DIFFERENTIAL GENE EXPRESSION IN THE BRAINS OF BEGGING POISON FROG TADPOLES

By

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July, 2020

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Parents often rely on offspring signals to adequately allocate resources to dependent offspring. The mimic poison frog, *Ranitomeya imitator,* has evolved elaborate parental care behaviors to raise their young. Their tadpoles develop in nutrient-poor pools where they beg to solicit trophic eggs from the mother. Previous research has shown a strong correlation between gene expression in the brain and social behaviors in a variety of taxa. This research focused solely on patterns of gene expression in the adult brain, providing a unique opportunity to investigate the genes that drive tadpole behaviors associated with parent-offspring interactions. In this experiment tadpoles of *Ranitomeya imitator* were induced to beg and differential gene expression was compared with that of non-begging, conspecific tadpoles as well as to tadpoles of another non-begging congener, *Ranitomeya variabilis*. RNAseq on the Illumina platform was used to construct transcriptomes and identify patterns of differential gene expression associated with different behaviors and with the highly divergent parental and offspring behaviors that have evolved in these two species with different modes of parental care. Six genes (*fdps, h2ax, mfsd9, gabrg3, scoc,* and *tc3a*) were found to be differentially expressed exclusively in *R. imitator.* Some of these genes are associated with social behavior, motor function behavior, and feeding

responses. For example, previous research on *mfsd9* shows that expression of this gene increased with food deprivation. Many tadpoles experience predation and thus may experience fear when larger individuals enter the breeding pool. *Gabrg3* is a gamma-aminobutyric acid receptor (GABA) known to have a calming effect in humans and was found to be expressed at a higher level in begging treatments. A preliminary cross-species analysis, filtering by a candidate list of genes associated with parental care, indicated that a higher number of social and feeding behavior genes were expressed in *R. imitator* than in *R. variabilis* tadpole brains. The results of these analyses provide evidence that *R. imitator* has evolved patterns of gene expression specifically associated with social behavior and parent-offspring communication in the context of trophic egg-feeding and begging behavior as a result of the evolution of more complex parental care strategies.

DIFFERENTIAL GENE EXPRESSION IN THE BRAINS OF BEGGING POISON FROG TADPOLES

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By

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Introduction

Offspring solicitation has been widely studied across many taxa (birds, mammals, amphibians, and insects). Offspring have evolved begging behaviors that indicate fitness or the need for additional investment (Kölliker & Richner, 2001; Smiseth et al., 2011), which is thought to have evolved from sibling competition (Rodríguez-Gironés et al., 1996). These offspring begging displays can be considered signaling mechanisms allowing parents to assess offspring need, which would otherwise remain hidden to parents that must make resource allocation decisions (Andrews & Smiseth, 2013; Godfray, 1991). Honest begging occurs when a parent allocates resources in accordance with the level of offspring signaling, which in turn reflect offspring need and the physiological cost of begging displays (Godfray, 1991). This benefits both offspring and parents by improving offspring condition (Mock et al., 2011). Extensive research indicates that basic parental care mechanisms gave rise to the evolution of more complex social behaviors (Fischer & O'Connell, 2017). Many social behaviors have a communicative function that rely on signal interpretation. These signals trigger neural responses, which in turn influence patterns of gene expression in the brain that ultimately affect behavior. The evolution of complex social behaviors has led to the evolution of gene expression patterns that are synchronized with social context (Drew et al., 2012; Fischer & O'Connell, 2017; Robinson et al., 2008).

Begging and offspring solicitation are considered to be intrinsic behavioral patterns influenced by gene expression and should be produced during begging displays (Kölliker & Richner, 2001; Velando et al., 2013). Previous research on birds, voles, mice and cichlids has shown a strong correlation between social interactions and gene expression in the brain (Robinson et al., 2008; Smiseth et al., 2011; Young et al., 1999). Songbirds, in particular, have served as important

models for gene expression related to social behavior (Balakrishnan et al., 2014). In the context of social behavior, transcriptomic analyses of differential expression suggest that social interactions have large and long-lasting effects on gene expression in the brain (O'Connell & Hofmann, 2011; Robinson et al., 2008). Experiments using genomic sequencing in a comparative context can elucidate the genetic, biochemical, and physiological mechanisms underlying parental care and parent-offspring communication. Furthermore, comparative studies provide insight into the evolution of many ecological adaptations involving behavior (Balakrishnan et al., 2014; Young et al., 1999).

In poison frogs (family Dendrobatidae), several clades have independently evolved complex parental care patterns, and parental care is thought to be ancestral within this family (Summers & McKeon, 2004). *Ranitomeya imitator* and *Ranitomeya variabilis* are two dendrobatid congeners that, despite having relatively similar ecological niches, have evolved two different parental care strategies associated with food allocation and offspring response. Their behavioral differences are associated with a transition in breeding pool size – originally from larger pools, transitioning to small pools with limited nutrient availability. This transition is also associated with a shift in mating system from promiscuity to monogamy, and a transition from male only parental care to biparental care (Brown et al., 2010; Summers & McKeon, 2004; Tumulty et al., 2014).

Ranitomeya imitator is one of the few monogamous and biparental species of poison frogs that regularly defends territories from both conspecifics and heterospecifics (Brown et al., 2008, 2009a). In pair-bonded frogs, males will lead the female to a suitable deposition location within their territory, where the female will lay up to four eggs on the surface of leaf axils. Eggs typically hatch within 7-14 days (Brown et al., 2010) after which the male will retrieve and carry the tadpoles individually, depositing each in an unoccupied axil of a *Heliconia* leaf within its

territory (Brown et al., 2008, 2010). The male will continue to care for the clutch and periodically inspects the tadpoles (roughly every 7-10 days) throughout their development (2-3 months) (Brown et al., 2010; Tumulty et al., 2014; Yoshioka et al., 2016).

Tadpoles of both *Ranitomeya imitator* and *Oophaga pumilio* exhibit a unique tactile begging behavior (described as rapid vibrations of the body within the water column) to communicate their need for supplemental feeding, in the form of unfertilized (trophic) eggs (Stynoski et al., 2018; Yoshioka et al., 2016). When a tadpole of *R. imitator* performs the begging behavior, the male parent will call for the female until she attends to the tadpole and deposits a single trophic egg (Brown et al., 2010; Yoshioka et al., 2016). Trophic egg feeding is an important evolved behavior in this species, one that requires repetitive parental recognition of visual and tactile cues exhibited by the offspring (Perry, et al., 2006). This behavior is associated with limited resource availability within tadpole rearing sites, since *R. imitator* raise their tadpoles in smaller phytotelmata (plant axil pools) than the sympatric species *Ranitomeya variabilis* (Brown et al., 2008, 2010). Research on this parent-offspring interaction has revealed parental recognition of nutritional need is based on the intensity and duration of tadpole begging, indicating that this behavior functions as an honest signal of long term nutritional need (Yoshioka et al., 2016). However, begging in this species is physiologically costly and is likely to elevate the risk of predation— costs that are likely to limit the exaggeration of this behavior (Mock et al., 2011; Stynoski et al., 2018; Yoshioka et al., 2016). The evolutionary importance of *R. imitator* biparental care mechanisms and trophic egg feeding is further illustrated by a decrease in tadpole survival when male parents were removed after tadpole deposition (Brown et al., 2009b; Tumulty et al., 2014).

Ranitomeya variabilis exhibits the promiscuity and uniparental male care found in most dendrobatid frogs, and males do not actively defend breeding territories, although they do show aggression towards other male frogs in proximity with a courting female (Brown et al., 2008, 2010; Summers & McKeon, 2004). Males will call to approaching females, leading them to the deposition location. Females typically lay up to six eggs and rely on the male to care for the clutch. *Ranitomeya variabilis* has less intensive parental care than *R. imitator* and uses larger bromeliads for egg and tadpole deposition (Brown et al., 2008). After the tadpoles hatch, the male retrieves and carries them individually to other phytotelmata, where chemical cues reduce the frequency of multiple tadpole deposition (Schulte & Mayer, 2017; Schulte et al., 2011). Parental care in this species involves placing tadpoles in breeding pools that have high densities of insect larvae and detritus. In some cases, small tadpoles are deposited with older larger tadpoles (the smaller tadpoles are often cannibalized). In some cases, this involves tactical parasitism, where tadpoles are deposited into pools with unrelated eggs (Brown et al., 2009; Schulte & Mayer, 2017). Some tadpoles will even attempt to elicit transportation from either conspecific or heterospecific adults to avoid cannibalistic tadpoles (Brown et al., 2008; Schulte & Mayer, 2017). Despite extensive sampling in the habitats occupied by both *R. variabilis* and *R. imitator*, studies have not revealed any occurrence of *R. variabilis* tadpoles being placed within pools utilized by *R. imitator*, possibly due to the inability of *R. variabilis* tadpoles to acquire adequate nutrition in these severely resource limited pools (Brown et al., 2009b). Since *R. variabilis* tadpoles must rely on nutrition found within their rearing pools and are not fed by the mother, the tadpoles of this species do not perform begging behavior to elicit supplemental feeding.

