

ASSESSMENT OF NON-LETHAL TOOLS TO ASSIGN SEX,
STAGE OF MATURITY, AND REPRODUCTIVE
CONDITION IN BURBOT *LOTA LOTA*

by

Lauren Marie McGarvey

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ABSTRACT

Non-lethal tools (plasma sex steroid concentrations and ultrasound) were assessed to assign sex, stage of maturity, and reproductive condition (non-reproductive and reproductive) in Burbot *Lota lota* from Lake Roosevelt, Washington. Gonadal tissue, plasma samples, and gonadal sonograms were collected from Burbot. Gonadal tissue was processed for histological analysis to describe gametogenesis and confirm sex, stage of maturity, and reproductive condition. Plasma testosterone (T) and estradiol-17 β (E2) concentrations were measured by radioimmunoassay. Plasma 11-ketotestosterone (11-KT) concentrations were measured by liquid chromatography-mass spectrometry. Gametogenesis was described by gonadal histology during the entire reproductive cycle. Plasma sex steroid profiles, gonadosomatic index, and ovarian follicle diameter were also described during the entire reproductive cycle. Plasma 11-KT concentrations were used to assign sex with 81% accuracy during the entire reproductive cycle, and plasma 11-KT and E2 concentrations were used to assign sex with 98% accuracy during the reproductive phase (i.e., November to March in Lake Roosevelt). In females, plasma T concentration, plasma E2 concentration, and month were used to assign stage of maturity with 87% accuracy, and plasma T concentration and plasma E2 concentration were used to assign reproductive condition with 98% accuracy. In males, plasma 11-KT concentration, girth at the urogenital pore, and month were used to assign stage of maturity with 73% accuracy, and plasma T concentration was used to assign reproductive condition with 90% accuracy. Ultrasound was used to assign sex with 97% accuracy, and ultrasound measurements of gonad size were a promising tool to assign stage of maturity and reproductive condition. Non-lethal tools (plasma sex steroid concentrations, gonad size measured by ultrasound, and ovarian follicle diameter) were also assessed to identify mass ovarian follicular atresia in female Burbot. Plasma T concentrations and ovarian follicle diameter were promising tools to identify mass ovarian follicular atresia. Non-lethal tools to assign sex, stage of maturity, and reproductive condition will enable fisheries biologists to assess indices of reproductive potential for the Burbot population in Lake Roosevelt. Indices of reproductive potential can be used to characterize and monitor population demographics, improve models of population growth, establish sustainable harvest regulations, monitor the effects of management actions, and monitor the effects of environmental stressors.

CHAPTER ONE

INTRODUCTION TO THESIS

Understanding the reproductive biology of fishes is essential in fisheries science and management because it determines the reproductive potential of a population (i.e., potential to produce recruitment) (Morgan 2008). A few indices of reproductive potential include sex ratio, reproductive structure (i.e., proportion of females and males in each stage of maturity), and spawning periodicity. These indices vary among species, populations, and within a population over time (Morgan 2008). Therefore, it is important to assess indices of reproductive potential that will influence population growth and resiliency to human activities, such as fishing pressure and habitat degradation (Morgan 2008).

Determining sex, stage of maturity, and reproductive condition in fishes is required to assess indices of reproductive potential. Gonadal histology is routinely used to determine sex, stage of maturity, and reproductive condition in fishes (Blazer 2002) but requires gametogenesis (i.e., the development of ovarian follicles and spermatozoa) to first be described which has never been fully described in Burbot *Lota lota*. The pre-vitellogenic (i.e., before yolk accumulation) and vitellogenic (i.e., yolk accumulation) stages of gametogenesis have been described by gonadal histology in female Burbot, and the spermatogenetic (i.e., sperm maturation) stage of gametogenesis has been described by gonadal histology in male Burbot (Schaefer et al. 2016). However, the post-ovulatory (i.e., post-spawning) and atretic (i.e., degradation and resorption of ovarian follicles)

stages of gametogenesis in female Burbot and the pre-spermatogenic (i.e., proliferation of spermatogonia) and post-spermiation (i.e., post-spawning) stages of gametogenesis in male Burbot remain undescribed.

Gonadal histology may be used to assign sex, stage of maturity, and reproductive condition but requires sacrificing the fish; therefore, fisheries biologists are interested in non-lethal tools. Non-lethal tools, such as quantifying plasma sex steroid concentrations and using ultrasound to examine gonadal morphology, have been used to assign sex, stage of maturity, and reproductive condition in fishes (Webb et al. 2002; Evans et al. 2004; Kohn et al. 2013) but must be validated by gonadal histology to ensure they are accurate. To our knowledge, non-lethal tools have not been assessed to assign sex, stage of maturity, and reproductive condition in Burbot, a species that does not exhibit sexual dimorphism (i.e., sex cannot be determined by external appearance) (Cott et al. 2013).

Plasma sex steroids have been used to assign sex, stage of maturity, and reproductive condition in modern teleosts and phylogenetically ancient sturgeons, such as Paiche *Arapaima gigas* (Chu-Koo et al. 2009), Hapuku *Polyprion oxygeneios* (Kohn et al. 2013), Cutthroat Trout *Oncorhynchus clarkii* (Bangs and Nagler 2014), Stellate Sturgeon *Acipenser stellatus* (Ceapa et al. 2002), White Sturgeon *Acipenser transmontanus* (Webb et al. 2002; Feist et al. 2004), Persian sturgeon *Acipenser persicus* (Viayeh et al. 2006), Lake Sturgeon *Acipenser fluvienscens* (Craig et al. 2009), and Chinese Sturgeon *Acipenser sinensis* (Du et al. 2017). Plasma sex steroids have distinct patterns of synthesis during gonadal development, known as plasma sex steroid profiles. For example, in female Rainbow Trout *Oncorhynchus mykiss*, plasma testosterone (T) and

estradiol-17 β (E2) concentrations peak prior to ovulation, with plasma T concentrations being much higher than plasma E2 concentrations (Scott et al. 1980a). Plasma 11-ketotestosterone (11-KT) concentrations also peak prior to ovulation but are much lower than plasma T and E2 concentrations (Scott et al. 1980a). During ovulation, plasma T concentrations are elevated whereas plasma E2 concentrations are low or non-detectable (Scott et al. 1980a). In male Rainbow Trout, plasma T concentrations increase until spermiation begins, while plasma 11-KT concentrations continue to increase until spermiation peaks (Scott et al. 1980b). Plasma E2 concentrations in male Rainbow Trout remain low throughout the reproductive cycle (Scott et al. 1980b). Describing the plasma sex steroid profiles for a population may enable individual sex, stage of maturity, and reproductive condition to be assigned by quantifying plasma sex steroid concentrations; however, there must be differences in steroid concentrations among classifications for this to be successful. To our knowledge, plasma sex steroid concentrations have not been assessed to assign sex, stage of maturity, and reproductive condition in Burbot.

Ultrasound has also been used to assign sex, stage of maturity, and reproductive condition in fishes, such as Atlantic Cod *Gadus morhua* (Karlsen and Holm 1994), Pacific Halibut *Hippoglossus stenolepis* (Loher and Stephens 2011), Murray cod *Maccullochella peelii* (Newman et al. 2008), Hapuku (Kohn et al. 2013), Cutthroat Trout (Bangs and Nagler 2013), and Stellate Sturgeon (Moghim et al. 2002). Ultrasound can accurately assign sex because of the gross morphological differences between ovaries and testes in fishes (Moghim et al. 2002; Loher and Stephens 2011). For example, sex can be assigned with 100% accuracy in Pacific Halibut because the ovaries are rounded and

tapered, whereas the testes are sickle-shaped and pointed (Loher and Stephens 2011). Ultrasound has been used to assign stage of maturity by detecting visual changes in developing gonads (e.g., appearance of oocytes, changes in tissue echogenicity, changes in gonad size) (Blythe et al., 1994; Evans et al. 2004; Novelo and Tiersch 2016). For example, ultrasound has been used to assign stage of maturity (pre-spawn and post-spawn) in adult steelhead *Oncorhynchus mykiss* (Evans et al. 2004). In females, ultrasound was used to detect morphological differences between pre-spawn and post-spawn ovaries. Pre-spawn ovaries had many ovarian follicles whereas post-spawn ovaries had only remnant ovarian follicles (Evans et al. 2004). In males, ultrasound was used to measure differences in gonad size between pre-spawn and post-spawn testes. Pre-spawn testes had a mean area of 2.86 cm² whereas post-spawn testes had a mean area of 0.62 cm² (Evans et al. 2004). Ultrasound can be used to assign stage of maturity in Burbot if there are detectable morphological differences in gonads among classifications. To our knowledge, ultrasound has not been assessed to assign sex, stage of maturity, and reproductive condition in Burbot.

Plasma sex steroid concentrations and ultrasound may also be used to identify mass ovarian follicular atresia in fishes. Ovarian follicular atresia is the breakdown and removal of ovarian follicles within the ovary to recover and recycle yolk materials (Grier et al. 2009; Wootton and Smith 2014). Plasma sex steroid concentrations have been used to identify mass ovarian follicular atresia in White Sturgeon *Acipenser transmontanus* (Talbot et al. 2011). Ovarian follicles undergoing atresia cease to synthesize sex steroids, resulting in decreased plasma sex steroid concentrations (Webb et al. 2001;

Talbott et al. 2011). Additionally, ovarian follicles undergoing atresia decrease in size as yolk is phagocytized and resorbed (Miranda et al. 1999), suggesting that gonad size or ovarian follicle diameter may be used to identify mass ovarian follicular atresia. To our knowledge, plasma sex steroid concentrations, gonad size measured by ultrasound, and ovarian follicle diameter have not been assessed to identify mass ovarian follicular atresia in female Burbot.

The Burbot in our study were collected from Lake Roosevelt, Washington. Lake Roosevelt supports the only known population of native Burbot with a stable abundance in Washington, despite population declines in other regions of their native range (e.g., Wyoming, Idaho, and British Columbia (Polacek et al. 2006; Golder Associates 2017). Lake Roosevelt supports a Burbot fishery, but the reproductive potential of the population is not well understood. Non-lethal tools to assign sex, stage of maturity, and reproductive condition will enable fisheries biologists to assess indices of reproductive potential (e.g., sex ratio, reproductive structure, and spawning periodicity) for the Burbot population in Lake Roosevelt to improve population forecasting models and determine sustainable harvest regulations of Burbot in Lake Roosevelt.

Overview of Thesis

In chapter two, gametogenesis was microscopically described in adult Burbot by gonadal histology during an entire reproductive cycle. Gametogenesis was also macroscopically described by gonadosomatic index (i.e., ratio of gonad weight to body weight) and ovarian follicle diameter during an entire reproductive cycle. Plasma sex

steroid profiles were described during an entire reproductive cycle. Non-lethal tools, including plasma sex steroid concentrations and ultrasound, were assessed to assign sex, stage of maturity, and reproductive condition in adult Burbot. Sex, stage of maturity, and reproductive condition assigned by plasma sex steroid concentrations and ultrasound were compared to sex, stage of maturity, and reproductive condition assigned by gonadal histology to determine the accuracy of each non-lethal tool.

In chapter three, gonad size (diameter and circumference) measured by ultrasound was assessed as a metric to assign stage of maturity and reproductive condition in adult Burbot. Stage of maturity and reproductive condition could not be assigned by ultrasound in chapter two (i.e., the ultrasound transducer could not detect echogenic or morphological changes among gonads); therefore, an additional study was designed to assess gonad size measured by ultrasound as a metric to assign stage of maturity and reproductive condition. Gonad diameter and circumference were measured monthly during the reproductive phase (i.e., November to March in Lake Roosevelt). The trends of gonad size measured by ultrasound during the reproductive phase are described. Gonad diameter measured by ultrasound was compared to excised gonad diameter measured by digital calipers, and gonad circumference measured by ultrasound was compared to excised gonad circumference measured by a measuring tape to assess if there was a difference between measurement types.

In chapter four, plasma sex steroid concentrations, gonad diameter measured by ultrasound, and ovarian follicle diameter were assessed as metrics to non-lethally identify mass ovarian follicular atresia in adult female Burbot. Histological analysis of ovarian

follicles was used to confirm mass ovarian follicular atresia. The trends in plasma sex steroid concentrations, gonad diameter measured by ultrasound, and ovarian follicle diameter during mass ovarian follicular atresia were described.

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CHAPTER TWO

DESCRIPTION OF GAMETOGENESIS AND ASSESSMENT OF NON-LETHAL
TOOLS TO ASSIGN SEX, STAGE OF MATURITY, AND REPRODUCTIVE
CONDITION IN BURBOT *LOTA LOTA*

Contributions of Authors and Co-Authors

Manuscript in Chapter 2

Author: Lauren M. McGarvey

Contributions: Conceived and implemented the study design, collected and analyzed data, and authored the manuscript.

Co-Author: Molly A. H. Webb

Contributions: Obtained funding, conceived the study design, discussed the implications of the results, and co-authored the manuscript.

Co-Author: Christopher S. Guy

Contributions: Obtained funding, conceived the study design, discussed the implications of the results, and co-authored the manuscript.

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Lauren M. McGarvey, Christopher S. Guy, Molly A. H. Webb

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ABSTRACT

Non-lethal tools (plasma sex steroid concentrations and ultrasound) were assessed to assign sex, stage of maturity, and reproductive condition in Burbot *Lota lota* from Lake Roosevelt, Washington. Gonadal tissue, blood plasma, and gonadal sonograms were collected monthly from November 2016 to March 2018. Gonadal tissue was processed for histological analysis to describe gametogenesis and confirm sex, stage of maturity, and reproductive condition. Plasma testosterone (T) and estradiol-17 β (E2) concentrations were measured by radioimmunoassay. Plasma 11-ketotestosterone (11-KT) concentrations were measured by liquid chromatography-mass spectrometry. Gametogenesis was described by gonadal histology during an entire reproductive cycle. Plasma sex steroid profiles, gonadosomatic index, and ovarian follicle diameter were also described during the entire reproductive cycle. Plasma 11-KT concentrations were used to assign sex with 81% accuracy during the entire reproductive cycle, and plasma 11-KT and E2 concentrations were used to assign sex with 98% accuracy during the reproductive phase (i.e., November to March in Lake Roosevelt). In females, plasma T concentration, plasma E2 concentration, and month were used to assign stage of maturity with 87% accuracy, and plasma T concentration and plasma E2 concentration were used to assign reproductive condition with 98% accuracy. In males, plasma 11-KT concentration, girth at the urogenital pore, and month were used to assign stage of maturity with 73% accuracy, and plasma T concentration was used to assign reproductive condition with 90% accuracy. Ultrasound was used to assign sex with 97% accuracy, but ultrasound could not be used to assign stage of maturity and reproductive condition. The non-lethal tools (plasma sex steroid concentrations and ultrasound) will enable fisheries biologists to assess indices of reproductive potential for the Burbot population in Lake Roosevelt. Indices of reproductive potential can be used characterize and monitor population demographics, improve models of population growth, establish sustainable harvest regulations, monitor the effects of management actions, and monitor the effects of environmental stressors.

Introduction

Gametogenesis, the development of ovarian follicles and spermatozoa, has never been fully described by gonadal histology in Burbot *Lota lota*. A single study has described portions of gametogenesis by gonadal histology (Schaefer et al. 2016), but the description did not include the entire reproductive cycle. The pre-vitellogenic (i.e., before yolk accumulation) and vitellogenic (i.e., yolk accumulation) stages of gametogenesis have been described in female Burbot, and the spermatogenic (i.e., sperm maturation) stage of gametogenesis has been described in male Burbot (Schaefer et al. 2016). However, the atretic (i.e., degradation and resorption of ovarian follicles) and the post-ovulatory (i.e., post-spawning) stages in female Burbot and the pre-spermatogenic (i.e., proliferation of spermatogonia) and post-spermiation (i.e., post-spawning) stages in male Burbot remain undescribed.

Gametogenesis can be macroscopically described by visual examination, gonadosomatic index (GSI), and ovarian follicle diameter but is most precisely described by gonadal histology (Tomkiewicz et al. 2003; Brown-Peterson et al. 2011). Gonadal histology provides a detailed description of the microscopic morphological changes in germ cells during gametogenesis (Cassel et al. 2017). Gonadal histology is routinely used for sex verification and identifying stage of maturity in fishes (Blazer 2002). Sex, stage of maturity, and reproductive condition can be reliably assigned by gonadal histology once gametogenesis has been completely described.

Burbot do not exhibit sexual dimorphism, meaning females and males cannot be differentiated by external appearance (Cott et al. 2013). Sex, stage of maturity, and

reproductive condition are only externally evident when fish release gametes. Histology may be used to assign sex, stage of maturity, and reproductive condition but requires sacrificing the fish. Non-lethal tools, such as quantifying plasma sex steroid concentrations and using ultrasound to examine gonadal morphology, have been used to assign sex, stage of maturity, and reproductive condition in fishes (e.g., Webb et al. 2002; Evans et al. 2004; Kohn et al. 2013) but have not been assessed in Burbot.

Plasma sex steroids have been used to assign sex, stage of maturity, and reproductive condition in modern teleosts and phylogenetically ancient sturgeons, such as Paiche *Arapaima gigas* (Chu-Koo et al. 2009), Hapuku *Polyprion oxygeneios* (Kohn et al. 2013), Cutthroat Trout *Oncorhynchus clarkii clarkii* (Bangs and Nagler 2014), Stellate Sturgeon *Acipenser stellatus* (Ceapa et al. 2002), White Sturgeon *Acipenser transmontanus* (Webb et al. 2002; Feist et al. 2004), Persian sturgeon *Acipenser persicus* (Viayeh et al. 2006), Lake Sturgeon *Acipenser fluvescens* (Craig et al. 2009), and Chinese Sturgeon *Acipenser sinensis* (Du et al. 2017). Plasma sex steroids are synthesized following the hypothalmo-pituitary-gonadal (HPG) axis (Webb and Doroshov 2011; Wotton and Smith 2015). Environmental cues and endogenous conditions act on the brain, stimulating the synthesis of gonadotropin-releasing hormone (GnRH) in the hypothalamus (Webb and Doroshov 2011). The GnRH then stimulates the synthesis of follicle stimulating hormone (FSH) and luteinizing hormone (LH) in the pituitary gland (Webb and Doroshov 2011). In females, the FSH and LH stimulate the synthesis of testosterone (T) and estradiol-17 β (E2) in the ovaries (Kagawa 2013; Wotton and Smith 2015). Some females may also synthesize 11-ketotestosterone (11-KT) in the ovaries,

but 11-KT is considered a minor sex steroid in females (Lokman et al. 2002; Matsubara et al. 2003). The T is enzymatically converted into E2, and E2 stimulates the synthesis of vitellogenin (VTG) in the liver (Pankhurst 2008; Wootton and Smith 2015). The VTG is taken up by ovarian follicles and cleaved into yolk proteins (Pankhurst 2008; Wootton and Smith 2015). After vitellogenesis is complete, the LH stimulates the synthesis of maturation inducing hormone (MIH) and initiates oocyte maturation (e.g., germinal vesicle breakdown, chromosome condensation, and formation of the first polar body) (Matsuyama 2002). In males, the LH stimulates the synthesis of T and 11-KT in the testes (Schulz et al. 2010). The 11-KT stimulates spermatogonial proliferation, and 11-KT and T initiate spermatogenesis (Pankhurst 2008; Schulz et al. 2010; Wootton and Smith 2015). The FSH stimulates structural and nutritional support of developing germ cells (Schulz et al. 2010).

Plasma sex steroids have distinct patterns of synthesis during gonadal development known as plasma sex steroid profiles. For example, in female Rainbow Trout *Oncorhynchus mykiss*, plasma T and E2 concentrations peak prior to ovulation, with plasma T concentrations being much higher than plasma E2 concentrations (Scott et al. 1980a). Plasma 11-KT concentrations also peak prior to ovulation but are much lower than plasma T and E2 concentrations (Scott et al. 1980a). During ovulation, plasma T concentrations are elevated whereas plasma E2 concentrations are low or non-detectable (Scott et al. 1980a). In male Rainbow Trout, plasma T concentrations increase until spermiation begins, while plasma 11-KT concentrations continue to increase until spermiation peaks (Scott et al. 1980b). Plasma E2 concentrations in male Rainbow Trout

remain low throughout the reproductive cycle (Scott et al. 1980b). Describing the plasma sex steroid profiles for a population may enable individual sex, stage of maturity, and reproductive condition to be assigned by quantifying plasma sex steroid concentrations; however, there must be differences in steroid concentrations among assignments for this to be successful. To our knowledge, plasma sex steroid concentrations have not been assessed to assign sex, stage of maturity, and reproductive condition in Burbot.

Ultrasound has been used to assign sex, stage of maturity, and reproductive condition in fishes, such as Atlantic Cod *Gadus morhua* (Karlsen and Holm 1994), Stellate Sturgeon (Moghim et al. 2002), Murray Cod *Maccullochella peelii* (Newman et al. 2008), Pacific Halibut *Hippoglossus stenolepis* (Loher and Stephens 2011), Cutthroat Trout (Bangs and Nagler 2013), and Hapuku (Kohn et al. 2013). Ultrasound can accurately assign sex because of the gross morphological differences between ovaries and testes in fishes (Moghim et al. 2002; Loher and Stephens 2011). For example, sex can be assigned with 100% accuracy in Pacific Halibut because the ovaries are rounded and tapered, whereas the testes are sickle-shaped and pointed (Loher and Stephens 2011). To our knowledge, ultrasound has not been assessed to assign sex in Burbot.

Ultrasound has been used to assign stage of maturity and reproductive condition by detecting visual changes in developing gonads (e.g., appearance of oocytes, changes in tissue echogenicity, changes in gonad size) (Evans et al. 2004; Novelo and Tiersch 2016). Ultrasound has been used to assign stage of maturity (pre-spawn and post-spawn) in adult steelhead *Oncorhynchus mykiss* (Evans et al. 2004). In females, ultrasound was used to detect morphological differences between pre-spawn and post-spawn ovaries. Pre-spawn

ovaries had many ovarian follicles whereas post-spawn ovaries had only remnant ovarian follicles (Evans et al. 2004). In males, ultrasound was used to measure differences in gonad size between pre-spawn and post-spawn testes. Pre-spawn testes had a mean area of 2.86 cm², whereas post-spawn testes had a mean area of 0.62 cm² (Evans et al. 2004). An ultrasound index of gonadal development in female Channel Catfish *Ictalurus punctatus* was also described using morphological and echogenic changes in ovaries during development (Novelo and Tiersch 2016). Undeveloped (i.e., non-reproductive) ovaries were differentiated by homogenous echogenic texture and lack of distinct oocyte structures, while developing (i.e., reproductive) ovaries had distinct oocyte structures (Novelo and Tiersch 2016). To our knowledge, ultrasound has not been assessed to assign stage of maturity and reproductive condition in Burbot.

The objectives of this study were to describe gametogenesis and the plasma sex steroid profiles in Burbot and assess non-lethal tools (plasma sex steroid concentrations and ultrasound) to assign sex, stage of maturity, and reproductive condition in Burbot from Lake Roosevelt, Washington. Non-lethal tools to assign sex, stage of maturity, and reproductive condition will enable fisheries biologists to assess indices of reproductive potential (e.g., sex ratio, reproductive structure, and spawning periodicity) for the Burbot population in Lake Roosevelt. Indices of reproductive potential can be used to characterize and monitor population demographics, model population growth, establish sustainable harvest regulations, monitor the effects of management actions, and monitor the effects of environmental stressors (Trippel 1993; Downs et al. 1997; Wildhaber et al. 2007; Power 2007; Morgan 2008; Schill et al. 2010).

Study Area

Lake Roosevelt is located in northeast Washington. The 209-km reservoir was formed after the construction of Grand Coulee Dam (GCD) on the Columbia River. The reservoir is typically 1-3 km wide with a maximum depth of 122 m extending 241 km upstream from GCD to the Canadian border (Polacek et al. 2006). At full pool elevation, the reservoir covers approximately 33,000 ha (Polacek et al. 2006). The reservoir supports many recreational fisheries including a Burbot fishery which may be underutilized and able to support greater harvest (CCT 2018). The reservoir is designated as a National Recreation Area by the National Park Service.

Methods

Fish Collection and Maintenance

Burbot were collected from Lake Roosevelt in November 2016, March 2017, June 2017, and October 2017 by the Confederated Tribes of the Colville Reservation. Baited cod traps were set at 10 m and pulled the following day. Cod traps were at 10 m to prevent barotrauma in captured fish. Each fish was tagged with a passive integrated transponder (PIT) and transferred to the Bozeman Fish Technology Center, Bozeman MT. Fish were maintained in tanks under a natural light cycle and a thermal profile matching Lake Roosevelt (Figure 2.1). Fish were fed live feed until satiation.

Histology

Gonadal tissue preserved in 10% phosphate buffered formalin for histological analysis (Webb and Erickson 2007). Gonadal tissue was embedded in paraffin wax and sectioned at 5 μ m. Gonadal tissue from November 2016 to March 2017 was stained with hematoxylin and eosin (H&E), and gonadal tissue from April 2017 to March 2018 was stained with periodic acid-Schiff (PAS). The PAS stain was used if post-ovulatory follicles were suspected to be present in ovarian tissue. Slides were examined under a Leica DM compound scope (250 – 450X) (Leica Biosystems Inc., IL, USA). Gametogenesis in Burbot was described following the descriptions of gametogenesis in teleost fishes from Brown-Peterson et al. (2011) and Grier et al. (2009).

Biological Sampling

Biological data were collected monthly from individual fish and included the following: body weight (± 0.01 g), gonad weight (± 0.01 g), total length (± 0.01 cm), girth at the pectoral fins and urogenital pore (± 0.01 cm), blood sample, ultrasound images of gonads, and gonadal tissue sample. Twelve fish (six females and six males) were euthanized by overdose of MS-222 (tricaine methanesulfonate) monthly from November 2016 to September 2017 (except for 13 fish in March 2017 and 16 fish in June 2017 to ensure at least six fish of each sex were included). Only seven fish were euthanized in October 2017 due to a limited number of Burbot. Six fish (three females and three males) were euthanized monthly from November 2017 to March 2018 to reduce the number of Burbot needed for the study.

Gonadosomatic Index and Ovarian Follicle Diameter

Gonads were excised and weighed (± 0.01 g) for calculation of gonadosomatic index ($GSI = [\text{gonad weight}/\text{total body weight}] \times 100$). Ovarian follicles were collected from excised ovaries and preserved in Ringers solution (Dettlaff et al. 1993). Fifteen ovarian follicles (Johnson 1971) from each sample were measured by image analysis using SPOT Advanced Software (SPOT Imaging, Michigan, USA). The diameter of each ovarian follicle was measured twice to increase measurement precision. If the difference between the two diameter measurements was greater than 3 μm , the measurements were repeated. The largest diameter measurement was recorded.

Plasma Sex Steroids

Blood was collected from the caudal vasculature using a heparinized syringe (3 cc with 22-gauge needle) (Webb et al. 2002). Plasma was separated by centrifugation (3400 rpm for 5 minutes) and stored at -80°C until analysis. Testosterone and E2 were extracted from the plasma following the methods of Fitzpatrick et al. 1987. Briefly, steroids from 100 μl of plasma were extracted twice with 2 ml of diethyl ether. Test tubes (12 x 75 mm) were vortexed vigorously with ether, and the aqueous phase was removed by snap-freezing in liquid nitrogen. Ether was evaporated overnight in a fume hood. Extracted steroids were resuspended in 1 ml of phosphate-buffered saline with gelatin (PBSG) the following day. Extraction recovery efficiencies for T and E2 were determined by adding tritiated steroids to tubes containing pooled plasma ($n = 4$), which were extracted as described above. Extraction recovery efficiencies were 87 – 94% for T

and 80 – 94% for E2. All steroid concentrations were corrected for the extraction recovery efficiency.

Plasma T and E2 concentrations were quantified by radioimmunoassay (RIA) as described by Fitzpatrick et al. (1986) and modified by Feist et al. (1990). Extracted steroids (0.05 ml) were assayed in duplicates for T and E2. Briefly, antibody (0.1 ml) was diluted in PBSG and added to all assay tubes except total count and non-specific binding tubes. Tubes were incubated at room temperature for 20 minutes. Tritiated steroid (0.1 ml) was added to all tubes. Tubes were incubated at room temperature for 90 minutes and then incubated in an ice bath for five minutes. Dextran charcoal suspension (6.25 g charcoal and 4.0 g dextran/l PBSG) was added in the appropriate volume (1.0 ml for T or 0.5 ml for E2). Tubes were incubated in an ice bath for 10 minutes and then centrifuged at 2200 g for 20 minutes. Aliquots from all tubes (0.5 ml) in scintillation cocktail (5 ml) were counted in a Beckman Coulter LS 6500 scintillation spectrophotometer (Beckman Coulter Inc., California, USA). Plasma sex steroid concentrations quantified by RIA were validated by verifying the serial dilutions were parallel to standard curves. Quantified plasma sex steroid concentrations below the minimum quantifiable concentration (MQC) of the RIA (i.e., the lowest concentration that was considered to be reliably quantified) were assigned the MQC (0.27 – 0.37 ng/mL for T and 0.15 – 0.19 ng/mL for E2). Non-detectable plasma sex steroid concentrations (i.e., not quantifiable) were assigned half of the MQC for statistical purposes (0.12 – 0.19 ng/mL for T and 0.06 – 0.09 ng/mL for E2) (Croghan and Egeghy 2003). The intra- and inter-assay coefficients of variation for all assays was less than 5 and 10%, respectively.

The 11-KT was extracted from plasma with methyl-tert-butyl ether. Plasma 11-KT concentrations were quantified by liquid chromatography-mass spectrometry (LC-MS) in the lab of Dr. Nancy Denslow at the University of Florida, Gainesville, Florida. Plasma 11-KT concentrations below the minimum quantifiable concentration (MQC) of the LC-MS analysis were assigned the MQC (0.02 ng/ml). Non-detectable 11-KT concentrations were assigned half of the MQC for statistical purposes (0.01 ng/ml) (Croghan and Egeghy 2003).

Ultrasound

A SonoSite Edge ultrasound (SonoSite Inc., Washington, USA) with a linear transducer (6-15 MHz) was used to capture images of gonads in euthanized fish. The Small Parts exam type was used with the optimization set to General. The ultrasound scanning depth was set between 1.8 and 2.2 cm. Fish were oriented ventrally, and three images were taken as follows: 1) transducer placed perpendicular to the midline at the urogenital pore, 2) transducer placed perpendicular to the midline and anterior to the urogenital pore, and 3) transducer placed parallel to the midline. All ultrasound images were archived. The accuracy of using ultrasound to assign sex and stage of maturity was assessed using paired gonadal histology and ultrasound images.

Data Analyses

Data were checked for normality using side-by-side boxplots and Q-Q plots. Body weight, total length, girth at the pectoral fins, and girth at the urogenital pore data were normally distributed, and parametric analyses (ANOVA) were used. The GSI,

ovarian follicle diameter, and plasma sex steroid concentration data were non-normally distributed, and non-parametric analyses (Kruskal-Wallis test) were used. The accepted significance level was $\alpha = 0.05$. Data are presented from June, the beginning of the annual reproductive cycle, to May, the end of the annual reproductive cycle. An AdaBoost algorithm (Alfaro et al. 2013) was used to determine the accuracy of assigning sex by plasma sex steroid concentrations. AdaBoost algorithms were then used to determine the accuracy of assigning stage of maturity and reproductive condition within a sex by plasma sex steroid concentrations and morphological parameters. The accuracy of assignment was determined by leave-one-out cross-validation. All statistical analyses were completed using R software (version 3.3.2).

Results

Histology

Gametogenesis was described by gonadal histology during an entire reproductive cycle. Nine stages of gonadal maturity were described for females (Table 2.1). Oogonial proliferation (Stage 1) ovaries were characterized by oogonia and potentially a few primary growth oocytes (i.e., growth before vitellogenesis) (Table 2.1). No females were observed in the oogonial proliferation stage of maturity; therefore, this stage of maturity was described following the description of gametogenesis in teleosts by Grier et al. (2009). Primary growth (Stage 2) ovaries were characterized by primary growth oocytes and oogonia (Table 2.1) (Figure 2.2.A). Thirty females were observed in the primary growth stage of maturity (Table 2.2). Cortical alveolar (Stage 3) ovaries were

characterized by ovarian follicles with cortical alveoli (Table 2.1) (Figure 2.2.B). Five females were observed in the cortical alveolar stage of maturity (Table 2.2). Early vitellogenic (Stage 4) ovaries were characterized by ovarian follicles with yolk granules accumulating on the periphery and a single layer of the zona radiata (Table 2.1) (Figure 2.2.C). Eight females were observed in the early vitellogenic stage of maturity (Table 2.2). Mid-vitellogenic (Stage 5) ovaries were characterized by ovarian follicles with yolk globules (mean diameter = $7.69\ \mu\text{m}$ ($5.97 - 9.89\ \mu\text{m}$; 95% CI)) accumulating towards the center (but may be present throughout), cortical alveoli on the periphery, and two layers of the zona radiata (Table 2.1) (Figure 2.2.D). Thirteen females were observed in the mid-vitellogenic stage of maturity (Table 2.2). Late vitellogenic (Stage 6) ovaries were characterized by ovarian follicles with larger yolk globules (mean diameter = $19.62\ \mu\text{m}$ ($14.55 - 24.69\ \mu\text{m}$; 95% CI)) throughout, two layers of the zona radiata, and a central germinal vesicle (i.e., nucleus) (Table 2.1) (Figure 2.2.E). Sixteen females were observed in the late vitellogenic stage of maturity (Table 2.2). Ripe (Stage 7) ovaries were characterized by ovarian follicles with fused yolk and an offset germinal vesicle (Table 2.1) (Figure 2.2.F). Three females were observed in the ripe stage of maturity (Table 2.2). Post-ovulatory (Stage 8) ovaries were characterized by the presence of post-ovulatory follicles, primary oocytes, and oogonia (Table 2.1) (Figure 2.2.G). Nineteen females were observed in the post-ovulatory stage of maturity (Table 2.2). Post-ovulatory follicles remained in the ovaries for many months following spawning and were observed in primary growth (Stage 2) females. Therefore, month was used to differentiate between post-ovulatory (Stage 8) and primary growth (Stage 2) females.

Females with post-ovulatory follicles present in the ovaries were classified as post-ovulatory (Stage 8) until the month of May, which is two months after spawning in March. Females with post-ovulatory follicles present in the ovaries after the month of May were classified as primary growth (Stage 2) because post-ovulatory follicles were collapsed, and oocytes were undergoing primary growth. Atretic (Stage 9) ovaries were characterized by more than 75% of ovarian follicles undergoing atresia (i.e., fragmentation, folding, and thickening of zona radiata) (Table 2.1) (Figure 2.2.H). Four females were observed in the atretic stage of maturity (Table 2.2).

Six stages of gonadal maturity were described for males (Table 2.1).

Spermatogonial proliferation (Stage 1) testes were characterized by testicular cysts containing only spermatogonia (Table 2.1) (Figure 2.3.A). Twenty males were observed in the spermatogonial proliferation stage of maturity (Table 2.3). Early spermatogenic (Stage 2) testes were characterized by testicular cysts containing spermatogonia and spermatocytes (Table 2.1) (Figure 2.3.B). Seventeen males were observed in the early spermatogenic stage of maturity (Table 2.3). Mid-spermatogenic (Stage 3) testes were characterized by more than 50% of testicular cysts containing spermatocytes and spermatids, with a few cysts containing spermatogonia (Table 2.1) (Figure 2.3.C). Ten males were observed in the mid-spermatogenic stage of maturity (Table 2.3). Ripe (Stage 4) testes were characterized by testicular cysts filled with spermatozoa and few testicular cysts (i.e., approximately 20%) beginning to empty of spermatozoa (Table 2.1) (Figure 2.3.D). Nine males were observed in the ripe stage of maturity (Table 2.3). Mid-spermiation (Stage 5) testes were characterized by testicular cysts with reduced

spermatozoa and testicular cysts empty of spermatozoa (Table 2.1) (Figure 2.3.E). Nine males were observed in the mid-spermiation stage of maturity (Table 2.3). Post-spermiation (Stage 6) testes were characterized by testicular cysts with residual spermatozoa and testicular cysts empty of spermatozoa (Table 2.1) (Figure 2.3.F). Six males were observed in the post-spermiation stage of maturity (Table 2.3).

Reproductive condition was characterized by the stages of maturity indicating a fish was reproductive (i.e., had initiated gonadal development to spawn or capable of spawning) or non-reproductive (i.e., had not initiated gonadal development to spawn or had already spawned) (Brown-Peterson et al. 2011). Females in the cortical alveolar (Stage 3), early vitellogenic (Stage 4), mid-vitellogenic (Stage 5), late vitellogenic (Stage 6), and ripe (Stage 7) stages of maturity were reproductive, whereas females in the oogonial proliferation (Stage 1), primary growth (Stage 2), post-ovulation (Stage 8), and atretic (Stage 9) stages of maturity were non-reproductive (Brown-Peterson et al. 2011). Males in the early spermatogenic (Stage 2), mid-spermatogenic (Stage 3), ripe (Stage 4), and mid-spermiation (Stage 5) stages of maturity were reproductive, whereas males in the spermatogonial proliferation (Stage 1) and post-spermiation (Stage 6) stages of maturity were non-reproductive (Brown-Peterson et al. 2011).

Biological Sampling

Female body weight varied from 460.30 to 1467.10 g and did not differ significantly by month ($F = 1.40$, $df = 11, 86$, $P = 0.189$) (Figure 2.4). Mean female body weight was 838.56 g (796.20 – 880.92 g; 95% CI). Male body weight varied from 436.90 to 2,420.60 g and differed significantly by month ($F = 2.00$, $df = 11, 59$, $P =$

0.044) (Figure 2.4). Mean male body weight was 835.78 g (764.47 – 907.09 g; 95% CI).

