© 2014. Published by The Company of Biologists Ltd | The Journal of Experimental Biology (2014) 217, 3221-3228 doi:10.1242/jeb.104430

RESEARCH ARTICLE

Social regulation of cortisol receptor gene expression

Wayne J. Korzan, Brian P. Grone and Russell D. Fernald*

ABSTRACT

In many social species, individuals influence the reproductive capacity of conspecifics. In a well-studied African cichlid fish species, *Astatotilapia burtoni*, males are either dominant (D) and reproductively competent or non-dominant (ND) and reproductively suppressed as evidenced by reduced gonadotropin releasing hormone (GnRH1) release, regressed gonads, lower levels of androgens and elevated levels of cortisol. Here, we asked whether androgen and cortisol levels might regulate this reproductive suppression. *Astatotilapia burtoni* has four glucocorticoid receptors (GR1a, GR1b, GR2 and MR), encoded by three genes, and two androgen receptors (ARα and ARβ), encoded by two genes. We previously showed that ARα and ARβ are expressed in GnRH1 neurons in the preoptic area (POA), which regulates reproduction, and that the mRNA levels of these receptors are regulated by social status. Here, we show that GR1, GR2 and MR mRNAs are also expressed in GnRH1 neurons in the POA, revealing potential mechanisms for both androgens and cortisol to influence reproductive capacity. We measured AR, MR and GR mRNA expression levels in a microdissected region of the POA containing GnRH1 neurons, comparing D and ND males. Using quantitative PCR (qPCR), we found D males had higher mRNA levels of ARα, MR, total GR1a and GR2 in the POA compared with ND males. In contrast, ND males had significantly higher levels of GR1b mRNA, a receptor subtype with a reduced transcriptional response to cortisol. Through this novel regulation of receptor type, neurons in the POA of an ND male will be less affected by the higher levels of cortisol typical of low status, suggesting GR receptor type change as a potential adaptive mechanism to mediate high cortisol levels during social suppression.

KEY WORDS: Cichlid, Glucocorticoid receptors, Social stress, Androgen receptors

INTRODUCTION

Social status hierarchies are a ubiquitous organizing principle of social systems in many animal species from ants (Wilson, 2000) to primates (Cheney and Seyfarth, 1990), including humans (Chiao et al., 2009). Such hierarchies typically regulate access to resources including territories, food and/or mates, granting individuals of high status access to these resources and requiring those of low status to find alternative solutions to survive. In many species, the reproductive capacity of low status individuals is reduced and mating is not possible because of behavioral and physiological changes (e.g. Willisch et al., 2012). Typically, aggressive encounters establish and maintain social rank, which, in turn, produces significant differences in reproductive capacity. In such social systems, levels of stress and reproductive hormones directly reflect the social rank of individuals and may play a causal role in behavioral and physiological changes.

The relationship between social dominance and reproductive physiology has been well studied in an African cichlid fish, *Astatotilapia burtoni* (Günther 1894), in which status regulates both reproductive access and competence. In *A. burtoni*, there are two types of adult males: those with and those without territories (Fernald, 1977). Dominant (D) males are brightly colored, whereas non-dominant (ND) males are cryptically colored, making them difficult to distinguish from the substrate and from females, which are similarly camouflaged. In their natural habitat, the shallow shore pools and river estuaries of Lake Tanganyika (Fernald and Hirata, 1977a; Coulter, 1991), *A. burtoni* live in a lek-like social system in which D males vigorously defend contiguous territories (Fernald and Hirata, 1977a; Fernald and Hirata, 1977b). This social system exerts potent control over the reproductive capacity of ND animals (Davis and Fernald, 1990; Francis et al., 1993). As is typical of many social hierarchies, D males have high levels of testosterone and low levels of the stress hormone cortisol while these levels are reversed in ND males (Fox et al., 1997). However, social status is not static and status reversals produce concomitant changes in reproductive capacity (Francis et al., 1993; Hofmann et al., 1999).

The stress response to social subordination inhibits the reproductive axis in many species and results in chronic elevation of stress hormones, which, if sustained, is generally considered detrimental (Webster et al., 2008; Kaplan and Manuck, 2004; Kaplan, 2008). Thus, it is a puzzle how socially suppressed individuals survive chronic elevation of glucocorticoid levels until an opportunity for social ascent occurs. One possible mechanism for modulating the responsiveness of non-dominant individuals to stress would be to change the amount and/or sensitivity of the cortisol receptors that mediate cortisol's potentially damaging effects on the body (Avitsur et al., 2001) and brain (Sapolsky, 1996).

Localizing androgen and cortisol receptors and quantifying their expression patterns is essential for discovering what role these hormones might play in social regulation of reproduction. In *A. burtoni* males, circulating testosterone is significantly more abundant than 11-ketotestosterone, a teleost-specific androgen, but D males have significantly higher levels of both hormones than ND males (Parikh et al., 2006a). *Astatotilapia burtoni* has two androgen receptors (ARs), ARα and ARβ (Harbott et al., 2007). *Astatotilapia burtoni* ARα is part of the ARa/AR1 group in teleosts, which has been shown to have a higher affinity for testosterone than 11-ketotestosterone (Pasmanik and Callard, 1988; Pottinger, 1987; Slater et al., 1995). In contrast, ARβ has been shown to have a higher affinity for 11-ketotestosterone than testosterone in other fish (Olsson et al., 2005).

Astatotilapia burtoni, like many other teleost species, has three receptor genes for cortisol: two glucocorticoid receptors (GR) and a mineralocorticoid receptor (MR) (Greenwood et al., 2003) [note that the nomenclature of the two GR genes has been changed since the original report (Greenwood et al., 2003) to be consistent with GR genes subsequently cloned from other species; i.e. GR1 is now GR2

Department of Biology, Neuroscience Program, Stanford University, Stanford, CA 94305, USA.

*Author for correspondence (rfernald@stanford.edu)

Received 24 February 2014; Accepted 18 June 2014

The Journal of Experimental Biology (2014) doi:10.1242/jeb.104430

and vice versa]. In mammals, MR binds cortisol and aldosterone equally (Funder et al., 1988); however, teleost fish do not produce aldosterone and MR is highly responsive to cortisol and may act as a cortisol or 11-deoxycortiscosterone receptor *in vivo* (Sturm et al., 2005).

One of the teleost GR genes (GR1) has two splice variants, GR1a and GR1b. These forms differ by a nine-amino acid insertion in the DNA-binding domain in GR1b (Greenwood et al., 2003; Stolte et al., 2006). This splice insertion has a strong influence on the transcriptional response to receptor binding. GR1b has been shown to act as a dominant negative inhibitor of transcription in zebrafish (Schaaf et al., 2008) and it drives significantly less reporter gene activity than GR1a in *A. burtoni* (Greenwood et al., 2003).

