Identification of neomales in South American catfish *Rhamdia quelen* on the basis of the sex ratio in the progeny

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Abstract

In the present study, we aimed to identify masculinized genotypic females of *Rhamdia quelen* on the basis of the sex ratio of the progeny. Masculinizing diets containing 3 levels of 17α-methyltestosterone (MT; 60, 90, or 120 mg kg^{-1}) were administered to *R. quelen* fry. Thereafter, 5 phenotypic males from each MT treatment were crossed with normal females, and their progenies were sexed. At least 1 male from each treatment group generated progeny with a higher female:male ratio. These males, designated M60, M90, and M120, were crossed again with 3 other normal females to re-evaluate the sex ratio of the progeny. Moreover, a normal male was used as a control. Again, we found a higher female:male ratio in the progeny of M60, M90, and M120. The control male produced 2 sets of balanced progeny and 1 with a lower female:male ratio. Although these breeders did not generate all-female progeny (79.75 ± 7.20% of females), our findings indicate that M60, M90, and M120 can be considered masculinized genotypic females of *R. quelen*, suggesting the involvement of autosomal genes or polygenic interactions in sex determination.

Keywords: 17α-methyltestosterone, indirect sex reversal, silver catfish.

Introduction

Monosex is a highly desired condition for many fish culture systems, primarily owing to differences in growth performance between males and females or unwanted reproduction during the production cycle (Dunham, 2004). Sex reversal is an important alternative approach available to prevent heterogeneous growth in fish farming, which can be induced by temperature (Baroiller *et al*., 1999) or hormone administration via dietary supplementation, immersion bath, injection, or implants (Pandian, 2013).

The South American catfish *Rhamdia quelen* (Siluriformes, Heptapteridae) is a gonochoristic species with a broad distribution in the Neotropics, and it is well adapted to aquaculture conditions (Barcellos *et al*., 2001). In recent years, commercial production of this fish has significantly increased in southern Brazil (Silveira *et al*., 2014). Owing to a higher rate of growth in females and the early maturation of males during the production cycle (Gomes *et al*., 2000; Fracalossi *et al*., 2002; Ghiraldelli *et al*., 2007), monosex female cultures have the potential to increase the production volume of this species.

Although sex reversal is generally induced directly in fish by administering steroids during the initial phases of development (Pandian, 2013), the use of synthetic steroids in farmed fish has sparked controversy regarding the potentially detrimental effects on human health and the environment. Moreover, it can negatively affect healthy fish development. For these reasons, this practice is prohibited in many European countries (Desprez *et al*., 2003; El-Sayed, 2006).

Without the use of exogenous hormones, female progeny might be obtained by crossing masculinized genotypic females that are phenotypically male (neomale breeders) with normal females (i.e., both phenotypically and genotypically females). This indirect method of sex reversal has been applied to commercial salmonid farming (Hunter *et al*., 1983; Piferrer and Donaldson, 1989; Feist *et al*., 1995), and it helps to avoid the use of hormones in fish raised for human consumption. Indirect sex reversal initially involves masculinizing genotypic females with an androgenic hormone to obtain neomale breeders and later fertilizing the oocytes from normal females with semen from neomales, which should theoretically produce offspring composed purely of normal females, depending on the sex determination system. These female offspring would be free of exogenous hormones.

In *R. quelen* from the Iguaçu River, Fenocchio *et al*. (2003), related that there are no differences in chromosomes between sexes. However, the existence of sex chromosomes in *R. quelen*, or the type of sex determination system is not known, to the best of our knowledge. In classical sex determination systems, females that are homogametic for the sex chromosome (XX/XY) model would theoretically produce all female progeny when crossed with neomales (XX; Kavumpurath and Pandian, 1994; Gomelsky, 2003). Likewise, for species in which the females are heterogametic (ZW/ZZ model), all female progeny would be generated by crossing a normal male (ZZ) with superfemales (WW), which would originate from neomales (ZW; genetically female and phenotypically male; Toledo-Filho *et al*., 1996). However, for some species, sex chromosomes and autosomal genes determine the sex, and the latter are influenced by genetic and environmental factors. This sex-determining mechanism would limit the development of all-female progeny using neomales or superfemale breeders (Devlin and Nagahama, 2002; Kirankumar and Pandian, 2002; Desprez *et al*., 2006).