A male with a body weight of 2,420.60 g was an extreme outlier in March.

Female total length varied from 43.60 to 64.00 cm and did not differ significantly by month ($F = 1.41$, $df = 11, 86$, $P = 0.182$) (Figure 2.5). Mean female total length was 52.33 cm (51.52 – 53.15 cm; 95% CI). Male total length varied from 36.00 to 69.00 cm and did not differ significantly by month ($F = 1.43$, $df = 11, 59$, $P = 0.183$) (Figure 2.5). Mean male total length was 51.77 cm (50.51 – 53.02 cm; 95% CI).

Female girth at the pectoral fins varied from 14.40 to 25.40 cm and differed significantly by month ($F = 3.22$, $df = 11, 86$, $P = 0.001$). Mean female girth at the pectoral fins increased from 17.82 cm (16.50 – 19.15 cm; 95% CI) in November to 21.68 cm (20.07 – 23.29 cm; 95% CI) in February and decreased to 19.50 cm (18.14 – 20.86 cm; 95% CI) in March (Figure 2.6). Female girth at the pectoral fins differed significantly by stage of maturity ($F = 3.53$, $df = 7, 90$, $P = 0.002$). Mean female girth at the pectoral fins increased from 17.80 cm (16.26 – 19.34 cm; 95% CI) during early vitellogenesis (Stage 4) to 21.41 cm (20.44 – 22.38 cm; 95% CI) during late vitellogenesis (Stage 6) and decreased to 18.93 cm (17.72 – 20.13 cm; 95% CI) during post-ovulation (Stage 8) (Figure 2.7). Mean female girth at the pectoral fins was 20.13 cm (13.39 – 26.86 cm; 95% CI) during atresia (Stage 9) and similar to ripe (Stage 7) females (Figure 2.7).

Male girth at the pectoral fins varied from 14.10 to 29.00 cm and differed significantly by month ($F = 3.04$, $df = 11, 56$, $P = 0.003$). Mean male girth at the pectoral fins increased from 17.26 cm (15.91 – 18.61 cm; 95% CI) in December to 21.34 cm

(19.27– 23.41 cm; 95% CI) in March and decreased to 19.70 cm (13.57– 25.83 cm; 95% CI) in April (Figure 2.8). Male girth at the pectoral fins differed significantly by stage of maturity ($F = 8.97$, $df = 5, 62$, $P = < 0.001$). Mean male girth at the pectoral fins increased from 17.56 cm (16.39 – 18.73 cm; 95% CI) during early spermatogenesis (Stage 2) to 21.56 cm (18.67 – 24.44 cm; 95% CI) during mid-spermiation (Stage 5) and decreased to 16.95 cm (15.11 – 18.79 cm; 95% CI) during post-spermiation (Stage 6) (Figure 2.9).

Female girth at the urogenital pore varied from 13.00 to 24.20 cm and differed significantly by month ($F = 5.09$, $df = 11, 86$, $P = < 0.001$). Mean female girth at the urogenital pore increased from 16.56 cm (13.93 – 19.18 cm; 95% CI) in November to 19.64 cm (18.29 – 21.00 cm; 95% CI) in February and decreased to 16.14 cm (14.39 – 17.89 cm; 95% CI) in April (Figure 2.10). Female girth at the urogenital pore differed significantly by stage of maturity ($F = 7.33$, $df = 7, 90$, $P = < 0.001$). Mean female girth at the urogenital pore increased from 15.50 cm (14.32 – 16.68 cm; 95% CI) during the appearance of cortical alveoli (Stage 3) to 19.59 cm (18.80 – 20.39 cm; 95% CI) during late vitellogenesis (Stage 6) and decreased to 16.02 cm (15.07 – 16.96 cm; 95% CI) during post-ovulation (Stage 8) (Figure 2.11). Mean female girth at the urogenital pore was 18.25 cm (12.44 – 24.06 cm; 95% CI) during atresia (Stage 9) and similar to late vitellogenic (Stage 6) females (Figure 2.11).

Male girth at the urogenital pore varied from 12.40 to 25.50 cm and differed significantly by month ($F = 4.38$, $df = 11, 56$, $P = < 0.001$). Mean male girth at the urogenital pore increased from 15.57 cm (14.77 – 16.36 cm; 95% CI) in November to

18.78 cm (16.99 – 20.57 cm; 95% CI) in January and remained elevated until decreasing to 13.90 cm (0.01 – 27.88 cm; 95% CI) in May (Figure 2.12). Male girth at the urogenital pore differed significantly by stage of maturity ($F = 13.31$, $df = 5, 62$, $P = < 0.001$). Mean male girth at the urogenital pore increased from 15.86 cm (14.95 – 16.77 cm; 95% CI) during early spermatogenesis (Stage 2) to 19.50 cm (18.57 – 20.43 cm; 95% CI) during mid-spermatogenesis (Stage 3) and remained elevated until decreasing to 15.17 cm (13.23 – 17.10 cm; 95% CI) during post-spermiation (Stage 6) (Figure 2.13).

Gonadosomatic Index and Ovarian Follicle Diameter

Female GSI varied from 0.24 to 16.46 and differed significantly by month ($H = 74.71$, $df = 11$, $P = < 0.001$). Median female GSI increased from 1.34 (0.77 – 1.93; min. – max.) in October to 13.75 (0.96 – 16.46; min. – max.) in March and decreased to 1.07 (0.51 – 2.44; min. – max.) in April (Figure 2.14). Female GSI differed significantly by stage of maturity ($H = 81.07$, $df = 7$, $P = < 0.001$). Median female GSI increased from 0.79 (0.24 – 1.16; min. – max.) during primary growth (Stage 2) to 14.00 (11.55 – 14.01; min. – max.) when ovarian follicles were ripe (Stage 7) and decreased to 0.87 (0.51 – 2.44; min. – max.) following spawning (Stage 8) (Figure 2.15). Female GSI was highly variable during atresia (Stage 9), varying from 2.39 to 13.64, because atresia was more advanced in some females than others (Figure 2.15).

Male GSI varied from 0.01 to 22.80 and differed significantly by month ($H = 57.99$, $df = 11$, $P = < 0.001$). Median male GSI increased from 1.83 (0.21 – 4.70; min. – max.) in November to 13.31 (6.45 – 15.49; min. – max.) in January and remained elevated at 12.78 (10.97 – 22.80; min. – max.) in February (Figure 2.16). Male GSI was

highly variable in March, varying from 0.78 to 19.78, because some males had initiated or completed spermiation while others had not (Figure 2.16). Male GSI differed significantly by stage of maturity ($H = 60.87$, $df = 5$, $P = < 0.001$). Median male GSI increased from 0.43 (0.01 – 0.76; min. – max.) during spermatogonial proliferation (Stage 1) to 15.15 (12.41 – 22.8; min. – max.) during mid-spermatogenesis (Stage 3) and steadily decreased to 0.82 (0.41 – 2.93; min – max) during post-spermiation (Stage 6) (Figure 2.17).

Ovarian follicle diameter varied from 116 to 894 μm and significantly differed by month ($H = 74.08$, $df = 11$, $P = < 0.001$). Median ovarian follicle diameter increased from 138 μm (123 – 140 μm ; min. – max.) in June to 804 μm (699 – 894 μm ; min. – max.) in March (Figure 2.18). Ovarian follicle diameter significantly differed by stage of maturity ($H = 71.68$, $df = 6$, $P = < 0.001$). Median ovarian follicle diameter increased from 147 μm (123 – 204 μm ; min. – max.) during primary growth (Stage 2) to 856 μm (827 – 894 μm ; min. – max.) when ovarian follicles were ripe (Stage 7) (Figure 2.19). Atretic (Stage 9) ovarian follicles could not be accurately measured because advanced atresia caused the ovarian follicles to become misshaped; therefore, atretic ovarian follicles were removed from the analysis.

Plasma Sex Steroids

Female plasma T concentrations varied from non-detectable to 39.86 ng/ml and differed significantly by month ($H = 89.67$, $df = 11$, $P = < 0.001$). Median female plasma T concentrations increased from 0.31 ng/ml in October to 27.04 ng/ml in February and decreased to non-detectable in April (Table 2.4). Female plasma T concentrations

differed significantly by stage of maturity ($H = 84.63$, $df = 7$, $P = < 0.001$). Median female plasma T concentration increased from 0.14 ng/ml during primary growth (Stage 2) to 25.99 ng/ml during late vitellogenesis (Stage 6) (Table 2.4). Median female plasma T concentration remained elevated when ovarian follicles were ripe (Stage 7) and decreased to non-detectable during post-ovulation (Stage 8) (Table 2.4). Female plasma T concentrations were variable during atresia (Stage 9), varying from non-detectable to 11.76 ng/ml (Table 2.4).

Female plasma E2 concentrations varied from non-detectable to 14.96 ng/ml and differed significantly by month ($H = 71.67$, $df = 11$, $P = < 0.001$). Median female plasma E2 concentration increased from 0.08 ng/ml in October to 6.00 ng/ml in January and decreased to non-detectable in April (Table 2.5). Female plasma E2 concentrations differed significantly by stage of maturity ($H = 76.68$, $df = 7$, $P = < 0.001$). Median female plasma E2 concentration increased from non-detectable during the appearance of cortical alveoli (Stage 3) to 6.50 ng/ml during late vitellogenesis (Stage 6) and decreased to non-detectable when ovarian follicles were ripe (Stage 7) (Table 2.5). Female plasma E2 concentrations were variable during atresia (Stage 9), varying from non-detectable to 2.8 ng/ml (Table 2.5).

Female plasma 11-KT concentrations varied from non-detectable to 0.18 ng/ml among all months and stages of maturity. Female plasma 11-KT concentrations are not further reported because of the non-detectable or low concentrations among all month and stages of maturity.

Male plasma T concentrations varied from non-detectable to 36.76 ng/ml and differed significantly by month ($H = 53.76$, $df = 11$, $P = < 0.001$). Median male plasma T concentration increased from 0.35 ng/ml in September to 20.52 ng/ml in February and decreased to non-detectable in April (Table 2.6). Male plasma T concentrations differed significantly by stage of maturity ($H = 57.68$, $df = 5$, $P = < 0.001$). Median male plasma T concentration increased from non-detectable during spermatogonial proliferation (Stage 1) to 23.75 ng/ml when testes were ripe (Stage 4) and decreased to non-detectable after spermiation (Stage 6) (Table 2.6).

Male plasma 11-KT concentrations varied from non-detectable to 79.63 ng/ml and differed significantly by month ($H = 44.71$, $df = 11$, $P = < 0.001$). Median male plasma 11-KT concentration increased from 0.60 ng/ml in October to 9.88 ng/ml in February and decreased to 0.04 ng/ml in April (Table 2.7). Male plasma 11-KT concentrations differed significantly by stage of maturity ($H = 41.56$, $df = 5$, $P = < 0.001$). Median plasma 11-KT concentration increased from 0.02 ng/ml during spermatogonial proliferation (Stage 1) to 18.09 ng/ml when testes were ripe (Stage 4) and decreased to 0.05 ng/ml after spermiation (Stage 6) (Table 2.7).

Male plasma E2 concentrations varied from non-detectable to 0.70 ng/ml among all months and stages of maturity. Male plasma E2 concentrations are not further reported because of the non-detectable or low concentrations among all months and stages of maturity.

Plasma 11-KT concentration was the best predictor of sex during the entire reproductive cycle (see appendix A). Sex was assigned with 97% accuracy in females

and 60% accuracy in males during the entire reproductive cycle (Table 2.8). The combination of plasma 11-KT concentration and plasma E2 concentration was the best predictor of sex during the reproductive phase (i.e., November to March in Lake Roosevelt) (see appendix A). Sex was assigned with 98% accuracy in both females and males during the reproductive phase (Table 2.8).

In females, the combination of plasma T concentration, plasma E2 concentration, and month was the best predictor of stage of maturity (see appendix A). Six stages of maturity were assigned with high accuracy including primary growth (Stage 2) (90%), cortical alveolar (Stage 3) (80%), early vitellogenic (Stage 4) (100%), mid-vitellogenic (Stage 5) (85%), late vitellogenic (Stage 6) (94%), and post-ovulatory (Stage 8) (95%) (Table 2.9). Two stages of maturity were assigned with low accuracy, including ripe (Stage 7) (67%) and atretic (Stage 9) (0%) (Table 2.9)

In females, the combination of plasma T concentration and plasma E2 concentration was the best predictor of reproductive condition (see appendix A). Non-reproductive condition was assigned with 97% accuracy and reproductive condition was assigned 100% accuracy (Table 2.10).

In males, the combination of plasma 11-KT concentration, girth at the urogenital pore, and month was the best predictor of stage of maturity (see appendix A). Three stages of maturity were assigned with high accuracy including spermatogonial proliferation (Stage 1) (85%), early spermatogenic (Stage 2) (93%), and mid-spermiation (Stage 5) (Table 2.11). Three stages of maturity were assigned with low accuracy

including mid-spermatogenic (Stage 3) (50%), ripe (Stage 4) (56%), and post-spermiation (Stage 6) (33.3%) (Table 2.11).

In males, plasma T concentration was the best predictor of reproductive condition (see appendix A). Non-reproductive condition was assigned with 92% accuracy and reproductive condition was assigned with 89% accuracy (Table 2.12).

Ultrasound

We observed distinct morphological characteristics between ovaries and testes in Burbot. Lobes of the ovaries were rounded and connected at the urogenital pore (Figure 2.20). Testes were angular and unconnected at the urogenital pore (Figure 2.21). The morphological differences between ovaries and testes were easily delineated using ultrasound during the spawning season. It is more difficult to differentiate ovaries and testes using ultrasound following the spawning season (Figure 2.22).

Sex was assigned with 99% accuracy in females using ultrasound (Table 2.13). Only a single female was misclassified in June when gonads were small and undeveloped. Sex was assigned with 95% accuracy in males using ultrasound (Table 2.13). Only two males were misclassified in June, and a single male was misclassified in each January 2017, March 2017, and April 2017. Sex was assigned with 100% accuracy in the remaining months using ultrasound (Table 2.13).

Ultrasound was not useful for assigning stage of maturity in this study. Morphological and echogenic differences among gonads of differing stages of maturity could not be detected using the SonoSite Edge ultrasound unit. For example, ovarian

follicles could not be detected because they were less than 1 mm in diameter when ripe, and the resolution of the ultrasound transducer was 1 mm.

Discussion

To our knowledge, we have documented the first complete histological description of gametogenesis in Burbot. A partial description of gametogenesis in Burbot has already been published (Schaefer et al. 2016), but this description included only the pre-vitellogenic and vitellogenic stages in females and the spermatogenic stages in males. In addition to the pre-vitellogenic, vitellogenic, and spermatogenic stages, our description of gametogenesis includes the post-ovulation and atretic stages in females and the spermatogonial proliferation and post-spermiation stages in males. Our complete description of gametogenesis enabled us to determine the Burbot collected for our study from Lake Roosevelt have an annual spawning periodicity and validate non-lethal tools (plasma sex steroid concentrations and ultrasound) to assign sex, stage of maturity, and reproductive condition by gonadal histology, which is considered the most accurate method to assign sex, stage of maturity, and reproductive condition in fishes (Blazer 2002).

The pattern of sex steroid synthesis in fishes (i.e., plasma sex steroid profiles) enables the successful assignment of sex, stage of maturity, and reproductive condition (e.g., Ceapa et al. 2002; Webb et al. 2002; Feist et al. 2004; Viayeh et al. 2006; Webb and Erickson 2007; Chu-Koo et al. 2009; Craig et al. 2009; Kohn et al. 2013; Bangs and Nagler 2014; Du et al. 2017). In Burbot, plasma 11-KT and E2 concentrations assigned

sex with 88% accuracy during the entire year and with 98% accuracy during the reproductive phase (i.e., November to March in Lake Roosevelt). Plasma 11-KT and E2 concentrations have been used to successfully assign sex in other fishes such as Stellate Sturgeon (Ceapa et al. 2002), Paiche (Chu-Koo et al. 2009), and Hapuku (Kohn et al. 2013). Plasma 11-KT and E2 concentrations were used to assign sex in Hapuku with 100% accuracy when fish were reproductive (i.e., two months prior to the spawning season and during the spawning season), but these steroids were not useful in assigning sex when fish were non-reproductive (i.e., post-spawn) (Kohn et al. 2013). We also observed a lower accuracy of assigning sex in non-reproductive Burbot using plasma 11-KT and E2 concentrations. Plasma sex steroids are not synthesized until fish have initiated gonadal development to spawn (Feist et al. 2004). In females, plasma T and E2 concentrations increase during vitellogenesis (i.e., accumulation of yolk in ovarian follicles) (Feist et al. 2004; Kagawa 2013; Wootton and Smith 2015). Plasma 11-KT concentrations may also increase during vitellogenesis depending on the species (Borg 1994). In males, plasma T and 11-KT concentrations increase with the initiation of spermatogenesis (i.e., meiosis) while plasma E2 concentrations remain low (Feist et al. 2004; Pankhurst 2008; Schulz et al. 2010; Wootton and Smith 2015). Both sexes have low plasma sex steroid concentrations before the initiation of a reproductive cycle and after spawning until initiation of the next reproductive cycle (Pankhurst 2008) making it difficult to assign sex using plasma sex steroid concentrations during these phases. Therefore, sampling time is an important consideration when using plasma sex steroids to assign sex in Burbot. Plasma samples should be collected during the reproductive phase

when sex steroid concentrations are elevated (i.e., November to March in Lake Roosevelt) to assign sex with the highest accuracy.

In female Burbot, plasma T and E2 concentrations were used to assign reproductive condition (non-reproductive and reproductive) with 98% accuracy. Plasma 11-KT concentrations were not used to assign reproductive condition because plasma 11-KT concentrations were non-detectable or low in all females as has been observed in other teleosts species (Borg 1994). In male Burbot, plasma T concentrations were used to assign reproductive condition with 90% accuracy. Plasma 11-KT concentrations can also be used to assign reproductive condition but with slightly lower accuracy. Plasma sex steroid concentrations have been used to assign reproductive condition in other fishes, such as White Sturgeon (Webb et al. 2002), Persian Sturgeon (Viayeh et al. 2006), Green Sturgeon *Acipenser medirostris* (Webb and Erickson 2007), and Cutthroat Trout (Bangs and Nagler 2014). Assigning reproductive condition can be used to determine the proportion of females and males spawning during an annual reproductive cycle, which has been documented to vary among Burbot populations (Pulliainen and Korhonen 1990; McPhail and Paragamian 2002). For example, approximately 30% of adult Burbot in Bothnian Bay, Finland were not developing to spawn during an annual reproductive cycle (Pulliainen and Korhonen 1990). Variation in the proportion of fish spawning may negatively affect the reproductive potential of a population and accounting for this variation may influence management decisions (Morgan 2008; Rideout and Tomkiewicz 2011). Sampling time is an important consideration when assigning reproductive condition to determine the proportion of females and males spawning during an annual

reproductive cycle. Plasma samples should be collected during the reproductive phase when sex steroid concentrations are elevated (i.e., November to March in Lake Roosevelt). Plasma samples should also be collected before spawning because sex steroid concentrations cannot be used to reliably differentiate fish that did not develop to spawn and post-spawn fish.

Plasma T did not improve the accuracy of assigning sex in Burbot but was useful in assigning reproductive condition. Plasma 11-KT and E2 concentrations were the best predictors of sex. In teleosts, 11-KT is considered the primary androgen (Borg 1994), and plasma 11-KT and E2 concentrations have been used to successfully assign sex in other teleosts (Chu-Koo et al. 2009; Kohn et al. 2013). Plasma T and E2 concentrations may be used to assign sex in Burbot but with a lower accuracy. Using plasma T and E2 concentrations to assign sex may be preferable because only two steroids would need to be analyzed to assign sex and reproductive condition, which may reduce the cost of analyzing samples. The choice of what plasma sex steroids to analyze will depend on the specific objectives and limitations of a fisheries program, such as acceptable accuracy of assignment and funding.

The plasma sex steroid profiles described in this study are similar to plasma sex steroid profiles described in other total spawning teleosts such as Rainbow Trout (Scott et al. 1980a; Scott et al. 1980b), Coho Salmon *Oncorhynchus kisutch* (Fitzpatrick et al. 1986), and Amago Salmon *Oncorhynchus masou* (Kagawa 2013). However, the plasma sex steroid profiles we observed differ from those previously observed in Burbot by Mustonen et al. (2002). Unlike our observations, plasma E2 concentrations remained

elevated in ripe (mean = 7.16 ng/ml) and post-ovulatory (mean = 5.36 ng/ml) female Burbot, and plasma T concentrations were much lower in ripe male Burbot (mean = 10.38 ng/ml) (Mustonen et al. 2002). Plasma sex steroid concentrations may differ among populations because of environmental contaminants which are known to disrupt the synthesis of sex steroids (Kime 1995; Nicolas 1999; Feist et al. 2005; Hinck et al. 2008). The models described can be applied to other Burbot populations; however, plasma sex steroid concentrations should first be quantified in a subset of the naïve population to determine if the plasma sex steroid profiles are similar to those we observed in the Lake Roosevelt population.

Ultrasound was used to assign sex with 97% accuracy in Burbot by detecting differences in gonadal morphology between females and males. Many studies have previously used ultrasound to accurately assign sex in fishes (e.g., Karlsen and Holm 1994; Moghim et al. 2002; Newman et al. 2008; Loher and Stephens 2011; Bangs and Nagler 2013; Kohn et al. 2013). Sex was assigned in reproductive Atlantic Cod (a member of the same taxonomic order as Burbot) using ultrasound to detect echogenic differences between ovaries and testes with 95% accuracy (Karlsen and Holm 1994). Sex was more difficult to assign in non-reproductive Atlantic Cod because there was little echogenic difference between ovaries and testes (Karlsen and Holm 1994). More recently, sex was assigned in non-reproductive and reproductive Pacific Halibut using ultrasound to detect morphological differences between ovaries and testes with 100% accuracy (Loher and Stephens 2011). In Pacific Halibut, ovaries are rounded and tapered, whereas testes are sickle-shaped and pointed (Loher and Stephens 2011). In this

study, we were also able to assign sex in both non-reproductive and reproductive Burbot using morphological differences between ovaries and testes, although accuracy decreased after spawning when the gonads were small and undeveloped (i.e., 81% in June). Therefore, sampling time should be considered when using ultrasound to assign sex in Burbot.

In this study, stage of maturity and reproductive condition could not be assigned using ultrasound because ovarian follicles (< 1 mm in diameter) were too small to be detected with the SonoSite Edge ultrasound unit. Ultrasound has been used to assign stage of maturity and reproductive condition in other female teleosts by detecting ovarian follicles, such as steelhead and Channel Catfish (Evans et al. 2004; Novelo and Tiersch 2016). However, ovarian follicles in ripe female steelhead were much larger (3 mm in diameter) than the ovarian follicles we observed in ripe female Burbot, making them easily detectable using ultrasound (Evans et al. 2004). Even though ovarian follicles in Burbot were too small to be detected using our ultrasound unit, we did notice an increase in the diameter of ovarian follicles collected during the reproductive phase. Ovarian follicle diameter can be used to assign stage of maturity and reproductive condition in female fishes because ovarian follicles increase in size during vitellogenesis (West 1990; Tyler and Sumpter 1996). Catheterization can be used to non-lethally collect ovarian follicles to measure diameter as an indication of stage of maturity and reproductive condition (Rottman et al. 1991; Markmann and Doroshov 1993). In chapter four, we demonstrated the diameter of ovarian follicles collected by catheterization is a promising tool to assign stage of maturity and reproductive condition in female Burbot.

Our observations of GSI in Burbot were similar to those previously observed in Burbot by Cott et al. (2013). Gonadosomatic index increased during the reproductive phase and was a metric of interest because it has been used to assign stage of maturity and reproductive condition in fishes (West 1990; Wootton and Smith 2015). However, calculating GSI requires sacrificing the fish to obtain a gonad weight. Because GSI is directly related to gonad size, we wanted to assess if gonad size measured by ultrasound could be used to non-lethally assign stage of maturity and reproductive condition in Burbot as has been done in other fishes (Blythe et al. 1994; Evans et al. 2004, Bryan et al. 2007). In chapter three, we demonstrated measuring gonad size by ultrasound is a promising tool to assign stage of maturity and reproductive condition in Burbot.

We demonstrated plasma sex steroid concentrations can be used to assign sex and reproductive condition in Burbot, and ultrasound can be used to assign sex in Burbot. Both tools are non-lethal and highly accurate, but there are differences in the cost and training associated with each tool. Collection of blood to quantify plasma sex steroid concentrations is minimally invasive and easy to collect, only requiring blood to be drawn from the caudal vasculature and centrifuged. However, the quantification of plasma sex steroid concentrations in a lab is expensive, varying from \$40 – 80 per sample, and the results are not immediate (Webb et al. 2017). Ultrasound is non-invasive, and the results are immediate. However, using ultrasound requires adequate training to become familiar with the visceral anatomy of the species of interest (i.e., 32 – 40 hours), and an ultrasound unit is expensive, varying from \$15,000 to 30,000 (Webb et al. 2017). The choice of tool will depend on the specific objectives and limitations of a

fisheries program, such as the duration of a project, funding, and need for immediate results.

The non-lethal tools described can be used to assign sex and reproductive condition in Burbot, enabling fisheries biologists to assess reproductive indices such as sex ratio, reproductive structure, and spawning periodicity. Reproductive indices are used in fisheries science and management to characterize and monitor population demographics, model population growth, establish sustainable harvest regulations, monitor the effects of management actions, and monitor the effects of environmental stressors (Trippel 1993; Downs et al. 1997; Wildhaber et al. 2007; Power 2007; Morgan 2008; Schill et al. 2010). By using the non-lethal tools we have described, fisheries biologists can assess reproductive indices of the Burbot population in Lake Roosevelt to inform and guide management decisions.

Table 2.1. Stages of gonadal development identified from histological analysis of gonadal tissue from Burbot. Stage numbers are referenced in parentheses. Germ cells were scored for stage of maturity using a modified protocol of Grier et al. (2009) and Brown-Peterson et al. (2011) to describe gametogenesis in Burbot.

Sex	Stage of Maturity	Description
Females	Oogonial Proliferation (1)	Oogonia and potentially a few primary growth oocytes
	Primary Growth (2)	Primary growth oocytes and oogonia
	Cortical Alveolar (3)	Cortical alveoli on periphery of ovarian follicles
	Early Vitellogenic (4)	Yolk granules accumulating in periphery of ovarian follicles, cortical alveoli on periphery of ovarian follicles, one layer of zona radiata
	Mid-Vitellogenic (5)	Yolk globules with a mean diameter of 7.93 μ m (5.97 – 9.89 μ m; 95% CI) accumulating towards center or may be present throughout ovarian follicles, few cortical alveoli on periphery of ovarian follicles, two layers of the zona radiata
	Late Vitellogenic (6)	Yolk fusing into larger globules with a mean diameter of 19.62 μ m (14.55 – 24.69 μ m; 95% CI) throughout ovarian follicle, two layers of zona radiata, central germinal vesicle (i.e., nucleus)
	Ripe (7)	Yolk fused but not completely coalesced in all ovarian follicles, offset germinal vesicle
	Post-Ovulatory (8)	Post-ovulatory follicles present with primary growth oocytes
	Atretic (9)	> 75% atretic ovarian follicles, no post-ovulatory follicles present with primary growth oocytes
Males	Spermatogonial Proliferation (1)	Cysts contain only spermatogonia
	Early Spermatogenic (2)	Cysts contain spermatogonia and spermatocytes
	Mid-Spermatogenic (3)	Few cysts contain spermatogonia, > 50% of the cysts contain spermatocytes and spermatids
	Ripe and Initiating Spermiation (4)	Cysts filled with spermatozoa, approximately 20% of cysts emptying of spermatozoa
	Mid-Spermiation (5)	Cysts with reduced or residual spermatozoa, some cysts may be empty
	Post-Spermiation (6)	Cysts with residual spermatozoa and cysts empty of spermatozoa

Table 2.2. Number of adult female Burbot in each stage of maturity determined by gonadal histology by month. No females were observed during oogonal proliferation (Stage 1).

Month	Oogonal proliferation (Stage 1)	Primary Growth (Stage 2)	Cortical Alveolar (Stage 3)	Early Vitellogenic (Stage 4)	Mid-Vitellogenic (Stage 5)	Late Vitellogenic (Stage 6)	Ripe (Stage 7)	Post-Ovulatory (Stage 8)	Atretic (Stage 9)
June		7							
July		7							1
August		8	1						1
September		6							
October		2	4						
November				8	1				
December					9				
January					2	5			1
February					1	8			
March						3	3	1	1
April								8	
May								10	
Total	0	30	5	8	13	16	3	19	4

Table 2.3. Number of adult male Burbot in each stage of maturity determined by gonadal histology by month.

Month	Spermatogonial Proliferation (Stage 1)	Early Spermatogenic (Stage 2)	Mid- Spermatogenic (Stage 3)	Ripe (Stage 4)	Mid- Spermiation (Stage 5)	Post- Spermiation (Stage 6)
June	8					1
July	3					1
August	2					
September	3					
October	1					
November	2	6				
December		8				
January		3	7			
February			3	6		
March				3	6	2
April					2	2
May	1				1	
Total	20	17	10	9	9	6

Table 2.4. Plasma testosterone (T) concentrations (ng/ml) in adult female Burbot by stage of maturity and month. Burbot were held at the Bozeman Fish Technology Center and exposed to a natural photoperiod and thermal profile similar to that in Lake Roosevelt, Washington. Data were collected from November 2016 to March 2018. Data are medians, ranges (min. – max.), and sample sizes (n). Plasma T concentrations below the minimum quantifiable concentration (MQC) of the radioimmunoassay were assigned the MQC (0.27 – 0.32 ng/ml). Non-detectable plasma T concentrations were assigned half of the MQC (0.12 – 0.19 ng/ml). No females were observed during oogonal proliferation (Stage 1).

Month	Primary Growth (Stage 2)	Cortical Alveolar (Stage 3)	Early Vitellogenic (Stage 4)	Mid-Vitellogenic (Stage 5)	Late Vitellogenic (Stage 6)	Ripe (Stage 7)	Post-Ovulatory (Stage 8)	Atretic (Stage 9)	Median by Month
June	0.12 n = 7								0.12 n = 7
July	0.12 (0.12 – 0.27) n = 7							0.12 n = 1	0.12 (0.12 – 0.27) n = 8
August	0.27 (0.14 – 0.27) n = 8	0.27 n = 1						0.27 n = 1	0.27 (0.14 – 0.27) n = 10
September	0.27 (0.27 – 0.32) n = 6								0.27 (0.27 – 0.32) n = 6
October	0.16 n = 2	0.44 (0.31 – 0.79) n = 4							0.31 (0.16 – 0.79) n = 6
November			1.41 (0.88 – 1.93) n = 8	2.36 n = 1					1.65 (0.88 – 2.36) n = 9
December				4.67 (1.73 – 8.11) n = 9					4.67 (1.73 – 8.11) n = 9
January				3.91 (2.82 – 5.00) n = 2	15.43 (4.06 – 21.42) n = 5			11.76 n = 1	12.34 (2.82 – 21.42) n = 8
February				8.21 n = 1	27.09 (12.25 – 37.03) n = 8				27.04 (8.21 – 37.03) n = 9
March					33.93 (29.28 – 39.25) n = 3	24.22 (5.35 – 39.86) n = 3	0.27 n = 1	0.47 n = 1	26.75 (0.27 – 39.86) n = 8
April							0.12 n = 8		0.12 n = 8
May							0.12 n = 10		0.12 n = 10
Median by Stage	0.14 (0.12 – 0.32) n = 30	0.31 (0.27 – 0.79) n = 5	1.41 (0.88 – 1.93) n = 8	4.67 (1.73 – 8.21) n = 13	25.99 (4.06 – 39.25) n = 16	24.22 (5.35 – 39.86) n = 3	0.12 (0.12 – 0.27) n = 19	0.37 (0.12 – 11.76) n = 4	

Table 2.5. Plasma estradiol-17 β (E2) concentrations (ng/ml) in adult female Burbot by stage of maturity and month. Burbot were held at the Bozeman Fish Technology Center and exposed to a natural photoperiod and thermal profile similar to that in Lake Roosevelt, Washington. Data were collected from November 2016 to March 2018. Data are medians, ranges (min. – max.), and sample sizes (n). Plasma E2 concentrations below the minimum quantifiable concentration (MQC) of the radioimmunoassay were assigned the MQC (0.15 – 0.17 ng/ml). Non-detectable plasma E2 concentrations were assigned half of the MQC (0.06 – 0.09 ng/ml). No females were observed during oogonial proliferation (Stage 1).

Month	Primary Growth (Stage 2)	Cortical Alveolar (Stage 3)	Early Vitellogenic (Stage 4)	Mid-Vitellogenic (Stage 5)	Late Vitellogenic (Stage 6)	Ripe (Stage 7)	Post-Ovulatory (Stage 8)	Atretic (Stage 9)	Median by Month
June	0.07 (0.07 – 0.15) n = 7								0.07 (0.07 – 0.15) n = 7
July	0.07(0.07 – 0.66) n = 7							0.07 n = 1	0.07(0.07 – 0.66) n = 8
August	0.08 n = 8	0.08 n = 1						0.08 n = 1	0.08 n = 10
September	0.08 (0.08 – 0.09) n = 6								0.08 (0.08 – 0.09) n = 6
October	0.08 n = 2	0.12 (0.08 – 0.63) n = 4							0.08 (0.08 – 0.63) n = 6
November			0.80 (0.26 – 1.22) n = 8	1.58 n = 1					0.84 (0.26 – 1.58) n = 9
December				4.20 (1.23 – 7.83) n = 9					4.20 (1.23 - 7.83) n = 9
January				7.62 (5.41 – 9.83) n = 2	6.58 (3.00 – 9.34) n = 5			2.80 n = 1	6.00 (2.80 - 9.83) n = 8
February				3.64 n = 1	7.60 (3.57 – 12.14) n = 8				5.58 (3.57 - 12.14) n = 9
March					6.42 (5.57 – 14.96) n = 3	0.08 (0.06 – 0.55) n = 3	0.06 n = 1	0.07 n = 1	0.32 (0.06 - 14.96) n = 8
April							0.08 n = 8		0.08 n = 8
May							0.08 n = 10		0.08 n = 10
Median by Stage	0.08 (0.07 – 0.66) n = 30	0.08 (0.08 – 0.63) n = 5	0.80 (0.26 – 1.22) n = 8	4.20 (1.23 – 9.83) n = 13	6.50 (3.00– 14.96) n =16	0.08 (0.06 – 0.55) n = 3	0.08 (0.06 – 0.08) n = 19	0.08 (0.07 – 2.80) n = 4	

Table 2.6. Plasma testosterone (T) concentrations (ng/ml) in adult male Burbot by stage of maturity and month. Burbot were held at the Bozeman Fish Technology Center and exposed to a natural photoperiod and thermal profile similar to that in Lake Roosevelt, Washington. Data were collected from November 2016 to March 2018. Data are medians, ranges (min. – max.), and sample sizes (n). Plasma T concentrations below the minimum quantifiable concentration (MQC) of the radioimmunoassay were assigned the MQC (0.27 – 0.37 ng/ml). Non-detectable plasma T concentrations were assigned half of the MCQ (0.11 – 0.16 ng/ml).

Month	Spermatogonial Proliferation (Stage 1)	Early Spermatogenic (Stage 2)	Mid-Spermatogenic (Stage 3)	Ripe (Stage 4)	Mid-Spermiation (Stage 5)	Post-Spermiation (Stage 6)	Median by Month
June	0.12 n = 8					0.12 n = 1	0.12 n = 9
July	0.12 n = 3					0.12 n = 1	0.12 n = 4
August	0.28 (0.27 – 0.28) n = 2						0.28 (0.27 – 0.28) n = 2
September	0.35 (0.27 – 0.37) n = 3						0.35 (0.27 – 0.37) n = 3
October	1.54 n = 1						1.54 n = 1
November	0.53 (0.16 – 0.90) n = 2	4.57 (0.72 – 6.43) n = 6					3.32 (0.16 - 6.43) n = 8
December		5.53 (3.47 – 9.24) n = 8					5.53 (3.47 – 9.24) n = 8
January		9.51 (7.14 – 11.12) n = 3	17.41 (12.83 – 32.36) n = 7				15.41 (7.14 - 32.36) n = 10
February			20.52 (14.18 – 27.46) n = 3	20.66 (12.59 – 35.58) n = 6			20.52 (12.59 - 35.58) n = 9
March				28.31 (14.45 – 36.76) n = 3	5.61 (0.11 – 8.97) n = 6	0.69 (0.11 – 1.27) n = 2	7.16 (0.11 - 36.76) n = 11
April					0.28 (0.12 – 0.44) n = 2	0.12 n = 2	0.12 (0.12 - 0.44) n = 4
May	0.12 n = 1				0.12 n = 1		0.12 n = 2
Median by Stage	0.12 (0.12 – 1.54) n = 20	5.52 (0.72 – 11.12) n = 17	17.83(12.83 – 32.36) n = 10	23.75 (12.59 – 36.76) n = 9	3.60 (0.11 – 8.97) n = 9	0.12 (0.11 – 1.27) n = 6	

Table 2.7. Plasma 11-ketotestosterone (11-KT) concentrations (ng/ml) in adult male Burbot by stage of maturity and month. Burbot were held at the Bozeman Fish Technology Center and exposed to a natural photoperiod and thermal profile similar to that in Lake Roosevelt, Washington. Data were collected from November 2016 to March 2018. Data are medians, ranges (min. – max.), and sample sizes (n). Plasma 11-KT concentrations below the minimum quantifiable concentration (MQC) were assigned the MQC (0.02 ng/ml). Non-detectable plasma 11-KT concentrations were assigned half of the MQC (0.01 ng/ml).

