Resumption of donor-origin spermatogenesis in senescent goldfish *Carassius auratus* (Linnaeus, 1758) following spermatogonial cell therapy

Sullip K Majhi [Corresp., 1] , **Mog Chowdhury** [1] , **Santosh Kumar** [1] , **Rajeev K Singh** [1] , **Vindhya Mohindra** [1] , **Kuldeep K Lal** [1]

[1] National Bureau of Fish Genetic Resources, Lucknow, India

Corresponding Author: Sullip K Majhi
Email address: skmajhi@nbfgr.res.in

Stem cell research has come into prominence because of its applications in assisted reproductive technology and the treatment of deadly diseases. In teleost fishes, spermatogonial stem cells have been effectively used to produce surrogate gametes and progeny through germ cell transplantation technique. The present study is the first report of an innovative application of stem cell therapy in fish species for revitalising the reproductive competence of senescent individuals. Senescent male goldfish, *Carassius auratus,* approximately 10 years of age were procured from a fish-breeding farm and were reared locally in the lab for an additional two years. The senescence of the individuals was then evaluated and confirmed using histological analysis, gonadal index assessment, and germ-cell specific *vasa* gene expression. Analyses revealed absence of spermatogonial cells and other germ cells in the testes of the senescent fish (n = 5). Spermatogonial cells from sexually immature *C. auratus* male donor were isolated using discontinuous percoll gradients, labelled with the fluorescent dye PKH-26, and transplanted into the gonads of senescent *C. auratus* males through urogenital papilla. Six months after the transplant, spermatozoa were collected through applying gentle manual pressure on the abdomen and were observed under a microscope. All *C. auratus* males with the transplant had produced spermatozoa from the transplanted cells. This was confirmed by the retention of PKH-26 in the spermatozoa and diagnostic SSR locus. Gravid *C. auratus* females were artificially inseminated with the spermatozoa of those senescent males and natural spawning was allowed. As a result viable progeny were produced. These observation suggests that the reproductive competence of senescent male fishes can be revitalised through spermatogonial stem cell therapy to produce functional gametes.

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3 **Short title**: Resumption of spermatogenesis in senescent fish

4 **Sullip Kumar Majhi*, Labrechai Mog Chowdhury, Santosh Kumar, Rajeev Kumar Singh,**
5 **Vindhya Mohindra and Kuldeep Kumar Lal**

6   **Abstract**

7   Stem cell research has come into prominence because of its applications in assisted reproductive

8   technology and the treatment of deadly diseases. In teleost fishes, spermatogonial stem cells have

9   been effectively used to produce surrogate gametes and progeny through germ cell

10   transplantation technique. The present study is the first report of an innovative application of

11   stem cell therapy in fish species for revitalising the reproductive competence of senescent

12   individuals. Senescent male goldfish, *Carassius auratus,* approximately 10 years of age were

13   procured from a fish-breeding farm and were reared locally in the lab for an additional two years.

14   The senescence of the individuals was then evaluated and confirmed using histological analysis,

15   gonadal index assessment, and germ-cell specific *vasa* gene expression. Analyses revealed

16   absence of spermatogonial cells and other germ cells in the testes of the senescent fish (n = 5).

17   Spermatogonial cells from sexually immature *C. auratus* male donor were isolated using

18   discontinuous percoll gradients, labelled with the fluorescent dye PKH-26, and transplanted into

19   the gonads of senescent *C. auratus* males through urogenital papilla. Six months after the

20   transplant, spermatozoa were collected through applying gentle manual pressure on the abdomen

21   and were observed under a microscope. All *C. auratus* males with the transplant had produced

22   spermatozoa from the transplanted cells. This was confirmed by the retention of PKH-26 in the

23   spermatozoa and diagnostic SSR locus. Gravid *C. auratus* females were artificially inseminated

24   with the spermatozoa of those senescent males and natural spawning was allowed. As a result

25   viable progeny were produced. These observation suggests that the reproductive competence of

26   senescent male fishes can be revitalised through spermatogonial stem cell therapy to produce

27   functional gametes.

28   **Key words: Senescence; Reproduction; Spermatogenesis; Aquaculture; Goldfish**

Introduction

31   Stem cells are described as undifferentiated cells that have the potential to renew

32   themselves and differentiate into a single cell type or multiple specialised cell types as they can

33   undergo numerous cycles of cell division while maintaining their undifferentiated state

34   (Takeuchi et al. 2004). Currently, stem cell research is one of the upcoming areas of research in

35   science. Since their discovery and the subsequent establishment of protocols for their successful

36   isolation and culture, researchers have used them for various purposes (Zhu et al. 2010),

37   including treatment of chronic diseases and reproductive ailments (Nazari et al. 2015). Stem cell

38   therapy has also shown the potential to revitalise or repair malfunctioning organs by using

39   unscathed donor cells, which can take repair the damaged organ through their potential of

40   differentiation to make the organ function normally and provide animals with an extended and

41   improved life (Min-Wen et al. 2016). In humans, this has opened a new avenue for regenerative

42   medical approaches and has become a viable treatment option in healing chronically damaged

43   organs (Zhao et al. 2014). However, in teleost fish, the use of stem cells for therapeutic purposes

44   has not been explored so far; nevertheless, this therapy has the potential to revitalise

45   malfunctioning reproductive organs of both sexually incompetent or senescent fishes that have a

46   high commercial value or have become critically endangered. Currently, in fish, stem cells are

47   widely used for surrogate brood stock development through germ-cell transplantation (Majhi et

48   al. 2009; Lacerda et al. 2015; Majhi et al. 2014). In this case, the donor cells obtained from

49   young hatchlings or adults are transplanted into the recipient fish at various developmental

50   stages, and on maturation, the recipient fish produce donor-derived gametes (Majhi et al. 2014;

51   Takeuchi et al. 2003). In this study, we demonstrate successful restoration of spermatogenesis in

52   senescent *C. auratus* through intragonadal stem cell therapy for the first time. The simple

53   approach described in this study ultimately leads to generation of viable functional spermatozoa

54   in senescent *C. auratus* males, which can fertilise eggs derived from young *C. auratus* females

55   and produce healthy progeny. Thus, this technique extends the reproductive lifespan of the fish

56   beyond the pubertal phase and effectively generates viable progeny.

