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ORIGINAL PAPER

The plus maze and scototaxis test are not valid behavioral assays for anxiety assessment in the South African clawed frog

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Abstract

There are no behavioral models for testing anxiety in amphibians, a group of animals widely used for developmental, ecotoxicological, and genetic research. We aimed to validate two common rodent paradigms, the plus maze and the scototaxis test, for use in the aquatic African clawed frog (*Xenopus laevis*). We predicted: (a) that frogs would prefer the dark, vs. light, portions of the testing arenas (face validity), (b) that this behavior could be altered with acute administration of anxio-selective drugs (construct validity), and (c) that time spent in the dark portions of the arenas would be positively correlated (predictive validity). Prior to testing, frogs were treated with fluoxetine (selective serotonin reuptake inhibitor [SSRI]), desipramine (serotonin- and norepinephrine-reuptake inhibitor), caffeine (methylxanthine, adenosine receptor antagonist, phosphodiesterase inhibitor), saline, or were left unmanipulated. Each drug was administered acutely (1 h prior to testing; caffeine) or subacutely (24, 3, and 1 h prior to testing; fluoxetine, desipramine) at one of three doses. Plus maze and scototaxis testing were separated by 1 week; each frog completed both behavioral tasks and was treated with the same drug regimen prior to testing. Overall, both tests showed face validity, however, data suggest these paradigms lack both construct and predictive validity.

Keywords Ecotoxicology · SSRI · Amphibian · Caffeine · PPCP

Abbreviations

PPCP	Pharmaceutical and personal care product
RDoC	Research Domain Criteria
SSRI	Selective serotonin reuptake inhibitor
TCA	Tricyclic antidepressant

Introduction

Anxiety is a complex and evolutionarily conserved (emotional) state associated with predictable changes in behavior that are generally dissociated from an external stimulus (McNaughton and Zangrossi 2008). A state of anxiety can arise from potential physical or psychological dangers in the environment (Perusini and Fanselow 2015; Clinchy et al. 2011; Steimer 2011; Harris and Carr 2016), and this state is experienced by multiple taxa, including nematode worms (Liu et al. 2018), crayfish (Fossat et al. 2014), fish (Stewart et al. 2012), rodents (Walf and Frye 2007), and humans (Craske et al. 2017). Anxiety, however, can be difficult to define clinically, and diagnosis is often based on subjective assessments, making studies of causes and consequences of anxiety difficult. The National Institute of Mental Health has proposed the use of Research Domain Criteria (RDoC) to better classify and define emotions and psychopathologies. The RDoC framework uses multiple biomarkers to develop specific constructs associated with psychological conditions to better understand and diagnose the physiological phenotypes of human behaviors and mental disorders (Insel 2014). The goal of this framework is that, in the future, biomarkers will be used to understand the distinguishing etiology of such conditions (Cuthbert and Insel 2013). It holds great promise, but due to ethical and logistical constraints, it is often difficult to completely study the biological underpinnings of anxiety in humans. Thus, using animal models to manipulate and measure anxiety and corresponding biomarkers is critical (Blanchard et al. 2013; Anderzhanova et al. 2017). In addition, by developing multiple animal

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models of anxiety, and thus having evolutionary (cross-species) validity, we will be able to elucidate the core traits and behaviors associated with various forms of anxiety (Kalueff et al. 2012; Stewart and Kalueff 2015).

Behavioral paradigms to assess anxiety in rodents are common and include the elevated plus maze, open- field test, light/dark (scototaxis) test, vogel conflict, and conditioned fear test (Griebel and Holmes 2013). The (elevated) plus maze test and the scototaxis (light/dark) test are the most widely used and extensively validated unconditioned response paradigms for studying the physiological and neural underpinnings of anxiety and the effects of anxioselective drugs in rodents (Pellow et al. 1985; Bourin and Hascoët 2003; Carobrez and Bertoglio 2005; Maximino et al. 2011; Griebel and Holmes 2013; Kumar et al. 2013). However, data from human and rodent studies cannot pinpoint the individual roles the prefrontal cortex and the limbic system play in aspects of anxiety as in these species both brain regions are important for this emotional state (Davidson 2002; Ressler and Mayberg 2007). In amphibians the limbic circuitry is conserved and operates independently of a brain cortex, allowing us to examine in an isolated way the subcortical pathways involved in initiating and maintaining anxiety (Sokolowski and Corbin 2012; Carr 2015).

To date, no amphibian anxiety behavioral paradigms exist. Here, we aimed to validate two different anxiety behavior assays, the plus maze test and the scototaxis test, for use in the aquatic amphibian the African clawed frog, *Xenopus laevis*. Assessing the validity of an animal behavioral model is critical for making data comparable between laboratories and across species, and thus is crucial for translational research (van der Staay et al. 2009; Maximino et al. 2010a; Blanchard et al. 2013; Stewart and Kalueff 2015). Therefore, to develop any animal model/behavioral paradigm, we must first assess its face, construct, and predictive validity (Willner 1984; Treit et al. 2009; Nestler and Hyman 2010; Belzung and Lemoine 2011; Goswami et al. 2013; Steward and Kalueff 2015). To do so, we use the validity definitions of Walf and Frye (2007).

The plus maze and scototaxis are ethological conflict-type tests (Kumar at el. 2013) and rely on innate light–dark preference. For light–dark-based anxiety tests, validation studies typically involve administering anxiogenic (e.g., caffeine, carbon dioxide, sodium lactate) or anxiolytic agents (e.g., selective-serotonin reuptake inhibitors [SSRI], tricyclic antidepressants [TCA], benzodiazepines, or alcohol) prior to behavioral testing with the expectation that these agents will alter the amount of time spend in the light (anxiogenic agents should decrease; anxiolytic agents should increase) (Belzung and Griebel 2001; Treit et al. 2009; Kumar et al. 2013). In humans, SSRIs are the frontline therapeutics (Hoffman and Mathew 2008) for treating multiple types of anxiety. Thus, scientifically valid animal models of anxiety should be sensitive to SSRIs (Borsini et al. 2002). Both the plus maze and the scototaxis test are clinical representations of the most common form of anxiety: generalized anxiety disorder (Griebel and Holmes 2013). Given that general anxiety disorder is routinely treated with serotonin-modifying drugs (e.g., SSRIs, 5HT-1A agonists, TCAs; Zohar and Westenberg 2000; Griebel and Holmes 2013), and that acute and chronic administration of these drugs can be effective in rodent models (see Borsini et al. 2002; Griebel and Holmes 2013), we chose to use an SSRI and a TCA in our study. Additionally, SSRIs and TCAs are both effective in the treatment of many types of anxiety and both are safer than benzodiazepines (Lembke et al. 2018), but SSRIs can treat a wider range of anxiety types (Zohar and Westenberg 2000). Caffeine is one of the most consumed psychoactive drugs in the world (James 1997) and can act as an anxiogenic agent (Smith 2002).

Thus, our goals were to determine whether (1) the plus maze and scototaxis behavioral paradigms pass face validity in *Xenopus laevis* and (2) these behavioral paradigms show construct and predictive validity in this species. Using anxiolytic (one SSRI and one TCA) and anxiogenic (caffeine) drugs, we determined if behavior in the plus maze and scototaxis test can be predictably altered. In addition to time in light/dark and entries into sections, we measured air gulps and thigmotaxis. We predicted that anxious animals would take fewer air gulps, as data in goldfish (Matsuda et al. 2013), zebrafish (Egan et al. 2009; Sackerman et al. 2010; Blaser and Rosemberg 2012), and *X. laevis* (Duggan et al. 2016) suggest that anxious aquatic animals spend more time away from the surface. We chose to categorize location position because thigmotaxic, or wall hugging, is typically assessed in anxiety assays as animals naturally tend to stay closer toward walls and avoid the open spaces i.e. they are centro-phobic (Simon et al. 1994; Treit et al. 2009; Kumar et al. 2013). For example, larval zebrafish exposed to caffeine, versus controls, spent more time at the tank edge (Richendrfer et al. 2012). This behavior is relevant for zebrafish (Maximino et al. 2010b) and likely relevant for *X. laevis* as Prater and colleagues recently showed that Xenopus injected with the anxiogenic peptide corticotropin-releasing factor increased time on the edge of the tank compared to unmanipulated frogs (Prater et al. 2018). Overall, we predicted that (1) control animals would spend the greatest duration of time in the dark portion of the testing arenas and, in the scototaxis test, spend a higher proportion of scans in the edge section (face validity), (2) in treated animals, compared to vehicle controls, fluoxetine- and desipramine-treated frogs would be less anxious (i.e., perform more air gulps, spend more time in the light portions, and in scototaxis in the center), while the caffeine-treated frogs would be more anxious (i.e., perform fewer air gulps, spend more time in the dark portions, and in the scototaxis at the edges)

(construct validity), and (3) time spent in the dark arm of plus maze would be positively correlated with time in the dark portion of the scototaxis test (predictive validity).

Methods

Animals

Xenopus laevis is a primarily aquatic frog species inhabiting freshwater ponds over a wide geographic area of Southern Africa. The frog remains submerged for most of its life and it can be preyed upon by birds, snakes, otters, and bass (Channing 2001). *X. laevis* is generally more active at night and feeds on a variety of slow-moving invertebrates as well as members of its own species (Measey et al. 1998). *X. laevis* is a good choice for a model organism for several reasons. The behavioral traits that are drawn from light–dark testing are considered to be phylogenetically conserved and can be seen across vertebrates, thus we expect these same behaviors to be present and robust in frogs. Additionally, genetic tools are available for this species (Tandon et al. 2016) and these tools could be useful for targeting specific biomarkers (but see Stewart et al. 2014). Finally, because of their environmental sensitivity and trophic importance, amphibians are good models for studying environmental disruptions (Hopkins 2007), and SSRIs, in particular, are a pharmaceutical and personal care product (PPCP) contaminant of concern for aquatic wildlife in general (Silva et al. 2012; Simmons et al. 2017). *X. laevis* is a widely used model organism in ecotoxicology (Gendron 2013), making a high throughput lab model of anxiety in *X. laevis* beneficial to regulators, environmental scientists, and other stakeholders assessing the SSRI and other PPCP risk to aquatic wildlife. If our model is supported, it could be helpful for understanding comparative neuroendocrinology, for investigating the neural and endocrine bases of human disease and psychopathology, and for ecotoxicology and PPCP studies.

