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1 Who is the best surrogate for germ stem cell transplantation in fish?

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16 **ABSTRACT**

17 Surrogate reproduction technology in fish has potential for aquaculture as well as endangered
18 species preservation and propagation. Species with some unfavourable biological characteristics for
19 culturing such as a late maturation or a large body size are ideal candidates for surrogate
20 reproduction using smaller and faster-maturing host. One of the general prerequisites for the
21 successful surrogacy and the pure donor-derived gamete production is the sterility of the host.
22 Various sterilization methods have been developed and used in fish surrogacy; however, a direct
23 comparison of available methods is missing. Such a knowledge gap hinders choice for the surrogate
24 in various fish species, including those in high commercial demand such as tuna or sturgeons,
25 where is a particular limitation from the point of the live material availability and difficulty to
26 perform a high throughput assessment of different surrogates. Yet, large sturgeons or tuna species
27 are one of the most prominent candidates for surrogacy. Zebrafish was utilized in this study as a
28 model species to answer whether and to which extent different sterilization strategies can affect the
29 surrogacy. Germ cell-depleted recipients (produced using knockdown of *dead end* gene), triploid
30 recipients, and zebrafish x pearl danio hybrid recipients were tested as they represent the most
31 frequently used types of surrogates. Spermatogonia isolated from vas::EGFP transgenic strain were
32 intraperitoneally transplanted into swim-up 5-day old zebrafish. Transplantation success, survival,
33 gonadal development, and reproductive output of the fish was analyzed. Germ cell-depleted
34 recipients with empty gonads were identified as the most convenient among tested sterilization
35 methods considering surrogacy induction success and reproductive output. The present study stands
36 as significant aid for selecting suitable surrogates in various fish species.

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38 ## 1 INTRODUCTION

39 Germ stem cell (GSC) manipulation in fish is still a relatively novel reproductive biotechnology.
40 The stem potential of GSCs in gonads is used for surrogate production of donor-derived gametes.
41 Isolated GSCs from an individual are transplanted into another individual, even from a relatively
42 distinct species. Transplanted GSCs are capable of migration and genital ridge colonization.
43 Afterwards, GSCs can undergo trans-differentiation when spermatogonia in a female body
44 environment switch to an oogonial fate and vice versa. Transplanted GSCs can proceed with
45 gametogenesis and give rise to donor-derived gametes (Goto and Saito, 2019). Surrogacy can be
46 accompanied by the inclusion of cryopreservation procedures when both male (Franěk et al., 2019a)
47 and female (Franěk et al., 2019b) GSCs can be cryopreserved efficiently and then recovered by
48 transplantation into surrogate hosts (Lee et al., 2013; Yoshizaki and Lee, 2018). Moreover, GSCs
49 manipulation technology in fish is recently being applied to produce genetically edited donor
50 gametes while avoiding eventual inviability of adult individuals because of induced mutation
51 (Zhang et al., 2021, 2020).

52 It is reasonable to presume that surrogate reproduction will be applied for species preservation or
53 aquaculture since the number of reports on GSCs manipulation in various species increases rapidly,
54 including aquaculture relevant species (Goto and Saito, 2019). Gametogenesis of large and late-
55 maturing species might be accelerated by GSCs transplantation into smaller and faster-maturing
56 recipients (Linhartová et al., 2015; Hamasaki et al., 2017; Baloch et al., 2019b). Reversely,
57 surrogacy can be utilized to increase the gamete production by transplantation of GSCs from
58 smaller to larger and potentially more fecund species, e.g. from goldfish (*Carassius auratus*) to
59 common carp (*Cyprinus carpio*) or from sterlet sturgeon (*Acipenser ruthenus*) to beluga (*Huso*
60 *huso*). Surrogacy also has potential to ameliorate breeding schemes via the distribution of the
61 germplasm from superior individuals via surrogates (Jin et al., 2021; Yang et al., 2021; Yoshizaki
62 and Yazawa, 2019).

63 Since surrogate reproduction in fish is a long term and laborious effort, whole technology needs to
64 be optimized to maximize its success. Optimal conditions for GSCs isolation (Shikina et al., 2013),
65 purification (Ryu and Gong, 2020), and *in vitro* expansion (Iwasaki-Takahashi et al., 2020; Xie et
66 al., 2019) were identified. Several studies already paid attention to the investigation of variables
67 related to used recipients. Optimal developmental stages for donors and recipients were investigated
68 in salmonids by primordial germ cells (PGCs) transplantation (Takeuchi et al., 2003) and in
69 zebrafish (*Danio rerio*) single PGCs transplantation into the blastula stage host. PGCs were shown
70 to lose their migratory potential progressively in zebrafish (Kawakami et al., 2010; Saito et al.,
71 2010). Similarly, differences were demonstrated on medaka (*Oryzias latipes*) in the age of

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72 recipients for spermatogonia transplantation (decreasing transplantation success with increasing
73 age) and in the positive effect of higher number of transplanted spermatogonia on the colonization
74 rate (Seki et al., 2017). Also, a short time window for transplanted GSCs to incorporate into the
75 host's genital ridge has been identified in rosy bitterling (*Rhodeus ocellatus*), suggesting certain
76 biological limitations of the transplantation procedure (Octavera and Yoshizaki, 2018).

77 However, many other factors affecting surrogacy success have been described initially, such as the
78 potential influence of the genetic relativeness between donor and recipient on the transplantation
79 success (Takeuchi et al., 2003). Or a comparison of the sterile and unsterile recipient (Marinović et
80 al., 2019) or a consequence of different sterilization methods on surrogacy success (Octavera and
81 Yoshizaki, 2018). Sterilization is crucial for successful surrogacy since introduced GSCs do not
82 have to compete for gonadal niche, and adult surrogates can produce only donor-derived gametes.
83 However, unlike in mammals, fish can be sterilized by diverse ways and mechanisms, resulting in
84 various levels of sterility ranging from germ cell-free gonads to decently developed gonads
85 incapable of producing motile spermatozoa.

86 Available methods for surrogate larvae sterilization are based on complete PGCs depletion via
87 targeting *dead end* (*dnd*) gene necessary for PGCs migration and maintenance (Baloch et al.,
88 2019a). PGCs depletion can be achieved by a temporal inhibition of translation using antisense
89 morpholino oligonucleotide proven to be effective in several fish species – sterlet sturgeon
90 (*Acipenser ruthenus*) (Linhartová et al., 2015), loach (*Misgurnus anguillicaudatus*) (Fujimoto et al.,
91 2010), goldfish (*Carassius auratus*) (Goto et al., 2012), cod (*Gadus morhua*) (Škugor et al., 2014),
92 zebrafish (Slanchev et al., 2005), rainbow trout (*Oncorhynchus mykiss*) (Yoshizaki et al., 2016).
93 More recently, gene editing methods such as CRISPR/Cas9 or zinc finger nucleases have been
94 employed to target *dnd* gene in sterlet sturgeon (Baloch et al., 2019b), Atlantic salmon (*Salmo*
95 *salar*) (Wargelius et al., 2016) and zebrafish (Li et al., 2017). Result of this sterilization method are
96 gonads utterly free of the GCs; however, the nature of this sterilization method can be considered
97 challenging and laborious since it requires individual embryo injection and known *dnd* sequence. It
98 is also necessary to be aware that sterilization by gene editing is considered as genetic modification
99 which might result in more strict regulations on maintenance and use of genetically modified fish.

100 Triploidization is another method of choice for sterility induction. Triploids are produced by
101 chromosome manipulation via the second polar body retention by a shock briefly after the
102 fertilization. Artificially induced triploids usually have impaired gametogenesis as a consequence of
103 odd chromosome number resulting in synapsis defects during meiosis (Piferrer et al., 2009).
104 Protocols for triploidy induction have been developed in many species (Piferrer et al., 2009), some
105 of them were also used as surrogates and successful donor-derived gametes were finally produced

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106 from triploid rainbow trout (Lee et al., 2013), Atlantic salmon (Hattori et al., 2019), medaka (Seki et
107 al., 2017) and zebrafish (Franěk et al., 2019c) recipients. Triploidy is suitable for large scale
108 production of recipients. However, there are also species for which triploidy does not guarantee
109 complete sterility (Murray et al., 2018), thus triploid recipients need to be used with cautions.

110 Last practically feasible method for sterilization for before intraperitoneal transplantation is an
111 interspecific hybridization. Fishes are, in most cases, external fertilizers which enable their simple
112 production, including hybridization. Hybridization has been attempted in many species, often
113 resulting in impaired reproductive performance. Reasons for altered gonadal development are
114 conditioned by genetic (in)compatibilities of two parental species (Fujimoto et al., 2008; Tichopád
115 et al., 2020), such as altered epistasis (Orr and Irving, 2001). Gonad in zebrafish (ZF) x pearl danio
116 (PD) (*Danio albolineatus*) hybrids can develop into male or female-like structures (Wong et al.,
117 2011). The presence of both sexes was also observed in the hybrid of two marine species, blue drum
118 (*Nibea mitsukurii*) and white croaker (*Pennahia argentata*), reporting arrested PGCs not
119 proliferating further (Yoshikawa et al., 2018). Both studies also tested infertile hybrids as
120 surrogates. They confirmed successful donor-derived gamete production showing that hybrid
121 sterility is GSC autonomous when supportive gonadal somatic cells are likely to remain unimpaired
122 by hybridization and can nurse transplanted GSCs. Hybridization among different tetra species
123 resulted in various patterns of gonadal development. Usually, diploid hybrids possessed gonads
124 with distinguishable male or female phenotypes with few germ cells; however, even advanced
125 stages of gonadal development might occur (Piva et al., 2018). Thus, hybridization is not a
126 universal approach for sterile surrogate production, and careful evaluation must be done in advance.
127 On the other hand, hybridization is suitable to facilitate large scale production of recipients.

128 There are several other methods for sterilization before germ cell transplantation, such as a
129 combination of thermal and cytostatic treatment. These methods are rather suited for intrapapillary
130 GSCs transplantations conducted in adult or juvenile fish (Lacerda et al., 2010; Nóbrega et al.,
131 2010). Similarly, sterilization is possible using specific transgenic lines by interfering PGCs
132 migration via ubiquitous expression of SDF1 (essential for PGCs migration) triggered by thermal
133 treatment (Wong and Collodi, 2013) or with nitroreductase expression in PGCs exclusively by
134 immersion into metronidazole enzyme resulting in its conversion into toxic metabolites targeting
135 only PGCs in zebrafish causing their depletion (Zhou et al., 2018). Transgenic medaka strain with
136 follicle-stimulating receptor mutation causing sterility in females was sex-reversed into phenotypic
137 males with subsequent spermatogonia transplantation into sterile hybrids of *Oryzias latipes* and *O.*
138 *curvinotus* to rescue egg production while maintaining the mutation transmission. Subsequently,
139 sperm from sex-reversed females homozygous for follicle-stimulating receptor mutation was used,

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140 and a system for production of all-female sterile progeny was established (Nagasawa et al., 2019).
141 All the above-mentioned transgenic strategies for sterilization are compelling and effective once
142 given transgenic line is established. Unfortunately, not all species are convenient for transgenesis
143 due to their long generation times and the lack of genomic data.

144 We presume that *dnd* knockdown, triploidization and hybridization are the most practical and
145 universal methods to sterilize recipients before the intraperitoneal germ cell transplantation.
146 Theoretically, completely germ cell less gonads might represent the best environment for
147 transplanted GSCs, as they do not need to compete for space with endogenous germ cells during
148 colonization. However, exhaustive comparison of different sterilization methods has not been
149 performed yet. The presented study aimed to provide a comprehensive analysis of different
150 sterilization treatment and their effect on surrogacy success in fish.

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152 ## 2 Material and methods

153 The study was conducted at the Faculty of Fisheries and Protection of Waters (FFPW), University
154 of South Bohemia in České Budějovice, Vodňany, Czech Republic. The facility has the competence
155 to perform experiments on animals (Act no. 246/1992 Coll., ref. number 16OZ19179/2016–17214).
156 The expert committee approved the methodological protocol of the current study of the Institutional
157 Animal Care and Use Committee of the FFPW according to the law on the protection of animals
158 against cruelty (reference number: MSMT-6406/119/2). The study did not involve endangered or
159 protected species. Authors of the study (RF, MF, VK, OL, MP) own the Certificate of professional
160 competence for designing experiments and experimental projects under Section 15d (3) of Act no.
161 246/1992 Coll. on the Protection of Animals against Cruelty.

