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Production of F_1 and F_2 diploid gynogenetic tilapias and analysis of the "Hertwig curve" obtained using ultraviolet irradiated sperm

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Summary. In this study, a Hertwig effect with a non-typical biphasic curve was obtained using sperm irradiated with increasing intensities of UV. The first phase of the UV curve appeared to be quite different from that normally demonstrated using γ or x-ray irradiation. This difference is characterised throughout the length of the first phase by (1) low and stable embryo hatching rates of about 3.5%, and (2) exclusive formation of haploid embryos at any irradiation intensity. Additionally, at both phases, the ability of the sperm to induce morula formation was not affected at all, and no aneuploidy nor chromosomal fragments could be seen. Therefore, it was suggested that in this fish the lethal effect of UV irradition on sperm is mainly expressed on early differentiative events during embryogenesis, which lead to a degeneration of the embryos during early stages of their development. The possible mechanism by which haploidy is achieved during the first phase is discussed. Two generations of diploid gynogenetic tilapias were induced by activating *Oreochromis aureus* eggs with UV-irradiated *O. niloticus* sperm and by using the heat-shock technique, at optimized conditions, for the prevention of the second polar body extrusion. Species specific dominant genetic markers (serum esterases and tail striation) were used to confirm the exclusive content of the maternal genome in gynogenetic offspring. Very low survival rates (0.36%) were shown in F_1 gynogenetic fish as well as a high incidence of malformations among survivors. In the second gynogenetic generation, both significantly higher survival rates (3.6%) and a considerably reduced incidence of malformations were obtained. We suggest that low frequencies of recombination occur in this species and cause a rapid increase in the inbreeding level. This is followed by the expression of lethal and defective genes that are considerably reduced after second generation selection.

Key words: Tilapia – Hertwig-effect – Haploidy – Gynogenesis – Inbreeding-level

Introduction

Gynogenesis and androgenesis are modes of reproduction in which offspring possess only one of the parental genomes. Gynogenesis occurs in nature in the all-female triploid viviparous population of fish (e.g. in Poeciliopsis; Schultz 1967), and in other amphibia and fish (for review see Cherfas 1981). In experimental gynogenesis eggs are activated with genetically inactive sperm cells and nonviable haploid embryos are usually formed. In order to obtain diploid gynogenetic offspring, diploidization of the haploid zygotes has to be achieved. The experimental induction of gynogenesis is carried out on the basis of the "Hertwig effect" (Hertwig 1911). The "Hertwig effect" is a paradoxical phenomenon in which high dose irradiated sperm were found more efficient in fertilizing normal eggs than low dose irradiated sperm. Successful induction of gynogenesis was performed in several species of amphibia and fish using either X-irradiation (Lasher and Rugh 1962; Stanley 1979), γ-irradiation (Purdom 1969; Nagy et al. 1978; Chourrout et al. 1980; Chourrout and Quillet 1982; Onozato 1982; Refstie et al. 1982) or UV irradiation (Pogany 1971; Ijiri and Egami 1980; Streisinger et al. 1981; Chourrout 1982; Chourrout and Itskovich 1983; Suzuki et al. 1985). Diploidization of haploid zygotes is currently achieved by preventing the second meiotic division or by interfering at the first mitotic devision. The most common techniques used for this purpose consist of treating the eggs by pressure shock (Streisinger et al. 1981; Chourrout 1984; Onozato 1984), heat shock (Chourrout and Quillet 1982; Chourrout and Itskovich 1983; Don and

Avtalion 1986) or cold shock (Purdom 1969; Nagy et al. 1978; Stanley 1969; Refstie et al. 1982; Suzuki et al. 1985), applied at the appropriate conditions for each species.

The possibility of developing a line with a high level of genotypic identity and a high coefficient of inbreeding, as determined by Nagy and Csanyi (1984), makes the effort put into this research worthwhile. Since Tilapia is a very important fish for aquaculture, the potential benefit of heterosis resulting from crossing two different gynogenetic lines might be of great importance.

Materials and methods

Fish

The *O. aureus* and *O. niloticus* Ein-Hamifratz lines, characterized by transferrin markers 6–8 and 7–9, respectively, were used for this study (Avtalion 1982; Mires 1982). The sera of fish were tested, using polyacrylamide gel electrophoresis, for their esterase isozyme and transferrin specific markers before the establishment of fish to families of 5–6 females and 1–2 males each. The fish were kept in aquaria at constant temperature (26.5 °C ± 1.5) as previously reported (Koiller and Avtalion 1985). The fish were fed with 40% protein-containing pellets quantitatively calculated as 2–4% of their weight/day.