Many social behaviors are complex and are reward or goal oriented; therefore, many genes responsible for regulating these functions code for genes in pathways associated with dopamine, serotonin, oxytocin and related neuropeptides (Guo, 2004; Keverne & Curley, 2004; Schneider et al., 2013). The expression levels of neuropeptides such as galanin, which is involved in both feeding behavior and parental care, have been shown to be positively correlated with parental care behavior (Fischer, Roland, Moskowitz, Tapia, et al., 2019). Previous research on mammals and amphibians (*Ranitomeya imitator*) has focused on some of the hormones and neuropeptides that mediate parental care, such as oxytocin, vasopressin, and androgens (Keverne & Curley, 2004; Schulte & Summers, 2017). The non-mammalian homologs of these neuropeptides are arginine-vasotocin, which has the same function as vasopressin, and mesotocin, which is functionally similar to oxytocin (Keverne & Curley, 2004; O'Connell & Hofmann, 2011; Schulte & Summers, 2017). These hormones and neuropeptides mediate cooperative behaviors (e.g. parental care) associated with intimate social interactions.

In association with their complex parental care, dendrobatid frogs have evolved advanced forms of cognitive mapping and spatial navigation abilities (Liu et al., 2019, 2020, in press; Pasukonis et al., 2013). Key aspects of these behaviors are linked to accurate learning and memory (Fischer et al., 2019; Liu et al., 2020). These abilities may also be important for the development of social reward-based behaviors as seen in trophic egg feeding, where poison frog tadpoles must first learn to distinguish and remember cues associated with predators from those associated with parental presence. They must then signal to parents (only) in order to illicit the desired response (trophic egg deposition) (Stynoski et al., 2018). Furthermore, it has been reported that tadpoles that do not beg are not provided with trophic eggs from the female parent (Dugas et al., 2017), which implies the presence of cognitive reward-based learning in

dendrobatid tadpoles. This type of learning, exhibited across many taxa, involves changing behavior in order to elicit the desired response, with the dopaminergic pathway being the predominant reward-based mechanism (Barron et al., 2010; Fischer, Roland, Moskowitz, Vidoudez, et al., 2019).

Due to the high energy demands of brain function, the digestive system is integral to the maintenance of brain activity and as such the pathways that regulate goal centered feeding behaviors are also related to social behavior and the evolution of complex parental care mechanisms (O'Connell & Hofmann, 2011). Previous research has revealed strong associations between feeding behaviors and brain regulation. Serpin family A member 3 (*serpina3*), lymphoid enhancer binding factor 1 (*lef1*), and paired-like homeodomain 2 (*pitx2*) regulated feeding behavior and efficiency in boars (Ding et al., 2017), whereas bone morphogenetic protein receptor 1a (*bmpr1a*) showed a similar function in mice (Peng et al., 2012). The neuropeptide galanin (*gal*) and the neuropeptide Y (*npy*) are known to be associated with feeding and social behavior (Fischer & O'Connell, 2017, 2020; Schneider et al., 2013). The agouti-related protein (*agrp*), in association with melanocortin receptor 4 (*mc4r*), is responsible for appetite, energy regulation, and food intake (Lu et al., 2017; Schneider et al., 2013). Finally, tachykinin precursor 3 (*tac3*) is highly expressed in the brain, stomach, and intestine and plays an important role in growth and digestive functions (Zhang et al., 2019).

A possible explanation for the costs associated with begging behavior are the effects of the hormones corticosterone and testosterone. Several studies have found that begging offspring suffer reduced growth rates (Buchanan et al., 2007; Stynoski et al., 2018; Yoshioka et al., 2016). Previous research indicates that testosterone is positively correlated with begging intensity but negatively associated with nestling growth in canaries (Buchanan et al., 2007). Increases of

these hormones led to increased levels of begging in chicks while also mediating a higher physiological deficit, enforcing the honesty of parent-offspring communication (Hinde et al., 2009; Kitaysky et al., 2001; Loiseau et al., 2008). In pied-flycatchers, testosterone affects begging intensity and duration, and increased testosterone leads to higher fledging success (possibly associated with increased metabolism and muscle mass) (Goodship & Buchanan, 2006; Lipar & Ketterson, 2000). Most research indicates that both corticosterone and androgens play a key role in begging behavior in the nestlings of many species of birds, likely due to the stress response correlated to short term food deprivation (Kitaysky et al., 2001). Increases in the hormone corticosterone have also been linked to energetic demands associated with parental care in frogs (Fischer & O'Connell, 2020).

The highly divergent parent and offspring behaviors that have evolved in *R. imitator* and *R. variabilis* should have led to divergent genetic, physiological, hormonal and neural mechanisms affecting tadpole behavior in the context of social interactions. Identifying the genes that are differentially expressed in the context of parent-offspring interactions within and between these two species should illuminate the mechanisms underlying the evolution of begging behavior in *R. imitator*. Restricted resource availability within tadpole breeding sites likely drives divergent patterns of selection on parental care differences between these species (trophic egg feeding versus detritus feeding).

Begging behavior constitutes a wide variety of modifications related to social behavior, learning, offspring bonding, feeding, and genes that mediate stress responses. My predictions concerning begging behavior can be described as follows:

(1) Tadpoles sampled after begging will show gene expression patterns related to social interaction, learning, and recognition.

(2) There will be a strong correlation between digestion and brain function, which will influence patterns of gene expression.
(3) Stress responses related to begging are also likely to influence patterns of gene expression

I hypothesize that there will be evidence of differential expression of genes associated with social behavior, learning, feeding, and stress responses (see Table 1) in begging compared to non-begging treatments in *R. imitator*, and between begging versus non-begging species (*R. imitator* versus *R. variabilis*). I also expect to find novel sets of genes that may influence begging behavior since relatively little research has been conducted on amphibian genes expressed in the brains of offspring.

Methods

Top Candidate Gene Table Construction

A top candidate genes list was organized to encompass the functions, hormones, and genes that may be associated with begging behavior (Table 1). These genes include those related to social behavior and learning, feeding and digestion, and stress responses. This table was used to compare genes found to be differentially expressed in *R. imitator* to other genes found in previous research associated with begging, parental behavior, and social interactions.

Candidate Genes	Gene Function	Description	Citation
Agrp	feeding behavior, weight homeostasis	agouti related neuropeptide	(Lu et al., 2017)
Ar	associated with greater begging intensity	adrogen receptor	(Hinde et al., 2009)
Avp	social behavior	arginine vasopressin	(Schulte & Summers, 2017), (Keverne & Curley, 2004)
Bmpr1a	critical to feeding behavior in mice	bone morphogenetic protein receptor	(Ding et al., 2017)
Crh	stress response, corticosterone related to begging	corticotropin releasing hormone	(Kitaysky et al., 2001)
Drd1	regulating food intake, mediates behavioral responses	dopamine receptor	(Guo 2004)
Drd3	associated with cognitive, emotional, and endocrine functions	dopamine receptor	(Guo 2004)
Drd4	associated with reward cues	dopamine receptor	(Guo 2004)
Egr1	associated with neuronal plasticity and social experience	early growth response 1	(Robinson et al., 2008)
Gal	appetite stimulating	galanin and GMAP prepropeptide	(Fischer and O'Connell, 2017)
Grp	encodes a member of the bombesin-like family of gastrin releasing peptides, associated with digestion	bombesin	(Fischer et al., 2019)
Htr1B	manages release of serotonin and domamine in the brain; associated with reward, learning, and memory	5-hydroxytryptamine receptor 1B	(Guo, 2004) (Fischer et al., 2019)
Lef1	increases production of energy related to changing feeding behavior	lymphoid enhancer	(Ding et al., 2017)
Mc4r	appetite	melanocortin 4 receptor	(Lu et al., 2017)
Npy	stress response, food intake	neuropeptide Y	(Fischer and O'Connell, 2017)
Oxtr	modulates stress, social memory and recognition, and bonding	oxytocin receptor	(Schulte & Summers, 2017), (Keverne & Curley, 2004)
Pitx2	food intake behavior	paired like homeodomain 2	(Ding et al., 2017)
Serpina3	control residual feed intake	serpin family	(Ding et al., 2017)
Tac3	associated with digestion	member of the tachykinin family	(Zhang et al., 2019)

Table 1. List of top candidate genes associated with begging, feeding, stress, and social behaviors. These genes have been identified and annotated in *Xenopus* transcriptome assemblages

Animal Husbandry

This project was conducted in a laboratory environment at East Carolina University, using a pre-established breeding colony of sexually mature *Ranitomeya* poison frogs, from March 2019 through October 2019. Adult frogs were kept and bred in pairs to ensure parental identity. Pairs were housed in 25.4x25.4x30.48 cm glass aquaria with sphagnum moss (New Zealand AAA Grade), and live golden pothos plants (*Epipremnum aureum*); small polyvinyl chloride pipes (breeding pools) were available to breeding adults for the deposition of eggs and/or tadpoles. The breeding colony was kept on a 12-hour light cycle and fed flightless fruit flies (*Drosophila melanogaster*) dusted with vitamin supplements (Repashy Calcium Plus) four times on a weekly basis. The ambient temperature was monitored and kept at 25°C and distilled water in each breeding pool was changed every 5-7 days to keep tadpole waste at a minimum.

