Female Mimicry by Sneaker Males Has a Transcriptomic Signature in Both the Brain and the Gonad in a Sex-Changing Fish

Erica V. Todd,*[,1] Hui Liu,[1] Melissa S. Lamm,[2] Jodi T. Thomas,[1] Kim Rutherford,[1] Kelly C. Thompson,[2] John R. Godwin,[2] and Neil J. Gemmell[1]

[1]Department of Anatomy, University of Otago, Dunedin, New Zealand

[2]Department of Biological Sciences and WM Keck Center for Behavioral Biology, North Carolina State University, Raleigh, NC

***Corresponding author:** E-mail: ericavtodd@gmail.com.

Associate editor: Yoko Satta

Abstract

Phenotypic plasticity represents an elegant adaptive response of individuals to a change in their environment. Bluehead wrasses (*Thalassoma bifasciatum*) exhibit astonishing sexual plasticity, including female-to-male sex change and discrete male morphs that differ strikingly in behavior, morphology, and gonadal investment. Using RNA-seq transcriptome profiling, we examined the genes and physiological pathways underlying flexible behavioral and gonadal differences among female, dominant (bourgeois) male, and female-mimic (sneaker) male blueheads. For the first time in any organism, we find that female mimicry by sneaker males has a transcriptional signature in both the brain and the gonad. Sneaker males shared striking similarity in neural gene expression with females, supporting the idea that males with alternative reproductive phenotypes have "female-like brains." Sneaker males also overexpressed neuroplasticity genes, suggesting that their opportunistic reproductive strategy requires a heightened capacity for neuroplasticity. Bourgeois males overexpressed genes associated with socio-sexual behaviors (e.g., *isotocin*), but also neuroprotective genes and biomarkers of oxidative stress and aging, indicating a hitherto unexplored cost to these males of attaining the reproductively privileged position at the top of the social hierarchy. Our novel comparison of testicular transcriptomes in a fish with male sexual polymorphism associates greater gonadal investment by sneaker males with overexpression of genes involved in cell proliferation and sperm quality control. We propose that morphological female-mimicry by sneaker male teleosts entails pervasive downregulation of androgenesis genes, consistent with low androgen production in males lacking well-developed secondary sexual characters.

Key words: **developmental plasticity, alternative male reproductive strategies, transcriptomics, phenotypic plasticity.**

Downloaded from https://academic.oup.com/mbe/article/35/1/225/4604... by guest on 28 April 2025

Introduction

Phenotypic plasticity in reproductive strategy as a response to environmental variation is widespread in nature. Such plasticity has clear implications for individual fitness while driving adaptive novelty and biological diversification at evolutionary scales (West-Eberhard 2003; Oliveira et al. 2008). Especially striking is the behavioral and sexual plasticity of many fishes, which includes functional sex change (female-to-male or male-to-female) and male intrasexual polymorphism, where territorial "bourgeois" and female-mimic "sneaker" males may arise from distinct developmental trajectories and differ strikingly in their behavioral ecology, external morphology, and gonadal investment (Taborsky 1998). However, the genomic and mechanistic basis of this demonstrable interplay between genes and environment remains elusive for most systems (Roff 2007; Aubin-Horth and Renn 2009; Feldmeyer et al. 2014).

Sexually plastic fishes offer excellent models for exploring the mechanistic basis of plastic development. Sexual transitions in fishes involve radical, often rapid, alterations in behavior, external morphology, and gonadal anatomy (Nakamura et al. 1989; Munday, Buston, et al. 2006; Sadovy de Mitcheson and Liu 2008; Godwin 2010; Todd, Liu, et al. 2016) in response to physiological (e.g., body size) and/or environmental (e.g., social) cues of future reproductive success (Ghiselin 1969; Warner 1988; Gross 1996; Taborsky and Brockmann 2010). However, the molecular mechanisms underpinning the amazing sexual plasticity of fishes remain largely unknown. Especially little is known of how the genome and environment intersect to trigger phenotypic transitions, or what expression states maintain plastic reproductive traits once an environmental cue has lapsed.

Bluehead wrasses (*Thalassoma bifasciatum*, Labridae) exhibit both female-to-male sex change and male sexual polymorphism (i.e., diandric protogyny). These small Caribbean reef fish live in social groups of one or a few dominant terminal-phase (TP) males and many females, plus a few initial-phase (IP) males (fig. 1A) (Warner and Swearer 1991; Munday, White, et al. 2006). Females and IP males mature directly from juveniles, are nonterritorial, and can each

© The Author 2017. Published by Oxford University Press on behalf of the Society for Molecular Biology and Evolution.
All rights reserved. For permissions, please e-mail: journals.permissions@oup.com

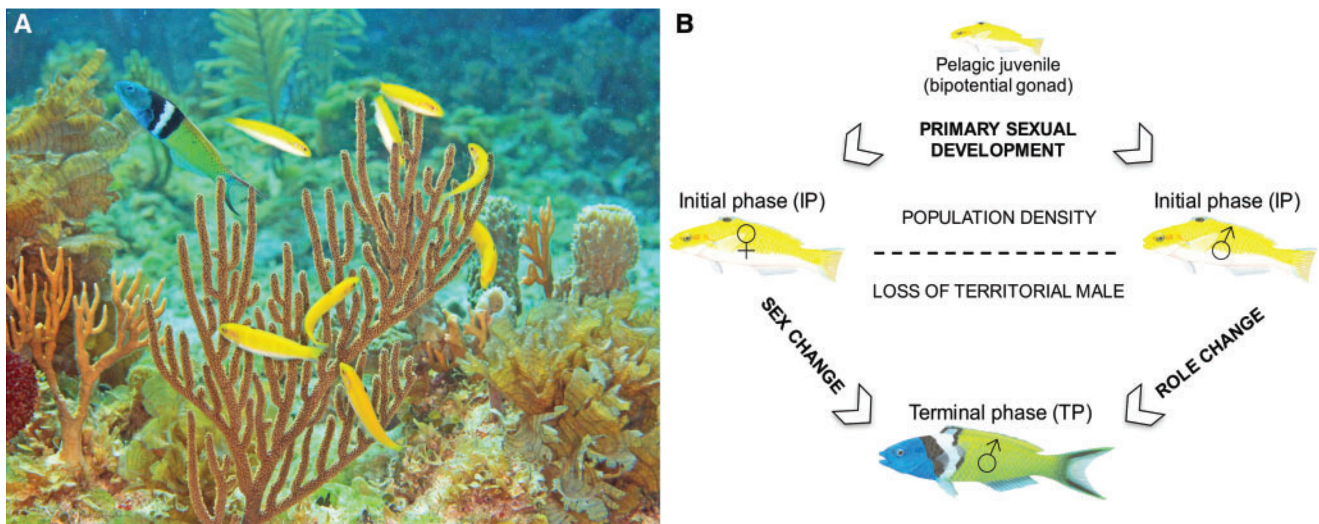

FIG. 1. Social structure and mating system of the bluehead wrasse, *Thalassoma bifasciatum*. (*A*) Distinctively colored terminal-phase (TP) males (top left) aggressively defend mating territories where they court groups of smaller females, which may also include cryptic "sneaker" males. Image credit: Kevin Bryant. (*B*) Sexual development is socially controlled. Pelagic juveniles settling on a home reef become either initial phase (IP) females or males. IP male development is rare except on larger reefs (>200 individuals), where up to 50% of juveniles become IP males (Warner 1984), presumably because TP males less-successfully monopolise mating opportunities at high population densities. Later in life and at a larger relative size, both IP phenotypes can become a terminal-phase (TP) male to assume a vacant dominant position in the social hierarchy (Warner and Swearer 1991).

become a brightly colored TP male later in life at a larger size and under appropriate social conditions (fig. 1*B*). Territorial TP males have primary access to females and focus considerable reproductive efforts on territorial defence, courtship, and distinctive coloration. By contrast, IP males mimic females in appearance and behavior and employ a range of subversive mating tactics to gain fertilizations that include sneaking (invading TP male territories to spawn with resident females), streaking (opportunistically releasing sperm as TP males pair spawn with females), and group-spawning (5–20 IP males with a single female) (Warner and Robertson 1978). Therefore, IP males almost always spawn under conditions of sperm competition and, in response, show significantly higher gonadal investment than TP males, both in terms of relative testis volume (3- to 4-fold larger) and sperm concentration (60% higher per spawn) (Warner and Robertson 1978; Taborsky 1998; Schärer and Robertson 1999).

Major differences in mating strategy, sexual ornamentation, and gonadal investment, as seen between TP and IP male blueheads, are typical of male sexual polymorphism across the animal kingdom (Taborsky 2001; Taborsky and Brockmann 2010). However, the key genes and biological systems that underlie these differences are not yet well-studied in any species. Transcriptomic studies in fishes have focused exclusively on the brain and suggest that reproductive behavior may be more important than gonadal sex in determining similarities in neural gene expression (Fraser et al. 2014; Schunter et al. 2014; Nugent et al. 2016; Partridge et al. 2016). Studies in other taxa are likewise limited to either single-organ (e.g., testes of squid producing dimorphic spermatozoa, Yoshida et al. 2014) or whole-body scales (e.g., "fighter" vs. "scrambler" male mites, Stuglik et al. 2014). Still uninvestigated at a transcriptional level are the striking differences in gonadal investment (higher in sneaker males), and secondary sexual characters and androgen production (higher in bourgeois males), that typify male sexual polymorphism in fishes and other animals (e.g., Sinervo et al. 2000), and which have a likely basis in the testis. For example, secondary sexual character development in bourgeois male teleosts is linked to high levels of the main teleost androgen 11-ketotestosterone (11KT), typically undetectable in females and female-mimic males (Cardwell and Liley 1991; Hourigan et al. 1991). No study has yet examined the genomic basis of behavioral and sexual plasticity in a species where both male and female development is plastic, that is, exhibiting both sex-change and male sexual polymorphism.

Bluehead wrasses are one of the best-studied models for understanding the social control, selective advantage, and neuroendocrine regulation of sexual plasticity (Warner 1975, 1984; Charnov 1982; Perry and Grober 2003; Lamm et al. 2015; Liu et al. 2017), and present an ideal system in which to study its genomic basis. We therefore used RNA sequencing (RNA-seq) to examine the transcriptome-wide expression states that define behavioral and gonadal sex differences among adult female, IP male, and TP male blueheads. Specifically, we sought to determine transcriptional signatures of male sexual polymorphism in this species to address the following questions: 1) does behavioral mimicry of females by IP males involve adoption of a feminized neural expression profile, 2) what are the key genes, in both brain and gonad, showing expression differences consistent with alternative mating strategies between territorial TP males and sneaker IP males, and 3) what key genes and functions contribute to differences in sperm production (higher in IP males), and secondary sexual characters and androgenesis (higher in TP males), among alternative male phenotypes?

Downloaded from https://academic.oup.com/mbe/article/35/1/225/4607869 by guest on 28 April 2025

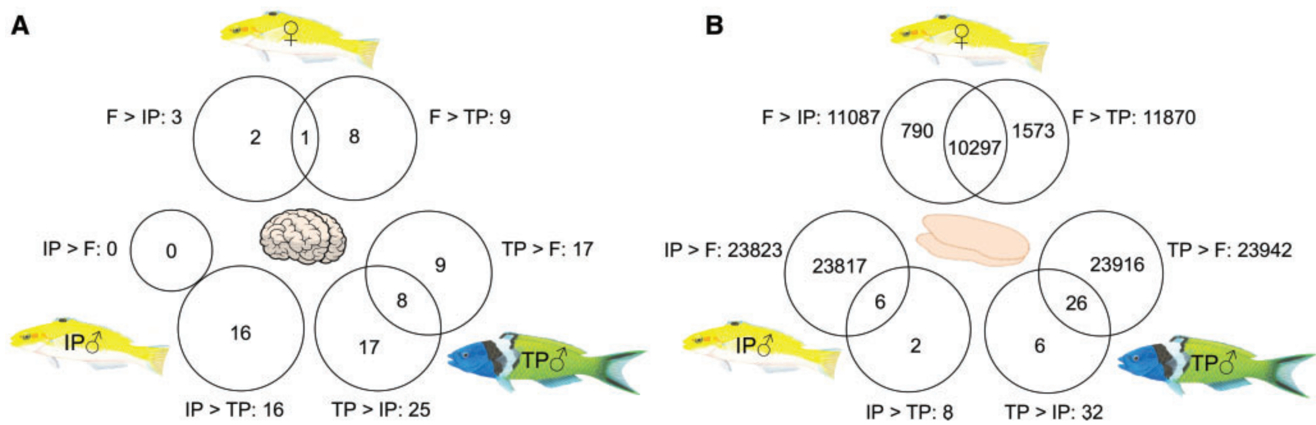

Fig. 2. Numbers of genes differentially expressed in (*A*) brain and (*B*) gonad among sexual phenotypes of the bluehead wrasse. For each phenotype, ellipses indicate the proportion of genes upregulated uniquely against one other phenotype, or both other phenotypes (overlap). Values indicate genes significant at FDR-*P* < 0.05, and in gonad only >log2-fold change. Annotation details for genes (extended list at FDR-*P* < 0.1) are shown in table 1 (brain) and table 2 (testis). F, female; TP, terminal-phase male; IP, initial-phase male.

