Corticosteroid receptor gene expression is related to sex and social behaviour in a social fish

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A B S T R A C T

Circulating corticosteroids have been related to social status in a variety of species. However, our understanding of corticosteroid receptor expression and its relationship with sociality is still in its infancy. Knowledge of variation in receptor expression is critical to understand the physiological relevance of differences in circulating corticosteroid concentrations. In this study, we examined corticosteroid receptor gene expression in relation to dominance rank, sex, and social behaviour in the highly social cichlid fish, Neolamprologus pulcher. We examined the relative gene expression of the three known teleost corticosteroid receptors: glucocorticoid receptor 1 (GR1), glucocorticoid receptor 2 (GR2), and the mineralocorticoid receptor (MR) in liver and brain tissue of dominant and subordinate N. pulcher males and females. Phylogenetic analysis revealed the N. pulcher gene originally described as GR2, clustered with other teleost GR1 genes, while the originally-described N. pulcher GR1 gene clustered with the GR2 genes of other teleosts. Therefore we propose a change in the original nomenclature of the N. pulcher GRs: GR1 (formerly GR2) and GR2 (formerly GR1) and adopt this new nomenclature throughout this manuscript. Liver MR transcript levels were higher in males than females, and positively related to submissive behaviour. Liver GR2 (formerly GR1) transcript levels were also higher in males than females. Collectively, the results demonstrate sex differences in corticosteroid receptor abundance, and suggest tissue- and receptor-specific roles for corticosteroid receptors in mediating aspects of social behaviour.

1. Introduction

Corticosteroids are involved in a suite of physiological maintenance functions including circadian rhythms, osmotic balance, and energy storage and mobilisation (see reviews by Mommsen et al., 1999; Sapolsky et al., 2000; Romero, 2004; Bonier et al., 2009). When an individual is faced with an acute challenge, circulating corticosteroid levels increase several-fold, and initiate many physiological and behavioural changes that are collectively termed a stress response. These responses promote individual survival during and after exposure to the challenging event (see reviews by Wingfield et al., 1998; Breuner et al., 2008). The corticosteroid response is controlled by negative feedback loops, and circulating corticosteroid levels return to baseline after the challenge subsides (see reviews by Sapolsky et al., 2000; Romero, 2004). However, if challenges are persistent or repeated, circulating corticosteroid levels can become chronically elevated, and are often associated with muscle catabolism, and suppression of immune function and reproduction (see reviews by Greenberg and Wingfield, 1987; Barton, 2002; Moore and Jessop, 2003). The negative feedback loops controlling corticosteroid secretion mean that individuals with chronically elevated corticosteroid levels are likely to have a reduced capacity to mount an acute stress response when faced with an immediate challenge (Sapolsky et al., 2000; Romero, 2004). Thus, circulating corticosteroids can have very different physiological effects depending on circulating concentration (i.e., baseline or stress-induced) and on the duration that a particular circulating level is maintained.

The general patterns of circulating corticosteroids in a social context have recently become more clear (see reviews by Creel, 2001; Goymann and Wingfield, 2004; Young et al., 2006; Schoech et al., 2007; Rubenstein and Shen, 2009). In dyadic aggressive encounters, losers typically display elevated corticosteroid levels relative to winners (see reviews by Gilmour et al., 2005; Sapolsky, 2005; although see Correa et al., 2003; Øverli et al., 1999; Buchner et al., 2004; Earley et al., 2006; Earley and Hsu, 2008 for exceptions). However, in stable social groups, dominance hierarchies readily emerge and within these established hierarchies the patterns of corticosteroid levels among individuals depend on a variety of social factors. In particular, the costs of group life shouldered by different individuals within the group are a reliable predictor of the variation in baseline corticosteroid secretion.
(Creel, 2001; Abbot et al., 2003; Goymann and Wingfield, 2004; Rubenstein and Shen, 2009). Costs of group living include behaviours such as maintaining the group territory, defending the group's territory against conspecific intruders and predators, caring for offspring (by parents and by non-reproductive subordinates), policing or reproductively suppressing other individuals within the group, and/or participating in agonistic interactions that maintain a dominance hierarchy with other group members. The sum of all such costly behaviours contributes to the overall physiological burden for the individual. The physiological cost associated with the adaptation to adverse social and physical situations is termed 'allostatic load' (McEwen and Wingfield, 2003; Goymann and Wingfield, 2004). Within a social group, individuals that bear a high allostatic load tend to display relatively higher baseline circulating corticosteroid levels compared with individuals with a lower allostatic load (Goymann and Wingfield, 2004; Rubenstein and Shen, 2009). Furthermore, subordinate individuals in highly social groups will often have low circulating corticosteroid levels compared to dominants, and this may be because they are able to effectively avoid direct aggression through the use of appeasing submissive displays (Bergmüller and Taborsky, 2005).

