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Research paper

Galanin and prolactin expression in relation to parental care in two sympatric cichlid species from Lake Tanganyika

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ABSTRACT

Our understanding of the hormonal mechanisms underlying parental care mainly stems from research on species with uniparental care. Far less is known about the physiological changes underlying motherhood and fatherhood in biparental caring species. Here, using two biparental caring cichlid species (*Neolamprologus caudopunctatus* and *Neolamprologus pulcher*), we explored the relative gene-expression levels of two genes implicated in the control of parental care, galanin (gal) and prolactin (prl). We investigated whole brain gene expression levels in both, male and female caring parents, as well as in non-caring individuals of both species. Caring males had higher prl and gal mRNA levels compared to caring females in both fish species. Expression of gal was highest when young were mobile and the need for parental defense was greatest and gal was lowest during the more stationary egg tending phase in *N. caudopunctatus*. The onset of parenthood was associated with lower expression of prl and higher expression of gal in *N. pulcher*, but this pattern was not observed in *N. caudopunctatus*. Our study demonstrates that gal gene expression is correlated with changes in parental care in two biparental cichlid species and extends both knowledge and taxonomic coverage of the possible neurogenetic mechanisms underlying parental care.

1. Introduction

Across species, parental care varies widely, with great diversity in both, the type of care performed and in the sex of the care giver (Clutton-Brock, 2002; Reynolds et al., 2002; Royle et al., 2012; Sefc, 2011). Our current understanding of the neural substrates underlying parental care largely comes from research on the brain of female mammals (Choleris et al., 2013; Dulac et al., 2014; Lee et al., 2009). Although studies of the mechanisms underlying paternal, biparental and alloparental care (e.g., care by non-parents or helpers-at-the-nest) are increasing, we still know little about the neural substrates associated with these less common forms of care (Bendesky et al., 2017; Bukhari et al., 2019; Dulac et al., 2014; Feldman et al., 2019; Kohl and Dulac, 2018).

Research conducted to date on the hormonal mechanisms underlying care suggests considerable evolutionary conservation across vertebrates with specific hormones like prolactin playing key roles in care modulation within and across species (Angelier and Chastel, 2009; Schradin and Anzenberger, 1999; Whittington and Wilson, 2013). Recently, another neuropeptide, galanin (*Gal*) has been shown to have a role in parental care regulation in mice (Wu et al., 2014). Wu et al. (2014) found that during parenting, a subset of *Gal* expressing neurons in the medial preoptic area of the brain (MPOA) were activated, and that genetic ablation of these neurons resulted in impairment of parental care. Further research by Kohl and colleagues (Kohl et al., 2018) showed that if virgin males, which normally attack pups, had optogenetic activation of these *Gal* expressing neurons, they instead groomed pups (Kohl et al., 2018; Wu et al., 2014). Expression of galanin is correlated to parental care in other taxa as well, including some fish species (Bukhari et al., 2019; Tripp et al., 2020); however, nearly all studies conducted to date have focused on species with uniparental care (but see Fischer et al., 2019 for an exception). To improve understanding of the neurogenomic mechanisms underlying biparental care and to expand taxonomic coverage of this topic, we used two cichlid fish species to explore the relative gene-expression levels of *prl* and *gal,* two genes potentially implicated in the control of parental care.

Prolactin is a 199 amino-acid peptide that is synthesized and released

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from lactotroph cells of the pituitary gland (Bole-Feysot et al., 1998; Fitzgerald and Dinan, 2008; Freeman et al., 2000). In rats (*Rattus norvegicus*), nulliparous, hypophysectomised females treated with ovine PRL showed maternal behavior when exposed to pups (Samuels and Bridges, 1983). In birds, the formation of crop "milk" in male and female ring doves (*Streptopelia capicola*) is dependent on the production of PRL (Buntin, 1996; Horseman and Buntin, 1995; Hu et al., 2016; Zhang et al., 2012) In fishes (Blue discus, *Symphysodon aequifasciata* and three-spined stickleback, *Gasterosteus aculeatus*), treatment with Prl increased mucus production (by epidermal cells that serve as food for young) as well as egg fanning behavior (Blüm and Fiedler, 1965; Chong et al., 2006; de Ruiter et al., 1986; Khong et al., 2009).

Galanin is a small 29 amino-acid peptide first isolated from the porcine intestine (Tatemoto et al., 1983) and subsequently was identified in birds, reptiles, and fishes (Wynick et al., 2001). Among its many functions, galanin stimulates the release of prolactin (Koshiyama et al., 1987), growth hormone (GH) (Murakami et al., 1987) and luteinizing hormone (LH) (López et al., 1991) from the pituitary gland, all of which have been associated with parental care (Mammals: Ziegler, 2000; for review see Ziegler, 2000; Birds: Sharp et al., 1988, 1979; for review see Buntin, 1996). The anterior commissural nucleus (ACN) in the MPOA revealed a positive response by *Gal* neurons during suckling behavior, increasing maternal motivation in rats (*Rattus norvegicus*) (Cservenák et al., 2017). Among teleost fishes, *gal* is highly expressed in the diencephalon (including the preoptic area) during the nest, egg, and early hatching stages of care in paternal male three-spined sticklebacks (Bukhari et al., 2019). However, the *gal* neurons in the POA-AH of parental midshipman fish (*Porichthys notatus*) were not highly activated by direct brood care (Tripp et al., 2020, 2019). Aside from these correlative findings from uniparental species, increased neural activation of galanin positive POA neurons has also been reported in parents during tadpole transport in a biparental species of poison frog (*Dendrobates imitator*, Fischer et al., 2019).

Among fishes, uniparental male-only care is the most common type of care, followed by female-only care, while biparental care is less common (Balshine, 2012; Sefc, 2011). In one large family of fishes, the cichlids, there is a variety of care patterns found, with 2/3 of cichlid species showing female-only uniparental care and 1/3 showing biparental care (Goodwin et al., 1998; Sefc, 2011). Cichlids have rapidly diverged (Gante et al., 2016) making it possible to find many closely related species that differ in care but that live in similar habitats and under similar ecological conditions. These characteristics make the cichlids an excellent model system for comparative studies on the proximate causes of parental care.

In this manuscript, we investigated the possibility that *prl1* and *gal* expression are linked to parenting behavior in both biparental and even in cooperative species, as has been previously shown for uniparental species. There are several forms or variants of prolactin in fishes; here we examined only *prl*1 as it is common to all vertebrates (Whittington and Wilson, 2013) while *prl*2 is highly expressed in the eye and is thought to be involved in retinal development (Huang et al., 2009). The *gal* gene structure is highly conserved across vertebrates (Mensah et al., 2010) and for this study, we used the long-form of the *gal* gene (Martins et al., 2014). We used two cichlid species from the same genus *N. caudopunctatus* and *N. pulcher*, that differ in caring strategies. In *N. caudopunctatus,* a male and a female raise the young together, and so this species is classified as a biparental breeding fish (Ochi and Yanagisawa, 1999). In contrast, in *N. pulcher* the breeding pair is joined by both related and unrelated non-reproducing group members in raising offspring and is classified as a cooperative breeder (Stiver et al., 2005; Wong and Balshine, 2011). In both of these cichlid species, the male and the female share the parental task of defending the brood and the territory (Cunha-Saraiva et al., 2019, 2018; Wong and Balshine, 2011). However, the role of non-breeding fish differs significantly between these species. In an established social group, non-breeding subordinate *N. pulcher* usually help look after young, while non-breeding *N. caudopunctatus* are voracious egg consumers and are chased off by the breeding pairs (Balshine et al., 2001; Cunha-Saraiva et al., 2018; Wong and Balshine, 2011).