Eleven *Ranitomeya imitator* and ten *R. variabilis* breeding pairs were selected, and two tadpoles from each pair were included in the experiment. Tadpoles included different color morphs from different source populations of *R. imitator* (Tarapoto, Varadero, Sauce, Huallaga, and cross breeds of each) and *R. variabilis* (Rodyll and Borja Ridge source populations). This project design allowed adults to breed and raise tadpoles under normal conditions until tadpoles were selected for trials. Tadpoles were raised until they reached a suitable age to exhibit strong begging behavior and an adequate size for easier brain extraction (larval stages 33-36, derived from Gosner, 1960 and Chakravarty et al., 2011). After tadpoles reached a suitable size, they were kept for 24 hours without food in order to elicit a strong hunger-driven begging response.

Seven *Ranitomeya variabilis* adult pairs were removed from the project because they did not produce a clutch and/or surviving tadpoles. Surrogate *R. variabilis* were obtained from a collaborating laboratory at Stanford University (the O'Connell lab) during the summer of 2019.

After a quarantine period of ten days, eight surrogate tadpoles were divided amongst four *R. variabilis* adult pairs in the East Carolina University frog lab (n = 4, control; n = 4, treatment). Surrogate tadpoles were placed with adult frog pairs to simulate natural adult-offspring interactions and to prevent biases between tadpoles raised naturally by parents and tadpoles without adult contact. Surrogates were placed in enclosures that either had previously completed trials or with pairs having younger tadpoles to prevent the transportation of smaller surrogate tadpoles to breeding pools with larger natural born tadpoles, avoiding the consumption of smaller tadpoles (Brown et al., 2009b; Schulte & Mayer, 2017; personal laboratory observations). Surrogate tadpoles were kept with foster parents for ≥ 2 weeks. Adult frogs were observed in surrogate pools on seven separate occasions during normal breeding pool water changes, which indicates that interactions took place between surrogate tadpoles and foster parents for the duration of tadpole rearing prior to the experimental trials. No surrogate or natural born tadpoles were killed or consumed during the duration of this project by either adult frogs or other tadpoles (personal observations).

Two *Ranitomeya imitator* breeding pairs were removed at the conclusion of the trial. One pair removal was due to the death of the male adult frog prior to having a second tadpole, and the other pair was removed because they did not have both surviving tadpoles at the conclusion of the project. Neither breeding pair was replaced in the experiment and the single tadpole from each pair was used during preliminary behavior and brain extraction trials. At the conclusion of the research period, 18 tadpoles from nine *R. imitator* pairs (n = 9, control; n = 9, treatment) and 14 tadpoles from three *R. variabilis* breeding pairs and surrogate additions (n = 7, control; n = 7, treatment) were included in the behavior and brain extraction experiment.

Behavior Experiment

An 18.93 liter food grade bucket was filled with 9.46 liters of distilled water, and an aquarium heater was placed at the bottom of the bucket with the temperature setting of 25°C. This temperature was used for optimal tadpole growth by researchers cataloging tadpole growth (Chakravarty et al. 2011). A plexiglass platform (with polyvinyl chloride pipe legs filled with river rocks and secured with aquarium sealant) was placed directly above the aquarium heater. A polystyrene rigid plastic cup measuring 7.62 cm by 7.62 cm was filled with 50 mL of distilled water secured with a Velcro plexiglass lid and placed on the platform. An aquarium thermometer was placed in the bucket at water level with the platform, and another thermometer was placed within the trial cup (Figure 1). The water in both the bucket and the trial cup was allowed to equilibrate for one hour and checked for temperature readings of 25°C prior to performing each trial. Six preliminary trials were completed between 04/27/2019 and 05/07/2019 to establish proper behavioral observation and euthanasia protocols. The first tadpole from each breeding pair that reached the appropriate Gosner stage, was randomized between control or treatment trials in order to prevent bias between trial selections. Behavior trials were completed at the lab in East Carolina University during May 2019 and October 2019 between 1000 and 1600 hours when these diurnal poison frogs are most active (Schulte, 1986; personal observation).

Figure 1. Behavior Trial Set up Includes: (1) trial bucket with two camera platforms, (2) plexiglass stand, (3) aquarium heater, (4) aquarium thermometer, and (5) two GoPro Hero3 Black Edition HD3.03.00) cameras (Photo by B. Geralds).

Begging Behavior experiment: After reaching the Gosner stage 33, tadpoles of *R. imitator* (n = 9) were collected and placed in the rigid plastic cup (previously described) with one of the parent frogs, with a preference for the female when it was possible to differentiate between the sexes (on two separate occasions the male parent was selected and was differentiated by calling within the testing chamber during the trial). The rigid plastic cup was secured with a clear piece of plexiglass to prevent escape. Both the parent frog and tadpole were allowed to acclimate to the environment for 10 minutes. Behaviors were recorded and remotely monitored by two GoPro (Hero3 Black Edition HD3.03.00) cameras, secured on platforms attached to the trial bucket, for 30 minutes. Immediately following the begging behavior trial tadpoles were anesthetized by administering 20% Benzocaine to the abdomen, then euthanized by immersion in a solution of 0.1% Tricaine methanesulfonate (MS-222) prepared in a 25 mm sodium bicarbonate. Euthanasia

occurred within 00:03:12 minutes ± 00:00:43 across all trials. This quick euthanasia was meant to prevent gene expression loss in the brains of the tadpoles. The brains of the tadpoles were extracted and preserved in RNAlater® RNA Stabilization Solution (Invitrogen, ThermoFisher Scientific) at 4°C for 24 hours and stored at -80°C for later transcriptome sequencing.

Control Begging Behavior experiment: After reaching Gosner stage 33, tadpoles from *R. imitator* (n = 9) were collected and placed in the rigid plastic cup without the presence of the adult parent frog. The rigid plastic cup was secured with a clear piece of plexiglass to prevent inconsistencies with the presence or absence of the glass. The tadpole was allowed to acclimate to the environment for 10 minutes. Tadpole behaviors were recorded and remotely monitored by two GoPro (Hero3 Black Edition HD3.03.00) cameras for 30 minutes as stated before in previous trial methods to ensure that begging did not occur in the absence of the adult parent frog. Immediately following the behavior trial, tadpoles were sacrificed as described above, and the brains were extracted and preserved in RNAlater at 4°C for 24 hours and stored at -80°C for later transcriptome sequencing.

Comparative experiment: After reaching Gosner stage 33, tadpoles from *R. variabilis* (n = 7, control; n = 9, treatment) were tested using the protocols described above for the begging behavior and control experiments. Brains from both comparative trials were collected and preserved as noted previously.

Behavioral Analysis

Trial videos were reviewed after brain extraction and behaviors were annotated and recorded for trial duration percent. Two *Ranitomeya imitator* treatment trials were excluded—one due to camera shifts that only allowed for partial view of the trial cup, and the other because both

cameras stop recording during the trial. One *R. variabilis* treatment trial affected by file corruption was not included in the review. Due to camera malfunctions (n = 9, control; n = 7, treatment), *R. imitator* and (n = 7, control; n = 6, treatment) *R, variabilis* trials were reviewed and annotated using the program Solomon Coder beta 19.08.02 (Péter, 2019). While some trials could not be incorporated in the behavior analysis, live streaming was used to confirm that all *R. imitator* tadpoles observed during treatment trials engaged in begging, whereas none of the control or *R. variabilis* tadpoles begged. An ethogram summarizing adult and tadpole behaviors and interactions is presented in Table 2. After visual analysis was completed using Solomon Coder, a table was created from each trial and the duration percentages from each behavior were calculated.

