Title: 'Sex change in the New Zealand spotty wrasse (*Notolabrus celidotus*), a temperate model species'

Running title (40 characters max.): Sex change in *Notolabrus celidotus*

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Abstract

Many studies of vertebrate sex change focus on subtropical and tropical teleosts. This study presents the protogynous New Zealand spotty wrasse, *Notolabrus celidotus*, as a temperate model. Captive fish were induced to change sex using either aromatase inhibition or manipulation of social groups. The endocrine and genetic cascade underlying this process was investigated using time-series sampling coupled with histological staging, sex steroid quantification and nanoString nCounter mRNA analysis. Seasonality affected sex ratios and sex steroid profiles; the likelihood of sex change increased when social manipulations were performed outside of the breeding season. Early-stage decreases in plasma 17β-estradiol (E2) concentrations or gonadal aromatase (*cyp19a1a*) expression were not detected in spotty wrasse, despite these being associated with the onset of sex change in many protogynous hermaphrodites. Gonadal expression of 21 candidate genes was examined in relation to gonadal histology and sex steroid concentrations across sex change. When compared to other species, some genes previously implicated in sex determination and differentiation showed typical sex-specific gonadal expression patterns (*foxl1*, *dmrt1*, *amh*), while other critical male- and female-pathway genes exhibited unexpected patterns (*cyp19a1a*, *rspo1*, *sox9a*). Moreover, expression of the masculinising factor *amh* (anti-Müllerian hormone) increased during early sex change, implying a potential role as a proximate trigger for sex change. Dynamic expression of DNA methyltransferase genes suggested a key role of epigenetic regulation during the ovary-to-testis transformation in this species. Collectively, these data provide a foundation for the spotty wrasse as a new teleost model to study sex change and cell fate in vertebrates.

Introduction

For most vertebrates, sex is genetically determined and remains constant throughout life. However, in reptiles and teleost fishes sexual state is often plastic. The direction and process of sex change differs greatly between species and taxa. Social environment and community structure tend to regulate sequential sex change, which is unique to teleosts (Reavis & Grober, 1999; Solomon-Lane et al. 2013; Sprenger et al., 2012). The fact that laboratory manipulation of social structure may induce natural sex change makes them convenient models to understand the mechanistic drivers of this transformation. These fish present *in vivo* opportunities to examine cell fate pathways, brain plasticity, and epigenetic regulation of life-history trajectory and reproductive status.

Current sex change research mostly focuses on tropical and warm-acclimated models within the Labridae (Godwin et al., 1996; Kojima et al., 2008; Lamm et al., 2015; Liu et al., 2017; Nakamura et al., 1989; Nozu et al., 2009; Ohta et al., 2003), Serranidae (Alam et al., 2008; Bhandari et al., 2003; Bhandari et al., 2005; Li et al. 2007; Chen et al. 2020) and Gobiidae (Kroon & Liley, 2000; Kroon et al. 2005; Maxfield & Cole, 2019). The influence of elevated water temperature and compressed light cues tends to elicit rapid sex change and extended reproductive phases in these species. In contrast, few studies focus on temperate sex changing fish that experience strong reproductive seasonality and a protracted period of sex change. These species, arguably, offer an extended window of graded change in which to tease out fine-scale modulation of physiological drivers.

The New Zealand (NZ) spotty wrasse, *Notolabrus celidotus*, is an endemic protogynous, temperate zone (35° – 47° S) labrid that is well suited to laboratory studies. These small (< 26 cm) fish are abundant and easily caught on shallow reefs and in harbours around the NZ coastline. They have dimorphic initial phase (IP) and terminal phase (TP) colour morphs,

characteristic of most wrasses (Choat, 1965; Jones, 1980). However, two male sexual strategies exist with IP sneaker males displaying female mimicry and behaviourally dominant TP males establishing defended breeding territories (Figure 1). Reproduction peaks in the austral spring but the exact length is likely to vary with latitude (Jones, 1980). The fish are physically hardy with a wide thermal tolerance (approximately 8° C – 25° C), they adapt well to captivity and tolerate experimental manipulation. Sexually mature fish will spawn in captivity and sex change is induced in IP fish through the manipulation of social structure. This proclivity to complete natural sex change under laboratory conditions is of particular significance as this is not possible with other model species such as the bluehead wrasse. Collectively, these attributes make spotty wrasse a convenient biological model to study sex change.

Sex change is effected through the reproductive axis, yet the underlying regulatory mechanisms are not well understood. The feminising and masculinising effects of the sex steroids, 17ß-estradiol (E2) and 11-ketotestosterone (11KT) on sex changing fish are clear (Frisch, 2004; Kroon and Liley, 2000; Todd et al., 2016). However, the molecular interplay modulating their balance is complicated. Recent studies indicate that cross-talk between the hypothalamic-pituitary-interrenal (HPI) and the hypothalamic-pituitary-gonadal (HPG) axes exists which suggests an influential role of stress in sex change (Liu et al., 2017; Todd et al., 2019). Furthermore, a suite of candidate genes involved in critical female (e.g. *cyp19a1a*, *foxl2a* and *ctnnb1*) and male (e.g. *amh*, *dmrt1* and *sox9a*) developmental pathways have also been implicated as having a regulatory role in sex change. The identification of an early molecular event acting as a key trigger of sex change is of special interest. Of these genes, *cyp19a1a*, the gonadal aromatase enzyme responsible for the bioconversion of testosterone into E2, is a strong candidate. An early decrease in E2 concentration has been associated with the onset of sex change in several protogynous species (Bhandari et al., 2003; Liu et al., 2017; Muncaster et al., 2013; Nakamura et

al., 1989). This is further supported by manipulative experiments to chemically inhibit the aromatase enzyme (Higa et al., 2003; Kroon et al., 2005; Lee et al., 2001; Nakamura et al., 2015; Nozu et al., 2009). With a network of candidate genes likely to influence (albeit indirectly) the gonadal sex steroid environment, a targeted approach to study their expression across sex change is warranted.

In this study, we present histological, endocrine and gene expression data to describe spotty wrasse as a new temperate-water model for the study of vertebrate sex change. We investigate molecular and endocrine pathways as well as potential triggers that may regulate gonadal restructure in these fish using both chemical and socially induced sex change.

Methods

Experimental set-up

Experiment 1: Induction of sex change in spotty wrasses by aromatase inhibition (AI2014)

In this experiment, the aromatase inhibitor (AI) fadrozole ($C_{14}H_{13}N_3$) was used to induce sex change in captive IP spotty wrasse individuals between August and September 2014. Fish were captured around high tide by hook and line off the coast of Tauranga, Bay of Plenty, New Zealand (37.6878° S, 176.1651° E) and subsequently maintained at the Aquaculture Centre at Toi Ohomai Institute of Technology, Tauranga. TP males were distinguished from IP males and females by external observation: IP fish have a large inky thumbprint spot in the middle of the body, whereas TP males have an irregularly shaped row of blackish spots and light electric blue wavy patterns on their cheeks (Choat, 1965). Thirty IP fish ranging from 154 – 229 mm total length (TL) were distributed across three 400-litre recirculating seawater systems under a natural photoperiod. Natural sex change was blocked by

153 placing a TP male in each tank, creating a socially inhibitory environment.
154 During the experiment, fish were fed frozen greenshell mussels (*Perna*
155 *canaliculus*) three times per week. Pellets containing 200 µg fadrozole
156 (Sigma-Aldrich) in a matrix of cholesterol:cellulose = 95:5 were made in-
157 house (as described in (Lokman et al., 2015; Sherwood et al., 1988)). The
158 release rate of fadrozole from these pellets was not tested. Sham pellets
159 (vehicle) contained matrix only. Following an acclimation period of three
160 weeks, on day 0 of the experiment all IP individuals were given a single
161 intramuscular fadrozole implant (n=16) or a sham implant without hormone
162 (n=14) using a Ralgun implanter (Syndel, Ferndale, WA).
163
164 All fish were removed from individual tanks on day 21 (Tank 1, n=11), day
165 39 (Tank 2, n=11) or day 60 (Tank 3, n=11; end of experiment). Fish were
166 anesthetised in an aerated bath containing 600 ppm 2-phenoxyethanol
167 (Sigma-Aldrich) and blood samples were collected from the caudal vein using
168 a 1 mL heparinised syringe. Fish were then euthanised by rapid decapitation.
169 The mid-section of one gonad was fixed in Bouin's solution (TP males) or 10%
170 neutral buffered formalin (IP individuals) overnight, and then stored in 70%
171 EtOH at room temperature until paraffin embedding for histological
172 analysis. The same location in the gonads was used in all fish when fixing
173 gonadal samples for histology. Body weight and length, and gonadal weight
174 were measured for each fish.
175
176 *Experiment 2: Social induction of sex change in spotty wrasses within their*
177 *breeding season (SI2016)*
178
179 Sex change was induced in captive spotty wrasses through manipulation of
180 social groups (i.e. removal of males from the treatment tanks) between
181 September and December 2016. Fifty IP and ten TP individuals ranging from
182 150 – 215 mm TL were captured and maintained as described in Experiment
183 1 (AI2014). Fish were distributed into groups across ten 400-litre
184 recirculating seawater systems such that each tank contained a hierarchy of

different sized IP fish and a single TP male (215 – 244 mm TL). After a 3-week acclimation, TP males were removed from the treatment tanks. Subsequently, the largest IP fish from each tank was terminally sampled on days 0, 30, 50, 60, 65, or 66 (end of experiment). Blood plasma collection, anaesthetic administration, tissue dissection and recording of morphometrics were conducted as described for AI2014.

Experiment 3: Social induction of sex change in spotty wrasses outside their breeding season (SI2018)

Social manipulation was used to induce sex change in captive spotty wrasses outside the breeding season between January and April 2018. Sixty five IP and twelve TP individuals ranging from 138 – 218 mm TL were captured and maintained as described in AI2014. Fish were distributed across twelve 400-litre recirculating seawater systems such that each tank contained a hierarchy of 4-5 different-sized IP fish and a single TP male (194 – 220 mm TL). Three control (5 IP females + 1 TP) tanks were maintained and nine manipulated (5 IP females - 1 TP) tanks had the males removed on day 0 after a 2-week acclimation period. A further five IP females were terminally sampled on day 0 to provide a baseline indication of reproductive status. Fish were sampled over a time series as follows; day1 (n=5), day 11 (n=5), day 26 (n=10), day 36 (n=10), day 55 (n=10), day 92 (n=9). Eleven mortalities occurred during the experiment.

Anaesthetic administration, blood plasma collection, and recording of morphometrics were conducted as described for AI2014. One gonad was flash frozen in ice-cold (on dry ice) isopentane (C_5H_{12}) (Sigma-Aldrich) and stored at -80 ºC for RNA analyses. The second gonad was preserved for histological analysis as described in AI2014. Fish in all three experiments were maintained and manipulated in accordance with New Zealand National Animal Ethics Advisory Committee guidelines (approved by the Animal Ethics Committee of Toi Ohomai Institute of Technology).

