



AQUAculture infrastructures for EXCELlence  
in European fish research towards 2020 —  
AQUAEXCEL2020

## **Deliverable 7.3:**

**Validation of the procedures for germ stem cell isolation, cryopreservation and transplantation in normal trout and carp lines. Integration of the procedures in WP3 standards**



## Table of contents

Executive Summary .....	4
<b>1 GERM CELL ISOLATION, CRYOPRESERVATION AND TRANSPLANTATION IN RAINBOW TROUT .....</b>	<b>6</b>
<b>1.1 Procedures for Isolation of adult male germ stem cell .....</b>	<b>6</b>
1.1.1 Isolation of total testicular cells .....	6
1.1.2 Preparation highly enriched germ stem cell fractions.....	6
<b>1.2 Procedure for cryopreservation of male germ stem cells .....</b>	<b>7</b>
<b>1.3 Procedures for germ stem cell transplantation .....</b>	<b>7</b>
<b>1.4 Results .....</b>	<b>7</b>
1.4.1 Simplification and improvements of the germ stem cell grafting procedure using a vital stain and triploid recipient embryos .....	7
1.4.2 Simplification and improvements of the germ stem cell grafting procedure using total testicular cells.....	8
1.4.3 Evaluation of the reproductive performances of grafted recipient females.....	9
1.4.3.1 Spawning period .....	9
1.4.3.2 Number of two-years-old recipient females spawning eggs:.....	10
1.4.3.3 Analysis of egg production.....	10
1.4.3.4 Analysis of egg sizes.....	11
1.4.3.5 Analysis of egg quality .....	11
1.4.3.6 Analysis of ovarian colonization by the donor germ stem cells.....	13
1.4.4 Evaluation of the reproductive performances of grafted recipient males	15
1.4.4.1 Number of sucessfully grated recipient males that spermiated .....	15
1.4.4.2 Sperm production in sucessfully grafted recipient males .....	16
1.4.4.3 Analysis of the testicular colonization by the donor germ stem cells	18
1.4.5 Control of the genetic origin of the gametes produced in the successfully grafted individuals.....	18
1.4.6 Cryopreservation of spermatogonial stem cells .....	19
<b>2 PROCEDURES FOR CARP GERM CELL ISOLATION, CRYOPRESERVATION AND TRANSPLANTATION.....</b>	<b>20</b>
<b>2.1 Procedures for the isolation of gonadal fragments.....</b>	<b>20</b>
2.1.1 Isolation of testicular fragments .....	20
2.1.2 Isolation of ovarian fragments .....	20
<b>2.2 Procedures for the cryopreservation of gonadal fragments .....</b>	<b>21</b>
2.2.1 Cryopreservation of testicular fragments .....	21
2.2.2 Cryopreservation of ovarian fragments .....	21

<b>2.3 Procedures for the transplantation of cells derived from frozen gonadal fragments.....</b>	<b>22</b>
<b>2.3.1 Cellular dissociation of the cryopreserved gonadal fragments.....</b>	<b>22</b>
<b>2.3.2 Sterilisation of the goldfish recipient embryos .....</b>	<b>22</b>
<b>2.3.3 Transplantation in goldfish recipient embryos .....</b>	<b>22</b>
<b>2.3.4 Germline chimera identification.....</b>	<b>23</b>
<b>2.4 Results .....</b>	<b>23</b>
<b>2.4.1 Cryopreservation of carp testicular fragments .....</b>	<b>23</b>
<b>2.4.2 Cryopreservation of carp ovarian fragments.....</b>	<b>24</b>
Conclusions .....	26
Glossary.....	27
Document information .....	28
Annex 1: Check list .....	29

## Executive Summary

### Background

Selection programs and additional research projects in the field of aquaculture have led to the production of numerous fish lines (selected lines, divergent lines, isogenic lines...) that show specific traits of interest for aquaculture research. The maintenance of these fish lines is sometimes difficult due to their susceptibility to water quality or pathogens. In consequence, there is a need to develop specific procedures to cryopreserve these genetic resources in experimental fish facilities. However, to date, none of the standard methods developed in the other animal production sectors (male and female gamete or embryo cryopreservation) can be used to faithfully regenerate fish lines as described below.

The epistatic interactions between the nuclear and mitochondrial genomes are important for animal performance traits. Unfortunately, only the maternal mitochondrial genome is transmitted to the offspring thanks to the accumulation of a large number of mitochondria into the oocytes and specific degradation mechanism of the sperm mitochondria, yet to be unraveled. After fertilization, paternal mitochondria are rapidly and specifically degraded so that sperm cryopreservation alone cannot regenerate the mitochondrial genome. In contrast to mammalian species, fish oocytes and embryos cannot be cryopreserved yet, which results in the absence of an appropriate procedure allowing a faithful regeneration of the candidate fish lines. Finally, the technique of nuclear transfer leads to unwanted heteroplasmy of the mitochondrial DNA in the few surviving fish.

### Rationale:

In fish, transplantation experiments have demonstrated that embryonic (primordial germ stem cells: PGC) and adult germ stem cells (spermatogonial or ovogonial stem cells: SSC or OSC) can colonize the gonads of a recipient fish, and normally differentiate to produce functional spermatozoa or oocytes depending on the sex of the recipient (Okutsu et al. 2006a; Yoshizaki et al. 2010). In addition, germ stem cells can be cryopreserved and stored for a long time at low temperature with maintenance of their stemness properties (Okutsu et al. 2006b). As a result, to date, germ stem cell grafting is the only method that can be used to faithfully cryopreserve and regenerate genetics resources in fish.

### Objectives

The present study was aimed to set up a standard and practical biotechnology based on germ stem cell (GSC) grafting that could be easily implemented in fish farms to cryopreserve and regenerate isogenic fish lines previously produced during the course of the first AQUAEXCEL project (rainbow trout and Atlantic salmon isogenic lines) or during the current AQUAEXCEL<sup>2020</sup> project.

In order to improve the practicability of germ stem cell grafting, we have explored different points:

- Test for the use of trypan blue in the injected cell suspensions in order to control the release of the cells into the peritoneal cavity of trout embryos.
- Test for the use of recipient trout embryos sterilised by triploidisation or injection of morpholinos to ensure that all gametes are derived from the donor germ cells only.
- Test for the use of a recipient trout strain (named golden trout strain in the present study) expressing a dominant phenotype character (yellow skin) to rapidly discriminate potential progenies derived from the endogenous recipient germ stem cells if any of these cells had survived after the sterilisation procedure.
- Test for the use of neomales as a source of undifferentiated A spermatogonia (adult male germ stem cells) instead of ovaries



- Test for the use of total testicular cells rather than enriched undifferentiated A spermatogonial fractions that requires a costly equipment (elutriator) and is time consuming.
- Develop the cryopreservation of adult germ stem cells and/or gonadal fragments in trout and common carp.

### Summary of main results:

In trout, we demonstrated that the use of triploid recipient embryos did not decrease the success rates of germ stem cell grafting and the success rate values remained high (about 70 to 80%) although diluted trypan blue was added to the injected cell suspensions. Transplantation of total testicular cells prepared from neomales belonging to different isogenic trout lines showed similar success rates compared to that of spermatogonial stem cell-enriched fractions. 70% of the grafted females spawned eggs whereas the percentages of recipient males producing milt were more heterogeneous and ranged from 40 to 80%. We observed that the success rates determined at the sexual maturation were only slightly different in recipient females (less than 15% of difference) but highly different (40 versus 80%) in recipient males when cell fractions were prepared from neomales belonging to two different isogenic trout lines. Further investigations will be required to determine whether the genetic backgrounds of the donor germ stem cells may affect the efficiency of the transplantation assays and/or spermatogenesis in males.

Analysis of reproductive performances of the recipients showed that the seasonality and production of eggs are rather similar to that of the recipient trout strain used. In contrast, egg size was rather similar to that observed in the donor isogenic trout line. Heterogeneity in egg size was higher in recipient females than in control diploid females. Egg quality was decreased in recipient females as evidenced by the lower survival rates of the progenies (about 50 versus 80% in diploid females on average). Milt production and sperm counts were greatly reduced in recipient males compared to diploid males belonging to the recipient trout strain (10 to 100 times lower). However, sperm production in most recipients was sufficient to ensure fertilization of thousands of eggs. Although survival rates were lowered, high numbers of progenies were generated by fertilizing the eggs of recipient females with the milt of recipient males. Molecular genotyping analyses showed that progenies were genetically identical to the donor isogenic trout lines.

In addition to the improvements in the protocols of transplantation assays and characterization of the reproductive performance of the recipients, we have developed a procedure to cryopreserve spermatogonial stem cell-enriched cell fractions. The best cryomedium tested allowed high cell viability compared to that observed with freshly prepared cells (75% versus 83% respectively). However, only 27% of the trout testicular cells were recovered after thawing. Similar efficiencies of germ stem cell grafting were observed (about 80%) using cryopreserved or freshly prepared spermatogonial cells indicating that cryopreserved spermatogonial stem cells maintained their stemness properties.

In carp, we have developed efficient procedures to cryopreserve testicular and ovarian fragments using slow freezing protocols. The cell viabilities observed using optimized procedures reached about 40 and 65% with testicular and ovarian fragments, respectively. Rapid freezing of testicular fragments led to lower cell viability (about 11%). Interspecific transplantation assays in sterile goldfish embryos were developed to rapidly assess the functional properties of the cryopreserved cells. Such assays showed that cryopreserved cell fractions contained surviving and functional adult germ stem cells since 40 and 70% of the recipients were colonized using testicular and ovarian cryopreserved cells, respectively. Such high transplantation efficiencies were similar to that obtained using freshly prepared but non-cryopreserved cells.

In summary, efficient procedures to cryopreserve adult germ stem cells and to graft these cells in recipients have been developed successfully in salmonids and cyprinids.

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## **1 GERM CELL ISOLATION, CRYOPRESERVATION AND TRANSPLANTATION IN RAINBOW TROUT**

### **1.1 Procedures for Isolation of adult male germ stem cell**

#### **1.1.1 Isolation of total testicular cells**

Trout testes should be collected from 1 year old immature males (belonging to standard or all-male populations) or sex reversed females (neomales). Testicular cells were dissociated in 50 ml L15 culture medium containing 2% collagenase and 40 µg/ml DNase I. The enzymatic dissociation was carried out for 6 hours at 12°C under shaking at 100 rpm. Cells were centrifuged for 15 minutes and suspended in 25 ml L15 supplemented with 2mM Glutamine, 1X antibiotics (penicillin, streptomycin, amphotericin), and 1% BSA. Cells were incubated overnight at 12°C under shaking (100 rpm). The next morning, cells were mechanically dispersed with a glass dounce homogenizer using the large clearance A pestle first, and then the small clearance B pestle. Cells were successively filtered on a 150 µm and 40 µm mesh, centrifuged for 15 minutes at 50 g, and suspended in L15 culture medium. Cells were loaded on a 45% percoll cushion in L15 culture medium solution, and centrifuged for 40 min at 500 g followed by 5 min at 50 g to remove cell clusters and erythrocytes. The upper floating cell layers were recovered, rinsed in fresh L15 culture medium and suspended in L15 containing antibiotics and 1% BSA to reach 250 000 cells/µl.

