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Short Communication

Production of YY-male of Nile tilapia *Oreochromis niloticus* (Linnaeus, 1758) from atypical fish

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ABSTRACT. Successful YY technology depends on the production of XY females. However, their identification is complicated because they are indistinguishable from normal females. Atypical fish could offer an alternative for a more rapid and precise identification. Progeny of atypical fish was evaluated in order to produce YY-males. In total, nine atypical fish and 18 normal males were selected. The fish were placed in 8 m^3 concrete tanks at a 2:1 sex ratio. The produced fry were collected and reared at 28 ± 1ºC in 85 L aquaria. Juveniles were placed in net cages for 30 days and finally in 8 m^3 concrete tanks until the age of 120 days. Progeny test was achieved evaluating differences between sexes in the genital papilla structure. Six of the nine atypical fish selected showed the 3:1 sex ratio (male-female) expected for sex-reversed females. A significantly higher proportion of males than the expected 3:1 distribution were observed in two atypical fish. This boost in male proportion could be attributed to a parental effect interacting with the water temperature during the fry stage. Use of atypical fish could help reduce the time and effort spent to identify XY females during the initial stage of YY technology.

Keywords: *Oreochromis niloticus*, progeny of atypical fish, YY-male, sex-reversed females, genital papilla.

Producción de machos YY de tilapia del Nilo *Oreochromis niloticus* (Linnaeus, 1758) a partir de peces atípicos

RESUMEN. La aplicación exitosa de la tecnología YY depende de la producción de hembras XY. Sin embargo, su identificación es complicada, ya que son indistinguibles de las hembras normales. Los peces atípicos podrían ofrecer una alternativa para una más rápida y precisa identificación. Se evaluó la progenie de nueve peces atípicos con la finalidad de producir machos YY. Los alevines obtenidos se criaron a 28 ± 1ºC en acuarios de 85 L. Los juveniles fueron colocados en jaulas flotantes por 30 días y, finalmente, en estanques de 8 m^3 hasta los 120 días de edad. La prueba de progenie se realizó evaluando las diferencias entre sexos en la estructura de la papila genital. Seis de los nueve peces atípicos seleccionados mostraron la proporción de sexos 3:1 (macho-hembra) esperada para hembras revertidas. Se observó una proporción de machos significativamente mayor a la distribución 3:1 esperada en dos peces atípicos. Este aumento en la proporción de machos puede ser atribuido a la interacción del efecto parental con la temperatura del agua durante la etapa de alevín. El uso de peces atípicos podría reducir el tiempo y esfuerzo empleados en la identificación de hembras XY durante la etapa inicial de la tecnología YY.

Palabras clave: *Oreochromis niloticus*, progenie de peces atípicos, macho YY, hembras revertidas, papila genital.

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The Nile tilapia *Oreochromis niloticus* (Linnaeus, 1758) is one of the most economically important species in worldwide finfish aquaculture (FAO, 2005; Nonglak *et al.*, 2012). In commercial farming of Nile tilapia, reproduction during grow-out is a major problem, leading to the presence of fry and juveniles that overpopulate ponds and ultimately result in a wide range of fish sizes at harvest, instead of the larger and more uniform fish expected from the original stocking (Mair *et al.*, 1997; Jiménez & Arredondo, 2000; Tariq-Ezaz *et al.*, 2004). For this reason, production of all-male populations of tilapia is desirable and for many years has been recognized as the most effective technique to increase production (Vera-Cruz *et al.*,

1996; Mair *et al.*, 1997; Jiménez & Arredondo, 2000; Müller & Hörstgen, 2007).

