Egyptian Journal of Aquatic Biology & Fisheries
Zoology Department, Faculty of Science,
Ain Shams University, Cairo, Egypt.
ISSN 1110 – 6131
Vol. 29(2): 2357 – 2369 (2025)
www.ejabf.journals.ekb.eg

Comparative Analysis of Sperm Quality and Fertilization Potential in Neomales (XX) and Normal Males (XY) of the Bonylip Barb *(Osteochilus vittatus)*

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ARTICLE INFO

Article History:
Received: June 7, 2024
Accepted: April 10, 2025
Online: April 19, 2025

Keywords:
Fertilizing ability,
Neomale,
Normal male,
Osteochlius vittatus,
Sperm quality

ABSTRACT

To enhance the production of all-female populations in aquaculture, it is essential to understand the reproductive differences between neomales and normal males, since this knowledge can significantly impact the efficiency and success of producing all-female offspring. The objective of this paper was to perform a comparative analysis of sperm quality and fertilization potential between neomales (XX genotype) and normal males (XY genotype) in the bonylip barb (*Osteochilus vittatus*). Fifteen neomales and fifteen normal males, each weighing 200–400g, were used as experimental subjects, with their gonad development monitored monthly. To evaluate fertilization ability, fifty females (weighing 200–550g) were spawned with both neomales and normal males. Sperm velocity and motility were analyzed using computer-assisted semen analysis (CASA) and included parameters, such as linearity (LIN), straightness (STR), wobble (WOB), curvilinear velocity (VCL), straight-line velocity (VSL), average path velocity (VAP), and percentage of motile sperm (% MOT). While there were no significant differences in sperm motility between neomales and normal males, the velocity parameters indicated that neomale sperm had higher percentages of straight-line velocity, linearity, straightness, and wobble. Significant differences ($P< 0.05$) were found in the amplitude of lateral head displacement and beat cross frequency values between the two groups. Additionally, sperm volume, fertilization rate, and hatching rate were significantly higher ($P< 0.05$) in neomales, with values of 14.33±2.08, 72.77±4.13, and 63.33±11.55, respectively. The findings suggest that neomales (genotype XX) in *Osteochilus vittatus* exhibit superior sperm quality and fertilization ability compared to normal males (genotype XY).

INTRODUCTION

In aquaculture, producing all-female offspring in certain species is crucial due to its potential to enhance growth performance and to reduce reproductive issues associated with mixed-sex populations. The generation of an all-female population presents a significant economic advantage in the aquaculture of the commercial rainbow trout

(*Oncorhynchus mykiss*) **(Nynca *et al.*, 2012)**, salmonid fish **(Judycka *et al.*, 2021)**, and neo-male mandarin fish (*Siniperca chuatsi*) **(Liu *et al.*, 2021)**. Achieving this goal often involves the utilization of neomales, which are individuals possessing XX chromosomes but exhibiting male phenotypic traits alongside normal males (XY) **(Rougeot *et al.*, 2004)**, to enhance hatchery production efficiency. Therefore, assessing the sperm quality and fertility capacity of neomales, compared to normal males, is crucial in determining their suitability for reproductive purposes in the context of producing all-female offspring.

Osteochilus vittatus, a bonylip barb species in the Cyprinidae family of freshwater fish, is native to Southeast Asia and is extensively found in Cambodia, Indonesia (Sumatra, Java, and Borneo), Laos, Malaysia, Myanmar, Thailand, and Vietnam **(Hasan *et al.*, 2019; Pamungkas *et al.*, 2022)**. The fish is bisexual, with XX chromosomes in females and XY chromosomes in males. Phenotypic females often exhibit advantages, such as faster weight and length growth **(Subagja *et al.*, 2017)**, making them preferred for enhancing production and reproductive efficiency in carp farming. Since *O. vittatus* can produce both eggs and caviar, an all-female population is necessary. Generating an all-female population offers considerable economic benefits in commercial *O. vittatus* aquaculture. Female monosex fish seeds were created by crossbreeding neomales with normal females. Hormonal treatment is one of the most common methods for producing neomales **(Subagja *et al.*, 2015)**. Masculinized females, also known as sex-reversed females (SRF) or neomales, display a male phenotype while maintaining a female genotype (XX). Consequently, all the sperm produced in their functional testes carry an X chromosome.

