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Neuroendocrine profiles associated with discrete behavioural variation in *Symphodus ocellatus*, a species with male alternative reproductive tactics

B. M. NUGENT,*† K. A. STIVER,*‡ S. H. ALONZO*§ and H. A. HOFMANN†

*Department of Ecology and Evolutionary Biology, Yale University, 165 Prospect St., New Haven, CT 06520, USA, †Department of Integrative Biology, Center for Computational Biology and Bioinformatics, The University of Texas at Austin, 2415 Speedway, Austin, TX 78712, USA, ‡Department of Psychology, Southern Connecticut State University, 501 Crescent St., New Haven, CT 06515, USA, §Department of Ecology and Evolutionary Biology, University of California, Santa Cruz, 1156 High St., Santa Cruz, CA 95064, USA

Abstract

The molecular mechanisms underlying phenotypic plasticity are not well understood. Identifying mechanisms underlying alternative reproductive tactics (ARTs) in species for which the behavioural and fitness consequences of this variation are well characterized provides an opportunity to integrate evolutionary and mechanistic understanding of the maintenance of variation within populations. In the ocellated wrasse *Symphodus ocellatus*, the behavioural phenotypes of three distinct male morphs (sneakers, satellites and nesting males), which arise from a single genome, have been thoroughly characterized. To determine the neuroendocrine and genomic mechanisms associated with discrete phenotypic variation and ARTs in *S. ocellatus* in their natural environment, we constructed a whole-brain *de novo* transcriptome and compared global patterns of gene expression between sexes and male morphs. Next, we quantified circulating cortisol and 11-ketotestosterone (11-kt), mediators of male reproductive behaviours, as well as stress and gonadal steroid hormone receptor expression in the preoptic area, ventral subpallial division of the telencephalon and dorsolateral telencephalon, critical brain regions for social and reproductive behaviours. We found higher levels of 11-kt in nesting males and higher levels of cortisol in sneaker males relative to other male morphs and females. We also identified distinct patterns of brain region-specific hormone receptor expression between males such that most hormone receptors are more highly expressed in satellites and nesting males relative to sneakers and females. Our results establish the neuroendocrine and molecular mechanisms that underlie ARTs in the wild and provide a foundation for experimentally testing hypotheses about the relationship between neuromolecular processes and reproductive success.

Keywords: alternative reproductive tactics, brain, gene expression, hormones

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Introduction

Defining and characterizing the basic biological mechanisms that give rise to phenotypic variation, and serve as a substrate for selection and ultimately species diversification, is a fundamental challenge in modern biology. Individuals of a single species can display discrete variation in behavioural repertoire, maximizing their reproductive success as a consequence. Across taxa, frequency- and condition-dependent reproductive success has selected for the presence of discrete morphological and behavioural phenotypes, referred to as alternative reproductive tactics (ARTs; Brockmann 2001; Taborsky *et al.* 2008). In some species, ARTs grant

Correspondence: B. M. Nugent, University of Pennsylvania, 380 S. University Avenue, Hill Pavilion, Philadelphia, PA 19104, USA, Fax: 215-573-5187; E-mail: bnugent@vet.upenn.edu

reproductive opportunities to subordinate animals, allowing them to circumvent direct competition with dominant competitors by adopting alternative behaviours (Brockmann 2001; Taborsky *et al.* 2008). Not surprisingly, ARTs are among the best-studied examples of behaviour patterns that show marked variation within sex, yet their molecular and physiological underpinnings are still largely unknown. Differences in hormone signalling and neural gene expression between reproductive types are presumed mechanisms and have been characterized in several fish species (Oliveira *et al.* 2001a, b; Knapp 2003; Aubin-Horth *et al.* 2005; Knapp & Neff 2007; Fraser *et al.* 2014; Stiver *et al.* 2015). Further characterization of the mechanisms underlying alternative reproductive phenotypes will be necessary to determine whether these mechanisms are conserved across taxa.

The well-characterized ocellated wrasse, *Symphodus ocellatus*, a teleost fish from the Mediterranean, has three distinct male phenotypes, each with its own behavioural repertoire and physical features (Warner & Lejeune 1985; Taborsky *et al.* 1987). Nesting males are large, colourful and socially dominant. As their name suggests, nesting males build nests out of algae and defend their territory against predators, takeover by neighbouring nesting males and parasitic spawning by sneaker and satellite males. Nesting males court and spawn with females for 3–5 days before transitioning to a paternal phase, wherein they remain in close proximity to their brood, fanning the eggs within their nest and chasing off predators (Warner & Lejeune 1985; Taborsky *et al.* 1987; Alonzo 2004). The second male morph, the satellite male, cooperates with nesting males by courting females and chasing off sneaker males, the third male morph (Stiver & Alonzo 2013). Satellites do not build nests or engage in paternal care, but they may assist nesting males in attracting females (Taborsky *et al.* 1987; Stiver & Alonzo 2013). Because the satellite engages in transient cooperation with the nesting male, the nesting male tolerates his presence, enabling the satellite to remain in close proximity to the nest where he engages in parasitic spawning behaviour, also known as 'sneaking' (Taborsky *et al.* 1987; Stiver & Alonzo 2013). Finally, sneaker males are small, cryptically coloured males resembling females, that engage in parasitic spawning at the nest and do not engage in any courtship or parental behaviours (Warner & Lejeune 1985; Taborsky *et al.* 1987). Females produce a new batch of eggs every few days and visit several nests before spawning (Warner & Lejeune 1985; Taborsky *et al.* 1987). Their nest choice is influenced by the presence of other females at a nest, referred to as mate-choice copying (Alonzo 2008).

An additional dimension of plasticity in the ocellated wrasse arises from the age dependency of their phenotype. Most *S. ocellatus* males transition between phenotypes across breeding seasons (Alonzo *et al.* 2000). Based on otolith analysis used to determine age, we know that in their first reproductive season, males are sneakers or satellites, or they remain nonreproductive. In their second season, sneaker males can transition to satellites or nesting males, and satellites and nonreproductive males can transition to nesting males (Alonzo *et al.* 2000). The precise environmental and/or social cues that determine these phenotypic transitions are unknown, but the developmental origin of phenotypic traits, and the ability to transition between morphs, suggests epigenetic underpinnings (Cardoso *et al.* 2015).

While differences in genetic background can determine reproductive behaviour and other traits in some species, they do not determine the life histories of juvenile male *S. ocellatus* (Alonzo *et al.* 2000). In many species with ARTs, developmental differences in environmental and physiological factors converge to determine an individual's phenotypic fate (West-Eberhard 2003; Oliveira *et al.* 2008). In *S. ocellatus*, developmental conditions leading to disparities in early growth rate likely influence which male morph a juvenile will adopt (Alonzo *et al.* 2000). Male ARTs are likely determined by early differences in growth and fall along two life history pathways. Some males breed as sneakers in the first year and as a satellite male in the second, while others switch from being a satellite or nonreproductive male to nesting in their second reproductive season (Alonzo *et al.* 2000).

Steroid hormones, such as androgens, oestrogens and glucocorticoids, have been consistently linked with reproductive, social and parental behaviours across phyla and are likely involved in the display of alternative reproductive behaviours. Steroid hormone receptors, such as oestrogen receptors alpha and beta (ERα and ERβ), the androgen receptor (AR), and the glucocorticoid and mineralocorticoid receptors (GR and MR), are all members of an extended family of nuclear transcription factors that, when bound to a ligand, can dimerize, enter the nucleus and directly regulate transcription (Evans 1988). Steroid hormones can also have rapid, nongenomic effects, which allows for immediate behavioural flexibility in specific social contexts (Schumacher 1990; Falkenstein *et al.* 2000). Thus, differences in circulating steroid hormones and steroid hormone receptor expression in the brain can have large impacts on gene expression, and ultimately the neural physiology producing differences in behaviour.