Hormonal sex-reversal by feeding fry with different hormones is the most common method used to produce all-male populations. However, the use of this method is increasingly being criticized. The accumulation of hormones in the environment, and an increasing number of consumers that are not interested in eating products that have been treated with hormones (Piferrer, 2001; Müller & Hörstgen, 2007; Leet *et al.*, 2011) have led to search alternative techniques for production of all-male populations. One viable alternative, on a commercial scale, is the production of genetically male tilapia (GMT^{TM}) based on crosses between YY-males and XX-females (Vera-Cruz *et al*., 1996; Mair *et al.*, 1997). This has been done on a significant scale in the Philippines for some time (Mair *et al.*, 1997). However, it has only become widespread in recent years to other countries.

The initial development of YY-males requires feminization of XY fry during their sexually undifferentiated stage and the identification of these newly created "sex-reversed females" (females XY) through a progeny test (Vera-Cruz *et al*., 1996; Mair *et al.*, 1997). However, identification of sex-reversed females is complicated and time consuming because their morphology, behavior and karyotypes are indistinguishable from that of their genetically female siblings (Mair *et al.*, 1997). A possible solution is the use of atypical fish. Several authors have reported the presence of atypical fish after a feminization process (Hopkins *et al.*, 1979; Jensen & Shelton, 1979; Calhoun & Shelton, 1983; Potts & Phelps, 1995). Atypical fish are characterized by having abnormal papillae or sometimes for having ovaries but a male papilla. In general, these fish are discarded because they are considered to lack a functional oviduct. However, previous experiments carried out in our laboratory showed that selected atypical fish were able to produce a viable number of fry with a high survival rate during the early stages of development. The present study evaluated the sex ratio obtained in crosses between selected atypical fish and normal males (XY). Our ultimate objective is the development of a breeding program to produce male genetic population through YY-male in the southern region of Mexico, one of the highest producers of tilapia.

Atypical fish and normal males of Nile tilapia used in this study were produced using locally available strains (Centro Acuícola de Temascal, Oaxaca and Sistema Cooperativo Integral, Veracruz) at the experimental aquaculture station of the Universidad del Papaloapan. Atypical fish were produced by feeding fry at swim-up stage with 120 mg kg^{-1} E_2-treated feed (53% protein) for 30 days. In our work, atypical fish had well-developed ovaries (identified after extraction) but retained a genital papilla similar to the male. No oviduct was observed in any of the fish analyzed, but only fish from which we obtained a viable sample of eggs via abdominal massage were selected as breeders for the production of YY males. In total, nine atypical fish and 18 randomly selected males of eight months of age were used in selected crosses.

O. niloticus spawners were stocked at a male-atypical fish ratio of 2:1 in nine 8 m^3 outdoor concrete tanks (one atypical fish per tank) supplied with fertilized water (28 to 30ºC). During this time they were fed with commercial floating pellets (32% protein). Hatched fry were collected 15 to 21 days later with a fine-mesh net after siphoning 90% of the water in the tanks. Fry were counted and transported to a closed recirculating system composed of 85 L acrylic aquaria. Individual spawns were stocked at an initial density of 2 fry L^{-1}. In spawns with a low number of fry, initial stocking density was determined according to the number of fry obtained. A photoperiod of 12L:12D was used and water temperature was maintained at 28 ± 1ºC. Fry were fed *ad libitum* eight times a day for 30 days. After this time, juveniles were nursed in outdoor 0.8 m^3 floating net cages and fed *ad libitum* six times a day (40% protein) for another 30 days and then transferred to 8 m^3 concrete tanks supplied with fertilized water and reared up to 120 days of age, when the sex ratios within these fingerlings could safely be determined by examination of the genital papilla. Fish were fed with a commercial diet at 35% protein (3.5 mm, Nutripec, Agribrands Purina) three times at day and subsequently a commercial diet at 25% protein (4.8 mm, Nutripec, Agribrands Purina) until the end of the experiment. In each individual spawn, sex of the progeny was determined in 20-30% of the population. In spawns with a low number of fish, progeny test was performed evaluating all the fish. Differences between sexes in the papilla structure were highlighted using dye (methylene blue at 1%). Additionally, when no sperm or eggs were obtained during progeny sexing, fish were sacrificed and the gonad was extracted in order to confirm the sex. Fish analyzed were classified as male, female, or undifferentiated.

