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Experimental hybridization of *Barbus barbus* and *Barbus meridionalis* : physiological, morphological, and genetic aspects

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Abstract

F1 hybrids were produced by crossing, in artificial conditions, female *Barbus barbus* and male *B. meridionalis*. Results from the genetic study describing enzymatic patterns produced after electrophoresis of experimental F1 hybrids confirmed a great part of the original interpretation of patterns observed in the natural population. Morphological studies which examined denticle length on the last hard ray of the dorsal fin (well developed in *B. barbus* and absent in *B. meridionalis*), showed that F1 hybrids' values were not exactly intermediate between the parental values. Up to two years old, experimental hybrids displayed identical growth rates and sexual dimorphism of length (in favour of the females) as *B. barbus* growing in the same conditions. At this age, in female hybrids, the ovaries matured and produced fertilizable ovules: in male hybrids, the testicles were morphologically normal but no sperm was produced. If this sterility is confirmed in the reciprocal cross (male *B. barbus* × female *B. meridionalis*), the phenomenon would be an essential feature in understanding this hybridization.

Keywords : Experimental hybridization, sterility, fish physiology, allozymes, *Barbus*, Cyprinidae.

Hybridation expérimentale de Barbus barbus *et* Barbus meridionalis : *aspects physiologiques, morphologiques et génétiques.*

Résumé

Le croisement, par reproduction artificielle, de *Barbus barbus* femelles et de *B. meridionalis* mâles, a permis d'obtenir des hybrides F1, introuvables dans le bassin de l'Hérault (France) où les deux espèces s'hybrident naturellement. L'étude génétique décrit les patterns enzymatiques de ces F1 expérimentaux et confirme en grande partie les interprétations déduites d'observations sur les hybrides naturels. L'étude morphologique portant sur la longueur des denticules du dernier rayon dur de la nageoire dorsale (denticules absents chez *B. meridionalis* et bien développés chez *B. barbus*) révèle que les valeurs hybrides ne sont pas intermédiaires entre les valeurs parentales. Jusqu'à l'âge de 24 mois, les hybrides expérimentaux ont la même croissance et présentent le même dimorphisme sexuel des tailles en faveur des femelles que des *B. barbus* élevés dans les mêmes conditions. Chez les femelles, les ovaires arrivent à maturité et produisent des ovules fécondables; chez les mâles, les testicules, bien que morphologiquement développés, ne produisent jamais de sperme (stérilité fonctionnelle). Si elle se confirme chez les hybrides mâles issus du croisement mâle *B. barbus* × femelle *B. meridionalis*, la stérilité des mâles F1 constitue une entrave à la fertilité des hybrides en milieu naturel et apparaît comme un phénomène très important dans la connaissance des mécanismes d'hybridation au sein des populations concernées.

Mots-clés : Hybridation expérimentale, stérilité, physiologie des poissons, allozymes, *Barbus*, Cyprinidae.

INTRODUCTION

Barbus barbus (L.) 1766, and *Barbus meridionalis* Risso 1826, hybridize naturally in the Hérault basin. The first description of the natural characteristics of this hybridization (Berrebi *et al.*, 1987) and its parasitological effects (Le Brun, 1988) was based on marker enzymes identified by electrophoresis on horizontal starch gel. The studies distinguished the genotypes of each of the parent species and the interspecific heterozygotes. Samples of the pure species were analyzed to determine genotypes, whereas hybrid genotypes (heterozygotes) were identified by deduction, *i.e.* their addition of genotypes of the two parent species. Identification of hybrid genotypes posed no problem in the case of systems with one locus, in which the species differed with respect to the position of their respective alleles (loci *Aat*-2, *Gda*, *Ldh*-3). In contrast, difficulties appeared in systems with more than one locus (the AK, MDH, 6-PDGH, PGM, and SOD systems), which can occur because of the tetraploidy of European barbels. A summary of the particularities of these organisms is given in Berrebi *et al.* (1990).

