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ORIGINAL ARTICLE

Predator presence decreases food consumption in juvenile *Xenopus laevis*

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Abstract
Predators impact prey in direct (lethal) and indirect (non-lethal) manners. Predator-avoidance models capitalize on the non-lethal effects of predators to study how predator-induced fear impacts prey behavior and physiology. Here, we aimed to develop a predator avoidance model to determine how predators alter feeding and anxiety-like behavior in the African clawed frog (*Xenopus laevis*). We determined (1) the repeatability of frog behavior over time, (2) the effect of a stimulus (nothing, a size-matched or a large conspecific [potential predator]) on frog behavior, and (3) the effect of a stimulus on frog behavior in the presence of food. Twelve juvenile experimental frogs were exposed to all three stimulus conditions over 1 week. We predicted that (1) frog baseline behavior would be repeatable, and that (2) the presence of the large frog, but not size-matched frog, would increase fear and anxiety-like behaviors (hiding and inactivity) and would decrease food consumption and the number of air gulps. In the presence of both food and stimulus, experimental frogs ate significantly less when exposed to a large (potential predator) vs. a size-matched and no frog and took more time to first contact the food. Time spent inactive and number of air gulps did not differ across conditions. Few frogs hid during the behavioral trials. Time spent exploring and inactive and tank locations were repeatable over time. Overall, our paradigm is a viable model for studying the effects of predators on prey behavior, especially as it relates to feeding.

Significance statement
Predators can induce fear and anxiety in prey and can alter prey behavior. We sought to develop a predator-avoidance model to study prey anxiety and feeding behavior in African clawed frogs. We determined (1) the repeatability of baseline behavior over time, (2) the effect of a stimulus (no, a size-matched, or a large [predatory] frog) on frog behavior, and (3) the effect of a stimulus on behavior in the presence of food. The presence of a potential predator alone did not significantly alter prey behavior. With predator + food, experimental frogs took longer to contact food, ate less, and spent less time feeding. Most activity and location behaviors were repeatable over time. This paradigm is a viable model for studying the effects of predators on prey feeding behavior.

Keywords Amphibian · Predation · Prey · Fear · Feeding · Predator-avoidance model

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Introduction

Every day, animals in the wild are threatened by predators. Predators can directly affect prey survival, and predation can have both individual- (loss of reproductive fitness) and population-level (population size, demographic) impacts on prey (Lima 1998; Abrams and Ginzburg 2000). Prey animals, in theory, should balance activities in order to minimize risk of predation while still maintaining adequate time for foraging and reproduction. This trade-off between food and safety governs many animal behaviors (McNamara and Houston 1986), and the presence of predators, or predator cues, can alter various aspects of prey foraging. For example, prey animals can

shift feeding to safer but less rewarding locations for foraging or spatially redistribute to areas with a perceived lower risk of predation (Ripple and Beschta 2004; Wirsing et al. 2007). Prey can adjust the time of day or amount of time in which to be active and feed (Clarke 1983; Caldwell 1986; Fenn and Macdonald 1995) and may spend less time foraging and more time hiding and being vigilant in the presence of a predator (Abramsky et al. 2002). When redistributing to different areas or foraging at different times of day, animals may find that this new setting provides a less abundant or less energy-rich food sources (Skilleter and Peterson 1994). All of these behavioral changes not only reduce the chances of a prey animal encountering and falling victim to a predator (Valeix et al. 2009) but can also decrease the animal's fitness (Sibly and Calow 2009).

Given that predators can alter many aspects of prey behavior, including foraging and reproduction, an appreciation for the non-lethal role of predators on prey has developed (Peckarsky et al. 1993; Krebs et al. 1995; Schmitz et al. 1997; Preisser et al. 2005). The near continual (non-lethal) predator threat on prey has been termed the ecology of fear (Kavaliers and Choleris 2001; Clinchy et al. 2011; Clinchy et al. 2013). This chronic threat, or fear, is a form of sustained psychological stress (or anticipatory stress; see Herman et al. 2003), as prey species are not in immediate danger of predation but are continually wary of possible attack. Predator-induced fear can play a major role in individual prey behavior and physiology, as well as in prey population dynamics and community structure. For example, emerging research has shown that increased predator pressure can inhibit brain cell proliferation in electric fish (*Brachyhypopomus occidentalis*; Dunlap et al. 2016) and can increase the neural and behavioral responses to predator-specific signals in crabs (*Neohelice granulate*; Magani et al. 2016). These studies suggest that predator threat can impact prey neural structure, thus providing a mechanism for how predator threat can alter behavior. Additionally, the mere (auditory) presence of a large carnivore can have ecosystem-wide impacts on community structure, an effect driven by the predator's fear-inducing capabilities (Suraci et al. 2016). On a broader scale, a meta-analysis found that the simple intimidation of prey by predators had as great or a greater effect than direct killing on prey demography (Preisser et al. 2005), highlighting the import role that predator-induced fear can play in prey species (Clinchy et al. 2013).

Predator-avoidance models of fear rely on exposing prey to non-life-threatening predator situations such as having a physical barrier placed between predator and prey or by exposing prey to predator urine or scent (Cohen and Zohar 2004; Adamec et al. 2007; Zoladz et al. 2008). By eliminating actual physical injury to the prey, these predator-avoidance models allow insight into the behavioral, physiological, and psychological cost of predator-induced fear on a prey animal. These models generally involve either acute (minutes to hours) or chronic (hours or repeated sessions) predator exposure and both methods have effects on various aspects of prey behavior and stress physiology (Adamec 1997; Cohen et al. 2003; Adamec et al. 2006; Hawlena and Schmitz 2010; Clinchy et al. 2013; Carr 2015; Abreu et al. 2016; Harris and Carr 2016). Overall, predator avoidance models have provided insight into predator-prey relationships (see Clinchy et al. 2013) and can be a valuable model for studying how predators impact physiology and behavioral trade-offs in prey.

