Neural Gene Expression Profiles and Androgen Levels Underlie Alternative Reproductive Tactics in the Ocellated Wrasse, *Symphodus ocellatus*

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Abstract

Discrete variation in reproductive behavior and physiology is observed in diverse taxa. Although it is known that most within-sex alternative reproductive tactics arise as a consequence of phenotypic plasticity, relatively little is known about differential neural gene expression among plastic alternative reproductive phenotypes. In the ocellated wrasse *Symphodus ocellatus*, males exhibit one of three alternative tactics (nesting, satellite, and sneaker) within a reproductive season, but switch tactics between years. Satellites and sneakers spawn parasitically in dominant (nesting) males' nests, but only nesting males provide parental care. Nesting and satellite males show transient cooperative defense of nests against sneakers. Here, we analyze circulating sex steroid hormone levels and neural gene expression profiles in these three male phenotypes and in females. 11-ketotestosterone (but not testosterone) was highest in nesting males, while estradiol was highest in females. Brain transcriptomes of satellites and females were most similar to each other and intermediate to nesting and sneaker males. Sneakers showed more total expression differences, whereas nesting males showed higher magnitude expression differences. Our findings reveal the surprising extent to which neural gene expression patterns vary across reproductive tactics that vary in a number of social traits, including aggression, territoriality, and cooperation, providing important insights into the molecular mechanisms that may underlie variation in cooperative and reproductive behavior.

Introduction

A fundamental issue in biology is explaining the evolutionary factors that maintain variation between individuals in the presence of selection and the mechanisms that underlie them. Discrete variation in reproductive behavior and physiology is found in many species, and extensive research has focused on understanding how this variation arises and is maintained within a population. Differences in reproductive traits within and between sexes are genetically fixed in some species, while in others environmental factors (e.g., incubation temperature, timing of birth, resource availability) can influence developmental processes (e.g., early growth rate) and result in divergent life history trajectories (e.g., as a male or female, Ross 1990; or as a particular mating tactic, Brockmann 2001). Plasticity in reproductive traits within the sexes, often referred to as alternative reproductive tactics (ARTs), can lead to the evolution

of discrete variation in behavior and morphology, due to frequency- and condition-dependent fitness outcomes (Brockmann 2001).

A better understanding of how reproductive plasticity arises from and is maintained by a common genome is a key to uncovering the mechanistic basis of phenotypic variance. Discrete variation in phenotype is associated with and arises from differences in hormonal, developmental, and physiological mechanisms (West-Eberhard 2003; Oliveira et al. 2008; Dijkstra et al. 2012a,b; Piferrer et al. 2012). Thus, individuals engaging in different reproductive tactics often also show clear phenotypic differences (Brockmann 2001), particularly as some behaviors may be physiologically or physically incompatible with one another (e.g., Scott 2006). Some of these differences (e.g., color, relative size) are easily observed, while others (e.g., circulating hormone and gene expression levels) are not as readily accessible. Documenting how morphological and physiological variation maps onto behavioral phenotypes increases our understanding of the mechanisms that underlie developmental and life history variation.

Among teleost fishes, there are many examples of reproductive plasticity. Teleosts show great variation in sex determination mechanisms (Devlin & Nagahama 2002) and also provide some of the clearest examples of alternative reproductive tactics (see Taborsky 1994, 2001). Individual life history can be similarly plastic, as individuals may reproduce both as a male and as a female at some point in their life (Ross 1990) or may engage in several alternative tactics across their reproductive life span (Taborsky 1994, 2001; Aubin-Horth et al. 2005a, 2009). As variation in physiology can be tied to variation in individual life history (particularly individual dominance, age, somatic growth, and sex), some specific differences in gene expression and hormone levels have been documented to covary with these traits (see Borg 1994; Brockmann 2001; Taborsky 2001; Aubin-Horth et al. 2007, 2009).

A common attribute relating both to sex roles and alternative reproductive tactics is an individual's dominance relative to others in their social environment (Taborsky 2001). Such differences in social dominance can be linked to hormonal and neural gene expression variation. Circulating levels of the fish-specific androgen 11-ketotestosterone (11-KT) are typically highest in the most dominant males and higher in males than in females (Borg 1994; Oliveira et al. 2001; Taves et al. 2009; Maruska & Fernald 2010a,b), and actions of this hormone have been tied to differences in male reproductive tactic (Oliveira et al.

2001). While generally not as important an androgen as 11-KT in fishes, testosterone (T) has been closely linked to dominance in one or both sexes in several fish species (all *Cichlidae*: e.g., Trainor & Hofmann 2006; Taves et al. 2009; Renn et al. 2012). Circulating estradiol (E) is generally higher in females than in males (Borg 1994); however, at least in some species, dominant males may also have very high E levels (again, these tend to be *Cichlidae*: e.g., Maruska & Fernald 2010a; O'Connell et al. 2012).

Modern genomic approaches have made it possible to examine the molecular underpinnings of behavioral variation among alternative phenotypes. Work in *Hymenoptera*, the group that contains perhaps the best known examples of species with discrete phenotypic classes (typically some are non-reproductive) most clearly reveals the power of this approach. One emerging pattern is the suggestion that the sterile (or predominantly sterile) worker caste may be of greater importance to the evolution of gene expression than the dominant queen caste (Ometto et al. 2011; Ferreira et al. 2013). Additionally, the importance of considering age and life stage has been demonstrated, as in some species developmental stage better explains differences in gene expression than do phenotype, sex, or even species (e.g., Ometto et al. 2011). Examining species with plastic phenotypes helps us to understand the dynamic and flexible nature of neural transcriptomes and identified gene modules associated with variation in social and reproductive behaviors in diverse species. In the eusocial wasp *Polistes canadensis*, a substantial fraction of the genome (9%) was differentially regulated between workers and queens, and castes showed striking differences in the direction of differential expression (workers generally showed upregulation of genes with castes-biased expression, while the majority of downregulated genes were observed in foundresses and callows; Ferreira et al. 2013). Differential expression is also often associated with changes from subordinate to dominant status (and non-reproductive to reproductive; Whitfield et al. 2006; Wurm et al. 2010). Non-reproductive workers of honeybee (*Apis mellifera*) societies provide another compelling example as they transition through a series of distinct behavioral tasks as they age. Specifically, substantial changes in brain gene expression (>85% of approximately 5,500 genes showed significant differences) are associated with the transition from nurse to forager, largely independent of age-related changes (Whitfield et al. 2006). Among fishes, several patterns that have been documented are similar to those observed among invertebrates. Social status has been associated with variation

in neural gene expression (for review, see Wong & Hofmann 2010). This has been particularly well documented in studies of cichlid fishes both on a genomic scale (Aubin-Horth et al. 2006; Renn et al. 2008; Schumer et al. 2011; O'Connell & Hofmann 2012) and at the level of candidate neuroendocrine gene expression (Greenwood et al. 2008; Maruska et al. 2011, 2012; O'Connell & Hofmann 2012).

