Oliveira, L.C., Broiz, A.C., de Macedo, C.E., Landeira-Fernandez, J., Brandão, M.L., 2007. 5-HT2 receptor mechanisms of the dorsal periaqueductal gray in the conditioned and unconditioned fear in rats. Psychopharmacology (Berl.) 191, 253–262. https://doi.org/10.1007/s00213-006-0653-3

Parra, K.V., Adrian, J.C., Gerlai, R., 2009. The synthetic substance hypoxanthine 3-N-oxide elicits alarm reactions in zebrafish (Danio rerio). Behav. Brain Res. 205, 336–341. https://doi.org/10.1016/j.bbr.2009.06.037

Perusini, J.N., Fanselow, M.S., 2015. Neurobehavioral perspectives on the distinction between fear and anxiety. Learn. Mem. 22, 417–425. https://doi.org/10.1101/lm.039180.115

Piato, A.L., Rosemberg, D.B., Capiotti, K.M., Siebel, A.M., Herrmann, A.P., Ghisleni, G., Vianna, M.R., Bogo, M.R., Lara, D.R., Bonan, C.D., 2011. Acute Restraint Stress in Zebrafish: Behavioral Parameters and Purinergic Signaling. Neurochem. Res. 36, 1876. https://doi.org/10.1007/s11064-011-0509-z

Quadros, V.A., Costa, F.V., Canzian, J., Nogueira, C.W., Rosemberg, D.B., 2018. Modulatory role of conspecific alarm substance on aggression and brain monoamine oxidase activity in two zebrafish populations. Prog. Neuropsychopharmacol. Biol. Psychiatry 86, 322–330. https://doi.org/10.1016/j.pnpbp.2018.03.018

Quadros, V.A., Silveira, A., Giuliani, G.S., Didonet, F., Silveira, A.S., Nunes, M.E., Silva, T.O., Loro, V.L., Rosemberg, D.B., 2016. Strain- and context-dependent behavioural responses of acute alarm substance exposure in zebrafish. Behav. Processes 122, 1–11. https://doi.org/10.1016/j.beproc.2015.10.014

Rosenzweig-Lipson, S., Zhang, J., Mazandarani, H., Harrison, B.L., Sabb, A., Sabalski, J., Stack, G., Welmaker, G., Barrett, J.E., Dunlop, J., 2006. Antiobesity-like effects of the 5-HT2C receptor agonist WAY-161503. Brain Res. 1073–1074, 240–251. https://doi.org/10.1016/j.brainres.2005.12.052

Salchner, P., Singewald, N., 2006. 5-HT receptor subtypes involved in the anxiogenic-like action and associated Fos response of acute fluoxetine treatment in rats. Psychopharmacology (Berl.) 185, 282–288. https://doi.org/10.1007/s00213-005-0247-5

Silva, P.I.M., Martins, C.I.M., Khan, U.W., Gjøen, H.M., Øverli, Ø., Höglund, E., 2015. Stress and fear responses in the teleost pallium. Physiol. Behav. 141, 17–22.

https://doi.org/10.1016/j.physbeh.2014.12.020

Silva, R.X. do C., Rocha, S.P., Herculano, A.M., Lima-Maximino, M.G., Maximino, C., 2020. Animal models for panic disorder. Psychol. Neurosci. 13, 1–18. https://doi.org/10.1037/pne0000177

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

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

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

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

Wolf, K., 1963. Physiological Salines for Fresh-Water Teleosts. Progress. Fish-Cult. 25, 135–140. https://doi.org/10.1577/1548-8659(1963)25[135:PSFFT]2.0.CO;2

Figure captions

Figure 1 – Experimental designs for (A) experiment 1 (CAS-elicited behavioral responses 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 during 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 after 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.