The watercourse in which this hybridization is active (the Lergue, a tributary of the Hérault in southern France) constitutes a "hybrid zone" as defined by Barton and Hewitt (1985). In this zone, which is only a few kilometers long, the barbel are all hybridized (recombined) to different degrees because of the fertility of the F1 descendants and subsequent generations. The phenomenon has been going on long enough to create a situation in which no F1 hybrid (heterozygous at all diagnostic loci) or individual of the pure species could be found among nearly 500 barbels captured at the location. The absence of F1 hybrids makes it impossible to confirm the hybrid character of certain genotypes.

It was of interest to obtain F1 hybrids experimentally, which could be used for genetic, morphological, physiological, and eco-ethological reference. In the present article, we report on this hybridization and preliminary data obtained.

MATERIALS AND METHODS

Artificial hybridization

Thirty specimens of wild *B. meridionalis* captured by electrofishing at an altitude of 150 m in the Tech (Berrebi *et al.*, 1988), an independent Mediterranean river at the foot of the Pyrenees in southern France. Then, they were transferred on June 25, 1987, to the pisciculture research station of the University of Liège at Tihange (Belgium) and stocked in rearing tanks filled with water taken from the river Meuse.

Morphological examination revealed the presence of several spermatogenic males. Hybridization was thus immediately carried out by artificial fertilization using five spermatogenic *B. meridionalis* males and two *B. barbus* females taken from a stock of fish of feral origin (from artificial fertilization in the field using parents captured in the Ourthe by electrofishing in May 1985) but raised in tanks from the egg stage. Artificial fertilization was carried out at 12:00 noon on June 26, 1987, according to the method previously used with *B. barbus* (Philippart, 1982; Philippart *et al.*, 1987; Poncin, 1988).

Breeding methods

Fertilized eggs were placed in a Zug bottle with water supplied by a thermostated (20°C) closed circuit. Larvae were raised on glass-rod trays in the same system. Alevins with resorbed yolk sacs obtained on July 6 were placed in a tank measuring $5 \times 2 \times 0.25$ m (2.5 m^3) supplied with well oxygenated (aerators) river water maintained at 20 to 25°C. The tank was emptied on October 6 (day 102) and the 512 surviving hybrid alevins, weighing on the average 1.8 g, were then transferred to a 4 m^2-1.5 m^3 tank supplied with water from a large-capacity semi-closed circuit used for raising *B. barbus* at 20 to 24°C. The tank was placed in a natural photoperiod.

Fish were given artificial feed for trout (46 to 50 % protein) distributed by an EWOS 505 automatic feeder. The daily ration was the same as that used for raising *B. barbus* (Poncin, 1988; Philippart *et al.*, 1989).

Between October 6, 1987 (day 102) and June 6, 1989 (day 711), survival (counts) and weight-increase (general weight) were followed up monthly, and size measurement (length to the fork = distance between the front tip of the head to the back of the caudal fin) was carried out every 3 months.

During the experiment, several density adjustments were made. On April 26, 1988 (day 306), the hybrids were divided into two groups, *i.e.* group A ($n = 316$; mean weight $W_m = 33.6$ g) and group B with a lower density ($n = 100$; $W_m = 35$ g). On September 23, (day 455), group A was again reduced by about 1/3 when 100 fish were removed for genetic and morphological study in Montpellier.

Comparison with a group of *B. barbus*

At the time artificial hybridization was carried out, we were fortunate enough to have a group of *B. barbus* at our disposal, raised from two feral females captured in the Meuse on June 5, 1987, and

Table 1. – Size structure of the hatchery reared populations of *Barbus barbus* and the hybrid *B. barbus* × *B. meridionalis* on June 6, 1989.

Body length (mm)	Hybrid		*B. barbus*	
	N total	*N* females*	*N* total	*N* males**
140	1	–	–	–
150	2	–	–	
160	6	–	–	–
170	25	–	1	1
180	30	-	9	9
190	31	–	8	8
200	28	3	16	16
210	24	6	12	10
220	14	5	20	19
230	9	5	15	13
240	12	5	12	8
250	7	4	10	5
260	11	4	12	3
270	21	13	17	1
280	14	9	22	2
290	10	8	9	
300	9	4	8	–
310	7	4	4	
320	2	2	4	
330			3	–
340	-	–	3	–
350	–	–	1	
TOTAL	263	72	186	95

* Females with ova or sexed by dissection.
** Males with milt.

males bred at Tihange (*table* 1). This group was raised under the same conditions as the hybrids and, despite an initial difference of 1 month in the spawning dates, they can be taken as a reference for long-term growth (observations completed on June 9, 1989, day 733), as well as structure (size), and sexual maturity.