Juvenile *X. laevis*, purchased from Xenopus1 (#4208 post-metamorphic frogs; Dexter, MI, USA), were group housed in a 175.26 cm × 41.91 cm × 55.88 cm trout tank (25 lux; Living Stream Systems, Frigid Units, Inc., Toledo, Ohio, USA) filled to between 25 and 50% max volume with deionized water conditioned with 0.3 g/L Instant Ocean (Spectrum Brands, Blacksburg, VA, USA). A total of 123 frogs were tested under our experimental paradigm. Final body mass averaged across all frogs was 6.91 g (range: 2.05–18.90), final snout vent length was 3.88 cm range: (1.40–5.20). Housing and testing were carried out in the same animal room (750 lux). We maintained lighting conditions at 12L:12D with lights off at 2 PM. Group housing stocking density ranged from 1 to 70 frogs, and frogs were group housed for 7 to 180 d prior to testing. Frogs received 3–4 pellets of post-metamorph frog brittle (Nasco, Atkinson, WI, USA) approximately 3 times per week after tank cleaning was performed. After completion of the behavioral experiments, frogs were euthanized in MS-222 (3 g/L dH_2O) matched with equal parts sodium bicarbonate ($NaHCO_3$), incisions were then made through the abdominal lining and muscle, and the frogs were fixed in 10% buffered formalin. Following 48 h in the fixative, frogs were rinsed in water and dissected to determine the sex of each animal (here: 71 males, 47 females, 5 undetermined; for sample size and sex per treatment group please see Tables 1 and 2) as frogs are not sexually dimorphic at this age and size (Carr et al. 2003). All procedures performed in studies involving animals were in accordance with ethical standards of the institution and were approved by the Texas Tech University Institutional Animal Care and Use Committee; Texas Tech University is an Association for Assessment and Accreditation of Laboratory Animal Care accredited institution.

Experimental design

Approximately 48 h prior to plus maze testing, frogs were isolated into individual clear plastic housing containers (50 lux; 30.48 cm × 15.24 cm × 20.32 cm), filled with 3.5 L of conditioned water (0.3 g InstantOcean/L dH_2O); frogs remained isolated in individual tanks for a total of 10 d. Frogs were treated with their respective drug regimen ($n = 7$–9 per treatment) on a standardized schedule (see details below). Frogs received one of three drugs, given at high, medium, or low dose (see below), or control saline (0.6% NaCl) injection, matched to drug injection time course, or no manipulation. Each frog was tested in only one treatment group. Throughout this protocol, frogs were fed and tanks were cleaned every 2–3 days depending on the day of the week. To ensure being placed in new water did not alter behavior (O'Neill et al. 2018), all frogs were allowed to acclimate for 10 min prior to plus maze testing and scototaxis testing (see details below) and behavior was subsequently recorded for 14 min. The plus maze test was followed by the scototaxis test 7 d later. All tests were carried out during the light portion of the daily cycle, between 9 AM and 2 PM. Behavioral trials were video recorded for later scoring. Due to video equipment failures and lighting system malfunction, not all frogs have data for each behavioral test. Out of the total 123 frogs, 111 trials from the plus maze testing and 97 trials from the scototaxis testing were scorable; a total of 85 frogs completed both behavioral assays.

Plus maze arena

The plus maze tests were carried out in 1 of 3 identical hand-built plus mazes (Fig. 1) measuring 30 cm (total

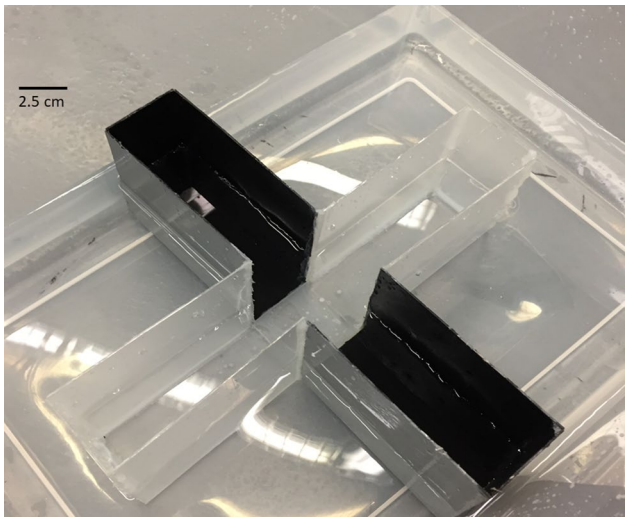

Fig. 1 Arial view of the custom-built aquatic plus maze in the testing chamber. Frogs were given 10 min to acclimate in the water surrounding the tank and were then carefully placed into the center for the start of the test

length across) × 5.5 cm (arm width) × 12.5 cm (arm height), placed inside of a clear open plastic container (58.4 cm × 41.3 cm × 31.4 cm), filled with 10 L of conditioned water (0.3 g InstantOcean/L dH_2O); temperature was recorded prior to each test. Plus maze arms (L × W × H; 12.5 cm × 5.5 cm × 12.5 cm) were painted black (5 lux) or left clear (95 lux); arms of the same color were placed on opposing sides. This testing arena design was based on the arena used for aquatic crayfish (Fossat et al. 2014). Each piece was cut from Plaskolite polystyrene sheeting (thickness 0.127 cm) and secured together using waterproof, 100% silicone sealant (Momentive Performance, Inc., Huntersville, NC, USA). Acclimation in the plus maze was carried out by allowing the frog to freely swim inside the container of water but outside of the plus maze, for 10 min prior to testing. Immediately following acclimation, frogs were placed in the center of the plus maze and recordings began.

Scototaxis arena

The scototaxis tests were carried out in 1 of 2 identical scototaxis arenas which were standard 40 L glass tanks (Fig. 2), 50.8 cm (length) × 25.4 cm (width) × 31.75 cm (height), with the outside painted half white (50 lux) and half black (15 lux) with spray paint. Design of the scototaxis tank was based on published experiments in zebrafish (Maximino et al. 2007, 2010b, 2011). For testing, we filled the tank with 10 L of conditioned water (0.3 g InstantOcean/L dH_2O) and recorded temperature prior to each test. Acclimation in the scototaxis tank was

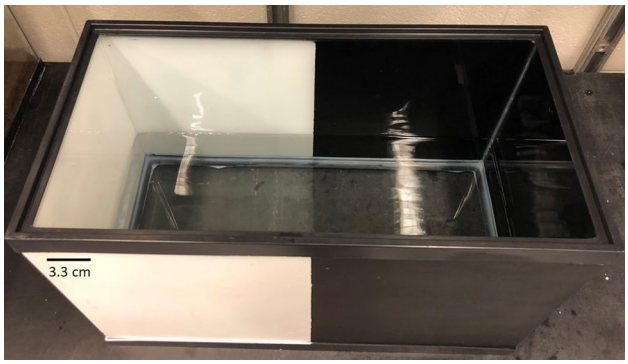

Fig. 2 Arial view of the custom-built scototaxis testing chamber. Frogs were given 10 min to acclimate within an opaque tube in the center and were then allowed to swim freely during testing

carried out by placing the frog into an opaque gray circular plastic tube (diameter = 7.5 cm) in the center of the tank, for 10 min prior to testing. Immediately following acclimation, the tube was carefully lifted up out of the tank, allowing the frog to become mobile and recordings began.

Drug administration

Drugs were administered via dorsal lymph sac injection (Foxon and Rowson 1956) using a 31G insulin syringe (BD Insulin Syringes, Franklin Lakes, NJ, USA). Injection volume was based on body mass and was given at a volume of 16 mL/kg. All drugs were dissolved in 0.6% saline (0.6 g NaCL into 100 mL sterile pyrogen-free, molecular biology grade water), aliquoted, and stored a − 20 °C until use. Injection aliquots were warmed to room temperature prior to injection. To our knowledge, no study to date has injected frogs with anxio-selective drugs and, therefore, we used data from rodents (see Borsini et al. 2002) and zebrafish (*Danio rerio;* Stewart et al. 2011a) to determine dosage and time course. Drugs were chosen based on the amount of previous data for comparison in these paradigms.

Caffeine

Anhydrous caffeine (27602, Sigma Aldrich, St. Louis, MO, USA), is a non-selective antagonist of adenosine receptors (Daly et al. 1994). Caffeine was injected immediately prior to the 10-min acclimation time. Time course was based on studies in zebrafish (Maximino et al. 2011) and mice (Jain et al. 1995). Low (5 mg/kg), medium (10 mg/kg), and high (15 mg/kg) doses were used. Our initial doses were much higher (150, 100, and 50 mg/kg) and were based on those found to be anxiogenic in zebrafish (100 mg/kg injection; Maximino et al. 2011) and rats (50 mg/kg injection; Hughes et al. 2014), but we subsequently lowered those doses due to the lethal effects of the higher doses.

Desipramine

Desipramine hydrochloride (D3900, Sigma Aldrich, St. Louis, MO, USA), at low (1 mg/kg), medium (10 mg/kg), or high (20 mg/kg) dosage was injected 24, 3, and 1 h prior to testing. Dosages were based on multiple rodent studies (see Borsini et al. 2002). Desipramine is a tricyclic antidepressant (TCA), classified as a secondary amine tricyclic, is a moderately selective inhibitor of the norepinephrine transporter and the serotonin transporter (Vetulani et al. 1976; Charney et al. 1984). Desipramine is an older drug used to treat anxiety but has been used extensively in animal paradigms (see Borsini et al. 2002), making it a good choice for comparison to other studies.

Fluoxetine

Fluoxetine hydrochloride (F132, Sigma Aldrich, St. Louis, MO, USA) was injected 24, 3, and 1 h prior to testing at one of three dosages: low (5 mg/kg), medium (10 mg/kg), and high (20 mg/kg). Fluoxetine is a canonical SSRI and second-generation antidepressant (Sommi et al. 1987) and is routinely used to treat many anxiety disorders (Zohar and Westenberg 2000; Griebel and Holmes 2013). While several studies have used chronic administration of SSRIs, various others have successfully used acute (1 administration) or subacute (3 administrations) time courses to alter behavior (see Borsini et al. 2002; Table Supplemental 1).

Behavioral scoring

Behavioral videos were manually scored using EthoVision XT (v13, Noldus Information Technology, Wageningen, The Netherlands). One scorer analyzed all plus maze videos and another scorer analyzed all scototaxis videos; both scorers were blind to the frog treatment groups during scoring. Behaviors scored and analyzed for the plus maze included each of the following: number of entries into each arm, time spent in each arm, and number of air gulps. For comparison with other studies, we also determined percent time in the light arm (light duration/[light duration + dark duration] × 100).

