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Rank- and sex-specific differences in the neuroendocrine regulation of glucocorticoids in a wild group-living fish

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ABSTRACT

Individuals that live in groups experience different challenges based on their social rank and sex. Glucocorticoids have a well-established role in coordinating responses to challenges and glucocorticoid levels often vary between ranks and sexes. However, the neuroendocrine mechanisms regulating glucocorticoid dynamics in wild groups are poorly understood, making it difficult to determine the functional consequences of differences in glucocorticoid levels. Therefore, we observed wild social groups of a cooperatively breeding fish (Neolamprologus pulcher) and evaluated how scale cortisol content (an emerging method to evaluate cortisol dynamics in fishes) and expression of glucocorticoid-related genes varied across group members. Scale cortisol was detectable in ~50% of dominant males (7/17) and females (7/15)-but not in any subordinates (0/16)-suggesting that glucocorticoid levels were higher in dominants. However, the apparent behavioural and neuroendocrine factors regulating cortisol levels varied between dominant sexes. In dominant females, higher cortisol was associated with greater rates of territory defense and increased expression of corticotropin-releasing factor in the preoptic and hypothalamic regions of the brain, but these patterns were not observed in dominant males. Additionally, transcriptional differences in the liver suggest that dominant sexes may use different mechanisms to cope with elevated cortisol levels. While dominant females appeared to reduce the relative sensitivity of their liver to cortisol (fewer corticosteroid receptor transcripts), dominant males appeared to increase hepatic cortisol breakdown (more catabolic enzyme transcripts). Overall, our results offer valuable insights on the mechanisms regulating rank- and sex-based glucocorticoid dynamics, as well as the potential functional outcomes of these differences.

1. Introduction

Living in groups can be advantageous, as group members often benefit from reduced predation risk (Hamilton, 1971; Wrona and Dixon, 1991), improved foraging efficiency (Evans et al., 2016; Ward and Zahavi, 1973), and a lightened workload (Ausband et al., 2016; Dornhaus et al., 2008). However, these benefits do not affect all group members equally because individuals often perform different tasks and behaviours (Loftus et al., 2021; Ulrich et al., 2018), have differential access to resources (Kokko and Johnstone, 1999; Metcalfe and Thomson, 1995), and experience different challenges (Abbott et al., 2003; Ray and Sapolsky, 1992). While this variation likely reflects interindividual differences in several physical (e.g., body size) and social factors (e.g., number and quality of affiliative relationships), two traits that are most consistently associated with this variation are individual differences in social rank and sex (Clutton-Brock et al., 2002; Goymann and Wingfield, 2004; Hellmann et al., 2015; Rubenstein and Shen, 2009).

Similarly, much of the physiological variation observed across members of social groups is thought to be a consequence of differences in social rank and sex, including differences in glucocorticoid levels

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(Abbott et al., 2003; Creel, 2001; Creel et al., 2013; Goymann and Wingfield, 2004). Glucocorticoids are steroid hormones that are primarily involved in the diurnal and stress-induced regulation of metabolism, and have a well-established role in coordinating behavioural and physiological responses to environmental and social challenges (Raulo and Dantzer, 2018; Tort and Teles, 2011). In social groups, basal glucocorticoid levels often vary between group members of different social rank and/or sex (Abbott et al., 2003; Creel, 2001; Creel et al., 2013; Goymann and Wingfield, 2004), and these differences are thought to reflect broader variation in the regulation of glucocorticoid dynamics (i.e. their production and breakdown) between group members. For example, elevated basal cortisol levels in subordinate male olive baboons (Papio anubis) compared to dominant males reflect an impaired ability by subordinates to regulate cortisol synthesis via negative feedback (Sapolsky, 1983). However, few studies have examined the mechanisms underlying differences in glucocorticoid dynamics within social groups (e.g., Creel et al., 1996; Michopoulos et al., 2012; Rubenstein, 2007; Saltzman et al., 2004), and this dearth of information makes it difficult to accurately predict the underlying causes and functional consequences of differences in glucocorticoid levels within social groups.

Glucocorticoid synthesis is regulated by the hypothalamic-pituitaryadrenal/interrenal (HPA/HPI) axis (Charmandari et al., 2005; Tort and Teles, 2011). Activation of the HPA/HPI axis is primarily regulated by corticotropin-releasing factor (CRF) neurons projecting from the hypothalamus and/or preoptic area, which promote the synthesis and release of adrenocorticotropic hormone (ACTH) from corticotropes located in the anterior pituitary (Aguilera, 1998; Bernier et al., 2009). Circulating ACTH stimulates glucocorticoid production within adrenal/interrenal tissue and causes systemic glucocorticoid levels to rise (Fridmanis et al., 2017; Tokarz et al., 2015). Across vertebrates, the effects of glucocorticoids are primarily mediated through glucocorticoid (GR) and/or mineralocorticoid (MR) receptors (Arterbery et al., 2011; Sacta et al., 2016). However, unlike other vertebrates, most teleost fishes possess an additional GR paralog and therefore have three cortisol receptors: GR1, GR2, and MR (Arterbery et al., 2011; Bury et al., 2003; Greenwood et al., 2003). While the functional consequences of this additional receptor remain unclear (Prunet et al., 2006; Sakamoto et al., 2018), GR2 and MR generally have a higher affinity for cortisol than does GR1 (Arterbery et al., 2011; Bury and Sturm, 2007; Greenwood et al., 2003) and the relative abundance of each receptor varies slightly across regions of the brain and in peripheral tissues (Bury et al., 2003; Greenwood et al., 2003; Korzan et al., 2014). Collectively, these data suggest that the regulation and/or actions of cortisol mediated via its receptors may vary in teleosts compared to other vertebrates. An additional way that the actions of glucocorticoids can be locally modulated in different tissues is by 11_β-hydroxysteroid dehydrogenase 2 (11_βHSD2), an enzyme which converts active glucocorticoids into inactive metabolites (Chapman et al., 2013; Tsachaki et al., 2017). However, while the basic neuroendocrine mechanisms that regulate glucocorticoid dynamics have been well studied in the laboratory, the social regulation of these mechanisms is poorly understood. Furthermore, we are unaware of any study that has comprehensively evaluated the neuroendocrine mechanisms underlying differences in glucocorticoid dynamics across group members in wild social groups (Creel et al., 2013).

