Title: 'Sex change in aquarium systems establishes the New Zealand spotty wrasse (*Notolabrus celidotus*) as a temperate model species for the investigation of sequential hermaphroditism'

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

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Sex change, wrasse, sex steroid, seasonality, aromatase

Summary statement (15 - 30 words):

The spotty wrasse, *Notolabrus celidotus*, is a new temperate model for the study of vertebrate sex change, this work characterises endocrine and genetic markers based on laboratory induced sex change.

Abstract

The stunning sexual transformation commonly triggered by age, size or social context in some fishes is one of the best examples of phenotypic plasticity thus far described. To date our understanding of this process is dominated by studies on a handful of subtropical and tropical teleosts, often in wild settings because sex change has been challenging to achieve in captivity. Here we have established the protogynous New Zealand spotty wrasse, *Notolabrus celidotus*, as a temperate model for the experimental investigation of sex change. Captive fish were induced to change sex using either aromatase inhibition or manipulation of social groups. Complete transition from female to male occurred over 60 days and time-series sampling was used to quantify changes in hormone production, gene expression and gonadal cellular anatomy using radioimmunoassay, nanoString nCounter mRNA and histological analyses, respectively. Early-stage decreases in plasma 17ß-estradiol (E2) concentrations or gonadal aromatase (*cyp19a1a*) expression were not detected in spotty wrasse, despite these being commonly associated with the onset of sex change in subtropical and tropical protogynous (female-to-male) hermaphrodites. In contrast, expression of the masculinising factor *amh* (anti-Müllerian hormone) increased during early sex change, implying a potential role as a proximate trigger for masculinisation. Expression of male-related genes responsible for androgen production *cyp11c1* and *hsd11b2* increased from mid sex change. Gonadal expression of the glucocorticoid and mineralocorticoid receptors *nr3c1* and *nr3c2*, putative mediators of the stress hormone cortisol, increased in late stages of sex change. Collectively, these data provide a foundation for the spotty wrasse as a temperate teleost model to study sex change and cell fate in vertebrates.

Introduction

For most vertebrates, sexual fate is genetically determined and remains fixed throughout life. However, for many teleost fishes sex is more plastic (Avise and Mank, 2009). The complete sex change that sequentially hermaphroditic fishes undergo during their reproductive lives, while unique among vertebrates, is taxonomically widespread across the teleost tree (Avise and Mank, 2009; Godwin, 2009; Munday et al., 2006). The direction and process of sex change differ greatly among teleosts (Gemmell et al., 2019; Goikoetxea et al., 2017; Todd et al., 2016) and appear to have evolved multiple times (Avise and Mank, 2009). The cellular and molecular processes that underpin sex change are slowly being determined for a few focal species (Todd et al., 2019). However, the extent to which these processes might be conserved and reused across the teleosts to achieve sex change is poorly understood (Ortega-Recalde et al., 2020).

Changes in the social environment and community structure often cue the timing of sex change (Reavis and Grober, 1999; Solomon-Lane et al., 2013; Sprenger et al., 2012). Species where manipulation of social structure can readily induce natural sex change are convenient models to understand the mechanistic drivers of this transformation. More broadly, these species present *in vivo* opportunities to examine cell fate pathways, neuro-endocrine plasticity, genetic and epigenetic regulation of life-history trajectory and reproductive status.

Current sex change research mostly focuses on tropical and subtropical 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; Chen et al., 2020b; Li et al., 2007) and Gobiidae (Kroon and Liley, 2000; Kroon et al., 2005; Maxfield and Cole, 2019). Unfortunately, such manipulations have frequently been limited to the wild because sex change in laboratory settings has proven challenging for many of these species. In addition, few studies have focused on temperate fishes that experience strong reproductive seasonality and a

99 protracted period of sex change. These species, arguably, offer an extended window
100 of graded change in which to tease out fine-scale modulation of physiological and
101 molecular drivers.
102
103 The New Zealand (NZ) spotty wrasse, *Notolabrus celidotus*, is an endemic
104 protogynous (female-to-male sex change) temperate zone (35° S – 47° S) labrid
105 that is well suited to laboratory studies. These small (< 26 cm) fish are abundant
106 and easily caught on shallow reefs and in harbours around the NZ coastline. They
107 have dimorphic initial phase (IP) and terminal phase (TP) colour morphs,
108 characteristic of most wrasses (Choat, 1965; Jones, 1980). As in other wrasses, two
109 male sexual strategies exist with IP sneaker males displaying female mimicry and
110 behaviourally dominant TP males establishing defended breeding territories (Fig.
111 1). Reproduction in the spotty wrasse peaks in the austral spring but the exact
112 timing varies with latitude (Jones, 1980), the NZ coastline spanning around 12 °
113 of latitude. This physically hardy species has a wide thermal tolerance
114 (approximately 8 – 25° C) and adapts well to captivity and tolerates experimental
115 manipulation. Sexually mature fish will spawn in captivity and sex change is
116 induced in IP fish through the manipulation of social structure (Thomas et al.,
117 2019). This proclivity to complete natural sex change under laboratory conditions
118 is of particular significance as other model species such as the bluehead wrasse
119 (*Thalassoma bifasciatum*) adapt poorly to captivity leading to most sex change
120 experiments being done in wild populations (Liu et al., 2015; Thomas et al., 2019;
121 Todd et al., 2019). Collectively, these attributes make spotty wrasse an excellent
122 biological model to study sex change.
123
124 Sex change is effected through the reproductive axis, yet the underlying regulatory
125 mechanisms are not well understood. The feminising and masculinising effects of
126 the sex steroids, 17ß-estradiol (E2) and 11-ketotestosterone (11KT) on sex
127 changing fish are clear (Frisch, 2004; Kroon and Liley, 2000; Todd et al., 2016).
128 However, the molecular interplay modulating their balance is complicated. Recent
129 studies indicate that the glucocorticoid stress hormone cortisol can influence sex
130 change. This is mediated via cross-talk between the hypothalamic-pituitary-

131 interrenal (HPI) and hypothalamic-pituitary-gonadal (HPG) axes and involves the
132 enzymes Cyp11c1 and Hsd11b2 (Liu et al., 2017; Todd et al., 2019). Studies in the
133 tropical model bluehead wrasse also suggest involvement of the glucocorticoid
134 (*nr3c1*) and mineralocorticoid (*nr3c2*) receptors (Todd et al., 2019). Indeed,
135 alongside a direct effect of cortisol on the gonad through activation of the
136 glucocorticoid receptors, the aforementioned adrenal enzymes could potentially
137 modulate the estrogen-androgen balance, which remains to be fully elucidated.
138 Comparing the interplay of these markers across the HPI and HPG axes of
139 different model species will be important for clarifying the mechanistic basis of
140 sex change.
141
142 The identification of an early molecular event that may trigger sex change is of
143 special interest. The gonadal aromatase enzyme, responsible for the bioconversion
144 of testosterone into E2 and encoded by *cyp19a1a,* is a strong candidate. An early
145 decrease in E2 concentration has long been associated with the onset of sex change
146 in several protogynous species (Bhandari et al., 2003; Liu et al., 2017; Muncaster
147 et al., 2013; Nakamura et al., 1989). This is further supported by manipulative
148 experiments where chemical inhibition of aromatase readily induces
149 masculinisation of females in various species (Higa et al., 2003; Kroon et al., 2005;
150 Lee et al., 2001; Nakamura et al., 2015; Nozu et al., 2009). The peptide anti-
151 müllerian hormone (Amh) is implicated in regulating spermatogonial proliferation
152 and male sex differentiation in fish (Li et al., 2015; Pfennig et al., 2015; Skaar et
153 al., 2011; Zhang et al., 2020), and has also been identified as a possible early
154 mediator of female-male sex change (Hu et al., 2015; Liu et al., 2017; Todd et al.,
155 2019). With a network of candidate genes likely to influence the gonadal sex
156 steroid environment, a targeted approach to characterise their expression across
157 sex change is warranted.
158
159 In this study, we present histological, endocrine and gene expression data to
160 characterise the sex change process in the spotty wrasse and establish this species
161 as a new temperate model for the study of vertebrate sex change. We investigate
162 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.