Behaviors scored and analyzed in the scototaxis test included: duration of time in the dark half, duration of time in the light half, duration of time in the initial start location (only scored initially), number of entries into each tank half, number of air gulps, and duration of time floating at the surface. Only 14 frogs (4 caffeine treated, 5 desipramine treated, 5 fluoxetine treated), out of the 97, spent any time floating at the surface, and thus this behavior was not analyzed statistically. In addition to durational and count data, we also performed instantaneous scans every 30 s to the determined location (center portion of tank or around edges) and locomotion (swimming or not swimming); these data were used to calculate the proportion of scans (out of 28) for each measure. In fish, thigmotaxis can be defined as time at tank edge, thrashing, stereotypy, or escape behavior (see Blaser et al. 2010), but here we used the classical definition as time at the tank edges. In the present study, we used a rectangular tank and thus the edge zones on all sides of the tank follow a mean distance from the wall (6.5 cm). We predicted that frogs would generally spend a greater proportion of scans in the edge zone vs the center, and that treatment with caffeine would increase time in edge zones whereas treatment with fluoxetine or desipramine would decrease time in edge zones.

Statistical analyses

Data from a total of 111 frogs were included in the analysis for the plus maze and data from 97 frogs were available for the scototaxis test scoring. Data were analyzed via non-parametric Wilcoxon signed-rank and Kruskal–Wallis analysis as residuals of the dependent variables failed tests of normality and various transformations (log and square root) did not improve normality as assessed by Shapiro Wilkes. For Kruskal–Wallis tests, effect sizes are presented as eta squared (η^2), $\chi^2/(N-1)$; Wilcoxon signed-rank test effect sizes are reported as r, $Z/\sqrt{N}$ (Rosenthal 1994). To test our first question, whether control frogs would spend more time in dark portions (plus maze: dark arm, scototaxis: dark half), we used a Wilcoxon signed-rank test to determine if the duration of time in light vs. dark differed in the control animals. Also, we used a Kruskal–Wallis test to compare the three control conditions (unmanipulated, saline for caffeine, saline for antidepressant) against one another to determine if the injection protocol impacted behavior. Next, we performed separate Kruskal–Wallis tests for each drug to determine the impact on behaviors, and each analysis contained 5 groups (unmanipulated, saline injected, dose 1, dose 2, and dose 3). Lastly, because we found no impact of dosage or control manipulation, we collapsed drug doses and control groups to form 4 groups (all control, all caffeine, all desipramine, all fluoxetine) and compared the three drugs treatments to controls. Finally, because no groups differed, we compared time in light and dark for all frogs using a Wilcoxon signed-rank test. After analyzing data from each behavioral test, we ran a Pearson's correlation between time spent in the dark sections (arms or half) to determine if frogs behaved consistently in each test and a Spearman's rho to determine if air gulping behavior was consistent. When comparing across groups, we determined if the proportion of scans during which the frog was located in the

center differed. But, when we determined if frogs preferred the edge over the center we used a Chi-squared goodness of fit to determine if the proportion of time at the edge was significantly greater than 0.64 because in our arena roughly 64% of the total area was edge whereas only 36% was center. Even though we had clear a priori predictions, we still used multiple statistical tests and comparisons for each study. Therefore, we followed the recommendation of Benjamin and colleagues (Benjamin et al. 2018) and have set an alpha of 0.05 as suggestive and 0.005 as significant.

Results

Plus maze behavior

Controls

Among all control animals (unmanipulated, saline caffeine, saline antidepressant), median duration of time spent in the dark arms was significantly greater than that in the light arms ($Z=3.14$, $P=0.002$, $N=26$, $r=0.62$; see Table 1 for all plus maze behavioral data, see Figs. S1–S4 for graphical representation of time in light data). Duration of time spent in the dark arm did not differ among control conditions ($X^2=0.50$, $P=0.975$, $df=2$, $N=26$, $\eta^2=0.02$). Control conditions did not differ in number of entries into the dark arm ($X^2=1.47$, $P=0.480$, $df=2$, $N=26$, $\eta^2=0.06$), number of entries into the light arm ($X^2=1.43$, $P=0.489$, $df=2$, $N=26$, $\eta^2=0.06$), percent of time in light arms ($X^2=0.05$, $P=0.975$, $df=2$, $N=26$, $\eta^2<0.01$), or number of air gulps ($X^2=1.81$, $P=0.404$, $df=2$, $N=26$, $\eta^2=0.07$). Thus, it did not appear that the injection protocol impacted behavior.

Caffeine

Duration of time spent in the dark arm did not differ among caffeine treatment groups ($X^2=1.25$, $P=0.869$, $df=4$, $N=48$, $\eta^2=0.02$). Caffeine treatment conditions did not alter the number of entries into the dark arm ($X^2=0.19$, $P=0.995$, $df=4$, $N=48$, $\eta^2=0.004$), number of entries into the light arm ($X^2=0.44$, $P=0.979$, $df=4$, $N=48$, $\eta^2<0.01$), percent of time in the light arm ($X^2=1.25$, $P=0.869$, $df=4$, $N=48$, $\eta^2=0.02$), or number of air gulps ($X^2=1.53$, $P=0.820$, $df=4$, $N=48$, $\eta^2=0.03$).

Desipramine

Duration of time spent in the dark arm did not differ among desipramine treatment groups ($X^2=0.72$, $P=0.948$, $df=4$, $N=45$, $\eta^2=0.02$). Desipramine treatment did not alter the number of entries into the dark arm ($X^2=6.46$, $P=0.167$, $df=4$, $N=45$, $\eta^2=0.15$), number of entries into the light arm ($X^2=3.78$, $P=0.437$, $df=4$, $N=45$, $\eta^2=0.09$), percent of time in the light arm ($X^2=0.73$, $P=0.948$, $df=4$, $N=45$, $\eta^2=0.02$), or in number of air gulps ($X^2=3.61$, $P=0.461$, $df=4$, $N=45$, $\eta^2=0.08$).

Fluoxetine

Duration of time spent in the dark arm did not differ among fluoxetine treatment groups ($X^2=5.02$, $P=0.286$, $df=4$, $N=45$, $\eta^2=0.11$). Fluoxetine treatment conditions did not differ in number of entries into the dark arm ($X^2=3.53$, $P=0.473$, $df=4$, $N=45$, $\eta^2=0.08$), entries into the light arm ($X^2=2.52$, $P=0.640$, $df=4$, $N=45$, $\eta^2=0.06$), percent of time in the light arm ($X^2=5.07$, $P=0.286$, $df=4$, $N=45$, $\eta^2=0.12$), or number of air gulps ($X^2=8.47$, $P=0.076$, $df=4$, $N=45$, $\eta^2=0.19$).

Collapsed groups

Among all animals (unmanipulated, saline, caffeine, antidepressant), the median duration of time spent in the dark arms was significantly greater than that in the light arms ($Z=6.53$, $P<0.001$, $N=111$, $r=0.62$). Collapsed treatment condition did not impact duration of time spent in dark arms ($X^2=0.82$, $P=0.843$, $df=3$, $N=111$, $\eta^2=0.007$) or number of air gulps ($X^2=3.68$, $P=0.298$, $df=3$, $N=111$, $\eta^2=0.03$). Groups did not differ in percent of time spent in light arms ($X^2=0.84$, $P=0.840$, $df=3$, $N=111$, $\eta^2<0.01$) or number of entries into the light arm ($X^2=3.58$, $P=0.311$, $df=3$, $N=111$, $\eta^2=0.03$); there was a trend for number of entries into the dark arm, but the result did not reach significance ($X^2=7.42$, $P=0.06$, $df=3$, $N=111$, $\eta^2=0.06$).

Scototaxis test behavior

Controls

Among all control animals combined (unmanipulated, saline caffeine, saline antidepressant), median duration of time spent in the dark side was suggestive with time spent in the dark marginally greater than in the light side ($Z=1.90$, $P=0.058$, $N=23$, $r=0.40$). Combined, control frogs spent more time at the edges that would be expected by random chance ($X^2=30.58$, $P<0.001$, $N=23$,), and spent more time not swimming/inactive versus swimming ($Z=4.23$, $P<0.001$, $N=23$, $r=0.88$). Among control conditions, duration of time spent in the dark half did not differ ($X^2=0.267$, $P=0.875$, $df=2$, $N=23$, $\eta^2=0.01$; all scototaxis data shown in Table 2, see Figs. S5–S8 for graphical representation of

Table 1 Aquatic plus maze behavioral data from all frogs. Results are presented by drug group as individual treatments and as a collapsed unit; total duration of behavioral test is 840 s

Treatment	*n* (M/F/u)	Light arm entry (count)	Duration of time in light arm (s)	Dark arm entry (count)	Duration of time in dark arm (s)	Percentage of time in light arm	Air gulps (count)
Control (all)	26 (12/13/1)	1 (1–4)	4.60 (0.95–840.00)	1 (0–3)	835.42 (0.00–839.06)	0.6 (0.1–100.0)	2.0 (0–9)
Unmanipulated	10 (6/4/0)	1 (1–2)	60.60 (1.52–212.12)	1 (1–2)	779.42 (627.90–838.50)	7.2 (0.2–25.3)	2.0 (0–8)
Saline Caffeine	9 (3/6/0)	1 (1–4)	4.48 (1.04–840.00)	1 (0–3)	835.54 (0.00–838.54)	0.5 (0.1–100.0)	2.0 (0–9)
Saline Antidepressant	7 (3/4/1)	1 (1–1)	4.60 (0.96–840.00)	1 (0–1)	835.419 (0.00–839.00)	0.5 (0.1–100.0)	5.0 (0–8)
Caffeine (all)	29 (20/9/0)	1 (1–7)	3.60 (0.20–840.00)	1 (0–7)	836.42 (0.00–839.80)	0.4 (0.0–100.0)	3.0 (0–13)
5 mg/kg	10 (7/3/0)	1 (1–4)	19.41 (0.24–840.00)	1 (0–4)	820.61 (0.00–839.78)	2.3 (0.0–100.0)	3.0 (0–13)
10 mg/kg	10 (7/3/0)	1 (1–6)	83.46 (0.97–840.00)	1 (0–6)	756.56 (0.00–839.06)	9.9 (0.1–100.0)	2.0 (0–12)
15 mg/kg	9 (6/3/0)	1 (1–7)	3.56 (0.77–840.00)	1 (0–7)	836.42 (0.00–839.26)	0.4 (0.1–100.0)	3.0 (0–12)
Desipramine (all)	28 (15/12/1)	1 (1–7)	7.50 (1.00–438.8)	1 (1–6)	832.50 (401.50–839.00)	0.9 (0.1–52.2)	4.0 (0–16)
1 mg/kg	10 (6/3/1)	1 (1–3)	4.27 (1.10–148.71)	1 (1–3)	835.75 (691.31–838.92)	0.5 (0.1–17.7)	3.5 (0–14)
10 mg/kg	9 (4/5/0)	1 (1–3)	10.24 (1.53–130.8)	1 (1–3)	829.78 (709.22–838.49)	1.2 (0.2 –15.6)	4.0 (2–6)
20 mg/kg	9 (5/4/0)	1 (1–7)	11.48 (1.00–438.80)	1 (1–6)	823.14 (401.23–839.00)	1.4 (0.1–52.2)	5.0 (1–16)
Fluoxetine (all)	28 (16/10/2)	1 (0–8)	3.60 (1.00–840.0)	1 (0–8)	836.40 (0.00–839.00)	0.4 (0.1–100.0)	3.0 (0–14)
5 mg/kg	9 (6/2/1)	1 (1–2)	3.67 (1.24–156.42)	1 (1–2)	836.35 (683.6–838.78)	0.4 (0.1–18.6)	2.0 (0–4)
10 mg/kg	10 (5/5/0)	1 (0–3)	2.34 (1.00–756.84)	1 (1–3)	837.68 (83.18–839.00)	0.3 (0.1–90.0)	3.0 (0–5)
20 mg/kg	9 (5/2/2)	1 (1–8)	75.44 (1.64–840.00)	1 (0–8)	764.58 (0.00–838.38)	9.0 (0.2–100.0)	6.0 (1–14)
All frogs	111 (63/44/4)	1 (0–8)	4.04 (1.00–840.00)	1 (0–8)	835.98 (0.00–839.00)	0.5 (0.0–100.0)	3.0 (0–16)