162 ### 2.1 Fish and production of recipients

163 Zebrafish broodstock was maintained as described previously (Franěk et al., 2019c). Zebrafish AB
164 line (descendants of fish purchased from European Zebrafish Resource Centre), vas::EGFP line
165 (EGFP expression is under control of vasa promotor) (descendants of fish purchased from
166 University of Liège, Belgium) and pearl danio (descendants of fish purchased from PetraAqua,
167 Czech Republic). All experimental groups were produced by *in vitro* fertilization. For each
168 transplantation trial, pooled eggs and pooled sperm was divided into four groups to establish
169 recipient groups from the same parents. PGCs depleted fish by *dnd*-MO (MO group) were produced
170 by injecting zebrafish embryos at 2-8 cell stage with 100 µM solution of antisense morpholino
171 diluted in 0.2 KCl with 1.5% Rhodamine B isothiocyanate–Dextran (10,000 MW) to ease the
172 confirmation of successful MO injection by fluorescence signal detection in the animal pole. To
173 produce triploids (3n group), an optimized heat-shock protocol (Franěk et al., 2019c) using 41.4 °C,
174 initiated 2 min post fertilization (mpf), lasting 2 min was performed. Hybrids between zebrafish
175 females and pearl danio males (H group) were produced as described previously (Wong et al.,
176 2011). Recipients were produced in three replicates at different timepoints (Fig. 1). Produced
177 embryos were cultured at 28.5 °C, swim-up larvae were fed from 5th-day post fertilization (dpf)
178 with paramecium *ad libitum*, from 10 dpf with *Artemia* sp. At the age of 4 weeks post-fertilization
179 (wpf) fish were transferred into zebrafish housing system and fed with a combination of dry diet
180 and *Artemia* sp. until the termination of the experiment.

181 ### 2.2 Germ cell donors and transplantation

182 Adult zebrafish donor males (6-8-month-old) from vas::EGFP transgenic line were over
183 anaesthetized in MS222, body was washed with 70% ethanol and decapitated. Testes were removed
184 carefully (8 males for one transplantation trial) and kept in phosphate-buffered saline (PBS). Testis

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185 were cut into small fragments in 2 ml tube with 0.1 ml of PBS and washed thoroughly by several
186 changes of PBS to facilitate sperm leakage. Afterwards, finely cut testes were digested in 8 ml of
187 dissociation media containing 0.1% trypsin in PBS on a laboratory shaker at room temperature for
188 60 min. During digestion, 0.5% DNase solution in distilled water was added when clumping or
189 agglutination of testis fragments was observed (usually, 70-100 µl of DNase was used). Digestion
190 was terminated by the addition of 7 ml L15 media with 20% fetal bovine serum (FBS). The
191 suspension was filtrated through a sterile 30µm mesh filter, centrifuged at 0.4 g for 10 min. The
192 supernatant was removed, and the pellet was resuspended in fresh L15 with 10% FBS and stored at
193 10 °C during transplantation.

194 Transplantation was performed at 5 dpf. Recipients were anaesthetized in 0.05% MS222 and placed
195 on agar coated petri dish. Microcapillary was polished on a grinder, filled with cell suspension and
196 mounted on micromanipulator with a pneumatic injector to keep equal injection pressure and
197 duration during transplantation. Seventy fish from each recipients group were transplanted in three
198 replicates. Always 10 recipients per group were transplanted, and then 10 recipients from another
199 group were transplanted to minimize the chance that some groups would be transplanted with
200 "aged" isolated testicular cells. Four transplanted groups were established – MO T, 3n T H T and
201 AB T. Controls were established from non-transplanted fish, 30 specimens for each recipient group
202 and replicate – MO C, 3n C, H C and AB C.

203 **Figure 1. Production of experimental and control groups.**

204 2.3. Identification of germline chimeras

205 Two weeks post-transplantation all surviving fish from transplanted groups were anaesthetized and
206 screened under a fluorescent stereomicroscope (Leica M205 FA) and separated to fish with EGFP
207 signal positive and negative. EGFP positive fish were evaluated for EGFP positive transplanted
208 cells distribution. All surviving transplanted adult fish from EGFP positive groups were prepared
209 for sperm collection as described previously (Franěk et al., 2019c). Sperm was collected
210 individually into 20 µl of E400 media (Cheng et al., 2021) and observed under a fluorescent
211 microscope (Olympus IX 83) to detect EGFP signal witnessing donor-derived origin. The
212 remainder of collected sperm was used for genotyping. DNA was extracted using the Hot-Shot
213 method and PCR with EGFP specific primers followed by gel electrophoresis as described
214 previously (Franěk et al., 2019c). Afterwards, confirmed chimeric males were reared separately
215 from males not producing donor-derived sperm.

216 2.4 Reproductive performance of germline chimeras

217 *2.4.1 Fertilization tests*

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218 Confirmed chimeric males were set up randomly into spawning aquaria (1l) with females from
219 control AB strain (1:1) as described for semi-artificial spawning (Franěk et al., 2019c) and allowed
220 to spawn for 4 hours. Eggs were collected, and their survival was monitored (fertilization rate, 24
221 hours post-fertilization (hpf), 48 hpf, hatching rate and swim-up rate). After hatching, 10 larvae
222 from each cross were frozen fixed for genotyping with EGFP specific primers. For each recipient
223 group, data from 15 successful spawnings were collected for assessment of spawning success, and
224 number of oviposited eggs (note that individual males were not spawned repeatedly) were collected.
225 For *in-vitro* fertilization, sperm from three males (note that males were not used repeatedly) from
226 each experimental group, including controls (also vas::EGFP strain), was collected individually,
227 then pooled and used to fertilize fraction from the mixture of stripped eggs from AB control
228 females. In total, *in-vitro* fertilization was repeated three times (with different males).

229 Females from AB T were firstly anaesthetized and screened under a fluorescent steromicroscope to
230 detect the presence of EGFP signal in the ovaries. EGFP positive AB T females were then
231 attempted to be spawned semi-artificially, collected eggs were separated according to EGFP signal,
232 and their fertilization and hatching rates were monitored.

233 *2.4.2 Spermatozoon motility, velocity and sperm concentration assessment*

234 Sperm samples collected from anaesthetized fish were stored immediately into 20 μl E400. Distilled
235 water containing 0.25% Pluronic F-127 (to prevent spermatozoon from adhering to microscope
236 slides) was used as the activation medium. Activation medium and sperm samples were stored on
237 ice before motility activation. Sperm was activated at room temperature (21 °C) by mixing the
238 immobilized sperm sample into 20 μl of the activation medium on a glass slide within 1 hour post
239 collection. The activated spermatozoa were directly recorded microscopically (UB 200i, PROISER,
240 Spain) at 10× using a negative phase-contrast condenser with an ISAS digital camera (PROISER,
241 Spain) setting at 25 frames/s. The Integrated System performed analyses of the sperm recordings for
242 Sperm Analysis software (PROISER, Spain) at 15 s post sperm activation. Computer-assisted
243 sperm analysis included the percentage of motile sperm (%), curvilinear velocity (VCL, μm/s),
244 straight-line velocity (VSL, μm/s), and spermatozoa rate with rapid motility (> 100 μm/s), medium
245 motility (46 to 100 μm/s), slow motility (10 to 45 μm/s), and static spermatozoa (< 10 μm/s).
246 Analyses of all samples (9 males per recipient group) were carried out in triplicate (each male
247 recorded three times).

248 Sperm concentration and the total number of sperm per male were evaluated for individual males in
249 E400 extender solutions. The sperm in E400 was diluted again 10-140 times according to the
250 density of sperm. The sperm concentration (expressed as 10^6/μl) was determined by a Bürker cell

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251 haemocytometer (Marienfeld, Germany, 12 squares counted for each male) using an optical phase-
252 contrast condenser and an ISAS digital camera (PROISER, Spain) under an Olympus microscope
253 BX 41 (4009). All measurements were repeated 3 times.

254 **2.5 Histology**

255 Sacrificed fish were firstly degutted, decapitated and photographed under a fluorescent microscope.
256 Trimmed torsos with gonads inside were fixed in Bouin's fixative overnight, washed in 70%
257 ethanol, and processed by resin sectioning and haematoxylin-eosin staining (Sullivan-Brown et al.,
258 2011). At least three specimens from transplanted and non-transplanted groups, including controls,
259 were processed.

260 **2.6 Electron microscopy**

261 Sperm collected in E400 media (from AB C and H C group) was fixed in 2.5% glutaraldehyde in
262 PBS. Samples were prepared for electron scanning microscopy as described previously (Franěk et
263 al., 2021) and observed on JEOL JSM-7401F scanning electron microscope.

264 **2.7 Confocal microscopy examination**

265 Dissected gonads were fixed in 4% paraformaldehyde (PFA) in PBS for 2h, washed 3 times in PBS
266 and immersed for 3h in 25% sucrose solution (in PBS). Specimens were incubated overnight in
267 Cryomount media (HistoLab), then placed and oriented in fresh Cryomount media into plastic
268 moulds and frozen on floating Styrofoam (1 cm height) in liquid nitrogen vapours and stored at -
269 80 °C until use. Frozen tissue blocks were equilibrated in the cryostat chamber at -25 °C for 30 min
270 before cutting and attached to metal chucks. Sections of 15 µm thickness were cut on cryostat,
271 attached on superfrost slides, allowed do dry at RT for 5 min and mounted in Fluoroshield™ with
272 DAPI histology mounting medium, sealed with coverslip and imaged with laser scanning confocal
273 microscope (Olympus FV 3000).

274 **2.8 Data evaluation and statistical analysis**

275 The data homogeneity of dispersion was evaluated using Levene's test. The difference in sperm
276 motility parameters, survival, 24 and 48 hours post-fertilization (hpf), hatching, swim-up among
277 groups was analyzed using a one-way ANOVA. LSD test determined all the differences among
278 means. Bar charts of survival rates in fertilization, 24 and 48 hpf, hatching and swim-up, survival
279 percentage in transplanted, 1, 7 and 14 days post-transplantation (dpt), 1, 3 and 6 months post-
280 transplantation (mpt), EGFP positive cells location (%) were drawn with mean ± standard deviation
281 of the mean (S.D.). All analyses were performed at a significant level of 0.05 by using R (R Core
282 Team, 2018).

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283 **3 RESULTS**

284 **3.1 Survival, transplantation success and colonization patterns**

285 The lowest survival during recipient production was observed in MO and 3n group which is
286 attributed to injection into embryos and heat shock treatment respectively. In hybrid group we
287 observed increased mortality prior to hatching (Fig. 2A). However, given the nature and easiness of
288 zebrafish breeding, we do not consider lower survival due to sterilization treatment as limiting.
289 Notably, post-transplantation survival in MO T group was comparable to the controls, while
290 survival performance of 3n T, H T and control groups was slightly lower. Altogether, overall
291 survival from transplantation to 6 months of age was in all groups (included transplanted groups)
292 from 65 to 85 % (Fig. 2B).

293 The transplantation success evaluated two-week post-transplantation showed consistent results
294 across different sterilization methods of the EGFP positive cells in recipients (Fig. 3A). Most of the
295 EGFP positive cells were located in the posterior or medial part of the body cavity (Fig. 3B1-2).
296 The anterior part was occupied by the EGFP positive cells rarely. This trend was prominent
297 primarily in MO and H recipients, while 3n and AB recipients showed more similar colonization
298 patterns between the posterior and medial part of the body cavity.

299

300 **Figure 2. Overall survival after different sterilization treatments.**

301

302 **Figure 3. Colonization and cell localization after vas::EGFP GSCs transplantation.**

303

304 **3.2 Gonadal development in sterilized controls and surrogates**

305 Sterilization treatments resulted in distinct patterns of gonadal development of adults. Testes of MO
306 C treated fish were small and free of germ cells. Stromal somatic cells formed cavities divided by
307 connective tissue into smaller compartments (Fig. 4A). In MO C group all (n=10) assessed controls
308 showed germ cell-free testis. Control triploids developed gonads with all spermatogenic stages but
309 with apparent defects in meiosis, resulting in aberrant spermatogenesis and the presence of only few
310 spermatozoa in testicular lumens (Fig. 4B). Gross appearance of 3n testis was smaller than normal
311 testis of diploid, while being more transparent due to lack of the high number of spermatozoa inside
312 (Fig. 4B2). Sections from triploids showed consistent testicular development in all assessed
313 specimens (n=10). More erratic development was observed in H C group. Gonads of hybrid males

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314 exhibited three phenotypes. H C had well-developed testis with few abnormally sized spermatozoa
315 (H I.) (Fig. 4C), nearly one third of hybrids had undeveloped testis lacking germ cells (H II.) (Fig.
316 4D) and few individuals showed combination of one developed and one undeveloped testis (H III.)
317 (Fig. 4E). Spermatozoa in developed hybrid testis were apparently large, which was later observed
318 with light microscopy. Gonadal phenotype in hybrids was presented mainly by H type I. (well-
319 developed gonads), H type II. males were less abundant, and only few individual fish were
320 identified as hybrid females (Fig. 5B5). Hybrid females developed gonads in decent size and were
321 determined by inner lamella-like structure forming empty cavities. Details on hybrid spermatozoa,
322 histology and incidence of gonadal phenotypes are given in figure 5. Hybrid ovaries were mostly
323 composed of homogenous mass of the larger cells suggesting oogonia or early-stage meiotic
324 oocytes (Fig. 4F). The incidence of hybrid females was rare and only 6 females were detected from
325 all adult surviving hybrids controls (N=59). Detailed description of gonadal phenotypes from
326 histological sections is given in figure 4.

