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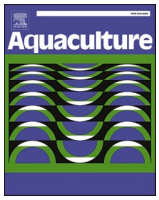

Stereological and histological characterization of spermatogenesis in Atlantic salmon (*Salmo salar)*

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ABSTRACT

Male Atlantic salmon display variation in life history strategies, where sexual maturation occurs during the freshwater phase as parr, after smoltification as jacks, as grilse after one, or after multiple winters at sea as anadromous adults, each strategy exhibiting evolutionary trade-offs. Spermatogenesis starts during puberty, salmon being a typical example for the unrestricted spermatogonia type of cystic spermatogenesis. If and how life history strategies affect the process of spermatogenesis is unknown. We characterized the germ cell stages appearing during spermatogenesis histologically and determined the increasing number of germ and Sertoli cells stereologically. We found 9 mitotic divisions and a loss of 64% of germ cells occurring during development, the highest ratio found in teleost fish yet. This may relate to the observation that salmon showed, with 207, the so far highest number of germ cells supported per Sertoli cell in vertebrates. We hypothesize that next to circulating hormones, such as follicle-stimulating hormone, local mechano-transduction pathways regulate changes in Sertoli cell number. Such changes accompany the development of spermatogenic cysts, apparently in response to the increasing and then again decreasing volume of individual germ cell clones. This coordinates Sertoli cell number with spermatogenic cyst maturation. No major differences in spermatogenesis were found between parr and two sea winter Atlantic salmon, consistent with a genetic fixation of the major traits of spermatogenesis, as found in most vertebrates.

1. Introduction

Since 2013, farmed salmonids have been the most valuable traded group of species of all aquaculture organisms worldwide. Atlantic salmon (*Salmo salar*) has experienced a rapid rise in demand due to a combination of marketing strategies, product innovation and technological advancements (FAO, 2024). In the wild; Atlantic salmon show different life history strategies; influenced by a combination of environmental and genetic factors (Hutchings and Jones, 1998; Imsland et al., 2014). Typically, Atlantic salmon, being anadromous, spawn in fresh water, where the offspring then grows until the parr stage. Alterations in photoperiod and water temperature trigger endocrine changes resulting in smoltification (Prunet et al., 1989) that enable salmon to transit from rivers to the marine environment where food is more abundant (Björnsson, 1997).

Puberty can be initiated in male Atlantic salmon before smoltification at the parr stage, which can develop into “sneaky spawners”, or after smoltification before one winter at sea (jacks), after one winter at sea (grilse) or after multiple winters at sea (multi-sea-winter [MSW] salmon) as anadromous adults (Hutchings and Jones, 1998). The exact mechanisms triggering maturation in parr remain unknown; but certain gene variants have been found associated with maturation (Pedersen et al., 2013). In jacks; grilse and MSW salmon; genotype-environment interactions modulate the timing of maturation. A considerable part of variation in time of maturation in these life history strategies can be attributed to alternative alleles for vestigial-like family member 3 (*vgll3)* (Barson et al., 2015) with evolutionary pressure seemingly favoring the homozygous *vgll3* late-maturing allele in male anadromous adults

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(Niemelä et al., 2022). Remarkably; maturing parr are reported to reach higher gonadosomatic index (GSI) values compared to grilse or MSW salmon (Gage et al., 1995; Vladić et al., 2019) even if parr are a fraction of the size of anadromous adults, a feature hypothesized to be a trade-off related to increased energy expenditure on reproduction for sneak-fertilizations by parr, instead of investing in courting and guarding behavior typical for anadromous adults (Vladić et al., 2002).

In salmon aquaculture, precocious puberty in male post-smolts and 1SW salmon (grilse) is associated with welfare issues and economic costs. Maturation induces osmoregulatory adaptations to freshwater, preparing the fish for the spawning migration into rivers, while the fish are confined to the marine environment in aquaculture facilities, leading to severe osmoregulatory maladaptation before harvest size is reached (Taranger et al., 2010). In addition; maturation negatively affects meat quality and growth; induces aggressive behavior; and increased mortality; jointly causing animal welfare issues and economic damage for the farmer (McClure et al., 2007). Moreover; escape of sexually competent salmon from aquaculture facilities is considered a major ecological risk regarding the potential for genetic introgression into wild populations (Besnier et al., 2022). The brain-pituitary-gonad (BPG) axis is the endocrine system responsible for initiating puberty and the development of fertility in all vertebrates (Schulz et al., 2024).

In male teleost fish, Sertoli cells provide the direct cellular environment supporting spermatogonia stem cells (SSC). The initial stage of development of a spermatogenic cyst is formed by a single Sertoli cell enveloping a single SSC. In addition, signaling molecules from other sources than Sertoli cells can contribute to the microenvironment characteristics, such as Leydig cells (Assis et al., 2016). Depending on the signaling "cocktail" in the SSC environment; it can remain quiescent; or – when cell cycling – undergo a self-renewal division; or embark on the path of spermatogenesis (Yoshizaki et al., 2024). Recent work has demonstrated that follicle-stimulating hormone (Fsh) signaling, a key-component of the BPG axis, is of crucial relevance in this regard in Atlantic salmon since loss of Fsh receptor function prevented the onset of puberty in males (Andersson et al., 2024). Work in zebrafish (*Danio rerio*) suggested that this is based on reduced self-renewal and a block of differentiating SSC cell cycling (Crespo et al., 2016; Nóbrega et al., 2015; Safian et al., 2017, 2018; Skaar et al., 2011).

When spermatogenesis starts towards differentiation, each differentiating SSC undergoes a mitotic division by producing a pair of spermatogonia interconnected by a cytoplasmic bridge; such bridges will connect all future descendants of a given stem cell, which thus forms a synchronously developing germ cell clone that is constrained in its mitotic activity to species-specific limits (Takamune et al., 2001; Yoshida et al., 2006). The germ cell clone then proceeds into meiosis (spermatocytes), finally forming spermatids that continue development without further proliferation into functional spermatozoa during a differentiation period known as spermiogenesis (Schulz et al., 2024). The increase in germ cell number and hence clone size, is associated with an increase in the number of the cyst-forming Sertoli cells, which stops after entering meiosis; Sertoli cells are required for survival (Matta et al., 2002), nurturing and regulating the development of "their" germ cell clone as well as for removing cell debris produced during spermatogenesis. This arrangement is referred to as the cystic type of spermatogenesis. Since the germ cell-supporting capacity of a single Sertoli cell is finite, the number of Sertoli cells in the testis determines the germ cell production capacity, which is generally more efficient in anamniote than in amniote vertebrates (França et al., 2015). Next to the number of mitotic divisions of germ cells and the number of supporting Sertoli cells, the total spermatogenic output of the testis is further determined by the number of spermatogenic cysts assigned to maturation, and finally by the number of germ cells becoming apoptotic during spermatogenesis. The loss of cells due to germ cell apoptosis typically accompanies normal spermatogenesis in vertebrates and amounts to losing 30–60% of germ cells in different vertebrates (Nóbrega et al., 2009).

For salmonids, the efficiency of spermatogenesis, as well as the number of Sertoli cells accompanying germ cell development have not been characterized. The start of puberty in Atlantic salmon postsmolts and grilse has previously been associated with an increase in the proliferation activity Sertoli cells as well as the earliest germ cell generation known as type A undifferentiated spermatogonia (A_{und}) (Melo et al., 2014; Schulz et al., 2019). In other teleosts, two morphologically different type A_{und} spermatogonia populations were discerned based on the morphology of the nucleus, such as in zebrafish (Leal et al., 2009) and in common carp (*Cyprinus carpio)* (Vigoya et al., 2025) the type A_{und}*, considered being the most undifferentiated germ cell type in the adult testis, is characterized by an irregular outline of the nuclear membrane forming several pockets often associated on the cytoplasmic side with nuage, electron-dense, non-membrane bound granules rich in RNA and RNA binding proteins (Lakshmi et al., 2025); while the nuclear membrane of type A_{und} single spermatogonia is smoother, is associated with less nuage, and shows a somewhat smaller cytoplasmic volume. Thus far, respective studies are missing in salmonids, which is relevant since SSCs form part of the A_{und} cell population. However, SSC-like properties of type A spermatogonia have been demonstrated by transplantation experiments in rainbow trout (*Oncorhynchus mykiss)* (Sato et al., 2017). Therefore; we examined the different generations of spermatogonia in detail in the present study. Respective knowledge may aid in future germ cell transplantation experiments in salmon (Kleppe et al., 2025), e.g., to accelerate breeding programs. The morphology of type A spermatogonia and further differentiated cell types, namely type B spermatogonia, meiotic (spermatocytes) and post-meiotic cells (spermatids and spermatozoa), have previously concisely been described in paraffin-embedded salmon testis of parr; postsmolts and grilse (Melo et al., 2014; Schulz et al., 2019; Skaftnesmo et al., 2021; Skjold et al., 2024). However, a detailed stereological and morphometrical analysis of the stages of spermatogenesis and accompanying Sertoli cells in Atlantic salmon is missing. To this end, the analysis of epoxy-embedded tissue is preferred over paraffin, offering higher quality and reliability of measurements and visualization (Copper et al., 2018). While the occurrence of apoptotic germ cell loss has been reported in postsmolts (Almeida, F.F.L. et al., 2022; Fjelldal et al., 2018; Skaftnesmo et al., 2021), particularly in type B spermatogonia and spermatocytes (Fjelldal et al., 2018), the average rate of apoptosis over all stages of spermatogenesis, Sertoli cell efficiency and mitotic generations that occur during spermatogenesis, as well as comparison of some of these parameters in different salmon life history strategies, so far have not been investigated. Consequently, effects of experimental treatments on spermatogenesis in salmon are difficult to evaluate in detail without baseline information.

Here, we describe spermatogenesis in under-yearling mature parr of less than 100 g body weight and compare it to spermatogenesis in two-sea-winter adult (2SW) salmon of more than 5 kg body weight. Through analysis of cysts at each stage of spermatogenesis, sectioned in their entirety, the nuclear diameter, cell diameter and total number of germ and Sertoli cells were determined, allowing us to describe the efficiency and morphometry of spermatogenesis in parr. The same parameters were described for the meiotic stages in 2SW, allowing to deduce the number of mitotic generations, Sertoli cell efficiency and germ cell loss also in adult 2SW males up until spermiogenesis.

2. Materials and methods / Experimental procedures

2.1. Experimental animals

adult Atlantic salmon of the AquaGen strain were reared under standard conditions at matre aquaculture Research Station (Matredal, Norway, 61°N, authorized by the Norwegian food safety authority, facility 110) and transferred to seawater in 2010. Rearing conditions mimicked standard farming conditions in commercial aquaculture. Since such conditions are subject to an exception within the Norwegian regulation on animal experimentation, no approval of the Norwegian animal research authority (NARA) was required. Testis tissue samples

were collected in June 2012. Maturing under-yearling salmon parr that hatched in spring 2012 were sampled in November 2012.

