Hormones and Behavior 64 (2013) 44–52

Contents lists available at SciVerse ScienceDirect

Hormones and Behavior

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

Arginine vasotocin and androgen pathways are associated with mating system variation in North American cichlid fishes

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ARTICLE INFO

Article history:
Received 19 November 2012
Revised 9 April 2013
Accepted 23 April 2013
Available online 30 April 2013

Keywords:
AVP
AVT
Herichthys
Monogamy
Polygamy
Prolactin
Vasopressin

ABSTRACT

Neuroendocrine pathways that regulate social behavior are remarkably conserved across divergent taxa. The neuropeptides arginine vasotocin/vasopressin (AVT/AVP) and their receptor V1a mediate aggression, space use, and mating behavior in male vertebrates. The hormone prolactin (PRL) also regulates social behavior across species, most notably paternal behavior. Both hormone systems may be involved in the evolution of monogamous mating systems. We compared AVT, AVT receptor V1a2, PRL, and PRL receptor PRLR1 gene expression in the brains as well as circulating androgen concentrations of free-living reproductively active males of two closely related North American cichlid species, the monogamous *Herichthys cyanoguttatus* and the polygynous *Herichthys minckleyi*. We found that *H. cyanoguttatus* males bond with a single female and together they cooperatively defend a small territory in which they reproduce. In *H. minckleyi*, a small number of large males defend large territories in which they mate with several females. Levels of V1a2 mRNA were higher in the hypothalamus of *H. minckleyi*, and PRLR1 expression was higher in the hypothalamus and telencephalon of *H. minckleyi*. 11-ketotestosterone levels were higher in *H. minckleyi*, while testosterone levels were higher in *H. cyanoguttatus*. Our results indicate that a highly active AVT/V1a2 circuit(s) in the brain is associated with space use and social dominance and that pair bonding is mediated either by a different, less active AVT/V1a2 circuit or by another neuroendocrine system.

Introduction

Across vertebrates, mating systems are remarkably variable (Shuster and Wade, 2003). General patterns of mating behavior include promiscuity (*i.e.*, individuals mate indiscriminately with multiple conspecifics), monogamy (male and female form a social bond and typically cooperate to care for their offspring), and polygamy (mate with several members of the opposite sex), which can occur as either polygyny or polyandry, depending on which sex is polygamous. Polygyny is much more common than polyandry and often involves resource-guarding males that control harems of females (Emlen and Oring, 1977; Heske and Ostfeld, 1990). A population's mating system may be genetically determined or may result when individuals plastically adjust their behavior according to local ecological or social conditions experienced at any given time (Fricke, 1980; Schradin et al., 2012). Actinopterygian (ray-finned) fishes exhibit diverse mating systems and therefore serve as good model organisms for study (Gross and Sargent, 1985). Heroine cichlid fishes are unusual in that most species form monogamous male–female pairs to mate and care for their offspring (Barlow, 1974).

The Rio Grande cichlid, *Herichthys cyanoguttatus*, is a typical monogamous heroine cichlid (Buchanan, 1971; Itzkowitz and Nyby, 1982) native to drainages of the Gulf Coast of northern Mexico and southern Texas (Brown, 1953; Miller et al., 2005) and is sexually monochromatic (Buchanan, 1971), a common trait of monogamous species. *Herichthys cyanoguttatus* pairs guard their offspring for 4–6 weeks before abandoning them and may remain together for multiple breeding cycles (Buchanan, 1971). The Cuatro Ciénegas cichlid, *Herichthys minckleyi*, is closely related to *H. cyanoguttatus* (Hulsey et al., 2010) and likely evolved when a recent ancestor of the two species invaded the spring-fed ponds and streams of the small, isolated desert valley of Cuatro Ciénegas to which it is endemic (Minckley, 1969). Interestingly, *H. minckleyi* is sexually dichromatic (Kornfield and Taylor, 1983), which is an indication of sexual selection; and Kornfield et al. (1982) captured lone females tending offspring and males guarding two nests simultaneously, suggesting that they might be polygynous. Recently we quantitatively compared mating

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0018-506X/$ – see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.yhbeh.2013.04.006

behavior in these species and determined that *H. minckleyi* males rarely tend their mates and offspring and when they are present they perform less parental care than *H. cyanoguttatus* males. Such a difference in mating behavior between closely related species is powerful because comparison between species is necessary to make conclusions about evolutionary change (Martins, 1996).

The nonapeptide arginine vasotocin (AVT) and its mammalian homolog arginine vasopressin (AVP) influence mating behavior in male vertebrates (reviewed by Goodson and Bass, 2001). AVP innervation throughout the brain and the distribution of the AVP V1a receptor subtype differ between monogamous and non-monogamous voles (Bamshad et al., 1993; Insel et al., 1994). Both pharmacological and genetic experiments have demonstrated a causal relationship between the AVP/V1a system and monogamous male behavior in the prairie vole, *Microtus ochrogaster* (Winslow et al., 1993; Young et al., 1999). In teleost fishes, AVT has been found to mediate mating behavior, aggression, and space use (Godwin and Thompson, 2012; Greenwood et al., 2008). Dewan et al. (2008, 2011) found that males of territorial, monogamous species of butterflyfishes (Chaetodontidae) had larger AVT-ir somata in the gigantocellular and magnocellular regions of the pre-optic area (POA) and higher AVT fiber densities in several nuclei of the telencephalon compared with males of non-territorial promiscuous species, but they were not able to determine whether the increased AVT-ir was related to pair-bonding or to territoriality.

One way to disentangle those factors would be to compare AVT and V1a expression between a monogamous species that maintains strong pair bonds and small territories with a polygynous species that maintains weak bonds and large territories. In the Amargosa pupfish, *Cyprinodon nevadensis amargosae*, Lema (2010) isolated and identified three AVT receptor subtypes as V1a1, V1a2 and V2 receptors. Using both *in situ* hybridization and immunohistochemistry Kline et al. (2011) and Huffman et al. (2012a) found almost identical distributions of the V1a2 subtype throughout the brain of the rockhind grouper, *Epinephelus adscensionis*, and the model cichlid *Astatotilapia burtoni*. In the grouper, the V1a2 subtype was much more highly expressed in the brain than the V1a1 subtype, and the expression of the V1a2 subtype was more closely associated with sex, reproduction, and behavior (Kline, 2010). We therefore decided to focus our study on this subtype. If AVT/V1a2 in *Herichthys* cichlids is associated with pair bonding then we would expect higher levels of AVT/V1a2 gene expression in the brain in reproductively active monogamous males than in non-reproductive males or polygynous males. If AVT/V1a2 is associated with aggressive territoriality then we would expect higher AVT/V1a2 expression in polygynous males than in non-reproductive males or monogamous males.