57   **Materials and Methods**

58   **Ethics statement**

59   This study was approved by the Animal Ethics Committee of National Bureau of Fish

60   Genetic Resources (#G/CPCSEA/IAEC/2015/2).

61   **Animals, age, and rearing protocols**

62   Male goldfish (*C. auratus*; n = 60; mean body weight ± standard deviation [SD] of 230 ±

63   20.5 g) were procured from Maharashtra, India, which were reported to be approximately 10

64   years old by the providers. The age of the fish was evaluated and confirmed by counting the

65   growth rings located at scale (the growth rings from the fish raised in the farm with known age

66   was used as reference; Supplementary Fig. 1). The fish were stocked in 500 L tanks after

67   procuring them from the farm at the density of 5.0 kg of fish per m^3 and reared in flow-through

68   fresh water system (temperature: 25 °C ± 2 °C; dissolved oxygen: 5.3–6.1 ppm; pH: 7.5–8.0;

69   hardness: 40–45 ppm) under a constant light cycle (12 h light and 12 h dark). The fish were

70   reared for an additional two years before confirmation of their senility through histological

71   analysis and germ-cell-specific *vasa* gene expression studies. The prepubertal donor *C. auratus*

72   males (three months old) were produced at the rearing facilities of the National Bureau of Fish

73   Genetic Resources, Lucknow. Both groups of animals were fed a commercially available

74   pelleted diet twice a day to satiation.

75 **Gonad histology**

76   After two years of rearing, five fish were randomly sampled and sacrificed using an

77 overdose of anaesthesia (2-phenoxyethanol; HiMedia, India), and their body weights were

78 recorded. The gonads of the fish were excised and weighed, macroscopically examined, and

79 photographed using a digital camera. The middle portion of the right and left gonads from each

80 fish were then immersed in Bouin's fixative for 24 h and preserved in 70% ethanol. The gonads

81 were processed for microscopic examination following routine histological procedures up to the

82 stage of preparing 5-μm thick cross-sections and staining them with hematoxylin–eosin.

83 Histological sections from each fish were examined under a microscope at magnifications

84 between 10× and 60×.

85 **Gene expression analysis**

86   Samples for real-time reverse transcription polymerase chain reaction (RT-PCR) of *vasa*

87 gene expression were obtained from the anterior region of the testes after the 2-year rearing

88 period (n=3) and at six months after spermatogonial cell therapy (n=3). The samples were stored

89 in RNA*later* (Sigma–Aldrich, St.Louis, MO, USA) at −80 ℃ until further processing. RNA was

90 extracted using TRIzol (Invitrogen Life Technology, Carlsbad, CA, USA) according to the

91 manufacturer's protocol. cDNA was synthesised using a first-strand cDNA synthesis kit

92 (Thermoscientific, USA). The primers for real-time RT-PCR were (5′-

93 AACCCTCATGTTCAGCGCCAC-3′ and 5′-TGGTTTCAACAAAGACCATCGTGC-3′). The

94 real-time PCRs were run in an ABI PRISM 7300 (Applied Biosystems, USA) system using

95 *Power* SYBR® Green PCR Master Mix in a total volume of 15 μL, which included 7.5 μL of 2×

96 Maxima™ SYBR Green qPCR Master Mix (Thermoscientific, USA), 20 ng of first-strand

97 cDNA and 5 pmol L^{-1} of each primer. *β*-actin (5′-GAC TTC GAG CAG GAG ATG G-3′ and 5′-

98 CAA GAA GGA TGG CTG GAA CA-3′) was used as an endogenous control. The comparative

99 Ct method was used for *vasa* mRNA quantification. The fold change in the expression level was

100 calculated using the $2-\Delta\Delta Ct$ method (Whelan et al. 2003).

Isolation and labelling of donor cells

102 The donor *C. auratus* males (n = 3) were sacrificed using an anaesthetic overdose, and

103 their testes were excised and rinsed in phosphate buffered saline (PBS; pH = 8.2). The testicular

104 tissue was finely minced and incubated in a dissociating solution containing 0.5% trypsin (pH

105 8.2; Worthington Biochemical Corp., Lakewood, NJ), 5% foetal bovine serum (JRH

106 Biosciences, Lenexa, KS), and 1mmol L^{-1} Ca^{2+} in PBS (pH 8.2) for 2 h at 22 °C. The dispersed

107 testicular cells were sieved through a nylon screen (mesh size 50 μm) to eliminate the non-

108 dissociated cell clumps, suspended in discontinuous percoll (Sigma–Aldrich, St. Louis, MO,

109 USA) gradients of 50%, 25%, and 12% and centrifuged at 200 ×*g* for 20 min at 20 °C (Majhi et

110 al. 2014). The bottom phase containing predominantly spermatogonial cells (determined during

111 preliminary trials which involved cell size measurement) was harvested, and the cells were

112 subjected to rinsing as well as a cell viability test through trypan blue (0.4% w/v) exclusion

113 assay. The cells were then exposed to the PKH-26 Cell Linker (Sigma–Aldrich, St.Louis, MO,

114 USA) at the concentration of 8 μmol/mL (room temperature, 10 min) to label the cells for

115 tracking their behaviour inside the senescent (recipient) *C. auratus* gonads. The staining

116 procedure was stopped by the addition of an equal volume of heat-inactivated foetal bovine

117 serum. The labelled cells were rinsed three times to remove the unincorporated dye, suspended in

118 Dulbecco Modified Eagle Medium (Life Technologies, Rockville, MD) with 10% foetal bovine

119 serum, and placed on ice until transplantation.

Cell therapy procedure

121 Forty fishes were first anaesthetised using 200 ppm phenoxyethanol and placed on a cell

122 transplantation platform, where they received a constant flow of oxygenated water containing

123 100 ppm of the anaesthetic drug. To prevent desiccation, the surface of the fish was kept

124 moisturised during the entire procedure of cell transplantation, which lasted for approximately 5–

125 7 min per fish on average. A micro syringe was used to inject the cell suspension into the

126 testicular lobe through genital papilla (Supplementary Fig.2). Each fish was injected with 100 μL

127 of a cell suspension containing approximately 5×10^4 cells, at the flow rate of approximately 20

128 μL/min. Trypan blue was added to the injection medium prior to transplantation to facilitate

129 visualisation of the cell suspension inside the needle and inside the gonads after injection. The

130 genital papilla region was topically treated with 10% isodine solution and the fish were returned

131 to clean water.

132 **Analysis of donor cells post therapy**

133 The fate of the donor cells after transplantation was analysed through fluorescent

134 microscopy at six and 12 weeks after injection. Then, the testes from the five animals chosen

135 randomly during each sampling were removed, washed in PBS (pH 8.2), macroscopically

136 examined for the degree of dispersion of the cell suspension (Fig.1), and immediately frozen in

137 liquid nitrogen. Cryostat (Leica CM 1500, Germany) sections 8 μm thick were cut from the

138 representative portions of these testes and observed under a fluorescent microscope (Nikon

139 Eclipse E600, Tokyo, Japan) for detecting the presence of PKH-26-labelled donor cells.

140 The fate of the donor cells was then examined by measuring the gonado-somatic index

141 (GSI; n = 5) and sperm density at six months after therapy. On each occasion, 20–30 μL of

142 sperm was collected from each of the 25 therapy-treated males and five control males. Sperm

143 was manually extracted by applying gentle abdominal pressure after careful removal of urine and

144   wiping the genital papilla. The sperm samples (10 μL) were then diluted 1,000 times with PBS

145   and the density of spermatozoa was counted using a haemocytometer (Marienfeld, Germany)

146   under a microscope. Some of the spreads of sperm used for counting were also observed under

147   the fluorescent microscope for the detection of PKH-26-labelled cells.