Here, we asked whether these steroid receptors might influence reproductive capacity in *A. burtoni*. Reproduction is controlled in *A. burtoni*, as in all vertebrates, by the hypothalamic preoptic area (POA) of the brain by gonadotropin releasing hormone (GnRH1)-containing neurons, named because their main product is the GnRH1 decapeptide. This peptide is delivered to the pituitary gland, where it causes the release of hormones that regulate the gonads. As these neurons are the critical control point through which the brain signals the gonads, we wanted to understand what hormonal signals might influence their activity. We previously used *in situ* hybridization to show that androgen receptors are expressed in GnRH1 neurons (Harbott et al., 2007), and here, we used this technique to localize GR and MR. To quantify effects, we used quantitative PCR (qPCR) on microdissected regions of the POA containing GnRH1 neurons to determine whether expression levels of the steroid receptor genes were changed in ND males.

RESULTS

Behavior

To quantify behavioral effects, the dominance index (DI) was calculated as the sum of the number of aggressive acts minus the number of submissive acts that occurred during a given observation period (White et al., 2002). DIs were averaged over the number of days an animal spent in one social setting, as were the number of reproductive displays. These daily mean values were used for comparison. As anticipated from previous studies (White et al., 2002), the DI of D males was significantly higher than that of ND males (N=17, t=6.476, P<0.0001; data not shown).

Steroid hormone levels

The levels of circulating hormones in males were compared as a function of social status. As expected, circulating cortisol levels were significantly elevated in ND males compared with D males (N=17, t=–3.181, P<0.006; Fig. 1). Circulating testosterone and 11-ketotestosterone levels were significantly higher for D males compared with ND males (N=18, t=6.317, P<0.0001 and N=15, t=5.717, P<0.0001, respectively; Fig. 1).

GnRH1 and GR/MR double *in situ* hybridization

We next performed double *in situ* hybridization to ask whether GR1, GR2 and/or MR were expressed within GnRH1-expressing neurons. Both GR1 and MR were highly expressed within the POA and also specifically within GnRH1 neurons (Fig. 2). GR2 was also expressed in the POA, but was missing or expressed only at very low levels within GnRH1 neurons (Fig. 2).

GnRH1 mRNA levels

GnRH1 mRNA levels in the microdissected POA were significantly higher in D males compared with ND males (N=13, t=–5.11, P<0.0001; Fig. 3), consistent with previous data from whole-brain analyses (Au et al., 2006) and from dissection of larger brain regions (Burmeister et al., 2007).

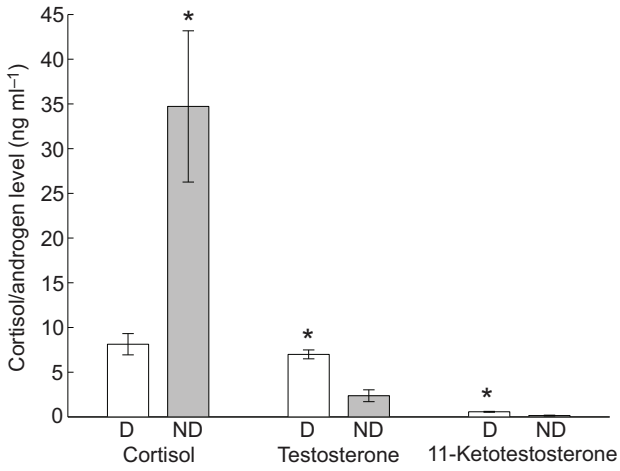

Fig. 1. Cortisol and androgen levels depend on social status. Non-dominant (ND) males have higher cortisol levels compared with dominant (D) males (P<0.006). In contrast, androgens [testosterone (P<0.0001) and 11-ketotestosterone (P<0.0001)] are significantly higher in D males compared with ND males.

Androgen receptors

ARα mRNA levels in the POA were significantly elevated in D males compared with ND males (N=11, t=2.705, P<0.024; Fig. 3) while ARβ mRNA levels in the POA were similar for D and ND males (Fig. 3).

Glucocorticoid and mineralocorticoid receptors

Dominant males had significantly higher levels of MR mRNA levels in the POA compared with ND males (N=11, t=5.885, P<0.0001; Fig. 4). GR2 mRNA was also significantly higher in D males (N=11, t=2.183, P<0.045). D males had significantly higher levels of GR1(a+b) mRNA expression in the POA compared with ND males as assessed by qPCR (N=10, t=3.546, P<0.002; Fig. 5). We used a primer situated within the splice insertion to selectively amplify GR1b, which revealed that GR1b is more highly expressed in ND males than in D males compared with all GR1 mRNA (Fig. 5). Because of individual variability of GR1b levels in males (especially ND males), the ratio of GR1b to all of GR1 was calculated to assess within each individual the potential contribution of splice variant GR1b (GR1b/GR1a+b, N=12, t=2.288, P<0.035; Fig. 5).

DISCUSSION

Androgen, glucocorticoid and mineralocorticoid receptors

Reproductive and stress hormones signaling through their receptors are important regulators of many behaviors and more generally for homeostatic balance. Mathematical models incorporating feedback and feedforward regulation of gene expression by high- and low-affinity nuclear cortisol receptors support the idea that these effects improve network robustness in the face of moderate-frequency stressors (Kolodkin et al., 2013). In *A. burtoni*, previous work has established that the mRNA levels of several receptors, including the main steroid receptors, are regulated by social status in distinct brain regions as well as in the pituitary and testes (reviewed in Maruska and Fernald, 2014). In the POA, ARα, ARβ and estrogen receptors ERβa and ERβb have all been shown to be upregulated in D males

