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Comparative investigation of biological indices and biochemical compounds of reproductive indicators in male and female *Pangasius nasutus*

DONALD TORSABO[1,2], GABRIEL AROME ATAGUBA[2], BENEDICT TERKULA IBER[1,2], NOORDIYANA MAT NOORDIN[1,3,♥], NURIZZATI IDRIS[1], IVAN CHONG CHU KOH[3], MUHAMMAD YAZED ABDUH[3], RUMEAIDA MAT PIAH[3,4], AMBOK BOLONG ABOL-MUNAFI[1]

[1]Higher Institution Centre of Excellence, Institute of Tropical Aquaculture and Fisheries, Universiti Malaysia Terengganu. 21030 Kuala Nerus, Terengganu, Malaysia. Tel.: +60-9864-6693, ♥email: diyananoordin@umt.edu.my
[2]Department of Fisheries and Aquaculture, Joseph Sarwuan Tarka University. Makurdi 970101, Benue, Nigeria
[3]Faculty of Fisheries and Food Science, Universiti Malaysia Terengganu. 21030 Kuala Nerus, Terengganu, Malaysia
[4]Institute of Oceanography and Environment, Universiti Malaysia Terengganu. 21030 Kuala Nerus, Terengganu, Malaysia

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Abstract. *Torsabo D, Ataguba GA, Iber BT, Noordin NM, Idris N, Koh ICC, Abduh MY, Piah RM, Abol-Munafi AB. 2024. Comparative investigation of biological indices and biochemical compounds of reproductive indicators in male and female* Pangasius nasutus*. Biodiversitas 25: 3105-3115.* *Pangasius nasutus* (Bleeker, 1863) is an emerging freshwater aquaculture species with known difficulties in the aspect of reproduction in captivity. This study examined biological, biochemical, and fatty acid profiles of *P. nasutus* obtained from cage culture systems in Pahang, Malaysia as indicators of sexual maturity. A total of 73 samples (43 females and 30 males) of fish were used to collect data on biological indices, biochemical composition, and fatty acid percentages. Otolith growth marks were examined and counted under a microscope for age determination. The results showed a female to male sex ratio of 1.5:1 and the age structure skewed towards three years old fish. The Gonadosomatic Index (GSI) was significantly high for females and with a significantly low difference in the Coelomic Fat Index (CFI) in comparison with the males. Protein and lipid were found to be highest in the testes of males and the liver of females respectively. Saturated Fatty Acids (SFA) and Monounsaturated Fatty Acids (MUFA) significantly differ between males and females in the liver and gonads, in addition to highest Polyunsaturated Fatty Acids (PUFA) also recorded in the gonads of 3 years fish. The current study reveals that *P. nasutus* reaches sexual maturity at a minimum age of 3 years, with an average body weight of 1099.27±21.5 g and an average total length of 48.36±0.45 cm. Significant differences between fatty acid groups highlight the need for lipid nutrition in the broodstock of *P. nasutus* for the supply of adequate energy and essential fatty acids for their gonad development.

Keywords: Age, captive stock, coelomic fat index, fatty acid, gonad development, *Pangasius nasutus*, otoliths

INTRODUCTION

Understanding the reproductive characteristics of fish is crucial for breeding success in captivity, whether for commercial purposes or conservation efforts (Tessaro et al. 2019). Effective management and development of cultured fish as broodstock hinge on key aspects of their life history, including age, growth, maturation age, and reproductive cycles (Nurdin et al. 2023). By monitoring these factors, aquaculturists can optimize harvesting sizes and the timing for artificial breeding, providing valuable insights into stock population dynamics. Reproduction in fish is closely tied to the accumulation and utilization of essential nutrients within their tissues, mobilized during reproductive activities (Ahmed and Mohammed-AbdAllah 2023). Factors influencing nutrient accumulation include age, size, and gender, along with external factors like water quality, seasons, and geographical location. The availability of food in an organism's environment is particularly critical for its survival. For reproductive success and offspring survival, essential fatty acids like Arachidonic Acid (ARA), Eicosapentaenoic Acid (EPA), and Docosahexaenoic Acid (DHA) are crucial (Ghosi Mobaraki et al. 2020). Highly Unsaturated Fatty Acid (HUFA) group, are crucial components of gametes and precursors for physiologically active molecules like prostaglandins and other eicosanoids, which significantly contribute to gonad maturation and egg release (Ramos-Júdez et al. 2023).

Despite the importance of these nutrients, many fish species face reproductive challenges in captivity, leading to reduced gamete fertilization, hatching rates, and larval survival. Many species such as common sole (*Solea solea* (Linnaeus, 1758)), Japanese flounder (*Paralichthys olivaceus* (Temminck & Schlegel, 1846)), lumpfish (*Cyclopterus lumpus* Linnaeus, 1758), flathead grey mullet (*Mugil cephalus* Linnaeus, 1758*)*, Malaysian mahseer (*Tor tambroides* (Bleeker, 1854)) Asian sea bass (*Lates calcarifer* (Bloch, 1790)) and common snook (*Centropomus undecimalis* (Bloch, 1792)) have shown reproductive dysfunctions in captive conditions (Pountney et al. 2022; Ramos-Judez et al. 2023). Studies on nutrient accumulation reveal variations in total lipids and the Gonadosomatic Index (GSI) during reproductive phases, emphasizing the need for optimized nutritional interventions. For instance, increases in total lipids during ovarian development and decreases in GSI post-spawning highlight the importance of targeted dietary management (Jan and Jan 2017).

Pangasius nasutus (Bleeker, 1863) also known as patin buah in Malaysia, native to peninsular Malaysia, is economically significant but faces declining populations due to overharvesting and habitat degradation (Abdul Halim et al. 2023). This species is found in the Rokan River drainage extending south to the Musi River drainage in Sumatra, in the Perak and Pahang River drainages of the Malay Peninsula, and from the Rajang River drainage to the Barito River drainage in Borneo (Vidthayanon and Ng 2020). This fish holds significant cultural and economic value, often considered a delicacy in Malaysia and commanding a high market price, estimated at around USD 16.02-68.52/kg, which is three times higher than that of local black *Pangasius* Valenciennes, 1840 catfish species such as *Pangasius micronema* Bleeker, 1846 and *Pangasius hypophthalmus* (Sauvage, 1878) (Sani et al. 2023). Efforts to culture *P. nasutus* have been ongoing, with local farmers expanding cage and pond cultures. The success of the artificial propagation program by the Department of Fisheries in 2004 marked a significant milestone, though continuous fry supply remains a challenge. Developing broodstock from existing cultures and selecting potential females and males for propagation are essential steps to address this bottleneck. Understanding reproductive indicators is vital for improving reproduction in conservation and restocking programs and developing biotechnologies for reproduction and aquaculture.

