Hormones and Behavior 80 (2016) 30–38

Contents lists available at ScienceDirect

Hormones and Behavior

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

Species-specific patterns of nonapeptide brain gene expression relative to pair-bonding behavior in grouping and non-grouping cichlids

Constance M. O'Connor [a,*], Susan E. Marsh-Rollo [a], Nadia Aubin-Horth [b], Sigal Balshine [a]

[a] Aquatic Behavioural Ecology Lab, Department of Psychology, Neuroscience, and Behaviour, McMaster University, 1280 Main Street West, Hamilton, Ontario L8S 4K1, Canada
[b] Département de Biologie and Institut de Biologie Intégrative et des Systèmes, Université Laval, Québec, Québec G1V 0A6, Canada

ARTICLE INFO

Article history:
Received 30 June 2015
Revised 18 October 2015
Accepted 24 October 2015
Available online 28 October 2015

Keywords:
Selective attachment bonding
Pair bonding
Social evolution
Lamprologine
Teleost

ABSTRACT

Comparative studies have revealed that vasopressin–oxytocin pathways are associated with both pair bonding and grouping behavior. However, the relationship between pair bonding and grouping behavior remains unclear. In this study, our aim was to identify whether two species that differ in grouping behavior display a corresponding difference in their pair bonds, and in the underlying vasopressin–oxytocin hormonal pathways. Using two species of cichlid fishes, the highly social *Neolamprologus pulcher* and the non-social *Telmatochromis temporalis*, we measured proximity of pairs during pair bond formation, and then measured social behaviors (proximity, aggression, submission, affiliation) and brain gene expression of isotocin and arginine vasotocin (the teleost homologues of oxytocin and vasopressin, respectively), as well as their receptors, after a temporary separation and subsequent reunion of the bonded pairs. Pairs of the social species spent more time in close proximity relative to the non-social species. Rates of aggression increased in both species following the separation and reunion treatment, relative to controls that were not separated. Overall, whole brain expression of isotocin was higher in the social species relative to the non-social species, and correlated with proximity, submission, and affiliation, but only in the social species. Our results suggest that both a social and a non-social cichlid species have similar behavioral responses to a temporary separation from a mate, and we found no difference in the brain gene expression of measured hormones and receptors based on our separation–reunion treatment. However, our results highlight the importance of isotocin in mediating submissive and affiliative behaviors in cichlid fishes, and demonstrate that isotocin has species-specific correlations with socially relevant behaviors.

Introduction

Pair bonding, or the preferential interaction of individuals to the exclusion of other potential partners, forms the basis for many social interactions, and therefore represents an interesting subset of social behavior. The processes that underlie the formation and maintenance of pair bonds are complex, and have been studied from both a behavioral and a mechanistic point of view (see review by Lim and Young, 2006). At the behavioral level, pair bonding can be broken down into three underlying components, all of which appear to be mediated at the mechanistic level by the vasopressin–oxytocin family of nonapeptide hormones (see review by Lim and Young, 2006). At the first stage of pair bonding, the individual must be motivated to approach a conspecific. Both oxytocin and vasopressin appear to modulate the motivation to approach other conspecifics. Rats (*Rattus norvegicus*) and mice (*Mus musculus*) treated with an oxytocin receptor antagonist reduced social approach, while socially defeated rats given oxytocin showed less social avoidance relative to controls (Lukas et al., 2011). In goldfish (*Carassius auratus*), treatment with isotocin (IT, the teleost fish homologue of oxytocin; Hoyle, 1999) increased social approach, while treatment with arginine vasotocin (AVT, the fish homologue of vasopressin; Hoyle, 1999) decreased social approach (Thompson and Walton, 2004). At the second stage of pair bonding, where individuals must be able to differentiate familiar and unfamiliar conspecifics, these two nonapeptides again appear to have important influence. For example, transgenic mice that lack the oxytocin gene are unable to recognize familiar individuals despite repeated exposure (Choleris et al., 2003; Ferguson et al., 2000), but their ability to recognize a familiar individual can be restored by treatment with oxytocin (Ferguson et al., 2001). Finally, at the third stage of social bonding, the individual must form a pair bond with the familiar conspecific such that the individual preferentially interacts with that conspecific, to the exclusion of other potential social partners (Lim and Young, 2006). Comparisons of closely related *Microtus* vole species suggest that differences in the distribution of oxytocin and vasopressin receptors are related to differences in the degree of social bonding (Insel and Shapiro, 1992; Insel et al., 1994; Young and Wang, 2004). In non-mammalian vertebrates, a general AVT/IT receptor antagonist delayed the formation of new pair bonds in the monogamous convict cichlid (*Amatitlania nigrofasciata*) but did not disrupt bonds in established pairs (Oldfield and Hofmann, 2011).

* Corresponding author.
E-mail address: coconn@mcmaster.ca (C.M. O'Connor).

http://dx.doi.org/10.1016/j.yhbeh.2015.10.015
0018-506X/

The relationship between the capacity for pair bonding and grouping behavior remains unclear, at both the behavioral and mechanistic level (Goodson, 2013). For group-living species with individual recognition, pair bonds form the basis for all subsequent within-group interactions. Therefore, it could be predicted that group-living animals will show stronger pair bonds relative to non-grouping animals, since they form multiple bonds with many individuals. Alternatively, it is possible that group-living animals have weakened pair bonds relative to non-grouping animals, since they routinely interact with many different individuals, and may be less selective in the choice of their social partners than more solitary animals that interact with only their mates and offspring. At the mechanistic level, the vasopressin–oxytocin family of nonapeptide hormones, as well as being linked to pair bonding behavior, has also been implicated as the mechanistic substrate for grouping behavior. For example, species-typical group size is related to nonapeptide receptor distribution in estrilid finches, and treating normally gregarious zebra finch (*Taeniopygia guttata*) with an oxytocin receptor antagonist reduced their preference for associating with large social groups (Goodson et al., 2009). Our aim in this study was therefore to identify whether species that vary in their grouping behavior display a corresponding difference in the strength and resilience of their pair bonds, and to understand the relationships among grouping, pair bonding, and vasopressin–oxytocin nonapeptide hormonal pathways.

In this study, we investigated the relationships among social system, the strength and resilience of pair bonds, and brain gene expression of the oxytocin–vasopressin family of nonapeptide hormones in two closely related species of Lamprologine cichlids, *Neolamprologus pulcher* and *Telmatochromis temporalis* (Day et al., 2007). *N. pulcher* is a group-living, cooperatively breeding cichlid that lives in permanent social groups comprised of a dominant breeding pair, and up to 20 subordinate conspecifics who jointly maintain and defend the territory (see review by Wong and Balshine, 2011). *N. pulcher* form pair bonds between the dominant male and female in each group, have social bonds among group members, and are strongly aggressive to non-group members. In contrast, *T. temporalis* is a non-grouping species that does not show any cooperative behaviors (Mboko and Kohda, 1999; Katoh et al., 2005; Heg and Bachar, 2006). *T. temporalis* forms pair bonds only between mates, and bonded pairs are aggressive to all other conspecifics, tolerating only their mate and very young offspring. However, the two species are otherwise similar. Both species are endemic to the rocky littoral zone of Lake Tanganyika, East Africa (Kuwamura, 1986; Brichard, 1989; Konings, 1998), approximately 4–6 cm long when mature, spawn under rocky shelters, and provide biparental care (Kuwamura, 1986; Brichard, 1989; Konings, 1998; Sefc, 2011). Both species can be monogamous (Kuwamura, 1986; Sefc, 2011), with opportunistic polygyny (Limberger, 1983; Mboko and Kohda, 1999; Desjardins et al., 2008; Wong et al., 2012). Females are socially monogamous, although there is genetic evidence of extra-pair paternity in both species (Katoh et al., 2005; Dierkes et al., 2008; Hellmann et al., 2015a, 2015b).

