J. Appl. Ichthyol. 24 (2008), 63–67

ISSN 0175–8659

Received: November 5, 2006
Accepted: February 18, 2007
doi:10.1111/j.1439-0426.2007.00915.x

Fertility in first-generation hybrids of roach, *Rutilus rutilus* (L.), and silver bream, *Blicca bjoerkna* (L.)

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Summary

Fertility in first-generation hybrids of roach, *Rutilus rutilus*, and silver bream, *Blicca bjoerkna*, was investigated. Sperm and egg production of hybrids at first sexual maturity were examined. Eggs from female hybrids were artificially fertilized with the sperm of a corresponding hybrid male; a hybrid male from the reciprocal crossbreeding; a parental species male *R. rutilus;* and a parental species male *B. bjoerkna*. The results revealed that gametogenesis was normal in female hybrids. However, in male hybrids, a low efficiency of gametogenesis was observed. The semen of male hybrids was extremely dilute, with spermatozoa concentration lower than that in parental species. Nevertheless, these F1 hybrids (males and females) from reciprocal crossbreeding were fertile. F2 and backcross generations were produced, but F2 crosses from the female hybrid and corresponding hybrid male displayed a drastically slower hatching rate. Also higher proportions of deformed embryos were hatched than in other post-F1-generation crosses.

Introduction

Roach, *Rutilus rutilus* L. and silver (or white) bream, *Blicca bjoerkna* L., have the same diploid chromosome complement (2n = 50–52) (Vasil'ev, 1980; Klinkhardt et al., 1995). The same number of chromosomes in these species may promote fertile hybrids because the chromosome constitution of both parents must be homologous so that pairing and segregation can occur at meiosis (Nikolukin, 1946).

Several hybrids in cyprinid fish have been studied but the majority of the research has concentrated almost entirely on their identification or description (Brassington and Ferguson, 1976; Bianco, 1982; Collares-Pereira and Coelho, 1983; Mir et al., 1988; Berrebi et al., 1989; Economidis and Wheeler, 1989; Golubtsov et al., 1990; Ünver and Erk'akan, 2005). Little scientific attention had been paid to the fertility of hybrids, especially, in hybrids produced in experimental conditions when compared with the voluminous research on hybrid descriptions. For hybrids of *R. rutilus* and *B. bjoerkna*, this situation reflects reality because these hybrids are probably not frequent in the wild. Their existence has been reported (Wheeler, 1969; Penczak, 1978; Swinney and Coles, 1982) and nevertheless constitutes a suitable reason to study them in all aspects of their biology, including their fertility.

It is, therefore, important to establish whether hybrids of *R. rutilus* and *B. bjoerkna* are capable of crossbreeding, either amongst themselves or with one or the other of their parental species. In the current study, we report the results of eight post-F1 crossbreeding experiments conducted under laboratory conditions, studying two types of female hybrids: the F1 hybrids between *B. bjoerkna* male × *R. rutilus* female and *R. rutilus* male × *B. bjoerkna* female.

Material and methods

Production of the spawners

Rutilus rutilus and *B. bjoerkna* specimens were captured in fish ladders at the Lixhe dam (Belgian Meuse River, 50°45′N; 5°40′E) during the reproductive migration in spring 2003. They were morphologically identified following the descriptions made by Regan (1911), Spillman (1961), Wheeler (1969) and Maitland (1972). Female eggs of each fish species were divided into two equal parts, each of which was artificially fertilized with the sperm of the other species (first-generation hybrids) or with the sperm of the conspecific male (intraspecific crossbreeding). These crossbreedings were designed for obtaining fish spawners; the characteristics of the fish used are indicated in Table 1. This procedure produces the hybrids of *B. bjoerkna* male × *R. rutilus* female ($B_m \times R_f$) and its reciprocal hybrids *R. rutilus* male × *B. bjoerkna* female ($R_m \times B_f$). Parental fish species (*R. rutilus* and *B. bjoerkna*) were produced starting from intraspecific crossbreedings. The hybrid and parent species used were reared in captivity (20°C, photoperiod 16 L / 8 D) at the Tihange aquaculture station in Belgium. These fish were sexually mature in 24 months.