The effects of corticosteroids are mediated through intracellular glucocorticoid (GR) and mineralocorticoid receptors (MR) that act as ligand-dependent transcription factors (Mommsen et al., 1999; Prunet et al., 2006; Stolte et al., 2006; Bury and Sturm, 2007). Teleost fish express both GRs and MRs (see reviews by Prunet et al., 2006; Bury and Sturm, 2007) and most fish species examined to date possess two GR isofoms (GR1 and GR2, Greenwood et al., 2003; Bury et al., 2003), with one known exception in zebrafish (Danio rerio), that possess only a single GR (Alsop and Vijayan, 2008). Accumulating evidence indicates that the gene expression of these receptors is influenced by circulating corticosteroid levels. For example, Johansen et al. (2011) recently demonstrated that rainbow trout (Oncorhynchus mykiss) bred for a low post-stress cortisol response exhibited higher brain MR mRNA transcript levels compared to fish bred for high cortisol responsiveness to a stressor. Similar patterns of GR mRNA abundance as a function of dominance rank, sex, and so-called social behaviours and cortisol concentrations, Bender et al. (2013) predicted a general pattern of reduced corticosteroid gene expression in those individuals typically associated with higher circulating corticosteroid levels. As dominant individuals display higher corticosteroid levels than subordinates (Mileva et al., 2009), we predicted that dominant individuals would display lower corticosteroid receptor gene expression than subordinates. Since females typically display more costly maintenance behaviours than males (Balshine et al., 2001; Mileva et al., 2009), we predicted that females would display lower corticosteroid receptor gene expression compared to males. We predicted a negative relationship between corticosteroid gene expression and maintenance behaviours (i.e., parental care and policing behaviours). Since submissive behaviour is related to lower circulating corticosteroid levels (Bender et al., 2006), we predicted a positive relationship between submissive behaviour and corticosteroid receptor gene expression.

2. Materials and methods

2.1. Experimental animals

All fish used in this experiment were adults from a breeding colony of N. pulcher held at McMaster University, Hamilton, Ontario, Canada. Fish were descendants of male and female breeding pairs caught in Lake Tanganyika, Zambia, in 2001 and 2002. Animals were housed in social groups consisting of a male and female dominant breeding pair with 1–20 subordinate helpers. Each social group inhabited a 189 L freshwater tank outfitted with a heater, thermometer, 2 foam filters, ~2 cm of coral sand substrate, a mirror placed at each end of the tank, and two inverted flowerpot halves for use as shelter and a spawning substrate. The light:dark cycle was kept constant at 13:11 h and water temperature was maintained at 26 ± 2 °C. Fish were fed 6 days per week with Nutrafin Basix commercial flake cichlid food.