148   Sperm and blood samples were collected at 18 months from the spermatogonia cell

149   transplanted senescent male (n=25). Total DNA was extracted using PureLink Genomic DNA kit

150   (Invitrogen Life Technology, Carlsbad, CA, USA) according to the manufacturer's protocol and

151   DNA   typing   was   done   using   *C. auratus*   SSR   locus   *J60**   (forward   5´-

152   CTGGCTGTCTGATCCTGCTGAT-3´   and   reverse   5´-

153   TGGCCAGAGTTTAAAAACCAGTCC-3´) (Yue and Orban 2002). PCR reaction was

154   performed in 25 μL consisted of 1x Taq buffer, 1.5 mM $MgCl_2$, 0.5 μm each primer, 200 μM of

155   dNTP, 0.75 U Taq DNA polymerase and 80 ng of the DNA template. The amplification was

156   done in a thermal Cycler (ThermoFisher Scientific, USA) and consisted of an initial denaturation

157   at 94 °C for 5 min, 25 cycles of 94 °C for 30 sec, 62 °C for 30 sec and 72 °C for 30 sec, followed

158   by elongation at 72 °C for 4 min. Amplified products was visualized by 10% polyacrylamide gel

159   electrophoresis (PAGE) and silver staining. Amplicon sizing was done on gel imaging and

160   analysis system (UVP) using Msp I digested pBR322 as ladder.

161   **Artificial fertilization and induced spawning**

162   After 6 months, the senescent therapy-treated *C. auratus* males (n=25) resumed

163   production of spermatozoa. Artificial insemination and natural spawning were performed using

164   eggs from wild *C. auratus* females. Approximately 20 μL of milt from each of the ten therapy-

165   treated males was used to fertilise a batch of *C. auratus* eggs. The batches of eggs were then

166   incubated under flowing water at 25 ºC until hatching. In the natural spawning trials, ten

167   senescent therapy-treated males and wild females were paired in a 100 L glass aquarium and

168   reared in fresh water (Temperature: 25 °C ± 2 °C; dissolved oxygen: 5.3–6.1 ppm; pH: 7.5–8.0;

169   hardness: 40–45 ppm) under a constant light cycle (12 h light and 12 h darkness). Every,

170   morning between 10:00 and 11:00 h, the tanks were examined for spawning and embryos were

171   collected. The fertilised eggs obtained from each cross were incubated at 25 ºC and observed

172   under a light microscope for the assessment of fertilisation, embryonic development, and

173   hatching.

174   **Statistical analysis**

175   The statistical significance of the differences in *vasa* gene expression levels, GSI values

176   and sperm densities among the groups is evaluated using one-way analysis of variance and

177   Tukey's multiple comparison test. Graphpad Prism ver. 4.00 (Graphpad Software, San Diego,

178   Carlifornia, USA) is used for statistical analyses. Data are presented as mean ± SD and the

179   differences among the groups are considered as statistically significant at $p < 0.05$.

180   **Results**

181   **Histological observation of germ cells**

182   Microscopic examination of the testes revealed that all the five senescent *C. auratus*

183   males aged 12 years exhibited shrunken gonads (Fig. 1A) and complete disappearance of all the

184   stages of germ cells (Fig. 2A-B) in all sections examined before stem cell transplantation. By

185   contrast, the control males (age >1 year) exhibited active spermatogenesis with a large cyst of

186   spermatogonial cells, all stages of germ cells (Fig. 2C-D) and their efferent duct contained

187   spermatozoa. These histological observations were also corroborated by the results of GSI

188   evaluation and real-time RT-PCR analysis, which showed that both GSI (Fig. 3) values and *vasa*

189   gene transcript levels (Fig. 4) were significantly lower in the senescent males compared to the

190   controls ($p < 0.05$).

Fate of transplanted cells in senescent testes

192   The transplanted donor spermatogonial cells were found randomly distributed throughout

193   the spermatogenic lobules in all the five senescent males examined 6 weeks after the therapy. At

194   12 weeks after the therapy, the donor spermatogonial stem cells had reached the blind end of the

195   lobules (cortical region of the testis; Fig. 5A-D) and had undergone proliferation to form a

196   network along the testicular lobule; this stage was observed in all the sampled fish (n=5; Fig.

197   5E). At six months after the therapy, the sperm could be collected by applying gentle pressure on

198   the abdomen of all 25 senescent *C. auratus* males (Fig. 5F). The sperm density, which was not

199   detectable before the therapy, had significantly increased after the therapy and was comparable

200   with that of the reproductive control males (Fig. 6).

Production of viable gametes and progeny from senescent males

202   Six months after cell therapy, the senescent *C. auratus* males resumed spermatogenesis

203   and produced spermatozoa from the transplanted cells; the origin of the spermatozoa (from the

204   transplanted cells) was confirmed by the existence of two different genotypes and presence of the

205   red fluorescent labels in the cells (Fig. 5F). Locus *J60** differentiated amplicons from blood

206   (recipient) and sperm (donor cell) DNA (Fig. 7 & 8). These therapy-treated males were then used

207   for artificial fertilization and for trials of induced spawning of eggs obtained from young *C.*

208   *auratus* females (Tables 1–2). These crosses resulted in normal embryonic development and

209   hatching, which were similar to those in the control fish. Breeding between the spermatozoa

210   from therapy-treated males and eggs from wild *C. auratus* females resulted in 88.6%–97.5%

211   hatching whereas 93.8%-97.7% result was obtained in the control (Table 1).On the other hand,

212   when the senescent males were coupled with wild *C. auratus* females for induced spawning, the

213   breeding resulted in 95.5%–99.5% hatching with normal embryonic development and viable

214   progeny; oppose to 97.7%–98.4% in control (Table 2). These observations suggest the viability

215   of the proposed approach in revitalising the reproductive competence of the males from

216   commercially valuable fish species that have aged and can no more impregnate the females.

217   **Discussion**

218   In the present study, spermatogonial cells harvested from prepubertal *C. auratus* testes

219   were successfully transplanted for restoring the reproductive competence of senescent *C. auratus*

220   males by using cell-therapy. As observed in mammals (Brinster and Zimmermann 1994; Ogawa

221   et al. 1999), the therapy resulted in recolonization of the seminiferous epithelium, resumption of

222   spermatogenesis, and production of functional spermatozoa. The simple but effective procedure

223   demonstrated in this study can be immediately applied in hatcheries and other seed production

224   facilities. This procedure has a considerable potential for extending the reproductive phase of

225   commercially valuable species.