Because of their ability to have both rapid and long-term effects on brain and behaviour, hormones are a likely candidate mechanism mediating ARTs (Knapp 2003). In males, high androgen levels are typically associated with male reproductive behaviours, aggressive behaviours and high social status (Oliveira *et al.* 2001a;

Parikh *et al.* 2006; Desjardins *et al.* 2008; Schradin *et al.* 2009; Taves *et al.* 2009; O'Connell & Hofmann 2011a). The dominant androgen in teleost fish, 11-ketotestosterone, is more often associated with differences in masculine behaviour than testosterone (Borg 1994), and this is also the case in *S. ocellatus* (Stiver *et al.* 2015). Within the brain, androgens can be aromatized to oestradiol (Naftolin 1994), which has also been shown to influence aggression and territorial behaviour (Nelson & Chiavegatto 2001; Trainor *et al.* 2006; O'Connell & Hofmann 2011a; Huffman *et al.* 2013). Circulating cortisol levels may also influence social status (DiBattista *et al.* 2005; Korzan *et al.* 2014). In addition to steroid hormones, peptide hormones are also critical mediators of social behaviours (Insel & Young 2000). The peptide hormone arginine vasotocin (AVT), homologue to the mammalian vasopressin, has been associated with social status, parental care, as well as courtship in teleost fish (Grober *et al.* 2002; Carneiro *et al.* 2003; Greenwood *et al.* 2008; Godwin & Thompson 2012; O'Connell *et al.* 2012; O'Connor *et al.* 2015).

The behavioural and physiological consequences of hormone receptor activation are determined by the region of the brain where receptor activation occurs, such that expression and activation of a given receptor in one region may result in different effects on behaviour than expression and activation of the same receptor in a different region (Bixo *et al.* 1995; Bale *et al.* 2001; Nomura *et al.* 2003). Numerous examples of region-specific effects of hormone receptor activation are seen in the rodent literature, for example oestradiol implants into the paraventricular nucleus of the hypothalamus (PVN) increase food intake and body weight with no impact on reproductive behaviours, whereas implants into the preoptic area (POA) or ventromedial hypothalamus (VMH) stimulate reproductive behaviours (Butera & Beikirch 1989). In addition, some neuroendocrine systems are highly localized to one brain region, such as AVT, which in teleosts is mainly expressed in the preoptic area, although its V1a receptor is widely distributed (Huffman *et al.* 2012). This is why examination of differences in hormone receptor expression must be conducted in a region-specific manner to gain insight into the molecular mechanisms underlying differences in social and sexual behaviours.

For most of the brain regions that are critical for social and reproductive behaviour homology, relationships have been inferred across the major vertebrate lineages (Insel & Young 2000; Goodson 2005; O'Connell & Hofmann 2012; Goodson & Kingsbury 2013). Together, these regions comprise the social decision-making (SDM) network, a network of brain regions important for social behaviours, such as reproduction, parental care and aggression, as well as reward processing (O'Connell & Hofmann 2012). Here, we focus on three central nodes of the SDM network to determine their role in ARTs. Arguably, the most critical region for the expression of male reproductive behaviours in vertebrates is the preoptic area (POA; Malsbury 1971; Arendash & Gorski 1983; Koyama *et al.* 1984; Moore & Lindzey 1992; Ball & Balthazart 2004; Goodson 2005). In teleost fish, activation of the POA has been linked to male reproductive behaviours, aggression and paternal care (Soma *et al.* 1996; O'Connell & Hofmann 2011b; O'Connell *et al.* 2012; Huffman *et al.* 2013), making it a critical candidate region for the control of behavioural differences across alternative male phenotypes (Foran & Bass 1999; Goodson & Bass 2000; Greenwood *et al.* 2008; O'Connell & Hofmann 2011a). The ventral subpallial division of the telencephalon (VS) is the putative homologue to the medial amygdala, another region implicated in control of male reproductive behaviours, aggression and fear (Koolhaas *et al.* 1990; Davis 1992; Newman 1999; Goodson 2005; O'Connell & Hofmann 2012; Goodson & Kingsbury 2013). Finally, the dorsolateral telencephalon (DL) is considered the homologue to the mammalian hippocampus and may be involved in learning, memory and stress responsivity (Rodrıguez *et al.* 2002; Goodson 2005; O'Connell & Hofmann 2011b; Goodson & Kingsbury 2013), all important processes mediating complex social interactions.

To determine the molecular and hormonal mechanisms controlling male ARTs, we quantified and correlated behaviours, circulating hormones and neural gene expression in wild *S. ocellatus* males and females. We constructed a *de novo* transcriptome using whole-brain RNA from females, sneakers, satellites and nesting males, and used this resource as a tool to conduct quantitative real-time PCR analysis of hormone receptor expression within distinct brain regions controlling social and sexual behaviours. Our goal was to determine whether hormone receptor expression in critical brain regions differs between male morphs and whether these differences are associated with particular behavioural repertoires. To understand the relationships between brain hormone receptor expression patterns and differences in circulating hormones across sexes and male morphs, we linked region-specific brain mRNA expression with circulating levels of the 11-ketotestosterone (11-kt), the dominant androgen in teleost fish, and the glucocorticoid cortisol. Finally, we associated variation in natural behaviours in wild fish with both region-specific gene expression and circulating hormones to identify potential neuroendocrine mediators of naturally occurring variation in male social and reproductive behaviours.

We predicted that circulating hormone levels and brain region-specific hormone receptor expression

underlie the marked behavioural variation in *S. ocellatus* and would show marked differences between females, sneakers, satellites and nesting males.

Materials and methods

Animals

All behavioural observations and sample collections were made during *Symphodus ocellatus*'s breeding season May–June at the University of Liege Marine Laboratory (La Station de Rescherches Sous-Marine et Oceanograhic, STARESO), near Calvi, Corsica, France. Samples collected during the 2010 field season were used to generate the *S. ocellatus* transcriptome and for whole-brain gene expression analysis for transcriptome validation. Samples collected during the 2013 field season were used for region-specific gene expression, behaviour and hormone quantification. Gene expression levels were not directly compared between these two cohorts. In the waters surrounding STARESO, *S. ocellatus* nests are built at depths ranging between 2 and 12 m. Only animals at nests with active spawning, with at least two sneakers and females, were chosen for inclusion in these studies. From each nest, one nesting male, satellite, sneaker and female were collected. The Yale University Institutional Animal Care and Use Committee approved all procedures involving animals.

