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Effects of 17α-ethinylestradiol (EE_2) on gonadal development and gene expression in larval mummichog (*Fundulus heteroclitus*)

By:

Carly Sing-Judge
B.Sc. Environmental Toxicology and Biology, University of Guelph, 2015

Thesis

Submitted to the Department of Biology
Faculty of Science

In partial fulfillment of the requirements for the Master of Science in Integrative Biology
Wilfrid Laurier University

2022

Carly Sing-Judge 2022©

Dedication

This thesis is proudly dedicated to my grandparents, Harry Clyde Sing and Barbara Joan Sing, who have helped me tremendously throughout my entire academic career. I would have never gone back to school to complete a Master’s degree without their love and encouragement to pursue these studies. My grandparents have always believed in my capabilities ever since I was young. I only wish I could have shared this milestone with them in person.

I miss you, Grandma and Papa.

Abstract

The mummichog (*Fundulus heteroclitus*) is a small-bodied estuarine fish that inhabits the North American east coast and is often used as a model adult saltwater fish in environmental bioassays. This study aimed to describe gonadal development and gene expression levels in five-week-old mummichog under control conditions and following 17α-ethinylestradiol (EE_2) treatment in order to better understand the susceptibility of sex determination (SD) and gonadal differentiation (GD) processes following contaminant exposure. In the first experiment, eggs were fertilized, collected and incubated in the laboratory for three weeks, then yolk-sac larvae were grown out for five weeks and sampled for gonadal development and gene expression levels in ovaries and testes. In a second experiment, yolk-sac larvae were reared for five weeks in one of three static (daily renewal) treatments [0, 2 and 10ng/L EE_2 (actual)] and then sampled for gonadal histology and gene expression analysis. In controls, the sex ratio approached 50/50 and EE_2 treatment did not change the sex ratios. However, altered gametogenesis and increased degeneration were observed in EE_2-treated ovaries and testes, indicating a possible downregulation of the HPG (hypothalamus-pituitary-gonadal) axis, thus, halting normal gonadal development. Several GD genes were identified for the first time in the mummichog that may be influencing female- and male- promoting networks. EE_2 treatment did not change ovarian gene expression, however, testis gene expression was severely altered by increased expression of female-promoting genes and anti-Mullerian hormone (*amh*) which may influence self-renewal in early-staged germ cells. The lack of effect of EE_2 on sex ratio was surprising and may have been a result of treating the fish after hatching instead of during embryogenesis. These results demonstrate that male

60 mummichog are more sensitive to EE_2 after hatching than females. This study also
61 demonstrates that GD mechanisms in the mummichog can be altered by environmentally-
62 relevant concentrations of EE_2 when exposed after hatching. This study suggests that in
63 mummichog SD is fixed prior to hatching but that GD mechanisms are susceptible to
64 environmental estrogen exposure during early development.

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I would first like to thank my thesis advisors Dr. Deborah MacLatchy at Wilfrid Laurier University and Dr. Glen Van Der Kraak at the University of Guelph. Thank you for being strong leaders throughout the COVID-19 pandemic and encouraging me not to give up on the writing process.

I would also like to acknowledge a few laboratory colleagues. Firstly, Dr. Robert Rutherford, a previous post-doc student in the MacLatchy lab, for his continuous support throughout my project both personally and professionally. Rob has inspired me to stay positive, even during the most difficult of times; I will never forget his sunny personality and the impact it made to the lab. As well, I would like to acknowledge Jacquie Matsumoto, from the Van Der Kraak lab, for providing exceptional technical advice and encouragement throughout my studies. Jacquie has been an amazing mentor since my undergraduate thesis project, I hope to one day provide the same kind of mentorship to other students. Lastly, I wanted to recognize Emily Corrigan, a previous technician from the MacLatchy lab, for her technical help with the project and providing me with the motivation that I needed to complete the lab work. It was always comforting having a close friend throughout my studies. I am gratefully indebted to them all for their very valuable guidance on this thesis.

Finally, I must express my very profound gratitude to my fiancé, Connor Simmons, for providing me with unfailing support and continuous encouragement throughout my years of study and through the process of researching and writing this thesis. Connor has always had my back through any rough day; this accomplishment would not have been possible without him. Thank you.

Carly Sing-Judge, B.Sc.

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145 # List of Abbreviations

146 11-KT - 11-ketotestosterone
147 28S - structural large ribosomal RNA of eukaryotic cytoplasmic ribosomes
148 18S - structural small ribosomal RNA of eukaryotic cytoplasmic ribosomes
149 β-actin - beta actin
150 AR - androgen receptor
151 amh - anti-Mullerian hormone
152 amhy - anti Mullerian hormone gene on the Y chromosome
153 amhr - amh receptor
154 BMP - bone morphogenetic protein
155 bmp15 - bone morphogenetic protein 15
156 BSD - behavioural sex determination
157 cDNA - clone DNA
158 Cq - cycle value
159 cyp11c1- cytochrome P450 family 11 subfamily C member 1
160 cyp19a1a - cytochrome P450 family 19 subfamily A group 1 subgroup A or aromatase
161 dax1- dosage-sensitive sex reversal, adrenal hypoplasia critical region, on chromosome x,
162 gene 1
163 dmy - DM-domain gene on the Y chromosome
164 dmrt1- double sex and Mab-3 related transcription factor 1
165 dph - days post hatch
166 DNA - deoxyribonucleic acid
167 E_1 - estrone
168 E_2 - 17β-estradiol
169 EDC - endocrine disruption chemical
170 EE_2 - 17α-ethinylestradiol
171 efl1α - elongation factor 1 alpha
172 ER - estrogen receptor
173 ERα - estrogen receptor alpha
174 ERβ - estrogen receptor beta
175 ESD- environmental sex determination
176 foxl2 - forkhead transcription factor 2
177 FSH - follicle stimulating hormone
178 GD - gonadal differentiation
179 gdf9 - gonadal differentiating factor 9
180 gdsf - gonadal derived soma factor
181 GnRH - gonadotropin releasing hormone
182 GSD - genetic sex determination
183 GTH - gonadotropin
184 H&E - hemoxylin and eosin stain
185 HPG - hypothalamic-pituitary-gonadal axis
186 LC MS/MS - liquid chromatography with tandem mass spectrometry
187 LH - luteinizing hormone

188 PCR - polymerase chain reaction
189 PGC - primordial germ cell
190 RNA - ribonucleic acid
191 RQI - RNA quality indicator number
192 RT-qPCR - Reverse transcription quantitative polymerase chain reaction
193 SD - sexual determination
194 SRY - sex-determining region on the Y chromosome, also known as testis determining
195 factor or testis determining factor
196 SOX9 - SRY-box transcription factor 9
197 T - testosterone
198 TMS - tricaine methanesulfonate
199 TGFβ - transforming growth factor beta superfamily
200 USEPA - United States Environmental Protection Agency
201 VTG - vitellogenin
202 wnt4 - wingless-related integration site family, member 4
203 wph - weeks post hatch
204 XX - homozygous female
205

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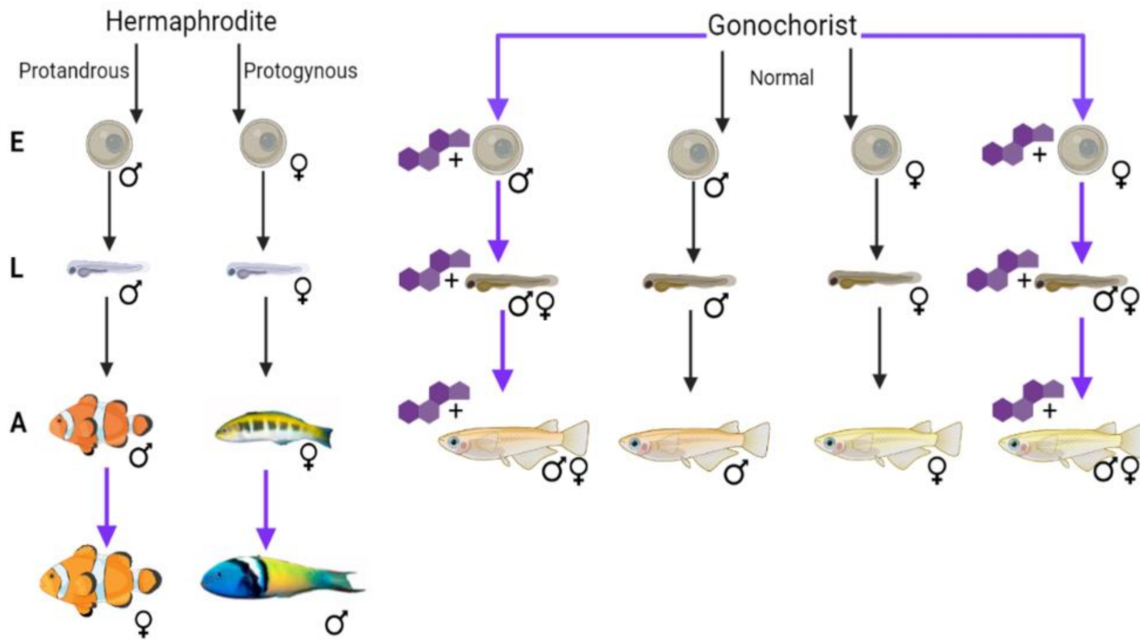
330

1.0 General introduction

331 Sexual development in fish is studied in the fields of aquaculture, ecotoxicology and
332 fundamental biology to understand normal differentiation and identify stressors impacting
333 this sensitive process. There are two closely related processes that influence sexual
334 development. Sex determination (SD) controls which developmental pathway, usually
335 male or female, an organism assumes, while gonadal differentiation (GD) encompasses
336 the processes controlling gonad development after sex has been determined. Both SD and
337 GD pathways are conserved among vertebrates, however, the specific genes involved
338 vary. This thesis is focused on describing gonadal morphology as well as identifying
339 genes, which are known to be involved in SD and GD in other species, in the mummichog
340 (*Fundulus heteroclitus*). This thesis utilized gonadal histology and reverse transcription
341 quantitative polymerase chain reaction (RT- qPCR) to examine morphology and gene
342 expression profiles in testis and ovary of five-week-old mummichog. Research has
343 demonstrated that SD and GD can be affected by endocrine disrupting compounds
344 (EDCs). This thesis also investigates the effects of a model estrogenic EDC (EE_2; 17α-
345 ethinylestradiol) on gonad morphology and gene expression in the developing
346 mummichog. The thesis begins with a detailed background on existing literature,
347 followed by the experimental work investigating SD and GD, and concludes with a
348 discussion of the results and a description of the integrative nature of the work.

1.1 Sexual development in fish

Sexual development in vertebrates includes the processes involved in transforming an immature embryo to an adult. The processes driving sexual development are not fixed, varying by species, genes/chromosomes, and environment (Devlin and Nagahama, 2002). Fish are good models for sexual development as they exhibit all sexual strategies (Norris and Lopez, 2011). Fish are generally gonochoristic (Bahamonde et al., 2013; Devlin and Nagahama, 2002), i.e., there are two sexes, and each individual develops into either a male (with testes) or female (with ovaries). One exception to this pattern is hermaphroditism, which represents an individual producing gametes from both sexes either simultaneously or in sequential stages of life (Todd et al., 2016). Another exception is intersex, which is described as the presence of both male and female gonadal tissue in a fixed sex individual. Intersex tends to be atypical in wildlife, occurring briefly in transitioning hermaphroditic fish and irregularly in some pre-spawning gonochoristic fish (Bahamonde et al., 2013). However, intersex has also been implicated as a signature effect caused by exposure to EDCs (Ibor et al., 2016) which will be further discussed later in the chapter. Each of these sexual strategies influences the sex of the adult fish (Figure 1.1). Sexual development is controlled by two closely related processes, SD and GD (Figure 1.2). Understanding these processes in fishes is valuable for better understanding the potential impacts anthropogenic wastes cause in fish populations via effects on sexual development (Courtenay et al., 2002).



369

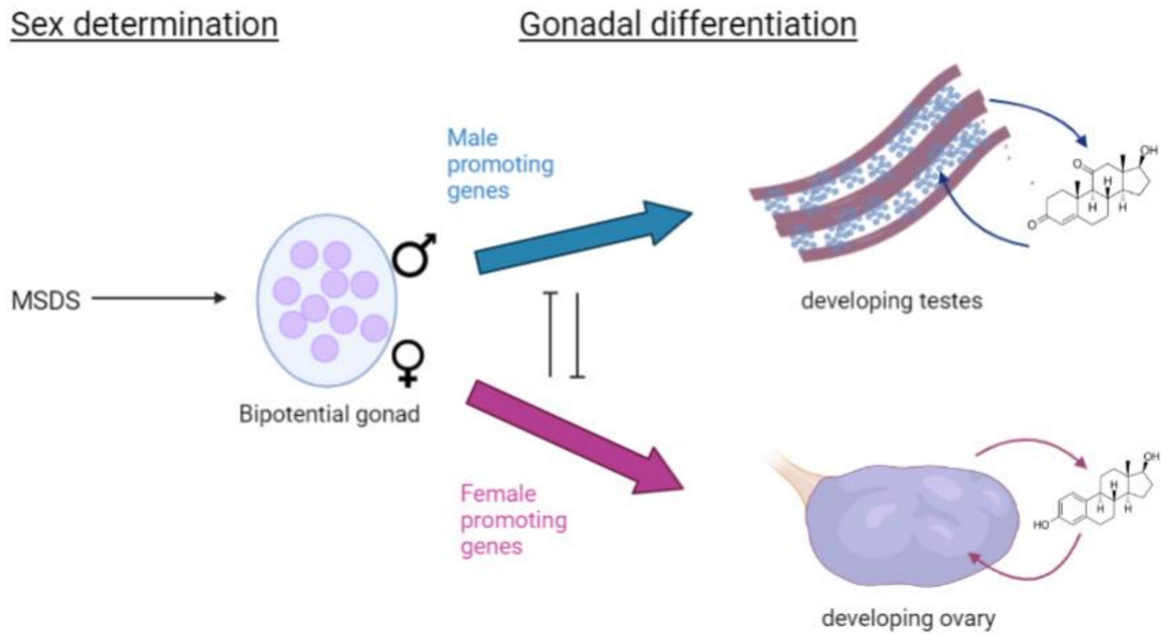
370 **Figure 1.1**: Sexual development strategies observed in fish, across embryonic (E), larval
371 (L) and adult (A) life stages. Hermaphroditic fish (left) are either protandrous, developing
372 into a mature male then transitioning to female [e.g., clownfish (*Amphiprion ocellaris*)],
373 or protogynous, developing first into a mature female then transitioning to male under
374 specific circumstances [e.g., bluehead wrasse (*Thalassoma bifasciatum*)]. The transition
375 period of hermaphroditic fish may involve a brief intersex phase (purple arrows).
376 Gonochoristic fish (right) will develop into one of two sexes [e.g., medaka (*Oryzias*
377 *latipes*)]. Unnatural intersex gonochorist (purple arrows) fish have been influenced by a
378 chemical stressor (e.g., effluents, DDT, or pharmaceuticals), disrupting normal
379 development and resulting in a gonad containing both male and female gametes. The
380 figure has been influenced by concepts from Dietrich and Krieger, 2009; Nagahama et al.,
381 2021. Image generated using BioRender.

382 **1.1.1 Sexual determination**

383 SD refers to the genes and molecular events that influence whether a male or female
384 sex-differentiating pathway will be followed during development (Devlin and Nagahama,
385 2002; Leet et al., 2011). Before gonads develop, the organism's unique genome at
386 fertilization influences factors that encourage the gonad to mature into a particular sex
387 (Norris and Lopez, 2011). There are three mechanisms of SD in fish: genetic sex
388 determination (GSD), behavioral sex determination (BSD) and environmental sex
389 determination (ESD). These mechanisms rely primarily on genetic components, social,

390 and environmental cues such as temperature, respectively. Fish may rely on one, two or
391 all three methods of SD for development, depending on the species (Devlin and
392 Nagahama, 2002; Leet et al., 2011). Due to this complexity of the different SD
393 mechanisms in fish, there are currently gaps in understanding the full physiological
394 mechanisms of how sex is determined (Devlin and Nagahama, 2002; Ortega-Recalde et
395 al., 2020).

396 Mammals have a master sex-determining gene identified which is the sex-
397 determining region on the Y chromosome, also known as testis determining factor (*SRY;*
398 Koopman et al., 1990). *SRY* promotes male development by upregulating SRY-box
399 transcription factor 9 (*SOX9*) expression, which then leads to a cascade of genetic factors
400 causing additional expression of genes that further male development, e.g., anti-Mullerian
401 hormone (*amh*). This sequence of events, known as the male differentiating pathway,
402 ultimately leads to the development of testes. While there is a good understanding of SD
403 in mammals, there is a need for more research on the control of GSD in fish. An analogue
404 of mammalian *SRY*, DM-domain gene on the Y chromosome (*dmy*), has been identified in
405 Japanese medaka (*Oryzias latipes*) as playing a key role in GSD. Since this discovery,
406 several other genes have been implicated in influencing SD and GD (Nagahama et al.,
407 2021), including gonadal derived soma factor (*gdsf*; Myosho et al., 2012), cytochrome
408 P450 family 11 subfamily c member 1 (*cyp11c1*; Todd et al., 2019) and *amh* on the Y
409 chromosome (*amhy*; Pan et al., 2019). While the number of genes impacting SD
410 continues to grow, there is currently no 'master gene' found across all teleost species.



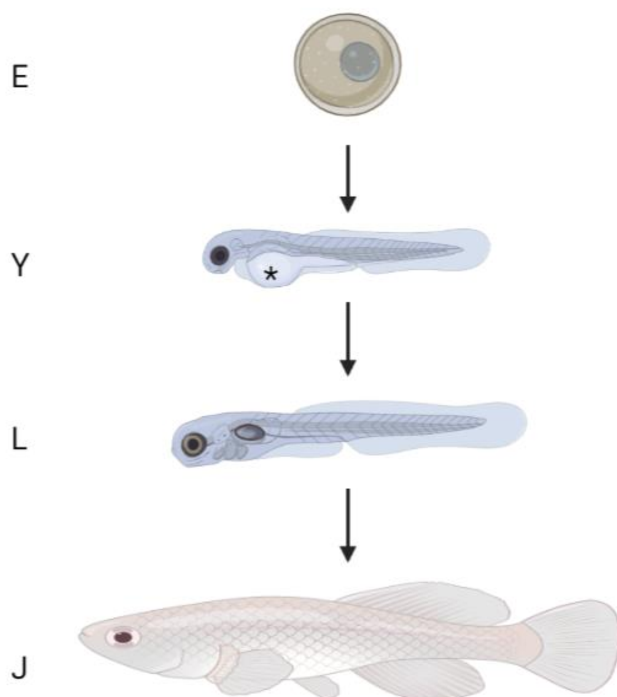
411

412 **Figure 1.2**: The two closely related processes, sexual determination (SD) and gonadal
413 differentiation (GD) are responsible for sexual development in fish. SD is governed by
414 the chromosome(s) and genes in the organism at fertilization. A master sex determining
415 switch (MSDS) is activated to promote an organism's development to either male or
416 female, e.g., *dmy* expression in the Japanese medaka. At the appropriate time, cells in the
417 undifferentiated gonad will express genes that further development of one sex while
418 simultaneously inhibiting the opposites sex's promoting network of genes. Somatic cells
419 in the developing gonad will then produce sex specific steroids that will further
420 development into a functioning gonad. Leydig cells (testes) produce 11-ketotestoserone
421 (11-KT) while granulosa cells (ovary) produce 17β-estradiol (E_2) to aid in development of
422 each sex. Adapted from Gonzalez et al. (2015) and generated using BioRender.

423 **1.1.2 Gonadal differentiation**

424 GD is the molecular and cellular processes that transform an immature gonad into a
425 testis (male) or ovary (female) after genetic sex has been determined (Devlin and
426 Nagahama, 2002; González et al., 2015; Leet et al., 2011). The timing of these processes
427 are species dependent; GD may occur during embryogenesis, larval, juvenile or adult
428 stages of life (Devlin and Nagahama, 2002; Dietrich and Krieger, 2009; Leet et al., 2011;
429 Piferrer, 2001). The transition into each life stage varies greatly from species to species.
430 In general, embryogenesis begins at the time of fertilization, encompassing development

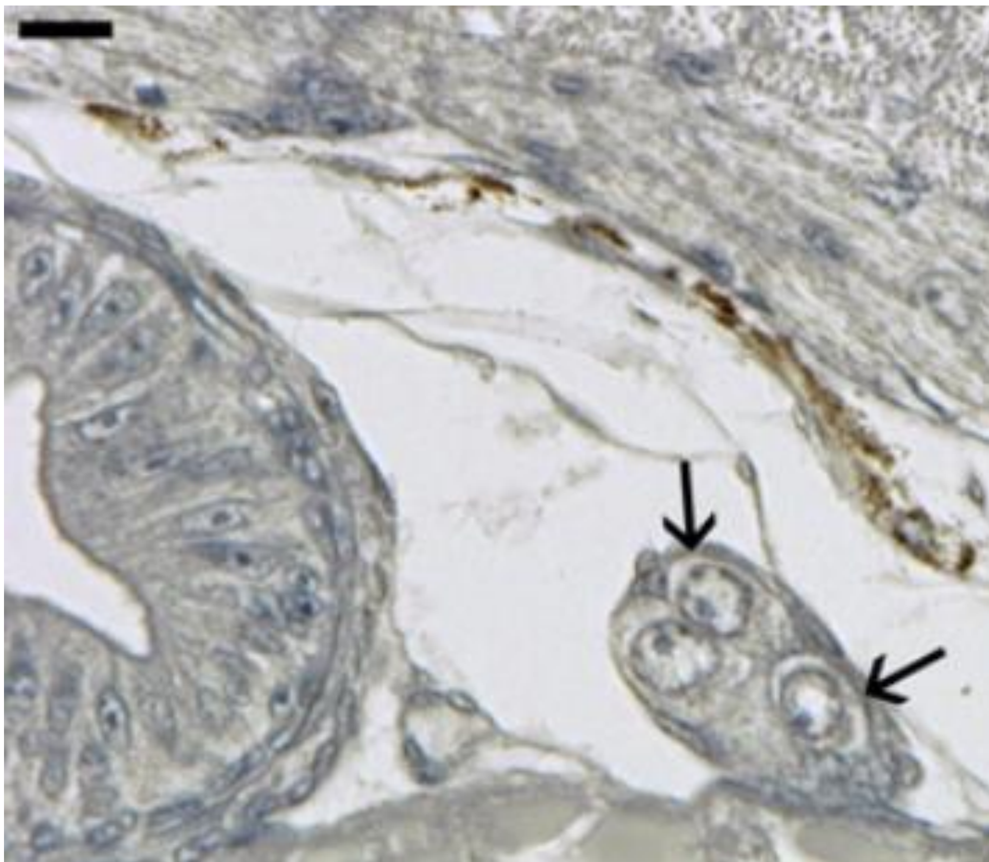
431 while inside the egg, and transitions into a free-swimming larva once free from the egg
432 and the yolk sac has been absorbed (Figure 1.3). In some cases, there is a yolk-sac larva
433 which is free-swimming but relies on feeding via the yolk sac. This stage is considered an
434 embryo until the yolk sac is completely absorbed. Larvae rely on feeding for nutrients and
435 typically have disproportionate features (e.g., large eyes compared to the body size).
436 Larvae transition into juvenile fish once unique features such as fin rays and scales are
437 apparent. This transition is typically followed by an increased growth rate. GD may occur
438 during any of these developmental stages (Devlin and Nagahama, 2002).



439

440 **Figure 1.3**: Typical order of fish development. Embryos (E, top) are contained in a
441 spherical egg where rapid cell proliferation occurs to create tissues and organs.
442 Eventually hatching enzymes and embryo movement degrades the egg layer resulting in a
443 hatched fish. Some species will hatch with a yolk sac (*) attached which will eventually
444 be consumed by the embryo (Y). The next stage, larvae (L), is characterized by the lack
445 of yolk sac as well as other distinct fish features such as pigmentation or fin rays. Fish
446 will transition into juveniles (J) when distinct features are visible followed by an
447 increased growth rate. Adapted from Alix et al., 2015; Armstrong and Child, 1965;
448 Nagahama et al., 2021. Image generated using BioRender.

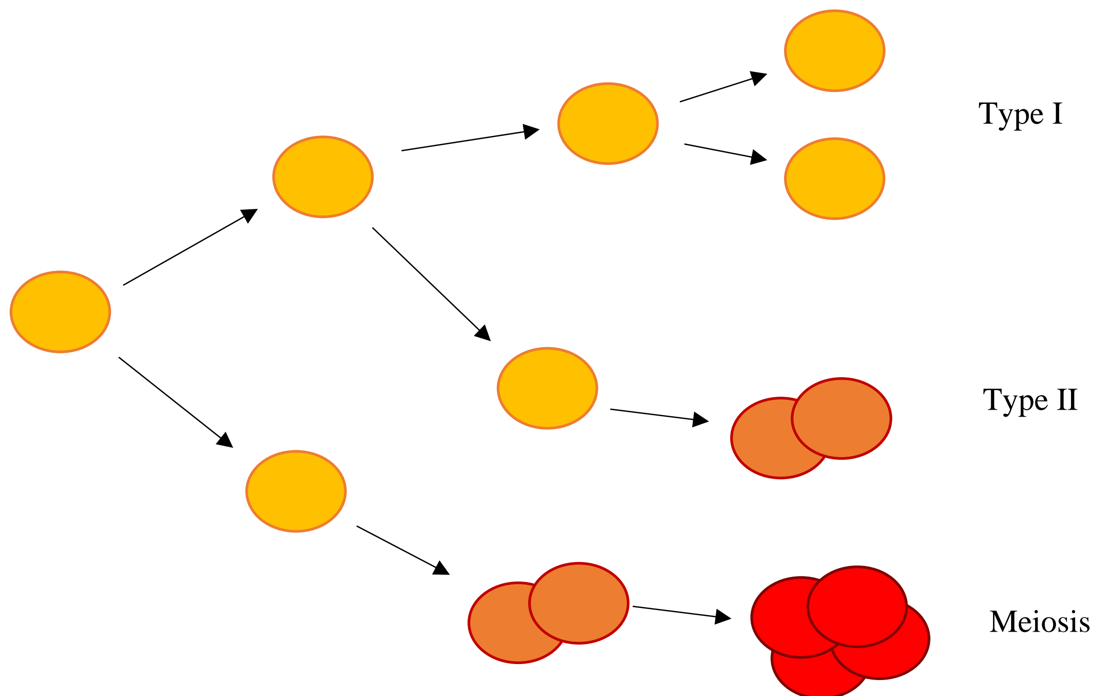
449 There are two main cell types within the gonad that contribute to its development:
450 germ cells, critical for conveying genetic information, and somatic cells, supporting the
451 growth and development of the germ cells (Nishimura and Tanaka, 2014). Typically, GD
452 begins once primordial germ cells (PGC), which are the precursor cells of gametes,
453 migrate from the dorsal wall of the coelomic cavity to the region of the presumptive
454 gonad (Devlin and Nagahama, 2002). Under the right conditions, these germ cells
455 proliferate and enlarge with their supporting somatic cells, to give rise to gonad tissue
456 (Figure 1.4).



457

458 **Figure 1.4**: Photomicrograph of undeveloped gonadal section of a newly hatched
459 mummichog (*Fundulus heteroclitus*) larva. Primordial germ cells (arrows) are in early
460 developmental stages. Immunostaining with anti-3β-hydroxysteroid dehydrogenase,
461 counterstaining with hematoxylin. Bar is 10μm. Photo taken from Shimizu et al. (2008)
462 with permission from Elsevier.
463

464 There are two different types of cellular divisions that can occur in PGCs: type I and
465 type II (Figure 1.5). Type I divisions are similar to stem cells where there is self-renewal,
466 each daughter cell produced is surrounded by somatic cells (Saito et al., 2007). Type II
467 divisions are characterized by an interconnection of the daughter cells and the onset of
468 meiosis, and, therefore, are committed to furthering gametogenesis processes (Saito et al.,
469 2007).



470

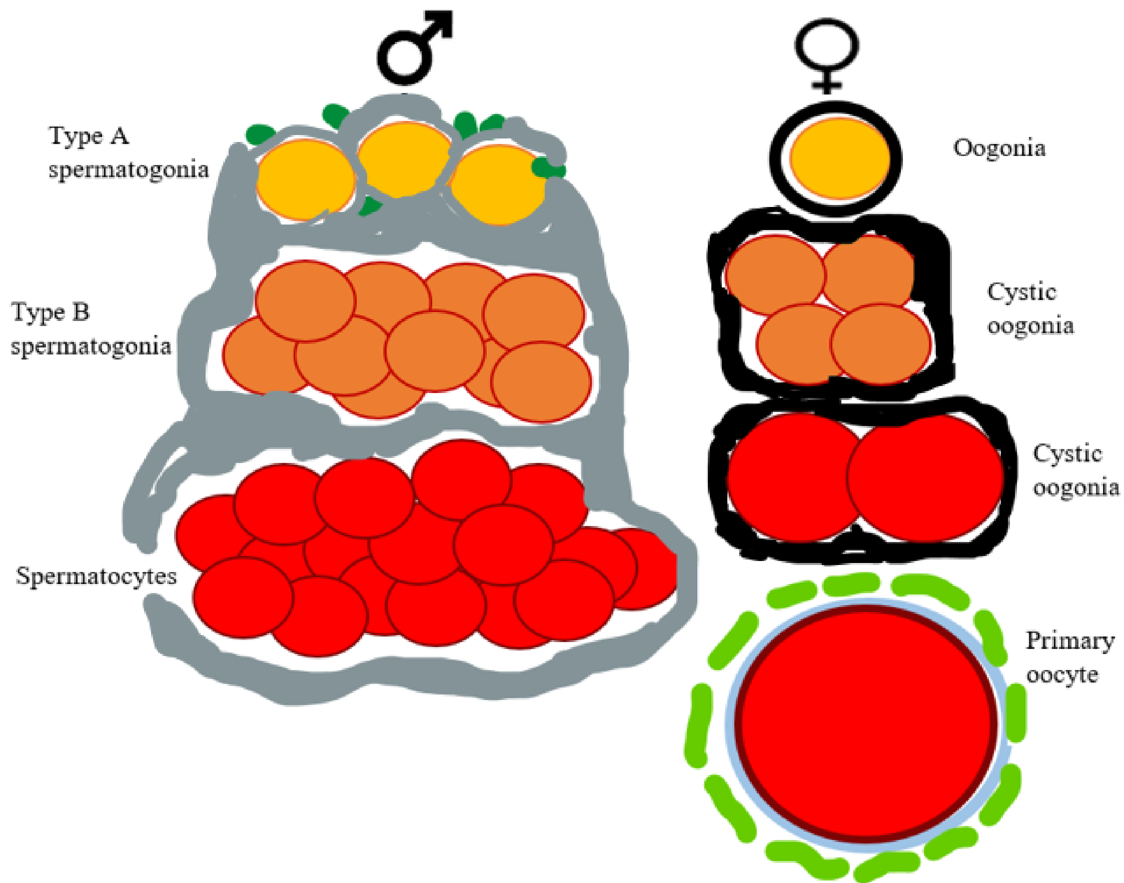
471 **Figure 1.5**: Representation of the types of germ cell divisions, type I and II with
472 subsequent meiosis. Type I (yellow) divisions are denoted by stem cell-like self renewal;
473 the daughter cells are completely separated. Type II (orange) divisions result in
474 interconnected daughter germ cells. The resulting daughter cells of type II divisions are
475 committed to meiosis (red) for gametogenesis. Adapted from Nishimura and Tanaka
476 (2014).

477 The morphological development of an immature gonad into ovaries or testes in
478 gonochoristic fish typically occurs during the early stages of life, either embryonic or
479 larval (Dietrich and Krieger, 2009). During this time, SD and GD engage in a

480 sophisticated relationship between the brain and gonad to facilitate this transition (Devlin
481 and Nagahama, 2002; Leet et al., 2011). Cellular communication between the brain and
482 gonad are carried out through the endocrine system's hypothalamic-pituitary-gonadal
483 (HPG) axis. Sensory information is collected from the internal and/or external
484 environment (neurotransmitters, social cues, temperature, photoperiod, etc.) which are
485 then processed and integrated in the brain by the hypothalamus. When appropriately
486 stimulated, the hypothalamus reacts by synthesizing and releasing neuropeptides called
487 gonadotropin releasing hormone (GnRH) which act on the anterior pituitary gland. The
488 anterior pituitary then releases the gonadotropins (GTH) follicle stimulating hormone
489 (FSH) and luteinizing hormone (LH) into the bloodstream. These GTHs act on the gonad
490 to stimulate the production of steroids through receptor-mediated signal transduction
491 which initiate and further the development of the gonad as well as relay negative
492 feedback to the brain to downregulate the axis (Biran and Levavi-Sivan, 2018).

493 ***1.1.2.1 Early gonadal development***

494 Early gonad development covers PGC migration and growth, development of the
495 gonadal ridge and the differentiation of the underdeveloped gonad (Norris and Lopez,
496 2011). In most gonochoristic fishes, this process is either differentiated, developing into
497 either an ovary or testis, or undifferentiated, develop an immature ovary before
498 transitioning to a mature ovary or testis (Norris and Lopez, 2011; Santos et al., 2017).
499 PGCs give rise to oogonia (female) or spermatogonia (male; Figure 1.6).



500

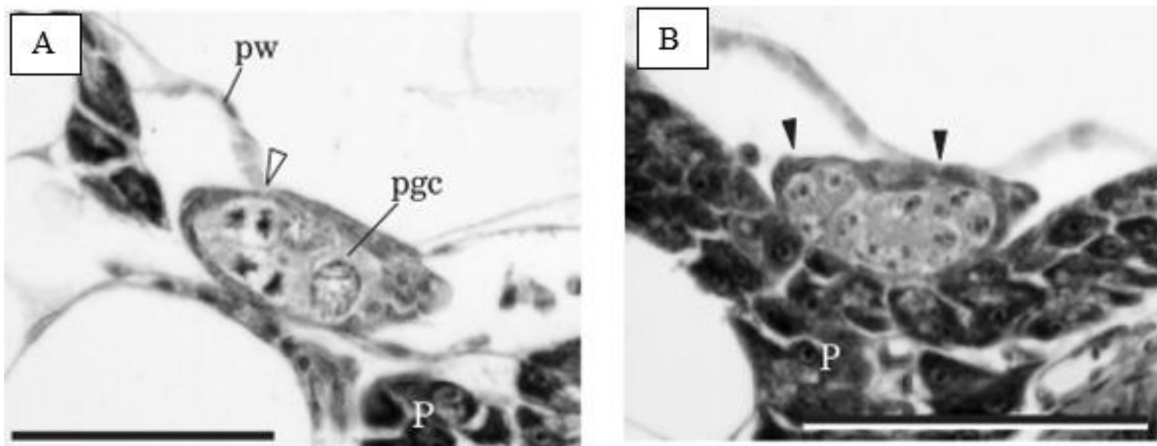
501 **Figure 1.6:** Early differentiation of male and female sex cells. Male primordial germ
502 cells differentiate into type A spermatogonia (top left) which are surrounded by Sertoli
503 (grey) and Leydig (dark green) cells. These cells undergo type II division generating type
504 B spermatogonia, which will divide meiotically to form spermatocytes. Spermatocytes are
505 enclosed in spermatocysts made of Sertoli cells to encourage further maturation of sex
506 cells. Female primordial germ cells differentiate into oogonia (top right) which are
507 surrounded by presumptive granulosa cells (black). Oogonia undergo type II divisions in
508 cyst-like capsules enclosed by somatic cells similarly to males however, most of the
509 daughter cells under atresia, a type of programmed cell death, to allow for resorption of
510 nutrients. Granulosa cells (blue) differentiate around the oocyte once it has differentiated
511 into a primary oocyte. Theca cells (light green) also surround the granulosa cells to form
512 the ovarian follicle which is necessary for further oocyte development. Adapted by Norris
513 and Lopez (2011) and Nishimura and Tanaka (2014).

514 ***1.1.2.2 Early ovarian differentiation***

515 Ovarian differentiation can be characterized by the onset of meiosis in germ cells and
516 the formation of the ovarian cavity, which can be distinguished histologically (Dietrich
517 and Krieger, 2009). Ovarian somatic cells provide factors for oogenesis, the development
518 of the ovum (egg). Oogenesis begins when PGCs differentiate into oogonia through a
519 mitotic division (Dietrich and Krieger, 2009), then undergo a series of steps including (1)
520 transformation into oocytes (2) growth of oocytes, (3) maturation, and (4) ovulation
521 (Dietrich and Krieger, 2009). The follicle layer consists of somatic cells enveloping the
522 oocyte which are necessary components providing factors throughout oogenesis (Dietrich
523 and Krieger, 2009). Thecal cells (outer layer) assist in ovarian development by supplying
524 testosterone (T) under the control of FSH (Norris and Lopez, 2011). T is then aromatized
525 to 17β-estradiol (E_2) in the granulosa cells. E_2 is essential for ovary development as it
526 required to produce vitellogenin (VTG), a characteristic of developing oocytes which is
527 essential to provide a food source to developing embryo (Dietrich and Krieger, 2009).

528 There are four early ovum stages: oogonia, chromatin nucleolar, perinucleolar, and
529 cortical alveolar, which are referred to as previtellogenic stages. Oogonia can be difficult
530 to distinguish from spermatogonia and are typically found in cysts (Figure 1.7A and 1.8).
531 Oogonia have not undergone meiosis, and expand in numbers through type 1 divisions.
532 Once meiosis has started, the germ cells then enter the chromatin nucleolar stage where
533 the chromosomes become arrested in the first meiotic prophase I (Figure 1.6 and 1.7B).
534 At this stage, the cell will appear to have a large nucleus with a thin layer of cytoplasm.
535 As the cell grows in size, the oocyte enters the next stage, by forming perinucleolar
536 oocytes (Figure 1.8). This stage is characterized by the appearance of multiple nucleoli at
537 the periphery of the nucleus. Once vacuoles become present in the cytoplasm, the oocyte

538 is entering the cortical alveolar stage (Figure 1.8). The vacuoles, or alveoli, are spherical
539 structures that appear empty with hematoxylin and eosin (H&E) staining. However, the
540 alveoli contain polysialoglycogproteins, which are vital to chorion development and
541 fertilization. Once yolk proteins are present in the cytoplasm, the oocyte is now beginning
542 vitellogenesis. Previous work by Urushitani et al. (2002) successfully identified these
543 stages of development in the mummichog using H&E staining and light microscopy
544 (Figure 1.8).

545 

546 **Figure 1.7**: Photomicrograph of transverse sections of gonads of larval fathead minnow
547 (*Pimephales promales*) at 10 days post hatch with hematoxylin and eosin stain. (A)
548 gonads containing cysts of pre-meiotic germ cells (oogonia, white arrow head), (B)
549 gonads where the somatic cells were located at the periphery of the gonad (black arrow
550 head), enclosing several germ cells at the chromatin nucleolar stage. pw, peritoneal wall;
551 pgc, primordial germ cell; P, pancreas. Bar is 50 µm. Image taken from Van Aerle et al.
552 (2004) with permission from Elsevier.
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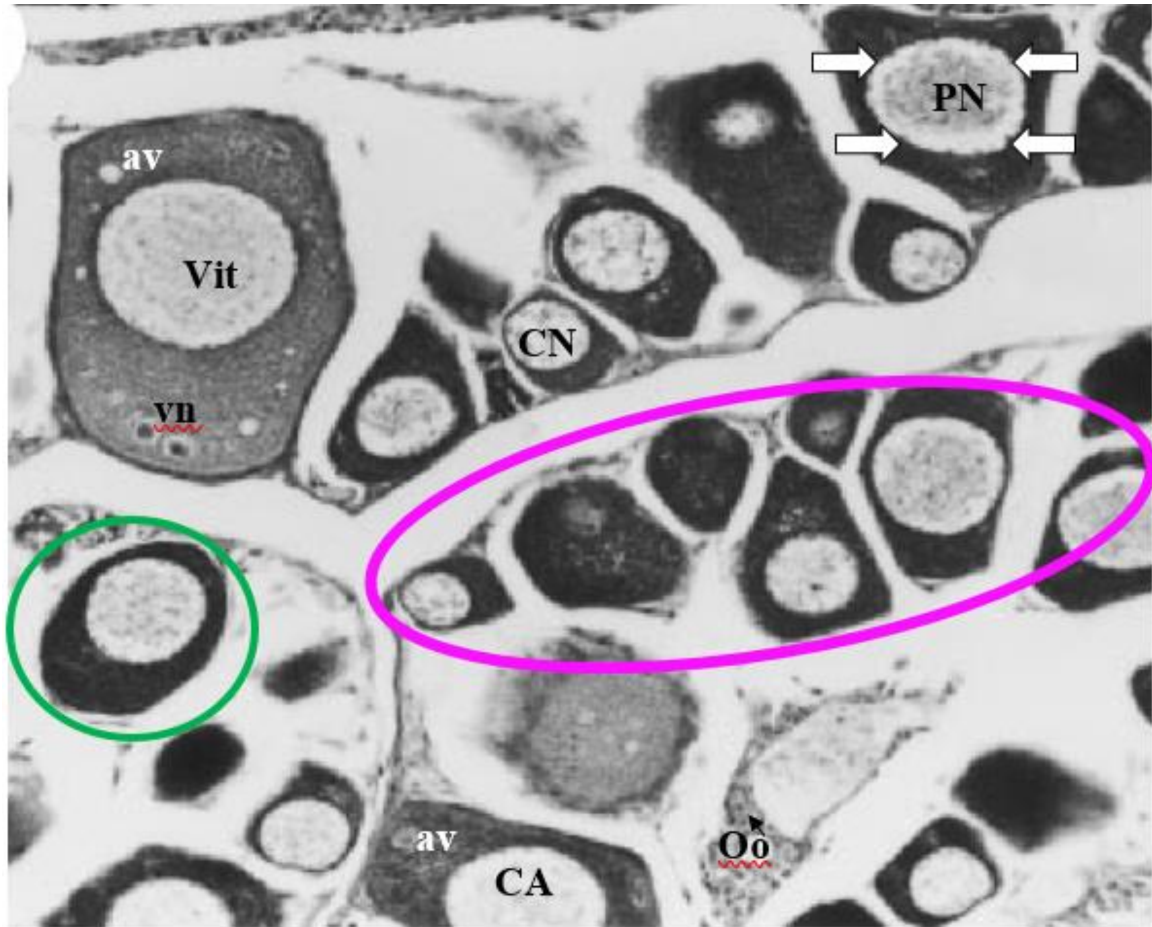
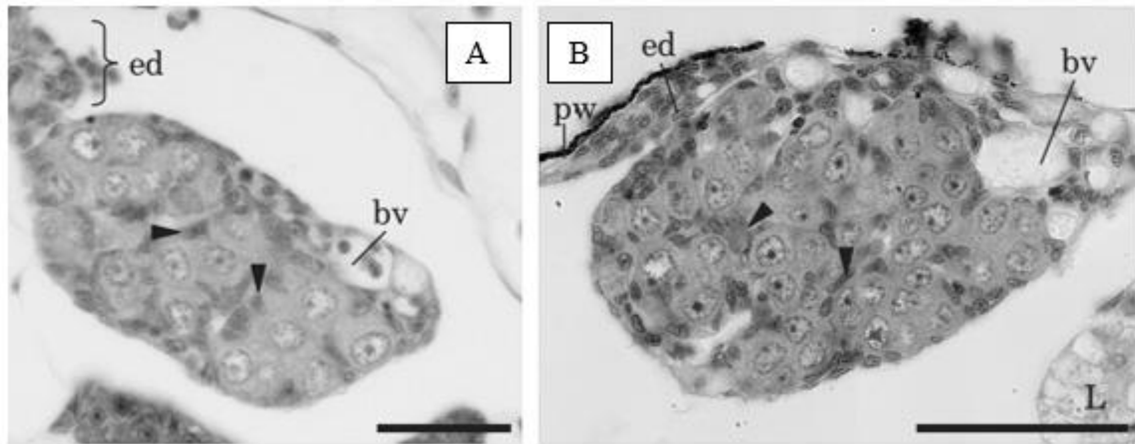


555 **Figure 1.8**: Photomicrograph of eight-week-old mummichog (*Fundulus heteroclitus*)
556 ovary under light microscope with hematoxylin and eosin stain at 50X magnification. The
557 epithelial folds (pink circle) help to keep the oocytes (green circle) organized. Photo
558 contains multiple developing oocytes at varying sizes and developmental stages. Oogonia
559 (Oo) are very small, and are easily identified by their large nucleus and minimal
560 cytoplasm. Chromatin nucleolar stage oocytes (CN) can be observed by their increased
561 size compared to Oo, large nucleus to cytoplasm ratio and no more than one nucleolus
562 (white arrow). Multiple nucleoli can be seen at the periphery of the nucleus in the
563 perinucleolar staged oocytes (PN), which are usually larger than the previous stage. The
564 alveoli vesicles (av) can be seen in the cytoplasm of one oocyte that is in the early cortical
565 alveolar (CA) stage. The farthest developed oocyte has entered the vitellogenic phase as
566 vitellogenin (vn) can be observed in the cytoplasm. Figure has been taken and modified
567 from Urushitani et al. (2002) with permission from John Wiley and Sons.

568 ##### *1.1.2.3 Early testis differentiation*

569 The first characteristic of gonochoristic male differentiation is typically that cell
570 maturation occurs later than females (Devlin and Nagahama, 2002). Testis differentiation
571 can be observed histologically by identifying male germ cells, somatic cells and efferent
572 ducts. Within the testis, sperm gametes are generated via spermatogenesis, which occurs
573 in the lobules. These lobules have an organized division appearance, which are connected
574 to efferent ducts (Figure 1.9A) leading to the urogenital pore (Norris and Lopez, 2011).
575 Germ cells reside in spermatocysts and move toward the testis lumen as they mature into
576 sperm. There are three stages of spermatogenesis, (1) spermatogonial (mitotic) phase, (2)
577 meiotic phase, and (3) spermiogenic phase (Norris and Lopez, 2011). During the first
578 stage, PGCs give rise to spermatogonia type A cells through mitotic divisions (Norris and
579 Lopez, 2011), influenced by the main teleost androgen 11-ketotestosterone (11-KT). This
580 hormone is produced in the somatic Sertoli cells, under the control of FSH and is critical
581 for spermatogonial development. When these cells are ready for further development, a
582 mitotic division into spermatogonia type B cells is promptly enveloped by Sertoli cells,
583 creating the testis lobule (Figure 1.9B). Leydig cells are located on the periphery of the
584 testis and are essential for testis maturation by generating T for Sertoli and germ cells
585 (Norris and Lopez, 2011). As the germ cells mature through phases 1-3, Sertoli cells
586 support germ cell survival, regulate development, and physiological functioning as the
587 germ cell matures into a spermatozoa (Dietrich and Krieger, 2009; Norris and Lopez,
588 2011). Mature spermatozoa are stored in the testis center until release into the aqueous
589 environment (Norris and Lopez, 2011).

590 Early testis differentiation can be identified histologically in the mummichog as
591 demonstrated by Shimizu et al. (2008; Figure 1.10). Mummichog testis differentiation
592 does not occur until three weeks after hatching, with the appearance of spermatogonia
593 (Shimizu et al., 2008). Mature spermatozoa are not typically seen until six weeks after
594 hatching (Figure 1.10). As gametes mature, they have a reduced size and can be observed
595 in the center of the testis tissue. Spermatozoa will develop a flagellum, a distinct feature
596 from the other stages.
597



598

599 **Figure 1.9**: Photomicrograph of transverse sections of fathead minnow (*Pimephales*
600 *promales)* testes at (A) 60 and (B) 90 days post hatch (dph) with hematoxylin and eosin
601 stain. At 60 dph, structural elements such as blood vessels (bv) and the efferent duct (ed)
602 can be observed, while germ cells have not matured. At 90 dph, lobules are more obvious
603 as the somatic cells (arrow heads) can be seen forming cysts around the germ cells. pw,
604 peritoneal wall; L, liver; P, pancreas. Bar is 25 µm. Image taken from Van Aerle et al.
605 (2004) with permission from Elsevier.
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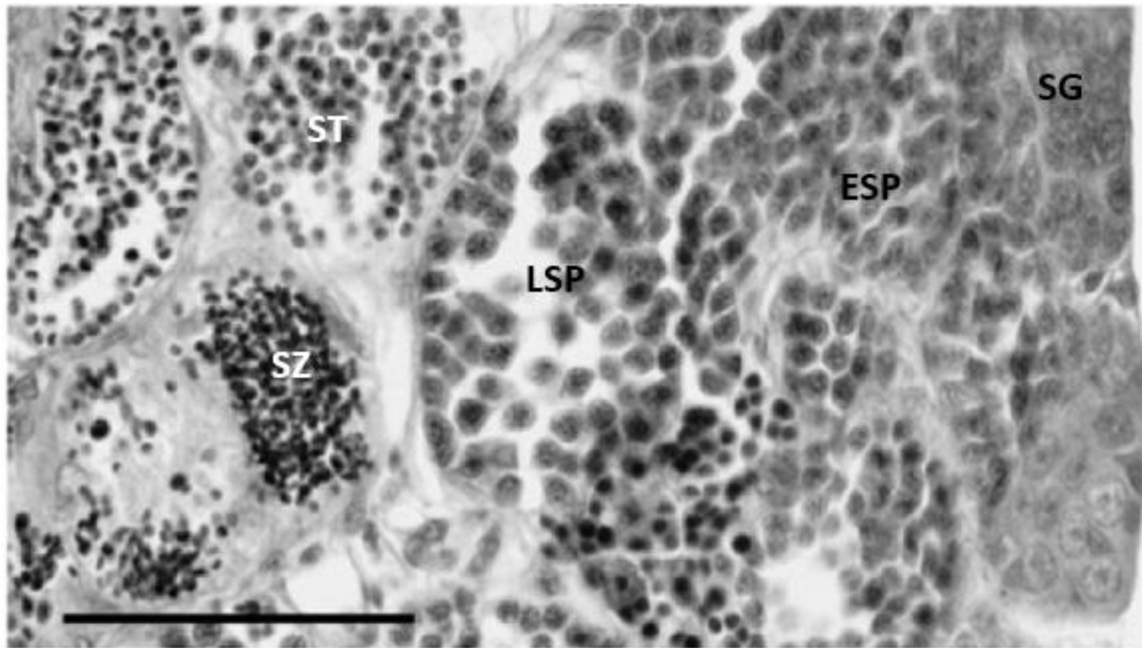


608 **Figure 1.10:** Photomicrograph of six-week-old mummichog (*Fundulus heteroclitus*)
609 testis. Periodic acid-Schiff and Mayer's hematoxylin staining. Spermatogonia (SG) are
610 located at the periphery of the gonad, during the first meiotic division, the cell is arrested
611 for one or two days as early spermatocytes (ESP) until the process is complete, giving rise
612 to late staged spermatocytes (LSP). Spermatids (ST) are derived from the second meiotic
613 division. The spermatocyst then releases the spermatids into the lumen of the testis where
614 they undergo maturation into spermatozoa (SZ). Bars is 50 µm. Photo taken and modified
615 from Shimizu et al. (2008) with permission from Elsevier.

616 **1.1.3 Steroids controlling sexual fate**

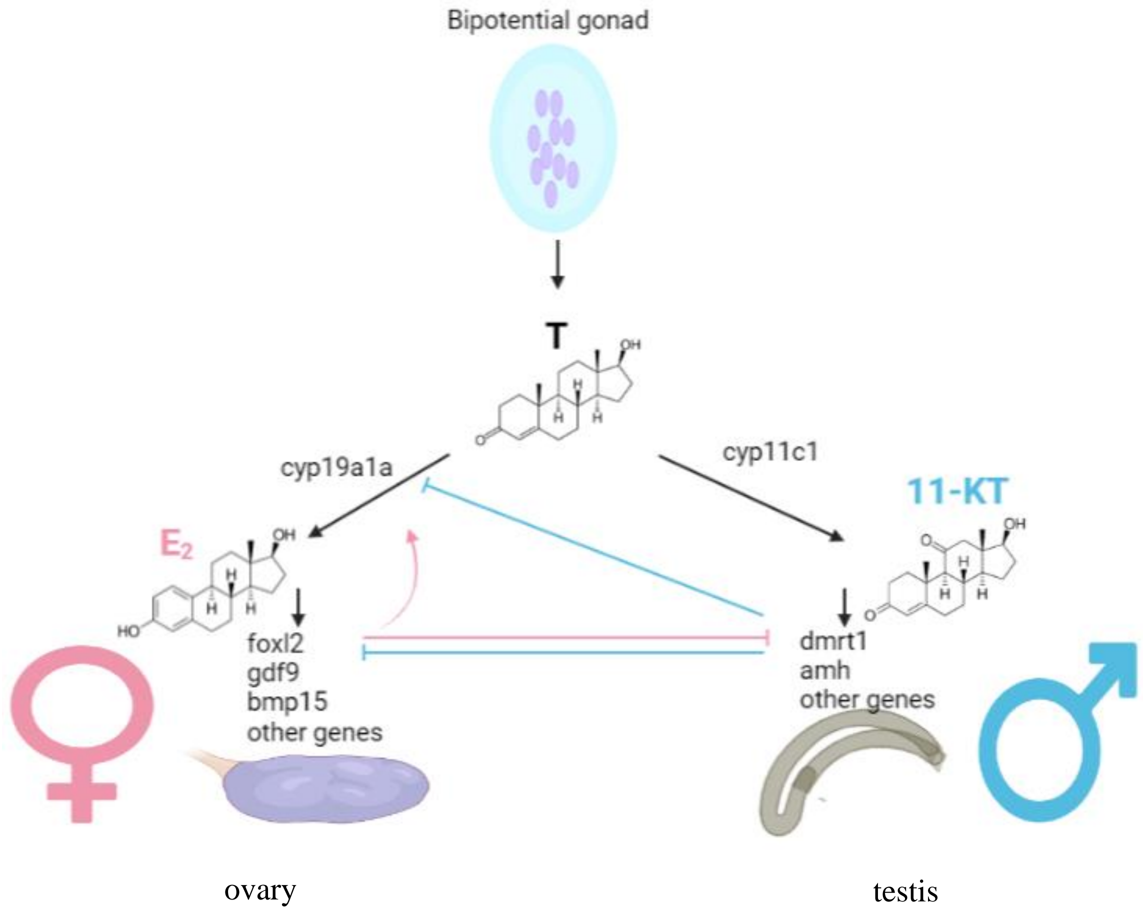
617 To initiate steroidogenesis in a developing gonad, GTHs from the brain stimulate the
618 gonad supporting somatic cells. FSH typically precedes LH activity in the gonad
619 (Dietrich and Krieger, 2009). FSH is responsible for early gonadal development and
620 vitellogenesis, while LH is responsible for later developmental processes such as oocyte
621 maturation, ovulation and spermiation (Peter and Yu, 1997; Weltzien et al., 2004).
622 Steroidogenesis produces estrogens and androgens; the balance between the two steroid
623 types controls sexual fate in fishes (Todd et al., 2016) In fish, a rise in production of the
624 dominant estrogen, E_2, promotes ovarian development. Likewise, an upsurge in the
625 dominant terminal androgen, 11-KT, will lead to testis development (Figure 1.11). The

626 balance of E_2 and 11-KT in fish influences the male and female molecular signaling
627 pathways (Todd et al., 2016).

628 **1.1.4 Genes controlling sexual fate**

629 Sex differentiating processes vary among fishes; however, the genes and cellular
630 networks involved in these processes are well conserved regardless of the sex-
631 determining mechanism (Alfaqih et al., 2009). Sexual fate can be described as a battle for
632 dominance between the male and female signaling pathways (Todd et al., 2016). Genes
633 that promote development of one sex may contribute to antagonistic effectors in the
634 opposite sex's network (Figure 1.11). Overall GD is influenced by the expression of
635 specific genes during a short period of time that is unique to each species. As an example,
636 *dmy* is expressed in Sertoli cells during early differentiation to facilitate the male pathway
637 in the medaka (Matsuda et al., 2002).

638 Genetic signaling pathways controlling sexual fate in fish have been linked to several
639 genes encoding transcription factors, steroidogenic enzymes and key receptors (Leet et
640 al., 2011). There are specific genes that have been found in association with promoting
641 both male and female signaling pathways, respectively (Leet et al., 2011; Todd et al.,
642 2016). While many have been identified, only genes pertinent to this thesis will be
643 discussed in detail.



ovary testis

Figure 1.11: Steroidogenesis in somatic cells generates testosterone (T) in the gonad. A fish destined to be female will convert T into 17β-estradiol (E_2) via aromatase which is encoded by *cyp19a1a*. E_2 will influence the expression of female promoting genes such as *foxl2*, *gdf9*, *bmp15* among others to facilitate ovary development and increase *cyp19a1a* expression to maintain the female signaling pathway. Female promoting genes such as *foxl2* have inhibitory effects on male promoting genes such as *dmrt1* to avert the male signaling pathway. Alternatively, a fish destined to be male will convert T into 11-KT via 11β-hydroxylase which is encoded by *cyp11c1*. 11-KT promotes the expression of male promoting genes such as *dmrt1* and *amh* to maintain the male differentiating pathway. Male promoting genes such as *dmrt1* directly inhibit female promoting genes (*cyp19a1a* and *foxl2*) to avert the female signaling pathway. Adapted from Leet et al. (2011) and Todd et al. (2016). Image generated using BioRender.

1.1.4.1 Female promoting genes

Bone morphogenetic protein 15 and growth and differentiating factor 9

Transforming growth factor beta superfamily (TGFβ) is composed of three major subfamilies: TGFβs, activin/inhibin/nodal, and bone morphogenetic proteins (BMP; Lin

663 et al., 2006; Yu et al., 2020). Members of this superfamily have been directly implicated

664 in follicle formation, granulosa cell differentiation, oocyte maturation, and

665 steroidogenesis in vertebrates (Moore et al., 2003; Spicer et al., 2008; Yu et al., 2020).

666 Strong expression of Bone morphogenetic protein 15 (*bmp15*) was found in zebrafish

667 (*Danio rerio*) during early oocyte development, but as the oocyte matured, expression of

668 *bmp15* was lost (Dranow et al., 2016). Loss of function studies of the *bmp15* gene in

669 mammals (sheep, human and mouse) found that granulosa cells could not differentiate,

670 therefore, follicles could not support the growth of the oocytes past the primary growth

671 stages, leading to infertility (Galloway et al., 2002; Galloway et al., 2000). Similar studies

672 in the zebrafish resulted in female to male sex reversal (Dranow et al., 2016), supporting

673 *bmp15* expression as being required for female sex determination. Recombinant *bmp15* in

674 zebrafish reduced *amh* gene expression (Chen et al., 2017), indicating that *bmp15*

675 expression also hinders the male differentiating pathway. Characterization of the

676 relationship between *bmp15* and growth and differentiating factor 9 (*gdf9*), investigated

677 using RT-qPCR, overexpression and knockout analysis, determined that both genes were

678 highly expressed in the ovary, especially in oogonia and early developed oocytes of the

679 Japanese flounder (*Paralichthys olivaceus*). Over expression analysis showed an

680 interesting relationship between the genes with steroidogenesis; *gdf9* expression could

681 increase steroidogenesis in the gonad while *bmp15* expression hindered steroidogenesis

682 (Yu et al., 2020). More research is needed on these genes in fish to gain insight on their

683 steroidogenic control.

684 ***Cytochrome P450 family 19 subfamily A member 1***

685 Cytochrome P450 family 19 subfamily A member 1 (*cyp19a1a*) encodes for

686 aromatase, the enzyme that converts T into E_2 in the gonad. E_2 is widely considered the

687 primary female-promoting steroid of gonochoristic fishes; its sharp increase in levels
688 during ovarian differentiation makes aromatase expression during this stage crucial
689 (Guiguen et al., 2010; Todd et al., 2016) and increased expression of *cyp19a1a* has been
690 documented in developing Nile tilapia (*Oreochromis niloticus*; Ijiri et al., 2008), rainbow
691 trout (*Oncorynchus mykiss*; Vizziano et al., 2007), and Japanese medaka (Nakamoto et
692 al., 2006). *Cyp19a1a* expression is transcriptionally regulated by FSH signaling and
693 forkhead transcription factor 2 (*foxl2*) expression in the Japanese flounder (Yamaguchi et
694 al., 2007). The *cyp19a1* gene contains a binding site for FOXL2. In the medaka, the
695 female pathway is controlled by dosage-sensitive sex reversal, adrenal hypoplasia critical
696 region, on chromosome X, gene 1 (*dax1;* Nakamoto et al., 2007). *Dax1* suppresses the
697 expression of *foxl2* which causes a downregulation of *cyp19a1a* (Nakamoto et al., 2007).
698 Experiments using aromatase blocking compounds prompted testis development in
699 honeycomb grouper (*Epinephelus merra;* Bhandari et al., 2004), Japanese flounder
700 (Kitano et al., 2000), golden rabbitfish (*Siganus guttatus;* Komatsu et al., 2006), and
701 European sea bass (*Dicentrarchus labrax*; Navarro-Martin et al., 2009). Blocking
702 aromatase production suppresses the female pathway to allow for male differentiation in
703 these fish.

704 ***Forkhead transcription factor 2***

705 The forkhead transcription factor family regulates numerous developmental
706 processes in vertebrates (Uhlenhaut et al., 2009). *Foxl2* is a well conserved gene
707 subfamily in vertebrates that is one of the earliest known markers for ovary differentiation
708 (Cocquet et al., 2002). Expression of *foxl2* in female fishes appears early in ovarian
709 differentiation and is maintained throughout adulthood, suggesting involvement in the
710 female differentiating pathway (Baron et al., 2004; Nakamoto et al., 2006; Yang et al.,

711 2017). In mammals, *foxl2* is required for granulosa cell differentiation (Schmidt et al.,
712 2004), and directly suppresses *sox9,* a male promoting factor (Uhlenhaut et al., 2009) as
713 demonstrated in mutational studies. In fish, sexually dimorphic expression of *foxl2* has
714 been observed in Nile tilapia (Ijiri et al., 2008) and zebrafish (Yang et al., 2017) showing
715 high expression in XX (homozygous females) individuals. Treatment with aromatase
716 inhibitors downregulated *foxl2* expression in rainbow trout, which suppresses the female
717 signaling pathway (Baron et al., 2004). Another study using the Japanese flounder found
718 that increased temperature resulted in males as well as low expression of *foxl2* and FSH
719 receptor, causing suppression of the female pathway (Yamaguchi et al., 2007). Further
720 analysis using luciferase transfection assays indicated an active binding site on the
721 promotor region of aromatase for FOXL2. These findings indicate that *foxl2* expression is
722 an important transcriptional regulator for aromatase in the Japanese flounder (Yamaguchi
723 et al., 2007), and important in maintaining the female pathway.

724 **1.1.4.2 Male promoting genes**

725 ***Anti-Mullerian hormone***

726 *Amh,* also known in fish as Mullerian-inhibiting factor, is a member of the TGFβ
727 superfamily of genes that is expressed in somatic cells of mammals and fish (Pfennig et
728 al., 2015). *Amh* in male goldfish (*Carassius auratus*) showed elevated expression during
729 gonadal development (Zheng et al., 2016). Expression of *amh* has been found in
730 undifferentiating gonads of both sexes of medaka (Klüver et al., 2007), Nile tilapia (Ijiri
731 et al., 2008), rainbow trout (Vizziano et al., 2007), and zebrafish (Rodríguez-Marí et al.,
732 2005). Researchers believe that *amh* expression correlates to the onset of meiosis in the
733 early gonad germ cells; meiosis began when *amh* expression in Sertoli and granulosa cells
734 decreased (Pfennig et al., 2015). In the hermaphroditic pejerrey (*Odontesthes*

735 *bonariensis*), *amh* has been identified as a male master sex-determining gene (Yamamoto
736 et al., 2014). An *amh* knockout study on pejerrey, showed an upregulation of female
737 promoting genes *foxl2* and *cyp19a1* expression which led to ovary development (Hattori
738 et al., 2012). In the crucian carp (*Cyprinus carpio*), *amh* is an important regulator for the
739 expression of *dax1* and *cyp19a1a* (Li et al., 2013a). Loss of *amh* function in zebrafish
740 causes a female biased sex ratio and abnormal male germ cell development (Lin et al.,
741 2017). These findings indicate that *amh* genes are conserved in teleost fish and their
742 transcriptional influence on the male or female differentiating pathways various slightly
743 from species to species.

744 ***Double sex and Mab-3 related transcription factor 1***

745 Double sex and Mab-3 related transcription factor 1 (*dmrt1*), is a transcription factor
746 implicated in male development in vertebrates, including teleost fish (Jeng et al., 2019; Li
747 et al., 2014). *Dmrt1* in fish is believed to be functionally analogous to the *SRY* gene in
748 mammals and is expressed exclusively in somatic cells (Kobayashi et al., 2004; Matsuda
749 et al., 2002). *Dmrt1* is a transcriptional regulator that activates male promoting genes
750 while simultaneously suppressing female signaling pathways, ultimately leading to male
751 development (Todd et al., 2016). Sexually dimorphic expression of *dmrt1* is higher in
752 males during gonad development in zebrafish, medaka, pejerrey, European sea bass, and
753 rainbow trout (Kobayashi et al., 2004; Marchand et al., 2000; Shen and Wang, 2014).
754 *Dmrt1* transcriptionally suppresses *cyp19a1a* expression, preventing estrogen production
755 via aromatase in Nile tilapia (Li et al., 2013b). In contrast, zebrafish show *dmrt1*
756 expression in both testis and early-stage oocytes. However, *dmrt1* mutants that lose *dmrt1*
757 function showed fish developing as female with normal ovary development (Webster et
758 al., 2017). Male fish from the same experiment were infrequent and exhibited sterility and

759 testis degeneration (Webster et al., 2017). *Dmrt1* expression seems to be expendable in
760 the ovary, while essential for males. Research on the Japanese eel (*Anguilla japonica*)
761 using immunofluorescence identified DMRT1 protein in the gonad which was localized
762 to spermatogonia type B cells, not type A or somatic Sertoli cells (Jeng et al., 2019).
763 These results demonstrate that *dmrt1* is not used for SD or GD, but rather regulating
764 spermiogenesis. Whether the primary role of *dmrt1* is in SD or GD, or instead linked to
765 proper spermiogenesis, is unknown for many teleost species.

766 **1.1.4.3 Other Genes**

767 While the aforementioned genes have been linked to promoting female- or male-
768 signaling pathways, there are other genes that may also promote GD (Table 1.1). While
769 the current thesis will focus on the discussed genes, these alternate genes may also play
770 critical roles in differentiation and could be incorporated into further studies. There are
771 currently gaps in the research fully describing the genes contributing to differentiation in
772 fish (Leet et al., 2011).

773 **Table 1.1**: Genes implicated in sex determination (SD), ovarian differentiation (OD),
774 testis differentiation (TD) and meiosis (ME) processes in different fish species. Genes
775 may appear in more than one category.
776

Genes	SD	OD	TD	ME
Anti-Mullerian hormone (*amh*)	P		Z, P	Z, M, R, T
Anti-Mullerian hormone receptor (*amhr*)			M	
Androgen receptor (*AR*)			F	
Cytochrome P450 family 11 subfamily C member 1 (*cyp11c1*)	B			
Cytochrome P450 family 19 subfamily A member 1 (*cyp19a1a*)		M		
dosage-sensitive sex reversal, adrenal hypoplasia critical region, on chromosome X, gene 1 (*dax1*)		Z	M	
double sex and Mab-3 related transcription factor 1 (*dmrt1*)	M		R, T, Z	
Estrogen receptor (*ER*)		Z		
forkhead transcription factor 2 (*foxl2*)		M, T, Z		
Gonadal derived soma factor (*gdsf*)			M	
SRY-box transcription factor 9 (*sox9a* and *b*)			T, Z, M	
wingless-related integration site family, member 4 (*wnt4*)		M, Z		

777 **Letter designation**: B: bluehead wrasse, E: Japanese eel, F: fathead minnow, M: medaka,
778 R; rainbow trout, T: tilapia, Z: zebrafish.