Results

Gene Expression Profiling by RNA Sequencing

To identify key genes and biological functions associated with behavioral and gonadal differences among plastic sexual phenotypes in the bluehead wrasse, we analyzed RNA sequencing (RNA-seq) data from forebrain and gonad of adult females, TP (bourgeois) males, and IP (sneaker) males (supplementary table S1, Supplemenatry Material online). We assembled a de novo transcriptome to provide a reference for expression quantification, which after redundancy removal contained 241,597 transcripts and 190,151 "genes" (supplementary table S2, Supplemenatry Material online). To identify global and gene-specific expression differences among phenotypes, we performed sample clustering and differential expression analyses. We investigated the functional relevance of genes differentially upregulated in each phenotype using Gene Ontology (GO) and KEGG pathway analyses.

Neural Expression Profiles Reflect Differences in Territorial versus Nonterritorial Behaviors

In forebrain, global expression differences among phenotypes were closely associated with differences in territorial behavior. The greatest number of differentially expressed (DE) genes was found between the two male morphs (41 DE genes FDR-*P* < 0.05, 65 DE genes FDR-*P* < 0.1), whereas IP males and females were highly similar (3 DE genes, FDR-*P* < 0.05 and 0.1) (fig. 2A) (table 1).

Genes differentially upregulated in TP males in comparisons against both IP males and females encode enzymes within the androgen (*hsd17b3*) and taurine (*cdo1*) production pathways, plus the hormone isotocin (*it*), which is linked to social dominance in teleosts (see Discussion). Other genes that were TP-male biased in forebrain against both nonterritorial phenotypes include those involved in adaptive (*MHC-1 ZBA*) and innate (*c7a*) immunity, regulators of actin cytoskeleton development (*rhpn2*, *rassf7a*), and transcriptional regulators of cellular differentiation (*creg1*).

When compared with IP males specifically, TP males showed significant upregulation of additional immunity genes (*cd9a*, *b2ml*), plus genes linked to neuroprotection from oxidative stress (*aldh2.2*, *foxo1a*) including ketogenesis (*hmgcll1*). Other genes upregulated in TP males relative to IP males relate to neuronal development, synaptic signaling, and behavior (*rem2*, *amer2*, *ppp3ca*, *gpsm1b*, *rgs5a*), including retinoid signaling (*cyp27c1*). Genes significantly upregulated in TP males against females alone include further immune response (*egfra*, *ik*) and neurogenesis (*swap70b*) genes, and regulators of neuroendocrine secretion (*pyya*, *proSAAS*).

Genes significantly upregulated in IP males against TP males include many that are functionally involved in processes relating to brain development and neurogenesis, including cell fate determination (e.g., *sox11a*, *cux2b*), lipid, and glucose metabolism (e.g., *abhd3*). Many are directly implicated in synaptic plasticity related to memory and learning (e.g., *shisa7b*, *HDAC1*, *atf4a*). Overexpression of protein kinase C (*prkcg*) in conjunction with numerous insulin signaling genes is significant, because insulin and prkc interact in numerous biochemical pathways underlying memory storage and learning (Nelson et al. 2008). No genes were significantly upregulated in IP males against females, highlighting the similarity in neural expression between these behaviorally more similar phenotypes.

Only three genes were differentially upregulated in females against IP males: brain aromatase (*cyp19a1b*) responsible for estrogen synthesis, a protein homologous with complement component C4, and an unannotated sequence significantly female-biased in comparisons against both male phenotypes. Brain aromatase was also significantly upregulated in females against TP males (although at FDR-*P* < 0.1), and in TP males against IP males. Against TP males, females significantly upregulated genes involved in transcription regulation and pre-mRNA processing (*scaf4*, *fosl2*), as well as neuronal connectivity, including gap junction formation (*cx35b*) but also axonal growth inhibition (*rtn4rl2*, *fign*).

Downloaded from https://academic.oup.com/mbe/article/35/1/225/4607869 by guest on 28 April 2025

Table 1. Genes Showing Significant Upregulation in Forebrain among Sexual Phenotypes of Bluehead Wrasse.

Symbol	Gene Name	Putative Function in Forebrain	Upregulated against (FDR-*P*)
Female biased			
Steroidogenesis			
cyp19a1b	Brain aromatase	Estrogen biosynthesis	IP (3.39E-10), TP (0.074)
Immunity			
c4	Complement component C4-like	Innate immunity	IP (0.010)
Neuronal connectivity			
si: ch73-233f7.5	Novel protocadherin protein	Cell adhesion and neuronal connectivity	TP (0.008)
rtn4rl2a	Reticulon 4 receptor-like 2 a	Regulation of axonal growth	TP (0.021)
rapgef5a	Rap guanine nucleotide exchange factor (GEF) 5a	Signal transduction	TP (0.040)
fign	Fidgetin	Microtubule severing protein, trims unstable neuronal connections	TP (0.046)
cx35b	Connexin 35b	Constituent of gap junctions	TP (0.074)
Other			
scaf4	SR-related CTD-associated factor 4a	Regulates transcription and mRNA splicing	TP (0.001)
fosl2	Fos-like antigen 2	Transcription regulation	TP (0.008)
si: ch73-390p7.2	Uncharacterized protein—At5g50100, mitochondrial-like	Unknown. Upregulated in parental vs. satellite males of bluegill sunfish (Partridge et al. 2016)	TP (0.090)
Unannotated genes: *n* =1 upregulated against IP males, *n* =4 upregulated against TP males			
TP male biased			
Steroidogenesis			
hsd17b3	Hydroxysteroid (17-beta) dehydrogenase 3	Androgen biosynthesis	F (1.73E-06), IP (0.008)
cyp19a1b	Brain aromatase	Estrogen biosynthesis	IP (0.033)
pyya	Peptide Yya	Regulates gonadotropin release	F (0.048)
proSAAS	Proprotein convertase subtilisin	Neuroendocrine secretion	F (0.089)
Neuroprotection and immunity			
c7a	Complement component 7a	Innate immunity	F (0.001), IP (2.27E-05)
si: dkey-51d8.3	Novel protein containing NLR C nacht domain	Regulates MHC gene expression	F (1.15E-05), IP (1.6E-04)
cdo1	Cysteine dioxygenase, type I	Biosynthesis of taurine, a powerful antioxidant and cytoprotectant with demonstrated antiaging effects in the brain and testis	F (3.57E-07), IP (5.80E-05)
mhc-1 zba	Major histocompatibility complex class I ZBA	Adaptive immunity	F (0.010), IP (0.045)
b2ml	Beta-2-microglobulin, like	Component of MHC-I immune complex, negatively regulates neurogenesis. Linked to cognitive decline in mammals	IP (0.007)
Ik	IK cytokine	Regulation of immune response	F (0.014)
cd9a	CD9 molecule (tetraspanin 29)	Mediates MHC adaptive immunity	IP (0.045)
egfra	Epidermal growth factor receptor a	Innate immunity	F (0.095)
aldh2.2	Aldehyde dehydrogenase 2 family	Metabolize toxic aldehydes to abrogate oxidative stress	IP (0.016)
hmgcll1	3-hydroxymethyl-3-methylglutaryl-CoA lyase-like 1	Ketogenesis during metabolic stress	IP (0.083)
sqstm1	Sequestosome 1	Protein turnover, oxidative stress response	IP (0.035)
foxo1a	Forkhead box class O1 a	Transcription factor acting in insulin signaling. Stimulates expression of antioxidant proteins. Represses txnip expression to regulate redox balance	IP (0.020)
npc2	Niemann-Pick disease, type C2	Cholesterol mobilization in control of lipid balance	IP (0.020)
txnipa	Thioredoxin interacting protein a	Inhibits oxidative stress response by reducing thioredoxin activity	IP (0.030)
rem2	RAS (RAD and GEM)-like GTP binding 2	Regulates insulin secretion. In addition, synapse development	IP (0.045)
ak5	Adenylate kinase 5	Cellular energy homeostasis	IP (0.045)
Socio-sexual behavior			
It	Isotocin	Socio-sexual behavior	F (2.92E-05), IP (5.76E-05)
rgs5a	Regulator of G-protein signaling 5a	Termination of G-protein signaling. May play a role in aggressive behaviors	IP (0.089)
gpsm1b	G-protein signaling modulator 1b	G-protein signaling. May regulate AVT signaling	IP (0.041)

(continued)

Downloaded from https://academic.oup.com/mbe/article/35/1/225/4607869 by guest on 28 April 2025

Table 1. Continued

Symbol	Gene Name	Putative Function in Forebrain	Upregulated against (FDR-*P*)
reep5	Receptor accessory protein 5	G-protein signaling	IP (*0.047*)
cyp27c1	Cytochrome P450, family 27, subfamily C, polypeptide 1	Retinoid signaling	IP (*0.001*)
RARRES3	Retinoic acid receptor responder 3	Retinoid signaling	IP (*0.005*)
ptgdsb.1	Prostaglandin D2 synthase b, tandem duplicate 1	Retinoid signaling and sexual behavior	IP (0.099)
Neuronal growth and signaling			
creg1	Cellular repressor of E1A-stimulated genes 1	Transcriptional control of cell growth and differentiation	F (*0.001*), IP (*0.040*)
rassf7a	Ras association (RalGDS/AF-6) domain family (N-terminal) member 7a	Regulates actin cytoskeleton organization	F (0.087), IP (*0.040*)
rhpn2	Rhophilin, Rho GTPase binding protein 2	Regulates actin cytoskeleton organization	F (*0.011*), IP (*0.049*)
swap70b	SWAP switching B-cell complex 70b subunit 70b	Regulates actin cytoskeleton organization	F (*0.016*)
tb4x	Thymosin, beta 4×	Actin sequestration, neuronal growth	IP (*0.023*)
amer2	APC membrane recruitment protein 2	Negative regulation of Wnt/b-catenin signaling, involved in synapse development	IP (0.056)
slc13a4	Solute carrier family 13 (sodium/sulfate symporter), member 4	Membrane transport of sodium ions	IP (0.068)
ppp3ca	Protein phosphatase 3, catalytic subunit, alpha isozyme	Catalytic subunit of calcineurin, a protein phosphatase that constrains synaptic transmission	IP (0.068)
Other			
hbbe1.1	Hemoglobin beta embryonic-1.1	Oxygen transport	IP (0.075)
ctsa	Cathepsin A	Protein degradation	IP (*0.030*)
Unannotated genes: *n* =7 upregulated against females, *n* =3 upregulated against IP males			
IP male biased			
Synaptic signaling, memory, and learning			
si: ch73-233f7.5	Novel protocadherin protein	Cell adhesion and neuronal connectivity	TP (*5.68E-05*)
inpp5l	Inositol polyphosphate-5-phosphatase L	Insulin signaling. Establishment and maintenance of neuronal networks	TP (*0.008*)
slc25a1a	Solute carrier family 25 (mitochondrial carrier; citrate transporter), member 1a	Insulin secretion and synapse formation	TP (*0.011*)
shisa7b	Shisa family member 7	Short-term synaptic plasticity	TP (*0.018*)
ppp1r14c	Protein phosphatase 1, regulatory (inhibitor) subunit 14C	Inhibits protein phosphatase, constraining synaptic transmission	TP (*0.023*)
hdac1	Histone deacetylase 1	Epigenetic repression of learning and memory-associated gene transcription	TP (*0.045*)
gng3	Guanine nucleotide binding protein (G protein), gamma 3	Encodes specificity-determining subunit, G-protein signaling	TP (0.056)
prkcg	Protein kinase C, gamma	Synaptic potentiation	TP (0.062)
ctdspl3	CTD (carboxy-terminal domain, RNA polymerase II, polypeptide A) small phosphatase like 3	Protein phosphatase activity. In addition, neuronal gene silencing	TP (0.062)
atf4a	Activating transcription factor 4a	Represses CREB-mediated transcription of gene expression required for memory formation	TP (0.068)
si: dkey-183j2.10	Probable glutamate receptor U1-like	Mediates postsynaptic excitation	TP (0.069)
Neurogenesis			
tmcc3	Transmembrane and coiled-coil domain family 3	Neurogenesis	TP (*0.033*)
sox11a	SRY (sex determining region Y)-box 11a	Transcription factor regulating neural progenitor cell proliferation and differentiation	TP (*0.035*)
mab21l1	Mab-21-like 1	Cell fate determination	TP (*0.035*)
cux2b	Cut-like homeobox 2b	Transcription factor regulating neural progenitor cell development	TP (*0.049*)
inab	Internexin neuronal intermediate filament protein, alpha b	Structural protein involved in neuronal development	TP (0.067)
rbp1	Retinol binding protein 1b, cellular	Retinol transport in developing neuronal structures	TP (0.069)
tmpob	Thymopoietin b	Regulates structural organization of nuclear lamina, cell cycle	TP (0.069)
gmcl1	Germ cell-less, spermatogenesis associated 1	Nuclear envelope protein involved in cell differentiation	TP (0.099)

