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Galanin neuron activation in feeding, parental care, and infanticide in a mouthbrooding African cichlid fish

Julie M. Butler*,[1], Erandi M. Herath, Arohan Rimal, Sarah M. Whitlow, Karen P. Maruska

Department of Biological Sciences, Louisiana State University, United States of America

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ABSTRACT

Galanin is a conserved neuropeptide involved in parental care and feeding. While galanin is known to mediate parental care and infanticide in rodents, its role in parental care and feeding behaviors in other taxa, particularly fishes, remains poorly understood. Mouthbrooding is an extreme form of parental care common in fishes in which caregivers carry offspring in their buccal cavity for the duration of development, resulting in obligatory starvation. In the cichlid fish *Astatotilapia burtoni*, females brood their young for ~2 wks and perform maternal care after release by collecting them into their mouth when threatened. However, females will cannibalize their brood after ~5 days. To examine the role of *gal* in feeding and maternal care, we collected mouthbrooding, fed, and starved females, as well as those displaying post-release maternal care and infanticide behaviors. Activation of *gal* neurons in the preoptic area (POA) was associated with parental care, providing the first link between *gal* and offspring-promoting behaviors in fishes. In contrast, activation of *gal* neurons in the lateral tuberal nucleus (NLT), the Arcuate homolog, was associated with feeding and infanticide. Overall, these data suggest *gal* is functionally conserved across vertebrate taxa with POA *gal* neurons promoting maternal care and Arc/NLT *gal* neurons promoting feeding.

1. Introduction

Parental care behaviors have evolved multiple times in animals and play an evolutionarily significant role in species persistence by increasing offspring survival (Clutton-Brock, 1991; Royle et al., 2012). A parent's decisions on whether or not to engage in parental care behaviors is influenced by both environmental and physiological factors, because many parental behaviors come at costs to the caregiver as they shift their energy allocation from self-promoting to offspring-promoting behaviors (Gross and Sargent, 1985; Liker and Székely, 2005; Manica, 2004; Nalepa, 1988; Smith and Wootton, 1995). When the costs of parental care outweigh the potential benefits or the environmental conditions interfere, animals can cease parental care and even engage in infanticide, or the cannibalization of one's own young (Hausfater, 1984). Infanticide is widespread across the animal kingdom, occurring in diverse taxa from invertebrates to all vertebrate groups, and can be performed by both male and female parents (Hausfater, 1984; Hrdy, 1979; Van Schaik and Janson, 2000). In teleost fishes, infanticide is often a result of small brood size, decreased physical condition of the parent, and mate availability (Manica, 2002a; Manica, 2002b; Ochi, 1985; Petersen, 1990). But less is known about the neural mechanisms underlying the decision to cannibalize ones young, especially in fishes, the most diverse and speciose group of vertebrates.

Teleost fishes display a diverse repertoire of parental care behaviors, from the complete absence of parental care, to egg and nest defense, to mouthbrooding (Blumer, 1982; Goodwin et al., 1998; Gross and Sargent, 1985). Mouthbrooding is an extreme form of parental care in which one or both parents carry the developing young for the partial or full duration of development (Oppenheimer, 1970). This timeframe can range from days to weeks and often results in obligatory starvation. Animals are partially unable and/or unwilling to feed due to the physical presence of the brood in their mouth, but the physiological and neural mechanisms associated with this obligatory starvation are poorly understood.

Components of parental care and feeding neural circuitry are shared and conserved across taxa (Fischer and O'Connell, 2017), with the neuropeptide galanin emerging as a candidate for mediating both parental care (Dulac et al., 2014) and feeding (Corwin et al., 1993; Crawley et al., 1990). Galanin is predominately found in the central nervous system and gastrointestinal tract of animals where it binds G-protein coupled Gal receptors. Teleost fish have four galanin receptors (*galr1*a, *galr1*b, *galr2*a, *galr2*b) that are found throughout the brain and in the testes of male Sea

* Corresponding author at: 371 Jane Stanford Way, Gilbert 304, Stanford, CA 94305-5020, United States of America.
E-mail address: jmbutler@stanford.edu (J.M. Butler).
[1] Present address: Biology Department, Stanford University, United States of America.

