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Inheritance of sex in two ZZ pseudofemale lines of tilapia *Oreochromis aureus*

Damien Desprez[a,b,c,*], Charles Mélard[c], Marie Claude Hoareau[a], Yohann Bellemène[a], Pierre Bosc[a], Jean François Baroiller[b,d]

[a] *ARDA, Freshwater Aquaculture Center, BP 16, ZI Les Sables, 97427 Etang Salé, Reunion Island, France*
[b] *CIRAD EMVT, Aquaculture Research Unit, BP 5095, 34033 Montpellier cedex1, France*
[c] *University of Liège, CEFRA, 10 chemin de la Justice, 4500 Tihange, Liège, Belgium*
[d] *INRA, Laboratory of Fish Physiology, Avenue du Général Leclerc, 35042 Rennes Cedex, France*

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Abstract

This paper reports a study on the sex determination system of the blue tilapia, *Oreochromis aureus*. Investigations were carried out using a pseudofemale line in two populations of *O. aureus*, known as Egyptian Population (EP) and Israel Population (IP). In *O. aureus*, males are the homogametic sex (ZZ/ZW), and sex reversal of fry with estradiol results in the production of some functional sex-reversed fish with a female phenotype and ZZ male genotype, known as pseudofemales or Δ-females. Crosses between ZZ pseudofemales and ZZ males theoretically should provide monosex ZZ male progeny only. We have studied the sex ratios of progeny from 43 IP (F_2 to F_3 generations) and 51 EP (F_1 to F_5 generations), pair-matings between normal males and pseudofemales. In IP, the male percentage in progenies ranged between 83% to 100% in F_2 and 66% to 100% in F_3. In EP, male percentage was more constant, varying from 88% to 100% in F_1, from 96% to 100% in F_3 and from 97% to 100% in F_5. In EP, F_2 and F_4 pseudofemales produced only monosex male progeny. This apparent difference in sex ratio frequency distributions between the two *O. aureus* pseudofemale lines could be due to the selection of males. EP pseudofemales were mated with their siblings for F_2 and F_3 pseudofemales or with closely related males for F_4 and F_5 pseudofemales. Conversely, IP pseudofemales were crossed with nonrelated males originating from research center broodstock, resulting in a higher proportion of females in sex ratio of progenies from successive generations of pseudofemales. The role of inbreeding is discussed in the context of predominantly monofactorial sex chromosome determination system operating in this species, influenced by other factors (genetic and environmental). The present study also shows that it is possible to fix the male sex determining factors

* Corresponding author. ARDA, Freshwater Aquaculture Center, BP 16, ZI Les Sables, 97427 Etang Salé, Reunion Island, France. Tel.: +262 2-62-26-50-82; fax: +262 2-62-26-50-01.
E-mail address: desprez.arda@guetali.fr (D. Desprez).

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(Z sex chromosome and genetic factors) in a line of pseudofemales, producing a high percentage of male progeny in five successive generations.

Keywords: *Oreochromis aureus*; Sex determination; Sex chromosome; Pseudofemale; Monosex progeny

