NTNU
Norwegian University of
Science and Technology

# Seasonal and inter-male variation in spermatogenesis in farmed Atlantic salmon (*Salmo salar*) during the last year of sexual maturation

**Jonni Juhani Virtanen**

Marine Coastal Development
Submission date: June 2016
Supervisor: Elin Kjørsvik, IBI

Norwegian University of Science and Technology
Department of Biology

# Abstract

Reproductive patterns in male teleost fish have received relatively little attention compared to those of their female counterparts. Testis maturation and progression in spermatogenesis are usually described with a combination of weight-based gonadosomatic index (GSI) and nominal classification based on histological analysis. Quantitative histological assessments are rare. In salmon aquaculture, breeding companies try to operate as efficiently as possible and additional knowledge of the timings and durations of the phases of spermatogenesis and the differences between individual fish could be useful in enhancing the production.

In the present study, the progression of spermatogenesis was observed in broodstock Atlantic salmon (*Salmo salar*) in the course of their last year of sexual maturation. A quantitative histological assessment was made by determining the spermatogenic maturity index (SMI) from 115 fish that were sampled during this period. SMI describes the maturation on a scale from 0 to 1 and is based on the area fraction estimations of different cell types in a histological section. The SMI was related to GSI and seasonal and inter-male variations were surveyed. The same fish were also examined with ultrasound and an ultrasound based GSI was established. The accuracy of ultrasound measurement was evaluated by comparing the ultrasound measurements to the true volumes and finally the applicability of ultrasound examination as a basis for sorting the fish to early and late spawners was evaluated.

The SMI correlated with GSI very well until the end of the meiotic phase when GSI reached peak mean values of 4.8 and started to decline, while SMI kept growing until the end of the experiment. Both indexes remained low until the fish were transferred to fresh water and rapid proliferation of germ cells begun. Largest variations in testis size between individual fish were found in July, when spermatocytes were the dominating cell type, all the fish were at meiotic phase and mean SMI was ~0.5. After the fish entered spermiogenic phase, GSI declined to a stable mean of 3.8 and SMI rose steadily to 0.91.

The ultrasound measurements were relatively inaccurate and there was too much inconsistency for reliable estimations on final milt volumes. However, the measurements could be easily improved and even now, the ultrasound could be used as a basis for sorting the fish to early and late spawners. It would be important to perform the sorting when the fish are at meiotic phase, when the individual differences are largest and the testis size has not started to decline, making it quite safe to assume that the biggest testes are the most mature ones.

# Acknowledgements

This Master's thesis was written at the Department of Biology, NTNU, Trondheim. It is a part of the NFR project "The application of ultrasound (US) technology in Atlantic salmon broodstock production" (Project number # 241398) currently conducted by AquaGen AS. The sampling was performed at AquaGen AS broodstock facility at Kyrksæterøra and the histological work was done at NTNU Centre of Fisheries and Aquaculture (Sealab).

First I would like to express my gratitude to my supervisors Elin Kjørsvik and Maren Mommens for guidance and feedback throughout the project. I also want to thank Tora Bardal for showing me the ropes around the lab and giving me guidance with the histology methods and Ingun Næve for instruction with the ultrasound measurements and sampling. I am also grateful to Maren Ranheim Gagnat for the help regarding statistics.

Another thank you goes to my fellow students for providing a pleasant studying atmosphere, with special thanks to Md. Enamul Hoque who has been working with the same fish as I.

Last but not least, I would like to thank the people closest to me, my family. My father, Teppo, who is the one to blame for my interest in aquaculture and my mother, Sinikka, who has always supported and encouraged me in my studies. I'm especially grateful to my sister, Henna, who has given me invaluable guidance on scientific writing on the last stages of my work.

Trondheim, May 2016

Jonni Virtanen

# Contents

**Abbreviations** ..... 1
**1 Introduction** ..... 2
1.1 Maturation of farmed salmon ..... 3
1.2 Spermatogenesis and spermiation in fish ..... 6
1.3 Aim of the study ..... 10
**2 Material and methods** ..... 11
2.1 Animals and sampling ..... 11
2.2 Testis histology ..... 14
2.3 Overall progression of spermatogenesis ..... 14
2.4 Spermatogenic maturity index, tissue types and area fraction estimations ..... 15
2.5 Ultrasound based testis volume and GSI ..... 17
2.6 Statistical analysis ..... 19
**3 Results** ..... 21
3.1 Fish and testis growth ..... 21
3.2 Overall progression of spermatogenesis ..... 24
3.3 Area fractions, SMI and GSI ..... 29
3.4 Ultrasound based GSI and volume calculations ..... 33
**4 Discussion** ..... 38
4.1 Progression of spermatogenesis ..... 38
4.2 SMI as a measure of maturity ..... 39
4.3 Ultrasound analyses ..... 41
4.4 Ultrasound examinations as a basis for sorting ..... 43
**5 Conclusions and future aspects** ..... 45
**6 References** ..... 46
**7 Appendixes** ..... 50
Appendix 1 – Ewos opal Breed 3500 fact sheet ..... 50
Appendix 2 – Temperature profile ..... 51
Appendix 3 – Hematoxylin and Eosin (H&E) staining protocol ..... 52
Appendix 4 – Staining and sectioning tests ..... 53
Appendix 5 – Area fraction and SMI calculations ..... 59
Appendix 6 – Sampling data ..... 63

# Abbreviations

| | |
|---|---|
| BPG-axis | Brain-pituitary-gonad axis |
| Ea | Excluded area |
| GnRHa | Gonadotropin releasing hormone analogue |
| GSI | Gonadosomatic index. The proportion of gonad mass from the total body mass |
| MIS | Müllerian-inhibiting substance, a hormone indicating final maturity |
| PIT | Passive Integrated Transponder, an internal tag for marking animals |
| SMI | Spermatogenic maturity index |
| Sc | Spermatocytes |
| Sg-A | Spermatogonia type A |
| Sg-B | Spermatogonia type B |
| St | Spermatids |
| Sz | Spermatozoa |
| Ts | Testicular somatic cells |
| US | Ultrasound |

# 1 Introduction

Over thirty thousand species of fish inhabit the earth and they display a vast amount of reproductive strategies (Nóbrega et al., 2009). Female reproduction has received substantially more attention than that of their male counterparts and knowledge of testis structure and spermatogenesis is still lacking, especially the quantitative aspects of it (Nóbrega et al., 2009). To have a better understanding of reproductive biology of male fish, it is useful to have knowledge of the duration of spermatogenesis, which in turn will help in explaining the functional and regulatory mechanisms of the reproductive strategies (Nóbrega et al., 2009). Aquaculture is one of the fastest growing branches in food production and highly dependent on successful production of fish larvae, making efficient operations at broodstock farms of utmost importance. Thus, the aim of this study is to provide information on Atlantic salmon (*Salmo salar*) spermatogenesis, which could be used to enhance the production at broodstock farms.

In salmon aquaculture, the fish within the same production batch have almost identical environmental conditions, age and size composition and access to food, making the batch more homogenous than their fellow salmon living in the wild. This is especially prominent in broodstock production, where much attention is given to individual fish and controlling the environmental conditions. Despite the relatively homogenous batches of fish, the progress in spermatogenesis has shown variation between individual males in broodstock brown trout (*Salmo trutta fario*) (Billard, 1983) and farmed Atlantic salmon (*Salmo salar*) (Melo et al., 2014) during the last year of sexual maturation. The most commonly used method for monitoring and determining the stage of maturation in aquaculture is palpation (Mylonas et al., 2010). Gentle abdominal pressure is applied and the stage of maturation is evaluated from the amount of milt acquired and how easily it was acquired. Other methods include sex hormone analysis for both sexes and analysing the amount of vitellogenin in blood for females (Mylonas et al., 2010). Ultrasonography has been previously used as a non-invasive method for sex identification and surveying gonadal growth in Atlantic salmon and various other species (Novelo and Tiersch, 2012). The ability to identify early, medium and late spawners based on their maturational progress as early as possible would be helpful in planning the production and marketing in broodstock farms. Salmon broodstock production has also developed in a direction where the genetic markers and DNA of individual brood fish are analyzed, making it increasingly important to gain the maximum sperm yield out of the best males.

This thesis is a part of the project "The application of ultrasound (US) technology in Atlantic salmon broodstock production" (NFR Project number # 241398) currently conducted by AquaGen AS, a salmon breeding company in Norway. One of the main objectives of this project is to use ultrasound for monitoring the maturation of Atlantic salmon during the last year before stripping. Ultrasound could potentially be used for identification of early, medium and late spawners at an earlier stage compared to palpation. The information of maturational progress achieved from the ultrasound examination could also be helpful in defining the correct timing for hormonal treatments that increase milt volumes. However, the data from ultrasound examinations needs to be verified by histological and hormonal analyses before ultrasound can be reliably used in production.

## 1.1 Maturation of farmed salmon

According to Mylonas et al., (2010), the reproductive cycle of male salmon can be separated into two major phases, spermatogenesis and spermiation. The germ cells proliferate, grow and differentiate during spermatogenesis and during spermiation, they are released to the sperm ducts and prepare for insemination. The corresponding phases for females would be vitellogenesis and oocyte maturation (Mylonas et al., 2010).

The age and size at maturation shows great variation between fish species and within strains of the same species (Taranger et al., 2010). The activation of brain-pituitary-gonad (BPG) axis (Figure 1) is controlling the onset of puberty and it is affected by numerous external and internal (for example adiposity and growth) factors. Sexual maturation usually causes a decline in growth, flesh quality and external appearance of the fish and early maturation is therefore problematic in many farmed fish (Taranger et al., 2010). Control over reproductive cycle is a crucial part for the success and sustainability of commercial aquaculture (Mylonas et al., 2010). The ability to control maturation and spawning is important for production of high quality larvae and for optimal and effective routines in commercial production.

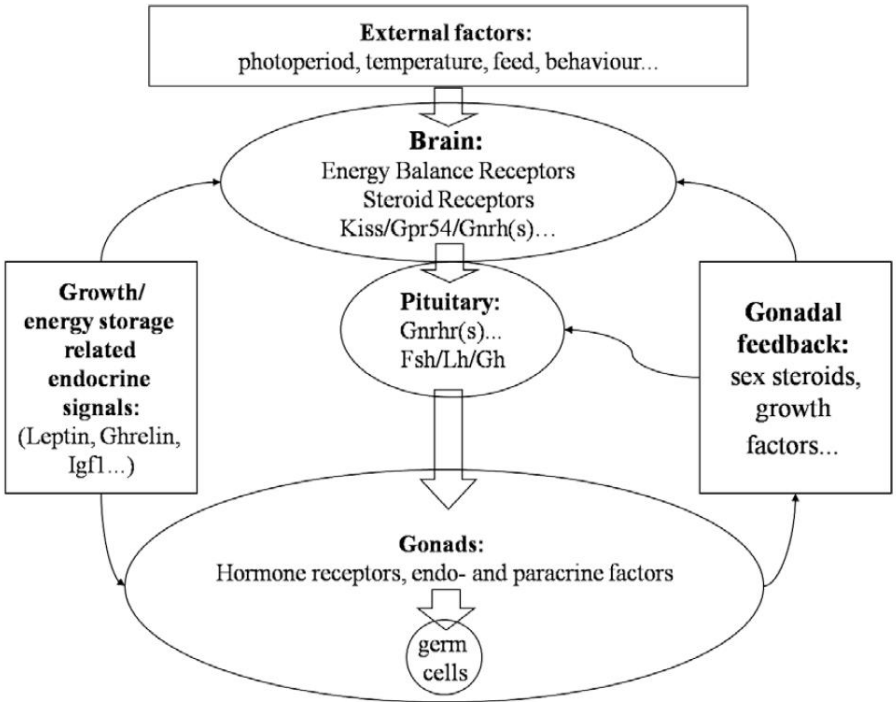



Figure 1. Reproductive axis in teleost fish. External factors cause the brain (hypothalamus) to produce gonadotropin releasing hormone which in turn causes the pituitary to produce gonadotropins, most importantly follicle stimulating hormone (Fsh) and luteinizing hormone. Image is from Taranger et al., 2010.

For Atlantic salmon, the main environmental cues that control the maturation are photoperiod and water temperature (Taranger et al, 2010). In nature, the winter water temperatures can be seen as a factor that determines the timing of spawning and photoperiod as a proximate cue that enables the initiation and completion of maturation at the correct time (Taranger et al, 2010). The spawning usually takes place in autumn and winter months, but shows considerable variations (Webb and McLay, 1996). The salmon adjusts the timing of spawning so that the egg and larval development progresses in a way that first feeding happens at a time when there is prey available (Taranger et al, 2010).

Earlier studies have shown that altering the photoperiod can be used to postpone the maturation to the next season in rainbow trout (Duston and Bromage, 1988) and Atlantic salmon (Taranger et al., 1998). Artificial photoperiods can also be used to advance or delay the timing of sperm formation and ovulation within the spawning season, enabling the production of off-season eggs, which is a step towards a year-round smolt production (Taranger et al., 1998). Taranger et al., (1998) observed that an abrupt change from natural lighting to continuous light in March accelerated the maturation of salmon and it was further accelerated by shortening the photoperiod in summer. In principle, long photoperiods or continuous light early in the season

and shorter photoperiods later in the season advance the maturation whereas short photoperiods early and long photoperiods later in the season delay it (Bromage et al., 2001) (Figure 2).

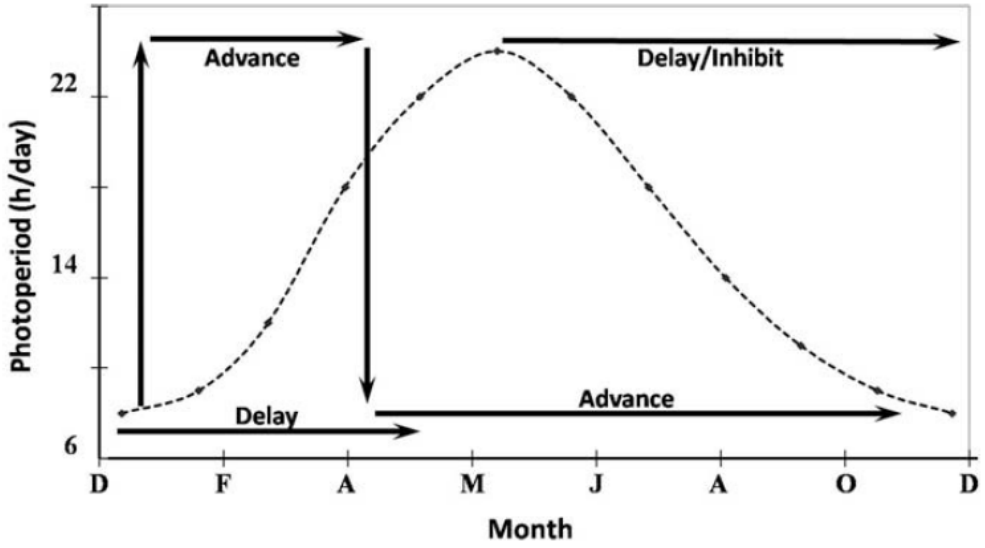



Figure 2. Concept of the effects of photoperiod manipulation to the timing of maturation. The dotted line is a representation of the natural light regime at high latitudes and the arrows depict the changes to photoperiod and their effects to maturation. Picture is taken from Taranger et al., 2010.

Since fish are poikilothermic, water temperature also has an important role as it can dictate the pace of all physiological processes in the fish (Taranger et al., 2010). Temperature can have an effect on the rate of gametogenesis and it can potentially allow or prevent the progression or completion of gametogenesis (Taranger et al., 2010). Usually if the temperatures are within the range of tolerated temperatures for the species, higher temperatures speed up the process without negative effects (Mylonas et al., 2010). However, high water temperatures close to the spawning time have shown to prevent both ovulation and sperm release in Atlantic salmon (Taranger et al., 2003). Taranger et al. (2003) also reported that exposing the salmon to cold water roughly one month prior to spawning accelerated both the sperm release and ovulation and at the same time improved the egg survival and synchrony in spawning.

Maturation can also be manipulated with hormonal treatment (Mylonas et al., 2010). Most of the hormonal treatment methods are not meant to induce spermatogenesis, but to induce spermiogenesis and enhance the production of seminal fluid, which in turn increases the amount of released spermatozoa (Mylonas et al., 1997). Gonadotropin releasing hormone analogue (GnRHa) treatment can also be used to override blockages in maturation and improve the synchrony of spawning between the fish (Taranger et al., 2003). GnRHa treatment has also resulted in increased milt volumes and acceleration of sperm release in Atlantic salmon (Taranger et al., 2003). The hormonal treatments have to be administered at the correct time,

since they are ineffective or inefficient if given to immature fish or adults that have not progressed far enough in their reproductive cycle, which makes accurate evaluation of maturity important (Mylonas et al., 2010).

## 1.2 Spermatogenesis and spermiation in fish

Sexual reproduction in animals is based on the production of haploid gametes in a process called gametogenesis (Schulz et al., 2010). According to Schulz et al., 2010, females produce a comparatively small amount of large eggs with rich nutrient reserves in a process called oogenesis while males produce a large number of small and motile genome vectors, spermatozoa, during the process of spermatogenesis.

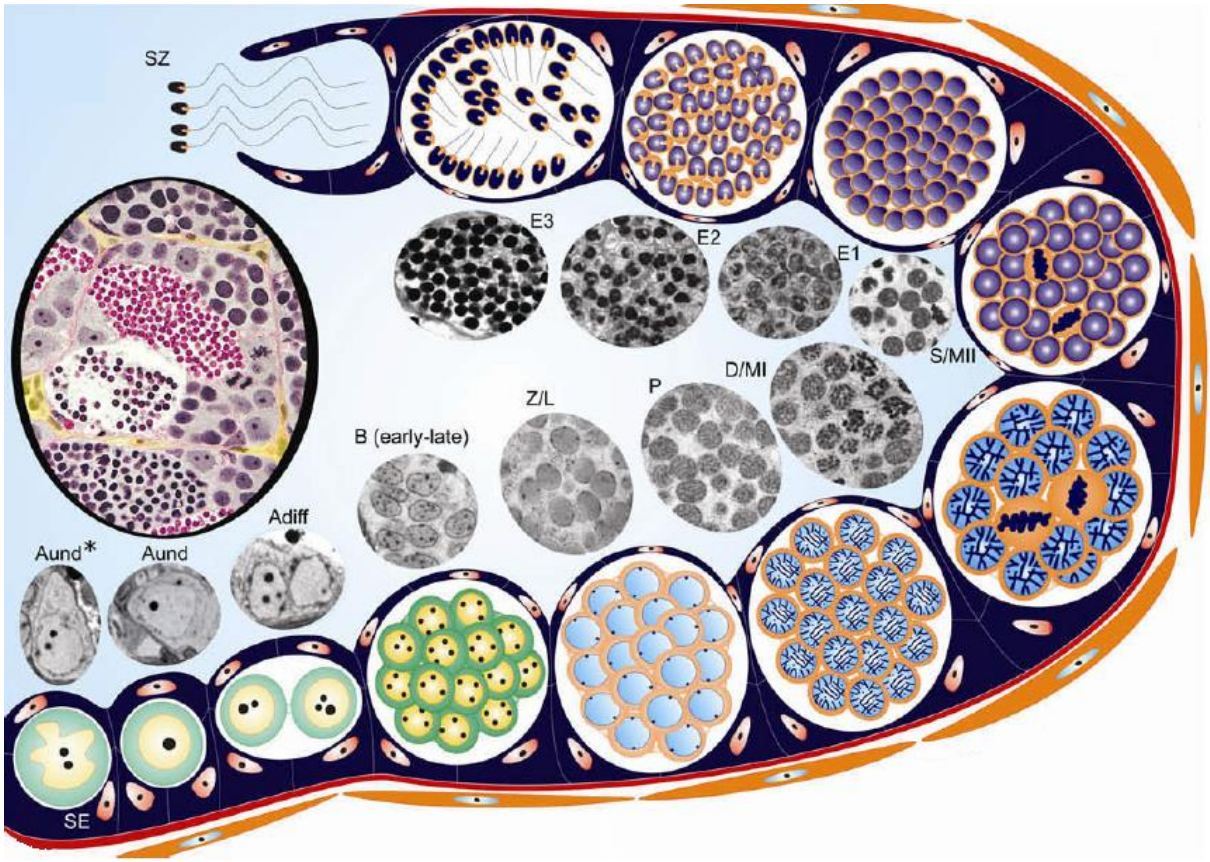



Figure 3. Progression of spermatogenesis in zebrafish. Undifferentiated spermatogonia A (Aund*) (possible stem cell); undifferentiated spermatogonia A (Aund); differentiated spermatogonia A (Adiff); spermatogonia type B [B (early–late)]; primary spermatocytes leptotenic/zygotenic stage (L/Z); primary spermatocytes pachytenic (P); diplotenic spermatocytes/metaphase I (D/MI); secondary spermatocytes/metaphase II (S/MII); early (E1), intermediate (E2) and final spermatids (E3); spermatozoa (SZ) and Sertoli cells (SE). Image is from Schulz et al., 2010.

Spermatogenesis (Figure 3) is a process where a small number of diploid spermatogonial stem cells proliferate and differentiate into a vast amount of haploid spermatozoa (Schulz et al., 2010). Unlike mammals that show a non-cystic type of spermatogenesis where a single Sertoli

cell supports germ cells of different stages, fish display a cystic form of spermatogenesis in which a group of Sertoli cells surrounds a single germ cell clone and follows it throughout the stages of spermatogenesis (Schulz et al., 2005). The duration and timing of spermatogenetic stages and the number of spermatogonial generations at mitotic phase varies between fish species, but the meiotic and spermiogenic phases of spermatogenesis are similar (Schulz et al., 2010). The process can be generally divided in three different phases: the mitotic (spermatogonial) phase, the meiotic phase and the spermiogenic phase (Nóbrega et al., 2009).

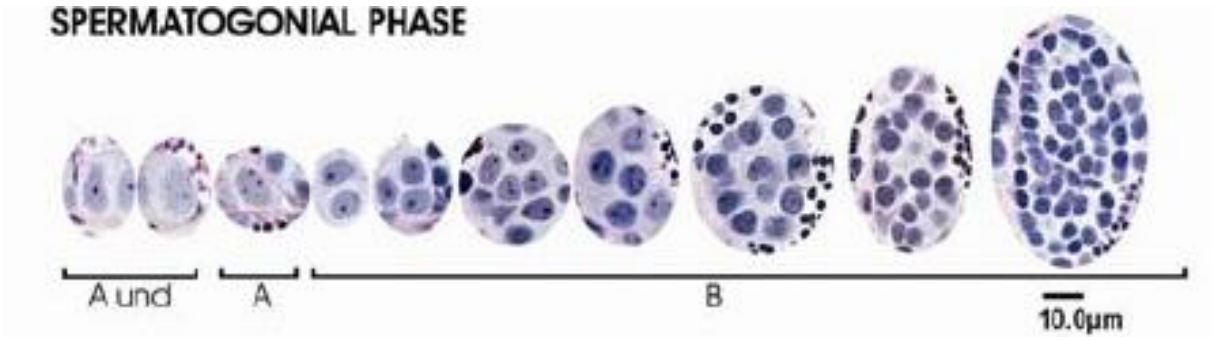



Figure 4. Mitotic phase of spermatogenesis in zebrafish. Undifferentiated spermatogonia A (A und), spermatogonia A (A) and different generations of spermatogonia B (B). Picture is modified from Nóbrega et al., 2009.

Spermatogenesis begins with the mitotic spermatogonial phase during which the undifferentiated A-type spermatogonia turn into differentiated spermatogonia A (Sg-A) (Figure 4). The two types share some morphological features, but the differentiated cells have much less self-renewal potential (Schulz et al., 2010). Type A spermatogonia are relatively large and reach a size of approximately 10 µm in diameter in many species. They have a clear homogenous nucleus, which contains one or two nucleoli (Miura, 1999). Differentiated spermatogonia A are followed by more swiftly proliferating type B spermatogonia (Sg-B), which usually have several generations depending on the fish species (Schulz et al., 2010). Nine generations have been found in zebrafish (Leal et al., 2009) and six in rainbow trout (Loir, 1999). The morphology of late Sg-B differs from their earlier counterparts. Early Sg-B resemble Sg-A while the late Sg-B have smaller mitochondria and their nucleus is denser and more heterogeneous (Miura, 1999).

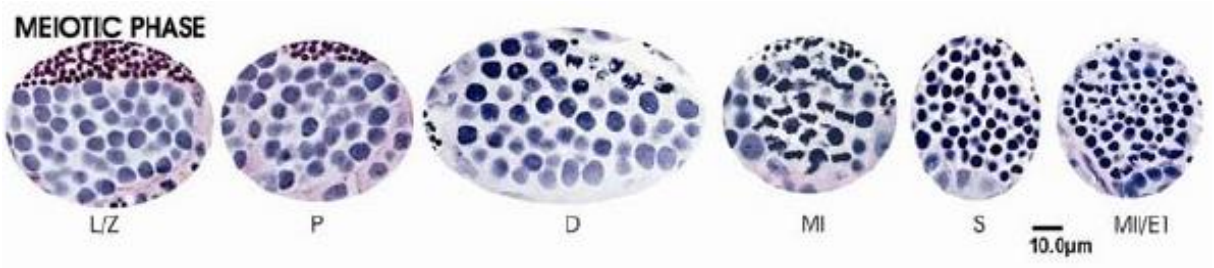



Figure 5. Meiotic phase of zebrafish spermatogenesis. Primary spermatocytes at leptotene/zygotene (L/Z), pachytene (P), diplotene (D), metaphase I (MI) and secondary spermatocytes (S), metaphase II/early spermatids (MII/E1). Figure modified from Nóbrega et al., 2009.

After the Sg-B are finished with their last mitotic division, they differentiate into primary (preleptotene) spermatocytes, initiating the meiotic phase (Figure 5). The primary spermatocytes go through several stages without proliferation before the first meiotic division (Schulz et al., 2010). The different stages can be identified under light microscopy by the properties of their nucleus, for example the shape and size and the degree of chromosome condensation (Schulz et al., 2010). Schulz et al. (2010) also reported that maximum cyst volumes in teleost testis can be observed when the germ cells have progressed into late meiosis. During the first meiotic division the homologous chromosomes are segregated into secondary spermatocytes, which rapidly go through the second meiotic division where the sister chromatids segregate and produce haploid spermatids, which contain one copy of each chromosome. One of the purposes of this process is to create genetic diversity by first recombining (crossing over) and then segregating the homologous chromosomes (Schulz et al., 2010).

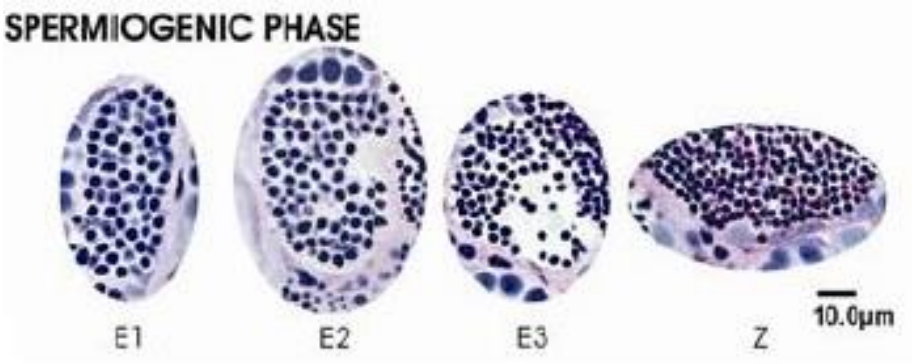



Figure 6. Spermiogenic phase of spermatogenesis in zebrafish. Early spermatids (E1), intermediate spermatids (E2), final spermatids (E3) and spermatozoa (Z). Figure modified from Nóbrega et al., 2009.