(continued)

Downloaded from https://academic.oup.com/mbe/article/35/1/225/4607869 by guest on 28 April 2025

Table 1. Continued

Symbol	Gene Name	Putative Function in Forebrain	Upregulated against (FDR-*P*)
Other			
scaf4	SR-related CTD-associated factor 4a	Regulates transcription and mRNA splicing	TP (*0.007*)
mpi	Mannose phosphate isomerase	Mannose–fructose metabolism upstream of glycolysis	TP (*0.030*)
abhd3	Abhydrolase domain containing 3	Phospholipid biosynthesis and metabolism	TP (*0.045*)
fabp2	Fatty acid binding protein 2, intestinal	Fatty acid metabolism and homeostasis	TP (*0.047*)
ahsa1b	AHA1, activator of heat shock protein ATPase homolog 1b	Protein regulation during stress response	TP (0.078)
Unannotated genes: n =0 upregulated against females, n =7 upregulated against TP males			

Note.—Annotated genes significant at FDR-$P < 0.1$ are shown. Values significant at FDR-$P < 0.05$ are italicized. FDR-P, significance value adjusted for false discovery rate; F, female; TP, terminal-phase male; IP, initial-phase male.

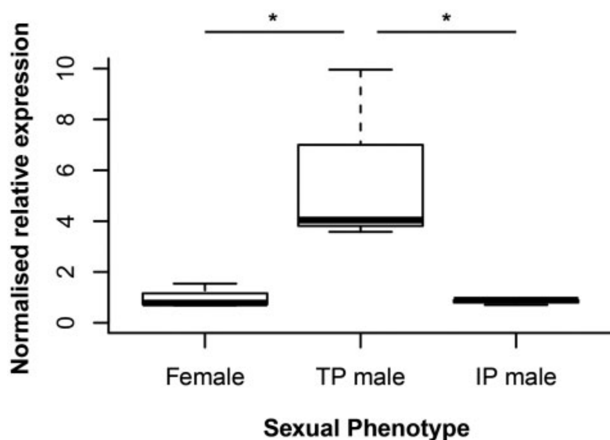

Fig. 3. *Isotocin* expression measured by qPCR. Normalized relative mRNA levels of *isotocin* in forebrain of female, TP male and IP male bluehead wrasse ($N = 3$ per phenotype). Data are presented as boxplots with the middle bold line representing the median, edges representing the upper and lower quartiles and vertical dotted lines ending in a horizontal bar representing the minimum and maximum values. *$P < 0.01$.

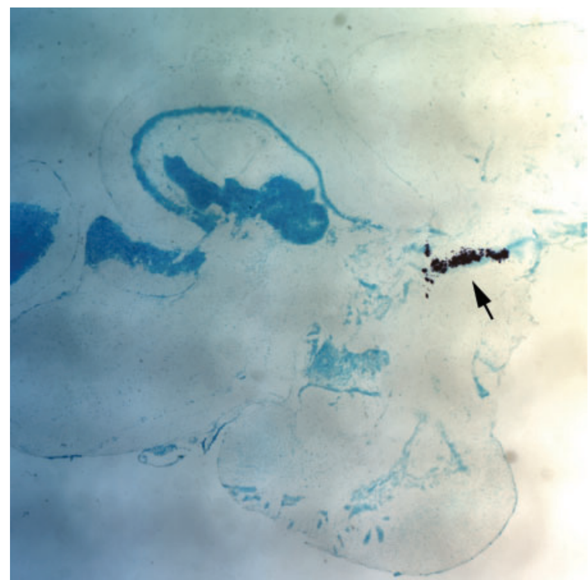

Fig. 4. In situ hybridization results for *isotocin*. Coronal section of a TP male bluehead wrasse brain indicating localization of hybridization signal for *isotocin* (dark area indicated by arrow) within the preoptic area.

Isotocin Expression Is Localized within the Preoptic Area

To technically validate the phenotype-specific expression pattern for *isotocin* observed in the RNA-seq data, we performed quantitative PCR (qPCR). qPCR data supported a significant effect of sexual phenotype on *isotocin* expression ($F2, 6 = 18.28$, $P < 0.01$) (fig. 3). Normalized relative *isotocin* mRNA levels were significantly higher in forebrain of TP males compared with IP males ($P < 0.01$) and females ($P < 0.01$). To identify the spatial pattern of *isotocin* expression within the forebrain, we also performed in situ hybridization (ISH) in a TP male. We found that *isotocin*-expressing neurons are localized within the gigantocellular, magnocellular, and parvocellular preoptic area of the hypothalamus (fig. 4).

Gonadal Gene Expression Is Strongly Sex-Biased

There were large, expected, sex-specific expression differences observed in the gonad. Tens of thousands of genes were significantly DE in comparisons between female ovary and either TP or IP male testis (>log2 FC and FDR-$P < 0.05$) (fig. 2*B*), representing ∼18% of all contigs in the reference assembly. Because there was substantial overlap in DE gene lists between IP or TP male testes and ovary (>76% of DE genes in common), functional enrichment analyses were performed for ovary and testis (see Materials and Methods).

Functional enrichment analysis of genes upregulated in ovary (2,644 unique Zebrafish genes) identified gene groups involved in egg–sperm interactions, mitochondrial and ribosomal structure and function, and RNA processing (15 significantly enriched GO terms, supplementary table S3, Supplemenatry Material online). The top enriched KEGG pathway in ovary was ribosome biogenesis (24 genes, supplementary table S4, Supplemenatry Material online).

Functional enrichment analysis of genes upregulated in testis (4,793 unique Zebrafish genes) identified gene groups

Downloaded from https://academic.oup.com/mbe/article/35/1/225/4607869 by guest on 28 April 2025

primarily involved in cilia/flagella biogenesis, plus other processes critical to sperm motility including calcium ion binding and ion channel activity (38 significantly enriched GO terms, supplementary table S3, Supplemenatry Material online). KEGG pathways significantly enriched in testis were relevant to spermatogenesis and included focal adhesion, phosphatydylinositol signaling system, and ECM-receptor and cytokine–cytokine receptor interaction (supplementary table S4, Supplemenatry Material online).

Testicular Expression Differences Reflect Differences in Androgen Production and Gonadal Investment

Forty genes were differentially regulated between TP and IP male testes: 32 upregulated in TP males and 8 upregulated in IP males (FDR-$P < 0.05$) (table 2). TP male-biased genes have functions in steroidogenesis and spermatogenesis. These include genes encoding the key enzymes involved in androgen biosynthesis (*cy17a1*, *cyp17a2*, *hsd3b1*), specifically 11KT conversion (*cyp11c1*, *hsd11b2*), plus the key steroidogenesis genes *star* and the luteinizing hormone/choriogonadotropin receptor (*lhcgr*, significant at FDR-$P < 0.1$). Other TP male-biased genes encode structural or membrane proteins functionally relevant to sperm maturation and egg–sperm interactions (e.g., *trpc6b*, *tspan35*, *vwde*, *acta2*). There were too few genes significantly DE between TP and IP male testes for statistically powerful enrichment analysis, although GO terms associated with TP male-biased genes relate primarily to steroid biosynthetic processes, lipid transport, calcium ion signaling, and membrane cellular components.

Genes upregulated in IP male testes (8 FDR-$P < 0.05$; 14 FDR-$P < 0.1$) encode proteins and transcription factors with roles in sperm quality (*kynu*) and cell proliferation processes, including ribosome biogenesis (*nsa2*, *surf6*, *zbed1*), DNA stability (*hist2h3ca1*), and DNA repair (*faap24*), plus several uncharacterized membrane proteins.

Discussion

Phenotypic plasticity is pervasive in nature, yet the mechanisms that regulate phenotypic transitions and maintain plastic traits remain elusive in most species. For many social fishes, switching among sexual phenotypes enables optimizing individual lifetime reproductive success in a dynamic social environment (Warner 1988; Munday, Buston, et al. 2006). Using whole-transcriptome gene expression profiling, we identify key genes, biological functions, and physiological systems associated with behavioral and gonadal sex differences among plastic sexual phenotypes in the bluehead wrasse, a model sex-changing fish with male sexual polymorphism. For the first time in any organism, we find that female mimicry by sneaker males has a transcriptional signature in both the brain and the gonad.

Neural expression differences closely reflected behavioral differences between territorial (TP males) and nonterritorial (females, IP males) phenotypes (fig. 2A) of bluehead wrasse. These data add to growing transcriptomic evidence supporting the idea that female-mimic males have “female-like” brains (Godwin 2010), both in fishes (Goodson and Bass 2000; Aubin-Horth et al. 2005; Renn et al. 2008; Fraser et al. 2014; Schunter et al. 2014; Partridge et al. 2016) and other animals (Stuglik et al. 2014). Therefore, feminized neural expression profiles may be a general neurogenomic feature of sneaker males that employ female behavioral mimicry as a reproductive tactic. Our data also reveal candidate genes for social dominance and suggest specific neuroplasticity processes may be active in each phenotype.

We present the first comparison of testicular transcriptomes between female-mimic and bourgeois males that invest differently in sperm production and secondary sexual characters. We propose a molecular basis for morphological female-mimicry in sneaker male teleosts that involves pervasive downregulation of androgenesis genes, consistent with low androgen (11-ketotestosterone) production in males lacking well-developed secondary sexual characters (Borg 1994). Our data also identify candidate genes overexpressed in sneaker males that may support greater gonadal investment and, potentially, chemical crypsis.

Isotocin as a Regulator of Teleost Socio-Sexual Behavior

Isotocin, the teleost homologue of *oxytocin* in mammals, showed an expression pattern consistent with mediating social dominance behaviors in bluehead wrasses. *Isotocin* expression was significantly TP-male biased, a pattern we confirmed with qPCR. Our ISH data localize *isotocin*-expressing neurons within the preoptic area of the hypothalamus (POA), a primary site for socio-sexual behavioral integration in vertebrates (O’Connell and Hofmann 2012). Isotocin is emerging as a major neuromediator of teleost sociality and social dominance hierarchies in particular (Almeida et al. 2012; Godwin and Thompson 2012; Lema et al. 2015). Experiments in cooperatively breeding cichlids (*Neolamprologus pulcher*) suggest that isotocin mediates social decision-making by enhancing sensitivity to social stimuli (Reddon et al. 2014): fish treated with exogenous isotocin were more responsive to social challenges from dominant individuals, and were more sensitive to the size of their opponents in aggressive interactions (Reddon et al. 2012). Reproductive success of TP males depends critically on their ability to establish and defend a mating territory (Warner and Schultz 1992). Effectively interpreting social cues will be critical in contests with competing TP males, and for courtship with females. Another possibility is that isotocin modulates anxiety, as oxytocin does in mammals (Neumann 2008). Increased isotocin and reduced anxiety in TP males may support territorial and courtship behaviors specific to this phenotype, compared with more “cautious” females and IP males.

Isotocin may also mediate behavioral transitions associated with socially cued sex change. Isotocin was one of very few neural genes found upregulated during female-to-TP male sex change in RNA-seq and qPCR time-series data from bluehead wrasse (Liu H, Todd EV, Lamm MS, Thomas JT, Godwin JR, Gemmell NJ, unpublished data). Isotocin was also differentially regulated across sex change in blue-banded goby (*Lythrypnus dalli*), a bidirectional sex-changer, although

Downloaded from https://academic.oup.com/mbe/article/35/1/225/4607869 by guest on 28 April 2025

Table 2. Genes Differentially Regulated between Testes of TP and IP Male Bluehead Wrasse.