#### **1.1.2 Preparation of highly enriched germ stem cell fractions**

Cells obtained after the percoll cushion step were subjected to a centrifugal elutriation (JE5 Beckman Instruments). Cell separation was performed at a constant rotation speed (2000 rpm) and increasing flow rates (6.6, 7.8, 8.6, 9.5, 10.5, 13.5, 15.1, 19, and 21 ml/min) in L15 medium containing 0.5% BSA. Collected fractions were rinsed, pelleted, and suspended in L15 medium. The male germ stem cells included in the undifferentiated spermatogonial cell fractions were obtained by pooling 19 and 21 ml/min elutriation fractions. Part of the cells were fixed in Bouin's solution and embedded in agarose for histological analysis and validation of the germ cell enrichment.

## 1.2 Procedure for cryopreservation of male germ stem cells

Spermatogonial stem cell fractions were first suspended in L15 medium containing 1% polyvinylpyrrolidone, 50 mM Sucrose, 30% Cryo3 #5617 (Stem Alpha), and 10% propanediol to reach  $2 \cdot 10^6$  cells/ml. 500  $\mu$ l cryopreservation straws were filled with the cell suspension, and loaded in a programmable freezer pre-cooled at 0°C. Cells were frozen following the different successive freezing speeds: -1°C/min until -7.4°C, -0.3°C/min until -40°C, and -2.5°C/min until -80°C. Straws were stored in liquid nitrogen until use.

## 1.3 Procedures for germ stem cell transplantation

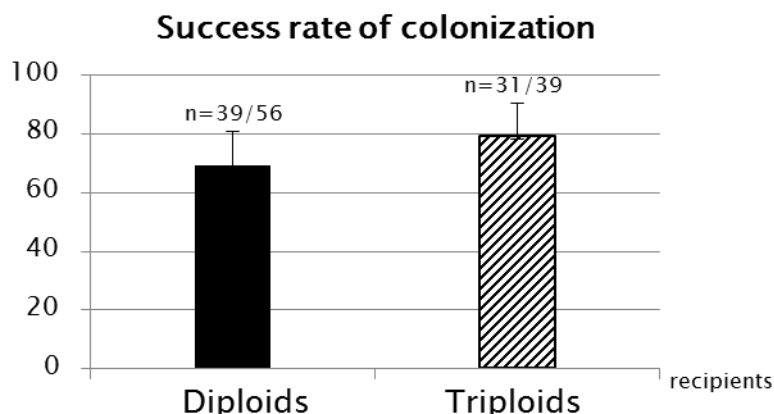
Embryos were anesthetized in the isotonic buffer containing 0.1% phenoxyethanol and maintained at 10°C. Cell suspensions (cryopreserved or freshly prepared germ stem cells) should be diluted at  $0.25 \cdot 10^6$  cells/ $\mu$ l. Cells should be injected into the abdominal cavity of female trout embryos the day of hatching (320 degree days). Embryos were injected respectively with cryopreserved and non-cryopreserved cells of the same cell preparation. Embryos were maintained at 10°C until the first feeding (550 degree days), and then reared at 12°C using standard farming procedures until 8 month of age. The survival rate of the group injected with cryopreserved germ stem cells was 76% at the first feeding and 55% at 8 month of age. Similar survival rates were observed at the first feeding (63%) and at 8 month of age (53%) for the group injected with the non-cryopreserved cells. The survival rate of the non-injected embryos was much higher (about 90%). This suggests that the transplantation assays induced an increased mortality but the latter did not vary drastically regardless of the cell suspension used.

## 1.4 Results

### 1.4.1 Simplification and improvements of the germ stem cell grafting procedure using a vital stain and triploid recipient embryos

In all fish species, triploid female fish are obviously sterile since no recruited or growing oocytes are observed in the ovaries. In contrast, triploid male fish produce few testicular spermatozoa but they are not capable to support embryo development. In the present study, we used the triploidisation procedure to obtain grafted animals producing functional gametes derived only from the transplanted germ stem cells. The triploidisation procedure is a standard farming practice, and it is therefore well disseminated. The triploidisation was also preferred to other sterilization procedures including morpholinos or CRISPR-Cas9 biotechnologies, because efficient triploidisation protocols were already available and the opacity and hardness of the egg chorion make the injection of morpholinos or guide RNA difficult and time consuming. In addition, the large size of the trout eggs was another limitation for the use of the morpholinos or guide RNA biotechnologies.

Enriched spermatogonial stem cells fractions were prepared using centrifuge elutriation from the gonads of 9 months aged male trout. We compared the success rate of the gonadal colonization between diploid and triploid recipient 6 months old embryos (Figure 1). We observed that the success rate of the germ stem cells grafting was similar between diploid and triploid recipient trout embryos. In consequence, all subsequent transplantation experiments were carried out using hatched triploid trout embryos.



**Figure 1: The success rate of the germ stem cells transplantation is similar between diploid and triploid recipient trout embryos.**

Undifferentiated spermatogonial cells were purified from testes collected from 9 month aged males belonging to the *mlc2:GFP* transgenic trout line as described previously (Bellaïche et al. 2014). Diploid or triploid hatched trout embryos belonging to the “Golden” strain were transplanted using a solution containing  $0.25 \cdot 10^6$  undifferentiated spermatogonial stem cells per ml. Germinal chimera were detected at 6 month of age by standard PCR using genomic DNA extracted from the two gonads and specific primers directed against the GFP gene. The successfully transplanted embryos and the total number of examined embryos are indicated at the top of each histogram.

The transplantation of the germ stem cells in the abdominal cavity of fish embryos requires a great dexterity of the technician and is time consuming. The use of a vital stain (0.04% trypan blue) in the injected cell suspension facilitates the visualization of the injection point and the spreading reproducibility of the injected cells suspension. The addition of trypan blue did not affect the success rate of the transplantation assays.

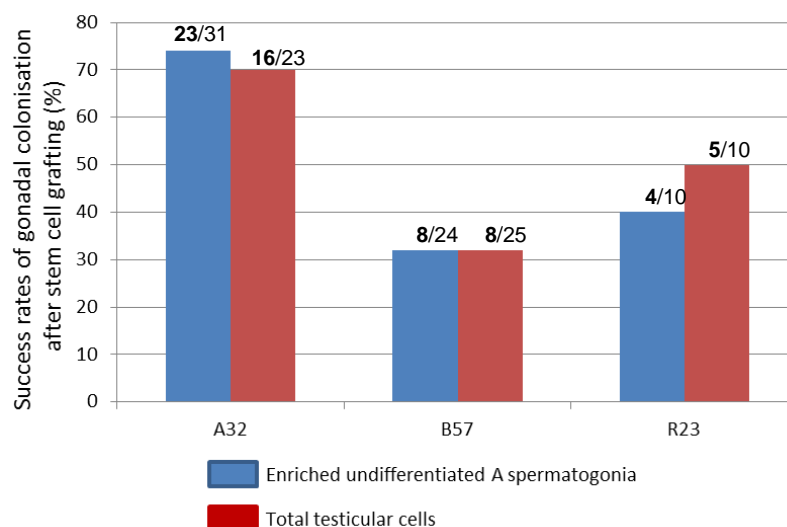
#### 1.4.2 Simplification and improvements of the germ stem cell grafting procedure using total testicular cells

We previously demonstrated that cell fractions containing 90% of undifferentiated A spermatogonia could be prepared using the centrifugal elutriation technique from testes of immature male trout (Bellaïche et al. 2014). Such cell fractions showed a high capacity to colonize the gonads of recipient trout embryos and to produce functional gametes (about 80% of success). However, the separation of undifferentiated A spermatogonia using the centrifugal elutriation technique required an expensive equipment and is laborious. In order to simplify the preparation of the cell suspensions, we compared the success rate of the gonadal colonization when using total testicular cells or enriched stem cell fractions containing approximatively 90% of undifferentiated A spermatogonia. The experiments were carried out using three different isogenic trout lines named A32, B57, and R23 (Figure 2). Germ stem cells fractions were collected from 12 months old neomales because isogenic trout lines are all-female populations and the amount of germ stem cells was expected to be higher in testes compared to ovaries. We observed that, for all isogenic trout lines used, the success rates of the gonadal colonization were similar between total testicular cells and undifferentiated A spermatogonia-enriched fractions. High rates of gonadal colonisation were also obtained using total testicular cells of the A03 isogenic trout line in two independent experiments (50 and 76%).

However, the success rate appeared highly different from one isogenic trout line to another. The difference may be caused by different factors including the proportion of germ stem cells accumulated in the testes of different isogenic trout lines. Further investigations will be necessary to evaluate whether the genetic background of the isogenic trout lines



influences the number of spermatogonial stem cells accumulated into the immature male gonads and/or the intrinsic properties of the germ stem cells to colonize the gonads.



**Figure 2: The success rate of germ stem cells grafting remains high when using total testicular cell fractions.**

Total testicular cell fractions and enriched germ stem cell fractions containing 90% of undifferentiated A spermatogonia were prepared from 9-12 months aged neomales belonging to three different isogenic trout lines named A32, B57 and R23. Cell fractions were injected independently in the abdominal cavity of triploid trout embryos at the hatching stage. The potential presence of germ cells derived from the injected donor germ stem cells were detected 9 months after the transplantation using different sets of microsatellites. The number of individuals carrying germ cells derived from the donor germ stem cell fractions (bold numbers) and the total numbers of individuals in each group are indicated on the top of each histogram. The success rate of germ stem cell grafting was similar between the two cell fractions suggesting that the procedure can be simplified using dissociated testicular cells containing from 20 to 40% of undifferentiated A spermatogonia.

Altogether, our data indicated that there is no need to use highly purified spermatogonial stem cell fractions to obtain high rates of gonadal colonization. However, it was necessary to demonstrate that the reproductive performance of the animals grafted with total testicular cells was not altered.

### 1.4.3 Evaluation of the reproductive performance of grafted recipient females

The success rate of the gonadal colonisation is an important indicator of germ stem cell grafting efficiency but we had to demonstrate the ability of the grafted animals to spawn and to produce high numbers of good quality gametes.

The reproductive performances of the grafted females were studied in the A32 and B57 groups by monitoring the spawning period, the number of spawning females, the number of spawned eggs, egg sizes and survival rate of hatched embryos.

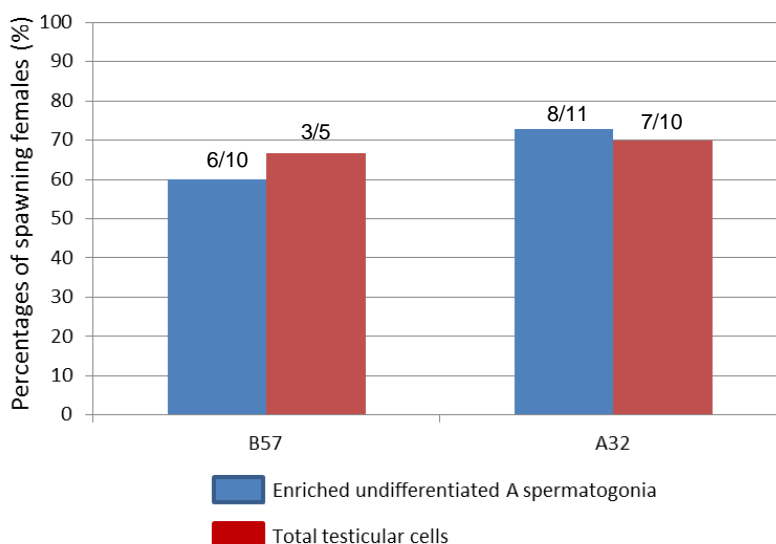
#### 1.4.3.1 Spawning period

Control Females belonging to the A32 or B57 isogenic trout lines ovulated from beginning to mid-January and from mid-December to the end of January, respectively. Interestingly, most of the females transplanted with A32 or B57 germ stem cells ovulated from the end of November to the beginning of January. This spawning period is similar to that of the golden synthetic trout strain that was used to produce the recipient triploid embryos. This observation indicates that the spawning period of the grafted females most likely depends on the recipient strain and not on the origin of the donor stem germ cells.