The survival of fish subjected to sex reversal and

those carrying the desired genotype until sexual maturation is typically very low (George and Pandian, 1996; Pandian, 2013). The sex reversal process thus demands a considerable investment of time and resources to produce a broodstock of sex-reversed individuals. Therefore, the total number of fish produced limits the use of lethal methods to determine the efficacy of the sex reversal process. However, progeny tests aimed at comparing the sex ratios of progeny from different parent fish allows the evaluation of sexual composition of the progeny of sex-reversed fishes and enables the formation of a broodstock of such breeders. Therefore, the present study aimed to identify masculinized genotypic female breeders of *R. quelen* using progeny tests based on the sex ratio.

Materials and Methods

The study was conducted at the Laboratory of Biology and Freshwater Fish Farming, (LAPAD; 27°43′44.4″ S, 48°30′32.8″ W) at the Federal University of Santa Catarina, Aquaculture Department, using experimental procedures approved by the Animal Ethics Committee (Protocol PP00788 - Development of Technology for the Farming of Native Fish).

The study was conducted in 3 steps: I) masculinization of the larvae over 21 days and growth of the masculinized fish for 240 days until the attainment of sexual maturation; II) first progeny test with a duration of 150 days to find breeders from the masculinization with a higher frequency of females in their progenies; III) the second progeny test started 30 days after the end of the first test, also over 150 days, to confirm the sexual proportion of the progenies generated by the masculinized females.

Biological material

R. quelen larvae used in the masculinizing treatments were obtained through the artificial reproduction of wild specimens captured in the upstream portion of the Uruguay River (27°31′32.0″ S, 51°47′21.2″ W) and were maintained in the LAPAD breeder bank. One specimen was deposited in the Museum of Zoology of the State University of Londrina, Paraná, Brazil (MZUEL 10549). Reproduction was induced by hypophysation (Woynarovich and Horváth, 1983), wherein pool from 2 females and 1 semen pool from 2 males were used. The fertilized eggs were incubated (cylinder-conical incubator) using a controlled water recirculation system at a temperature of 25.2 ± 0.2°C.

Masculinization

17α-methyltestosterone (MT; Sigma, St Louis, MO, USA) was incorporated into a commercial diet (Guabi® Pirá alevinos 55, Campinas, São Paulo, Brazil) at doses of 60, 90, or 120 mg kg^{-1} of feed according to the ethyl alcohol evaporation method (Shelton *et al.*, 1981). All diets were sieved (using mesh sizes of 0-250, 250-650, and 650-850 μm), individually packed, and refrigerated until the first (mesh of 0-250 μm), second (mesh of 250-650 μm), and third (mesh of 650-850 μm) weeks of feeding. Larvae at the onset of exogenous feeding, i.e., 2 days after hatching (DAH), were split into 3 groups (1 group per dosage), and each group was administered a different dose of MT 5 times a day for 21 days. After the masculinizing treatment period, the fish were in a controlled water recirculation system at an average temperature of 25.3 ± 1.4°C and under a 12 h light-dark cycle until sexual maturation. Eight-month-old phenotypic males from each dosage group were stimulated to release semen by applying light pressure to the abdomen and were individually marked with passive integrated transponder tags (Mini Transponder Trovan®, Santa Barbara, CA, USA) for identification purposes during the progeny tests.

Progeny tests

Two progeny tests were conducted to identify neomales. In the first test, semen from each male coming from masculinization treatments was used to fertilize a pool of oocytes from 2 wild females, and the males that produced progeny with a prevalence of females ($P < 0.05$) were selected. In the second test, the males selected from the first test were used to fertilize separate aliquots of oocytes manually collected from 3 wild females (different from those used during the first test) to confirm the prevalence of females in the progeny.

Test I

In this test, we used 5 phenotypic males from each MT dose group (60, 90, and 120 mg kg^{-1} of feed) with mean weights $\pm$ SD of 156.54 ± 46.11 g, 316.20 ± 98.67 g, and 277.18 ± 59.08 g, respectively, and 2 females from the broodstock of wild breeders with a mean weight of 650.20 ± 212.06 g. The selected females had a swollen abdomen and reddish genital papilla, which are typical characteristics of females suitable for artificial reproduction. Spawning was induced in all fish through hypophysation (Woynarovich and Horváth, 1983). The females received 2 doses (0.5 and 5.0 mg kg^{-1}) of carp pituitary extract (CPE; Danúbio Aquacultura, Blumenau, Santa Catarina, Brazil) with a 12 h interval between doses, and the males received 1 dose (4.0 mg kg^{-1}) at the time the second dose was applied to the females. After manual collection and pooling of the oocytes from the females, 5 g aliquots were individually fertilized with each collected semen sample. The fertilized eggs were separately incubated in a conical-cylinder incubator connected to a controlled water recirculation system at 24.9 ± 0.4°C.