Gonadal tissue processing for histology

Histological analysis of gonadal tissues was used to characterise cellular changes occurring across sex change. Tissues from AI2014 and SI2016, fixed in Bouin's solution (TP males) or 10% neutral buffered formalin (IP individuals), were processed for routine embedding in paraffin (New Zealand Veterinary Pathology, Hamilton Laboratory, New Zealand). SI2018 gonadal tissues were embedded in paraffin (Otago Histology Services Unit, Department of Pathology, Dunedin School of Medicine, University of Otago, New Zealand). Sections were cut at 3 µm and stained with Mayer's haematoxylin and eosin.

Gonadal sections were examined under light microscope to confirm the sex of each individual and samples were subsequently classified into sex-change stages as outlined in Table 1 (modified from (Thomas et al., 2019)). As spotty wrasses are seasonal breeders, females were classified as either non-breeding females (NBF) or breeding females (BF), depending on the presence of maturing oocytes. Transitioning individuals were classified as being in an early (ET), mid (MT) or late (LT) stage of transition.

Table 1. Histological stages of gonadal sex change in New Zealand spotty wrasse.

Stage	Abbreviation	Characteristics
Non-Breeding Female	NBF	Ovary with predominantly healthy previtellogenic oocytes. Clearly atretic later-stage oocytes may also be present
Breeding/Recrudescent Female	BF	Presence of healthy previtellogenic and vitellogenic oocytes. May also include hydrating oocytes
Early Transitioning	ET	Atretic oocytes of all stages, including previtellogenic oocytes, common. Nests of gonial cells, yellow-brown bodies and stromal cells often evident
Mid-Transitioning	MT	Spermatogenic cysts containing mainly spermatogonia or spermatocytes evident.

Stage	Abbreviation	Characteristics
		Many atretic oocytes often still present. Stromal cells and cellular debris common
Late Transitioning	LT	Evidence of lobular structure forming. Male germ cells dominate. Yellow-brown bodies and cellular debris common
Terminal Phase Male	TP	Fully structured testis with spermatogenic cysts arranged into lobules. Sperm ducts developed peripherally inside the tunica albuginea and presence of central remnant lumen. All stages of spermatogenic germ cells may be present depending on season. A few degenerating oocytes may persist.
Initial Phase Male	IP	Presence of lobule formation containing spermatogenic cysts. No evidence of degenerating oocytes. No central lumen and sperm ducts tend to be aggregated centrally

Steroid measurements

Blood was centrifuged at 13,500 rpm for 3 minutes to obtain plasma, which was stored at -20 °C until steroid analysis. Measurement of blood plasma concentrations of E2 and 11KT across sex change were conducted by radioimmunoassay (RIA) after routine steroid extraction following procedures described in (Kagawa et al., 1981; Kagawa et al., 1982; Young et al., 1983). Assays were validated for spotty wrasse plasma, serially diluted plasma behaving in a manner similar to the standard curve (parallel displacement). Tritiated 11KT was synthesised using the methodology described in (Lokman et al., 1997), whereas label for the E2 assay was acquired from Perkin Elmer. Antiserum against E2 was purchased from MyBioSource and antiserum against 11KT was kindly donated by Professor Yoshitaka Nagahama, Emeritus Professor, National Institute for Basic Biology, Okazaki, Japan. After incubation and separation of antibody-bound and -unbound steroid by charcoal-dextran solution (0.5% dextran/charcoal), tubes were centrifuged (15 min, 2000g), the supernatant was decanted, and radioactivity measured using a MicroBeta® Trilux scintillation counter (Wallac 1450, Perkin Elmer). Samples from each experiment were run in

separate assays with a minimum detectable level of 40 pg/tube (0.08 ng/mL) (E2) and 50 pg/tube (0.10 ng/mL) (11KT) for AI2014, 35 pg/tube (0.07 ng/mL) (E2) and 120 pg/tube (0.24 ng/mL) (11KT) for SI2016, and 50 pg/tube (0.10 ng/mL) (E2) and 70 pg/tube (0.14 ng/mL) (11KT) for SI2018. Extraction efficiencies were 46% (E2) and 82% (11KT) for AI2014; 61% (E2) and 87% (11KT) for SI2016; and 69% (E2) and 40% (11KT) for SI2018.

Due to non-normality of the RIA data, the non-parametric Kruskal-Wallis test (Kruskal and Wallis, 1952) was used to compare plasma steroid concentrations between sexual stages, for E2 or 11KT separately. If stage was found to have a significant effect, *post hoc* comparisons using Dunn's tests (Dunn, 1961) with Benjamini Hochberg correction for multiple comparisons (Benjamini and Hochberg, 1995) were performed between stages to determine where the significance lay, carried out in R (v. 1.1.453) (Core Team, 2013).

RNA extraction from gonadal tissues

Gonadal samples were homogenised using a power homogeniser before RNA extraction. For AI2014 and SI2016 samples, RNA was extracted with TRIzol™ (Thermo Fisher Scientific) using chloroform as the phase separation reagent, before DNase-treatment (TURBO DNA-free Kit, Thermo Fisher Scientific) and total RNA clean-up (RNA Clean & Concentrator, Zymo Research). For SI2018 samples, RNA was extracted with Direct-zol RNA MiniPrep Plus (Zymo Research) without phase separation (on column DNase treatment).

RNA concentration was measured using a Qubit 2.0 Fluorometer (Life Technologies) and RNA integrity was evaluated on a Fragment Analyzer (Advanced Analytical Technologies Inc.). The RNA profiles of sex-changing gonads presented a strong peak of low molecular weight RNA. This is considered to be a result of massive 5S rRNA amplification in ovaries (Liu,

2016), and masks the 18S and 28S rRNA peaks used to calculate the RNA Integrity Number (RIN) values, making them unreliable estimates of RNA integrity. Similar patterns have been observed in ovaries and/or intersex gonads of thicklip gray mullet (*Chelon labrosus* (Diaz de Cerio et al., 2012)), sharsnout seabream (*Diplodus puntazzo* (Manousaki et al., 2014)) and bluehead wrasse (Liu, 2016). Therefore, in spotty wrasses, RNA integrity for such samples was confirmed by visual inspection of 18S and 28S rRNA peaks.

Gene expression analysis with nanoString nCounter™ technology

A probe array of 24 candidate genes was designed for spotty wrasse (Table 2). Spotty wrasse-specific transcript sequences were identified from a draft spotty wrasse transcriptome assembly (EV Todd & NJ Gemmell, 2015, unpublished data) available in the Gemmell lab, Anatomy Department, University of Otago, New Zealand, using sequence similarity searches. Reference transcripts from zebrafish (*Danio rerio*) were downloaded from GenBank or Ensembl for each target gene, and *actb1*, *eef1a1a* and *g6pd* as potential housekeeping genes. Downloaded transcripts were then used to identify the corresponding spotty wrasse sequences from the transcriptome via local BLASTn and tBLASTx (translated the query nucleotide sequences, and the spotty wrasse transcriptome into deduced amino acid sequences in all six possible frames, which were then compared by local alignment). The best matching spotty wrasse contig was chosen and its identity confirmed using online nucleotide BLAST (BLASTn) against the NCBI database (http://www.ncbi.nlm.nih.gov/).

These sequences were submitted to nanoString Technologies for probe design, and nanoString nCounter™ CodeSet gene expression quantification was delivered by the Otago Genomics Facility, Biochemistry Department, University of Otago, New Zealand. Gonadal RNA (100 ng) from 5 control females sampled on day 0 of the SI2018 experiment (CF), 19 ET, 9 MT, 9 LT and 5 TP also from SI2018 was used to perform gene expression profiling.

Two approaches, RefFinder (https://www.heartcure.com.au/reffinder/?type=reference) (Xie et al., 2012) and BestKeeper (https://www.gene-quantification.de/bestkeeper.html) (Pfaffl et al., 2004) were used to determine the stability of gene expression of the potential housekeeping genes (*g6pd*, *eef1a1a* and *actb1*) and their suitability as reference genes for the normalisation of nanoString results. However, neither RefFinder or BestKeeper considers potential differences in reference gene expression between experimental groups, which can confound results and lead to an inaccurate interpretation (Dheda et al., 2004; Setiawan and Lokman, 2010). Therefore, candidate reference genes were also evaluated for differences in target molecule counts between sex-change stages. Due to non-normality of the raw nanoString data, the non-parametric Kruskal-Wallis test (Kruskal and Wallis, 1952) was used on the *actb1*, *eef1a1a* and *g6pd* counts, as well as the geometric mean of all possible combinations of these three genes, to determine whether stage had a significant effect on expression levels of each candidate housekeeping gene.

342 Table 2. Genes analysed in spotty wrasse gonad using the nanoString nCounter™ CodeSet technology. Abbreviations: high
343 mobility group (HMG), murine-mammary-tumour virus (MMTV), sex-determining region-Y (SRY).

Gene symbol	Gene description	Contig ID	Reference transcript ID
Housekeeping genes			
actb1	ß-actin, cytoplasmic 1	c58053_g1_i1	NM_131031.1
eef1a1a	eukaryotic translation elongation factor 1 alpha 1a	c58053_g1_i1	NM_200009.2
g6pd	glucose-6-phosphate dehydrogenase	c39960_g1_i1	ENSDART00000104834.6
Steroidogenic enzymes and hormone receptors			
cyp19a1a	aromatase a (gonad isoform)	c52027_g1_i1	NM_131154.3
cyp11c1/b2	steroid 11ß-hydroxylase	c62027_g1_i1	NM_001080204.1
hsd11b2	11ß-hydroxysteroid dehydrogenase type 2	c67035_g1_i1	NM_212720.2
nr3c1	glucocorticoid receptor	c36910_g2_i1	NM_001020711.3
nr3c2	mineralocorticoid receptor	c49976_g1_i2	NM_001100403.1
Key sex-related transcription factors			
foxl2a	forkhead box L2a	c53356_g1_i1	NM_001045252.2
dmrt1	doublesex and mab-3 related transcription factor 1	c66498_g1_i1	NM_205628.2
amh	anti-Müllerian hormone	c51546_g1_i1	NM_001007779.1
sox9a	SRY-related HMG box 9a	c53707_g2_i1	NM_131643.1
sox8b	SRY-related HMG box 8b	c60549_g2_i1	NM_001025465.1
Rspo1/Wnt/ß-catenin pathway			
wnt4a	wingless type MMTV integration site family, member 4a	c52640_g1_i1	NM_001040387.1
ctnnb1	catenin (cadherin-associated protein), beta 1	c47984_g1_i2	NM_131059.2
wnt4b	wingless type MMTV integration site family, member 4b	c60281_g1_i1	NM_131500.1

