An assay to assess predator avoidance behaviors in tadpoles of the African clawed frog (*Xenopus laevis*)

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Abstract

An animal's behavioral response to various sensory inputs affects survival probability. Aquatic species have a lateral line system that provides information on water wave disturbances through receptor cells called neuromasts. I conducted this study to test a modified behavioral assay from Claas and Dean (2006) with *Xenopus laevis* tadpoles as a model species. Intensity of behavioral response to an air puff stimulus was recorded for 45 tadpoles staged 51-54 (Nieuwkoop and Faber, 1994) for 0.5 centimeter height intervals spanning from the surface of the water up to 7 centimeters above the surface of the water. A response threshold was at 5 centimeters above the surface of the water for 44 of the 45 tadpoles with response intensity decreasing steadily as stimulus height increased. The behavioral assay was predictable and repeatable for the *X. laevis* tadpoles and provided a pattern of behavioral responses that can be used to understand movement decisions linked to survival probability.

Introduction

Animals rely on their sensory system to obtain and process environmental and internal cues to evaluate behavioral responses and act appropriately to the stimulus. Animals combine information from several sensory inputs, such as vision, electroreception, auditory, olfaction, and lateral line systems, to navigate the trade-offs between energy expenditure to power movement and behaviors that promote survival. Before moving, animals receive motivation from some internal factor, determine how and where to move, and deal with any external factors that may influence their movement (Nathan et al., 2008).

For aquatic species, behavioral response to water disturbances is critical to locate prey (Gorner, 1973; Junges et al., 2010; Kanter and Coombs, 2003), detect predators (Bleckmann,

1980), navigate obstacles (Burt de Perera, 2004), schooling (Katz et al., 1981) and for station holding (Schimdt et al., 2011). The lateral line system is a mechanosensory system utilized by many aquatic species to sense water disturbances including currents, water displacement by another animal, and vibrations from sound waves (Dijkgraaf, 1962). Lateral line organs contain receptor cells called neuromasts with kinocilia on one side of each neuromast to collect information on wave location and direction (Shelton, 1970). In the African clawed frog (*Xenopus laevis*), sensitivity of the neuromast is connected to which side the kinocilia is on and the lateral line organs are oriented so that half of the neuromasts have the kinocilia on one side and the remaining half on the opposite side (Gorner, 1973).

Sensory systems other than the lateral line also provide sensory input used in water wave disturbance detection and response mechanisms. For example, the lateral line input in adult *X. laevis* was used to inform decisions on turning and approaching a prey item farther away while visual input was more relevant for close prey items and food capture (Claas and Dean, 2006). Indeed, multiple sources of simultaneous sensory inputs can be involved in behavioral responses (Montgomery et al., 2013). Visual input can be used in conjunction with lateral line input as an external frame of reference for rheotactic behaviors in the torrentfish (*Cheimarrichthys fosteri*), the Mexican cave fish (*Astyanax fasciatus*) and the antarctic fish (*Pagothenia borchgrevinki*) (Montgomery et al., 1997). Also, *A. fasciatus* make use of tactile stimuli as a reference point to guide orientation behaviors (Baker and Montgomery, 1999).

Many studies have looked into the effects of leisoned lateral line systems and orientation accuracy and have found that despite large absences of lateral line organs, accurate behavioral responses can still be made from the input that is received (Elephandt, 1982; Buck et al., 2012; Claas and Munz, 1996; Junges et al., 2010; Simmons et al., 2015). The lateral line system input

in adult X. laevis is used as the primary provider for information of water disturbances while other sensory systems are used only when the lateral line is absent (Elepfandt, 1984). Other suggested sensory inputs used in water wave detection include magnetic fields in zebrafish (*Danio rerio*) (Cresci et al., 2017), somatosensory system in *X. laevis* (Claas et al., 1993), and the labyrinth receptors in the inner ear of *X. laevis* (Gorner, 1976).

Aquatic species interpret sensory input in ways reflective of their ecology and morphological conditions such that the same stimuli will result in different behavioral responses in different species (Elepfandt and Simm, 1985). *X. laevis* tadpoles are suspension feeders and prefer midwaters which affects their behavioral responses observed after surface water disturbances (Brown and Simmons, 2016). *X. laevis* are known to respond to surface water waves by detecting the disturbance with their lateral line system which encodes it in a way different from the touch sense (Harris and Milne, 1966). Although *X. laevis* remain fully aquatic as adults, changes in the lateral line system are present through metamorphosis (Shelton, 1971). Behavioral implications of the structural changes of the lateral line system across metamorphosis have been under-examined (Simmons et al., 2004).