327

328 **Figure 4. Different sterilization treatments affect gonadal development.**

329

330 **Figure 5. Abnormal spermatozoa morphology in ZF x PD hybrids.**

331

332 **3.3 Success in germline chimera male induction**

333 The highest incidence of adult germline chimeras was observed in the MO T group, followed by 3n
334 T group. Interestingly, % of adult germline chimeras in H T and AB T group were almost equal
335 (Fig. 6A). Intraperitoneally transplanted GSCs were capable of establishing donor-derived
336 spermatogenesis in all tested sterilization treatments as well in non-sterilized AB recipients.
337 Dissection of adult germline chimeras showed influence of sterilization treatments on the extent of
338 testicular development. Various patterns of gonadal development were observed across tested
339 sterilization treatments and were reflected in GSI when compared to their respective controls (Fig.
340 6B). Observed gross gonadal development was prominent MO T group which gained the largest
341 increment in gonadal development (comparing transplanted group with their respective sterilization
342 control). In MO T group, transplanted GSCs were able to frequently reconstitute spermatogenesis
343 unilaterally (Fig. 6 D3, 6 D4) or even bilaterally into fully developed testes in term of length and
344 width (Fig. 6 D9). Similar capacity was also observed in 3n T and H T group, but with less
345 incidence of fully developed gonad and higher incidence of only spatially localized EGFP positive
346 spermatogenesis not reaching full length and width of the testes (Fig. 6D). Developed recipient-

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347 derived spermatogenesis in AB T group largely limited EGFP positive spermatogenesis when only
348 spatially restricted EGFP positive areas of testicular tissue were observed macroscopically (Fig. 6
349 D5-6).

350 Sterilization treatments promoted donor's germ cells development. Introduced GSCs could expand
351 and occupy whole testis cross-section, which was reflected by EGFP signal detection on
352 cryosections (Fig. 7). Interestingly, triploid and non-sterilized recipients showed that introduced
353 GSCs must compete for the testicular niches with endogenous germ cells since cryosections in
354 germline chimeras showed presence of spermatocysts lacking EGFP signal (Fig. 7B). Germline
355 chimeras from AB T group exhibited more erratic distribution of exogenous germ cells which was
356 limited spatially and EGFP positive cells were not able to occupy the recipient testes completely
357 (Fig. 7D-E). Histological analysis of identified germline chimeras showed testis with similar
358 morphology to donor and recipient controls (Fig. 8). However, in some individuals from 3n T and H
359 T group we have identified signs about partial spermatogenesis of recipient's germ cells e.g.
360 spermatocysts with meiotic germ cells arrested in pachytene (3n T) as well as individual
361 spermatozoa with abnormally large heads and empty testicular lumens in H T group (Fig. 8 C4).

362

363 **Figure 6. Adult germline chimeras.**

364

365 **Figure 7. Distribution of transplanted germ cells from vas::EGFP donors in the recipients'**
366 **gonads.**

367

368 **Figure 8. Gonadal development in germline chimeras.**

369

370 **3.4 Occurrence of chimeric females producing donor-derived eggs**

371 Several females from AB T group were identified to have EGFP signal in their ovaries. Those fish
372 were attempted for spawning with control AB males. Production of viable donor-derived eggs in
373 zebrafish was confirmed in 7 from 10 spawned females. Individual AB T females produced EGFP
374 positive eggs in various ratios, but their proportion was significantly lower compared to recipient-
375 derived eggs (Fig. 9A). Eggs and later embryos from donor-derived EGFP eggs showed similar
376 viability to the recipient-derived (endogenous) eggs. (Fig. 9B). The presence of oogenesis derived
377 from transplanted male GSCs was also confirmed on ovarian cryosection by detection of EGFP
378 signal (Fig. 10). Interestingly, overall sex ratio in AB T compared to AB C was slightly biased in
379 favour of females (Fig. 9D) but without significant difference.

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380

381 **Figure 9. Female germline chimeras in AB T group.**

382

383 **Figure 10. Detection of chimeric gonads in non-sterilized AB females.**

384

385 **3.5 Reproductive performance of chimeric males**

386 The sperm concentration and total amount of produced sperm in germline chimeras was influenced
387 by the fact that the testes comprised of donor-derived germ cells are not reaching their full size
388 compared to controls. All sterilization methods interfered with the sperm motility, curvilinear and
389 straight-line velocity (11A-C) which were usually significantly lower than in AB C group. This fact
390 is clearly visible in Fig. 11D, where the largest proportion of fast spermatozoa were found in the
391 recipient control. Only MO T group retained statistically comparable level of motility to donor
392 strain and outperformed 3n and H T group, yet without significant differences. Also, it was apparent
393 that motility performance in 3n T and H T groups was more dispersed showing very well and
394 poorly performing males compared to other assessed groups. Results showed that MO recipients
395 males produced highest volume of sperm (Fig. 11E), concentration of spermatozoa (Fig. 11F), total
396 number of spermatozoa (Fig. 11G) and finally also total motile spermatozoa (Fig. 11H) among
397 tested sterilized recipients. Overall results from sperm analysis are given in Figure 11.

398

399 **Figure 11. Reproductive performance of chimeric males with donor and recipient controls.**

400

401 **3.6 Fertilization trials**

402 Semi artificial fertilization trials conducted individually (one experimental male with one control
403 female) resulted in similar success of males to induce oviposition (number of spawning females)
404 when over 70% of pairs attempted for spawning actually spawned (Fig. 12A), and number of
405 oviposited eggs (Fig. 12B) was statistically comparable between transplanted groups and their
406 respective controls (e.g. MO T and MO C group). Non-transplanted (sterile) MO C, 3n C and H C
407 controls were able to induce oviposition, but no surviving progeny (reaching swim up stage) was
408 obtained when most oviposited eggs were unfertilized or died during embryonic development.
409 Comparison of transplanted recipients with AB and vas::EGFP controls showed poor performance
410 of H T recipients, which was especially prominent in semi-artificial fertilization trials, while MO T
411 and 3n T males showed performance comparable to one of the controls (AB C or vas::EGFP) (Fig.

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412 12C). In vitro fertilization resulted in higher progeny production in all groups including controls
413 (Fig. 12D). Importantly, the percentage of swim-up larvae was statistically comparable amongst all
414 groups except the H T group.

415 Genotyping of hatched larva originating from crosses of chimeric males and AB control females
416 showed 100% germline transmission detected by EGFP specific primers in MO T, 3n T and H T
417 group. In the AB T group, only a low germline transmission rate was detected 12 ± 6 % (Mean ±
418 SD). The complete dataset for individual males and their germline transmission rate is in
419 Supplementary file 1.

420

421 **Figure 12. Reproductive success of chimeric males.**

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422 **4. DISCUSSION**

423 The present study aimed to thoroughly assess different sterilization strategies for surrogacy in fish
424 using zebrafish model, and their consequences on the reproductive output. We tested three types of
425 sterilization for intraperitoneally transplanted zebrafish spermatogonia to help with the direct
426 selection of the sterilization methods for species, which are not fully established in the laboratories.
427 Similar kind of the study presented here (from recipient embryo to donor-derived gamete
428 production) would be difficult to conduct in species such as tuna or sturgeons.

429 One of the first issues in choosing a convenient recipient is its availability and survival, which goes
430 hand in hand with necessary efforts to achieve the given type of sterilization. Hybridization and
431 triploidization are methods of choice for large scale sterility induction. However, further problems
432 can appear in adult chimeras since their endogenous germ cells can proceed through gametogenesis.
433 PGCs depletion during early embryonic development by targeting *dnd* gene requires precise
434 injection of each embryo which is time consuming, demanding and suitable only for certain types of
435 experiments because developing embryos have to be micromanipulated within limited time.

436 Each sterilization strategy has its pros and cons, which needs to be considered and evaluated
437 carefully. Besides differently altered survival dependent upon the sterilization method, we were able
438 to identify striking differences in gonadal development and reproductive output of differently
439 sterilized surrogates. Once the gonads are free of endogenous GCs, it is likely to obtain the most
440 consistent results without interference with gonadal development because there is no competition
441 between exo- and endogenous GCs. Later observed reproductive differences might be the ultimate
442 decisive factor for choosing the most perspective sterilization strategy to obtain germline chimeras
443 with closest characteristics to the original donor strain.

444 The sterilization treatments negatively influenced survival prior to transplantation. Heat shock for
445 triploidy induction and MO injection had a severe impact on the survival rate. This can be
446 considered as expected based on previous results (Delomas and Dabrowski, 2018; Franěk et al.,
447 2019b). Low survival due to the temperature treatment for triploidization group needs to be
448 considered, however, it can be mitigated by using large amount of fertilized eggs. Situation with
449 low survival in MO group is more challenging to be tackled since the number of injected embryos is
450 limited by the skills of the personnel performing microinjection and developmental speed of
451 fertilized eggs. However, it is also possible to alternate laborious MO delivery by microinjection
452 using immersion of fertilized zebrafish eggs *in vivo* MO (capable of penetration and transport
453 through cell membranes) (Wong and Zohar, 2015).

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454 Post-transplantation survival is also an important aspect for the selection of suitable recipients and
455 sterilization strategy. MO T and 3n T groups with their respective controls showed comparable
456 post-transplantation survival to control groups from donor and recipient strains over the duration of
457 the experiment. Usually, larvae malformed due to the sterilization treatments could not proceed
458 embryogenesis or they did not reach swim-up feeding stage. Thus, only healthy and feeding larvae
459 are used for transplantation and they do not further interfere with survival in case of MO and 3n
460 recipients. H T group experienced the lowest survival post transplantation. We presume that this can
461 be attributed to the hybridization itself caused by partial genetic incompatibility of parental species.
462 Also, influence of transplanted cells is possible, since H C group showed higher post-
463 transplantation survival. Interestingly, comparison between transplanted and control group in MO
464 and 3n recipients showed that transplantation procedure did not interfere with the survival rate.
465 Lower robustness of the ZFxPD hybrids was probably challenged by transplantation. In general,
466 post-transplantional survival should be of larger concern than survival before transplantation. Due
467 to low survival in the H T group, some germline chimeras were lost during ongrowing. This can
468 represent a severe issue when amount of available donor's GSCs is limited. In other words, it is
469 more reasonable to sacrifice lower survival during embryogenesis for the sake of the post-
470 transplantation survival until adulthood.

471 All tested recipients developed gonads capable to support transplanted GSCs including non-
472 sterilized AB recipients. Transplantation success evaluated by the colonization rate was found as
473 only a preliminary indicator because it did not show differences among assessed groups, which
474 were later obvious in adult fish. Although we have used sterilization treatment including complete
475 PGCs ablation, transplantation into non-sterilized AB recipients still resulted in the EGFP positive
476 cells in more than 30% of the transplanted fish (2wpt). This finding clearly shows that colonization
477 rate assessed few days or weeks post-transplantation does not guarantee high success since
478 incidence of chimeric gonads in non-sterile AB T adults was indeed low. Similar pattern was also
479 observed in hybrid recipients showing high colonization rate but low incidence of adult germline
480 chimera. However, the low number of adult germline chimera in H T was attributed to their low
481 survival during the experiment. In other species it was also documented that recipient's gonads with
482 endogenous GCs can be colonized with transplanted cells. However, introduced cells are later
483 losing the pace of the recipient's gametogenesis and are finally outcompeted (Yoshizaki et al.,
484 2016). There are probably two scenarios for transplanted GSCs, which are dependent on the sterility
485 level and determine the success of the transplanted cells. First, when the GSCs are introduced into
486 the PGCs depleted gonads, they are not in competition for the space (germinal niches) and
487 transplantation success can be evaluated early. The second one takes place in PGCs non-depleted
488 recipients (non-sterile control, 3n or H) when introduced cells colonized the gonad, however,

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489 introduced GSCs might be limited until endogenous GCs proceed to affected gametogenesis stage.
490 If endogenous GCs do not experience developmental problems (e.g. arrest in meiosis), the relative
491 proportion of exogenous cells decreases, and they cannot further occupy more testicular niches. We
492 presume that eventual loss of transplanted cells in the competitive environment of the unsterile
493 gonads takes place during more advanced stages of the gonadal development. It would be very
494 informative to identify this period precisely and to find mechanistic and molecular reasons behind
495 this loss of the introduced cells and later use it for interventions to increase transplantation success.