Month	Spermatogonial Proliferation (Stage 1)	Early Spermatogenic (Stage 2)	Mid-Spermatogenic (Stage 3)	Ripe (Stage 4)	Mid-Spermiation (Stage 5)	Post-Spermiation (Stage 6)	Median by Stage
June	0.02 (0.01 – 0.09) n = 8					0.01 n = 1	0.02 (0.01 - 0.09) n = 9
July	0.02 (0.02 – 0.03) n = 3					0.02 n = 1	0.02 (0.02 - 0.03) n = 4
August	0.04 (0.02 – 0.06) n = 2						0.04 (0.02 - 0.06) n = 2
September	0.13 (0.12 – 0.16) n = 3						0.13 (0.12 – 0.16) n = 3
October	0.60 n = 1						0.60 n = 1
November	0.02 n = 2	1.17 (0.71 – 3.63) n = 4					0.83 (0.02 - 3.63) n = 6
December		2.30 (0.01 – 8.84) n = 8					2.30 (0.01 – 8.84) n = 8
January		6.09 (5.05 – 9.74) n = 3	11.14 (3.73 – 16.31) n = 5				8.63 (3.73 - 16.31) n = 8
February			7.21 (4.67 – 15.02) n = 3	12.26 (0.01 – 49.39) n = 6			9.88 (0.01 - 49.39) n = 9
March				55.12 (24.52 – 79.63) n = 3	6.16 (0.60 – 9.24) n = 6	0.73 (0.19 – 1.27) n = 2	6.98 (0.19 - 79.63) n = 11
April					0.12 (0.01 – 0.23) n = 2	0.04 (0.01 – 0.07) n = 2	0.04 (0.01 - 0.23) n = 4
May	0.02 n = 1				0.09 n = 1		0.06 (0.02 - 0.09) n = 2
Median by Stage	0.02 (0.01 – 0.60) n = 20	2.85 (0.01 – 9.74) n = 15	9.33 (3.73 – 16.31) n = 8	18.10 (0.01 – 79.63) n = 9	2.87 (0.01 – 9.24) n = 9	0.05 (0.01 – 1.27) n = 6	

Table 2.8. Accuracy of assigning sex in adult Burbot using an AdaBoost algorithm with plasma 11-ketotestosterone (11-KT) concentration as a predictor during the entire reproductive cycle (i.e., entire year) and with the combination of plasma 11-KT concentration and plasma estradiol-17 β (E2) concentration as a predictor during the reproductive phase (i.e., November to March in Lake Roosevelt). Values in bold are the percentage of fish correctly assigned, whereas values not in bold are the percentage of fish incorrectly assigned. Sample sizes (n) are in parentheses.

Actual	Predicted		Total
	F	M	
Entire Year			
F	97 (88)	3 (3)	100 (91)
M	40 (27)	60 (40)	100 (67)
Nov. – Mar.			
F	98 (41)	2 (1)	100 (42)
M	2 (1)	98 (41)	100 (42)

Table 2.9. Accuracy of assigning stage of maturity in adult female Burbot using an AdaBoost algorithm with the combination of plasma testosterone (T) concentration, plasma estradiol-17 β (E2) concentration, and month as a predictor. Values in bold are the percentage of fish correctly assigned, whereas values not in bold are the percentage of fish incorrectly assigned. Samples sizes (n) are in parentheses.

Actual	Predicted								Total
	Primary Growth (Stage 2)	Cortical Alveolar (Stage 3)	Early Vitellogenic (Stage 4)	Mid-Vitellogenic (Stage 5)	Late Vitellogenic (Stage 6)	Ripe (Stage 7)	Post-Ovulatory (Stage 8)	Atretic (Stage 9)	
Primary Growth (Stage 2)	90 (27)	3 (1)						7 (2)	100 (30)
Cortical Alveolar (Stage 3)	20 (1)	80 (4)							100 (5)
Early Vitellogenic (Stage 4)			100 (8)						100 (8)
Mid-Vitellogenic (Stage 5)				85 (11)	15 (2)				100 (13)
Late Vitellogenic (Stage 6)				6 (1)	94 (15)				100 (16)
Ripe (Stage 7)						67 (2)		33 (1)	100 (3)
Post-Ovulatory (Stage 8)							95 (18)	5 (1)	100 (19)
Atretic (Stage 9)	50 (2)				25 (1)		25 (1)	0 (0)	100 (4)

Table 2.10. Accuracy of assigning reproductive condition in adult female Burbot using an AdaBoost algorithm with the combination of plasma testosterone (T) concentration and plasma estradiol-17 β (E2) concentration as a predictor. Values in bold are the percentage of fish correctly assigned, whereas values not in bold are the percentage of fish incorrectly assigned. Sample sizes (n) are in parentheses.

Actual	Predicted		Total
	Non-Reproductive	Reproductive	
Non-Reproductive	97 (56)	3 (2)	100 (58)
Reproductive	0 (0)	100 (40)	100 (40)

Table 2.11. Accuracy of assigning stage of maturity in adult male Burbot using an AdaBoost algorithm with the combination of plasma 11-ketotestosterone concentration (11-KT), girth at the urogenital pore, and month as a predictor. Values in bold are the percentage of fish correctly assigned, whereas values not in bold are the percentage of fish incorrectly assigned. Samples sizes (n) are in parentheses.

Actual	Predicted						Total
	Spermatogonial Proliferation (Stage 1)	Early Spermatogenic (Stage 2)	Mid-Spermatogenic (Stage 3)	Ripe (Stage 4)	Mid-Spermiation (Stage 5)	Post-Spermiation (Stage 6)	
Spermatogonial Proliferation (Stage 1)	85 (17)					15 (3)	100 (20)
Early Spermatogenic (Stage 2)		93 (14)		7 (1)			100 (15)
Mid-Spermatogenic (Stage 3)			50 (4)	50 (4)			100 (8)
Ripe (Stage 4)			44 (4)	56 (5)			100 (9)
Mid-Spermiation (Stage 5)	11 (1)				78 (7)	11 (1)	100 (9)
Post-Spermiation (Stage 6)	33.3 (2)				33.3 (2)	33.3 (2)	100 (6)

Table 2.12. Accuracy of assigning reproductive condition in adult male Burbot using an AdaBoost algorithm with plasma testosterone (T) concentration as a predictor. Values in bold are the percentage of fish correctly assigned, whereas values not in bold are the percentage of fish incorrectly assigned. Samples sizes (n) are in parentheses.

Actual	Predicted		Total
	Non-Reproductive	Reproductive	
Non-Reproductive	92 (24)	8 (2)	100 (26)
Reproductive	11 (5)	89 (40)	100 (45)

Table 2.13. Accuracy of assigning sex using ultrasound in adult Burbot by month. The percentage of fish correctly assigned is reported with sample sizes (n) in parentheses.

*November 2016 was a training month for assigning sex by ultrasound in Burbot; therefore, the November 2016 accuracy was excluded from the mean accuracy calculation.

Month	Year	Ultrasound Accuracy (%)	
		Females	Males
*November	2016	67 (6)	80 (5)
December	2016	100 (6)	100 (5)
January	2017	100 (5)	83 (6)
February	2017	100 (6)	100 (6)
March	2017	100 (5)	88 (8)
April	2017	100 (8)	75 (4)
May	2017	100 (10)	100 (2)
June	2017	86 (7)	78 (9)
July	2017	100 (8)	100 (4)
August	2017	100 (10)	100 (2)
September	2017	100 (6)	100 (3)
October	2017	100 (6)	100 (1)
November	2017	100 (3)	100 (3)
December	2017	100 (3)	100 (3)
January	2018	100 (3)	100 (4)
February	2018	100 (3)	100 (3)
March	2018	100 (3)	100 (3)
Mean		99	95

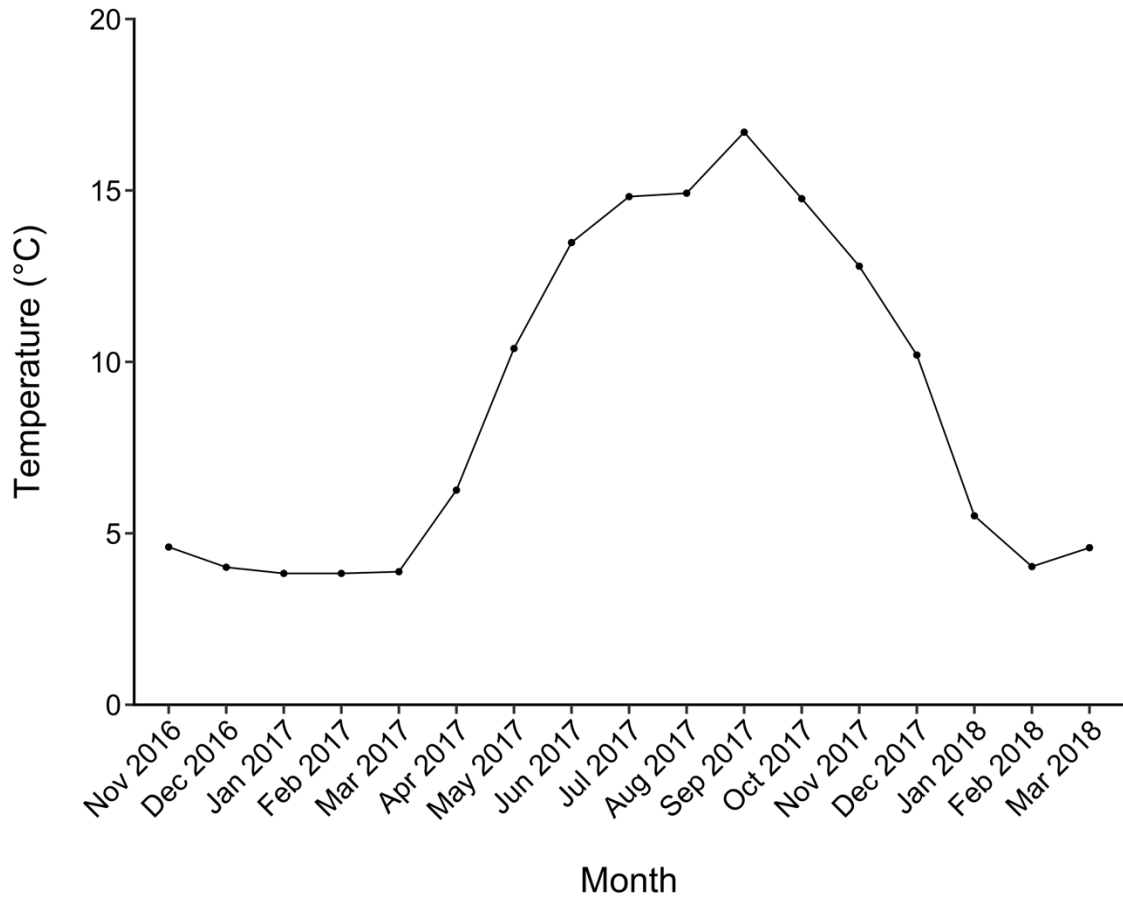


Figure 2.1. Thermal profile on which Burbot were maintained at the Bozeman Fish Technology Center to replicate the thermal profile of Lake Roosevelt, Washington. Data are the mean monthly water temperatures (°C).

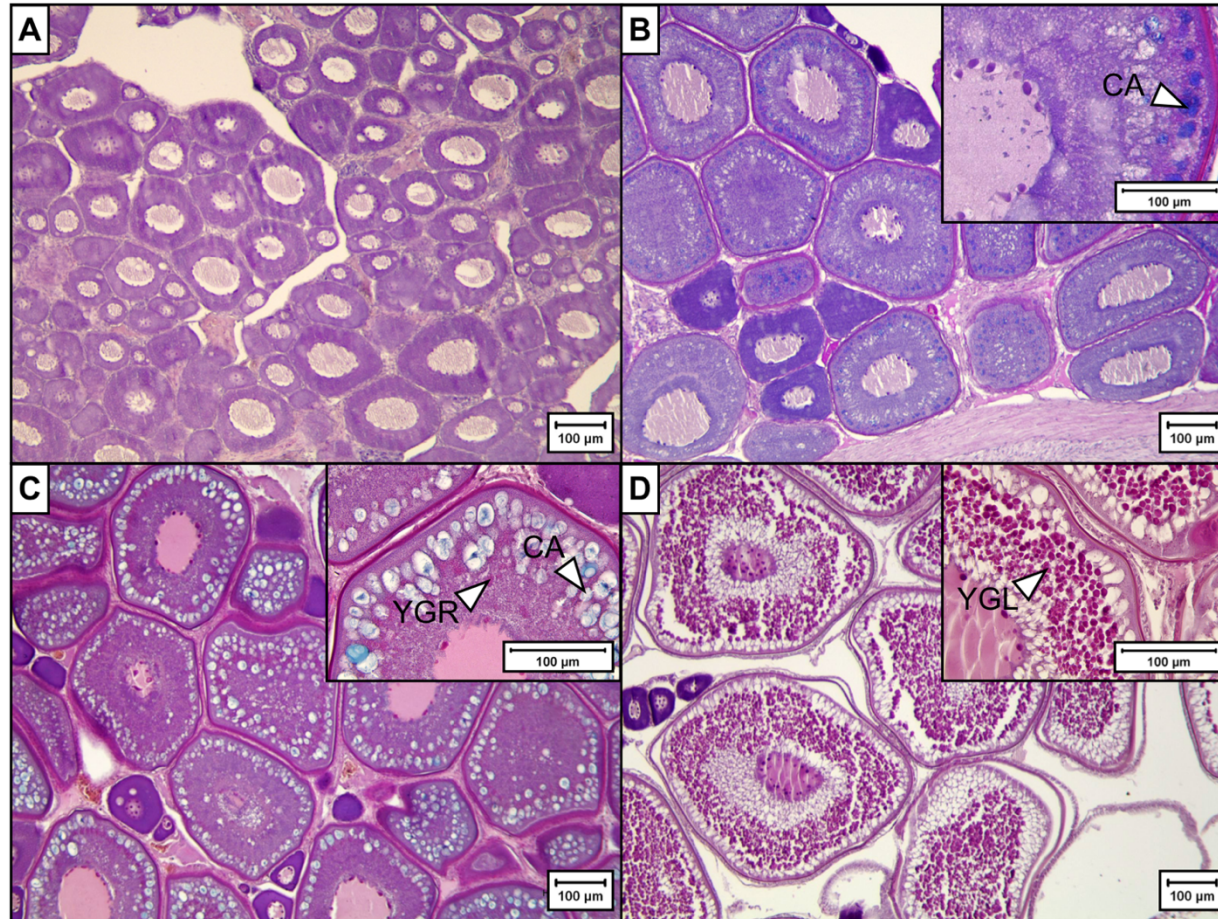


Figure 2.2. Histological description of gametogenesis in female Burbot. **A.** Primary growth (Stage 2) oocytes with oogonia. H&E stain. **B.** Cortical alveolar (Stage 3) ovarian follicles with cortical alveoli (CA) on periphery. PAS stain. **C.** Early vitellogenic (Stage 4) ovarian follicles accumulating yolk granules (YGR) and cortical alveoli (CA) on periphery. H&E stain. **D.** Mid-vitellogenic (Stage 5) ovarian follicles with yolk globules (YGL) (mean diameter = 7.69 μm (5.97 – 9.89 μm ; 95% CI)) accumulating towards center and few cortical alveoli on periphery. H&E stain.

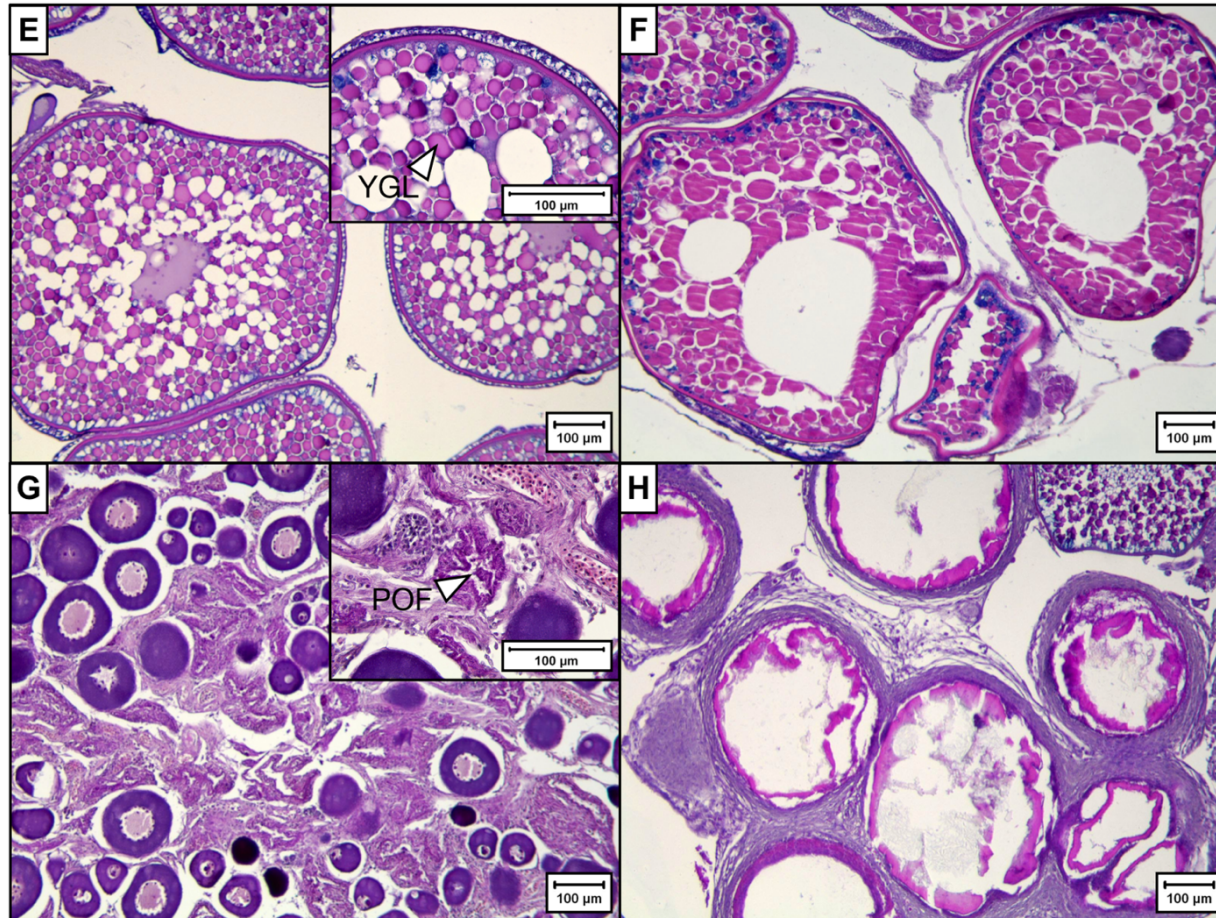


Figure 2.2 Continued. **E.** Late vitellogenic (Stage 6) ovarian follicles with yolk globules (YGL) (mean diameter = $19.62\ \mu\text{m}$ ($14.55 - 24.69\ \mu\text{m}$; 95% CI)) throughout. PAS stain. **F.** Ripe (Stage 7) ovarian follicles with fused yolk. PAS stain. **G.** Post-ovulatory (Stage 8) follicles (POF) with primary growth oocytes. PAS stain. **H.** Atretic (Stage 8) follicles. PAS stain.

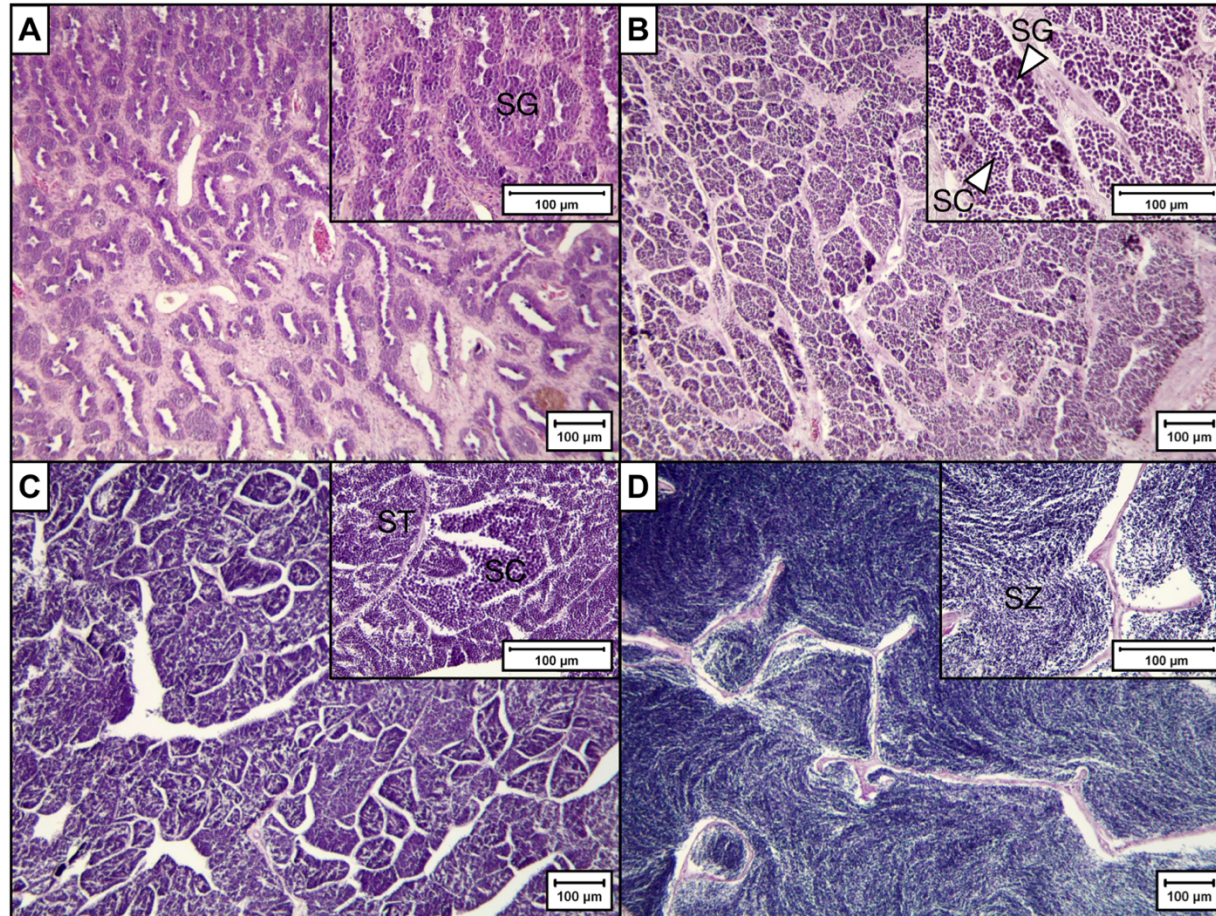


Figure 2.3. Histological description of gametogenesis in male Burbot. **A.** Spermatogonial proliferation (Stage 1) cysts with only spermatogonia (SG). PAS stain. **B.** Early spermatogenic (Stage 2) cysts with spermatocytes (SC) and spermatogonia (SG). H&E stain. **C.** Mid-spermatogenic (Stage 3) cysts with spermatids (ST) and spermatocytes (SC). Spermatogonia may be present. H&E stain. **D.** Ripe (Stage 4) cysts filled with spermatozoa (SZ). H&E stain.

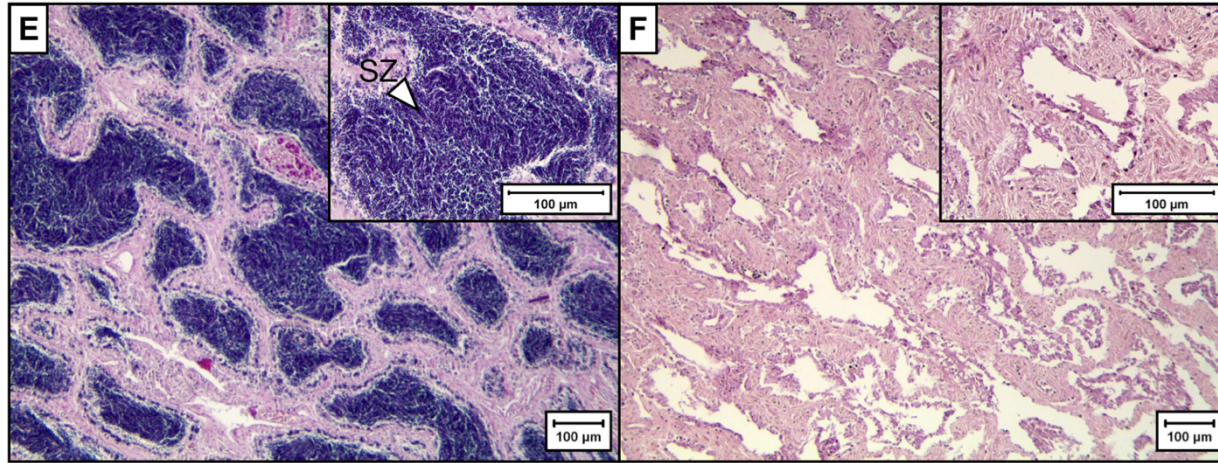


Figure 2.3 Continued. **E.** Mid-spermiation (Stage 5) cysts with reduced spermatozoa (SZ). PAS stain. **F.** Post-spermiation (Stage 6) cysts empty of spermatozoa. PAS stain.

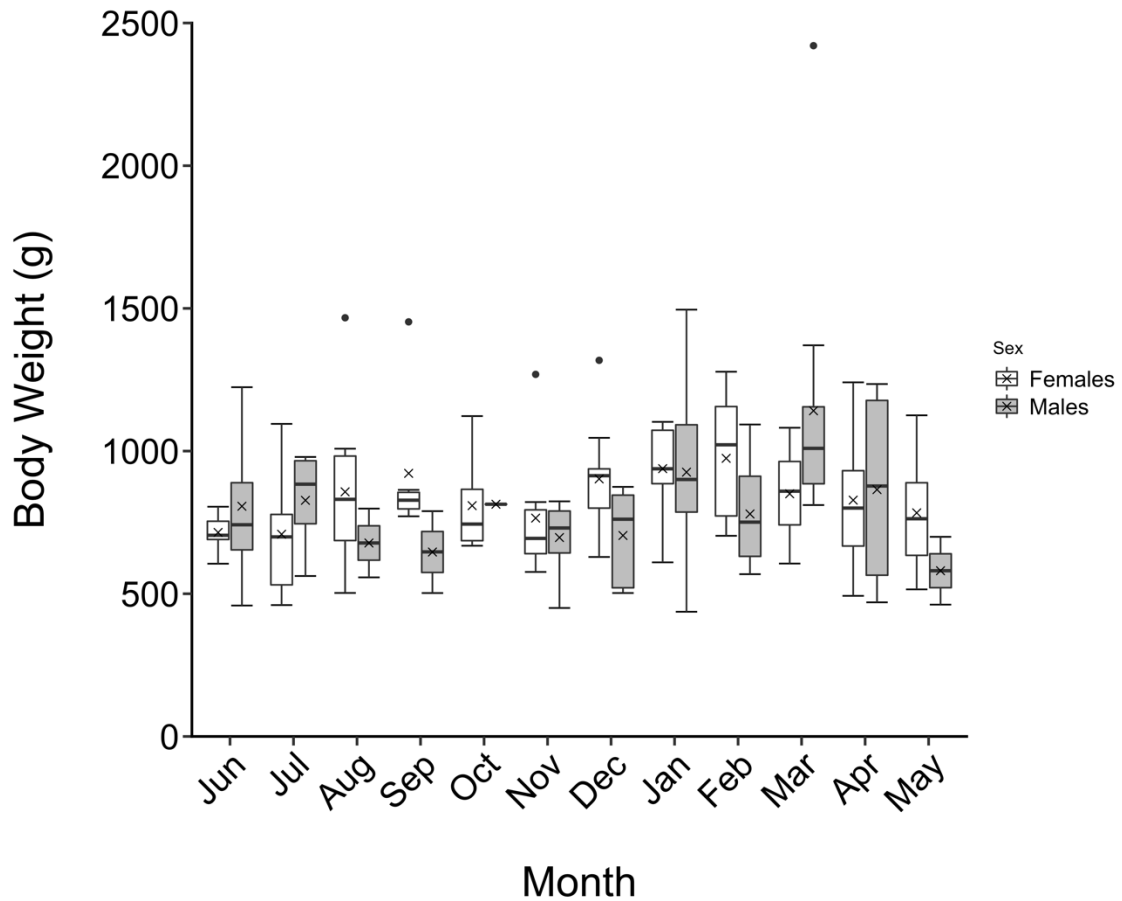


Figure 2.4. Body weight (g) of adult female and male Burbot by month. Burbot were held at the Bozeman Fish Technology Center and exposed to a natural photoperiod and thermal profile similar to that in Lake Roosevelt, Washington. Data are the observed values of body weight. The box encompasses data from the first to third quartiles (i.e., 25th to 75th percentiles). The horizontal line within a box represents the median. The black cross represents the mean. The whiskers represent the minimum and maximum values. Outliers are represented by individual points. If there are outliers, the whiskers represent the minimum value within 1.5 times the interquartile range below the first quartile or the maximum value within 1.5 times the interquartile range above the third quartile. A single male was observed in October.

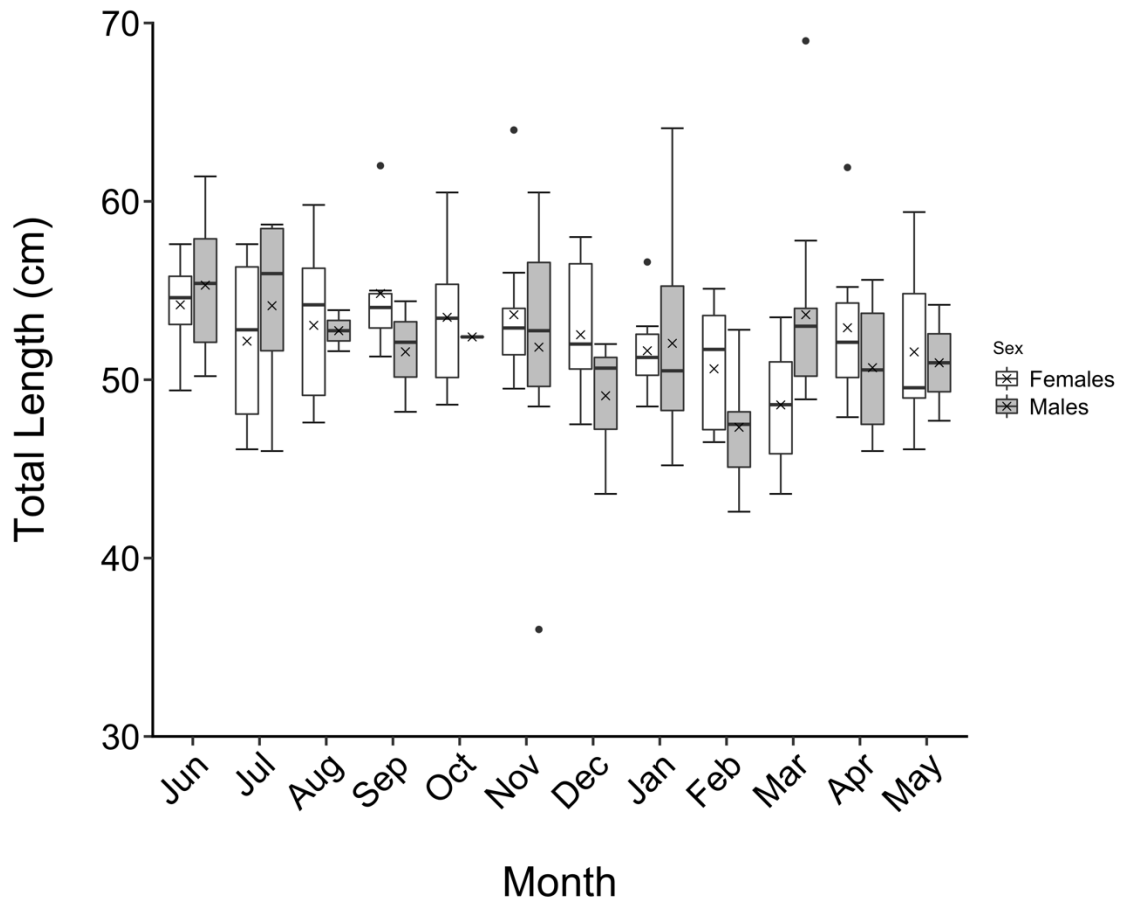


Figure 2.5. Total length (cm) of adult female and male Burbot by month. Burbot were held at the Bozeman Fish Technology Center and exposed to a natural photoperiod and thermal profile similar to that in Lake Roosevelt, Washington. Data are the observed values of body weight. The box encompasses data from the first to third quartiles (i.e., 25th to 75th percentiles). The horizontal line within a box represents the median. The black cross represents the mean. The whiskers represent the minimum and maximum values. Outliers are represented by individual points. If there are outliers, the whiskers represent the minimum value within 1.5 times the interquartile range below the first quartile or the maximum value within 1.5 times the interquartile range above the third quartile. A single male was observed in October.

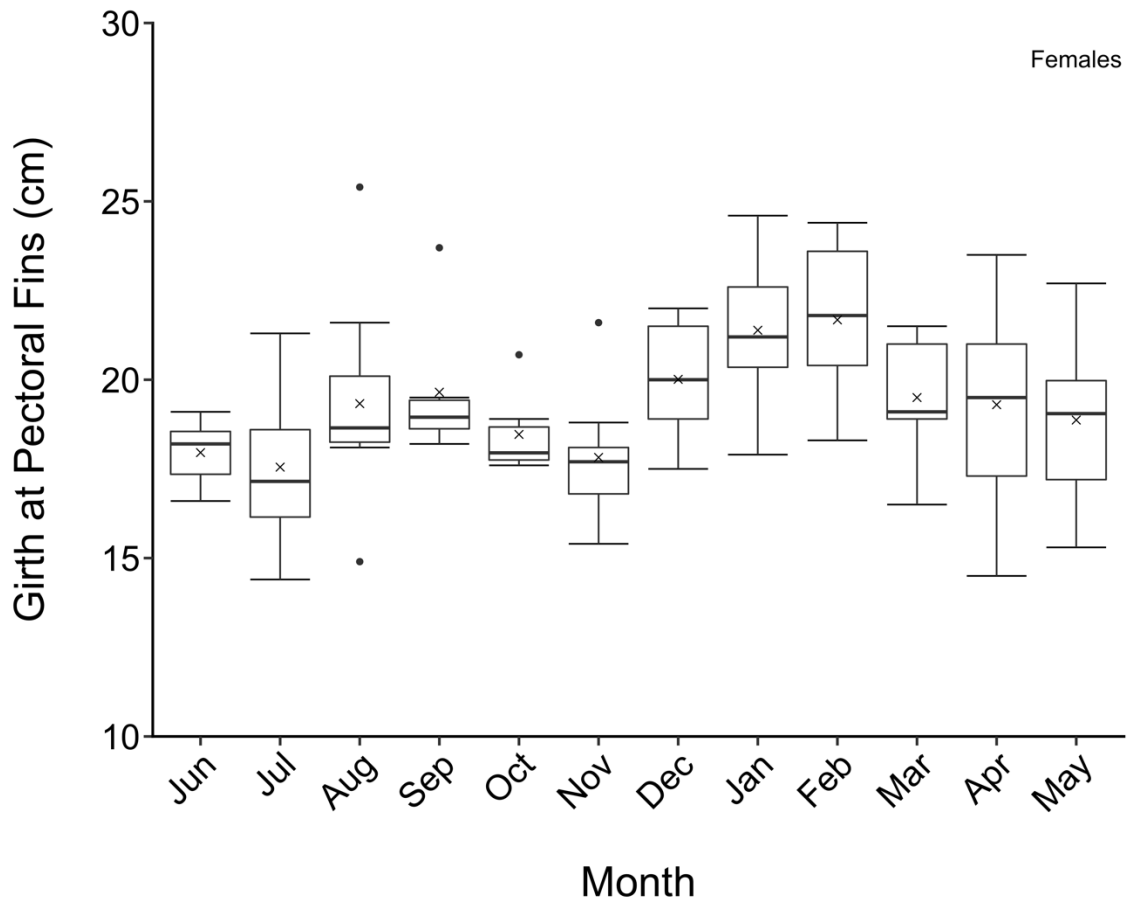


Figure 2.6. Girth at pectoral fins of adult female Burbot by month. Burbot were held at the Bozeman Fish Technology Center and exposed to a natural photoperiod and thermal profile similar to that in Lake Roosevelt, Washington. Data are the observed values of body weight. The box encompasses data from the first to third quartiles (i.e., 25th to 75th percentiles). The horizontal line within a box represents the median. The black cross represents the mean. The whiskers represent the minimum and maximum values. Outliers are represented by individual points. If there are outliers, the whiskers represent the minimum value within 1.5 times the interquartile range below the first quartile or the maximum value within 1.5 times the interquartile range above the third quartile.

