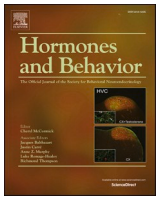
Hormones and Behavior 161 (2024) 105521

Contents lists available at ScienceDirect

Hormones and Behavior

journal homepage: www.elsevier.com/locate/yhbeh



Social regulation of arginine vasopressin and oxytocin systems in a wild group-living fish

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ARTICLE INFO

Keywords:
Arginine vasopressin
Brood care
Cichlid
Dominance
Neolamprologus pulcher
Oxytocin

ABSTRACT

The neuropeptides arginine vasopressin (AVP) and oxytocin (OXT) are key regulators of social behaviour across vertebrates. However, much of our understanding of how these neuropeptide systems interact with social behaviour is centred around laboratory studies which fail to capture the social and physiological challenges of living in the wild. To evaluate relationships between these neuropeptide systems and social behaviour in the wild, we studied social groups of the cichlid fish *Neolamprologus pulcher* in Lake Tanganyika, Africa. We first used SCUBA to observe the behaviour of focal group members and then measured transcript abundance of key components of the AVP and OXT systems across different brain regions. While AVP is often associated with male-typical behaviours, we found that dominant females had higher expression of *avp* and its receptor (*avpr1a2*) in the preoptic area of the brain compared to either dominant males or subordinates of either sex. Dominant females also generally had the highest levels of leucyl-cystinyl aminopeptidase (*lnpep*)—which inactivates AVP and OXT—throughout the brain, potentially indicating greater overall activity (i.e., production, release, and turnover) of the AVP system in dominant females. Expression of OXT and its receptors did not differ across social ranks. However, dominant males that visited the brood chamber more often had lower preoptic expression of OXT receptor a (*oxtra*) suggesting a negative relationship between OXT signalling and parental care in males of this species. Overall, these results advance our understanding of the relationships between complex social behaviours and neuroendocrine systems under natural settings.

1. Introduction

Ample evidence from a variety of vertebrate taxa shows that the nonapeptides oxytocin (OXT; often called isotocin in fish and mesotocin in birds/reptiles/frogs) and arginine vasopressin (AVP; often called arginine vasotocin in non-mammalian vertebrates) regulate social behaviour (Goodson and Thompson, 2010; Insel, 2010; Kelly and Goodson, 2014). Specifically, OXT primarily plays a stimulatory or motivational role, enhancing behaviours that require social approach—such as affiliation and care—while AVP largely influences withdrawal activities—such as aggression and defense (Goodson and Thompson, 2010; Kelly and Goodson, 2014; Lee et al., 2009). Consistent with the relationship between these nonapeptides and social behaviour, AVP levels are usually higher in more aggressive individuals (e.g., males and/or socially dominant individuals; Albers, 2015; Goodson and Thompson, 2010; Kelly and Goodson, 2014). These differences are thought to, at least in part, reflect higher androgen levels in dominant individuals and males because reductions in brain AVP levels in castrated males are reversed following testosterone administration (De Vries et al., 1984; Hillsman et al., 2006). In contrast, status-based differences in OXT levels tend to be more variable and reflect species-specific differences in affiliative and caring tendencies (Godwin and Thompson, 2012; Goodson and Thompson, 2010; Kelly and Goodson, 2014). Dominants and subordinates can also differentially modulate the activity of these

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https://doi.org/10.1016/j.yhbeh.2024.105521
Received 17 November 2023; Received in revised form 9 February 2024; Accepted 26 February 2024
Available online 6 March 2024

systems in specific brain regions by either altering receptor density or adjusting the abundance of their inactivating enzyme, leucyl-cystinyl aminopeptidase (LNPEP; Tsujimoto et al., 2021). While many studies have investigated status-based differences in receptor densities (e.g., Grieb et al., 2021; Lee et al., 2019; Lema et al., 2015), only a handful of studies have investigated whether LNPEP is socially regulated. One of the few examples comes from the Amargosa pupfish (*Cyprinodon nevadensis amargosae*), where hypothalamic transcript abundance of *lnpep* was higher in subordinate versus dominant females (but not males) and was negatively correlated with aggression (Elkins et al., 2017). In another study, male (but not female) mice that lack LNPEP were more social than wild-type mice (Burns et al., 2019), which is consistent with apparent sex-based differences in the activity of these nonapeptide systems.

Despite several decades of research into how AVP and OXT systems affect social behaviour—and how these neuropeptides can differ across social ranks and between sexes—to date, much of our knowledge of these neuropeptides comes from laboratory studies. Furthermore, many studies which have investigated AVP/OXT regulation in wild animals have solely focused on levels of the neuropeptides themselves (e.g., Cardoso et al., 2015; Reddon et al., 2017; Semsar and Godwin, 2003; Stiver et al., 2015) and not other components of these neuropeptide systems. For instance, far fewer studies have investigated changes in receptor abundance (e.g., Huang et al., 2021; Nowicki et al., 2020; Oldfield et al., 2013) and we are not aware of any study which has investigated LNPEP in a wild animal. Such knowledge is necessary because wild animals must contend with a myriad of competing needs that laboratory animals often do not face, and decisions related to these needs generally have greater reproductive and/or survival consequences for wild animals. For example, wild animals must carefully decide when and how to find a mate, how to best protect their territory and/or young, and whether to remain in or leave a social group. Therefore, an enhanced understanding of the relationship between these neuropeptide systems and social behaviours under the social complexity of the wild is sorely needed.