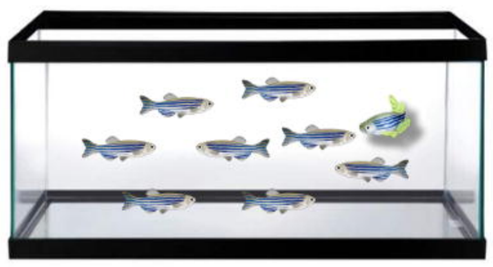
Figure 4 – Effects of WAY-161503 on behavior during CAS exposure. (A) Time spent on top third of the tank. (B) Time spent on bottom third of the tank. (C) Erratic swimming. (D) Total time spent freezing. (E) Swimming speed. Different letters represent statistical

892 differences at the $p < 0.05$ level; similar letters indicate lack of statistically significant
893 differences. Data are presented as individual data points (dots) superimposed over the
894 median ± interquartile ranges. Dashed lines on panels A and B represent change levels.
895 Dots connected by lines represent group means. CTRL = controls (water-exposed
896 animals); CAS = conspecific alarm substance. Final sample sizes: CTRL + VEH: n = 10
897 animals; CTRL + 1 mg/kg WAY-161503: n = 21 animals; CAS + VEH: n = 21 animals;
898 CAS + 1 mg/kg WAY-161503: n = 21 animals.
899 Figure 5 – Effects of WAY-161503 on behavior after CAS exposure. (A) Time spent on top third
900 of the tank. (B) Time spent on bottom third of the tank. (C) Erratic swimming. (D) Total
901 time spent freezing. (E) Swimming speed. Different letters represent statistical
902 differences at the $p < 0.05$ level; similar letters indicate lack of statistically significant
903 differences. Data are presented as individual data points (dots) superimposed over the
904 median ± interquartile ranges. Dashed lines on panels A and B represent change levels.
905 Dots connected by lines represent group means. CTRL = controls (water-exposed
906 animals); CAS = conspecific alarm substance. Final sample sizes: CTRL + VEH: n = 10
907 animals; CTRL + 1 mg/kg WAY-161503: n = 21 animals; CAS + VEH: n = 21 animals;
908 CAS + 1 mg/kg WAY-161503: n = 21 animals.
909 Figure 6 – Effects of RS-10221 on behavior during CAS exposure. (A) Time spent on top third of
910 the tank. (B) Time spent on bottom third of the tank. (C) Erratic swimming. (D) Total time
911 spent freezing. (E) Swimming speed. Different letters represent statistical differences at
912 the $p < 0.05$ level; similar letters indicate lack of statistically significant differences. Data
913 are presented as individual data points (dots) superimposed over the median ±
914 interquartile ranges. Dashed lines on panels A and B represent change levels. Dots
915 connected by lines represent group means. CTRL = controls (water-exposed animals);
916 CAS = conspecific alarm substance. Final sample sizes: CTRL + VEH: n = 10 animals;
917 CTRL + 2 mg/kg RS-10221: n = 21 animals; CAS + VEH: n = 20 animals; CAS + 2
918 mg/kg RS-10221: n = 21 animals.
919 Figure 7 – Effects of RS-10221 on behavior after CAS exposure. (A) Time spent on top third of
920 the tank. (B) Time spent on bottom third of the tank. (C) Erratic swimming. (D) Total time
921 spent freezing. (E) Swimming speed. Different letters represent statistical differences at
922 the $p < 0.05$ level; similar letters indicate lack of statistically significant differences. Data
923 are presented as individual data points (dots) superimposed over the median ±
924 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.