We tested the hypotheses that a highly social, group-living species would form pair bonds more quickly, re-establish pair bonds better following a perturbation, and show larger differences in brain nonapeptide hormone gene expression after re-establishing a pair bond compared to more solitary, non-grouping species. To do so, we performed a laboratory study in which we measured behavior first during pair bond formation, and then following a temporary separation and subsequent reunion between the bonded mates. We then measured whole brain gene expression in the same individuals. We predicted that pairs of the social species would spend more time in close proximity during the initial pair bond formation, and would attempt to re-establish a pair bond more quickly following a temporary separation relative to the non-social species. Since IT and AVT pathways have been related to social behavior such as aggression, submission, and affiliation across many species, including *N. pulcher* (Aubin-Horth et al., 2007; Reddon et al., 2012, 2014, 2015; Hellmann et al., 2015a, 2015b), we predicted that whole brain expression of these genes would show a more pronounced change following the temporary separation and reunion in the social species relative to the non-social species (i.e., that there would be variation in genomic reaction norms; Aubin-Horth and Renn, 2009). Finally, beyond these predicted differences at the species level, we predicted that we would see correlations between social behavior and brain nonapeptide gene expression at the individual level.

Methods

Study animals and experimental design

The experiment was conducted January–February 2014 at McMaster University in Hamilton, ON, Canada. Fish were sexually mature, laboratory-reared descendants of wild-caught fish from Lake Tanganyika. All fish were measured for body size (standard length, SL) using calipers, and body mass using an electronic scale, and sexed by examination of the external genital papillae. Each fish was given a unique dorsal fin clip for identification, which does not adversely affect behavior and grows back within two weeks (Stiver et al., 2004). To form pairs, one male and one female of the same species that were previously unfamiliar with one another were placed together in a 200 L aquarium containing 3 cm of coral sand substrate, a water filter, heater, a thermometer, and 2 flowerpot halves as shelters. Pairs were formed such that the male was always 5–15% larger than the female, which is the range of sexual dimorphism observed in wild pairs (Balshine et al., 2001). Since *N. pulcher* live and breed in social groups, each *N. pulcher* pair was also housed with 2–4 small (SL < 20 mm) sexually immature individuals. The water temperature of all aquaria was held at 26 ± 2 °C, and all fish were fed dried prepared cichlid food ad libitum six times per week, and kept on a 13:11 light:dark cycle.

Bonding score

To assess how rapidly each species forms pair bonds, each pair was observed during the early phase of pair bond formation. For 3 days after the pairs were first introduced, each pair was observed once per day for 2 min, and scored as either 'together' (within a body length of each other, using the body length of female as the reference; Dey et al., 2013) or 'apart' (more than a body length apart from each other) for the majority of the observation period.

Social bond disruption

After a 7–9 day pair bond formation phase, the pairs were randomly assigned to either a 'separation' or a 'control' treatment. In the 'separation' treatment, pairs were separated for 60 min by an opaque barrier. The barrier was then removed and the fish observed for 10 min by an experienced observer. In the 'control' treatment, the fish remained together with no disruption, and then were similarly observed for 10 min. For the observation, the fish were scored as either 'together' or 'apart' using the criteria described above. The behaviors were also scored throughout the 10 min observation period based on an ethogram (Supplementary Table 1). Briefly, behaviors recorded included aggressive, submissive, and affiliative behaviors. Aggressive behaviors were displays such as aggressive head-down postures and frontal displays, as well as overt aggressive acts with physical contact, such as chases, rams, bites, or mouth wrestles. Submissive behaviors are produced by these cichlids in response to aggression from another individual, and consist of head-up submissive postures, quivering submissive displays, as well as fleeing from the aggressor. Affiliative behaviors are spontaneously produced towards another individual, and include behaviors such as swimming closely in parallel, and soft touches. Both submissive and affiliative behaviors represent appeasement gestures, and function to reduce aggression between group members (Bergmüller and Taborsky, 2005; Dey et al., 2013).

Tissue sampling

The fish were given an additional 50 min to allow for any changes in brain gene expression as a result of the separation and reunion treatment (i.e., 50 min after the behavioral observation for all fish; 60 min total following the reunion for the separation treatment fish). Each pair was then captured, stunned by submersion in an ice bath, and the heads severed and brains removed and preserved in RNAlater (Invitrogen, Carlsbad, CA). Fish were dissected, the liver and the gonads weighed, and sex confirmed. Vials containing the brains were refrigerated at 4 °C for 12 h before being transferred to a −20 °C freezer for storage until analysis of brain gene expression levels. See Table 1 for sample sizes and measured traits of the fish used.

Analysis of gene expression by quantitative real-time PCR

We follow the guidelines of the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE; Bustin et al., 2009). Brain gene expression levels of isotocin (IT) and arginine vasotocin (AVT), as well as of their receptors, were quantified using quantitative real-time PCR (RT-qPCR). Cichlids possess several paralogous receptors that bind AVT: V1a1, V1a2, V1b, and V2 receptors (Lema, 2010). The V1a2 receptor has been the most widely implicated in social behavior in a range of fish species (Lema, 2010; Kline et al., 2011; Huffman et al., 2012; Oldfield et al., 2013). Thus, we examined the brain gene expression of the AVT receptor V1a2 (AVTR). There are two paralogous isotocin receptors in fish (O'Connor et al., 2015), IT receptor 1 (ITR1) and IT receptor 2 (ITR2), and we measured both. See O'Connor et al. (2015) for sequences of all the five candidate genes studied, as well as the 18S reference gene, for *N. pulcher* and *T. temporalis*. We used RT-qPCR-specific primers that were designed for use in both species, optimized for the same conditions, and with known efficiencies based on previous experiments (O'Connor et al., 2015).

All assays were performed February–March 2014. Brains were thawed and individually homogenized, and total RNA extracted using the standard TRIzol reagent protocol (Invitrogen). The concentration and quality of RNA were analyzed using a Nanodrop spectrophotometer (Thermo Scientific, Wilmington, DE) and a subset from each species was checked for integrity using an Aligent RNA analysis kit (Aligent Technologies, Santa Clara, CA). All samples had an RNA Integrity Number (RIN) higher than 8.0, which is considered acceptable for qPCR analysis (Fleige et al., 2006; Schroeder et al., 2006). Prior to cDNA synthesis, aliquots of 2000 ng of RNA were treated with amplification grade DNase (Invitrogen) to eliminate genomic DNA contamination. First strand cDNA was then synthesized from DNase-treated total RNA using SuperScript II Reverse Transcriptase (Invitrogen), with RNaseOUT recombinant ribonuclease inhibitor (Invitrogen), and a mix of random hexamer (100 ng μL^{-1}) and oligo dT primers (500 ng μL^{-1}), using a 96-well PCR machine (MasterCycler, Eppendorf, Mississauga, ON) with a 42 °C incubation and 70 °C inactivation temperature.

Gene expression of individuals was measured using a 384-well plate RT-qPCR machine (Light Cycler, Roche, Basel, Switzerland) using a scaled-down version of the manufacturer's protocol, with a total volume of 15 μL comprised of 5 μL of cDNA, 7.5 μL of SYBR Green PCR Master Mix (Invitrogen), 1.5 μL of nuclease free water (Ambion, Carlsbad, CA), and 1 μL of primer pairs. All individuals were assayed in triplicate for a given gene on a single 384-well plate prepared using an EpMotion liquid handler (Eppendorf, Hamburg, Germany), and the mean Cq value was used for each fish. For purposes of comparison, mRNA abundance of each focal individual for each gene of interest was calculated relative to the mean mRNA abundance of the control males of the non-social species. Relative mRNA abundance of the gene of interest was then expressed against the relative reference gene 18S, and calculated according to the $\Delta\Delta C_t$ method (Pfaffl, 2001).

