Sensitivity to inbreeding and sperm cryopreservation in the catfish *Heterobranchus longifilis* Valenciennes, 1840

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Introduction

The African Clariidae, *Heterobranchus longifilis* Valenciennes 1840, is characterized by a high fecondity (up to 120.000 ovules per kg of body weight). Its growth rate (8 to 12 g per day) makes it a fish of the future for aquaculture. *Heterobranchus longifilis* is being reproduced at the Centre de Recherches Océanologiques (CRO) since 1984, and its reproductive cycle is now fully understood in captivity. This species has been the object of an electrophoretic study (TEUGELS *et al.*, 1992) carried out on 13 loci using six wild and six domestic individuals from the third generation (F3), which showed that the domestic individuals lost a large part of the genetic variability present in the wild population from which they are descended (three polymorphic loci in the 13 studied).

Numerous studies carried out on other fish species, showed that domestication could lead to an important decrease in genetic variability (TANIGUCHI *et al.*, 1983; VUORINEN, 1984; KRIEG and

GUYOMARD, 1985). This loss of variability can sometimes be observed from the first domestic generation (TANIGUCHI *et al.*, 1983). It can, in certain cases, lead to a modification in rearing performances (FERGUSSON, 1992; DANZMANN *et al.*, 1985; DANZMANN *et al.*, 1987).

Species with high fecundity, for which a single pair can beat the origin of a domestic population, risk firstly a decrease ingenetic variability (due to the small necessary effective size needed to maintain a culture population), and secondly a decrease in the heterozygosity rate (due to inbreeding). Numerous studies have shown the relationship existing between the number of heterozygous loci of an individual and certain of its biological characteristics: growth rate and oxygen consumption (KOEHN and SHUMWAY, 1982), weight loss rate during starving (RODHOUSE and GAFFNEY, 1984; DIEHL *et al.*, 1985). Generally speaking, heterozygotes have a more efficient metabolism than do homozygotes (KOEHN and SHUMWAY, 1982).

In aquaculture the availability of gametes throughout the year is important to ensure a constant supply of fish. From that point of view, *H. longifilis* presents a definite interest for aquaculture since its gametogenesis is continuous once sexual maturity is reached. However, the males have to be sacrificed and the testes dissected out to collect the sperm as the semen cannot be easily obtained by stripping unlike the ovules in females.

Several means, including long term storage of gametes maybe used to improve fish farm stocks. Cryopreservation of sperm can facilitate artificial insemination and allow a better brood stock management.

Two consecutive studies were carried out. The objective of the first one was to determine whether or not the loss of polymorphysm resulting from successive consanguine crosses in this species is accompanied by differences in zootechnical performances (fecundity, larval survival and growth) of farmed individuals. The aim of the second study was to assess the fertilizing capacity of the sperm after cryopreservation, and to show if this technique can be used to restore genetic variability in farmed strains.

Material and methods

Biological material

The domestic brood stock of *H. longifilis* used were collected at the Layo Station (CRO). They were 3 years old and sexually mature since the age of 1 year. These fish are descendants of a wild stock that had spontaneously colonized the station ponds in 1982 (LEGENDRE, 1983) and were raised in lagoon pens. These domestic individuals (F3), are descendants of three consanguine crosses of one female and one male. Wild broodstock were aged from 2 to 3 years and were captured at the approximate age of 6 months (25 cm) in the lagoon or the neighboring swampy areas and raised at the Layo Station in lagoon cage-pens.

Study of genetic variability

Two samples of 50 individuals from F1 and F4 descending respectively from wild broodstock and from broodstock hatched incaptivity were studied. For each individual, an eye, and a piece of liver and muscle (1 cm^3) were taken. Tissues were preserved at -30°C for several weeks then ground just before analysis. Isoenzymatic variations at 23 loci were examined (AGNESE *et al.*, 1995).

