A high-throughput test of anxiety for zebrafish (*Danio rerio*) larvae: the larval diving response (LDR)

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Abstract

A high level of anxiety is a characteristic symptom across the anxiety spectrum disorders and several other affective disorders, and is often detected early in a child's development. Zebrafish are widely used in translational studies for drug discovery in anxiety research, where the species' naturalistic diving response to a new environment is a reliable and validated marker for anxiety-like behavior. Here, we present for the first time a method for examining diving behavior in zebrafish larvae to assess anxiety-like behaviors at early-stages (7 days-post-fertilization). Anxiogenic (caffeine 100 mg/L) and anxiolytic (diazepam 5 mg/L) drugs were used to pharmacologically validate the protocol. Larvae were pretreated in drug (or fresh water) for 30-min, then transferred to cuvettes containing fresh water and habituated for a further 30-min. Larval diving response (LDR) was then recorded using an automated system (Zantiks) for 30-min. As with adult fish, diazepam was anxiolytic, significantly reducing the time spent in bottom of the novel environment, and increasing the time spent in the top. Meanwhile, caffeine induced anxiogenic-like effects by increasing the time spent in the bottom zone of the environment. This new automated and high-throughput screening tool has the potential use for screening of anxiogenic and anxiolytic compounds, and for studies aiming to understand the mechanisms underlying affective disorders.

Keywords: psychopharmacology; larval diving response; LDR; drug discovery; novel tank; zebrafish

1. Introduction

Anxiety exists either as a state (fear or nervousness, typically associated with a negative effect) or as a trait, in which anxiety proneness is stable across the lifespan(Hovenkamp-Hermelink, Jeronimus, Myroniuk, Riese, & Schoevers, 2021). Trait anxiety can reach clinical levels, and represents a transdiagnostic trait observed across many affective disorders (Demetriou et al., 2021; Newby, McKinnon, Kuyken, Gilbody, & Dalgleish, 2015) and across the lifespan (Beesdo, Knappe, & Pine, 2009). The emergence of anxiety traits can occur from as young as 6 years old, and can lead to an increased risk of developing subsequent mood disorders, substance misuse, disruptive behaviors, suicidal behavior, educational underachievement, and later adult economic disadvantage (Asselmann, Wittchen, Lieb, & Beesdo-Baum, 2018). Despite pathological anxiety being common -- up to 33.7% of people are affected by anxiety disorders during their life time (Bandelow & Michaelis, 2015) -- the available drugs for treating anxiety are only palliative (i.e. not curative), and are associated with several unwanted side effects, such as fatigue, restlessness, sexual dysfunctions and abuse liability (Ator, 2005; Bandelow, Michaelis, & Wedekind, 2017). It is therefore crucial to develop treatments high in efficacy and without significant side effects, and animal models are an essential vehicle for progressing this.

Zebrafish (*Danio rerio)* has been widely used in translational neuroscience, and several behavioral tasks have been developed to better understand anxiety-like behavior (Cachat et al., 2010; Maximino et al., 2010; Stewart, Gaikwad, Kyzar, & Kalueff, 2012). Its unique characteristics as a vertebrate, such as external fertilization, high fecundity, low cost, behavioral complexity and embryo transparency, have collectively put this species center stage as a model for psychiatric drug discovery model across developmental stages (Cassar et al., 2020). Zebrafish naturally display anxiety upon introduction to a novel environment, and novel tank tests (NTT) have been commonly used to evaluate locomotion and anxiety-like behavior by exploiting the fish's natural diving response in response to a novel environment, thus showing good face validity

(Blaser & Rosemberg, 2012; Levin, Bencan, & Cerutti, 2007). These tasks also have construct validity with both anxiolytic and anxiogenic drugs reducing and exacerbating the responses, respectively (Egan et al., 2009; Parker, Millington, Combe, & Brennan, 2012; Rosemberg et al., 2012). However, although the NTT has high reproducibility and easy validation in different laboratorial conditions (low-cost and maintenance task), its use has thus far been limited to adult zebrafish, and little is known about the diving response of younger animals.

The development of behavioral techniques that can reliably assess anxiety levels at early-stages could help further understand mechanisms associated with anxiety in childhood and allow the development of drugs that might help to target this. Thus, in this preregistered study, we designed a new high-throughput test designed for zebrafish larvae (the larval diving response [LDR], carried out at 7 days post-fertilization (dpf)). The test was further validated by using anxiolytic and anxiogenic drugs (diazepam and caffeine, respectively). Both diazepam and caffeine are widely used to evaluate anxiety levels in adults and zebrafish larvae (Bencan, Sledge, & Levin, 2009; Maeda, Hasumi, & Yoshida, 2021; Richendrfer, Pelkowski, Colwill, & Creton, 2012; Rosa et al., 2018; Tran et al., 2017).

We had two primary pre-registered (https://osf.io/sy9bf/) hypotheses: 1) Time spent in the upper third of the apparatus will be increased following pre-treatment by an anxiolytic drug (diazepam); 2) Time spent in the upper third of the apparatus will be decreased by pre-treatment by an anxiogenic drug (caffeine). In addition, we sought to examine the best conditions for inducing anxiety responses in the larvae. To do this, we first characterized the wild-type behavioral responses over a 2-h period and selected the times of maximum activity.

2. Material and Methods

2.1. Animal husbandry and experimental design

Larval AB wild-type zebrafish (total n = 300 in which n = 60 larvae were used for pilot experiments) were bred in-house using a marble system from multiple tanks (4 tanks/breeding). After egg collection, groups of ~50 embryos were randomly assigned to petri dishes (100 x 15

mm) and reared up to 7 dpf. Larvae were fed daily from day 5 (ZM000, ZM Fish Food & Equipment, Winchester, UK). Fish were kept on a 14/10-hour light/dark cycle (lights on at 9:00 a.m.; pH 8.4; 28 °C (±1 °C)). Behavioral experiments were performed between 10:00 and 16:00 h. Prior to analysis, data from 4 animals were removed due to tracking errors. We performed a power analysis (G* Power) based on pilot data and found an effect size of $\eta_p^2 = .95$ ($f = 4.4$). With power = .8 ($p = 0.05$) we required 18 zebrafish larvae per condition. As our aim here was to develop a protocol, to ensure data reliability, we ran four independent batches ($n = 20$ per group in each batch). All behavioral testing was carried out in a fully randomized order, choosing fish at random from one of six housing dishes, and subsequently from four groups of petri dish treatments.