The Journal of Experimental Biology (2014) doi:10.1242/jeb.104430

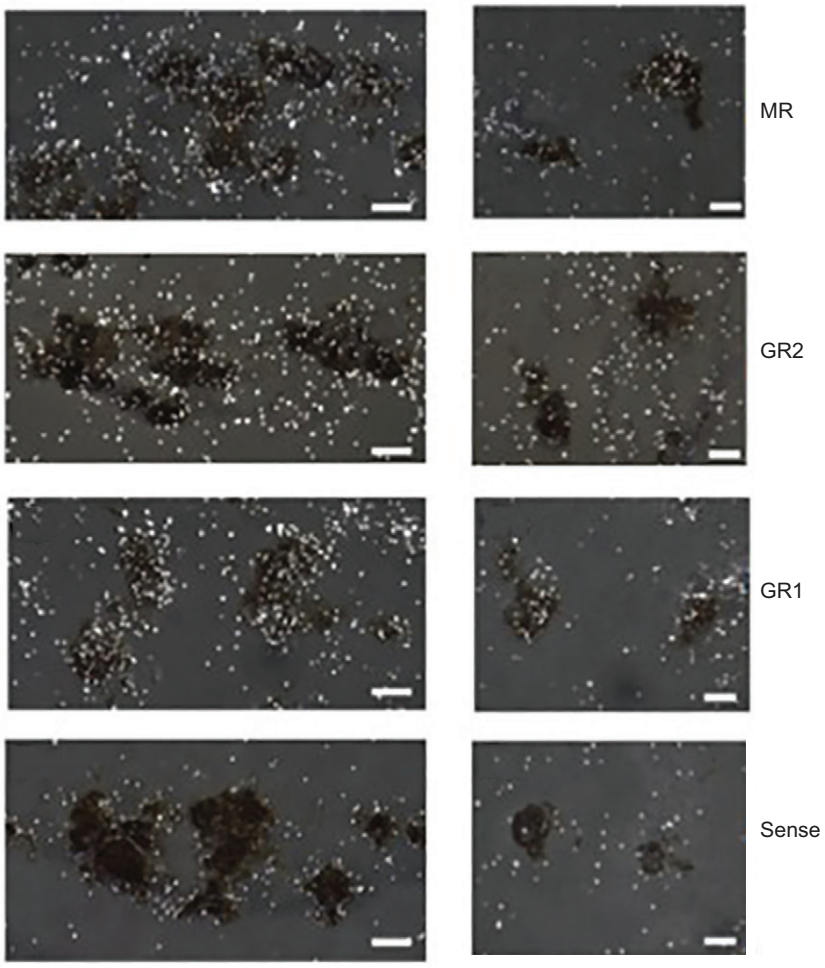

Fig. 2. Cortisol receptor expression within gonadotropin releasing hormone (GnRH1) neurons. Photomicrographs of the preoptic area (POA) containing diaminobenzidine (DAB)-labeled GnRH1 neurons (brown). Left and right panels show separate locations in the field. The receptors responsive to cortisol are labeled by *in situ* hybridization using ^{3}H-thymidine where silver grains show double label with mineralocorticoid receptor (MR) and glucocorticoid receptor GR1, but not GR2. *In situ* labeling with a sense probe for GR1 was used as a control. Scale bars, 20 µm.

relative to the levels found in ND males. This regulatory action extends to GnRH1 and its receptors, suggesting it is an integral part of the mechanisms through which animals respond to changes in social status. The data presented here are an extension of the general principle of receptor regulation as we show that the relative abundance of a particular GR receptor subtype can also be altered as a function of social status.

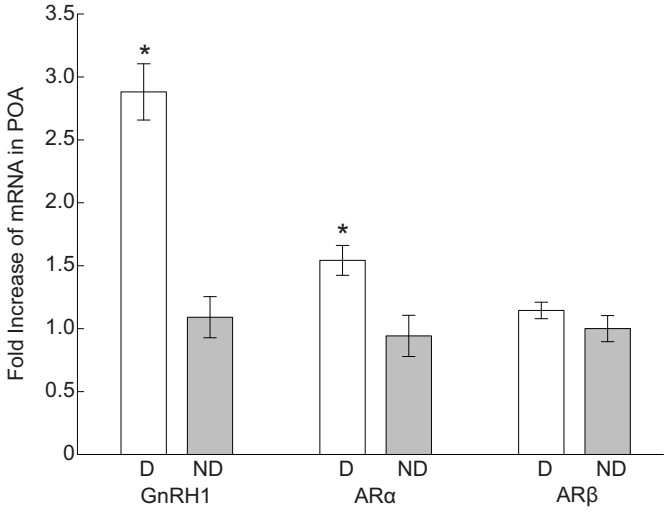

Fig. 3. GnRH1 and androgen receptor mRNA levels in microdissected POA regions. D males have significantly higher levels of GnRH1 (P<0.0001) and androgen receptor ARα mRNA compared with ND males (P<0.024), but ARβ mRNA levels do not differ. mRNA levels are normalized to those of housekeeping genes (see Materials and methods) and given as fold increases.

Glucocorticoid and mineralocorticoid receptor expression in the POA

We found GR1, GR2 and MR mRNA are expressed in the POA both using *in situ* hybridization and by qPCR. In addition, GR1 and MR are expressed in GnRH1 neurons, as revealed by double *in situ* hybridization, and are thus poised to play a direct role in the regulation of GnRH1 neurons by cortisol as a function of social status.

Using microdissected POA regions, we found that MR levels are higher in D males compared with ND males. A clearly defined role for MR in fish remains elusive. In vertebrates, cortisol binds and activates both GR and MR, but MR has 10 times higher sensitivity to cortisol than GR and can also be activated by aldosterone (Bury and Sturm, 2007; Krozowski and Funder, 1983). Plasma aldosterone levels are usually lower than those of cortisol in tetrapods such as humans (Baker et al., 2007). In contrast, in all teleosts tested to date, aldosterone levels are at or close to non-detectable levels (Prunet et al., 2006). It has been proposed that MRs are occupied by cortisol under basal conditions for both tetrapods and fish (Bury and Sturm, 2007; Krozowski and Funder, 1983). Sturm et al. (Sturm et al., 2005) have shown that an alternative glucocorticoid, 11-deoxycorticosterone (DOC), exhibits a higher affinity for MR compared with cortisol. DOC levels circulating in teleosts are comparable to resting cortisol levels and a potential role of DOC signaling through MRs has been proposed to counter increased

The Journal of Experimental Biology (2014) doi:10.1242/jeb.104430

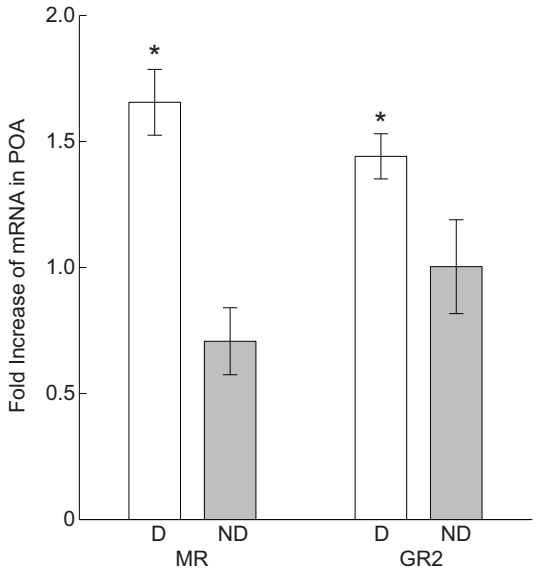

Fig. 4. MR and GR2 mRNA levels in microdissected POA regions. Expression levels of MR (P<0.0001) and GR2 (P<0.045) mRNA levels, normalized to those of housekeeping genes (see Materials and methods) and given as fold increase, are elevated in D males compared with ND males.

cortisol levels following stressful events (Sturm et al., 2005). MRs are thought to be occupied by cortisol (or another glucocorticoid like DOC) under basal conditions and in theory could decrease the signaling of cortisol through GRs. In the case of D males, an increase of MR levels in the POA with low levels of cortisol available could increase neuronal glucocorticoid signaling compared with ND males, possibly during transient increases caused by fighting.

mRNA levels of MR in the brain of juvenile rainbow trout are higher than in any other tissues measured using RT-PCR (Sturm et al., 2005). Recent work by Johansen et al. (Johansen et al., 2011) showed that MR mRNA levels are elevated in the hypothalamus of rainbow trout that have been selectively bred for low cortisol response to stress compared with trout that were bred for high cortisol release. These low-response fish tend to become dominant when paired in agonistic interactions with high-response fish (Johansen et al., 2011). Thus, MR may play a conserved role in regulating dominance or cortisol levels in *A. burtoni* and rainbow trout.