Reproduction

Oocyte maturation and ovulation were induced by a single intramuscular injection of human chorionic gonadotropin (hCG) at adose of 1.5 I.U. per g of body weight. The females used were selected firstly on the basis of their stoutness and the softness of their belly, and mainly on the basis of their oocyte diameter. Oocyte diameters from selected females were measured from a sample of 30

to 40 oocytes per female collected by intra ovarian biopsy before hormonal inducement.

After ovulation, each female was stripped of the maximum of her ovules. For each female, all ovules were weighed, then 300 to 400 ovules were weighed and counted to determine fecundity (number of ovules collected). Male broodstock received no hormonal treatment. The sperm collected after sacrificing the males and dissecting the testicles was kept on ice after a one-tenth dilution with 0.9% NaCl. For the strain comparison, six wild females and four wild males on one hand and four F3 females and four F3 males on the other hand were used.

Evaluation of zootechnical performances

Each female was fertilized using pooled sperm obtained from a mixture of the milt from the different males of the same generation. The quality of ovules harvested was evaluated using hatching percentages on lots of 200 to 300 ovules fertilized with 200 µl of diluted sperm. At hatching (24 hours after fertilization), the proportions of normal and deformed fry obtained from each lot were determined by observation and counting on a light table. Modal oocyte diameter, hatching percentage and the proportion of normal and deformed fry were determined for each female.

Early fry growth was followed for a period of 14 days from D1 (one day after hatching). The experiment was carried out in Two PVC tanks subdivided into six compartments of a working capacity of 50 l and attached to a closed circuit. For the different groups of broodstock (domestic and wild) six lots of 300 fry (after yolk absorption) were taken from a pool made proportionally using the percentage of normal fry from each female. In the comparison of performance between the F1 and F4 strains, three replicates of 300 fry from each strain were placed in six compartments of 50 l (six fry per liter).

Fry were fed *ad libitum* at a rhythm of 6 meal per 24-h period. From the second day after hatching and until the eighth, the fry were fed only *Artemia salina* nauplii. From the 9th to the 11th day

the nauplii were progressively replaced by artificial feed (Trouvit), which became the sole feed until the 14th day.

To follow the evolution of fry weight of the two generations, 24 fry were taken from each compartment and weighed after draining on the 5th, 8th, 11th and 14th day. At the end of the experiment, a count of all individuals was made to establish the survival rate in each compartment.

Cryopreservation of sperm

The fish and the sperm

The study was conducted with gametes collected from sexually mature 3 to 5 year-old individuals coming from a F1generation.

Five males of *H. longifilis* were sacrificed and the sperm was collected by dissecting the testes. The sperm from all the males was pooled and a sample of the pooled milt was deep-frozen in liquid nitrogen for 8 months prior to the beginning of the breeding experiment.

The milt from a second group of five males was collected and pooled using the same procedure as described above. A sample of this milt was deep-frozen in liquid nitrogen for 1 hour prior to insemination. Another sample of the same milt was stored in a glass tube kept sealed on crushed ice until use as fresh milt.

Cryopreservation techniques and sperm quality

The diluent tested in this study was based on that of MOUNIB'S (1978) (125 mM sucrose, 100 mM potassium bicarbonate, 6.5mM reduced glutathion) to which were added 5 % DMSO (Dimethylsulfoxide), 5 % Glycerol and 10 % hen Egg yolk.

The sperm was mixed with the diluent at a ratio of 1:3 and placed in 5 ml straws and allowed to freeze 3 cm above the level of liquid nitrogen for 20 mn before transfer into liquid nitrogen (OTEME *et al.*, 1996). The motility of the sperm was evaluated before and after freezing.

The fertilizing ability of the sperm was evaluated using hatching percentages on batches of 200 to 300 ovules collected from one female *H. longifilis* and artificially inseminated respectively with fresh sperm, sperm (from the same pool) thawed after 1 hour of cryopreservation and with sperm cryopreserved in liquid nitrogen for 8 months, at a ratio of 200 µl of milt (diluted 1:10 in 0.9 % NaCl solution) for 400 mg of ovules.