In this study, we investigated how glucocorticoid levels and the neuroendocrine factors regulating glucocorticoids varied across members of wild social groups of *Neolamprologus pulcher*; a cooperatively breeding cichlid fish from Lake Tanganyika, Africa. These fish live in groups consisting of a dominant male-female breeding pair and 1–20 mixed sex subordinates (Balshine et al., 2001; Heg et al., 2004a). In the wild, group membership is relatively stable and dominant group members typically maintain these positions for many months (Dierkes et al., 2005; Stiver et al., 2004). Dominant males are often polygynous and spend much of their time shuttling between the different territories that they hold (Desjardins et al., 2008a; Wong et al., 2012), while dominant

females remain on one territory and generally perform the highest rates of territory defense and brood care of any group member (Desjardins et al., 2008b,c). Dominant females are assisted in these tasks by subordinate members of the group (Taborsky, 1984; Taborsky and Limberger, 1981; Wong and Balshine, 2011). Previous laboratory studies of social groups of N. pulcher have found contrasting effects of social rank on cortisol levels; dominants can have cortisol levels that are either lower than (Culbert et al., 2018), higher than (Mileva et al., 2009), or equivalent to subordinates (Bender et al., 2006, 2008). Additionally, females exhibit lower expression of the cortisol receptors gr2 and mr in their livers compared to males irrespective of rank (O'Connor et al., 2013), but no sex differences in basal or stress-induced cortisol levels were observed in these laboratory studies (Antunes et al., 2021; Bender et al., 2008; Mileva et al., 2009). However, cortisol levels-and the mechanisms regulating their dynamics-have never been evaluated in wild social groups of N. pulcher.

We first observed the behaviour of-and then collected-dominant males, dominant females, and subordinates in wild social groups of N. pulcher using SCUBA. To evaluate differences in glucocorticoid levels we quantified scale cortisol content, an emerging method to evaluate glucocorticoid dynamics in fishes (Gormally and Romero, 2020). Previous studies have shown that scale cortisol content increases $\sim 1-2$ weeks after experiencing chronic (but not acute) stress and remains elevated for at least a month after the stressor subsides (Aerts et al., 2015; Carbajal et al., 2019; Hanke et al., 2019; Laberge et al., 2019). Therefore, scale cortisol content provides an integrative measure to evaluate long-term cortisol dynamics between social group members. We predicted that cortisol levels of dominant N. pulcher would be greater than subordinates, owing to the elevated energetic demands associated with maintaining dominance (Creel et al., 1996; Goymann and Wingfield, 2004; Romero et al., 2009). In addition, we predicted that cortisol dynamics might differ between dominant males and dominant females because dominant sexes perform different tasks (Taborsky and Limberger, 1981; Wong and Balshine, 2011) and while females hold only one territory, males often hold several (Desjardins et al., 2008a; Wong et al., 2012). To evaluate the neuroendocrine mechanisms regulating cortisol dynamics within social groups, we measured transcript abundance of a suite of genes critically involved in cortisol regulation $(11\beta hsd2, crfb$ (the teleost homolog of CRF in mammals; Cardoso et al., 2016; Grone and Maruska, 2015), gr1, gr2, and mr). In the brain, we assessed differences in the preoptic (primary site of HPI axis regulation in teleosts (Bernier et al., 2009)), hypothalamic (secondary site of HPI axis regulation in teleosts (Bernier et al., 2009)), and telencephalic (contains limbic centers that can affect the HPI axis (de Kloet et al., 2005; Joëls et al., 2008)) regions. We also evaluated whether there were transcriptional differences in the liver because the liver is a major target of cortisol's metabolic actions and the primary site of cortisol catabolism. We predicted that dominants would employ mechanisms to reduce the effects of elevated cortisol levels in their liver (e.g., elevated 11β hsd2 and/or reduced receptor abundance), which may help them minimize energy reserve depletion owing to the catabolic effects of cortisol (Faught and Vijayan, 2016; Mommsen et al., 1999).

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, 30 social groups were located between 6 and 8 m depth and used for this study. Groups contained an average (mean \pm SEM) of 7.6 \pm 0.5 individuals.