Gamete collection and insemination

Eggs and sperm were gently stripped from females and males immediately before spawning. Artificial insemination of eggs and incubation of the embryos, up to yolk sac absorption, together with determination of the viability rates at four different developmental stages (morula, M; pigmentation, P; hatching, H and viability beyond yolk sac absorption, V) were carried out as previously described (Don and Avtalion 1986; Yeheskel and Avtalion 1987).

UV irradiation of sperm

An irradiation cell equipped with two G15 T8 15w UV lamps (Philips) was used for the sperm irradiation. The intensity of UV irradiation applied to the sperm samples was modulated by modifying the duration (in sec) of the irradiation applied at a pre-optimized distance of 8 cm between the sperm sample and the UV lamps. Ultraviolet meter measurements (UVX radiometer, UVP International, USA), at this distance showed an irradiation intensity of 3,070 $\mu w/cm^2$. Sperm samples of 0.8 ml were added to disposable tissue culture petri dishes with an internal diameter of 3.9 cm, in order to obtain a sperm layer of 0.66 mm thick. When being irradiated, the sperm samples were kept under gently magnetic stirring as suggested by Chourrout (1982). The irradiated sperm samples were then used for artificial insemination as described earlier.

Temperature shock

Heat-shock treatments were applied to the eggs using a system of 4 Zuger bottles of 250 ml each, supplied with hot circulating water and thermoregulated as desired. For diploidizing the haploid zygotes we applied the optimal conditions which were previously determined for the induction of triploidy in *O. aureus*, using a heat shock of 3.5–4 min at 39.5 °C and at the zygotic age of 3 min (Don and Avtalion 1986).

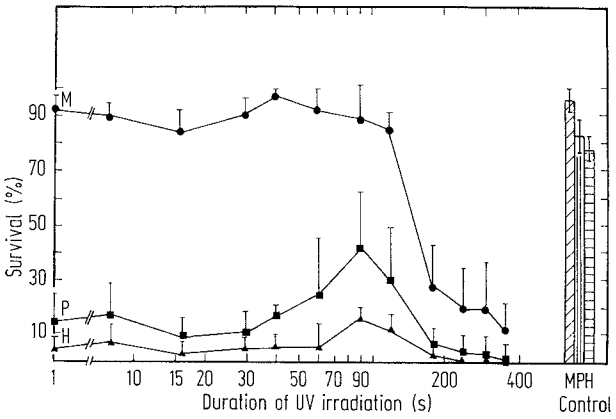

Fig. 1. Survival of haploid *O. aureus* embryos at the three developmental stages: morula – *M* (●); pigmentation – *P* (■) and hatching – *H* (▲), after fertilization with *O. niloticus* sperm irradiated by UV-irradiation for increasing durations and at a constant distance of 8 cm

Identification of ploidy

Ploidy was determined by counting chromosomes of 50 karyotypes per slide, prepared from a pool of 10–20 embryos taken from each group as previously reported (Don and Avtalion 1986).

Results

Egg activation with UV irradiated sperm

Aliquots of sperm collected from *O. niloticus* active males were exposed to UV irradiation for different periods of time, as detailed in "Materials and methods". Eggs were artificially inseminated with the irradiated sperm aliquots and incubated as previously reported (Don et al. 1987). Results showed that in the first phase of the curve (1–60 sec; Fig. 1), the irradiated sperm conserved its potential to activate a very high proportion of the eggs (about 95%) to complete second meiosis and to reach the morula stage (6–8 successive mitotic divisions). In contrast, a sharp reduction of the embryo viability was observed at the P stage (14%–26%) and at the H stage (3.5%–5%), throughout the length of this phase (Fig. 1). On the other hand, sperm irradiated for 90 sec (second phase), while slightly less active for the induction of the morula (90%), were paradoxically more efficient in activating the eggs through pigmentation (42%) and in development beyond the hatching stage (18%). From this stage on, embryo viability decreased in relation to the increase of sperm exposure to UV irradiation, until reaching 0% viability for P and H stages and 12% for the M stage when sperm was irradiated for 6 min (Fig. 1). All of the hatching embryos, at any irradiation intensity, were haploids ($1N = 22$) without any hyper-haploids or