In our present study, we aimed to develop a predator avoidance model in *Xenopus laevis (*African-clawed frog), with the specific goal of investigating the impact of a predator on feeding and anxiety-like behaviors. The majority of existing predator avoidance models have used rodents as focal animals (e.g., *Rattus norvegicus* [McGregor and Dielenberg 1999; Bramley et al. 2000; Adamec et al. 2006]; *Mus musculus* [Kavaliers and Choleris 2001]). Recently, though, fish have been used as subjects (zebrafish [*Danio rerio*] [Bass and Gerlai 2008; Gerlai et al. 2009; Ahmed et al. 2011; Luca and Gerlai 2012]; threespot damselfish [*Stegastes planifrons*] [Helfman 1989]). Fish models have enabled cross-species analysis of conserved affective phenotypes (Kalueff et al. 2007; Stewart and Kalueff 2014; Stewart et al. 2014) and are beneficial for comparative studies as neural structures associated with fear (the habenula and limbic system), and many predator detection pathways are evolutionary conserved (Carr 2015; Loonen and Ivanova 2015). Expanding predator avoidance models to additional species would be beneficial for comparative studies and for understanding predator-prey dynamics in different ecological contexts. Thus, we used *X. laevis* in our model.

X. laevis offer many advantages for studying anxiety and fear-related behaviors because they are genetically tractable and have a well characterized developmental period. Due to their popularity as a study species, much is known about their physiology and neuroanatomy, and several genetic and molecular tools are available. Lastly, because many of the sensory pathways for detecting predators in *X. laevis* are also present in mammals, *X. laevis* can be an excellent model for providing insight into subcortical pathways triggering fear and anxiety (Carr 2015). A predator avoidance model using *X. laevis* could also provide information on the neuroendocrine underpinnings of trade-offs between feeding and fleeing behavior and could provide additional insight into ways in which predators can alter neural substrates which regulate prey behavior. Despite their popularity as research subjects, there is a surprising lack of available models for studying fear, anxiety, and stress-related behavior in *X. laevis*.

In this study, we exposed juvenile frogs to a large conspecific (potential predator), a size-matched conspecific, or nothing and measured food consumption and multiple behaviors. We chose large frogs as predators due to the fact that in the wild, large *X. laevis* are indiscriminant feeders and will attack and consume most anything, including smaller *X. laevis*

(Tinsley and Kobel 1996). Size-matched frogs were used so we could determine effects of a predatory vs a non-predatory conspecific. Our experimental design allowed us to (1) estimate repeatability of baseline frog behavior over time, (2) to determine the effect of the presence of a conspecific (size-matched or large) on frog behavior, and (3) to determine the effect of a conspecific (size-matched or large) on behavior in the presence of food. We predicted that (1) frog behavior would be repeatable, (2) the presence of the large frog would increase fear/anxiety-like behaviors (hiding, inactivity) and decrease air gulping, and (3) the presence of the large (predatory), but not size-matched frog, would decrease consumption of food and increase fear/anxiety-like behaviors (e.g., hiding, air gulps, inactivity) in juvenile *X. laevis*. Repeated surface breaches would make the juvenile frogs easier targets for a bottom- or surface-sitting large *X. laevis* (Chum et al. 2013), thus, we expected air gulping/surface breaches to decrease. Preference for the lower parts of the tank has been exhibited in zebrafish and goldfish (Khor et al. 2013; Matsuda et al. 2013), and treatment with an anxiogenic peptide (corticotropin-releasing factor [CRF]) resulted in an increase in time to come to the surface (Matsuda et al. 2013). Thus decreased surface breaches could be a sign of increased anxiety in *X. laevis* as well. If large, but not size-matched, frogs decrease feeding and increase anxiety-like behaviors, then this paradigm is the first step in developing a robust predator-avoidance model for this species.

Methods

Animals

A total of 12 Nieuwkoop-Faber stage 66 frogs (Nieuwkoop and Faber 1994) were purchased from Xenopus Express (Homosassa, FL, USA) and were group housed ($n = 5$–8 frogs per tank at 20 °C) in 20-L glass aquaria containing 4 L deionized water conditioned with 1.25 g of Instant Ocean (Oceanic Natural Sea Salt Mix, Instant Ocean, Blacksburg, VA). By definition, Stage 66 *Xenopus* have completed metamorphosis (Nieuwkoop and Faber 1994) and are classified as juvenile frogs. Frogs were maintained in the laboratory for approximately 52 days (range 33–61) prior to testing. Starting body mass was 2.18 ± 0.24 g (mean ± SEM) and did not differ by sex (female 2.31 g, male 2.12 g). Ending snout-vent length was 2.63 ± 0.09 cm. Prior to experimentation, frog tanks were cleaned and frogs were fed Frog Brittle (Nasco) floating pellets three times per week. The light regimen was 12L:12D. Following behavioral experiments, juvenile frogs were euthanized in MS-222 (1 g/L dH_2O, Sigma-Aldrich, St. Louis, MO, USA) with equal parts sodium bicarbonate ($NaHCO_3$, EM Science, Gibbstown, USA), a small incision was made through the abdominal skin and musculature, and the frogs were fixed in 10 % neutral buffered formalin (NBF, EMD Chemicals, Inc., Gibbstown, USA). Following 48 h in NBF fixative, frogs were rinsed in water and dissected to determine sex (four female, eight males) as external genitalia were not yet visible at this stage. Gonads of all frogs were photographed by a Nikon SMZ 1500 microscope with Nikon DXM 1200F CCD for record keeping. Large stimulus frogs (all female; average body mass 99.75 ± 5.50 g [mean ± SEM]) were from our in-house colony (originally purchased from a *Xenopus* supplier) and kept in large, flow-through 160-L aquaria at a stocking density of 20 per tank. Size-matched stimulus frogs were housed separately from experimental frogs but were approximately the same age and size as experimental animals.