226   The testes recovered from the senescent *C. auratus* males exhibited shrinkage of

227   testicular lobes, absence of germ cells, and deposition of adipose and connective tissue. The

228   germinal epithelium was continuous, and the testicular cross-section was noticeably smaller than

229   that observed in reproductive males. Notably, this observation is contrary to that in senescent

230   guppy (*Lepistes reticulatus*) in which histological changes in the testes revealed a progressive

231   increase in the percentage of lobules containing spermatids and spermatozoa and an increase in

232   the deposition of melanin as well as adipose and connective tissue (Woodhead and Elett 1969).

233   However, in senescent mosquito fish (*Gambusia affinis*) and European bitterling (*Rhodeus*

234   *amarus*), partial testicular degeneration was observed to occur with a considerable reduction in

235 the number of germ cells (Woodhead 1979; Rasquin and Hafter 1951; Haranghy et al. 1977).

236 According to Finch (1990) such variations in the gonadal germ cell profiles and testicular

237 morphologies of senescent fishes are due to their growth patterns and origins of habitation. Thus,

238 the characteristics of senescence in fishes cannot be generalised and vary widely depending on

239 their habitation history and other physico-chemical parameters of their ecosystem (Liu and

240 Walford 1966). For instance, tropical fish species grow faster and attain senescence

241 comparatively earlier than do temperate fish species (Patnaik et al. 1994). The experimental fish

242 used in this study were reared in a tropical environment (temperature range: 25 °C–32 °C) for 10

243 years; probably this may explain why germ cell degeneration in the senescent individuals was

244 considerably more severe than that previously reported in other fish species.

245 Regardless of the differences between sterile (natural or experimentally induced) and

246 senescent testes, the process of recolonization by the transplanted spermatogonial cells was

247 similar to the previously reported process (Majhi et al. 2009; Lacerda et al. 2015; Majhi et al.

248 2014; Ogawa et al. 2000). For instance, after cell therapy, many PKH-26-labelled

249 spermatogonial cells eventually settled along the blind end of the seminiferous lobules and began

250 proliferation within weeks of transplantation. Considering that only spermatogonial stem cells

251 can migrate and settle at the basement membrane and resume the process of spermatogenesis

252 (Ogawa 2001), we can surmise that the cells used for therapy were spermatogonial stem cells.

253 This conclusion is also proved by the fact that when the senescent *C. auratus* males (26 months

254 after cell therapy) were repeatedly paired with wild *C. auratus* females for natural spawning,

255 they continued to produce viable progeny to date. Transmembrane protein molecules present at

256 the junctional complex located in the seminiferous tubules have been reported to transduce

257 signals, maintain cell polarity, and mediate germ cell migration (Wang and Cheng 2007).

258   Although we did not examine the endocrine regulation involved in the migration, proliferation

259   and differentiation of the donor spermatogonial cells inside the gonads of senescent *C. auratus*

260   males, the results obtained in the present study (time-course observations of donor

261   spermatogonial cells after therapy) indicated that the donor spermatogonial cells might have

262   sensed and responded to the molecules released from the blind end of the seminiferous lobules in

263   therapy-treated senescent *C. auratus* males. Consequently, these spermatogonial cells migrated

264   and settled down near the basement membrane and resumed spermatogenesis. However, the

265   duration for which the *C. auratus* males treated with spermatogonial cell therapy retain their

266   reproductive competence and produce functional gametes remains to be determined.

267   The present study demonstrated that spermatozoa derived from therapy-treated senescent

268   *C. auratus* males exhibited functional properties similar to those of the control spermatozoa in

269   terms of fertilisation, embryonic development and hatching, and no evidence of defective

270   spermatozoa was observed. Defective spermatogeneis has been reported to occur most

271   prominently when the donor and recipient animals are phylogenetically distant (Nantel et al.

272   1996). In this study, the spermatogonial cells derived from pre-pubertal *C. auratus* males were

273   transplanted into senescent *C. auratus* males; thus, the transplanted cells were probably

274   immunologically compatible with the gonadal environment of the senescent *C. auratus* males.

275   Moreover, the progeny produced using the spermatozoa derived from therapy-treated *C. auratus*

276   males exhibited a similar growth pattern to that of the progeny produced from the control males.

277   These observations suggest that the reproductive competence of senescent *C. auratus* males

278   could be successfully revitalised using spermatogonial cell therapy. Currently, we are examining

279   the feasibility of the cell-therapy approach in revitalising the reproductive competence of female

280   *C. auratus*.

281   In conclusion, the most remarkable achievement of this study is the production of

282   functional spermatozoa from senescent *C. auratus* males after spermatogonial cells derived from

283   the pre-pubertal *C. auratus* donor were transplanted into the testes of the senescent males

284   through the genital papilla. This approach, which to the best of our knowledge has been validated

285   for the first time in fish in this study, is a method to generate functional gametes for additional

286   years after they have aged beyond reproductive capability. This is a crucial development in the

287   breeding of rare and/or commercially valuable fish species that are developed as brooders and

288   require sizeable investments in terms of feed and health care. Such fish species are used for only

289   a few years by the hatcheries for seed production and are invariably discarded when they lose

290   their reproductive competence because of age.

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Figure 1

Visualization of the dispersal of the cell suspension through the gonad of senescence *C. auratus* after transplantation.

A) Macroscopic appearance of the testis 48 hours after spermatogonia cell transplantation (note the diffusion of the marker Trypan blue throughout the testis). B) Appearance of the testis 6 months after cell transplantation (note the transplanted cells in senescence testis have undergone proliferation and differentiation to match the testis of a sexually mature individual). Scale bars indicate 2 cm.