Genetic analysis

One hundred individuals were taken from group A for enzyme electrophoresis. They were frozen and transported from the pisciculture station to the genetics laboratory where they were dissected to remove 1.5 g of skeletal muscle and the whole liver.

These organs were homogenized and centrifuged at 15,000 r.p.m. The supernatant (soluble enzyme extract) was placed in starch gel for electrophoresis. After migration, the gel was stained with specific solutions to locate proteins carrying each of the enzyme functions sought. Only the ten diagnostic enzyme functions (showing differences between species) were analyzed according to the method of Pasteur *et al.* (1987) adapted by Berrebi *et al.* (1988).

To characterize the group of fish used for analysis, several morphological parameters were also examined: total length (Lt), sex, and maximum length of the denticles on the last hard ray of the dorsal fin (LDmax). The same denticles are about 1 mm long on *B. barbus* measuring 190 mm (mean total length of hybrids analyzed) and are completely absent on *B. meridionalis*. These measurements were used to check the variation of this character, which is representative of the intermediate morphology of F1 hybrids.

RESULTS

First phases of development

The initial objective of the experiment was to obtain F1 hybrids for genetic studies. Since artificial hybridization was done in extreme circumstances, and we could not predict how the hybrids would develop, we decided to forgo any examination (or sampling) of eggs or alevins (counting, measurement) that might increase mortality and reduce the chances of obtaining a sufficient number of hybrids for subsequent observations over the next few years. Consequently we obtained no quantitative data on the first phases of development (rates of hatching, growth, larval survival, etc.) although the pattern was comparable to the artificial reproduction of *B. barbus*.

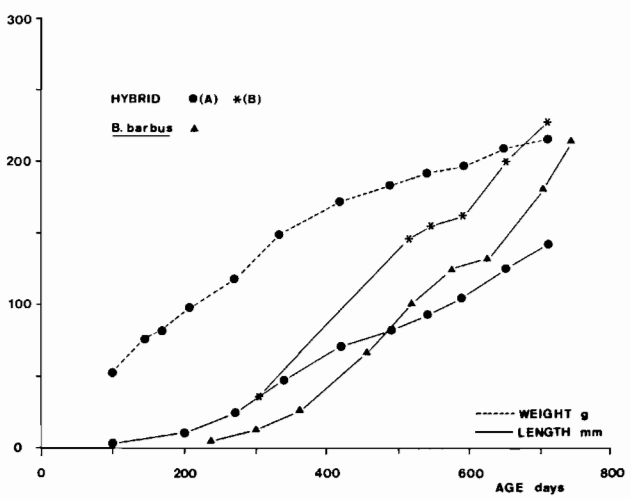

Figure 1. – Growth in length (fork length) and weight of populations of *B. barbus* × *B. meridionalis* hybrid (experiment A : high stocking density; experiment B: low stocking density) and *B. barbus* reared in tank from June 1987 to June 1989

Growth

Figure 1 shows the time courses of weight- and length- increase of the hybrids in group A (high density) and B (low density), and the *B. barbus* group. At the end of the experiment in early June 1989 (day 711 for the hybrids, day 743 for *B. barbus*), the mean weight and length were respectively 143 g and

Table 2. – Data on the fecundity of female hybrids *B. barbus* × *B. meridionalis*.

