

Galanin expression varies with parental care and social status in a wild cooperatively breeding fish

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ABSTRACT

As many busy parents will attest, caring for young often comes at the expense of having time to feed and care for oneself. Galanin is a neuropeptide that regulates food intake and modulates parental care; however, the relative importance of galanin in the regulation of feeding versus caring by parents has never been evaluated before under naturalistic settings. Here, we assessed how expression of the galanin system varied in two brain regions, the hypothalamus (which regulates feeding) and the preoptic area (which modulates social behaviours including care) in a wild cichlid fish, *Neolamprologus pulcher*. Females with young had higher hypothalamic expression of galanin receptor 1a, and the highest expression of galanin and galanin receptor 1a was observed in females that foraged the least. However, expression of five other feeding-related neuropeptides did not change while females were caring for young suggesting that changes in the hypothalamic galanin system may not have been directly related to changes in food intake. The preoptic galanin system was unaffected by the presence of young, but preoptic galanin expression was higher in dominant females (which are aggressive, regularly reproduce and care for young) compared to subordinate females (which are submissive, rarely reproduce but often help care for young). Additionally, preoptic galanin expression was higher in fish that performed more territory defense. Overall, our results indicate that galanin has brain-region-specific roles in modulating both parental care and social status in wild animals.

1. Introduction

Some species produce numerous offspring and provide little to no care (e.g., many fishes and insects), while other species produce few offspring but provide each with abundant care (e.g., most mammals and birds; Balshine, 2012; Clutton-Brock, 1991). Reproductive tactics and investment strategies can also vary considerably within a species—or even within the same individual across time—depending on differences in individuals' internal state and/or surrounding environments (Alonso-Alvarez and Velando, 2012; Johnstone, 2000). This variation in care tactics occurs because provisioning young and providing high-quality care requires considerable investment in terms of energy and time (Alonso-Alvarez and Velando, 2012; Clutton-Brock, 1991; Webb et al.,

2002) and can even reduce adult lifespan in some species (Downing et al., 2021; Gross and Sargent, 1985; Tuomi et al., 1983). Often individuals must undergo many physiological and behavioural changes to provide efficient and effective care (Alonso-Alvarez and Velando, 2012). While several hormones and neuropeptides are involved in coordinating these changes (Kenkel et al., 2017; Kohl and Dulac, 2018; Rogers and Bales, 2019), recent cross taxa evidence has pointed to galanin as a key neuroendocrine regulator of parental care in vertebrates (Bukhari et al., 2019; Butler et al., 2020; Fischer et al., 2019; Wu et al., 2014).

Galanin is a neuropeptide that is mainly expressed in the brain and in the gut (Marcos and Coveñas, 2021; Mensah et al., 2010) and is most often associated with the neuroendocrine regulation of food intake (Fang et al., 2011; Marcos and Coveñas, 2021). However, recent work in

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mammals (Kohl et al., 2018; Wu et al., 2014), amphibians (Fischer et al., 2019), and fishes (Bukhari et al., 2019; Butler et al., 2020) has also implicated galanin as a major neuroendocrine regulator of parental care (Fischer and O'Connell, 2017; Zilka et al., 2017). Specifically, populations of galanin-expressing neurons located in the medial preoptic area of the brain are activated during periods of courtship, mating, and care provisioning, with distinct populations of neurons being activated by each of these different acts (Kohl et al., 2018; Tripp et al., 2020). For instance, in fishes that exhibit male alternative reproductive tactics, males that court females and provide care have higher galanin levels compared to cuckold males that sneak copulations and do not provide care (Partridge et al., 2015; Tripp et al., 2020, 2018). The preoptic galanin system has also been directly implicated in the regulation of aggression (Wu et al., 2014; Yamashita et al., 2020), which can be another important element of parental care in many species, taking the form of defending young from predators. In addition to its role in regulating behaviours associated with reproduction and parental care, galanin can also influence an individual's reproductive state via interactions with the endocrine reproductive axis (Cheung et al., 1996; Lopez and Negro-Vilar, 1990). Therefore, the roles of the preoptic galanin system in regulating parental care, as well as reproduction more generally, appear to be functionally and phylogenetically widespread.

While the preoptic galanin system appears to help regulate parental care and social behaviours more generally, the hypothalamic galanin system regulates food intake by stimulating feeding behaviours (Crawley et al., 1990; Tachibana et al., 2008; Volkoff and Peter, 2001). For many animals, providing care and provisioning young comes at the cost of having less time to feed oneself (Balshine-Earn, 1995; Bose et al., 2016; Burke et al., 2015; Crowl and Alexander, 1989; Hanson et al., 2009). Therefore, while the hypothalamic galanin system is unlikely to be involved in the direct regulation of parental care behaviours, it could be involved in the regulation of foraging behaviours while individuals provide care. However, we are aware of only a single laboratory-based study (Butler et al., 2020) that has specifically assessed the possible region-specific involvement of the galanin system in the brain of caring parents (Preoptic Area = Parental Care; Hypothalamus = Foraging). Despite the fitness consequences of effectively regulating parental care and feeding likely being of more immediate and direct importance in the wild, to our knowledge no field-based study has directly evaluated the dual contributions of the galanin system towards parental care and foraging.

In this study, we investigated how the preoptic and hypothalamic galanin systems are regulated in wild female *Neolamprologus pulcher* from Lake Tanganyika, Africa. These fish live in cooperatively breeding social groups consisting of a dominant male-female breeding pair and 1–20 mixed sex subordinate helpers (Balshine et al., 2001; Heg et al., 2004). Unlike other teleost fishes where offspring cannibalism is common (Manica, 2002; Pereira et al., 2017; Smith and Reay, 1991), *N. pulcher* rarely cannibalize their young and provide high quality care towards them (Heg and Hamilton, 2008; Jindal et al., 2017; von Siemens, 1990). Dominant females produce the majority of young within each group (Dierkes et al., 2005; Hellmann et al., 2015), and while all group members help care for young, dominant females usually provide the most care (Balshine et al., 2001; Desjardins et al., 2008; Heg and Hamilton, 2008; Taborsky and Grantner, 1998). These differences in reproduction and care provisioning likely contribute to the energetic costs incurred by dominants, which spend less time feeding (Sopinka et al., 2009) and maintain lower energy reserves than subordinates (Hellmann et al., 2016; Sopinka et al., 2009). Given the combination of reproductive, parental, and overall energetic demands that dominant females face, evaluating the relationship of the galanin system with each of these demands will help to determine the role(s) of this neuroendocrine system.