1. Introduction

Several different sex determination mechanisms have been observed in fish species, including hermaphroditism (Price, 1984; Chourrout, 1988), polygenic systems (Kallman, 1984), monofactorial sex determination systems (Chourrout, 1988) or environmental sex determination (Conover and Heins, 1987). In the genus *Oreochromis*, both male homogamety (ZZ/WZ) and female homogamety (XX/XY) have been proposed, based on experimental sex ratios from intraspecific crosses, involving normal or sex-reversed parents (Clemens and Inslee, 1968; Jalabert et al., 1974; Guerrero, 1975; Jensen and Shelton, 1979). Experimental results have demonstrated that monosex progeny were obtained when sex-reversed fish of the homogametic sex were mated with normal fish of the same sex genotype (Clemens and Inslee, 1968 in *O. mossambicus*; Yamamoto, 1969 in *Oryzia latipes*; Jalabert et al., 1974 in *O. niloticus*). In *Oreochromis aureus*, males are homogametic (ZZ/ZW), and sex reversal of fry with estradiol results in the production of some functional sex-reversed fish with a female phenotype and ZZ male genotype, known as pseudofemales (Jensen and Shelton, 1979; Mélard, 1995) or Δ-females (Mair et al., 1987). First generation pseudofemales (F_1 pseudofemales) can be identified by progeny testing: the cross between a ZZ pseudofemale and a normal ZZ male theoretically provides a monosex ZZ male progeny. Conversely, breeding ZW females gives 50% ZZ males and 50% ZW females. Sex-reversal treatment with estradiol, applied to monosex male progeny from F_1 pseudofemales, leads to second generation pseudofemales (F_2 pseudofemales) (Mair et al., 1991b; Mélard, 1995), which do not need to be identified by progeny testing. This technique can be repeated through successive pseudofemale generations (F_3, F_4...). If *O. aureus* utilised a monofactorial sex chromosome determination system, crosses of ZZ pseudofemales × ZZ males should give systematically ZZ male progenies only. Therefore, pseudofemale lines are of interest in studying the sex determination system, because deviations from expected (♂/♀) sex ratio (1:0) in progenies can be easily identified. These deviations suggest the influence of an additional factor or factors (environmental or genetic) other than sex chromosomes also play a role in the sex determination in this species. Several authors have observed significant deviations from expected sex ratios in crosses involving *O. aureus* (Shelton et al., 1983; Mair et al., 1991b; Mélard, 1995), and hypotheses have been suggested to explain results, including a polygenic system of sex determination, epsitatic autosomal genes or environmental influence on sex differentiation (Majumdar and Mc Andrew, 1983; Lester et al., 1989; Mair et al., 1991b; Wohlfarth and Wedekind, 1991; Baroiller et al., 1995; Baroiller, 1996).

The present study was carried out using pseudofemales lines in two populations of *O. aureus*, known as Egyptian population (EP) and Israel population (IP). We have studied the sex ratio of progenies in F_1 to F_3 generations of the IP pseudofemale line and in F_1 to F_5 of

the EP pseudofemale line. We have also analysed the influence of inbreeding on sex ratio variability, mating pseudofemales with closely related or nonrelated males. Inbreeding results were discussed in the context of a predominantly monofactorial sex determination system, influenced by additional factors (genetic and environmental). This study helps to further elucidate the sex determination system and the inheritance of the genetic sex determination in the genus *Oreochromis* and more especially in *O. aureus*. Understanding genetic sex determination systems in the genus *Oreochromis* contributes to the development of methods for controlling sex through genetic means, like the pseudofemale line in *O. aureus* to produce monosex male progeny.

2. Materials and methods

2.1. Origin of fish

The *O. aureus* Israel population (IP) originates from the Dor station in Israel. This population has been acclimated since 1979 in the experimental station of University of Liège (Belgium). Hulata et al. (1993) made it clear that the Dor station stock derived from the original founders collected in Lake Hula (North Israel) in about (1958). The *O. aureus* Egypt population (EP) originates from Lake Manzala in Egypt. This population was first transferred to the Ivory Coast (Institut des Savanes, Bouaké) and from there to ARDA-Freshwater Aquaculture center in Reunion Island (France, Indian Ocean) in 1995. This study was carried out in University of Liège (IP) and in ARDA-Freshwater Aquaculture Center (EP).

2.2. Pseudofemale line production

We describe below the different production stages of successive pseudofemale generations in the two populations, IP and EP. The method of 17α-ethynylestradiol sex-reversal treatment and the progeny testing to identify the F_1 pseudofemale were described in Mélard (1995). In this study, the pseudofemales were identified when sex ratio of progeny was significantly different ($P<0.05$) from an expected sex ratio (♂/♀) of 1:1.

Israel population (IP) pseudofemale line: F_1 pseudofemales were produced by sex reversal (100 to 200 mg of 17α-ethynylestradiol kg^{-1} of food for 40 days) of fry from three progenies of normal *O. aureus*. To obtain F_2 pseudofemale broodstock, six progenies from five F_1 pseudofemales crossed with three males were submitted to a hormonal treatment of 150 mg of 17α-ethynylestradiol kg^{-1} of food for 40 days (Mélard, 1995). This treatment was applied to three progenies from two F_2 pseudofemales crossed with three males to produce F_3 pseudofemale broodstock (Table 1).