Gene symbol	Gene description	Contig ID	Reference transcript ID
rspo1	R-spondin-1 (precursor)	c50451_g1_i2	NM_001002352.1
E3 ubiquitin-protein ligase			
znrf3	zinc and ring finger 3	c68386_g1_i1	NM_001308555.1
fancl	Fanconi anaemia complementation group L	c63372_g2_i1	NM_212982.1
Epigenetic regulatory factors			
dnmt1	DNA methyltransferase 1	c43163_g1_i1	NM_131189.2
dnmt3aa	DNA methyltransferase 3aa	c59097_g4_i2	NM_001018134.1
Jumonji gene family			
jarid2b	jumonji, AT rich interactive domain 2b	c67175_g1_i2	NM_001202459.1
kdm6bb	lysine (K)-specific demethylase 6B, b	c52506_g1_i1	NM_001030178.2
Pluripotency factor			
pou5f3	POU domain, class 5, transcription factor 1	c63041_g1_i1	NM_131112.1

The geometric mean of gene pair *actb1*|*g6pd* was selected as the reference gene for data normalisation (see Results). Normalisation calculations were performed automatically in the nanoString nSolver Analysis software (version 4.0), and normalised data were log base-2 transformed prior to analysis. Due to non-normality of the normalised nanoString data, the non-parametric Kruskal-Wallis test (Kruskal and Wallis, 1952) was run separately for each target gene to determine whether stage had a significant effect on gene expression level, and *post hoc* comparisons using Dunn's tests (Dunn, 1961) with Benjamini Hochberg correction for multiple comparisons (Benjamini and Hochberg, 1995) were performed to determine where the significance, if any, lay between stages. All analyses were performed in R (v. 3.3.2) (Core Team, 2013). NanoString Expression Data Analysis Guidelines (MAN-C0011-04) were followed to determine an expression threshold, set as the $\log_2$ of the geometric mean of the negative control counts plus two standard deviations.

In addition, Principal Component Analysis (PCA) (scaled) was used to visualise overall mRNA expression variation among samples considering all genes, within and across sex-change stages in R (v. 1.1.453) (Core Team, 2013). To identify the genes contributing most to observed patterns represented by the first two principal components, component loadings (defined as eigenvectors scaled by the square root of the respective eigenvalues) were represented as coordinates in a Cartesian plane.

Results and Discussion

Histological analysis of gonadal sex change

Experiment 1: Induction of sex change in spotty wrasses by aromatase inhibition (AI2014)

Aromatase inhibition successfully induced sex change in 93% of the surviving (2 mortalities) captive female spotty wrasses held under socially inhibitory

conditions. Histological analysis confirmed that among the AI-implanted females, 12 fish reached ET stage (day 21, n=2; day 39, n=5; day 60 n=5), and one reached LT stage (day 60). A single fadrozole-implanted fish remained female. In contrast, none of the control females showed signs of ovarian atresia or sex change. None of the fish examined were IP males.

Experiment 2: Social induction of sex change in spotty wrasses within their breeding season (SI2016)

The manipulation of social groups, through male removal, successfully promoted sex change (81%) in female spotty wrasses during the 2016 breeding season. Histology confirmed that among the socially manipulated females, 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 found to be a fully TP male (day 60). Four of the socially manipulated fish remained female (day 30, n=2; day 66, n=2). Ovarian atresia was evident in four of the control females (day 30, n=3; day 66, n=1). However, it was impossible to elucidate whether this was indicative of early sex change or gonadal resorption following the breeding season (Thomas et al., 2019). Histological analysis also confirmed that five of the initial 50 IP individuals captured were IP males (10.0% frequency), a slightly higher ratio than reported in the wild (4.1 – 5.7%) (Jones, 1980).

Experiment 3: Social induction of sex change in spotty wrasses outside their breeding season (SI2018)

Male removal conducted outside the breeding season also induced sex change in female spotty wrasses. All socially manipulated females showed histological signs of ovarian degeneration or sex change. Histology confirmed that 18 females reached ET stage (day 1, n=3; day 11, n=2; day 26, n=3; day 36, n=3; day 55, n=4; day 96, n=3), 12 MT stage (day 1, n=1; day 11, n=3; day 26, n=4; day 36, n=1; day 55, n=3), 13 LT stage (day 26, n=2; day 36, n=6; day 55, n=3; day 92, n=2) and 6 became full TP males (day 1, n=1; day 92, n=5). No IP males were found (Figure

2). Unfortunately control tanks with male fish present experienced poor health and reduced survival. This confounded the efficacy of socially induced sex change in this experiment. Nonetheless, the results of the other two experiments (AI2014 & SI2016) indicate that both chemical and social manipulation increases sex change compared to control female fish that have a male present (Fisher's exact test, $p < 0.001$). This is also supported by previous studies with this species (Muncaster, unpublished data).

Incidence of sex change depending on seasonality

The breeding season of spotty wrasses in northern New Zealand lasts from late July until the end of November and peaks in the austral spring (Jones, 1980). Of the socially manipulated spotty wrasses, the greatest number of mid-transitional and fully transformed males occurred during the post-spawning period (SI2018) in summer and early autumn (January to April; Figure 3). A total of 55% of these fish had MT through to TP stage gonads. In contrast, less than 25% of the fish socially manipulated during the October-December breeding season (SI2016) presented the same stage gonads. Sex change is often seasonally biased with the greatest occurrence following the breeding season in temperate and warm water species, but it may occur all year-round in some tropical species (Alonso-Fernández et al., 2011; Li et al., 2007; Muncaster et al., 2013; Sadovy and Shapiro, 1987; Thomas et al., 2019). Our results support observations in wild spotty wrasses, in which natural sex change has been documented during the non-reproductive months between November and May (Jones, 1980). This indicates that for experimental purposes, post-spawned fish present the best candidates for socially manipulated sex change. However, we have also demonstrated that male removal can lead to sex change within the breeding season.

Hormonal profile analysis

Plasma E2 concentrations showed a general decreasing trend from female to male stages (Figure 4A). Fish treated with an aromatase inhibitor (AI2014) had mean

plasma E2 concentrations ranging from 0.08 ng/ml to 0.34 ± 0.25 SD ng/ml for LT and ET stages respectively. In comparison, non-manipulated control fish had a lower mean plasma E2 concentration of 0.45 ± 0.46 SD ng/ml, although this was not statistically different. Social manipulation during the breeding season (SI2016) showed significantly higher plasma E2 concentrations in control females (CF) (0.39 ± 0.40 SD ng/mL) compared to TP (0.07 ± 0.00 SD ng/mL, $p < 0.001$) and IP males (0.07 ± 0.00 SD ng/mL, $p < 0.05$). However, when social manipulation was conducted after the spawning season (SI2018), plasma E2 concentrations were minimal in all fish with no significant differences between sexual stages. Similar patterns of reduced E2 concentrations immediately after the breeding season have been observed in other protogynous species (Bhandari et al., 2003; Li et al., 2007; Muncaster et al., 2010). This is not surprising considering the importance of E2 in driving seasonal oocyte growth in teleosts (Jalabert, 2005; Lubzens et al., 2010; Nagahama, 1994; Patiño and Sullivan, 2002). After reproduction and the conclusion of active oogenesis, temperate fish often enter a period of gonadal resorption and quiescence characterised by ovarian atresia and reduced sex steroid concentrations (Scott et al., 1984).

While E2 has a clear role in maintaining ovarian function, there is no obvious relationship between plasma E2 concentrations and the initiation of sex change in spotty wrasses. A marked decrease in E2 concentrations has been implicated as a critical initiator of sex change in many protogynous species (Bhandari et al., 2003; Liu et al., 2017; Muncaster et al., 2013; Nakamura et al., 1989). Yet, despite a 2.4-fold decrease, there was no significant difference in plasma E2 concentration between CF and ET (0.16 ± 0.16 SD ng/mL) fish during the breeding season (SI2016). Similarly, there was no significant decrease of plasma E2 concentration between female and early transitional spotty wrasses from the other experiments (AI2014 & SI2018). While this may, in part, relate to sample size or seasonal influence, similar results were also reported in the bambooleaf wrasse (Ohta et al., 2008) and orange-spotted grouper (Chen et al., 2020). Undetectable E2 concentrations existed from MT to male (TP & IP) stages in nearly all fish. Therefore, while there is no evidence of a minimum plasma E2 threshold required

to initiate gonadal transition in spotty wrasse, a general reduction is associated with the process of sex change in this species. Indeed, E2 depletion leads to masculinisation in hermaphroditic and gonochoristic species alike (Bhandari et al., 2004; Li et al., 2019; Nozu et al., 2009; Paul-Prasanth et al., 2013; Takatsu et al., 2013).

Elevated plasma 11KT concentrations were observed in individual fish towards the transitional and male stages in all three experiments (AI2014, SI2016 and SI2018; Figure 4B). While variability in the data and reduced statistical power made the detection of discernible differences impossible, the timing of these observations coincides with the histological appearance of spermatogenic cysts (see Table 1). Increased 11KT concentrations were also evident from mid transition onwards in other protogynous species (Bhandari et al., 2003; Nakamura et al., 1989; Nakamura et al., 2005). Fish treated with AI (AI2014) had minimal 11KT values in the earlier sexual stages. This was evident in a single BF (0.18 ng/ml) and ET (0.22 ± 0.17 SD ng/ml) fish while a later stage LT individual presented with 1.00 ng/ml. In comparison, sham-treated CF had minimal plasma 11KT concentrations (0.17 ± 0.03 SD ng/ml). Fish socially manipulated during the breeding season (SI2016) showed remarkably similar plasma 11KT concentrations regardless of sexual stage. These values ranged from 0.13 ± 0.02 SD ng/ml in CF and BF to 0.23 ± 0.31 SD ng/ml in TP. All of the transitional and IP fish had either identical mean or individual 11KT concentrations of 0.14 ng/ml. Many of the androgen concentrations of fish socially manipulated after the breeding season (SI2018) remained minimal as expected during quiescence. Minimum 11KT concentrations (0.13 ± 0.02 SD ng/ml) existed in CF and mean values were slightly higher in subsequent stages such as ET (0.15 ± 0.03 SD ng/ml) and MT fish (0.19 ± 0.15 SD ng/ml). The greatest plasma 11KT values were in LT (0.29 ± 0.32 SD ng/ml) fish, while slightly reduced concentrations were recorded in TP individuals (0.22 ± 0.18 SD ng/ml). This disparity of androgen concentrations in late-stage fish most likely reflects the chronology of the experiment. TP fish were removed at the beginning of the experiment when spermatogenesis should be minimal or non-

existent. The occurrence of several LT fish coincided with seasonal gonadal recrudescence.

