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Short Communication

Atypical feminized male's agonistic behavior relative to males and females of Nile tilapia (*Oreochromis niloticus* L.)

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ABSTRACT. Early maturity during tilapia culture is a recurring problem. To avoid this, a series of techniques have been developed, including the production of YY-males. This technique involves the use of hormones to produce phenotypic females (XY genotype). However, incomplete transformations are frequently observed and the produced atypical feminized males (AFM) could display an ambiguity in the phenotypic expression of behavioral patterns. The aim of this study was to measure the frequency and intensity of aggressive behavior as well as the role that initial residence plays when involving three phenotypes (males, females and AFM). The experiment consisted of three stages. Resident fish were AFM in the first stage, males in the second and females in the third. In each stage the resident fish confronted males, females and AFM acting as intruders. Aggressive behavior was exercised more frequently by resident fish. Intersexual confrontations showed higher levels of aggression compared to intrasexual confrontations. The frequency of confrontations was not significantly different in confrontations involving AFM, however, differences were observed in intensity of aggression. It is possible that an incomplete transformation at physiological level could be responsible for an inaccurate decoding of signal during confrontations.

Keywords: *Oreochromis niloticus*, agonistic behavior, atypical feminized males, aquaculture.

Comportamiento agonístico de machos feminizados atípicos en relación a machos y hembras de tilapia del Nilo (*Oreochromis niloticus* L.)

RESUMEN. La madurez temprana durante el cultivo de tilapia es un problema recurrente. Para evitarlo, existe una serie de técnicas, incluyendo la producción de machos-YY. Esta técnica implica el uso de hormonas para producir hembras fenotípicas (genotipo XY). Sin embargo, son frecuentes transformaciones incompletas y los machos feminizados atípicos (MFA) producidos podrían mostrar ambigüedad en la expresión fenotípica de los patrones de comportamiento. El objetivo del presente trabajo fue determinar la frecuencia e intensidad del comportamiento agresivo, así como el papel que juega la residencia inicial al involucrar tres fenotipos (machos, hembras y MFA). El experimento abarcó tres etapas. En la primera etapa, MFA actuaron como residentes, en la segunda machos, y en la tercera, hembras. En cada etapa el residente enfrentó a machos, hembras y MFA que actuaron como intrusos. El comportamiento agresivo lo ejercieron con mayor frecuencia los peces residentes. Las confrontaciones intersexuales mostraron mayores niveles de agresión en comparación con las intrasexuales. La frecuencia de confrontaciones no fue significativamente diferente en confrontaciones que incluyeron MFA, sin embargo, se observaron diferencias en la intensidad de la agresión. Es posible que una transformación incompleta a nivel fisiológico pueda ser responsable de una decodificación incorrecta de señales durante las confrontaciones.

Palabras clave: *Oreochromis niloticus*, comportamiento agonístico, machos feminizados atípicos, acuicultura.

In Mexico, tilapia represents 60% of the production of farmed fish and is estimated to reach an annual production of 200,000 ton by the year 2020 (Norzagaray *et al.*, 2013). Tilapia culture, specially the

one of Nile tilapia (*O. niloticus*), offers many advantages over other fish species. However, and despite its relatively low fecundity (Phelps & Popma, 2000), the control of its early maturity is a recurring problem. In mixed-sex cultures, Nile tilapia fish reach

sexual maturity (30-50 g) long before commercial size (350-400 g) (Jiménez & Arredondo, 2000; Arboleda-Obregón, 2005). To avoid this, a series of techniques have been developed in recent years. One of the more promising techniques involves the production of YY-males, which, when combined with normal females (XX) produce progenies composed of 100% genetic males. While the ultimate goal of YY-technology is to reduce the use of hormones, the first step of this technology still involves using hormones to feminize normal male fry (XY). This produces a phenotypic transformation, *i.e.* the morphology and function of adult females from male fingerlings (Vera & Mair, 2000; Ovidio *et al.*, 2002; Desprez *et al.*, 2003).

During this process, it is common to observe the presence of atypical fish, in this case atypical feminized males (AFM), which are normally characterized by having either abnormal genital papilla or male papilla and with ovaries. AFM are usually discarded due to the suspicion of not having a functional oviduct resulting from a morphologically incomplete transformation. Previous experiments performed in our laboratory have shown that AFM selected were not only able to generate a number of viable fry, but also a sex ratio that confirms the presence of an XY genotype (Alcántar-Vázquez *et al.*, 2014). However, the number of spawns obtained from crosses between AFM and normal males was lower compared to those where normal females are used and in some cases it was possible to observe severely beaten AFM when they were placed in a reproduction tank with one male of similar size, suggesting some physiological disorder.