Materials and 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 (IPM) 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) (Fig. 1). 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 placing a TP male in each tank, creating a socially inhibitory environment. During the experiment, fish were fed frozen greenshell mussels (*Perna canaliculus*) three times per week. Pellets containing 200 μg fadrozole (Sigma-Aldrich) in a matrix of cholesterol:cellulose = 95:5 were made in-house (as described in (Lokman et al., 2015; Sherwood et al., 1988)). Sham pellets (vehicle) contained matrix only. Following an acclimation period of three weeks, on day 0 of the experiment all IP individuals were given a single intramuscular fadrozole implant (n=16) or a sham implant without hormone (n=14) using a Ralgun implanter (Syndel, Ferndale, WA).

All fish were removed from individual tanks on day 21 (Tank 1, n=11), day 39 (Tank 2, n=11) or day 60 (Tank 3, n=11; end of experiment). Fish were anesthetised in an aerated bath containing 600 ppm 2-phenoxyethanol (Sigma-Aldrich) and blood samples were collected from the caudal vein using a 1 mL heparinised syringe. Fish were then euthanised by rapid decapitation. The mid-section of one gonad was fixed in Bouin's solution (TP males) or 10% neutral buffered formalin (IP individuals) overnight, and then stored in 70% EtOH at room temperature until paraffin embedding and sectioning for histological analysis. The same location in the gonads was used in all fish when fixing gonadal samples for histology. Body weight and length, and gonadal weight were measured for each fish.

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

Sex change was induced in captive spotty wrasses through manipulation of social groups (i.e., removal of males from the treatment tanks) between September and December 2016. Fifty IP and ten TP individuals ranging from 150 – 215 mm TL were captured and maintained as described in Experiment 1 (AI2014). Fish were distributed into groups across ten 400-litre 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 either removed from the treatment tanks (n = 5) or left in the control tanks (n=5). 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)

225 Social manipulation was used to induce sex change in captive spotty wrasses
226 outside the breeding season between January and April 2018. Sixty-five IP and
227 twelve TP individuals ranging from 138 – 218 mm TL were captured and
228 maintained as described in AI2014. Fish were distributed across twelve 400-litre
229 recirculating seawater systems such that each tank contained a hierarchy of 4 – 5
230 different-sized IP fish and a single TP male (194 – 220 mm TL). Three control (5
231 IP females + 1 TP) tanks were maintained and nine manipulated (5 IP females - 1
232 TP) tanks had the males removed on day 0 after a 2-week acclimation period. A
233 further five IP females were terminally sampled on day 0 to provide a baseline
234 indication of reproductive status. Fish were sampled over a time series as follows;
235 day 1 (n=5), day 11 (n=5), day 26 (n=10), day 36 (n=10), day 55 (n=10), day 92
236 (n=9). Eleven mortalities occurred during the experiment.
237
238 Anaesthetic administration, blood plasma collection, and recording of
239 morphometrics were conducted as described for AI2014. One gonad was flash
240 frozen in ice-cold (on dry ice) isopentane (C_5H_{12}) (Sigma-Aldrich) and stored at -80
241 ºC for RNA analyses. The second gonad was preserved for histological analysis as
242 described in AI2014.
243
244 Fish in all three experiments were maintained and manipulated in accordance
245 with New Zealand National Animal Ethics Advisory Committee guidelines. Ethics
246 applications were approved by the Animal Ethics Committee of Toi Ohomai
247 Institute of Technology and independently reviewed by the University of Otago
248 Animal Ethics Committee.
249
250 **Gonadal tissue processing for histology**
251
252 Histological analysis of gonadal tissues was used to characterise cellular changes
253 occurring across sex change. Tissues from AI2014 and SI2016, fixed in Bouin's
254 solution (TP males) or 10% neutral buffered formalin (IP individuals), were
255 processed for routine embedding in paraffin (New Zealand Veterinary Pathology,
256 Hamilton Laboratory, New Zealand). SI2018 gonadal tissues were embedded in

257 paraffin (Otago Histology Services Unit, Department of Pathology, Dunedin
258 School of Medicine, University of Otago, New Zealand). Sections were cut at 3 - 4
259 µm and stained with Mayer's haematoxylin and eosin.
260
261 Gonadal sections were examined under light microscope to confirm the sex of each
262 individual and samples were subsequently classified into a series of stages to
263 demonstrate the transition from initial stage female through to terminal phase
264 male (Table 1).
265
266 **Steroid measurements**
267
268 Blood was centrifuged at 13,500 rpm for 3 minutes to obtain plasma, which was
269 stored at -20 ºC until steroid analysis. Measurement of blood plasma
270 concentrations of E2 and 11KT across sex change were conducted by
271 radioimmunoassay (RIA) after routine steroid extraction following procedures
272 described in (Kagawa et al., 1981; Kagawa et al., 1982; Young et al., 1983). Assays
273 were validated for spotty wrasse plasma, serially diluted plasma behaving in a
274 manner similar to the standard curve (parallel displacement). Tritiated 11KT was
275 synthesised using the methodology described in Lokman et al. (1997), whereas
276 label for the E2 assay was acquired from Perkin Elmer. Antiserum against E2 was
277 purchased from MyBioSource and antiserum against 11KT was kindly donated by
278 Emeritus Professor Yoshitaka Nagahama, National Institute for Basic Biology,
279 Okazaki, Japan. After incubation and separation of antibody-bound and -unbound
280 steroid by charcoal-dextran solution (0.5% dextran/charcoal), tubes were
281 centrifuged (15 min, 2000g), the supernatant was decanted, and radioactivity
282 measured using a MicroBeta® Trilux scintillation counter (Wallac 1450, Perkin
283 Elmer). Samples from each experiment were run in separate assays with a
284 minimum detectable level of 40 pg/tube (0.08 ng/mL) (E2) and 50 pg/tube (0.10
285 ng/mL) (11KT) for AI2014, 35 pg/tube (0.07 ng/mL) (E2) and 120 pg/tube (0.24
286 ng/mL) (11KT) for SI2016, and 50 pg/tube (0.10 ng/mL) (E2) and 70 pg/tube (0.14
287 ng/mL) (11KT) for SI2018. Extraction efficiencies were 46% (E2) and 82% (11KT)

288 for AI2014; 61% (E2) and 87% (11KT) for SI2016; and 69% (E2) and 40% (11KT)
289 for SI2018.
290
291 Due to non-normality of the RIA data, the non-parametric Kruskal–Wallis test
292 (Kruskal and Wallis, 1952) was used to compare plasma steroid concentrations
293 between sexual stages, for E2 or 11KT separately. If stage was found to have a
294 significant effect, *post hoc* comparisons using Dunn's tests (Dunn, 1961) with
295 Benjamini–Hochberg correction for multiple comparisons (Benjamini and
296 Hochberg, 1995) were performed between stages to determine where the
297 significance lay, carried out in R (v. 1.1.453) (Core Team, 2013).
298
299 **RNA extraction from gonadal tissues**
300
301 Gonadal tissues from the SI2018 experiment were used to assess sex change-
302 related gene expression. Samples were homogenised using a power homogeniser
303 before RNA extraction. RNA was extracted with Direct-zol RNA MiniPrep Plus
304 (Zymo Research) without phase separation (on column DNase treatment).
305
306 RNA concentration was measured using a Qubit 2.0 Fluorometer (Life
307 Technologies) and RNA integrity was evaluated on a Fragment Analyzer
308 (Advanced Analytical Technologies Inc.). The RNA profiles of sex-changing gonads
309 presented a strong peak of low molecular weight RNA. This is considered to be a
310 result of massive 5S rRNA amplification in ovaries (Liu, 2016), and masks the 18S
311 and 28S rRNA peaks used to calculate RNA Integrity Number (RIN) values,
312 making them unreliable estimates of RNA integrity. Similar patterns have been
313 observed in ovaries and/or intersex gonads of thicklip gray mullet (*Chelon labrosus*
314 (Diaz de Cerio et al., 2012)), sharsnout seabream (*Diplodus puntazzo* (Manousaki
315 et al., 2014)) and bluehead wrasse (Liu, 2016). Therefore, in spotty wrasses, RNA
316 integrity for such samples was confirmed by visual inspection of 18S and 28S
317 rRNA peaks.
318