The androgen profiles from the fish in this study do not show a clear statistical relationship between sexual stages. However, the role of 11KT in driving spermatogenesis is well established in teleosts (Miura et al., 1991; Nagahama, 1994; Nakamura et al., 1989; Schulz et al., 2010). Much of this androgen activity is likely to be paracrine in nature with steroidogenic somatic cells stimulating local germ cells both directly and indirectly within the gonadal compartment (Schulz, 1986). The additional role of 11KT in expressing seasonal male secondary sexual characteristics, such as morphometric and behavioural modifications, also exists (Borg, 1994; Semsar and Godwin, 2004). This likely requires elevated plasma concentrations for remote, effective target cell signalling and may in part explain substantial elevations of 11KT prior to breeding in many male teleosts. The absolute concentrations of androgen required to stimulate spermatogenesis are conceivably much lower. Furthermore, prior to these peak physiological concentrations the actual concentrations of 11KT within the gonad are likely to be higher than in the plasma. It is, therefore, possible that the absolute concentrations of 11KT required to initiate spermatogenesis during gonadal restructure are not reflected in the spotty wrasse plasma samples. This issue could be investigated using *in vitro* explant culture systems.

Comparison of housekeeping gene stabilities

The ranking of candidate reference genes by RefFinder, Δ CT and NormFinder was *actb1* > *g6pd* > *eef1a1a*. The BestKeeper ranking was *g6pd* > *actb1* > *eef1a1a*, based on both SD and r (Tables S1A and S1B). These results suggest that *actb1* and *g6pd* should be used as reference genes for normalisation of the current nanoString data.

The gonadal mRNA levels of candidate reference genes *actb1* and *eef1a1a* were found to be significantly affected by sex-change stages using the non-parametric

Kruskal-Wallis test (*actb1*, X^2 (4) = 23.94, p < 0.001; *eef1a1a*, X^2 (4) = 29.53, p < 0.001). The geometric mean of the mRNA levels of all three genes and that of gene pairs *actb1* | *eef1a1a* and *eef1a1a* | *g6pd* were also significantly influenced by stage (*actb1* | *eef1a1a* | *g6pd*, X^2 (4) = 16.77, p < 0.005; *actb1* | *eef1a1a*, X^2 (4) = 27.53, p < 0.001; *eef1a1a* | *g6pd*, X^2 (4) = 13.82, p < 0.01). Candidate reference gene *g6pd* mRNA levels (X^2 (4) = 7.97, p = 0.09) and the geometric mean of gene combination *actb1* | *g6pd* mRNA levels were not significantly affected by stage; (X^2 (4) = 9.03, p = 0.06) (Figure S1). Consequently, gene pair *actb1* | *g6pd* was selected to normalise the target gene expression data (i.e. highest p-value for a gene combination observed) (Figure S1E).

NanoString gene expression analysis

Several genes (*wnt4a*, *wnt4b* and *sox8b*) were excluded from analysis as their expression was below the detection threshold. It remains unknown whether this reflects low biological expression in the spotty wrasse gonad, or if it is a consequence of probe design.

PCA clustering of samples based on the collective gene set revealed that gonadal samples strongly cluster by sexual stage (Figure 5). Samples were most strongly organised by sex-change stage and progressing from female to male (PC1, 50.7% variation explained). Secondly, gonadal transcriptomes were organised by developmental commitment (PC2, 19.4% variation explained), separating transitional gonads from those in a more advanced state of differentiation, representing fully differentiated ovaries of control females and testes of TP males. This discrete PCA clustering of the sexual stages provides validation of the histological staging criteria (Table 1).

The spotty wrasse data show a striking resemblance to transcriptome-wide data from transitioning bluehead wrasse gonads (Todd et al., 2019). This demonstrates the relevance of this suite of 18 genes to describe the genetic regulation of the female-to-male transition in spotty wrasse. However, some overlaps between

sexual stages are evident, in particular between ET and MT, and LT and TP males. This reinforces the concept of a continuous progression of gonadal restructure, rather than a transition punctuated by larger transformative changes (Muncaster et al., 2013; Todd et al., 2019).

Sexual dimorphic expression of genes encoding steroidogenic enzymes and hormone receptors

The temporal regulation of steroid hormones is essential for the coordination of sex differentiation, sexual maturation and behaviour in vertebrates. They are also potent mediators of gonadal sex change in teleosts (Guiguen et al., 2010; Higa et al., 2003; Nakamura et al., 1989). As expected *cyp19a1a* expression was greatest in CF and was gradually downregulated across sex change (Figure 6A). Expression did not differ significantly in these stages and there was no evidence of an early, rapid downregulation that has been thought to trigger sex change in other species (Gemmell et al., 2019; Liu et al., 2017; Todd et al., 2016). Levels of *cyp19a1a* expression remained similar across ET, MT and LT stages (2018) while in comparison, plasma E2 concentrations were negligible by mid transition in all three experiments (AI2014, SI2016 and SI2018). While *cyp19a1a* is an unlikely proximate trigger of sex change in spotty wrasse, a more distant connection exists nonetheless. This is evident in the number of fish that changed sex following aromatase inhibition (AI2014) as well as the occurrence of sex change in fish socially manipulated after the breeding season (SI2018) when plasma E2 concentrations were minimal. Considering the potent feminising action of E2, a reduction of gonadal concentrations may act as a gateway to facilitate the progression of transition rather than acting as an early trigger. This action may be through the release of steroid induced suppression on male-pathway genes (Guiguen et al., 2010).

In teleosts, testosterone (T) can be converted into 11KT by 11ß-hydroxylase (Cyp11c1) and 11ß-hydroxysteroid dehydrogenase type 2 (Hsd11b2) (Frisch, 2004). Upregulation of *cyp11c1* was observed across sex change (Figure 6B), with

599 significantly (X^2 (4) = 32.39, p < 0.001) greater expression in TP spotty wrasses
600 than CF (median 2.6-fold greater) and ET (median 2.1-fold greater) fish. Albeit
601 less pronounced, *hsd11b2* expression increased in a similar pattern across sex
602 change (X^2 (4) = 32.13, p < 0.001; Figure 6C). The simultaneous upregulation of
603 both *cyp11c1* and *hsd11b2* from MT through to TP stages coincides with the
604 presence of spermatogenic cysts in the gonad and likely reflects an increase in
605 gonadal 11KT production. Since plasma 11KT concentrations were not greatly
606 elevated at this time of year (see Figure 4B), these expression patterns may
607 indicate either a subtle paracrine action of 11KT in the early testis or the as-yet
608 untranslated proteins.
609
610 Both *cyp11c1* and *hsd11b2* are involved in the teleost stress response through the
611 production of cortisol and its subsequent inactivation to cortisone, respectively
612 (Arterbery et al., 2010; Goikoetxea et al., 2017). Cross talk between the interrenal
613 and reproductive axes through the upregulation of these enzymes has been
614 implicated as influencing masculinisation in teleosts (Goikoetxea, 2020; Liu et al.,
615 2017). Genes encoding the glucocorticoid (*nr3c1*) and mineralocorticoid (*nr3c2*)
616 receptors in the spotty wrasse gonad showed elevated expression in the LT to TP
617 stages (*nr3c1*, X^2 (4) = 28.40, p < 0.001; *nr3c2*, X^2 (4) = 13.02, p < 0.05) (Figures 6D
618 and 6E). This differs from bluehead wrasse in which opposing sex-specific
619 expression patterns were observed for *nr3c1* (male-biased expression) and *nr3c2*
620 (female-biased expression) (Liu et al., 2015; Todd et al., 2019). Early-stage
621 expression of *cyp11c1*, *hsd11b2* and *nr3c2* was thought to indicate a role for
622 cortisol in triggering sex change in bluehead wrasse (Todd *et al.*, 2019).
623 Upregulation of these genes in spotty wrasse, occurred in later stage gonads
624 showing no clear link for cortisol in the initiation of sex change in this species.
625 However, (Chen et al., 2020) report a marked transient increase of cortisol during
626 the early stages of protogynous sex change in orange-spotted grouper. Similar to
627 spotty wrasse in this study, these fish do not show a significant reduction of E2
628 during sex change. Further investigation into the role of cortisol and the interrenal
629 axis during sex change in spotty wrasse is warranted.
630

Expression of major sex determination and differentiation genes

Core feminising (e.g. *foxl2* and Rspo1/Wnt/ß-catenin signalling pathway genes) and masculinising (e.g. *dmrt1*, *sox9*, *amh*) networks direct development towards ovarian or testicular fate and are highly-conserved across vertebrates (Herpin and Schartl, 2011b; Munger and Capel, 2012). Transcription factor forkhead box L2 (Foxl2) and ovarian specific Rspo1/Wnt/ß-catenin signalling pathway genes are essential for ovarian maintenance in mammals (Yang et al., 2017) and their role in the promotion of female development in teleost fish is well established (Harris et al., 2018; Li et al., 2013; Liu et al., 2015). In spotty wrasse, *foxl2a* expression was significantly reduced in TP fish (X^2 (4) = 15.05, $p < 0.005$; Figure 7A). This indicates that downregulation of *foxl2a* is important in the late stages of testicular development and is not a proximate trigger for sex change in this species. Although sexually dimorphic expression of *foxl2* was not evident in three-spot wrasse (Kobayashi et al., 2010), similar expression patterns to that of the spotty wrasse have been observed in honeycomb grouper (*Epinephelus merra*) and bluehead wrasse (Alam *et al.*, 2008; Liu, 2016).This may indicate a species-specific function of *foxl2a* in protogynous teleosts (Liu, 2016).

Studies to date confirm a conserved role of ß-catenin (*ctnnb1*) in the establishment and maintenance of ovarian differentiation in vertebrates (Chassot et al., 2011). A consistent female-biased pattern of *ctnnb1* expression was observed in spotty wrasse with significant downregulation evident in MT and male fish (X^2 (4) = 36.72, $p < 0.001$) (Figure 7B)., In contrast, expression of *rspo1* was significantly upregulated during sex change (MT) and male stages (X^2 (4) = 35.07, $p < 0.001$) (Figure 7C), indicating a role in testicular development of spotty wrasses. While *rspo1* activates the Wnt/ß-catenin signalling pathway in female mammalian development, current evidence suggests that this is less conserved in teleosts (Herpin et al., 2013; Liu et al., 2015; Manousaki et al., 2014; Zhou et al., 2012). Male-biased expression of *rspo1* was observed in the protogynous bluehead wrasse (Liu et al., 2015) and gonochoristic East African cichlid fishes (Böhne et al., 2013), while in medaka (*Oryzias latipes*), Rspo1 activates ovarian differentiation and

maintenance (Zhou et al., 2012). Collectively, these data suggest that *rspo1* may participate in the development of both ovaries and testes in teleost fishes.

E3 ubiquitin-protein ligase zinc and ring finger 3 (Znrf3) has been reported to have a testis-determining function in mammals (Harris et al., 2018), where it can act as an inhibitory regulator of the Rspo1/Wnt/ß-catenin signalling pathway through the ubiquitination and subsequent degradation of Wnt receptor complex components (Hao et al., 2012). Male mice gonads lacking *znrf3* will undergo partial or complete sex reversal (Harris et al., 2018). Expression of *znrf3* was significantly downregulated in ET through to TP spotty wrasse compared to control females (X^2 (4) = 18.55, $p < 0.001$; Figure 7D). This provides novel information on the potential role of *znrf3* in downregulation of ovarian function at the onset of teleost sex change.