Ovulation conditions and post-F1 crossbreedings

Selected females were exclusively F1 hybrids and gravid. Two types of female hybrids, – (i) the F1 hybrids between *B. bjoerkna* male × *R. rutilus* female (BR) and (ii) *R. rutilus* male × *B. bjoerkna* female (RB) – were studied. Males consisted of a corresponding hybrid male of the female; a hybrid male from the reciprocal crossbreeding; a parental species male *R. rutilus;* and a parental species male *B. bjoerkna*. All males produced milt. Ovulation was observed under environmental conditions at water temperature 20 ± 0.2°C, dissolved oxygen 7.9 ± 0.5 mg l^{-1}, pH 8.1 ± 0.5, photoperiod 16 L / 8 D, spawning substrate and mixture of male and female hybrids. In each type of female hybrid, two groups of fish were considered for spawning examinations. In the first group, two females and four males were mixed (range: females 145–155 mm Fork length (FL), 44–63 g; and males 140–155 mm FL, 51–60 g for BR and RB, respectively). The second group was composed of three females and six males (range: females 135–155 mm FL, 37–61 g; and males 123–155 mm FL, 26–54 g for BR and RB, respectively). For the post-F1 crossbreeding experiments, two

Table 1
Crossbreeding and characteristics of F0 spawners used to obtain F1 generation (fork length, weight and age)

F1 crossbreedings	Characteristics of F0 spawners				
Male (m) × female (f)	Fish	Fork length (mm)	Weight (g)	Age (years)	Soft rays of anal fin (n)
$R_m \times R_f$	R_f	196	115	5	11
$B_m \times R_f$	R_m	250	251	5	11
$R_m \times B_f$	B_f	216	226	5	20
$B_m \times B_f$	B_m	230	228	5	20

R, *Rutilus rutilus*; B, *Blicca bjoerkna*.

groups of one female and two males were formed in each type of female hybrids.

Female hybrids and their corresponding male hybrids were placed in a $0.92 \times 0.40 \times 0.40$-m experimental nylon basket installed in a $6.00 \times 1.00 \times 0.67$-m tank (closed loop). It was equipped with a bullor for oxygenation and a 0.16×0.16-m synthetic spawning substrate simulating vegetation. The female hybrid was generally mature the following day and was taken on spawning substrate. The eggs were stripped and divided into four equal parts. Each part was artificially fertilized with the sperm of one of the following: a corresponding hybrid male; a hybrid male from the reciprocal crossbreeding; a parental species male *R. rutilus;* or a parental species male *B. bjoerkna*, at 1 ml 100 g^{-1} of spawn. This post-F1 crossbreeding procedure was repeated twice for each type of female hybrid. Eight post-F1 crossbreeding experiments (Table 2) were conducted with these two types of female hybrid. The characteristics of the hybrids and parental species used in post-F1 crossbreedings are detailed in Table 3. Incubation took place in 1-L Zug jars at 21 ± 0.2°C water temperature, 7.7 ± 0.6 mg l^{-1} dissolved oxygen, 8.3 ± 0.3 pH, and 0.03 ± 0.2 and 0.29 ± 0.2 mg l^{-1}, nitrites and ammonium, respectively.

Table 2
Post-F1 crossbreeding descriptions

F1 female hybrid	Post-F1 crossbreedings Male × female
BR	B × BR
	BR × BR
	RB × BR
	R × BR
RB	B × RB
	BR × RB
	RB × RB
	R × RB

R, *Rutilus rutilus*; B, *Blicca bjoerkna*; BR, F1 hybrid of male *B. bjoerkna* and female *R. rutilus*; RB, F1 hybrid of male *R. rutilus* and female *B. bjoerkna*.

Egg, sperm and embryo analysis

In each type of female hybrid, the spawning rate was defined as the percentage of completely ovulated female hybrids of the five females sampled. The gonadosomatic index (GSI) was expressed as the percentage of egg weights spawned per total body weight. Absolute fecundity was considered as the total number of eggs spawned per female and calculated from two samples of eggs (1 g). For egg diameters, samples of 60 eggs per female hybrid were individually measured using a microscope provided with an ocular micrometer.

Sperm concentration was determined from samples of 10 males per type of hybrid (mean ± SD: 141 ± 12.0 mm FL, 39 ± 10.2 g and 133 ± 9.6 mm FL, 30 ± 7.9 g for BR and RB, respectively) or parental species (131 ± 7.2 mm FL, 37 ± 6.4 g and 167 ± 16.4 mm FL, 65 ± 16.8 g for *B. bjoerkna* and *R. rutilus*, respectively). Milk was extracted with a syringe and 200-fold diluted with an extender, a bicine solution at pH 7.8 (Moore, 1996). Sperm concentration was estimated by counting spermatozoa in 30 random cases (0.0025 mm^2) of a hemocytometer (Bürker's cell) on a phase contrast microscope (400×). Sperm concentration was expressed as the number of spermatozoa per milliliter of semen (Rougeot et al., 2004).