Tag number	Date	Spawn number	Length (mm)	Weight (g)	Ova weight (g)	(%)
DN 8878	7/4	–	227	154	2,0	1.30
DN 3883	7/4		261	230	9	3.91
DN 8890	7/4	–	260	220	7	3.18
DN 8806	20/4	–	273	272	3	1.10
DN 8897	27/4	2	200	98	3	3.06
DN 8847	27/4	2	219	128	8	6.25
DN 8888	27/4	–	214	128	9	7.03
DN 8898	27/4	–	286	270	+	+
DN 8877	6/6	2	271	270	11	4.07
DN 6290	6/6	-	275	272	<1	+
DN 6289	6/6	–	274	253	<1	+
DN 8876	13/7		284	276	6,1	2,21
-	13/7	2	240	176	4,6	2,61
DN 8874	6/6	2	286	–	spent	–
DN 8865	6/6	2	279		spent	–

215 mm (± 6, confidence interval: 95%) in group A, 228 g and 250 mm (± 10) in group B, and 214 g and 352 mm (± 6) in the *B. barbus* group. The final sizes of the hybrids (groups A and B combined) and *B. barbus* are shown in *table* 2.

The growth curves show that the hybrids (e.g. group B) have a growth rate comparable (parallel slopes) to that of *B. barbus*. This result is confirmed by the more precise analysis represented in *figure* 2, which shows the relationship between the mean weight of the fish over periods of 1 month and the maximum growth observed.

Reproductive biology

Sexual maturity

In the *B. barbus* group, the first spermatogenic males were found in July 1988 (age ≈ 400 days), which corresponds to many previous observations (Poncin, 1988). No spermatogenic males were found among the hybrids at this time, and dissected males were found to be immature. In contrast, the ovaries of the females were either immature or at a stage of development (vacuolization and vitellogenesis; *see* Poncin, 1988) that was not as advanced as in *B. barbus* individuals of the same age, but which nevertheless suggested that maturation would occur in early 1989.

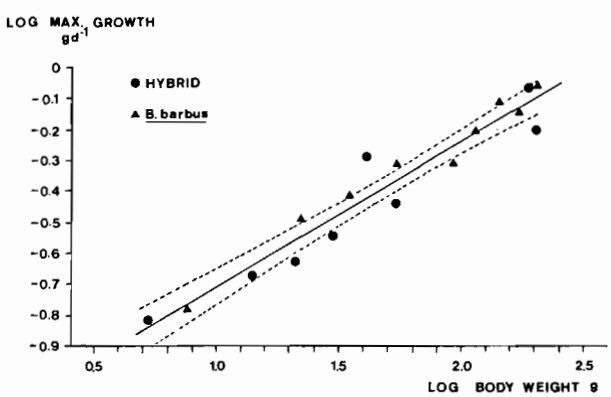

Figure 2. – Relationship between maximum growth in weight and body weight in *B. barbus* × *B. meridionalis* hybrid and *B. barbus* reared in tank (4 m²-1,5 m³) at 20-24°C and given artificial feed. Equation of the regression line is log maximum growth (g/day) = −1.176 + 0.467 Log body weight (g) Dotted lines indicates the 95% confidence bands for the mean of log max growth.

In mid-January 1989, the hybrid males examined had a gonadosomatic index (GSI, the percentage of gonad weight with respect to total body weight) varying from 0.3 to 1.5% (mean 0.99%). Several females had ovaries that were in an advanced state of vitellogenesis, a sign of approaching ovulation. Indeed, during an examination on April 7, 1989, 3 (10.3%) mature females produced ovules after abdominal pressing and 26 (89.7%) females measuring 200 to 307 mm had passed the ovulation stage several days earlier (all these females were identified by double marking: the tip of the left pelvic fin was removed and a numbered ring was attached to the hard ray of the dorsal fin). However, no spermatogenic hybrid males were found, whereas there were 94 (50.2%) spermatogenic males 160 to 280 mm long in the *B. barbus* group, accompanied by 31 (16.6%) mature or post-ovulatory females 240 to 340 mmm long and 62 (33.2%) individuals 170 to 340 mm long whose sex could not be determined by external examination. Dissection showed most of them to be immature or maturing females.

At the end of the experiment on June 6, 1989 (see *table* 1), no spermatogenic male hybrids were found in either group. Thirty-two females had spawned in group A (17.9%) and 18 in group B (21.2%). The smallest hybrid female was 20 cm long, the largest 31 cm. At the same time, the *B. barbus* group included 95 spermatogenic males and 91 mature and immature females, corresponding to a balanced sex ratio (M/F) of 1.04.