496 Importantly, PGCs depletion and triploidization treatment clearly showed to promote chimeric
497 gonad incidence and development in adults. This finding is striking, especially in triploids since
498 they have quite well-developed testes with complete, although impaired, spermatogenesis with
499 lower GCs numbers. Thus, we suggest that fish GSCs are in the host environment opportunistic and
500 capable of utilizing the gonadal environment once they experience some developmental problems in
501 gametogenesis. Therefore, the extent of gonadal development in the sterile host might not be always
502 crucial for surrogacy success. Incidence of adult AB T male chimeras was about 10% and
503 transplanted cells were capable of establishing spermatogenesis in tiny part of the testes resulting in
504 low production of donor-derived sperm reflected by low germline transmission rate. On the other
505 hand, dissection of all sterilized recipients showed potential of the transplanted cells to establish and
506 expand spermatogenesis on a large scale. Macroscopically, MO T group showed the most
507 developed chimeric testes. 3n T and H T group could also create a considerable area with EGFP
508 positive cells. However, full bilateral or unilateral development of chimeric testes was rare in 3n T
509 an H T groups, when cells were localized rather spatially not occupying full length of the testes.
510 These findings lead us to presumption about competition between exo- and endogenous germ cells,
511 which has further consequences to the proportion of adult germline chimeras and their reproductive
512 performance.

513 Reasons causing low sperm performance in 3n T and H T groups are challenging to interpret.
514 Presence of recipient-derived sperm in HT was confirmed in the testicular lumen by histology;
515 however we roughly estimated less than 1% incidence of abnormally sized spermatozoa. At light
516 and fluorescent microscopy level, we could identify only few individual abnormally sized and
517 EGFP negative spermatozoa among hundreds of spermatozoa showing donor-derived
518 characteristics. Therefore, it is not probable, that overall low sperm performance in HT group was
519 caused by recipient-derived sperm. Partial genetic incompatibility of parental species has clear
520 consequences on endogenous gametogenesis. Although transplanted cells could establish normal
521 spermatogenesis, some molecular alteration conditioning final spermatogenic stages might be
522 present, and in turn, resulted in poor performance of some 3n T and H T chimeric males. Hybrid

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523 testes likely need to cope with increased apoptosis of germ cells, as shown previously due to failure
524 in homologous chromosome pairing (Ponjarat et al., 2019). We can speculate that pathways
525 responsible for removing defective germ cells in the hybrid and triploid testes have further negative
526 consequences on the final spermiogenesis phase, causing low sperm performance in some males.

527 Observed differences in sperm quality and quantity were reflected in both fertilization trials.
528 Importantly, lower sperm performance and fertilization success in semi-artificial tests was less
529 striking during *in vivo* fertilization trials. The potential problem and risks of 3n and especially H
530 recipients are dispersed motility rates. About 25% of spermatozoa from 3n T and H T male
531 chimeras had extremely low velocity (<10 μm/s). Moreover, presence of well performing males as
532 well as bad performing males was evident. Sperm performance in 3n T and H T groups in the
533 combination of sperm quantity and motility indicates the fact that these groups produce on average
534 about 5 to 20 times less motile spermatozoa than the MO T group. Sperm from low performing
535 males can compromise well-performing sperm once they are pooled during collection. It is
536 important for *in vitro* fertilization, always collecting sperm individually and pooling it only when
537 fertilizing the eggs. If the sperm is pooled immediately after sperm collection, it rapidly decreases
538 sperm motility and fertility (Cheng et al. 2021).

539 To our surprise, females from AB T group produced donor-derived eggs giving rise to viable
540 embryos. This finding represents first report on surrogate egg production in zebrafish, because
541 triploid or PGCs depleted zebrafish are all male only (Delomas and Dabrowski, 2018; Slanchev et
542 al., 2005). Proportion of produced donor-derived EGFP positive eggs was rather low (about 20% on
543 average). Therefore, it is clear that non-sterilized ovaries constitute very competitive environment.
544 Interestingly, total proportion of chimeric males and chimeric females in AB T group was 19 and 17
545 individuals, respectively. This finding suggests that the trans-differentiation of male GSCs to female
546 GSCs in the ovarian environment is not decisive for successful intraspecific surrogacy.

547 Interestingly, incidence of hybrid females in control groups was rare and no chimeric hybrid female
548 was observed in this study. Similarly, Wong and Saito (2011) also did not record any hybrid
549 chimeric females after ovarian cell transplantation. In overall, low incidence of hybrid females in
550 danio species was recently reported in cross of zebrafish and spotted danio (*Danio nigrofasciatus*)
551 (Endoh et al., 2020). It is evident that female hybrids are likely to experience more severe
552 gametogenesis alteration than males. Therefore, it is reasonable to expect that poorly developed
553 ovaries in hybrid females cannot provide proper environment for transplanted cells and production
554 of donor-derived zebrafish eggs can be achieved only through non-sterilized female recipients as it
555 is for the first time described in the present study.

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556 Predictable and stable gonadal phenotype development was identified as a concern in hybrid male
557 recipients when three distinct phenotypes were observed. Previous study utilizing ZF x PD hybrid
558 recipients showed lack of spermatozoa in the hybrid testes (Wong and Saito, 2011) while our study
559 confirmed that hybrid GCs are capable to proceed throughout entire spermatogenesis resulting in
560 the production of extremely abnormal spermatozoa. On the other hand, gonadal phenotypes in PGC
561 ablated fish and triploids were consistent. Fish produced by *dnd* gene targeting developed empty
562 gonads composed of the solely somatic cells. Further female or male fate differentiation of the
563 sterile gonad is species specific. Germ cell less zebrafish and medaka have been shown to develop
564 into phenotypical males only (Kurokawa et al., 2007; Slanchev et al., 2005; Tzung et al., 2015).
565 Otherwise, several species have germ cell independent sex differentiation such as loach (Fujimoto
566 et al., 2010), goldfish (Goto et al., 2012), trout (Yoshizaki et al., 2016), Atlantic salmon (Wargelius
567 et al., 2016) or rosy bitterling (Octavera and Yoshizaki, 2018). Zebrafish (Franěk et al., 2019) and
568 rosy bitterling surrogates (Octavera and Yoshizaki, 2018) showed only male development after
569 germ cell transplantation, meaning that introduced additional GSCs are not capable of rescuing
570 female fate of the gonad. Majority of induced triploid surrogates can differentiate into both sexes,
571 including salmonids (Lee et al., 2013; Okutsu et al., 2007), medaka (Seki et al., 2017), grass puffer
572 (*Takifugu niphobles*) (Hamasaki et al., 2017), or Nibe croaker (*Nibea mitsukurii*) (Yoshikawa et al.,
573 2017). Hybrids in this study showed gonadal phenotypes with developed testes, altered ovaries, and
574 empty gonads resembling germ cell-depleted MO phenotype.

575 Strain specific differences or different age of the assessed fish can probably play an important role
576 in gonadal phenotype of hybrids. Similar variance in testicular phenotype was described in
577 mackerel hybrid of *Scomber australasicus* x *S. japonicus,* when part of the hybrid males could
578 proceed through spermatogenesis while the second phenotype was germ cell less (Kawamura et al.,
579 2020). Consequently, semi fertility of the hybrid could interfere with the fertilization since endo-
580 and exogenous gametes are in the competition for the ova. Therefore, suitability of hybrids for
581 surrogacy needs to be verified thoroughly in particular species. The hybridization itself is very
582 convenient tool for recipient production for surrogacy because it requires only fertilization without
583 further manipulation. However, since mechanisms causing occurrence of sterile and GCs producing
584 gonadal phenotypes are unknown it should be evaluated with cautions.

585 **CONCLUSION**

586 GSCs manipulation is potent biotechnology to ameliorate breeding of aquaculture species and
587 preserve valuable genetic resources in environmentally relevant or even endangered species. This
588 study aimed to identify best sterilization treatment - essential factor influencing the surrogacy
589 success rate. The presented study assessed various sterilization treatments in fish for surrogates

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590 preparation and their influence on gonadal development and reproductive output in germline
591 chimeras. Of the utmost importance, germ cell-free gonads were identified as the best environment
592 for transplanted cells yielding the highest transplantation success and gonadal development.
593 Importantly, reproductive performance of males including quantity and motility parameters and
594 fertilization rate clearly favors germ cell depleted recipients. The use of triploid and hybrid males
595 from the point of view of the production of sufficient quantity and quality sperm proves to be risky
596 to achieve stable results. Moreover, only germ cell depleted recipient retained reproductive
597 characteristics of the donor strain. Presented findings should help in decision on what type of
598 sterilization should be used prior to transplantation and surrogacy induction, especially in non-
599 model fish species.

600 The overall suitability and versatility of the zebrafish surrogate model can be utilized to provide
601 deeper insights into the mechanism of GCs behaviour in the recipient's gonads and dissect specific
602 factors influencing promotion of the exogenous GCs development. Our interest should also be
603 directed to the molecular aspects of surrogacy. Nowadays, GSCs manipulations and surrogacy were
604 performed in wide range of species. However, we know only little about the lifetime or
605 transgenerational consequences of gametes produced from surrogate parents and how they can
606 possibly influence resulting progeny and its performance.

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AUTHOR CONTRIBUTIONS

Conceptualization: RF; Data curation and Formal Analysis: YC; Funding acquisition: VK, OL, IŠ, MP; Investigation: RF, YC, MF, XX, MAS, VK, OL, IŠ, MP; Methodology: RF, YC, IŠ; Project administration: RF; Resources: RF, YC, MF, OL, IŠ, MP; Supervision: RF; Validation: RF, YC; Visualization: RF, YC; Writing – original draft: RF; Writing – review & editing: all authors contributed.

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809 **FIGURE CAPTIONS**

810 **Figure 1. Production of experimental and control groups.**

811 **Figure 2. Overall survival after different sterilization treatments.** Survival from fertilization to
812 swim-up stage (A) and survival from transplantation to 6 months of age (B). (mean ± S.D.).
813 Different letter denotes statistically significant difference between groups at each developmental
814 stage ($P < 0.05$, one-way analysis of variance (ANOVA) followed by an LSD test for post hoc
815 multiple comparisons).

816 **Figure 3. Colonization and cell localization after vas::EGFP GSCs transplantation.** A)
817 Comparison of transplantation success evaluated in all recipient groups at 2 wpt. No significant
818 differences were detected among groups. B1) Localization patterns of EGFP positive cells in the
819 body cavity of the recipients. Different superscripts, i.e. a, b, and c, indicate statistical differences
820 between location for the same group whereas different superscripts, i.e. x and y, indicate statistical
821 differences between the group at the same location ($P < 0.05$, one-way analysis of variance
822 (ANOVA) followed by an LSD test for post hoc multiple comparisons). B2, B3) Evaluation of
823 EGFP positive cells localization in the body cavity. B2) vas:EGFP control individual. B3) MO T
824 individual. Cells in the white dashed rectangle are example of medial-posterior colonization.