This study aims to assess the reproductive status and biochemical compounds, including fatty acid profiles, of male and female *P. nasutus* under captive culture. By comparing these profiles against their otolith structure-based age, this research seeks to provide insights into the optimal timing for artificial breeding. This knowledge is crucial for enhancing *P. nasutus* propagation, ensuring a stable fry supply for cage and pond stocking, and supporting the sustainable management of this valuable species.

MATERIALS AND METHODS

Sampling area

Pangasius nasutus samples were collected in a cage culture facility at the river bank in Pekan, Pahang State, Malaysia, situated in the East Coast region of Peninsular Malaysia, 3.4921°N, 103.3895°E (Figure 1). The cage water temperature and dissolved oxygen were 30.36°C and 4.45 mg/L respectively. The samples were collected and processed in situ, and analysis of the samples was conducted in laboratory facilities at Universiti Malaysia Terengganu, Terengganu, Malaysia.

Ethical approval

This study received ethical approval from the Institutional Animal Care and Use Committee (IACUC), Research Management and Innovation Centre (CRIM), Universiti Malaysia Terengganu (UMT), Kuala Terengganu, Terengganu (CRIM_IACUC UMT V1/2020).

Sample collection

Seventy-three (73) specimens of *P. nasutus* (43 females and 30 males) were randomly obtained from the cage in the month of May. The choice of sampling time in May was informed by the onset of spawning observed in other *Pangasius* species during this month. Upon collection, the fish was placed in 50 L fiber tanks and ice was slowly introduced to immobilize the fish before sampling procedures. Ethical approval for this study was obtained from the Institutional Animal Care and Use Committee (IACUC), research management and innovation center, University Malaysia Terengganu. Fish were dissected in situ, where gonads, and liver were taken. Before dissection, fish were pitted at the base of temporal bones directly to the brain using a pointed probe. A section of gonads and liver were taken and quick-frozen in liquid nitrogen and subsequently stored in a -80 freezer for further analysis. The fish heads were detached, placed in an ice box for preservation, and transported to the laboratory for otolith extraction and age determination.

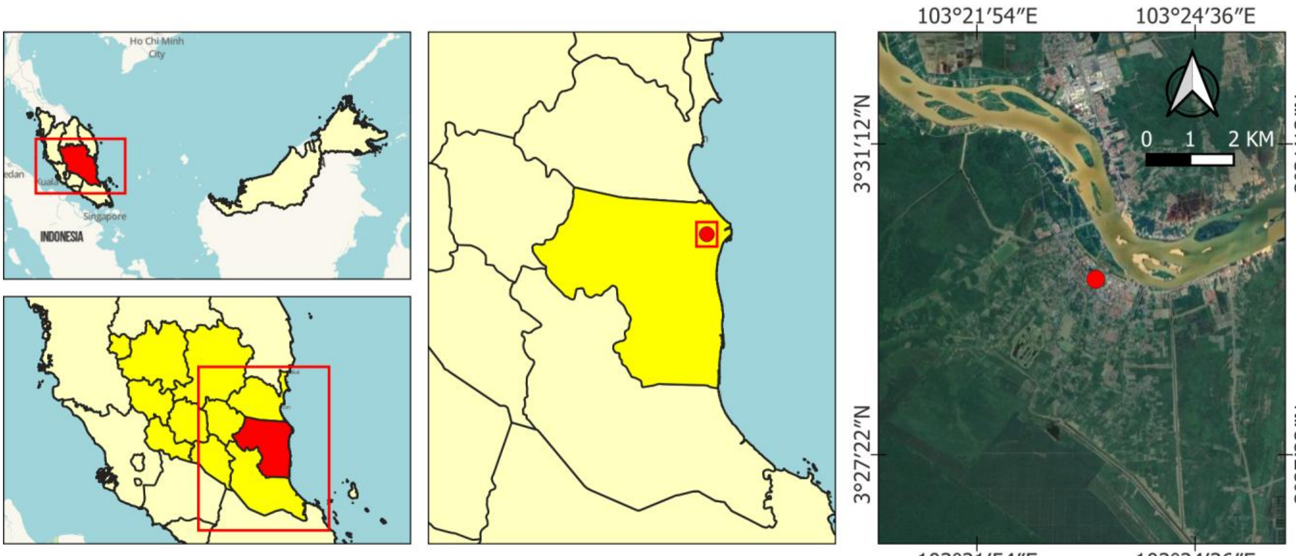

Figure 1. Map of the study site at the river bank in Pekan, Pahang State, Malaysia, situated in the East Coast region of Peninsular Malaysia. Image generated with Google Earth™ mapping service

Age determination

Age estimation analysis was conducted on a combined sample of 73 fish, consisting of 30 males and 43 females from a captive population, otoliths were extracted from the upper head of the males and females of *P. nasutus* washed with distilled water to remove attached tissues and left to dry before being preserved in cryovials for age determination. Otoliths from the right side of the fish head were embedded in epoxy resin using molds and kept for two weeks then cut transversely, measuring 0.4 mm thick through the nucleus and mounted on microscope slides for observation under a microscope in reflected light against a dark background (Lepak et al. 2012). Growth marks were visible as alternating translucent (light) and opaque (dark) zones. The fish age was estimated by counting the number of opaque zones.

Biological indices

Biometric parameters such as Body Weight (BW) and Total Length (TL) of the fish specimens were determined using an electronic balance (Shimadzu ATX224, USA) with 0.01 kg precision and a meter board with an accuracy of 0.1 cm respectively. The specimens were dissected, and the gonads, liver, coelomic fat, and visceral were collected and weighed for the computations of the Gonadosomatic Index (GSI), Hepatosomatic Index (HSI), Viscerosomatic Index (VSI), Coelomic Fat Index (CFI), and Condition Factor (K) respectively.

The following formulas were applied as described by Zardo and Behr (2015), Murgas et al. (2018), and Abdo et al. (2018).

$$GSI = 100 \times gonad\ weight\ (g)/body\ weight\ (g)$$
$$HSI = 100 \times liver\ weight\ (g)/body\ weight\ (g)$$
$$VSI = 100 \times visceral\ weight\ (g)/body\ weight\ (g)$$
$$CF = 100 \times coelomic\ fat\ weight\ (g)/body\ weight\ (g)$$
$$K = 100\left({}^{W}/_{L^3}\right)$$

Where: W: the body weight of fish in g; L: the total length of fish in cm.

Biochemical analysis

Biochemical analysis for crude lipid, crude protein, moisture, and ash followed the methods outlined by the Association of Official Analytical Chemists, AOAC (2006). To summarize, moisture content was determined by oven drying the sample at 105°C for 24 hours until a constant weight was achieved. Ash content was measured using a muffle furnace at 550°C for 8 h. Crude protein content was determined using the Kjeldahl method by measuring nitrogen (N×6:25). Crude lipid content was determined using soxhlet apparatus with Petroleum ether at a boiling temperature of 60-80°C.