The round spermatids go through significant morphological changes related to the development of the flagellum, midpiece and sperm head as they mature into spermatozoa during the spermiogenic phase (Figure 6) (Miura, 1999). Spermatids can be classified into early,

intermediate and final spermatids based on their nuclear condensation (Schulz, 2005). The structure of spermatozoa displays considerable variation between teleost species. Spermatozoa of salmonids have a cylinder shaped, slightly elongated head while tilapia spermatozoa for example have spherical heads (Miura, 1999). These notable changes to the cells may explain the decrease in cyst volumes observed in tilapia (Schulz et al., 2005) and zebrafish (Leal et al., 2009) during this phase. Melo et al. (2014) observed a decrease in testis size also in Atlantic salmon as more germ cells developed into spermatozoa. Apoptotic loss of germ cells may also be most significant during this phase (Schulz et al., 2010). At the final stretch of the spermiogenic phase, Sertoli cell structures change and cause the opening of cyst walls, which leads to the release of spermatozoa into the lobule lumen (Schulz et al., 2010). The opening of cyst walls and release of spermatozoa in the end of spermiogenesis initiates the spermiation stage (Schulz and Miura, 2002).

It is common that the spermiation period for males is longer than the spawning period of females, making it possible for the males to fertilize eggs of several females (Mylonas et al., 2010). The final maturation (hydration/capacitation) of sperm takes place in the end of spermiation (Schulz et al., 2010). In some species spermatozoa that have completed spermiogenesis are still not capable of fertilizing eggs (Miura, 1999). In salmonids it is the high pH (~8.0) of the seminal plasma in the sperm duct that induces sperm motility and if spermatozoa from the sperm duct are diluted with fresh water, they gain motility whereas physically mature spermatozoa from the testis will remain immotile (Miura, 1999).

The germinal epithelium contains only two types of cells: germ cells and somatic Sertoli cells (Schulz et al., 2010). In addition to serving as structural support when forming the spermatocysts, Sertoli cells have many other important roles during spermatogenesis (Schulz et al., 2010). Their main purposes are to maintain germ cell survival, development and physiological functioning and they are responsible for secreting the fluid that generates the tubular lumen (Schulz et al., 2010). According to Schulz et al. (2010), they are also extraordinarily effective as phagocytes and remove and recycle apoptotic germ cells and cellular debris very quickly.

## 1.3 Aim of the study

Efficient operation in broodstock production is one of the fundamental factors for the growing aquaculture industry. The ability to identify early and late spawners earlier in the production would improve the efficiency in broodstock farms. With increased effort put into mapping the DNA of individual brood fish, gaining the maximum sperm yield from the best individuals is getting more and more important. Information of the progression of spermatogenesis could be useful in improving the timing of treatments that are used for enhancing the sperm production.

The research questions for this study are:

- How is the progression of spermatogenesis in farmed Atlantic salmon and what kind of individual differences are there between the males?
- How could ultrasound examinations be used as a basis for sorting the fish into early and late spawners?

A histological assessment of the progression of spermatogenesis in broodstock Atlantic salmon males during the last year before stripping will be conducted. A quantitative analysis that is based on the relative percentages of the sperm cells at different stages will be made and the duration and timing of the stages of spermatogenesis will be explored. The variation in maturational progress between individual males will also be studied. The histological assessment, which is based on the maturation of germ cells, will be related to the conventional, weight based GSI.

The same fish will also be examined by ultrasound in order to estimate the testis volume. These volume calculations will then be used to establish an ultrasound based GSI. The accuracy of ultrasound based GSI will be evaluated by comparing the volumes calculated from the ultrasound measurements to the true volumes of the testis.

Finally, the information gained from both the histological and ultrasound examinations will be put together to evaluate the potential of using ultrasound as a basis for sorting the fish in early and late spawners.

# 2 Material and methods

## 2.1 Animals and sampling

The Atlantic salmon (*Salmo salar*) used in this study were provided by AquaGen AS and kept in sea cages in Hemnefjorden. The sea cages were 90 meters in circumference and 15 meters in depth (volume 9669 $m^3$, fish density 20 $kg/m^3$). The fish were fed with regular commercial dry feed (Ewos Opal on-growing feed) until 5.9.2014, when the diet was changed to a special broodstock diet Ewos Opal Breed 3500 (Appendix 1). Starting in September 2014, 10-20 two and a half year old males were randomly selected from the cage and sampled each month (Table 1). The fish were kept under constant additional light from 3.3.2015 onward which has been shown to accelerate sexual maturation (Taranger et al., 2010). The feeding was stopped on 5.4.2015 and on 20.5.2015 the fish were moved to a circular (60 $m^3$, fish density 70 $kg/m^3$) freshwater tank in Kyrksæterøra. The water temperature in the tank was gradually increased from ~7 to ~15°C in ten days. While indoors, the fish were kept under 8 h light, 16 h dark photoperiod. Starting on the first week of August (7.8.2015), the sampling was done on a weekly basis and five fish were sampled each time from this point on. To accelerate and improve the synchrony of final maturation (Taranger et al., 2003), a temperature drop was initiated on 9.8.2015. The temperature was gradually decreased from ~15 to ~7 °C with the use of heat exchangers during the course of ten days and maintained at around 7 °C until the end of the experiment. Full temperature profile in Appendix 2.

The fish were euthanized with an overdose of anaesthetic (Aqui-S, AQUI-S New Zealand Ltd) and the body weight (to the nearest 10 g) and fork length (to the nearest 0.5 cm) were measured. The testes were dissected out and weighed to the nearest 0.1 g and the length of the left testis was measured. Gonadosomatic index, GSI, is the proportion of gonad mass from the total body mass and it was calculated with the equation:

$$GSI = \frac{gonad\ weight\ (g)}{body\ weight\ (g)} * 100.$$

Volume of the left testis was measured to the nearest 1 ml by water displacement. From the left testis, a tissue sample was taken approximately from the first third at the cranial end and fixated in formaldehyde for histological analyses.

Before the testes were dissected out, ultrasound pictures were taken using an Esaote MyLabAlpha device and a linear probe “SL1543” and the length of the left testis was measured

by ultrasound. The fish was placed on the table left side up and the ultrasound probe was placed at the basis of the pectoral fin. The testis was then located and a ruler was placed at the upper basis of the pectoral fin. The length of the left testis was measured and depending on the size of the testis, one to four pictures were taken along the length of the testis. The pictures were taken at positions where the testis was clearly visible with the ultrasound and the placement of the probe was written down for each picture taken. July samples had an average of two pictures per fish and the samples before that had one. During August and September, 2-4 pictures were taken from each fish.

A total number of 185 fish were sampled and every fish was taken into account for the morphometric measurements (Table 1). For histological analyses, the least emphasis was put on the earliest samples with undeveloped testis and the number of analysed samples was reduced. For the first six months of sampling, five samples from each month were randomly selected. From March to June, five samples from fish with the lowest and five from fish with the highest GSI were chosen and starting from July, every sample was analysed. The ultrasound images were analysed for every fish that had a clear enough image for accurate measurements.

Table 1. Mean water temperatures from daily recordings and numbers of samples taken and analysed. The temperature measurements were taken at a depth of 6 meters during sea phase and the values given are means (ranges). The photoperiod row indicates the light treatment (daily hours in light and darkness).

| | **2014** | | | | **2015** | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **2. Sep** | **6. Oct** | **11. Nov** | **1. Dec** | **6. Jan** | **2. Feb** | **2. Mar** | **8. Apr** | **4. May** | **9. Jun** | **1. Jul** | **7. Aug** | **13. Aug** | **20. Aug** | **27. Aug** | **2. Sep** | **Total** |
| **Temp. (°C)** | 15 (13–16) | 13 (10–14) | 11 (9–12) | 9 (7–10) | 7 (6–8) | 6 (6–7) | 6 (6–7) | 6 (6–7) | 8 (7–9) | 15 (15–16) | 15 (14–16) | 15 (14–15) | 12 (10–15) | 8 (7–9) | 7 (7–7) | 7 (7–8) | |
| **Photo-period** | natural | natural | natural | natural | natural | natura | LD 24:0 | LD 24:0 | LD 24:0 | LD 8:16 | LD 8:16 | LD 8:16 | LD 8:16 | LD 8:16 | LD 8:16 | LD 8:16 | |
| **Fish sampled (N)** | 16 | 19 | 10 | 12 | 11 | 14 | 14 | 12 | 12 | 20 | 20 | 5 | 5 | 5 | 5 | 5 | **185** |
| **Histological analyses (n)** | 5 | 5 | 5 | 5 | 5 | 5 | 10 | 10 | 10 | 10 | 20 | 5 | 5 | 5 | 5 | 5 | **115** |
| **Ultrasound analyses(n)** | 0 | 0 | 0 | 0 | 0 | 2 | 9 | 6 | 9 | 17 | 19 | 4 | 5 | 5 | 5 | 5 | **86** |

Salmon in sea cages (sea phase)

Salmon in fw tanks (fresh water phase)

Salmon in fw tanks, temperature dropped (cold water phase)

## 2.2 Testis histology

A variety of biological stains can be used to make cells visible or to improve the details in histological sections. Hematoxylin and Eosin (H&E) staining is one of the most common techniques used in histology and it has been in use for over a century (Fischer et al., 2006). Hematoxylin stains nucleic acids deep blue and Eosin gives a pink color to proteins. In the photomicrographs of tissues in this study, cytoplasm (especially in blood cells) and connective tissues have varying shades of pink and nuclei are dark blue/purple.

Small scale tests were performed to find out if minor changes to the staining protocol (Appendix 3) would result in getting better images for the histological analysis. Different section thicknesses (1-4 µm) and timings in hematoxylin and eosin were tried out and paraffin was compared to a plastic (Technovit® 7100, Kulzer, Germany) as the embedding material.

Changes to the staining protocol didn't improve or change the images significantly. Thinner sections were slower and more difficult to make and the images were not significantly better. Technovit embedding was slower to perform than paraffin embedding and the images were not significantly better. Images and more detailed descriptions of the tests are presented in Appendix 4.

## 2.3 Overall progression of spermatogenesis

The tissue samples were dehydrated (Leica TP1020 tissue processor) and embedded in paraffin. The embedded tissues were then sectioned to 4 µm thickness with a microtome (Leica 2055 Autocut) using steel knives. The sections were mounted on glass microscope slides and stained with hematoxylin and eosin (Appendix 3). The stained sections were scanned with a digital slide scanner (Hamamatsu NanoZoomer) with 20x magnification. The scanned slides were then examined with Hamamatsu NDP.view2 software and 10 images were taken along each section. Each picture represented an area of approximately 0.71 $mm^2$. The software has a tracking map which was used to ensure that the images wouldn't contain the same area more than once.

## 2.4 SMI, tissue types and area fraction estimations

Spermatogenic maturity index (SMI), introduced by Tomkiewicz et al. (2011), is a relatively new method for quantitative assessment of testis maturity. It is based on area fractions of somatic tissues and germ cells at different stages of spermatogenesis. The tissue types are given a weight factor that is used as a multiplier for the area fractions. The results are then summed up which gives a value ranging from 0 to 1. This value can then be conveniently used to evaluate testis development or to find correlations with physiological and morphological parameters. Spermatogenic maturity index has previously been successfully used to describe testis development and progression of spermatogenesis in European eels (Tomkiewicz et al., 2011).

The cells in the testis tissues were identified according to several sources (Dziewulska & Domagala, 2003; Dziewulska & Domagala, 2005; Leal et al., 2009; Schulz et al., 2010 and Melo et al., 2014) and assigned into five groups to match the SMI method presented by Tomkiewicz et. al. 2011. Germ cells were divided in to four groups: spermatogonia (Sg), spermatocytes (Sc), spermatids (St) and spermatozoa (Sz). Fifth group were testicular somatic cells (Ts), which included connective tissue, blood cells, Leydig and Sertoli cells. Areas with no tissue were excluded.

To estimate the area fractions of different cell types in a section, a point grid of 112 points, where one point represented an area of 6500 $\mu m^2$, was placed on the image with ImageJ software's (National Institute of Mental Health, Bethesda, Maryland, USA) Grid plug-in. Tissue type under each point was identified and marked using ImageJ's Cell Counter plug-in. An illustration of this kind of method is presented in Figure 7. The estimations for area fractions were obtained by dividing the sum of points identified to a tissue type by the total number of points that hit testis tissue in the image. This was done to three images that were randomly selected from the 10 images taken from each sample and the mean value represented the area fractions for the different cell types in the tissue sample. Example of area fraction calculation is presented in Table 2.

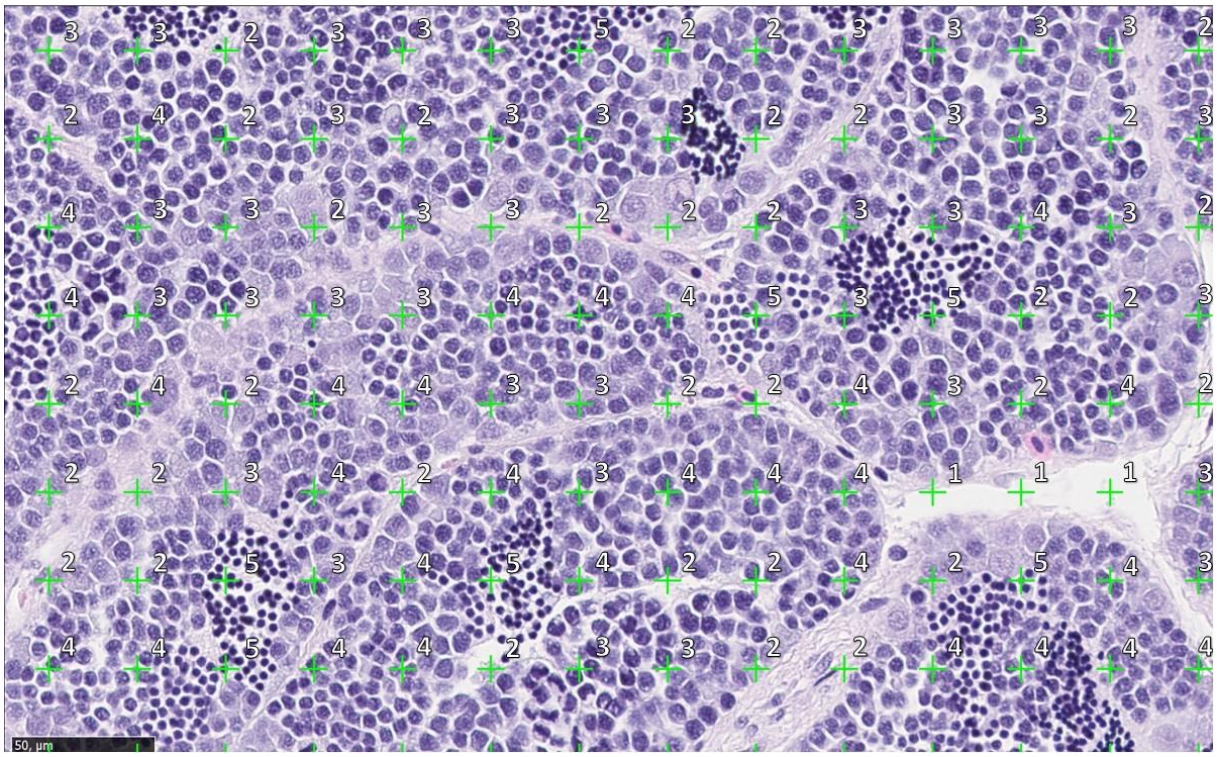



Figure 7. Photomicrograph of a salmon testis (40x magnification) section stained with hematoxylin and eosin. A point grid has been added with ImageJ's Grid plug-in. The number next to each cross identifies the cell type present at the cross intersection. The cell types were counted and marked with ImageJ's Cell Counter plug-in. The cell types and an example of an area fraction estimation is shown in Table 2

To calculate the SMI according to Tomkiewicz et al. (2011), a weight factor (w=0.0, 0.25, 0.5, 0.75 or 1.0) that increases with progressing development was assigned to each tissue type. The area fractions per tissue type were then multiplied with the corresponding weight factor and summed up:

$$SMI = 0.0F_{Ts} + 0.25F_{Sg} + 0.5F_{Sc} + 0.75F_{St} + 1.0F_{Sz} \ ,$$

where F is the area fraction of the corresponding tissue type [testicular somatic cell (Ts), spermatogonia (Sg), spermatocytes (Sc), spermatids (St) or spermatozoa (Sz)]. The value of the index varies between 0 when only Ts are found and 1.0 when all the germ cells have developed into spermatozoa. Illustration of an SMI calculation is provided in Table 2.

Table 2. Examples of area fraction and spermatogenic maturity index (SMI) calculations. The cell types are excluded area (Ea), testicular somatic cells (Ts), spermatogonia (Sg), spermatocytes (Sc), spermatids (St) and spermatozoa (Sz). The point counts (n) per tissue category (i) are based on the numbers from the 112-point grid on the photomicrograph of salmon testis (Figure 7). F is the area fraction and w is the weight factor assigned for the cell type. The spermatogenic maturity index of the sample is 0.27.

| | | | Area fraction calculation | | SMI calculation | |
|---|---|---|---|---|---|---|
| Cell type | i | $n_i$ | $n_i/(n_{total}-n_1)$ | F | w | F*w |
| Ea | 1 | 3 | | | | |
| Ts | 2 | 34 | 34/(112-3) | 0.31 | 0 | 0.00 |
| Sg | 3 | 39 | 39/(112-3) | 0.36 | 0.25 | 0.09 |
| Sc | 4 | 29 | 29/(112-3) | 0.27 | 0.5 | 0.13 |
| St | 5 | 7 | 7/(112-3) | 0.06 | 0.75 | 0.05 |
| Sz | 6 | 0 | 0 | 0.00 | 1 | 0.00 |
| Total | | 112 | | 1.00 | | 0.27 |

## 2.5 Ultrasound based testis volume and GSI

To estimate the testis volume, the ultrasound pictures that were taken during sampling were examined with eSaote MyLab™Desk3 software (Figure 8). The left testis was located from the picture and the area of the cross-section was measured by tracing along the edges of the testis with the software's area measurement tool "Trace". The software automatically gives the area of the outlined object. The images where the testis outline was too unclear to define or the testis exceeded the image borders were excluded.

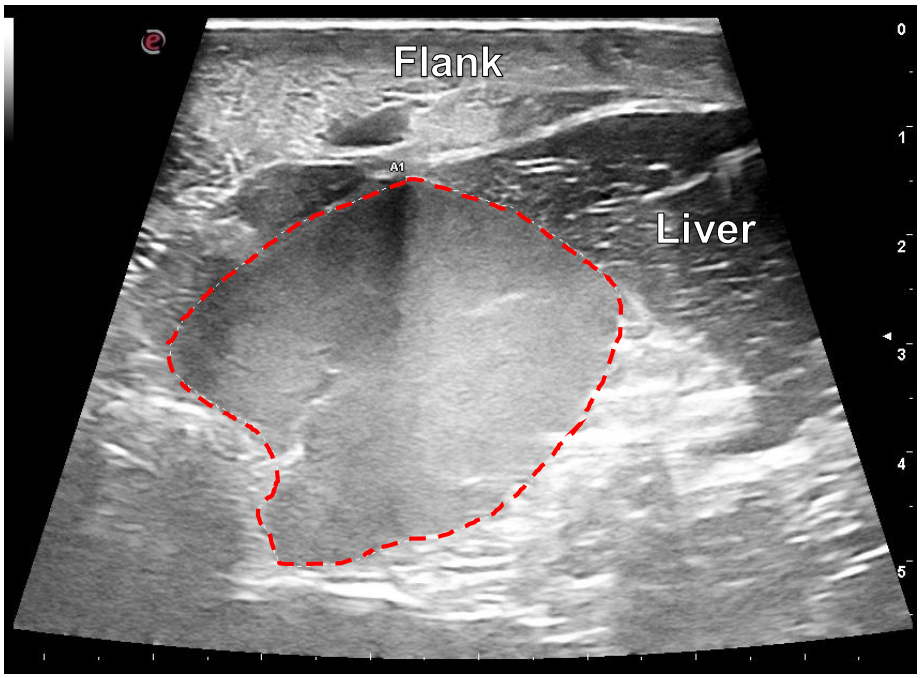



Figure 8. An image of a transverse cross-section of a gonad, digitally captured with ultrasound. The image is examined with eSaote MyLab™Desk3 software. The testis is outlined with the dotted line.

Three different geometric shapes were used to represent the testis and the volumes were estimated by using the cross-section area and ultrasound length measurements in the corresponding equations (Figure 9). The volume was also estimated by using only the ultrasound length measurements.

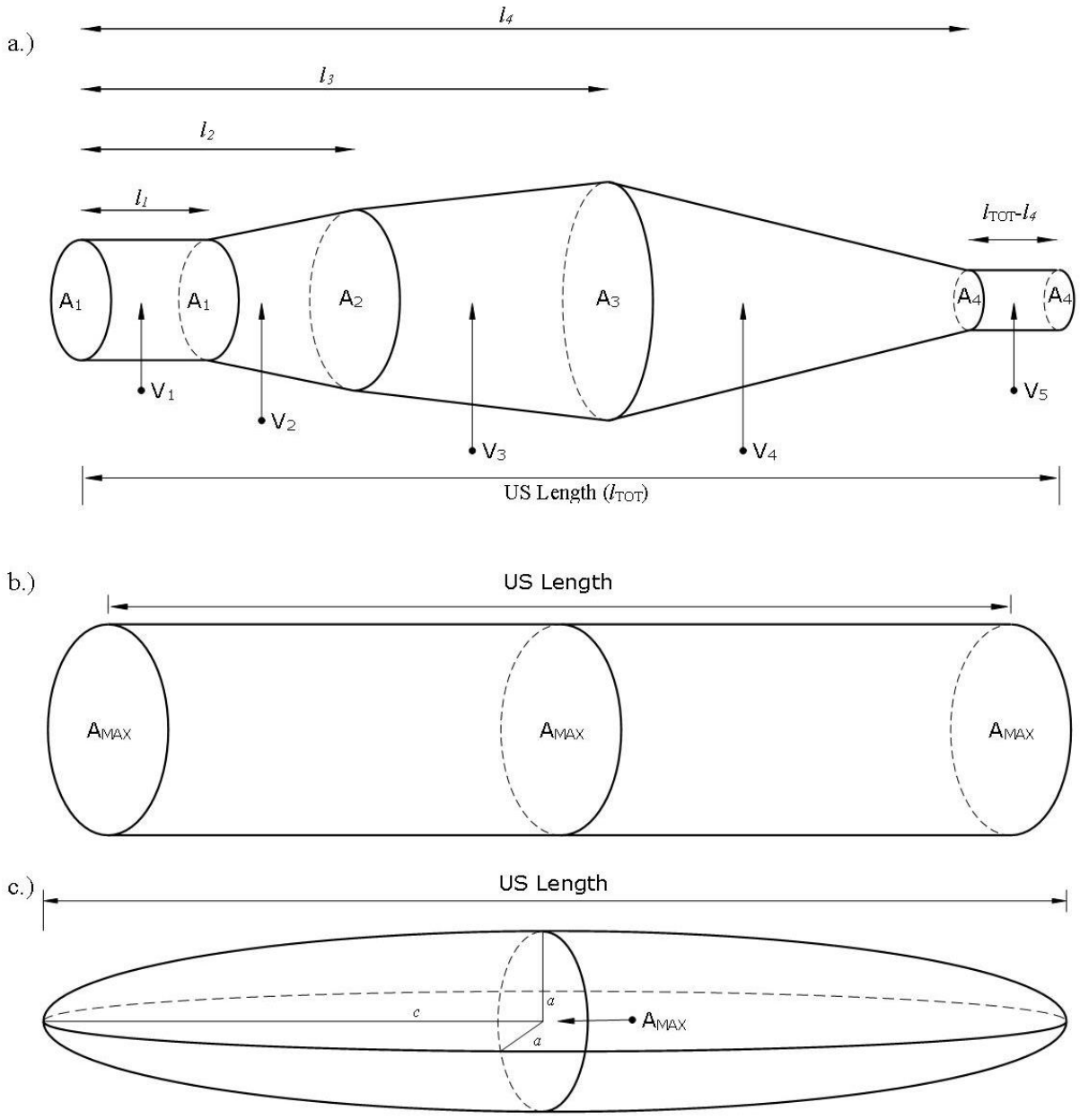



Figure 9. Generic shapes used for different ultrasound volume calculations: a.) Sectioned tube, b.) straight tube and c.) ellipsoid.

The equations used for the volume calculations were:

a) $V_{TOT} = V_1 + V_2 + V_3 + V_4 + V_5$ Number of sections depends on how many US pictures there were. Maximum was 5 sections.

   $V_1 = A_1 * l_1$

   $V_2 = (l_2 - l_1) * [(A_1 + A_2)/2]$

   $V_3 = (l_3 - l_2) * [(A_2 + A_3)/2]$

   $V_4 = (l_4 - l_3) * [(A_3 + A_4)/2]$

   $V_5 = (l_{TOT} - l_4) * A_4$

b) $V = A_{MAX} * USlength$

c) $V = \frac{4}{3}\pi * a^2 * c$ . Also, the volume of an ellipsoid is two thirds the volume of an elliptic cylinder or in this case a tube so $V = \frac{2}{3} * A_{MAX} * USlength$ could also be used.

To establish an ultrasound based GSI, the testis weight was estimated by using the ultrasound volume calculations and average testis density of the samples. It was also taken into account that the left testis was a bit more than half the total gonad weight on average. The gonad weight used in ultrasound based GSI was calculated with the equation:

$$weight = \rho * V_{tot} \text{ , where } V_{tot} = \frac{V_{left}}{(\frac{\%Left}{100})}$$

The density, ρ, was 1,05 g/ml and %Left was 55.5 % on average in this study. The density and left testis proportion values are mean values from the whole sampling data (Appendix 6).

## 2.6 Statistical analysis

Before deciding on using 112 points in the point grid, SMI calculations were made from three samples using a very dense grid with 405 points as the control and 180, 144 and 112 points as the other groups. An analysis of variance, Two-way ANOVA, with sample and the number of points as the sources of variation, was then used to assess if there is a significant difference in SMI calculation results depending on the number of points used. Shapiro-Wilk was used to test the normality of data and Holm-Sidak method was used afterwards for comparisons versus the control group. Significant difference between using 405 and 112 points in the point grid for SMI calculations was not found (p=0.43) so 112 points (6500 µm$^2$ per point) was selected as the point density.

For the fish and testis growth and area fractions of cell types and SMI, all data were $\log_{10}$ transformed and normality was tested with Shapiro-Wilk and homogeneity of variance with Levene test. Normal distribution was not found on any parameter so non-parametric tests were used. Homogeneity of variance was not found on all samples so Mood's median test was used to test differences between months and if differences were found, homogeneous subsets were identified with stepwise step-down multiple comparisons.

All GSI values for ultrasound GSI calculations were $\log_{10}$ transformed. The normality was tested for each month with Shapiro-Wilk test ($p<0.05$) and the homogeneity of variance with Levene test. The data was normally distributed and had homogeneity of variance so one-way ANOVA ($p=0.05$) was used to analyse if there were differences in means between the GSI values from different methods for each month. If a difference was found, Student-Newman-Keuls post-hoc test was used to see which of the GSI measurements had significant differences.