2.2. Experimental protocol

The social groups used in this study represented a subset of the social groups used in Mileva et al. (2009). In total, n = 30 social groups were observed for Mileva et al. (2009), and n = 7 of these groups were randomly selected for the current study. The dominant breeding pair and the two largest subordinate helpers from each social group (mean group size 10.1 ± 1.5 standard error of the mean [SEM]) were identified through the use of ethograms available for this species (e.g., Buchner et al., 2004; Sopinka et al., 2009). To accurately track individuals during detailed behavioural observations, all focal fish were netted from their home tanks, sexed by examination of external features, weighed, measured (standard length and mass), and uniquely fin-clipped before being returned to their home tank. Fin clipping does not adversely affect behaviour (Stiver et al., 2004). Detailed behavioural observations were carried out 3–7 days following the measurement and fin clipping of focal individuals. Each individual was observed three times, in 10 min intervals between 8:00 and 13:00 h. All behaviours were recorded for each focal individual following the ethogram outlined in Sopinka et al. (2009). Briefly, the behaviours scored were aggressive behaviours, including aggressive displays (head-up posture or frontal displays with the operculum flared), chasing, biting, and ramming (making contact with another
fish with the mouth closed); submissive behaviours, including sub-
misive displays (head-up postures or quivering displays); affiliative
behaviours, including soft touches, parallel swims, or follows (one fish
follows another); parental or alloparental care behaviours, including
visiting the flowerpot halves, micromanipulating eggs (making contact
with the mouth to remove debris from the eggs), defence and guarding of
young, and fanning the eggs; and territory maintenance activities, in-
cluding digging and carrying sand. Additionally, time spent by the
focal fish behind filters and in the flowerpot halves, as well as the fre-
quency with which each individual performed locomotive (swimming,
shing) were recorded.

2.3. Analysis of gene expression by real-time RT-PCR

Brain and liver samples from 28 focal fish (14 dominant and 14
subordinate individuals; Table 1) were individually homogenised in
TRIzol reagent (Invitrogen, Carlsbad, CA, USA) using a 21-gauge need-
le attached to a 3 mL syringe until the mixture could pass easily
through the needle. Total RNA was extracted from homogenised tis-
uce samples using TRIzol reagent according to the manufacturer’s
 specifications. The concentration and quality of RNA were analysed
at a wavelength of 260 nm using a NanoDrop 1000 spectrophotome-
ter (ThermoFisher Scientific, Waltham, MA, USA). Prior to cDNA syn-
thesis, aliquots of 2 μg of RNA were treated twice with ampli
fication (Invitrogen) and ligated into a plasmid (pDrive vector, Qiagen) and amplified in DH5α Escherichia coli compet-
tent cells (Invitrogen) and ligation was confirmed with blue-white
screening. White plasmids were isolated (QIAprep Spin Miniprep kit,
Qiagen) and sequenced.

A 2 μL aliquot of cDNA was used in a 12.5 μL real-time RT-PCR reac-
tion containing forward and reverse primers, and a SYBR green master
mix (Stratagene, Santa Clara, CA, USA). Samples were analysed using
a Mx3000P Real-Time PCR System with associated MxPro 4.01 software
(Stratagene). The composition of the reaction and the settings used
for the thermocycler were those suggested by the manufacturer, with
the exception that the reaction volume was scaled to 12.5 μL from
25 μL. Standard curves were generated for all genes using pooled liver
cDNA to assess the efficiencies of the primer reactions (Table 2). Pooled
samples were serially diluted (1 in 5) in RNase/DNase-free water
(Sigma-Aldrich, St. Louis, MO, USA) for a total of 6 standards. A set
of ‘no reverse transcriptase’ and ‘no template’ control samples was
included in every plate to verify that the generated amplicons did not
originate from genomic contamination. The ‘no reverse transcriptase’
templates were created by omission of reverse transcriptase during
cDNA synthesis. The resulting cycle threshold (Ct) values were linearly
regressed against the relative template concentration and reaction effi-
ciencies were deemed acceptable if they fell in the range 85–115% and
had an R² value of at least 0.97 (Table 2). For purposes of comparison
against social status, mRNA abundance of each focal individual (domi-
nant female, dominant male, subordinate male and subordinate female)
was calculated relative to the mean mRNA abundance of the gene of in-
terest (i.e., the corticosteroid receptor of interest, or the 18S gene) in
the dominant male group. Relative mRNA abundance of the gene of interest
was then expressed against the relative reference gene 18S (template
diluted 1000-fold) and calculated according to Pfaffl’s ΔΔCt method
(Pfaffl, 2001).