### 1.4.3.2 Number of two-year-old recipient females spawning eggs:

The number of grafted females that spawned eggs was determined at two years in two groups of animals transplanted with either a mix of total testicular cells or enriched undifferentiated A spermatogonia. Two independent experiments were conducted using donor cells collected from the A32 or B57 isogenic trout lines (Figure 3). In both experiments, the percentage of females that spawned was high and similar (from 60 to 70%) whatever the cell fractions used.

The percentage of females spawning A32 oocytes (about 70%) was similar to the success rate of the gonadal colonisation (70%) determined at 9 month of age (Figure 2). In contrast, the percentage of females (about 60%) spawning B57 eggs was much higher than the success rate of the gonadal colonisation (30%) previously determined using a molecular genotyping approach at 9 months of age (Figure 2). The difference could result from the low number of B57 germ stem cells colonising and spreading into the gonad at 9 months of age. In addition, one cannot exclude false negative detection due to a lower efficiency of the primer set used to detect the B57 genotype (OMM1087 microsatellite).



**Figure 3: Percentages of female recipients spawning eggs**

Cell fractions containing a mix of testicular cells or enriched undifferentiated A spermatogonia were prepared from 9-12 months aged neomales belonging to B57 or A32 isogenic trout lines. Cell fractions were injected independently in the abdominal cavity of triploid golden embryos at the hatching stage. Female recipients were reared until their sexual maturation at two years. Ovulating females were monitored each week from mid-November to mid-January by practicing a gentle abdominal massage and squeezing out few ovocytes.

### 1.4.3.3 Analysis of egg production

Eggs were collected from female recipients two days after the detection of ovulation and quantified (Figure 4A). The numbers of eggs produced by the A32 or B57 recipient females was similar regardless of the donor germ stem cells fractions. Interestingly, egg production by the females of the B57 and A32 isogenic trout lines was much higher compared to that of the B57 and A32 grafted golden recipient females (4000 versus 1700 eggs/kg body weight). On the other hand, egg production by the A32 and B57 recipient females was similar to that observed with two-year-old females of the golden strain (about

2000 eggs/kg body weight). This suggests that egg production could depend on the performance of the recipient strain.

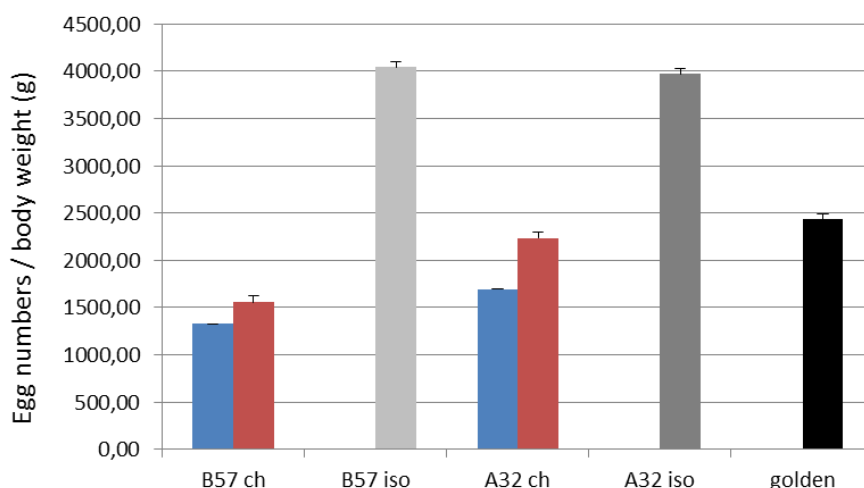
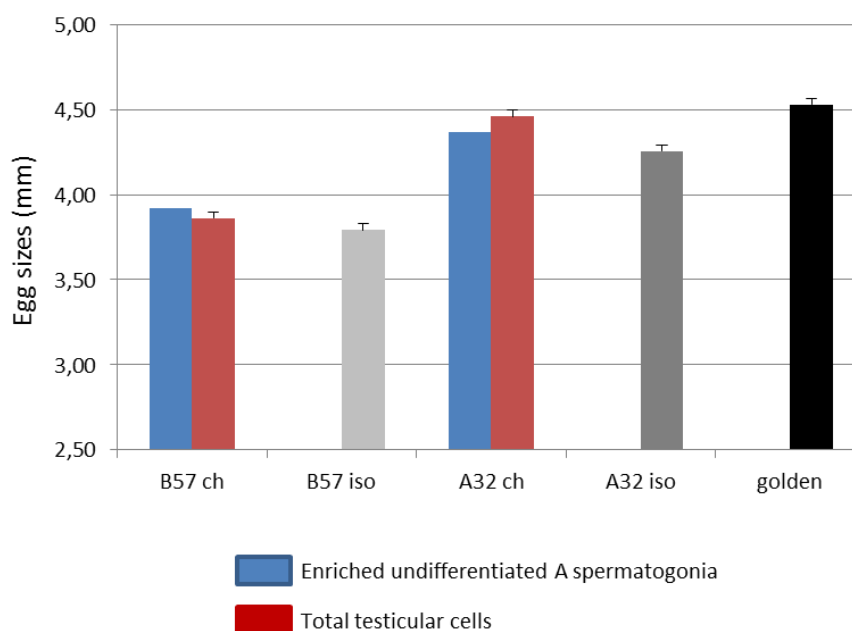
#### 1.4.3.4 Analysis of egg sizes

Egg sizes were determined using the VISEGG method (Bugeon et al. 2016) that is based on the automated processing of 2D images (Figure 4b). The average size of the eggs collected from the A32 recipient females was similar to that of the standard A32 isogenic trout line and golden recipient trout line. In contrast, the eggs collected from the B57 recipient females showed an average size much smaller than that of the golden strain (3.8 mm versus 4.5 mm). However, the average size of the egg produced in the B57 recipient females was similar to that of the standard B57 isogenic trout line. Our data indicate that the size of the eggs produced in the grafted females is not likely to be dependent on the recipient strain but rather on the genetic background of the donor germ stem cells.

The size of the oocytes produced in the recipient females showed higher variability than that of diploid females of recipient strain (not shown).

#### 1.4.3.5 Analysis of egg quality

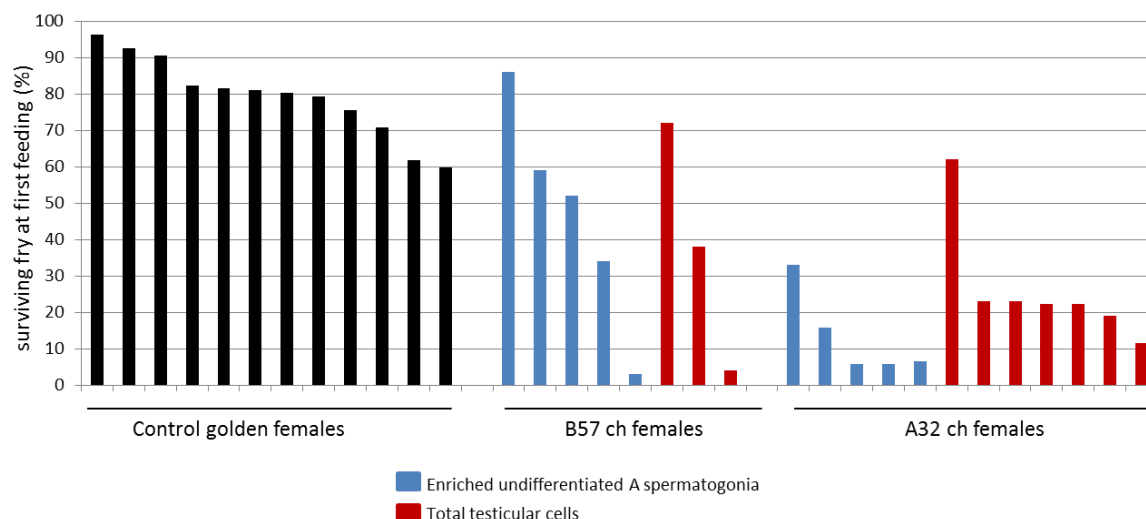
Eggs were collected from grafted females and fertilized using the milt of normal males to assess their ability to support embryo development until the first feeding stage (Figure 5). Eggs originating from the transplantation of total testicular cells or from the transplantation of undifferentiated A spermatogonia showed a similar variability of egg quality regardless of the B57 or A32 donor germ stem cells. Egg quality varied from 3 to 85% depending on the females. Egg quality of two year-old females belonging to the recipient golden trout strain was also variable from one female to another but to a lower extend (60 to 95%). In summary, the quality of the eggs produced in two-year old grafted female recipients appeared to be reduced but remained high enough to produce large progenies.

**A egg numbers****B egg sizes****Figure 4: egg production and egg sizes in grafted females**

Cell fractions containing a mix of testicular cells or enriched undifferentiated A spermatogonia were prepared from 9-12 months aged neomales belonging to B57 or A32 isogenic trout lines. Cell fractions were injected independently in the abdominal cavity of triploid trout embryos at the hatching stage. Female recipients were reared until their sexual maturation at two years. Eggs were collected from mid-November to mid-January by practicing a gentle abdominal massage. A) Egg numbers were determined by dividing the weight of each egg laying (without coelomic liquid) by the average weight of an egg previously determined by weighing a known number of eggs (100 to 300 eggs on average). Egg sizes were determined using the VISEGG system based on 2D image processing. Briefly, 100 to 300 unfertilized eggs were placed in a large Petri dish containing Cortland medium and images were taken using a digital camera (Canon EOS 1000 D). An algorithm was then developed with the Visilog® software (Thermo Fisher Scientific) to automatically separate eggs and measured individual parameters (average

area). The average egg diameter was calculated using the following equation 
$$\text{Diameter} = \sqrt{\frac{\text{egg area}}{\pi}} \times 2$$
 B57ch: female recipients grafted with B57 germ stem cells; A32 ch: female recipients grafted with A32 germ stem cells; B57 iso: normal females from the B57 isogenic trout line, A32 iso: normal females from the A32 isogenic trout line golden: normal females of the golden recipient strain.