The fertilization rate was expressed as the percentage of 'eyed' embryos 1 day after fertilization. In each crossbreeding experiment, two replications of 100 eggs sampled were observed under a microscope.

The hatching rate was evaluated by the percentages of embryos hatched 4 days after fertilization from samples of 500 eggs (two replications per post-F1 crossbreeding).

The embryos were measured directly upon hatching from samples of 50 embryos per post-F1 crossbreeding in duplicate. Embryos hatched were individually measured (total length ± 1 mm on a microscope) and weighed (body weight ± 0.1 mg).

At hatching, deformed embryos were identified and counted by microscopic examination in samples of 50 hatched embryos (two replications per post-F1 crossbreeding). The deformed embryos were presented as the percentage of deformed embryos per total of embryos hatched.

Table 3
Characteristics of breeders used to obtain post-F1-generation hybrids

		Characteristics of R1–R2					
Breeders	Sex	Fork length (mm)	Weight (g)	Soft rays of anal fin (n)	Sperm density (cells × 10^9 ml^{-1})	GSI (%)	Absolute fecundity (eggs × 10^3)
B	Male	122–134	31–41	20–20	14.0–13.2	–	–
BR	Male	130–145	25–37	15–15	2.6–4.0	–	–
RB	Male	140–130	34–25	15–15	2.9–3.0	–	–
R	Male	165–160	64–53	11–11	33.3–24.5	–	–
BR	Female	155–152	62–55	15–15	–	19.6–17.0	8.8–7.2
RB	Female	155–157	59–61	15–15	–	15.6–16.6	7.9–9.5

R, *Rutilus rutilus*; B, *Blicca bjoerkna*; BR, F1 hybrid of male *B. bjoerkna* and female *R. rutilus*; RB, F1 hybrid of male *R. rutilus* and female *B. bjoerkna*; GSI, gonadosomatic index at spawn.
Range – values of first (R1) and second (R2) replications.

Statistical analysis

The fertilization rate, hatching rate and proportions of embryos deformed in post-F1-generation hybrids were compared using Fisher's exact probability (FEP)-test. Mean performance of embryos hatched (total length and body weight) and spermatozoa density were analyzed with the Kruskal–Wallis (KW)-test followed by multiple paired comparisons tests using the Mann–Whitney *U*-test. For all statistical analyses, probability values <0.05 were considered significant.

Results

In this study, all types of female hybrids completely ovulated (100%) under our experimental conditions (Table 4). Neither the GSI nor the absolute fecundity were significantly different (Mann–Whitney *U*-test, $P > 0.05$) between BR and RB (respectively, median values = 16.5% and 15.6% for GSI and 7.2×10^3 and 7.9×10^3 eggs for fecundity). However, for egg diameters, the difference was significant (*U*-test, $P < 0.0001$) between BR and RB (mean ± SD = 1.2 ± 0.1 and 1.3 ± 0.1 mm, respectively).

The comparative sperm density in the parental species and the first-generation hybrids between *R. rutilus* and *B. bjoerkna* is summarized in Fig. 1. The KW-test showed a significant effect of the F1 crossbreedings on the sperm density (d.f. = 3, $H = 31.575$, $P < 0.0001$). In paired comparisons tests, statistical analysis indicated that the sperm density of hybrids was substantially lower (*U*-test, $P < 0.001$) than in parental species. In hybrids, the sperm density was not significantly different (*U*-test, $P > 0.05$) between BR and RB (median values = 2.6×10^9 and 3.0×10^9 spermatozoa ml^{-1}, respectively). In parental species, the sperm density was significantly different (*U*-test, $P < 0.05$) between *R. rutilus* and *B. bjoerkna* (31.1×10^9 and 12.4×10^9 spermatozoa ml^{-1}, respectively).