We predicted that caring individuals would have higher *prl*1 mRNA levels than non-caring individuals in both species. Given that the optogenetic activation of *gal* neurons is connected with the inhibition of infanticidal behaviour in male virgin rats (Kohl et al., 2018; Wu et al., 2014), we also predicted that *gal* would be down-regulated in non-caring individuals compared to caring individuals. Furthermore, we predicted that correlated *prl1* and *gal* gene transcript expression levels would be linked with the degree of parental care behaviours performed.

2. Material and methods

2.1. Study animals and housing conditions

Fifty-six *Neolamprologus caudopunctatus* (28 males and 28 females) and 42 *Neolamprologus pulcher* (21 males and 21 females) were used in the study. Biparental *N. caudopunctatus* pairs excavate a breeding cavity in crevices or under stones where they spawn and guard their young together for up to six weeks (Ochi and Yanagisawa, 1999). *N. pulcher* is also biparental, both parents look after young by providing brood care and defense, but they live in social groups and breed cooperatively. Groups consist of a dominant breeding pair and 1–20 smaller subordinates that typically don't breed (Hellmann et al., 2015; Stiver et al., 2005). Both breeders and subordinates participate in territory defense, territory maintenance, direct brood care of the young (by cleaning and fanning the eggs and defending the young) and can remain in the same territory for several years (Balshine et al., 2001; Jungwirth et al., 2019; Stiver et al., 2005). All fish were wild caught from the most southern tip of Lake Tanganyika/Republic of Zambia in autumn 2015 with an unknown parental history.

Each fish was sexed and measured for standard length (SL), total length (TL), and mass (M) (see Supplementary Table 1 for morphological details). Fish were fed daily with either frozen food (a mixture of artemia, cyclops, and daphnia species plus red mosquito larvae) or with tropical fish flakes. Holding and experimental tanks were maintained at a constant water temperature of 26 ± 1 °C under a 12/12 h light/dark cycle. Prior to using the fish in experiments, the fish were housed in single-sex stock tanks (400 L) containing a maximum of 40 individuals for the smaller *N. caudopunctatus* and a maximum of 30 individuals for the larger *N. pulcher*. In the behavioral assays described below, the F1 sexually immature juvenile offspring from *N. caudopunctatus* wild-caught parents (fish that were between 30 and 45 mm, standard length SL) and *Telmatochromis vittatus* were used as territorial intruders. Two different intruder species were used because previous studies have identified sexually immature *N. caudopunctatus* juveniles as both territory intruder and egg/offspring consumers that elicit a strong defensive response from *N. caudopunctatus* parents (Cunha-Saraiva et al., 2019, 2018; Ochi and Yanagisawa, 1999; Schaedelin et al., 2015). In the case of *N. pulcher* pilot studies in our lab showed that *T. vittatus* elicits a strong defensive response from *N. pulcher* parents and subordinate helpers of this species and a similar response to *T. vittatus* has been reported in a closely related and also cooperative species, *N. savoryi* (Josi et al., 2019).

2.2. General procedures and experimental protocols

A male and female pair of *N. caudopunctatus* were each placed in a 45 L experimental tank (n = 21) containing a breeding shelter (half a flowerpot), a sponge filter, a heater and a 3 cm sand layer. Breeding pairs in the experimental tanks were checked daily for signs of nest preparation and maintenance, spawning, pair stability and general health. Pairs were given a minimum of 7-days prior to any behavioral observations and were randomly selected for observation at different stages of the breeding cycle (Fig. 1a). Randomization was performed by giving each pair a unique ID, which was used to design a randomization

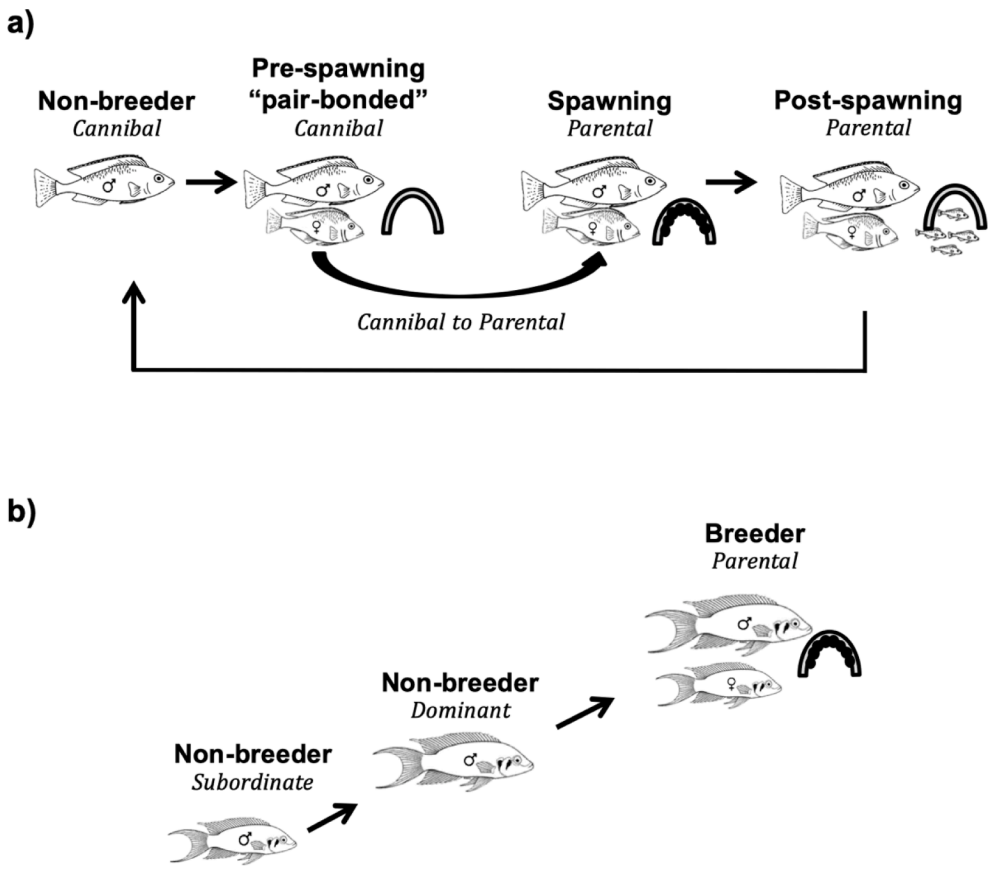

Fig. 1. a) A diagram illustrating the different reproductive stages of *Neolamprologus caudopunctatus*'s breeding cycle. Non-breeding individuals and pre-spawning breeding pairs are egg cannibals, while spawning and post-spawning pairs are non-cannibals (Cunha-Saraiva et al., 2018); **b)** The diagram illustrates the different social and reproductive stages of *Neolamprologus pulcher*'s breeding cycle. Non-breeding individuals live in a social group with a breeding pair that is always dominant to the non-breeders (Wong and Balshine, 2011).