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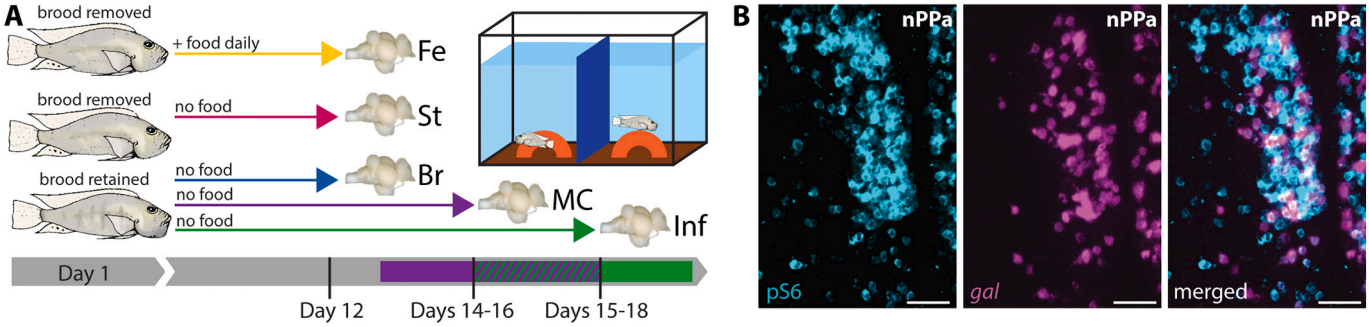

Fig. 1. Experimental protocol for collecting animals (A). Mouthbrooding females were identified on the first day of brooding in community tanks. Some females had their brood removed while others were allowed to retain their brood before being transferred to holding tanks (inset in A). Fed females were fed daily (yellow), but starved females were not (pink). Fed, starved, and brooding females (blue) were collected after 12 days. Females for the maternal care (purple) and infanticide (green) groups were collected after 14–18 days when they were displaying their respective behaviors. Hatched bar in (A) represents an overlap in collection timeframe between the maternal care and infanticide groups. Brains were stained for the neural activation marker pS6 (cyan) and *gal* (magenta), and co-labeled cells (white) were quantified (B). Scale bars represent 25 μm. nPPa: anterior part of the parvocellular preoptic area. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

bass *Dicentrarchus labrax* (Martins et al., 2014) and likely have different functional roles (e.g. feeding, parental care, coloration; Eskova et al., 2020). Activation of galanin neurons in the medial preoptic area (mPOA) of rodents promotes parental care behaviors in both sexes while ablation of galanin mPOA neurons inhibits parental care and increases offspring-directed aggression (Wu et al., 2014). Parental poison frogs have more galanin neurons than nonparental frogs, independent of sex, but in a species-dependent manner (Fischer et al., 2019). Other work has found that mPOA galanin neurons are activated during reproductive interactions (Bakker et al., 2002; Tripp et al., 2020) and are involved in rat sexual behavior (Bloch et al., 1996, 1993; Poggioli et al., 1992). In midshipman fishes, POA galanin neurons are differentially activated in type I males compared to type II males during courtship, but not during nest defense (Tripp et al., 2020). In contrast to its role in parental care and reproduction in the POA, galanin neurons in the arcuate nucleus of mammals mediate feeding behavior by stimulating food intake (Corwin et al., 1993; Crawley et al., 1990) and galanin promotes feeding behaviors in goldfish (de Pedro et al., 1995; Volkoff and Peter, 2001). Because of its involvement in both parental care behaviors and feeding, galanin is an excellent candidate for mediating the obligatory-starvation associated with mouthbrooding.

