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1 # Clonal gametogenesis is triggered by intrinsic stimuli in the hybrid's
2 germ cells but is dependent on sex differentiation

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19 interpretation, and draft preparation. R.F. performed MO injection, transplantation, and spawning. MDK
20 performed and analyzed microsatellites data. DD and AM performed meiotic investigations. K.H. was
21 responsible for fish collection, identification, and ploidy measurement. CH.S. performed histology. K.J. &
22 M.P conceived the study, participated on crossing experiments and analysis of progeny, and co-drafted
23 the MS with T.T.

24 **Competing Interest Statement:** The authors declare that they have no known competing financial
25 interests or personal relationships that could have appeared to influence the work reported in this paper.

26 **Keywords:** asexuality, hybridization, gonial stem cell transplantation, premeiotic reduplication

27 **This PDF file includes:**

28 Main Text
29 Figures 1 to 4
30 Tables 1 to 1
31

32 **Abstract**

33 Interspecific hybridization may trigger the transition from sexual reproduction to asexuality, but
34 mechanistic reasons for such a change in a hybrid's reproduction are poorly understood.
35 Gametogenesis of many asexual hybrids involves a stage of premeiotic endoreduplication

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36 (PMER), when gonial cells duplicate chromosomes and subsequent meiotic divisions involve
37 bivalents between identical copies, leading to production of clonal gametes. Here, we
38 investigated the triggers of PMER and whether its induction is linked to intrinsic stimuli within a
39 hybrid's gonial cells or whether it is regulated by the surrounding gonadal tissue.

40 We investigated gametogenesis in the *Cobitis taenia* hybrid complex, which involves sexually
41 reproducing species (*Cobitis elongatoides* and *C. taenia*) as well as their hybrids, where females
42 reproduce clonally *via* PMER while males are sterile. We transplanted spermatogonial stem cells
43 (SSCs) from *C. elongatoides* and triploid hybrid males into embryos of sexual species and of
44 asexual hybrid females, respectively, and observed their development in an allospecific gonadal
45 environment. Sexual SSCs underwent regular meiosis and produced normally reduced gametes
46 when transplanted into clonal females. On the other hand, the hybrid's SSCs lead to sterility
47 when transplanted into sexual males, but maintained their ability to undergo asexual
48 development (PMER) and production of clonal eggs, when transplanted into sexual females.

49 This suggests that asexual gametogenesis is under complex control when somatic gonadal tissue
50 indirectly affects the execution of asexual development by determining the sexual differentiation
51 of stem cells and once such cells develop to female phenotypes, hybrid germ cells trigger the
52 PMER from their intrinsic signals.

53 **Significance Statement**

54

55 Although sexual reproduction is a dominant trait among all eukaryotes, many taxa have evolved
56 the ability to reproduce asexually. While asexuality appears to be linked to interspecific
57 hybridization, it remains unknown how the coexistence of diverged genomes may initiate such a

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58 swap in reproduction. In our study, we transplanted germ cells between asexual hybrids and their
59 parents. On one hand, the ability of clonal gametogenesis occurred exclusively in hybrid germ
60 cells, suggesting that asexual development is directly triggered by the hybrid genomic
61 constitution of the cell. On the other hand, clonality was observed only in cells transplanted into
62 females, suggesting that the execution of clonal development is influenced by signals from the
63 gonadal environment and regulated by somatic factors.

64

65 **Main Text**

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67 **Introduction**
68

69 Although sexual reproduction is a predominant characteristic of all multicellular eukaryotes, based on
70 conserved molecular machinery controlling meiotic divisions, it has been disrupted many times. This has
71 resulted in a variety of the so-called asexual reproductive modes that occur in most animal and plant
72 phyla. Asexual lineages not only allow key questions about ultimate advantages and disadvantages of sex
73 to be tested, but due to their clonal multiplication, unusual gametogenic and developmental pathways,
74 they have proved to be appealing models for many biological disciplines concerned with ecology, cell
75 biology and molecular genetics (1). Yet, despite intensive research in asexual organisms, frustratingly
76 large gaps remain in our understanding of mechanisms that trigger such a switch from sex to various
77 forms of asexuality. This is apart from some straightforward cases, such as *Wolbachia*-induced asexuality
78 (2) or the presence of candidate "asexuality genes" in a few model taxa (3, 4).

79 A promising class of theories aiming to identify some common mechanisms underlying the
80 emergence of asexuality builds on the fact that many asexual organisms are of hybrid origin. It has been
81 proposed that abandonment of sex may be stimulated by aberrant interactions between orthologous copies
82 of individual genes (5, 6), chromosomes (7) or even entire regulatory networks brought together by
83 hybridization between distinct but not co-adapted genomes (8). Unfortunately, the scarcity of empirical
84 studies prevents any clear-cut conclusions about the role of hybridization in triggering asexuality. Indeed,
85 if hybridization is supposed to initiate asexuality, it is difficult to explain why meiosis is affected in
86 similar ways across diverse taxa.

87 For instance, one gametogenic pathway that is relatively common among independently arisen
88 asexual animals and plants in *sensu lato* is premeiotic endoreplication (PMER) (9–16). This pathway,
89 depicted in Fig. 1, is characterized by duplication of chromosomes in oogonia before meiosis.
90 Consequently, subsequent meiotic divisions occur in a polyploid gamete with bivalents forming between

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91 homologues. Such a process alleviates potential problems in pairing of orthologous chromosomes in
92 hybrids (9, 17) and simultaneously leads to the production of clonal progeny because bivalent pairing and
93 crossovers occur between identical sister copies of duplicated chromosomes.

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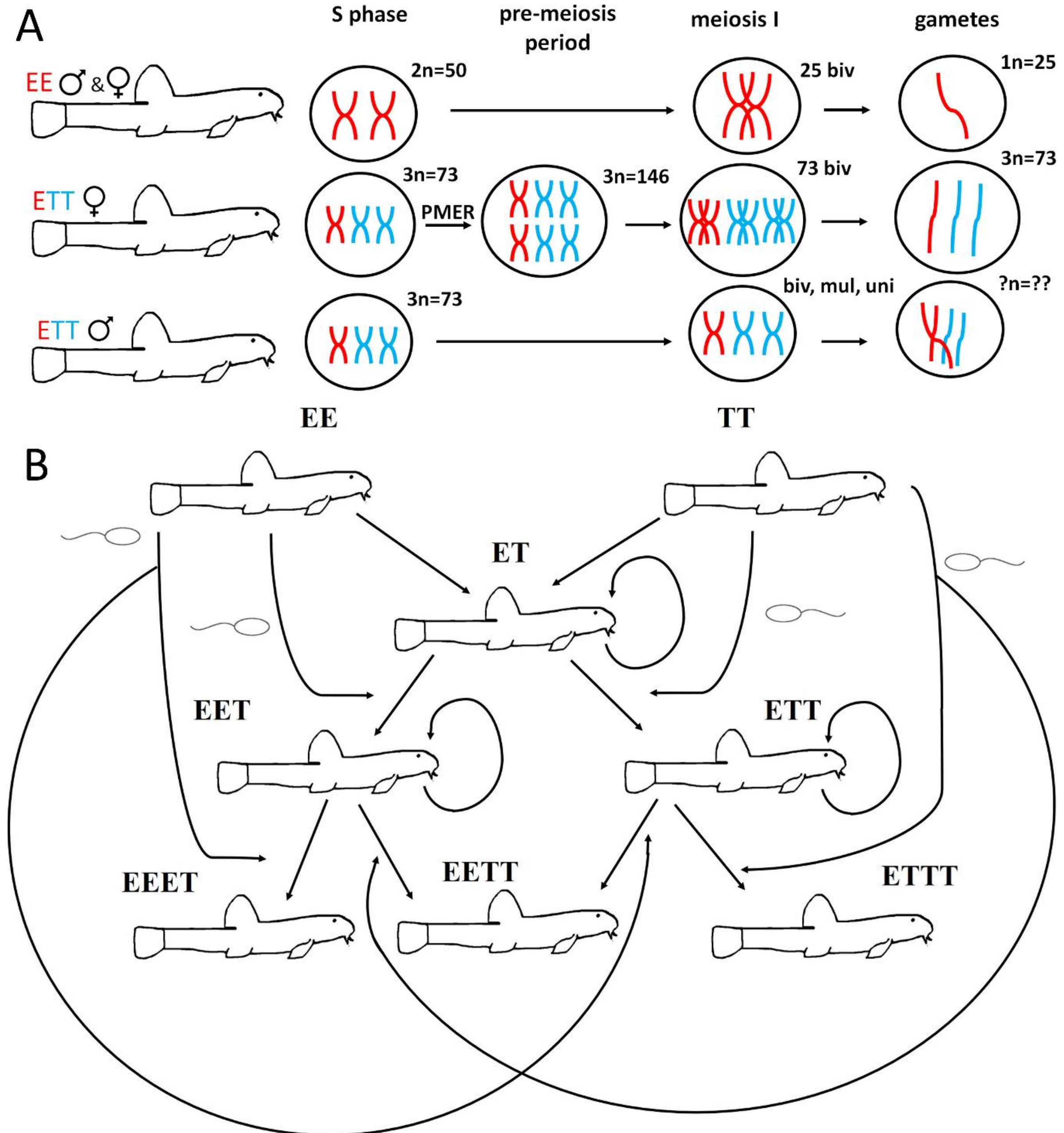