There is close association between monogamous mating behavior and paternal care (Barlow, 1991; Keenleyside, 1991). The hormone prolactin (PRL) stimulates paternal behavior in male vertebrates (birds: Buntin et al., 1991; mammals: Gubernick and Nelson, 1989; reviewed by Schradin and Anzenberger, 1999). In male fishes, exogenous PRL increases paternal fanning behavior and decreases fighting (Blüm and Fiedler, 1965; de Ruiter et al., 1986; Páll et al., 2004). While tetrapods possess only one PRL receptor (PRLR), two subtypes, PRLR1 and PRLR2, have been found in a cichlid fish (Fiol et al., 2009). Those authors found that PRLR2 was closely related to osmoregulation during times of hyperosmotic stress and was expressed in the brain at much lower levels than PRLR1. We reasoned that any PRL effects on parental behavior are more likely mediated by PRLR1 and thus decided to focus our study on this receptor subtype. In *H. cyanoguttatus*, both the female and male fan their offspring (Buchanan, 1971), suggesting that neural PRL pathways might be more active in monogamous *H. cyanoguttatus* males than in polygynous *H. minckleyi* males.

The androgens testosterone and (in actinopterygians) 11-ketotestosterone (11-KT) have been associated with social behavior in males and vary according to mating system in vertebrates (Wingfield et al., 1990), including fishes (Aubin-Horth et al., 2007; Hirschenhauser and Oliveira, 2006; Kindler et al., 1989; O'Connell and Hofmann, 2012a; Pankhurst, 1995; Parikh et al., 2006; Ros et al., 2003; Taves et al., 2009; Trainor and Hofmann, 2006). We expected that there might be differences in androgen levels between male *H. cyanoguttatus* and *H. minckleyi*.

In the current study we compared the social structure of *H. minckleyi* to that of *H. cyanoguttatus* in natural and semi-natural environments. We then tested in wild caught animals whether males of the polygynous *H. minckleyi* have higher levels of androgens than *H. cyanoguttatus* males. Finally, we hypothesized that mRNA expression levels of AVT, PRL, and their receptors in the telencephalon and hypothalamus vary between the two species according to mating system.

Materials and methods

Behavior analysis

To characterize breeding behavior in male *H. minckleyi* and understand how it differs from *H. cyanoguttatus*, we conducted field observations of breeding adults of both species in the summer of 2008. Observations were made of *H. cyanoguttatus* in Shoal Creek in Austin, Texas (30.283085° N, 97.751727° W) and in the San Marcos River in the wetland area of Spring Lake at Aquarena Springs in San Marcos, Texas (29.89096° N, 97.933466° W). The water in Shoal Creek ranged from approximately 1 to 10 m across and from a few centimeters to 1.0 m deep. The water in the San Marcos River was approximately 0.5 m deep. In the San Marcos River, territoriality and pair formation begin in mid-February; spawning begins in late March, peaks in mid-late April, and ends in early September (Buchanan, 1971). Observations were made of *H. minckleyi* at The University of Texas at Austin's Pickle Campus in semi-natural populations established June 24, 2000 in two outdoor concrete pools (30.388420° N, 97.724900° W; 24 × 24 m; 2 m deep) that contained nothing but water, fish, a detritus substrate, and dense stands of aquatic plants (*Typha* sp. [Typhaceae], *Potamogeton* sp. [Potamogetonaceae], *Chara* sp. [Characeae]). This habitat was similar to the natural environment in their native Cuatro Ciénegas (Minckley, 1969). There are no data for spawning seasonality for *H. minckleyi*.

Observations were made in a manner similar to that of Itzkowitz and Nyby (1982). The observer stood at the water's edge (on a floating boardwalk at the San Marcos River) and recorded observations on a clipboard. Data collection began when a female cichlid was observed guarding a brood-site or offspring and continued for 15 min, during which time the presence or absence of a male mate was recorded. In order to further clarify the social structure of *H. minckleyi*, an observer entered the pools with snorkeling gear and an underwater clipboard and recorded the general behavior of reproductively active males and females on a total of 10 days between June 17 and July 13, 2010. Once a large, territorial female or male was located, it was focally observed for up to 15 min and its use of space in the pool recorded on a hand drawn map. Territorial male *H. minckleyi* were individually recognizable and could be followed from one observation day to the next. Large, territorial males associated with brooding females and seemed to be reproductively active, but smaller, non-territorial males were not reproductively successful — in one case a smaller, non-territorial male–female pair attempted to spawn and was mobbed by dozens of conspecifics that rapidly consumed their eggs. There was no indication that the presence of the observer affected the social behavior of the fish at any of the locations after a short acclimation period.

We prepared maps for each species to show the typical densities and territory sizes of reproductively active individuals. The numbers of reproductively active females observed with a male mate and the number observed without a male mate during the 15 min observation period were compared between the two species with a Fisher exact probability test. To determine if males desert their mates as

their offspring get older, these categories were then subdivided based on age of the offspring (early stage: offspring not yet produced, eggs, or wrigglers; late stage: free-swimming fry) and compared again. Finally, to determine if any species differences in paternal care varied according to the developmental stage of the offspring, a Mantel–Haenszel–Cochran test for multiple 2 × 2 tables was performed.

Tissue collection

To obtain tissues for isolation and sequencing of the genes of interest, we used hook and line with earthworms as bait to collect three *H. cyanoguttatus* from Shoal Creek and two *H. minckleyi* from Pickle Campus. For the quantitative real-time PCR (qPCR) analyses, both non-reproductive and reproductively active males were targeted for collection. The reproductive status of each fish was recorded at the time of capture. *H. cyanoguttatus* were determined to be non-reproductive if they were not associated with a female and not in breeding colors; reproductive if they were associated with a female and were in breeding colors. *H. minckleyi* were individually recognized as reproductive based on previous behavior observations. Other, smaller males were recorded as non-reproductive. Fourteen male *H. cyanoguttatus* were collected from Shoal Creek between 18:30 and 20:30 h on May 18 and 21, 2012 by using either a seine or a backpack electroshocker (Smith Root LR-24). Eight of these were non-territorial, non-reproductive males and six were territorial reproductive males. Eight male *H. minckleyi* were collected from the Pickle Campus pools using gillnets between 19:00 and 21:00 h on July 13, 2010. Three were non-territorial, non-reproductive males. Five were large, territorial, reproductive males. The non-territorial males of both species were large enough to be physiologically capable of reproducing based on aquarium observations (pers. obs., R.G.O.), but were not reproductively active when captured.