Statistical analyses were made using SigmaPlot 13.0 (Systat Software, San Jose, CA) and IBM SPSS Statistics for Windows, Version 22.0. (IBM Corp. Armonk, NY). Graphs and tables were made with Microsoft Office Excel 2016.

# 3 Results

## 3.1 Fish and testis growth

In the beginning of the experiment (2.9.2014) the fish had a mean weight of 6.6 ± 0.7 kg and a mean length of 82 ± 2 cm (Table 3). The condition factor in the beginning was 1.2 ± 0.07 on average. The fish reached peak mean weights in April (15.3 ± 1.7 kg), but the mean weights were not significantly different between 8.4.2015 and 2.9.2015. Highest mean lengths (109 ± 5 cm) were observed 7.8.2015 and no significant differences were found between the samplings between 4.5.2015 and 2.9.2015. The fish had the highest mean condition factor on 8.4.2015 (1.45 ± 0.11) and from 9.6.2015 onwards it was significantly lower.

The gonads remained relatively small during the sea phase (2.9.2014 – 4.5.2015), but there was a significant increase in gonad weight as the mean weight grew from 12.9 ± 5.3 g to 33.1 ± 8.4 g (Table 3). In the first sampling of the fresh water period (9.6.2015) a significant increase in weight was also observed and the mean gonad weight had reached 98.6 ± 60.6 g. The most notable changes in gonad weight between two consecutive samplings happened between 9.6.2015 and 1.7.2015. The average gonad weight reached 542.7 ± 232.1 g on 1.7.2015 and there was significant growth between the samplings. The maximum mean values for gonad weight were observed on 7.8.2015 (688.1 ± 189.9 g), but there were no significant differences in gonad weights between 1.7.2015 and 2.9.2015.

Gonad density was relatively close to 1.0 throughout the experiment, but varied between means of 0.94 ± 0.2 and 1.16 ± 0.1 g/cm$^3$ (Table 3). Mean density from whole data was 1.05 ± 0.11 g/cm$^3$. The correlation coefficients (Pearson) were low between GSI and density (0.43) or gonad weight and density (0.41). The left testis proportion from total gonad weight fluctuated between means of 51.9 ± 2.9 and 59.7 ± 7.0 %, but there were no significant differences between months ($p>0.05$). The mean left testis proportion from the whole data was 55.5 ± 0.11 %.

Table 3. Selected parameters (mean ± SD) and numbers of samples taken and analysed. Gonad density is calculated from the true volume (measured by water displacement) and left testis weights that are represented in (Appendix 6 – Sampling data). Left testis lengths are the true values from ruler measurements after dissection of testis. Left testis proportion is the proportion of left testis weight from the total gonad weight. The total number of sampled fish was 185 and the letters indicate significant differences.

| | **2014** | | | | **2015** | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **2. Sep** | **6. Oct** | **11. Nov** | **1. Dec** | **6. Jan** | **2. Feb** | **2. Mar** | **8. Apr** | **4. May** | **9. Jun** | **1. Jul** | **7. Aug** | **13. Aug** | **20. Aug** | **27. Aug** | **2. Sep** |
| **Weight (kg)** | 6,6 ± 0,7$^{a}$ | 7,2 ± 1,2$^{ab}$ | 9,1 ± 1,6$^{bc}$ | 9,5 ± 1,9$^{cd}$ | 9,4 ± 1,7$^{c}$ | 12,6 ± 2,5$^{de}$ | 12,2 ± 3,2$^{cde}$ | 15,3 ± 1,7$^{e}$ | 14,0 ± 2,4$^{de}$ | 13,5 ± 1,9$^{de}$ | 13,3 ± 2,1$^{de}$ | 14,2 ± 1,3$^{e}$ | 13,2 ± 1,0$^{de}$ | 12,8 ± 1,9$^{de}$ | 13,2 ± 1,2$^{de}$ | 12,2 ± 2,4$^{de}$ |
| **Length (cm)** | 82 ± 2$^{a}$ | 86 ± 4$^{ab}$ | 90 ± 3$^{bc}$ | 90 ± 4$^{bc}$ | 92 ± 4$^{cd}$ | 96 ± 6$^{cde}$ | 97 ± 4$^{de}$ | 102 ± 3$^{ef}$ | 102 ± 5$^{fg}$ | 104 ± 4$^{fg}$ | 105 ± 5$^{fg}$ | 109 ± 5$^{g}$ | 107 ± 5$^{g}$ | 106 ± 3$^{fg}$ | 108 ± 2$^{g}$ | 106 ± 6$^{g}$ |
| **Cond. factor (Fulton)** | 1.18 ± 0.07 abcd | 1.12 ± 0.10 ab | 1.24 ± 0.11 bcde | 1.27± 0.15 efg | 1.18 ± 0.13 bcde | 1.40 ± 0.10 fg | 1.30 ± 0.25 cdef | 1.45 ± 0.11 f | 1.31 ± 0.10 def | 1.20 ± 0.09 bcd | 1.14 ± 0.08 abc | 1.09 ± 0.05 a | 1.09 ± 0.11 a | 1.08 ± 0.09 ab | 1.06 ± 0.11 ab | 1.02 ± 0.08 a |
| **GSI** | - | - | - | 0.13 ± 0.04$^{a}$ | 0.14 ± 0.04$^{ab}$ | 0.17 ± 0.04$^{abc}$ | 0.23 ± 0.09$^{c}$ | 0.21 ± 0.06$^{bc}$ | 0.24 ± 0.07$^{c}$ | 0.73 ± 0.46$^{d}$ | 4.13 ± 1.71$^{e}$ | 4.83 ± 1.17$^{e}$ | 3.77 ± 0.36$^{de}$ | 3.75 ± 0.27$^{e}$ | 3.75 ± 0.51$^{e}$ | 3.92 ± 0.68$^{e}$ |
| **Gonad weight (g)** | - | - | - | 12.9 ± 5.3$^{a}$ | 13.6 ± 4.8$^{a}$ | 22.0 ± 6.7$^{ab}$ | 28.1 ± 13.4$^{b}$ | 32.3 ± 8.8$^{b}$ | 33.1 ± 8.4$^{b}$ | 98.6 ± 60.6$^{c}$ | 542.7 ± 232.1$^{d}$ | 688.1 ± 189.9$^{d}$ | 495.7 ± 45.5$^{d}$ | 484.4 ± 102.7$^{d}$ | 496.2 ± 90.8$^{cd}$ | 467.2 ± 52.2$^{d}$ |
| **Gonad density (g/cm³)** | - | - | - | - | - | 0.94 ± 0.2$^{a}$ | 0.96 ± 0.1$^{ab}$ | 1.03 ± 0.1$^{abc}$ | 1.05 ± 0.1$^{bcd}$ | 1.04 ± 0.0$^{bc}$ | 1.16 ± 0.1$^{e}$ | 1.07 ± 0.0$^{bcde}$ | 1.09 ± 0.0$^{cde}$ | 1.10 ± 0.0$^{de}$ | 1.11 ± 0.0$^{de}$ | 1.08 ± 0.0$^{de}$ |
| **Left testis length(cm)** | - | - | - | - | - | 20 ± 4$^{b}$ | 13 ± 4$^{a}$ | 13 ± 2$^{a}$ | 15 ± 3$^{ab}$ | 16 ± 3$^{ab}$ | 25 ± 5$^{c}$ | 29 ± 3$^{c}$ | 27 ± 4$^{c}$ | 26 ± 3$^{c}$ | 29 ± 4$^{c}$ | 28 ± 6$^{c}$ |
| **Left testis proportion** | - | - | - | 54.7 ± 7.0 | - | 56.1 ± 5.9 | 54.5 ± 4.6 | 54.1 ± 4.5 | 59.7 ± 7.0 | 54.7 ± 6.5 | 56.6 ± 4.5 | 54.1 ± 5.2 | 52.8 ± 4.9 | 51.9 ± 2.9 | 56.5 ± 2.4 | 55.5 ± 3.2 |
| **Fish sampled (N)** | 16 | 19 | 10 | 12 | 11 | 14 | 14 | 12 | 12 | 20 | 20 | 5 | 5 | 5 | 5 | 5 |

Salmon in sea cages (sea phase)

Salmon in fresh water tanks (fresh water phase)

Salmon in fresh water tanks, temperature dropped (cold water phase)

The largest differences in gonad weight between the fish from the same sampling were found on 1.7.2015, where the largest gonads weighed ~880 g and the smallest ~210 g while the difference in body weight of the fish in question was only ~1 kg (Figure 10). The correlation between fish weight and gonad weight was low (0.31).

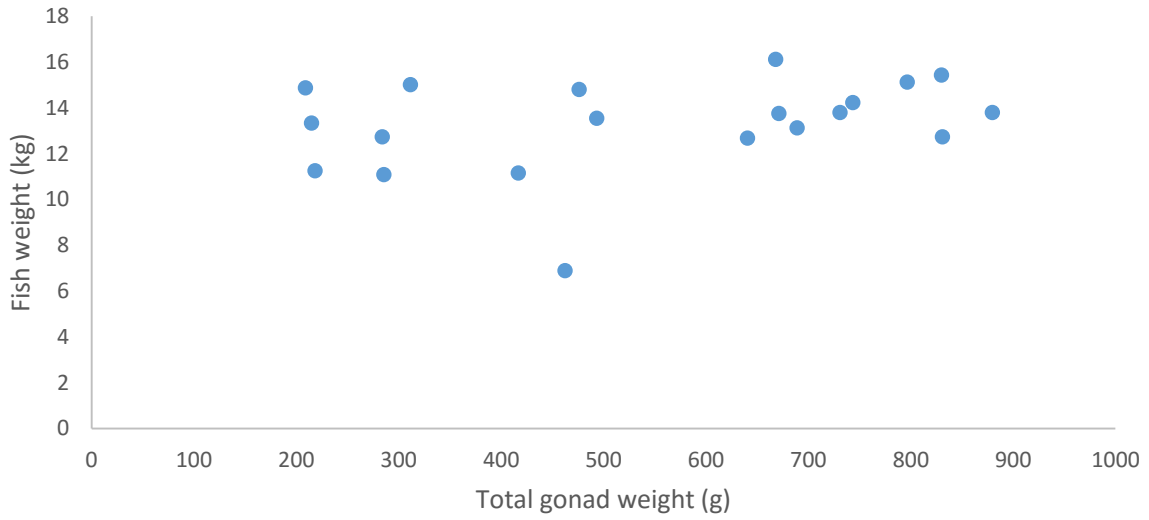



Figure 10. Total gonad weight plotted against fish weight from the fish sampled on 1.7.2015 shows considerable variations in total gonad weight.

## 3.2 Overall progression of spermatogenesis

During the first nine months of the experiment when the salmon were kept at sea, the gonads remained small and the tissue consisted mainly of testicular somatic cells. The only germ cells present were spermatogonia (Figure 11). In the first sampling of the experiment only A-type spermatogonia were observed. B-type spermatogonia were present in some samples from October onwards. The amount of spermatogonia and the number of germ cells in the cysts increased quite steadily throughout the sea phase. Sertoli cells could be clearly seen surrounding the germ cells and forming the spermatocysts during the first four months of the sea phase, but were not so distinctively seen after that.

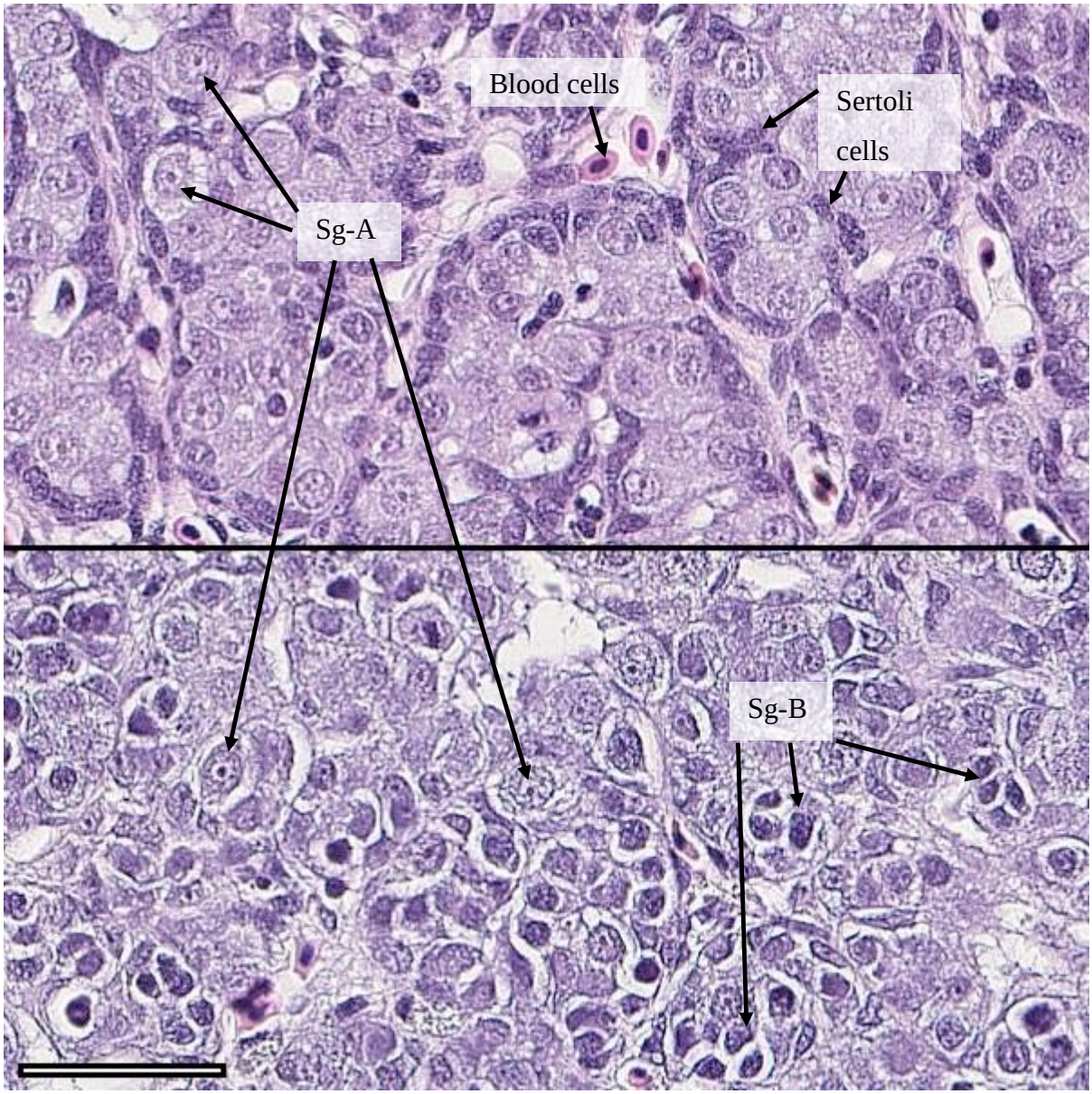



Figure 11. Photomicrographs (scale bar = 50 µm) from the samplings in the beginning (upper, 2.9.2014) and the end (4.5.2015) of the sea phase. A-type spermatogonia (Sg-A) were the most common cells and B-type spermatogonia (Sg-B) were the most developed germ cells present.

Rapid proliferation of germ cells was observed soon after the fish were transferred to fresh water. Clear changes from the previous sampling were observed in testis size and tissue composition. Most of the spermatocysts contained B-type spermatogonia and the number of cells in the cysts had increased (Figure 12). Average gonad weight had tripled in one month and meiotic divisions, where spermatogonia B become primary spermatocytes, had begun in three of the ten samples. Spermatocytes were distinctly present in these three samples with over 25 % of the germ cells being at spermatocyte stage. The same three samples also contained a small amount of spermatids (1-3 % of all germ cells).

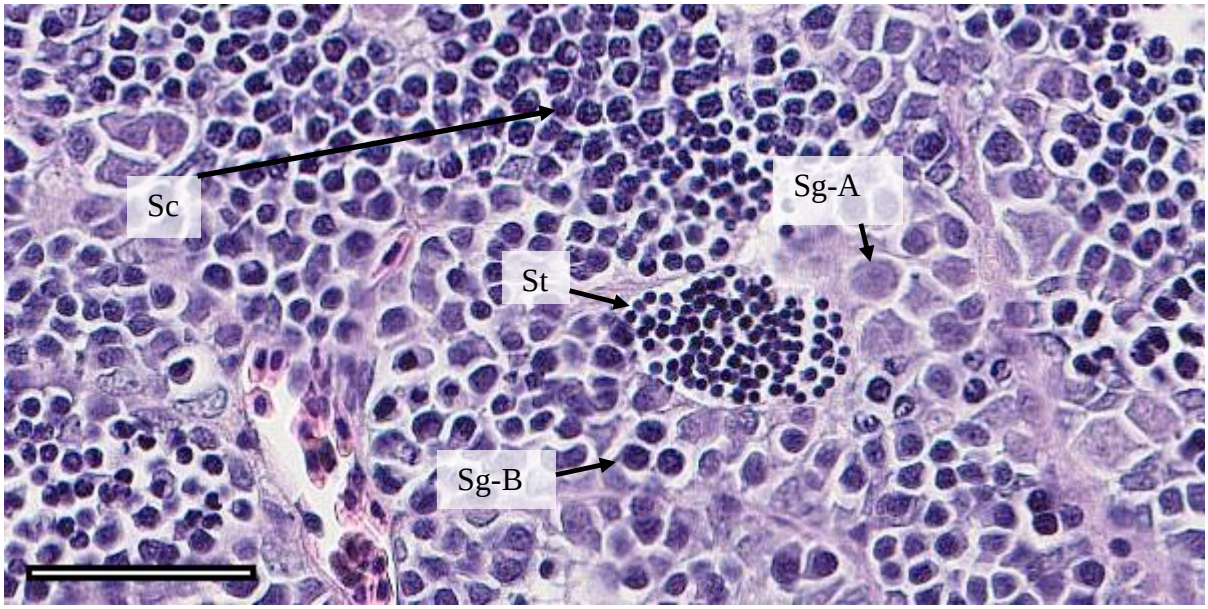



Figure 12. Sample from June (9.6.2015). Scale bar = 50 µm. Spermatogonia A and B (Sg-A, Sg-B), spermatocytes (Sc) and spermatids (St). Spermatogonia were still the most abundant germ cells, but spermatocytes were already found on half of the samples. The number of Sg-B in the cysts was seemingly higher than in previous samples.

In July, all the fish had reached meiotic stage in spermatogenesis and spermatocytes were the dominating cell type in every sample (Figure 13). Second meiotic divisions had also taken place since spermatids were observed in all but one fish. Secondary spermatocytes were rarely found. The first spermatozoa were observed in this sampling and they were absent in only three of the twenty samples.

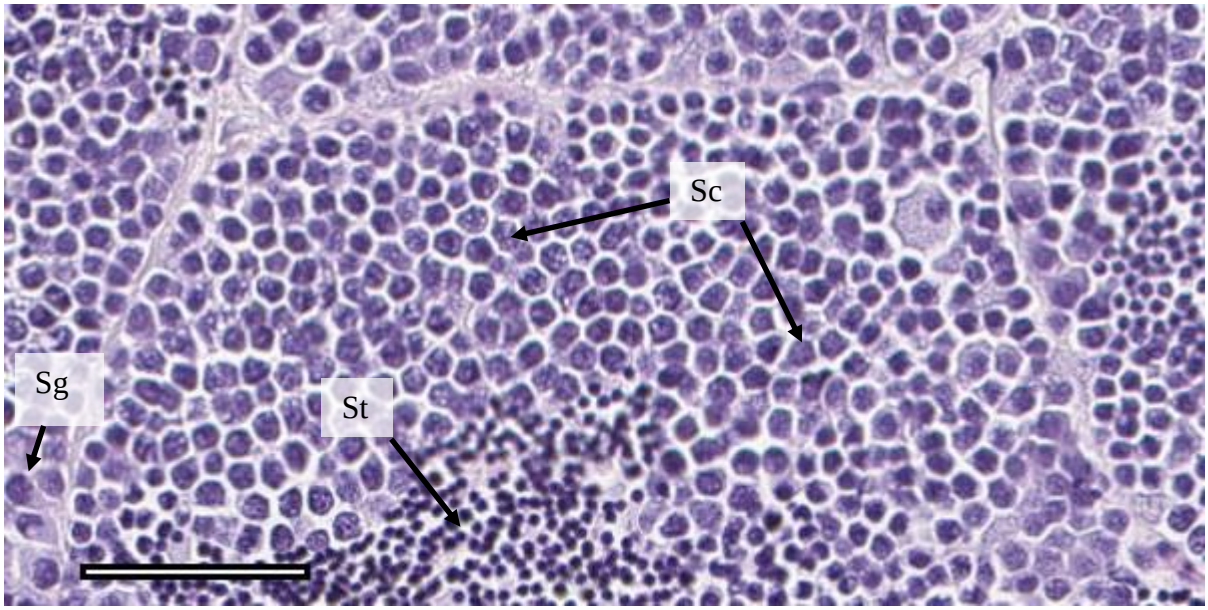



Figure 13. Spermatogonia (Sg) were no longer the most common cell type in July, but they were still found in small amounts, mainly in between the cysts. Spermatocytes (Sc) were the dominating cell type and spermatids (St) were found in all but one sample. Scale bar = 50 µm.

In the first weekly sampling on August (7.8.2015), considerable individual variation was found in both the testis size and progression of spermatogenesis. Three of the five fish had mature spermatozoa as the dominating cell type and they had reached the spermiogenic stage. The cyst walls had mostly broken off and spermatozoa had been released into the lobule lumen. Spermatocysts containing germ cells from earlier developmental phases, mainly spermatocytes and spermatids, were also present in these three samples. The other two samples were still at meiotic phase and consisted mostly of spermatocytes, but they were the two largest testis in this sampling (Figure 14).

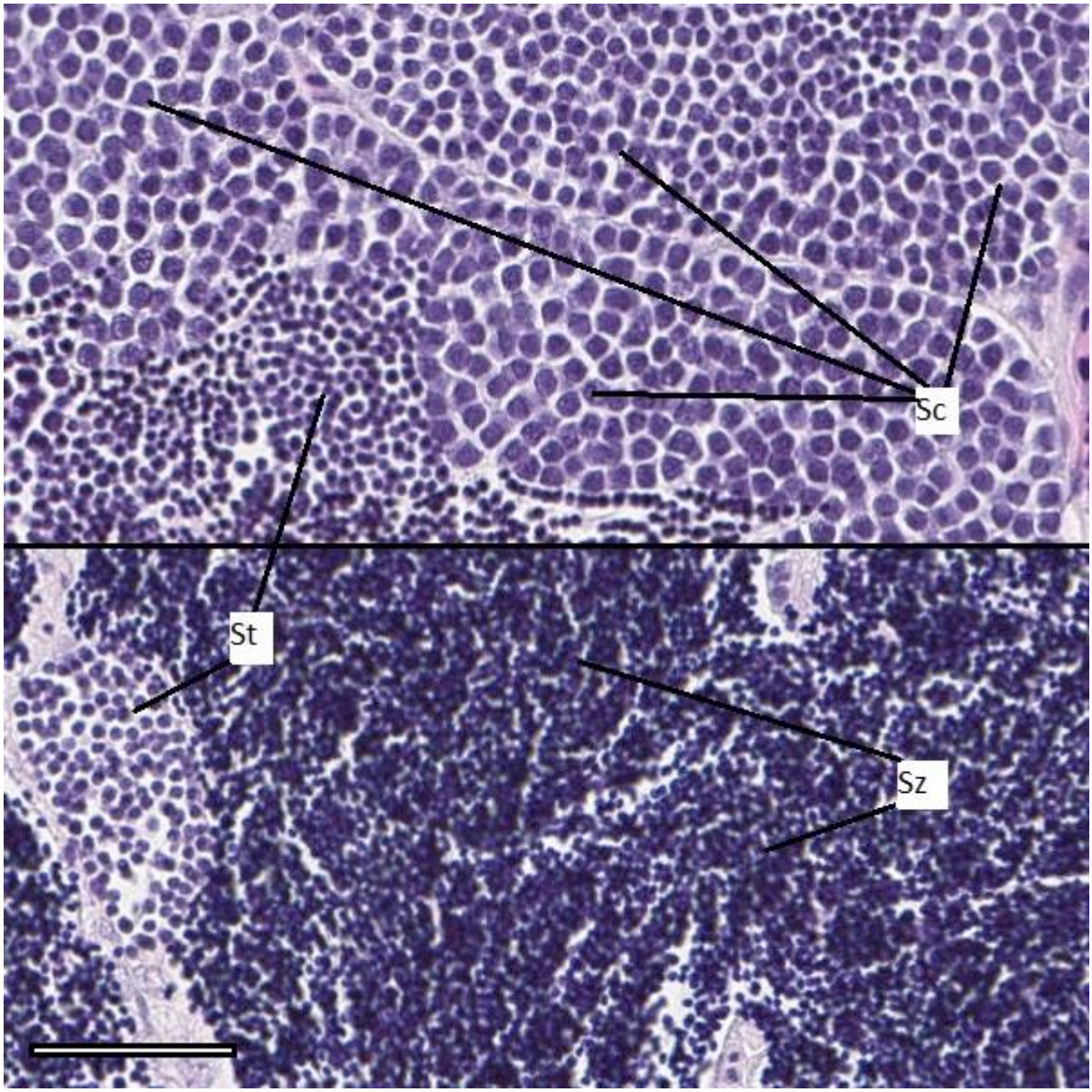



Figure 14. Two samples from the first week of August (7.8.2015). In the bigger testis (upper), the cyst walls are still intact and spermatocytes (Sc) and spermatids (St) are the dominating cell types. In the smaller testis (lower), cyst walls are mostly broken and mature spermatozoa (Sz) are freely in the lobule lumen. Scale bar = 50 µm.

During the last four samplings (13.8.; 20.8.; 27.8. and 2.9.2015) when the temperature had been dropped, the proportion of spermatocytes and spermatids decreased as they developed into spermatozoa (Figure 15). Spermatozoa were the dominating cell type in all except one sample that hadn't reached the spermiogenic stage. However, a few cysts with spermatids, spermatocytes or both were found in every sample. Spermatogonia were only found as the A-type, mainly in between the cysts.

