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Conservation and diversity in expression of candidate genes regulating socially-induced female-male sex change in wrasses

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Fishes exhibit remarkably diverse, and plastic, patterns of sexual development, most striking of which is sequential hermaphroditism, where individuals readily reverse sex in adulthood. How this stunning example of phenotypic plasticity is controlled at a genetic level remains poorly understood. Several genes have been implicated in regulating sex change, yet the degree to which a conserved genetic machinery orchestrates this process has not yet been addressed. Using captive and in-the-field social manipulations to initiate sex change, combined with a comparative qPCR approach, we compared expression patterns of four candidate regulatory genes among three species of wrasses (Labridae) - a large and diverse teleost family where female-to-male sex change is pervasive, socially-cued, and likely ancestral. Expression in brain and gonadal tissues were compared among the iconic tropical bluehead wrasse (*Thalassoma bifasciatum*) and the temperate spotty (*Notolabrus celidotus*) and kyusen (*Parajulus poecilepterus*) wrasses. In all three species, *cyp19a1a* (encoding gonadal aromatase that converts androgens to oestrogens) and *amh* (encoding anti-müllerian hormone that primarily regulates male germ cell development) were downregulated and upregulated, respectively, at the initiation of gonadal sex change, and may act concurrently to orchestrate ovary-testis transformation. In the brain, our data argue against a role for brain aromatase (*cyp19a1b*) in initiating behavioural sex change, as its expression trailed behavioural changes. However, we find that isotocin (*it*, that regulates teleost socio-sexual behaviours) expression correlated with dominant male-specific behaviours in the bluehead wrasse, suggesting *it* upregulation mediates the rapid behavioural sex change characteristic of blueheads and other tropical wrasses. However, *it*

expression was not sex-biased in temperate spotty and kyusen wrasses, where sex change is more protracted and social groups may be less tightly-structured. Together, these findings suggest that while key components of the molecular machinery controlling gonadal sex change are phylogenetically conserved among wrasses, neural pathways governing behavioural sex change may be more variable.

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Abstract

Fishes exhibit remarkably diverse, and plastic, patterns of sexual development, most striking of which is sequential hermaphroditism, where individuals readily reverse sex in adulthood. How this stunning example of phenotypic plasticity is controlled at a genetic level remains poorly understood. Several genes have been implicated in regulating sex change, yet the degree to which a conserved genetic machinery orchestrates this process has not yet been addressed. Using captive and in-the-field social manipulations to initiate sex change, combined with a comparative qPCR approach, we compared expression patterns of four candidate regulatory genes among three species of wrasses (Labridae) - a large and diverse teleost family where female-to-male sex change is pervasive, socially-cued, and likely ancestral. Expression in brain and gonadal tissues were compared among the iconic tropical bluehead wrasse (*Thalassoma bifasciatum*) and the temperate spotty (*Notolabrus celidotus*) and kyusen (*Parajulus poecilepterus*) wrasses. In all three species, *cyp19a1a* (encoding gonadal aromatase that converts androgens to oestrogens) and

amh (encoding anti-müllerian hormone that primarily regulates male germ cell development) were downregulated and upregulated, respectively, at the initiation of gonadal sex change, and may act concurrently to orchestrate ovary-testis transformation. In the brain, our data argue against a role for brain aromatase (*cyp19a1b*) in initiating behavioural sex change, as its expression trailed behavioural changes. However, we find that isotocin (*it*, that regulates teleost socio-sexual behaviours) expression correlated with dominant male-specific behaviours in the bluehead wrasse, suggesting *it* upregulation mediates the rapid behavioural sex change characteristic of blueheads and other tropical wrasses. However, *it* expression was not sex-biased in temperate spotty and kyusen wrasses, where sex change is more protracted and social groups may be less tightly-structured. Together, these findings suggest that while key components of the molecular machinery controlling gonadal sex change are phylogenetically conserved among wrasses, neural pathways governing behavioural sex change may be more variable.

Introduction

Most animals irreversibly differentiate as either male or female, yet some species exhibit remarkable sexual plasticity. This is true for teleost fishes, the only vertebrate lineage to display sequential hermaphroditism, in which individuals begin life as one sex but can change to the opposite sex sometime later in their life cycle [1, 2]. Sex change is typically cued by changes in social structure or by reaching a threshold age or size [3, 4], and characteristically involves radical changes in behaviour, external colouration and gonadal anatomy [5, 6]. Three patterns are observed; protogyny (female-to-male), protandry (male-to-female), and bidirectional sex change [7]. Protogyny is most common, although the widespread and patchy distribution of sequential hermaphroditism across the teleost phylogeny implies multiple evolutionary origins and frequent transitions to and from gonochorism (stable separate sexes) [8].

Despite significant research effort, the genetic cascades that orchestrate sex change remain elusive [6]. Numerous genes involved in vertebrate sexual development have been investigated for their potential roles in sex change [6]. Genes that exhibit expression changes early on in sex change are of particular interest as proximal molecular regulators of the process. One such gene is c*yp19a1a*, encoding the aromatase enzyme that converts testosterone (T) to 17β-estradiol (E2) in the female gonad to maintain ovarian function [2, 9]. Aromatase expression is rapidly arrested in transitioning females and this occurs in parallel with a sharp decline in plasma E2 levels and the onset of ovarian atresia [10]. Treatment with aromatase inhibitors reliably induces complete sex reversal in teleosts, whereas co-administration with E2 is preventative [11-14]. Thus, arrested *cyp19a1a* expression may initiate gonadal sex change in protogynous species by interrupting a positive E2 feedback loop that in fishes maintains both feminising gene expression and ovarian function [6, 15].

The most well-studied potential initiator of the male-specific expression pathway in sex-changing species is *dmrt1*, a gene that encodes a transcription factor critical for promoting male

gonadal development in animals as diverse as flies and humans [16]. A paralogue of *dmrt1* (*dmy*) has also become the male sex-determining gene in several fish species [17-19]. However, in protogynous hermaphrodites studied to date, changes in *dmrt1* expression regularly appear downstream of other genes, suggesting that *dmrt1* may be more important in progressing rather than initiating sex change [22, 23].

Anti-Müllerian hormone, Amh, a multifunctional member of the transforming growth factor-ß (TGF-ß) family, also plays a key role in regulating germ cell development in vertebrates, especially in males [24-26]. Amh is the male-determining factor in Patagonian pejerrey (*Odontesthes hatcheri*) [27] and Nile tilapia (*Oreochromis niloticus*) [28], while the Amh receptor, Amhr2, determines maleness in several species of *Takifugu* pufferfish [29]. A transcriptome-wide expression analysis of bluehead wrasse found *amh* and *amhr2* to be the earliest male-pathway genes upregulated during female to male sex change, concurrent with arrested expression of *cyp19a1a* and prior to the appearance of male tissues [23]. Expression of *amh* also increased during early protogynous sex change in ricefield eel (*Monopterus albus*) [30], and decreased during protandrous sex change in Red Sea clownfish (*Amphiprion bicinctus*) [31]. Therefore, Amh is emerging as a key initiator of maleness in gonochoristic and sex-changing fish.

Most studies focus on gonadal gene expression, yet social cues for sex change are visual and induce rapid neurochemical changes in the brain to initiate behavioural responses that precede, and likely trigger, gonadal changes [32-35]. Teleost fishes are unique in having a duplicated, brain-specific paralogue of the aromatase gene, *cyp19a1b*, responsible for local oestrogen production that plays a key role in brain sexualisation [36]. Paralleling gonadal *cyp19a1a* activity, forebrain *cyp19a1b* expression is downregulated in transitioning female bluehead wrasse [23]. Treatment with exogenous E2 also stimulates *cyp19a1b* expression and prevents behavioural sex change in this species [37].

A further gene of growing interest is isotocin (*it*) [38, 39]. Homologous to mammalian oxytocin, *it* appears to regulate teleost sociosexual behaviours [40-46]. Transcriptomic analyses in the bluehead wrasse have found forebrain *it* expression to be specific to terminal-phase males, implicating *it* in social dominance and sex change [47].

Protogyny is most pervasive, and likely ancestral, in labrid fishes [48]. The Labridae are the second largest marine fish family, encompassing the wrasses, parrotfish and hogfish with over 500 species in 70 genera [49, 50]. Protogyny is best studied in wrasses, which present a powerful model to study the evolution and functioning of sex change and explore the degree to which molecular control of this process is conserved. Labrids have a characteristic lek-like mating system, and are often diandric with two colour morphs; initial phase (IP) individuals consist of similarly coloured females and less abundant primary males (female-mimics), which can sex or

role change respectively, to replace the dominant terminal phase (TP) male upon its death or removal (Figure 1A) [5, 51]. Sex change occurs year-round in tropical wrasses, but follows a discreet spawning season in temperate species and occurs more slowly and from an already regressed ovary with low oestrogen production [52]. Thus, an important question is whether aromatase downregulation plays a pivotal role initiating sex change in both tropical and temperate wrasses.

