

Masculinized dominant females in a cooperatively breeding species

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Abstract

The molecular mechanisms underlying complex social behaviours such as dominance are largely unknown. Studying the cooperatively breeding African cichlid *Neolamprologus pulcher*, we show that dominant females were similar to dominant males in dominance behaviour, high testosterone levels and brain arginine vasotocin expression (a neuropeptide involved in vertebrate territorial, reproductive and social behaviours) compared to subordinate helpers, but had lower levels of 11-ketotestosterone than males. Furthermore, brain gene expression profiles of dominant females were most similar to those of the males (independent of social rank). Dominant breeder females are masculinized at the molecular and hormonal level while being at the same time reproductively competent, suggesting a modular organization of molecular and endocrine functions, allowing for sex-specific regulation.

Keywords: behaviour, cooperative breeding, DNA microarray, dominance, gene expression, hormone, integrative biology

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Introduction

In cooperatively breeding species, breeding pairs are assisted in raising offspring by individuals who live with them in the social group. Social status within a group is a major determinant of reproduction and survival. Understanding the ecological and evolutionary factors favouring cooperation and conflict in social groups has been a long-standing focus of biology (West-Eberhard 1975; Moehlman 1979; Riedman 1982; Koenig *et al.* 1992; Balshine-Earn *et al.* 1998; Baglione *et al.* 2002). Behavioural and endocrine studies have been conducted on group-living species to determine the striking differences in behaviour, hormones and life cycle that result from different dominance status

(Jarvis 1981; Taborsky & Limberger 1981; Schoech *et al.* 1991; Ziegler 2000; Clutton-Brock *et al.* 2001; Creel 2001; Khan *et al.* 2001; Oliveira *et al.* 2003; Bender *et al.* 2006). However, the molecular substrates underlying complex social behaviour such as dominance hierarchies and social affiliation remain largely unknown in many species. Knowledge of the molecular basis of dominance is crucial to get an integrated picture of how the diverse life history requirements associated with social status are balanced to result in a robust dominant organism. As social dominance can change throughout an animal's life, regulation of gene activity and changes in physiological measures are likely to be extremely important mechanisms for the control and implementation of changes in social status (Whitfield *et al.* 2003; Aubin-Horth *et al.* 2005a; Burmeister *et al.* 2005; Stiver *et al.* 2006). We used the cooperatively breeding fish *Neolamprologus pulcher* from Lake Tanganyika to explore how differences in social status are associated with a suite of physiological, hormonal and molecular traits. In this species, dominant breeding pairs aggressively defend a nest and care for offspring together with subordinate helpers (Buchner *et al.* 2004). Both male and female breeders actively defend the territory (Balshine-Earn *et al.* 1998; Taborsky

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Table 1 Univariate two-way analysis of variance of the effects of dominance (Dom), sex (Sex) and their interaction (Dom * Sex) on behavioural, physiological and hormonal measures

Dependent variable	<i>F</i>			<i>P</i> value		
	Dom	Sex	Dom * Sex	Dom	Sex	Dom * Sex
Dominance Index	9.028	1.017	0.042	0.005	0.319	0.839
Feeding	2.375	2.375	3.994	0.131	0.131	0.053
Helping	1.325	0.030	0.030	0.257	0.864	0.864
Social	0.733	0.243	1.183	0.397	0.625	0.283
Aggressive	1.213	0.315	0.892	0.278	0.578	0.351
Length	66.644	2.220	0.108	< 0.0001	0.144	0.745
Condition factor	60.1	0.491	0.002	< 0.0001	0.488	0.967
Gonado-somatic index	15.692	3.022	0.723	< 0.0001	0.090	0.400
Testosterone	6.429	3.286	1.490	0.020	0.086	0.237
11-ketotestosterone	1.504	17.952	1.951	0.229	< 0.0001	0.172
Progesterone	0.264	0.021	0.259	0.612	0.886	0.616

& Grantner 1998; Desjardins *et al.* 2006). Therefore, a powerful aspect of this model system is the ability to study social status in both sexes and thus partition the effects of dominance and sex, which gives a broader insight into the control of complex social behaviour. In this study, we simultaneously measured behaviour, body condition, reproductive investment, hormone levels (testosterone, 11-ketotestosterone, progesterone) and brain gene expression using a custom-made cichlid fish complementary DNA (cDNA) microarray in individuals in 14 social groups. We analysed differences between dominants and subordinates of both sexes for these traits. We determined if the different components of social status measured on individual fish covary. We also examined whether individual brain gene expression profiles were similar across dominance ranks, sexes, or both.