Egypt population (EP) pseudofemale line: Three progenies of normal female EP were submitted to a sex-reversal treatment (125 mg of 17α-ethynylestradiol kg^{-1} of food for 40 days). Then, the same hormonal treatment was applied to two progenies from the same F_1 pseudofemale and to one progeny from an F_2 pseudofemale in order to produce F_2 and F_3 pseudofemale broodstock, respectively (Table 2). Fry from successive crossings of one F_3 pseudofemale with two males were sex reversed (125 mg of 17α ethynylestradiol kg^{-1} of food for 40 days) to produce F_4 pseudofemale broodstock (Table 2). The same treatment

Table 1
Proportion of males in progeny of repeat mated F_2 and F_3 pseudofemale *O. aureus* Israel Population (IP)

Spawning number		1	2	3	4	5	6	7
Pseudofemale identification	Generation	Male %	Male %	Male %	Male %	Male %	Male %	Male %
1	F_2	99	**100**	100	**100**	94	100	
2	F_2	100	100	89	95	100	100	
3	F_2	96	99	100	100	100	100	100
4	F_2	100	**100**	100	100	97	100	
5	F_2	98	100					
6	F_2	80 (a)	83 (a)	96 (b)	93 (b)			
7	F_2	86 (a)						
8	F_2	98 (a)						
9	F_3	70						
10	F_3	98 (c)	77 (d)					
11	F_3	100						
12	F_3	97						
13	F_3	95						
14	F_3	90						
15	F_3	71						
16	F_3	66						
17	F_3	77						

a, b, c, d: Male identification.
Bold results: progenies sex-reversed to produce F_3 pseudofemale generation.

was applied to two progenies from two F_4 pseudofemales crossed with two males to produce F_5 pseudofemale broodstock (Table 2).

To each 17α-ethynylestradiol-treated group, we systematically added a control group that had not been submitted to hormonal treatment. After a period of 60 to 90 days or an average weight of >3 g, samples of 30 fish in treated groups and 100 fish in control groups were sexed by the aceto-carmine squash method (Guerrero and Shelton, 1974). At this stage of development, oocytes are easily identifiable in their auxocytosis or previtellogenesis stages and a typical lobular configuration is observed in the testes (Baroiller, 1996). Only the sex-reversed progenies of pseudofemales that produced 100% male in control group were retained for broodstock.

2.3. Progeny testing

IP F_1 to F_3 pseudofemales and EP F_1 to F_5 pseudofemales were progeny tested by crossing with related or nonrelated males. Some pseudofemales crossed several times ($n = 2$ to 7) with the same or different normal males to check the sex ratio stability in the successive progeny. IP pseudofemales were crossed with largely unrelated IP males, taken from the *O. aureus* broodstock of the experimental station of University of Liège. Conversely, the EP males came from the control group of hormonal treatment. Thus, EP pseudofemales were mated with their siblings (F_2 and F_3) or with closely related breeders (F_4 and F_5). All tested males and pseudofemales were individually tagged with passive integrated transponder tags (Fish Eagle pit tag I.D. system).

Table 2
Proportion of males in progeny of repeat mated F_1, F_2, F_3, F_4 and F_5 pseudofemale *O. aureus* Egypt Population (EP)

Spawning number		1	2	3	4	5	6	7
Pseudofemale identification	Generation	Male %	Male %	Male %	Male %	Male %	Male %	Male %
18	F_1	100	**100**	**100**				
19	F_1	100	98	88				
20	F_1	100						
21	F_1	100						
22	F_1	100						
23	F_1	100						
24	F_2	**100** (e)						
25	F_2	100 (e)						
26	F_2	100 (e)						
27	F_2	100 (e)						
28	F_2	100 (e)						
29	F_3	100 (f)						
30	F_3	100 (f)	100 (g)	**100** (g)	98 (g)	100 (h)	**100** (h)	100 (h)
31	F_3	100 (f)	100 (g)	100 (h)				
32	F_3	100 (f)	100 (g)	99 (h)	98 (h)	97 (h)		
33	F_3	100 (f)	100 (g)	100 (g)				
34	F_3	100 (f)	96 (g)	100 (h)				
35	F_3	100 (f)						
36	F_4	100 (i)	**100** (i)					
37	F_4	100 (j)	**100** (j)					
38	F_5	100 (k)	100 (k)	100 (l)				
39	F_5	100 (l)	100 (l)					
40	F_5	97 (l)	100 (l)	100 (l)				
41	F_5	98 (l)						