In addition to egg quality, sperm quality is vital for the reproductive success of fish. Some studies have indicated that sperm quality is comparable among XX masculinized males, XY males, and YY males **(Rurangwa *et al.*, 2004a; Gennotte *et al.*, 2012)**. Evaluating and verifying semen quality before using it in artificial propagation is essential for achieving high fertilization rates **(Figueroa *et al.*, 2018)**. In fish, the key semen quality parameters are motility **(Parodi *et al.*, 2015)**, total abnormalities **(Dadras *et al.*, 2017)**, and concentration **(Yang *et al.*, 2018)**. Semen membrane integrity and adenosine triphosphate (ATP) concentration are also commonly measured **(Geffen & Evans, 2000)**. Nevertheless, semen motility is considered the most critical factor for assessing semen quality **(Parodi *et al.*, 2015)**. Sperm motility is a crucial factor in determining semen quality and fertilization capability. Fertilization capacity, which serves as a reliable indicator for evaluating sperm quality, is considered the most definitive test. The use of computer-assisted semen analysis (CASA) is increasingly recognized as an easy, fast, and accurate method for assessing various semen motility indices **(Kowalski *et al.*, 2011; Gallego & Asturiano, 2018)**.

The objective of this study was to evaluate the sperm quality and fertilization potential of neomales (XX genotype) compared to normal males (XY genotype) to improve the production of all-female offspring in hatcheries. Through comprehensive evaluations of sperm motility, velocity, fertilization rate, and hatching success, this study aimed to elucidate the reproductive potential of neomales relative to normal males. By comparing these parameters between the two male types, valuable insights can be gained to inform breeding strategies aimed at maximizing the efficiency and productivity of *O. vittatus* hatchery operations. Furthermore, understanding the reproductive performance of neomales in comparison to normal males is essential for the development of sustainable aquaculture practices that prioritizes genetic improvement and population control. The results of this study could greatly benefit *O. vittatus* aquaculture by offering insights into using neomales to produce all-female offspring, which would improve the economic success and environmental sustainability of *O. vittatus* farming.

MATERIALS AND METHODS

The study was carried out at the Research Institute for Fish Breeding (RIFB) under the Ministry of Marine Affairs and Fisheries in West Java, Indonesia. *Osteochilus vittatus* specimens were sourced from fish populations in Bogor.

Experimental fish

The experiment utilized fifteen neomales and fifteen normal males, each with a body weight ranging from 200 to 400 grams. Neomales were produced by treating larvae (aged 4 days) with 500µg/ kg of 17α-methyltestosterone for 21 days, as reported by **Subagja *et al.* (2015)**. The population of the neomale bonylip barb fish were maintained until it reached sexual adulthood (produces sperm), and then a progeny test was carried out. The progeny test of functional male fish (neomale) produces larvae of all females. Fifty females, weighing between 200 and 550 grams, were utilized to assess the fertilization ability of the males. The fish were housed in concrete ponds at the RIFB in Subang, West Java, Indonesia, and fed a diet containing 38 percent protein. Monthly monitoring of gonad development was conducted. Fish with mature gonads were subjected to spawning, and their reproductive performance was monitored. Both normal males and neomales were paired with the females for spawning. The fertilization and hatching rates were recorded. At the end of the study, sperm motility, velocity, fertilization rate, and hatching rate were all evaluated.

Collection of semen

Sperm from the broodstock fish was extracted by manually stripping the fish abdomen. The collected sperm was then transferred into glass vials and was stored in insulated containers at 4°C, ensuring no direct contact with ice.