It is possible that in the AFM, an ambiguity in the phenotypic expression provoked by the differences between morphology and functionality of sexual structures could have a profound behavioral effect, for example in the aggression behavior, which is relevant to fish populations in terms of social structure, levels of stress and, consequently, in the overall performance of fish farming (Øverli *et al.*, 2002; Huntingford *et al.*, 2006; Boscolo *et al.*, 2011). In the context of animal behavior and particularly in what is known as fight theory (Maynard-Smith, 1982; Riechert, 1998; Arnott & Elwood, 2009; Mowles & Ord, 2012), the AFM represents an interesting opportunity to observe how to resolve conflicts against opponents with similar fight capabilities (RHP) based in their phenotype (females) but with probable differences arising from its genotype (males).

During a conflict between fish of either the same sex (intrasexual) or the opposite sex (intersexual), aggression can be defined as a behavior directed to harm or intimidate another individual. It is known that in vertebrates the mechanism of hormonal regulation acts

on the central nervous system, closely interacting with sex determination at the embryonic level (Salame-Méndez, 1998) and with how aggression is expressed between fish (Rosvall *et al.*, 2012). The objective of this work is to measure both the frequency of certain behavioral components used as indicators of confrontation intensity as well as the role that initial residence (dominance of a site) plays when involving the three phenotypes usually present in a population of Nile tilapia subjected to the sex reversal process. Considering that the feminization process involves morphological and functional (physiological) transformation from male to female, it is assumed that the change extends to the behavioral level. Typically, females are less aggressive than males, which are more commonly involved in confrontational behavior related to the dominance of a given site (Bradbury & Vehrencamp, 1998). Considering a likely modified physiological profile in sex-reversed fish in a context of confrontation dyads, in this work we predict that AFM will display behavior different from normal females in both frequency and intensity of aggressive behavior against normal male and female fish.

The experimental Nile tilapia fish (*O. niloticus*) fish used in this study was males, females and atypical feminized males (AFM) from the same batch. All were produced using locally available strains (Centro Acuicola de Temascal, Oaxaca and Sistema Cooperativo Integral, Veracruz) in the experimental aquaculture station from the Universidad del Papaloapan (18°06'N, 95°53'W; at a height of 30 m above sea level) and maintained in 3 m diameter concrete tanks with recirculating fertilized water. AFM were produced by feeding fry at swim-up stage with 120 mg of E₂ kg⁻¹ of powdered tilapia pellets for 30 days. Before starting the experiment, fish were reared up to sexual maturity (six months of age) and fed with a commercial diet according to their stage of development. In total, 72 fish were used for this experiment on confrontations.

The study was conducted in a recirculating water system composed of 12-85 L acrylic aquaria. Water in the recirculating system was filtered with a mechanical filter (Hayward, Model S310T2, Hayward Pool Products Inc., Elizabeth, NJ, USA) and a biofilter containing only plastic Bio-Balls (Aquatic Eco-System, Model CBB1, Pentair Ltd., Apopka, FL, USA) and then passed through a UV lamp (Lumiaction, Model BE1X20, Lumiaction Co. Ltd., Taipei, Taiwan). During the period of treatment, a photoperiod of 12L:12D was used and water temperatures were thermostatically controlled and maintained at 28 ± 1°C. All aquaria were covered with non-reflective paper on three sides and

separated 2 cm from each other to prevent the fish could see or interact with the fish from other aquaria.

The experiment consisted of three stages. Resident fish were AFM in the first stage (designated AR), males in the second (designated MR) and females in the third (designated FR). In each stage the resident fish confronted males, females and AFM all acting as intruders. Each combination was carried out in quadruplicate form and randomly assigned. A resident was defined as the fish that occupied each aquarium in the first instance. Before each stage, residents remained in the aquaria for a period of seven to ten days for acclimatization. During this period, fish were fed with commercial diet at 25% protein two times a day. Each intruder was previously weighed using a digital scale (± 0.01) (Scout Pro, Ohaus) to ensure that its size were as similar as possible (± 20 g) to the size of the resident. Additionally, considering that the ability to fight may involve the length or weight of the contender, we decided to use an index that considers both simultaneously, so standard and total length were obtained using an ictiometer, in order to calculate the fish condition factor (Froese, 2006).