Methods

Social groups studied

We used 14 established social groups of the Tanganyikan cichlid *Neolamprologus pulcher* in this study. The groups were part of a colony housed at McMaster University in Hamilton, Ontario, Canada. All fish used were wild-caught breeders collected from the southern shores of Lake Tanganyika in 2001 and 2002 and their progeny. Each social group was housed in a 50-gallon tank. None of the groups had fry during this experiment. A breeding pair and two helpers were studied in each social group. All social groups were formed at least 3 weeks prior to beginning the study. Breeders were always larger in body size (length and mass) than helpers (2-way ANOVA, $P < 0.0001$, Table 1).

Assessing dominance status

Four focal individuals from each group, a breeding male and female and two helpers of unknown sex were observed for 10 min on three separate occasions, recording the frequency of all behaviours on Psion organizers using the OBSERVER package (Noldus). Behaviours included feeding, aggressive or dominant behaviours (bites, chases, rams, head shakes and mouth fights), submissive behaviours (submissive postures and displays), helping (egg cleaning, digs, carries, defence, guards, fanning, number of visits to the brood chamber/nest) and social behaviours (nudges, follows) [see Buchner *et al.* (2004) for a recent and detailed ethogram for this species]. At least one observation was conducted in the morning (8:00–11:00 h) and one in the afternoon (13:00–16:00 h) to account for diurnal differences in behaviour (Balshine-Earn *et al.* 1998; Werner *et al.* 2003). The dominance index (DI) was calculated for each individual by subtracting the average number of subordinate behaviours from the average number of dominant behaviours ($\Sigma_{\text{dom}} - \Sigma_{\text{sub}}$). Following the third set of behavioural observations in each group, the fish were all measured. We minimized the number of fish to be sacrificed and 43 individuals (mostly from 10 groups) were quickly killed using an overdose of benzocaine. A blood sample was taken by caudal severance and the brains were rapidly removed and stored in RNA later (Ambion).

Physiological measures

We removed the gonads. These were weighed and stored in ethanol. A condition factor (CF), a measure of overall energy reserve (Helfman *et al.* 1997), was calculated as the weight of the fish minus the weight of the gonads,

divided by its standard length. The gonadosomatic index (GSI), a measure of reproductive investment, was calculated as the weight of the gonads divided by the weight of the fish.

Hormone levels

The blood was collected in heparinized microcapillary tubes. Plasma was removed after centrifugation and frozen at -20°C . The following hormones were measured by radioimmunoassay as described in (Desjardins *et al.* 2006): testosterone, 11-ketotestosterone and progesterone. Testosterone and 11-ketotestosterone are known to have important effects on reproductive and aggressive and dominant behaviour in males (Wingfield *et al.* 1990). A fish-specific androgen, 11-ketotestosterone is thought to be the most potent male androgen in fishes (Borg 1994). Progesterone was analysed because of its important role in female reproduction and aggression in other vertebrates as well as paternal care in rodents (Nelson 2005). Because of the small fish size and some sample loss, enough plasma was collected to estimate testosterone levels for 23 individuals, 11-ketotestosterone levels for 36 individuals and progesterone levels for 26 individuals.

Brain gene expression

Microarray used. We studied gene expression in the brains of 18 of the 43 individuals sampled for behaviour. We used a DNA microarray containing 4573 elements constructed from a brain-specific *Astatotilapia burtoni* cDNA library [Renn *et al.* 2004; National Center for Biotechnology Information (NCBI) GEO platform GPL528] because the brain is central to the control of reproduction, physiology, and behaviour. These clones have been annotated as described in Renn *et al.* (in preparation). Estimates of divergence time suggest that *N. pulcher* and *A. burtoni* diverged relatively recently around 3 millions years ago (Salzburger *et al.* 2005) and heterologous hybridization, targeting RNA to an array constructed for a different species, has previously been shown to give biologically meaningful results with this microarray platform and specifically with *N. pulcher* (Renn *et al.* 2004; Aubin-Horth *et al.* 2005b). In total, the brains of six breeding individuals (3 males and 3 females) and 12 helpers (6 males and 6 females) were used in the microarray experiment.

