Development of the visual system in social poison frog tadpoles

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ABSTRACT

The ways in which animals sense the world around them change throughout development. Young of many species have absent or limited visual capabilities, but still make complex decisions about individuals with whom they interact. Poison frog tadpoles display complex social behaviors that have been suggested to rely on vision despite a century of research indicating tadpoles have poorly-developed visual systems. Here, we examined visual system development in tadopoles of the Mimetic Poison Frog (*Ranitomeya imitator)* that use begging displays to stimulate egg feeding from their mothers. Neural activation in the retina increased in begging metamorphic tadpoles, but not in begging pre-metamorphic tadpoles. Molecular profiling of active eye neurons during begging identified numerous differentially expressed development-related transcripts, suggesting that developmental stage, not begging, was driving gene expression profiles. Using the neural tracer neurobiotin, we found that connections between the eye and brain proliferate during metamorphosis, with little retinotectal connections in recently-hatched tadpoles. To assess visual capabilities of tadpoles, we used a light/dark preference assay in early, middle, and late stages. All tadpoles showed a preference for the dark side, but the strength of preference increased with developmental stage and eyes were not required for this behavior. Taken together, these data indicate visual ontology of poison frog tadpoles is similar to that of other frogs, with poor visual capabilities at hatching and immense morphological and physiological changes occurring during metamorphosis. More broadly, this highlights the importance of multimodal cues, including photodetection via the pineal structure, in tadpole social interactions.

INTRODUCTION

Vision is one of the main sensory modalities animals use for social communication. However, the young of many species have limited or absent visual capabilities. For example, cats and dogs are born with their eyes closed and are functionally blind. Mice pups do not open their eyes until they are 11-12 days old (UCSF Lab Animal Resource Center). Tadpoles hatch with externally visible, partially developed eyes, but their visual capabilities are not well-developed (Hoff et al., 1999). Despite poor or absent vision, these young animals make complex social decisions by relying on information from other sensory modalities. Although it is assumed if an animal can see or not based on the eyes being open or sealed shut, as is often the case with mammalian neonates, this is less clear in young animals of other taxa, whose eyes remain visible throughout development and after hatching. Understanding how the visual system develops is an important step towards understanding how neonates and juveniles recognize and communicate with other individuals in their environment.

From as early as the mid 1800s, frogs and toads were fundamental in visual neuroscience (for review, see Donner & Yovanovich, 2020). Adults have a fully developed and complex retina similar to other vertebrates but are more easily accessible. Anurans have a plethora of ecological diversity and yet the ontogeny of the visual systems is relatively well conserved. Vision is also important in tadpoles and has been implicated in schooling behaviors (Caldwell, 1989; Katz et al., 1981), predator avoidance (Hettyey et al., 2012), conspecific identification (Gouchie et al., 2008), and habitat assessment (Hettyey et al., 2012; Rot-Nikcevic et al., 2006). However, many tadpoles are hatched with under-developed eyes and are thought to have poor vision (Hoff et al., 1999). Grant et al. (1980) divided early development of the tadpole retina into four stages, with visual function being present at the end of the first stage shortly after hatching, but noted that a mature retina was not present until there is significant hindlimb development. In addition to changes in the retina, retinal projections to brain regions important for visual processing, such as the optic tectum, are still forming throughout metamorphosis (Fujisawa, 1987). However, it is unclear how retinal development coincides with these complex visual behaviors observed in tadpole behavioral ecology.

Poison frogs (Dendrobatidae) are emblematic taxa for amphibian visual systems, as their bright displays advertise their chemical defenses to predators. These taxa display a wide range of parental care strategies, notably tadpole transport and egg provisioning (Summers & Earn, 1999; Summers & Tumulty, 2014; Weygoldt, 2009). Tadpoles of these species also show an impressive diversity of complex behaviors, such as aggression and begging for egg meals from their parents. Recently, it was hypothesized that poison frog tadpoles, and likely other social tadpoles, are dependent on visual cues, as their pools are often murky and found in low-light environments (Fouilloux et al., 2022; Stynoski & Noble, 2012). Indeed, research in the Strawberry poison frog (*Oophaga pumilio*), suggests that tadpoles use visual cues to recognize parents apart from heterospecifics (Fouilloux et al., 2022; Stynoski & Noble, 2012) and sexually imprint on the color morph of their caregiver (Yang et al., 2019). However, the ontogeny of the visual system in poison frog tadpoles, especially those displaying complex social behaviors, has not been well studied.

Here we examined visual system development in Mimetic poison frog (*Ranitomeya imitator*) tadpoles. In this species, dads transport hatched tadpoles to individual pools in

bromeliad leaves. Every few days, the parents will check on the tadpole, and if hungry, the tadpole will beg for food by rapidly vibrating its body (Yoshioka et al., 2016). Tadpoles must also attempt to avoid predation from spiders and other tadpoles, as sympatric *Ranitomeya variabilis* tadpoles cannibalize *R. imitator* tadpoles (Brown et al., 2008). In *Oophaga pumilio* tadpoles, predation accounts for as much as 67% of tadpole mortality, emphasizing the importance of detecting and avoiding potential predators (Maple, 2002). As such, *R. imitator* tadpoles must make complex decisions about whether a visitor to their pool is a potential caregiver or predator. We tested the hypothesis that vision facilitates begging behavior, but that young tadpoles have overall poor visual capabilities.

METHODS

Experimental Animals

All *Ranitomeya imitator* tadpoles were captive bred in our poison frog colony from adult breeding pairs using standard animal procedures in our laboratory. Briefly, reproductive male and female *R. imitator* were housed in a glass terrarium (12x12x18 inch) containing several water pools, greenery, and a moss-substrate floor. Water pools were checked regularly for deposited tadpoles. Transported tadpoles were housed individually in circular containers (5 cm diameter) in a large aquarium (5 gallon) maintained at 26-28°C with constant recirculation. Tadpoles were fed brine shrimp flakes or tadpole pellets (Josh's Frogs, Owosso, MI, USA) three times weekly. Each rearing container contained sphagnum moss and tadpole tea leaves as extra sources of nutrients. We used adult *R. imitator* females from actively reproducing pairs as stimulus animals. All procedures were approved by Harvard University Animal Care and Use Committee (protocol #17-02-293) and Stanford Administrative Panel on Laboratory Animal Care (protocol #33097).