Table 1
Sample sizes, masses, and behavioural scores for all focal Neolamprologus pulcher
included in the study. All values are presented as means±standard error of the mean
(SEM). See Materials and methods for an explanation of how the behaviour scores were
 calculated.

<table>
<thead>
<tr>
<th>Status</th>
<th>Sex</th>
<th>Sample size</th>
<th>Mass (g)</th>
<th>Maintenance score</th>
<th>Submission score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dominant</td>
<td>Male</td>
<td>7</td>
<td>10.86±1.15</td>
<td>10.59±1.96</td>
<td>0.00±0.00</td>
</tr>
<tr>
<td>Dominant</td>
<td>Female</td>
<td>7</td>
<td>7.14±0.66</td>
<td>17.05±4.00</td>
<td>1.48±1.04</td>
</tr>
<tr>
<td>Subordinate</td>
<td>Female</td>
<td>6</td>
<td>3.23±0.19</td>
<td>2.95±0.61</td>
<td>1.88±1.50</td>
</tr>
<tr>
<td>Subordinate</td>
<td>Male</td>
<td>8</td>
<td>3.96±0.47</td>
<td>2.17±0.70</td>
<td>2.33±0.99</td>
</tr>
</tbody>
</table>

Table 2
Forward (F) and reverse (R) real-time RT-PCR primers used to analyse corticosteroid receptor gene expression in Neolamprologus pulcher brain and liver. All sequences are listed in
the 5′–3′ direction. Reverse primers (R) are listed as the reverse complement sequence of the original DNA template. GenBank accession numbers are provided for the sequences against which the primers were designed.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Accession #</th>
<th>Primer</th>
<th>Sequence 5′–3′</th>
<th>Efficiency of real-time RT PCR reaction (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GR1</td>
<td>EF661652</td>
<td>F</td>
<td>GCC CCA GCC CCC ACC ATT AGC AAC AT</td>
<td>112.4</td>
</tr>
<tr>
<td></td>
<td>(formerly GR2)</td>
<td>R</td>
<td>CTT GCC CCA CTC GAC TGC AGA CAC A</td>
<td></td>
</tr>
<tr>
<td>GR2</td>
<td>EF661651</td>
<td>F</td>
<td>TGC GCT TGT CAG TGC CAC CAG GT</td>
<td>113.1</td>
</tr>
<tr>
<td></td>
<td>(formerly GR1)</td>
<td>R</td>
<td>AGT CGT CTT CAG AAG TAA GT</td>
<td></td>
</tr>
<tr>
<td>MR</td>
<td>EF661650</td>
<td>F</td>
<td>GGC CTC TAA CGG TGG CCA AAC AT</td>
<td>110.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R</td>
<td>CAC ATG GAG GCC AGA AAA GGT</td>
<td></td>
</tr>
<tr>
<td>18S</td>
<td>AF337051</td>
<td>F</td>
<td>ATG GCC CTT CAG TGC TG</td>
<td>103.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R</td>
<td>CTC AAT CTC GTC TGT CGT AA</td>
<td></td>
</tr>
</tbody>
</table>
2.4. Sequence analysis