We hypothesized that the presence of young would be associated with transcriptional changes within both the preoptic and hypothalamic galanin systems in dominant females. Specifically, we predicted that the

preoptic galanin system of dominant females would be activated to promote high levels of parental care when young are present, whereas activity of the hypothalamic galanin system in dominant females would be reduced to discourage foraging while caring for their young. To test these predictions, we compared transcript abundance of galanin and its receptors in both the preoptic area and hypothalamus of caring dominant females from social groups where young (i.e., free-swimming fry) were present as well as non-caring dominant females from groups where young were absent. To further elucidate the potential mechanisms responsible for regulating foraging during periods of care, we measured hypothalamic transcript abundance of several additional anorexigenic (corticotropin-releasing factor b, *crfb*; proopiomelanocortin a1, *pomc-a1*) or orexigenic (agouti-related peptide, *agrp*; hypocretin/orexin, *hcr*; neuropeptide Y, *npY*) neuropeptides that have previously been implicated in regulating food intake while providing care (Fischer and O'Connell, 2017). We only measured levels of these transcripts in the hypothalamus because it is the primary central regulator of food intake in vertebrates (Anubhuti, 2006; Volkoff, 2016). We then assessed whether hypothalamic transcript abundance of the galanin system and other feeding-related neuropeptides was related to foraging rates of dominant females. Lastly, we also assessed whether activity of the galanin system was greater in more dominant and/or territorial individuals because previous work has suggested a positive relationship between dominance/aggression and galanin in fish (e.g. Partridge et al., 2015; Renn et al., 2008; Tripp et al., 2018; Yamashita et al., 2020). Accordingly, we predicted that dominant females would have higher transcript abundance of galanin compared to subordinate females, specifically in the preoptic area because of its important role in regulating aggression and social behaviours more generally (Goodson, 2005; O'Connell and Hofmann, 2011a, 2011b). Additionally, we assessed whether preoptic galanin expression correlated with how aggressive/submissive individuals were within their social group or how aggressive they were towards territory intruders (either heterospecifics or conspecifics from other social groups).

2. Methods

2.1. Field site and animals

This study was conducted off the shore of Mutondwe Island, Lake Tanganyika, Zambia (8°42'45" S, 31°7'27" E) in December 2019. Using SCUBA, 26 social groups were located between 6 and 8 m depth and used for this study. Groups contained an average (mean ± SEM) of 7.6 ± 0.6 individuals.

2.2. Behavioural observations

In the field, the behaviour of focal dominant females ($N = 13$; SL = 63 ± 0.7 mm) and large subordinate female helpers ($N = 13$; SL = 54 ± 1.7 mm) was observed over two 10 min observation periods conducted on separate days (mean of 31 h between observation periods; range of 21–96 h). Group members are readily identifiable based on individual differences in body size, unique markings on their body and face, behaviour, and discrete home ranges within each group's territory (Werner et al., 2003), allowing us to accurately observe the behaviour of individuals across several days. Following a 2 min acclimation period at each group, we scored all aggressive (chases, bites, rams, opercular flares, aggressive postures, and lateral displays) and submissive behaviours (submissive postures, tail quivers, j-hooks, and flees) that our focal fish exchanged with groupmates (see Sopinka et al., 2009 for further details on these behavioural displays and acts). We also scored how many aggressive acts each focal fish performed towards intruding heterospecifics and conspecifics from other groups (territory defense), as well as how much time (s) each fish spent in the brood chamber (a proxy for brood care) and how much time each fish spent feeding in the water column. To assess how dominant an individual was within their social

group, we calculated a dominance index (see Aubin-Horth et al., 2007; Fitzpatrick et al., 2008) for each focal fish by subtracting the combined number of aggressive acts received and submissive acts given from the total number of aggressive acts given and submissive acts received (Dom Index = (Agg Given + Sub Rec) - (Agg Rec + Sub Given)). The presence or absence of newly hatched young (i.e., fry) was also confirmed during these two focal observation periods (fry were present in 5/13 (dominant females) and 0/13 (subordinate females) groups at the time of collection).

2.3. Capture and sampling

Within 72 h of the second observation period, focal fish were captured using fence nets and hand nets. It took an average of 7.0 ± 0.2 min from when a diver initially approached a territory to lay out the fence net to the point where the fish was processed on the surface of the water. Once caught, fish were placed in a mesh collection bag and sent to a boat waiting on the surface using a custom floatation apparatus. At the surface, each fish was retrieved by a team member (BMC) and was immediately euthanized via terminal anaesthesia (0.5 g L^{-1} ethyl-p-aminobenzoate; Sigma-Aldrich). On the boat, fish were measured (to the nearest 0.1 cm), sex was identified via visual examination of the gonads, and the brain was removed and placed into RNA-later (Ambion). Brains in RNA-later were initially kept at $4 \text{ }^\circ\text{C}$ for 12 h, after which they were transferred to $-20 \text{ }^\circ\text{C}$ for later measurement of transcript abundance.

To reduce the impact that our experiment had on individual groups, we removed dominant females in half of the observed social groups ($N = 13$) and subordinate female helpers from the other half of groups ($N = 13$). Additionally, one dominant female whose behaviour was not recorded was opportunistically collected from a group that neighbored one of our focal groups. We successfully confirmed that the correct focal individuals were removed by revisiting each group and identifying all remaining group members (see Section 2.2) following removals.