Tadpole Development

All tadpoles used in behavior trials were measured for body mass, body length (mouth to tail peduncle), and total length. We developed a *R. imitator* staging guide based on Gosner staging (**Supplementary Doc**; Gosner, 1960). A numerical stage (based on hindlimb development) was recorded for all tadpoles. When appropriate, we grouped tadpoles based on stage. Early-stage tadpoles had minimal pigmentation, no hindlimb development, and consisted of only stage 25 tadpoles. Middle-stage tadpoles were partially pigmented with a tan/gray coloring, had minimal hindlimb development (<1 mm), and consisted of tadpoles between stages 26-29. Late-stage tadpoles had full pigmentation, including some adult-typical pigmentation, significant hindlimb development, forelimbs had not emerged, and ranged from stage 30-40.

Begging Behavior Trials and Analysis

To examine begging behaviors, we exposed naive tadpoles of various developmental stages to reproductive females. On the morning of the trial (8-10 AM), tadpoles were placed into a circular arena (5 cm diameter, 10 cm height) filled with 100 mL of prewarmed frog water or square acrylic arena (5x5x5 cm) filled with 45 ml of frog water and allowed to acclimate for 10

minutes. Arenas were placed on an LED lightpad and imaged from above using a GoPro camera. After an acclimation period, we recorded a 10 min baseline for each tadpole when no stimulus was present. We then added an *R. imitator* female and allowed them to interact for 20 minutes. Based on live observations, each tadpole was assigned as begging (at least two bouts of begging during 20 min trial) or non-begging (no begging during 20 min trial). To assess how development correlates with behavior, 10 tadpoles were tested once a week for 6 consecutive weeks in begging trials. For all other experiments, early, middle, and late-stage tadpoles were only used once in behavior and immediately collected.

Videos were scored using BORIS (Friard & Gamba, 2016). We quantified the number of bouts and time tadpoles spent begging, swimming or moving. Swimming behavior was quantified as the amount of time a tadpole spent swimming around the arena and had to involve multiple back and forth tail movements. In contrast, a "movement" was quantified when a tadpole performed a single tail flick to change position in the arena and was included in activity measures as 0.5 sec. Begging behavior involves the rapid vibration of the body/tail, often with the tail straight, and is performed with the tadpole at a >45˚ angle to the female (Summers & Tumulty, 2014).

Light Preference Trials

To assess tadpole visual capabilities and light environmental preferences across development, we tested early, middle, and late-stage tadpoles in a light/dark preference arena. The behavioral arena was constructed from two petri dishes: 9 cm and 14 cm diameter. We burned a small hole in the middle of the large petri dish and painted half of the bottom and sides of the larger petri dish black with multiple coats of black acrylic paint to ensure that no light passed through it. The other half was left unpainted. The dish was then sprayed with Kyrlon Fusion Clear Gloss to seal it from water and create a slight "frost" on the unpainted side. This helped to reduce reflections on the unpainted side (**Supplemental Info**). A screw and bolt were used to attach the small petri dish to the lid of the large petri dish. The painted dish was placed between the two, with the screw going through a hole burned in the larger dish, so that the light/dark dish could be rotated. This setup allows us to change the light environment (flip the light/dark sides) without disrupting the tadpole in the small petri dish. The behavioral setup was placed on an LED light pad set to full power and a GoPro camera was used to record from above.

On the morning of the trials, tadpole containers were moved to a procedure room and allowed to acclimate to the room for ~10 min. The small petri dish (tadpole arena) was filled with 40 ml of frog water. Tadpoles were transferred to the middle of the arena and behaviors were recorded for 3 minutes. We then flipped the light arena and recorded for an additional 3 minutes. All tadpoles were used in the light preference tests on two consecutive days, with eye removal and neurobiotin injections (see below for details) immediately following the first trial.

We scored each video for time spent in each light environment, as well the amount of activity (swim duration and number of movements) that occurred on each side of the arena. We also measured the latency to activity and the latency to enter the dark side of the arena. Since most tadpoles settled onto the dark side of the arena early in the trial, we also recorded if the tadpole "tracked" to the dark side of the arena after the sides were flipped and used this as an indication of a true preference being displayed.

Neurobiotin Injections

To examine when connections between the retina and brain are established in *R. imitator*, we injected neurobiotin into the optic nerve of early, middle, and late-stage tadpoles. Following the light preference trial, each tadpole was anesthetized in 0.01% MS-222 in frog water and the eyes were removed. Approximately 0.1 µl of 10% neurobiotin solution was placed in the eye cup onto the optic nerve. Tadpoles were placed into recovery containers in fresh frog water and allowed to recover overnight. The following morning, blinded tadpoles were run through the light preference trials before being collected. All blinded tadpoles recovered by the following morning, had normal swimming behavior, and responded to a water puff.

Tissue Collection

For tadpoles used in begging trials, the female was quickly removed from the arena at the end of the trial, the light turned off, and the tadpole incubated in the arena for 30 min. For tadpoles used for neurobiotin tracing, tadpoles were euthanized immediately after the second color preference test. To collect brains for immunohistochemistry, tadpoles were then euthanized with an overdose of benzocaine, the brain exposed, and the whole head fixed in 4% paraformaldehyde (PFA) prepared in 1x phosphate buffered saline (PBS) at 4°C overnight. The tadpole head was then rinsed in 1x PBS for 24 h, and cryoprotected in 30% sucrose prepared in 1x PBS at 4°C. Once dehydrated, tadpoles were embedded in OCT mounting media (Tissue-Tek® O.C.T. Compound, Electron Microscopy Sciences, Hatfield, PA, USA), rapidly frozen, and stored at -80°C until cryosectioning. We sectioned the whole tadpole head, including the brain and eyes, at 14 µm and collected sections into 4 sets of alternate series of SuperFrost Plus microscopy slides (VWR International, Randor, PA, USA). Slides were allowed to dry completely and stored at -80°C until processing. For eyes collected for phosphoTRAP, the tadpole was euthanized as above, but the eyes were quickly removed from the head and immediately snap frozen in phosphoTRAP dissection buffer. Both eyes from three tadpoles were pooled together for each sample.

Immunohistochemistry

To compare neural activation in the retina of begging and non-begging tadpoles, we stained cryosectioned tadpoles for the phosphorylated ribosomes (pS6). Similar to immediate early genes, pS6 labels recently-activated neurons (Knight et al., 2012). A western blot for pS6 in *R. imitator* tadpoles shows a band at the appropriate size, but this band is absent in protein samples treated with protein phosphatase 1, indicating it is specific for only phosphorylated S6 ribosomes (**Supplemental Info**). Slides were allowed to come to room temperature, washed with 1x PBS 3x10min, blocked in 1x PBS with 5% normal goat serum (NGS) and 0.3% triton-X, incubated in 1:5000 rabbit anti-pS6 (Invitrogen; cat# 44-923G) prepared in blocking solution overnight at 4°C. The following morning, slides were washed with 1x PBS for 3x10 min, incubated in 1:200 AlexaFluor 488 goat anti-rabbit secondary antibody prepared in 1x PBS with 5% NGS for 2 hours, rinsed 1x PBS for 3x10 min, incubated in DI water for 10 min, and coverslipped with DAPI hardset mounting media. All slides were stored flat at 4°C until imaging.