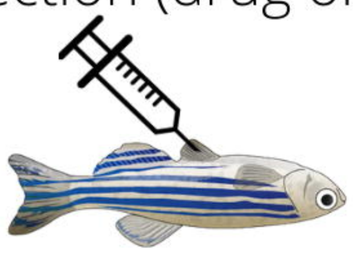
A



Quarantine (>14d)

20 min

Injection (drug or VEH)

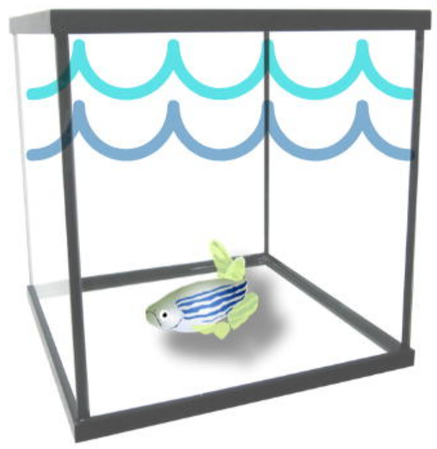


CAS exposure

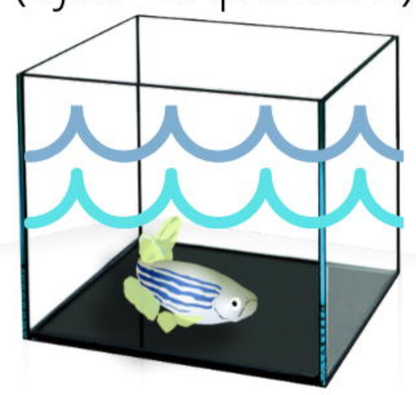
6 min

Washout (1 min)

Behavioral observation (*during* exposure)



Behavioral observation (*after* exposure)



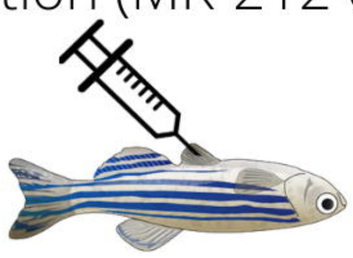
6 min

B

Quarantine (>14d)

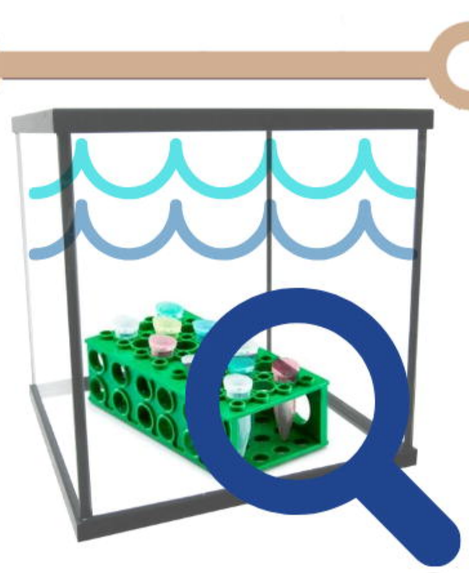
20 min

Injection (MK-212 or VEH)