The masculinising factor *dmrt1* (doublesex and mab-3 related transcription factor 1) is believed to act antagonistically to *foxl2* in order to suppress *cyp19a1a* and other female pathway genes while activating male-promoting pathways (Herpin and Schartl, 2011a; Kobayashi et al., 2013; Matson and Zarkower, 2012; Matson et al., 2011; Minkina et al., 2014). Expression of *dmrt1* was significantly upregulated in LT and TP stage spotty wrasses compared to earlier transitional and female stages (X^2 (4) = 19.39, $p < 0.001$; Figure 7E). Dmrt1 disruption in male Nile tilapia caused upregulation of *foxl2* and *cyp19a1a* expression while Foxl2 deficiency caused females to sex reverse (Li et al., 2013). A similar gene interaction may exist in spotty wrasse, where downregulation of *foxl2a* and *cyp19a1a* approximates the upregulation of *dmrt1* in later stage fish. This genetic interplay may regulate the maintenance of the new sexual phenotype rather than the initiation of sex change.

Anti-Müllerian hormone, expressed by *amh*, is strongly associated with male vertebrate sex differentiation (Josso, di Clemente and Gouédard, 2001; Pfennig et al. 2015). This masculinising association was also evident in spotty wrasse with an approximately linear increase in *amh* expression across all gonadal stages.

Expression of *amh* was greatest in TP males (2.2-fold, 1.6-fold and 1.4-fold greater in TP than CF, ET and MT, respectively; X^2 (4) = 38.71, p < 0.001; Figure 7F). The apparent upregulation of *amh* with ET and MT stage spotty wrasses indicates a pivotal role in the initial stages of sex change. Furthermore, *amh* demonstrated the greatest contribution to PC1 (ovary-to-testis transition) of the PCA analysis. This is consistent with other studies that show upregulation of *amh* expression at the onset of protogynous sex change and complementary downregulation in the early stages of protandrous sex change (Hu et al., 2015; Liu et al., 2017; Wu et al., 2015). A recent mechanistic model for socially induced sex change in the protogynous bluehead wrasse suggests that early *amh* activation may be induced by the stress hormone cortisol (Todd et al., 2019). This indicates the value of further research into the role of cortisol in spotty wrasse.

Transcription factor *sox9* is another key component of male developmental pathways in vertebrates (Harris et al., 2018), and is thought to be activated by *dmrt1* in fish (Herpin and Schartl, 2011a; Herpin and Schartl, 2011b). In contrast, *sox9a* expression showed unexpected female-bias in spotty wrasse, with significant downregulation in testis compared to ovary and transitional gonads (X^2 (4) = 11.23, p < 0.05; Figure 7G). Fish possess two *sox9* paralogs, *sox9a* and *sox9b,* due to the teleost whole-genome duplication (Chiang et al., 2001). Gene duplication mechanisms have been proposed to contribute to the diversity of sex determination mechanisms and sexual plasticity of fishes compared to other vertebrate systems (Ortega-Recalde et al., 2020). Neofunctionalisation, whereby a gene acquires a new function after a duplication event, has been observed in sex-changing fish (Glasauer and Neuhauss, 2014; Todd et al., 2019). For example, in the protogynous bluehead wrasse, duplicate copies of female-pathway genes (e.g. *foxl2a/b*, *wnt4a/b*) showed opposing expression patterns across female-to-male sex change (Liu et al., 2015; Todd et al., 2019). While both *sox9a* and *sox9b* are upregulated across sex change in bluehead wrasse (Liu et al., 2015), expression patterns of *sox9a* may be either female (Böhne et al., 2013; Yokoi et al., 2002) or male-biased (Baron et al., 2008; Chiang et al., 2001; Ijiri et al., 2008) in gonochoristic species. In spotty wrasses, *sox9a* may have acquired a female-

specific role due to such neofunctionalisation. While expression patterns support a connection to sex change regulation, they also highlight the need to examine *sox9b* expression to determine whether this paralog has conserved male-specific function in spotty wrasse gonads.

Epigenetic modifiers are dynamically expressed during sex change

The epigenetic regulation of sex differentiation and sex change has been described in several species. These epigenetic modifications have emerged as a critical liaison between environmental changes, such as temperature or social hierarchy, and sexual development (Domingos et al., 2018; Ellison et al., 2015; Navarro-Martín et al., 2011; Piferrer, 2013; Strömqvist et al., 2010; Todd et al., 2019; Wen et al., 2014; Wu et al., 2016; Zhang et al., 2013; Zhong et al., 2014). The DNA methyltransferase genes *dnmt1* and *dnmt3aa*, responsible for the maintenance and *de novo* methylation of DNA, respectively (Todd et al., 2019), showed significant changes in gonadal expression across sex change in the spotty wrasse (*dnmt1*, X^2 (4) = 36.91, $p < 0.001$; *dnmt3aa*, X^2 (4) = 30.11, $p < 0.001$) (Figures 8A and 8B). Although functionality cannot be inferred, the downregulation of *dnmt1* and simultaneous upregulation of *dnmt3aa* in transitional gonads indicate their involvement in the genetic cascade regulating sex change in spotty wrasse. This expression pattern is consistent with bluehead wrasse (Todd et al., 2019). Together, these data suggest epigenetic reprogramming via changes in sexually dimorphic DNA methylation is a key element orchestrating sexual fate transitions in sequential hermaphrodites.

Expression of the chromatin modifier genes *jarid2b* and *kdm6bb* was significantly downregulated during spotty wrasse sex change and is evident in the early to mid-transitional stages. Females had a median 1.4-fold greater expression of *jarid2b* (X^2 (4) = 34.47, $p < 0.001$; Figure 8C) and median 1.2-fold greater expression of *kdm6bb* (X^2 (4) = 23.41, $p < 0.001$; Figure 8D) than TP males. Sex-biased expression of *jarid2b* and *kdm6bb* in spotty wrasses adds further weight to growing evidence that these genes play important regulatory roles in sex change.

In bluehead wrasse, *jarid2* expression was transiently downregulated during sex change (Todd et al., 2019). The expression of *jarid2b* and *kdm6bb* is regulated by stress in mammalian systems (Bovill et al., 2008; Williams et al., 2014), and a temperature-dependant epigenetic regulation of these genes has been suggested to control sex reversal in the Australian bearded dragon (*Pogona vitticeps*), through *jarid2b*/*kdm6bb* differential intron retention induced by extreme temperatures (Deveson et al., 2017). Moreover, KDM6B promotes transcription of Dmrt1 to regulate temperature-dependant sex determination in the red-eared slider turtle, *Trachemys scripta elegans* (Ge et al., 2018). The relationship between *kdm6bb* and *dmrt1* is yet to be explored in teleosts and may be worth further investigation.

Emerging regulators of sex change in teleosts

E3 ubiquitin-protein ligase Fancl (Fanconi anaemia complementation group L) is associated with DNA repair pathways (Meetei et al., 2003). This gene generated interest after reports that Tp53-mediated germ cell apoptosis following a mutation in *fancl* jeopardised oocyte survival and induced female-to-male sex reversal in zebrafish (Rodríguez-Marí et al., 2010). However, in spotty wrasses, *fancl* expression decreased in LT and TP individuals (X^2 (4) = 31.31, $p < 0.001$; Figure 8E), well after apoptosis of most oocytes in the early and mid-transitional gonads. Therefore, rather than initiating sex change via oocyte apoptosis, *fancl* is more likely to be involved in the progression of gonadal transition in spotty wrasses.

Stem cell maintenance and pluripotency may be important for sex change in wrasses, where no male germ cells are identifiable prior to sex change (Todd et al., 2019). Vertebrate pluripotency factor *pou5f3* (POU domain, class 5, transcription factor 1) is critical for the maintenance and regulation of stem cell pluripotency in teleosts (Gao et al., 2017; Lacerda et al., 2019; Xiaohuan et al., 2016). Furthermore, it is able to reprogram somatic cells to become pluripotent cells in medaka (Tapia et al., 2012). In spotty wrasses, gonadal expression of *pou5f3* was downregulated across sex change, with CF and ET fish showing a median 2-fold

and 1.9-fold greater expression than TP males, respectively (X^2 (4) = 36.22, p < 0.001; Figure 8F). Gao et al. (2017) found the greatest *pou5f3* expression in Japanese flounder ovary compared to testis. Expression was evident throughout the oocyte cytoplasm while it was restricted to spermatogonial germ cells in the testis. This sexually dimorphic expression in Japanese flounder is consistent with the graded decrease in *pou5f3* expression observed across sex change in spotty wrasse. While this is the first report of *pou5f3* expression in a sequentially hermaphroditic fish, further investigation into this gene could reveal interesting information, particularly if retention of pluripotency exists in spermatogonial cells and allows for the possibility of sex reversion. Male to female reversion is currently unknown in spotty wrasse but has been reported in other protogynous wrasses (Kuwamura et al., 2002; Kuwamura et al., 2007; Ohta et al., 2003).

Conclusion

This study provides a comprehensive characterisation of the anatomical, endocrine and molecular events during sex change in the temperate-water New Zealand spotty wrasse. Sex change was successfully induced using either chemical (aromatase inhibition) or social manipulation. Seasonality influenced sex change, with a greater number of individuals undergoing gonadal transition when reduced plasma sex steroid concentrations occurred following the breeding season. NanoString gene expression analysis showed a number of gene targets followed sexually dimorphic patterns typically observed in teleosts (*foxl2a*, *dmrt1, amh*), while atypical patterns were observed for others (*rspo1*, *sox9a*, *znrf3*, *kdm6bb*, *pou5f3*). Importantly, these data support increasing evidence for the involvement of epigenetic regulation of gonadal sex change. Analysis of these genetic data verified that transitional fish can be accurately categorised into staged categories based on gonadal histology. Furthermore, it implicates *amh* as a proximate trigger for sex change and a beneficial marker to elucidate the difference between females undergoing seasonal ovarian atresia and ET fish. Further investigations should consider manipulative whole-organism and *in vitro* studies, including gene

822 knockdown approaches to identify specific gene functions during gonadal sex
823 change.
824
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Figure legends:

Figure 1. Life cycle of New Zealand spotty wrasse (*Notolabrus celidotus*). Juveniles first develop into either initial phase (IP) females or males, which can then become terminal phase (TP) males via sex or role change, respectively. Adapted from (Thomas et al., 2019; Todd et al., 2018). IP spotty wrasse image by Allan Burgess, TP spotty wrasse image by Jodi Thomas.

Figure 2. Histological stages of gonadal development observed in spotty wrasses from the SI2018 experiment: a) control non-breeding females: ovary filled with previtellogenic oocytes; b) early transitioning fish: visible nests of gonial cells, yellow-brown bodies and stromal cells; c) mid-transitioning fish: evidence of proliferation of spermatogenic cysts; d) late transitioning fish: male germ cells dominate over female structures; e) terminal phase male: mature testes showing cysts of spermatocytes and spermatozoa. Abbreviations: atretic previtellogenic oocyte (APVO), gonial cell (GC), previtellogenic oocyte (PVO), stromal cell (SC), spermatocyte (SPC), spermatogonia (SPG), spermatozoa (SPZ), yellow-brown body (YBB). Scale bar: 100 μm.