table by using the cluster_ra function from the randomizr package in R. 1) Seven pairs were observed and sampled before egg laying began (classified as pre-spawning pairs); 2) seven pairs were observed and sampled within the first 24 hrs following egg laying (classified as spawning pairs) and 3) seven pairs were sampled 10 days after egg laying, during the free-swimming care phase of offspring development (classified as post spawning pairs). To compare breeding pairs to *non-paired* non-breeding individuals we also sampled 4) seven non-paired males and seven non-paired females from same-sex 160 L holding stock tanks (classified as non-breeding individuals). All fish used in this experiment were kept under similar environmental conditions and water quality and fish general health were checked daily. A series of behavioral assays were performed with the fish in the different treatment or classification groups, and then immediately afterwards the fish were sampled. The sampling time points across the breeding cycle are described above and took into consideration tight connection between egg laying and the cessation of egg cannibalism in *N. caudopunctatus* (Cunha-Saraiva et al., 2018). These discrete sampling points (non-paired, pre-spawning, spawning and post-spawning) allowed the genomic dynamics of *prl1* and *gal* expression and possible connections with parental care to be explored.

We created 7 *N. pulcher* breeding pairs, by selecting males and females from the single sex holding tanks (400 L). One male and one female were placed together in an 80 L experimental tank (O'Connor et al., 2015). The experimental tanks contained 3 cm of sand as substrate, half a flowerpot as a breeding shelter, a heater and a sponge filter. In each pair the male breeder was always at least 5 mm larger than the female breeder as this is the size difference found in natural pairs (Balshine et al., 2001). *N. pulcher* were placed in larger tanks because they are larger and have larger territory sizes compared to the *N. caudopunctatus* (Balshine et al., 2001; Schädelin et al., 2012). The breeding pairs were checked daily, for territory maintenance, spawning, pair stability and general health for a period of 7-days before behavioral observations. Once spawning (within the first 24 hrs of egg laying) was observed, a series of behavioral assays were performed with the seven breeding pairs and then the fish were terminally sampled.

To compare breeding *N. pulcher* pairs to non-breeders (Fig. 1b) and to disentangle the effect of social status on whole-brain mRNA levels of *prl1* and *gal*, a well-established laboratory protocol was followed (Taborsky et al., 2012; von Siemens, 1990; Zöttl et al., 2013) where 7 male-male and 7 female-female social dyads of non-reproductive individuals were established. One individual of the dyad was always 5 mm larger than the other as this distinct size difference leads to a quick and consistent dominance hierarchy, with the larger fish of the same sex dyad emerging as dominant and the other one as the subordinate (Hamilton et al., 2005; Heg et al., 2008; Reddon et al., 2011; Wong and Balshine, 2011). These dyads were kept together (two fish in 80 L experimental tanks) for 7 days and then they were terminally sampled.

2.3. Behavioral assays and scoring

To control for diurnal variation in behavior and physiology (Desjardins et al., 2011; Lema et al., 2010; Werner et al., 2003), behavioral observations always took place between 10 h and 13 h. Parental behavior was observed and scored ~ 1 h before the whole-brain sampling by conducting 10-min behavioral observation followed by a 2-min nest intrusion assay. To ensure that the fish were not disturbed by observer presence, the observer sat still 1 m from the tank and the observation period only began after a two-minute habituation period to the presence of the observer. Following the habituation period each pair was observed for 10 min and all behaviors including any aggressive (e.g., head down or opercula spread) and submissive behaviors (e.g., tail quiver or flee), as well as any parental care/brood care (e.g., egg cleaning and fanning) and territory maintenance (e.g., nest cleaning and

building) behaviors were recorded. A full description of all the behaviors recorded for *N. caudopunctatus* can be found in Cunha-Saraiva et al., 2018, and for *N. pulcher* in Sopinka et al., 2009. Thereafter, a nest defense assay was performed by placing three juvenile *N. caudopuncatus* or two adults *Telmatochromis vittatus* in a transparent plexiglas cylinder (diameter × height: 18 × 40 cm) placed at ~ 10 cm from the entrance of the breeding shelter in *N. caudopunctatus* or *N. pulcher* pair tanks, respectively. The nest defense assay lasted 2 min and began as soon as one of the fishes performed an aggressive display or act towards the egg predators in the cylinder (mean latency to attack the cylinder: ~30 s, range: 0 – 120 s). All aggressive behaviors, such as head-down display, approach or opercula spread, towards the *N. caudopunctatus* juveniles and towards the *T. vittatus* were recorded and classified as defense behavior.

2.4. Tissue sampling and processing

One hour after the observations the individuals were captured and euthanized with an overdose of anesthetic (MS222, Sigma, 1000 mg/L) and then the spinal cord was severed. Fish were then measured, dissected, and their sex was confirmed. The whole brains were preserved in vials of RNAlater (EURx, Gdansk, Poland) that were refrigerated at 4 °C for 12 hrs before storing at −80 °C. Brains were defrosted and homogenized and total RNA was isolated using a Maxwell ® 16 total RNA Purification kit. RNA integrity and purity were assessed by 1% agarose gel electrophoresis and was quantified using a NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific, USA). Prior to cDNA synthesis, total RNA was treated with DNase (DNA-free kit, Ambion, UK) to eliminate genomic DNA contamination, according to the manufacturer's instructions. First strand cDNA was synthesized from 500 ng DNase-treated total RNA in a 20 µl reaction containing 500 ng of DNase-treated RNA, 200 ng of random hexamers (Jena Biosciences, Germany), 100 U of RevertAid (Fermentas, Thermo Fischer Scientific, USA) reverse transcriptase and 8 U of RiboLockRNase Inhibitor (Fermentas). Reactions were incubated for 10 min at 25 °C and 60 min at 42 °C, followed by enzyme inactivation for 10 min at 70 °C, and then stored at – 20 °C until use.