The objective of this study is to develop a simple and repeatable design of a behavioral assay, adapted from Claas and Dean (2006), useful for assessing lateral line system-mediated responses to water wave disturbance in amphibians using *X. laevis* tadpoles as a model. Several studies have been conducted with *X. laevis* to study rheotactic behaviors with various test apparatuses and stimuli, including, dipping a thin glass rod in the water (Elepfandt, 1982; Elepfandt, 1984; Gorner, 1973; Okazawa and Funahashi, 2013), producing an air puff with a loudspeaker (Behrend et al., 2008; Claas and Munz, 1996; Zittlau et al., 1986), and dropping water from a stopcock (Gorner et al., 1984). Aquatic species such as striped panchax

(*Aplocheilus lineatus*) (Bleckmann, 1980) and *A. fasciatus* (Baker and Montgomery, 1999; Burt de Perera, 2004) have also been used to investigate the role of the lateral line system in rheotactic behaviors. Other studies have investigated the effects of current on rheotactic behavior in a variety of species including: *X. laevis* (Simmons et al., 2004), mottled sculpin (*Cottus bairdi*) (Kanter and Coombs, 2002), and bullfrogs (*Rana catesbeiana*) (Schmidt et al., 2011; Brown and Simmons, 2016). As water wave disturbances are associated with behaviors that promote survival, such as predator detection and prey detection, understanding how amphibians utilize sensory input from the lateral line system with a simple assay can be beneficial to establish standard behavioral patterns. From these behavioral patterns, different environmental conditions and stressors can be investigated in their effect on behavioral responses that deviate from the species-specific pattern.

Methods

Study Subjects and Husbandry

Xenopus laevis tadpoles, stages 51-54 (Nieuwkoop and Faber, 1994), were purchased from Xenopus1 (Dexter, MI, USA). Tadpoles were communally housed in two rectangular glass tanks filled with dechlorinated, aerated water. Temperature ranged from 21-24°C and the tadpoles were maintained under a 12:12 hour light:dark cycle. Tadpoles were fed a 60 g/L Sera Micron solution.

Behavioral Testing

Testing was done with 45 tadpoles stimulated by surface water waves to elicit a predator detection response. Tadpoles were transferred to a 1.5 cm height and 60 mm diameter petri dish filled with dechlorinated water for behavioral testing. This test apparatus was designed to keep

the tadpole close to the water surface to minimize water depth effects on stimulus perception as observed with adult *X. laevis* by Elepfandt and Wiedemer (1987). Tadpoles were given a 5-minute acclimation period before trials began.

A two-mL plastic pipette connected to a nine-inch glass Pasteur pipette was used to produce an air puff by fully compressing the plastic bulb to create the surface water wave stimulus similar to the stimulus used by Claas and Dean (2006) (Figure 1). A testing session consisted of the tadpole receiving an air puff stimulus five times per height over 0.5 cm height intervals starting at the surface of the water to seven centimeters above the surface. The tadpoles were given ten seconds between air puffs to minimize residual effects of the previous stimulus as the lateral line system has been found to filter out predictive stimulus (Montgomery et al., 2013). Also, *X. laevis* retains short-term memory of turn amplitude when orienting towards a stimulus that can affect subsequent stimulus responses (Okazawa and Funahashi, 2013). Tadpoles were given 20 seconds after each height change to minimize possible effects from the disturbance caused by moving the pipette. Stimulus was given only when the animal was at rest because swimming inhibits the stimulation of the lateral line system in *X. laevis* (Shelton, 1971). From preliminary observations, tadpoles often stopped responding around seven centimeters from the water surface so height was tested up to this point. Each tadpole was tested throughout the range of stimulus heights and the order of heights was randomized to account for diminishing response as the testing session progressed.

Data was collected on response intensity for each stimulus given. Response intensity was split into five categories. A level 0 response was defined as no observable behavioral movement. A level 1 response was defined as a flinch movement in which the movement was within one-body length from the original position of the tadpole. A level 2 response was defined as a

movement in which a tadpole moved more than one-body length away from its original position at a relaxed swimming speed. A level 3 response was defined as a movement in which a tadpole moved more than one-body length away from its original position at a fast swimming speed. A level 4 response was defined as the highest intensity response where a tadpole swam at a rapid pace around the petri dish.