2.4. Transcript abundance analysis by real-time polymerase chain reaction

Transcript abundance was measured via semi-quantitative real-time polymerase chain reaction (qPCR) using gene-specific primers (Table 1). Prior to RNA extraction, the hypothalamus (HYP) and preoptic area

(POA) were dissected out (as described in Culbert et al., 2021) and the two regions of the brain were processed separately. Total RNA was extracted using RiboZol reagent (VWR) according to the manufacturer's instructions. RNA concentration was determined spectrophotometrically (NanoDrop 2000; Thermo-Fisher Scientific) and RNA integrity was assessed using an Agilent TapeStation 4150. Complementary DNA (cDNA) was synthesized using a commercial kit (qScript; Quanta Bio-Sciences) using $1 \mu\text{g}$ of DNase 1-treated RNA (PerfeCTa; Quanta Bio-Sciences) as template. Following cDNA synthesis, we performed qPCR using SYBR green (SsoAdvanced Universal; BioRad) with a CFX96 system (BioRad). All samples were run in triplicate and negative controls, including no template controls (where cDNA was replaced with water) and no reverse transcriptase controls (where qScript was replaced with water during cDNA synthesis) were included. Each reaction contained $10 \mu\text{l}$ of SYBR green, $5 \mu\text{l}$ of combined forward and reverse primers ($[0.2 \mu\text{M}]$ each), and $5 \mu\text{l}$ of $10\times$ diluted cDNA. Cycling parameters included a 30 s activation step at $95 \text{ }^\circ\text{C}$, followed by 40 cycles consisting of a 3 s denaturation step at $95 \text{ }^\circ\text{C}$ and a combined 30 s annealing and extension step at $60 \text{ }^\circ\text{C}$. To confirm the specificity of each reaction, a melt curve analysis was conducted at the end of each run. Primer sets were designed using Primer-BLAST (NCBI; Ye et al., 2012) based on the annotated genome of *N. brichardi* (a sub- or sister-species of *N. pulcher*; Brawand et al., 2014; Duftner et al., 2007; Gante et al., 2016). Standard curves for each primer set (using serial dilutions ($4\times$) of pooled cDNA) were constructed for each gene to account for differences in amplification efficiency (see Table 1). To correct for differences in primer amplification efficiency, the average threshold cycle value for each individual was fit to the antilog of each gene-specific standard curve. To correct for minor differences in template input and transcriptional efficiency, we measured the transcript abundance of beta actin (β -actin) and elongation factor 1 alpha ($ef1a$) as reference genes. Expression of these two genes was strongly correlated in both regions of the brain ($R^2 = 0.87$ and 0.81 in preoptic area and hypothalamus, respectively) and we normalized each gene of interest to the transcript abundance of β -actin because it was slightly more stable than $ef1a$ across groups. Data are expressed as fold-changes relative to the mean value of dominant females.

Because there are multiple galanin receptors in teleost fishes ($galr1a$, $galr1b$, $galr2a$, and $galr2b$) which are differentially expressed across tissues and between different regions of the brain (Cohen et al., 2020; Kim et al., 2014; Martins et al., 2014), we initially assessed which paralogs of $galr$ ($galr1a$, $galr1b$ and $galr2a$) were most abundant in the brain of

Table 1
Gene specific primers used for the real-time qPCR analyses.

Gene	Primer sequence (5' to 3')	Amplicon Size (bp)	Efficiency (%)	Accession number	Reference
β -actin	F: CGCTCCTCGTGTCTCTTC R: TCTTCTCCATGTCATCCCAGTTG	107	105	XM_006797985	(Culbert et al., 2019)
$agrp$	F: TGCTACTGCCGCTTCTCAA R: AGTTTCTGCCTTGGCTCCTC	112	103	XM_006779687	Current Study
$crfb$	F: ATCACCTTCCATCTTCAACAG R: CTGGACATCTCCATCATCTC	204	108	JX134406	(Taborsky et al., 2013)
$ef1a$	F: AAGAAGATCGGCTACAACCCC R: AGCCCATCTTGTCACTGGTC	94	116	XM_035909298	(Culbert et al., 2021)
gal	F: CTGCCTCCTTCTGTACATCA R: GGGTCAGTCAGTCAGATGGT	198	103	XM_006782226	Current Study
$galr1a$	F: GATACGCCGCCCTCAACTAA R: ACTGGTGCCTTATCCCCTCT	195	95	XM_006794868	Current Study
$galr1b$	F: GCGTCATCTGGACGATGTCT R: CTCCCAGCAGAAAGTTCCGT	92	92	XM_006780450	Current Study
$hcrt$	F: ATCCTCACTCTGGCAAACG R: TCAGAAATCCCTGCTGCTTGG	100	95	XM_006798238	Current Study
npy	F: ACGTTCACAGTCAAGATATGA R: GACGTAAGTGACGGACGTGT	119	99	XM_006787635	Current Study
$pomc-a1$	F: TGTAATCGTAGTGGGGCTGG R: GGGATGATGGGCTTCTCGTT	141	105	XM_035915633	Current Study

β -actin, beta actin; $agrp$, agouti-related peptide; $crfb$, corticotropin-releasing factor b; $ef1a$, elongation factor 1 alpha; gal , galanin; $galr1a$, galanin receptor 1a; $galr1b$, galanin receptor 1b; $hcrt$, hypocretin/orexin; npy , neuropeptide y; proopiomelanocortin a1, $pomc-a1$.