Confrontations were recorded at different times of day (except at night) for a full minute using a digital camera (Sony Handycam DCR-DVD 610). Each recording session consisted of the recording made at a specific time of all combinations presented in the aquaria. Each stage lasted for a period of 24-h, consisting of a variable number of sessions (5 in AR, 6 on MR and 7 in FR). The first session of recording in each stage lasted for 2 min with the objective to measure the latency, that is, the elapsed time (in seconds) from the moment the intruder was placed into the aquaria until the first observed interaction between resident and intruder. After completing each stage, the fish were returned to 3 m diameter quarantine tanks for recovery before being transferred along with the rest of the population. Recordings were cataloged and sorted to obtain the following behavioral patterns: number of interactions in a minute of observation (in the first session, data were taken during the second minute), number of interactions for each fish (resident/intruder), and intensity level of aggression (I to V scale, based to the operational definitions of Evans *et al.* (2008), level I: body facing the opponent and erect dorsal fin; level II: fast and directed movement towards the opponent without physical contact; level III: short and lateral movements making contact with the mouth on the side of the opponent; level IV: chasing the opponent; level V: contact with both fish mouths, pushing the opponent). Finally, the number of times that an individual recoiled after the attack (called submission) was included. These observations were performed for each opponent (focal observations).

Aggressive behavior was calculated by subtracting the number of submissions from the number of aggressions recorded for each fish. This value can be positive (high aggressiveness), negative (high submission) or zero (aggressiveness-submission in equal terms). Median, quartiles and extreme values were calculated. Since in most fights, the majority of confrontations occurred in the first observation session (in the rest, the number of confrontations represent only 13%), data were pooled for each category of contenders and compared as independent groups (different aquaria) using a Wilcoxon or Kruskal Wallis test. We calculated the proportion of each intensity level of aggression with respect to the total number of confrontations and then compared in terms of the residence roles and sex categories. A chi-square test of independence was applied in a contingency table to measure the relative frequencies between level of aggression and type of confrontation. The proportion values obtained were arcsine transformed and then analyzed using an ANOVA test for each type of confrontation, using as classification variable the levels of intensity of aggression (Zar, 1984). Condition factor was calculated using basic Fulton's equation ($K = 100 \times W/L^3$; see Froese, 2006), and length (L) and weight (W) data. Comparisons between contenders were applied using t Welch test. All tests were performed at 0.05 significant level.

All of our comparisons showed no significant differences in terms of condition factor (*t*-Student statistic, all $P > 0.05$) and time of latency between categories of contenders ($H = 7.19$; $DF = 8$; $P = 0.517$). However, time of latency showed a tendency to be longer and more variable when resident females were faced with female intruders.

Each resident fish showed an increase in aggressive activity once a second fish, an intruder, was placed into the aquarium. Aggressive behavior (in relation to submissive behavior) was exercised more frequently by resident fish (Fig. 1). Intrasexual confrontations (male-male and female-female) showed a lower median number of attacks when compared to intersex confrontations (male-female), however, no significant differences were detected ($H = 2.81$; $df = 3$; $P = 0.422$). In confrontations where AFM were involved either as residents or as intruders, the median number of attacks was not significantly different ($H = 4.05$; $df = 4$; $P = 0.399$) to that observed in confrontations between normal males and females. As with the number of attacks, no significant differences ($W = 11,153.5$; $P = 0.78$) were observed in the frequency of attacks between confrontations with or without AFM. However, confrontations involving AFM displayed a tendency toward a higher frequency of attacks.

In general, when comparing the role of resident-intruder against the intensity of aggression in all com-