In this study, four candidate genes are evaluated as proximate regulators of protogynous sex change, in the gonad (*cyp19a1a*, *amh*) and brain (*cyp19a1b*, *it*), using a comparative approach across three diandric protogynous wrasses (Figure 1B): the tropical Caribbean bluehead wrasse (*Thalassoma bifasciatum*), and the temperate New Zealand spotty wrasse (*Notolabrus celidotus*) and Japanese kyusen wrasse (*Parajulis poecilepterus*). We sought to 1) investigate whether evolutionarily conserved molecular mechanisms underlie protogynous sex change in wrasses, and 2) examine potential differences among tropical versus temperate species. Specifically, we were interested in the importance of changes in *aromatase* expression in initiating gonadal sex change and *isotocin* expression in initiating behavioural sex change in temperate species, in which sex change proceeds from post-spawning, already regressed ovaries, and in which social hierarchies may be less tightly structured.

Materials & Methods

Sample collection

Experiment 1: Social induction of sex change in wild bluehead wrasse

Sex change was induced in wild bluehead wrasse social groups by social manipulation on patch reefs off the coast of Key Largo, Florida, between May and June 2014. These experiments are described in detail elsewhere [47]. Three individuals representing each of six stages of sex change (described below), plus control females, IP males and TP males were used in the current study. Experiments were conducted in accordance with approval from North Carolina State University (12-069-0).

Experiment 2: Social induction of sex change in captive spotty wrasse

A social manipulation experiment was conducted to induce sex change in captive female spotty wrasse between August and December 2016, towards the end of the spawning season and overlapping the period when sex change is documented in the wild (November – May) [53]. Fish were collected from Tauranga Harbour (37° 40’ 29’ S; 176° 10’ 20’ E) by hook and line. Fifty IP fish were evenly distributed into groups across ten 500 L tanks containing recirculating seawater (35 ppt). IP individuals ranged from 149 mm – 217 mm total length (TL) and were distributed such that each tank contained a hierarchy of different sized fish plus a single TP male (size range 222 mm – 247 mm TL). Ambient light was available through semi-translucent roof panels and was supplemented with overhead fluorescent lighting (Sylvania Cool White De Luxe, Osram Sylvania Ltd) on a 12:12 light:dark daily cycle. Fish were fed a combination of thawed mussel,

Perna canaliculus, and commercial marine fish feed (Ridley Aquafeed, Ridley Corporation) to satiation three times per week.

Following an acclimation period of three weeks, TP males were removed from five of the 10 tanks (day 0), creating a permissive social environment for sex change. As a control on day 0, the largest IP fish were removed from each of the control tanks, and also served as a baseline. Subsequently, the largest IP fish per tank was removed at day 30, 50, 60, 65, and 66 post TP male removal. Fish were immediately anaesthetised in an aerated bath containing 6 ml L^{-1} 2-phenoxyethanol (Sigma Aldrich) before being euthanized by decapitation. A gonad section (mid-section of one paired gonad) and the whole brain were preserved in RNAlater (Invitrogen™, Thermo Fisher Scientific), chilled at -18°C for 24 hours, then stored at -80°C until RNA extraction. An additional gonad section (mid-section of second gonad) was fixed in either Bouin's (testis and transitional gonads) or neutral-buffered formalin (ovary), and subsequently dehydrated by submersion in ethanol (70, 80, 96 and 100%) followed by xylene. Gonadal tissue was paraffin-embedded and 3 µm sections were stained with hematoxylin and eosin (H&E) for light microscopy (New Zealand Veterinary Pathology, Hamilton) to determine sexual status. Experiments were conducted in accordance with approval from the New Zealand National Animal Ethics Advisory (2015_02).

Survey 1: Opportunistic sampling of spotty wrasse

Seven fish were caught by hook and line off Portobello Wharf, Dunedin, New Zealand, and an additional seven fish were obtained from the nearby New Zealand Marine Studies Centre, during the non-breeding season between March and May 2013. Fish were euthanized with an overdose of benzocaine (0.3g/L) and the brain and gonads dissected immediately. One gonad and the whole brain were preserved in RNAlater (Life Technologies, Inc.) on ice, before storage at -80°C until RNA extraction. The second gonad was preserved in 10% formalin for histological analysis. Gonadal tissue was paraffin-embedded and 5 µm sections were stained with H&E to determine sexual status (Histology Services Unit, University of Otago). Experiments were conducted in accordance with approval from the New Zealand National Animal Ethics Advisory (92-10).

Survey 2: Wild-caught kyusen wrasse

Fish were caught by hook and line from Oomura bay (n = 2) and Chijiwa bay (n = 29), Kyushu Island, Japan, at the end of the breeding season between September and November 2010. Fish were euthanized with an overdose of 2-phenoxyethanol and the brain and gonads dissected out immediately. A gonad section and the brain were preserved in RNAlater (Life Technologies, Inc.) on ice, or flash frozen in liquid nitrogen, before storage at -80°C until RNA extraction. An additional gonad section was preserved in Bouin's fixative for histological analysis. Gonadal tissue was paraffin-embedded and 5 µm sections were stained with H&E to determine sexual

status. Experiments were conducted in accordance with approval from the Animal Care and Use Committee of the Institute for East China Sea Research, Nagasaki University, Japan (#15-06).

Histological analysis of the gonad

For bluehead wrasse, transitioning fish were grouped into six stages as per the classification system of Nakamura, Hourigan [10]. As seasonal breeders, female spotty and kyusen wrasses were classified as either non-breeding female (NBF) or breeding female (BF), depending on the presence of maturing oocytes. Transitioning animals were classified into early transitional (ET), mid transitional (MT) or late transitional (LT) stages (see Table S1). In spotty and kyusen wrasse, the ET, MT and LT stages broadly correspond to stages 2, 3-4 and 5-6 as outlined by Nakamura, Hourigan [10] and used to classify bluehead wrasse.

RNA extraction

Due to samples being obtained from several sources and processed at different times, different extraction protocols were used and are summarised in Table S2. For spotty and bluehead brain samples, the hindbrain (corpus cerebelli, pons, and medulla) was removed prior to RNA extraction. The forebrain/midbrain was prioritised for analysis as it is expected to contain key neural circuits involved in socially regulated sex change [54].

Reverse transcription

Total RNA was quantified by Qubit 2.0 Fluorometer (Qubit RNA HS Assay Kit, Life Technologies), and RNA purity was measured by spectrophotometer (NanoDrop 2000c, ThermoFisher Scientic). Bluehead RNA was reverse transcribed in a Mastercycler Pro S thermal cycler (Eppendorf) with the following protocol: 37°C (15 mins), 85°C (5 secs), 4°C until removal. For spotty and kyusen, reverse transcription reactions were performed in a SureCycler 8800 (Agilent Technologies) with the following protocol: 25°C (10 mins), 37°C (120 mins), 85°C (5 mins), 4°C until removal. Further details are provided in Table S2.

Determination of gene sequences

Preliminary sequence data for four target genes (*cyp19a1a*, *amh*, *cyp19a1b*, and *it*) and three potential reference genes (*ef1a*, *18S*, and *g6pd*) were obtained from transcriptome assemblies for bluehead wrasse [47] and spotty wrasse (Todd et al., unpublished) representing gonad and brain tissues. Bluehead wrasse *it* and *ef1a* sequences were previously published (Genbank MF279538.1 and MF279537.1, respectively) [39]. Contigs were partially verified using PCR. PCR primers were designed against the contig sequence for each gene in bluehead and spotty wrasse using Primer3 (Untergasser et al., 2012), and are shown in Table S3. Reactions (20 µL) contained 10 ng cDNA, 1x MyTaq reaction buffer (Bioline), 1x MyTaq DNA Polymerase (Bioline), and 0.5 µM forward and reverse primers. Reactions were run in a Mastercycler Pro S thermal cycler (Eppendorf) with the following protocol: 95 °C (3 mins) followed by 35 cycles of 95 °C (30 secs), annealing at 5 °C below primer melting temperature (see Table S3) (30 secs),

and 72 °C (45 secs), with a final extension at 72 °C (15 mins). PCR products were visualised by electrophoresis through a 1% agarose gel using SYBR Safe DNA Gel Stain (Invitrogen). Amplicons of expected sizes were gel-extracted using a NucleoSpin gel and PCR clean-up kit (Macherey-Nagel). Extracted products were Sanger sequenced (Genetic Analysis Services, Department of Anatomy, University of Otago) in both directions using the respective PCR primers. For kyusen wrasse, bluehead wrasse PCR primers were used to determine partial gene sequences. Forward and reverse amplicons were aligned to create a consensus kyusen sequence for each gene in Geneious R10 [55]. Primers for *amh* and *it* did not amplify kyusen DNA. However, qPCR primers designed for bluehead wrasse were successful in kyusen, as described below.

Quantitative real-time PCR

For each gene, species-specific primers were designed nested within the verified partial gene sequences in Primer3 (see Table S4). Primers were designed to cross exon-exon boundaries to avoid amplifying residual contaminating DNA.