To address this gap, we conducted a field study on a cichlid fish from Lake Tanganyika in Africa, *Neolamprologus pulcher*. These fish are one of few fishes which breed cooperatively and live in permanent social groups (Dey et al., 2017; Taborsky and Limberger, 1981). Each social group consists of a dominant male and female breeding pair with 1–20 subordinates of both sexes (Wong and Balshine, 2011). Most subordinates are reproductively suppressed (Fitzpatrick et al., 2006; Heg, 2008; Heg and Hamilton, 2008), but will assist the breeding pair with brood care, as well as territory maintenance and defense (Taborsky, 1984; Taborsky and Limberger, 1981; Wong and Balshine, 2011). However, of all group members it is usually the dominant female that performs the highest rates of territory defense and brood care (Desjardins et al., 2008b, 2008c). Dominant group members usually maintain their social status and position for 3–12 months (Dierkes et al., 2005; Stiver et al., 2004), allowing for the behaviour of individuals of different status and sex to be reliably tracked for extended periods. Therefore, this group-living species offers a useful model for investigating relationships between AVP/OXT systems and social factors, some of which have already been examined in previous laboratory-based studies. For example, OXT generally tends to promote submission and social attentiveness in subordinate but not dominant *N. pulcher* in the lab (O'Connor et al., 2016; Reddon et al., 2012, 2014; but see Reddon et al., 2015). However, dominant and subordinate brains do not contain different amounts of OXT peptide (Reddon et al., 2015). In contrast, dominants have higher whole brain abundance of AVP transcripts (Aubin-Horth et al., 2007) but less AVP peptide than subordinates (Reddon et al., 2015), suggesting status-based differences in AVP turnover and/or storage. Finally, sex-based differences in the expression of AVP have been reported (Aubin-Horth et al., 2007; O'Connor et al., 2016), but the direction of this difference varied between these two previous laboratory studies. Despite the wealth of information on the social roles of AVP/OXT systems in laboratory-reared *N. pulcher*, there has been only a single investigation of wild *N. pulcher* which reported that OXT injections increased rates of submission in subordinates (Hellmann et al., 2015). Therefore, additional studies of these neuropeptide systems in wild *N. pulcher* groups are necessary to fully understand the role of these peptides in relation to their behaviour within an ecologically valid context.

We used SCUBA to observe the behaviour of wild dominant and subordinate members of *N. pulcher* social groups and then collected these fish to measure the expression of AVP, OXT, and their receptors across different brain regions. We also measured expression of LNPEP because it can further tune the activity of both nonapeptide systems (Tsujimoto et al., 2021) and has previously been linked to social behaviour (Burns et al., 2019; Elkins et al., 2017). We specifically focused on the preoptic area (POA) and hypothalamus (HYP) because these are the regions of the brain where AVP (both regions) and OXT (only in the POA) are centrally synthesized in teleosts (Cerdá-Reverter and Canosa, 2009; Godwin and Thompson, 2012) and both regions are involved with the regulation of social behaviour and reproduction (Kelly and Goodson, 2014; O'Connell and Hofmann, 2011). Similarly, we also investigated the telencephalon (TEL) because it contains homologs of brain structures involved in the regulation of social behaviours in mammals (e.g., amygdala and lateral septum; O'Connell and Hofmann, 2011). Given the intimate link between social dominance/aggression and activity of the AVP system (Goodson and Thompson, 2010), we predicted that expression of AVP and its receptors would be higher in dominants and positively related to within-group aggression and territory defense. In contrast, since OXT activity is often associated with affiliative and brood care (Kelly and Goodson, 2014), we predicted that expression of OXT and its receptors would be higher in subordinates and positively associated with levels of affiliation and brood care. Finally, because activity of AVP and OXT systems often differ between sexes (Albers, 2015; Caldwell, 2018), we also predicted that expression of the OXT system would be greater in females and that expression of the AVP system would be greater in males.

2. Methods

2.1. Field site, behavioural observations, and animal collection

This study was conducted off the shore of Mutondwe Island, Lake Tanganyika, Zambia (8°4245′ S, 31°727′ E) in December 2019. 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.

Using SCUBA, divers located 30 social groups on territories located between 6 and 8 m depth. Each group contained between 5 and 10 individuals (mean ± SEM of 7.6 ± 0.5) that live and defend a small territory (~1 m in diameter) together. To minimize impacts on each individual social group, we targeted dominant females for removal in half of the groups ($N = 15$) and dominant males and subordinate helpers from the other half of groups (N = 15). To confirm the social status of focal group members, we observed the behaviour of individual fish during two 10 min observation periods conducted on separate days (mean of 31 h apart; range of 1–4 days). Such observation protocols provide reliable and repeatable behavioural data in *N. pulcher* (Chervet et al., 2011; Schürch et al., 2010; Witsenburg et al., 2010). Specifically, following a 2 min acclimation period, all affiliative (follows, parallel swims, and soft touches), aggressive (chases, bites, rams, opercular flares, aggressive postures, and lateral displays), submissive (submissive postures, tail quivers, j-hooks, and flees), and workload [brood chamber visits and combined aggression towards intruding con- and heterospecifics (i.e., territory defense)] behaviours were scored as described by Sopinka et al. (2009). We calculated a dominance index (Aubin-Horth et al., 2007; Fitzpatrick et al., 2008) for each focal fish by subtracting the

total 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})]. All affiliative acts performed or received were combined to provide a global affiliation index for each focal fish.