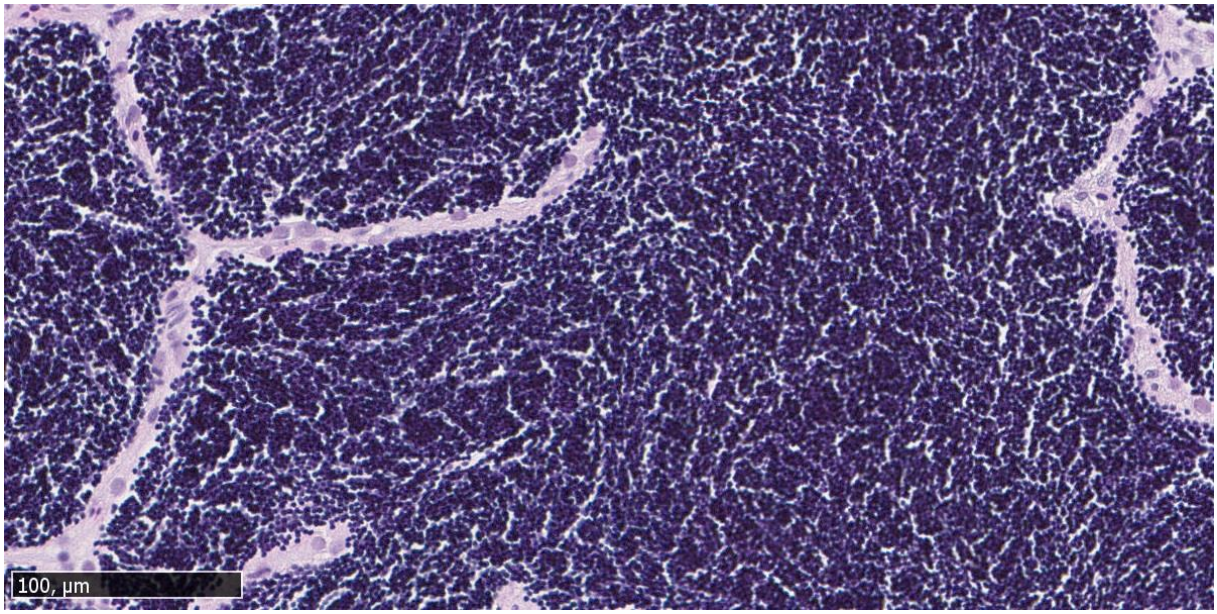



Figure 15. Photomicrograph of a mature Atlantic salmon testis. Spermatozoa are clearly the dominating cell type and they are released in the lobule lumen.

## 3.3 Area fractions, SMI and GSI

In the first sampling of the experiment (2.9.2014), the mean spermatogenic maturity index, SMI, was 0.03 ± 0.01 and testis tissue consisted of mean 11.4 ± 3.6 % spermatogonia while the rest of the area was testicular somatic cells (Figure 16). There were no significant changes in the spermatogonia proportion between the first five months, but after 2.2.2015 the mean fraction of spermatogonia started to increase and reached a mean proportion of 36.8 ± 10.9 % in the last sampling before the fresh water transfer (Table 4). At the same time B-type spermatogonia were found in increasing proportions and SMI had a mean of 0.09 ± 0.03.

Rapid changes in testis structure were observed after the fish were transferred to fresh water (Figure 16). The first spermatocytes appeared on 9.6.2015 and their area fraction reached a peak on 1.7.2015 (mean 66.0 ± 9.8 %), followed by a significant decrease to a mean level of 24.8 ± 28.7 % on 7.8.2015 (Table 4). Spermatids also first appeared on 9.6.2015 with a mean proportion of 0.6 ± 1.1 %. The highest mean area fractions for spermatids were observed on 1.7.2015 (19.7 ± 8.9 %), but there were no significant differences between 1.7.2015 and 7.8.2015. Spermatozoa appeared on 1.7.2015 with a mean proportion of 3.4 ± 3.4 % but on 7.8.2015 their proportion had significantly increased and they were the dominating cell type with a mean proportion of 48.2 ± 36.3 %. These rapid changes in the testis tissue composition were reflected in the SMI as it quickly rose significantly from a mean of 0.19 ± 0.08 to 0.75 ± 0.14 during the fresh water period (9.6.2015 - 7.8.2015).

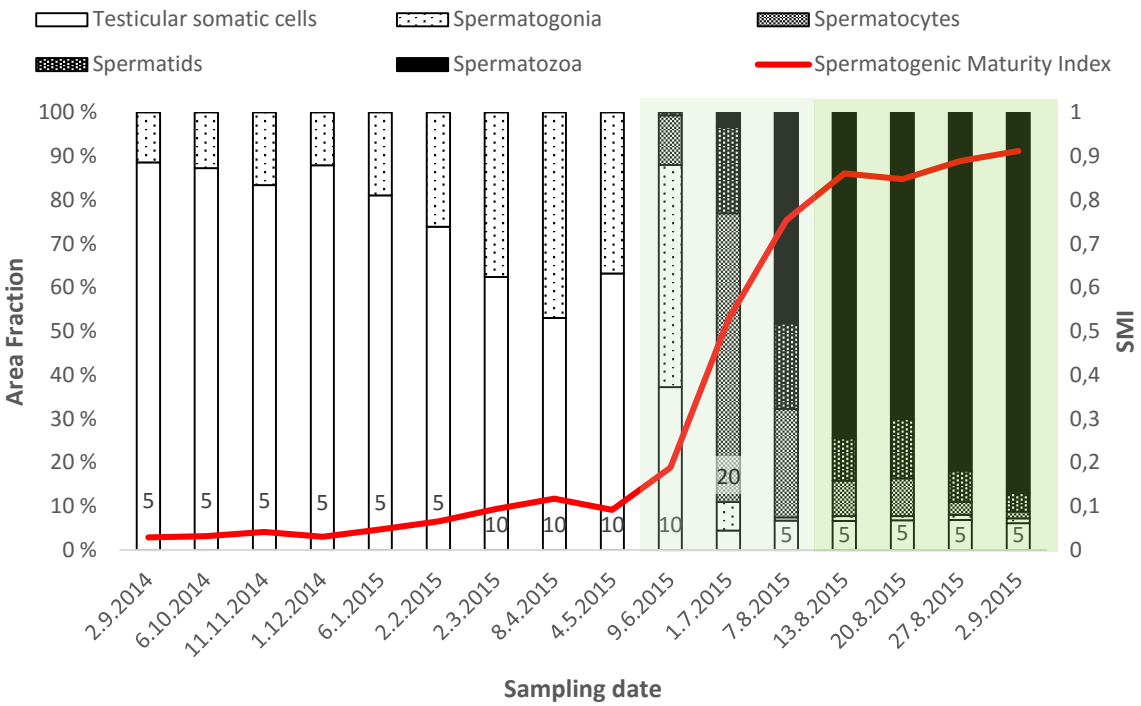



Figure 16. Progression of spermatogenesis in salmon during the last year of maturation. Area fraction estimations for different cell types are represented as bars and spermatogenic maturity index (SMI) as the solid line for different sampling dates. The numbers represent the number of fish and the background colouring indicates the production phase (Table 4).

During the cold water phase (13.8.2015 – 2.9.2015), the changes in area fractions and SMI between consecutive samplings were not as dramatic as before since the sampling was done on a weekly basis and the fish begun to reach full maturity (Figure 16). Spermatozoa were the dominating germ cells throughout the cold water phase and their average area fraction increased from a mean of 74.5 ± 12.9 % to a maximum mean of 87.0 ± 8.8 % by the end of this period. However, significant differences in spermatozoa proportions were not found during this period (Table 4). Area fractions for spermatids fluctuated between means of 4.2 ± 4.6 and 13.5 ± 11.4 % and for spermatocytes they were between 1.6 ± 2.8 and 8.6 ± 9.1 %. Significant differences between samplings were not found in either cell type during the cold water phase. Mean SMI increased slowly until the end from 0.86 ± 0.05 (13.8.2015) to a peak value of 0.91 ± 0.05 (2.9.2015), but significant differences in SMI between the samplings were not found.

Table 4. Area fractions of different germ cells as percentages and the SMI and GSI from the same fish. Values are mean ± SD and the letters indicate significant differences between months.

| | 2014 | | | | 2015 | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **2. Sep** | **6. Oct** | **11. Nov** | **1. Dec** | **6. Jan** | **2. Feb** | **2. Mar** | **8. Apr** | **4. May** | **9. Jun** | **1. Jul** | **7. Aug** | **13. Aug** | **20. Aug** | **27. Aug** | **2. Sep** |
| **Sperma-togonia (%)** | 11.4 ± 3.6$^{bcd}$ | 12.7 ± 2.4$^{cde}$ | 16.6 ± 2.9$^{cde}$ | 12.1 ± 2.0$^{cd}$ | 18.9 ± 2.4$^{de}$ | 26.1 ± 4.7$^{ef}$ | 37.6 ± 6.6$^{fg}$ | 47.0 ± 6.1$^{gh}$ | 36.8 ± 10.9$^{fgh}$ | 50.8 ± 8.8$^{h}$ | 6.5 ± 7.4$^{bc}$ | 0.7 ± 0.9$^{a}$ | 1.1 ± 0.8$^{abc}$ | 1.0 ± 0.8$^{a}$ | 1.2 ± 0.5$^{a}$ | 1.1 ± 1.0$^{ab}$ |
| **Sperma-tocytes (%)** | - | - | - | - | - | - | - | - | - | 11.4 ± 18.3$^{ab}$ | 66.0 ± 9.8$^{b}$ | 24.8 ± 28.7$^{a}$ | 8.1 ± 9.3$^{a}$ | 8.6 ± 9.1$^{a}$ | 3.0 ± 2.6$^{a}$ | 1.6 ± 2.8$^{a}$ |
| **Sperma-tids (%)** | - | - | - | - | - | - | - | - | - | 0.6 ± 1.1$^{a}$ | 19.7 ± 8.9$^{b}$ | 19.6 ± 12.9$^{b}$ | 9.7 ± 8.6$^{ab}$ | 13.5 ± 11.4$^{ab}$ | 7.1 ± 6.2$^{a}$ | 4.2 ± 4.6$^{a}$ |
| **Sperma-tozoa (%)** | - | - | - | - | - | - | - | - | - | - | 3.4 ± 3.4$^{a}$ | 48.2 ± 36.3$^{ab}$ | 74.5 ± 12.9$^{b}$ | 70.1 ± 22.2$^{b}$ | 81.8 ± 8.9$^{b}$ | 87.0 ± 8.8$^{b}$ |
| **SMI** | 0.03 ± 0.01$^{a}$ | 0.03 ± 0.01$^{ab}$ | 0.04 ± 0.01$^{ab}$ | 0.03 ± 0.01$^{a}$ | 0.05 ± 0.01$^{ab}$ | 0.07 ± 0.01$^{bc}$ | 0.09 ± 0.02$^{cd}$ | 0.12 ± 0.02$^{de}$ | 0.09 ± 0.03$^{cd}$ | 0.19 ± 0.08$^{e}$ | 0.53 ± 0.05$^{f}$ | 0.75 ± 0.14$^{fg}$ | 0.86 ± 0.05$^{g}$ | 0.85 ± 0.09$^{g}$ | 0.89 ± 0.03$^{g}$ | 0.91 ± 0.05$^{g}$ |
| **GSI** | - | - | - | 0.12 ± 0.02$^{a}$ | 0.13 ± 0.04$^{a}$ | 0.18 ± 0.03$^{ab}$ | 0.24 ± 0.11$^{ab}$ | 0.22 ± 0.07$^{ab}$ | 0.24 ± 0.07$^{ab}$ | 0.82 ± 0.65$^{b}$ | 4.13 ± 1.71$^{c}$ | 4.83 ± 1.17$^{c}$ | 3.77 ± 0.36$^{c}$ | 3.75 ± 0.27$^{c}$ | 3.75 ± 0.51$^{c}$ | 3.92 ± 0.68$^{c}$ |
| **Fish sampled (N)** | 5 | 5 | 5 | 5 | 5 | 5 | 10 | 10 | 10 | 10 | 20 | 5 | 5 | 5 | 5 | 5 |

Salmon in sea cages (sea phase)

Salmon in fresh water tanks (fresh water phase)

Salmon in fresh water tanks, temperature dropped (cold water phase)

The progress of GSI aligned fairly well with the development of SMI (Figure 17). GSI remained small throughout the sea phase and reached a mean of 0.24 ± 0.07 by 4.5.2015. Gonad size and GSI started increasing rapidly after the fish were moved to fresh water. By the first sampling of the fresh water phase (9.6.2015), GSI had reached an average of 0.82 ± 0.65. In the last sampling before cold water phase (7.8.2015), GSI reached the highest mean values for this experiment (4.83 ± 1.17). The gonad weight increased sixfold between the first and last sampling of the fresh water phase. Major difference between the developments of the two indexes (SMI and GSI) happened during the cold water phase. Mean GSI declined from the peak value from 7.8.2015 to a mean of 3.77 ± 0.36 by the next sampling (13.8.2015). However, significant differences in GSI were not found between these two samplings or any other samplings during the cold water phase (Table 4). Mean SMI kept increasing until the and reached a mean of 0.91 ± 0.05 on the last sampling (2.9.2015), but significant differences in SMI between the samplings during the cold water phase were not found.

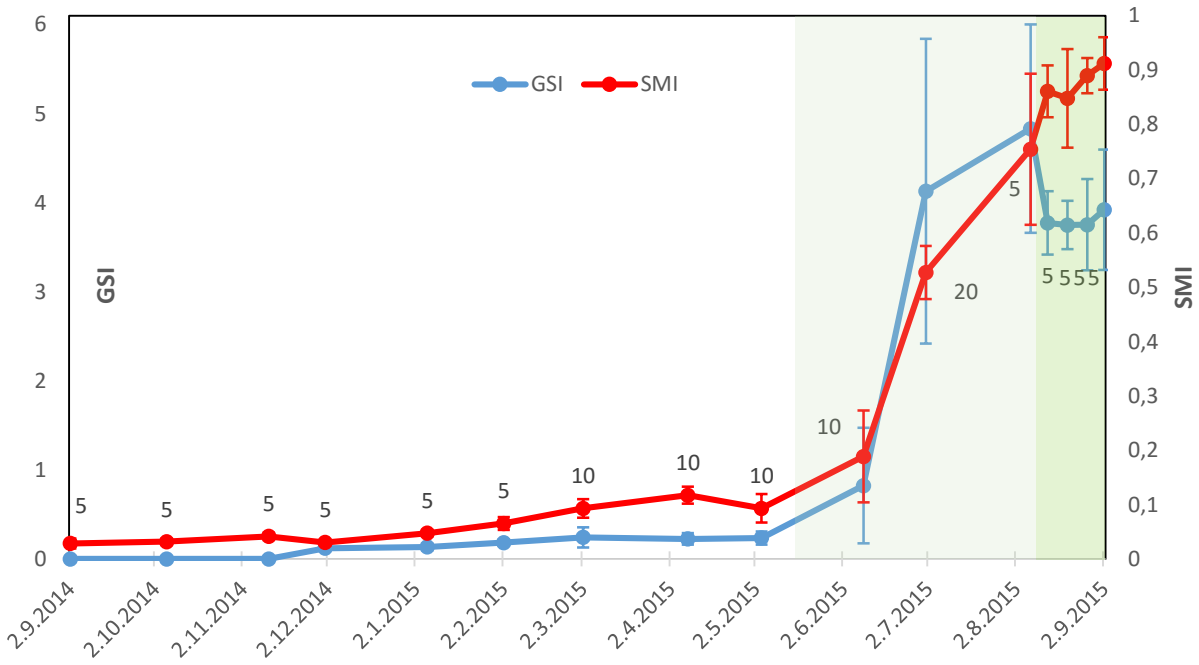



Figure 17. Salmon testis development during the last year of maturation. SMI and the corresponding gonadosomatic maturity index (GSI) for the same fish. Data is based on the mean values from each sampling and error bars represent ±SD. The dates on x-axis represent a time scale, not the actual sampling dates. The numbers represent the number of fish and the background colouring indicates the production phase (Table 4).

Some differences were also observed in the amounts of variation in GSI and SMI in the fish between months (Figure 18). The highest variations were found on samplings during the fresh water period (9.6.; 1.7. and 7.8.2015). In June and first week of August both indexes had relatively high fluctuation. In July only GSI had considerable variation and SMI was relatively

stable around 0.5-0.6. The relationships of GSI and SMI for individual fish also showed considerable differences between the samplings during the fresh water phase. In June, the general trend was that the fish with the higher GSI also had higher SMI. In July the SMI stayed relatively stable despite the considerable fluctuation in GSI. In the first sampling of August, the fish with highest GSI values had the lowest SMI.

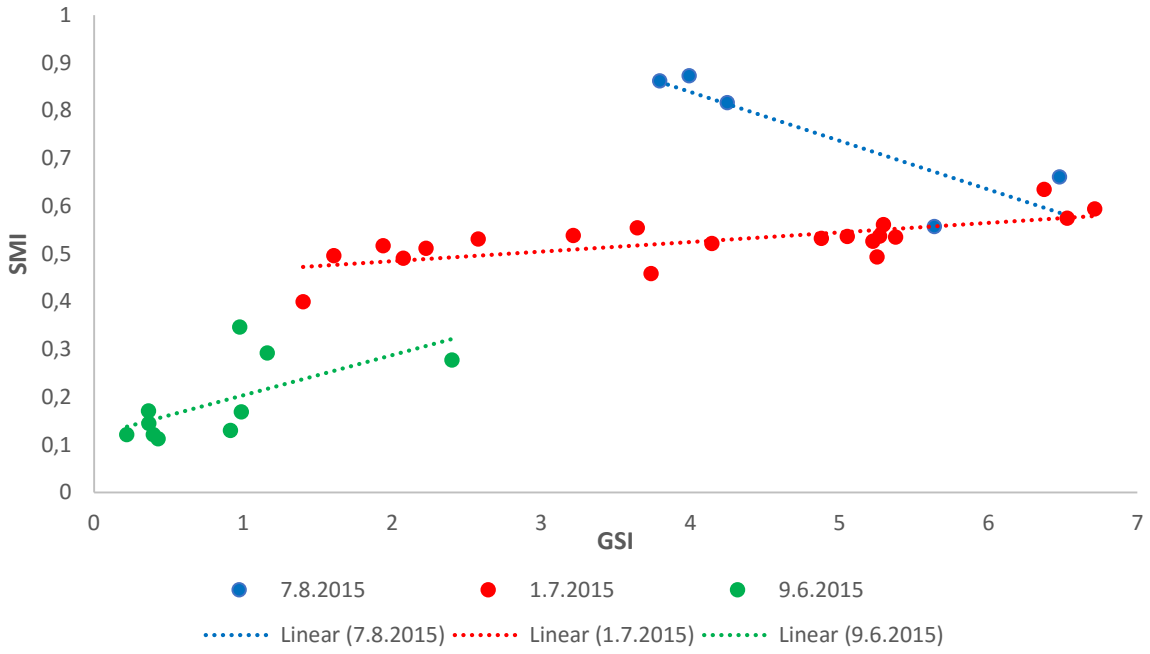



Figure 18. SMI plotted against GSI on individual fish from the first three samplings of the fresh water phase.

## 3.4 Ultrasound based GSI and volume calculations

All ultrasound methods underestimated the GSI during the first five months when gonad volumes were fairly small (Figure 19). The graphs from ultrasound GSI followed the progression of true GSI quite well until July, when the differences between methods became more apparent. The ultrasound method graphs didn't follow the changes of true GSI very well but they had similar changes with each other. The sectioned tube calculations had a tendency for underestimation except for the samplings on 1.7.2015 and 27.8.2015, where the mean estimations were almost spot-on. The straight tube equation gave quite good mean estimations for every sampling except for the underestimation on the first sampling of August and the overestimation on the last week of August.

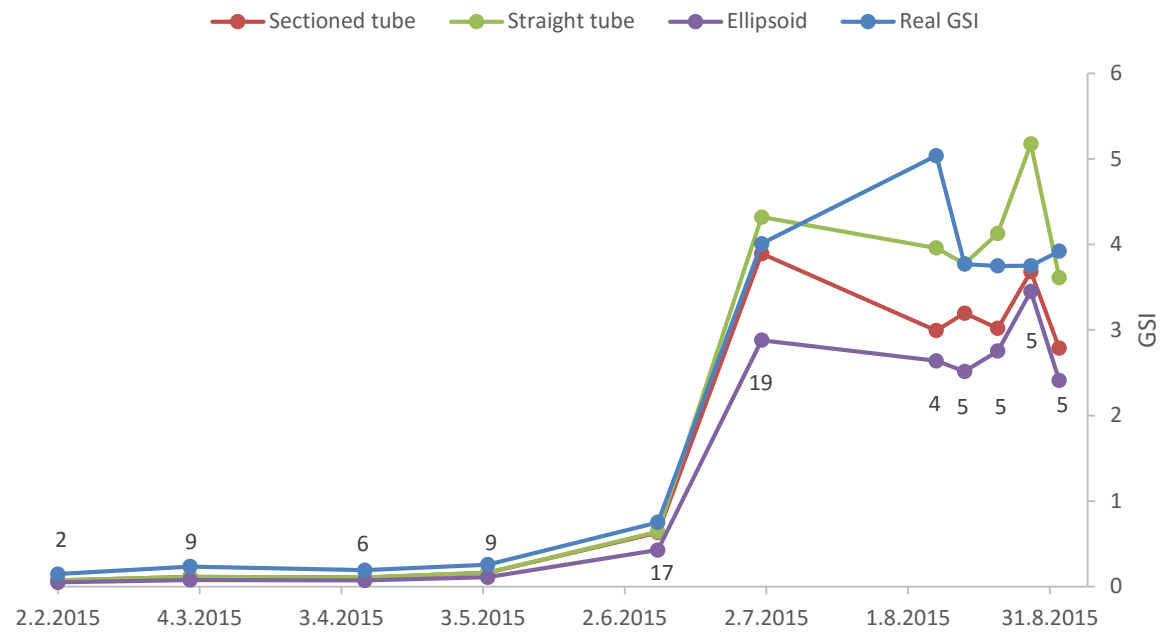



Figure 19. Development of the real GSI compared to ultrasound based GSI during the experiment. The GSIs are mean values that were calculated from every sample that had a clear enough ultrasound picture (numbers indicate the number of fish). The Real GSI values are from the same samples as the calculated ones. X-axis indicates a timeline, not sampling dates.

True GSI differed from every ultrasound GSI value in three of the eleven sampling dates ($p<0.05$) (Table 5). From all the equations used, the ellipsoid equation values were most often significantly different from the true GSI and there were only four months where significant difference was not found. Straight tube equation had seven and sectioned tube six months without significant differences to the true GSI. There were only two dates where the sectioned tube and straight tube GSI values had a significant difference with each other (20.8.2015 and 27.8.2015).

Table 5. GSI (mean ± SD) from true values from weighing and from the different ultrasound equations. Letters indicate significant differences between treatments within each month.

| Date | GSI (true) | GSI (Sectioned tube) | GSI (straight tube) | GSI (ellipsoid) |
|---|---|---|---|---|
| 2.2.2015 | 0,15 ± 0,0 | 0,07 ± 0,0$^{a}$ | 0,07 ± 0,0$^{a}$ | 0,05 ± 0,0$^{a}$ |
| 2.3.2015 | 0,23 ± 0,1 | 0,11 ± 0,1$^{a}$ | 0,11 ± 0,1$^{a}$ | 0,08 ± 0,1$^{a}$ |
| 8.4.2015 | 0,19 ± 0,0$^{a}$ | 0,11 ± 0,1$^{ab}$ | 0,11 ± 0,1$^{ab}$ | 0,07 ± 0,1$^{b}$ |
| 4.5.2015 | 0,26 ± 0,1 | 0,16 ± 0,1$^{a}$ | 0,16 ± 0,1$^{a}$ | 0,11 ± 0,1$^{a}$ |
| 9.6.2015 | 0,75 ± 0,5 | 0,63 ± 0,7 | 0,64 ± 0,7 | 0,43 ± 0,5 |
| 1.7.2015 | 4,01 ± 1,7$^{ab}$ | 3,89 ± 1,5$^{ab}$ | 4,32 ± 1,7$^{a}$ | 2,88 ± 1,1$^{b}$ |
| 7.8.2015 | 5,04 ± 1,2$^{a}$ | 2,99 ± 1,0$^{b}$ | 3,96 ± 0,8$^{ab}$ | 2,64 ± 0,5$^{b}$ |
| 13.8.2015 | 3,77 ± 0,4 | 3,20 ± 1,1 | 3,77 ± 1,0 | 2,52 ± 0,6 |
| 20.8.2015 | 3,75 ± 0,3$^{a}$ | 3,02 ± 0,5$^{b}$ | 4,13 ± 0,5$^{a}$ | 2,75 ± 0,3$^{b}$ |
| 27.8.2015 | 3,75 ± 0,5$^{a}$ | 3,68 ± 0,6$^{a}$ | 5,18 ± 0,9$^{b}$ | 3,45 ± 0,6$^{a}$ |
| 2.9.2015 | 3,92 ± 0,7$^{a}$ | 2,79 ± 0,8$^{ab}$ | 3,61 ± 1,0$^{ab}$ | 2,41 ± 0,6$^{b}$ |

The coefficient of determination ($R^2$-value) for linear regressions between true volume and ultrasound volume was highest on the straight tube and ellipsoid calculations ($R^2$=0.88), but not so much lower on the sectioned tube calculations ($R^2$=0.83) (Figure 20). The ultrasound method slightly underestimated the length of the testis throughout the experiment. Using only the testis length measurements to estimate gonad volume was not as accurate as when the cross section area was taken into account. Linear regression between ultrasound length and real volume resulted in $R^2$=0.70 and the variation increased along with the testis volume.

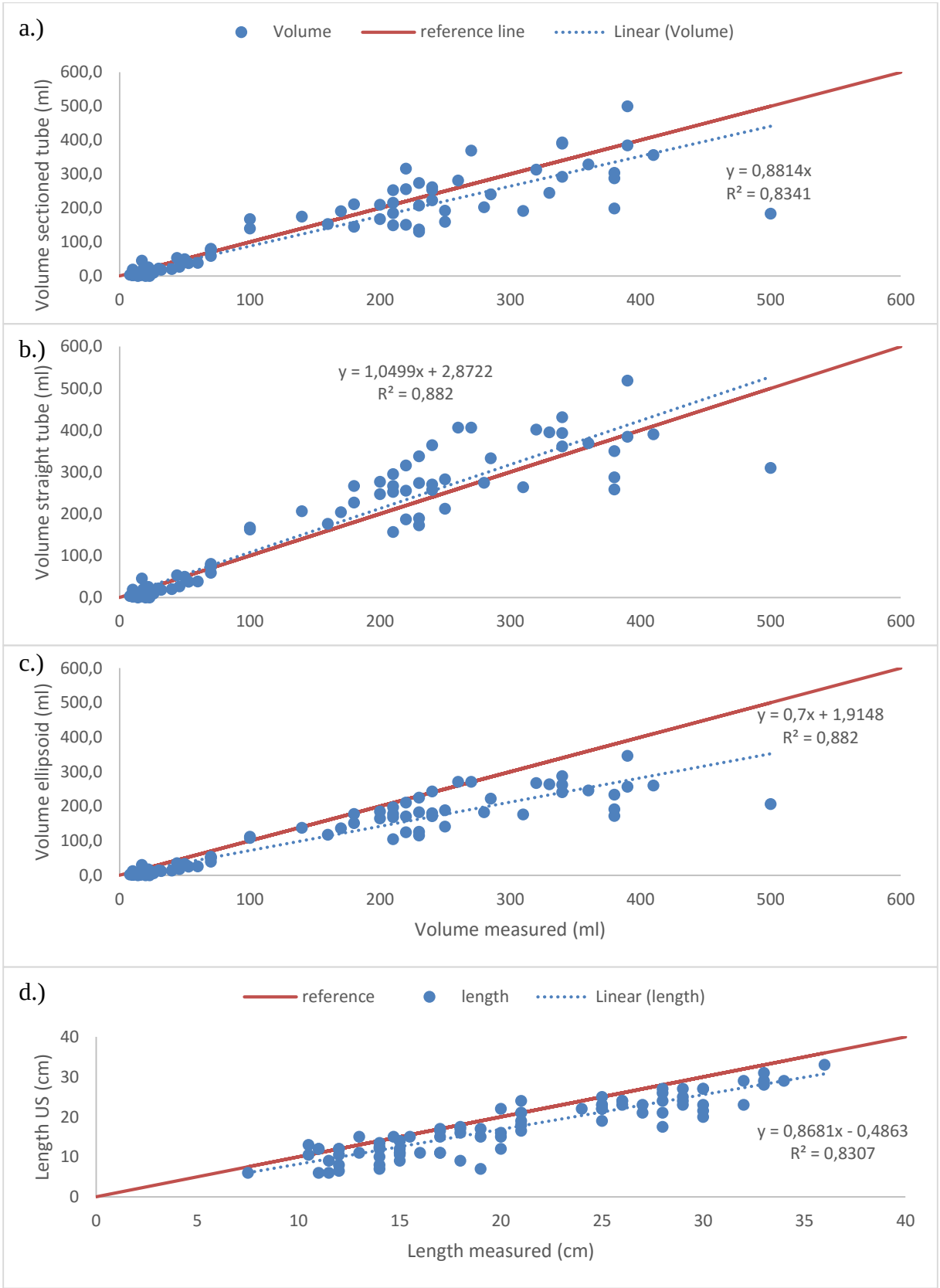



Figure 20. Regression relationships of conventional (water displacement method) and ultrasound based volume measurements (a-c) and conventional and ultrasound length measurements (d.). The dashed line represents the regression line and the solid line represents the 1:1 line.