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96 **Figure 1.** *Cobitis taenia* (TT) and *Cobitis elongatoides* (EE) hybridization scheme. A) Gametogenesis in

97 EE, triploid ETT female and male. Red color represents chromosomes derived from parental species EE,

98 blue those from parental species TT. In hybrid females, the premeiotic endoreplication (PMER) results in

99 doubling of chromosomes and pairing between identical copy produces proper bivalents with

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100 crossovers. However, in males, PMER does not occur. Therefore chromosomes cannot properly pair
101 leading to bivalents (biv), multivalents (mul) and univalents (uni). Parental species produce haploid
102 gametes, hybrid females produce clonal eggs and males either cannot finish meiosis properly or final
103 spermatozoa are often aneuploid or polyploid with disrupted motility. B) F1 hybrids are produced by
104 natural or artificial spawning between TT and EE individuals. ET hybrid males are sterile while hybrid
105 females are fertile using gynogenesis as reproductive mode. In some cases, sperm from either parental
106 species can fertilize the egg giving rise to the triploid hybrids (ETT or EET depending on sperm), which
107 are also either sterile (males) or fertile (females). Fertilization of triploid eggs is also possible but natural
108 occurrence of EEET, EETT, and ETTT is very rare and tetraploids appear unable to reproduce.

109

110 Analysis of the speciation process in spined loaches (*Cobitis*; Actinopterygii) demonstrated that
111 emergence of hybrid asexuality mechanistically coincided with hybrid sterility (9, 18). Hence, the
112 emergence of asexual gametogenesis in general, and PMER in particular, may represent an alternative
113 type of reproductive incompatibility promoting speciation among hybridizing species (18, 19). There are
114 notable analogies between the emergence of hybrid asexuality and classical speciation models assuming
115 the accumulation of postzygotic reproductive incompatibilities. Indeed, the emergence of both hybrid
116 asexuality and sterility effectively restricts interspecific geneflow thereby promoting speciation, and scale
117 with genomic divergence between parental species. Furthermore, in a similar way to hybrid sterility,
118 asexual gametogenesis also arises asymmetrically with respect to the sex of the hybrids so that most
119 known asexual vertebrates exhibit strongly female-biased sex ratios for which they are sometimes also
120 referred to as 'all-female' or 'unisexual' (20, 21).

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121 Unfortunately, there are only a few studies investigating functional differences between male and
122 female hybrids in taxa where asexuality occurs. Available data indicate that when hybrid females are
123 fertile and asexual (often employing PMER), their hybrid brothers are usually sterile (11, 19, 22).
124 However, some exceptions exist, *e.g.* in hybridogenetic taxa like *Pelophylax* (23) or *Squalius* (24).
125 Crossing experiments within several asexual complexes of fishes and reptiles further demonstrate that
126 asymmetry between female hybrid asexuality and male sterility is directly linked to the merging of
127 parental genomes and already occurs in the F1 hybrid generation (9, 11, 25–27). Yoshikawa *et al.* (28)
128 further examined *Misgurnus* spp. (loaches), where hybrid females typically reproduced clonally with
129 PMER, and artificially reverted clonal diploid female progenies into males. They reported that PMER
130 occurred also in spermatogonia of these artificially sex-reverted males from females, which was
131 surprising since natural *Misgurnus* male hybrids are sterile (11). Such a finding therefore indicates that
132 asexual gametogenesis is somehow linked to one sex (females), and seems to depend on genetic sex
133 determination rather than on phenotypic sex.

134 PMER thus appears to be a crucial cellular deviation in the evolution of many natural hybrid and
135 allopolyploid lineages, making it important in furthering our understanding of genetic and cellular
136 mechanisms which underly its occurrence. Unfortunately, how and why the hybrid germ cells switch
137 their developmental pathway towards PMER are unknown. For instance, it remains unclear whether
138 PMER results from endomitosis, which involves mitotic replication of chromosomes without cell
139 division, or endoreduplication, *i.e*. replication of chromosomes without initiation of mitosis. It has been
140 shown that red crucian carp × common carp [*Carassius auratus* (red variety) x *Cyprinus carpio*] hybrids
141 produce tetraploid oogonia by germ cell fusion, rather than by multiplication of their chromosomes (29).

142 Loaches of the family Cobitidae (Cypriniformes, Teleostei) have been shown to be a suitable
143 model organism to understand the mechanisms underlying hybrid sterility and asexuality (10, 17, 18, 28,

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144 30). In particular, the so-called *C taenia* hybrid complex is distributed in Europe and comprises sexual
145 species of *Cobitis taenia* (TT) and *Cobitis elongatoides* (EE) that diverged ~9 million years ago (18) but
146 are frequently hybridizing, producing sterile males and hybrid females, which reproduce clonally using
147 PMER (9, 31). Hybrid females are gynogenetic and hence sperm is required to trigger their gametes'
148 development but this does not generally contribute to the progeny's genome. However, their oocytes
149 sometimes incorporate the sperm's genome leading to a new generation of sterile triploid males and
150 gynogenetic females with a triploid genome composition (Figure 1). Consequently, natural populations of
151 spined loaches are generally composed of sexual host species (often occurring in a minority), diploid, and
152 mainly triploid clonal hybrid females (32) .