Fatty acids analysis

Fatty acids were determined using one-step method (Abdulkadir and Tsuchiya 2008). Briefly, the freeze-dried samples of liver, gonads, and muscle were milled into powder form. 200 mg of the samples were introduced into a 50 mL centrifuge tube in triplicates using 4 ml of hexane alongside 1 mL of internal standard solution (19:0, Nonadecanoic acid ≥98% purity, Sigma-Aldrich, CAS,646-3-0, Germany). Nitrogen gas was used to purge the headspace of the tube after adding 2 mL of 14% BF_3 in methanol and a magnetic stirring bar and then tightly closed with a teflon-lined screw cap. The sealed tube was heated on a hot plate at 100°C with continuous stirring for 120 min. Upon cooling to room temperature, 1 mL of hexane and 2 mL of distilled water was introduced. After vigorous shaking for 1 minute, the tube underwent centrifugation at 650 g for 3 min. The top phase generated was a hexane layer containing the Fatty Acid Methyl Esters (FAMEs). Afterward, approximately, 1-2 mL of the hexane layer was pipetted into a clean sample vial and injected into the GC-FID for FAME analysis. A comparative analysis was conducted on key fatty acids, including arachidonic acid (C20:4n-6), eicosapentaenoic acid (C20:5n-3), and docosahexaenoic acid (C22:6n-3). These fatty acids were selected due to their significant roles in fish reproductive physiology, making them valuable indicators of sexual maturity and spawning readiness.

Data analysis

Statistical analysis was conducted using SPSS version 28.0.0. Data were presented as means ± standard deviation, with statistical significance set at $P < 0.05$ for all tests. Normal distribution was assessed using the Shapiro-Wilk test, and homogeneity of variance was checked using Levene's test. One-way ANOVA was employed for data analysis, followed by Tukey's Honest Significant Difference (HSD) test for post hoc comparisons. The student's t-test was used to compare body lengths between female and male fish.

RESULTS AND DISCUSSION

Biological indices

The percentage count of sampled *P. nasutus* showed that 60% of the total sampled fish were female. The age structure of *P. nasutus* (Figure 2) shows that among the total fish sampled, 41 fish were 3 years old, 13 were 5 years old, 12 were 2 years old, and 7 fish were 4 years old. Females were more prevalent in age classes two and three, while males were more common in age classes four and five among the fish.

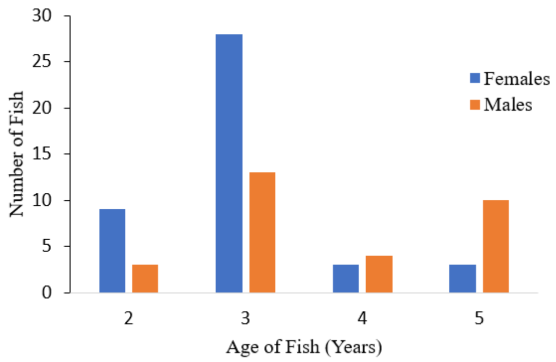

Figure 2. Age structure of males and females captive *Pangasius nasutus*

The biological indices of *P. nasutus* are presented in Table 1. The BW and TL showed no significant difference between males and females but significantly ($p<0.05$) differ among ages. The result showed that *P. nasutus* in the cage culture system matures at the average BW of 1099.27±21.5 g and TL of 48.36±0.45cm. Female *P. nasutus* have a significantly higher GSI compared to males. At the same time, GSI differs significantly among ages with 3- and 4-years old fish recording higher values followed by 5 years old fish, while the least value for GSI was recorded among 2 years old fish. HSI and VSI showed no significant difference between males and females and among the specimens different ages. Fish showed significant differences in CFI between males and females and among age groups with males having significantly higher CFI than females. There was a significant difference in condition factor K between ages, with age 5 recording the highest K compared to 4, 3, and 2-year-old fish. Matured male and female *P. nasutus* gonads are presented in Figure 3.

The interaction of sex, age, and GSI is presented in Figure 4. The plot shows an interaction between the sex, age, and the gonad-somatic index in males and females and across all ages. The plot shows that the GSI is consistently higher in females than in males across all age groups. The difference in GSI between sexes tends to increase with age, with the most significant difference observed at age 4.

Biochemical analysis

Proximate analysis of the liver and gonads of male and female *P. nasutus* is presented in Table 2. The result shows that the protein content of the testes is significantly higher compared to the ovaries while the protein content of the liver in both males and females shows no significant difference ($p<0.05$). Lipid content in the liver of female *P. nasutus* is higher compared to the male liver, though with no significant difference ($p<0.05$), while the lipid content of the testes was higher compared to the ovaries with no significant difference ($p<0.05$). Moisture content was higher in the liver of females with no significant difference compared to those of males, while the moisture content in the ovaries was significantly higher compared to the testes ($p<0.05$). The testes recorded significantly higher ash content compared to the ovaries while there was no significant difference in the ash content of the liver in male and female fish respectively.

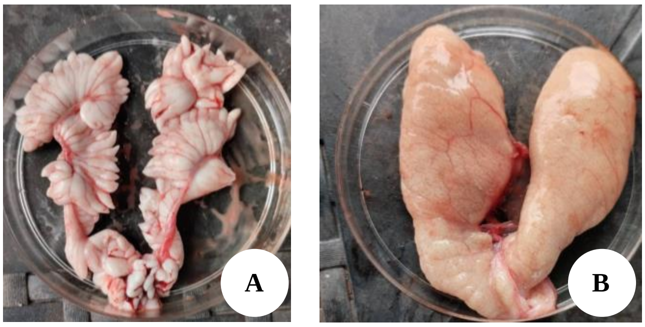

Figure 3. Matured gonads of *Pangasius nasutus* during the time of sampling. A) male; B) female

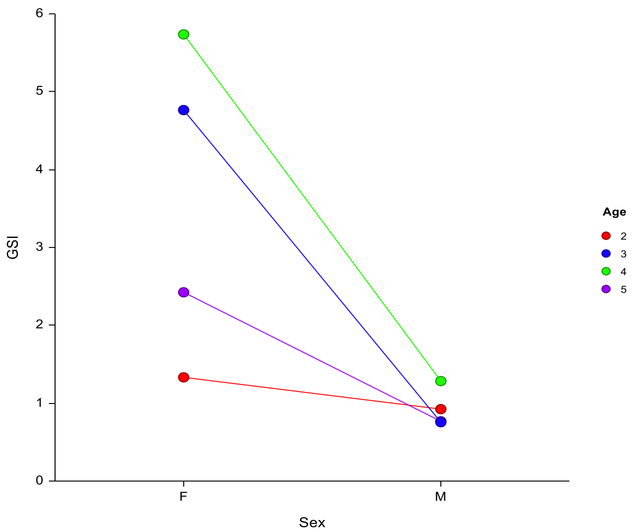

Figure 4. Interaction plot between age, sex, and GSI of *P. nasutus*

Table 1. Biological indices of male and female *Pangasius nasutus* with the interaction between GSI, sex, and age