Gene expression studies have also informed us about the evolution of alternative life histories associated with discrete reproductive phenotypes. Comparison of gene expression in related species that differ in plasticity of mating tactics reveals that behavioral plasticity carries a substantial physiological cost that can favor the evolution of fixed life histories associated with different tactics over maintenance of tactic plasticity (Fraser et al. 2014). Expression differences among these discrete phenotypes reflect what we know about how they differ in behavior and life history (Aubin-Horth et al. 2005a; Fraser et al. 2014; Schunter et al. 2014). The behavioral differences of reproductive phenotypes are reflected in the specific gene expression of each (Fraser et al. 2014; Schunter et al. 2014). Growth is known to be involved in the development of reproductive phenotype in some species, and the need for future growth is associated with individual age, which can covary with reproductive phenotype (Alonzo et al. 2000). Accordingly, variation in expression of growth-related genes reflects the differential need for growth between alternative male reproductive phenotypes (Aubin-Horth et al. 2005a). Sex may be less important than reproductive tactic in terms of similarities among phenotypes (e.g., expression differences are greater between alternative male black-faced blenny phenotypes than between males and females; Schunter et al. 2014). These past findings form a framework for further examinations of how physiology covaries with individual life history, both for general patterns of differences between phenotypes, and for differences in expression of specific genes.

Here, we examine physiological variation in the ocellated wrasse (*Symphodus ocellatus, family Labridae*), a non-sex changing teleost fish with a complex mating system (Warner & Lejeune 1985; Taborsky et al. 1987). Males of this species engage in one of three reproductive tactics during a given reproductive season as follows: nesting, satellite, or sneaker. Dominant nesting males build nests, court, and spawn with females, provide parental care, and cooperate with satellite males (Soljan 1930; Warner & Lejeune 1985; Taborsky et al. 1987; Stiver & Alonzo 2013). Satellites cooperate with the nesting

male to defend the nest against sneakers or other competitors, and court females; however, they also engage in parasitic spawning at that nest (Warner & Lejeune 1985; Taborsky et al. 1987; Stiver & Alonzo 2013). Sneaker males spawn parasitically without courting, providing parental care, or being territorial, and they are not cooperative (Warner & Lejeune 1985; Taborsky et al. 1987). Accordingly, nesting males are the largest and most colorful, satellite males are medium in size and coloration, and sneaker males are the smallest and least colorful (Warner & Lejeune 1985; Taborsky et al. 1987). The continuum of male size and coloration is reflected in relative dominance and territoriality: nesting males are the most dominant and territorial, and sneaker males the least.

While males utilize a single tactic per reproductive season, they can transition to other tactics between seasons. Specifically, males display three potential life history trajectories depending on early growth prior to their first winter or first reproductive year (Alonzo et al. 2000). Specifically, they might (1) breed as a 1-yr-old sneaker, then as 2-yr-old satellite, (2) breed as a 1-yr-old satellite, then as 2-yr-old nesting male, or (3) remain non-reproductive as a 1-yr-old before breeding as a 2-yr-old nesting male (Alonzo et al. 2000). Sneakers have the highest gonadal investment relative to their body weight (followed by satellite males, Warner & Lejeune 1985; Taborsky 1994) and release significantly more sperm per spawn than either nesting males or satellites (Alonzo & Warner 2000). While no significant differences in the spawning rate of all three male phenotypes across the season have been found (Warner & Lejeune 1985), variation in gonadal investment, sperm production, velocity, and motility between male types has been documented (Warner & Lejeune 1985; Taborsky 1994; Alonzo & Warner 2000; SH Alonzo, KA Stiver & SE Marsh-Rollo, unpubl. data). While nesting males father the majority of the young at their nest (Alonzo & Heckman 2010), variation in total reproductive success between either sneakers and satellites at a nest or among all male phenotypes across the reproductive season has not been quantified, although work is ongoing.

The life history of female ocellated wrasses is much less variable. Females reach reproductive maturity after 1 yr and have a maximum documented life span of 2 or 3 yr (Alonzo et al. 2000). Females are non-territorial, do not participate in nest-building or parental care, display little aggressive behavior toward either sex, and sometimes travel in small groups with other females. Females

appear to base their spawning decisions primarily on nest success and therefore copy the mate choice of other females (Alonzo 2008). Females lack the colorful operculum indicative of males in this species, but their physical appearance is otherwise similar to that of sneakers.

Here, we document body and gonad size differences and examine variation in circulating sex steroid hormones and brain gene expression patterns to better understand the plasticity in reproductive behavior among the three distinct male strategies along with females. As the four reproductive phenotypes in *S. ocellatus* show interesting contrasts in reproductive behavior, we hope to identify broad patterns of expression differences, and some potential specific candidate genes, that may relate to this behavioral variation. Using a database with information on over 900 individuals collected over 4 yr, we first document discrete size variation (a marker for investment in somatic growth) between the four reproductive phenotypes. Then, we determine the correlates between the observed behavioral, physiological, and neurogenomic differences among reproductive phenotypes, specifically asking how gene expression variation corresponds to differential investment in territoriality, aggression, and cooperation, and to variation in mating strategy.

In documenting the variation among phenotypes, we add to a growing literature on how gene expression variation underlies variation in behavior, particularly discretely different reproductive phenotypes (e.g., Aubin-Horth et al. 2005a; Fraser et al. 2014; Schunter et al. 2014). As our focal species shows an overlap in reproductive behavior between phenotypes as well as behaviors that are unique to a type, patterns of gene expression variation mapped to this behavioral variation may better inform us of how variant patterns of reproductive investment can arise through differential expression of a common genome. Information about variation in *S. ocellatus* is particularly valuable, as the male phenotypes differ not only in levels of aggression and territoriality, but also in whether or not they engage in cooperation (which involves tolerance and continued positive interaction with an active competitor by both satellites and nesting males, and the satellites engaging in costly aggression against sneakers, and actively exposing themselves to potential aggression from the nesting male). Additionally, we attend to the potential for different strategies of differential expression (greater number of genes versus greater magnitude of expression difference) that correlate with clear differences in individual behavior.

Methods

Variation in Body Size and Condition Collections

Research was conducted at the University of Liège (Belgium) Marine Laboratory (La Station de Recherches Sous-marines et Océanographiques, STARESO), in the Baie de Revellata near Calvi, Corsica, France, on SCUBA at <10 m depth. Morphological data were collected from 974 individuals of known phenotype (nesting, satellite and sneaker males, and females) caught for use in other behavioral or physiological studies in June 2009, and in May–June 2010–2012. These wild-living fish were sampled prior to any physiological sampling or manipulation. Standard length was measured for all 974, and weight was obtained for 931 of these fish. Both metrics were used to examine between-phenotype differences in size and somatic investment.