Fertility

At the beginning of July 1989, a large number of males were dissected to determine the development of testes in hybrids and *B. barbus*. The testes of the hybrids were generally lighter (mean GSI = 1.87% ± 0.37, c.i.: 95%, $n = 32$) than those of *B. barbus* individuals (GSI = 3.06% ± 0.41, $n = 23$)

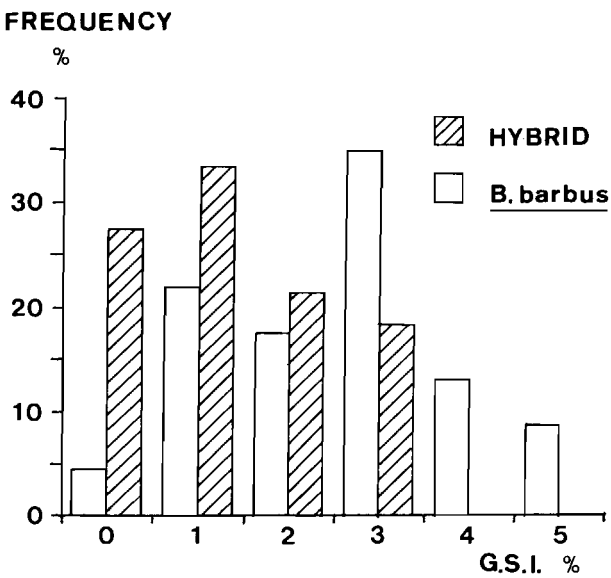

Figure 3. – Frequency distribution of the gonadosomatic index (GSI, % body weight) values in male *B. barbus* × *B. meridionalis* hybrids ($n = 32$) and *B. barbus* ($n = 23$) in early June 1989 (age 700-750 days). GSI values are grouped into classes: class 0 = 0-0.99; class 1 = 1-1.99, etc.

(*fig* 3). The testes of the hybrids were often irregular (absence of a lobe, marked longitudinal asymmetry) and contained no liquid milt.

Initial data on the fertility of the female hybrids are given in *table* 2. On first examination, the female hybrids would appear to be less fertile than the *B. barbus* females characterized by Poncin (1988), but it should be noted that our observations concern first spawns, which are often smaller than subsequent spawns. The fertility of hybrid and *B. barbus* females will be strictly compared during their second year of maturity in 1990.

Lastly, it should be mentioned that six female hybrids spawned at least twice during the reproduction period in 1989. This phenomenon of multiple spawning is comparable to that observed in *B. barbus* and has the same causes (high level of feeding at 20 to 24°C under experimental breeding conditions; Poncin, 1988).

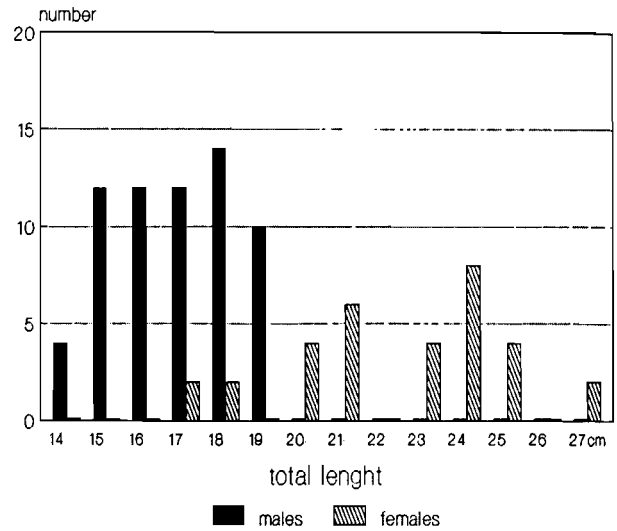

Figure 4. – Histogram of sizes (total length) according to the sex of 100 F1 hybrids analyzed by electrophoresis. The sexual dimorphism is quite evident, with a mean size of 172 mm for the males and 213 mm for the females.

