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Is there convergence in the molecular pathways underlying the repeated evolution of sociality in African cichlids?

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ABSTRACT

Despite wide variation in the complexity of social interactions across taxa, the basic behavioral components of sociality appear to be modulated by conserved hormone pathways. Specifically, the nonapeptide hormones oxytocin and vasopressin and their receptors have been implicated in regulating diverse social behaviors across vertebrates. Here, we took advantage of the repeated evolution of cooperative breeding in African cichlids to investigate whether there are consistent brain gene expression patterns of isotocin and arginine vasotocin (teleost homologues of oxytocin and vasopressin), as well as their receptors, between four closely related pairs of social (cooperative) and non-social (non-cooperative) species. We first found that the coding sequences for the five genes studied were highly conserved across the eight species. This is the first study to examine the expression of both isotocin receptors, and so we performed a phylogenetic analysis that suggests that these two isotocin receptors are paralogues that arose during the teleost genome duplication. When we then examined brain gene expression patterns relative to social system, we found that there were whole-brain gene expression differences between the social and non-social species in many of the species pairs. However, these relationships varied in both the direction and magnitude among the four species pairs. In conclusion, our results suggest high sequence conservation and species-specific gene expression patterns relative to social behavior for these candidate hormone pathways in the cichlid fishes.

Introduction

Social behavior is taxonomically widespread and ranges in complexity from temporary aggregations to permanent groups with cooperation among group members (Rubenstein and Kealey, 2010). However, despite this wide variation in both the extent and complexity of social interactions, sociality is thought to have evolved from a combination of more simple social behaviors, and gradual changes to these basic behaviors (Soares et al., 2010). These basic behaviors underlying complex sociality include the tendency to approach others, the recognition and discrimination of friends and foes, and the use of tactics that minimize the costs of antagonistic interactions (Soares et al., 2010). Small behavioral changes, mediated by small differences in the underlying molecular and physiological pathways, can then build to form complex social phenotypes (Goodson, 2005, 2013; Donaldson and Young, 2008; Soares et al., 2010; O'Connell and Hofmann, 2011; Zayad and Robinson, 2012).

Converging evidence from a range of species and social contexts points towards a highly conserved set of molecular pathways having an important role in modulating a wide variety of both basic and complex social behaviors (Goodson, 2005, 2013; Donaldson and Young, 2008; Soares et al., 2010; O'Connell and Hofmann, 2011; Zayad and Robinson, 2012). In vertebrates, the hormones oxytocin and vasopressin and their receptors have been strongly linked to both social and anti-social behaviors (Goodson, 2013). These hormones are remarkable in the diversity of social behaviors that they regulate, including social approach (Goodson et al., 2009; Lukas et al., 2011), social recognition (Bielsky and Young, 2004), social bonding (Young and Wang, 2004; Ross and Young, 2009; Insel and Young, 2001; Klatt and Goodson, 2012), social cognition (Donaldson and Young, 2008), as well as dominance-related behavior (Goodson and Bass, 2001; Larson et al., 2006), and a suite of cooperative behaviors (Madden and Clutton-Brock, 2011; Soares et al., 2012). However, both the strength and direction of the specific relationships between social behavior and these hormones vary widely depending on species, sex, social context, and the specific behavior being examined. Thus, a general framework describing the function of these hormones in relation to sociality has remained elusive (Goodson, 2013).

Studying convergence at the molecular level within an ecological framework can provide novel information about the evolution of a species or trait (Elmer and Meyer, 2011). Because of the diversity of behaviors associated with oxytocin and vasopressin, comparative studies have been invaluable in uncovering the evolution of the molecular

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pathways underlying social behavior variation (Insel, 2010; Phelps et al., 2010). For example, classic studies with *Microtus* voles demonstrated that both oxytocin and vasopressin, and in particular the distribution and abundance of their receptors, differ between monogamous and polygynous species of voles (reviewed in Wang et al., 1999). Comparative studies in estrilid finches have demonstrated that the mesotocin and vasotocin molecular pathways and receptor distributions vary with the degree of species-typical grouping behavior (the avian equivalents of oxytocin and vasopressin, respectively; Goodson et al., 2009; Goodson and Kingsbury, 2011).

Fishes show considerable diversity in social systems (Godin, 1997; Nelson, 2006), and are excellent candidates for extensive, replicated comparative work to study the evolution of the molecular pathways underlying sociality (Larsen et al., 2011). In fishes, measurements and pharmacological manipulations of isotocin (IT) and arginine vasotocin (AVT, the teleost equivalents of oxytocin and vasopressin, respectively; Hoyle, 1999) have revealed a role for these hormones in dominance and social behavior modulation across a range of species. As in other vertebrates, results again are species- and context-specific (e.g., Thompson and Walton, 2004; Aubin-Horth et al., 2007; Renn et al., 2008; Reddon et al., 2012, 2014; Oldfield et al., 2013). Thus, comparisons of how these hormones and their receptors differ in expression, in association with evolutionary divergence in social behavior across fish species, will expand our understanding of the molecular basis of sociality on a broad scale.

To explore the link between these hormonal pathways and the evolution of social behavior, we compared the brain gene expression of these hormones and their receptors between eight species of social and non-social Lamprologine cichlid fishes, endemic to Lake Tanganyika, Africa. The rapid and repeated radiation of African cichlids has resulted in over 1650 formally described taxa, one of the largest of the vertebrate families, within a short divergence time (Kocher, 2004). This rapid divergence has made African cichlids in general an excellent model for comparative research (Kocher, 2004; Seehausen, 2006). Within the African cichlids, the Lamprologines are particularly interesting as these fish have radiated relatively recently (5.3 million years ago; Sturmbauer et al., 2010), and are the only fish tribe where cooperative breeding has been described (Heg and Bachar, 2006; Wong and Balshine, 2011). Although the ancestral state of the Lamprologines is non-social, cooperative breeding has arisen multiple times in this lineage (Heg and Bachar, 2006), making these fishes an ideal model to explore the mechanistic underpinnings of complex sociality within a comparative framework. We selected four social cichlids that live in permanent social groups and display cooperative breeding behavior (Heg and Bachar, 2006) such that four evolutionary transitions to cooperative breeding were represented (Heg and Bachar, 2006). For each of these social species, we then selected as a comparison a closely related species that does not show any grouping or cooperative behaviors (Konings, 1998; Kuwamura, 1986, 1997; Brichard, 1989). We selected social and non-social species pairs such that each social and non-social species within a given pair are phylogenetically close (based on published Lamprologine cichlid phylogenies using both mitochondrial and nuclear gene sequences; Sturmbauer et al., 1994; Day et al., 2007; Sturmbauer et al., 2010; Fig. 1), and have similar ecology outside of the differences in social system (Table 1). This approach of studying variation in sociality (i.e., group living and cooperative versus non-grouping and non-cooperative) within a comparative framework provides a useful baseline for understanding the role of IT and AVT in fish social behavior evolution.