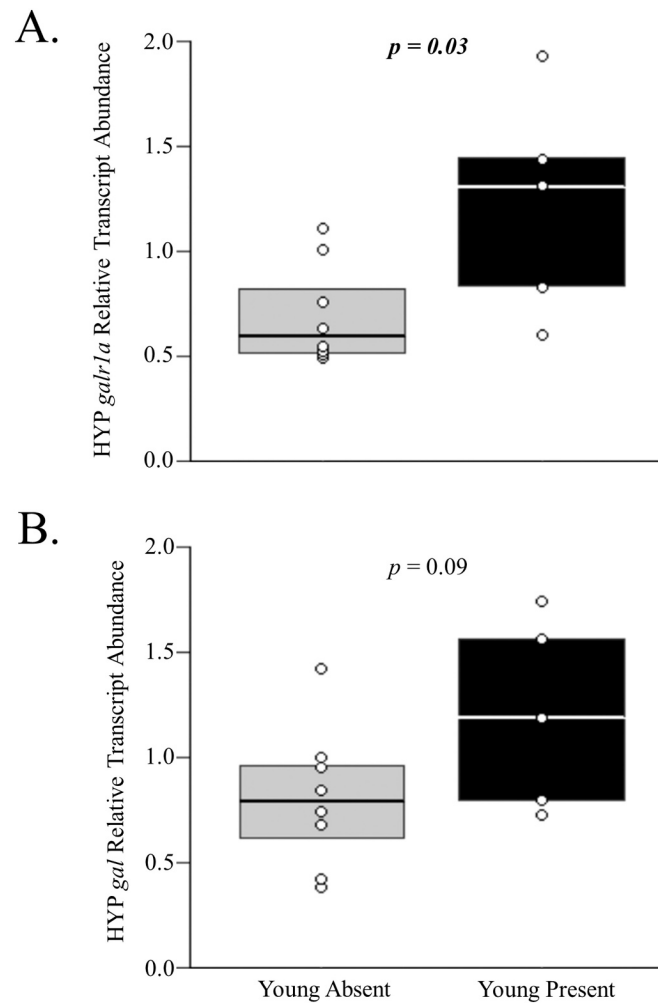


Fig. 1. Differences in transcript abundance of a) *galr1a* and b) *gal* in the hypothalamus (HYP) of dominant female *N. pulcher* when young were absent (grey) or present (black). Values are presented as medians and 1st and 3rd quartiles; points represent individual values. Statistical results are indicated on each graph (see statistical analysis section for further details).

Table 2

Transcript abundance of genes in the preoptic area and hypothalamus of dominant female *N. pulcher* when young were absent or present on their territory. Data are expressed relative to the mean value of all dominant females combined and are reported as means \pm SEM. Significant differences ($p < 0.05$) are indicated with bold font.

		Young Absent (N = 8)	Young Present (N = 5)	η^2	F	p
Preoptic area	<i>gal</i>	0.86 \pm 0.39	0.90 \pm 0.36	0.04	0.41	0.54
	<i>galr1a</i>	0.95 \pm 0.15	1.08 \pm 0.06	0.09	1.02	0.33
	<i>galr1b</i>	0.92 \pm 0.12	1.09 \pm 0.10	0.08	0.96	0.35
Hypothalamus	<i>gal</i>	0.80 \pm 0.12	1.20 \pm 0.20	0.23	3.37	0.09
	<i>galr1a</i>	0.70 \pm 0.09	1.22 \pm 0.23	0.35	5.99	0.03
	<i>galr1b</i>	0.96 \pm 0.08	0.92 \pm 0.09	0.01	0.10	0.76
	<i>agrp</i>	0.91 \pm 0.19	1.14 \pm 0.56	0.01	0.05	0.82
	<i>crfb</i>	0.98 \pm 0.17	0.97 \pm 0.14	0.01	0.01	0.97
	<i>hcrt</i>	1.06 \pm 0.12	0.90 \pm 0.14	0.06	0.71	0.42
	<i>npv</i>	1.11 \pm 0.21	0.82 \pm 0.15	0.08	0.97	0.35
	<i>pomc-a1</i>	1.09 \pm 0.22	0.83 \pm 0.18	0.05	0.54	0.48