153 In this study, we investigated whether the initiation of PMER is autonomously regulated in the
154 hybrid's germ cells or whether it depends on extrinsic stimuli from surrounding somatic cells and the
155 tissue in which they occur. We also tested whether PMER is strictly confined to female sex determination,
156 or whether the germline originating from males may also undergo such a pathway. To do so, we
157 performed the following study, the design of which is depicted in Figure 2. We transplanted testicular
158 cells containing spermatogonial stem cells (SSCs) between sexually reproducing species and their asexual
159 hybrids from the *C. taenia* hybrid complex and investigated the development of such cells in the host's
160 body. Specifically, we extracted spermatogonial stem cells from *C. elongatoides* males (sexual species)
161 and sterile allotriploid males with the *Cobitis elongatoides-taenia-taenia* genomic constitution (see Figure
162 1 for an explanation of the hybrid origins). These cells were reciprocally transplanted into juvenile
163 recipients of both sexes that were sterilized by oligonucleotide morpholino treatment prior to
164 transplantation. Therefore, two groups of fish were obtained: 1) triploid recipients of gonial cells from
165 diploid donors (hereafter called TrDd) and 2) diploid recipients of gonial cells from triploid donors
166 (hereafter called DrTd). Recipients were kept until adulthood and allowed to spawn in order to investigate

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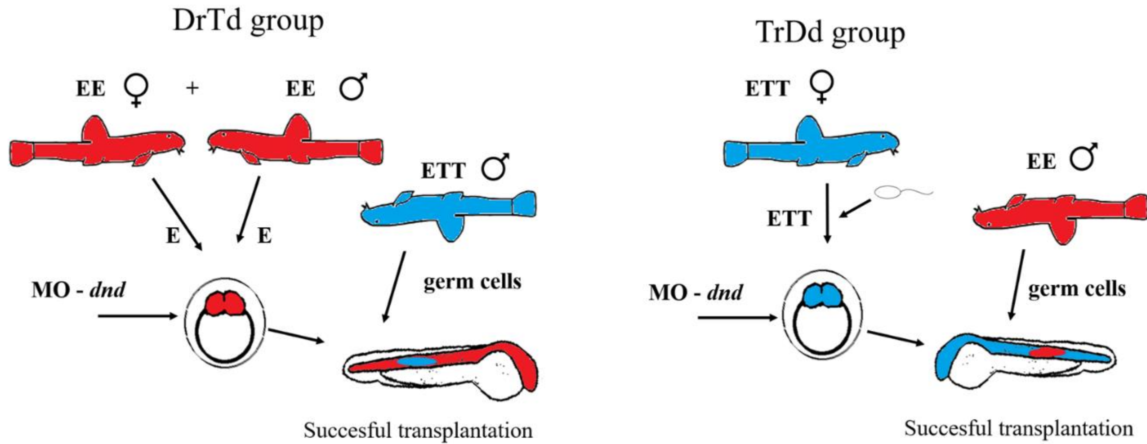
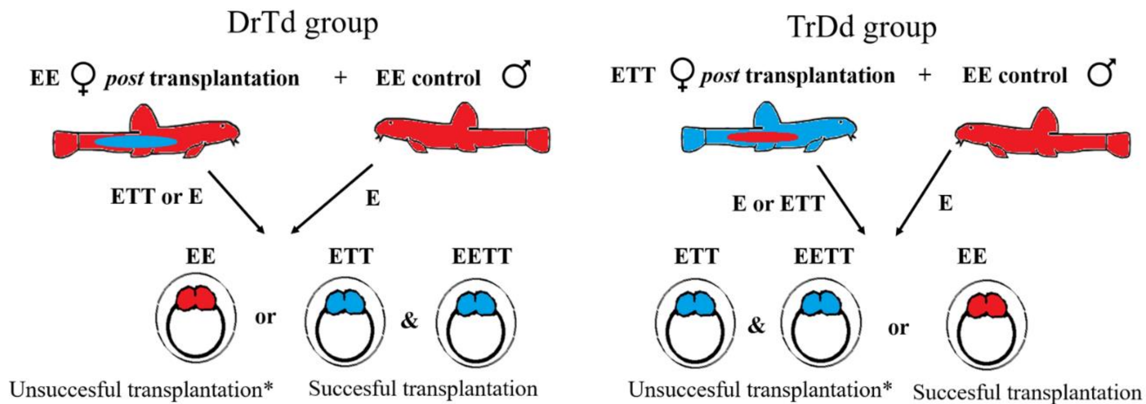
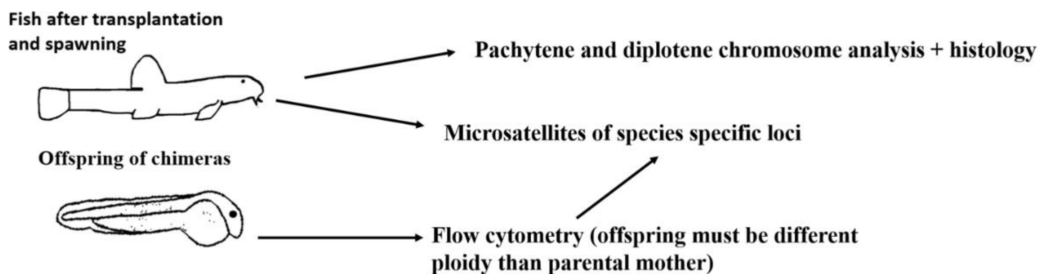
167 their fertility and inheritance patterns in their progeny. After spawning, the recipients' gonads were
168 investigated using cytogenetic methods to check for meiotic patterns and potential presence of PMER.







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170 **Figure 2.** The experimental design. 1) The design of reciprocal transplantation between two groups:
171 diploid recipient and triploid donor (DrTd), and triploid recipient and diploid donor (TrDd). In the DrTd
172 group, parental species of *Cobitis elongatoides* (EE – red color) were spawned and their early embryos (2
173 cells stage) were injected with morpholino (MO) against the *dnd* gene to terminate development of
174 parental gonads. Transplantation was undertaken using the germ stem cells from adult allotriploid male
175 *Cobitis elongatoides-taenia-taenia* (ETT – blue color). In the second group TrDd, eggs of ETT females
176 were activated with goldfish (*Carassius auratus*) (sperm symbol). Embryos were treated with anti-*dnd*
177 MO and later transplanted with germ cells from adult EE males. 2) Two years after transplantation,
178 experimental fish from both groups were spawned with the EE males. In the DrTd group, EE fish after
179 successful transplantation should produce triploid ETT eggs where two scenarios can occur: either eggs
180 are activated only (gynogenesis) producing ETT offspring or sperm can be incorporated into the eggs
181 thus producing EETT. In the TrDd group, successfully transplanted ETT fish should produce haploid E
182 gamete which must be fertilized with sperm and produce parental species EE. *In the case of
183 unsuccessful morpholino treatment and transplantation, fish would produce their natural biotype, *i.e*.
184 haploid eggs in the case of parental species and triploid eggs in case of hybrids. 3) Confirmation of
185 successful transplantation. Offspring of chimeric fish from the DrTd group carried the *Cobitis taenia*
186 microsatellite loci in the case of successful transplantation. On the other hand, *C. taenia* loci were
187 absent in offspring from the TrDd group.