We first sequenced the genes for IT and AVT in all eight of our social and non-social species pairs, to determine if there were any sequence differences between social and non-social species, and as a prerequisite to measure gene expression in these non-model species. Since differences in expression of the receptors of these hormones appear to be as informative as the differences in expression of the hormones themselves in many systems (Insel, 2010; Turner et al., 2010), we also sequenced the genes for the AVT and IT receptors. We then compared whole brain gene expression of AVT, IT, and the receptors, in each social species relative to their closely related non-social species. Fish possess several receptors that bind AVT: V1a1, V1a2, V1b, and V2 receptors (Amores et al., 1998; Lema, 2010; Godwin and Thompson, 2012). We examined the brain gene expression of the V1a2 AVT receptor (AVTR, termed "V1a" in Huffman et al., 2012), because 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). Two IT receptors are found in many fish species, which may be the result of the teleost whole genome duplication event (Van De Peer et al., 2009), but very little is known about the function of these two receptors. Increased gene duplication retention is a feature of African cichlid genomes (Machado et al., 2014; Brawand et al., 2014) and has been proposed as a basis for functional novelty (Lynch and Force, 2000). Therefore, we constructed a gene tree of the known IT receptor sequences in teleosts, including the eight new sequences we generated for the two IT receptors for the Lamprologine cichlids, in order to better understand the relationship between the receptors, and measured the expression of both IT receptors found in Lamprologine cichlids, IT receptor 1 (ITR1), and IT receptor 2 (ITR2, termed "ITR" in Huffman et al., 2012). This study therefore provides a rare look at the gene expression of both the hormones and receptors that have been so often associated with social behaviors within the same individuals, and within a comparative framework in fishes. Overall, we hypothesized that a consistent pattern of differential brain gene expression of these hormones and their receptors across the four social and non-social species pairs would provide strong evidence of similar selection pressure on these molecular pathways in modulating social behavior in fishes.

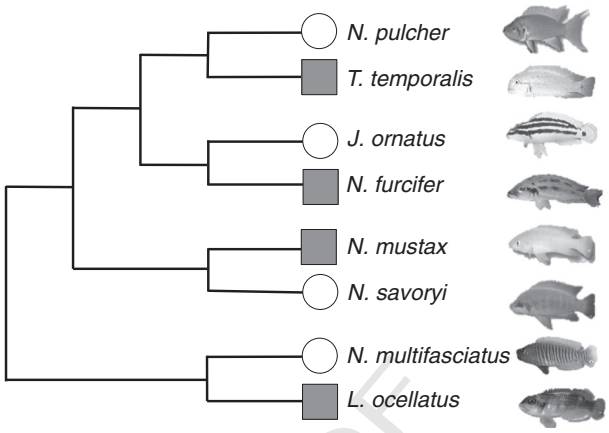

Fig. 1. Simplified phylogenetic relationship among the four species pairs of social (grouping and cooperatively breeding) and non-social (non-grouping and non-cooperative) Lamprologine fishes included in our study. This simplified phylogeny is redrawn from mitochondrial and nuclear phylogenies presented in Day et al. (2007) and Sturmbauer et al. (2010). White circles represent social species, and gray squares represent non-social species.

Methods

Study species and study site

The species pairs selected for comparison were: 1) *Neolamprologus pulcher* and *Telmatochromis temporalis*; 2) *Julidochromis ornatus* and *Neolamprologus furcifer*; 3) *Neolamprologus savoryi* and *Neolamprologus mustax*; and, 4) *Neolamprologus multifasciatus* and *Lamprologus ocellatus* (Fig. 1). All eight species hold permanent territories, breed year-round, and are similar in general appearance and ecology (Konings, 1998; Kuwamura, 1986, 1997; Brichard, 1989), but diverge strongly

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Table 1
Ecological characteristics, mating systems, and parental care systems of the Lamprologine cichlid fishes (*Neolamprologus pulcher*, *Telmatochromis temporalis*, *Julidochromis ornatus*, *Neolamprologus furcifer*, *Neolamprologus savoryi*, *Neolamprologus mustax*, *Neolamprologus multifasciatus*, and *Lamprologus ocellatus*) used in the current study. Information on diet and habitat is from Konings (1998), and Brichard (1989). Information on mating and parental care systems is from Kuwamura (1986, 1997), and Brichard (1989).

Species	Social system	Mating system	Parental care	Diet	Breeding habitat
N. pulcher	Cooperative	Polygynous	Biparental	Suspended zooplankton	Rocky substrate
T. temporalis	Non-cooperative	Polygynous	Maternal	Substrate algae and invertebrates	Rocky substrate
J. ornatus	Cooperative	Polygynandrous	Biparental	Substrate algae and invertebrates	Rocky substrate
N. furcifer	Non-cooperative	Monogamous	Biparental	Substrate algae and invertebrates	Rocky substrate
N. savoryi	Cooperative	Polygynous	Biparental	Substrate algae and invertebrates	Rocky substrate
N. mustax	Non-cooperative	Polygynous	Maternal	Substrate algae and invertebrates	Rocky substrate
N. multifasciatus	Cooperative	Polygynous	Biparental	Suspended zooplankton	Snail shells
L. ocellatus	Non-cooperative	Polygynous	Maternal	Substrate algae and invertebrates	Snail shells