Immediately after capture, fish were measured for standard length (SL) and weighed on a portable electronic balance (Ohaus). Fish were killed in the field by rapid cervical dissection within minutes of capture and blood was collected from the dorsal aorta using a heparinized SURFLO Winged Infusing Set (Fisher), transferred to a microcentrifuge tube containing a drop of heparin, and placed on ice for subsequent centrifugation for 15 min at 3000 rpm (1500 rcf). Plasma was stored at −80 °C until hormone assays were performed. Brains were dissected within ca. 5 min of euthanasia and stored in RNAlater (Ambion, USA) on ice. A few hours later, we used a sterile petri dish filled with RNAlater and a dissecting microscope to dissect telencephalon and hypothalamus from the rest of the brain and stored the tissues separately in RNAlater at 4 °C overnight and then at −20 °C until RNA extraction. Finally, gonads were placed in 100% ethanol for transportation to the lab and were weighed within hours, and the gonadosomatic index (GSI) calculated ([gonad weight/body weight] × 100). All procedures were approved by the Institutional Animal Care and Use Committee at The University of Texas at Austin.

Hormone assays

The following hormones were measured by EIA assays according to Kidd et al. (2010) and the manufacturers' instructions: 11-ketotestosterone (Cayman Chemicals, USA) and testosterone (Assay Designs). Plasma samples were diluted 1:30 with assay buffer from the respective EIA system. All samples and standards were assayed in duplicate. Optical Density (OD) was measured using a Beckman Coulter DTX 880 Multimode Detector or a Molecular Devices Spectramax M3 plate reader, and the OD readings for each sample were compared to the standard curve for quantification. The intra-assay coefficients of variation were less than 2% for T and 10% for 11-KT; the inter-assay CVs were 14% and 16% for T and 11-KT, respectively.

Isolation and phylogenetic analysis of AVT, V1a2, PRL and PRLR1 cDNA

Because the AVT, V1a2, PRL, PRLR1, and 18S sequences had not been described in either study species, we designed primers based on homologous teleost sequences (GenBank accession nos.: *Astatotilapia burtoni* AVT: AF517935; *A. burtoni* V1a2: AF517936; *Oncorhynchus mykiss* [Salmonidae] PRL: AAA49611; Nile tilapia, *Oreochromis niloticus* [Cichlidae], PRL: AAA53282; Pufferfish, *Takifugu rubripes* [Tetraodontidae], PRL: NP_001072092; Goldfish, *Carassius auratus* [Cyprinidae] PRL: AAB47156; *A. burtoni* PRLR: U67333) (see Table S1 for primer sequences). Whole brain cDNA from either *H. cyanoguttatus* or *H. minckleyi* was used as a template for touchdown PCR. After confirmation of correct fragment size by electrophoresis on a 1% agarose gel, PCR products were purified and submitted for direct sequencing at the University of Texas at Austin ICMB DNA Sequencing Facility. The partial mRNA sequences have been submitted to GenBank (accession nos. for *H. cyanoguttatus* AVT: HQ694776, V1a2: HQ694777, PRL: HQ694778, PRLR1: HQ694779, 18S: HQ694775, and *H. minckleyi* AVT: HQ694781, V1a2: HQ694782, PRL: HQ694783, PRLR1: HQ694784, 18S: HQ694780).

Based on the partial mRNA sequences described above, we determined the *H. cyanoguttatus* and *H. minckleyi* AVT, V1a2, PRL, and PRLR1 amino acid sequences for comparison with homologous amino acid sequences of multiple species. Using Mega 4 (http://www.megasoftware.net/mega.html), we aligned the sequences with ClustalW and generated bootstrapped neighbor-joining gene trees to confirm that we had cloned the correct orthologs (Fig. S1).

RNA extraction and cDNA synthesis

Total RNA was extracted from the telencephalon and hypothalamus of male *H. cyanoguttatus* and male *H. minckleyi* using Trizol Reagent (Invitrogen, USA) and treated with DNase I (Turbo DNase, Ambion) according to the manufacturer's instructions. RNA was reverse transcribed with Superscript III reverse transcriptase (Invitrogen) using oligo(dT) and random primers (Invitrogen). In negative controls the reverse transcriptase was omitted. The transcription reactions were purified using Microcon YM30 columns (Millipore).

Quantitative real-time PCR

qPCR primers were designed to span an exon–exon boundary and bind to a region in the sequence that is identical in both species (Table S2). Primer uniqueness was confirmed by performing BLAST search (NCBI, http://blast.ncbi.nlm.nih.gov). All qPCR primers were 19–23 base pairs in length, with GC contents of 40–60% and melting temperatures between 60 and 63 °C (<1 °C difference for each pair).

For each sample, transcript levels of candidate and reference genes were measured in triplicate on a ViiA7 real-time PCR system (Applied Biosystems) using Platinum SYBR Green qPCR Super Mix-UDG (Invitrogen). No-template controls for each primer mix and no-reverse-transcription controls were also run in triplicate. cDNA standard curves were calculated from a serial dilution of pooled cDNA from both species. Efficiency was calculated using the formula $E = 10^{(-1/\text{slope})} - 1$. A melting curve analysis from 60 °C to 95 °C with continuous fluorescence measurement concluded the end of the cycling protocol. Baseline and threshold values were automatically determined for all reactions using ViiA7 software (Applied Biosystems), and the results were exported to Microsoft Excel for further analysis. The threshold cycle (Ct) values for a sample were used to calculate the absolute quantity of cDNA based on the gene-specific linear standard curve of $\log_{10}$ ng total cDNA *vs.* Ct. Quantity was normalized to 18S.

Data analysis

We analyzed 11 dependent variables: mRNA levels of four genes (AVT, V1a2, PRL, PRLR1) from hypothalamus and from telencephalon, plasma concentrations of the androgens 11-KT and testosterone, and gonadosomatic index. Kolmogorov–Smirnov tests found data in several cells to significantly depart from normality ($p \leq 0.05$). Therefore, all dependent variables were $\log_{10}$-transformed after changing several 0 values in PRL and PRLR1 measures to 1×10^{-x}, with x = one decimal place smaller than the smallest value for each variable (*i.e.*, 1×10^{-7} for telencephalic and hypothalamic PRL and 1×10^{-2} for hypothalamic PRLR1). After the transformation, no variables significantly differed from normality (Kolmogorov–Smirnov $Z \geq 0.57$, $p > 0.05$).