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191 **Results**

192

193 **Transplantation efficiency**

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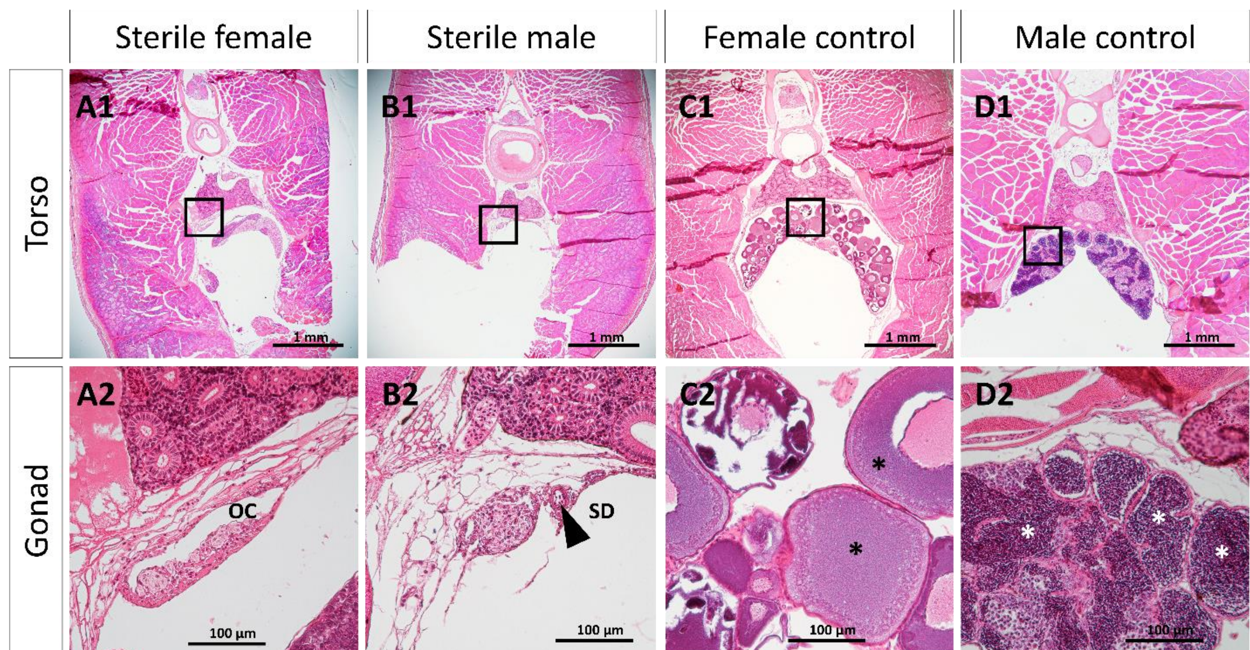
194 Morpholino was injected into embryos between the one and four cell stage of development. After 2
195 years, we collected 31 representative fish of both experimental groups for further examinations, which
196 included experimental crossing, histology and cytogenetic analysis of meiotic chromosomes. In total, we
197 examined 23 fish of the DrTd group (12 females and 11 males) and eight fish of the TrDd group. Among
198 these, we observed that four out of 12 DrTd females were not sterilized completely due to morpholino
199 failure, six were sterilized but without successful transplantation (Figure 3) and two appeared as
200 chimaeras after successful sterilization and transplantation. Similarly, sterilization failed in four out of 11
201 DrTd males. In five we observed sterilization without transplantations (Figure 3) and two appeared as
202 successful chimaeras. In the TrDd group, six hybrid females were successfully sterilized and transplanted
203 (Table 1) while two were succesfully sterilized but with no success in transplantation



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205 **Figure 3.** Results of histology analysis after MO-*dnd* treatment designed for *Cobitis taenia* in *Cobitis*
206 *elongatoides*. A1 shows the whole body histology of a sterile female after morpholino treatment. A2 is a
207 magnification of the ovarian cavity. B1 shows the whole body histology of a sterile male after
208 morpholino treatment. B2 shows enlarged fragment from B1 of the sperm duct (SD), *i.e.* the connection

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209 between each testis to a urogenital opening, the testes themselves are not present. C1 and C2 show
210 fertile females with vitellogenic eggs (black asterisks), while D1 and D1 show fertile males with gametes
211 (white asterisks).
212
213
214 **Genetic profiling of experimental fish and their offspring based on microsatellite markers**

215 After reaching sexual maturity, 23 of the recipient fish were spawned with males of *C. elongatoides*
216 and their offspring were profiled together with their parents. Genetic profiling of fish was performed by
217 analyses of selected species specific (*C. elongatoides v. C. taenia*) microsatellite loci. Since we used two
218 types of fish, diploid *C. elongatoides* and triploid *C. elongatoides-taenia-taenia*, the success of
219 transplantation was indicated by the presence of *C. taenia* specific loci in the juveniles of the DrTd group
220 analyzed (where parental species *C. elongatoides* produce juveniles with *C. taenia* loci) or by its absence
221 in juveniles of TrDd (where hybrids *C. elongatoides-taenia-taenia* produce juveniles without *C. taenia*
222 loci). Altogether, from 10 potential chimeric females (six *C. elongatoides* and four *C. elongatoides-*
223 *taenia-taenia*), we obtained 136 juvenile individuals for analysis. Successful transplantation was
224 confirmed in two out of six diploid *C. elongatoides* females of the DrTd group, which altogether
225 produced 23 juveniles with a foreign allelic profile corresponding to the donor´s genotype (ETT).
226 Moreover, in six out these 23 juveniles, we observed incorporation of paternal *C. elongatoides* sperm,
227 leading to an increased ploidy level of progeny (EETT). This confirms their successful transplantation
228 and demonstrates that such transplanted oogonia maintain their ability to produce clonal progenies. In the
229 remaining four *C. elongatoides*, transplantation was not successful. In a reciprocal experiment, four *C.*
230 *elongatoides-taenia-taenia* females from the TrDd group yielded 47 juveniles, whose genotype
231 corresponded to one haploid set of their *C. elongatoides* donor and a second haploid set of the *C.*
232 *elongatoides* father. This indicated successful transplantation in the TrDd group and suggested that *C.*

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233 *elongatoides* germ cells transplanted into ETT females conserved the ability to properly divide into
234 apparently normal EE-type gametes.

235 **Histology**

236 Histological analysis of chimeric fish was mostly directed to the males. Hybrid male sterility in
237 *C. elongatodies x C. taenia* was represented by meiotic arrest leading to an aberrant germ cell population,
238 lacking functional spermatozoa, while hybrid females produced fertile eggs. Nevertheless, it was possible
239 to obtain supporting evidence of successful transplantation in diploid males (Figure 4A) when compared
240 to gonadal tissue from diploid (Figure 4B) and triploid controls (Figure 4C). The DrTd males and triploid
241 control males contained germ cells in premeiotic stages and a small number of postmeiotic abnormal
242 cells, while diploid controls contained mainly functional spermatozoa. In females, diploid controls, DrTd
243 and TrDd groups showed normal gametogenesis with late vitellogenic oocytes. However, histology itself
244 was not used as an indicator of successful transplantation.

245 **Meiosis analysis of chimeric fish**

246 In order to investigate the particular gametogenic mechanisms adopted by the fish and their
247 gametes, we isolated chromosomes during the pachytene and diplotene meiosis stages. We analyzed 67
248 pachytene spreads obtained from two *C. elongatoides* sperm lacking males in the DrTd group. We
249 observed abnormal pairing with several bivalents and univalents (Figure 4D) and compared these with
250 controls from 2n and F1 3n males (Figure 4E and 4F, respectively). We did not observe any cells with
251 duplicated genomes. We additionally checked another six males from the same group; however, we did
252 observe cells with 25 bivalents during pachytene, suggesting no success in transplantation. In females
253 from the DrTd group, we did observe oocytes with improper pairing (Figure 4I) but also with duplicated
254 genomes as 73 bivalents indicating the occurrence of PMER in chimeric fish during gametogenesis

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255 (Figure 4J). Figure 4K shows pachytene chromosomes from the 2n female controls. We did not find
256 mispaired chromosomes in cells which underwent PMER. From four chimeric triploid females in the
257 DdTr group, we successfully observed diplotene chromosomes with 25 bivalents (Figure 4M), and for one
258 female we additionally managed to obtain chromosomes during both the pachytene and diplotene stages.
259 In total, we examined 61 oocytes which included 25 bivalents of *C. elongatoides*. This suggests
260 successful transplantation followed by the normal formation of haploid gametes of *C. elongatoides*.
261 Triploid control fish can be seen in Figure 4N with 73 bivalents.