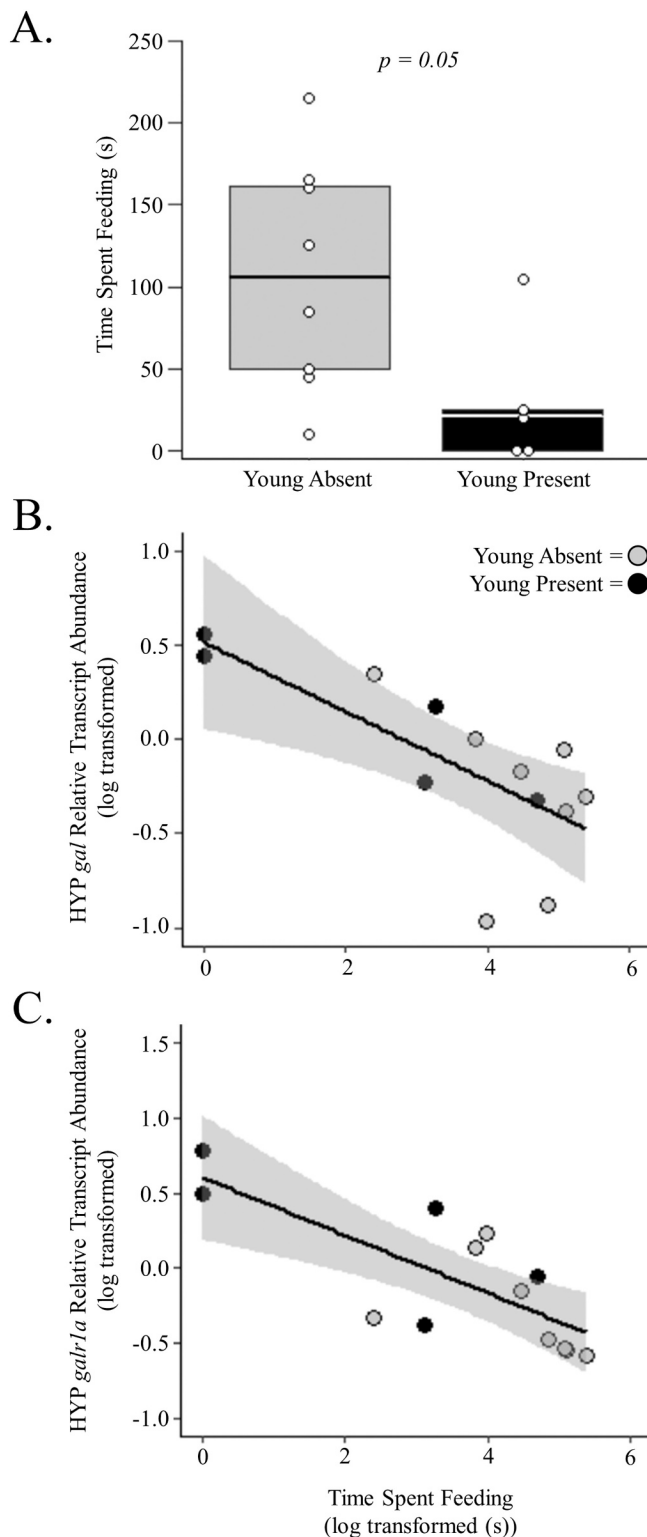


Fig. 2. Differences in a) time spent feeding between dominant female *N. pulcher* when young were absent (grey) or present (black), as well as the relationship between time spent feeding and transcript abundance of b) *gal* and c) *galr1a* in the hypothalamus (HYP) of dominant females. Values are presented as medians and 1st and 3rd quartiles; points represent individual values. Linear regressions were fitted, and the shaded area shows the 95 % confidence interval of the regression line. Statistical results are indicated on each graph (see statistical analysis section for further details).

Table 3

Relationship between transcript abundance of feeding-related peptides in the hypothalamus and the amount of time that dominant female *N. pulcher* with or without young spent foraging. All data were log transformed. Significant differences ($p < 0.05$) are indicated with **bold font**.

	Model term	η_p^2	F	p
gal	Foraging	0.52	5.80	0.04
	Young	0.01	0.02	0.89
	Foraging*Young	0.01	0.04	0.85
galr1a	Foraging	0.61	6.29	0.03
	Young	0.06	0.56	0.47
	Foraging*Young	0.01	0.01	0.99
<i>galr1b</i>	Foraging	0.03	0.77	0.40
	Young	0.06	0.57	0.47
	Foraging*Young	0.03	0.26	0.63
<i>agrp</i>	Foraging	0.04	0.36	0.56
	Young	0.01	0.04	0.85
	Foraging*Young	0.03	0.29	0.61
<i>crfb</i>	Foraging	0.10	1.18	0.31
	Young	0.03	0.23	0.64
	Foraging*Young	0.01	0.08	0.93
<i>hcrt</i>	Foraging	0.02	0.01	0.99
	Young	0.03	0.32	0.59
	Foraging*Young	0.08	0.76	0.41
<i>npv</i>	Foraging	0.01	0.32	0.59
	Young	0.07	0.73	0.42
	Foraging*Young	0.06	0.55	0.48
<i>pomc-a1</i>	Foraging	0.01	0.18	0.69
	Young	0.02	0.18	0.69
	Foraging*Young	0.01	0.01	0.94

N. pulcher to help guide us in determining which galanin receptors to target in the current study. We did not assess the abundance of *galr2b* because GALR2b is not appreciably activated by galanin in teleosts (Cohen et al., 2020; Kim et al., 2014). We found that *galr2a* was 64× and 16× less abundant than *galr1a* and *galr1b*, respectively, in both the hypothalamic and preoptic areas of the brain. These findings are consistent with previous studies of *galr* distribution in teleost brains (Cohen et al., 2020; Martins et al., 2014). Because of these differences in abundance, combined with galanin generally being a more potent agonist for GALR1 versus GALR2 across vertebrates (Cohen et al., 2020; Kim et al., 2014), we focused our transcriptional analysis of the galanin receptors on *galr1a* and *galr1b*.

2.5. Statistical analysis

Statistical analyses were performed using R (v. 3.6.3; R Core Team, 2022) and a significance level (α) of 0.05 was used for all tests. The assumptions of all models were evaluated visually using the 'performance' package (Lüdtke et al., 2021). When analyzing group differences, data were log-transformed in some cases (territory defense and transcript abundance of preoptic *gal*, as well as hypothalamic *galr1a* and *agrp*) to address issues with normality. Additionally, all correlative analyses were conducted using log-transformed data because this reduced skew and improved the overall fit of most models. All models were fit using the `lm` function and overall differences were determined using the `Anova` function in the 'car' package (Fox and Weisberg, 2011). We estimated effect sizes by calculating either eta-squared (η^2) or partial eta-squared (η_p^2) values using the 'effectsize' package (Ben-Shachar et al., 2020). To investigate whether providing care affected transcript abundance of galanin system components (preoptic area and hypothalamus) or feeding peptides (hypothalamus) in dominant females, we used general linear models (LMs) that included the caring state (young present or absent) as a fixed factor. We also assessed whether the amount of time that dominant females spent feeding was related to hypothalamic transcript abundance of either the galanin system or other feeding peptides using LMs that included caring state and the interaction term between caring state and time spent feeding. We then determined whether transcript abundance of the galanin system varied between