All the ultrasound methods were very inaccurate when the gonads were small (2.2.-4.5.2015) and underestimated the testis volume (Table 6). High inaccuracy was also observed in sectioned tube and ellipsoid calculations when the gonads were at their largest (7.8.2015). The ultrasound measurements were the most accurate when the gonad volume was around 250 ml, but the errors had relatively high standard deviations. For example, on 13.8.2015 the average errors for sectioned tube and straight tube calculations were below ±10 % but they had high standard deviations. The directions of errors (positive or negative) and standard deviations were not very consistent for any method and the method with the least amount of error differed between months. The most consistency was found on ellipsoid equation, since it underestimated the volumes every month, but the fluctuation in SD was also relatively high.

Table 6. Monthly breakdown of average error when volumes calculated from ultrasound measurements were compared to the true volume. The values describe the error as a percentage (mean ± SD) from the true volume from that month.

| | | | Percentage error | | |
|---|---|---|---|---|---|
| **Sampling date** | **n** | **True volume (mean, ml)** | **Sectioned tube (%)** | **Straight tube (%)** | **Ellipsoid (%)** |
| 2.2.2015 | 2 | 9 | -64 ± 7 | -64 ± 7 | -76 ± 5 |
| 2.3.2015 | 9 | 16 | -56 ± 19 | -56 ± 19 | -71 ± 13 |
| 8.4.2015 | 6 | 15 | -48 ± 37 | -48 ± 37 | -65 ± 25 |
| 4.5.2015 | 9 | 18 | -34 ± 47 | -33 ± 48 | -55 ± 32 |
| 9.6.2015 | 17 | 52 | -27 ± 31 | -27 ± 31 | -51 ± 21 |
| 1.7.2015 | 19 | 258 | 9 ± 26 | 21 ± 26 | -19 ± 17 |
| 7.8.2015 | 4 | 380 | -37 ± 24 | -17 ± 21 | -45 ± 14 |
| 13.8.2015 | 5 | 240 | -7 ± 34 | 9 ± 30 | -27 ± 20 |
| 20.8.2015 | 5 | 230 | -11 ± 7 | 22 ± 8 | -19 ± 6 |
| 27.8.2015 | 5 | 252 | 2 ± 17 | 43 ± 14 | -5 ± 10 |
| 2.9.2015 | 5 | 239 | -28 ± 9 | -6 ± 19 | -37 ± 13 |

# 4 Discussion

The aim of this study was to describe the progression of spermatogenesis and how it varies between individual Atlantic salmon males. A histological assessment was made and compared to conventional GSI. The same males were also examined with ultrasound and the accuracy of ultrasound measurements was evaluated. The information gained from both examinations was put together to evaluate if ultrasound examinations could be a viable basis for sorting the fish to early and late spawners at an earlier stage of the production cycle.

## 4.1 Progression of spermatogenesis

From a histological perspective, the maturational progress of the salmon in this study was very similar to the patterns described earlier in salmonids by Dziewulska and Domagala (2003) and in Atlantic salmon by Melo et al. (2014). Spermatogenesis can be roughly divided into three phases: the mitotic phase with different types of spermatogonia, the meiotic phase with primary and secondary spermatocytes and spermiogenic phase with spermatids and spermatozoa (Nóbrega et al., 2009).

During the sea phase (2.9.2014-2.5.2015), which could be considered as the mitotic phase for the fish in this experiment, the gonad weight remained low and only low levels of germ cell proliferation were observed. Some of this proliferation may be associated with the allometric growth of the gonads and can also be found in non-maturing male testes (Melo et al., 2014). However, proliferation of Sertoli cells and presence of B-type spermatogonia was observed in the sampled fish already in the sampling on October 2014, demonstrating that the fish were committed to maturation. Atlantic salmon has two developmental switches that control gonad maturation (Mangel, 1994). Based on the physiological state of the fish and the environment, the fish makes its first decision on whether to continue gonad growth or switch it off approximately twelve months before spawning. The second switch takes place in the spring when the fish has to decide either to continue with the gonad growth or to abort it. This second decision has been hypothesized to coincide with the onset of meiosis (Campbell et al., 2003).

The onset of meiosis occurred after the fish were moved to fresh water tanks indoors. The transfer took place on 20.5.2015 and the first sampling of fresh water phase was 9.6.2015 Regardless of the short acclimation time and stress caused to the fish from transport, clear changes from the previous sampling were observed. Type B spermatogonia had proliferated and the number of cells in the cysts had increased. The most challenging identification task in

this study was to differentiate late spermatogonia B from early spermatocytes. However, the cystic structure of the testis was more apparent during the meiotic phase than during the earlier mitotic phase and it was easier to estimate the numbers of cells in the cysts. In salmonids, cysts of spermatogonia B contain up to 64 cells (Murza, 1983) (cited in Dziewulska and Domagala, 2003), which was sometimes used as a deciding factor when choosing between Sg-B and Sc. In July all the fish had advanced well into the meiotic phase and all but one fish had germ cells at spermatid stage. Secondary spermatocytes were rarely observed which is quite common as they go through the second meiotic division very quickly (Dziewulska and Domagala, 2003). During the last sampling before the cold water phase (7.8.2015), the fish could be clearly divided into two groups based on the stage of their spermatogenesis. Three of the samples were in spermiogenic phase with majority of the germ cells in spermatozoa stage and the two other samples were still in the meiotic stage with spermatocytes as the dominating cell type.

During the cold water phase (13.8.2015-2.9.2015), all the fish were at the spermiogenic stage. The cyst walls formed by Sertoli cells are broken down at the end of spermiogenesis and the spermatozoa are released in the lobule lumen. According to Schulz et al. (2010), the spermatozoa in the testis are still incapable of fertilizing eggs as they acquire motility only after the passage through the sperm duct. The process of gaining motility doesn't involve morphological changes in the spermatozoa (Schulz et al., 2010).

## 4.2 SMI as a measure of maturity

The importance of fecundity and egg production has caused female reproductive strategies and ovarian development to receive substantial attention compared to the study of male spermatogenesis (Tomkiewicz et al., 2011). The maturation of male fish has mainly been assessed by using the gonadosomatic index (GSI) and nominal classification based on microscopic observations (Kayaba et al., 2015). GSI alone has proven to be inadequate to describe the testis maturity due to overlapping within maturation classes (Dziewulska and Domagala, 2005). In previous histological studies (Hiroi and Yamamoto, 1968; Dziewulska and Domagala, 2005 and Melo et al., 2014) the males have been nominally classified to 5-9 maturity classes based on for example the most advanced germ cell found or other histological criteria. Spermatogenic maturity index (SMI) provides a quantitative scale based on the area fractions of different tissue types, which can be used to describe the maturational status of wild-captured or farmed fish (Tomkiewicz et al., 2011). Even though the progression of spermatogenesis varies from cyst to cyst, the maturation of different parts of the testis has been

found to be quite synchronous in chum salmon, *Oncorhynchus keta* (Hiroi and Yamamoto, 1968) and european eel, *Anguilla anguilla* (Tomkiewicz et al., 2011). Dziewulska and Domagala (2003) have reported that the histological changes are similar between three salmonids (sea trout, *Salmo trutta trutta*; brown trout, *Salmo trutta fario* and salmon, *Salmo salar L.*), which supports the theory that the area fractions from one section can accurately represent the whole testis in this study.

In the present study, the changes in average SMI followed the progression of GSI fairly well until the fish begun to reach spermiogenic stage (7.8.2015) and mean GSI started to decline (Figure 17). The correlation coefficients (Pearson) between SMI and GSI were 0.90 between 2.9.2014 and 7.8.2015 and 0.25 between 13.8.2015 and 2.9.2015. Identifying additional cell types (Sg-B and different stages of spermatocytes and spermatids) and adding separate weight factors to them might improve the correlation before spermiogenic phase when the testes are still growing, but not after the weight starts decreasing. Identifying the different stages accurately would also be a lot more laborious and probably require electron microscopy.

A decrease in testis weight before completing maturation has been observed earlier in chum salmon (Hiroi and Yamamoto, 1968) and brown trout (Billard, 1983). Spermatocysts in teleost testis have reached maximum size when the germ cells are well into the meiotic stage (Schulz et al., 2005). Highest GSI was also observed in brown trout (*Salmo trutta fario*) when the amount of spermatocytes was at its highest (Billard, 1983). All these statements support the theory that there is an actual decline in GSI before final maturation even though a statistically significant decrease in GSI was not found in this study. The main reasons for not finding a significant difference are most likely the low sample numbers and high standard deviation observed in 1.7.2015 and 7.8.2015.

The decline in GSI can be explained by the lack of cell divisions after the second meiotic division where secondary spermatocytes turn into spermatids. The spermatids go through a three stage metamorphosis during which they shed their so-called residual bodies before becoming mature spermatozoa (Schulz et al., 2010). Loss of cellular material also happens due to apoptosis. Although teleost spermatogenesis is a relatively efficient process, up to 30 % germ cell loss during spermiogenesis has been detected in tilapia (Vilela et al., 2003). Apoptotic cells were rarely observed in the histological sections in the present study, which is quite common since Sertoli cells are very effective at removing cellular debris (Schulz et al., 2010). Some reduction in gonad weight may also be caused by running milt, but that does not explain the

whole drop, since many testes had empty sperm ducts even during the samplings where the fish were at spermiogenic stage (Figure 21). The SMI kept increasing as the proportion of mature spermatozoa increased, indicating that in the final stretch of maturation, SMI is a better indicator of maturity than GSI.

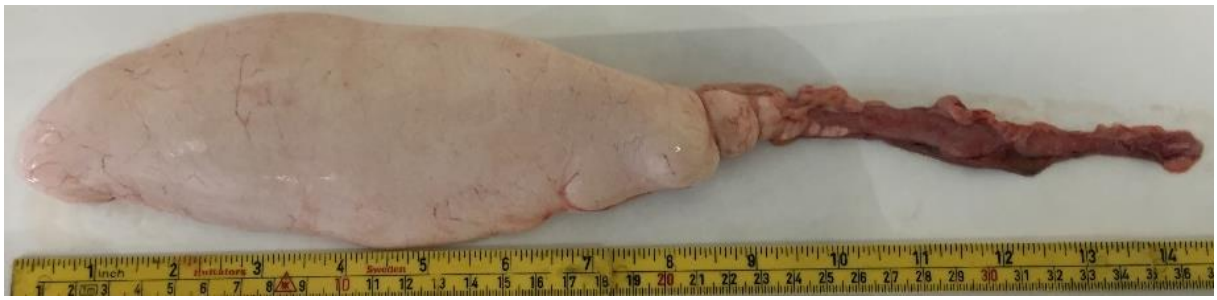


Figure 21. Dissected testis of a mature salmon. Picture is taken on 13.8.2015 when all the fish were at spermiogenic phase. The empty sperm duct is visible on the right side of the picture.

During July, the SMI was relatively stable (mean 0.53 ± 0.05) and spermatocytes were the dominating cell type in every sample. However, considerable variation in GSI was observed between individual fish as the values ranged from ~1.4 to ~6.7. High variation in GSI was also observed in brown trout one month before the GSI reached peak values, indicating that the progress of spermatogenesis is not strictly synchronous (Billard, 1983). The reason why SMI did not show such fluctuation is most likely the fact that SMI calculation formula gives the same weight factor to spermatocytes of all stages (preleptotene primary spermatocytes, leptotene/zygotene primary spermatocytes, pachytene spermatocytes, diplotene spermatocytes and secondary spermatocytes). Secondary spermatocytes are rarely observed and the two later stages of primary spermatocytes (pachytene and diplotene) have shown significantly larger cyst volumes in tilapia (Schulz et al., 2005) and zebrafish (Leal et al., 2009). According to Schulz et al. (2010), the meiotic and spermiogenic phases of spermatogenesis are relatively similar between teleosts, which supports the assumption that prior to spermiogenic stage, fish with higher GSI are also more mature in the present study. This also means that if the two indexes measuring maturity (SMI and GSI) are compared, there is no clear answer to which one is better but instead they complement one another.

## 4.3 Ultrasound analyses

Testis volume and GSI were estimated from a total of 86 fish by using the gonad length measured by ultrasound and testis cross section area measurements from ultrasound images. Three different geometric shapes with corresponding volume equations were assigned to represent the testis and the volumes and GSI were compared to the true values from sampling.

In the earlier samples (2.2.2015 - 4.5.2015) (Table 6) the gonads were difficult to identify from the ultrasound pictures since they were really small and dark in color, making them harder to differentiate from adjacent organs. The rib bones also caused shadows which sometimes partly covered the testis in the ultrasound images. Since the cross sections were very small, even slight deviations while tracing the testis edges with the software caused relatively large errors to the volume calculations, making the volume estimations very inaccurate early on. As the gonads got larger, they also got lighter in color in the ultrasound pictures and easier to point out. Small deviations while outlining the testis did not cause as large relative errors for volume calculations. However, problems emerged in the form of testis exceeding the borders of the ultrasound images when the testis were at their largest. This caused underestimation to the volume calculations especially with the ellipsoid and sectioned tube equations. Pictures with large testis that exceeded the borders were excluded and the remaining images for that sample were used. Gonad length was underestimated with ultrasound by 3 cm on average, which may also cause some underestimation (Figure 20 d). The underestimation in length compared to the ruler measurements might be due to removing the testis from its natural state inside the body cavity and straightening it out on the table, possibly making the testis slightly longer for the ruler measurements.

In the present study, emphasis was put on getting clear ultrasound images during the sampling and not so much on how the images represented the testis in question. The pictures were also not taken at positions where the testis was clearly too big to fit within the ultrasound image borders. In order to get accurate volume estimations from using the straight tube equation, the image should be taken at a position where the testis is at its average thickness (average cross section area). For most accurate estimations with the ellipsoid calculation, the image should be taken at a point where the testis is at its thickest. Mattson (1991) defined the thickest point by moving the ultrasound probe back and forth along the belly while looking at the screen of the ultrasound device. The thickest point (maximum cross section area) might be easier to define than the average point, making the ellipsoid equation potentially more convenient to use than the straight tube equation. The sectioned tube equation would most likely be more accurate if the pictures were taken at equidistant intervals in relation to the gonad length and at least three pictures were taken from each sample. A method similar to this (three pictures from equidistant points of the gonad) resulted in slight underestimations (-16.57 %) in gonad volume of Pallid sturgeon (*Scaphirhynchus albus*) (Bryan et al., 2007).

According to the graph (Figure 19) and the $R^2$-values in the regression analyses (Figure 20), the straight tube equation seems to give values closest to the true value. The $R^2$-value is a little bit higher ($R^2$=0.88) in both straight tube and ellipsoid calculations than the sectioned tube ($R^2$=0.83), but the linear trend line is closest to the reference line in straight tube equation. Some decline to the $R^2$-value of the sectioned tube estimations is due to different numbers of ultrasound images taken from each sample. However, the $R^2$-value only tells how well the data points fit the trend line equation and nothing about how close the values are to the true volumes. It is also questionable how useful the information of volumes from the early samples is and if only samples from July onwards are taken into account in the regression analyses, the $R^2$-values drop to below 0.5 with all three equations. If the ultrasound analyses are used to estimate the final volumes of milt that can be stripped from the males, the loss of cellular material during spermiogenesis has to be taken into account if the ultrasound measurements are done before the fish are at that stage.

## 4.4 Ultrasound examinations as a basis for sorting

Ultrasound could be a useful tool in sorting the fish into different cohorts, for example early and late spawners. Since the ultrasound only gives information about the gonad size, the images should be taken at a time when the differences in gonad size between individual fish is largest. In the present study the best timing would have been during the fresh water phase (9.6.-7.8.2015), when the fish were in meiotic phase. Earlier than that the differences in gonad size were really small and predicting the maturation speed would probably be impossible so far prior to spawning. After 7.8.2015 the individual variation in gonad size and maturation was not so large anymore, which would make it difficult to sort the fish by using ultrasound.

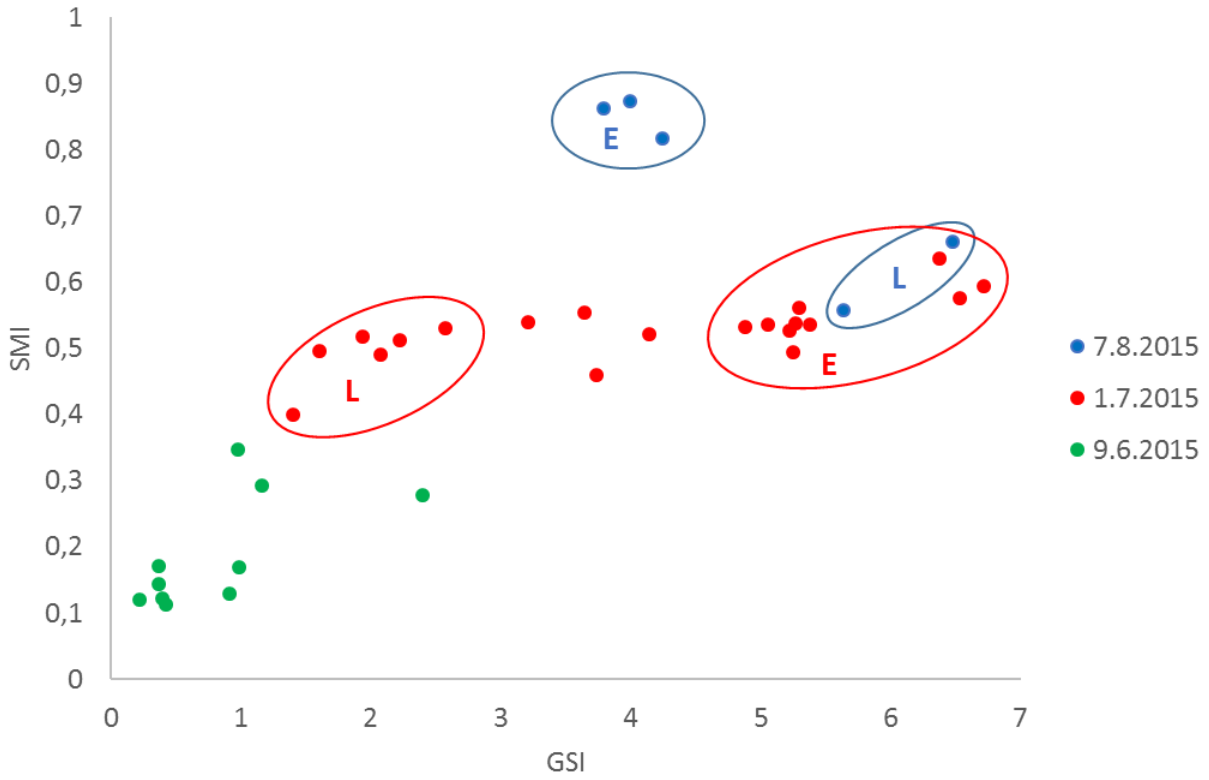



Figure 22. Relationship between GSI and SMI during the fresh water phase. The circles illustrate possible cohorts for the fish. Circles marked with E could be considered as early spawners or being further in maturation and circles with L are later spawners or less mature. Blue circle only concerns blue dots and red circle only red dots.

The size differences in June are still quite small and reliable sorting would be difficult (Figure 22). On 7.8.2015 three fish were already in spermiogenic phase so their gonad size had declined, making the fish with smaller GSI the earlier spawners of this batch. This is also supported by the hormone analysis done from the same fish by Hoque (2016), which showed the highest levels of MIS, a hormone for final maturation, on fish that had a GSI of roughly 3-4.

Since there is no way of telling the stage of spermatogenesis from the ultrasound image with the current knowledge, it would be somewhat impossible to reliably sort the fish if some of them were already at spermiogenic phase. In July the size differences of gonads in the fish with high and low GSI were so significant that they could be easily sorted based on that. As the fish were not yet in the spermiogenic phase, it would be quite safe to assume that the bigger testes would also be more mature, making the fish with high GSI the early spawners. During the time when the fish were at spermiogenic phase, the mean GSI was ~3.8 and there were no fish with GSI less than 3, which also supports the theory that the fish labelled as early in July (Figure 22) were still growing their gonads.

# 5 Conclusions and future aspects

The progression of spermatogenesis was fairly slow until the fish were transferred to fresh water indoors. After roughly 40 days in fresh water (20.5.2015-1.7.2015), all the fish had reached meiotic phase and the differences in maturation between the fish were greatest during that phase. In order to evaluate the timing and duration of the meiotic phase, it would have been beneficial to increase the sampling frequency already in June. After approximately 85 days in fresh water, all but one fish had reached spermiogenic phase and it would be relatively safe to initiate hormonal treatment. However, since so many factors affect the maturation (temperature, light, genetics, timing of fresh water transfer etc.), further studies would be needed to estimate the initiation and duration of the different spermatogenic phases for other batches of fish.

Ultrasound could be used as a basis for sorting the fish, but knowing the time when the fish are at meiotic phase in spermatogenesis would be essential. In the meiotic phase the differences in gonad size were the largest between males and it would be the easiest time to sort them. However, it would be important to know that there would be no fish that had passed the meiotic stage because the testis size decreases during spermiogenic phase, making it impossible to sort the fish to early and late spawners based on the testis size.

The accuracy of ultrasound measurements was not good enough to give accurate estimations of the final sperm yield. The accuracy could be improved by a more systematic way of taking the pictures. The ultrasound equipment/settings should be selected so that pictures could be taken even from the biggest testes without them exceeding the image borders. An additional ultrasound study could be made to study the development of testis in individual males by doing repeated measures on the same males with the use of PIT tags for example. This would possibly provide more information on the degree of the reduction of testis size during spermiogenic phase and the maximum testis size in the end of meiotic phase. Since the colour of testis tends to go lighter in the ultrasound images as the fish matures, additional information could be gained from analysing the connections between colour and SMI/GSI.

The fish that were studied in this experiment were also analysed for sex hormone concentrations by another master student (Hoque, 2016). The relationships between the hormone concentrations and histological assessment could provide additional information on the progress and timing of spermatogenesis.

# 6 References

Billard, R. (1983) A quantitative analysis of spermatogenesis in the trout, *Salmo trutta fario. Cell Tissue Res* 230, 495-502

Bromage, N., Porter, M. and Randall, C. (2001) The environmental regulation of maturation in farmed finfish with special reference to the role of photoperiod and melatonin. *Aquaculture* 197, 63–98

Bryan J.L., Wildhaber M.L., Papoulias D.M., DeLonay A.J., Tillit D.E. and Annis M.L., (2007) Estimation of gonad volume, fecundity, and reproductive stage of shovelnose sturgeon using sonography and endoscopy with application to the endangered pallid sturgeon. *J. Appl. Ichthyol*. 23, 411–419. Blackwell Publishing Ltd.

Campbell, B., Dickey, J.T. and Swanson, P. (2003) Endocrine changes during onset of puberty in male spring chinook salmon, *Oncorhynchus tshawytscha. Biology of reproduction* 69, 2109–2117

Duston, J. and Bromage, N. (1988) The entrainment and gating of the endogenous circannual rhythm of reproduction in the female rainbow trout (*Salmo gairdneri*). *J Comp Physiol A* 164, 259-268

Dziewulska, K. and Domagala, J. (2003) Histology of salmonid testes during maturation. *Reproductive Biology* 3(1), 47–61

Dziewulska, K. and Domagala, J. (2005) Differentiation of gonad maturation in sibling precocious males of the sea trout (*Salmo trutta m. trutta L*.) in their first year of life. *Aquaculture* 250, 713–725

Fischer, A.H., Jacobson, K.A., Rose, J. and Zeller, R. (2006) Hematoxylin and Eosin Staining of tissue and cell sections. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, USA.