The 2SW adults were kept under continuous light from first feeding in spring 2009 until October 2009, when the photoperiod changed to a simulated natural photoperiod (SNP). This was maintained until transfer to sea cages in may 2010, where the fish experienced the natural photoperiod. The water temperature was kept at 12 °C from first feeding until the summer solstice in 2009, followed by the natural freshwater and seawater temperatures thereafter. Parr were raised in the same way, with first feeding in the spring of 2012, until sampling took place in November 2012 in freshwater under SNP conditions.

Testis tissue of four parr and four 2SW adults was used for histological and stereological analysis. Fish were anesthetized, weighed and decapitated, according to standard animal welfare guidelines in Norway. After opening the body cavity, the testes were extracted, weighed, and parts of each testis were processed for histology (see below). The gonadosomatic index (GSI) was calculated as the percentage of gonad weight from total body mass.

2.2. Histological and stereological analyses

Both testes of each fish were extracted and weighed (Table S1). The region between the anterior one-third and two-third total length of the testis was used for the analysis of spermatogenesis. Slices of ~5 mm thickness were cut and placed in an at least 20-fold volume of 0.1 M phosphate-buffered 4% glutaraldehyde fixative, pH 7.3 at 4 °C overnight. Following standard dehydration, the tissue was embedded in Technovit 7100 (Kulzer Technik) and sectioned using a Microm HM 340 E microtome. First, we measured the size of the nuclei of the different germ cell types in a pilot study, to determine the section thickness required to avoid counting the same nucleus twice on serial sections, following a previously described procedure (Matta et al., 2002). For the stereological analysis; serial sectioning of the testes was thus performed at 4 μm thickness to count mitotic and meiotic germ cell nuclei; and at 2 μm thickness to count spermatid nuclei; respectively. The sections were stained with 1% toluidine blue (Leal et al., 2009). From all individuals, cysts were identified that were sectioned in their entirety, so that within each cyst the number of Sertoli cells, Sertoli cell nuclear size, number of germ cells, germ cell nuclear size and the cell diameter (from which cell volume is derived) could be determined. From these endpoints, also the Sertoli cell efficiency, Volumetric Sertoli cell efficiency (VSE) and germ cell survival were calculated.

Sertoli cell efficiency was calculated by dividing the number of germ cells per cyst by the number of Sertoli cells of the same cyst, after which the average of the Sertoli cell efficiency of each cyst was taken. Germ cell survival was calculated by dividing the number of germ cells by the theoretical number of germ cells, multiplied by 100%. Calculations for volumes were based on measurements of the nuclear diameter and cellular diameter with the formula: $V = \pi d^3 / 6$. Standard error of the mean of calculated values was obtained through propagation of uncertainty.

Four parr and four 2SW salmon were analyzed. To calculate the average germ cell and Sertoli cell nuclear diameters, each nucleus was measured twice using an ocular micrometer, calibrated with a stage micrometer, and the average was taken. For each of the parr fish, twenty nuclear diameter measurements were performed per germ cell type (A_{und}*, A_{und}, $A_{diff}1$, $A_{diff}2$, $A_{diff}3$, $A_{diff}4$, B1, B2, B3, B4, preleptotene/leptotene/zygotene, pachytene, diplotene, secondary spermatocyte, spermatid 1, spermatid 2, spermatid 3), thus a total of 80 measurements were performed in total per germ cell type for the parr group (Table S2).

Sertoli cell nuclear diameter was measured twice as described for germ cells and the average was taken. For each parr, 5 Sertoli cell nuclear diameter measurements were made per cyst type (measuring the nuclei of Sertoli cells forming these cysts), i.e., 20 measurements were made in total. Next, the number of cells in one cyst type was determined by counting the number of nuclei found in each section (thickness: 4 μm for mitotic and meiotic germ cell cysts, 2 μm for spermiogenic germ cell cysts) across the whole cyst. This was performed for 5 cysts per cyst type per fish. Since one nucleus may be present in multiple sections, the total number was adjusted with a correction factor, calculated as follows:

$$CorrectionFactor = Avg.nuclear\ diameter\ (of\ germ\ cell\ type) / section\ thickness.$$

Dividing the number of nuclei counted by the correction factor yields the adjusted number of cells per cyst. Since our main question concerned the number of spermatogonia generations preceding meiosis, cell counting was only done at the spermatocyte stage in 2SW salmon (Table S3). In addition, nuclear size was measured using an ocular micrometer for both meiosis and spermiogenesis, to infer possible size differences in the two life stages. Like in parr, 20 germ cell nuclear diameter measurements were made per cyst type per fish in the 2SW group, 5 nuclear diameter measurements for Sertoli cells per cyst type per fish and to determine germ cell and Sertoli cell number per cyst, 5 cysts were assessed per fish. The correction factors used for each cell type and salmon life stage are given in Table S4.

2.3. Statistical analysis

All experimental data were analyzed using two-way ANOVA (Student-Newman-Keuls test) in R 4.4.1 with aid of first the *leveneTest* from the *car* package (for variance), then the *Shapiro.test* function from the *stats* package (for normality), and finally the *SNK.test* function from the *Agricolae* package and *aov* function from the *Stats* package (R Core Team, 2024). Data was visualized using the *ggplot2* package (Wickham, 2016) and *ggpubr* package (Kassambara, 2025). Data regarding "*no. of germ cells per cyst*" was log2-transformed (to linearize data at each step of cell division), and data regarding "cell volume", "cyst volume", "Sertoli cell efficiency" and "VSE" were natural-log-transformed before further statistical testing. For each metric, normality and homoscedasticity between cyst types were established before testing for significance, by application of the Shapiro-Wilk test and the Levene's test, respectively. In tables and figures, non-transformed data is always shown. Differences were considered significant at a level of $\alpha < 0.05$ and were visualized in this publication using compact letter display. All quantitative data is given as mean ± SEM (standard error of the mean).

3. Results and discussion

3.1. Testis anatomy

The testes in Atlantic salmon are paired, elongated structures running along the length of the body cavity, ventral of the trunk kidney and bilateral from the medially located swim bladder (Fig. S1). As described previously (Grier et al., 1980), no polarity or maturational gradient for spermatogenesis was found in testis tissue; and in maturing males, spermatogenic cysts with germ cells at all stages of development were randomly distributed over the different spermatogenic tubules, so that the salmon testis can be grouped among the unrestricted spermatogonia testis type (Schulz et al., 2024). Spermatogenic tubules form an anastomosing network that connects to an efferent duct (Fig. S1), a caudal extension of each testis that is composed of tubules lined with a Sertoli cell-like epithelium. The two ducts fuse caudally into a single sperm duct shortly before connecting to the urogenital sinus, which opens into the urogenital pore where also the urinary duct ends.

In immature salmon, testes are thin, threadlike organs (see Oldham et al., 2023: Fig. 1). With the initiation of puberty, testes show superallometric growth, reflected in an increase in the gonadosomatic index (GSI) in both parr and adult salmon (Ciani et al., 2021; Schulz et al., 2019). With the appearance of significant numbers of germ cells in meiotic and post-meiotic stages, the testes adopt a whitish colour and efferent ducts become distinguishable (Fig. S1).

Testis weight for parr averaged at 5.6 ± 1.0 g (SEM), for 2SW males

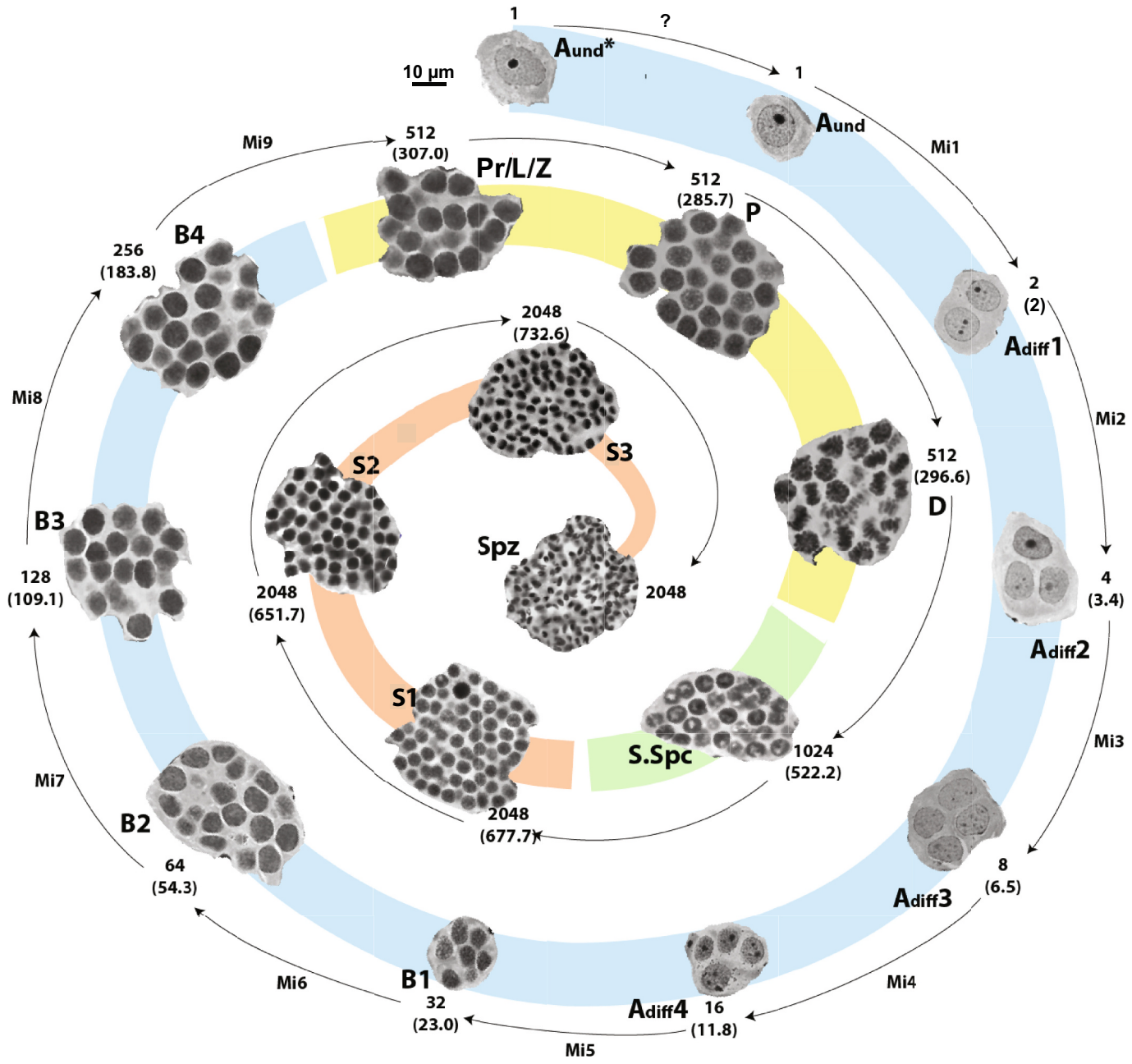

Fig. 1. Progression of germ cell differentiation within a cyst during spermatogenesis in Atlantic salmon. Numbers indicate theoretical cell numbers if no germ cell loss would take place, numbers in brackets indicate the actual (counted) average cell numbers found. Spermatogenesis starts at the A_{und}* stage as the most undifferentiated, single cell type, from where an unknown transition, indicated by a question mark, leads to a single A_{und} cell. Germ cells then undergo 9 mitotic cell divisions (Mi1 – Mi9, blue), differentiating from A_{und} to type A_{diff} and subsequently type B spermatogonia. After the final mitotic cell cycle, B4 spermatogonia differentiate into preleptotene/leptotene/zygotene (Pr/L/Z) primary spermatocytes, thereby entering meiosis. After reaching the pachytene (P) and diplotene (D) stages, the two meiotic divisions occur, first to secondary spermatocytes (S.Spc), and then to spermatids. Spermatids differentiate without cell cycling through three (arbitrary) stages (S1, S2, S3) into mature spermatozoa (Spz) which are released by Sertoli cells during spermiation and are found as free cells in the lumen of the spermatogenic tubule. In blue, yellow, green and orange, the phases mitosis, meiosis I, meiosis II, and spermiogenesis are indicated, respectively. All images are to scale. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