The progeny of each male was reared separately for 150 days in circular tanks (60 L) using a controlled water recirculation system with an initial density of 6 larvae per liter of water. These fry were fed exclusively with newly hatched nauplii of *Artemia* sp. for 15 days (Inve Aquaculture Inc., Salt Lake City, UT, USA) that had previously been maintained for 30 min in an immersion bath containing an enriched solution (1 L distilled water, 12 g bovine liver powder, 9 g casein, 2 g choline chloride, 10 g vitamin and mineral PREMIX, 40

ml crude fish oil, and 60 ml sunflower oil) to improve larval nutrition. The ingredients and amounts of the enriched solution were adapted from Cardoso *et al*. (2004) and Dias *et al*. (2011). Between days 15 and 30, the larvae were fed enriched *Artemia* simultaneously with a sieved mixture (1:1; 650-850 μm) of bovine liver powder and commercial flocculated micro-feed (Alcon® Alevinos, Camboriú, Santa Catarina, Brazil). After 30 days, the *Artemia* were removed from the feed, and only the mixture of bovine liver powder and commercial flocculated micro-feed was provided until the juveniles reached 2 months of age, when they began to receive commercial extruded feed (Guabi Pirá 40®, Guabi Nutrição e Saúde Animal SA, Campinas, São Paulo, Brazil).

At 150 DAH, 30 fish from each progeny group were randomly sampled and euthanized by submersion in 100 mg L^{-1} eugenol (Iodontosul® Eugenol, Porto Alegre, Rio Grande do Sul, Brazil) for 5 min prior to the removal of the gonads for sex determination. For the groups that exhibited a significant deviation from a 1:1 sex ratio in favor of females (P < 0.05), the sampling was extended to 90 fish or to all fish in the experimental unit when the number of fish was less than 90.

The gonads were removed by ventral incision, and sex was macroscopically determined (Fig. 1a, 1b). For fish with small gonads that could not be sexed macroscopically, sex was determined using an imprint of gonadal tissue on glass slides (without the use of dyes), which was observed under a light microscope (LED Leica® DM 3000, Bannockburn, IL, USA; Fig. 1c, 1d). The fish with undifferentiated gonads were excluded, i.e., only the fish with gonads that could be sexed were considered in the evaluation of the sex ratio. At the time of sexing, individual biometrics, including fish weight (g) and total length (cm), were determined.
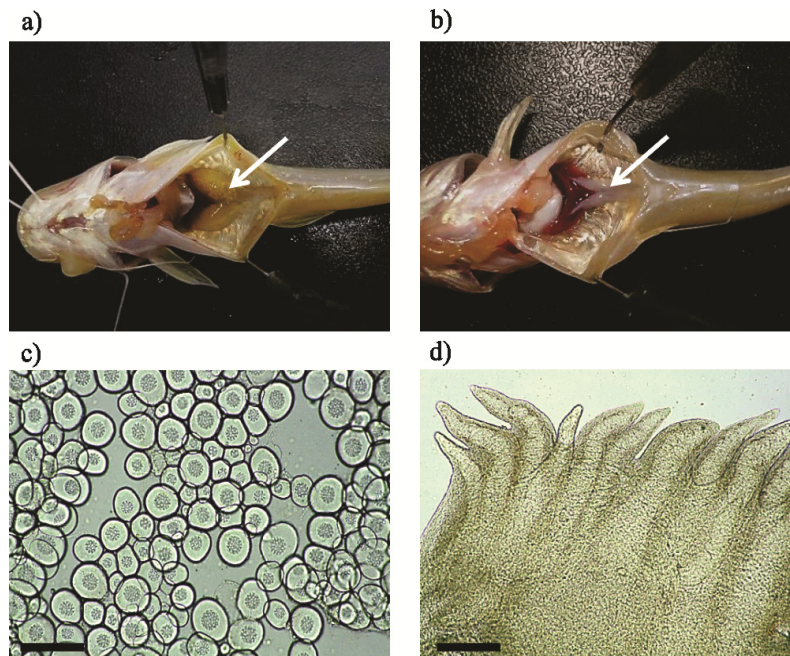

Figure 1. View of the gonads used for sexing the progeny of *Rhamdia quelen* generated from crosses between normal females and neomales. a) Female gonads with paired saculiform structures of a translucent yellowish color. b) Male gonads with paired elongated whitish structures. c) Apparent oocytes produced using the imprint of gonadal tissue on a glass slide. d) Digitiform projections of the male gonads produced from the imprint of gonadal tissue on a glass slide. Bars: 200 μm.