Data Analyses

Categories of response intensity observed in the tadpoles was totaled for the 15 height intervals. At each height interval, percentage of tadpoles showing a response regardless of the level of intensity was calculated. Average category of response intensity for the tadpoles was determined for each height interval with standard error measurements.

Results

All trials were conducted within one week in which no mortality was experienced in the study group of tadpoles. Water temperature in the test apparatus ranged between 21.0-24.2°C. All 45 tadpoles responded with an intensity category of 1 or higher to the air puff stimulus in between the stimulus height intervals up to 2 centimeters above water surface (Table1; Figure 2). Two-thirds of the tadpoles displayed a level 0 response to the air puff stimulus at 7 centimeters above the water surface. A response threshold appears to be at 5 centimeters above the water surface for 44 of the 45 tadpoles. One tadpole had only a response of level 1 intensity or higher for the stimulus heights between the water surface to 2 centimeters above the water surface and at 3 centimeters above water surface with a level 0 response for the remaining height intervals. As the height intervals increased above 5 centimeters above the water surface, the percentage of tadpoles responding with an intensity category of 1 or higher declined rapidly. Between 5

centimeters and 6 centimeters, 10% fewer tadpoles responded with an intensity category of 1 or higher with each 0.5 centimeters height increase. Between 6 centimeters and 7 centimeters, about 15-20% fewer tadpoles responded with an intensity category of 1 or higher with each 0.5 centimeters height increase.

The mean category of response intensity tended to steadily decrease with increasing stimulus height (Table 2; Figure 3). With every 2 centimeter increase in stimulus height, the mean response intensity increased by approximately 1. Variation was present in response intensity among the tadpoles while adhering to the overall trend of the response, gradually decreasing in intensity as the stimulus was produced farther from the surface of the water.

Discussion

Overall, the results show a gradient of response intensity as stimulus height above the water increased, with greater heights eliciting lower intensity reactive behaviors in the tadpoles (Figure 3). Tadpoles filtered out responses to sensory inputs above about 5 centimeters, suggests a threshold beyond which they filter out the stimulus (Figure 2). Other species have shown similar responses. For example, Mexican blind cave fish (*Astyanax mexicanus*) did not respond behaviorally to the lowest water disturbance they can detect with the lateral line system (Montgomery et al., 2013). Lower frequency stimuli could be detected in the striped panchax (*Aplocheilus lineatus*) without causing a behavioral response as the sensory input was filtered out (Bleckmann, 1980). Lastly, large water disturbances near the head of *X. laevis* may elicit a physiological response without detectable movement (Russell, 1971).

Individual tadpoles varied in their responses, as expected, but overall, showed repeatability across treatment levels. Nonetheless, there are often varying degrees of

responsiveness among individual animals. Some of the differing behavioral responses observed in the rheotactic behavior of zebrafish (*Danio rerio*) to magnetic fields as influence from two general personality types: reactive and proactive (Cresci et al., 2018). Variability in responses can be dampened by effects of multiple height intervals in the assay. Animals react behaviorally to sensory input as appropriate to increase survival probability while balancing the energy needed to create that response. By testing behavioral responses across stimulus heights, a pattern can be developed, and deviations from this pattern are useful for evaluating the effect of environmental conditions, such as water toxicity, on behavioral responses. These deviations can be used to further discriminate the effect of physiological conditions, such as neuromast loss.

Several factors may confound the results observed due to the particulars of the experimental design. The tadpoles used in the experiment were lab-bred which can result in different behavioral responses than those that would be observed in wild caught *X. laevis* (Chum et al., 2013). An air puff stimulus given above the body of the tadpoles is known to cause activation of the lateral lines over the entire body (Okazama and Funahashi, 2013). Thus, directional detection can be difficult to sense accurately with wide spread activation of the neuromasts, however, since the air puff was designed to mimic a predator, the directional component of the response is not as important as with the adult *X. laevis* who would orient to the stimulus as if a food item. As the trial sessions progressed, the lateral line system may have been fatigued (Sand, 1937) or the tadpole may have decided against reacting behaviorally as the system filtered out predictive input (Montgomery et al., 2013), even as the order of height intervals tested was randomized. Also circadian effects could have affected the responses of the tadpoles (Simmons et al., 2015) as trials were run throughout the day in this study. Although lateral line organs were not quantified in the tadpoles, differences in number of neuromast organs

among the tadpoles is not likely to influence the ability to detect and appropriately gauge a behavioral response to the surface water wave (Simmons et al., 2015).