Testing chamber

Predator-avoidance tests were conducted in 1 of 3 glass aquaria (50 L × 25 W × 30 H cm) externally lined by black plastic to prevent outside visual stimulation. Tanks were separated into two 25-cm chambers by commercially available tank dividers (#TDMBX, Aqua Life, Hauppauge, NY); dividers were made of clear plastic with holes measuring ~0.16 cm in diameter and ~0.64 cm apart (Fig. 1). To provide shelter, a 3.81-cm PVC elbow was spray painted black (Truck Bed Coating, Rust-Oleum, Vernon Hills, IL) and secured to a strip (6.35 × 25 cm) of Plaskolite acrylic sheeting using waterproof sealant (Marine Adhesive Sealant 05203, 3M, St. Paul, MN). The PVC hides were placed midway between the divider and the back wall of the tank, roughly 7.62 cm from either side; the openings of the PVC elbow faced the divider. Chicken liver (1 g; Pilgrim's Pride Corporation, Greenly, CO) was adhered (HFT Super Glue, Harbor Freight Tools, Camarillo, CA) to two galvanized steel washers that were glued together (Gorilla Glue, The Gorilla Glue Company, Cincinnati, OH) and painted black with the same paint used on the PVC elbows (total size 4.45 cm in diameter, 0.64 cm high). The washer with liver was placed 3.81 cm from the divider, between the divider and the PCV elbow, and 12.7 cm from either sidewall. The prey's side of the tank was visually divided into thirds using tape underneath the tank bottom: the first third (front) being near the divider and, when applicable, contained the food; the second third (middle) contained the hide; and the back third (back) was located farthest from the divider. Tanks were filled with 12 L of water (0.3 g Instant Ocean/L), and water temperature was recorded after each test.

Experimental design

A total of 12 juvenile frogs were exposed in a Latin square randomized fashion over 1 week to a control (no frog), a size-matched conspecific, or a large conspecific (potential

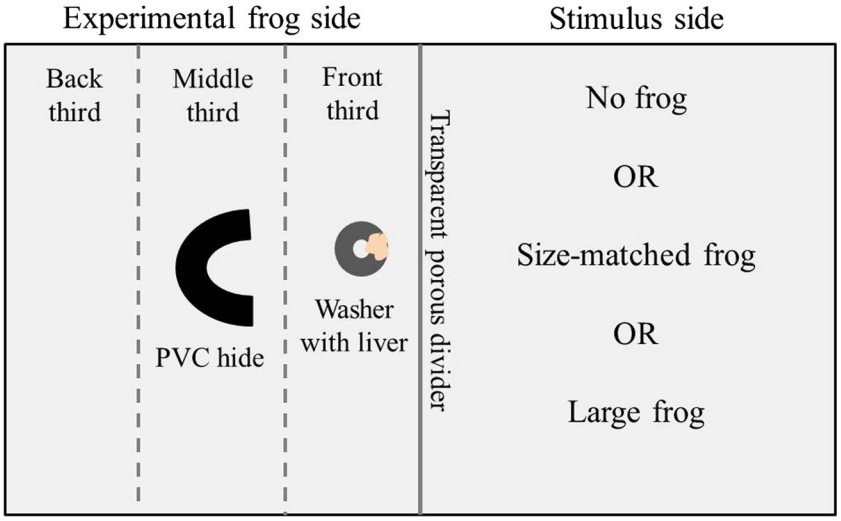

Fig. 1 Schematic of predator avoidance testing arena. For testing, juvenile experimental frogs (n = 12) were confined to the side of the tank with the PCV elbow hide and, when applicable (during part c only), the washer with chicken liver; the stimulus (no frog, size-matched frog, or large frog) was located on the other side of the transparent porous divider. The experimental frog's side was divided, via tape under the tank, into thirds. Each experimental frog was exposed to all three stimuli over 1 week

predator) for 50 min, and behavior was recorded. One third (4) of the experimental frogs were exposed to the large frog first; one third were exposed to the small frog first; and the remaining one third were exposed to the no stimulus condition first. The behavioral session was broken into three parts (a, b, and c). Experimental frogs were first given 10 min to become acclimated to the tank (part a). Immediately following the acclimation period, a stimulus frog was carefully lowered into the stimulus section (see Fig. 1) of the tank with a fish net, and experimental frog behavior was recorded for 40 min. The first 10 min of stimulus exposure was used to measure experimental frog response to only the stimulus (part b), the remaining 30 min was used to measure food consumption (1 g chicken liver) in the presence of the stimulus (part c). The chicken liver was attached to a washer to prevent the frog from taking the food to the hide, and was carefully added to the tank using a gloved hand immediately following part b of the experiment. Forty-eight hours prior to experimentation the focal frog was first isolated in a 30 L × 15 W × 20 H-cm tank with 1 L of water and 0.3 g of Instant Ocean and then fed 1 Nasco sinking pellet. Trials were conducted in the dark using an infrared light and infrared camera (Panasonic WV-CP604, Kadoma, Japan), and the 50-min behavioral video recordings were later scored using JWatcher V1.0. Behavioral videos were collected and scored by two of the authors, and each experimental frog was exposed to all three conditions. Thus, complete blinded procedures were not possible because the stimulus frog was present in parts b and c of the trial. Scorers did, however, train together and follow a specific and carefully constructed ethogram. To minimize variation, they also randomized the order in which they scored trials and each animal's behavior was scored by a single person. Behaviors scored and analyzed included the following: latency to feed (part c only), latency to hide, exploring the tank edges (duration), inactive (duration), open swimming (duration), air gulps (count), hiding (bouts, duration), and feeding (part c only; bouts, duration). Additionally, every 30 s, the experimental frog's location (tank third) and directionality (facing divider, or not) were scored. After the trial, frogs were returned to their home tank and fed.