Quantitative real-time PCR (qPCR) was used to measure mRNA levels for each gene in either gonad or brain using the QuantStudio 5 Real-Time PCR system (ThermoFisher). All samples, including standards and negative controls, were run in duplicate (bluehead wrasse) or triplicate (all other samples) in a 96-well plate. An inter-plate calibrator (cDNA from 6 randomly chosen individuals) was run in triplicate for each spotty qPCR assay. Target gene DNA previously obtained by PCR was used to create standard curves consisting of seven 10-fold dilutions. Reactions (10 µL) contained 20 ng cDNA (except for *18S*, 0.2 ng), 1 µM forward and reverse primers, 1x SYBR® Premix Ex Taq™ II (Tli RNaseH Plus) (Takara), and 1x ROX reference dye (Takara). Bluehead wrasse samples were run without ROX reference dye. Cycling conditions were 95°C (2 mins) followed by 40 cycles of 95°C (5 secs), annealing temperature (see Table S4) (10 secs), and 72°C (5 secs). Melt curve analysis was run to verify the production of a single product which was then confirmation-sequenced (Genetic Analysis Services, Department of Anatomy, University of Otago). Further qPCR details are supplied in a MIQE table (see Table S4).

Statistical Analysis

Due to non-normality of the raw qPCR data, the non-parametric Kruskal-Wallis test was used, followed by *post hoc* comparisons using Dunns tests, with Benjamini Hochberg correction for multiple comparisons, in R [56] (Data S1 and S2). Expression of *ef1a, 18S* and *g6pd* as well as the geometric mean of all possible combinations, was tested for use as reference genes. The reference gene(s) showing no significant difference in distribution across sexes, and the flattest expression profile across sexes was chosen to normalise the results (see Table S5 for chosen reference genes). Results are presented for un-normalised data, due to all reference genes (and combinations) showing a significant difference in distribution across sexes in one experiment,

and the normalisation drastically changing the trend of the results in another (see Table S5). Results for normalised data are available as supplemental materials (see Fig. S1 for gonadal genes and Fig. S2 for brain genes). For each experiment, graphed results are presented as expression relative to control females (i.e. all other sample quantities are expressed as an n-fold difference relative to the control female group).

Phylogenetic Analysis

Robust fine-scale phylogenies support comparative analyses of labrids [49]. However, as these do not yet include the spotty wrasse, we undertook phylogenetic analyses to resolve the relationship of spotty wrasse to the bluehead and kyusen wrasses. Sequences of the 12S and 16S mitochondrial ribosomal genes were produced for all three species, using PCR primers from Westneat and Alfaro [50], and they were combined with sequences from 296 labrid taxa analysed in Baliga and Law [49] (kindly provided by Dr. Vikram Baliga). Genomic DNA was extracted from ovary (kyusen and bluehead wrasse) and liver (spotty) samples using a standard lithium-chloride protocol [57]. Mitochondrial ribosomal genes *12S* and *16S* were PCR-amplified using reactions (20 µL) containing 10 ng DNA, 1x NH_4 reaction buffer (Bioline), 1x BIOTAQ DNA Polymerase (Bioline), 2mM $MgCl_2$ solution, 1mM dNTP mix, and 1 µM forward and reverse primers. Reactions were run in a Sure Cycler 8800 (Agilent Technologies) with the following protocol: 94 °C (2 mins) followed by 30 cycles of 94 °C (30 secs), 60 °C (12S) or 49 °C (16S) (30 secs), and 72 °C (55 secs), with a final extension at 72 °C (2 mins). PCR products were visualised by electrophoresis through a 1% agarose gel using SYBR Safe DNA Gel Stain (Invitrogen), purified using AcroPrep Advance 96-well filter plates (Pall Corporation), and Sanger sequenced in both directions using the respective PCR primers (Genetic Analysis Services, Department of Anatomy, University of Otago).

Phylogenetic relationships within the Labridae were reconstructed using Bayesian inference in MrBayes 3.2.6 [58], using the CIPRES Science Gateway v3.3. *12S* and *16S* sequences were concatenated following determination of the best-fit model of nucleotide substitution for each gene (GTR + I + G, based on AIC, BIC and DT scores) in jModelTest 2.0 [59] (Data S3). A partitioned analysis was carried out with four separate runs, each from a different random starting tree. Default settings were used as priors, and four Markov chains were sampled every 10, 000 generations over 71.2 million Markov chain Monte Carlo generations. Convergence was supported by the average standard deviation of split frequencies of independent runs falling below 0.01. Bayesian posterior probabilities were calculated after discarding the first 25% of sampled trees burn-in. The 50% majority rule consensus tree was prepared in FigTree v1.4.3 (http://tree.bio.ed.ac.uk/).

Results

Labridae phylogeny

Spotty wrasse was placed with strong statistical support (>90% bootstrap support) within the Pseudolabrines, together with other *Notolabrus* spp. This group is resolved as sister to the Labrichthyines and Julidines, which contains the bluehead and kyusen wrasse. Our analysis places the Labrichthyines as sister to the Julidines, as in previous labrid phylogenies (Westneat and Alfaro [50] Cowman, Bellwood [60]), whereas the Baliga and Law [49] topology positions the Labrichthyines within the Julidines.

Sex change

Experiment 1: Social induction of sex change in wild bluehead wrasse

Social manipulations successfully induced sex change in wild female bluehead wrasses, and form part of whole-transcriptome analyses described elsewhere [39, 47]. Three samples representative of each sex change stage, plus control females, TP and IP males were analysed herein (Figure 3).

Experiment 2: Social induction of sex change in captive spotty wrasse

Removal of TP males readily induced sex change in captive female spotty wrasse (Figure 4). Histological analysis revealed that in the manipulated tanks (TP male removed), 15 fish reached ET stage (day 30 n = 2, day 50 n = 3, day 60 n = 4, day 65 n = 3, day 66 n = 3), one reached MT stage (day 50), one LT stage (day 50), and one was classified as fully TP male (day 60). Only four females within the manipulated tanks showed no histological signs of sex change upon sampling (day 30 n = 2, day 66 n = 2). There was no conclusive evidence of sex change by females in control tanks (TP male present). However, four control females (day 30 n = 3, day 66 n = 1) showed evidence of early ovarian atresia indicative of an ET stage, although this may represent normal atresia following the breeding season. Across the entire experiment, five of the original 53 IP fish were found to be IP males after histological analysis.

Survey 1: Opportunistic sampling of spotty wrasse

Among the opportunistically caught spotty wrasse, fish were found at a range of stages (NBF n = 6, ET n = 3, MT n = 2, LT n = 2, TP male n = 1) (Figure 5).

Survey 2: Wild-caught kyusen wrasse

Wild-caught kyusen wrasse were mostly females (NBF n = 7) and TP males (TP n = 11), plus a few ET females (n = 3) and IP males (n = 3) (Figure 6). The ET fish were sampled in late September.

Quantitative real-time PCR

Gonad: cyp19a1a

In all three species, *cyp19a1a* expression was highest in ovaries and near-zero in TP and IP male testes (Figure 7). In bluehead and spotty wrasses, *cyp19a1a* expression decreased across progressive sex change stages.

359
360 *Experiment 1: Social induction of sex change in wild bluehead wrasse*
361 Sex had a significant effect on *cyp19a1a* expression ($X^2(8) = 25.08$, $p < 0.01$). At the onset of
362 behavioural sex change (stage 1), a spike in *cyp19a1a* expression occurred (median 1.8 fold
363 higher than control females (CF)), followed by near zero expression from stage 2 onwards (onset
364 of ovarian atresia). There was a significant difference in the distribution of *cyp19a1a* expression
365 between control females and TP (median 0.0001-fold that of CF, $p < 0.05$) and IP males (median
366 0.00007-fold that of CF, $p < 0.05$), but not between control females and stages 1 – 6. However,
367 there was a clear trend of decreasing expression (Figure 7).
368
369 *Experiment 2: Social induction of sex change in captive spotty wrasse*
370 Sex significantly affected *cyp19a1a* expression ($X^2(7) = 38.35$, $p < 0.0001$). Decreased
371 *cyp19a1a* expression was first observed among females at the ET stage (median 0.3-fold lower
372 than C BF D0, non-significant; median 0.28-fold lower than C BF, $p < 0.01$). The single MT fish
373 had a median *cyp19a1a* expression 0.25-fold that of C BF D0, while the single LT individual had
374 near-zero *cyp19a1a* expression (median 0.005-fold that of C BF D0). Distribution of gonadal
375 *cyp19a1a* expression was significantly reduced in TP and IP male testes compared with ovaries
376 of all control females (median in both males 0.02-fold that of C BF D0, $p < 0.01$).
377
378 *Survey 1: Opportunistic sampling of spotty wrasse*
379 Sex did not have a significant effect on *cyp19a1a* expression ($X^2(7) = 9.23$, $p = 0.06$). However,
380 a clear trend was observed with *cyp19a1a* expression at near-zero levels in MT (median 0.004-
381 fold that of NBFs) and LT stage fish (median 0.03-fold that of NBFs), and in the single TP male
382 (median 0.003-fold that of NBFs). Gonadal *cyp19a1a* expression in three ET samples ranged
383 from 0.3 to 4.3-fold higher than that seen among the NBFs.
384
385 *Survey 2: Wild-caught kyusen wrasse*
386 Sex significantly affected *cyp19a1a* expression ($X^2(3) = 17.02$, $p < 0.001$). There was no
387 difference in *cyp19a1a* expression between NBFs and the three samples staged as ET. Although
388 *cyp19a1a* expression was near-zero in TP and IP males (both median 0.1-fold that of NBF), only
389 TP males showed a significant difference in distribution compared with females ($p < 0.01$).
390
391 *Gonad: amh*
392 Gonadal *amh* expression showed a pattern opposite to that of *cyp19a1a*. In all three species, *amh*
393 expression was near-zero in females and highest in TP males, with a clear trend of increasing
394 expression across sex change (Figure 7). In all experiments, sex had a significant effect on *amh*
395 expression (bluehead wrasse: $X^2(8) = 21.46$, $p < 0.01$, spotty wrasse experiment: $X^2(7) = 33.18$,
396 $p < 0.0001$, spotty wrasse survey: $X^2(4) = 9.63$, $p < 0.05$, kyusen wrasse: $X^2(3) = 18.46$, $p <$
397 0.001).
398

Experiment 1: Social induction of sex change in wild bluehead wrasse

Increased *amh* expression was obvious from stage 2 (median 3-fold higher than CF), and steadily increased to a significantly higher distribution in TP (median 13-fold higher than CF, $p < 0.05$) and IP males (median 18-fold higher than CF, $p < 0.05$).