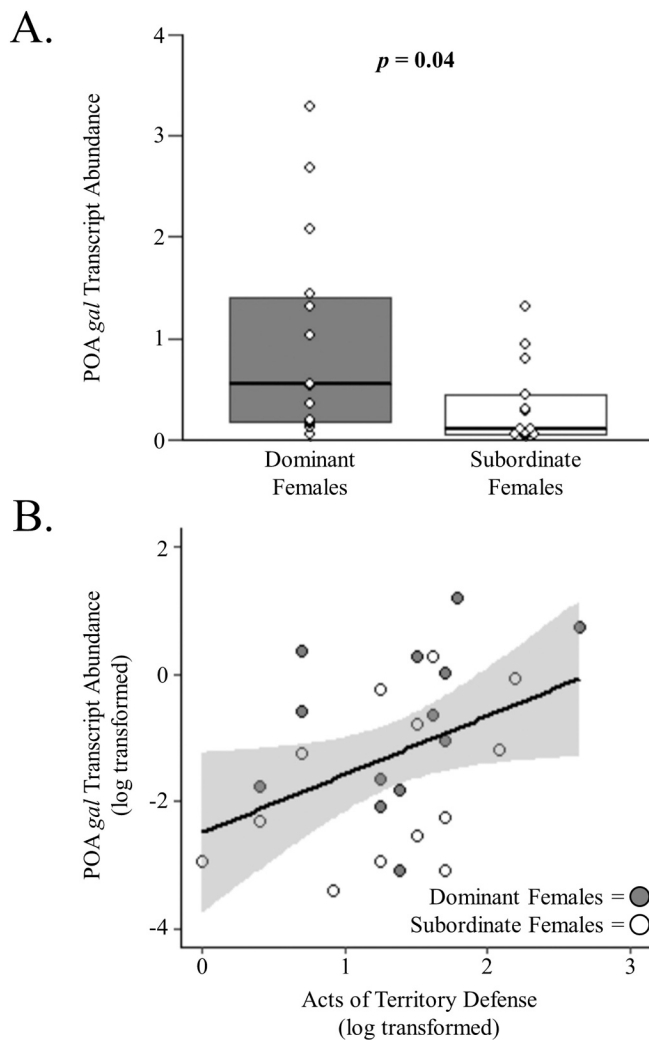


Fig. 3. a) Relative transcript abundance of *gal* in the preoptic area (POA) of the brain and b) the relationship between preoptic *gal* and the amount of territory defense performed by dominant (dark grey) and subordinate (white) female *N. pulcher*. Values are presented as medians and 1st and 3rd quartiles; points represent individual values. A linear regression was fitted, and the shaded area shows the 95 % confidence interval of the regression line. Significant differences between groups are indicated using an asterisk (see statistical analysis section for further details).

social ranks using LMs that included social rank (dominant or subordinate female) as a fixed factor. Finally, to assess whether preoptic galanin expression was related to either how aggressive individuals were towards their groupmates (dominance index scores) or intruding con- and heterospecifics (amount of territory defense performed), we used LMs that included social rank and the interaction term between social rank and the focal behaviour.

3. Results

3.1. Associations with parental state

In the hypothalamus, dominant females with young had ~70 % greater transcript abundance of *galr1a* (Fig. 1A; Table 2) compared to dominant females without young ($p = 0.03$). Dominant females with young also had ~50 % greater transcript abundance of *gal* (Fig. 1B; Table 2) compared to dominant females without young, but this difference did not reach statistical significance ($p = 0.09$). No differences were detected between dominant females with and without young in

expression of *galr1b* in the hypothalamus or any target component of the galanin system in the preoptic area (Table 2). Dominant females did not differ in terms of how behaviourally dominant they were in their groups, how much territory defense they performed, or how much time they spent in the brood chamber, based on whether young were present or not (Supp. Table 1). Dominant females with young spent ~70 % less time foraging than females without young (Fig. 2A); however, this difference also did not quite reach significance ($p = 0.05$; Supp. Table 1). Dominant females that foraged the least had the highest hypothalamic expression of both *gal* (Fig. 2B; Table 3) and *galr1a* (Fig. 2C; Table 3), but not *galr1b* (Table 3). Hypothalamic transcript abundance of several other neuropeptides implicated in the regulation of feeding (*agrp*, *crfb*, *galr1b*, *hcrt*, *npv*, and *pomc-a1*) did not vary based on the presence or absence of young (Table 2), and transcript abundance of these other feeding neuropeptides was unrelated to the amount of time that dominant females spent feeding (Table 3).

3.2. Associations with social status

Transcript abundance of *gal* in the preoptic area was ~3× higher in dominant females compared to subordinate females (Fig. 3A; Table 4). However, no differences in preoptic galanin receptor expression (*galr1a* or *galr1b*) or expression of any target component of the hypothalamic galanin system were observed between dominant and subordinate females (Table 4). Dominant females were more aggressive and less submissive than subordinate females, as reflected by their higher dominance index scores (Supp. Table 2), but no differences were observed between dominants and subordinates in territory defense, foraging rates, or time spent in the brood chamber (Supp. Table 2). Across all females (dominants and subordinates) *gal* expression in the preoptic area was positively correlated with the amount of territory defense that fish performed (Fig. 3B; Defense: $\eta_p^2 = 0.19$, $F_{1,22} = 4.48$, $p = 0.04$; Social Status: $\eta_p^2 = 0.14$, $F_{1,22} = 3.56$, $p = 0.07$; Defense*Status: $\eta_p^2 = 0.01$, $F_{1,22} = 0.01$, $p = 0.98$). However, preoptic *gal* expression was not related to how aggressive a fish was within their social group as reflected by their dominance index scores (Dominance: $\eta_p^2 = 0.02$, $F_{1,22} = 0.04$, $p = 0.85$; Social Status: $\eta_p^2 = 0.12$, $F_{1,22} = 3.11$, $p = 0.09$; Dominance*Status: $\eta_p^2 = 0.02$, $F_{1,22} = 0.51$, $p = 0.49$).

4. Discussion

Galanin is emerging as a key neuroendocrine regulator of parental care across vertebrates (Fischer and O'Connell, 2017; Zilkha et al., 2017), but it is still unclear how populations of galanin neurons located in distinct regions of the brain coordinate to alter the behaviour and/or physiology of caring individuals. In contrast to our prediction that activity of the hypothalamic galanin system would be dampened in caring females to discourage feeding, we found that the hypothalamic galanin system—specifically, transcript abundance of galanin receptor 1a—was upregulated when dominant females were caring for young, and that this upregulation was negatively correlated with foraging rates. We also did not find any evidence that the preoptic galanin system was more transcriptionally active in caring versus non-caring females. However, preoptic galanin was related to aggression/territoriality because dominant females had higher preoptic galanin expression compared to subordinate females and preoptic galanin levels were higher in fish that performed greater amounts of territory defense. Overall, our results suggest that the galanin system has a nuanced, brain-region-specific role in regulating care, feeding, aggression, and social rank in wild animals.