Within 72 h of the second observation period, fish were captured using fence nets, sent to the surface, and were immediately euthanized via terminal anaesthesia (0.5 g L^{-1} ethyl-p-aminobenzoate). We were unable to capture one focal dominant male. The average duration from initially approaching a territory to brain removal was 7.0 ± 0.2 min. Fish were measured (to the nearest 0.1 cm), sex was assigned by examining the gonads, and the entire brain was removed and placed into RNA-later (Ambion). To confirm that the correct focal individuals were removed from each group, we revisited all groups following removals. Using individual differences in body size, unique body markings, and discrete home ranges within each group's territory (Werner et al., 2003), we identified all remaining group members and successfully confirmed that the correct fish was removed in all but one group (where a large female helper was accidently captured instead of the targeted dominant female). Additionally, three dominant males and one dominant female were opportunistically collected from groups that neighboured our focal groups; however, no behavioural data was collected for these fish. In total, we collected brains from 17 dominant males (N = 14 with behavioural data), 15 dominant females (N = 14 with behavioural data), and 16 large subordinate helpers (13 females, 3 males; N = 15 with behavioural data). Our results did not change whether the few subordinate males that were collected were included in our analyses or not, indicating that sex was not a major contributor to differences that were observed between dominants and subordinates.

2.2. Transcript abundance measurement

We measured *avp, oxt,* and *lnpep* (which all have only a single paralog in teleosts; Elkins et al., 2017; Theofanopoulou et al., 2021), as well as both paralogs of *oxtr*—*oxtra* and *oxtrb* (previously named isotocin receptor 2 and isotocin receptor 1, respectively; O'Connor et al., 2015) in all brain regions. Teleost fishes possess at least 5 distinct AVP receptors (Lema, 2010; Ocampo Daza et al., 2022; Theofanopoulou et al., 2021); however, we specifically focused on receptor AVPR1a2 as it is broadly important for regulating social behaviours and reproduction in teleosts (Huffman et al., 2015; Lema et al., 2012; Oldfield et al., 2013; Yokoi et al., 2015). We recognize that taxa-specific nomenclature is often used to describe the AVP and OXT systems in different animal groups (e.g., the use of arginine vasotocin and isotocin in fishes), but we have opted to follow the nomenclature suggested by the Zebrafish Nomenclature Committee (ZNC) which emphasizes the shared evolutionary origins of these peptide families across vertebrates (Ocampo Daza et al., 2022; Theofanopoulou et al., 2021).

We separated the HYP, POA, and TEL under a stereo microscope using micro-dissecting scissors (see Culbert et al., 2021 for additional details) based on the brain atlas of the closely-related cichlid *Astatotilapia burtoni* (Fernald and Shelton, 1985). Transcript abundance was measured in each individual brain region via semi-quantitative real-time polymerase chain reaction (qPCR) using gene-specific primers (Table 1). Briefly, total RNA was extracted using RiboZol reagent (VWR) and 1 μg of RNA that had been treated with PerfeCTa DNase (Quanta Biosciences) was used to synthesize complementary DNA (cDNA) using a commercial kit (qScript; Quanta BioSciences). We were unable to extract sufficient RNA from two samples (1 dominant female POA and 1 subordinate HYP). Following cDNA synthesis, we performed qPCR using a CFX96 system (BioRad) with SYBR green (SsoAdvanced Universal; BioRad). All samples were run in triplicate for 40 cycles and no template and no reverse transcriptase controls were included. Melt curve analysis was conducted at the end of each run to confirm the specificity of each reaction. Input values for each gene were obtained by fitting the average threshold cycle value to the antilog of each gene-specific standard curve to account for differences in primer amplification efficiency. We measured transcript abundance of beta actin (*β-actin*) and elongation factor 1α (*ef1α*) as candidate reference genes, and data were normalized to *β-actin* because it was the most stable across groups. Data are expressed as fold-changes relative to dominant males.

2.3. Statistical analysis

Statistical analyses were performed using R (v. 4.3.0; R Core Team, 2023) and a significance level (α) of 0.05 was used for all tests. When data did not meet the assumptions of normality and/or equal variance, data were either log or square-root transformed to meet these assumptions. All models were fit using the lmer function in the 'lme4' package (Bates et al., 2015), and when overall differences were detected using the Anova function in the 'car' package (Fox and Weisberg, 2018), post hoc Tukey's tests were performed using the 'emmeans' package (Lenth, 2016). Effect sizes were estimated by calculating eta-squared (η^2) or R^2 values using the 'effectsize' package (Ben-Shachar et al., 2020). To investigate how transcript abundance of individuals varied across social ranks, general linear models (LMs) were fit with social rank (dominant male, dominant female, or subordinate) as a fixed factor. We also used LMs to evaluate relationships between: a) the preoptic AVP system (*avp* and *avpr1a2*) and aggressive/reproductive behaviours (dominance index scores and rates of territory defense); b) the preoptic OXT system

Table 1
Gene specific primers used for real-time polymerase chain reaction (qPCR).