Factor	BW (g)	TL (cm)	GSI	HSI	VSI	CFI	K
Sex							
F	1415.48±74.9	50.55±0.71	3.81±0.38^{a}	0.92±0.03	1.27±0.06	3.69±0.44^{b}	1.08±0.03
M	1116.71±47.2	47.89±0.54	0.88±0.15^{b}	0.85±0.03	1.24±0.06	5.15±0.38^{a}	1.00±0.02
p-value	0.637	0.528	0.000	0.143	0.536	0.000	0.988
Age							
2	932.08±31.8^{c}	45.73±0.46^{b}	0.99±0.25^{ab}	0.86±0.06	1.32±0.13	4.06±0.55^{b}	0.98±0.03^{b}
3	1099.27±21.5^{bc}	48.36±0.45^{b}	3.01±0.43^{a}	0.92±0.03	1.26±0.06	3.87±0.38^{b}	0.98±0.02^{b}
4	1294.29±114^{b}	49.61±1.15^{b}	2.56±0.92^{a}	0.82±0.07	1.18±0.08	4.36±0.94^{ab}	1.04±0.04^{b}
5	1954.12±86.5^{a}	54.15±1.12^{a}	2.23±0.42^{b}	0.86±0.04	1.22±0.05	5.74±0.84^{a}	1.24±0.05^{a}
p-value	0.000	0.000	0.004	0.486	0.775	0.002	0.000
Interaction (Sex*Age)							
p-value	0.314	0.765	0.040*	0.532	0.897	0.180	0.440

Note: Means in the same column of factors followed by different superscripts differ significantly ($p<0.05$). BW: Body Weight, TL: Total Length, GSI: Gonadosomatic Index, HIS: Hepatosomatic Index, VSI: Viscerosomatic Index (VSI), CFI: Coelomic Fat Index, K: Condition Factor

Table 2. Biochemical content of liver and gonads of male and female *Pangasius nasutus*

Parameters (%)	**Male**		**Female**	
	Liver	**Testes**	**Liver**	**Ovaries**
Protein	42.65±0.23^{c}	75.49±0.88^{a}	46.00±0.26^{c}	64.69±5.83^{b}
Lipid	30.95±3.27^{a}	15.05±0.00^{b}	35.04±0.48^{a}	12.82±7.98^{b}
Moisture	5.46±0.14^{a}	1.44±0.00^{c}	5.49±0.14^{a}	4.74±0.056^{b}
Ash	5.56±0.56^{c}	13.01±0.00^{a}	4.79±0.76^{c}	9.24±0.89^{b}

Note: Data presented as mean ± SD. Means in the same row marked with different superscripts indicate significant differences (p>0.05)

Table 3. Fatty acids (%) composition of male and female *Pangasius nasutus* liver at different age classes

Fatty acids	**Age (years)**							
	2		**3**		**4**		**5**	
	Male	**Female**	**Male**	**Female**	**Male**	**Female**	**Male**	**Female**
C16:0	27.9±0.01^{d}	28.2±0.01^{c}	26.4±0.03^{e}	25.7±0.01^{f}	39.33±0.06^{a}	37.43±0.33^{b}	27.34±0.10^{d}	28.56±0.19^{a}
C18:0	17.81±0.06^{b}	18.41±0.32^{a}	9.82±0.04^{e}	8.53±0.03^{f}	13.28±0.02^{c}	13.34±0.22^{c}	11.70±0.03^{d}	11.60±0.05^{c}
C20:0	2.62±0.04^{e}	2.72±0.21^{d}	5.15±0.00^{b}	5.46±0.00^{a}	2.93±0.08^{c}	2.76±0.12^{d}	2.32±0.02^{f}	2.74±0.01^{b}
C22:0	3.02±0.03^{c}	2.98±0.02^{d}	4.19±0.00^{a}	4.52±0.01^{a}	2.56±0.00^{b}	3.72±0.33^{b}	2.11±0.01^{e}	2.31±0.02^{c}
C16:1n-9	2.86±0.00^{b}	3.53±0.03^{a}	2.56±0.00^{b}	3.26±0.00^{a}	2.67±0.00^{b}	2.74±0.01^{b}	3.23±0.05^{a}	3.18±0.02^{a}
C18:1n-7	25.34±0.11^{b}	24.43±0.22^{d}	25.41±0.01^{c}	25.23±0.01^{c}	17.97±0.03^{e}	16.77±0.23^{f}	38.96±0.02^{a}	34.56±0.06^{b}
C18:2n-6	12.19±0.04^{c}	12.78±0.02^{c}	17.43±0.05^{a}	18.73±0.05^{a}	2.55±0.01^{d}	2.92±0.01^{d}	13.77±0.04^{b}	13.67±0.07^{b}
C22:2n-6	0.30±0.03^{c}	0.35±0.05^{c}	0.52±0.00^{c}	0.86±0.00^{c}	1.43±0.11^{a}	1.45±0.01^{a}	1.00±0.17^{b}	1.05±0.00^{b}
C20:4n-6	2.03±0.02^{c}	2.05±0.02^{c}	3.02±0.00^{b}	4.05±0.00^{a}	0.35±0.09^{e}	0.38±0.29^{d}	0.03±0.02^{f}	0.05±0.01^{f}
C18:3n-3	4.52±0.15^{b}	4.64±0.32^{b}	5.18±0.12^{a}	5.54±0.06^{a}	0.21±0.00^{d}	0.24±0.12^{d}	0.39±0.02^{c}	0.34±0.01^{d}
C20:3n-3	0.08±0.02^{c}	0.07±0.01^{c}	0.10±0.00^{c}	0.30±0.00^{b}	0.15±0.00^{b}	0.17±0.00^{c}	0.22±0.52^{a}	0.33±0.24^{a}
C20:5n-3	2.76±0.03^{c}	2.32±0.01^{d}	3.34±0.03^{b}	4.56±0.01^{a}	3.21±0.00^{b}	2.98±0.03^{c}	1.05±0.01^{e}	1.01±0.02^{e}
C22:6n-3	1.26±0.04^{a}	1.71±0.42^{a}	1.38±0.00^{a}	1.92±0.43^{a}	1.03±0.06^{a}	1.01±0.01^{a}	0.49±0.01^{b}	0.59±0.01^{b}
∑ n-6	12.49±0.54^{d}	13.13±0.13^{c}	17.95±0.31^{b}	19.59±0.31^{a}	3.41±0.30^{e}	3.72±0.33^{c}	13.80±1.03^{c}	13.72±1.03^{c}
∑ n-3	5.86±0.54^{b}	6.42±0.22^{b}	6.66±0.31^{a}	7.76±0.11^{a}	0.33±0.30^{e}	0.41±0.20^{e}	4.15±1.03^{c}	3.97±1.03^{d}
n-3/n-6	0.45±0.01^{a}	0.49±0.02^{a}	0.38±0.00^{a}	0.40±0.00^{a}	0.10±0.00^{c}	0.11±0.00^{c}	0.30±0.01^{b}	0.29±0.01^{b}
∑ SFA	53.44±0.16^{a}	53.31±0.34^{a}	47.15±0.05^{b}	44.21±0.25^{b}	55.54±0.16^{a}	53.53±0.11^{a}	43.47±0.11^{c}	37.77±0.14^{d}
∑ MUFA	27.95±0.11^{c}	28.01±0.14^{b}	28.17±0.00^{b}	28.54±0.12^{b}	21.63±0.04^{d}	20.51±0.07^{e}	42.24±0.01^{a}	42.77±0.01^{a}
∑ PUFA	18.37±0.13^{d}	19.55±0.23^{c}	24.66±0.06^{b}	27.05±0.21^{a}	3.34±0.23^{g}	4.16±0.11^{f}	17.95±0.12^{e}	17.69±0.15^{e}