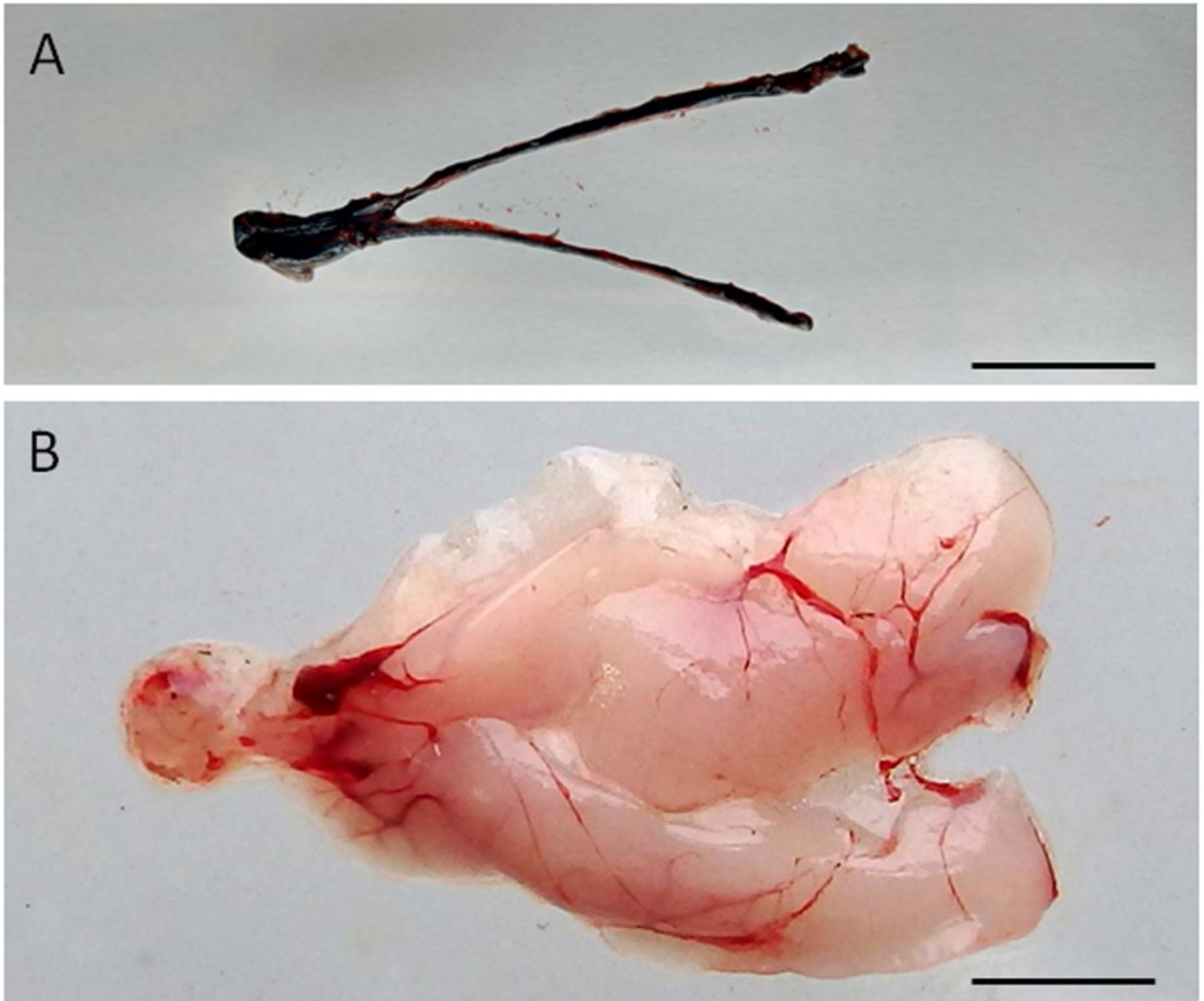



Figure 2

Histological appearance of senescence and control *C. auratus* gonads

Panels on the right are high magnifications of insets in the left panels. A,B) Twelve year old senescence testis showing empty niche (arrows) with complete absence of spermatogonia and other germ cells. C,D) A sexually mature control testis indicating large cysts of spermatogonia in the blind end of the spermatogenic lobules (arrows), spermatocytes (arrowhead) and spermatids (star) depicting an active spermatogenesis within the lobules. Scale bars indicate 100 µm (A and C) and 20 µm (B and D).

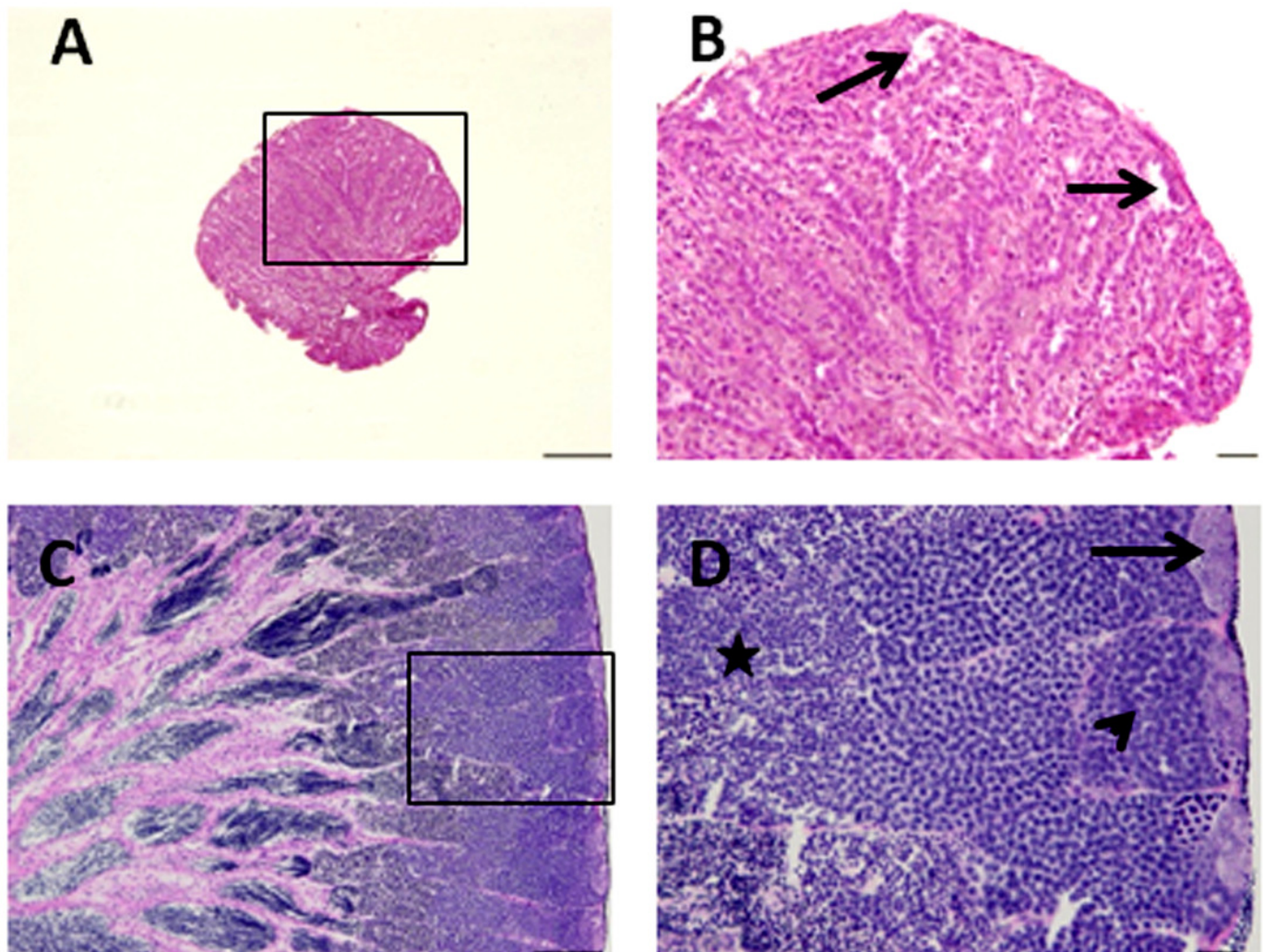



Figure 3

Changes in the gonado-somatic index of senescence and control gonads between 0 day
(A) and six months (B).