319 **Gene expression analysis**

320

321 A probe array of 18 candidate genes was designed for spotty wrasse based on the
322 nanoString nCounter™ technology (Table S1). Spotty wrasse-specific transcript
323 sequences were identified from a draft spotty wrasse transcriptome assembly (EV
324 Todd & NJ Gemmell, 2015, unpublished data, now superseded by a fully
325 annotated genome https://vgp.github.io/genomeark/Notolabrus_celidotus/).
326 Reference transcripts from zebrafish (*Danio rerio*) were downloaded from
327 GenBank or Ensembl for each target gene, and *actb1*, *eef1a1a* and *g6pd* as
328 potential housekeeping genes (see Supplemental Materials for details on the
329 determination of the housekeeping genes' stability). Transcripts were used to
330 identify the corresponding spotty wrasse sequences from the transcriptome via
331 local BLASTn and tBLASTx (translated the query nucleotide sequences, and the
332 spotty wrasse transcriptome into deduced amino acid sequences in all six possible
333 frames, which were then compared by local alignment). For the best matching
334 spotty wrasse contig, gene identity was confirmed using online nucleotide BLAST
335 (BLASTn) against the NCBI database (http://www.ncbi.nlm.nih.gov/).

336

337 Best-match spotty wrasse contig sequences were submitted to nanoString
338 Technologies for probe design. NanoString nCounter™ CodeSet gene expression
339 quantification was performed on a panel of 18 sex change-related genes at the
340 Otago Genomics Facility, Biochemistry Department, University of Otago, New
341 Zealand. These data were generated using 100 ng gonadal RNA from the SI2018
342 fish (5 control females (day 0; F), 19 ET, 9 MT, 9 LT and 5 TP). The full suite of 18
343 genes were subjected to PCA analysis (see below) to help verify the gonadal
344 staging. Here, we also present gene expression profiles for six gonadal genes
345 relating to the endocrine function of the reproductive and stress axes (Table 2).
346 The remaining expression data, including more novel targets, will be addressed in
347 future work. The geometric mean of gene pair *actb1*|*g6pd* was selected as the
348 reference gene for data normalisation (see Results). Normalisation calculations
349 were performed automatically in the nanoString nSolver Analysis software
350 (version 4.0), and normalised data were log base-2 transformed prior to analysis.

351 Due to non-normality of the normalised nanoString data, the non-parametric
352 Kruskal–Wallis test (Kruskal and Wallis, 1952) was run separately for each target
353 gene to determine whether stage had a significant effect on gene expression level,
354 and *post hoc* comparisons using Dunn's tests (Dunn, 1961) with Benjamini–
355 Hochberg correction for multiple comparisons (Benjamini and Hochberg, 1995)
356 were performed to determine where the significance, if any, lay between stages.
357 All analyses were performed in R (v. 3.3.2) (Core Team, 2013). NanoString
358 Expression Data Analysis Guidelines (MAN-C0011-04) were followed to determine
359 an expression threshold, set as the $\log_2$ of the geometric mean of the negative
360 control counts plus two standard deviations.
361
362 Principal Component Analysis (PCA) (scaled, R (v. 1.1.453) (Core Team, 2013))
363 was used to visualise variation in the overall mRNA expression across the
364 expanded suite of 18 sex change-related genes (Table S1) in relation to sexual
365 stage and assess clustering of the histological defined in Table 1. To identify the
366 genes contributing most to observed patterns represented by the first two
367 principal components, component loadings (defined as eigenvectors scaled by the
368 square root of the respective eigenvalues) were represented as coordinates in a
369 Cartesian plane.
370
371 **Results and Discussion**
372
373 The definition and delineation of sexual stages differs between studies of
374 protogynous fish. In this study, we define five gonadal stages to describe the sexual
375 transition from female to terminal phase male endpoints in the temperate spotty
376 wrasse. While these stages are based on gonadal histology the classification is also
377 supported by the PCA analysis of the gonadal gene expression data. These samples
378 showed clusters arranged in the sequential order of the sexual stages from female
379 through to male (PC1, 50.7% variation explained; Fig. 2).
380
381 The spotty wrasse gonadal gene set was also clustered according to the degree of
382 their developmental commitment (PC2, 19.4% variation explained). Fully

differentiated ovary and testis were separated from less differentiated transitional gonads. These patterns show a striking resemblance to whole transcriptome gonadal expression profiles from transitioning tropical bluehead wrasse (Todd et al., 2019). This demonstrates the importance and relevance of the spotty wrasse gene panel to studying sexual transition in protogynous wrasses more broadly.

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 experimental fish were found to be IPM.

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). No conclusive evidence of sex change was found in any of the control females, although ovarian atresia was observed in four of these individuals (day 30, n=3; day 66, n=1). Histological analysis also confirmed that five of the

414 starting 50 IP individuals were IPM (10.0% frequency), a slightly higher ratio than
415 reported previously (4.1 – 5.7%) (Jones, 1980).
416
417 *Experiment 3: Social induction of sex change in spotty wrasses outside their*
418 *breeding season (SI2018)*
419
420 Male removal conducted outside the breeding season also induced sex change in
421 female spotty wrasses. All socially manipulated females showed histological signs
422 of ovarian degeneration or sex change. Histology confirmed that 18 females
423 reached ET stage (day 1, n=3; day 11, n=2; day 26, n=3; day 36, n=3; day 55, n=4;
424 day 96, n=3), 12 MT stage (day 1, n=1; day 11, n=3; day 26, n=4; day 36, n=1; day
425 55, n=3), 13 LT stage (day 26, n=2; day 36, n=6; day 55, n=3; day 92, n=2) and 6
426 became full TP males (day 1, n=1; day 92, n=5). No IPM were found (Fig. 3).
427 Unfortunately control tanks with male fish present experienced an unknown
428 infection and reduced survival in the later stages of the experiment. This
429 confounded the efficacy of socially induced sex change in this experiment.
430 Nonetheless, the results of the other two experiments (AI2014 & SI2016) indicate
431 that both chemical and social manipulation increases sex change compared to
432 control female fish that have a male present (Fisher's exact test, $p < 0.001$). This
433 is also supported by previous studies with this species (Muncaster, unpublished
434 data).
435
436 <u>Incidence of sex change depending on seasonality</u>
437
438 The breeding season of spotty wrasses in northern New Zealand lasts from late
439 July until the end of November and peaks in the austral spring (Jones, 1980). Of
440 the socially manipulated spotty wrasses, the more mid-transitional and fully
441 transformed males were recorded in the experiment conducted during the post-
442 spawning period (SI2018) in summer and early autumn (January to April; Fig. 4).
443 In this experiment, 55% of IP fish had MT through to TP stage gonads, whereas
444 less than 25% of fish socially manipulated during the October-December breeding
445 season (SI2016) presented the same stage gonads. Sex change is often seasonally

