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YY super males have better spermatic quality than XY males in red tilapia *Oreochromis niloticus*

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Abstract

The influence of sex-related chromosomal arrangement in an YY and XY population of *Oreochromis niloticus* males was investigated in this study. A better spermatic mobility, motility, viability, confluence and less abnormality were found in double Y-chromosome males (YY-males), compared to XY-males; however, this better spermatic quality did not correlate with the body weight of individuals which was significantly better in XY-males. This study establishes the influence of YY-related sequences over spermatic quality and indicates the usefulness of the YY technology as a strategy to obtain better fish breeders and genetically male individuals for a tilapia farming program.

Keywords: *Oreochromis niloticus*; tilapia; spermatic quality; YY males.

1. Introduction

Oreochromis niloticus is a bisexual fish with the sexual chromosome arrangement of XX for females and XY for males. Phenotypic males often show better advantages such as faster weight and length gain (Githukia *et al*., 2015) and it is preferred to ensure high productivity and reproductive capability in tilapia farming. Weight records of monosex and mixed populations demonstrate the male monosex superiority (Omasaki *et al*., 2016). One of the most used strategies to ensure monosex individuals is the use of hormonal treatments (Gale *et al*., 1999; Beaven and Muposhi, 2012; Megbowon and Mojekwe 2014). Other methods that are safer for the environment include hybrid crosses of *Oreochromis niloticus* females with *Oreochromis aureus* males (Lozano *et al*., 2014) or between YY super male with XX female individuals to ensure genetically determined males (Alcántar-Vásquez *et al*., 2014; Schill *et al*., 2016). Monosex males obtained by the latter case exhibit many advantages over the hormonal treatment, including faster development and homogeneous growth, reducing farming costs and possible immunological detriments in the male population caused by hormonal treatments (Beardmore *et al*, 2001). Although the YY super male technology is spreading widely over the world, only few reports have analyzed gamete quality in tilapia males which, despite the fact that they are phenotypically males, have the YY or XY arrangement that could potentially influence in their reproductive capability.

Besides the quality of the eggs, spermatic quality is a key factor for the reproductive success of tilapia. Few studies have reported the same sperm quality of XX masculinized-males, XY males and YY males (Rurangwa *et al*., 2004; Gennotte *et al*., 2012). However, even if the genetic

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background of the stock is similar, we asked whether the sexual chromosome arrangement and the introduction to a new environment could potentially influence the spermatic quality of male monosex tilapia.

There exist several lineages of *O. niloticus*, including *O. niloticus* grey, *O. niloticus* red and *O. niloticus* silver, which are broadly distributed in several countries and show different phenotypes. In Peru, the predominant lineage is *O. niloticus* grey, that have been farmed and bred empirically without major scientific strategies. The introduction of exogenous lineages with favorable phenotypes, along with the use of monosex technology, may represent a good strategy to increase productivity. However, it is necessary to assess the reproductive characteristics of newly introduced lineages, since they may respond differently to the new environment and exhibit variations in its phenotype.

In order to delineate strengthens of the YY technology for a breeding program and also to better understand the influence of the sex genotypes on spermatic quality, the spermatic quality of an introduced population of male red tilapia *O. niloticus* with YY and XY genetic rearrangement was compared.

2. Material and methods

Fishes

Live specimens of *Oreochromis niloticus* red were obtained from Tilaqua (The Netherlands). Twenty 21-month-old individuals of each type, YY and XY male, were selected for the study. All individuals were kept at 24 ± 2 °C of temperature, 5 – 8 mg/l of oxygen, 7 of pH, 45 cm of water transparency and 14 h light / 10 h darkness photoperiodic regime. Feeding was carried out three times a day with a commercial available diet composed by 40% proteins, 8% lipids, 4% fiber, 13% humidity and 10% ashes.

Genotyping

The presence of Y-related and X-related sequences was confirmed as described by Sun *et al.* (2014). Briefly, DNA was extracted from caudal fins with standard procedures and subjected to PCR with the primers
SCAR-5F TAAATTAATGACATTTCAGTTATG
and
SCAR-5R-Y TTACAGCAGCACCCAGAGTCAT
for the Y-related sequence; and
SCAR-5-X CTGGTTTGCAATAGTTAGGGTGCT
and
SCAR 5R CAGAAATGTAGACGCCCAGGTATC
for the X-related sequence.