in their social behavior. *N. pulcher*, *J. ornatus*, *N. savoryi*, and *N. multifasciatus* are cooperatively breeding fish that live in permanent groups that co-defend a shared territory (Rossiter, 1993; Konings, 1998; Heg et al., 2005a; Heg and Bachar, 2006; Wong and Balshine, 2011), while *T. temporalis*, *N. furcifer*, *N. mustax*, and *L. ocellatus* exhibit the ancestral social system for cichlids (Day et al., 2007; Goodwin et al., 1998), with no grouping or cooperative behavior (Heg and Bachar, 2006). Fish were collected between September and December 2008 from three sites in Lake Tanganyika near Mpulugu, Zambia. *N. pulcher*, *T. temporalis*, *N. savoryi*, and *J. ornatus* were all collected in Kasakalwe Bay (8°46′ S, 31°5′ E). *N. mustax*, *N. multifasciatus* and *L. ocellatus* were collected near Mbita Island (8°43′ S, 31°7′ E), while *N. furcifer* were collected near Wonzye Point (8°43′ S, 31°8′ E). Individuals from all eight species were located and captured with fence and hand nets using SCUBA between depths of 8–14 m, and brought slowly to the surface in mesh collection bags. Only sexually mature males were collected, in order to minimize the potential variation based on sex or reproductive state. At the surface, fish were transferred to aerated opaque plastic barrels (189 L each). *N. pulcher*, *T. temporalis*, *N. savoryi*, *J. ornatus* and *N. furcifer* were processed the same day that they were collected. *N. mustax*, *N. multifasciatus* and *L. ocellatus* were held overnight in the aerated barrels, and processed the following morning. All fish were weighed using an electronic scale and standard length (SL) was measured using calipers. Fish were first stunned by submersion in an ice bath, and then the head was severed and brain removed and preserved in RNAlater (Invitrogen, Carlsbad, CA). Vials were kept at room temperature for 12 h before being transferred to a −20 °C freezer for later analysis of brain gene expression levels. See Table 2 for sample sizes and measured traits for all fish used in this study.

Sample processing

All brains were thawed and individually homogenized, and total RNA was extracted using the standard TRIzol reagent protocol (Invitrogen). The concentration and purity of RNA were analyzed for all samples using a Nanodrop spectrophotometer (Thermo Scientific, Wilmington, DE) and a subset of each species was checked for integrity using either an Experion RNA Analysis Kit (Experion Technologies, Kerala, India) or a 2100 Bioanalyzer instrument (Agilent Technologies, Santa Clara, CA). Prior to cDNA synthesis, aliquots of 2000 ng of RNA were treated with amplification grade DNase I (Invitrogen) to eliminate genomic DNA contamination. First strand cDNA was then synthesized from DNase-treated total RNA using SuperScript II Reverse Transcriptase (Invitrogen) with a mix of random hexamer (Invitrogen, 100 ng per reaction) and oligo $(dT)_{12–18}$ primers (Invitrogen, 500 ng per reaction).

Table 2
Measured characteristics (standard length and mass) and sample sizes of the sexually mature male Lamprologine cichlid fishes (*Neolamprologus pulcher*, *Telmatochromis temporalis*, *Julidochromis ornatus*, *Neolamprologus furcifer*, *Neolamprologus savoryi*, *Neolamprologus mustax*, *Neolamprologus multifasciatus*, and *Lamprologus ocellatus*) used in the current study. Values are presented as mean ± standard error of the mean.

Species	Sample size	Standard length (mm)	Mass (g)
N. pulcher	11	58.0 ± 1.1	4.7 ± 0.4
T. temporalis	12	59.8 ± 1.0	4.8 ± 0.2
J. ornatus	9	60.4 ± 2.8	4.5 ± 0.5
N. furcifer	10	90.1 ± 4.8	15.6 ± 2.4
N. savoryi	10	56.3 ± 1.0	4.4 ± 0.3
N. mustax	8	64.5 ± 2.1	7.4 ± 0.7
N. multifasciatus	14	26.7 ± 0.8	0.4 ± 0.0
L. ocellatus	12	42.7 ± 0.9	1.8 ± 0.1

Sequencing candidate genes in species pairs

We studied brain gene expression levels of IT and AVT, as well as of their receptors, using quantitative real-time PCR (RT-qPCR). The sequences for these five candidate genes were not available for these eight species, and so we designed primers to PCR-amplify a partial cDNA sequence for each of our genes of interest. Primers were designed based on sequences of the African cichlid *Astatotilapia burtoni* obtained from NCBI (Supplementary Table 1), with the exception of the sequence for the isotocin receptor ITR1, which was obtained from the genome sequence of *Neolamprologus brichardi* (http://cichlid.umd.edu/cichlidlabs/kocherlab/bouillabase.html). Primers were tested in silico using Primer 3 (Rozen and Skaletzky, 2000) and Amplify 3 (Engels, 2005). The primers were then used with cDNA samples from each species in a PCR using the manufacturer's protocol for TAQ DNA polymerase (Life Technologies, Carlsbad, CA). Amplicons were verified for size and specificity on a 1.2% agarose electrophoresis gel stained with SybrSafe (Life Technologies) and then purified using ExoSAP-IT (MJS Bio-Lynx, Brockville, ON). cDNA sequences were obtained by Sanger sequencing of these PCR products (Plate-forme d'Analyses Génomiques, Institut de Biologie Intégrative et des Systèmes, Université Laval). Sequences were verified using 4Peaks 4.1 (Griekspoor and Groothuis, 2006) and compared between species pairs to detect SNPs that could affect annealing efficiency of the primers with Serial Cloner 2.1 (Serial Basics, 2009) and ClustalX (Larkin et al., 2007). The sequences for each gene were then compared among all eight of our cichlid species, to determine sequence similarity among all species, and to determine whether there were any differences in the sequence of any of the genes between social and non-social species. Sequences were then used to design RT-qPCR-specific primers that were usable in both species of each species pair, and optimized for the same conditions (Supplementary Table 2). All partial cDNA sequences were submitted to NCBI (Supplementary Table 3).