Teleost corticosteroid receptor sequences were retrieved from the National Center for Biotechnology Information (NCBI) nucleotide database (http://www.ncbi.nlm.nih.gov/nucleotide/) through a series of BLASTN searches using the N. pulcher GR1 (GenBank Accession # EF661651.1), GR2 (EF661652.1), and MR (EF661650.1) as queries. Sequences from the following species were collected from the resulting BLASTN queries: Astatotilapia burtoni (GR1 AF263738.1, GR2a AF263739.1, GR2b AF263740.1, MR AF263741.1), Cyprinus carpio (GR1, GR2 a1 AF218424.1, GR3 EF436284.1, MR EF436284.1), Dicentrarchus labrax (GR AM549305.1), Oncorhynchus mykiss (GR1, NM_001124730.1, GR1, Z52410.1, GR2 AY495372.1, MR AY495584.1, MR AY495585.1), Opsanus beta (GR HQ424878.1), Oreochromis mossambicus (GR GU296354.1, MR HM769956.1), Oreochromis niloticus (GR XM_003445902.1, MR XM_003449675.1), Oryzias dancena (GR1, HM598068.1, GR2, HM598069.1), Oryzias latipes (GR AB284183.1, MR AB284184.1), Paralichthys olivaceus (GR AB013444.1), Parichthys notatus (GR EF902836.2, GR2, HM164445.1, MR GU384923.1), Salmo trutta (GR AY836149.1, MR EF859777.1), and Sparus aurata (GR DQ486890.1). Additional corticosteroid receptor sequences were identified in the protein ENSEMBL protein database (http://www.ensembl.org/index.html) and sequences were retrieved for the following species: Gasterosteus aculeatus (GR1, ENSGACT00000027452, GR1, ENSGACT00000027453, MR ENSGACT000000022752), Gadus morhua (GR1 ENSCMOT00000006200, GR2 ENSCMOT000000195605), Takifugu rubripes (GR1, ENSTRUC00000006599, GR1, ENSTRUC00000015715, GR2 ENSISTRUT00000018489, MR ENSISTRUT00000035810), and Tetrodorn nigroviridis (GR1 ENSINT00000021610, GR2 ENSINT0000001990, MR ENSINT0000002111). All nucleotide sequences were aligned using ClustalW (http://www.ebi.ac.uk/Tools/msa/clustalw2/). Following the alignment, the sequences were manually truncated to the ligand-binding domain of the receptor, covering 1032 characters: the region of the receptor that encompasses the partial sequences of all N. pulcher corticosteroid receptors. A phylogenetic tree was created by means of the maximum likelihood method based on the Tamura 3-parameter model (Tamura, 1992) using Mega 5.05 software (Tamura et al., 2011). Gene clusters were analysed through bootstrap analyses using 1000 pseudo-replicates (Felsenstein, 1985). All analyses were carried out using the Petromyzon marinus corticosteroid receptor (AY028457.1) as an outgroup.

2.5. Statistical analysis of the patterns corticosteroid receptor gene expression

Two behavioural measures were calculated for each focal individual, based on the three 10-min behavioural observations per individual. First, a measure of costly maintenance behaviour (‘maintenance score’) was calculated as the sum of parental or alloparental care behaviour (maintenance of brood chamber and eggs) and policing behaviour (the number of aggressive acts directed towards other social group members). Second, a measure of submissive behaviour (‘submission score’) was calculated as the sum of the submissive acts performed by each individual in response to an aggressive act received from any social group member (see Table 1).

General linear models were used to examine the relationship between dominance rank (dominant or subordinate), sex (male or female) and social behaviour (maintenance score, submission score) and relative corticosteroid receptor gene expression. For all models, source tank (i.e., the individual’s social group) was included as a random effect to account for non-independence of individuals within a given social group (Briffa and Elwood, 2010). Variables were scaled by their standard deviation and centered by their means to make estimates comparable for all model terms (Schielzeth, 2010). In all cases, $\alpha = 0.05$. Unless otherwise noted, values are presented as mean ± 1 standard error of the mean (SEM). All analyses were performed using R version 2.14.0.