2.2. Behavioural observations

The behaviour of focal dominant males (N = 14; standard length

 $(SL) = 71 \pm 0.7 \text{ mm}$, dominant females (N = 14; $SL = 63 \pm 0.6 \text{ mm}$), and large subordinate helpers (N = 15: 12 females and 3 males; SL = 53 \pm 1.5 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). Previous studies have shown that similar observation protocols are sufficient to obtain reliable and repeatable behavioural data for individual N. pulcher (Chervet et al., 2011; Schürch et al., 2010; Schürch and Heg, 2010; Witsenburg et al., 2010). Following a 2 min period for fish to acclimate to the presence of the observer, we scored all affiliative (follows, parallel swims, and soft touches), aggressive (chases, bites, rams, opercular flares, aggressive postures, and lateral displays), and submissive behaviours (submissive postures, tail quivers, j-hooks, and flees), as well as each fish's workload (moving substrate, brood chamber visits, and aggression towards intruding conspecifics (nongroup members) and heterospecifics; see Sopinka et al., 2009 for further behavioural details). We calculated a dominance index (see 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})). Additionally, although fish mostly socially interact on their territory, they spend variable amounts of time in the water column feeding and may even briefly visit other territories (Bergmüller et al., 2005; Desjardins et al., 2008a). Therefore, we also measured the amount of time that focal fish spent away from their group's territory.

2.3. Capture and sampling

Within 72 h of the second observation period, focal fish whose behaviour had previously been observed were captured using fence nets and hand nets. The average time from a diver's initial approach of a territory to lay out the fence net to the point of processing of the fish on the surface of the water was 7.0 ± 0.2 min. Once caught, fish were 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 and liver were removed and placed into RNA-later (Ambion). Tissues in RNA-later were kept at 4 °C for 12 h, after which they were transferred to -20 °C for later measurement of transcript abundance. Additionally, all scales from each fish were removed and stored at -20 °C for later quantification of cortisol.

To reduce the impact that our experiment had on individual groups, we targeted dominant females in half of the observed social groups (N = 15) and dominant males and subordinate helpers from the other half of groups (N = 15). Additionally, three dominant males and one dominant female whose behaviour was not recorded were opportunistically collected from groups that neighboured our focal groups. To confirm that the correct focal individuals were removed we revisited each group following removals. Using individual differences in body size, unique markings, behaviour, 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).

2.4. Scale cortisol quantification

Scale cortisol was extracted and measured as described in Laberge et al. (2019). We used a monoclonal mouse, anti-cortisol antibody (XM210; Abcam) that is highly specific for cortisol ($K_a = 1.7 \times 10^9 \text{ M}^{-1}$) and has limited cross-reactivity (<1%) with other steroid hormones according to the manufacturer. To validate the cortisol radioimmuno-assay for *N. pulcher*, scale cortisol levels were measured in laboratory-raised fish (N = 9) that had been held in a 527-litre stock tank

containing ~75 mixed-sex adults at McMaster University in Hamilton, Ontario, Canada. A serial dilution of extract from a pool of scales yielded a displacement curve that was parallel to the standard curve (Supp. Fig. 1) indicating that the scale extract did not contain any substances that interfered with the assay. Owing to the limited amount of scales that could be collected from these small fish (average body weight of $6.7 \pm$ 0.4 g with an average of 113.1 ± 5.5 mg of dried scales; N = 48), samples were run in singleton using extract from ~100 mg of scales (to minimize detection bias between ranks because of differences in body size between dominants and subordinates). The lower detection limit of the assay was 0.22 mg g⁻¹ and the intra- and inter-assay coefficients of variation for an internal standard were 6.2% and 9.5%, respectively. Scale cortisol content values are presented as nanograms of cortisol per gram of dried scale and are corrected for the loss of cortisol during the extraction process (extraction efficiency = 85%).

2.5. 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 processing, the telencephalon (TEL), preoptic area (POA), and hypothalamus (HYP) were dissected out (using the brain atlas of the closely-related cichlid Astatotilapia burtoni (Fernald and Shelton, 1985); see Supp. Fig. 2 for a diagram of the dissection protocol) and each region was processed separately. Total RNA was extracted from brain (TEL, POA and HYP) and liver samples using RiboZol reagent (VWR) as per the manufacturer's instructions. Extracted RNA was quantified using a NanoDrop (Thermo-Fisher Scientific) and RNA integrity was assessed using an Agilent TapeStation 4150. We were unable to extract sufficient RNA from two samples (1 dominant female POA and 1 subordinate female HYP). Complementary DNA (cDNA) was generated from 1 µg of RNA that had been treated with PerfeCTa DNase 1 (Quanta BioSciences), using a commercial kit (qScript; Quanta BioSciences). Following cDNA synthesis, we performed qPCR using a CFX96 system (BioRad) with SYBR green (SsoAdvanced Universal; 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 a total of 20 µl, which consisted of 10 µl of SYBR green, 5 µl of combined forward and reverse primers (0.2 μ M), and 5 μ l of 10–20 \times diluted cDNA. Cycling parameters included a 30 s activation step at 95 °C, followed by 40 cycles consisting of a 3 s denaturation step at 95 °C and a combined 30 s annealing and extension step at 60 °C. Melt curve analysis was conducted at the end of each run to confirm the specificity of each reaction. To account for differences in amplification efficiency, standard curves were constructed for each gene using serial dilutions $(4\times)$ of pooled cDNA. Input values for each gene were obtained by fitting the average threshold cycle value to the antilog of the gene-specific standard curve, thereby correcting for differences in primer amplification efficiency. To correct for minor variations in template input and transcriptional efficiency, we measured transcript abundance of beta actin (β -actin) and elongation factor 1α (ef1 α) as reference genes. Transcript abundance of each gene of interest was normalized relative to the expression of β -actin in all regions of the brain and relative to the geometric mean of the two reference genes in the liver, as these two approaches yielded the greatest stability across social ranks in their respective tissue. Data are expressed as fold-changes relative to the mean value of dominant males.