779 # 1.2 Endocrine disruption in fish

780 Endogenous hormones are crucial for sexual development in fish. This hormonal
781 balance is highly susceptible to interference by exogenous hormones, mimics or
782 antagonists, commonly known as EDCs. According to the US Environmental Protection
783 Agency (USEPA), EDCs can interact with multiple targets within fish including changing
784 the natural rate of hormone synthesis, transport, secretion, receptor agonism/antagonism
785 or elimination (USEPA, 2006). These compounds include diverse chemical structural
786 classes such as steroids, organochlorines, dioxins, polychlorinated biphenyls, alkyl
787 phenolic surfactants, flavonoids and other phytochemicals, inorganic anions, and metals
788 (Norris and Lopez, 2011). EDCs can be natural or artificially synthesized.

789 Anthropogenic EDCs enter the environment in a variety of ways including discharge
790 of waste effluents from industries, such as pulp and paper production, wastewater
791 treatment (sewage), and agricultural activities (Leet et al., 2011). EDCs have been
792 documented in all types of aquatic environments around the globe, including marine,
793 estuarine, and freshwater. A study in Luxemburg quantifying various pharmaceuticals in
794 sewage effluents showed 5 ng/L estrone (E_1), 46 ng/L E_2, and 12 ng/L EE_2, among other
795 pharmaceuticals (Pailler et al., 2009). In Eastern Asia, a three-year study quantifying
796 various estrogenic EDCs in surface waters revealed E_1 and E_2 between 1.3-19.8 ng/L
797 (Duong et al., 2010). In the United States, a study measuring the occurrence of steroids in
798 the Elkhorn River found synthetic estrogens [1.7 ng/L (EE_2), 7.4 ng/L (E_1)] and
799 androgens [0.9 ng/L (T) and 5.3 ng/L (androstenedione)] in surface waters (Kolok et al.,

800 2007). Due to the abundance of EDCs found in the aquatic environment, there is ongoing
801 concern of the impacts these compounds may cause to resident organisms.

802 An abundance of literature demonstrates that EDCs cause negative effects to fish in
803 the receiving environments (Ankley et al., 2003; Bosker et al., 2009; Chen et al., 2016;
804 Lister et al., 2011). One of the most concerning aspects of these disruptions is alteration
805 of SD and/or GD mechanisms leading to abnormal gonadal development, reproductive
806 dysfunction and eventual population collapse (Leet et al., 2011). Most fish retain a
807 bipotential ability even after gonadal differentiation has occurred, allowing EDCs to
808 potentially disrupt reproductive processes and alter gonad development before, during or
809 after maturation. This interference can result in masculinization or feminization of fishes
810 and their populations (Devlin and Nagahama, 2002; Li et al., 2019; Todd et al., 2016).

811 Exogenous steroids may alter the expression of genes that are working to control SD
812 and GD in the fish by interacting directly with nuclear receptors. Once these hormones
813 enter a cell and complex with a hormone receptor [such as an androgen receptor (AR) or
814 an estrogen receptor (ER)], they can act on hormone response elements controlling
815 transcription and translation of specific genes, eliciting physiological responses in the cell
816 or the organism (Leet et al., 2011). There is substantial research available investigating
817 the effects of estrogens on various fish species and for the purposes of this thesis, effects
818 of exogenous estrogens will be the primary focus.

819 **1.2.1 Estrogens and their effects on fish**

820 EE_2 is a common estrogenic EDC is found in the birth control pill. After ingestion,
821 EE_2 is metabolized into a biologically inactive, water-soluble sulfate or glucuronide
822 conjugate which is then excreted in urine (Nieto et al., 2008) or remains in the gut as EE_2
823 due to *Escherichia coli* bacterium that deconjugate these metabolites from β-

824 glucuronidase and sulfatase activity (Atkinson et al., 2012; Lai et al., 2000). This results
825 in humans excreting biologically active EE_2 into the wastewater systems, which is
826 eventually processed by sewage treatment facilities via activated sludge or biofilms,
827 before entering the receiving environment in effluent (Adeel et al., 2017). In a 2021
828 review, researchers reported that EE_2 in surface waters from 32 different countries
829 averaged between 0-33 ng/L, with some countries having over 17 000 ng/L (Tang et al.,
830 2021). Between 1999 and 2020, the average levels of EE_2 in Canadian waters were found
831 to be 1.1 ng/L, with a maximum of 2.5 ng/L (Tang et al., 2021).

832 EDCs downstream of sewage wastewater facilities are bioavailable to aquatic
833 organisms that inhabit receiving areas (Leet et al., 2011; Lister et al., 2011). Even though
834 sewage treatment facilities are >90% effective at removing EE_2 from water, the minute
835 concentrations in the effluent can nevertheless cause negative effects to downstream fish
836 (Chimchirian et al., 2007). Estrogens have been implicated in causing various
837 reproductive effects in fishes, including increased E_2 and VTG plasma concentrations
838 (Filby et al., 2006; Orrego et al., 2010), reduced gonad development (Ali et al., 2018;
839 Nash et al., 2004), disrupted spermatogenesis (Van Aerle et al., 2002), decreased
840 fecundity and fertility (Filby et al., 2006), pathological changes in gonads (Dietrich and
841 Krieger, 2009), decreased male secondary sexual characteristics (Filby et al., 2006), and
842 female skewed sex ratios (Fenske et al., 2005; Hahlbeck et al., 2004).

843 **Estrogenic effects on gonad morphology**

844 The effects of estrogenic EDCs on fish gonadal development have been evaluated
845 histologically (Dietrich and Krieger, 2009; Leet et al., 2011). Pathological abnormalities
846 affecting the gonad of individuals include: inhibition of gametogenesis, necrosis and
847 apoptosis of germ and/or somatic cells, fibrosis (wound healing where connective tissue

848 replaces functional tissue), partial or complete inhibition of gonadal duct formation,
849 increased atresia (programmed cell death, apoptosis, of oocytes), and somatic cell
850 hypertrophy (increased cell size) and hyperplasia (increased cell numbers; Dietrich and
851 Krieger, 2009). These abnormalities are typically evaluated quantitatively or qualitatively
852 using the entire gonad from an individual. EE_2 is routinely used as an estrogenic model
853 EDC. The follow sections on estrogen induced effects to fish gonad will have a focus on
854 EE_2.

855 ***A) Hindering gametogenesis***

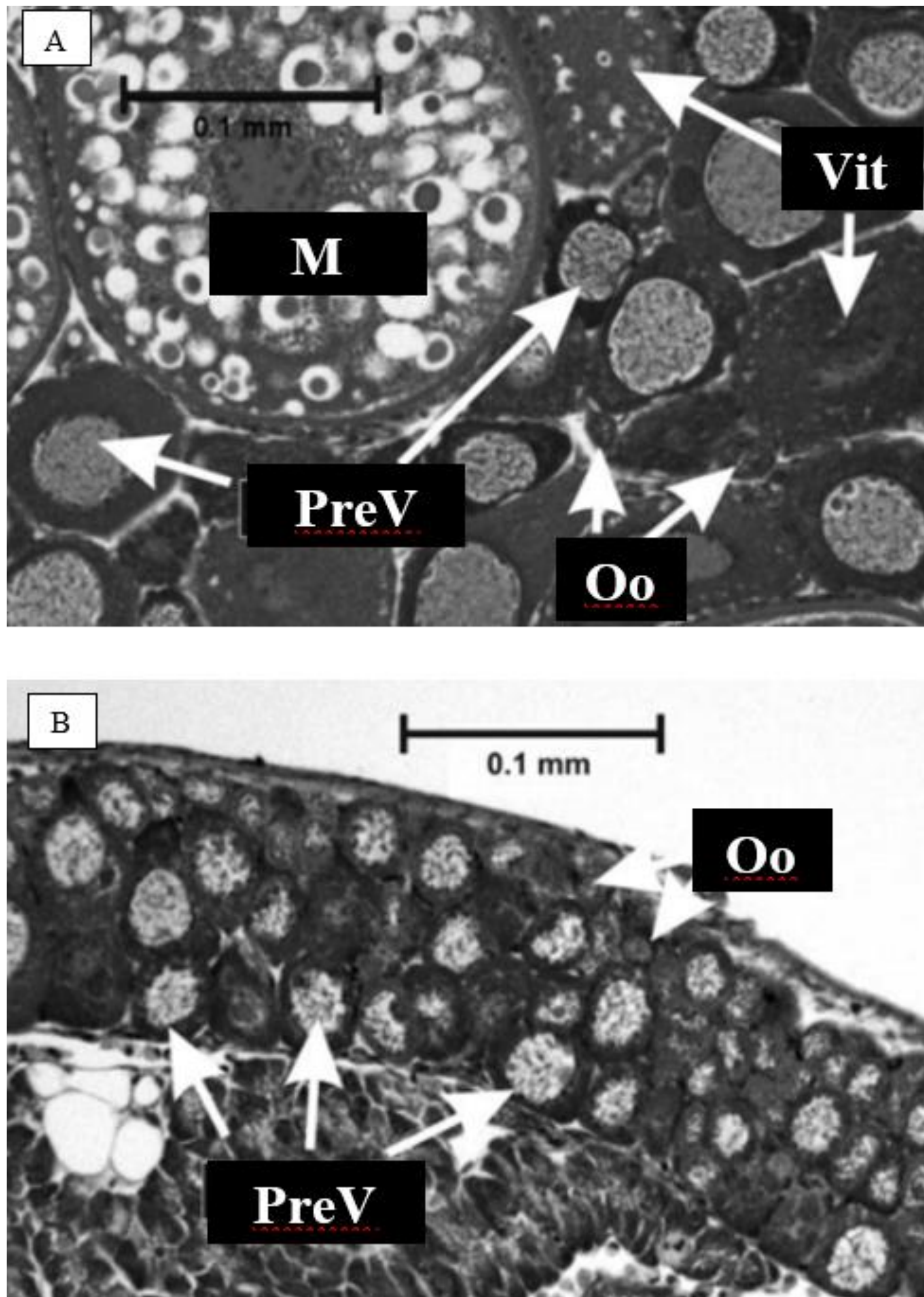
856 EE_2 has been reported to cause accelerated gametogenesis in female mummichog
857 (Chehade, 2012) and inhibitory effects in several other fish, regardless of sex [Table 2.1
858 (male) and 2.2 (female)]. These effects may cause reproductive issues for individual fish
859 in adulthood (Dietrich and Krieger, 2009), which has been demonstrated in the fathead
860 minnow (*Pimephales promales*; Van Aerle et al. 2002).

861 ***EE_2 effects on oogenesis***

862 Laboratory research studies involving high concentrations of EE_2 provide evidence of
863 germ cell regression or inhibited maturation, decreased number of germ cells, and
864 inhibited follicular development of the ovary (Dietrich and Krieger, 2009). These
865 observations suggest negative feedback to the HPG axis, resulting in downregulation that
866 hinders oogenesis (Leet et al., 2015). As an example, EE_2 has a higher binding affinity to
867 ERβ than E_2 which demonstrates a mechanism that can potentially downregulate the HPG
868 axis when bound (Katsu et al., 2007). Least killifish (*Heterandria formosa)* exposed to 25
869 ng/L EE_2 for 12 weeks during gonadal development resulted in hindered oogenesis; on
870 average treated ovaries were composed of approximately 80% previtellogenic oocytes

871 while controls contained significantly more developed oocytes – vitellogenic or mature
872 (Jackson et al., 2019). Several other researchers have reported EE_2 prevented oogenesis in
873 the zebrafish (Weber et al., 2003), fathead minnow (Weisbrod et al., 2007), rare minnow
874 (*Gobiocypris rarus*; Zha et al., 2008; Zha et al., 2007), and medaka (Seki et al., 2002).
875 Weber et al. (2003) reported EE_2 treated ovaries containing only previtellogenic follicles,
876 which are significantly under-developed compared to the mature oocytes in the control
877 group (Figure 1.12).

878



879 **Figure 1.12**: Photomicrographs of hematoxylin and eosin-stained ovary sections from
880 zebrafish (*Danio rerio*) exposed from 2 to 60 dph to acetone solvent (A; control) or 1 ng/l
881 of 17α-ethinylestradiol (B; EE_2) at 200X magnification. Control ovaries contained
882 multiple oocytes that were vitellogenic (Vit) and mature (M). While EE_2 treated ovaries
883 did not contain any vitellogenic oocytes, only previtellogenic (PreV) and oogonia (Oo)
884 were observed. Figure taken and modified from Weber et al. (2003) with permission from
885 Elsevier.

886 ***EE_2 effects on spermatogenesis***

887 Several studies using high concentrations of EE_2 provide evidence of germ cell stage
888 absence and inhibition of gametogenesis, among other effects, in the testis (Dietrich and
889 Krieger, 2009). This disrupted gonadal development is caused from altered endocrine
890 communication on normal cellular signaling (Dietrich and Krieger, 2009). This effect has
891 been frequently noted in the fathead minnow (Dietrich and Krieger, 2009). As an
892 example, 5 ng/L EE_2 caused an arrest of spermatogenesis in fathead minnow exposed
893 during the first year of a three year-long study (Kidd et al., 2007). Researchers later
894 reported population collapse after three years of exposure. Another study by Van Aerle et
895 al. (2002) exposing fathead minnow to 10 ng/L EE_2 during various short windows of
896 development showed increased percentages of early-stage spermatocytes in treatments
897 groups when sampled at 100 dph (Figure 1.13). Both studies indicate that EE_2 has the
898 potential to disturb spermatogenesis in fathead minnow, whether for a short period of
899 time or over a lifetime (Kidd et al., 2007; Van Aerle et al., 2002). Several other studies
900 have reported hindered spermatogenesis from EE_2 exposure in the zebrafish (Weber et al.,
901 2003), rare minnow (Zha et al., 2007), guppy (*Poecilia reticulata*; Nielsen and Baatrup,
902 2006) and least killifish (Jackson et al., 2019).

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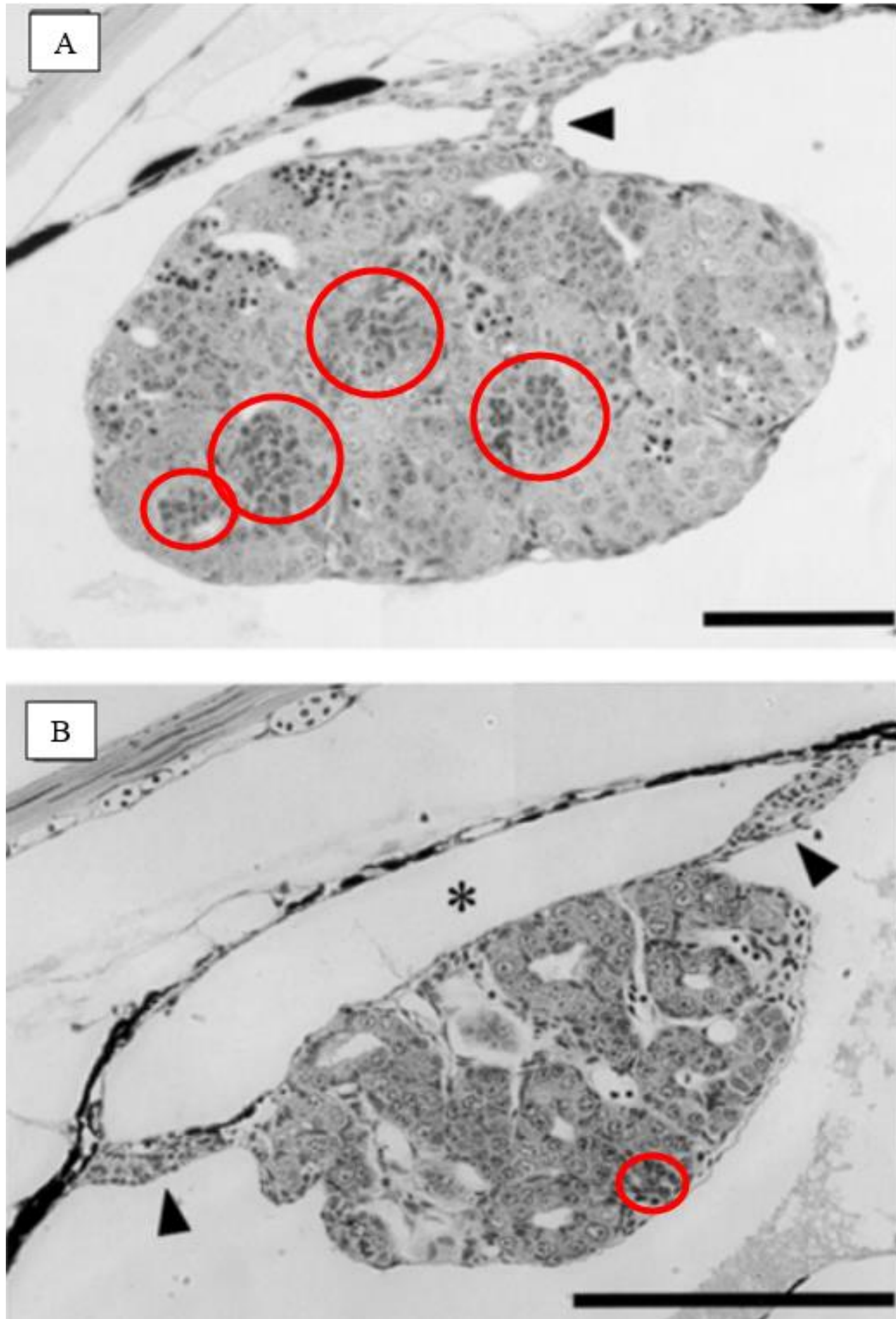


904 **Figure 1.13:** Photomicrograph of fathead minnow (*Pimephales promales*) testis in
905 control (A) and 10 ng/L 17α-ethinylestradiol-treated (EE_2, B) at 100 dph. Control male
906 showing sperm duct (arrowhead) and late spermatocytes (red circle). Treatment male with
907 fully formed ovarian-like cavity (*) attached to the peritoneal wall by duct cells
908 (arrowhead) and with few late spermatocytes. Bar is 100 µm. Photo taken from Van Aerle
909 et al. (2002) with permission from Springer Nature.

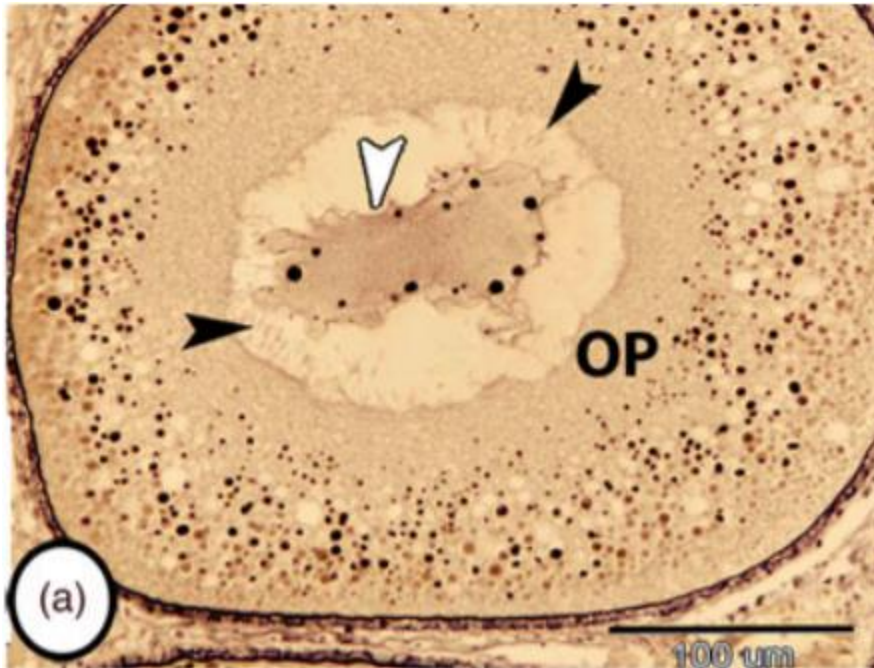
910 ***B) Degeneration***

911 Degeneration is often referred to as tissue that deteriorates or loses functional ability.
912 Degeneration is associated with traumatic injury, aging as well as general wear and tear.
913 EE_2 causes various gonadal tissue degeneration effects in both male and female fish
914 (Table 1.2 and 1.3).

915 ***Ovarian degeneration via EE_2***

916 Atresia is a common process in a healthy ovary, one that allows de-recruitment of
917 malfunctioning oocytes and preservation of energy (Dietrich and Krieger, 2009). Atretic
918 follicles can be identified histologically (Figures 1.14 and 1.15). Exposure to various
919 estrogenic compounds, including EE_2, increase frequency of atretic follicles. This drastic
920 effect has led to a decrease in follicles reaching maturity and consequently reduced
921 fecundity in zebrafish (Weber et al., 2003) and medaka (Seki et al., 2002). While not fully
922 understood, research has identified a possible mechanism in which exogenous estrogens
923 may cause atresia. Vitellogenic follicles undergo atresia when E_2 and gonadotropins are
924 reduced (Wood and van der Kraak, 2002). Under the influence of an estrogen and/or
925 mimic, such as EE_2, the HPG axis is expected to downregulate, which could produce
926 decreased levels of E_2 and gonadotropins in the ovary leading to increased atresia
927 frequency (Dietrich and Krieger, 2009). One study that exposed zebrafish to EE_2 and then
928 implemented a recovery period found that while treated ovaries recuperated from various
929 histopathological issues, time to atresia recovery was the longest (Luzio et al., 2016).
930 Researchers from this study concluded that the prolonged atresia causes negative impacts
931 to reproductive function of the given population.

932



933 **Figure 1.14**: The initial stage of ovarian follicular atresia of mature Redbelly tilapia
934 (*Coptodon zillii*) using Verhoff's stain. Shrinkage and degradation of the nuclear
935 membrane (white arrowhead) resulting in the dispersing of chromatin (black arrowheads)
936 in the ooplasm (OP). Figure taken from Mokhtar and Hussein (2020; publisher
937 Cambridge University).
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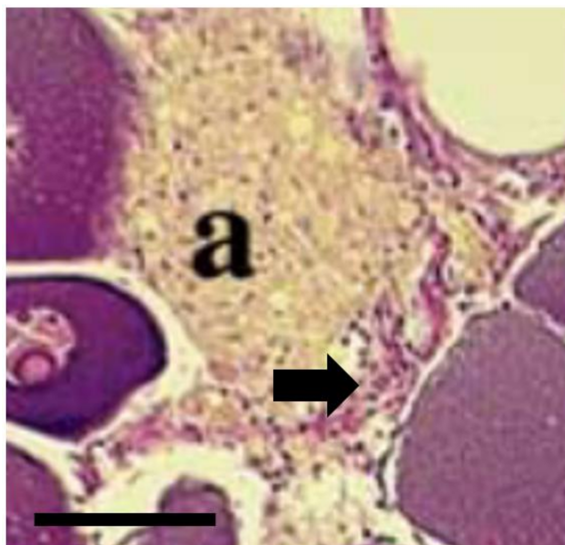

940 **Figure 1.15**: Photomicrograph of late-stage atresia in the ovaries of the common carp
941 (*Cyprinus carpio)* with hemoxylin and eosin staining. Yellowish pigment (a) is
942 surrounded by fibroblast cells (arrow). Bar is 200 µm. Figure taken from Blazer et al.
943 (2002) with permission from Springer Nature.

944 ***Testis degeneration via EE_2***

945 Common degeneration effects reported from EE_2 exposure in males include testis
946 inflammation, hypertrophic interstitial tissue and necrosis (Dietrich and Krieger, 2009).
947 These effects have been reported in the fathead minnow (Palace et al., 2002), zebrafish
948 (Schafers et al., 2007; Weber et al., 2003), three spined stickleback (*Gasterosteus*
949 *aculeatus*; Figure 1.16; Bjorkblom et al., 2009), and rare minnow (Zha et al., 2007). As an
950 example, in male zebrafish exposed to 1-10 ng/L EE_2 for 60 dph showed severe fibrosis
951 as well as acellular areas (Weber et al., 2003). Researchers believe the acellular area was
952 a result of necrosis with no fibrotic cellular repair. In a separate study by Luzio et al.
953 (2016), zebrafish were allowed a long recovery period after a chronic exposure to EE_2
954 from hatching. The results from this study showed that zebrafish gonad have a remarkable
955 capability to regenerate following necrosis and fibrosis damage (Luzio et al., 2016).
956 Studies providing evidence of EE_2 induced degeneration in fish report their findings in a
957 variety of ways, with little consistency (Dietrich and Krieger, 2009). However, the overall
958 conclusions provided agree this damage may lead to reproductive dysfunction of affected
959 fish and/or their populations (Dietrich and Krieger, 2009; Schafers et al., 2007; Weber et
960 al., 2003).

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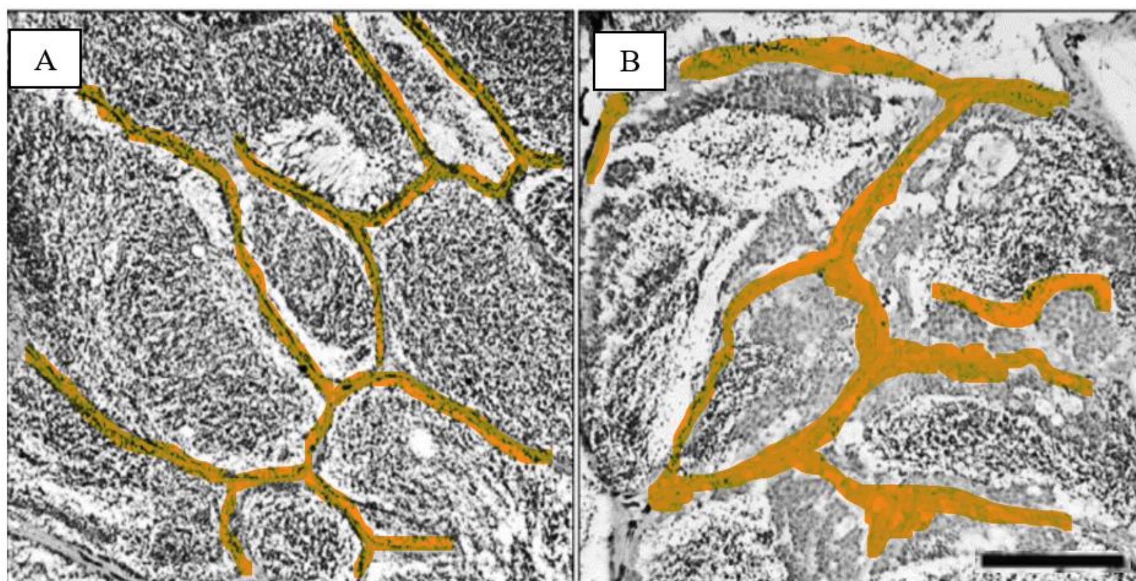


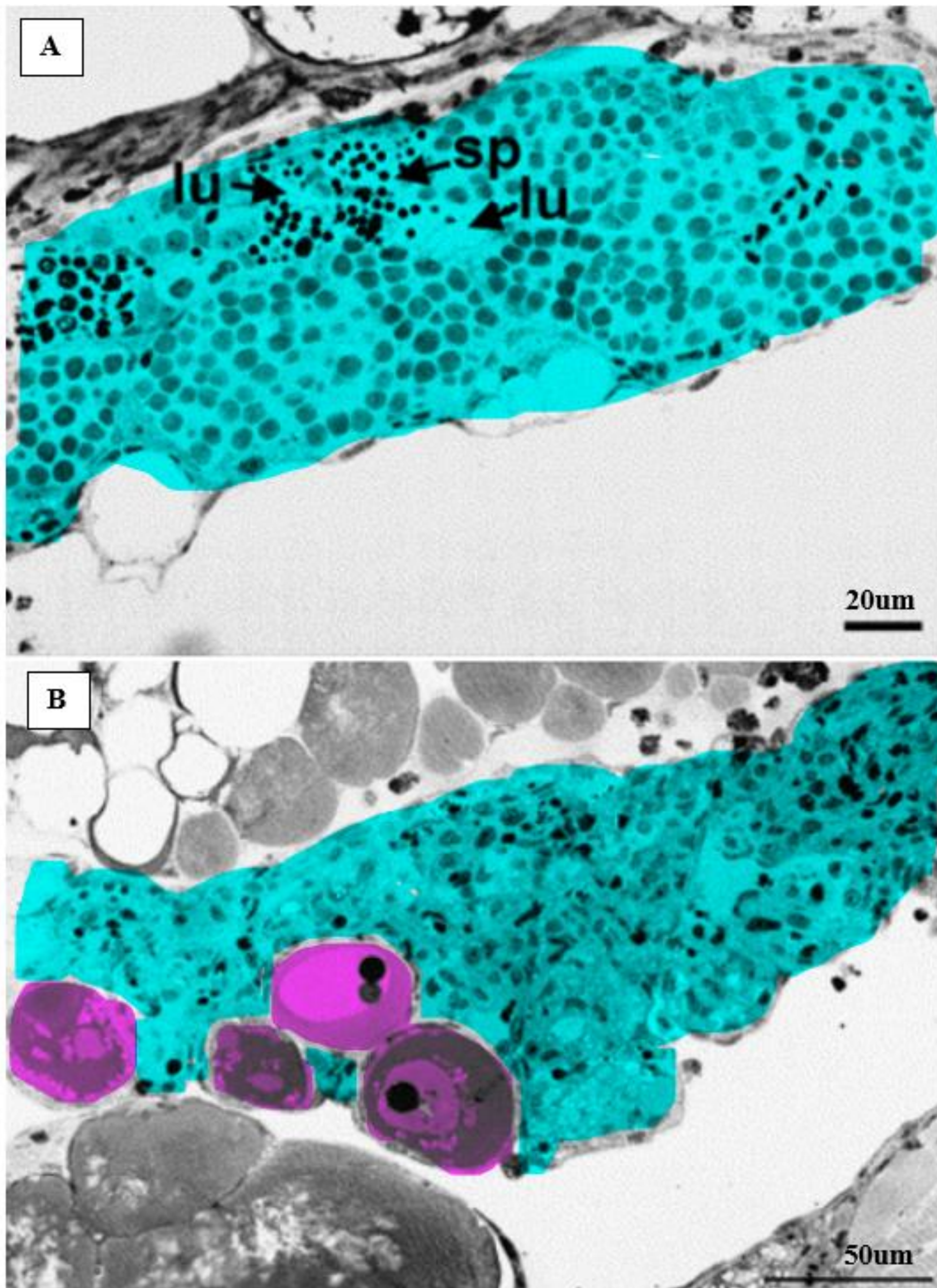
962 **Figure 1.16:** Photomicrographs of hematoxylin and eosin-stained histological sections of
963 testes from male three-spined stickleback (*Gasterosteus aculeatus*) exposed for four
964 weeks: (A) Mature testis, control fish; (B) severely affected testis with disrupted lobular
965 arrangement due to increased fibrosis, fish exposed to 20 ng/L of 17α-ethinylestradiol.
966 Basal membrane thickness (orange) has been highlighted to show increase in interstitial
967 tissue from treatment. Bar is 100 µm. Figure taken and modified from Björkblom et al.
968 (2009) with permission from John Wiley and Sons.

969 ***C) Altered sex determination***

970 Experiments using high concentrations of EDCs can contribute to complete sex
971 reversal in fish or atrophy of the gonad tissue (Dietrich and Krieger, 2009). A study
972 investigating various EE_2 effects on developing mummichog after fertilization used
973 gonadal histology to evaluate treatment effects on sex ratios. Results from this study
974 showed a female skewed sex ratio; mummichog exposed to 0, 10, 50 and 250 ng/L EE_2
975 contained 60%, 90%, 100% and 80% females, respectively, at 10 wph (Chehade, 2012).
976 These results indicate mummichog are sensitive to EE_2 during the GD process. Another
977 study using EE_2 (100 ng/L), exposed adults and their offspring from fertilization to 52
978 wph found an >80% female skewed sex ratio in the offspring (Peters et al., 2010).
979 Feminized sex ratios have been reported from environmentally-relevant EE_2 exposures in

980 zebrafish (Luzio et al., 2016) and fathead minnow (Van Aerle et al., 2002) indicating that
981 supraphysiological concentrations of EE_2 are not necessary to cause changes to sex in
982 fish. Luzio et al. (2016) exposed <24 h post fertilized zebrafish embryos to 4 ng/L EE_2 for
983 90 days and demonstrated a 60%:40% female to male ratio. Van Aerle et al. (2002)
984 exposed various stages of fathead minnow embryo and larvae to 10 ng/L EE_2 for short
985 intervals of time (fertilized egg to 5, 5-10, 10-15, 15-20 dph) as well as fertilized eggs
986 through to 20 dph and then fish were sampled at sexual maturity (100 dph). There was a
987 female sex bias in the embryo to 5 dph as well as the embryo to 20 dph EE_2 treatment and
988 no changes to sex ratios were reported in the treatments that started after hatching.

989 Intersex is a condition where the gonad of a fixed sex individual contains both male
990 and female gametes. This condition is atypical in nature and is routinely associated with
991 fish exposed to EDCs both in field and laboratory studies (Bahamonde et al., 2013).
992 Intersex has been implicated as a common effect from estrogen exposure; oocytes will
993 typically develop in testis tissue, commonly referred to as testis-ova (Figure 1.17). EE_2 in
994 particular induces intersex by generating testis-ova in male fathead minnow (Kidd et al.,
995 2007; Lange et al., 2001), medaka (Metcalfe et al., 2001; Seki et al., 2002), zebrafish
996 (Nash et al., 2004), rare minnow (Zha et al., 2007), least killifish (Jackson et al., 2019)
997 and the three spined stickleback (Hahlbeck et al., 2004) at environmentally-relevant and
998 higher concentrations (0.1-50 ng/L). Male fish develop oocytes because pharmaceutical
999 estrogens, like EE_2, directly interact with receptors, change gene expression, alter
1000 available receptors (AR or ER), and alter HPG axis feedback (Leet et al., 2011). These
1001 disruptions could lead to feminized intersex gonadal development if female promoting
1002 genes are upregulated in testis tissue. The intersex condition is important to monitor in
1003 fish as it can lead to reproductive dysfunction in fish and affect fish populations.



1004

1005 **Figure 1.17**: Light micrograph of zebrafish (*Danio rerio*) with normally developed testis
1006 (40 days post hatch; dph) under control conditions (A) and intersexed testis (testis-ova)
1007 following 25 ng/L EE_2 for 20 days starting at 20 dph (B). A is showing a healthy testis
1008 (blue highlight) with visible spermatids (sp) developing near the lumen (lu). B is showing
1009 an intersexed gonad with testis and ovary (pink highlight) tissue. Image taken and
1010 modified from Örn et al. (2003) with permission from Elsevier.

1011 The concern with exogenous estrogens is that they may feminize male fish, resulting
1012 in female skewed sex ratios (Leet et al., 2011). Currently, there are limited studies
1013 investigating environmentally-relevant estrogen exposure to fish population sex ratios.
1014 One study exposing developing zebrafish to 1 ng EE_2/L for 40 days reported no intersex,
1015 but a significantly female-skewed sex ratio (Örn et al., 2006). Another exposure,
1016 conducted over the lifetime of zebrafish, used 5 ng/L EE_2 and found a 50% reduction to
1017 fecundity, with complete population failure stemming from no fertilization in the
1018 subsequent generation (Nash et al., 2004). The male signaling pathway was completely
1019 shut down, producing only functionally female offspring (Nash et al., 2004). Another
1020 experiment using least killifish showed that a 12-week exposure to 25 ng/L of EE_2
1021 resulted in a female biased sex ratio (Jackson et al., 2019). A significant number of males
1022 from the same treatment were intersexed, resulting in declined spermatogenesis (Jackson
1023 et al., 2019). The results from these studies would support the need for further
1024 investigation on environmentally-relevant EDC exposure on fish since major changes to
1025 sex ratios in wild fish will affect the continued survival of their population (Dietrich and
1026 Krieger, 2009).

1027 **Estrogen effects on gene expression**

1028 Given the important role of genes in the sexual development of fish, evaluating EDC
1029 effects on SD and GD gene expression is warranted. Relative gene expression analysis
1030 can be used to help determine mechanisms of action and whether EDCs are affecting
1031 male or female signaling pathways in fish. One study using adult fathead minnow
1032 exposed to 10 ng EE_2/L for 21 days found *ERα, ERβ* and *AR* expression in males and
1033 females to be significantly downregulated (Filby et al., 2007). Chronic studies exposing
1034 fathead minnows (Feswick et al., 2016; Leet et al., 2015) and zebrafish (Liang et al.,

1035 2017) to EE_2 reported decreased *cyp19a1a* expression. Interestingly, studies using adult
1036 mummichog detected no changes in *cyp19a1a* expression (Doyle et al., 2013;
1037 Kanagasabesan, 2018). It has been suggested that high levels of E_2 in a developing female
1038 mummichog ovary could explain the decreased sensitivity of mummichog to
1039 environmentally-relevant EE_2 concentrations (Kanagasabesan, 2018). These results are
1040 not showing a well-established correlation with *cyp19a1a* and E_2 in mummichog as
1041 shown in other fish species, indicating that *cyp19a1a* may have other transcriptional
1042 controls that need to be investigated (Rutherford et al., 2020). Studies evaluating gene
1043 expression in the mummichog have focused on adult models, with limited research
1044 available on the early developmental stages. More research is required for understanding
1045 whether a particular EDC could be causing estrogenic gene expression disturbances in
1046 fish during the SD and GD developmental window. SD and GD fish studies using model
1047 compounds, like EE_2, are encouraged to determine sensitivities to these processes. More
1048 recently, studies investigating both histological and gene expression endpoints have been
1049 carried out; sex ratios including intersex, within a population can be proven histologically
1050 while preserving the remaining gonadal tissue for mRNA analysis (Chen et al. 2017).
1051 These results are advantageous as they could bring valuable mechanistic insights to the
1052 pathological abnormalities observed.

1053 **Research gaps**

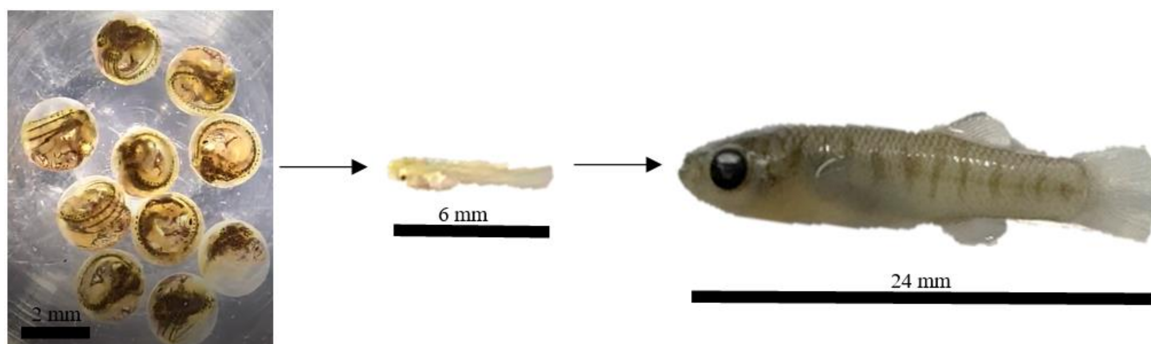
1054 There are several limitations in fish sexual development research that should be
1055 addressed. Firstly, many studies using EE_2 contain supraphysiological concentrations,
1056 which are typically higher than those found in aquatic environments (Leet et al., 2011).
1057 Research using environmentally-relevant EE_2 concentrations would provide better
1058 predictions for EE_2 exposed fish populations. Another research gap would be the lack of

1059 studies on estrogenic effects in developing fish; most research available focusses on
1060 estrogenic effects in adult fish models. Early life staged fish in particular have been
1061 shown to be more sensitive than adult fish to contaminants (Mohammed, 2013). The
1062 USEPA encourages using early life stages in toxicity testing as the data offers results on
1063 the most vulnerable life stage and best predicts effects of all life stages in a given
1064 environment (Mohammed, 2013). Finally, there is the lack of research on SD and GD in
1065 saltwater fish. There is extensive SD and GD research available on freshwater fish. The
1066 absence of saltwater fish development research is surprising since saltwater fish represent
1067 17% of meat in the food industry (Costello et al., 2020). This statistic is expected to
1068 increase to 25% by 2050 (Costello et al., 2020). Therefore, maintaining saltwater fish
1069 populations will be a valuable asset to the industry. There is a need to understand sexual
1070 development mechanisms in developing saltwater fish and how they respond to estrogens
1071 at environmentally-relevant concentrations.

1072 ## 1.3 *Fundulus heteroclitus*: a good saltwater fish toxicology model

1073 There is a need for an early life stage estuarine model for toxicologically studies; the
1074 mummichog (Figure 1.18) is a potential candidate to fill this gap. The mummichog is the
1075 common killifish that can be found along the Atlantic coast of North America. Its small
1076 size is easily accommodated in a laboratory setting. Its ability to adapt to varying
1077 salinities, oxygen content and temperature makes it a good model species in physiological
1078 studies (Rutherford et al., 2020). Mummichog are gonochoristic and breeding is cyclical;
1079 gonad size, measured as a percentage of total body weight, and steroid hormone levels
1080 have been shown to coincide with spawning events on the full and new moons during the
1081 summer months (Taylor et al., 1979; Cochran, 1987; Hsiao and Meier, 1989). Gonadal

1082 recrudescence (a period of active gametogenesis and growth of the gonad) and spawning
1083 may be induced in the lab through manipulation of photoperiod and temperature allowing
1084 for a year-round supply of reproductive mummichog and embryos for development
1085 studies (MacLatchy et al., 2003). Mummichog have been used as a model fish species to
1086 study reproductive effects from complex effluents (Bosker et al., 2010), estrogenic EDCs
1087 (Meina et al., 2013), and androgenic EDCs (Rutherford et al., 2015). Historically, adult
1088 mummichog have been shown to be less sensitive to EE_2 compared to other fish species
1089 (Rutherford et al., 2020). Mummichog exposed to 100ng/L of EE_2 for 28 days showed no
1090 effects to egg production (Bosker et al., 2016). However, there is limited knowledge on
1091 gonadal development pathways and their susceptibility to EDC interference.
1092 Understanding EDC effects has factually been investigated in freshwater systems more so
1093 than saltwater systems (Leet et al., 2011).



1094

1095 **Figure 1.18**: Mummichog (*Fundulus heteroclitus*) embryos 15 days post fertilization
1096 (left; 2X magnification), post-hatch yolk-sac larvae (middle) 10 min after hatching and a
1097 five-week-old juvenile (right, average length was 23.59 ±1.06 mm, N=45).

1098 The mechanisms that drive SD and GD in the mummichog are not yet fully
1099 understood. Morphological SD can be observed two-weeks post hatch with the presence
1100 of primary oocytes, which was previously reported by Shimizu et al. (2008). That study
1101 provided insight during the GD process in juvenile mummichog from one-week post
1102 hatch to thirty-six weeks post hatch using immunohistochemistry techniques to visualize
1103 GD and highlight the roles of LH and FSH in the gonad tissue (Shimizu et al., 2008). FSH
1104 production early in the GD process is believed to initiate steroidogenesis, while LH
1105 production was not found until further into sexual development. FSH signaling in the
1106 early gonad is likely crucial for GD in the mummichog (Shimizu et al., 2008). Gene
1107 expression studies would be an asset in understanding the mechanisms of whether
1108 estrogens are capable of disturbing natural SD and GD processes in a developing fish.
1109 Significant questions remain regarding which genes or signaling pathways are being
1110 expressed or blocked during the SD stage to drive gonadal development.

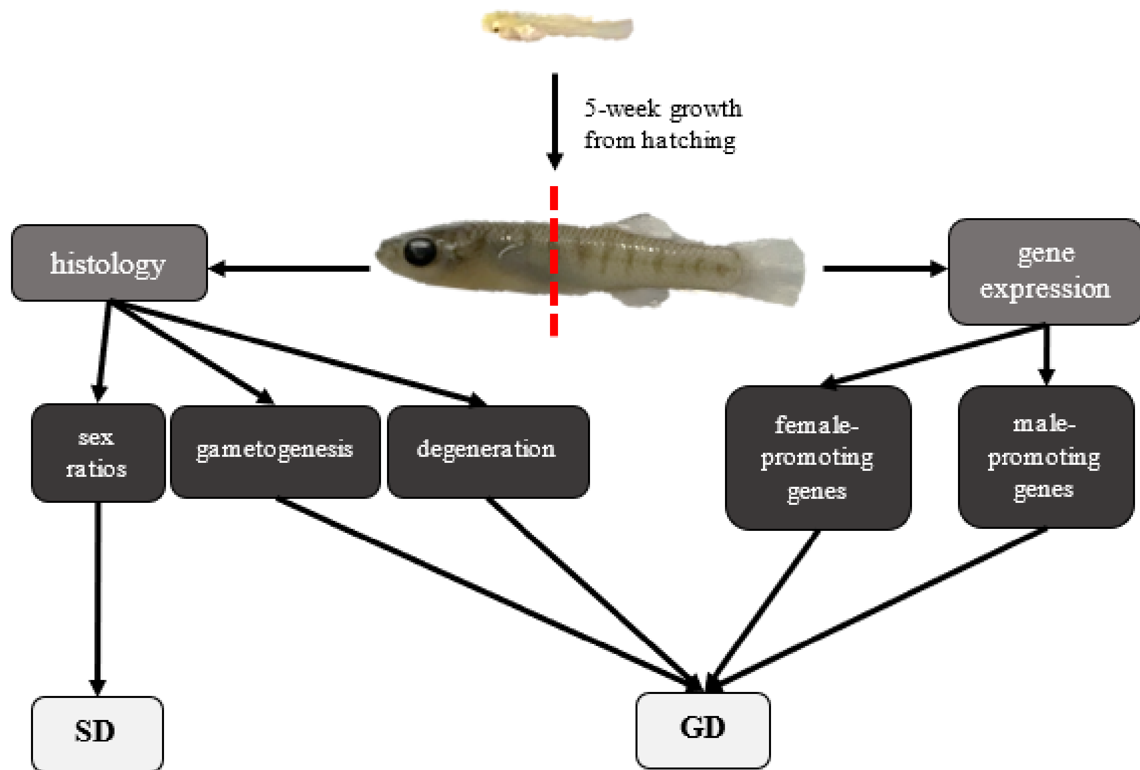
1111 There are a few studies available that indicate estrogenic effects on developing
1112 mummichog exposed to EE_2. Mummichog exposed to EE_2 8 h after fertilization
1113 continuously until sampling at later development have shown feminized (>80%) sex
1114 ratios (Chehade, 2012; Peters et al., 2010), accelerated onset of ovarian differentiation
1115 (Chehade, 2012) and reported no intersex (Chehade, 2012; Peters et al., 2010). These
1116 studies indicate mummichog are sensitive to environmentally-relevant and
1117 supraphysiological EE_2 concentrations during development. However, the window of
1118 sensitivity during development has not been investigated. Other endpoints should be
1119 explored to help determine the physiological mechanisms that are being altered, including
1120 gametogenesis, degeneration and gene expression.

1121 # 1.4 Research Objectives

1122 My thesis focuses on characterizing SD and GD mechanisms of five-week-old
1123 *Fundulus heteroclitus*, using histology to sex the fish and gene expression to examine the
1124 expression of genes implicated in GD using previously optimized mummichog primers.
1125 To achieve this, individual mummichog were grown to five weeks and then cut in half;
1126 one half was used for gonadal histological analysis and the other half for gene expression
1127 endpoints to characterize GD mechanisms (Figure 1.19). Histology allows for gonadal
1128 sexing and description of gonadal development. The sex of the fish is crucial to establish
1129 for pooled gene expression analysis of each sex separately. A focus will be placed on
1130 female promoting genes, *cyp19a1a*, *foxl2*, *bmp15* and *gdf9*, and male promoting genes,
1131 *amh* and *dmrt1*, as they have been shown to drive female and male differentiation in other
1132 fish species (Table 1.1). The sex ratio of five-week-old mummichog under standard
1133 laboratory conditions is expected to be 50% female:male with no intersex. Male and
1134 female mummichog are expected to show differential expression of male and female
1135 promoting genes. Mummichog testis should have higher expression *amh* and *dmrt1* while
1136 ovaries should have higher expression of *cyp19a1a*, *foxl2, bmp15 and gdf9.*

1137 The second objective is to determine sensitivity of exposure to environmentally-
1138 relevant EE_2 after hatching. Post hatch, yolk-sac larvae were exposed to 0, 2 and 10 ng/L
1139 of EE_2 until five weeks old and then cut in half to collect gonadal tissue for both gonadal
1140 histology and gene expression. Histology will be used to determine the sex of the fish,
1141 including the prevalence of intersex, and whether EE_2 is affecting gametogenesis and/or
1142 degeneration of the gonad. EE_2 is expected to alter gonadal development in male and
1143 female five-week-old mummichog. Fish exposed to EE_2 are predicted to contain a dose

1144 dependent increase in females due to the activation of the female signaling pathway in
1145 fish. The presence of intersexed fish is not expected because this condition has not been
1146 previously documented in field or laboratory studies on mummichog. EE_2 induction of
1147 altered gametogenesis and increased degeneration is expected in both sexes since the
1148 excess estrogen will have a direct effect on the developing gonads. Gonads from EE_2-
1149 treated fish are predicted to contain fewer developed germ cells. Histology will be used to
1150 determine whether EE_2 is contributing to higher frequencies of atretic follicles in ovaries,
1151 and increased area of necrosis and hypertrophic interstitial tissue in testis. Male
1152 mummichog exposed to EE_2 after hatch for five weeks are predicted to show increased
1153 expression of female promoting genes, *cyp19a1a*, *foxl2, bmp15, and gdf9,* as well as a
1154 decrease in expression of male promoting genes, *dmrt1* and *amh*, in the testes due to the
1155 activation of the female differentiating pathway which will consequently suppress the
1156 male differentiating pathway. Female mummichog exposed to EE_2 after hatch for five
1157 weeks will show decreased expression of female promoting genes, *cyp19a1a*, *foxl2,*
1158 *bmp15, and gdf9,* as well as an increase in male promoting gene expression, *dmrt1* and
1159 *amh* in the ovary due to downregulation of the female differentiating pathway. The
1160 female signaling pathway will be overwhelmed and shut down in the female fish.



1161
1162 **Figure 1.19:** Representation of research objectives for Experiments 1 and 2. After five
1163 weeks of growth from hatching, fish are euthanized and cut in half in between the anal
1164 and pelvic fins. The anterior half will be used for histological analysis under normal
1165 (Experiment 1) and 17α-ethinylestradiol (EE_2) treatment (Experiment 2) conditions while
1166 the posterior half will be used for gene expression analysis under the same conditions.
1167 Sex ratios will help to determine normal sex ratios of mummichog under standard
1168 laboratory conditions and whether sex determining mechanisms (SD) are vulnerable to
1169 EE_2 treatment. Gametogenesis and degeneration analysis will only be conducted for
1170 Experiment 2, which will determine whether EE_2 treatment is affecting gametogenesis
1171 and/or inducing degeneration of the gonad (GD). Gene expression will be used to identify
1172 male- or female-promoting genes in the mummichog that may be influencing gonadal
1173 differentiation (GD) and to determine whether these genes are changing from EE_2
1174 treatment.

1175 **Table 1.2**: Comparison of various male fish species exposed to environmentally-relevant
1176 concentrations of 17α-ethinylestradiol (EE_2) in various experimental designs including
1177 stage of development [hours (hph), days (dph) or weeks (wph) post hatch, or adult]
1178 duration of exposure (years=y, months=mo, weeks=w, days=d), and route of exposure.
1179 Effects caused by EE_2 exposure are listed as testis-ova (intersex), effects of
1180 spermatogenesis, presence of fibrosis or apoptosis (degeneration).

Species	Conc. (ng/L)	Stage of development	Duration	Route of Exposure	Results	Reference
Fathead minnow (*Pimephales promales)*	5-6	All stages	7 y	Aqueous Experimen-tal Lake	Testis-ova and arrested spermatogenesis (in first year only)	(Kidd et al., 2007)
	0.2-64	<24hr	305 d	Aqueous flow through	Testis-ova, testicular apoptosis	(Lange et al., 2001)
	4 – 8.1	Adult	113 d	Aqueous	Delayed spermatogenesis fibrosis	(Palace et al., 2002)
	10	Before and after hatching	5-10 or 20 d	Aqueous	Inhibited spermatogenesis	(Van Aerle et al., 2002)
Medaka (*Oryzias latipes*)	0.1-1000	1 dph	85-110 d	Aqueous	Testis-ova	(Metcalfe et al., 2001)
	32.6-488	Adult	21 d	Aqueous flow through	>63.9 ng/L: testis ova	(Seki et al., 2002)
Zebrafish (*Danio rerio*)	5	Egg stage	Lifelong (220 d)	Aqueous flow through	Few testis ova	(Nash et al., 2004)

	1-10	2 dph	60 d	Aqueous	Fibrosis, regressed testes	(Weber et al., 2003)
	9.3	0 dph	177 d	Aqueous flow through	Fibrosis	(Schafers et al., 2007)
Rare minnow (*Gobiocypris rarus)*	1-25	Adult	28 d	Aqueous flow through	Testis-ova, inhibited spermatogenesis lesion (degeneration)	(Zha et al., 2007)
Three spined stickleback (*Gasterosteus aculeatus)*	50	0 dph	39-58 d	Aqueous semi static	Testis-ova	(Hahlbeck et al., 2004)
	20	Adult	4 w	Aqueous flow through	Inhibited spermatogenesis increased lobular disorganization, fibrosis	(Bjorkblom et al., 2009)
Guppy (*Poecilia reticulata*)	10-200	Birth	3.5 mo	Aqueous flow through	10 ng/L: Fewer spermatocytes, >10 ng/L: fewer spermatids	(Nielsen and Baatrup, 2006)
Least killifish (*Heterandria formosa*)	5-25	<1 w	12-23 w	Aqueous	Intersexed males with delayed spermatogenesis	(Jackson et al., 2019)

1181

1182 **Table 1.3**: Comparison of various female fish species exposed to environmentally-
1183 relevant concentrations of 17α-ethinylestradiol (EE_2) in various experimental designs
1184 including stage of development [hours (hph), days (dph) or weeks (wph) post hatch, or
1185 adult] duration of exposure (years=y, months=mo, weeks=w, days=d), duration of
1186 exposure (years=y, months=mo, days=d, weeks=w), and route of exposure. Effects
1187 caused by EE_2 exposure are listed as sex ratios, effects of oogenesis, presence of fibrosis
1188 or atresia (degeneration).

Species	Conc. (ng/L)	Stage of development	Duration	Route of Exposure	Results	Reference
Zebrafish (*Danio rerio*)	5	Egg stage	Lifelong (220 d)	Aqueous flow through	Arrest of development/ no functional females	(Nash et al., 2004)
	1-10	2 dph	60 d	Aqueous	Regressed ovary previtellogenic follicles	(Weber et al., 2003)
	9.3	0 dph	177 d	Aqueous flow through	Follicular atresia	(Schafers et al., 2007)
Fathead minnow (*Pimephales promales*	50	Adults	15 d	Aqueous	Increase in previtellogenic follicles	(Weisbrod et al., 2007)
Medaka (*Oryzias latipes*)	32.6-488	Adult	21 d	Aqueous flow through	Atresia, regressed ovary previtellogenic follicles	(Seki et al., 2002)
Rare minnow	1-25	Adult	28 d	Aqueous flow through	Degenerated ovary	(Zha et al., 2007)

(*Gobiocypris rarus)*	1, 4	2 hpf	180 d	Aqueous flow through	Degenerated ovary	(Zha et al., 2008)
Mummichog (*Fundulus heteroclitus*)	10-250	Egg stage	10 wph	Aqueous semi static	100% female sex ratio, accelerated ovary development	(Chehade, 2012)
	1-100	Parents, embryo and larvae	61 wph	Aqueous semi static	>80% female sex ratio,	(Peters et al., 2010)
Least killifish (*Heterandria Formosa*)	5-25	<1 w	12-23 w	Aqueous	Delayed oogenesis	(Jackson et al., 2019)

1189

2.0 Materials and methods

2.1 Adult fish collection and husbandry

Wild mummichog were collected from an estuary site near Little Shemogue Harbour, NB (N 46°10, W 64°08) considered free of known contaminants. A beach seine net (1/4″ mesh size) and corralling technique were used to capture the fish, which were immediately transported in a truck to the Center for Cold Regions and Water Science Building (CCRWS, Wilfrid Laurier University, Waterloo, ON) in aerated plastic totes.

The fish were kept in 425 L plastic recirculating saltwater tanks (Aquabiotech, Coaticook, QC) and were held under standard laboratory conditions (16 ppt salinity, >80% dissolved oxygen, 16 h light:8 h dark; MacLatchy et al., 2003). Salt water was made in lab using reverse osmosis (RO) and city dechlorinated water mixed with Crystal Sea Marinemix salt (Marine Enterprises International, Baltimore, MD); salinity was confirmed daily using a YSI Pro 1030 handheld conductivity meter (Cole-Parmer Canada Company, Montreal, QC). Dissolved oxygen was confirmed using a YSI pro 2030 oxygen probe (Cole-Parmer Canada Company). The water temperature and feeding conditions varied throughout the year to control gonad recrudescence and regression. Warmer temperatures and increased feeding stimulate the fish to spawn, which naturally occurs in the summer (June-August) in Eastern Canada. For spawning conditions, temperature of the water was increased from 15ºC to 22-23ºC. Fish were fed commercial trout pellets (Fish Farm Supply, Elmira, ON, Canada) until satiated.

1210 ## 2.2 Fertilization, collection and incubation

1211 To obtain gametes for fertilization, adult fish were transferred into a recirculating
1212 aquatic housing unit connecting multiple tanks to one filtration system where the water
1213 was maintained at 22-23°C under spawning conditions (Section 2.1). Male and female
1214 fish were separated during the acclimation period in 10 and 20 L glass aquaria,
1215 respectively. Once acclimated, male fish were combined into the 20 L aquaria with the
1216 females for gamete release and ova fertilization. The water inflow into the 20 L aquaria
1217 was increased to transfer the eggs out of the aquaria and onto a polyester felt filter (1
1218 micron width) above the sump. Eggs were collected from the filter daily using a scoopula,
1219 cleaned with salt water (16 ppt) and transferred to a plastic container (dimensions: 32 cm
1220 X 26 cm X 12 cm) with a lid and 2.54 cm wide foam inserts (Coulon et al., 2012) to
1221 sandwich the embryos. Collections were done over a three-day period and the eggs were
1222 combined. Plastic containers holding mummichog embryos were incubated at 21°C and
1223 16 h:8 h light:dark cycle for 18-21 days in a controlled environmental chamber (LTCB-19
1224 BioChambers, Winnipeg, MB). While incubating, the containers were removed from the
1225 incubator daily to visually inspect embryos for viability, and to spray the foam with salt
1226 water (16 ppt) to keep the embryos damp. Embryos that were unviable (cloudy, white
1227 appearance) were immediately discarded.

1228 ## 2.3 Fish growth

1229 Methods used were influenced by protocols developed in the MacLatchy Lab
1230 (MacLatchy et al., 2003; Peters et al., 2010). At hatching, 60 yolk-sac larvae (18-21 days
1231 from fertlization) were randomly allocated into 6 L (22 cm X 22 cm X 14 cm) glass
1232 aquaria with a handheld brine shrimp aquarium net (0.40”; Penn Plax, Hauppauge, NY).

1233 Fish were kept under standard laboratory conditions (Section 2.1) and were fed live

1234 *Artemia nauplii* (brine shrimp; Brine Shrimp Direct, Ogden, UT) for five weeks twice

1235 daily until satiated. Fish were inspected daily to confirm adequate health as per the

1236 Wilfrid Laurier Animal Care Committee.

1237 The ammonia concentration in each tank was determined every three days to ensure

1238 <1 mg NH_3/L using TNT 831 Nitrogen, Ammonia kit (Hach Company, London, ON)

1239 following the manufacturer's instructions on the DR3900 Laboratory Spectrophotometer

1240 (Hach Company).

1241 2.4 Sampling

1242 At five weeks old, fish were euthanized using an overdose of tricaine

1243 methanesulfonate (TMS; Sigma Aldrich, Saint Louis, MO). The first twenty fish sampled

1244 were measured for total length and weight. Fish were then cut between the anal and pelvic

1245 fins using a double-edged blade (Derby double edged razor blades, Royal Island

1246 Enterprise LLC, Garfield, NJ). The anterior half of the fish was placed into a microfuge

1247 tube (1.7 mL microfuge tube, Diamed Laboratory Supplies Inc., Mississauga, ON)

1248 containing 10% buffered formalin (Sigma-Aldrich), while the posterior half was placed

1249 into another microfuge tube containing RNAlater™ Stabilization Solution (Life

1250 Technologies, Carlsbad, CA, USA). All microfuge tubes were left at room temperature

1251 for 24 h before further processing or longer-term storage conditions in the -20°C freezer.

1252 2.5 Histology cassette preparation and processing

1253 After storage in formalin for 24 h, fish anterior halves were dyed using Davidson

1254 Marking black, green, yellow, orange and blue tissue dye for unique identification of each

1255 fish (Bradley Products, Inc., Bloomington, MN, USA). Once dried, five different

1256 coloured samples were placed into Tissue Path™ Microsette™ Six Compartment Biopsy
1257 Cassettes (Fisher Scientific Company, Ottawa, ON). Cassettes were immediately sent to
1258 Susan Lapos at the animal health laboratory (AHL) histology department at University of
1259 Guelph, Guelph, ON. Fish dye colour and cassette number corresponded to a specific fish
1260 (Figure A1.1). Cassettes were processed and embedded in paraffin wax longitudinally.
1261 Three slides were taken from each block (5 µm thickness) with 60-80 µm sectioning
1262 between each slide and stained with H&E (Sigma Aldrich). This revealed a cross section
1263 view of the fish. Each block was sectioned three to five times to ensure >90% of fish
1264 gonads were visible on slides.

1265 2.6 Analysis

1266 2.6.1 Histology

1267 **Sex ratio**

1268 Mummichog were sexed based on gonad morphology. Categories of gonadal sex
1269 included: 1) female, 2) male, or 3) intersex (male and female gametes in the same fish).
1270 Ovaries were classified by the presence of oogonia, chromatin nucleolar, perinucleolar or
1271 cortico alveolar staged oocytes, and somatic granulosa and theca cells (Figure 2.1). Testes
1272 were identified by the presence of spermatogonia, spermatocytes, spermatids, and somatic
1273 Sertoli cells and Leydig cells (Figure 2.2).

1274 **Female gonadal development**

1275 Ovarian gametogenesis and degeneration were evaluated to determine gonad
1276 developmental changes in Experiment 2 only. Slides without a full cross section of the
1277 ovary were omitted.

Gametogenesis

Ten fish slides were randomly selected (3-4/replicate tank) from each treatment for analysis (A.1.2.1-A1.2.30). Each oocyte in the field of view was classified into a developmental stage: oogonia, chromatin nucleolar, perinucleolar and cortico alveolar (Figure 2.1). An overall percentage of each developmental stage was calculated by dividing the total number of oocytes in the given stage by the total number of oocytes in the field of view.

$$\textbf{Developmental stage percentage (\%)} = \frac{\text{Count of developmental stage in ovary}}{\text{Total count of oocytes in ovary}} * 100$$

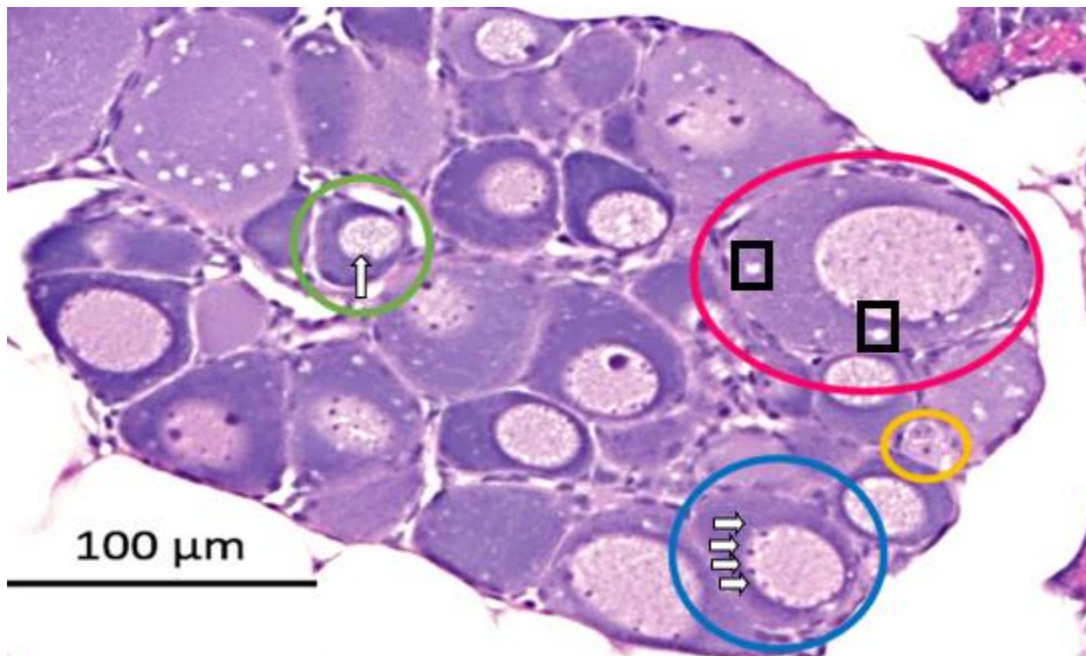


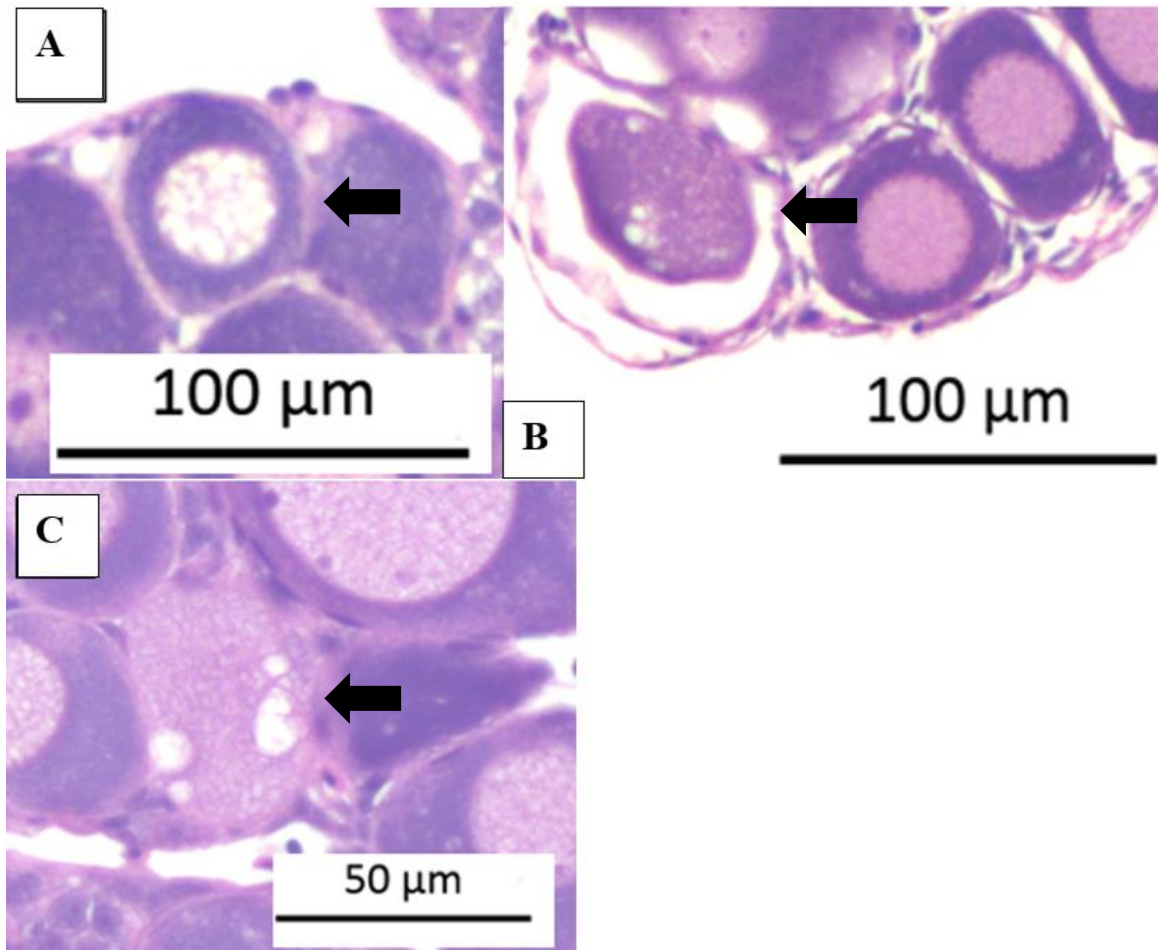
Figure 2.1: Five-week-old mummichog (*Fundulus heteroclitus*) ovary under light microscope with hemoxylin and eosin staining. Oocytes at different stage of development were found in the ovary of an individual fish. Oogonia (yellow circle) were early staged oocytes that have differentiated from primary germ cells (undifferentiated sex cells). Oogonia were identified by their small size and small cytoplasm to nucleus area ratio. As oocytes develop, their overall size and cytoplasm to nucleus area ratio increases. Chromatin nucleolar oocytes (green circle) were the next stage after oogonia; these cells were identified by their large nucleoli (arrow) at the periphery of the nucleus. Perinucleolar oocytes (blue circle) were identified by multiple nucleoli at the periphery of the nucleus. Fat globules (square) indicate the cortico alveolar oocyte (red circle). The

1297 follicular envelope surrounds each oocyte which were composed of somatic theca and
1298 granulosa cells. 10 X magnification.
1299
1300 ***Degeneration***

1301 To calculate degeneration in the ovary, atretic follicles were identified and counted in

1302 all slides. The three stages of atresia that were identified histologically are: nuclear

1303 disintegration, follicular dissociation, and fibrosis (Figure 2.2).