Proportions of males identified in progeny of atypical fish per normal male crosses were tested against the 3:1 expectation using the chi-square test at a probability level of 0.1% ($P < 0.001$). Progeny sex ratios approximating 3:1 (males:females) are indicative of a maternal XY genotype.

Results showed that six of the nine atypical fish analyzed produced progenies with the 3:1 sex ratio (male-female) expected for functional sex-reversed

females (Table 1). Atypical fish three and four showed a proportion of males significantly higher ($P < 0.001$) than the predicted 3:1 sex ratio (Table 1). A significantly lower ($P < 0.001$) proportion of males than predicted were observed in the first atypical fish. Proportion of males in this atypical fish was similar to that observed in progenies provided by normal females in our laboratory (54 to 58%) at similar rearing temperatures (26-29ºC). The number of fry produced per atypical fish was within the previously observed ranges for normal females of *O. niloticus* in our laboratory. However, atypical fish three, five and six produced few fry in relation to their body weight (Table 1).

Confirmation of the XY status in selected sex-reversed-females during the development of the YY technology is critical and should be conducted with maximum care since their morphology, behavior and karyotypes are indistinguishable from that of a normal female (Mair *et al.*, 1997). The importance of XY females lies in the fact that, in theory, their progeny would be composed of 75% males, of which 50% would possess an XY genotype, while the other 25% an YY genotype (Vera-Cruz *et al.*, 1996; Mair *et al.*, 1997; Müller & Hörstgen, 2007). Therefore, the presence of a phenotypic trait that allows us to more precisely identify females with XY genotype could help reduce the time and effort spent during this stage while increasing the probability that fish selected are indeed sex-reversed females. Piferrer (2001) suggests that if such phenotypic trait is consistent, progeny testing can be eliminated, since it allows the segregation of genotypic and phenotypic females. Several species, including rainbow trout, coho salmon and chinook salmon have shown phenotypic differences (missing or incomplete ducts and differences in the shape of the genital papilla) after sex reversal procedures (Johnstone *et al.*, 1979; Piferrer, 2001). However, these features have not been used for the identification of sex-reversed females (Piferrer, 2001). In our work, atypical fish had a genital papilla which retained mainly its male form, with no obvious oviduct, but that could expel viable eggs after an abdominal massage (through a remaining male *vas deferens* or a quasi-oviduct near the tip of the papilla -based on observations made in our laboratory-), allowing us to use this trait to select potential sex-reversed females. Our results showed that eight of the nine atypical fish selected based on the shape of the genital papilla produced sex ratios strongly skewed towards male, suggesting an XY genotype.

In an atypical fish, the feminization process is probably completed at a physiological level, but the channel associated with the oviduct is not properly formed and the genital papilla retain its male form. This incomplete feminization at the morphological level could be the result of low levels of hormone uptake or the interaction with environmental variables, such as rearing temperature. Devlin & Nagahama (2002) suggest that complete sex transformation is affected by a genetic influence on the ability to respond to exogenous hormones. This could explain the presence (observed in previous experiments) of different proportion of atypical fish (13 to 40%) between families treated at the same dosage, duration and timing of hormonal treatment.

Although atypical fish are more easily identified externally, the presence of a quasi-oviduct not properly formed or a remaining male *vas deferens* not suitable for expelling eggs could prevent the correct expulsion of the eggs. These morphological abnormalities could explain the reduced number of fry observed in three of the eight atypical fish that showed a high proportion of males. Feist *et al.* (1995) report similar results in the rainbow trout *Oncorhynchus mykiss*, in this case, a high proportion of sex-reversed males showed either a lack of or incomplete sperm ducts and semen had to be removed surgically. Difficulty in obtaining an egg sample during the abdominal massage in some atypical fish with well-developed ovaries (identified after extraction) could support this. However, other factors could affect the number of fry collected; age of the atypical fish, nutritional status, genetic interactions, the fact that it is the first time that they reproduce and the possibility that the atypical fish eat part of the eggs during the incubation in the mouth period.