Construction of the isotocin receptor gene tree

To learn about the relationship between the two IT receptors in Lamprologine cichlids, and in teleost fishes more generally, we constructed a gene tree of the IT receptor sequences that we had produced for our 8 study species, as well as any available IT receptor gene sequences for teleost fishes. We collected as many teleost IT receptor sequences as possible by conducting BLAST (Basic Local Alignment Search Tool; http://blast.ncbi.nlm.nih.gov/Blast.cgi) searches using the

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N. pulcher ITR1 and ITR2 partial cDNA sequences, as well as by searching the NCBI nucleotide sequence database for 'isotocin receptor' and 'oxytocin receptor' and compiling all matches from teleost fishes. We included the oxytocin receptor sequence from a mammal (humans, *Homo sapiens*), an amphibian (the cane toad, *Bufo marinus*), and a bird (red junglefowl, *Gallus gallus*) as outgroup sequences. See Supplementary Table 4 for GenBank accession numbers of all sequenced collected from NCBI.

Nucleotide sequences were aligned using MUSCLE (Edgar, 2004a,b) and viewed in MESQUITE (Maddison and Maddison, 2011) to verify the alignment of sequences, and trim the alignment such that only the region with overlapping sequence data for all species was retained. The resulting alignment of 735 base pairs (bp) was then used to estimate phylogenetic relationships and divergence times among gene sequences. The software program BEAST v1.8 (Drummond and Rambaut, 2007; Drummond et al., 2012) was used to perform 80 million generations of Bayesian Markov chain Monte Carlo, employing an uncorrelated lognormal relaxed molecular clock (Drummond et al., 2006) and HKY substitution model with gamma + invariant site heterogeneity. The model of nucleotide evolution was selected as the most appropriate using Akaike information criterion (AIC; Posada and Buckley, 2004) in the program jModelTest 2 (Guindon and Gascuel, 2003; Darriba et al., 2012). The divergence date of the outgroup taxa (mammals, amphibians, and birds) from teleost fishes was used as a calibration point, using the estimate of approximately 476 million years ago (Mya; Blair and Hedges, 2005) for their most recent common ancestor. Thus, the root of the tree was set at 476 Mya, with a standard deviation (SD) of 12 (20 million years, Ma), assuming a normal distribution. The program Tracer v1.5 (Rambaut et al., 2014) was used to analyze the output from BEAST v1.8 and confirm estimated sample size (ESS) values >200. Within BEAST v1.8, the program TreeAnnotator was used to find the maximum clade credibility (MCC) tree, using a burnin of 8 million trees (10%) set with a posterior probability limit of zero. The mean heights of each node were set so the MCC tree would have the mean height at each node of all the samples (Drummond et al., 2006). The final tree was plotted in R using the ape (Paradis et al., 2004) and phyloch (Heibl, 2008) packages in R version 3.0.2 (R Development Core Team, 2013).

Analysis of gene expression by quantitative real-time PCR

For each species pair, RT-qPCR primers (Supplementary Table 2) and whole brain cDNA were then used in a quantitative real-time PCR experiment following a scaled-down version (final volume of 25 μl) of the Quantitect SYBRGreen PCR kit manufacturer's protocol (Qiagen, Toronto, ON) in a 96-well RT-qPCR machine (Realplex^2 EP gradient S instrument, Eppendorf, Mississauga, ON). For each gene, a cDNA standard curve (six 1:10 dilutions) was quantified in duplicate for each species, followed by a melting curve analysis (50 °C to 90 °C) along with negative controls (no primers, no template). Gene expression of individuals was measured by RT-qPCR using the same instruments and reagents in triplicate for each species pair, and the mean Cq value was used for each individual. 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 non-social species within each species pair. Relative mRNA abundance of the gene of interest was then expressed against the relative reference gene 18S (primers based on the cichlid *Oreochromis esculentus*; O'Connor et al., 2013), and calculated according to the $\Delta\Delta C_t$ method (Pfaffl, 2001). See Supplementary Tables 2 and 3 for cDNA concentrations, hybridization temperatures and efficiency for each gene in each species pair.

Statistical analyses

RT-qPCR produces relative data, and gene expression values for each individual therefore represent the expression of the candidate gene relative to the average expression of the non-social species in each species pair, and relative to the 18S control gene. All samples for each species pair were extracted and reverse-transcribed together, and each gene was measured on a single plate for each species pair. The relative values for each gene can therefore be directly compared within each species pair. However, it is possible that there is variation in the extraction efficiency or measurement efficiency of the target genes relative to the 18S control genes among species pairs, and any comparisons of gene expression among all eight species must take this potential variability into account. Accordingly, we first separately compared the relative expression of each candidate gene between the social and non-social species within each species pair using linear models. We then examined general patterns in the relative abundance of candidate genes by comparing gene expression between social and non-social species using linear mixed models with 'species' nested within 'pair' included as random effects, to control for the non-independence of individuals within a given species, and to control for potential variation based on differences in extraction and measurement efficiency among species pairs. We did not include an explicit statistical phylogenetic control in these analyses, since the relative expression of a candidate gene between two species is not a trait under selection, and these values are therefore not appropriate to assess using a statistical phylogenetic control. However, the use of closely related and paired social and non-social species provides a level of phylogenetic control in the experimental design. All data were rank-transformed prior to analyses to achieve equality of variance among all species. All analyses were performed in R version 3.0.2 (R Core Team, 2013). Linear mixed models were performed using the nlme package (Pinheiro et al., 2013). The level of significance for all tests was assessed at $\alpha = 0.05$.

Ethical note

During all procedures, we took care to minimize handling time and stress as much as possible for the study animals. 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. 06-10-59) and the Zambian Department of Fisheries. All procedures adhered to both Canadian and Zambian laws, as well as the guidelines of the Canadian Council for Animal Care and the Animal Behavior Society/Association for the Study of Animal Behaviour.

Results

Sequencing candidate genes

All candidate genes were expressed in the brains of the eight studied species, and we successfully obtained partial cDNA sequences for each candidate gene (Supplementary Table 2). For the portion of the coding sequence that we obtained, all genes were highly conserved among all eight species (95–100% identity match among all species, for all 5 sequenced genes; Supplementary Table 5).