Hormone Analyses

Blood/Plasma collection

We collected blood from euthanized individuals during the May–June field season either via caudal severance (in 2011; N = 22) or directly from the dorsal aorta (in 2012; N = 36) using a heparinized 25G butterfly syringe (Terumo Surflo, Fisher Scientific). Blood was placed on ice following collection, spun for 10 min at 1177 g to separate plasma, and the plasma separated and frozen (maintained at −20°C) for storage and shipment.

Hormone analyses

Free 11-ketotestosterone (11-KT), testosterone (T), and 17β-estradiol (E) were measured for each individual using ELISA (Cayman Chemical Cat. No.: 582751, 582701, and 58225, respectively). Plasma samples were processed as previously described (Kidd et al. 2010) with the modification that samples were diluted 1:64 for 11-KT and 1:32 for T and E. We validated the ELISAs by assessing parallelism between concentration standards provided by the manufacturer and serial dilutions ranging from 1:2 to 1:64 from a 115 µL pool of plasma, created from samples of all four phenotypes. The resulting dilution curves were parallel to the respective standard curves for 11-KT (comparison of slopes: $t_9 = 0.097$, p = 0.462) and E ($t_8 = −1.600$, p = 0.148), but not for T ($t_9 = 3.565$, p = 0.003). Even though the T measurements should therefore be considered less reliable, there were no significant differences between phenotypes

($F_{3,38}$ = 1.3, p = 0.30). Intra-assay coefficients of variation were 15.9% for 11-KT, 14.6% for T, and 9.6% for E, respectively; interassay coefficients of variation for 11-KT and T were 6.5% and 5.5%, respectively. Only one assay plate was run for E. Somewhat surprisingly, intra-assay variation was greater than interassay variation for the androgens, possibly because the plasma samples were obtained in the field and may thus have experienced suboptimal storage and shipping conditions, whereas the control samples used to determine interassay variation were obtained in the laboratory. Nevertheless, the variation lies within the acceptable range.

Gene Expression Analyses

Brain collections

In June 2009, nesting males, satellite males, sneaker males, and females (N = 10 each) were collected from 13 nests. Nests were observed for 10 min prior to capture of any individuals to determine individual phenotype, to confirm the identity of the nesting male and satellite, and to ensure that the nests were in the actively spawning phase with a high number (>2) of sneakers present. Following observation, individuals at the nest were caught with hand nets, transferred to shore, and promptly euthanized in MS-222 within 19 ± 10 min (mean ± SD) after capture. Individuals of the different phenotypes were captured and processed in haphazard order to control of order effects of sampling. Then, body mass and standard length were recorded. Brains were rapidly dissected and stored in RNAlater (Ambion) at room temperature for 24 h, and then placed into a −20°C freezer until subsequent RNA extraction. Brain dissection of all individuals was completed within 10 minutes of death (mean post-euthanasia dissection time ± SD = 418 ± 116 s). Brains from all phenotypes were collected, sampled, and stored in the same manner, and whenever possible, we included individuals of each phenotype sampled from a single nest to decrease the potential of between-nest variation confounding differences among phenotypes (nine nests contributed three or more individuals of the different phenotypes to the final analysis).

RNA extraction, and microarray hybridization and analysis

RNA was extracted from whole brains using TRIzol (Invitrogen) according to the manufacturer's instructions. RNA from 10 individuals from each type was pooled to create an aggregate sample for each of the four reproductive phenotypes for transcriptome comparison between phenotypes. Samples were prepared as previously described (Renn et al. 2008), and all samples were competitively hybridized with dye reversal to a custom-made cDNA array (GEO platform ID: GLP6416) constructed from brain-specific and mixed tissue libraries of the model cichlid *Astatotilapia burtoni*, representing a total of 17 712 features (Renn et al. 2004; Salzburger et al. 2008). This platform and its predecessor have been shown to give biologically meaningful results for several other cichlid and non-cichlid species (Renn et al. 2004; Aubin-Horth et al. 2005b, 2007; Cummings et al. 2008; Schumer et al. 2011). Due to the challenges associated with collecting samples in the field (specifically, collection of tissue from all four phenotypes immediately following behavioral observation, and the limited amount of RNA were able to extract from smaller individuals), there was no opportunity to examine expression variance between individuals. Therefore, we pooled brains for expression analysis, and our design featured four (pooled) independent samples and 6 (12 with dye swapping) interlinked microarray comparisons (see Fig. 1). Note that two pool replicates per phenotype would not be sufficiently large for estimating between-pool variance. Also, the effect of an outlier within a pool of five individuals would be considerable, whereas the effect of an outlier within a pool of ten is likely not problematic. Thus, we used single pools of ten. Following hybridization, all array slides were scanned and subjected to initial processing using the GenePix 4000B array scanner using GenePix 5.1 software. Features with erratic signal intensity and features with average intensity less than 2 SD above the average background intensity were omitted from the analysis. This filtering left 8078 features (45.6% of array features) available for expression analysis.
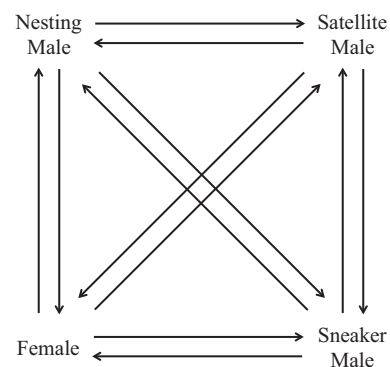
Fig. 1: Diagram outlining the pairwise comparisons (including dye swaps) in gene expression made between the four reproductive phenotypes of *Symphodus ocellatus*.

Microarray data were normalized as in Townsend et al. (2003), and Zhang & Townsend (2010). Relative gene expression was estimated and compared between phenotypes using the Bayesian Analysis of Gene Expression Levels software (BAGEL; Townsend & Hartl 2002; Townsend 2004), using posterior probability (PP) thresholds of PP ≥0.99 or PP ≥0.95 to determine statistical significance of differential expression. BAGEL can yield estimates with appropriately wide confidence intervals based on modest experiments conducted when samples are challenging to acquire. BAGEL estimate variance based on differences between the results of dye swap comparisons and among transitive inferences across reproductive types (see Fig. 1). The variance estimated does not include any estimate of biological variation within reproductive types, but accurately estimates mean expression between reproductive types and the (technical) variance between array results arising from RNA extraction, reverse transcription, hybridization, and scanning.

To adjust for multiple hypothesis testing, we assessed the false discovery rate (FDR) according to Benjamini & Hochberg (1995). Owing to increased variance obtained with heterologous hybridizations (see Renn et al. 2004 for an in-depth discussion of these issues), we did not expect to achieve statistical significance for many features and, as a consequence, fewer still that remain after adjustment for FDR. Not surprisingly, of the 1113 features that showed significant differences between at least two phenotypes at PP ≥0.95, few genes (N = 1) were significant after FDR. To compare expression profiles for the genes with a significant difference of PP ≥0.95 across phenotypes, we performed unsupervised hierarchical clustering with Euclidean distance as the similarity metric and complete linkage using the hclust function in R/Bioconductor (R Development Core Team 2008). Additional statistical tests were conducted using SPSS 19.0 or JMP 10.0.