Symbol	Gene Name	Putative Function in Testis	FDR-*P*
TP male biased			
Steroidogenesis			
hsd11b2	Hydroxysteroid (11-beta) dehydrogenase 2	11-ketotestosterone conversion	1.79E-06
cyp17a1	Cytochrome P450, family 17, subfamily A, polypeptide 1	Androgen biosynthesis	1.30E-05
star	Steroidogenic acute regulatory protein	Cholesterol transport to initiate steroidogenesis in response to LH surges	0.001
cyp17a2	Cytochrome P450, family 17, subfamily A, polypeptide 2	Androgen biosynthesis	0.001
hsd3b1	Hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 1	Androgen biosynthesis	0.008
cyp11c1	Cytochrome P450, family 11, subfamily C, polypeptide 1	11-ketotestosterone conversion	0.008
ugt5e1	UDP glucuronosyltransferase 5 family, polypeptide E1	Steroid metabolism	0.028
lhcgr	Luteinizing hormone/choriogonadotropin receptor	LH signaling to initiate steroidogenesis	0.072
Sperm maturation, sperm–egg interactions			
CABZ01041812.1	Putative ferric-chelate reductase 1/mucin-like	Ion transport	0.002
trpc6b	Transient receptor potential cation channel, subfamily C, member 6b	Transmembrane ion channel important for sperm function	0.005
tspan35	Tetraspanin 35	Transmembrane protein involved in cell motility and fertilization	0.024
vwde	von Willebrand factor D and EGF domains	Calcium ion binding, cell adhesion, and cell–cell interaction	0.029
acta2	Actin, alpha 2, smooth muscle, aorta	Cell motility	0.057
Other			
tnnt2e	Troponin T2e, cardiac	Calcium signaling	1.30E-05
METTL21C (1 of many)	METTL21C—Protein-lysine methyltransferase	Protein regulation	2.39E-04
sin3ab	SIN3 transcription regulator family member Ab	Transcriptional repressor	0.003
s100a10b	S100 calcium binding protein A10b	Calcium binding protein	0.007
–	Predicted receptor-type tyrosine-protein phosphatase N2 (UniRef90_UPI00054BEDEC)	Transmembrane receptor	0.029
zgc: 91890	Predicted riboflavin transporter 2-like	Riboflavin transport	0.029
mal	Mal, T-cell differentiation protein	Lipid-binding transmembrane protein/T-cell maturation	0.037
sez6b	Seizure related 6 homolog b	Cell–cell recognition	0.038
–	Predicted transcriptional regulator Myc-B-like isoform X1	Regulation of cell proliferation	0.039
loxa	Lysyl oxidase a	Collagen and elastin	0.040
si: dkey-266f7.1	Predicted nuclear GTPase SLIP-GC-like	Genome stability	0.040
si: ch211-202f3.3	Predicted calpain-1 catalytic subunit-like	Calcium binding/signal transduction	0.040
–	Uncharacterized intermediate filament protein (OrthoDB ENSSHAP00000001381)	Cytoskeleton	0.050
si: zfos-943e10.1	Novel GRAM domain-containing protein 3	Membrane protein	0.072
PARP9	Predicted poly [ADP-ribose] polymerase 9 isoform X1	Transferase	0.072
si: dkey-36i7.3	FXYD domain-containing ion transport regulator 12	Membrane ion channel	0.078
mal	Mal, T-cell differentiation protein	Membrane protein	0.091
Unannotated genes: $n = 12$			
IP male biased			
Ribosome biogenesis			
NSA2	NSA2 ribosome biogenesis homolog	Ribosome biogenesis, cell proliferation, cell cycle regulation	4.07E-04

(continued)

Downloaded from https://academic.oup.com/mbe/article/35/1/225/4607869 by guest on 28 April 2025

Table 2. Continued

Symbol	Gene Name	Putative Function in Testis	FDR-*P*
surf6	Surfiet 6	Nucleolar matrix protein, DNA/RNA binding, ribosome biosynthesis	0.064
ZBED1	Zinc finger BED domain-containing protein 1-like	Transcription factor regulating ribosomal protein genes and cell proliferation	0.050
DNA stability and repair			
si: dkey-23a13.21	Novel protein similar to Histone 2, H3ca1	Transcription regulation, DNA stability, and repair	*0.010*
faap24	Fanconi anaemia core complex associated protein 24	Protection against DNA damage, recruits the Fanconi anaemia core complex to damaged DNA	0.062
Sperm quality/sperm–egg interaction			
kynu	Kyureninase	Tryptophan catabolism along the kynurenine pathway. Important for sperm quality control. Implicated as a sex pheromone in salmon	*0.024*
Other			
si: dkey-11f4.16	Uncharacterized membrane protein	Membrane protein, immune response	*0.010*
si: dkey-12l12.1	Uncharacterized membrane protein	Membrane protein	*0.019*
NETO1	Neuropilin and tolloid-like protein 1	Membrane protein	0.072
DHDH (1 of many)	Dihydrodiol dehydrogenase (dimeric)	Oxidation–reduction activity	0.087
Unannotated genes: $n = 4$			

Note.—Annotated genes significant at FDR-$P < 0.1$ are shown. Values significant at FDR-$P < 0.05$ are italicized. FDR-*P*, significance value adjusted for false discovery rate; TP, terminal-phase male; IP, initial-phase male.

in the opposite direction: isotocin-immunoreactive cells were more numerous in the forebrain of females than dominant males, and decreased during female-to-male sex change (Black et al. 2004). Limited current data suggest complex and potentially polymodal effects of isotocin on teleost sociality depending on sex, dosage, timing, and social context (Thompson and Walton 2004; Braida et al. 2012; Sokołowska et al. 2013; Lema et al. 2015). Although generalizations are not yet possible, the clear role isotocin plays in regulating teleost sociality, including socially cued phenotypic transitions, warrants further investigation.

Our data also reveal other genes that merit further investigation as candidate regulators of aggression and dominance behavior in teleosts. The G-protein signaling genes *rgs5a* and *gpsm1b* are upregulated in territorial male blueheads (this study) and black-faced blennies (Schunter et al. 2014). In mammals, *rgs* genes are implicated in male aggression (Oliveira-dos-Santos et al. 2000) and *gpsm1b* mediates signaling from vasoconstrictive hormones including vasopressin (homologue of teleost vasotocin). Major neuropeptides like vasotocin, gonadotropin-releasing hormone, and kisspeptin, plus monoamine neurotransmitters norepinephrine, dopamine, and serotonin, have all been implicated in mediating sex-specific behaviors and sex-change in bluehead wrasses and other fishes (Godwin et al. 2000; Semsar et al. 2001; Grober et al. 2002; Larson et al. 2003; Semsar and Godwin 2003; Lorenzi et al. 2009; Mechaly et al. 2013) (reviewed by Godwin 2010; Godwin and Thompson 2012). Curiously, no transcriptomic studies of males with alternative mating phenotypes, including ours, have found significant phenotypic differences in expression for the relevant encoding genes. Studying expression differences at a whole-brain/forebrain level could obscure meaningful differences at individual nuclei (see Conclusions and Future Directions). Nevertheless, *gpsm1b* is a promising upstream regulator of these neuropeptide genes and may well be involved in coordinating some of the neuroplasticity seen among sexual phenotypes in fish.

IP Males Upregulate Genes Involved in Neuroplasticity, Memory, and Learning

The opportunistic mating strategy of IP males likely requires significant behavioral flexibility (group-spawning, sneaking, streaking), and we find evidence that dynamic neuroplasticity processes affecting memory and learning are active in the forebrain of these males compared with females and TP males (pair-spawning) (table 1). These include genes encoding protein kinases (*prkcg*) and phosphatases (*ppp1r14c*, *inpp5l*, *ctdspl3*) that regulate brain plasticity by strengthening and weakening the efficacy of synaptic transmission, respectively (Winder and Sweatt 2001). IP males also upregulate several transcription factors that either promote neurogenesis (e.g., *sox11* and *cux2b*, Iulianella et al. 2008; Wang et al. 2013) or negatively regulate learning- and memory-associated expression programs (*HDAC1* and *artf4a*, Lee and Silva 2009; Whittle and Singewald 2014). The balance between processes that promote or constrain neuroplasticity is believed to be the basis for learning and memory (Abbott and Nelson 2000). Earlier transcriptomic studies have also associated sneaking behavior in male teleosts with overexpression of learning- and memory-associated genes, suggesting that sneaking is the more cognitively demanding strategy (Aubin-Horth et al. 2005; Fraser et al. 2014).

Females and TP male blueheads also uniquely upregulated genes associated with neurogenesis and plasticity, suggesting that distinct neuroplasticity processes are active depending on the sexual phenotype. Females overexpressed genes

Downloaded from https://academic.oup.com/mbe/article/35/1/225/4607869 by guest on 28 April 2025

involved in the formation or remodeling of specific neuronal connections, whereas TP males overexpressed genes largely related to neuroprotection. The teleost brain is unique among vertebrates in displaying high neurogenic activity throughout life, modulated by local steroid production (reviewed by Diotel et al. 2011; Pellegrini et al. 2016). The tight link between neurogenesis and steroidogenesis in the adult teleost brain is likely important in facilitating the unique behavioral and sexual plasticity of fishes (Le Page et al. 2010), and may explain our observation of distinct neuroplasticity-related expression among sexual phenotypes of bluehead wrasse.

Its Tough at the Top—TP Males Upregulate Genes Involved in Neuroprotection and Stress

Overexpression of numerous genes related to immunity and neuroprotection (table 2) suggests that the brain of TP males may be differentially affected by oxidative stress and aging. These include β2-microglobulin (*B2M*), a component of MHC-I molecules identified as a proaging factor in the blood of humans and mice (Villeda et al. 2011; Smith et al. 2015). Neurodefensive genes upregulated in TP males include cysteine dioxygenase (*cdo1*), essential for the biosynthesis of taurine—a powerful antioxidant and cytoprotectant with demonstrated antiaging effects in the brains of mice (El Idrissi et al. 2013) and the testes of rats and fish (Yang et al. 2010a, 2015; Higuchi, Celino, Shimizu-Yamaguchi, et al. 2012; Higuchi, Celino, Tamai, et al. 2012). The brain's immune system modulates the structural remodeling processes that enable neural circuits to adapt and change with experience (Stevens et al. 2007; Elmer and McAllister 2012; Lee et al. 2014). However, oxidative stress and aging are both associated with immune hyperactivity, with detrimental effects on brain plasticity and neurogenesis (Boulanger and Shatz 2004; Yirmiya and Goshen 2011).

The defining features of bourgeois male teleosts—social dominance, higher androgen levels, and often older age—are all associated with increased oxidative stress (von Schantz et al. 1999; Alonso-Alvarez et al. 2007; Garratt and Brooks 2012; Olsson et al. 2012; Beaulieu et al. 2014; Georgiev et al. 2015a). However, dominant individuals are often also reported to have heightened antioxidant defences and a superior oxidative status (Haddad et al. 2007; Isaksson et al. 2011; Georgiev et al. 2015b). Our data not only suggest that TP male brains are more affected by age and oxidative stress but that they may compensate by upregulating neuroprotective systems.

A direct relationship between oxidative stress and social dominance still requires empirical testing in fishes. However, overexpression of genes encoding antioxidant defences (e.g., aldehyde dehydrogenases) is now demonstrated for dominant males of several species (e.g., blueheads, current study; black-faced blenny, Schunter et al. 2014; African cichlids, Renn et al. 2008). Increased oxidative load may represent an unrecognized physiological cost of attaining the reproductively privileged position of a bourgeois male at the top of the social hierarchy, relevant to better understanding the trade-offs of sexual plasticity.

Downregulation of Androgenesis May Underlie Morphological Crypsis in IP Males

Expression differences between IP and TP male testes were expected to reflect phenotype-specific differences in gonadal investment (higher in IP males) (Schärer and Robertson 1999) and production of 11-ketotestosterone (11KT) (Hourigan et al. 1991), the main teleost androgen (higher in TP males). Lack of secondary sexual characters in cryptic male teleosts is associated with low 11KT production (testosterone acts primarily as a prohormone in teleosts, Borg 1994), and we provide the first comprehensive molecular data showing there is pervasive downregulation of androgen pathway genes in a male fish with female mimicry. We observed that genes encoding the near-complete complement of enzymes within the teleost androgen biosynthesis pathway were significantly overexpressed in TP male relative to IP male testes, and also female ovaries. These included both enzymes responsible for testosterone-11KT conversion (*cyp11c1* and *hsd11b2*), plus key genes regulating steroidogenesis and lipid (steroid) signaling (table 2). 11KT is synthesized in the teleost testis and promotes male-specific functions including secondary sexual character development, reproductive behaviors and spermatogenesis (Borg 1994). Males lacking well-developed secondary sexual characters have consistently low or undetectable levels of circulating 11KT, similar to females, a feature thought to permit morphological crypsis in sneaker males (reviewed by Brantley et al. 1993; Borg 1994). Pervasive downregulation of androgen pathway genes in IP male bluehead wrasse provides a genetic basis for altered androgen production and morphological crypsis in a sneaker male with female mimicry.