RNA extraction and cDNA preparation. After brain tissue homogenization (Tissue Tearor, Biospec Products), total RNA was extracted according to a standard Trizol protocol (Invitrogen). Four independent reverse transcriptions were performed on total mRNA extracted from each brain sample according to a standard protocol (Invitrogen). These four cDNA samples from one individual were then

pooled and aliquoted, thus averaging potential effects of reverse transcription over all replicates to minimize the source of technical error due to variation in reverse transcription (Gibson *et al.* 2004). Complementary DNA from each fish was kept separate.

Microarray hybridization. Complementary DNA aliquots from two fish were labelled, respectively, with Cy3 and Cy5 fluorescent dyes (Amersham) according to a standard amino-allyl attachment method protocol and competitively hybridized on a microarray slide, also according to standard protocol (detailed in Aubin-Horth *et al.* 2005b). Complementary DNA from each fish was used four times (technical replicates, including dye-swaps, some fish were used two or six times depending on the quantity of RNA available) in a loop-design comparison, such that an individual of a given category (breeding male, breeding female, helper male, helper female) was directly compared to other individuals without the need for a reference sample (see Table 2). This type of design takes advantage of additional information obtained from transitive comparisons of individuals while minimizing the number of arrays needed, an important factor when the total amount of RNA that can be obtained per sample is limited (Churchill 2002; Townsend 2003). Thirty-one microarrays were used to compare 62 independent labelling reactions.

Hybridization data analysis. Arrays were scanned with an Axon 4000B scanner (Axon Instruments) using GENEPIX 5.0 software (Axon Instruments). Spots were examined individually and flagged as 'bad' if irregularities occurred. Raw data (after flag filtering and removal of spots with intensities lower than the local background intensity plus two standard deviation of this background intensity) was imported into R software version 1.9 (R Development Core Team 2004) and normalized using the Linear Models for Microarray Data package (LIMMA version 1.6.5; Smyth *et al.* 2003). Background-subtracted mean intensities (using the minimum method) were normalized using within-array loess normalization. Ratios of intensities were calculated as Cy5 intensities/Cy3 intensities. Ninety-six per cent of the spots on the microarray passed the filter and gave a usable intensity signal (4378 out of a possible 4573 spots), again demonstrating the feasibility and validity of heterologous hybridization with this species.

Statistical analyses

Behaviour, physiology, hormone levels. Univariate two-way analyses of variance were used to determine the effect of dominance status, sex and the interaction of these two factors on variation in behaviours, physiology and hormones. Variables were log-transformed if distribution were not normal based on a Kolmogorov–Smirnov test.

Table 2 Microarray hybridizations performed between pairs of individuals. Individuals in the left column were labelled with a Cy5 dye and individuals in the top row were labelled with a Cy3 dye. D, dominant; S, subordinate; M, male; F, female. For example, cDNA labelled with a Cy5 dye from dominant male 2 (DM2) was competitively hybridized with cDNA labelled with a Cy3 dye from subordinate female 3 (SF3)

		Cy3																		
		DF1	DF2	DF3	DM1	DM2	DM3	SF1	SF2	SF3	SF4	SF5	SF6	SM1	SM2	SM3	SM4	SM5	SM6	
Cy5	DF1	—																1	1	
	DF2		—		1	1		1												
	DF3			—			1													
	DM1	1			—			1												
	DM2			1		—			1				1							
	DM3	1					—													
	SF1		1					—						1						
	SF2								—											
	SF3									—			1				1			
	SF4			1			1				—									
	SF5											—		1						
	SF6											1	—							
	SM1				1									—		1		1		
	SM2									1					—					
	SM3														1	—				
	SM4					1		1									—			
	SM5																1	—	1	
	SM6							1		1									—	