Results

Variation in Body Size and Condition

Body size data confirm that the three male types differ significantly in mean standard length (one-way ANOVA, $F_{3,970}$ = 1686.8, N = 974, p < 0.0001) and weight ($F_{3,927}$ = 1982.4, N = 931, p < 0.0001; see Table 1), such that nesting males are larger than satellites, which are in turn larger than sneakers. Females do not differ from sneakers in mean standard length, but are intermediate to satellites and sneakers in weight.

An examination of body condition (residuals of the regression of body mass) revealed that nesting males have the highest investment in mass relative to length among males, followed by sneakers and then by satellites; females are intermediate to nesting males and sneakers and do not significantly differ from either ($F_{3,927}$ = 84.1, N = 931, p < 0.0001; see Table 1).

Variation in GSI (gonad weight/body weight ×100) among males followed previously reported patterns (Warner & Lejeune 1985, Taborsky 1994) and confirmed the phenotype/reproductive status of examined individuals. Sneakers had the largest gonads relative to their body size (N = 10, range: 6.0–11.3%, mean ± SE = 8.3 ± 0.58% of body weight) followed by satellites (N = 9, range: 3.2–8.0%, mean ± SE = 5.6 ± 0.50%) and then by nesting males (N = 10, range: 1.0–2.2%, mean ± SE = 1.5 ± 0.14%; one-way ANOVA, $F_{2,26}$ = 60.23, p < 0.001). Gonadal data reported here are of the individuals used in the gene expression analysis (one satellite whose absolute gonad weight fell within the normal satellite range was omitted due to an imprecise body weight); these data are part of a larger dataset examined in SH Alonzo, KA Stiver & SE Marsh-Rollo (unpubl. data), and thus, test of the full dataset is not included here. Similarly, reproductive status of females was confirmed by their GSI (N = 10, range: 4.0–7.4%, mean ± SE = 5.4 ± 0.29%).

Variation in Circulating Hormone Levels

Nesting males had significantly higher levels of circulating 11-KT than satellite males, sneaker males, and females (one-way ANOVA, $F_{3,31}$ = 9.93, p < 0.001; Fig. 2a), while the other three phenotypes did not differ from one another. Females had significantly higher 17β-estradiol levels than all three male types ($F_{3,26}$ = 65.9, p < 0.001; Fig. 2b), while males did not differ from one another.

Genome-Wide Gene Expression Differences

Of the 8078 array features that could be examined in *S. ocellatus*, 6246 showed above-threshold expression levels for all four phenotypes. All subsequent analyses were conducted using this shared set. Differential expression between at least two phenotypes was detected for 1113 (17.8%) of these features at p < 0.05 and 218 (3.5%) at p < 0.01. Clustergrams revealed striking differences in gene expression between phenotypes (Fig. 3a), and a principal components analysis examining all genes with significant expression variation between at least two phenotypes

Table 1: (A) A summary of the mean and standard error of the measures of size and magnitude of gene expression for each reproductive phenotype. (B) A summary of the post-hoc tests (p-values) associated with tests of differences in size and magnitude of gene expression differences between reproductive phenotypes. All are Tukey's HSD

A) Phenotype	Standard length	Weight	Body condition	Magnitude of gene expression difference
Nest	74.97 ± 0.16	9.38 ± 0.06	0.23 ± 0.04	1.51 ± 0.014
Sat	61.09 ± 0.37	4.91 ± 0.09	−0.66 ± 0.03	1.40 ± 0.015
Sneak	40.39 ± 0.38	2.51 ± 0.07	0.01 ± 0.04	1.42 ± 0.010
Fem	50.68 ± 0.53	2.92 ± 0.11	0.11 ± 0.04	1.36 ± 0.013
B) Phenotypes compared	Standard length	Weight	Body condition	Magnitude of gene expression difference
Nest to Sat	<0.001	<0.001	<0.001	< 0.001
Nest to Sneak	<0.001	<0.001	<0.001	< 0.001
Nest to Fem	<0.001	<0.001	0.173	< 0.001
Sat to Sneak	<0.001	<0.001	<0.001	0.728
Sat to Fem	<0.001	<0.001	<0.001	0.298
Sneak to Fem	0.054	0.006	0.365	0.005

Nest, nesting males; Sat, satellite males; Sneak, sneaker males; Fem, females.
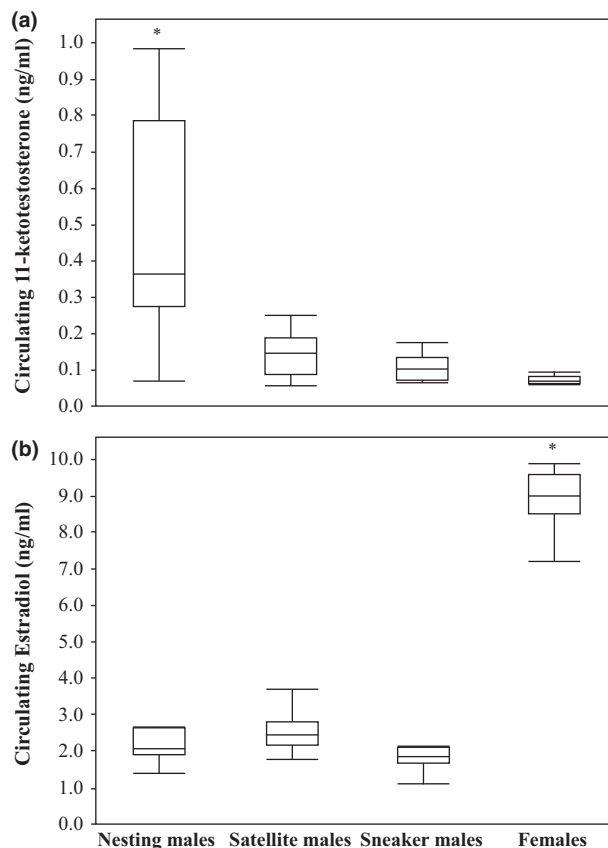
Fig. 2: Variation in circulating hormone levels among the four phenotypes. Differences were calculated using one-way ANOVA (Tukey's HSD post-hoc tests). Asterisks indicate phenotypes for which levels were significantly higher than in the other three phenotypes. (a) Nesting males have a higher level of circulating 11-ketotestosterone than the other three phenotypes. (b) Females have a higher level of circulating estradiol than any of the male phenotypes.

revealed that the expression profiles of sneakers and nesting males were the most divergent, while satellite males and females were most correlated (Fig. 3b). Examination of the number of genes that showed differential expression between any two phenotypes (irrespective of the magnitude of the fold difference) revealed more differences between sneakers and the other phenotypes than between any other pairwise comparisons: sneakers and females had the most genes showing differential expression (12.5% of features), followed by sneakers and nesting males (9.4%), then sneakers and satellites (8.4%). The fewest differences in expression were observed between satellites and females (1.7%), followed by nesting males and satellites (2.7%), then nesting males and females (3.5%).