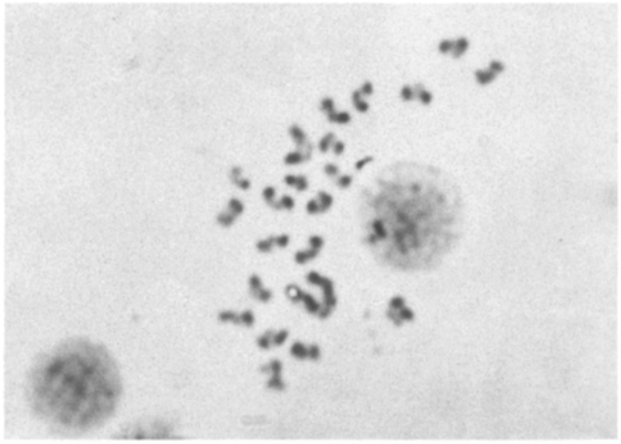

Fig. 2. Metaphase karyotype of haploid ($1N=22$, $\times 630$) embryos (direct Giemsa staining)

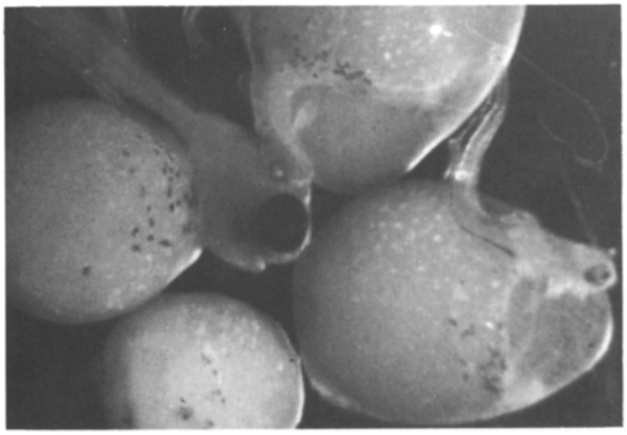

Fig. 3. Normal diploid embryo and haploid embryos, obtained after fertilization with irradiated sperm. The diploid embryo and the haploid embryos, from the same female and the same batch of eggs, were incubated under similar conditions. ($\times 60$)

Table 1. Percent of viability of diploid F_1 and F_2 gynogenetic tilapias and controls at hatching (H) and beyond yolk sac adsorption (V)

		Experimental diploid Gynogenetic	Controls		
			Haploid gynogenetic	Normal diploid	Triploid
F_1	$\sum n$[a]	5 000	400	400	400
	H[b]	4.25±5.2	13.2±11.4	65.7± 9.7	40.0± 7.8
	V[c]	0.36±0.27	0.0	57.7±12.3	35.5±10.8
F_2	$\sum n$[a]	7 000	600	600	600
	H[b]	7.7±5.5	20.5±11.9	61.4±15.0	30.5±23.0
	V[c]	3.6±2.1	0.0	50.4±11.9	23.2±24.1

[a] $\sum n$ = Total number of treated eggs
[b] H = Mean survival rate (in %) ±SD at the hatching stage
[c] V = Mean survival rate (in %) ±SD beyond yolk sac absorption

hypo-diploids and without any additional chromosomal fragments (Fig. 2). They were all morphologically deformed showing a typical haploid syndrome (Fig. 3).

Diploidization of haploid activated eggs

Diploidization of haploid gynogenetic embryos was achieved using the technique previously used to induce triploidy in *O. aureus* by heat shock (Don and Avtalion 1986). *O. aureus* eggs were activated by *O. niloticus* sperm, irradiated for 1.5 min as detailed above, and subjected to a heat shock of 39.5±0.2°C for 3.5–4 min at the precise zygotic age of 3 min. Three different control experiments were also performed: (1) all haploids in which the eggs were activated with the irradiated sperm as in the experimental group, but were not heat shocked, 1N control; (2) all triploids in which the eggs were fertilized with non-irradiated sperm but submitted to heat shock under the same conditions as the fertilized eggs in the experimental group, 3N control; and (3) all diploids in which normal diploid hybrids were formed, 2N control.

Karyological and morphological examinations showed that all embryos of the 1N control and 3N control experiments were haploids or triploids, respectively, as predicted. Therefore, all diploid embryos that survived from the experimental group were expected to be diploid gynogenetic fish. The mean hatching rate, as well as the mean survival rate (MSR) beyond yolk sac absorption, of these three groups are presented in Table 1. It could be observed that the hatching rates of the 1N control were consistently higher than that of the gynogenetic embryos, but the final survival rates of the 1N control were always 0%, in contrast to the MSR of the F_1 diploid gynogenetic embryos which was 0.36%±0.27%. This survival rate was determined as a mean result from 4 different experiments carried out with 5,000 eggs that were activated and diploidized.