Note, six months after therapy the gonado-somatic index value of senescence *C. auratus*
males have significantly increased to match the sexually mature control. Data are presented
as mean ± SD. Columns with double asterisk vary significantly (ANOVA - Tukey test, *p*<0.05).

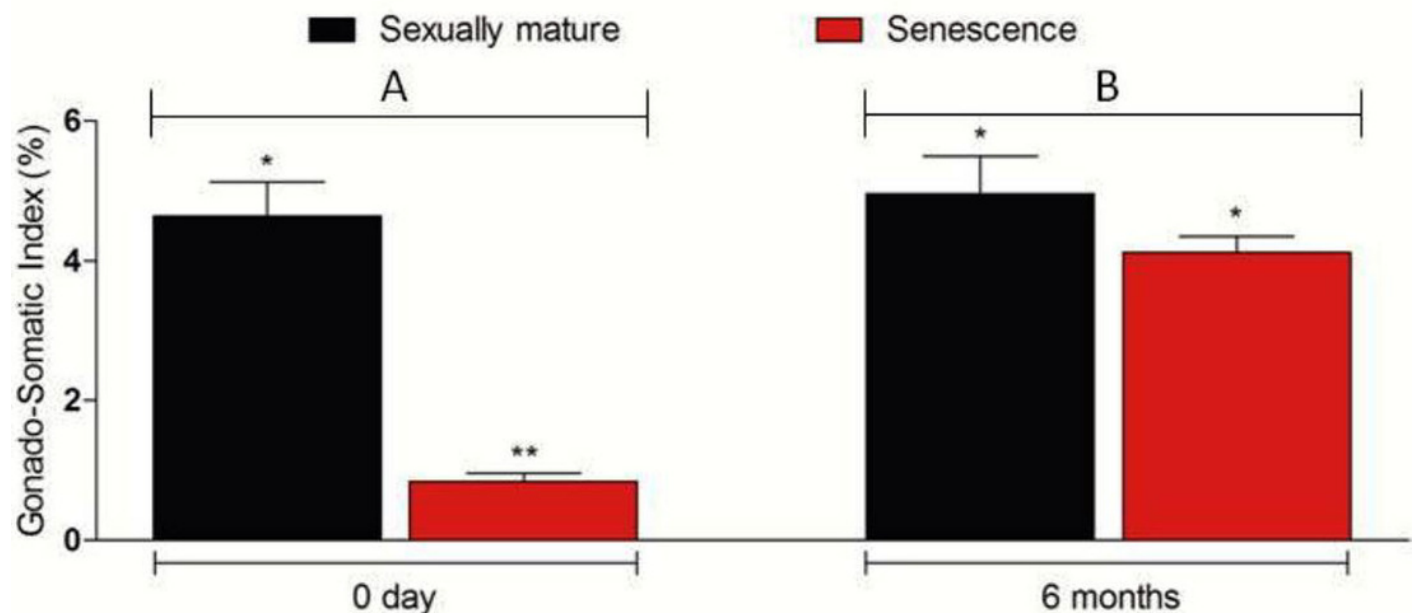



Figure 4

Changes in *vasa* gene transcript levels in senescence and control *C. auratus* gonads between 0 day (A) and six months (B).

Note, six months after therapy the *vasa* gene transcript levels in senescence *C. auratus* males have significantly increased to match the reproductive control. Data are presented as mean ± SE. Columns with double asterisk vary significantly (ANOVA - Tukey test, $p<0.05$).

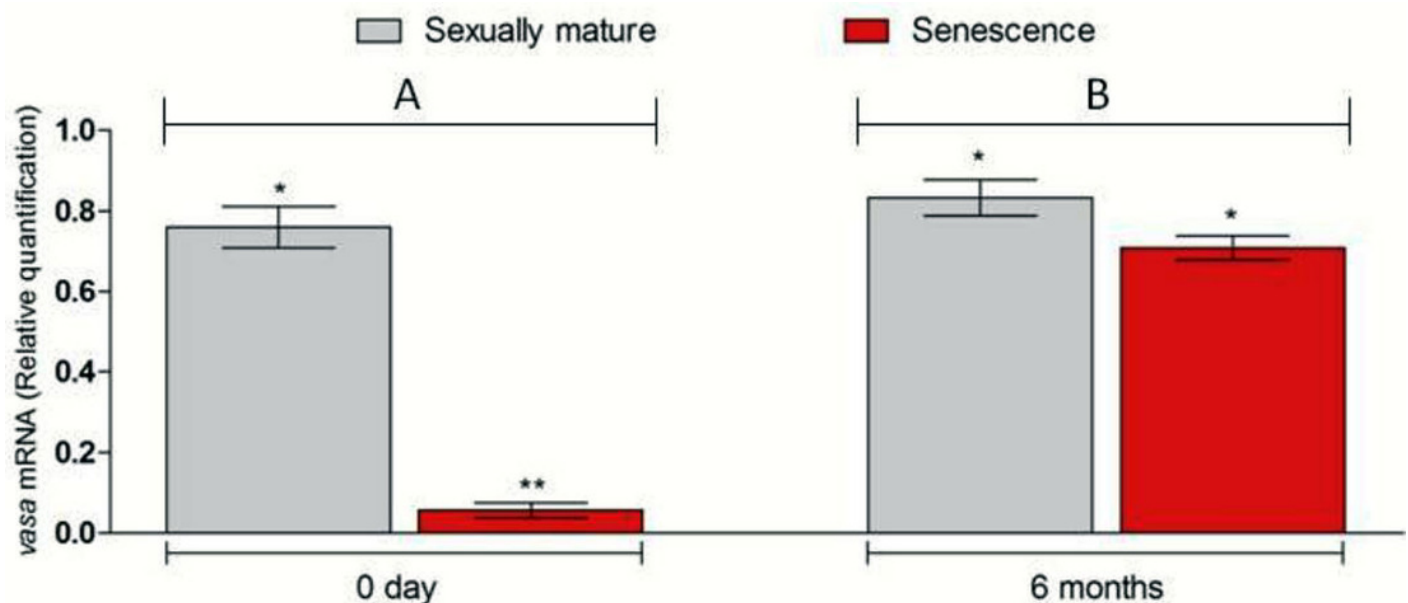



Figure 5

Fate of PKH-26-labeled donor spermatogonia cells in senescent *C. auratus* males examined between four weeks and six months after transplantation.