Time-calibrated gene tree

Our analyses of the IT receptor sequences (Fig. 2) suggest an evolutionary ancient divergence between ITR1 and ITR2 at 351.9 Ma, with a highest posterior density (HPD) 95% confidence interval of 250.1–462.6 Ma (this divergence is labeled as Node 2 in Fig. 2; see also Supplementary Table 6). This node in the phylogeny is well supported, with a posterior probability of 1.0, and corresponds to the likely timing of the teleost whole genome duplication event, dated to ~350 Mya (see review by Meyer and Van de Peer, 2005). Not all clades in the gene tree could be resolved with certainty, particularly the more recent divergences within Lamprologine cichlids (Fig. 2), which is likely because the partial sequences that we obtained were so highly conserved

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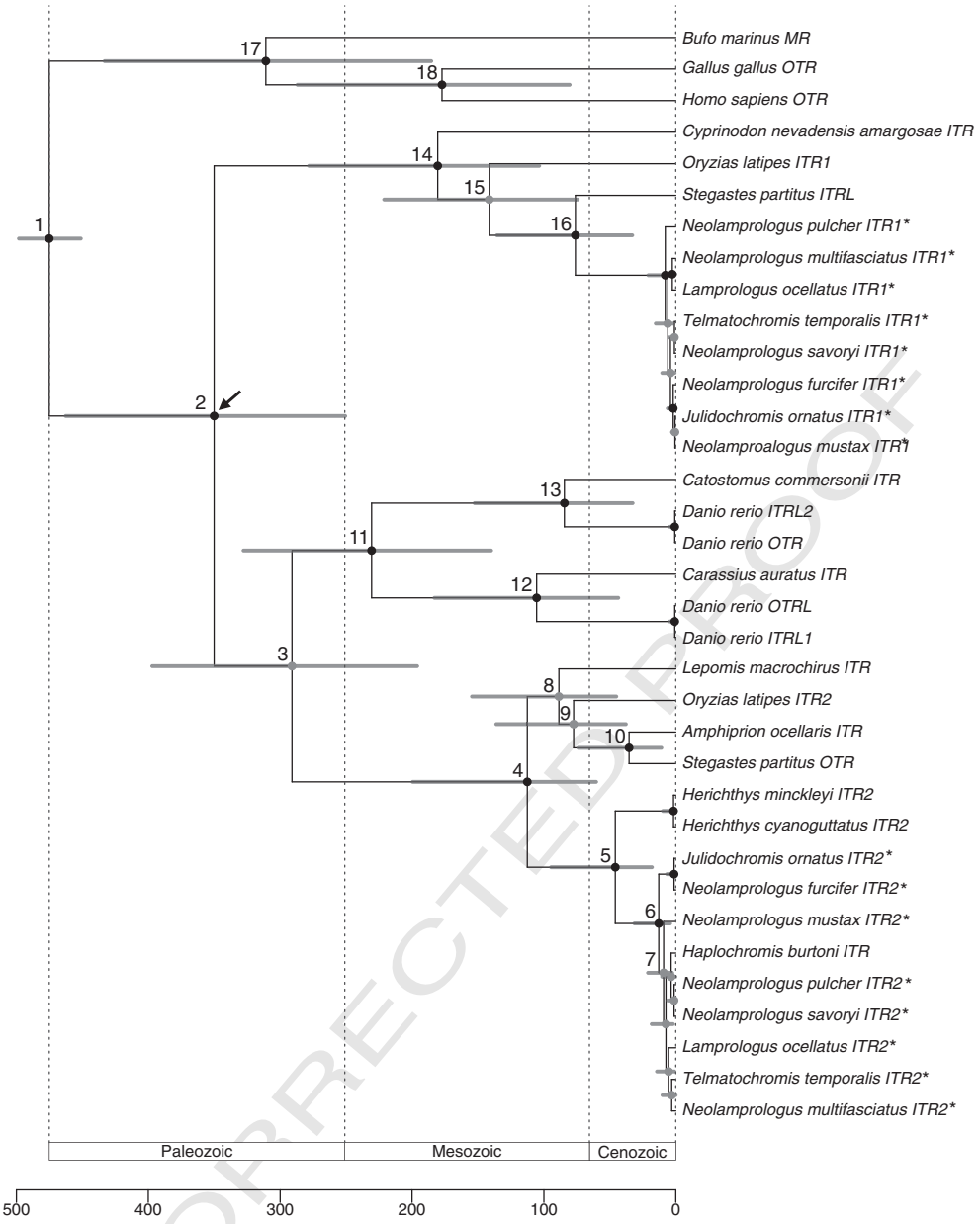

Fig. 2. Time-calibrated phylogenetic tree of isotocin receptor sequences from teleost fish, constructed in BEAST and rooted with outgroup sequences from a mammal, bird, and amphibian. Genes for the Lamprologine cichlids (*Neolamprologus pulcher*, *Telmatochromis temporalis*, *Julidochromis ornatus*, *Neolamprologus furcifer*, *Neolamprologus savoryi*, *Neolamprologus mustax*, *Neolamprologus multifasciatus*, and *Lamprologus ocellatus*) were sequenced in the current study, and are indicated on the tree by asterisks (*). All other sequences were obtained from GenBank (Supplementary Table 4). Taxon names are followed in capital letters by the gene name given on GenBank (MR = mesotocin receptor; OTR = oxytocin receptor; OTRL = oxytocin receptor-like; ITR = isotocin receptor; ITRL = isotocin receptor-like; ITR1 = isotocin receptor 1; ITR1 = isotocin receptor 2; ITRL1 = isotocin receptor-like 1; ITRL2 = isotocin receptor-like 2). Nodes are annotated to indicate posterior probabilities of >0.95 (black dots) or <0.95 (gray dots). Error bars (95% highest posterior density, HPD) for all divergences in gray. Nodes with estimated divergences >10 million years (Ma) are labeled numerically, and Supplementary Table 6 presents estimated divergences and posterior probabilities for all labeled nodes. Node 2 (indicated by an arrow, ↙) represents the divergence between the two isotocin receptors. The dating of this divergence corresponds to the likely timing of the teleost whole genome duplication event ~350 million years ago (Mya, see review by Meyer and Van de Peer, 2005).