446 biased with the greatest occurrence following the breeding season in temperate
447 and warm water species, but occurs year-round in many tropical species (Alonso-
448 Fernández et al., 2011; Li et al., 2007; Muncaster et al., 2013; Sadovy and Shapiro,
449 1987; Thomas et al., 2019). Our results support observations in wild spotty
450 wrasses, in which natural sex change has been documented during the non-
451 reproductive months between November and May (Jones, 1980). This indicates
452 that for experimental purposes, post-spawned fish present the best candidates for
453 socially manipulated sex change. However, we have also demonstrated that male
454 removal can lead to sex change within the breeding season.
455
456 Steroid production in relation to sex change
457
458 Plasma E2 concentrations showed a general decreasing trend from female to male
459 stages (Fig. 5A). Fish treated with an aromatase inhibitor (AI2014) had mean
460 plasma E2 concentrations ranging from 0.08 ng/ml to 0.34 ± 0.25 SD ng/ml for LT
461 and ET stages respectively. In comparison, female fish had a higher mean plasma
462 E2 concentration of 0.42 ± 0.46 SD ng/ml, although this was not statistically
463 different. Social manipulation during the breeding season (SI2016) showed
464 significantly higher plasma E2 concentrations in females (0.38 ± 0.39 SD ng/mL)
465 compared to males (0.07 ± 0.00 SD ng/mL, $p < 0.001$ for TP and 0.07 ± 0.00 SD
466 ng/mL, $p < 0.05$ for IPM). However, plasma E2 concentrations were minimal in all
467 sexual stages when social manipulation was conducted after the completion of the
468 spawning season (SI2018). Similar reduced E2 concentrations after breeding were
469 observed in other protogynous species (Bhandari et al., 2003; Li et al., 2007;
470 Muncaster et al., 2010). Gonadal resorption and quiescence often follows
471 reproduction in temperate fish and this is characterised by ovarian atresia and
472 reduced sex steroid concentrations (Scott et al., 1984). This is not surprising
473 considering the importance of E2 in driving seasonal oocyte growth in teleosts
474 (Jalabert, 2005; Lubzens et al., 2010; Nagahama, 1994; Patiño and Sullivan,
475 2002).
476

477 While E2 has a clear role in maintaining ovarian function, there is no obvious
478 relationship between plasma E2 concentrations and the initiation of sex change in
479 spotty wrasses. A marked decrease in E2 concentrations has been implicated as a
480 critical initiator of sex change in many protogynous species (Bhandari et al., 2003;
481 Liu et al., 2017; Muncaster et al., 2013; Nakamura et al., 1989). Yet, despite a 2.3-
482 fold decrease, there was no significant difference in plasma E2 concentration
483 between F and ET (0.16 ± 0.16 SD ng/mL) fish during the breeding season
484 (SI2016). Similarly, there was no significant decrease of plasma E2 concentration
485 between female and early transitional spotty wrasses from the other experiments
486 (AI2014 & SI2018). While this may, in part, relate to sample size or seasonal
487 influence, similar results were also reported in the bambooleaf wrasse
488 (*Pseudolabrus sieboldi*) (Ohta et al., 2008) and orange-spotted grouper
489 (*Epinephelus coioides*) (Chen et al., 2020b). Undetectable E2 concentrations
490 existed from MT to male (TP & IP) stages in nearly all fish. Therefore, while there
491 is no evidence of a minimum plasma E2 threshold required to initiate gonadal
492 transition in spotty wrasse, a general reduction is associated with the process of
493 sex change in this species. Indeed, E2 depletion leads to masculinisation in
494 hermaphroditic and gonochoristic species alike (Bhandari et al., 2004; Li et al.,
495 2019; Nozu et al., 2009; Paul-Prasanth et al., 2013; Takatsu et al., 2013).
496
497 Elevated plasma 11KT concentrations were observed in individual fish towards
498 the transitional and male stages in all three experiments (AI2014, SI2016 and
499 SI2018; Fig. 5B). While variability in the data and reduced statistical power made
500 the detection of discernible differences impossible, the timing of these observations
501 coincides with the histological appearance of spermatogenic cysts (see Table 1).
502 Increased 11KT concentrations were also evident from mid transition onwards in
503 other protogynous species (Bhandari et al., 2003; Nakamura et al., 1989;
504 Nakamura et al., 2005). Fish treated with AI (AI2014) had minimal 11KT values
505 in the earlier sexual stages. This was evident in the F (0.17 ± 0.03 ng/ml) and ET
506 (0.22 ± 0.17 SD ng/ml) fish while a later stage LT individual presented with 1.0
507 ng/ml. Fish socially manipulated during the breeding season (SI2016) showed
508 remarkably similar plasma 11KT concentrations regardless of sexual stage. These

509 values ranged from 0.13 ± 0.02 SD ng/ml in F to 0.23 ± 0.31 SD ng/ml in TP. All of
510 the F, transitional, and IPM had either identical mean or individual 11KT
511 concentrations of 0.14 ng/ml. Many of the androgen concentrations of fish socially
512 manipulated after the breeding season (SI2018) remained minimal as expected
513 during quiescence.
514

515 The lowest mean 11KT concentrations (0.13 ± 0.02 SD ng/ml) presented in F and
516 were only slightly higher in subsequent stages such as ET (0.15 ± 0.03 SD ng/ml)
517 and MT fish (0.19 ± 0.15 SD ng/ml). Highest plasma 11KT values were observed
518 in LT (0.29 ± 0.32 SD ng/ml) fish, while slightly reduced concentrations were
519 recorded in TP individuals (0.22 ± 0.18 SD ng/ml). This disparity of androgen
520 concentrations in late-stage fish most likely reflects the chronology of the
521 experiment. TP fish were removed from the socially manipulated tanks at the
522 beginning of the experiment immediately after the breeding season. Collection of
523 these tissue and plasma samples would likely have been when spermatogenesis
524 was minimal or non-existent. The occurrence of several LT fish occurred later in
525 the experiment, coinciding with seasonal gonadal recrudescence.

526 The androgen profiles from the fish in this study do not show a clear statistical
527 relationship between sexual stages. However, the role of 11KT in driving
528 spermatogenesis is well established in teleosts (Miura et al., 1991; Nagahama,
529 1994; Nakamura et al., 1989; Schulz et al., 2010). Much of this androgen activity
530 is likely to be paracrine in nature with steroidogenic somatic cells stimulating local
531 germ cells both directly and indirectly within the gonadal compartment (Schulz,
532 1986). The additional role of 11KT in expressing seasonal male secondary sexual
533 characteristics, such as morphometric and behavioural modifications, also exists
534 (Borg, 1994; Semsar and Godwin, 2004). This likely requires elevated plasma
535 concentrations for remote, effective target cell signalling and may in part explain
536 substantial elevations of 11KT prior to breeding in many male teleosts. The
537 absolute concentrations of androgen required to stimulate spermatogenesis are
538 conceivably much lower. Furthermore, prior to these peak physiological
539 concentrations the actual concentrations of 11KT within the gonad are likely to be

540 higher than in the plasma. It is, therefore, possible that the absolute
541 concentrations of 11KT required to initiate spermatogenesis during gonadal
542 restructure are not reflected in the spotty wrasse plasma samples. This issue may
543 be further investigated using *in vitro* explant culture systems (Goikoetxea et al.,
544 2020). Alternatively, gonadal expression of key steroidogenic enzymes should also
545 reflect, in proxy, the androgen activity across different sexual stages.
546
547 Sexually dimorphic expression of gonadal endocrine genes
548
549 To obtain a more sensitive assessment of the endocrine regulation of gonadal sex
550 change, the expression of three genes encoding gene products involved in steroid
551 biosynthesis, two steroid receptors, and anti-Mullerian hormone were measured.
552 The temporal regulation of steroid hormones is essential for the coordination of
553 sex differentiation, sexual maturation and behaviour in vertebrates. They are also
554 potent mediators of gonadal sex change in teleosts (Guiguen et al., 2010; Higa et
555 al., 2003; Nakamura et al., 1989). As expected *cyp19a1a* expression was greatest
556 in F and was gradually downregulated across sex change (Fig. 6A). Expression did
557 not differ significantly across stages. As reflected in the E2 data, there was no
558 evidence of an early, rapid downregulation that has been thought to trigger sex
559 change in other species (Gemmell et al., 2019; Liu et al., 2017; Todd et al., 2016).
560 Levels of *cyp19a1a* expression remained similar across ET, MT and LT stages
561 (2018) while in comparison, plasma E2 concentrations were negligible by mid
562 transition in all three experiments (AI2014, SI2016 and SI2018). While *cyp19a1a*
563 is an unlikely proximate trigger of sex change in spotty wrasse, a more distant
564 association exists nonetheless. This is evident in the number of fish that changed
565 sex following aromatase inhibition (AI2014) as well as the occurrence of sex
566 change in fish socially manipulated after the breeding season (SI2018) when
567 plasma E2 concentrations were minimal. Considering the potent feminising action
568 of E2, a reduction of gonadal concentrations may act as a gateway to facilitate the
569 progression of transition rather than acting as an early trigger. This action may
570 be through the release of steroid-induced suppression on male-pathway genes
571 (Guiguen et al., 2010).