The African cichlid fish *Astatotilapia burtoni* is an ideal system for investigating how feeding and parental care neural circuitry intersect (Grone et al., 2012; Maruska and Fernald, 2018; Porter et al., 2017; Renn et al., 2009). Females make a dramatic shift from self-feeding immediately before spawning to obligatory starvation for the duration of brooding (~14 days). While mouthbrooding, females make critical decisions on a daily basis on whether to continue brooding and food-restriction or to release or consume their brood in favor of food intake. After approximately two weeks, females release fully-developed juveniles and will defend their brood for days after release by collecting their young back into the buccal cavity. However, some females will slowly cannibalize their brood in the days following release, presenting a unique opportunity to study the role of galanin in parental care, feeding, and infanticide behaviors.

The goal of this study was to test the hypothesis that galanin neurons are differentially activated during distinct parental care and feeding states in a mouthbrooding fish using pS6, which labels recently activated cells. By comparing activation of *gal* neurons in mouthbrooding, fed, and starved fish, as well as those displaying post-release maternal care and infanticide, we were able to demonstrate galanin cell population-specific activation related to feeding and parental care, similar to that seen in rodents. These results provide function-based evidence for the conservation of galanin in parental care behaviors across taxa and suggest that the neural correlates of infanticide and offspring-directed aggression may be evolutionarily conserved.

2. Materials and methods

2.1. Animals

Adult *Astatotilapia burtoni* derived from a wild-caught stock from Lake Tanganyika, Africa in the 1970s were raised under laboratory conditions similar to their natural environment (~28 °C, pH 8.0, 12-h light/12-h dark cycle). Fish were fed daily with cichlid flakes (AquaDine, Healdsburg, CA, USA) and twice weekly with brine shrimp (Sally's Frozen Brine Shrimp, San Francisco, CA, USA). Prior to experiments, fish were maintained in community aquaria in mixed sex groups. Aquaria contained gravel at the bottom, halved terracotta pots to serve as shelters, and several dominant, territorial males. All experimental protocols were approved by the Institutional Animal Care and Use Committee (IACUC) at Louisiana State University, Baton Rouge, LA, and were in accordance with the guidelines set forth by the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals, 2011.

2.2. Experimental procedures

To examine the role of the neuropeptide galanin in feeding and maternal care states, mouthbrooding females were collected from community tanks in the early brooding phase (within 24 h of brood onset) and transferred to 38 L experimental tanks. Each tank was divided in half by a clear, acrylic barrier, and only one female was placed in each compartment. Females were randomly assigned to one of five experimental groups: mouthbrooding, fed, starved, post-release maternal care, or infanticide (Fig. 1A). Mouthbrooding females were transferred on day 1 of brooding, allowed to retain their brood, and were not fed. To generate the fed and starved groups, mouthbrooding females were stripped of their brood on day one of mouthbrooding. Fed females were fed 1–2 cichlid flakes daily, but starved females were not fed. Brooding, fed, and starved females were collected after 12 days in the experimental setup. Some mouthbrooding females were allowed to retain their broods until they naturally released them (12–14 dpf). After females released fully-developed fry, the number of fry were counted. Females collected for the post-release maternal care group were collected 1–3 days post release and when > 80% of their brood remained (Fig. S1). Females were collected for the infanticide group 4–6 days post-release, when < 60% of the brood remained, indicating they consumed > 40% of their released offspring. Females in all experimental groups were checked daily to monitor for released fry and infanticide behaviors by counting the number of juveniles swimming around the tank. Upon dissection, the number of consumed juveniles in the stomach and intestines were estimated to verify infanticide occurred.

A total of 35 females were collected, 7 per each group. All females were collected at the same time of day (9–11 am). Females were measured for standard length (SL) and body mass (BM) before being sacrificed by rapid cervical transection. The ovaries were removed and weighed (OM, ovary mass) to calculate gonadosomatic index (GSI = (OM/BM)*100). The brain was exposed, fixed in the head in 4% paraformaldyhde (PFA) prepared in 1× phosphate buffered saline (1× PBS) at 4 °C overnight, and rinsed in 1× PBS for ~24 h. Cryoprotected brains (30% sucrose in 1×PBS at 4 °C overnight) were embedded in OCT media and sectioned in the transverse plane with a cryostat (Leica CM1850 or Cryostar NX50) at 20 μm and collected onto alternate sets of charged slides.