2.6. Statistical analysis

Statistical analyses were performed using R (v. 3.6.3; R Core Team, 2021) 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 log-transformed to meet these assumptions. All models were

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
11β hsd2	F: ATCACCCAGCCACAACAAGT	162	106	XM_006779684	Current study
	R: ATTGACCTCCATGCACCCAC				
β -actin	F: CGCTCCTCGTGCTGTCTTC	107	111	XM_006797985	(Zhao and Fernald, 2005)
	R: TCTTCTCCATGTCATCCCAGTTG				
<i>crfb</i>	F: ATCACCTTCCATCTTCAACAG	204	108	JX134406	(Taborsky et al., 2013)
	R: CTGGACATCTCCATCATCTC				
ef1a	F: AAGAAGATCGGCTACAACCCC	94	116	XM_035909298	Current study
	R: AGCCCATCTTGTCACTGGTC				
gr1	F: GCTGATCAAGATGAAAGTGC	198	105	EF661652	(Taborsky et al., 2013)
	R: AGAGTAGACATGAGCCGTGA				
gr2	F: TCGTTCCAACAATGTTATCC	204	107	EF661651	(Taborsky et al., 2013)
	R: GCAGAGTCATCTGATCATCC				
mr	F: CTCATGGATGTGTCTGTCCT	201	113	EF661650	(Taborsky et al., 2013)
	R: GAGAGGAACTCGTCGTAGGT				

11βhsd2, 11β-hydroxysteroid dehydrogenase type 2; β-actin, beta actin; crfb, corticotropin-releasing factor b; ef1α, eukaryotic elongation factor 1 alpha; gr1, glucocorticoid receptor 1; gr2, glucocorticoid receptor 2; mr, mineralocorticoid receptor.

fit using the lm function and when overall differences were detected using the Anova function in the 'car' package (Fox and Weisberg, 2011), post hoc Tukey's tests were performed using the 'emmeans' package (Lenth, 2016). We also estimated effect sizes for overall models by calculating eta-squared values (η^2) using the 'effectsize' package (Ben-Shachar et al., 2020) and by calculating Cohen's *d* values (*d*) for post hoc contrasts using the 'emmeans' package (Lenth, 2016). To investigate how the behaviour and 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. Subordinates were not analyzed separately based on sex because only three subordinate males were collected. Exclusion of these three subordinate males did not affect our results and therefore all fish were included in our analyses.

Because scale cortisol content was only detectable in a subset of dominant males and females, we conducted additional analyses using only dominant individuals to probe which behavioural and transcriptional factors varied with cortisol detectability. We specifically focused on agonistic behaviour (aggression towards groupmates, conspecifics from neighbouring groups, and heterospecifics) and the number of visits to the brood chamber (a proxy for brood care) because these behaviours represent the majority of behavioural energy expenditure in dominant N. pulcher (Grantner and Taborsky, 1998; Taborsky and Grantner, 1998). Additionally, previous laboratory studies have suggested a relationship between agonistic behaviours and cortisol levels in N. pulcher (Culbert et al., 2018, 2019b; Ligocki et al., 2015; Mileva et al., 2009). While territory maintenance also comprises a major component of behavioural energy expenditure in dominant females, acts of territory maintenance were rarely observed (only five acts across all behavioural observations) and were therefore not assessed. These analyses were performed using LMs that included dominant sex (dominant male or dominant female), scale cortisol content (detected or not detected) and their interaction term as fixed factors.

3. Results

3.1. Social behaviour across ranks

Dominance index scores varied across social ranks (Table 2; $F_{2.41} =$ 5.58, p = 0.007). Dominant males and females obtained scores that were ~ $3 \times$ higher than subordinates (dominant males: t = 2.92, d = 1.07, p =0.02; dominant females: t = 2.80, d = 1.03, p = 0.02), but the dominance index scores of dominant males and females did not differ from one another (t = 0.11, d = 0.04, p = 0.99). Similarly, we detected differences between social ranks in workload scores (Table 2; $F_{2,41} = 4.46$, p = 0.02) and in time spent away from the territory (Table 2; $F_{2,41} = 4.95$, p =0.01). Dominant females performed approximately twice the workload of dominant males (t = 2.92, d = 1.10, p = 0.02), but subordinates performed intermediate workload levels that did not differ from either dominant males (t = 2.03, d = 0.74, p = 0.12) or dominant females (t =0.99, d = 0.36, p = 0.59). Dominant males spent approximately twice as much time off of the group's territory compared to both dominant females (t = 2.81, d = 1.06, p = 0.02) and subordinates (t = 2.66, d = 0.97, p = 0.03), whereas subordinates and dominant females spent similar amounts of time off territory (t = 0.25, d = 0.09, p = 0.97).