Hiroi, O. and Yamamoto, K. (1968) Studies on the maturation of salmonid Fishes-I. Changes in the testis of the Chum salmon, *Oncorhynchus keta*, during anadromous migration. *Laboratory of Freshwater Fish Culture, Faculty of Fisheries, Hokkaido University*

Hoque, E.Md. (2016) Changes in hormone profiles in relation to ultrasound-determined gonadal maturation during a reproductive cycle in male Atlantic salmon (*Salmo salar*). *M.Sc. thesis, Norwegian University of science and technology (NTNU), Trondheim*

Kayaba, T., Wada, T., Murakami, O., Sawaguchi, S. and Kawabe, R. (2015) A new method for identifying the male spawning period of fish using sperm duct volume as an index. *Fisheries Research* 170, 199–204

Leal, M.C., Cardoso, E.R., Nóbrega, R.H., Batlouni, S.R., Bogerd, J., França, L.R. and Schulz, R.W. (2009) Histological and stereological evaluation of zebrafish (*Danio rerio*) spermatogenesis with an emphasis on spermatogonial generations. *Biology of reproduction* 81, 177–187

Loir, M. (1999) Spermatogonia of rainbow trout: II. In Vitro study of the influence of pituitary hormones, growth factors and steroids on mitotic activity. *Molecular reproduction and development* 53, 434–442

Mangel, M. (1994) Climate change and salmonid life history variation. *Deep-Sea Research II* 41, 75-106

Mattson, N.S. (1991) A new method to determine sex and gonad size in live fishes by using ultrasonography. *Journal of Fish Biology* 39, 673-677

Melo, M.C., Andersson, E., Fjelldal, P.G., Bogerd, J., França, L.R., Taranger, G.L. and Schulz, R.W. (2014) Salinity and photoperiod modulate pubertal development in Atlantic salmon (*Salmo salar*). *Journal of Endocrinology* 220, 319–332

Miura, T. (1999) Spermatogenetic cycle in fish. *Encyclopedia of reproduction* 4, 571–578. Academic press

Mylonas, C.C., Scott, A.P., Vermeissen, E.L.M. and Zohar, Y. (1997) Changes in plasma gonadotropin II and sex steroid hormones, and sperm production of striped bass after treatment with controlled-release gonadotropin-releasing hormone agonist-delivery systems. *Biology of reproduction* 57, 669–675

Mylonas, C.C., Fostier, A. and Zanuy, S. (2010) Broodstock management and hormonal manipulations of fish reproduction. *General and Comparative Endocrinology* 165, 516–534

Novelo, N.D. and Tiersch, T.R. (2012) A review of the use of ultrasonography in fish reproduction. *North American Journal of Aquaculture* 74, 169–181

Nóbrega, R.H., Batlouni S.R. and França, L.R. (2009) An overview of functional and stereological evaluation of spermatogenesis and germ cell transplantation in fish. *Fish Physiol Biochem* 35, 197–206

Schulz, R.W. and Miura, T. (2002) Spermatogenesis and its endocrine regulation. *Fish Physiology and Biochemistry* 26, 43–56

Schulz, R.W., Menting, S., Bogerd, J., França, L.R., Vilela, D.A.R. and Godinho, H.P. (2005) Sertoli cell proliferation in the adult testis—Evidence from two fish species belonging to different orders. *Biology of reproduction* 73, 891–898

Schulz, R.W., França, L.R., Lareyre, J-J., LeGac, F., Chiarini-Garcia, H., Nóbrega, R.H. and Miura, T. (2010) Spermatogenesis in fish. *General and Comparative Endocrinology* 165, 390–411

Taranger, G.L., Haux, C., Stefansson, S.O., Björnsson, B.T., Walther, B.T. and Hansen, T. (1998) Abrupt changes in photoperiod affect age at maturity, timing of ovulation and plasma testosterone and oestradiol-17β profiles in Atlantic salmon, *Salmo salar*. *Aquaculture* 162, 85–98

Taranger, G.L., Vikingstad, E., Klenke, U., Mayer, I., Stefansson, S.O., Norberg, B., Hansen, T., Zohar, Y. and Andersson, E. (2003) Effects of photoperiod, temperature and GnRHa treatment on the reproductive physiology of Atlantic salmon (*Salmo salar L*.) broodstock. *Fish Physiology and Biochemistry* 28, 403–406

Taranger, G.L., Carrillo, M., Schulz, R.W., Fontaine, P., Zanuy, S., Felip, A., Weltzien, F-A., Dufour, S., Karlsen, Ø., Norberg, B., Andersson, E. and Hansen, T. (2010) Control of puberty in farmed fish. *General and Comparative Endocrinology* 165, 483–515

Tomkiewicz, J. and Kofoed, T.M.N., 2011. Assesment of testis development during induced spermatogenesis in the European Eel *Anguilla anguilla. Marine and Coastal Fisheries: Dynamics, Management, and Ecosystem Science* 3, 106–118

Vilela, D.A.R., Silva, S.G.B., Peixoto, M.T.D., Godinho, H.P. and França, L.R. (2003) Spermatogenesis in teleost: insights from the Nile tilapia (*Oreochromis niloticus*) model. *Fish Physiology and Biochemistry* 28, 187–190

Webb, J.H. and McLay, A. (1996) Variation in the time of spawning of Atlantic salmon (*Salmo salar*) and its relationship to temperature in the Aberdeenshire Dee, Scotland. *Can. J. Fish. Aquat. Sci.* 53, 2739–2744

# 7 Appendixes

*Appendix 1 – Ewos opal Breed 3500 fact sheet*

OPAL

## EWOS opal Breed

Ekstrudert, fullverdig fôr til laks og ørret

***Sammensetning g/kg fôr:***

| | **3500** |
|---|---|
| **Protein:** | 380-420 |
| **Fett:** | 315-355 |
| **Vann:** | 50-80 |
| **Aske:** | 55-70 |
| **Fiber:** | 7-15 |
| **Nitrogenfrie forbindelser:** | 135-160 |
| **Astaxanthin (mg/kg):** | 50 |

***Energi, MJ/kg***

| | |
|---|---|
| **Brutto energi, MJ/kg:** | 24,3-25,7 |

***Råvarer:***

Fiskemel, fiskeolje, hvete, soyaproteinkonsentrat, hvetegluten, solsikke, mineraler, vitaminer, astaxanthin, Prebiosal

***Brukeranbefaling:***

Anbefalt fôr til stamfisk, fra 12 til 9 måneder før kjønnsmodning, til stryking.

| **Fôrtype** | **Fiskestørrelse** |
|---|---|
| 3500 | 3500 g -> |

Gjenstand for mindre endringer

Dato: Juni 16, 2010

**EWOS AS**
Adresse: Postboks 4, Sentrum, 5803 Bergen

EWOS

## *Appendix 2 – Temperature profile*

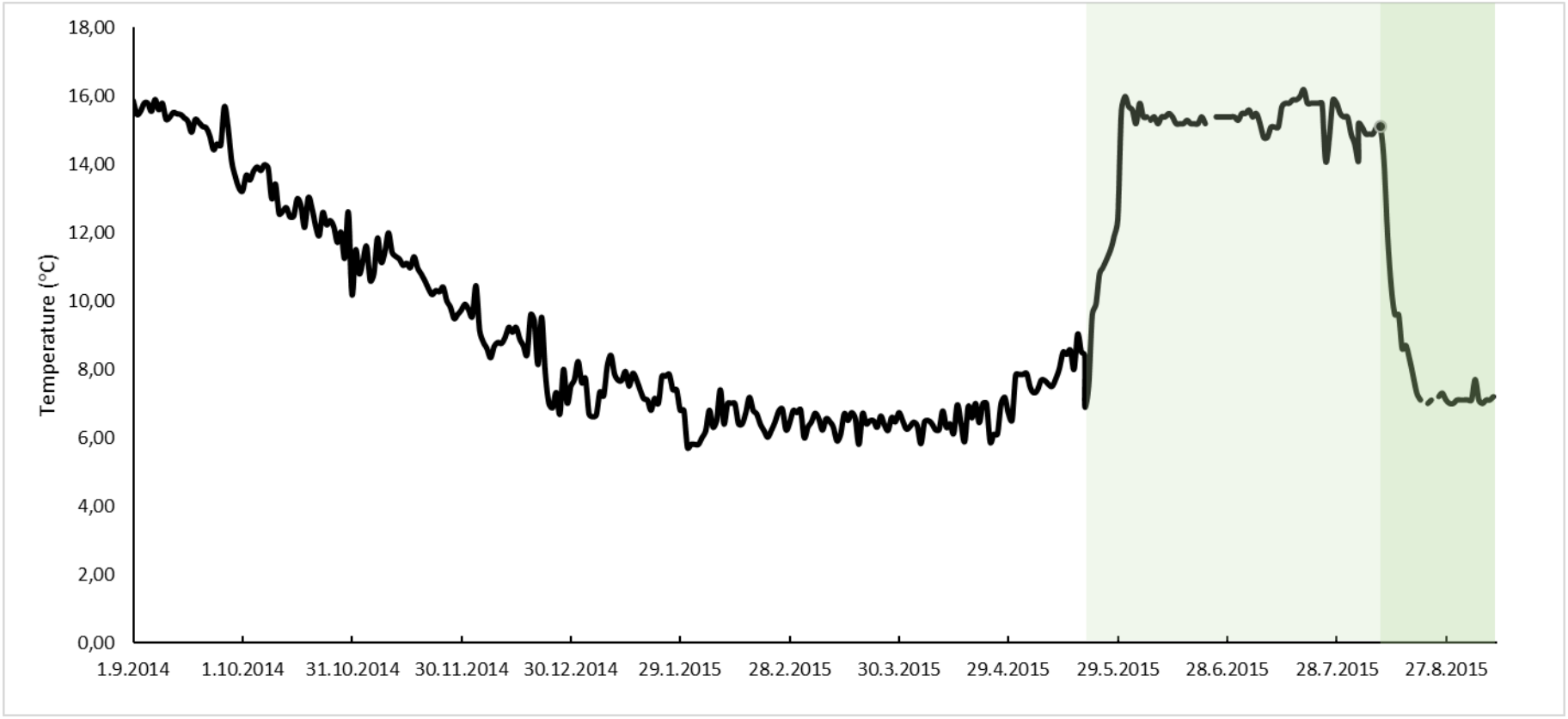


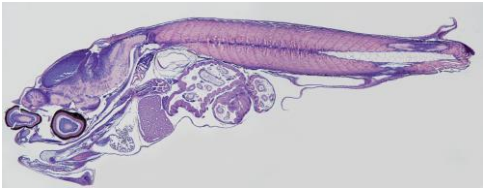

Figure 23. Temperature profile of the whole experiment. Background colour indicates the production phase (Table 1) and temperatures during sea phase were taken at a depth of 6 m.

## *Appendix 3 – Hematoxylin and Eosin (H&E) staining protocol*

•Place slides containing paraffin sections in a slide holder

•Deparaffinise and rehydrate sections:

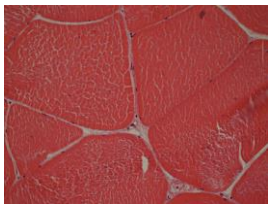
| | | |
|---|---|---|
| TissueClear | | 5 min |
| TissueClear | | 5 min |
| TissueClear | | 5 min |
| 100 % ethanol | | 2 min |
| 100 % ethanol | | 2 min |
| 96 % ethanol | | 2 min |
| 70 % ethanol | | 2-3 min |
| Distilled $H_2O$ | | 5 min |
| Mayers Hem. | | 3 min (2-5 min) |
| Tap water | running | 3 min (to allow stain to develop) |
| 1% HCl in 70 % ethanol (acid alcohol 4 ml 6M HCl + 200 ml 70 % ethanol ) | | Dip 5x (fast) (to destain) |
| Tap water | running | 3 min |
| 0,5% eosin | | 2 min |
| Tap water | | dip |
| Distilled $H_2O$ | | dip |



Air dry or proceed to dehydration.

<u>Dehydration:</u>

| | |
|---|---|
| 70 % ethanol | dip |
| 100 % ethanol | 30 sek |
| 100 % ethanol | 2 x 2 min |
| TissueClear | 5 min |
| TissueClear | 5 min |



•Coverslip slides using Tissue-Mount
•Angle the coverslip and let it fall gently onto the slide. Allow the mounting medium to spread beneath the coverslip, covering all the tissue.
•Dry TissueMount 10-15 min at 60°C

Nuclei - blue
Cytoplasm, connective tissue, muscle etc. - varying shades of pink

## *Appendix 4 – Staining and sectioning tests*

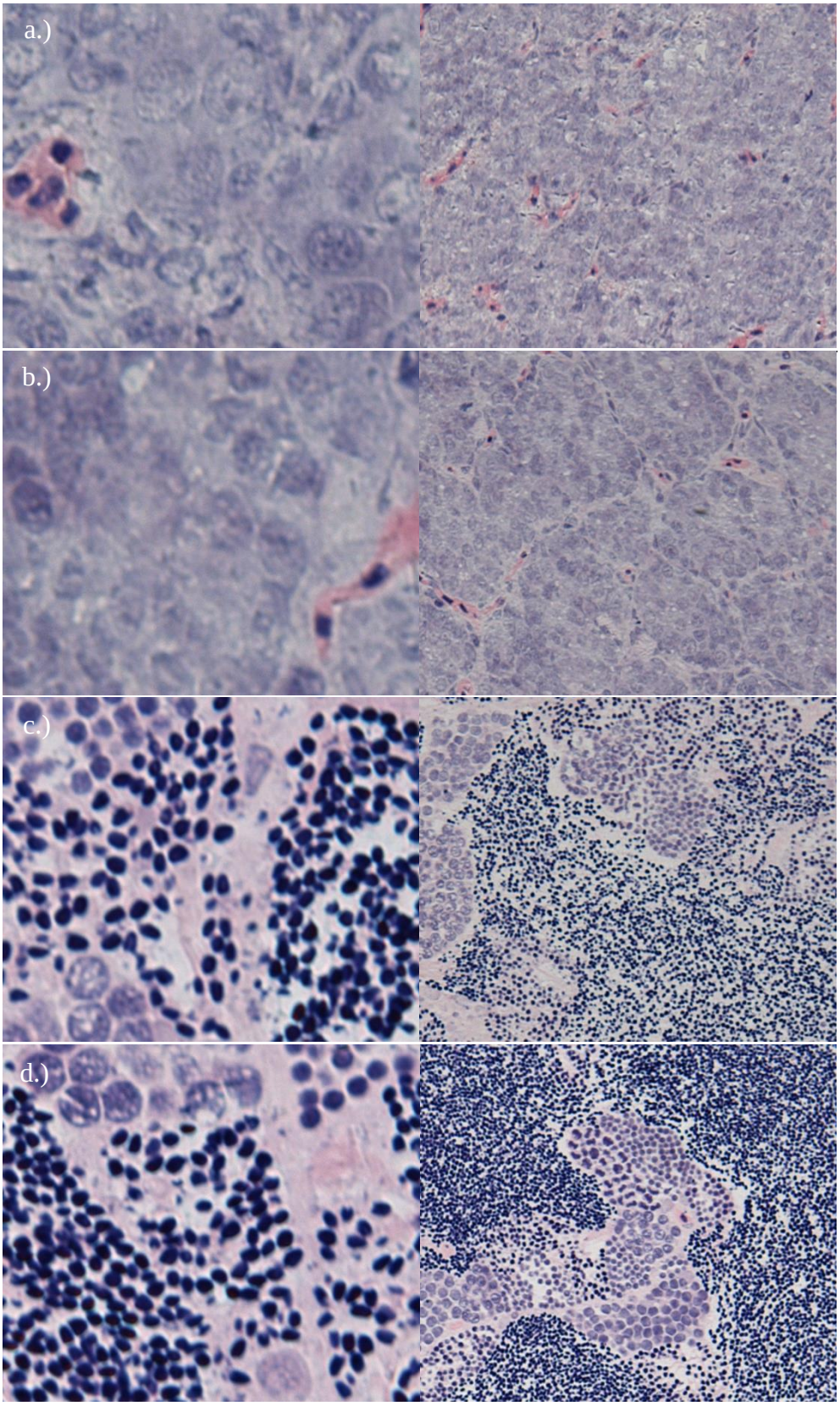



Figure 24. Technovit embedded samples (M72 a, b. and M228 c, d.) in 1µm sections stained with H&E. Staining was done according to the protocol with some changes in timings: Hematoxylin 10 min and Eosin 4 min in a. and c. and hematoxylin 15 min, eosin 5 min in b. and d. The left picture is with x64 magnification and the right is x16.

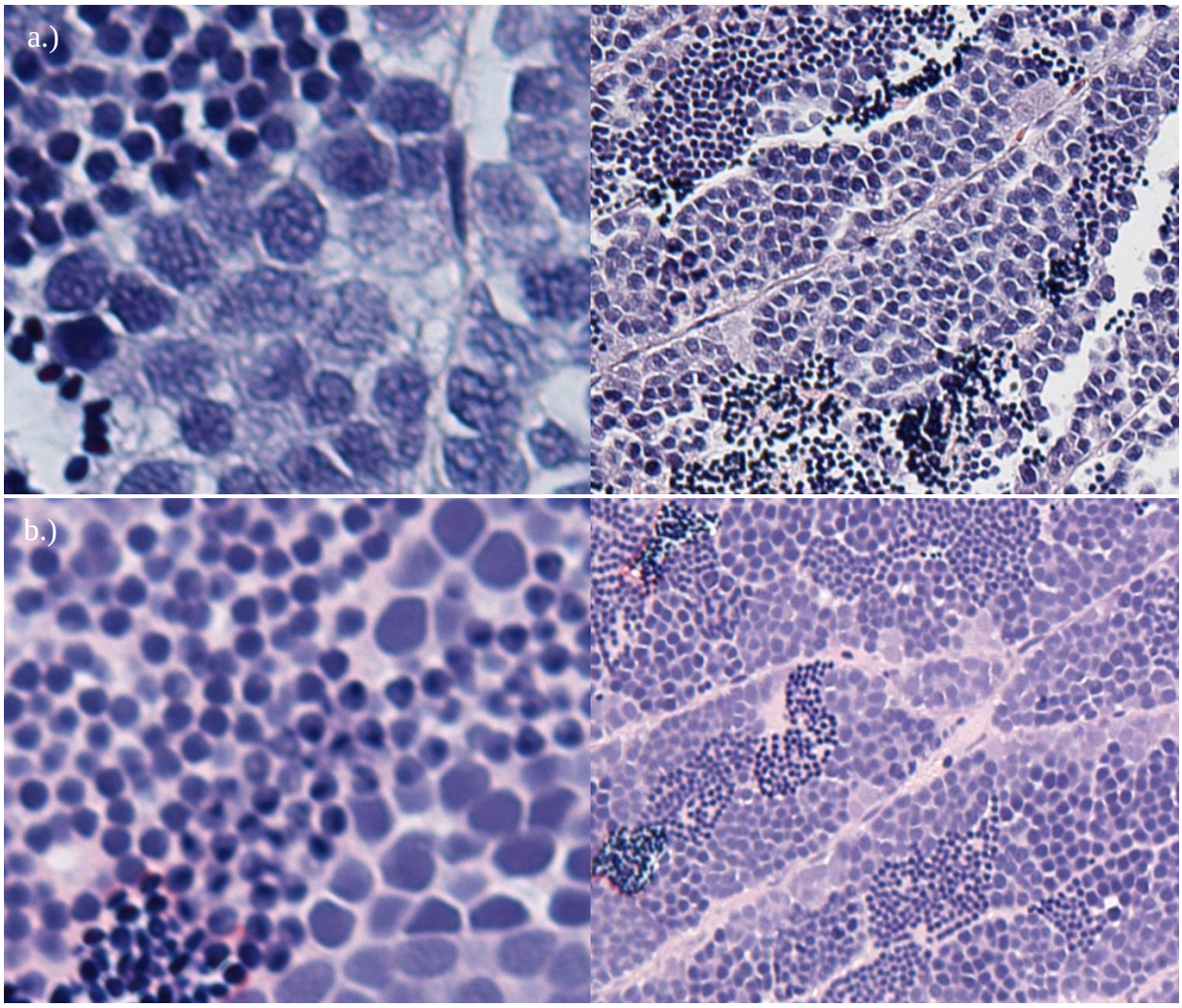



Figure 25. A comparison between paraffin (a.) and technovit (b.) embedding. Sample ID M202 sectioned to 2 µm thickness and stained with H&E according to the protocol.

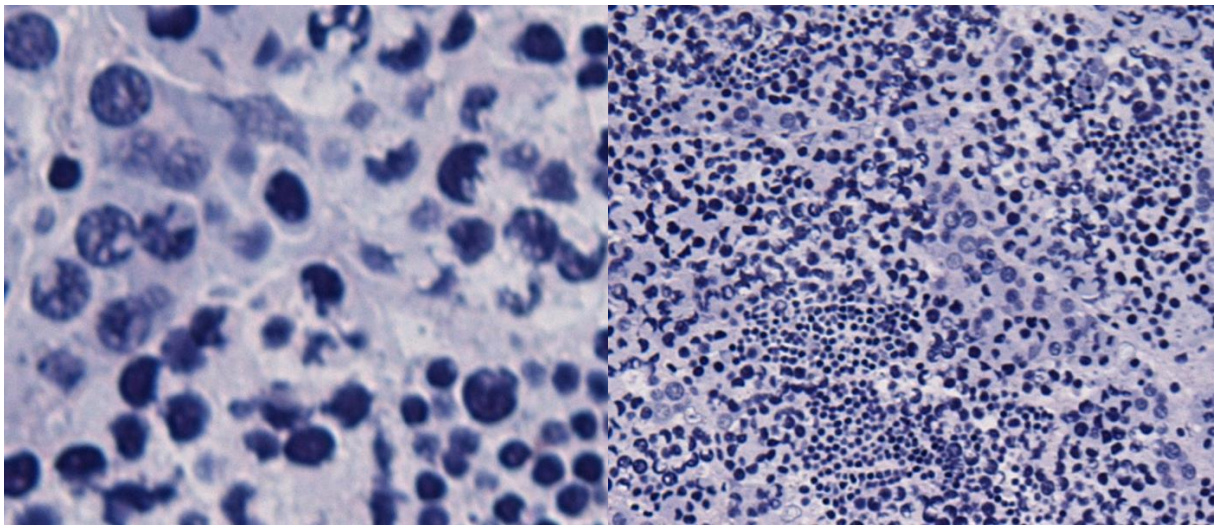


Figure 26. Sample ID M189 embedded in technovit, sectioned in 2 µm and stained according to H&E protocol, but skipping the acid alcohol phase.

Paraffin embedded samples sectioned to 2-4 µm thicknesses and stained with H&E according to the protocol (Appendix 1)

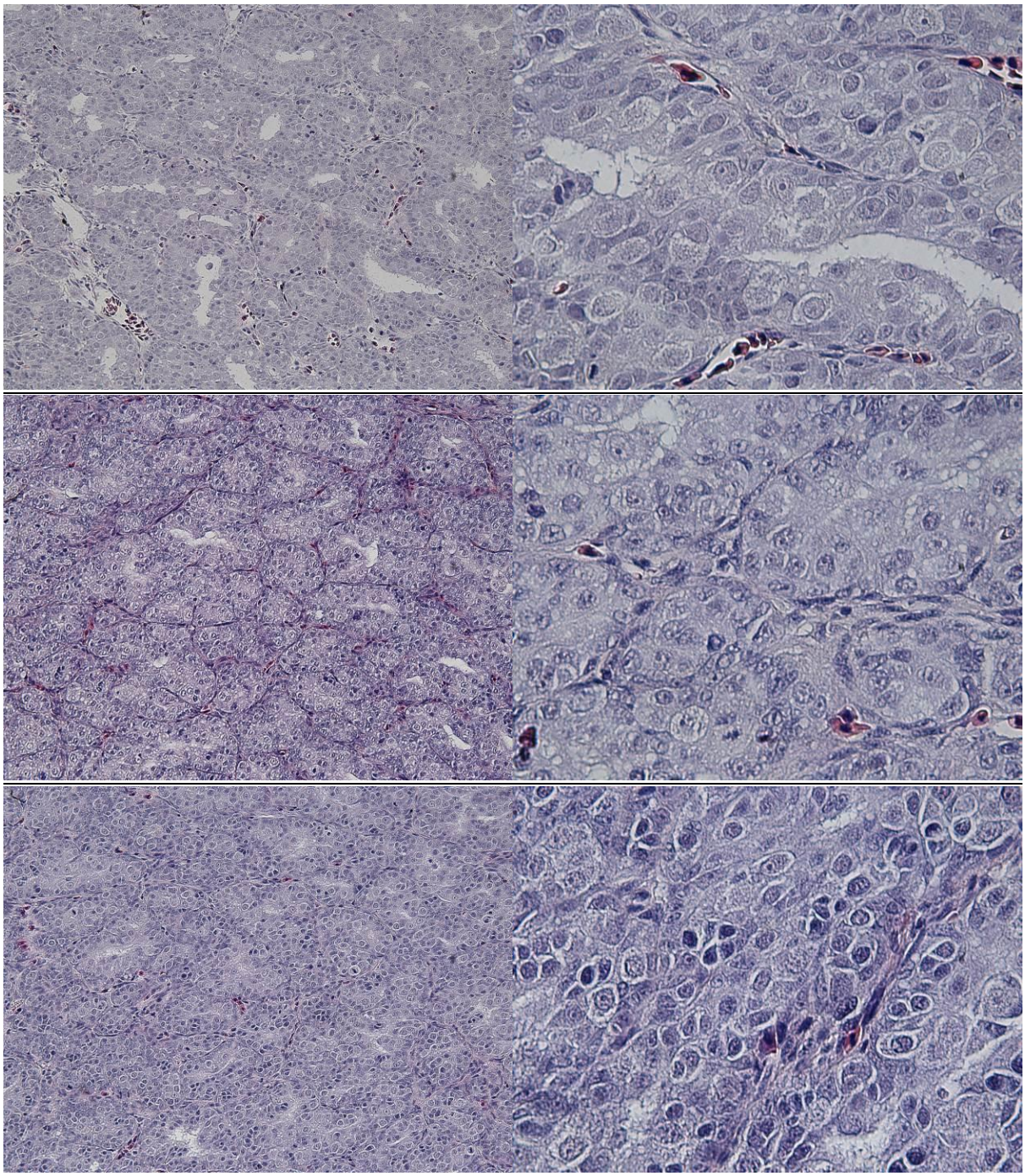


Figure 27. M82 2 µm (upper), 3 µm (mid), 4 µm (lower) in x16 and x64 magnification.

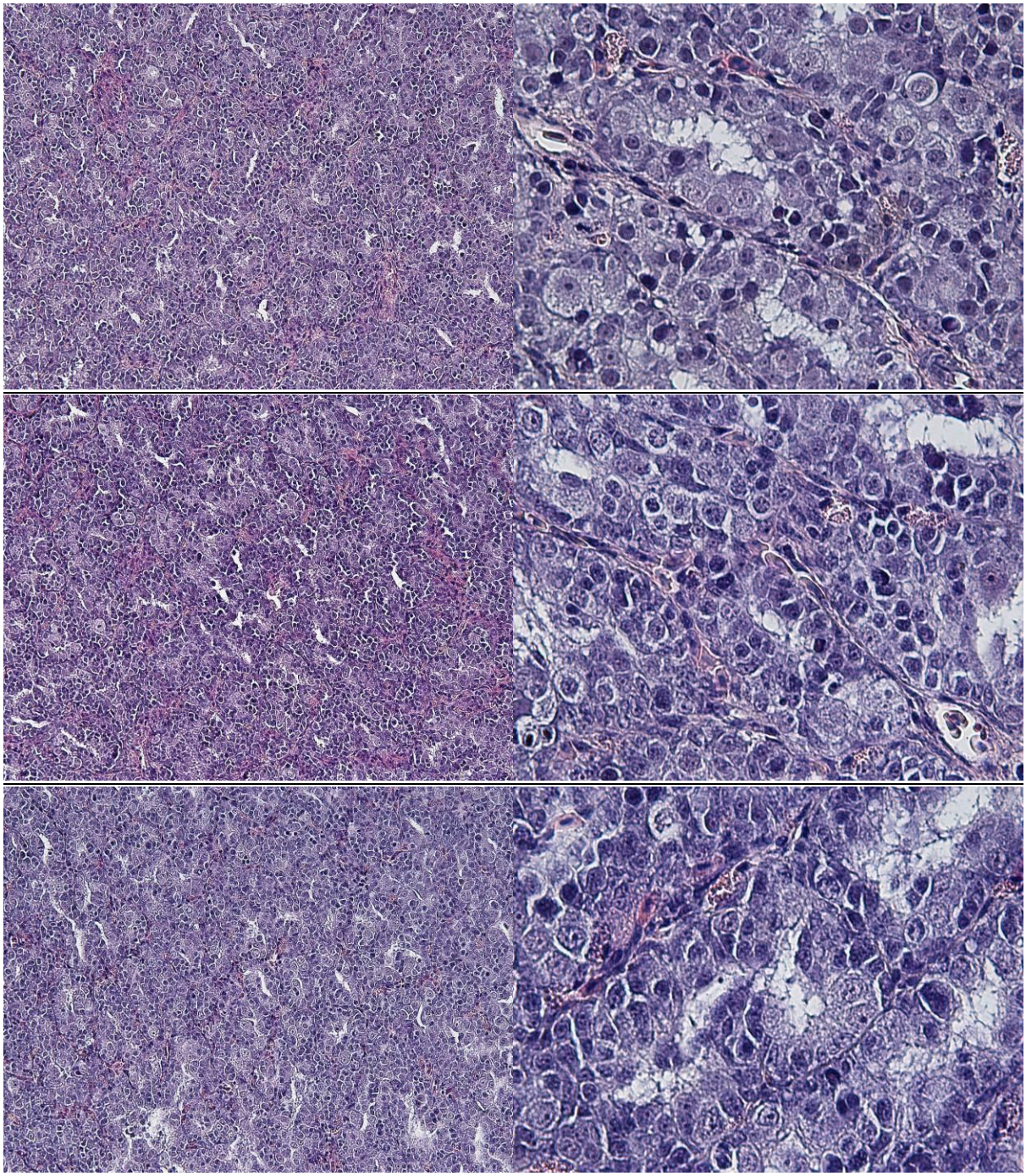


Figure 28. M171 2 µm (upper), 3 µm (mid), 4 µm (lower) in x16 and x64 magnification.