Differential regulation of androgen pathway genes is likely of central importance for maintaining morphological and behavioral differences between bourgeois and cryptic males, but also for orchestrating phenotypic transitions in sexually plastic fishes. A recent time-series RNA-seq analysis across sex change in bluehead wrasse found that these same androgenesis genes are steadily upregulated in the transitioning gonad (Liu H, Todd EV, Lamm MS, Godwin JR, Gemmell NJ, unpublished data). Our data and that of Liu et al. are consistent with the steady rise in serum 11KT as TP males develop their characteristic coloration during sex or role change, in blueheads and other diandric protogynous hermaphrodites (Nakamura et al. 1989; Cardwell and Liley 1991). 11KT treatment can also readily induce secondary sexual character development in female and cryptic male blueheads (Semsar and Godwin 2004) and other fishes with male sexual polymorphism (Oliveira, Canario, et al. 2001; Oliveira, Carneiro, et al. 2001).

Without high androgen production, the factors that support testicular function in cryptic males, whose testes may be 2- to 3-fold larger than those of bourgeois males (see Introduction), were unclear. In our data, genes encoding enzymes required for testosterone-11KT conversion were significantly overexpressed in IP male testes compared with female ovaries (*cyp11c1*, *hsd11b2*; i.e., TP > IP > F). This suggests that IP males produce low but biologically meaningful levels of 11KT, sufficient to support healthy

Downloaded from https://academic.oup.com/mbe/article/35/1/225/4607869 by guest on 28 April 2025

spermatogenesis but not secondary sexual character development. Other sex steroids, including estrogens and progestins, also play a role in teleost spermatogenesis (Schulz et al. 2010; Kobayashi et al. 2011). However, aromatase expression (indicative of estrogen synthesis) was higher in the gonad and brain of TP males over IP males.

Greater Gonadal Investment by IP Males Is Associated with Overexpression of Cell Proliferation and Sperm Quality Genes

Sneaker males show greater investment in sperm production and gonadal volume to support a mating strategy characterized by high levels of sperm competition (see Introduction). Our transcriptomic data from bluehead wrasse testes provide novel evidence that biological processes potentially supporting increased spermatogenesis in IP males include ribosome biogenesis, genome integrity, and sperm quality (table 2). Increased ribosome biogenesis characterizes proliferating cells and is essential during gametogenesis (Sanchez et al. 2016). Enhanced DNA-stability, DNA-repair, and immunoprotection functions will also be critical for maintaining sperm integrity during spermatogenesis. Genes upregulated in IP male testes include components of the Fanconi anemia DNA-repair pathway (*faap24*), which acts in male germ cells to maintain genome integrity during spermatogenesis in humans (Jamsai et al. 2015) and zebrafish (Titus et al. 2009), and kynureninase (*kynu*), which is involved in tryptophan catabolism along the kynurenine pathway that in mammals regulates an immune-tolerant environment in the testis and is critical for sperm quality control (Jrad-Lamine et al. 2011; Guiton et al. 2013). We provide the first molecular evidence for enhanced genome integrity and sperm quality control processes in testes of a cryptic male teleost with increased sperm production.

IP and TP Male Testes Differentially Regulate Genes Associated with Sex Pheromone Production and Egg–Sperm Interactions

Our data imply that female mimicry by IP males may involve active chemical subterfuge. IP male testes and female ovaries both overexpressed kynureninase (*kynu*) relative to TP males, one of only two genes to show this pattern in our data (the second gene was unannotated). Aside from a role in sperm quality control (discussed earlier), products of the kynurenine pathway are found to be potent male attractants in aquatic broadcast spawners (Riffell et al. 2004), including female masu salmon, which release L-kynurenine as a sex pheromone attractive to spermiating males (Yambe et al. 2006). If kynurenine is a female sex pheromone in bluehead wrasse, as it is in salmon, IP males may use it as a form of chemical disguise. In the black goby (*Gobius niger*), sneaker male ejaculate was shown to be pheromonally inconspicuous to dominant males, thereby increasing mating opportunities through parasitic spawning (Locatello et al. 2002). Chemical mimicry of females by males has been reported in only a few other instances (e.g., rove beetles, Peschke 1987; garter snakes, Shine et al. 2000; diandric ants, Cremer et al. 2002) and is far less well-studied than morphological mimicry, or the sex pheromone production by bourgeois males that is usually considered to be androgen dependent (Borg 1994) (e.g., produced from specialized anal glands in dominant male blennies that are absent in parasitic males, Serrano et al. 2008). Further experiments are needed to confirm whether cryptic male teleosts produce specific chemical signals designed to deceive aggressive males, but our data suggest chemical mimicry may be more widely employed than currently appreciated.

Differential expression of membrane protein genes and genes involved in egg–sperm interactions suggests that molecular-level factors may be influencing sperm competition between TP and IP males. For example, several genes functionally important for sperm activation and fertilization were upregulated in TP male testes, and both male phenotypes upregulated different uncharacterized genes encoding predicted membrane proteins (table 2). Many of the former are conserved across metazoans, yet little is known regarding their potential role in nonmammalian vertebrate reproduction. These genes warrant further investigation for their potential involvement in cellular-level sperm competition.

Expected Patterns

There were also many expected patterns in the transcriptome data reflecting known differences in hormone production, behavior, and physiology among sexual phases of bluehead wrasse. For example, the extensive sex-biased differences in gonadal gene expression relate to physiological differences in the production of eggs versus sperm that are widely reported in testis–ovary transcriptome comparisons (Small et al. 2009; Manousaki et al. 2014), including previously for female versus TP male bluehead wrasse (Liu et al. 2015).

In the brain, there were significant expression differences in genes involved in local sex steroid production. Genes responsible for local testosterone (*hsd17b3*) and estrogen (*cyp19a1b*) synthesis were overexpressed in TP males and females, respectively. Interestingly, aromatase expression was significantly higher in TP males over IP males (i.e., F > TP > IP), a pattern described in other teleosts with male sexual polymorphism (Gonçalves et al. 1996; Schunter et al. 2014; Partridge et al. 2016). These data are consistent with conserved roles for these genes in maintaining neural sex differences in teleosts, including reproductive behavior (Le Page et al. 2010; Diotel et al. 2011).

TP males also overexpressed several genes known to regulate neuroendocrine secretion (*pyya*, *proSAAS*, *cdo1*). Cysteine dioxygenase (*cdo1*), essential in the biosynthesis of taurine, is known to stimulate testosterone production in the mammalian and teleost testis (Yang et al. 2010b). Peptide YY (*pyya*), although poorly studied in teleosts, shares structural homology with neuropeptide Y, a neurotransmitter regulating neuroendocrine release that also reduces anxiety and stress (Dumont et al. 1992). Interestingly, administration of NPY may induce sex change in bluehead wrasse (Kramer and Imbriano 1997). These genes are candidates for supporting local androgen production and influencing TP male-specific reproductive behavior.

Downloaded from https://academic.oup.com/mbe/article/35/1/225/4607869 by guest on 28 April 2025

Conclusions and Future Directions

We identify the expression states that define alternative reproductive phenotypes in a sex-changing fish with male sexual polymorphism, and generate testable hypotheses regarding the key genes and physiological processes that regulate sexual plasticity in the bluehead wrasse. For the first time in any species, we show that behavioral and morphological female-mimicry by sneaker males has a molecular signature in both the brain and the gonad. Our data support the premise that sneaker males have "female-like" brains, and provides the first evidence that morphological mimicry by these males involves pervasive downregulation of androgenesis genes in the gonad.

Testing some of our hypotheses in a free-living nonmodel species will be challenging, and verifying these patterns in independent animal systems will be important. A valuable approach for future work will be finer-scale anatomical sampling, as current transcriptomic studies in nonmodel species are limited in their resolution because of sampling at whole-organ or whole-organism levels. As we and others have shown, organ-level analyses readily detect broad-scale expression differences and generate rich data sets for hypothesis development. However, in heterogeneous tissues like the brain, meaningful yet subtle, transient, or highly localized (e.g., region- or cell-specific) expression differences will be impossible to detect without anatomically targeted sampling (see Todd, Black, et al. 2016). Therefore, it may be unsurprising that studies to date have not found significant expression differences for key neuropeptide genes known to regulate socio-sexual behavior and reproduction in teleosts (see above). Coupling anatomically guided tissue sampling, for example, using detailed immunohistochemical methods, with increasingly accessible and sophisticated sequencing approaches (e.g., low-input or single cell RNA-seq, Kolodziejczyk et al. 2015) should yield much greater insight into the molecular underpinnings of behavioral and sexual variation in these intriguing model systems.

Materials and Methods

Sample Collection

TP males, IP males, and females of *T. bifasciatum* ($N = 5$ per phenotype) were collected in June 2014 from patch reefs in the Florida Keys. Fish were euthanized with an overdose of MS-222 (Sigma–Aldrich) within 2 min of capture and the brain and gonads dissected immediately. The whole brain and one of the paired gonadal lobes were preserved immediately in RNAlater (Life Technologies). Samples were kept on ice before transfer to −20 °C for up to 1 week, then at −80 °C until RNA extraction. To confirm sexual phenotype of each individual and ensure that no sampled females were undergoing sex change, histological analysis was performed on the second gonadal lobe. Tissue was fixed in 4% paraformaldehyde/1× PBS overnight at 4 °C then stored in 1× PBS until examination via paraffin sectioning and Hematoxylin and Eosin (HE) staining (Histology Laboratory, College of Veterinary Medicine, NCSU). Sample collection was performed under NOAA permit FKNMS-2014-056 in accordance with guidelines established by the Institutional Animal Care and Use Committee at North Carolina State University.

RNA Extraction, Library Preparation, and Sequencing

Total RNA was isolated from one gonadal lobe and the forebrain of each individual. Forebrain only (including midbrain, excluding hindbrain consisting of corpus cerebelli, pons, and medulla) was used for RNA extraction, as this region contains neural circuits involved in the regulation of social behavior and decision-making that are likely important for driving phenotypic behavioral differences (O'Connell and Hofmann 2011). Individual tissue samples were homogenized in 1-ml TRIzol Reagent (Life Technologies) with 0.5-mm zirconium oxide beads in a Bullet Blender (Next Advance) for 5 min (brain samples) or using a TissueLyser II (Qiagen) (gonad samples). Phase separation was performed using a 1/10 volume of 1-Bromo-3-Chloropropane (Sigma–Aldrich), incubated for 5 min at room temperature before centrifugation at 12,000×g for 15 min. RNA was precipitated from the upper aqueous phase using an equal volume of 70% ethanol, then purified using a Norgen Total RNA purification kit (Millennium Science) including on-column DNase treatment (Millennium Science). RNA concentrations were measured by Qubit 2.0 Fluorometer RNA assay HS kit (Life Technologies) and standardized to 100 ng/μl. RNA integrity was assessed on an Agilent 2100 Bioanalyzer. All samples had RIN $\geq$ 7.2 (mean 8.7, SD 0.80) (supplementary table S1, Supplemenatry Material online). A single sample with RIN 6.1 was excluded from analysis (see below).

The 30 samples (forebrain and gonad for each of 5 biological replicates per phenotype) were prepared as individually barcoded Illumina TruSeq stranded mRNA libraries and sequenced in a pool of 86 total libraries (additional libraries are from a related study) over four lanes of an Illumina HiSeq 2500 (V4 chemistry, 125-bp paired-end reads) at the Otago Genomics and Bioinformatics Facility at the University of Otago, under contract to New Zealand Genomics Limited (NZGL). Libraries were pooled prior to multi-lane sequencing to avoid technical biases due to lane effects (Todd, Black, et al. 2016). Raw RNA-seq data are available at NCBI's Sequence Read Archive under BioProject PRJNA376835 (IP males and females) and PRJNA293777 (TP males).