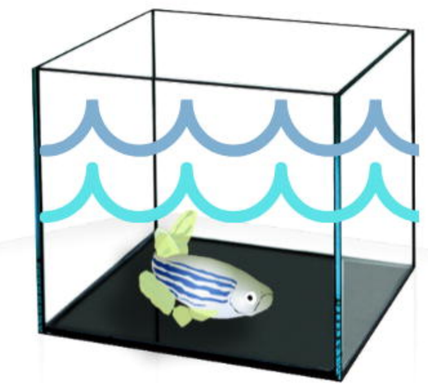


ARS

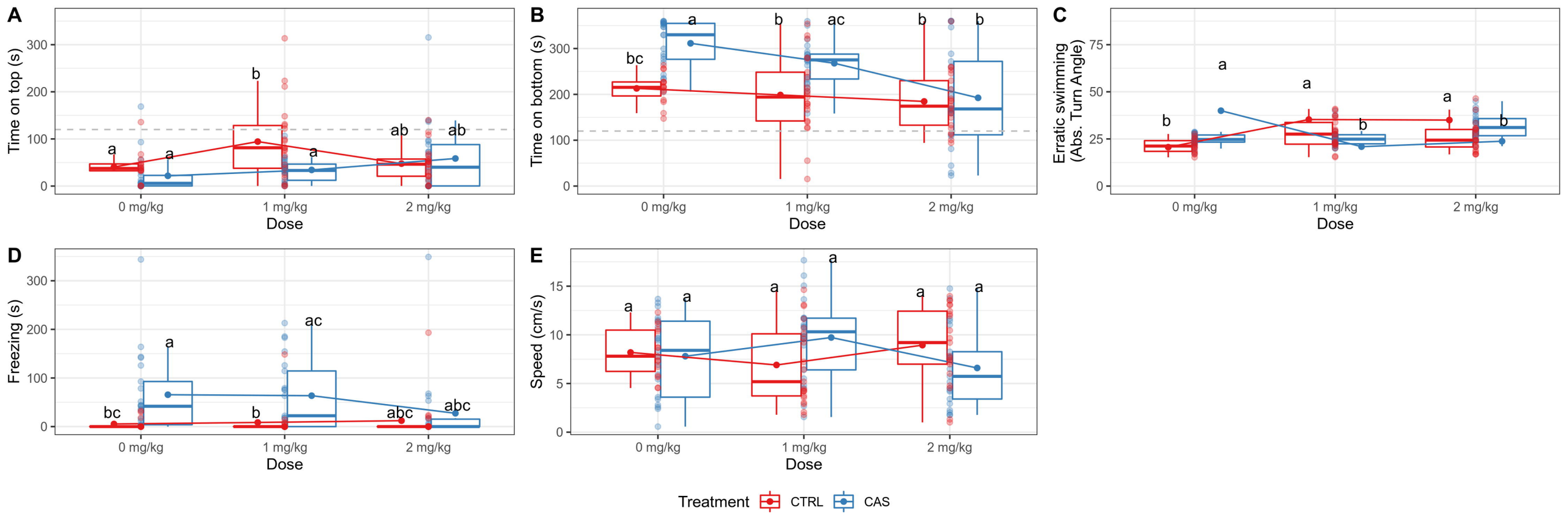
90 min



Behavioral observation (*after* exposure)