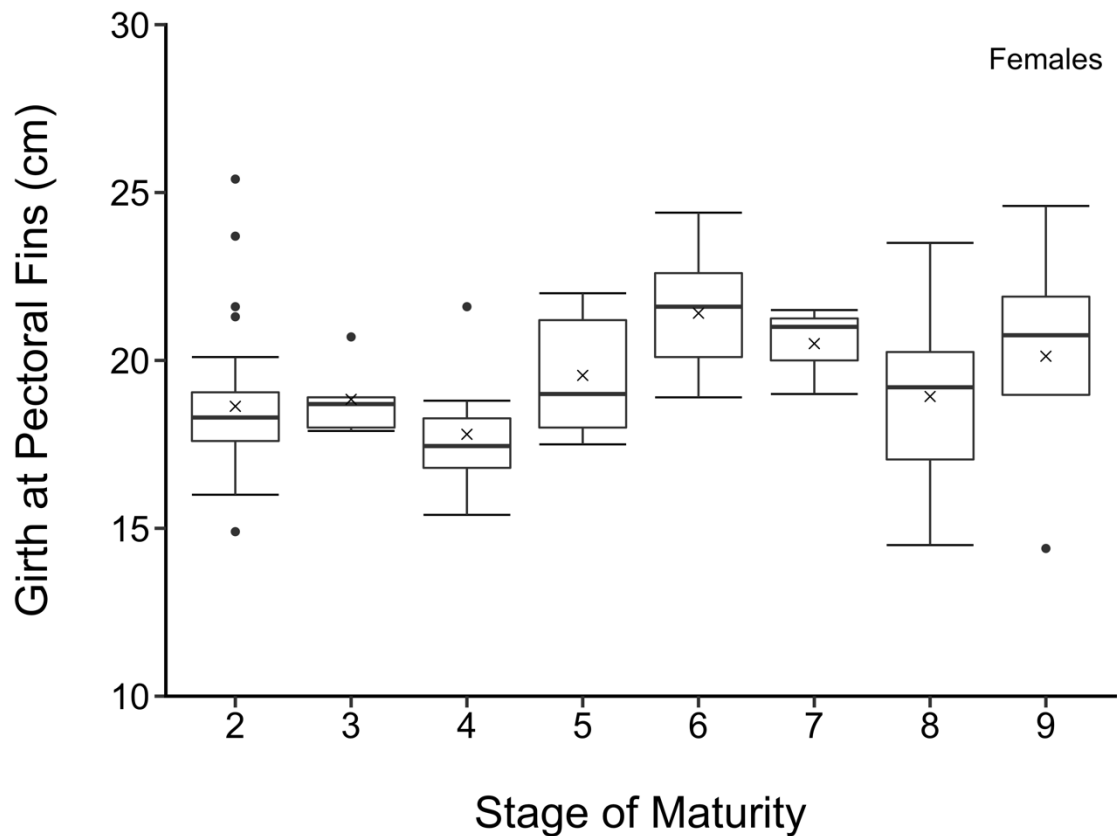


Figure 2.7. Girth at pectoral fins of adult female Burbot by stage of maturity. Burbot were held at the Bozeman Fish Technology Center and exposed to a natural photoperiod and thermal profile similar to that in Lake Roosevelt, Washington. Data are the observed values of body weight. The box encompasses data from the first to third quartiles (i.e., 25th to 75th percentiles). The horizontal line within a box represents the median. The black cross represents the mean. The whiskers represent the minimum and maximum values. Outliers are represented by individual points. If there are outliers, the whiskers represent the minimum value within 1.5 times the interquartile range below the first quartile or the maximum value within 1.5 times the interquartile range above the third quartile.

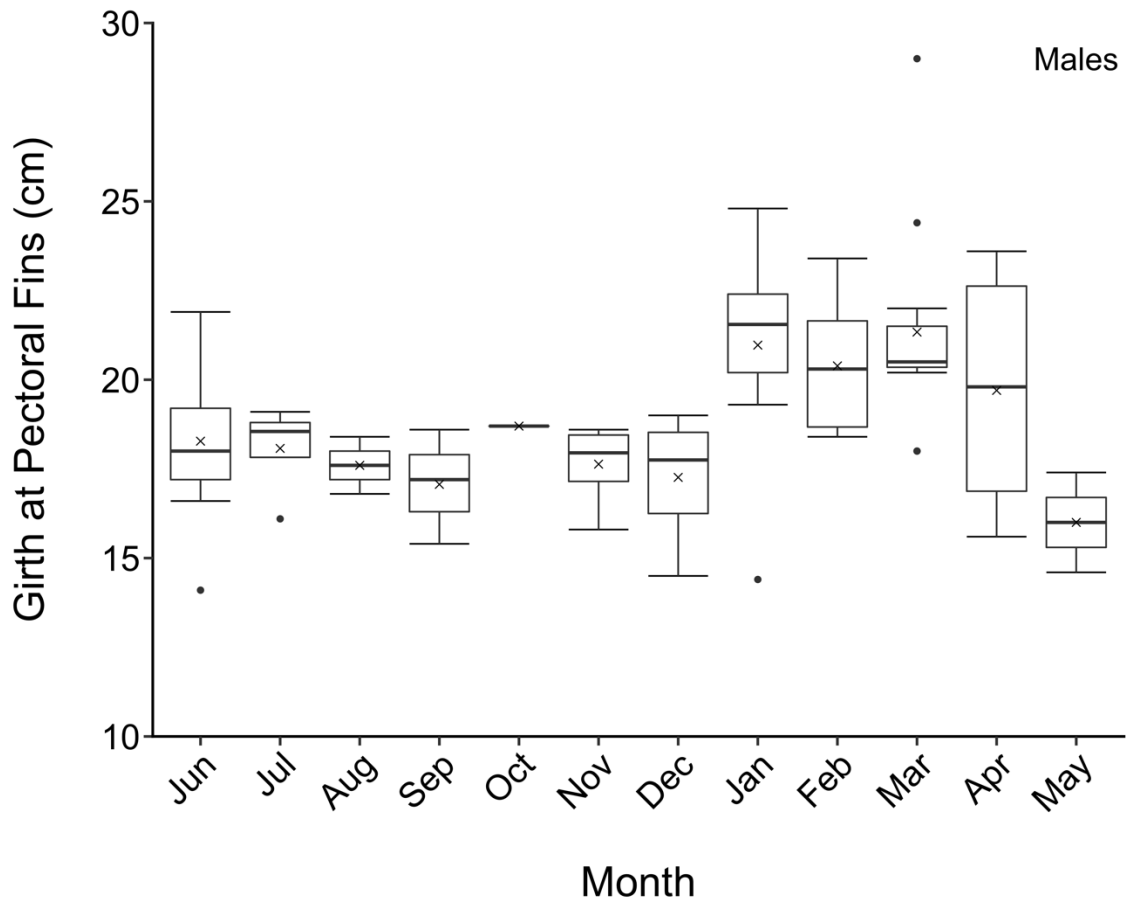


Figure 2.8. Girth at pectoral fins of adult male Burbot by month. Burbot were held at the Bozeman Fish Technology Center and exposed to a natural photoperiod and thermal profile similar to that in Lake Roosevelt, Washington. Data are the observed values of body weight. The box encompasses data from the first to third quartiles (i.e., 25th to 75th percentiles). The horizontal line within a box represents the median. The black cross represents the mean. The whiskers represent the minimum and maximum values. Outliers are represented by individual points. If there are outliers, the whiskers represent the minimum value within 1.5 times the interquartile range below the first quartile or the maximum value within 1.5 times the interquartile range above the third quartile. A single male was observed in October.

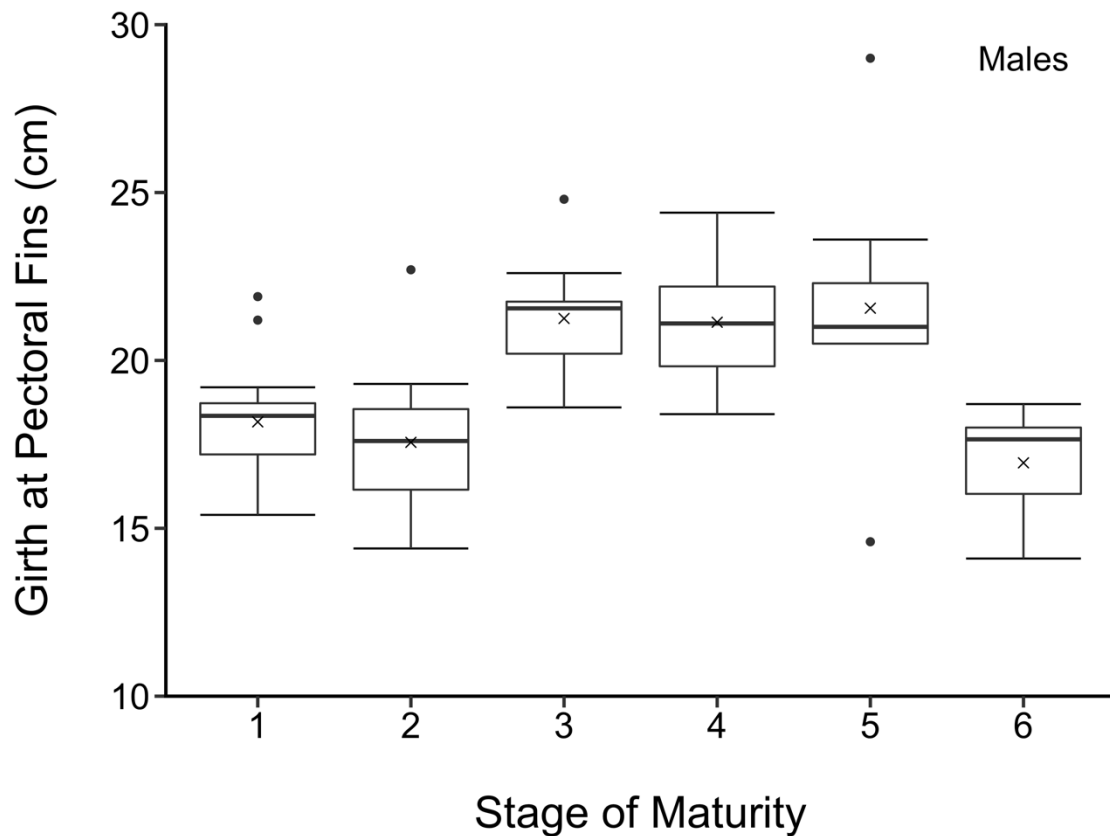


Figure 2.9. Girth at pectoral fins of adult male Burbot by stage of maturity. Burbot were held at the Bozeman Fish Technology Center and exposed to a natural photoperiod and thermal profile similar to that in Lake Roosevelt, Washington. Data are the observed values of body weight. The box encompasses data from the first to third quartiles (i.e., 25th to 75th percentiles). The horizontal line within a box represents the median. The black cross represents the mean. The whiskers represent the minimum and maximum values. Outliers are represented by individual points. If there are outliers, the whiskers represent the minimum value within 1.5 times the interquartile range below the first quartile or the maximum value within 1.5 times the interquartile range above the third quartile.

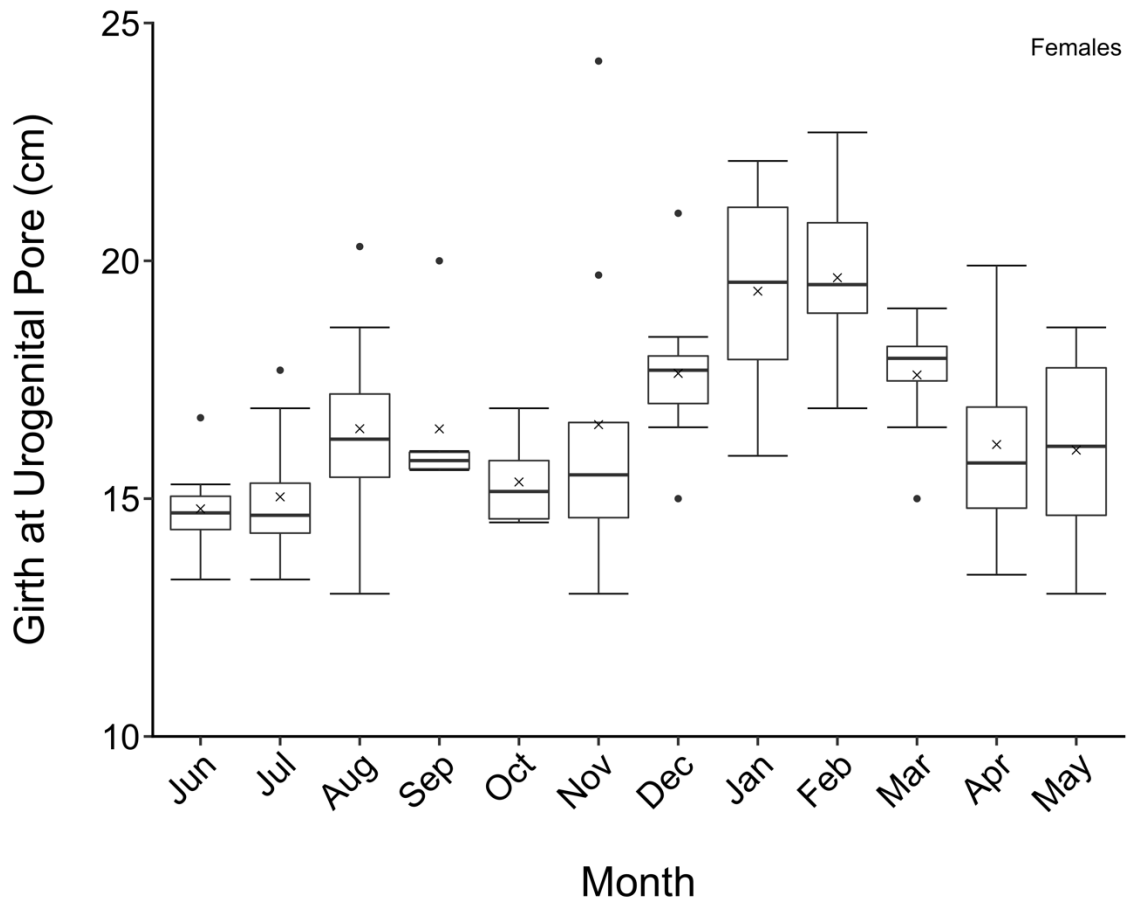


Figure 2.10. Girth at urogenital pore of adult female Burbot by month. Burbot were held at the Bozeman Fish Technology Center and exposed to a natural photoperiod and thermal profile similar to that in Lake Roosevelt, Washington. Data are the observed values of body weight. The box encompasses data from the first to third quartiles (i.e., 25th to 75th percentiles). The horizontal line within a box represents the median. The black cross represents the mean. The whiskers represent the minimum and maximum values. Outliers are represented by individual points. If there are outliers, the whiskers represent the minimum value within 1.5 times the interquartile range below the first quartile or the maximum value within 1.5 times the interquartile range above the third quartile.

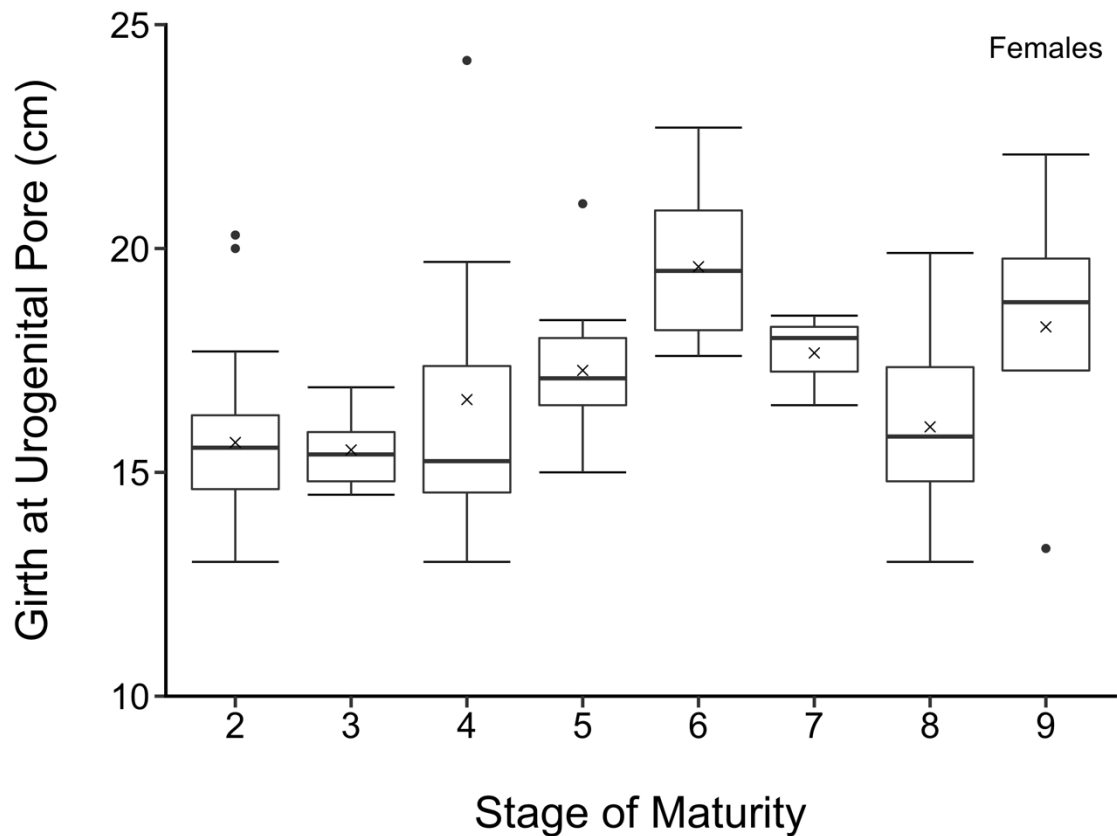


Figure 2.11. Girth at urogenital pore of adult female Burbot by stage of maturity. Burbot were held at the Bozeman Fish Technology Center and exposed to a natural photoperiod and thermal profile similar to that in Lake Roosevelt, Washington. Data are the observed values of body weight. The box encompasses data from the first to third quartiles (i.e., 25th to 75th percentiles). The horizontal line within a box represents the median. The black cross represents the mean. The whiskers represent the minimum and maximum values. Outliers are represented by individual points. If there are outliers, the whiskers represent the minimum value within 1.5 times the interquartile range below the first quartile or the maximum value within 1.5 times the interquartile range above the third quartile.

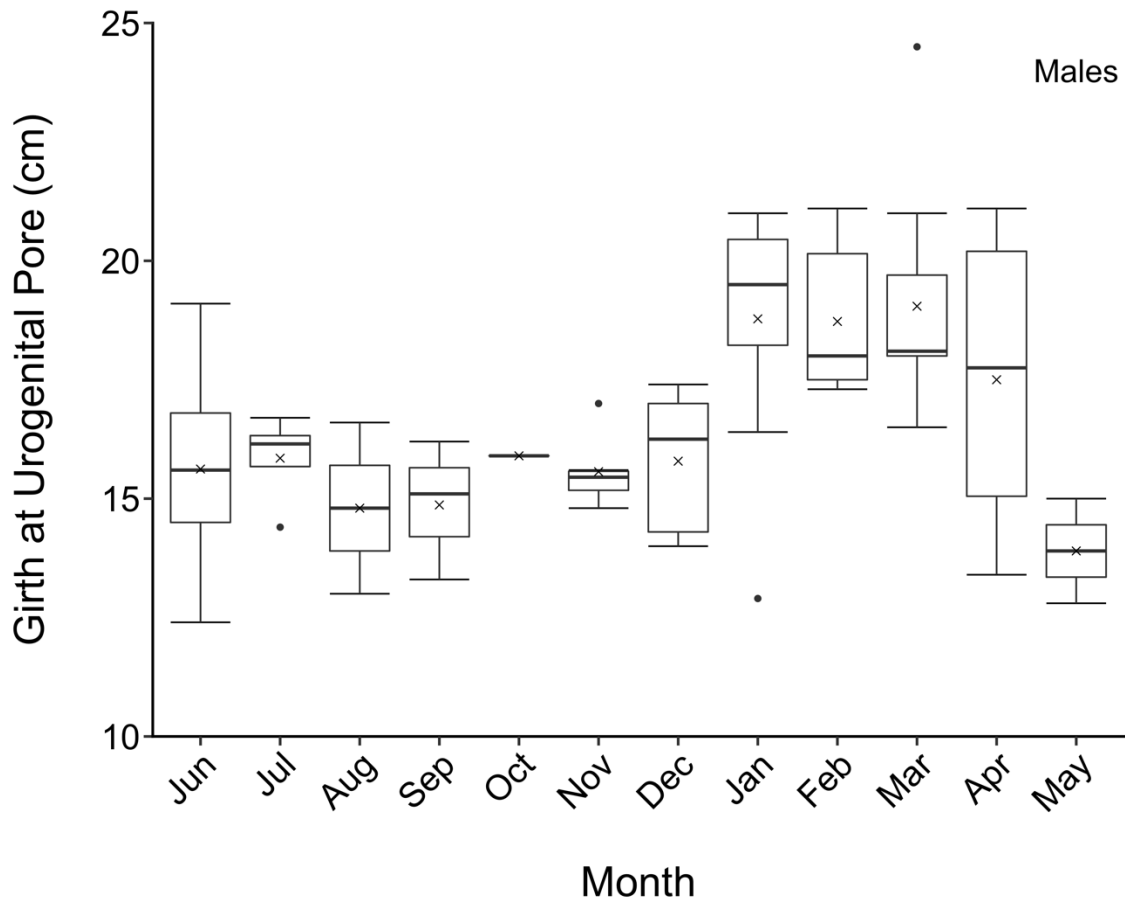


Figure 2.12. Girth at urogenital pore of adult male Burbot by month. Burbot were held at the Bozeman Fish Technology Center and exposed to a natural photoperiod and thermal profile similar to that in Lake Roosevelt, Washington. Data are the observed values of body weight. The box encompasses data from the first to third quartiles (i.e., 25th to 75th percentiles). The horizontal line within a box represents the median. The black cross represents the mean. The whiskers represent the minimum and maximum values. Outliers are represented by individual points. If there are outliers, the whiskers represent the minimum value within 1.5 times the interquartile range below the first quartile or the maximum value within 1.5 times the interquartile range above the third quartile. A single male was observed in October.

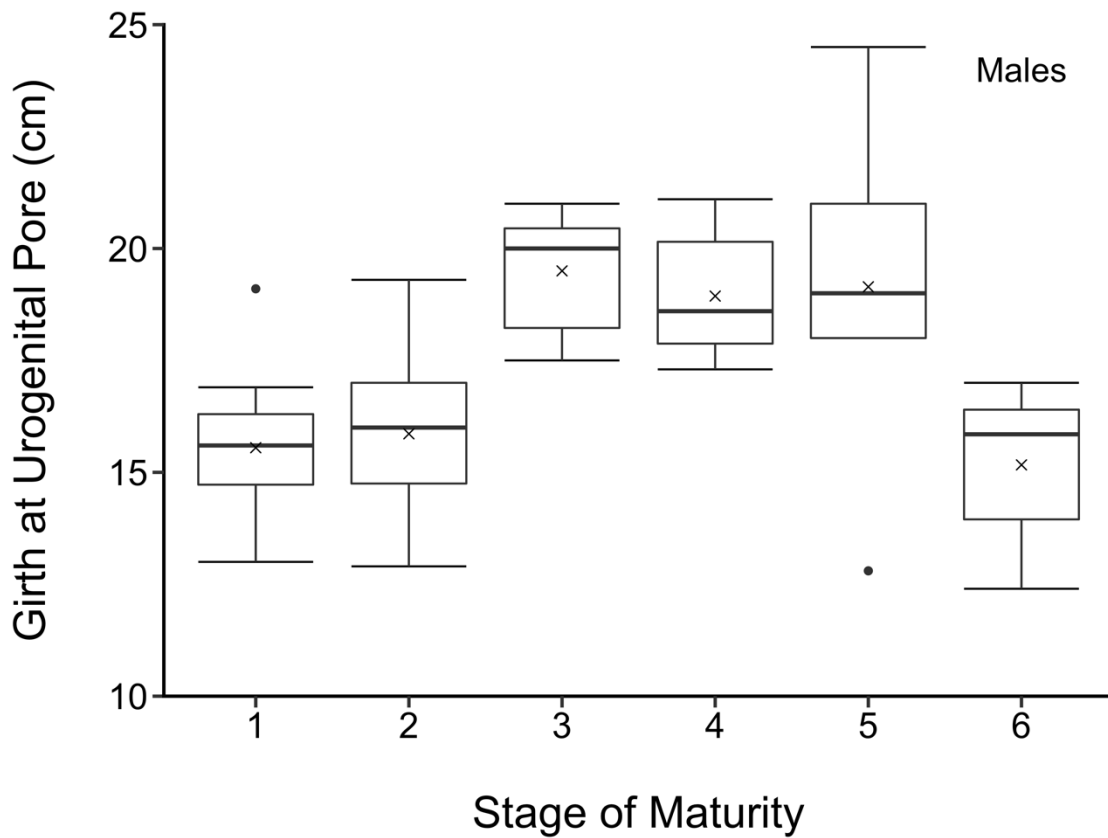


Figure 2.13. Girth at urogenital pore of adult male Burbot by stage of maturity. Burbot were held at the Bozeman Fish Technology Center and exposed to a natural photoperiod and thermal profile similar to that in Lake Roosevelt, Washington. Data are the observed values of body weight. The box encompasses data from the first to third quartiles (i.e., 25th to 75th percentiles). The horizontal line within a box represents the median. The black cross represents the mean. The whiskers represent the minimum and maximum values. Outliers are represented by individual points. If there are outliers, the whiskers represent the minimum value within 1.5 times the interquartile range below the first quartile or the maximum value within 1.5 times the interquartile range above the third quartile.

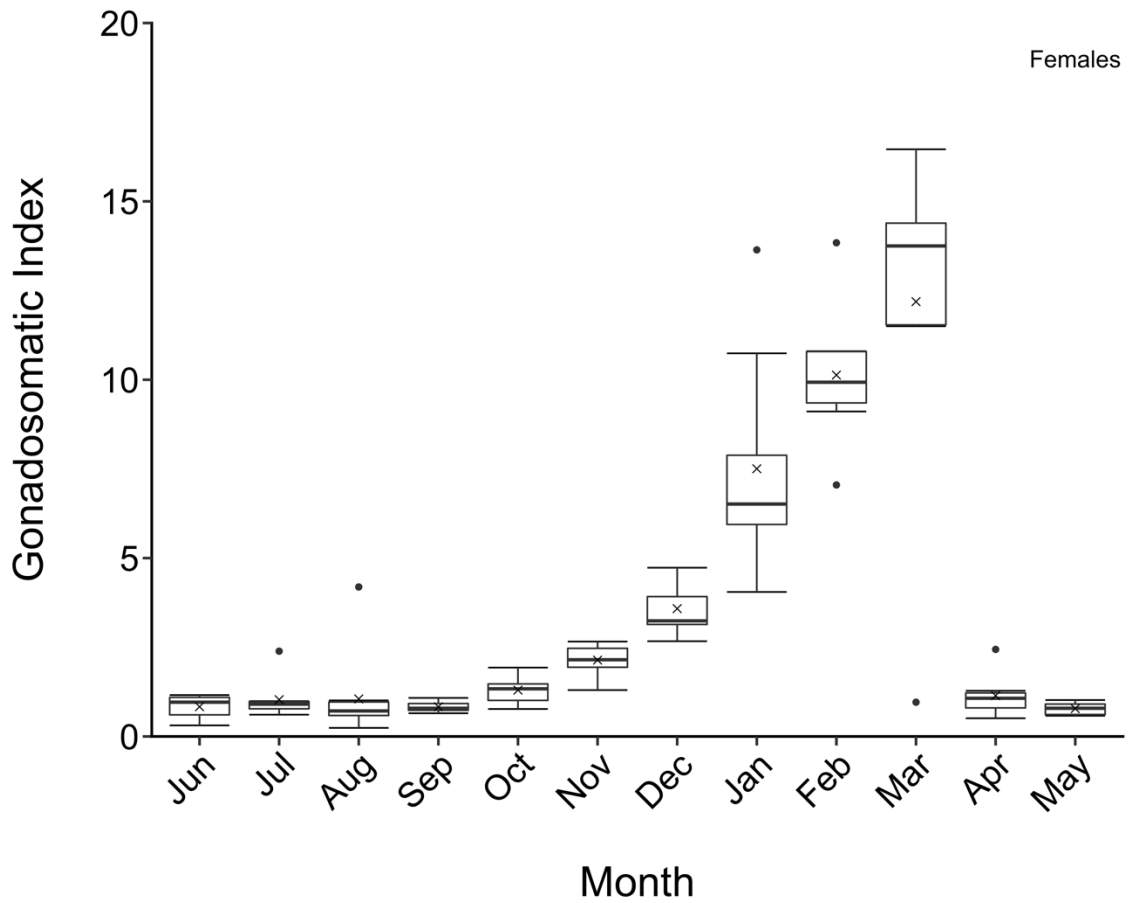


Figure 2.14. Gonadosomatic index (GSI) of adult female Burbot by month. Burbot were held at the Bozeman Fish Technology Center and exposed to a natural photoperiod and thermal profile similar to that in Lake Roosevelt, Washington. Data are the observed values of GSI. The box encompasses data from the first to third quartiles (i.e., 25th to 75th percentiles). The horizontal line within a box represents the median. The black cross represents the mean. The whiskers represent the minimum and maximum values. Outliers are represented by individual points. If there are outliers, the whiskers represent the minimum value within 1.5 times the interquartile range below the first quartile or the maximum value within 1.5 times the interquartile range above the third quartile.

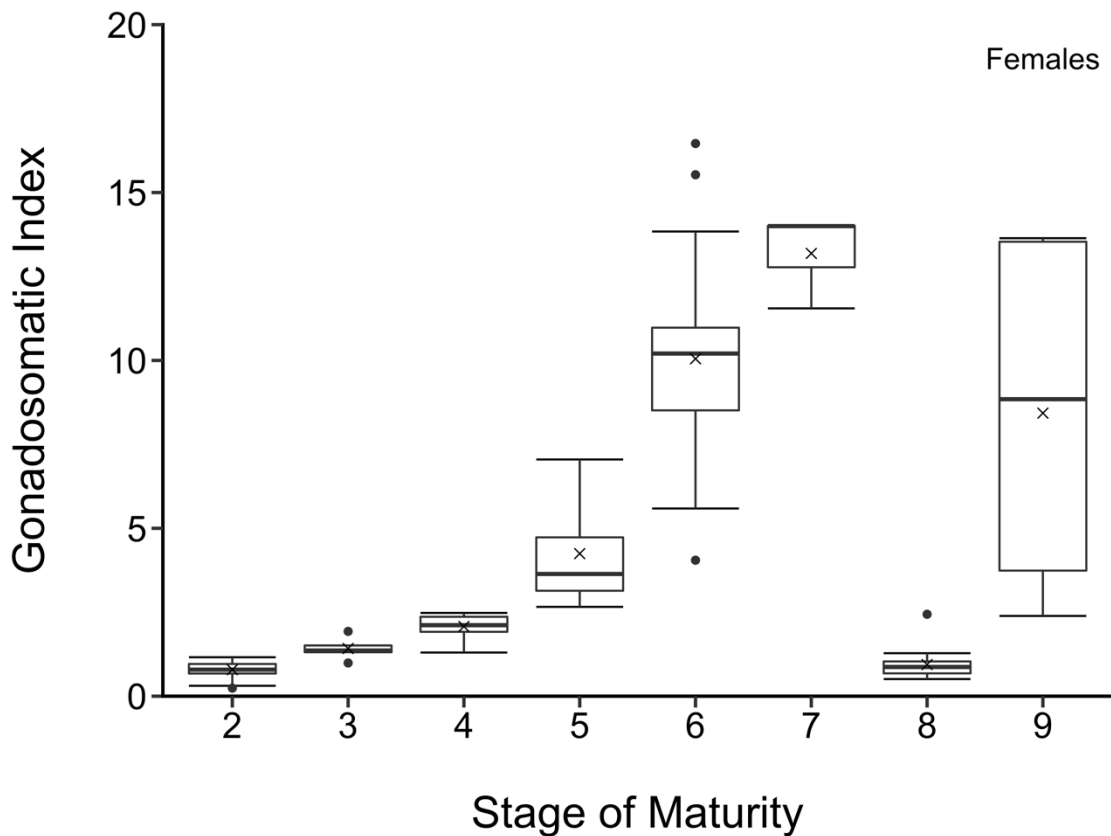


Figure 2.15. Gonadosomatic index (GSI) of adult female Burbot by stage of maturity. Burbot were held at the Bozeman Fish Technology Center and exposed to a natural photoperiod and thermal profile similar to that in Lake Roosevelt, Washington. Data are the observed values of GSI. No females were observed during oögonial proliferation (Stage 1). The box encompasses data from the first to third quartiles (i.e., 25th to 75th percentiles). The horizontal line within a box represents the median. The black cross represents the mean. The whiskers represent the minimum and maximum values. Outliers are represented by individual points. If there are outliers, the whiskers represent the minimum value within 1.5 times the interquartile range below the first quartile or the maximum value within 1.5 times the interquartile range above the third quartile.

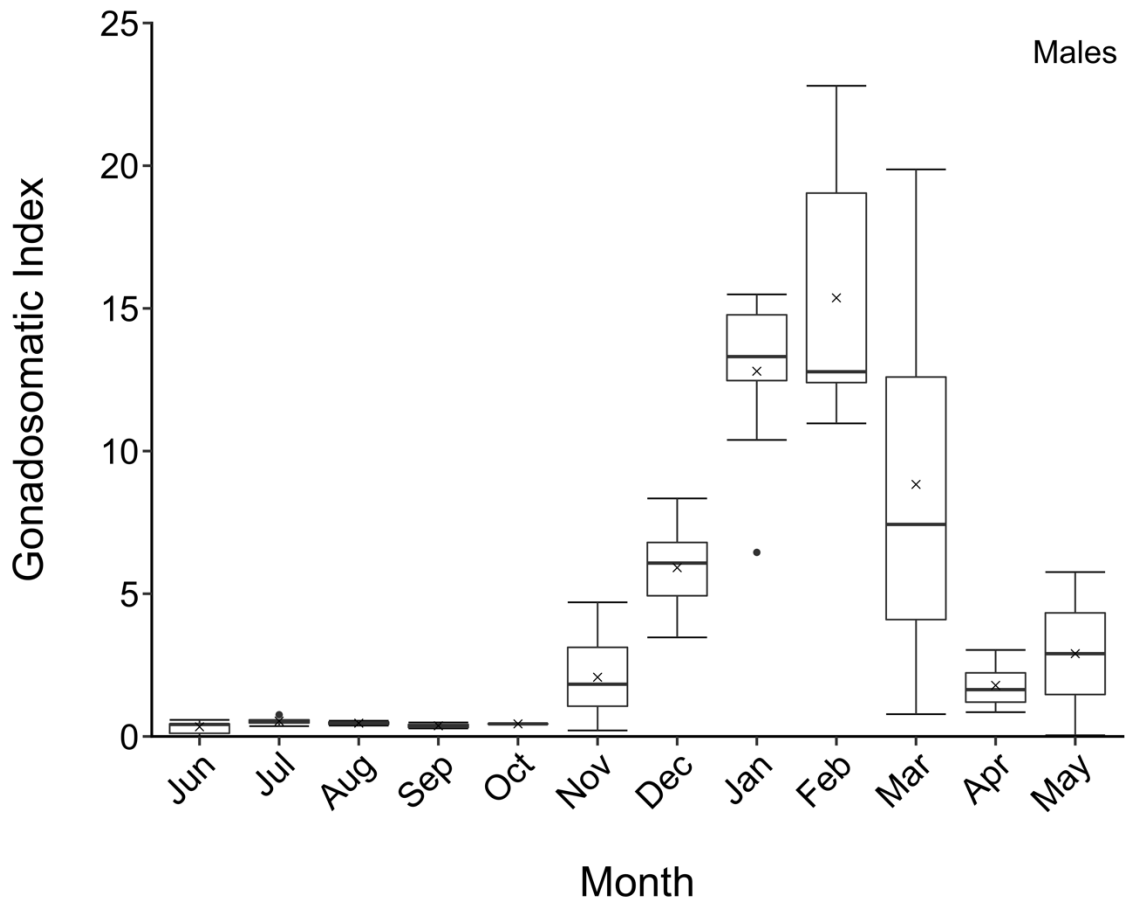


Figure 2.16. Gonadosomatic index (GSI) of adult male Burbot by month. Burbot were held at the Bozeman Fish Technology Center and exposed to a natural photoperiod and thermal profile similar to that in Lake Roosevelt, Washington. Data are the observed values of GSI. The box encompasses data from the first to third quartiles (i.e., 25th to 75th percentiles). The horizontal line within a box represents the median. The black cross represents the mean. The whiskers represent the minimum and maximum values. Outliers are represented by individual points. If there are outliers, the whiskers represent the minimum value within 1.5 times the interquartile range below the first quartile or the maximum value within 1.5 times the interquartile range above the third quartile. A single male was observed in October.