Data are presented as median (min–max) for count and durational data. *n* = total number of frogs per group

M male, *F* female, *u* undetermined

Table 2 Scototaxis test behavioral data from all frogs. Results are presented by drug group as individual treatments and as a collapsed unit; total duration of behavioral test is 840 s

Treatment	*n* (M/F/u)	Light entry (count)	Duration in light (s)	Dark entry (count)	Duration dark (s)	Air gulps (count)	Proportion in center zone	Proportion swimming
Control (all)	23 (13/9/1)	1.0 (0–6)	35.40 (0.00–839.10)	1 (0–6)	662.58 (0.00–839.00)	2.0 (0–13)	0.04 (0.00–1.00)	0.04 (0.00–0.32)
Unmanipulated	9 (4/5/0)	2.0 (0–5)	9.88 (0.00–768.78)	1 (0–6)	344.67 (0.00–839.00)	3.0 (0–13)	0.04 (0.00–1.00)	0.04 (0.00–0.29)
Saline Caffeine	7 (6/1/0)	1.0 (0–4)	154.276 (0.00–755.80)	1 (0–4)	662.58 (0.00–837.86)	2.0 (1–5)	0.00 (0.00–0.21)	0.04 (0.00–0.07)
Saline Antidepressant	7 (3/3/1)	0.0 (0–6)	0.00 (0.00–839.10)	1 (0–6)	717.20 (0.00–837.70)	2.0 (0–4)	0.04 (0.00–0.14)	0.07 (0.04–0.32)
Caffeine (all)	21 (16/5/0)	3.0 (0–6)	589.99 (0.00–768.79	3 (0–6)	199.23 (0.00–839.00)	3.0 (0–5)	0.07 (0.00–1.00)	0.11 (0.00–0.25)
5 mg/kg	7 (5/2/0)	2.0 (0–6)	651.18 (0.00–696.58)	1 (0–6)	179.14 (0.00–835.78)	1.0 (0–3)	0.14 (0.00–1.00)	0.11 (0.00–0.18)
10 mg/kg	7 (6/1/0)	3.0 (0–4)	602.43 (0.00—768.79)	3 (1–3)	199.23 (66.40–839.00)	3.0 (2–4)	0.07 (0.00–0.61)	0.14 (0.07–0.25)
15 mg/kg	7 (5/2/0)	2.0 (0–6)	236.87 (0.00–750.77)	3 (1–5)	601.71 (88.25–836.09)	2.0 (1–5)	0.04 (0.00–0.07)	0.11 (0.00–0.25)
Desipramine (all)	27 (15/11/1)	1.0 (0–9)	42.08 (0.00–782.67)	2 (1–10)	672.98 (54.32–839.10)	2.0 (0–9)	0.04 (0.00–0.93)	0.07 (0.00–0.46)
1 mg/kg	8 (4/3/1)	2.5 (0–9)	212.42 (0.00–672.93)	2 (1–10)	604.21 (156.09–837.18)	2.0 (0–4)	0.13 (0.00–0.93)	0.11 (0.00–0.46)
10 mg/kg	9 (4/5/0)	1.0 (0–6)	1.00 (0.00–782.67)	1 (1–6)	618.87 (54.32–839.10)	2.0 (0–4)	0.04 (0.00–0.43)	0.04 (0.00–0.39)
20 mg/kg	10 (7/3/0)	1.0 (0–5)	11.11 (0.00–617.63)	2 (1–4)	696.03 (216.00–838.19)	3.0 (0–9)	0.00 (0.00–0.18)	0.07 (0.00–0.25)
Fluoxetine (all)	26 (16/7/2)	1.0 (0–6)	44.51 (0.00–806.54)	1 (1–7)	733.40 (27.20–838.34)	1.5 (0–8)	0.04 (0.00–0.71)	0.07 (0.00–0.25)
5 mg/kg	8 (5/3/0)	2.0 (0–4)	130.55 (0.00–624.62)	2 (1–4)	684.99 (206.36–821.64)	2.0 (0–5)	0.02 (0.00–0.46)	0.07 (0.00–0.14)
10 mg/kg	9 (6/3/0)	0.0 (0–6)	0.00 (0.00–179.68)	1 (1–7)	829.50 (46.48–838.15)	1.0 (0–3)	0.04 (0.00–0.71)	0.04 (0.04–0.25)
20 mg/kg	9 (6/1/2)	0.0 (0–6)	0.00 (0.00–806.54)	1 (1–6)	833.78 (27.20–838.34)	2.0 (0–8)	0.00 (0.00–0.71)	0.11 (0.04–0.21)
All frogs	97 (60/32/4)	0.0 (0–9)	103.36 (0.00–839.10)	1 (0–10)	662.58 (0.00–839.20)	2.0 (0–13)	0.04 (0.00–1.00)	0.07 (0.00–0.46)

Data are presented as median (min–max) for count, duration, and proportion data. *n* = total number of frogs per group

M male, *F* female, *u* undetermined

time in light data); neither did time spend in the initial middle spot of the tank ($X^2 = 0.172$, $P = 0.917$, $df = 2$, $N = 23$, $\eta^2 = 0.007$). Controls did not differ in the number of air gulps ($X^2 = 0.596$, $P = 0.742$, $df = 2$, $N = 23$, $\eta^2 = 0.03$), proportion of scans in the center ($X^2 = 0.575$, $P = 0.750$, $df = 2$, $N = 23$, $\eta^2 = 0.02$), or proportion of scans spent swimming ($X^2 = 5.56$, $P = 0.062$, $df = 2$, $N = 23$, $\eta^2 = 0.25$). Control animals did not differ the number of entries into the light half ($X^2 = 0.28$, $P = 0.868$, $df = 2$, $N = 23$, $\eta^2 = 0.01$) or dark half ($X^2 = 0.16$, $P = 0.923$, $df = 2$, $N = 23$, $\eta^2 = 0.007$). Thus, overall, it does not appear that saline injection altered behavior.

Caffeine

Duration of time spent in the dark half did not differ among caffeine treatment groups ($X^2 = 1.36$, $P = 0.851$, $df = 4$, $N = 37$, $\eta^2 = 0.03$); neither did time spend in the initial middle spot of the tank ($X^2 = 2.94$, $P = 0.568$, $df = 4$, $N = 37$, $\eta^2 = 0.08$). Caffeine treatment did not alter the number of air gulps ($X^2 = 4.35$, $P = 0.361$, $df = 4$, $N = 37$, $\eta^2 = 0.12$), proportion of scans in the center ($X^2 = 4.91$, $P = 0.297$, $df = 4$, $N = 37$, $\eta^2 = 0.14$), or scans spent swimming ($X^2 = 8.43$, $P = 0.077$, $df = 4$, $N = 37$, $\eta^2 = 0.23$). Caffeine treatment did not alter the number of entries into the light half ($X^2 = 2.05$, $P = 0.726$, $df = 4$, $N = 37$, $\eta^2 = 0.06$) or dark half ($X^2 = 3.61$, $P = 0.461$, $df = 4$, $N = 37$, $\eta^2 = 0.10$).

Desipramine

Duration of time spent in the dark half did not differ among desipramine treatment groups ($X^2 = 1.41$, $P = 0.842$, $df = 4$, $N = 43$, $\eta^2 = 0.03$); neither did time spend in the initial middle spot of the tank ($X^2 = 1.07$, $P = 0.899$, $df = 4$, $N = 43$, $\eta^2 = 0.03$). Desipramine treatment did not alter the number of air gulps ($X^2 = 3.32$, $P = 0.506$, $df = 4$, $N = 43$, $\eta^2 = 0.08$), proportion of scans spent in the center of the tank ($X^2 = 7.26$, $P = 0.123$, $df = 4$, $N = 43$, $\eta^2 = 0.17$), or proportion of scans spent swimming ($X^2 = 1.31$, $P = 0.859$, $df = 4$, $N = 43$, $\eta^2 = 0.03$). Desipramine treatment did not alter the number of entries into the light half ($X^2 = 3.11$, $P = 0.539$, $df = 4$, $N = 43$, $\eta^2 = 0.07$) or dark half ($X^2 = 2.01$, $P = 0.734$, $df = 4$, $N = 43$, $\eta^2 = 0.05$).