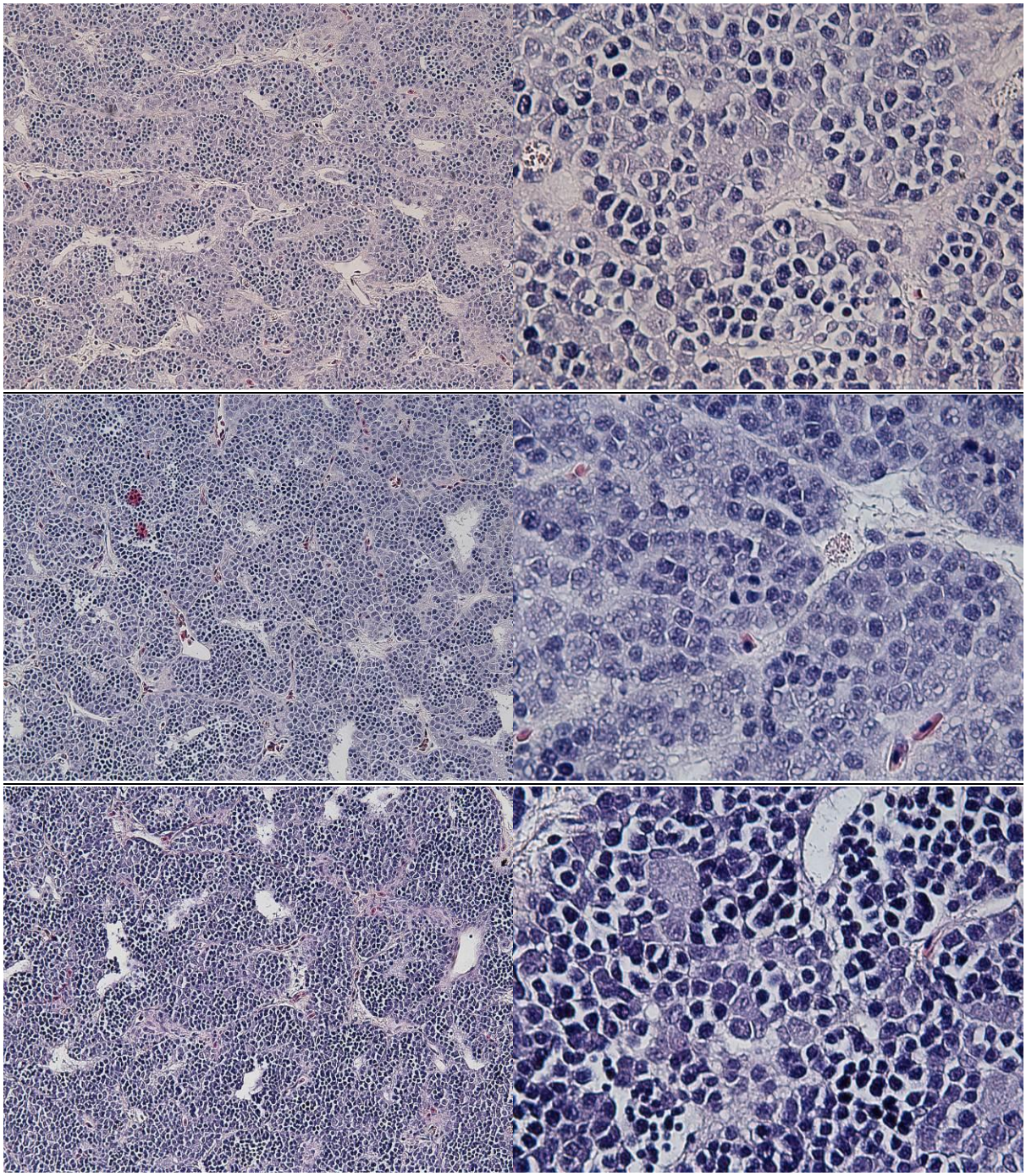


Figure 29. M194 2 µm (upper), 3 µm (mid), 4 µm (lower) in x16 and x64 magnification.

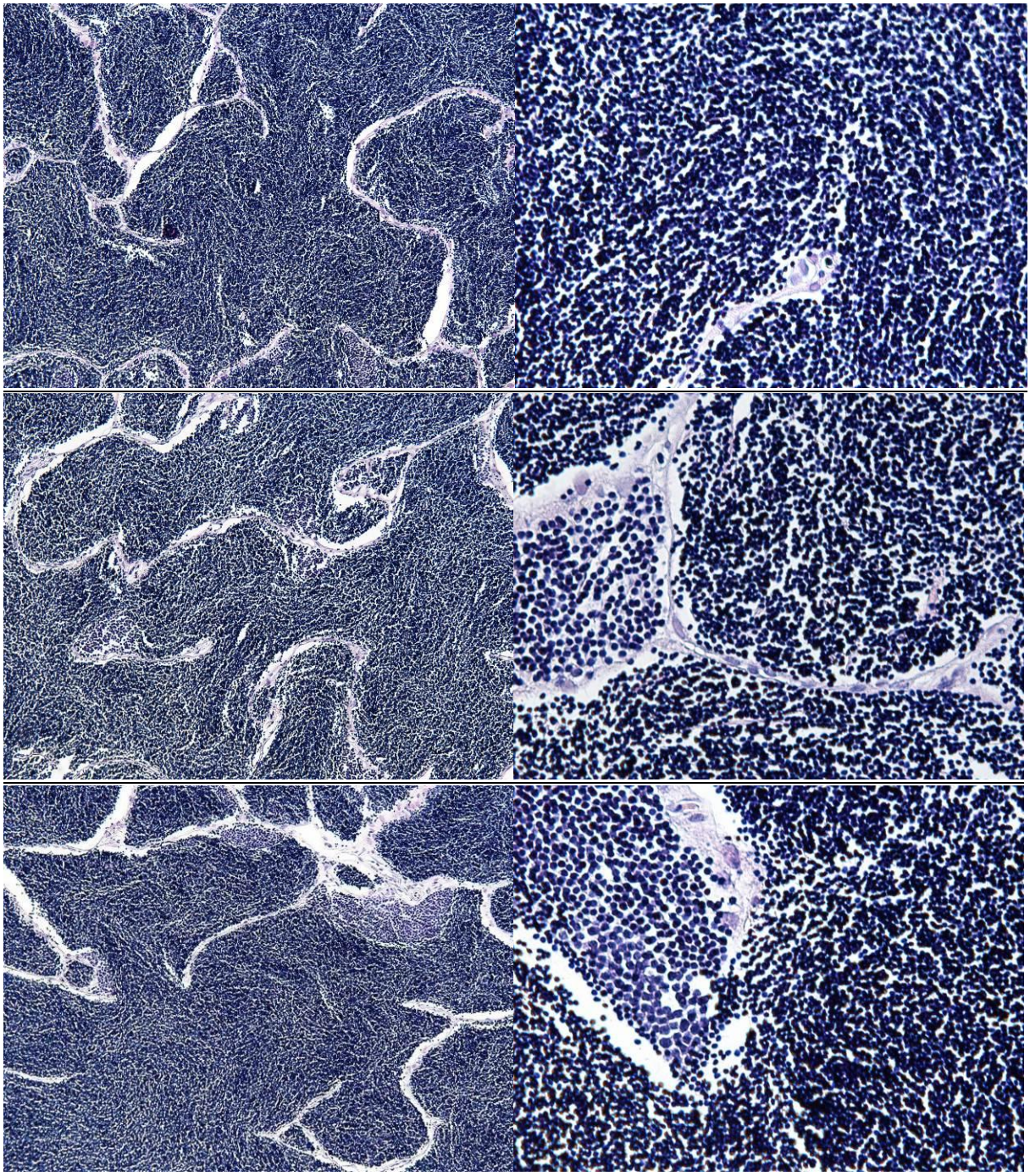


Figure 30. M243 2 µm (upper), 3 µm (mid), 4 µm (lower) in x16 and x64 magnification.

## *Appendix 5 – Area fraction and SMI calculations*

Table 7. Area fractions of different cell types and SMI by sample. The bolded values on the same row with the date are averages from that date.

| Sample ID | Excluded area | Testicular somatic cells | Spermato-gonia | Spermato-cytes | Sperma-tids | Sper ma-tozoa | SMI | GSI |
|---|---|---|---|---|---|---|---|---|
| **2.9.2014** | **4 %** | **89 %** | **11 %** | **0 %** | **0 %** | **0 %** | **0.03** | |
| M6 | 3 % | 92 % | 8 % | 0 % | 0 % | 0 % | 0.02 | |
| M9 | 2 % | 89 % | 11 % | 0 % | 0 % | 0 % | 0.03 | |
| M12 | 6 % | 91 % | 9 % | 0 % | 0 % | 0 % | 0.02 | |
| M15 | 3 % | 89 % | 11 % | 0 % | 0 % | 0 % | 0.03 | |
| M16 | 3 % | 83 % | 17 % | 0 % | 0 % | 0 % | 0.04 | |
| **6.10.2014** | **1 %** | **87 %** | **13 %** | **0 %** | **0 %** | **0 %** | **0.03** | |
| M26 | 1 % | 87 % | 13 % | 0 % | 0 % | 0 % | 0.03 | |
| M29 | 1 % | 85 % | 15 % | 0 % | 0 % | 0 % | 0.04 | |
| M35 | 1 % | 90 % | 10 % | 0 % | 0 % | 0 % | 0.02 | |
| M38 | 2 % | 89 % | 11 % | 0 % | 0 % | 0 % | 0.03 | |
| M39 | 1 % | 86 % | 14 % | 0 % | 0 % | 0 % | 0.04 | |
| **11.11.2014** | **0 %** | **83 %** | **17 %** | **0 %** | **0 %** | **0 %** | **0.04** | |
| M43 | 0 % | 85 % | 15 % | 0 % | 0 % | 0 % | 0.04 | |
| M44 | 1 % | 87 % | 13 % | 0 % | 0 % | 0 % | 0.03 | |
| M45 | 0 % | 81 % | 19 % | 0 % | 0 % | 0 % | 0.05 | |
| M46 | 0 % | 81 % | 19 % | 0 % | 0 % | 0 % | 0.05 | |
| M48 | 0 % | 83 % | 17 % | 0 % | 0 % | 0 % | 0.04 | |
| **1.12.2014** | **1 %** | **88 %** | **12 %** | **0 %** | **0 %** | **0 %** | **0.03** | **0.12** |
| M64 | 1 % | 88 % | 12 % | 0 % | 0 % | 0 % | 0.03 | 0.12 |
| M66 | 0 % | 87 % | 13 % | 0 % | 0 % | 0 % | 0.03 | 0.11 |
| M67 | 0 % | 90 % | 10 % | 0 % | 0 % | 0 % | 0.03 | 0.14 |
| M68 | 1 % | 90 % | 10 % | 0 % | 0 % | 0 % | 0.03 | 0.10 |
| M70 | 1 % | 85 % | 15 % | 0 % | 0 % | 0 % | 0.04 | 0.14 |
| **6.1.2015** | **0 %** | **81 %** | **19 %** | **0 %** | **0 %** | **0 %** | **0.05** | **0.13** |
| M82 | 0 % | 82 % | 18 % | 0 % | 0 % | 0 % | 0.05 | 0.16 |
| M83 | 1 % | 85 % | 15 % | 0 % | 0 % | 0 % | 0.04 | 0.12 |
| M84 | 0 % | 79 % | 21 % | 0 % | 0 % | 0 % | 0.05 | 0.11 |
| M85 | 0 % | 81 % | 19 % | 0 % | 0 % | 0 % | 0.05 | 0.09 |
| M91 | 0 % | 79 % | 21 % | 0 % | 0 % | 0 % | 0.05 | 0.19 |
| **2.2.2015** | **1 %** | **74 %** | **26 %** | **0 %** | **0 %** | **0 %** | **0.07** | **0.18** |
| M103 | 0 % | 75 % | 25 % | 0 % | 0 % | 0 % | 0.06 | 0.14 |
| M106 | 1 % | 80 % | 20 % | 0 % | 0 % | 0 % | 0.05 | 0.19 |
| M107 | 2 % | 67 % | 33 % | 0 % | 0 % | 0 % | 0.08 | 0.23 |
| M110 | 1 % | 73 % | 27 % | 0 % | 0 % | 0 % | 0.07 | 0.18 |
| M112 | 1 % | 75 % | 25 % | 0 % | 0 % | 0 % | 0.06 | 0.18 |

| Sample ID | Excluded area | Testicular somatic cells | Spermato-gonia | Spermato-cytes | Sperma-tids | Sper ma-tozoa | SMI | GSI |
|---|---|---|---|---|---|---|---|---|
| **2.3.2015** | **0 %** | **62 %** | **38 %** | **0 %** | **0 %** | **0 %** | **0.09** | **0.24** |
| M122 | 0 % | 52 % | 48 % | 0 % | 0 % | 0 % | 0.12 | 0.22 |
| M123 | 0 % | 60 % | 40 % | 0 % | 0 % | 0 % | 0.10 | 0.19 |
| M125 | 0 % | 74 % | 26 % | 0 % | 0 % | 0 % | 0.06 | 0.32 |
| M127 | 0 % | 61 % | 39 % | 0 % | 0 % | 0 % | 0.10 | 0.18 |
| M128 | 0 % | 65 % | 35 % | 0 % | 0 % | 0 % | 0.09 | 0.18 |
| M129 | 0 % | 60 % | 40 % | 0 % | 0 % | 0 % | 0.10 | 0.14 |
| M130 | 0 % | 65 % | 35 % | 0 % | 0 % | 0 % | 0.09 | 0.21 |
| M132 | 0 % | 57 % | 43 % | 0 % | 0 % | 0 % | 0.11 | 0.19 |
| M133 | 0 % | 71 % | 29 % | 0 % | 0 % | 0 % | 0.07 | 0.24 |
| M134 | 0 % | 59 % | 41 % | 0 % | 0 % | 0 % | 0.10 | 0.51 |
| **8.4.2015** | **0 %** | **53 %** | **47 %** | **0 %** | **0 %** | **0 %** | **0.12** | **0.22** |
| M141 | 0 % | 49 % | 51 % | 0 % | 0 % | 0 % | 0.13 | 0.17 |
| M142 | 0 % | 55 % | 45 % | 0 % | 0 % | 0 % | 0.11 | 0.14 |
| M143 | 0 % | 54 % | 46 % | 0 % | 0 % | 0 % | 0.12 | 0.18 |
| M144 | 0 % | 52 % | 48 % | 0 % | 0 % | 0 % | 0.12 | 0.19 |
| M146 | 1 % | 38 % | 62 % | 0 % | 0 % | 0 % | 0.15 | 0.18 |
| M147 | 1 % | 58 % | 42 % | 0 % | 0 % | 0 % | 0.11 | 0.36 |
| M149 | 0 % | 57 % | 43 % | 0 % | 0 % | 0 % | 0.11 | 0.30 |
| M150 | 0 % | 51 % | 49 % | 0 % | 0 % | 0 % | 0.12 | 0.24 |
| M151 | 0 % | 58 % | 42 % | 0 % | 0 % | 0 % | 0.10 | 0.19 |
| M152 | 0 % | 58 % | 42 % | 0 % | 0 % | 0 % | 0.10 | 0.23 |
| **4.5.2015** | **0 %** | **63 %** | **37 %** | **0 %** | **0 %** | **0 %** | **0.09** | **0.24** |
| M161 | 0 % | 65 % | 35 % | 0 % | 0 % | 0 % | 0.09 | 0.19 |
| M162 | 0 % | 55 % | 45 % | 0 % | 0 % | 0 % | 0.11 | 0.27 |
| M163 | 0 % | 56 % | 44 % | 0 % | 0 % | 0 % | 0.11 | 0.14 |
| M165 | 0 % | 81 % | 19 % | 0 % | 0 % | 0 % | 0.05 | 0.32 |
| M166 | 0 % | 83 % | 17 % | 0 % | 0 % | 0 % | 0.04 | 0.18 |
| M167 | 0 % | 65 % | 35 % | 0 % | 0 % | 0 % | 0.09 | 0.27 |
| M168 | 1 % | 52 % | 48 % | 0 % | 0 % | 0 % | 0.12 | 0.23 |
| M169 | 0 % | 62 % | 38 % | 0 % | 0 % | 0 % | 0.09 | 0.32 |
| M171 | 0 % | 54 % | 46 % | 0 % | 0 % | 0 % | 0.12 | 0.34 |
| M172 | 0 % | 58 % | 42 % | 0 % | 0 % | 0 % | 0.10 | 0.15 |

| Sample ID | Excluded area | Testicular somatic cells | Spermato-gonia | Spermato-cytes | Sperma-tids | Sper ma-tozoa | SMI | GSI |
|---|---|---|---|---|---|---|---|---|
| **9.6.2015** | **1 %** | **37 %** | **51 %** | **11 %** | **1 %** | **0 %** | **0.19** | **0.82** |
| M186 | 1 % | 20 % | 46 % | 32 % | 2 % | 0 % | 0.29 | 0.98 |
| M187 | 0 % | 52 % | 48 % | 0 % | 0 % | 0 % | 0.12 | 0.37 |
| M188 | 0 % | 34 % | 63 % | 3 % | 0 % | 0 % | 0.17 | 0.40 |
| M189 | 1 % | 22 % | 48 % | 27 % | 3 % | 0 % | 0.28 | 0.91 |
| M191 | 1 % | 34 % | 64 % | 2 % | 0 % | 0 % | 0.17 | 0.43 |
| M193 | 0 % | 55 % | 45 % | 0 % | 0 % | 0 % | 0.11 | 0.99 |
| M194 | 2 % | 48 % | 52 % | 0 % | 0 % | 0 % | 0.13 | 2.40 |
| M196 | 0 % | 51 % | 49 % | 0 % | 0 % | 0 % | 0.12 | 0.37 |
| M198 | 1 % | 42 % | 58 % | 0 % | 0 % | 0 % | 0.14 | 0.22 |
| M200 | 5 % | 14 % | 35 % | 50 % | 1 % | 0 % | 0.35 | 1.16 |
| **1.7.2015** | **3 %** | **4 %** | **6 %** | **66 %** | **20 %** | **3 %** | **0.53** | **4.13** |
| M201 | 11 % | 6 % | 0 % | 57 % | 24 % | 13 % | 0.59 | 2.58 |
| M202 | 1 % | 5 % | 7 % | 68 % | 18 % | 2 % | 0.51 | 5.23 |
| M203 | 3 % | 2 % | 0 % | 49 % | 39 % | 10 % | 0.64 | 5.38 |
| M204 | 1 % | 6 % | 6 % | 74 % | 12 % | 2 % | 0.49 | 1.40 |
| M205 | 2 % | 5 % | 3 % | 66 % | 25 % | 1 % | 0.53 | 3.22 |
| M206 | 2 % | 4 % | 2 % | 72 % | 18 % | 4 % | 0.54 | 6.53 |
| M207 | 2 % | 4 % | 11 % | 69 % | 15 % | 1 % | 0.50 | 5.30 |
| M208 | 0 % | 2 % | 11 % | 75 % | 12 % | 0 % | 0.49 | 5.27 |
| M209 | 10 % | 6 % | 5 % | 89 % | 0 % | 0 % | 0.46 | 1.94 |
| M210 | 2 % | 4 % | 4 % | 71 % | 20 % | 1 % | 0.52 | 3.64 |
| M211 | 2 % | 2 % | 3 % | 68 % | 24 % | 3 % | 0.55 | 4.15 |
| M212 | 1 % | 4 % | 12 % | 65 % | 13 % | 7 % | 0.52 | 3.74 |
| M213 | 1 % | 1 % | 2 % | 78 % | 16 % | 2 % | 0.54 | 5.25 |
| M214 | 2 % | 2 % | 3 % | 69 % | 21 % | 5 % | 0.56 | 1.61 |
| M215 | 3 % | 2 % | 1 % | 64 % | 31 % | 2 % | 0.57 | 5.05 |
| M216 | 2 % | 4 % | 8 % | 60 % | 25 % | 3 % | 0.54 | 4.88 |
| M217 | 1 % | 8 % | 34 % | 51 % | 6 % | 2 % | 0.40 | 2.08 |
| M218 | 10 % | 11 % | 3 % | 52 % | 30 % | 4 % | 0.53 | 6.37 |
| M219 | 5 % | 5 % | 3 % | 68 % | 24 % | 0 % | 0.53 | 2.23 |
| M220 | 5 % | 6 % | 11 % | 56 % | 21 % | 7 % | 0.53 | 6.71 |
| **7.8.2015** | **3 %** | **7 %** | **1 %** | **25 %** | **20 %** | **48 %** | **0.75** | **4.83** |
| M221 | 3 % | 8 % | 0 % | 10 % | 23 % | 59 % | 0.82 | 6.48 |
| M222 | 2 % | 11 % | 1 % | 1 % | 5 % | 82 % | 0.86 | 3.99 |
| M223 | 3 % | 3 % | 2 % | 66 % | 26 % | 2 % | 0.56 | 5.64 |
| M224 | 4 % | 9 % | 0 % | 4 % | 7 % | 80 % | 0.87 | 3.79 |
| M225 | 2 % | 3 % | 0 % | 43 % | 36 % | 18 % | 0.66 | 4.25 |

| Sample ID | Excluded area | Testicular somatic cells | Spermato-gonia | Spermato-cytes | Sperma-tids | Sper ma-tozoa | SMI | GSI |
|---|---|---|---|---|---|---|---|---|
| **13.8.2015** | **2 %** | **7 %** | **1 %** | **8 %** | **10 %** | **74 %** | **0.86** | **3.77** |
| M226 | 2 % | 6 % | 2 % | 25 % | 12 % | 56 % | 0.78 | 3.55 |
| M227 | 0 % | 6 % | 0 % | 4 % | 7 % | 83 % | 0.90 | 3.50 |
| M228 | 3 % | 4 % | 1 % | 6 % | 24 % | 65 % | 0.86 | 4.31 |
| M229 | 4 % | 10 % | 1 % | 2 % | 2 % | 85 % | 0.88 | 3.54 |
| M230 | 1 % | 7 % | 2 % | 4 % | 5 % | 82 % | 0.88 | 3.96 |
| **20.8.2015** | **3 %** | **7 %** | **1 %** | **9 %** | **13 %** | **70 %** | **0.85** | **3.75** |
| M231 | 2 % | 5 % | 1 % | 2 % | 6 % | 86 % | 0.92 | 4.17 |
| M232 | 4 % | 10 % | 1 % | 24 % | 33 % | 33 % | 0.69 | 3.78 |
| M233 | 1 % | 7 % | 1 % | 5 % | 7 % | 81 % | 0.89 | 3.78 |
| M234 | 2 % | 7 % | 0 % | 2 % | 6 % | 84 % | 0.90 | 3.53 |
| M235 | 6 % | 6 % | 2 % | 10 % | 15 % | 67 % | 0.84 | 3.48 |
| **27.8.2015** | **2 %** | **7 %** | **1 %** | **3 %** | **7 %** | **82 %** | **0.89** | **3.75** |
| M236 | 2 % | 7 % | 1 % | 1 % | 1 % | 91 % | 0.92 | 3.52 |
| M237 | 0 % | 6 % | 1 % | 1 % | 3 % | 89 % | 0.92 | 4.14 |
| M238 | 2 % | 7 % | 1 % | 2 % | 5 % | 84 % | 0.89 | 3.05 |
| M239 | 4 % | 6 % | 1 % | 5 % | 15 % | 74 % | 0.88 | 4.35 |
| M240 | 3 % | 8 % | 2 % | 6 % | 13 % | 71 % | 0.84 | 3.69 |
| **2.9.2015** | **2 %** | **6 %** | **1 %** | **2 %** | **4 %** | **87 %** | **0.91** | **3.92** |
| M241 | 2 % | 2 % | 0 % | 0 % | 0 % | 98 % | 0.98 | 3.74 |
| M242 | 2 % | 6 % | 2 % | 2 % | 10 % | 80 % | 0.89 | 3.93 |
| M243 | 3 % | 5 % | 0 % | 0 % | 2 % | 93 % | 0.95 | 3.94 |
| M244 | 1 % | 11 % | 1 % | 0 % | 1 % | 88 % | 0.88 | 3.06 |
| M245 | 2 % | 7 % | 2 % | 6 % | 8 % | 77 % | 0.86 | 4.94 |

## *Appendix 6 – Sampling data*

Table 8. Sampling data from all samples.