Statistical analyses

During the early pair formation period, the observation periods that the pair of fish was scored as 'together' were summed, and this was used as a 'bonding' score. During the post-treatment period, the number of times that the pair of fish was scored as 'together' during the observation was summed, and this was used as a 'proximity' score. The total number of aggressive behaviors, submissive behaviors, and affiliative behaviors performed by each individual was summed, and was considered 'aggression', 'submission', and 'affiliation', respectively. Bonding scores during the early pair formation period were compared between species, and proximity scores during the post-treatment observation period were compared between species and treatments, using cumulative link models for ordinal data in the 'ordinal' package (Christensen, 2015). Total aggression, submission, and affiliation during the post-treatment observation period were compared between sexes, species, and treatments, with 'pair' included as a random effect to account for non-independence of data, using generalized linear mixed models with negative binomial error distributions and log-link functions, using the 'MASS' package (Venables and Ripley, 2002; Ripley et al., 2015).

Relative brain gene expression was compared between sexes, species, and treatments using general linear mixed models, with pair included as a random effect. The relationship between behavior (proximity score, aggression, submission, affiliation) and brain gene expression was also considered using general linear mixed models. For only those genes where we found an effect of sex, species, or treatment (see above), this effect and the interaction effect were included in the model. For all models, pair was included as a random effect.

All initial models contained two-way interaction effects, and if no interaction effects were significant (all $p > 0.05$), then the interaction terms were dropped from the final models. All statistics were performed in R version 3.2.0 (R Development Core Team, 2015) within RStudio (Racine, 2012).

Ethical note

We minimized handling time and stress as much as possible during all procedures. The methods described for animal capture, housing, and euthanasia were assessed and approved by the Animal Research Ethics Board of McMaster University (Animal Utilization Protocol No. 14-02-

Table 1
Measured morphological characteristics (standard length and mass) and sample sizes of the fishes used in the current study. Data from real-time quantitative PCR (RT-qPCR) comes from the same individuals used for behavioral data collection. However, with the exception of *N. pulcher* control females and *N. pulcher* separation males, RT-qPCR data were not obtained from all individuals, which leads to different sample sizes for the behavioral data and RT-qPCR data. All values are presented as mean ± standard error of the mean (SEM).

Species	Treatment	Sex	Data type	Sample size	Standard length (mm)	Mass (g)
N. pulcher	Control	Male	Behavior	11	75.0 ± 1.6	12.4 ± 0.9
			RT-qPCR	7	76.9 ± 1.7	12.8 ± 1.1
		Female	Behavior & RT-qPCR	11	68.7 ± 1.6	9.4 ± 0.7
	Separation	Male	Behavior & RT-qPCR	9	76.7 ± 2.4	12.3 ± 0.9
		Female	Behavior	9	68.3 ± 1.9	9.1 ± 0.7
			RT-qPCR	8	67.6 ± 2.0	8.9 ± 0.8
T. temporalis	Control	Male	Behavior	10	67.4 ± 2.3	9.1 ± 0.5
			RT-qPCR	8	68.2 ± 2.9	9.0 ± 0.6
		Female	Behavior	10	57.1 ± 1.5	5.0 ± 0.4
			RT-qPCR	8	57.4 ± 1.8	4.8 ± 0.5
	Separation	Male	Behavior	11	67.3 ± 1.8	8.9 ± 0.6
			RT-qPCR	10	67.1 ± 1.9	8.7 ± 0.7
		Female	Behavior	11	55.9 ± 2.1	5.1 ± 0.4
			RT-qPCR	9	57.0 ± 2.2	5.2 ± 0.5

Table 2
Statistical output from generalized linear mixed models investigating differences in bonding scores based on species (social *N. pulcher* vs. non-social *T. temporalis*); proximity scores based on species and treatment (separation vs. control); and aggression, submission, and affiliation based on species, treatment, and sex (male vs. female). For bonding scores and proximity, a single score was used for each pair. For aggression, submission, and affiliation, 'pair' was included as a random effect to account for non-independence of mated pairs. The full sample of individuals was used for all tests (see Table 1 for sample sizes). For all tests, two-way interactions were included in the original models, and dropped from the final models if they were non-significant. Bold italics indicate significant model terms ($\alpha = 0.05$). See Methods for full statistical details.

Behavior	Model term	Estimate	Standard error	z-Value	p-Value
Early pair formation bonding score	***Species***	***−2.07***	***0.65***	***−3.20***	***0.001***
Post-treatment proximity score	***Species***	***−1.23***	***0.59***	***−2.07***	***0.04***
	Treatment	−0.54	0.58	−0.94	0.35
Aggression	Species	−0.51	0.46	−1.12	0.26
	Treatment	***1.17***	***0.46***	***2.53***	***0.01***
	Sex	***1.08***	***0.46***	***2.36***	***0.02***
Submission	Species	−0.02	0.44	−0.04	0.97
	Treatment	0.36	0.44	0.81	0.42
	Sex	***−2.90***	***0.60***	***−4.82***	***<0.001***
Affiliation	Species	−0.48	0.30	−1.57	0.12
	Treatment	0.26	0.30	0.87	0.39
	Sex	−0.20	0.30	−0.67	0.50

05), and adhered to Canadian laws, as well as the guidelines of the Canadian Council for Animal Care and the Animal Behaviour Society/Association for the Study of Animal Behaviour.

Results

During the early pair formation period, pairs of the social species, *N. pulcher*, spent more time within a body length of one another compared to pairs of the non-social species (Table 2; Fig. 1A). Pairs from the social species also spent more time in close proximity following a temporary separation and reunion (Table 2; Fig. 1B), with the separation–reunion treatment having no effect on proximity scores in either species (Table 2).

Males performed more overall aggression towards their mates than females (Table 2; Fig. 2A), and fish subjected to the separation treatment performed more aggression towards their mate when reunited relative to the control fish that were never separated (Table 2; Fig. 2B). Females were more submissive towards their mates than males (Table 2; Fig. 2C), but there was no effect of the separation and reunion treatment on rates of submission (Table 2; Fig. 2D). We found no differences in aggression or submission based on species (Table 2), and no differences in rates of affiliation between the pairs based on species, sex, or treatment (Table 2).

IT brain gene expression was higher in the social *N. pulcher* relative to the non-social *T. temporalis*, with no effect of sex or treatment (Table 3; Fig. 3A). AVT was higher in males than in females, again with no effect of species and treatment (Table 3; Fig. 3B). We found no differences in any of the measured receptors based on species, sex, or treatment (Table 3).

Brain gene expression of IT was not related to aggression (Table 4; Fig. 4B). Brain gene expression of IT was, however, significantly positively correlated with proximity scores (Table 4; Fig. 4A), rates of submission (Table 4; Fig. 4C), and rates of affiliation (Table 4; Fig. 4D), but this pattern was observed only in the social species, *N. pulcher* (Table 4; Fig. 4C and D). There were no significant relationships between any behavior recorded and brain gene expression of AVT, or with any of the studied receptors (Tables 4–5).

Discussion

Are there differences in the strength or resilience of pair bonds between a social and non-social species?

In this study, we found few differences in pair bonding behavior between the highly social *N. pulcher*, and the non-social *T. temporalis*. Our behavioral results therefore suggest that pair bonding and grouping are not correlated, but instead may be additive behavioral processes. We found that pairs of the group-living, highly social *N. pulcher* spent more time within a body length of one another relative to pairs of the non-social non-grouping *T. temporalis* during pair bond formation, once pair bonds were established, and following a disruption to the pair bond. Since proximity is a common measure of the strength of pair bonds (Williams et al., 1992), this result provides some evidence that the social *N. pulcher* form stronger pair bonds than the non-social *T. temporalis*. However, we found no difference between *N. pulcher* and *T. temporalis* in the specific behaviors that were performed following a temporary separation and subsequent reunion, which suggests that the two species use similar behavioral strategies to re-establish a pair bond when it is perturbed. We reconcile these findings by suggesting that *N. pulcher* are more gregarious overall, and have a greater propensity to associate with conspecifics in close proximity relative to *T. temporalis*, but this is not associated with a difference in pair bonding between mates. Complex group-living behavior is thought to have evolved from a combination of more simple social behaviors, and