1304

1305 **Figure 2.2:** Atretic follicle stages (black arrow) that can be identified histologically in a
1306 five-week-old mummichog (*Fundulus heteroclitus*). In order of development: nuclear
1307 disintegration/ granulation (A; 10 X magnification), follicular dissociation (B; 10 X
1308 magnification), and fibrosis (C; 20 X magnification) were the three stages that were used
1309 to identify ovarian degeneration in the mummichog.

1310 **Male gonadal development**

1311 Testis gametogenesis, degeneration and abnormal cavity presence were evaluated to
1312 determine testis developmental changes in Experiment 2. Slides that did not contain at
1313 least one full testis were omitted.

1314 ***Gametogenesis***

1315 Sixteen fish slides were randomly selected (5-6/replicate tank) from each treatment
1316 for analysis (A.1.3.1-A1.3.30). Sample numbers were increased from 10 to 16 for this
1317 analysis to increase significance level of the results. Each gamete in the testes were
1318 counted using a handheld tally counter and classified into a developmental stage (Figure
1319 2.3). An overall percentage of each developmental stage in the testis was calculated by
1320 dividing the total number of cells in the given stage divided by the total number of testis
1321 cells in the testis slide.

$$\textbf{Developmental stage percentage (\%)} = \frac{\text{Count of developmental stage in testis}}{\text{Total count of all stages in testis}} * 100$$

1322

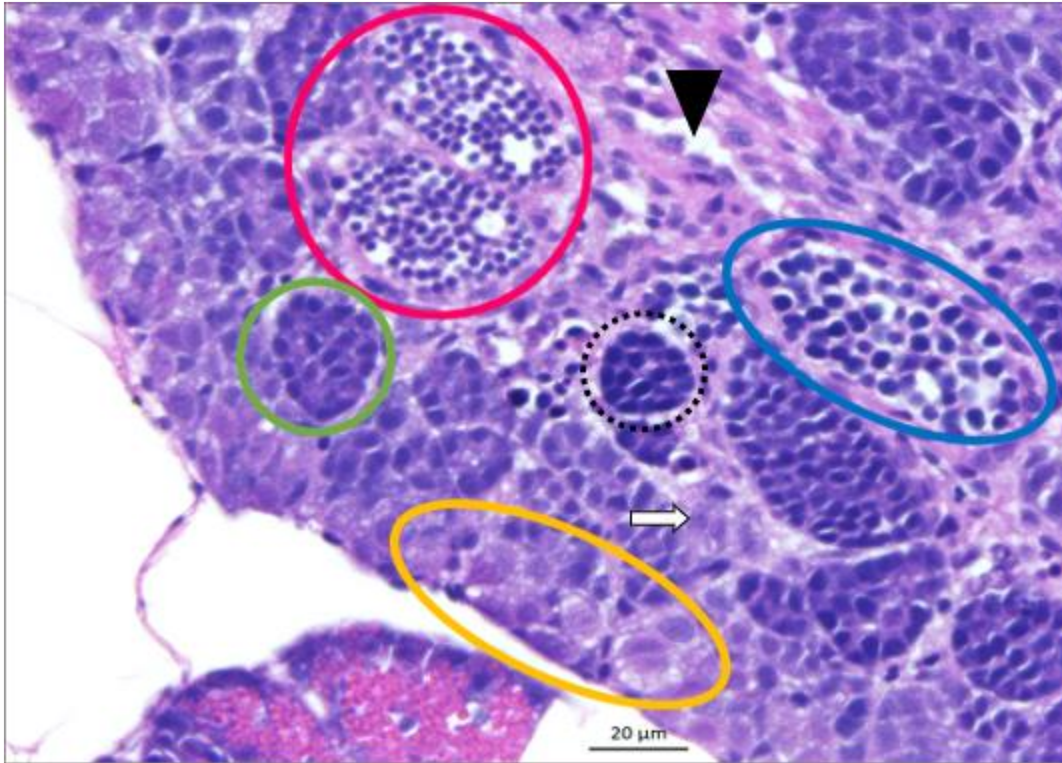


Figure 2.3: Five-week-old mummichog (*Fundulus heteroclitus*) testis under light microscope with hemoxylin and eosin staining. A testis showing various stages of sperm cell development (circles) and spermatocysts (dotted line circle) which enclose the developing cells in somatic Sertoli cells. Spermatogonia (yellow) were located at the periphery of the testis. Typically, spermatogonia were the largest cells in the testis and as cells divide and mature, their size decreases as the spermatocysts move toward the efferent duct (black arrowhead) in the lumen. Spermatogonia give rise to spermatocytes; early spermatocytes (green) were found close to spermatogonia and were tightly packed together. Late spermatocytes (blue) were identifiable by the increased space in the spermatocyst and rounded shape. Spermatid (red) were easily identified by their small size (about 50% of spermatocyte size). Leydig cells (white arrow), which assist in steroidogenesis, were perceptible throughout the testes. 40 X magnification.

1336 ***Degeneration***

1337 Necrotic and hypertrophic interstitial tissue were identified histologically and used to

1338 determine percent degeneration in all testes slides (Figure 2.4). ImageJ

1339 (https://imagej.nih.gov/ij/index.html) was used to calculate the area of necrotic and

1340 hypertrophic interstitial tissue in the testes as well as the total area of the testes. An

1341 overall percentage was calculated by dividing the area of necrosis and fibrosis by the total

1342 area of the testes in the field of view.

$$\textbf{Percentage degeneration (\%)} = \frac{\text{Area of necrotic and fibrotic tissue in testes}}{\text{Total area of testes}} * 100$$

1343

1344 A degeneration index was used to categorize the severity of degeneration in the testis

1345 tissue (Figure 2.4).

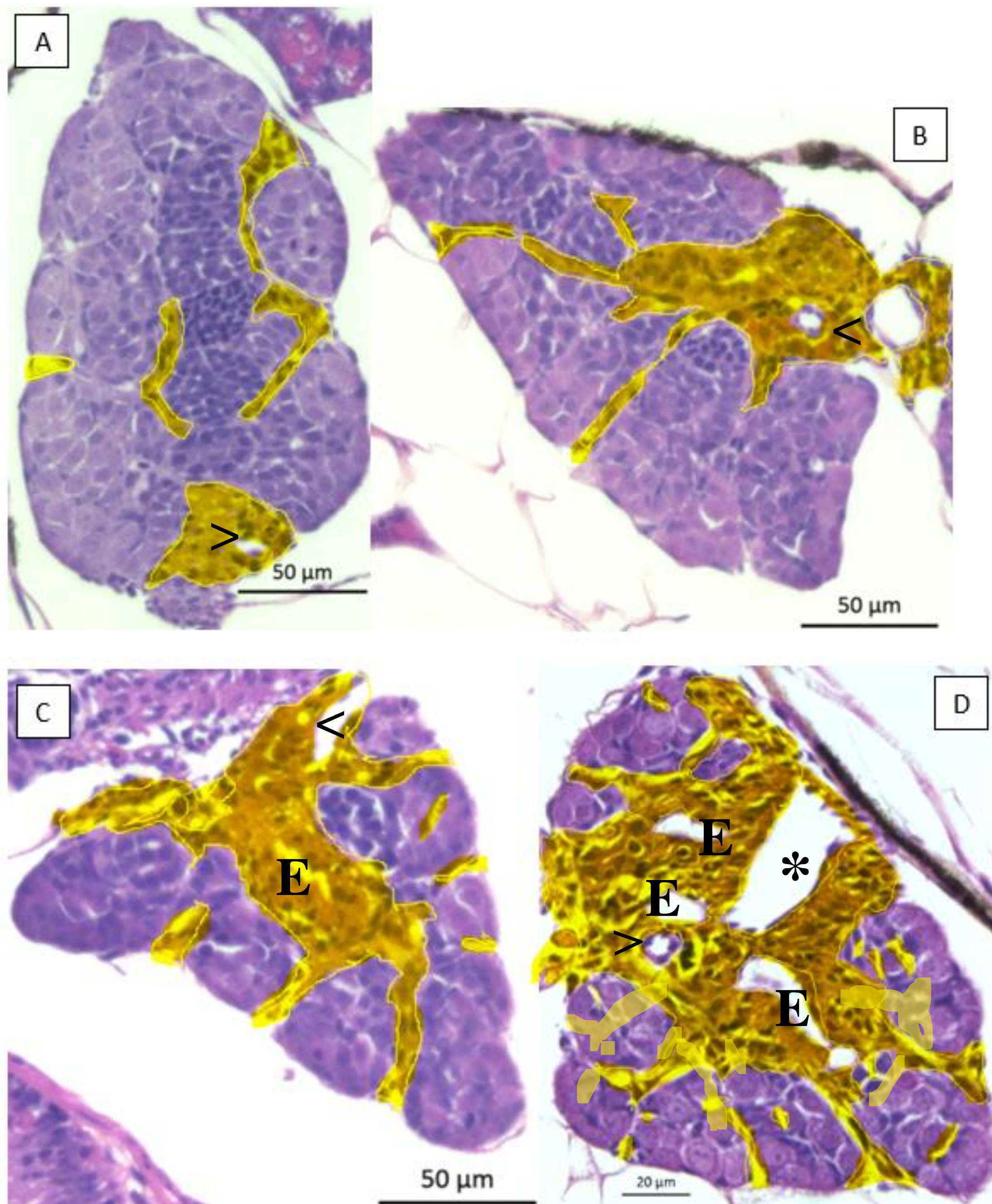
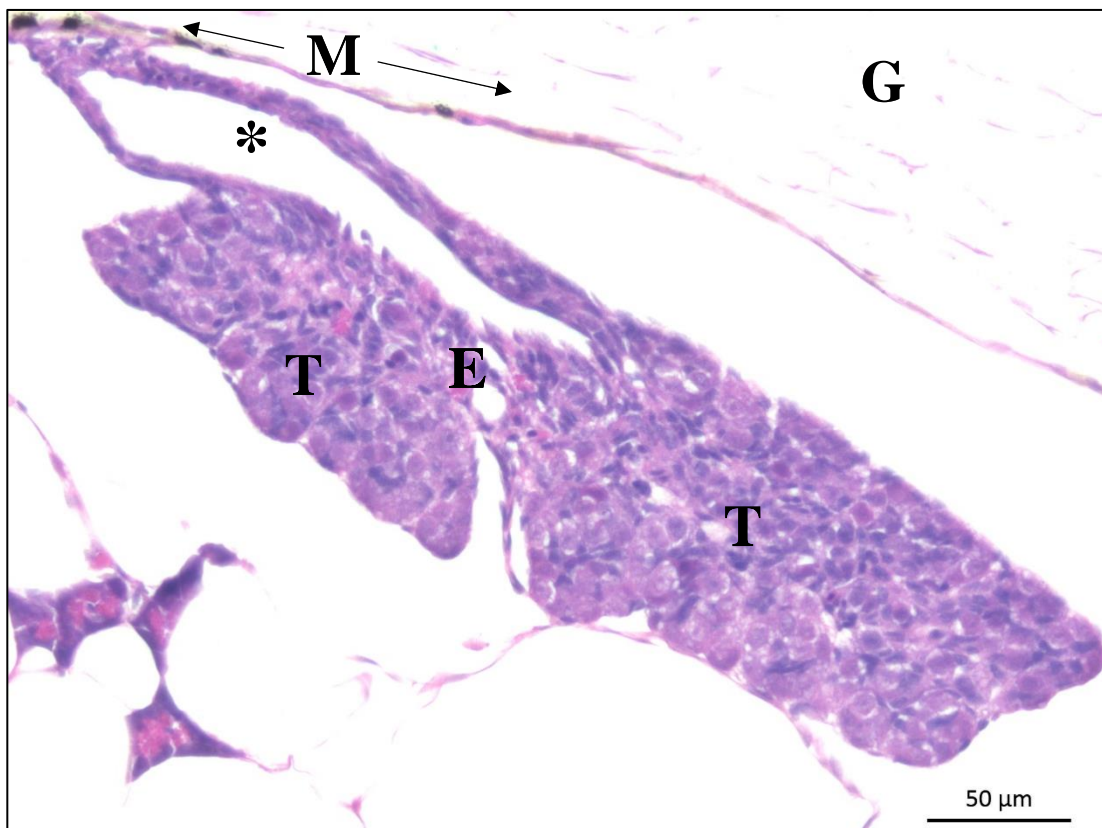


Figure 2.4: Degeneration index to evaluate severity of necrosis and hypertrophic interstitial tissue in mummichog (*Fundulus heteroclitus*) testis treated with 17α-ethinylestradiol (EE_2) for five weeks post hatch. Histological degeneration percentage calculated using ImageJ program on histological testis tissue, shown in yellow on figures. Area of necrosis and hypertrophic interstitial tissue divided by total area of testis tissue multiplied by 100. Example photos and identification of mummichog testis provided from Experiment 2. Categories are A <20% degeneration (control male, 20X), B = 20-40% degeneration (low EE_2 concentration treatment, 20X, male), C = 40-60% (high EE_2 concentration treatment, 20X, male) and D >60% degeneration (high EE_2 concentration treatment, 40X, male). E, efferent ducts. Arrowhead, blood vessels. *, abnormal cavity.

1357 ***Abnormal Cavity***

1358 The presence of an abnormal cavity was counted from sixteen randomly selected
1359 testis slides from each treatment. The abnormal cavity was identified by its large size,
1360 which was greater than the efferent duct size, and attached to the mesentery (Figure 2.5).



1361
1362 **Figure 2.5**: Photomicrograph of a five-week-old mummichog (*Fundulus heteroclitus*)
1363 testis under light microscope with hemoxylin and eosin staining. Fish were exposed to
1364 daily renewal of 10 ng/L 17α-ethinylestradiol (EE_2) for five weeks post hatch. The
1365 abnormal cavity (*) was observed in several EE_2 treatment exposed mummichog; which
1366 was easily distinguishable from the efferent duct (E). Each abnormal cavity was attached
1367 to both testis lobes (T) and the mesentery (M), which separates the gut with the gas
1368 bladder (G).
1369

1370 **2.6.2 Gene expression**

1371 ***Dissection***

1372 Fish were held on top of 4% agar (Select Agar™, Invitrogen, Waltham, MA) and the
1373 gonad was removed from the posterior body cavity under a dissecting microscope using

1374 very fine tip forceps (Tip dimensions: 0.05 x 0.02 mm; Dumont #55 forceps, Fine Science
1375 Tools Inc., North Vancouver, BC) and immediately placed onto dry ice. Four ovaries and
1376 seven to eight testes were pooled to generate a pooled ovary and testis sample,
1377 respectively, and then placed in a microfuge tube.

1378 ***RNA extraction***

1379 Total RNA was extracted from the frozen pooled gonad samples using PureZOL™
1380 (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The protocol used followed the
1381 manufacturer instructions with minor modifications from (Toni et al., 2018).

1382 To start, 500 µL of PureZOL™ (Bio-Rad) was added to one microfuge tube
1383 containing a pooled sample and was then immediately homogenized using a BD Luer-
1384 Lok™ Syringe (3 mL) with attached needle (21G; BD – Canada, Mississauga, ON) by
1385 inhaling and expelling the liquid and tissue into the syringe. One sample was processed at
1386 a time to prevent degradation of RNA. Once all samples were homogenized, samples
1387 were incubated at room temperature for 5 min. Next, 150 µL of chloroform (Sigma
1388 Aldrich) was added to each tube and invert mixed for 30 s. Samples were incubated for 3
1389 min at room temperature then centrifuged at 2°C for 20 min at 12000 X *g*. The upper
1390 aqueous phase of each sample was then carefully transferred into a fresh microfuge tube.
1391 Then 1.5 µL UltraPure™ Glycogen (Thermofisher Scientific, Mississauga, ON) was
1392 gently mixed into each sample followed by 300 µL of isopropanol (Sigma Aldrich).
1393 Samples were then pulse vortexed and transferred into the -20°C freezer for overnight
1394 precipitation.

1395 Samples were removed from the freezer and placed directly into the centrifuge at 2°C
1396 for 20 min at 12000 X *g*. Once removed from the centrifuge, the supernatant was

1397 discarded. To wash the samples, 800 µL of chilled 75% ethanol was added to each tube
1398 and vortexed. Samples were placed back into the centrifuge for 10 min at 7500 X *g*, 2°C.
1399 This wash step was repeated twice. Samples were then air dried at room temperature in
1400 the fume hood for 3 min. Samples were then reconstituted in 10 µL of sterile molecular
1401 grade water (Wisent Inc., Saint-Jean-Baptiste, QC) and incubated at 55°C for 10 min.
1402 Samples were vortexed before transfer into the -80°C freezer.

1403 ***RNA purity, integrity and yield***

1404 RNA purity and yield were determined using a Nanodrop 8000 (Thermo Fisher
1405 Scientific, Burlington, ON) using 2 µL of 10X diluted sample. Samples yielding <31.25
1406 ng/µl of RNA in the original sample were eliminated due to insufficient concentration.
1407 RNA purity was determined using 260/280 nm absorbance ratios; samples with ratios
1408 below 1.7 were eliminated due to phenol contamination. The Experion™ (Bio-Rad) was
1409 used to evaluate RNA integrity and confirm yield. Analysis was conducted using the
1410 RNA Highsens analysis kit (Bio-Rad) following the manufacturer's instructions. Samples
1411 were diluted to 2 ng/µL as per Nanodrop results. The RQI (RNA quality indicator
1412 number) was used to determine RNA integrity; this method offers a robust assessment of
1413 RNA integrity using an algorithm that compares three regions of an electrophoretic
1414 profile to a series of degradation standards. The RQI algorithm within the Experion
1415 system compares the electropherogram of RNA samples to data from a series of
1416 standardized [28S (structural large ribosomal RNA of eukaryotic cytoplasmic ribosomes),
1417 18S (structural small ribosomal RNA of eukaryotic cytoplasmic ribosomes)], degraded
1418 RNA samples and automatically returns a number between 10 (intact RNA) and 1

1419 (degraded RNA) for each sample. Samples containing an RQI value ≤ 6.9 were

1420 eliminated due to degradation.

1421 RQI equation:

1422 $I_S = I_i \alpha_i$

1423 Where $I_i \in \{I\}^M_{i=1}$ is a set of integral numbers assigned to the standards

1424 $I = \min\{\alpha_i\}^M_{i=1}$, where $\alpha_i = // P(S^S, S^I) //$, where S^S is sample signal and S^I and

1425 standard signal

1426 ***DNase treatment***

1427 Samples were treated with DNase I (Sigma-Aldrich, St. Louis, MO) to remove any

1428 genomic DNA. To start the reaction, 250 ng of RNA was transferred into corresponding

1429 PCR tubes (0.2 ml; Bio-Rad) with 1 µL of DNase enzyme (1 U/µL) for 15 min following

1430 the manufacturer's instructions. Samples were incubated using the C1000 Touch™

1431 Thermal Cycler (Bio-Rad) at 70°C for 10 min with 1 µL of stop solution. Once complete,

1432 samples were chilled on ice and immediately reverse transcribed.

1433 ***Reverse transcription reaction***

1434 Samples were reverse transcribed using iScript™ Reverse Transcription Supermix

1435 for RT-qPCR (Bio-Rad) following the manufacturer's instructions. Samples were

1436 incubated using the C1000 Touch™ Thermal Cycler (Bio-Rad). Samples were primed for

1437 5 min at 25°C, then reverse transcribed for 20 min at 46°C followed by inactivation at

1438 95°C for 1 min.

1439 ***qPCR***

1440 For RT-qPCR analysis, SsoAdvanced™ Universal Inhibitor-Tolerant SYBR®Green

1441 Supermix (Bio-Rad) was used following the manufacturer's instructions. Each sample

1442 was run in duplicate containing 2.5 μL of template 25X diluted cDNA with 5 μL of

1443 SYBR®Green Supermix and 1.25 μL of forward and reverse primers complementary to

1444 the genes of interest (Table 2.1). The CFX96 Touch™ Real-Time PCR Detection System

1445 (Bio-Rad) was used for the PCR reaction and melt curve analysis (section A1.4). The

1446 PCR reaction protocol followed: polymerase activation and denaturing at 98°C for 2 min,

1447 then amplification steps including denaturing for 15 s at 98°C, annealing at 60°C for 60 s

1448 followed by a plate reading. The amplification steps were repeated 39 times. Melt curve

1449 analysis was conducted starting at 60°C and increasing 0.5°C every 2 s until 95°C.

1450 ***qPCR analysis***

1451 Raw data provided by the CFX96 Touch™ Real-Time PCR Detection System for

1452 each gene of interested was normalized to reference genes *βactin* and *ef1α* (elongation

1453 factor 1 alpha) using Bio-Rad Maestro gene study software version 5.3.

1454 The program uses the $2^{-\Delta\Delta cq}$ method to determine the normalized expression of the gene of

1455 interest.

1456
1457
1458

$$\text{Normalized expression}_{\text{sample (GOI)}} = \frac{RQ_{\text{sample (GOI)}}}{(RQ_{\text{sample (Ref 1)}} \times (RQ_{\text{sample (Ref 2)}} \ldots \times (RQ_{\text{sample (Ref n)}}))^{1/n}}$$

1459 Where:

1460 RQ = the relative quantity

1461 GOI = gene of interest

1462 Ref = reference gene

1463

1464 The gene study software provides target stability values [Coefficient of Variation

1465 (CV) and M-value (M)] to calculate the quality of the reference genes (A1.4.1 and

1466 A1.4.2).

1467 ### 2.6.3 Primer development optimization and sequencing

1468 Primers to identify mummichog gene of interest sequences were designed and
1469 optimized in the Van Der Kraak Lab at the University of Guelph using adult mummichog
1470 gonadal tissue (Table 2.1). Primer optimization was confirmed in the MacLatchy lab
1471 using five-week-old mummichog gonadal tissue. Final PCR products from the Van Der
1472 Kraak lab were sequenced. Sequences were run on BLAST using Ensembl.org to identify
1473 the location of the products on the mummichog genome and map introns and exons.
1474 Products were found to be located on one exon. Therefore, DNase treatment was critical
1475 to eliminate DNA contamination.

1476 **Table 2.1:** Genes studied with their accession numbers. Forward and reverse primer
1477 sequences designed for qPCR analysis, primer efficiency and R^2 from pooled five-week-
1478 old mummichog (*Fundulus heteroclitus*) mixed gonadal tissue standard curve. *β-actin* and
1479 *ef1α* were reference genes. Product size base pairs (bp) were provided from sequencing
1480 results via the Van Der Kraak laboratory. Product sequences compared on Ensembl.org
1481 were found on one exon.

Gene	Accession number	Forward sequence 5'-3'	Reverse sequence 5'-3'	Product size (bp)	Efficiency (%)	R^2
β-actin	AF397164	GCACGGTATTGTACCAA	GGGTGTTGAAGGTCTCAA	NA	94.3	0.998
ef1α	AY430091	GTGAGCACCTACATCAAGA	CTTCCAGCCTTTGAACCA	NA	95.3	0.999
cyp19a1a	AY428665	GTCCACTCTTGTCTTATTTG	CTCTCCTCTCCATTGATC	NA	93.1	0.987
foxl2	XM_012863233.2	GGACCAAATGGCCTTAATGA	TGCCAGCCTTTCTTGTTCTT	246	93.9	0.985
bmp15	XM_012874987.2	AAAGATTCTTCGCTGGACGA	AATGCCAGGTTGAAGGAGTG	158	90.9	0.992
gdf9	XM_012856823.2	GCAAGCGTGGAGATAAAAGC	CCGTAGATGAAGCCCATTGT	202	93.2	0.997
dmrt1	XM_021307184.1	CACGGCATGTCCTCTCAGTA	GCAGGTTATGGTGGAGTCGT	180	90.2	0.988
amh	XM_012864864.2	CGTTCATGCGCTCTTATTGA	GTGAGCACCACAGCTTCAAA	158	90.4	0.989

1482

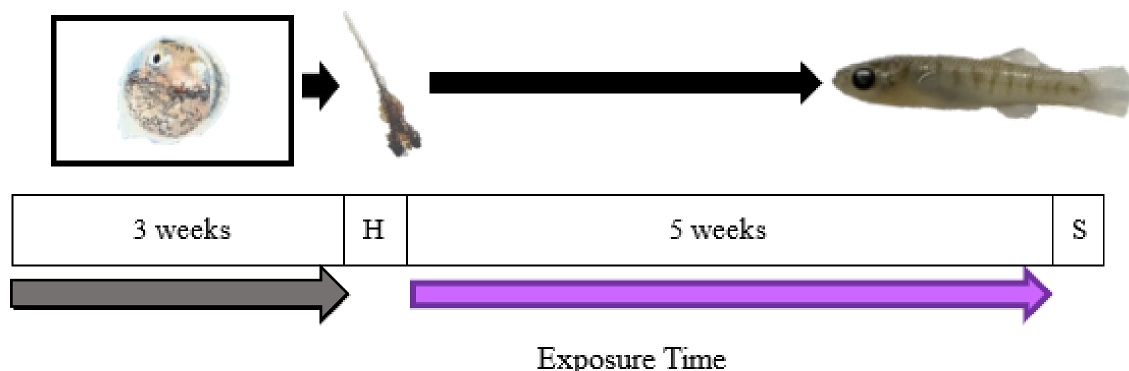
1483 ### 2.6.4 Experiment 1: Pilot study design

1484 The purpose of this experiment is to establish normal gonadal development, sex
1485 ratios and gene expression levels in female and male five-week-old mummichog. Eggs
1486 were fertilized, collected and incubated as per section 2.2 and reared as per section 2.3.
1487 Twenty-eight mummichog were randomly sampled at five-weeks-old from a filtered tank
1488 as per section 2.4.

1489 ### 2.6.5 Experiment 2: EE_2 exposure design

1490 The purpose of this experiment is to establish how gonad morphology, sex ratios and
1491 gene expression levels were affected following EE_2 exposure after hatching for five
1492 weeks (Figure 2.6). Mummichog egg were fertilized, collected and incubated as
1493 previously described (Section 2.2). Three days before mummichog eggs hatched, the
1494 dosing and experimental tanks were pretreated with corresponding EE_2 treatment (Section
1495 2.6.5.1; Figure 2.7). After hatching, the fish were randomly allocated into one of nine
1496 experimental tanks and kept under the conditions listed in Section 2.3 (Figure 2.7). Tanks
1497 received a daily treatment renewal for five weeks until sampling.

1498

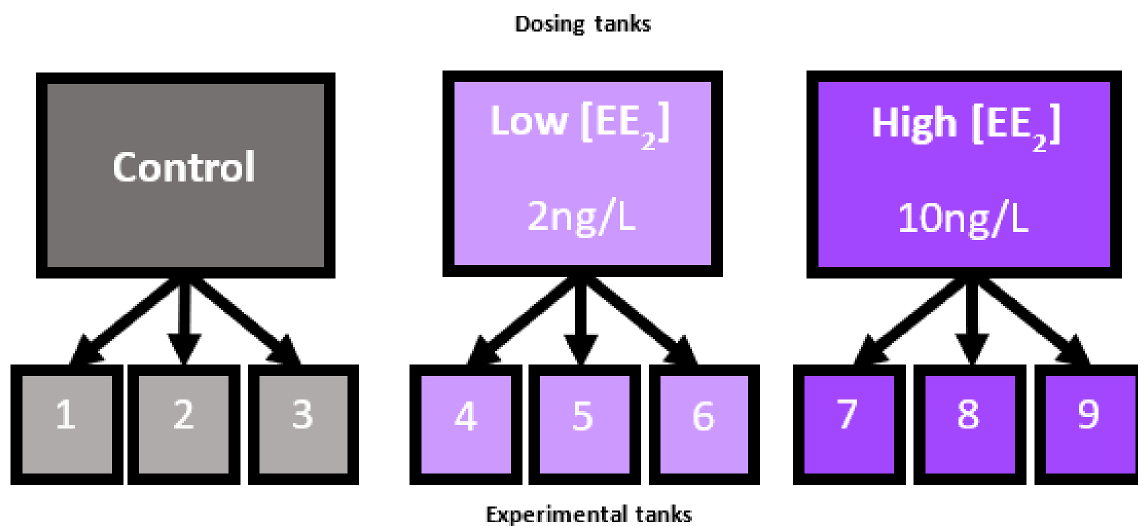


1499
1500 **Figure 2.6**: Experiment two timeline: Fertilized embryos were collected from a mesh
1501 filter in tanks holding male and female adult mummichog (*Fundulus heteroclitus*; section
1502 2.2). Eggs were incubated (grey arrow) for three weeks at 21°C and hatched all at once
1503 (H). On day one of 17α-ethinylestradiol (EE_2) exposure (purple arrow), freshly hatched

1504 yolk-sac larvae were randomly allocated into 1 of 9 experimental tanks, totaling 60 per
1505 tank. Fish were kept in their tanks, receiving daily treatment renewal from hatching to
1506 five weeks and were then sampled (S).

1507 **2.6.5.1 Treatments**

1508 A stock solution of EE_2 (Sigma Aldrich, Saint Louis, MO) was prepared in
1509 anhydrous ethanol (Commercial Alcohols, Brampton, ON) at 1 mg/mL and stored at -
1510 20°C. The working stocks for each treatment were designed so that a 0.5 mL aliquot of
1511 working stock would reach a desired final EE_2 concentration in 15 L of salt water in the
1512 dosing aquaria. Final treatment concentrations were intended to be 0 ng/L (control), an
1513 environmentally-relevant concentration: 10 ng/L, and a supraphysiological high
1514 concentration: 100 ng/L. Actual exposure concentrations were measured to be 0, 2 and 10
1515 ng/L EE_2. Treatment was renewed in the experimental tanks daily. After removing 80%
1516 of the day-old treatment out of each experimental tank, 4.5 L of fresh treatment was then
1517 added into the corresponding experimental tank (Figure 2.7).



1518

1519 **Figure 2.7:** Experiment 2 treatment experimental design. Each dosing tank was filled
1520 with 15 L of saltwater then mixed with 0.5 ml of ethanol and or the corresponding 17α-
1521 ethinylestradiol (EE_2) treatment. Each experimental tank contained randomly allocated
1522 mummichog (*Fundulus heteroclitus*) kept under standard laboratory conditions. During
1523 daily water changes, 80% of the treatment water in the experimental tank was removed

1524 with a siphon, freshly mixed treatment from the dosing tank was slowly added to the
1525 experimental tanks. Measured final exposure concentrations were 0, 2 and 10 ng/L in the
1526 control, low concentration and high concentration treatments, respectively.

1527 2.6.5.2 Treatment water sampling

1528 Water samples (500 mL) were taken weekly to determine the EE_2 concentration
1529 using liquid chromatography with tandem mass spectrometry (LC MS/MS; Servos lab,
1530 University of Waterloo, Waterloo, ON). Water samples were stabilized using 1 g/L
1531 sodium azide (Sigma Aldrich) and 50 mg/L of ascorbic acid (Sigma Aldrich). Upon
1532 arrival at the Servos lab, samples were filtered, extracted, dried using nitrogen gas and
1533 stored at -20°C until analysis using Agilent autosampler with a cooled sample tray,
1534 Agilent 1260 binary pump LC, and Agilent 6460 triple quadrupole mass spectrometer MS
1535 (Agilent Technologies Inc., Santa Clara, CA; Srikanthan, 2019). Method detection limit
1536 was 0.5 ng/L.

1537 2.7 Statistics

1538 Statistical analyses were conducted using SPSS (IBM, Inc.) computer software.
1539 Sex ratios and abnormal cavity counts (testis only) in control and treatment groups were
1540 evaluated using a Pearson's Chi^2. P-values $\leq$0.05 were considered statistically different
1541 from the null hypothesis. Both histological gametogenesis and degeneration of male and
1542 female mummichog were investigated separately for differences among treatments.
1543 Assumptions of normality and homogeneity of variance were tested using Shapiro-Wilk
1544 and Levene's tests, respectively. Data were $\log_{10}$ transformed and/or $\log_{10}(x+1)$ and re-
1545 tested when assumptions failed. A one-way ANOVA test was used to identify any
1546 differences in gonadal development (gametogenesis and degeneration) among EE_2
1547 treatments. Results showing significant differences (p-value$\leq$0.05) among treatments

1548 were followed by Tukey's post hoc test to identify the specific treatments with

1549 significantly different gonadal development. Data sets that failed Shapiro-Wilk and/or

1550 Levene's tests (p-value≥0.05), even after transformation, were evaluated with a non-

1551 parametric Games-Howell post hoc test for significant differences in gonadal

1552 development. Gene expression Cq values were normalized (ΔΔCq) to two reference

1553 genes, *βactin* and *efl1α*, on the Bio-Rad Gene study CFX Manager software. Assumptions

1554 of normality and homogeneity of variance were tested using Shapiro-Wilk and Levene's

1555 tests, respectively. Data were $\log_{10}$ transformed and retested when assumptions failed. A

1556 one-way ANOVA test was used to identify any differences in normalized gene expression

1557 among EE_2 treatments. Results showing significant differences (p-value≤0.05) among

1558 treatments were followed by Tukey's post hoc test to identify the specific treatments with

1559 significantly different normalized gene expression. Data sets that failed Shapiro-Wilk

1560 and/or Levene's test (p-value≥0.05), even after data transformations, were evaluated with

1561 a non-parametric Games-Howell post hoc test for significant differences in normalized

1562 gene expression among treatments.

1563 # 3.0 Results

1564 ## 3.1 Fish health

1565 The health of all fish in both experiments appeared normal during the five-week

1566 growth period. No obvious external signs of disease, malformation, or other health issues

1567 were observed. No significant differences in mortality were recorded among the groups.

1568 At most, one mortality was recorded in each tank.

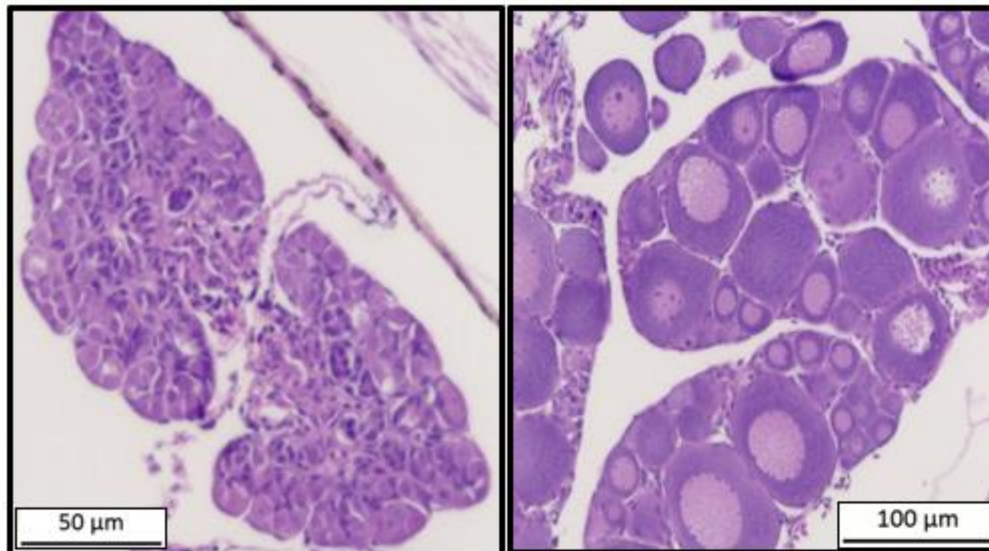
1569 ## 3.2 Experiment 1: Pilot study

1570 ### Sex ratio

1571 Based on histology (Figure 3.1), five-week-old mummichog were 46% female:54%

1572 male (12 female:14 male) that were sampled from a single tank (Table 3.1). No intersex

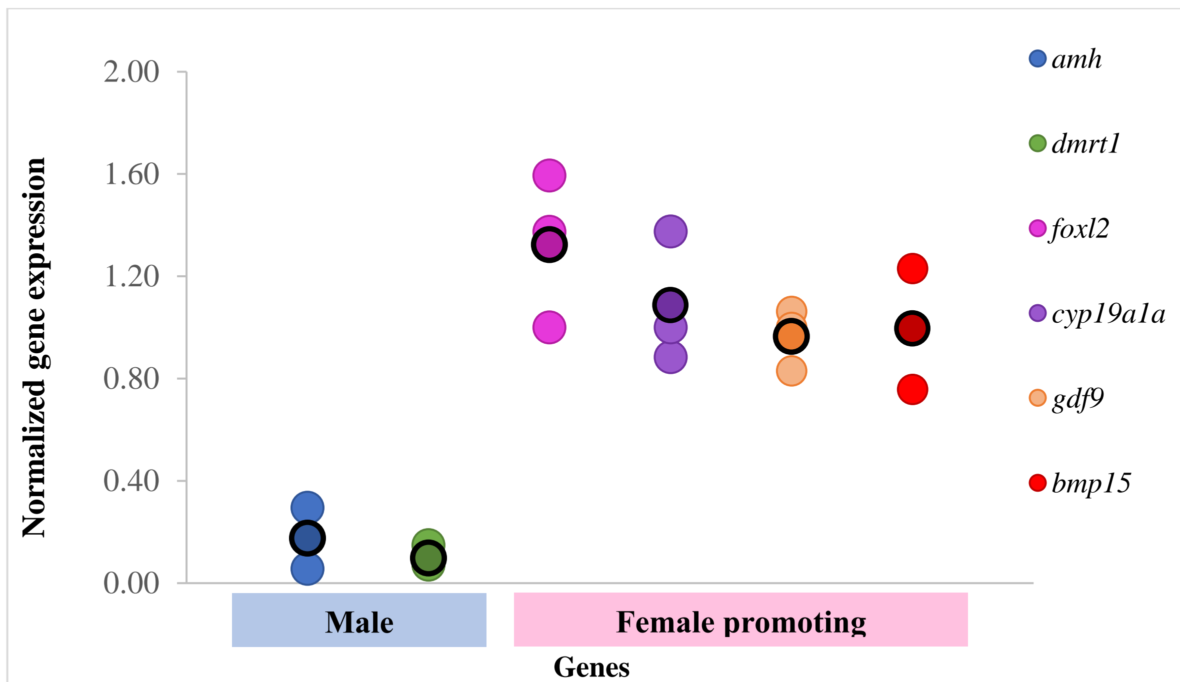
1573 fish were identified.



1574
1575 **Figure 3.1**: Photomicrograph of five-week-old mummichog (*Fundulus heteroclitus*)
1576 testes (left) and ovary (right) under light microscope with hemoxylin and eosin staining.
1577 Presence of testis or ovary on slide was used to determine sex of fish in Experiment 1 to
1578 understand the normal sex ratio of mummichog under standard laboratory conditions and
1579 to sex fish for gene expression analysis.
1580

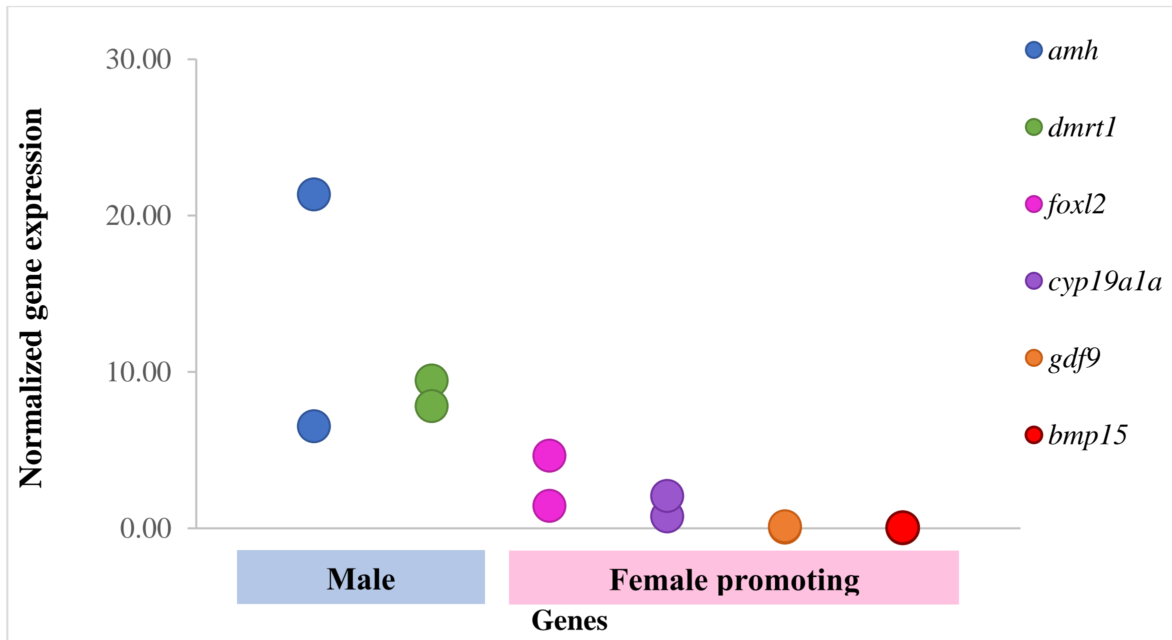
1581 **Gene expression**

1582 Samples were dissected and pooled based on histological sexing. All six genes of
1583 interest, *cyp19a1a*, *foxl2*, *gdf9*, *bmp15*, *dmrt1* and *amh*, were expressed in pooled
1584 mummichog ovaries (n=3 pools of 4 individuals; Figure 3.2). Normalized gene
1585 expression appears higher for female promoting genes compared to male promoting
1586 genes, although, no statistical analysis was conducted.



1587
1588 **Figure 3.2**: Normalized gene expression of *amh*, *dmrt1*, *foxl2*, *cyp19a1a*, *gdf9*, and
1589 *bmp15* from pooled five-week-old mummichog (*Fundulus heteroclitus*) ovaries under
1590 standard laboratory conditions. RNA (250ng) was used for cDNA synthesis. Gene
1591 expression was normalized to *β-actin* and *ef1a*. Circles without outline are actual
1592 normalized sample values, circles with black outline are mean normalized gene
1593 expression from RT-qPCR. N=3 pools containing ovaries from 4 individuals.

1594 Samples were dissected and pooled based on histological sexing (n=2 pools of testis
1595 from 7 individuals). All six genes of interest, *cyp19a1a*, *foxl2*, *gdf9*, *bmp15*, *dmrt1* and
1596 *amh,* were expressed in pooled mummichog testes (Figure 3.3). Normalized gene
1597 expression appears higher for male promoting genes compared to female promoting
1598 genes, although, appropriately, no statistical analysis was conducted



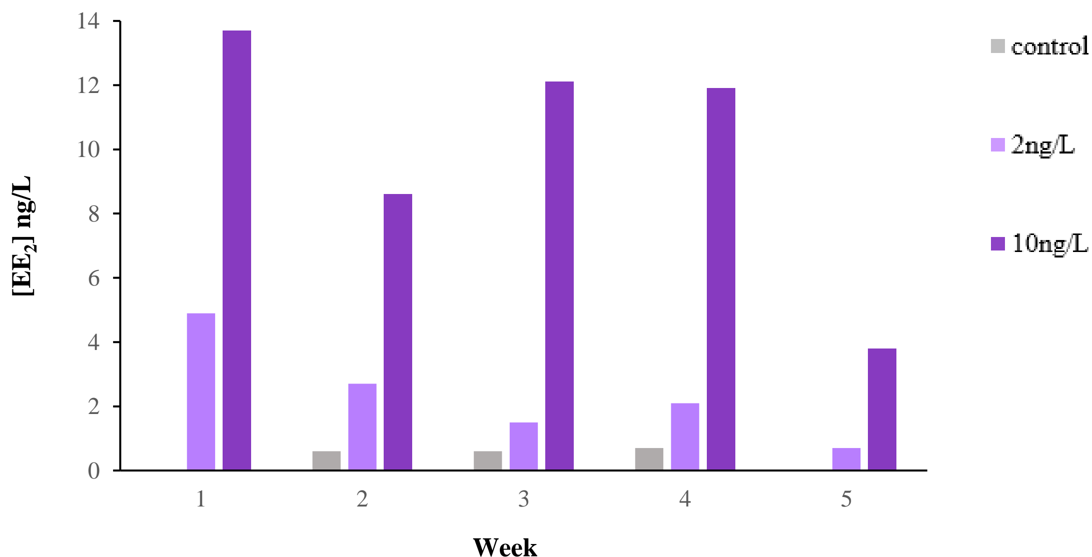
1599

1600 **Figure 3.3**: Normalized gene expression of *amh*, *dmrt1*, *foxl2*, *cyp19a1a*, *gdf9*, and
1601 *bmp15* from pooled five-week-old mummichog (*Fundulus heteroclitus*) testis (7) under
1602 standard laboratory conditions. 250 ng RNA was used for cDNA synthesis. Gene
1603 expression was normalized to *β-actin* and *ef1a*. N=2 pools of testis from 7 individuals.

1604 ## 3.3 Experiment 2: EE_2 exposure

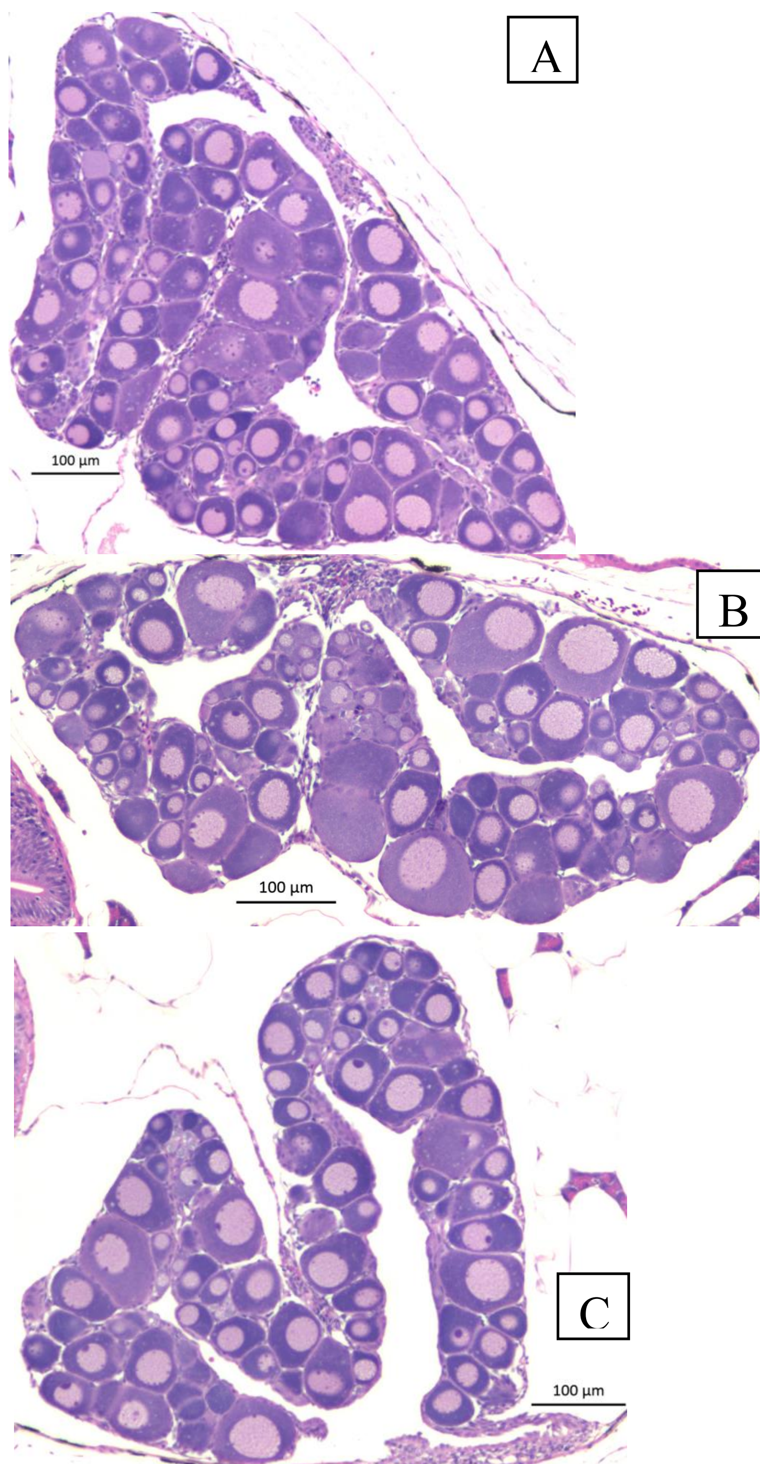
1605 ### EE_2 concentration

1606 Samples of treatment water (1 L) were taken from one replicate tank before water
1607 changes from each treatment weekly to verify EE_2 concentration over five weeks (Figure
1608 3.4). Average EE_2 concentration in each treatment was <0.5 ng/L (control), 2.34 ng/L
1609 (low) and 10.02 ng/L (high). EE_2 levels varied slightly each week, with a decline in EE_2
1610 concentration on week five in the high concentration treatment (3.8 ng/L). The control
1611 treatment contained low levels of EE_2 in weeks 2-4, with the highest amount detected in
1612 week 4 at 0.7 ng/L.



1613
1614 **Figure 3.4:** Treatment water 17α-ethinylestradiol (EE_2) concentration (ng/L) as
1615 determined LC/MS/MS analysis (method detection limit = 0.5 ng/L). Water samples (500
1616 ml) were obtained weekly from each treatment (one experimental replicate tank) to
1617 confirm treatment concentration for five weeks.

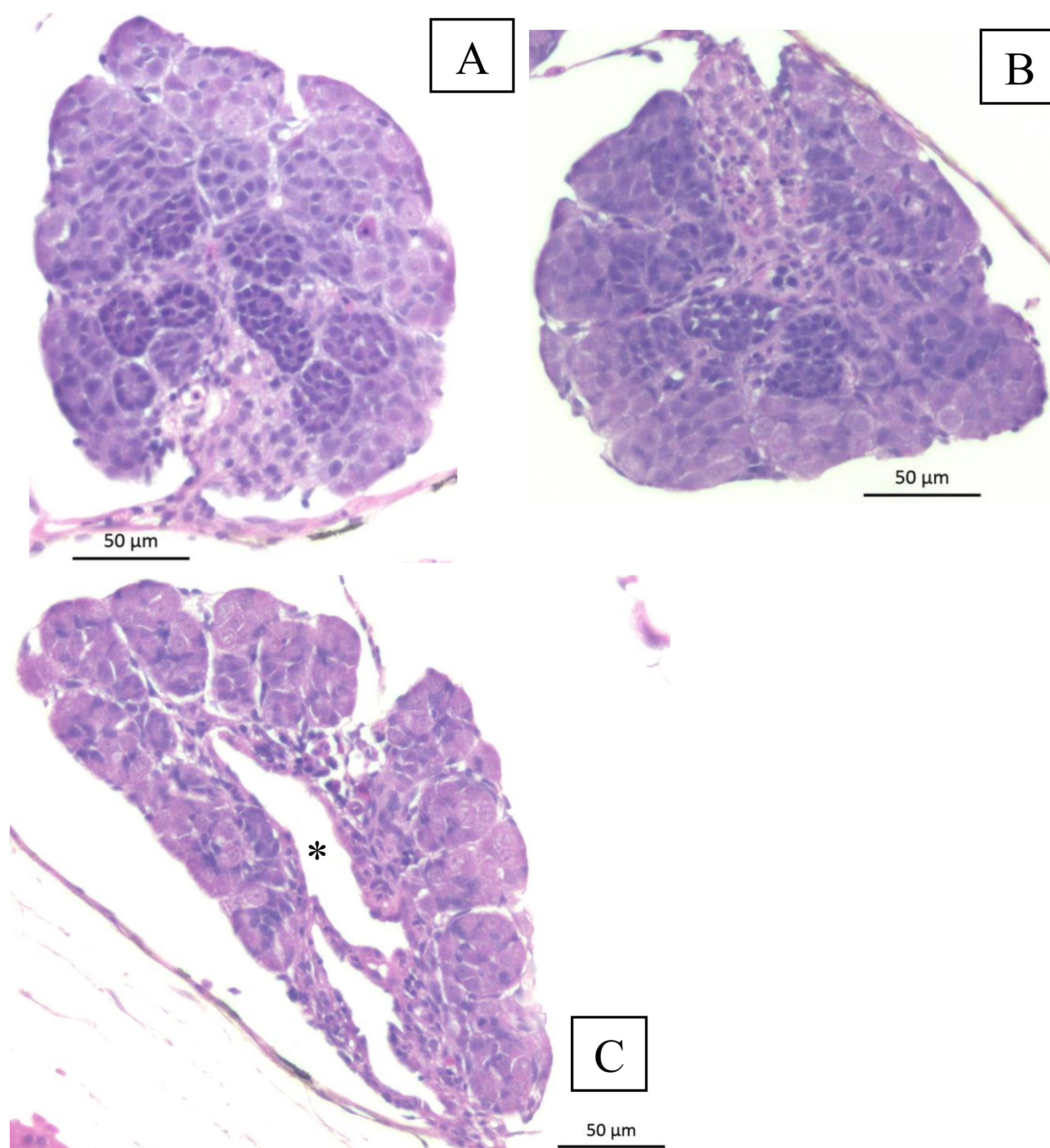
1618



1619

1620 **Figure 3.5:** Photomicrographs of cross sectioned five-week-old female mummichog
1621 (*Fundulus heteroclitus*) ovary exposed to daily renewal of 17α-ethinylestradiol (EE_2) for
1622 five-weeks post hatch under light microscope (10X) with hemoxylin and eosin staining
1623 (Experiment 2). EE_2 treatments are control (0 ng/L, A), low EE_2 concentration (2 ng/L;
1624 B), and high EE_2 concentration (10 ng/L; C).

1625



1626

1627 **Figure 3.6:** Photomicrographs of cross sectioned five-week-old male mummichog
1628 (*Fundulus heteroclitus*) testis exposed to daily renewal of 17α-ethinylestradiol (EE_2) for
1629 five-weeks post hatch under light microscope (20X) with hemoxylin and eosin staining
1630 (Experiment 2). EE_2 treatments are control (0 ng/L, A), low EE_2 concentration (2 ng/L;
1631 B), and high EE_2 concentration (10 ng/L; C) which is also demonstrating an abnormal
1632 cavity (*).

1633 **Sex ratio**

1634 Sex ratios did not change among EE_2 treatment in five-week-old mummichog (Table
1635 3.1). Mummichog sampled at five weeks in each treatment contained 53.8%: 46.2%: 0%
1636 (control; female: male: intersex), 46.2%: 53.8%: 0% (low EE_2 concentration), and 48.6%:
1637 51.4%: 0% (high EE_2 concentration). No intersex fish were found in five-week-old
1638 mummichog gonad in any of the EE_2 treatments. There were no significant differences in
1639 sex frequency with EE_2 treatment (Pearson's Chi^2 test; p-value 0.351).

1640 **Table 3.1**: Five-week-old mummichog (*Fundulus heteroclitus*) sex ratios (%) from
1641 Experiments 1 and 2. Fish sex classified histologically by presence of male or female
1642 gonadal tissue or intersexed gonad (both male and female gametes) or undefined (no
1643 gonad was found). Chi^2 results revealed no significant differences were found between
1644 male and female sex ratio percentages. No intersex fish were found in either experiment.
1645 Experiment 1 values are from 28 fish sampled from a single tank. Experiment 2 values
1646 are the mean ± SEM of three experimental tanks from three 17α-ethinylestradiol (EE_2)
1647 treatments; 0 ng/L (control, N=178), 2 ng/L (low EE_2 concentration, N= 179) and 10 ng/L
1648 (high EE_2 concentration, N=179). Histology slides that did not contain a gonad were
1649 excluded from analysis.

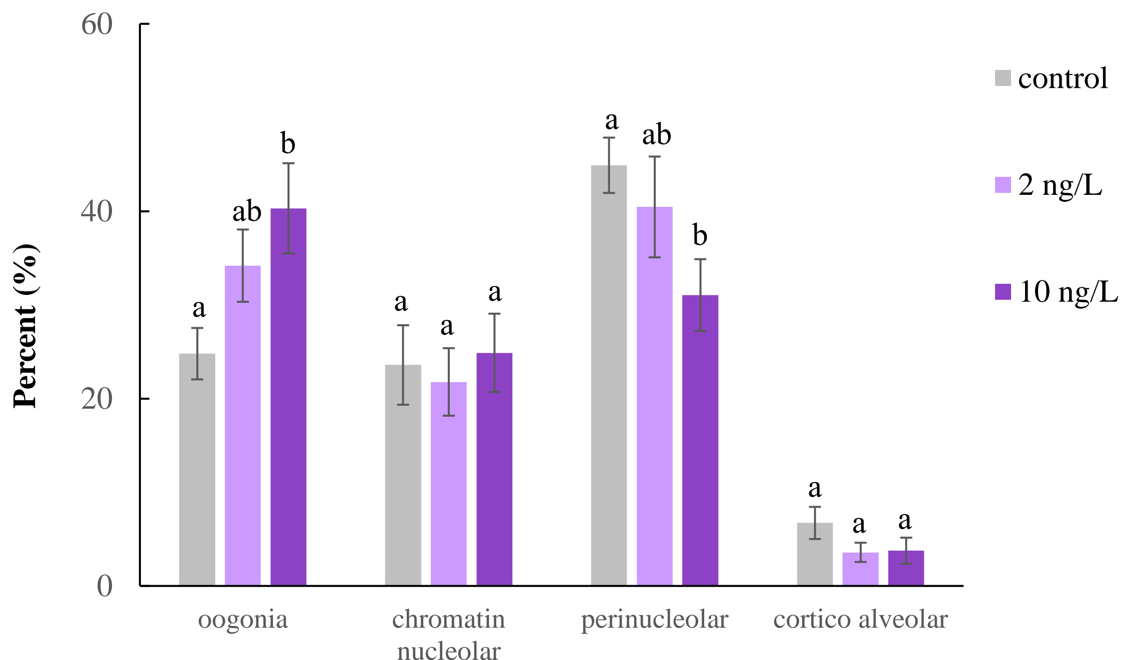
		Sex ratio percentage (%)			
Experiment #	**Treatment**	**Female**	**Male**	**Intersex**	**Undefined**
1	16ppt salt water	43.0	50.0	0	7.0
2	Control	51.1±2.8	43.8±2.2	0	5.1±1.0
	Low EE_2 concentration (2ng/L)	43.6±1.6	50.8±2.1	0	5.6±0.5
	High EE_2 concentration (10 ng/L)	47.0±2.2	49.6±4.3	0	3.4±2.6

1650

1651 **Female gonadal development**

1652 ***Gametogenesis***

1653 All four stages of developing oocytes (oogonia, chromatin nucleolar, perinucleolar
1654 and cortico alveolar) were identified in all three treatments (Figure 3.5 and 3.7). Oogonia
1655 percentages increased from 25% in the control treatment to 34% in the low EE_2
1656 concentration treatment and significantly increased to 40% in the high EE_2 concentration
1657 treatment (p-value 0.03). Perinucleolar oocyte percentages showed a decreasing trend
1658 with 45% in the control, 40% in the low EE_2 concentration treatment and significantly
1659 decreased to 31% in the high EE_2 concentration treatment (p-value 0.031). There were no
1660 significant differences in percentage of chromatin nucleolar and cortico alveolar oocytes
1661 among treatments (chromatin nucleolar p-value 0.861, cortico alveolar p-value 0.225).



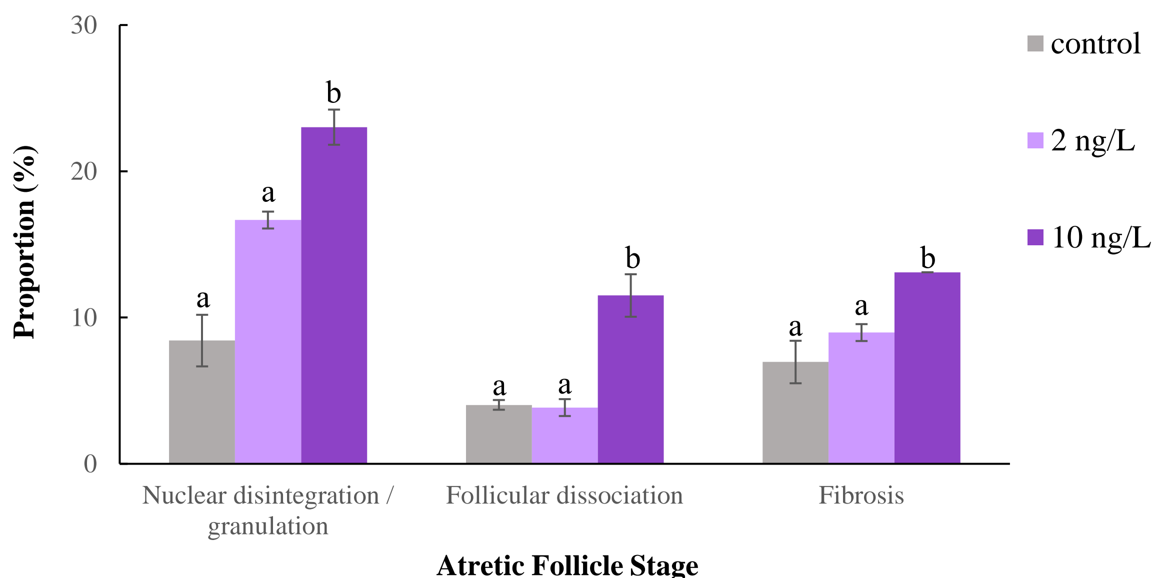
1662
1663 **Figure 3.7**: Distribution of early developmental stage oocytes from five-week-old
1664 mummichog (*Fundulus heteroclitus*) ovary exposed to daily renewal of control (0 ng/L),
1665 low (2 ng/L) and high (10 ng/L) concentrations of 17α-ethinylestradiol (EE_2) from
1666 hatching. From left to right, oocyte stages displayed are in order of earliest to latest in

1667 development. Values displayed are mean percentages ± SEM from 10 randomly selected
1668 histology slides per treatment. Letters designate statistical differences (one-way ANOVA
1669 and Games-Howell post hoc test; p-value≤0.05).

1670 ***Degeneration***

1671 All categories of atresia (nuclear disintegration/granulation, follicular dissociation,
1672 and fibrosis) were observed in all three treatments of EE_2 (Figure 3.8). Nuclear
1673 disintegration / granulation proportions were 8.4 (control), 16.7 (low EE_2 concentration
1674 treatment), and 23.0 % (high EE_2 concentration treatment) indicating increasing nuclear
1675 atresia with treatment (p-value 0.002). Follicular dissociation proportions were 4.0
1676 (control), 3.9 (low EE_2 concentration treatment), and 11.5 (high EE_2 concentration
1677 treatment) indicating increasing follicular dissociation atresia with treatment (p-value
1678 0.004). Fibrosis proportions were 7.0 (control), 9.0 (low EE_2 concentration treatment),
1679 and 13.1 (high EE_2 concentration treatment) indicating increasing fibrosis atresia with
1680 treatment (p-value 0.021). There were no significant differences of atretic follicle counts
1681 between control and low EE_2 concentration treatment in any category.

1682



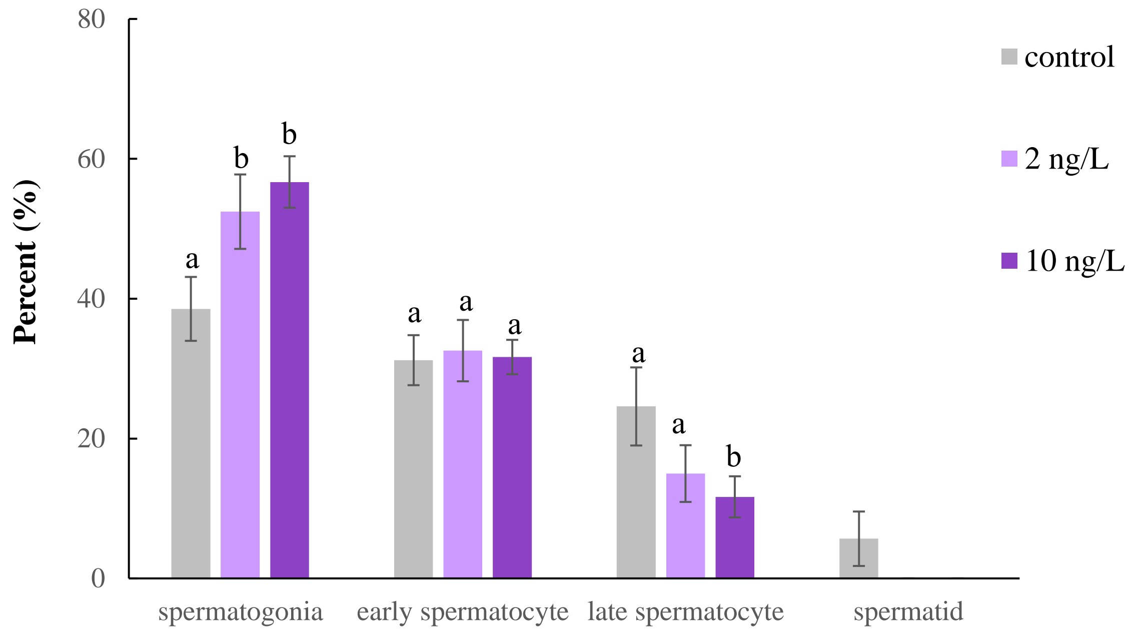
1683

1684 **Figure 3.8**: The proportion of different classifications of atretic follicles observed in five-
1685 week-old mummichog (*Fundulus heteroclitus*) ovary exposed to daily renewal of control
1686 (0 ng/L), low (2 ng/L) and high (10 ng/L) concentration of 17α-ethinylestradiol (EE_2)
1687 from hatching. Atretic follicles stages include nuclear disintegration/granulation,
1688 follicular envelope dissociation, and fibrosis. Values displayed are mean ± SEM counts of
1689 atretic follicles from all three treatments. Letters designate statistical differences (Tukey's
1690 post hoc test; p-value≤0.05). Ovary sample numbers are 91, 78 and 84 in the control, low
1691 and high EE_2 concentration groups respectively.