A second generation (F_2) of gynogenetic tilapias was established by applying the technique described above to 8-month-old sexually mature F_1 gynogenetic females. The MSR of the F_2 gynogenetic embryos (3.6%±2.1%) indicates a significantly ($P<0.01$) higher survival rate, beyond yolk sac adsorption, in comparison to the MSR obtained in the F_1 gynogenetic fish (0.36%). The MSR of the F_2 embryos was the result of experiments carried out with 7,000 eggs used in 8 different experiments. No statistically significant differences could be seen between the viability of F_1 and F_2 2N and 3N controls ($P<0.2$), as well as between the hatching rates of the 1N controls in F_1 and F_2 ($P>0.2$).

Genetic markers

Biochemical and morphological genetic markers of the donor species of the eggs and sperm were used to confirm

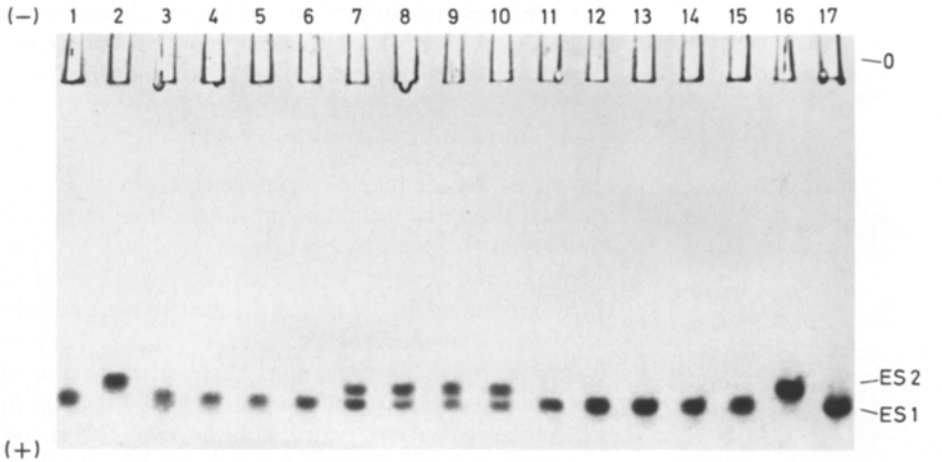

Fig. 4. Electrophoretic picture of serum esterases as a genetic marker. Samples of maternal parent (*O. aureus*) are shown in *lanes 1 & 17* (*ES-1*) and of paternal parent (*O. niloticus*) in *lanes 2 & 16* (*ES-2*). Gynogenetic offsprings show only the maternal pattern (*ES-1*) in *lanes 3–6* and *11–15*. Normal hybrids of these two parents (*lanes 8–10*) and the triploid hybrid (*lane 7*) show the two isoenzymes (*ES-1 + ES-2*)

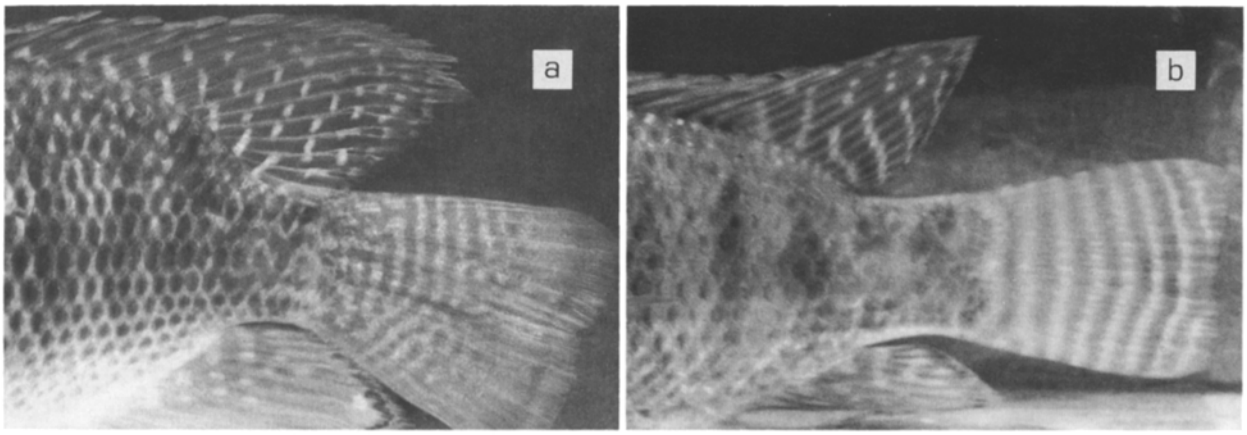

Fig. 5. (**a**) Unstriped tail of *O. aureus* and (**b**) striped tail of *O. niloticus*