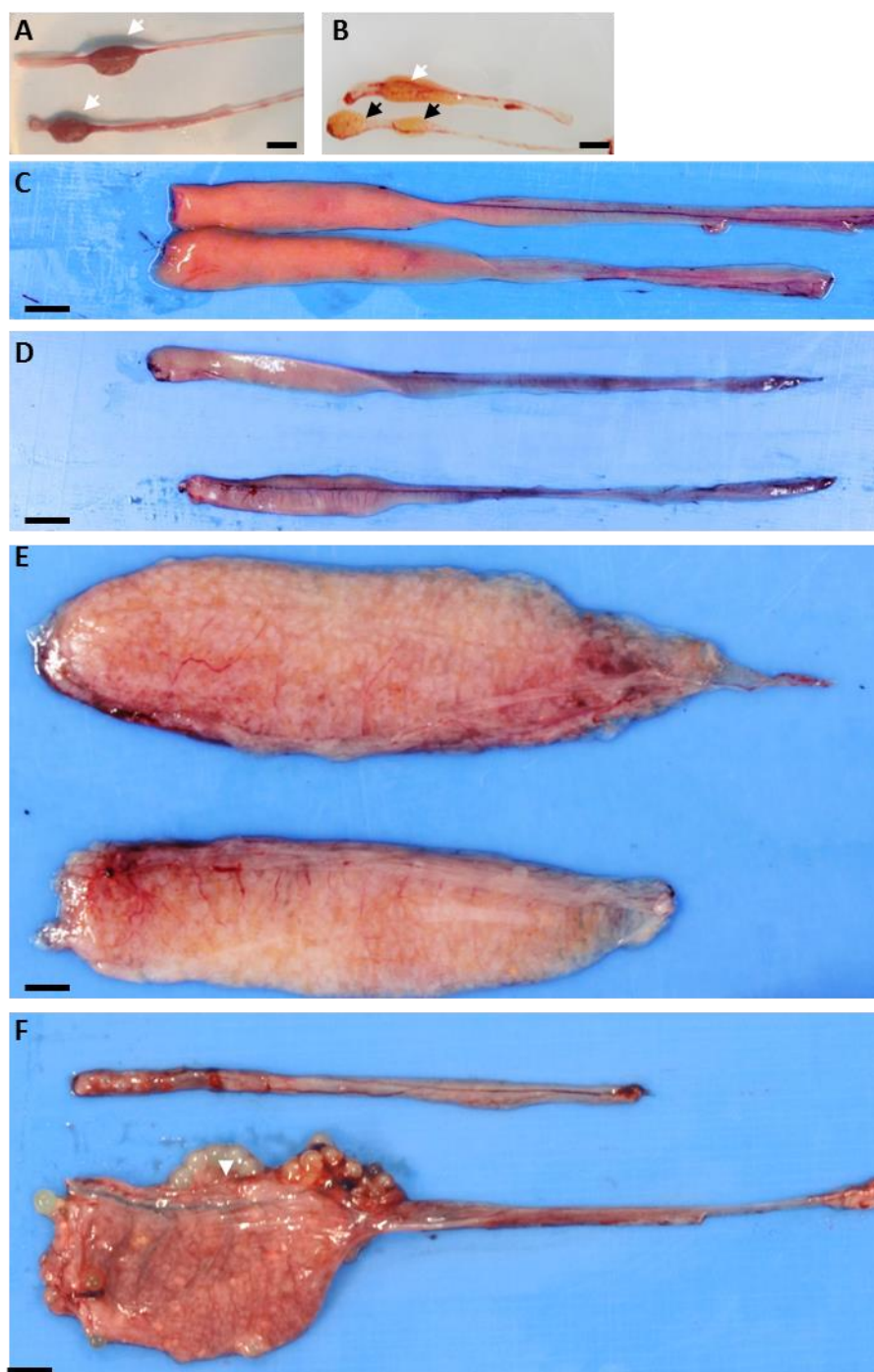
**Figure 5: Quality of the eggs produced in grafted recipient females.**

Eggs were first collected from females grafted with the different germ stem cell fractions. For each lay, a known number of eggs was fertilized using a pool of milt collected from three standard diploid male trout of an autumnal strain. The percentages of surviving fry were determined just before fry reached the first feeding stage. Control golden females: eggs collected from two-year old females of the golden trout strain; B57 ch: egg collected from females grafted with B57 germ stem cells; A32 ch: eggs collected from females grafted with A32 germ stem cells. The blue and red colors distinguish the females grafted with either total testicular cells or enriched undifferentiated A spermatogonia.

#### 1.4.3.6 Analysis of ovarian colonization by the donor germ stem cells

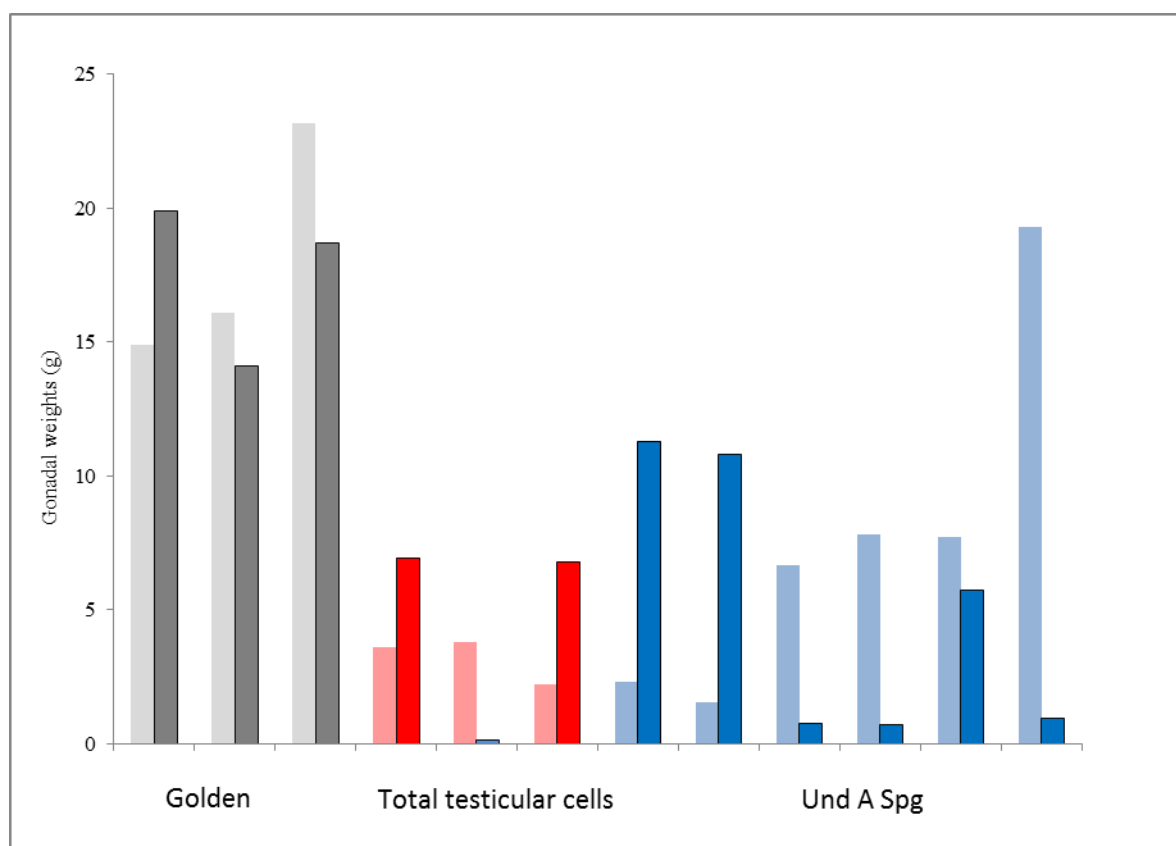
Analysis of the gonads collected from successfully grafted recipient females showed that the gonadal colonization was clearly heterogeneous from one female to another regardless of the germ stem cell fractions used in the transplantation assays. Some females showed only one colonized gonad while others showed one to three areas of growing oocytes in one of the two ovaries (Figure 6). This observation combined with the fact that the number of oocytes collected from each female reached normal values (Figure 4) suggests that an unexpected compensatory mechanism has occurred leading to the vitellogenic growth and ovulation of a normal number of oocytes even when only one gonad is colonized. In contrast to the ovaries of the standard golden females, the weights of the left and right ovaries were lower and highly heterogeneous in most of the ovulated B57 and A32 recipient females (Figure 7). It has been previously observed that considerable numbers of degenerating oocytes remain in the ovaries after the first spawning in trout (Tyler et al. 1990). This indicates that females produce a surplus of previtellogenic oocytes but only part of them will complete vitellogenesis and oocyte maturation. The loss of weight of the ovaries in post-ovulated recipient females implies a drastic reduction of the stock of previtellogenic oocytes previously formed to generate the season's batch of eggs. However, the lowered stock of previtellogenic oocytes remained sufficient to produce a normal number of mature oocytes released after ovulation (Figure 4).

Altogether, our observations suggest that the production of previtellogenic oocytes stock is likely not a limiting step for egg production but rather the active vitellogenic phase of oocyte growth as observed in standard females.



**Figure 6: Description of the heterogeneous colonization of the gonads in grafted recipient females.**

Ovaries were collected from 14 (A and B) or 23 months (F) aged females grafted with enriched undifferentiated A spermatogonia of the A32 isogenic trout line. A) both gonads of the female recipient were colonized and only one major area of growing vitellogenic oocytes was observed in each gonad (white arrowheads). B) Both gonads of the recipient females were colonized and two major areas of growing oocytes were observed in one of the two gonads (black arrowheads). C) Ovaries of a control diploid female that did not mature at two years of age. D) Ovaries of a control non-grafted triploid female at two years of age. E) Ovaries of a diploid female that sexually matured and ovulated at two years of age. Numerous previtellogenic oocytes are present in the gonads. D) Ovaries of a grafted A32 recipient female. Only one gonad (at the button of the image) was colonized and produced oocytes. Note that a lower number of vitellogenic oocytes remained into this ovary compared to standard diploid females (E). Scale bars represent 1 cm.



**Figure 7: Heterogeneous weights of the ovaries removed from ovulated B57 recipient females.**

Ovulation of the B57 recipient females was monitored regularly once a week. After egg collection, females were euthanized and the weights of the left (clear color) and right (dark color) ovaries were weighed independently. Ovaries collected from standard females of the golden trout strain (black and grey histograms) showed similar weights indicating that both ovaries contributed equally to the production of the oocytes stock. In contrast, most of the ovulated B57 recipient females showed ovaries with lower and highly different weights regardless of the germ stem cell fractions used in the transplantation assays. Our observation suggests that the reservoir of vitellogenic oocytes could be reduced in successfully grafted recipient females compared to standard females. Und A spg: donor cell fraction enriched in undifferentiated A spermatogonia.

## 1.4.4 Evaluation of the reproductive performance of grafted recipient males

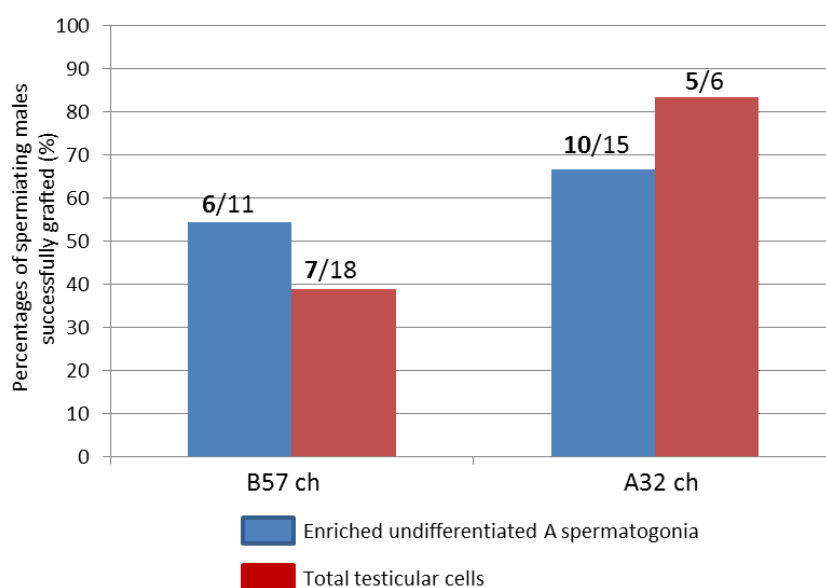
### 1.4.4.1 Number of successfully grafted recipient males that spermated

Successfully grafted recipient males were determined using flow cytometry analyses by detecting haploid cells (spermatozoa) or diploid cells in the gonads. Note that no spermatozoa was detected in the milt of non-grafted triploid recipients using flow cytometry. In addition, sperm DNA was genotyped using a microsatellites panel discriminating the genotype of the donor germ stem cell fractions from that of the recipient embryos. The percentage of triploid males that released spermatozoa was determined at two years in two groups of recipients grafted with either a mix of total testicular cells or enriched undifferentiated A spermatogonia (Figure 8). Two independent experiments were conducted using donor cells collected from the A32 or B57 isogenic trout lines. In both experiments, the percentage of recipient males that produced spermatozoa were similar whatever the donor cell fractions used and were rather high (from 40-55 to 65-80% for the B57 and A32 recipient groups respectively). However, one can notice that the percentages of spermating recipient males were lowered using the B57 cell fractions (from 40 to 55%) compared to that of the A32 cell fractions (from 65 to 83%). The marked difference between the B57 and A32 total testicular cells (40 and 83% respectively) could result from the different genetic background

or a lower number of germ stem cells in the B57 immature neomales compared to that of the A32 neomales. Further investigations will be required to confirm the different assumptions.