Data QC, De Novo Transcriptome Assembly, and Expression Quantification

Read quality was assessed using FastQC v0.11.5 (Andrews 2010). Raw reads were error corrected using Rcorrector v1.0.2 (Song and Florea 2015) with default parameters. Adapter removal and light quality trimming at PHRED < 5 was performed in TRIMMOMATIC v0.35 (Bolger et al. 2014), as recommended for RNA-seq data (MacManes 2016).

In the absence of a well-annotated reference genome for bluehead wrasse, a de novo transcriptome was assembled as a reference for read mapping and expression quantification. To avoid the detrimental effects of sequence polymorphism on assembly quality (MacManes 2016), our assembly was based on brain and gonad libraries from a single individual per phenotype (6 libraries, 66.0 million trimmed paired reads). Error

Downloaded from https://academic.oup.com/mbe/article/35/1/225/4607869 by guest on 28 April 2025

corrected, trimmed reads were assembled in Trinity v2.2.0 (Grabherr et al. 2011; Haas et al. 2013) using default parameters. To reduce redundancy in the assembled transcripts, CD-HIT-EST v4.6.6 (Li and Godzik 2006) was used to collapse contigs with at least 98% identity (e.g., representing alternative alleles from the same locus). Assembly quality and completeness were evaluated before and after redundancy removal using BUSCO v1.22 (Simao et al. 2015) and TransRate v1.0.3 (Smith-Unna et al. 2016). BUSCO was run using the vertebrate ortholog database of 3,023 genes. Optional contig filtering within TransRate was not employed because this negatively affected assembly completeness, reducing complete BUSCO genes to 76%.

Gene expression estimates for each sample were obtained using the "align_and_estimate_abundance.pl" script from the Trinity package: for each library, corrected trimmed read pairs were mapped against the transcriptome assembly using Bowtie2 v2.2.9 (Langmead and Salzberg 2012) and transcript abundances were estimated using RSEM v1.2.29 (Li and Dewey 2011) with default settings. The Trinity de novo transcriptome assembler reconstructs genes and their putative alternative splice isoforms. However, the inherent difficulty in correctly inferring isoforms without a well-annotated reference genome means that many isoforms are erroneously inferred from de novo assemblies, such that there is lower performance of differential expression analyses performed at the transcript level (Vijay et al. 2013). Therefore, we performed all downstream analyses at the "gene" level, using Trinity clusters as a proxy for genes (representing the summed counts of alternative isoform expression per gene). Gene-level analysis facilitates detecting differences in relative isoform usage between conditions (differential transcript usage), but cannot identify expression changes specific to individual isoforms. Although this may omit biologically meaningful differential expression between specific gene isoforms (e.g., an expression change in the opposite direction but of the same magnitude), gene-level expression analysis has been shown to produce more accurate, powerful, and interpretable results than transcript-level analysis (Soneson et al. 2015).

Differential Expression

Sample clustering and differential expression analyses were performed for forebrain and gonad separately using a generalized linear model (GLM) framework in DESeq2 v1.12.4 (Love et al. 2014) within R v3.3.0 (R Core Team 2016). To explore among-sample and within-group variation in transcriptome-wide expression, samples were clustered using heatmap and PCA approaches following regularized log transformation (see supplementary figure S5, Supplemenatry Material online). These analyses identified one IP male gonad sample and one IP male brain sample as potential outliers. Histological examination of the former suggested inadvertent sampling of intestinal tissue, and the brain sample corresponded to lower quality input RNA (RIN 6.1). Both samples were excluded from further analysis as outliers may compromise statistical power to detect true differential expression by artificially increasing variance (biological noise) among samples. This is especially detrimental when true expression differences are subtle, as they are in the brain (Todd, Black, et al. 2016). Therefore, brain and gonad differential expression were performed using 5 TP male, 4 IP male, and 5 female samples as biological replicates (14 final samples per tissue).

For each tissue, a single GML was fit to estimate size factors and dispersion across all 14 samples, before performing three pairwise contrasts of differential expression using the Wald test for significance of GLM coefficients. The three pairwise comparisons were performed for each tissue to identify genes characteristically expressed in each phenotype: TP male versus female, IP male versus female, TP male versus IP male. To reduce the false-discovery burden and optimize the number of significant results returned in each pairwise comparison, default independent filtering and outlier exclusion were performed prior to differential expression (DE) analysis within DESeq2. False Discovery Rate (FDR) was controlled at 5% to account for multiple testing, and an adjusted significance value (FDR-*P*) of <0.05 was used to define genes as significantly differentially expressed (DE) (fig. 2). Genes significant at FDR-$P < 0.1$ were also screened to investigate general patterns of gene expression between groups and are shown in tables 1 and 2.

Transcriptome Annotation, Functional Enrichment, and Gene Pathway Analysis

Primary annotation of assembled contigs was achieved using BLASTX homology searches against the Ensembl zebrafish (*Danio rerio*) proteome (vGRCz10), chosen as the teleost genome of highest quality and completeness. Homology to known Zebrafish proteins (e-values $<$ 1e-10) was found for 23% of contigs in the final assembly. It is typical for the majority of contigs in de novo assemblies from nonmodel species to lack annotation information (e.g., Schunter et al. 2014, 21% annotated; Whittington et al. 2015, 35% annotated). However, almost half of all contigs found significantly DE in our analysis were annotated (e.g., 49%, FDR-$P < 0.05$). Significantly differentially expressed contigs are more likely to be highly expressed in at least one condition and, therefore, more correctly assembled. Many unannotated and non-DE contigs may represent transcription artefacts, lowly expressed mRNA variants or microRNAs, as well as misassemblies.

Contigs were also searched against the comprehensive UniRef90 (release 2016_09) protein database using the ultra-fast sequence aligner DIAMOND v0.8.24 (Buchfink et al. 2015) in –sensitive mode. Further annotation was performed using dammit! v0.3 (www.camillescott.org/dammit; last accessed November 13, 2017), which utilizes multiple databases for transcriptome annotation: Pfam-A v30.0 to identify protein domains together with TransDecoder to identify coding regions, Rfam v12.1 for noncoding RNAs, and OrthoDB v9 and BUSCO v1.22 ("vertebrata") for orthologous genes. In all cases, an e-value cut-off of 1e−10 was used, retaining the best hit.

To investigate the functional relevance of genes differentially upregulated in each phenotype, Gene Ontology (GO) annotation and KEGG pathway analyses were performed in DAVID v6.8 (Huang et al. 2009a,b), using Ensembl gene IDs for homologous zebrafish genes. Significantly enriched GO terms

Downloaded from https://academic.oup.com/mbe/article/35/1/225/4607869 by guest on 28 April 2025

and KEGG pathways were determined against the zebrafish genomic background using a default EASE of 0.1 and Benjamini-corrected *P* values to account for multiple testing. Functional Annotation Clustering was used to group significantly enriched GO terms (medium stringency).

Of the very large number of genes DE in comparisons between male and female gonad, only genes DE ≥ log2FC 2.0 were used in enrichment analyses. Due to extensive overlap in DE gene lists in comparisons between females versus either TP or IP males (>76%), enrichment analyses were performed at the level of ovary-enriched (significantly upregulated in female against IP and TP male) or testis-enriched (significantly upregulated in IP and TP males against females). There were too few genes significantly DE in other comparisons for statistically powerful GO analysis, and DAVID GO assignment was used for exploratory purposes only.

Isotocin Expression: Technical Validation by qPCR and Localization by In Situ Hybridization

Quantitative real-time PCR (qPCR) was used to independently measure forebrain *isotocin* mRNA levels among TP males, IP males, and females ($N = 3$ per phenotype). To localize *isotocin*-expressing neurons within the bluehead wrasse brain, in situ hybridization (ISH) was used to visualize the spatial expression pattern of *isotocin* mRNA in the bluehead wrasse brain. Detailed qPCR and ISH methods are presented in supplementary S6, Supplemenatry Material online.

Supplementary Material

Supplementary data are available at *Molecular Biology and Evolution* online.

Acknowledgments

We are grateful for constructive feedback from two anonymous reviewers that helped to improve the final manuscript. We thank Simon Fisher, Chris Friesen, and Simon Muncaster for insightful discussions regarding neuroplasticity, oxidative stress, and the role of 11KT in mediating male secondary sexual characters, respectively. We also thank Mik Black for statistical advice and Bill Tyler for field support. This work was supported by the Royal Society of New Zealand Marsden Fund (UOO1308 to N.J.G.) and the National Science Foundation (1257791 to J.R.G. and 1257761 to Bill Tyler at Indian River State College, Florida). E.V.T. is supported by a Health Sciences Postdoctoral Fellowship from the University of Otago.

References

Abbott LF, Nelson SB. 2000. Synaptic plasticity: taming the beast. *Nat Neurosci.* 3(Supp):1178–1183.

Almeida O, Gozdowska M, Kulczykowska E, Oliveira RF. 2012. Brain levels of arginine-vasotocin and isotocin in dominant and subordinate males of a cichlid fish. *Horm Behav.* 61(2):212–217.

Alonso-Alvarez C, Bertrand S, Faivre B, Chastel O, Sorci G. 2007. Testosterone and oxidative stress: the oxidation handicap hypothesis. *Proc Biol Sci.* 274(1611):819–825.

Andrews S. 2010. FastQC: a quality control tool for high throughput sequence data. Available from: http://www.bioinformatics.babraham.ac.uk/projects/fastqc, last accessed November 13, 2017.

Aubin-Horth N, Landry CR, Letcher BH, Hofmann HA. 2005. Alternative life histories shape brain gene expression profiles in males of the same population. *Proc Biol Sci.* 272(1573):1655–1662.

Aubin-Horth N, Renn SCP. 2009. Genomic reaction norms: using integrative biology to understand molecular mechanisms of phenotypic plasticity. *Mol Ecol.* 18(18):3763–3780.

Beaulieu M, Mboumba S, Willaume E, Kappeler PM, Charpentier MJE. 2014. The oxidative cost of unstable social dominance. *J Exp Biol.* 217(Pt 15):2629–2632.

Black MP, Reavis RH, Grober MS. 2004. Socially induced sex change regulates forebrain isotocin in *Lythrypnus dalli*. *Neuroreport* 15(1):185–189.

Bolger AM, Lohse M, Usadel B. 2014. Trimmomatic: a flexible trimmer for Illumina Sequence Data. *Bioinformatics* 30(15):2114–2120.

Borg B. 1994. Androgens in teleost fishes. *Comp Biochem Physiol C Pharmacol Toxicol Endocrinol.* 109(3):219–245.

Boulanger LM, Shatz CJ. 2004. Immune signalling in neural development, synaptic plasticity and disease. *Nat Rev Neurosci.* 5(7):521–531.

Braida D, Donzelli A, Martucci R, Capurro V, Busnelli M, Chini B, Sala M. 2012. Neurohypophyseal hormones manipulation modulate social and anxiety-related behavior in zebrafish. *Psychopharmacology* 220(2):319–330.

Brantley RK, Wingfield JC, Bass AH. 1993. Sex steroid levels in Porichthys notatus, a fish with alternative reproductive tactics, and a review of the hormonal bases for male dimorphism among teleost fishes. *Horm Behav.* 27(3):332–347.

Buchfink B, Xie C, Huson DH. 2015. Fast and sensitive protein alignment using DIAMOND. *Nat Methods* 12(1):59–60.

Cardwell JR, Liley NR. 1991. Hormonal control of sex and color change in the stoplight parrotfish, *Sparisoma viride*. *Gen Comp Endocrinol.* 81(1):7–20.

Charnov E. 1982. The theory of sex allocation. Princeton, USA: Princeton University Press.

Cremer S, Sledge MF, Heinze J. 2002. Chemical mimicry: male ants disguised by the queen's bouquet. *Nature* 419(6910):897–897.

Diotel N, Do Rego J-L, Anglade I, Vaillant C, Pellegrini E, Vaudry H, Kah O. 2011. The brain of teleost fish, a source, and a target of sexual steroids. *Front Neurosci.* 5:137.

Dumont Y, Martel J-C, Fournier A, St-Pierre S, Quirion R. 1992. Neuropeptide Y and neuropeptide Y receptor subtypes in brain and peripheral tissues. *Prog Neurobiol.* 38(2):125–167.

El Idrissi A, Shen CH, L'amoreaux WJ. 2013. Neuroprotective role of taurine during aging. *Amino Acids* 45(4):735–750.

Elmer BM, McAllister AK. 2012. Major histocompatibility complex class I proteins in brain development and plasticity. *Trends Neurosci.* 35(11):660–670.