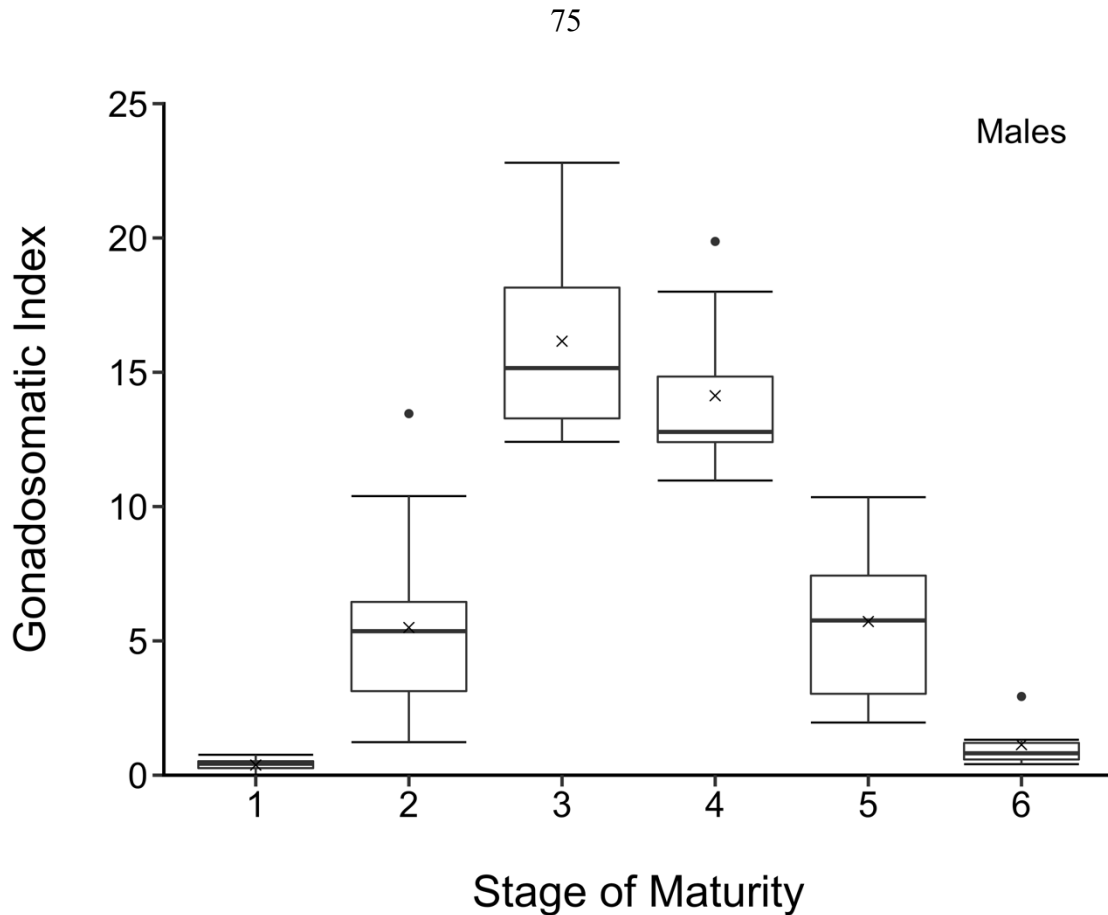


Figure 2.17. Gonadosomatic index (GSI) of adult male Burbot by stage of maturity. Burbot were held at the Bozeman Fish Technology Center and exposed to a natural photoperiod and thermal profile similar to that in Lake Roosevelt, Washington. Data are the observed values of GSI. The box encompasses data from the first to third quartiles (i.e., 25th to 75th percentiles). The horizontal line within a box represents the median. The black cross represents the mean. The whiskers represent the minimum and maximum values. Outliers are represented by individual points. If there are outliers, the whiskers represent the minimum value within 1.5 times the interquartile range below the first quartile or the maximum value within 1.5 times the interquartile range above the third quartile.

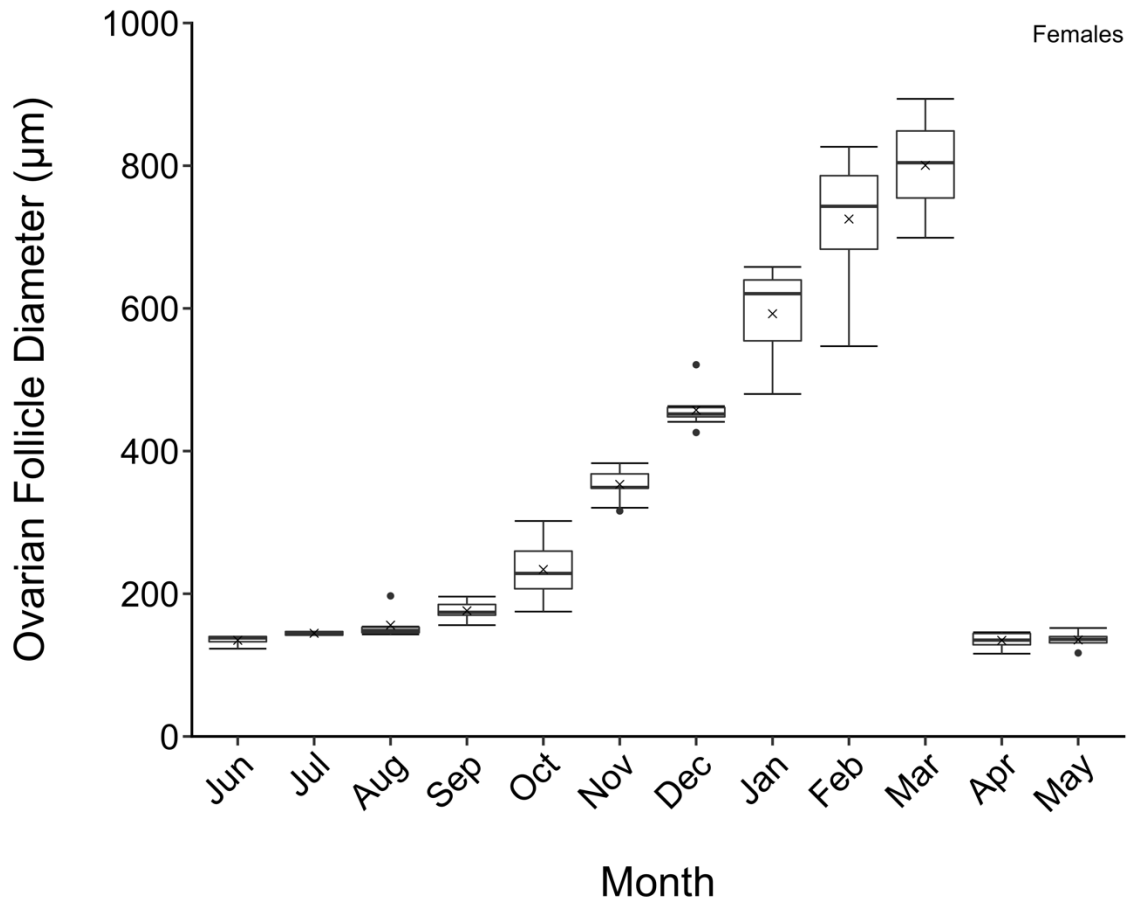


Figure 2.18. Ovarian follicle diameter (µm) of adult female Burbot by month. Burbot were held at the Bozeman Fish Technology Center and exposed to a natural photoperiod and thermal profile similar to that in Lake Roosevelt, Washington. Data are the average diameter (µm) of 15 ovarian follicles from each individual. The box encompasses data from the first to third quartiles (i.e., 25th to 75th percentiles). The horizontal line within a box represents the median. The black cross represents the mean. The whiskers represent the minimum and maximum values. Outliers are represented by individual points. If there are outliers, the whiskers represent the minimum value within 1.5 times the interquartile range below the first quartile or the maximum value within 1.5 times the interquartile range above the third quartile.

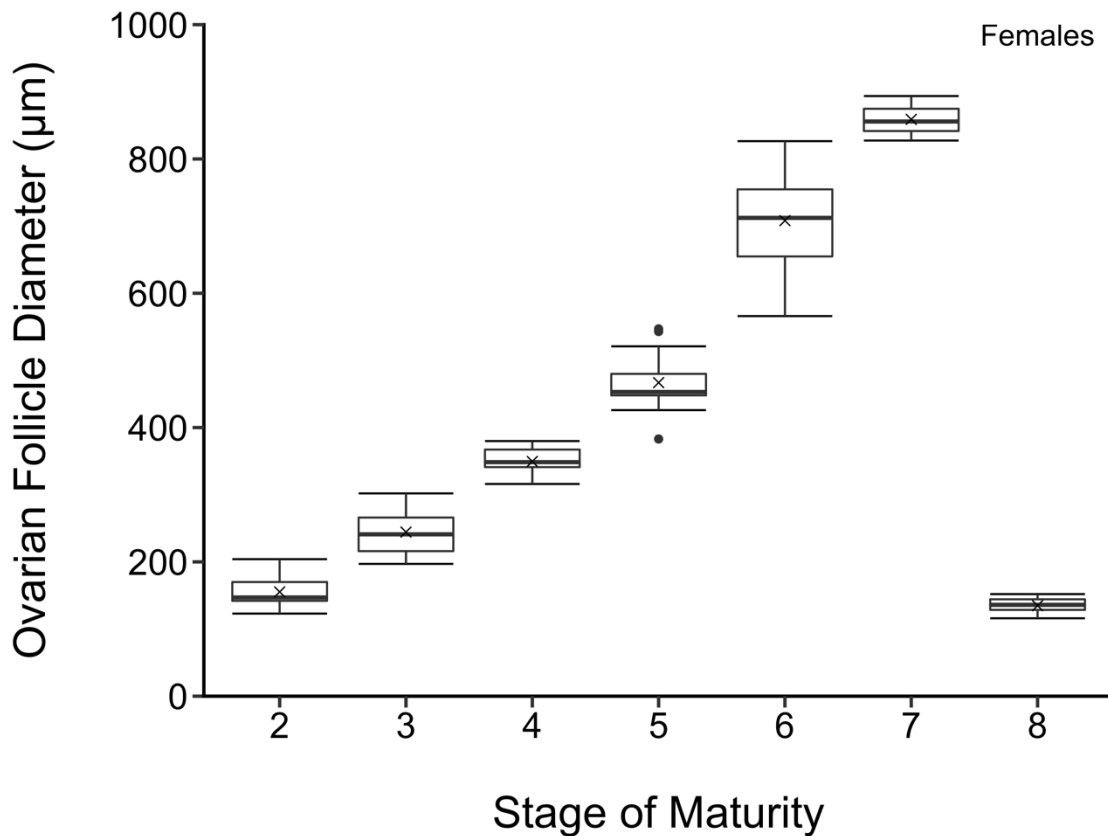


Figure 2.19. Ovarian follicle diameter (μm) of adult female Burbot by stage of maturity. Burbot were held at the Bozeman Fish Technology Center and exposed to a natural photoperiod and thermal profile similar to that in Lake Roosevelt, Washington. Data are the average diameter (μm) of 15 ovarian follicles from each individual. The box encompasses data from the first to third quartiles (i.e., 25th to 75th percentiles). The horizontal line within a box represents the median. The black cross represents the mean. The whiskers represent the minimum and maximum values. Outliers are represented by individual points. If there are outliers, the whiskers represent the minimum value within 1.5 times the interquartile range below the first quartile or the maximum value within 1.5 times the interquartile range above the third quartile. No females were observed during oogonal proliferation (Stage 1).



Figure 2.20. Ultrasound image of ovarian tissue (Stage 6) from an adult female Burbot in February. The ultrasound transducer was placed perpendicular to the midline at the urogenital pore to capture the image. The white line delineates the ovaries. The lobes of the ovaries were rounded and clearly connected.

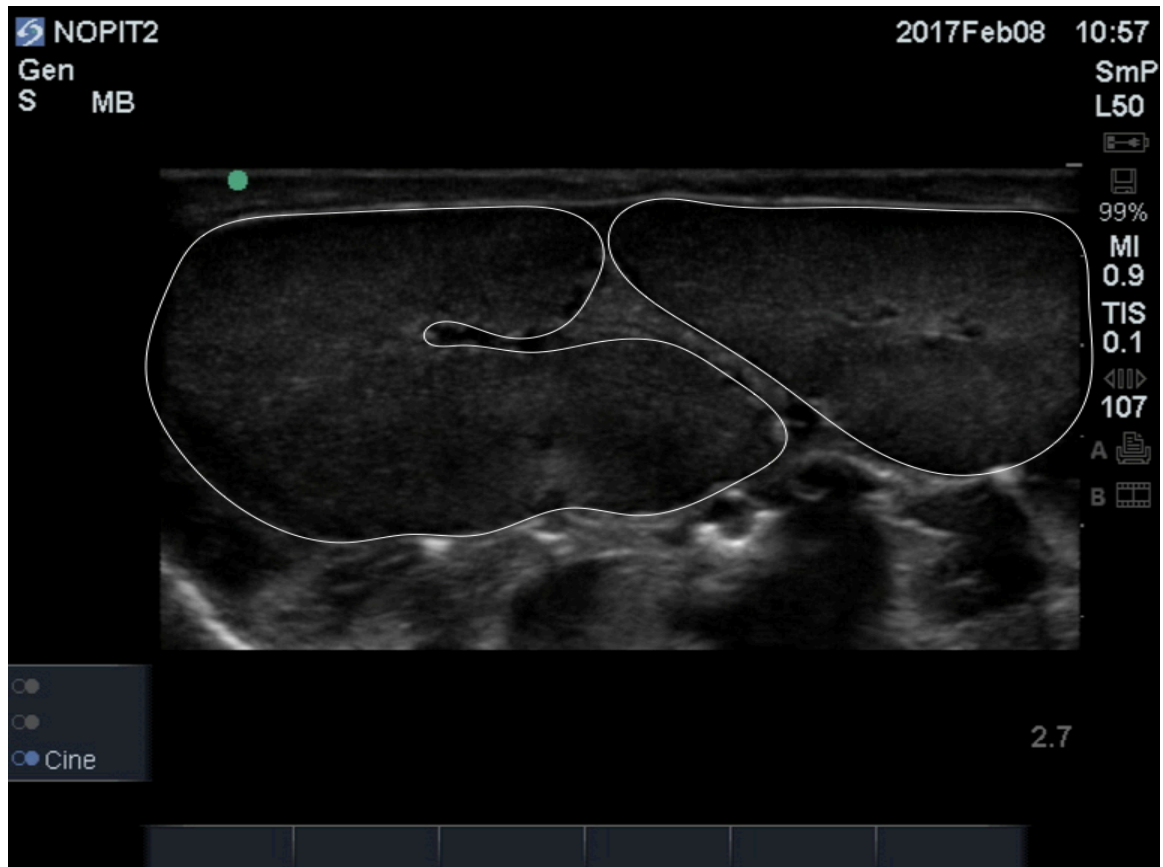


Figure 2.21. Ultrasound image of ripe (Stage 4) testicular tissue from an adult male Burbot in February. The ultrasound transducer was placed perpendicular to the midline at the urogenital pore to capture the image. The white lines delineate the testes. The testes were angular and unconnected.

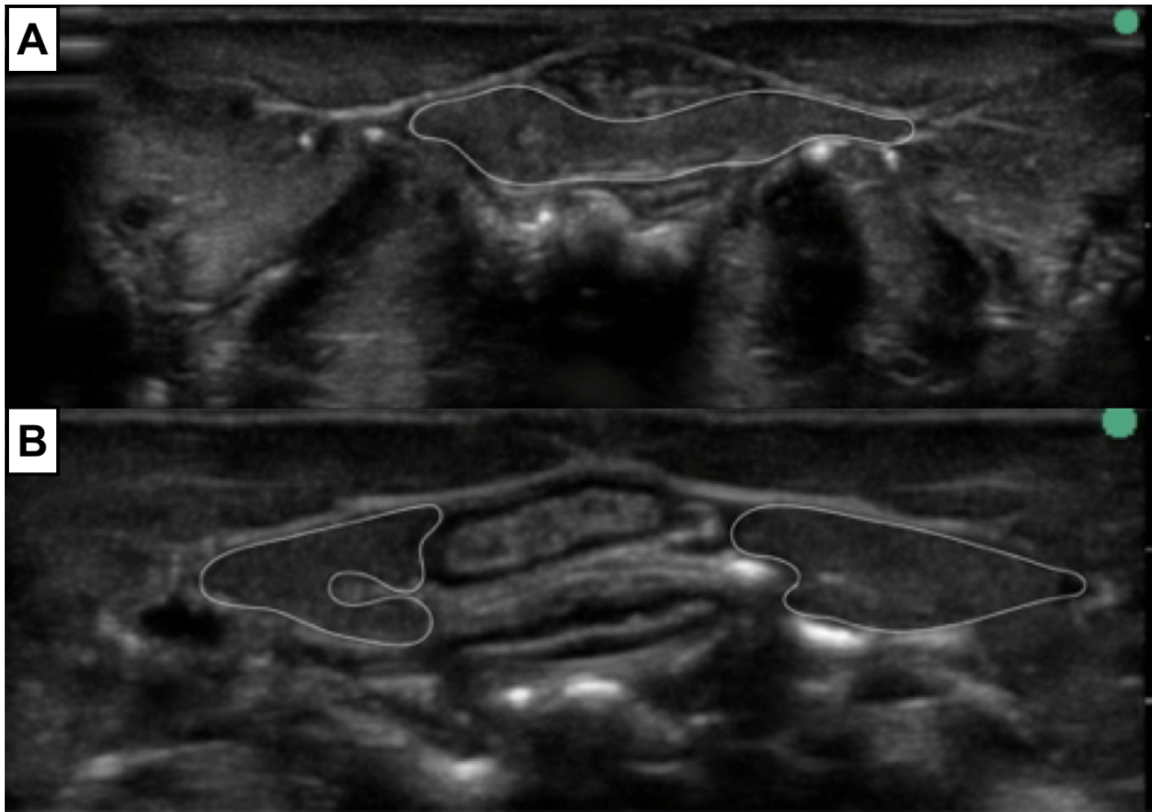


Figure 2.22. Ultrasound image of primary growth (Stage 2) ovarian tissue from an adult female Burbot (A) and spermatogonial proliferation (Stage 1) testicular tissue from an adult male Burbot (B) in June. The ultrasound transducer was placed perpendicular to the midline at the urogenital pore to capture the images. The white lines delineate ovarian tissue (A) and testicular tissue (B). Ovaries were undeveloped and had a similar appearance to testes. However, the lobes of the ovaries were clearly connected whereas the testes were unconnected.

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CHAPTER THREE

ASSESSMENT OF GONAD SIZE MEASURED BY ULTRASOUND TO
ASSIGN STAGE OF MATURITY AND REPRODUCTIVE
CONDITION IN BURBOT *LOTA LOTA*

Contributions of Authors and Co-Authors

Manuscript in Chapter 3

Author: Lauren M. McGarvey

Contributions: Conceived and implemented the study design, collected and analyzed data, and authored the manuscript.

Co-Author: Molly A. H. Webb

Contributions: Obtained funding, conceived the study design, discussed the implications of the results, and co-authored the manuscript.

Co-Author: Christopher S. Guy

Contributions: Obtained funding, conceived the study design, discussed the implications of the results, and co-authored the manuscript.

Manuscript Information Page

Lauren M. McGarvey, Christopher S. Guy, Molly A. H. Webb

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ABSTRACT

Gonad size (diameter and circumference) measured by ultrasound was assessed as a metric to assign stage of maturity and reproductive condition (non-reproductive and reproductive) in Burbot *Lota lota* from Lake Roosevelt, Washington. Paired gonadal tissue and ultrasound measurements were collected monthly from November 2017 to March 2018. Gonadal tissue was processed for histological analysis to confirm sex and stage of maturity. Gonad diameter and circumference were measured by ultrasound. Excised gonad diameter (i.e., true) was measured by digital calipers, and excised gonad circumference (i.e., true) was measured by a measuring tape. All late vitellogenic (Stage 6) females had a gonad diameter measured by ultrasound greater than 3.90 cm, suggesting a value of 3.90 cm could be used to characterize late vitellogenic (Stage 6) females. There was no overlap in the distribution of gonad diameter measured by ultrasound between non-reproductive and reproductive stages of maturity in males, suggesting gonad diameter measured by ultrasound can be used to assign reproductive condition in males. Gonad circumference measured by ultrasound in males is not reported because some testes were wider than the ultrasound transducer and could not be measured. Measurements of female gonad circumference and male gonad diameter did not differ significantly between measurement methods (ultrasound and true), but measurements of female gonad diameter differed significantly between measurement methods ($t = -2.32$; $df = 28$; $P = 0.028$). Gonad diameter and circumference measured by ultrasound were highly correlated with gonadosomatic index and ovarian follicle diameter, suggesting gonad size measured by ultrasound is an appropriate index of gonadal development in Burbot. Using ultrasound to assign stage of maturity and reproductive condition will enable fisheries biologists to non-lethally assess indices of reproductive potential for the Burbot population in Lake Roosevelt. Indices of reproductive potential can be used to characterize and monitor population demographics, improve models of population growth, establish sustainable harvest regulations, monitor the effects of management actions, and monitor the effects of environmental stressors.

Introduction

Ultrasound has been used to non-lethally assign sex, stage of maturity, and reproductive condition in fishes by detecting morphological and echogenic differences among gonads (Shields et al. 1993; Moghim et al. 2002; Colombo et al. 2004; Evans et al. 2004; Novelo and Tiersch 2016). In a previous study, we assessed the use of ultrasound to assign sex, stage of maturity, and reproductive condition in adult Burbot *Lota lota* (see chapter two). Ultrasound assigned sex with 97% accuracy but could not be used to assign stage of maturity and reproductive condition by detecting morphological or echogenic differences among gonads. For example, ripe ovarian follicles are less than 1 mm in diameter and could not be delineated using our ultrasound transducer as has been done in previous studies (Shields et al. 1993; Evans et al. 2004; Novelo and Tiersch 2016). However, there was a noticeable change in gonad size during development to spawn.

Gonad size measured by ultrasound has previously been used to assign stage of maturity and reproductive condition in fishes (Mattson 1991; Blythe et al. 1994; Evans et al. 2004; Newman et al. 2008; Whittamore et al. 2010; Du. et al. 2017; Naeve et al. 2018). Gonad diameter measured by ultrasound was proposed as a metric to assign stage of maturity (ripe and non-ripe) in adult Striped Bass *Morone saxatilis* (Blythe et al. 1994). Ultrasound was used to determine sex and measure gonad diameter at the perceived maximum area (i.e., maximum area determined by scanning the entire gonad with an ultrasound transducer). In female Striped Bass, a gonad diameter measured by ultrasound greater than 3.0 cm was suggested to characterize ripe females. In male

Striped Bass, a gonad diameter measured by ultrasound greater than 2.0 cm was suggested to characterize ripe males. Gonad area measured by ultrasound has also been used as a metric to assign stage of maturity (pre-spawn and post-spawn) in adult male steelhead *Oncorhynchus mykiss* (Evans et al. 2004). Ultrasound was used to measure gonad area at the perceived maximum area. Gonad area was $2.86 \pm 0.73 \text{ cm}^2$ (mean $\pm$ S.E.M.) in pre-spawn males and $0.62 \pm 0.24 \text{ cm}^2$ (mean $\pm$ S.E.M.) in post-spawn males. In male steelhead, a gonad area of 1.25 cm^2 or greater was suggested to characterize pre-spawn males. To our knowledge, gonad size measured by ultrasound has never been assessed as a metric to assign stage of maturity in Burbot.

The objective of this study was to assess gonad size (diameter and circumference) measured by ultrasound as a metric to non-lethally and non-invasively assign stage of maturity and reproductive condition in Burbot. Using ultrasound to assign sex (see chapter two), stage of maturity, and reproductive condition will enable fisheries biologists to assess indices of reproductive potential (e.g., sex ratio, reproductive structure, and spawning periodicity) for the Burbot population in Lake Roosevelt. Indices of reproductive potential can be used to characterize and monitor population demographics, model population growth, establish sustainable harvest regulations, monitor the effects of management actions, and monitor the effects of environmental stressors (Trippel 1993; Downs et al. 1997; Wildhaber et al. 2007; Power 2007; Morgan 2008; Schill et al. 2010).

Study Area

Lake Roosevelt is located in northeast Washington. The 209-km reservoir was formed after the construction of Grand Coulee Dam (GCD) on the Columbia River. The reservoir is typically 1-3 km wide with a maximum depth of 122 m extending 241 km upstream from GCD to the Canadian border (Polacek et al. 2006). At full pool elevation, the reservoir covers approximately 33,000 ha (Polacek et al. 2006). The reservoir supports many recreational fisheries including a Burbot fishery which may be underutilized and able to support greater harvest (CCT 2018). The reservoir is designated as a National Recreation Area by the National Park Service.

Methods

Fish Collection and Maintenance

Burbot from Lake Roosevelt were collected in October 2017 by the Confederated Tribes of the Colville Reservation. Baited cod traps were set at 10 m and pulled the following day. Cod traps were set at 10 m to prevent barotrauma in captured fish. Each fish was tagged with a passive integrated transponder (PIT) and transferred to the Bozeman Fish Technology Center, Bozeman MT. Fish were maintained in tanks with a natural light cycle and temperature profile matching Lake Roosevelt (see chapter two). Fish were fed live feed until satiation.

Biological Sampling

Six fish were euthanized by overdose of MS-222 (tricaine methanesulfonate) monthly from November 2017 to March 2018. An equal portion of females and males were selected using ultrasound (see chapter two). Biological data were collected from individual fish including the following: body weight (± 0.01 g), gonad weight (± 0.01 g), ultrasound measurements of gonad diameter and circumference (± 0.01 cm), measurements of excised gonad diameter and circumference (± 0.01 cm), and gonadal tissue.

Histology

Gonadal tissue was preserved in 10% phosphate buffered formalin for histological analysis (Webb and Erickson 2007). Gonadal tissue was embedded in paraffin wax and sectioned at 5 μ m. Gonadal tissue was stained with periodic acid-Schiff (PAS). Slides were examined under a Leica DM compound scope (250 – 450X) (Leica Biosystems Inc., IL, USA). Germ cells in gonadal tissue were assigned stage of maturity using our description of gametogenesis in Burbot (see chapter two) (Table 3.1). Stage of maturity was used to assign reproductive condition. Females in the cortical alveolar (Stage 3), early vitellogenic (Stage 4), mid-vitellogenic (Stage 5), late vitellogenic (Stage 6), and ripe (Stage 7) stages of maturity were reproductive, whereas females in the oogonial proliferation (Stage 1), primary growth (Stage 2), post-ovulation (Stage 8), and atretic (Stage 9) stages of maturity were non-reproductive (Brown-Peterson et al. 2011). Males in the early spermatogenic (Stage 2), mid-spermatogenic (Stage 3), ripe (Stage 4), and mid-spermiation (Stage 5) stages of maturity were reproductive, whereas males in the

spermatogonial proliferation (Stage 1) and post-spermiation (Stage 6) stages of maturity were non-reproductive (Brown-Peterson et al. 2011).

Ultrasound

A SonoSite Edge ultrasound (SonoSite Inc., Washington, USA) with a linear transducer (6-15 MHz) was used to measure gonad size in euthanized fish. The Small Parts exam type was used with the optimization set to General. The ultrasound scanning depth was set between 2.2 and 2.8 cm. Fish were oriented ventrally, and the ultrasound transducer was placed on the abdomen to locate the right gonad. The right gonad was always measured for standardization. The gonad was scanned until the maximum area was in view (Blythe et al. 1994; Evans et al. 2004). Gonad diameter (i.e., the maximum distance from the left to right edge) was measured by ultrasound using the caliper function (Figure 3.1). A value of 5.11 cm (the width of the ultrasound transducer) was assigned if the gonad was wider than the ultrasound transducer and could not be measured. Gonad circumference was measured by ultrasound using the caliper function on manual trace (Figure 3.3). Circumference could not be measured if the gonad was wider than the ultrasound transducer. Measurements were repeated three times for each individual, and mean measurements were used in analyses. All sonograms with measurements were archived.

True Measure of Gonad Diameter and Circumference

Gonads were excised to measure true diameter using Mitutoyo digital calipers (150 mm range $\pm$ 0.01 mm) (Mitutoyo American Corp., Illinois, USA) and true

circumference using a measuring tape (± 0.1 mm). Measurements were repeated three times for each individual, and mean measurements were used in analyses. Gonad diameter measured by ultrasound was compared to excised gonad diameter measured by calipers, and gonad circumference measured by ultrasound was compared to excised gonad circumference measured by a measuring tape.

Gonadosomatic Index and Ovarian Follicle Diameter

Gonads were excised and weighed (± 0.01 g) for calculation of gonadosomatic index ($GSI = [\text{gonad weight}/\text{total body weight}] \times 100$). Ovarian follicles were collected from excised ovaries and preserved in Ringers solution (Dettlaff et al. 1993). Fifteen ovarian follicles (Johnson 1971) from each individual were measured using SPOT Advanced image analysis software (SPOT Imaging, Michigan, USA). The diameter of each ovarian follicle was measured twice to increase measurement precision. If the difference between the two diameter measurements was greater than 3 μm , the measurements were repeated. The largest diameter measurement was recorded.

Data Analyses

Data were checked for normality using side-by-side boxplots and Q-Q plots. All data were normally distributed, and parametric statistical analyses were used. One-way analysis of variance (ANOVA) was used for mean comparisons of gonad size measured by ultrasound among months and stages of maturity followed by pairwise comparisons with a Bonferroni correction. A two-sample t-test was used for mean comparisons of gonad size between measurement methods (ultrasound and true). The accepted

significance level was $\alpha = 0.05$. Correlation coefficients (r) were calculated to assess the linear relationship between gonad size measured by ultrasound and indices of gonadal development. All statistical analyses were conducted in R software (version 3.3.2).

Results

Histology

Females were observed in the early vitellogenic (Stage 4) ($n = 3$), mid-vitellogenic (Stage 5) ($n = 6$), and late vitellogenic (Stage 6) ($n = 6$) stages of maturity. No females were observed in the oogonial proliferation (Stage 1), primary growth (Stage 2), cortical alveolar (Stage 3), ripe (Stage 7), post-ovulatory (Stage 8), or atretic (Stage 9) stages of maturity.

Males were observed in spermatogonial proliferation (Stage 1) ($n = 2$), early spermatogenic (Stage 2) ($n = 7$), mid-spermatogenic (Stage 3) ($n = 4$), and ripe (Stage 4) ($n = 3$) stages of maturity. No males were observed in the mid-spermiation (Stage 5) or post-spermiation (Stage 6) stages of maturity.

Ultrasound

Female gonad diameter measured by ultrasound varied from 2.19 to 4.97 cm and differed significantly among months ($F = 15.48$; $df = 4, 10$; $P = < 0.001$). Mean female gonad diameter measured by ultrasound increased from 2.44 cm in December to 4.54 cm in March (Figure 3.4) (Table 3.2). Female gonad diameter measured by ultrasound differed significantly among stages of maturity ($F = 19.99$; $df = 2, 12$; $P = < 0.001$).

Mean female gonad diameter measured by ultrasound increased from 2.46 cm during early vitellogenesis (Stage 4) to 4.42 cm during late vitellogenesis (Stage 6) (Figure 3.5) (Table 3.2).

Female gonad circumference measured by ultrasound varied from 5.57 to 11.40 cm and differed significantly among months ($F = 17.13$; $df = 4, 10$; $P = < 0.001$). Mean female gonad circumference measured by ultrasound increased from 5.84 cm in December to 10.46 cm in March (Figure 3.6) (Table 3.3). Female gonad circumference measured by ultrasound differed significantly among stages of maturity ($F = 17.73$; $df = 2, 12$; $P = < 0.001$). Mean female gonad circumference measured by ultrasound increased from 5.81 cm during early vitellogenesis (Stage 4) to 10.12 cm during late vitellogenesis (Stage 6) (Figure 3.7) (Table 3.3).

Male gonad diameter measured by ultrasound varied from 0.88 to 5.11 cm (the width of the ultrasound transducer) and differed significantly among months ($F = 10.56$; $df = 4, 10$; $P = 0.001$). Mean male gonad diameter measured by ultrasound increased from 1.54 cm in November to 5.11 cm in March (Figure 3.8) (Table 3.4). All testes in March were too large to be measured by ultrasound and assigned a diameter of 5.11 cm. Male gonad diameter measured by ultrasound differed significantly among stages of maturity ($F = 20.10$; $df = 3, 12$; $P = < 0.001$). Mean male gonad diameter measured by ultrasound increased from 0.99 cm during spermatogonial proliferation (Stage 1) to 5.11 cm when testes were ripe (Stage 4) (Figure 3.9) (Table 3.4). All ripe testes (Stage 4) were too large to be measured by ultrasound and assigned a diameter of 5.11 cm. Male

gonad circumference measured by ultrasound is not reported because the circumference of testes wider than the ultrasound transducer could not be measured.

True Measure of Gonad Diameter and Circumference

All ultrasound measurements of gonad diameter and circumference, regardless of month and stage of maturity, were compared to all true measurements of excised gonad diameter and circumference (i.e., measured by calipers and a measuring tape, respectively). Measurements of female gonad diameter differed significantly between the two methods ($t = -2.32$; $df = 28$; $P = 0.028$) (Figure 3.10). Mean female gonad diameter measured by ultrasound was 3.45 cm (2.93 – 3.98 cm; 95% CI), and mean female gonad diameter measured by calipers was 2.74 cm (2.34 – 3.14 cm; 95% CI). There was a 21% difference between the two measurement methods.

Measurements of female gonad circumference did not differ significantly between the two methods ($t = -0.77$; $df = 28$; $P = 0.450$) (Figure 3.11). Mean female gonad circumference measured by ultrasound was 8.05 cm (6.88 – 9.21 cm; 95% CI), and mean female gonad circumference measured by a measuring tape was 7.49 cm (6.45 – 8.53 cm; 95% CI). There was a 7% difference between the two measurement methods.

Measurements of male gonad diameter did not differ significantly between the two methods ($t = -0.21$; $df = 29$; $P = 0.838$) (Figure 3.12). Mean male gonad diameter measured by ultrasound was 3.68 cm (2.87 – 4.48 cm; 95% CI), and mean male gonad diameter measured by calipers was 3.56 cm (2.61 – 4.50; 95% CI). There was a 3% difference between the two measurement methods.

Gonadosomatic Index and Ovarian Follicle Diameter

Female GSI and ovarian follicle diameter increased from early vitellogenesis (Stage 4) to late vitellogenesis (Stage 6) (see chapter two). Female gonad diameter and circumference measured by ultrasound were strongly correlated with GSI ($r = 0.91$) and ovarian follicle diameter ($r = 0.89$) (Figure 3.13).

Male GSI increased from spermatogonial proliferation (Stage 1) to mid-spermatogenesis (Stage 3) (see chapter two). Male gonad diameter was strongly correlated with GSI ($r = 0.90$) (Figure 3.14). Male gonad circumference could not be correlated with GSI because the circumference of all testes could not be measured.

Discussion

We have demonstrated that ultrasound is a promising non-lethal tool to assign stage of maturity and reproductive condition in Burbot. Ultrasound has been used to assign stage of maturity and reproductive condition by measuring reproductive structures in other fishes such as Atlantic Salmon *Salmo salar* (Mattson 1991; Naeve et al. 2018), Striped Bass (Blythe et al. 1994), steelhead (Evans et al. 2004), Murray Cod *Maccullochella peelii* (Newman et al. 2008), Small-Spotted Catshark *Scyliorhinus canicular* (Whittamore et al. 2010), Thornback Ray *Raja clavate* (Whittamore et al. 2010), and Chinese Sturgeon *Acipenser sinensis* (Du et al. 2017). Our results are similar to other studies that found gonad size measured by ultrasound was highly correlated with GSI and ovarian follicle diameter, two commonly used indices of gonadal development in fishes (e.g., Blythe et al. 1994; Newman et al. 2008; Naeve et al. 2018). For example,

gonad diameter measured by ultrasound was strongly correlated ($r = 0.92$) with ovarian follicle diameter in Striped Bass (Blythe et al. 1994), similar to our results. The strong correlation between gonad size measured by ultrasound and commonly used indices of gonadal development (GSI and ovarian follicle diameter) suggests gonad size measured by ultrasound is a non-lethal and non-invasive index of gonadal development for fishes.

Only a few studies have documented how gonad size measured by ultrasound changes with stage of maturity determined by gonadal histology (e.g., Newman et al. 2008; Du et al. 2017). In Murray Cod, gonad diameter measured by ultrasound differed significantly among stages of maturity but with a high degree of overlap, indicating the assignment of stage of maturity based solely on an ultrasound measurement of gonad diameter was prone to error (Newman et al. 2008). We also observed overlap in the distributions of gonad diameter measured by ultrasound among stages of maturity. However, we did not observe overlap in the distributions of gonad diameter measured by ultrasound between non-reproductive stages of maturity and reproductive stages of maturity in males, suggesting that gonad diameter measured by ultrasound could be used to assign reproductive condition. Unfortunately, females were only observed in the reproductive stages of maturity; therefore, we could not compare gonad diameter measured by ultrasound between non-reproductive and reproductive stages of maturity. We suggest gonad diameter measured by ultrasound can be used to assign reproductive condition in Burbot, but a study including all stages of maturity during the annual reproductive cycle and larger sample sizes would be useful to validate our initial results.

In females, late vitellogenic (Stage 6) gonad diameter measured by ultrasound differed significantly from early vitellogenic (Stage 4) and mid-vitellogenic (Stage 5) gonad diameter measured by ultrasound. However, there was only a 0.15 cm difference between the distribution of late vitellogenic (Stage 6) gonad diameter measured by ultrasound and the distributions of early vitellogenic (Stage 4) and mid-vitellogenic (Stage 5) gonad diameter measured by ultrasound. Characterizing late vitellogenic (Stage 6) females would be of interest because these females are capable of spawning (Brown-Peterson et al. 2011). Values of gonad diameter have been proposed in other fishes to characterize females capable of spawning (Blythe et al. 1994; Whittamore et al. 2010; Du et al. 2017). All late vitellogenic (Stage 6) females had a gonad diameter measured by ultrasound greater than 3.90 cm; therefore, we suggest a gonad diameter of 3.90 cm may be used to characterize late vitellogenic (Stage 6) females, but an additional study with larger sample sizes should further assess this value.