Test II

The phenotypic males from test I that generated the highest percentage of female progeny were used in test II, 6 months after the initiation of the first test. Semen from each male was used to fertilize separate 3 mature wild females, with a weight of 733.49 ± 94.48 g, derived from a broodstock of breeders (different from those used in the first test). During this progeny test II, the semen of 1 male from the broodstock of wild breeders (weight of 310.0 g) that did not receive a masculinizing treatment was used as a control. Reproduction was induced in all fish. The progeny were reared and sexed following the protocols used in the first test. However, in test II, all fish present in the experimental units were sampled; the minimum number of fish analyzed was 50.

Statistical analyses

The male to female ratio was evaluated using the chi-square test and an expected sex ratio of 1:1 (Zar, 2010).

In the first progeny test, the male to female ratios of all progeny groups were subjected to the chi-square heterogeneity test (α = 0.05) to test the hypothesis (H$_0$) that the male to female ratio was independent of the male breeder (Zar, 2010). The test was applied again because of the presence of heterogeneity, and 2 progeny groups were compared: I) groups with a higher male:female ratio or a balanced (1:1) ratio and II) groups with a higher female:male ratio.

In the second progeny test, the progeny of each male breeder was subjected to the heterogeneity test to test the hypothesis that the sex ratios between the progeny groups were similar regardless of the breeding females.

Results

Of the fish sexed during the progeny tests, 8% exhibited undifferentiated gonads. Crosses between normal females and neomales produced progeny with

79.75 ± 7.20% females.

Test I

The mean weight and total length ± SD of all generated progeny were 6.56 ± 4.17 g and 9.89 ± 1.81 cm, respectively. One phenotypic male from each MT dosage group produced progeny with a higher female:male ratio ($P < 0.05$), whereas the sex ratio was 1:1 ($P > 0.05$), or the percentage of males was higher ($P < 0.05$) in the other progeny groups (Table 1). Only 1 male (from the 60 mg kg^{-1} feed MT treatment) did not respond to induction with CPE and did not release semen for fertilization.

The heterogeneity test indicated that the effect of the male breeder on the sex ratio of the progeny was significant ($\chi^2 = 68.92$; $df = 13$; $P < 0.05$). When the progenies were combined, as described in section 2.3.3, and the sex ratios compared, no effect of the male breeder was observed between the pooled group consisting of the progeny groups with a 1:1 sex ratio or a higher male:female ratio ($\chi^2 = 9.62$; $df = 10$; $P = 0.47$) and the pooled group consisting of progeny groups with a higher female:male ratio ($\chi^2 = 3.25$; $df = 2$; $P = 0.20$).

Therefore, the heterogeneity resulting from the combination of all progeny can be attributed to the prevalence of females in the progeny generated by 3 males, 1 from each masculinizing dose, which were designated M60, M90, and M120.

Test II

The mean weight and total length ± SD of all generated progeny were 10.14 ± 3.46 g and 10.74 ± 1.29 cm, respectively. The percentage of females was significantly higher ($P < 0.05$) in the progeny from each of the phenotypic males in all masculinization treatments (M60, M90, and M120), corroborating the results obtained in the first progeny test (Fig. 2). The prevalence of females obtained in the heterogeneity test was consistent with the progeny obtained from each male regardless of the breeding female, demonstrating that these specimens of *R. quelen* are masculinized genotypic females (neomale breeders). In the crosses between the control male and breeding wild females, 2 progenies exhibited a 1:1 sex ratio, and 1 had a higher male:female ratio, resulting in sex ratio heterogeneity ($\chi^2 = 9.99$; $df = 2$; $P < 0.05$).