825 **Figure 4. Different sterilization treatments affect gonadal development. A) Morpholino**
826 **treated male**. Both gonads are developed as empty testes (A1 – black arrowheads) lacking germ
827 cells only from stromal cells (A2 – turquoise arrowhead) forming empty lumen-like structure (A2 –
828 grey arrowheads). Gonads are macroscopically thin (A3 – black arrowheads) and without EGFP
829 expression (A4). **B) Triploid male** with developed testis (B1 – black arrowheads). Only few
830 individual spermatozoa are present in lumens (B2 – blue arrowheads), most of the lumens are sperm
831 free and undeveloped (B2 – grey arrowhead). Meiotic germ cell arrested pachytene (B2 – brown
832 arrowhead) and spermatids (B2 – orange arrowhead) are frequently observed as well as early-stage
833 germ cells (B2 – green arrowheads). Gonads are macroscopically well developed, but without
834 typical white colour (B3), lacking EGFP expression (B4). **C) Hybrid male with type I. gonads.**
835 Testes are well developed (C1 - black arrowheads). Lumens are large with several dozens of
836 spermatozoa with various head size (C2 – blue arrowhead), several empty and small lumens are
837 present as well (C2 – grey arrowheads). Spermatocysts have clear structure and are mostly filled
838 with meiotic germ cells with regular morphology (C2 – brown arrowhead) and with meiotic germ
839 cells showing aberrant nuclei morphology (C2 – red arrowhead). Early-stage germ cells are
840 frequently observed (C2 – green arrowheads). Gonads are macroscopically well developed (C3 –
841 black arrowheads) and lacking EGFP signal (C4). **D) Hybrid male with type II. gonads – fully**

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842 **sterile.** Testes are undeveloped (D1 – black arrowheads) with similar morphology to MO treated
843 male. Only stromal cells are present (D2 – turquoise arrowhead) forming empty compartments
844 lacking spermatozoa (D2 – grey arrowheads). Gonads are macroscopically undeveloped and thin
845 (D3 – black arrowheads) and lacking EGFP signal (D4). **E) Hybrid male type III. gonads.** One
846 testis is poorly developed with similar structure to type II gonads, but few spermatozoa are present
847 (E2 – blue arrowhead). Second testis is over developed with same structure and macroscopical
848 appearance as in hybrid gonad type I (see C2 and C3) EGFP signal is not detected (D4). **F) Hybrid**
849 **female.** Both ovaries are large with several empty inner compartments (F1 – grey arrowheads).
850 Ovarian cells are forming typical lamella-like structure (F2 – black arrowheads), only early meiotic
851 oocytes (F2 – yellow arrowheads) and early-stage germ cells are present (F2 – green arrowhead).
852 Macroscopic structure is similar to hybrid male type I. gonads (F3 – black arrowheads), no EGFP
853 signal is detected (F4). **Control recipient AB male (G) and donor vas::EGFP male (H).** Testes
854 are well developed, lumens are densely filled with spermatozoa (G2, H2 – blue arrowheads),
855 several spermatocysts with meiotic spermatocytes are detected on the sections (G2, H2 – yellow
856 arrowheads) as well as early-stage germ cells (G2, H2 – green arrowheads). Gonads are
857 macroscopically well developed with typical white compartments indicating presence of sperm in
858 large amount (H3, G3 – black arrowheads). Strong EGFP signal is detected in vas::EGFP testis (H4
859 – white arrowheads).

860 **Figure 5. Abnormal spermatozoa morphology in ZF x PD hybrids.** A) Spermatozoa appearance
861 in AB control. A1) Light microscopy caption. A2) SEM caption showing normal spermatozoa
862 morphology. A3) Traversal testicular section. B) Spermatozoa appearance in hybrid with gonadal
863 type I. B1, B2) Light microscopy showing abnormally large and shaped heads of the spermatozoa,
864 both flagellums are very short. B3) Transversal testicular section. Note the size of the cells in the
865 lumen (black arrowhead) compared to size of cells in A3. B4) SEM caption of abnormal
866 spermatozoa, with double head and two flagellums. B5) Percentage occurrence of different gonadal
867 phenotypes (described in Fig. 4) in control hybrid group. Different letter denotes statistically
868 significant difference ($P < 0.05$, one-way analysis of variance (ANOVA) followed by an LSD test
869 for post hoc multiple comparisons).

870 **Figure 6. Adult germline chimeras.** A) Overall % proportion of confirmed germline chimeras (in
871 case of AB T also chimeric females were detected) (producing EGFP positive gametes) from
872 surviving adults. B) GSI in experimental males and their respective control groups. C) Lateral
873 patterns of testicular development in adult male chimeras. D) Ventral view on dissected germline
874 chimeric male and donor control showing lateral and spatial patterns of testicular development.

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875 Values with different letters are significantly different ($P < 0.05$, one-way analysis of variance
876 (ANOVA) followed by an LSD test for post hoc multiple comparisons).

877 **Figure 7. Distribution of transplanted germ cells from vas::EGFP donors in the recipients'**
878 **gonads. A)** Morpholino treated male with exogenous spermatogenesis with EGFP signal occupying
879 whole testis. **B)** Triploid male with most of the testes occupied by vas::EGFP positive
880 spermatogenesis. B1-B3 red dashed line indicates spermatocysts with germ cells lacking EGFP
881 signal. **C)** Hybrid male recipient with exogenous spermatogenesis occupying whole testis. **D, E)**
882 AB transplanted males with only partial colonization of recipient testis by exogenous
883 spermatogenesis. Note the red dashed lines depicting part of the testis with endogenous
884 spermatogenesis lacking EGFP signal especially in E1-E3. **F)** vas::EGFP control specimen with
885 well-organized spermatocysts and EGFP expression through whole section. **G)** AB control male
886 with complete spermatogenesis and lumens filled with spermatozoa. **H)** Morpholino treated male
887 lacking germ cells with developed empty lumens (white arrowheads). **I)** Triploid male specimen
888 with developed testis with few individual spermatozoa (yellow arrowheads), poorly developed
889 lumen (white arrowhead) and meiotic germ cells with aberrant morphology (red arrowhead). **J)**
890 Hybrid male with developed testis and spermatogenesis. with few individual spermatozoa (yellow
891 arrowheads), poorly developed lumen (white arrowhead) and meiotic germ cells with aberrant
892 morphology (red arrowhead). **K)** Hybrid male with undeveloped testis lacking germ cells only with
893 empty lumens (white arrowheads). White asterisks indicate lumen filled with spermatozoa. Note
894 that EGFP signal intensity is strongest in the early-stage germ cells and decreasing by
895 differentiation towards spermatozoa.

896 **Figure 8. Gonadal development in germline chimeras.** A) MO T chimeric male. A1-2)
897 Colonized testis is apparent in the opened body cavity, while the second testis is very thin.
898 Histological sections are showing colonized testis with filled lumens (black asterisks) (A4), while
899 the second testis has typical structure of germ cell free gonad (A5). B) 3n T chimeric male. Both left
900 and right testis are colonized in the medial/anterior part (B1-2). Histological sections are showing
901 lumens filled with spermatozoa suggesting presence of spermatogenesis from donor-derived cells.
902 C) H T chimeric male. C1-2) Developed testis are large and expressing EGFP signal. However,
903 histological sections (C3-4) shows that many lumens are empty (red asterisk) suggesting that the
904 encompassing germ cells are not undergoing proper gametogenesis D) AB T chimeric males with
905 testis colonized in the very anterior part (D2). Testes are well filled with spermatozoa (D3-4).

906 **Figure 9. Female germline chimeras in AB T group.** A) Proportion of produced recipient-derived
907 and donor-derived oocytes. B) Survival rate of recipient-derived and donor-derived oocytes. C)
908 Example of donor derived oocyte with strong EGFP signal (white arrowhead) and recipient-derived

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909 oocytes (black arrowhead) produced by AB T female. D) Overall incidence of females in AB T and
910 AB C group. Asterisk stands for statistically significant difference (T-test, $^{**}P < 0.01$), while "ns"
911 stand for no statistical difference (T-test, $P > 0.05$).

912 **Figure 10. Detection of chimeric gonads in non-sterilized AB females. A)** Control female from
913 AB strain, only DAPI signal is detected on the whole ovary cryosections (A1-A6), no EGFP signal
914 is detected in opened body cavity (A7-A10). **B)** Control female from vas::EGFP donor strain, DAPI
915 as well EGFP signal is detected on the whole section of the ovaries. Early stage (small) oocytes
916 have strong EGFP signal which is apparent on cryosections (B1-B6) as well on view on the opened
917 body cavity (B7-B10). **C)** AB female germline chimera, ovarian germ cells derived from
918 transplanted spermatogonia are occupying considerable part of the ovary indicated by red dashed
919 line (C2-C3, C8 and C10). Magnified view on cryosection (C6) shows that endogenous oocytes
920 positive only for DAPI signal (small oocytes indicated by blue arrow, advanced oocytes by blue
921 asterisk) are developing in close contact with exogenous EGFP positive oocytes (small oocytes
922 indicated by white arrow, advanced oocytes by white asterisk). View on opened body cavity shows
923 anterior localization of donor derived oocytes indicated by red dashed line (C8 and C10). Images
924 with 1,2 and 3 numerals were stitched from XY stack and images with 4, 5 and 6 numerals are
925 magnified captions respectively. Images with 9 and 10 numerals are magnified captions of images
926 with 7 and 8 numerals respectively.

927 **Figure 11. Reproductive performance of chimeric males with donor and recipient controls.**
928 Motility rate (%) (a), curvilinear velocity (µm/s) (b), straight-line velocity (µm/s) (c), percentage of
929 sperm motility from total motility of spermatozoa evaluated at 15 s of PSA: rapid motility (>100
930 µm/s), medium motility (46 to 100 µm/s), slow motility (10 to 45 µm/s) and static spermatozoa
931 (<10 µm/s) of tested groups at 15 s post activation. Total sperm volume collected from males (e),
932 sperm concentration (x 10^6/µl), total sperm number (x 10^6) and total motile sperm number (x 10^6).
933 Values with a different lowercase letter are significantly different ($P < 0.05$, one-way analysis of
934 variance (ANOVA) followed by an LSD test for post hoc multiple comparisons).

935 **Figure 12. Reproductive success of chimeric males.** A) Spawning success of males from
936 experimental and control groups including sterile controls. Successful spawning of the given pair
937 was recorded when 20 and more eggs were observed. B) Number of oviposited eggs during semi-
938 artificial fertilization. Survival rates after semi-artificial (C) and in vitro fertilization tests (D) (mean
939 ± S.D.). Values with different letters are significantly different among all groups (B) and within
940 each development stage (C and D) ($P < 0.05$, one-way analysis of variance (ANOVA) followed by
941 an LSD test for post hoc multiple comparisons).

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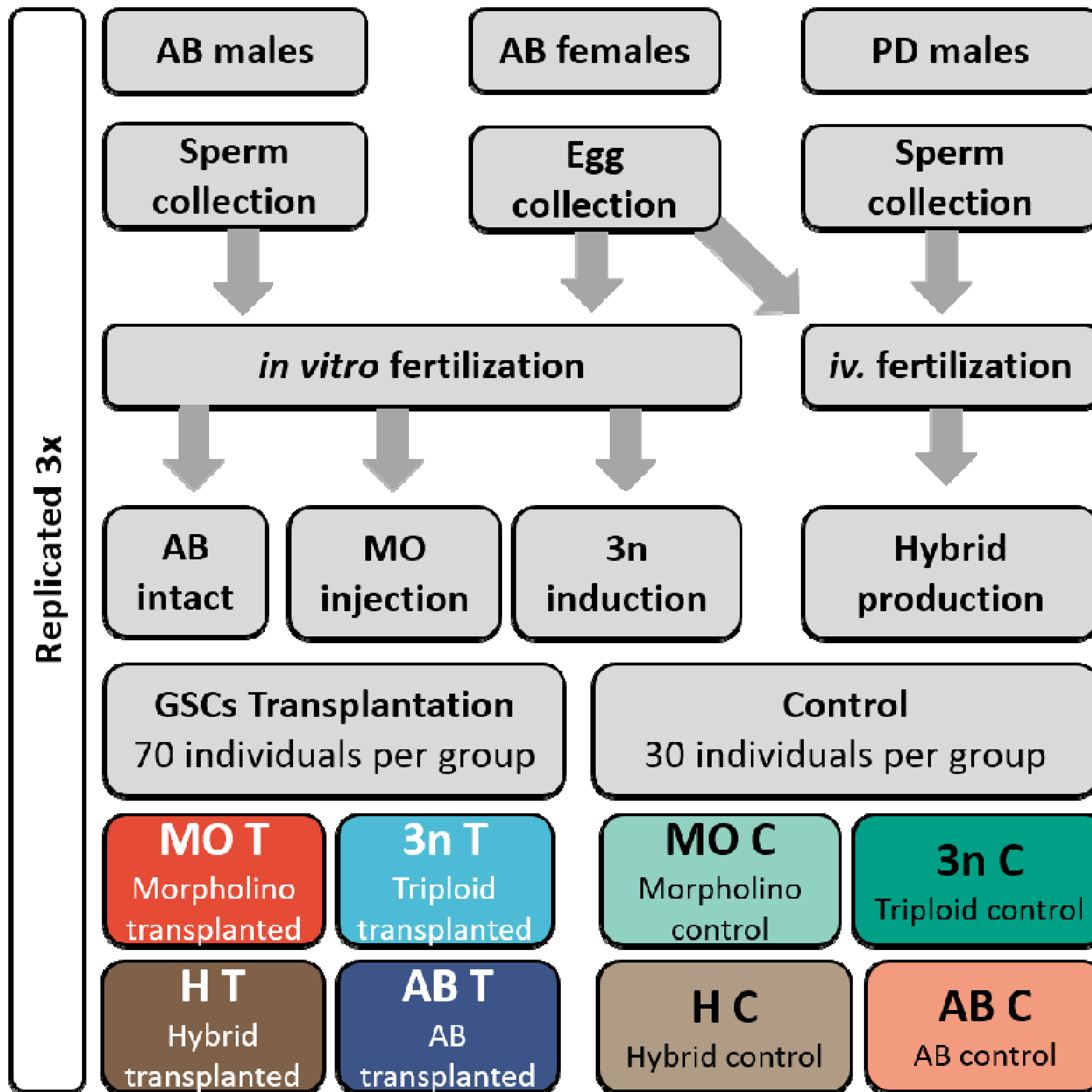
943 Figure 1