at 333.8 ± 82.8 g (Table S1). However, we found an average GSI of 6.7 ± 1.0% for under-yearling parr and 4.0 ± 0.4% for 2SW salmon at the time of sampling (Table S1). As expected, based on these GSI values (Ciani et al., 2021; Schulz et al., 2019), the testes contained all stages of germ cell development, including free sperm in the tubular lumina.

3.2. Cystic spermatogenesis, number of mitotic divisions and germ cell loss

One of the hallmark features of animal germ cells going through differentiating cell divisions is that cytokinesis is incomplete, so that the differentiating daughter cells derived from a given stem cell remain interconnected by cytoplasmic bridges, thus forming a synchronously developing germ cell clone (Sertoli, 1878). In the cystic type of spermatogenesis seen in fishes and amphibians, different clones are segregated from each other by Sertoli cells, such that a group of Sertoli cells envelopes a germ cell clone to form a spermatogenic cyst (Pudney, 1993). By analyzing serial sections and counting the nuclei of cells in cysts in different stages of development, starting from the initial cyst of a

single type A spermatogonium enveloped by a single Sertoli cell, we determined the total number of the cysts' Sertoli cells and germ cells throughout cyst development. This also allowed deducing the number of mitotic cell cycles salmon germ cells go through before entering meiosis. Moreover, by comparing the theoretical number of germ cells after each mitosis with the actual number counted, which was always lower than the theoretical number (see below), we were able to estimate the efficiency of salmon spermatogenesis in two ways: first, by determining the fraction of cells lost, probably to apoptosis (Baum et al., 2005), and by determining at what specific developmental step(s) these losses were occurring, and second by calculating the germ cell/Sertoli cell ratio. Finally, this information allowed ranking of the germ cell generations into successive stages forming a developmental path, in turn enabling us to identify stage-specific morphological characteristics (Fig. 1; Fig. 2A - K). The complete set of stereological results obtained (e.g., nuclear and cellular diameters and volumes as well as cell numbers) are shown in Table S2 for parr and Table S3 for 2SW adults; selected aspects are also shown in Figs. 3–6.

3.3. Cysts with single spermatogonia

In the testis of parr, two types of cysts were found that were composed of a single germ cell, A_{und}* or A_{und}, each enveloped by one Sertoli cell (Table S2; Fig. 1; Fig. 2A and B). The germ cell types present in such cysts are the largest found in the testis in terms of cell and nuclear size. Type A_{und}* spermatogonia show the largest nuclear diameter (Table S2: germ cells - nuclear diameter) and cell size (Table S2: cell volume) of all germ cell types, with little heterochromatin and a convoluted nuclear envelope, a large cytoplasmic volume and many darkly stained nuage particles (aka peri-nuclear germ granules) associated with the external side of the convoluted nuclear membrane (Fig. 1, Fig. 2A). In the light of these features and the fact that type A_{und}* cells resemble primordial germ cells, this type is considered being the most undifferentiated germ cell type, as also found in other fish species (Schulz et al., 2010). The A_{und} germ cell type, in contrast, shows a rounded nucleus, slightly smaller cell and nuclear size, and nuage particles are less frequent (Fig. 1; Fig. 2B). At the A_{und} stage, the total cyst volume is at its minimum (Table S2: cyst volume).

Subtypes of A_{und} have been described in other teleosts, among which zebrafish (*Danio rerio*) (Leal et al., 2009; Nóbrega et al., 2010), yellowtail tetra (*Astyanax altiparanae*) (de Paiva Camargo et al., 2017); yellow croaker (*Larimichthys polyactis*) (Yang et al., 2024); black rockfish (*Sebastes melanops*) (Jin et al., 2024) and rainbow trout (Bellaiche et al., 2014). Also in mammals (especially primates), subtypes of single germ cells, A_{dark} and A_{pale} are distinguished, A_{pale} showing a higher proliferation activity, while A_{dark} is considered the reserve SSC population (van Alphen et al., 1988).

Recently, a study investigated the transcriptome of early spermatogonia in a seasonally reproducing fish where, like Atlantic salmon, the gonads regress after the spawning season, to then regenerate and mature again (Jin et al., 2024). Based on transcriptomic, not morphological criteria, two subtypes; 'undiff 1' and 'undiff 2' were identified that, according to pseudo-time analysis, transition along the same gradient, succeeded by subsequent differentiating stages of spermatogenesis. Comparing regressing and regenerating testis, the authors reported that the numbers of both, 'undiff 1' and 'undiff 2' cells, were higher in the regenerating phase. However, the 'undiff 2' population increased at a higher rate and exhibited increased gene activity related to proliferation (Jin et al., 2024). In zebrafish, studies using testes recovering from busulfan treatment that induced substantial germ cell loss, found that the ratio between A_{und}*:A_{und} shifted from 1:1 (control) to 1:3 (recovering), suggesting A_{und} may constitute the population with higher proliferation activity (Nóbrega et al., 2010). If A_{und} indeed are like 'undiff2' requires further investigation clarifying the relation between morphological and transcriptomic stages of the type A spermatogonia.

In Atlantic salmon an increase in the proliferation activity of undifferentiated type A spermatogonia was previously observed, however whether A_{und}* or A_{und} account for this increase was not investigated (Schulz et al., 2019). In recent studies on Fsh-receptor (*fshr)* knock-out salmon (Andersson et al., 2024); which fail to enter puberty; both A_{und}* and A_{und} were found; the latter becoming the dominating germ cell type in this mutant testis tissue with time. It is not known yet if a cell cycle separates these two morphological variants or if they represent different stages of a single cell cycle. Regardless; we understand the way A_{und} germ cells are formed and accumulate in the *fshr* mutant salmon testes with time as supporting the sequence A_{und}* à A_{und} regarding an increasing status of differentiation (Andersson et al., 2024).

Transcriptome profiling of the 'undiff2' cell population showed a higher expression of genes related to oxidative phosphorylation pathways and proliferation (Jin et al., 2024), generally associated with differentiation (Tsogtbaatar et al., 2020). 'Undiff1' on the other hand expressed genes related to (anaerobic) glycolysis. This is in line with findings in Atlantic salmon, where an upregulation of anaerobic pathways was reported in testis tissue with undifferentiated A spermatogonia showing increased self-renewal proliferation together with increased Sertoli cell proliferation at the start of puberty (Crespo et al., 2019). Similarly, single-cell RNA-sequencing (scRNA-seq) experiments in mouse and human germ cells indicated that glycolysis is more prominent in SSCs (Hermann et al., 2018).

In zebrafish germ cells, a protein called meiosis specific with coiled-coil domain (Meioc) locates to the nuage granules, where it binds to and prevents the release of another protein called Piwi-like RNA-mediated gene silencing 1 (Piwil1) from the granule to the nucleolus. When having access to the nucleolus, Piwil1 downregulates ribosomal RNA expression, thereby reducing the cell's protein production capacity, which is correlated with preventing differentiation of spermatogonia in zebrafish (Kawasaki et al., 2025). The relevance of the Piwil1 protein also for the survival and development of Atlantic salmon germ cells has been demonstrated in genetic studies (Almeida, F.F.L. et al., 2022). The amount of nuage in salmon germ cells changes from A_{und}* to $A_{und,}$ and further the $A_{diff,}$ as suggested by our studies (Fig. 1: A_{und}*, $A_{und,}$ A_{diff}). As such, rRNA expression, influenced by peri-nuclear granule constituents, may potentially aid as a distinguishing feature bridging morphology and transcriptome data in future studies, also in salmon.

3.4. Cysts with multiple spermatogonia

SSCs that undergo self-renewal yield two independent cells, one remaining associated with its supporting Sertoli cell, while the second can form a new cyst by associating with an idle Sertoli cell, thereby expanding the SSC population and the number of initial spermatogenic cysts. In contrast, when SSCs undergo a differentiating division, cytokinesis at the end of the differentiating cell cycle is incomplete such that a cytoplasmic bridge remains to connect the two differentiating daughter cells, which now form a pair of cells surrounded by their cyst-forming Sertoli cell. All subsequent cell cycles show the same incomplete cytokinesis, such that differentiating germ cells derived from a given stem cell form an interconnected, synchronously developing germ cell clone that shows geometrically increasing cell numbers (i.e., 2, 4, 8, 16 etc.) with each cell cycle (Xie et al., 2020). The cytoplasmic bridges keep connecting the cell clone members until the completion of spermiogenesis, shortly before spermiation occurs and the cyst wall opens; however; in some flatfish species, germ cell individualization occurs already at the beginning of spermiogenesis (Schulz et al., 2024). This definition of stem cell vs. differentiating spermatogonia differs somewhat from the one established in mammals, where the term SSC also refers to the stemness of germ cells. Stemness is defined as the capacity of germ cells to establish donor-derived spermatogenesis in the testis of an infertile recipient after germ cell transplantation. While single A_{und} have the highest stemness, also pairs and even clones of 4 type A spermatogonia can show some stemness in experiments with rodent models