3.2. Scale cortisol content across ranks

We successfully detected cortisol in the scales of all laboratory fish (Fig. 1A; 9/9; 2.05 ± 0.53 ng cortisol per g scale (0.35–5.19 ng g⁻¹)), but we were only able to detect cortisol in the scales of ~50% of wild dominant males (7/17; 1.49 ± 0.36 ng g⁻¹ (0.51–2.94 ng g⁻¹)) and females (7/15; 3.50 ± 1.31 ng g⁻¹ (0.76–11.00 ng g⁻¹)), and 0% of the wild subordinates (0/17).

3.3. Transcript abundance of cortisol-related genes across ranks

Hepatic transcript abundance of 11β hsd2 varied across social ranks (Fig. 2A; $F_{2,45} = 24.61$, p < 0.001), with dominant males having $\sim 15 \times$ more transcripts than dominant females (t = 6.84, d = 2.42, p < 0.001) and $\sim 5 \times$ more than subordinates (t = 4.62, d = 1.61, p < 0.001). No difference was detected between dominant females and subordinates (t

Table 2

Social behaviours of dominant male, dominant female, and subordinate *N. pulcher*. Values for an individual fish represent the average of two 10 min observation periods. Behaviours are reported as means \pm SEM. Significant differences (p < 0.05) are indicated in **bold** and letters indicate differences between groups based on post hoc analysis.

	Dominant males ($N = 14$)	Dominant females ($N = 14$)	Subordinates ($N = 15$)	F	р	η^2
Dominance index Workload Time spent off territory (s)	$\begin{array}{l} 2.00 \pm 0.53^a \\ 4.64 \pm 0.81^a \\ 226.0 \pm 38.9^a \end{array}$	$\begin{array}{l} 1.89 \pm 0.90^a \\ 8.14 \pm 0.90^b \\ 103.2 \pm 21.2^b \end{array}$	$\begin{array}{l} -0.81 \pm 0.60^{\rm b} \\ 7.00 \pm 0.78^{\rm ab} \\ 113.6 \pm 28.1^{\rm b} \end{array}$	5.58 4.46 4.95	0.007 0.02 0.01	0.21 0.18 0.19



Fig. 1. Proportion of fish originating from the laboratory (Laboratory Stock) and the field (Dominant Males, Dominant Females, and Subordinates) where scale cortisol content could be detected. A dashed line is used separate fish originating from the laboratory versus the field.

= 2.26, d = 0.81, p = 0.07). Expression of gr1 (Fig. 2B; $F_{2,45} = 6.36$, p = 0.004) and gr2 (Fig. 2C; $F_{2,45} = 5.99$, p = 0.005) in the liver also varied across social ranks, with dominant females having ~30% lower levels of each compared to dominant males (gr1: t = 3.48, d = 1.23, p = 0.003; gr2: t = 3.36, d = 1.19, p = 0.005) and subordinates (gr1: t = 2.48, d = 0.89, p = 0.04; gr2: t = 2.46, d = 0.89, p = 0.046). Hepatic transcript abundance of gr1 (t = 0.98, d = 0.34, p = 0.59) and gr2 (t = 0.88, d = 0.31, p = 0.66) did not differ between dominant males and subordinates. Hepatic transcript abundance of mr did not differ across ranks (Table 3; $F_{2,45} = 2.48$, p = 0.09).

In the hypothalamus (HYP), we detected variation across social ranks in transcript abundance for both $11\beta hsd2$ (Fig. 3A; $F_{2,43} = 4.01$, p =0.03) and *crfb* (Fig. 3B; $F_{2,43} = 7.14$, p = 0.002). Transcript abundance of $11\beta hsd2$ was ~30% lower in subordinates compared to dominant males (t = 2.81, d = 1.00, p = 0.02), while dominant females had intermediary levels that did not differ from either subordinates (t = 1.11, d = 0.41, p =0.51) or dominant males (t = 1.62, d = 0.58, p = 0.25). Conversely, transcript abundance of *crfb* was ~60% and ~30% higher in subordinates versus dominant males (t = 3.71, d = 1.31, p = 0.002) and dominant females (t = 2.53, d = 0.92, p = 0.04), respectively. Transcript abundance of *crfb* did not differ between dominant males and females (t =1.10, d = 0.39, p = 0.52). No other transcriptional differences were detected across social ranks in the HYP or other regions of the brain (Table 3).

3.4. Variation in scale cortisol content of dominant sexes

3.4.1. Relationship between scale cortisol and social behaviours

Rates of aggression performed against neighbouring conspecifics varied with the interaction between focal fish sex and scale cortisol detection (Fig. 4A; $F_{1,28} = 5.06$, p = 0.03). Dominant females with detectable scale cortisol levels performed $\sim 10 \times$ higher rates of aggression against intruding conspecifics compared to dominant females with undetectable scale cortisol levels (t = 3.03, d = 1.64, p = 0.03). Similar differences based on cortisol detection were not observed in dominant males (t = 0.2, d = 0.11, p = 0.99) and no other behavioural differences were found between fish with detectable and undetectable cortisol levels (Supp. Table 1).

3.4.2. Relationship between scale cortisol and expression of cortisol-related genes

Transcript abundance of *crfb* in the POA (Fig. 4B; $F_{1,27} = 8.46$, p =



Fig. 2. Relative transcript abundance of 11β hsd2 (A), gr1 B), and gr2 C) in the liver of dominant male, dominant female, and subordinate *N. pulcher*. Values are presented as medians and 1st and 3rd quartiles; points represent individual values. Significant differences between groups are indicated using different letters based on post hoc analyses (see Statistical analysis section for further details).