Number of Differentially Expressed Genes

Sneaker males differed from at least one other phenotype in the expression of 1048 genes (16.8%), followed by females (916 genes: 14.7%), nesting males (720 genes: 11.5%), and finally satellites males (710 genes: 11.4%). Similarly, when the number of genes that were uniquely differentially expressed by each phenotype was examined, sneaker males again showed the greatest difference, with more features with significantly different expression than *all* other phenotypes (4.5% of features) compared to the number of uniquely expressed genes in nesting males (1.2%), females (0.19%), and satellites (0.16%; see Fig. 4a). Up- and downregulation of genes was symmetric in nesting males (sign test, p = 0.55), whereas satellites
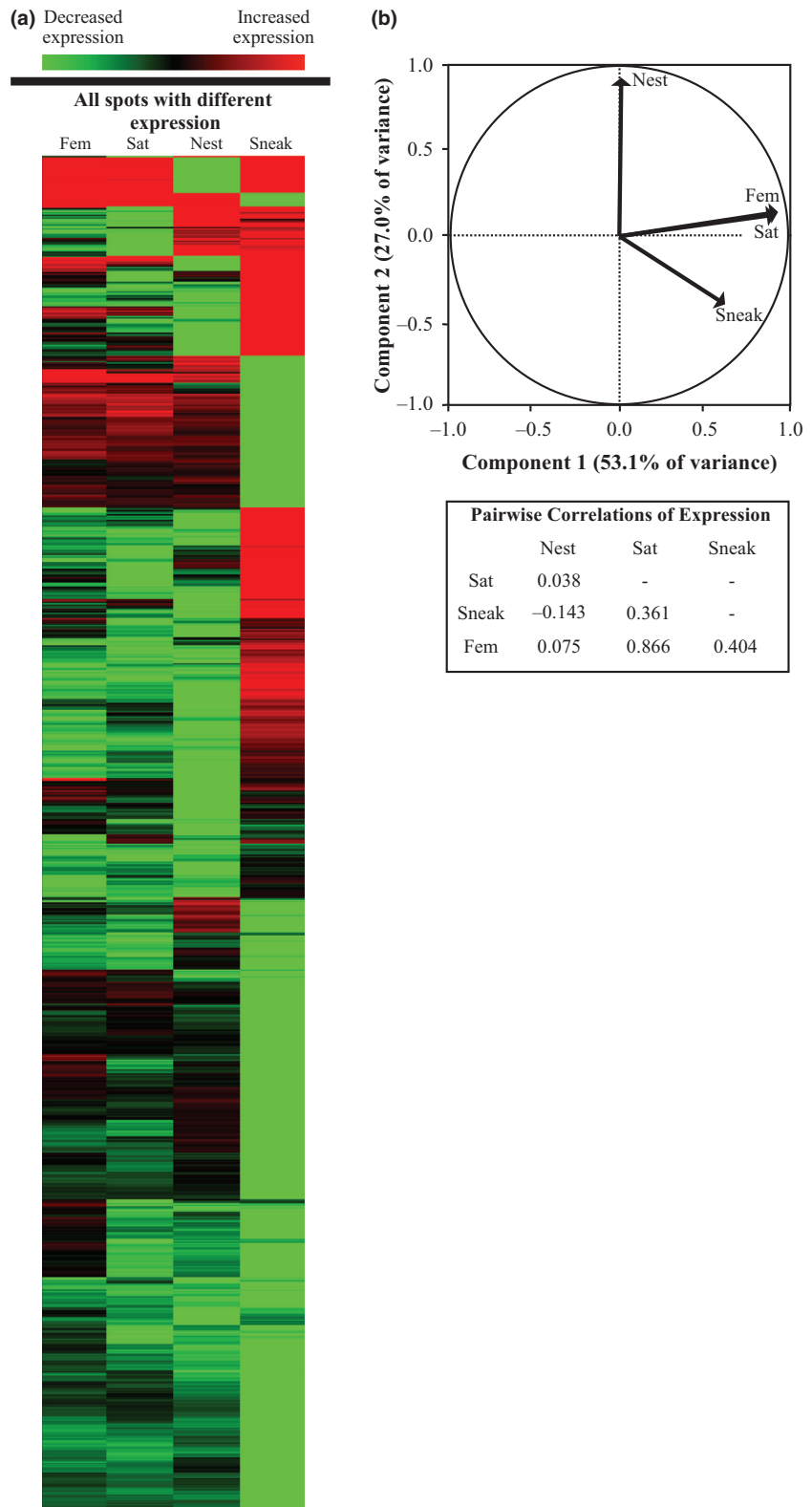
Fig. 3: Overall, nesting (Nest) and sneaker (Sneak) males showed the greatest differences in expression, with females (Fem) and satellite (Sat) males intermediate to these two phenotypes in expression. Below are two examinations of expression differences based on features where at least two phenotypes differed in expression with a posterior probability (PP) threshold of PP ≥0.95 (N = 1113). (a) The heat map was produced by Hierarchical Clustering Explorer 3.5 (hierarchical clustering with Euclidian distance and complete linkage). Red indicates increased relative expression, and green indicates decreased relative expression. (b) A principle components analysis confirmed that satellites and females had gene expression that was most similar to one another, and sneaker and nesting males most dissimilar (image shows the clustering of phenotypes with regard to components 1 and 2). Expression clustered along two principle components that accounted for 53.1% and 27.0% of the variance in gene expression. Also reported are the correlations in gene expression levels between the four phenotypes.

(a)