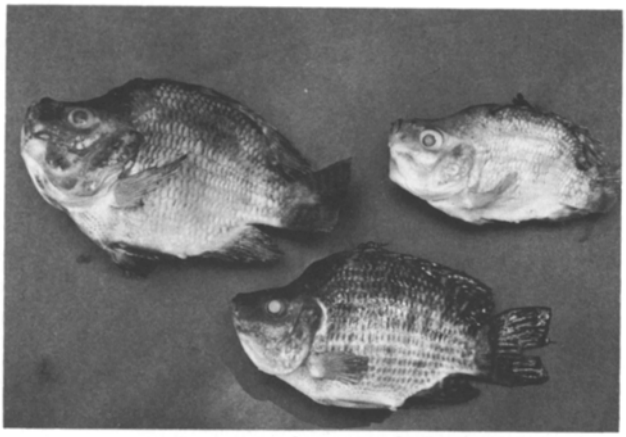

Fig. 6. Deformed fish obtained among F_1 diploid gynogenetic offsprings

that the surviving fish from the experimental groups were indeed gynogenetics, (i.e. possessing the maternal genome only). We tested the serum esterase electrophoretic pattern of both parents (*O. niloticus* male and *O. aureus* female) of the surviving fish from the experimental groups and of the 2N and 3N control groups. Results showed that each parent possessed its species specific serum esterases as expected. In the serum of fish from the experimental groups only the maternal esterase was present, in contrast to the 2N and 3N controls that showed both isozymes (Fig. 4). Triploid control Es-1 seems to be enhanced in comparison with the normal diploid control (Fig. 4). The tail striation of *O. niloticus* and the unstriped red-edged tail of *O. aureus* (male and female) were also used as a dominant genetic marker. According to our observations on hundreds of hybrids between

these two species (including both reciprocal hybrids), all F_1 hybrids showed the tail striation inherited from the *O. niloticus* parent and red-edged tail of the *O. aureus* parent (these traits become more apparent at the spawning season). All 2N and 3N controls matched our expectations and had striped tails. However, all the fish that we considered gynogenetic had only the characteristics of *O. aureus* (Fig. 5a–b).

It is noteworthy that a major part (9 out of 18) of the surviving F_1 gynogenetic offspring showed different body malformations such as disjunction of jaws from the body, megallocephaly and other sublethal malformations (Fig. 6), while the surviving F_2 gynogenetic offspring did not show any malformations.

Discussion

Analysis of the "Hertwig curve" obtained using UV-irradiated sperm

A "Hertwig effect" with a non-typical biphasic curve was demonstrated in the present study (Fig. 1). In the typical "Hertwig curve", the first phase is characterized by a decrease in embryo survival rates as the irradiation intensity of the sperm increases. In the second phase, a paradoxical increase of the embryo survival rates appears, while the irradiation intensity continues to increase. Such typical curves were reported in salmonids using either γ-irradiation (Chourrout et al. 1980; Onozato 1982) or UV-irradiation (Chourrout 1982), and in *Oryzias latipes* using UV-irradiation (Ijiri and Egami 1980). The current explanation of this paradoxical curve is that at the first phase an increasing destruction of sperm chromatin occurs in correlation with the increase of irradiation intensity. Consequently, decreasing amounts of diploid and viable aneuploid embryos are the result of fertilization with sperm cells that escaped the immediate lethal effects of irradiation. At the second phase when γ-rays are used, the higher irradiation intensities destroy the paternal chromatin completely, and haploid embryos possessing exclusively the maternal genome are formed (Lasher and Rugh 1962; Ijiri and Egami 1980; Chourrout et al. 1980; Chourrout 1982; Onozato 1982). However, with the use of UV-irradiation, it was suggested that the syngamy of parental pronuclei does not occur probably due to a massive pyrimidine dimerization that leads to nucleoplasm coagulation of paternal chromatin (Ijiri 1980; Ijiri 1983). Recent findings, which point to the occurrence of paternal chromosomal residues in karyotypes of embryos obtained using sperm irradiated with low dose of γ-rays, seem to confirm this explanation (Chourrout and Quillet 1982; Onozato 1982, Thorgaard et al. 1985).