Behaviour quantifications

Nests were observed (and in 2013 video recorded) for 10 min prior to capture of each nest's nesting and satellite male, along with one sneaker and female per nest. The number of sneakers and females at each nest and the nesting male's proximity to the nest were quantified in addition to nesting male and satellite behaviours (n = 8–10/phenotype). The behaviours quantified were the number of spawns, aggressive behaviours, parental behaviours and courtship behaviours, sneaks and submissive behaviours (operationally defined in Warner & Lejeune 1985; Alonzo & Warner 2000; Stiver & Alonzo 2013). Since sneakers and females often transiently visit and leave nests, and because differentiating individual sneakers and females is not always feasible at very active nests, behavioural data for individual sneakers and females were not scored. Following capture, fish were rapidly transported to an above-water researcher who collected blood, brains and gonads from each animal. Collections were completed within 25 min of behavioural observations (the mean time from catch to sample collection was 18 min). Brains and gonads were stored in RNAlater (Ambion) for 24 h at room temperature, then at −20 °C until processed further. Behavioural data were analysed by two-tailed, t-test to compare behaviour counts between satellite and nesting males using $P < 0.05$ as a criteria for statistical significance in R (R Development Core Team 2011); however, figures were made using PRISM 6 (GraphPad, Inc).

Hormone assays

Commercially available enzyme immunoassay (EIA) systems were used to quantify free plasma cortisol (Enzo Life Sciences) and 11-ketotestosterone levels (Cayman Chemical) as per the manufacturer's instructions and as previously described (Kidd *et al.* 2010). Plasma from the 2013 cohort of *S. ocellatus* samples was diluted 1:30 for 11-kt measurement and 1:50 for cortisol quantification. Hormone data were analysed by two-tailed, one-way ANOVA to compare levels across morphs using $P < 0.05$ as a criteria for statistical significance (n = 4–6/group) in R and figures were made using PRISM 6 (GraphPad, Inc).

RNA-seq and de novo transcriptome assembly

Whole-brain RNA was extracted from 7 to 8 brains/morph using a standard TRIZOL (Life Technologies) protocol. Briefly, whole brains were homogenized in 1 mL of TRIZOL and incubated for 5 min at room temperature (RT). Two hundred microlitre of chloroform (Sigma) was added to each sample, vortexed, incubated at RT for 2–3 min and centrifuged at 12,000 rpm for 15 min at 4 °C. Aqueous phase was mixed with an equal amount of isopropanol, incubated at RT for 10 min and centrifuged at 12,000 rpm for 10 min at 4 °C. RNA pellets were washed twice with 1 mL of 75% EtOH, air dried and resuspended in 30 μL of RNase-free water. Sample quality and concentration were assessed on a Bioanalyzer (Agilent) using a RNA 6000 Nano chip. Four morph-specific cDNA libraries were constructed using the NEBNEXT mRNA Library Prep Master Mix Set for Illumina (New England Biolabs) according to the manufacturer's protocol. Five samples per morph, with RNA integrity numbers (RIN) above 5, were pooled and mRNA was isolated using a MICROPOLY(A) Purist Small Scale mRNA Purification kit (Ambion), according to the manufacturer's protocol. Two hundred and fifty ng of mRNA per library was fragmented to 350 bp, and 45 ng of fragmented mRNA was added to cDNA synthesis reactions. Following second-strand cDNA synthesis, end repair, dA-tailing and adaptor ligation, each sample was purified/size selected using Ampure XP magnetic beads (Agencourt). The final cDNA libraries were 250 bp each with concentrations ranging from 3.03 to 4.92 ng/μL.

Paired-end sequencing (100 bp) of barcode-ligated libraries was conducted on an Illumina HiSeq 2000 at the University of Texas Genome Sequencing and

Analysis Facility. Reads (NM: 43.5 million, SAT: 51.8 million, SN: 30.5 millions and FEM: 35.6 million) were sorted, barcodes were clipped using custom python scripts, and read quality was assessed by FASTQC (Andrews 2010). *De novo* transcriptome assembly was performed with Trinity (Haas *et al.* 2013) using reads from all four phenotypes. Lowly abundant transcripts with an FPKM <0.5 were filtered by RSEM, resulting in an assembly with 80,103 contigs with an N50 of 2119. To annotate the *S. ocellatus* transcriptome, we employed BLASTX (using a conservative *E*-value cut-off of 1×10^{-10}) on protein sequences for *Oryzias latipes* and *Takifugu rubripes* obtained from the ENSEMBL BIOMART database, which resulted in the alignment of 25,856 contigs to the *O. latipes* genome and 25,213 contigs to the *T. rubripes* genome. Contigs that aligned with high confidence to more than one database were annotated based on the lowest *E*-value and highest % identity to either reference genome, resulting in an assembly where roughly 32% of high-quality contigs were successfully annotated, similar to recent reports of *de novo* transcriptome assembly in the teleost brain (Schunter *et al.* 2014).

Normalized reads from each morph's pooled mRNA were mapped back to the transcriptome using Bowtie (Langmead & Salzberg 2012). SAM files were converted to BAM files using SAMTOOLS (Li *et al.* 2009). Pairwise differential expression estimations were performed using EDGER with default settings for an exact test for the negative binomial distribution with a dispersion value of 0.4 (Robinson *et al.* 2010). As RNA was pooled prior to library generation, $n = 1$/group. PANTHER pathway analysis (Mi *et al.* 2013) was used to assess potential biological processes associated with differential gene expression in genes with a log fold change >1.5 and *P* value <0.05. Heatmap of differentially expressed genes (Figure 1, Supporting information) was made using gplots (Warnes *et al.* 2009) in R with default options for hierarchical clustering (i.e. 'complete' linkage method in hclust).

Quantitative real-time PCR

We first confirmed the gene expression patterns obtained by transcriptome analysis from whole brains collected from a separate cohort of fish ($n = 7$/phenotype). RNA was extracted using TRIZOL as described above and assayed using quantitative real-time PCR (qPCR, as described below). The results confirmed transcriptome expression estimates for 4 out of 5 randomly selected genes, validating our overall approach (Figure 2, Supporting information).

For region-specific gene expression analysis, RNA was extracted from tissue punches (0.3 μm diameter, 300 μm thickness) using a QUICK-RNA MICROPREP kit (Zymo Research) according to the manufacturer's instructions. To increase RNA yield, a proteinase K digestion was performed by adding 20 μL of proteinase K (20 mg/mL) to tissue punches in RNA/DNA shield (Zymo Research) and incubating at 55 °C for 2 h prior to performing the extraction. cDNA was synthesized using the GOSCRIPT Reverse Transcription system (Promega) with random primers and oligo(dT). All primers for whole-brain transcriptome validation and region-specific gene expression analysis (Table 1) were designed against sequences in the *S. ocellatus* transcriptome, except for vasotocin 1a receptor (V1aR), which was not identified in our transcriptomic analysis. To generate primers for V1aR, previously published degenerate primers designed to consensus sequences from teleosts (Lema *et al.* 2012) were used to amplify *S. ocellatus* whole-brain cDNA. The PCR product was purified and sequenced at the University of Texas at Austin DNA Sequencing Facility. V1aR primers were designed based on partial cDNA sequences for *S. ocellatus*. For region-specific gene expression, $n = 8$ sneakers, $n = 9$ females and nesting males, and $n = 10$ satellites. Data points were not removed in any analysis unless they were too low to detect by qPCR.

Transcript expression was quantified in triplicate for each gene on a VIIA7 Real-time PCR System (Life Technologies) using GOTAQ qPCR Master Mix (Promega). Standard curves for each gene were generated from serial dilutions of purified PCR products for each gene. Following the cycling protocol, continuous fluorescence was measured to generate a melting curve from 60 to 95 °C. VIIA7 software automatically generates baseline and threshold values for each gene, and the threshold cycle (Ct) values for each sample were used to determine cDNA quantity. Primer amplification efficiencies and relative expression levels were determined using MCMC.QPCR Bayesian analysis package in R (Matz *et al.* 2013), with GTP-binding protein (GTPbp) as a control gene (see Figure 3, Supporting information). Normalized relative expression values were analysed by one-way ANOVA across morphs using $P < 0.05$ as a cut-off for statistical significance. Pearson's correlation coefficients with $P < 0.05$ were considered significant in the gene behaviour and gene hormone correlation analyses. Correlation data were analysed using the HMISC (Harrell 2015) package in R, and *P*-values were Bonferroni corrected using the stats package in R (R Development Core Team 2011).