To detect neurobiotin in tadpole brains, slides of cryosectioned brains were reacted with a fluorescently-labeled streptavidin. Slides were brought to room temperature, washed with 1x PBS 3x10 min, and treated with streptavidin (1:400 prepared in 1x PBS; Streptavidin, Alexa

Fluor 568 conjugate; Invitrogen, S11226) for 1 hour in the dark. Slides were then washed in 1x PBS for 3x5min, rinsed in DI water, and coverslipped with DAPI hardset mounting media. Slides were allowed to dry at RT in the dark for 1 hour, sealed with clear nail polish, and stored at 4°C until imaging.

Microscopy and Cell Counts

To visualize pS6 labeled neurons, stained eye sections were imaged at 20x resolution on a Leica DM6B microscope using a DFC9000 digital camera controlled by LASX software. All images were taken with approximately the same exposure and intensity settings. To quantify the number of labeled cells, images were loaded into FIJI image analysis software (Schindelin et al., 2012). Cell counts were done in a single eye for each tadpole. We quantified sections immediately adjacent to the optic disk (± 5 sections) for a total of 6-8 sections per tadpole. pS6-stained cells were visible in both the ganglion cell layer and inner nuclear layer, so each layer was quantified separately. The inner nuclear layer likely included multiple cell types, including amacrine cell, bipolar cells, and horizontal cells, and was roughly separated into a middle and top sub-section. For each image, the region of interest was circled, the area quantified, and the number of pS6-labeled cells in each region was quantified. The total number of pS6-positive cells in a layer was then divided by the total area of the region quantified to create a cell density.

To measure the amount of neurobiotin present in the tecum, we imaged tadpole brains as described above, with the same exposure and intensity settings used for all animals. Only one hemisphere of the entire tectum was imaged. We used the threshold function in FIJI to highlight pixels representing neurobiotin staining. Threshold was based on the background levels of each image. Once the pixels were highlighted, we used FIJI to measure the percent of the region of interest highlighted to calculate a “relative projection density”. This method controls for differences in tectal size/thickness. We also completed these measurements on control tadpoles that were blinded but did not have neurobiotin injected into the optic nerve. The relative projection density was below 1% for all controls. Because tectal layers are not apparent until later developmental stages, we quantified the tectum in whole, instead of by layers. Tectal thickness was measured as the distance from the outside of the tectum to the inner edge along the optic ventricle. The two widest measurements were averaged together for each animal.

Molecular Profiling of Active Neurons

We used phosphoTRAP to molecularly profile active neurons in the eye during begging, which uses an antibody for neural activation marker pS6 to purify transcripts being translated by phosphorylated ribosomes at the time of collection. RNAseq is then performed on the total (TOT) input RNA sample and the immunoprecipitated (IP) sample. RNA samples were processed and purified as previously described (Fischer et al., 2019; Knight et al., 2012). RNA samples were purified using a SMART-Seq v4 Ultra Low Input RNA Kit for Sequencing (Takara, Mountain View, CA, USA), followed by library preparation using the Nextera XT DNA Library Prep kit (Illumina, San Diego, CA, USA), both according to manufactures’ protocols. Pooled equimolar library samples were run on an Illumina Hi-Seq 2500. Sequencing results were aligned to a *R. imitator* transcriptome built from tadpole brain, eyes, and gut samples. Count data was analyzed in R using paired t-tests on each transcript between the TOT and IP samples, similar to that described in Tan et al. (2016; **Supplemental Info**). Fold changes were

also calculated for each transcript as log2 (IP count / TOT count). Differentially expressed genes were defined as having a p-value under 0.05 and a fold change greater than 1.5 in either direction. A list of differentially expressed genes was generated for begging and non-begging tadpoles. We chose to use paired t-tests of transcripts within each group because this better reflects changes in expression associated with begging and reduces the variation present due to intrinsic variables (developmental stage, hunger, etc) that affect total count data. We also did not correct for multiple hypothesis testing in phosphoTRAP data due to the small sample size (N=3 per group) and large number of transcripts. Bonferroni corrections or similar procedures reduce statistical power and increase the chance of type II errors, especially in small sample sizes. While these tests do reduce type I errors, their unacceptable effects on statistical power can hide potential biologically relevant results (Nakagawa, 2004).

Statistical Analyses

All statistics were performed in R (v4.0.2). We used student's t-tests and generalized linear mixed models (GLMM; Package: glmmTMB; Brooks et al., 2017) to compare behaviors among groups, with animal ID as a repeated, random factor when appropriate. Similarly, pS6 data was analyzed using a GLMM with layer as a repeated factor and animal ID as a random factor. When appropriate, Tukey's post-hoc tests were used to parse differences among groups. Correlations were done using Pearson correlations. All data were checked for statistical outliers prior to analyses and removed. The only outlier detected was in the begging group of pS6 staining in the retina, in which one individual had activation three times higher than all other animals.

All graphs were produced in R using ggplot2 (Wickham 2016). Box plots are used throughout for data visualization. All data points are represented as closed circles, data mean as an 'X', and data median as a solid line. Boxes extend to the furthest data points within the 25/75th quartiles, and whiskers extend to the furthest data points in the 5/95th percentile. We used a volcano plot for visualizing phosphoTRAP data. Data points were plotted as -log10(p-value) and log2(fold change) for each transcript. Lines as Y=1.3, X=-0.58, and X=0.58 represent significant cutoffs of $P<0.05$ and $FC>1.5$, respectively.

RESULTS

Older tadpoles are more likely to beg

To lay a foundation for understanding the sensory contributions to begging behavior, we conducted begging behavior assays with randomly selected tadpoles and reproductive females. We initially classified tadpoles as begging or non-begging independent of stage. In general, ~70% of tadpoles begged during behavior trials (**Fig. 1A**), with an average begging duration of 85.652 sec (**Fig. 1B**). However, when we accounted for tadpole stage, there was uneven distribution of stage within each group (**Fig. 1C**). Begging tadpoles were more developed than non-begging tadpoles, suggesting age might impact the likelihood to beg. To examine this further, we tested tadpoles in begging trials weekly for 6 consecutive weeks. Tadpoles are more likely to beg after 3 weeks of development (**Fig. 1D**), which roughly corresponds to the transition from early (stage 25) to middle developmental stages. Although the number of begging bouts

did not statistically differ, tadpoles spent more time begging to females during the trials occurring weeks 5-6 compared to those in weeks 1-3 (**Fig. 1E**).