Experiment 2: Social induction of sex change in captive spotty wrasse

There was a trend of progressive *amh* upregulation beginning in the single fish staged as MT (20-fold higher than C BF D0), continuing in the LT individual (47-fold higher than C BF D0) and reaching a significantly higher distribution in TP males (median 36-fold higher than C BF D0, $p < 0.01$). IP males showed a distribution of *amh* expression intermediate to that of all fish with an intact ovary, and TP males (median 7.23-fold higher than C BF D0).

Survey 1: Opportunistic sampling of spotty wrasse

Despite a significant effect of sex on *amh* expression, *post hoc* analysis showed no significant differences between individual sex stages. ET fishes showed similar expression levels to NBFs, while MT and LT stage fish showed a trend of increased *amh* expression (median 10-fold and 20-fold higher than NBFs, respectively). Expression of *amh* in the single TP male was 3.7-fold higher than in NBFs.

Survey 2: Wild-caught kyusen wrasse

ET fish showed similar *amh* expression to NBFs (median 2-fold higher). TP males had a significantly higher distribution of *amh* expression (88-fold higher than NBF, $p < 0.001$), while *amh* mRNA levels in IP male were intermediate to those of NBFs and TP males (35-fold higher than NBFs).

Brain: cyp19a1b

For all three species, sex did not have a significant effect on *cyp19a1b* expression (bluehead wrasse: $X^2(8) = 13.64$, $p = 0.09$, spotty experiment: $X^2(7) = 2.88$, $p = 0.90$, spotty survey: $X^2(4) = 3.13$, $p = 0.54$, kyusen wrasse: $X^2(3) = 3.00$, $p = 0.28$) (Figure 8). However, in the bluehead wrasse a subtle trend is evident similar to that of *cyp19a1a* expression in the gonad; expression peaks transiently at stage 1 (median 1.8 fold higher than CF), then decreases at stage 2 (median 0.3-fold that of CF), remaining at low levels at subsequent sex change stages and in TP (median 0.4-fold that of CF) and IP males (median 0.3-fold that of CF). No clear trends in *cyp19a1b* expression were evident in brain samples from spotty or kyusen wrasses.

Brain: it

In bluehead wrasse, sex did have a significant effect on *it* expression ($X^2(8) = 18.12$, $p < 0.05$). However, *post hoc* analysis showed no significant differences between individual sex stages. There was a trend of increasing *it* expression in fore/midbrain of bluehead wrasse, beginning at stage 1 (median 2-fold higher than CF) and progressively increasing to highest levels in TP

males (median 3-fold higher than CF). IP male *it* expression was similar to that of control females (median 1.1-fold higher than CF). In spotty and kyusen wrasses, sex did not have a significant effect on *it* expression (spotty experiment: $X^2(7) = 2.82$, p = 0.90, spotty survey: $X^2(4) = 4.00$, p = 0.98, kyusen wrasse: $X^2(3) = 1.87$, p = 0.60), nor were there any clear trends.

Discussion

In order to understand sex change from a functional and evolutionary standpoint, an important question is to what degree genetic systems regulating sex change are conserved or different among species. Using a comparative qPCR approach across three wrasse species which share protogyny as an ancestral state, we investigated the roles of *cyp19a1a* and *amh* as proximal regulators of gonadal sex change, and *cyp19a1b* and *it* as regulators of behavioural sex change in the brain. We evaluate whether these genes may form part of a conserved molecular machinery underlying protogynous sex change in wrasses, and whether any differences exist among tropical and temperate species differing in the seasonality of sex change and the rigidity of social hierarchies.

Gonadal sex change - *cyp19a1a* and *amh* as proximal regulators

In protogynous hermaphrodites, interrupted *cyp19a1a* expression has been suggested as the molecular switch that initiates ovarian atresia and gonadal sex change [6, 38]. Experimental studies have shown that treatment of adult females with aromatase inhibitors can induce complete sex change, both in year-round [11, 12] and seasonal [13, 14, 61] breeders. However, whether *cyp19a1a* downregulation acts as a proximal switch initiating natural gonadal sex change broadly in protogynous species has been unclear. Firstly, because prior studies in other species have not examined atretic ovaries from females during earliest sex change, i.e. before proliferation of male tissues, they could not confirm whether *cyp19a1a* downregulation occurs coincidentally with the initiation of gonadal sex change in year-round [62, 63] and seasonal [61, 64] breeders. Secondly, in seasonally-breeding species where sex change proceeds from an already regressed ovary with lower oestrogen production and aromatase activity [65], *cyp19a1a* downregulation may be less important.

Our spotty and kyusen wrasse samples include early transitioning females with advanced ovarian atresia prior to the appearance of male tissues (ET stage). In spotty wrasse socially manipulated to change sex in captivity, *cyp19a1a* mRNA levels in ET ovaries are intermediate to those of control breeding females and males (Figure 7B). In both species caught from the wild, *cyp19a1a* mRNA levels in ET ovaries are within the range recorded for non-breeding females (Figure 7C and D). Therefore, downregulation of aromatase expression compared to breeding females appears an important event in the initiating stages of gonadal sex change in temperate and tropical wrasses alike. However, unlike bluehead wrasse, many spotty and kyusen non-sex changing females had near-zero *cyp19a1a* mRNA levels and this may reflect the seasonal atresia that occurs in these temperate species following the breeding season. Thus, for temperate

wrasses that breed seasonally, reduced aromatase expression is not a conclusive marker to distinguish early stage sex changers from non-breeding females with atretic ovaries. Due to this seasonal atresia, there is also a degree of ambiguity in delineating early sex changers from non-breeding individuals with atretic ovaries.

Amh is an important early initiator of male phenotype in fishes, regulating germ cell proliferation and differentiation in the testis of numerous species [66-69]. Our data indicate that *amh* is upregulated during sex change with significantly greater *amh* expression evident in TP males compared to that of females in all three wrasses. Testicular production of Amh occurs primarily in Sertoli cells surrounding type A undifferentiated spermatogonia, where it suppresses germ cell proliferation and differentiation as well as steroidogenesis in the interstitial Leydig cells [66, 70]. As such, *amh* expression may be expected to increase with spermatogonial recruitment during sex change. Increased numbers of spermatogonial cysts were observed throughout the developing testis in all three wrasses. Therefore, our data support *amh* as an important proximal regulator of the male phenotype in protogynous wrasses.

In all three wrasses, *amh* was upregulated coincidentally with the downregulation of *cyp19a1a* at early sex change from IP female to TP male. An inverse relationship between *amh* and *cyp19a1a* expression is widely reported in fishes [66], including zebrafish (*Danio rerio*) [71, 72], Japanese flounder (*Paralichthys olivaceus*) [73], pejerrey (*Odontesthes bonariensis*) [74], Southern flounder (*Paralichthys lethostigma*) [75] and rainbow trout (*Oncorhynchus mykiss*) [76]. Together, these data suggest a bidirectional antagonism between *amh* and *cyp19a1a* may operate to control sexual fate in fishes [6], presumably acting within a broader antagonistic framework between core feminising (e.g. *cyp19a1a*, *foxl2*, *wnt4*) and masculinising (e.g. *dmrt1*, *sox9*, *amh*) gene networks known to be responsible for directing and maintaining sexual fate in vertebrates [77]. Furthermore, because *amh* upregulation was a consistent feature of sex changers from at least mid-transitional stages, *amh* upregulation may be a more useful marker of transitioning phenotypes than downregulation of *cyp19a1a*, which could not distinguish sex-changers from non-breeding females.