Results

Behaviour quantifications in wild Symphodus ocellatus males

Prior to collecting the fish used in the present study, we quantified the behaviour of satellite and nesting males at each nest (Fig. 1). Sneakers and females were found

Table 1 Primer sequences used for quantitative real-time PCR in whole-brain and region-specific analyses

Gene	Primer	Sequence (5′–3′)	Amplicon size (bp)	Efficiency 1
AR	Forward	TGCGAGATAACTGCTGGTCA	173	1.90
	Reverse	ATGACTCCTGCTCGTTTCCT		
ERa	Forward	TGGGATGCTAAAAGAGGGA	200	1.99
	Reverse	GTCGGGCATGGCAAATAACT		
ER|3	Forward	GAGGCACAGTCCGAAATTCC	208	1.94
	Reverse	TCCTCCAGTCCAGAAAGTG		
ankrd22	Forward	TGCTGAAGTACAACGAGGGT	168	1.85
	Reverse	GCACCACAAGTCAAAGCTCT		
EBF3	Forward	ACCCGACAAGAGAGGAGTTG	186	2.06
	Reverse	CGATGTAACCGTCACCGTTC		
CYP2K1	Forward	CAGTGGCCCAGTACAACAAC	174	1.96
	Reverse	CTGACATAGCGTTGTGTGGG		
EPAS1	Forward	GACCGAACGCCTCTAAGTCT	186	1.97
	Reverse	GCACACCGTTATCCAGTGTAC		
VI aR	Forward	GGAATGAGGAGGTGGCTCAA	150	2.02
	Reverse	CCAGGCTCAGGTGTTTGATG		
MR (nr3c2)	Forward	GTCCTTCTAGTGGGCTTGGT	150	1.92
	Reverse	GTCCTTCTAGTGGGCTTGGT		
GR	Forward	CCCACGACGAGTATTTGTGC	179	1.96
	Reverse	GAGCTGATAGAAACGCTGCC		
EF1	Forward	ATGAATCACAAACAGGGCCG	184	1.93
	Reverse	CTGCAGGTGGATGAAGAACG		
GTPbp	Forward	GGGCATTTTGTTCCACCGAT	151	1.94
	Reverse	ATGAAGCGGAAGTGGACTGA		
BDNF	Forward	AAAAAGTCCCTGTCCCCAAT	192	2.01
	Reverse	TTATAAACCGCCAGCCAATC		

in high density at focal nests and individuals visited and left frequently, and therefore we did not score their behaviour. As previously reported, *Symphodus ocellatus* nesting males and satellites display differences in their behavioural repertoires (Warner & Lejeune 1985; Taborsky *et al.* 1987; Stiver & Alonzo 2013). However, both male types engage in courtship and aggression, which is typically directed towards sneaker males or encroaching foreign satellites or nesting males. Courtship is typically more frequent in satellites than nesting males, which was consistent in the current cohort ($t_{(17)} = 3.407$, $P < 0.01$). We found no differences in the number of aggressive behaviours displayed by satellites and nesting males (Fig. 2; $t_{(17)} = 0.5241$, $P = 0.61$). Satellite males often display submissive behaviours towards the nesting male and occasionally attempt to sneak spawns in the nest. The vast majority of spawning events were accomplished by nesting males compared to satellites ($t_{(16)} = 4.056$, $P < 0.001$). Paternal behaviour was solely displayed by nesting males (Fig. 1).

Circulating 11-kt and cortisol levels differ between sexes and male morphs

Nesting males had significantly higher levels of circulating 11-kt than females and sneakers (Fig. 2A; $F_{(3,19)} = 4.906$, $P < 0.05$). However, we did not find a statistically significant difference in 11-kt levels between satellites and nesting males ($P = 0.062$). Plasma cortisol levels were higher in sneakers compared to females, satellites and nesting males (Fig. 2B; $F_{(3,20)} = 6.553$, $P < 0.01$).

Transcriptomics in the S. ocellatus brain

De novo construction of the ocellated wrasse whole-brain transcriptome enabled gross evaluation of global gene expression patterns across sexes and morphs. Clustering analysis of total gene expression indicated that females and nesting males had the most divergent patterns of gene expression in the brain (Figure 1a, b, Supporting information). There were a total of 258 genes with significant differences [logFC > 1.5, $P < 0.05$; top DE genes provided in Table 1 (Supporting information)] in expression level between nesting males and females, with 163 genes expressed at higher levels in nesting males compared to females and 95 with higher expression levels in females. PANTHER biological process gene ontology (GO) analysis indicated that about half of the DE genes in nesting males vs. females and nesting males vs. the other male morphs are involved in metabolic and cellular processes (Figure 1c, Supporting information). Our

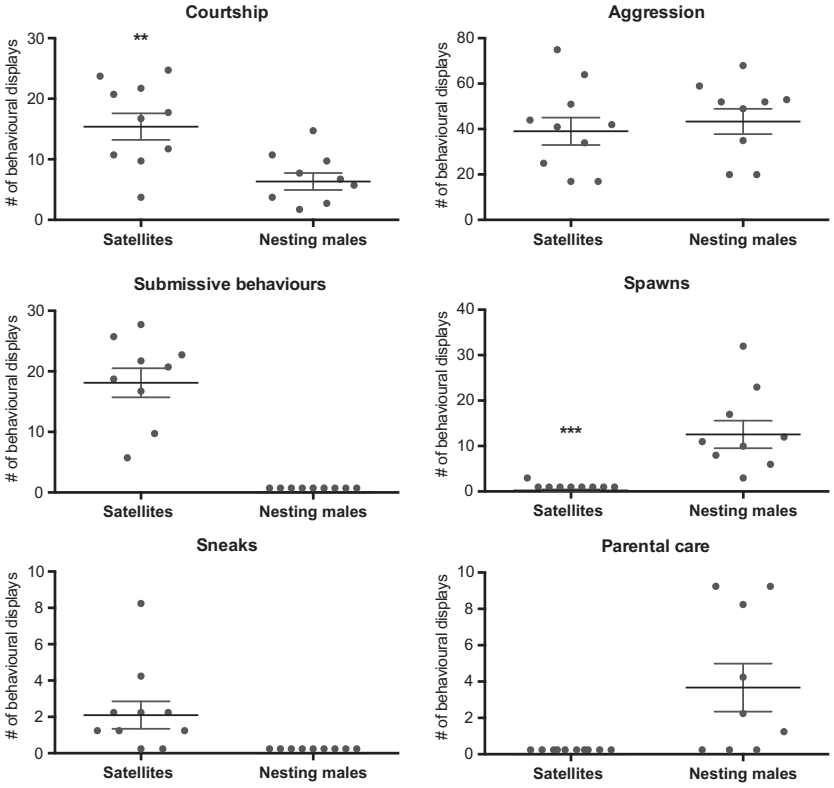

Fig. 1 Behavioural repertoires of wild satellite and nesting male *S. ocellatus*. Behaviour was quantified for each satellite and nesting male prior to collection for analysis of circulating cortisol and 11-kt, and neural gene expression. Both male morphs engage in courtship and aggression, but more courtship displays were present in satellites compared to nesting males. Satellites often engage in submissive behaviours and sneaking spawns, whereas nesting males do not. Spawning behaviour is significantly more frequent among nesting males compared to satellites. Satellites do not engage in paternal behaviours as nesting males are the sole providers of parental care. Error bars indicate mean ± s.e.m. $^{**}P < 0.01$, $^{***}P < 0.001$.

analysis indicated that the groups with the most similar patterns of gene expression and the fewest DE genes were females and satellite males, which had only a total of 63 differences in gene expression (Figure 1a, b, Supporting information).