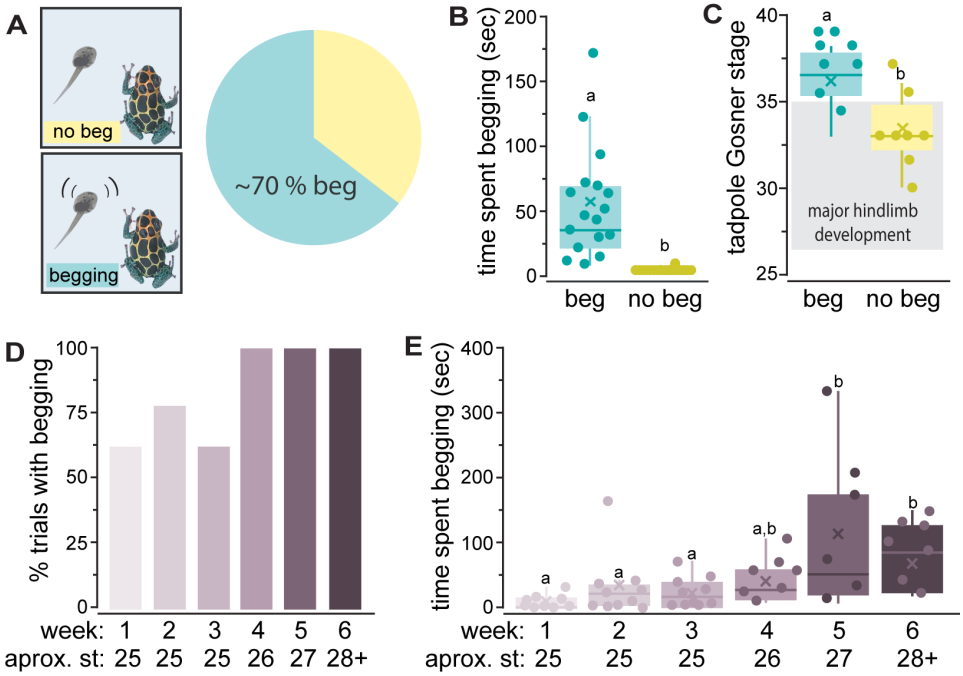

Figure 1. Tadpole begging increases during development. (**A**) When exposed to a reproductive female, ~70% of tadpoles display begging behavior for an average time of 85.652 sec (**B**). (**C**) Begging tadpoles were of later developmental stages than non-begging tadpoles. (**D**) As tadpoles enter metamorphosis (st 26+; weeks 4-6), they are more likely to beg. (**E**) Older tadpoles spent more time begging. Different lowercase and uppercase letters represent significant differences (P<0.05).

Tadpole stage and begging impact retinal neural activation

As vision has been proposed to be important for begging behavior (Stynoski & Noble, 2012), we quantified neural activation in the retina of begging and non-begging tadpoles. Begging tadpoles had higher activation in both the ganglion cell layer and inner nuclear layer compared to non-begging tadpoles (**Fig. 2A-B**; beg v no beg: $F_{1,15}$=8.701, P=0.026; layer: $F_{2,15}$=2.888, P=0.095). However, there was a significant positive correlation between tadpole stage and neural activation (R=0.557, P=0.025; **Fig. 2C**). To account for the developmental differences in begging and non-begging tadpoles, we also included a group of younger tadpoles from a separate study on begging and aggression. Within this group of stage 25 tadpoles, there was no statistical difference in neural activation in either the ganglion cell or inner nuclear layers among begging, aggressive, and control tadpoles (**Fig. 2D**, $F_{4,41}$=6.122; P<0.001).

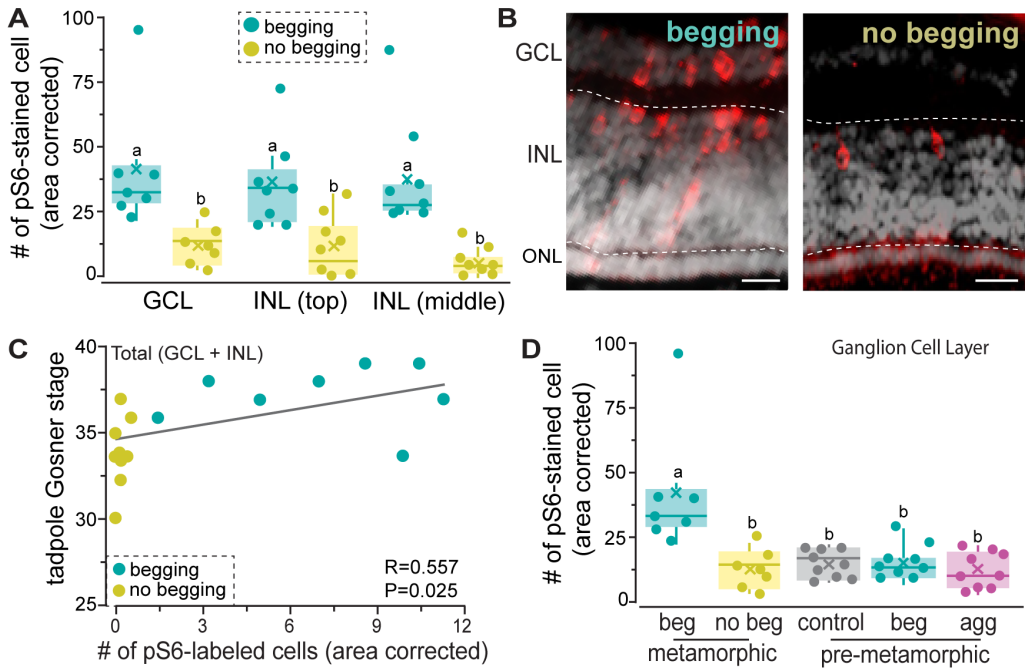

Figure 2. Neural activation varies with stage and begging. (**A**) Begging tadpoles had higher pS6 staining in the ganglion cell layer (GCL) and inner nuclear layer (INL) compared to non-begging tadpoles. (**B**) Photomicrographs showing pS6 staining in the GCL and INL, but not in the outer nuclear layer (ONL). Dotted lines approximately separate layers. Scale bar = 10 um. (**C**) The number of pS6-stained cells positively correlated to tadpole stage. (**D**) While metamorphic begging tadpoles had higher activation in the ganglion cell layer, there was no difference in pS6 staining in the retina of control, begging, and aggressive pre-metamorphic tadpoles. Different lowercase letters represent significant differences (P<0.05).