Quantifying thresholds used by animals as they navigate sensory inputs is valuable in understanding the balance of weighing energy costs with promoting survival. Differences in behavioral responses in different species to lateral line input should be further examined to gain a greater understanding of the differences in behavioral ecology and morphology of the different species (Brown and Simmons, 2016). Also, thresholds observed in species under normal conditions are valuable to determine and predict effects of potential conservation issues such as water toxicity (Haselman et al., 2018). Deviations from an animal's system of stimuli filtration is useful to understand how physiological effects disrupt behavioral responses that may reflect a lower survival probability.

Using a simple behavioral assay with common equipment can be a helpful tool in determining sensory thresholds of aquatic species as they detect water wave disturbances. From a standard threshold, explorations on the effects of environmental and morphological factors that influence behavioral responses to stimuli at the water surface can be valuable for understanding a species' ecology. It is also important to understand how different sensory systems interact and the order of information obtained from each system when an animal is making movement decisions as it pertains to survival tactics. The behavioral assay provided a system with common equipment that produced a pattern of behavioral response intensity with a threshold that was consistent for a majority of the tadpoles tested. The behavioral assay was useful to discriminate a pattern of behavioral responses with overall predictable results while reflecting the expected variability among the tadpoles.

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Figure 1. Test apparatus used to produce an air puff stimulus to *Xenopus laevis* tadpoles to test predator avoidance behavioral thresholds.
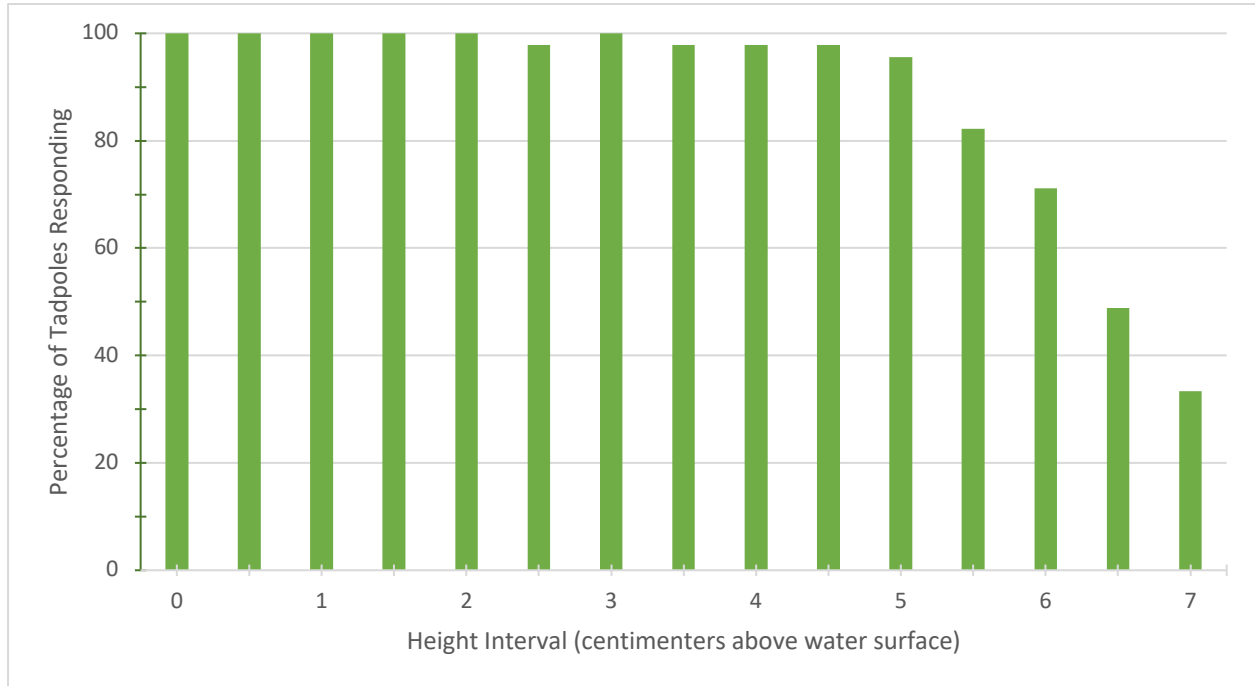
Figure 2. Percentage of *Xenopus laevis* tadpoles responding behaviorally to an air puff stimulus at 15 height intervals. For each height interval, n=45.