Sperm quality parameters

Sperm was obtained through extrusion or massage on the abdominal region as previously described by Piñeros-Piñeros and Cala-Cala (1991). Sperm sampling and handling from all YY and XY individuals was performed in the same scheduling time. The quality parameters assessed were: volume, mobility, motility, viability, morphology and sperm density as described by Bastardo *et al.* (2004) and Sánchez-Rodríguez and Billard (1977). The sperm volume was measured using a graduated-1ml syringe. The mobility was determined by assessing 100 cells in 8 – 10 fields and classified as rapid and linear progressive movement (Bastardo *et al.*, 2004), slow progressive movement, *in situ* movement, and immobile sperm. The motility was obtained using an optic microscope at 100X magnification after activation with farming water at 24 ± 2 ºC with oxygen 4.77 mg/l. The degree of motility was estimated using arbitrary units as described by Sánchez-Rodríguez and Billard (1977). Viability was performed by dead cells exclusion using propidium iodide (PI) and evaluated using a fluorescent microscope with specific filters and differential interference contrast (DIC). 100 cells were counted from 5 fields from 3 different individuals, and the DIC pictures were also used to assess sperm morphology.

Statistical analysis

Mean values comparisons were performed using t student test with a significance level of $\alpha = 0.05$.

3. Results and discussion

Besides a morphological assessment to determine the phenotypic gender, XY and YY genotypes were identified with the markers SCAR-5F-X/5R and SCAR-5F/5R-Y by conventional PCR (Figure 1A) as previously described (Sun *et al*., 2014). Then, a morphological analysis was performed to study the gender genotype influences on important economical parameters such as weight and length. There was found that XY males had more than 1.5-fold significant higher weight average compared to YY males (Figure 1B). However, body length of XY and YY males were similar (Figure 1C and 1D).

Later, the work was focused on assessing the sperm quality parameters. First of all, the spermatic volume was measured (Figure 2A) and sperm viability from XY and YY males was evaluated using propidium iodide (PI) as indicator of dead cells, finding no significant differences (Figure 2B). However, the sperm confluence of the YY males was about 2.4-fold higher than the confluence from XY males sperm collected (Figure 2C).

Spermatic cells were classified and quantified according to its mobility as previously described (Bastardo *et al*., 2004) (Figure 3A): rapid linear movement (RL), slow movement (S), *in situ* movement (IS) and immobile (I). Spermatic cells derived from either YY or XY males were categorized as I in over 90 per cent. Although were found no significant differences among the percentage of cells with S, IS and I, a higher percentage with RL was found in YY sperm cells, compared to XY phenotypes. In this research, XY males had better phenotype regarding to its weight gain; however, XY males had lower reproductive capability as demonstrated with lower sperm viability, volume, confluence and mobility.

The sperm confluence reported in this study was the highest reported for YY male tilapia (Gennotte *et al*., 2012; Bombardelli *et al*., 2010; Mataveli *et al*., 2007). However, this sperm confluence from YY males was still lower when compared to that from other species (Gennotte *et al*., 2012). Previous reports established a low sperm confluence in *Oreochromis niloticus* (Gennotte, *et al*., 2012), compared to *Oncorhynchus mykiis* (Piñeros-Piñeros and Cala-Cala *et al*., 1991), *Eremophilus mutisii* (Montejo *et al*., 2002), and *Brycon amazonicus* (Cruz-Casallas *et al*., 2006).

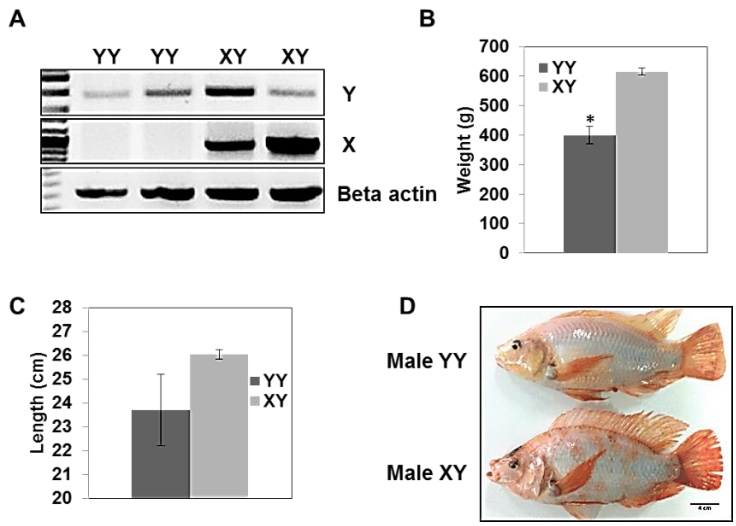

Figure 1. Characterization of YY and XY males *Oreochromis niloticus* red tilapia. A. PCR markers SCAR-5F/5R-Y and SCAR-5F-X/5R, β-actin internal control. B. Weight mean ± SD (n = 20), * $p < 0.05$ t test. C. Length average ± SD (n = 20) D. YY and XY males, age 21 months. Scale bar = 4 cm.