2.3. Double-label staining

To identify and quantify galanin (*gal*) neurons activated in the brain, double label in situ hybridization/immunohistochemistry staining was used as done previously (Butler and Maruska, 2019). Briefly, sectioned brains were first stained with a riboprobe specific for *A. burtoni gal* and reacted with SigmaFast Red for 2 h. Slides were then washed with 1× PBS in the dark and incubated with pS6 antibody (pS6 ribosomal protein S235/236 rabbit mAb, Cell Signaling 4858s, 1:1500 final dilution concentration) overnight at 4 °C. Slides were rinsed with 1× PBS and incubated in goat anti-rabbit secondary antibody (Alexa Fluor 488; 1:277) at room temp for 2 h. Slides were coverslipped with DAPI fluorogel for counterstaining and to aid in visualization of brain cytoarchitecture. Staining with a sense *gal* probe did not produce any staining. Preabsorption controls for pS6 were previously reported for *A. burtoni* (Butler et al., 2018), and showed no staining when the antibody was preabsorbed with pS6 blocking peptide. A western blot for pS6 produced a single band at the correct size (32 kDa). pS6 has been successfully used in *A. burtoni* for examining neural activation following a variety of experimental conditions (Butler et al., 2018, Butler et al., 2019; Maruska et al., 2020). Importantly, these studies show that pS6 is sensitive to steady-state differences (Maruska et al., 2020) as well as dynamic enough to show differences in neural activity related to a novel stimulus (Butler et al., 2019).

2.4. Imaging and quantification

Imaging was done with a Nikon Eclipse Ni microscope and monochrome camera (Nikon DS QiMc) controlled by Nikon Elements software. Images were taken in the DAPI, TxRed, and FITC channels and loaded into imageJ for processing. All images were pseudocolored, and the contrast and brightness were adjusted. The total number of *gal*-expressing cells was counted for each brain region. To examine the percent of *gal* cells activated, we merged images of galanin and pS6 staining, and the number of cells co-expressing *gal* and pS6 was counted for the entire brain region throughout its rostro-caudal extent. Data are expressed as the percentage of *gal* cells co-labeled with pS6. We also included the number of sections with *gal* staining per region as a covariate to account for differences in brain/body size and any potential missing or damaged sections. If more than two consecutive sections were missing or damaged per region, that animal was excluded from analyses for that region. Only the preoptic area (POA) and lateral tuberal nucleus (NLT) were quantified because they contain abundant *gal* neurons and are involved in parental care and feeding behaviors in vertebrates (Hu et al., 2016; Porter et al., 2017; Tripp and Bass, 2020; Wu et al., 2014).

2.5. Statistical analysis

Statistical analyses were performed in R v3.6.2. A linear mixed model (package: lme4; Bates et al., 2015) was used to analyze the number of *gal* cells and *gal*-pS6 co-labeled cells. Brain region was included as a within-subject factor and condition as a between-subject factor. To account for variation in brain/body size, we included the number of brain sections per region that was quantified as a covariate. Physical characteristics (SL, BM, GSI) were analyzed with a one-way ANOVA, and are reported in supplemental information. Effect sizes (partial eta squared) were calculated based on sample sizes and F statistics. Tukey's posthoc testing was done to isolate potential differences and adjusted for multiple testing via Bonferroni correction. Discriminant function analysis was done using the MASS package for R (Venables and Ripley, 2002), with prior probabilities determined based on sample sizes and missing values replaced with group means.

3. Results

Similar to that observed in other fishes and previously shown in *A. burtoni* (Hu et al., 2016), *gal* expression was found predominately in the anterior preoptic area and ventral part of the hypothalamus (Tripp and Bass, 2020) (Fig. S2). While scattered *gal* cells were observed in other brain regions both here and previously by Hu et al., our focus is on *gal* neuron activation in these large cell populations of the anterior part of the parvocellular preoptic area (nPPa) and lateral tuberal nucleus, ventral and intermediate subdivisions (NLTv, NLTi).

