



SELECTION OF POTENTIAL YY MALES OF NILE TILAPIA *Oreochromis niloticus* (LINNAEUS, 1758) AND EVALUATION OF THE PERCENTAGE OF MALES OBTAINED[†]

[SELECCIÓN DE POTENCIALES MACHOS YY DE TILAPIA DEL NILO *Oreochromis niloticus* (LINNAEUS, 1758) Y EVALUACIÓN DEL PORCENTAJE DE MACHOS OBTENIDO]

Juan Pablo Alcántar-Vázquez^{1*}, Luis Pozos-Dauzón², Daniel Calzada-Ruiz³, Alfonso Álvarez-González³, Alvaro Perez-Carbajal¹, Rocio López-Hinojosa¹, Carolina Antonio Estrada¹ and Raúl Moreno-de la Torre¹

¹Laboratorio de Acuicultura, Des: Ciencias Agropecuarias. Universidad del Papaloapan (UNPA). Av. Ferrocarril s/n, Col. Ciudad Universitaria, Loma Bonita, Oaxaca, 68400. México. Email: jupasoul@hotmail.com, alvaro.perez.carbajal@gmail.com, roccy97@hotmail.com, carolina_ibq210780@hotmail.com, raulbuzo@hotmail.com

²Granja Unidad de Producción del Tesechoacan. Sistema Cooperativo Integral (SISCOIN). Aquiles Serdan No. 28. Apartado postal 13. Col. Centro, José Azueta, Veracruz, 95580. México. Email: lupoda499@gmail.com

³Laboratorio de Acuicultura Tropical, División Académica de Ciencias Biológicas. Universidad Juárez Autónoma de Tabasco. Carretera Villahermosa-Cárdenas Km. 0.5 S/N, Entronque a Bosques de Saloya. Villahermosa, Tabasco, C.P. 86150, México. Email: calzada.rd@hotmail.com, alvarez_alfonso@hotmail.com

*Corresponding author

SUMMARY

Successful development of YY technology depends not only in the identification of YY males (YYM), but also on the evaluation of the percentage of males obtained from them. The progeny of selected potential YYM was evaluated individually in order to determine its genetic status. In total, 12 potential YYM and 12 normal females (XX) were selected. Potential YYM were selected taking into account external color, shape of the genital papillae and size. After individual crossing, a confirmed YYM was crossed consecutively with three XX females and three XY females to further assess the variation in the percentage of males obtained. A significant deviation from the 1:1 sex ratio expected for normal males (XY) was observed for ten of the twelve potential YYM that were evaluated, confirming YY status. Variation ranking between 88 to 93% in the percentage of males was observed in crosses between YYM and XX females. Finally, failure to obtain only 100% males was observed in crosses between YYM and XY females. This variation could be the result of the interaction of minor genetic parental factors or the water temperature during fry period. External parameter used for selection of potential YYM need to be optimized in further work.

Keywords: Nile tilapia; progeny test; XY females; YY males.

RESUMEN

El desarrollo exitoso de la tecnología YY depende no sólo de la identificación de machos YY (YYM), sino también de la evaluación de los porcentajes de machos obtenidos. La progenie de potenciales YYM seleccionados se evaluó individualmente para determinar el porcentaje de machos obtenido. En total, se seleccionaron 12 potenciales YYM y 12 hembras normales (XX). Los potenciales MYY fueron seleccionados considerando su coloración externa, forma de la papila genital y tamaño. Después de terminadas las cruza individuales, un YYM confirmado fue cruzado consecutivamente con tres hembras XX y tres hembras XY para determinar la variación en el porcentaje de machos. Se observó una desviación significativa de la proporción de sexos 1:1 esperada para machos normales en diez de los doce potenciales YYM evaluados, confirmando su estatus YY. Se observó una variación en el porcentaje de machos de 88 a 93% en cruza entre YYM y hembras XX. Finalmente, no fue posible obtener 100% de machos en las cruza entre YYM y hembras XY. Esto probablemente fue resultado de la interacción de factores genéticos parentales

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menores o la temperatura del agua durante el periodo de alevín. Los parámetros externos utilizados para seleccionar potenciales YYM necesitan ser optimizados en trabajos futuros.