A,B) Cryostat section of a transplanted testis at four weeks showing the presence of transplanted spermatogonia cell at the blind end of the spermatogenic lobules (arrow; B is a high magnification of the box in A). C,D) Cryostat section of a non-transplanted, control testis at four weeks showing the approximate location of the blind end of the spermatogenic lobules (D is a high magnification of the box in C). E) Whole-mount preparation of a transplanted testis at twelve weeks showing the proliferation of donor-derived cells (highlighted) along the length of the gonad. F) Whole-mount preparation of spermatozoa cell derived from the senescent male 6 months after the procedure (Note, the spermatozoa produced were of donor-origin that was characterized by retention of PKH-26 dye). Scale bars indicate 100 μm (A and C), 20 μm (B and D), 500 μm (E) and 50 μm (F).

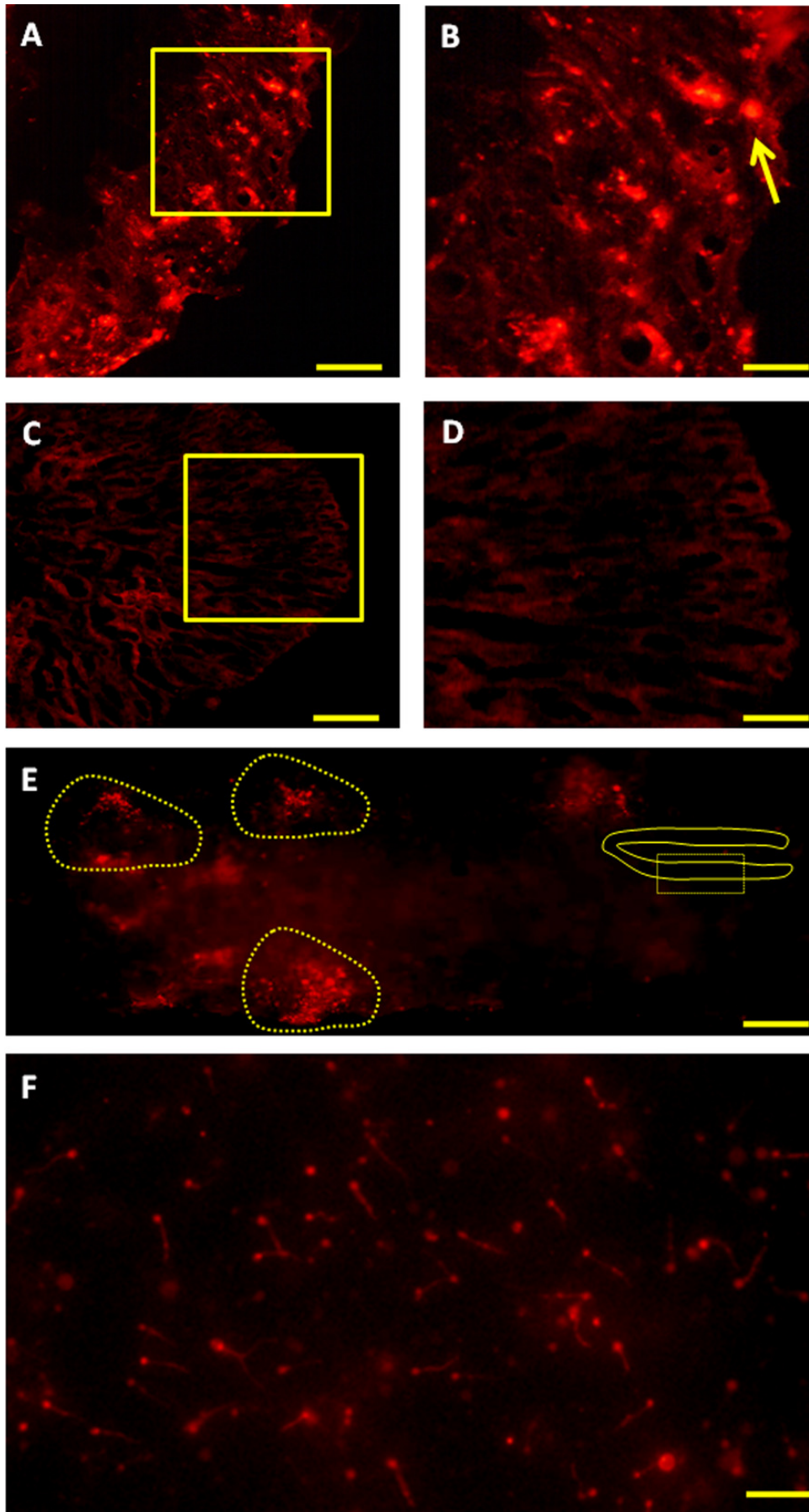



Figure 6

Sperm density in senescent recipients and non-transplanted (negative control) *C. auratus* between 0 day (A) and 6 months (B) after spermatogonial cell therapy.

Note, the sperm count in the senescent males had significantly increased after spermatogonial cell therapy. Data are presented as mean ± SD. Columns with double asterisk vary significantly (Tukey's multiple comparison test, *p*<0.05).