Morphological and genetic variation of 100 F1 hybrids (*figure* 6)

Figures 4 and 5 show histograms of size (Lt) and denticle length (LDmax), respectively, according to the sex of 100 hybrids in group A on day 455. The sexual dimorphism is very striking, with mean lengths of 172 mm for the males, and 213 mm for the females. The sex ratio is unbalanced (Chi-2 = 4.66 with d.f = 1), with two males for one female (M/F = 2.09).

The length of the denticles on the last hard ray of the dorsal fin, size-independent in that sample, varied considerably, *i.e.* 0.12 to 0.37 mm on the males, and 0.14 to 0.30 on the females. These values are not intermediate between the parental values (in the same class of size, LDmax = 0 mm in the paternal species *B. meridionalis*, and about 1 mm in the maternal species *B. barbus*). There was no sexual dimorphism with regard to this character, since the denticles were even slightly smaller on the females, although the females were larger than the males.

The genetic study clearly defined the enzymatic patterns of the F1 hybrids. The intuitive interpretation used in previous studies was correct except for two cases: (i) the fastest part of the PGI system (loci *Pgi*-2 and *Pgi*-3, *see* Berrebi *et al.*, 1988), and (ii) locus *Es*-5, whose determinism remains unknown and calls for further research, *e.g.* using a different electrophoretic gels.

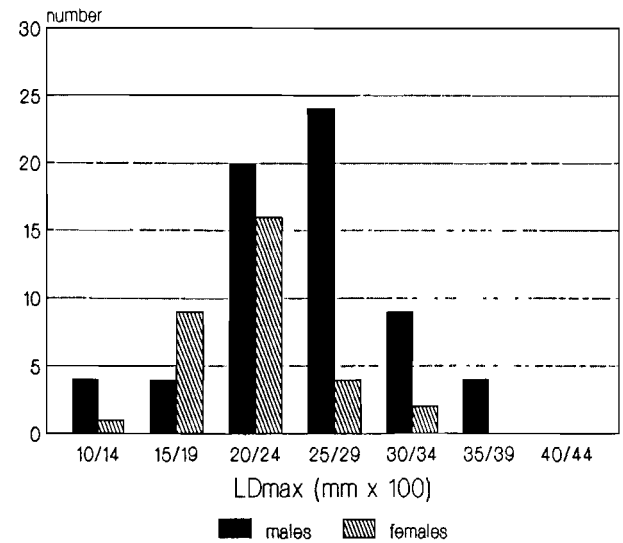

Figure 5. – Histogram of the lengths of denticles on the last hard ray of the dorsal fin (LDmax) of 100 F1 hybrids analyzed by electrophoresis.

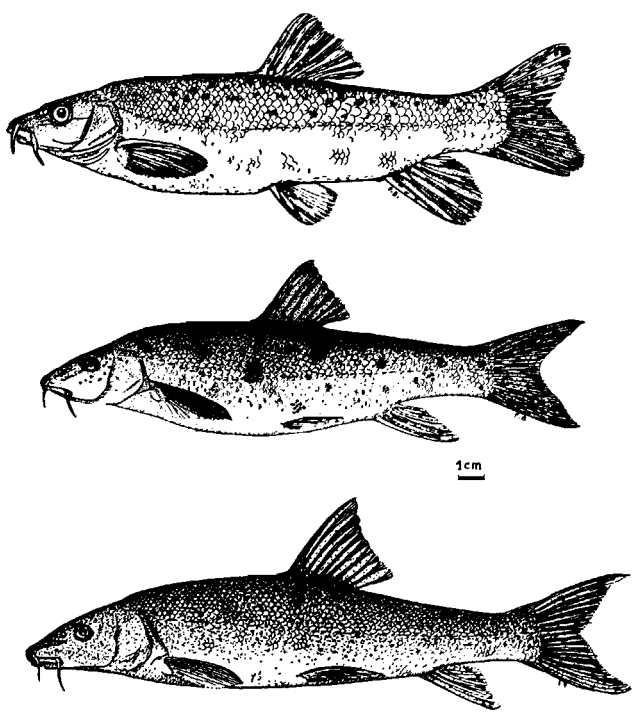

Figure 6. – Morphology of *B. barbus* (below), *B. meridionalis* (above) and the F1 hybrid (middle). The external characters that most distinguish the two species are intermediate in the hybrid, *i. e.* the concave/convex profile of the dorsal fin, the pointed/round tips of the dorsal, caudal, and anal fins, the pointed/round form of the snout, the number of scales on the lateral line, the dorsal brown spots, the presence of denticles behind the last bony ray of the dorsal fin (not shown on the drawing).