Q1 The geological timescale is from Gradstein et al. (2004).

among species (see "Sequencing candidate genes" section in the Results section, above). However, there is high posterior support (>0.95) for all but four nodes that are older than 10 Ma (Supplementary Table 6).

Gene expression between social and non-social species

There was no consistent pattern of brain expression of any of the measured genes between social and non-social species (Table 3). Within the species pairs, we observed significant differences in brain gene expression between species, but there was variation in both the magnitude and direction of the pattern among pairs. In the first species pair, the social species (*N. pulcher*) had higher brain gene expression of all of measured genes (IT, ITR1, ITR2, AVT, AVTR) relative to the non-social species (*T. temporalis*, Fig. 3A–E). In the second species pair, expression of ITR1 (Fig. 3B) was higher in *J. ornatus* (the social species) relative to *N. furcifer* (the non-social species), with no difference in brain gene expression of IT, ITR2, AVT, or AVTR. In the third species pair, there were no significant differences in brain gene expression between the social species, *N. savoryi*, relative to the non-social species, *N. mustax*. In the fourth species pair, the patterns were reversed, and IT (Fig. 3A) and ITR2 (Fig. 3C) expression were lower in the social species, *N. multifasciatus*, relative to the non-social species, *L. ocellatus*,

Table 3
Results of linear models comparing rank-transformed relative brain gene expression between eight species of social (grouping and cooperatively breeding) and non-social (non-grouping and non-cooperative) Lamprologine cichlid fishes. There were no differences in relative brain expression of measured genes between the social and non-social species ($\alpha = 0.05$).

Gene	All species combined	
	t-Value	p-Value
IT	0.50	0.65
ITR1	1.34	0.27
ITR2	−0.77	0.52
AVT	2.26	0.11
AVTR	−0.33	0.76

with no differences in expression for ITR1, AVT and AVTR. See Table 4 for the full statistical results.

Discussion

In this study, we sequenced five candidate genes for eight species of social and non-social Lamprologine cichlid fishes. We identified that the sequences for each gene were highly conserved among species. We then examined the whole brain gene expression of these five candidate genes in the eight species, and hypothesized that consistent patterns in gene expression would suggest convergent evolution acting on these molecular pathways relative to social behavior. However, while we did find significant differences in brain gene expression between the social and non-social species of many species pairs, we did not find consistent patterns between the social and non-social species pairs examined. Together, our results do not provide support for convergent evolution of these candidate pathways, but instead highlight species-specific patterns of expression.

We predicted that a common expression pattern would be found for these strong candidates genes, because IT has been found to play an important role in social approach (Thompson and Walton, 2004) and attention to social stimuli (Reddon et al., 2012), while AVT has been found to play an important role in dominance-related behavior including aggression and hierarchy formation (e.g., Thompson and Walton, 2004; Santangelo and Bass, 2006, 2010; Braida et al., 2012). However, we did not find a common expression pattern for IT and AVT and their receptors in the social species relative to the non-social species. We found considerable variation among the species pairs, and a high degree of species-specificity. While we found that both IT and AVT were higher in two of the social species examined relative to their non-social species, we also found no difference in IT expression in one species pair, and no difference in AVT expression in two species pairs. One of the most interesting species-specific patterns was the *L. ocellatus* and *N. multifasciatus* species pair, which displayed opposite gene expression patterns to the other species pairs, such that the social species had lower IT expression relative to the non-social species. This species pair has a different ecology (these are shell-dwelling species while the other species pairs breed under rocks; Table 1), and is phylogenetically more distant than the