Gene	Primer Sequence (5′ to 3′)	Amplicon Size (bp)	Efficiency (%)	Accession Number	Reference
avp	F: TGCAATTCTGAGGGCTGTATG R: TCTTGAGCAGCAGATGGACG	182	106	KT266574	O'Connor et al., 2015
avpr1a2	F: GGAAATCACCTTCCGCTTCTAC R: GACTGCTGTAGGGTTTTCAGAG	156	101	XM_006782475	O'Connor et al., 2015
β-actin	F: CGCTCCTCGTGCTGTCTTC R: TCTTCTCCATGTCATCCCAGTTG	107	111	XM_006797985	Zhao and Fernald, 2005
ef1α	F: AAGAAGATCGGCTACAACCCC R: AGCCCATCTTGTCACTGGTC	94	116	XM_006794500	Culbert et al., 2021
lnpep	F: GTGGAATGACCTGTGGCTCA R: GCGTGCGAGGAATTTAGTGC	150	115	XM_006784166	Current Study
oxt	F: GCTTCGGGCCAAGTATC R: GTGCAGCTCTCTGCATCA	181	103	KT277231	O'Connor et al., 2015
oxtra	F: CTTTGCACAGGAGAAAAGAC R: TCAACCCATAACAAATGCTC	237	104	XM_006806706	O'Connor et al., 2015
oxtrb	F: CTCTTAGGGAGGTCGGTAATG R: GAGTGAGAGCCAAAAAGTGC	207	101	XM_006783124	O'Connor et al., 2015

avp, arginine vasopressin; *avpr1a2*, arginine vasopressin receptor 1a2; *β-actin*, beta actin; *ef1α*, eukaryotic elongation factor 1 alpha; *lnpep*, leucyl–cystinyl aminopeptidase; *oxt,* oxytocin; *oxtr,* oxytocin receptor.

(*oxt*, *oxtra*, and *oxtrb*) and prosocial behaviours (affiliation index scores and number of brood visits); and c) preoptic nonapeptide inactivating activity (*lnpep*) and all aggressive, reproductive, and prosocial behaviours (affiliative and dominance index scores, rates of territory defense, and number of brood visits). Specifically, all models included the behaviour of interest, social rank, and the interaction term between social rank and the behaviour of interest as fixed effects. When a significant interaction term was observed, we performed follow-up analyses of each social rank individually to examine status-specific relationships.

3. Results

3.1. Arginine vasopressin system

Dominant females had the highest expression of both *avp* (Fig. 1A; Table 2, $p = 0.02$) and *avpr1a2* (Fig. 1B; Table 2, $p = 0.02$) in the POA. Levels of *avp* in the POA of dominant females were ~ 100 % higher than subordinates ($p = 0.03$) and ~ 80 % higher than dominant males ($p = 0.04$); expression of *avp* did not differ between subordinates and dominant males ($p = 0.99$). For *avpr1a2*, dominant females had 60 % more transcripts than dominant males ($p = 0.02$) and 40 % more than subordinates ($p = 0.04$). No difference in *avpr1a2* expression was detected between subordinates and dominant males ($p = 0.99$). We also observed higher expression of *avp* in the HYP of dominant females, but this difference did not quite reach statistical significance (Fig. 1C; Table 2, $p = 0.051$). No differences in expression of *avpr1a2* were observed in either the hypothalamus or the telencephalon (Table 2). In addition, no significant relationships between preoptic abundance of either *avp* or *avpr1a2* and aggressive behaviours were detected (Supp. Table 1).

3.2. Oxytocin system

We did not detect any statistical differences in the expression of *oxt* or its receptors (*oxtra* and *oxtrb*) between social ranks in any of the brain regions that we evaluated (Table 2). However, preoptic levels of *oxtra* were strongly negatively associated with the number of times that dominant males visited the brood chamber (Fig. 2; $R^2 = 0.55$, $p = 0.003$; Supp. Table 1). No such relationship between brood chamber visits and *oxtrb* expression was detected in dominant females ($R^2 = 0.01$, $p = 0.86$) or subordinates ($R^2 = 0.16$, $p = 0.13$). Similarly, no other significant relationships with prosocial behaviours were detected (Supp. Table 1).