Analysis

Data were analyzed to answer three main questions. First, how repeatable is experimental frog baseline behavior (part a) prior to each stimulus conditions (no, size-matched, or large frog)? Second, what is the effect of the stimulus (no, size-matched, or large frog) on experimental frog behavior (comparison of part b data across conditions)? And third, what is the effect of the stimulus on food-related and non-food-related behavior (comparison of part c data across conditions)? All data were checked for normality using Shapiro-Wilks and Kolmogorov-Smirnov tests and when appropriate, sphericity (using Mauchly's test). To estimate repeatability, we used the methods of Lessells and Boag (1987) to calculate an intraclass correlation coefficient (ICC) for each behavior during part a (baseline) across the three treatment conditions. For each analysis, we include the ICC, the F statistic, and the P value. Repeatability, determined via ICC, is the amount of behavioral variation that comes from differences between different individuals. If an individual animal behaves consistently over time, and differently from another animal, that behavior is repeatable. Behavioral repeatability can be used to estimate the genetic basis or heritability of a behavioral trait or to assess whether individuals consistently behave differently from one another, a measure useful when determining how different

(ecological or environmental) variables influence behavioral consistency (see Bell et al. 2009 for a full discussion). Here, we were interested in repeatability of baseline behavior over time in individual frogs. If baseline behaviors are repeatable, this suggests that order of stimulus presentation does not alter baseline measures of behavior and that frogs behave consistently in our testing paradigm. Durational data (time spent: inactive, exploring tank edges, open swimming, hiding, and feeding) were analyzed using repeated measures (RM) ANOVA with stimulus presentation as a covariate. Count and proportion data (number of air gulps; proportion of 30-s scans in front third, middle third, and back third of tank; feeding bouts in part c) were analyzed using Friedman ANOVA tests. For part c data, latency to contact the liver, grams of liver consumed/body mass of the focal frog for each 30-min food trial, and grams of liver consumed per minute spent feeding (30 min—latency)/body mass of the focal frog were compared across stimulus conditions using RM ANOVA with order of stimulus presentation as a covariate. Latency to contact the liver data were log10 transformed to improve normality (values presented as untransformed data for ease of interpretation).

Effect size estimates are presented for each main effects analysis; for RM ANOVA, the partial eta squared is reported and Kendall's W is reported for the Friedman test. Due to a priori predictions, significant main effects were followed up with Fisher's LSD (for ANOVA) and Wilcoxon signed rank (for Friedman's) tests.

Results

Question 1: consistency of baseline behavior over trials

Behavior from part a (10 min of undisturbed recording) was compared across all trial conditions, prior to addition of the stimulus, to see if baseline behaviors varied over time. Due to technical issues, one video was lost from this dataset and thus data from 11 frogs are included here. Behavioral data are presented in Table 1.

ICCs for measured behaviors ranged from −0.06 to 0.36 and are in line with previously published repeatability estimates for behavior (Bell et al. 2009; Wolak et al. 2012). Individual behavioral ICC results are as follows: duration of time spent exploring the tank edges ICC = 0.32, $F_{11,23} = 2.42$, $P = 0.035$; duration of time inactive ICC = 0.36, $F_{11,23} = 2.71$, $P = 0.020$; duration of time swimming in the open tank ICC = −0.06, $F_{11,23} = 0.83$, $P = 0.615$; air gulps ICC = 0.06, $F_{11,23} = 1.19$, $P = 0.347$; proportion of 30-s scans spent in front third ICC = 0.31, $F_{11,23} = 2.37$, $P = 0.039$; middle third ICC = 0.01, $F_{11,23} = 1.02$, $P = 0.459$ or back third ICC = 0.34, $F_{11,23} = 2.54$, $P = 0.023$; and proportion of scans facing the tank divider ICC = 0.26, $F_{11,23} = 2.04$, $P = 0.072$. No frogs hid during part a videos, and therefore, no hiding data were available for analysis.

Question 2: behavioral response to a stimulus frog only

Behavior from part b (10 min of stimulus presentation) was compared across stimulus conditions to determine if the presence of a conspecific altered frog behavior. Stimulus condition significantly affected the number of air gulps ($\chi^2 = 9.04$, $P = 0.011$, Kendall's $W = 0.377$), and the proportion of scans in which the experimental frog was facing the divider ($\chi^2 = 7.70$, $P = 0.021$, Kendall's $W = 0.321$). Experimental frogs spent more time facing the divider when there was a small frog present vs. no frog ($Z = 2.941$, $P = 0.003$); no other comparisons differed (no frog vs. large $Z = 0.71$, $P = 0.480$; small vs. large $Z = 1.65$, $P = 0.099$). For air gulps, however, following Wilcoxon signed rank post hoc tests, no groups actually differed in this behavior, although there is a marginal non-significant trend for the no frog vs. large frog comparison (no frog > large) and the large vs. small comparison (small > large; nothing vs. small $Z = 1.61$, $P = 0.107$; no frog vs. large $Z = 1.789$, $P = 0.074$; small vs. large $Z = 1.77$, $P = 0.077$). Type of stimulus did not alter the proportion of scans spent in different thirds of the cage (front third, $\chi^2 = 5.61$, $P = 0.061$, Kendall's $W = 0.234$; middle third, $\chi^2 = 1.76$, $P = 0.416$, Kendall's $W = 0.073$; back third, $\chi^2 = 4.44$, $P = 0.108$, Kendall's $W = 0.185$).