Fish have two GR1 isoforms that differ in the spacing between two DNA zinc finger binding domains because of an insertion (see Greenwood et al., 2003). These isoforms show differential activation via a reporter construct *in vitro* (Greenwood et al., 2003) and similar insertions have been found in GR receptors of other teleost species (Lethimonier et al., 2002; Takeo et al., 1996). We found that the ratio of GR1b to total GR1 levels was significantly higher in ND males, suggesting a differential role for GR1b versus GR1a in the regulation of social status.

It is possible that the GR1a and GR1b isoforms recognize different promoter sequences, and thereby have different target genes. It is interesting to speculate that GR1b might be upregulated in ND males because a different suite of target genes is expressed in ND versus D males. An alternative scenario is that changes in cortisol receptor mRNA in the POA might directly or indirectly effectively protect the POA region of ND males from the chronically elevated levels of cortisol they endure. Interestingly, both GR2 and MR are downregulated in ND males. However, GR2 expression levels are very low to undetectable in GnRH1 neurons, suggesting that GR2 expression in neighboring neurons could modify GnRH1 release but not directly within GnRH1 neurons. In addition, the GR1 isoform that is upregulated in ND males is one that shows reduced DNA transcription (Greenwood et al., 2003). If the effect (direct or indirect) is protection of the POA and other regions of the telencephalon, it could be adaptive for a species with a flexible reproductive strategy.

A recent study in another teleost compared GR mRNA levels of males by status. Similar to *A. burtoni*, plainfin midshipman (*Porichthys notatus*) males occur in one of two phenotypes: a D male and a ND male (sneaker male). ND plainfin midshipman have twofold more circulating cortisol than D males (Arterbery et al., 2010) and also exhibit elevated GR1b mRNA levels in all brain

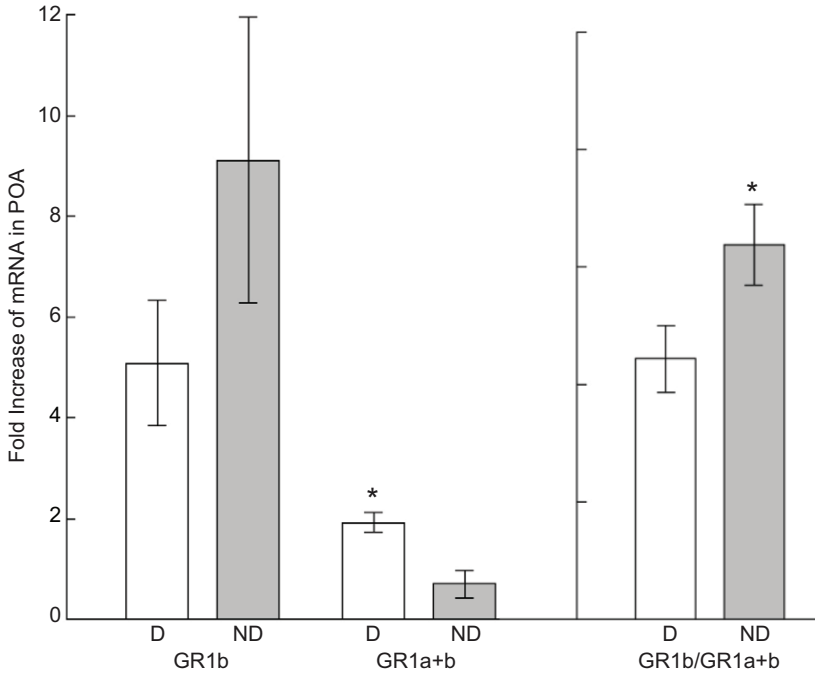

Fig. 5. mRNA levels for GR1b, GR1a+b and GR1b/GR1a+b. The mRNA quantity, normalized to that of housekeeping genes (see Materials and methods) and given as fold increase, of GR1b and the combined receptor GR1a+1b is plotted. The ratio of GR1b mRNA to GR1a mRNA in the POA is significantly higher (P<0.05) in ND males compared with D males.

The Journal of Experimental Biology (2014) doi:10.1242/jeb.104430

regions tested. Moreover, when GR mRNA levels were measured in the vocal muscles, Genova et al. (Genova et al., 2012) found increased GR mRNA in non-singing males. Modulation of teleost GR1b expression may provide an explanation for the survival of ND males and modulation of their neurons, especially in brain nuclei that are critical for the behavioral and physiological plasticity exhibited in male *A. burtoni*.

Androgen receptor ARα and ARβ expression in the POA

Similar to other teleosts, cichlids have two androgen receptor subtypes, ARα and ARβ (Harbott et al., 2007). Previous measurements on *A. burtoni* using qPCR has shown that both ARα and ARβ are present in the brain (Burmeister et al., 2007) and *in situ* hybridization revealed that they are co-localized with GnRH1 neurons in the POA of *A. burtoni* (Harbott et al., 2007). *In situ* data show high levels of expression of both ARα and ARβ in the POA of male *A. burtoni* (Harbott et al., 2007). Moreover, when whole brains from D males were macrodissected into three different regions, Burmeister et al. (Burmeister et al., 2007) found that the anterior portion, which contains the POA, had elevated ARα and ARβ mRNA levels compared with ND males. In our study, microdissection of the POA revealed that D males had significantly higher mRNA levels of ARα compared with ND males (Fig. 3). However, unlike in the larger brain regions, ARβ levels were not different in the POA of D and ND males. This may be due to elevated ARβ expression in non-POA regions in the anterior brain. Similarly to D male *A. burtoni*, D male plainfin midshipman also have elevated circulating levels of 11-ketotestosterone and express higher levels of ARα mRNA in vocal muscles compared with ND males (Genova et al., 2012). Our data combined with previous work showing that the higher levels of testosterone were coupled with the increased affinity for testosterone by ARα and increased ARα expression in the POA suggest it may be the key androgen receptor associated with maintenance of GnRH1 neuron size and elevated androgen levels in D males (Fig. 6).