Brain region-specific gene expression across sexes and morphs

Using our *de novo* transcriptome as a tool to identify potential genetic contributors to differential social and sexual behaviours, we quantified mRNA levels of several hormone receptors known to control social and sexual behaviours in brain regions within the neural SDM (O'Connell & Hofmann 2012), namely the POA, VS (medial/central amygdala homologue) and DL (hippocampus homologue).

We quantified ERα, ERβ, AR and V1aR mRNA in the POA of all morphs, as shown in Fig. 3A. Levels of ERα in the POA were significantly higher in satellite and nesting males compared to both females and sneakers ($F_{(3,29)} = 49.05$, $P < 0.0001$). Levels of ERβ were too low to reliably detect in the female and sneaker POA, and there was not a significant difference in ERβ mRNA expression between satellites and nesting males ($t_{(16)} = 0.6106$, $P = 0.55$, satellite mean = 6.38, s.e.m. = 0.39; nesting male mean = 6.7, s.e.m. = 3.247). Females had significantly lower levels of AR in the POA compared to satellite and nesting males ($F_{(3,29)} = 7.276$, $P < 0.001$), suggesting that, in general, steroid hormone receptor expression is highest in older, socially dominant males compared to females and sneaker males. Although V1aR was highly expressed in the POA in all groups, there were no statistically significant differences across sex or male morph ($F_{(3,34)} = 0.5390$, $P = 0.66$).

In the VS, the putative medial amygdala/extended central amygdala homologue, we measured ERα, ERβ, AR and V1aR (Fig. 3B). As in the POA, ERα was significantly higher in the satellite and nesting male VS compared to sneakers and females ($F_{(3,25)} = 39.56$, $P < 0.0001$). AR expression displayed the same pattern as ERα in the VS and was significantly higher in satellite and nesting males ($F_{(3,25)} = 38.87$, $P < 0.0001$). Levels of ERβ and V1aR were too low to reliably detect in the sneaker and female VS. However, we found higher mRNA expression levels of both of these receptors in the nesting male VS compared to satellites (V1aR $t_{(14)} = 4.470$, $P < 0.001$, satellite mean = 4.38, s.e.m. = 0.27; nesting male mean = 5.8, s.e.m. = 0.17; ERβ $t_{(14)} = 2.506$, $P < 0.05$, satellite mean = 6.83, s.e.m. = 0.19; nesting male mean = 7.36, s.e.m. = 0.08).

In the putative hippocampus homologue DL, we quantified the adrenal hormone receptors GR and MR, as well as the neurotrophin brain-derived neurotrophic

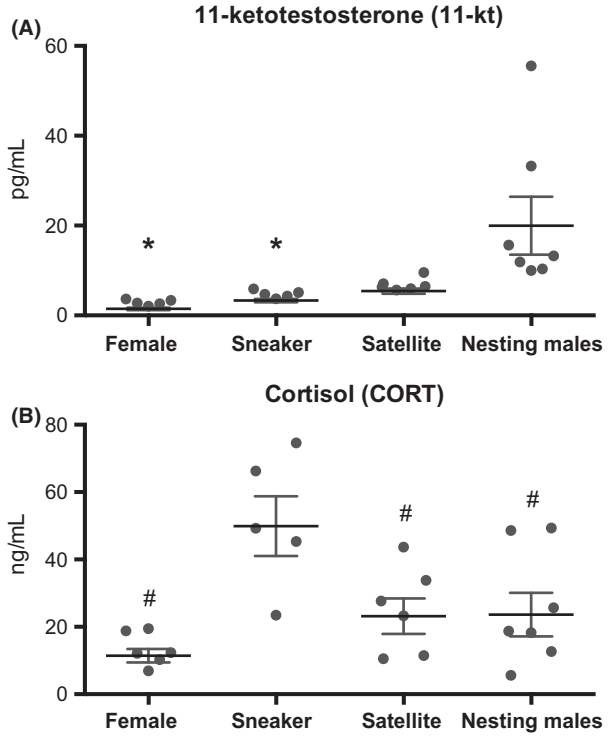

Fig. 2 Circulating 11-kt and cortisol levels in wild *S. ocellatus*. (A) 11-kt levels were significantly higher in nesting males relative to females and sneakers, whereas (B) sneakers had the highest levels of circulating cortisol compared to females and other male morphs. Error bars indicate mean ± s.e.m. $^{*}P < 0.05$ compared to nesting males and $^{\#}P < 0.05$ compared to sneakers.

factor (BDNF), a critical regulator of synapse formation in the hippocampus that is regulated by gonadal and adrenal hormones (reviewed in Pluchino *et al.* 2013; Fig. 3C). We found large differences and low variability in GR expression across morphs. Females and sneakers had very low GR expression in the DL compared to satellites and nesting males ($F_{(3,27)} = 1221$, $P < 0.0001$). Although in whole brain, MR expression was highest in nesting males (Figure 2, Supporting information), satellite males had the highest levels of MR in the DL compared to all other groups ($F_{(3,27)} = 176.8$, $P < 0.0001$). MR was also higher in nesting males compared to females and sneakers ($P < 0.001$). BDNF was higher in the nesting male and satellite DL compared to females and sneakers ($F_{(3,28)} = 91.57$, $P < 0.0001$). Importantly, there were no significant differences in expression of our control gene, GTPbp across groups in any brain region (Figure 3, Supporting information).

Associations between male behaviours, hormones and neural gene expression

We sought to identify the biological links between region-specific gene expression levels, circulating hormone levels and variation in behaviour. We correlated hormone levels and region-specific gene expression among all animals sampled (Fig. 4A) and performed an additional correlation analysis adding satellite and nesting male behavioural data (Fig. 4B) to identify relationships between genes, hormones and behaviours. Levels of 11-kt and cortisol did not significantly correlate with expression of any hormone receptor gene when analysed across all morphs (Fig. 4A; Table 2, $n = 36$), although we identified many correlations between genes in the brain (Fig. 4A; Table 2).

When data from satellite and nesting males were combined ($n = 19$), we found statistically significant correlations between GR in the DL and ERα ($R^2 = -0.88$, $P < 0.01$) and AR ($R^2 = -0.88$, $P < 0.01$) in the VS (Fig. 4B; Table 3). ERα and AR expression levels in the VS were strongly linked ($R^2 = 1$, $P < 0.001$) in satellites and nesting males. ERβ levels also positively correlated with V1aR in the VS ($R^2 = 0.81$, $P < 0.05$). In addition, we found a statistically significant correlation in courtship behaviours with MR in the DL ($R^2 = 0.81$, $P < 0.01$; Fig. 4B; Table 3).