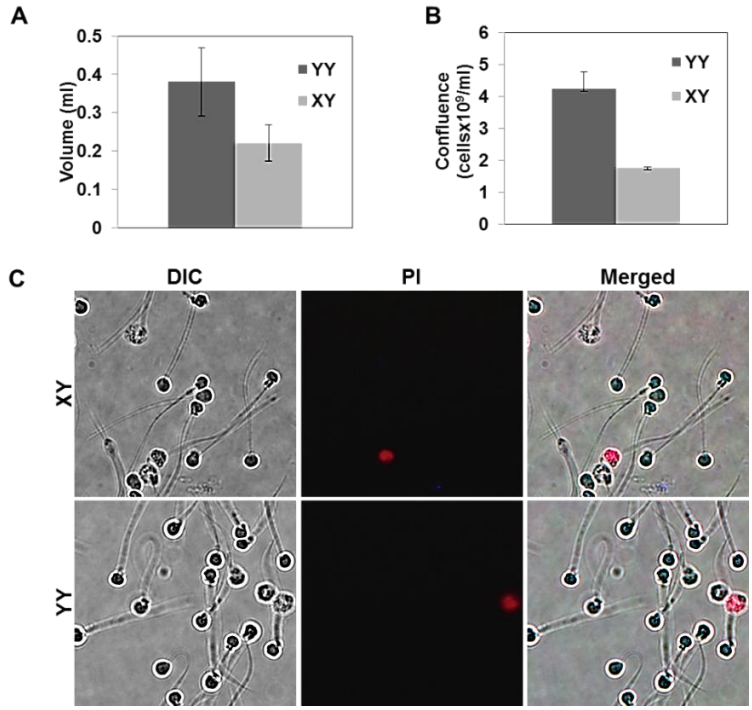

Figure 2. Spermatic quality of YY and XY males of *Oreochromis niloticus* red tilapia. A. Spermatic volume, mean ± SD (n = 20), $p < 0.05$ t test. B. Spermatic concentration, mean ± SD (n = 20), $p > 0.05$. C. Sperm viability evaluated with propidium iodide (PI), representative image.

Spermatic cells were then exposed to distilled or farming water to assess its motility. It has been previously shown that spermatic cells increased its motility after exposure to hypotonic water, in a process called "activation" (Billard *et al*., 1995; Valdebenito *et al*., 2009). The average time by which the spermatic cells remain activated with either farming or distilled water was significantly higher in those derived from YY genotypes (Figure 3B). The activation time with farming water was in average 32.6 minutes for XY and 54.7 minutes for YY; and with distilled water 21.9 minutes and 36.3 minutes for XY and YY males, respectively (Figure 3B).

Sperm mobility without activation is an important parameter that indicates fecundation capability (Bastardo *et al*., 2004). High levels of sperm cells classified as immobile (90%) for both, XY and YY males, is an expected feature previously registered (Gennotte *et al*., 2012). Nevertheless, the high percentage of sperm cells from YY males with RL, compared to those XY males, strongly indicates the better reproductive capability of YY males. This is consistent with the results published by Herrera *et al*. (2001) where YY males showed greater spermatogenic and primordial germ cells, faster sexual maturity and thicker gonadal tissue than XY male fishes. Contrary to what was stated by Gennotte *et al*. (2012).

In respect of regulatory mechanism for the testicle development, there would be many miRNA involved which are differentially expressed in YY an XY males, causing differences in testicular gonads histology, structure and function among YY an XY male fishes (Jing *et al*., 2014; Wu *et al*., 2015).

Moreover, in the activation assay, the motility of YY sperm cells lasted in average 54.7 ± 30.42 min, significantly longer that the of XY-males. These values were higher than the ones prior reported in

the study published by Gennotte *et al*. (2012), where the motility of sperm cells lasted 24'52 " ± 10'40".

To evaluate the morphology of spermatic cells, DIC pictures were used. Cells derived from XY males had a lower percentage of normal cells compared to those derived from YY males, with 78.24% and 99.71% respectively (Figure 4A and Figure 4B). Head and tail abnormalities were quantified.

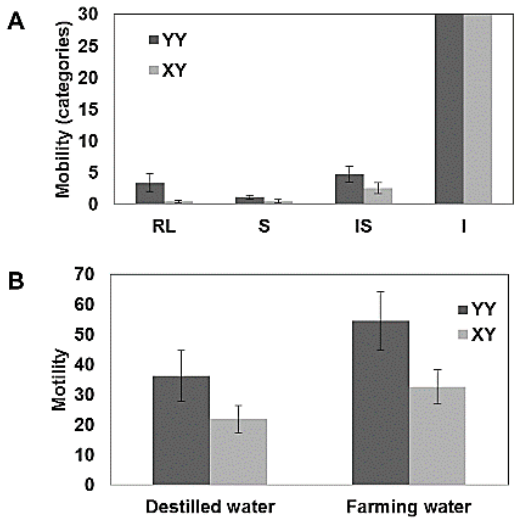

Figure 3. Spermatic mobility and motility of YY and XY males of *Oreochromis niloticus* red tilapia. A. Mobility (RL: progressive linear rapid movement, S: progressive slow movement, IS: *in situ* movement and I: immobile). B. Motility using distilled water (left) and farming water (right). Mean ± SD (n = 20), $p > 0.05$.