Feldmeyer B, Elsner D, Foitzik S. 2014. Gene expression patterns associated with caste and reproductive status in ants: worker-specific genes are more derived than queen-specific ones. *Mol Ecol.* 23(1):151–161.

Fraser BA, Janowitz I, Thairu M, Travis J, Hughes KA. 2014. Phenotypic and genomic plasticity of alternative male reproductive tactics in sailfin mollies. *Proc Biol Sci.* 281(7):20132310.

Garratt M, Brooks RC. 2012. Oxidative stress and condition-dependent sexual signals: more than just seeing red. *Proc Biol Sci.* 279(1741):3121–3130.

Georgiev AV, Thompson ME, Mandalaywala TM, Maestripieri D. 2015a. Oxidative stress as an indicator of the costs of reproduction among free-ranging rhesus macaques. *J Exp Biol.* 218(Pt 13):1981–1985.

Georgiev AV, Muehlenbein MP, Prall SP, Emery Thompson M, Maestripieri D. 2015b. Male quality, dominance rank, and mating success in free-ranging rhesus macaques. *Behav Ecol.* 26(3):763–772.

Ghiselin MT. 1969. The evolution of hermaphroditism among animals. *Q Rev Biol.* 44(2):189–208.

Godwin J. 2010. Neuroendocrinology of sexual plasticity in teleost fishes. *Front Neuroendocrinol.* 31(2):203–216.

Godwin J, Sawby R, Warner RR, Crews D, Grober MS. 2000. Hypothalamic arginine vasotocin mRNA abundance variation across

Downloaded from https://academic.oup.com/mbe/article/35/1/225/4607869 by guest on 28 April 2025

sexes and with sex change in a coral reef fish. *Brain Behav Evol.* 55(2):77–84.
Godwin J, Thompson R. 2012. Nonapeptides and social behavior in fishes. *Horm Behav.* 61(3):230–238.
Gonçalves EJ, Almada VC, Oliveira RF, Santos AJ. 1996. Female mimicry as a mating tactic in males of the blenniid fish *Salaria pavo*. *J Mar Biol Assoc UK.* 76(02):529–538.
Goodson JL, Bass AH. 2000. Forebrain peptides modulate sexually polymorphic vocal circuitry. *Nature* 403(6771):769–772.
Grabherr MG, Haas BJ, Yassour M, Levin JZ, Thompson DA, Amit I, Adiconis X, Fan L, Raychowdhury R, Zeng Q, et al. 2011. Full-length transcriptome assembly from RNA-Seq data without a reference genome. *Nat Biotechnol.* 29(7):644–U130.
Grober MS, George AA, Watkins KK, Carneiro LA, Oliveira RF. 2002. Forebrain AVT and courtship in a fish with male alternative reproductive tactics. *Brain Res Bull.* 57(3–4):423–425.
Gross MR. 1996. Alternative reproductive strategies and tactics: diversity within sexes. *Trends Ecol Evol.* 11(2):92–98.
Guiton R, Henry-Berger J, Drevet JR. 2013. The immunobiology of the mammalian epididymis: the black box is now open! *Basic Clin Androl.* 23:8.
Haas BJ, Papanicolaou A, Yassour M, Grabherr M, Blood PD, Bowden J, Couger MB, Eccles D, Li B, Lieber M, et al. 2013. De novo transcript sequence reconstruction from RNA-seq using the Trinity platform for reference generation and analysis. *Nat Protoc.* 8(8):1494–1512.
Haddad LS, Kelbert L, Hulbert A. 2007. Extended longevity of queen honey bees compared to workers is associated with peroxidation-resistant membranes. *Exp Gerontol.* 42(7):601–609.
Higuchi M, Celino FT, Shimizu-Yamaguchi S, Miura C, Miura T. 2012. Taurine plays an important role in the protection of spermatogonia from oxidative stress. *Amino Acids* 43:2359–2369.
Higuchi M, Celino FT, Tamai A, Miura C, Miura T. 2012. The synthesis and role of taurine in the Japanese eel testis. *Amino Acids* 43:773–781.
Hourigan TF, Nakamura M, Nagahama Y, Yamauchi K, Grau EG. 1991. Histology, ultrastructure, and in vitro steroidogenesis of the testes of two male phenotypes of the protogynous fish, *Thalassoma duperrey* (labridae). *Gen Comp Endocrinol.* 83(2):193–217.
Huang DW, Sherman BT, Lempicki RA. 2009a. Bioinformatics enrichment tools: paths toward the comprehensive functional analysis of large gene lists. *Nucleic Acids Res.* 37:1–13.
Huang DW, Sherman BT, Lempicki RA. 2009b. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nat Protoc.* 4:44–57.
Isaksson C, While GM, McEvoy J, Van De Crommenacker J, Olsson M, Groothuis TG, Komdeur J, Wapstra E. 2011. Aggression, but not testosterone, is associated to oxidative status in a free-living vertebrate. *Behaviour* 148(5):713–731.
Iulianella A, Sharma M, Durnin M, Vanden Heuvel GB, Trainor PA. 2008. Cux2 (Cutl2) integrates neural progenitor development with cell-cycle progression during spinal cord neurogenesis. *Development* 135(4):729–741.
Jamsai D, O'Connor AE, O'Donnell L, Lo JCY, O'Bryan MK. 2015. Uncoupling of transcription and translation of Fanconi anemia (FANC) complex proteins during spermatogenesis. *Spermatogenesis* 5(1):e979061.
Jrad-Lamine A, Henry-Berger J, Gourbeyre P, Damon-Soubeyrand C, Lenoir A, Combaret L, Saez F, Kocer A, Tone S, Fuchs D, et al. 2011. Deficient tryptophan catabolism along the kynurenine pathway reveals that the epididymis is in a unique tolerogenic state. *J Biol Chem.* 286(10):8030–8042.
Kobayashi Y, Nozu R, Nakamura M. 2011. Role of estrogen in spermatogenesis in initial phase males of the three-spot wrasse (*Halichoeres trimaculatus*): effect of aromatase inhibitor on the testis. *Dev Dyn.* 240(1):116–121.
Kolodziejczyk AA, Kim JK, Svensson V, Marioni JC, Teichmann SA. 2015. The Technology and Biology of Single-Cell RNA Sequencing. *Mol Cell* 58(4):610–620.
Kramer CR, Imbriano MA. 1997. Neuropeptide Y (NPY) induces gonad reversal in the protogynous bluehead wrasse, *Thalassoma bifasciatum* (Teleostei: Labridae). *J Exp Zool.* 279(2):133–144.
Lamm MS, Liu H, Gemmell NJ, Godwin JR. 2015. The need for speed: neuroendocrine regulation of socially-controlled sex change. *Integr Comp Biol.* 55(2):307–322.
Langmead B, Salzberg SL. 2012. Fast gapped-read alignment with Bowtie 2. *Nat Methods* 9(4):357–359.
Larson ET, Norris DO, Summers CH. 2003. Monoaminergic changes associated with socially induced sex reversal in the saddleback wrasse. *Neuroscience* 119(1):251–263.
Le Page Y, Diotel N, Vaillant C, Pellegrini E, Anglade I, Mérot Y, Kah O. 2010. Aromatase, brain sexualization and plasticity: the fish paradigm. *Eur J Neurosci.* 32(12):2105–2115.
Lee H, Brott BK, Kirkby LA, Adelson JD, Cheng S, Feller MB, Datwani A, Shatz CJ. 2014. Synapse elimination and learning rules co-regulated by MHC class I H2-Db. *Nature* 509(7499):195–200.
Lee Y-S, Silva AJ. 2009. The molecular and cellular biology of enhanced cognition. *Nat Rev Neurosci.* 10(2):126–140.
Lema SC, Sanders KE, Walti KA. 2015. Arginine vasotocin, isotocin and nonapeptide receptor gene expression link to social status and aggression in sex-dependent patterns. *J Neuroendocrinol.* 27(2):142–157.
Li B, Dewey CN. 2011. RSEM: accurate transcript quantification from RNA-Seq data with or without a reference genome. *BMC Bioinformatics* 12:323.
Li W, Godzik A. 2006. Cd-hit: a fast program for clustering and comparing large sets of protein or nucleotide sequences. *Bioinformatics* 22(13):1658–1659.
Liu H, Lamm MS, Rutherford K, Black MA, Godwin JR, Gemmell NJ. 2015. Large-scale transcriptome sequencing reveals novel expression patterns for key sex-related genes in a sex-changing fish. *Biol Sex Differ.* 6(1):26.
Liu H, Todd EV, Lokman PM, Lamm MS, Godwin JR, Gemmell NJ. 2017. Sexual plasticity: a fishy tale. *Mol Reprod Dev.* 84(2):171–194.
Locatello L, Mazzoldi C, Rasotto M. 2002. Ejaculate of sneaker males is pheromonally inconspicuous in the black goby, *Gobius niger* (Teleostei, Gobiidae). *J Exp Zool A Ecol Genet Physiol.* 293(6):601–605.
Lorenzi V, Carpenter RE, Summers CH, Earley RL, Grober MS. 2009. Serotonin, social status and sex change in the bluebanded goby *Lythrypnus dalli*. *Physiol Behav.* 97(3–4):476–483.
Love MI, Huber W, Anders S. 2014. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol.* 15(12):550.
MacManes MD. 2016. Establishing evidenced-based best practice for the de novo assembly and evaluation of transcriptomes from non-model organisms. *bioRxiv* 035642; doi: https://doi.org/10.1101/035642.
Manousaki T, Tsakogiannis A, Lagnel J, Sarropoulou E, Xiang JZ, Papandroulakis N, Mylonas CC, Tsigenopoulos CS. 2014. The sex-specific transcriptome of the hermaphrodite sparid sharpsnout seabream (*Diplodus puntazzo*). *BMC Genomics* 15:655–655.
Mechaly AS, Vinas J, Piferrer F. 2013. The kisspeptin system genes in teleost fish, their structure and regulation, with particular attention to the situation in Pleuronectiformes. *Gen Comp Endocrinol.* 188:258–268.
Munday PL, Buston PM, Warner RR. 2006. Diversity and flexibility of sex-change strategies in animals. *Trends Ecol Evol.* 21:89–95.
Munday PL, White JW, Warner RR. 2006. A social basis for the development of primary males in a sex-changing fish. *Proc Biol Sci.* 273:2845–2851.
Nakamura M, Hourigan TF, Yamauchi K, Nagahama Y, Grau EG. 1989. Histological and ultrastructural evidence for the role of gonadal steroid hormones in sex change in the protogynous wrasse *Thalassoma duperrey*. *Environ Biol Fishes.* 24(2):117–136.
Nelson TJ, Sun M-K, Hongpaisan J, Alkon DL. 2008. Insulin, PKC signaling pathways and synaptic remodeling during memory storage and neuronal repair. *Eur J Pharmacol.* 585(1):76–87.

Downloaded from https://academic.oup.com/mbe/article/35/1/225/4607869 by guest on 28 April 2025

Neumann ID. 2008. Brain oxytocin: a key regulator of emotional and social behaviours in both females and males. *J Neuroendocrinol.* 20(6):858–865.

Nugent BM, Stiver KA, Alonzo SH, Hofmann HA. 2016. Neuroendocrine profiles associated with discrete behavioural variation in *Symphodus ocellatus*, a species with male alternative reproductive tactics. *Mol Ecol.* 25(20):5212–5227.

O'Connell LA, Hofmann HA. 2011. Genes, hormones, and circuits: an integrative approach to study the evolution of social behavior. *Front Neuroendocrinol.* 32:320–335.

O'Connell LA, Hofmann HA. 2012. Evolution of a vertebrate social decision-making network. *Science* 336:1154–1157.

Oliveira RF, Canario AVM, Grober MS. 2001. Male sexual polymorphism, alternative reproductive tactics, and androgens in combtooth blennies (Pisces: Blenniidae). *Horm Behav.* 40:266–275.

Oliveira RF, Carneiro LA, Goncalves DM, Canario AV, Grober MS. 2001. 11-Ketotestosterone inhibits the alternative mating tactic in sneaker males of the peacock blenny, *Salaria pavo*. *Brain Behav Evol.* 58:28–37.

Oliveira RF, Taborsky M, Brockmann HJ. 2008. Alternative reproductive tactics: an integrative approach. Cambridge, UK: Cambridge University Press.

Oliveira-dos-Santos AJ, Matsumoto G, Snow BE, Bai D, Houston FP, Whishaw IQ, Mariathasan S, Sasaki T, Wakeham A, Ohashi PS, et al. 2000. Regulation of T cell activation, anxiety, and male aggression by RGS2. *Proc Natl Acad Sci U S A.* 97(22):12272–12277.