Aromatase and isotocin in the brain

In species where sex change is socially cued, complex neurochemical changes in the brain presumably translate visual social information into behavioural and reproductive responses necessary for sex change. Prior work has identified several neuropeptides as likely regulators of behavioural sex change in social tropical wrasses, including arginine vasotocin, Cyp19a1b, It, and gonadotropin-releasing hormone (reviewed in [35, 78, 79]).
Our data do not strongly support a prominent role for *cyp19a1b* in initiating behavioural sex change in protogynous wrasses. In spotty and kyusen wrasses, *cyp19a1b* expression was neither sex-specific nor showed any clear trend across sex change. In bluehead wrasse, although *cyp19a1b* expression clearly decreased with sex change, the trend was non-significant and only

noticeable from stage 2, after behavioural changes first occur at stage 1. Likewise, Black, Balthazart [80] found whole brain aromatase activity declined only after behavioural changes in female-to-male sex change of the bluebanded goby (*Lythrypnus dalli*). Expression of *cyp19a1b* in bluehead wrasse fore/midbrain closely parallels gonadal *cyp19a1a* mRNA levels, and may reflect peripheral changes in E2 via putative oestrogen response elements in the *cyp19a1b* promotor [36]. However, exogenous E2 treatment of bluehead wrasse stimulated brain *cyp19a1b* expression and prevented behavioural sex change under socially permissive conditions [37]. Brain gene expression patterns are highly heterogeneous and it remains possible that localised *cyp19a1b* expression changes are important, but would not be detected in studies at a whole brain or fore/midbrain level.

Isotocin plays an important role in modulating teleost socio-sexual behaviours, and social dominance hierarchies in particular [34, 81, 82]. Our data for bluehead wrasse show that *it* expression is TP male biased and upregulated across sex change, beginning with a median 2-fold increase at stage 1 when behavioural changes first occur. Our qPCR data validate the same patterns reported in recent whole-transcriptome analyses in this species [38, 39]. In tropical wrasses, stage 1 is characterised by rapid (minutes to hours) increases in aggression and male-typical courtship behaviours in transitioning females [5] that are presumably critical for establishing dominance as the new TP male before gonadal sex change ensues. An opposite pattern for *it* was observed in the bluebanded goby, a bidirectional hermaphrodite in which high social status is also a critical cue for female-male sex change, yet isotocin-immunoreactive cells in the pre-optic area decreased across female-male sex change [83]. These data and studies in social cichlids indicate isotocin can have species-specific and context-dependent roles in social behaviour [84-86]. Expression of *it* was not associated with sexual phenotype or sex change in spotty or kyusen wrasses. Although our social manipulation experiment provides the first evidence confirming sex change is socially cued in spotty wrasse, behavioural markers of sex change have not been characterised in either species. Overall, our data and those of Black, Reavis [83] support isotocin as an important proximal mediator of behavioural transitions in sequential hermaphrodites with strict social hierarchies. Further work is necessary to clarify whether *it* also regulates socially-cued sex change in temperate wrasses, and may show seasonal fluctuations.

Conclusions

This research investigated whether evolutionarily conserved molecular mechanisms underlie protogynous sex change in wrasses. In this first comparison of candidate gene expression in tropical versus temperate protogynous species, we find both conservation and diversity in the regulatory machinery underlying sex change. Our data support conserved roles for *cyp19a1a* and *amh* as important proximal regulators of gonadal sex change in protogynous wrasses - these genes may act concurrently to orchestrate the ovary-testis transition by controlling ovarian atresia and testicular development, respectively. However, differences in timing of expression

changes relative to the appearance of male tissues may reflect differences between tropical and temperate species in the seasonality or duration of sex change. In the brain, our data do not support a role for brain aromatase, *cyp19a1b*, in initiating behavioural sex change, as expression changes for this gene trailed rapid behavioural changes. Brain isotocin expression strongly correlated with TP male-specific behaviours and the rapid behavioural changes characterising the onset of sex change in the bluehead wrasse, but not spotty or kyusen wrasses. Characterising behavioural and molecular markers of sex change in temperate wrasses will be important for understanding how visual social cues are transduced to initiate the sex change cascade. Future work employing macro-dissection of the brain will be important, as our sampling of the whole brain or fore/midbrain may have obscured important region-specific signals. Future manipulative experiments will also be important in determining specific functions of these genes in regulating sex change.

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Figure 1

Life history of protogynous wrasses

(**A**) Generalised life cycle of protogynous fishes. Juveniles with a bipotential gonad undergo primary sexual development as either initial phase (IP) females or males. Terminal phase (TP) males develop via sex change by IP females, or role change by IP males, following appropriate social cues. Figure adapted from [39]. (**B**) Progression of sex change in bluehead, spotty and kyusen wrasses. Sex change in the bluehead wrasse is classified into 6 stages as previously described [10], and occurs remarkably fast; behavioural change occurs within 0.5-2 days and complete ovary-to-testis transformation is completed in 8-10 days [5]. Sex change in spotty and kyusen wrasses is classified into early, mid and late stages, broadly corresponding to stages 2, 3-4 and 5-6 in the bluehead wrasse, respectively. Sex change in these seasonal breeders may take up to several months. Figure adapted from [38]. Bluehead wrasse male image open access by Evan D'Alessandro, courtesy Oregon State University; bluehead wrasse female image open access; spotty male image by JT; spotty female image with permission by Allan Burgess fishingmag.co.nz ; kyusen images with permission by Keoki Stender, www.marinelifephotography.com .

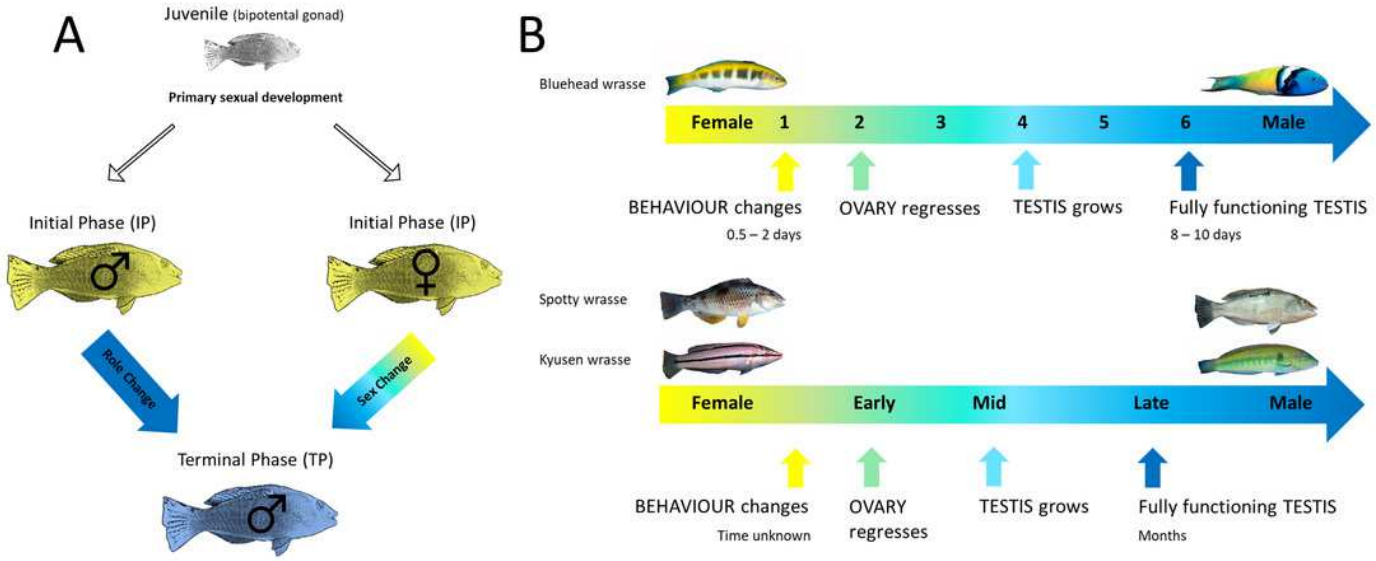


Figure 2(on next page)

Majority rule consensus tree from Bayesian MCMC analyses.

The tree is simplified to show relationships between the bluehead, spotty, and kyusen wrasses. Unlabelled nodes have Bayesian posterior probabilities > 0.90. Tip labels are the species or genus names, with the number of species sampled in brackets. A triangular tip indicates the clade has been collapsed.