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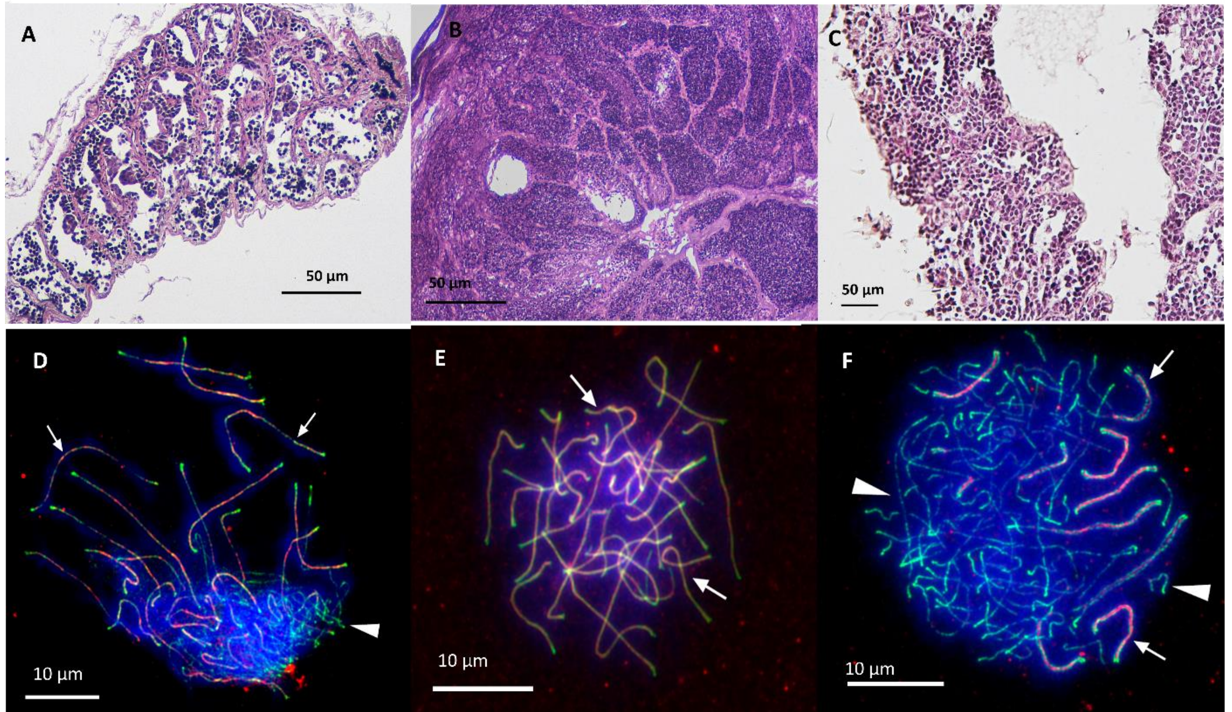

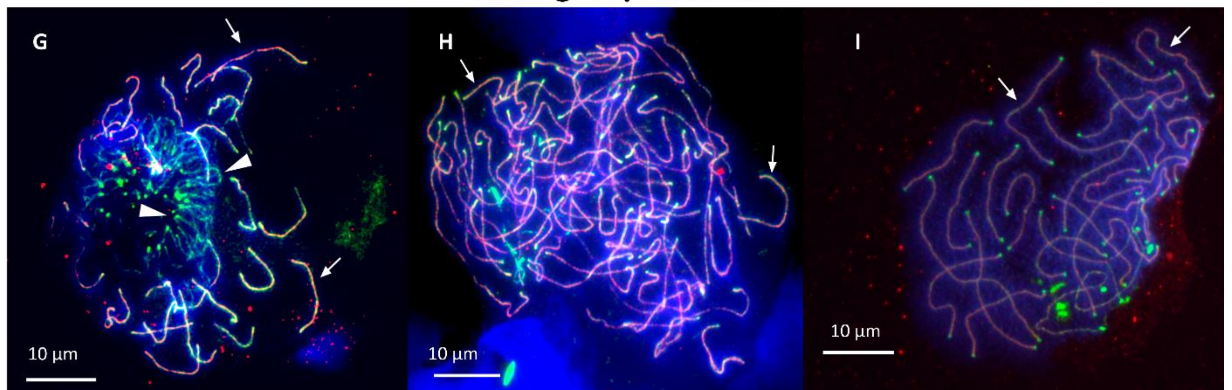

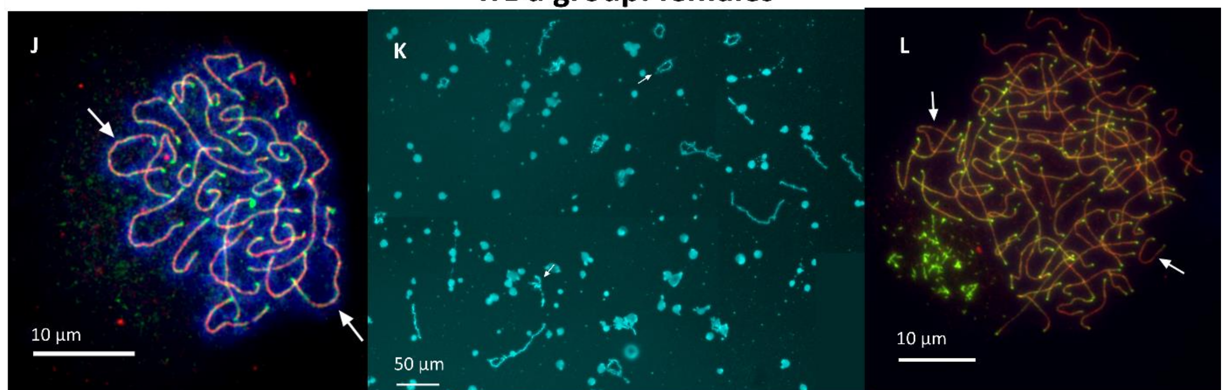

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263 **Figure 4.** Results of histology and pachytene analysis from a diploid recipient with a triploid donor
264 (DrTd, figures A-I), and a triploid recipient with a diploid donor (TrDd, figures J-L). A-C shows
265 comparisons of histology of diploid chimeric fish (A) with a diploid control (B) and a triploid control (C).
266 D-F show the pachytene analysis from the same fish samples. Diploid chimeric male (D) had an improper
267 pairing during the pachytene stage leading to many univalents, while the diploid control (F) shows 25
268 bivalents in proper pairing. The triploid male control also had improper pairing which is similar to the
269 diploid chimeric fish. Pachytene analysis of the female in the DrTd group shows a phenomenon typical of
270 triploid females described by Dedukh (9, 40), *i.e*. either improper pairing (G) or properly paired 74
271 bivalents as result of premeiotic endoreplication (H). The diploid female control (I) shows 25 bivalents.
272 (J) and (K) show the pachytene and diplotene chromosome analysis with 25 bivalents, respectively, in
273 triploid fish from the TrDd group. Synaptonemal complexes were immunolabeled with antibodies
274 against SYCP3 protein (green) and SYCP1 protein (red); chromosomes are stained with DAPI (blue). In K
275 microphotograph, diplotene chromosomes are stained with DAPI (cyan).

276

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277 **Table 1.** Summary of the chimeric experiment. DrTd group, diploid recipient, triploid donor; TrDd group,
278 triploid recipient, diploid donor; Biotype EE, parental species diploid *Cobitis elongatodies*; ETT, triploid
279 hybrid of *Cobitis elongatodies-taenia-taenia*. Genetic profiling is based on microsatellite loci from
280 chimeric offspring where the first number represents embryos derived from the recipient germ line and
281 the second number from the donor germ line (*i.e*. successful transplantation). Note that the term
282 "ordinary" in the histology analysis column stands for no difference from the control groups. We show
283 only successful chimeric fish.