A multivariate general linear model (GLM) was constructed in SPSS 20 that included all of the transformed dependent variables and also included processing time from capture until death as a covariate. Species was included as a fixed factor, as was social status (non-reproductive *vs.* reproductive). Processing time did not have an effect in the model so it was omitted from the final analysis. Because multivariate GLMs in SPSS omit all data from each replicate that is missing a value for one or more dependent variables, in order to include all individual animals we replaced missing values with the mean of the values observed in the cell (Zar, 1999, pg. 245–248). Only eight (3.3%) of 242 data points total (11 dependent variables for each of 22 animals) were missing and thus had to be substituted. Mean substitution of missing values preserves the mean value of each cell, and while underestimating variance and overrepresenting sample size, it is widely accepted to not seriously affect analyses as long as the fraction of substituted data points is below 10–15% (Schafer and Graham, 2002). Because several variables differed significantly in variance between fixed factor levels, we used Pillai's trace to interpret significance of the multivariate model.

Univariate between-subjects F-tests that indicated the effect of each fixed factor and interaction between fixed factors on each dependent variable were also produced by the GLM routine in SPSS. An interaction was found for several of the dependent variables, so for each variable we performed simple-effects tests to determine where the differences lay.

Simple-effects tests were performed on the $\log_{10}$-transformed data by comparing levels of one fixed factor using *t*-tests while holding the other fixed factor constant. Simple-effects tests were especially important because any differences that occur in the reproductive biology of these species we would expect to be pronounced in reproductive individuals and subdued in non-reproductive individuals.

Additionally, we used Minitab 16 to perform Pearson's correlations to describe relationships between $\log_{10}$-transformed dependent variables. Both species were included, and both non-reproductive and reproductive males were included. We employed a false discovery rate p-value threshold (Benjamini and Hochberg, 1995), which provides adjusted alpha values to account for multiple comparisons (Table S3). Therefore, we report the exact p-value produced for each correlation analysis and indicate whether it fell below its adjusted alpha.

Results

As expected, reproductively active male and female *H. cyanoguttatus* formed pairs that guarded small territories approximately 1 m in diameter (Fig. 1A). Reproductively active male *H. minckleyi*, however, maintained large territories several meters in diameter (Fig. 1B). Although each pool contained several hundred individuals, we observed only four large, territorial males within each pool. Each territory contained one or more nests, which were deep cylindrical holes in the vegetation and detritus substrate, and one or more brooding females were observed in each male territory. Brooding females were reclusive until their offspring became free-swimming, at which time they were seen slowly swimming with their offspring, although they appeared to remain within the same male's territory day after day. Affiliative and paternal behavior typical of paired cichlids (Oldfield and Hofmann, 2011) was regularly observed in *H. cyanoguttatus* pairs, but was never observed between territorial *H. minckleyi* males and females, although males occasionally came into close vicinity of parental females.

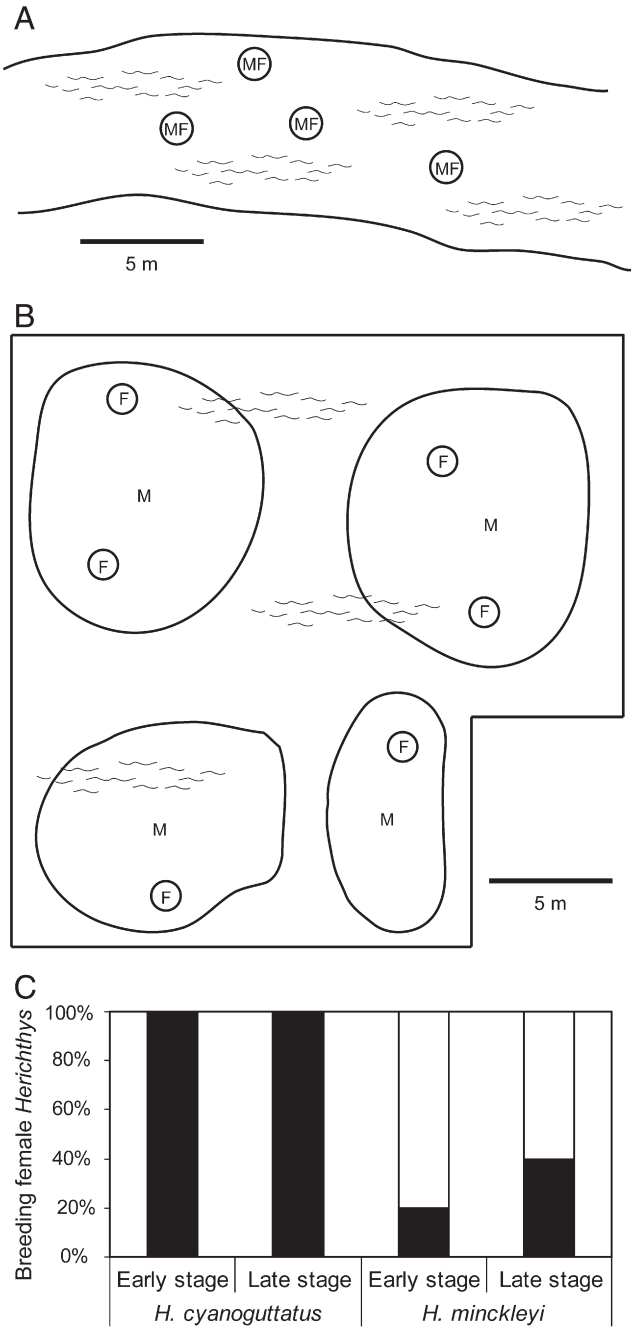

Fig. 1. Maps of habitats of reproductively active (A) *H. cyanoguttatus* and (B) *H. minckleyi* showing territories and locations of males (M), females (F), and pairs (MF). Reproductively active male and female *H. cyanoguttatus* formed pairs that guarded small territories (approximately 1 m). A relatively smaller number of reproductively active male *H. minckleyi* maintained large territories (several m) within which they mated with multiple females. (C) Distributions of reproductively active *Herichthys* females that were observed with a male mate (black bars) or alone (white bars) throughout a 15-min observation period. Female *H. cyanoguttatus* were observed with males significantly more often than were *H. minckleyi*, irrespective of developmental stage of offspring. Early stage: offspring not yet produced, eggs, or wrigglers. Late stage: free-swimming fry.

Total numbers of brooding females attended by males *vs.* brooding females unattended by males differed significantly between species (Fisher exact probability test, two-tailed: $p < 0.0005$). Brooding *H. cyanoguttatus* females were always observed with a male mate, while brooding *H. minckleyi* females were usually observed without a male mate. We also compared numbers of attended and unattended brooding females in the early stages of brooding (*i.e.*, before free-swimming offspring are present) separately from those in the later stage of brooding (free-swimming fry) (*H. cyanoguttatus*: early stage n = 5, late stage n = 10; *H. minckleyi*: early stage n = 5, late stage n = 5). The two species differed in numbers of attended females and unattended females at both the early stage ($p < 0.05$) and late stage ($P = 0.02$); and developmental stage of offspring had no effect on male presence (Mantel–Haenszel–Cochran test for multiple 2×2 tables: Common odds $ratio_1 = 9.89$, $p = 0.002$). These results indicate that although male *H. minckleyi* did not constantly associate with a particular brooding female, they maintained ongoing relationships with those females with which they had mated (Fig. 1C).