0.007) and HYP (Fig. 4C; $F_{1,28} = 5.02$, p = 0.03) varied with the interaction between sex and cortisol detection. In the POA, dominant females with detectable levels of scale cortisol had ~60% higher transcript abundance of *crfb* compared to females with undetectable cortisol levels (t = 3.52, d = 1.88, p = 0.008), males with undetectable scale cortisol levels (t = 3.23, d = 1.73, p = 0.02), and males with undetectable scale cortisol levels (t = 3.03, d = 1.49, p = 0.03). Similarly, transcript abundance of *crfb* in the HYP of dominant females with detectable levels of scale cortisol was ~50% higher than females with undetectable scale cortisol levels (t = 2.99, d = 1.55, p = 0.03). Females with detectable scale cortisol levels also tended to have higher expression of *crfb* in the

Table 3

Transcript abundance of genes in the livers and brains (preoptic area, hypothalamus, and telencephalon) of dominant male, dominant female, and subordinate helper *N. pulcher*. Data are expressed relative to the mean values for dominant males and are reported as means \pm SEM. Significant differences (p < 0.05) are indicated in **bold** and letters indicate differences between groups based on post hoc analysis.

		Dominant males ($N = 17$)	Dominant females ($N = 15$)	Subordinates ($N = 16$)	F	р	η^2
Liver	11β hsd2	1.00 ± 0.31^{a}	$0.07\pm0.02^{\rm b}$	$0.19\pm0.06^{\rm b}$	24.61	<0.001	0.52
	gr1	$1.00\pm0.06^{\rm a}$	$0.70\pm0.07^{\rm b}$	$0.92\pm0.06^{\rm a}$	6.36	0.004	0.22
	gr2	$1.00\pm0.06^{\rm a}$	$0.72\pm0.06^{\rm b}$	$0.91\pm0.06^{\rm a}$	5.99	0.005	0.21
	mr	1.00 ± 0.06	0.80 ± 0.13	1.12 ± 0.11	2.48	0.09	0.10
Preoptic area	11β hsd2	1.00 ± 0.18	0.99 ± 0.31	0.97 ± 0.19	0.22	0.80	0.01
	crfb	1.00 ± 0.08	1.22 ± 0.12	1.21 ± 0.09	1.71	0.19	0.07
	gr1	1.00 ± 0.05	1.07 ± 0.05	1.03 ± 0.06	0.50	0.61	0.02
	gr2	1.00 ± 0.06	1.07 ± 0.06	1.06 ± 0.05	0.51	0.61	0.02
	mr	1.00 ± 0.06	1.16 ± 0.06	1.12 ± 0.07	1.86	0.17	0.08
Hypothalamus	11β hsd2	$1.00\pm0.11^{\rm a}$	0.79 ± 0.10^{ab}	$0.66\pm0.09^{\rm b}$	4.01	0.03	0.16
	crfb	$1.00\pm0.07^{\rm a}$	$1.17\pm0.12^{\rm a}$	$1.58\pm0.14^{\rm b}$	7.14	0.002	0.24
	gr1	1.00 ± 0.03	1.00 ± 0.03	0.95 ± 0.05	0.66	0.52	0.03
	gr2	1.00 ± 0.03	0.96 ± 0.05	1.01 ± 0.04	0.43	0.65	0.02
	mr	1.00 ± 0.04	0.91 ± 0.04	1.01 ± 0.05	1.50	0.23	0.06
Telencephalon	11β hsd2	1.00 ± 0.10	0.87 ± 0.15	0.82 ± 0.13	1.76	0.18	0.07
	gr1	1.00 ± 0.04	1.05 ± 0.04	0.96 ± 0.04	1.32	0.28	0.06
	gr2	1.00 ± 0.02	0.98 ± 0.03	0.98 ± 0.05	0.07	0.93	0.01
	mr	1.00 ± 0.04	0.89 ± 0.05	1.00 ± 0.05	1.67	0.20	0.07



Fig. 3. Relative transcript abundance of $11\beta hsd2$ (A) and *crfb* (B) in the hypothalamus of dominant male, dominant female, and subordinate *N. pulcher*. Values are presented as medians and 1st and 3rd quartiles; points represent individual values. Significant differences between groups are indicated using different letters based on post hoc analyses (see Statistical analysis section for further details).

HYP compared to males with either detectable (t = 2.57, d = 1.37, p = 0.07) or undetectable scale cortisol levels (t = 2.67, d = 1.32, p = 0.06), but these differences did not reach statistical significance. No other differences in transcript abundance in the brains or livers of fish with detectable and undetectable cortisol levels were observed (Supp. Table 2).