Note: Results are presented as a percent of total FAME content (%). Results are presented as mean ± SD. n=3 Means in the same row with different superscripts are significantly different (p<0.05)

Fatty acid (%) composition of liver and gonad of *P. nasutus*

The fatty acid (%) composition of male and female *P. nasutus* livers varied significantly across different age classes, as shown in Table 3. A comparative analysis of key fatty acids for fish reproductive physiology such as Arachidonic acid, C20:4n-6; Eicosapentaenoic acid, C20:5n-3; and Docosahexaenoic acid, C22:6n-3 are presented in Figure 5. Arachidonic acid, C20:4n-6 levels peaked in 3-year-old females (4.05±0.00%) and declined markedly in older fish, with the lowest levels observed in 5-year-olds (0.03±0.02% to 0.05±0.01%). Similarly, Eicosapentaenoic acid, C20:5n-3 was highest in 3-year-old females (4.56±0.01%) and generally decreased with age, reaching the lowest levels in 5-year-olds (1.01±0.02% to 1.05±0.01%). While, Docosahexaenoic acid, C22:6n-3 content remained relatively stable across ages, with slight variations but no consistent age-related trend observed. Total n-6 fatty acids (n-6) were highest in 3-year-olds (19.59±0.31% in females) and lowest in 4-year-olds (3.41±0.30% to 3.72±0.33%). Total n-3 fatty acids (n-3) showed a similar pattern, peaking in 3-year-olds (7.76±0.11% in females) and decreasing notably in 4-year-olds (0.33±0.30% to 0.41±0.20%). The n-3/n-6 ratios were highest in 2-year-olds (0.45±0.01 to 0.49±0.02) and lowest in 4-year-olds (0.10±0.00 to 0.11±0.00).

The fatty acid (%) composition of male and female *P. nasutus* ovary and testis varied significantly across different age classes, as shown in Table 4. Testes generally contained higher proportions of C18:0, C22:0, C18:2n-6, and C20:4n-6 compared to ovaries. Conversely, ovaries showed higher levels of C18:1n-7, C18:3n-3, C20:3n-3, and C20:5n-3 across different age groups. The plot (Figure 6) shows that arachidonic acid (C20:4n-6) increases from age 2 to 4 in ovaries, peaking at age 4, then slightly decreases, while in testes, it peaks at age 3 before declining. Eicosapentaenoic acid (C20:5n-3) rises significantly from age 2 to 3 in both ovaries and testes, then declines, indicating its importance in early reproductive stages. Docosahexaenoic acid (C22:6n-3) increases from age 2 to 4 in ovaries, peaking at age 4, then drops significantly at age 5, while in testes, it peaks at age 3 and decreases thereafter.

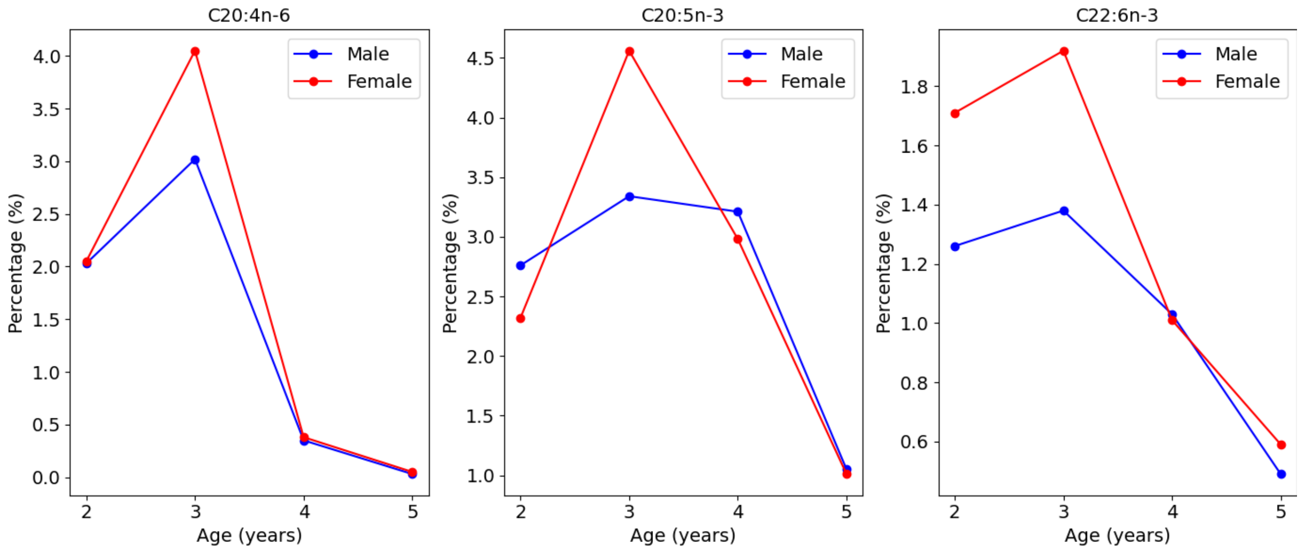

Figure 5. Comparative analysis of key fatty acids (arachidonic acid, C20:4n-6; eicosapentaenoic acid, C20:5n-3; and docosahexaenoic acid, C22:6n-3) in the liver of male and female *Pangasius nasutus* across different age classes