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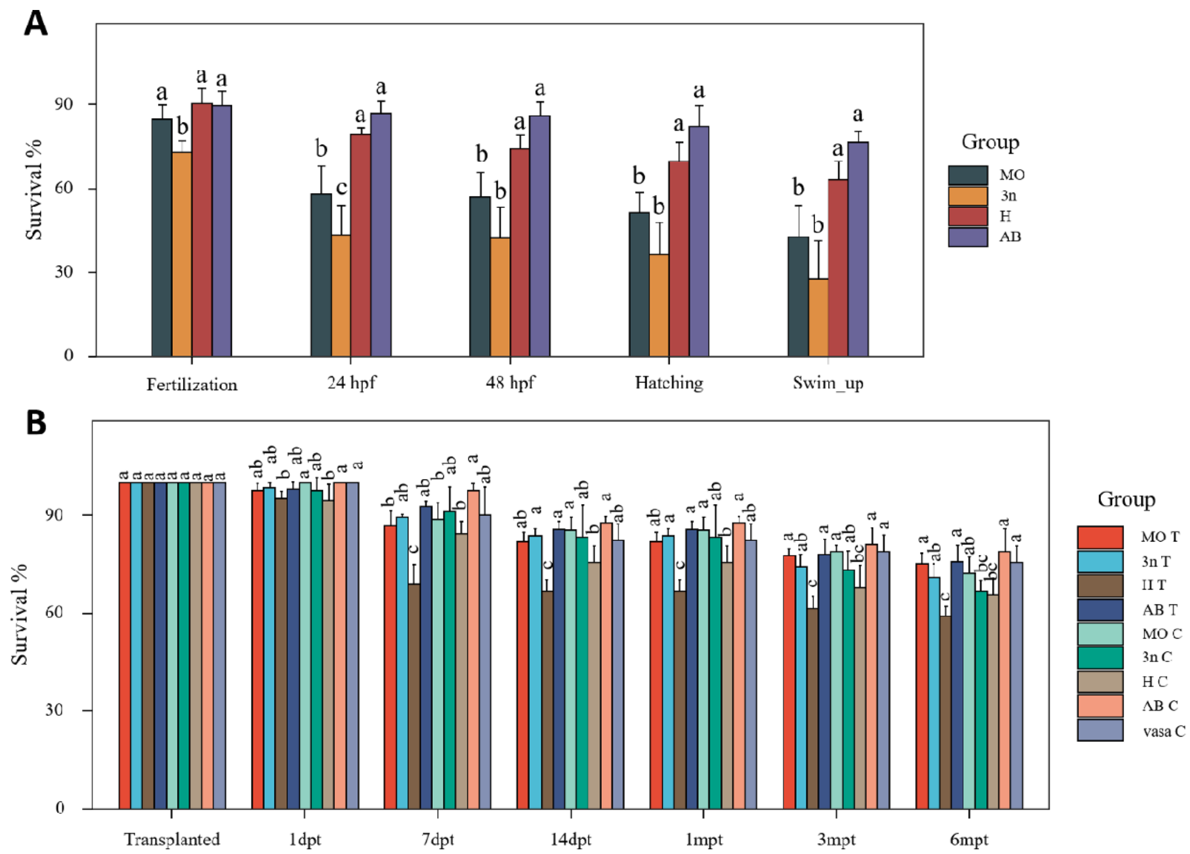
946 Figure 2



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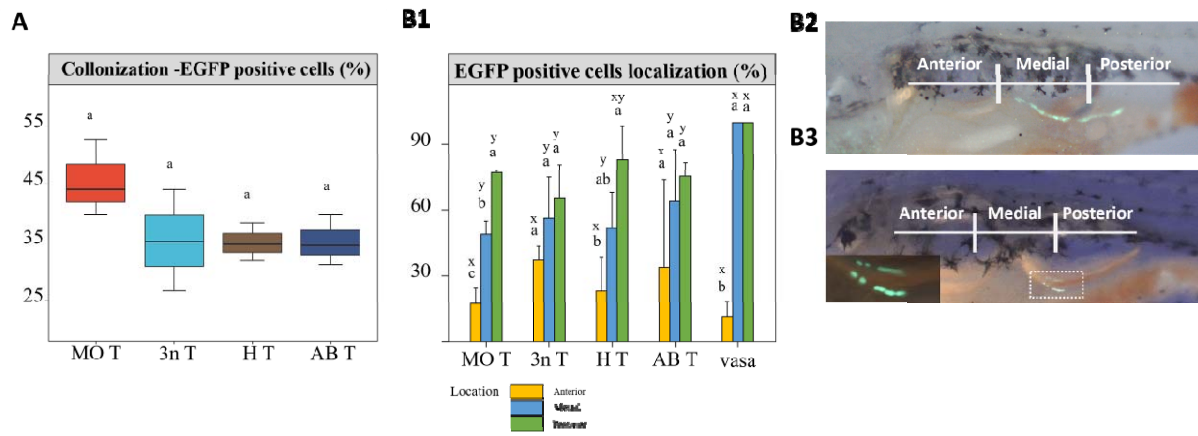
949 Figure 3



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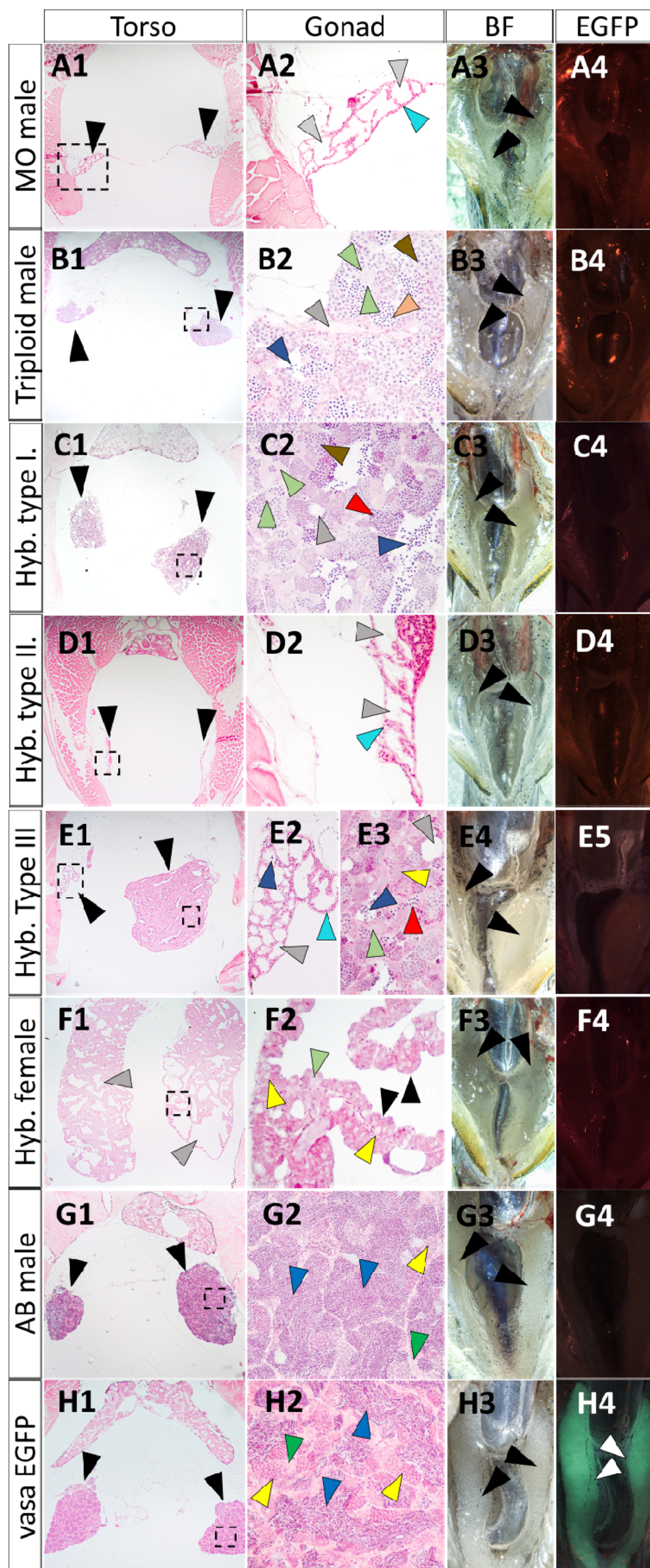
952 Figure 4



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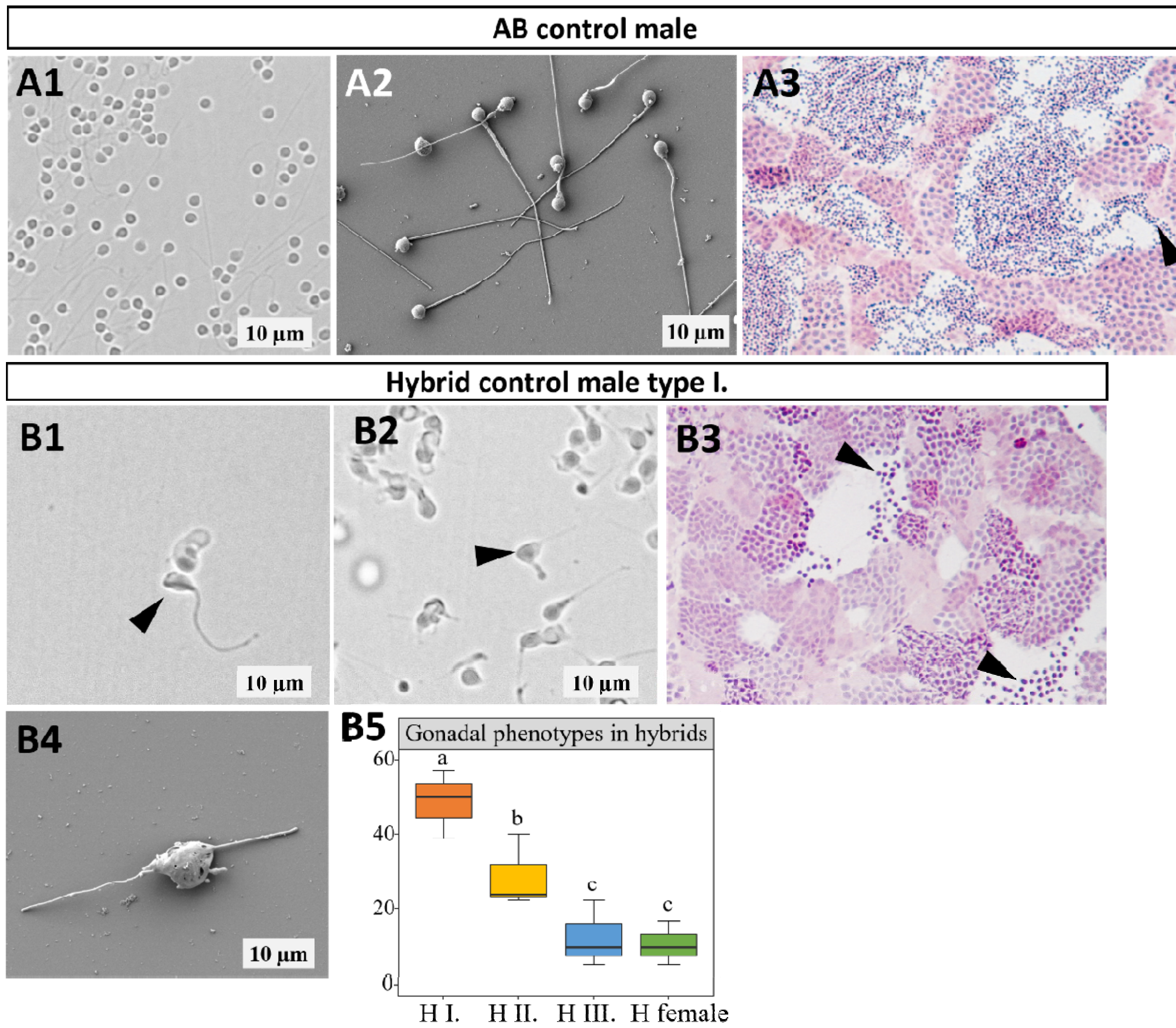
954 Figure 5