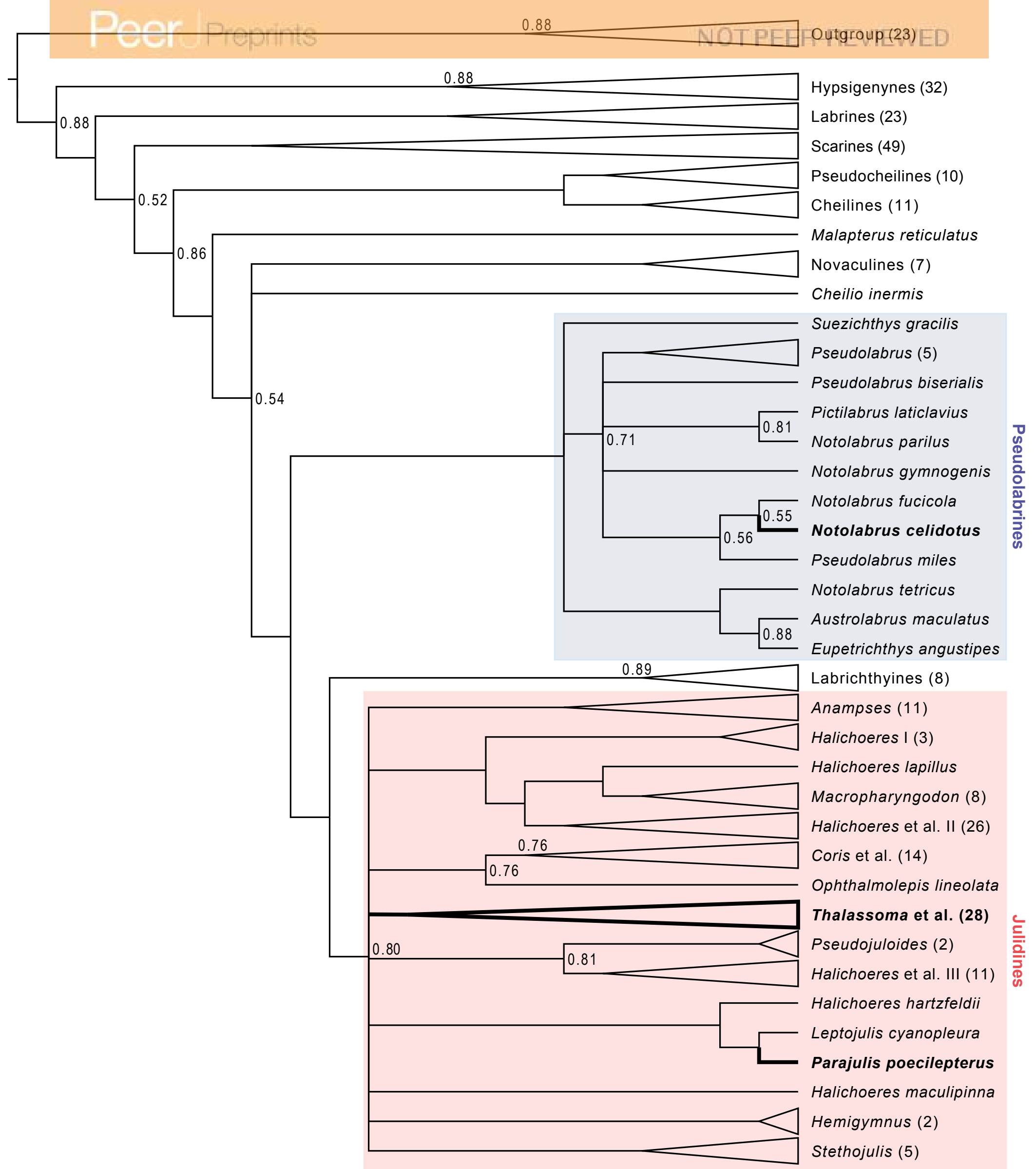
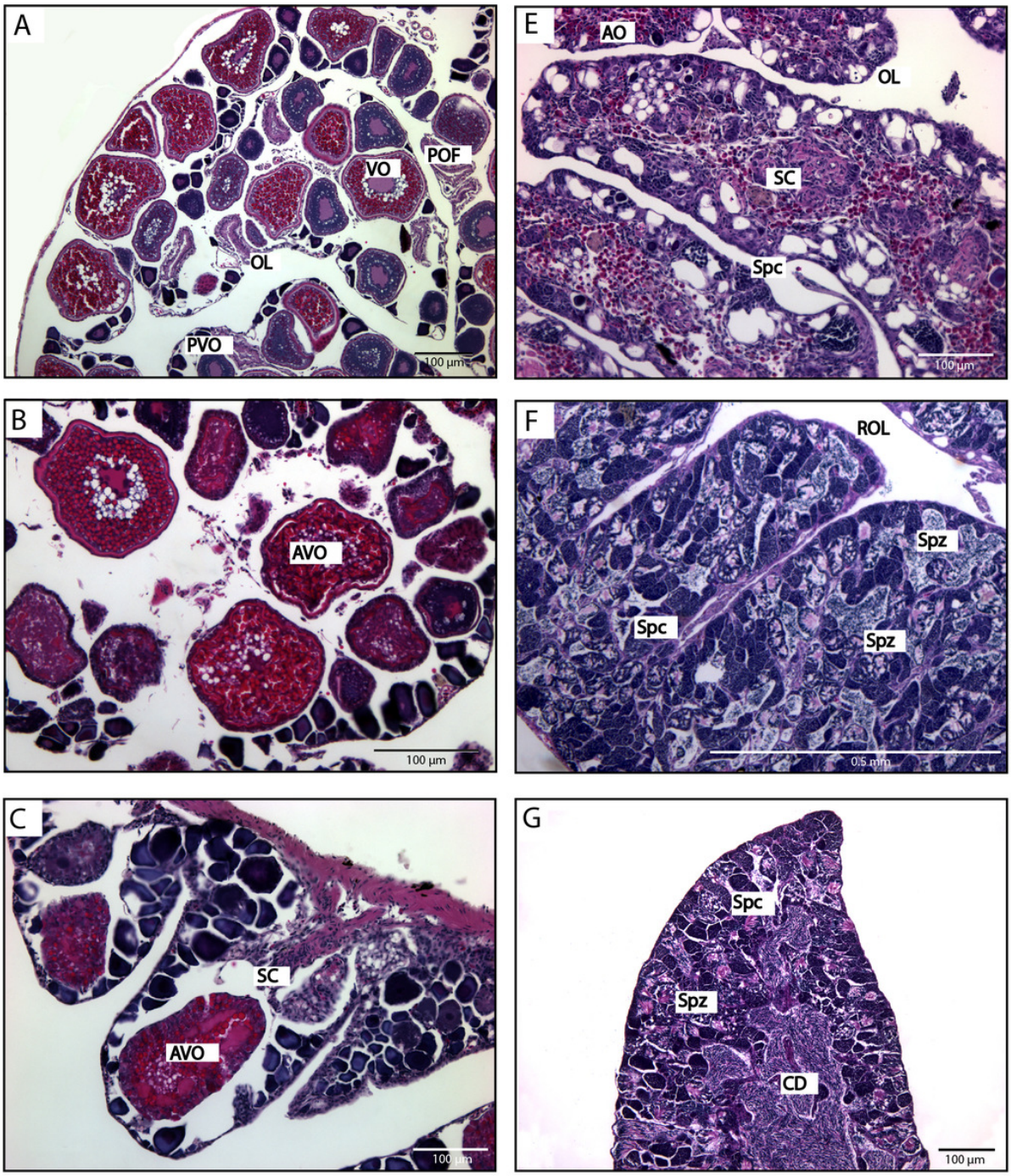


Figure 3

Histological stages of gonadal sex change in the bluehead wrasse

(**A**) Stage 1, breeding female with mature ovary containing pre-vitellogenic and vitellogenic oocytes. (**B**) Stage 2, atresia of vitellogenic oocytes. (**C**) Stage 3, atresia of pre-vitellogenic and vitellogenic oocytes and clustering of stromal cells. (**D**) Stage 4, proliferation of presumed spermatogonia. (**E**) Stage 5, spermatogenesis begins. (**F**) Stage 6, mature testis with spermatozoa and a residual ovarian lumen. (**G**) Initial phase male containing spermatozoa, where absence of a residual ovarian lumen suggests this fish has not sex changed and is an initial phase male. Scale bar, 100 µm (A, B, C, D, E, G), 0.5 mm (F). Stages follow the classification of [10].



Abbreviations for wrasse histology
Oog = oogonia
PVO = previtellogenic oocyte
VO = Vitellogenic oocyte
AVO = atretic vitellogenic oocyte
AO = atretic oocyte
Spg = spermatogonia
PSpg = presumed spermatogonia
Spc = spermatocytes
Spz = spermatozoa
OL = ovarian lumen
ROL = Residual ovarian lumen
CD = Collecting duct
ECD = Efferent collecting duct
SC = stromal cells
YBB = yellow brown bodies
POF = Post ovulatory follicle

Figure 4

Time course of sex change in the spotty wrasse following social manipulation in captivity (Experiment 2)

Points represent the sex change stage of each sampled female on each sampling day. Blue circles are samples from control tanks (TP male present - non-permissive environment), and red triangles from manipulated tanks (TP male removed - permissive environment).