The number of *gal* cells differed among brain regions but not female groups (group: $F_{4,24} = 3.43$, $P = 0.137$, eta = 0.118; region: $F_{2,12} = 68.109$, $P < 0.001$, eta = 0.569; group*region: $F_{8,48} = 0.342$, $P = 0.947$, eta = 0.007; Fig. S3). The nPPa contained the most *gal* cells, followed by the NLTi, then NLTv. In contrast, activation of *gal* cells was dependent on both female condition and brain region (Fig. 2; group: $F_{4,24} = 7.035$, $P < 0.001$, eta = 0.540; region: $F_{2,12} = 71.436$, $P < 0.001$, eta = 0.922; group*region: $F_{8,48} = 8.140$, $P < 0.001$, eta = 0.575). In the POA, females displaying post-release maternal care had the greatest percentage of activated *gal* cells (Fig. 2A, B). Starved and infanticide groups had the lowest percentage of activated *gal* cells, with fed females as an intermediate not different from any of the groups. In the NLTv, animals displaying maternal care (brooding and post-release maternal care) had fewer activated *gal* neurons than those without parental care (fed, starved, and infanticide groups; Fig. 2C, D). In the NLTi, fed females had the highest percentage of activated *gal* cells, brooding and maternal care females had the lowest, and starved and infanticide females were an intermediate between the two (Fig. 2E, F).

Discriminate function analysis (DFA) was used to assess female group similarity resulting from *gal* cell number and activation across the three brain regions. A DFA of *gal* cell number did not produce any significant functions, while a DFA of co-labeled *gal*-pS6 cells produced two significant functions (Fig. 3). The first function explained 76.2% of the variance, separated groups with parental care from those without, and was positively loaded by the nPPa and negatively loaded by the NLT subdivisions. Function 2 explained 14.1% of the variance, separated fed females from starved and infanticide females, and was positively loaded by the NLTi and nPPa. The DFA correctly classified 74.3% of fish overall. All brooding and 85% of maternal care and fed fish were correctly predicted. In contrast, the DFA only correctly predicted 57% of infanticide and 43% of starved females, commonly misclassifying these two groups with each other.

4. Discussion

Parental care is a costly behavior that often requires animals shifting from self-promoting to offspring promoting behaviors (Royle et al., 2012). Here, we found that neurons expressing galanin, a neuropeptide implicated in parental care and feeding in mammals, are differentially activated during maternal care and infanticide behaviors in a mouthbrooding cichlid fish. This provides the first evidence linking galanin to parental care and infanticide in non-mammalian taxa and suggests the role of galanin in female parental care is rooted deep in evolutionary history.