3.3. Nonapeptide inactivation

Expression of the nonapeptide inactivating enzyme *lnpep* displayed status-specific responses across the POA (Fig. 3A; Table 2, $p = 0.003$), the TEL (Fig. 3B; Table 2, $p = 0.02$), and the HYP (Fig. 3C; Table 2, $p = 0.02$). More specifically, preoptic expression of *lnpep* in dominant females was ~20 % greater than in either subordinates ($p = 0.006$) or dominant males ($p = 0.01$). Dominant females also had ~10 % higher levels of *lnpep* in the TEL than subordinates ($p = 0.02$), with a similar tendency for elevated levels in dominant females compared to dominant males ($p = 0.06$). No differences in *lnpep* levels were detected between dominant males and subordinates in either the POA ($p = 0.96$) or the TEL ($p = 0.93$). In contrast, expression of *lnpep* in the HYP was ~12 % lower in subordinates compared to either dominant females ($p = 0.04$) or dominant males ($p = 0.03$), but levels of *lnpep* in the HYP did not differ between dominant females and males ($p = 0.99$). We did not detect any significant relationships between expression of *lnpep* in the POA and any aggressive or prosocial behaviours (Supp. Table 1).

4. Discussion

We found that in wild social groups of the African cichlid, *N. pulcher*,

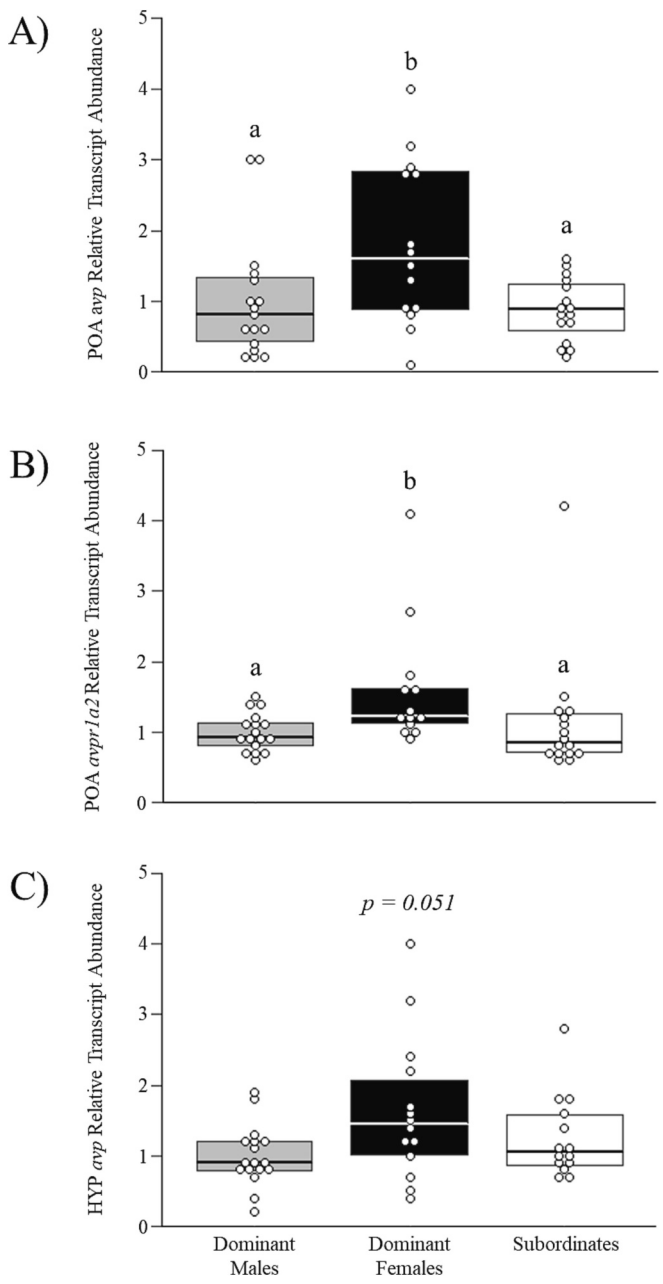

Fig. 1. Relative transcript abundance of *avp* (a) and *avpr1a2* (b) in the preoptic area (POA), and *avp* (c) in the hypothalamus (HYP) of dominant male, dominant female, and subordinate *N. pulcher*. Data are presented as medians and 1st and 3rd quartiles; points represent individual values. Differences between groups are indicated using letters (see text for further details).

the AVP system of dominant females was more active than in either dominant males or subordinates. Specifically, dominant females had higher expression of *avp* in the POA (with a similar pattern in the HYP), as well as elevated preoptic levels of *avpr1a2*. A previous lab-based study of *N. pulcher* observed similar patterns with dominant females having higher whole brain expression of *avp* (Aubin-Horth et al., 2007). These authors suggested that elevated *avp* levels in females were driven by the high rates of aggression and territoriality displayed by female *N. pulcher*. While dominant female *N. pulcher* are submissive towards their dominant male partner (Wong et al., 2012), dominant females are generally more aggressive towards all other group members (Desjardins et al., 2008c), as well as territory intruders (Desjardins et al., 2008a, 2008b, 2008c). This is especially true when a female has a polygynous partner