Because older, begging tadpoles had higher neural activation in the retina, we next molecularly profiled these active neurons in eyes from begging and non-begging tadpoles using phosphoTRAP. There were 83 transcripts that were enhanced in neurons active during begging, but 2389 transcripts that were depleted in neurons active during begging (**Fig. 3A**). Of the transcripts that were up-regulated, 52 were *crystallin*-related, which is an important structural component of the lens (**Fig. 3B**). *Grifin*, a lens-specific protein, was also enhanced. Other potentially relevant up-regulated transcripts include several related to retinoic acid signaling and *sortilin*, an important regulator of neuron growth. Among the depleted or down-regulated transcripts associated with begging, there was a high number of transcripts associated with ribosome biosynthesis and maturation (266 transcripts), cell division (102 transcripts), lysosome regulation (48 transcripts), axonal transport (34 transcripts), and neurogenesis and nervous system development (24 transcripts). Another potentially important depleted transcript was *ephrin*, which is an important modulator of retinotectal connections and organization (McLaughlin et al., 2003). Despite more than 2400 transcripts enhanced or depleted in begging tadpoles, there were only 4 up-regulated and 18 down-regulated genes in the eyes of non-begging tadpoles. None of these genes overlapped between begging and non-begging tadpoles.

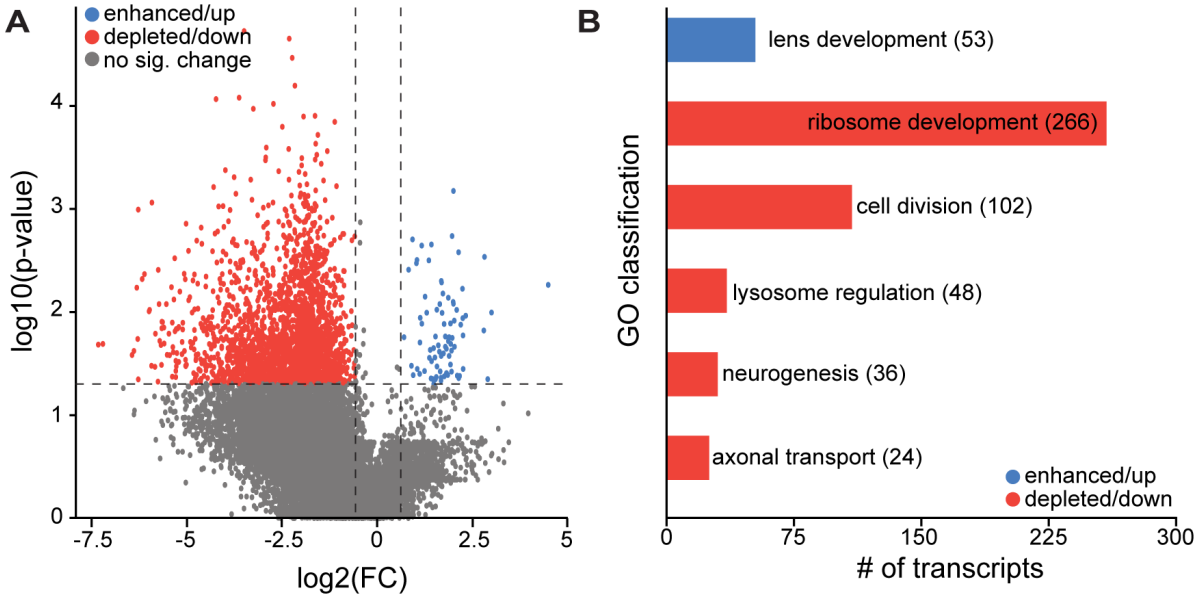

Figure 3. Enhanced and depleted transcripts in the eyes of begging tadpoles. (**A**) Volcano plot of enhanced (blue) and depleted (red) transcripts in the eyes of begging tadpoles. (**B**) Gene ontology classification of enhanced and depleted transcripts reveals that over half of the enhanced transcripts are related to lens development, with cell development-related transcripts dominating depleted transcripts. The number of transcripts in each category follows the classification name.

Retinotectal connections increase during metamorphosis

To visualize connectivity between the retina and brain, we next applied neurobiotin, an anterograde neuronal tracer, to the optic nerve and quantified the amount of neurobiotin present in the tectum (**Fig. 4A, B**). This serves as a proxy for the extent of retinotectal connections and/or axonal branching in the optic tectum. Late-stage tadpoles had more neurobiotin present in their tectum than younger tadpoles ($F_{2,21}$=22.326; P<0.001), but middle tadpoles still had more than early-stage tadpoles (**Fig. 4C**). Relative projection density was positively correlated with tadpole stage in late-stage tadpoles (R=0.905; P<0.001; **Fig 4D**). Little to no fluorescence was observed in un-injected tadpoles (average density <1%). Neurobiotin density was controlled for by tectum size, since older tadpoles had wider tectums than younger tadpoles ($F_{2,21}$=88.476; P<0.001), and middle-stage tadpoles as an intermediated between early and late-stage tadpoles (**Fig. 4E**). Although not quantified, the lens of younger tadpoles appeared to have more DNA present (visualized via DAPI staining) than in older tadpoles, suggesting that it is still developing.