e, f, g, h, i, j, k, l: Male identification.
Bold results: progenies sex-reversed to produce next pseudofemale generation.

Israel population (IP) pseudofemale line: Pools of 10 to 15 pseudofemales and 1 male of the same generation were placed in spawning tanks (4 m^2/1.6 m^3) supplied with warm water (25–27 °C). The fertilised eggs were collected once a week and incubated in a small hatchery at a temperature of 27 ± 1 °C. After the yolk sac resorption, fry were reared in small aquaria (60 l) in a re-circulating system. Temperature minima and maxima for these experiments were 25.9 and 28.2 °C, respectively. A progeny was identified by parental male and pseudofemale tag numbers. Some spawnings of F_2 and F_3 pseudofemales were also collected in spawning pools of 10 pseudofemales and 3 males (tanks of 4 m^2/1.6 m^3 supplied with warm water, 25–27 °C). In this case, the paternal origin of progenies was unknown.

Egypt population (EP) pseudofemale line: One male and five to six pseudofemales of the same generation were maintained in a 400 l thermo-regulated aquarium (27 ± 1 °C) in a re-circulating system. The reproductive status was checked twice a day by detection of female mouthbrooding behaviour. Mouthbrooding females were isolated in their respective aquaria and progenies were removed from the mouth of female 9–10 days after spawning. Each progeny was identified by male and female tag numbers and was reared in a small

tank (100 l) within a re-circulating system. Temperature minima and maxima for these experiments were 26.5 and 30.1 °C, respectively.

Fry were sexed as described above, and a sample of 100 fish from each progeny was analysed. The sex ratio of the pseudofemale progeny was compared to the theoretical sex ratio (♂/♀) (1:0; 1:1 or 3:1) using a 2×2 contingency χ^2 test.

3. Results

3.1. Israel population (IP) pseudofemale line

Results on F_1 pseudofemales were presented already in Mélard (1995). In brief, the F_1 pseudofemales ($n = 22$) produced percentage of males ranging from 80% to 100% and seven F_1 pseudofemales systematically produced monosex male progeny in successive crossings. Progeny sex ratios from repeated pair-matings were not significantly different in the successive crossings, except for five pseudofemales presenting heterogeneous results (maximum difference: 16.4%).

In the present study, six and two pair-matings, respectively, in F_2 and F_3 pseudofemales were tested. Additional results (35 pair-matings) were also obtained from 13 pseudofemales (F_2 and F_3), in which, the male parent was not identified (Table 1). F_2 and F_3 pseudofemales produced mean male percentages of $97.1 \pm 5.2\%$ and $84.1 \pm 12.5\%$ (Table 1), respectively.

The male percentage ranged between 80.0% and 100.0% in progenies from F_2 pseudofemales, and F_2 progeny sex ratios from 57.6% ($n = 19$) pseudofemales crosses were 1:0. Crossing F_2 pseudofemale "6", the percentage of males in progeny involving male "b" (93 and 96%) was significantly higher than with male "a" (80% and 83%) (χ^2 test, $P < 0.05$). Sex ratios from crosses of male "a" with three F_2 pseudofemales produced three different sex ratios (Table 1), one not significantly different from a 3:1 (pseudofemale 6, $\chi^2 = 0.72$, $P > 0.05$), one significantly different from 3:1 and 1:0 (pseudofemale 7; $\chi^2 = 3.85$ and 15.05, $P < 0.05$), and the last one not significantly different from 1:0 (pseudofemale 8, $\chi^2 = 2.02$, $P > 0.05$).