2.5. Candidate gene primer validation

Whole-brain gene expression levels of *prl*1 and *gal* were studied, using quantitative real-time PCR (RT-qPCR). Primers for PCR-amplification of a partial cDNA sequence for each of the genes of interest were designed based on the sequences of *prl*1 and *gal* of four cichlid species (*Oreochromis niloticus*, *Neolamprologus brichardi*, *Astatotilapia burtoni* and *Pundamilia nyererei*; Supplementary Material Table 2). Multiple sequence alignments were performed using ClustalX (v2.0.11) (Larkin et al., 2007), the alignments were edited using GeneDoc version 2.7.0 and the conserved 5' and 3' flanking regions were selected for primer design. The selected primers were then used to amplify the ORF of *prl*1 and *gal* from a pool of brain cDNA. RT-PCR amplification was done using 1 µl of cDNA, 50 µM dNTPs, 10 pmol of each primer and 0.5 U DreamTaq DNA Polymerase (Fermentas). Cycling conditions were 5 min at 95 °C followed by 35 cycles of denaturation for 20 s at 95 °C, 20 s of annealing at the optimized temperature for primer pairs and 1 min of extension at 72 °C, followed by a final extension of 5 min at 72 °C. The amplified fragments were purified using a GFX DNA purification kit (GE healthcare) and sequenced to confirm their identity. The percentage of identity of the *N. caudopunctatus* and *N. pulcher prl*1 and *gal* nucleotide sequence, respectively was confirmed using the BLASTN and BLASTX tools to search against the Genebank database. Multiple-sequence alignments with other cichlid *prl*1 and *gal* were executed as described above to determine sequence identity and confirm gene identity.

2.6. Analysis of gene expression by quantitative real-time PCR

Transcript levels of *prl*1 and *gal* (candidate genes) and β-Actin and 18S (reference genes, (Ahi et al., 2017) were measured by real time quantitative PCR (RT-qPCR) in a CFX Connect qPCR thermocycler (BioRad). A sample of 14 fish were analyzed at each point in the reproductive cycle: for *N. caudopunctatus* (non-breeding, pre-spawning, spawning and post-spawning pairs) and for *N. pulcher* (breeding pairs, non-breeding dominant and subordinate). Quantification of transcripts was carried out in duplicate using the relative standard curve method and EvaGreen chemistry, following the procedure described in Martins et al. (2014). Briefly, *prl*1, *gal*, β-actin (*actb*) and 18S mRNA levels were measured in each sample in duplicate using 11 µl reactions that contained 2 µl of each cDNA (diluted 1:2), 300 nM of each specific primer (Table 3, Supplementary material), 34 mM of Mili-Q water and 5 mM EvaGreen Supermix (Bio-Rad Laboratories, USA). The cycling conditions were 30 s at 95 °C, 35 cycles of 5 s at 95 °C and 10 s at the optimized annealing temperature (Supplemental Material Table 3), followed by a final melting curve between 65 and 95 °C. Specificity of qPCR reactions for *prl*1 and *gal* was confirmed by the presence of a single peak in melt curves, amplification of a product of the expected size (*prl1*: 129 bp, *gal*: 120 bp, *actb*: 189 bp, 18S: 103 bp) and the sequence of the amplified products. A cDNA control sample (a standard cDNA synthesis reaction without the addition of reverse transcriptase, −RT) was included for both genes to confirm there was no genomic DNA contamination and no product was detected. Standard curves relating amplification cycle with initial template quantity were generated using serial dilutions of specific RT-PCR products for each gene under quantification. The efficiency of qPCR reactions ranged from 91% to 98% for both *prl*1 and *gal*, with an $R^2 > 0.98$. Relative expression levels were calculated using the comparative C_T method ($2_{-\Delta\Delta CT}$, Livak and Schmittgen, 2001) and the expression of *prl*1 and *gal* were relative to *actb* for *N. caudopunctatus* and relative to 18S for *N. pulcher*. Different reference genes were used due to the lack of primer efficiency of *actb* for *N. pulcher*, notwithstanding, both reference genes did not vary in expression between samples. All samples for each of the species were extracted and reverse transcribed as a group, and each gene was measured on a single PCR plate for each species. The relative values for each gene can therefore be directly compared between each sampling point for each species separately.

2.7. Statistical analysis

We used R statistical software 3.6.1 to perform the analyses. Prior to any statistical test, all dependent variables were categorized according to their distribution and transformed when necessary to reach normal distribution of residuals and equal variance. *prl*1 mRNA levels were log-transformed to meet normality for both *N. caudopunctatus* and *N. pulcher,* whereas untransformed *gal* mRNA levels followed a gaussian distribution for both species (Shapiro-Wilk test, $P > 0.05$). To determine how *prl*1 and *gal* mRNA levels change across the defined sampling points (*N. caudopunctatus:* non-breeding, pre-spawning, spawning and post-spawning; *N. pulcher:* non-breeder subordinate, non-breeder dominant, and breeding) for each species (see Section 2.2 of the methods), an ANOVA (aov function, from the stats package) was run for each gene with sampling point, sex (male and female) and their interactions as factors. Each model was validated by assessing the distribution of its residuals and variance (Johnson and Omland, 2004). Effect sizes estimates were computed as follows: Eta-square for analysis of variance (etaSquared function, from the lsr package; Navarro, 2015) and Cohen's d for pair-wise comparisons (cohen.d function, from the effsize package; Torchiano, 2018).

To test for differences between the different stages of the breeding cycle, we used glht as a general linear hypothesis testing function in R (Hothorn et al., 2008) and employed user-defined contrasts (*N. caudopunctatus:* non-breeding vs spawning; pre-spawning vs spawning; spawning vs post-spawning; non-breeding vs post-spawning;

N. pulcher: breeding vs non-breeding dominant; breeding vs non-breeding subordinate; non-breeding dominant vs non-breeding subordinate). This is a single-step method test, which adjusts the p-values to the number of comparisons using Tukey contrasts. User-defined contrasts were established as such to provide a comparison between the different sequential phases of the species' breeding cycle as described in Fig. 1a, b.

To test if males and females differed in the amount of parental care behavior provided, a brood defense score was calculated for the defense assay (the sum of all observed aggressive behaviors, such as head down, approach and opercula spread, towards the nest intruders) and a brood care score (the sum of all observed care behaviors including egg cleaning, fanning and cavity visits) for each individual in the study. Since egg cleaning/fanning and cavity visits were strongly correlated (Spearman correlation, rho = 0.65, $P < 0.001$, corr.test function was performed using the psych package, Revelle, 2019), we used cavity visits as our brood care measure. To analyze behaviors, all the behavioral variables were SQRT transformed and then used as the response variable in a generalized linear model. No statistical difference was found between the spawning and post-spawning pairs in terms of brood care or defense (**Brood care:** ANOVA, F-test = 0.01, P = 0.89; **Brood defense:** ANOVA, F-test = 0.40, P = 0.52). Thus, we explore the link between gene expression levels and the behavioral sex differences in *N. caudopunctatus*. We focused on the most active and long phase of care,