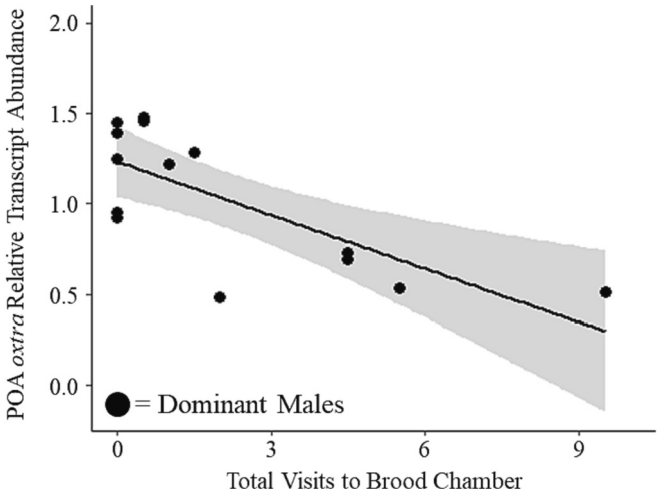

Fig. 2. Relationship between the number of visits to the brood chamber and *oxtra* expression in the preoptic area (POA) of dominant males. Points represent individual values. Linear regressions were fitted, and the shaded area shows the 95 % confidence interval of the regression line (see statistical analysis section for further details).

that is dominant over multiple territories and social groups—which was the case for most groups in our study population (observation by BMC, IYL, MGS, MYLW, and SB)—because polygynous males spend less time on a given territory and contribute less towards territory defense (Culbert et al., 2021; Desjardins et al., 2008a; Jungwirth et al., 2016; Wong et al., 2012). However, while dominant females in our study performed high levels of territory defense (Culbert et al., 2021), we did not detect any relationship between transcript abundance of components of the AVP system and rates of either territory defense or within group aggression (dominance index scores). Therefore, it is possible that the observed differences in gene expression were not directly related to aggression or defense.

Instead of being related to aggression, activation of the AVP system in dominant females may be related to the reproductive actions of AVP (Joy and Chaube, 2015; Ramachandran et al., 2023; Ramallo et al., 2012; Singh and Joy, 2009) either in the brain (e.g., regulates reproductive behaviours and gonadotropin release from the pituitary) or the gonads (e.g., regulates synthesis of sex steroids and oocyte maturation). Dominant female *N. pulcher* invest proportionately more in their gonads and have higher circulating testosterone levels than dominant males or subordinates of either sex (Aubin-Horth et al., 2007; Desjardins et al., 2008a). While 11-ketotestosterone is considered the primary reproductive androgen in male teleost fish (Borg, 1994; Oliveira et al., 2002), several studies have reported a positive relationship between circulating testosterone levels and rates of aggression/territoriality in female *N. pulcher* (Desjardins et al., 2006, 2008c; Taves et al., 2009). In mammals, the conversion of testosterone into estradiol via aromatase in the brain promotes central AVP production and improves the capacity for social recognition (Nomura et al., 2002; Pierman et al., 2008; Plumari et al., 2002). A link between AVP and aromatase activity in the brain has also recently been reported in zebrafish (Shaw et al., 2023), as well as several studies describing positive relationships between brain AVP levels and reproduction more broadly in teleosts (Kalamarz-Kubiak et al., 2017; Sokołowska et al., 2015). Thus, it is possible that similar regulatory mechanisms are also influencing AVP expression in dominant female *N. pulcher*, whereas such a mechanism may be less prominent in dominant males because 11-KT cannot be converted into estradiol by aromatase (Tokarz et al., 2015). Taken together, these data suggest that the AVP system results of the current study may reflect interactions between sex steroid synthesis and behaviour. Interestingly, Greenwood et al. (2008) suggested that aggression and reproductive processes were