3. Results

3.1. Phylogenetic analysis

The maximum likelihood tree revealed that teleost corticosteroid receptors fall into three main gene clusters: GR1, GR2 and MR. The gene tree also revealed that the N. pulcher gene originally described as GR1 (EF661651.1) by comparison with the sequences of another cichlid Astatotilapia burtoni, reported by Greenwood et al. (2003), had a greater sequence similarity to other piscine GR2 isoforms, and the GR2 isoform of N. pulcher (EF661652.1) clustered with the GR1 isoforms of other teleosts (Fig. 1). The clustering of MR genes was more straightforward. The N. pulcher MR sequence showed a strong sequence similarity to other piscine MRs and grouped within the MR gene cluster (Fig. 1). Bootstrap analysis of the phylogenetic tree revealed only weak statistical support for some of the nodes, likely because these analyses were undertaken using sequences that covered the ligand-binding domain of the corticosteroid receptor genes, which covers approximately 35% of the full-length sequence. Greater statistical support would be expected if full sequences were available for all genes from all species.

3.2. Patterns in corticosteroid receptor gene expression

None of the corticosteroid receptor gene expression values mapped onto social status (Tables 3 and 4). However, sex was significantly correlated to corticosteroid receptor relative gene expression in liver tissue (Tables 3 and 4) with female fish displaying lower liver MR relative transcript levels (Fig. 2a) and lower liver GR2 (formerly GR1) relative transcript levels than male fish (Fig. 2b). Submission scores were positively correlated with liver MR relative gene expression (Fig. 3). There was no influence of any of the measured parameters on liver GR1 (formerly GR2) relative gene expression (Table 4), or on any of the brain corticosteroid receptor gene expression levels (Table 4).

4. Discussion

In the current study, we confirmed that a change in nomenclature is necessary for N. pulcher glucocorticoid receptors. We further documented differences in corticosteroid gene expression patterns in relation to sex and social behaviour in this cooperatively breeding teleost fish.

4.1. What can we learn from the phylogenetic analysis?

The maximum likelihood tree revealed that the N. pulcher gene originally described as GR1 (EF661651.1) had a greater sequence similarity to other piscine GR2 isoforms, while the GR2 isoform of N. pulcher (EF661652.1) clustered with GR1 isoforms. Maruska and Fernald (2010) documented the same pattern in A. burtoni, and Arbeyr and colleagues also detected mismatches using partial sequences for N. pulcher corticosteroid receptors (Arbeyr et al., 2011). Maruska and Fernald (2010) suggested a change in terminology of the two GR receptors for A. burtoni, and we similarly suggest this change in nomenclature for the N. pulcher corticosteroid receptors: GR1 (formerly GR2; EF661652.1) and GR2 (formerly GR1; EF661651.1). In this manuscript, we adopt the new consistent nomenclature for these GR genes. The clustering of MR genes was more straightforward, and the N. pulcher MR sequence showed a strong sequence similarity to other piscine MRs and grouped within the MR gene cluster. Our data indicate that a more rigorous analysis of corticosteroid receptor evolution in fish using longer
Fig. 1. A phylogenetic tree illustrating the relationship between the *Neolamprologus pulcher* glucocorticoid receptor isoforms (GR1 and GR2; highlighted in black) and mineralocorticoid receptor (MR; highlighted in black). The phylogenetic analysis was carried out on partial sequences covering 1032 nucleotide characters (representing the ligand-binding domain of the receptors) and was created using maximum likelihood methods based on the Tamura 3-parameter model with the *Petromyzon marinus* corticosteroid receptor (CR) as the outgroup. The branch lengths are scaled to represent the relative number of substitutions occurring along each branch. The statistical support for the nodes is indicated as percentage obtained from bootstrap analysis using 1000 pseudoreplicates. Bootstrap values below 50% are absent from the tree, denoting poor branch support. Sequences for the piscine GRs, MRs, and CRs were obtained from GenBank and Ensembl genomes (see Materials and methods for accession numbers and ENSEMBL IDs).
sequences from more species would be a valuable contribution to the literature on corticosteroid receptors.

4.2. What can we learn from the patterns of corticosteroid receptor gene expression in relation to social status, sex, and social behaviour?