1692 ## Male gonadal development

1693 ### *Gametogenesis*

1694 The three earliest stages of developing sperm cells, spermatogonia, early
1695 spermatocyte, and late spermatocyte, were identified in all three treatments (Figure 3.6
1696 and 3.9). Spermatids (the most advanced sperm developmental stage observed) were only
1697 found in control testes, therefore, no statistical analysis was conducted on this stage.
1698 There was a significant increase in spermatogonia percentage following EE_2 treatment,
1699 39% in control, 52% in the low EE_2 concentration and 57% in the high EE_2 concentration
1700 treatment (p-value 0.004). There was a significant decrease in late spermatogonia
1701 percentage through EE_2 treatment, 25% in control, 15% in the low EE_2 concentration and
1702 12% in the high EE_2 concentration treatment (p-value 0.039). There were no significant
1703 differences in percent early spermatocytes among treatments (p-value 0.935).

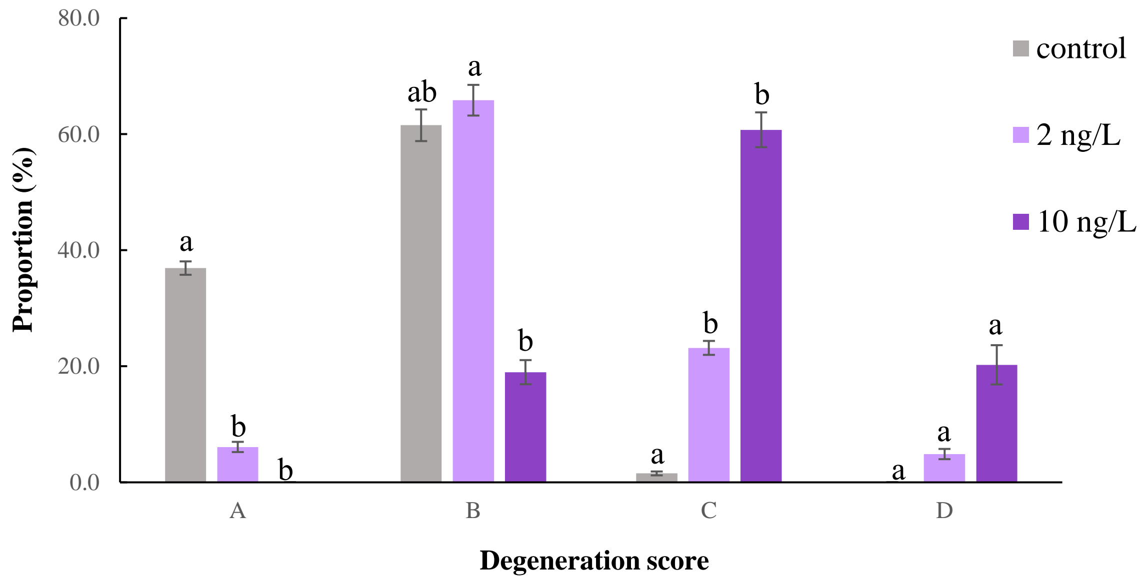


1704
1705 **Figure 3.9**: Distribution of early sperm developmental stages in five-week-old
1706 mummichog (*Fundulus heteroclitus*) testis treated with daily renewal of control (0 ng/L),
1707 low (2 ng/L) and high (10 ng/L) concentrations of 17α-ethinylestradiol (EE_2) from
1708 hatching. Early sperm cell stages in order from left to right in development. Letters
1709 designate statistical differences (Tukey's post hoc test; p-value≤0.05). Spermatid stage
1710 did not undergo statistical analysis as most of the testes examined did not contain
1711 spermatid cells. Random subset of testis samples used for analysis, N=16 from each
1712 treatment.
1713

Degeneration

All testis samples contained areas of degeneration, which were then categorized (Figure 3.10; A;<20%, B; 20-40%, C; 40-60%, D; >60%). The proportion of testis with <20% degeneration was 36.9% in the control, 6.1% in the low EE_2 concentration treatment, and 0.0% in the high EE_2 concentration treatment indicating significantly lower degeneration in the control group (p-value 0.001). The proportion of testis with 20-40% degeneration was 61.5% in the control, 65.9% in the low EE_2 concentration treatment, and 19.0% in the high EE_2 concentration treatment (p-value 0.028). The proportion of testis with 40-60% degeneration was 1.5% in the control, 23.2% in the low EE_2 concentration treatment, and 60.8% in the high EE_2 concentration treatment indicating significantly higher degeneration in the high concentration treatment (p-value 0.003). The proportions of testis with >60% degeneration was 0.0% in the control, 4.9% in the low EE_2 concentration treatment, and 20.3% in the high EE_2 concentration treatment (p-value 0.231). There were no statistical differences among treatments in category D, which is likely due to the low values and high variation among replicate tanks.

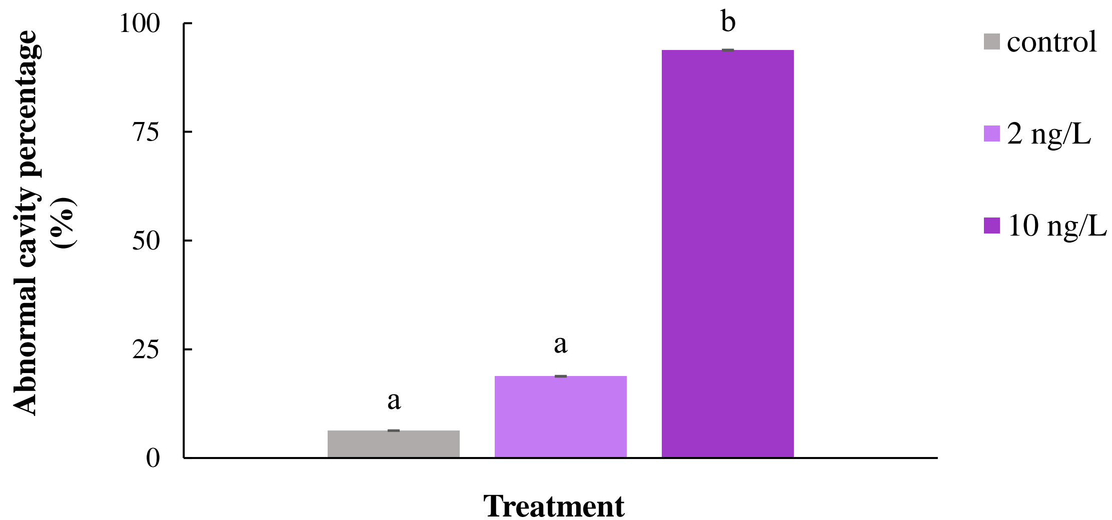
1730



1731
1732 **Figure 3.10**: Average degeneration score in five-week-old mummichog (*Fundulus*
1733 *heteroclitus*) testis exposed to daily renewal of control (0 ng/L), low (2 ng/L), and high
1734 (10 ng/L) concentration of 17α-ethinylestradiol (EE_2) after hatching. Degeneration scores:
1735 A≤ 20%, B= 20-40%, C= 40-60%, D≥ 60% degeneration calculated by dividing the total
1736 area of necrotic and hypertrophic interstitial tissue area by the whole testis area using
1737 ImageJ image processing software. Values displayed are mean proportions ± SEM of
1738 testis samples in each degeneration score category from all testis samples. Letters
1739 designate statistical differences (Tukey's post hoc test used for scores A and B; Games-
1740 Howell post hoc test used for scores C and D; p-value≤0.05). Number of testis examined
1741 were 78, 91 and 89 in the control, low and high EE_2 concentration groups, respectively.

1742 ***Abnormal Cavity***

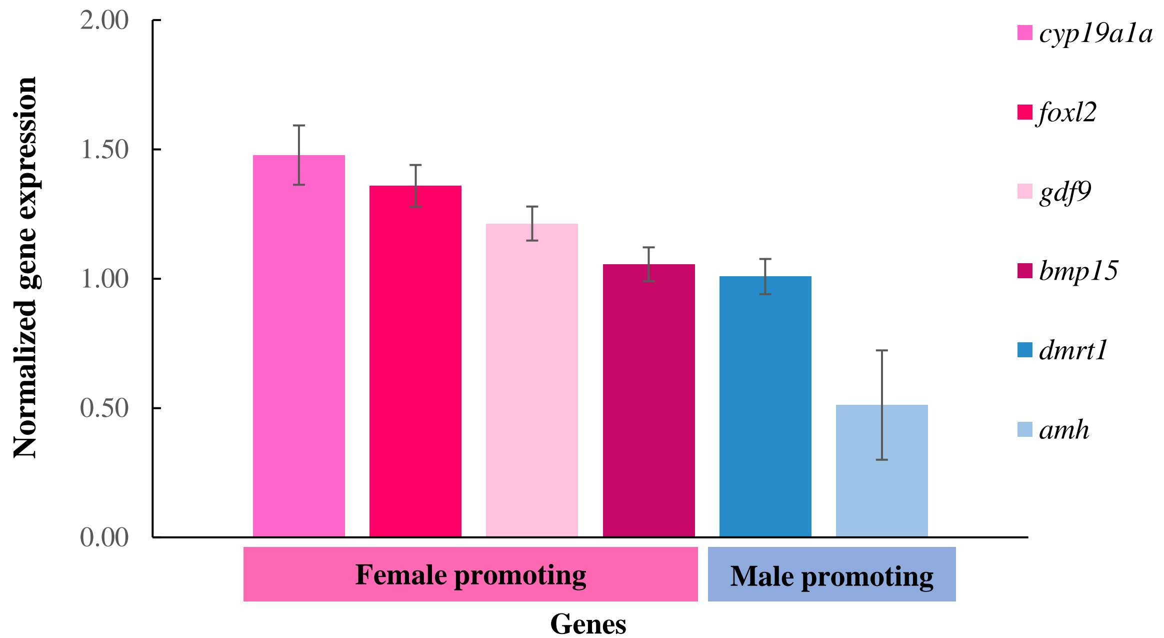
1743 Male mummichog contained an abnormal cavity, indicating a morphological
1744 irregularity following EE_2 treatment (Figure 3.10). The presence of an abnormal cavity in
1745 the testis tissue calculated from a subset (n=16) of samples showed 6% in control, 18% in
1746 low EE_2 concentration treatment and 94 % of high EE_2 concentration treatment, which
1747 was statistically higher among treatments (p-value < 0.001; Pearson's Chi^2; Figure 3.11).



1748
1749 **Figure 3.11:** Proportion of abnormal cavities found in five-week-old mummichog
1750 (*Fundulus heteroclitus*) testes exposed to daily renewal of control (0 ng/L), low (2 ng/L)
1751 and high (10 ng/L) 17α-ethinylestradiol (EE_2) concentration treatments for five weeks
1752 after hatching. Letters designate statistical differences (Pearson's chi square test; p-
1753 value≤0.05). N = 16.

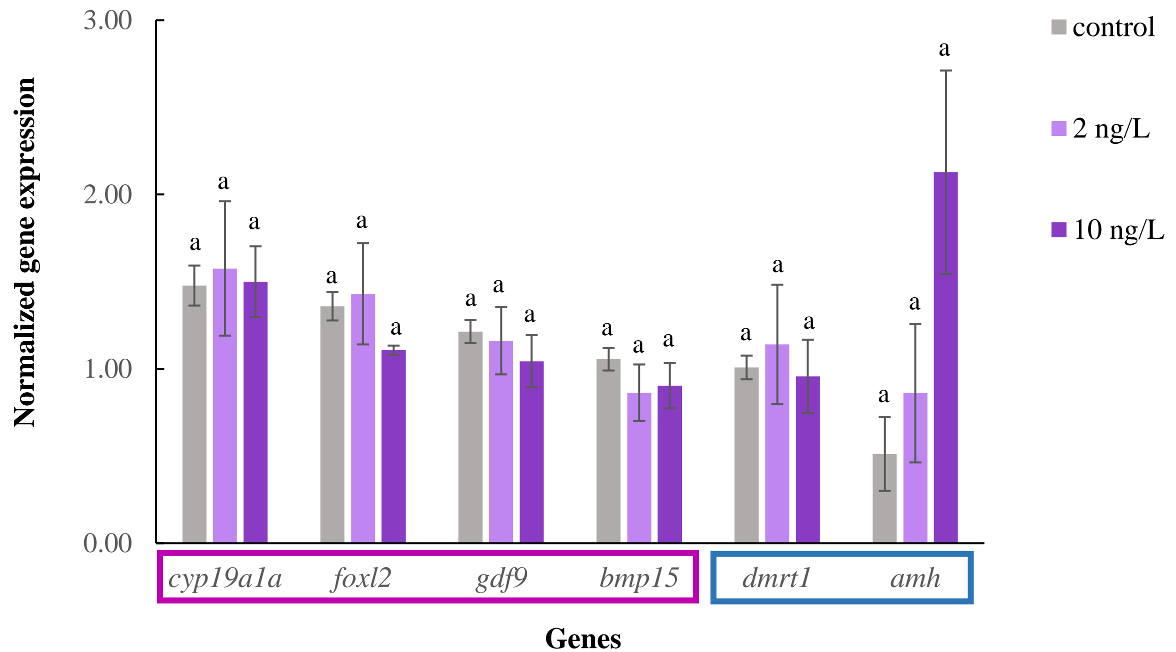
1754 **Ovarian gene expression**

1755 Pooled ovary samples contained four randomly dissected ovaries from the same tank.
1756 Three pseudoreplicate samples were used from each tank (n=3 pools of 4 individuals).
1757 When Experiment 2 control gene expression (Figure 3.12) is compared with the pilot
1758 study (Figure 3.2) ovary results, normalized gene expression appeared similar except for
1759 *dmrt1*, which was higher than expected. Appropriately, no statistical analysis was
1760 conducted to compare between the two experiments.



1761
1762 **Figure 3.12:** Normalized gene expression of *cyp19a1a*, *foxl2*, *gdf9*, *bmp15*, *dmrt1* and
1763 *amh* from pooled five-week-old mummichog (*Fundulus heteroclitus*) ovaries under
1764 standard laboratory conditions. Results displayed are control treatment from Experiment
1765 2. RNA (250 ng) was used for cDNA synthesis. *β-actin* and *ef1a* used as reference genes.
1766 Values displayed are mean ± SEM from RT-qPCR. N=3 pools of 4 individuals.

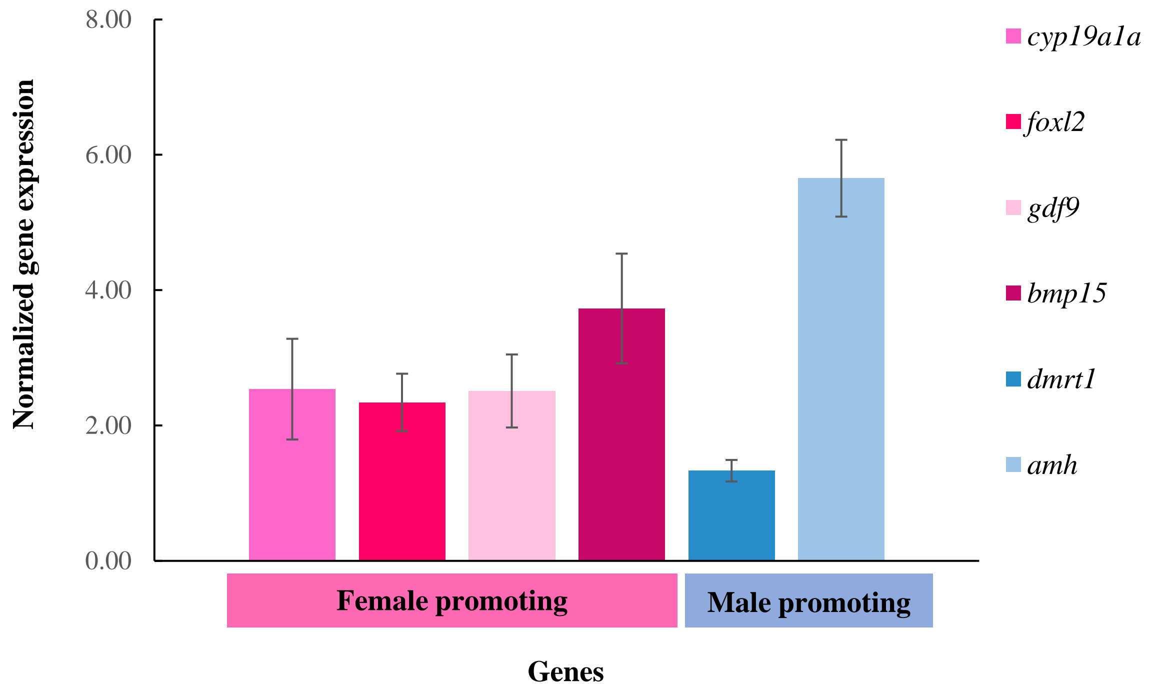
1767 There were no differences in expression due to EE_2 treatment (Figure 3.13). One-way
1768 ANOVA results indicate that there were no differences in ovarian *cyp19a1a* (p-value
1769 0.962), *foxl2* (p-value 0.400), *gdf9* (p-value 0.716), *bmp15* (p-value 0.555), *dmrt1* (p-
1770 value 0.855) or *amh* (p-value 0.079) gene expression among EE_2 treatments (Figure 3.13).



1771
1772 **Figure 3.13**: Normalized gene expression of gonadal differentiating genes in pooled five-
1773 week-old mummichog (*Fundulus heteroclitus*) ovaries from daily renewal of control (0
1774 ng/L), low (2 ng/L) and high (10 ng/L) 17α-ethinylestradiol (EE_2) concentration
1775 treatments from hatching. RNA (250 ng) was used to generate cDNA for RT-qPCR, N=3
1776 pools of 4 individuals. Gene expression normalized to *β-actin* and *ef1a.* Letters designate
1777 significant differences in gene expression among treatments (one-way ANOVA).

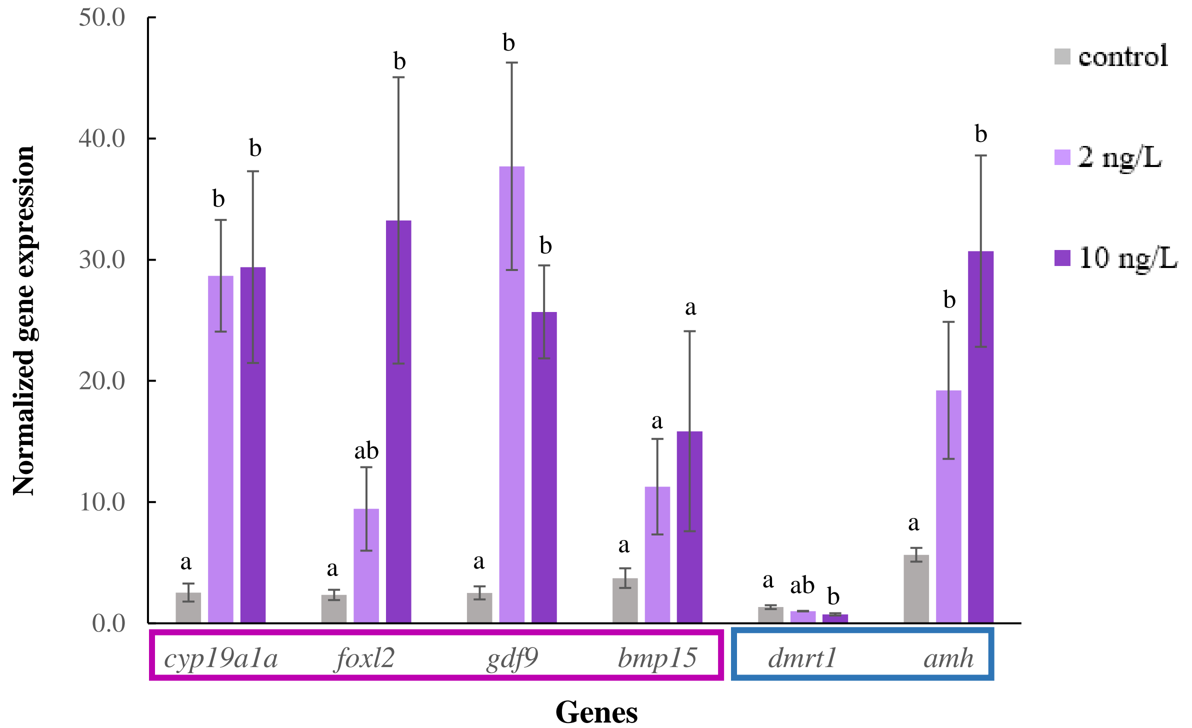
1778 **Testis gene expression**

1779 Pooled testes samples contained eight randomly dissected testes from the same tank.
1780 Three pseudoreplicate samples were used from each tank (n=3 pools of 8 individuals).
1781 When Experiment 2 control gene expression (Figure 3.14) is compared with the pilot
1782 study (Figure 3.3) results, normalized gene expression appeared similar except for *dmrt1*,
1783 which was lower than expected. Appropriately, no statistical analysis was conducted
1784 between experiments.



1785
1786 **Figure 3.14**: Normalized gene expression of *cyp19a1a*, *foxl2*, *gdf9*, *bmp15*, *dmrt1* and
1787 *amh* from pooled five-week-old mummichog (*Fundulus heteroclitus*) testes (8) under
1788 standard laboratory conditons. Results displayed are control treatment from Experiment 2.
1789 RNA (250 ng) used for cDNA synthesis. *β-actin* and *ef1a* used as reference genes. Values
1790 displayed are mean ± SEM from RT-qPCR. N=3 pools of 8 individuals.

1791 Most genes showed significant differences in normalized expression following EE_2
1792 treatment (Figure 3.15). One-way ANOVA results indicated that EE_2 treatment
1793 significantly increased gene expression in *cyp19a1a* (p-value 0.006), *foxl2* (p-value
1794 0.049), *gdf9* (p-value <0.001) and *amh,* (p-value 0.003) while *dmrt1* gene expression was
1795 significantly decreased (p-value 0.014). There were no significant differences in *bmp15*
1796 gene expression among treatments (p-value 0.136), although an increasing trend was
1797 observed.



1798
1799 **Figure 3.15:** Normalized gene expression of gonadal differentiating genes in pooled five-
1800 week-old mummichog (*Fundulus heteroclitus*) testes from daily renewal of control (0
1801 ng/L), low (2 ng/L) and high (10 ng/L) 17α-ethinylestradiol (EE_2) concentration
1802 treatments from hatching. RNA (250 ng) was used to generate cDNA for RT-qPCR, N=3
1803 pools containing testis from 8 individuals. Gene expression normalized to *β-actin* and
1804 *ef1a.* Letters designate significant differences in gene expression among treatments (one-
1805 way ANOVA, Tukey's post hoc test; p-value≤0.05)
1806

4.0 Discussion

This study is the first of its kind to investigate SD and GD of mummichog as an early life stage estuarine model. Mummichog exposed to environmentally-relevant concentrations of EE_2 for five weeks post-hatch did not show any feminization; however, females demonstrated inhibited gametogenesis and increased follicular atresia. Male mummichog demonstrated inhibited gametogenesis, increased area of necrosis and hypertrophic interstitial tissue while also displaying an abnormal cavity. The expression of genes that have been associated in SD and GD mechanisms in other fish (*cyp19a1a*, *foxl2*, *gdf9*, *bmp15*, *dmrt1*, and *amh*) were measured by RT-qPCR. Gene expression in five-week-old female mummichog were not affected by EE_2. In five-week-old males, expression of *cyp19a1a*, *foxl2*, *gdf9* and *amh* were upregulated while *dmrt1* was downregulated by EE_2 exposure.

4.1 Environmentally-relevant EE_2

The LC/MS/MS analysis showed that the actual EE_2 concentrations after 24 h in this experiment were 0 ng/L (control), 2 ng/L (low concentration treatment) and 10 ng/L (high concentration treatment), with a larger decline in EE_2 levels during week five. While these concentrations are environmentally-relevant, they were lower than expected. Working stock concentrations were designed for target EE_2 treatments to be 10 (low concentration) and 100 ng/L (high concentration). EE_2 can be lost during an experiment to fish uptake, microbial activity, photodegradation, and/ or adhesion to the aquarium/ equipment used (Bjorkblom et al., 2009). To account for EE_2 loss, this experiment pretreated all equipment for three days before fish were added to the system as well as replenished EE_2 treatments daily with an 80% water change. There was likely EE_2 loss to

1830 equipment used as well as loss to fish absorption. Since EE_2 is hydrophobic, and has a
1831 half-life of 33 ± 13 h, it can be difficult maintaining a consistent concentration throughout
1832 experimentation (Roggio et al., 2014). As an example, one study that renewed various
1833 EE_2 treatments (0, 10, 75 and 150 ng/L) daily for five male fish in a 5 L aquarium found
1834 ~50% loss over the 24 h period (Roggio et al., 2014), so it is not surprising that EE_2 loss
1835 was significant in this experiment with 60 fish per tank. Previous studies on fathead
1836 minnow (Lange et al., 2001), three spined stickleback (Bjorkblom et al., 2009) and
1837 mummichog (Hogan et al., 2010) have shown that nominal concentrations versus actual
1838 concentrations for EE_2 can vary between 50 and 90%. The current results indicate an 85-
1839 95% treatment loss over a 24 h period. Further precautions should be taken in the future
1840 to account for this loss; an experimental design with a lower fish density may help
1841 prevent such drastic EE_2 loss; as well, a treatment flow-through protocol can help
1842 maintain a constant desired concentration (Al-Ansari et al., 2013; Bjorkblom et al., 2009).
1843 The final exposure concentrations were determined to be environmentally-relevant. A
1844 recent review showed EE_2 levels from 32 countries to range from 0-33 ng/L (Tang et al.,
1845 2021). Earlier studies have measured EE_2 levels in Canadian water to be in similar ranges
1846 compared to this study; 0.1-10 ng/L EE_2 were reported in sewage effluents (Desbrow et
1847 al., 1998; Ternes et al., 1999). Studies using environmentally-relevant concentrations of
1848 EE_2 during development in the fathead minnow, zebrafish, medaka among others, have
1849 induced a variety of histological gonadal effects including changes to sex ratios (Table
1850 1.2 and 1.3). Previous studies using developing mummichog have not demonstrated these
1851 changes using environmentally-relevant EE_2 concentrations, instead >10 ng/L was
1852 required to cause physiological changes (Peters et al., 2010). The results from the current

1853 study are the first to report physiological changes in the mummichog to environmentally-

1854 relevant concentrations of EE_2.

1855 4.2 Sex ratios

1856 This study confirmed that under standard laboratory conditions normal SD

1857 mechanisms in mummichog lead to a 50:50 sex ratio. Similar results have been

1858 documented in other studies investigating mummichog sex ratios under standard

1859 laboratory conditions (Chehade, 2012; Peters et al., 2007; Urushitani et al., 2002).

1860 Aqueous EE_2 exposure at environmentally-relevant concentrations of newly-hatched fish

1861 for five weeks did not alter sex ratios in the current experiment. In previous studies, EE_2

1862 skewed the sex ratio of mummichog toward female when exposure began before hatching

1863 (Chehade, 2012; Peters et al., 2010). Nominal exposure concentration of 100 ng/L EE_2 for

1864 61 weeks post hatch (wph), beginning with reproducing adults through fertilization,

1865 embryo, larval and juvenile stages, skewed histological sex toward female (86%; Peters et

1866 al., 2010). In a later study (Chehade, 2012), mummichog were exposed to various EE_2

1867 treatments after fertilization and continuously for 10 wph; juvenile fish sampled at 5 wph

1868 were skewed 90% female in 10 ng/L EE_2 and 100% female at 50 and 250 ng/L EE_2. The

1869 lack of a feminizing effect in the current experiment could be due to the timing of the EE_2

1870 exposure. In the current experiment, yolk-sac larvae were exposed <24 h after hatching

1871 then were continually exposed to a daily renewal of EE_2 treatment for 5 weeks; this

1872 experimental design is different from the previous studies where EE_2 treatment started at

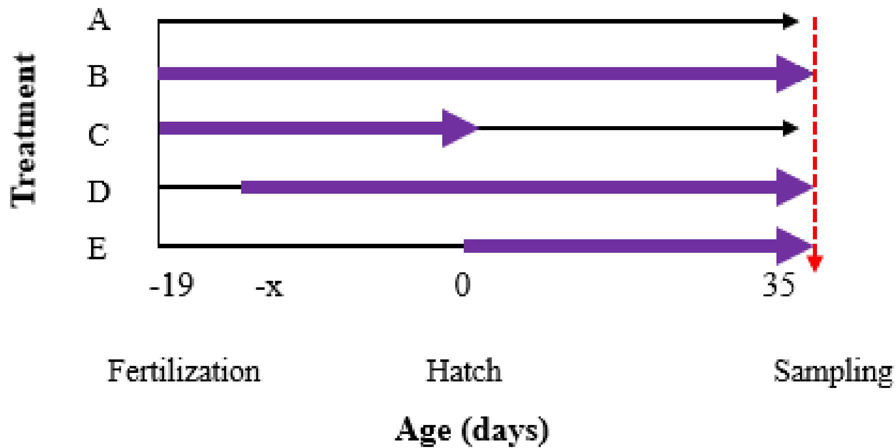
1873 fertilization. There may be an important window of sensitivity in mummichog

1874 development to exogenous estrogen. The timing of chemical exposure before gonadal

1875 differentiation is the most influential for fish gonadal sex (Devlin and Nagahama, 2002;

1876 Piferrer, 2001). As an example, medaka eggs injected with EE_2 produced sex reversal in
1877 genetic males (Papoulias et al., 1999), while another study demonstrated that exposing
1878 medaka two days after hatching led to intersex condition, with no sex reversal (Balch et
1879 al., 2004). Normal mummichog gonadal differentiation has been reported to occur two
1880 weeks after hatching (Chehade, 2012; Shimizu et al., 2008); however, exposure during
1881 this period at waterborne concentrations of 2 and 10 ng/L did not alter gonadal sex in the
1882 current study. Whether this is due to exposure concentration and/or exposure only
1883 occurring post-hatch is not known; the sex-determining mechanisms in mummichog
1884 could be initiated prior to hatch.

1885 There is high variation in sex-determining mechanisms among fish species
1886 (Nagahama et al., 2021). The SD mechanisms in mummichog are currently unknown and
1887 may, as in other species, occur during embryogenesis (i.e., prior to hatching). In the
1888 zebrafish, brain *cyp19a1b* or aromatase B has been identified as a potential sex-
1889 determining mechanism; it is directly influenced during embryogenesis by activation of
1890 the estrogen receptor (Leet et al., 2011; Trant et al., 2001). Future EE_2 exposure
1891 experiments in mummichog should include the embryonic developmental stage from
1892 fertilization to provide further insight on the window of sensitivity in mummichog most
1893 susceptible to EE_2. To differentiate among mechanisms activated during embryogenesis
1894 and post-hatch, exposures that include: fertilization and embryo; fertilization, embryo and
1895 post-hatch; embryo and post-hatch; and post-hatch only could be performed (Figure 4.1) -
1896 a similar approach was conducted by Van Aerle et al. (2002). Understanding the
1897 sensitivity of mummichog at various developmental stages to EE_2 could provide valuable
1898 insight into SD and GD mechanisms.



1899

1900 **Figure 4.1:** Proposed exposure experiment to understand window of sensitivity in a
1901 developing mummichog to 17α-ethinylestradiol (EE_2). All treatment group eggs will be
1902 artificially fertilized (day -19), incubated for three weeks to hatch (day 0) and then
1903 sampled (day 35). The different treatments are denoted with letters on the left which will
1904 differ based on exposure time to EE_2 (purple arrow). Treatment A represents a control
1905 group with no EE_2 exposure. Treatment B represents treating embryos right at
1906 fertilization and continuously until sampling. Treatment C represents treating embryos at
1907 fertilization until hatching. Treatment D represents exposing fish during embryonic
1908 development, in between fertilization and hatching (-x), and continuously until sampling.
1909 Treatment E represents treating yolk-sac larvae after hatching until sampling.

1910 4.3 Intersex

1911 This experiment did not find intersex in either male or female mummichog
1912 developing normally or under the influence of EE_2. Similar results were observed in
1913 early-life stage estrogen exposure studies using mummichog (E_2; Urushitani et al. 2002,
1914 EE_2; Chehade 2012, Shimizu et al. 2008). These results further demonstrate that
1915 mummichog have a unique resistance to the intersex condition (Rutherford et al., 2020).
1916 Because intersex is commonly found in other species, such as the zebrafish (Nash et al.,
1917 2004), fathead minnow (Lange et al., 2001) and medaka (Metcalfe et al., 2001) exposed
1918 to estrogenic EDCs (Dietrich and Krieger, 2009), the current results confirm that
1919 mummichog do not make an ideal model for intersex studies in fish.

1920 Typically, intersex, or testis-ova, is observed in male fish that have been exposed to
1921 estrogenic compounds; the female steroid, or mimic, is capable of inducing the female
1922 pathway (Dietrich and Krieger, 2009). Testis-ova has been associated with increased
1923 *cyp19a1a* expression, E_2, FSH and LH in the tilapia (Ibor et al., 2016). While testis
1924 *cyp19a1a* expression was increased in males in the present study, it was not linked to
1925 intersex. In a separate study using least killifish, researchers found that 5 ng/L EE_2
1926 treatment caused higher incidences of intersex than the 25 ng/L treatment (Jackson et al.,
1927 2019). The suggestion was that the EE_2 caused a downregulation of receptor binding and
1928 reduced the overall intersex impact (Jackson et al., 2019). The mechanism explaining
1929 why mummichog are resistant, or why other fish are more susceptible, to intersex is
1930 currently unknown. There is a bias in intersex studies; approximately 84% of studies are
1931 using freshwater models while the remainder are estuarine and marine models (Abdel-
1932 Moneim et al., 2015). One estuarine model, the thicklip grey mullet (*Chelon labrosus*),
1933 has been reported to display intersexed gonads in contaminated waters containing E_2,
1934 EE_2, and other known estrogenic EDCs (Bizarro et al., 2014). Researchers found that
1935 adult intersexed thicklip grey mullet contained increased P450 aromatase activity and
1936 significantly higher transcripts of *cyp19a1a* than the normal males, which also closely
1937 matched the levels found in females (Sardi et al., 2015). These results indicated that the
1938 development of oocytes in the testis tissue is triggered by an increase of *cyp19a1a*
1939 expression and aromatase activity. Further investigation is warranted here since the
1940 current study found increased *cyp19a1a* expression in males yet reported no intersex. It
1941 would be advantageous for researchers to investigate this resistance to intersex by
1942 understanding other mechanisms of intersex in fish, especially estuarine models.

1943 ## 4.4 Abnormal cavity

1944 An unexpected finding in this experiment was the presence of an abnormal cavity in
1945 EE_2-treated male mummichog. A significant increase in abnormal cavity frequency was
1946 observed in the 10 ng/L EE_2 treatment. This observation has not been previously
1947 documented in male mummichog exposed to EE_2 or any other EDC. One study using
1948 fathead minnow found an abnormal cavity in males exposed briefly to EE_2 during various
1949 critical SD and GD developmental points before and after hatching (Van Aerle et al.,
1950 2002). Researchers noted the abnormal cavity was likely an ovarian cavity due to the
1951 physical similarities observed in the females (Van Aerle et al., 2002). Currently, there is
1952 limited information available on mummichog duct and cavity formation, therefore,
1953 determining the cause of the abnormal cavity was not possible. In the roach (*Rutilus*
1954 *rutilus*), ovarian development has been more thoroughly described (Nolan, 2001;
1955 Rodgers-Gray et al., 2001). The roach ovarian cavity is located between the dorsal wall of
1956 the gonad and the coelomic epithelium, with two points of attachment to the mesentery
1957 (Nolan, 2001). These descriptive guidelines enable intersex or sex reversal identification
1958 in early gonadal development (Nolan, 2001). It is not known whether the abnormal cavity
1959 noted in the mummichog testis tissue in the present study is an ovarian cavity; future
1960 studies are encouraged to investigate the entire reproductive tissue to understand normal
1961 vs abnormal duct and cavity formation as a potential early marker for EE_2-induced
1962 feminization. Rogers-Gray et al. (2001) exposed young roach (50 dph) to sewage effluent
1963 which resulted in feminizing effects, including males developing ovarian cavities. Even
1964 after a period of depuration, the ovarian cavity remained in the male gonad indicating a
1965 permanent effect from exposure to effluent before GD differentiation had occurred

1966 (Rodgers-Gray et al., 2001). Abnormal duct development in the roach has been shown to
1967 compromise reproductive capabilities and consequently impacting fish populations
1968 (Jobling et al., 2002). Sexually mature roach gonads contained gametes, however, due to
1969 duct disruptions, fish were unable to release gametes into the aqueous environment
1970 (Jobling et al., 2002). The current study is unable to determine whether the abnormal
1971 cavity would have long lasting reproductive effects on adults. Further investigation into
1972 mummichog duct development and whether an abnormal cavity derived from EE_2
1973 exposure is permanent would improve current knowledge of mummichog GD.

1974 **4.5 Gametogenesis**

1975 In the current study, environmentally-relevant concentrations of EE_2 altered
1976 gametogenesis of both sexes. Treatment with 10 ng/L EE_2 inhibited oogenesis; treated
1977 ovaries were composed of significantly higher percentages of oogonia and lower
1978 percentages of perinucleolar oocytes. Low EE_2 concentrations (1-50 ng/L) halted
1979 oogenesis in medaka (Seki et al., 2002), fathead minnow (Weisbrod et al., 2007) and
1980 zebrafish (Weber et al., 2003). Previous work on mummichog demonstrated that EE_2
1981 treatment accelerates ovarian development, causing treated ovaries to appear one week
1982 earlier compared to controls (Chehade, 2012). Therefore, it was surprising to find
1983 inhibited oogenesis in the presence of EE_2 in the current study. However, the timing of
1984 exposure could be playing a significant role in the discrepancy observed between these
1985 two studies. Timing of exposure plays an important role in the outcome of gonadal
1986 development (Piferrer, 2001). In general, the timing before differentiation is the most
1987 susceptible to contaminants (Dietrich and Krieger 2009). Therefore, because mummichog
1988 ovaries are not identified histologically until 2 wph (Chehade, 2012; Shimizu et al.,

1989 2008), mechanisms influencing ovarian differentiation are likely occurring before
1990 hatching.
1991 In males, a significant increase in percent spermatogonia and decrease of percent late
1992 spermatocytes in the testis were observed and indicate delayed development. The most
1993 developed stage, spermatid, was only observed in control testes. These results are aligned
1994 with previous studies showing that EE_2 inhibited spermatogenesis in male fathead
1995 minnow (Palace et al., 2002), zebrafish (Weber et al., 2003), rare minnow (Zha et al.,
1996 2007), least killifish (Jackson et al., 2019) and guppy (Nielsen and Baatrup, 2006). E_2 has
1997 been demonstrated to be vital in spermatogonial self-renewal by inducing type 1 cell
1998 divisions (Schulz et al., 2010). A type I division results in two separate daughter cells,
1999 while type II divisions result in interconnected daughter cells that are destined for
2000 meiosis. EE_2 treatment could enhance spermatogonial self-renewal in mummichog testis
2001 by binding to ER in spermatogonia; which could explain the significant increase in
2002 spermatogonia percentages. Type 1 self-renewal divisions do not lead to meiosis,
2003 therefore preventing further development of the germ cells.
2004 Environmentally-relevant EE_2 (10 ng/L) altered gametogenesis of both sexes of
2005 mummichog by preventing the sex cells from developing at the same rate as the control
2006 fish. Estrogens and other steroids, including androgens and progestins, have a vital role in
2007 early gonadal differentiation in both sexes (Norris and Lopez, 2011). While estrogens
2008 play an important role in oocyte development and growth, they also have the capability of
2009 downregulating the HPG axis which orchestrates sexual development (Lin and Ge, 2009).
2010 EE_2 can overwhelm the HPG axis causing a downregulation of gonadotropin FSH
2011 production. Since FSH stimulates steroidogenesis in gonadal somatic cells, this may
2012 explain why cell development and differentiation was inhibited. Without the influence of

2013 androgens and progestins on early developing gametes, these cells may not grow,
2014 proliferate or differentiate. Spermatogonial proliferation in the Japanese eel, including
2015 meiosis and further differentiation, was dependent on the androgen 11-KT from Leydig
2016 cells to induce mediators in Sertoli cells (Miura and Miura, 2003). Without 11-KT, the
2017 Sertoli cells were not able to provide the spermatogonia with the necessary factors for
2018 proliferation, resulting in halted spermatogenesis (Miura and Miura, 2003). Shimizu et al.
2019 (2008) showed that FSH signaling was probably important in early GD in the
2020 mummichog; immunohistochemistry showed that FSH signaling was found in the
2021 mummichog gonad before, during and after GD. Researchers believe that FSH is
2022 responsible for initiating steroidogenesis in the gonad in early development, contributes to
2023 the formation of cortico alveoli in oocytes and then cooperation between both FSH and
2024 LH supports later stages of development (e.g., vitellogenesis and spermiation; Shimizu et
2025 al., 2008). Downregulation of FSH and downstream steroidogenesis may be one
2026 mechanism by which EE_2 affected gonadal development in the current study. To confirm
2027 this theory, further testing could be conducted to measure plasma steroid levels in early
2028 stages mummichog exposed to EE_2 to confirm whether steroid production is being shut
2029 down. Further depuration studies could determine whether gametogenesis in mummichog
2030 can recover with removal of the treatment.

4.6 Degeneration

Increased atresia was found in ovarian tissue from EE_2 (10 ng/L) exposure during GD. While atresia is a normal process, extensive atresia can significantly impact GD in the ovary as germ cells, and their follicles, are decimated. Atresia mechanisms in the mummichog are currently unknown. Estrogens alter rates of ovarian atresia in mammals (Billig et al., 1993), and have been linked to enhanced ovarian atresia in fish species (Zillioux et al., 2001). EE_2 exposure has been reported to cause increased atresia during GD in the zebrafish (10 ng/L; Schafers et al., 2007) and the rare minnow (0.2 ng/L; Zha et al., 2008). Similar to altered gametogenesis, significant atresia has been linked to reduction in gonadotropins FSH and LH (Dietrich and Krieger, 2009).

Areas of necrosis and hypertrophic interstitial tissue also increased in EE_2 treated males. Degeneration by EE_2 has been demonstrated in testes in zebrafish (Luzio et al., 2016; Schafers et al., 2007), rare minnow (Zha et al., 2007), fathead minnow (Lange et al., 2001; Palace et al., 2002), as well as the protandrous *Lates calcarifer* (barramundi; Banh et al., 2020). The endpoints measured in these aforementioned studies included necrosis, apoptosis and/or widespread fibrosis. Studies on zebrafish have reported degeneration effects from EE_2, similar to the effects observed in the present experiment (Schafers et al., 2007; Weber et al., 2003), with increased necrosis and hypertrophic interstitial tissue. Experimental analysis and result reporting vary between studies; there is no standardized method for reporting degeneration across studies (Dietrich and Krieger, 2009).

Degeneration observed in both male and female mummichog could be a result of HPG axis downregulation. Depressed gonadotropins have been linked to increased atresia

2054 (Papoulias et al., 1999) and necrosis/apoptosis of testis somatic cells (Weber et al., 2004)
2055 from EE_2 exposure. In the latter study, depressed androgen production in the somatic cells
2056 of fathead minnow facilitated altered homeostasis of Leydig and Sertoli cells, which
2057 ultimately led to significant cell death in the testis (Dietrich and Krieger, 2009; Weber et
2058 al., 2004). Somatic cells are required to support germ cell growth; without gonadal
2059 somatic cells, germ cell death is inevitable. Germ cells are necessary for normal gonadal
2060 development.

2061 With significant cell death occurring, as seen in the current study, the injured area is
2062 replaced with fibrotic tissue to maintain gonadal structure. This tissue or scarring was
2063 observed in both male and female gonadal tissue of the mummichog in the present study
2064 and is considered to be non-functional. Luzio et al. (2016) reported significant
2065 degeneration in testes of zebrafish exposed to EE_2, however, a recovery period reversed
2066 some of these degeneration effects. Future studies investigating the recovery period in
2067 mummichog would be of value as fish may not be exposed to contaminants in their
2068 environments throughout their lifecycle. As well, periods of depuration following
2069 contaminant exposures may provide more mechanistic understanding.

4.7 Ovarian gene expression

In this study, mummichog gonadal female and male promoting genes were characterized in control and EE_2 environments. To our knowledge, the gene expression of *foxl2*, *gdf9*, *bmp15*, *dmrt1* and *amh* in the ovary are the first to be documented in the mummichog. These genes were anticipated to be expressed in the ovary during GD as they have been identified in many other fish (Nagahama et al., 2021). One of the genes investigated, *cyp19a1a*, has been previously reported in adult mummichog ovaries (Doyle et al., 2013; Kanagasabesan, 2018).

Experiment 1 of the current study showed that ovaries express several genes, classified in other species as male- and female-promoting genes. Female-promoting genes *cyp19a1a*, *foxl2*, *gdf9* and *bmp15* showed higher expression than the male-promoting ones, *dmrt1* and *amh*, however, statistical analysis is not possible across genes since the primer efficiencies were different (Table 2.1). When these results are compared with Experiment 2, the level of expression was similar. In Experiment 2, the expression of *dmrt1* was higher than expected, indicating there may be more variation than observed in the initial study. Zebrafish also express *dmrt1* during early oocyte differentiation (Guo et al., 2005) indicating "male"- and "female"-promoting descriptors may not be completely defining of action. The current study design used a low sample number, therefore, increasing sample size from 3 in future experiments is warranted for *dmrt1* expression under standard laboratory conditions in the ovary to increase precision and power of analysis.

In Experiment 2, neither EE_2 treatment caused changes to ovarian gene expression. These results are in agreement with current mummichog EE_2 literature (Rutherford et al.,

2093 2020). Researchers did not find EE_2 induced changes to *cyp19a1a* expression in adult
2094 mummichog exposed to supraphysiological 50 ng/L (Doyle et al., 2013; Meina et al.,
2095 2013), 74 ng/L (Kanagasabesan, 2018) or 100 ng/L EE_2 (Bosker et al., 2016). It is known
2096 that many fish exhibit a downregulation of ovarian *cyp19a1a* when exposed to EE_2, such
2097 as zebrafish (Cosme et al., 2015; Urbatzka et al., 2012) and fathead minnow (Feswick et
2098 al., 2016; Leet et al., 2015). This downregulation is presumed to be due to negative
2099 feedback of the HPG axis (Leet et al., 2015). It is well established that adult female
2100 mummichog are resistant to EE_2 exposure as adults in that they continue spawning while
2101 other species of fish shut down reproduction; whether the mechanisms in developing
2102 juveniles and reproductive adults are linked requires more experimental studies.
2103 However, the results from this study support that female mummichog are resistant to EE_2
2104 exposure even at a young stage in life. Therefore, the negative histological results
2105 observed in ovaries may not be physiological responses to female-promoting gene
2106 expression network alterations.

2107 Estrogen needs to complex with ERs to carry out physiological effects on cells.
2108 Kanagasabesan (2018) results show that ERα expression is relatively low in
2109 previtellogenic oocytes, then significantly increases into vitellogenesis. *Cyp19b* (brain
2110 aromatase) contains an estrogen response element (ERE), which allows for estrogens to
2111 bind and increase expression of *cyp19b* in zebrafish (Trant et al., 2001). However,
2112 *cyp19a1* in zebrafish does not contain an ERE (Trant et al., 2001) and is therefore not
2113 directly regulated by estrogen. The absence of changes in gene expression in the post-
2114 hatch juvenile mummichog ovary via either direct EE_2 action or indirectly through the
2115 HPG axis, indicates a lack of sensitivity to exogenous estrogen. More studies are needed

2116 to confirm these preliminary findings for the suite of genes studied, including brain
2117 aromatase.

2118 4.8 Testis gene expression

2119 Experiment 1 revealed higher expression of male-promoting genes compared to
2120 female-promoting genes in testes. Similar to the ovarian results, statistical analysis was
2121 not conducted among genes in the testes. When results were compared to Experiment 2,
2122 there was a similar level of expression observed between the experiments, except for
2123 *dmrt1*, which was lower. The sample sizes used were 2 (Experiment 1) and 3 (Experiment
2124 2), were low. Increasing sample sizes in future experiments is warranted for *dmrt1*
2125 expression under standard laboratory conditions in the testis to increase precision and
2126 power of analysis.

2127 Experiment 2 demonstrated that 10 ng/L EE_2 treatment downregulates *dmrt1* gene
2128 expression in the testis. This downregulation of *dmrt1* did not alter the male GD pathway.
2129 This is contrary to what has been observed in zebrafish, in which *dmrt1* gene expression
2130 is indispensable for male differentiation (Webster et al., 2017). EE_2 downregulated *dmrt1*
2131 gene expression in zebrafish exposed during GD, shutting down the male differentiating
2132 pathway and resulting in ovary differentiation (Schulz et al., 2007).

2133 *Dmrt1* is a strong male differentiation transcription factor that influences the
2134 expression of male- and female-promoting genes in many fish species (Tenugu et al.,
2135 2021). In the zebrafish, *dmrt1* upregulates male promoting *amh* and *sox9* genes while
2136 simultaneously suppressing female-promoting *foxl2* and *cyp19a1a* genes. Results from
2137 the current study suggest this may not be the case for mummichog, as *cyp19a1a*, *foxl2*
2138 and *amh* were all significantly upregulated in the presence of EE_2. It is possible that

2139 *dmrt1* may not play a significant role in male differentiation as shown in other fish at this
2140 stage in development. There is limited research on male-promoting gene expression in the
2141 developing mummichog, including this study which only investigated two genes. There
2142 are several other male-promoting genes that could be investigated in the mummichog,
2143 such as *sox9.* As previously mentioned, *sox9* is a typical male promoting gene that is
2144 commonly associated with upregulating other male promoting genes such as *amh* in
2145 mammals (Morais de Silva et al., 1996; Rodríguez-Marí et al., 2005). Researchers have
2146 demonstrated that male differentiation in the zebrafish is influenced by *sox9* (Rodríguez-
2147 Marí et al., 2005), *dmrt1* and *amh* (Schulz et al., 2007) using in situ hybridization and
2148 RT-qPCR. Identifying male differentiating genes in the mummichog would help to
2149 understand the mechanisms controlling male differentiation.

2150 The female promoting genes here are the first to be reported in mummichog testis;
2151 *foxl2*, *cyp19a1a*, *gdf9* and *bmp15* have not been previously reported. Results from
2152 Experiment 2 demonstrated that both EE_2 treatments significantly increased gene
2153 expression of *cyp19a1a* and *gdf9* in testis. *Foxl2* expression was only significantly
2154 upregulated in the high EE_2 concentration treatment. Increased *foxl2* and *cyp19a1a* have
2155 been observed in rainbow trout exposed to EE_2 during GD (Guiguen et al., 2010). It is
2156 possible that the overwhelming effect of EE_2 in the testis was causing upregulation of
2157 female-promoting gene expression in the current study. In many fish species, E_2, *foxl2*
2158 and *cyp19a1a* have a positive relationship in that increases in their production (Leet et al.,
2159 2011; Tenugu et al., 2021). E_2 encourages *foxl2* expression, which increases *cyp19a1* to
2160 produce more E_2. Therefore, EE_2 is likely mimicking E_2 and cascading the expression of
2161 female promoting genes in the testis. *Bmp15* expression did not change, although there is
2162 an increasing trend which can be observed. A power analysis revealed that a sample size

2163 of 12 would be needed to show that the *bmp15* gene expression is significantly different
2164 between the control and high concentration group (significance = 0.05, power = 0.8).

2165 These increased gene expression results are different from adult fish exposed to EE_2
2166 which showed no changes to *cyp19a1a* gene expression in male zebrafish (Urbatzka et
2167 al., 2012), fathead minnow (Leet et al., 2015) and *Jenynsia multidentata* (a Rio de la Plata
2168 one-sided livebearer; (Roggio et al., 2014). Gene expression pathways controlling steroid
2169 hormones have differing sensitivities to exogenous hormones before, during, and after
2170 GD (Leet et al., 2015). Additionally, exposure duration, especially during critical periods
2171 of development, has been identified as an important factor that influences results (Leet et
2172 al., 2015). The results from this experiment emphasize the importance of further
2173 investigation on various life stages and exposure conditions in different fish models to
2174 untangle different modes of action and organism sensitivity to exogenous hormone
2175 exposure.

2176 In the presence of EE_2, *amh* gene expression was significantly upregulated. This
2177 result contradicts other studies that reported downregulation in the fathead minnow (Filby
2178 et al., 2007; Filby et al., 2006) and zebrafish (Schulz et al., 2007). The present results
2179 suggest that *amh* is likely not a male-promoting gene during early mummichog GD. A
2180 review by Pfennig (2015) implicates *amh* as a potential regulator of the onset of gonadal
2181 development in some fish, a process which was first identified in mammals. In medaka,
2182 mutant *amh* was found to prematurely initiate testis development (Morinaga et al., 2007).
2183 In addition, weak *amh* signaling in female Japanese flounder was detected before GD,
2184 and was then absent during the onset of germ cell type II divisions in GD (Yoshinaga et
2185 al., 2004).

2186 EE_2 treatment inhibited spermatogenesis and oogenesis in the current study. The
2187 hindered gametogenesis observed may correlate to the increasing expression of *amh*, as
2188 there were more early-staged germ cells, likely from type I divisions, in the high EE_2
2189 treatment. Further studies should be conducted sampling mummichog at earlier and later
2190 points in gonadal development to gain a better understanding of *amh* function in both
2191 sexes of mummichog.
2192 In the presence of EE_2, gene expression that normally facilitates the male
2193 differentiating pathway is inhibited or altered (Leet et al., 2011). The results from this
2194 study confirm, in general, that mummichog respond during development similarly to
2195 other species, because most female promoting genes were upregulated and male
2196 promoting *dmrt1* was downregulated in the testis. EE_2 acts as a mimic of E_2 facilitating
2197 the female-differentiating pathway and consequently suppressing the male-differentiating
2198 pathway (Leet et al., 2015). Altered male-differentiating pathway gene expression has
2199 been implicated in estrogenic EDC exposure, including EE_2 (Leet et al., 2011). While EE_2
2200 was not capable of altering female mummichog gene expression, male expression was
2201 feminized. However, histological results of both sexes do not indicate feminization, rather
2202 demonstrating an effect on the HPG axis that is halting gonadal development. Research
2203 taking into account plasma steroid levels, gene expression, and histological analysis
2204 would expand on this fundamental mummichog GD knowledge.

4.9 Conclusions

SD and GD processes in fish are very complex, varying greatly from species to species (Devlin and Nagahama, 2002). However, these mechanisms are commonly demonstrated to be altered by EE_2 (Leet et al., 2011). This study provided evidence that mummichog exposed to environmentally-relevant EE_2 after hatching severely altered GD mechanisms, while SD mechanisms remain fixed. The present study demonstrates that environmentally-relevant EE_2 concentrations can significantly disrupt gonadal development in the mummichog, hinder gametogenesis, increase degeneration and alter gene expression. The likely mechanism for these changes is the downregulation of the HPG axis, subsequently affecting development of the gonad. There is a delicate paracrine relationship between gametes and their supporting somatic cells (Devlin and Nagahama, 2002); this study provides evidence showing that EE_2 is preventing gonadal development which may be a result of improper HPG axis communication. As a result, gametogenesis of both males and females were significantly reduced from EE_2 treatment. Surprisingly, sex ratios were unaffected by treatment which is likely due to the EE_2 exposure after hatching; an investigation comparing EE_2 sensitivity to sex ratios is encouraged for further investigation on the window of sensitivity. SD mechanisms may be established during embryogenesis as feminized sex ratios have been previous reported in mummichog exposed before hatching (Chehade, 2012; Peters et al., 2010). As expected, intersex was not observed in this study, which was not surprising because mummichog have not been documented to show this form of gonadal determination naturally or artificially from any EDC exposure to date (Rutherford et al., 2020). Mummichog may not be as sensitive to intersex SD mechanisms as demonstrated in other fish species. EE_2

2228 treatment affected male mummichog more so than in females. Female gene expression
2229 pathways did not change from EE_2 treatment; however, testis gene expression profiles
2230 were significantly altered. Male fish also displayed an abnormal cavity in the testis, while
2231 ovaries showed no signs of abnormal morphology. The results from this study have added
2232 to pre-existing mummichog gonadal development knowledge and provide useful baseline
2233 work for future studies.

2234 To strengthen future work and further SD and GD mechanisms in the mummichog,
2235 further studies should be conducted. Going forward, a higher EE_2 concentration treatment
2236 should be utilized in experiments since mummichog respond to supraphysiological high
2237 EE_2 concentrations. To investigate SD mechanisms, research should focus on whether sex
2238 ratios can be altered by EE_2 when mummichog are exposed before and after hatching
2239 (Figure 4.1). Sex ratios did not change from treatment in the current experiment, which
2240 was not predicted. Experimentation exposing embryos to EE_2 before hatching should be
2241 conducted to gain valuable insight into the SD mechanisms and when they are susceptible
2242 to interference. To investigate GD mechanisms, research should focus on before, during
2243 and after gonadal differentiation time points. From previous work, mummichog GD has
2244 been established to occur at two weeks old in females and three weeks old in males
2245 (Shimizu et al., 2008). Increasing sampling timepoints in future experiments would
2246 capture before, during and after GD has occurred. There is little knowledge on
2247 mummichog duct development. Establishing normal and adverse duct development in the
2248 mummichog could help to identify whether the abnormal cavity seen in this experiment is
2249 a feminizing effect i.e., an ovarian cavity. Gene expression profiles before, during and
2250 after differentiation would allow for further understanding of which genes and
2251 mechanisms are orchestrating GD. This methodology could also provide insight on

2252 reproductive effects in adult mummichog that have been exposed to EE_2 during
2253 development. Gametogenesis or other common reproductive endpoints such as VTG
2254 induction and reproductive success could be measured after a period of depuration to
2255 determine whether EE_2 exposure during a critical time in development contributes to long
2256 term effects in individuals that may lead to negative effects on the population.

5.0 Thesis Relation to Integrative Biology

The Wilfrid Laurier Biology Department defines integrative biology as an interdisciplinary approach to biological research that allows one to explore the answers to complex questions from a perspective that bridges the traditional sub-disciplines of biology, across diverse taxa, over time scales ranging from shorter (physiological) to longer (evolutionary).

There were many different facets of biology that were used to accomplish this work, resulting in an integrative thesis. This project has taken a holistic approach by considering a link between the environment and the effects that manifest at different levels of biological activity (cellular/tissue and molecular). EE_2 causes a variety of developmental effects to various fish species, ranging from body size, skeletal growth, and sexual development (Chehade, 2012; Dietrich and Krieger, 2009; Leet et al., 2011). At the tissue level of organization, the current thesis demonstrates adverse effects of environmentally-relevant EE_2 in mummichog by showing halted gametogenesis, degeneration and abnormal testis morphology. At the molecular level, GD genes in the mummichog have been successfully identified for the first time, including *cyp19a1a*, *foxl2*, *gdf9*, *bmp15*, *dmrt1* and *amh*, which have been identified in other fish species as driving male or female development. These genes were demonstrated to be specifically altered by EE_2 in males, demonstrating their vulnerability to estrogen chemicals in the water.

This thesis analyzed results following a five-week period of experimentation, and the results are considered to be a physiological response. Adverse effects on various levels of biological organization can be quantified using standardized toxicological tests such as early lifecycle bioassays. This study was in association with an ongoing project in the

2280 MacLatchy lab which involves developing an in-lab fish early lifecycle bioassay for
2281 industry to use to evaluate the potential effects of pulp mill effluents on estuarine
2282 ecosystems. There is currently limited research focused on development of an estuarine
2283 fish model for coastal environmental monitoring, in comparison to the amount of research
2284 on suggested freshwater fish models. This large-scale project involves collaboration with
2285 government, industry and experts in the fields of toxicology, physiology and ecology.
2286 This thesis, on the nature of the processes mediating reproductive development in
2287 mummichog down to the tissue and molecular levels, provides fundamental information
2288 that can be used in future in the early lifecycle bioassay being developed to assess how
2289 industrial discharges affect fish reproduction, development and growth. Estrogenic effects
2290 on both male and female mummichog, e.g., can now be evaluated at a very early age,
2291 which would allow for decreased testing time and space in the laboratory. Ultimately, it is
2292 hoped results in laboratory bioassays can be extrapolated to determine ecological risk to a
2293 population or community.

2294 By understanding how chemicals, such as EE_2, affect fish and their communities,
2295 scientists can better inform industry and policy makers on acceptable chemical
2296 management strategies for ecosystem protection. The research in this thesis provides one
2297 small piece of the puzzle by demonstrating how fundamental studies on biological
2298 mechanisms can underpin such extrapolative work across biological levels of
2299 organization.

2300

2301 # 6.0 References

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2828

2829 # Appendices

2830 ## A1: Methodology

2831 ### A1.1 Histology cassette preparation & processing:

2832 After storage in formalin for 24 h, fish anterior halves were dyed using Davidson
2833 Marking black, green, yellow, orange and blue tissue dye (Bradley Products, Inc.,
2834 Bloomington, MN, USA) and placed into Tissue Path™ Microsette™ Six Compartment
2835 Biopsy Cassettes (Fisher Scientific Company, Ottawa, ON). Cassettes were immediately
2836 sent to Susan Lapos at the animal health laboratory (AHL) histology department at
2837 University of Guelph, Guelph, ON. Fish dye colour and cassette number corresponded to
2838 a specific fish (Figure A1.1A). Cassettes were processed and embedded in paraffin wax
2839 longitudinally (Figure A1.1B), then each block was sectioned (60-80µm) perpendicular to
2840 the fish onto a standard glass slide and stained with H&E (Sigma-Aldrich; Figure A1.1C).
2841 This revealed a cross section view of the fish (Figure A1.1C). Each block was sectioned
2842 three to five times to ensure all fish gonads were visible on slides.

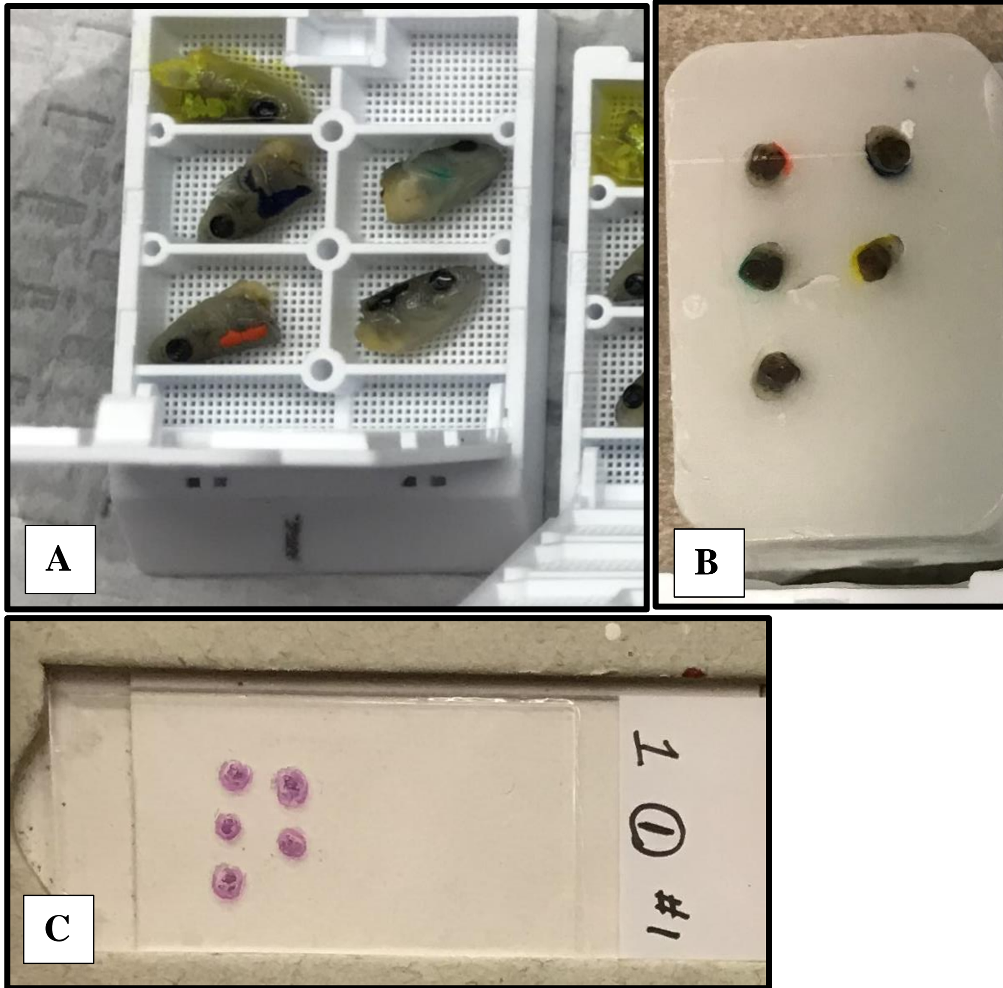
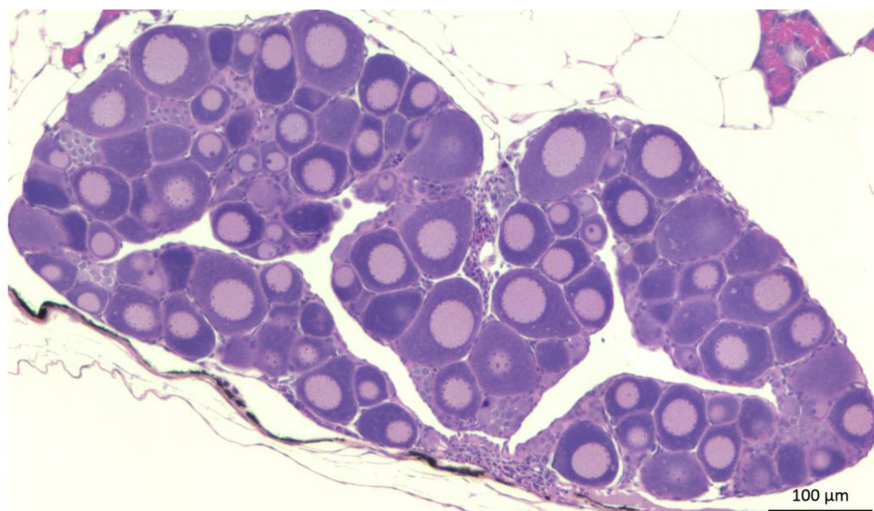


Figure A1.1: Sampled anterior five-week-old mummichog dyed in cassettes (A), paraffin wax blocks (B) and sectioned histological slide (C) of transverse cross sectioned mummichog with hemoxylin and eosin staining. Mummichog dye colour and cassette number were essential for fish identification.