Additionally, stimulus type did not alter duration of time spent swimming in open water ($F_{2,22} = 0.31$, $P = 0.738$, partial eta squared = 0.030), and although not statistically significant, there was a trend for a stimulus effect on duration of time spent exploring ($F_{2,22} = 3.29$, $P = 0.058$, partial eta squared = 0.248) and time spent inactive ($F_{2,22} = 3.21$, $P = 0.062$, partial eta squared = 0.243). Only 1 of the 12 frogs hid during exposure to the large stimulus frog; no other frogs hid during any other part b trials. Due to this, we did not analyze hiding behavior statistically (but see Table 1 for data summary).

Question 3: behavioral responses to food and a stimulus frog

Behavior from part c (30 min of stimulus presentation + liver) was compared across stimulus conditions to determine if the presence of a conspecific altered frog feeding and non-feeding behaviors.

Latency to contact liver

Type of stimulus significantly affected the latency to contact the liver ($F_{2,20} = 4.12$, $P = 0.032$, partial eta squared = 0.292; Fig. 2A); stimulus presentation order did not significantly influence results ($F_{2,20} = 1.34$, $P = 0.285$). Experimental frogs

Table 1 Behavioral data from predator avoidance experiments

Behavior	Part a (600 s)			Part b (600 s)			Part c (1800 s)		
	Nothing	(pre) small	(pre) large	Nothing	Small	Large	Nothing	Small	Large
Durational (s)									
Inactive	350.44 ± 29.48	350.79 ± 21.37	385.90 ± 26.94	301.63 ± 32.94	421.36 ± 30.65	508.61 ± 31.56	1110.27 ± 139.21	1141.61 ± 109.66	1423.07 ± 68.01
Open-water swimming	12.64 ± 3.97	8.30 ± 2.38	12.01 ± 4.31	4.54 ± 3.13	6.42 ± 1.46	4.85 ± 2.12	78.17 ± 11.90	128.90 ± 128.56	54.44 ± 15.35
Explore tank edges	230.32 ± 33.71	237.56 ± 21.50	197.29 ± 26.83	281.29 ± 35.62	169.33 ± 30.11	68.47 ± 27.96	249.08 ± 80.63	276.53 ± 41.80	224.15 ± 61.74
Latency to hide	600.00 (600.00–600.00)	600.00 (600.00–600.00)	600.00 (600.00–600.00)	600.00 (600.00–600.00)	600.00 (600.00–600.00)	600.00 (399.82–600.00)	1800 (119.15–1800)	1800 (399.82–1800)	1800 (576.48–1800)
Hiding*	0.00 (0.00–0.00)	0.00 (0.00–0.00)	0.00 (0.00–0.00)	0.00 (0.00–0.00)	0.00 (0.00–0.00)	0.00 (0.00–178.47)	0.00 (0.00–178.47)	0.00 (0.00–14.29)	0.00 (0.00–472.17)
Latency to contact liver	–	–	–	–	–	–	**422.85 ± 140.61a**	**387.61 ± 150.71a**	**1130.10 ± 210.63b**
Feeding	–	–	–	–	–	–	**782.28 ± 114.65a**	**515.68 ± 108.39a, b**	**189.96 ± 103.80b**
Counts/bouts*									
Hiding	0.00 (0.00–0.00)	0.00 (0.00–0.00)	0.00 (0.00–0.00)	0.00 (0.00–0.00)	0.00 (0.00–0.00)	0.00 (0.00–1.00)	0.00 (0.00–2.00)	0.00 (0.00–4.00)	0.00 (0.00–1.00)
Air gulps	7.50 (0.00–19.00)	9.00 (3.00–34.00)	9.00 (0.00–21.00)	12.00 (0.00–24.00)	6.50 (0.00–15.00)	1.00 (0.00–30.00)	11.50 (3.00–59.00)	15.00 (2.00–43.00)	8.00 (3.00–20.00)
Feeding	–	–	–	–	–	–	3.50 (0.00–7.00)	4.00 (0.00–14.00)	1.00 (0.00–13.00)
30-s scans*									
Location									
In front third of tank	0.48 (0.19–0.86)	0.48 (0.19–0.62)	0.48 (0.00–0.86)	0.48 (0.10–0.86)	0.67 (0.10–0.90)	0.26 (0.00–0.81)	**0.73 (0.51–0.95)a**	**0.66 (0.25–0.82)a**	**0.41 (0.02–0.89)b**
In middle third of tank	0.17 (0.05–0.24)	0.14 (0.10–0.29)	0.12 (0.00–0.43)	0.10 (0.00–0.19)	0.10 (0.00–0.52)	0.10 (0.00–0.33)	**0.05 (0.02–0.16)a**	**0.10 (0.02–0.49)b**	**0.18 (0.02–0.46)b**
In back third of tank	0.38 (0.00–0.67)	0.40 (0.14–0.71)	0.40 (0.00–0.52)	0.38 (0.14–0.76)	0.24 (0.00–0.48)	0.55 (0.10–1.00)	0.17 (0.03–0.43)	0.25 (0.08–0.57)	0.48 (0.03–0.95)
Directionality									
Facing tank divider	0.45 (0.24–0.76)	0.50 (0.19–0.71)	0.45 (0.00–0.67)	**0.43 (0.05–0.81)a**	**0.67 (0.48–0.95)b**	**0.55 (0.00–1.00)a, b**	0.50 (0.25–0.61)	0.52 (0.41–0.74)	0.50 (0.05–0.84)
Facing away from tank divider	0.55 (0.19–0.76)	0.50 (0.24–0.81)	0.48 (0.00–0.71)	0.57 (0.19–0.95)	0.33 (0.05–0.52)	0.45 (0.00–1.00)	0.48 (0.39–0.75)	0.48 (0.26–0.59)	0.47 (0.16–0.95)