GnRH1

The role of the hypothalamic-pituitary–gonadal axis in regulating reproductive competence is well documented in vertebrates (e.g. Sower et al., 2009). GnRH1-producing cells, located in the POA of the hypothalamus, project into the portal vasculature in mammals and directly into the pituitary in fish (Fridberg and Ekengren, 1977), where they cause release of gonadotropin hormones (follicle-stimulating hormone and luteinizing hormone). In *A. burtoni*, social status regulates reproductive competence directly (Davis and Fernald, 1990; Fernald, 2012) and our data measuring local GnRH1 levels in the hypothalamus showed a twofold increase in GnRH1 mRNA expression in the POA of D compared with ND males (Fig. 3). These levels are consistent with previous measurements that sampled larger brain regions (Au et al., 2006; Burmeister et al., 2007).

Hormones and behavior

Our data (not shown) confirmed previous laboratory studies reporting significant differences in agonistic and reproductive behavior as well as differences in the levels of stress and reproductive hormones between D and ND male *A. burtoni*. D males had higher levels of aggressive and reproductive behaviors than ND males. Similarly, we also confirmed previously reported glucocorticoid (cortisol) and androgen (testosterone and 11-ketotestosterone) level differences between D and ND males (Fig. 1). Taken together, behavioral endocrine data verify the key characteristics of male *A. burtoni* when they are housed in pairs.

Conclusions

The social regulation of reproductive capacity is an important evolutionary adaptation in *A. burtoni*. We have shown behavioral, hormonal and mRNA expression level differences between the two distinct social states of males, consistent with previous data using larger tissue samples from the brain. Our data suggest that in D males, increased expression of ARα, MR and GR2 occurs during normal feedback signaling for reproductive stimulation in the POA, a region involved in both neuroendocrine control of the gonads and regulation of male reproductive behaviors. The low levels of cortisol may require increased production of a highly active receptor for proper feedback signaling during normal daily cycling of cortisol. In ND males, decreases of MR and GR2 and an increase of GR1b expression may be important for protection of neurons, including

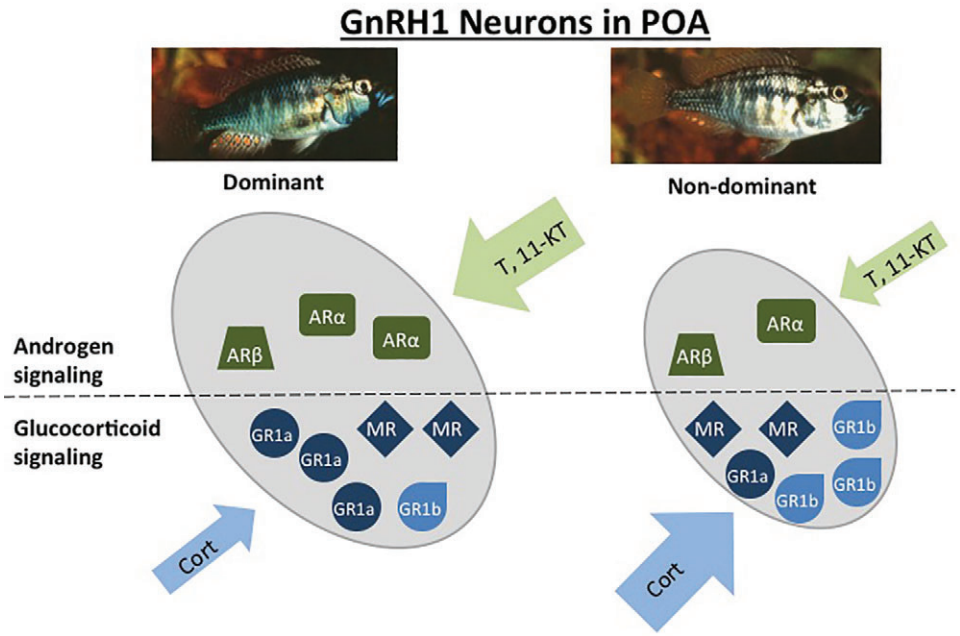

Fig. 6. Dominant males have higher amounts of both testosterone and 11-ketotestosterone, larger GnRH1 neurons and higher ARα mRNA levels compared with ND males. D males also have higher mRNA levels for MR and GR1a. ND males have a reduced level of GR1a mRNA (high transcriptional response receptors in GnRH1 neurons) and high levels of GR1b (low transcriptional response receptors). Cort, cortisol; T, testosterone; 11-KT, 11-ketotestosterone.

The Journal of Experimental Biology (2014) doi:10.1242/jeb.104430

those containing GnRH1, from high cortisol levels, and thus allow the neuronal circuit important for future social opportunity to remain intact (Fig. 6). We hypothesize that possibly other brain areas are similarly protected though we do not have data to test that hypothesis.

As noted, we measured mRNA levels to assess the changes in expression of the receptors of interest. How are these changes in GR mRNA related to GR protein expression? Though this is not known in *A. burtoni* directly, it is known that cortisol levels and their receptors are tightly controlled in the homeostatic regulation of the hypothalamic-pituitary–adrenal (HPA) axis. For example, in response to an acute cortisol antagonist, rainbow trout GR protein levels decrease and, in response, GR mRNA levels increase (Alderman et al., 2012), consistent with the tight regulation of the HPA axis. Correspondingly, chronic treatment with GR agonists generally results in a coincident decrease in both GR mRNA and GR protein levels, suggesting that using mRNA as a proxy for protein levels is appropriate (e.g. Svec and Rudis, 1981; Okret et al., 1986). These data suggest that mRNA levels are reasonable predictors of protein levels for the GR receptors.

Our results suggest a novel potential mechanism for the regulation of the HPA axis. In their dynamic social interactions, *A. burtoni* are subjected to rapid and dramatic changes in their social interactions from social dominance to social suppression with concomitant cortisol level changes. Modulation of cortisol receptor subtype expression that could mitigate the consequences of socially induced increases in cortisol levels in ND males.

MATERIALS AND METHODS

Animals

Astatotilapia burtoni bred from wild-caught individuals were housed in aquaria under conditions mimicking their natural habitat: 29°C, pH 8, 12 h:12 h light:dark cycle with full spectrum illumination (Fernald and Hirata, 1977b). A layer of gravel (~3 cm) covered the bottom of the aquaria and terracotta pots in each tank facilitated the establishment and maintenance of territories by males. Animals were fed *ad libitum* every morning with cichlid pellets and flakes (AquaDine, Healdsburg, CA, USA). Animals were marked using randomized combinations of colored beads attached just beneath the dorsal fin. All animals were treated in accordance with the Stanford University Institutional Animal Care and Use Committee (IACUC) guidelines.