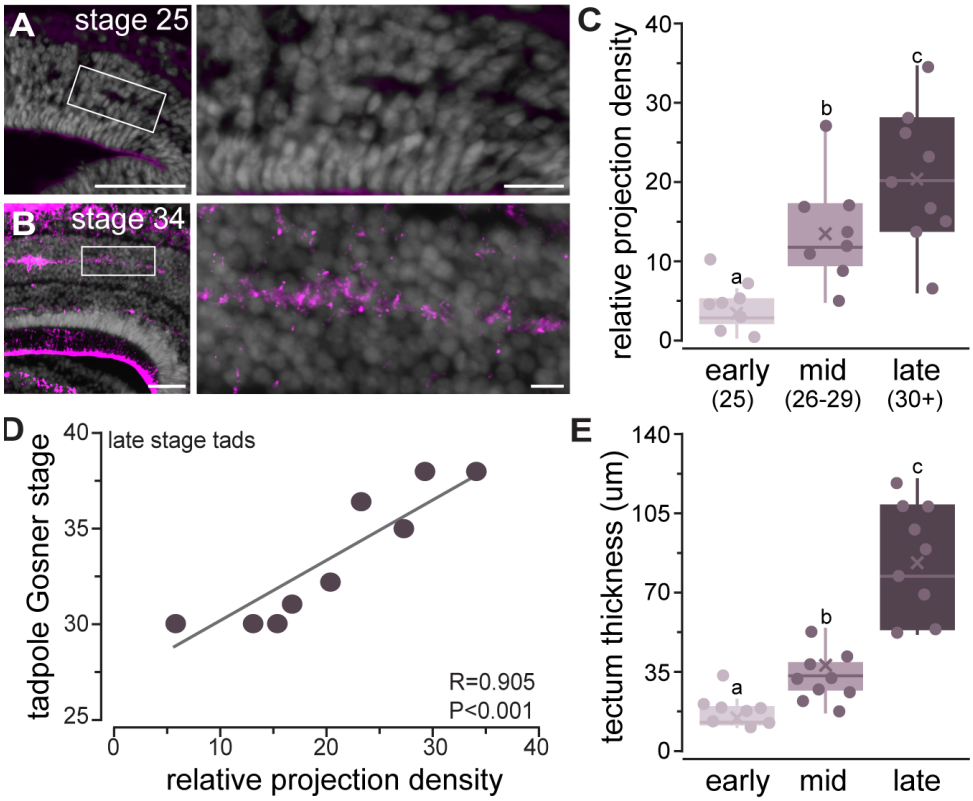

Figure 4. Retinotectal projections increase during metamorphosis. (**A-B**) Photomicrographs of fluorescently-detected neurobiotin in the tectum after it was injected into the optic nerve of an early (A) and late (B) stage tadpole. (**C**) More neurobiotin was detected in the tectum as tadpole stage increased, indicating increased retinotectal projections and/or branching. (**D**) In late-stage tadpoles, stage positively correlates with the amount of retinotectal connections. (**E**) Thickness of the tectum also increased with tadpole stage. Scale bars represent 50 um and 12.5 um left and right images in A-B, respectively. Different lowercase letters represent significant differences (P<0.05). Numbers in parentheses in the x-axis of C represent the developmental stages included in each group, which are the same in E.

Tadpoles prefer dark environments

Early, middle, and late-stage tadpoles were tested in a light/dark preference arena (**Fig. 5A**), where the environment was flipped halfway through the trial to see if tadpoles tracked to one side over the other (**Fig. 5B**). All tadpoles, independent of stage, showed a preference for the dark side of the arena, as evident by the time spent on the dark side and the ability to track to the dark side when the arena was flipped (**Fig. 5C, D**). The strength of the preference increased with age, with late-stage tadpoles spending more time on the dark side than early and middle-stage tadpoles ($F_{2,68}$=6.263; P=0.003). Further, all late-stage tadpoles tracked to the dark side of the arena after the sides were flipped, but only 65% and 75% of early and middle stage tadpoles, respectively, displayed side tracking behavior. Late-stage tadpoles tracked to the dark side sooner than early and middle stage tadpoles ($F_{2,63}$=3.707; P=0.030). When first entering the arena, late-stage tadpoles also entered the dark side sooner ($F_{2,68}$=4.812; P=0.011) than younger tadpoles (**Fig. 5E**) and all late-stage tadpoles explored the dark side while only

81% of younger tadpoles initially explored the dark side of the arena. This cannot be explained by activity, as there is no significant difference in the total activity time ($F_{2,68}$=1.436; P=0.245) among the different stages. In addition, the amount of activity on the dark side of the arena did not statistically change with tadpole stage ($F_{2,68}$=2.182; P=0.120). As such, the higher preference for the dark side displayed by older tadpoles is likely due to either increased light detection capabilities and/or increased motivation.

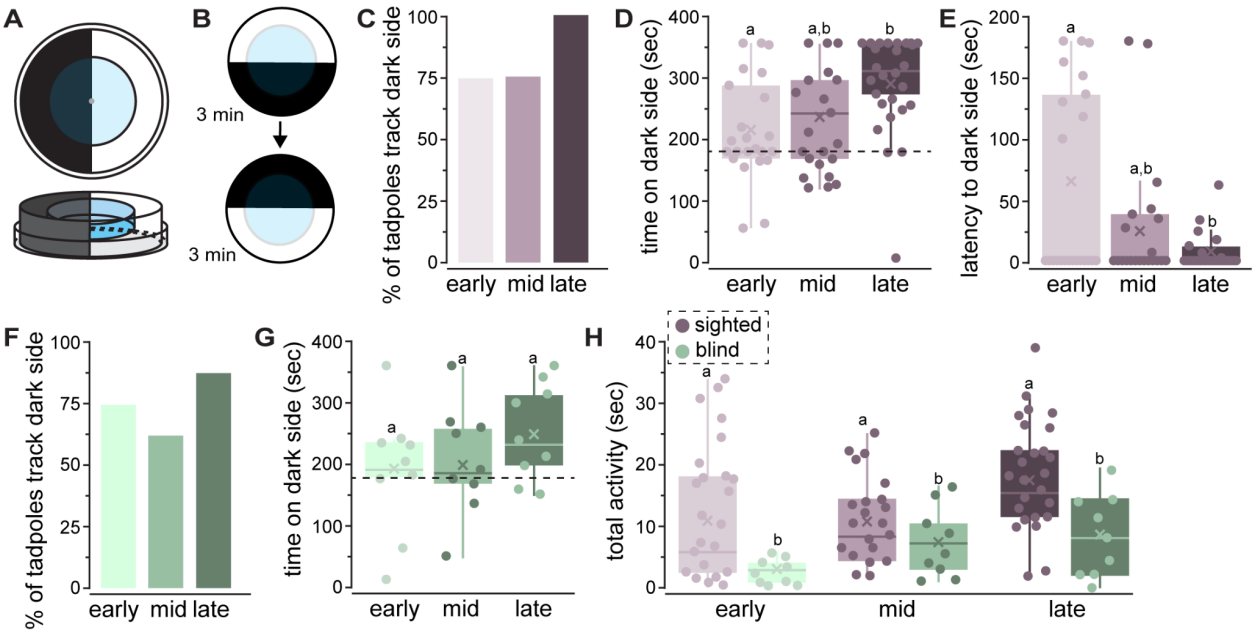

Figure 5. Older tadpoles show a stronger preference for dark environments, independent of sight. (**A-B**) A behavioral arena was constructed so that the light environment could be flipped halfway through the trial without touching the tadpole dish. (**C**) All late-stage tadpoles tracked to the dark side after the arena flip, but only ~75% of younger tadpoles displayed this behavior. (**D-E**) Late-stage tadpoles spent more time in the dark environment and entered it earlier compared to early-stage tadpoles. (**F-G**) Blind tadpoles also display a preference for the dark environment. (**H**) Blind tadpoles were less active than sighted tadpoles, but activity did not differ with stage. Dotted lines in D and G are placed at 180 sec, or 50% of the total trial time. Different lowercase letters represent significant differences (P<0.05).