(b)
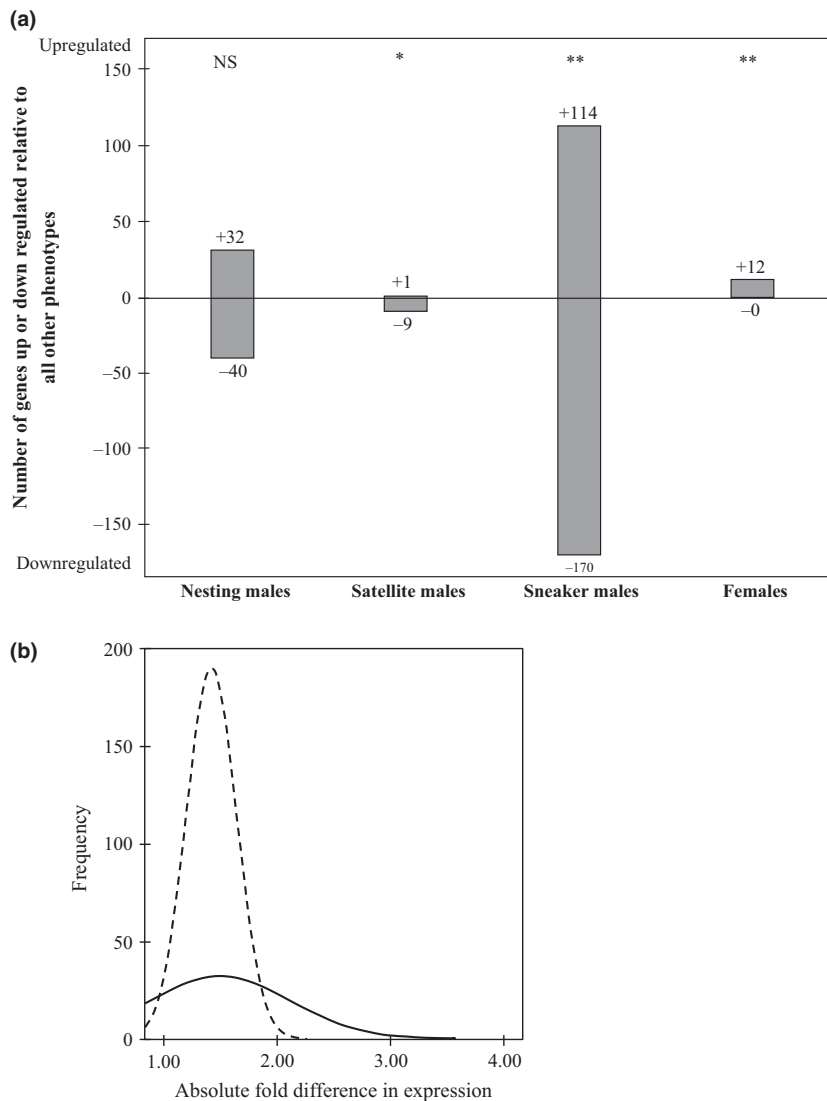
Fig. 4: Summary of the differences in gene expression between the four phenotypes, in terms of number of features, and magnitude of expression difference. (a) Overall, sneaker males showed more pairwise differences in expression from the other three phenotypes than did any other type. This graph depicts the number of genes for which each phenotype showed significantly different expression from all other phenotypes. Also tested was the degree to which a difference in expression was biased toward up- or downregulation (binomial tests: NS: $p > 0.05$; *: $p < 0.05$, **: $p < 0.01$). (b) The average magnitude of expression differences for nesting males was larger than those of the other three phenotypes (using absolute value of difference). This figure shows two normal curves, the first generated from pairwise phenotype comparisons that involved the nesting male (solid line; range = 1.04–7.25, mean ± SE = 1.5 ± 0.02), and the second from pairwise phenotype comparisons that did not involve the nesting male (dashed line; range = 1.04–2.43, mean ± SE = 1.4 ± 0.005). When the four phenotypes were compared using an ANOVA, the absolute magnitude difference in expression was greater for nesting males relative to all other phenotypes, and for sneakers relate to females (see table 1).

($p = 0.02$) and sneakers ($p = 0.001$) displayed a significant bias toward downregulation, and female-specific genes were significantly more often upregulated ($p = 0.0005$).

Magnitude of Differential Expression

Examination of the fold differences in expression revealed that nesting males showed the greatest variation in absolute fold difference in expression (Levene's test, all phenotypes: $F_{3,4780} = 51.9$, $p < 0.0001$; see Fig. 4b) and the highest mean fold difference in expression (mean expression of sneakers was also higher than that of females: repeated-measures one-way ANOVA, $F_{3,4780} = 20.1$, $P < 0.0001$; see Table 1).

Discussion

Variation in Body Size and Condition

Our reported size differences confirm that the alternative reproductive phenotypes of the ocellated wrasse show distinct differences in morphology and patterns of growth over their lifetime. Furthermore, a sex difference in growth investment is suggested, as terminal size of males exceeded that of females despite a similar expected life span (typically 2 yr, Alonzo et al. 2000).

Variation in Circulating Hormone Levels

Hormonal variation among phenotypes also mirrors expectations based on phenotypic behavior and hor-

mone function. 11-KT is the major androgen in many fish species (Borg 1994), and it is typically increased in dominant males, as seen here in the elevated 11-KT in nesting males relative to sneakers and satellites (Oliveira et al. 2001; Maruska & Fernald 2010a,b; Oldfield et al. 2013; Taves et al. 2009). Testosterone levels did not vary among phenotypes, which is a common finding among fish species given the role of 11-KT as the functional analog for T (but see Desjardins et al. 2008; Parikh et al. 2006; Trainor & Hofmann 2006; Taves et al. 2009; Renn et al. 2012 which represent exceptions to this general pattern, all from *Cichlidae* species). Increased estradiol in females relative to males is typical given the role of estradiol in vertebrates (e.g., Borg 1994; but see Maruska & Fernald 2010a; O'Connell et al. 2012). Therefore, variation in circulating hormone levels among the phenotypes is as expected based on the biological function of these hormones in fishes.

Genome-Wide Gene Expression Differences

General differences among phenotypes

In terms of the sheer number of differentially expressed genes, sneaker males showed both the highest number from at least one other phenotype, and the most uniquely expressed genes. However, nesting males showed the greatest fold difference in expression for those genes where they differed. Thus, while sneakers showed greater differential expression in terms of kind of difference, nesting males showed a greater degree of a difference. This variation in mode of differential expression may represent different 'solutions' to the problem of differentiating from a shared genome.

Expression differences among males were in accordance with their relative dominance and overlap in reproductive behaviors, as nesting males were most dissimilar from sneakers, and satellite males were intermediate to both (note, however, that this also reflects the age structure of the phenotypes, see below). These results present some interesting comparison to some of the broader patterns arising from studies of gene expression in invertebrate species. For example, although subordinate *S. ocellatus* are reproductive, the increased differential expression we observed among sneakers is reminiscent of similar patterns of the importance of the molecular changes in subordinate *Hymenopteran* castes in social evolution in these species (see Ometto et al. 2011; Ferreira et al. 2013). Similarly, the four *S. ocellatus* phenotypes showed differences in the direction of the gene expression differences (i.e., phenotypes differed in

their tendency toward up- or downregulation) that reflected similar differences in expression found among castes (Ferreira et al. 2013).

The similarity in gene expression profiles of satellites and females is perhaps surprising given the profound differences in reproductive behavior between these two phenotypes; however, it does reflect work in other fishes that revealed variation in gene expression among male types to exceed that between males and females (Schunter et al. 2014; but see our comments below in '*Possible limitations*' regarding the high dissimilarity between females and sneaker males). Future work will examine specific explanations for this similarity. One possibility is that similarity in expression could reflect the similarity in mixed-age structure of these two phenotypes: satellites and females are 1 or 2 yr old, compared to sneakers which are all 1 yr of age, and nesting males which are all two. Again, this importance of age/developmental stage has been similarly identified in studies of *Hymenoptera* (e.g., Ometto et al. 2011). Alternatively, the similarity could reflect their similar social experiences at the nest: both are tolerated (or encouraged to be) at the nest by nesting males, but both also experience aggression from the nesting male. In contrast, nesting males receive aggression only during dominance contests, while sneakers are never tolerated and receive aggression from both the nesting males and satellite males.