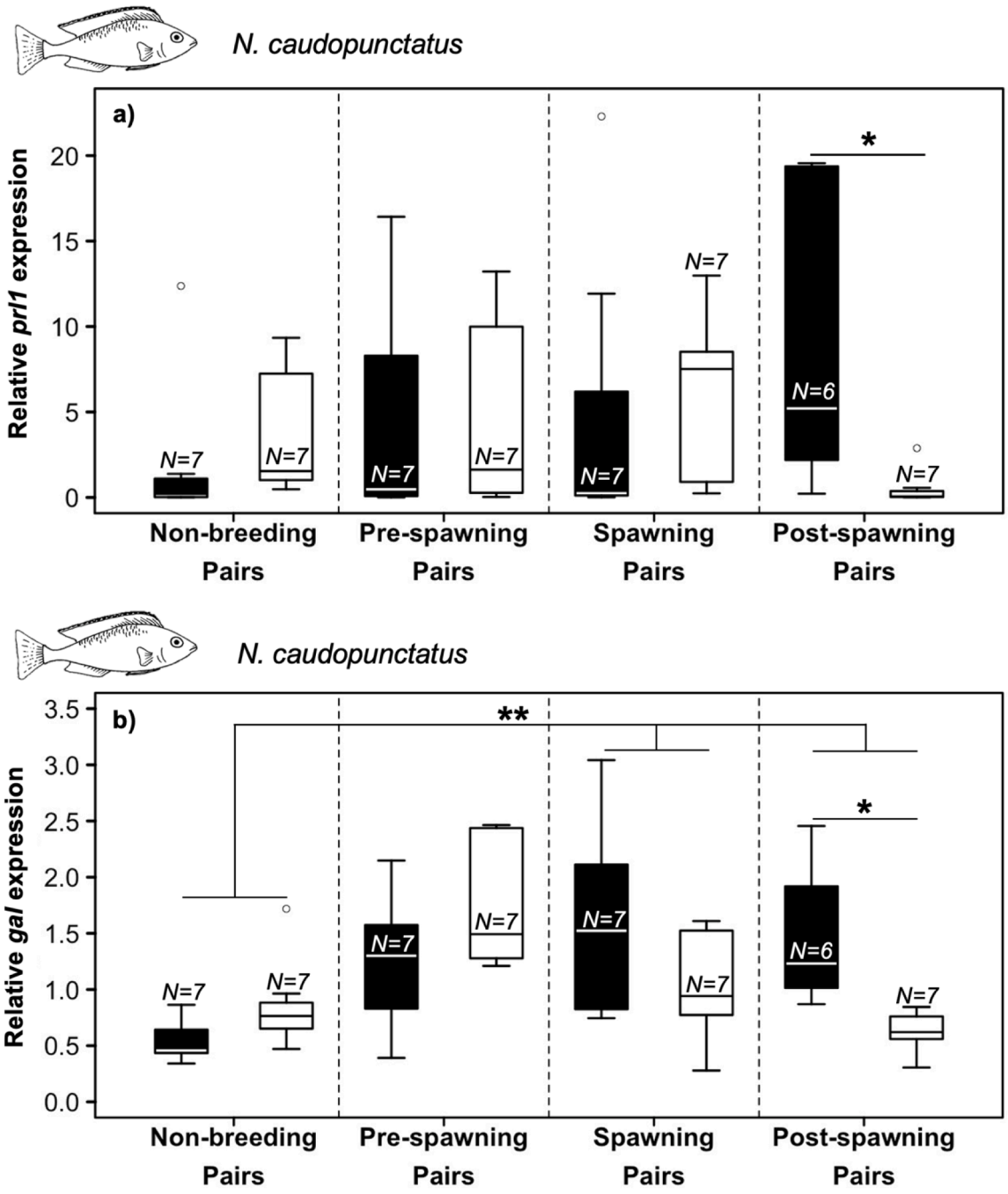

Fig. 2. a) *prl*1 and **b)** *gal* relative mRNA levels for both male and female *Neolamprologus caudopunctatus* at each reproductive stage. Black boxes represent males (M) and white boxes represent females (F). Box-plots show the interquartile range (IQR) of each group analyzed with whiskers extending to 1.5 × the IQR. Horizontal lines represent medians. Outliers were included in the analysis and are depicted on the graphs as white circles. Significant differences as revealed by Tukey Contrasts are depicted on the graph as bold stars, all other contrasts were not significant. *$P < 0.05$, **$P < 0.01$.

the post-spawning phase which last roughly 40-days (Cunha-Saraiva et al., 2019; Ochi and Yanagisawa, 1999). Each model was validated by assessing the distribution of its residuals and variance (Johnson and Omland, 2004).

Finally, to explore how the relative expression of each candidate gene might be linked to specific behaviors, a spearman rank correlation was performed. Because gene expression data was compared to several different behaviors (e.g. brood care and defense), correlations were corrected for multiple comparisons using the "holm" P-value adjustment method (Aickin and Gensler, 1996). More specifically, the relative mRNA levels of *prl*1 and *gal* were correlated with 1) brood care and 2) defense in both *N. caudopunctatus* and *N. pulcher*.

2.8. Ethical note

The procedures described in this study were approved by the University of Veterinary Medicine Vienna institutional ethics and animal welfare committee and in conformity with the Austrian national authority's procedures according to paragraph 26 of the Animal Experiment Act, Tierversuchsgesetz 2012-TVG 2012 (permits Austria: GZ 68.205/0145- WF/V/3b/ 2016; 68.205/0064-WF/V/3b/2017; 68.205/0093-WF/V/3b/2018).

3. Results

3.1. prl1 and gal mRNA expression in Neolamprologus caudopunctatus

Breeding male *N. caudopunctatus* had higher *prl*1 mRNA levels compared to breeding females, but this difference was driven entirely by the most active phase of parental care, the post-spawning stage, when young are mobile and free-swimming (Fig. 2a, Table 1; post-spawning: M vs F, t = 2.99, P = 0.01, Cohen's D = 1.01). For no other stage of the reproductive cycle were *prl*1 mRNA levels significantly different between males and females (Fig. 2a, Table 1; non-breeder: M vs F, t = −2.15, P = 0.13, Cohen's D = 0.44; pre-spawning: M vs F, t = −0.83, P = 0.87, Cohen's D = 0.03; spawning: M vs F, t = −1.61, P = 0.37, Cohen's D = 0.08). *gal* mRNA levels were lowest during the non-breeding stage and increased significantly in the parental care phase (both spawning and post-spawning phase, Fig. 2b, Table 1; non-breeding vs spawning, t = −3.61, P = 0.003, Cohen's D = 1.04; non-breeding vs post-spawning, t = −3.0, P = 0.01, Cohen's D = 0.59). For no other stage of the reproductive cycle were *gal* mRNA levels significantly different (Fig. 2b, Table 1; non-breeder vs pre-spawning, t = −2.37, P = 0.08, Cohen's D = 1.51; pre-spawning vs spawning, t = −1.24, P = 0.57, Cohen's D = 0.34; spawning vs post-spawning, t = 0.47, P = 0.95, Cohen's D = 0.43).