Data displayed as average ± SEM or median (range) from all three parts of the experimental protocol. Behaviors that differed significantly are bolded; different letters denote group differences. * denotes median (range) data.

Part a acclimation to tank, *Part b* stimulus only, *Part c* stimulus + food

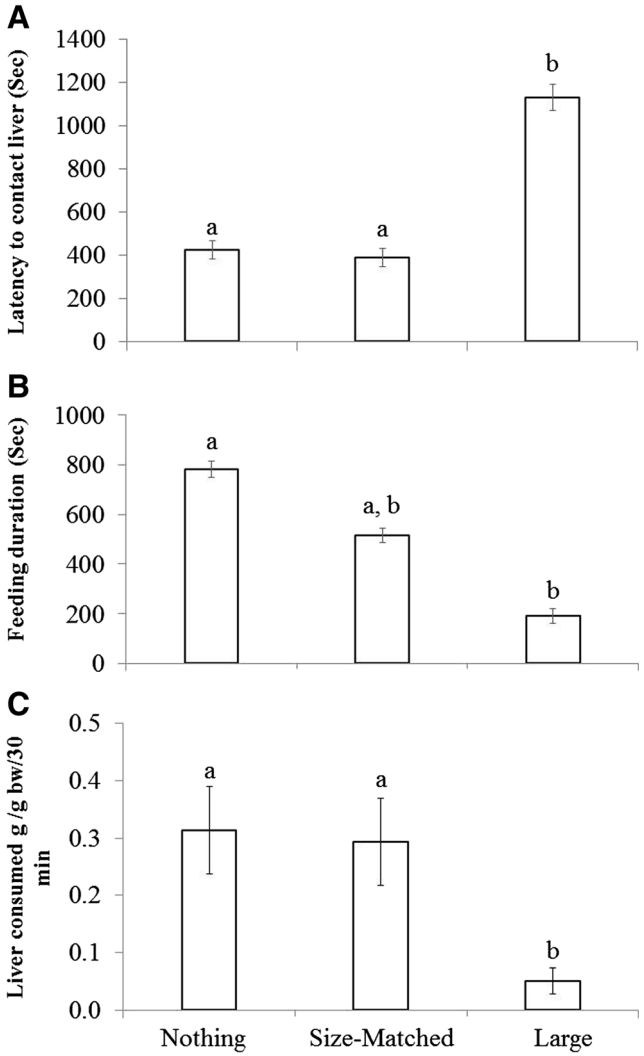

Fig. 2 Effect of stimulus condition on experimental frog (N = 12). **A** Latency to contact liver, **B** duration of time spent feeding during the 30-min trial, and **C** mass of liver consumed, adjusted for frog body mass, during a 30-min behavioral trial. Date presented are mean ± SEM; *different letters* represent significantly different groups

approached the liver more quickly when exposed to no frog (control) vs. a large frog (t = 2.91, P = 0.016), and also approached food more quickly when exposed to a size-matched frog than a large frog (t = 3.42, P = 0.007); however, there was no difference in latency to contact liver when frogs were exposed to no frog vs. size-matched stimulus frogs (t = 1.01, P = 0.333).

Feeding duration

Stimulus presentation significantly altered amount of time spent feeding ($F_{2,20}$ = 5.08, P = 0.016; partial eta squared = 0.33; Fig. 2B); stimulus presentation order did not have an effect of feeding duration ($F_{1,10}$ = 0.95, P = 0.352). Experimental frogs spent less time feeding in the presence of large frogs vs. no frog (t = 3.87, P = 0.003); no other stimulus conditions differed (small vs. large t = 2.12, P = 0.060; small vs. no frog t = 1.93, P = 0.083). Despite differences in duration, stimulus condition did not affect number of feeding bouts (χ^2 = 5.04, P = 0.080).

Liver consumption

Type of stimulus significantly affected the body-mass-adjusted mass of liver consumed by the experimental frog ($F_{2,20}$ = 5.49, P = 0.013, partial eta squared = 0.354; Fig. 2C); stimulus presentation order did not significantly influence results ($F_{1,10}$ = 0.15, P = 0.707). Experimental frogs ate significantly more food when exposed to no frog (control) vs. a large frog (t = 3.61, P = 0.005) and when exposed to a size-matched frog than a large frog (t = 2.92, P = 0.015); no frog vs. size-matched stimulus frog conditions did not differ (t = 0.45, P = 0.658).