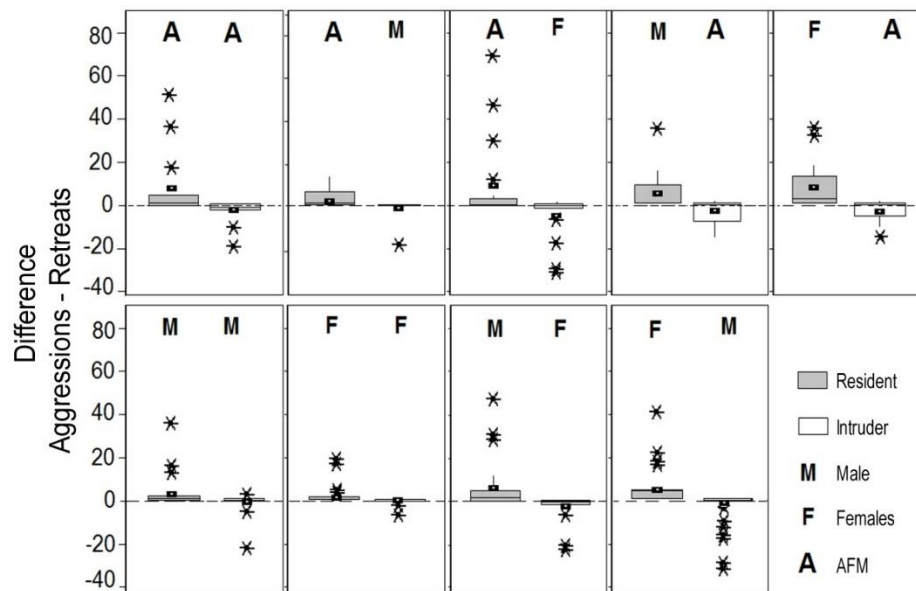


Figure 1. Relative differences between aggression and retreats (submission), according the role of resident or intruder of each contestant (mean: black box, median: horizontal line; quartiles: box, extreme values: asterisks) ($P > 0.05$). The top panel refers to interactions with atypical feminized males (AFM). The bottom panel refers to inter and intrasexual confrontations between normal males and females.

binations recorded, significant differences (chi-square = 178.03; $df = 24$; $P < 0.001$) were observed in the relative frequency of each intensity level of aggression and the mean proportion of different aggression levels (F values are showed in Fig. 2). Two levels of intensity were in proportion more frequent: the less intense aggression (level I) and one in which there was physical contact with the mouth on the side of the opponent (level III). Intrasexual confrontations showed almost no increase in intensity of aggression, while intersexual confrontation showed in each confrontation an increase in intensity (level III). Meanwhile, in confrontations that included AFM, it was observed that, as residents, they showed a higher level of aggression (level III) only toward other AFM. As intruders, AFM were attacked with more intensity (level III) in almost all cases by both males and females. In some confrontations, male aggression toward AFM reached a level IV (Fig. 2).

There are few references to aggressive behavior of sex-reversed fish, particularly males transformed into females. According to our results, AFM behave like normal females but with differences at inner aggressive behavior, something known as behavioral pattern (Lehner, 1996). This is supported by the fact that AFM were not shown to be more aggressive than normal females or males with respect to frequency of aggressive behavior but did prove to be so in terms of intensity level. This result contrasts with that observed by Ovidio *et al.* (2002), who reported that aggressive

behavior in feminized males was higher when compared to that observed in normal females, a result based only on the frequency of confrontations and not, as was observed in our study, on the differential occurrence of certain behavioral acts.

In our work, AFM had genital papilla which retained mainly the male form with no obvious oviduct but that could expel viable eggs after abdominal massage. It is possible that this incomplete transformation at morphological level could be associated with an incomplete transformation at the physiological level, which in turn could be responsible for the altered agonistic behavior observed in AFM (Alcántar-Vázquez *et al.*, 2014).

According to Varadaraj (1989), partial transformations can occur, apparently caused by variations in dose, and to a lesser degree, by duration of exposure to the hormone. Therefore, partial transformation could produce not only an incomplete sex-reversal from male to female at physiological level but also an incomplete transformation at tissue level, more specifically at gonadal tissue, generating intersex individuals (with both types of gonadal tissues at the same time). Chemical signals translated to behaviors coming from this intersex gonad could be mixed and ambiguous in AFM.

There is evidence that aggressive behavior in mice is the result of hormonal action rather than chromosomal differences between sexes (Canastar *et al.*, 2008).