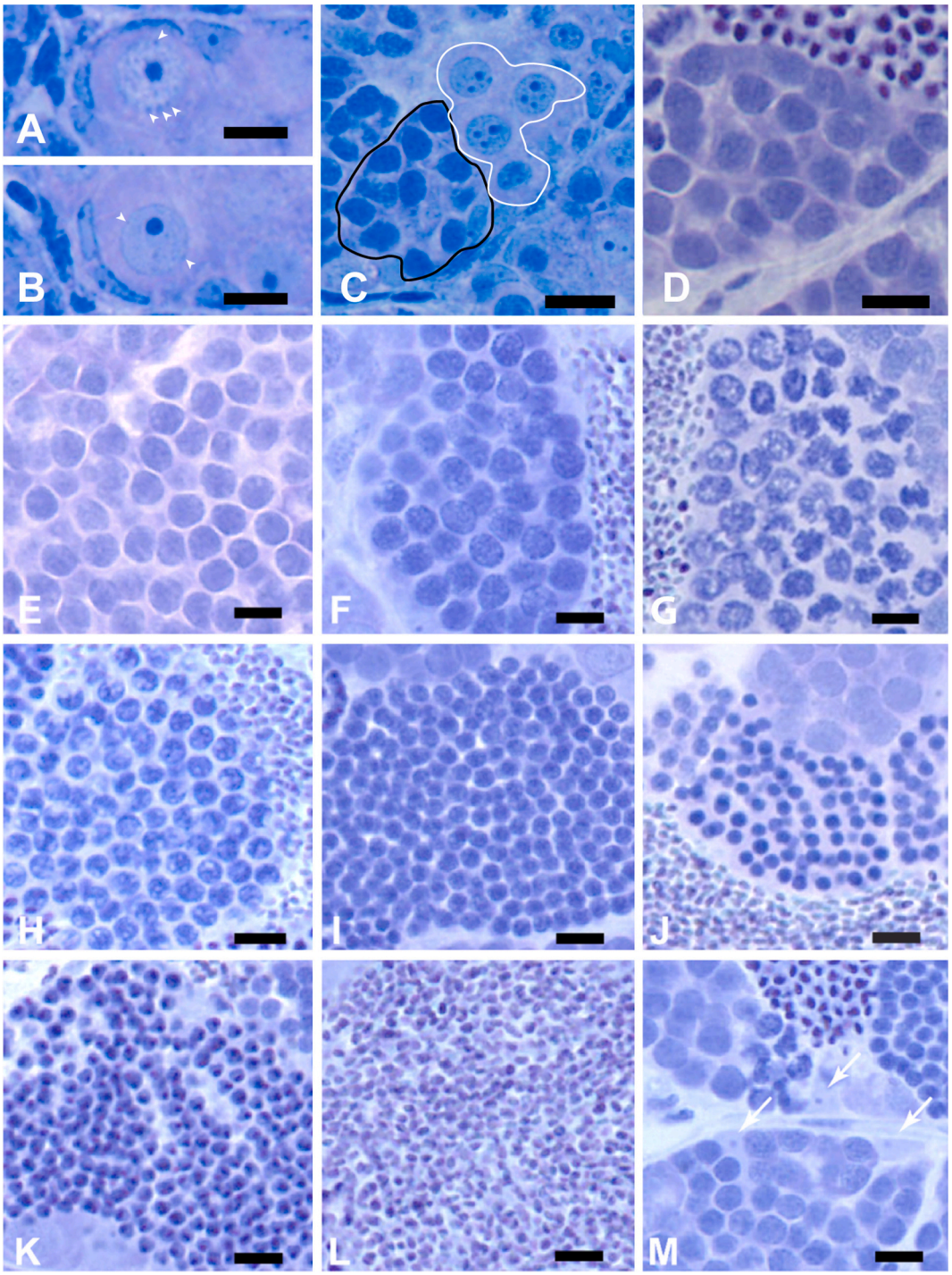

Fig. 2. Toluidine blue staining of Atlantic salmon testis sections. A - L show the morphology of the germ cell types and steps of spermatogenesis, M shows Sertoli cells. A) Type A_{und}* spermatogonia. Cytoplasmic nuage particles visible near the somewhat convoluted nuclear envelope (white circles). B) Type A_{und} spermatogonia, where the nuclear membrane is smooth and nuage particles (white circles) are less abundant and less pronounced than in A_{und}* C) A group of type A_{diff} spermatogonia (white boundary) is situated next to a group of type B spermatogonia (black boundary). D) Type B spermatogonia. E) Preleptotene/leptotene/zygotene spermatocytes; homologous chromatids start to pair, appearing as darkly stained threads in the nucleus. F) Pachytene spermatocytes; pairing and condensing of chromatids result in thick, darkly stained threads in the nucleus that contact the nuclear membrane. G) Diplotene spermatocytes; homologous chromatids start to separate, and the lightly colored nucleoplasm becoming visible between chromatids. H) Secondary spermatocytes; this cell type shows condensed chromosomes, quickly completes the second meiotic division and therefore is rare on sections. I) S1 spermatids. J) S2 spermatids. K) S3 spermatids. L) spermatozoa. M). Sertoli cell nuclei (white arrows) appear triangular or bean-shaped, often containing a single nucleolus. Thinly stretched cytoplasmic extensions form the boundary of spermatogenic cysts or line the lumen of spermatogenic tubules. Scale bars are 10 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(Lord and Oatley, 2024). Since transplantation studies are only starting to be established in Atlantic salmon (Kleppe et al., 2025), it is possible that the present definition of undifferentiated single A_{und*}/A_{und} vs. A_{diff} spermatogonia, i.e., clones of two or more cells, will change when functional results based on transplantation studies will become available.

Before entering meiosis, differentiating spermatogonia proceed through a genetically determined number of mitotic divisions morphologically distinguishable as type A_{diff} spermatogonia and type B spermatogonia (De Rooij and Russell, 2000). These morphological differences are found in all studied vertebrates (Leal et al., 2009) and can be characterized as follows. Type A_{diff} spermatogonia show a small amount of heterochromatin in the nucleus; one or a few nucleoli; larger nuclear size and a larger cytoplasmic volume than type B spermatogonia (Fig. 1: $A_{diff}1–4$; Fig. 2C) and resembles the nucleus in A_{und} spermatogonia; albeit smaller in size. Type B spermatogonia generally show two or more nucleoli and stain darker with nuclear dyes (Fig. 1: B1–4; Fig. 2D) due to a higher amount of heterochromatin (Lacerda et al., 2014). $A_{und}*$, A_{und} and some A_{diff} also can be found in immature testis (Melo et al., 2014). In zebrafish, differentiation of spermatogonia depended on an increased transcription of rRNA, involving Meioc and Piwil1 proteins, as discussed above (Kawasaki et al., 2025). In *meioc* loss-of-function mutant zebrafish testes, only spermatogonia type A_{und} and A_{diff}, but not type B, can be found. This is in line with our previous observation that type B spermatogonia are present only in pubertal salmon testes (Schulz et al., 2019), opening the possibility that Piwil1 may have an alike function in the puberty-associated production of type B spermatogonia in salmon. However, this is preceded by an increased single cell proliferation of type A spermatogonia and of Sertoli cells, together with elevated plasma androgen levels, hallmark features of entering puberty in male salmon (Crespo et al., 2019) depending on Fsh receptor-mediated signaling (Andersson et al., 2024).

In the present study, we found cysts of differentiating type A spermatogonia, in which between one and four differentiating mitotic divisions had occurred. The first of such divisions result in a cyst with two germ cells (Fig. 3A) which are smaller in both nucleus and cell size than their type A_{und} precursors (Table S2: germ cells - nuclear diameter, cell volume, Fig. 2C), but still clearly type A based on morphology and hence characterized as type $A_{diff}1$ (Fig. 1: $A_{diff}1$). Interestingly, Sertoli cell number did not significantly increase compared to A_{und} (Fig. 3B; Table S2: no. of Sertoli cells), whereas in other species, Sertoli cell numbers increased after the first differentiating division (Leal et al., 2009; Rodrigues et al., 2017; Schulz et al., 2005).

While in total four generations of differentiating type A spermatogonia occur with a geometrically increasing germ cell number and a gradually increasing Sertoli cell number (Fig. 3A, B; Table S2: germ cell number, Sertoli cell number), cyst volume increased from the nadir at A_{und} but stabilized until the A3 stage (Fig. 4A), probably related to a certain loss in cell volume and some germ cell loss (Table S2: cyst volume, cell size). Germ cell loss is first observed in $A_{diff}2$ (Fig. 5), with an average loss of 0.6 cells, accounting at this early stage for 14.6% of total theoretical spermatogenic output, had these cells proceeded with subsequent cell divisions and differentiated into spermatozoa.

Germ cell nuclear diameter generally decreases with differentiating spermatogenic divisions (Leal et al., 2009; Schulz et al., 2005). This is also the case in Atlantic salmon (Table S2: germ cells - nuclear diameter). Interesting to note is that the nuclear diameter in salmon spermatogonia is the largest described to date in teleost fish (Table S2: germ cells - nuclear diameter), possibly related to the additional genome duplication event that occurred in the salmonid lineage (Macqueen and Johnston, 2014). Similarly sized nuclei have been described in spermatogonia of other salmonids; such as rainbow trout (Loir, 1999). At the end of the type A spermatogonia divisions, nearly a fifth of the theoretical germ cell production has been lost (Table S2: germ cell survival), a total cell loss at this point of ~3 of theoretically 16 cells (Fig. 5).

Spermatogenic cysts with more than 16 spermatogonia showed a different nuclear morphology that allowed classifying them as type B spermatogonia, based on the characteristics described above (Fig. 1: B1–4, Fig. 2D). By counting the cell numbers in each cyst, four generations of type B spermatogonia (B1 – B4) could be distinguished. Nuclear diameter, a reliable method to differentiated between different generations of type B spermatogonia in other species (De Melo Dias et al., 2017), shows considerable overlap between generations in Atlantic salmon (Table S2: germ cells - nuclear diameter). Only the nuclear diameter of stage B1 significantly differs from the three other type B spermatogonia, that, peculiarly, is smaller than either A4 or B2 spermatogonia. Since there is also little other morphological variation between type B spermatogonia generations (Fig. 1: B1–4), the total cell number in each cyst is so far the only reliable parameter for identifying the exact spermatogenic cell stage (see Table S2: no. of germ cells per cyst).

Based on the reduced cell dimensions type B1 spermatogonia cysts also show a slightly lower volume, despite a near doubling of cell numbers (Fig. 3A), while apoptosis plays a negligible part in this regard, as germ cell loss is less than 2% in this step (Fig. 5). Accordingly, Sertoli cell efficiency doubles, since the Sertoli cell number per cyst remained stable, despite supporting twice the number of germ cells (Fig. 3A, B; Table S2: Sertoli cell efficiency). On the other hand, the total cyst volume did not change significantly since A3 spermatogonia, suggesting that the demand for Sertoli cell support remained stable, as reflected by an also stable volumetric Sertoli cell efficiency (VSE) (Table S2).