Results and discussion

Genetic variability

Of the 23 loci studied, only two were revealed as polymorphic : Mdh-1 and Pgm. At locus Mdh-1, two alleles with almost identical frequencies were observed in the two populations (F1 and F4) : Mdh-1 f (fast) at a frequency of 75% and Mdh-1 s (slow) at a frequency of 25% in the F1 population, Mdh-1 f at a frequency of 73% and Mdh-1 s at a frequency of 27% in the F4 population. At locus Pgm, two alleles were observed only in the F1 population, the allele Pgm s at the frequency of 95% and the allele Pgm f at the frequency of 5%. The observed rate of polymorphism (average heterozygosity), H, is equal to 2% for the F1 strain and 1.7% for the F4 strain.

The results obtained can be compared to those of TEUGELS *et al.* (1992). The enzymatic activities of nine additional loci were observed (Aat-2, Adh, Ak, Es-1, Es-2, Fbp, Icdh, Iddp-2, Ldh-3) as compared to this study, but none of these loci were shown to be polymorphic. At locus Mdh-1, these authors observed two alleles in the wild population, Mdh-1 100 and Mdh-1 75. These two alleles most certainly correspond to the observed alleles Mdh-1 f and Mdh-1 s. The frequencies of these two alleles are very similar in the F1 (75%/25%) and F4 (73%/28%) populations. These values are also comparable to those observed by TEUGELS *et al.* (1992) for the wild

population (60%/40%). However, these results show that the F3 population studied by TEUGELS *et al.* (1992) was not monomorphic for the allele Mdh-1 100 as their results seem to suggest since the two alleles were found in the F4 population.

At locus Pgm, TEUGELS *et al.* (1992) observed three alleles only in the wild population : Pgm 100 (30%), Pgm 85 (60%) and Pgm 60 (10%), the F3 population being monomorphic for the allele Pgm 85. Only two of these alleles were observed in the F1 population of our study, Pgm f (fast) and Pgm s (slow) and one single one in the F4 population, Pgm s. It is very likely that the allele Pgm s present in the heterozygous state in the F4 population is the allele Pgm 85 present in the homozygous state in the F3 population (TEUGELS *et al.*, 1992). The allele Pgm f observed in our study is, most probably, the allele Pgm 100.

The F4 population differs from the F1 population by an absence of polymorphism at the locus Pgm. The F1 population differs from the wild population by lower number of alleles at the locus Pgm (2 instead of 3). In all cases, a loss over successive generations of the least frequent alleles, namely Pgm 60 (10% in the wild population) and the Pgm 100 (30% in the wild population) is observed. Only the locus Mdh-1 of which the alleles are both at high frequencies in the wild population (60% and 40%) continues to be represented by two alleles at high frequencies in domestic populations. The heterozygosity rate observed for the wild population is equal to 8.5%. If H values for the F1 and F4 populations are calculated considering only the 12 loci common to both our study and that of TEUGELS *et al.* (1992), we obtain 3.9% and 3.2% respectively. The main cause of this loss of polymorphism is the small necessary effective size (½ effective population size) of stocks, which is to say, the real number of broodstock used to create a new generation.

Evaluation of zootechnical performances

Table I summarizes the results obtained during artificial fertilization. No significant differences (Duncan test at a fixed significance of 5%) were observed between the two strains in egg diameter (P = 18.5%), hatching percentage (P = 61%) and

percentage of normal fry (P = 58%). Only comparison of the percentages of deformed fry showed a significant difference (P = 3%). Deformed fry were more numerous in the F4 population; however, percentages remained small (less than or equal to 2%).

Results of the comparison of the body weight are shown in table II. At the 14th day (D14), the survival of the F1 population was very significantly higher than that of the F4 population (P =1%). Mean weight also were significantly higher for the F4 population than for the F1 generation (P = 2%).