The gonad diameter of one mid-spermatogenic (Stage 3) and all ripe (Stage 4) males was wider than the ultrasound transducer and assigned a value of 5.11 cm (i.e., the width of the ultrasound transducer). Our results suggest a gonad diameter measured by ultrasound of 5.11 cm could be used to characterize mid-spermatogenic (Stage 3) and ripe (Stage 4) males, both of which would be expected to spawn during the current reproductive cycle (Brown-Peterson et al. 2011). However, the shape and frequency of the ultrasound transducer should be considered if further investigating the use of gonad size measured by ultrasound to assign stage of maturity and reproductive condition in male Burbot. In large female Striped Bass, the entire cross-section of the ovary could not

be delineated using a linear transducer (6-8 MHz) but could be delineated using a curved transducer (3-5 MHz) (Will et al. 2002). A curved transducer may be used to measure the entire gonad in male Burbot by providing a wider field of view at a lower resolution (Will et al. 2002; Jennings et al. 2005).

Female gonad circumference and male gonad diameter were similar between ultrasound and true measurements. Several studies have observed a high correlation between ultrasound and true measurements of gonad size (e.g., Mattson 1991; Jennings et al. 2005; Bryan et al. 2007; Whittamore et al. 2010; Naeve et al. 2018). However, female gonad diameter differed significantly between ultrasound and true measurements. The difference between measurement methods may be attributed to flattening of the gonad when applying the ultrasound transducer; therefore, it is critical to be aware of the pressure exerted on the fish by the ultrasound transducer. A few other studies have also observed a difference in ultrasound and true measurements of reproductive structures (Bryan et al. 2007; Whittamore et al. 2010). For example, ultrasound measurements were 52% smaller than true measurements of ovarian follicle diameter in Shovelnose Sturgeon *Scaphirhynchus platyrhynchus* (Bryan et al. 2007). The discrepancy between measurement methods was attributed to the ellipsoid shape of ovarian follicles in sturgeon, making it difficult to measure the longest axis (Bryan et al. 2007). An adjustment for the ultrasound error (i.e., the average difference between the two methods) was applied to all ultrasound measurements of ovarian follicle diameter (Bryan et al. 2007). A similar adjustment could be added to measurements of female gonad diameter in Burbot; however, the high correlation observed between female gonad diameter

measured by ultrasound and indices of gonadal development (GSI and ovarian follicle diameter) indicates it is an appropriate index of gonadal development.

Ultrasound can be used to assign sex (see chapter two) and is a promising tool to assign stage of maturity and reproductive condition in Burbot. Ultrasound is non-lethal and non-invasive, therefore, eliminating the need to sacrifice fish and reducing stress experienced by fish compared to other indices of gonadal development (i.e., gonadal histology, GSI, ovarian follicle diameter, and plasma sex steroids) (Blythe et al. 1994). Ultrasound offers immediate results unlike other indices of gonadal development that require time to analyze samples in a lab. However, an ultrasound unit is very expensive, varying from \$15,000 to 30,000, and using ultrasound requires adequate training to become familiar with the visceral anatomy of the species of interest (i.e., 32 – 40 hours) (Webb et al. 2017). Despite the associated cost and training, ultrasound is a preferable tool when sacrificing fish is not acceptable, such as for threatened and endangered species, or when results are desired immediately. Ultrasound will enable fisheries biologists to non-lethally assess indices of reproductive potential such as sex ratio, reproductive structure, and spawning periodicity. Ultrasound may also be further assessed to determine fecundity in Burbot as has been done in other fishes (Will et al. 2002; Bryan et al. 2005; Bryan et al. 2007). Indices of reproductive potential are used in fisheries science and management to characterize and monitor population demographics, model population growth, establish sustainable harvest regulations, monitor the effects of management actions, and monitor the effects of environmental stressors (Trippel 1993; Downs et al. 1997; Wildhaber et al. 2007; Power 2007; Morgan 2008; Schill et al. 2010).

By using ultrasound as we have described, fisheries biologists can non-lethally assess indices of reproductive potential of the Burbot population in Lake Roosevelt to inform and guide management decisions.

Table 3.1. Description of gonadal stages of maturity in Burbot assigned by histological analysis of gonadal tissue.

Sex	Stage of Maturity	Description
Females	Oogonial Proliferation (1)	Oogonia and potentially a few primary growth oocytes
	Primary Growth (2)	Primary growth oocytes and oogonia
	Cortical Alveolar (3)	Cortical alveoli on periphery of ovarian follicles
	Early Vitellogenic (4)	Yolk granules accumulating in periphery of ovarian follicles, cortical alveoli on periphery of ovarian follicles, one layer of zona radiata
	Mid-Vitellogenic (5)	Yolk globules with an average diameter of 7.93µm (5.97 – 9.89 µm; 95% CI) accumulating towards center or may be present throughout ovarian follicles, few cortical alveoli on periphery of ovarian follicles, two layers of the zona radiata
	Late Vitellogenic (6)	Yolk fusing into larger globules with an average diameter of 19.62 µm (14.55 – 24.69 µm; 95% CI) throughout ovarian follicle, two layers of zona radiata, central germinal vesicle (i.e., nucleus)
	Ripe (7)	Yolk fused but not completely coalesced in all ovarian follicles, offset germinal vesicle
	Post-Ovulatory (8)	Post-ovulatory follicles present with primary growth oocytes
	Atretic (9)	> 75% atretic ovarian follicles, no post-ovulatory follicles present with primary growth oocytes
Males	Spermatogonial Proliferation (1)	Cysts contain only spermatogonia
	Early Spermatogenic (2)	Cysts contain spermatogonia and spermatocytes
	Mid-Spermatogenic (3)	Few cysts contain spermatogonia, > 50% of the cysts contain spermatocytes and spermatids
	Ripe and Initiating Spermiation (4)	Cysts filled with spermatozoa, approximately 20% of cysts emptying of spermatozoa
	Mid-Spermiation (5)	Cysts with reduced or residual spermatozoa, some cysts may be empty
	Post-Spermiation (6)	Cysts with residual spermatozoa and cysts empty of spermatozoa

Table 3.2. Gonad diameter (cm) measured by ultrasound in adult female Burbot by month and stage of maturity. No females were observed in the primary growth (Stage 1), cortical alveolar (Stage 2), ripe (Stage 7), post-ovulatory (Stage 8), or atretic (Stage 9) stages of maturity. Data are means (95% CI) and sample sizes (n).

Month	Early Vitellogenic (Stage 4)	Mid-Vitellogenic (Stage 5)	Late Vitellogenic (Stage 6)	Mean by Month
November	2.46 (1.87– 3.04) n = 3			2.46 (1.87– 3.04) n = 3
December		2.44 (2.27 – 2.61) n = 3		2.44 (2.27 – 2.61) n = 3
January		3.70 (2.30– 5.10) n = 2	4.06 n = 1	3.82 (3.25 – 4.40) n = 3
February		3.24 n = 1	4.40 (1.60 – 7.20) n = 2	4.01 (2.26 – 5.76) n = 3
March			4.54 (3.24 – 5.84) n = 3	4.54 (3.24 – 5.84) n = 3
Mean by Stage	2.46 (1.87– 3.04) n = 3	2.99 (2.33 – 3.66) n = 6	4.42 (3.99 – 4.84) n = 6	

Table 3.3. Gonad circumference (cm) measured by ultrasound in adult female Burbot by month and stage of maturity. No females were observed in the primary growth (Stage 1), cortical alveolar (Stage 2), ripe (Stage 7), post-ovulatory (Stage 8), or atretic (Stage 9) stages of maturity. Data are means (95% CI) and sample sizes (n).

Month	Early Vitellogenic (Stage 4)	Mid-Vitellogenic (Stage 5)	Late Vitellogenic (Stage 6)	Mean by Month
November	5.81 (5.23 – 6.40) n = 3			5.81 (5.23 – 6.40) n = 3
December		5.84 (5.45 – 6.23) n = 3		5.84 (5.45 – 6.23) n = 3
January		8.45 (5.78 – 11.12) n = 2	9.30 n = 1	8.73 (7.40 – 10.06) n = 3
February		8.15 n = 1	10.02 (0 – 24.18) n = 2	9.39 (5.54 – 13.24) n = 3
March			10.46 (7.78 – 13.14) n = 3	10.46 (7.78 – 13.14) n = 3
Mean by Stage	5.81 (5.23 – 6.40) n = 3	7.10 (5.64 – 8.55) n = 6	10.12 (8.98 – 11.25) n = 6	

Table 3.4. Gonad diameter (cm) measured by ultrasound in adult male Burbot by month and stage of maturity. No males were observed in the mid-spermiation (Stage 5) or post-spermiation (Stage 6) stage of maturity. Data are means (95% CI) and sample sizes (n). Gonad diameters wider than the ultrasound transducer could not be measured and were assigned a value of 5.11 cm (the width of the ultrasound transducer).

Month	Spermatogonial Proliferation (Stage 1)	Early Spermatogenic (Stage 2)	Mid-Spermatogenic (Stage 3)	Ripe (Stage 4)	Mean by Month
November	0.99 (0.01 – 2.39) n = 2	2.65 n = 1			1.54 (0.01 – 3.94) n = 3
December		2.72 (0.68 – 4.76) n = 3			2.72 (0.67 – 4.76) n = 3
January		3.73 (1.22 – 6.24) n = 3	5.11 n = 1		4.08 (2.36 – 5.79) n = 4
February			4.80 (3.77 – 5.82) n = 3		4.80 (3.77 – 5.82) n = 3
March				5.11 n = 3	5.11 n = 3
Mean by Stage	0.99 (0 – 2.39) n = 2	3.14 (2.28 – 4.00) n = 7	4.88 (4.28 – 5.47) n = 4	5.11 n = 3	

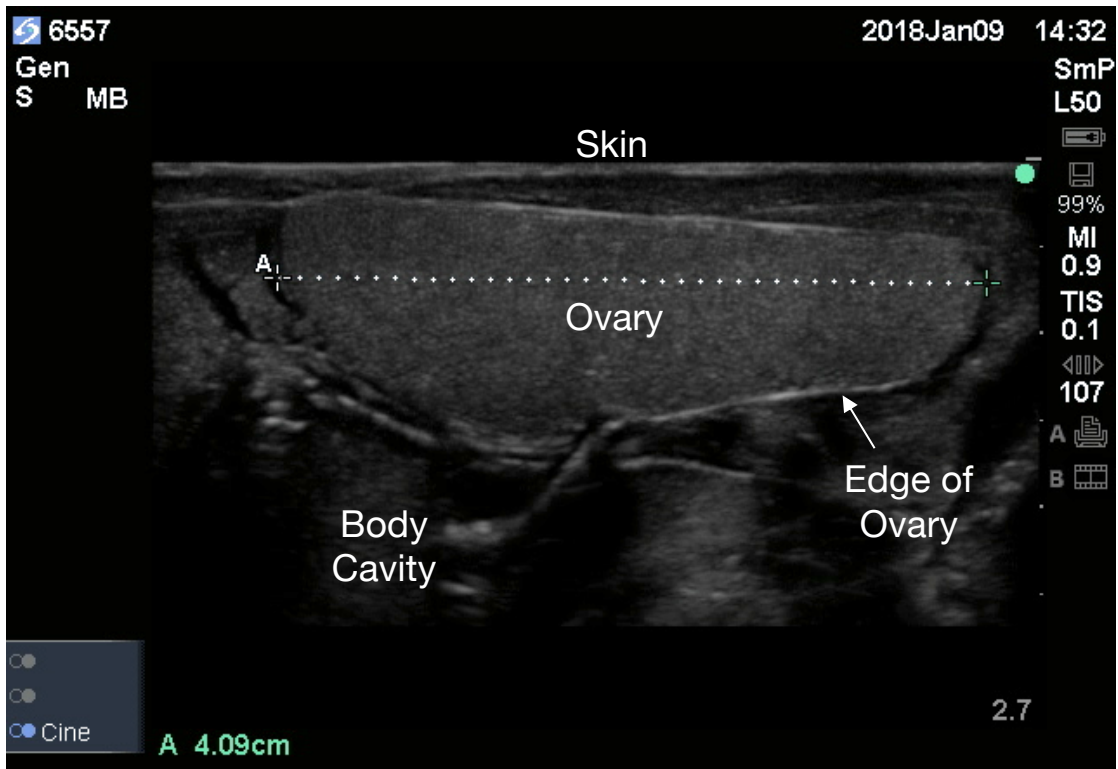


Figure 3.1. Sonogram showing the diameter of a late vitellogenic (Stage 6) ovary in January. The dashed, white line represents the gonad diameter.

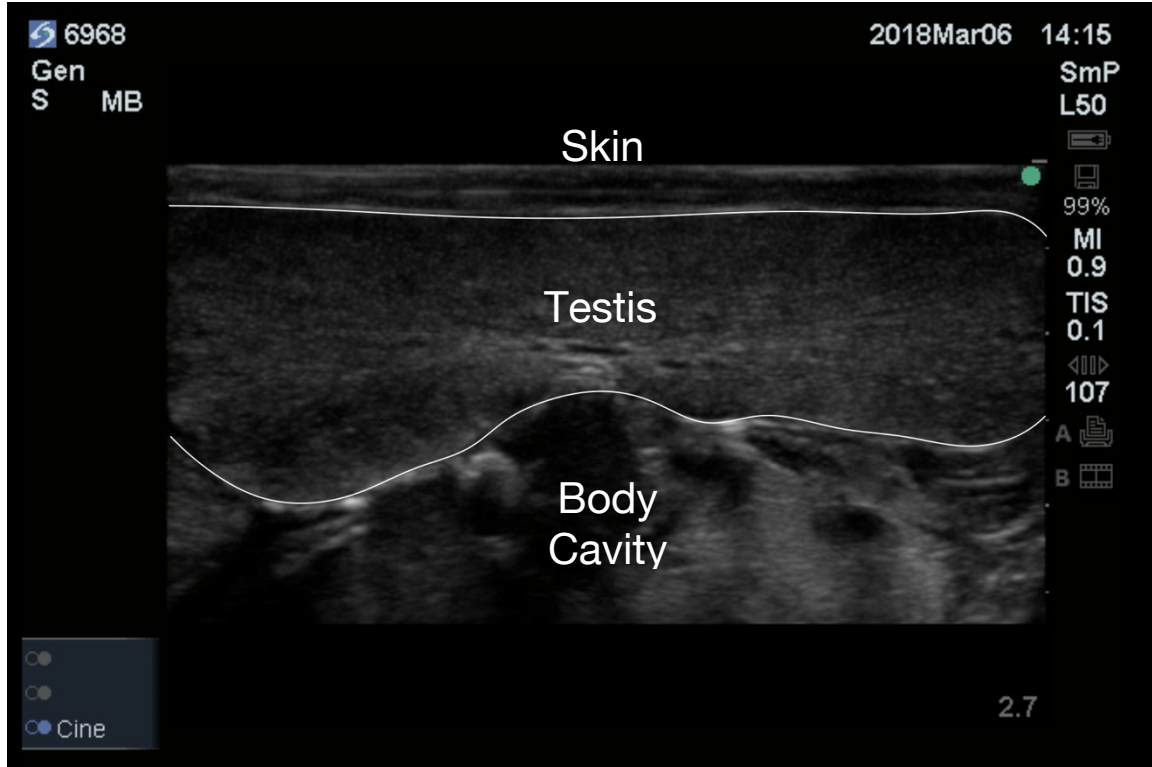


Figure 3.2. Sonogram taken at the maximum area of a ripe (Stage 4) testis in March. The white line delineates testicular tissue. The testis was wider than the ultrasound transducer; therefore, a value of 5.11 cm (the width of the ultrasound transducer) was assigned for gonad diameter measured by ultrasound.

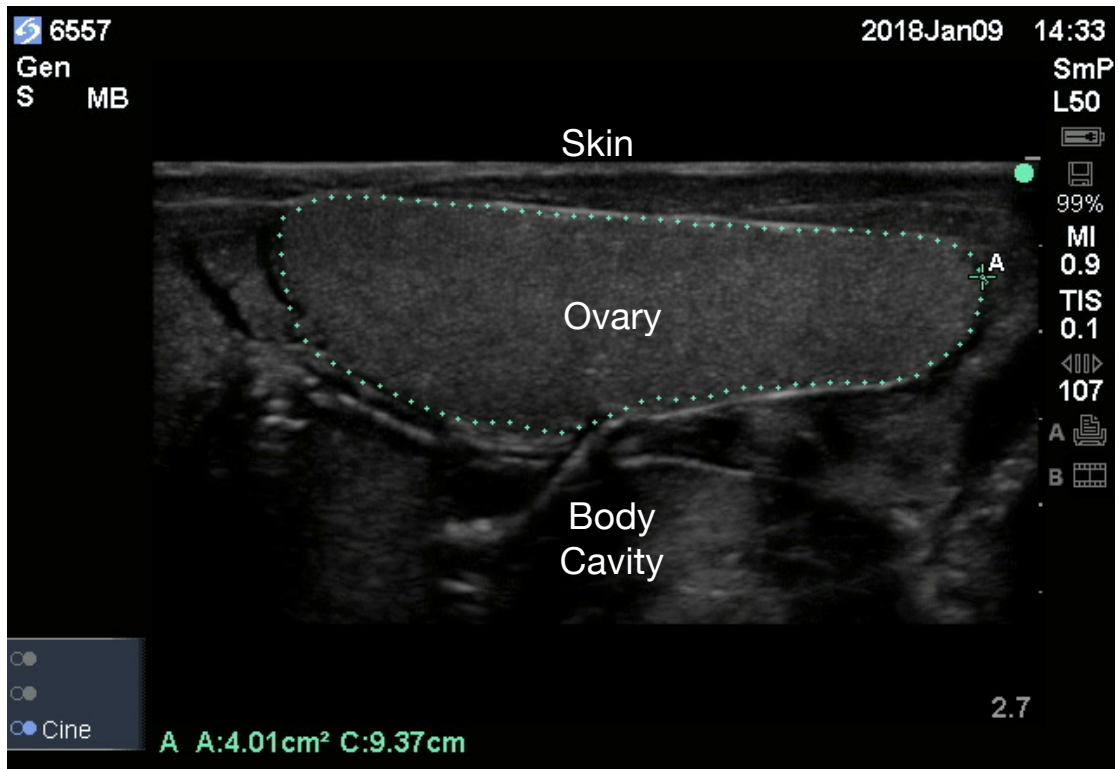


Figure 3.3. Sonogram showing the circumference of a late vitellogenic (Stage 6) ovary in January. The green dots represent the gonad circumference.

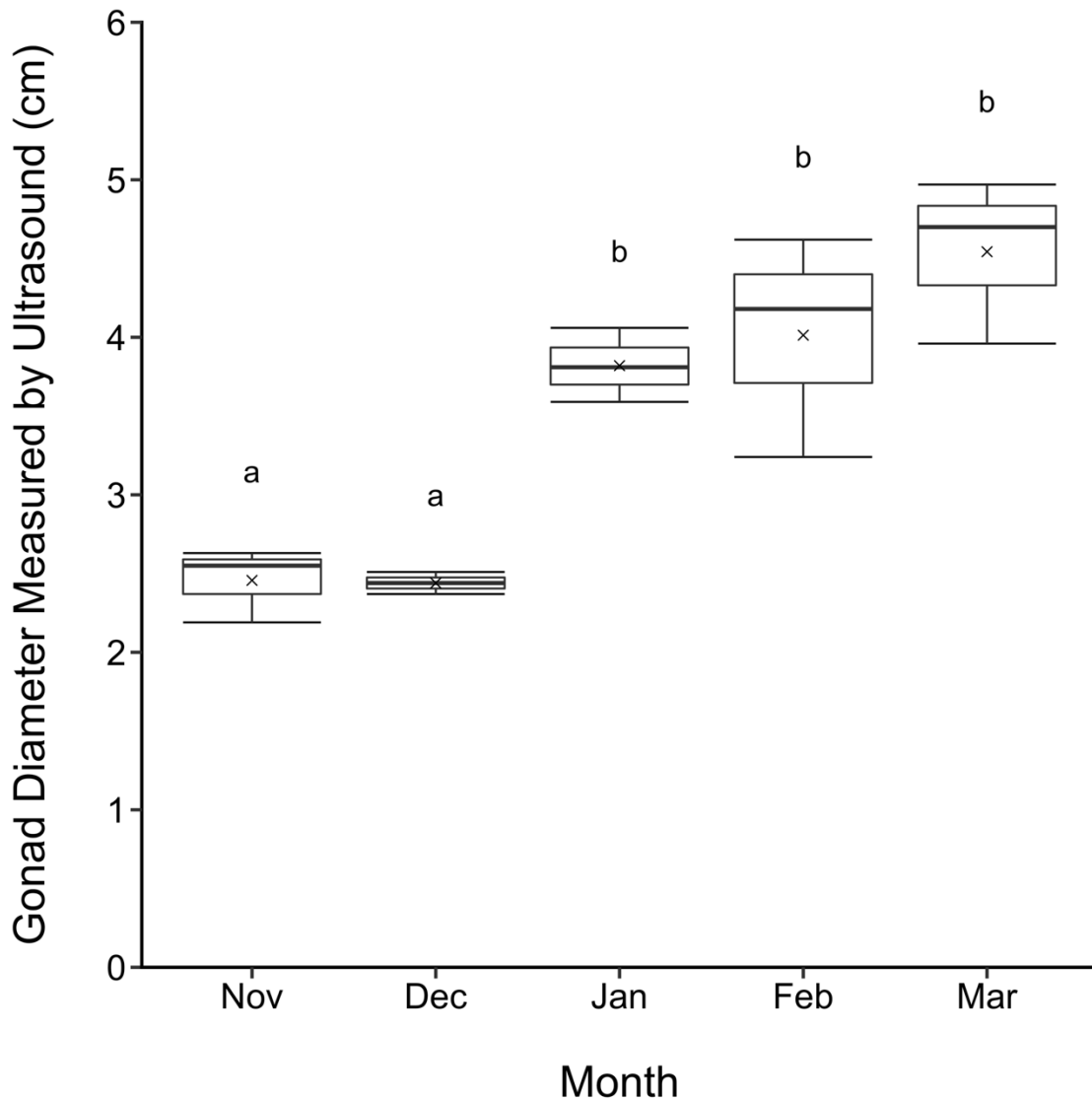


Figure 3.4. Gonad diameter measured by ultrasound (cm) of adult female Burbot by month. Burbot were held at the Bozeman Fish Technology Center and exposed to a natural photoperiod and thermal profile similar to that in Lake Roosevelt, Washington. The box encompasses data from the first to third quartiles (i.e., 25th to 75th percentiles). The horizontal line within a box represents the median. The cross represents the mean. The whiskers represent the minimum and maximum value. Different letters indicate significant differences among months.

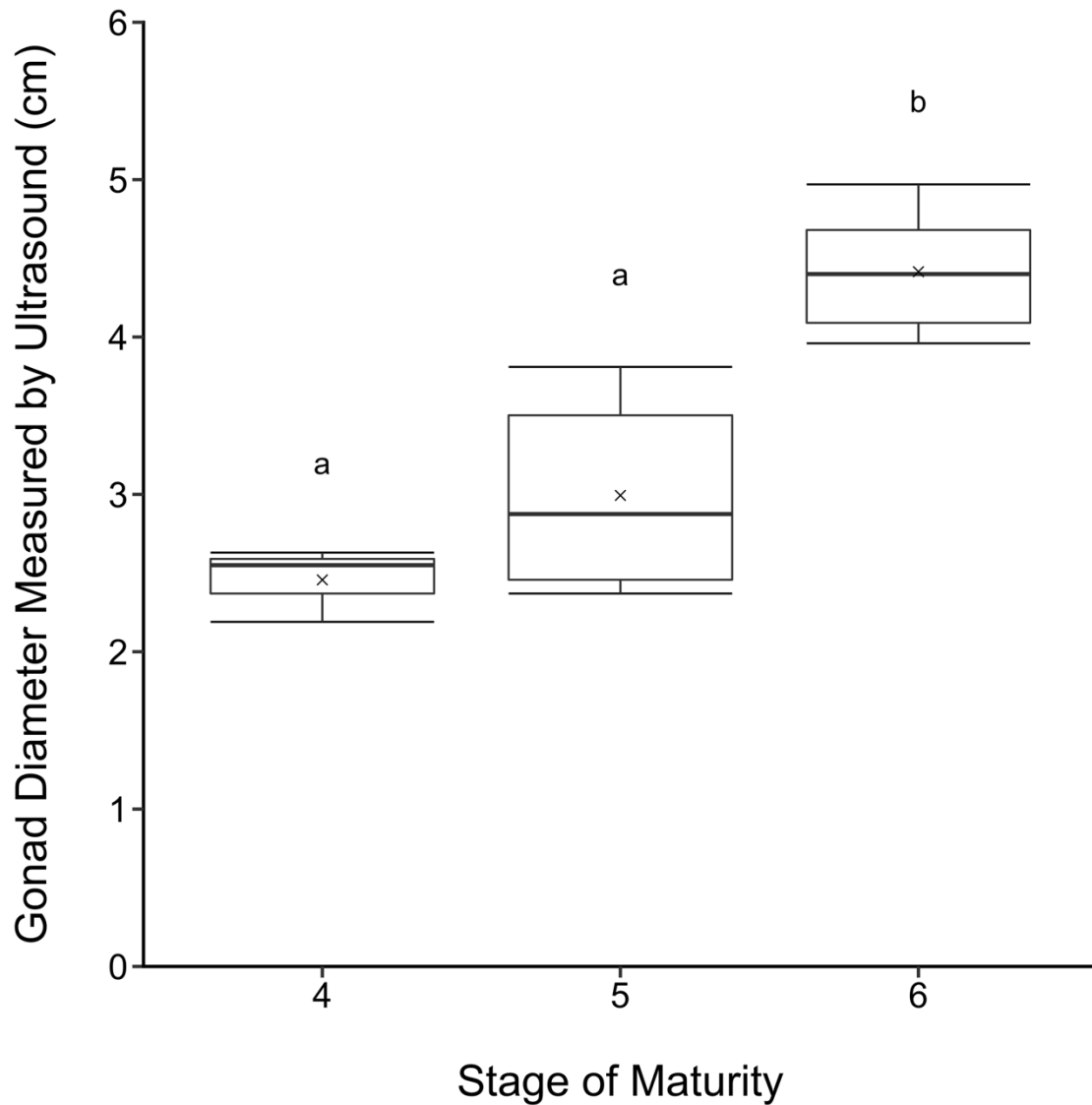


Figure 3.5. Gonad diameter measured by ultrasound (cm) of adult female Burbot by stage of maturity. Burbot were held at the Bozeman Fish Technology Center and exposed to a natural photoperiod and thermal profile similar to that in Lake Roosevelt, Washington. The box encompasses data from the first to third quartiles (i.e., 25th to 75th percentiles). The horizontal line within a box represents the median. The cross represents the mean. The whiskers represent the minimum and maximum value. Different letters indicate significant differences among months. Females were only observed during early vitellogenesis (Stage 4), mid-vitellogenesis (Stage 5), and late vitellogenesis (Stage 6).

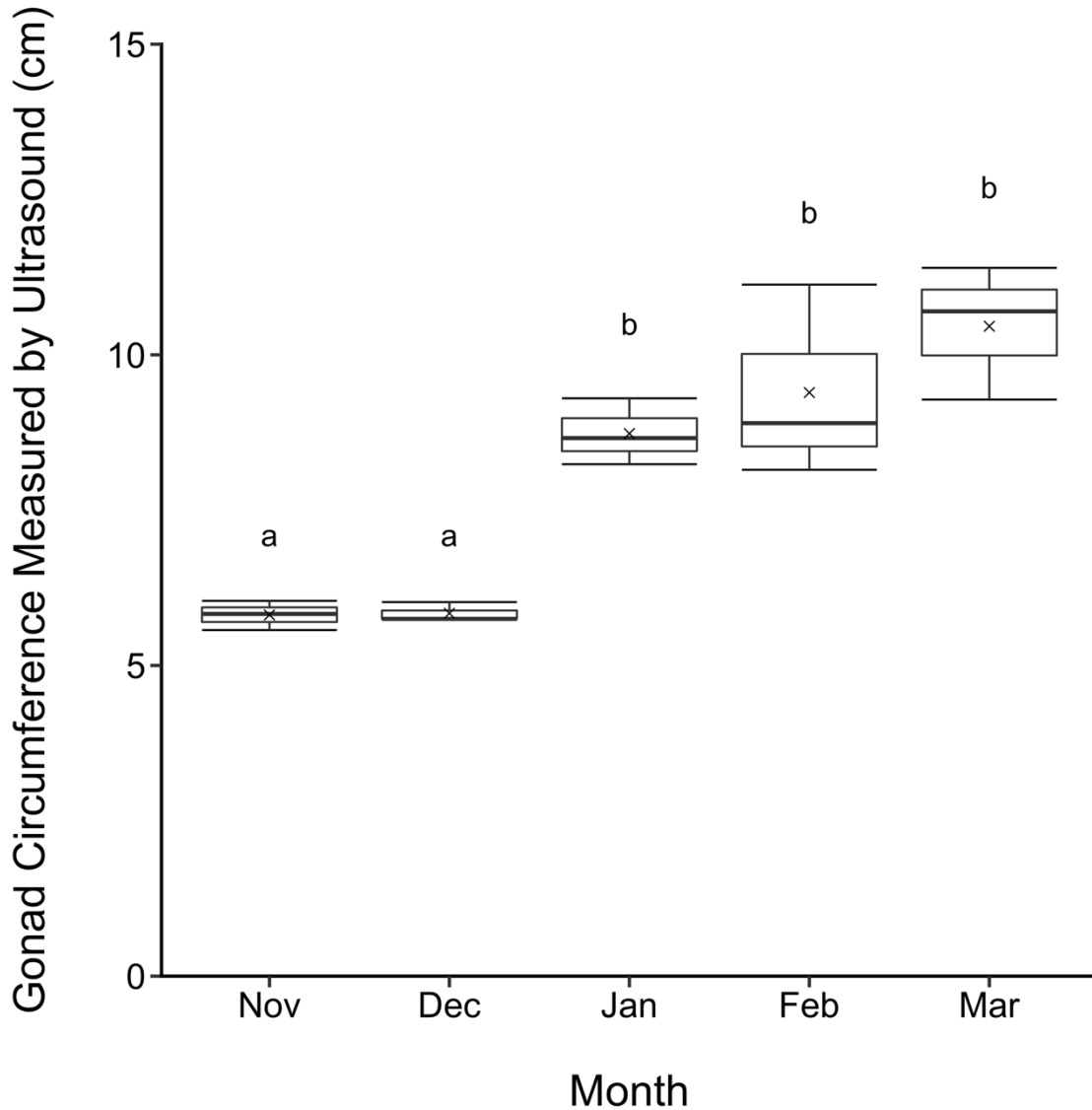


Figure 3.6. Gonad circumference measured by ultrasound (cm) of adult female by month. Burbot were held at the Bozeman Fish Technology Center and exposed to a natural photoperiod and thermal profile similar to that in Lake Roosevelt, Washington. The box encompasses data from the first to third quartiles (i.e., 25th to 75th percentiles). The horizontal line within a box represents the median. The cross represents the mean. The whiskers represent the minimum and maximum value. Different letters indicate significant differences among months.

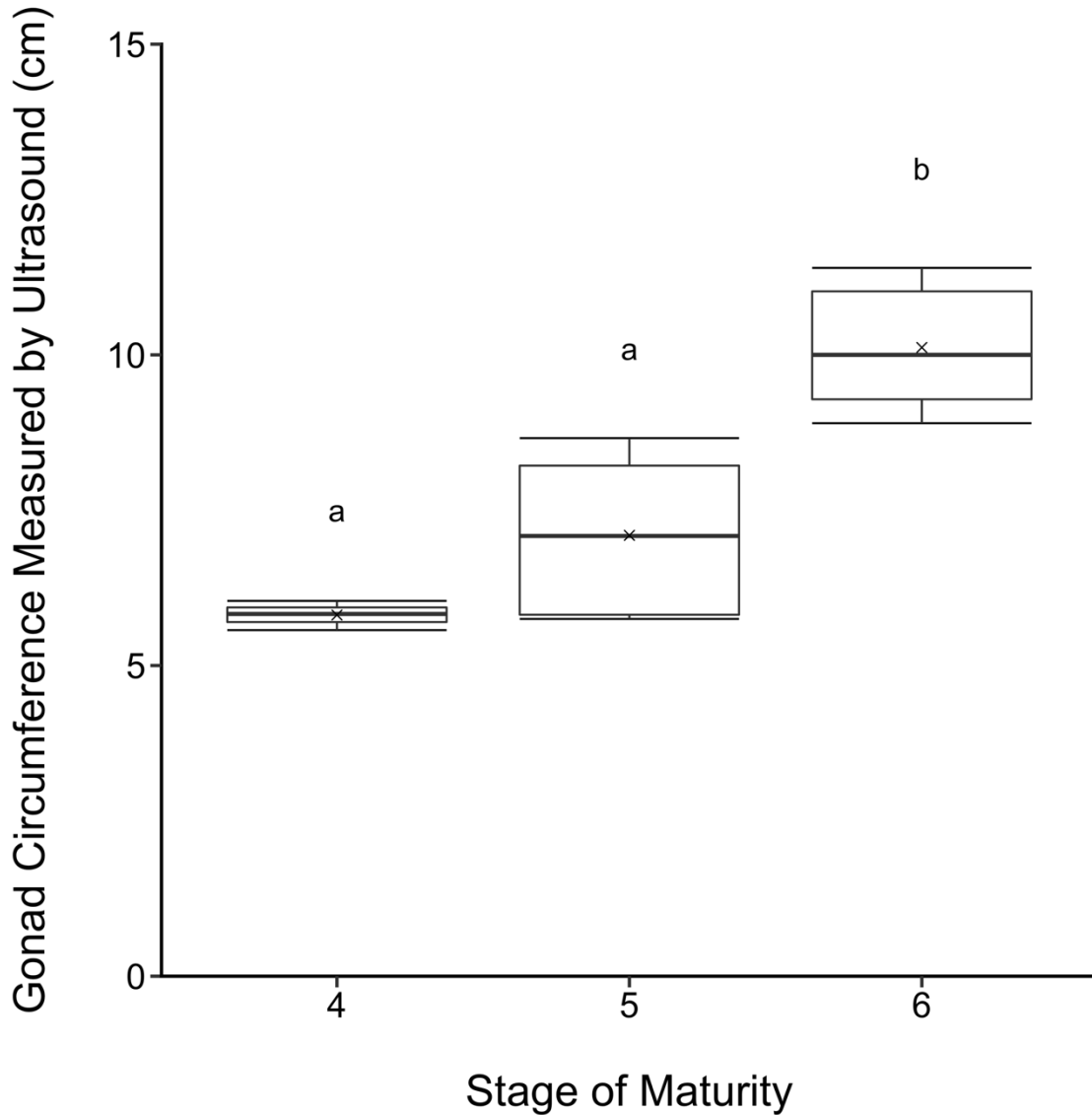


Figure 3.7. Gonad circumference measured by ultrasound (cm) of adult female Burbot by stage of maturity. Burbot were held at the Bozeman Fish Technology Center and exposed to a natural photoperiod and thermal profile similar to that in Lake Roosevelt, Washington. The box encompasses data from the first to third quartiles (i.e., 25th to 75th percentiles). The horizontal line within a box represents the median. The cross represents the mean. The whiskers represent the minimum and maximum value. Different letters indicate significant differences among months. Females were only observed during early vitellogenesis (Stage 4), mid-vitellogenesis (Stage 5), and late vitellogenesis (Stage 6).

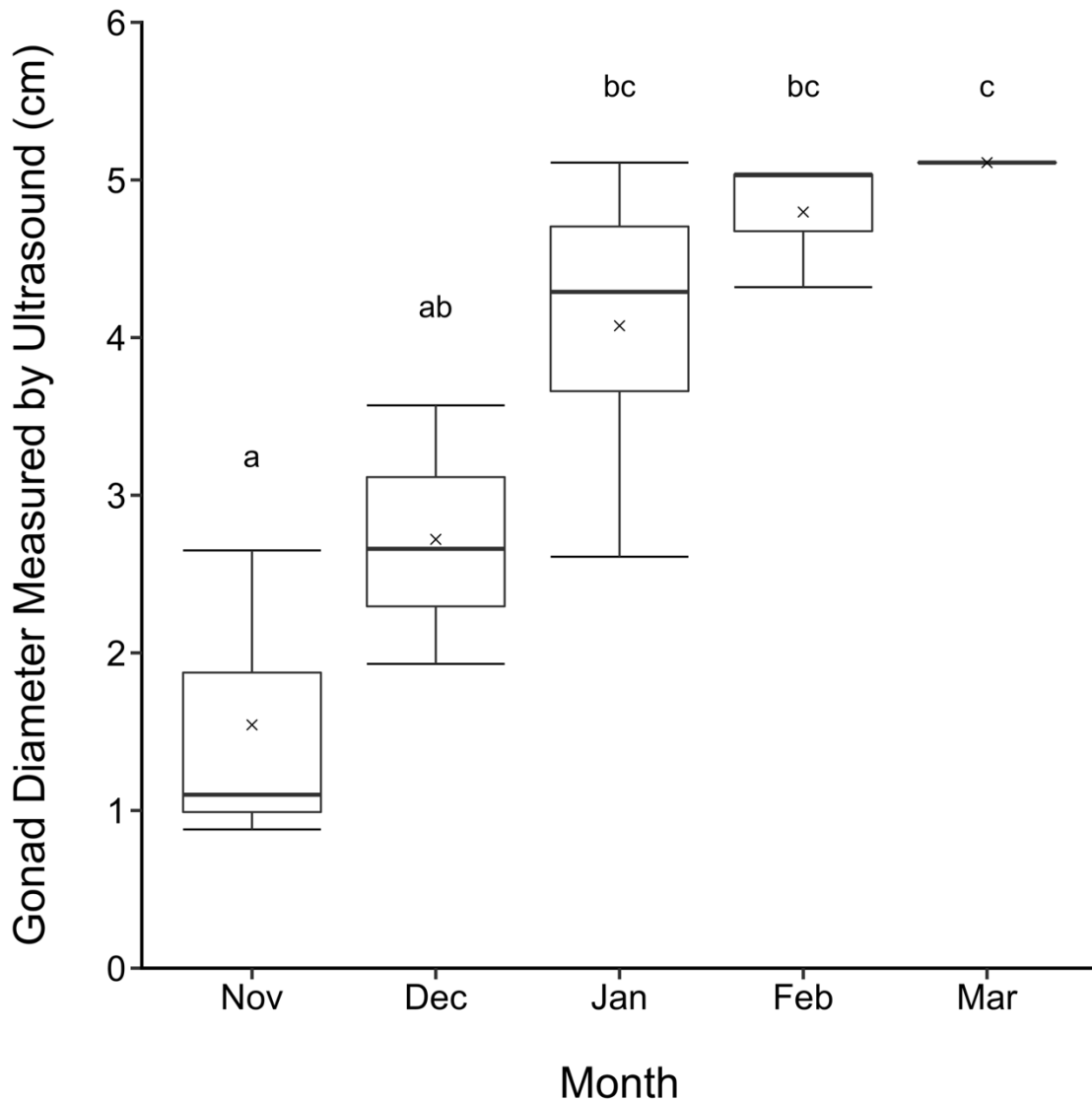


Figure 3.8. Gonad diameter measured by ultrasound (cm) of adult male Burbot by month. Burbot were held at the Bozeman Fish Technology Center and exposed to a natural photoperiod and thermal profile similar to that in Lake Roosevelt, Washington. The box encompasses data from the first to third quartiles (i.e., 25th to 75th percentiles). The horizontal line within a box represents the median. The cross represents the mean. The whiskers represent the minimum and maximum value. Different letters indicate significant differences among months. Gonad diameters greater than 5.11 cm (the width of the ultrasound transducer) could not be measured by ultrasound and were assigned a value of 5.11 cm. All gonad diameters in March were assigned a value of 5.11 cm.