Populations of galanin neurons located in the hypothalamus are involved in the regulation of food intake across vertebrates (Fang et al., 2011; Marcos and Coveñas, 2021), including fishes (Butler et al., 2020; Unniappan et al., 2004). In the current study, we found that dominant females that were caring for young had higher hypothalamic abundance of *galr1a* compared to dominant females without young, and that expression of *gal* and *galr1a* was higher in females that spent less time

Table 4

Transcript abundance of genes in the preoptic area and hypothalamus of dominant and subordinate female *N. pulcher*. Data are expressed relative to the mean values for dominant females and are reported as means \pm SEM. Significant differences ($p < 0.05$) are indicated with **bold font**.

		Dominant females (N = 14)	Subordinate females (N = 13)	η^2	F	p
Preoptic area	<i>gal</i>	1.00 \pm 0.28	0.35 \pm 0.11	0.16	4.93	0.04
	<i>galr1a</i>	1.00 \pm 0.09	0.84 \pm 0.09	0.06	1.73	0.20
	<i>galr1b</i>	1.00 \pm 0.08	0.92 \pm 0.04	0.04	0.95	0.34
Hypothalamus	<i>gal</i>	1.00 \pm 0.11	0.94 \pm 0.13	0.01	0.10	0.75
	<i>galr1a</i>	1.00 \pm 0.13	1.26 \pm 0.18	0.04	1.01	0.33
	<i>galr1b</i>	1.00 \pm 0.06	1.05 \pm 0.08	0.01	0.30	0.59

foraging. This is surprising because galanin typically promotes food intake in teleosts (de Pedro et al., 1995; Guijarro et al., 1999; Volkoff and Peter, 2001), suggesting that caring dominant females tended to reduce foraging efforts despite higher activity of a neuropeptide system which promotes feeding. While the mechanism of action for the orexigenic effects of galanin in teleosts remains unclear (Jensen, 2001; Mensah et al., 2010), our data tend to support the hypothesis of Li et al. (2013)—that the relationship between feeding and the galanin system is mediated by GALR1a in teleosts—because hypothalamic expression of both *gal* and *galr1a* (but not *galr1b*) were correlated with the amount of time that dominant females spent feeding. In goldfish (*Carassius auratus*) the appetite-stimulating actions of galanin are at least partially mediated by interactions with hypocretin/orexin (HCRT) and neuropeptide Y (NPY; Volkoff and Peter, 2001). However, we found that the presence of young was not associated with differences in hypothalamic transcript abundance of either *hcrt* or *npy*, nor was the expression of these neuropeptides correlated with foraging (with similar findings for *agrp*, *crfb*, and *pmc-a1*). This indicates that transcriptional activation of the hypothalamic galanin system was not associated with parallel stimulation of the downstream effectors of galanin's orexigenic actions, or other feeding-related peptides more generally. In contrast, several of these feeding-related peptides (e.g., AGRP, HCRT, NPY, POMCa1) have been implicated in the regulation of food intake during maternal care in a closely related, mouthbrooding cichlid (*Astatotilapia burtoni*; Grone et al., 2012; Porter et al., 2017). It remains possible that changes in levels of the receptors for these other feeding peptides—which were not measured in the current study but have previously been shown to change during care in *A. burtoni* (e.g., NPY receptors; Grone et al., 2012)—may help to explain why we did not observe a relationship between levels of these feeding peptides with either feeding or activity of the hypothalamic galanin system while females provided care. However, our current data suggest that the observed relationship between the hypothalamic galanin system and feeding may have been indirect and activation of the hypothalamic galanin system in caring females could instead be related to other functions of the hypothalamic galanin system.

Several galanin positive neuronal tracts that originate from the lateral tuberal nucleus located in the hypothalamus project to the pituitary (Anglade et al., 1994; Magliulo-Cepriano et al., 1993; Olivereau and Olivereau, 1991; Prasada Rao et al., 1996). Many of these hypothalamic galanin neurons project to gonadotropes and/or lactotropes (Batten et al., 1990; Magliulo-Cepriano et al., 1993; Moons et al., 1991, 1989; Olivereau and Olivereau, 1991), both of which produce hormones (gonadotropins and prolactin, respectively) that are involved in the regulation of reproduction and parental care across vertebrates (Buntin, 1996; Whittington and Wilson, 2013; Ziegler, 2000). In mammals, galanin generally stimulates the release of luteinizing hormone from gonadotropes (Lopez et al., 1991; Lopez and Negro-Vilar, 1990; Sahu et al., 1994; Wynick et al., 1993) and a similar role in stimulating the release of gonadotropins from the pituitary has been observed in fishes (Pinto et al., 2017; Prasada Rao et al., 1996). Galanin also stimulates the release of prolactin from lactotropes in mammals (Arvat et al., 1995; Baratta et al., 1997; Lopez et al., 1993; Wynick et al., 1998), but we are unaware of any study that has evaluated whether galanin also regulates prolactin release in teleosts. While changes in the release of

gonadotropins and/or prolactin during periods of care have not been assessed in *N. pulcher*, levels of gonadotropins and prolactin both change during periods of care in several mouth-brooding cichlids (Specker and Kishida, 2000; Tacon et al., 2000; Weber and Grau, 1999) suggesting that similar changes might also occur in *N. pulcher*. Overall, while the hypothalamic galanin system is clearly activated while dominant females care for young, the precise cause(s) of this upregulation requires further evaluation.