Palabras clave: Tilapia del Nilo; prueba de progenie; hembras XY; machos YY.

INTRODUCTION

In commercial farming of Nile tilapia, uncontrolled 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 and Arredondo, 2000; Beardmore *et al.*, 2001; Tariq-Ezaz *et al.*, 2004). A great deal of research effort has been invested in the development of monosex populations of Nile tilapia to exploit their benefits for aquaculture (Beardmore *et al.*, 2001; Abucay and Mair, 2004). Production of a monosex, all-male population in Nile tilapia culture eliminates uncontrolled reproduction and allows for the production of marketable-sized fish (Varadaraj, 1989) and therefore has been recognized for many years as the most effective technique to increase tilapia production (Vera-Cruz *et al.*, 1996; Mair *et al.*, 1997; Müller and Hörstgen, 2007; Phumy *et al.*, 2012).

Several approaches have been developed to achieve monosex, all-male populations, the most commonly applied in the industry today being direct hormonal sex-reversal by feeding fry with different hormones, (Mair *et al.*, 1997; Piferrer, 2001). However, reports of accumulation of hormones in the environment as well as the increasing number of consumers not interested in eating products that have been treated with hormones (Piferrer, 2001; Müller and Hörstgen, 2007; Filby *et al.*, 2007; Leet *et al.*, 2011) have led to the search for alternative techniques for the production of all-male tilapia populations. One of the developments from this search, and a viable alternative on a commercial scale, is YY technology derived from novel YY “supermales” which allows for the production of genetically male tilapia based on crosses between YY males (YYM) and normal females (XX) (Vera-Cruz *et al.*, 1996; Mair *et al.*, 1997; Abucay and Mair, 2004; Müller and Hörstgen, 2007). The development of YY-male technology is well described by Mair *et al.* (1997).

Production of YY males (YYM) at the Universidad del Papaloapan (Alcántar-Vázquez *et al.*, 2014) has led to the optimization of YY technology and its integration with commercial culture of Nile tilapia in the region. However, failure to efficiently separate YYM from XY males and the reported variations in

the percentage of males obtained from YYM could delay this. To this day, only Abucay and Mair (2004) have tried to identify YYM using external characteristics. In their work they evaluated two methods for identifying males with the YY genotype (feminization of the YYM and morphometric and meristic characters); however, although both methods failed to reach a 100% confidence in routine identification of YYM, the form (mainly the width) of the genital papillae showed significant differences between genotypes, with the papillae of the YYM being thinner.

The group of potential YYM produced from a first batch of XY females (Alcántar-Vázquez *et al.*, 2014) at the Universidad del Papaloapan was divided in two main groups using external parameters and the present work is part of the current evaluation of individual and in groups (under commercial conditions in regional farms) of potential YYM.

The present work was undertaken to make individual identifications of the percentage of males obtained from potential YYM selected using only external parameters and to assess the variation in the percentage of males obtained crossing YYM with XX and XY females. Although in theory YYM should produce only genetic males, there appears to be a genetic basis for the occurrence of occasional females in the progeny of YYM (Mair *et al.*, 1997; Baroiller *et al.*, 2009).

MATERIAL AND METHODS

Fish.

The *O. niloticus* potential YYM used in this experiment were produced in the aquaculture station of the Universidad del Papaloapan by crossing XY females (confirmed by progeny test) (Alcántar-Vázquez *et al.*, 2014) with normal males (XY). Potential YYM obtained from XY females were divided in two groups taking into account external color (belly and breast red colored, and all body dark grey –no red-), shape of the genital papillae (thin and short, width and large) and size (in both groups we only selected the fish located in the 10th percentile of size). Using these external parameters, we formed two groups with the larger potential YYM, but one composed of fish with reddish color and width genital papillae, and the other of fish with dark grey color and thin genital papillae. Based in the information

obtained from Abucay and Mair (2004), in this work we selected potential YYM from the group with dark grey color and thin genital papillae to be tested. In total, 12 potential YYM (1000-1300 g) were used for progeny testing. They were fed twice a day with commercial pellets (32% protein, Nutripec, Agribrands Purina, Irapuato Gto. Mexico).