Gene expression. Ratios of intensities were used to determine significant differences in gene expression between dominance ranks using a Bayesian analysis (BAGEL version 3.6; Townsend & Hartl 2002). For each gene, we obtain a relative gene expression level for each node defined by a dominance/sex combination (breeder males, breeder females, helper males, helper females) as well as a Bayesian posterior probability (BPP) of significant differences between two nodes. This method was used preferentially over a traditional analysis of variance to determine genes with significant variation in expression since it is robust to data sets with missing data and variation in replication (Meiklejohn & Townsend 2005). To determine an appropriate significance threshold, we estimated the false-positive rate associated with a given BPP by performing a permutation analysis (Meiklejohn *et al.* 2003; Meiklejohn & Townsend 2005). Specifically, we created a randomized gene expression data set by sampling intensity ratios with replacement from the entire original data matrix and performed the same Bayesian analysis as with the original data set. We repeated these two steps three times. The average number of significant differences in gene expression among dominance/sex combination nodes found in these three random data sets, in which no significant difference should be detected, gives us the level of false-positives to be expected from our data set at a given BPP value. A BPP of 0.9995 gave 0.34% of all the clones as significant by chance. This cut-off was used to declare gene expression variation

to be statistically significant if a BPP was higher than this value between two nodes, as it represented the best trade-off in terms of balancing type I and type II errors (false-positive vs. false-negative rates; Sokal & Rohlf 1995).

Once a gene was identified as significantly differentially expressed between two nodes at BPP 0.9995, we combined information from all the clones present on the microarray representing that gene, if more than one was available, to determine if other comparisons between nodes were significant. We combined the BPP for each clone by calculating the probability of differential expression of the clones (the product of the BPP) divided by the sum of this BPP product + the product of the probability that a clone is not differentially expressed ($1 - \text{BPP}$) as in this formula: $abc / [abc + (1 - a)(1 - b)(1 - c)]$ where a , b and c are the BPPs associated with different clones of the same gene. This new combined probability had to be above 0.9995 to declare the difference in expression significant between two dominance/sex combination nodes. No comparison that was originally significant based on a single clone lost significance after combining BPPs from all the clones associated with the same contig. For a gene to be categorized as variable in expression with dominance independent of sex, it had to show significantly different expression between dominant breeders of both sexes and subordinate helpers of both sexes. A gene that was significantly different between only one sex of dominant individuals vs. subordinates (or vice-versa) was therefore not included in that list of genes.

Covariation of individual characteristics

After determining which genes varied significantly in expression with dominance, we examined the relationship between individual arginine vasotocin (AVT) gene expression and testosterone levels. We determined gene expression level for each of the 18 individuals by re-analysing the microarray data using the same Bayesian analysis method as described above, but with each fish treated as a node. We calculated a Pearson correlation between AVT gene expression levels in individual brains and plasma testosterone measurements obtained individually for each fish. For the 18 individuals used in the microarray study, testosterone data was available for five dominant (2 females, 3 males) and five subordinate (3 females, 2 males) individuals.

Individual expression profiles. Individual gene expression levels were also used in a hierarchical clustering and a principal component analysis (PCA) to determine if individual expression profiles are most similar across dominance status, sex, or both. We collected information on expression level for each individual for a core set of genes that showed significant differential expression when comparing gene expression of the four groups in the original analysis (therefore including differences between males and females of a given dominance status, such that expression differences were not necessarily related to dominance, and included genes that varied in expression with dominance for one sex only), at the same stringent cut-off BPP of 0.9995. This data set is thus larger than the list of genes that vary strictly with dominance independently of sex. This cut-off resulted in data available at the individual expression level for 36 genes. We first performed a clustering analysis of these gene expression profiles using the 'heatmap' function of the 'stats' package in R (version 1.9; (R Development Core Team 2004). Hierarchical clustering of individual expression profiles was based on the dissimilarity between expression levels for a given gene using the complete linkage agglomeration method. Euclidian distance, which integrates effects of amplitude of ratios as well as direction (correlation) in patterns, was used to calculate the dissimilarity matrix.