4. Discussion

Variation in glucocorticoid levels across social group members has been widely documented in group-living vertebrates, but no studies on wild animals have evaluated the neuroendocrine factors underlying these differences. Consequently, it has been difficult to determine the functional consequences of differences in glucocorticoid dynamics within social groups. Here, we evaluated the potential behavioural and neuroendocrine factors regulating glucocorticoid dynamics in wild social groups of N. pulcher in Lake Tanganyika, Africa. We found that cortisol levels were higher in dominants of both sexes (~50% detection rate) compared to subordinates (0% detection rate), consistent with the hypothesis that cortisol levels in wild N. pulcher reflect the elevated metabolic costs associated with maintaining dominance (Creel et al., 1996; Goymann and Wingfield, 2004; Romero et al., 2009). However, male dominants and female dominants appeared to differ in the way they adjusted their liver to cope with higher cortisol levels. While dominant females had low hepatic levels of gr1 and gr2 (reducing cortisol sensitivity), dominant males instead had high hepatic levels of 11β hsd2 (increasing cortisol breakdown). Additionally, the factors regulating cortisol dynamics differed between dominant sexes. High cortisol levels in dominant females reflected increased abundance of crfb in the hypothalamic and preoptic areas of the brain (indicative of increased HPI axis activity) and higher rates of aggression towards conspecific territory neighbours, but no clear relationships were observed in dominant males. Overall, our data support rank- and sexbased differences in the behavioural and neuroendocrine regulation of glucocorticoid dynamics in this group-living fish.

While feathers (Bortolotti et al., 2008; Freeman and Newman, 2018) and fur/hair (Davenport et al., 2006; Heimbürge et al., 2019) have been widely used for over a decade to evaluate long-term glucocorticoid dynamics in behavioural/ecological studies, similar techniques for fish have lagged. Recent studies have suggested that scale cortisol content may provide a similar means of monitoring long-term variation in



Fig. 4. Rates of aggression performed towards conspecific neighbours (A), and relative transcript abundance of *crfb* in the preoptic area (B) and in the hypothalamus (C) of dominant male and dominant female *N. pulcher* when scale cortisol content was either detectable (black boxes) or undetectable (white boxes). Values are presented as medians and 1st and 3rd quartiles; points represent individual values. Significant differences between groups are indicated using different letters based on post hoc analyses (see Statistical analysis section for further details).

glucocorticoid dynamics of fish (Gormally and Romero, 2020; Laberge et al., 2019); however, the potential utility of scale cortisol content as a tool for behavioural studies had not been assessed before. Our finding that cortisol was more readily detectable in the scales of dominant versus subordinate *N. pulcher* represents the first evidence that scale cortisol content—an emerging technique for evaluating long-term glucocorticoid dynamics in fishes (Gormally and Romero, 2020; Laberge et al., 2019)—varies with social status, supporting the use of scale cortisol as a tool for future behavioural studies. Consistent with the differences in cortisol levels, we also detected transcriptional differences

across social ranks for several genes involved in regulating the actions of cortisol.

One of the major roles of glucocorticoids is the regulation and mobilization of energy reserves (Tort and Teles, 2011). In N. pulcher, dominants of both sexes have lower energy reserves than subordinates (Culbert et al., 2019a; Hellmann et al., 2016; Sopinka et al., 2009). Consequently, dominants may require mechanisms to minimize the catabolic effects associated with elevated cortisol levels (Charmandari et al., 2005; Mommsen et al., 1999) and prevent complete exhaustion of their already impaired energy reserves (Gregory and Wood, 1999; Madison et al., 2015). Indeed, dominant males and dominant females in the current study both exhibited transcriptional changes consistent with avoiding energy depletion in their livers, which is one of the major targets for the metabolic actions of glucocorticoids (Charmandari et al., 2005; Mommsen et al., 1999). Dominant males displayed higher transcript abundance of hepatic 11\betahsd2 suggesting that their livers may have moderated the effects of elevated cortisol levels through increased cortisol catabolism (Chapman et al., 2013). 11_βHSD2 is thought to function as an intracellular regulator of MR and/or GR activity in teleosts, possibly acting to prevent overstimulation of corticosteroid receptors when cortisol is high (Alderman and Vijayan, 2012; Tokarz et al., 2013). Therefore, dominant males may be utilizing elevated 11BHSD2 to buffer against elevated cortisol levels. Interestingly, dominant females instead had reduced hepatic transcript abundance of the cortisol receptors gr1 and gr2 suggesting reduced sensitivity to cortisol. These findings are similar to the laboratory findings reported in O'Connor et al. (2013), where female N. pulcher had lower hepatic transcript abundance of gr2 and mr compared to males-although, unlike the current study, expression of corticosteroid receptors did not differ between social ranks. However, rank-specific downregulation of hepatic corticosteroid receptor abundance has been observed in laboratory studies of socially subordinate rainbow trout (Oncorhynchus mykiss; Jeffrey et al., 2012) and mice (Mus musculus; Wu et al., 2011), both of which maintain chronically elevated cortisol levels and exhibit a catabolic energy state in their liver (Chuang et al., 2010; Gilmour et al., 2012; Kostyniuk et al., 2018). Thus, reduced hepatic cortisol signaling-whether through reduced receptor abundance or increased cortisol catabolism-appears to be critical for coping with rank-specific elevations of glucocorticoid levels in both the laboratory and field.