Expression profiles of candidate genes

We summarized genes with shared expression that appeared to be associated with specific social behaviors that might be similar across certain reproductive phenotypes (Table 2; see also Fig. 5). For example, nesting and satellite males are more territorial, aggressive, and cooperative than females and sneaker males, whereas only satellite and sneaker males spawn parasitically (Warner & Lejeune 1985; Taborsky et al. 1987). Five genes were significantly downregulated in satellite and nesting males compared with females and sneaker males, and eight genes were differentially expressed between sneakers and satellites compared to nesting males and females. Future work is aimed at better illuminate the role of these genes in the reproductive and social behaviors associated with each reproductive phenotype.

Specific differences between annotated genes are summarized in Supplementary Tables S1 (a summary grouped by apparent function) and S2 (an examination of specific genes for which there are directional predictions; Aubin-Horth et al. 2005a, 2007; Renn

Table 2: A summary of the genes that show up- or downregulation of expression between phenotypes that share some component of reproductive behavior. Reported are the unique ID, GenBank accession number (if assigned; annotation where possible in italics), the two groups that showed upregulation, the relative expression for each phenotype, and the specific significant differences in expression between the four phenotypes

| Unique ID, Accession Number *Annotation*	Upregulated in	Nest	Sat	Sneak	Fem	Specific differences
hh_Ab_Pinky2003_000010595, DY631259	Sneak, Fem	1.00	1.24	1.69	1.41	(Sneak > Fem) > (Sat>Nest)
hh_Ab_Pinky2003_000010774, —	Sneak, Fem	1.01	1.00	1.60	1.26	(Sneak > Fem) > (Sat = Nest)
hh_Ab_Pinky2003_000011493, DY631480 *Apolipoprotein N-acyltransferase (partial)*	Sneak, Fem	1.00	1.05	1.61	1.22	(Sneak > Fem) > (Sat = Nest)
hh_Ab_Pinky2003_000016264, DY632304 *Peptide-N(4)-(N-acetyl-beta-glucosaminyl) asparagine amidase, Danio rerio (Zebrafish) (partial)*	Sneak, Fem	1.00	1.09	2.25	1.28	(Sneak > Fem) > (Sat = Nest)
hh_Ab_Pinky2003_000014749, DY629703	Sneak, Fem	1.00	1.00	1.39	1.13	(Sneak > Fem) > (Sat = Nest)
hh_Ab_Brain2000_000000039, —	Sat, Sneak	1.00	1.20	1.39	1.13	(Sneak > Sat) > (Fem > Nest)
hh_Ab_Pinky2003_000016371, —	Sat, Sneak	1.00	1.27	1.46	1.04	(Sat = Sneak) > (Nest = Fem)
hh_Ab_Pinky2003_000019851, —	Sat, Sneak	1.02	1.18	1.32	1.00	(Sat = Sneak) > (Nest = Fem)
hh_Ab_Pinky2003_000012499, DY626482	Sat, Sneak	1.00	1.32	1.40	1.06	(Sat = Sneak) > (Nest = Fem)
hh_Ab_Pinky2003_000012931, DY626393	Sat, Sneak	1.00	1.59	2.20	1.14	(Sat = Sneak) > (Nest = Fem)
hh_Ab_Pinky2003_000010420, CN468620 *Cynoscion arenarius 12S rRNA (partial); tRNA-Val gene (complete); 16S rRNA (partial)*	Nest, Fem	1.28	1.06	1.00	1.28	(Nest = Fem) > (Sa = Sneak)
hh_Ab_Pinky2003_000014840, Y629622 *Squalus acanthias internal transcribed spacer 2; 28S rRNA gene (partial)*	Nest, Fem	1.23	1.09	1.00	1.26	(Nest = Fem) > (Sat = Sneak)
hh_Ab_Pinky2003_000011889, —	Nest, Fem	1.55	1.13	1.00	1.35	(Nest > Fem) > (Sat = Sneak)

Nest, nesting males; Sat, satellite males; Sneak, sneaker males; Fem, females.

et al. 2008; Schumer et al. 2011). Similar to Schunter et al. 2014; we found differential expression in a number of genes associated with transport; however, we did not find consistent upregulation in associating with sneaking strategies that they found. Growth-related genes (ribosomal proteins, histone, and proteasome-related genes; Renn et al. 2008) were generally more highly expressed in those phenotypes with greater need for current or future growth (based on age and life history, sneakers are expected to have the greatest needs, followed by satellites and females, and finally by nesting males; Alonzo et al. 2000).

A few of the better understood dominance-related genes (gonadotropin α-subunit and arginine vasotocin (AVT); Gen et al. 2003; Aubin-Horth et al. 2007; Renn et al. 2008; Schumer et al. 2011) showed differential expression, although not generally in the expected directions. The α-subunit of the gonadotropins follicle-stimulating hormone (FSH) and luteinizing hormone (LH) is crucially important to reproductive physiology, including gametogenesis and synthesis of sex steroid hormones in the gonads (e.g., see Maruska et al. 2011 for an examination of how these hormones increase with an increase in social status). Expression of gonadotropin α-subunit was highest in the nesting males and lowest in sneakers. This difference is consistent with the differences in circulating levels of 11-KT, but did not reflect the relative investment in gonadal tissue and sperm production (both of which are highest in sneakers; Warner & Lejeune 1985; Taborsky 1994; Alonzo & Warner 2000; SH Alonzo, KA Stiver & SE Marsh-Rollo, unpubl. data).

Expression of AVT was expected to show a positive correlation with dominance based on previous whole brain expression studies in cichlid fishes (Aubin-Horth et al. 2007; Renn et al. 2008; Schumer et al. 2011). However, in the present study, the only significant difference in whole brain AVT expression was increased expression in sneakers relative to females.

High territoriality/Aggression/Cooperation

Low territoriality/Aggression/Cooperation
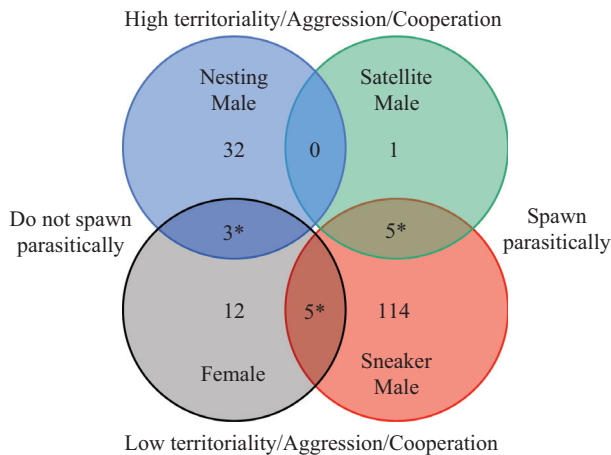
Fig. 5: Venn diagram indicating the number of feature that one (or two) phenotypes showed significant upregulation relative to the other three (or two) phenotypes. The phenotypes are placed with regard to shared behaviors of interest. Satellites and sneakers both spawn parasitically, while nesting males and females do not. Nesting males and satellites are both aggressive, territorial, and cooperative, while sneakers and females are not. * indicates features that are also summarized in table 2.