We also performed a PCA (SPSS software) with the 18 individuals as the variables and the relative expression level of the 36 genes as observations for each variable. After verifying that assumptions for this type of analysis were met by our data set [Kaiser-Meyer-Olkin (KMO) measure of sampling adequacy and Bartlett's test of sphericity], we analysed the correlation matrix and retained the principal components from the initial unrotated extraction with an eigenvalue above 2, based on a scree plot.

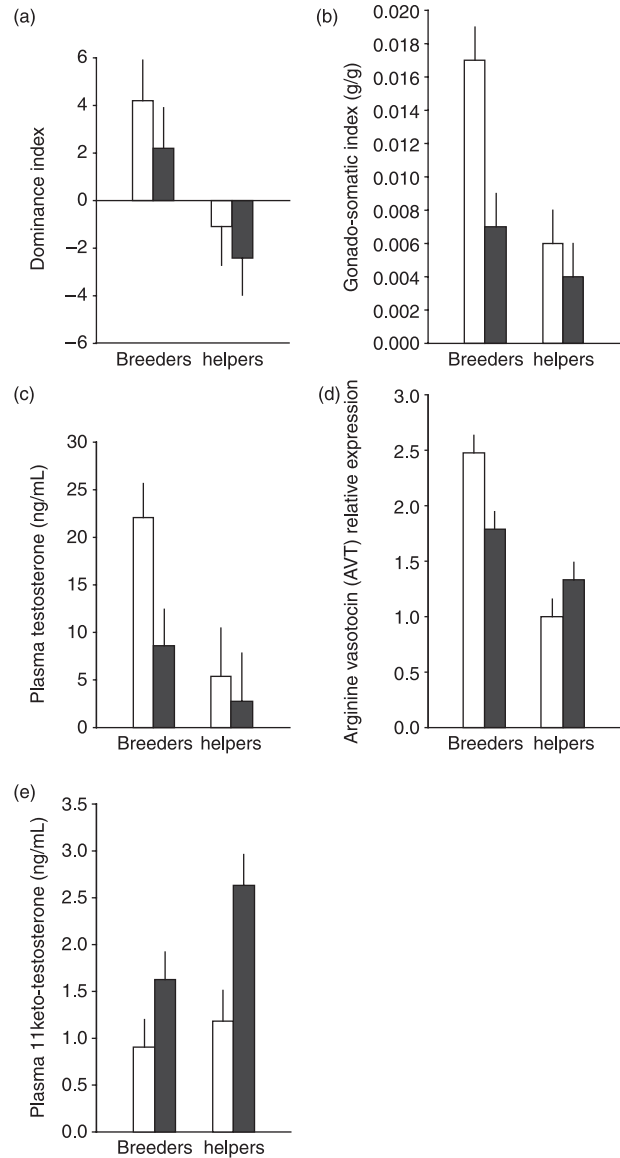


Fig. 1 Dominant breeders and subordinate helpers show differences in behaviour, physiology, hormones and gene expression. Females are represented by open bars and males by filled bars. Individuals of different social ranks differ in terms of their (a) dominance index, (b) reproductive investment, (c) testosterone plasma levels and (d) AVT brain expression. (e) 11-ketotestosterone varies between sexes. Vertical bars represent 1 SE except (d) when bars represent credible intervals.

Results

Each of the 14 *Neolamprologus pulcher* social groups used in this study contained a breeding pair and at least two helpers within a stable dominance hierarchy. We first showed that dominance index, based on the sum of all aggressive behaviours minus the sum of submissive displays of an individual, was higher in breeders than in helpers (2-way ANOVA, $P < 0.005$, Fig. 1a). Condition factor,

Table 3 Genes that vary in expression in the brain of dominant breeders compared to subordinate helpers, independent of sex. The different clones on the array that represent a given gene that were significantly differentially expressed are listed using the TIGR contig number (TC) or the clone ID (hh_Ab) when no TIGR contig number is available