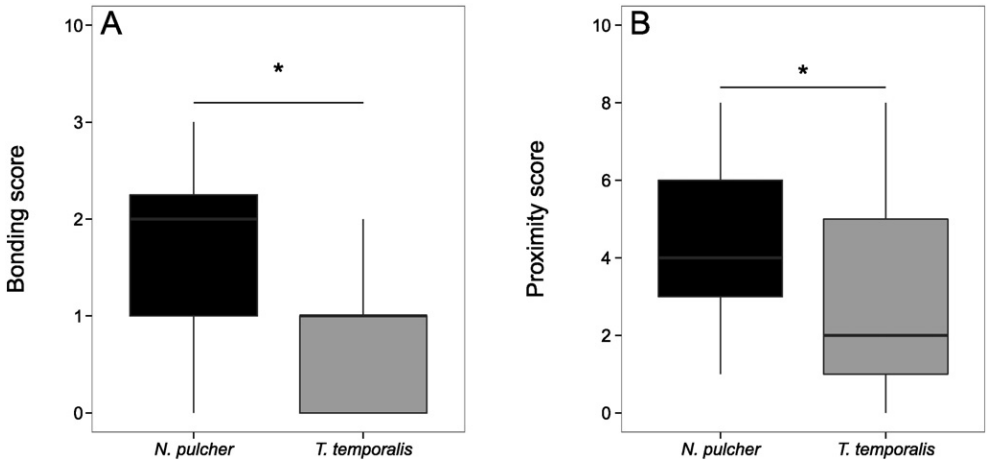

Fig. 1. Bonding and proximity scores, which are indicators of how much time pairs of fish spend within a body length of one another and therefore have the potential to interact, for social *N. pulcher* and non-social *T. temporalis* cichlid fishes. Asterisks indicate significant differences between groups. Both (A) bonding scores during the early pair formation period and (B) proximity scores during the post-treatment observation period were higher in the social *N. pulcher* relative to the non-social *T. temporalis*. See Methods and Table 2 for full statistical details.

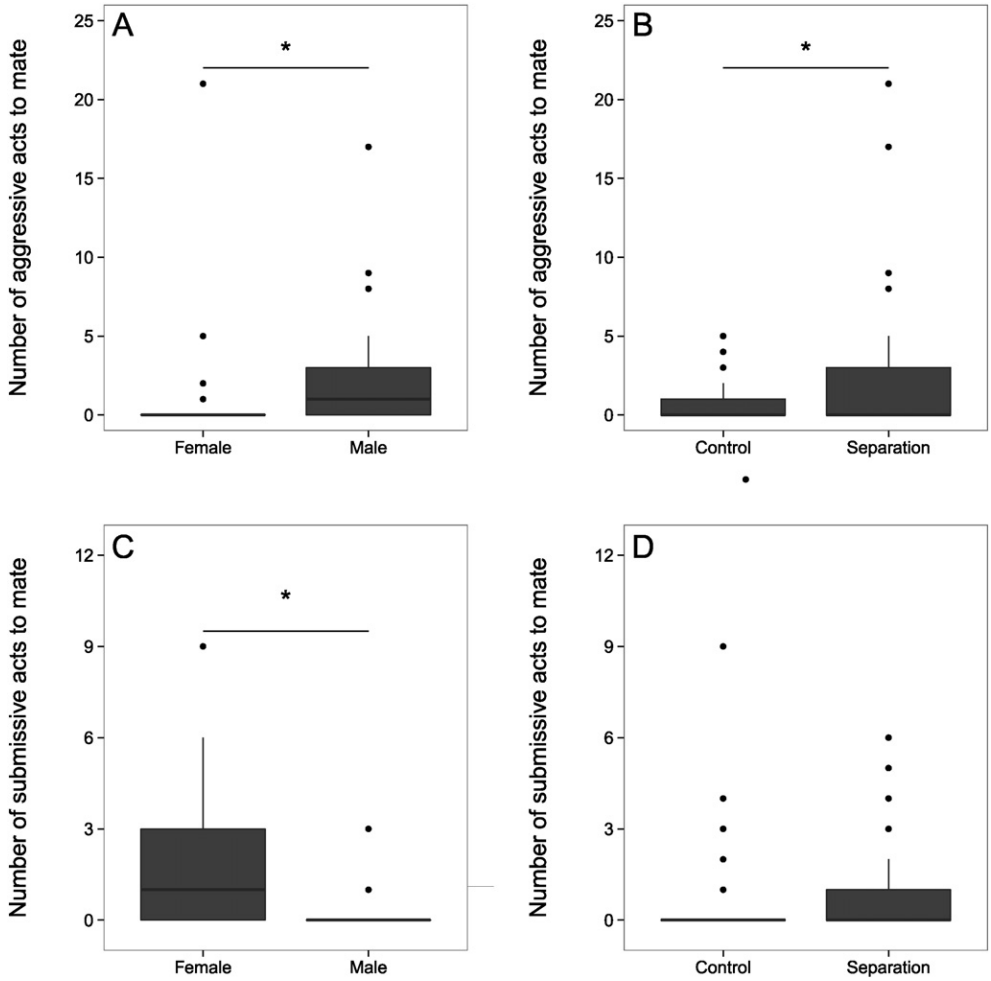

Fig. 2. Behavior of male and female social *N. pulcher* and non-social *T. temporalis* cichlid fishes that were either temporality separated from their mate (i.e., separation treatment) or left together (i.e., control). Asterisks indicate significant differences between groups. There was no effect of species on fish behavior. However, (A) males produce more aggression than females, and (B) fish separated from the mates produce more aggression than control fish. (C) Females are more submissive than males, (D) with no effect of treatment on submission. See Methods and Table 2 for full statistical details.

Table 3
Statistical output from general linear mixed models investigating differences in relative brain gene expression based on species (social *N. pulcher* vs. non-social *T. temporalis*), treatment (separation vs. control), and sex (male vs. female). For each test, 'pair' was included as a random effect to account for non-independence of mated pairs. A subset of individuals was used for all tests (see Table 1 for an explanation of the sample sizes). For all tests, two-way interactions were included in the original models, and dropped from the final models if they were non-significant. Bold italics indicate significant model terms ($\alpha = 0.05$). See Methods for full statistical details.

Gene	Model term	Estimate	Standard error	t-Value	p-Value
IT	***Species***	***−1.21***	***0.55***	***−2.21***	***0.03***
	Treatment	0.44	0.55	0.80	0.43
	Sex	−0.51	0.55	−0.92	0.36
ITR1	Species	−0.28	0.19	−1.51	0.14
	Treatment	0.02	0.19	0.12	0.90
	Sex	−0.16	0.19	−0.86	0.40
ITR2	Species	0.11	0.13	0.85	0.40
	Treatment	0.13	0.13	0.98	0.33
	Sex	−0.18	0.13	−1.39	0.17
AVT	Species	−0.13	0.44	−0.30	0.76
	Treatment	0.10	0.44	0.23	0.82
	Sex	***1.02***	***0.44***	***2.34***	***0.02***
AVTR	Species	0.12	0.16	0.71	0.48
	Treatment	−0.07	0.16	−0.42	0.67
	Sex	−0.20	0.16	−1.20	0.23

gradual changes to these basic behaviors (Soares et al., 2010), and in Tanganyikan cichlids, pair bonding is the ancestral state (Sefc, 2011), while gregarious grouping behavior is derived. Thus, while the pair bonds explored in the current study may form a building block in the evolution of grouping behavior, the increased number of bonds necessitated by group-living and cooperation in species such as *N. pulcher* does not appear to either strengthen or dampen the basic pair bond between mates. It has been suggested that social grouping in primates represents an extension of pair bonding mechanisms to include non-pair individuals (Dunbar and Shultz, 2007). Therefore, it would be valuable to compare the strength and nature of selective bonds between mates in social and non-social species with the bonds observed between other group members in the social species.