In contrast to cysts containing A3 to B1 spermatogonia, nuclear diameter and cyst volume increased 2.6-fold in B2 cysts (Table S2: germ cells - nuclear diameter, cyst volume), accompanied by a significant increase in Sertoli cell numbers (Table S2: no. of Sertoli cells per cyst, cyst volume). A drop, followed by an increase in nuclear size in type B spermatogonia, was not observed in guppy (*Poecilia reticulata)* (Billard and Escaffre, 1969); common carp *(Cyprinus carpio)* (Hulak et al., 2008); bamboo-leaf wrasse (*Pseudolabrus sieboldi*) (Kitano et al., 2011); tilapia (*Oreochromis niloticus*) (Schulz et al., 2005); rainbow trout (Loir, 1999); yellow-tail tetra (Rodrigues et al., 2017) and zebrafish (Leal et al., 2009); and thus may be specific to Atlantic salmon. A possible explanation for smaller sized B1 cells is assuming a comparatively short cell-cycle time from B1 to B2, resulting in a shorter time window, in which cell growth can be realized before the next cell division comes up. B3 cysts again nearly doubled in cyst volume (1.87×) associated with a cell division. Sertoli cell numbers again remained identical to those found in B2 cysts (Fig. 3B; Table S2: no. of Sertoli cells per cyst). The fourth and final B spermatogonia cyst type identified before meiosis showed a less steep increase in cyst volume (1.4× increase in volume). Sertoli cell number further increased to approach the maximum number (Fig. 3B; Table S2: no. of Sertoli cells per cyst). This is in accordance with other studies reporting that Sertoli cell proliferation mainly increased with spermatogonia proliferation (Leal et al., 2009; Schulz et al., 2005). While type B spermatogonia so far showed little germ cell loss, this changed for the final generation before entering meiosis, such that germ cell loss at the end of all mitotic generations is 42.1%, an absolute cell loss of 74 cells until this stage (Fig. 5). A slight increase in germ cell numbers is seen at B2 and B3 (Fig. 5), reflecting the presence of some larger cysts at the B2 and B3 generations of spermatogonia containing a higher number of germ and Sertoli cells. As will be discussed further below, these cysts may have originated theoretically from the fusion of two spermatogenic cysts, an additional round of mitosis, or from exceptionally low apoptotic cell loss.

During the mitotic phase, a total of 9 cell cycles took place before meiosis started (Table S2: no. of germ cells per cyst, Fig. 1), although currently, it is not known whether $A_{und}*$ divide to form two independent, single $A_{und}*$ or two independent, single A_{und}, or one $A_{und}*$ and one A_{und}, or if an $A_{und}*$ differentiates without cell cycling into an A_{und}; also, it cannot be excluded that depending on the conditions, permutations between these pathes may occur. Within the family of salmonids, the number of mitotic divisions shows some variation. In the genus of

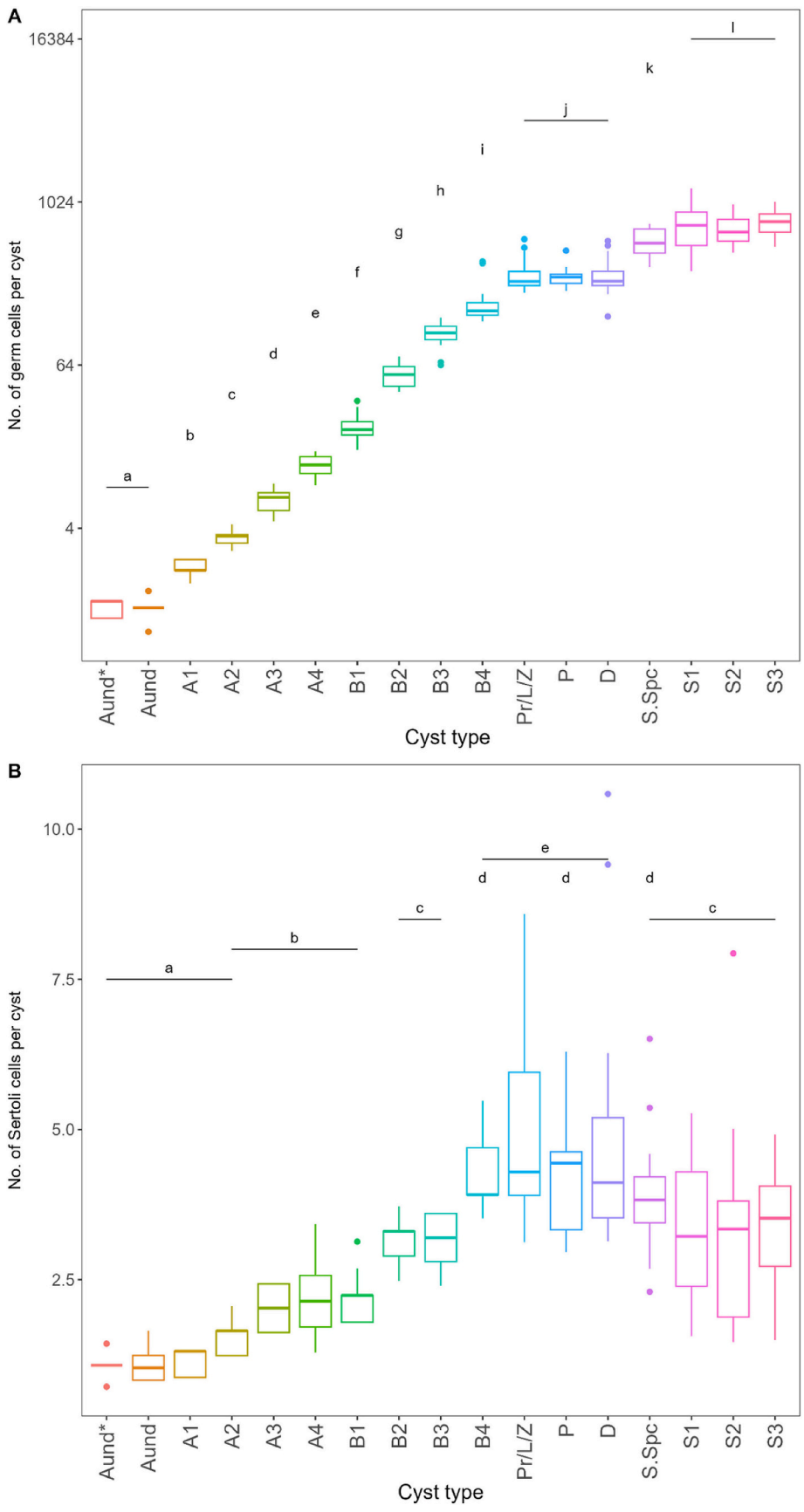

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Fig. 3. A) Logarithmic (log2) scaling of germ cell numbers for each germ cell type. Relative spread of cell number was lower during the mitotic phase of spermatogenesis, compared to meiosis I and spermiogenesis, when a larger variation in germ and Sertoli cell numbers was observed. B) Sertoli cell number per cyst. Variation increased during meiosis and spermiogenesis. Horizontal bars indicate the median. Different characters indicate significant ($\alpha < 0.05$) differences. A_{und}* = single undifferentiated type A spermatogonia, A_{und} = single undifferentiated type A spermatogonia, A1 – A4 = four generations of differentiating type A spermatogonia, B1 – B4 = four generations of type B spermatogonia, Pr/L/Z = preleptotene, leptotene and zygotene spermatocytes during meiosis I, P = pachytene spermatocytes during meiosis I, D = diplotene spermatocytes during meiosis I, S.Spc = secondary spermatocyte, S1 – S3 = stages of maturing spermatids.

Oncorhynchus, the Pacific salmonids, rainbow trout and masu salmon (*Oncorhynchus masou*) show 6 and 10 mitotic divisions, respectively (Ando et al., 2000; Loir, 1999), while sakhulin taimen (*Parahucho perryi*) go through 8 mitotic divisions (Ando et al., 2000). In the *Salmo* genus; Atlantic salmon is the only described species so far, with 9 mitotic divisions. The number of mitotic divisions usually is specific for a given species and is genetically encoded (França et al., 1998), but there are also examples for some variation in the number of pre-meiotic cell divisions, like in *Xenopus* (Takamune et al., 2001), mouse (Yoshida et al., 2006) and primates (Plant, 2010). A hypothetical, alternative explanation for considerably higher germ cell and Sertoli cell number in some cysts, compatible with an extra mitosis, is that two cysts in the same stage of development may have fused. So far, there is no experimental evidence in Atlantic salmon supporting either an extra round of mitosis or the fusion of cysts.

Parr invest a relatively higher amount of energy in spermatogenesis, as indicated by their higher GSI, and forgo investment in somatic tissues, increasing reproductive odds. Since there is some flexibility in the number of mitotic divisions in some species (see above), it appeared possible that this differential investment in spermatogenesis was connected to differences in the number of mitotic divisions between parr and 2SW. While our observations do not support this notion (Table S2: no. of germ cells per cyst, Table S3: no. of germ cells per cyst), some uncertainty remains. Spermatogonia cysts in 2SW males were not analyzed directly but the number of mitotic cell cycles was deduced from the number of primary spermatocytes. If, for example, the loss of spermatogonia to apoptosis was much lower in 2SW males, only 8 mitotic divisions might lead to a similar number of primary spermatocytes. However, this seems unlikely considering that 1SW grilse regularly showed apoptotic spermatogonia in their testes (Skaftnesmo et al., 2021), resembling the situation in precocious parr and potentially also in 2SW salmon.

3.5. Meiosis I

Generally, the different stages of the prophase of meiosis I are distinguishable based on nuclear morphology, such as chromosome condensation and pairing, as described in previous studies (Leal et al., 2009; Schulz et al., 2005). In salmon, identification of preleptotene, leptotene and zygotene stages was difficult, as chromosomal structures after toluidine blue staining were in part obscured by a haze in these earlier stages of prophase (Fig. 1: Pr/L/Z; Fig. 2E) and only became more clearly visible at the pachytene stage (Fig. 1: P; Fig. 2F). This might be the result of the metachromatic properties of toluidine blue, possibly staining one or more nuclear components present in early salmon spermatocytes but not or much less found in other species. Henceforth, preleptotene, leptotene and zygotene stages were grouped and analyzed as one stage (Pr/L/Z) and were distinguishable from type B spermatogonia and pachytene spermatocytes by darkly stained chromosomal threads contacting the inner nuclear membrane (Fig. 1: Pr/L/Z; Fig. 2E), though less condensed compared to the pachytene stadium. Further distinguishable characteristics of Pr/L/Z are the absence of the small nucleoli seen in type B spermatogonia, the total cell number contained in one cyst, and the nuclear diameter which further decreased to a value slightly smaller than measured in B4 spermatogonia (Table S2: germ cells - nuclear diameter, no. of germ cells per cyst). At this stage the maximum cyst size is reached, accompanied by reaching the maximum number of ~5 Sertoli cells during spermatogenesis (Table S2: cyst volume, no. of Sertoli cells). No further increase in Sertoli cell number is observed (Table S2: no. of Sertoli cells per cyst) as is typical for teleost meiosis (Leal et al., 2009; Matta et al., 2002; Schulz et al., 2005). Although no cell barrier studies have been performed in salmon, tight junction formation between Sertoli cells usually coincides with reaching maximum Sertoli cell numbers and cyst volume values before entry into spermiogenesis (França et al., 2015).