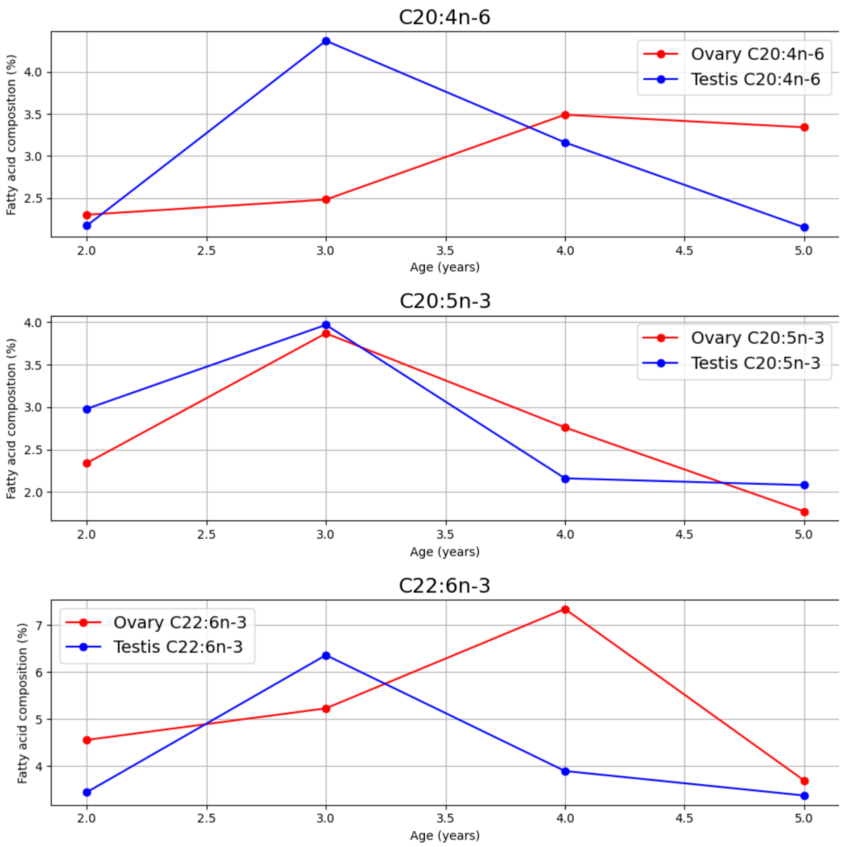

Figure 6. Comparative analysis of key fatty acids (arachidonic acid, C20:4n-6; eicosapentaenoic acid, C20:5n-3; and docosahexaenoic acid, C22:6n-3) in the ovary and testis *Pangasius nasutus* across different age classes

Table 4. Fatty acids composition of *Pangasius nasutus* ovary and testes at different ages

	Ovary				Testis			
	Age (years)				Age (years)			
Fatty acids	**2**	**3**	**4**	**5**	**2**	**3**	**4**	**5**
C16:0	18.90±0.05[b]	22.27±0.27[a]	20.27±0.27[a]	19.83±0.08[b]	13.45±0.56[d]	14.88±0.02[c]	24.49±0.27[a]	19.43±0.22[b]
C18:0	16.30±0.04[a]	6.00±0.15[c]	6.00±0.15[c]	15.03±0.07[b]	11.19±0.88[d]	22.69±0.04[a]	16.93±0.86[c]	18.66±0.22[b]
C20:0	3.87±0.01[b]	3.91±0.06[b]	10.98±2.15[a]	3.15±0.02[b]	1.72±0.35[b]	0.73±0.01[c]	1.56±0.04[b]	1.14±0.11[b]
C22:0	7.04±0.03[c]	8.26±0.13[b]	15.22±0.83[a]	7.71±0.03[c]	5.59±0.14[c]	10.00±0.03[a]	6.26±0.08[b]	6.82±0.10[b]
C16:1n-9	0.66±0.01[b]	1.07±0.02[a]	0.05±0.01[d]	0.98±0.02[c]	1.62±0.23[a]	1.47±0.00[b]	1.61±0.03[a]	1.08±0.02[b]
C18:1n-7	21.09±0.09[a]	21.67±0.88[a]	12.70±0.58[c]	20.59±0.20[b]	14.95±0.51[c]	9.98±0.11[d]	17.63±0.42[b]	19.31±0.28[a]
C18:2n-6	12.71±0.02[c]	13.66±0.07[b]	12.42±0.38[d]	14.07±0.03[a]	15.55±0.65[a]	9.66±0.01[d]	10.26±0.16[c]	14.11±0.15[b]
C22:2n-6	0.03±0.00	0.16±0.03	0.10±0.02	0.02±0.00	0.55±0.05[a]	0.08±0.01[b]	0.10±0.01[b]	0.08±0.03[b]
C20:4n-6	2.30±0.02[c]	2.48±0.01[c]	3.49±0.01[a]	3.34±0.01[b]	2.17±0.00[c]	4.37±0.01[a]	3.16±0.00[b]	2.15±0.03[c]
C18:3n-3	0.37±0.01[b]	0.45±0.00[b]	1.20±0.10[a]	0.45±0.01[b]	1.06±0.12[a]	0.18±0.02[b]	0.26±0.01[b]	0.34±0.06[b]
C20:3n-3	13.22±0.15[c]	15.09±0.24[b]	12.06±1.18[a]	13.26±0.07[c]	11.29±0.80[d]	22.59±0.21[a]	16.52±0.16[b]	13.38±1.04[c]
C20:5n-3	2.34±0.01[c]	3.87±0.01[a]	2.76±0.01[b]	1.77±0.01[d]	2.98±0.01[b]	3.97±0.01[a]	2.16±0.01[c]	2.08±0.03[c]
C22:6n-3	4.56±0.02[c]	5.23±0.08[b]	7.34±0.22[a]	3.70±0.01[d]	3.45±0.67[b]	6.36±0.06[a]	3.90±0.01[b]	3.38±0.09[b]
∑ n-6	13.01±0.02[c]	14.14±0.00[b]	16.01±0.10[a]	14.41±0.03[b]	16.1±0.05[a]	10.11±0.01[c]	10.52±0.11[c]	14.19±0.02[b]
∑ n-3	18.15±0.20[c]	20.77±0.01[b]	23.36±0.02[a]	17.41±0.07[d]	15.8±0.02[d]	29.13±0.02[a]	20.68±0.00[b]	17.18±0.01[c]
n-3/n-6	1.40±0.03[b]	1.74±0.02[a]	1.46±0.00[b]	1.20±0.00[c]	0.98±0.01[d]	2.88±0.00[a]	1.97±0.00[b]	1.21±0.10[c]
∑ SFA	46.11±0.03[c]	40.44±0.40[d]	52.03±1.02[a]	45.72±0.18[b]	31.95±0.22[c]	48.30±0.06[a]	49.24±0.37[a]	46.05±0.43[b]
∑ MUFA	21.75±0.05[b]	22.77±0.69[a]	12.91±0.48[c]	21.59±0.17[b]	16.57±0.28[c]	10.62±0.07[d]	18.40±0.36[b]	20.54±0.24[a]
∑ PUFA	31.16±0.09[b]	34.91±0.29[a]	34.61±0.88[a]	31.70±0.02[b]	31.90±0.23[b]	39.24±0.07[a]	31.20±0.00[b]	31.37±0.65[b]