In the multivariate GLM analysis, the independent factor 'species' showed significant effects on gene expression and hormonal variables (Pillai's Trace = 0.84; $F_{11,8} = 3.68$; $p = 0.04$). The independent factor 'reproductive status' did not show significant effects on gene expression and hormonal variables in the multivariate GLM (Pillai's Trace = 0.32; $F_{11,8} = 0.35$; $p = 0.95$), and there was no significant interaction between 'species' and 'reproductive status' (Pillai's Trace = 0.60; $F_{11,8} = 1.10$; $p = 0.46$). The univariate between-subjects effects tests comparing between species and between non-reproductive and reproductive males for each dependent variable revealed both 'species' and 'interaction' effects. Specifically, these tests found species differences in 11-KT and in testosterone, an interaction between the two factors in the production of telencephalic AVT, an effect of species and an interaction effect on hypothalamic V1a2, species and interaction effects on telencephalic PRLR1, and a species effect on hypothalamic PRLR1. All of these effects were supported by a significant value in the corrected model, except the telencephalic AVT interaction (Table 1).

11-KT was higher in reproductive male *H. minckleyi* than in reproductive male *H. cyanoguttatus* (Fig. 2A). On the other hand, testosterone was higher in reproductive male *H. cyanoguttatus* than in reproductive male *H. minckleyi* (Fig. 2B). Although no interaction was indicated in the GLM or univariate between-subjects effects tests, simple-effects *t*-tests suggested that these differences existed between reproductive males only and that there was no species difference for non-reproductive males (Figs. 2A,B). Gonadosomatic index (GSI) did not differ according to species or reproductive status.

Simple-effects tests revealed no differences between groups in telencephalic AVT or V1a2 (Fig. 3A), although hypothalamic V1a2 was

Table 1
Results of univariate between-subjects effects tests comparing gene expression in different regions of the brain and plasma hormone concentrations between non-reproductive and reproductive male monogamous *H. cyanoguttatus* and polygynous *H. minckleyi* (d.f. = 3,18 for corrected model; 1,18 for each dependent variable). Bold font indicates $P < 0.05$. The multivariate Pillai's trace value was found to be significant for species (see text). Hyp = hypothalamus, Tel = telencephalon.

	Corrected model		Species		Social status		Interaction	
	F	p	F	p	F	p	F	p
11-KT	5.01	**0.01**	13.44	**0.002**	0.16	0.69	0.08	0.78
T	6.83	**0.003**	20.27	**<0.001**	0.58	0.46	0.001	0.98
GSI	0.57	0.64	0.002	0.97	1.46	0.24	0.001	0.97
Tel AVT	1.88	0.17	0.02	0.88	1.99	0.18	4.68	**0.04**
Tel V1a2	0.54	0.66	0.22	0.64	0.15	0.70	0.87	0.36
Hyp AVT	0.17	0.92	0.09	0.77	0.38	0.55	0.01	0.91
Hyp V1a2	4.85	**0.01**	7.80	**0.01**	0.70	0.41	4.58	**<0.05**
Tel PRL	0.47	0.71	0.77	0.39	0.01	0.91	0.52	0.48
Tel PRLR1	6.99	**0.003**	16.74	**0.001**	0.69	0.42	1.05	0.32
Hyp PRL	0.74	0.54	0.72	0.41	1.26	0.28	1.18	0.29
Hyp PRLR1	4.32	**0.02**	5.70	**0.03**	0.77	0.39	4.75	**0.04**