572

573 In teleosts, testosterone (T) can be bioconverted into 11KT by the enzymes 11ß-
574 hydroxylase (Cyp11c1) and 11ß-hydroxysteroid dehydrogenase type 2 (Hsd11b2)
575 (Frisch, 2004). Upregulation of *cyp11c1* was observed across sex change (Fig. 6B),
576 with significantly (X^2 (4) = 32.39, $p < 0.001$) greater expression in TP spotty
577 wrasses than F (median 2.6-fold greater) and ET (median 2.1-fold greater) fish.
578 Albeit less pronounced, *hsd11b2* expression increased in a similar pattern across
579 sex change (X^2 (4) = 32.13, $p < 0.001$; Fig. 6C). The simultaneous upregulation of
580 both *cyp11c1* and *hsd11b2* from MT through to TP stages coincides with the
581 presence of spermatogenic cysts in the gonad and likely drives the increase in
582 gonadal 11KT production. Although this is not evident in plasma 11KT
583 concentrations (see Fig. 5B), these expression patterns may indicate either
584 undetected paracrine action of 11KT within the early testis or the accumulation
585 of enzyme transcripts prior to translation.
586

587 Another contemporary hypothesis for a universal trigger of sex change involves
588 stress, Cyp11c1 and Hsd11b2 are key players in the teleost stress response, being
589 necessary for the interrenal biosynthesis of cortisol and for its subsequent
590 inactivation to cortisone, respectively (Arterbery et al., 2010; Fernandino et al.
591 2013; Goikoetxea et al., 2017). Cross-talk between the interrenal and reproductive
592 axes through the upregulation of these enzymes has been implicated as
593 influencing masculinisation in teleosts (Chen et al. 2020; Fernandino et al, 2013;
594 Goikoetxea, 2020; Hattori et al. 2013; Liu et al., 2017, Solomon-Lane et al. 2013),
595 particularly through the production of weak interrenal-derived androgens,
596 released as part of the stress response, which could conceivably serve as
597 precursors for 11KT synthesis (Lokman et al., 2002). As cortisol exerts its effects
598 by activating its receptors, among which Nr3c1 and Nr3c2 are best characterised,
599 the genes encoding these receptors present interesting targets. These genes have
600 opposing expression patterns in bluehead wrasse, with *nr3c1* showing a male bias
601 while *nr3c2* is female-biased (Liu et al., 2015; Todd et al., 2019). However, gonadal
602 expression of both *nr3c1* (X^2 (4) = 28.40, $p < 0.001$) and *nr3c2* (X^2 (4) = 13.02, $p <$
603 0.05) was elevated in the LT to TP stages of spotty wrasse (Figs. 6D and 6E).

604 Expression of these genes did not differ between F and TP fish, which is likely
605 connected to sample size-related variability, particularly in female fish. Early-
606 stage expression of *cyp11c1*, *hsd11b2* and *nr3c2* was thought to indicate a role for
607 cortisol in triggering sex change in the bluehead wrasse model (Todd et al., 2019).
608 In contrast, upregulation of these genes occurred in later stage gonads in the
609 spotty wrasse. This suggests a relationship between cortisol and sex change may
610 exist but not necessarily as an initiator in this species. Chen et al. (2020a) report
611 a marked transient increase of cortisol concentrations during the early stages of
612 protogynous sex change in orange-spotted grouper. Similar to what we observed
613 in spotty wrasse, these fish do not show a significant reduction in levels of E2
614 during sex change. As such, the precise role of cortisol and the stress response
615 during sex change warrants further investigation.
616
617 Anti-Müllerian hormone, encoded by *amh*, is strongly linked to male vertebrate
618 sex differentiation (Josso et al., 2001; Pfennig et al., 2015). This was reflected in a
619 linear function across sex change stages in spotty wrasse. *Amh* expression was
620 greatest in TP males (2.2-fold, 1.6-fold and 1.4-fold greater in TP than F, ET and
621 MT, respectively; X^2 (4) = 38.71, $p < 0.001$; Fig. 6F). The apparent upregulation of
622 *amh* in ET and MT stage fish indicates its importance in the initiating stages of
623 sex change in this species. Furthermore, *amh* demonstrated the greatest
624 contribution to PC1 (ovary-to-testis transition) of the PCA analysis. This is
625 consistent with other studies showing upregulation of *amh* at the onset of
626 protogynous sex change and complementary downregulation in the early stages of
627 protandrous sex change (Hu et al., 2015; Liu et al., 2017; Wu et al., 2015). The
628 expression pattern of *amh* provides further validation for the histological staging
629 of ET individuals as resolving cellular differences between ET fish and post-
630 spawned females with regressing ovaries can be difficult in sex changing fish. A
631 recent mechanistic model for socially induced sex change in the protogynous
632 bluehead wrasse highlights an association between early *amh* activation and the
633 stress hormone, cortisol (Todd et al., 2019). Therefore, *amh* may form part of the
634 molecular trigger that initiates sex change, and its upregulation could be a useful
635 early molecular marker for protogynous species.

636 **Conclusion**

637

638 This study validates the chemical and social induction of sex change in captivity
639 and describes the anatomical, endocrine and molecular events during sex change
640 to establish the New Zealand spotty wrasse as a temperate-water model for sex
641 change research. Sex change is most readily induced following the reproductive
642 season when plasma sex steroid concentrations are low.

643

644 In general, the female-related markers E2 and *cyp19a1a* declined during sex
645 change while the male-related markers 11KT, *cyp11c1*, *hsd11b2* increased. The
646 enzymes encoded by these latter genes are also actively involved in the interrenal
647 stress response. The gonadal expression of *nr3c1* and *nr3c2* increased in the later
648 stages of sex change in spotty wrasse. This could suggest a putative role for stress
649 during sex change but there is no clear evidence for cortisol as an early-stage
650 activator of sex change. Similarly, E2 did not seem to be an initiator of sex change
651 in this model, but *amh* showed early-stage upregulation. Apart from being an
652 interesting target to better understand early activators of sex change, *amh* may
653 prove to be a beneficial marker to resolve ET stage fish from females undergoing
654 seasonal ovarian atresia

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917 **Figure legends:**

918

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

924

925 Figure 2. PCA (18 genes) of gonad samples. The transition from females to males
926 is captured along PC1 (50.7% variance), whereas PC2 (19.4% variance) extremes
927 represent sexually differentiated gonads (bottom) and undifferentiated
928 transitionary gonads (top). Sample sizes: F n = 5, ET n = 19, MT n = 9, LT n = 9,
929 TP n = 5. Abbreviations: control female (F), early transitioning fish (ET), late
930 transitioning fish (LT), mid-transitioning fish (MT), terminal phase male (TP).

931

932 Figure 3. Histological stages of gonadal development in spotty wrasse: A) female;
933 B) early transitioning fish; C) mid-transitioning fish; D) late transitioning fish; E)
934 terminal phase male. Abbreviations: atretic oocyte (AtO), blood vessel (BlV),
935 collagen (Col), gonial cells (GoC), gonadal lumen (Lum), previtellogenic oocyte
936 (PvO), oogonia (Oog), stromal cells (StC), sperm collecting duct (SCD),
937 spermatocytes (Spc), spermatogonia (Spg), spermatozoa (Spz), tunica albuginea
938 (TAl), yellow-brown body (YBB). Scale bars in A, B, E = 200 μm; C, D = 100 μm.