In this paper the first phase of the curve seems to have an atypical shape, showing (1) low and stable embryo hatching rates of about 3.5% throughout the length of this phase, and (2) that the hatching embryos, independent of the irradiation intensity, were exclusively haploids.

These findings, which are in agreement with the results reported by Chourrout and Itskovich (1983) in another species of tilapia, are not consistent with the above mentioned explanation for the first phase. Therefore, it seems to us that in tilapias the UV damaged sperm chromatin exert toxic effects [perhaps due to the formation of thymidine dimers as suggested by Ijiri (1983)] resulting in degeneration of the tilapia embryos at early stages of their development. However, this damaged sperm is still able to activate the eggs for morula development, even with high irradiation intensities (Fig. 1). This indicates that the lethal effect of UV-irradiation is mainly expressed on early differentiative events during embryogenesis, and might be the reason why we could not detect aneuploidy or chromosomal fragments at the hatching stage. However, the mechanism by which the haploid embryos are formed along the first phase is more difficult to explain. It is possible that for a low and constant percentage of UV-irradiated sperm either asyngamy or activation of the eggs without sperm penetration might occur.

Production of F_1 and F_2 diploid gynogenetic embryos

The results obtained in the control groups (1N and 3N), where 100% of the embryos in each group were haploids or triploids, indicate that the surviving diploid embryos of the experimental groups could be considered as diploid gynogenetic fish. This consideration was confirmed using known electrophoretic (esterases) and morphological (tail striation) dominant genetic markers specific to the donor species of eggs and sperm (Figs. 4 and 5). In fact, when we tested the sera of our adult fish, expected to be gynogenetic, and their tails, only the maternal esterase was present and no paternal tail striation could be seen. In contrast, the 2N and 3N controls showed both isozymes (Fig. 4) and the typical paternal tail striation. It is noteworthy that the enhanced dosage of Es-1 in the triploid control (Fig. 4, lane 7) might be a result of the two maternal alleles expression.

A very high mortality rate (Table 1) and a high incidence of distortions (Fig. 6) were observed in these gynogenetic tilapias. The phenomenon of increased mortality and increased incidence of deformation and embryonic weakness in induced gynogenetic fish has been widely reported in literature. This is probably the result of a rapid increase in the inbreeding level that enables the expression of lethal and defective recessive genes (Purdom 1969; Nagy et al. 1978; Chourrout and Quillet 1982; Refstie et al. 1982; Onozato 1984; Suzuki et al. 1985).

The level of mortality in F_1 diploid gynogenetic fish seems to be species dependent (reviewed by Don and Avtalion 1987). This might be attributed to: (1) differences in the recombination frequencies between species; or (2) occurrence of duplicated gene loci within a single haploid set of chromosomes, which is common in some fish with tetraploid ancestries (Allendorf and Thorgaard 1984). As to the former possibility, it is well known that the lower the recombination frequency, the higher the level of inbreeding and mortality (Purdom 1969; Nagy and Csanyi 1984). In the second case, mutual compensation between these loci contributes to higher survival rates of the F_1 gynogenetic embryos, even if the inbreeding coefficient is equal or almost equal to 1, as suggested in salmonids (Guyomard 1984). The low survival rates (0.36%) obtained in this work in *O. aureus* and by Penmam et al. (1986) in the same species might, therefore, be due to the lack of such double gene systems and to a high inbreeding coefficient. In fact, the inbreeding level that probably characterises this line of *O. aureus* was further demonstrated in scale transplantation tests (Avtalion et al. 1987). Further investigation is needed to determine the actual recombination rates for specific loci. However, this seems not to be the rule in all the tilapia species, since higher rates of viability (40% hatching) were shown in *O. niloticus* (Chourrout and Itscovitch 1983).

The survival rates in our F_2 gynogenetic fish were significantly higher (3.6%, $P<0.01$) than those of the first generation (Table 1), as well as the incidence of malformation, which was significantly decreased. Moreover, all the living offspring from one of the F_1 gynogenetic females had a typical striker-like shape pointing to a high level of genotypic identity. These results support the suggestion that deleterious recessive alleles were purged in the F_1 gynogenetic fish.

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