Female and male *N. caudopunctatus* were not overall different in *gal* mRNA expression levels (Table 1), however, similar to *prl*1, parental males showed higher *gal* mRNA levels compared to females at the stage of care when young were mobile and free-swimming (post-spawning) (Fig. 2b, Table 1; post-spawning: M vs F, t = 2.71, P = 0.03, Cohen's D = 2.03; for no other stage of the reproductive cycle were *gal* mRNA levels significantly different between males and females, non-breeder: M vs F, t = −1.08, P = 0.72, Cohen's D = 1.01; pre-spawning: M vs F, t = −1.63, P = 0.36, Cohen's D = 0.63; spawning: M vs F, t = 1.84, P = 0.73, Cohen's D = 0.73).

Contrary to the patterns of male biased territorial defense reported previously in the field and in the lab for this species (Ochi and Yanagisawa, 1999; Cunha-Saraiva et al., 2018), in this study, male *N. caudopunctatus* did not defend the brood more than females (Table 2). However, as expected based on previous laboratory and field results, females did care for eggs and tended the nest more than males (Table 2; see also Supplemental Table 4 for behavioral descriptive statistics).

*prl*1 appeared to be negatively correlated with brood care in post-spawning males, but not females (Fig. 4, Table 3), while *gal* mRNA levels were not correlated with brood care or offspring defense during the post-spawning phase of care for both males and females (Table 3). Moreover, *prl*1 and *gal* mRNA levels were not correlated in post-spawning pairs (Spearman correlation, N = 13, rho = 0.22, P = 0.45).

3.2. prl1 and gal mRNA expression in Neolamprologus pulcher

Non-breeding subordinate *N. pulcher* had the highest *prl1* mRNA levels, and they were considerably higher than both non-breeding dominant fish and breeding *N. pulcher,* whilst no clear difference was found between breeding pairs and non-breeding dominant fish (Fig. 3a, b, c; Table 1, breeding pairs vs non-breeding subordinates, t = 4.46, P < 0.001, Cohen's D = 0.68; non-breeding dominants vs non-breeding subordinates, t = 6.14, P < 0.001, Cohen's D = 1.19; breeding pairs vs non-breeding dominants, t = −1.62, P = 0.25, Cohen's D = 0.001). *N. pulcher* males and females differed in *prl1* mRNA levels (Table 1), however, the effect of sex on *prl1* mRNA levels was dependent on the time point of sampling. Male *N. pulcher* breeders had higher *prl1* mRNA levels compared to breeder females whereas non-breeding subordinate females had higher *prl1* mRNA levels compared to non-breeding subordinate males (Fig. 3a and c; Table 1; breeding: M vs F, t = 2.67, P = 0.03, Cohen's D = 0.52; non-breeding dominants: M vs F, t = −1.02, P = 0.66; non-breeding subordinates: M vs F, t = −3.08, P = 0.01).

gal mRNA levels were also dependent on the social or breeding status as spawning individuals had higher *gal* mRNA expression levels compared to non-breeders, both dominants and subordinate's females and males (Fig. 3d, e, f; Table 1; breeders vs non-breeding subordinates,

Table 1
ANOVA[b] analysis and effect size estimates (Eta-square) for both *prl*1 and *gal* for each species, *N. caudopunctatus* and *N. pulcher*.

Species	Gene	Factors	Effect size	d. f.	F-value	P-value
N. caudopunctatus	*prl*1	Sampling point	0.006	3	0.13	0.93
		Sex	0.009	1	0.65	0.42
		Interaction	0.24	3	5.42	**0.002**
	gal	Sampling point	0.20	3	5.25	**0.003**
		Sex	0.01	1	0.84	0.36
		Interaction	0.17	3	4.59	**0.006**
N. pulcher	*prl*1	Sampling point	0.74	2	79.1	**<0.001**
		Sex	0.01	1	0.60	0.44
		Interaction	0.08	2	9.17	**0.006**
	gal	Sampling point	0.20	2	4.27	**0.02**
		Sex	0.01	1	0.84	0.36
		Interaction	0.18	2	4.92	**0.01**

[b] Potential factors influencing mRNA levels: sampling point = *N. caudopunctatus*: Non-breeding vs Spawning; Pre-spawning vs Spawning; Spawning vs Post-spawning; Non-breeding vs Post-spawning; *N. pulcher:* Breeding vs Non-breeding dominant; Breeding vs Non-breeding subordinate; Non-breeding dominant vs Non-breeding subordinate. Significant P-values are highlighted in bold font.

Table 2
ANOVA analysis and effect size estimates (Eta-square) for each parental behavior category (brood care and defense) for both species, *N. caudopunctatus* and *N. pulcher*.

Species	Behavior	Factors	Effect size	d. f.	F-value	P-value
N. caudopunctatus	Defense	Sex	0.008	1	0.25	0.61
	Brood care	Sex	0.52	1	27.4	**<0.001**
N. pulcher	Defense	Sex	0.13	1	1.51	0.24
	Brood care	Sex	0.02	1	0.22	0.64

Significant P-values are highlighted in bold font.

Table 3
Sex, sample sizes, Spearman rank correlation coefficients and *P*-values (Holm correction applied) for the behavioral responses and whole brain mRNA levels.

		*prl*1				*gal*			
Species	Measure	sex	n	rho	*P*	sex	n	rho	*P*
N. caudopunctatus	Defense	M	6	− 0.52	0.23	M	6	0.23	0.66
		F	7	− 0.63	0.13	F	7	− 0.05	0.91
	Brood care	M	6	− 0.79	**0.04**	M	6	0.36	0.44
		F	7	− 0.58	0.18	F	7	− 0.36	0.43
N. pulcher	Defense	M	5	− 0.15	0.79	M	6	0.26	0.61
		F	6	0.65	0.17	F	6	0.25	0.65
	Brood care	M	6	− 0.65	0.65	M	6	− 0.80	**0.04**
		F	5	− 0.78	0.11	F	6	− 0.37	0.46

Significant P-values are highlighted in bold font.

t = −3.26, P = 0.006, Cohen's D = 0.97; breeders vs non-breeding dominants, t = −2.93, P = 0.01, Cohen's D = 0.31; non-breeding dominants vs non-breeding subordinates, t = −0.20, P = 0.97, Cohen's D = 0.89). Moreover, *N. pulcher* male breeders had higher *gal* mRNA levels compared to female breeders while no such sex differences were found between male and female non-breeding dominants or subordinate individuals (Fig. 3d, e, f; Table 1, breeders: M vs F, t = 2.71, P = 0.02, Cohen's D = 1.08; non-breeding dominants: M vs F, t = −1.73, P = 0.24, Cohen's D = 0.98; non-breeding subordinates: M vs F, t = 0.27, P = 0.99, Cohen's D = 0.27).

Breeder male and female *N. pulcher* in this study performed similar amounts of brood care and defense (Table 2; see also Supplemental Table 4 for behavioral descriptive statistics). Brood care (e.g., fanning, cleaning and cavity visits combined) was negatively correlated with *gal* mRNA levels in males but not females (Table 3, Fig. 4), but no relationship was observed with defense (Table 3). No relationship was detected between *prl*1 mRNA levels and parental behaviors in either males or females (Table 3). Notwithstanding, *prl1* and *gal* mRNA levels were positively correlated in breeding pairs (Spearman correlation, N = 14, rho = 0.69, P = 0.01).