2.2 Materials

At 7dpf, the LDR was tested using Zantiks AD automated tracking systems (Zantiks Ltd., Cambridge, UK). Caffeine (100 mg/L; Sigma Aldrich, UK) and Diazepam (5 mg/L; Sigma Aldrich, UK) were used as anxiolytic and anxiogenic drugs, respectively (doses based on previous research from our lab and others) (Bencan et al., 2009; Egan et al., 2009; Rosa et al., 2018). In adult zebrafish, the NTT is used to measure exploration, locomotion, boldness and anxiety-like behavior (Egan et al., 2009; Kalueff et al., 2013; Levin et al., 2007; Wong et al., 2010). For this experiment, zebrafish larvae (7 dpf) were individually pipetted into standard cuvettes (45 mm height x 12.5 mm depth x 12.5 mm width) containing 3.5 mL of aquarium water (**Fig. 1A**). The behavioral activity was analyzed using the Zantiks LT system (Zantiks Ltd., Cambridge, UK).

2.3. Larval Diving Response (LDR)

Zebrafish behavior in the tank diving response is typically analyzed for 5-6 min (Egan et al., 2009; Parker et al., 2012; Rosemberg et al., 2012). Here larvae were incubated in drug solution (or fresh aquarium water) for 30 min in groups of $n = 5$ per petri-dish. They were then added to cuvettes for habituation in fresh water (see below) for 30-min, after which they were filmed for

their responses for a further 30 minutes (**Fig. 1B**). These timings were determined following pilot experiments, where untreated larvae (7dpf) showed a significant increase in movement patterns only after 30 min of habituation ($p^* = 0.0309$), and this formed a fundamental step when looking at diving response of zebrafish larvae (**Fig. 1C**). Diazepam also showed a significant effect only when comparing the data within groups at the time-period of 30 – 60 min ($p^* = 0.0257$) and the caffeine effect was significantly increased when comparing 0 – 30 *vs.* 30 – 60 min ($p^* = 0.0267$) (**Fig. 1D**). The tank was separated into three virtual areas (bottom, middle and top) to provide a detailed evaluation of vertical activity and total distance traveled (mm), number of entries, and time spent (s) in each of the tank zones. Drug response across time was also analyzed by looking at time spent in each zone across the full duration of the test (30 min).

2.4. Statistics

Normality of data and homogeneity of variance were analyzed by Kolmogorov–Smirnov and Bartlett's tests, respectively. In the pilot study, T-test was performed to compare distance traveled and time spent in the bottom during preliminary observations, comparing 0 – 30 min *vs.* 30 – 60 min. The analysis of how 0 – 30 *vs.* 30 – 60 min of test affect drug response was also performed in the pilot experiments by using two-way (2 x 3) ANOVA as a function of time-period (0 – 30 min *vs.* 30 – 60 min) and treatment (control, 5mg/L diazepam and 100mg/L caffeine). In the full experiment, one-way analysis of variance (ANOVA) with treatment as an independent variable (three levels – control *vs.* caffeine *vs.* diazepam) was used to analyze distance travelled. Two-way (3 x 3) ANOVA was used to analyze the average number of entries and average time spent in each zone as a function of treatment (three levels – control *vs.* caffeine *vs.* diazepam) and zone (three levels – bottom *vs.* middle *vs.* top) as independent variables. Two-way repeated measures ANOVA was used for analysis across time using time (29 levels: 29 x 1 min time bins) and treatment (control *vs.* caffeine *vs.* diazepam) as factors. Tukey's test was used as post-hoc analysis, data was represented as mean and error of the mean (SEM), and results were considered significant when $p \leq 0.05$. Because both drugs did not affect zebrafish larvae locomotion, we have

used ANOVA rather than ANCOVA (with distance traveled as covariate) as initially planned in the preregistration of this study.

3. Results

3.1. Pilot study

In **Fig. 1C** a significant increase in the distance travelled was found for control animals when comparing initial 0 – 30 min time-period *vs.* 30 – 60 min ($F_{(15, 15)} = 2.567$, $p^* = 0.0309$). Although time spent in the bottom was not significant for controls when comparing time periods (**Fig. 1C**; $F_{(15, 15)} = 1.448$, $p = 0.0555$), when looking at the pilot data comparing time-period and treatments (**Fig. 1D**), a significant effect for time-period ($F_{(1, 108)} = 6.998$, $p^{**} = 0.0094$) and treatment ($F_{(2, 108)} = 11.76$, $p < 0.0001$) was found. No interaction effect between time-period and treatment as factors was observed ($F_{(2, 108)} = 2.784$, $p = 0.0662$). Tukey`s post-hoc analysis yielded a significant difference for diazepam exposed larvae when compared to controls at 30 – 60 min ($p^* = 0.0257$). Meanwhile, no effect is observed for the diazepam exposed larvae at 0 – 30 min compared to the control group 0 – 30 min ($p = 0.8222$). Caffeine effect was also higher at 30 – 60 min, where a significant increase between caffeine 0 -30 min *vs.* caffeine 30 – 60 min was observed ($p^* = 0.0276$).

3.2. Zebrafish larvae (7dpf) show anxiety responses which are validated by exposure to anxiolytic and anxiogenic drugs

In **Fig.2** the behavioral patterns of zebrafish larvae were investigated when exposed to anxiolytic (diazepam) and anxiogenic (caffeine) drugs. For time spent in each zone, a significant interaction between treatment and zone ($F_{(4,711)} = 27.85$, $p^{****} < 0.0001$) and a significant zone effect ($F_{(3,711)} = 74.83$, $p^{****} < 0.0001$) were found. No significant main effect was observed for treatment ($F_{(2,711)} = 0.1228$, $p = 0.8845$). Briefly, Tukey's *pos hoc* test showed a significant difference between time spent in bottom for controls when compared to diazepam ($p^{**} = 0.0081$) and caffeine ($p^* = 0.0115$). Significant differences for time spent in the top zone were only observed for controls *x* diazepam ($p^{****} < 0.0001$) and no significant differences were observed

for control *x* caffeine for time spent in the top zone (p = 0.7157). No significant differences were observed for time spent in the middle when comparing caffeine (p = 0.3307) and diazepam (p = 0.300) to control group (**Fig. 2B**). One-way ANOVA yielded no significant changes when comparing different treatments for larvae distance travelled ($F_{(2,237)}$ = 0.1992, p = 0.8195)(**Fig. 2A**). When looking at number of entries (mean entries per minute) at each third of the tank, no significant effect was observed for interaction (treatment*zone; $F_{(4,711)}$ = 0.5241, p = 0.7181) and for zone effect ($F_{(2,711)}$ = 0.7796, p = 0.4590). A significant treatment effect ($F_{(2,711)}$ = 3.386, p^* = 0.0344) was found for the number of entries per zone, however *post hoc* test showed no difference among groups.