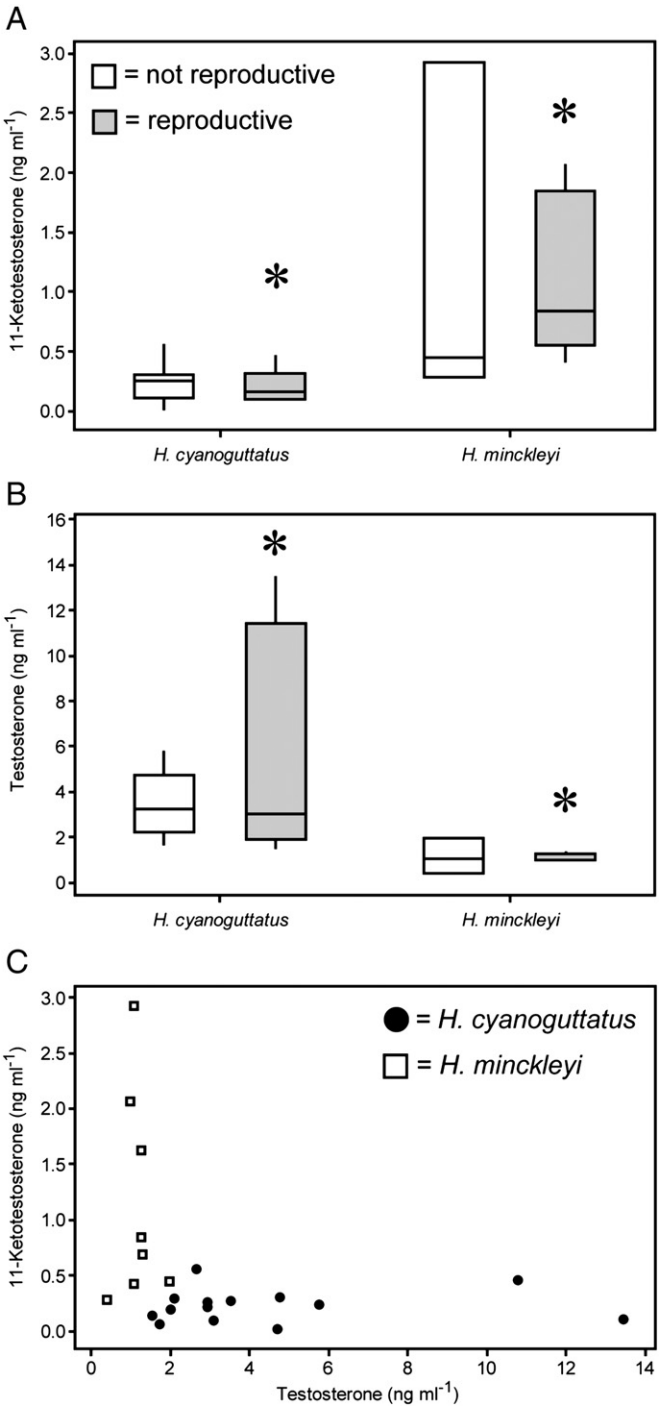

Fig. 2. Boxplots showing circulating androgens of non-reproductive (white bars) and reproductive (gray bars) males of monogamous *H. cyanoguttatus* and polygynous *H. minckleyi*. (A) 11-ketotestosterone, * = simple-effects two-tailed *t*-test $p = 0.002$. (B) Testosterone, * = simple-effects two-tailed *t*-test $p = 0.02$. Boxplots represent medians and first and third quartiles; whiskers represent upper and lower limits. (C) Scatterplot of 11-ketotestosterone *vs.* testosterone. Filled circles represent non-reproductive and reproductive male *H. cyanoguttatus* and open squares represent non-reproductive and reproductive male *H. minckleyi*. A correlation analysis that included combined $\log_{10}$-transformed data from both species was not significant after false discovery rate (FDR) adjustment for multiple comparisons (Benjamini and Hochberg, 1995): Pearson statistic −0.44, $p = 0.04$, FDR $\alpha = 0.01$, but the pattern suggests a negative relationship between the two variables.

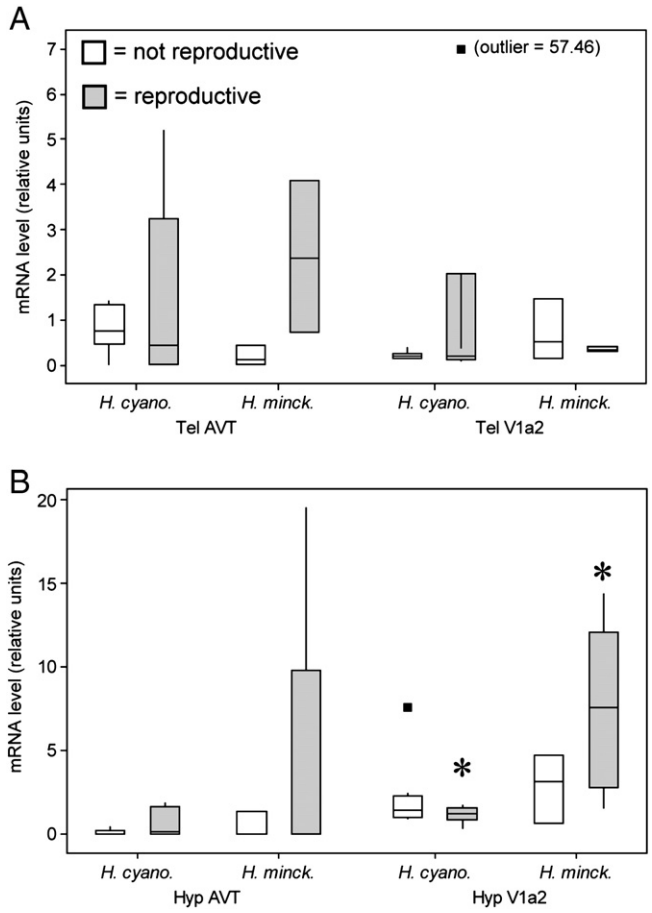

Fig. 3. Boxplots showing AVT and V1a2 gene expression (mRNA) in (A) telencephalon (Tel) and (B) hypothalamus (Hyp) of non-reproductive (white bars) and reproductive (gray bars) male monogamous *H. cyanoguttatus* and polygynous *H. minckleyi*. Boxplots represent medians and first and third quartiles; whiskers represent upper and lower limits. Squares = outliers. * indicates a significant result ($p = 0.01$) of a simple-effects two-tailed *t*-test.

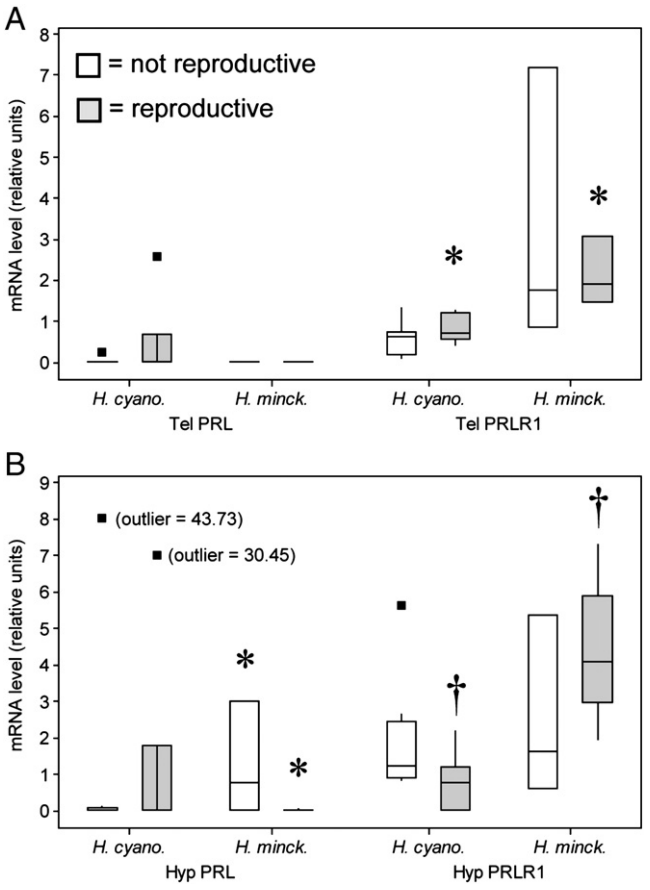

Fig. 4. Boxplots showing PRL/PRLR1 gene expression (mRNA) in (A) telencephalon (Tel) and (B) hypothalamus (Hyp) of non-reproductive (white bars) and reproductive (gray bars) male monogamous *H. cyanoguttatus* and polygynous *H. minckleyi*. Simple-effects two-tailed *t*-tests in A: * = $p < 0.001$; in B: * = $p < 0.01$, † = $p = 0.03$. Boxplots represent medians and first and third quartiles; whiskers represent upper and lower limits. Squares = outliers.

higher in reproductive *H. minckleyi* than in reproductive *H. cyanoguttatus*. No such species difference was found in non-reproductive males (Fig. 3B).

Expression of PRLR1 in both the telencephalon and hypothalamus was higher in *H. minckleyi* than in *H. cyanoguttatus* in reproductive males but not in non-reproductive males according to simple-effects tests (Fig. 4). Although the fixed factors showed no effect on PRL expression in any brain region in the univariate between-subjects tests, simple-effects tests suggested that expression was higher in non-reproductive male *H. minckleyi* than in reproductive male *H. minckleyi* in the hypothalamus.