Fluoxetine

Duration of time spent in the dark half did not differ among fluoxetine treatment groups ($X^2 = 2.11$, $P = 0.715$, $df = 4$, $N = 42$, $\eta^2 = 0.05$); neither did time spend in the initial middle spot of the tank ($X^2 = 1.83$, $P = 0.765$, $df = 4$, $N = 42$, $\eta^2 = 0.04$). Fluoxetine treatment did not alter the number of air gulps ($X^2 = 2.03$, $P = 0.728$, $df = 4$, $N = 42$, $\eta^2 = 0.05$); proportion of scans in the center ($X^2 = 3.21$, $P = 0.523$, $df = 4$, $N = 42$, $\eta^2 = 0.08$) or proportion of scans spent swimming ($X^2 = 3.71$, $P = 0.447$, $df = 4$, $N = 42$, $\eta^2 = 0.09$). Fluoxetine treatment did not impact the number of entries into the light half ($X^2 = 1.87$, $P = 0.758$, $df = 4$, $N = 42$, $\eta^2 = 0.05$) or dark half ($X^2 = 0.87$, $P = 0.929$, $df = 4$, $N = 42$, $\eta^2 = 0.02$).

All frogs and collapsed groups

Among all animals (unmanipulated, saline, caffeine, antidepressant) combined, median duration of time spent in the dark side was significantly higher than that in the light side ($Z = 4.67$, $P < 0.001$, $N = 97$, $r = 0.47$). Overall, all frogs displayed more thigmotaxis than would be expected by random chance ($X^2 = 27.78$ $P < 0.001$, $N = 97$), and spent more time not swimming/inactive vs. swimming ($Z = 8.58$, $P < 0.001$, $N = 97$, $r = 0.87$). Collapsed treatment conditions (all control, all caffeine, all desipramine, all fluoxetine) did not impact duration of time spent in dark half ($X^2 = 7.23$, $P = 0.067$, $df = 3$, $N = 97$, $\eta^2 = 0.08$), nor did they impact time spend in the initial middle spot of the tank ($X^2 = 2.01$, $P = 0.569$, $df = 3$, $N = 97$, $\eta^2 = 0.02$). Collapsed treatment groups did not differ from one another in the number of air gulps ($X^2 = 1.86$, $P = 0.601$, $df = 3$, $N = 97$, $\eta^2 = 0.02$); proportion of scans in the center ($X^2 = 2.01$, $P = 0.571$, $df = 3$, $N = 97$, $\eta^2 = 0.02$) or proportion of scans spent swimming ($X^2 = 2.20$, $P = 0.528$, $df = 3$, $N = 97$, $\eta^2 = 0.02$). Collapsed groups did not differ in the number of entries into the light half ($X^2 = 3.68$, $P = 0.298$, $df = 3$, $N = 97$, $\eta^2 = 0.04$) or dark half ($X^2 = 4.32$, $P = 0.229$, $df = 3$, $N = 97$, $\eta^2 = 0.05$).

Correlation between plus maze and scototaxis test

Even though in both tests frogs preferred the dark tank sections over the light, surprisingly, there was no correlation between time spent in the dark arm of the plus maze and time spent in the dark half of the scototaxis tank (Pearson's r; $r = 0.138$, $P = 0.209$, $N = 85$; Fig. 3). However, a suggestive result in number of air gulps was found as they were positively correlated between tests (Spearman's rho; $\rho = 0.257$, $P = 0.018$, $N = 85$). Additionally, the number of air gulps was positively related to number of entries into the dark portion for the plus maze (Spearman's rho; $\rho = 0.427$, $P < 0.001$, $N = 111$) and the scototaxis test (Spearman's rho; $\rho = 0.485$, $P < 0.001$, $N = 97$), and suggestively related to the number of entries into the light portion (plus maze: $\rho = 0.390$, $P = 0.018$, $N = 85$; scototaxis: $\rho = 0.362$, $P < 0.001$, $N = 97$). Suggesting that frogs that are moving around the tank during testing more take more breaths, and thus that air gulps may not be representative of anxiety in this species.

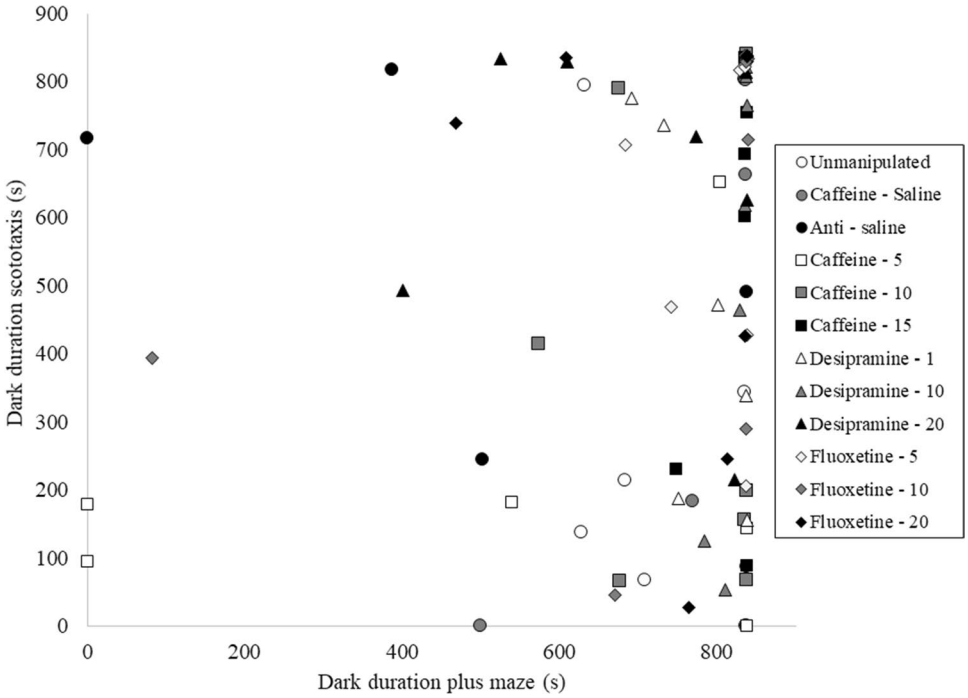

Fig. 3 Duration of time (out of 840 s test) that frogs spent in the dark portion of each testing arena. Despite the fact the frogs preferred dark to light in each test, there was no correlation between time spent in the dark arm of the plus maze and time spent in the dark half of the scototaxis tank (Pearson's r; $r = 0.138$, $P = 0.209$, $N = 85$)

Discussion

Frogs prefer dark to light areas: behavioral paradigms have face validity

Our aim was to determine if the (submerged) plus maze and scototaxis paradigms are valid behavioral assays for assessing anxiety behavior in frogs. We predicted that (1) control animals would spend the greatest duration of time in the dark portion of the testing arenas and, in the scototaxis test, spend a higher proportion of scans in the edge section (face validity), (2) in treated animals, compared to vehicle controls, fluoxetine- and desipramine-treated frogs would spend more time in the light, and in scototaxis in the center, while the caffeine-treated frogs would spend more time in the dark, and in the scototaxis at the edges (construct validity), and (3) time spent in the dark arm of plus maze would be positively correlated with time in the dark portion of the scototaxis test (predictive validity). When conditions were collapsed, frogs spent significantly more of their time in the dark compared to the light portions of the tank in both behavioral paradigms, but there was no statistical difference between time spent in dark and light portions among any of the treatment groups vs the control animals in either test. Additionally, when looking at control frogs only, they did not spend significantly more time in the dark in the scototaxis arena, but they did in the plus maze. Frogs treated with caffeine did not increase time in dark portions or time at the edge (scototaxis only), and frogs treated with fluoxetine and desipramine did not increase time spent in light portions or center (scototaxis only). No treatment altered the number of entries into either the light or the dark section of the arenas. In the scototaxis test, frogs spent significantly more time at the edge versus the center and not swimming/immobile versus swimming as measured by instantaneous scan. Thus, overall, we were able to show that frogs prefer dark tank sections to the light tank sections and that in the scototaxis test frogs prefer the edges to the center and spend more time not swimming/immobile, suggesting that individually both tests have face validity. However, we were not able to alter this preference with drug administration suggesting our paradigms failed in terms of construct validity. Moreover, some of the frogs showed low levels of locomotion (scototaxis) and exploratory behavior (both tests), as measured by entries into light/dark tank sections. It should be noted that in this species sitting immobile is a common behavior and thus this traditional rodent marker of anxiety-like behavior may not be informative here. However, thigmotaxis behavior may be relevant. Additionally, and somewhat surprisingly, the duration of time spent in the dark arms of the plus maze was not correlated with the duration of time spent in the dark portion of the scototaxis test, suggesting these paradigms also lack predictive validity.

Possible explanations for why plus maze & scototaxis failed predictive validity and experimental design considerations

Initially, we were surprised that time spent in the dark portion of the arenas by each frog was not positively correlated across test paradigms. However, upon greater inspection of

the literature, it appears this seems to be a common finding when multiple paradigms are compared. For example, when assessing anxiety in zebrafish, Blaser and Rosemberg (2012) found that two common tests—the tank diving test and the scototaxis test—assess different aspects of anxiety and may assess different constructs (see also Maximino et al. 2012). Additionally, even though the plus maze and open field are both unconditioned response paradigms based on the avoidance of lit areas, factor analysis in various rodent studies has shown they do not produce a common anxiety-related factor (File 1991; Trullas and Skolnick 1993; Ramos et al. 1997, 1998; Vendruscolo et al. 2003) and thus may get at different aspects of anxiety-like behavior. Additionally, in Lewis rats, chlordiazepoxide had anxiolytic effects in the plus maze but not in the open field (see Ramos 2008). The reasons for lack of correlation when using individual data are unclear but may stem from a few major issues. First, it is important to realize these behavioral paradigms are tests of state (i.e., situational) anxiety and not trait anxiety per se and thus behavioral outcomes could differ from one point in time to the next based on intra-individual differences (see Lister 1990; Ramos 2008). Thus, while we found that in both tests frogs preferred the dark portion, behavior was variable across testing days and data were more variable in the scototaxis test than in the plus maze. Second, it may be important to assess various anxiety test paradigms as partially overlapping constructs where manipulation (e.g., genetic, drug, environmental) may influence performance in one test but not in another (see Fig. 1 in Ramos 2008). Third, even though we used published methods to design our arenas (Maximino et al. 2007, 2010b, 2011; Fossat et al. 2014), the set-up here might not have been ethologically relevant for our frogs. Xenopus are nocturnal and juvenile frogs of this species tend to live in muddy ponds and are preyed upon by predators located in the water, at the water's edge, and in the air. Thus, using fully open dark arm and dark tank side design might have impacted frog behavior. Additionally, testing during the light versus the dark period may have influenced results, but we chose to test in the light so we could maximize the contrast between the light and dark tank sections (also see Maximino et al. 2010c for the rationale of testing zebrafish in these paradigms during lights-on). Future studies should determine if different testing arenas or time of day of testing impact outcomes. Lastly, there was considerable variation in behavioral data among frogs, while behavioral variation is not uncommon and can be important (e.g., Williams 2008) it may make finding effects more difficult. However, recent data suggest that individual behaviors are repeatable in amphibians (Duggan et al. 2016; Kelleher et al. 2018). In future trials, using the triple test paradigm proposed by Ramos and adding more individuals may help to address these issues (Ramos et al. 2008).