939

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

946

947 Figure 5. Plasma E2 (A) and 11KT (B) concentrations in females, early, mid- and
948 late transitioning fish, and TP and IP males obtained across the three experiments,

949 AI2014, SI2016 and SI2018. Each red (AI2014), yellow (SI2016) and blue (SI2018)
950 line represents the variation in mean E2 or 11KT concentrations across groups per
951 experiment while the black line represents the variation in overall mean
952 concentrations for the three experiments altogether. Sample sizes: E2, F n = 36,
953 ET n = 42, MT n = 8, LT n = 14, TP† n = 17, IP n = 5; 11KT, F n = 40, ET n = 41,
954 MT n = 9, LT n = 15, TP† n = 26, IP n = 4. Abbreviations: 11-ketestosterone (11KT),
955 aromatase inhibition experiment 2014 (AI2014), initial phase female (F), 17β-
956 estradiol (E2), early transitioning fish (ET), initial phase male (IPM), late
957 transitioning fish (LT), mid-transitioning fish (MT), social induction experiment
958 2016 (SI2016), social induction experiment 2018 (SI2018), terminal phase male
959 (TP). †Both control males used throughout the experiments to create a socially
960 inhibitory environment (SI2016: E2 n = 10, 11KT n = 10; SI2018: E2 n = 0, 11KT
961 n = 10), and males obtained through sex change of manipulated females (SI2016:
962 E2 n = 1, 11KT n = 1; SI2018: E2 n = 5, 11KT n = 5) were grouped altogether as
963 TP males for the purpose of this analysis.
964
965 Figure 6. Relative gonadal expression of *cyp19a1a* (A), *cyp11c1* (B), *hsd11b2* (C),
966 *nr3c1* (D), *nr3c2* (E) and *amh* (F) mRNA. Expression levels are compared among
967 control females sampled on day 0, transitioning individuals and TP males. In the
968 boxplots, each point represents an individual fish, the middle bold line represents
969 the median, the edges of the box represent the upper and lower quartiles, and
970 vertical lines represent the minimum and maximum values. Letters denote a
971 significant difference in distribution between groups. Sample sizes: F n = 5, ET n
972 = 19, MT n = 9, LT n = 9, TP† n = 5. Abbreviations: control female (F), early
973 transitioning fish (ET), late transitioning fish (LT), mid-transitioning fish (MT),
974 terminal phase male (TP). †Both a male used during the acclimation period of the
975 experiment (n = 1), and males obtained through sex change of socially manipulated
976 females (n = 4) were grouped altogether as TP males for the purpose of this
977 analysis.

Figures:

Figure 1

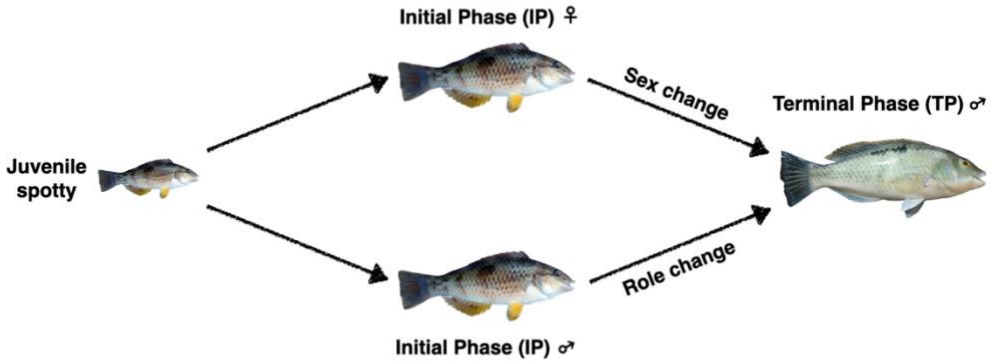
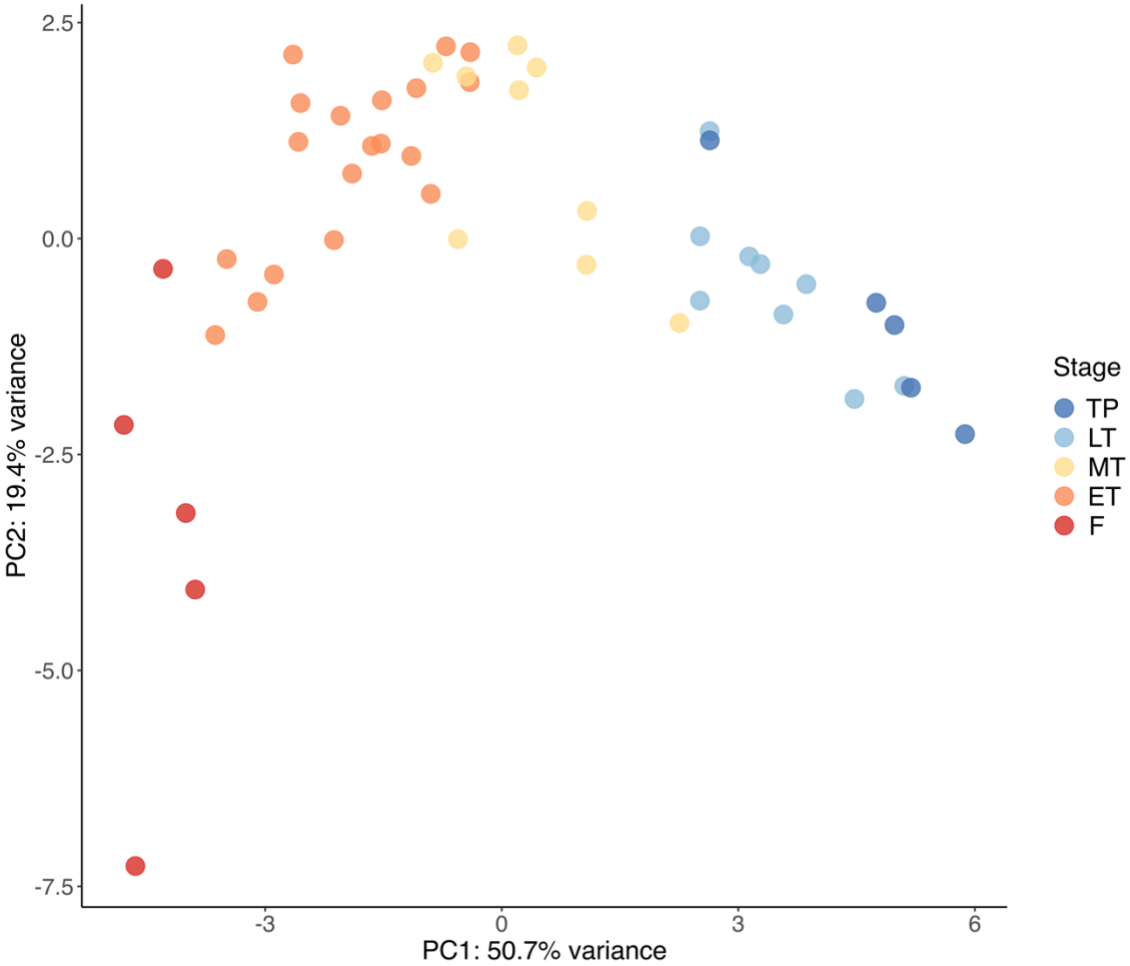


Figure 2


2.5
0.0
-2.5
-5.0
-7.5
PC2: 19.4% variance
-3
0
3
6
PC1: 50.7% variance
Stage
TP
LT
MT
ET
F