In addition to calculating the amount of liver consumed overall, we also determined how much of the liver was consumed adjusted for the latency to contact liver (30 min trial—latency to contact liver) per gram of frog. Results mirrored those found above. Type of stimulus significantly affected the time-feeding and body-mass-adjusted mass of liver consumed by the experimental frog ($F_{2,20}$ = 4.88, P = 0.019, partial eta squared = 0.328); stimulus presentation order did not significantly affect results ($F_{1,10}$ = 0.481, P = 0.504). Experimental frogs ate significantly more food when exposed to no frog (control) vs. a large frog (t = 3.66, P = 0.003) and when exposed to a size-matched frog than a large frog (t = 2.5, P = 0.025); no frog vs. size-matched stimulus frog conditions did not differ (t = 0.50, P = 0.582).

Hiding

Regardless of stimulus, experimental frogs mostly chose not to utilize the provided hide during the stimulus + food portion of the test. During each stimulus condition only 25 % (3/12), experimental frogs hid for any duration of time. No frog hid during every trial. Due to the low number of frogs that hid, data were not analyzed statistically but medians are presented in Table 1 (for easier comparison, mean ± SEM in seconds for no, size-matched, and large frog conditions, respectively, are given here 7.80 ± 6.22, 2.39 ± 1.35, 43.38 ± 39.07).

Additional behaviors

Stimulus condition affected which third of the cage experimental frogs spent more time in (front third, χ^2 = 9.50, P = 0.009, Kendall's W = 0.396; middle third, χ^2 = 9.04, P = 0.011, Kendall's W = 0.377; back third, χ^2 = 1.50, P = 0.472, Kendall's W = 0.063). Compared to the no frog condition, experimental frogs spent less time in the front third of the tank (the part nearest to the stimulus frog) when

exposed to a large frog ($Z = 2.71$, $P = 0.007$) but not to a small frog ($Z = 1.81$, $P = 0.071$); experimental frogs were more likely to be found in the front third of the tank when there was a small stimulus frog vs. a large stimulus frog ($Z = 2.51$, $P = 0.012$). Additionally, experimental frogs were more likely to be in the middle third of the tank (the part with the hide) when there was no stimulus frog present (no frog vs. small $Z = 2.31$, $P = 0.021$; no frog vs. large $Z = 2.83$, $P = 0.005$; small vs. large $Z = 0.94$, $P = 0.349$).

Stimulus condition did not alter duration of time the experimental from spent swimming in open water ($F_{2,20} = 2.51$, $P = 0.106$, partial eta squared = 0.201), exploring the tank edges ($F_{2,20} = 0.09$, $P = 0.913$, partial eta squared = 0.009), or time inactive ($F_{2,20} = 2.65$, $P = 0.095$, partial eta squared = 0.210). Nor did it alter the number of air gulps ($\chi^2 = 2.09$, $P = 0.353$, Kendall's $W = 0.087$) or the proportion of scans facing the divider ($\chi^2 = 0.55$, $P = 0.758$, Kendall's $W = 0.023$).

Discussion

We set out to design a predator avoidance paradigm in order to answer three specific questions: (1) how repeatable is frog baseline behavior over time?; (2) what is the effect of the presence of a conspecific (size-matched or large [potential predator]) on frog behavior?; and (3) what is the effect of a conspecific (size-matched or large) on behavior in the presence of food? In a Latin squares randomized fashion, we exposed our frogs to either no frog, a size-matched, or a large frog (potential predator), with and without food present, and recorded experimental frog behaviors. Our results suggest that it is not the presence of a predator alone that influences the behavior of experimental frogs, but rather the combination of both predator and food. Continued use of this paradigm will allow us, for the first time, to study in depth the non-lethal effects that predators have on the feeding/fleeing behaviors in *X. laevis*.

In order to insure that our design can be developed into a robust predator avoidance assay, we estimated repeatability of frog baseline behavior across days. Baseline levels of exploring and inactivity, as well as proportion of time spent in the front third and back third, were repeatable, suggesting that these behaviors do not change markedly over time when frogs are in an undisturbed tank. With these findings, we can conclude that, in general, *X. laevis* have a measurable set of behaviors that remain consistent over trials.

When comparing behavior across only stimulus conditions (part b), we found that only directionality differed significantly (frogs more likely to face the divider when the stimulus as a small frog vs. no frog). However, there were non-significant trends for decreased air gulp number and increased time spent inactive in the presence of the large (predatory) frog. These results suggest that predators alone are not enough to influence the behavior of experimental frogs in this scenario. With the addition of food, however, marked changes in experimental frog behavior were noted (part c). Experimental frogs took longer to first contact the food, ate significantly less, and spent less time eating while in the presence of large (predatory) frogs when compared to no stimulus and to size-matched frogs. In addition to feeding behaviors, experimental frogs spent less time in the part of the tank near the divider (front third) when in the presence of the large frog and food, and spent more time in the area of the tank closest to the divider in the presence of a size-matched frog and food. Combined, data from parts b and c suggest that experimental frogs were able to detect the presence of the large frog across the tank divider.

The sensory modality/modalities used by experimental frogs to discriminate between the small vs. large (predatory) conspecific are not known. It is possible that experimental frogs used olfactory, auditory, and lateral line cues, either separately or in combination, as we do not expect visual cues to play a major role in this specific paradigm (*Xenopus* are better at detecting out-of-water, as opposed to in-water, visual cues; Tinsley and Kobel 1996). Future work to determine the mechanisms by which detection/discrimination occurs would be informative and could provide insight into how predators may influence prey physiology and behavior (Harris and Carr 2016; Magani et al. 2016).