Blinded tadpoles still show a dark preference

Tadpoles that were blinded for neurobiotin injections still showed a preference for the dark environment (**Fig 5F, G**). In total, 75% of blinded tadpoles displayed side tracking behavior after the arena was flipped, with late-stage tadpoles the most likely (88%) to track to the dark side. Even though blinded tadpoles were able to track to the dark light environment, their activity was dramatically reduced compared to sighted trials (**Fig. 5H**; blind v sight: $F_{1,53}$=8.196, P=0.021; stage $F_{2,53}$=3.418, P=0.058), but this decrease in activity did not vary significantly by stage ($F_{2,53}$=0.101, P=0.904). There were no significant differences among the three stages of blinded tadpoles for time spent in the dark environment ($F_{2,23}$=0.793; P=0.466), latency to enter the dark environment ($F_{2,23}$=1.477; P=0.251), or activity ($F_{2,23}$=3.131; P=0.066).

DISCUSSION

Animals rely on sensory information to carry out vital life processes, even at a young age. Visual capabilities are well documented to vary throughout life, where neonates of several species have absent or reduced vision. However, even these youngest members of species have to make complex decisions about their environment to distinguish between threats and caregivers. Anurans are of historical importance for neuroscience and visual science research, where tadpoles have poorly developed eyes that continue to develop through metamorphosis (Grant et al., 1980; Hoff et al., 1999). Here, we examined the visual system in *Ranitomeya imitato*r, a dendrobatid poison frog species with complex social behaviors observed between parents and offspring (Brown et al., 2008; Summers & Tumulty, 2014).

Begging behavior changes across development

Mimetic poison frog tadpoles make crucial decisions with each visitor to their nursery, as they must assess each visitor to decide whether or not it is appropriate to beg for food. This process is required for feeding, but is also energetically costly (Stynoski et al., 2018; Yoshioka et al., 2016), and begging to a non-caregiver may increase predation risk (Stynoski & Noble, 2012). Strawberry poison frog (*Oophaga pumilio*) tadpoles need multimodal cues to elicit begging (Stynoski & Noble, 2012), and the authors of that study emphasized the importance of visual cues. In addition, *O. pumilio* tadpoles are thought to imprint on their parents' coloration (Yang et al., 2019), further suggesting visual cues are important for tadpole social recognition. In laboratory conditions, *R. imitator* tadpoles reliably beg for food from any reproductive female (~75% in these experiments), but some tadpoles do not. This variation in begging was at least partially related to tadpole stage, since begging tadpoles were of a later developmental stage than those that did not beg. In addition, metamorphic tadpoles were more likely to beg than pre-metamorphic tadpoles. When they did beg, they begged for a longer period of time, which might increase their chance of being fed (Yoshioka et al., 2016). This result aligns with previous research showing that *R. imitator* tadpoles increased their begging with age, but that the increased begging is dependent on nutritional need. It is important to note that the studies in *O. pumilio* were done using metamorphic tadpoles (Stynoski & Noble, 2012: Gosner stages 30-40; Stynoski et al., 2018: stage 28+). In one previous *R. imitator* begging paper, the authors noted that they only used tadpoles >14 days post-hatch, as younger tadpoles did not display begging (Yoshioka et al., 2016). A similar pattern of increased begging with age can be seen in many birds, with Kilner (2001) noting that "begging displays become increasingly flamboyant as chicks near independence." House wrens (*Troglodytes aedon)* increase their begging with age, but the rate of increase is dependent on offspring fitness and brood size (Bowers et al., 2019). It is possible that the increase in begging observed in metamorphic tadpoles reflects higher nutritional needs associated with early metamorphosis (Pandian & Marian, 1985).

Changes in eye structure and function across development

Tadpoles are hatched with under-developed eyes and are thought to have poor vision (Hoff et al. 1999). Grant et al. (1981) divided early development of the tadpole retina into four stages, with visual function being present at the end of the first stage (pre-metamorphic) but

noted that a mature retina was not present until the end of the metamorphosis. In *Xenopus* tadpoles, the eyes undergo the most pronounced morphological and physiological changes during early metamorphic stages. This developmental work can be juxtaposed with the behavioral ecology literature, where a recent study suggested that vision is important for poison frog tadpoles during social interactions (Fouilloux et al., 2022), who often develop in water environments with low visibility. This suggestion, combined with the fact that even recently hatched tadpoles display aggressive and begging behaviors, seems at odds with the well-documented research on *Xenopus* visual system ontology. If vision is important for tadpole begging behaviors, we hypothesized that begging tadpoles would have higher neural activation in the retina compared to non-begging tadpoles. Although metamorphic begging tadpoles did have higher neural activation in both the ganglion cell layer and inner nuclear layer, pre-metamorphic begging, control, and aggressive tadpoles had similar levels of neural activation in the retina. The ganglion cell layer, which consists of neurons whose axons comprise the optic nerve, is mostly developed by tadpole hatching, whereas cells are continually added to the inner nuclear layer during tadpole development (Hollyfield, 1968). Neuromodulatory amacrine cells in the inner nuclear layer do not develop until early metamorphosis, which coincides with the onset of expression of neuromodulators important for retina processing, such as tyrosine hydroxylase (synthesizes dopamine; Huang & Moody, 1995; Reh & Tully, 1986; Sarthy et al., 1981) and neuropeptide Y (Hiscock & Straznicky, 1990; Huang & Moody, 1995). In our study, neural activation in our older metamorphic tadpoles significantly correlated with developmental stage. One possibility is that the cellular mechanisms and/or circuitry leading to the higher activation in begging metamorphic tadpoles is simply not present in pre-metamorphic tadpoles. Future studies should examine the types of cells activated in the retina of begging metamorphic tadpoles compared to younger begging tadpoles.