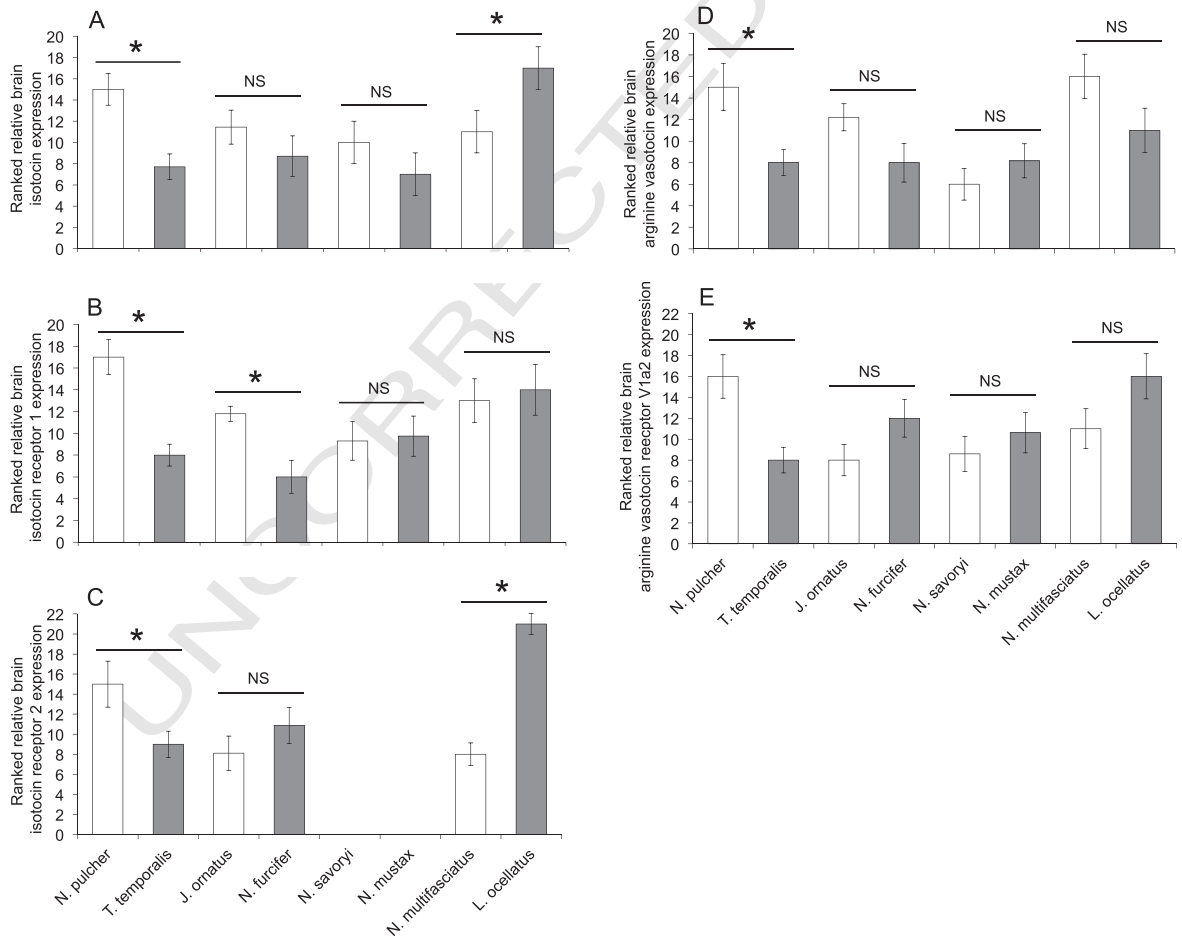

Fig. 3. Ranked relative brain expression of the measured candidate genes: (A) isotocin (IT); (B) isotocin receptor 1 (ITR1); (C) isotocin receptor 2 (ITR2); (D) arginine vasotocin (AVT); (E) arginine vasotocin receptor V1a2 (AVTR). We measured these genes in eight species of social (grouping and cooperatively breeding) and non-social (non-grouping and non-cooperative) Lamprologine fishes (*Neolamprologus pulcher*, *Telmatochromis temporalis*, *Julidochromis ornatus*, *Neolamprologus furcifer*, *Neolamprologus savoryi*, *Neolamprologus mustax*, *Neolamprologus multifasciatus*, and *Lamprologus ocellatus*), with the exception of ITR2, where we were not able to obtain data for *N. savoryi* and *N. mustax*. Data are presented as mean ± standard error of the mean (SEM). Asterisks indicate significant ($\alpha = 0.05$) differences between the social and non-social species for each pair of fish.

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Table 4
Results of linear models comparing rank-transformed relative brain gene expression between social (grouping and cooperatively breeding) and non-social (non-grouping and non-cooperative) species of selected closely related pairs of Lamprologine cichlid fishes (*Neolamprologus pulcher* and *Telmatochromis temporalis*; *Julidochromis ornatus* and *Neolamprologus furcifer*; *Neolamprologus savoryi* and *Neolamprologus mustax*; *Neolamprologus multifasciatus* and *Lamprologus ocellatus*). Bold italics indicate a significant ($\alpha = 0.05$) difference in relative brain gene expression between the social and non-social species of a given species pair.

Gene	Species pair							
	N. pulcher and *T. temporalis*		*J. ornatus* and *N. furcifer*		*N. savoryi* and *N. mustax*		*N. multifasciatus* and *L. ocellatus*	
	t-Value	p-Value	t-Value	p-Value	t-Value	p-Value	t-Value	p-Value
IT	***2.80***	***0.01***	1.07	0.30	−1.08	0.30	***−2.28***	***0.03***
ITR1	***3.98***	***<0.001***	***3.11***	***0.007***	0.17	0.86	−0.46	0.65
ITR2	***2.28***	***0.03***	−1.11	0.28	N/A	N/A	***−8.41***	***<0.001***
AVT	***2.92***	***0.008***	1.72	0.10	1.00	0.34	1.47	0.15
AVTR	***3.24***	***0.004***	−1.52	0.15	0.79	0.44	−1.83	0.08

other three species pairs (Fig. 1), which may be factors explaining the differences in brain gene expression. In another interesting species pair, *N. pulcher* and the *T. temporalis*, we found higher brain gene expression of every measured gene and receptor in the social species, *N. pulcher*, relative to the non-social species, *T. temporalis*. *N. pulcher* was the first documented cooperatively breeding cichlid species (Taborsky and Limberger, 1981), lives in colonies composed of distinct 2–200 social groups (Wong and Balshine, 2011), and has the largest species-typical group size of all of the species investigated. Social groups frequently contain up to 20 individuals (Heg et al., 2005b), and groups as large as 61 individuals have been reported (Heg et al., 2005a). *N. savoryi* form mixed-species colonies with *N. pulcher*, but occur at lower densities within the colonies than the *N. pulcher*, and the largest reported group is 36 individuals (Heg et al., 2005a). *N. multifasciatus* is also a colonial species, but both *N. multifasciatus* and *J. ornatus* live in much smaller social groups, typically comprised of fewer than 10 individuals (Kohler, 1998; Heg et al., 2005a; Heg and Bachar, 2006). Thus, it is possible that the highly gregarious nature of *N. pulcher* contributes to relatively higher brain gene expression of IT, AVT, and the respective receptors, in this species. Further research on patterns of candidate gene expression relative to gregariousness and species-typical group sizes within the Lamprologine cichlids is warranted.

We studied the AVT receptor V1a2, as the most likely receptor to be associated with complex social behavior in fish (Lema, 2010; Kline et al., 2011; Huffman et al., 2012; Oldfield et al., 2013). Our results suggest that the repeated association observed between affiliation and higher vasopressin receptor expression in between-species comparative studies in mammals (see review by Young et al., 1998) may not hold true for teleost fishes. However, we should note that many of the observed patterns with IT, AVT, and their receptors relative to social behavior in other studies were specific to certain brain regions (O'Connell and Hofmann, 2011; Godwin and Thompson, 2012; Young et al., 1998). In particular, areas of the hypothalamus and the ventral telencephalon, as well as the periaqueductal gray area, have been termed the 'social behavior network' (Newman, 1999; Goodson, 2005; O'Connell and Hofmann, 2011). Increased density of oxytocin receptors within this 'social behavior network' characterizes monogamous *Microtus* voles relative to polygynous species (reviewed in Wang et al., 1999), and gregarious estrilid finches relative to more solitary species (Goodson et al., 2009; Goodson and Kingsbury, 2011). Thus, is it possible that our social and non-social cichlids displayed similar patterns within these brain regions, but our use of whole-brain gene expression may have diluted region-specific patterns. Further, both IT and AVT have peripheral functions in fish (e.g., Pang, 1977; Balment et al., 2006; Amer and Brown, 1995), and any differences between social and non-social species within the 'social behavior network' may have been masked by the similarities in other areas related to basic physiological processes. Finally, although the hormonal pathways we chose were very strong candidates for having a role in the evolution of sociality, it is possible that other molecular pathways may show a convergent pattern in expression in social Lamprologines. Overall, the variation in the pattern of brain gene expression suggests that despite the known implication of these hormones in modulating specific social behaviors in fishes, consistent patterns in the expression of these genes at the whole-brain level is neither necessary nor sufficient during the evolution of group-living and cooperation in Lamprologines. Instead, our results suggest that the repeated evolution of sociality in Lamprologines involved differential expression of these candidate pathways in specific brain regions, during specific developmental periods that were not captured in our adult male fish, or through differential remodeling of other molecular pathways.