Our results support the notion that a separation perturbed the social pair bond relationship in both species, and behavioral strategies were necessary to re-establish this relationship. We found that both species were affected by a perturbation to the pair bond, and displayed increased aggression towards their mate following a temporary separation and subsequent reunion. The strategies used were more biased towards aggressive behaviors than we had a priori predicted, based on previous research in mammalian (e.g., Boissy and Le Neindre, 1997; Shepherd and French, 1999) and avian (e.g., Remage-Healey et al., 2003; Lendvai and Chastel, 2008) systems, which typically reports

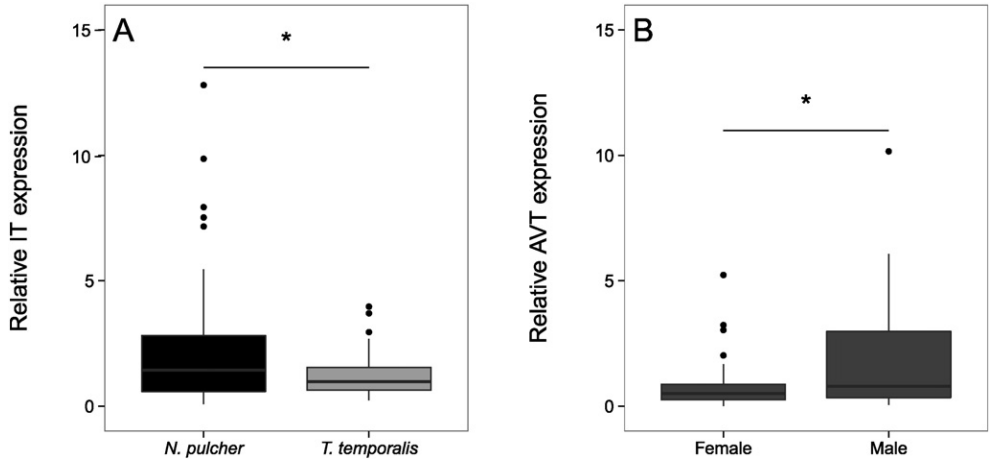

Fig. 3. Brain gene expression of male and female social *N. pulcher* and non-social *T. temporalis* cichlid fishes. Asterisks indicate significant differences between groups. (A) Isotocin (IT) is higher in the social *N. pulcher* relative to the non-social *T. temporalis*. (B) Arginine vasotocin (AVT) is higher in males than females. See Methods and Table 3 for full statistical details.

that mates have increased affiliation when reunited following a separation. We had predicted that the social *N. pulcher* would display increased submission following a temporary separation relative to control fish, and relative to *T. temporalis*. *N. pulcher* use more submissive displays than *T. temporalis* during staged territorial contests between same-sex conspecifics, and *N. pulcher* are especially likely to use submissive displays when the contestants are familiar (Hick et al., 2014). Submissive displays are used as a signal that an individual is willing to take on a subordinate position, and are an important step in the evolution of grouping and cooperative behavior because they allow for the establishment and maintenance of a stable dominance hierarchy with minimal physical aggression (Reddon and Reader, 2015). In this study, since we found no difference in submission rates between mated pairs based on the separation and reunion treatment, and no difference in submission rates between the social and more solitary species, we hypothesize that subordinate displays may be specific to hierarchy formation, and are needed as an additional step in the evolution of group formation, beyond the behaviors used to establish pair bonding. Future research that compares the use of submissive displays in *N. pulcher* during the pair bonding process between group members (e.g., when a new group member joins the group) and between pair bonded mates is necessary to confirm this hypothesis.

Table 4
Statistical output from general linear mixed models investigating differences in the relationship between relative brain gene expression and behavior, for genes where an overall effect of species, sex, or treatment was found (see Table 3). For each test, 'pair' was included as a random effect to account for non-independence of mated pairs. A subset of individuals was used for all tests (see Table 1 for an explanation of the sample sizes). For all tests, two-way interactions were included in the original models, and dropped from the final models if they were non-significant. Bold italics indicate significant model terms ($\alpha = 0.05$). See Methods for full statistical details.

Gene	Behavior	Estimate	Standard error	t-Value	p-Value
IT	***Proximity***	***0.52***	***0.18***	***2.94***	***0.005***
	Species	1.50	1.04	1.44	0.16
	Proximity × species	***−0.63***	***0.22***	***−2.87***	***0.006***
	Aggression	−0.01	0.07	−0.10	0.92
	Species	***−1.21***	***0.55***	***−2.22***	***0.03***
	Submission	***0.69***	***0.24***	***2.88***	***0.005***
	Species	−0.46	0.59	−0.78	0.44
	Submission × species	***−0.82***	***0.30***	***−2.73***	***0.008***
	Affiliation	***0.38***	***0.09***	***4.08***	***<0.001***
	Species	0.45	0.67	0.67	0.51
	Affiliation × species	***−0.42***	***0.13***	***−3.23***	***0.002***
AVT	Proximity	−0.08	0.08	−1.00	0.32
	Sex	***1.00***	***0.43***	***2.33***	***0.03***
	Aggression	−0.02	0.06	−0.34	0.74
	Sex	***1.04***	***0.44***	***2.38***	***0.02***
	Submission	−0.04	0.13	−0.28	0.78
	Sex	***0.96***	***0.48***	***2.00***	***0.05***
	Affiliation	−0.01	0.06	−0.12	0.91
	Sex	***1.00***	***0.44***	***2.29***	***0.03***

Are there differences in the molecular response to pair bond disruption between a social and non-social species?

We found no differences in the genomic reaction norms to the separation and reunion treatment between the two species (e.g., Aubin-Horth and Renn, 2009). The functional relationship between the studied hormonal pathways and aggression, submission, and affiliation in various species initially led us to predict that expression levels of these genes would be more affected by pair bond disruption in the social species, *N. pulcher*, relative to the non-social *T. temporalis*. However, we found no differences in the molecular response to pair bond disruption between the social and non-social species. We found that both species had increased aggression following the separation and reunion treatment, but we found no differences in AVT based on the treatment, despite some evidence that AVT is relevant for aggression in these cichlids. We found that male fish were more aggressive, less submissive, and had higher whole brain gene expression of AVT relative to females, in both species. Previous studies have found differences in AVT in *N. pulcher* based on social status (Reddon et al., 2015), as well as social status and sex (Aubin-Horth et al., 2007). The consistent factor is that AVT appears to be important in mediating aggressive behavior in *N. pulcher*. The sex differences found in the current study are also consistent with previous research indicating that AVT appears to play an important role in mediating male aggression in other fish species (Foran and Bass, 1998; Godwin et al., 2000; Semsar et al., 2001; Huffman et al., 2015), as well as in avian (Goodson, 1998) and mammalian systems (Stribley and Carter, 1999; Wersinger et al., 2002). However, we found no differences in AVT based on the separation and reunion treatment, despite the increase in aggression in both species following the separation and reunion treatment relative to controls. It is possible that the time period that we chose for sampling did not capture transient changes in brain gene expression resulting from our treatment. Further, many relationships between socially-relevant behavior and nonapeptides occur in specific brain regions (e.g., Young et al., 1998; Greenwood et al., 2008; O'Connell and Hofmann, 2011; Godwin and Thompson, 2012). Therefore, it is possible that there were effects of our treatment that occurred in specific brain regions, but were not apparent at the whole brain level. Further studies examining the response