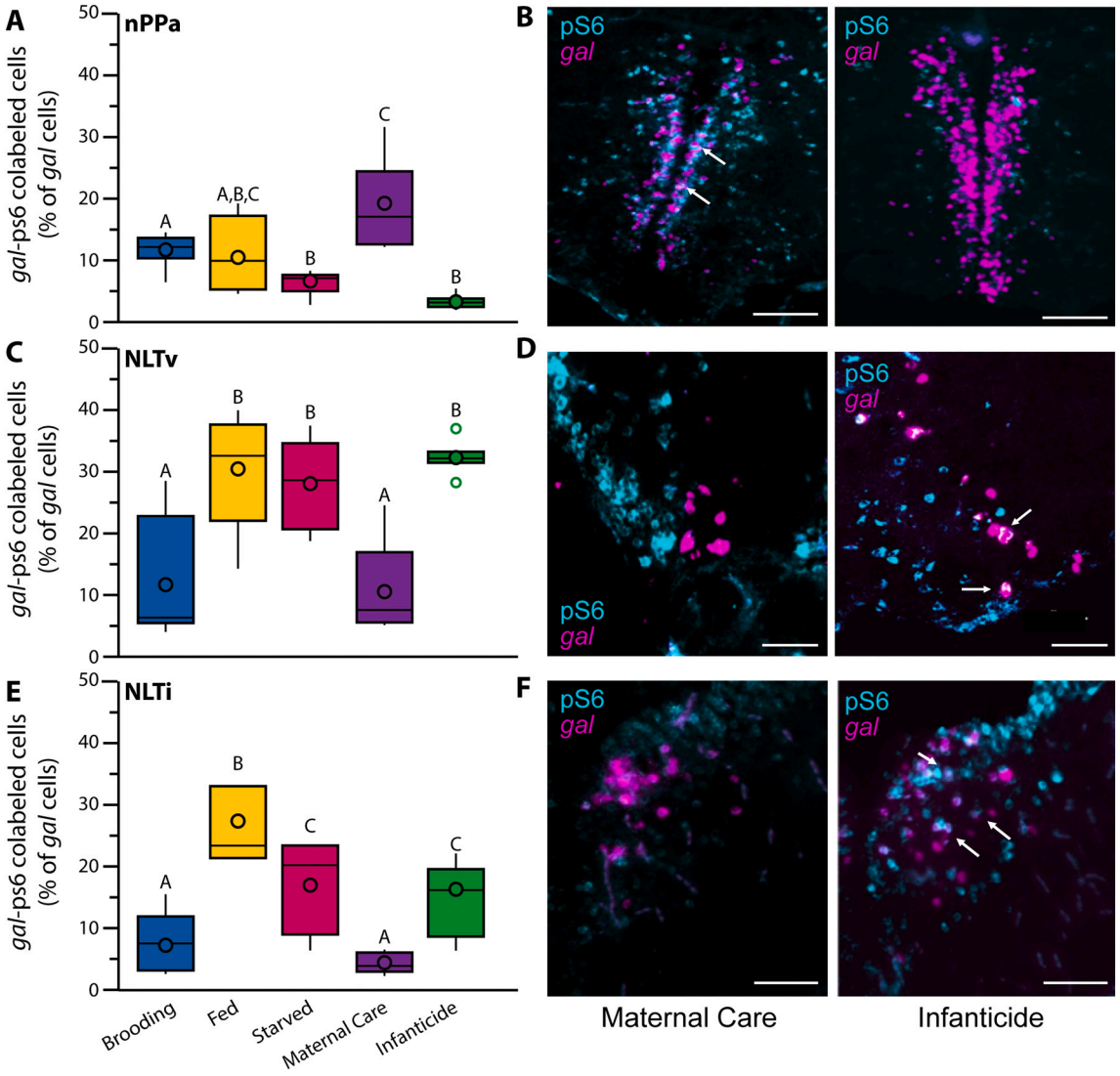

Fig. 2. Brain region and female state dependent activation of *gal* neurons. *Gal* activation was dependent on maternal care and feeding state in the nPPa (A), NLTv (C), and NLTi (E). Representative photomicrographs of *gal* and pS6 staining in maternal care (left) and infanticide (right) fish in the nPPa (B), NLTv (D), and NLTi (F). Co-labeled cells appear white, with arrows indicating examples of co-labeled cells. Different letters indicate statistical significance at $P < 0.05$. For boxplots, box extends to the furthest data points within the 25th/75th percentiles, and whiskers extend to the furthest data points within 1.5× the interquartile range. Outliers (beyond 1.5× the interquartile range) are designated by open circles. Data median is represented by a solid line and data mean by an open circle within the bars. $N = 5–7$ females per group per region. Scale bars represent 50 μm (B) and 25 μm (D, F). nPPa: anterior part of the parvocellular preoptic area; NLTv: lateral tuberal nucleus, ventral subdivision; NLTi: lateral tuberal nucleus intermediate subdivision.