Nine F_3 pseudofemales were progeny tested. The results are shown in Table 1. Only the pseudofemale "11" gave monosex male progeny. The percentage of males in the other F_3 pseudofemale progenies ranged from 66% to 98%. F_3 pseudofemale "10" produced successively 98% and 77% males ($\chi^2 = 20.16$, $P < 0.05$) with males "c" and "d", respectively (Table 1).

3.2. Egypt population (EP) pseudofemale line

Fifty one pair-matings were tested, involving F_1 to F_5 pseudofemales. The five successive generations produced mean sex ratios of $98.6 \pm 3.6\%$, 100%, $99.5 \pm 1.1\%$, 100% and $99.4 \pm 1.1\%$ of males. The mean sex ratio in EP F_3 pseudofemale ($99.5 \pm 1.1\%$ of males) was significantly higher than in IP F_3 pseudofemale ($84.2 \pm 12.5\%$) (Mann and Whitney test, $U = 27.5$; $n = 10$; $m = 23$; $P < 0.05$). Opposite of that, the other possible comparisons of sex ratio between the pseudofemale generations were not significantly different (Mann and Whitney test, $P > 0.05$).

The percentages of male monosex (1:0) progeny from F_1, F_2, F_3, F_4 and F_5 EP pseudofemales were respectively 80.0% ($n=10$ progeny tested), 100.0% ($n=5$), 78.3% ($n=23$), 100.0% ($n=4$) and 77.8% ($n=9$) (Table 2). Sex ratios in progeny from F_1 to F_5 pseudofemales were not significantly different from a sex ratio of 1:0, except when crossing F_1 pseudofemale "19" (88% males, $\chi^2=12.76$, $P<0.05$) and F_3 pseudofemale "34" with male "g" (96% males, $\chi^2=4.08$, $P<0.05$) (Table 2). There was no significant difference in progeny sex ratios from repeated crosses of pair-matings, except with F_1 pseudofemale "19" and F_3 pseudofemale "34" (χ^2 test, $P<0.05$) (Table 2).

4. Discussion

The present study show that it is possible to obtain high male progeny in successive generations of pseudofemales in *O. aureus* by progeny testing and hormonal sex reversal. The monofactorial sex determination system with two sex chromosomes (Z,W) in *O. aureus* is supported by our results on the analysis of progeny sex ratios from pseudofemales. Since deviations from all-male sex ratios were obtained in progeny from F_2 and F_3 IP pseudofemales. Similar deviations from monosex ratios have also obtained in *O. aureus* (Mair et al., 1987, 1991b) and in *O. niloticus* (Mair et al., 1991a, 1997; Pham et al., 1999). To explain these results in *O. aureus*, which are not consistent with a monofactorial sex determination system, Mair et al. (1991b) proposed a more complex system, based on the existence of an autosomal recessive gene (F,f) epistatic to the main sex chromosomes (Z,W) in *O. aureus*. The combination of a homozygous autosomal recessive allele with either pair of sex chromosomes (ZZ or WZ) would produce a phenotypic female (i.e. genotype ZZ/ff gives a phenotypic female). This hypothesis is in part in accordance with our results, and could explain the deviating sex ratios in F_2 and F_3 pseudofemale progeny of IP which were not significantly different from 3:1. As suggested by Wohlfarth and Wedekind (1991) in *O. niloticus*, the variability of sex ratio in relation to monofactorial sex determination system results from autosomal loci influencing sex determination. The results on IP F_2 pseudofemale "6" also suggest the influence on sex determination of an additional genetic factors other than sex chromosomes (Table 1). These genetic factors would interfere with sex differentiation regulated by the expression of genes on the sex chromosomes. Thus, the sex determination in *Oreochromis* will not depend only on sex chromosomes, but will equally be influenced by one or more other genetic factors. However, the present study also shows that it is possible to fix male sex determining factors (Z sex chromosome and genetic factor(s)) in a line of *O. aureus* pseudofemale.