Assessment of sperm motility, velocity, fertilization efficiency, and hatching success

The volume of sperm was determined using a calibrated 1mL syringe. Sperm motility and velocity were evaluated through CASA, with findings presented as the percentage of motile sperm (%MOT), curvilinear velocity (VCL), straight-line velocity

(VSL), average path velocity (VAP), linearity (LIN), straightness (STR), and wobble (WOB).

The fertilization rate and hatching rate were calculated using the following equations:

$$\text{Fertilization rate (\%)} = \frac{\text{Number of fertilized eggs}}{\text{Total number of eggs}} \times 100$$

$$\text{Hatching rate (\%)} = \frac{\text{Number of hatched eggs}}{\text{Total number of fertilized eggs}} \times 100$$

(Chalde *et al*., 2014; Gárriz & Miranda, 2020)

Statistical analysis

The data regarding sperm motility, velocity, fertilization rate, and hatching rate were subjected to statistical analysis using Microsoft Excel 2016 and the SPSS program (version 25). An independent sample t-test was employed for data analysis.

RESULTS

The sperm motility analysis (Table 1) showed that the percentage of progression motility and velocity had no significant differences between neomales and normal males. The percentage of fast-progressive (type a) normal males is significantly higher than that of neomales. No significant differences were observed in the average values of velocity parameters such as curvilinear velocity (VCL), average path velocity (AVP), and beat cross frequency (BCF) (Tables 2, 3). However, in terms of other velocity parameters, neomale sperm exhibited significantly higher percentages (P< 0.05) of straight-line velocity, linearity, straightness, and wobble (Table 2) **(Kime *et al*., 2001)**.

Table 1. Motility analysis of sperm on neo-male and normal male of *Osteochillus vittatus*

Progression	Neomales (%)	Normal males (%)
Static	3.07 ± 2.31^{a}	1.50 ± 1.64^{a}
Non-progressive motile	68.33 ± 6.18^{a}	74.57 ± 2.80^{a}
Progressive motile	28.60 ± 8.49^{a}	23.90 ± 3.82^{a}
Velocity		
Rapid	4.07 ± 3.91^{a}	8.97 ± 4.76^{a}
Medium	53.17 ± 7.16^{a}	56.67 ± 2.51^{a}
Slow	40.93 ± 8.52^{a}	32.90 ± 4.99^{a}
Static	1.83 ± 2.23^{a}	1.50 ± 1.64^{a}
W.H.O		
Fast progressive (type a)	0.37 ± 0.31^{a}	2.40 ± 0.53^{b}
Slow progressive (type b)	37.12 ± 9.54^{a}	28.03 ± 7.48^{a}
Non-progressive (type c)	60.70 ± 7.85^{a}	68.07 ± 6.50^{a}
Immotile (type d)	1.83 ± 2.23^{a}	1.50 ± 1.64^{a}

Mean values in the same row with different superscript letters indicate significant differences between the groups (P<0.05).

The lateral head displacement amplitude (Table 3) was greater in normal males compared to neomales (P<0.05). There were no statistical differences in the percentage of hyperactive sperm between neomales and normal males (Table 4).

Table 2. Average values of the velocity parameters of *Osteochillus vittatus* sperm