3.3. Larvae diving response is consistent across time and changes depending on drug exposure

Fig. 3 shows zebrafish larvae response to different anxiogenic/anxiolytic drugs across time in a diving response task. Two-way RM ANOVA yielded a significant effect for interaction between factors (treatment*group; $F_{(56, 6636)}$ = 1.386, p^* = 0.0302), time ($F_{(28, 6636)}$ = 10.11, p^{****} < 0.0001), group ($F_{(2, 237)}$ = 20.14, p^{****} < 0.0001) and individuals ($F_{(237, 6636)}$ = 44.32, p^{****} < 0.0001). Briefly, analysis across time showed that control animals increased the time spent in the bottom across time when comparing 27, 28 and 29 min to the first minute of the test (p^* = 0.0144, p^{**} = 0.0093 and p^* = 0.0334, respectively). Diazepam did not affect time spent in bottom across time: no significant effect was observed between any of the time bins (p's > 0.05). Animals exposed to caffeine for 30 minutes showed a significant increase in the time spent in the bottom zone from 5 minutes compared to the first minute of test (p^{**} = 0.0066), where from time bin 12 to 29 a minimum p effect observed was p^{***} < 0.001. No significant effect was observed for caffeine time bin 1 *vs.* 2 (p = 0.9207), 3 (p = 0.1922) and 4 (p = 0.1710). When comparing groups, diazepam had a significant decrease on the time spent in bottom starting at 2 minutes of behavioral recording (p^* = 0.0123) and, peaked at 4 min (p^{**} = 0.0012), 9 min (p^{**} = 0.0025) and 28 min (p^{**} = 0.0071). Caffeine increasing time spent in bottom started at 6 min (p^* =

0.0165) reaching the highest difference at 24 min (p^{***} = 0.0006). The overall difference between groups was described in the previous section (**Fig. 3**).

Two-way RM ANOVA showed no significant interaction (treatment*time; $F_{(56, 6636)}$ = 0.8912, p = 0.7030) effect. However, a significant time ($F_{(28, 6636)}$ = 1.577, p^{*} = 0.0271), treatment ($F_{(2, 237)}$ = 21.01, p^{****} < 0.0001) and between individuals ($F_{(237, 6636)}$ = 11.27, p^{****} < 0.0001) effect was observed. No effect was observed after the Tukey`s *pos hoc* analysis for time spent in the middle zone across time. Lastly, although no significant interaction between treatment and time was observed for time spent in the top zone ($F_{(56, 6636)}$ = 0.9063, p = 0.6726), a significant effect was seen for time ($F_{(28, 6636)}$ = 8.749, p^{****} < 0.0001), treatment ($F_{(2, 237)}$ = 17.03, p^{****} < 0.0001) and between individuals ($F_{(237, 6636)}$ = 48.25, p^{****} < 0.0001). Control animals did not show any differences in the time spent in the top zone across time, however diazepam exposed animals showed a significant increase in the time spent in the top zone from 6 min (*vs.* 1 min; p^{**} = 0.0077) with a minimum p value of *** < 0.001 from 7 – 29 min time bins. Meanwhile, caffeine exposed larvae showed a significant decrease in the time spent in the top zone comparing 1 *vs.* 7 (p^{*} = 0.0138) to 29 (1 *vs.* 29; p^{**} = 0.0013), except for time bin 14 (p = 1178), 24 (p = 0.0681) and 26 (p = 0.0534). When comparing difference between groups, diazepam significantly increased time spent in the bottom compared to controls at 4 min (p^{*} = 0.0125) peaking at 15 min (p^{****} < 0.0001) and 27 min (p^{****} < 0.0001). Caffeine did now show any differences compared to control when performing caffeine *vs.* controls analysis in specific time points (**Fig 3**).

4. Discussion

Here, we have developed an automated method to evaluate anxiety-related behaviors in 7dpf zebrafish larvae (the Larval Diving Response: LDR). This task has good face validity, exploiting the naturalistic diving response of zebrafish. The task also has construct validity, as larvae exposed to 5mg/L diazepam (anxiolytic) show decreased anxiety-like behavior, and larvae exposed to 100mg/L caffeine (anxiogenic) show increased anxiety-like behavior while, critically, neither of the drugs affected locomotion. This is, to our knowledge, the first time that diving

responses have been evaluated, quantified, and validated in zebrafish larvae, and we have demonstrated this using a fully automated, commercially available system that will allow scientists to efficiently perform high-throughput screening using larvae at an early stage of 7 dpf.

Anxiety and locomotion are often studied in zebrafish larvae. The open field test is one of the most commonly used methods, where researchers assess different parameters by placing animals in multi-well plates and recording their movements for 5 - 10 min (Ingebretson & Masino, 2013; Lange et al., 2013; Lange et al., 2012; MacPhail et al., 2009; Ulhaq, Orn, Carlsson, Morrison, & Norrgren, 2013). The locomotor patterns studied in multi-well plates usually aim to investigate the total distance swum, meanwhile the time spent close to the walls (thigmotaxis) is used as a parameter to measure anxiety-related behavior (Kalueff et al., 2013; Stewart et al., 2012). It could be argued, however, that thigmotaxis as a measure of anxiety in fish lacks face validity: '*wall hugging*' as a protective behavior is hard to envisage in a fishes' natural environment(Kallai et al., 2007; Simon, Dupuis, & Costentin, 1994; Walz, Mühlberger, & Pauli, 2016). In addition, despite several researchers using thigmotaxis in a multi-well plate to investigate anxiety-related behaviors in larvae, this behavioral endpoint has been criticized as being a poor predictor of anxiety in adult zebrafish (Blaser, Chadwick, & McGinnis, 2010). For example, adult zebrafish confined in an avoidance chamber (white or black) display increased anxiety-related behaviors, but thigmotaxis and locomotor behavior were not correlated to fish avoidance (Blaser et al., 2010). Furthermore, caffeine at 50 and 100 mg/L significantly decreased time spent in the top of the NTT, indicating that those doses are anxiogenic, but thigmotaxis at both doses was not affected (Rosa et al., 2018). These data further demonstrate that thigmotaxis may not be a reliable parameter when looking at anxiety-like behaviors and that the use of complementary analysis, such as diving response may help researchers to better understand the effects of anxiogenic and anxiolytic drugs.