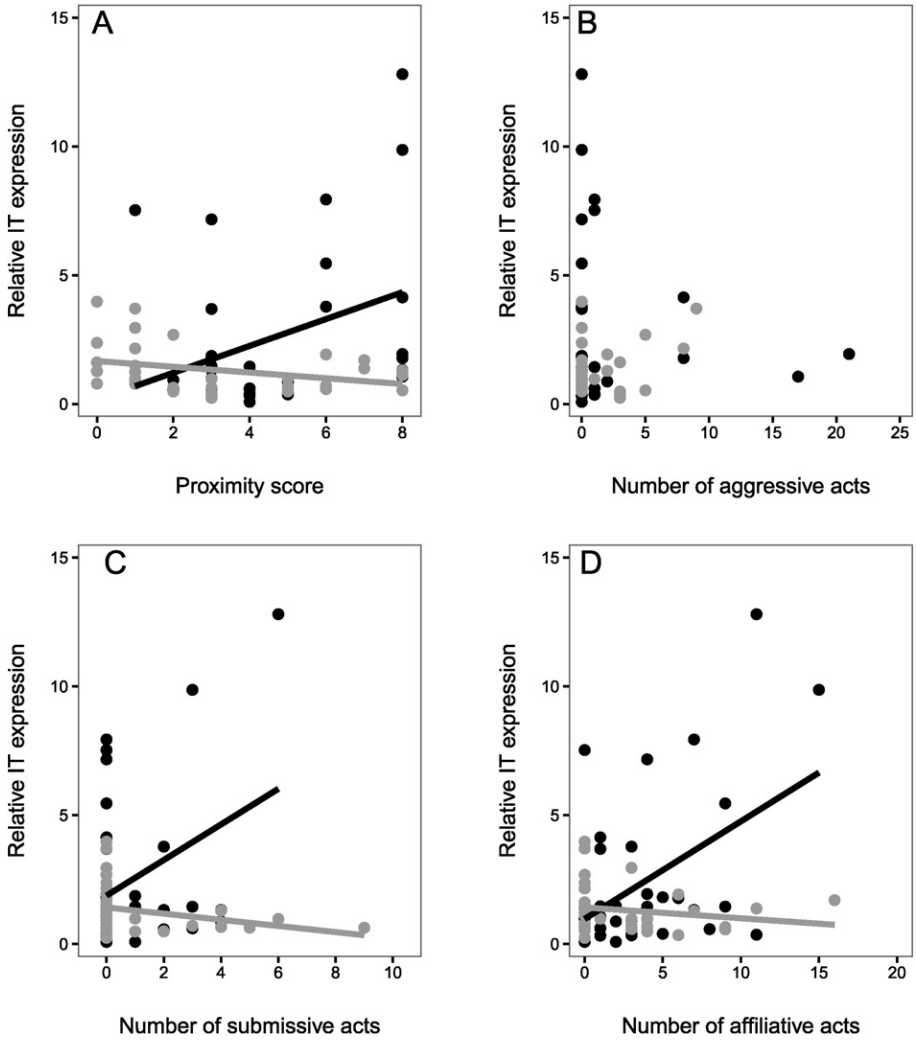

Fig. 4. Correlations between behavioral metrics and brain gene expression in social *N. pulcher* (black) and non-social *T. temporalis* (light gray) cichlid fishes. Isotocin (IT) is unrelated to (B) aggression, but is positively correlated with (A) proximity, (C) submission, and (D) affiliation, in only the social *N. pulcher*. See Methods and Table 4 for full statistical details.

to separation and reunion at various time courses following manipulation and in specific brain regions would be valuable.

We did find that brain gene expression of the nonapeptide hormone IT was higher in the social *N. pulcher* relative to the non-social *T. temporalis*, in accordance with its hypothesized functional role in social behavior modulation. A recent field study of the whole brain gene expression of nonapeptide hormones and receptors revealed higher brain gene expression of all measured nonapeptides and receptors in wild *N. pulcher* relative to wild *T. temporalis* (O'Connor et al., 2015). We are uncertain why in the current laboratory study, we found no differences in AVT, or in any of the measured receptors. An investigation of relative levels of nonapeptides and receptors between *N. pulcher* and *T. temporalis* across different social contexts would be useful. However, higher brain gene expression of IT in *N. pulcher* relative to *T. temporalis* is a consistent pattern between laboratory and field populations. Since both species form pair bonds, but only *N. pulcher* displays grouping behavior, and has concurrent higher IT expression, our nonapeptide results suggest that the two behaviors may be modulated independently at the neuromolecular hormonal level. This supports our hypothesis based on the behavioral data that pair bonding and grouping may be additive rather than correlated behavioral processes.

Table 5
Statistical output from general linear mixed models investigating differences in the relationship between relative brain gene expression and behavior, for genes where no effects of species, sex, or treatment were found (see Table 3). For each test, 'pair' was included as a random effect to account for non-independence of mated pairs. A subset of individuals was used for all tests (see Table 1 for an explanation of the sample sizes). See Methods for full statistical details.

Gene	Behavior	Estimate	Standard error	t-Value	p-Value
ITR1	Proximity	−0.06	0.04	−1.74	0.09
	Aggression	−0.00	0.03	−0.03	0.97
	Submission	−0.02	0.05	−0.44	0.66
	Affiliation	−0.01	0.03	−0.59	0.56
ITR2	Proximity	−0.03	0.03	−1.26	0.21
	Aggression	−0.01	0.02	−0.50	0.62
	Submission	−0.00	0.04	−0.10	0.92
	Affiliation	0.00	0.02	0.23	0.82
AVTR	Proximity	0.02	0.03	−0.55	0.59
	Aggression	−0.01	0.02	−0.53	0.60
	Submission	0.03	0.04	0.65	0.52
	Affiliation	0.01	0.02	0.42	0.68

Species-specific correlations between behavior and nonapeptide brain gene expression

We found that whole brain gene expression of IT was positively correlated with affiliation and submission in the highly social species, *N. pulcher*. Previous research has reported a negative correlation

between circulating IT in the brain and affiliative behavior (Reddon et al., 2015), and suggests that more submissive and affiliative *N. pulcher* are both producing higher levels of IT in the brain, and turning over (i.e., up-taking, breaking down, or exporting) IT at higher rates. In either case, more submissive and affiliative *N. pulcher* are both producing and using higher levels of IT compared to less submissive and affiliative *N. pulcher*. Further, a previous study showed that IT treatment increases the attention to social stimuli in *N. pulcher* during staged contests (Reddon et al., 2012) as well as increasing submissive displays in both natural group settings in the laboratory (Reddon et al., 2014) and in the field (Hellmann et al., 2015a, 2015b). Taken together, these results support a strong role for IT in mediating submissive and affiliative behaviors in the social cichlid *N. pulcher*. Since the action of IT on socially-relevant behaviors is likely mediated through specific brain regions rather than at the whole-brain level (Young et al., 1998; Greenwood et al., 2008; O'Connell and Hofmann, 2011; Godwin and Thompson, 2012), it is remarkable that we are able to find consistent relationships using a whole-brain approach in the current study. This result emphasizes the importance of IT in mediating submissive and affiliative behaviors in the highly social, group-living and cooperatively breeding *N. pulcher*. Interestingly, the *T. temporalis* show similar behaviors towards their mate when compared with *N. pulcher*. However, the *T. temporalis* have lower whole brain gene expression of IT, and no relationship was found between their social behavior and brain gene expression. This suggests that there is not a simple up-regulation of IT brain gene expression and a corresponding increase in affiliation and submission towards a mate. Instead, there is a complex, species-specific relationship, with a relationship between IT and behavior only appearing in the social species. We found no differences in expression levels of the receptors, but there could potentially be difference in spatial patterns of these receptors in specific brain regions (e.g., Insel and Shapiro, 1992); such a spatial pattern difference could contribute to differences in behavior observed between species.

Conclusion

Our aim in this study was to identify whether species that vary in their grouping behavior display a corresponding difference in the strength and resilience of their pair bonds, and to understand the relationships among grouping, pair bonding, and vasopressin–oxytocin nonapeptide hormonal pathways. We found that the two species use similar behavioral strategies to re-establish a pair bond when it is perturbed, with no difference in the resilience of pair bonds between the social and the non-social species. However, we found higher brain gene expression of IT in the social *N. pulcher* relative to the non-social *T. temporalis*, with a species-specific correlation between behavior and whole brain gene expression of IT, but only in the highly social species. Our results highlight the importance of isotocin in mediating submissive and affiliative behaviors in cichlid fishes, and demonstrate that isotocin has species-specific correlations with socially relevant behaviors. Our results contribute to an increasing body of literature (see review by Goodson, 2013) demonstrating that the oxytocin–vasopressin family of nonapeptides has species-specific effects on social behavior, and it is important to consider the life history of the species under study when interpreting results.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.yhbeh.2015.10.015.