Behavior	Description
looking at adult	at a distance > 3.73mm on screen, tadpole snout is directly pointed at adult
looking at tadpole	at a distance > 3.73mm on screen, adult snout is directly pointed at tadpole
looking at both *	at a distance > 3.73mm on screen, both tadpole and adult snouts are directly pointed at each other
movement	tadpole is swimming, not in the direction or facing adult
movement(2)	adult is moving, not in the direction or facing tadpole
stationary	tadpole is not moving, not facing adult
stationary(2)	adult is not moving, not facing tadpole
begging*	rapid movement/vibration of tadpole body
proximity*	adult is at or below water surface ≤ 3.73mm on screen distance from tadpole; includes facing or not facing
misc	technical difficulties or anything not previously listed

Table 2. Behavior Ethogram: used to analyze trial videos. Each behavior is restricted as stated and is divided into individual interaction or multiple simultaneous interaction that cannot be annotated concurrently. Behaviors annotated with * cannot occur with any other behavior.

Tadpole Brain extraction

Dissections were completed using a Konus Crystal-45 dissecting microscope. Euthanized tadpoles were placed in a dry petri dish. Using dissecting scissors to make an angled cut towards the mouth region (to avoid cutting into the digestive organs), the head was removed from the

body. With size 4 forceps, the outer layer of skin was removed from the posterior to anterior region of the head. Each eye was removed by placing the tip of the forceps below each eye and lifting the eye from the socket. The tadpole was then flipped so the ventral region was facing upwards, and the tip of the forceps was placed within the mouthparts so that the lower jaw could be removed. The head was again flipped so the dorsal region was facing upward, and the remaining nonbrain material was removed. Using the tip of the forceps, a small incision was made at the anterior region of the brain (to avoid puncturing the soft white brain material) in the thin black membrane covering the brain (Figure 2). The tip of the forceps was placed within the skull beneath the brain, and the brain was lifted from the head. The brain was preserved in RNAlater at 4°C for 24 hours before transferring the sample to -80°C for later transcriptome sequencing.

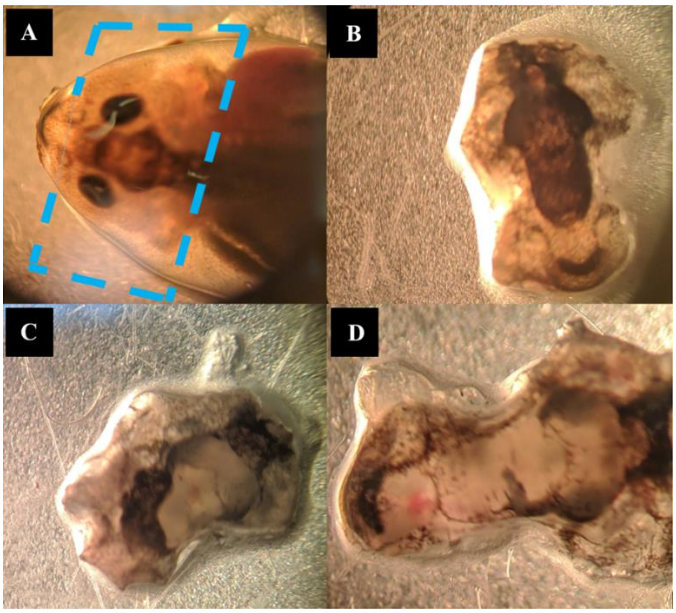

Figure 2. Tadpole Brain Extraction: (A) Illustrates the angle of the cut made to preserve brain tissue and avoid other organs (B) eyes, lower jaw, and remaining non-brain material has been removed, leaving only the black membrane covering the brain (C) non-brain material is further

removed and black membrane as been pulled back to reveal brain (D) final result of extraction before brain removal from skull (Photo by B. Geralds).

RNA extraction and sample sequencing

Ranitomeya samples were randomized to prevent batch bias, and RNA extractions followed the TRIzol RNA extraction protocol. Sample quality was checked using Qubit RNA high sensitivity assay kits, and RNA was quantified with RIN scores using an Agilent 2100 Bioanalyzer at the Genomic Core Facility (East Carolina University). Samples were sent to Novogene (Hong Kong, China) for library prep and sequencing in March 2020. At the recommendation of Novogene, three samples (two control *R. imitator* control and one *R. variabilis* treatment) were not sequenced due to low sample quantity and/or substandard RIN values (after sample removal; n = 7, control; n = 9, treatment for *R. imitator*; and n = 7, control; n = 6, treatment for *R. variabilis*). Sample sequencing was completed and returned for analysis in April 2020.

Read quantification, transcriptome annotation, and differential expression analysis

A combined dataset of one randomly selected treatment and control sample was used to build *de novo* transcriptomes for each species using the Oyster River Protocol version 2.2.8 (MacManes, 2018). Initial error correction using RCorrector 1.01 (Song & Florea, 2015) was followed by a robust removal of Illumina adaptors from sequences and mild quality trimming using trimmomatic version 0.36 (at a Phred score of ≤ 3; (Bolger et al., 2014)). When more rigorous trimming methods are used, transcriptome assemblages have reduced completeness (MacManes, 2014). The Oyster River Protocol assembles a final transcriptome by using a series of different single transcriptome assemblers, and also multiple kmer lengths, before merging them into a

final transcriptome (MacManes, 2018). Assemblies were conducted using Trinity version 2.8.5 (Grabherr et al., 2011), Transabyss version 2.0.1 (Robertson et al., 2010), and SPAdes assembler version 3.13.1, with a kmer length of 55 and a second time with a kmer length of 75 (Bankevich et al., 2012). These separately built transcriptomes (using the three aforementioned assemblers) were merged using OrthoFuser (MacManes, 2018). Unique contigs that were dropped in Orthofuser were retrieved using a reciprocal blast search of the final assembly against the three individual assemblies for unique contigs. All contigs were removed with expression lower than one Transcript Per Million (TPM) using the TPM = 1 flag in the ORP. Transcriptome quality was assessed using BUSCO version 3.0.1 (Simão et al. 2015) and TransRate 1.0.3 (Smith-Unna et al. 2016). Finally, the reference transcriptome was annotated using Diamond with the Swiss Prot database (The UniProt Consortium, 2019).

Raw sample reads were trimmed using trimmomatic (Bolger et al., 2014) in order to remove Illumina adaptors and maintain high-quality reads. The abundance of transcripts from the trimmed reads were quantified and pseudoaligned to the reference transcriptome using Kallisto version 0.44.0 (Bray et al., 2016). The average proportion of read sequences pseudoaligned to the reference transcriptome for *Ranitomeya imitator* and *R. variabilis* was 44.5% and 45.4% respectively. Differential gene expression was tested in Rstudio version 3.6.3 using Sleuth version 0.30.0 (Pimentel et al., 2017). Likelihood ratio tests were used to test the differences between the control (non-begging) and treatment (begging) samples, and were corrected for multiple comparisons associated with genetic sequencing with a Benjamini-Hochberg-adjusted False Discovery Rate using q-value ≤ 0.05 as the significance threshold to incorporate statistical significance for *R. imitator* and *R. variabilis*. This threshold was later modified for *R. imitator* (q-value ≤ 0.3) in an exploratory attempt to locate genes with biological significance (Boehm et

al., 2006). Final threshold values included p-value ≤ 0.01 for both *R. imitator* and *R. variabilis* samples. Transcripts without an associated gene name were annotated using Geneious biologics software version 2019.0.4 (Geneious Prime 2019.0.4 https://www.geneious.com) to find the corresponding protein sequence before using the Basic Local Alignment Search Tool tblastn and tblastx (National Center for Biotechnology Information, 1988) to identify matching gene sequences.

Preliminary Parental Candidate Gene Analysis

A preliminary qualitative approach was used to compare transcripts per million (TPM) for genes of interest between *Ranitomeya imitator* and *R. variabilis.* Combined control and treatment sample transcripts from *R. imitator* (n = 7, control; n = 9, treatment) and *R. variabilis* (n = 7, control; n = 6, treatment) were annotated using the SwissProt database. Annotated transcripts were filtered using a candidate gene list (derived from (Fischer et al., 2019; Fischer et al., 2019)). Research from these projects found genes that likely influenced parental care in poison frogs (tadpole transport and maternal provisioning). Feeding genes of interest were also selected from the top candidate genes list (Table 1). The average TPM was calculated for each transcript that matched to a gene name from the candidate gene list (Table 5, Figure 11).

Results

Behavior Analysis

Ranitomeya imitator tadpole behaviors were analyzed and each behavior scored for the duration of time the behavior was exhibited. Behavioral durations were used to calculate the percent total of the 30-minute trial. Proximity and begging behaviors were treated as interactions between

adults and offspring. Individuals begged for an average of 2.9% ± 4.031 and were in proximity 6.7% ± 4.636 of trial duration.