1015 Figure 3

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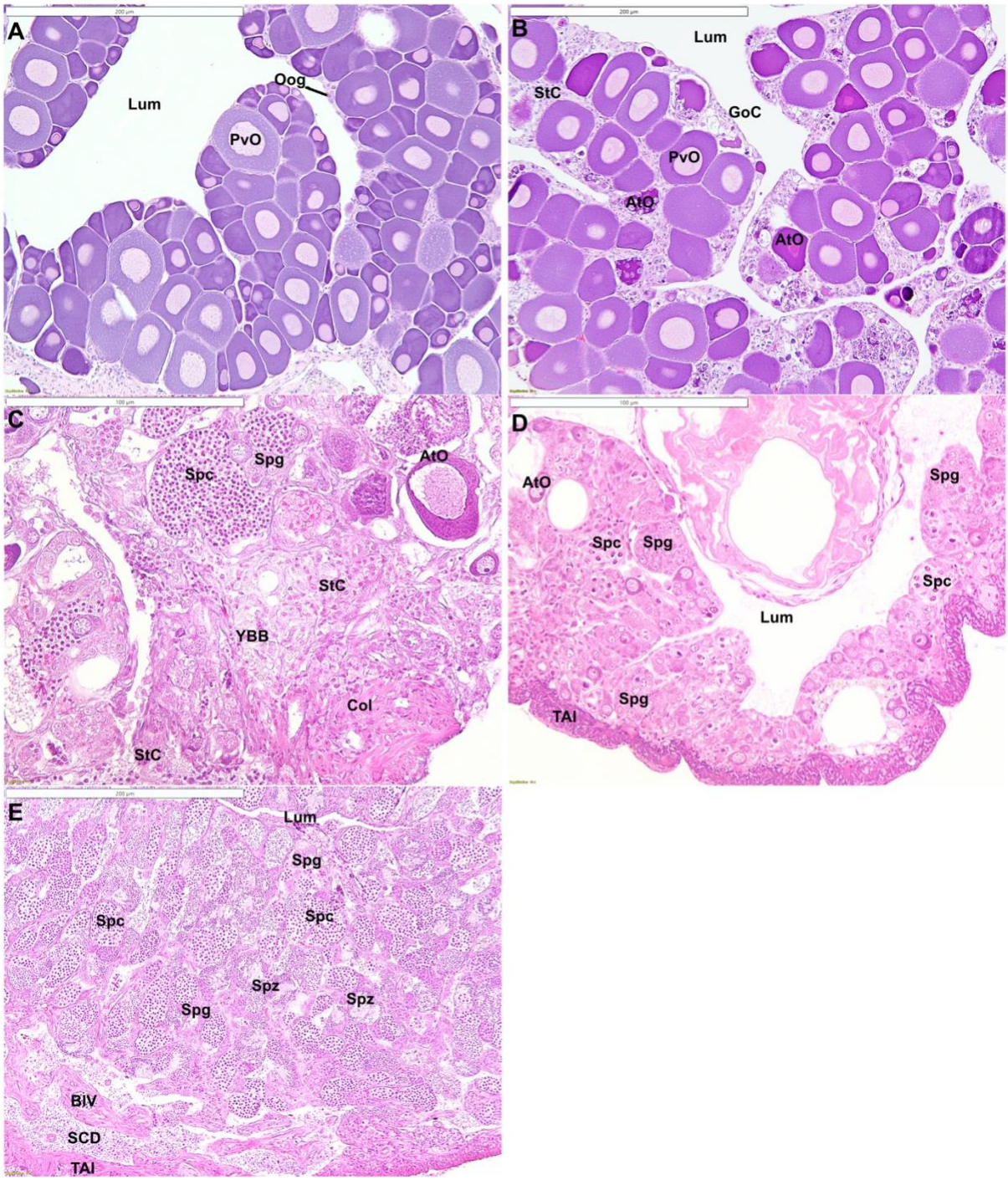

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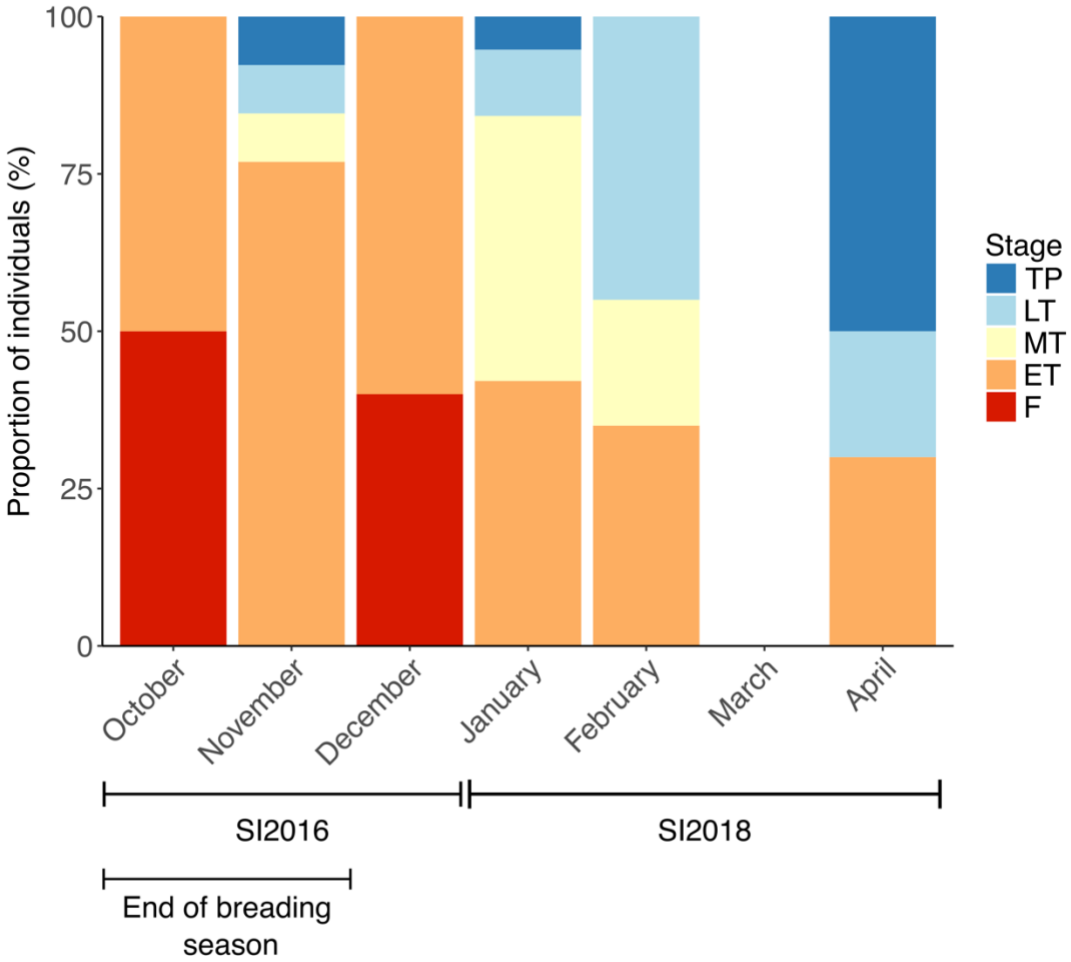
Figure 4


100
75
50
25
0
Proportion of individuals (%)
October
November
December
January
February
March
April
SI2016
SI2018
End of breading season
Stage
TP
LT
MT
ET
F