Significant comparisons	Gene annotation	TIGR Contig or clone ID no.	Other significant comp.
Dominant > subordinate	Arginine vasotocin	TC310	BRF > BRM, HM > HF
		h_Ab_StanfordCol_000005672	
		hh_Ab_StanfordCol_000005673	
		hh_Ab_StanfordCol_000005674	
		hh_Ab_StanfordCol_000005705	
		hh_Ab_StanfordCol_000005706	
Subordinate > dominant	Myelin basic protein	TC22	HF > HM
		CD59-like protein	HF > HM
		No annotation	BRM > BRF
		TC25/TC26*	

*TC25 and TC26 probably represent the same gene, based on a BLAST analysis of the two sequences (analysis not shown).

which relates body mass to body length, was significantly higher in dominant breeders than in subordinate helpers (2-way ANOVA, $P < 0.0001$) as was the gonadosomatic index, a measure of investment in reproductive function (2-way ANOVA, $P < 0.0001$, Fig. 1b).

We found that the variation in physiological traits between dominance ranks was reflected in circulating hormone levels and brain gene expression. Testosterone levels in the blood were higher in dominant breeders (both males and females) than in subordinate helpers (2-way ANOVA, $P = 0.02$, Table 1, Fig. 1c). There was significant variation of 11-ketotestosterone with sex, with male breeders and helpers having higher circulating levels than females (2-way ANOVA, $P < 0.0001$, Table 1, Fig. 1e) while progesterone did not vary significantly with social status or sex (2-way ANOVA, $P > 0.6$, Table 1). Four genes were found to vary in expression between the brains of dominant and subordinate fish independently of sex (Table 3) using a conservative Bayesian posterior probability of 0.9995. AVT gene expression was significantly higher in dominant individuals compared to subordinates (Table 3, Fig. 1d). Furthermore, dominant breeder females had significantly higher AVT expression than dominant breeder males, while subordinate helper males had higher expression than subordinate helper females. The three other dominance-related genes, a myelin basic protein, a CD59-like protein and one gene with no significant BLAST hit [represented by The Institute for Genomic Research (TIGR) contigs TC25 and TC26] were significantly higher in expression in subordinate helpers than in dominant breeders (Table 3). We found that the covariation between individual testosterone level and AVT expression was high and significant ($r = 0.86$, $P = 0.001$). As this correlation includes both dominant and subordinate individuals, it indicates that there is variation in these traits not only among but also within the dominance ranks.

Because we were interested in whether an individual's gene expression profiles might be similar to other individuals of the same sex and social status, we then used unsupervised hierarchical clustering to explore whether and how individual fish associate according to the similarity in their gene expression profiles. We used information on gene expression level for each individual for a set of 36 genes that showed significant differences between breeders of one or both sexes when compared to helpers of one or both sexes, as well as differences between sexes within a dominance rank (cut-off BPP of 0.9995). We found that two major clusters of individual expression profiles resulted from this analysis (Fig. 2a). One cluster was composed of the expression profiles of all the helper females and one helper male, while the other cluster included dominant males, dominant females and all the other helper males. We also performed a PCA to uncover the pattern of interrelations among these individuals. We found that two dimensions in the component space accounted for 59.3 [principal component 1 (PC1)] and 17.4% (PC2) of the variance, respectively. The component plots (Fig. 2b) revealed that two distinct groups of individuals loaded on both principal components: the first group contains dominant breeders of both sexes and helper males and the second group contains helper females and one helper male, the same helper male as in the hierarchical clustering (Fig. 2b). We did not find a behaviour (feeding, social, dominant, subordinate, aggressive, helping) or physiological variable (size, sexual maturity, liver size) that would also group this subordinate male with subordinate females rather than with other subordinate males. We repeated this analysis without the expression data for AVT to determine if this gene was driving the groupings and found similar results (data not shown). Therefore, two different analyses show that dominant females were more similar in their brain gene expression profiles to males than to helper females.