The next phase of prophase I, pachynema, is generally characterized by condensing chromosomes (Fig. 1: P; Fig. 2F); nuclear size and cell size significantly increase (Table S2 - germ cells: nuclear diameter, cell volume) as seen in bamboo leaf wrasse (Kitano et al., 2011); zebrafish (Leal et al., 2009); yellow-tail tetra (Rodrigues et al., 2017) and tilapia (Schulz et al., 2005). However; in Atlantic salmon the nucleus size is larger in Pr/L/Z spermatocytes (Table S2: germ cells - nuclear diameter); like in guppy (Billard and Escaffre, 1969). In accordance with nucleus size; cyst size in other species reaches its peak at the pachytene stage (Leal et al., 2009; Schulz et al., 2005); while cyst size remains stable during meiosis in salmon (Table S2: cyst volume). Following pachynema; diplotene spermatocytes contain condensed chromosomes (Fig. 2G) that start to separate from their homologous chromatid and exhibit the smallest nuclear diameter of all prophase I stadia (De Melo Dias et al., 2017; Leal et al., 2009), also in salmon (Table S2: nuclear diameter). Like observed in other teleosts (Leal et al., 2009; Schulz et al., 2005), salmon do not experience significant germ cell loss throughout prophase I (Fig. 5).

Like germ cells (Fig. 3A), variation in Sertoli cell numbers is larger during meiosis I than during most of the mitotic phase, indicative of larger variety in individual cyst sizes (Fig. 3B), as has been discussed already above regarding the latest spermatogonia type B generations. During meiosis I, a Sertoli cell efficiency of about 65 germ cells per Sertoli cell is reached (Table S2: Sertoli cell efficiency), roughly double the Sertoli cell efficiency in zebrafish and tilapia at this stage (Leal et al., 2009; Schulz et al., 2005) and higher than; but in the range of; guppy (Billard and Escaffre, 1970).

3.6. Meiosis II

Cyst volume and Sertoli cell numbers start to decrease after completion of meiosis I (Table S2: cyst volume; no. of Sertoli cells per cyst). Secondary spermatocytes are typically short-lived, as DNA synthesis is skipped and the cells quickly proceed to meiosis II (Schulz et al., 2010). As chromosome homologs segregate during meiosis I, DNA content and nucleus size is reduced in secondary spermatocytes (Table S2: germ cells - nuclear diameter). This stage is therefore distinguishable by a halving in cell size, and the presence of condensed chromosomes (Fig. 1: S. Spc; Fig. 2H), in anticipation for the second meiotic cell division (Schulz et al., 2010). Sertoli cell number significantly decreases (Table S2: Sertoli cell number per cyst), along with a minor, insignificant, loss of germ cells and decrease in cyst size (Table S2: no. of germ cells per cyst, cyst size).

Interestingly, during meiosis I and II, significant differences between nucleus size in parr and 2SW are found (Table S2, Table S3: germ cells - nuclear diameter) with 2SW skewing on the larger side. There is some evidence suggesting larger male-guppies produce longer spermatozoa (Skinner and Watt, 2007). Change in sperm size under sperm competition is observed in several other species (Gomendio et al., 1997; Montoto et al., 2011; Skinner and Watt, 2007), where it affects sperm length and head size. However, that nuclear size in meiotic cells relate to sperm

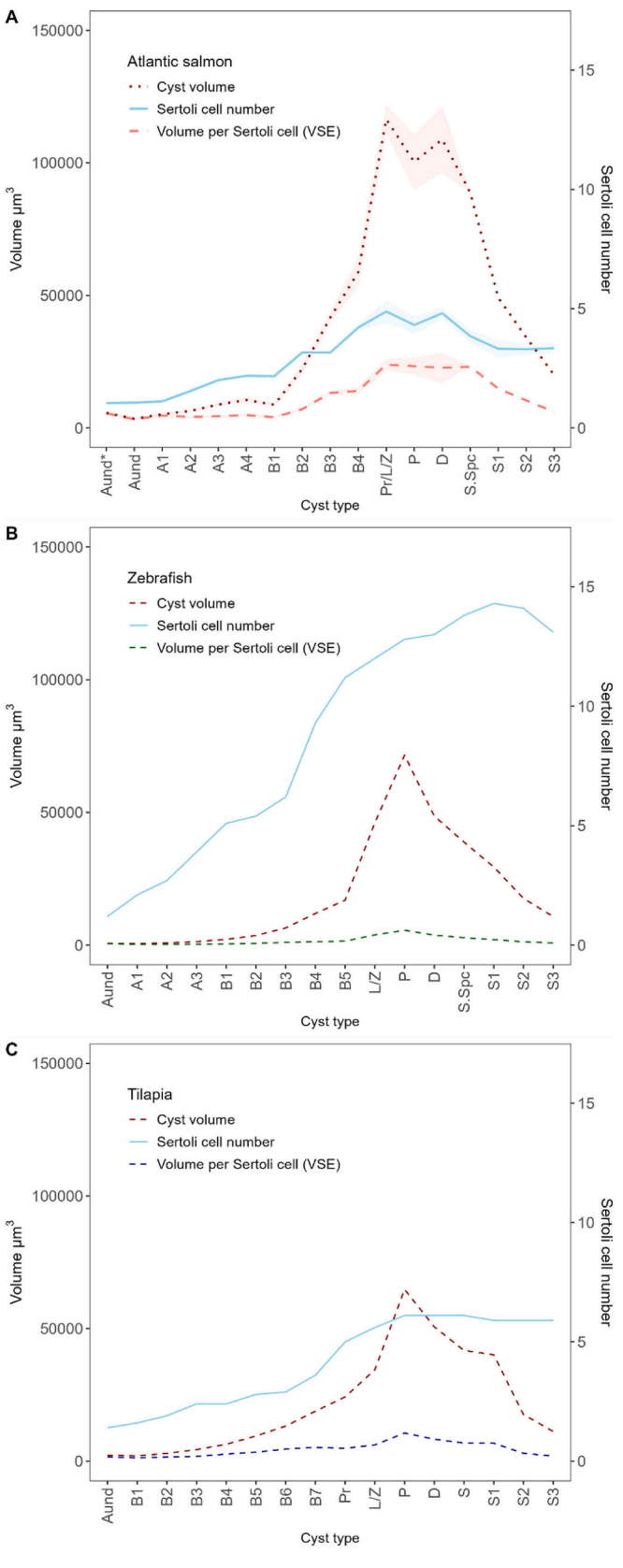

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Fig. 4. The volumetric Sertoli cell efficiency (VSE) plotted against Sertoli cell number and cyst volume during spermatogenesis in salmon (A), zebrafish (B) or tilapia (C). The VSE is calculated as the ratio between cyst volume and Sertoli cell number per cyst. Statistically significant differences between cyst types in salmon can be found in Table S2. The data for zebrafish and tilapia was taken from (Leal et al., 2009) and (Schulz et al., 2005), respectively. In (A) rose-colored ribbons indicate the SEM, if not too small for the scale. A_{und} = single undifferentiated type A spermatogonia, A1 – A4 = four generations of differentiating type A spermatogonia, B1 – B4 = four generations of type B spermatogonia, Pr = preleptotene spermatocytes during meiosis I, L = leptotene spermatocytes during meiosis I, Z = zygotene spermatocytes during meiosis I, P = pachytene spermatocytes during meiosis I, D = diplotene spermatocytes during meiosis I, S.Spc = secondary spermatocyte, S1 – S3 = (arbitrary) stages of spermatids undergoing spermiogenesis. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

competition and sperm tail length and hence sperm competition in anadromous adults and parr (Vladić et al., 2002) is not likely since during the last stages of spermiogenesis (S2; S3); nuclear size did not differ between parr and 2SW adults. Rather; inter-male variation may be an effect of the sample size in each life history group ($N = 4$) and might disappear at larger sample sizes. Such individual variation has been reported for Atlantic salmon sperm previously (Gage et al., 1998).

3.7. *Spermiogenesis*

The end of meiosis II marks the last cell division of spermatogenesis, which continues with spermiogenesis. During this process, the haploid spermatids differentiate into spermatozoa. We defined 3 steps, based on morphological criteria during spermiogenesis (Fig. 1: S1 – S3, Fig. 2I - K). S1 spermatids are distinguished by a round nucleus with light, granular nuclear staining and a smaller nuclear diameter (~4 µm, Fig. 2I; Table S2: germ cells - nuclear diameter) than found in secondary spermatocytes. S2 spermatids show round nuclei with dark, homogenous staining, and slightly smaller nucleus size than S1 (~3.5 µm, Fig. 2J; Table S2: germ cells - nuclear diameter). S3 spermatids are identified by an oval shaped, highly condensed darkly stained nucleus (Fig. 2K), but cannot be discerned based on nucleus size from S2 spermatids. Finally, after S3 spermatids have completed differentiation, the resulting spermatozoa are released by the surrounding Sertoli cells into the lumen of the seminiferous tubule in a process known as spermiation (Schulz et al., 2024). Spermatozoa are characterized by a highly compacted; oval nucleus; and an oblong-shaped head with a slight indentation (Fig. 1: Spz; Fig. 2K). In rainbow trout; development of the different spermatid stages was reported to not always occur synchronously (Billard, 1983), a phenomenon not encountered in the Atlantic salmon testis samples analyzed here (Fig. 2I - K).