DISCUSSION

The experimental hybridization described here is part of a much larger research program concerning the physiology and evolutionary biology of the genus *Barbus* and the role of hybridization in the evolution of these organisms.

To elucidate the mechanisms operating in the hybrid zone of the Lergue, it is essential to know the relative fecundities of intra- and interspecific crosses. It is already evident that the hybridization occurs easily and the descendants are numerous. However, the slightest lack of fecundity can jeopardize the development of the phenomenon, as shown by Dowling and Moore (1985) in the case of two species of North American cyprinid (genus *Notropis*).

Consequently, the fate of the hybrid population must also be determined, and in this respect the functional sterility of the experimental F1 hybrid males is a very important phenomenon. After the formation of this hybrid zone, any individual of the pure species entering the zone is likely to have encountered a hybrid with variable degree of introgression. The level of sterility in those cases of crosses is not yet determined.

The origin of the hybrid sterility remains to be determined, and it is essential to verify: (i) whether this characteristic will persist in the next reproductive period (in 1991), (ii) whether the phenomenon is related to individual characteristics of the females used for artificial fertilization in 1987 (implying that the experiment will have to be repeated with other *B. barbus* females), and (iii) whether the same phenomenon will occur when a *B. barbus* male is crossed with a *B. meridionalis* female. During these experiments, the early stages of development will be observed in detail (length of incubation according to temperature, survival from hatching, etc.) in the two types of hybrids and in the parent species.

Apart from the sterility of the males, the hybrids raised in captivity resembled *B. barbus* individuals, *i.e.* they had the same growth rate, the same marked sexual dimorphism favoring larger females, the same early acquisition of female sexual maturity (starting at 20 cm and during the second year of life), and the same multiple spawning by females during the reproductive period. With regard to fecundity (which appears to be lower in the hybrids than in *B. barbus*) and the size of the ovules (smaller in the hybrids), it would be premature to draw conclusions from the data collected in 1989, considering the wide natural variation of fertility. These aspects of reproductive biology will have to be investigated in further studies.

Lastly, special attention should be given to an eco-ethological study of the hybrids in the natural environment (*see* Baras and Cherry, 1990) and in the aquarium. Procedures allowing *B. barbus* reproduction in large aquariums (Gougnard *et al.*, 1987) make it possible to study the reproductive behavior of the