In contrast to our prediction, we did not detect a relationship between *gal* expression in the preoptic area and parental care because dominant females with young did not have higher preoptic expression of *gal* compared to dominant females without young. These findings are counter to those reported in a laboratory-based study of female *A. burtoni* where galanin neurons located within the parvocellular preoptic area (nPPa) were more active when females were providing care (Butler et al., 2020). These conflicting results could reflect differences in how offspring care is divided in *N. pulcher* versus *A. burtoni*. Female *A. burtoni* provide solo care for their offspring for 10–14 days (Butler et al., 2020; Fernald and Hirata, 1979), but while dominant female *N. pulcher* provide the majority of care towards their offspring, they are usually assisted by both the dominant male and subordinate helpers (Desjardins et al., 2008; Taborsky, 1984, 1985). As such, the physiological changes associated with the provisioning of care by female *N. pulcher* may be muted compared to responses in non-cooperatively breeding species. These contrasting results might also reflect differences in the analytical techniques used to assess galanin system activity. While we assessed galanin system activity by quantifying relative transcript abundance within the entire preoptic area using qPCR, Butler et al. (2020) quantified the number of galanin-positive neurons located in the nPPa that were translationally active. Because we focused our analysis on the entire preoptic area, it remains possible that the transcriptional activity of the galanin system in discrete neuronal populations within the preoptic area (e.g., the nPPa) changes when female *N. pulcher* provide care. However, our results are consistent with a recent laboratory-based study of *N. pulcher* which found that whole brain expression of *gal* was not positively correlated with the levels of care that individuals provided (Cunha-Saraiva et al., 2021). While more studies are needed, data from the laboratory and field suggest that preoptic galanin neurons may not directly promote parental care in *N. pulcher*.

While preoptic *gal* expression did not appear to be related to parental care, *gal* expression in the preoptic area was higher in dominant females (which were more aggressive) compared to subordinate females and was positively correlated with the amount of territory defense that fish performed towards intruders. Aggression towards territory intruders can serve as a form of parental care in *N. pulcher* and it is therefore possible that the observed relationship between preoptic *gal* levels and territory defense could (at least in part) reflect interindividual differences in caring tendencies. In general, these data are consistent with previous studies that have reported higher galanin expression in dominant/territorial individuals of both sexes across several fishes (Cunha-Saraiva et al., 2021; Partridge et al., 2015; Renn et al., 2008; Tripp et al., 2018; but see Pavlidis et al., 2011). Additionally, previous work in male medaka (*Oryzias latipes*) found that activation of galanin neurons located in the medial preoptic (which were far less abundant in females)

promoted aggression between conspecific males in an androgen-dependent manner (Yamashita et al., 2020). The results of the current study suggest a similar association between dominance/aggression/territory defense and the activation of galanin neurons in the preoptic area in female *N. pulcher*. While Cunha-Saraiva et al. (2021) did not observe a relationship between levels of territory defense and whole brain transcript abundance of *gal* in *N. pulcher* held in the laboratory, these contrasting results could be due to analytical (e.g., whole brain expression versus preoptic area expression) and/or ecological (e.g., temporary introduction of intruders in the laboratory versus regular territory intrusions in the field) differences between the laboratory-based study of Cunha-Saraiva et al. (2021) and the current study that was conducted in the field. It is also worth noting that the apparent positive relationship between the preoptic galanin system and aggression in fishes contrasts with previous work in mice, where activation of galanin neurons in the medial preoptic area is associated with reduced inter-male aggression and lower levels of pup-directed aggression displayed by virgins of either sex (Kohl et al., 2018; Wu et al., 2014). However, further investigation of the relationship between the preoptic galanin system and aggression in additional species will be necessary to establish whether these findings represent a conserved difference between mammals and fishes.

The elevated levels of *gal* observed in the preoptic area of dominant females compared to subordinate females could also be related to the elevated reproductive potential of dominants. As is the case for many cooperative breeders (Faulkes and Bennett, 2001; Montgomery et al., 2018; Riehl, 2017), subordinate *N. pulcher* females have smaller gonads given their body size than dominant females (Aubin-Horth et al., 2007; Sopinka et al., 2009) and reproduce much less frequently than dominants (Heg, 2008; Heg and Hamilton, 2008; Hellmann et al., 2015). This reproductive suppression could partly reflect the observed differences in preoptic galanin levels because galanin-expressing neurons in the preoptic area project towards gonadotropes and/or lactotropes (Batten et al., 1990; Magliulo-Cepriano et al., 1993; Moons et al., 1991, 1989; Olivereau and Olivereau, 1991); both of which influence reproductive cycles in teleosts (Levavi-Sivan et al., 2010; Whittington and Wilson, 2013; Zohar et al., 2010). Therefore, the higher expression of *gal* in the preoptic area of dominant females observed in this study may be at least partially related to differences in reproductive state, in addition to variation in aggression.

Taken together, our data suggest that the regulation of parental care in these wild cichlids appears to involve the hypothalamic galanin system. In addition, preoptic galanin levels are associated with social status—and possibly territory defense—in female *Neolamprologus pulcher*. Follow-up studies involving manipulations of food availability and/or galanin activity (via pharmacological approaches) during periods of care, as well as assessing whether the observed transcriptional differences reflect changes at the protein level, are now warranted to confirm the relationships that we observed. Additionally, since several results in the current study bordered on statistical significance—reflecting the modest sample sizes used in this study conducted on a wild population—future studies would benefit from using larger sample sizes to provide greater statistical power. Overall, our results provide important insights into the nuanced relationships between neuropeptides and social behaviour in wild vertebrates.

Ethics

All protocols were approved by the Animal Research Ethics Board of McMaster University (Animal Utilization Protocol No. 18-04-16) and the Zambian Department of Fisheries and followed the guidelines of the Canadian Council on Animal Care.

CRediT authorship contribution statement

BMC, IYL, MGS, MYLW, and SB conducted the behavioural

observations and collected the fish. BMC performed the laboratory analyses under the supervision of NJB and SB. BMC also analyzed the data and wrote the first draft of the manuscript. All authors contributed to the design of the experiment, discussed results, provided feedback on the manuscript, and approved the final draft.

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Declaration of competing interest

The authors declare no competing interests.

Data availability

Supporting data can be found in the attached Supplemental file.

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Appendix A. Supplementary data

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