4. Discussion

This study revealed high *gal* mRNA levels, but not high *prl*1 mRNA levels, in the breeders of two cichlid species the biparental *N. caudopunctatus* and the cooperative breeder, *N. pulcher*. The caring males of both species expressed significantly higher *prl*1 and *gal* mRNA levels than did caring females. *prl*1 was negatively correlated with the frequency of brood care in male *N. caudopunctatus*, while the expression of *gal* was negatively correlated with the frequency of brood care in male *N. pulcher*.

Several studies have shown that increased *gal* neuron activation is associated with reproductive behavior, such as the onset of caring in mice (Bukhari et al., 2019; Kohl et al., 2018; Wu et al., 2014), and the switch from a subordinate non-reproductive status to a reproductively active dominance status in male convict cichlids (*Astatotilapia burtoni)* (Renn et al., 2008). The increase in the relative expression of *gal* mRNA in breeder *N. pulcher* and caring *N. caudopunctatus*, might indicate that *gal* is also associated with reproductive behaviors in biparental species (Cunha-Saraiva et al., 2018).

Prolactin has long been associated with parental care (Bachelot and Binart, 2007). In uniparental male bluegill (*Lepomis macrochirus*), prolactin implants resulted in an increase in caring behavior while 11-ketotestosterone (11-KT) implants increased aggression towards a brood predator (Cunha et al., 2019). Cunha and colleagues' study (Cunha et al., 2019) revealed a trade-off between aggression and caring; a parent cannot be both caring and highly aggressive. *prl*1 was negatively correlated with brood care in male *N. caudopunctatus*, in the phase of post-spawning care, when free-swimming offspring are mobile and therefore extremely vulnerable to predators, with non-breeding conspecifics representing one of the biggest threats (Ochi and Yanagisawa, 1999). Therefore, caring males and females should be allocating most of their effort and energy to defending their offspring from predators. The reasons for higher *prl*1 expression in *N. caudopunctatus* post-spawning males, but not in females, is not known and does not seem to be linked to increased brood care as described in the bluegill (Cunha et al., 2019). Moreover, the endocrine correlates we have identified in this study might be related to the regulation of specific *gal* or *prl1* receptors (Bouret et al., 2000; Power, 2005). Although we don't have information about *gal* or *prl1* receptors for the two cichlid species studied (but see Martins et al., 2014), these are obvious candidates for future research and additional studies are still needed to infer causality between the neuroendocrine system and parental behavior.

In the wild, dominant breeding *N. pulcher* males do not typically participate in direct caring behaviors (i.e., egg cleaning or fanning), their time is mostly spent protecting the territory (Balshine et al., 2001; Desjardins et al., 2008). Egg tending is performed by both dominant breeding *N. pulcher* females and non-breeding subordinate individuals (Wong and Balshine, 2011). However, as our laboratory based behavioral paradigm did not fully replicate a natural *N. pulcher* social group, the lack of subordinate helpers might have resulted in males sharing the load, performing more direct care behaviors compared to what is typically observed in the wild. This may explain the absence of typical sex differences in brood care behavior observed in the *N. pulcher* of our study. In spawning parental males *gal* mRNA levels were significantly higher than in females, and the reasons for the negative relationship between care and *gal* mRNA are currently unknown. Future studies are needed to explore the function of *gal* in sex-specific parental care behaviors and how the dynamics of gal expression might be synchronized across the different phases of the breeding cycle.

The observation that males, but not females, in *N. caudopunctatus* and *N. pulcher*, expressed higher levels of *prl*1 within the first 24 hrs. after egg-laying is intriguing and conflicts with previous results that reported lower levels of *prl*1 in *N. pulcher* males compared to females (Bender et al., 2008). Several factors may explain the divergence between our results and those from the previous study. For example, Bender et al., 2008 sampled breeding *N. pulcher* pairs within social groups, while we sampled breeding pairs without a social group. There are also technical considerations linked to the PCR approach taken to measure gene expression in the two studies. The almost 200 times more *prl*1 measured in non-breeding subdominant individuals compared to the levels observed in caring parental fish may be associated with social position and stress. In support of this idea is the finding of similarity in *prl*1 levels in the dominant non-breeders and breeders. Similar, high *prl*1 mRNA levels in non-breeding subordinate individuals in comparison with breeding parents have previously been reported in stable social groups of *N. pulcher* (Bender et al., 2008) and other cooperatively breeding species (e.g. Meerkats, *Suricata suricatta*, Carlson et al., 2006; Florida scrub-jay, *Aphelocoma coerulescens,*Schoech, 1998; Schoech et al., 1996; Mexican Jay, *Aphelocoma wollweberi*, Brown and Vleck, 1998; Common marmosets, *Callithrix jacchus*, Roberts et al., 2001; and Harris' Hawks, *Parabuteo unicinctus*, Vleck and Goldsmith, 1991; but see Schradin and Pillay, 2004 for a counter example). As we did not measure the cortisol levels of the fish, we cannot rule out that the differences that we