Pairing of males

Reproductively active adult *A. burtoni* were kept in aquaria (85 cm length×53.7 cm width for 30 days). Two pairs of size-matched D males (N=24) from different aquaria were introduced with females to a new experimental aquarium, identical to their former aquarium except that a perforated divider separated the tank into two equal sections with a pair of males and three females in each section. Within minutes of being transferred to the aquaria, one male asserted his prior D status and the other became ND. They were kept for 30 days in this new condition (Fox et al., 1997) and then killed. Males were used only once in each experiment. Tissue collection as well as pairing of animals and all other behavioral procedures followed procedures described in detail previously (Korzan et al., 2008).

Behavior

Behavior of the paired males and their neighboring pair was recorded with a digital video camera (MiniDV, JVC) for 30 min, 6 h after lights were turned on each day for the first 3 days (days 1–3) as well as the final three consecutive days (days 28–30) of the experiment. Aggressive behavior for each male was coded by observers blind to the experiment and ranked using a dominance index, calculated from the sum of aggressive behavioral acts (e.g. bites and chases) minus subordinate behavioral acts (e.g. flee) performed per 10 min interval (White et al., 2002). Immediately after the final observation, both pairs of D and ND males in a tank were killed and brains and plasma were collected. Plasma was collected using a hypodermic needle (25 gauge) coated with heparin, inserted on the midline ~5 mm behind the anal fin into the caudal vein (Fox et al., 1997). Approximately 200 µl of blood was collected and centrifuged for 3 min (13.5×10^3 ***g***) to separate plasma from blood cells. The plasma was then transferred into a clean collection tube (1.5 ml). Plasma and brains were immediately frozen on dry ice and stored at −80°C until assayed. Behavioral data for some pairs were excluded because of a significant recording time (more than 5 min) outside the field of view of the recording equipment.

Steroid hormone measurements

Testosterone, 11-ketotestosterone and cortisol levels in *A. burtoni* plasma were measured using commercially available reagents (testosterone and cortisol: Assay Design, Ann Arbor, MI, USA; 11-ketotestosterone: Cayman Chemical, Ann Arbor, MI, USA) following protocols established previously (Parikh et al., 2006a; Parikh et al., 2006b). Plasma cortisol concentrations were measured in triplicate from blood plasma using a standard competitive immunoassay. Cortisol levels were measured using standard procedures, calculating the concentration from the standard curve. Similar protocols were used for 11-ketotestosterone and testosterone quantification with the addition of an extraction step utilizing diethyl ether and ethyl acetate/hexane, respectively, to remove the hormone from the whole plasma (Korzan et al., 2008). Because of the small size of *A. burtoni*, collecting blood samples is a challenge and very small volumes (<100 µl) are normally obtained. Therefore, the volume of plasma needed to run multiple tests was not always possible.

In situ hybridization co-localization of glucocorticoid receptor mRNA and GnRH1 mRNA in POA

In situ hybridization followed standard procedures as used in the Fernald laboratory (Burmeister et al., 2007; Grens et al., 2005). Male brains were frozen in Tissue-Tek OCT compound (Ted Pella, Redding, CA, USA) inside Peel-A-Way plastic molds (Polysciences, Inc., Warrington, PA, USA) on dry ice and stored at −80°C. Brain tissue was sectioned coronally in three series at 14 µm using a Microm HM 550 cryostat (Thermo Scientific, Waltham, MA, USA) and mounted on glass charged slides that were then stored at −80°C until use. Slides were brought to room temperature, fixed for 10 min in 4% paraformaldehyde in phosphate-buffered saline (PBS), rinsed twice for 3 min each in PBS, immersed in 0.1 mol l^{-1} triethanolamide (TEA) buffer for 3 min, acetylated in 0.25% acetic anhydride in 0.1 mol l^{-1} TEA for 10 min, rinsed twice for 3 min each in 2× sodium citrate sodium chloride (SSC) buffer, dehydrated in an ethanol series, and air dried. Radioactive riboprobes labeled with ^{35}S-UTP were diluted to 5×10^6 cpm ml^{-1}, and DIG probes were diluted to 1 ng ml^{-1} in hybridization solution (Sigma-Aldrich, St Louis, MO, USA) supplemented with 1 g ml^{-1} dithiothreitol (DTT). Preheated probe mix was added to each slide and then slides were coverslipped and immersed overnight in a 60°C mineral oil bath. After removing slides from the mineral oil, residual oil was removed by immersion in chloroform. Probe and coverslips were removed in two rinses of 4× SSC, and then washed with 2× SSC with DTT. To detect the GnRH1 probe, slides were incubated in anti-DIG-peroxidase primary antibody (Roche, Indianapolis, IN, USA), then amplified using biotinyl tyramide (Tyramide Signal Amplification kit, NEN Life Sciences, Boston, MA, USA), incubated in avidin-HRP and stained using 0.05% diaminobenzidine (DAB). Finally, slides were dehydrated in ethanol, air dried and dipped in nuclear emulsion diluted 1:1 in water (NBT-2; Eastman Kodak, Rochester, NY, USA), air dried, and stored in a light-tight box at 4°C for 3–4 weeks. Following development, slides were stained with Cresyl Violet, dehydrated in an ethanol/xylene series and coverslipped. We used sense versions of each radioactive probe to test the specificity of our *in situ* hybridization results, and in no instance was any signal above background evident.

Microdissection of GnRH1 brain regions and mRNA isolation/quantification

Brains were cut coronally at 300 µm and freeze-thawed onto uncoated specimen slides (Korzan et al., 2000). The part of the POA containing GnRH1 neurons, the anterior parvocellular preoptic nucleus (aPPn), was identified using reference images from prior *in situ* and immunohistochemistry

experiments in this species (Harbott et al., 2007; Burmeister and Fernald, 2005) and an anatomical map of GnRH1 expression (White et al., 1995). The POA, including the aPPn, was microdissected from coronal slices using a 300 μm diameter punch (Korzan et al., 2000).

Microdissected punches were expelled into lysis buffer (RNeasy Plus Micro, Qiagen) and RNA was isolated using the protocol provided (Purification of total RNA from microdissected cryosections; Qiagen kit no. 74034), a standard procedure that removes DNA from the samples. We also treated the samples with a DNase (Turbo DNA-free, Ambion, Austin, TX, USA) before synthesizing cDNA using random hexamer primers and reverse transcription (Roche Applied Science, Indianapolis, IN, USA).