Zebrafish larvae develop excellent vision at 5 dpf (Neuhauss, 2003), displaying a robust set of behaviors already at the end of the first week of development (5 – 7 dpf) (Dadda, Domenichini, Piffer, Argenton, & Bisazza, 2010; Facchin, Argenton, & Bisazza, 2008). Zebrafish

larvae were previously shown to increase bottom dwelling (diving behavior) as an escape response to light stimulus, meanwhile, olfactory stimuli do not alter diving responses (Bishop, Spence-Chorman, & Gahtan, 2016). These data, therefore, suggest that the increased diving response to a threatful light-evoked stimulus may be an innate defense of zebrafish larvae against predation, and increases in bottom dwelling could indicate an anxiogenic response. Indeed, in the classical (adult) NTT, zebrafish spend most of their time at the bottom of the tank and gradually explore the top zone of the tank (Blaser & Rosemberg, 2012; Egan et al., 2009; Mezzomo, Silveira, Giuliani, Quadros, & Rosemberg, 2016; Rosemberg et al., 2012; Wong et al., 2010). Here, we aimed to investigate and fully characterize the LDR, we first analyzed larval behavioral activity across 60 min of free exploration (no drugs) and found that activity significantly increases following 30 min of habituation to the tank. This habituation to the environment therefore appears to be a necessary step when analyzing LDR. Moreover, once animals are moved to the test tank, the drug effect of caffeine and diazepam are higher after those 30 mins of habituation. Although in adults this habituation time eliminates the novel factor as a stressor, we have found the diving responses in larvae appear later (after 30-min habituation) and remain stable for ~30 min and validated this with anxiogenic (caffeine at 100 mg/L) and anxiolytic (diazepam at 5 mg/L) drugs: diazepam significantly decreases time spent in the bottom of the tank and increases time spent in the top zone, while caffeine increases the time that larvae spend in the bottom of the tank during the behavioral testing time.

Diazepam is a benzodiazepine that plays a major role in the GABAergic system, causing anxiolytic and sedative effects (Briley & Langer, 1978; Calcaterra & Barrow, 2014). In adult zebrafish, diazepam is known for decreasing time spent in the bottom zone where low doses of 1.2 and 5 mg/L induce an anxiolytic effect (Bencan et al., 2009). Importantly, depending on the dose, diazepam can play an inhibitory effect on zebrafish locomotor activity (Tran et al., 2017). Caffeine is also often used for the validation of novel anxiety-related tasks being an agent that induces anxiogenic effects through its antagonistic effect on adenosine receptors (Alsene, Deckert, Sand, & de Wit, 2003; van Calker, Biber, Domschke, & Serchov, 2019). Similar to diazepam,

caffeine is also known for modulating anxiety-like behavior depending on dose, where higher doses were shown to significantly affect zebrafish locomotor activity following a 3D analysis of zebrafish behavior in a novel environment (Rosa et al., 2018). Here, both diazepam and caffeine did not affect larvae distance traveled, indicating that the concentrations used for this experiment lead to anxiolytic and anxiogenic effects without affecting locomotion, supporting the relevance of diving response at early-stages for the study of anxiety-related behaviors.

The use of zebrafish earlier in the lifespan has several advantages, both practically and ethically. Practically, their small size means the larvae can be screened in large numbers increasing the throughput, and the short rearing period means that multiple generations can be screened in a short period of time. As well as throughput, this has significant cost implications, with animals only being grown to a very young age therefore not requiring food and tank space. Ethically, only growing fish to a young (juvenile) age means that they are not kept in laboratory conditions beyond a few days old, thus reducing the cumulative life experience of the animals(Honess & Wolfensohn, 2010; Mendl, Burman, & Paul, 2010).

One potential limitation of this task is that, in adult zebrafish, the critical factor in the NTT is the novelty of the environment. Specifically, when introduced to a novel environment, there is an increase in stress-related physiological and behavioral markers (Cachat et al., 2010; Kysil et al., 2017). Although here, the zebrafish larvae went through a 30 min habituation, zebrafish larvae displayed significantly different diving responses when exposed to pharmacological agents such as caffeine and diazepam. In fact, caffeine only started to show a significant increase in the time spent in the bottom zone from 6 min, reaching its highest effect at 24 min. Meanwhile, diazepam effects also increased across time where higher significant values can be observed after 6 – 7 min of the behavioral testing. It should be noted that this was following a 30-min treatment incubation, suggesting that the pharmacodynamics in larvae may be an important consideration here.

5. Conclusion

In summary, here we have characterized a new test to evaluate anxiety-like behavior in zebrafish larvae considering the species naturalistic diving response when facing potential threats. The method was validated using anxiogenic and anxiolytic drugs, where 5 mg/L diazepam reduced, and 100 mg/L caffeine increased, anxiety-like behaviors in 7dpf zebrafish. Abnormal anxiety levels have been detected in childhood, and this new task may be used as a translational tool to better understand anxiety at early-life stages. Moreover, coupled with the numerous advantages of using zebrafish larvae in research, this new fully-automated, commercially available, high-throughput screening method could accelerate the discovery of new drugs for treating neuropsychiatric disorders that affect anxiety.

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Author's contributions

BDF and MOP designed the experimental procedures; MOP contributed for reagents/analysis tools; MOP performed the experiment; BDF and MOP analyzed the data and contributed for the manuscript writing.

Declarations

Ethics Approval

The protocols described here are part of a preregistered study. All experiments were carried out following approval from the University of Portsmouth Animal Welfare and Ethical

Review Board, and under license from the UK Home Office (Animals (Scientific Procedures) Act, 1986) [PPL: PP8708123].

Consent for Publication

All authors have read the paper and agreed to publish it.

Conflict of Interest/Competing Interests

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Open Practices Statement and Data Availability

The data for all experiments are available at (https://osf.io/kpmvh) and more info about the study preregistration can be found at https://osf.io/qxpu8.