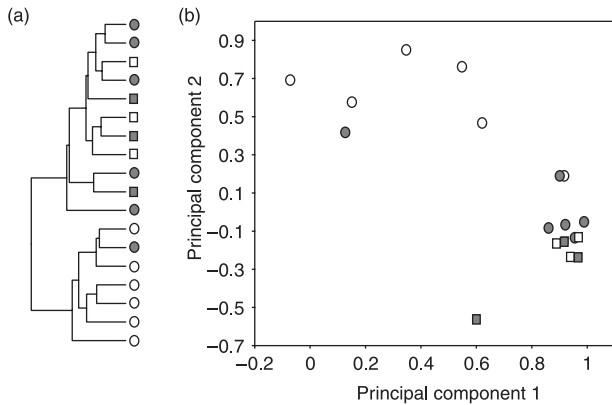


Fig. 2 Unsupervised hierarchical clustering and PCA of gene expression uncovers the masculinized gene expression profiles of dominant breeder females. (a) The main cluster of individuals groups dominant females with males of both dominance status and separates subordinate helper females from other individuals. (b) The component plot shows that two distinct groups of individuals load on both principal components: the first group contains dominant breeders of both sexes and helper males and the second group contains helper females and one helper male, the same one as in the hierarchical clustering analysis in panel a. Females are represented by open symbols and males by filled symbols with dominants represented by a square and subordinates by a circle.

Genes used in the clustering and PCA were the ones that varied significantly in expression between any two dominance/sex combination groups, including between males and females of the same dominance status, indicating that this classification of dominant females' brain expression profiles as 'male-like' is robust.

Discussion

We found a striking similarity between dominant males and females in dominance behaviour, testosterone levels and AVT gene expression compared to subordinate helpers. Moreover, expression profiles of dominant females were most similar to males of both social ranks, resulting in masculinized dominant females that are at the same time reproductively competent. *Neolamprologus pulcher* female breeders, which face the same environment and perform similar behaviours as dominant breeder males, appear to do so by initiating a series of changes in specific molecular pathways that ultimately masculinize their brain and endocrine system to a considerable extent.

The high testosterone level in dominant females can seem puzzling, since androgens typically play an important role in male sexual differentiation and behaviour (Simon 2002). However, in *N. pulcher*, both sexes of the breeding pair aggressively defend the territory against intruders (Balshine-Earn *et al.* 1998; Balshine *et al.* 2001; Desjardins *et al.* 2006).

In fact, dominant females perform the most defence of the territory (Desjardins *et al.* submitted) and both testosterone and 11-ketotestosterone levels have been shown to increase in dominant females in response to a territory challenge by a conspecific intruder (Desjardins *et al.* 2006). It is also known in certain cases that artificial increases of testosterone levels in females of other species of fishes and birds increase their agonistic behaviours (Munro & Pitcher 1985; Ketterson *et al.* 2005; Zysling *et al.* 2006). Taken together, these results suggest that the atypical intense territorial defence behaviour found in dominant females of this species is in part achieved by, and results in, high levels of testosterone comparable to what is found in dominant males. In this context, it is interesting that 11-ketotestosterone (which cannot be aromatized) was significantly lower in dominant and subordinate females than in males of both dominance status. In the same line, it is an interesting paradox that in order to show the array of traits associated with dominance in males, dominant females were male-like in their expression profile for genes that do not vary with dominance in males. Indeed, males of both dominance statuses and dominant females grouped together in the clustering and PCAs of gene expression profiles. In summary, dominant females share dominance-related traits with dominant males (behaviour, testosterone levels, AVT expression), can be characterized as male-like (brain gene expression profiles) and simultaneously female-like (reproductive capacity, 11-ketotestosterone levels). As these dominant females are reproductive, these results hint at a modular organization of molecular and endocrine functions that can be regulated differently in males and females to result in a dominance phenotype, perhaps to allow each sex to cope with specific molecular and endocrine constraints. Cooperatively breeding species such as *N. pulcher* therefore provide a powerful model system for separating the effects of sex and dominance on traits ranging from molecules to behaviour within an organismal context to further our understanding of the evolution of social behaviour. Furthermore, our analysis of dominant females in addition to males challenges established notions of male-specific behavioural, endocrine and molecular profiles, which may be the result of the lack of molecular and endocrine studies on species where females are territorial and aggressive.