In the present study, only the sex-reversed progeny of pseudofemales that produced 100% male in the control group was retained for broodstock, effectively representing selection against sex modifying factors. According to the results, this selection of pseudofemales may have been more effective in the EP pseudofemale line than in the IP, because the mean sex ratio in EP F_3 pseudofemale ($99.5 \pm 1.1\%$) was higher than in IP F_3 pseudofemale ($84.2 \pm 12.5\%$). This difference could be due to the selection of males, since EP pseudofemales were mated with their siblings (pseudofemales F_2 and F_3) or with closely related breeders (pseudofemales F_4 and F_5), whereas IP pseudofemales were crossed with nonrelated males originating from unselected broodstock. A high degree of inbreeding in

the EP line may have furthered selection of autosomal male determining factors. Conversely, nonrelated males, crossed with IP pseudofemales, could have carried female determining factors. The balance of these autosomal sex factors could influence sex chromosome expression and their effects could have increased in successive generations of pseudofemales, explaining the decrease in male percentage in progeny of the F_2 and F_3 IP pseudofemales. Complex models of sex determination, as proposed by Mair et al. (1991b), cannot be applied to all species of genus *Oreochromis* (Mair et al., 1991a,b; Wohlfarth and Wedekind, 1991) and to all populations of a species (Shelton et al., 1983; Mair et al., 1991b; the present study). Furthermore, there are inherent differences in sex ratio variability between populations in *O. niloticus* (Shelton et al., 1983; Mair et al., 1991a; Pham et al., 1999) and in *O. aureus* (Shelton et al., 1983; Mair et al., 1991b). This inherent difference between populations could explain the sex ratio variability obtained in EP and IP lines. These results also suggest that autosomal sex factors would be specific to each species or to each population. Crossing-over and recombination of sex determining genes also could account for these unpredicted sex ratios (Avtalion and Don, 1990; Mair et al., 1991a,b; Müller-Belecke and Hörstgen-Schwark, 1995).

A number of studies have demonstrated that high temperatures (34–36 °C) strongly skewed the sex ratio in favour of male (Lester et al., 1989; Baroiller et al., 1995; Abucay et al., 1999 in *O. niloticus*; Desprez and Mélard, 1998 in *O. aureus*) and high proportions of males can be produced in *O. aureus* (95–100%) (Desprez and Mélard, 1998) and *O. niloticus* (98–100%) (Baroiller and Clota, 1997). However, it is unlikely that temperature was involved in the present study because during the rearing of progenies, the temperature has never above 30.0 °C in EP and 28.5 °C in IP which was below the minimum level (34 °C) for temperature influence, determined by Baroiller et al. (1995) in *O. niloticus*. However, the effects of other environmental factors (pH, day length, salinity...) on sex determination cannot be discounted. Low pH has also been observed to affect sex ratio in Cichlids and Poeciliids (Rubin, 1985), e.g. in *Apistogramma* (Teleostei, Cichlidae) (Römer and Beisenherz, 1996). In *O. niloticus*, varying levels of salinity did not significantly affect sex ratio but brought about a slight difference (Abucay et al., 1999). The effects of some environmental factors could result more in low-marked variation, than temperature, and explain either these slight variations of sex ratio or differences in repeated crossings in *O. aureus* pseudofemale lines.

Although temperature and other factors influence sex determination in *Oreochromis*, the conclusion must be that sex in *O. aureus* is mainly determined by a monofactorial sex chromosome determination system with an influence of other genetic (autosomal factors, crossing-over). The present study shows that it is possible to obtain a line of pseudofemales, producing a high percentage of male progeny in five successive generations. Several studies showed that genetic approach can be used to produce progeny with a high percentage of males: ZZ pseudofemale line in *O. aureus* (Jensen and Shelton, 1979; Lahav, 1993; Desprez et al., 1995) and YY super male line in *O. niloticus* (Scott et al., 1989; Mair et al., 1997). The genetic approach avoids the hormonal sex-reversal treatment on a mass scale and the problem of androgen residues in market-sized fish. The present study suggests that the application of a genetic process to aquaculture requires strict selection of both sexes (male and female) and not just of one sex (super-male YY or pseudofemale ZZ). The existence of a complex sex determination mechanism involving sex chromosomes and other genetic

factors in *Oreochromis* species justifies that very selection. Although further studies on sex determination will be necessary to optimise the selection.

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