| Sampling date | Salmon ID | Fish weight (kg) | Fish length (cm) | Weight left testis (g) | Total testis weight | Testis length (cm) | Testis length US (cm) | Left testis volume (ml) | Condition factor (Fulton) | GSI | Gonad density (g/cm^3) | left testis-% |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 2.9.2014 | M1 | 7.02 | 83 | 3.9 | | | | | 1.23 | | | |
| 2.9.2014 | M2 | 5.72 | 82 | 3.4 | | | | | 1.04 | | | |
| 2.9.2014 | M3 | 6.60 | 82 | 10.0 | | | | | 1.20 | | | |
| 2.9.2014 | M4 | 6.52 | 83 | 4.0 | | | | | 1.14 | | | |
| 2.9.2014 | M5 | 6.54 | 82 | 2.9 | | | | | 1.19 | | | |
| 2.9.2014 | M6 | 7.08 | 85 | 4.0 | | | | | 1.15 | | | |
| 2.9.2014 | M7 | 6.66 | 85 | 8.0 | | | | | 1.08 | | | |
| 2.9.2014 | M8 | 7.70 | 85 | 11.3 | | | | | 1.25 | | | |
| 2.9.2014 | M9 | 7.44 | 84 | 3.1 | | | | | 1.26 | | | |
| 2.9.2014 | M10 | 6.76 | 81 | 6.3 | | | | | 1.27 | | | |
| 2.9.2014 | M11 | 6.84 | 84 | 4.0 | | | | | 1.15 | | | |
| 2.9.2014 | M12 | 7.00 | 82 | 3.2 | | | | | 1.27 | | | |
| 2.9.2014 | M13 | 6.10 | 82 | 4.7 | | | | | 1.11 | | | |
| 2.9.2014 | M15 | 5.96 | 79 | 3.5 | | | | | 1.21 | | | |
| 2.9.2014 | M16 | 5.47 | 77 | 3.2 | | | | | 1.20 | | | |
| 2.9.2014 | M18 | 5.48 | 80 | 4.1 | | | | | 1.07 | | | |
| 6.10.2014 | M21 | 6.92 | 84 | 3.0 | | | | | 1.17 | | | |
| 6.10.2014 | M22 | 5.12 | 78 | 2.6 | | | | | 1.08 | | | |
| 6.10.2014 | M23 | 6.32 | 83 | 2.6 | | | | | 1.11 | | | |
| 6.10.2014 | M24 | 7.02 | 85 | 4.1 | | | | | 1.14 | | | |
| 6.10.2014 | M26 | 5.68 | 77 | 3.0 | | | | | 1.24 | | | |
| 6.10.2014 | M27 | 7.28 | 89 | 5.1 | | | | | 1.03 | | | |
| 6.10.2014 | M28 | 8.66 | 90 | 5.1 | | | | | 1.19 | | | |
| 6.10.2014 | M29 | 9.68 | 90 | 5.4 | | | | | 1.33 | | | |

| Sampling date | Salmon ID | Fish weight (kg) | Fish length (cm) | Weight left testis (g) | Total testis weight | Testis length (cm) | Testis length US (cm) | Left testis volume (ml) | Condition factor (Fulton) | GSI | Gonad density (g/cm^3) | left testis-% |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 6.10.2014 | M30 | 7.42 | 88 | 2.9 | | | | | 1.09 | | | |
| 6.10.2014 | M31 | 6.72 | 86 | 3.4 | | | | | 1.06 | | | |
| 6.10.2014 | M32 | 4.78 | 82 | 3.5 | | | | | 0.87 | | | |
| 6.10.2014 | M33 | 8.12 | 87 | 3.3 | | | | | 1.23 | | | |
| 6.10.2014 | M34 | 8.38 | 90 | 4.7 | | | | | 1.15 | | | |
| 6.10.2014 | M35 | 7.98 | 90 | 5.4 | | | | | 1.09 | | | |
| 6.10.2014 | M36 | 5.86 | 84 | 3.9 | | | | | 0.99 | | | |
| 6.10.2014 | M37 | 7.24 | 84 | 5.7 | | | | | 1.22 | | | |
| 6.10.2014 | M38 | 7.76 | 87 | 4.0 | | | | | 1.18 | | | |
| 6.10.2014 | M39 | 7.16 | 87 | 4.8 | | | | | 1.09 | | | |
| 6.10.2014 | M40 | 8.04 | 90 | 3.7 | | | | | 1.10 | | | |
| 11.11.2014 | M42 | 6.86 | 85 | 3.4 | | | | | 1.12 | | | |
| 11.11.2014 | M43 | 10.24 | 91 | 4.5 | | | | | 1.36 | | | |
| 11.11.2014 | M44 | 6.95 | 86 | 5.2 | | | | | 1.09 | | | |
| 11.11.2014 | M45 | 8.77 | 89 | 5.2 | | | | | 1.24 | | | |
| 11.11.2014 | M46 | 10.23 | 93 | 5.1 | | | | | 1.27 | | | |
| 11.11.2014 | M47 | 8.79 | 91 | 4.7 | | | | | 1.17 | | | |
| 11.11.2014 | M48 | 8.35 | 88 | 5.1 | | | | | 1.23 | | | |
| 11.11.2014 | M49 | 10.51 | 92 | 8.6 | | | | | 1.35 | | | |
| 11.11.2014 | M50 | 11.69 | 94 | 9.3 | | | | | 1.41 | | | |
| 11.11.2014 | M51 | 8.56 | 91 | 9.0 | | | | | 1.14 | | | |
| 1.12.2014 | M61 | 6.14 | 86 | 2.7 | 4.9 | | | | 0.97 | 0.08 | | 55 % |
| 1.12.2014 | M62 | 9.52 | 88 | 5.5 | 9.2 | | | | 1.40 | 0.10 | | 60 % |
| 1.12.2014 | M63 | 11.3 | 95 | 12.8 | 21.3 | | | | 1.32 | 0.19 | | 60 % |
| 1.12.2014 | M64 | 9.46 | 89 | 7.8 | 12.9 | | | | 1.34 | 0.14 | | 60 % |
| 1.12.2014 | M65 | 11.04 | 93 | 9.0 | 17.4 | | | | 1.37 | 0.16 | | 52 % |
| 1.12.2014 | M66 | 9.52 | 90 | 5.5 | 9.7 | | | | 1.31 | 0.10 | | 57 % |

| Sampling date | Salmon ID | Fish weight (kg) | Fish length (cm) | Weight left testis (g) | Total testis weight | Testis length (cm) | Testis length US (cm) | Left testis volume (ml) | Condition factor (Fulton) | GSI | Gonad density (g/cm^3) | left testis-% |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1.12.2014 | M67 | 5.3 | 82 | 2.9 | 7.3 | | | | 0.96 | 0.14 | | 40 % |
| 1.12.2014 | M68 | 10.22 | 94 | 7.0 | 11.2 | | | | 1.23 | 0.11 | | 63 % |
| 1.12.2014 | M69 | 10.06 | 92 | 8.2 | 14.3 | | | | 1.29 | 0.14 | | 57 % |
| 1.12.2014 | M70 | 9.52 | 88 | 5.5 | 11.1 | | | | 1.40 | 0.12 | | 50 % |
| 1.12.2014 | M71 | 11.6 | 94 | 10.0 | 22.5 | | | | 1.40 | 0.19 | | 44 % |
| 1.12.2014 | M72 | 9.86 | 92 | 7.3 | 12.4 | | | | 1.27 | 0.13 | | 59 % |
| 6.1.2015 | M81 | 12.58 | 100 | | 21 | | | | 1.26 | 0.17 | | |
| 6.1.2015 | M82 | 9.12 | 96 | | 17.1 | | 13 | | 1.03 | 0.19 | | |
| 6.1.2015 | M83 | 9.66 | 95 | | 8.9 | | 13 | | 1.13 | 0.09 | | |
| 6.1.2015 | M84 | 9.56 | 92 | | 10.8 | | 14 | | 1.23 | 0.11 | | |
| 6.1.2015 | M85 | 10.66 | 95 | | 12.7 | | 8 | | 1.24 | 0.12 | | |
| 6.1.2015 | M86 | 9.44 | 90 | | 16.7 | | 11 | | 1.29 | 0.18 | | |
| 6.1.2015 | M87 | 9.32 | 91 | | 15.1 | | 12 | | 1.24 | 0.16 | | |
| 6.1.2015 | M88 | 6.92 | 87 | | 7.1 | | 10 | | 1.05 | 0.10 | | |
| 6.1.2015 | M89 | 9.92 | 92 | | 18.1 | | 12.5 | | 1.27 | 0.18 | | |
| 6.1.2015 | M90 | 6.36 | 88 | | 6.4 | | | | 0.93 | 0.10 | | |
| 6.1.2015 | M91 | 9.8 | 90 | | 15.6 | | 14.5 | | 1.34 | 0.16 | | |
| 2.2.2015 | M101 | 13.52 | 98 | 19.4 | 32.8 | 30 | 18 | 20 | 1.44 | 0.24 | 0.97 | 59 % |
| 2.2.2015 | M102 | 17.56 | 105 | 17.3 | 31.7 | 26 | | 19 | 1.52 | 0.18 | 0.91 | 55 % |
| 2.2.2015 | M103 | 13.72 | 101 | 13.1 | 24.3 | 20 | | 14 | 1.33 | 0.18 | 0.94 | 54 % |
| 2.2.2015 | M104 | 13.18 | 100 | 9.6 | 18.8 | 21 | 11 | 10 | 1.32 | 0.14 | 0.96 | 51 % |
| 2.2.2015 | M105 | 7.78 | 85 | 7.5 | 13.2 | 19 | 17 | 8 | 1.27 | 0.17 | 0.94 | 57 % |
| 2.2.2015 | M106 | 14.34 | 100 | 14.9 | 26.3 | 18 | | 15 | 1.43 | 0.18 | 0.99 | 57 % |
| 2.2.2015 | M107 | 10.14 | 94 | 12.6 | 23.5 | 22 | 11 | 12 | 1.22 | 0.23 | 1.05 | 54 % |
| 2.2.2015 | M108 | 8.89 | 86 | 7.6 | 10.9 | 17 | 11 | 10 | 1.40 | 0.12 | 0.76 | 70 % |
| 2.2.2015 | M109 | 12.02 | 96 | 9.8 | 21.9 | 20 | | 11 | 1.36 | 0.18 | 0.89 | 45 % |
| 2.2.2015 | M110 | 11.68 | 91 | 12.0 | 21.8 | 17 | 12 | 8 | 1.55 | 0.19 | 1.50 | 55 % |

| Sampling date | Salmon ID | Fish weight (kg) | Fish length (cm) | Weight left testis (g) | Total testis weight | Testis length (cm) | Testis length US (cm) | Left testis volume (ml) | Condition factor (Fulton) | GSI | Gonad density (g/cm^3) | left testis-% |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 2.2.2015 | M111 | 12.68 | 97 | 8.7 | 14 | 17 | | 11 | 1.39 | 0.11 | 0.79 | 62 % |
| 2.2.2015 | M112 | 12.36 | 95 | 10.0 | 17.1 | 19 | | 14 | 1.44 | 0.14 | 0.71 | 58 % |
| 2.2.2015 | M113 | 14.58 | 103 | 13.1 | 22 | 24 | 15 | 16 | 1.33 | 0.15 | 0.82 | 60 % |
| 2.2.2015 | M114 | 13.62 | 95 | 14.6 | 29.1 | 15 | 14 | | 1.59 | 0.21 | | 50 % |
| 2.3.2015 | M121 | 11.36 | 100 | 10.6 | 22.7 | 10 | 10 | 12 | 1.14 | 0.20 | 0.88 | 47 % |
| 2.3.2015 | M122 | 12.12 | 98 | 32.5 | 61.9 | 15 | 9 | 32 | 1.29 | 0.51 | 1.02 | 53 % |
| 2.3.2015 | M123 | 13.6 | 102 | 17.6 | 32.2 | 15 | 12 | 18 | 1.28 | 0.24 | 0.98 | 55 % |
| 2.3.2015 | M124 | 7.9 | 92 | 8.8 | 16.9 | 8 | 6 | 10 | 1.01 | 0.21 | 0.88 | 52 % |
| 2.3.2015 | M125 | 9.88 | 93 | 10.5 | 19.1 | 12 | 12 | 10 | 1.23 | 0.19 | 1.05 | 55 % |
| 2.3.2015 | M126 | 7.62 | 94 | 7.8 | 14.4 | 11 | | 8 | 0.92 | 0.19 | 0.98 | 54 % |
| 2.3.2015 | M127 | 12.92 | 102 | 14.4 | 27.4 | 14 | 8 | 14 | 1.22 | 0.21 | 1.03 | 53 % |
| 2.3.2015 | M128 | 11.72 | 95 | 9.1 | 16.7 | 14 | 7 | 10 | 1.37 | 0.14 | 0.91 | 54 % |
| 2.3.2015 | M129 | 13.24 | 99 | 12.6 | 24.2 | 12 | 9 | | 1.36 | 0.18 | | 52 % |
| 2.3.2015 | M130 | 7.32 | 92 | 8.3 | 13.5 | 11 | 10.5 | 10 | 0.94 | 0.18 | 0.83 | 61 % |
| 2.3.2015 | M131 | 17.8 | 102 | 21.0 | 35 | 17 | 11 | 20 | 1.68 | 0.20 | 1.05 | 60 % |
| 2.3.2015 | M132 | 13.95 | 97 | 27.1 | 45.3 | 24 | 24 | 28 | 1.53 | 0.32 | 0.97 | 60 % |
| 2.3.2015 | M133 | 17.01 | 101 | 15.4 | 32.7 | 13 | 11 | 17 | 1.65 | 0.19 | 0.91 | 47 % |
| 2.3.2015 | M134 | 14.14 | 96 | 19.1 | 31.5 | 15 | 14 | 20 | 1.60 | 0.22 | 0.96 | 61 % |
| 8.4.2015 | M141 | 16.52 | 102 | 21.7 | 38.5 | 15 | 14.0 | 22 | 1.56 | 0.23 | 0.99 | 56 % |
| 8.4.2015 | M142 | 15.94 | 102 | 14.6 | 29.6 | 10 | 10.0 | 16 | 1.50 | 0.19 | 0.91 | 49 % |
| 8.4.2015 | M143 | 14.76 | 102 | 20.9 | 36.1 | 12 | 14.0 | 24 | 1.39 | 0.24 | 0.87 | 58 % |
| 8.4.2015 | M144 | 17.40 | 105 | 26.8 | 52.8 | 14 | 11.0 | 28 | 1.50 | 0.30 | 0.96 | 51 % |
| 8.4.2015 | M145 | 13.28 | 98 | 15.9 | 25 | 12 | 6.0 | 14 | 1.41 | 0.19 | 1.14 | 64 % |
| 8.4.2015 | M146 | 11.72 | 95 | 22.9 | 42 | 11 | 10.0 | 22 | 1.39 | 0.36 | 1.04 | 55 % |
| 8.4.2015 | M147 | 17.32 | 103 | 14.4 | 30.6 | 10 | | 12 | 1.59 | 0.18 | 1.20 | 47 % |
| 8.4.2015 | M148 | 15.84 | 103 | 15.5 | 29.5 | 15 | 11.0 | 16 | 1.45 | 0.19 | 0.97 | 53 % |
| 8.4.2015 | M149 | 13.64 | 105 | 13.6 | 26.4 | 15 | 15.0 | 12 | 1.20 | 0.19 | 1.13 | 52 % |

| Sampling date | Salmon ID | Fish weight (kg) | Fish length (cm) | Weight left testis (g) | Total testis weight | Testis length (cm) | Testis length US (cm) | Left testis volume (ml) | Condition factor (Fulton) | GSI | Gonad density (g/cm^3) | left testis-% |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 8.4.2015 | M150 | 16.32 | 101 | 15.5 | 29.9 | 12 | 8.0 | 14 | 1.58 | 0.18 | 1.11 | 52 % |
| 8.4.2015 | M151 | 15.00 | 102 | 12.1 | 20.9 | 16 | | 12 | 1.41 | 0.14 | 1.01 | 58 % |
| 8.4.2015 | M152 | 15.46 | 102 | 14.3 | 25.7 | 12 | 6.5 | 14 | 1.46 | 0.17 | 1.02 | 56 % |
| 4.5.2015 | M161 | 14.22 | 103 | 11.2 | 21 | 11 | 12.0 | 10 | 1.30 | 0.15 | 1.12 | 53 % |
| 4.5.2015 | M162 | 13.80 | 104 | 26.5 | 47.3 | 13 | 15.0 | 26 | 1.23 | 0.34 | 1.02 | 56 % |
| 4.5.2015 | M163 | 14.24 | 104 | 26.7 | 45.4 | 11 | 12.0 | 24 | 1.27 | 0.32 | 1.11 | 59 % |
| 4.5.2015 | M164 | 14.30 | 99 | 19.9 | 34.4 | 19 | 15.0 | 22 | 1.47 | 0.24 | 0.90 | 58 % |
| 4.5.2015 | M165 | 15.16 | 106 | 19.4 | 34.4 | 19 | 7.0 | 18 | 1.27 | 0.23 | 1.08 | 56 % |
| 4.5.2015 | M166 | 9.02 | 93 | 14.1 | 24.7 | 13 | 11.0 | 14 | 1.12 | 0.27 | 1.01 | 57 % |
| 4.5.2015 | M167 | 13.40 | 102 | 13.4 | 24.4 | 14 | 10.0 | 12 | 1.26 | 0.18 | 1.12 | 55 % |
| 4.5.2015 | M168 | 11.12 | 94 | 21.4 | 35.8 | 11 | 13.0 | 20 | 1.34 | 0.32 | 1.07 | 60 % |
| 4.5.2015 | M169 | 18.59 | 109 | 21.0 | 26 | 13 | 14.0 | 20 | 1.44 | 0.14 | 1.05 | 81 % |
| 4.5.2015 | M170 | 16.30 | 104 | 24.6 | 41.1 | 16 | 15.0 | 23 | 1.45 | 0.25 | 1.07 | 60 % |
| 4.5.2015 | M171 | 12.48 | 98 | 20.4 | 33.6 | 17 | | 20 | 1.33 | 0.27 | 1.02 | 61 % |
| 4.5.2015 | M172 | 15.36 | 106 | 17.9 | 29.4 | 20 | | 17 | 1.29 | 0.19 | 1.05 | 61 % |
| 9.6.2015 | M181 | 14.82 | 106 | 49.9 | 94.6 | 15 | 11 | 46 | 1.24 | 0.64 | 1.08 | 53 % |
| 9.6.2015 | M182 | 14.35 | 106 | 41.7 | 88.2 | 20 | 12 | 42 | 1.20 | 0.61 | 0.99 | 47 % |
| 9.6.2015 | M183 | 14.99 | 107 | 53.7 | 80.4 | 17 | 17 | 53 | 1.22 | 0.54 | 1.01 | 67 % |
| 9.6.2015 | M184 | 15.59 | 110 | 72.9 | 135.5 | 14 | 13.5 | 70 | 1.17 | 0.87 | 1.04 | 54 % |
| 9.6.2015 | M185 | 16.00 | 110 | 59.9 | 118.9 | 16 | 12 | 60 | 1.20 | 0.74 | 1.00 | 50 % |
| 9.6.2015 | M186 | 12.14 | 101 | 74.2 | 141.1 | 14 | 12.5 | 70 | 1.18 | 1.16 | 1.06 | 53 % |
| 9.6.2015 | M187 | 11.76 | 101 | 19.4 | 26 | 11 | 6 | 16 | 1.14 | 0.22 | 1.21 | 75 % |
| 9.6.2015 | M188 | 16.01 | 107 | 33.2 | 58.6 | 17 | | 32 | 1.31 | 0.37 | 1.04 | 57 % |
| 9.6.2015 | M189 | 12.99 | 101 | 177.4 | 312.1 | 20 | 22 | 170 | 1.26 | 2.40 | 1.04 | 57 % |
| 9.6.2015 | M190 | 14.59 | 103 | 51.3 | 96.3 | 17 | 15 | 50 | 1.34 | 0.66 | 1.03 | 53 % |
| 9.6.2015 | M191 | 9.87 | 103 | 46.5 | 97.7 | 12 | 12 | 44 | 0.90 | 0.99 | 1.06 | 48 % |
| 9.6.2015 | M192 | 12.45 | 103 | 54.9 | 95.8 | 18 | | 52 | 1.14 | 0.77 | 1.06 | 57 % |

| Sampling date | Salmon ID | Fish weight (kg) | Fish length (cm) | Weight left testis (g) | Total testis weight | Testis length (cm) | Testis length US (cm) | Left testis volume (ml) | Condition factor (Fulton) | GSI | Gonad density (g/cm^3) | left testis-% |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 9.6.2015 | M193 | 9.73 | 97 | 20.2 | 41.8 | 12 | 10.5 | 20 | 1.07 | 0.43 | 1.01 | 48 % |
| 9.6.2015 | M194 | 15.96 | 110 | 72.8 | 146 | 18 | 16 | 70 | 1.20 | 0.91 | 1.04 | 50 % |
| 9.6.2015 | M195 | 12.78 | 102 | 31.2 | 55.2 | 14 | 12 | 30 | 1.20 | 0.43 | 1.04 | 57 % |
| 9.6.2015 | M196 | 12.45 | 102 | 28.0 | 49.6 | 18 | 9 | 26 | 1.17 | 0.40 | 1.08 | 56 % |
| 9.6.2015 | M197 | 15.58 | 108 | 47.6 | 97.8 | 20 | 16 | 46 | 1.24 | 0.63 | 1.03 | 49 % |
| 9.6.2015 | M198 | 12.93 | 102 | 25.2 | 47.7 | 15 | 10.5 | 26 | 1.22 | 0.37 | 0.97 | 53 % |
| 9.6.2015 | M199 | 13.75 | 102 | 41.6 | 72.4 | 16 | 11 | 40 | 1.30 | 0.53 | 1.04 | 57 % |
| 9.6.2015 | M200 | 11.92 | 99 | 63.7 | 116.7 | 20 | 15 | 60 | 1.23 | 0.98 | 1.06 | 55 % |
| 1.7.2015 | M201 | 6.89 | 90 | 261.7 | 462.6 | 27 | 21 | 210 | 0.96 | 6.71 | 1.25 | 57 % |
| 1.7.2015 | M202 | 12.74 | 103 | 164.9 | 283.9 | 21 | 18 | 160 | 1.18 | 2.23 | 1.03 | 58 % |
| 1.7.2015 | M203 | 13.8 | 106 | 475.9 | 879.7 | 25 | 25 | 440 | 1.18 | 6.37 | 1.08 | 54 % |
| 1.7.2015 | M204 | 15.01 | 107 | 206.2 | 311.5 | 26 | 24 | 180 | 1.23 | 2.08 | 1.15 | 66 % |
| 1.7.2015 | M205 | 13.76 | 106 | 378.4 | 671.4 | 26 | 24 | 340 | 1.16 | 4.88 | 1.11 | 56 % |
| 1.7.2015 | M206 | 12.68 | 104 | 380.0 | 640.8 | 34 | 29 | 340 | 1.13 | 5.05 | 1.12 | 59 % |
| 1.7.2015 | M207 | 13.34 | 103 | 119.0 | 214.8 | 21 | 16.5 | 100 | 1.22 | 1.61 | 1.19 | 55 % |
| 1.7.2015 | M208 | 13.12 | 107 | 365.0 | 689.2 | 21 | 19.0 | 310 | 1.07 | 5.25 | 1.18 | 53 % |
| 1.7.2015 | M209 | 11.15 | 103 | 270.6 | 416.8 | 26 | 23.0 | 240 | 1.02 | 3.74 | 1.13 | 65 % |
| 1.7.2015 | M210 | 16.12 | 111 | 383.0 | 668.2 | 30 | 27.0 | 340 | 1.18 | 4.15 | 1.13 | 57 % |
| 1.7.2015 | M211 | 13.54 | 106 | 264.8 | 493.4 | 30 | 21.5 | 210 | 1.14 | 3.64 | 1.26 | 54 % |
| 1.7.2015 | M212 | 11.25 | 101 | 120.0 | 218.2 | 18 | 17.5 | 100 | 1.09 | 1.94 | 1.20 | 55 % |
| 1.7.2015 | M213 | 15.12 | 109 | 414.2 | 796.8 | 25 | 22.0 | 380 | 1.17 | 5.27 | 1.09 | 52 % |
| 1.7.2015 | M214 | 13.8 | 107 | 432.4 | 731 | 29 | 24.0 | 390 | 1.13 | 5.30 | 1.11 | 59 % |
| 1.7.2015 | M215 | 12.73 | 100 | 430.2 | 831.2 | 25 | 22.0 | 410 | 1.27 | 6.53 | 1.05 | 52 % |
| 1.7.2015 | M216 | 14.81 | 110 | 260.2 | 476.2 | 17 | 16.5 | 220 | 1.11 | 3.22 | 1.18 | 55 % |
| 1.7.2015 | M217 | 14.88 | 108 | 98.8 | 208.8 | 18 | 17.0 | 70 | 1.18 | 1.40 | 1.41 | 47 % |
| 1.7.2015 | M218 | 15.44 | 106 | 497.0 | 830.2 | 29 | 23.0 | 390 | 1.30 | 5.38 | 1.27 | 60 % |
| 1.7.2015 | M219 | 14.23 | 109 | 413.6 | 743.6 | 30 | 23.0 | 380 | 1.11 | 5.23 | 1.09 | 56 % |

| Sampling date | Salmon ID | Fish weight (kg) | Fish length (cm) | Weight left testis (g) | Total testis weight | Testis length (cm) | Testis length US (cm) | Left testis volume (ml) | Condition factor (Fulton) | GSI | Gonad density (g/cm^3) | left testis-% |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1.7.2015 | M220 | 11.08 | 102 | 175.0 | 285.6 | 28 | 17.5 | 140 | 1.04 | 2.58 | 1.25 | 61 % |
| 7.8.2015 | M221 | 15.59 | 114 | 410.4 | 662.2 | 28 | 21 | 380 | 1.05 | 4.25 | 1.08 | 62 % |
| 7.8.2015 | M222 | 14.49 | 112 | 291.6 | 549.8 | 27 | 23 | 280 | 1.03 | 3.79 | 1.04 | 53 % |
| 7.8.2015 | M223 | 13.9 | 108 | 372.8 | 783.6 | 30 | 20 | 360 | 1.10 | 5.64 | 1.04 | 48 % |
| 7.8.2015 | M224 | 12.14 | 102 | 290.0 | 484.8 | 27 | 19 | 260 | 1.14 | 3.99 | 1.12 | 60 % |
| 7.8.2015 | M225 | 14.82 | 110 | 537.0 | 960 | 33 | 29 | 500 | 1.11 | 6.48 | 1.07 | 56 % |
| 13.8.2015 | M226 | 12.03 | 98 | 248.0 | 476.6 | 21 | 24 | 230 | 1.28 | 3.96 | 1.08 | 52 % |
| 13.8.2015 | M227 | 14.81 | 111 | 292.8 | 524.2 | 28 | 24 | 270 | 1.08 | 3.54 | 1.08 | 56 % |
| 13.8.2015 | M228 | 13.01 | 108 | 253.4 | 561 | 24 | 22 | 230 | 1.03 | 4.31 | 1.10 | 45 % |
| 13.8.2015 | M229 | 13.22 | 110 | 268.6 | 463.2 | 32 | 23 | 250 | 1.01 | 3.50 | 1.07 | 58 % |
| 13.8.2015 | M230 | 12.79 | 107 | 239.6 | 453.6 | 28 | 26 | 220 | 1.06 | 3.55 | 1.09 | 53 % |
| 20.8.2015 | M231 | 10.7 | 102 | 203.4 | 372.8 | 21 | 21 | 180 | 1.01 | 3.48 | 1.13 | 55 % |
| 20.8.2015 | M232 | 13.5 | 107 | 227.6 | 477 | 28 | 27 | 210 | 1.12 | 3.53 | 1.08 | 48 % |
| 20.8.2015 | M233 | 11.3 | 105 | 214.0 | 426.6 | 25 | 23 | 200 | 0.99 | 3.78 | 1.07 | 50 % |
| 20.8.2015 | M234 | 13.2 | 106 | 266.0 | 499.4 | 29 | 27 | 240 | 1.11 | 3.78 | 1.11 | 53 % |
| 20.8.2015 | M235 | 15.5 | 109 | 348.2 | 646 | 29 | 25 | 320 | 1.20 | 4.17 | 1.09 | 54 % |
| 27.8.2015 | M236 | 12 | 110 | 260.0 | 443 | 33 | 28 | 240 | 0.90 | 3.69 | 1.08 | 59 % |
| 27.8.2015 | M237 | 12.4 | 107 | 310.0 | 539 | 33 | 31 | 260 | 1.01 | 4.35 | 1.19 | 58 % |
| 27.8.2015 | M238 | 13 | 106 | 224.0 | 397 | 30 | 27 | 200 | 1.11 | 3.05 | 1.12 | 56 % |
| 27.8.2015 | M239 | 15.2 | 109 | 362.0 | 630 | 25 | 23 | 330 | 1.17 | 4.14 | 1.10 | 57 % |
| 27.8.2015 | M240 | 13.4 | 106 | 248.0 | 472 | 25 | 25 | 230 | 1.13 | 3.52 | 1.08 | 53 % |
| 2.9.2015 | M241 | 7.99 | 96 | 225.0 | 395 | 25 | 22 | 210 | 0.90 | 4.94 | 1.07 | 57 % |
| 2.9.2015 | M242 | 14.13 | 109 | 242.0 | 432 | 21 | 21 | 220 | 1.09 | 3.06 | 1.10 | 56 % |
| 2.9.2015 | M243 | 13.03 | 109 | 307.0 | 513 | 32 | 29 | 285 | 1.01 | 3.94 | 1.08 | 60 % |
| 2.9.2015 | M244 | 13.04 | 109 | 272.0 | 513 | 36 | 33 | 250 | 1.01 | 3.93 | 1.09 | 53 % |
| 2.9.2015 | M245 | 12.93 | 106 | 250.0 | 483 | 25 | 19 | 230 | 1.09 | 3.74 | 1.09 | 52 % |