Author contributions

CMO, SMR, and SB conducted the behavioral observations. CMO and NAH conducted the laboratory assays. CMO performed the statistical analyses, and wrote the first draft of the manuscript, with input and guidance from SMR, NAH, and SB.

Data accessibility

All data including RT-qPCR values and behavioral scores are available on Dryad (http://dx.doi.org/10.5061/dryad.8405j).

Acknowledgments

The authors thank Isabelle Giguère for assistance with RT-qPCR, and Adam Reddon for helpful comments on an earlier version of this manuscript. Research was supported by the Discovery Grants from the Natural Sciences and Engineering Research Council of Canada to SB and NAH. CMO is supported by an NSERC Postdoctoral Fellowship. SB is supported by the Canada Research Chairs Program.

References

Aubin-Horth, N., Renn, S.C., 2009. Genomic reaction norms: using integrative biology to understand molecular mechanisms of phenotypic plasticity. Mol. Ecol. 18, 3763–3780.
Aubin-Horth, N., Desjardins, J.K., Martei, Y.M., Balshine, S., Hofmann, H.A., 2007. Masculinized dominant females in a cooperatively breeding species. Mol. Ecol. 16, 1349–1358.
Balshine, S., Leach, B., Neat, F., Reid, H., Taborsky, M., Werner, N., 2001. Correlates of group size in a cooperatively breeding cichlid fish (*Neolamprologus pulcher*). Behav. Ecol. Sociobiol. 50 (2), 134–140.
Bergmüller, R., Taborsky, M., 2005. Experimental manipulation of helping in a cooperative breeder: helpers 'pay to stay' by pre-emptive appeasement. Anim. Behav. 69, 19–28.
Boissy, A., Le Neindre, P., 1997. Behavioral, cardiac and cortisol responses to brief peer separation and reunion in cattle. Physiol. Behav. 61, 693–699.
Brichard, P., 1989. Cichlids and All the Other Fishes of Lake Tanganyika. THF Publications, Neptune City, NJ.
Bustin, S.A., Benes, V., Garson, J.A., Hellemans, J., Huggett, J., Kubista, M., Mueller, R., Nolan, T., Pfaffl, M.W., Shipley, G.L., Vandesompele, J., Wittwer, C.T., 2009. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin. Chem. 55, 611–622.
Choleris, E., Gustafsson, J.Å., Korach, K.S., Muglia, L.J., Pfaff, D.W., Ogawa, S., 2003. An estrogen dependent micronet mediating social recognition: a study with oxytocin- and estrogen receptor α- and β-knockout mice. Proc. Natl. Acad. Sci. U. S. A. 100, 6192–6197.
Christensen, R.H.B., 2015. Ordinal: Regression Models for Ordinal Data (URL:) http://www.cran.r-project.org/package=ordinal/.
Day, J.J., Santini, S., Garcia-Moreno, J., 2007. Phylogenetic relationships of the Lake Tanganyika cichlid tribe Lamprologini: the story from mitochondrial DNA. Mol. Phylogenet. Evol. 45, 629–642.
Desjardins, J.K., Fitzpatrick, J.L., Stiver, K.A., Van der Kraak, G.J., Balshine, S., 2008. Costs and benefits of polygyny in the cichlid *Neolamprologus pulcher*. Anim. Behav. 75, 1771–1779.
Dey, C.J., Reddon, A.R., O'Connor, C.M., Balshine, S., 2013. Network structure is related to social conflict in a cooperatively breeding fish. Anim. Behav. 85, 395–402.
Dierkes, P., Taborsky, M., Achmann, R., 2008. Multiple paternity in the cooperatively breeding fish *Neolamprologus pulcher*. Behav. Ecol. Sociobiol. 62, 1581–1589.
Dunbar, R.I., Shultz, S., 2007. Evolution in the social brain. Science 317 (5843), 1344–1347.
Ferguson, J.N., Aldag, J.M., Insel, T.R., Young, L.J., 2001. Oxytocin in the medial amygdala is essential for social recognition in the mouse. J. Neurosci. 21, 8278–8285.
Ferguson, J.N., Young, L.J., Hearn, E.F., Matzuk, M.M., Insel, T.R., Winslow, J.T., 2000. Social amnesia in mice lacking the oxytocin gene. Nat. Genet. 25, 284–288.
Fleige, S., Walf, V., Huch, S., Prgomet, C., Sehm, J., Pfaffl, M.W., 2006. Comparison of relative mRNA quantification models and the impact of RNA integrity in quantitative real-time RT-PCR. Biotechnol. Lett. 28, 1601–1613.
Foran, C.M., Bass, A.H., 1998. Preoptic AVT immunoreactive neurons of a teleost fish with alternative reproductive tactics. Gen. Comp. Endocrinol. 111, 271–282.
Godwin, J., Thompson, R., 2012. Nonapeptides and social behavior in fishes. Horm. Behav. 61, 230–238.
Godwin, J., Sawby, R., Warner, R.R., Crews, D., Grober, M.S., 2000. Hypothalamic arginine vasotocin mRNA abundance variation across sexes and with sex change in a coral reef fish. Brain Behav. Evol. 55, 77–84.
Goodson, J.L., 1998. Territorial aggression and dawn song are modulated by septalvasotocin and vasoactive intestinal polypeptide in male field sparrows (*Spizella pusilla*). Horm. Behav. 34, 67–77.
Goodson, J.L., 2013. Deconstructing sociality, social evolution and relevant nonapeptide functions. Psychoneuroendocrinology 38, 465–478.
Goodson, J.L., Schrock, S.E., Klatt, J.D., Kabelik, D., Kingsbury, M.A., 2009. Mesotocin and nonapeptide receptors promote estrildid flocking behavior. Science 325, 862–866.
Greenwood, A.K., Wark, A.R., Fernald, R.D., Hofmann, H.A., 2008. Expression of arginine vasotocin in distinct preoptic regions is associated with dominant and subordinate behaviour in an African cichlid fish. Proc. R. Soc. B 275, 2393–2402.
Heg, D., Bachar, Z., 2006. Cooperative breeding in the Lake Tanganyika cichlid *Julidochromis ornatus*. Environ. Biol. Fish 76, 265–281.
Hellmann, J.K., Ligocki, I.Y., O'Connor, C.M., Reddon, A.R., Garvy, K.A., Marsh-Rollo, S.E., Gibbs, H.L., Balshine, S., Hamilton, I.M., 2015a. Reproductive sharing in relation to group and colony-level attributes in a cooperative breeding fish. Proc. R. Soc. B (in press).