6 min


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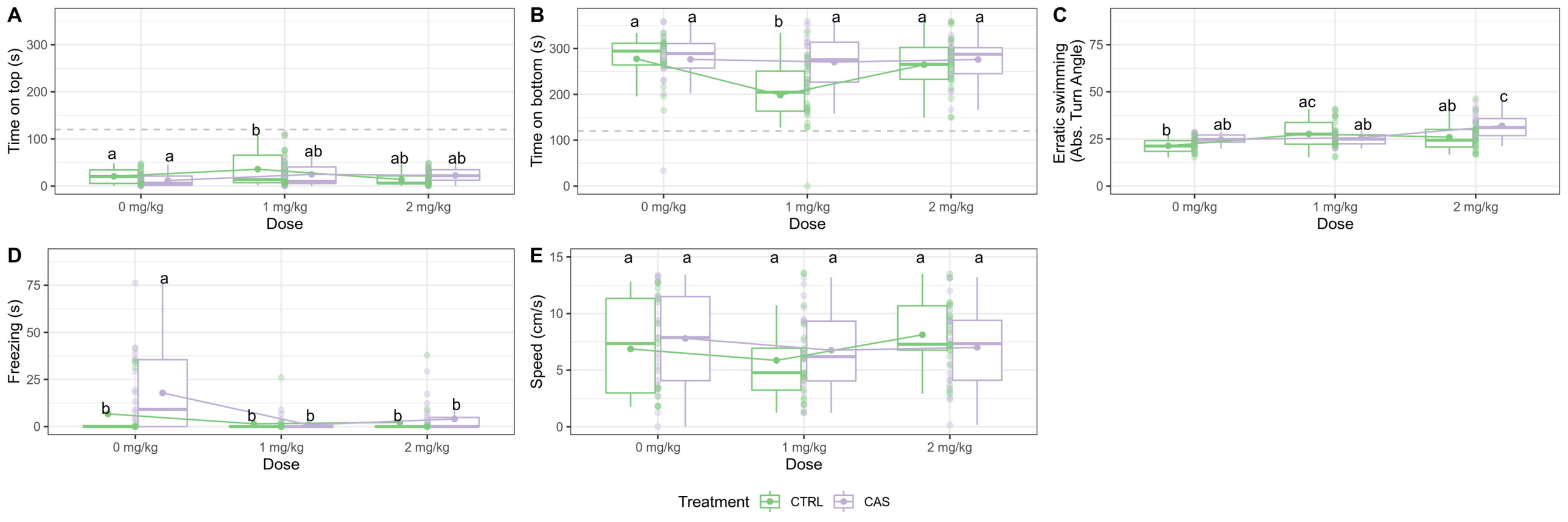
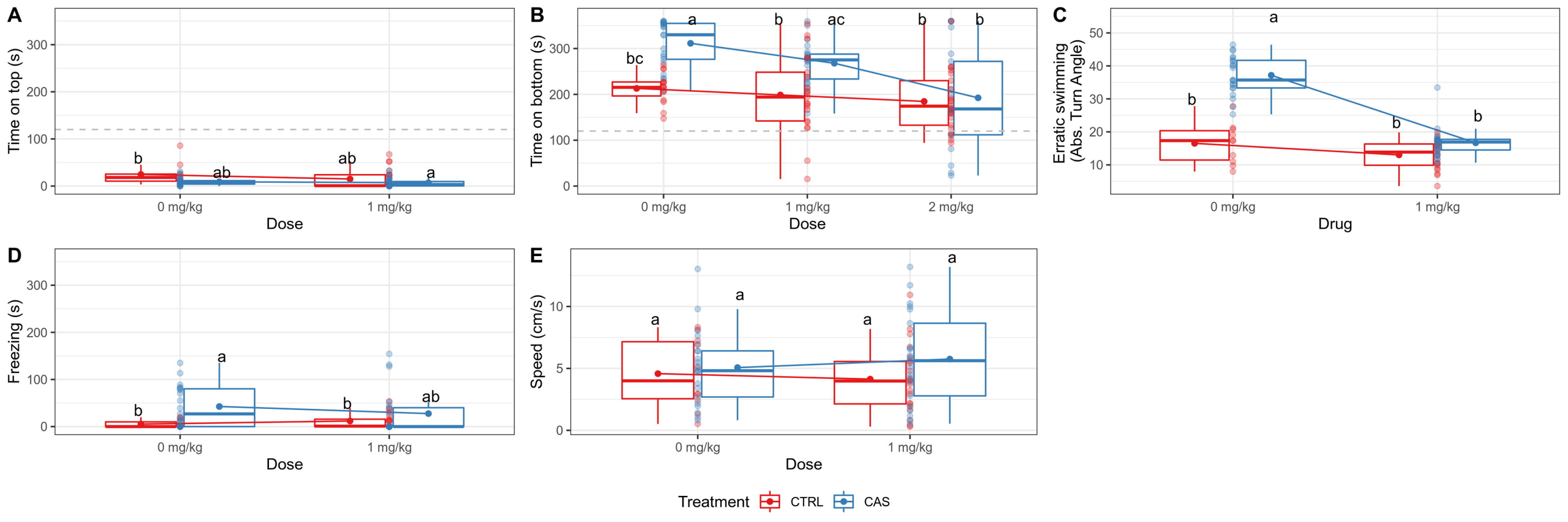
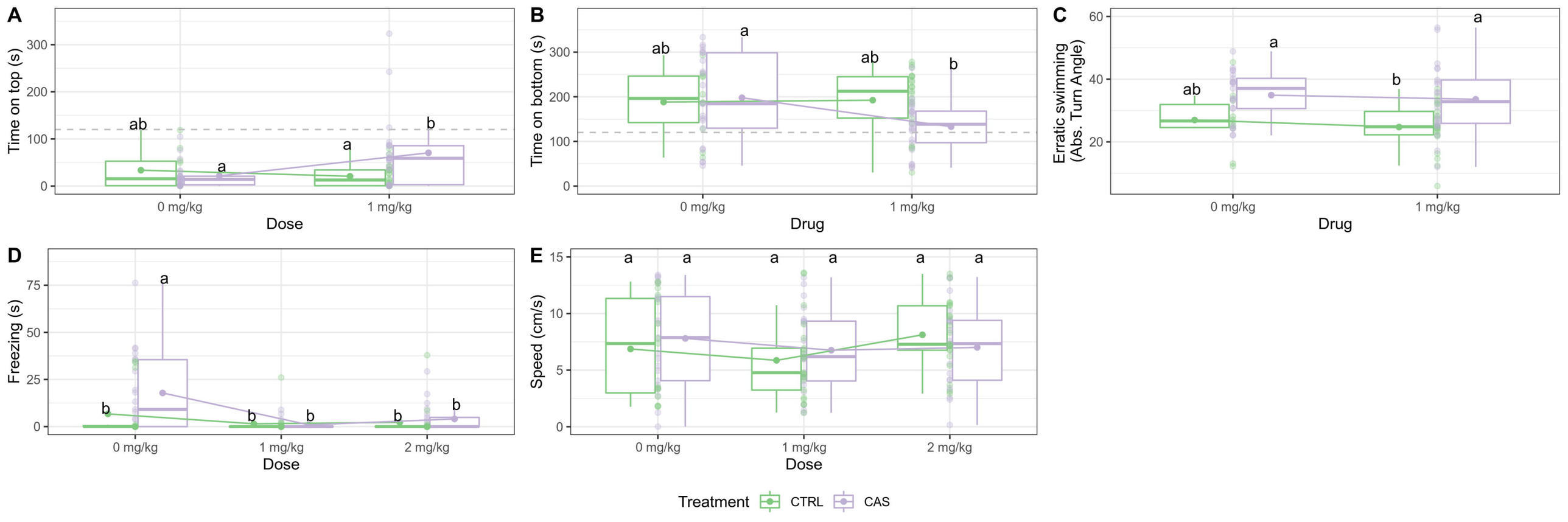
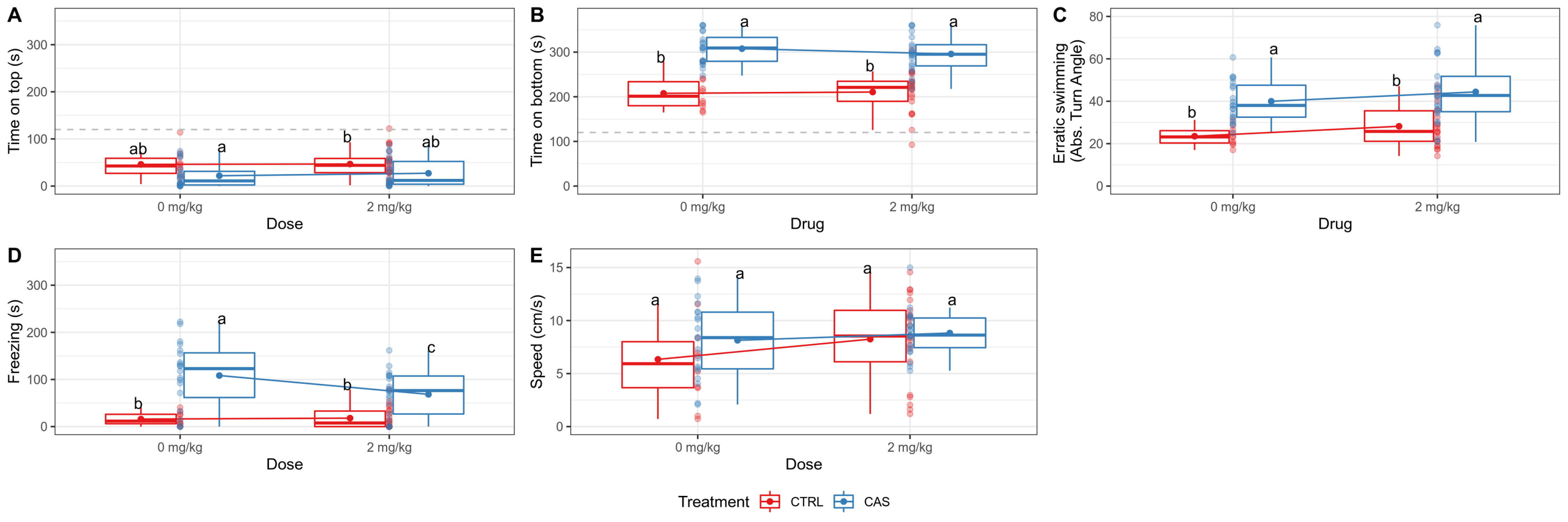