As predicted, we documented lower liver MR and GR2 (formerly GR1) gene expression levels in females than males. This finding is consistent with the differences in maintenance behaviours (i.e., parental care, defence behaviours) between males and females (Balshine et al., 2001; Desjardins et al., 2008; Mileva et al., 2009), with females performing more of these behaviours. This may also be explained by differences in other aspects of physiology between males and females. For example, the physiological systems that control the production and secretion of corticosteroids also influence, and are influenced by, the processes controlling the production and secretion of reproductive hormones (Greenberg and Wingfield, 1987; Moore and Jessop, 2003; Fuzzen et al., 2011). It is therefore conceivable that differences between males and females in corticosteroid receptor gene expression reflect interactions between the pathways controlling stress responses and reproduction. This study is among the first to examine sex effects in

Table 3
Corticosteroid receptor relative gene expression for the Neolamprologus pulcher focal individuals included in the current study. Individual values are relative to the mean value for dominant males, and then corrected for 18S abundance. All values are presented as means ± standard error of the mean (SEM).

<table>
<thead>
<tr>
<th>Status</th>
<th>Sex</th>
<th>Liver relative gene expression</th>
<th>Brain relative gene expression</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>GR1 (formerly GR2)</td>
<td>GR2 (formerly GR1)</td>
</tr>
<tr>
<td>Dominant</td>
<td>Male</td>
<td>2.49±1.20</td>
<td>3.12±1.36</td>
</tr>
<tr>
<td>Dominant</td>
<td>Female</td>
<td>1.11±0.15</td>
<td>1.01±0.06</td>
</tr>
<tr>
<td>Subordinate</td>
<td>Male</td>
<td>1.04±0.19</td>
<td>1.76±0.38</td>
</tr>
<tr>
<td>Subordinate</td>
<td>Female</td>
<td>2.32±1.29</td>
<td>1.17±0.18</td>
</tr>
</tbody>
</table>

For example, the physiological systems that control the production and secretion of corticosteroids also influence, and are influenced by, the processes controlling the production and secretion of reproductive hormones (Greenberg and Wingfield, 1987; Moore and Jessop, 2003; Fuzzen et al., 2011). It is therefore conceivable that differences between males and females in corticosteroid receptor gene expression reflect interactions between the pathways controlling stress responses and reproduction. This study is among the first to examine sex effects in

Fig. 2. Liver (A) mineralocorticoid receptor (MR) and (B) glucocorticoid receptor 2 (GR2; formerly GR1) rank-transformed relative gene expression as a function of sex and status in Neolamprologus pulcher. Sex was significantly correlated with both liver GR2 and MR abundance (p<0.05). For full statistical details, see Table 4.
corticosteroid receptor gene expression levels in teleost fishes, and the difference between the sexes that we document warrants further investigation.

As predicted, submissive behaviour was positively correlated with liver MR gene expression. In *N. pulcher*, subordinate individuals can appease dominant individuals by exhibiting a high degree of submissive behaviour (Bermüller and Taborsky, 2005), and the most submissive individuals also display the lowest circulating corticosteroid levels (Bender et al., 2006). Submissive behaviour may serve as a behavioural mechanism for subordinates to avoid aggressive interactions and thereby reduce their circulating corticosteroid levels. There is some evidence to support this idea. For example, in rats and mice, increased MR activation in the brain reduces anxiety-like behaviour (Herrero et al., 2006; Rozeboom et al., 2007), which is consistent with a general pattern of increased submission and reduced anxiety with increasing receptor density and/or sensitivity. Although the pattern is compelling, there are still clear knowledge gaps, particularly since in the current study we documented differences in liver MR gene expression, rather than brain MR gene expression. Explicit experimental manipulation of receptor sensitivity and measurement of submissive and aggressive behaviour in dominant and subordinate individuals is now warranted to further elucidate the links among behaviour, circulating cortisol concentrations and corticosteroid receptor gene expression.