Fertilization and hatching rates in post-F1-generation hybrids (Table 5), respectively assessed 1 and 4 days after fertilization, revealed that F2-generation hybrids (range: 83–97%) were not significantly different (FEP-test, $P > 0.05$) from the backcrosses (82–96%) in terms of the fertilization rate. However, for the hatching rate, F2-generation hybrids from the female hybrid and its corresponding hybrid male (0–1.8%) were substantially lower (FEP-test, $P < 0.0001$) than those of other types of F2-generation hybrids (35–62%) and backcrosses (10–65%).

For the total length and body weight of embryos hatched, a significant effect of post-F1 crossbreedings was observed in female hybrid RB (KW-test for length and weight: d.f. = 3, $H = 98.689$, $P < 0.0001$ and d.f. = 3, $H = 47.815$, $P < 0.0001$). In female hybrid BR, the post-F1 crosses showed a significant effect on the weight (KW-test, d.f. = 3, $H = 197.838$, $P < 0.0001$), but no significant difference was found in the total length (KW-test, d.f. = 3, $H = 5.379$, $P = 0.1461$). Between the post-F1 crossbreedings, in female hybrid RB, the F2 crossbreeding from the female hybrid and its corresponding male hybrid (RB × RB, mean values = 5.1 mm and 7.8×10^{-1} mg, total length and weight,

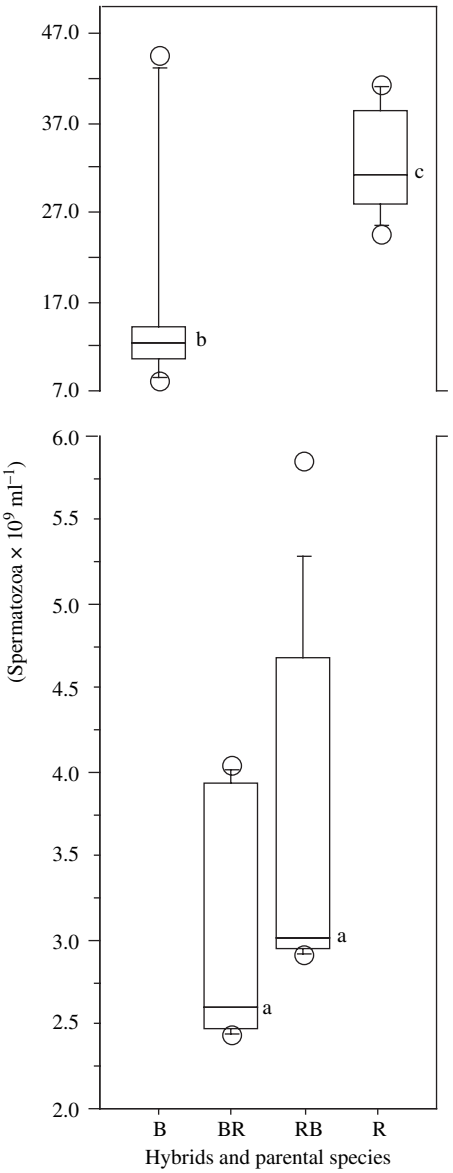

Fig. 1. Comparison of sperm density for hybrids and parent species. R, *Rutilus rutilus*; B, *Blicca bjoerkna*; BR, F1 hybrid of male *B. bjoerkna* and female *R. rutilus*; RB, F1 hybrid of male *R. rutilus* and female *B. bjoerkna*; values are median, percentiles 5, 25, 75 and 95; horizontal line inside the box marks the position of the median; circles indicate minimal and maximal values, n = 10; hybrids or parental species marked with same letter are not significantly different (Mann–Whitney *U*-test, $P < 0.05$)

Table 4
Spawning rate, gonadosomatic index at spawn (GSI), absolute fecundity and egg diameters of female hybrids

Female hybrid	n	Spawning rate (%)	GSI (%)		Absolute fecundity, (eggs × 10^3)		Egg diameters, (mm), n = 300	
			Median	Range	Median	Range	Mean	Range
RB	5	100	15.6	15.5–21.4	7.9	7.6–15.5	1.3	0.8–1.5
BR	5	100	16.5	15.0–19.6	7.2	4.0–8.8	1.2	1.0–1.6

BR, F1 hybrid of male *B. bjoerkna* and female *Rutilus rutilus*; RB, F1 hybrid of male *R. rutilus* and female *B. bjoerkna*.
Range – extreme values.