Discussion

Dynamic neuroendocrine control of gene expression is associated with phenotypic plasticity across taxa. Here, we describe the development of a *de novo* transcriptome, hormone profiles and brain region-specific gene expression profiles in the ocellated wrasse, *Symphodus ocellatus*, a reef fish with thoroughly characterized ecology and social dynamics (Warner & Lejeune 1985; Taborsky *et al.* 1987; Stiver & Alonzo 2013). Previous analysis of circulating hormones in *S. ocellatus* revealed higher levels of oestradiol in females compared to males, with no differences among male morphs; 11-kt levels were highest in nesting males compared to both females and other male morphs, while testosterone did not vary significantly across morphs or sexes (Stiver *et al.* 2015). In the current cohort, we confirmed that nesting males had significantly higher levels of circulating 11-kt compared to females and sneakers. Although 11-kt was lower in satellites than nesting males as previously described (Stiver *et al.* 2015), differences did not reach statistical significance in the current cohort. Elevated 11-kt levels have been associated with paternal care and enhanced aggression in other teleost species (Rodgers *et al.* 2006; Pradhan *et al.* 2014), and therefore higher 11-kt in nesting males relative to other morphs is consistent with their behavioural repertoire. However, satellite males also exhibit high levels of aggression towards sneakers despite their low levels of circulating 11-kt. Thus, the biological mechanisms controlling aggressive behaviours in *S. ocellatus* males

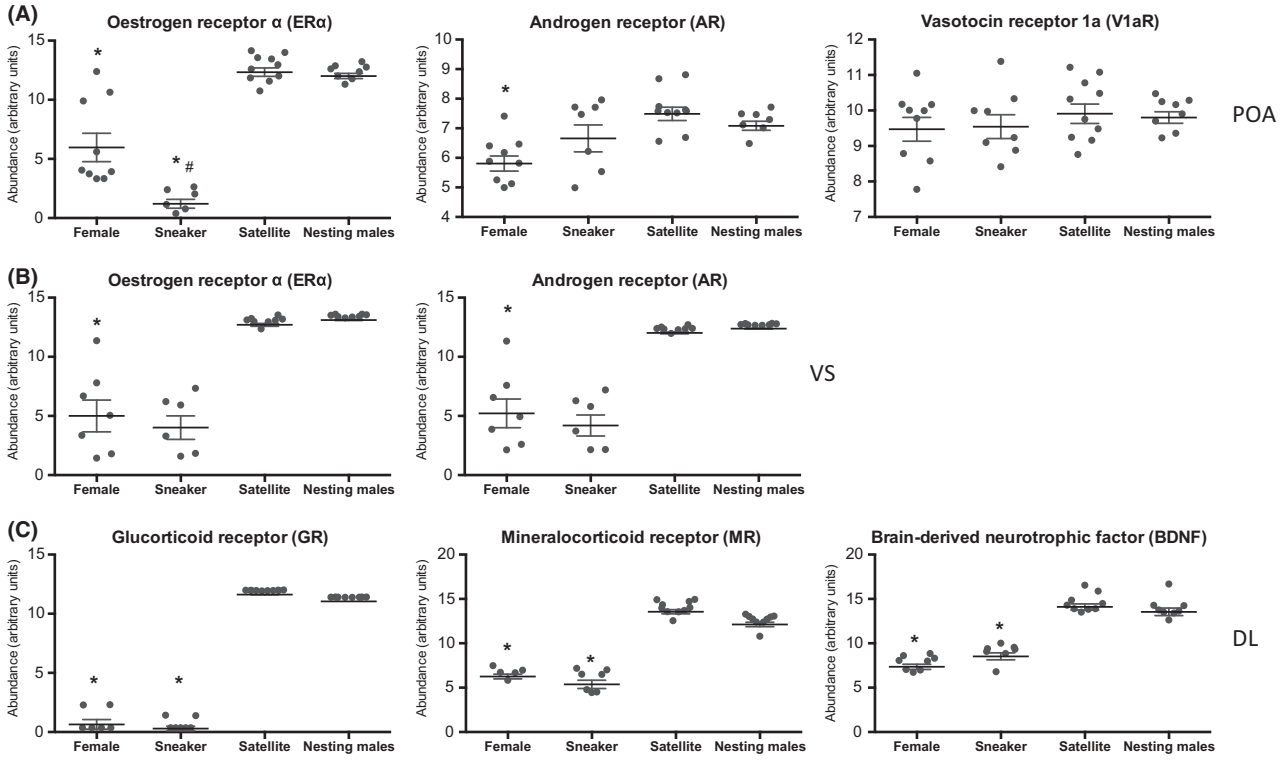

Fig. 3 Brain region-specific gene expression of key mediators of social and sexual behaviours. (A) In the POA, ERα mRNA expression was higher in satellites and nesting males compared to females and sneakers. AR was higher in satellites and nesting males compared to females but did not significantly differ from levels in sneaker males. There were no differences in V1aR mRNA across groups in the POA. (B) In the VS, ERα and AR mRNA levels showed the same patterns, where satellites and nesting males had higher levels compared to females and sneakers. (C) In the DL, both cortisol receptors were expressed at higher levels in satellites and nesting males compared to females and sneakers. The neurotrophin BDNF showed similar expression patterns. Error bars indicate mean ± s.e.m. $^{*}P < 0.05$ compared to satellites and nesting males and $^{\#}P < 0.05$ compared to females.

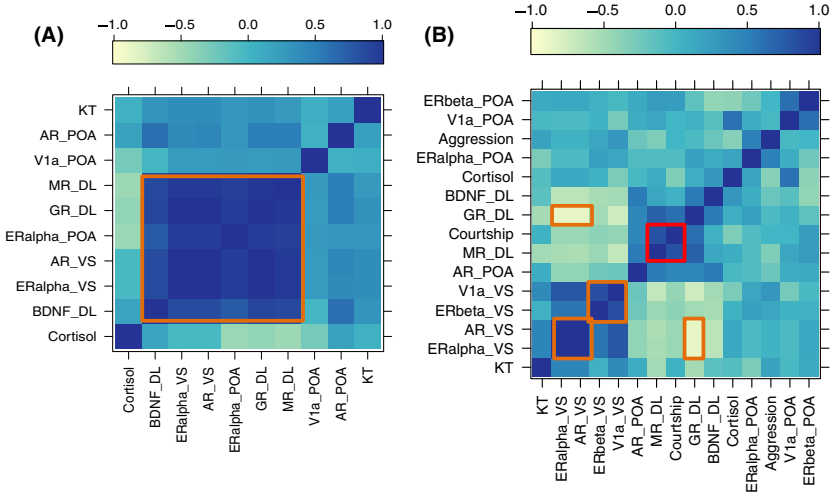

Fig. 4 Neuroendocrine and behavioural correlates in wild *S. ocellatus*. (A) When all groups are combined, there are no statistically significant correlations between cortisol or 11-kt and neural hormone receptor mRNA levels, although the expression levels of many genes correlate with each other likely due to differences across groups in hormone receptor expression levels. (B) To identify patterns of hormones and gene expression that correlate with behaviour in wild *S. ocellatus*, we correlated behaviours expressed by both satellites and nesting males (aggression and courtship) with mRNA expression and hormone levels across groups. Orange boxes highlight significant correlations between genes. Red boxes indicate statistically significant correlations between gene expression and behaviour.