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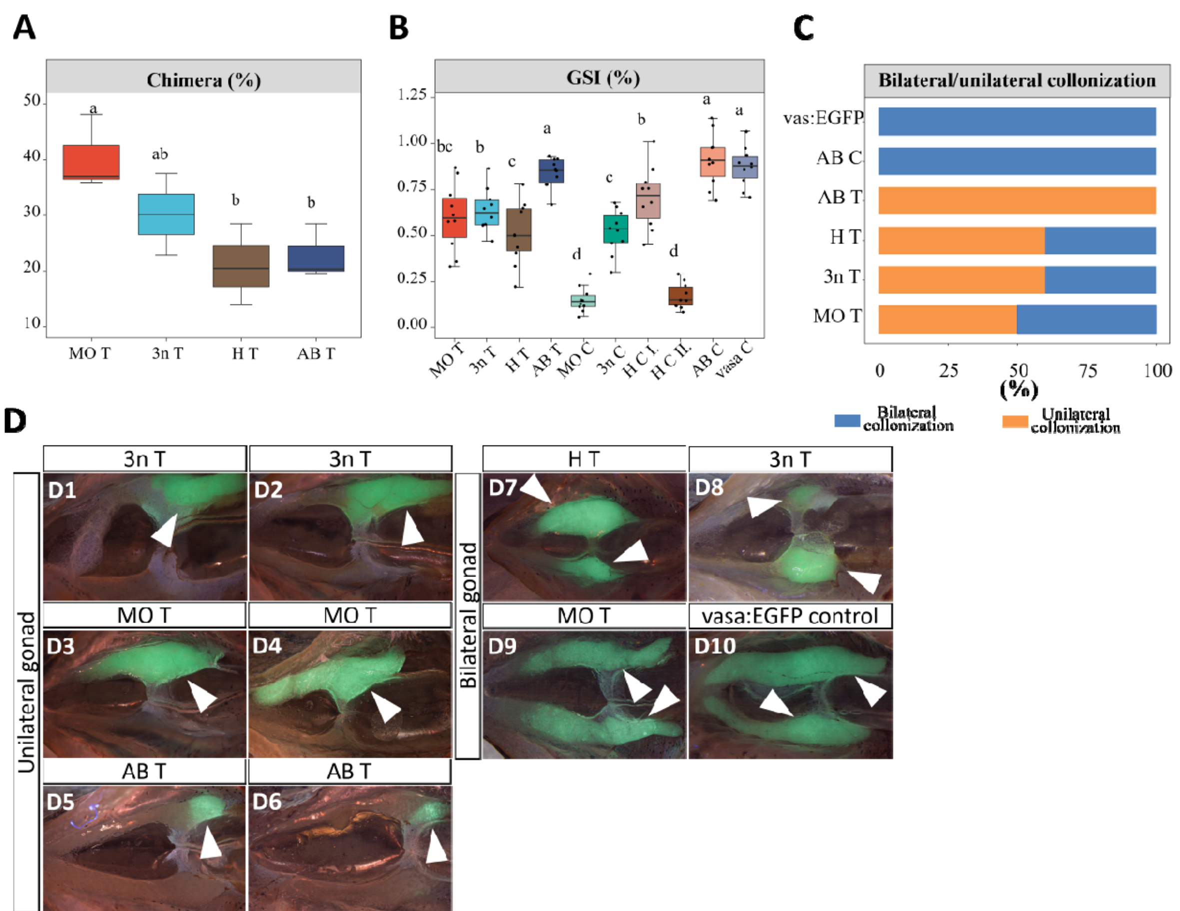
957 Figure 6



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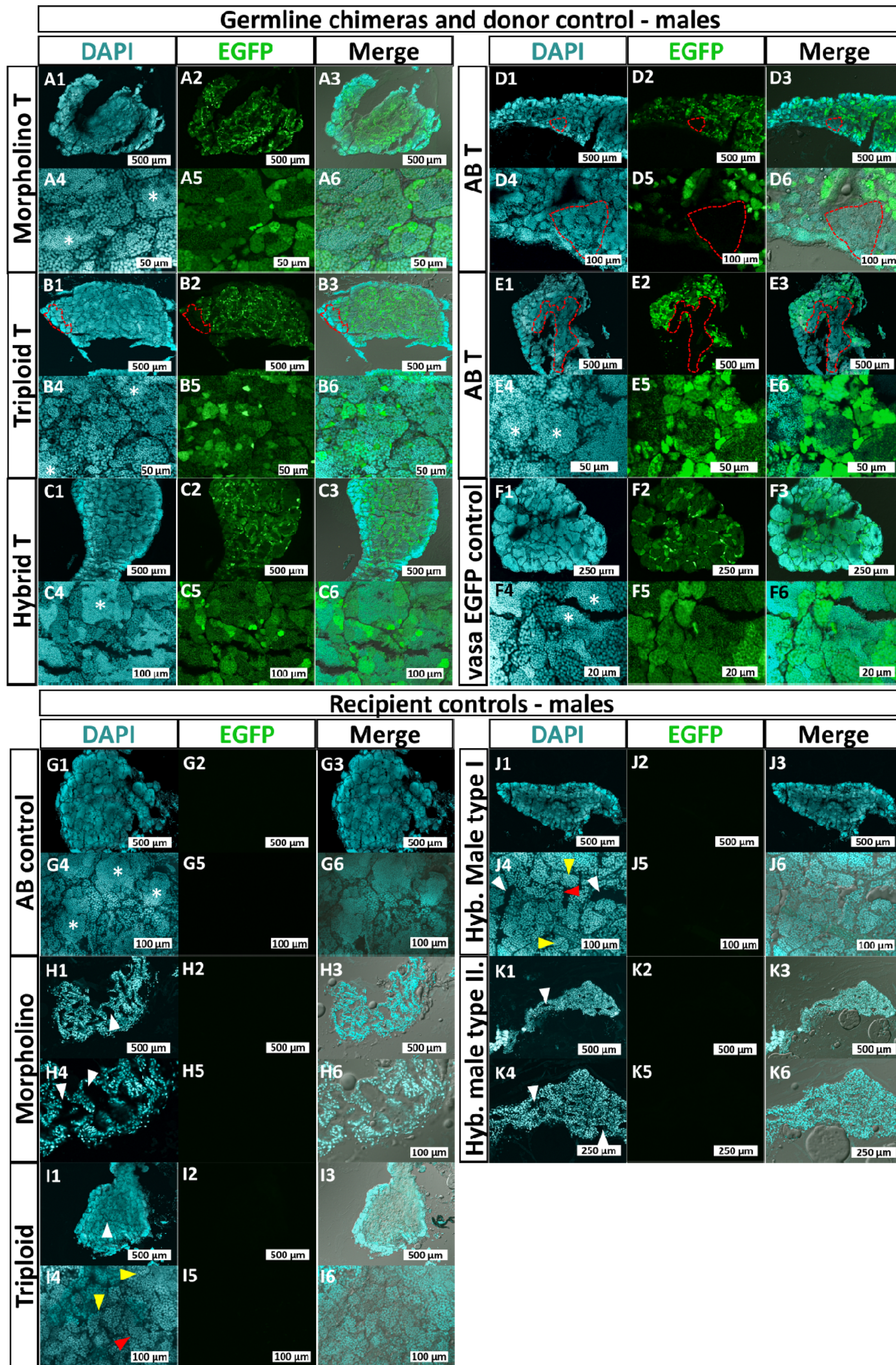
960 Figure 7



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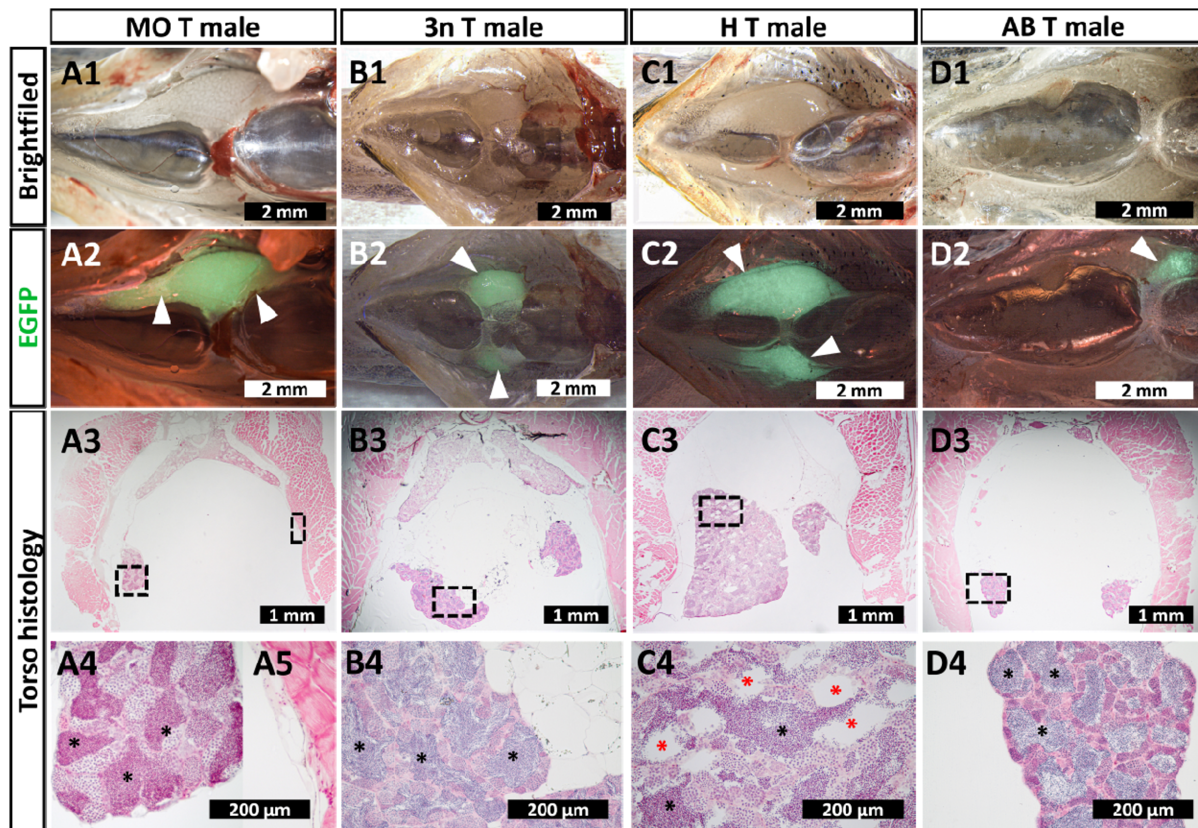
962 Figure 8