The substantial reduction in individual cell volume characterizing spermiogenesis (Table S2: cell volume) reflects the exocytosis of excess cellular material from spermatids as so-called residual bodies. Furthermore, the last meiotic division is associated with moderate germ cell loss (Fig. 5; Table S2: no. of germ cells). Cyst volume decreases threefold due to these changes (Table S2: cyst volume), yet Sertoli cell number remains unchanged from meiosis II onwards (Fig. 3B). Consequently, after a last increase in germ cell number during the last cell division, the highest Sertoli cell efficiency of Atlantic salmon spermatogenesis is reached during spermiogenesis. With an average of ~207 germ cells supported by one Sertoli cell (Table S2: Sertoli cell efficiency), this efficiency is higher than in any other vertebrate reported to date, with guppy exhibiting the second highest reported number with a Sertoli cell efficiency of 167, and human with the lowest Sertoli cell efficiency of 3 (Billard and Escaffre, 1970; Johnson et al., 1984). Throughout spermatogenesis, salmon show relatively high Sertoli cell efficiency, indicating that this metric is not related to a specific stage of spermatogenesis, but rather an inherent trait of salmon

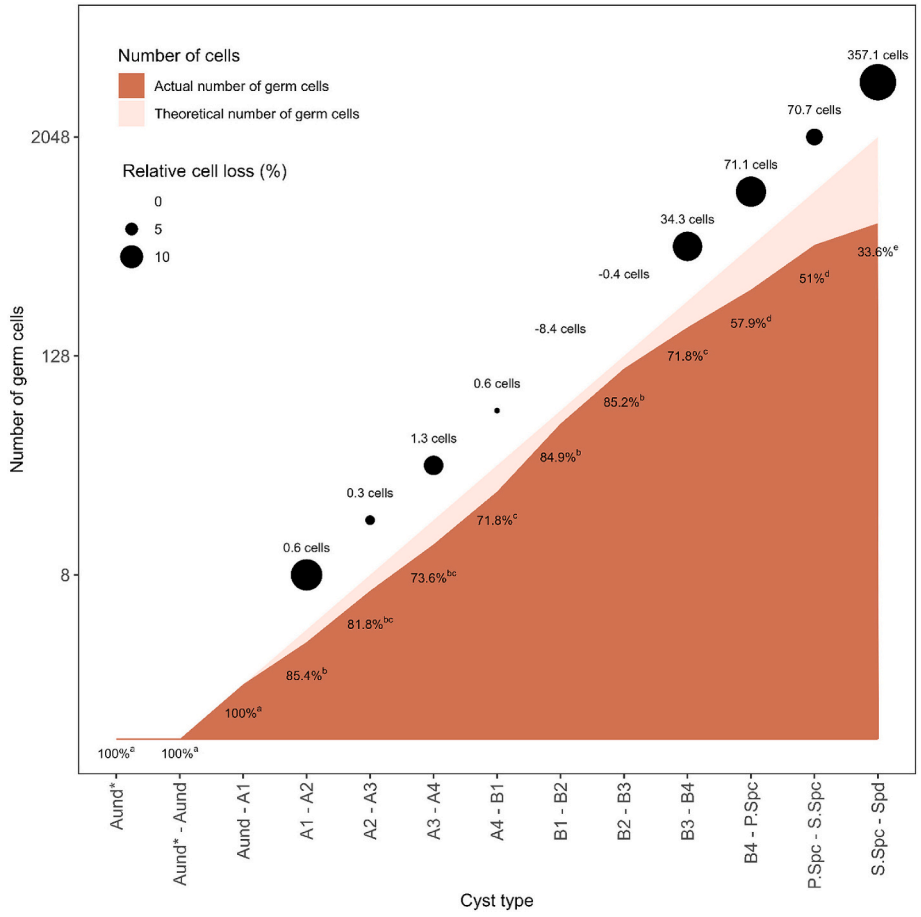

Fig. 5. The theoretical number of germ cells when no cell loss would take place (rose) compared to the actual number of germ cells for each spermatogenic step (red). As the scale is log2-transformed, the area of cell loss at each step is in proportion with more advanced steps (and not skewed by the effect of cell multiplications and an increase in total number of cells). The size of the black points above the graph visualizes the relative percent decrease in cell numbers at each individual step of spermatogenesis and thus indicates the cell loss at each step on the total output of spermatogenesis. Annotated at each black circle is the average number of cells lost at this step. A_{und}* = undifferentiated type A spermatogonia, A_{und} = undifferentiated type A spermatogonia, A1 – A4 = four generations of differentiating type A spermatogonia, B1 – B4 = four generations of type B spermatogonia, P.Spc, primary spermatocyte; S.Spc; secondary spermatocyte; Spd; spermatid. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

spermatogenesis.

It is currently not known which tasks or traits of the Sertoli cell determine their germ cell-supporting capacity. In addition to the uptake of residual bodies, phagocytotic activity by Sertoli cells is also required for the removal of apoptotic germ cells. Salmon primary spermatocytes and spermatids are not subject to significant cell loss (Table S2: no. of germ cells, apoptosis). Therefore, to compare cell loss with other species (since spermatid characteristics may be arbitrarily defined between species), data on cell numbers during meiosis I and spermiogenesis were pooled to investigate which phase of spermatogenesis yields the strongest decline in germ cell number. At the end of salmon spermatogenesis, total germ cell loss has reached 66.4% (Fig. 5). Compared to other fish species (Fig. 6A), total germ cell loss in Atlantic salmon spermatogenesis is high (Almeida et al., 2008; Billard and Escaffre, 1969; Kitano et al., 2011; Leal et al., 2009; Schulz et al., 2005) and more in line with values found in mammals (França et al., 2016).

The phase of spermatogenesis, in which the highest percentage of germ cells is lost, differs per species (Fig. 6A), but for all species the highest phagocytic activity to remove apoptotic cells is required during spermiogenesis, when the highest absolute numbers of germ cells are lost (Fig. 6B). Since in teleost fish earlier cyst types are enveloped by fewer Sertoli cells, the number of apoptotic cells per Sertoli cell was compared as a measure of the phagocytotic activity required by one Sertoli cell to tidy up the cyst for each stage of spermatogenesis. Per Sertoli cell, the largest germ cell loss occurs after meiosis II or during spermiogenesis in all investigated species (Billard and Escaffre, 1969; Leal et al., 2009; Schulz et al., 2005), with Atlantic salmon roughly losing comparatively 5- to 10-fold the number of germ cells per Sertoli cell. Especially when considering the volume loss of individual cells (Table S2: cell volume), resulting in the production of residual bodies in addition to apoptosis, it is expected that the highest phagocytotic activity will take place during spermiogenesis. Apoptotic cells in zebrafish were rarely found in sections (Leal et al., 2009); whereas in sections of salmon testis apoptotic cells are commonly observed (Skaftnesmo et al., 2021). The contrast of apoptotic cells between zebrafish and salmon sections may be related to differences in environmental temperature, Sertoli cell efficiency, and/or the duration of spermatogenesis, potentially resulting in differently balanced rates of phagocytosis and apoptosis.

A higher Sertoli cell number, i.e., a lower Sertoli cell efficiency and VSE may be required for species that proceed through spermatogenesis more rapidly or where germ cells exhibit higher metabolic demands than in salmon. However, this cannot explain the comparatively high germ cell loss rates in salmon, unless the Sertoli cell efficiency is suboptimal for germ cell survival. Stimulation of Sertoli cell numbers in tilapia yielded higher germ cell survival rates and lower Sertoli cell efficiencies (Matta et al., 2002); indicating Sertoli cell numbers in fish are not optimized for germ cell survival; and perhaps another limiting factor

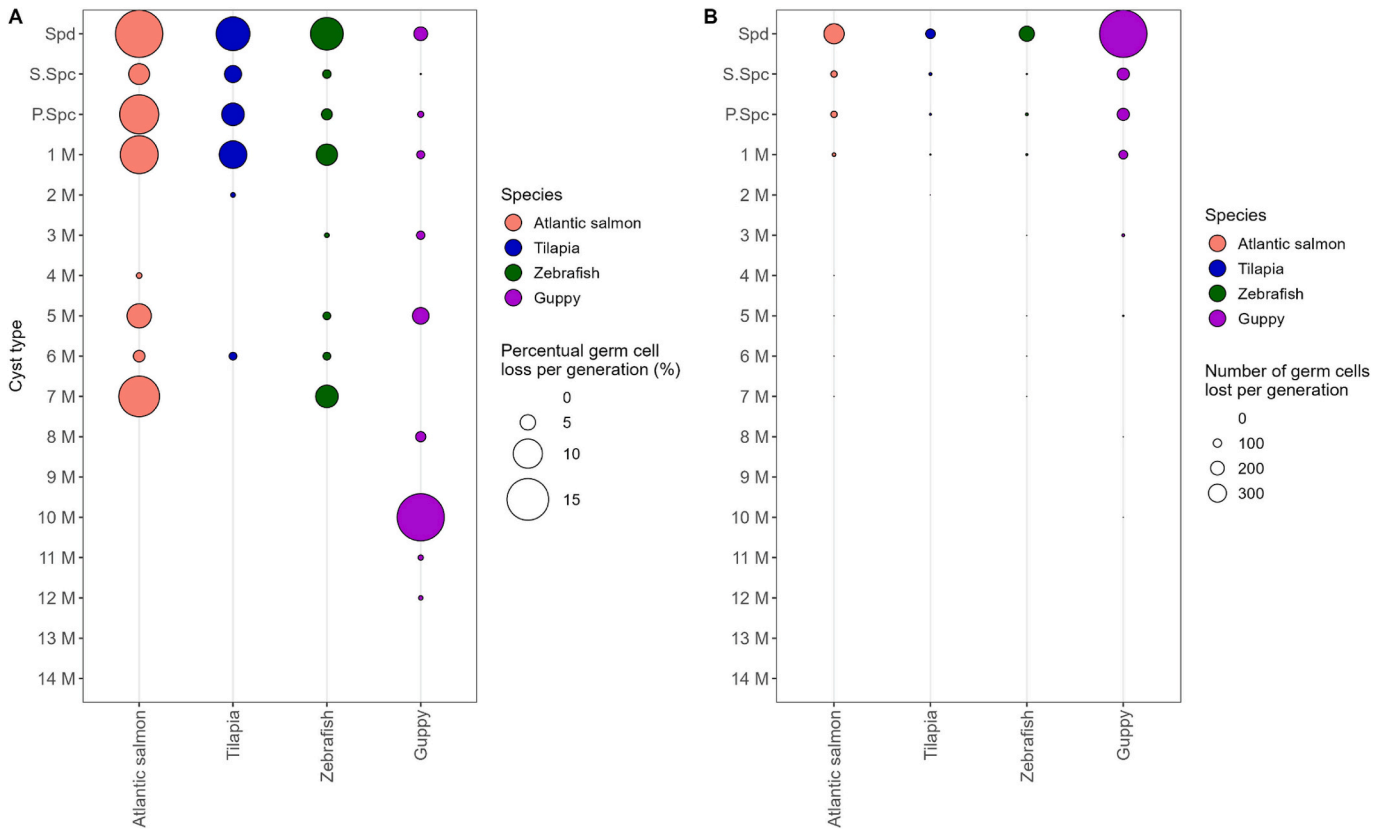

Fig. 6. Comparison of germ cell loss between Atlantic salmon (present study), tilapia (Schulz et al., 2005); zebrafish (Leal et al., 2009) and guppy (Billard et al., 1969), expressed as number of cells lost at each step of spermatogenesis (A), or percent germ cell loss of the total spermatogenic output at each germ cell generation (B). Because the number of mitotic divisions leading up to meiosis differs between species, the mitotic generations are indicated as the number of cell divisions each cyst undergoes until entering meiosis. (8 M for eight total mitotic divisions as in tilapia, 9 M for nine total mitotic divisions as in salmon and zebrafish and 14 M for guppy), followed by primary spermatocytes (P.Spc), secondary spermatocytes (S.Spc) and finally maturing spermatids (Spd). In terms of absolute (A) germ cell loss, all four species show the majority of germ cell loss starting from the end of mitosis, through meiosis and into spermiogenesis (with ~360, ~120, ~230 and ~ 1450 for salmon, tilapia, zebrafish and guppy, respectively), while the maximum relative (B) germ cell loss is concentrated in the spermiogenic phase.

is at work. Exposure to thyroid hormone stimulation like in zebrafish (Morais et al., 2013), if also resulting in higher Sertoli cell numbers per cyst in salmon, might further unravel the factors influencing high Sertoli cell efficiency and cell loss. In addition, the study of Sertoli cell efficiency in species closely related to Atlantic salmon, or with similar life histories and reproductive strategies (i.e., cold habitats, seasonally breeding), may further illuminate which traits determine Sertoli cell efficiency and germ cell loss rates.