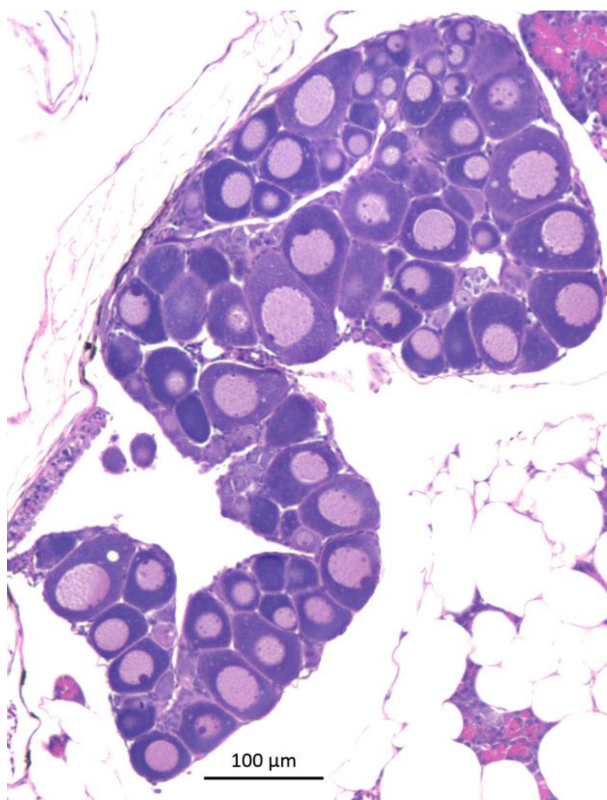
A1.2 Ovarian histology figures

Figures in this section (A.1.2.1-A1.2.30) represent the histological slides (10X) used to evaluate the female mummichog gametogenesis data for Experiment 2. Each slide includes fish identification being Tank number [T1-3 are control treatment (0 ng/L EE_2); T4-6 are high EE_2 concentration treatment 10 ng/L EE_2; T7-9 are low EE_2 concentration treatment (2 ng/L EE_2)], cassette number and dye used to colour coordinate the fish (A1.1).

2856 ***Control Treatment***

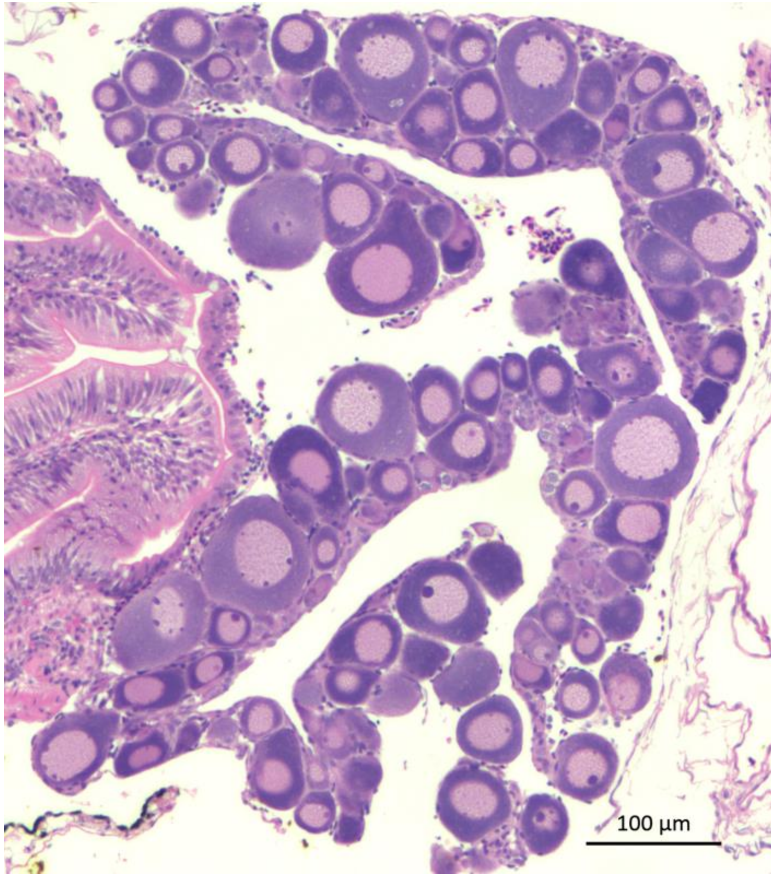


2857
2858 **Figure A.1.2.1:** Fish identification T1-5 yellow, female ovary (10X)
2859



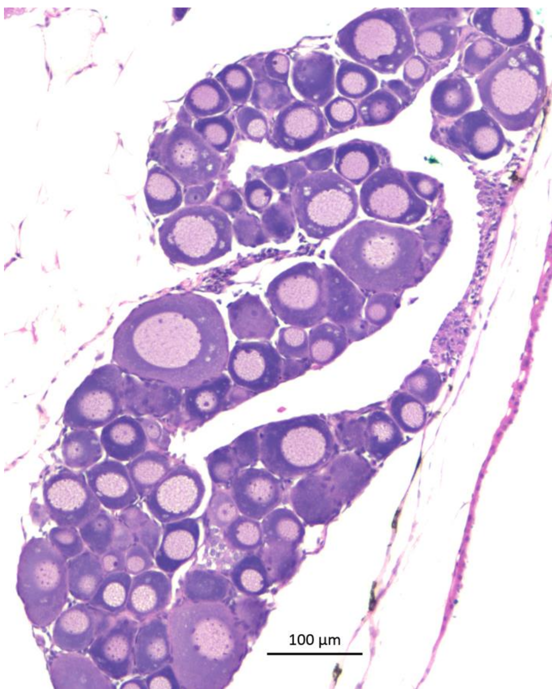
2860
2861 **Figure A.1.2.2:** Fish identification T1-7 green, female ovary (10X).

2862

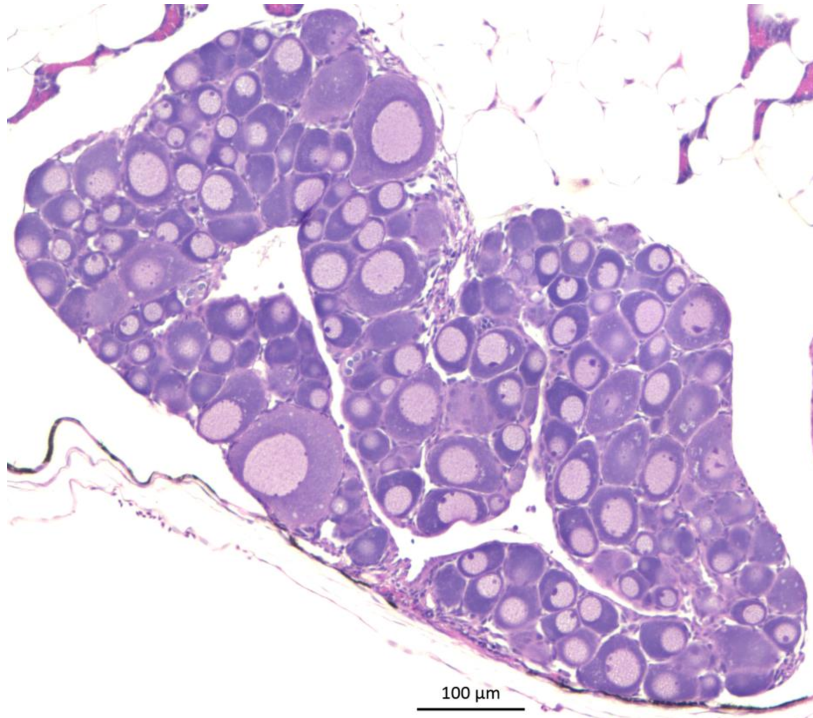


2863 **Figure A.1.2.3:** Fish identification T1-3 yellow, female ovary (10X)
2864

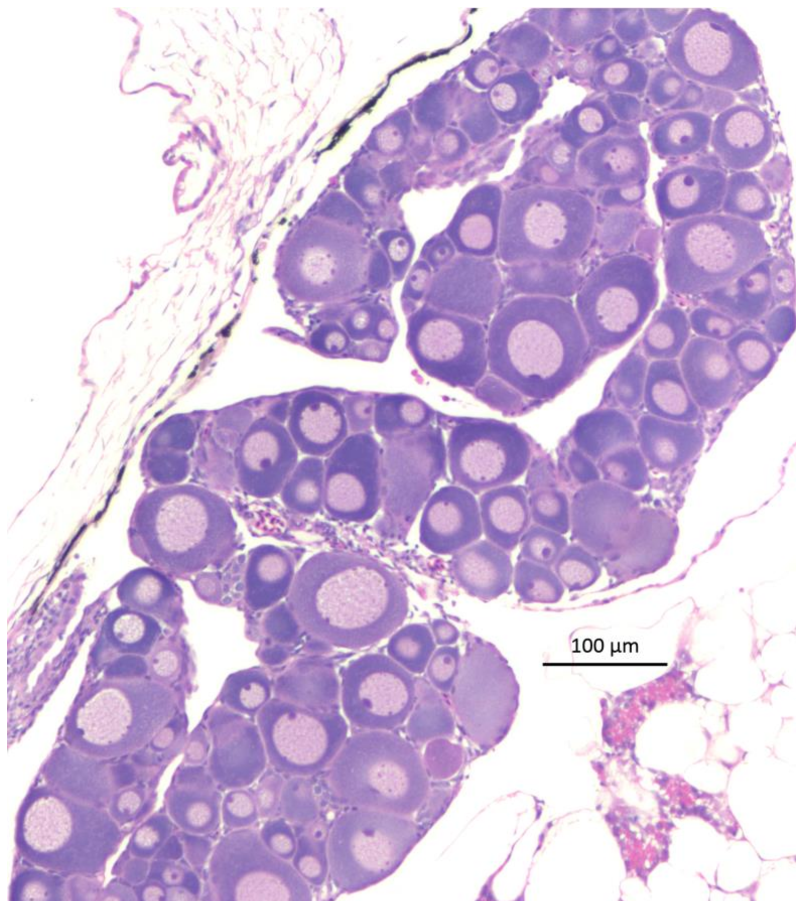
2865



2866 **Figure A.1.2.4:** Fish identification T2-5 green, female ovary (10X)

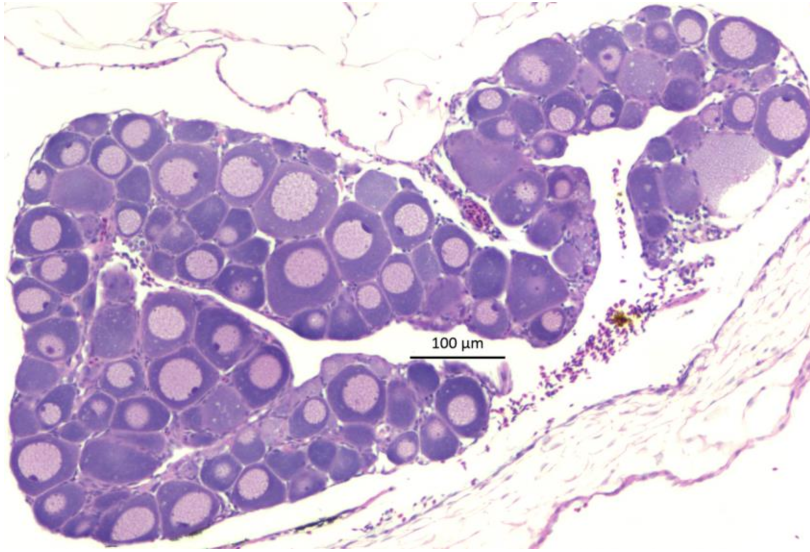
2867


2868 **Figure A.1.2.5:** Fish identification T2-6 green, female ovary (10X)
2869

2870


2871 **Figure A.1.2.6:** Fish identification T2-3 black, female ovary (10X)

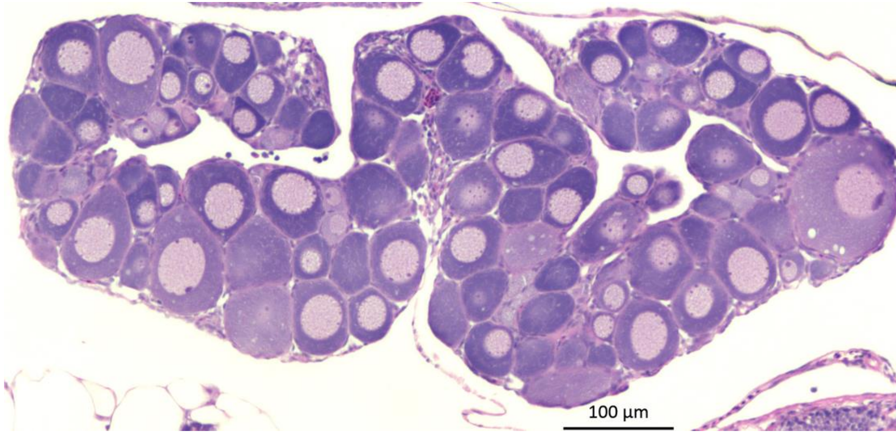
2872



2873 **Figure A.1.2.7:** Fish identification T3-1 yellow, female ovary (10X)

2874

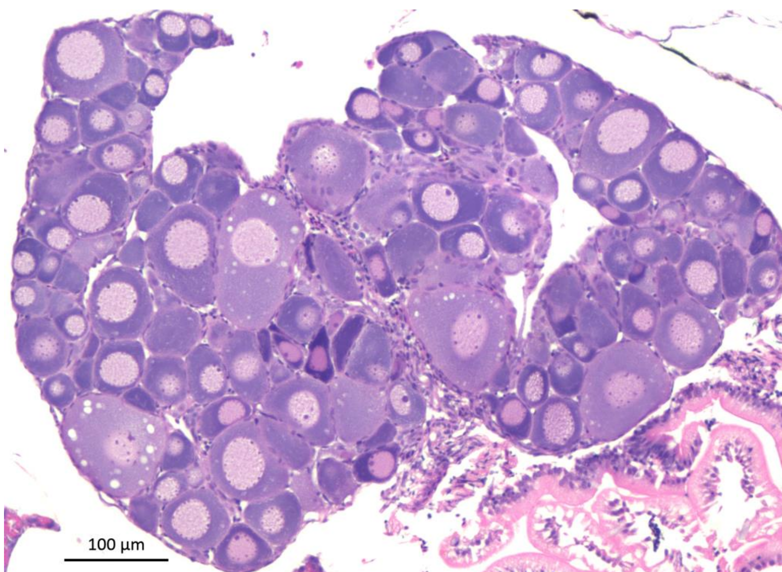
2875



2876 **Figure A.1.2.8:** Fish identification T3-11 orange, female ovary (10X)

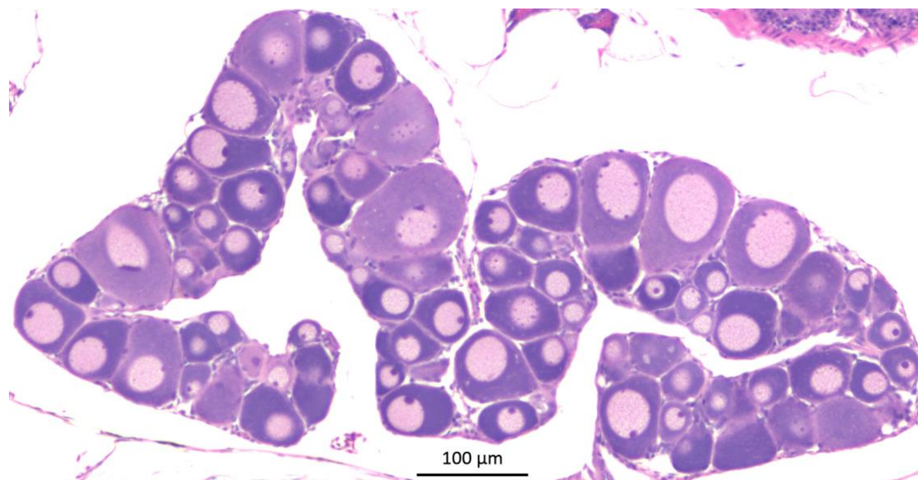
2877

2878



2879 **Figure A.1.2.9:** Fish identification T3-12 yellow, female ovary (10X)

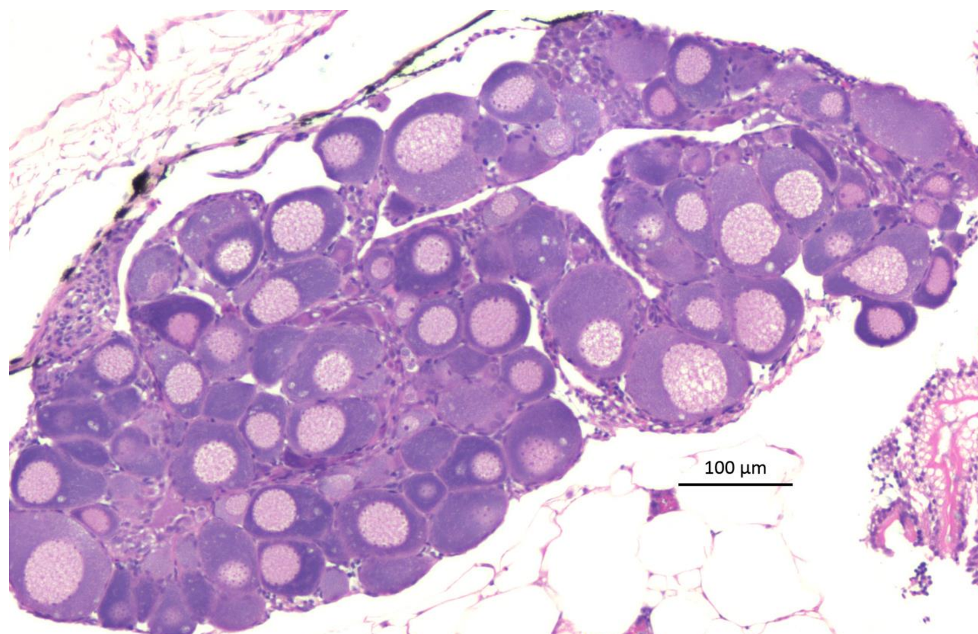
2880



2881
2882 **Figure A.1.2.10:** Fish identification T3-4 blue, female ovary (10X)

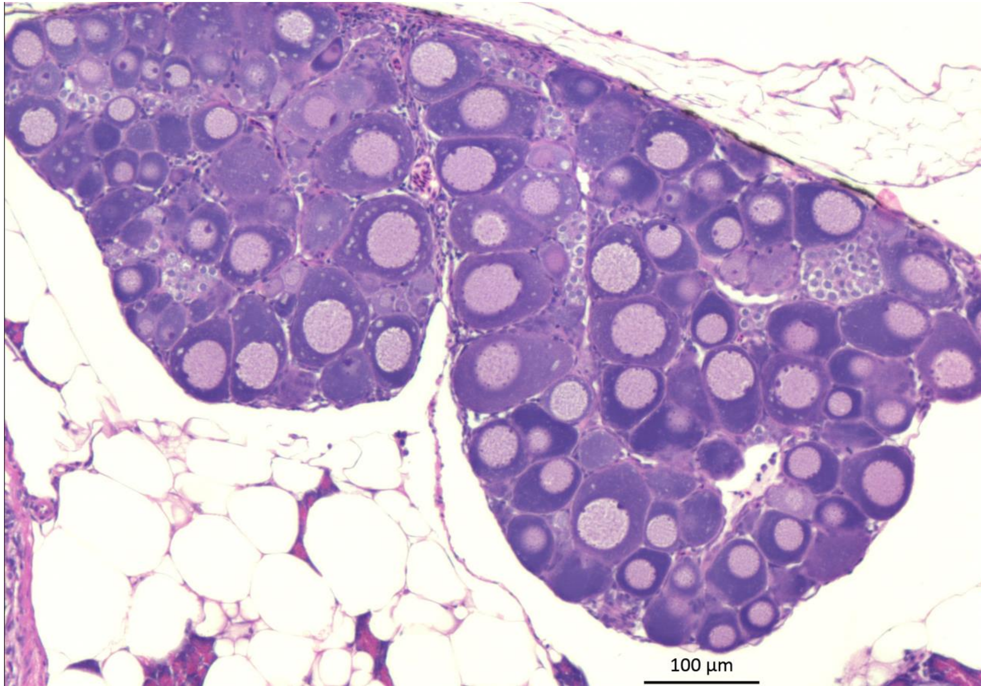
2883

2884 ***Low EE_2 concentration treatment***



2885
2886 **Figure A.1.2.11:** Fish identification T7- 1 black, female ovary (10X)

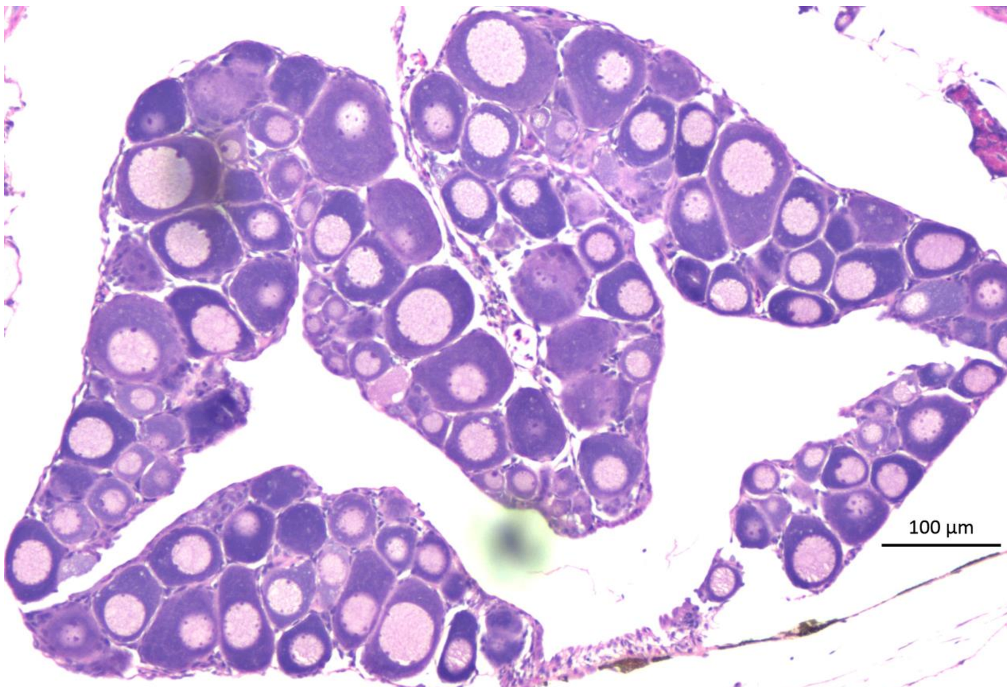
2887



2888 **Figure A.1.2.12:** Fish identification T7-3 yellow, female ovary (10X)

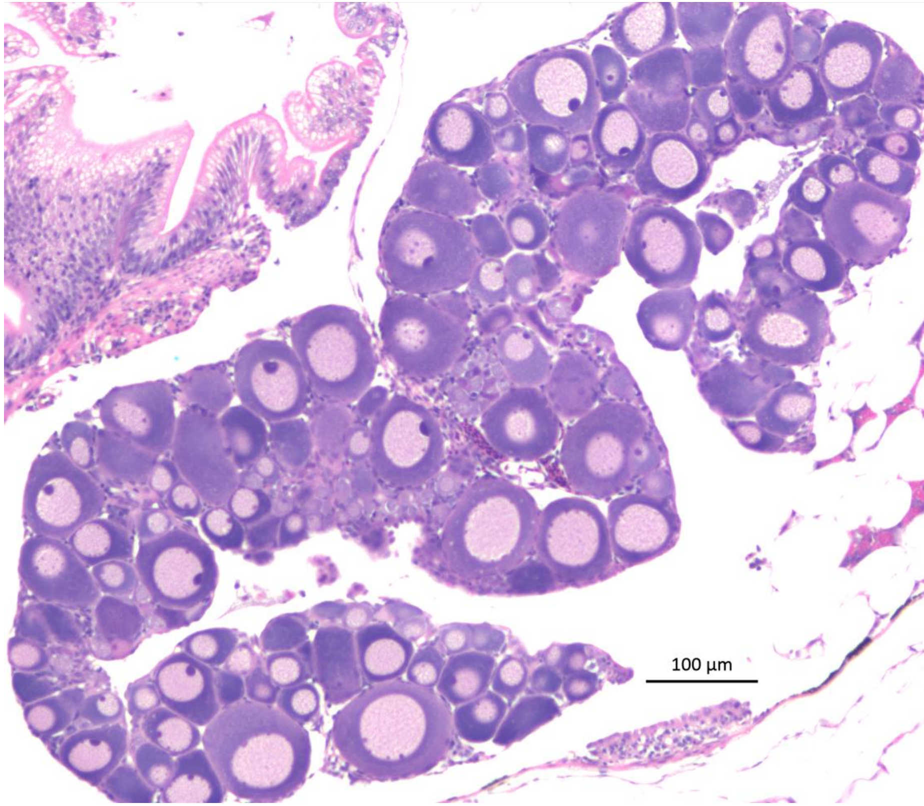
2889

2890



2891 **Figure A.1.2.13:** Fish identification T7-4 yellow, female ovary (10X)

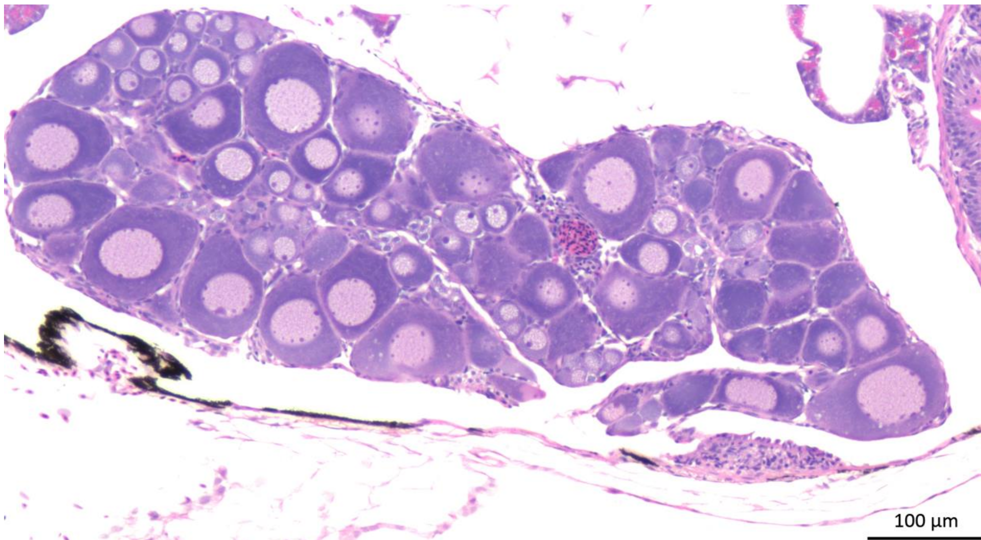
2892



2893 **Figure A.1.2.14:** Fish identification T7-6 green, female ovary (10X)

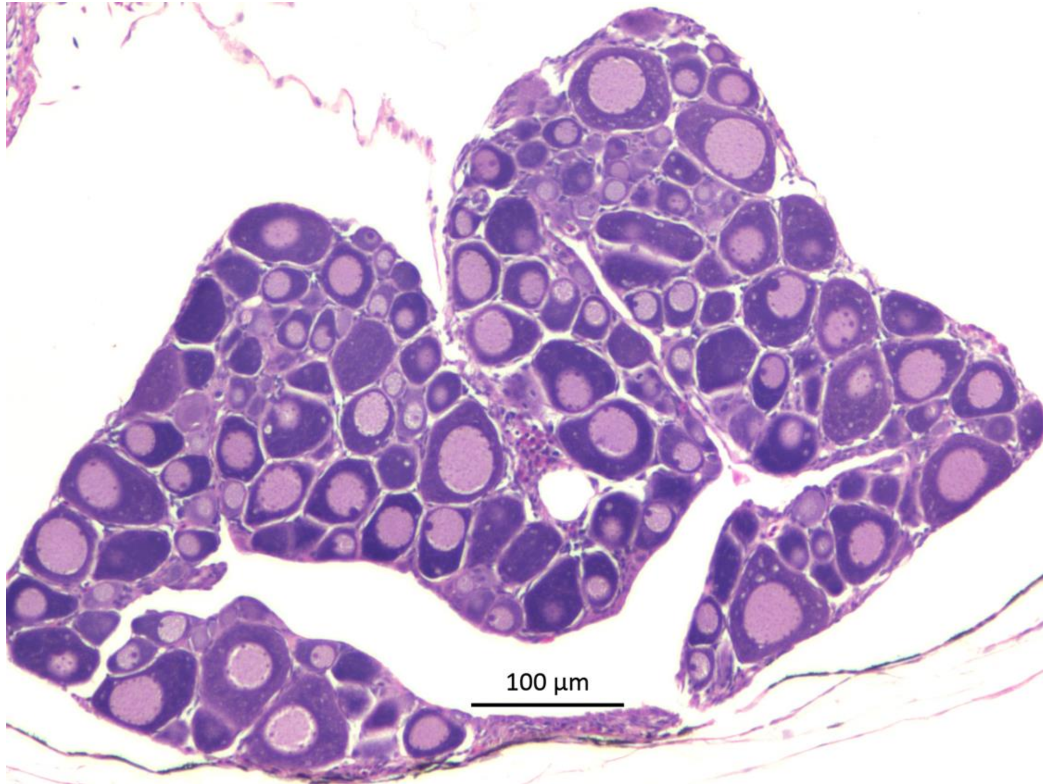
2894

2895



2896 **Figure A.1.2.15:** Fish identification T8-3 black, female ovary (10X)

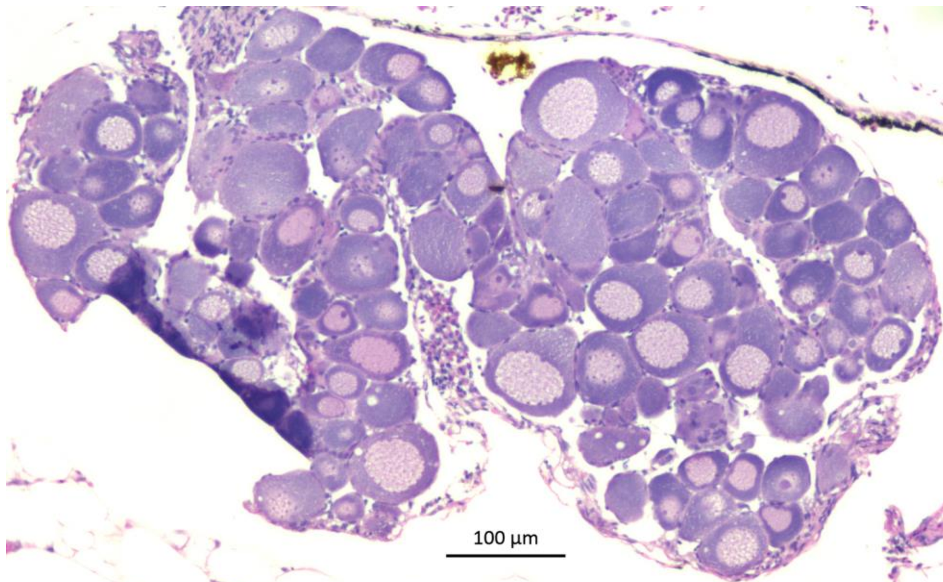
2897



2898 **Figure A.1.2.16:** Fish identification T8-8 orange, female ovary (10X)

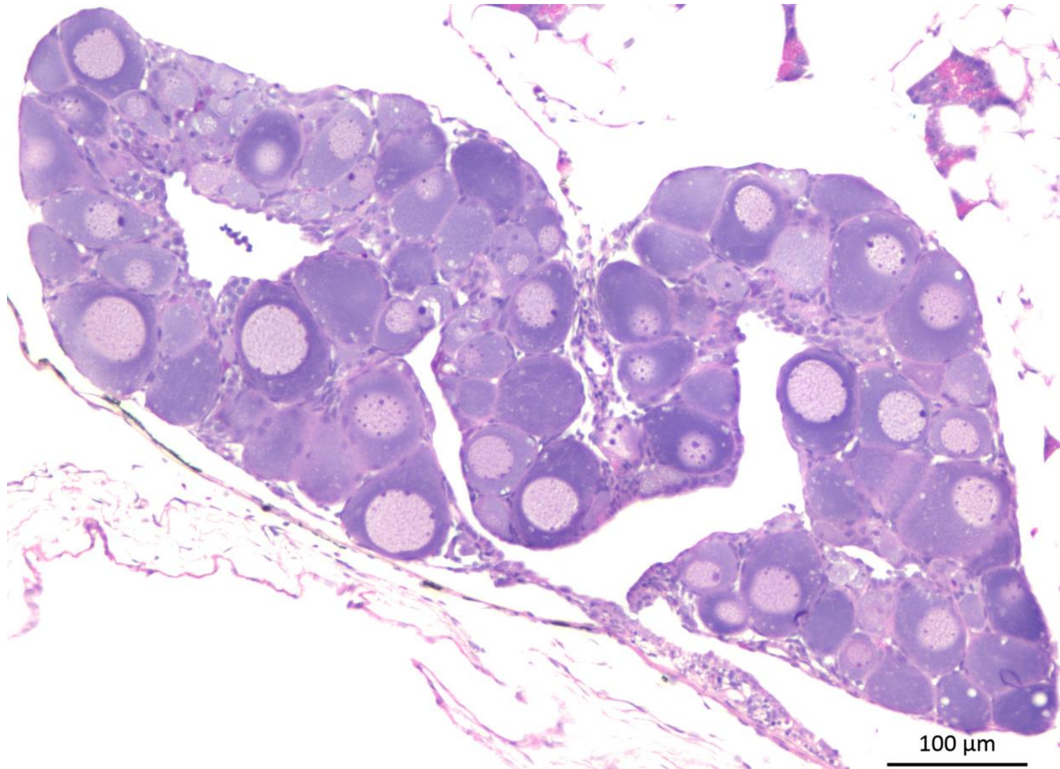
2899

2900



2901 **Figure A.1.2.17:** Fish identification T8-11 yellow, female ovary (10X)

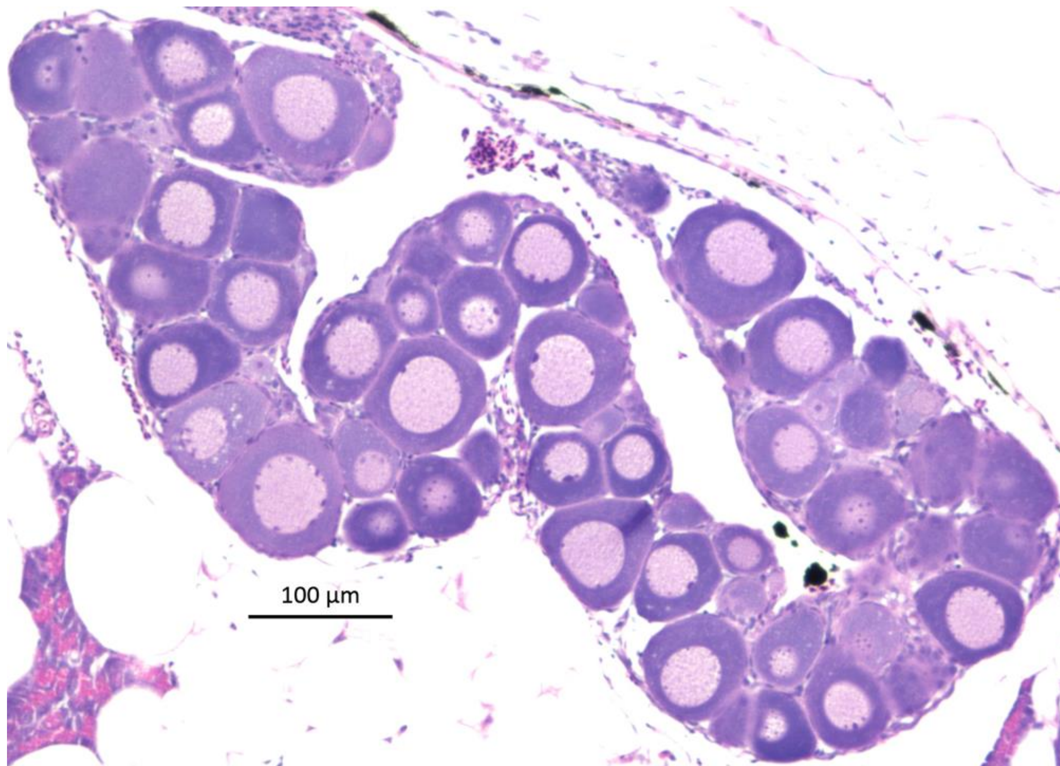
2902



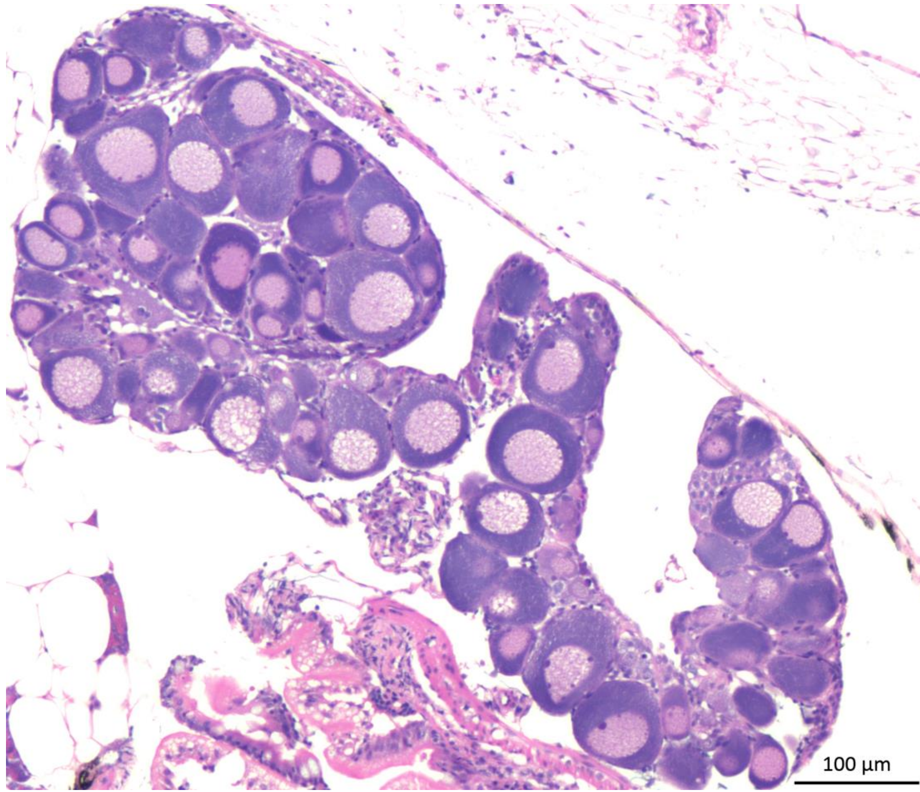
2903 **Figure A.1.2.18:** Fish identification T9-3 orange, female ovary (10X)

2904

2905



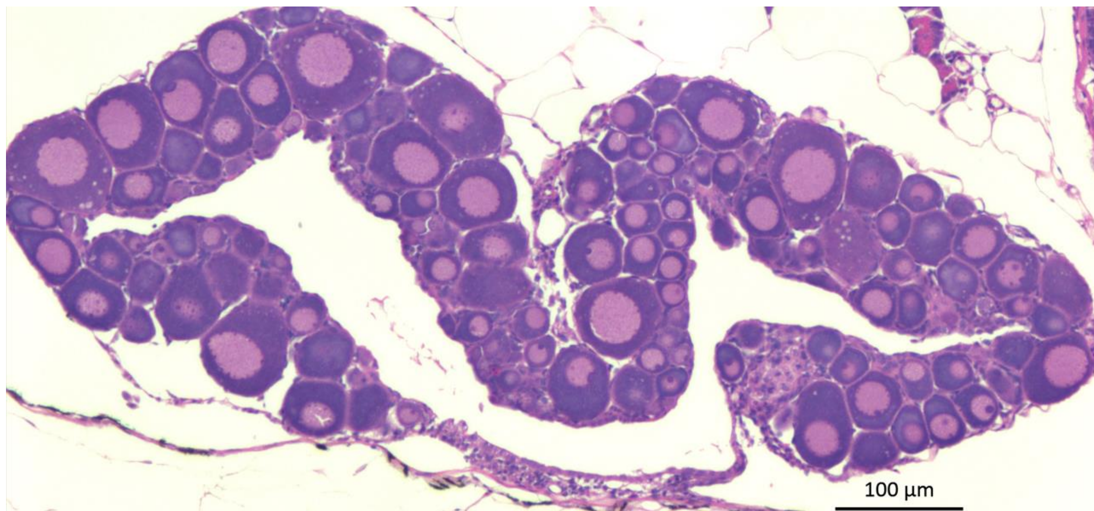
2906 **Figure A.1.2.19:** Fish identification T9-8 yellow, female ovary (10X)

2907


2908 **Figure A.1.2.20:** Fish identification T9-11 green, female ovary (10X)

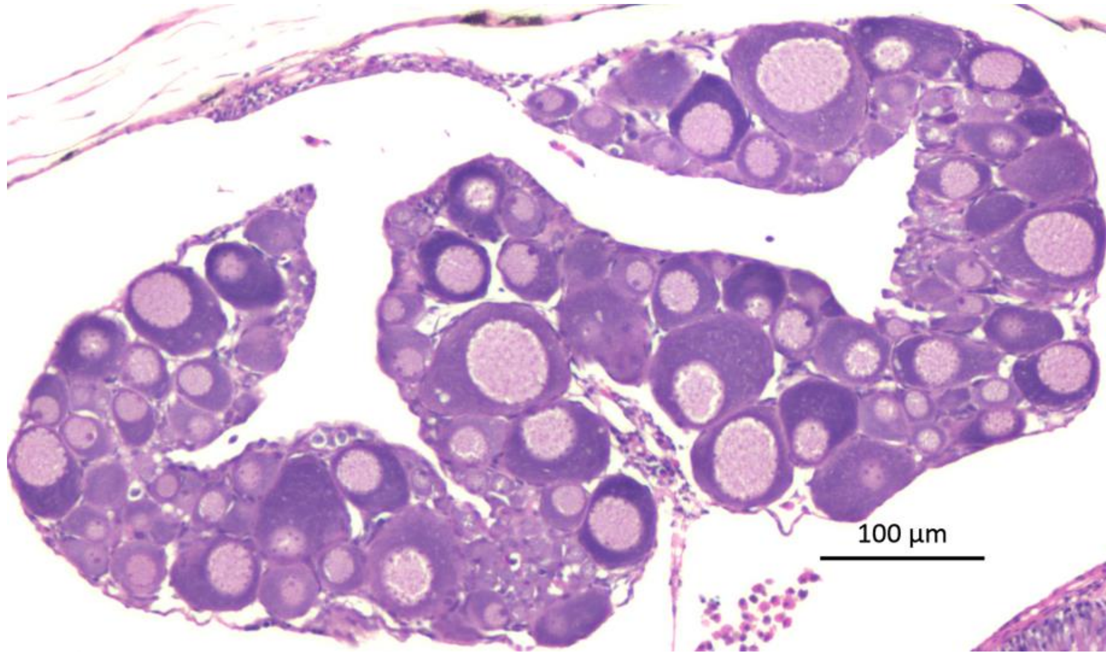
2909

2910 ***High EE_2 concentration treatment***

2911


2912 **Figure A.1.2.21:** Fish identification T4-11 yellow, female ovary (10X)

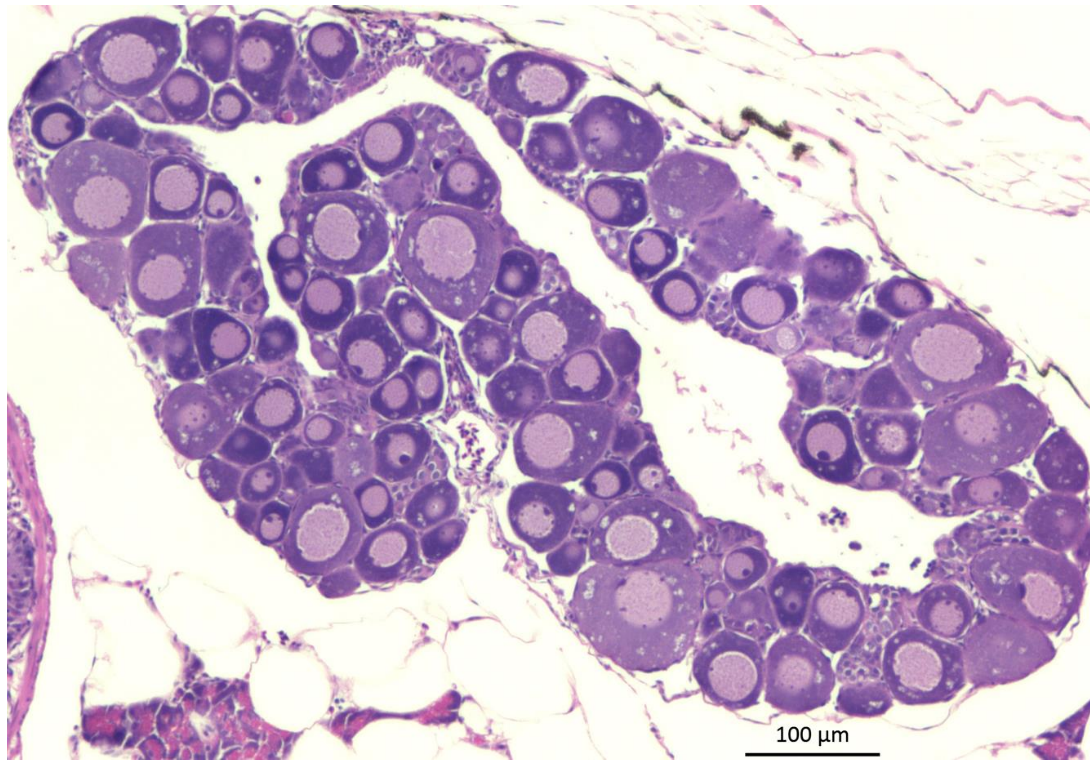
2913



2914 **Figure A.1.2.22:** Fish identification T4-12 blue, female ovary (10X)

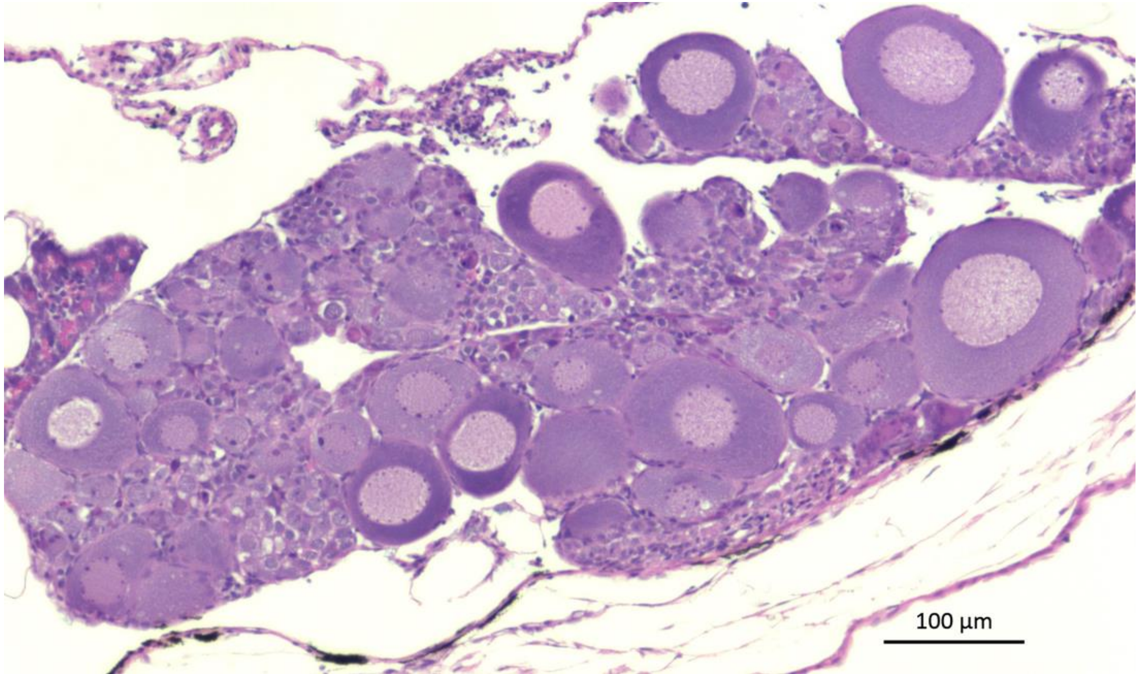
2915

2916



2917 **Figure A.1.2.23:** Fish identification T4-3 yellow, female ovary (10X)

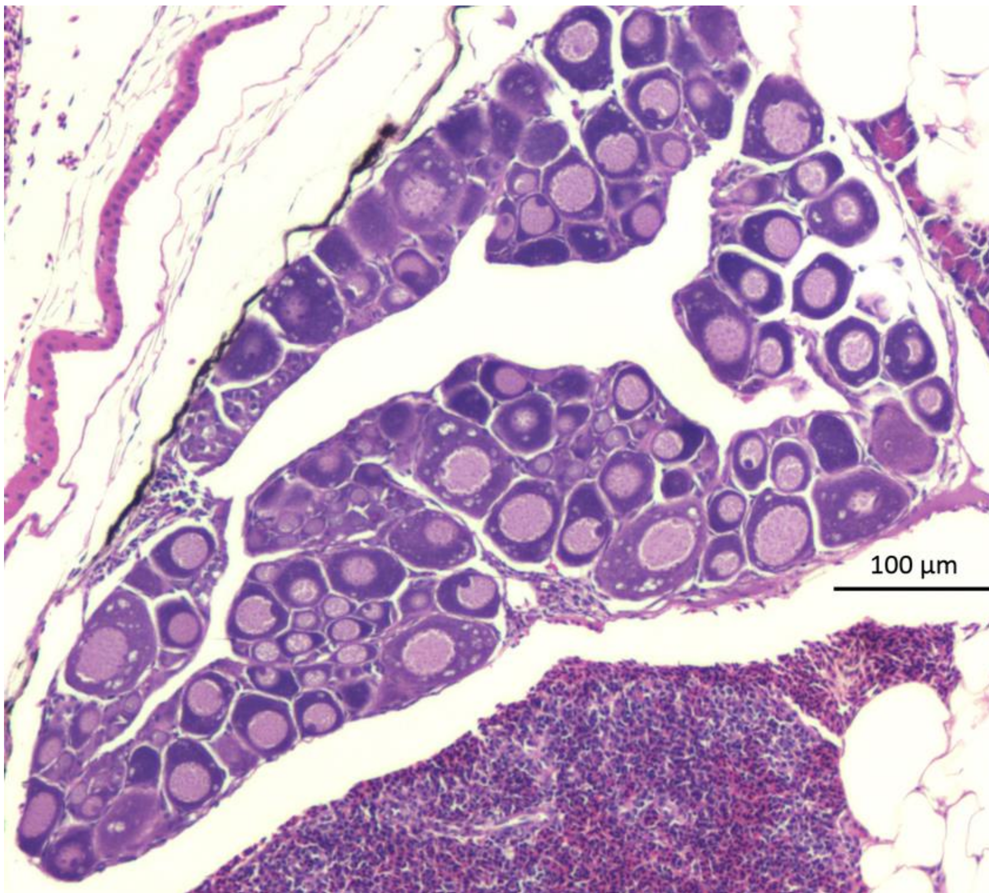
2918



2919 **Figure A.1.2.24:** Fish identification T5-1 green, female ovary (10X)

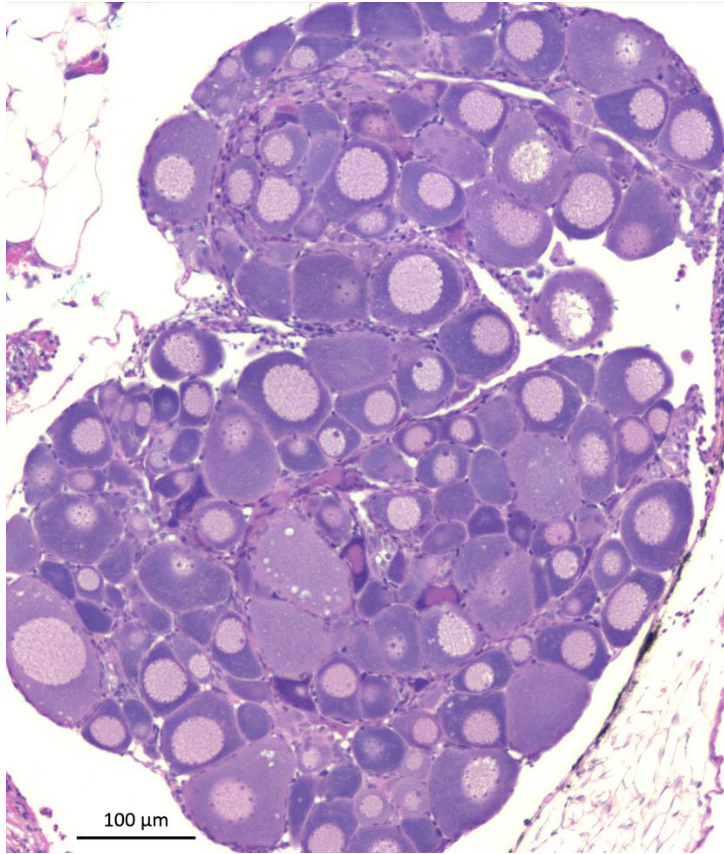
2920

2921



2922 **Figure A.1.2.25:** Fish identification T5-7 green, female ovary (10X)

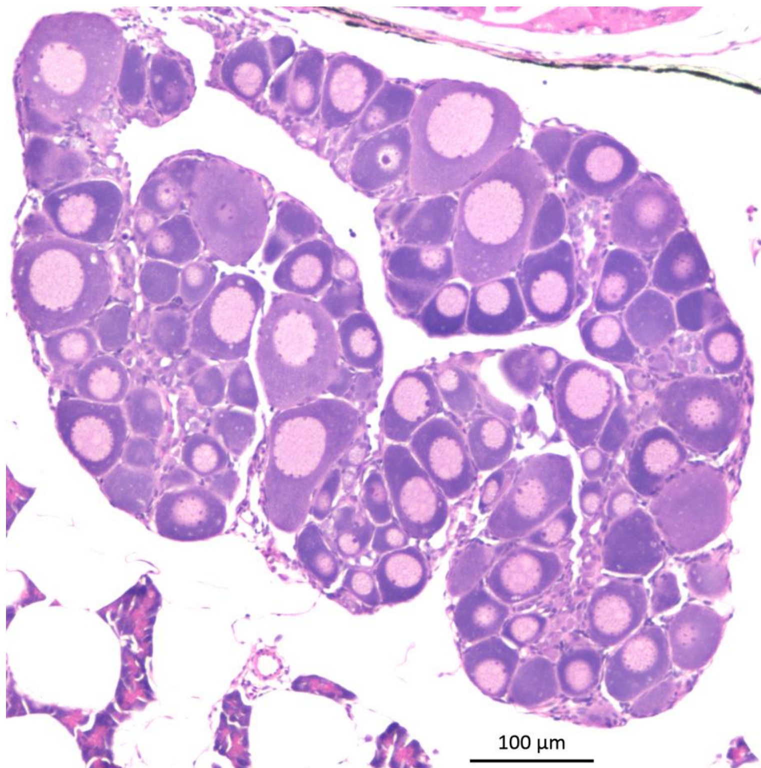
2923



2924 **Figure A.1.2.26:** Fish identification T5-3 green, female ovary (10X)

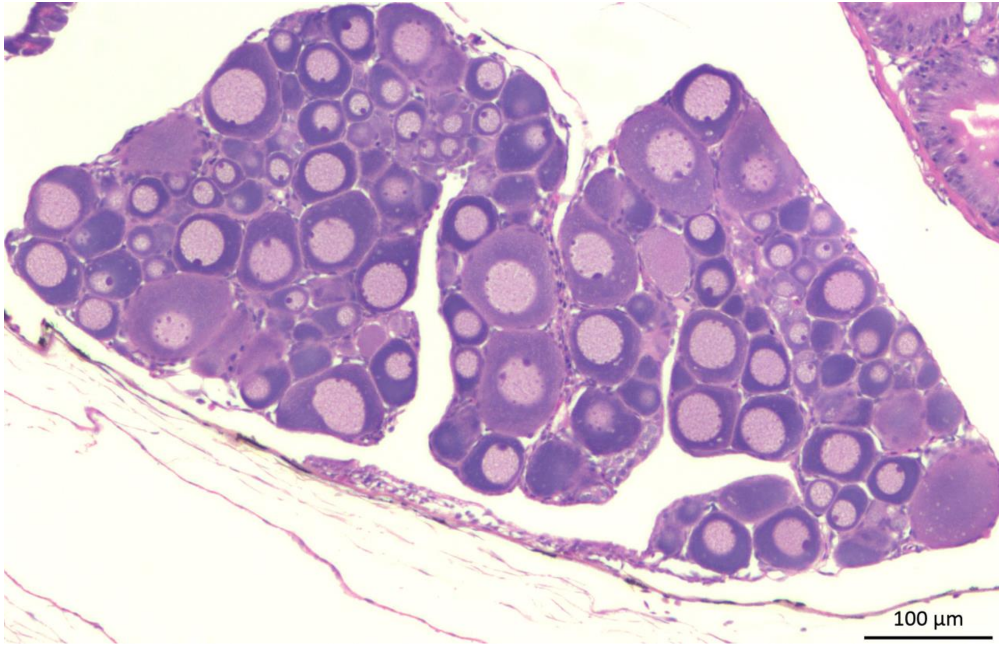
2925

2926



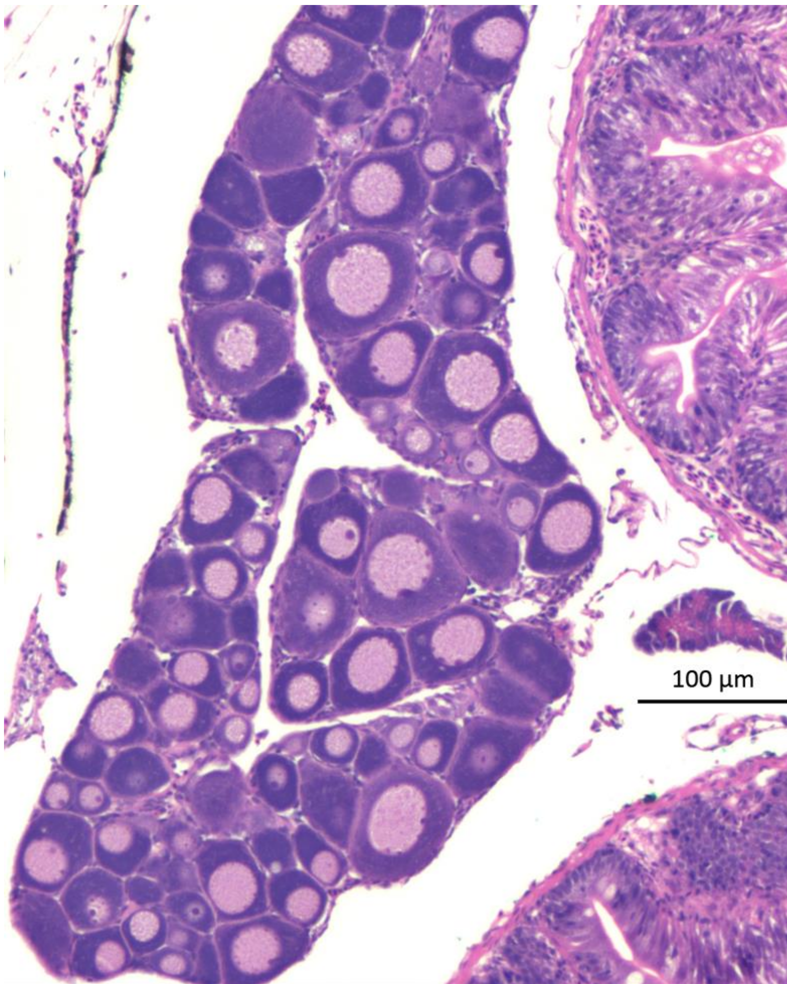
2927 **Figure A.1.2.27:** Fish identification T6-7 orange, female ovary (10X)

2928



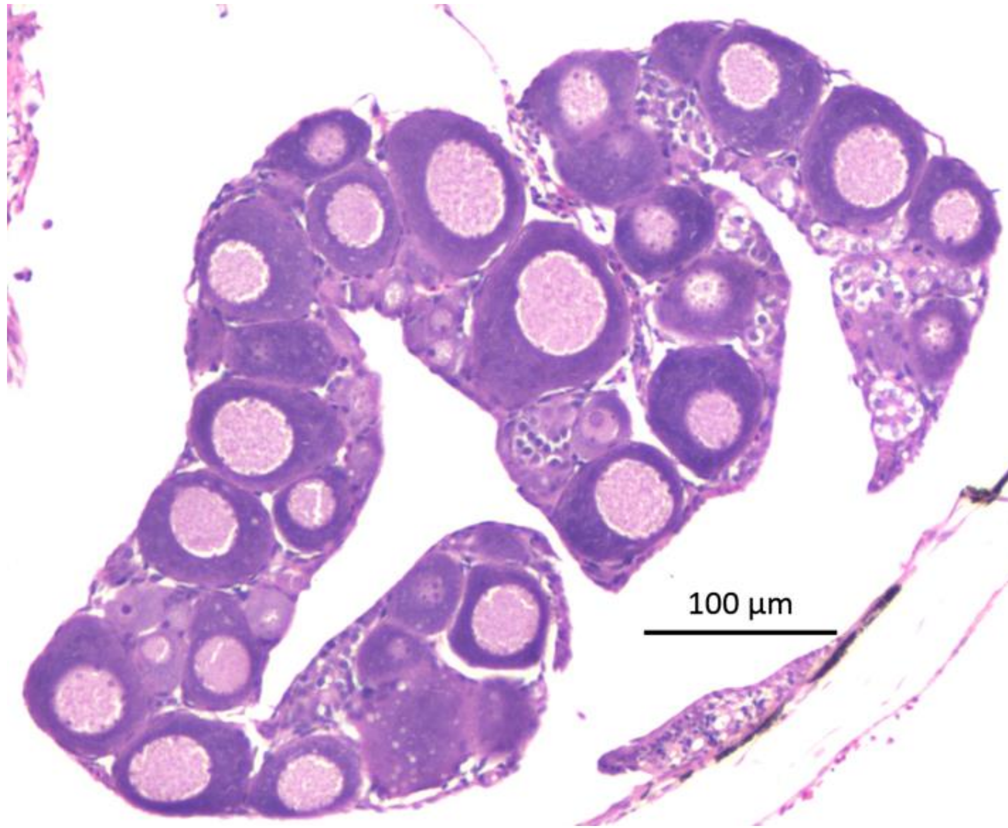
2929 **Figure A.1.2.28:** Fish identification T6-1 black, female ovary (10X)

2930



2931

2932 **Figure A.1.2.29:** Fish identification T6-11 green, female ovary (10X)

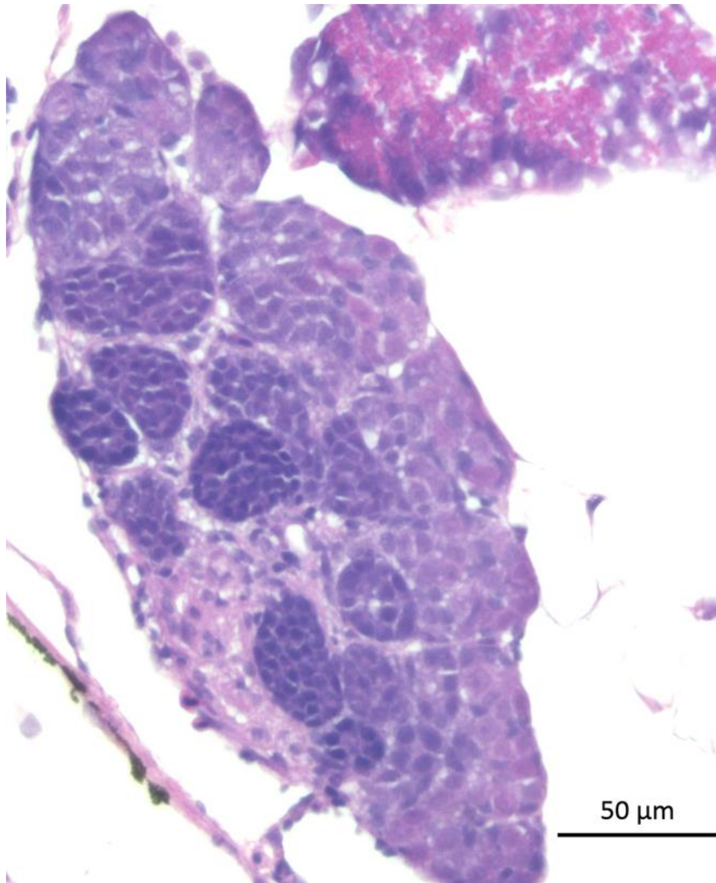


2933
2934 **Figure A.1.2.30:** Fish identification T6-2 green, female ovary (10X)
2935

2936 ## A1.3 Testis histology figures

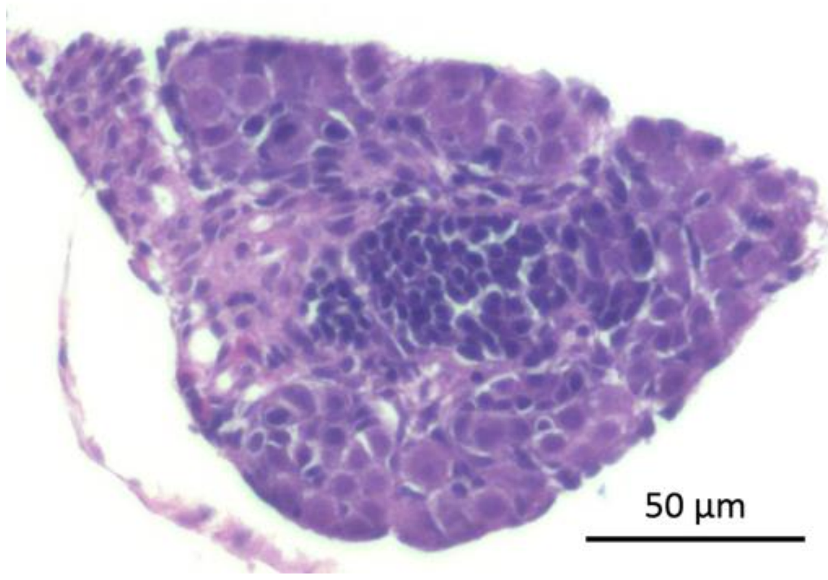
2937 Figures in this section (A.1.3.1-A1.3.30) represent the histological slides (20X or 40X)
2938 used to evaluate the male mummichog gametogenesis data for Experiment 2. Each slide
2939 includes fish identification being Tank number [T1-3 are control treatment (0 ng/L EE_2);
2940 T4-6 are high EE_2 concentration treatment 10 ng/L EE_2; T7-9 are low EE_2 concentration
2941 treatment (2 ng/L EE_2)], cassette number and dye used to colour coordinate the fish
2942 (A1.1).
2943