Table 2 Gene/hormone correlates in wild *S. ocellatus*. Pearson's correlation coefficients corrected for multiple comparisons are shown

	Via POA	AR_POA	ERalpha_POA	ERalpha_VS	AR_VS	GR_DL	MR_DL	BDNF_DL	Cortisol
Via POA									
AR POA	0.03								
ERalpha_POA	0.25	0.28							
ERalpha_VS	0.24	0.41	0.93***						
AR VS	0.24	0.41	0.93***	1.00***					
GR_DL	0.24	0.51	0.94***	0.99***	0.99***				
MR DL	0.27	0.5	0.92***	0.97***	0.97***	0.98***			
BDNF DL	−0.02	0.60*	0.74***	0.85***	0.85***	0.88***	0.85***		
Cortisol	−0.29	0.18	−0.45	−0.05	−0.05	−0.42	−0.48	0.15	
KT	0.06	0.2	0.27	0.37	0.37	0.32	0.27	0.29	0.03

Data from all groups are combined ($n = 36$) *$P < 0.05$. ***$P < 0.001$.

warrant further exploration in future studies. Cortisol levels were highest in sneaker males, which is consistent with findings in other teleost species in which sneaker and subordinate-type males have high cortisol relative to their dominant conspecifics (Fox *et al.* 1997; Knapp & Neff 2007; Bender *et al.* 2008; Arterbery *et al.* 2010). Surprisingly, we found no statistical associations between circulating hormone levels and brain hormone receptor gene expression despite the well-documented ability of circulating hormones to regulate the expression of their own, and related, receptors (Evans 1988; Beato 1993; Beato *et al.* 1995).

The use of microarray platforms, next-generation sequencing and *de novo* transcriptome construction has already enabled analysis of gene expression patterns in several nontraditional model species with ARTs (Renn *et al.* 2004; Aubin-Horth *et al.* 2005, 2007; Cummings *et al.* 2008; Schumer *et al.* 2011; Fraser *et al.* 2014; Schunter *et al.* 2014; Stuglik *et al.* 2014; Feng *et al.* 2015; Stiver *et al.* 2015). Whole-brain transcriptome analysis by RNA-Seq in the current study confirmed our previous microarray-based analyses of whole-brain gene expression in this species, with sneakers and nesting males displaying the most divergent patterns of gene expression across both males and females, and females and satellites exhibiting similar patterns of gene expression (Stiver *et al.* 2015). Greater variation in whole-brain gene expression within sex compared to across sex is surprising, but has been reported previously in the teleost brain (Schunter *et al.* 2014; Stiver *et al.* 2015). There is, at this point, no good explanation for the similarities in whole-brain, global gene expression profiles between females and satellites. We previously hypothesized that these differences could be due to their comparable social status at the nest (Stiver *et al.* 2015), wherein their presence is encouraged by nesting males while being subordinate targets for nesting male aggression (Stiver & Alonzo 2013). The distinct behavioural and hormonal profiles of sneakers and nesting males are mimicked by their large differences in neural gene expression. As sneakers had significantly higher levels of cortisol, and nesting males had significantly higher levels of 11-kt compared to satellites and females, the observed patterns of whole-brain gene expression across morphs and sexes might indicate the influence of these hormones on transcriptional regulation, resulting in similar patterns of gene expression in satellites and females, which have lower levels of both of these hormones. Neural gene expression patterns might to some extent also be due to age (Stiver *et al.* 2015), which is well established in mammals (Somel *et al.* 2006). Sneakers are all roughly 1 year old around the breeding season, whereas satellites and females are roughly 1–2 years old, and nesting males are all 2+ years old (Alonzo *et al.* 2000). However, our region-specific candidate gene analysis did not provide any evidence for age-dependent gene expression patterns, suggesting that socially relevant gene expression is not strongly associated with age, but rather influenced by sex and phenotypic status.

Our whole-brain transcriptome allowed us to identify sequences of critical hormone receptor genes in *S. ocellatus*. This enabled use of a candidate gene approach to quantify differential patterns of hormone receptor expression in the POA, VS and DL, regions associated with reproductive behaviours, aggression, parental care and stress responsivity. Hormone receptor signalling in discrete brain regions is critical for the display of complex social and sexual behaviours. We predicted that hormone receptor expression likely contributes to differences in discrete behavioural variation displayed by *S. ocellatus* males and that hormone receptor mRNA expression levels would vary greatly between sexes and male morphs. We observed higher levels of hormone receptor mRNA expression in satellites and nesting males across brain regions in nearly all genes of interest. Further, when data were correlated across sexes

Table 3 Gene/hormone/behaviour correlates in wild satellite and nesting male *S. ocellatus*

	V1a_POA	AR_POA	ERalpha_POA	ERbeta_POA	ERbeta_VS	V1a_VS	ERalpha_VS	AR_VS	GR_DL	MR_DL	BDNF_DL	Cortisol	KT	Courtship
V1a POA														
AR POA	0.08													
ERalpha_POA	0.07	−0.09												
ERbeta POA	0.6	0.1	−0.25											
ERbeta VS	0.02	−0.25	0.2	0.04										
V1a VS	−0.28	−0.23	0.15	−0.03	0.81*									
ERalpha_VS	−0.07	−0.41	−0.05	0.13	0.55	0.75								
AR VS	−0.07	−0.41	−0.05	0.12	0.55	0.75	1.00***							
GR DL	0.04	0.43	0.21	−0.16	−0.51	−0.74	−0.88**	−0.88**						
MR DL	0.14	0.53	−0.08	0.24	−0.46	−0.68	−0.55	−0.55	0.69					
BDNF DL	−0.12	0.52	0.08	−0.43	−0.55	−0.49	−0.64	−0.64	0.52	0.14				
Cortisol	0.59	−0.03	0.15	−0.4	0.03	−0.31	0.12	0.12	0.06	0.09	0.27			
KT	−0.04	0.07	−0.3	0.1	0.05	0.4	0.43	0.43	−0.52	−0.5	−0.13	−0.1		
Courtship	−0.01	0.42	−0.08	0.22	−0.41	−0.43	−0.45	−0.45	0.59	0.81**	0.04	−0.31	0.02	
Aggression	−0.03	−0.22	0.49	0.01	0.13	0.28	0.02	0.03	−0.05	−0.32	−0.22	−0.35	0.19	0

Pearson's correlation coefficients corrected for multiple comparisons are shown. Satellite and nesting male data are combined ($n = 19$) $^{*}P < 0.05$. $^{**}P < 0.01$, $^{***}P < 0.001$.

and male morphs, we found strong correlations between expression levels of hormone receptors. Specifically, we found strong associations between DL glucocorticoid signalling, important in social cognition and sexual/aggressive behaviours (Maruska *et al.* 2013) with sex steroid signalling in VS and POA, which have both been implicated in sexual behaviour (O'Connell & Hofmann 2011a). These expression patterns may suggest that gonadal and adrenal steroid hormone signalling work together across multiple brain regions to regulate behaviour. A more likely explanation derives from our findings that, in general, satellites and nesting males have higher levels of hormone receptor expression relative to sneakers and females. This means that intersex and morph correlations may be skewed by sex and male age/morph.

The consistent elevation of hormone receptor gene expression levels in satellites and nesting males was not due to technical or experimental error, since we found no differences in AR and V1aR in the POA (Fig. 3) and varying patterns of expression in randomly selected genes used to validate expression patterns in our transcriptome (Figure 2, Supporting information). These results are likely indicative of enhanced hormonal signalling activity in the satellite and nesting male brain. V1aR expression in the POA did not differ between groups despite the well-known role of AVT signalling in this region in dominance, aggressive behaviours and paternal behaviours in teleosts (Greenwood *et al.* 2008; Backström & Winberg 2009; Almeida *et al.* 2012; O'Connell & Hofmann 2012; Huffman *et al.* 2015).