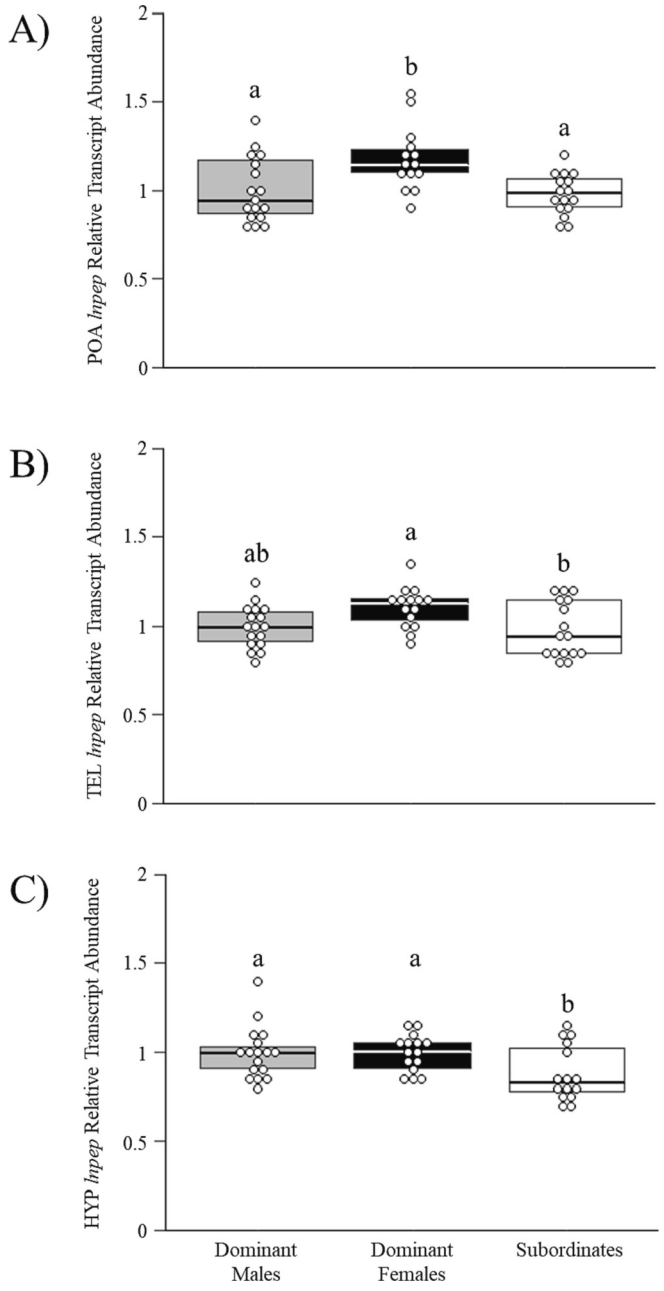

Fig. 3. Relative transcript abundance of *lnpep* in the preoptic area (a; POA), hypothalamus (b; HYP), and telencephalon (c; TEL) of dominant male, dominant female, and subordinate *N. pulcher*. Data are presented as medians and 1st and 3rd quartiles; points represent individual values. Differences between groups are indicated using letters (see text for further details).

specifically regulated by AVP production in the parvocellular region of the POA in males of a closely related cichlid *Astatotilapia burtoni*. Consequently, future studies should focus on determining which population(s) of AVP-expressing neurons in the POA are upregulated in dominant females to better understand the cause(s) of these social status-based differences in AVP activity.

Activity of OXT systems often differ with social rank across vertebrates (Goodson and Thompson, 2010; Kelly and Goodson, 2014), including in fishes (Kleszczyńska et al., 2012; Lema et al., 2015). However, we did not observe any differences in the expression of OXT or its receptors between dominant and subordinate members of wild *N. pulcher* social groups, which is consistent with other laboratory-based findings of *N. pulcher* where OXT peptide levels also did not differ

Table 2
Transcript abundance of genes in brain regions (preoptic area, hypothalamus, and telencephalon) of dominant male, dominant female, and subordinate *N. pulcher*. Data are expressed relative to the mean values for dominant males and are reported as means ± SEM. Significant differences ($p < 0.05$) as determined using linear models are indicated in **bold** and statistical trends ($p < 0.1$) are indicated with *italics*. Letters indicate differences between groups based on post hoc analysis.

Brain Region	Gene	Dominant Males ($N = 17$)	Dominant Females ($N = 15$)	Subordinates ($N = 16$)	η^2	F	p
Preoptic Area	***avp***	**1.00 ± 0.21[a]**	**1.82 ± 0.31[b]**	**0.88 ± 0.11[a]**	**0.16**	**4.28**	**0.02**
	avpr1a2	**1.00 ± 0.07[a]**	**1.60 ± 0.24[b]**	**1.14 ± 0.22[a]**	**0.18**	**4.57**	**0.02**
	oxt	1.00 ± 0.18	1.08 ± 0.17	0.79 ± 0.16	0.03	0.73	0.49
	oxtra	1.00 ± 0.09	1.21 ± 0.10	1.07 ± 0.08	0.06	1.39	0.26
	oxtrb	1.00 ± 0.08	1.13 ± 0.11	0.87 ± 0.06	0.10	2.31	0.11
	lnpep	**1.00 ± 0.05[a]**	**1.18 ± 0.05 [b]**	**0.99 ± 0.03[a]**	**0.23**	**6.45**	**0.003**
Hypothalamus	*avp*	*1.00 ± 0.11*	*1.64 ± 0.27*	*1.27 ± 0.16*	*0.13*	*3.20*	*0.051*
	avpr1a2	1.00 ± 0.06	1.11 ± 0.07	1.05 ± 0.04	0.04	0.97	0.39
	oxtra	1.00 ± 0.05	0.99 ± 0.06	0.96 ± 0.04	0.01	0.23	0.79
	oxtrb	1.00 ± 0.18	1.10 ± 0.18	0.96 ± 0.14	0.01	0.23	0.80
	lnpep	**1.00 ± 0.04[a]**	**0.99 ± 0.03[a]**	**0.88 ± 0.04[b]**	**0.17**	**4.51**	**0.02**
Telencephalon	*avpr1a2*	1.00 ± 0.05	0.95 ± 0.05	1.02 ± 0.10	0.01	0.23	0.80
	oxtra	1.00 ± 0.07	0.89 ± 0.07	0.86 ± 0.10	0.04	0.86	0.43
	oxtrb	1.00 ± 0.09	0.91 ± 0.07	0.85 ± 0.06	0.05	1.06	0.35
	lnpep	**1.00 ± 0.03[ab]**	**1.11 ± 0.03[b]**	**0.98 ± 0.04[a]**	**0.16**	**4.13**	**0.02**