None of the correlations remained significant after false discovery rate correction (Benjamini and Hochberg, 1995) (Table S3). However, the pattern of association between 11-KT and testosterone suggested a negative relationship (Fig. 2C). To further examine the relationships between hormone levels, gonadal size, and gene expression, we used the iGraph package in R (R Core Team, 2012) to create association networks based on those Pearson correlation coefficients that produced p-values < 0.05. This analysis enabled us to explore potential functional networks that may contribute to mating system and space use variation in *Herichthys* species. Specifically, the variables related to polygyny and large territories, including 11-KT, V1a2, PRLR1, and even AVT, were associated with each other and were negatively associated with the variables related to monogamy and small territories, testosterone and PRL (Fig. 5).

Discussion

In the current study we compared males of a monogamous cichlid species that maintains strong pair bonds and forms small territories with males of a closely related polygynous species that maintains weak bonds but forms large territories. We examined circulating androgen levels along with the activity of AVT and PRL pathways in both hypothalamus and telencephalon. Our results show that circulating 11-KT levels along with hypothalamic V1a2 expression and hypothalamic and telencephalic PRLR1 expression are more strongly associated with territoriality and social dominance than with pair bonding in this system.

Social structure differed considerably between the two species. In each pool four large male *H. minckleyi* divided all available space into large territories that each included dozens of smaller subordinate males, while *H. cyanoguttatus* pairs maintained small territories from which they excluded rival males. Despite being in a non-native environment for 10 years, the prevalence of unattended females in *H. minckleyi* was consistent with observation we made previously in their natural habitat. This consistency in social organization, along with the sexual dichromatism indicative of sexual selection in *H. minckleyi*, strongly indicates that our observed differences in behavior, physiology, and gene transcription are evolutionary differences and not merely plastic adjustments to local environmental conditions. Furthermore, species differences in physiology and gene transcription were observed in reproductive males but not in non-reproductive males, suggesting that the differences were related to the different

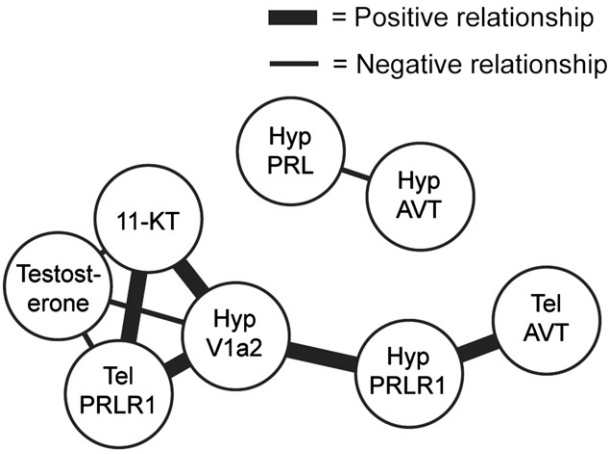

Fig. 5. A covariance network that integrates circulating androgens and neural gene expression of male *H. cyanoguttatus* and *H. minckleyi*. Lines represent Pearson correlations of $p < 0.05$ (although no p-values fell below adjusted alphas after accounting for multiple comparisons, Benjamini and Hochberg, 1995). Fat lines = positive relationship, thin lines = negative relationship. Hyp: Hypothalamus; Tel: Telencephalon.

mating strategies and were not due to other aspects of life history, habitat, or an artifact. Interestingly, we previously found that frequency of aggressive brood defense performed by reproductive males was higher in *H. cyanoguttatus* than *H. minckleyi*. Therefore, the current study distinguished social dominance from simple rates of aggression and identified physiological factors that are more closely associated with social dominance (Francis et al., 1992; Hamilton et al., 2005).

Circulating 11-KT levels were higher in *H. minckleyi*, which formed large territories, and testosterone was higher in *H. cyanoguttatus*, which remained close to their mates and more actively defended their offspring. This suggests that 11-KT is more involved than testosterone is in mediating territoriality and social dominance. In several teleost species, territoriality has been associated with 11-KT and sometimes with testosterone (Huffman et al., 2012b; O'Connell and Hofmann, 2012a; Pankhurst, 1995; Parikh et al., 2006; Rodgers et al., 2006; Trainor and Hofmann, 2006). In the plainfin midshipman, *Porichthys notatus*, 11-KT was higher in large, territorial males but testosterone was higher in smaller, non-territorial, sneaker males (Brantley et al., 1993). In bluegill sunfish, *Lepomis macrochirus* [Centrarchidae], both testosterone and 11-KT were associated with spawning in dominant territorial individuals, but in small, non-territorial cuckolders only testosterone was elevated (Kindler et al., 1989). In the St. Peter's cichlid, *Sarotherodon galilaeus*, 11-KT levels in males were elevated when fish were maintained at high-competition male-biased operational sex ratios, but there was no difference between monogamous and polygamous males (Ros et al., 2003). In the cooperatively breeding cichlid *Neolamprologus pulcher*, dominant males had higher levels of 11-KT but similar levels of testosterone compared to subordinate males (Taves et al., 2009). On the other hand, testosterone may have been higher in *H. cyanoguttatus* because (1) it is more involved in producing acts of aggressive behavior in brood defense, (2) it may mediate courtship or reproductive physiology because males were in constant contact with females, or (3) it simply may have been higher because it had not been converted to 11-KT. Quantitative measures of brood defense tested for correlation with testosterone and 11-KT, and pharmacological androgen administration, would be required to clarify the roles of androgens in *Herichthys* mating systems. In other vertebrates testosterone is the predominant androgen and has been associated with aggressive behavior and mating system (Klose et al., 2009; Lynn, 2008).

Gonad size did not vary according to species or social status. In diverse vertebrates small testes are often found in polygynous (harem-forming) and monogamous species, and large testes are found in species that mate promiscuously (Harcourt et al., 1981; Heske and Ostfeld, 1990; Stockley et al., 1997). Furthermore, some studies have found larger gonads in territorial, reproductive male cichlids than in non-reproductive males (Hofmann and Fernald, 2000). However, in wild-caught Midas cichlids, *Amphilophus citrinellus*, Oldfield (2011) found mature spermatozoa in all adult males, even those that were considerably smaller than the largest, presumably territorial, males. In this way *Herichthys* males are probably similar to *Amphilophus* males. In addition, the testes of *Herichthys* males seem to typically be capable of producing enough spermatozoa to fertilize the eggs of more than one female.