Table 1. Numbers of male and female progeny from progeny test I in which normal *Rhamdia quelen* females of a wild-breeding broodstock (the pool from 2 females) were crossed with phenotypic males produced in masculinizing treatments using 17α-methyltestosterone (MT). *Significantly different sex ratio using the chi-square test ($P < 0.05$), considering an expected ratio of 1:1. -- Specimen that did not respond to hormonal induction with CPE to release semen.

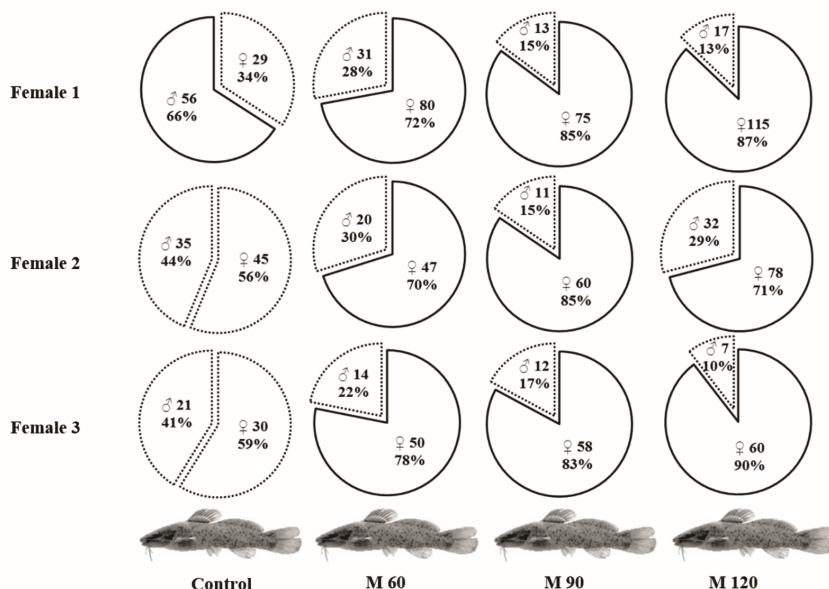| Breeding males | MT kg of feed^{-1} | | | | | |
| | 60 mg kg^{-1} | | 90 mg kg^{-1} | | 120 mg kg^{-1} | |
	♂ (%)	♀ (%)	♂ (%)	♀ (%)	♂ (%)	♀ (%)
1	16 (53)	14 (47)	18 (60)	12 (40)	13 (43)	17 (57)
2	23*(77)	07 (23)	18 (60)	12 (40)	18 (60)	12 (40)
3	22 (24)	68*(76)	18 (60)	12 (40)	12 (13)	78*(87)
4	21*(70)	09 (30)	24*(80)	06 (20)	23*(77)	07 (23)
5	--	--	24 (27)	64*(73)	12 (48)	13 (52)

Figure 2. Sex ratio of the progeny of *Rhamdia quelen* generated in progeny test II of normal females with individuals masculinized with 1 of 3 doses of 17α-methyltestosterone-60 (M60), 90 (M90), or 120 (M120) mg kg^{-1} of feed-and a control male. In all the 3 treatments, solid lines indicate a higher presence of females, and dotted lines indicate a lower presence of males. In the controls, solid lines represent a higher presence of males, and dotted lines represent a balanced (1:1) sex ratio using the chi-square test ($P < 0.05$), considering an expected sex ratio of 1:1.

Discussion

The phenotypic males produced in the different treatment groups and evaluated in the progeny tests were maintained in a water recirculation system at 25.3 ± 1.4°C and under a 12 h light-dark cycle from the initial phases of life. Under these conditions, they matured at the age of 8 months, which is similar to the age at maturity observed in *R. quelen* in soil fish ponds in southern Brazil (Ghiraldelli *et al*., 2007). In contrast, neofemales (feminized males) of *Betta splendens* show delayed sexual maturation compared with normal females (Pandian *et al*., 1994). However, the testicular development of *R. quelen* neomales mirrors that of normal males, which was demonstrated by both types of males releasing semen at the same age. Apparently, the formation of the sperm duct in *R. quelen* neomales occurs without abnormalities (another important characteristic of *R. quelen* neomales breeders), in contrast to that observed in *Oncorhynchus mykiss*, in which neomales do not have sperm ducts and need to be euthanized to collect semen (Kowalski *et al*., 2011).