ID	Sex of recipient	Biotype of recipient	Biotype of donor	Genetic profiling of offspring (donor derived/recipient dervied)	Pachytene analysis	Histology analysis
DrTd group						
5	F	EE	ETT	ETT 16 & EE 0	73 bivalents	ordinary gonad with eggs
6	F	EE	ETT	ETT 7 & EE 0	not done	NA
13	M	EE	ETT	not spawned	bivalents with univalents	reduced number of germ cells: no spermatozoa
16	M	EE	ETT	now spawned	bivalents and univalents	reduced number of germ cells: no spermatozoa
TrDd group						
17	F	ETT	EE	not spawned	25 bivalents	ordinary gonad with eggs
18	F	ETT	EE	EE 8 & 0 ETT	25 bivalents	ordinary gonad with eggs

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19	F	ETT	EE	EE 7 & 0 ETT	25 bivalents	ordinary gonad with eggs
20	F	ETT	EE	EE 17 & 0 ETT	not done	NA
21	F	ETT	EE	EE 15 & 0 ETT	not done	ordinary gonad with eggs
22	F	ETT	EE	Not spawned	25 bivalents	NA

284
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287
288 **Discussion**
289

290 Although asexual organisms are important models for many biological disciplines, the
291 mechanisms triggering clonal reproduction of gametes remain generally unclear. To test whether stimuli
292 for asexual development are intrinsic to differentiating germ cells or depend on their gonadal
293 environment, we performed reciprocal transplantation of spermatogonial cells between triploid hybrids
294 (*C. elongatoides-taenia-taenia*) and one of their parental sexual species (*C. elongatoides*).

295 Prior to the experimental transplantation of the donor's germ cells, the recipients' gonads had to
296 be sterilized. This goal was achieved with morpholino antisense RNA against the *Dnd* gene, which is a
297 routinely applied procedure in many cell transplantation experiments. This is quite challenging when
298 employed in hybrids and allopolyploids, because of the need to successfully target all gene copies coming
299 from diverged parental subgenomes in a hybrid (33). Nevertheless, although *Cobitis* hybrids combine
300 genomes that diverged as long as ~9 Mya (18), we achieved a reasonable rate of successful sterilization
301 ranging between 65-100 %, which is comparable to other studies (34, 35) and indicated that our MO
302 design based on one parental genome is sufficient to targets all orthologous alleles in such situations.

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303 After their successful transplantation, we recognized that male germ stem cells from male donors,
304 either *C. elongatoides* or the triploid hybrid, transdifferentiated into oogonia when transplanted into
305 female recipients. This demonstrated that both types of male germ stem cells were sensitive to the
306 gonadal environment and their sexual differentiation is largely driven by the recipient's body. This
307 observation is consistent with a hypothesis that fish germ stem cells are sexually plastic (so-called
308 sexually bipotent) and after transplantation, they may transdifferentiate into both oocytes and
309 spermatogonia, respectively, depending on the particular gonadal environment they occur in (36–39). By
310 contrast, we found no effect of the recipient's gonadal environment on the ability or inability of
311 transplanted germ cells to undergo clonal development. In particular, *C. elongatoides* male stem germ
312 cells transplanted into ETT female recipients consistently developed into reduced oocytes that gave rise to
313 recombining pure-bred progeny, while triploid male stem germ cells demonstrated the ability of PMER
314 when transplanted into *C. elongatoides* females and produced purely clonal progeny, or tetraploid
315 progeny with clonally transmitted maternal genome and incorporated sperm. This suggests that ability to
316 undergo PMER is inherent to the hybrid constitution of the asexual gonial cells rather than being affected
317 by their gonadal environment.

318 Our data also provided interesting insight into asymmetrical reproduction patterns of hybrids with
319 respect to sex, since the hybrids' spermatogonia transplanted into *C. elongatoides* males apparently didn't
320 express the ability of PMER and arrested their development in metaphase I due to abnormal pairing of
321 orthologous chromosomes. These results are in exact agreement with previous analyses of natural and
322 experimental ET and ETT hybrids (9, 40), which reported that PMER is confined to hybrid females and
323 occurs already in the F1 generation, while all hybrid males are sterile with spermatogonia arrested at
324 metaphase I (9, 30, 31, 41). How and why should the sex affect the initiation of PMER remains unclear,
325 however. Our data demonstrated that gonial cells originating from male-determined juveniles are able to
326 undergo PMER and develop into clonal progeny when transplanted into the ovary where they

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327 transdifferentiate into oogonia. This implies that in *Cobitis* hybrids, the ability of PMER reflects the
328 phenotypic sex of gonads and does not necessarily depend on genetic sex determination. By contrast,
329 investigation of hybrids from the related genus *Misgurnus*, (42, 43) demonstrated that female hybrids
330 which were sex-reversed into males maintained the ability to produce unreduced fertile sperm *via* PMER,
331 while natural hybrid males remained sterile (11). This would suggest that the cell's capability of PMER
332 may depend on genetic sex determination even when developing into male phenotypes.

333 The apparent discrepancy between our and Yoshikawa *et al.*'s (43) study may be due to
334 several reasons. First, asexual hybrids in both genera evolved independently from different parental
335 species and hence the particular type of cell deregulation leading to PMER may be different. Second,
336 there are to date no robust data on genetic sex determination in Cobitidae, albeit male heterogamy
337 (X1X2Y and X0, respectively) has been indicated in two species of the family (44, 45). Hence, the type of
338 sex determination may vary between both *Cobitis* and *Misgurnus* genera. Finally, Yoshikawa *et al*. (43)
339 investigated the development of female-originated gonial cells in phenotypic males, while we investigated
340 the fate of male-originated gonial cells transplanted into recipients of both sexes. It is thus theoretically
341 possible that hybrid primordial germ cells genetically determined as females maintain their capability of
342 PMER even when turned into spermatogonia [as in (43)], while primordial germ cells genetically
343 determined as males gain such a capability only when turned into female phenotypes, and produce sterile
344 gametes when maintaining their original sex (as in present study).

345 Either way, our findings, together with previously gathered information about germ cell development in
346 fishes, and in Cobitidae in particular, lead us to propose the following hypothesis related to the triggering
347 of asexuality: the capability of clonal gametogenesis (at least the one based on PMER) is rather
348 independent of the gonadal environment and appears triggered by intrinsic stimuli within asexual gonial
349 cells, which is causally linked to the hybrid constitution of their genomes. Nonetheless, the very execution

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350 of PMER appears to be primarily bound to an oogonial developmental pathway. It is at this level when
351 the gonadal environment affects development and asexuality, since primordial germ cells are sexually
352 bipotent and their differentiation into oogonia is decisively affected by the environment in which they
353 occur. Once the developmental pathway into male or female germlines is decided, the hybrid's gonial
354 cells develop into sterile spermatocytes, while the testes or fertile oocytes are capable of PMER while in
355 the ovary. This hypothesis implies two sets of crucial questions for future research in asexual organisms:
356 (1) How does the hybridization *per se* trigger PMER? and (2) Why is it usually linked to one sex in
357 hybrids? With currently available knowledge, we may so far offer only speculative answers.