Olsson M, Tobler M, Healey M, Perrin C, Wilson M. 2012. A significant component of ageing (DNA damage) is reflected in fading breeding colors: an experimental test using innate antioxidant mimetics in painted dragon lizards. *Evolution* 66(8):2475–2483.

Partridge CG, MacManes MD, Knapp R, Neff BD. 2016. Brain transcriptional profiles of male alternative reproductive tactics and females in bluegill sunfish. *PLoS One* 11(12):e0167509.

Pellegrini E, Diotel N, Vaillant-Capitaine C, Perez Maria R, Gueguen MM, Nasri A, Cano Nicolau J, Kah O. 2016. Steroid modulation of neurogenesis: focus on radial glial cells in zebrafish. *J Steroid Biochem Mol Biol.* 160:27–36.

Perry AN, Grober MS. 2003. A model for social control of sex change: interactions of behavior, neuropeptides, glucocorticoids, and sex steroids. *Horm Behav.* 43(1):31–38.

Peschke K. 1987. Cuticular hydrocarbons regulate mate recognition, male aggression, and female choice of the rove beetle, *Aleochara curtula*. *J Chem Ecol.* 13(10):1993–2008.

R Core Team. 2016. R: a language and environment for statistical computing. Vienna (Austria): R Foundation for Statistical Computing.

Reddon AR, O'Connor CM, Marsh-Rollo SE, Balshine S. 2012. Effects of isotocin on social responses in a cooperatively breeding fish. *Anim Behav.* 84(4):753–760.

Reddon AR, Voisin MR, O'Connor CM, Balshine S. 2014. Isotocin and sociality in the cooperatively breeding cichlid fish, *Neolamprologus pulcher*. *Behaviour* 151(10):1389–1411.

Renn SCP, Aubin-Horth N, Hofmann HA. 2008. Fish and chips: functional genomics of social plasticity in an African cichlid fish. *J Exp Biol.* 211(18):3041–3056.

Riffell JA, Krug PJ, Zimmer RK. 2004. The ecological and evolutionary consequences of sperm chemoattraction. *Proc Natl Acad Sci U S A.* 101(13):4501–4506.

Roff DA. 2007. Contributions of genomics to life-history theory. *Nat Rev Genet.* 8(2):116–125.

Sadovy de Mitcheson Y, Liu M. 2008. Functional hermaphroditism in teleosts. *Fish Fish* 9(1):1–43.

Sanchez CG, Teixeira FK, Czech B, Preall JB, Zamparini AL, Seifert JRK, Malone CD, Hannon GJ, Lehmann R. 2016. Regulation of ribosome biogenesis and protein synthesis controls germline stem cell differentiation. *Cell Stem Cell* 18(2):276–290.

Schantz TV, Bensch S, Grahn M, HasselquistD, Wittzell H. 1999. Good genes, oxidative stress and condition-dependent sexual signals. *Proc Soc Biol.* 266(1414):1–12.

Schärer L, Robertson DR. 1999. Sperm and milt characteristics and male v. female gametic investment in the Caribbean reef fish, *Thalassoma bifasciatum*. *J Fish Biol.* 55:329–343.

Schulz RW, de França LR, Lareyre J-J, Le Gac F, LeGac F, Chiarini-Garcia H, Nobrega RH, Miura T. 2010. Spermatogenesis in fish. *Gen Comp Endocrinol.* 165(3):390–411.

Schunter C, Vollmer SV, Macpherson E, Pascual M. 2014. Transcriptome analyses and differential gene expression in a non-model fish species with alternative mating tactics. *BMC Genomics* 15(1):13.

Semsar K, Godwin J. 2003. Social influences on the arginine vasotocin system are independent of gonads in a sex-changing fish. *J Neurosci.* 23:4386–4393.

Semsar K, Godwin J. 2004. Multiple mechanisms of phenotype development in the bluehead wrasse. *Horm Behav.* 45(5):345–353.

Semsar K, Kandel FL, Godwin J. 2001. Manipulations of the AVT system shift social status and related courtship and aggressive behavior in the bluehead wrasse. *Horm Behav.* 40(1):21–31.

Serrano RM, Lopes O, Hubbard PC, Araújo J, Canário AVM, Barata EN. 2008. 11-ketotestosterone stimulates putative sex pheromone production in the male peacock blenny, *Salaria pavo* (Risso 1810). *Biol Reprod.* 79(5):861–868.

Shine R, Harlow P, LeMaster MP, Moore IT, Mason RT. 2000. The transvestite serpent: why do male garter snakes court (some) other males? *Anim Behav.* 59(2):349–359.

Simao FA, Waterhouse RM, Ioannidis P, Kriventseva EV, Zdobnov EM. 2015. BUSCO: assessing genome assembly and annotation completeness with single-copy orthologs. *Bioinformatics* 31(19):3210–3212.

Sinervo B, Miles DB, Frankino WA, Klukowski M, DeNardo DF. 2000. Testosterone, endurance, and Darwinian fitness: natural and sexual selection on the physiological bases of alternative male behaviors in side-blotched lizards. *Horm Behav.* 38(4):222–233.

Small CM, Carney GE, Mo Q, Vannucci M, Jones AG. 2009. A microarray analysis of sex- and gonad-biased gene expression in the zebrafish: evidence for masculinization of the transcriptome. *BMC Genomics* 10:579.

Smith LK, He Y, Park J-S, Bieri G, Snethlage CE, Lin K, Gontier G, Wabl R, Plambeck KE, Udeochu J, et al. 2015. β2-microglobulin is a systemic pro-aging factor that impairs cognitive function and neurogenesis. *Nat Med.* 21(8):932–937.

Smith-Unna R, Boursnell C, Patro R, Hibberd J, Kelly S. 2016. TransRate: reference free quality assessment of de novo transcriptome assemblies. *Genome Res.* 26(8):1134–1144.

Sokołowska E, Kleszczyńska A, Kalamarz-Kubiak H, Arciszewski B, Kulczykowska E. 2013. Changes in brain arginine vasotocin, isotocin, plasma 11-ketotestosterone and cortisol in round goby, *Neogobius melanostomus*, males subjected to overcrowding stress during the breeding season. *Comp Biochem Physiol A Mol Integr Physiol.* 165(2):237–242.

Soneson C, Love MI, Robinson MD. 2015. Differential analyses for RNA-seq: transcript-level estimates improve gene-level inferences. *F1000Research* 4:1521.

Song L, Florea L. 2015. Rcorrector: efficient and accurate error correction for Illumina RNA-seq reads. *GigaScience* 4:48.

Stevens B, Allen NJ, Vazquez LE, Howell GR, Christopherson KS, Nouri N, Micheva KD, Mehalow AK, Huberman AD, Stafford B, et al. 2007. The classical complement cascade mediates CNS synapse elimination. *Cell* 131(6):1164–1178.

Stuglik MT, Babik W, Prokop Z, Radwan J. 2014. Alternative reproductive tactics and sex-biased gene expression: the study of the bulb mite transcriptome. *Ecol Evol.* 4(5):623–632.

Taborsky M. 1998. Sperm competition in fish: 'bourgeois' males and parasitic spawning. *Trends Ecol Evol.* 13:222–227.

Taborsky M. 2001. The evolution of bourgeois, parasitic, and cooperative reproductive behaviors in fishes. *J Hered.* 92:100–110.

Taborsky M, Brockmann HJ. 2010. Alternative reproductive tactics and life history phenotypes. In: Kappeler P, editor. Animal behaviour: evolution and mechanisms. Berlin, Germany: Springer Berlin Heidelberg. p. 537–586.

Downloaded from https://academic.oup.com/mbe/article/35/1/225/4607869 by guest on 28 April 2025

Thompson RR, Walton JC. 2004. Peptide effects on social behavior: effects of vasotocin and isotocin on social approach behavior in male goldfish (*Carassius auratus*). *Behav Neurosci.* 118(3):620–626.
Titus TA, Yan YL, Wilson C, Starks AM, Frohnmayer JD, Bremiller RA, Canestro C, Rodriguez-Mari A, He X, Postlethwait JH. 2009. The Fanconi anemia/BRCA gene network in zebrafish: embryonic expression and comparative genomics. *Mutat Res.* 668(1–2):117–132.
Todd EV, Black MA, Gemmell NJ. 2016. The power and promise of RNA-seq in ecology and evolution. *Mol Ecol.* 25:1224–1241.
Todd EV, Liu H, Muncaster S, Gemmell NJ. 2016. Bending genders: the biology of natural sex change in fish. *Sex Dev.* 10(5–6):223–241.
Vijay N, Poelstra JW, Kunstner A, Wolf JBW. 2013. Challenges and strategies in transcriptome assembly and differential gene expression quantification. A comprehensive in silico assessment of RNA-seq experiments. *Mol Ecol.* 22(3):620–634.
Villeda SA, Luo J, Mosher KI, Zou B, Britschgi M, Bieri G, Stan TM, Fainberg N, Ding Z, Eggel A, et al. 2011. The ageing systemic milieu negatively regulates neurogenesis and cognitive function. *Nature* 477(7362):90–94.
Wang Y, Lin L, Lai H, Parada LF, Lei L. 2013. Transcription factor Sox11 is essential for both embryonic and adult neurogenesis. *Dev Dyn.* 242(6):638–653.
Warner RR. 1975. The adaptive significance of sequential hermaphroditism in animals. *Am Nat.* 109(965):61–82.
Warner RR. 1984. Mating Behavior and Hermaphroditism in Coral Reef Fishes. *Am Sci.* 72:128–136.
Warner RR. 1988. Sex change and the size-advantage model. *Trends Ecol Evol.* 3(6):133–136.
Warner RR, Robertson DR. 1978. Sexual patterns in the labroid fishes of the western Caribbean I: the wrasses. *Smithson Contrib Zool.* 254:1–27.
Warner RR, Schultz ET. 1992. Sexual selection and male characteristics in the bluehead wrasse, *Thalassoma bifasciatum*: mating site acquisition, mating site defense, and female choice. *Evolution* 46(5): 1421–1442.
Warner RR, Swearer SE. 1991. Social control of sex change in the bluehead wrasse, *Thalassoma bifasciatum* (Pisces: Labridae). *Biol Bull.* 181(2):199–204.
West-Eberhard MJ. 2003. Developmental plasticity and evolution. New York, USA: Oxford University Press.
Whittington CM, Griffith OW, Qi W, Thompson MB, Wilson AB. 2015. Seahorse brood pouch transcriptome reveals common genes associated with vertebrate pregnancy. *Mol Biol Evol.* 32(12):3114–3131.
Whittle N, Singewald N. 2014. HDAC inhibitors as cognitive enhancers in fear, anxiety and trauma therapy: where do we stand? *Biochem Soc Trans.* 42(2):569–581.
Winder DG, Sweatt JD. 2001. Roles of serine/threonine phosphatases in hippocampel synaptic plasticity. *Nat Rev Neurosci.* 2(7):461–474.
Yambe H, Kitamura S, Kamio M, Yamada M, Matsunaga S, Fusetani N, Yamazaki F. 2006. L-Kynurenine, an amino acid identified as a sex pheromone in the urine of ovulated female masu salmon. *Proc Natl Acad Sci U S A.* 103(42):15370–15374.
Yang J, Wu G, Feng Y, Lv Q, Lin S, Hu J. 2010a. Effects of taurine on male reproduction in rats of different ages. *J Biomed Sci.* 17(Suppl 1):S9.
Yang J, Wu G, Feng Y, Sun C, Lin S, Hu J. 2010b. CSD mRNA expression in rat testis and the effect of taurine on testosterone secretion. *Amino Acids* 39(1):155–160.
Yang J, Zong X, Wu G, Lin S, Feng Y, Hu J. 2015. Taurine increases testicular function in aged rats by inhibiting oxidative stress and apoptosis. *Amino Acids* 47(8):1549–1558.
Yirmiya R, Goshen I. 2011. Immune modulation of learning, memory, neural plasticity and neurogenesis. *Brain Behav Immun.* 25(2): 181–213.
Yoshida M-A, Yamada L, Ochi H, Iwata Y, Tamura-Nakano M, Sawada H, Sauer WHH, Ogura A, Hirohashi N. 2014. Integrative omics analysis reveals differentially distributed proteins in dimorphic euspermatozoa of the squid, *Loligo bleekeri*. *Biochem Biophys Res Commun.* 450(3):1218–1224.

Downloaded from https://academic.oup.com/mbe/article/35/1/225/4607869 by guest on 28 April 2025