While we attempted to match the species pair with respect to phylogeny and ecological niche, there was some variation in both mating and parental care systems across the species pairs (Table 1). This may explain some of the inconsistencies in hormone and receptor expression among the species pairs, given that both monogamy (e.g., Insel et al., 1998; Oldfield et al., 2013) and parental care (e.g., O'Connell et al., 2012) are influenced by these, and other, candidate hormone pathways. In the current study, the relative nature of RT-qPCR data means that the experimental unit is effectively the pair, rather than the species, and so we cannot test for variation among our species based on factors that do not vary consistently between the two species within each pair. However, the Lamprologine cichlids display a diversity of mating systems, parental care systems, and social systems (e.g., Goodwin et al., 1998; Heg and Bachar, 2006; Sefc, 2011), which provides great potential to disentangle these various factors. Expanding this comparative model to a greater number of Lamprologine species may resolve some of these discrepancies, and would be a useful next step in this promising model system. The holding conditions were also not identical for all of the species pairs, as *N. pulcher*, *T. temporalis*, *N. savoryi*, *J. ornatus* and *N. furcifer* were processed in the evening, while *N. mustax*, *N. multifasciatus* and *L. ocellatus* were held overnight in aerated barrels, and then processed in the morning. There is evidence that AVT exhibits diurnal cycles in fish, such as rainbow trout (*Oncorhynchus mykiss*), where AVT increases throughout the day to reach maximal levels at sunset although the same studies reports no diurnal patterns in circulating IT (Kulczykowska and Stolarski, 1996; Kulczykowska, 1999). Therefore, we might predict that the species processed in the evening could exhibit higher AVT expression than those processed in the morning. For three of our species pairs this is unlikely an issue, since both species of each sister species pair were handled identically. However, for *N. savoryi* and *N. mustax*, the difference in handling may have masked potential differences in AVT expression. Stressors have also been shown to elevate circulating AVT, although not IT, in rainbow trout (Kulczykowska, 2001). Our fish processed in the morning may have been either more stressed (i.e., suffering from chronic holding stress) or less stressed (i.e., recovered following the capture stress) relative to the fish processed in the evening after capture. Therefore, we might also expect that differences in AVT expression between *N. savoryi* and *N. mustax* could have been masked by the differences in handling between the two species. Thus, the differences in

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handling mean that we must use caution when interpreting the AVT data from the *N. savoryi* and *N. mustax* species pair.

Divergence in the function of paralogous genes has been found in fish (Harris et al., 2014). Further, increased gene duplication retention is a feature of African cichlid genomes (Machado et al., 2014; Brawand et al., 2014) and has been proposed as a basis for functional novelty (Lynch and Force, 2000). However, no research to date has examined the relationship between the two teleost IT receptors and their gene expression, or explored whether one receptor is more relevant for social behavior than the other. We therefore first constructed a gene tree that placed the divergence between ITR1 and ITR2 at 352 Mya, which corresponds to the likely timing of the teleost whole genome duplication event of ~350 Mya (see review by Meyer and Van de Peer, 2005). Thus, our gene tree supports that these two IT receptors are indeed paralogues, and likely arose as a result of the teleost whole genome duplication event. In many species, only a single receptor has been sequenced (Fig. 2), but this likely reflects a research gap rather than widespread loss of one of the receptor paralogues. In Lamprologine cichlids, both of these IT receptor paralogues are still present, and appear to be associated with social system, although with variation in the direction of the association. We found higher ITR1 brain gene expression in two of the social species relative to their non-social relatives, and inconsistent patterns of ITR2 expression between the social and non-social species examined. It is therefore possible that the relationship between the IT molecular pathway and sociality in fishes is regulated through both of these receptors, in addition to the implication of IT itself. The fact that we found differential expression of the two receptors among species pairs suggests that there could be sub-functionalization or neo-functionalization in these receptors in cichlids or in fish more generally (Lynch and Force, 2000; Postlethwait et al., 2004). Additionally, the naming of the IT receptors has been inconsistent to date (Fig. 2), and we suggest that future studies identify whether one or both receptors are present in a given species, and use the nomenclature for ITR1 and ITR2 presented in this study. Consistency in nomenclature will identify more clearly which receptor is being examined, and will help to better understand the potential sub-functionalization of these two paralogues and their role in the evolution of social behavior in teleosts. Taken together, our results provide a strong impetus for future research investigating both receptors relative to social behavior.

In summary, this study contributes to an emerging and complex picture regarding the isotocin and vasotocin pathways relative to social system. Comparing the expression patterns of these molecular pathways in four occurrences of the evolution of sociality within Lamprologine cichlids shows considerable species-specificity in expression patterns of these nonapeptide hormones and their receptors, and no overall consistent pattern of gene expression associated with social behavior. Our study highlights the usefulness of the Lamprologine cichlids as a model for social evolution, provides one of the first comparisons of brain gene expression in the repeated evolution of cooperative breeding in vertebrates, and highlights promising avenues for future research.

Uncited reference

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Oldfield and Hofmann, 2011

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.yhbeh.2015.07.008.

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