1036 Figure 5

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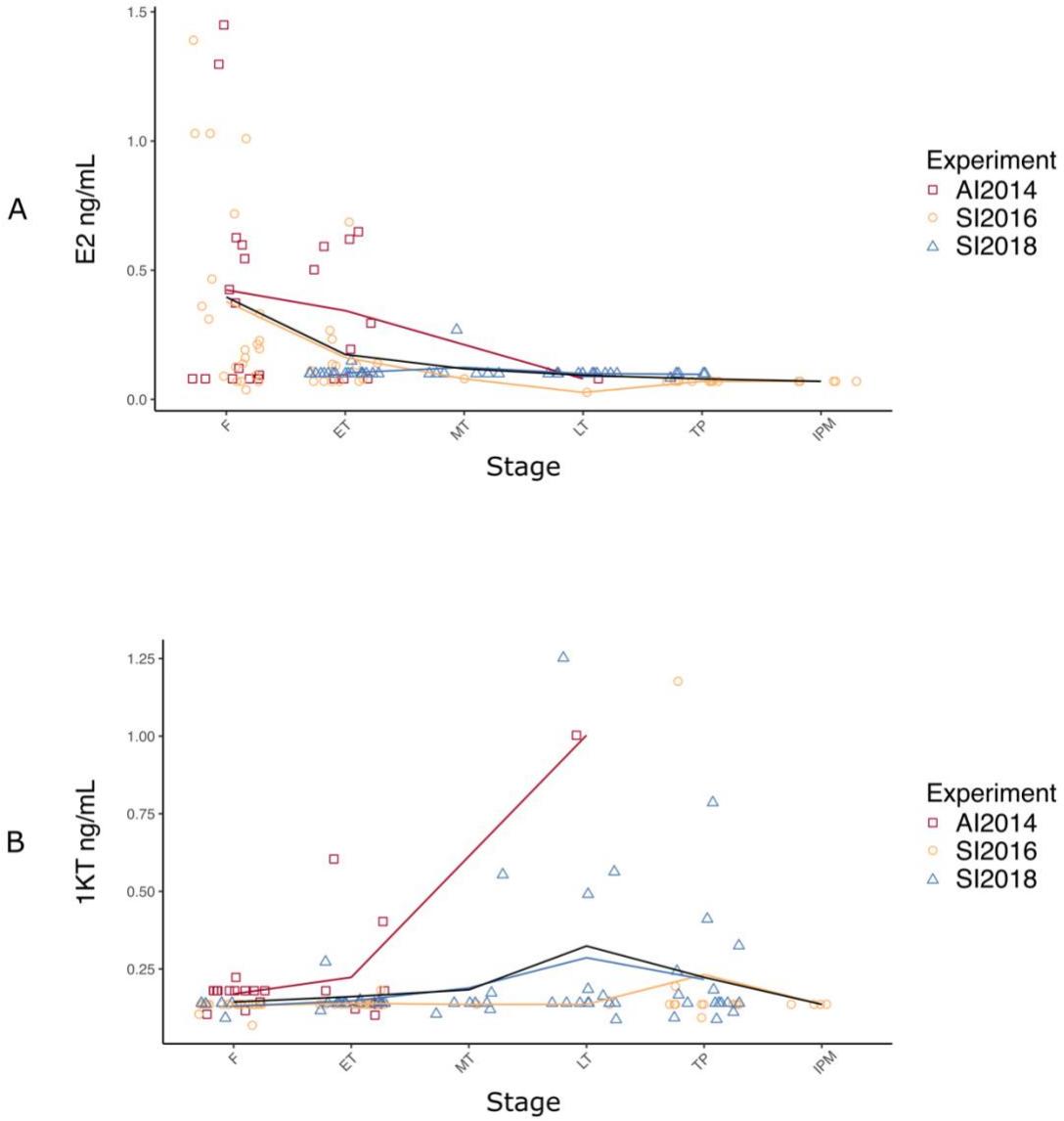

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1046 Figure 6

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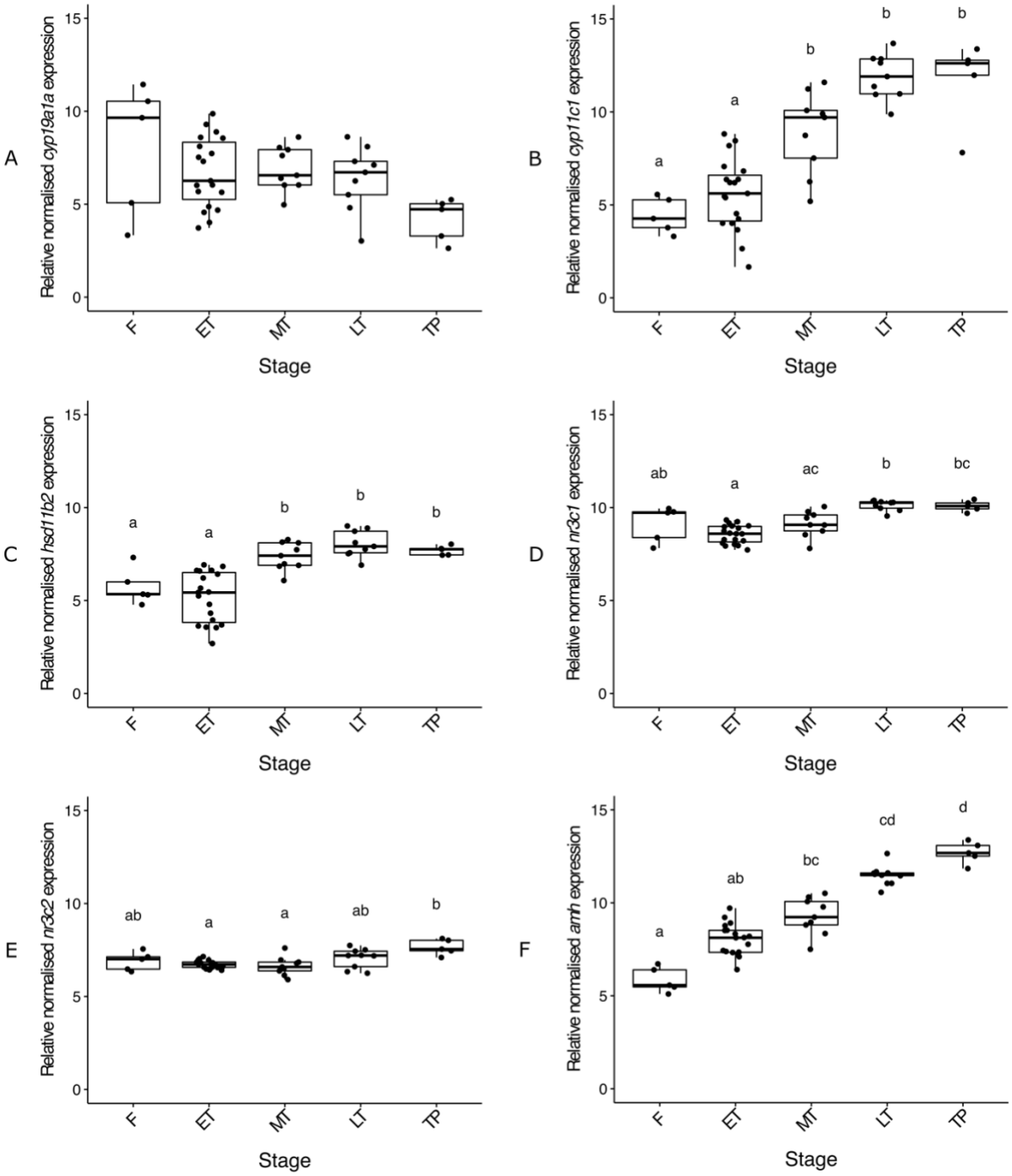

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1055 Table 1

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Stage	Abbreviation	Characteristics
Initial Phase Female	F	Ovary is dominated by healthy oocytes. Depending on season, these may be predominantly previtellogenic stage oocytes or may also include vitellogenic and maturing oocytes. Atretic late-stage oocytes may be common post-spawning.
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. 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	IPM	Presence of lobule formation containing spermatogenic cysts. No evidence of degenerating oocytes. No central lumen and sperm ducts tend to be aggregated centrally.

1057

1058 Table 2

1059

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			
amh	anti-Müllerian hormone	c51546_g1_i1	NM_001007779.1

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1061