2944 ***Control treatment***



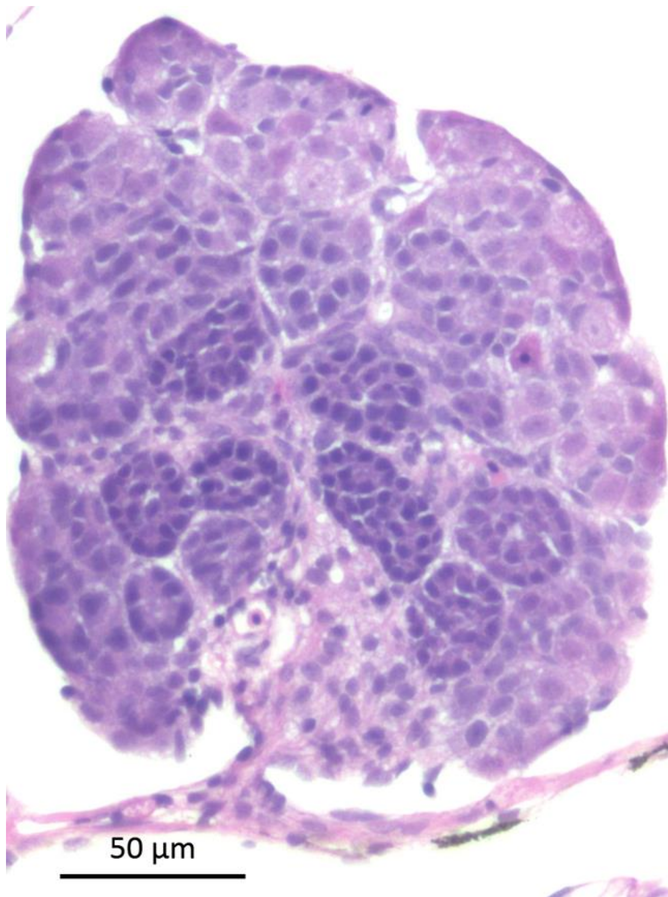
2945
2946 **Figure A.1.3.1:** Fish identification T1-7 yellow, male testis (20X)

2947



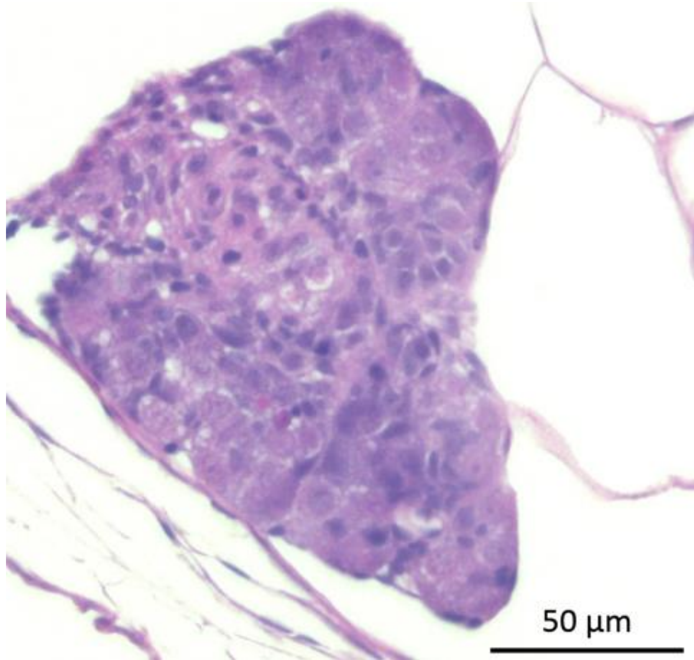
2948 **Figure A.1.3.2:** Fish identification T1-12 blue, male testis (20X)

2949



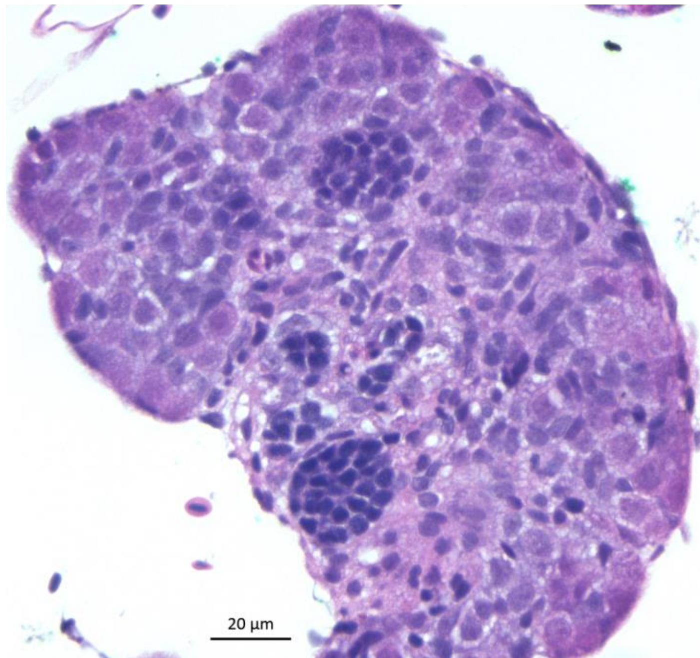
2950 **Figure A.1.3.3:** Fish identification T1-12 green, male testis (20X)

2951



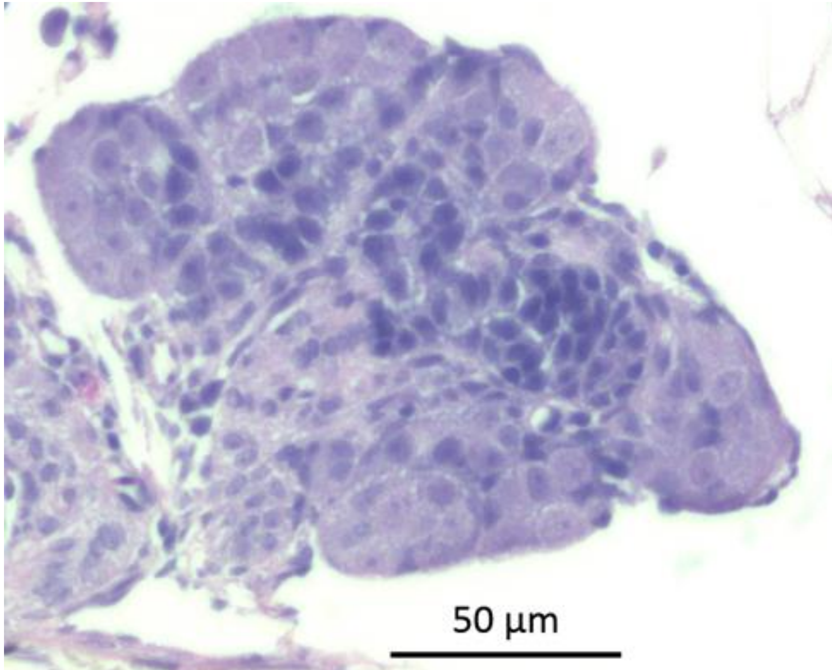
2952 **Figure A.1.3.4:** Fish identification T2-10 yellow, male testis (20X)

2953



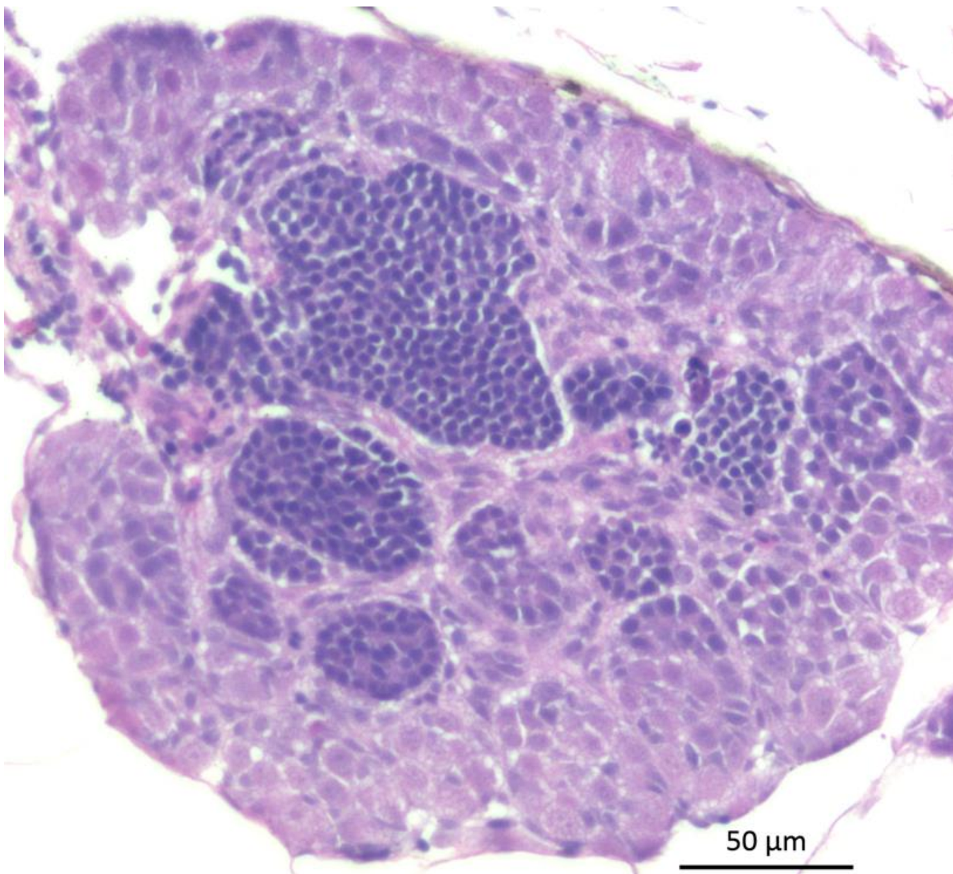
2954 **Figure A.1.3.5:** Fish identification T2-1 green, male testis (40X)

2955



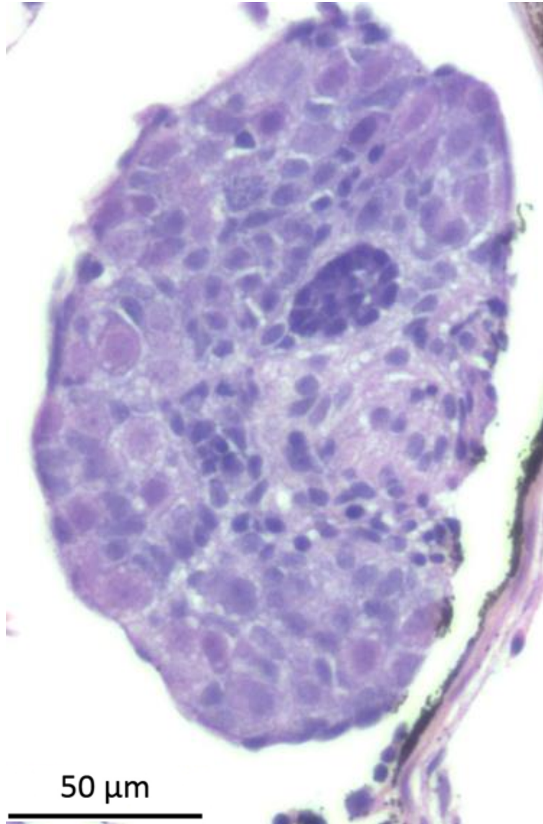
2956 **Figure A.1.3.6:** Fish identification T2-11 blue, male testis (20X)

2957



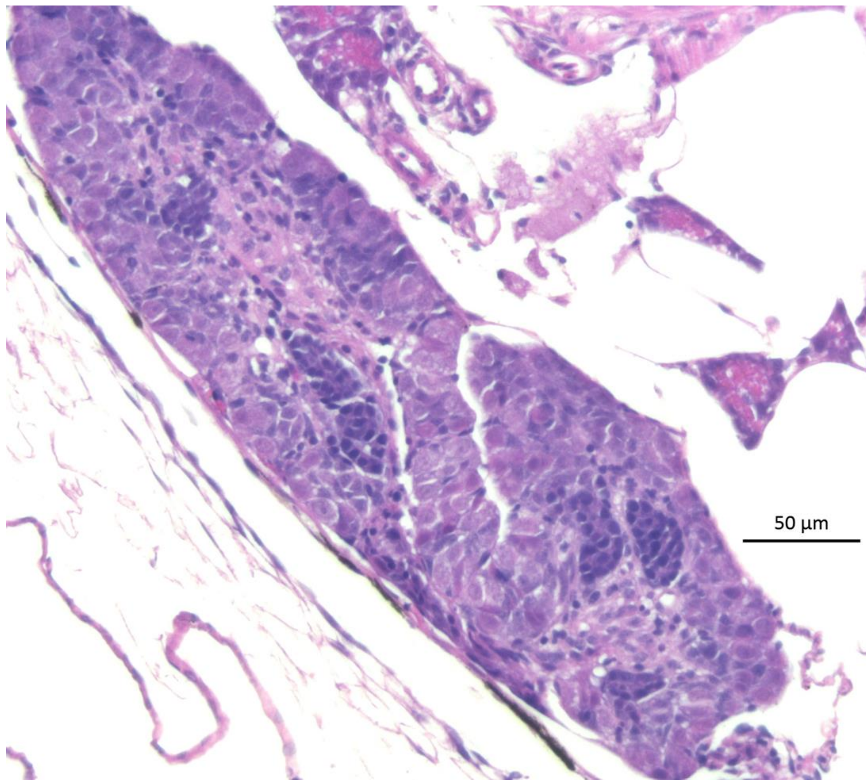
2958 **Figure A.1.3.7:** Fish identification T3-4 black, male testis (20X)

2959



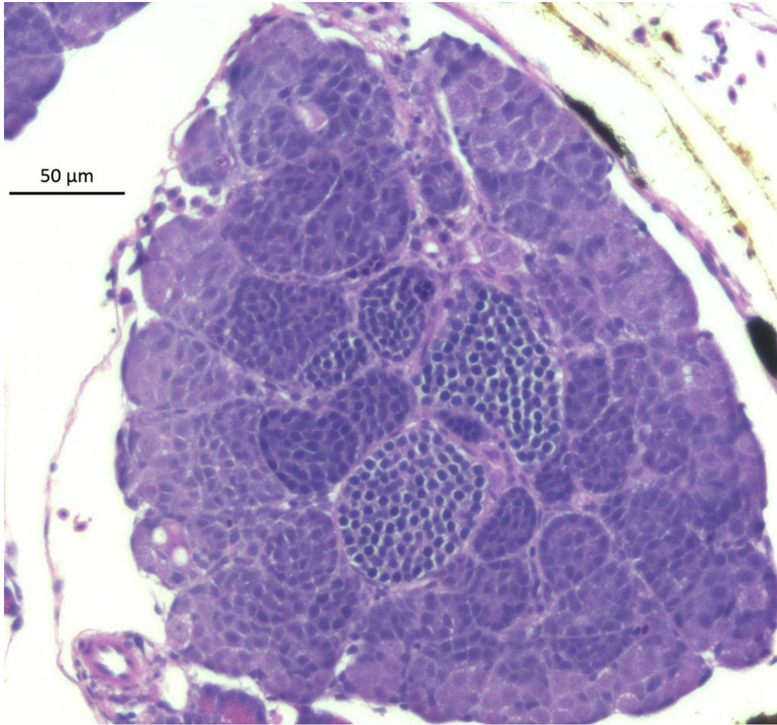
2960 **Figure A.1.3.8:** Fish identification T3-7 orange, male testis (20X)

2961



2962 **Figure A.1.3.9:** Fish identification T3-10 green, male testis (20X)

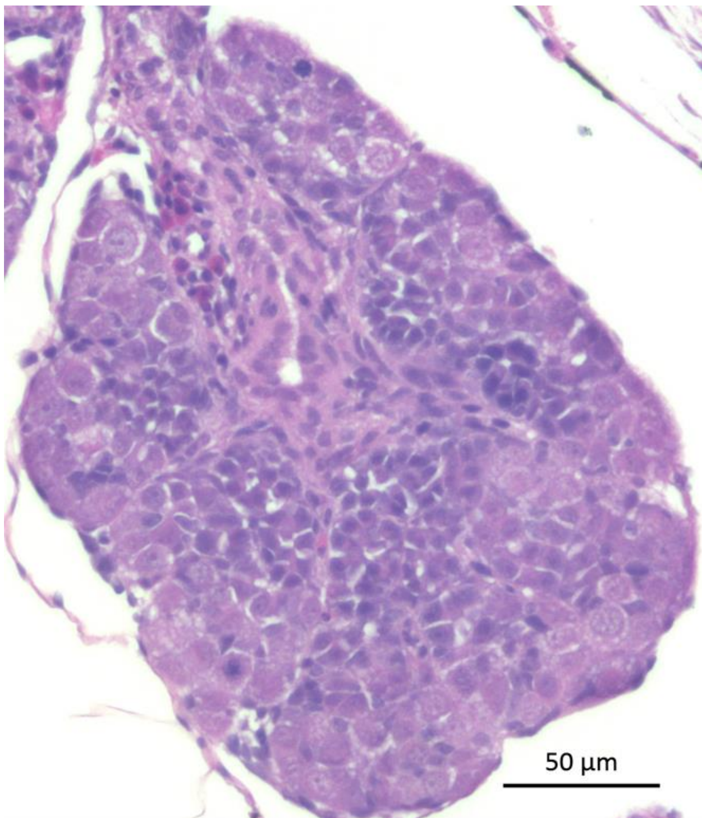
2963



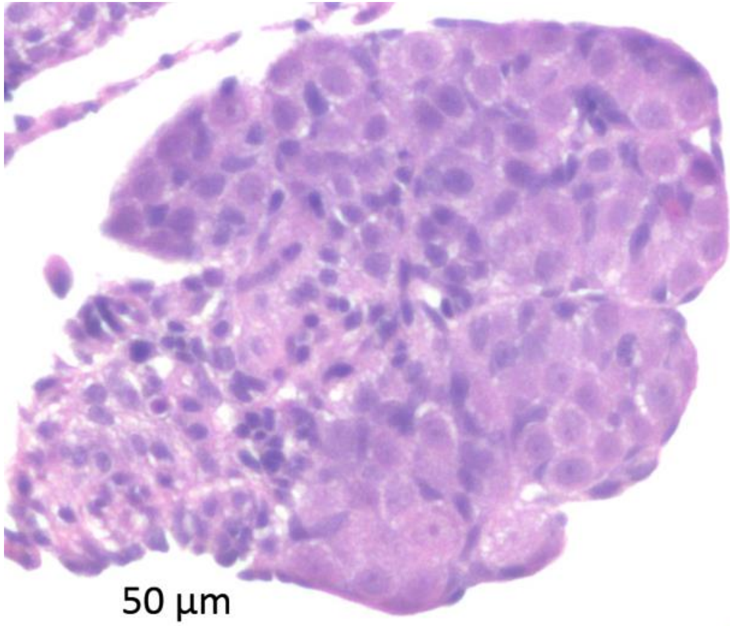
2964 **Figure A.1.3.10:** Fish identification T3-8 yellow, male testis (20X)

2965 ***Low EE_2 concentration treatment***

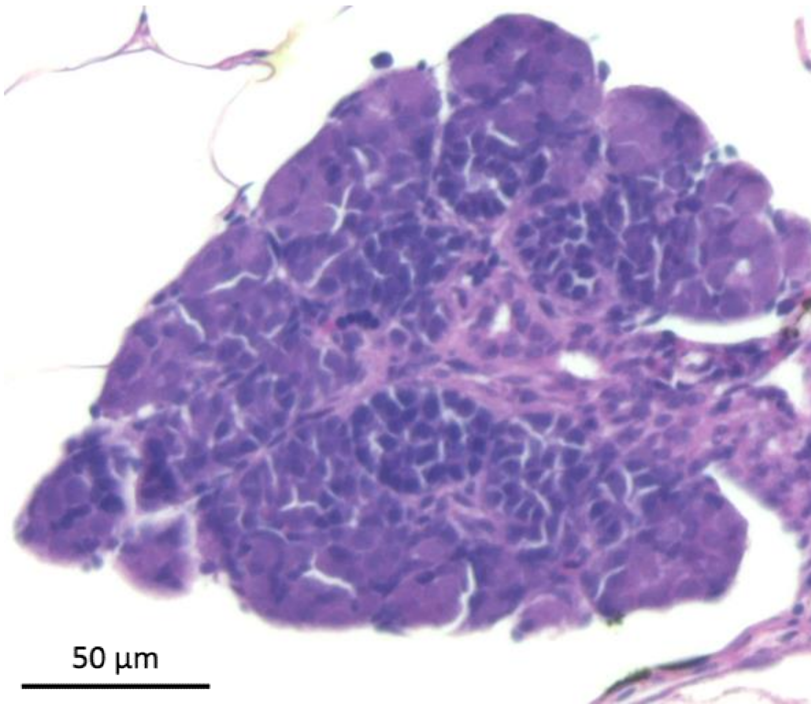
2966



2967 **Figure A.1.3.11:** Fish identification T7-2 yellow, male testis (20X)

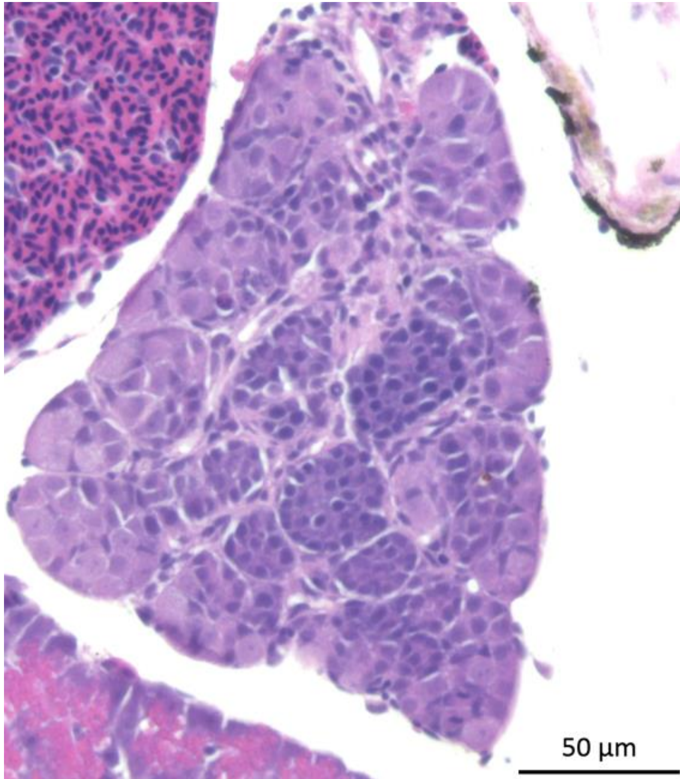


2968
2969 **Figure A.1.3.12:** Fish identification T7-8 blue, male testis (20X)



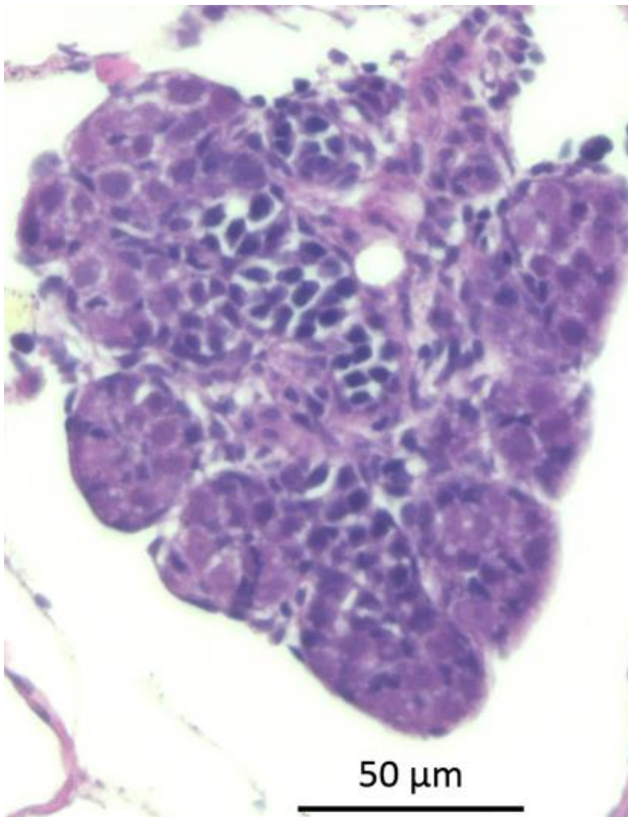
2970
2971 **Figure A.1.3.13:** Fish identification T7-11 green, male testis (20X)

2972

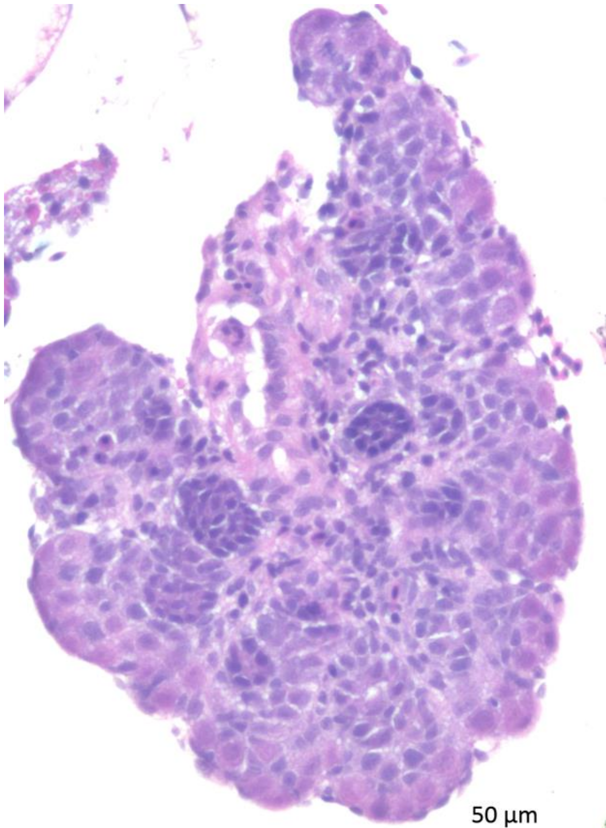


2973 **Figure A.1.3.14:** Fish identification T7-8 yellow, male testis (20X)

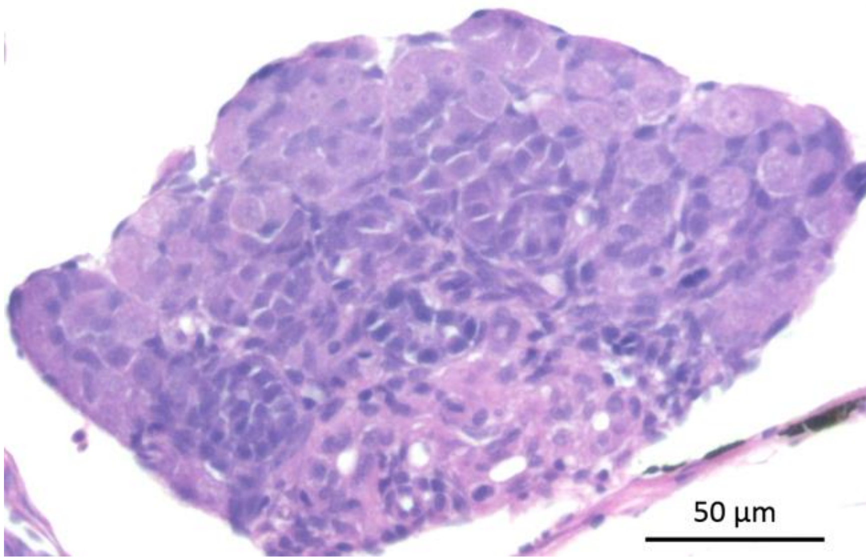
2974



2975 **Figure A.1.3.15:** Fish identification T8-8 green, male testis (20X)

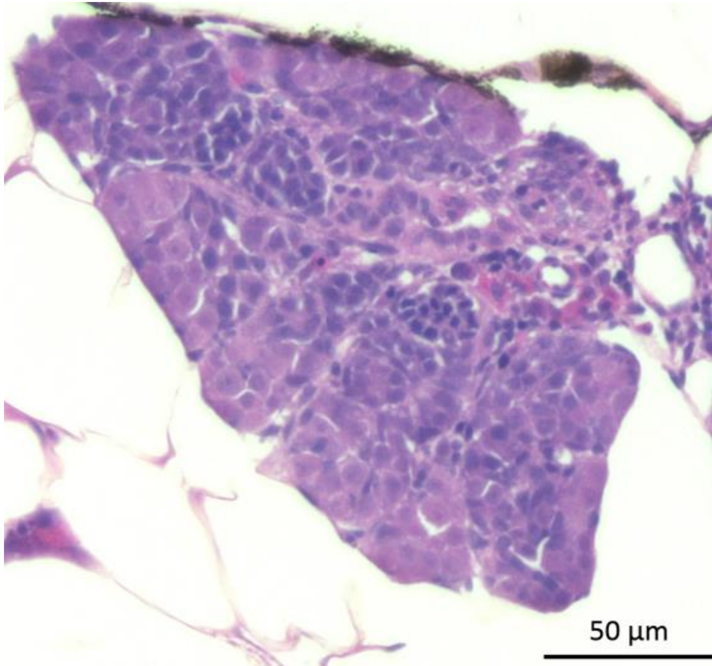
2976


2977 **Figure A.1.3.16:** Fish identification T8-1 blue, male testis (20X)

2978


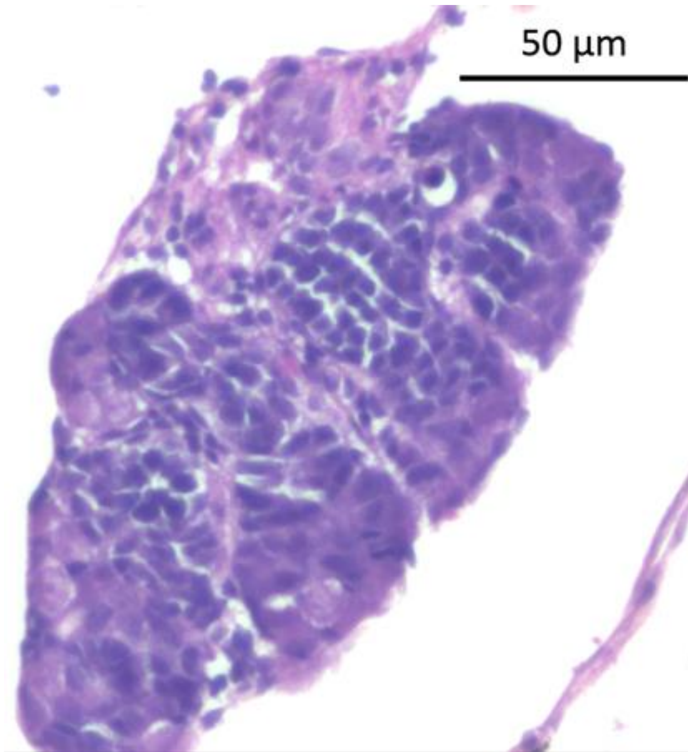
2979 **Figure A.1.3.17:** Fish identification T8-5 yellow, male testis (20X)

2980



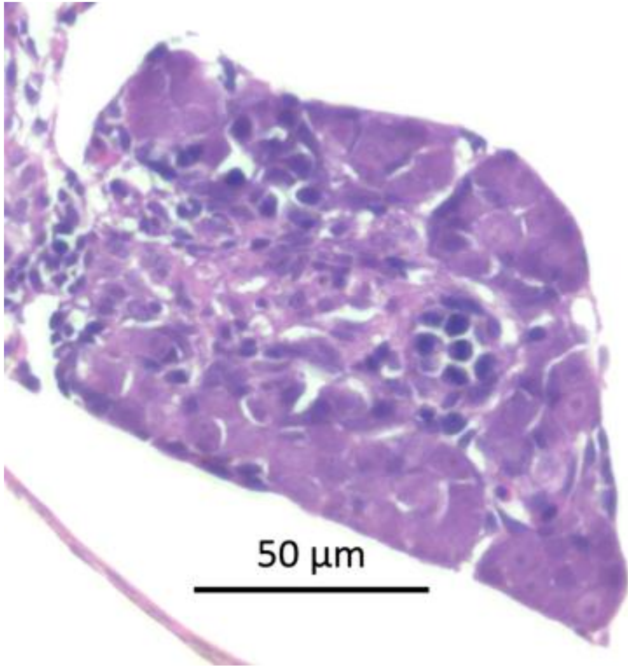
2981 **Figure A.1.3.18:** Fish identification T9-4 orange, male testis (20X)

2982



2983 **Figure A.1.3.19:** Fish identification T9- 6 blue, male testis (20X)

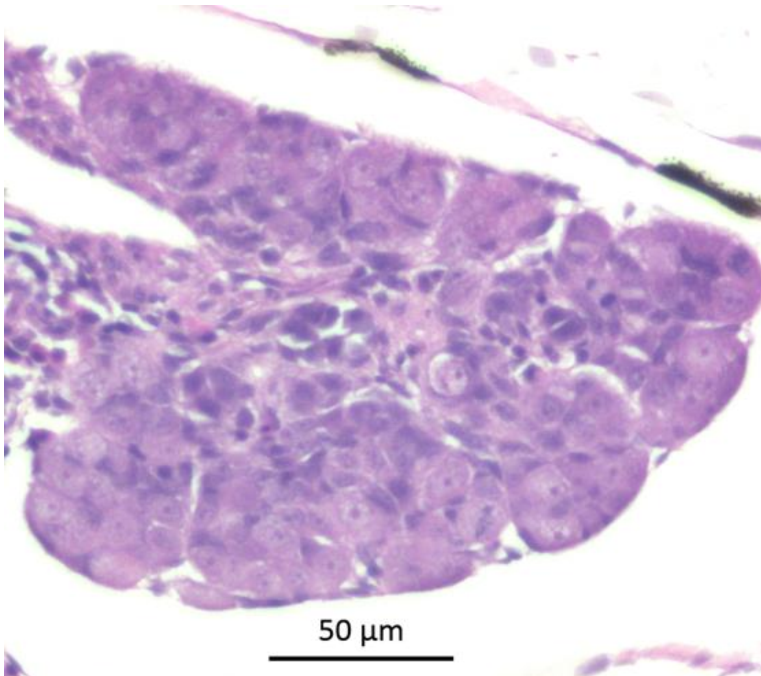
2984



2985 **Figure A.1.3.20:** Fish identification T9-11 black, male testis (20X)

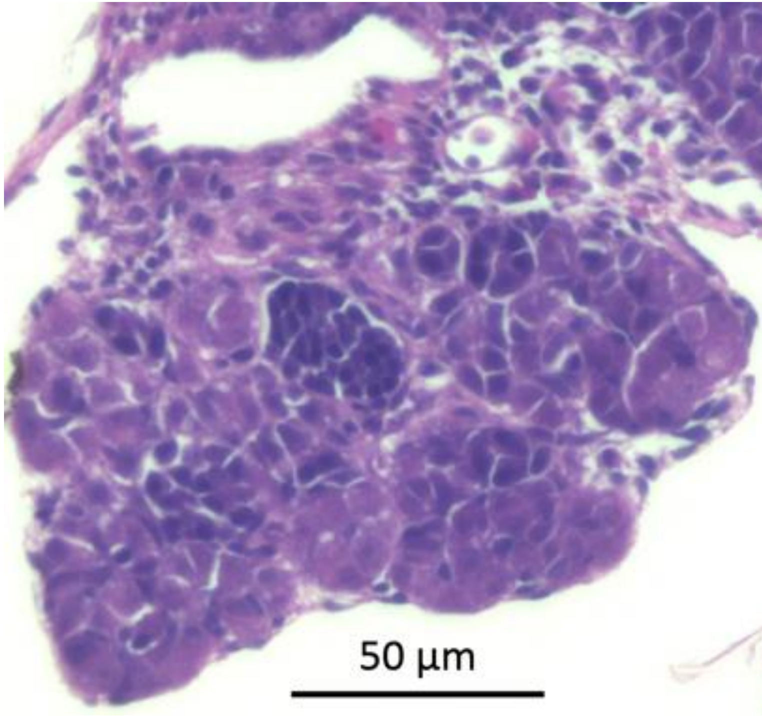
2986 ***High EE_2 concentration treatment***

2987



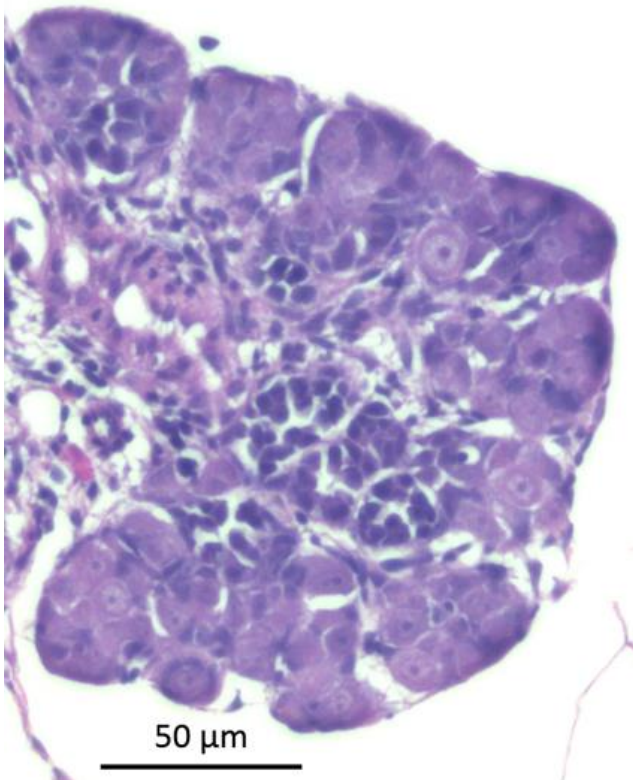
2988 **Figure A.1.3.21:** Fish identification T4-8 orange, male testis (20X)

2989

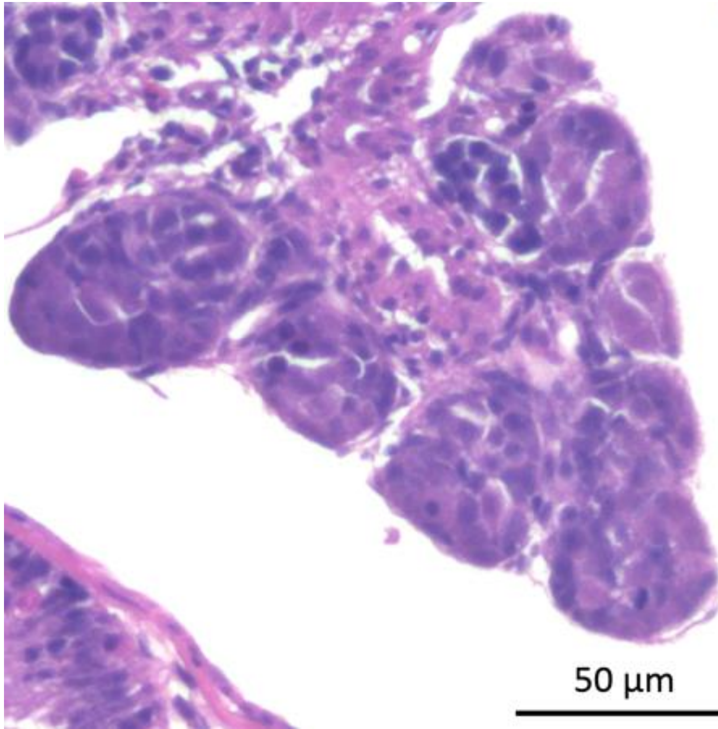


2990 **Figure A.1.3.22:** Fish identification T4-1 orange, male testis (20X)

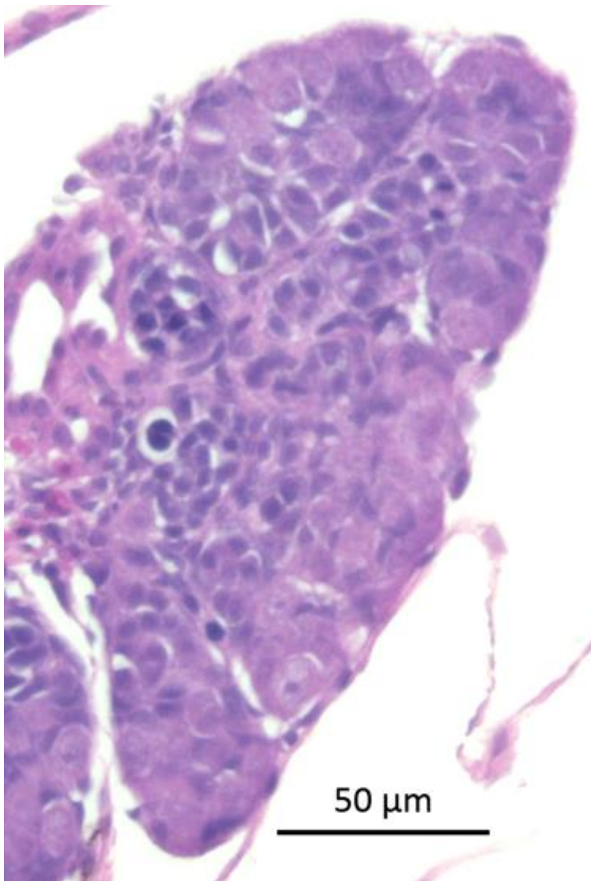
2991



2992 **Figure A.1.3.23:** Fish identification T4-9 green, male testis (20X)

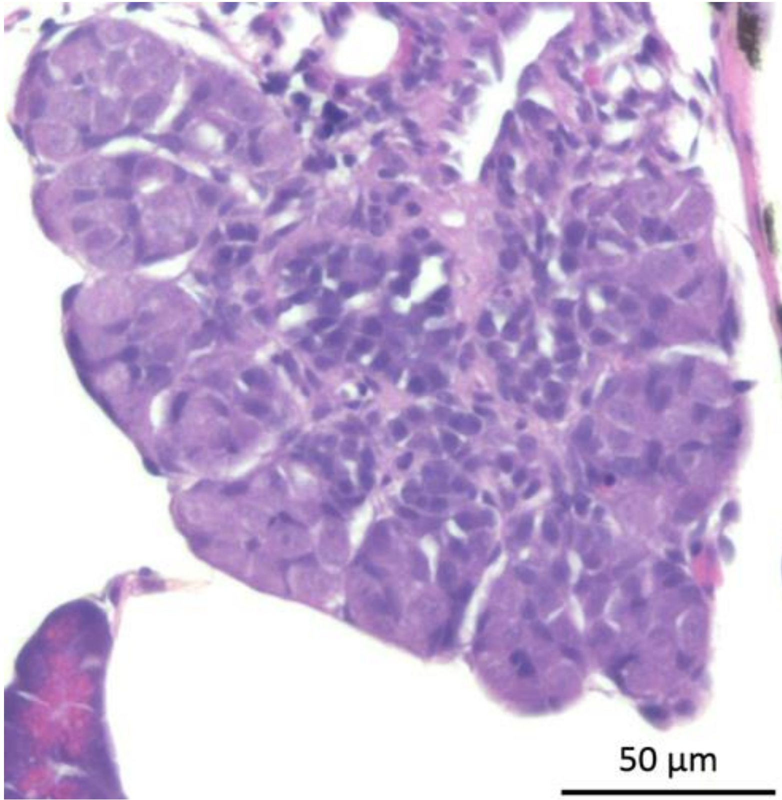
2993


2994 **Figure A.1.3.24:** Fish identification T5-2 blue, male testis (20X)

2995


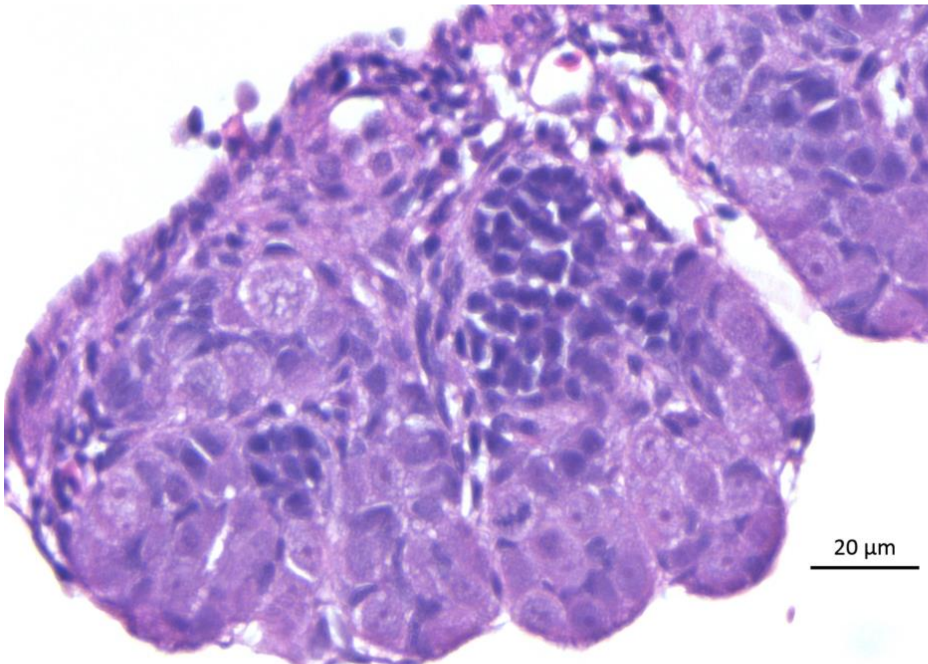
2996 **Figure A.1.3.25:** Fish identification T5-10 blue, male testis (20X)

2997



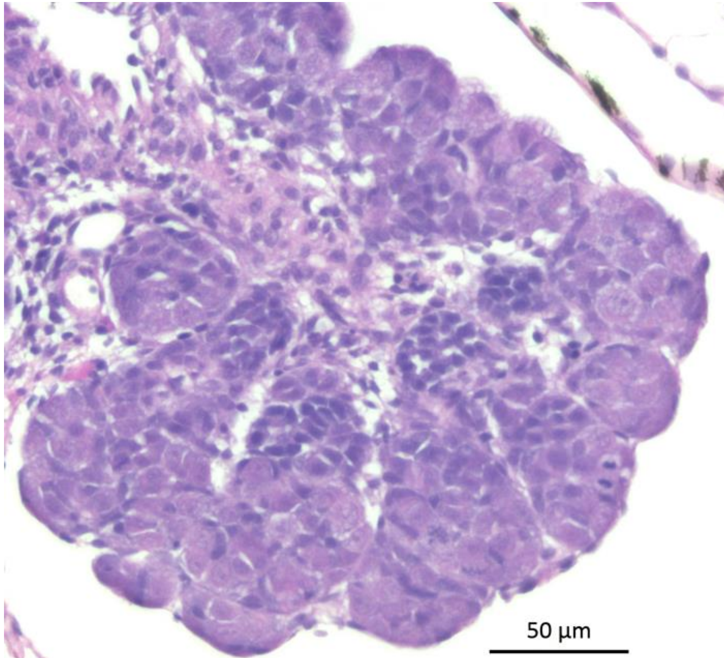
2998 **Figure A.1.3.26:** Fish identification T5-1 black, male testis (20X)

2999



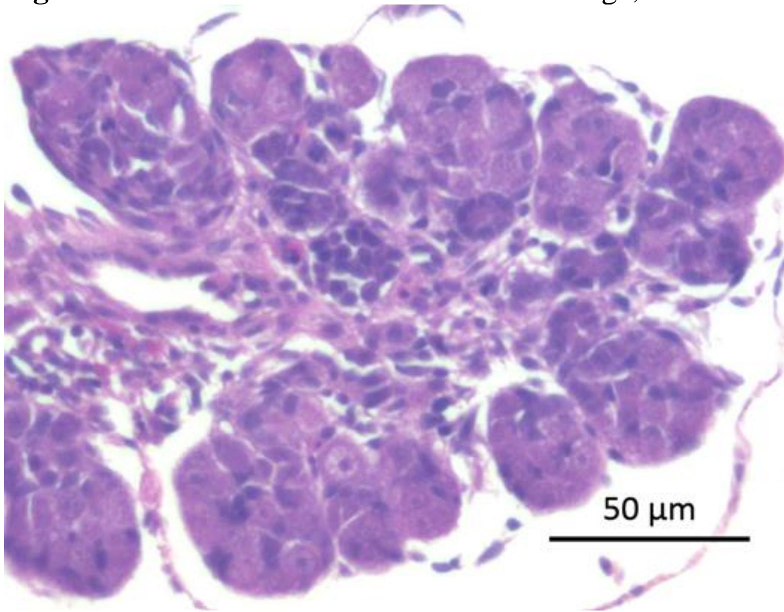
3000 **Figure A.1.3.27:** Fish identification T5-4 orange, male testis (40X)

3001



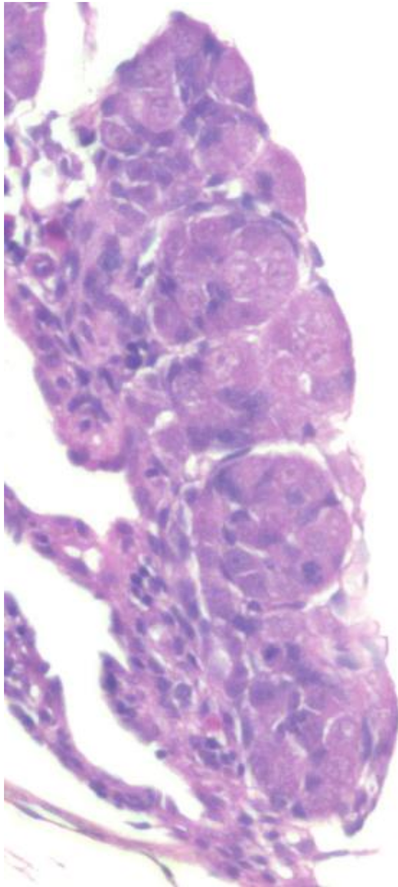
3002 **Figure A.1.3.28:** Fish identification T6-4 orange, male testis (20X)

3003



3004 **Figure A.1.3.29:** Fish identification T6-8 yellow, male testis (20X)

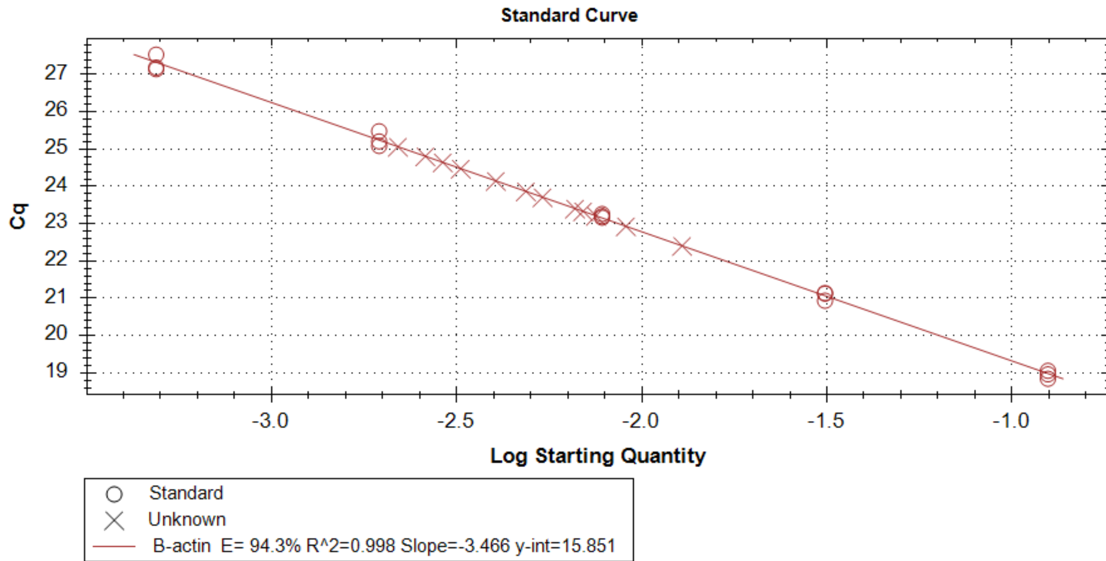
3005



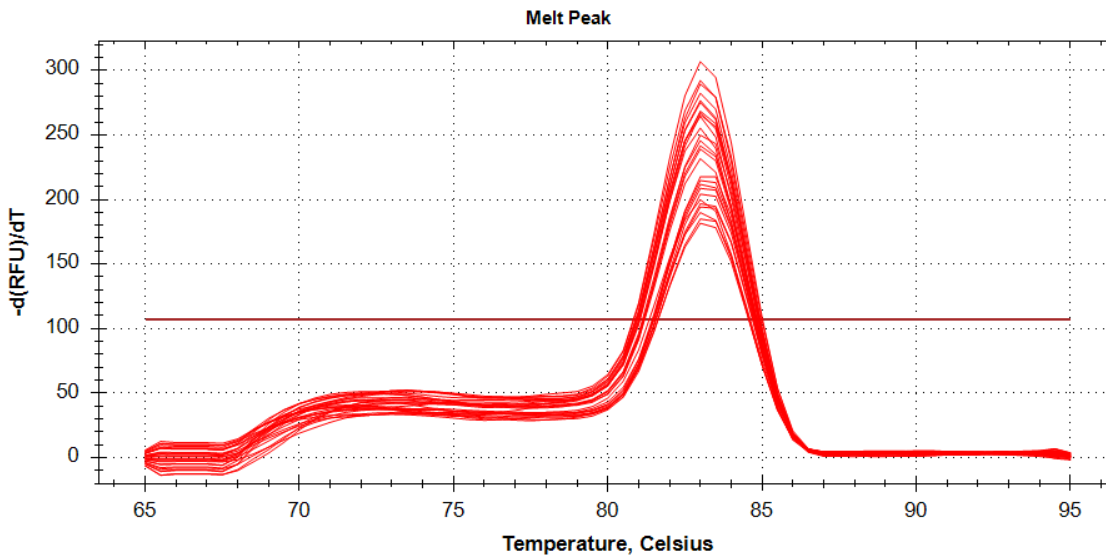
3006 **Figure A.1.3.30:** Fish identification T6-1 green, male testis (20X)

3007

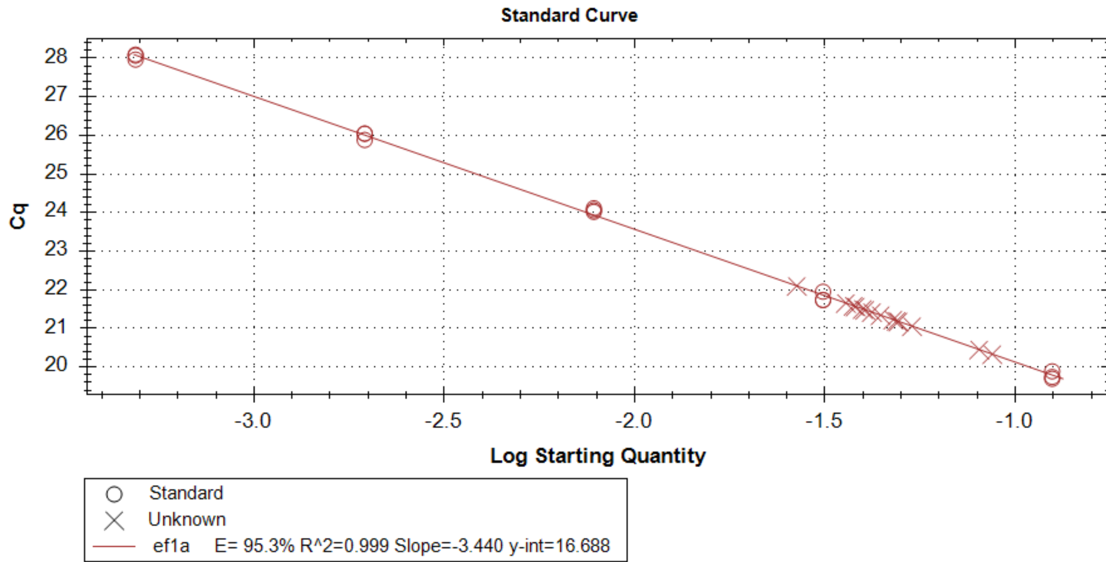
3008 ## A1.4 Reference gene expression
3009



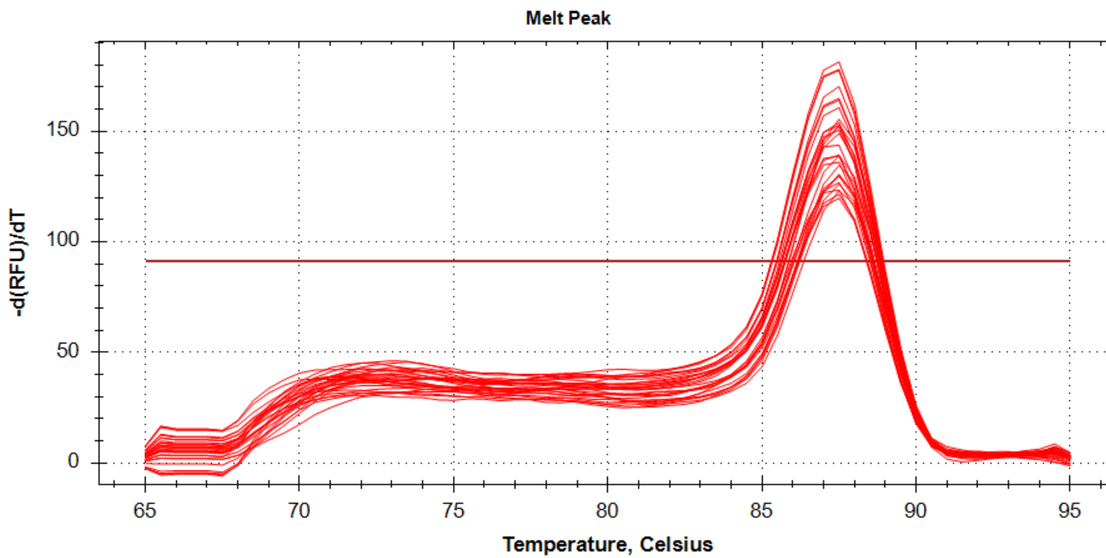
3010
3011 **Figure A.1.4.1:** Testis *βactin* gene expression Cq (cycle value). Average Cq is 23.84±
3012 0.24 (SEM) for testis samples from Experiment 2.



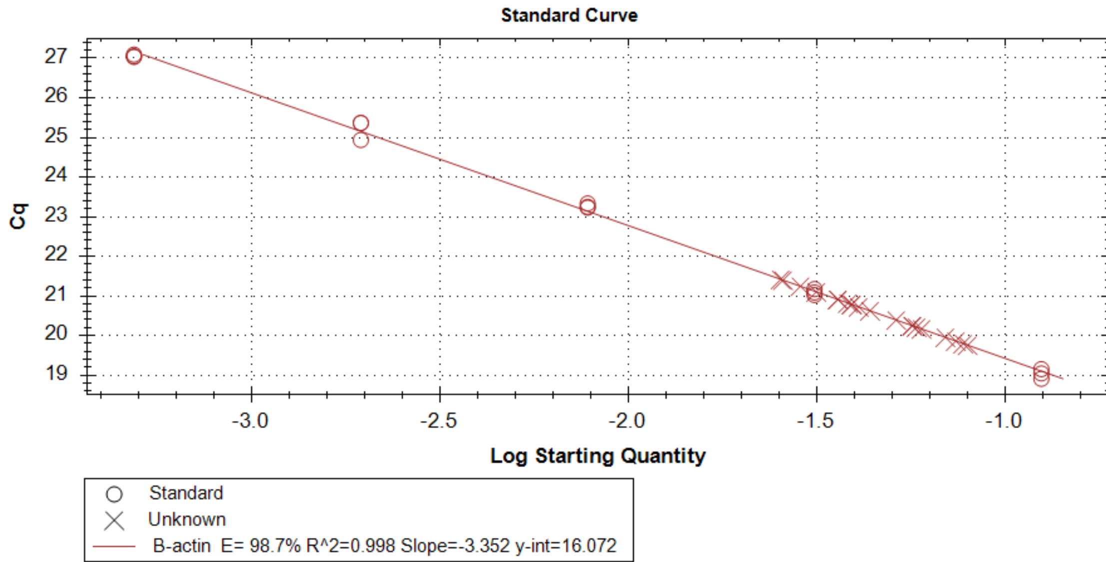
3013
3014 **Figure A.1.4.2:** Testis *βactin* melt curve indicating one product at 83°C.



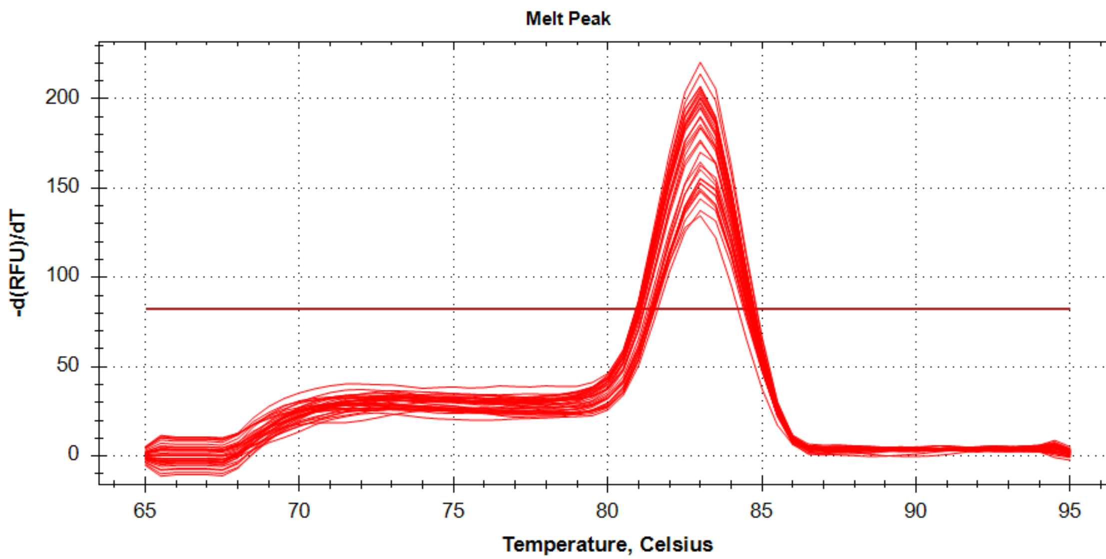
3015
3016 **Figure A.1.4.3:** Testis *ef1a* gene expression Cq (cycle value). Average Cq is 21.30± 0.12
3017 (SEM) for testis samples from Experiment 2.



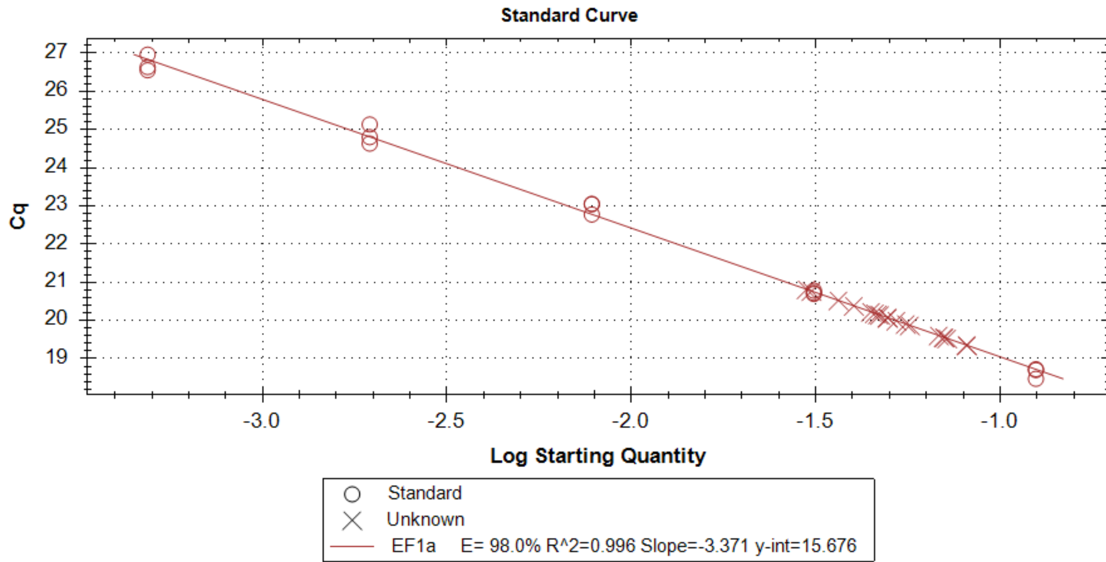
3018
3019 **Figure A.1.4.4:** Testis *ef1a* melt curve indicating one product at 87.5°C.



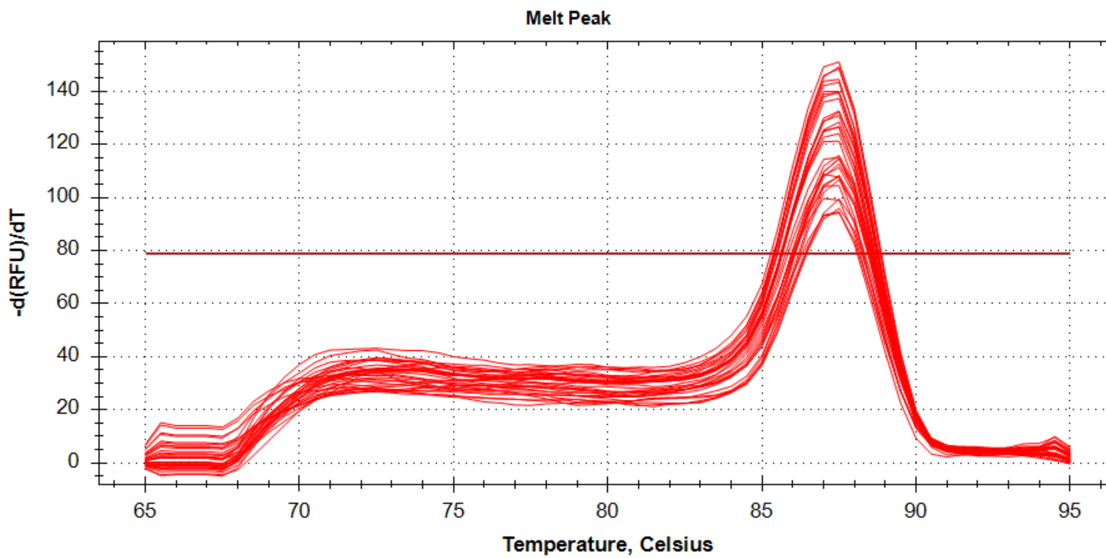
3020
3021 **Figure A.1.4.5:** Ovary *βactin* gene expression Cq (cycle value). Average Cq is 20.57±
3022 0.12 (SEM) for ovary samples from Experiment 2.
3023



3024
3025 **Figure A.1.4.6:** Ovary *βactin* melt curve indicating one product at 83°C.



3026
3027 **Figure A.1.4.7:** Ovary *ef1a* gene expression Cq (cycle value). Average Cq is 20.06± 0.10
3028 (SEM) for ovary samples from Experiment 2.



3029
3030 **Figure A.1.4.8:** Ovary *ef1a* melt curve indicating one product at 87.5°C.
3031

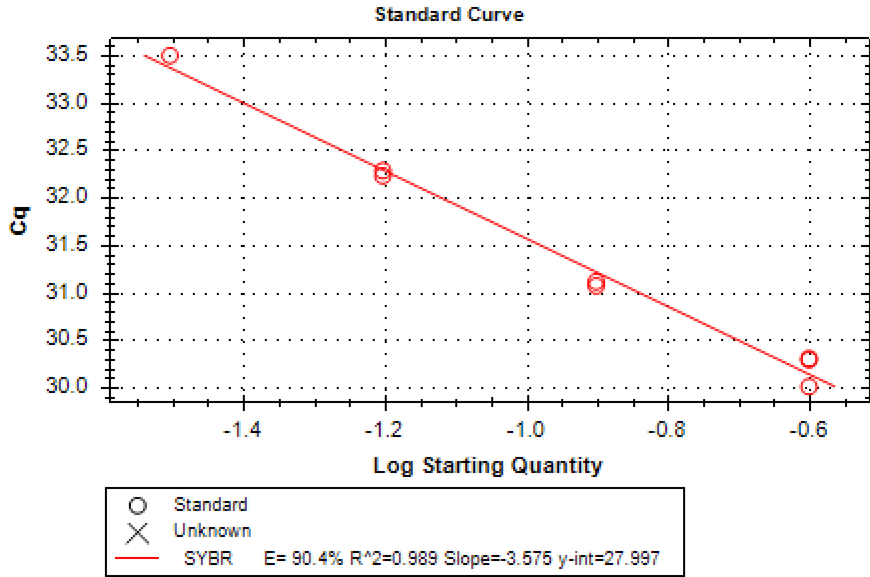
3032 **Table A4.1**: Stability values of the reference genes, *βactin* and *ef1a*, on pooled ovarian
3033 tissue provided by BIORAD gene study program.