We found that AVT mRNA levels did not differ according to species or social status. AVP/AVT activity in the hypothalamus is often associated with aggression and territoriality in diverse vertebrates (reviewed by Goodson and Thompson, 2010). In teleosts, whole brain AVT levels are generally higher in aggressive individuals (Aubin-Horth et al., 2007; Greenwood et al., 2008; Renn et al., 2008). However, in teleosts AVT-producing neurons are only found in the parvo-, magno-, and gigantocellular portions of the pre-optic area (POA) and (to a much lesser extent) the lateral tuberal nucleus of the ventral hypothalamus (Goodson and Bass, 2000; Greenwood et al., 2008). Given the location of the POA between telencephalon and rostral hypothalamus, it is likely that our hypothalamic samples contained the magno- and gigantocellular nuclei of the POA (see also Burmeister et al., 2007; Greenwood et al., 2008), where increased AVT expression has been associated with social dominance or, more generally, increased male aggression (Dewan et al., 2008, 2011; Godwin et al., 2000; Greenwood et al., 2008; Larson et al., 2006; Lema, 2006; Santangelo and Bass, 2010). Our telencephalic AVT measures, on the other hand, likely reflect expression in the parvocellular POA (Burmeister et al., 2007; Greenwood et al., 2008), where previous findings in other teleosts showed increased parvocellular AVT activity to be associated with subordinate behavior (Greenwood et al., 2008; Larson et al., 2006; Ramallo et al., 2012) or, more generally, decreased aggression and/or increased shoaling behavior (Dewan and Tricas, 2011; Lema, 2006; Santangelo and Bass, 2010). We did not find any differences in hypothalamic (*i.e.*, putatively magno- and/or gigantocellular) AVT levels. We also found no differences in telencephalic AVT or V1a2 expression. However, our results indicate that territoriality in *Herichthys* may be regulated, at least in part, by V1a2 in the hypothalamus (Lema et al., 2012).

Pair bond formation may involve an AVT/V1a2 circuit separate from a circuit regulating territoriality (Goodson, 2008). There is an association between AVT/AVP/V1a and courtship and reproductive behavior in teleosts (Bastian et al., 2001; Greenwood et al., 2008; Grober et al., 2002; Pickford and Strecker, 1977; Salek et al., 2002; Semsar et al., 2001) and in diverse tetrapod taxa (Goodson and Bass, 2001). Young and Wang (2004) proposed that pair bonding in prairie voles involves AVP-V1a binding in the ventral pallidum that regulates partner preference. It is possible that in pair-bonded *H. cyanoguttatus* AVT or V1a2 is up-regulated in a particular region of the telencephalon or hypothalamus, but that such an up-regulation was masked by even stronger up-regulation of a territoriality circuit in *H. minckleyi* and was therefore not discernible in the current study. On the other hand, pair bond formation may be regulated through a separate pathway involving isotocin, which was not examined in the current study. In either case, our recent findings in the convict cichlid, *Amatitlania nigrofasciata*, that nonapeptides regulate the formation of the pair-bond (Oldfield and Hofmann, 2011) and that isotocin in particular regulates paternal behavior (O'Connell et al., 2012) suggest that a role of these pathways in forming a pair bond might arise from a conserved ancestral function in social recognition (Bielsky and Young, 2004; van Wimersma Greidanus and Maigret, 1996). Clearly, in order to elucidate in more detail the neural circuits involved in mating system evolution, future studies using *Herichthys* will need to focus on specific fore- and midbrain regions involved in social decision-making (O'Connell and Hofmann, 2011a,b, 2012b).

Male *H. cyanoguttatus* spent more time close to their offspring than did male *H. minckleyi*. We therefore expected them to have higher PRL expression, given the role of this hormone in stimulating paternal care in other vertebrates. For example, male three-spine sticklebacks, *Gasterosteus aculeatus* [Gasterosteidae], treated with PRL increased paternal behavior (fanning eggs) and reduced courtship displays (zig-zag dances) (Páll et al., 2004). PRL has also been positively associated with fanning in several species of heroine cichlids (Blüm and Fiedler, 1965). Surprisingly, we did not see elevated PRL in pair-bonded *H. cyanoguttatus* males. How can we explain this surprising result? Our *H. cyanoguttatus* collection occurred after an intense rain that seemed to have washed most offspring from the stream, and we believe that most of our reproductive *H. cyanoguttatus* males were therefore in the early stages of the reproductive cycle and may not yet have been performing parental care. On the other hand, aspects of our results suggest down-regulation of PRL in reproductive polygynous *H. minckleyi*. PRL in the hypothalamus was lower in reproductive than in non-reproductive *H. minckleyi* while PRLR1 in the telencephalon and hypothalamus was higher in reproductive *H. minckleyi* than in reproductive *H. cyanoguttatus*.

The network analysis (Fig. 5) suggests potential functional relationships that may lead to new hypotheses about the neuroendocrine and molecular basis of variation in mating systems. We found associations between physiology and hypothalamic *vs.* telencephalic candidate gene expression. Across vertebrates, androgens have been associated with AVT and V1a expression (reviewed by Goodson and Bass, 2001; in fishes, Aubin-Horth et al., 2007). Follicle stimulating hormone (FSH) and luteinizing hormone (LH), which stimulate gonadal androgen production, are known to inhibit the effects of PRL (Blüm and Fiedler, 1965). It is therefore possible that in *H. minckleyi* the conversion of testosterone to 11-KT is somehow related to the production of AVT, V1a2, and PRLR1, and that the increase in PRLR1 inhibits the production of PRL, resulting in decreased parental care. Even though many other factors not considered here are likely involved, and the relationships may well differ in specific brain regions, these patterns nevertheless suggest distinct functional modules that may govern space use behavior and therefore play a role in shaping mating systems. They also provide strong predictions that *H. cyanoguttatus* and *H. minckleyi* would respond to 11-KT manipulations by modifying territory size and fanning behavior.

Conclusions

We found in the present study that V1a2 in the hypothalamus is associated with space use, social dominance, and mating system in two teleost fish species. Future studies employing controlled pharmacological experiments, brain region-specific analyses, and phylogenetic comparisons, will be necessary to better understand molecular pathways involved in aggression, space use, pair bonding, and the evolution of mating systems in fishes, and to identify those mechanisms that might be similar across vertebrate taxa.

Acknowledgments

We thank members of the Hofmann laboratory for discussions, Sean Maguire and Bryan Matthews for technical assistance, and Nadia Aubin-Horth, Mike Benard, Sheryl Petersen, and Scott Small for commenting on earlier versions of the manuscript. Jakob and Jonathan Dornhofer, Adam Cohen and Ben Labay aided in collecting fish. This work was supported by an Academic Careers in Science and Engineering plus NSF-ADVANCE Opportunity grant to R.G.O., and National Science Foundation Grant IOS-0843712, an Alfred P. Sloan Foundation Fellowship, the Dwight W. and Blanche Faye Reeder Centennial Fellowship in Systematic and Evolutionary Biology, and an Institute for Cellular & Molecular Biology Fellowship to H.A.H.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.yhbeh.2013.04.006.

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