Only 8% of all fish sexed during the progeny tests exhibited undifferentiated gonads, indicating that at 150 days old, the gonads of most fish could be identified macroscopically or microscopically by using the imprint of gonadal tissue on glass slides. In *Oreochromis niloticus*, sexing can be performed after the age of 90 days, regardless of the method used: macroscopic examination of the urogenital papilla, microscopic examination of gonads freshly stained with acetate-carmine, or histological analysis of the gonads (Makino *et al*., 2009).

A 1:1 sex ratio or a higher male:female ratio in the progeny generated in test I by males was consistent with those obtained from crosses with the untreated wild male (male control) in the second progeny test, demonstrating that the crosses occurred with phenotypic and genotypic males. In contrast, the prevalence of females among the progeny of the phenotypic males from the treatment groups M60, M90, and M120 in both progeny tests demonstrates that these animals can be considered neomale breeders. Although a full female progeny was not produced, each of these neomales participated in 4 crosses, and the prevalence of females was significant in the progeny regardless of the wild female breeder used.

Entirely female progeny is expected in a cross between a normal homogametic female (XX) and a neomale (XX), as is observed in salmonids (Donaldson and Devlin, 1996). This outcome does not occur in some species owing to the influence of other genetic and environmental factors (particularly temperature) on sex determination, in addition to the influence of sex chromosomes (Devlin and Nagahama, 2002; Kirankumar and Pandian, 2002; Desprez *et al*., 2006). Progeny tests with XX neomales of *Betta splendens* produced 13% males in the progeny, indicating the possible participation of autosomal genes in sex determination (Kirankumar and Pandian, 2002). In *Perca fluviatilis*, crosses between normal females and neomales result in progeny comprising 95-100 % females, whereas normal males of this species generate an unbalanced sex ratio that could be in favor of either sex (Rougeot *et al*., 2002). The authors of the study suggest that a model with homogametic females (XX) is affected by autosomal or polygenic factors in sex determination.

Water temperature is one of the main factors that can affect physiological sexual differentiation in fish (Nakamura *et al*., 1998; Ospina-Álvarez and Piferrer, 2008). However, different temperatures (19, 25, and 30°C) at the time of egg fertilization and incubation (Longo and Nuñer, 2010) or during the early developmental stage (19, 24, and 29°C; Sulis-Costa *et al*., 2013) did not alter the sex ratio of *R. quelen*.

Because sex determination involves complex pathways, the participation of autosomal genes is expected in cases in which the sex ratio of the progeny is unbalanced (Devlin and Nagahama, 2002). Considering that the presence of sex chromosomes has not been observed for *R. quelen* and that the sex determination system of this species has not been fully elucidated, sex determination might depend on a set of autosomal chromosomes, as occurs in *Pseudoplatystoma corruscans*, another Siluriformes species (Piferrer, 2001). However, belonging to the same order or genus does not necessarily imply similarities in the mechanisms involved in sex determination. The genus *Oreochromis* has 2 sex determination models: homogametic males (ZZ/ZW) and homogametic females (XX/XY) (Mair *et al*., 1991; Müller-Belecke and Hörstgen-Schwark, 1995). However, deviations in the sex ratios of the progeny suggest the involvement of other factors in sex determination in this genus (Mair *et al*., 1991; Baroiller and D'Cotta, 2001; Desprez *et al*., 2003).

Some studies have found that the identification of the genes involved in the gonadal differentiation of each sex is fundamental to a better understanding of the sex determination mechanism in fish (Siegfried, 2010). These genes have been identified in a few species (Matsuda *et al*., 2002; Kamiya *et al*., 2012; Yano *et al*., 2012) and have been used as chromosomal markers to distinguish pairs of sex chromosomes (Okutsu *et al*., 2015). In addition, functional genomic studies (transcriptomics) currently applied to fish have identified genes that are complementary to those considered essential for sex determination (Zhang *et al*., 2011; Yano *et al*., 2012; Sun *et al*., 2013; Tao *et al*., 2013; Lu *et al*., 2014).

In the present study, crosses between wild females and masculinized genotypic females on average produced progeny with 79.75 ± 7.20% females, and that too without the use of exogenous hormones. Because these cohorts were not entirely female, it is possible that autosomal genes or polygenic interactions were involved in the sex determination of *R. quelen*. Overall, the female:male ratio achieved using neomales of *R. quelen* indicates that the farming of this species is feasible because the growth rate of females is 30% higher than that of males (Baldisserotto and Radünz, 2004), which would lead to a considerable increase in biomass at the end of the production cycle.

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