Quantification of mRNA levels was performed using standard quantitative PCR procedures (e.g. Harbott et al., 2007). PCR primer pairs for GnRH1 and ARα and ARβ (Burmeister et al., 2007), MR, GR1a+b, GR1b and GR2 (Greenwood et al., 2003) were synthesized and used to amplify the samples using SYBR Green Supermix and the MyIQ system (Bio-Rad). Results were normalized to the geometric mean of the internal control genes actin (Zhao and Fernald, 2005), 18S (Burmeister et al., 2007) and G3PDH (Greenwood et al., 2003) for quantification and comparison. mRNA levels were calculated from the raw data using the Real Time PCR Miner program (Zhao and Fernald, 2005). GR1 ratio was calculated based on mRNA levels from qPCR reactions for primers that select for GR1a+b and primers for GR1b, then calculating the ratio of GR1b/(GR1b+GR1a+b). For all qPCR reactions, the extremely small amount of tissue and mRNA extracted from punches did not allow for reanalysis of some reactions that failed.

Data analysis

DI, hormone levels and qPCR data of ND and D male pairs were compared using two-tailed paired *t*-tests. The level of significance was $P<0.05$ (SPSS 13.0, SPSS Inc., Chicago, IL, USA). All error bars for figures are s.e.m.

Acknowledgements

We thank Dr Anna Greenwood for contributing the GR and MR *in situ* hybridization data and Caroline Hu for providing Fig. 6.

Competing interests

The authors declare no competing financial interests.

Author contributions

W.J.K. and R.D.F. designed the study. W.J.K. and B.P.G. performed the experiments and collected and analyzed the results. W.J.K. and R.D.F. wrote the manuscript.

Funding

This research was supported in part by National Institutes of Health NRSA 1 F32MH074222-01 to W.J.K. and National Institutes of Health NINDS 034950 to R.D.F. Deposited in PMC for release after 12 months.

References

Alderman, S. L., McGuire, A., Bernier, N. J. and Vijayan, M. M. (2012). Central and peripheral glucocorticoid receptors are involved in the plasma cortisol response to an acute stressor in rainbow trout. *Gen. Comp. Endocrinol.* **176**, 79-85.

Arterbery, A. S., Deitcher, D. L. and Bass, A. H. (2010). Corticosteroid receptor expression in a teleost fish that displays alternative male reproductive tactics. *Gen. Comp. Endocrinol.* **165**, 83-90.

Au, T. M., Greenwood, A. K. and Fernald, R. D. (2006). Differential social regulation of two pituitary gonadotropin-releasing hormone receptors. *Behav. Brain Res.* **170**, 342-346.

Avitsur, R., Stark, J. L. and Sheridan, J. F. (2001). Social stress induces glucocorticoid resistance in subordinate animals. *Horm. Behav.* **39**, 247-257.

Baker, M. E., Chandsawangbhuwana, C. and Ollikainen, N. (2007). Structural analysis of the evolution of steroid specificity in the mineralocorticoid and glucocorticoid receptors. *BMC Evol. Biol.* **7**, 24.

Burmeister, S. S. and Fernald, R. D. (2005). Evolutionary conservation of the egr-1 immediate-early gene response in a teleost. *J. Comp. Neurol.* **481**, 220-232.

Burmeister, S. S., Kailasanath, V. and Fernald, R. D. (2007). Social dominance regulates androgen and estrogen receptor gene expression. *Horm. Behav.* **51**, 164-170.

Bury, N. R. and Sturm, A. (2007). Evolution of the corticosteroid receptor signalling pathway in fish. *Gen. Comp. Endocrinol.* **153**, 47-56.

Cheney, D. L. and Seyfarth, R. M. (1990). The representation of social relations by monkeys. *Cognition* **37**, 167-196.

Chiao, J. Y., Harada, T., Oby, E. R., Li, Z., Parrish, T. and Bridge, D. J. (2009). Neural representations of social status hierarchy in human inferior parietal cortex. *Neuropsychologia* **47**, 354-363.

Coulter, G. W. (1991). *Lake Tanganyika and its Life.* New York, NY: Oxford University Press.

Davis, M. R. and Fernald, R. D. (1990). Social control of neuronal soma size. *J. Neurobiol.* **21**, 1180-1188.

Fernald, R. D. (1977). Quantitative behavioral observations of haplochromis-burtoni under semi natural conditions. *Anim. Behav.* **25**, 643-653.

Fernald, R. D. (2012). Social control of the brain. *Annu. Rev. Neurosci.* **35**, 133-151.

Fernald, R. D. and Hirata, N. R. (1977a). Field study of haplochromis-burtoni quantitative behavioral observations. *Anim. Behav.* **25**, 964-975.

Fernald, R. D. and Hirata, N. R. (1977b). Field study of Haplochromis burtoni: habitats and co-habitants. *Environ. Biol. Fishes* **2**, 299-308.

Fox, H. E., White, S. A., Kao, M. H. and Fernald, R. D. (1997). Stress and dominance in a social fish. *J. Neurosci.* **17**, 6463-6469.

Francis, R. C., Soma, K. and Fernald, R. D. (1993). Social regulation of the brain-pituitary-gonadal axis. *Proc. Natl. Acad. Sci. USA* **90**, 7794-7798.

Fridberg, G. and Ekengren, B. (1977). The vascularization and the neuroendocrine pathways of the pituitary gland in the Atlantic salmon, *Salmo salar. Can. J. Zool.* **55**, 1284-1296.

Funder, J. W., Pearce, P. T., Smith, R. and Smith, A. I. (1988). Mineralocorticoid action: target tissue specificity is enzyme, not receptor, mediated. *Science* **242**, 583-585.

Genova, R. M., Marchaterre, M. A., Knapp, R., Fergus, D. and Bass, A. H. (2012). Glucocorticoid and androgen signaling pathways diverge between advertisement calling and non-calling fish. *Horm. Behav.* **62**, 426-432.

Greenwood, A. K., Butler, P. C., White, R. B., DeMarco, U., Pearce, D. and Fernald, R. D. (2003). Multiple corticosteroid receptors in a teleost fish: distinct sequences, expression patterns, and transcriptional activities. *Endocrinology* **144**, 4226-4236.

Grens, K. E., Greenwood, A. K. and Fernald, R. D. (2005). Two visual processing pathways are targeted by gonadotropin-releasing hormone in the retina. *Brain Behav. Evol.* **66**, 1-9.

Harbott, L. K., Burmeister, S. S., White, R. B., Vagell, M. and Fernald, R. D. (2007). Androgen receptors in a cichlid fish, *Astatotilapia burtoni*: structure, localization, and expression levels. *J. Comp. Neurol.* **504**, 57-73.

Hofmann, H. A., Benson, M. E. and Fernald, R. D. (1999). Social status regulates growth rate: consequences for life-history strategies. *Proc. Natl. Acad. Sci. USA* **96**, 14171-14176.

Johansen, I. B., Sandvik, G. K., Nilsson, G. E., Bakken, M. and Overli, O. (2011). Cortisol receptor expression differs in the brains of rainbow trout selected for divergent cortisol responses. *Comp. Biochem. Physiol.* **6D**, 126-132.