References

Alsene, K., Deckert, J., Sand, P., & de Wit, H. (2003). Association Between A2a Receptor Gene Polymorphisms and Caffeine-Induced Anxiety. *Neuropsychopharmacology, 28*(9), 1694-1702. doi:10.1038/sj.npp.1300232

Asselmann, E., Wittchen, H. U., Lieb, R., & Beesdo-Baum, K. (2018). Sociodemographic, clinical, and functional long-term outcomes in adolescents and young adults with mental disorders. *Acta Psychiatr Scand, 137*(1), 6-17. doi:10.1111/acps.12792

Ator, N. A. (2005). Contributions of GABAA Receptor Subtype Selectivity to Abuse Liability and Dependence Potential of Pharmacological Treatments for Anxiety and Sleep Disorders. *CNS Spectrums, 10*(1), 31-39. doi:10.1017/S1092852900009883

Bandelow, B., & Michaelis, S. (2015). Epidemiology of anxiety disorders in the 21st century. *Dialogues in clinical neuroscience, 17*(3), 327-335. doi:10.31887/DCNS.2015.17.3/bbandelow

Bandelow, B., Michaelis, S., & Wedekind, D. (2017). Treatment of anxiety disorders. *Dialogues in clinical neuroscience, 19*(2), 93-107. doi:10.31887/DCNS.2017.19.2/bbandelow

Beesdo, K., Knappe, S., & Pine, D. S. (2009). Anxiety and Anxiety Disorders in Children and Adolescents: Developmental Issues and Implications for DSM-V. *Psychiatric Clinics of North America, 32*(3), 483-524. doi:https://doi.org/10.1016/j.psc.2009.06.002

Bencan, Z., Sledge, D., & Levin, E. D. (2009). Buspirone, chlordiazepoxide and diazepam effects in a zebrafish model of anxiety. *Pharmacology Biochemistry and Behavior, 94*(1), 75-80. doi:https://doi.org/10.1016/j.pbb.2009.07.009

Bishop, B. H., Spence-Chorman, N., & Gahtan, E. (2016). Three-dimensional motion tracking reveals a diving component to visual and auditory escape swims in zebrafish larvae. *Journal of Experimental Biology, 219*(24), 3981-3987. doi:10.1242/jeb.147124

Blaser, R. E., Chadwick, L., & McGinnis, G. C. (2010). Behavioral measures of anxiety in zebrafish (Danio rerio). *Behavioural Brain Research, 208*(1), 56-62. doi:https://doi.org/10.1016/j.bbr.2009.11.009

Blaser, R. E., & Rosemberg, D. B. (2012). Measures of anxiety in zebrafish (Danio rerio): dissociation of black/white preference and novel tank test. *PLoS One, 7*(5), e36931. doi:10.1371/journal.pone.0036931

Briley, M. S., & Langer, S. Z. (1978). Influence of GABA receptor agonists and antagonists on the binding of 3H-diazepam to the benzodiazepine receptor. *European Journal of Pharmacology, 52*(1), 129-132. doi:https://doi.org/10.1016/0014-2999(78)90031-6

Cachat, J., Stewart, A., Grossman, L., Gaikwad, S., Kadri, F., Chung, K. M., . . . Kalueff, A. V. (2010). Measuring behavioral and endocrine responses to novelty stress in adult zebrafish. *Nature Protocols, 5*(11), 1786-1799. doi:10.1038/nprot.2010.140

Calcaterra, N. E., & Barrow, J. C. (2014). Classics in Chemical Neuroscience: Diazepam (Valium). *ACS Chemical Neuroscience, 5*(4), 253-260. doi:10.1021/cn5000056

Cassar, S., Adatto, I., Freeman, J. L., Gamse, J. T., Iturria, I., Lawrence, C., . . . Zon, L. I. (2020). Use of Zebrafish in Drug Discovery Toxicology. *Chemical Research in Toxicology, 33*(1), 95-118. doi:10.1021/acs.chemrestox.9b00335

Dadda, M., Domenichini, A., Piffer, L., Argenton, F., & Bisazza, A. (2010). Early differences in epithalamic left-right asymmetry influence lateralization and personality of adult zebrafish. *Behav Brain Res, 206*(2), 208-215. doi:10.1016/j.bbr.2009.09.019

Demetriou, E. A., Park, S. H., Pepper, K. L., Naismith, S. L., Song, Y. J., Thomas, E. E., . . . Guastella, A. J. (2021). A transdiagnostic examination of anxiety and stress on executive function outcomes in disorders with social impairment. *J Affect Disord, 281*, 695-707. doi:https://doi.org/10.1016/j.jad.2020.11.089

Egan, R. J., Bergner, C. L., Hart, P. C., Cachat, J. M., Canavello, P. R., Elegante, M. F., . . . Kalueff, A. V. (2009). Understanding behavioral and physiological phenotypes of stress and anxiety in zebrafish. *Behav Brain Res, 205*(1), 38-44. doi:10.1016/j.bbr.2009.06.022

Facchin, L., Argenton, F., & Bisazza, A. (2008). Lines of Danio rerio selected for opposite behavioural lateralization show differences in anatomical left-right asymmetries. *Behavioural Brain Research, 197*, 157-165. doi:10.1016/j.bbr.2008.08.033

Honess, P., & Wolfensohn, S. (2010). The extended welfare assessment grid: a matrix for the assessment of welfare and cumulative suffering in experimental animals. *Altern Lab Anim, 38*(3), 205-212. doi:10.1177/026119291003800304

Hovenkamp-Hermelink, J. H. M., Jeronimus, B. F., Myroniuk, S., Riese, H., & Schoevers, R. A. (2021). Predictors of persistence of anxiety disorders across the lifespan: a systematic review. *The Lancet Psychiatry, 8*(5), 428-443. doi:https://doi.org/10.1016/S2215-0366(20)30433-8

Ingebretson, J. J., & Masino, M. A. (2013). Quantification of locomotor activity in larval zebrafish: considerations for the design of high-throughput behavioral studies. *Front Neural Circuits, 7*, 109. doi:10.3389/fncir.2013.00109

Kallai, J., Makany, T., Csatho, A., Karadi, K., Horvath, D., Kovacs-Labadi, B., . . . Jacobs, J. W. (2007). Cognitive and affective aspects of thigmotaxis strategy in humans. *Behav Neurosci, 121*(1), 21-30. doi:10.1037/0735-7044.121.1.21

Kalueff, A. V., Gebhardt, M., Stewart, A. M., Cachat, J. M., Brimmer, M., Chawla, J. S., . . . Schneider, H. (2013). Towards a comprehensive catalog of zebrafish behavior 1.0 and beyond. *Zebrafish, 10*(1), 70-86. doi:10.1089/zeb.2012.0861