Fry production and experimental conditions.

Each potential YYM to be tested was stocked with a single female (XX) in a 3-m diameter outdoor geomembrane tank (28-30 °C) supplied with fertilized water. Recently hatched and sexually undifferentiated fry weighing 0.1 g and measuring 8 mm in length were collected 18 to 21 days later with a fine-mesh net after siphoning 90% of the water from the tanks. The fry from each individual spawn was counted and transported to a closed recirculating system composed of 3-m diameter outdoor concrete tanks at an initial stocking density of 1 fry 45 L⁻¹ (one tank per spawn).

Fry were fed *ad libitum* six times a day for 30 days (53% protein, < 0.35 mm, Nutripec Purina®) after which juveniles were fed *ad libitum* four times a day (50% protein, 1.0 mm, Nutripec Purina®) for another 20 days. Fish were reared up to approximately 110 days of age when sex ratios for these fingerlings could be safely determined by removing the gonad. Fish were fed four times a day with commercial diet (44 and 40% protein, Nutripec Purina®) for approximately 30 days, followed by a commercial diet with 35% protein (Nutripec Purina®) protein three times a day for another 20 days and finally a commercial diet with 25% protein (Nutripec Purina®) until the end of the experiment.

Evaluation of sex percentages.

Sex of approximately 40-50% of the population of each individual spawn was evaluated taking into account external and internal characteristics, under a protocol developed in our laboratory (Alcántar-Vázquez *et al.*, 2014; Alcántar-Vázquez *et al.*, 2015; Marín-Ramírez *et al.*, 2016; Juárez-Juárez *et al.*, 2017). First, differences between sexes in the papilla structure were highlighted using dye (methylene blue at 1%). Second, a gentle abdominal massage was applied to try to obtain sperm or eggs. Third, fish were sacrificed, and the gonad was removed along with the spermatoc/ovarian ducts in order to confirm the sex. Translucent gonadal tissue accompanied with yellow eggs (mature) or white eggs (immature) was indicative of a female fish, while whitish (mature) or reddish (immature) gonadal tissue was indicative of a male fish. Finally, in order to discard intersex individuals, each gonad was observed externally in

detail. In dubious cases, the gonad was cut open and a sample of tissue was taken to analyze it using a microscope.

All fish from each individual spawn were counted, weighed and measured for the calculation of survival rate and average wet weight and total length.

Crosses between YYM and females with XX and XY genotype.

A confirmed YYM that produced 100% male progeny in the individual crosses with XX females was selected for assess variation in the percentage of males obtained. For this, the YYM was individually crossed with three XX females and three XY females. Each individual spawn was treated the same way as described for the individual spawns of potential YYM. Between individual spawns, the YYM was left to recover for approximately one month.

Statistics.

Proportion of males identified in each individual spawn of potential YYM was tested against the 1:1 expectation using a chi-square test. Progeny sex ratios approximating 1:0 (male: female) are indicative of a paternal YY genotype. All tests were performed at 0.05, 0.01 and 0.001 significant levels in order to minimize the chance of making an error in confirming the YY status. Percentage of males obtained from confirmed YYM was compared against the 1:0 expectation using a chi-square test performed at 0.001 significant level.

RESULTS AND DISCUSSION

The percentage of males produced per potential YYM is shown in Table 1. Only ten of the twelve potential YYM selected managed to produce progenies significantly skewed toward male with a sex ratio that deviated significantly from the 1:1 sex ratio expected in crosses between normal males (XY) and normal females (XX). However, only eight potential YYM (number 2, 5, 6, 7, 9, 10, 11 and 12) were significantly different from 1:1 at a significant level of $P < 0.001$. The other two YYM (number 1 and 4) were significantly different from 1:1 only at a significant level of $P < 0.01$. Finally, potential YYM numbers 3 and 8 produced sex ratios expected for normal crosses and were designated normal males (XY).