between social ranks within groups (Reddon et al., 2015). Similarly, while we predicted that activity of the OXT system would be positively related to prosocial and caring behaviours, we found no evidence to support such a behavioural relationship. In fact, dominant males with the highest preoptic *oxtra* expression visited the brood chamber (a caring behaviour) the least, suggesting—if anything—a negative relationship between OXT activity and care. This finding contrasts with previous results in both mammals (Keebaugh et al., 2015) and fish (DeAngelis et al., 2017; O'Connell et al., 2012), where greater OXTr signalling is often associated with higher amounts of parental care. Furthermore, whole brain expression of *oxtra* was higher in male clownfish (*Amphiprion ocellaris*) that were actively providing care (DeAngelis et al., 2018). However, our results are consistent with previous laboratory-based studies of *N. pulcher,* where OXT generally appears to negatively influence prosocial behaviours. For example, pharmacological manipulation of OXTr activity suggests that preferences for larger groups are negatively associated with OXTr activity in *N. pulcher* (Reddon et al., 2014), and peptide levels of OXT were higher in the brains of less affiliative fish (Reddon et al., 2015). Consistent with the apparent negative relationship between OXT and prosociality in *N. pulcher*, Reddon et al. (2017) reported that dominant males from highly social, group-living cichlids in the wild (including *N. pulcher*) had fewer OXT neurons in the parvocellular region of the preoptic area when compared with species that are their close relatives but are less social and live in pairs instead of groups. Interestingly, O'Connor et al. (2016) found that *oxt* transcript abundance was positively correlated with affiliative and submissive behaviours when *N. pulcher* were held in pairs (i.e., when no subordinates were present). Thus, the relationship between OXT and prosocial behaviours likely depends on the social context and characteristics of the overall social environment, such as group composition.

Most studies to date have focused on the role of nonapeptides and their receptors in mediating the behavioural effects of these neuropeptide systems; however, a role of the nonapeptide degrading enzyme LNPEP is slowly emerging in both mammals (Burns et al., 2019) and fish (Elkins et al., 2017). In general, levels of LNPEP in the brain seem to be positively associated with aggression/stress, and negatively associated with prosocial behaviours in the few species studied to date (Burns et al., 2019; Elkins et al., 2017). Specifically, male LNPEP-knockout mice (but not females) were more sociable towards a stranger and were less behaviourally stressed in a novel environment (Burns et al., 2019), and transcript levels of *lnpep* in the hypothalamus of female Amargosa pupfish (but not males) was lower in more aggressive and dominant fish (Elkins et al., 2017). In the current study, we did not find any significant relationships between preoptic levels of *lnpep* and social behaviours (either aggressive or prosocial). However, *lnpep* levels were generally highest in dominant females across all three brain regions. Since dominant females also had elevated expression of several components of the AVP system, it is possible that LNPEP is more associated with regulating AVP activity (and less associated with OXT activity) in *N. pulcher*. Despite growing support for a relationship between LNPEP and social behaviour, contrasting patterns across different species currently paint a conflicting picture, and additional studies are needed to determine the source(s) of these differences.

In summary, we conducted one of the first studies to investigate how nonapeptide systems—including levels of peptides, receptors, and their degrading enzyme—are related to social factors in a wild animal and found that the AVP system was more affected by social rank than the OXT system. Specifically, dominant females generally had higher levels of *avp*, *avpr1a2*, and *lnpep* than dominant males and subordinates. However, we found few links between the activity of either nonapeptide system and social behaviours, potentially reflecting the complexity of the social environment and/or physical habitats encountered in the wild. Future studies should also measure levels of AVP and OXT peptides in the brains of wild *N. pulcher* to determine whether the transcriptional changes observed in the current study represent changes at the protein level (Sokołowska et al., 2020). Overall, our results emphasize the importance of conducting behavioural neuroendocrinology studies under natural conditions to better understand the co-evolution of complex social behaviours and neuroendocrine pathways.

Funding

This material is based upon work supported by Natural Sciences and Engineering Research Council of Canada (NSERC) Discovery grants provided to NJB (RGPIN-2015-04498) and SB (RGPIN-2016-05772), and by the National Science Foundation under grant No. 1557836 provided to IMH. BMC was supported by a NSERC Doctoral Canadian Graduate Scholarship (CGS—D) and an Ontario Graduate Scholarship, and MGS was supported by an Ontario Graduate Fellowship.

CRediT authorship contribution statement

Brett M. Culbert: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing – original draft, Writing – review & editing. **Isaac Y. Ligocki:** Investigation, Methodology, Writing – review & editing. **Matthew G. Salena:** Investigation, Methodology, Writing – review & editing. **Marian Y.L. Wong:** Investigation, Methodology, Writing – review & editing. **Ian M. Hamilton:** Funding acquisition, Supervision, Writing – review & editing. **Nicholas J. Bernier:** Funding acquisition, Investigation,

Methodology, Supervision, Writing – review & editing. **Sigal Balshine:** Conceptualization, Funding acquisition, Investigation, Methodology, Supervision, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors declare no competing interests.

Data availability

Data are attached as a supplemental file

Acknowledgments

We would like to thank Dr. Cyprian Katongo at the University of Zambia, the Mpulungu Department of Fisheries, and the residents of Chikonde village for their support of our research. We also appreciate the guidance and helpful comments provided by Dr. Nadia Aubin-Horth during the early development of this project.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.yhbeh.2024.105521.

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