Kysil, E. V., Meshalkina, D. A., Frick, E. E., Echevarria, D. J., Rosemberg, D. B., Maximino, C., . . . Kalueff, A. V. (2017). Comparative Analyses of Zebrafish Anxiety-Like Behavior Using Conflict-Based Novelty Tests. *Zebrafish, 14*(3), 197-208. doi:10.1089/zeb.2016.1415

Lange, M., Neuzeret, F., Fabreges, B., Froc, C., Bedu, S., Bally-Cuif, L., & Norton, W. H. (2013). Inter-individual and inter-strain variations in zebrafish locomotor ontogeny. *PLoS One, 8*(8), e70172. doi:10.1371/journal.pone.0070172

Lange, M., Norton, W., Coolen, M., Chaminade, M., Merker, S., Proft, F., . . . Bally-Cuif, L. (2012). The ADHD-susceptibility gene lphn3.1 modulates dopaminergic neuron formation and locomotor activity during zebrafish development. *Mol Psychiatry, 17*(9), 946-954. doi:10.1038/mp.2012.29

Levin, E. D., Bencan, Z., & Cerutti, D. T. (2007). Anxiolytic effects of nicotine in zebrafish. *Physiol Behav, 90*(1), 54-58. doi:10.1016/j.physbeh.2006.08.026

MacPhail, R. C., Brooks, J., Hunter, D. L., Padnos, B., Irons, T. D., & Padilla, S. (2009). Locomotion in larval zebrafish: Influence of time of day, lighting and ethanol. *Neurotoxicology, 30*(1), 52-58. doi:10.1016/j.neuro.2008.09.011

Maeda, H., Hasumi, A., & Yoshida, K.-i. (2021). Caffeine-induced bradycardia, death, and anxiety-like behavior in zebrafish larvae. *Forensic Toxicology, 39*(2), 427-436. doi:10.1007/s11419-021-00577-8

Maximino, C., de Brito, T. M., da Silva Batista, A. W., Herculano, A. M., Morato, S., & Gouveia, A., Jr. (2010). Measuring anxiety in zebrafish: a critical review. *Behav Brain Res, 214*(2), 157-171. doi:10.1016/j.bbr.2010.05.031

Mendl, M., Burman, O. H. P., & Paul, E. S. (2010). An integrative and functional framework for the study of animal emotion and mood. *Proceedings of the Royal Society B: Biological Sciences, 277*(1696), 2895-2904. doi:10.1098/rspb.2010.0303

Mezzomo, N. J., Silveira, A., Giuliani, G. S., Quadros, V. A., & Rosemberg, D. B. (2016). The role of taurine on anxiety-like behaviors in zebrafish: A comparative study using the novel tank and the light-dark tasks. *Neurosci Lett, 613*, 19-24. doi:10.1016/j.neulet.2015.12.037

Neuhauss, S. C. (2003). Behavioral genetic approaches to visual system development and function in zebrafish. *J Neurobiol, 54*(1), 148-160. doi:10.1002/neu.10165

Newby, J. M., McKinnon, A., Kuyken, W., Gilbody, S., & Dalgleish, T. (2015). Systematic review and meta-analysis of transdiagnostic psychological treatments for anxiety and depressive disorders in adulthood. *Clinical Psychology Review, 40*, 91-110. doi:https://doi.org/10.1016/j.cpr.2015.06.002

Parker, M. O., Millington, M. E., Combe, F. J., & Brennan, C. H. (2012). Housing conditions differentially affect physiological and behavioural stress responses of zebrafish, as well as the response to anxiolytics. *PLoS One, 7*(4), e34992. doi:10.1371/journal.pone.0034992

Richendrfer, H., Pelkowski, S. D., Colwill, R. M., & Creton, R. (2012). On the edge: Pharmacological evidence for anxiety-related behavior in zebrafish larvae. *Behavioural Brain Research, 228*(1), 99-106. doi:https://doi.org/10.1016/j.bbr.2011.11.041

Rosa, L. V., Ardais, A. P., Costa, F. V., Fontana, B. D., Quadros, V. A., Porciúncula, L. O., & Rosemberg, D. B. (2018). Different effects of caffeine on behavioral neurophenotypes of two zebrafish populations. *Pharmacology Biochemistry and Behavior, 165*, 1-8. doi:https://doi.org/10.1016/j.pbb.2017.12.002

Rosemberg, D. B., Braga, M. M., Rico, E. P., Loss, C. M., Cordova, S. D., Mussulini, B. H., . . . Souza, D. O. (2012). Behavioral effects of taurine pretreatment in zebrafish acutely exposed to ethanol. *Neuropharmacology, 63*(4), 613-623. doi:10.1016/j.neuropharm.2012.05.009

Simon, P., Dupuis, R., & Costentin, J. (1994). Thigmotaxis as an index of anxiety in mice. Influence of dopaminergic transmissions. *Behav Brain Res, 61*(1), 59-64. doi:10.1016/0166-4328(94)90008-6

Stewart, A. M., Gaikwad, S., Kyzar, E., & Kalueff, A. V. (2012). Understanding spatio-temporal strategies of adult zebrafish exploration in the open field test. *Brain Research, 1451*, 44-52. doi:https://doi.org/10.1016/j.brainres.2012.02.064

Tran, S., Fulcher, N., Nowicki, M., Desai, P., Tsang, B., Facciol, A., . . . Gerlai, R. (2017). Time-dependent interacting effects of caffeine, diazepam, and ethanol on zebrafish behaviour. *Progress in Neuro-Psychopharmacology and Biological Psychiatry, 75*, 16-27. doi:https://doi.org/10.1016/j.pnpbp.2016.12.004

Ulhaq, M., Orn, S., Carlsson, G., Morrison, D. A., & Norrgren, L. (2013). Locomotor behavior in zebrafish (Danio rerio) larvae exposed to perfluoroalkyl acids. *Aquat Toxicol, 144-145*, 332-340. doi:10.1016/j.aquatox.2013.10.021

van Calker, D., Biber, K., Domschke, K., & Serchov, T. (2019). The role of adenosine receptors in mood and anxiety disorders. *Journal of Neurochemistry, 151*(1), 11-27. doi:https://doi.org/10.1111/jnc.14841