The percentage of A32 recipient males that successfully produced sperm (66 to 83%) was similar to the success rate of the gonadal colonisation (about 70%) previously determined using a molecular genotyping approach at 9 months of age (Figure 2). In contrast, the percentage of B57 recipient males that released spermatozoa at two years was slightly higher (40 to 50%) compared to the success rate of the gonadal colonization (30%) determined at 9 months of age (Figure 2). This difference could result from the low sampling and/or from a lower sensitivity of the genotyping PCR that was based on the use of the OMM1087 microsatellite.

Note that the percentage of spermiating recipient males was rather similar to the percentage of recipient females spawning eggs for each corresponding experimental group (Figure 3).



**Figure 8: Percentage of spermiating recipient males successfully grafted.**

Cell fractions containing a mix of testicular cells or enriched undifferentiated A spermatogonia were prepared from 9-12 months aged neomales belonging to B57 or A32 isogenic trout lines. Cell fractions were injected independently in the abdominal cavity of triploid golden embryos at the hatching stage. Male recipients were reared until their sexual maturation at one or two years of age. Spermiating was monitored from mid-November to mid-January by practicing a gentle abdominal massage.

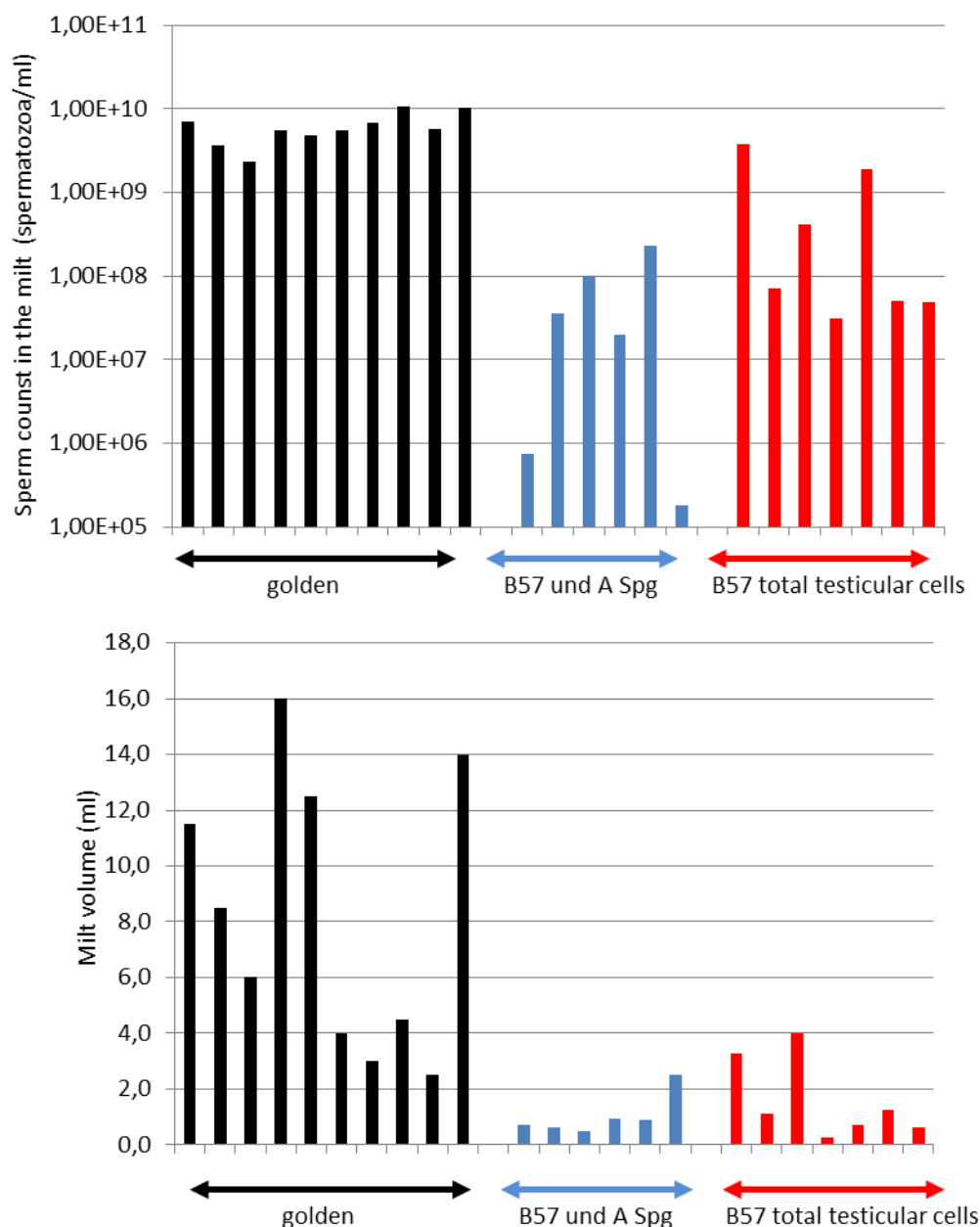
#### 1.4.4.2 Sperm production in successfully grafted recipient males

Sperm production was monitored in successfully grafted recipient males and compared to standard diploid males belonging to the golden trout recipient strain. Sperm concentrations in control diploid males were rather homogenous with values ranging from  $10^9$  to  $10^{10}$  spermatozoa/ml.

Our data showed that recipient males successfully grafted with B57 donor germ cells produced milt, but sperm concentrations greatly varied from  $10^5$  to about  $10^9$  spermatozoa/ml. The mean sperm concentration was 10 to 100 times lower than observed in control diploid males in the recipient groups grafted with an enriched fraction of undifferentiated A spermatogonia and total testicular cells, respectively. The volume of milt was highly variable in each experimental group, including control diploid males. The volume of milt collected from control diploid males ranged from 2 to 16 ml. However, the largest volumes of milt collected from grafted recipient males did not exceed 2 to 4 ml.



Altogether, our data indicate that sperm production is significantly reduced in successfully grafted recipient males compared to control diploid males. However, about 50% of the recipient males produced sufficient amounts of sperm (1 ml of milt at least with a sperm concentration greater than  $10^7$  spermatozoa/ml) to fertilize thousands of eggs. In addition, testicular sperm could be prepared from the recipient males whenever higher numbers of sperm are needed.



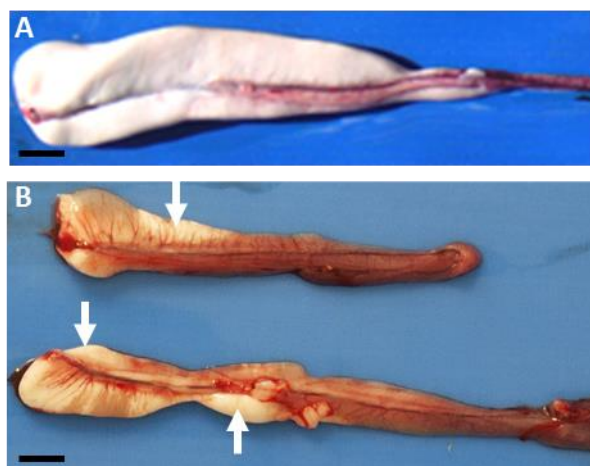
**Figure 9: Production of spermatozoa in successfully B57 grafted recipient males.**

Cell fractions containing a mix of total testicular cells or enriched undifferentiated A spermatogonia were prepared from 9-12 months aged neomales belonging to the B57 isogenic trout lines. Cell fractions were injected independently in the abdominal cavity of triploid trout embryos at the hatching stage. Male recipients were reared until their sexual maturation at two years. Milt was collected by gentle abdominal massage from mid-November to mid-January. Sperm concentrations were determined by detecting haploid cells using flux cytometry. Each histogram represents values acquired from one individual.

Golden: standard diploid males belonging to the recipient golden trout strain; B57 und A Spg: recipient males grafted with an enriched fraction of undifferentiated A spermatogonia; B57 total testicular cells: recipient males grafted with a fraction of total testicular cells.

#### 1.4.4.3 Analysis of the testicular colonization by the donor germ stem cells

In grafted triploid recipient males, we observed that the production of spermatozoa was limited to a narrow area (Figure 10). This suggests that few germ stem cells may enter the gonads just after transplantation and they likely divide progressively to colonize the adjacent area.



**Figure 10: Regionalized sperm production in successfully grafted male recipients.**

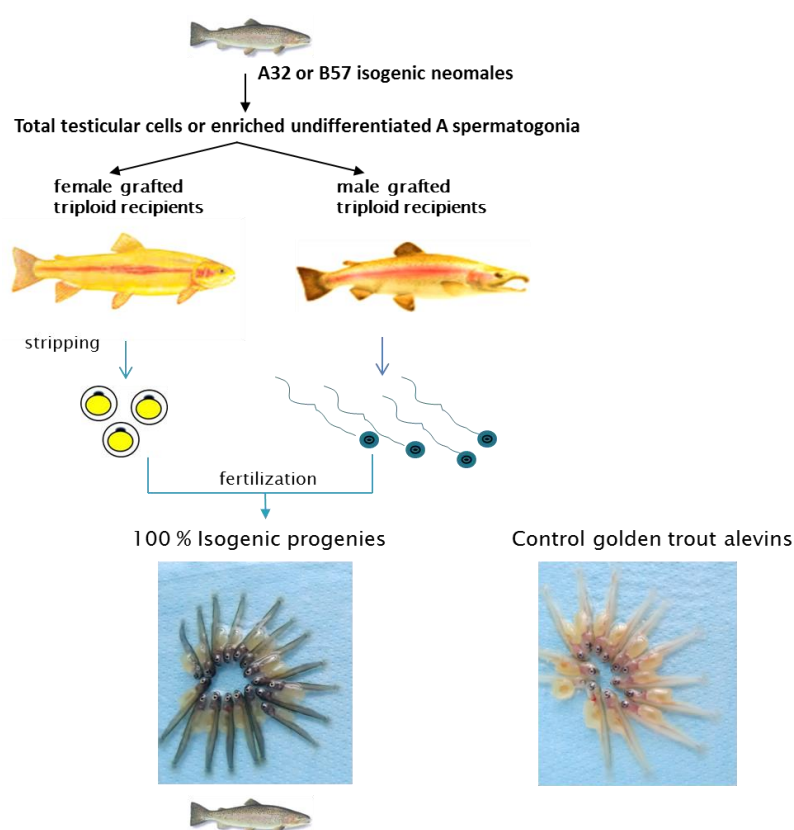
Sexually mature males were euthanized while they were releasing sperm following a gentle abdominal massage. A) testis collected from a control diploid male and showing a massive production of spermatozoa as revealed by the white color of the tissue. B) testes of a successfully A32 grafted males showing narrowed area of sperm production (white arrows).