Note: Results are presented as a percent of total FAME content (%). Results are presented as mean ± SD. n=3 Means in the same row for ovaries and testes with different superscripts are significantly different ($p<0.05$)

Discussion

Biological indices

In this study, the composition of females was observed to be higher in the sampled population than the males (1.5:1) which completely deviated from the hypothetical distribution of 1:1 in normal population distribution. Sex ratio differences within a population can be influenced by a combination of factors, including mortality, growth, behavioral differences between males and females, higher predation rates of one sex over the other, and selective harvesting can affect sex ratio (Hagen et al. 2022; Bautista et al. 2023). In the wild, any deviation from the normal ratio of 1:1 is linked to certain factors such as mortality or even the effects of environmental changes (Hagen et al. 2022). However, in our study, no definite conclusion is reached as our samples were based on cage-cultured stock, which could be prone to selective stocking of specific sex over the other. *P. nasutus* was observed to be sexually matured in the cage culture system in this study at an average body weight of 1099.27±21.5 g and at a total length of 48.36±0.45cm and at 3 years old. Age 3 is identified as the age of sexual maturity, based on indicators such as GSI and elevated levels of key fatty acids responsible for reproductive development found in 3-year-old fish. To the best of our knowledge; this has not been reported in any literature for *P. nasutus*. In contrast to our findings, two catfish closely related to the species in our study; stripped catfish, *P. hypophthalmus* and African catfish, *Clarias gariepinus* (Burchell, 1822) in the wild were reported to be sexually matured at a TL of 21.32 cm and 32.80 cm, respectively (Hossain et al. 2016). Though in captive condition *P. hypophthalmus* was reported to be sexually matured at a BW of ≥989.70±59.98 g and a TL of ≥46.99±1.04 cm and at the age of 13-14 months (Kabir et al. 2019), which is earlier compared to *P. nasutus* in captive condition. Pangas catfish, *Pangasius pangasius* (Hamilton, 1822) recorded its first sexual maturity at the age of 4.5+ years old and a BW of 4400±0.8 g and a TL of 71.8±3.1 cm (Rahman et al. 2020). The age, BW, and TL at maturity compiled from these reports show that the sexual maturation of fish is species-specific and might be influenced by genetic factors, environmental conditions or differences in aquaculture practices in captive conditions. Genetic factors influence growth and reproductive strategies, while environmental conditions such as temperature and food availability affect growth rates and maturation timing. Differences in aquaculture practices, such as feeding regimes and stocking densities, further contribute to these variations. Precise aging techniques might reveal more nuanced differences in maturation timing (Mobley et al. 2021). Additionally, cage-cultured conditions provide stable food supply, reduced predation risk, and controlled water quality, leading to enhanced growth rates and potentially earlier or more consistent maturation patterns compared to wild environments, were fluctuating conditions and predators impact growth and reproductive indices (Smith et al. 2024).

In this study, differences in age structure were observed, revealing a shift in the demographic makeup of *P. nasutus* in the sampled population, with 3-year-old and 5-year-old fish dominating in numbers. The existence of older individuals within the population can have a positive impact on productivity, particularly in terms of recruitment (Ohlberger et al. 2022). This finding suggests positive reproductive potential for this fish species, as age 3 is identified as a reproductively viable age based on other observed indicators. However, it is important to note that our study's results could be strengthened through a more comprehensive examination of the wild population, as our sample collection was limited to cage-bred animals and did not cover an extended period.

In the current study, it was observed that the weight, length, and condition factor of *P. nasutus* in cage culture increased with age, with no significant difference between males and females. These findings align with Yimer et al. (2024). Sex and age seem to be a factor of influence on the GSI of *P. nasutus*, as females in this study recorded higher GSI than males and 2 years old fish recorded the lowest GSI which is an indication that at this age *P. nasutus* might not be sexually matured. In comparison, 3-year-old fish showed higher GSI which could probably represent the reproductive age for *P. nasutus* in the captive condition. In comparison, at 4 years old and 5 years old the GSI begins to decrease though the weight of the fish continues to increase. Similar results were reported for armored catfish, *Loricariichthys melanocheilus* Reis & Pereira, 2000 (Zardo and Behr 2015). The decrease in the GSI of *P. nasutus* at age 4 and age 5 in this study could be that they are at the spent stage, which demonstrates the inactive reproductive stage. Consequently, considering the age of fish, older female fish not only have higher GSI than younger fish, but they also produce larger eggs and larvae that survive and grow faster and are more resistant to starvation (Hixon et al. 2014)

In our study, the coelomic fat index of *P. nasutus* was higher in males than females across different ages. This outcome may be linked to the fish's capacity to mobilize and utilize reserve fat content for reproductive purposes. Notably, the Gonadosomatic Index (GSI) of female *P. nasutus* was higher than that of males in this study, possibly indicating female utilization of reserve fat for reproductive activities. The behaviour of CFI suggests its involvement in ovarian development in *P. nasutus* females, with intense uptake during gonadal maturation (Murgas et al. 2018). The disparity in CFI between male and female *P. nasutus* in our study may be attributed to the less complex nature of male gonadal maturation and gamete discharge, primarily involving cell division. In contrast, females undergo more intricate processes, such as vitellogenin production (Ramos-Júdez et al. 2023). Similarly, Abdo et al. (2018) reported a lower coelomic fat index in *Prochilodus hartii* Steindachner, 1875 with gonadal development at Stage 3 and Stage 4 compared to those at stage 1 and stage 2. Age-wise CFI of *P. nasutus* varies between ages as the more viable reproductive age recorded a lower coelomic fat index than the immature 2-year-old fish and spent 4- and 5-year-old fish with higher CFI content. The difference in Coelomic Fat Content (CFI) between *P. nasutus* of different ages is likely due to the lack of reproductive activity at age 2, compared to ages 4 and 5 when the fish are recovering CFI after reproduction, aided by abundant food supply.