358 First, PMER occurs already in F1 generations (9, 25, 40) and hence it is unlikely that this trait
359 evolves by accumulated mutations during evolution of hybrid populations. Instead, it is more likely that
360 the execution of PMER is based on developmental programs that have already existed in cells of sexual
361 progenitors of hybrid asexuals but are just triggered by the hybrid nature of the gamete. Possibly, the
362 initiation of PMER is driven by accumulated incompatibilities between genomes brought together by
363 hybridization, which fail to properly regulate gametic development and cell division leading to aberrant
364 chromosome duplications (5, 6, 18). For instance, the very nature of PMER, *i.e.* multiplication of the
365 genome without cell division, makes it at least superficially analogous to endopolyploidy, which is a
366 common mechanism how various organisms, including fishes, modify the genomic content of specific
367 cell types or tissues. Cellular mechanisms ensuring the alternation of S and G phases are relatively
368 conserved among various animal lineages (46), suggesting that cells of most organisms are capable of
369 endopolyploidy under the proper regulatory stimulus (47). Extrapolating Carman's model (8) of
370 asexuality, it is possible that PMER occurs in hybrid lineages when a particular type of misregulation
371 between admixed parental subgenomes generates endopolyploidy specifically in gonial tissue, leading to
372 stabilization of clonal lineage. The situation may be quite complex, however, and the result may crucially
373 depend on other traits than hybridization, *e.g.* the hybrid's ploidy affecting the stoichiometric ratio of

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374 orthologous alleles and their products. For instance, in *Poeciliopsis* spp. (mollies), diploid hybrids are
375 hybridogenetic (*i.e*. clonally transmit only one parental genome and exclude the other's before meiosis),
376 while triploid hybrids between the same parental species change the reproductive mode to gynogenesis
377 and clonality (48) Similarly, diploid hybrid *Misgurnus* spp. reproduce gynogenetically *via* PMER, while
378 tetraploid hybrids between the same parental species produce reduced gametes (30)

379 The second question may have a lot in common to fundamental differences between male and
380 female types of gametogenesis. Such differences may translate into the timing of DNA methylation in
381 male and female gametogenesis (49, 50). There is also evidence for differences in patterns of epigenetic
382 regulation between SSCs and derived oocytes from SSCs (51), which suggest an artificial epigenetic
383 restart of our transplanted SSCs. It may thus be proposed that in the hybrid's spermatogonia transplanted
384 into female recipients, the cell-cell communication between female somatic cells and transplanted SSCs
385 has led to the establishment of gonadal tissue according to the recipient's sex determination (52), thus
386 epigenetic reprogramming to female-like patterns and ultimate awakening of PMER. In that scenario, the
387 SSCs transplanted into male recipients would not undergo such a process. They would thus not gain the
388 ability of PMER.

389 The present findings demonstrated that the investigation of gametic development is likely to
390 provide crucial insights in understanding asexual reproduction and the establishment of interspecific
391 reproductive barriers in the speciation process. Namely, this study indicated that ability to perform
392 asexual gametogenesis *via* PMER is causally linked to hybrid composition gonial cells and is triggered by
393 factors intrinsic to these cells and developmental programs inherited from parental species. On the other
394 hand, it also appears that the execution of PMER is exclusive to the female germline, whose
395 determination apparently depends on cell-cell communication with surrounding gonadal tissue. Thus,
396 even in hybrid females, whose fertility is restored by PMER, the sex-specific factors of surrounding

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397 somatic tissue that control gametic development contribute to the postzygotic barrier, since PMER
398 prevents the hybrid's effective backcrossing to parental species.

399

400
401
402 Materials and Methods
403
404 Experimental protocol was approved by Ministry of Agriculture of the Czech Republic (reference
405 number: 55187/2016-MZE-17214).

406 Sterilization by *dead end* antisense oligonucleotide

407 Morpholino oligonucleotide was designed based on the *dnd* gene sequence of *Misgurnus*
408 *anguillicaudatus* (AB531494.1). The *dnd* sequence of *Cobitis taenia* obtained from
409 DDBJ/EMBL/GenBank transcriptome (GGJF00000000.1) was aligned against AB531494.1 by muscle
410 software v.3.8.1551 to validate specificity to the *Cobitis* genus. Alignment with the highlighted MO
411 target and phylogenetic maximum likelihood tree was performed with iqtree, 1.6.10 (53) and
412 approximate Bayes test (54). MFP+MERGE model selection was based on the *dnd* gene of *M.*
413 *anguillicaudatus* (AB531494.1), *Danio rerio* (AY225448.1), *Carrasius auratus* (JN578697.1:44-1140),
414 *Gobiocypris rarus* (KM044011.1), *Paedocypris progenetica* (KY828447.1), *Sinocyclocheilus*
415 *rhinocerous* (XM_016576295.1), *Rhodeus ocellatus* (MG995743.1). SNPs were checked at probe
416 position for any interspecific variability. Morpholino oligonucleotide was synthesized by Gene Tools,
417 LLCTM (Philomath, Oregon, U.S.A.).

418 The final solution for sterility induction was composed of 100 µM of MO and 300 ng/µL of
419 mRNA in combination with GFP and zebrafish (*D. rerio*) nos1 3´UTR and diluted in 0.2 M KCl (55).
420 The control group solution received only 300 ng/µL of mRNA diluted in 0.2 KCl. Solutions were loaded
421 into a microcapillary mounted on a micromanipulator (M-152 Narishige, Japan) with an automatic

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422 microinjector (FemtoJet Eppendorf, Germany). Each embryo was injected into a blastodisc at the one to
423 four cell stage. Altogether, 50 embryos were injected in each group.
424

425 **Histology analysis**

426 Either whole body segments or gonadal tissue were fixed overnight in Bouin's fixative.
427 Specimens were immersed in 70% ethanol, dehydrated and cleared in an ethanol–xylene series, embedded
428 into paraffin blocks, and cut transversally into 4 μm thick sections using a rotary microtome (Leica
429 RM2235; Wetzlar, Germany). Paraffin slides were stained with hematoxylin and eosin by using a staining
430 machine (Tissue-Tek DRS 2000; Sakura Finetek USA, Inc., Torrance, California) according to
431 standard procedures. Histological sections were photographed using a microscope (Nikon Eclipse Ci;
432 Tokyo, Japan) with a mounted camera (Canon EOS 1000D; Ōta, Tokyo, Japan). In the case of
433 morpholino treated fish, the sex identification was based on Fujimoto *et al*. (34) and Goto *et al*. (56).

434

435 **Induction of germline chimeras and donor-derived gametes production**

436

437 Five diploids *C. elongatoides* and two triploids *C. elongatoides-taenia-taenie* donor male specimens were
438 over anesthetized in tricaine solution (MS222), disinfected with 70% ethanol and decapitated. The body
439 cavity was carefully opened, and the gonads were removed and placed in ice-cold phosphate-buffered
440 saline (PBS). Testes were cut into smaller fragments to allow leakage of the sperm and serially washed in
441 PBS. Gonad fragments were transferred into 15 ml tubes and well chopped with scissors. Gonadal tissue
442 was enzymatically digested in 5 ml of PBS with 0.15% trypsin with a laboratory shaker at room
443 temperature for 1.5 h. DNase I (Sigma Aldrich 10104159001; Merck, Burlington, Massachusetts, USA)
444 (aliquoted to 5% stock solution in RNase free water) was added continuously when clumping was

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445 observed. Afterwards, digestion was terminated by the addition of 5 ml L15 with 20% fetal bovine serum,
446 filtrated through 30μm filters, and centrifuged at 400*g* for 10 min. The supernatant was removed, and the
447 pellet was carefully resuspended. The cell suspension was loaded into a pulled glass microcapillary
448 mounted on a micromanipulator with a pneumatic injector.