Kaplan, J. R. (2008). Origins and health consequences of stress-induced ovarian dysfunction. *Interdiscip. Top. Gerontol.* **36**, 162-185.

Kaplan, J. R. and Manuck, S. B. (2004). Ovarian dysfunction, stress, and disease: a primate continuum. *ILAR J.* **45**, 89-115.

Kolodkin, A., Sahin, N., Phillips, A., Hood, S. R., Bruggeman, F. J., Westerhoff, H. V. and Plant, N. (2013). Optimization of stress response through the nuclear receptor-mediated cortisol signalling network. *Nat. Commun.* **4**, 1792.

Korzan, W. J., Summers, T. R. and Summers, C. H. (2000). Monoaminergic activities of limbic regions are elevated during aggression: influence of sympathetic social signaling. *Brain Res.* **870**, 170-178.

Korzan, W. J., Robison, R. R., Zhao, S. and Fernald, R. D. (2008). Color change as a potential behavioral strategy. *Horm. Behav.* **54**, 463-470.

Krozowski, Z. S. and Funder, J. W. (1983). Renal mineralocorticoid receptors and hippocampal corticosterone-binding species have identical intrinsic steroid specificity. *Proc. Natl. Acad. Sci. USA* **80**, 6056-6060.

Lethimonier, C., Tujague, M., Kern, L. and Ducouret, B. (2002). Peptide insertion in the DNA-binding domain of fish glucocorticoid receptor is encoded by an additional exon and confers particular functional properties. *Mol. Cell. Endocrinol.* **194**, 107-116.

Maruska, K. P. and Fernald, R. D. (2014). Social regulation of gene expression in the African cichlid fish *Astatotilapia burtoni*. *Oxford Handbook of Psychology*, 1-22. doi:10.1093/oxfordhb/9780199753888.013.012.

Okret, S., Poellinger, L., Dong, Y. and Gustafsson, J. A. (1986). Down-regulation of glucocorticoid receptor mRNA by glucocorticoid hormones and recognition by the receptor of a specific binding sequence within a receptor cDNA clone. *Proc. Natl. Acad. Sci. USA* **83**, 5899-5903.

Olsson, P.-E., Berg, A. H., von Hofsten, J., Grahn, B., Hellqvist, A., Larsson, A., Karlsson, J., Modig, C., Borg, B. and Thomas, P. (2005). Molecular cloning and characterization of a nuclear androgen receptor activated by 11-ketotestosterone. *Reprod. Biol. Endocrinol.* **3**, 37.

Parikh, V. N., Clement, T. S. and Fernald, R. D. (2006a). Androgen level and male social status in the African cichlid, *Astatotilapia burtoni*. *Behav. Brain Res.* **166**, 291-295.

Parikh, V. N., Clement, T. S. and Fernald, R. D. (2006b). Physiological consequences of social descent: studies in *Astatotilapia burtoni*. *J. Endocrinol.* **190**, 183-190.

Pasmanik, M. and Callard, G. V. (1988). A high abundance androgen receptor in goldfish brain: characteristics and seasonal changes. *Endocrinology* **123**, 1162-1171.

Pottinger, T. G. (1987). Androgen binding in the skin of mature male brown trout, *Salmo trutta* L. *Gen. Comp. Endocrinol.* **66**, 224-232.

Prunet, P., Sturm, A. and Milla, S. (2006). Multiple corticosteroid receptors in fish: from old ideas to new concepts. *Gen. Comp. Endocrinol.* **147**, 17-23.

Sapolsky, R. M. (1996). Stress, glucocorticoids, and damage to the nervous system: the current state of confusion. *Stress* **1**, 1-19.

Schaaf, M. J. M., Champagne, D., van Laanen, I. H. C., van Wijk, D. C. W. A., Meijer, A. H., Meijer, O. C., Spaink, H. P. and Richardson, M. K. (2008). Discovery of a functional glucocorticoid receptor beta-isoform in zebrafish. *Endocrinology* **149**, 1591-1599.

Slater, C. H., Fitzpatrick, M. S. and Schreck, C. B. (1995). Characterization of an androgen receptor in salmonid lymphocytes: possible link to androgen-induced immunosuppression. *Gen. Comp. Endocrinol.* **100**, 218-225.

Sower, S. A., Freamat, M. and Kavanaugh, S. I. (2009). The origins of the vertebrate hypothalamic-pituitary-gonadal (HPG) and hypothalamic-pituitary-thyroid (HPT) endocrine systems: new insights from lampreys. *Gen. Comp. Endocrinol.* **161**, 20-29.

Stolte, E. H., van Kemenade, B. M., Savelkoul, H. F. and Flik, G. (2006). Evolution of glucocorticoid receptors with different glucocorticoid sensitivity. *J. Endocrinol.* **190**, 17-28.

Sturm, A., Bury, N., Dengreville, L., Fagart, J., Flouriot, G., Rafestin-Oblin, M. E. and Prunet, P. (2005). 11-deoxycorticosterone is a potent agonist of the rainbow trout (*Oncorhynchus mykiss*) mineralocorticoid receptor. *Endocrinology* **146**, 47-55.

Svec, F. and Rudis, M. (1981). Glucocorticoids regulate the glucocorticoid receptor in the AtT-20 cell. *J. Biol. Chem.* **256**, 5984-5987.

Takeo, J., Hata, J., Segawa, C., Toyohara, H. and Yamashita, S. (1996). Fish glucocorticoid receptor with splicing variants in the DNA binding domain. *FEBS Lett.* **389**, 244-248.

Webster, R. I., Majnemer, A., Platt, R. W. and Shevell, M. I. (2008). Child health and parental stress in school-age children with a preschool diagnosis of developmental delay. *J. Child Neurol.* **23**, 32-38.

White, S. A., Kasten, T. L., Bond, C. T., Adelman, J. P. and Fernald, R. D. (1995). Three gonadotropin-releasing hormone genes in one organism suggest novel roles for an ancient peptide. *Proc. Natl. Acad. Sci. USA* **92**, 8363-8367.

White, S. A., Nguyen, T. and Fernald, R. D. (2002). Social regulation of gonadotropin-releasing hormone. *J. Exp. Biol.* **205**, 2567-2581.

Willisch, C. S., Biebach, I., Koller, U., Bucher, T., Marreros, N., Ryser-Degiorgis, M. P., Keller, L. F. and Neuhaus, P. (2012). Male reproductive pattern in a polygnyous ungulate with a slow life-history: the role of age, social status and alternative mating tactics. *Evol. Ecol.* **26**, 187-206.

Wilson, E. O. (2000). *Sociobiology: The New Synthesis*. Cambridge, MA: Harvard University Press.

Zhao, S. and Fernald, R. D. (2005). Comprehensive algorithm for quantitative real-time polymerase chain reaction. *J. Comput. Biol.* **12**, 1047-1064.

The Journal of Experimental Biology