Biochemical analysis

The proximate analysis of *P. nasutus* gonads in both males and females in this study revealed a high protein content, underscoring the importance of protein in gonad development and egg production. The accumulation of protein in the testes and ovaries indicates its involvement in vitellogenesis during female gonad development and cell division in male fish (Zhou et al. 2017). Li et al. (2014) observed an increase in protein content in the eggs of wild Chinese sturgeon compared to those of matured cultured Chinese sturgeon, suggesting a regulatory system that may impact reproductive success negatively. In our study, the lipid content in the male liver of *P. nasutus* was higher than in females, potentially reflecting differing energy demands between male and female *P. nasutus* and their utilization of stored energy during the reproductive process. Additionally, the GSI of females was higher than that of males, highlighting a greater demand for lipids during female gonadal development compared to males. Furthermore, the lipid content in *P. nasutus* male gonads (testes) exceeded that in female gonads (ovaries), supporting the idea that females may utilize a higher proportion of their lipid reserves for gonad development, potentially leading to lipid depletion. This aligns with findings in the female Chinese sturgeon, *Acipenser sinensis* Gray, 1835, where lipid levels in gonads steadily increased from stage 2 to 3, then gradually declined with sex maturation, a phenomenon attributed to the energy-intensive process of egg maturation, with lipids being the primary energy source via β-oxidation (Song et al. 2014). High protein content in the gonads and differing lipid content between male and female livers and gonads correlate with reproductive status and health in *P. nasutus*, as higher protein levels typically indicate active gametogenesis and optimal reproductive health, while lipid content, including essential fatty acids like n-3 and n-6 PUFAs, is crucial for energy storage and hormone synthesis, reflecting distinct reproductive roles and energy demands between sexes (Zhou et al. 2022). Although specific thresholds are not universally established, high Gonadosomatic Index (GSI), balanced lipid content, and elevated protein levels collectively point to a robust reproductive condition.

Moreover, in the maturation phase, mugilid *Liza aurata* utilized dietary lipids and body reserves, including the liver and muscle, to support gonadal nourishment (Quirós-Pozo et al. 2023). The moisture content in the gonads in our current study was found to be lower compared to the liver. This aligns with findings in the Chinese sturgeon, *Acipenser sinensis* Gray, 1835, where ovaries showed low moisture content during stages 3 and 4 of development (Zhou et al. 2017). The low moisture content observed in this study suggests that more energy reserves and nutrients are required for gonadal development in *P. nasutus*, and the significantly higher ash content in the gonads compared to the liver in both males and females indicates that the gonads store more energy and nutrients for maturity, while the liver functions to deliver these reserves to the gonads for development and maturation, consistent with findings in bream where testes and ovaries also showed higher ash content than the liver and muscle (Payuta and Flerova 2021).

Fatty acids composition

Fatty acids play crucial roles in providing metabolic energy for various physiological processes in fish, including growth and reproduction (Torsabo et al. 2022). Long-Chain Polyunsaturated Fatty Acids (LC-PUFA), such as arachidonic acid (C20:4n-6), Docosahexaenoic Acid (DHA: 22:6n-3) and Eicosapentaenoic Acid (EPA: 20:5n-3), are essential for nutritional influence, particularly during sexual maturation and gonad development (Kottmann et al. 2020; Bhat et al. 2022). In our study C20:4n-6 showed an increasing trend from age 2 to 4 in ovaries, peaking at age 4, and then slightly decreasing, while in testes, it peaks at age 3 before declining (Figure 3). This pattern suggests that arachidonic acid plays a crucial role in the reproductive maturation of fish, as it is a precursor for prostaglandins, which are vital for ovulation and spermatogenesis (Shen et al. 2023). Studies in other fish species such as *Pangasianodon hypophthalmus* (Sauvage, 1878) (Kabir et al. 2019), Blue gourami, *Trichopodus trichopterus* (Pallas, 1770) have shown similar patterns where elevated levels of arachidonic acid are linked to reproductive activities. Eicosapentaenoic acid (C20:5n-3) rises significantly from age 2 to 3 in both ovaries and testes, then declines, indicating its importance in early reproductive stages (Sharma et al. 2022). This is consistent with findings in other aquatic species, where C20:5n-3 is essential for the development of reproductive tissues and successful spawning (Torsabo et al. 2024). The decline in later stages suggests a reduced demand for this fatty acid as the fish reach reproductive maturity. Docosahexaenoic acid (C22:6n-3) increases from age 2 to 4 in ovaries, peaking at age 4, then drops significantly at age 5, while in testes, it peaks at age 3 and decreases thereafter. DHA is crucial for the structural integrity of cell membranes in reproductive tissues, and its high levels during critical reproductive periods support the development and function of these tissues (Mejri et al. 2017). The observed decline after the peak suggests that as the fish mature and the reproductive tissues are fully developed, the demand for DHA decreases. The influence of Polyunsaturated Fatty Acids (PUFA) in reproductive processes has been documented in various fish species, including common carp (*Cyprinus carpio* Linnaeus, 1758) (Xu et al. 2017), zebrafish (*Danio rerio* (Hamilton, 1822)) (Diogo et al. 2015), and European eel (*Anguilla Anguilla* (Linnaeus, 1758)) (Kottmann et al. 2020).

Three-year-old fish have the highest levels of the key fatty acids in both the liver, ovary, and testis including arachidonic acid (C20:4n-6), eicosapentaenoic acid (C20:5n-3), and total n-6 and n-3 fatty acids. The abundance of these fatty acids in three-year-old fish can be attributed to the fact that this age likely represents a peak in reproductive maturity and activity. At this stage, fish are in optimal health and reproductive condition, requiring higher levels of these fatty acids to support reproductive processes, cell membrane integrity, and overall physiological function. The subsequent decline in fatty acid levels in older fish may reflect a natural reduction in reproductive activity and metabolic demands as fish age. These findings highlight the species-specific variations in the utilization of LC-PUFA and the importance of specific types of PUFA for the reproductive performance of fish. Grasping these subtleties can offer valuable perspectives into dietary needs and nutritional strategies necessary for optimizing fish reproductive activities, ultimately contributing to the development of sustainable aquaculture practices.

In conclusion, the current investigation indicates that the age of sexual maturation for both male and female *P. nasutus* is 3 years. Reproductive indicators suggest that at this age, females exhibit better sexual maturity compared to males. Reproductive indicators such as the Gonadosomatic Index (GSI), biochemical contents of proteins and lipids, and key fatty acids involved in fish reproductive physiology were found to be optimal in *P. nasutus* at 3 years old, indicating sexual maturity. The analysis of essential fatty acid accumulation in *P. nasutus* organs underscores a significant need for n-3 and n-6 PUFA in reproductive activities. Furthermore, the study recommends a thorough understanding of the dietary requirements for *P. nasutus* broodstocks, particularly the optimal combination of lipids and essential fatty acids, as these factors significantly influence gonad development. This information can be crucial in addressing challenges associated with gonad development in captivity. The findings stress the importance of emphasizing nutrition, lipid supply, and a balanced dietary fatty acid profile for farmed *P. nasutus* broodstocks to ensure adequate lipids and essential fatty acids for gonadal development. The study provides foundational data for further research into the reproductive physiology of captive *P. nasutus*. Future studies should compare sex differences in maturity, optimize dietary lipids and fatty acids, explore environmental impacts on reproduction, develop non-invasive health monitoring methods, and investigate genetic markers for superior traits. Additionally, they should examine the effects of captive breeding on genetic diversity and reproductive health for sustainable breeding and conservation.

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