Possible explanations for why anxio-selective drugs did not alter behavior

The exact reasons why our drug treatments did not alter frog behavior is unknown, but theoretically at least, it could be due one of many reasons, including the drug classes used (e.g., SSRI and TCA vs benzodiazepine), a ceiling effect of behavior, the time course of injection, the drug doses, or the neural correlates of anxiety in amphibians. Benzodiazepines, namely diazepam, were the gold standard for anxiety models for many years, but this class of drugs is only truly effective for human generalized anxiety disorder (see Rodgers et al. 1997). While many studies have used these GABA-modifying drugs to test the construct validity of anxiety behavior assays, several studies have determined the efficacy of serotonin-modifying drugs and TCAs in unconditioned response anxiety paradigms with both chronic and acute administration (Griebel 1995; Borsini et al. 2002; Varty et al. 2002; Griebel and Holmes 2013). However, SSRIs can have different effects on anxiety-like behaviors (see Borsini et al. 2002), including anxiolytic (Kurt et al. 2000), anxiogenic (Kshama et al. 1990), and ineffective (Beaufour et al. 1999), and some of these results differ by time course. Acute and chronic fluoxetine or desipramine treatment can produce different results with no consistent result as to which time course or dose is more effective (see Table S1); it is possible that chronic administration would be effective in *X. laevis*. More studies are warranted to better understand the efficacy of these drugs in animal models if we hope to bridge the translational divide between animal models and emotional disorders in humans (Lampis et al. 2011).

In addition to not seeing a reduction in anxiety-like behaviors with fluoxetine and desipramine, we did not see an anxiogenic response following administration of caffeine. In zebrafish, acute caffeine treatment (100 mg/L of tank water) can produce anxiogenic effects in the novel tank test (Egan et al. 2009; Wong et al. 2010) and in the scototaxis test (100 mg/kg injection 10 min prior to test, Maximino et al. 2011; 100 mg/L tank water, Stewart et al. 2011b). Acute high-doses of caffeine are anxiogenic in rodents (see Correa and Font 2008 for review), however, various authors have also noted anxiolytic effects of caffeine (see Introduction of Hughes et al. 2014). In our study, caffeine did not appear to be anxiogenic or anxiolytic, but a ceiling effect may have occurred. Control frogs spent a large portion of their time in dark sections of the tank: median 835 out of 840 possible seconds in the dark arms of the plus maze, median of 662 out of 840 possible seconds in the dark half of the scototaxis tank. Thus, it may not have been possible to increase the time in dark arms under these conditions.

Comparative discussion of role of serotonin in neural underpinnings of anxiety

Given that our dosages and time course of fluoxetine, desipramine, and caffeine treatments all failed to elicit changes in behavior it is possible that the dose or time course was not sufficient, or that these drugs do not impact anxiety-like behaviors in *X. laevis*. Doses were spread over ranges and were chosen as they have been shown to be effective in rodents and zebrafish (see Table S1), but, as mentioned above, duration of administration may play a role. Additionally, it is possible that species differ in drug metabolism, a phenomenon which is important for variability of response to drugs in humans (Cregg et al. 2013). The neural substrates and brain regions underlying anxiety are highly evolutionarily conserved (Shin and Fishman 2002; Adhikari 2014; Carr 2015;) and are present, in at least some form, in animals ranging from lampreys to primates (Loonen and Ivanova 2015; Tovote et al. 2015). Based on the available literature, it seems unlikely that *X. laevis* have neural circuits underlying anxiety that are completely different from other organisms. For example, *X. laevis* has a well-developed raphe nucleus from an early age (van Mier et al. 1986) suggesting that the chemoarchitecture for serotonergic involvement in anxiety is present in the brain. Moreover, fluoxetine has been shown to act as a serotonin reuptake blocker in *Xenopus tropicalis* (long-term housing in 1 or 10 µg/L tank water; Berg et al. 2013), although there does appear to be age- and/or stage-dependent sensitivity in this species. Additionally, it is likely that serotonin plays a role in anxiety across both invertebrate (Tierney 2018) and vertebrate (Lowry et al. 2005) taxa. For example, conserved anxiety-related neural network and signaling have been established in nematodes (Liu et al. 2018), zebrafish (Jesuthasan 2012), and rodents (Tovote et al. 2015), and human anxiolytic drugs have been effective in these models. Nematodes treated with the SSRI sertraline (Zoloft) no longer responded to predator threat (Lui et al. 2018), and slugs treated with fluoxetine were more likely to seek out a parasitic nematode than were untreated slugs (Morris et al. 2018). Both behaviors could be consistent with reduced anxiety (increased riskiness), and these data suggest fear and anxiety pathways are conserved and that SSRIs work on invertebrates. Data from fishes show both benzodiazepines and serotonin-modifying drugs can produce anxiolytic effects (Stewart et al. 2011a; Brodin et al. 2013; Brooks 2014; Simmons et al. 2017). Interestingly acute injection of the benzodiazepine chlordiazepoxide (15 ug/g) into the crayfish (*Procambarus clarkia*) was anxiolytic in the plus maze, whereas acute injection of serotonin (5 µg/g) was anxiogenic as it increased light avoidance behaviors (Fossat et al. 2014), thus suggesting that increases in serotonin may not always be anxiolytic. In mice, two distinct dorsal raphe-derived serotonergic sub-systems differentially impact anxiety-like behavior and challenge-coping behavior (Ren et al. 2018), suggesting global manipulation of serotonin may not target anxiety specifically and may explain discrepancies. Finally, it does not appear that the anxio-selective effects of these drugs are due to solely to cortical versus limbic system processing as they have produced effects in other taxa that lack cortical development. Studies using additional serotonin-modifying compounds and benzodiazepines in *X. laevis* would be illuminating.

Conclusion

In summary, we showed the (submerged) plus maze and scototaxis test have face validity in juvenile *Xenopus laevis* as frogs spent more time in the dark versus the light portions of the arena in both tests. Even though our behavior paradigms did not appear to pass conventional construct validation with three classes of anxio-selective drugs they may still be useful. For example, it is important to ask what can be learned about novel treatments or underpinnings of anxiety when these classical drugs fail (Rodgers et al. 1997; Ennaceur 2014). In rodents, the plus maze is highly sensitive to benzodiazepines but results with SSRIs are mixed (see Ennaceur 2014), despite this discrepancy the plus maze is still regarded as a valid and gold-standard anxiety test for rodents and it is known that SSRIs are effective for anxiety clinically. When human clinical use of antidepressant drugs for anxiety treatment began to rise in the 1990s, many of the traditional rodent behavior paradigms that were developed for use with benzodiazepines were not sensitive to antidepressants and thus these drugs were labeled as non-effective or even anxiogenic (Treit et al. 2009). This highlights the pitfalls of using select classes of drugs as the gold standard as this approach can be overly restrictive and limit the discovery of new drugs (see Treit et al. 2009). Similarly, it has been argued that with the absence of complex understanding of what biological substrates drive various anxiety states in humans, it is unwarranted to disregard an animal model just because it does pass pharmacological validation (construct validity) using a specific set of traditional drugs (Rodgers et al. 1997), thus creating a catch 22 for validation studies. Building on our studies using different conditions, for example, adding treatment with benzodiazepines, additional serotonin-modifying drugs, priming frogs with a stressor (Clinchy et al. 2013; Fossat et al. 2014; Duggan et al. 2016), altering the arena set-up, conducting tests in the dark, or incorporating novel classes of anxiolytics [e.g., 3,4-methylendioxymethamphetamine [MDMA]; fatty acid amide hydrolase [FAAH] inhibitors (Gaetani et al. 2003); psychedelics (Carhart-Harris et al. 2018); gallic acid (Mansouri et al. 2014), or other peptides (Rotzinger et al. 2010; Harris et al. 2019)] could be beneficial for understanding the neural bases of anxiety.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

References

Adhikari A (2014) Distributed circuits underlying anxiety. Front Behav Neurosci 8:112

Anderzhanova E, Kirmeier T, Wotjak CT (2017) Animal models in psychiatric research: the RDoC system as a new framework for endophenotype-oriented translational neuroscience. Neurobiol Stress 7:47–56

Beaufour CC, Ballon N, Le Bihan C, Hamon M, Thiébot MH (1999) Effects of chronic antidepressants in an operant conflict procedure of anxiety in the rat. Pharmacol Biochem Behav 62(4):591–599

Belzung C, Griebel G (2001) Measuring normal and pathological anxiety-like behaviour in mice: a review. Behav Brain Res 125(1–2):141–149

Belzung C, Lemoine M (2011) Criteria of validity for animal models of psychiatric disorders: focus on anxiety disorders and depression. Biol Mood Anxiety Disord 1(1):9

Benjamin DJ, Berger JO, Johannesson M, Nosek BA, Wagenmakers EJ, Berk R, Bollen KA, Brembs B, Brown L, Camerer C, Cesarini D, Chambers CD, Clyde M, Cook TD, De Boeck P, Dienes Z, Dreber A, Easwaran K, Efferson C, Fehr E, Fidler F, Field AP, Forster M, George EI, Gonzalez R, Goodman S, Green E, Green DP, Greenwald A, Hadfield JD, Hedges LV, Held L, Hau Ho T, Hoijtink H, Hruschka DJ, Imai K, Imbens G, Loannidis JPA, Jeon M, Holland Jones J, Kirchler M, Laibson D, List J, Little R, Lupia A, Machery E, Maxell SE, McCarthy M, Moore S, Morgan SL, Munafo M, Nakagawa S, Nyhan B, Parker TH, Pericchi L, Perugini M, Rouder J, Rousseau J, Savalei V, Schonbrodt FD, Sellke T, Sinclair B, Tingley D, Van Zandt T, Vazire S, Watts DJ, Winship C, Wolpert RL, Xie Y, Young C, Zinman J, Johnson VE (2018) Redefine statistical significance. Nat Hum Behav 2(1):6–10

Berg C, Backström T, Winberg S, Lindberg R, Brandt I (2013) Developmental exposure to fluoxetine modulates the serotonin system in hypothalamus. PLoS ONE 8(1):e55053