Velocity Parameters	Neomale				Normal male			
	Total	Slow	Medium	Rapid	Total	Slow	Medium	Rapid
Curvilinear Velocity/VCL ('µm/s)	50.99±5.10a	33.99±3.29a	61.03±2.52a	96.58±22.07a	57.90±5.04a	32.87±1.80a	65.00±0.87a	111.63±5.10a
Straight-line Velocity/VSL ('µm/s)	32.57±3.59a	27.33±5.83a	39.33±2.50a	37.17±23.18a	29.97±3.61a	18.77±4.52a	33.53±1.85b	56.87±11.29a
Average Path Velocity/VAP ('µm/s)	40.24±2.69a	30.96±4.44a	48.11±3.04a	60.18±14.26a	43.10±4.61a	24.87±3.37a	48.23±1.80a	84.20±4.50a
Linearity/LIN (%)	64.06±5.58a	81.89±12.18a	69.82±6.54a	42.34±16.34a	51.70±2.27b	56.73±10.26a	51.57±2.68b	51.23±12.14a
Straightness/STR (%)	80.9±4.99a	87.7±6.02a	81.9±5.66a	58.4±23.14a	69.50±1.30b	74.67±7.49a	71.13±4.21a	71.20±7.97a
Wobble/WOB (%)	79.13±3.32a	90.95±7.34a	78.82±4.09a	62.61±7.07a	74.33±1.89a	75.60±5.88b	74.17±2.34a	75.57±6.18a

Mean values in the same row with different superscript letters indicate significant differences between the groups (*P*<0.05).

Table 3. Amplitudo of lateral, head displacement and beta/cross frequency value of sperm on neomale and normal male *Osteochillus vittatus*

Parameters	Neomale			Normal male			Unit
	Total	Medium Progresive.	Rapid Progresive.	Total	Medium Progresive.	Rapid Progresive.	
Amplitude of lateral head displacement (ALH)	1.7±0.43a	1.7±0.47a	3.9±1.92a	2.73±0.25b	2.60±0.30a	3.23±0.91a	mm
Beat/Cross Frequency (BCF)	4.90±0.53a	4.91±0.54a	2.94±1.65a	4.37±5.11a	4.43±0.35a	3.30±0.75a	Hz

Mean values in the same row with different superscript letters indicate significant differences between the groups (*P*<0.05).

Table 4. Percentage of hyperactive value on neo-male and normal male sperm *Osteochillus vittatus*

Parameter	Neomale		Normal male	
	Total	Percentage (%)	Total	Percentage (%)
Hyperactive	58.33±91.54a	4.74±6.38a	64.33±42.67a	5.73±4.89a

Mean values in the same row with different superscript letters indicate significant differences between the groups (*P*<0.05).

The observations on sperm volume, fertilization rate, and hatching rate between neomales and normal males revealed significant differences (Table 5). The mean values for sperm volume, fertilization rate, and hatching rate were higher in neomales compared to normal males.

Table 5. Sperm volume, fertilization rate and hatching rate of neomale and normal male *Osteochillus vittatus*

Parameter	Neomale	Normal male
Sperm volume (mL)	14.33±2.08a	6.67±2.89b
Fertilization rate (%)	72.77±4.13a	27.57±20.26b
Hatching rate (%)	63.33±11.55a	48.33±7.64a

Mean values in the same row with different superscript letters indicate significant differences between the groups (P<0.05).

DISCUSSION

Ensuring the quality of gametes, including both sperm and eggs, is a crucial priority in the aquaculture industry. This applies to both economically valuable species and potential new candidates **(Cabrita *et al.*, 2014; Samarin *et al.*, 2017)**. The quality of fish sperm holds equal importance to that of female eggs for the production of viable offspring. While induced spawning in female fish has been extensively studied, comparatively less attention has been paid to males. Parameters such as sperm morphology, density, volume, motility, fertilizing capacity, as well as the composition and osmolality of seminal plasma, are commonly assessed to gauge sperm quality in fish **(Linhart *et al.*, 2005; Alavi *et al.*, 2006; Ochokwu *et al.*, 2015)**. The ability of sperm to yield viable embryos upon fertilizing high-quality eggs in suitable conditions is closely linked to sperm quality **(Duarte *et al.*, 2009; Jane Ochokwu, 2015)**. Therefore, an effective management of fish reproduction necessitates the selection of superior males as breeders to enhance aquaculture practices or conserve wild stocks **(Bokor *et al.*, 2021)**.