Across taxa, an animal's physiological state, including nutritional status, has modulatory roles on its social behaviors. Parental care and feeding are intimately linked, with species-specific behavioral rules governing feeding and reproductive efforts (Fischer and O'Connell, 2017; Royle et al., 2012). In mammals and birds, parents will increase food intake during pregnancy and after birth to allow for food provisioning (Beekman et al., 2019; Cripps and Williams, 1975; Douglas et al., 2007; Meiri, 2019). In other cases, such as mouthbrooding, food intake is reduced during pregnancy or parental care to the benefit of both the parent and offspring. Mouthbrooding results in physiological and neural responses that are different from starvation (Grone et al., 2012; Maruska et al., 2020), suggesting integration of feeding and parental care neural circuitry. *Gal* neuron activation is likely due to integration of nutritional/feeding state and parental care, with cell population-specific roles.

We found more activated *gal* neurons in the nPPa of female cichlids engaged in maternal care, and fewer in females performing infanticide. Importantly, the total number of *gal* neurons does not differ across female groups in any brain region, but the percentage of activated cells does. Activation of Gal neurons in the mPOA of rodents induces parental care and attenuates infanticide and pup-directed aggression (Wu et al., 2014). From the mPOA, Gal neurons in rodents project to the periaqueductal grey, ventral tegmental area (VTA), medial amygdala, and paraventricular nucleus, with each projection group controlling a particular aspect of parental behaviors (Kohl et al., 2018). By stimulating galanin release into these target areas, Kohl et al. (2018) found that Gal projections to the ventral tegmental area (VTA) stimulate motivation to retrieve pups while Gal projections to the PAG regulate motor control of pup behavior. Interestingly, we previously found that the periventricular nucleus of the posterior tuberculum (TPp), a putative partial homolog of the mammalian VTA, is highly activated in mouthbrooding females but not fed or starved females (Maruska et al., 2020). Although untested, *gal* neurons in the POA could activate dopaminergic TPp neurons to stimulate reward pathways for the continuation of brooding and maternal care behaviors.

While preoptic area Gal neurons are involved in parental care in

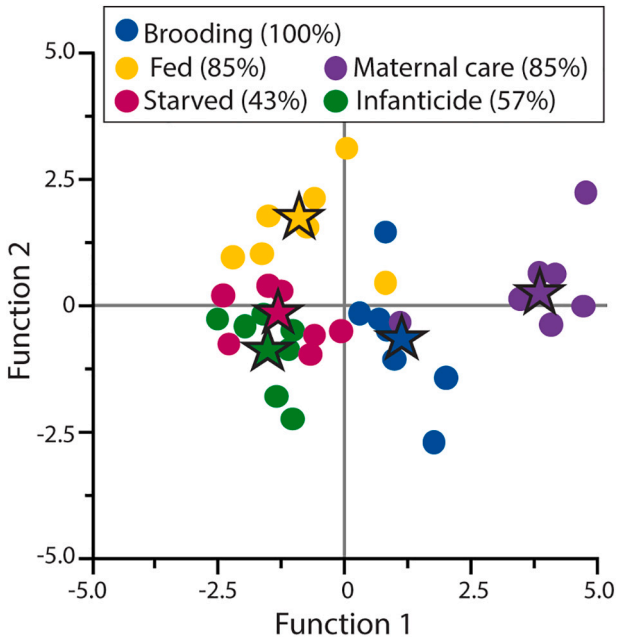

Fig. 3. Discriminant function analysis (DFA) clearly distinguished female groups with parental care from those without parental care based on *gal* cell activation alone (B). Circles represent individual animals and stars represent group centroids. Percentages in legend are the percentage of animals in each female group correctly predicted by the DFA.

rodents (Wu et al., 2014), no association of parental nest defense and Gal neurons was found in midshipman fish (Tripp et al., 2020). One possible explanation of why galanin appears to be involved in parental behaviors in cichlids but not midshipman, could be due to differences in the type of parental behaviors performed or the sex of the caregiver. In rodents and in *A. burtoni*, the female caregiver is providing physical care to their young, either through pup retrieval or brooding, while nest defense in male midshipman may be more neurobiologically similar to aggression than to parental care. Further work is needed on other fishes with different strategies of parental care to examine these relationships. In the POA of *A. burtoni* females, more *gal* neurons are activated in the maternal care group, which are commonly performing fry re-uptake when threatened, a behavior that could be partially analogous to pup retrieval. Thus, *gal* neuron activity in the preoptic area likely helps to maintain parental behaviors, and connections to feeding/energetic circuitry may regulate the switch to infanticide when conditions warrant.