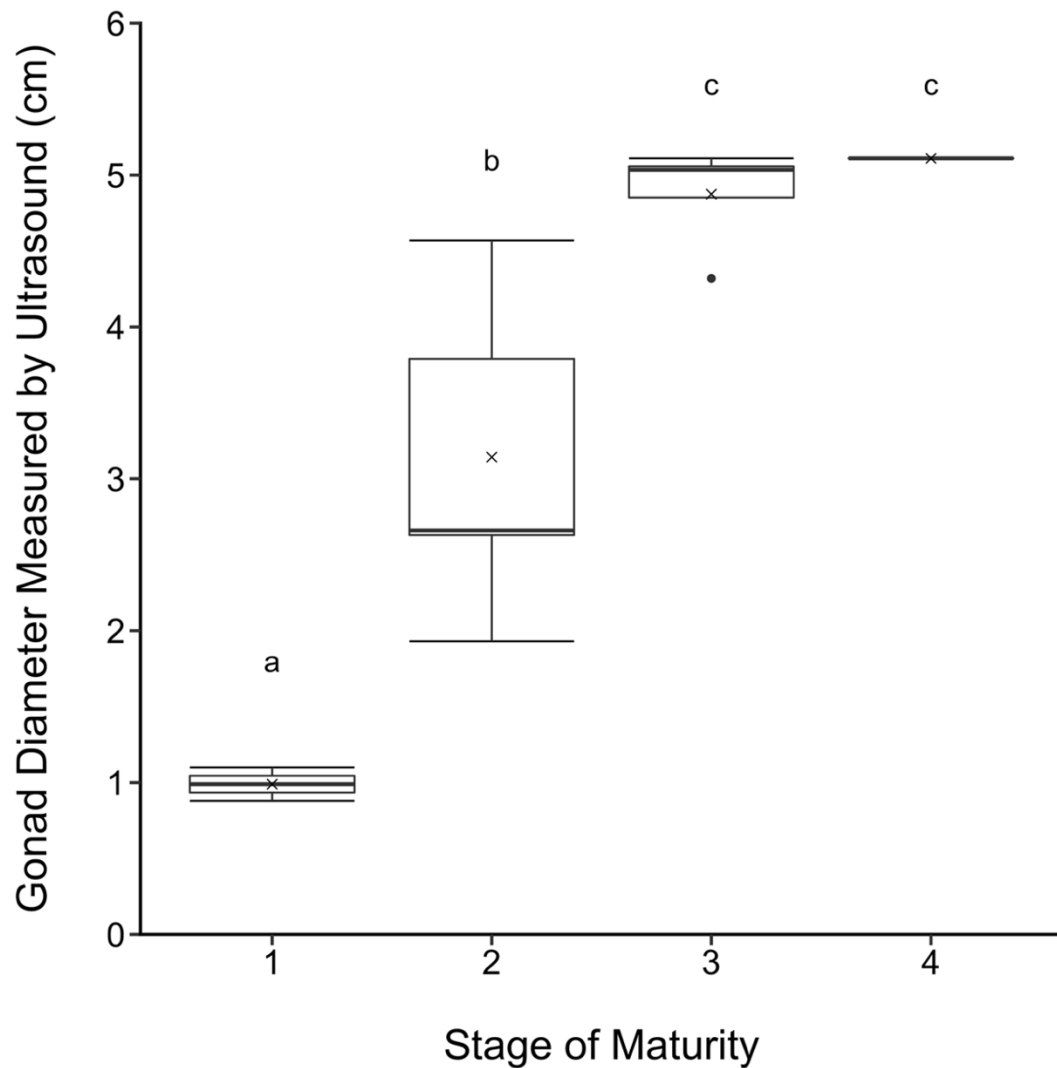


Figure 3.9. Gonad diameter (cm) of adult male Burbot by stage of maturity. Burbot were held at the Bozeman Fish Technology Center and exposed to a natural photoperiod and thermal profile similar to that in Lake Roosevelt, Washington. The box encompasses data from the first to third quartiles (i.e., 25th to 75th percentiles). The horizontal line within a box represents the median. The cross represents the mean. The whiskers represent the minimum and maximum value. Outliers are represented by individual points. If there are outliers, the whiskers represent the minimum value within 1.5 times the interquartile range below the first quartile or the maximum value within 1.5 times the interquartile range above the third quartile. Different letters indicate significant differences among months. Males were only observed during spermatogonial proliferation (Stage 1), early spermatogenesis (Stage 2), mid-spermatogenesis (Stage 3), and while ripe (Stage 4). Gonad diameters greater than 5.11 cm (the width of the ultrasound transducer) could not be measured by ultrasound and were assigned a value of 5.11 cm.

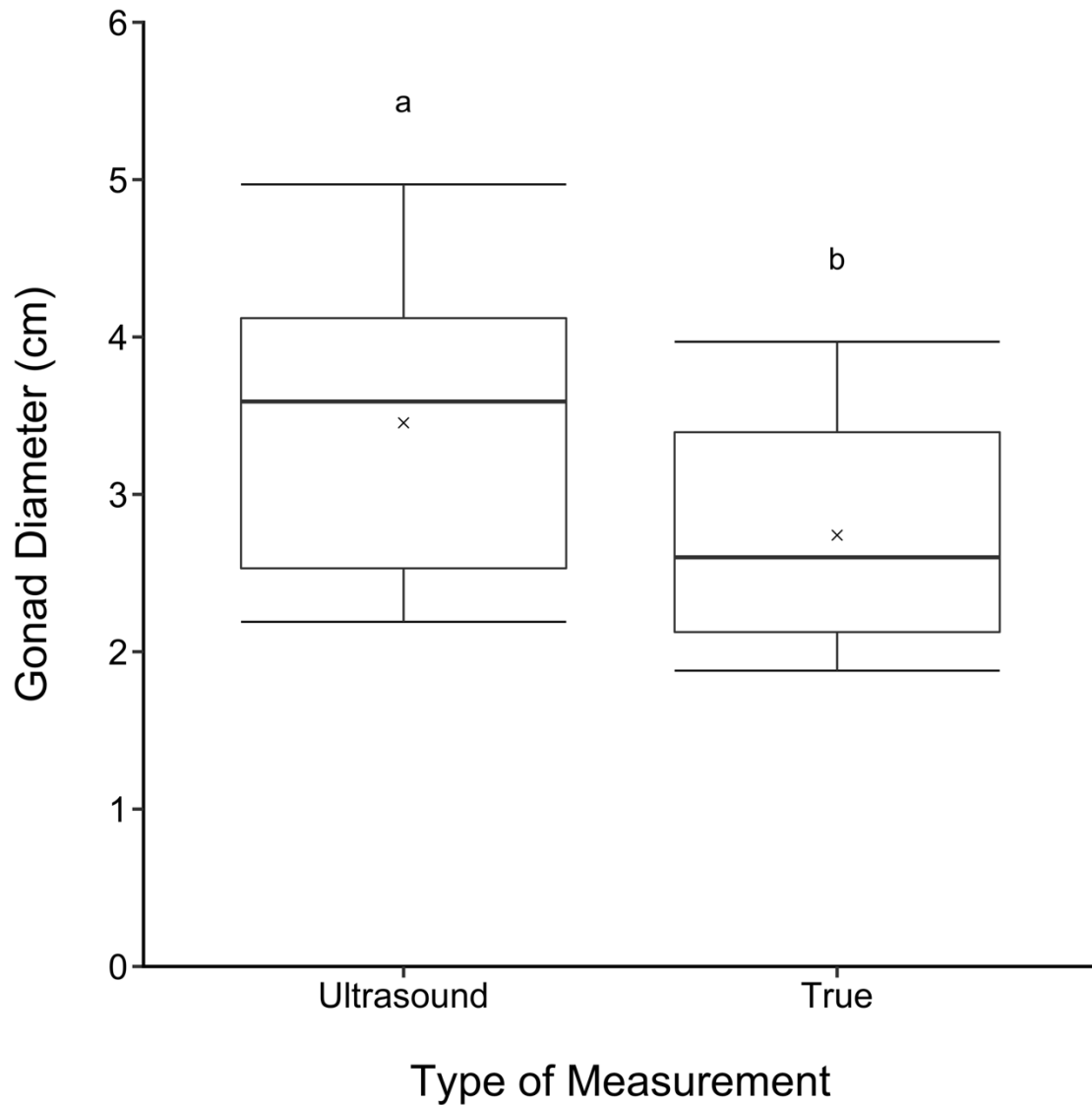


Figure 3.10. Gonad diameter measured by ultrasound (cm) and excised gonad diameter measured by calipers (cm) (i.e., true) of adult female Burbot. The box encompasses data from the first to third quartiles (i.e., 25th to 75th percentiles). The horizontal line within a box represents the median. The cross represents the mean. The whiskers represent the minimum and maximum value. Different letters indicate a significant difference between measurement methods.

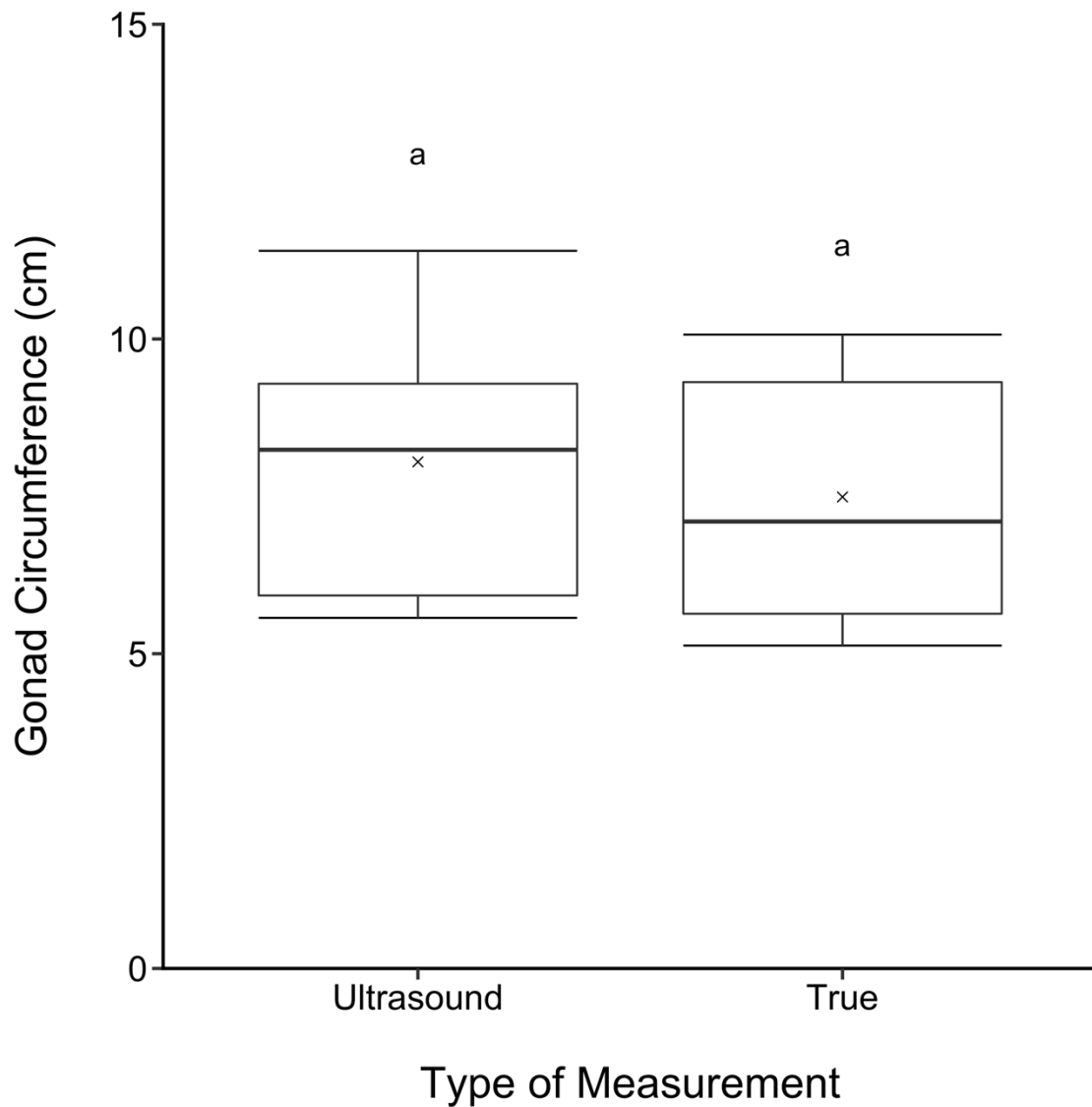


Figure 3.11. Gonad circumference measured by ultrasound (cm) and excised gonad circumference measured by a measuring tape (cm) (i.e., true) of adult female Burbot. The box encompasses data from the first to third quartiles (i.e., 25th to 75th percentiles). The horizontal line within a box represents the median. The cross represents the mean. The whiskers represent the minimum and maximum value. Similar letters indicate no significant difference between measurement methods.

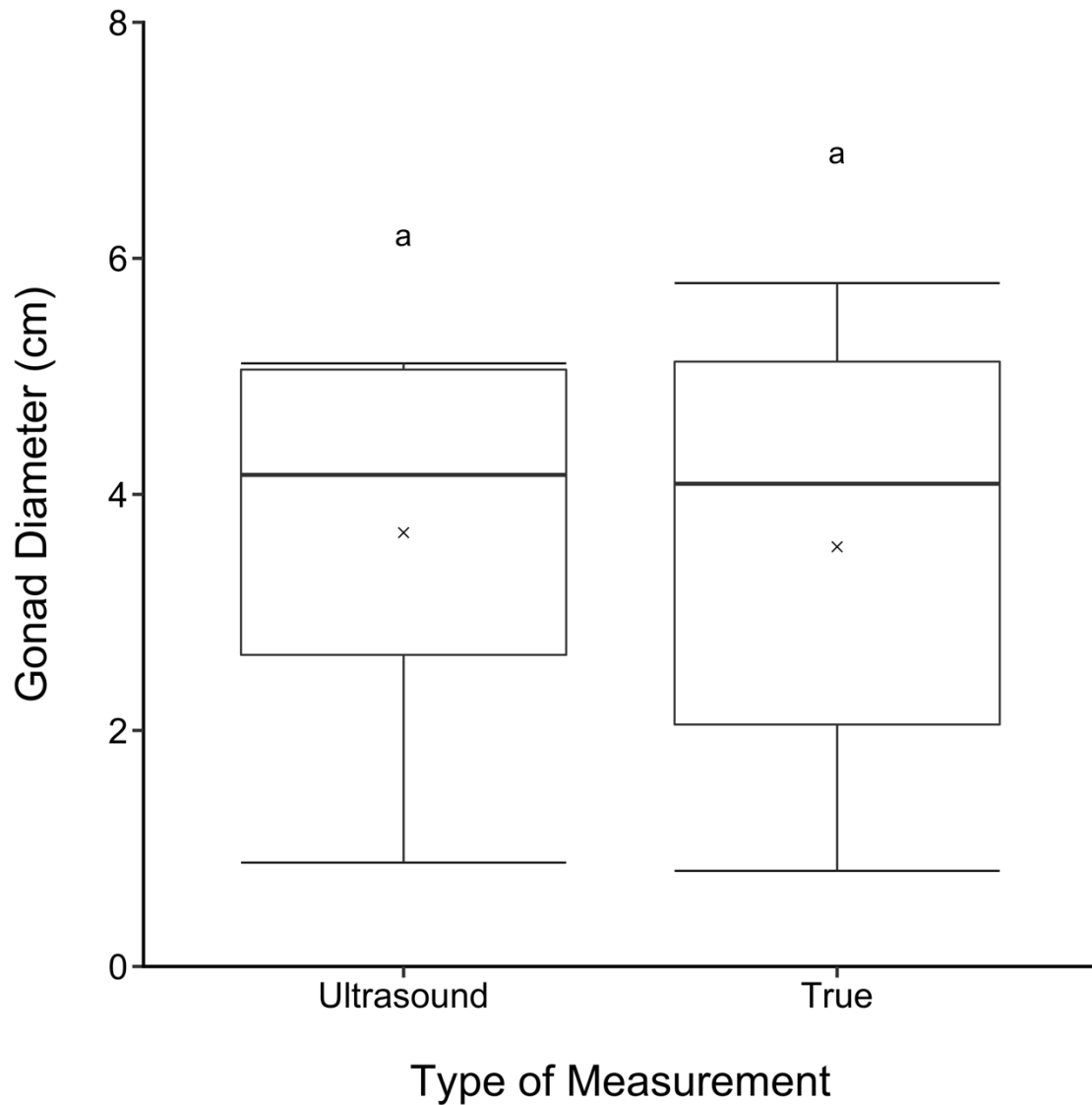


Figure 3.12. Gonad diameter measured by ultrasound (cm) and excised gonad diameter measured by calipers (cm) (i.e., true) of adult male Burbot. The box encompasses data from the first to third quartiles (i.e., 25th to 75th percentiles). The horizontal line within a box represents the median. The cross represents the mean. The whiskers represent the minimum and maximum value. Gonad diameters greater than 5.11 cm (the width of the ultrasound transducer) could not be measured by ultrasound and were assigned a value of 5.11 cm. Similar letters indicate no significant difference between measurement methods.

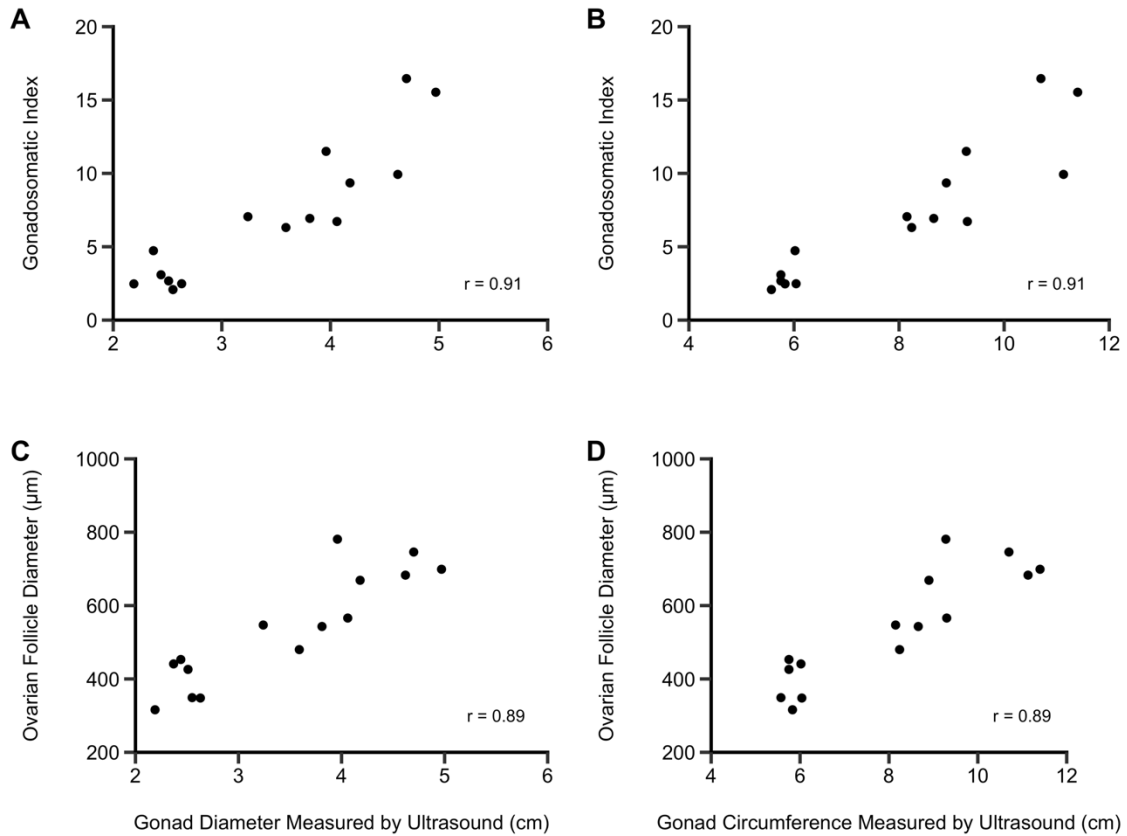


Figure 3.13. Correlations of gonad size measured by ultrasound and indices of gonadal development. **A.** Correlation ($r = 0.91$) between gonad diameter measured by ultrasound (cm) and gonadosomatic index (GSI) of adult female Burbot. **B.** Correlation ($r = 0.91$) between gonad circumference measured by ultrasound (cm) and gonadosomatic index (GSI) of adult female Burbot. **C.** Correlation ($r = 0.89$) between gonad diameter measured by ultrasound (cm) and ovarian follicle diameter (μm) of adult female Burbot. **D.** Correlation ($r = 0.89$) between gonad circumference measured by ultrasound (cm) and ovarian follicle diameter (μm) of adult female Burbot.

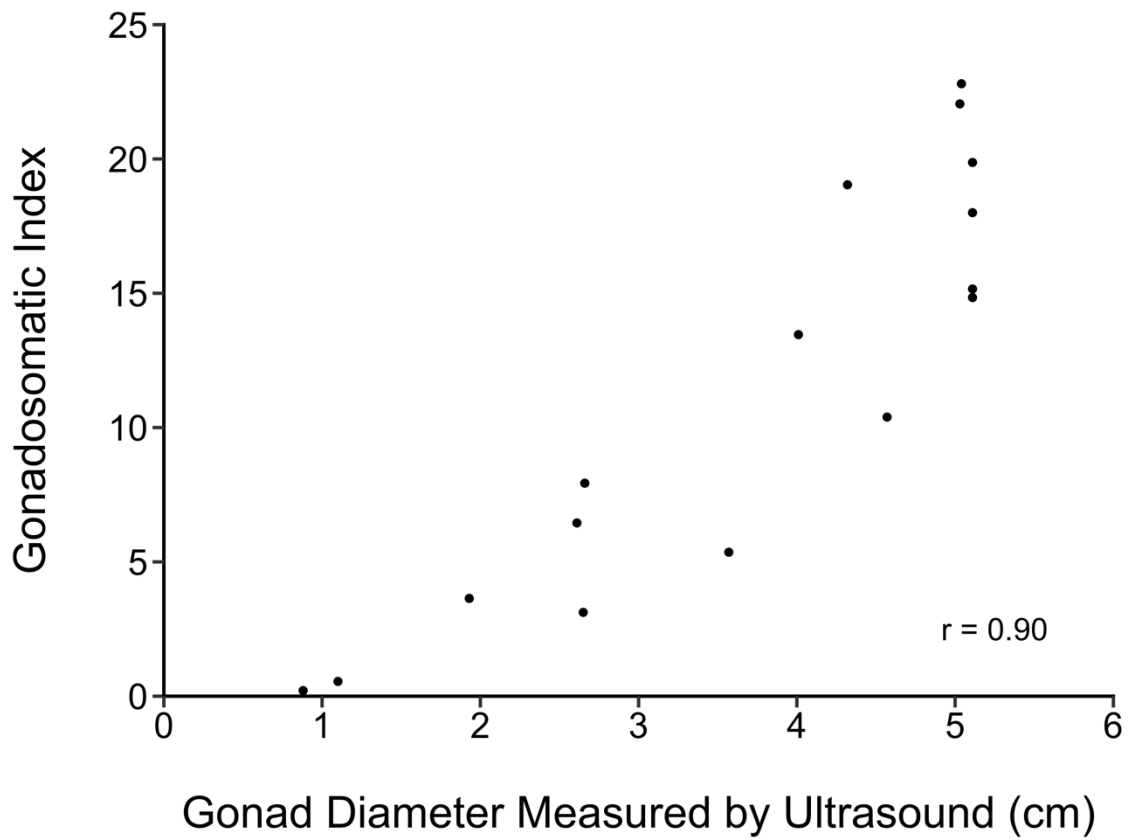


Figure 3.14. Correlation ($r = 0.90$) between gonad diameter measured by ultrasound (cm) and gonadosomatic index (GSI) of adult male Burbot.

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CHAPTER FOUR

THE ASSESSMENT OF NON-LETHAL TOOLS TO IDENTIFY
MASS OVARIAN FOLLICULAR ATRESIA
IN BURBOT *LOTA LOTA*

Contributions of Authors and Co-Authors

Manuscript in Chapter 4

Author: Lauren M. McGarvey

Contributions: Conceived and implemented the study design, collected and analyzed data, and authored the manuscript.

Co-Author: Molly A. H. Webb

Contributions: Obtained funding, conceived the study design, discussed the implications of the results, and co-authored the manuscript.

Co-Author: Christopher S. Guy

Contributions: Obtained funding, conceived the study design, discussed the implications of the results, and co-authored the manuscript.

Manuscript Information Page

Lauren M. McGarvey, Christopher S. Guy, Molly A. H. Webb

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ABSTRACT

Plasma sex steroid concentrations, gonad size measured by ultrasound, and ovarian follicle diameter were assessed as metrics to non-lethally identify mass ovarian follicular atresia (i.e., non-annual spawning) in Burbot *Lota lota* from Lake Roosevelt, Washington. Ovarian follicles, blood plasma, and gonadal sonograms were collected weekly from January 28, 2018 to March 25, 2018. Ovarian follicles were processed for histological analysis to confirm stage of maturity. Plasma sex steroid concentrations, testosterone (T) and estradiol-17 β (E2), were measured by radioimmunoassay. Gonad diameter was measured by ultrasound. Ovarian follicle diameter was measured by image analysis. Mean plasma T concentration decreased significantly ($F = 94.57$, $df = 1$, $P < 0.001$) from 8.94 ng/ml during late vitellogenesis (Stage 6) to 1.83 ng/ml during atresia (Stage 9) suggesting that plasma T concentrations may be used to identify mass ovarian follicular atresia. We recommend not using plasma E2 concentrations to identify mass ovarian follicular atresia because plasma E2 concentrations rapidly decreased during the completion of vitellogenesis or the initiation of atresia in teleosts; therefore, plasma E2 may not accurately identify mass ovarian follicular atresia. Mean diameter of atretic ovarian follicles decreased significantly during the last two weeks of the study suggesting that ovarian follicle diameter may be used to identify advanced mass ovarian follicular atresia. Non-lethal tools (plasma sex steroid concentrations and ultrasound) will enable fisheries biologists to assess indices of reproductive potential for the Burbot population in Lake Roosevelt. Indices of reproductive potential can be used characterize and monitor population demographics, improve models of population growth, establish sustainable harvest regulations, monitor the effects of management actions, and monitor the effects of environmental stressors.

Introduction

Ovarian follicular atresia is the breakdown and removal of ovarian follicles from the ovary to recover and recycle ovarian follicle materials (Grier et al. 2009; Wootton and Smith 2014). Ovarian follicular atresia is commonly observed in female teleosts and is thought to maintain ovarian homeostasis by controlling the number of developing ovarian follicles (Tyler and Sumpter 1996; Lubzens et al. 2010). However, increased levels of ovarian follicular atresia can be initiated by factors such as stress, fasting, environmental contaminants, light, temperature, confinement, or inadequate hormone levels (Miranda et al. 1999; Lubzens et al. 2010). Mass ovarian follicular atresia (i.e., the majority of ovarian follicles undergoing atresia) has been observed in female teleosts resulting in individuals omitting from spawning during an annual reproductive cycle (i.e., spawning non-annually) (Rideout et al. 2000; Rideout et al. 2005). One-third of Atlantic Cod *Gadus morhua*, a member of the same taxonomic order as Burbot, in Smith Sound, Newfoundland were undergoing mass ovarian follicular atresia and spawning non-annually (Rideout et al. 2000). Variation in spawning periodicity has also been observed in several Burbot *Lota lota* populations. Approximately 15% of adult female Burbot in the Tanana River, Alaska and 30% of adult female Burbot in the Bothnian Bay, Finland were predicted to spawn non-annually (Evenson 1990; Pulliainen and Korhonen 1990); however, it is not clear whether these females did not enter the reproductive phase or initiated mass ovarian follicular atresia. Variation in spawning periodicity affects the reproductive potential of a population by the direct loss of gametes (Rideout et al. 2000),

and the occurrence of mass ovarian follicular atresia in female Burbot in Lake Roosevelt, Washington is unknown.

Gonadal histology is the most accurate method to identify mass ovarian follicular atresia in fishes but requires sacrificing the fish (Rideout et al. 2005). Plasma sex steroid concentrations have been used to non-lethally identify mass ovarian follicular atresia in White Sturgeon *Acipenser transmontanus* (Talbot et al. 2011). Ovarian follicles undergoing atresia cease to synthesize sex steroids resulting in decreased plasma sex steroid concentrations (Webb et al. 2001; Linares-Casenave et al. 2002; Talbot et al. 2011) and suggesting that plasma sex steroid concentrations may be used to non-lethally identify mass ovarian follicular atresia in female Burbot. Additionally, ovarian follicles undergoing atresia decrease in size as yolk is phagocytized and resorbed (Miranda et al. 1999) suggesting that gonad size or ovarian follicle diameter may be used to identify mass ovarian follicular atresia in female Burbot. To our knowledge, plasma sex steroid concentrations, gonad diameter measured by ultrasound, and ovarian follicle diameter have never been assessed to identify mass ovarian follicular atresia in female Burbot.

The objective of this study was to assess plasma sex steroid concentrations, gonad size (diameter and circumference) measured by ultrasound, and ovarian follicle diameter as metrics to non-lethally identify mass ovarian follicular atresia in female Burbot. Using non-lethal tools to detect mass ovarian follicular atresia will enable fisheries biologists to assess indices of reproductive potential (e.g., reproductive structure and spawning periodicity) for the Burbot population in Lake Roosevelt. Indices of reproductive potential can be used to characterize and monitor population demographics, model

population growth, establish sustainable harvest regulations, monitor the effects of management actions, and monitor the effects of environmental stressors (Trippel 1993; Downs et al. 1997; Wildhaber et al. 2007; Power 2007; Morgan 2008; Schill et al. 2010).

Study Area

Lake Roosevelt is located in northeast Washington. The 209-km reservoir was formed after the construction of Grand Coulee Dam (GCD) on the Columbia River. The reservoir is typically 1-3 km wide with a maximum depth of 122 m extending 241 km upstream from GCD to the Canadian border (Polacek et al. 2006). At full pool elevation, the reservoir covers approximately 33,000 ha (Polacek et al. 2006). The reservoir supports many recreational fisheries including a Burbot fishery which may be underutilized and able to support greater harvest (CCT 2018). The reservoir is designated as a National Recreation Area by the National Park Service.

Methods

Fish Collection and Maintenance

Burbot were collected from Lake Roosevelt by the Confederated Tribes of the Colville Reservation in October 2017. Baited cod traps were set at 10 m to prevent barotrauma and pulled the following day. Each fish was tagged with a passive integrated transponder (PIT) and transferred to the Bozeman Fish Technology Center, Bozeman MT. Fish were maintained in tanks under a natural light cycle and fed live feed until satiation. Burbot spawn at temperatures of 1 - 4°C in February or March (McPhail and

Paragamian 2000); therefore, the water temperature was increased from 5 to 11°C during a three-week period prior to the spawning season to induce mass ovarian follicular atresia (Figure 4.1).

Biological Sampling

Ten females in the late stages of gametogenesis were repeatedly sampled each week from January 29, 2018 to March 25, 2018. Fish were anesthetized using MS-222 (tricaine methanesulfonate), and fresh water was continuously supplied to the gills during sampling. Biological data was collected from each individual and included the following: blood sample, gonadal sonogram, and ovarian follicle sample.

Histology

Ovarian follicles were collected by inserting a flexible catheter through the urogenital pore into the ovary and applying suction (Rottmann et al. 1991) (Figure 4.2). Ovarian follicles were preserved in 10% phosphate buffered formalin for histological analysis (Webb and Erickson 2007). Ovarian follicles were embedded in paraffin wax and sectioned at 5 µm. Ovarian follicles were stained with periodic acid-Schiff (PAS). Slides were examined under Leica DM 2000 compound scope (250 – 450X) (Leica Biosystems Inc., IL, USA). Ovarian follicles were scored for stage of maturity using our previous description of gametogenesis in female Burbot (see chapter two) (Table 4.1). Signs of atresia (i.e., thickening and deformed follicular layers) in more than 75% of the ovarian follicles observed histologically was required to classify fish as atretic (Stage 9) (see chapter two).

Plasma Sex Steroids

Blood was collected from the caudal vasculature using a heparinized syringe (3 cc with 22-gauge needle) (Webb et al. 2002). Plasma was separated by centrifugation (3400 rpm for 5 minutes) and stored at -80°C until analysis. Testosterone and E2 were extracted from the plasma following the methods of Fitzpatrick et al. 1987. Briefly, steroids from 100 µl of plasma were extracted twice with 2 ml of diethyl ether. Test tubes (12 x 75 mm) were vortexed vigorously with ether, and the aqueous phase was removed by snap-freezing in liquid nitrogen. Ether was evaporated overnight in a fume hood. Extracted steroids were resuspended in 1 ml of phosphate-buffered saline with gelatin (PBSG) the following day. Extraction recovery efficiencies for T and E2 were determined by adding tritiated steroids to tubes containing pooled plasma (n = 4), which were extracted as described above. Extraction recovery efficiencies were 87 – 91% for T and 80 – 87% for E2. All steroid concentrations were corrected for the extraction recovery efficiency.

Plasma T and E2 concentrations were quantified by radioimmunoassay (RIA) as described by Fitzpatrick et al. (1986) and modified by Feist et al. (1990). Extracted steroids (0.05 ml) were assayed in duplicates for T and E2. Briefly, antibody (0.1 ml) was diluted in PBSG and added to all assay tubes except total count and non-specific binding tubes. Tubes were incubated at room temperature for 20 minutes. Tritiated steroid (0.1 ml) was added to all tubes. Tubes were incubated at room temperature for 90 minutes and then incubated in an ice bath for five minutes. Dextran charcoal suspension (6.25 g charcoal and 4.0 g dextran/l PBSG) was added in the appropriate volume (1.0 ml

for T or 0.5 ml for E2). Tubes were incubated in an ice bath for 10 minutes and then centrifuged at 2200 g for 20 minutes. Aliquots from all tubes (0.5 ml) in scintillation cocktail (5 ml) were counted in a Beckman Coulter LS 6500 scintillation spectrophotometer (Beckman Coulter Inc., California, USA). Plasma sex steroid concentrations quantified by RIA were validated by verifying the serial dilutions were parallel to standard curves. Quantified plasma sex steroid concentrations below the minimum quantifiable concentration (MQC) of the RIA (i.e., the lowest concentration that was considered to be reliably quantified) were assigned the MQC (0.37 ng/mL for T and 0.14 – 0.19 ng/mL for E2). Non-detectable plasma sex steroid concentrations (i.e., not quantifiable) were assigned half of the MQC for statistical purposes (no concentrations were non-detectable for T and 0.07 ng/ml for E2) (Croghan and Egeghy 2003). The intra- and inter-assay coefficients of variation for all assays was less than 5 and 10%, respectively.

Ultrasound

A SonoSite Edge ultrasound (SonoSite Inc., Washington, USA) with a linear transducer (6-15 MHz) was used to measure gonad size in anesthetized fish. The Small Parts exam type was used with the optimization set to General. The ultrasound scanning depth was set between 2.2 and 2.8 cm. Fish were oriented ventrally, and the ultrasound transducer was placed on the abdomen to locate the right gonad. The right gonad was always measured for standardization. The gonad was scanned until the perceived maximum area was in view (Blythe et al. 1994; Evans et al. 2004). Gonad diameter (i.e., the maximum distance from the right to left edge) was measured using the caliper

function (see chapter three). A value of 5.11 cm (the width of the ultrasound transducer) was assigned if the gonad edges extended beyond the width of the ultrasound transducer (i.e., the gonad was too large to measure) (see chapter three). Circumference was also measured by ultrasound using the caliper function on manual trace (see chapter three). Circumference was not measured if the gonad was wider than the ultrasound transducer. All sonograms with measurements were archived.