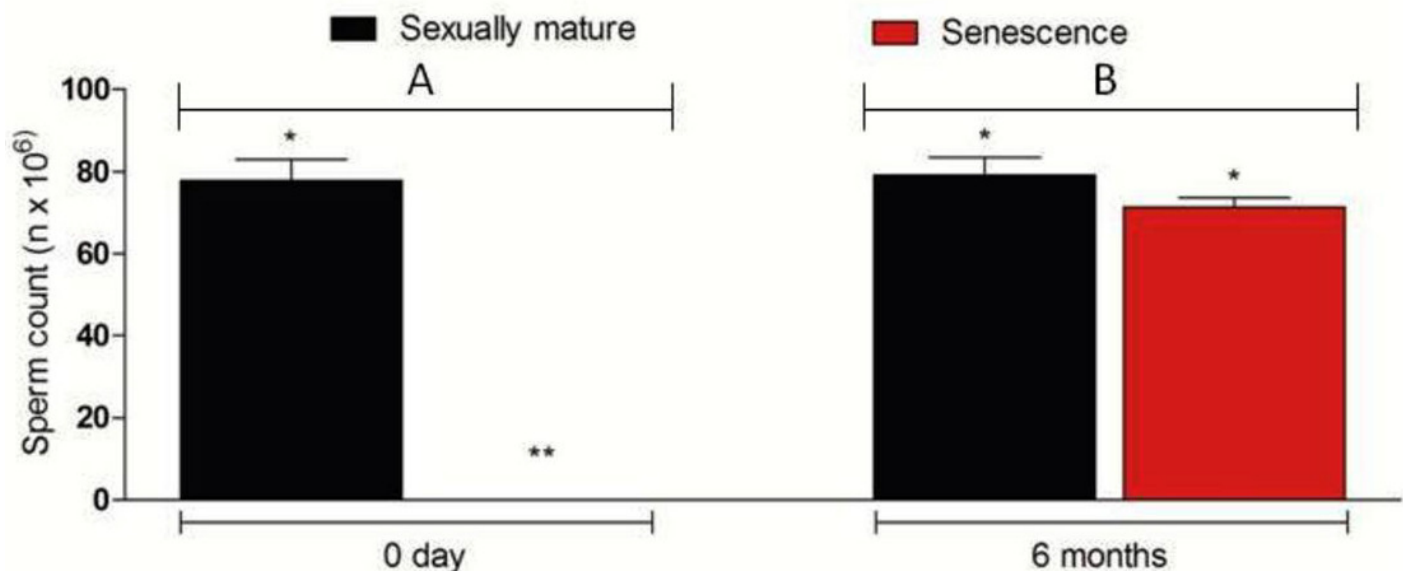
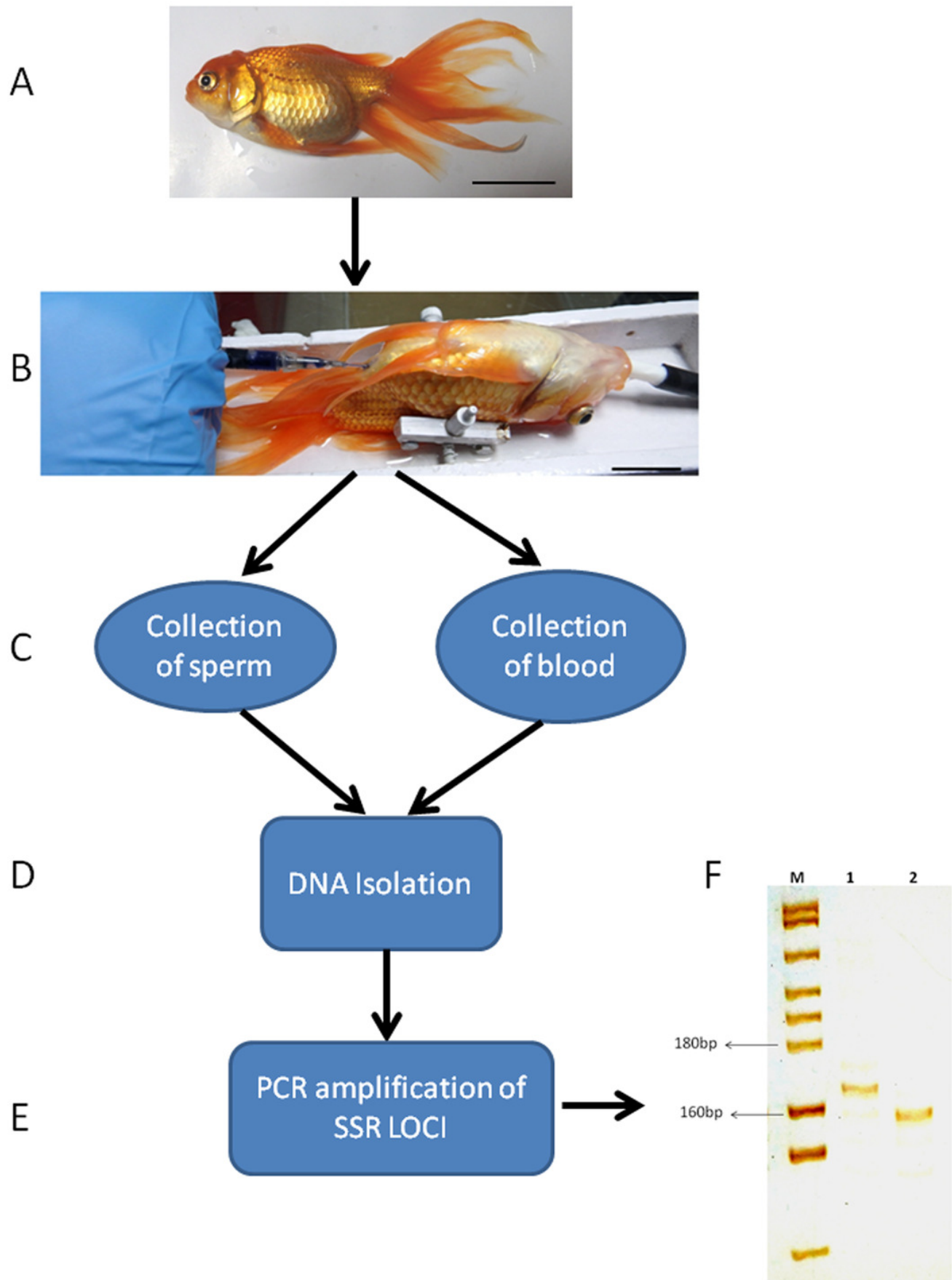



Figure 7

Genotyping of sperm and blood collected from a senescent *C. auratus* males 18 months after the cell transplantation.

A) The senescent *C. auratus* male used in the experiment. B) The donor spermatogonial cell transplantation in senescent *C. auratus* male. C) Collection of sperm and blood samples from senescent *C. auratus* males 18 months after the cell transplantation. D) DNA isolation from the samples using PureLink Genomic DNA kit according to the manufacturer's protocol. E) The microsatellite primers were used for amplification of DNA for genotyping. F) Lanes include molecular marker (M), blood of senescent male (1) and sperm (2). Note, the donor-derived spermatozoa were detected in the sperm of senescent recipient shown in lane 2.

Results of artificial fertilization.

The eggs derived from wild gravid *C. auratus* females with sperm from ten senescence *C. auratus* males (n=10; transplanted with spermatogonia derived from pre-pubertal *C. auratus* donors) and control (crosses between sexually mature *C. auratus* males and females). Data presented are mean ± SD.

Fertilization trail	Eggs (n) from wild gravid *C. auratus* females	Fertilization rate (n; %)	Hatching rate (n; %)
senescence *C. auratus* males (n=10)	476±107	453±112	432±109
Control (n=3)	503±99	476±91	460±95

Results of induced spawning.

The cross between senescence *C. auratus* males (n=10; transplanted with spermatogonia derived from pre-pubertal *C. auratus* donors) with wild gravid *C. auratus* females and a control (paired between sexually mature *C. auratus* males and females). Data presented are mean ± SD.

Fertilization trail	Total number of embryo collected (n)	Fertilization rate (n; %)	Hatching rate (n; %)
senescence *C. auratus* males paired with gravid *C. auratus* females (n=10)	1068±271	1046±273	1026±277
Control (n=3)	1091±140	1068±128	1047±127

Figure 8

Schematic illustration of species-specific microsatellite loci J60* amplifying both senescent (recipient) and donor genomic DNA.

The species-specific microsatellite loci J60* (Yue and Orban, 2002) amplifying both senescent (recipient) and donor genomic DNA and visualization through silver staining. The two genotypes (A) senescent 166/166 and (B) donor 158/158) were electrophoretically resolved on polyacrylamide gel using Msp I digested pBR322 as ladder (M).