In studies currently underway, atypical fish have shown different patterns of antagonistic behavior compared to those displayed in normal males and females, and in some cases an absence of reproductive behavior in the presence of normal males. This abnormal behavior could be responsible for the low number of fry produced in three of the atypical fishes analyzed.

In theory, crosses between sex-reversed females and normal males should produce a proportion of 75% males, however, variations to this proportion are frequently observed. In our case, progeny sex ratios of males in the eight atypical fish with an XY genotype varied from 69 to 91% but with a mean close to 78%. These variations could be explained by the interaction of the three components that governed sex in the Nile tilapia, a complex genetic sex determination system with a major determinant locus (sex chromosomes XX/XY) and some minor genetic factors (parental factors), as well as the influence of the temperature (environmental factors) (Baroiller *et al.*, 2009). Wang & Tsai (2000) report that the exposure of 10-day-old fry to water temperatures from 28º to 32°C could induce

Table 1. Atypical fish (AF), total weight of atypical fish (g) (TWA), fry produced per atypical fish (FPA), survival (S), number of fish evaluated (NFE) and sex ratio observed in the progeny of the atypical fish of *O. niloticus*. *Significantly different from the expected 3:1 distribution ($P < 0.001$).

AF	TWA	FPA	S	NFE	Sex ratio		
					% Males	% Females	% Undifferentiated
1	288	254	91.2	58	55*	42	3
2	294	243	90.3	72	78	18	4
3	354	47	92.2	44	88*	12	--
4	256	166	88.9	58	91*	9	--
5	385	37	91.9	34	69	31	--
6	420	28	90.6	25	71	29	--
7	350	385	92.7	102	76	21	3
8	450	585	88.2	113	70	28	2
9	431	478	86.3	82	79	15	6

gonadal masculinization and therefore an increase in the proportion of males. Baroiller *et al.* (2009), mention that this temperature sensitivity of Nile tilapia during sex differentiation is not seen in all progenies. Some male or female breeders provide progenies displaying a high sensitivity to temperature giving a high proportion of males in their sex ratio, while others gave an insensitive balanced sex ratio. A clear parental effect on this thermosensitivity with an influence of both parents has been demonstrated by Baroiller & D´Cotta (2001). This supports the idea that the pronounced and significant excess of males observed in atypical fish three and four could be a consequence of a genetic parental effect interacting with the water temperature during the fry stage (28 ± 1ºC). It is possible that the breeders (the atypical fish or the males) that were used displayed a genetic tendency toward the gonadal masculinization at moderately elevated water temperatures, boosting the male proportion observed in the progeny of two of the atypical fish analyzed.

Previous experiments carried out in our laboratory at 28 ± 1ºC have shown several control groups with a significant deviation from the expected 1:1 sex ratio. Similar results have been observed by Contreras-Sánchez (2001) and Wessels & Hörstgen-Schwark (2011) in control groups of Nile tilapia reared at a water temperature of 28ºC. Although the water temperature during the fry stage could have influenced the final proportion of males observed, we cannot rule out the possibility that the combination of breeders used could be the only factor responsible for the variation observed, especially in atypical fish three and four. An important parental effect may be responsible in accordance with that proposed by Baroiller *et al.* (2009) for the high proportion of males observed. Further work needs to be done to establish whether these variations are the result of a genetic tendency involving the water temperature or simply the product of the parental genetics.

At present, progeny of selected atypical fish has been reduced to only a few potential YY-males that would be crossed with normal females. These males were selected based in their phenotypic traits, including the shape (width) of the genital papilla which, according to Abucay & Mair (2004), could be used as a strong discriminator between the two genotypes. Use of atypical fish could help the segregation of genotypic and phenotypic females, reducing the time and effort spent in select sex-reversed females.

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