Computer-assisted sperm analysis (CASA) enables the assessment of sperm motility characteristics, enhances reproducibility, and simplifies result documentation. CASA evaluation holds significant importance in predicting sperm's ability to achieve fertilization, as the percentage of motile sperm and sperm progressive velocity serve as the primary predictors of fertility **(Kime *et al.*, 1996, 2001; Rurangwa *et al.*, 2001)**. According to **Rurangwa *et al*. (2001)**, it has been established using the CASA system that progressive sperm motility velocities (VCL, VSL, and VAP) exhibit stronger correlations with fertilization rates than other movement parameters in the African catfish. Similar associations between sperm motility and fertilization capacity were also observed in the rainbow trout **(Lahnsteiner *et al.*, 1998)**, turbot **(Dreanno *et al.*, 1999)**, and carp **(Linhart *et al.*, 2000)**.

This study provides a comparative analysis of sperm quality between neomales (XX) and normal males (XY) of *O. vittatus*. Differences in reproductive parameters were observed between the two genotypes, particularly in sperm motility, velocity, and fertilization capability. The comprehensive quantification of neomale sperm represents a novel contribution to the understanding of this species, suggesting potential avenues for enhancing semen quality through refined hatchery production techniques, including the management of broodstock, their conditioning, and fertilization methods.

The percentage of sperm showing motility (MOT) and the motile concentration (MOC), indicating the proportion of motile sperm in a sample, are effective markers for sperm count. Fertility might be influenced by both the quantity of motile sperm and their velocity. Thus far, other motion parameters generated by the tracker have not provided additional useful information regarding sperm quality in fish **(Rurangwa *et al.*, 2004a)**.

In the Nile tilapia, males with unusual sexual genotypes (XX and YY) are indistinguishable in appearance from normal XY males. They produce and release functional sperm, and can naturally reproduce with females. However, as observed *in O. aureus*, the sexual genotype may impact the reproductive performance of the Nile tilapia breeders **(Desprez *et al.*, 2008)**. Differences in reproductive success among males could arise from variations in sperm quality or in courtship and mating behavior. The findings of the study on tilapia sperm revealed that sexual genotype did not affect sperm quality in the Nile tilapia, and there were no substantial differences in any aspects of tilapia sperm between XX, XY, and YY males **(Gennotte *et al.*, 2012)**.

In our study, there were no significant differences observed in sperm motility and velocity between neomales (XX) and normal males (XY) of *O. vittatus*, indicating that sexual genotype does not impact sperm quality. **Geffen and Evans (2000)** reported comparable motility rates in rainbow trout neomales (XX) and males (XY). Similarly, various studies focusing on the sperm characteristics of male and sex-reversed female teleosts have failed to establish a correlation between sperm quality and sexual genotype or sex reversal treatment. **Rougeot *et al.* (2004)** found no disparities in terms of GSI, sperm density, motility, velocity, and fertilization rate between XY and XX males in perch. **Fitzpatrick *et al.* (2005)** demonstrated in coho salmon (*Oncorhynchus kisutch*) that sperm density, motility, and velocity from functional XX males were comparable to those of normal XY males when milt was extracted.

The sperm of the majority of teleost fish is typically characterized by straight-line motility, evidenced by nearly identical values of VSL (straight-line velocity) and VCL (curvilinear velocity) **(Kime *et al.*, 2001)**. However, in the rainbow trout, VSL values tend to be notably lower than VCL values, leading to a low LIN (LIN = VSL/VCLx100%) **(Dietrich *et al.*, 2005; Dietrich *et al.*, 2005)**.

VCL (curvilinear velocity) is a crucial factor in the reproductive success of male fish **(Lahnsteiner *et al.*, 1998; Gage *et al.*, 2004)**. In the rainbow trout, VCL values have been reported to range from 106 to 120µm s-1, with an average of 144.7µm s-1 in stripped sperm samples from XY males, which is higher than previously reported values **(Lahnsteiner *et al.*, 1998; Dietrich *et al.*, 2005a; Dietrich *et al.*, 2005b)**. This variation may be due to differences in genetic fish stocks or measurement techniques. Our study found no significant difference in VCL values between neomale and normal male sperm, which were 50.99±5.10 and 57.90±5.04µm s-1, respectively.