Femelle	Weight (g)	ED (mm)	H (%)	DF(%)
Wild	4050	1.52	87.3	1.5
Wild	8100	1.49	93.5	1.8
Wild	4600	1.56	95.2	1.4
Wild	6400	1.67	87.9	1.6
Wild	5250	1.45	94.9	1.5
Wild	4250	1.50	99.0	1.6
F3	5100	1.51	97.7	1.7
F3	5600	1.47	74.6	1.7
F3	3000	1.47	93.3	1.8
F3	6450	1.44	95.9	2.0

Table I
Results of artificial fertilization to obtain F1 and F4 populations of *H. longifilis*. ED, egg diameter ; H, hatching percentage ; DF, deformed fry.

Because of the very different survival rates of the two generations, the corresponding densities in the culture tanks were not the same. Because of this, the observed differences in growth rates may be a genotype result (if one exists) or an effect of the density.
The body weight which appeared greater, of F4 individuals may

therefore be explained in part (or perhaps in totality) by the difference in rearing density resulting from a higher mortality observed in the F4 population. The correlation determined between initial rearing density and final body weight suggests that the mortality observed in the F4 population occurs very early during rearing.

Génération	D1	D5	D8	D11	D14	IN	FN
F1	2	22	60	97	129	300	282
F1	2	20	55	86	131	300	246
F1	2	23	59	93	150	300	261
F4	2	21	63	109	174	300	149
F4	2	18	54	99	181	300	171
F4	2	23	63	88	174	300	174

Table II
Results of comparison of growth between three lots of generation 1 (F1) individuals and three lots of generation 4 (F4) individuals of *H. longifilis*. D5, D8, D11, D14, weight at the 5th, 8th, 11th, 14th day ; IN, initial number of fry ; FN, final number of fry.

Sperm cryopreservation

Sperm motility

The sperm motility measured before and after cryopreservation showed that the motility was altered by the freezing-thawing process. Fresh sperm exhibited a percentage of motile spermatozoa ranging from 70 to 80 % ten sec after dilution, while cryopreserved sperm only showed motility percentages comprised between to 20 and 30 %.

Hatching of fertilized eggs

Total hatching rates obtained were 78.9%, 81.1%, and 83.4% respectively for the fresh sperm, the sperm cryopreserved for one

hour in liquid nitrogen and the sperm cryopreserved for 8 months in liquid nitrogen.

The observed hatching rates of deformed fry were 6.2%, 5.8% and 6.0% respectively for the fresh sperm, the sperm cryopreserved for 1 hour and 8 months in liquid nitrogen.

These results show that the cryopreserved sperm was as effective as the fresh sperm in fertilization trials and that *H.longifilis* sperm can be cryopreserved for at least several months with no effect on its fertilizing ability. Similar results were obtained with *Clarias gariepinus* by STEYN and VAN VUREN (1987) who reported that the sperm of this species could be cryopreserved for 28 months with no deterioration of its fertizing ability.

Conclusion

The results show that *H. longifilis* is a species highly sensitive to domestication. In four generations, a significant decrease in fry survival rate appears. The origin of this higher mortality must be determined. Several hypotheses can be proffered: the existence of lethal genes which might be expressed shortly after hatching, the influence of individual heterozygosity rates, an emphasized cannibalism phenomenon (behavior modification), a greater variability in growth rates favoring cannibalism (without modification of intrinsic population behavior). The apparent increase in the rate of deformed fry in the F4 population is very small, the maximum percentage observed being 2%. Considering the very high fecundity of this species, this rate is insignificant. This species is at once very prolific and possesses very high growth rate (about 10 g per day). For these reasons, its culture is developing in Africa and in Europe. Growers need to pay particular attention to the necessary effective size of their populations. A loss of genetic variation via a reduction of the number of broodstock (genetic drift), or an increase in the number of homozygotes

(inbreeding), may noticeably alter the performances of cultured populations. The fertilizing ability of *H. longifilis* sperm is not affected by cryopreservation. This offers the possibility not only of limiting the quantity of male individuals sacrificed or operated for reproduction, but also of constituting a gene bank in order to limit inbreeding, to maintain, and if necessary to improve the quality of broodstocks through a selection programme.

Acknowledgements
This work was made possible thanks to funding from Orstom (l'Institut français de recherche scientifique pour le développement en coopération).

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