3.8. *Changes in Sertoli cell number per cyst may involve mechanotransduction signaling in response to changes in cyst volume*

From A_{und} to A2 and from A3 – B1 germ cell number quadruples, while Sertoli cell number and cyst volume do not increase significantly (Fig. 4A; Table S2). This suggests that a cyst's germ cell number per se is of limited relevance for the cyst's Sertoli cell number, in contrast to the cyst's volume. Therefore, we determined the relation between Sertoli cell number and cyst volume, the VSE (Volumetric Sertoli cell efficiency) (Fig. 4A; Table S2), integrating changes in Sertoli cell number and cyst volume during spermatogenesis. We propose that the VSE is an indicator for the metabolic demand per Sertoli cell and for the degree of stretching exerted by germ cells on Sertoli cells.

Initially, we found a relatively constant VSE reflecting concomitant increases in cyst volume and Sertoli cell number, lasting from A_{und*} until B1 spermatogonia (Fig. 4A). Maintaining this low VSE throughout the period that type A and B1 spermatogonia generations develop suggests a requirement for this high level of Sertoli cell support, potentially needed to support the repeated rounds of mitotic cell cycling that are known to be associated with a switch from anaerobic to aerobic metabolism in germ cells and an up-regulation of their cellular protein synthesis machinery (Jin et al., 2024; Kawasaki et al., 2025). After its nadir in B1, the VSE increased to reach maximum levels, an overall 6-fold increase, in early primary spermatocytes, and stayed on this high plateau until completion of meiosis. Finally, a rapid 6-fold decrease was observed, when the VSE returned to starting levels (Fig. 4A; Table S2) during spermiogenesis. Ostensibly, this VSE profile suggests that B spermatogonia and meiotic cells can develop with a lower level of Sertoli cell support than type A spermatogonia and spermatids. However, it appears relevant to consider aspects of both, time and time of the year, in this context as well.

Meiosis I takes more time than the mitotic cell cycles of type A spermatogonia in zebrafish (Leal et al., 2009). If this should also apply to salmon; then the lower level of support associated with high VSE levels during meiosis may be compensated by the longer period the support is provided. Moreover; the proliferation of spermatogonia starts in late winter/early spring; while meiotic stages become prominent in late spring/early summer (Ciani et al., 2021; Schulz et al., 2019), when increasing water temperatures allow for higher metabolic rates (Claireaux et al., 2000) that also may facilitate sufficient germ cell support despite comparatively low Sertoli cell numbers. The return of VSE to the levels of high support during spermiogenesis; like those seen in type A spermatogonia; may reflect a high metabolic demand associated with processing apoptotic cells (Fig. 6B) and residual bodies during spermiogenesis. After all; on the whole organ level; GSI levels become

halved from the seasonal maximum towards completion of spermiogenesis in salmon before the spawning season starts (Melo et al., 2014).

When spermiogenesis is completed, spermatozoa are released by opening of the cyst (spermiation), i.e., the spermatogenic cyst ceases to exist. The fate of the cyst-forming Sertoli cells has not been clarified yet in teleost fish. Part of the Sertoli cells may contribute to the single-layered epithelium covering the lumen of the spermatogenic tubules also housing scattered single A_{und*} cells, the stem cell reserve for the following reproductive season. Moreover, since spermiation of individual cysts occurs over an extended period of ~7 months starting in February in Atlantic salmon in this study, it seems possible, in particular during the rapid testicular growth period, that 'freed' Sertoli cells may join another cyst still undergoing development. Also, part of the freed Sertoli cells may become apoptotic and be cleared by other Sertoli cells. In any case, in view of the about 50-fold changes of GSI values in successive reproductive seasons, it is reasonable to assume that during the post-spawning regression of the testis, also many Sertoli cells will be lost and therefore must be formed anew during the next reproductive season.

Irrespective of the mechanism providing Sertoli cells, expansions in cyst volume are followed by incorporating additional Sertoli cells into the growing cyst in salmon (Fig. 4A), zebrafish (Fig. 4B) and tilapia (Fig. 4C) testis (Leal et al., 2009; Schulz et al., 2005). Since increases in Sertoli cell numbers occurred in a predictable manner per cyst, we assume that these changes reflect the integration of systemic endocrine and local (per cyst) regulatory triggers. Although experimental stimulation of Sertoli cell numbers in tilapia was followed by additional germ cell proliferation, the ratio between germ and Sertoli cells decreased (Matta et al., 2002), suggesting that a higher Sertoli cell number alone is insufficient to fully exploit the additional germ cell supporting capacity of these Sertoli cells. We hypothesize that a mechanotransduction mechanism may be involved in locally coordinating Sertoli cell numbers with cyst volume expansions, i.e., increases in germ cell number per cyst, in an optimized, efficient manner. Support for this assumption comes from high correlations ($R > 0.7$; data not shown) between germ and Sertoli cell numbers starting with A4 cysts, also when including the rarely observed cysts with about twice the number of germ cells at certain stages (Table S2: no. of germ cells, no. of Sertoli cells); after all, they also contained more Sertoli cells.

A local mechanism adapting Sertoli cell number to cyst size could rely on tensile forces transduced through cell-cell junctions in the Sertoli cells (for example through the involvement of cadherins (Benham-Pyle et al., 2015)), stimulating an increase in numbers when thresholds are reached. Alternatively, stretching activated channels (SACs) which could also sense compression, for example in germ cells (Gudipaty et al., 2017), and may trigger the integration of additional Sertoli cells into an existing cyst. And finally, the Hippo signaling pathway, known for regulating cell proliferation, apoptosis and stem cell activity (in general; organ size), may operate in salmon Sertoli cells, considering that Vgll3, a transcriptional cofactor antagonizing Hippo signaling in the nucleus, is expressed by Sertoli cells and changes in expression when salmon enter puberty (Kjærner-Semb et al., 2018). Future work will have to show if some of these mechanisms operate in cystic spermatogenesis and contribute to regulating Sertoli cell number.

Next to locally operating systems, a candidate for a systemic, endocrine signal regulating Sertoli cell numbers via proliferation is the pituitary gonadotropin Fsh, known to stimulate Sertoli cell proliferation in all vertebrates (Franca et al., 2015). At the beginning of a reproductive season, salmon show an elevated level of single cell proliferation in the testis, involving Sertoli cells and type A spermatogonia, leading to an expansion of the number of initial spermatogenic cysts, which is associated with increased pituitary Fsh production and elevated plasma androgen levels (Schulz et al., 2019). These hallmarks of entering puberty fail to occur in salmon missing a functional Fsh receptor (Andersson et al., 2024), suggesting that proliferation of Sertoli cells and single type A spermatogonia to produce new cysts is a functional domain of Fsh, also involving elevated levels of androgen production. This is in line with the observations made after unilateral castration of adult male catfish (*Clarias gariepinus*) (Schulz et al., 2012), where 4 days after surgery androgen production was up-regulated in the remaining testis, associated with an elevated level of *fshr* mRNA. Interestingly, next to Sertoli cells, only single type A spermatogonia, but not groups of 2 or more spermatogonia, showed elevated levels of proliferation in the catfish testis. This suggested that the immediate response to the removal of one testis triggered, via Fsh signaling, a wave of producing new spermatogenic cysts, while existing cysts continued their regular developmental path. Collectively, this suggests that Fsh is an important regulator of Sertoli cell proliferation in terms of producing new spermatogenic cyst, while future work should aim at investigating, if the local mechanisms discussed above also require a (perhaps permissive) level of Fsh stimulation, to facilitate the growth of already existing cysts.

3.9. Spermatogenic efficiency in parr and 2SW

Based on research indicating the trade-offs between early-maturing and late-maturing life history paths in Atlantic salmon, parr were expected to invest more energy in spermatogenesis than 2SW adults (Gage et al., 1995; Vladić et al., 2010). Some of these reported investments are likely not reflected in the GSI or process of spermatogenesis. Although the GSI in wild parr is generally higher than in anadromous adults, we did not find differences in the number of mitotic divisions, apoptosis rate or Sertoli cell efficiency between the two life history types in salmon. In this regard, it would be interesting to compare maturing parr with adult anadromous males that also matured as parr and then reverted, smoltified, to return later as anadromous adults, as reported to occur previously (Fjelldal et al., 2018).

We consider here two possible explanations regarding the apparent lack of alterations during spermatogenesis between salmon life histories: First, the genetic diversity in farmed salmon may not reflect the one in wild populations, as breeding programs usually do not select on sperm competition. Hence, farmed strains may have lost some of the sperm competition traits found in the wild (Gage et al., 1995). Second, parr invest more energy in testis growth, relative to their body size, however spermatogenesis follows a standard, genetically encoded pattern, suggesting that the number of spermatogenic cysts committed to differentiation is regulated, but not the spermatogenic process itself.

In conclusion, the mitotic phase of Atlantic salmon spermatogenesis holds 9 mitotic divisions and – while the overall (mitotic, meiotic and spermiogenic phases) germ cell loss of 64% is one of the highest reported in a teleost – salmon still are highly efficient in their germ cell and volumetric supporting capacity provided by Sertoli cells, as indicated by the so far highest germ to Sertoli cell ratio found in vertebrates. No major differences were found in spermatogenesis between parr and 2SW male salmon. The morphological characterization of spermatogenesis may aid future investigations on Atlantic salmon male fertility, such as unraveling factors impacting spermatogenic efficiency or the further elucidation of endocrine and paracrine regulation of spermatogenesis and in how far direct interactions between germ and Sertoli cells are instrumental in this regard.

CRediT authorship contribution statement

Martijn D.A. Luksenburg: Writing – review & editing, Writing – original draft, Visualization, Investigation, Formal analysis. **Michelle C. Melo:** Investigation, Formal analysis, Data curation. **Eva Andersson:** Resources, Data curation. **Per Gunnar Fjelldal:** Resources, Investigation, Data curation. **Jan Bogerd:** Supervision, Project administration, Funding acquisition. **Geir Lasse Taranger:** Supervision, Resources, Project administration, Funding acquisition, Conceptualization. **Rüdiger W. Schulz:** Writing – review & editing, Supervision, Project administration, Methodology, Funding acquisition, Conceptualization.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aquaculture.2026.743973.

Data availability

Data will be made available on request.

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