In this study, VSL, LIN, STR, and WOB values were higher in neomales compared to normal males. The ALH value of sperm from neomales was lower than that of normal males, measuring 1.7±0.43 and 2.73±0.25µm, respectively. **Dietrich *et al.* (2005)** reported an ALH value of about 8.5µm for the stripped XY rainbow trout, which is higher than the ALH values for normal male fish (XY) observed in this study. Low VSL and LIN values combined with high ALH rates in mammalian sperm indicate hyperactivity **(Shivaji *et al.*, 1995)**. For freshwater fish, such sperm movement might indicate hypoosmotic shock occurring before fertilization **(Ravinder *et al.*, 1997)**.

The beat cross frequency (BCF) was comparable between neomales and normal males, with a value of 4-5 Hz, consistent with the findings of other researchers studying rainbow trout **(Kime *et al.*, 2001)**. Additionally, it has been noted that during the final stages of spermatic duct maturation, the BCF value remains stable in the movement characteristics of rainbow trout sperm.

It is well established that sperm velocity parameters (VSL and VCL) are strongly correlated with fertilization success **(Lahnsteiner *et al.*, 1998; Rurangwa *et al.*, 2004b)**. **Gage *et al*. (2004)** recently discovered that in Atlantic salmon, sperm velocity, rather than sperm count or duration of motility, is the primary factor influencing fertilization success. CASA parameters like ALH and LIN are valuable for estimating sperm swimming trajectories. **Ravinder *et al.* (1997)** and **Lahnsteiner *et al.* (1998)** found that sperm exhibiting a circular motility pattern, typical at the end of the rainbow trout sperm movement, had significantly higher ALH values compared to linearly moving spermatozoa.

Our study revealed significant differences in sperm volume, fertilization rate, and hatching rate between neo-male and normal male fish. Neo-males exhibited higher values in these parameters compared to normal males, indicating a greater reproductive potential. This suggests that neo-males could play a crucial role in aquaculture and fisheries management. Additionally, our findings indicate that sex reversal treatment does not affect the reproductive potential of neo-male fish. Similarly, **Liu *et al.* (2019)** reported that in mandarin fish (*Siniperca chuatsi*), the fresh sperm motility and fertilization rate of sex-reversed females were approximately 83 and 70%, respectively, showing no significant difference from normal males. Previous studies have also shown no significant difference in sperm quality between sex-reversed females and normal males **(Geffen & Evans, 2000; Rougeot *et al.*, 2004; Gennotte *et al.*, 2012)**. The study indicates that neomales in *Osteochilus vittatus* possess superior sperm quality and fertilization capability compared to normal males. Although there were no significant differences in sperm motility, neomale sperm exhibited better velocity parameters, higher sperm volume, fertilization rate, and hatching rate. The presence of two X chromosomes in neomales does not adversely affect sperm quality, highlighting their potential for improving hatchery production. The research emphasizes the importance of sperm quality for fertilization success and suggests further exploration to optimize the use of neomales. The findings on the sperm quality and fertilization ability of neomales and normal males in *Osteochilus vittatus* have significant implications for hatchery production of all-female offspring, potentially leading to increased efficiency, reduced costs, and genetic improvement opportunities.

CONCLUSION

The findings of this study reveal differences in sperm quality and fertilization potential between neomales (XX) and normal males (XY) in *Osteochilus vittatus*. Neomales show higher sperm volume, fertilization rate, and hatching rate compared to normal males, suggesting their potential to enhance the production of all-female offspring in *O. vittatus* aquaculture. Further research is recommended to explore the mechanisms behind these differences and to optimize breeding strategies for better aquaculture practices.

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