We used phosphoTRAP to examine transcripts being actively translated in the eyes of begging and non-begging tadpoles. We initially expected that eyes of begging tadpoles would have enhanced expression of some socially-relevant neuromodulatory transcripts. While there were over 2400 enhanced and depleted transcripts in begging tadpoles, many of these were related to development. For example, begging tadpoles had a high number of enhanced transcripts associated with lens development. The increased expression of *crystallin*-related genes in the older group of tadpoles that displayed begging could reflect ongoing lens maturation. In *Xenopus* tadpoles, the size and density of the lens does not change during metamorphosis (Polansky & Bennett, 1973), but the expression of *crystallins* does change across developmental stages (Mizuno et al., 1999; Polansky & Bennett, 1973). Lens development in fish is largely complete before hatching, and it only grows in size as the animal develops and grows (Greiling & Clark, 2009). However, ongoing lens development in *R. imitator* tadpoles is consistent with what has been observed in other tadpole species (e.g., Polansky & Bennett, 1973). We also noted that older, begging tadpoles had a high number of depleted transcripts associated with development. This could reflect the developmental stage difference between begging and non-begging tadpoles, and suggests that tadpole stage, not the display of begging behavior, was driving our phosphoTRAP analysis. Overall, our data suggest that *R. imitator* tadpole eyes undergo morphological and physiological changes through late metamorphic stages, similar to that described in other frogs.

Retinotectal projection development

We used the neural tracer neurobiotin to examine connections between the eye and the optic tectum. The optic tectum is the primary target of retinal ganglion cells in the eye, with immature connections between the eye and tectum forming within hours of fertilization in *Xenopus* tadpoles (Liu et al., 2016). Some neurobiotin was detected in early-stage *R. imitator* tadpoles, indicating that connections between the eye and brain are present. Despite loading similar amounts of tracer into the optic nerve of early, middle, and late-stage tadpoles, the amount of tracer detected in the tectum increased with developmental stage in *R. imitator* tadpoles. This result suggests that retinotectal projections increase during metamorphosis, which is further supported by the correlation between the tracer abundance and gosner developmental stage. One explanation for the increased neurobiotin observed in older tadpoles could be due to increased axonal branching and the establishment of circuitry within the tectum (Fawcett, 1981; Fujisawa, 1987; Holt & Harris, 1983). In both *Xenopus* tadpoles and poison frog tadpoles, retinotectal projections rapidly expand during metamorphosis with only minimal retinotectal connections and axonal branching present at hatching.

Tadpole phototaxis behavior

We sought to test visual capabilities using a light/dark behavioral assay. Light preference assays are a common behavioral tool to assess exploratory, anxiety-like behaviors, and visual capabilities (Maximino et al., 2010; Takao & Miyakawa, 2006). For example, rodents display pro-exploratory behaviors in novel environments, but also have an aversion to bright open areas (Bourin & Hascoët, 2003; Shimada et al., 1995). When placed in the arena, most tadpoles initially chose one side and displayed some exploratory behaviors on that side. Midway through the trials, we flipped the light environment to test if tadpoles would track to the same light environment, indicating a true preference. In general, all tadpoles displayed a preference for the dark environment. The strength of this preference was strongest in late-stage tadpoles that all tracked the dark environment and spent more time on and entered the dark side sooner. In tadpoles, light preference and phototactic behaviors seem to vary with species. *Xenopus* tadpoles show a preference for the white side of a white/black preference assay, but when the optic nerves are severed, this preference goes away (Moriya et al., 1996; Viczian & Zuber, 2014). This preference is dependent on both developmental stage and light environment during development. All young tadpoles show a preference for the white environment, but metamorphic *Xenopus* tadpoles lose this preference, and froglets all display a black preference (Moriya et al., 1996). The light preference in *Xenopus* has been tied to retinal photosensors (type II opsins), with their expression increasing during development (Bertolesi et al., 2021). Generally, tadpoles display a preference for brighter light environments because they are often more nutrient rich (Jaeger & Hailman, 1976), but some species may choose a light environment that allows them to be inconspicuous and thus reduces predation risk (Bertolesi et al., 2021; Eterovick et al., 2020). Since *R. imitator* tadpoles are raised in small pools in bromeliad leaves, predator avoidance, especially from above, may be the dominant motivating factor for the observed dark side preference. However, this idea needs to be tested in future experiments.

We found that eyes were not needed for a phototactic response in *R. imitator* tadpoles. In *Xenopus* tadpoles, a phototactic response towards the light side has been attributed to the photosensitive cells in the pineal structure (Adler, 1976; Foster & Roberts, 1982; Mrosovsky &

Tress, 1966). However, severing the optic nerve in some studies removed the preference for the white background environment (Viczian & Zuber, 2014). It is important to note that these studies used a white/black assay, not a light/dark assay. Although a black arena will have lower light levels than the neighboring white side, the difference in illumination between the two sides is much greater in a light/dark assay that uses backlighting. This subtle difference could explain why eyes are important in some behavioral conditions, but not others, although, to our knowledge, this difference has not been experimentally tested. Light levels detected via the pineal structure are also involved in swimming and cementing behaviors in *Xenopus* (Jamieson & Roberts, 1999, 2000; Li et al., 2014). In our experiments, even blinded tadpoles displayed a behavioral preference for the dark side of the arena, indicating eyes are not needed for their phototactic response. However, blinding tadpoles removed the stronger dark side preference exhibited in late-stage tadpoles, suggesting that eyes likely play some role in light detection in late-stage tadpoles. The role of the pineal in detecting light is especially interesting given the natural behaviors of these poison frog tadpoles, where individual young are reared in small pools in bromeliad leaves. The parents visit the tadpole in its nursery, often approaching from above. This likely creates some sort of shadow that the tadpoles can detect, potentially via the pineal structure, as has been observed in blind cavefish (Yoshizawa & Jeffery, 2008). This result also emphasizes that, although vision is likely important for tadpole behavior, they rely on multisensory information from their environment (Rot-Nikcevic et al., 2006; Saidapur et al., 2009; Stynoski & Noble, 2012). Non-visual information may be especially important in young tadpoles with poorly developed visual systems or in animals that live in light-limited environments.

Conclusions

Although vision is likely important in poison frog tadpoles, full visual capabilities likely do not emerge until metamorphosis. This pattern is supported by the presence of development genes in phosphoTRAP data, changes in retina morphology, and retinotectal connections. Despite an underdeveloped visual system, even young tadpoles display begging behaviors towards parents, suggesting they might rely on other sensory modalities for caregiver recognition. Mimetic poison frog tadpoles also do not rely on eyesight for light/dark detection, although removing the eyes does remove the slightly stronger preference for the dark side in late-stage tadpoles, suggesting that vision may play some role in mediating this preference in later metamorphic stages. Together, this work emphasizes the importance of examining sensory system development in social animals and highlights the importance of multisensory interactions.

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DATA STATEMENT

All data associated with phosphoTRAP has been uploaded as supplemental information or to data repositories. The transcriptome will be uploaded to Dryad upon acceptance. Other data is available upon reasonable request.

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