Differences in AVT expression may be spatially localized: specifically, different populations of AVT-expressing neurons likely serve different, and possibly opposing, functions in behavioral control (Ota et al. 1999; Miranda et al. 2003; Larson et al. 2006; Greenwood et al. 2008). Future studies will focus on α-subunit expression, particularly how it relates to 11-ketotestosterone levels and individual reproductive investment, and will also examine AVT expression at higher spatial resolution.

General gene expression variation summary

Overall, variation in reproductive and social behavior explained transcriptome variation among phenotypes better than sex did, although other factors (such as the age-dependent expression) could affect observed patterns as well. Satellite males and females were most similar in neural gene expression, and both were intermediate in expression to nesting and sneaker males. Sneaker and nesting males showed strikingly different patterns of expression compared to the other phenotypes: sneakers had a higher total number of differentially expressed genes, whereas nesting males showed the greatest of gene expression, suggesting two alternative manners of behavioral differentiation via differential expression (by kind versus by degree). Consistent with previous work, similarity of expression between male types followed in accordance with

the dominance of the types (Aubin-Horth et al. 2005a,b, 2007; Renn et al. 2008; Schumer et al. 2011).

Not all observed expression patterns of specific candidate genes were expected (most notably, AVT expression did not follow the expected pattern). In the ocellated wrasse, dominance covaries with the behaviors that define the different male reproductive tactics (territoriality, aggression, cooperation, and parental care; Warner & Lejeune 1985; Taborsky et al. 1987; Stiver & Alonzo 2013); thus, interpreting specific expression differences with regard to dominance generally can be difficult. In contrast, expectations of differential expression with regard to sex and differences in current and future growth of the phenotypes were more consistent with past findings. Finally, examination with regard to the specific behaviors that define the phenotypes revealed a subset of genes that may contribute to those particular behaviors and thus warrant further examination.

Possible limitations of whole brain expression profiling using heterologous hybridization

Measuring gene expression in whole brains has limitations as any given gene may show opposing expression patterns in different brain regions. Also, genes expressed in only a small subset of cells may not be detected reliably. However, whole brain transcriptome analyses have been very informative in a variety of systems (Whitfield et al. 2006; Renn et al. 2008), especially those with a rich ecological and evolutionary literature but limited mechanistic knowledge (e.g., Aubin-Horth et al. 2005a, 2007; Cummings et al. 2008; Wang et al. 2008; Machado et al. 2009; Ferreira et al. 2013). Future studies will focus on candidate brain regions implicated in the regulation of social behavior (Newman 1999; Goodson 2005; O'Connell & Hofmann 2012).

Another possible limitation of our study arises from the fact that we performed heterologous hybridizations to an array platform custom-made for the model cichlid fish *A. burtoni* (Renn et al. 2004, 2008). This platform has repeatedly been shown to give biologically meaningful results for several other cichlid (*Enantiopus melanogenys, Neolamprologus pulcher/brichardi, Oreochromis nigrensis*) and non-cichlid (*Xiphophorus nigrensis, Poecilia reticulata, Salmo salar, Danio rerio*) species (Renn et al. 2004; Aubin-Horth et al. 2005b, 2007; Cummings et al. 2008; Schumer et al. 2011). Renn et al. (2004) analyzed the effect of sequence divergence (as a proxy for evolutionary distance) on the ability to obtain biologically meaningful results

with heterologous hybridization. Their results demonstrated that even subtle gene expression differences can be detected across perciform fishes (including wrasses) and beyond (e.g., platyfish and guppy). Nevertheless, a substantial number (ca. 50%) of array features did not yield hybridization above-threshold intensities, likely due to sequence divergence between the platform species, the cichlid *A. burtoni* and *S. ocellatus*. Additionally, as mentioned above, Schunter et al. (2014) found that phenotypic difference among males was more associated with differential gene expression than was sex. While we found the greatest similarity between satellite males and females, sneaker males and females showed the highest number of features with differential expression. As our analysis was biased toward slowly evolving genes, we may have missed rapidly evolving genes of potential relevance for behavioral plasticity in the context of alternative reproductive tactics. Ongoing studies using RNA sequencing are designed to overcome this limitation.

Conclusions

Complex variation in individual behavior between members of the same species often arises due to plasticity in expression of a common genome. We have outlined the role of genes that have been suggested to relate to dominance and reproduction (e.g., gonadotropin α-subunit), by identifying differential expression, and hormonal variation (elevated 11-KT in nesting males, and E in females) that reflects this neural expression difference. Additionally, we have identified a short list of candidate genes that are potentially involved in the expression of complex social behaviors, such as cooperation between reproductive competitors. We found that similarity in male gene expression patterns frequently mirrored similarity in reproductive behaviors: nesting and sneaker males showed the most striking differences in behavior and brain gene expression profiles. However, some candidate genes did not reflect the role in reproduction and social interaction revealed in past research in other species (e.g., AVT), suggesting a greater complexity to their role in this species. Further work is needed to understand brain gene expression and behavior when individuals are faced with potentially conflicting physiological demands; for example, in nesting males, there may be conflicting physiological requirements for aggression and cooperation. Understanding the management of potential trade-offs may potentially illuminate the evolution of the alternative tactics in this species. Future research will also

examine brain region-specific variation in expression to better understand the role of candidate genes identified here and in studies on other species, and also examine how gene expression correlates with behavior within a reproductive phenotype.

Finally, there is increasing attention to how individual phenotype is influenced by genotype and gene expression of others in the social environment. For example, differential expression of the 'queen mandibular pheromone' (QMP) by queen bees directly controls the variation in worker behavior (including social behavior; Grozinger et al. 2003). Similarly, examination of the *Gp-9* genotypes in the fire ant *Solenopsis invicta* revealed that worker gene expression was less related to their individual genotype than it was to the genotypic composition of the colony (Wang et al. 2008). Similar examinations in vertebrate species such as *Symphodus ocellatus* will reveal the influence of individual genotype and phenotype on gene expression on others in the social environment, and allow us to better understand the major contributing factors to individual variation.

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Supporting Information

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

Table S1: A summary, grouped by apparent function, of annotated genes differently expressed with a significance of p < 0.01 between at least two phenotypes compared.

Table S2: Predicted and observed patterns of expression for a subset of candidate genes that with directional predictions (Aubin-Horth *et al*. 2005a,b, 2007; Renn *et al*. 2008; Schumer *et al*. 2011), and differential expression between at least two phenotypes.