Figure 3. Proportion of SI2016 and SI2018 spotty wrasses in different sexual phases depending on month of sampling. Abbreviations: breeding female (BF), early transitioning fish (ET), late transitioning fish (LT), mid-transitioning fish (MT), non-breeding female (NBF), social induction experiment 2016 (SI2016), social induction experiment 2018 (SI2018), terminal phase male (TP). Sample sizes: October n=4, November n=13, December n=5, January n=20, February n=20, March n=0, April n=9.

Figure 4. Plasma E2 (A) and 11KT (B) concentrations in control females, manipulated breeding females, early, mid- and late transitioning fish, and TP and IP males obtained across the three experiments, AI2014, SI2016 and SI2018. Each red (AI2014), yellow (SI2016) and blue (SI2018) line represents the variation in mean E2 or 11KT concentrations across groups per experiment while the black line

represents the variation in overall mean concentrations for the three experiments altogether. Sample sizes: E2, CF n = 32, BF n = 4, ET n = 42, MT n = 8, LT n = 14, TP† n = 17, IP n = 5; 11KT, CF n = 35, BF n = 5, ET n = 41, MT n = 9, LT n = 15, TP† n = 26, IP n = 4. Abbreviations: 11-ketestosterone (11KT), aromatase inhibition experiment 2014 (AI2014), breeding female (BF), control female (CF), 17ß-estradiol (E2), early transitioning fish (ET), initial phase male (IP), late transitioning fish (LT), mid-transitioning fish (MT), social induction experiment 2016 (SI2016), social induction experiment 2018 (SI2018), terminal phase male (TP). †Both control males used throughout the experiments to create a socially inhibitory environment (SI2016: E2 n = 10, 11KT n = 10; SI2018: E2 n = 0, 11KT n = 10), and males obtained through sex change of manipulated females (SI2016: E2 n = 1, 11KT n = 1; SI2018: E2 n = 5, 11KT n = 5) were grouped altogether as TP males for the purpose of this analysis.

Figure 5. PCA (18 genes) of gonad samples. The transition from females to males is captured along PC1 (50.7% variance), whereas PC2 (19.4% variance) extremes represent sexually differentiated gonads (bottom) and transitionary gonads (top). Sample sizes: CF n = 5, ET n = 19, MT n = 9, LT n = 9, TP n = 5. Abbreviations: control female (CF), early transitioning fish (ET), late transitioning fish (LT), mid-transitioning fish (MT), terminal phase male (TP).

Figure 6. Relative gonadal expression of *cyp19a1a* (A), *cyp11c1* (B), *hsd11b2* (C), *nr3c1* (D) and *nr3c2* (E) mRNA. Expression levels are compared among control females sampled on day 0, transitioning individuals and TP males. In the boxplots, each point represents an individual fish, the middle bold line represents the median, the edges of the box represent the upper and lower quartiles, and vertical lines represent the minimum and maximum values. Letters denote a significant difference in distribution between groups. Sample sizes: CF n = 5, ET n = 19, MT n = 9, LT n = 9, TP† n = 5. Abbreviations: control female (CF), early transitioning fish (ET), late transitioning fish (LT), mid-transitioning fish (MT), terminal phase male (TP). †Both a male used during the acclimation period of the experiment (n =

1267 1), and males obtained through sex change of socially manipulated females (n = 4)
1268 were grouped altogether as TP males for the purpose of this analysis.
1269
1270 Figure 7. Relative gonadal expression of *foxl2a* (A), *ctnnb1* (B), *rspo1* (C), *znrf3* (D),
1271 *dmrt1* (E), *amh* (F) and *sox9a* (G) mRNA. Expression levels are compared among
1272 control females sampled on day 0, transitioning individuals and TP males. In the
1273 boxplots, each point represents an individual fish, the middle bold line represents
1274 the median, the edges of the box represent the upper and lower quartiles, and
1275 vertical lines represent the minimum and maximum values. Letters denote a
1276 significant difference in distribution between groups. Sample sizes: CF n = 5, ET n
1277 = 19, MT n = 9, LT n = 9, TP† n = 5. Abbreviations: control female (CF), early
1278 transitioning fish (ET), late transitioning fish (LT), mid-transitioning fish (MT),
1279 terminal phase male (TP). †Both a male used during the acclimation period of the
1280 experiment (n = 1), and males obtained through sex change of socially manipulated
1281 females (n = 4) were grouped altogether as TP males for the purpose of this
1282 analysis.
1283
1284 Figure 8. Relative gonadal expression of *dnmt1* (A), *dnmt3aa* (B), *jarid2b* (C),
1285 *kdm6bb* (D), *fancl* (E) and *pou5f3* (F) mRNA. Expression levels are compared
1286 among control females sampled on day 0, transitioning individuals and TP males.
1287 In the boxplots, each point represents an individual fish, the middle bold line
1288 represents the median, the edges of the box represent the upper and lower
1289 quartiles, and vertical lines represent the minimum and maximum values. Letters
1290 denote a significant difference in distribution between groups. Sample sizes: CF n
1291 = 5, ET n = 19, MT n = 9, LT n = 9, TP† n = 5. Abbreviations: control female (CF),
1292 early transitioning fish (ET), late transitioning fish (LT), mid-transitioning fish
1293 (MT), terminal phase male (TP). †Both a male used during the acclimation period
1294 of the experiment (n = 1), and males obtained through sex change of socially
1295 manipulated females (n = 4) were grouped altogether as TP males for the purpose
1296 of this analysis.
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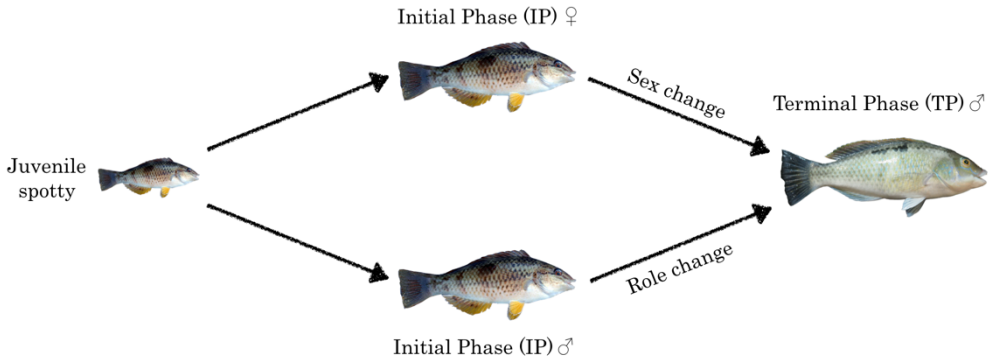
1298

Figure 1:



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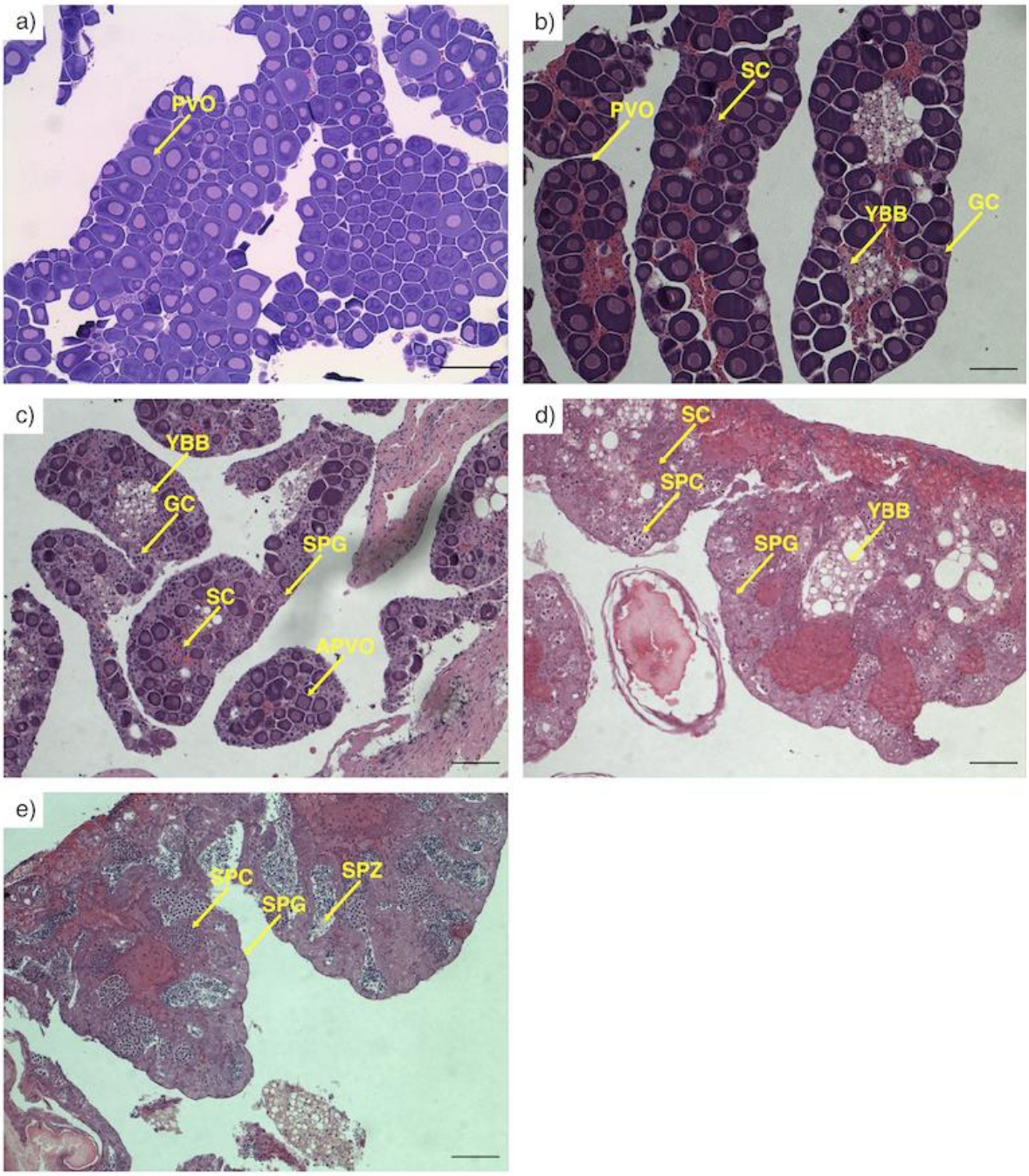