Walz, N., Mühlberger, A., & Pauli, P. (2016). A Human Open Field Test Reveals Thigmotaxis Related to Agoraphobic Fear. *Biol Psychiatry, 80*(5), 390-397. doi:10.1016/j.biopsych.2015.12.016

Wong, K., Elegante, M., Bartels, B., Elkhayat, S., Tien, D., Roy, S., . . . Kalueff, A. V. (2010). Analyzing habituation responses to novelty in zebrafish (Danio rerio). *Behav Brain Res, 208*(2), 450-457. doi:10.1016/j.bbr.2009.12.023

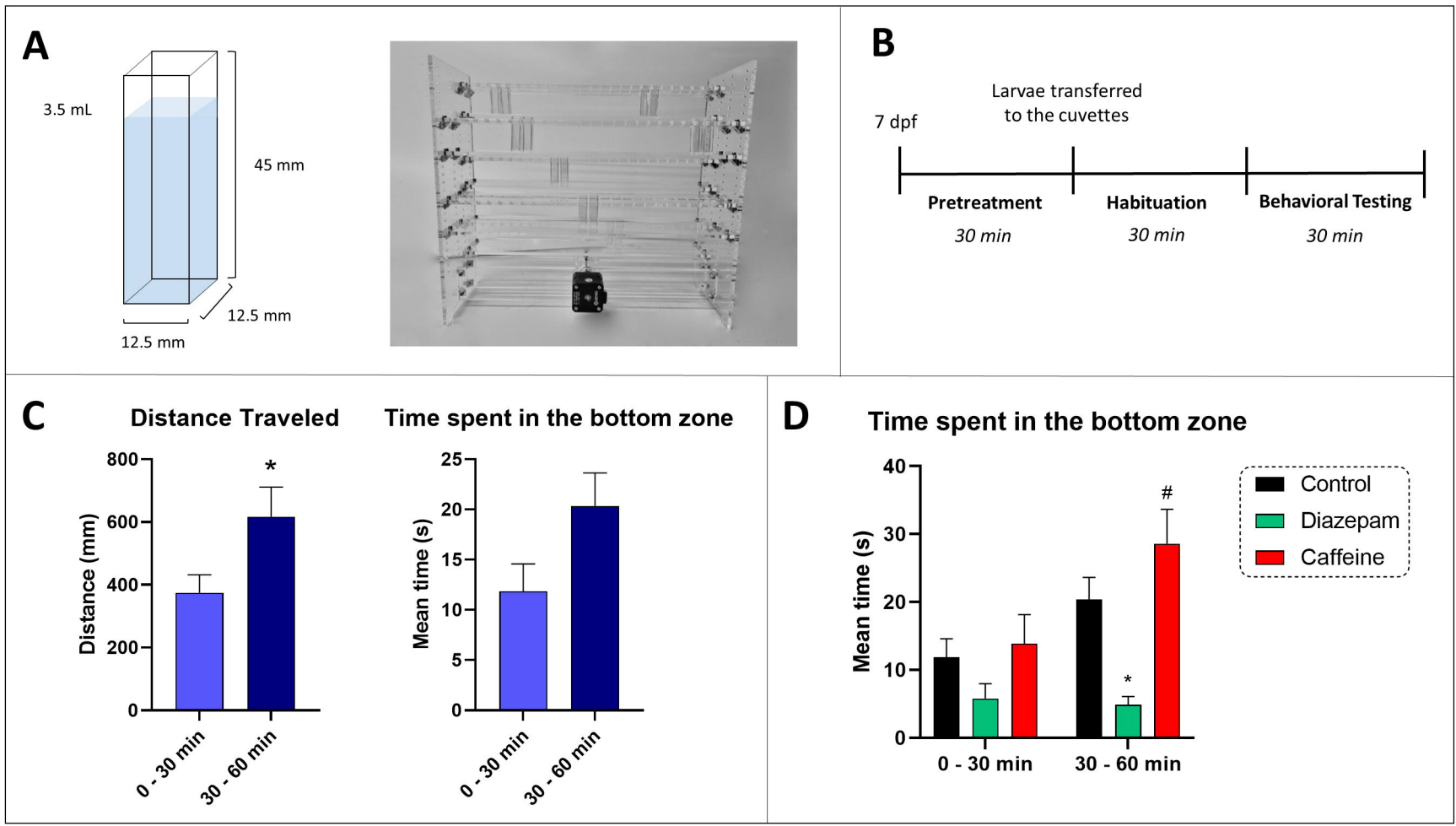
Figure legends

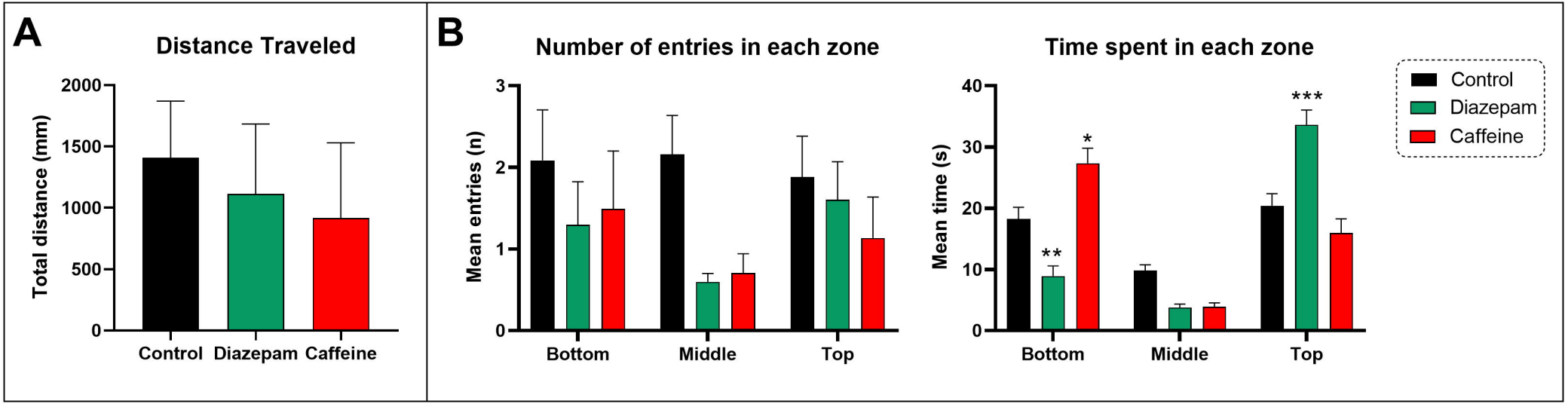
Figure 1. (A) Illustration showing the cuvette dimensions used as the behavioral apparatus for the larvae tank diving test (LDR) and a picture of the tank set up for larvae behavioral testing. **(B)** Summary of the experimental design used in this work. **(C)** Shows that larvae have a significant increase in their activity after 30 min of behavioral testing ($p^* < 0.05$; $n = 16$) and an effect of $p = 0.0555$ for time spent in the bottom (0 – 30 *vs.* 30 – 60 min; $n = 16$). **(D)** Initial data collection, showing that caffeine has a higher effect after 30 min of habituation ($p^* = 0.0276$) and that diazepam only show a significant effect compared to controls after 30 min of habituation ($p^* = 0.0257$). Data were represented as mean ± S.E.M and analyzed by two-way ANOVA (treatment and testing time), followed by Tukey's test multiple comparison test. Asterisks indicate significant difference when compared to controls within testing time ($p^* < 0.05$) and octothorp when comparing 0 – 30 *vs.* 30 – 60 min within treatment ($p^{\#} < 0.05$).