The presence of two males classified as XY males it indicates that external parameters (especially external color and shape of genital papilla) used for separating YYM from XY males needs to be reconsidered in

order to eliminate any XY males from the groups of YY breeders formed. Although we selected these external parameters based partially in the work of Abucay and Mair (2004) and the external differences observed during the growth of potential YYM, our successful rate for YYM selection was only 83%. This could explain the percentage (92-96%) of males observed in the progeny of groups of 30 potential YYM (selected using the same external parameters) transferred to commercial Nile tilapia farms and tested under commercial conditions (in a reproduction tank with 90 normal females).

Although in some cases the identification of YYM is possible through molecular markers (Sun *et al.*, 2014), this generally only works with a 100% confidence for the genetic line where the YYM were obtained. The genetic line we used to produce the YYM is one developed in the region, so the molecular markers need to be developed for our own line in order to make a precise identification of YYM. This work is currently being done in our laboratory; however, the approach investigated in this work for the identification of YYM is based solely on external parameters.

Evaluation of the percentage of males obtained from YYM during the development of YY technology is critical since masculinization of undifferentiated XY fry normally produces male percentages close to 100%. If tilapia farmers are asked to adopt YY technology, the percentage of males produced per YYM should be similar or superior to that obtained through masculinization by exogenous steroids. In this work, male percentages observed in the potential YYM classified as YYM (including those at significant levels of 0.01 and 0.001) were in accordance with those predicted by the hypothesis of multifactorial (genetic and environmental elements) sex determination proposed for Nile tilapia (Baroiller *et al.*, 2009). All YYM, except number five, produced a percentage of males lower than the expected 100%. These results are in accordance with previous results obtained by Mair *et al.* (1991), Mair *et al.* (1997) and especially with those by Müller and Hörstgen (2007) who observed a percentage of females as high as 20% in the progeny of a male classified as YY male. Taking into account only the eight males classified as YY males at a significant level of 0.001, the overall percentage of males from these YYM reached approximately 94%, similar to the 95% observed by Mair *et al.* (1997) in the YY males classified at a significant level of 0.001 during their experiments.

Table 1. Percentage of survival, final wet weight, final total length, and percentage of males and females obtained from potential YY males of Nile tilapia (*Oreochromis niloticus*). Data collected at 110 days of age.

pYYM	NFE	S (%)	FWW (g)	FTL (cm)	Sex distribution	
					Male (%)	Female (%)
1	60	88	70.4	15.5	68**	32
2	51	87	90.9	15.8	96***	4
3	76	86	67.0	13.4	57 ^{ns}	43
4	85	88	96.8	16.8	68**	32
5	44	82	96.6	16.5	100***	--
6	64	98	82.3	15.8	77***	23
7	58	95	99.7	17.1	97***	3
8	53	79	73.2	15.2	36 ^{ns}	64
9	48	91	89.4	15.9	96***	4
10	44	87	101.3	17.2	93***	7
11	66	89	95.5	16.4	96***	4
12	49	93	98.0	16.8	95***	5

pYYM = Potential YY male, NFE = Number of fish evaluated, S = Survival, FWW = final wet weight, FTL = final total length, ns = not significant, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

The percentage of males obtained in the crosses between YYM number 5 (100% males) and females with genotype XX and XY is shown in Table 2. It was impossible to obtain 100% males in any of the individual spawns evaluated, including the ones obtained from crosses between YYM and XY females. However, all individual spawns presented a sex ratio expected for crosses between YYM (1 male: 0 female) and both type of females (XX and XY) at a significant level of $P < 0.001$. Variation between individual spawns was low (1-5%), specially in the YYM with XY female crosses (2%).