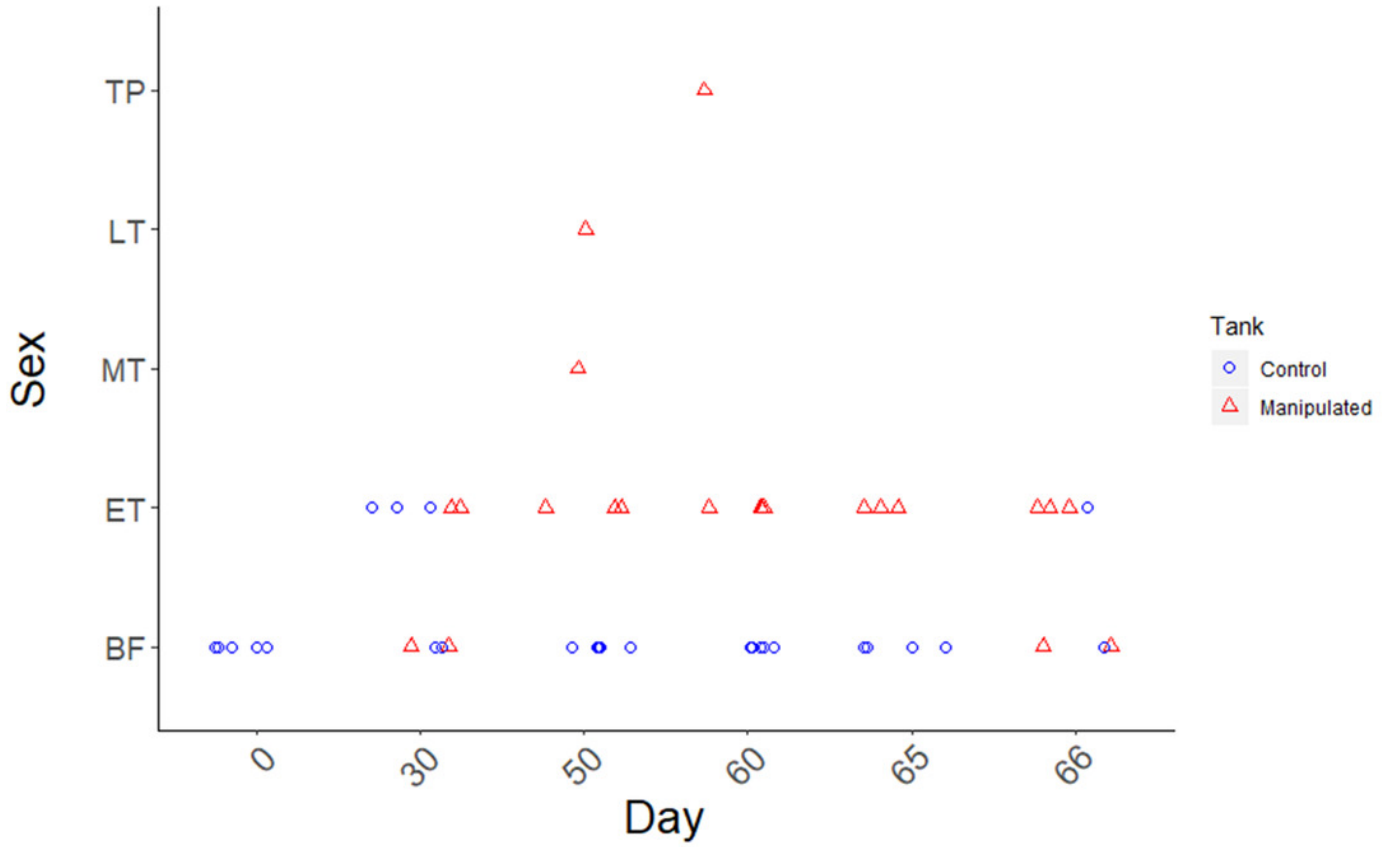


Figure 5

Histological stages of gonadal sex change in the spotty wrasse.

(**A**) Breeding female with pre-vitellogenic and vitellogenic oocytes, (**B**) early transitional; atresia of oocytes. (**C**) Mid transitional; oocyte numbers diminished and ovarian follicles were largely atretic, with proliferation of spermatogonia. (**D**) Late transitional; spermatogenic cysts predominate over atretic oocytes. (**E**) Terminal phase male; mature testis with spermatozoa in cysts arranged into seminiferous tubules with presence of a residual ovarian lumen. (**F**) Initial phase male containing spermatozoa, where absence of a residual ovarian lumen suggests this fish has not sex changed and is an initial phase male. Scale bar, 0.1 mm. See Fig. 3 for abbreviations.

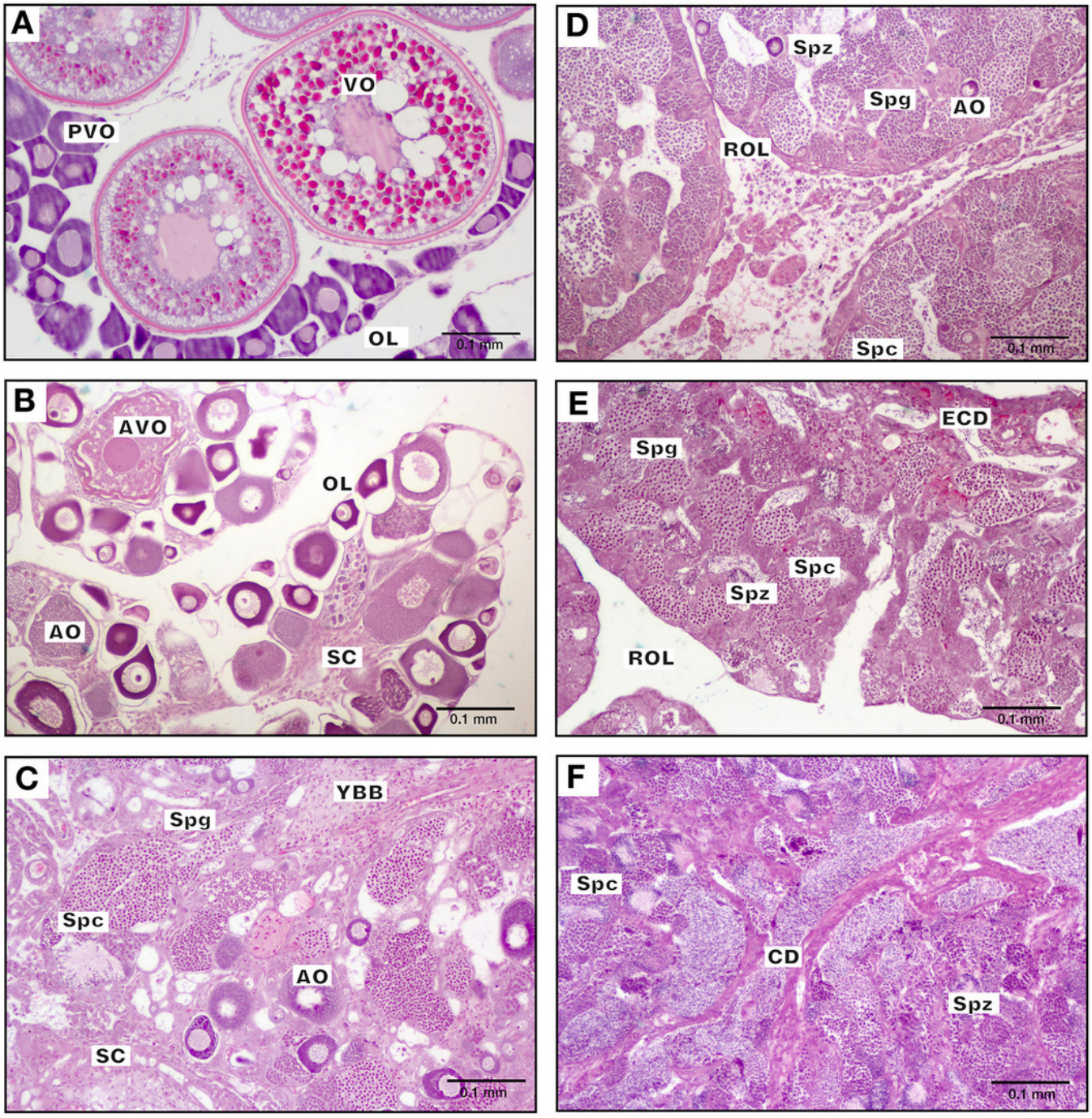


Figure 6

Histological stages of gonadal sex change in the kyusen wrasse

(**A**) Non-breeding female with pre-vitellogenic oocytes. (**B**) Early transitional; atretic oocytes. (**C**) Late transitional; proliferation of spermatogonia. (**D**) Terminal phase male; mature testis with lobular structure and a residual ovarian lumen. Scale bar, 100 μm (A, B), 50 μm (C, D). See Fig. 3 for abbreviations.