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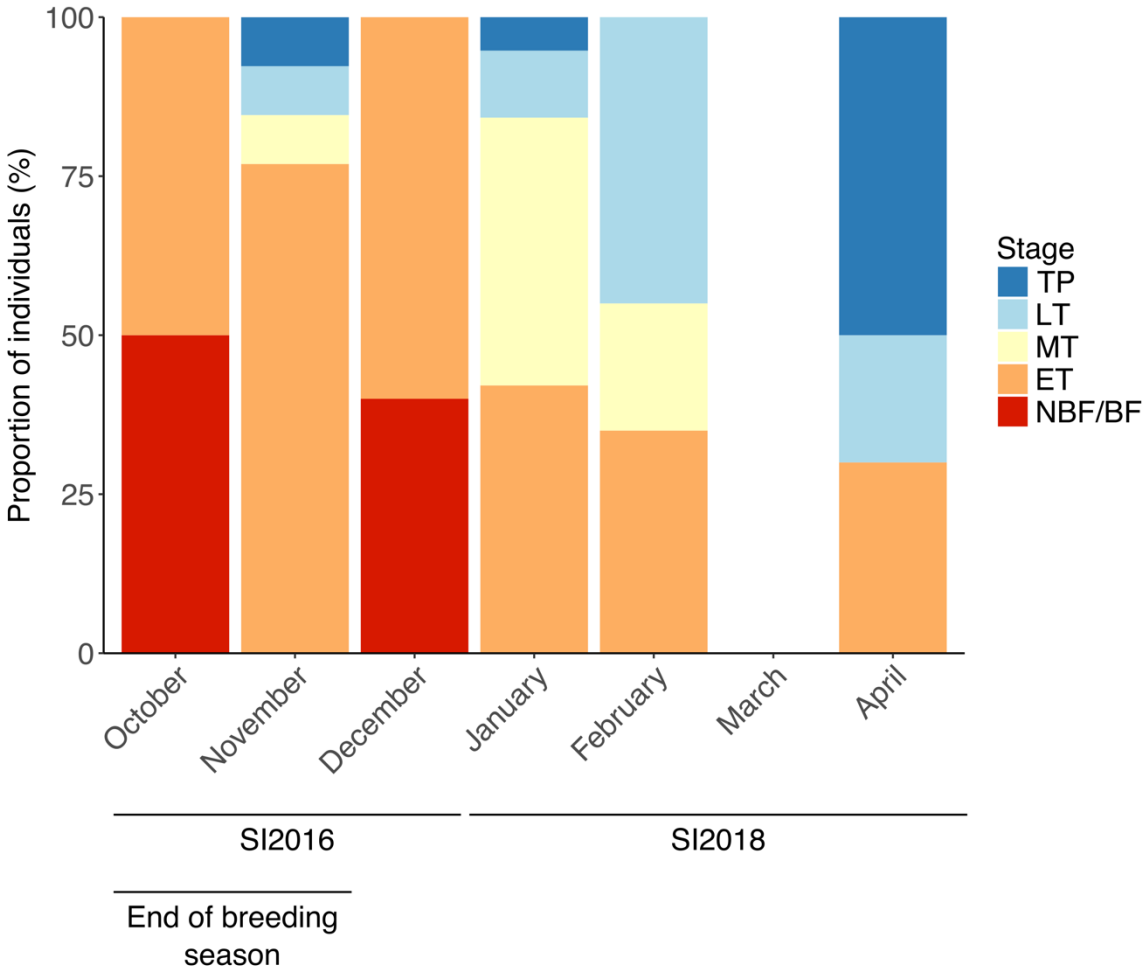

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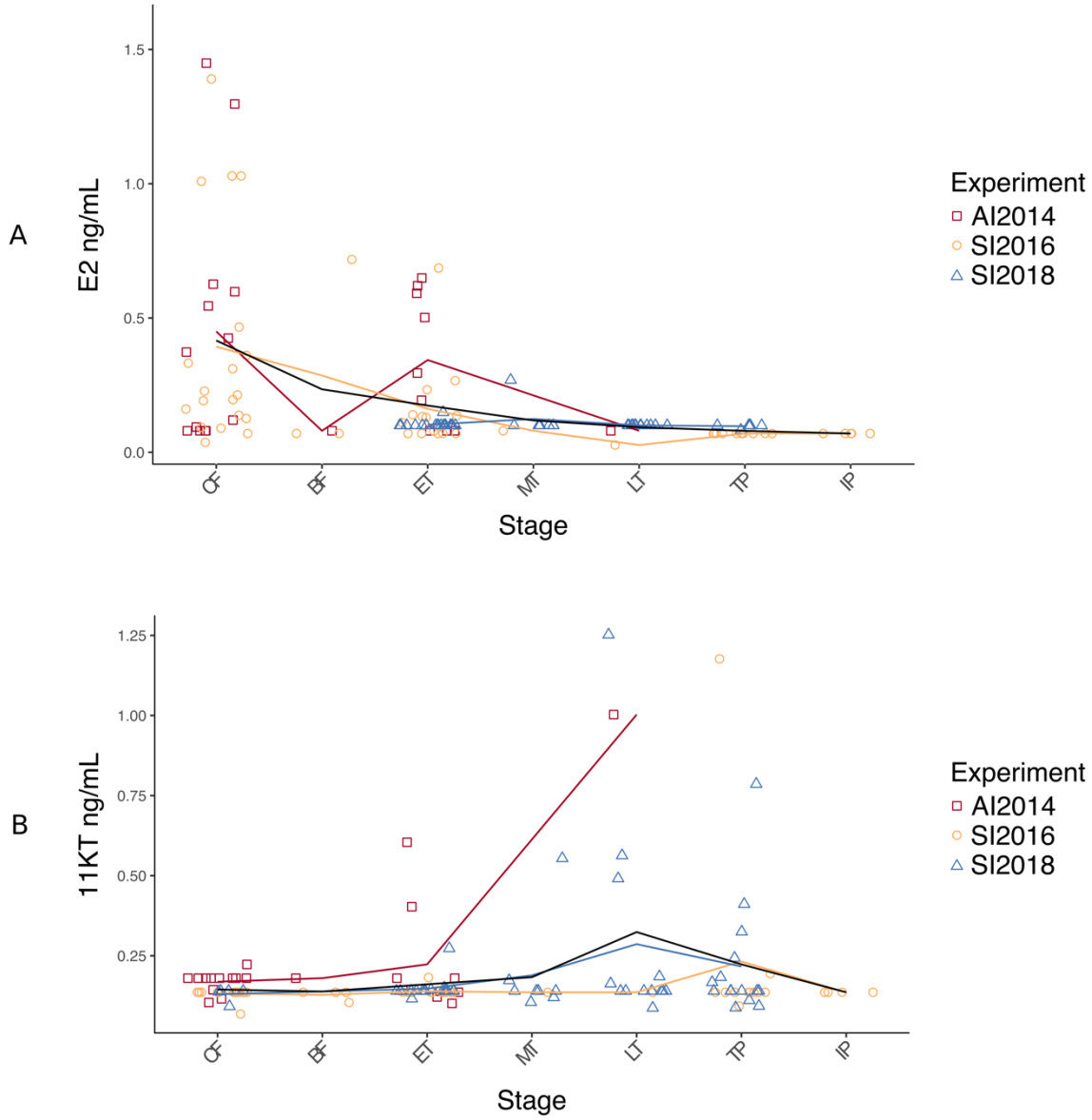

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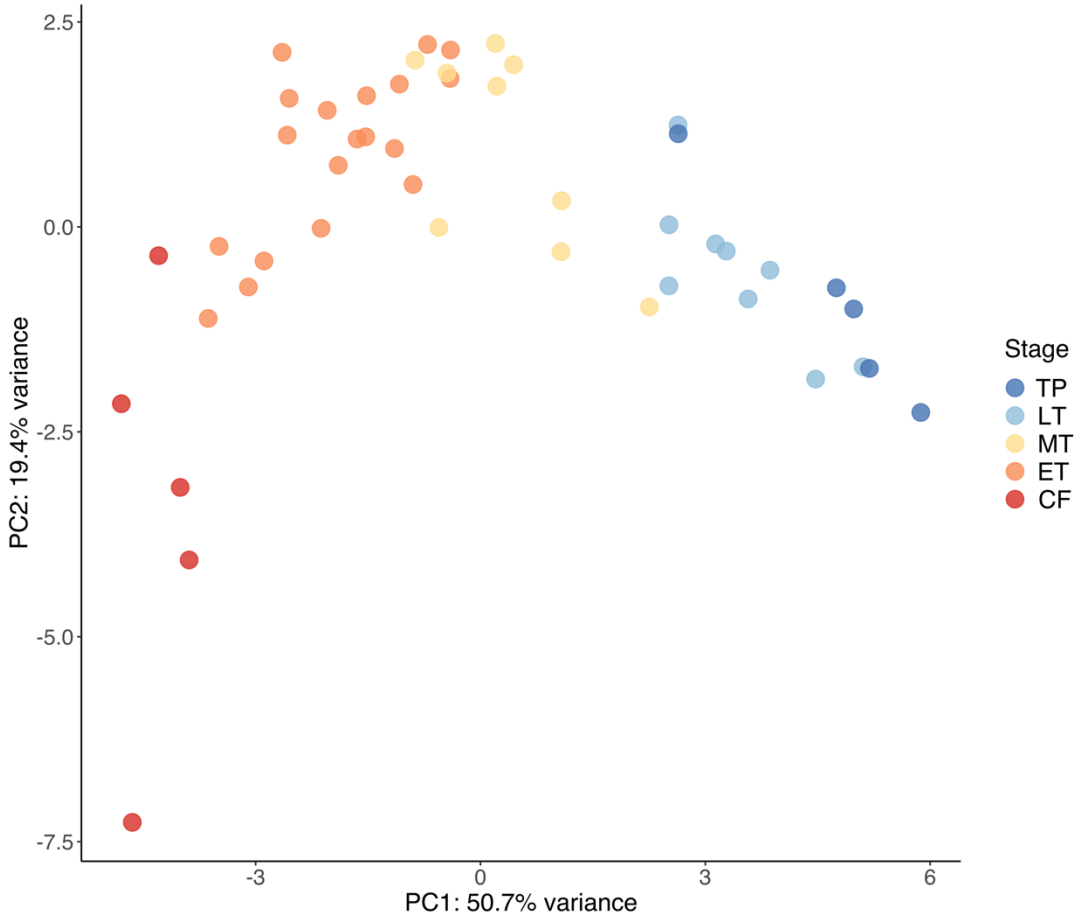