We did not find lower gene expression levels in dominant relative to subordinate individuals, or the expected negative relationship between corticosteroid receptor gene abundance and costly workload behaviours. It is possible that circulating cortisol levels and corticosteroid receptor gene abundance reflect different timescales of stress physiology. Circulating cortisol levels generally provide a snapshot of an individual’s current state, while corticosteroid receptor gene abundance reflects an individual’s response to the current state, and preparation for future state. Finally, a lack of information on the relationship between receptor relative gene expression and protein level limits interpretation of our data. In the absence of receptor-specific antibodies, receptor relative gene expression was measured in the present study as the closest proxy to the levels of protein present in the tissues of interest. However, researchers who have simultaneously compared gene expression to functional protein levels have not always found a perfect correlation between the two (see review by Greenbaum et al., 2003). A better understanding of receptor physiology and specifically the relationship between gene expression and protein levels would shed further light on the functional significance of our findings.

4.3. Why are there differences among tissues and receptors?

The teleost GRs and MR differ in molecular sequence, ligand affinity, transactivation properties and tissue distribution (Stolte et al., 2006; McCormick et al., 2008; Arterbery et al., 2011). In *N. pulcher*, GR1 (formerly GR2) is cortisol-specific, while both GR2 (formerly GR1) and MR are activated by both aldosterone and cortisol (Arterbery et al., 2011). It has been suggested that GR2 is activated by baseline cortisol concentrations, while GR1 is more involved in the cortisol stress response (see review by Prunet et al., 2006). In rainbow trout, transactivation assays revealed differences between GR1 and GR2 in sensitivity to cortisol, with GR2 being more sensitive to lower cortisol concentrations (Bury et al., 2003). In *A. burtonii*, a cichlid species closely related to *N. pulcher*, transactivation assays did not reveal any differences in sensitivity to cortisol between GR1 and GR2, but the response of GR1 (formerly GR2; Maruska and Fernald, 2010) to cortisol was greater than the response of GR2 (formerly GR1) (Greenwood et al., 2003). Thus, it is possible that differences between tissues and receptors reflect differences in sensitivity or response to baseline versus stress-induced levels of circulating cortisol. Additionally, MRs in teleost fish are likely activated by cortisol (McCormick et al., 2008), but the specific function(s) of MR remain uncertain. However, without further research into the specific function and sensitivity of these receptors in different tissues, this suggestion remains speculative.

Corticosteroid receptor gene expression was examined in brain tissue because the differences observed in social behaviour across social ranks are generated by the brain, with its capacity to centrally integrate external and internal information. However, we found no differential patterns in corticosteroid receptor gene expression in brain tissue. Specific brain regions have been associated with social behaviour (Goodson, 2005; O’Connell and Hofmann, 2012), and in future studies, examination of specific brain regions, particularly those associated with social behaviour, may prove more informative than whole brain homogenates. We also examined transcript abundance in the liver because it plays a key role in growth and metabolic responses to stress, both of which differ between dominant and
subordinate *N. pulcher* (Taborsky, 1984; Mileva et al., 2009; Sopinka et al., 2009). Given that glucocorticosteroids play a role in metabolism, we expect that differences in CR and GR2 gene expression in the liver may reflect differences in metabolic requirements among individuals. Studies examining how metabolic costs and specific receptor gene expression change during social ascent (see Balshine and Buston, 2008) would be useful in confirming this supposition. Such studies would also help to disentangle the direct and indirect influences of social status on corticosteroid receptor gene expression, and would broaden our understanding of the differences between receptor gene expression patterns in different tissues.

5. Conclusions

This study expands our understanding of the relationships among teleost corticosteroid receptors, and aligns the nomenclature for *N. pulcher* glucocorticoid receptor isoforms with that of other teleost fish. Furthermore, this study presents the first exploration of corticosteroid receptor expression patterns in a cooperatively breeding species, and documents differences in liver MR and GR2 gene expression in relation to sex, as well as differences in liver MR gene expression in relation to submissive behaviour. While interpretation of these results is currently constrained by our limited understanding of the differences in function and sensitivity of the corticosteroid receptors in fishes, this study contributes to our understanding of the role that the corticosteroid system plays in social systems, and highlights profitable areas for future research.

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