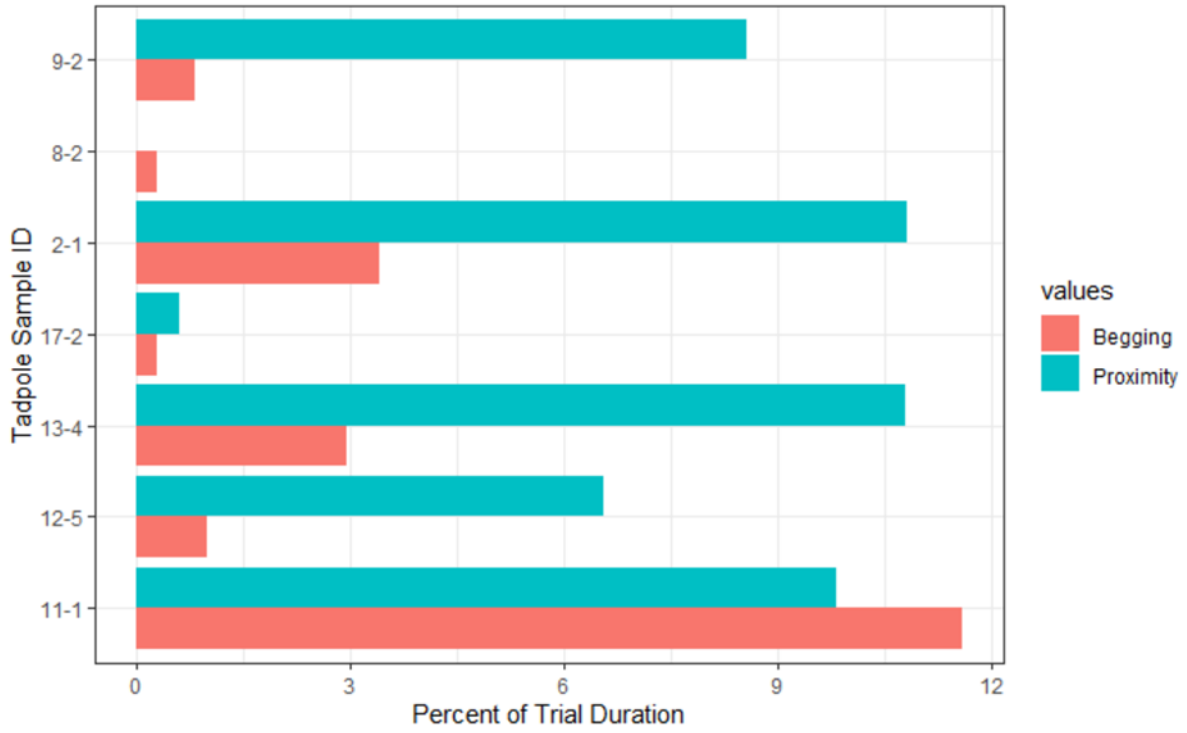

Figure 3. Begging and Proximity Bar Graph: showing the duration of begging displays by tadpoles in the begging trials and the duration tadpoles in this treatment type spent interacting with parent frogs.

Differential Expression Analysis

Initially, no significant differentially expressed genes were found between the *Ranitomeya imitator* control and treatment samples. However, when begging samples with performance behavior durations < 1% were excluded from the analysis, four statistically significant genes were found (q-value ≤ 0.05) with two additional biologically significant genes after threshold

modification (q-value ≤ 0.3) (Table 3). It should be noted that two additional transcripts were significantly differentially expressed but could not be matched to an annotated gene.

Gene Symbol	p-value	q-value	Gene Function
fdps	3.15E-05	0.219359	enzyme coding gene resulting product is key for cholesterol and sterol biosynthesis
h2ax	9.86E-07	0.00825	protein coding gene and DNA repair protein that regulates motor behavior and has strong influences in the brain and behavior
mfsd9	1.88E-10	7.87E-06	protein coding gene detected in food regulatory regions of the brain that may have involvment in nutrient intake and regulation
gabrg3*	1.02E-09	2.13E-05	protein coding gene that encodes for a gamma-amniobutyric acid (GABA) receptor, inhibitory neurotransmitter related to decreases in anxiety
scoc*	4.70E-05	0.245907	protein coding gene that plays a role in neurogenesis and domaminergic neurotransmission; positive regulator of amino acid starvation-induced autophagy
tc3a	9.61E-07	0.00825	expression results in frequent excision and transposition of endogenous TC3 elements
NODE_46781_length_986_cov_5.996707_g27054_i0	9.20E-07	0.008245	unknown
TRINITY_DN3863_c0_g1_i1	4.55E-05	0.245907	unknown

Table 3. *Ranitomeya imitator* Significant Genes: Biologically significant and statistically significant genes differentially expressed between control and treatment samples. Gene functions were generated using Uniprot database, NCBI database, and previous research (Crestani et al., 1999; Gerra et al., 2018; Perland et al., 2017; Romanelli et al., 2009; Shekhar et al., 1990; Weyemi et al., 2018). Genes with associated * symbol indicates genes that were not previously annotated and were manually recovered using BLAST. The last two entries are transcripts that could not be matched to an annotated gene.

Differential transcripts were used to generate a heat map according to highest fold change (Figure 4). Treatment samples had higher fold change expression in the genes *gabrg3, mfsd9, fdps* and *scoc* whereas *tc3a* and *h2ax* had lower fold change.

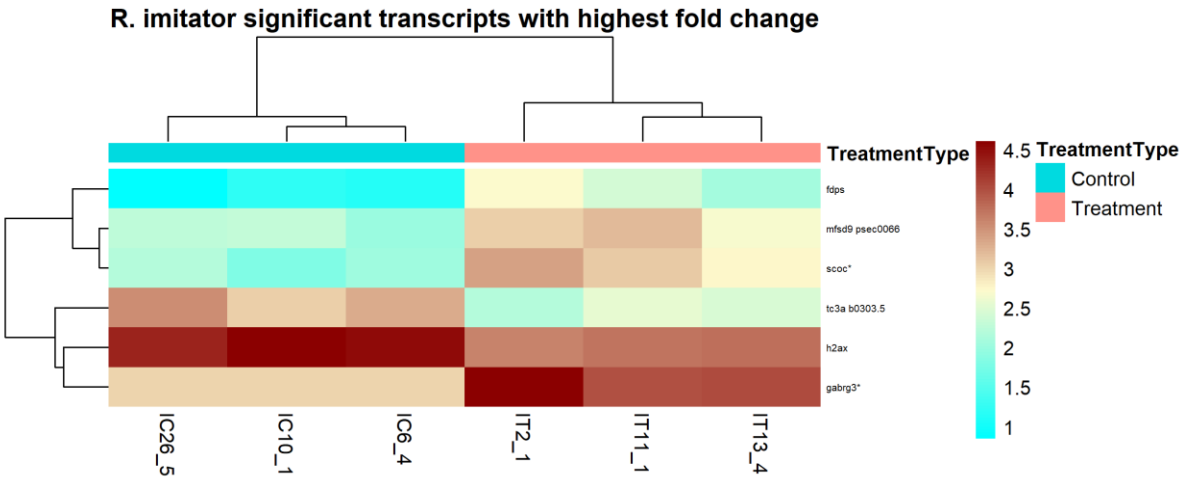

Figure 4. Heat map: generated in Sleuth describing differential expression between control and treatment samples in *Ranitomeya imitator*.

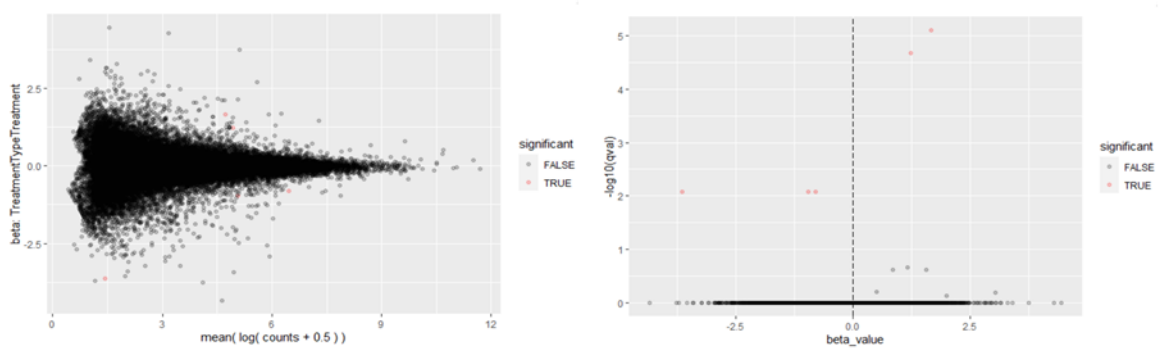

Figure 5. MA plot (left) and volcano plot (right) for *Ranitomeya imitator* transcripts.