#### 1.4.5 Control of the genetic origin of the gametes produced in the grafted recipients

Eggs collected from B57 or A32 grafted female recipients were fertilized with the milt of the corresponding B57 or A32 grafted male recipients, respectively (Figure 11). All progenies harboured a black color similar to that of the B57 or A32 isogenic trout lines but different from the yellow color of the skin of the recipient golden trout strain. The yellow color of the skin is a dominant phenotypic trait of the golden trout recipient strain caused by a single gene yet to be discovered. Therefore, if one parent had produced gametes from their own germ stem cells, we would have observed fry with a yellow body. Our data indicated that all fry were black, and therefore, originated from the fractions of donor germ stem cells. To confirm our observations, additional genetic analysis were carried out. More than 10 fry were genotyped from each egg-laying using informative microsatellites distinguishing the genotype of the grafted parents and that of the isogenic donor germ stem cells. Our data showed that all fry were carrying homozygous alleles corresponding to those observed in the corresponding isogenic trout lines (data not shown).

Finally, the progenies were also genotyped to detect the putative presence of the SdY gene that is the master gene controlling the sexual differentiation of the male gonads in trout. Note that the A32 and B57 donor male germ stem cells (spermatogonial stem cells) used in the present study did not harbour the SdY gene because they were collected from neomales (genetic females sex-reversed using 17 alpha methyl testosterone, a synthetic androgen). As expected, we did not detect the SdY gene in the progenies indicating that they were all females.

In summary, the phenotypic observations and genetic analyses of the offspring demonstrated that the eggs and sperm collected from the grafted triploid recipients were only derived from the donor germ stem cells.



**Figure 11: Gametes collected from grafted triploid recipients derived only from the fractions of donor germ stem cells.**

Eggs were collected from ovulated recipient females and fertilized with the milt of recipient males. Embryos were reared until the first feeding and the black color of their body was observed as expected for embryos deriving from the grafted isogenic donor germ stem cells. Note that the yellow color of the recipient golden trout strain is a dominant trait.

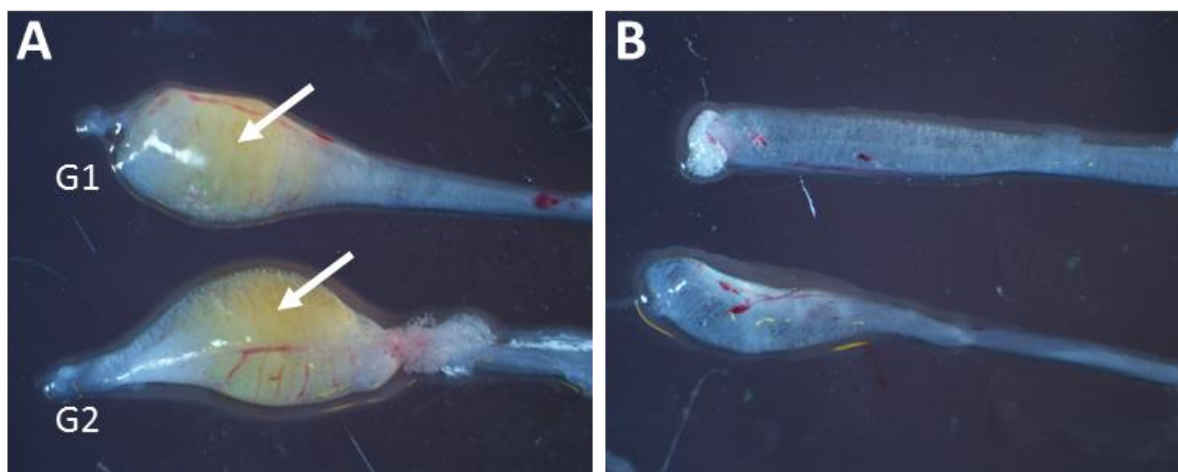
#### 1.4.6 Cryopreservation of spermatogonial stem cells

Cryomedia containing different cryoprotectants and sugars were tested (data not shown) to cryopreserve spermatogonial stem cell-enriched fractions. For the best cryomedium tested (L15 supplemented with 1% polyvinylpyrrolidone, 50 mM Sucrose, 30% Cryo3 (Stem Alpha), and 10% propanediol), the percentage of cell recovery (initial number of cells submitted to the cryopreservation procedure divided by the number of cells recovered after thawing) was about 27% and the viability reached 75%. In order to demonstrate that cryopreserved cells maintained their stemness properties, cells were transplanted into the abdominal cavity of female triploid trout embryos at the hatching stage. 62 and 60 embryos were injected respectively with cryopreserved and non-cryopreserved cells of the same initial cell preparation. Embryos were reared using standard farming procedures until 8 months of age. The survival rate of the group injected with cryopreserved germ stem cells was 76% at the first feeding and 55% at 8 months old. Similar survival rates were observed at the first feeding (63%) and at 8 months old (53%) for the group injected with the non-cryopreserved cells. The survival rate of the non-injected embryos was much higher (90%). This suggests that the transplantation induced an increased mortality but the latter did not vary drastically regardless of the cell suspension used.

Recipient females were euthanized at 8 months old and triploidy was checked from blood samples using flux cytometry. Ovaries were collected and observed for the presence of growing oocytes using a stereomicroscope (Figure 12). The success rate reached 89% using

the cryopreserved germ stem cells. This was similar to what was observed with a freshly prepared but non non-cryopreserved germ stem cell fraction (80%). The colonization was confirmed by molecular genotyping using genomic DNA extracted from the gonads of the recipient and a specific primer set amplifying the sex-determining gene in trout (*sry* gene). The success rates reached 59 and 56% for the non-cryopreserved and cryopreserved cells, respectively. One can note lower success rates compared to the other method based on the direct detection of growing oocytes. This is likely due to the lower sensitivity of the molecular genotyping when few oocytes are developing in the ovaries.

Taken together, our data demonstrate our capability to cryopreserve and transplant efficiently male germ stem cells in trout.



**Figure 12: Detection of growing oocytes in the ovaries of successfully grafted female recipient.**

A) Purified male germ stem cells were injected in triploid embryos of an all-female progeny and ovaries were collected at 8 months of age. The presence of recruited and growing oocytes (white arrows) was observed under the AZ100 multizoom macroscope equipped with the Di1 digital camera (Nikon). Note that only the anterior (G1) or medium (G2) area of the gonads were obviously colonized. B) No recruited oocyte was observed in control non-injected triploid embryos at 8 months of age.

## **2 PROCEDURES FOR CARP GERM CELL ISOLATION, CRYOPRESERVATION AND TRANSPLANTATION**

### **2.1 Procedures for the isolation of gonadal fragments**

#### **2.1.1 Isolation of testicular fragments**

Common carp males (age 1+ year, BW:  $128 \pm 34$  g) were euthanized by an overdose of 2-phenoxyethanol and decapitated. Testes were aseptically excised, washed in phosphate buffered saline (PBS) and cleaned of large blood vessels and adjacent connective tissue. Testes were then cut into small fragments, approximately weighing 50 to 150 mg.

#### **2.1.2 Isolation of ovarian fragments**

Ovarian tissue was excised aseptically from 7 months aged females (BW:  $7.3 \pm 1.8$  g) and washed in phosphate buffered saline (PBS) with 100 U/mL penicillin and 0.1 mg/ml streptomycin. Ovaries were cut and pieces weighing 50 to 150 mg were selected depending on the experiments.



## 2.2 Procedures for the cryopreservation of gonadal fragments

Optimization of the freezing protocol was conducted in sequential experiments where one cryopreservation parameter was changed in each experiment and the best outcome was used in the subsequent experiment. The different sizes of gonadal fragments tested (50, 100 and 150 mg), the different cryoprotectants (Dimethyl sulfoxide (Me2SO), Methanol (MeOH), Ethylene glycol (EG), Glycerol (Gly), Propylene glycol (PG)) used at different concentrations, sugar supplementations (Glucose, Fructose, Thehalose, Sucrose), equilibration times, and the different freezing rates tested are reported in two articles (Franek et al. 2019a; Franek et al. 2019b). Only the optimized procedures are reported below.

### 2.2.1 Cryopreservation of testicular fragments

#### Slow freezing protocol of testicular fragments

100 mg tissue fragments were loaded into 1.8 ml cryotubes (Nunc) filled with 1 ml of cryomedium (PBS supplemented with 2M Me2SO, 0.3M glucose, 1.5% BSA and 25 mM HEPES). Samples were equilibrated for 30 min on ice, and subsequently placed into CoolCell (Biocision) freezer boxes and into a deep freezer (-80°C) which enabled cooling rates of -1°C/min. Alternatively, samples could be frozen using a Controlled rate freezer (IceCube 14S programmable freezer; IceCube Series v. 2.24, Sy-Lab, Neupurkersdorf, Austria). Samples frozen in freezer box were after 4 h plunged into liquid nitrogen for at least 1 day of storage. Samples frozen in controlled rate freezer were plunged into liquid nitrogen right after completing the freezing program. Samples were thawed in a 26°C water bath and tissue fragments were washed three times in L-15 where they remained until further work. In these conditions, cell viability determined using trypan blue reached 40.7±9.2%.

#### Rapid freezing protocol (vitrification) of testicular fragments

Vitrification was conducted by utilizing needle immersed vitrification (NIV) methodology as described previously (Marinovic et al. 2018). Briefly, 50 to 100 mg testicular fragments were pinned to an acupuncture needle and immersed into two media prior to cryopreservation: the equilibration solution (ES) and the vitrification solution (VS). The best viability was observed when samples were successively immersed for 15 min in the ES solution (L15 medium supplemented with 10% FBS, 25 mM HEPES, 0.5M Trehalose, 1.5M Propylene glycol, and 1.5M Me2SO) and for 1.5 to 2 min in VS (1.5M MeOH and 5.5M Me2SO). Tissue fragments on the needles were briefly put on paper wipes to remove excess of ES and VS and the needles were plunged into liquid nitrogen. After at least 1 day of storage, tissue pieces were warmed in three subsequent warming solutions at room temperature containing L-15 supplemented with 10% FBS and various concentrations of sucrose (WS1: 3M; WS2: 1M; WS3: no sucrose). In these conditions, cell viability determined using trypan blue reached 11.4 ± 4.9%.

### 2.2.2 Cryopreservation of ovarian fragments

100 mg ovarian fragments were equilibrated for 60 min and then frozen in a cryomedium containing 1.5% BSA in PBS supplemented 1.5M Me2SO and 0.3M glucose. In these standard conditions cell viability reached 65%.