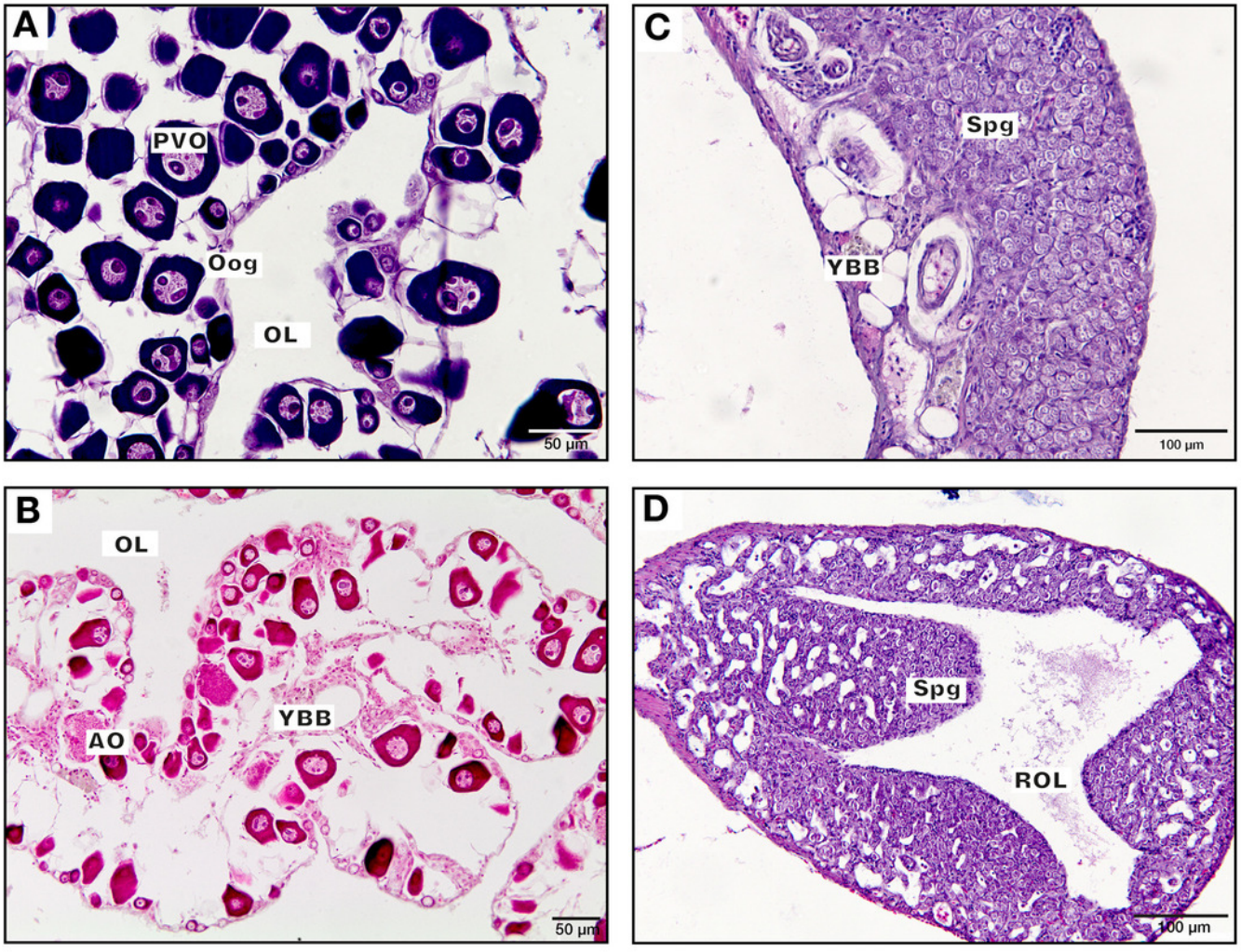


Figure 7 (on next page)

Relative gonadal expression of *cyp19a1a* (left) and *amh* (right) mRNA.

Expression levels are compared among females, transitioning fish, TP males and IP males. (**A**) Bluehead wrasse induced to change sex in the wild (Experiment 1). (**B**) Spotty wrasse induced to change sex in captivity (Experiment 2). (**C**) Wild-caught spotty wrasse (Survey 1). (**D**) Wild-caught kyusen wrasse (Survey 2). Points represents individual fish. Boxplots represents the median, lower and upper quartile values, and 1.5-fold the interquartile range. Yellow, blue and grey points indicate expression is significantly female-biased, male-biased, and non-significantly different, respectively. Letters denote a significant difference in distribution between groups and 'a' indicates overall significance without significant pairwise. Sample sizes: bluehead wrasse n = 3, all groups; spotty wrasse socially induced to change sex in captivity C BF D0 n = 5, C BF n = 16, M BF n = 4, ET n = 20, MT n = 1, LT n = 1, TP n = 11, IP n = 5; spotty wrasse opportunistically caught NBF n = 6, ET n = 3, MT n = 2, LT n = 2, TP n = 1; kyusen wrasse NBF n = 7, ET n = 3, TP n = 11, IP n = 3. Abbreviations: C BF D0 = breeding female from control tank (TP male present) at experiment day 0, C BF = breeding female from control tank (TP male present) removed at progressive time points throughout the experiment, CF = control female, ET = early transitional, IP = initial phase male, LT = late transitional, M BF = breeding female from manipulated tanks (TP male removed) removed at progressive time points throughout experiment, MT = mid transitional, NBF = non-breeding female, S1-6 = stages 1-6, TP = terminal phase male.

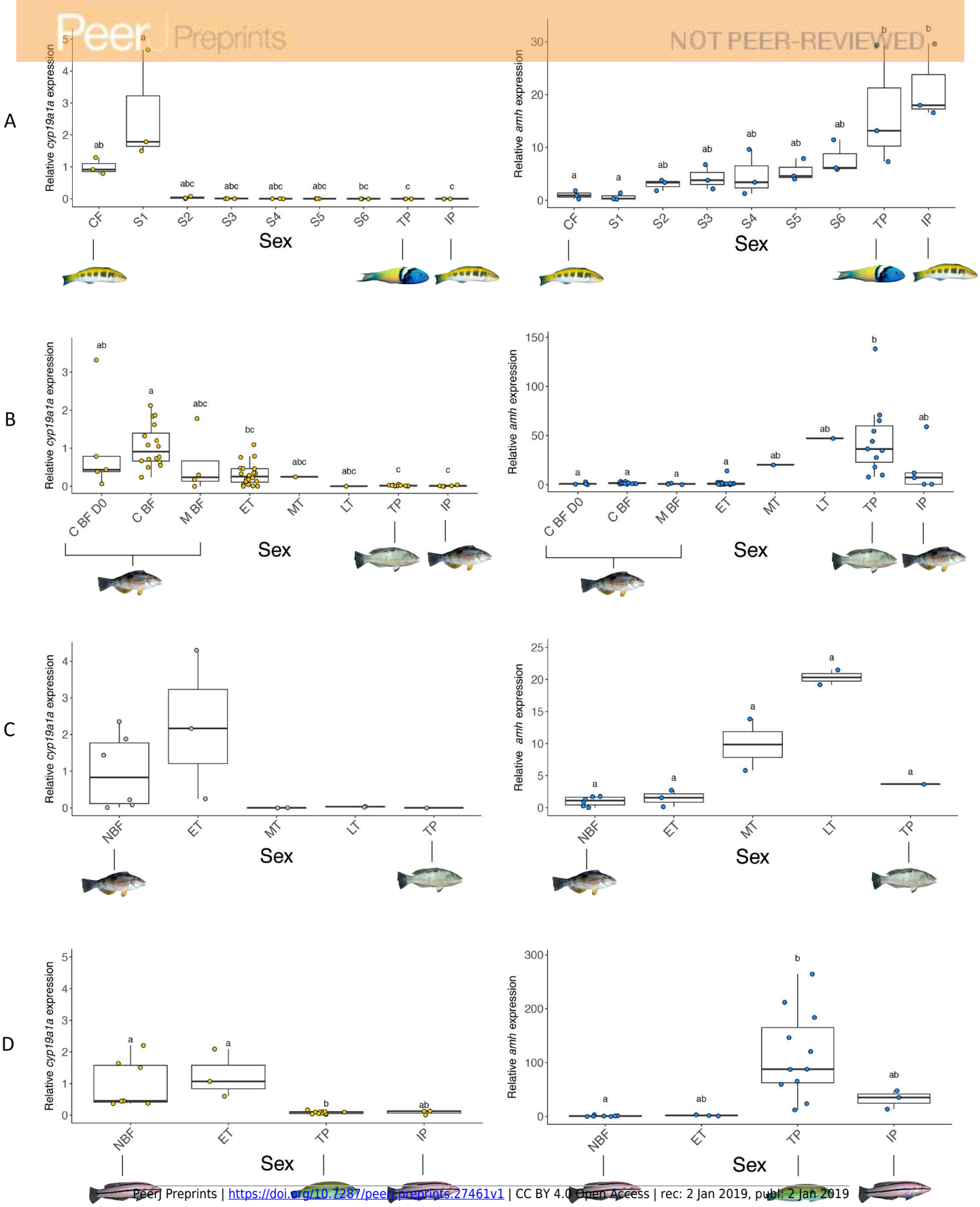


Figure 8(on next page)

Relative brain expression of *cyp19a1b* (left) and *it* (right) mRNA.

Expression levels are compared among females, transitioning fish, TP males and IP males. (**A**) Bluehead wrasse induced to change sex in the wild (Experiment 1). (**B**) Spotty wrasse induced to change sex in captivity (Experiment 2). (**C**) Wild-caught spotty wrasse (Survey 1). (**D**) Wild-caught kyusen wrasse (Survey 2). Points represents individual fish. Boxplots represents the median, lower and upper quartile values, and 1.5-fold the interquartile range. Yellow, blue and grey points indicate expression is significantly female-biased, male-biased, and non-significantly different, respectively. Letters denote a significant difference in distribution between groups and 'a' indicates overall significance without significant pairwise. Sample sizes: bluehead wrasse n = 3 all groups; spotty wrasse socially induced to change sex in captivity C BF D0 n = 5, C BF n = 16, M BF n = 4, ET n = 20, MT n = 1, LT n = 1, TP n = 11; IP n = 5, spotty wrasse opportunistically caught NBF n = 6, ET n = 2, MT n = 2, LT n = 3, TP n = 1; kyusen wrasse NBF n = 6, ET n = 5, TP n = 7, IP n = 4. Abbreviations: C BF D0 = breeding female from control tank (TP male present) experimental day 0, C BF = breeding female from control tank (TP male present) removed at progressive time points throughout the experiment, CF = control female, ET = early transitional, IP = initial phase male, LT = late transitional, M BF = breeding female from manipulated tanks (TP male removed) removed at progressive time points throughout experiment, MT = mid transitional, NBF = non-breeding female, S1-6 = stages 1-6, TP = terminal phase male.