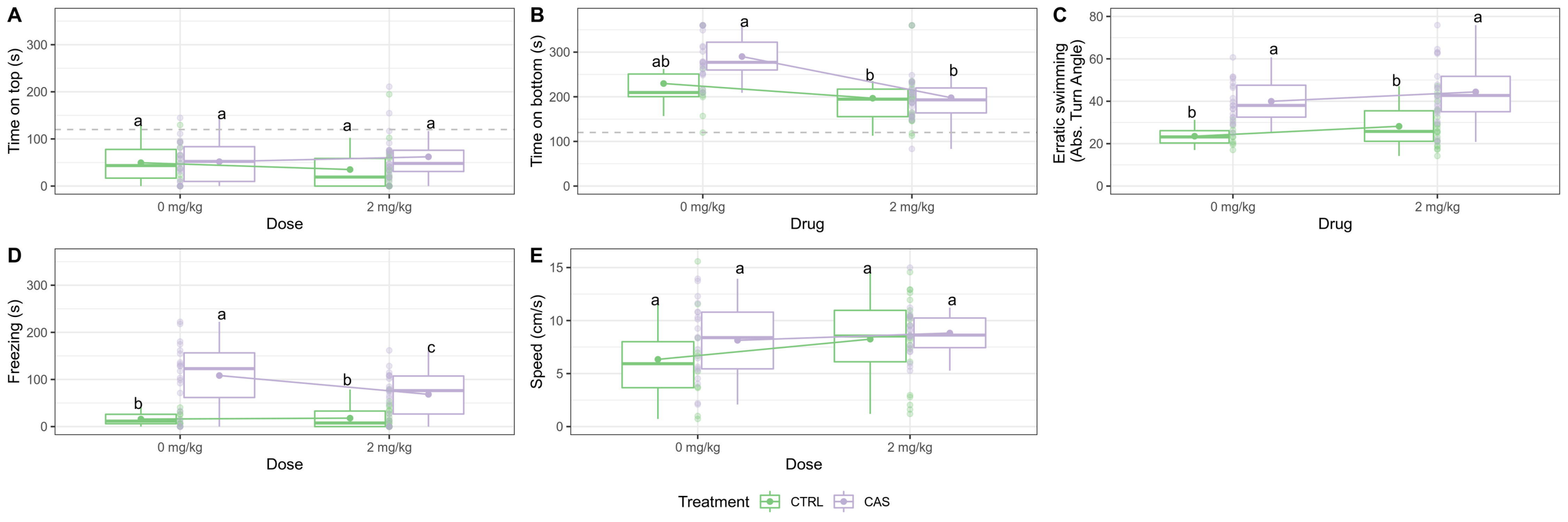
0 mg/kg
1 mg/kg
2 mg/kg
Dose
B
Time on bottom (s)
300
200
100
0
bc
a
b
ac
b
b
0 mg/kg
1 mg/kg
2 mg/kg
Dose
C
Erratic swimming (Abs. Turn Angle)
75
50
25
0
b
a
a
b
a
b
0 mg/kg
1 mg/kg
2 mg/kg
Dose
D
Freezing (s)
300
200
100
0
bc
a
b
ac
abc
abc
0 mg/kg
1 mg/kg
2 mg/kg
Dose
E
Speed (cm/s)
15
10
5
0
a
a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