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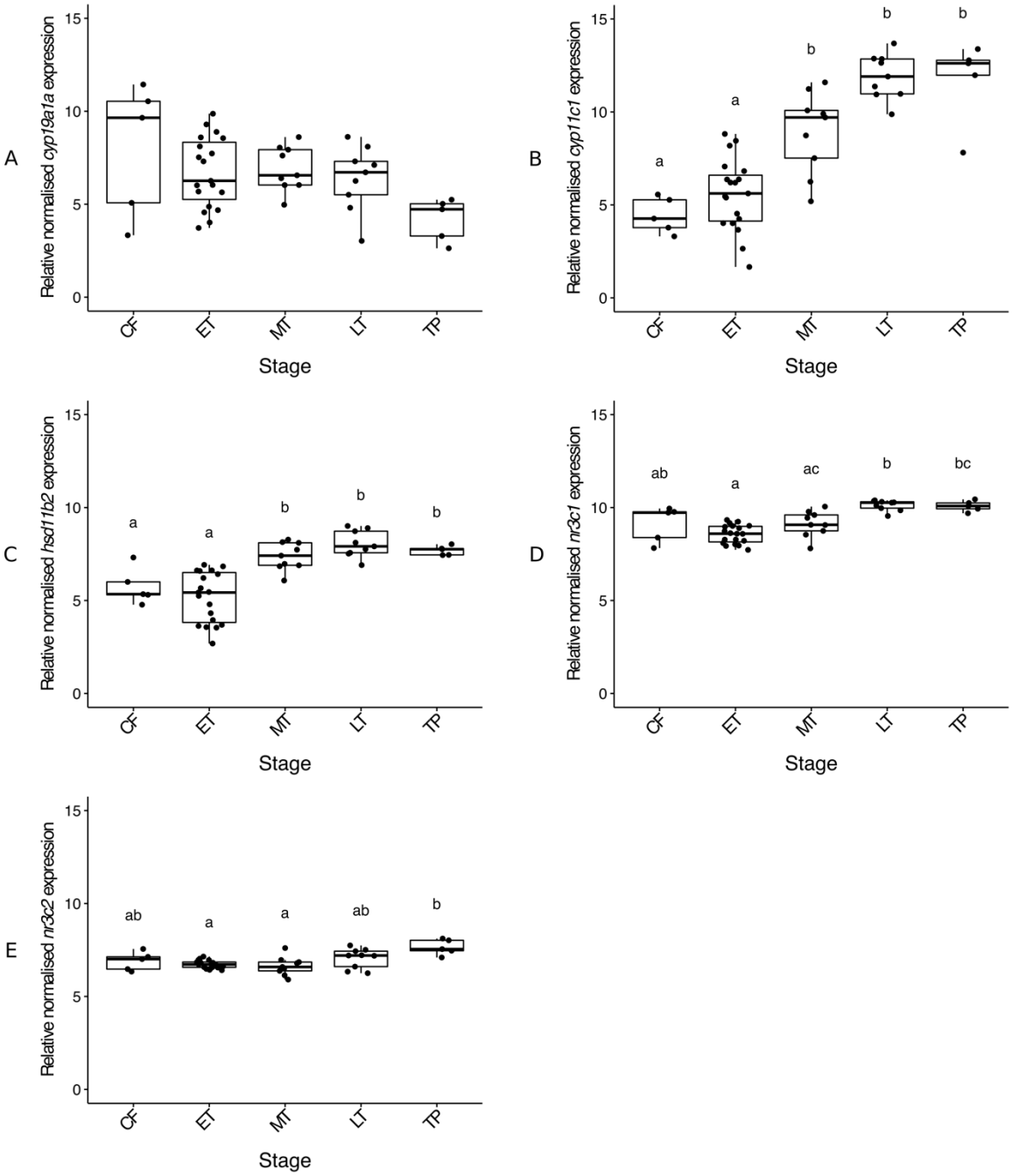

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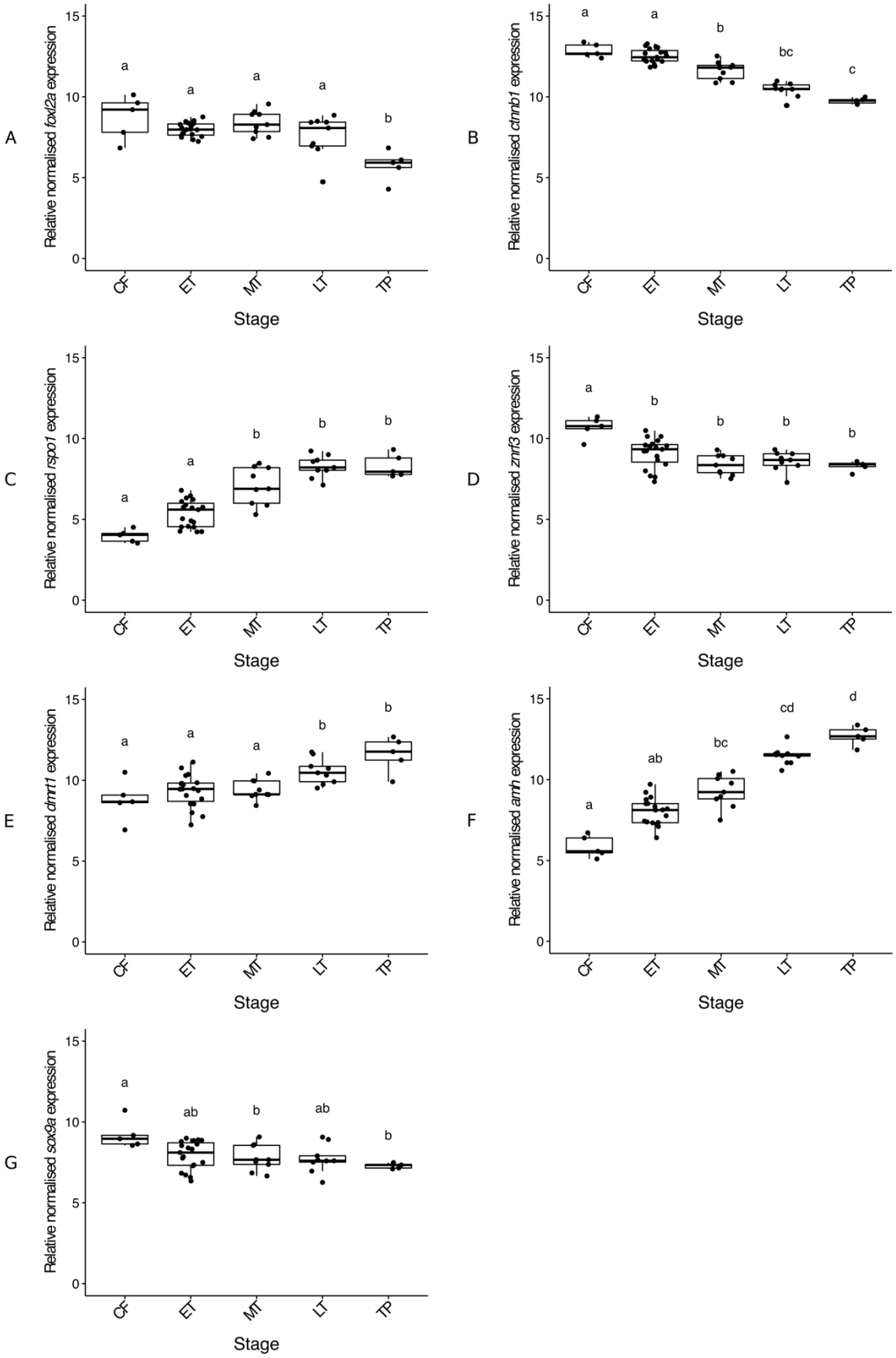

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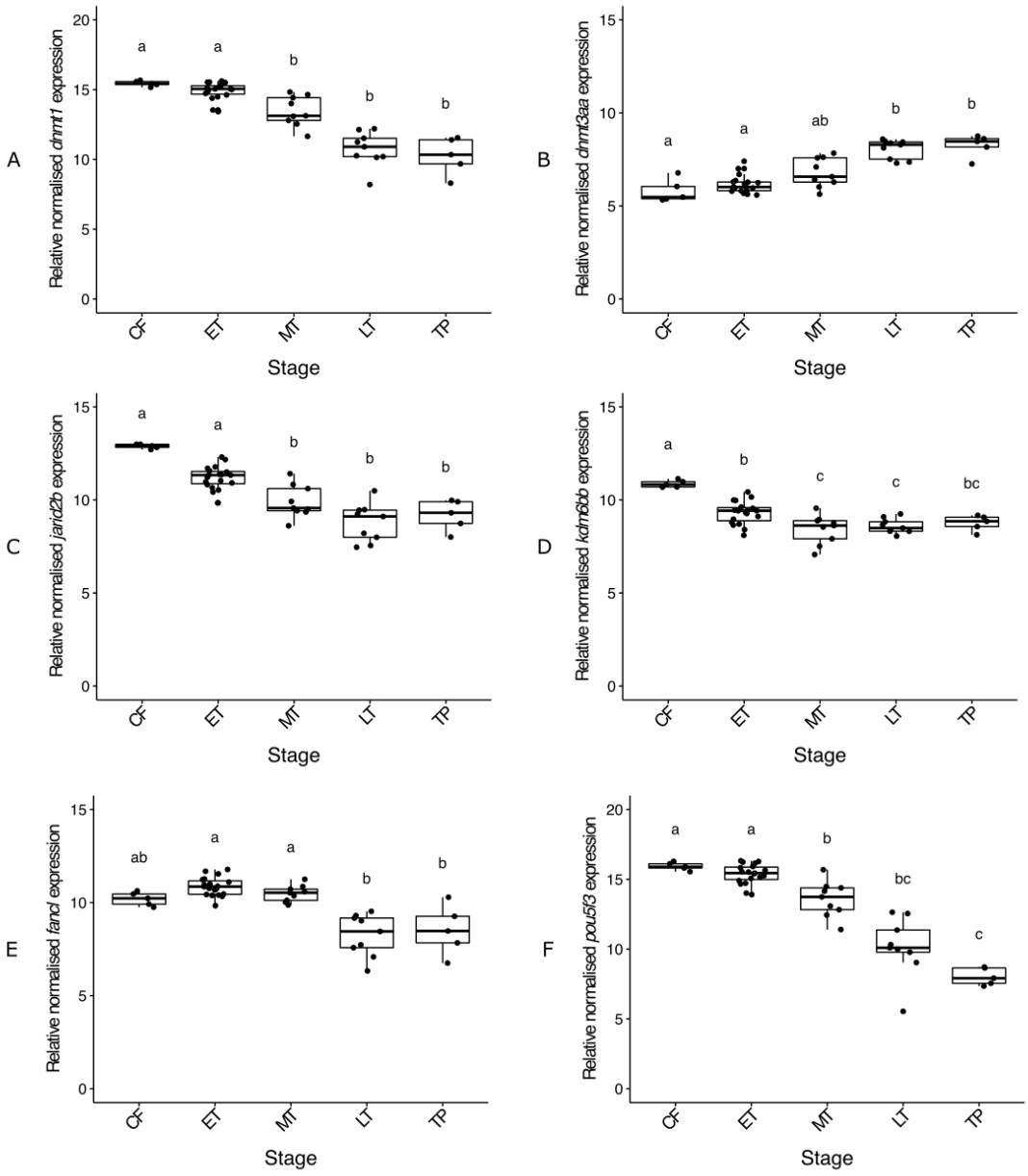

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