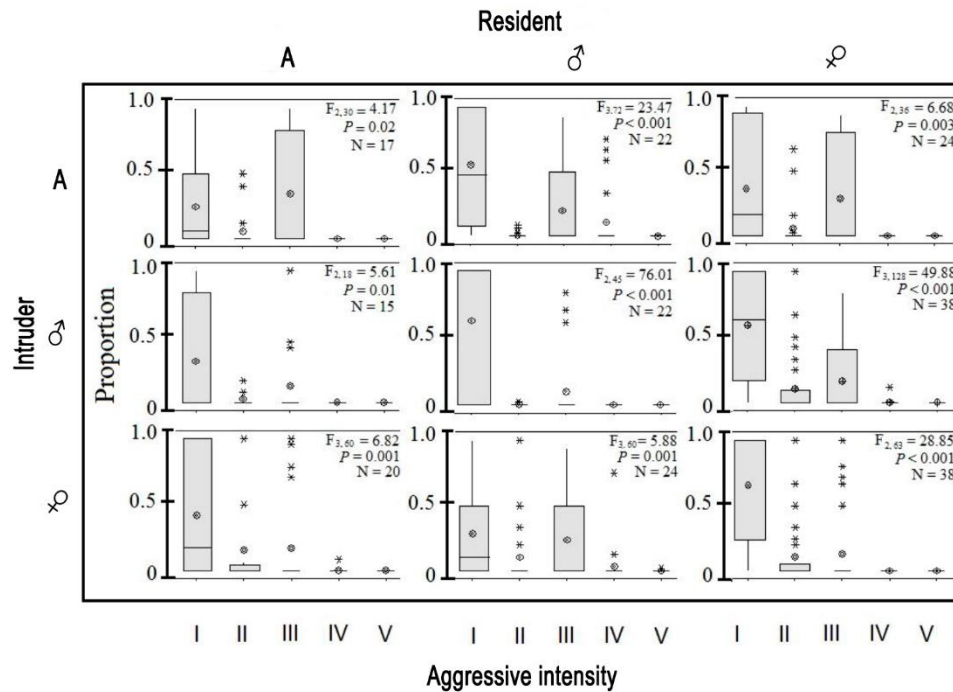


Figure 2. Proportion of behavioral components (levels of aggression) for different contender's combinations (role of resident-intruder and sex categories). Mean median, quartiles, and extreme values are presented. Statistic values for ANOVA tests among levels of aggression are presented for each type of confrontation. ♂ = male, ♀ = female, A = atypical feminized males (AFM).

It is possible then to consider that in other vertebrate groups such as fish something similar would occur and the underlying differences between the experimental groups analyzed would be related only to the effect of the levels of sexual hormones. Therefore, it is possible that the incomplete feminization reached at the physiological level could be responsible for increasing intensity of the aggressive behavior displayed by and toward the AFM. This could provoke, as our data suggests, that AFM behave similarly to females or males depending on the sex of the resident with whom they share the aquarium.

Although an incomplete feminization could be responsible for the agonistic behavior observed in the AFM, we cannot rule out the effect on hormonal patterns and therefore on behavior of the interaction of the three components that govern sex in the Nile tilapia: a complex genetic sex determination system with a major determinant locus, some minor genetic factors, as well as the influence of temperature (Baroiller *et al.*, 2009). Baroiller & D'Cotta (2001) mention that fish have certain plasticity during sex differentiation since several functional sex phenotypes can be generated by diverse mechanisms, including sex-reversal. Therefore, a sex-reversed fish, especially an atypical one, would show altered hormonal patterns caused by the combination of gene expression of sex genes of both

sexes, in this case, probably male sexual genes that were not completely inactivated due to an incomplete feminization at genetic level. These altered hormonal patterns will finally be reflected in an altered behavior of AFM (more aggressive in intensity) or in the sending of mixed signals (combination of the expression of genes of both sexes) that would cause further aggression against them.

Aggressive behavior was issued by the resident toward the intruding fish. In most cases the lower level of aggression was the most frequent (dorsal erection). Based in our results on time of latency, residents probably did not make distinctions when evaluating (to confront) different types of intruders. Therefore the conflict, by the dominance, usually was resolved quickly and with little effort made by the resident.

In our work, it was possible to observe that male and female residents increased the intensity of confrontation only toward intruders of the opposite sex while AFM acted similarly only against other AFM. In the first case, it is probable that confrontations emerged between sexes had an origin based on a conflict associated with the reproductive process (Shuster & Wade, 2003). In the second case, it is possible that the aggression intensity rose due to the fact that they faced fish whose responses to agonistic signals were ambiguous. In consequence, fish that escalated con-

frontation by showing more aggression, reiterated and even magnified signals originally issued in low aggression levels (Payne & Pagel, 1997). This emphasizes why behavioral acts such as "persecution" (level IV of aggression) have predominantly occurred in cases of confrontation with AFM.

In this experiment, it was not the confrontation frequency but rather the way each aggressive confrontation was displayed (different proportion of behavioral components) that determined the patterns of aggressive behavior observed in males, females and AFM. Our experiment suggests one of two possible scenarios for the confrontation dynamics observed: in the first scenario, the resident fish was required to escalate to a higher level of aggression even if the intruder decided not to repel the attack, probably because the intruder showed signs (perhaps chemical) received by the resident fish as a challenge to dominance of the territory (aquarium). In the other scenario, it is probable that resident fish fell into a mechanized behavior (stereotypy) and exerted unnecessary and unavoidable higher levels of aggression. It is not clear why, in the case of *O. niloticus*, a dominant fish can apply a level of aggression that results in death of a subordinate fish.

Although some studies have insisted on the artificiality that surrounds this kind of experimental observations made in conditions of captivity, particularly in the case of aggressive behavior as a response variable (Sloman & Armstrong, 2002), our experiment showed the relevance of the resident status or intruder on the outcome of fights and that the AFM analyzed possess a behavioral ambiguity, suggesting that these fish probably display altered communication codes that could exert some unbalanced influence in the natural or artificial social environment of fishes. Further investigation would be required to clarify and explain this scenario.

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