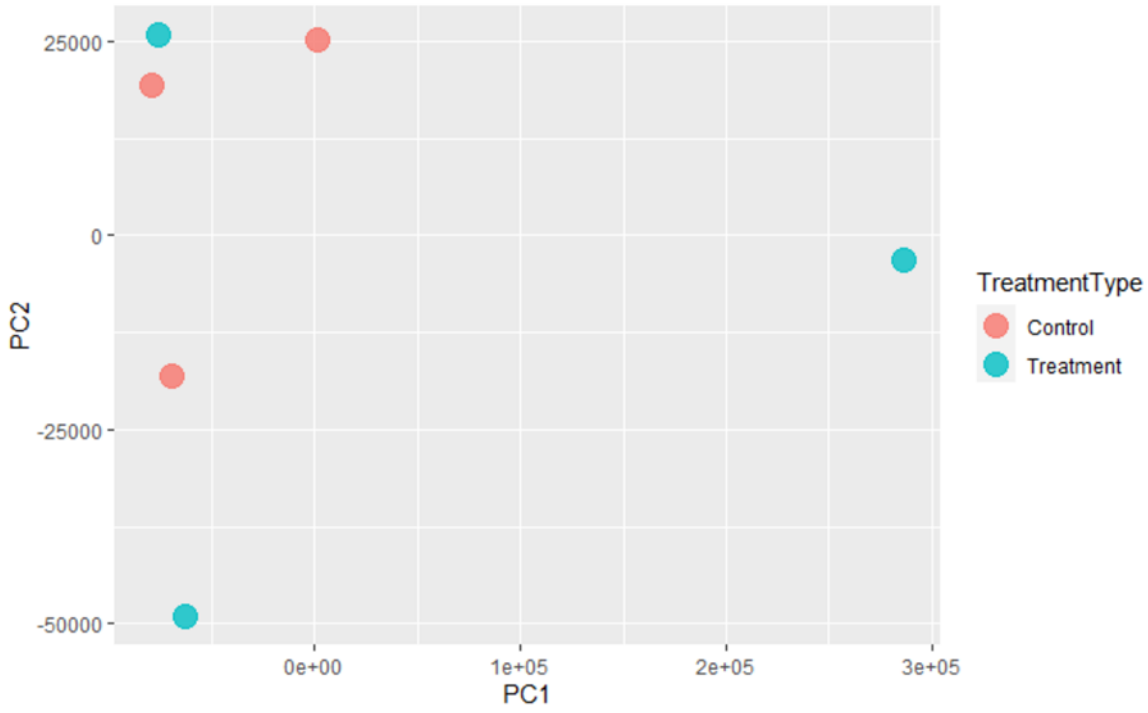

Figure 6. PCA plot: describing the principal components affecting variation in abundance between control and treatment samples of the species *Ranitomeya imitator*.

There were 11 significant differentially expressed genes found in *R. variabilis* meeting the significant threshold criteria ($q \leq 0.05$) (Table 4). These genes were not differentially expressed in *R. imitator*.

Gene Symbol	p-value	q-value	Gene Function
srrm1	6.80E-07	0.009279	protein coding gene involved in numerous pre-mRNA processing events
cdc42bpa	3.19E-06	0.012889	protein coding gene that plays a role in the regulation of cytoskeleton reorganization and cell migration, upregulated in many cancers
papolg	4.37E-06	0.012889	protein coding gene related pathways are mRNA surveillance pathway
tmem184b	3.62E-06	0.012889	protein coding gene that may activate the MAP kinase signaling pathway
ptprf	1.38E-05	0.029801	regulates a variety of cellular processes (cell growth, differentiation, mitotic cycle, and oncogenic transformation)
scn8a	2.56E-05	0.045482	protein coding gene involved in rapid membrane depolarization that occurs during the formation of the action potential in neurons
prkce	2.66E-05	0.045482	protein coding involved in cellular signalling pathways
gpatch8*	9.12E-10	3.74E-05	protein coding related to RNA processing
adgrl3*	2.48E-06	0.012889	protein coding gene that may function in both cell adhesion and signal transduction
fmn1*	1.97E-05	0.036904	protein coding gene that plays a key role in limb morphenogenesis
wnk2*	3.07E-05	0.047257	protein coding gene that may be important in the regulation of electrolyte homeostasis, cell signaling survival, and proliferation

Table 4. *Ranitomeya variabilis:* Statistically significant genes differentially expressed between control and treatment samples. Gene functions were generated using Uniprot database and NCBI database. Genes with associated * symbol indicates genes that were not previously annotated and were manually recovered using BLAST.

Differential expression was also illustrated with a heatmap (Figure 7).

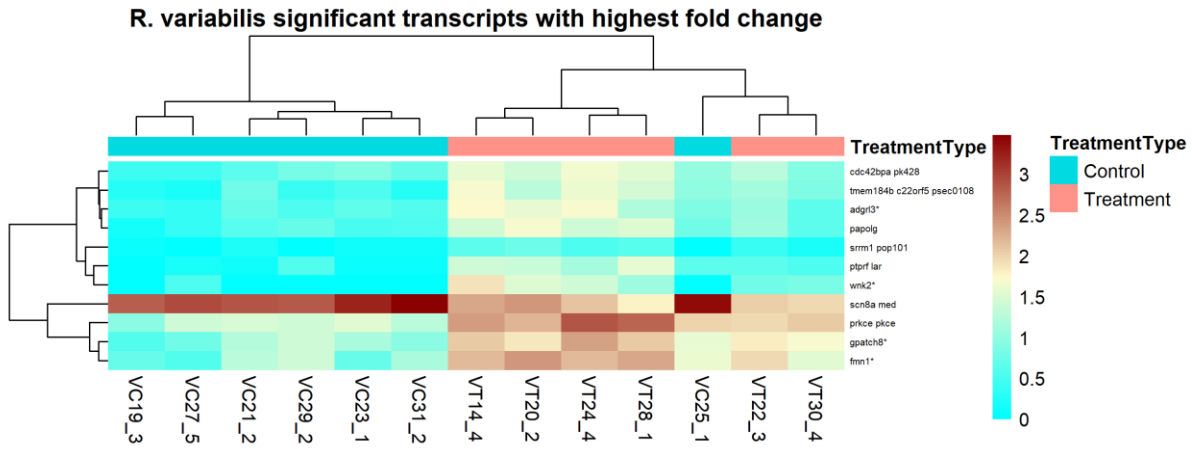

Figure 7. Heat map: generated in Sleuth describing differential expression between control and treatment samples in *Ranitomeya variabilis.*

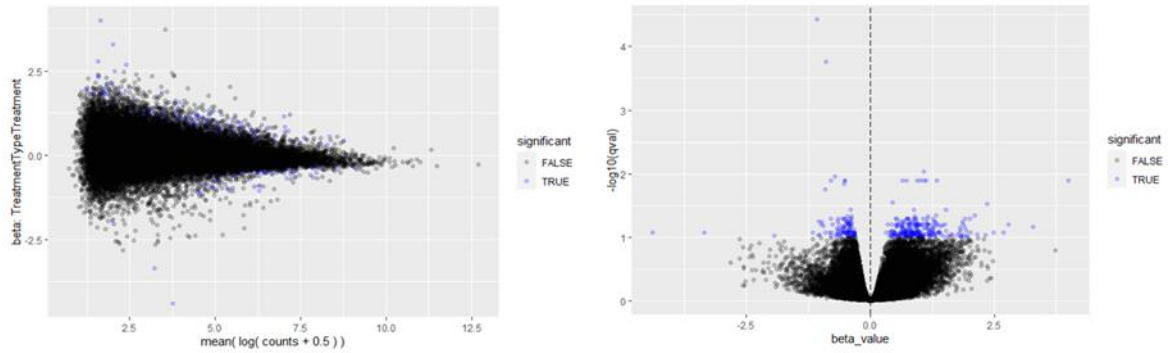

Figure 8. MA plot (left) and volcano plot (right) for *Ranitomeya variabilis* transcripts.