While populations of corticotropin-releasing factor (CRF) neurons in the hypothalamus project onto corticotropes (Trudeau and Somoza, 2020), the relative role of these neurons in regulating the HPI axis is thought to be minor (Bernier et al., 2009). Indeed, the transcriptional differences that we observed in the hypothalamus (higher crfb and reduced 11β hsd2 levels in subordinates) appear unrelated to rank-based differences in cortisol dynamics. Instead, these changes may relate to differences in the regulation of food intake between subordinates and dominants, which is centrally regulated by populations of neurons located in the hypothalamus (Anubhuti, 2006; Volkoff, 2016). Both CRF and cortisol are potent inhibitors of food-intake in teleosts (Bernier, 2006; Conde-Sieira et al., 2018; Gregory and Wood, 1999; Madison et al., 2015; Matsuda et al., 2008) and the combination of elevated crfb and reduced 11phsd2 (indicative of decreased cortisol breakdown) suggests that the local actions of CRF and cortisol in the hypothalamus were greater in subordinates. In social groups with size-based dominance hierarchies, subordinates often restrict their growth to remain smaller than dominant individuals (Buston, 2003; Huchard et al., 2016; Thorley et al., 2018; Wong et al., 2008), including in N. pulcher (Heg, 2010; Heg et al., 2004b). This suppression of growth reduces the threat of subordinates physically challenging more dominant group members and reduces intragroup conflict (Ang and Manica, 2010; Hamilton et al., 2005; Wong et al., 2007). In fishes, socially-regulated growth is thought to reflect strategic reductions in food intake by subordinates when they approach the size of dominants (Ang and Manica, 2010; Wong et al., 2008). While it is unknown whether subordinate N. pulcher make similar socially regulated adjustments in food intake, the transcriptional data

presented in the current study are consistent with the strategic suppression of food intake by subordinates.

While dominant males and females displayed similar cortisol levels, they exhibited distinct behavioural phenotypes. Specifically, dominant females spent more time on the group's territory and performed a greater workload than dominant males. In fact, the relative amount of aggression that dominant females performed towards conspecific neighbours was higher in females with detectable levels of scale cortisol suggesting that contributions to territory defense-and perhaps social conflict more generally-may account for much of the variation in glucocorticoid dynamics across dominant females in the wild. Consistent with the apparent positive relationship between social conflict and cortisol levels in N. pulcher, previous work has reported that rates of cortisol excretion were lower in subordinates that were more submissive towards dominant group members in the laboratory (Bender et al., 2006). This relationship does not appear to be a consequence of cortisol directly promoting social conflict because experimentally elevated cortisol levels do not promote aggression in wild subordinate N. pulcher (Culbert et al., 2021). Instead, this relationship likely reflects the metabolic demands associated with agonistic interactions. Even brief agonistic interactions can cause circulating glucocorticoid levels to rise across vertebrates (Elias, 1981; Øverli et al., 1999; Summers et al., 2005) and the performance of agonistic behaviours increases routine metabolic rate by $\sim 4 \times$ in N. pulcher held in the laboratory (Grantner and Taborsky, 1998). In contrast to dominant females, none of the behaviours assessed in the current study explained variation in cortisol levels across dominant males. Dominant males are often polygynous, holding several territories each with their own social group and in general spend less time on any one territory than either dominant females or subordinates (Desjardins et al., 2008a; Wong et al., 2012). In the current study, almost all dominant N. pulcher males observed were polygynous (personal observation; BMC, IYL, MGS, MYLW, and SB) and males spent approximately twice as much time away from a group's territory than either dominant females or subordinates. Accordingly, because males spent less time on the group's territory our observations of dominant male social behaviours were sparse compared to dominant females and subordinates. Therefore, while it is possible that social behaviours are also associated with cortisol dynamics in dominant males, the specific behavioural factors influencing their cortisol levels remain uncertain.

In addition to apparent differences in the behavioural factors influencing glucocorticoid dynamics between dominant males and females, we also noted sex differences in the neuroendocrine regulation of the HPI axis. While crfb mRNA levels were higher in both the preoptic area and hypothalamus of dominant females with detectable scale cortisol levels compared to those in which scale cortisol content could not be detected (indicating elevated HPI axis activity), similar differences were not observed in dominant males. In teleosts, CRF neurons are the primary regulator of HPI axis activity and cortisol synthesis (Bernier et al., 2009); however, several other neuropeptides can also influence HPI axis activity. In mammals, arginine vasopressin (AVP) becomes the primary regulator of HPA axis activity during chronic stress (Aguilera, 1998), but we found no relationship between scale cortisol detection and expression of arginine vasotocin (AVT; the teleost homolog of AVP (Godwin and Thompson, 2012)) in the preoptic area or hypothalamus of either sex (Culbert et al., unpublished data). Additionally, glucocorticoid synthesis can also be modulated directly at the adrenal/interrenal tissue by various hormones (Arnold-Reed and Balment, 1994; Lim et al., 2013; Nussdorfer, 1996), and it is possible that such local regulation assumed a more prominent role in regulating cortisol synthesis in dominant males. Clearly further investigation into possible sex-specific differences in the neuroendocrine regulation of glucocorticoid dynamics is warranted.

In conclusion, we conducted the first assessment of the neuroendocrine mechanisms regulating glucocorticoids in members of wild social groups and report rank- and sex-specific differences in both the behavioural and neuroendocrine factors regulating glucocorticoid dynamics. This work represents an important step in assessing the potential functional consequences of differences in glucocorticoid dynamics within social groups and enhances our understanding of the physiological underpinnings of complex social lifestyles in vertebrates.

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 NAH, 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.

Declaration of competing interest

The authors declare no competing interests.

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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.

Data accessibility

Supporting data can be found in the attached Supplemental file.

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Appendix A. Supplementary data

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