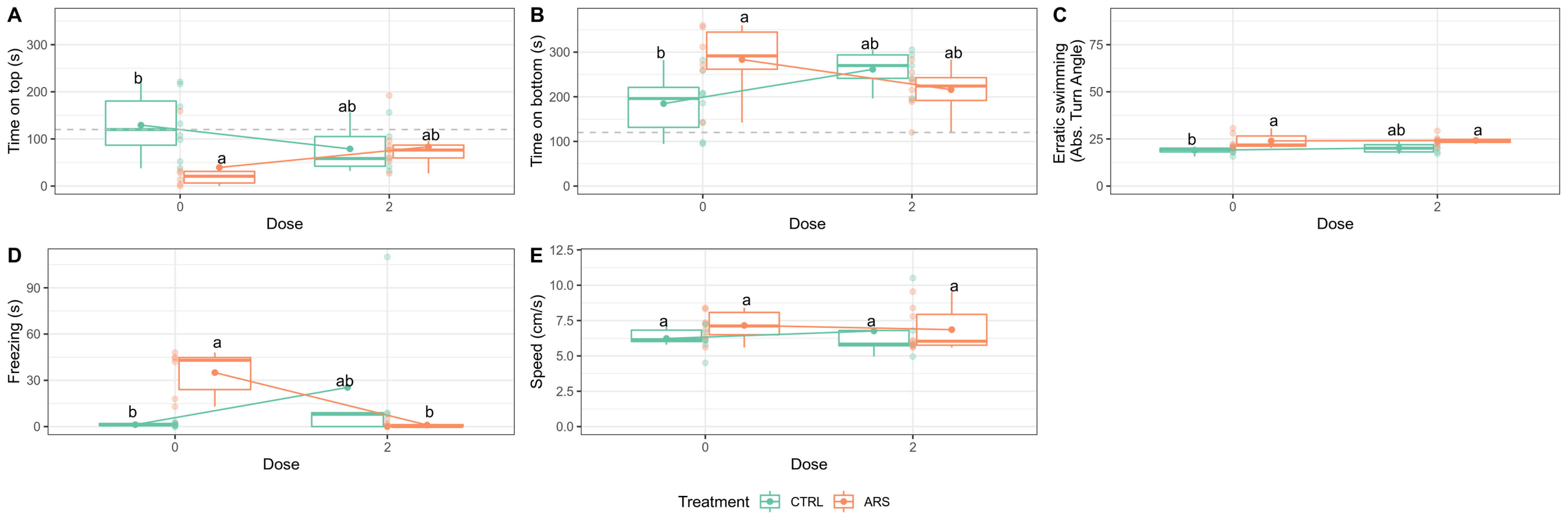

A
Time on top (s)
300
200
100
0
a
a
b
ab
ab
ab
0 mg/kg
1 mg/kg
2 mg/kg
Dose
B
Time on bottom (s)
300
200
100
0
a
a
b
a
a
a
0 mg/kg
1 mg/kg
2 mg/kg
Dose
C
Erratic swimming (Abs. Turn Angle)
75
50
25
0
b
ab
ac
ab
ab
c
0 mg/kg
1 mg/kg
2 mg/kg
Dose
D
Freezing (s)
75
50
25
0
b
a
b
b
b
b
0 mg/kg
1 mg/kg
2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