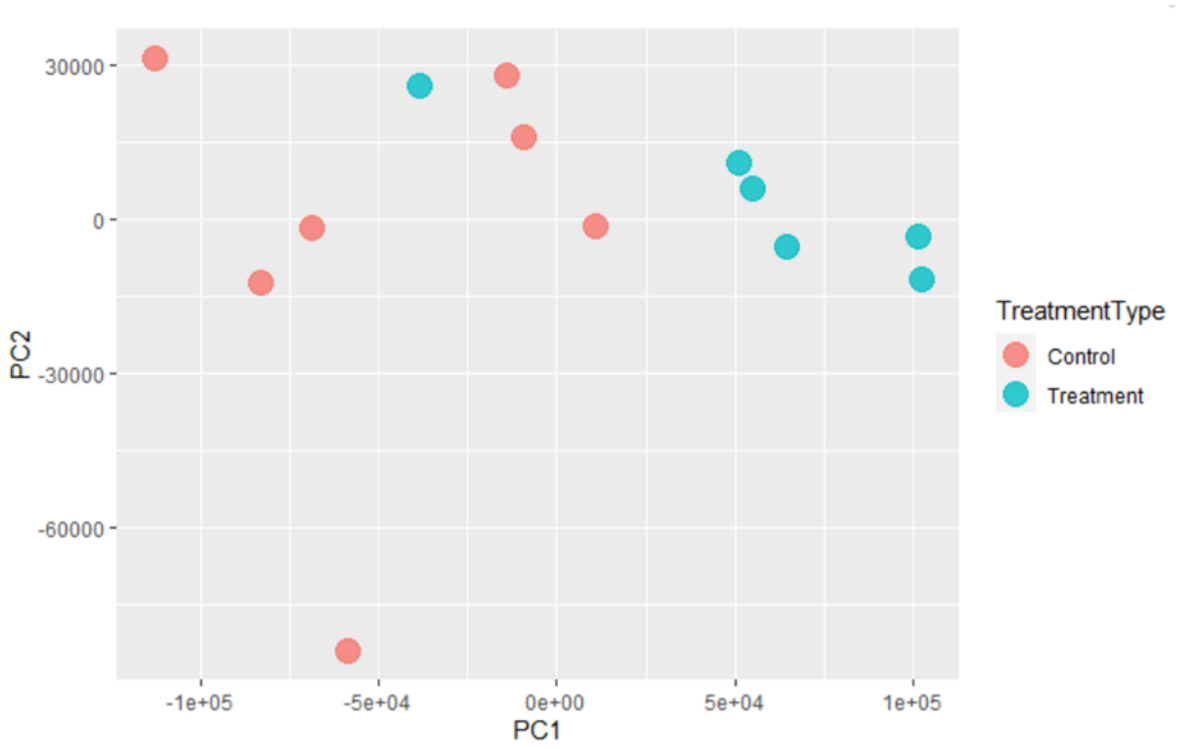

Figure 9. PCA plot: describing the principal components affecting variation in abundance between control and treatment samples of the species *Ranitomeya variabilis.*

None of the six genes (*fdps*, *h2ax*, *mfsd9*, *gabrg3*, *scoc*, and *tc3a*) found to be significantly differentially expressed in *R. imitator* were found to be significantly differentially expressed in *R. variabilis* (Figure 10).

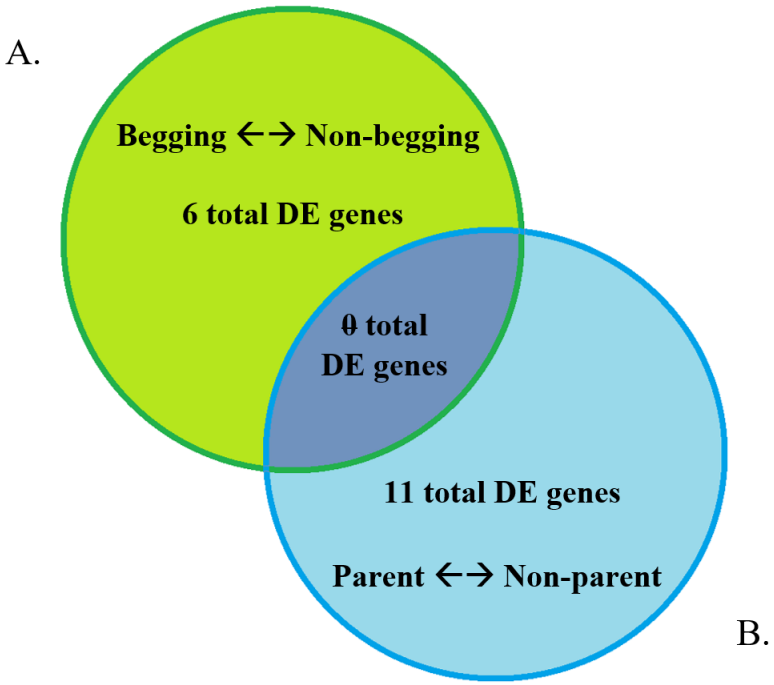

Figure 10. DE Venn diagram: genes differentially expressed between *Ranitomeya imitator* control and treatment samples (top left) and between *Ranitomeya variabilis* control and treatment samples (bottom right).

Preliminary Parental Candidate Gene Analysis

Ranitomeya imitator had more transcripts match to the genes associated with feeding and parental care behavior (29 genes) than *R. variabilis* (six genes, five of which were shared with *R. imitator*. *Ranitomeya variabilis* had a higher average of TPM for three of these shared genes (Table 5, Figure 11).

Gene Symbol	R. imitator ($\bar{x}$ TPM)	R. variabilis ($\bar{x}$ TPM)
grp	2.628	9.93
htr1a	21.251	18.543
igf1r	0.599	0.223
npy	9.532	16.031
pomc	6.729	8.601
drd1	—	0.577
aqp4	0.266	—
baiap2	1.743	—
calcr	2.942	—
cbln1	2.857	—
cck	0.815	—
dbh	0.097	—
drd4	1.166	—
gabra1	0.08	—
gal	34.68	—
gnrhr2	1.45	—
htr1b	14.896	—
htr1d	0.099	—
htr5a	16.208	—
mbp	7.084	—
myh11	0.822	—
nlgn3	5.523	—
nos1	4.161	—
npy2r	0.36	—
nts	2.032	—
pgc	0.513	—
prlr	1.881	—
srm	4.306	—
syt2	0.552	—
trhr	4.654	—

Table 5. Parental Care Candidate Gene List: Average TPM annotated and filtered using a candidate gene list all samples in *Ranitomeya imitator* and *Ranitomeya variabilis*.

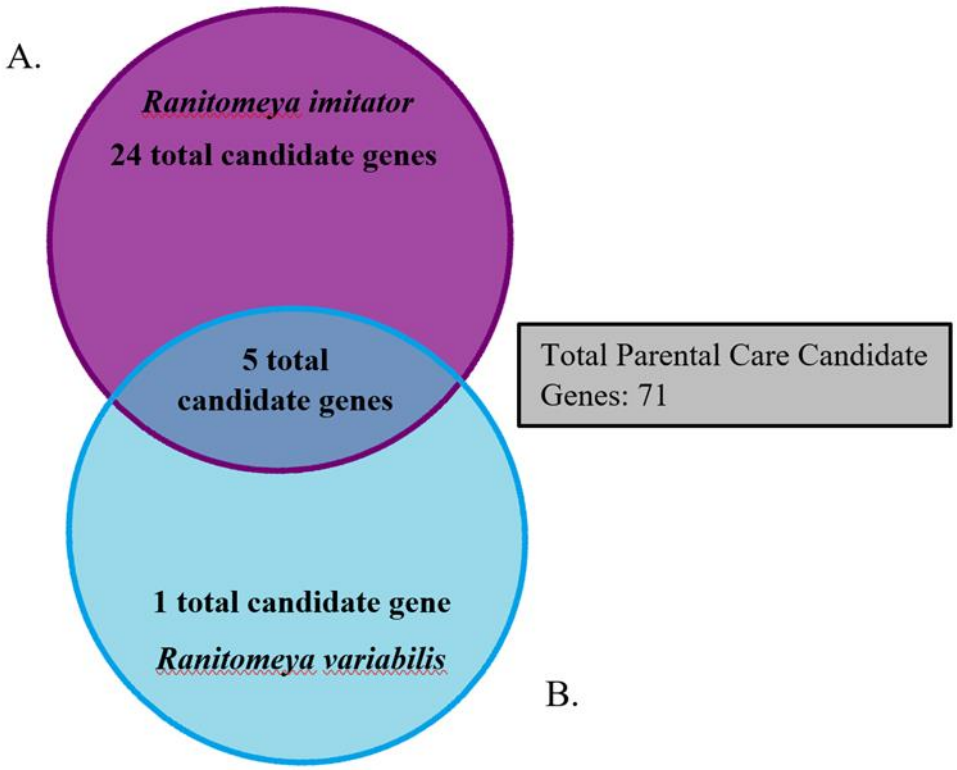

Figure 11. Parental Candidate Gene Venn Diagram: In both species 30 of the 71 parental genes were found, but only 5 of those were shared between species.