449 Prior to cell transplantation, primordial germ cells were depleted and, 2n and 3n recipients were
450 prepared by injection of antisense morpholino designed against the *dead-end* gene. Sterilized recipients
451 were anesthetized in 0.05% tricaine solution, placed on an agar coated Petri dish and cells were injected
452 into the coelomic cavity. Recipients were transferred into fresh water to recover. Germline chimeras were
453 cultured at room temperature in aquaria with controlled cooling and water filtration and fed *ad libitum*
454 with brine shrimps (*Artemia* sp.), bloodworms (*Tubifex* sp.), and a dry diet. Two groups of chimeras were
455 made: 1) diploid recipient and triploid male donor (DrTd) and 2) triploid recipient and diploid male donor
456 (TrDd).
457
458 **Spawning**

459 Six months prior to spawning, water temperature was slowly decreased (-2 °C per day) to 14 °C
460 and kept at this level for 3 months. In the following 3 months, water temperature was increased (+2 °C
461 per day) to final temperature of 22 °C and kept till spawning with increased bloodworm feeding.
462 Transplanted fish were exactly 2 years old at the time of spawning. Transplanted fish together with
463 control fish (*C. elongatoides*) were injected twice (24 and 12 h prior to spawning) with Ovopel (Interfish
464 Kf, Budapest, Hungary). A solution for the first injection was made from one Ovopel pill per 20 mL of
465 0.9% NaCl. The solution for the second injection was made from one Ovopel pill per 5 mL of 0.9% NaCl.
466 In both cases, the volume of the Ovopel solution directly injected into a fish body was 0.05 mL per 10 g
467 of fish weight. Eggs were carefully removed from spined loaches and put in a dried Petri dish. Sperm

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468 were added to the eggs together with fresh water. The number of fish in each group was as follows: 14
469 fish in the DrTd group (six females, eight males), eight fish in TrDd (eight females, zero males), and six
470 males of *C. elongatoides* as controls.

471 **Identification of chimeras**

472 In this study, we used parental species *C. elongatoides* (EE – composition of genomes) and
473 triploid hybrids between *C. elongatoides and C. taenia* (ETT). Diploid parental species produce haploid
474 gametes while hybrids produce oocytes which contain both genomes; therefore presence/absence of C.
475 *taenia* (TT) species in offspring can prove the success of transplantation. Successfully transplanted 2n EE
476 fish will produce ETT eggs while 3n ETT fish will produce haploid eggs with genome E. Indication of
477 five specific loci Cota068, Cota111, Cota010, Cota093, Cota032 of *C. taenia* (57) in offspring from
478 parental species of EE means that diploid fish possess hybrid gonads. On the other hand, triploid fish ETT
479 producing haploid eggs represent the occurrence of diploid gonads in triploid fish. To support our results,
480 we also used flow cytometry analysis and meiosis analysis on both parental species and offspring.

481 **DNA extraction and analysis of microsatellites**

482 Whole genomic DNA from individuals tested (*C. elongatoides* and *C. elongatoides-taenia-*
483 *taenia*) were extracted from a dorsal fin in adults or part of larvae using a commercial Tissue DNA
484 Isolation Kit (Geneaid Biotech, Taipei, Taiwan) following the manufacturer's protocol. Genotype
485 determination in the fishes was performed by analyses of selected microsatellite species specific loci (31,
486 57). Fragment-length analyses were performed on an ABI 3730 Avant capillary sequencer (Applied
487 Biosystems, Foster City, California, USA) with an internal size standard (GeneScan-500 LIZ, Thermo
488 Fisher Scientific, Waltham, Massachusetts, USA); the alleles were scored manually with GeneMapper
489 v. 3. 7 (Applied Biosystems, Zug, Switzerland).

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490 **Flow cytometry analysis**

491 The level of ploidy was determined as the relative DNA content of fin clip cells *via* flow
492 cytometry (Partec CCA I; Partec GmbH, Munster, Germany with a UV mercury lamp for excitation and
493 an emission level of 435/500 nm) using standard CyStain® DNA 1-step solution (Sysmex CZ s.r.o., Brno,
494 Czech Republic) containing 49.6-diamidino-2-phenylindol (DAPI). As a reference standard, we used a fin
495 clip of diploid *C. elongatoides*.

496 **Pachytene chromosomes with immunofluorescent staining**

497 Pachytene chromosomes were obtained from males and females according to protocols described by
498 Moens (58) and Araya-Jaime *et al*. (59). Ovaries were homogenized manually in 1× PBS solution.
499 Afterwards, 20 µl of cells suspension was put on SuperFrost® slides (Menzel Gläser; Thermo Fisher
500 Scientific) followed by addition of 40 µl of 0.2 M sucrose and 40 µl of 0.2% Triron X100 for 7 min. The
501 samples were fixed for 16 min by adding 400 µl of 2% PFA. Testes were homogenized manually
502 followed by dropping 1 µl of suspension into 30 µl of hypotonic solution (1/3 of 1× PBS) and then
503 dropped onto SuperFrost® slides (Menzel Gläser; Thermo Fisher Scientific). The samples were fixed in
504 400 µl of 2% PFA for 4 min. After fixation, slides with the pachytene samples from males and females
505 were air dried and washed in 1× PBS.

506 Slides were stored until immunofluorescent staining of synaptonemal complexes (SC). Lateral
507 components of synaptonemal complexes (SCs) were visualized by rabbit polyclonal antibodies (ab14206,
508 Abcam) against SYCP3 protein while the central component of SCs was detected by chicken polyclonal
509 antibodies against SYCP1 protein (a gift from Sean M. Burgess). Fresh slides were incubating with 1%
510 blocking reagent (Roche) in 1× PBS and 0.01% Tween-20 for 20 min followed by the addition of primary
511 antibody for 1h at RT. Slides were washed 3 times in 1× PBS at RT and incubated in the combination

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512 with secondary antibodies Alexa 488-conjugated goat anti-rabbit IgG (H+L) (Molecular Probes) and
513 Alexa-594-conjugated goat anti-chicken IgG (H+L) (Molecular Probes) for 1h at RT. Slides were washed
514 in 1× PBS and mounted in Vectashield/DAPI (1.5 mg/ml) (Vector, Burlingame, Calif., USA).

515 Diplotene chromosomal samples (also known as "lampbrush chromosomes") were prepared from
516 parental and hybrid females according to an earlier published protocol (60). Vitellogenetic oocytes of 0.5–
517 1.5 mm in diameter were taken from females in the OR2 saline [82.5 mM NaCl, 2.5 mM KCl, 1 mM
518 MgCl2, 1 mM CaCl2,1mM Na2HPO4, 5 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic
519 acid); pH 7.4]. Isolation of the oocytes` nuclei was performed manually in the isolation medium "5:1" (83
520 mM KCl, 17 mM NaCl, 6.5 mM Na2HPO4, 3.5 mM KH2PO4, 1mM MgCl2, 1 mM DTT (dithiothreitol);
521 pH 7.0–7.2) using jeweler forceps (Dumont, Switzerland). Nuclear envelopes were manually removed in
522 a quarter strength "5:1" medium with the addition of 0.1% paraformaldehyde and 0.01% 1M MgCl2 in
523 glass chambers attached to a slide. After this procedure, we obtained chromosome samples from
524 individual oocytes in each chamber. Slides with oocyte nuclei contents were subsequently centrifuged for
525 20 min at +4 °C, 4000 rpm, fixed for 30 min in 2% paraformaldehyde in 1x PBS, and post-fixed in 70%
526 ethanol overnight (at +4°C).

527 Pachytene and diplotene chromosomes were investigated using a Provis AX70 Olympus
528 microscope with standard fluorescence filter sets. Microphotographs were captured by CCD camera
529 (DP30W Olympus; Tokyo, Japan). Olympus Acquisition Software was used for capturing the images
530 followed by their adjustment and arrangement in Adobe Photoshop, CS6 software.

531

532
533
534 **Acknowledgments**
535
536 This research was funded by Institutional Research Concept RVO67985904 and by Czech Science Foundation,
537 projects No. 17-09807S (to M.P. & K.H.) and 19-21552S & 21-25185S (to Lab. of Fish Genetics). The

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538 transplantation and fish manipulation were financially supported by the Ministry of Education, Youth and Sports of
539 the Czech Republic - project CENAKVA (LM2018099) and Biodiversity (CZ.02.1.01/0.0/0.0/16_025/0007370).
540
541 Prof. Sean Burgess for sharing primary antibodies against SYCP1.
542
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