In this study, several genes with diverse functions varied in expression between the brains of dominant and subordinate individuals. Some, such as AVT, were candidate genes, while others that have biological functions not usually associated with social behaviour also differed, such as genes related to neural growth and ageing/immune reaction (myelin basic protein and CD59-like protein). TIGR contigs TC25 and TC26 probably represent the same gene, since these two contig sequences assemble into one larger contig (analysis not shown). This gene, which is

over-expressed in subordinate individuals independent of sex, has no known homologue and no functional annotation in the public sequence databases, which makes it a novel and interesting gene to investigate further (e.g. by determining its distribution in the brain). AVT and its mammalian homologue AVP have been linked to aggressive (dominance, territoriality) and reproductive behaviours in fishes and birds, and to male-male aggression, social affiliation and partner preference in mammals (Dantzer *et al.* 1988; Goodson & Bass 2001; Insel & Young 2001; Semsar *et al.* 2001; Goodson *et al.* 2004; Semsar & Godwin 2004; Oliveira *et al.* 2005). *Neolamprologus pulcher* shows both territoriality and pair bonding, social behaviours that are similar to what is found in mammal species such as voles that have been the focus of studies on the link between social affiliation and AVP. Our results bridge a gap between mammalian and other vertebrate systems and open an entirely new avenue to study the functional role of AVT in the context of both aggression and social affiliation in the same fish model system. Previous studies in fishes, birds and mammals have found a relationship between testosterone or 11-ketotestosterone levels, AVT/AVP and behaviour, while other studies have not (see for example Delville *et al.* 1996; Goodson & Bass 2001; Viglietti-Panzica *et al.* 2001; Semsar & Godwin 2004; Oliveira *et al.* 2005). The combination of the observed strong positive association between testosterone levels and AVT gene expression across individuals in our study, the various results from these other independent reports and the proposition that selection may act differentially on testosterone levels depending on the social mating system (Ketterson *et al.* 2005) call for further functional studies to assess if general rules can be outlined for different social systems, both for AVT and steroid hormones. Pharmacological manipulations of AVT, of its receptors, and of androgens in *N. pulcher* will help to tease apart the functional significance of higher AVT expression and testosterone levels in dominant individuals and to determine whether the AVT system is involved in pair bonding in fish species.

Investigating a system at different levels of biological organization, from behaviour to hormones and gene expression, provides new insights into vertebrate social dominance in general. The molecular and endocrine masculinization of the female brain depending on social status is likely not limited to fishes. This finding underscores the need for a comparative approach in a wide range of vertebrates with diverse patterns of social organization to determine where similar molecular and endocrine substrates regulate social life and where they have evolved independently. The astonishing diversity of social systems and life histories found in the nearly 2000 species of African cichlids (Barlow 2000), together with their genetic homogeneity and the ease of laboratory manipulation, makes

these fish uniquely suited for deciphering the molecular basis and evolution of vertebrate social behaviour.

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Authors' contribution: N.A.H., J.K.D., S.B. and H.A.H. conceived of the project and designed the experiment. J.K.D. performed the behaviour experiments. J.K.D. performed the radio-immuno assays. N.A.H. and Y.M.M. performed the microarray experiments. N.A.H. and J.K.D. analysed the data. N.A.H. performed the statistical and bioinformatics analyses. N.A.H. wrote the manuscript together with H.A.H. and S.B. We are grateful to Kelly Stiver for conducting some of the behavioural observations and brain extractions, to Susan Marsh-Rollo for assistance with feeding and maintaining the fish groups, to Christian R. Landry for advice on bioinformatics analyses and to Nina Duftner, Anna Greenwood, Christian R. Landry, Suzy Renn and Kelly Stiver for comments on earlier versions of this manuscript. This work was supported by a postdoctoral fellowship from the Natural Science and Engineering Research Council of Canada (NSERC) (N.A.H.); an NSERC graduate fellowship (J.K.D.); a Harvard College Research Program grant and a summer research internship award from the Bauer Center for Genomics Research (Y.M.M.); an NSERC discovery grant, a Canadian Foundation for Innovation (CFI) and an Ontario Innovation Trust (OIT) New Opportunities Awards (S.B.); as well as by a National Institutes of Health grant NIGMS GM068763 and the Bauer Center for Genomics Research (H.A.H.).

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