Height Interval (centimeters above water surface)	Percentage of Tadpoles Responding to Stimulus
0.0	100.0%
0.5	100.0%
1.0	100.0%
1.5	100.0%
2.0	100.0%
2.5	97.78%
3.0	100.0%
3.5	97.78%
4.0	97.78%
4.5	97.78%
5.0	95.56%
5.5	82.22%
6.0	71.11%
6.5	48.89%
7.0	33.33%

Table 1. Percentage of *Xenopus laevis* tadpoles responding to air puff stimulus at 15 height intervals. For each height interval, n = 45.

Height Interval (mm from water surface)	Mean Response Intensity ± SE
0	3.7 ± .05
0.5	3.4 ± .08
1.0	3.2 ± .09
1.5	3.1 ± .10
2.0	2.6 ± .10
2.5	2.3 ± .11
3.0	2.0 ± .11
3.5	2.3 ± .12
4.0	1.6 ± .11
4.5	1.5 ± .11
5.0	1.3 ± .09
5.5	1.3 ± .12
6.0	0.78 ± .09
6.5	0.52 ± .08
7.0	0.26 ± .12

Table 2. Mean (± SE) response intensity for *Xenopus laevis* tadpoles to air puff stimulus at 15 height intervals for five response intensity categories. For each height interval, n=45.
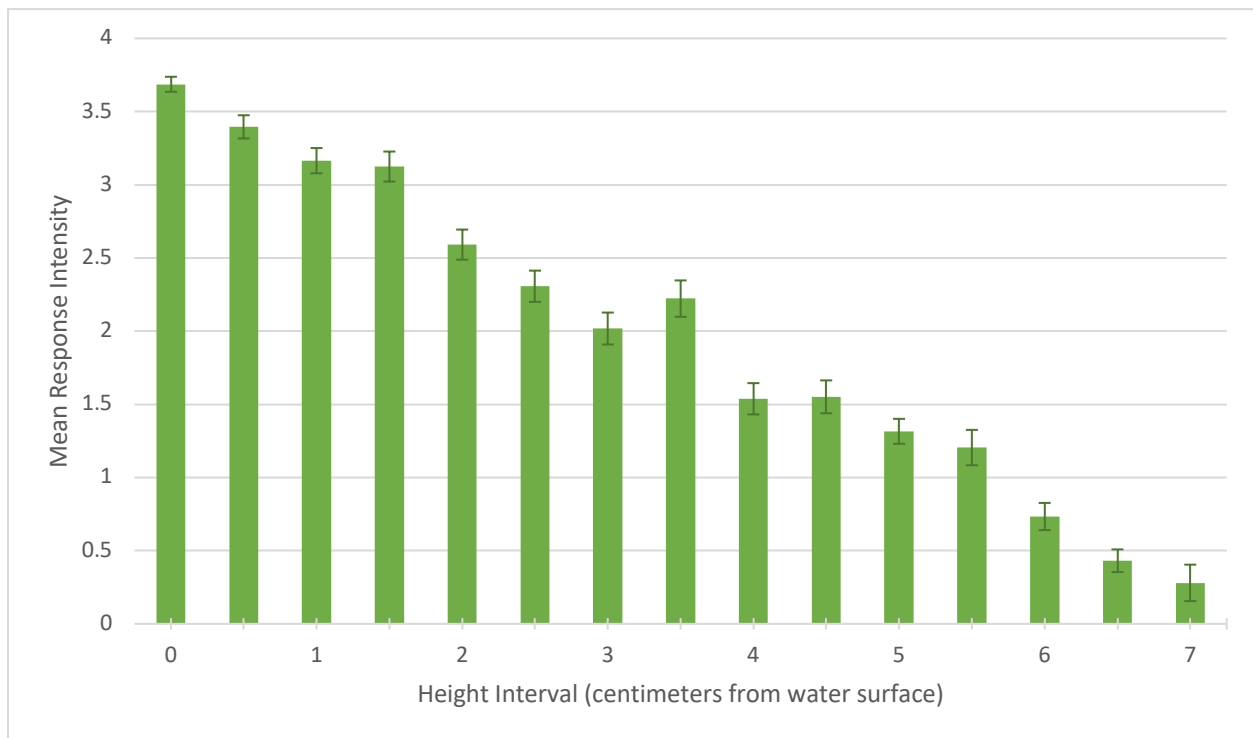

Figure 3. Mean response intensity for Xenopus laevis tadpoles to an air puff stimulus at 15 height intervals for five response intensity categories. For each height interval, n=45.