Blanchard DC, Summers CH, Blanchard RJ (2013) The role of behavior in translational models for psychopathology: functionality and dysfunctional behaviors. Neurosci Biobehav Rev 37(8):1567–1577

Blaser RE, Rosemberg DB (2012) Measures of anxiety in zebrafish (*Danio rerio*): dissociation of black/white preference and novel tank test. PLoS ONE 7(5):e36931

Blaser RE, Chadwick L, McGinnis GC (2010) Behavioral measures of anxiety in zebrafish (*Danio rerio*). BehavBrain Res 208(1):56–62

Borsini F, Podhorna J, Marazziti D (2002) Do animal models of anxiety predict anxiolytic-like effects of antidepressants? Psychopharmacol 163(2):121–141

Bourin M, Hascoët M (2003) The mouse light/dark box test. Eur J Pharmacol 463(1–3):55–65

Brodin T, Fick J, Jonsson M, Klaminder J (2013) Dilute concentrations of a psychiatric drug alter behavior of fish from natural populations. Science 339(6121):814–815

Brooks BW (2014) Fish on Prozac (and Zoloft): ten years later. Aquatic Toxicol 151:61–67

Carhart-Harris RL, Roseman L, Haijen E, Erritzoe D, Watts R, Branchi I, Kaelen M (2018) Psychedelics and the essential importance of context. J Psychopharmacol 32(7):725–731

Carobrez AP, Bertoglio LJ (2005) Ethological and temporal analyses of anxiety-like behavior: the elevated plus-maze model 20 years on. Neurosci Biobehav Rev 29(8):1193–1205

Carr JA (2015) I'll take the low road: the evolutionary underpinnings of visually triggered fear. Front Neurosci 9:414

Carr JA, Gentles A, Smith EE, Goleman WL, Urquidi LJ, Thuett K, Kendall RJ, Giesy JP, Gross TS, Solomon KR, Van Der Kraak G (2003) Response of larval *Xenopus laevis* to atrazine: assessment of growth, metamorphosis, and gonadal and laryngeal morphology. Environ Toxicol Chem 22(2):396–405

Channing AC (2001) Amphibians of central and Southern Africa. Protea House, Pretoria, p 470

Charney DS, Heninger GR, Sternberg DE (1984) Serotonin function and mechanism of action of antidepressant treatment: effects of amitriptyline and desipramine. Arch Gen Psychiatry 41(4):359–365

Clinchy M, Schulkin J, Zanette LY, Sheriff MJ, McGowan PO, Boonstra R (2011) The neurological ecology of fear: insights neuroscientists and ecologists have to offer one another. Front Behav Neurosci 5:21

Clinchy M, Sheriff MJ, Zanette LY (2013) Predator-induced stress and the ecology of fear. Funct Ecol 27(1):56–65

Correa M, Font L (2008) Is there a major role for adenosine A2A receptors in anxiety. Front Biosci 13:4058–4070

Craske MG, Stein MB, Eley TC, Milad MR, Holmes A, Rapee RM, Wittchen HU (2017) Anxiety disorders. Nat Rev Dis Primers 3:17100

Cregg R, Russo G, Gubbay A, Branford R, Sato H (2013) Pharmacogenetics of analgesic drugs. Br J Pain 7(4):189–208

Cuthbert BN, Insel TR (2013) Toward the future of psychiatric diagnosis: the seven pillars of RDoC. BMC Med 11(1):126

Daly JW, Shi D, Nikodijevic O, Jacobson KA (1994) The role of adenosine receptors in the central action of caffeine. Pharmacopsychoecologia 7(2):201

Davidson RJ (2002) Anxiety and affective style: role of prefrontal cortex and amygdala. Biol Psychiatry 51(1):68–80

Duggan PE, Prater C, Carr JA, Harris BN (2016) Predator presence decreases food consumption in juvenile *Xenopus laevis*. Behav Ecol Sociobio 70(12):2005–2015

Egan RJ, Bergner CL, Hart PC, Cachat JM, Canavello PR, Elegante MF, Elkhayat SI, Bartels BK, Tien AK, Tien DH, Mohnot S (2009) Understanding behavioral and physiological phenotypes of stress and anxiety in zebrafish. Behav Brain Res 205(1):38–44

Ennaceur A (2014) Tests of unconditioned anxiety—pitfalls and disappointments. Physiol Behav 135:55–71

File SE (1991) The biological basis of anxiety. In: Meltzer HY, Nerozzi D (eds) Current practices and future developments in the pharmacotherapy of mental disorders. Elsevier Publishing, Amsterdam, pp 159–165

Fossat P, Bacqué-Cazenave J, De Deurwaerdère P, Delbecque JP, Cattaert D (2014) Anxiety-like behavior in crayfish is controlled by serotonin. Science 344(6189):1293–1297

Foxon GEH, Rowson KEK (1956) The fate of 'Thorotrast' (thorim dioxide) injected into the dorsal lymph sac of the frog, *Rana temporaria*. Q J Microscop Sci 3(37):47–57

Gaetani S, Cuomo V, Piomelli D (2003) Anandamide hydrolysis: a new target for anti-anxiety drugs? Trends in Mol Medicine 9(11):474–478

Gendron A (2013) Amphibian ecotoxicology. In: Férard JF, Blaise C (eds) Encyclopedia of aquatic ecotoxicology. Springer, Dordrecht

Goswami S, Rodríguez-Sierra O, Cascardi M, Paré D (2013) Animal models of post-traumatic stress disorder: face validity. Front Neurosci 7:89

Griebel G (1995) 5-Hydroxytryptamine-interacting drugs in animal models of anxiety disorders: more than 30 years of research. Pharmacol Thers 65(3):319–395

Griebel G, Holmes A (2013) 50 years of hurdles and hope in anxiolytic drug discovery. Nat Rev Drug Dis 12(9):667–687

Harris BN, Carr JA (2016) The role of the hypothalamus-pituitary-adrenal/interrenal axis in mediating predator-avoidance trade-offs. Gen Comp Endocrinol 230:110–142

Harris BN, Hohman ZP, Campbell CM, King KS, Tucker CA (2019) FAAH genotype, CRFR1 genotype, and cortisol interact to predict anxiety in an aging, rural Hispanic population: a Project FRONTIER study. Neurobiol Stress 10:100154

Hoffman EJ, Mathew SJ (2008) Anxiety disorders: a comprehensive review of pharmacotherapies. Mount Sinai JJ Medicine: A J TranslPers Medicine 75(3):248–262

Hopkins WA (2007) Amphibians as models for studying environmental change. ILAR J 48(3):270–277

Hughes RN, Hancock NJ, Henwood GA, Rapley SA (2014) Evidence for anxiolytic effects of acute caffeine on anxiety-related behavior in male and female rats tested with and without bright light. Behav Brain Res 271:7–15

Insel TR (2014) The NIMH research domain criteria (RDoC) project: precision medicine for psychiatry. Am J Psychiatry 171(4):395–397

Jain N, Kemp N, Adeyemo O, Buchanan P, Stone TW (1995) Anxiolytic activity of adenosine receptor activation in mice. Br J Pharmacol 116(3):2127–2133

James JE (1997) Understanding caffeine: a biobehavioral analysis. Sage Publications, Beverley Hills

Jesuthasan S (2012) Fear, anxiety, and control in the zebrafish. Devel Neurobiol 72(3):395–403

Kalueff AV, Stewart AM, Kyzar EJ, Cachat J, Gebhardt M, Landsman S (2012) International Zebrafish Neuroscience Research Consortium. Time to recognize zebrafish 'affective' behavior. Behaviour 149(10–12):1019–1036

Kelleher SR, Silla AJ, Byrne PG (2018) Animal personality and behavioral syndromes in amphibians: a review of the evidence, experimental approaches, and implications for conservation. Behav Ecol Sociobiol 72(5):79

Kshama D, Hrishikeshavan HJ, Shanbhogue R, Munonyedi US (1990) Modulation of baseline behavior in rats by putative serotonergic agents in three ethoexperimental paradigms. Behav Neural Biol 54(3):234–253

Kumar V, Bhat ZA, Kumar D (2013) Animal models of anxiety: a comprehensive review. J Pharmacol Toxicol Methods 68(2):175–183

Kurt M, Arik AC, Celik S (2000) The effects of sertraline and fluoxetine on anxiety in the elevated plus-maze test. J Basic Clin Physiol Pharmacol 11(2):173–180

Lampis V, Maziade M, Battaglia M (2011) Animal models of human anxiety disorders: reappraisal from a developmental psychopathology vantage point. Pediatric Res 69(5 Pt 2):77R–84R

Lembke A, Papac J, Humphreys K (2018) Our other prescription drug problem. N Eng J Med 378(8):693–695

Lister RG (1990) Ethologically-based animal models of anxiety disorders. Pharmacol Ther 46(3):321–340

Liu Z, Kariya MJ, Chute CD, Pribadi AK, Leinwand SG, Tong A, Curran KP, Bose N, Schroeder FC, Srinivasan J, Chalasani SH (2018) Predator-secreted sulfolipids induce defensive responses in C. elegans. Nat Commun 9(1):1128

Loonen AJ, Ivanova SA (2015) Circuits regulating pleasure and happiness: the evolution of reward-seeking and misery-fleeing behavioral mechanisms in vertebrates. Front Neurosci 9:394

Lowry CA, Johnson PL, Hay-Schmidt A, Mikkelsen J, Shekhar A (2005) Modulation of anxiety circuits by serotonergic systems. Stress 8(4):233–246

Mansouri MT, Soltani M, Naghizadeh B, Farbood Y, Mashak A, Sarkaki A (2014) A possible mechanism for the anxiolytic-like effect of gallic acid in the rat elevated plus maze. Pharmacol Biochem Behav 117:40–46

Matsuda K, Hagiwara Y, Shibata H, Sakashita A, Wada K (2013) Ovine corticotropin-releasing hormone (oCRH) exerts an anxiogenic-like action in the goldfish, *Carassius auratus*. Gen Comp Endocrinol 188:118–122

Maximino C, Marques T, Dias F, Cortes FV, Taccolini IB, Pereira PM, Colmanetti R, Lozano R, Gazolla RA, Tenoria R, Tavares de Lacerda RI, Rodrigues STK, de Oliveira Valéria, Coelho Lameirao S, Pontes AAA, Romao CF, Prado VM, Gouveia A (2007) A comparative analysis of the preference for dark environments in five teleosts. Int J Comp Psychol 20(4):351–367