Hellmann, J.K., Reddon, A.R., Ligocki, I.Y., O'Connor, C.M., Garvy, K.A., Marsh-Rollo, S.E., Hamilton, I.M., Balshine, S., 2015b. Group response to social perturbation: impacts of isotocin and the social landscape. Anim. Behav. 105, 55–62.
Hick, K., Reddon, A.R., O'Connor, C.M., Balshine, S., 2014. Strategic and tactical fighting decisions in cichlid fishes with divergent social systems. Behaviour 151, 47–71.
Hoyle, C.H., 1999. Neuropeptide families and their receptors: evolutionary perspectives. Brain Res. 848, 1–25.
Huffman, L.S., Hinz, F.I., Wojcik, S., Aubin-Horth, N., Hofmann, H.A., 2015. Arginine vasotocin regulates social ascent in the African cichlid fish *Astatotilapia burtoni*. Gen. Comp. Endocrinol. 212, 106–113.
Huffman, L.S., O'Connell, L.A., Kenkel, C.D., Kline, R.J., Khan, I.A., Hofmann, H.A., 2012. Distribution of nonapeptide systems in the forebrain of an African cichlid fish, *Astatotilapia burtoni*. J. Chem. Neuroanat. 44, 86–97.
Insel, T.R., Shapiro, L.E., 1992. Oxytocin receptor distribution reflects social organization in monogamous and polygamous voles. Proc. Natl. Acad. Sci. U. S. A. 89, 5981–5985.
Insel, T.R., Wang, Z.X., Ferris, C.F., 1994. Patterns of brain vasopressin receptor distribution associated with social organization in microtine rodents. J. Neurosci. 14, 5381–5392.
Katoh, R., Munehara, H., Kohda, M., 2005. Alternative male mating tactics of the substrate brooding cichlid *Telmatochromis temporalis* in Lake Tanganyika. Zool. Sci. 22, 555–561.
Kline, R.J., O'Connell, L.A., Hofmann, H.A., Holt, G.J., Khan, I.A., 2011. The distribution of an AVT V1a receptor in the brain of a sex changing fish, *Epinephelus adsensionis*. J. Chem. Neuroanat. 42, 72–88.
Konings, A., 1998. Cichlids in Their Natural Habitat. Cichlid Press, El Paso, TX.
Kuwamura, T., 1986. Parental care and mating systems of cichlid fishes in Lake Tanganyika: a preliminary field survey. J. Ethol. 4, 129–146.
Lema, S.C., 2010. Identification of multiple vasotocin receptor cDNAs in teleost fish: sequences, phylogenetic analysis, sites of expression, and regulation in the hypothalamus and gill in response to hyperosmotic challenge. Mol. Cell. Endocrinol. 321, 215–230.
Lendvai, Á.Z., Chastel, O., 2008. Experimental mate-removal increases the stress response of female house sparrows: the effects of offspring value? Horm. Behav. 53, 395–401.
Lim, M.M., Young, L.J., 2006. Neuropeptidergic regulation of affiliative behavior and social bonding in animals. Horm. Behav. 50, 506–517.
Limberger, D., 1983. Pairs and harems in a cichlid fish *Lamprologus brichardi*. Z. Tierpsychol. 62, 115–144.
Lukas, M., Toth, I., Reber, S.O., Slattery, D.A., Veenema, A.H., Neumann, I.D., 2011. The neuropeptide oxytocin facilitates pro-social behavior and prevents social avoidance in rats and mice. Neuropsychopharmacology 36, 2159–2168.
Mboko, S.K., Kohda, M., 1999. Piracy mating by large males in a monogamous substrate-breeding cichlid in Lake Tanganyika. J. Ethol. 17, 51–55.
O'Connell, L.A., Hofmann, H.A., 2011. Genes, hormones, and circuits: an integrative approach to study the evolution of social behavior. Front. Neuroendocrinol. 32, 320–335.
O'Connor, C.M., Marsh-Rollo, S.E., Cortez Ghio, S., Balshine, S., Aubin-Horth, N., 2015. Is there convergence in the molecular pathways underlying the repeated evolution of sociality in African cichlids? Horm. Behav. 75, 160–168.
Oldfield, R.G., Hofmann, H.A., 2011. Neuropeptide regulation of social behavior in a monogamous cichlid fish. Physiol. Behav. 102, 296–303.
Oldfield, R.G., Harris, R.M., Hendrickson, D.A., Hofmann, H.A., 2013. Arginine vasotocin and androgen pathways are associated with mating system variation in North American cichlid fishes. Horm. Behav. 64, 44–52.
Pfaffl, M.W., 2001. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res. 29, e45.
R Development Core Team, 2015. R: A Language and Environment for Statistical Computing URL: http://www.R-project.org/.
Racine, J.S., 2012. RStudio: a platform-independent IDE for R and Sweave. J. Appl. Econ. 27, 167–172.
Reddon, A.R., Reader, S.M., 2015. The role of submissive behaviour in reducing the costs of conflict and facilitating group living (Manuscript in preparation).
Reddon, A.R., O'Connor, C.M., Marsh-Rollo, S.E., Balshine, S., 2012. Effects of isotocin on social responses in a cooperatively breeding fish. Anim. Behav. 84, 753–760.
Reddon, A.R., O'Connor, C.M., Marsh-Rollo, S.E., Balshine, S., 2014. Isotocin and sociality in a cooperatively breeding cichlid fish. Behavior 15, 1389–1411.
Reddon, A.R., O'Connor, C.M., Marsh-Rollo, S.E., Balshine, S., Gozdowska, M., Kulczykowska, E., 2015. Brain nonapeptide levels are related to social status and affiliative behaviour in a cooperatively breeding cichlid fish. R. Soc. Open Sci. 2, 140072.
Remage-Healey, L., Adkins-Regan, E., Romero, L.M., 2003. Behavioral and adrenocortical responses to mate separation and reunion in the zebra finch. Horm. Behav. 43, 108–114.
Ripley, B., Venebles, B., Bates, D.M., Hornik, K., Gebhardt, A., Firth, D., 2015. MASS: Modern Applied Statistics With S (URL:) http://cran.r-project.org/package=MASS.
Schroeder, A., Mueller, O., Stocker, S., Salowsky, R., Leiber, M., Gassmann, M., Lightfoot, S., Menzel, W., Granzow, M., Ragg, T., 2006. The RIN: an RNA integrity number for assigning integrity values to RNA measurements. BMC Mol. Biol. 7, 3.
Sefc, K.M., 2011. Mating and parental care in Lake Tanganyika's cichlids. Int. J. Evol. Biol. 2011, 470875.
Semsar, K., Kandel, F.L.M., Godwin, J., 2001. Manipulations of the AVT system shift social status and related courtship and aggressive behavior in the bluehead wrasse. Horm. Behav. 40, 21–31.
Shepherd, R.E., French, J.A., 1999. Comparative analysis of sociality in lion tamarins (*Leontopithecus rosalia*) and marmosets (*Callithrix kuhli*): responses to separation from long-term pairmates. J. Comp. Psychol. 113, 24.
Soares, M.C., Bshary, R., Fusani, L., Goymann, W., Hau, M., Hirschenhauser, K., Oliveira, R.F., 2010. Hormonal mechanisms of cooperative behavior. Proc. R. Soc. Lond. B 365, 2737–2750.
Stiver, K.A., Dierkes, P., Taborsky, M., Balshine, S., 2004. Dispersal patterns and status change in a co-operatively breeding cichlid *Neolamprologus pulcher*: evidence from microsatellite analyses and behavioural observations. J. Fish Biol. 65, 91–105.
Stribley, J.M., Carter, C.S., 1999. Developmental exposures to vasopressin increases aggression in adult prairie voles. Proc. Natl. Acad. Sci. U. S. A. 96, 12601–12604.
Thompson, R.R., Walton, J.C., 2004. Peptide effects on social behavior: effects of vasotocin and isotocin on social approach behavior in male goldfish (*Carassius auratus*). Behav. Neurosci. 118, 620–626.
Venables, W.N., Ripley, B.D., 2002. Modern Applied Statistics With S. Fourth edition. Springer, New York, NY.
Wersinger, S.R., Ginns, E., O'Carroll, A.-M., Lolait, S.J., Young III, W.S., 2002. Vasopressin V1b receptor knockout reduces aggressive behavior in male mice. Mol. Psychol. 7, 975–984.
Williams, J., Catania, K., Carter, C., 1992. Development of partner preferences in female prairie voles (*Microtus ochrogaster*): the role of social and sexual experience. Horm. Behav. 26, 339–349.
Wong, M.Y.L., Balshine, S., 2011. The evolution of cooperative breeding in the African cichlid fish, *Neolamprologus pulcher*. Biol. Rev. 86, 511–530.
Wong, M.Y.L., Jordan, L.A., Marsh-Rollo, S., St-Cyr, S., Reynolds, S.J.O., Stiver, K.A., Desjardins, J.K., Fitzpatrick, J.L., Balshine, S., 2012. Mating systems in cooperative breeders: the roles of resource dispersion and conflict mitigation. Behav. Ecol. 23, 521–530.
Young, L.J., Wang, Z., 2004. The neurobiology of pair bonding. Nat. Neurosci. 7, 1048–1054.
Young, L.J., Wang, Z., Insel, T.R., 1998. Neuroendocrine bases of monogamy. Trends Neurosci. 21, 71–75.