Morphological abnormalities are usually reported in sperm cells and influence its fecundation capability (Bastardo *et al*., 2004). Male fishes considered as breeders should ideally have less than 30% of sperm abnormalities. In this respect, both YY and XY males outreached this parameter, however, the 99.71% of normal sperm cells from YY males compared to 78.24% from XY males indicates a better capability of fecundation and also ideal individuals for a breeding program.

For the sperm quality assessment, particularly as regards amount of sperm, it is important to keep in mind the reproductive cycle since the spermatogenesis would be synchronized with the female egg maturity, in case of male and female joint breeding. To achieve this synchronization, it would be required a communication through sounds (Longrie *et al.*, 2013) or a chemical communication using pheromones (Li and Buchinger 2014).

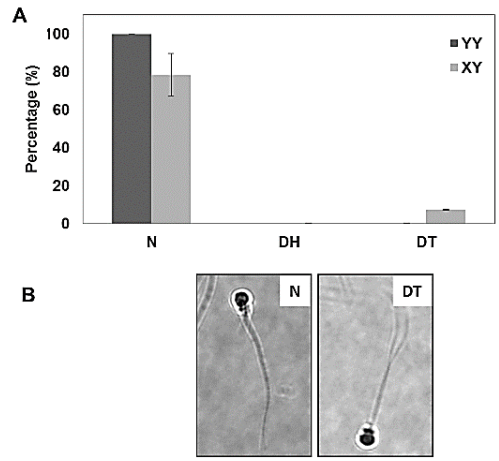

Figure 4. Sperm morphology of YY and XY males of *Oreochromis niloticus* red tilapia. A. Average percentage normal upset sperms, N: normal, DH: defect at the top, DT: defect in tail, mean ± SD (n = 20), $p < 0.05$. B: Representative image of defect in tail.

It is known that environmental factors influence the spermatic quality of fishes, such as temperature, light exposure, pH and osmotic changes (Rurangwa *et al*, 2004, Biswas *et al*., 2005; Bobe and Labbé, 2010; Legendre *et al*., 2016), especially when they are farmed in captivity (Zohar and Mylonas, 2001). The meiotic phase of spermatogenesis in *O. niloticus* at 30 °C is accelerated, whereas at 20 °C the spermatocytes are stopped in late pachytene, consequently varying the production of sperm (Vilela *et al*., 2003). Other aspect that also affect spermatic quality are the amount of protein (Moraes *et al*., 2015; Mewes *et al*., 2015), hormonal treatments (Mylonas *et al*., 2017) and, probably, fatty acids in the diet as reported in *Anguilla anguilla* where it is shown the matching between the fatty acids levels and changes on sperm production and

spermatic speed (Baeza *et al*., 2015). Variations in sperm quality depend not only on external changes, but also on the lineage genomic status and epigenetic effects in the regions of DNA involved in primordial cells production and therefore the sperm amount and quality (Robles *et al*., 2017).

Another aspect that is not greatly studied in fishes is the expression of the microRNA types (miRNA) in male germ cells, that was reported abundantly available in testes of rainbow trout (Farlora *et al.*, 2015), suggesting that the miRNA could be correlated to the spermatogenesis and function of the sperm (Kun-Tong *et al*., 2015). The significant association between DNA integrity and sperm quality and fertility was reported in other species (Sheikh *et al.*, 2008; Dietrich *et al*., 2010). The DNA integrity can be affected by environmental or endogenous mutagen during spermatogenesis or spermiogenesis. In this latter process, there is a change in the DNA configuration by an exchange of histones for protamines (Chiva *et al*., 2011; Gou *et al*., 2017); therefore, the DNA sequences could be a target for genotoxic agents.

The possible interaction between the sex-determine DNA sequences and different environmental factors influencing spermatic quality is beyond the scope of this study. Spermatic quality changes over seasonal changes with the subsequent variations in temperature and light exposure.

4. Conclusions

Although the XY males had a better performance in terms of weight gain, YY males have better reproductive capabilities based on sperm amount and quality. The YY technology is a valuable and useful strategy to obtain YY breeders with high reproductive capabilities and to ensure a homogeneous population of male monosex XY with great advantages in weight and length gain. Moreover, it remains to be investigated whether the additional copy of the Y-related sequence and/or the absence of the X-related sequence are key factors that influence the better spermatic quality observed in this study.

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