## 2.3 Procedures for the transplantation of cells derived from frozen gonadal fragments

### 2.3.1 Cellular dissociation of the cryopreserved gonadal fragments

#### Dissociation procedure of thawed testicular fragments:

Each tissue fragment was weighed before dissociation, and subsequently transferred into the dissociation medium containing L-15 (L5520-100ML, Lot: SLBU2824, Sigma-Aldrich Inc., St. Louis, MO) supplemented with 2 mg/ml collagenase (17100-017, Lot: 1371008, Gibco, Life Technologies Czech Republic s.r.o., Czech Republic), 1.5 mg/ml trypsin (T4799-25G, Lot: SLBN0947V, Sigma-Aldrich Inc., St. Louis, MO) and 40 µg/ml of DNase I (10104159001, Lot: 22067800, Roche Diagnostics GmbH, Germany, minced into small pieces and incubated for 1.5 h at room temperature (RT; 22°C) on a shaking plate. Digestion was terminated after addition of 1ml L-15 medium with 10% Fetal bovine serum (FBS) (v/v) (F9665, Lot: BCBS4908V, Sigma-Aldrich Inc., St. Louis, MO). In order to obtain a single cell suspension, samples were filtered through 30 µm CellTrics filters (04-0042- 2316, Sysmex, Germany) and centrifuged for 10 min at 200 xg. The supernatant was removed and the pellet was resuspended by a gentle pipetting with addition of an appropriate volume of L-15 medium.

#### Dissociation procedure of thawed ovarian fragments:

50 to 150 mg ovarian fragments were thawed in a 26 °C water bath and tissue pieces were rehydrated and washed 3 times in L-15. Each ovarian fragment was dissociated in 1 ml of digestion medium containing PBS with 0.15% trypsin, 0.05% DNase grade II (Roche) at 22 °C, on a laboratory shaker (30 rpm). Digestion was terminated after 1.5 h by addition of 10% (v/v) Fetal bovine serum (FBS) and 500 µl L-15 medium. The suspension was filtered through CellTrics® 30 µm (Sysmex, Germany) filters. The suspension was centrifuged for 10 min at 500g, the supernatant was removed and the pellet was resuspended by gentle pipetting with addition of an appropriate volume of L-15 medium (30–100 µl).

### 2.3.2 Sterilisation of the goldfish recipient embryos

Goldfish (*Carassius auratus*) embryos were injected under the blastodisc at the 2-cell stage without dechoriation with 100 mM solution of antisense dead end morpholino (dnd-MO: GenBank accession no. JN578697, target sequence: 5' CATCACAGGTGGACAGCGGCATGGA 3') purchased from Gene Tools LLC (Philomath, OR, USA) and diluted in 0.2M KCl. The injection was carried out using a micromanipulator (M-152, Narishige, Japan) and FemtoJet 4x microinjector (Eppendorf, Germany). Injection pressure and pressure duration were set to inject dnd-MO in approximate volume of 10% of the total yolk volume. Part of embryos injected with dnd-MO was co-injected with GFP-nos1 3'UTR mRNA to confirm successful depletion of primordial germ cells. Water was changed daily until hatching.

### 2.3.3 Transplantation in goldfish recipient embryos

Transplantation was conducted into 11 dpf dnd-MO treated recipient larvae. Two different test groups were defined: (1) a recipient group in which fresh cells were injected and (2) a recipient group into which cryopreserved/thawed cells were injected. Due to the higher overall viability obtained by freezing ( $40.7 \pm 9.2\%$ ) compared to vitrification ( $11.4 \pm 4.9\%$ ), only spermatogonia frozen with the optimized protocol based on 2M Me2SO and 0.3M Glucose using Cool Cell Box were transplanted alongside freshly isolated cells into the recipient goldfish larvae. Spermatogonia were enriched using 30% Percoll gradient (P1644-100ML, Lot: SLBS3410V, Sigma-Aldrich Inc., St. Louis, MO). Recipient larvae were

anesthetized in a 0.05% tricaine solution (A40-25G, Lot: MKBV1603V, Sigma-Aldrich Inc., St. Louis, MO) and approximately 5000 cells were injected into the peritoneal cavity of the recipients. Injected larvae (100 larvae per group) were transferred into fresh water and left to recover for two weeks and fed with *Artemia* nauplii. Germline chimeras were then transferred into aquaria and fed with an artificial food (SCARLET, Alltech Coppens, The Netherlands). Water temperature was constantly held at 23°C after the transplantation in order to prevent sex bias.

### 2.3.4 Germline chimera identification

The success rate of the spermatogonial stem cell grafting was determined at 3 months post-transplantation (BW: about 5.6 g). Firstly, gonads were visually inspected for signs of development under a light microscope. Expression of the carp *ddx4* and *dnd1* genes was investigated to confirm the presence of germ cells originating from the donor germ stem cells.

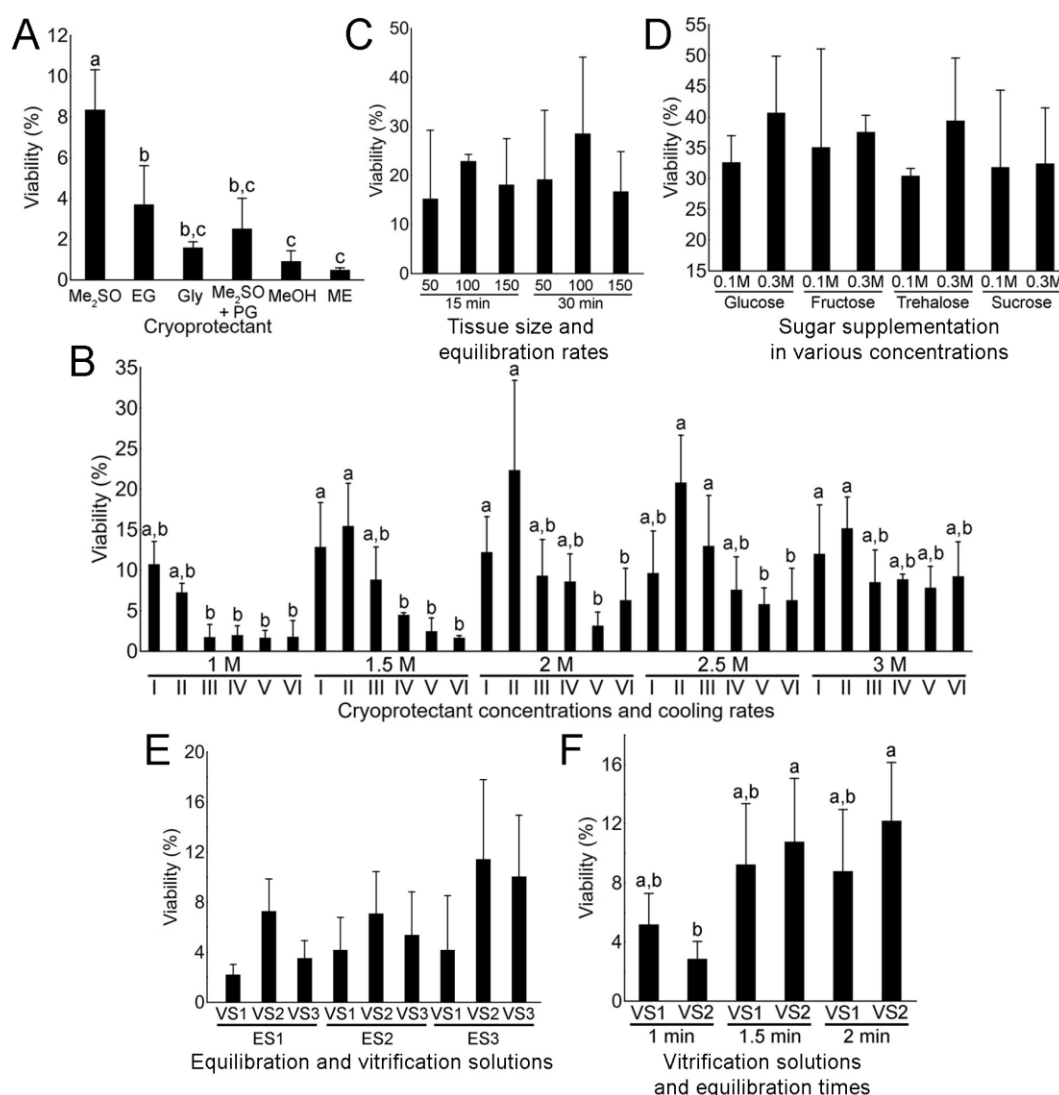
The success rate of ovarian stem cell grafting was determined at 1 month post-transplantation using grafted PKH26 labelled cells. Gene expression analysis of carp *vasa* and *dnd1* genes was investigated at 2 months post-transplantation.

## 2.4 Results

### 2.4.1 Cryopreservation of carp testicular fragments

The different experiments carried out to optimize the cryopreservation of carp testicular fragments are summarized in Figure 13. The highest cell viability was obtained using a slow freezing protocol. Testicular fragments were equilibrated for 30 minutes in the cryomedium containing L15 supplemented with 2M Me<sub>2</sub>SO, 0.3M glucose, 1.5% BSA and 25 mM HEPES. A freezing rate of -1°C/min was applied to reach -80°C and samples were then frozen in liquid nitrogen. In these conditions, cell viability determined using trypan blue reached 40.7 ± 9.2%. The rapid freezing protocols tested showed lower cell viabilities (11.4 ± 4.9% at the best).

The preservation of the stemness properties of cryopreserved spermatogonial stem cells was demonstrated using transplantation assays in goldfish recipient embryos. The success rate of the spermatogonial stem cell grafting was determined at 3 months post-transplantation (BW: about 5.6 g). Firstly, gonads were visually inspected for signs of development under a light microscope. Approximately 50% of recipients injected with fresh spermatogonia displayed developing gonads. Similarly, 40% of the recipients injected with cryopreserved carp spermatogonia displayed developing gonads. Subsequently, expression of the carp *ddx4* and *dnd1* genes was investigated to confirm the presence of germ cells originating from the donor germ stem cells (not shown). All goldfish recipients were true germinal chimera since they expressed carp *ddx4* and *dnd1* genes in their gonads. Altogether, data indicate that cryopreserved carp spermatogonial stem cells remain capable of colonizing the gonads of a surrogate species.



**Figure 13 : Optimization of the freezing (A-D) and vitrification (E, F) protocols for common carp spermatogonia.** (A) Viability of spermatogonia after freezing with dimethyl sulfoxide (Me<sub>2</sub>SO), ethylene glycol (EG), glycerol (Gly), Me<sub>2</sub>SO and propylene glycol at ratio 1:1 (Me<sub>2</sub>SO+PG), methanol (MeOH) and methoxyethanol (ME). (B) The effects of Me<sub>2</sub>SO concentrations (1–3 M) and cooling rates of 0.5 (I), 1 (II), 2.5 (III), 5 (IV), 7.5 (V) and 10 (VI)°C/min on spermatogonia viability. (C) Viability of spermatogonia after exposing 50, 100 or 150 mg tissue fragments for 15 or 30 min to the cryomedium. (D) Effect of sugar supplementation of spermatogonia viability. Effects of different equilibration (ES) and vitrification (VS) solutions (E) and exposures (1–2 min) to different VS (F) on spermatogonia viability after NIV. All values are presented as mean ± SD. Different letters above the SD lines indicate statistical significance (Tukey's HSD,  $p < 0.05$ ), while the lack of such letters indicates the lack of statistical significance.