Table 5
Fertilization rate, hatching rate, total length and weight for embryos hatching in post-F1-generation hybrids

Breeders		Fertilization rate (%), n = 100 eggs		Hatching rate (%), n = 500 eggs			Embryos hatched (mean ± SD)	
Male	Female hybrid	Mean	Range	Mean	Range	n	Total length (mm)	Weight (10^{-1} mg)
B	BR	92	90–93	61	56–65	100	5.9^{a} ± 0.3	8.0^{b} ± 0.9
	RB	90	88–92	38	24–47	103	5.6^{b} ± 0.2	8.8^{b} ± 0.8
BR	BR	86	83–88	0.7	0–1	53	5.9^{a} ± 0.1	9.5^{d} ± 0.8
	RB	91	89–92	51	35–62	101	5.6^{b} ± 0.3	9.0^{c} ± 1.1
RB	BR	94	92–96	47	44–49	108	6.4^{a} ± 1.2	6.0^{a} ± 0.1
	RB	95	96–97	1.5	1.0–1.8	31	5.1^{a} ± 0.3	7.8^{a} ± 0.8
R	BR	95	94–96	47	32–58	100	6.0^{a} ± 0.1	9.1^{c} ± 1.0
	RB	85	82–89	15	10–18	104	5.8^{c} ± 0.3	8.7^{b} ± 1.1

B, *Blicca bjoerkna*; BR, R, *Rutilus rutilus*; F1 hybrid of male *B. bjoerkna* and female *R. rutilus*; RB, F1 hybrid of male *R. rutilus* and female *B. bjoerkna*.
Mean values – mean values of four replications from duplicates of crossbreeding experiments in each type of female hybrid; range – extreme values of replicates; n – number of embryos observed.
By female hybrid, mean values with same superscripts in same column not significantly different (Mann–Whitney *U*-test or Fisher's exact probability test, $P < 0.05$).

respectively) displayed the smallest size (*U*–test, $P < 0.0001$). However, the largest size was observed in R × RB (5.8 mm) for the total length and in BR × RB (9.0×10^{-1} mg) for the weight. In female hybrid BR, the highest weight (*U*–test, $P < 0.05$) was found in the F2 crossbreeding from the female hybrid and its corresponding male hybrid (BR × BR, 9.5×10^{-1} mg) and the smallest weight (*U*–test, $P < 0.0001$) in RB × BR (6.0×10^{-1} mg). The mean values in F2-generation hybrids (5.1–6.4 mm) were not significantly different (*U*–test, $P > 0.05$) from backcrosses (5.6–6.0 mm). As for the total length, the mean values of weight in F2-generation hybrids (6.0×10^{-1}–9.5×10^{-1} mg) were not significantly different (*U*-test, $P > 0.05$) from backcrosses (8.0×10^{-1}–9.1×10^{-1} mg).

The proportions of deformed embryos at hatching (Fig. 2) revealed that with F2 crossbreeding in particular, the female hybrid and its corresponding male hybrid (mean value, 34.0% and 37.5%, respectively, for BR × BR and RB × RB) were much higher (FEP-test, $P < 0.001$) than for other type of F2-generation hybrids with the female hybrid and its reciprocal hybrid male (10.0% for RB × BR and 5.0% for BR × RB) and backcrosses (6.0% and 7.0%, respectively, for B × BR and B × RB and, 7.5% and 6.5% for R × BR and R × RB).

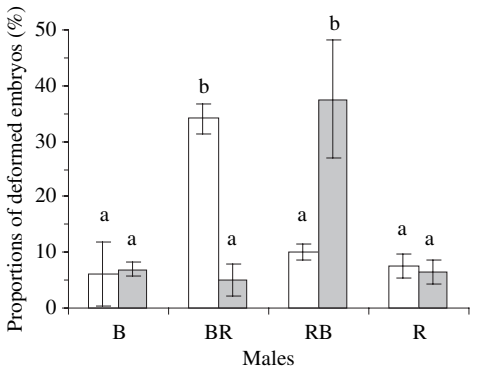

Fig. 2. Proportions of deformed embryos at hatching (mean values) in F2 cross and backcrosses. Vertical bars indicate range of crossbreeding replicates; R, *Rutilus rutilus*; B, *Blicca bjoerkna*; BR, F1 hybrid of male *B. bjoerkna* and female *R. rutilus*; RB, F1 hybrid of male *R. rutilus* and female *B. bjoerkna*; values across common bars with different letters differ significantly (Fisher's exact probability test, $P < 0.05$); n = 200 for all post-F1 crossbreedings except BR × BR n = 46 and RB × RB n = 36; □ – female hybrid BR; ■ – female hybrid RB

Discussion

A successful spawning rate was observed in female hybrids under our experimental conditions. This reveals that these female hybrids were capable of ovulating completely in captivity without injection of hormonal inductors. In addition, success on the GSI, fecundity and egg sizes of female hybrids suggests that these female hybrids have a normal gametogenesis. In particular, in this study, female hybrids were at their first sexual maturity: first spawns are often smaller than subsequent spawns.