Ovarian Follicle Diameter

Fifteen ovarian follicles (Johnson 1971) from each sample were measured by image analysis using SPOT Advanced Software (SPOT Imaging, Michigan, USA). The diameter of each ovarian follicle was measured twice to increase measurement precision. If the difference between the two diameter measurements was greater than 3 μm , the measurements were repeated. The largest diameter measurement was recorded.

Data Analyses

Data were assessed for normal distributions using side-by-side boxplots and Q-Q plots. Steroid (T and E2) data were non-normally distributed and normalized using the $\log_e$ transformation for analyses. Gonad diameter and ovarian follicle diameter data were normally distributed. Mixed effect models were used for mean comparisons (plasma sex steroid concentrations, gonad diameter, and ovarian follicle diameter) by week to account for repeated measures and followed by pairwise mean comparisons using a Bonferroni correction. Mixed effect models were used for mean comparisons (plasma sex steroid concentrations, gonad diameter, and ovarian follicle diameter) by stage of maturity to

account for repeated measures. The accepted significance level was $\alpha = 0.05$. All statistical analyses were completed using R software (version 3.3.2).

Results

Histology

All females were late vitellogenic (Stage 6) on January 28, the beginning of the study (Table 4.2). Females initiated atresia at varying times of the study, but all females were atretic (Stage 9) by March 19 (Table 4.2).

Plasma Sex Steroids

Plasma T concentrations varied from 0.35 to 23.47 ng/ml and differed significantly among weeks ($F = 16.49$, $df = 8$, $P = < 0.001$). Mean plasma T concentration increased from 10.46 ng/ml on January 28 to 13.91 ng/ml on February 5 and steadily decreased to 1.65 ng/ml on March 25 (Table 4.3). Plasma T concentrations differed significantly between stages of maturity ($F = 94.57$, $df = 1$, $P = < 0.001$). Mean plasma T concentration decreased from 10.30 ng/ml during late vitellogenesis (Stage 6) to 2.41 ng/ml during atresia (Stage 9) (Table 4.3).

Plasma E2 concentrations varied from non-detectable to 12.61 ng/ml and differed significantly among weeks ($F = 26.93$, $df = 8$, $P = < 0.001$). Mean plasma E2 concentration steadily decreased from 5.62 ng/ml on January 28 to 0.20 ng/ml on March 25 (Table 4.4). Plasma E2 concentrations differed significantly between stages of maturity ($F = 60.49$, $df = 1$, $P = < 0.001$). Mean plasma E2 concentration decreased from

3.34 ng/ml during late vitellogenesis (Stage 6) to 0.29 ng/ml during atresia (Stage 9) (Table 4.4).

Ultrasound

Gonad diameter measured by ultrasound varied from 2.55 to 5.11 cm (the maximum value capable of being measured by the ultrasound transducer) and differed significantly among weeks ($F = 2.27$; $df = 8$; $P = 0.035$). Mean female gonad diameter was 4.05 cm (3.66 – 4.45 cm; 95% CI) on January 28, remained elevated until March 6, and steadily decreased to 3.43 cm (2.87 – 3.98 cm; 95% CI) on March 25 (Figure 4.3). Gonad diameter measured by ultrasound differed significantly between stages of maturity ($F = 7.58$; $df = 1$; $P = 0.008$). Mean gonad diameter measured by ultrasound decreased from 4.05 cm (3.87 – 4.23 cm; 95% CI) during late vitellogenesis (Stage 6) to 3.65 cm (3.46 – 3.85 cm; 95% CI) during atresia (Stage 9) (Figure 4.3). Gonad circumference measured by ultrasound is not reported because some ovaries were too wide to measure.

Ovarian Follicle Diameter

Ovarian follicle diameter varied from 501 to 825 μm and differed significantly among weeks ($F = 12.66$, $df = 8$, $P = <0.001$). Mean ovarian follicle diameter increased from 648 μm (627 – 670 μm ; 95% CI) on January 28 to 750 μm (713 – 789 μm ; 95% CI) on March 5 and steadily decreased to 552 μm (490 – 614 μm ; 95% CI) on March 25 (Figure 4.4). Ovarian follicle diameter did not differ significantly between stages of maturity ($F = 1.73$, $df = 1$, $P = 0.19$). Mean ovarian follicle diameter decreased from 677

μm (665 – 690 μm ; 95% CI) during late vitellogenesis (Stage 6) to 655 μm (616 – 694 μm ; 95% CI) during atresia (Stage 9) (Figure 4.4).

Discussion

We have demonstrated that plasma sex steroid concentrations are a promising tool to non-lethally identify mass ovarian follicular atresia in Burbot. Research regarding plasma sex steroid concentrations and ovarian follicular atresia has focused on commercially raised sturgeon species (e.g., Webb et al. 2001; Linares-Casenave et al. 2002; Talbott et al. 2011) in which yield and quality of caviar are negatively affected by ovarian follicular atresia (Talbott et al. 2011). In White Sturgeon, plasma T concentrations were used to identify mass ovarian follicular atresia (Talbott et al. 2011). Plasma E2 concentrations were not recommended to identify mass ovarian follicular atresia because plasma E2 concentrations were non-detectable in both maturing and atretic females (Talbott et al. 2011). We observed a similar trend of both late vitellogenic (Stage 6) and atretic (Stage 9) female Burbot having very low plasma E2 concentrations. Plasma E2 concentrations rapidly decrease once vitellogenesis is complete in female teleosts (Scott et al. 1980; Fitzpatrick et al. 1986; Kime 1993; Kagawa 2013); therefore, plasma E2 concentrations may decrease due to the completion of vitellogenesis or the initiation of atresia. We suggest plasma T concentrations be used to identify mass ovarian follicular atresia in female Burbot because plasma T concentrations remain elevated after vitellogenesis is complete (see chapter two), enabling a decrease in plasma T concentrations to be a reliable indicator of mass ovarian follicular atresia. Identifying

mass ovarian follicular atresia in Burbot is important because females spawning non-annually will affect the reproductive potential of the population and can be indication of a non-optimal environmental conditions (Rideout et al. 2005; Linares-Casenave et al. 2002; Lubzens et al. 2010; Talbott et al. 2011; Webb and Doroshov 2011)

Time is an important consideration when using plasma T concentrations to identify atretic (Stage 9) females. Decreased plasma T concentrations can be used to identify atretic (Stage 9) females, but post-ovulatory (Stage 8) females also have decreased plasma T concentrations (see chapter two). Collecting plasma samples before spawning will prevent the misidentification of post-ovulatory (Stage 8) females as atretic (Stage 9) females when using plasma T concentrations to identify mass ovarian follicular atresia.

The plasma sex steroid concentrations (T and E2) in late vitellogenic (Stage 6) female Burbot in this study were lower than plasma sex steroid concentrations previously observed in late vitellogenic (Stage 6) female Burbot (see chapter 2). In our previous description of gametogenesis in Burbot, we observed late vitellogenic (Stage 6) females with a median plasma T concentration of 25.99 ng/ml and a median plasma E2 concentration of 6.50 ng/ml (see chapter two). In the current study, we observed late vitellogenic (Stage 6) females with a median plasma T concentration of 9.29 ng/ml and a median plasma E2 concentration of 2.61 ng/ml (medians are not reported in the this chapter but were calculated to make a comparison to previous data). Plasma sex steroid concentrations observed in the current study were likely lower than plasma sex steroid concentrations observed previously because females had initiated atresia (i.e.,

approximately 20% of ovarian follicles exhibited signs of atresia). Plasma sex steroid concentrations have been documented to decrease when histological signs of atresia were evident in White Sturgeon (Webb et al. 2001; Linares-Casenave et al. 2002; Talbott et al. 2011). More than 50% of females had initiated atresia by February 19th, but our classification of atresia (Stage 9) required more than 75% of ovarian follicles to be exhibiting signs of atresia. Therefore, females initiating atresia and decreasing plasma sex steroid production were classified as late vitellogenic (Stage 6), accounting for the lower plasma sex steroid concentrations observed in late vitellogenic (Stage 6) females.

Ovarian follicular atresia is typically associated with decreased fish condition (Rideout et al. 2000; Kraus et al. 2008; Skjaeraasen et al. 2009); however, few studies have described how gonad size and ovarian follicle diameter change during mass ovarian follicular atresia. Gonadosomatic index (GSI), which is directly related to gonad size, and ovarian follicle diameter during mass ovarian follicular atresia have been described in Piau *Leporinus reinhardti*, a total spawning teleost like Burbot (Miranda et al. 1999). Both GSI and ovarian follicle diameter decreased during mass ovarian follicular atresia, but the complete absorption of ovarian follicles occurred over a four-month period. The present study occurred over a nine-week period until the initial signs of mass ovarian follicular atresia were observed in all fish. Gonad diameter measured by ultrasound differed significantly between late vitellogenic (Stage 6) and atretic (Stage 9) females; however, we only observed a 0.40 cm decrease in mean gonad diameter measured by ultrasound which we do not consider biologically significant. We only observed a 22 μ m decrease in mean ovarian follicle diameter between late vitellogenic (Stage 6) and atretic

(Stage 9) females because all stages of ovarian follicular atresia (i.e., initial to advanced) were included in our study. A more marked decrease in gonad size and ovarian follicle diameter would likely have been observed in females undergoing advanced ovarian follicular atresia, when yolk is phagocytized and resorbed (Miranda et al. 1999). The distributions of gonad diameter measured by ultrasound and ovarian follicle diameter overlapped between late vitellogenic (Stage 6) and atretic (Stage 9) females indicating both tools may misidentify atretic (Stage 9) females. However, ovarian follicle diameter differed significantly during the last two weeks of the study, suggesting ovarian follicle diameter may be used to identify advanced ovarian follicular atresia in female Burbot.

Plasma sex steroid concentrations and ovarian follicle diameter are promising non-lethal tools to identify mass ovarian follicular atresia in female Burbot. Non-lethal tools to identify mass ovarian follicular atresia will enable fisheries biologists to non-lethally assess reproductive indices such as reproductive structure and spawning periodicity. Reproductive indices are used in fisheries science and management to characterize and monitor population demographics, model population growth, establish sustainable harvest regulations, monitor the effects of management actions, and monitor the effects of environmental stressors (Trippel 1993; Downs et al. 1997; Wildhaber et al. 2007; Power 2007; Morgan 2008; Schill et al. 2010). By using the tools described, fisheries biologists can non-lethally assess reproductive indices of the Burbot population in Lake Roosevelt to inform and guide management decisions.

Table 4.1. Description of gonadal stages of maturity in female Burbot assigned by histological analysis of ovarian follicles.

Sex	Stage of Maturity	Description
Females	Oogonial Proliferation (1)	Oogonia and potentially a few primary growth oocytes
	Primary Growth (2)	Primary growth oocytes and oogonia
	Cortical Alveolar (3)	Cortical alveoli on periphery of ovarian follicles
	Early Vitellogenic (4)	Yolk granules accumulating in periphery of ovarian follicles, cortical alveoli on periphery of ovarian follicles, one layer of zona radiata
	Mid-Vitellogenic (5)	Yolk globules with a mean diameter of 7.93 μ m (5.97 – 9.89 μ m; 95% CI) accumulating towards center or may be present throughout ovarian follicles, few cortical alveoli on periphery of ovarian follicles, two layers of the zona radiata
	Late Vitellogenic (6)	Yolk fusing into larger globules with a mean diameter of 19.62 μ m (14.55 – 24.69 μ m; 95% CI) throughout ovarian follicle, two layers of zona radiata, central germinal vesicle (i.e., nucleus)
	Ripe (7)	Yolk fused but not completely coalesced in all ovarian follicles, offset germinal vesicle
	Post-Ovulatory (8)	Post-ovulatory follicles present with primary growth oocytes
	Atretic (9)	> 75% atretic ovarian follicles, no post-ovulatory follicles present with primary growth oocytes

Table 4.2. Number of females in each stage of maturity determined by gonadal histology during each week. *Ovarian follicles could not be histologically analyzed from two females on February 19 and one female on March 19. Blood could not be collected from one female on February 26. Plasma could not be analyzed from one female on March 12. Two females died before sampling on March 5 and March 19.

Week	Late Vitellogenic (Stage 6)	Atretic (Stage 9)	Total by Week
Jan 28	10		10
Feb 5	10		10
Feb 12	10		10
Feb 19	8		8*
Feb 26	6	3	9*
Mar 5	1	7	8*
Mar 12	1	6	7*
Mar 19		5	5*
Mar 25		6	6
Total by Stage	46	27	

Table 4.3. Plasma testosterone (T) concentrations (ng/ml) in female Burbot exposed to increasing water temperatures prior to the spawning season to initiate mass ovarian follicular atresia by stage of maturity and week. Data were collected weekly from January 28 to March 25, 2018. Females were observed in the late vitellogenic (Stage 6) and atretic (Stage 9) stages of maturity. Data are means (95% CI) and sample sizes (n). Different letters indicate significant differences in mean plasma T concentrations by week and stage of maturity.

Week	Late Vitellogenic (Stage 6)	Atretic (Stage 9)	Mean by Week
Jan 28	10.46 (8.25 – 12.66) n = 10		10.46 (8.25 – 12.66) ^{ab} n = 10
Feb 5	13.91 (9.94– 17.89) n = 10		13.91 (9.94 – 17.89) ^a n = 10
Feb 12	11.48 (7.72 – 15.24) n = 10		11.48 (7.72 – 15.24) ^a n = 10
Feb 19	7.93 (3.72 – 12.13) n = 8		7.93 (3.72 – 12.13) ^{abc} n = 8
Feb 26	6.46 (2.82 – 10.10) n = 6	2.84 (0.01 – 7.53) n = 3	5.25 (2.62 – 7.88) ^{bcd} n = 9
Mar 5	6.39 n = 1	2.87 (1.83 – 3.91) n = 7	3.31 (1.95 – 4.67) ^{cde} n = 8
Mar 12	6.99 n = 1	2.40 (0.01 – 4.88) n = 6	3.06 (0.50 – 5.62) ^{de} n = 7
Mar 19		2.44 (0.61 – 4.27) n = 5	2.44 (0.61 – 4.27) ^{de} n = 5
Mar 25		1.65 (0.30 – 2.99) n = 6	1.65 (0.30 – 2.99) ^e n = 6
Mean by Stage	10.30 (8.80 – 11.81) ^a n = 46	2.41 (1.78 – 3.04) ^b n = 27	

Table 4.4. Plasma estradiol (E2) concentrations (ng/ml) in female Burbot exposed to increasing water temperatures prior to spawning season to initiate mass ovarian follicular atresia by stage of maturity and week. Data were collected weekly from January 28, 2018 to March 25, 2018. Females were observed in the late vitellogenic (Stage 6) and atretic (Stage 9) stages of maturity. Data are means (95% CI) and sample sizes (n). Different letters indicate significant differences in mean plasma E2 concentration by week and stage of maturity.

Week	Late Vitellogenic (Stage 6)	Atretic (Stage 9)	Mean by Week
Jan 28	5.62 (4.46 – 6.78) n = 10		5.62 (4.46 – 6.78) ^a n = 10
Feb 5	5.84 (3.44 – 8.24) n = 10		5.84 (3.44 – 8.24) ^a n = 10
Feb 12	2.52 (1.22 – 3.82) n = 10		2.52 (1.22 – 3.82) ^a n = 10
Feb 19	0.71 (0.23 – 1.19) n = 8		0.71 (0.23 – 1.19) ^b n = 8
Feb 26	0.81 (0.03 – 1.59) n = 6	0.43 (0.01 – 2.00) n = 3	0.68 (0.15 – 1.22) ^b n = 9
Mar 5	2.06 n = 1	0.43 (0.16 – 0.70) n = 7	0.63 (0.10 – 1.17) ^b n = 8
Mar 12	1.48 n = 1	0.17 (0.01 – 0.35) n = 6	0.36 (0.01 – 0.84) ^b n = 7
Mar 19		0.29 (0.01 – 0.72) n = 5	0.29 (0.01 – 0.72) ^b n = 5
Mar 25		0.20 (0.01 – 0.41) n = 6	0.20 (0.01 – 0.41) ^b n = 6
Mean by Stage	3.34 (2.48 – 4.21) ^a n = 46	0.29 (0.17 – 0.42) ^b n = 27	

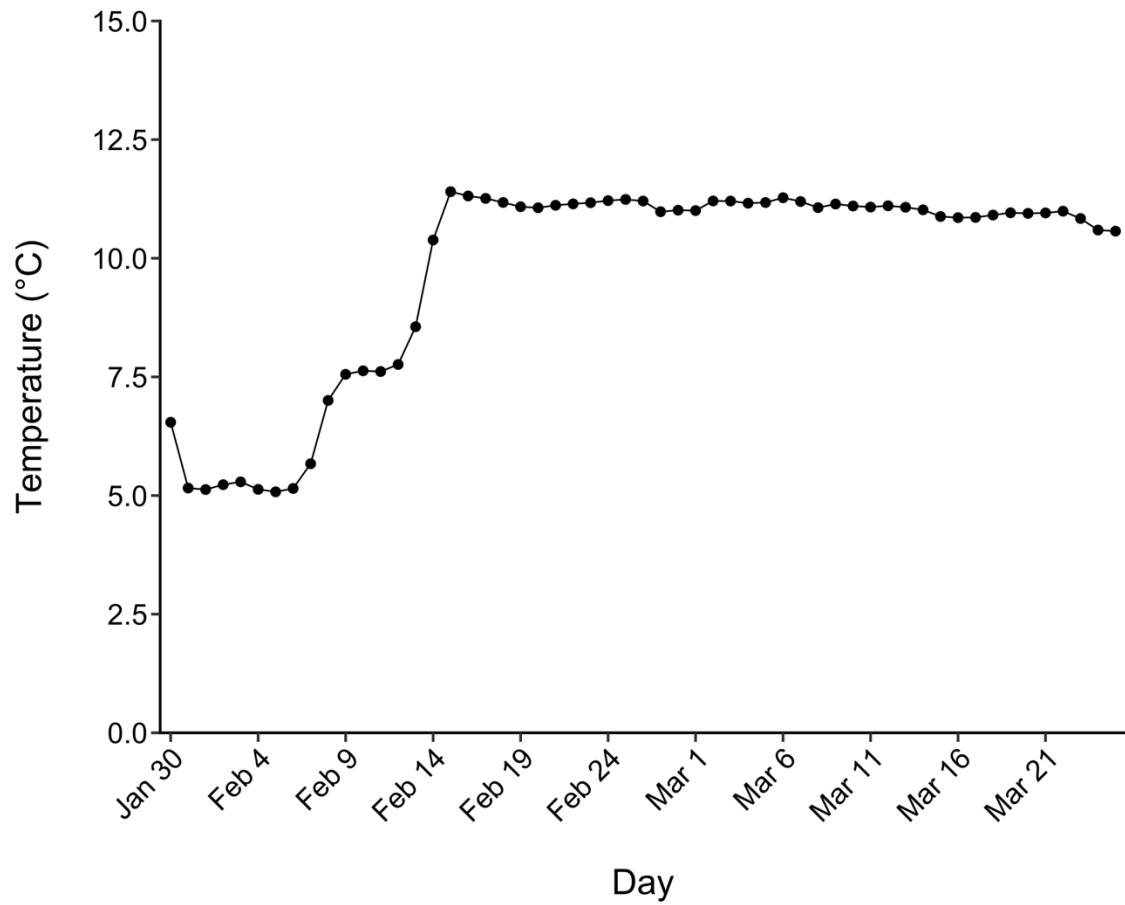


Figure 4.1. Thermal profile on which adult female Burbot were maintained at the Bozeman Fish Technology Center. Water temperature was increased from 5°C to 10°C during a three-week period prior to the spawning season to initiate mass ovarian follicular atresia.

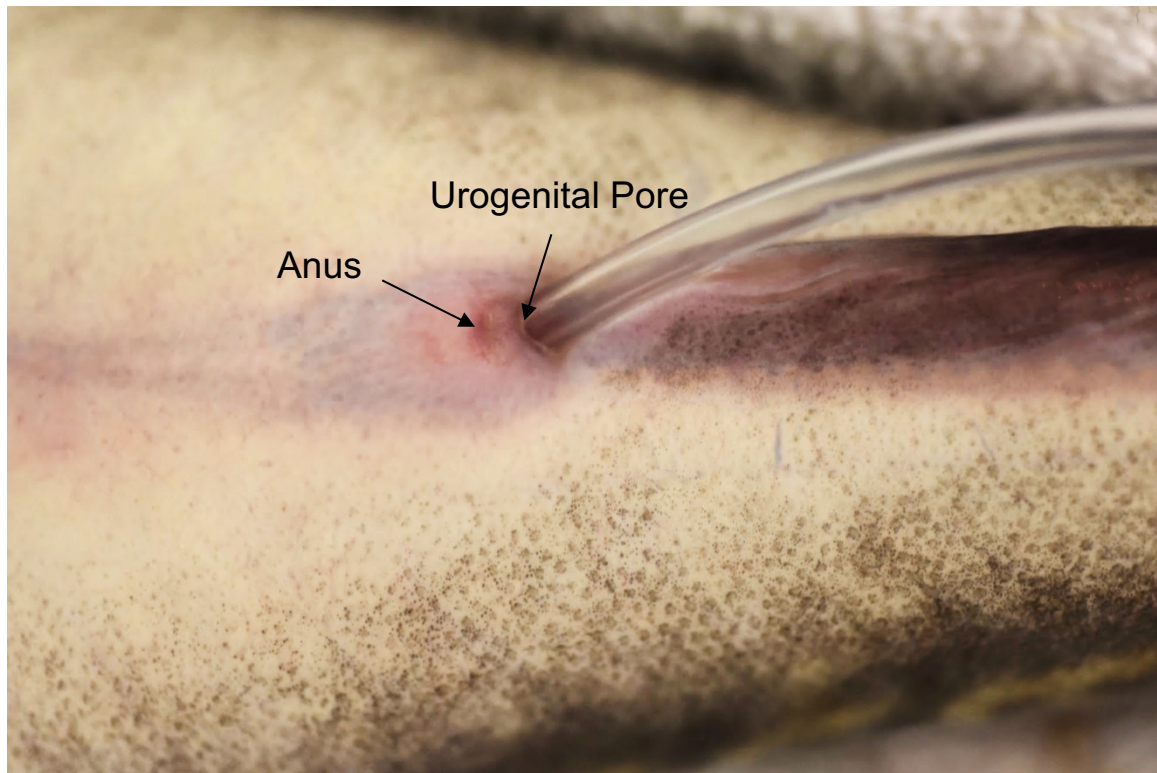


Figure 4.2. Collection of ovarian follicles by the insertion of a flexible catheter through the urogenital pore into the ovary of an adult female Burbot.

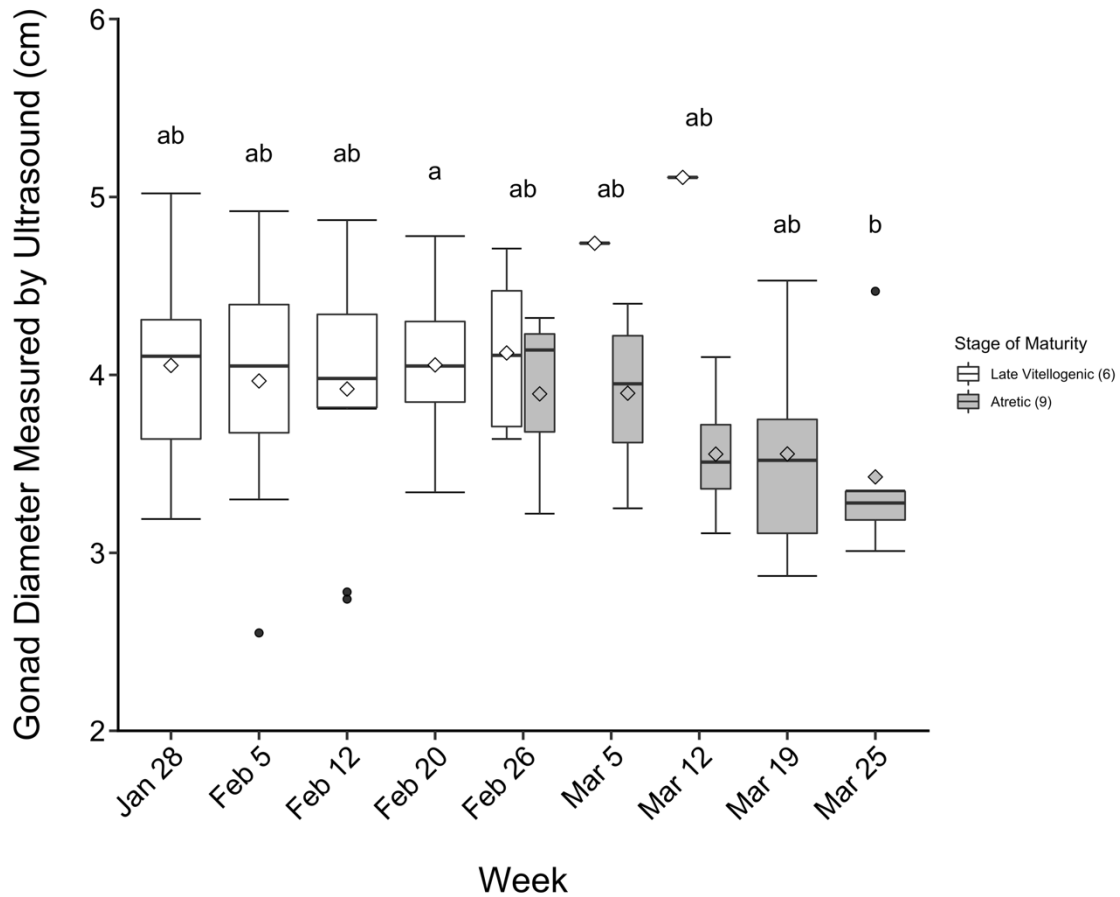


Figure 4.3. Gonad diameter measured by ultrasound (cm) of adult female Burbot by week and stage of maturity. Burbot were held at the Bozeman Fish Technology Center and exposed to increasing water temperatures to initiate mass ovarian follicular atresia. The box encompasses data from the first to third quartiles (i.e., 25th to 75th percentiles). The horizontal line within a box represents the median. The diamond represents the mean. The whiskers represent the minimum and maximum value. Outliers are represented by individual points. If there are outliers, the whiskers represent the minimum value within 1.5 times the interquartile range below the first quartile or the maximum value within 1.5 times the interquartile range above the third quartile. A single female was observed in late vitellogenesis (Stage 6) on March 5 and 12 represented by the white diamonds. Different letters indicate significant differences in gonad diameter among weeks regardless of stage of maturity.

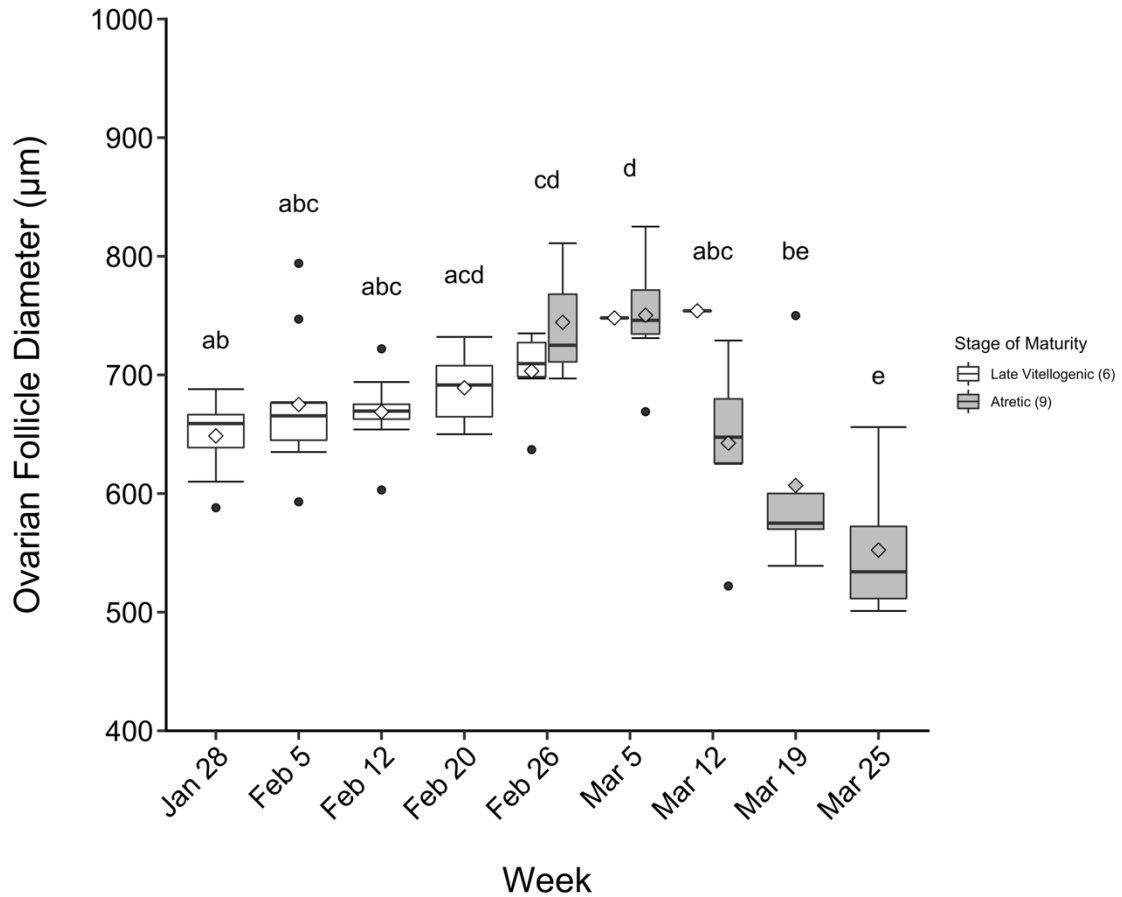


Figure 4.4. Ovarian follicle diameter (µm) of adult female Burbot by week and stage of maturity. Burbot were held at the Bozeman Fish Technology Center and exposed to increasing water temperatures to initiate mass ovarian follicular atresia. The box encompasses data from the first to third quartiles (i.e., 25th to 75th percentiles). The horizontal line within a box represents the median. The diamond represents the mean. The whiskers represent the minimum and maximum value. Outliers are represented by individual points. If there are outliers, the whiskers represent the minimum value within 1.5 times the interquartile range below the first quartile or the maximum value within 1.5 times the interquartile range above the third quartile. A single female was observed in late vitellogenesis (Stage 6) on March 5 and 12 represented by the white diamonds. Different letters indicate significant differences in gonad diameter among weeks regardless of stage of maturity.

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CHAPTER FIVE

CONCLUSIONS

The objective of this study was to assess non-lethal tools to assign sex and stage of maturity in adult Burbot from Lake Roosevelt. The specific objectives of chapter two were to describe gametogenesis, describe the plasma sex steroid profiles, and assess non-lethal tools (plasma sex steroid concentrations and ultrasound) to assign sex, stage of maturity, and reproductive condition in Burbot. The specific objective of chapter three was to assess gonad diameter measured by ultrasound as a metric to assign stage of maturity and reproductive condition in Burbot. The specific objective of chapter four was to assess non-lethal tools (plasma sex steroid concentrations, gonad diameter measured by ultrasound, and ovarian follicle diameter) to identify mass ovarian follicular atresia in female Burbot.

This is the first study to describe gametogenesis by gonadal histology during the entire reproductive cycle in Burbot. Gonadal histology was used to validate non-lethal tools to assign sex, stage of maturity, and reproductive condition in Burbot. Plasma 11-ketotestosterone (11-KT) concentration was used to assign sex with 81% accuracy during the entire reproductive cycle, and plasma 11-KT and estradiol-17 β (E2) concentrations were used to assign sex with 98% accuracy during the reproductive phase (i.e., November to March in Lake Roosevelt). Stage of maturity and reproductive condition within a sex were assigned by plasma sex steroid concentrations. In females, plasma testosterone (T) concentration, plasma E2 concentration, and month were used to assign stage of maturity

with 87% accuracy, and plasma T and E2 concentrations were used to assign reproductive condition (i.e., non-reproductive and reproductive) with 98% accuracy. In males, plasma 11-KT concentration, girth at the urogenital pore, and month were used to assign stage of maturity with 73% accuracy, and plasma T concentration was used to assign reproductive condition with 90% accuracy. Ultrasound was used to assign sex in Burbot with 97% accuracy. A combination of plasma sex steroid concentrations and ultrasound may be used as an effective method to assign sex and stage of maturity in Burbot.

We have demonstrated that ultrasound is a promising tool to assign stage of maturity and reproductive condition in Burbot by measuring gonad size. Morphological and echogenic criteria that have been used to assign stage of maturity and reproductive condition in other fishes (e.g., ovarian follicle diameter and tissue echogenicity) could not be detected in Burbot during this study; however, an ultrasound unit with higher resolution may be able to detect these criteria. We have described gonad diameter measured by ultrasound over a portion of the reproductive cycle in both female and male Burbot, and we propose that gonad diameter measured by ultrasound may be used as an index of gonadal development. An additional study with larger sample sizes including all stages of maturity would be useful to determine values of gonad diameter measured by ultrasound to characterize all stages of maturity in female and male Burbot.

We have described several non-lethal tools fisheries biologists may use to identify mass ovarian follicular atresia in female Burbot. Decreased plasma T concentrations may be used to identify mass ovarian follicular atresia before the spawning season. The

diameter of ovarian follicles collected by catheterization may also be used to identify the advanced stages of mass ovarian follicular atresia.

There are several important considerations to be accounted for when using the non-lethal tools we have described to assign sex, stage of maturity, and reproductive condition in Burbot. The plasma sex steroid concentrations, gonadosomatic index (GSI), gonad diameter, and ovarian follicle diameter data presented are representative of Burbot from Lake Roosevelt. Lake Roosevelt contains pollutants, such as metals and chlorinated organic compounds, which have been documented to lower plasma sex steroid concentrations, GSI, and ovarian follicle diameter in teleosts (Kime 1995). Other populations of Burbot may exhibit similar trends in plasma sex steroid concentrations, GSI, gonad diameter, and ovarian follicle diameter; however, the values may vary among populations.

Several studies have documented stress from confinement to lower plasma sex steroid concentrations, GSI, and ovarian follicle diameter in teleosts (Pickering et al. 1993; Barton and Iwama 1991). Stress from confinement was minimized by providing a natural light cycle, matching the thermal profile of the tank to the thermal profile of Lake Roosevelt (except in chapter four when tank temperatures were increased to induce mass ovarian follicular atresia), and feeding fish to satiation. Fish actively fed and spawned during the studies indicating that stress from confinement was minimal.

The non-lethal tools described will enable fisheries biologists to assess indices of reproductive potential (e.g., sex ratio, reproductive structure, and spawning periodicity)

for the Burbot population in Lake Roosevelt to improve population forecasting models and determine sustainable harvest regulations of Burbot in Lake Roosevelt.

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APPENDIX A

ACCURACY OF PREDICTORS TO ASSIGN SEX, STAGE OF MATURITY, AND
REPRODUCTIVE CONDITION IN BURBOT *LOTA LOTA*

Accuracy (%) of assigning sex, stage of maturity, and reproductive condition in adult female and male Burbot using AdaBoost algorithms with various predictors. T = testosterone; E2 = estradiol-17 β ; 11-KT = 11-ketotestosterone; UGP Girth = girth at the urogenital pore.

Assignment	Predictor	Accuracy (%)
Sex (Entire year)	T	64
	E2	72
	T + E2	78
	11-KT	81
	11-KT + E2	78
	T + 11-KT	78
	T + E2 + 11-KT	80
Sex (November to March)	T	60
	E2	94
	T + E2	91
	11-KT	94
	E2 + 11-KT	98
	T + 11-KT	93
	T + E2 + 11-KT	98
Female Stage of Maturity	T	67
	E2	49
	T + E2	78
	T + E2 + UGP Girth	77
	T + Month	85
	E2 + Month	79
	T + E2 + Month	87
	T + E2 + UGP Girth + Month	84
Female Reproductive Condition	T	97
	E2	91
	T + E2	98
	T + E2 + UGP Girth	97
	T + Month	97
	E2 + Month	94
	T + E2 + Month	98
	T + E2 + UGP Girth + Month	97

Accuracy (%) of assigning sex, stage of maturity, and reproductive condition in adult female and male Burbot using AdaBoost algorithms with various predictors. T = testosterone; E2 = estradiol-17 β ; 11-KT = 11-ketotestosterone; UGP Girth = girth at the urogenital pore.

Assignment	Predictors	Accuracy (%)
Male Stage of Maturity	T	49
	UPG Girth	45
	T + Month	68
	T + UGP Girth	61
	T + UGP Girth + Month	69
	11-KT	49
	11-KT + Month	66
	11-KT + UGP Girth	49
	11-KT + UGP Girth + Month	73
	T + 11-KT	51
	T + 11-KT + UGP Girth	55
	T + 11-KT + Month	70
	T + 11-KT + UGP Girth + Month	73
Male Reproductive Condition	T	90
	UPG Girth	79
	T + Month	89
	T + UGP Girth	86
	T + UGP Girth + Month	86
	11-KT	87
	11-KT + Month	87
	11-KT + UGP Girth	85
	11-KT + UGP Girth + Month	90
	T + 11-KT	90
	T + 11-KT + UGP Girth	88
	T + 11-KT + Month	88
	T + 11-KT + UGP Girth + Month	90