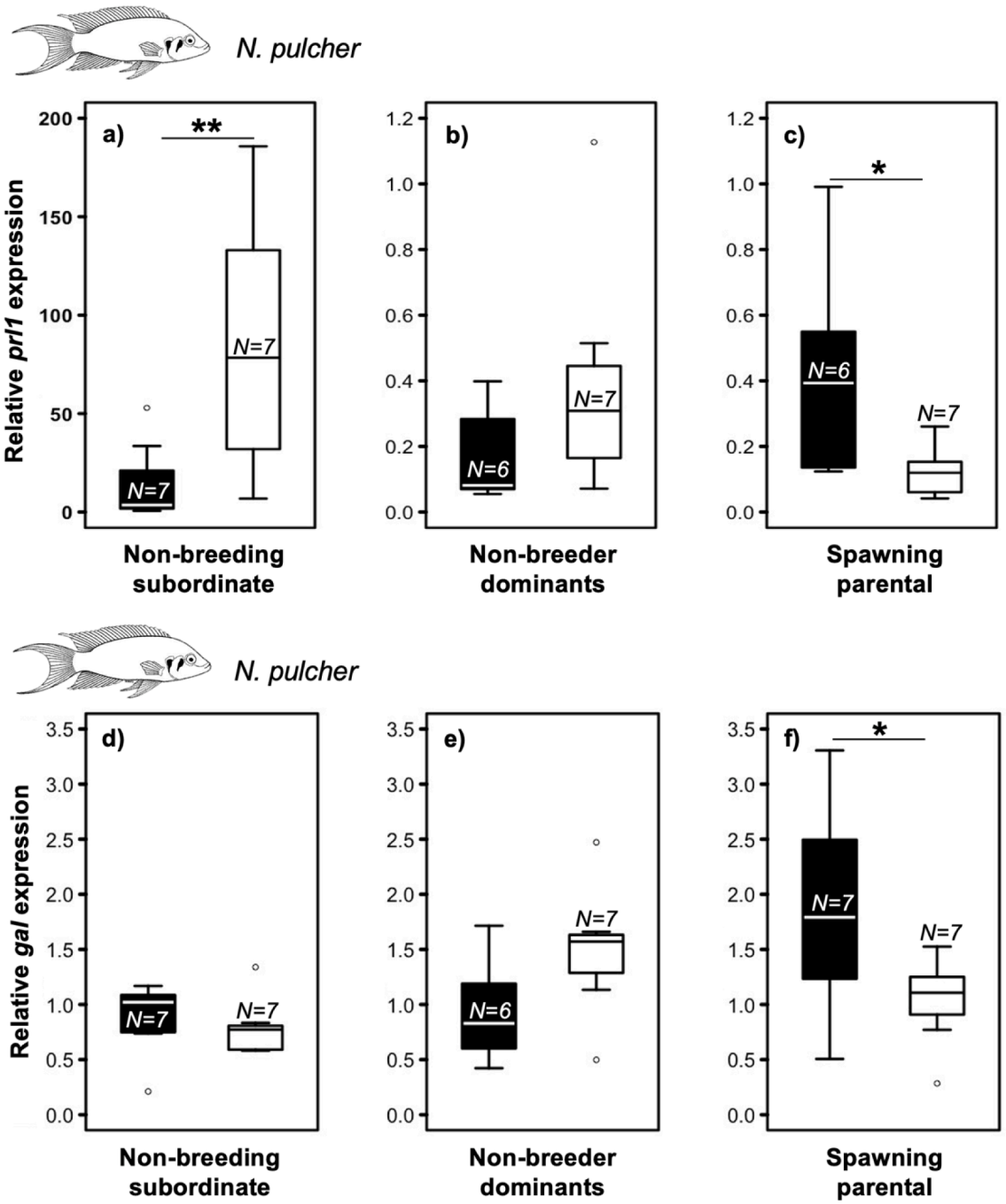

Fig. 3. The *prl*1 mRNA expression levels for **a)** non-breeding subordinate *Neolamprologus pulcher*. Note, the y-axis is depicted in bold to highlight the difference in scale for *prl*1 relative expression in comparison with the other groups, **b)** non-breeding dominants and **c)** breeding individuals. The *gal* mRNA levels for **d)** non-breeding subordinates, **e)** non-breeding dominants and **f)** breeding pairs of *Neolamprologus pulcher*. Black boxes represent males and white boxes represent females. Box-plots show the interquartile range (IQR) of each group analyzed with whiskers extending to 1.5 × the IQR. Horizontal lines represent medians. Outliers were included in the analysis and are depicted on the graphs as white circles. Sample sizes are depicted in the graphs. Significant differences between male and female of each sampling are depicted on the graph as bold stars. *$P < 0.05$.

observed in *prl*1 expression in the present study are due to stress.

No *prl*1 gene expression difference existed between non-breeding and breeding biparental *N. caudopunctatus*. However, gal mRNA levels were higher in breeding than non-breeding *N. caudopunctatus*, suggesting that an increase in *gal* might be associated with the transition from a non-reproductive non-caring individual into a care giver (Cunha-Saraiva et al., 2018). Notwithstanding, we did find that, at the most intense and longest phase of care (at 7-days after egg laying), males had higher levels of both *prl*1 and *gal* when compared to females.

The results obtained in the present study together with our recent study (Cunha-Saraiva et al., 2019) in which post-spawning females of *N. caudopunctatus* had higher whole-brain bioactive arginine-vasotocin (AVT) levels compared to post-spawning males, contribute to increase knowledge of endocrine correlates of parental care (Aubin-Horth et al., 2007). AVT appears to be more important for maternal behavior and Gal appears to be important for paternal behavior in this biparental cichlid species (Butler et al., 2021), which suggests that although both parents perform brood care and defense the mechanism modulating care for

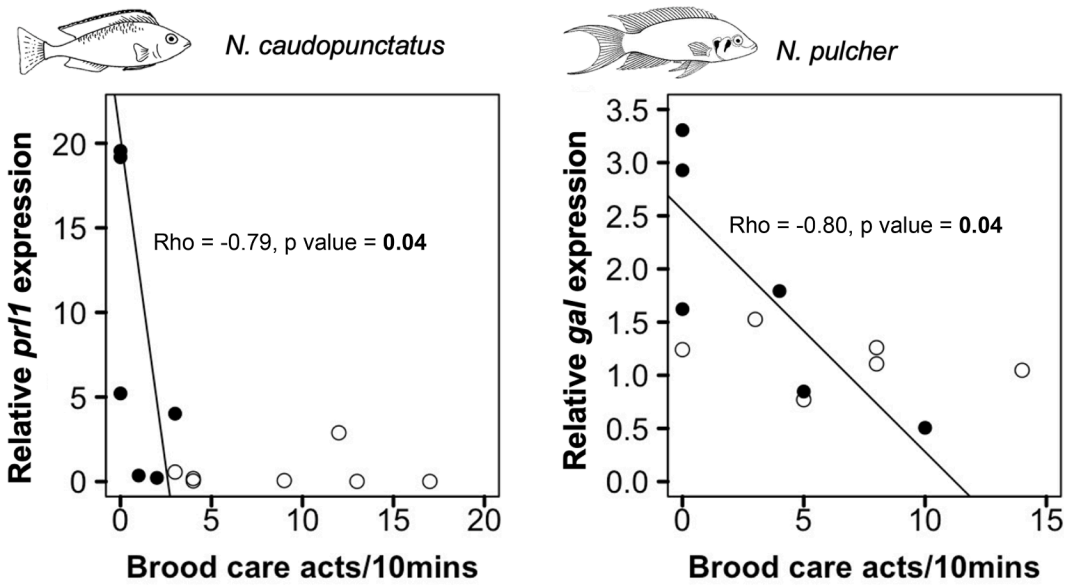

Fig. 4. The negative relationship between **a)** *prl*1 and brood care in *N. caudopunctatus* males (black circles) but not females (white circles), and **b)** *gal* and brood care in *N. pulcher* males (black circles) but not females (white circles). The significant male regression line is depicted in the graph as a bold line. Regression coefficient and p-value are also depicted in the graph.

each sex may be different.

5. Conclusion

In conclusion, *gal* expression is associated with parental care and thus might be involved in the regulation of parental care in both *N. caudopunctatus* and *N. pulcher*, two common cichlid fishes endemic to Lake Tanganyika. As these species show somewhat different breeding systems, our results emphasize the importance of *gal* signaling in caring parents. Our results also underscore that *gal* expression might not only be important for the onset of caring in uni-parental species but also for biparental or cooperative species. Here the investigation of two behaviorally distinct species, has facilitated a more comprehensive understanding of the mechanisms underlying parental care.

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Declaration of Competing Interests

We have no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ygcen.2021.113785.

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