Stability values: ovary	**Coefficient Variance**	**M value**
Acceptable value	*<.5*	*<1*
βactin	0.1736	0.4156
ef1a	0.1216	0.4156

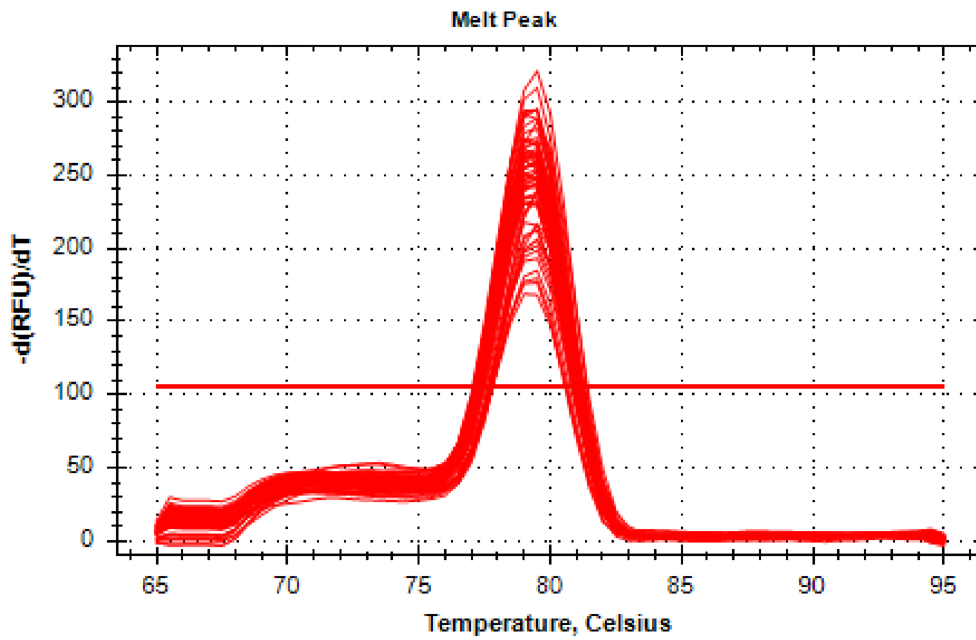
3034
3035 **Table A4.2**: Stability values of the reference genes, *βactin* and *ef1a*, on pooled testis
3036 tissue provided by BIORAD gene study program.

Stability values: testis	**Coefficient Variance**	**M value**
Acceptable value	*<.5*	*<1*
βactin	0.2373	0.8982
ef1a	0.473	0.8982

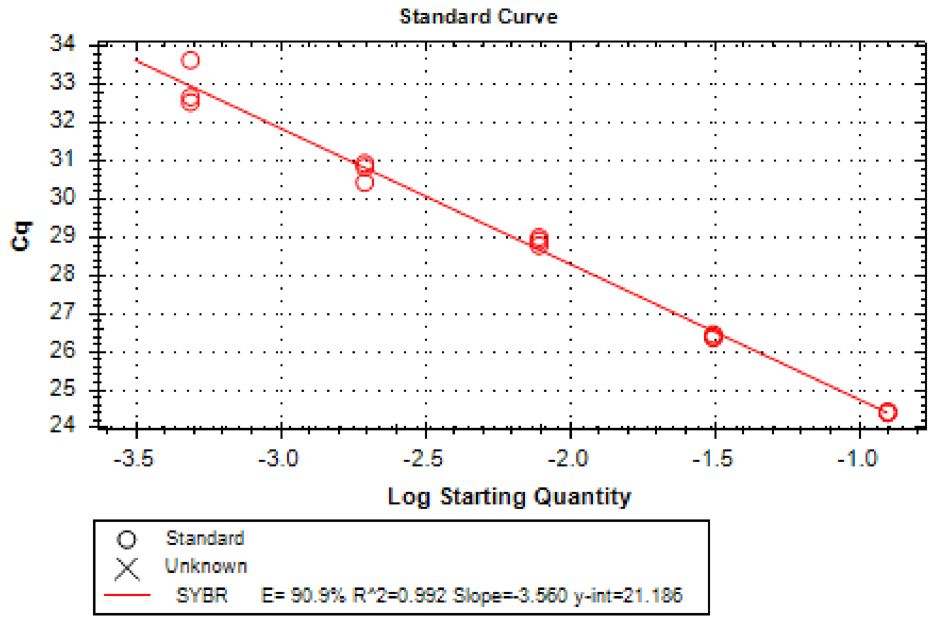
3037



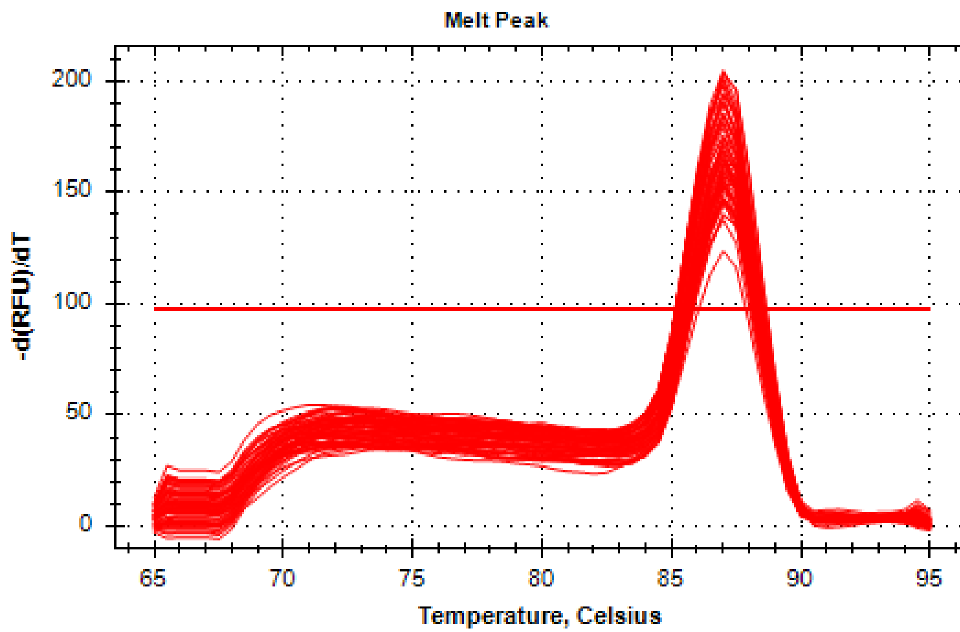
3038
3039 **Figure A.1.4.9:** *amh* gene expression Cq (cycle value) standard curve.
3040



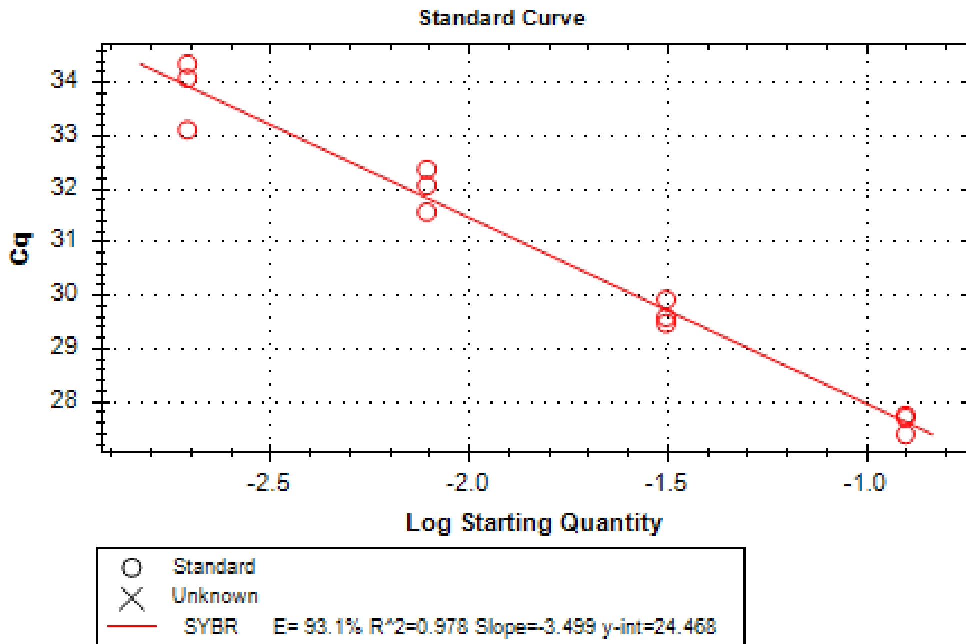
3041
3042 **Figure A.1.4.10:** *amh* melt curve indicating one product at 79.5°C.
3043



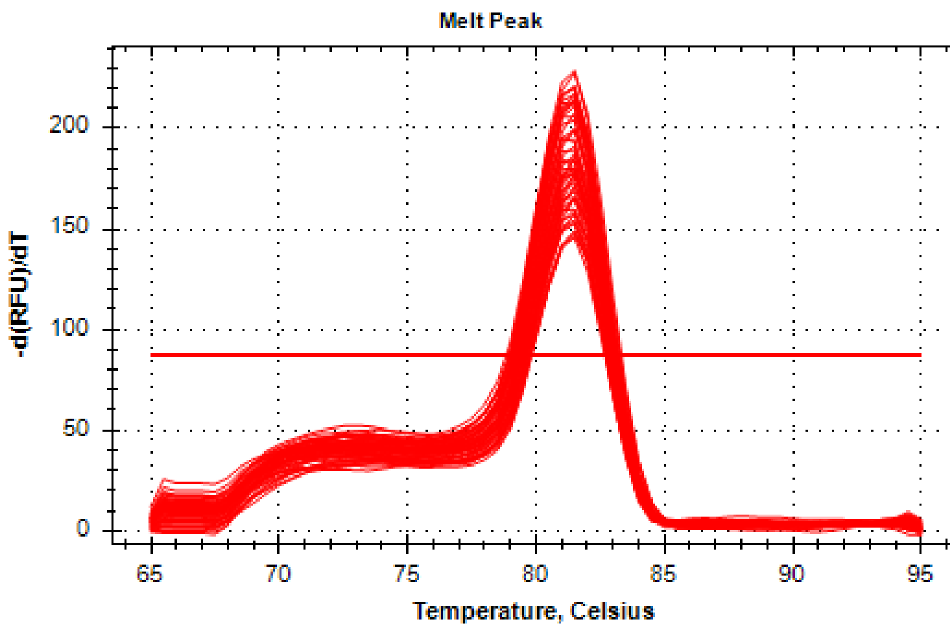
3044
3045 **Figure A.1.4.11:** *bmp15* gene expression Cq (cycle value) standard curve.



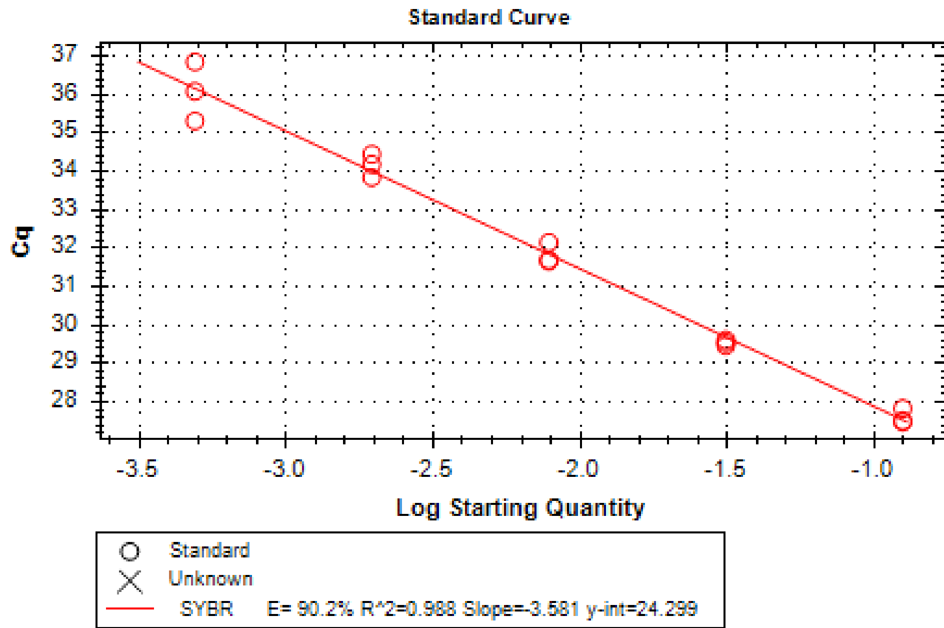
3046
3047 **Figure A.1.4.12:** *bmp15* melt curve indicating one product at 87°C.
3048



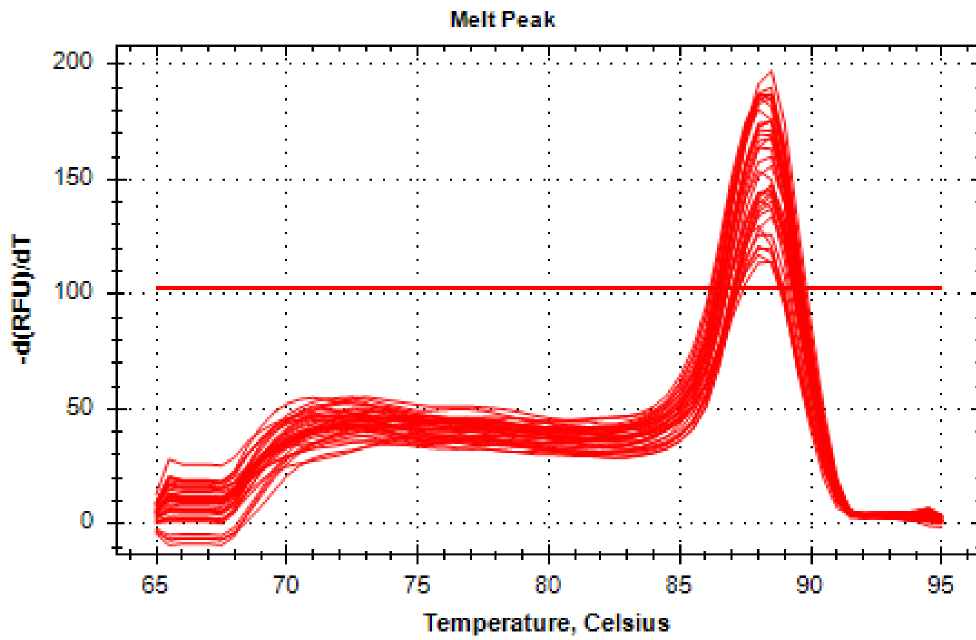
3049
3050 **Figure A.1.4.13:** *cyp19a1a* gene expression Cq (cycle value) standard curve.
3051



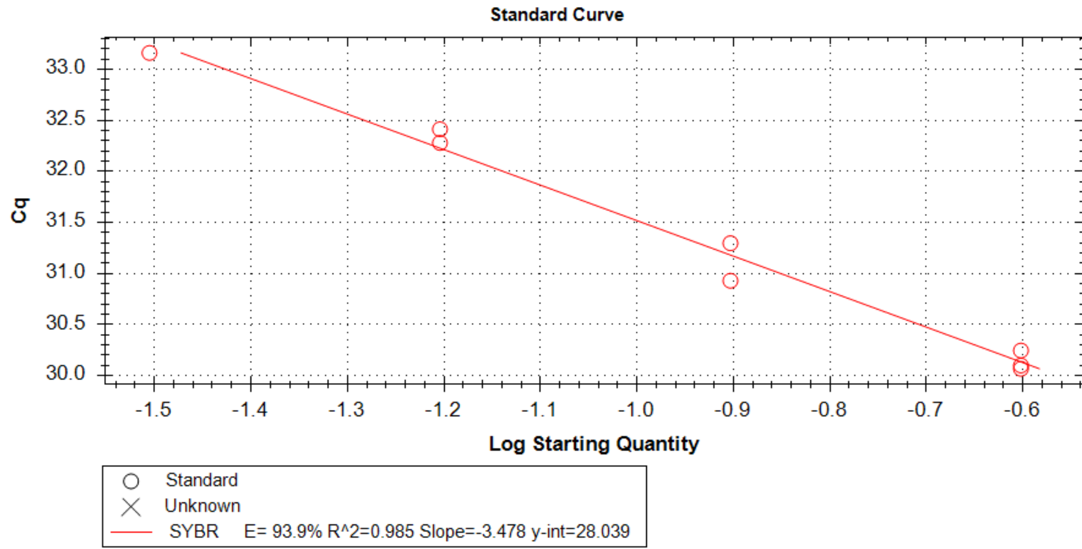
3052
3053 **Figure A.1.4.14:** *cyp19a1a* melt curve indicating one product at 81.5°C.



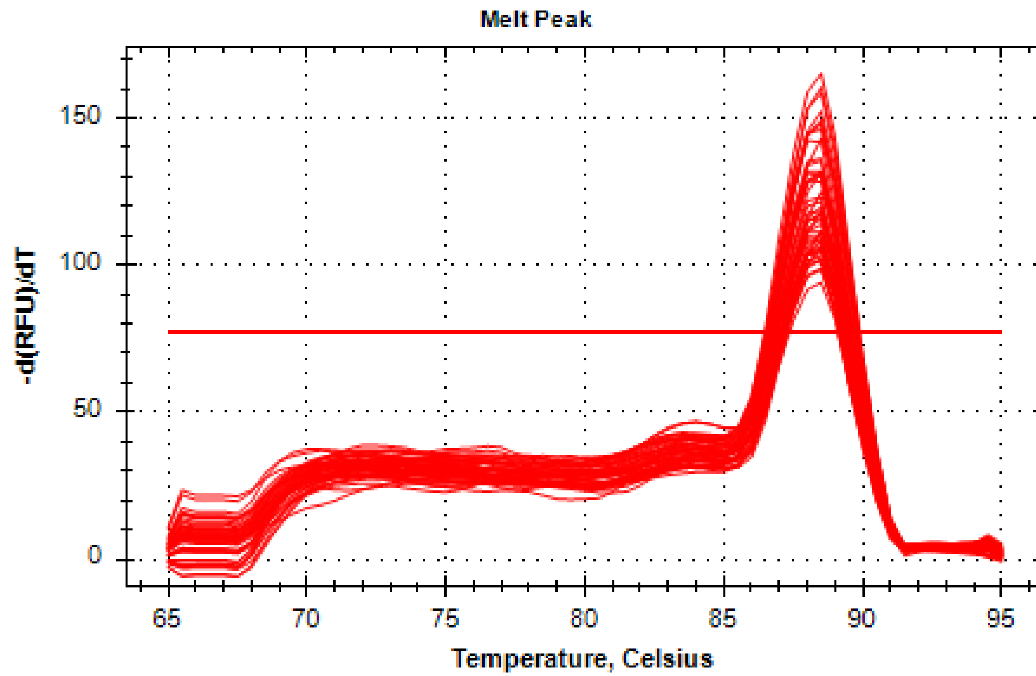
3054
3055 **Figure A.1.4.15:** *dmrt1* gene expression Cq (cycle value) standard curve.



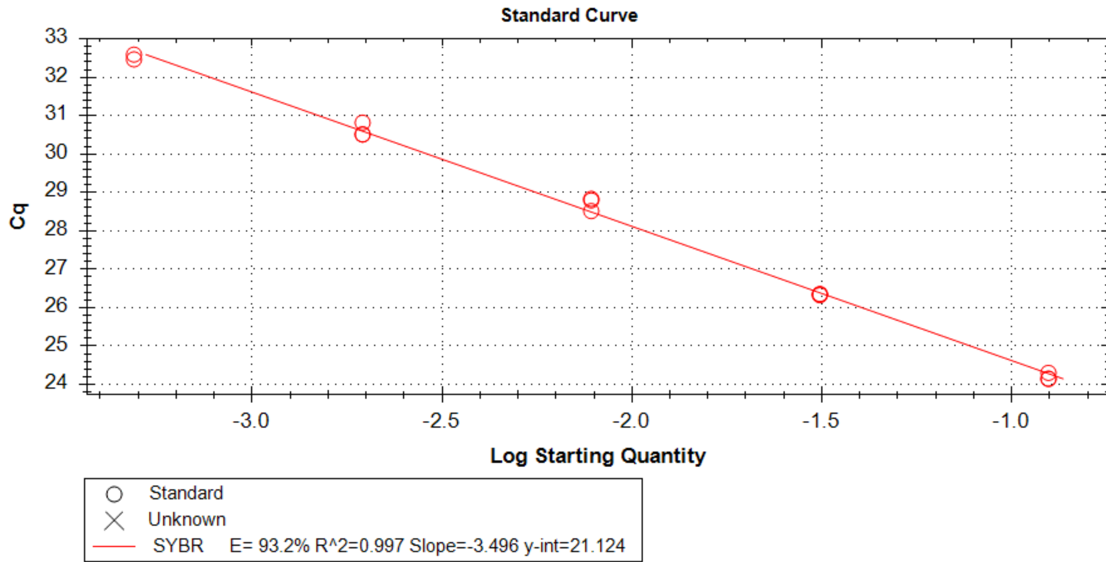
3056
3057 **Figure A.1.4.16:** *dmrt1* melt curve indicating one product at 88°C.
3058



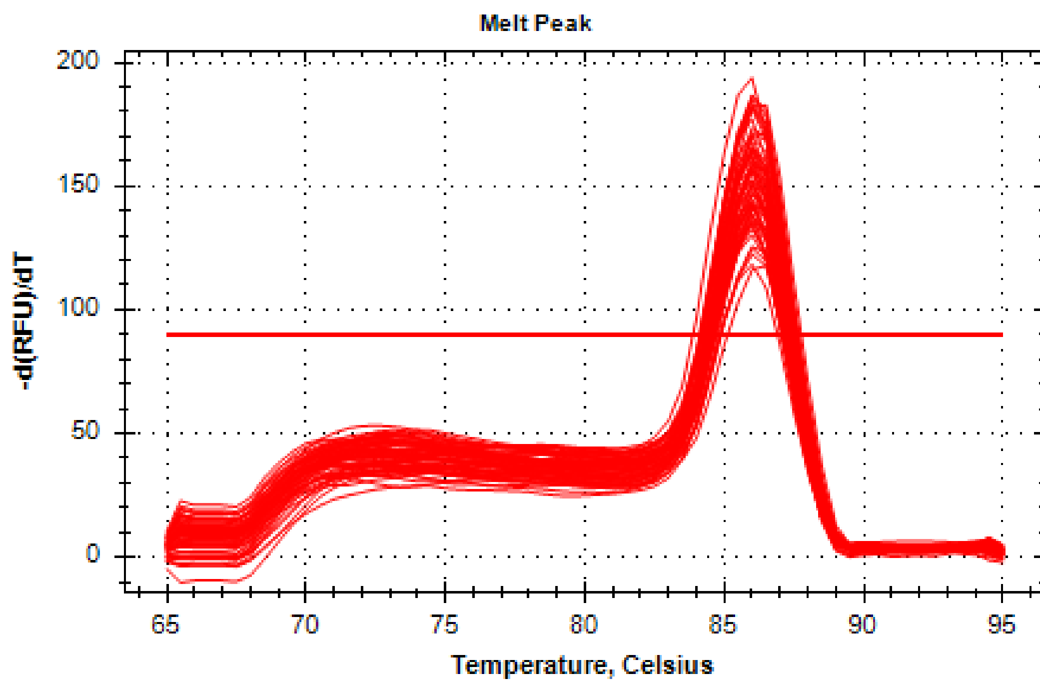
3059
3060 **Figure A.1.4.17:** *foxl2* gene expression Cq (cycle value) standard curve.



3061
3062 **Figure A.1.4.18:** *foxl2* melt curve indicating one product at 88.5°C



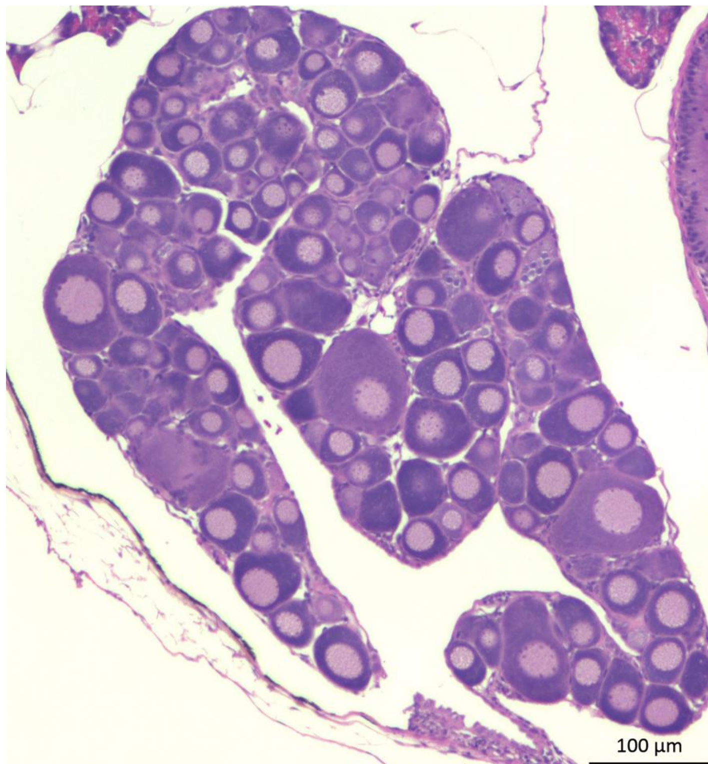
3063
3064 **Figure A.1.4.19:** *gdf9* gene expression Cq (cycle value) standard curve.
3065



3066
3067 **Figure A.1.4.20:** *gdf9* melt curve indicating one product at 86°C
3068

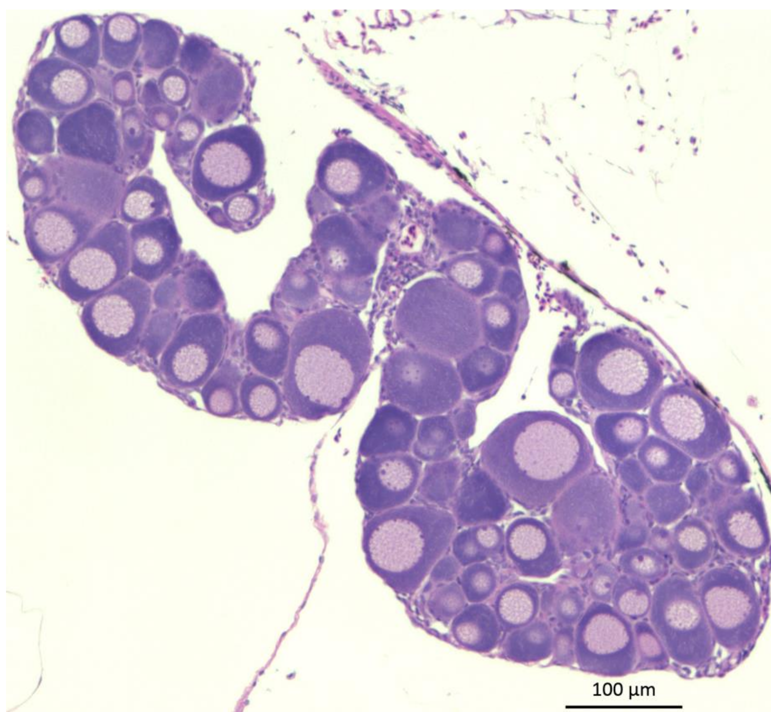
3069 **A2: Extra histology figures**

3070



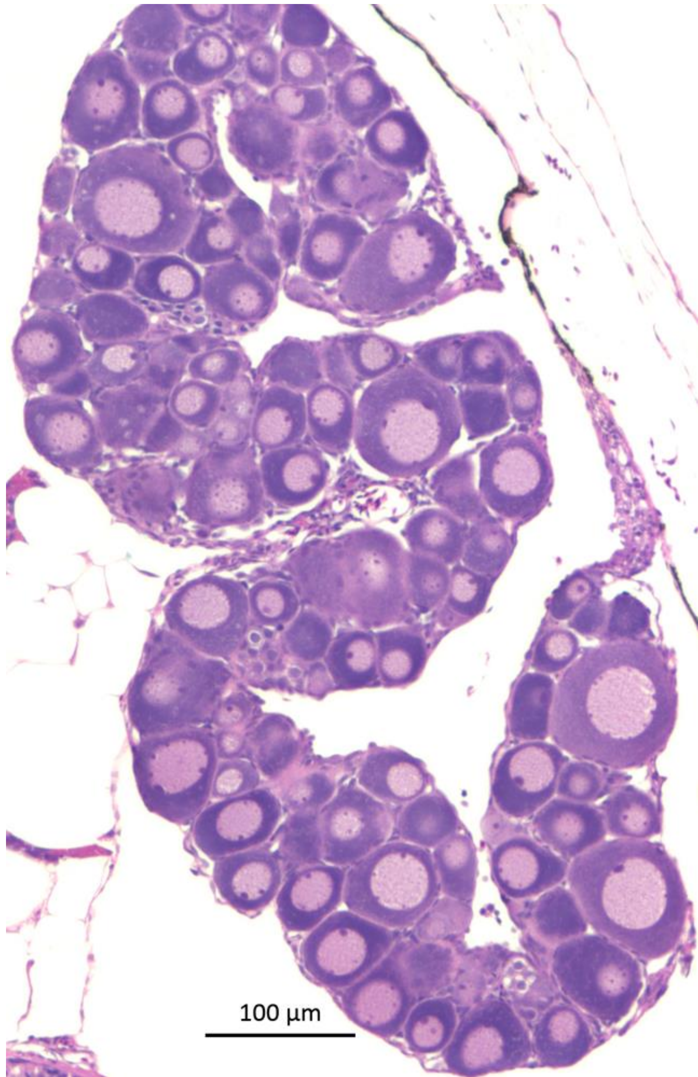
3071

3072 **Figure A.2.1:** Fish identification T1-4 yellow, female ovary (10X)



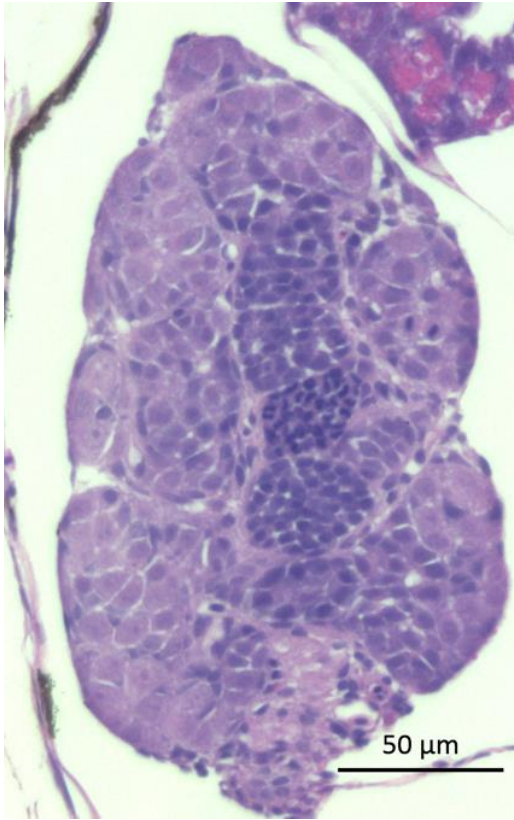
3073

3074 **Figure A.2.2:** Fish identification T1-5 yellow, female ovary (10X)



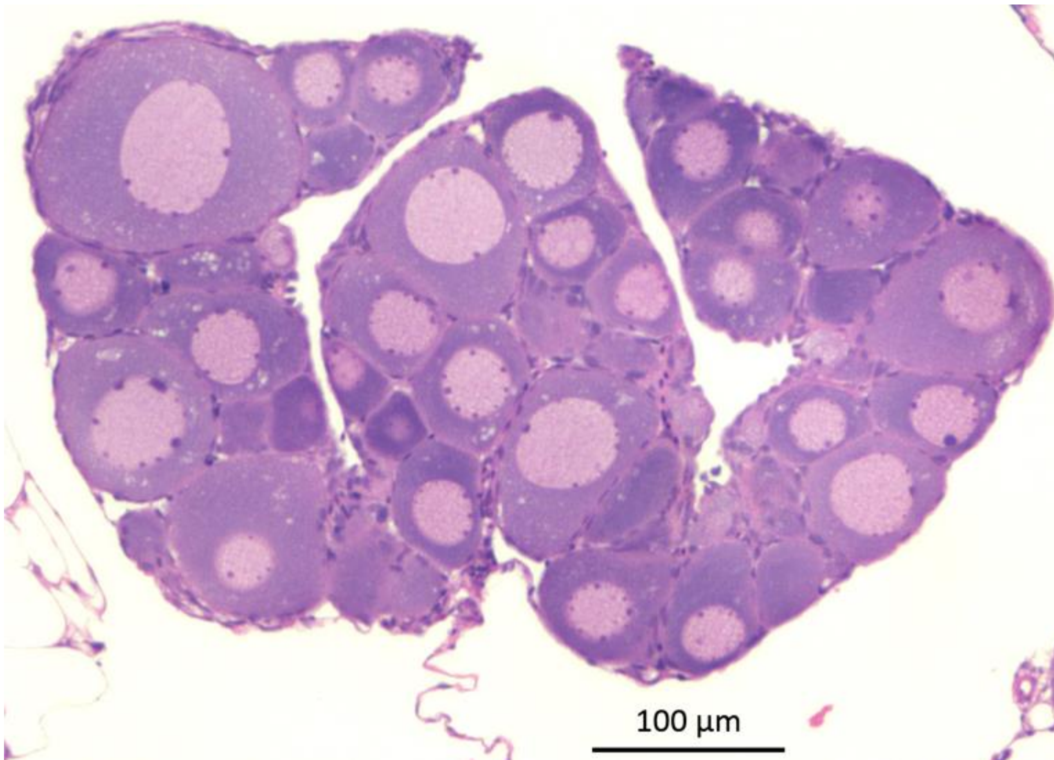
3075
3076 **Figure A.2.3:** Fish identification T1-6 blue, female ovary (10X)
3077

3078



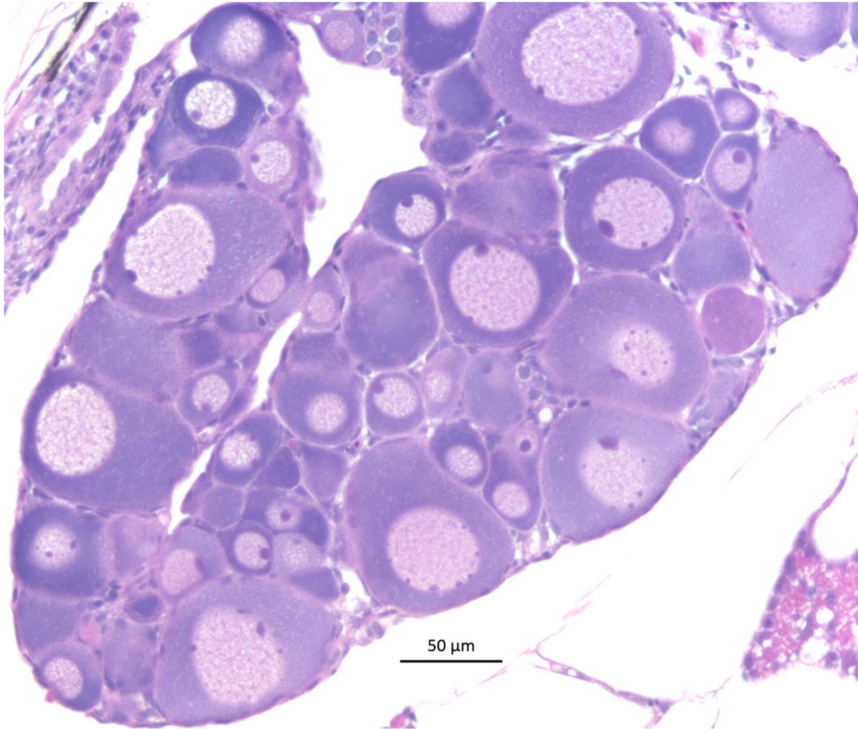
3079 **Figure A.2.4:** Fish identification T1-2 yellow, male testis (20X)

3080



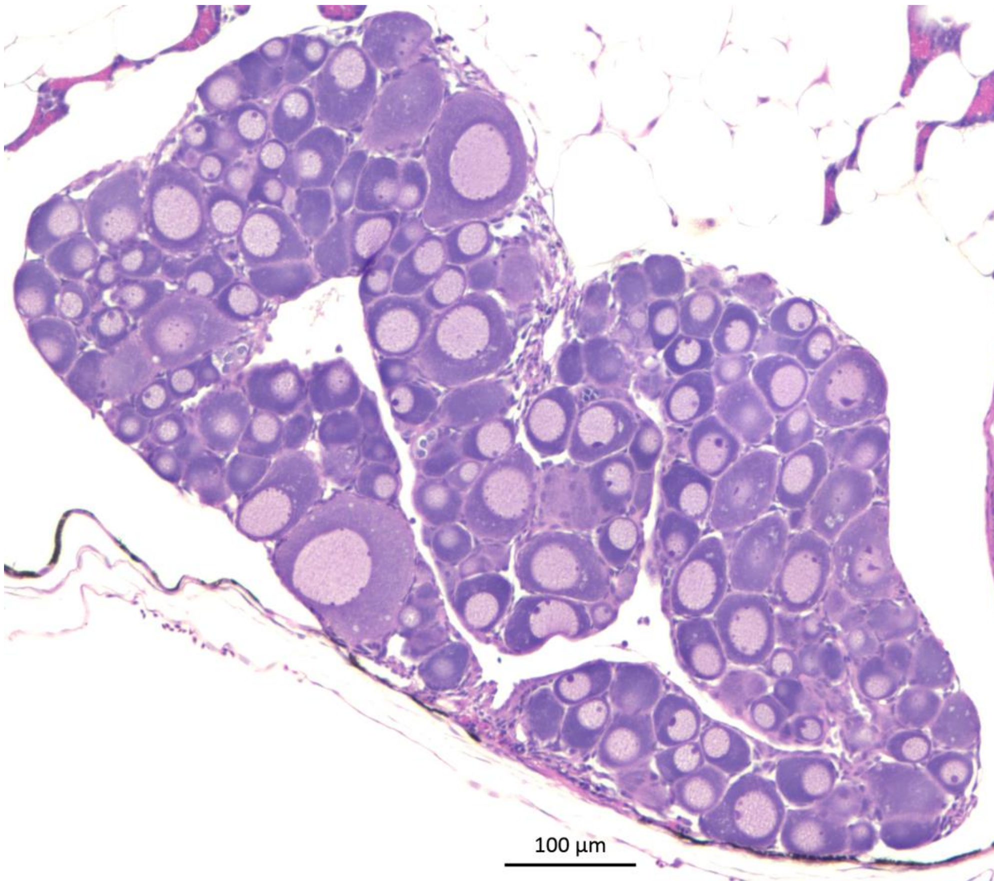
3081 **Figure A.2.5:** Fish identification T2-2 yellow, female ovary (10X)

3082



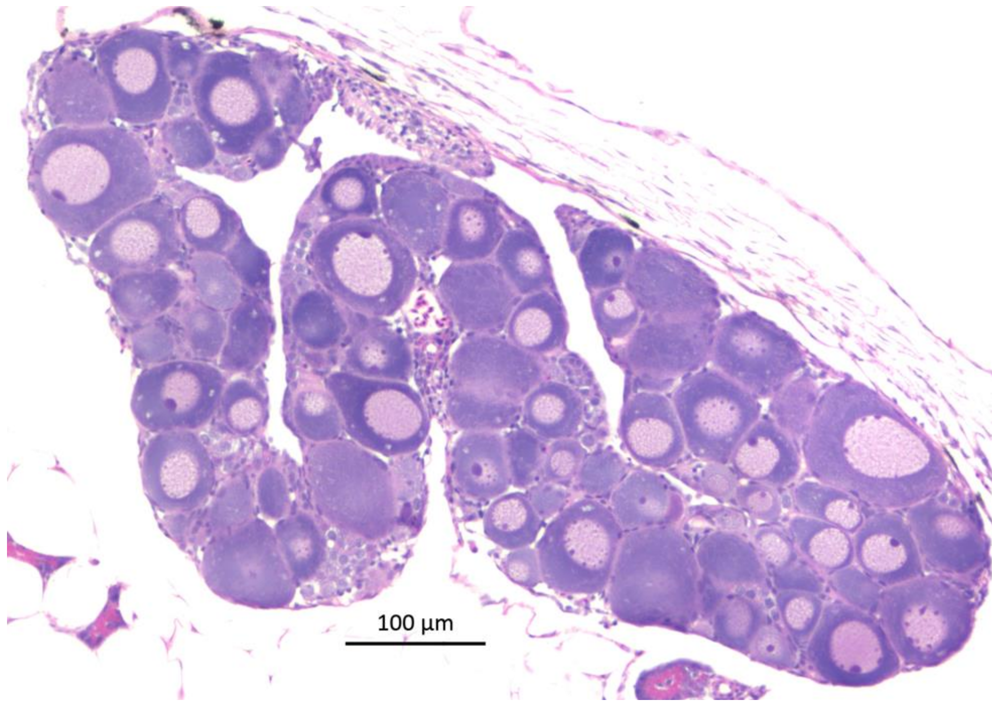
3083 **Figure A.2.6:** Fish identification T2-3 black, female ovary (20X)

3084



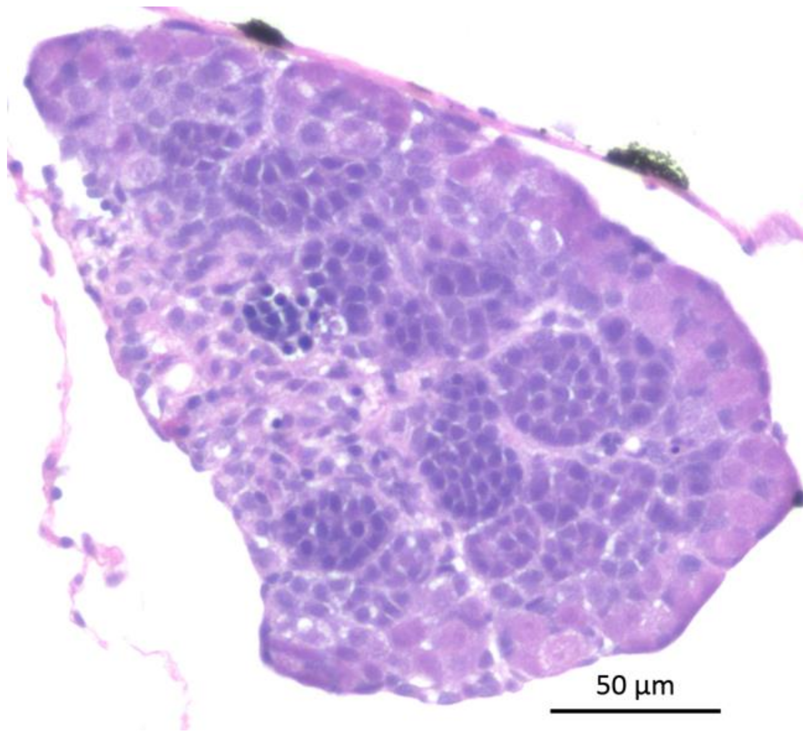
3085 **Figure A.2.7:** Fish identification T2-6 green, female ovary (10X)

3086

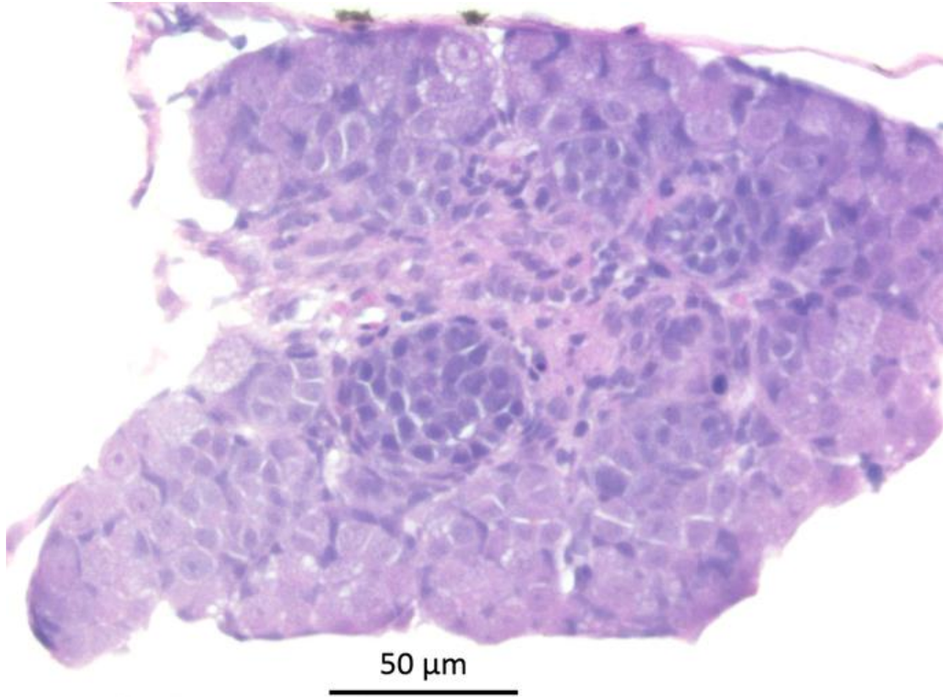


3087 **Figure A.2.8:** Fish identification T2-8 green, female ovary (10X)

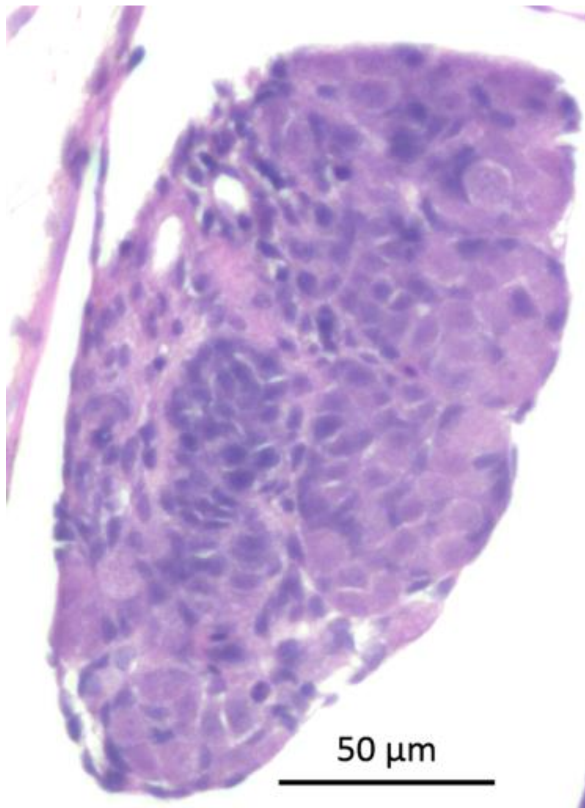
3088



3089 **Figure A.2.9:** Fish identification T2-6 black, male testis (20X)

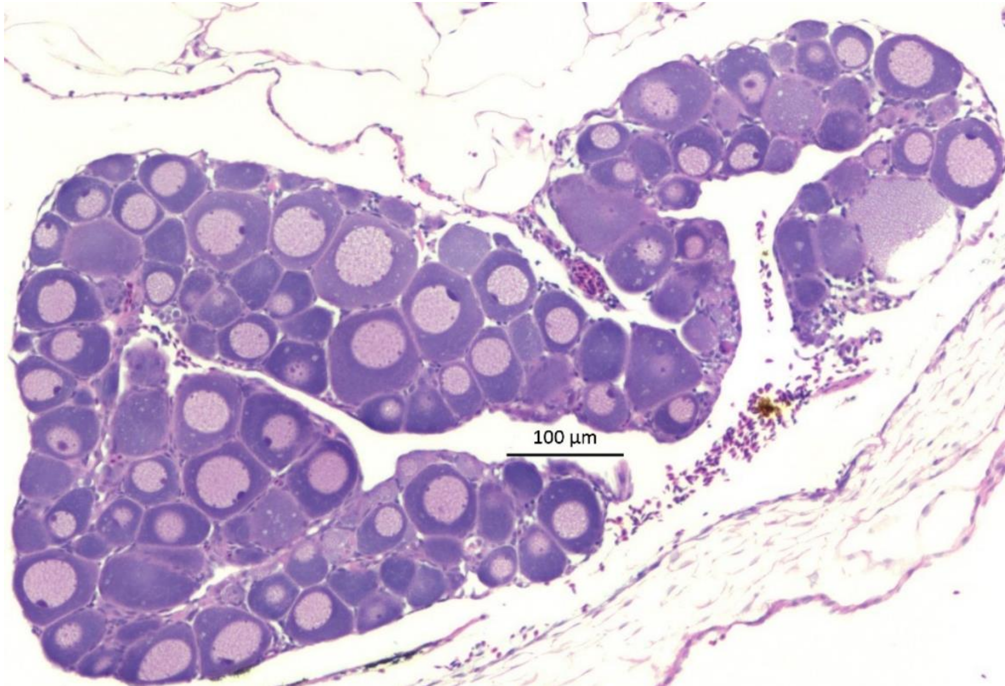


3090
3091 **Figure A.2.10:** Fish identification T2-7 orange, male testis (20X)



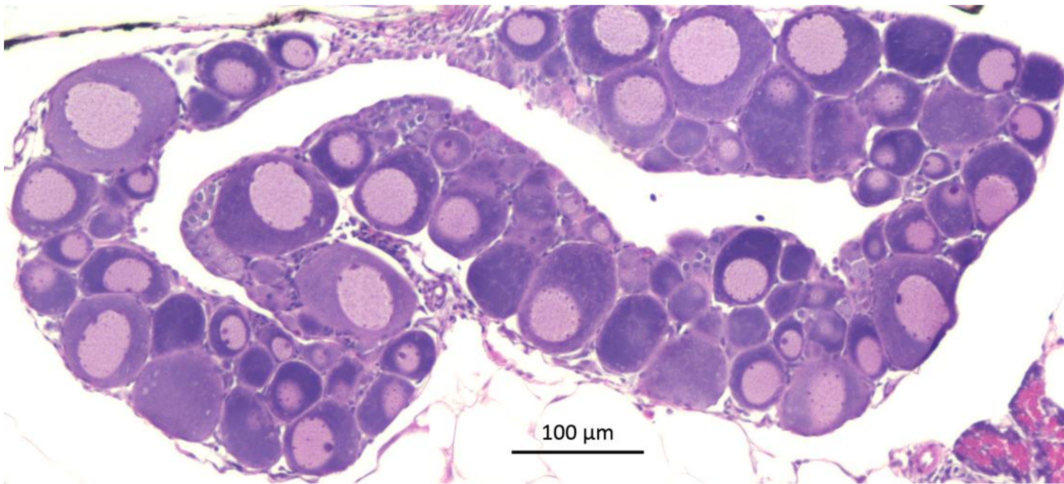
3092
3093 **Figure A.2.11:** Fish identification T2-12 yellow, male testis (20X)

3094



3095 **Figure A.2.12:** Fish identification T3-1 yellow, female ovary (10X)

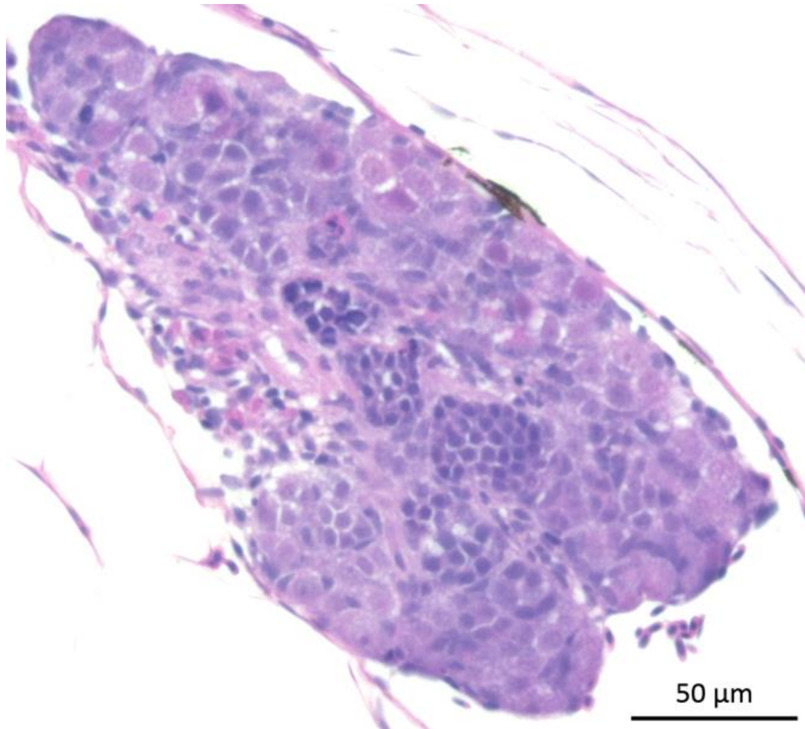
3096



3097 **Figure A.2.13:** Fish identification T3-3 green, female ovary (10X)

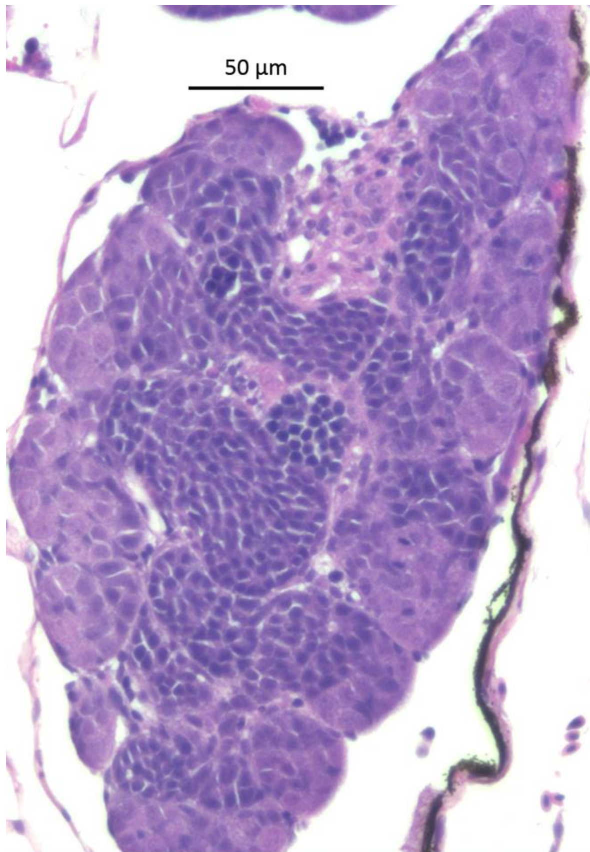
3098

3099



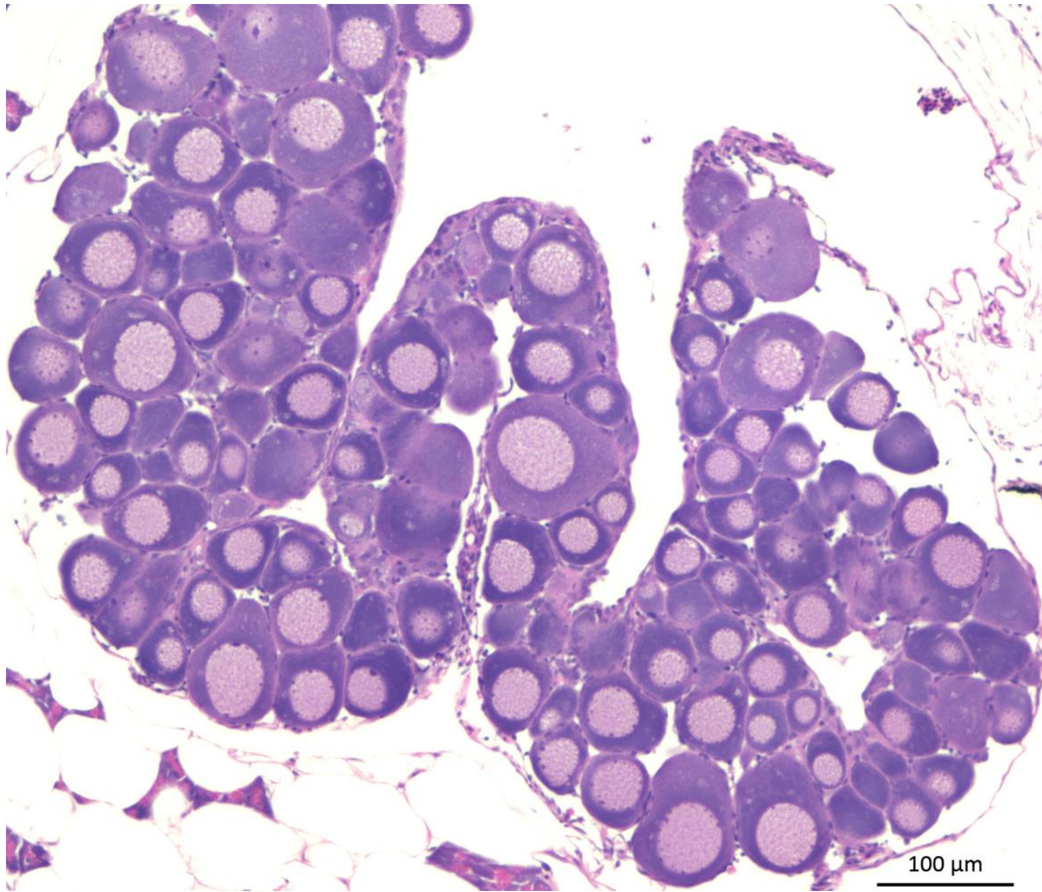
3100 **Figure A.2.14:** Fish identification T3-1 blue, male testis (20X)

3101



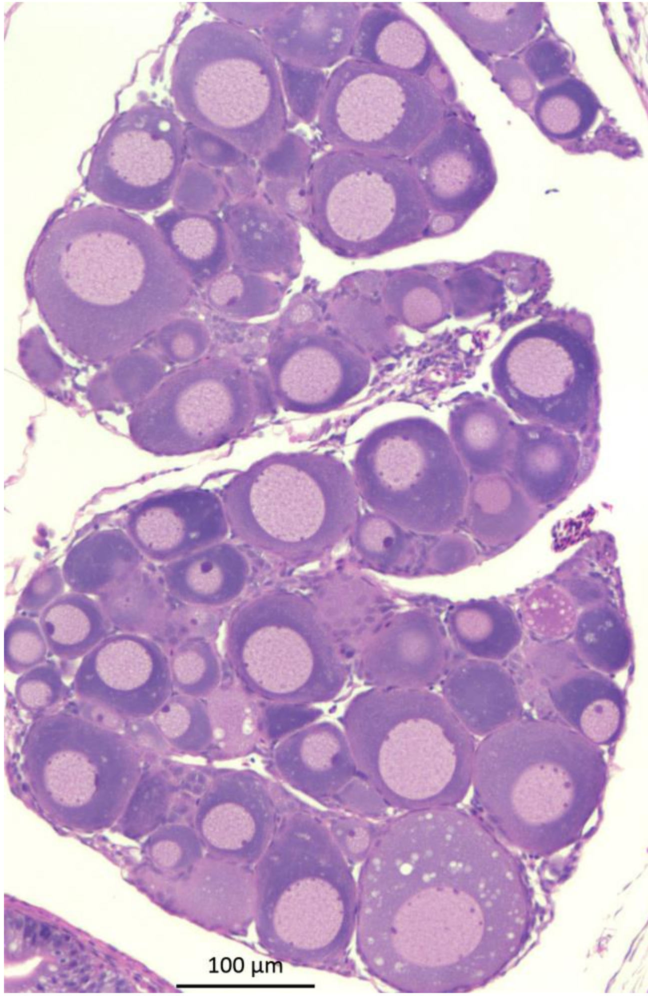
3102 **Figure A.2.15:** Fish identification T3-10 yellow, male testis (20X)

3103



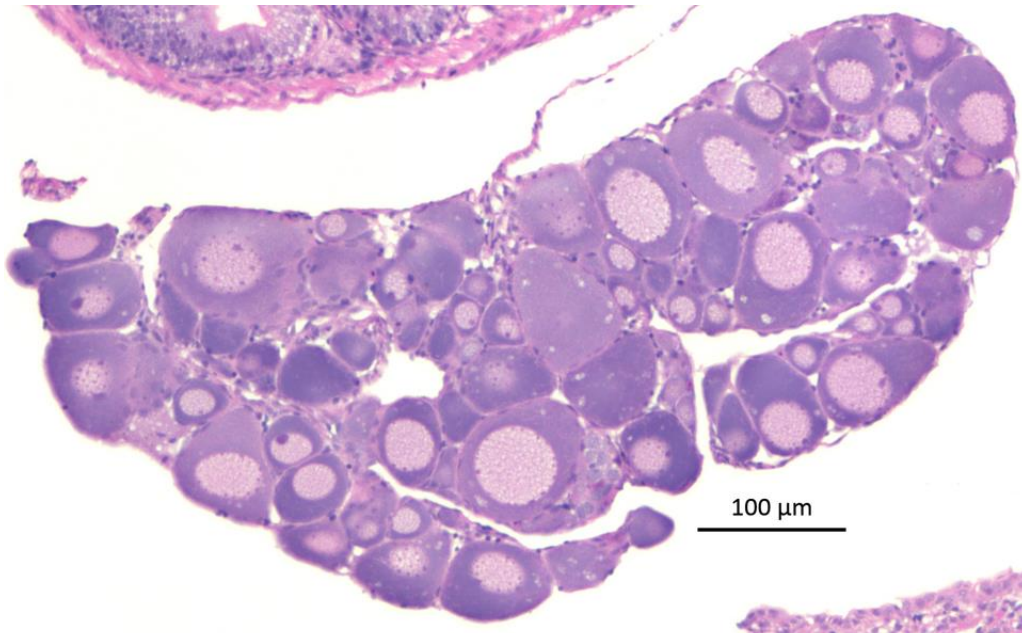
3104 **Figure A.2.16:** Fish identification T4-4 green, female ovary (10X)

3105



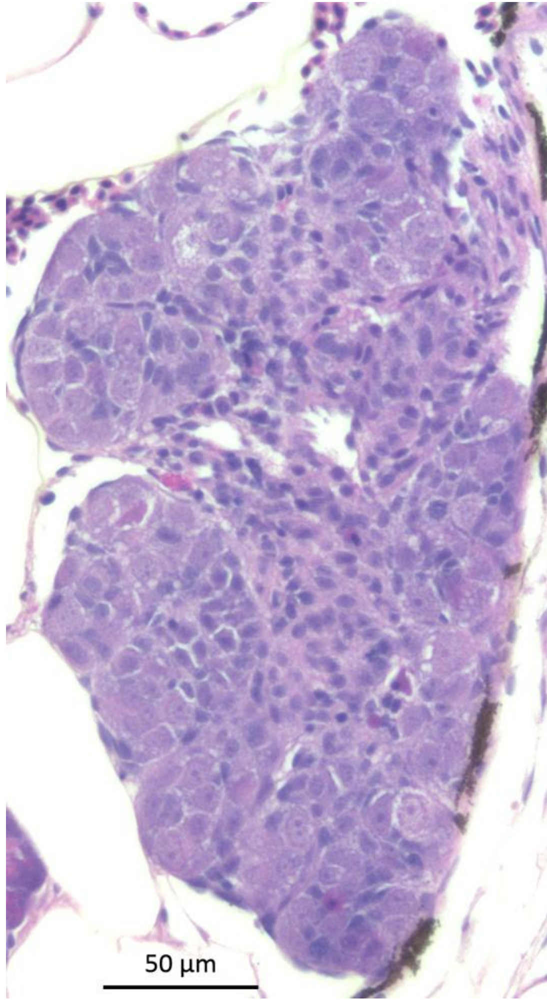
3106 **Figure A.2.17:** Fish identification T4-6 blue, female ovary (10X)

3107



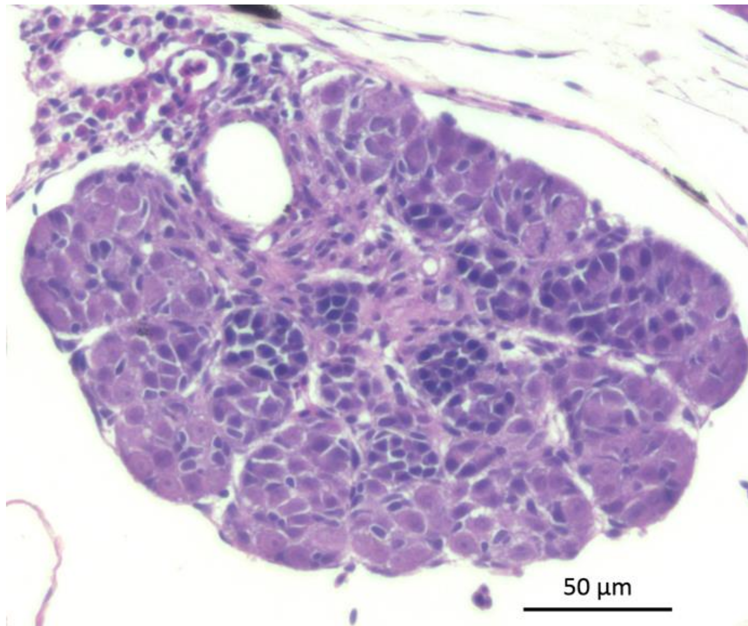
3108 **Figure A.2.18:** Fish identification T4-10 orange, female ovary (10X)

3109



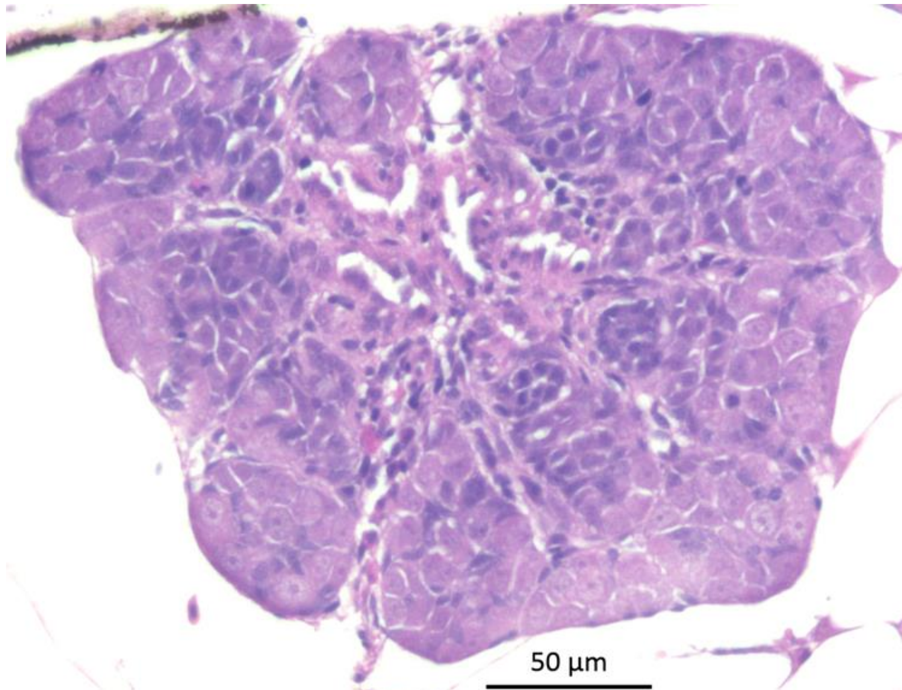
3110 **Figure A.2.19:** Fish identification T4-1 blue, male testis (20X)