In addition to its role in parental care, galanin in the arcuate nucleus stimulates food intake (Corwin et al., 1993; Crawley et al., 1990; Qualls-Creekmore et al., 2017), possibly through dopaminergic neuron activation to increase fat ingestion (Lee et al., 1994). We found relatively few activated *gal* neurons in the NLT of brooding and maternal care females. In the NLT, an area with known orexigenic and anorexigenic neuropeptides (Porter et al., 2017), fed, starved, and infanticide fish had similar activation, while brooding and post-release maternal care fish had fewer activated *gal* neurons. Further, fish engaged in infanticide had a greater percentage of activated *gal* neurons than those displaying maternal care behaviors, despite having similar physiological states and feeding opportunities. This could suggest that brood presence or maternal care has an inhibitory effect on *gal* neurons in the NLT. Overall, these data support the hypothesis that galanin has a functionally conserved role in parental care, infanticide, and feeding across taxa.

Unexpectedly, the infanticide group closely resembled starved fish, with the DFA unable to distinguish infanticide and starved fish but able to clearly distinguish the other three female groups. Infanticide is often due to environmental or physiological constraints that render parental care too costly for the caregiver (Manica, 2002a; Marconato et al., 1993). Based on our data, engaging in infanticide behaviors is not the same as simply feeding, an important distinction that was previously unknown. The distinct reversal in the percentage of activated *gal* neurons in females showing maternal care (high nPPa, low NLT) compared to those practicing infanticide (low nPPa, high NLT) suggests that galanin may be involved in the neurocircuitry regulating the motivational switch to abandon offspring care in favor of selfcare (i.e. infanticide). Both feeding and parental care are rewarding stimuli, and galanin is known to interact with dopaminergic reward circuitry (Kohl et al., 2018; Lee et al., 1994), but there are likely motivational thresholds influenced by internal and external conditions that mediate decisions between performing care and feeding behaviors. Further work is needed to analyze how the neural circuitry governing feeding and parental care integrate and influence critical choices about self-promoting and offspring-promoting behaviors in terms of reward processing and infanticide.

We, and many of the studies discussed here, used staining for neurons containing either Gal peptide or mRNA. Importantly, this does not directly examine galanin release from neurons or the role of galanin receptors. It is possible that galanin simply serves as a marker for cell types involved in parental care and feeding, and that other neurochemicals are responsible for the behaviors observed. Future work is need to directly manipulate the galanin system (e.g. knockout Gal receptors in targeted cell groups) across taxa to truly determine if it has a functionally-conserved role in feeding and parental care.

5. Conclusions

Activation of *gal* neurons is dependent on maternal care and feeding states in a mouthbrooding cichlid fish. The obligatory starvation associated with mouthbrooding and parental care in *A. burtoni* creates an ideal scenario to study how galanin functions at the intersection of feeding and parental care neural circuitry. Our analyses reveal that *gal* is functionally conserved across distant vertebrate lineages, with cell population-specific activation related to parental care and feeding. For the first time, we found that *gal* neurons are activated during parental care in fishes, suggesting that it is more evolutionarily rooted than previously known. Our results also indicate that engaging in infanticide is not neurologically the same as eating food, a previously unknown and important distinction that opens avenues of future research into understanding the neurobiological underpinnings of infanticide. Collectively, this work provides evidence towards better understanding how parental care and feeding neural circuitry have co-evolved.

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Data availability

The datasets supporting this article have been uploaded as part of the supplementary material. Code for analyses will be made available upon request.

Declaration of competing interest

Authors have no financial or competing interests.

Authors contributions

JB, SM, EH, and AR carried out all experimental work. JB performed data analysis and drafted the manuscript. JB and KM conceived and designed the study. KM provided guidance and funding. All authors gave final approval for publication and agree to be held accountable for the work performed therein.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.yhbeh.2020.104870.

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