Contrary to our predictions, we did not see a difference in time spent hiding in any portion of our experiment; however, few frogs chose to hide in the provided tubes, possibly due to the tube's large size. Frogs that did hide did so in a pattern in line with our prediction, with the most hiding occurring in the presence of the large frog. In addition to changes in hiding behavior, we predicted that predator presence would decrease air gulps and activity. We did see a significant main effect of stimulus alone (no food present) on air gulps, and data show a trend for decreasing air gulps from no-frog to small-frog to large-frog trials, however, post hoc tests were not significant. Additionally, when food and stimulus were present, the median number of gulps was lowest in the presence of a large conspecific, even though results were not statistically significant. We also observed a (non-significant) trend for time spent inactive in the presence of a stimulus only (no food present) and stimulus + food, with data mirroring our prediction of frogs spending more time inactive in the presence of the large frog. Combined, the results of our study show that the addition of food in the presence of a predator has a measurable effect on experimental frog behavior that is not produced in the presence of a predator alone. In addition, the order of stimulus presentation was randomized, and stimulus order was not significant in any of the analyses, suggesting that results obtained are not due to an order effect.

Other previously conducted predator avoidance studies have results which both agree with and contrast the findings from our study. Monclús found no significant difference in the amount of activity between European rabbits (*Oryctolagus cuniculus*) exposed to predator odor and those that were not, mirroring our findings (Monclús et al. 2005). Additionally, much like our study, Monclús found that on average, rabbits exposed to predator odor consumed less food, but unlike our experiment, there was no significant difference in the amount of time that rabbits spent feeding. Lastly, much like our experiment, zebrafish exposed to a predator did not alter their general activity levels but did spend significantly more time in the area of the tank furthest from the predator (Luca and Gerlai 2012). However, unlike our study, zebrafish exposed to a predator displayed erratic predator avoidance movement and decreased the distance swam (Luca and Gerlai 2012). Overall, our study mirrors finding from other studies in mammals and fish, suggesting that changes in predator-induced behaviors measured here are consistent with those in other predator exposure models.

Prey should respond to predator presence by increasing the use of hides or environmental refuges (Werner et al. 1983; Kotler 1984; Sih 1986, 1992); therefore, we were surprised to find that our experimental frogs did not hide in the presence a predator. In addition to the potential reason listed above (tube size), frogs may have not utilized the hide due to the fact that there was not a significant enough contrast between light and dark because tests were performed in the dark phase. Previous scototaxis test experiments using fishes have determined that fish prefer dark areas over light areas (Maximino et al. 2010). Time spent in the dark area is associated with increased anxiety, as pre-treatment with anti-anxiety medications, such as diazepam, increase the time the fish spends in the light area (Matsuda et al. 2011). Since *X. laevis* are nocturnal, we performed our study in the dark cycle. Repeating our paradigm in the light phase may be more similar to a scototaxis-like test and may result in increased hiding behavior.

Our final question was to determine the effect of a conspecific (size-matched or large) on feeding behavior. Our results clearly show that the presence of a potential predator impacts feeding behavior in juvenile *X. laevis*. Several research studies (Krebs et al. 1995; Zanette et al. 2003; Clinchy et al. 2004) suggest a synergistic effect of a predator and food on prey ecology and behavior. These studies aimed to understand the role that both predator pressure and food availability play in annual reproductive success. Rather than the traditionally accepted additive effects of predator and food, these studies showed there to be interactive effects between the two that produced a much greater ecological and behavioral response. An additive model would for example assign a value X to food and Y to predator pressure and predict reproductive success to be the value $X + Y$. However, studies (Krebs et al. 1995; Zanette et al. 2003; Clinchy et al. 2004) found that when food was added and predator pressure was lowered, the annual reproductive success increased to double of what was predicted from an additive model. This interactive effect may explain why in our experiment behaviors differed significantly only in the presence of both food and predator. This synergistic effect may be the key to understanding the behavioral responses of the frogs in our study and the mechanisms responsible for these behaviors.

Additionally, prey animal age might play a role in the behavioral response to a predator. We chose juvenile developmental frogs due to their relative size compared to larger frogs so that the larger frogs would represent a viable environmental predator. However, our results might indicate a unique set of behaviors for juveniles who are expected to show a robust response to the trade-off between feeding and hiding due to their increased need for nutrients to support their growth and development (Monaghan 2008; Crespi and Unkefer 2014). It is well documented that prolonged periods of hiding can lead to malnutrition and increased hunger and can cause a prey animal to be less vigilant and bolder in its foraging when threatened by predation (Lima 1998), and small frogs have fewer energy reserves than do large (older) ones. Our juvenile frogs may not have hidden in response to the large, predatory frogs due to increased hunger/need for energy. Thus, results from our behavioral model may differ in older frogs, due to different trade-off pressures, to learning, or to age effects (see Putman et al. 2015).

Conclusion

In summary, we have shown that (1) baseline frog activity and location is repeatable over time and (2) the combined presence of food and a predator decreased juvenile frog feeding behavior and food intake. These findings suggest that this paradigm is a viable model for studying the effects of predators on prey behavior, especially as it relates to feeding. Future mechanistic studies will focus on the sensory modalities used for potential predator detection, the role of anxiety-related brain regions (e.g., amygdala), stress (e.g., hypothalamic-pituitary-interrenal axis) hormones, satiety neuropeptides, and the use of anxiolytic drugs on the behaviors measured within our paradigm. We also plan to use more robust behavioral analysis software (e.g., Ethovision) that will allow us to determine distance traveled and average velocity; as in zebrafish, these measures were affected by a predator (Luca and Gerlai 2012). Future research may also use experimental frogs of varying ages to determine if predator-related feeding behavior differs across age class in this species.

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Compliance with ethical standards

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

Ethical approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All procedures followed the Guide for the Care and Use of Laboratory Animals and were reviewed and approved by the IACUC of Texas Tech University. This article does not contain any studies with human participants performed by any of the authors.

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