An important parental effect may be responsible in accordance with that proposed by Baroiller *et al.* (2009) for variations observed in male percentages between the selected YYM. Variations observed in the crosses between YYM (number 5) and XX and XY females appear to confirm this. Mair *et al.* (1997) suggest that a series of autosomal sex modifying genes, acting as a threshold trait, may be responsible for the occurrence of these small percentages of females and that these autosomal genes could be

selected against to further increase the proportion of males in the progeny of YYM. However, we cannot rule out the effect of water temperature on gonadal differentiation during the fry period. This is well supported by observations made by several authors that have worked with Nile tilapia (Mair *et al.*, 1997; Contreras-Sánchez, 2001; Wessels and Hörstgen-Schwark, 2011; Lazaro-Velasco, 2014; Alcántar-Vázquez *et al.*, 2014). Wang and Tsai (2000) report that the exposure of recently hatched fry to water temperatures from 28° to 32°C could induce gonadal masculinization and therefore an increase in the proportion of males, while water temperatures close to 20°C induce gonadal feminization and an increase in the proportion of females. Baroiller *et al.* (2009) mention that this sensitivity to water temperature during sex differentiation is not observed in all progenies of Nile tilapia. Additionally, Baroiller and D’Cotta (2001) demonstrated that there is a clear parental effect on this thermosensitivity with an influence of both parents. Some male or female breeders would provide progenies displaying a high sensitivity to temperature giving a high proportion of males or females in their sex ratio, while others would give an insensitive balanced sex ratio (Baroiller and D’Cotta, 2001; Ospina-Álvarez and Piferrer, 2008). It is possible that the variations observed in the male percentages in the YYM could have been caused by variations in the water temperature during fry period. Previous observations made in our laboratory using the same genetic line (from which YYM were produced) have shown a clear thermosensitivity in the sex ratios obtained. Further work needs to be done with the selected YYM to establish whether these variations are the product of the interaction of parental genetics or water temperature during early stages of culture.

Regardless of the factor or factors causing the appearance of small proportion of females in the progeny of YYM, selection of YYM that consistently produce percentages of 100% males should be performed. Mair *et al.* (1991) recommend crossing the selected YYM only with selected females (preferably with the ones that also produce high male percentages), while Beardmore *et al.* (2001) suggest that maintenance of broodstock is essential and that for this technology in order to be profitable needs to be coupled with other forms of genetic improvement. One of these genetic improvement techniques is gynogenesis. Recently, our laboratory acquired a UV chamber and we began inducing this technique using eggs of XY females with the hope to obtain a significant number of YYM in only one generation.

Table 2. Percentage of survival, final wet weight, final total length, and percentage of males and females obtained from crosses between a YY male with XX, and XY females of Nile tilapia (*Oreochromis niloticus*). Data collected at 110 days of age.

FM	NFE	S (%)	FWW (g)	FTL (cm)	Sex distribution	
					Male (%)	Female (%)
XX ₁	42	91	102.4	17.4	93*	7
XX ₂	49	94	99.3	17.1	92*	8
XX ₃	50	95	94.2	16.8	88*	12
XY ₁	55	96	89.2	16.4	95*	5
XY ₂	51	92	93.3	16.7	95*	5
XY ₃	46	94	99.9	17.1	93*	7

FM = Female, NFE = Number of fish evaluated S = Survival, FWW = final wet weight, FTL = final total length, * $P < 0.001$

CONCLUSIONS

Although external parameters used in the present work failed to separate with a 100% efficacy YYM from XY, the success rate was sufficiently high to allow us to think that its optimization could in further works reach a close to 100% success rate. The variation observed in the percentage of males was lower than expected, especially in the spawns between YYM and XY females. However, the fact that none of the spawns reach a 100% male progeny, support the fact that for the successful integration of YY technology to commercial culture of Nile tilapia occurs is necessary to mass produce YYM (through gynogenesis) and select only the ones that produce routinely progenies with a percentage of males close to 100%.

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