Eggs and sperm were produced by female and male hybrids of *R. rutilus* and *B. bjoerkna*, respectively. This indicates that these inter-generic hybrids have the biological capability to reproduce. In hybrids, as in fish species or other animals, the reproductive success principally depends on the quality of both male and female gametes. However, in male hybrids, the semen was extremely diluted, with spermatozoa concentration lower than that in parent species. This suggests a low efficiency of gametogenesis in male hybrids. Indeed, Stoumboudi et al. (1993) suggested that the spermatozoan index is a more accurate indicator of both testicular activity and the timing of reproductive activity than the GSI. The low sperm density of these male hybrids could result from genetic factors. In hybrids of *Scardinius erythrophtalmus* (L.) and *Abramis brama* (L.), the absence of spermatozoa was observed in some individual hybrids and has been explained by the conditions of artificial rearing (Kopiejewska et al., 2004). For Chevassus (1983), it could also result from the gametic sterility that is found in hybrids of fish. In artificial hybrids of *Clarias gariepinus* Burchell (2n = 56) and *Heterobranchus longifilis* Valenciennes (2n = 54), the low efficiency of gametogenesis observed has been explained by the unequal parental haploid numbers, which may have induced problems in chromosome pairing during meiosis (Legendre et al., 1992). The unsuccessful pairing of the homologous chromosomes in natural sterile hybrids of *Barbus longiceps* Valenciennes and *Capoeta damascina* Valenciennes had probably prevented spermatogenesis from extending beyond the pachytene of the first meiotic division (Stoumboudi and Abraham, 1996). In these male hybrids, the male germ cell protective barrier was absent.

The high fertilization rate in post-F1-generation hybrids observed in this study indicates that the first-generation hybrids (males and females) are fertile. The success of the fertilization rate could be attributed to the genetic affinity of

parental species and their resemblance in chromosomal numbers or formulas. However, the low hatching rate and the high proportion of deformed embryos in F2-generation hybrids from the female hybrid and its corresponding hybrid male could result from genetic factors. This low hatching rate is probably caused by the presence of lethal recessive allele pairing and their expression, as in inbreeding depression (Mrakovac and Haley, 1979; Park et al., 2006), and the difficulty in proteolytic enzyme secretions that destroy the chorion protein layers and thus hatching. In hatched embryos, vertebrate anomalies such as scoliosis and lordosis were observed. These anomalies in fish are generally caused by water temperatures (Gabriel, 1944), hereditary factors (Orska, 1962; Gill and Fisk, 1966) and toxic environmental factors at the egg stage or the embryonic developing stage (Bucke, 1974). In this investigation, the causes of these abnormalities have not been identified, but it is possible that genetic factors were implicated. Moreover, in this study, crossbreeding for obtaining post-F1-generation hybrids was done based on spawners that have strong parental bonds. Nevertheless, for several authors, hybrid sperm always led to a lower hatching rate and a higher proportion of deformed larvae than semen from pure species (Wood and Jordan, 1987; Legendre et al., 1992).

To conclude from the data obtained, we have demonstrated that the F1 hybrids (males and females) from reciprocal crossbreeding of these species were normally viable. This could indicate an absence of an asymmetrical hybridization gene. On the contrary, in hybrids of *Barbus barbus* (L.) and *Barbus meridionalis* (L.) only female hybrids are fertile (Philippart and Berrebi, 1990). Normal embryos obtained in all cases of F2 or backcross fertilizations proved, despite various abnormalities, that male and female hybrids were not sterile. Thus, the risk of genetic contamination of natural fish stocks by post-F1-generation hybrids cannot be overlooked. In addition, there remains the problem of their identification in the natural environment because the variation in observable characteristics in post-F1 hybrid offspring is increased. Evidently, at the ecological level, this situation has implications on the genetic integrity of parent species, the preservation of biodiversity and the population dynamics of fish stocks.

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