3111



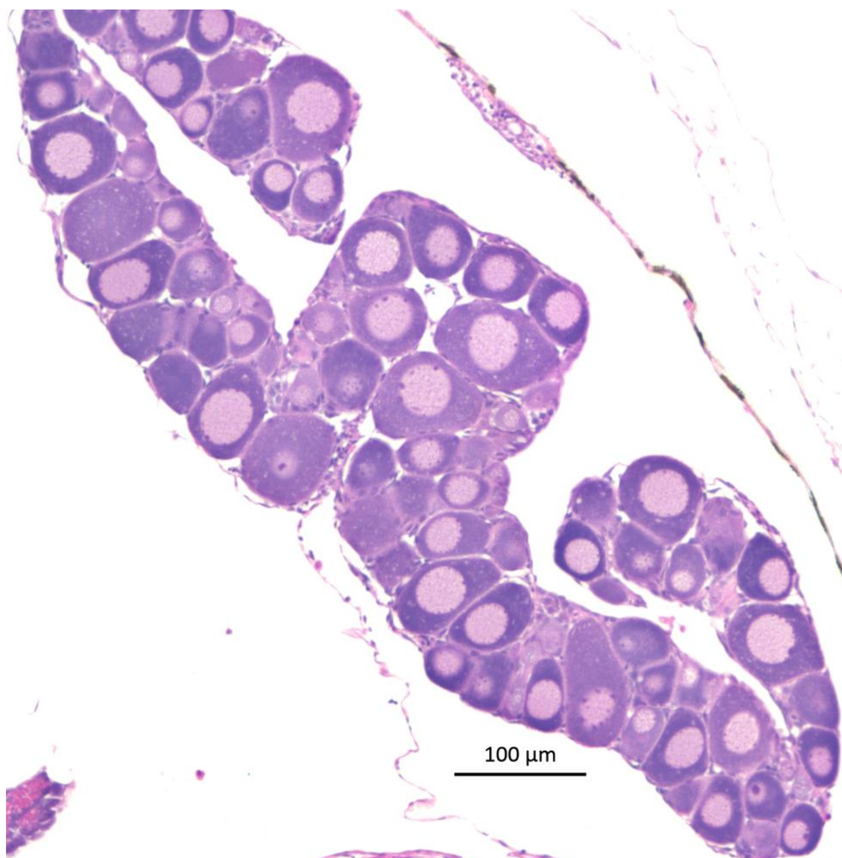
3112 **Figure A.2.20:** Fish identification T4-1 yellow, male testis (20X)

3113

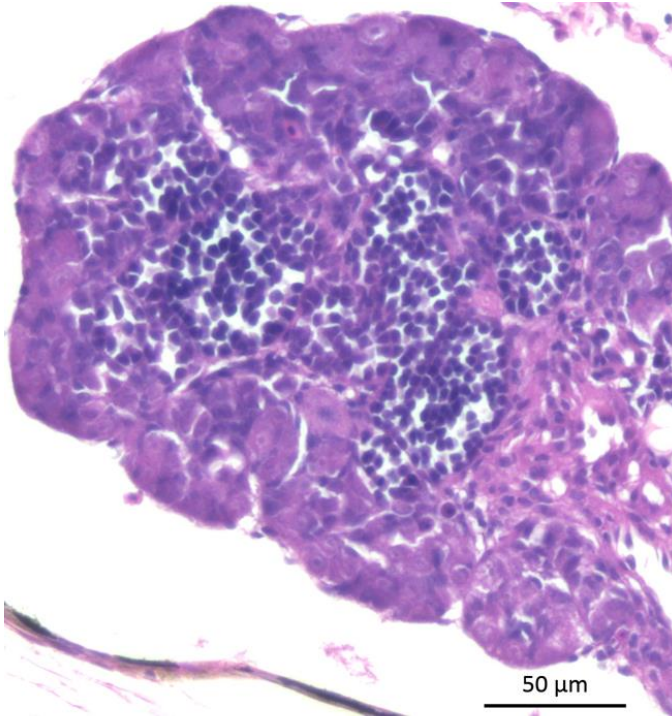


3114 **Figure A.2.21:** Fish identification T4-4 orange, male testis (20X)

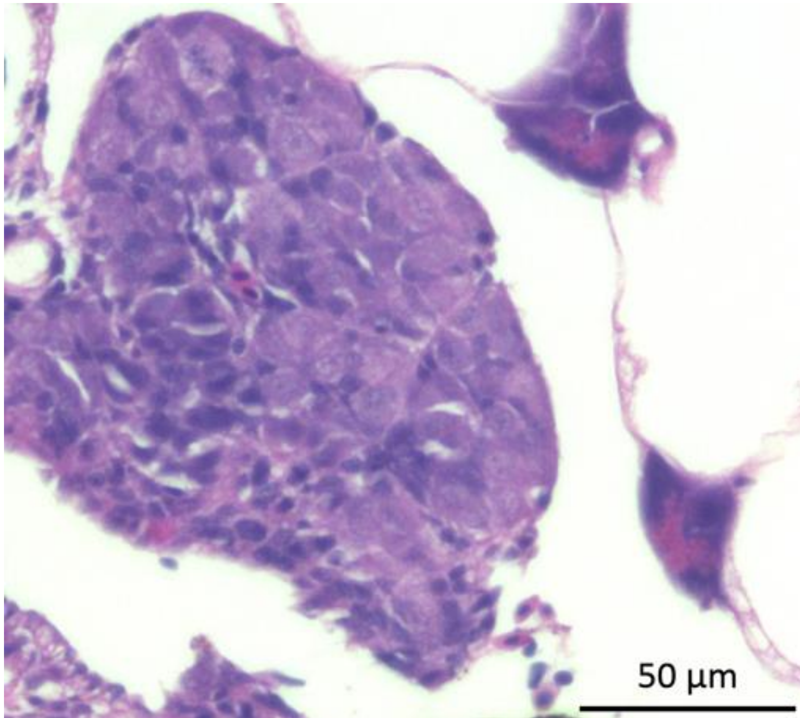
3115



3116 **Figure A.2.22:** Fish identification T5-10 black, female ovary (10X)

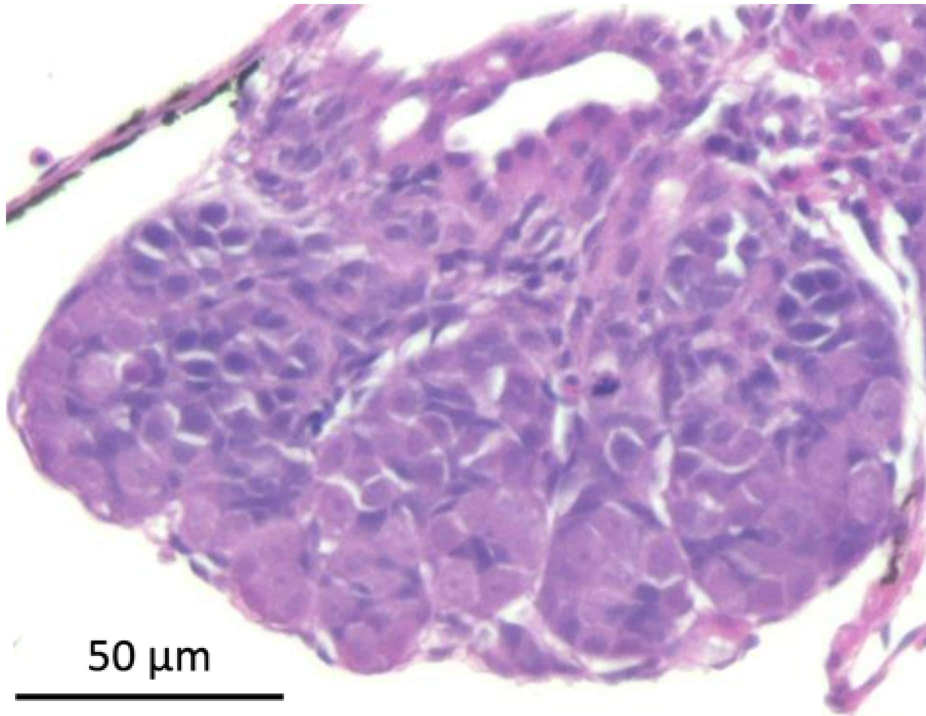
3117


3118 **Figure A.2.23:** Fish identification T5-2 black, male testis (20X)

3119


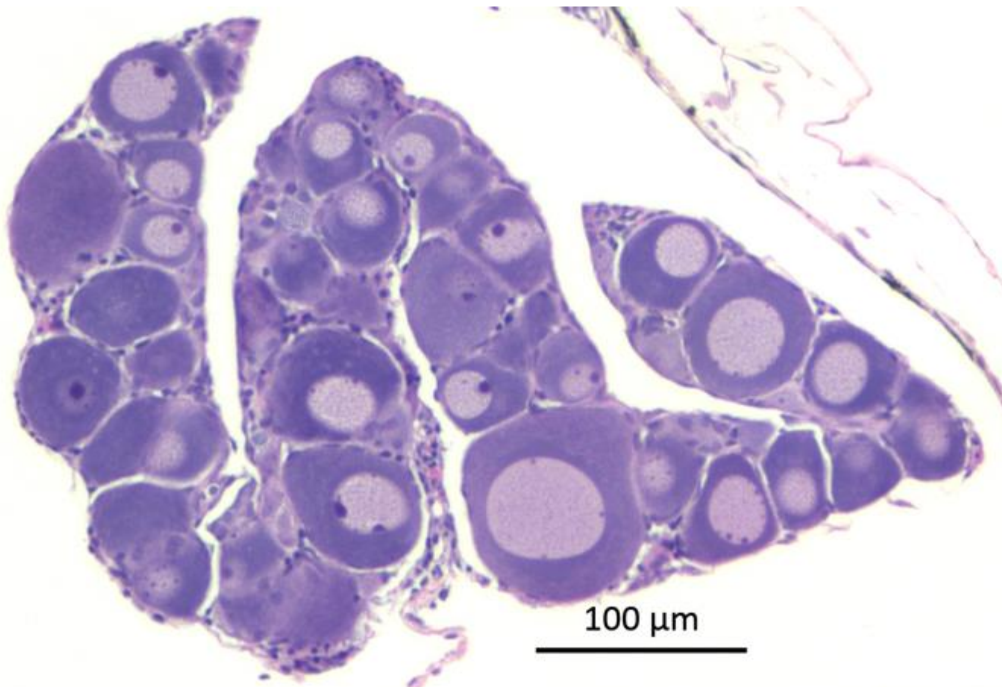
3120 **Figure A.2.24:** Fish identification T5-6 blue, male testis (20X)

3121



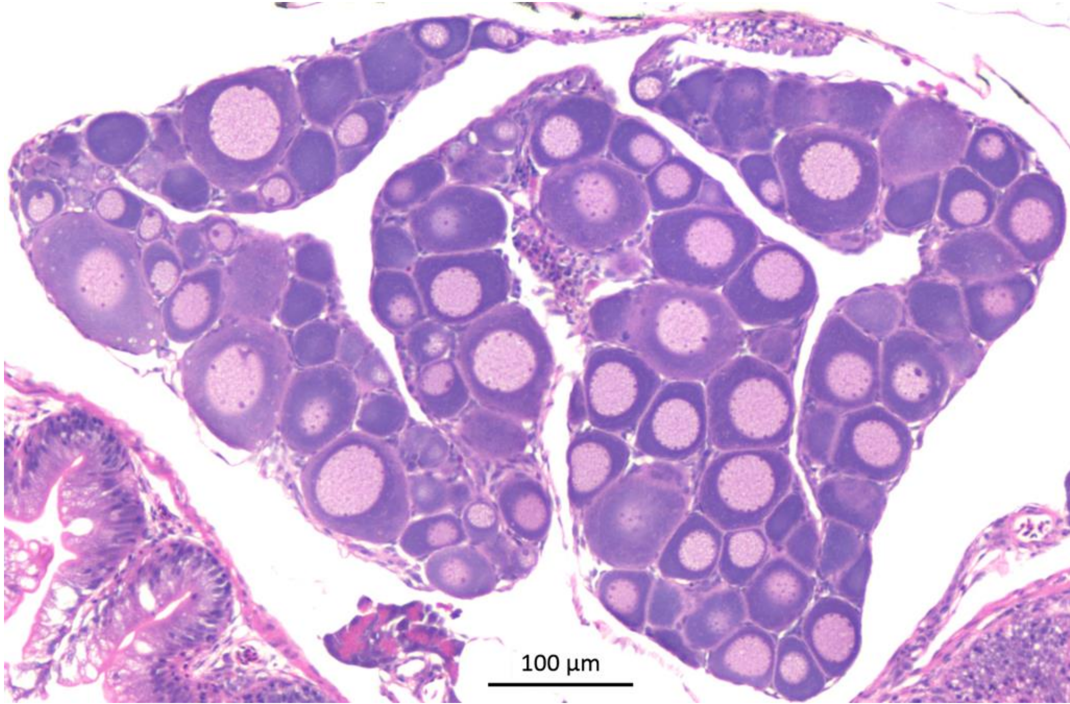
3122 **Figure A.2.25:** Fish identification T5-10 blue, male testis (20X)

3123



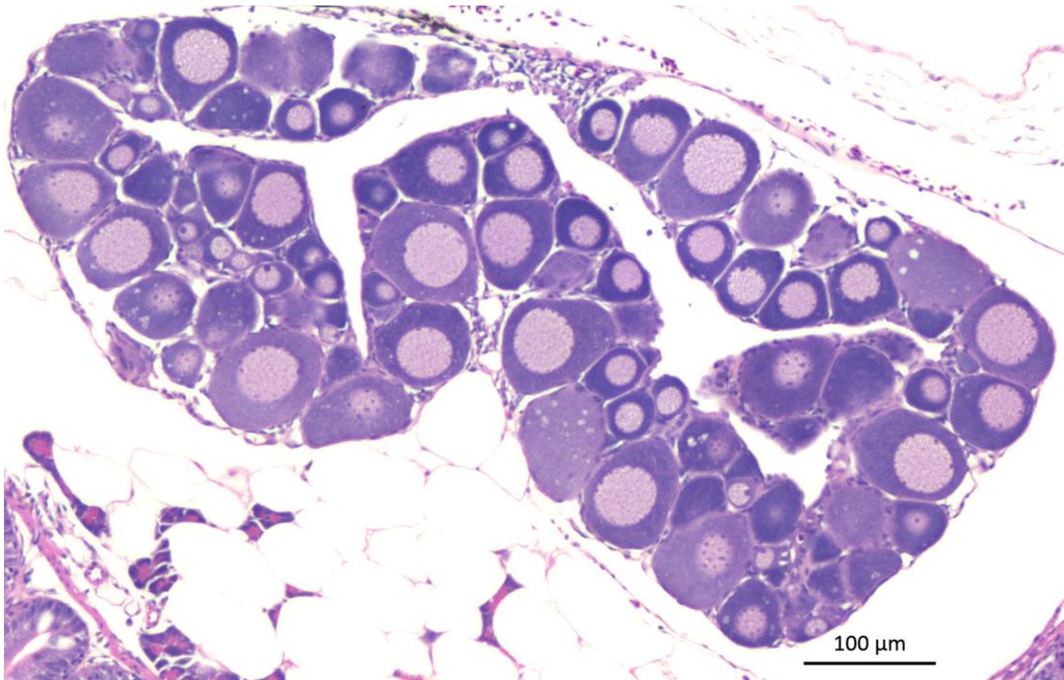
3124 **Figure A.2.26:** Fish identification T6-5 black, female ovary (10X)

3125



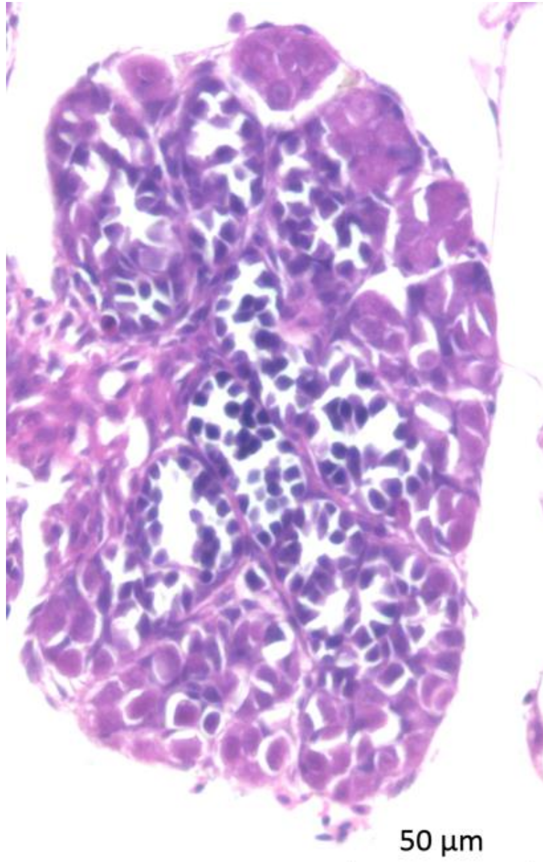
3126 **Figure A.2.27:** Fish identification T6-9 orange, female ovary (10X)

3127



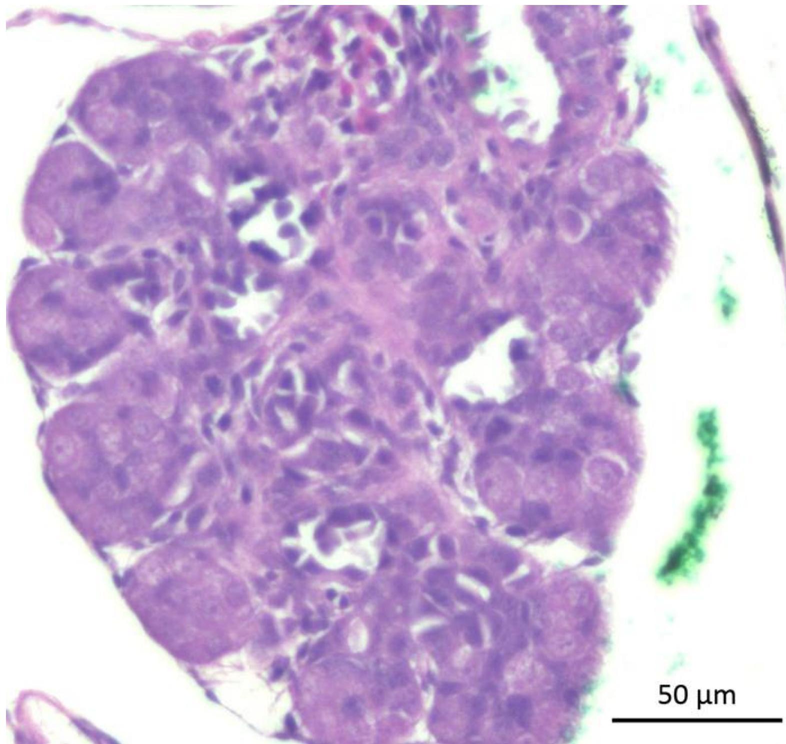
3128 **Figure A.2.28:** Fish identification T6-10 orange, female ovary (10X)

3129



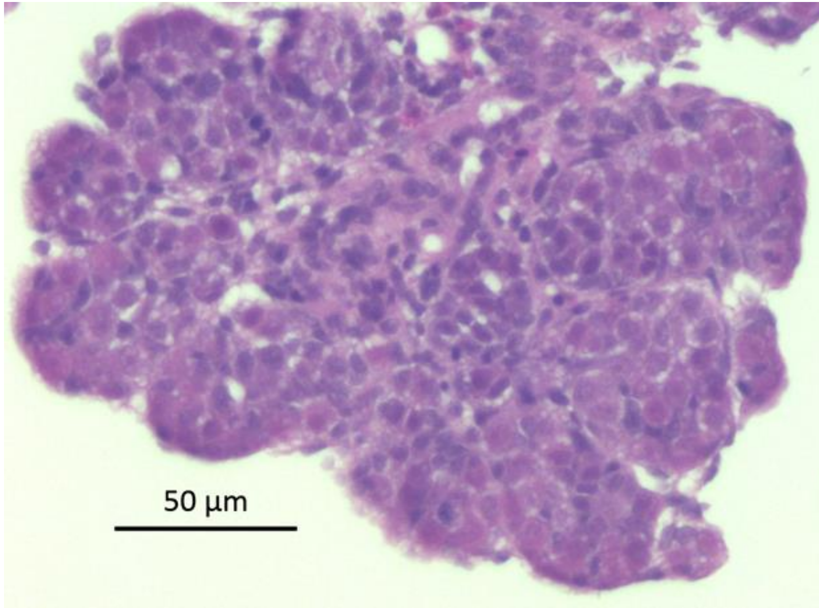
3130 **Figure A.2.29:** Fish identification T6-2 black, male testis (20X)

3131



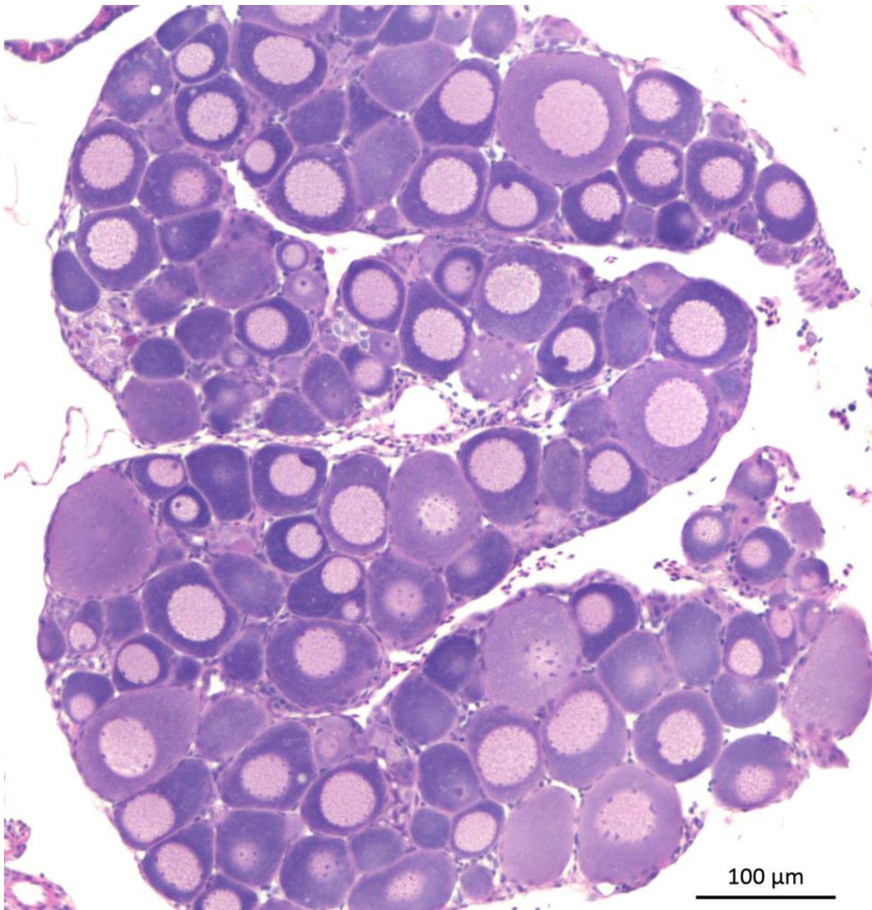
3132 **Figure A.2.30:** Fish identification T6-4 green, male testis (20X)

3133

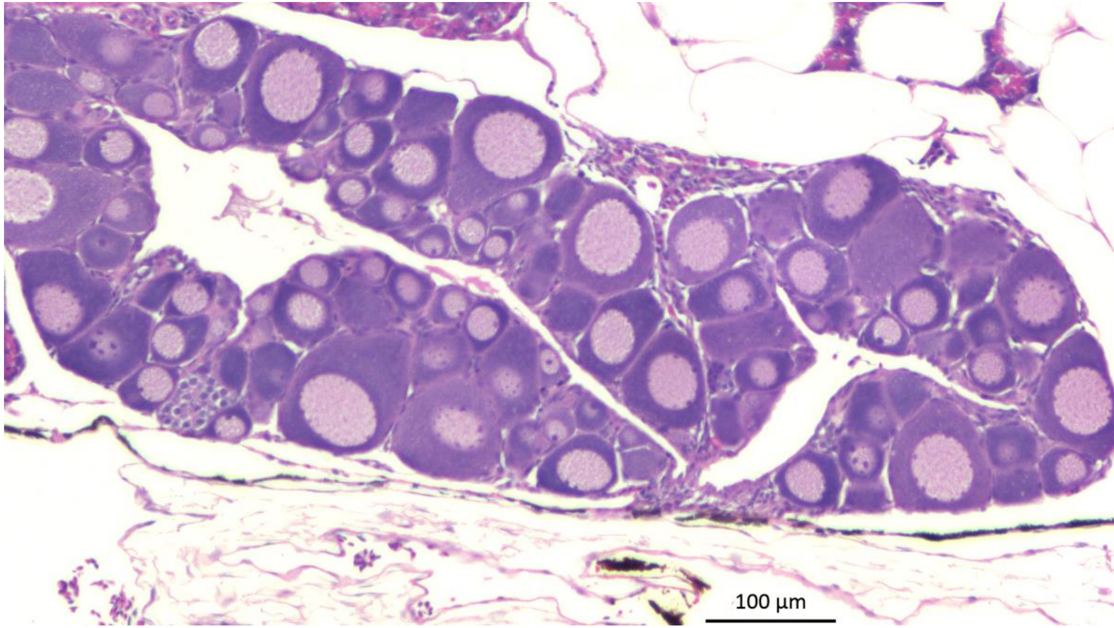


3134 **Figure A.2.31:** Fish identification T6-7 yellow, male testis (20X)

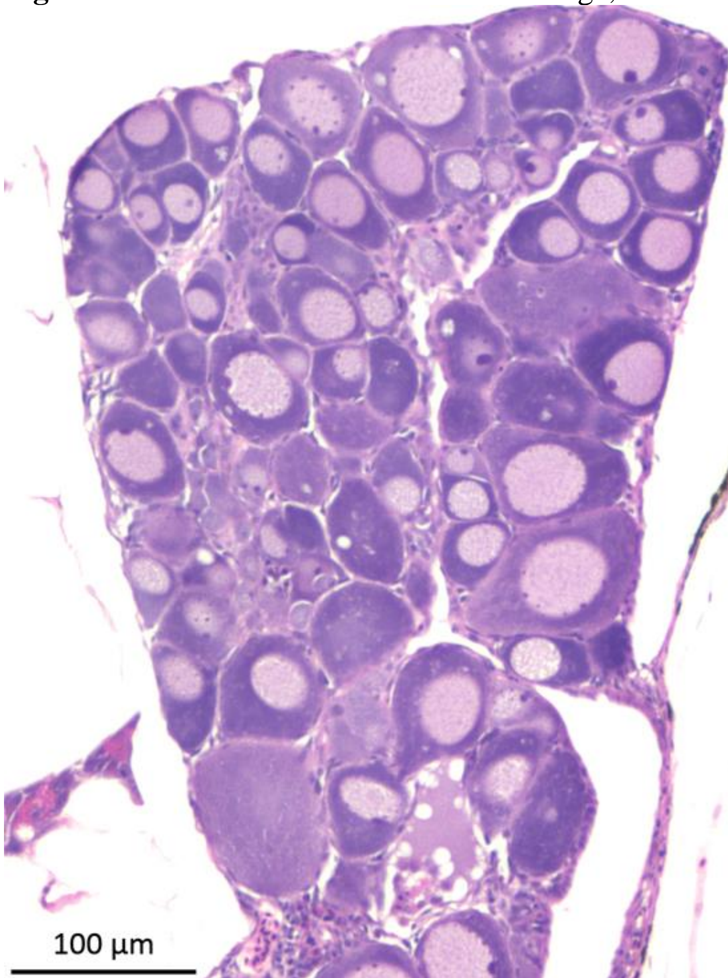
3135



3136 **Figure A.2.32:** Fish identification T7-1 yellow, female ovary (10X)

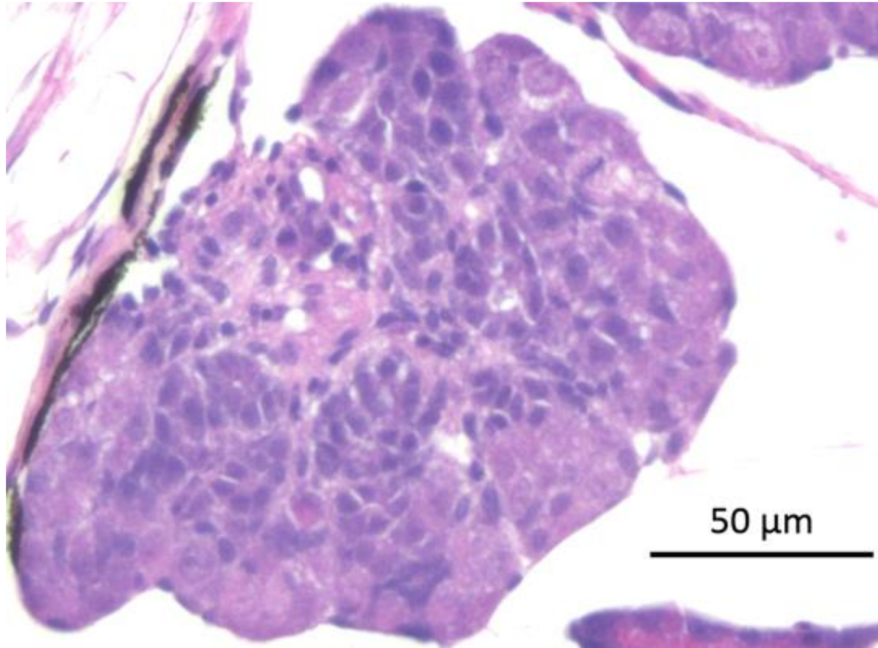


3137
3138 **Figure A.2.33:** Fish identification T7-5 orange, female ovary (10X)



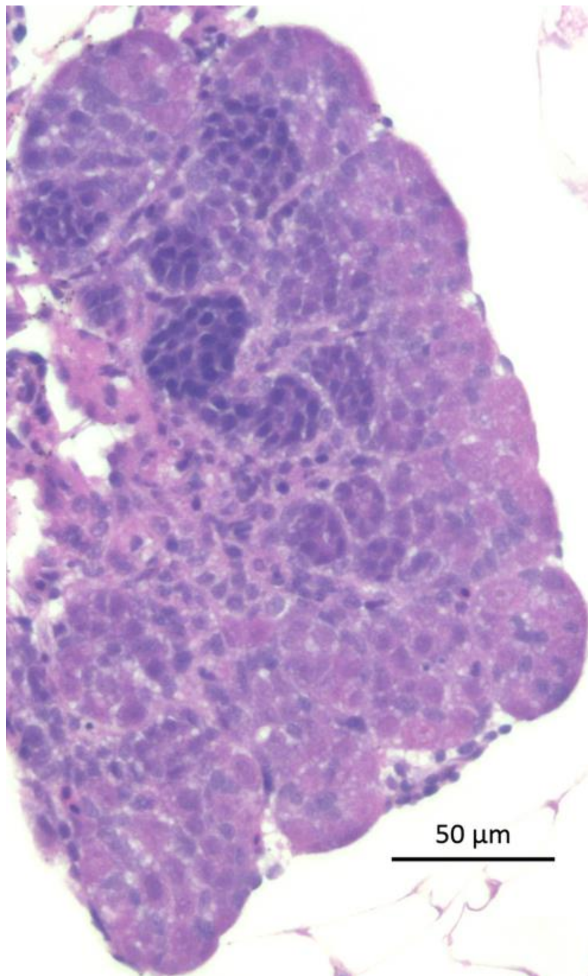
3139
3140 **Figure A.2.34:** Fish identification T7-8 orange, female ovary (10X)

3141



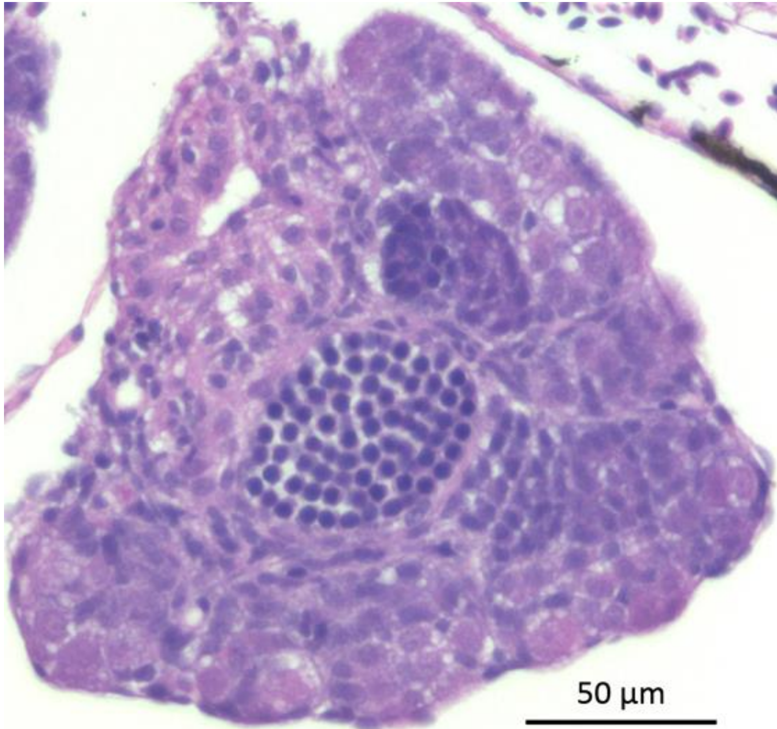
3142 **Figure A.2.35:** Fish identification T7-1 blue, male testis (20X)

3143



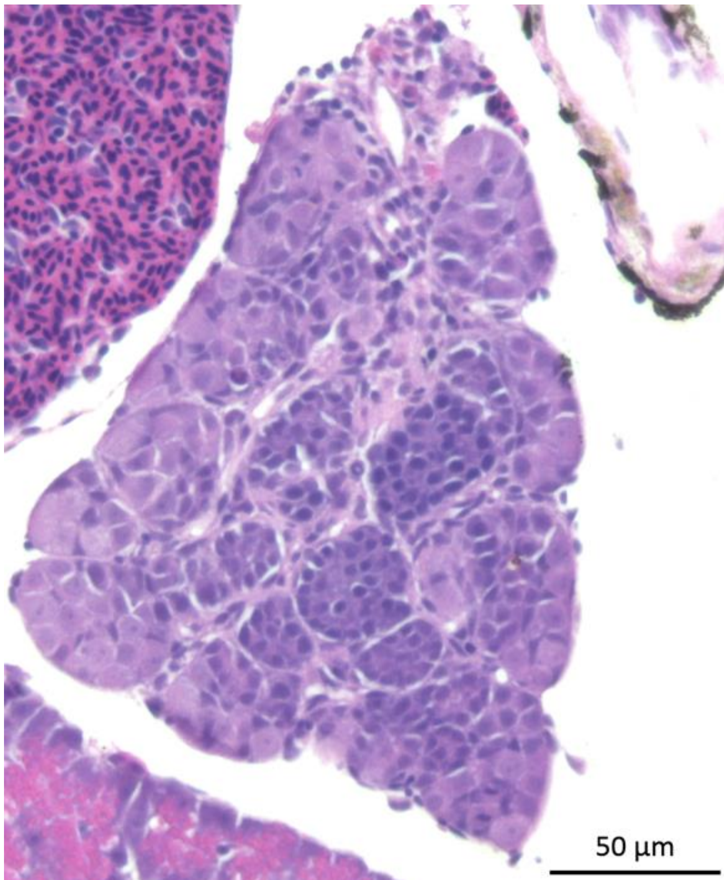
3144 **Figure A.2.36:** Fish identification T7-2 green, male testis (20X)

3145



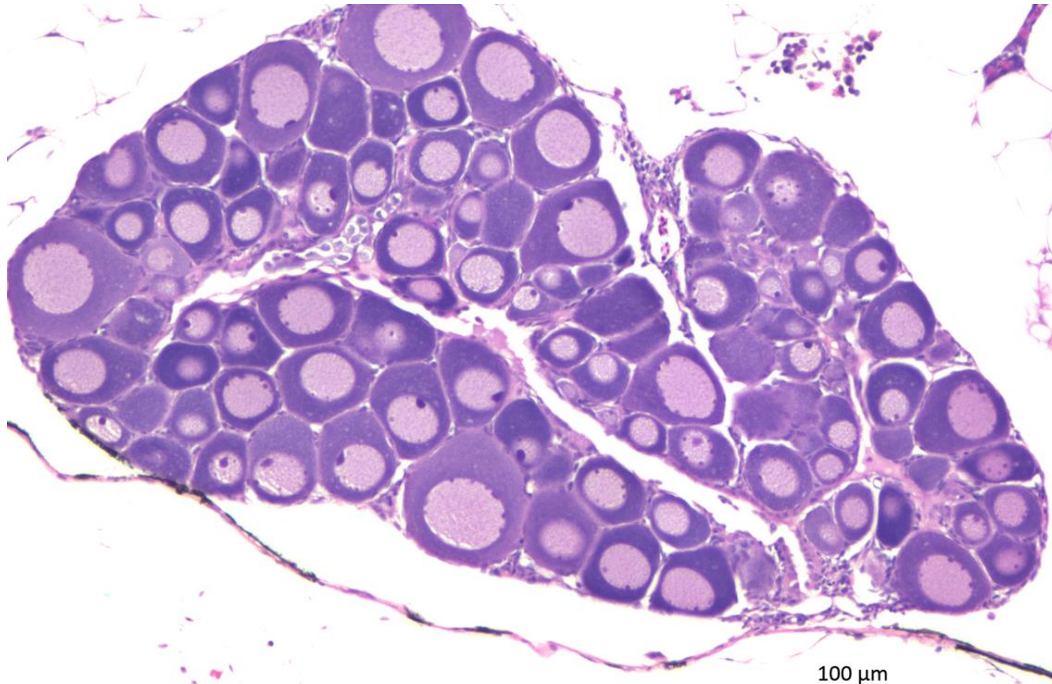
3146 **Figure A.2.37:** Fish identification T7-3 green, male testis (20X)

3147



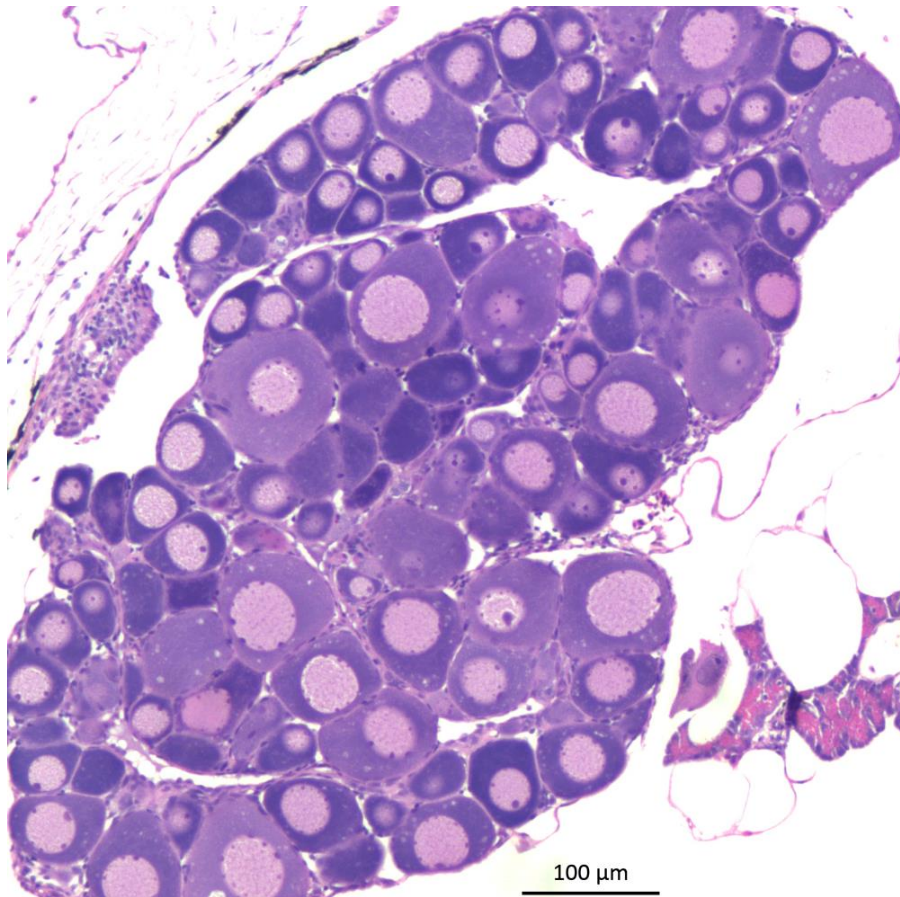
3148 **Figure A.2.38:** Fish identification T7-8 yellow, male testis (20X)

3149



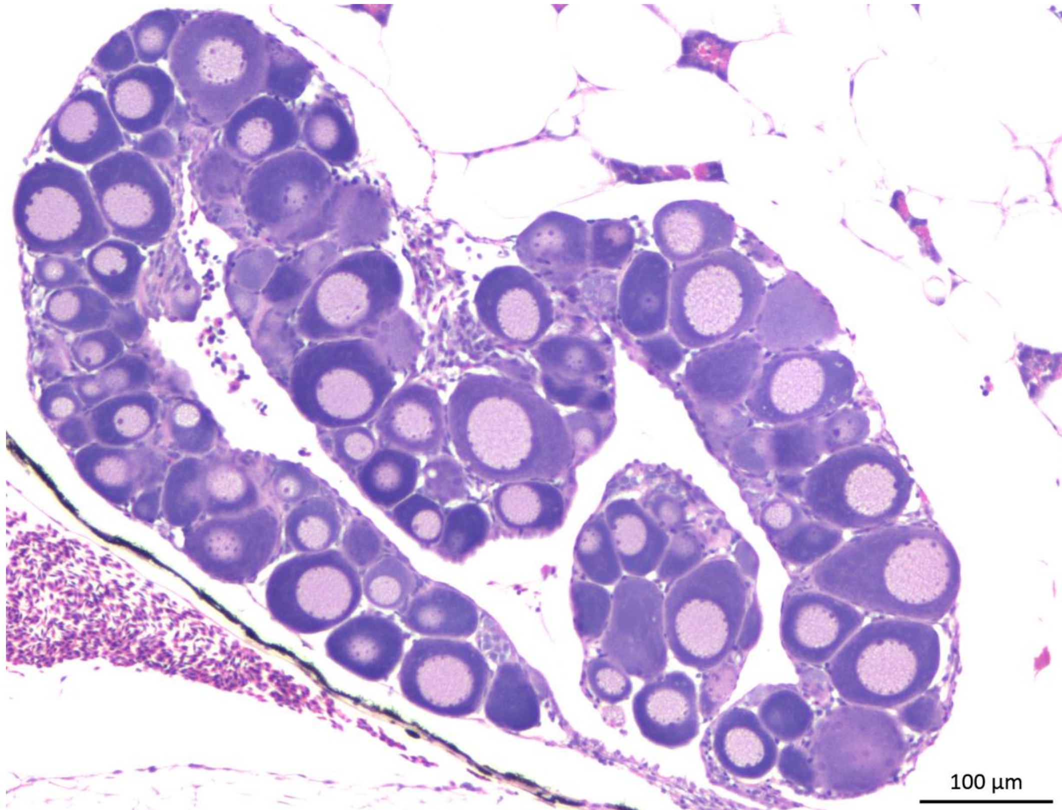
3150 **Figure A.2.39:** Fish identification T8-2 green, female ovary (10X)

3151



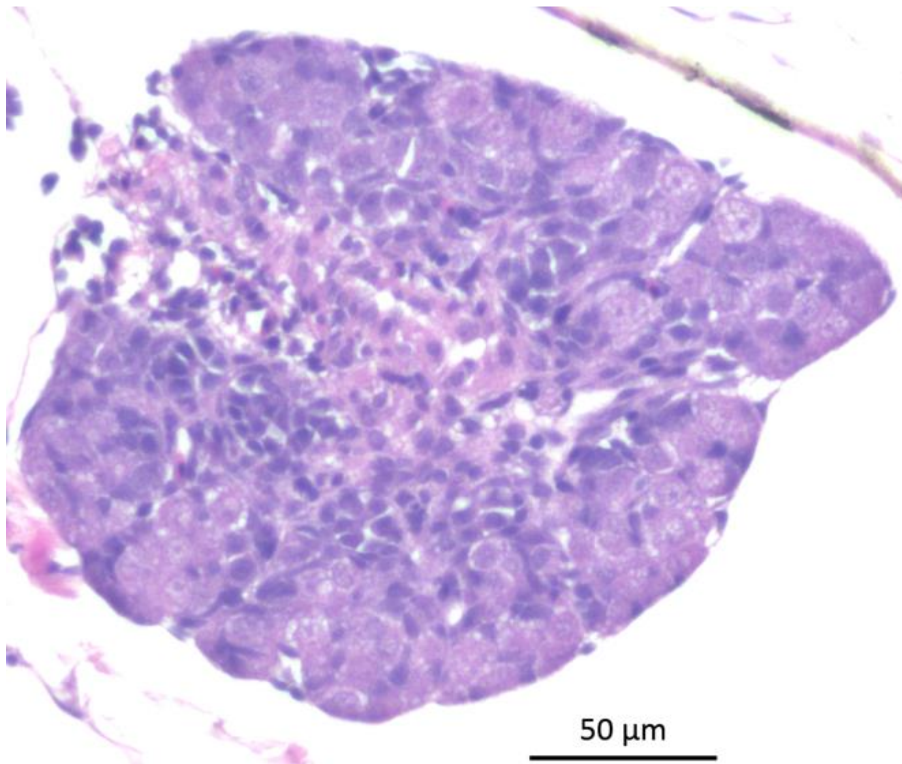
3152 **Figure A.2.40:** Fish identification T8-4 black, female ovary (10X)

3153

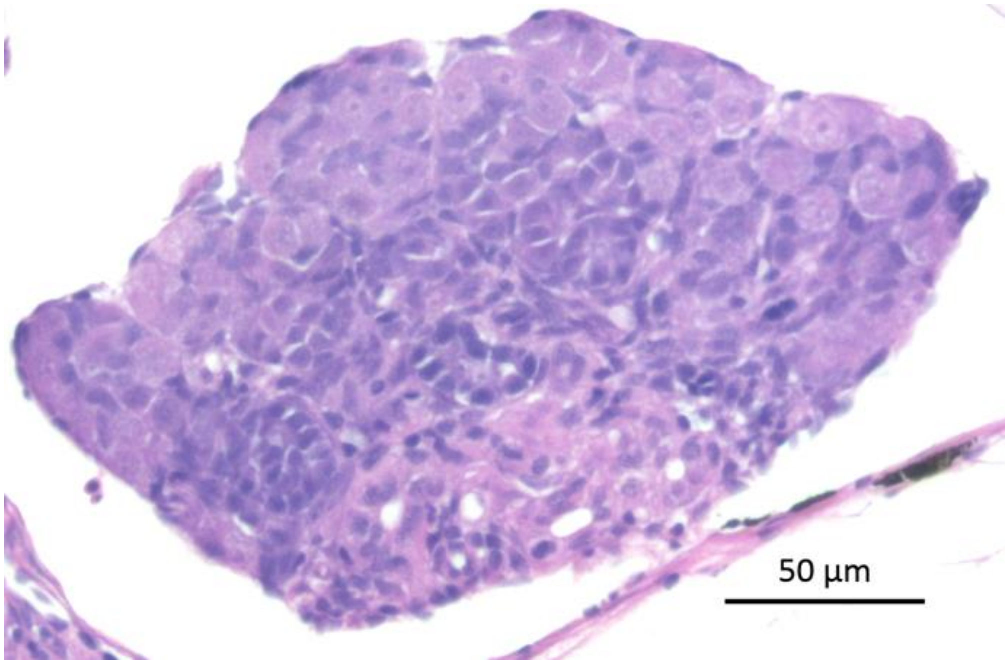


3154 **Figure A.2.41:** Fish identification T8-5 black, female ovary (10X)

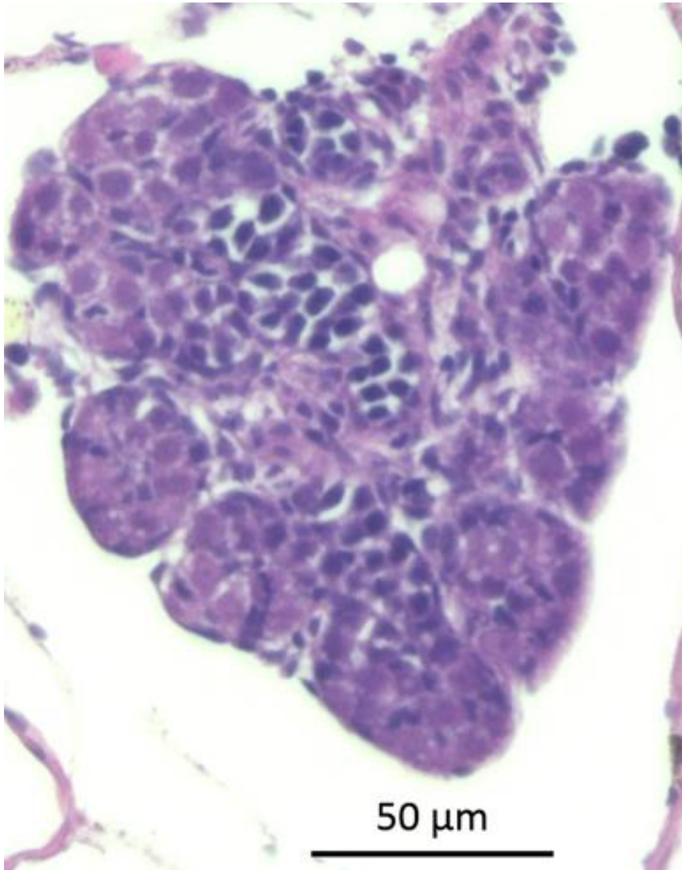
3155



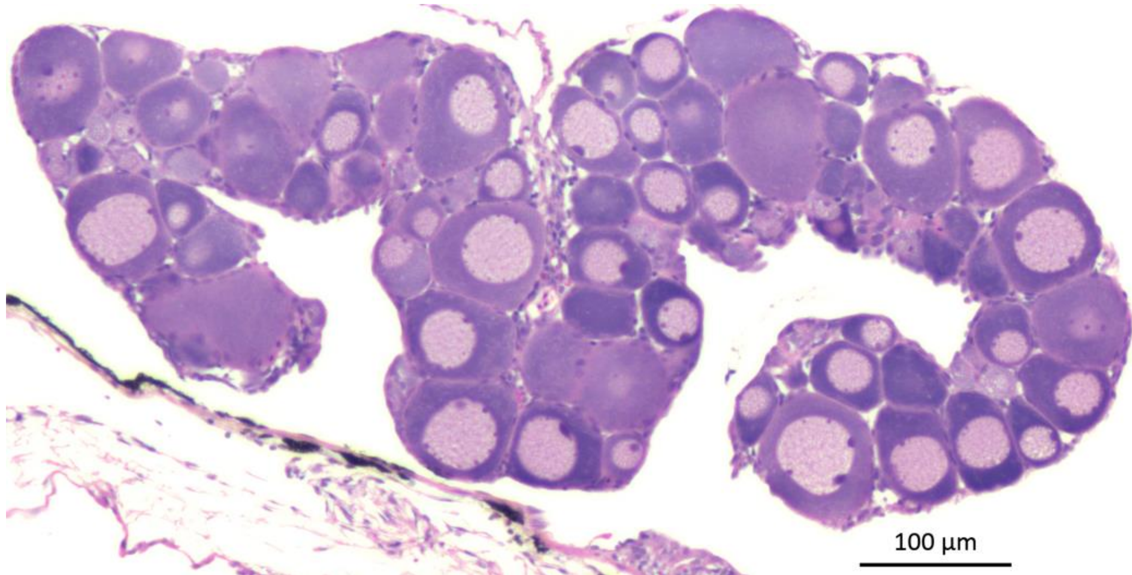
3156 **Figure A.2.42:** Fish identification T8-1 black, male testis (20X)

3157


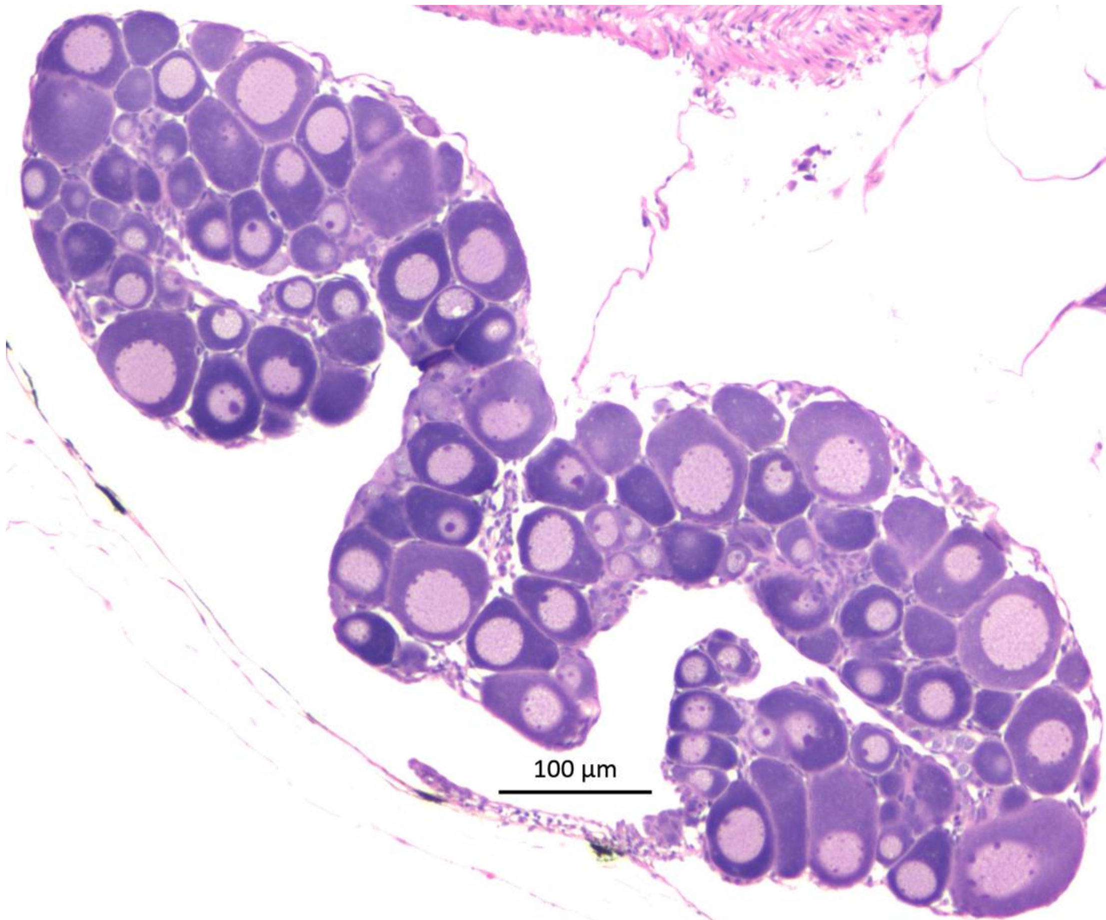
3158 **Figure A.2.43:** Fish identification T8-5 yellow, male testis (20X)

3159


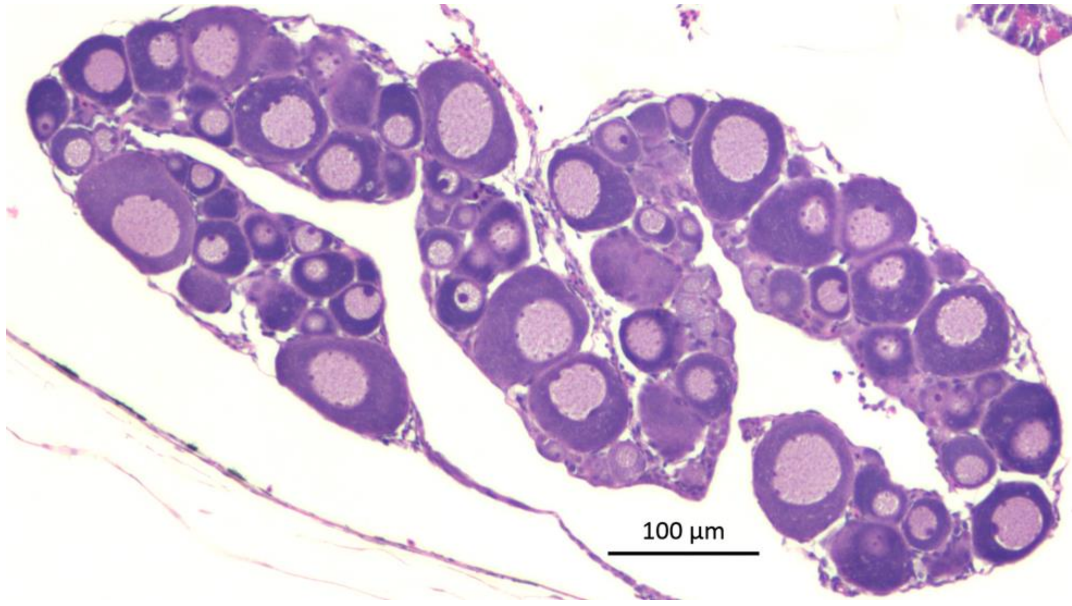
3160 **Figure A.2.44:** Fish identification T8-8 green, male testis (20X)



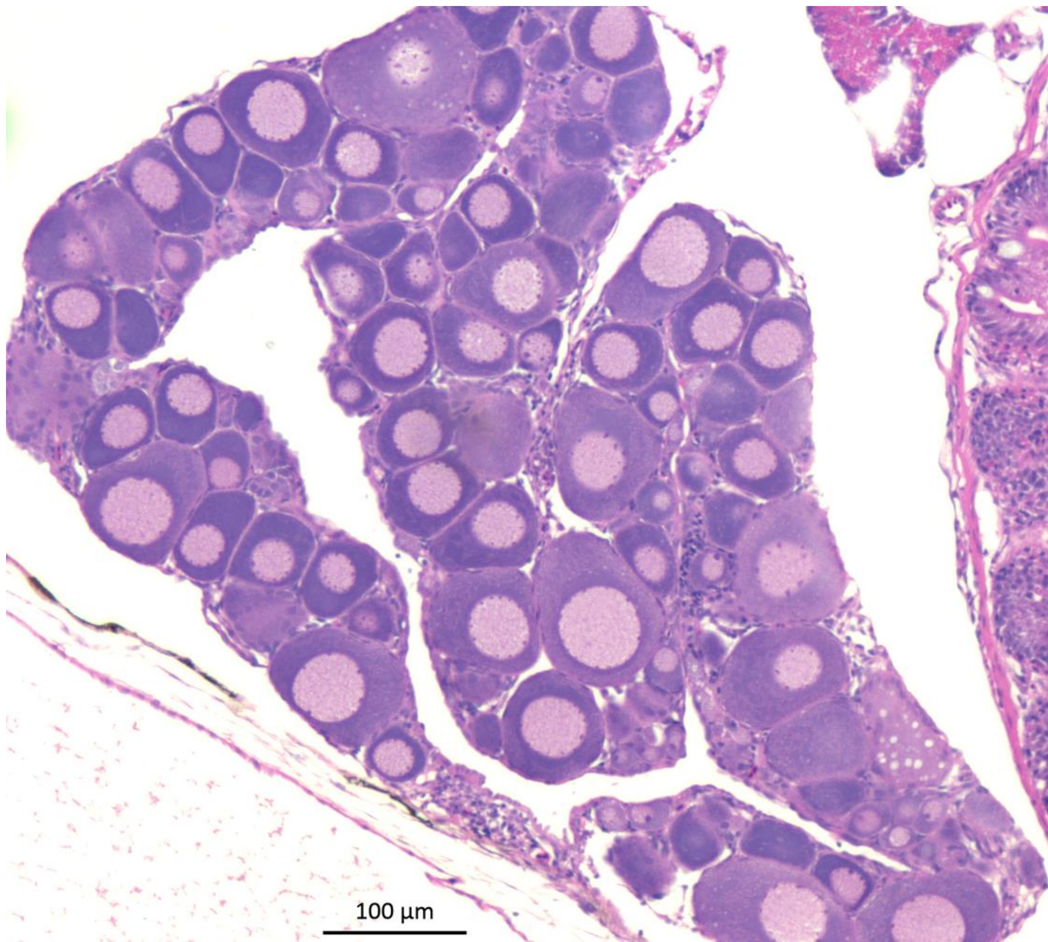
3161
3162 **Figure A.2.45:** Fish identification T9-2 orange, female ovary (10X)



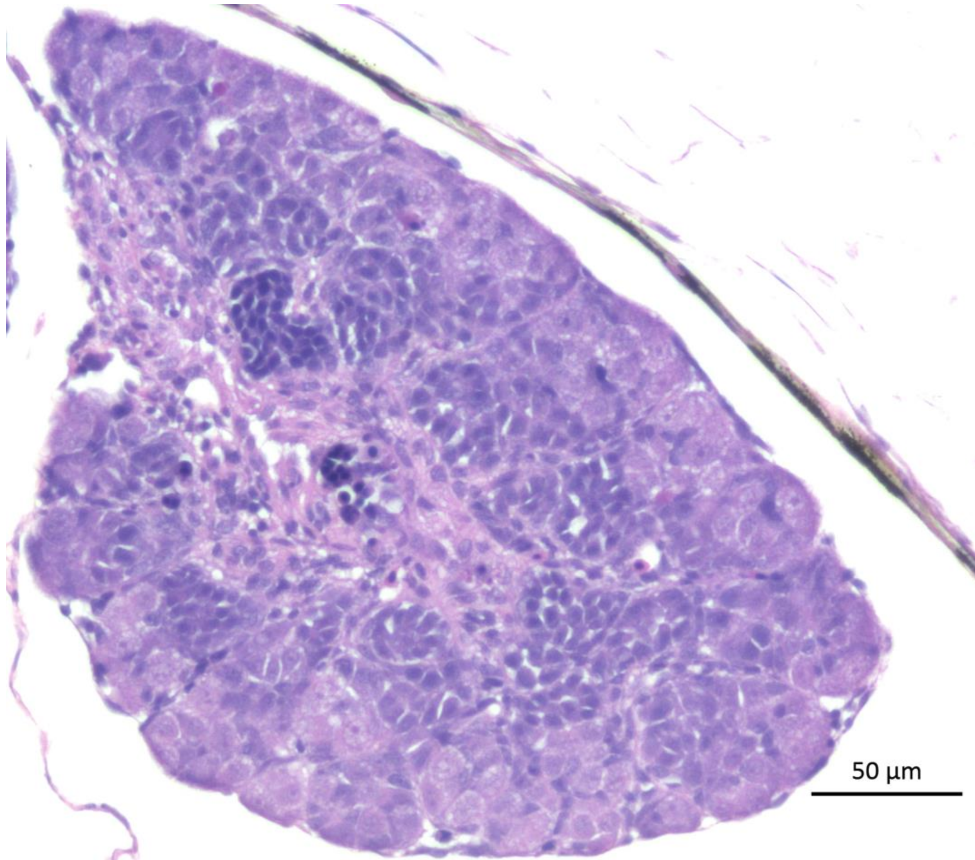
3163
3164 **Figure A.2.46:** Fish identification T9-9 blue, female ovary (10X)



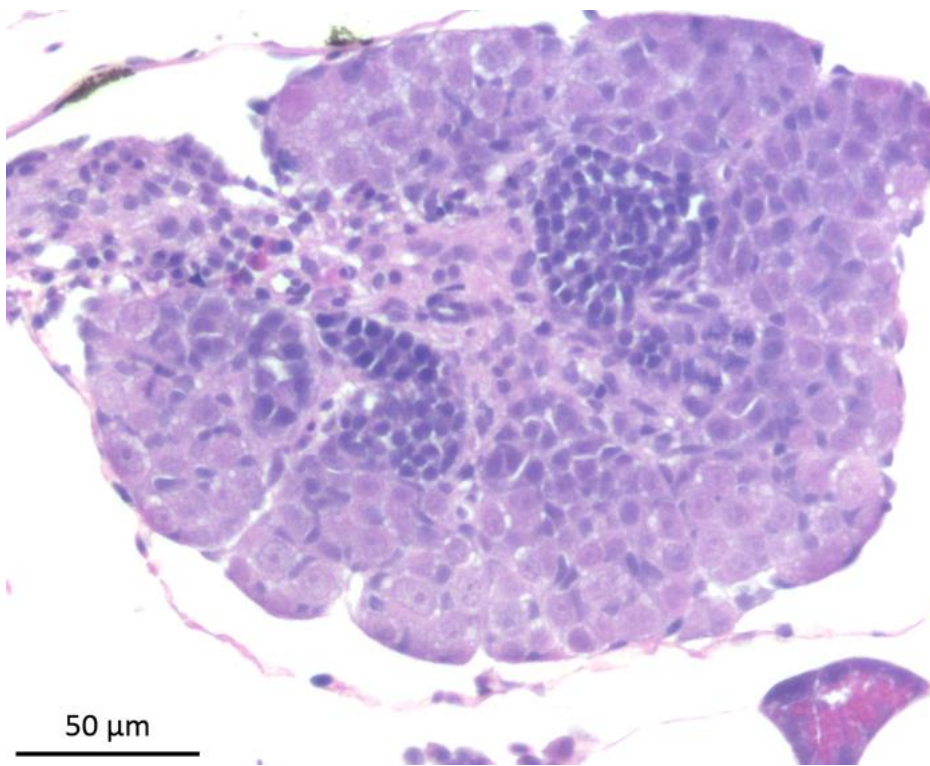
3165
3166 **Figure A.2.47:** Fish identification T9-10 black, female ovary (10X)



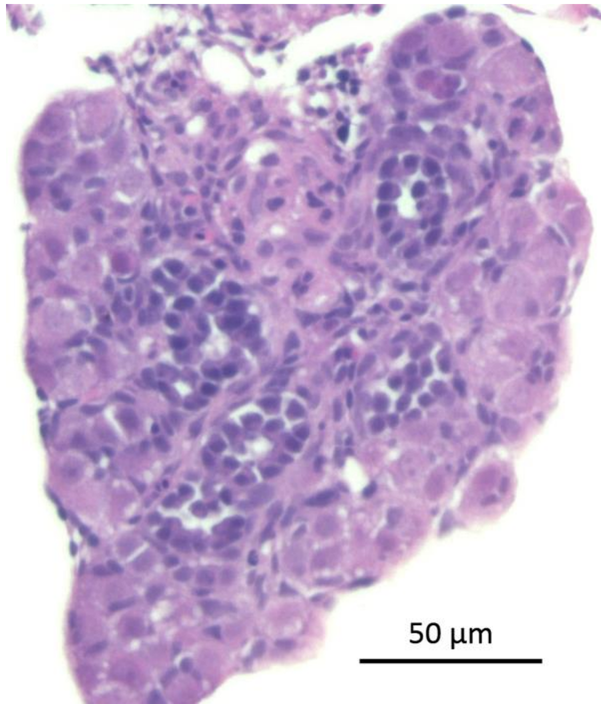
3167
3168 **Figure A.2.48:** Fish identification T9-12 blue, female ovary (10X)



3169
3170 **Figure A.2.49:** Fish identification T9-1 black, male testis (20X)



3171
3172 **Figure A.2.50:** Fish identification T9-5 black, male testis (20X)



3173
3174 **Figure A.2.51:** Fish identification T9-9 orange, male testis (20X)
3175
3176