Satellites and nesting males display distinct, yet similarly complex behaviours (Fig. 1). The behaviours displayed by both male morphs necessitate neuroendocrine signalling, yet result in divergent physiological outcomes to produce distinct behavioural phenotypes. Satellites and nesting males both display courtship behaviour and aggression towards sneakers. When we correlated the frequency of these behaviours in both male morphs combined with neural hormone receptor expression, we found a statistically significant association between MR and courtship behaviours. Courtship and appetitive reproductive behaviours have been associated with cortisol signalling in other taxa (Rose *et al.* 1993; Cease *et al.* 2007; Lutterschmidt & Maine 2014), suggesting that *S. ocellatus* may share a common physiological mechanism controlling courtship behaviour.

Although hormone receptor expression levels are high in both satellites and nesting males, these receptors are associated with different behaviours and physiology in each male type, creating specific neuroendocrine profiles for each. These divergent neuroendocrine profiles may have been selected to

maximize morph-specific behaviour, creating male alternatives. For future studies, a larger sampling of satellite and nesting males will enable within-morph characterization of neuroendocrine control of behaviour.

Male *S. ocellatus* transition between morphs across breeding season, suggesting that differences in circulating hormones and brain hormone receptor expression are developmentally dependent but plastic. The cues that initiate phenotypic transitions are currently unknown, but epigenetic phenotypic control likely underlies both developmental programming determining life history path and phenotypic plasticity in *S. ocellatus*. Epigenetic mediators of gene expression have been identified as critical regulators of phenotypic plasticity across taxa (Kucharski *et al.* 2008; Smith *et al.* 2011; Foret *et al.* 2012). Epigenetic processes, such as DNA methylation and histone modifications, are plausible mechanisms for enabling enough control of gene expression to drive discrete variation in behaviour and physiology, while maintaining the ability to display marked plasticity and are therefore likely contributors to the aetiology of plasticity in male alternatives. Steroid hormones have recently been shown to influence DNA methylation patterns responsible for long-term programming of gene expression in the mammalian brain (Nugent *et al.* 2015), which may also underlie phenotypic programming and plasticity in teleosts. In addition, age-related changes of DNA methylation have been reported on hormone receptor promoters and other genes in the brain (Schwarz *et al.* 2010; Hernandez *et al.* 2011). Manipulation studies are necessary to determine whether epigenetic processes are involved in *S. ocellatus* phenotypic plasticity and to further characterize the contribution of specific neuroendocrine mechanisms in the physiological and behavioural differences in *S. ocellatus* males. These studies will provide insight into the importance of particular hormones or hormone receptors in the reproductive success of each morph.

Conclusions

Massive genomic plasticity in the absence of genetic differences underlies phenotypic plasticity in some species with ARTs (Renn *et al.* 2004; Aubin-Horth *et al.* 2005, 2007; Cummings *et al.* 2008; Schumer *et al.* 2011; Fraser *et al.* 2014; Schunter *et al.* 2014; Stuglik *et al.* 2014; Feng *et al.* 2015; Stiver *et al.* 2015). In the ocellated wrasse, this genomic plasticity, and the changes in reproductive morph associated with divergent brain transcriptomic and neuroendocrine profiles, might be age-related. Our data suggest that as *S. ocellatus* males transition between morphs, they undergo large changes in hormone production as well as brain hormone receptor expression, giving rise to striking differences in physiology and behaviour. The question remains as to how these differences are developmentally programmed and maintained, and whether the mechanisms controlling phenotypic plasticity in this and other teleosts are similar to those found in other taxa.

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B.M.N., S.H.A. and H.A.H. designed the study. All molecular techniques (transcriptome library prep, quantitative PCR, hormone assays) and subsequent data analysis were performed by B.M.N. Brains for transcriptomics were collected in the field by K.A.S. and S.H.A. Brains and blood for region-specific qPCR and hormone assays were collected in the field by B.M.N., K.A.S. and S.H.A. K.A.S. analysed behavioural data. H.A.H. provided additional support for molecular studies and analysis. B.M.N., S.H.A. and H.A.H. wrote the manuscript.

Data accessibility

Raw reads are available at the Sequence Read Archive (SRA), BioProject: PRJNA316102, BioSamples: SAMN047 6443-6. Reference transcriptome is available at Dryad: doi:10.5061/dryad.fj34d.

Supporting information

Additional supporting information may be found in the online version of this article.

Fig. S1 Transcriptomics in *S. ocellatus* brain reveal differences in gene expression patterns across morphs. (A) Summary table of gene expression differences in ocellated wrasse whole brain. Column headers indicate group with higher gene expression relative to group indicated by row header (i.e. 163 genes were significantly higher in nesting male whole brain compared to females), based on a fold change >2 and $P < 0.05$. (B) Heatmap of all genes with differences in gene expression across 2 or more morphs. (C) Pie charts of GO pathways associated with genes with differential expression in the nesting males vs. females, and nesting males vs. satellites and sneakers.

Fig. S2 qPCR Transcriptome Validation in Whole Brain. Five genes indicated as differentially expressed between sexes/morphs were chosen for differential expression validation in a separate cohort of wrasse brains. In our transcriptome, we detected a greater number of reads corresponding to *nr3c2*, the gene that encodes the mineralocorticoid receptor (MR), in nesting males compared to other groups. qPCR analysis for *nr3c2*

revealed significantly higher expression in both nesting males and satellite males compared to females and sneakers (ANOVA; $F_{(3,20)} = 34.95$, $P < 0.0001$). Ankyrin repeat domain 22 (*ankrd22*) was most abundant in sneakers compared to other groups in the wrasse transcriptome, and there was a statistical trend towards higher expression when quantified by qPCR ($F_{(3,24)} = 2.854$, $P = 0.0584$). The monooxygenase cytochrome p450 (CYP2K1) was significantly higher in the nesting male brain compared to sneakers ($F_{(3,24)} = 4.655$, $P < 0.05$), confirming transcriptome patterns. EPAS1, a transcription factor also known as HIF2a, was significantly higher in sneakers compared to females ($F_{(3,23)} = 4.549$, $P < 0.05$), yet qPCR results did not reach significance between sneakers and satellites and sneakers and nesting males by Tukey HSD post hoc test. We did not confirm significant differences in expression of the transcription factor EBF3 ($F_{(3,24)} = 1.564$, $P = 0.224$), when measured by qPCR. Transcriptome read counts are displayed below their respective group. Error bars indicate mean ± s.e.m. $^{*}P < 0.05$ compared to females, $^{\#}P < 0.05$ compared to sneakers.

Fig. S3 No differences in control gene, GTPbp across groups in any brain region. We found no statistical differences in our control gene, GTP-binding protein (GTPbp) in any of our analyses. POA $F_{(3,31)} = 1.062$, $P = 0.3794$; DL $F_{(3,28)} = 0.2706$, $P = 0.846$; VS $F_{(3,28)} = 1.144$, $P = 0.3485$. Error bars indicate mean ± s.e.m.

Table S1 Top DE genes in *S. ocellatus* whole brain. Contig tag #s, gene symbols, statistics, and relative comparisons are provided for differentially expressed genes among all groups. Differences with FDR values >0.5 are shown.