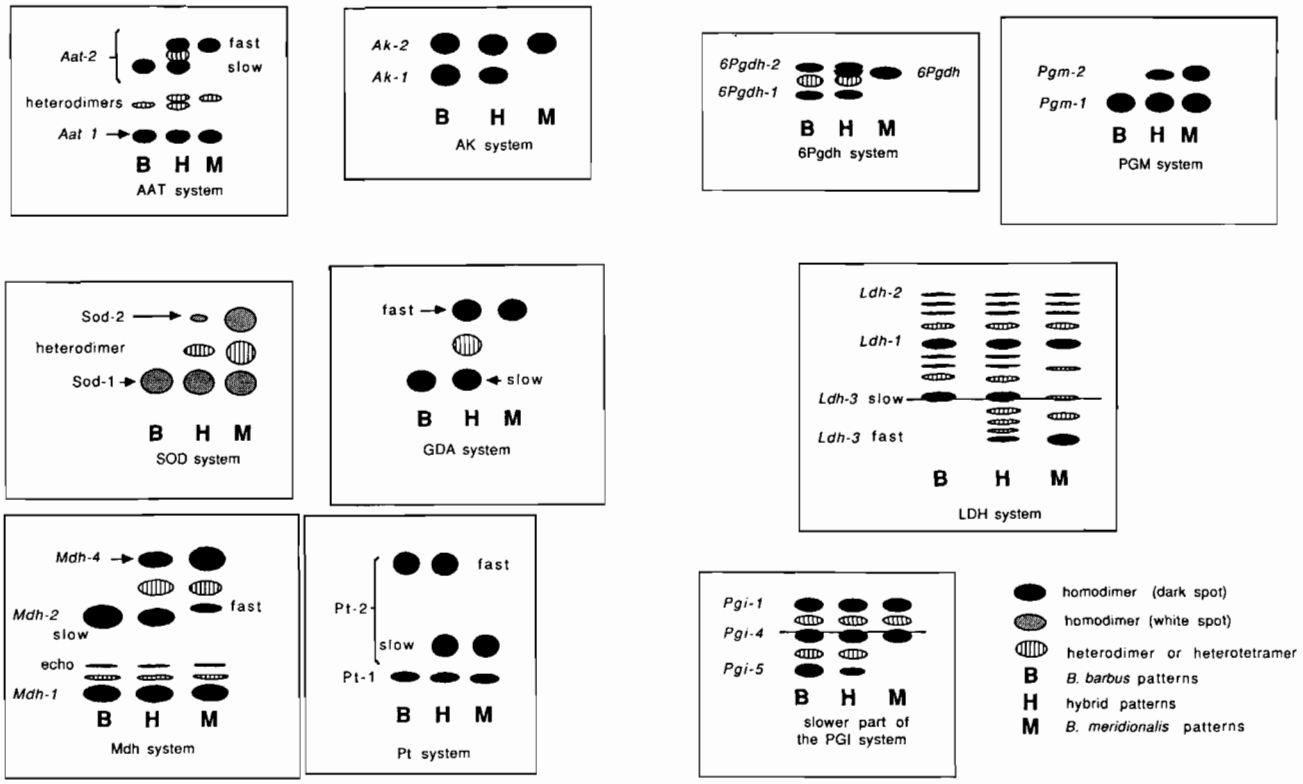

Figure 7. – Enzyme patterns of the ten clearly defined systems. Fastest part of the PGI system and *Es*-5 locus require further research and confirmation.

hybrids with regard to their congeners (how do sterile male hybrids behave in the presence of fertile female hybrids?) and the parent species. It would also be of interest to study the reproductive hormones involved (for *B. barbus*, *see* Poncin, 1988).

Among the mechanisms of evolution, hybridization is increasingly considered to be an important means of speciation. Intra- and interspecific hybridization is frequent in fishes, especially in the Cyprinidae (for reviews, *see* Buth, 1984 and Ferguson, 1980). According to Berrebi *et al.* (1989), there are two extreme cases in hybridization phenomena. The most frequent case yields strictly sterile hybrids. In the other extreme case, the hybrids have the same fertility as each of the parent species. The hybridization of the two barbel species is close to the second case, but we emphasize that obstacles to fertility are indicated (sterile F1 males).

Hybridizations producing fertile descendants have a strong evolutionary impact, since the integrity of the parent species is threatened and introgression can occur. For example, following climatic changes, hybridization between two species having no reproductive barrier can lead to the formation of a third, synthetic species. Such cases are rarely reported because they are difficult to determine, considering that the parent species usually disappear. Menzel (1976) has attributed several species of the genus *Notropis* to this phenomenon.

Although *B. barbus* and *B. meridionalis* are considered to be authentic species from a taxonomic viewpoint (Wright, 1978), the term "semi-species" is more appropriate according to Buth (1979) and Dowling *et al.* (1984). We cannot predict the fate of the introgression, but two unpublished observations are of importance. This hybridization has been observed in large rivers of southern France, such as the Rhône and the Garonne, and in the main watercourse of the Hérault, *B. barbus* shows a uniform 15% introgression by *B. meridionalis*. Lastly, since a subspecies (*B. meridionalis petenyi* Heckel) hybridizes with *B. barbus* in Yugoslavia (Stratil *et al.*, 1983) it is likely that hybridization occurs wherever the two species cohabit.

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