Discussion

Behavior Analysis

Significant differentially expressed genes were not found when individuals that performed begging behavior <1% of the total trial duration were included in the analysis. One possibility for this discrepancy could be that *R. imitator* tadpoles have a constitutively higher expression of some neurological genes than *R. variabilis* regardless of the parental condition tadpoles experience. However, additional comparative analyses between both species is needed to determine if this is the case.

Another possibility is associated with the observation that begging individuals have many begging sessions throughout a 3.5 hour period under natural conditions (Yoshioka et al., 2016), and each begging session lasts for short bursts with a duration of only a few seconds (personal laboratory observation). Tadpoles may beg for several bursts that would sum to a substantial amount of begging; however, such short bursts may not be adequately represented by the limited trial duration employed here.

Feeding events orchestrated by parent frogs typically occur every 7.3 days (Yoshioka et al., 2016) under natural conditions. Parent frogs in the laboratory were allowed to raise and feed tadpoles *ad libitum* until tadpoles reached a suitable stage for trials. Tadpoles were set aside for 24 hours in order to elicit a begging response during trials. However, due to the extended time span of naturally occurring feeding events, it may be more effective to increase the time tadpoles are set aside to stimulate the intensity of begging behavior more strongly.

Preliminary Parental Candidate Gene Analysis

Preliminary analysis qualitatively comparing average TPM between all individuals of both species shows evidence that *R. imitator* expresses more social genes regardless of parental interaction or environmental condition. Since these hormones and neuropeptides are expressed in adult poison frogs during parental care, these genes may also be constitutively upregulated in the brains of tadpoles in species that frequently experience social interaction in the context of parent-offspring communication (begging). More rigorous analyses will need to be performed to determine if this preliminary finding can explain differences of gene expression between species.

Significant Differentially Expressed Genes in Ranitomeya imitator

The genes that were significantly differentially expressed in the *R. imitator* tadpole brains did not match those in the list of top candidate genes associated with begging, feeding, stress, and social behaviors identified in previous research on parental care and begging (Table 1). However, the majority of the genes that did show differential expression are associated with learning and social behavior (*h2ax, scoc* and *gabrg3*), as well as digestion and brain function (*mfsd9*), and stress (*fdps*).

Transposable element Tc3 transposase (*tc3a*) is an anomaly with respect to the other significantly expressed genes found in *R. imitator.* This gene is expressed in a species of nematode (*Caenorhabditis elegans*), resulting in recurrent removal and rearrangement of TC3 elements (Uniprot Database, 2020). One explanation could be that artifactual RNA from nematodes commonly found in poison frog habitats was sequenced as part of the tadpole brain material. However, this is an unlikely explanation because treatment tadpoles had consistently lower expression of this gene while control tadpoles had higher expression. One would expect to find a more sporadic representation between individuals if this phenomenon were due to artifacts in the brain tissue, which indicates the need to further investigate this gene and its role in *R. imitator.*

Farnesyl diphosphate synthase (*fdps*) codes for an enzyme responsible for cholesterol biosynthesis and the modification and synthesis of steroid hormones in the isoprenoid pathway (Romanelli et al., 2009). One of the key steroid hormones is corticosterone, which has been implicated in some begging experiments as a regulator, and cost, of begging (Kitaysky et al., 2001; Loiseau et al., 2008). Major facilitator superfamily domain containing 9 (*mfsd9*) is a protein coding gene which has been detected in regions of the brain associated with hunger and feeding. Expression of (*mfsd9*) increased with food deprivation in the brains of mice which

suggests an association with mechanisms related to feeding (Perland et al., 2017). The Short Coiled-Coil Protein (*scoc*) is involved in neurogenesis and dopaminergic neurotransmission (Gerra et al., 2018). The dopamine pathway is the most characterized pathway associated with behavioral reward systems (i.e. the experience of pleasure upon performing particular behaviors) (Sapolsky, 2017). Gene knockout studies have shown that the *h2ax* gene (H2A.X Variant Histone) is vital to responses to oxidative stress and motor function behaviors (Weyemi et al., 2018). Knockout studies should be considered in *R. imitator* tadpoles because there were higher levels of differential expression in the control tadpoles (that did not beg) (Figure 4).

One interesting, highly expressed gene found in this study (*gabrg3*) encodes for a GABA receptor and is highly expressed in the brain in the context of social interactions (especially those associated with decreased social anxiety), and is also linked to autism in humans (Crestani et al., 1999; Wang et al., 2018). The expression of receptor genes during development and the density of receptors in brain cells are important in the initiation and regulation of numerous physiological responses (Stone, 1998). As behaviors (begging) are continually induced through parental responses, more receptors are created to allow a stronger response, both across development and in response to immediate stimulation (Crespo et al., 2009). The binding of GABA to its receptors regulates the reduction of anxiety levels and threat avoidance (Crestani et al., 1999; Shekhar et al., 1990) and may play an important role in begging tadpoles. Tadpoles generally have no defense against predators and thus must be able to properly ascertain threat levels when a foreign body enters their breeding pool. In the case of *R. imitator*, the parent frequently enters the pool for trophic egg feeding events, so the expression of *gabrg3* may be crucial for reduced anxiety about approaching adults, facilitating the evolution of begging behaviors.

Previous research indicates the possibility that *gabrg3* may be a partially imprinted gene, possibly involved in parent-offspring and sexual conflict mediated by genomic imprinting effects. In humans, this locus is associated with autism and especially Angelman Syndrome, which is associated with high demands by the child on the mother (Wang et al., 2018). The kinship theory of imprinting (Haig, 2000) posits that this is because genes expressed on the paternal chromosome (and suppressed, or "imprinted" on the maternal chromosome) in offspring will promote the tendency to demand more resources from the mother, at the expense of siblings (because over the course of evolutionary history paternally-derived gene copies are less likely to have copies in "siblings" than genes derived from the mother (maternal gene copies)). In *R. imitator*, which has evolved biparental care (but with some opportunity for multiple mating (Brown et al. 2010)), it would be expected that paternal genes would be expressed in the tadpole brain to cause more vigorous and persistent begging, whereas maternal genes should have the reverse effect. With respect to *gabrg3*, this could mean that there is a bias towards high paternal expression, low maternal expression of this gene, because GABA binding with its receptors is associated with a reduction in social anxiety and (presumably) an increased willingness to approach and beg from the mother. Note one would not expect to see imprinting of this gene in *R. variabilis* because there is no begging, so no opportunity for paternal genes to affect tadpole behavior to obtain more resources from the mother. This is speculative and would need further investigation to determine the effects that this mother-father intergenomic conflict may have on begging behavior. This particular gene was not immediately annotated using the SwissProt database; it was necessary to use tblastx (NCBI) to annotate the associated transcript. The query length of the nucleotide sequence was 576, with a max score of 43.2, a total score of 2649, query cover was 99%, and there was a significant E value of 0.003 associated with this gene match.

Further research using a poison frog genome (when available) will be necessary to confirm the expression of *gabrg3* as a homolog (or paralog with similar function) in *R. imitator*.

The roles of the genes that were significantly expressed only in *R. imitator* (*fdps, h2ax, mfsd9, gabrg3, scoc,* and *tc3a*) in social behavior, hunger and digestion, and stress response lend support to the possibility that they influence begging behavior and thus may have likely evolved in the context of trophic egg-feeding and the associated begging behavior.

Conclusions

Although a small part of a larger research project in illuminating genes associated with parent-offspring interactions, the results of this research indicate that several genes associated with feeding, social behavior, neurological functions, and motor function behaviors may play crucial roles in the initiation and persistence of tadpole begging in *R. imitator*. These findings should help further our understanding of the evolution of begging behavior in tadpoles who are fed by their parents. This project used *de novo* transcriptomes to compare differential expression. One future plan includes conducting a more rigorous cross species comparison of expression level between *R. imitator* and *R. variabilis* against a candidate gene list comprised of known social context (Fischer et al., 2019; Fischer, et al., 2019) to determine whether *R. imitator* tadpoles have constitutively higher levels of social gene expression. This will also involve conducting a control analysis comparing a random set of genes of the same size, to determine if *R. imitator* has a higher standard expression for any gene. Another future plan involves mapping the reads from the transcriptomes to a newly developed genome assembly for *R. imitator* for additional differential expression analysis. Further research opportunities should include similar examination of the tadpoles of another dendrobatid species, *Oophaga pumilio*, which also beg for trophic egg allocation.

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