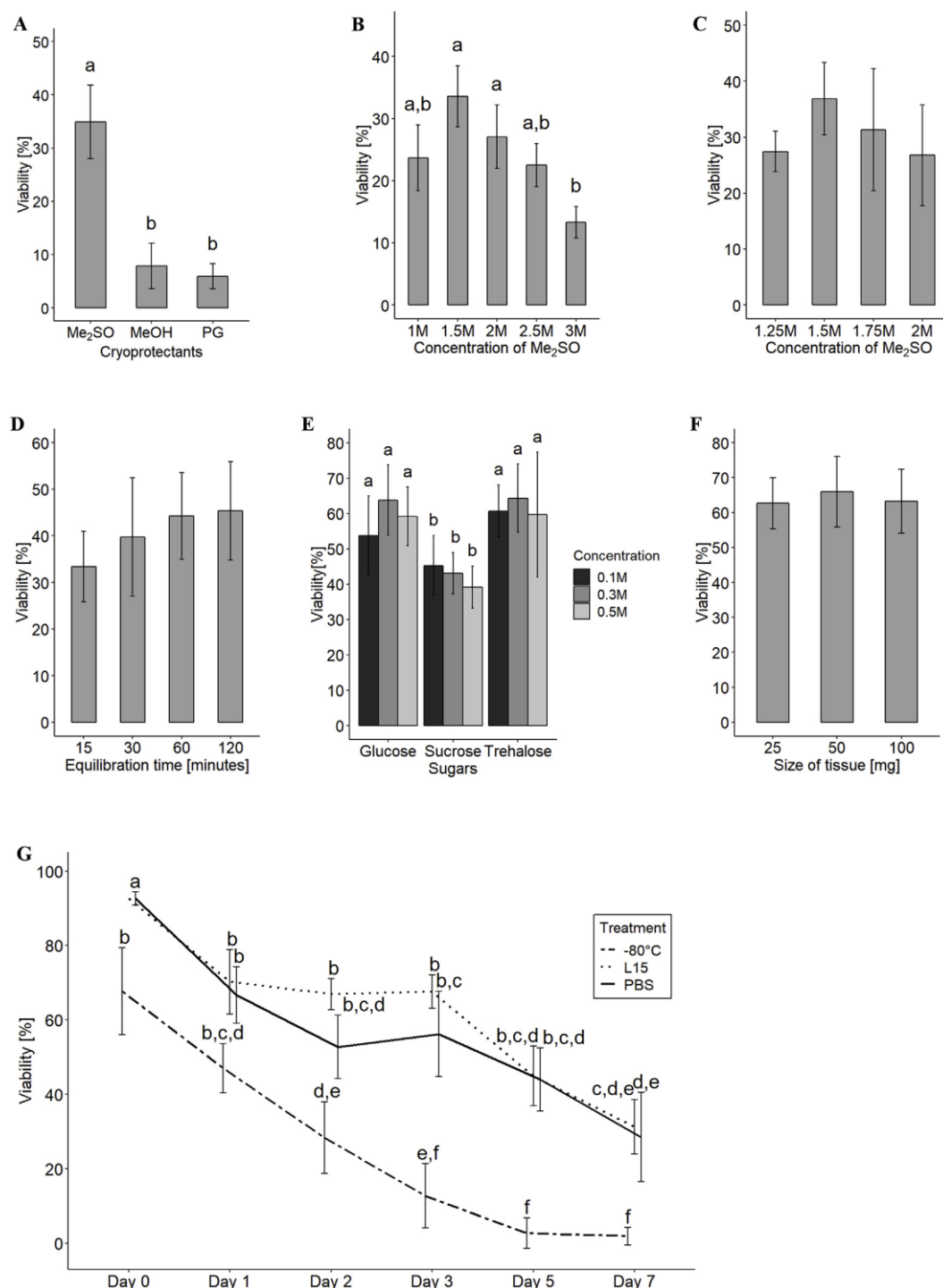
## 2.4.2 Cryopreservation of carp ovarian fragments

The different experiments carried out to optimize the cryopreservation of carp ovarian fragments are summarized in Figure 14. The highest cell viability was obtained using a slow freezing protocol. 50 mg ovarian fragments were equilibrated for 60 min on ice and then frozen in a cryomedium containing 1.5% BSA in PBS supplemented with 1.5M Me<sub>2</sub>SO and 0.3M glucose using a freezing rate of -1°C/min. In these standard conditions cell viability reached 67.5%.

The success rate of ovarian stem cell grafting was determined at 1 month post-transplantation using PKH26 labelled cells and gene expression analysis of carp *vasa* and *dnd1* genes was investigated at 2 months post-transplantation. 73% of the recipients were successfully grafted using cryopreserved carp ovogonia. This is similar to what was obtained



using freshly prepared ovogonia (76%). Altogether, data indicate that cryopreserved carp oogonial stem cells remain capable of colonizing the gonads of a surrogate species.



**Figure 14: Optimization of the slow-rate freezing (-1 °C/min) and short term storage (at -80 and 4 °C) protocols for common carp oogonial stem cells (OSCs).** (A-F) Post thaw viability after freezing ovarian tissue fragments after varying different cryobiological parameters. (A) Viability of OSCs after freezing with dimethyl sulfoxide (Me<sub>2</sub>SO), methanol (MeOH) and propylene glycol (PG) (B, C). Viability of OSCs after utilizing different molar concentrations of Me<sub>2</sub>SO. The effects of equilibration time (D), sugar supplementation (E) and tissue size (F) on OSC viability. (G) Short-term storage of ovarian tissue fragments at -80 °C or under hypothermic conditions (4 °C) in an L-15 or PBS-based media. All data are presented as mean ± SD. Different letters above the SD bars indicate statistical significance (Tukey's HSD,  $p < 0.05$ ).

## Conclusions

In trout, we have established efficient procedures based on germ stem cells grafting that will allow to cryopreserve and to faithfully regenerate genetic resources. This knowledge was recently transferred to European research infrastructures, including PEIMA (France) and IMR Matre (Norway) that maintain several isogenic trout and salmon lines, respectively.

We demonstrated that triploid recipient trout are suitable recipients for transplantation assays. High success rates of the grafting assays were obtained using triploid recipients, and about 60% of the sexually mature triploid recipients produced functional gametes derived only from the donor germ stem cells. Because triploidisation is a standard farming practice this knowledge can be quickly transferred to and applied by the industry.

We showed that there is no need to further purify the rare germ stem cells from the dissociated testicular cells extracted from 9-month old neomales. This observation will facilitate the use and reduce the cost of transplantation in the experimental research infrastructures. Although the percentage of successfully grafted male and female recipients remained high regardless of the A32 and B57 isogenic trout lines used, further experiments will be required to determine whether the efficiency of the transplantation assays could vary depending on the genetic background of the donor germ stem cells or depending on the genetic background of recipient fish strain.

Successfully grafted trout recipient males showed lower sperm count and volume of milt compared to standard genetic males. This indicates that male fertility is reduced in a great number of recipient males. If necessary, this limitation could be overcome by using testicular sperm. The spawning season and egg production of the successfully grafted recipient females were rather similar to that of the diploid females belonging to the recipient strain. However, the lower survival rate of the progenies indicates that egg quality is decreased in recipient females. Despite the decreased reproductive performances observed in successfully grafted male and female recipients at two years of age, many progenies were generated and were found genetically identical to the genetic characteristics of the donor germ stem cells. Future research directions will aim to understand the mechanisms responsible for the lower reproductive performances of the grafted male and female trout.

A procedure allowing the cryopreservation of cell fractions highly enriched undifferentiated A spermatogonia has been successfully established in trout. However, this technique requires expensive equipment and is laborious. Although a technical sheet reporting this procedure has been referenced in WP3, additional experiments are undergoing to optimize a protocol that could be easily carried out in all European research infrastructures at low cost. Such experiments are aimed to cryopreserve large amounts of trout testicular fragments using a rapid freezing method similar to vitrification.

In carp, procedures that allow an easy and rapid cryopreservation of gonadal fragments have been established to cryopreserve both spermatogonial and oogonial stem cells. In addition, cryopreserved carp germ stem cells were successfully transplanted in recipient goldfish previously sterilized using *anti-dead end* morpholinos. Further investigations will aim to demonstrate whether functional carp gametes could be produced through interspecific transplantation in goldfish.

## Glossary

AQUAEXCEL<sup>2020</sup>: AQUAculture Infrastructures for EXCELlence in European Fish Research towards 2020

Germinal chimera: grafted recipients carrying gonads made of its own cells and germ stem cells originating of the donor.

Oogenesis: female gametogenesis that results in the production of ovocytes and ova.

Ovarian stem cells (OSC). Adult stem cells of the female gametogenesis

Spermatogenesis: male gametogenesis that results to the production of spermatozoa

Spermatogonial stem cells (SSC): Adult stem cells of the male gametogenesis

## Document information

<b>EU Project N°</b>	652831	<b>Acronym</b>	AQUAEXCEL <sup>2020</sup>
<b>Full Title</b>	AQUAculture Infrastructures for EXCELlence in European Fish Research towards 2020		
<b>Project website</b>	<a href="http://www.aquaexcel.eu">www.aquaexcel.eu</a>		

<b>Deliverable</b>	<b>N°</b>	D7.3	<b>Title</b>	Validation of the procedures for germ stem cell isolation, cryopreservation and transplantation in normal trout and carp lines.
<b>Work Package</b>	<b>N°</b>	7	<b>Title</b>	<b>Standardisation and maintenance of fish lines</b>

<b>Date of delivery</b>	<b>Contractual</b>	Month 48	<b>Actual</b>	Month 54
<b>Dissemination level</b>	X	<b>PU Public, fully open, e.g. web</b>		
		<b>CO Confidential, restricted under conditions set out in Model Grant Agreement</b>		
		<b>CI Classified, information as referred to in Commission Decision 2001/844/EC.</b>		

<b>Authors (Partner)</b>	Partners 1 (INRA) and 9 (JU)			
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<b>Version log</b>			
<b>Issue Date</b>	<b>Revision N°</b>	<b>Author</b>	<b>Change</b>

## Annex 1: Check list

Deliverable Check list (to be checked by the “Deliverable leader”)

	Check list		Comments
BEFORE	I have checked the due date and have planned completion in due time		<i>Please inform Management Team of any foreseen delays</i>
	The title corresponds to the title in the DOW		<i>If not please inform the Management Team with justification</i>
	The dissemination level corresponds to that indicated in the DOW		
	The contributors (authors) correspond to those indicated in the DOW		
	The Table of Contents has been validated with the Activity Leader		<i>Please validate the Table of Content with your Activity Leader before drafting the deliverable</i>
	I am using the AQUAEXCEL <sup>2020</sup> deliverable template (title page, styles etc)		<i>Available in “Useful Documents” on the collaborative workspace</i>
<b>The draft is ready</b>			
AFTER	I have written a good summary at the beginning of the Deliverable		<i>A 1-2 pages maximum summary is mandatory (not formal but really informative on the content of the Deliverable)</i>
	The deliverable has been reviewed by all contributors (authors)		<i>Make sure all contributors have reviewed and approved the final version of the deliverable. You should leave sufficient time for this validation.</i>
	I have done a spell check and had the English verified		
	I have sent the final version to the WP Leader, to the 2 <sup>nd</sup> Reviewer and to the Project coordinator (cc to the project manager) for approval		<i>Send the final draft to your WPLLeader, the 2<sup>nd</sup> Reviewer and the coordinator with cc to the project manager on the 1<sup>st</sup> day of the due month and leave 2 weeks for feedback. Inform the reviewers of the changes (if any) you have made to address their comments. Once validated by the 2 reviewers and the coordinator, send the final version to the Project Manager who will then submit it to the EC.</i>



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