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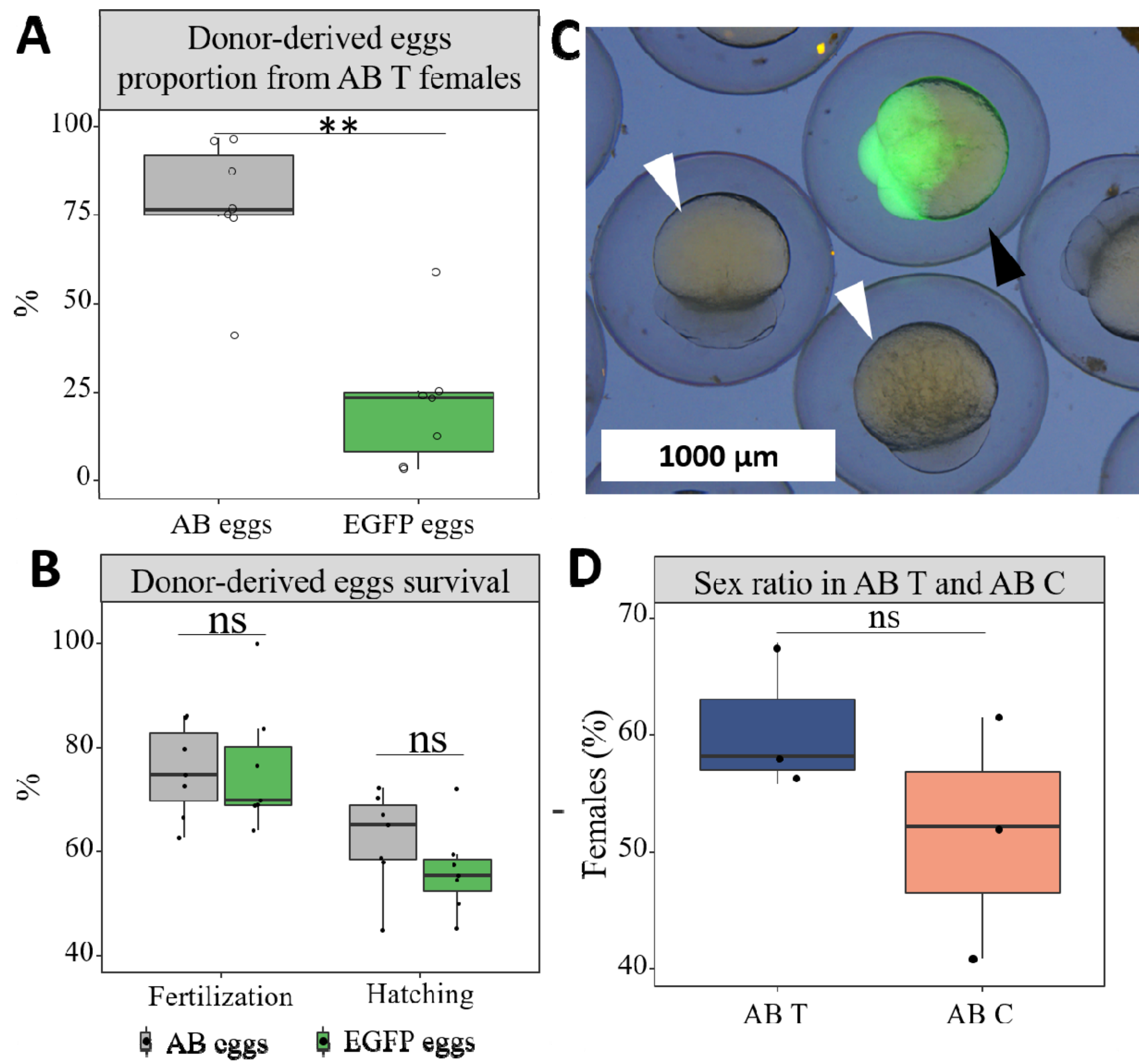
965 Figure 9



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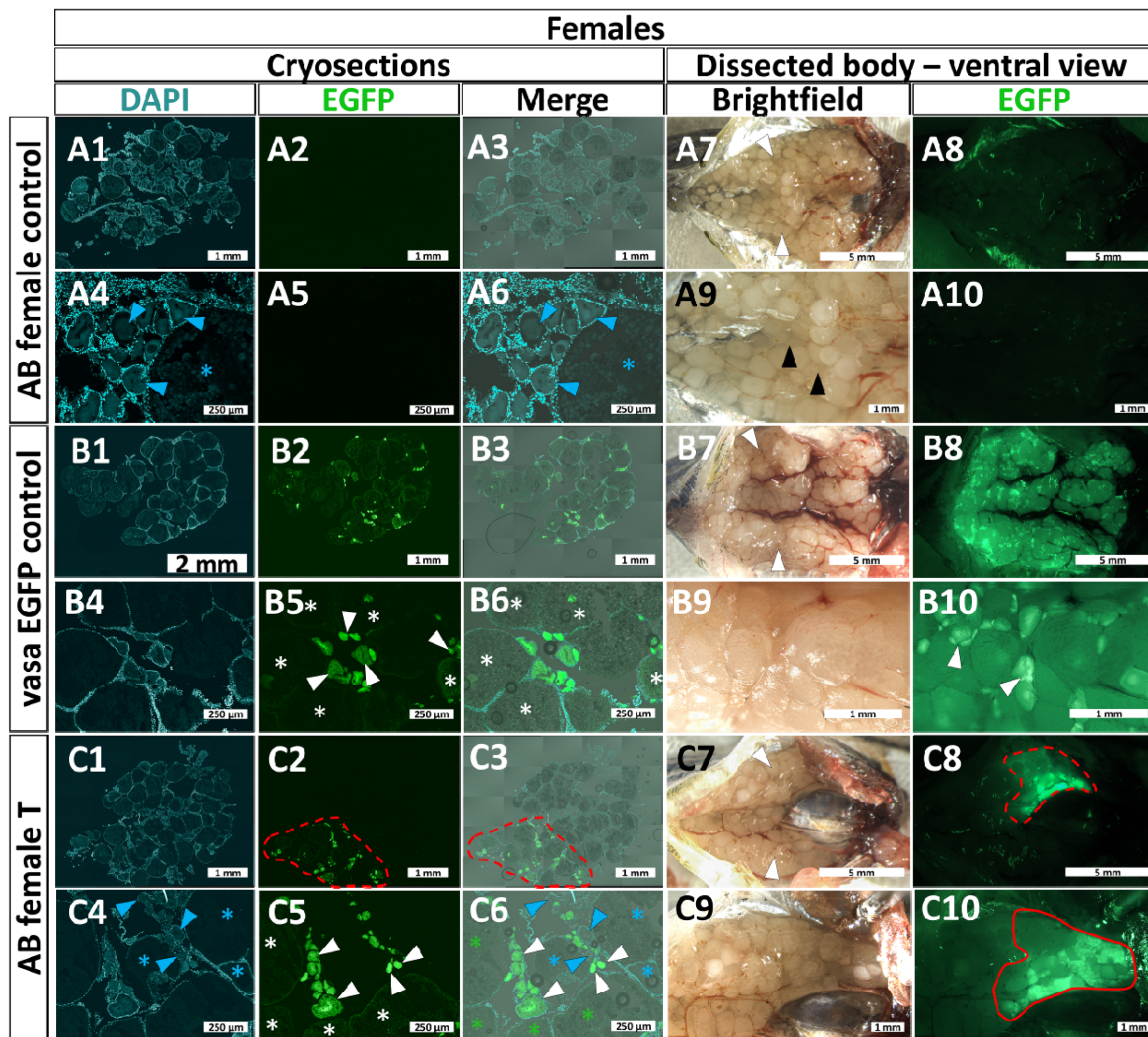
968 Figure 10



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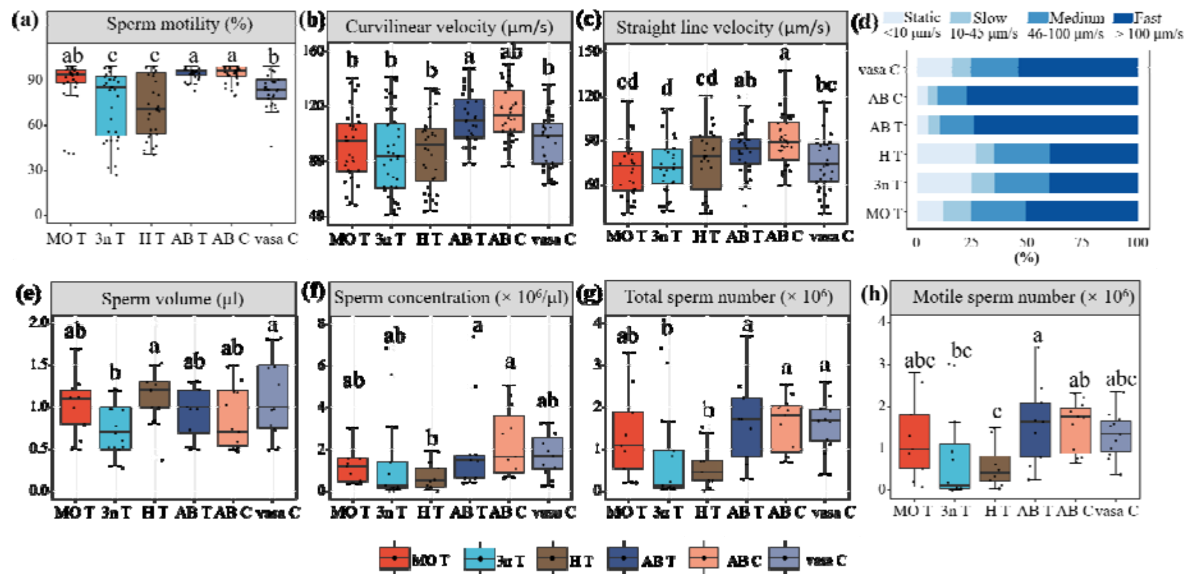
971 Figure 11



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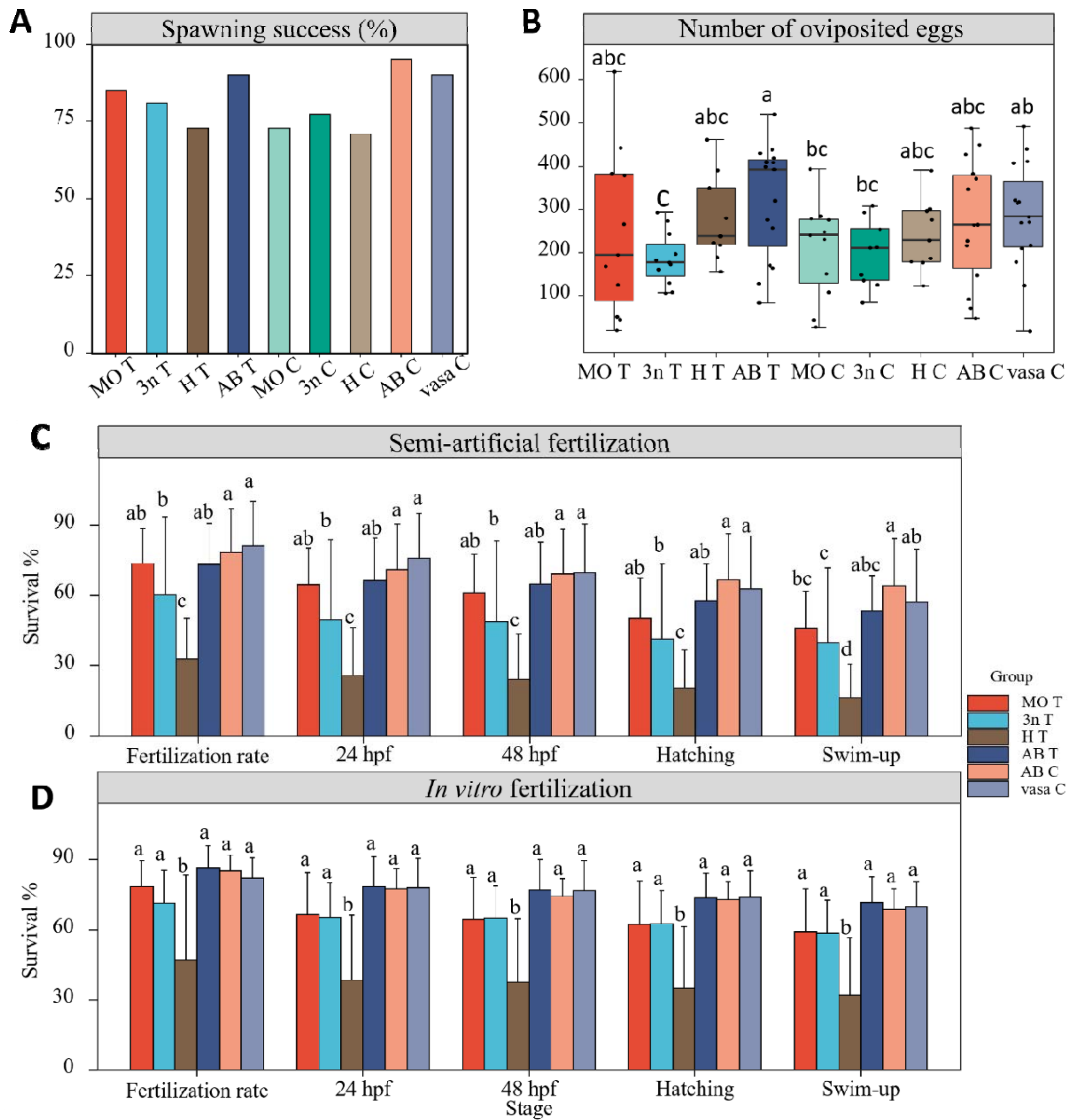
974 Figure 12



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