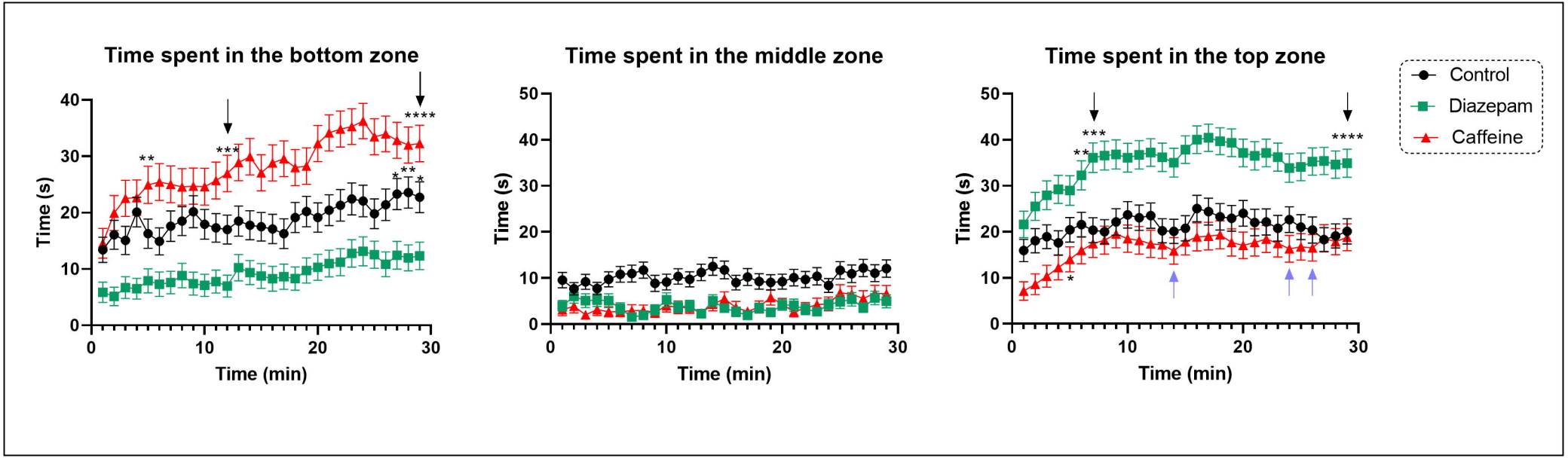
Figure 2. Effects of diazepam and caffeine on a larvae tank diving test. **(A)** Diazepam and caffeine did not alter larvae locomotion (*n = 80 per group)*. Data were represented as mean ± S.E.M and analyzed by one-way ANOVA, followed by Tukey's test post hoc test. **(B)** Diazepam and caffeine alter diving response in zebrafish larvae by decreasing and increasing anxiety, respectively (*n = 80 per group)*. Data were represented as mean ± S.E.M and analyzed by two-way ANOVA (treatment and zone as main factors), followed by Tukey's test multiple comparison

test. Asterisks represent statistical differences when compared to controls of each zone ($p^* < 0.05$, $p^{**} < 0.005$, $p^{***} < 0.001$).

Figure 3. Analysis across-time of tank diving response of animals exposed to caffeine (100 mg/L) and diazepam (5 mg/L). When looking at the time spent in the bottom, control animals have a significant increase in time spent at the bottom during the final minutes of the test. Diazepam did not affect time spent in the bottom across time. Meanwhile caffeine shows a significant increase in time spent in bottom from minute 5. Black arrows indicate statistical significance higher than $p^{***} < 0.001$ from time bin 12 to 29. No significant changes were observed for the middle zone. An increased response to diazepam starts at time bin 6 and a minimum p value of < 0.001 is observed from time bin 7 to 29 (Black arrows). Blue arrows indicate a lack of statistical difference after the start of the caffeine effects for time spent in the top zone. Data were represented as mean ± S.E.M and analyzed by two-way RM ANOVA, followed by Tukey's test multiple comparison test (*n = 80 per group*). Asterisks represent statistical differences when compared to time bin 1 of each treatment ($p^* < 0.05$, $p^{**} < 0.005$, $p^{***} < 0.001$, $p^{****} < 0.0001$).


A
3.5 mL
45 mm
12.5 mm
12.5 mm
B
7 dpf
Larvae transferred
to the cuvettes
Pretreatment
30 min
Habituation
30 min
Behavioral Testing
30 min
C
Distance Traveled
Distance (mm)
800
600
400
200
0
*
0 - 30 min
30 - 60 min
Time spent in the bottom zone
Mean time (s)
25
20
15
10
5
0
0 - 30 min
30 - 60 min
D
Time spent in the bottom zone
Mean time (s)
40
30
20
10
0
#
*
0 - 30 min
30 - 60 min
Control
Diazepam
Caffeine


A
Distance Traveled
Total distance (mm)
2000
1500
1000
500
0
Control
Diazepam
Caffeine
B
Number of entries in each zone
Mean entries (n)
3
2
1
0
Bottom
Middle
Top
Time spent in each zone
Mean time (s)
40
30
20
10
0
**
*
***
Bottom
Middle
Top
Control
Diazepam
Caffeine


Time spent in the bottom zone
Time spent in the middle zone
Time spent in the top zone
Time (s)
Time (min)
Control
Diazepam
Caffeine