Maximino C, de Brito TM, da Silva Batista AW, Herculano AM, Morato S, Gouveia A (2010a) Measuring anxiety in zebrafish: a critical review. Behav Brain Res 214(2):157–171

Maximino C, de Brito TM, Colmanetti R, Pontes AAA, de Castro HM, de Lacerda RIT, Morato S, Gouveia A Jr (2010b) Parametric analyses of anxiety in zebrafish scototaxis. Behav Brain Res 210(1):1–7

Maximino C, de Brito TM, de Mattos Dias CAG, Gouveia A Jr, Morato S (2010c) Scototaxis as anxiety-like behavior in fish. Nat Protoc 5(2):209

Maximino C, da Silva AWB, Gouveia A, Herculano AM (2011) Pharmacological analysis of zebrafish (*Danio rerio*) scototaxis. Prog Neuro-Psychopharmacol Biolo Psychiatry 35(2):624–631

Maximino C, Benzecry R, Oliveira KRM, de Batista OBE, Herculano AM, Rosemberg DB, de Oliveira DL, Blaser R (2012) A comparison of the light/dark and novel tank tests in zebrafish. Behaviour 149(10–12):1099–1123

McNaughton N, Zangrossi H (2008) Theoretical approaches to the modeling of anxiety in animals. In: Blanchard DC, Griebel G, Nutt DJ (eds) Handbook of anxiety and fear, vol 17. Elsevier Publishing, Amsterdam, pp 11–27

Measey GJ (1998) Diet of feral *Xenopus laevis* (Daudin) in South Wales, UK. J Zool 246(3):287–298

Morris A, Green M, Martin H, Crossland K, Swaney WT, Williamson SM, Rae R (2018) A nematode that can manipulate the behaviour of slugs. Behav Process 151:73–80

Nestler EJ, Hyman SE (2010) Animal models of neuropsychiatric disorders. Nat Neurosci 13(10):1161

O'Neill SJ, Williamso JE, Tosetto L, Brown C (2018) Effects of acclimatisation on behavioural repeatability in two behaviour assays of the guppy *Poecilia reticulata*. Behav Ecol Sociobiol 72(10):166

Pellow S, Chopin P, File SE, Briley M (1985) Validation of open: closed arm entries in an elevated plus-maze as a measure of anxiety in the rat. J Neurosci Methods 14:149–167

Perusini JN, Fanselow MS (2015) Neurobehavioral perspectives on the distinction between fear and anxiety. Learn Mem 22(9):417–425

Prater CM, Harris BN, Carr JA (2018) Tectal CRFR1 receptors modulate food intake and feeding behavior in the South African clawed frog *Xenopus laevis*. Horm Behav 105:86–94

Ramos A (2008) Animal models of anxiety: do I need multiple tests? Trends Pharmacol Sci 29(10):493–498

Ramos A, Berton O, Mormède P, Chaouloff F (1997) A multiple-test study of anxiety-related behaviours in six inbred rat strains. Behav Brain Res 85(1):57–69

Ramos A, Mellerin Y, Mormede P, Chaouloff F (1998) A genetic and multifactorial analysis of anxiety-related behaviours in Lewis and SHR intercrosses. Behav Brain Res 96(1–2):195–205

Ramos A, Pereira E, Martins GC, Wehrmeister TD, Izídio GS (2008) Integrating the open field, elevated plus maze and light/dark box to assess different types of emotional behaviors in one single trial. Behav Brain Res 193(2):277–288

Ren J, Friedmann D, Xiong J, Liu CD, DeLoach KE, Ran C, Pu A, Sun Y, Weissbourd B, Neve RL, Horowtiz M, Luo L (2018) Anatomically defined and functionally distinct dorsal raphe serotonin sub-systems. Cell 175(2):472–487. https://doi.org/10.1016/j.cell.2018.07.043

Ressler KJ, Mayberg HS (2007) Targeting abnormal neural circuits in mood and anxiety disorders: from the laboratory to the clinic. Nat Neurosci 10(9):1116

Richendrfer H, Pelkowski SD, Colwill RM, Creton R (2012) On the edge: pharmacological evidence for anxiety-related behavior in zebrafish larvae. Behav Brain Res 228(1):99–106

Rodgers RJ, Cao BJ, Dalvi A, Holmes A (1997) Animal models of anxiety: an ethological perspective. Braz J Medical Bioll Res 30:289–304

Rosenthal R (1994) Parametric measures of effect size. In: Cooper H, Hedges LV (eds) The handbook of research synthesis. Russell Sage Foundation, New York, pp 231–244

Rotzinger S, Lovejoy DA, Tan LA (2010) Behavioral effects of neuropeptides in rodent models of depression and anxiety. Peptides 31(4):736–756

Sackerman J, Donegan JJ, Cunningham CS, Nguyen NN, Lawless K, Long A, Benno RH, Gould GG (2010) Zebrafish behavior in novel environments: effects of acute exposure to anxiolytic compounds and choice of *Danio rerio* line. Int J Comp Psychol 23(1):43

Shin JT, Fishman MC (2002) From zebrafish to human: modular medical models. Ann Rev Genom Hum Genet 3(1):311–340

Silva LJ, Lino CM, Meisel LM, Pena A (2012) Selective serotonin re-uptake inhibitors (SSRIs) in the aquatic environment: an ecopharmacovigilance approach. Sci Total Environ 437:185–195

Simmons DBD, McCallum ES, Balshine S, Chandramouli B, Cosgrove J, Sherry JP (2017) Reduced anxiety is associated with the accumulation of six serotonin reuptake inhibitors in wastewater treatment effluent exposed goldfish *Carassius auratus*. Sci Rep 7(1):17001

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

Smith A (2002) Effects of caffeine on human behavior. Food Chem Toxicol 40(9):1243–1255

Sokolowski K, Corbin JG (2012) Wired for behaviors: from development to function of innate limbic system circuitry. Front Mol Neurosci 5:55

Sommi RW, Crismon ML, Bowden CL (1987) Fluoxetine: a serotonin-specific, second-generation antidepressant. Pharmacother J Hum Pharmacol Drug Ther 7(1):1–14

Steimer T (2011) Animal models of anxiety disorders in rats and mice: some conceptual issues. Dialog Clin Neurosci 13(4):495–506

Stewart AM, Kalueff AV (2015) Developing better and more valid animal models of brain disorders. Behav Brain Res 276:28–31

Stewart A, Wu N, Cachat J, Hart P, Gaikwad S, Wong K, Utterback E, Gilder T, Kyzar E, Newman A, Carols D, Chang K, Hook M, Rhymes C, Caffery M, Greenberg M, Zadina J, Kalueff AV (2011a) Pharmacological modulation of anxiety-like phenotypes in adult zebrafish behavioral models. Prog Neuro-Psychopharmacol Biol Psychiatry 35(6):1421–1431

Stewart A, Maximino C, De Brito TM, Herculano AM, Gouveia A, Morato S, Cachet JM, Gaikwad S, Elegante MF, Hart PC, Kalueff AV (2011b) Neurophenotyping of adult zebrafish using the light/dark box paradigm. In: Kalueff AV, Cachat J (eds) Zebrafish neurobehavioral protocols. Humana Press, Clifton, pp 157–167

Stewart A, Gaikwad S, Kyzar E, Green J, Roth A, Kalueff AV (2012) Modeling anxiety using adult zebrafish: a conceptual review. Neuropharmacol 62(1):135–143

Stewart AM, Braubach O, Spitsbergen J, Gerlai R, Kalueff AV (2014) Zebrafish models for translational neuroscience research: from tank to bedside. Trends Neurosci 37(5):264–278

Tandon P, Conlon F, Furlow JD, Horb ME (2016) Expanding the genetic toolkit in *Xenopus*: approaches and opportunities for human disease modeling. Devel Biol 426(2):325–335

Tierney AJ (2018) Invertebrate serotonin receptors: a molecular perspective on classification and pharmacology. J Exp Biol 221(19):jeb184838

Tovote P, Fadok JP, Lüthi A (2015) Neuronal circuits for fear and anxiety. Nat Rev Neurosci 16(6):317–331

Treit D, Engin E, McEown K (2009) Animal models of anxiety and anxiolytic drug action. Behavioral neurobiology of anxiety and its treatment. Springer, Berlin, pp 121–160

Trullas R, Skolnick P (1993) Differences in fear motivated behaviors among inbred mouse strains. Psychopharmacol 111(3):323–331

van der Staay FJ, Arndt SS, Nordquist RE (2009) Evaluation of animal models of neurobehavioral disorders. Behav Brain Funct 5(1):11

Van Mier P, Joosten HWJ, Van Rheden R, Ten Donkelaar HJ (1986) The development of serotonergic raphespinal projections in *Xenopus laevis*. Int J Devel Neurosci 4(5):465–475

Varty GB, Morgan CA, Cohen-Williams ME, Coffin VL, Carey GJ (2002) The gerbil elevated plus-maze I: behavioral characterization and pharmacological validation. Neuropsychopharmacol 27(3):357–370

Vendruscolo LF, Takahashi RN, Brüske GR, Ramos A (2003) Evaluation of the anxiolytic-like effect of NKP608, a NK1-receptor antagonist, in two rat strains that differ in anxiety-related behaviors. Psychopharmacol 170(3):287–293

Vetulani J, Stawarz RJ, Dingell JV, Sulser F (1976) A possible common mechanism of action of antidepressant treatments. Naunyn-Schmiedeberg's Archi Pharmacol 293(2):109–114

Walf AA, Frye CA (2007) The use of the elevated plus maze as an assay of anxiety-related behavior in rodents. Nat Prot 2(2):322

Williams TD (2008) Individual variation in endocrine systems: moving beyond the 'tyranny of the Golden Mean'. Philos Trans R Soc Lond B Biol Sci 363(1497):1687–1698

Willner P (1984) The validity of animal models of depression. Psychopharmacol 83(1):1–16

Wong K, Elegante M, Bartels B, Elkhayat S, Tien D, Roy S, Goodspeed J, Suciu C, Tan J, Grimes C, Chung A, Rosenberg M, Gaikwad S,

Denmark A, Jackson A, Kadri F, Chung KM, Stewart S, Glider T, Beeson E, Zapolsky I, Wu N, Cachat J, Kalueff AV (2010) Analyzing habituation responses to novelty in zebrafish (*Danio rerio*). Behav Brain Res 208(2):450–457

Zohar J, Westenberg HGM (2000) Anxiety disorders: a review of tricyclic antidepressants and selective serotonin reuptake inhibitors. Acta Psychiatr Scand 101(S403):39–49