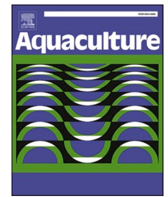
Aquaculture 576 (2023) 739832

Contents lists available at ScienceDirect

Aquaculture

journal homepage: www.elsevier.com/locate/aquaculture



An insight into milt antioxidant, fatty acid, amino acid composition and testis histology of an anadromous euryhaline fish *Tenualosa ilisha,* Ham. 1822 for its conservation and aquaculture perspectives

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ARTICLE INFO

Keywords:
Tenualosa ilisha
Antioxidants
Amino acids
Fatty acid
Histology

ABSTRACT

Hilsa, *Tenualosa ilisha,* Ham. 1822*,* is the prime table fish and important commercial fish in the Indo-Pacific region. However, the species has recently declined drastically due to various factors. Our earlier studies recorded a significant association between the breeding season and its migration. Studies on milt and its biochemical properties are essential to ensure its successful conservation and artificial breeding. Here, milt samples of the hilsa of varied sizes were evaluated for their antioxidant property, fatty acid and amino acid content, and the histology of the testis. Analysis of milt antioxidants was done using free radicals DPPH, FRAPS & ABTS methods and compared with the free-radical quenching properties of the synthetic antioxidants. High performance liquid chromatography (HPLC) and gas chromatography and mass spectrometry (GC/MS) determined the composition of amino acids and fatty acids, respectively. The highest and lowest antioxidant activity was exhibited by hilsa milt samples M3 (b.wt 358.6 ± 0.75 g) i.e. 84.59 ± 0.48% and M12 (b.wt 168.35 ± 0.12 g) i.e. 27.53 ± 3.59%. Most notably, the antioxidant activity was found to follow a body weight gradient. Omega (ω)-3 polyunsaturated fatty acids (PUFAs), docosahexaenoic acid (C22:6, DHA) and eicosapentaenoic acid (C20:5, EPA) were the predominant PUFAs and among the amino acids, arginine (0.70 g/100 g) was present in the highest quantity followed by glutamic acid (0.14 g/100 g) and proline (0.14 g/100 g). The histology study of the testis reveals the changes in the cells during the process of spermatogenesis. Histological images demonstrated the different stages of testis maturation of males sampled at the spawning sites. This baseline information generated is of its first kind in hilsa, a transboundary migratory fish in South Asian countries supporting conservation and artificial breeding for commercial production.

1. Introduction

The hilsa shad, *Tenualosa ilisha* is a significant transboundary migratory economic fish of the Indo-Pacific region. The species is found in marine, estuarine, and riverine waterways, and it has a wide range of habitats. It spends most part of its life cycles at sea but migrates to freshwater rivers for spawning; it has a great nutritional value and contains omega (ω)-3 polyunsaturated fatty acids (ω-3 PUFAs), docosahexaenoic acid (DHA), and eicosapentaenoic acid (EPA) (Mohanty et al., 2012). However, from a fisheries perspective, the majority of counts are captured by Bangladesh, Myanmar, and India. The fish is found in the Indian riverine habitat in eastern and western India namely the lower stretch of the Ganga, Hooghly Rupnarayan, Bhagirathi, Godavari, Brahmaputra, Tapti, Narmada and other coastal rivers (Bhaumik, 2012). The hilsa production (~ 80%) of India is from the Ganga-Brahmaputra-Meghna (GBM) basin of West Bengal. Hilsa migrates towards freshwater regions periodically for spawning. The peak time for freshwater migration of the fish starts from in July and continues up to November. A vital correlation between the season of migration and the species' breeding season was noticed (Ahsan et al., 2014). During spermatogenesis, spermatozoa are produced in the fish sperm and seminal plasma is developed by the gonad and spermatic ducts (Krol et al., 2006). Fertilization ability depends on the concentration and motility of spermatozoa (Cejko et al., 2008). In the external

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https://doi.org/10.1016/j.aquaculture.2023.739832
Received 23 December 2022; Received in revised form 9 June 2023; Accepted 22 June 2023
Available online 24 June 2023
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fertilization process, the movement of the spermatozoa begins when it comes in contact with water. Fish sperm study offers many benefits and provides adequate information on motility and fertility rates (Cabrita et al., 2011; Martínez-Páramo et al., 2017). The preservation and potentiality of hilsa milt are vital for its successful artificial propagation. The fish milt's fatty acid and amino acid content marks the performance traits and fertility. Hilsa is a migratory fish that moves quickly and perishes as soon as it is caught. As a result, when enough adult females are available, mature males are not available simultaneously. In future, cyropreservation like techniques of semen should support the conservation strategies. For development of cryopreservation methods basic data on semen biology are necessary. In light of the observed decline of hilsa in the Ganga and the pennisular river systems, measures have been taken to enhance its availability in the river system, with the aim of preserving its population. Cryopreservation like technique plays a key role in this aspect, which would subsequently increase the breeding efficiency of the female hilsa as and when found in the riverine ecosystem in course of migration. However, this technique might compromise sperm quality. Hence, we have tried to characterize sperm in terms of amino acid and fatty acid content, free radical scavenging potential, and histological modificatiions of testis to evaluate sperm quality of hilsa for the river stretch. Amino acids are involved in a variety of biological processes and serve as a precursor for lipids, steroids, glycogen, and nucleotides (Wu, 2009). Intercellular matrix protein hydrolysates yield amino acids or short peptides that serve as indicators of the timing of sperm maturation. (Kawabata et al., 1992). Additionally, they provide sperm with substrates easily oxidised (Mann and Lutwak-Mann, 1981). The proliferation and differentiation of sperm cells in fish are correlated with the proteins rich in amino acid called protamines that are found inside sperm nuclei (Balhorn, 2007), thereby indicating an important role of amino acids in the spermatogenesis process (Wu, 2009). The growing demand for animal protein has appraised aquaculture practices more intensive. The cultured fishes are exposed to frequent transportation stress, temperature fluctuations, and hence oxidative stress (Shiva et al., 2011). Previous studies have recorded that fish germ cells tend to lose antioxidant capacity due to oxidative stress (Pan et al., 2019; Félix et al., 2020; Bibi et al., 2021). The scanty antioxidants render the spermatozoa reproductive potential to deteriorate drastically. As a matter of fact, the control of fish sperm quality is a major aspect of a successful aquaculture practice. It enhances the production of commercial fish species as well as the viability of the newer species (Wischhusen et al., 2019). The antioxidant quantification of the sperm is a direct measurement of the male brooder's performance and a qualitative assessment of reproductive success and broodstock improvement (Len et al., 2019; Wang et al., 2022). The current research work aims to investigate the total antioxidant content, fatty acid and amino acid composition of mature hilsa milt. The histological study is used to understand spermatogonial development.

2. Material and methods

2.1. Sampling sites

A total of 31 numbers of live male hilsa with an average weight of 219.98 g and an average length of 27.63 cm were collected from Kalinagar (22° 26′ 25.49″ N;88° 6′ 9.86″ E) Naldari (22° 23′ 3.62″ N;88° 6′ 56.19″ E), Burul (22° 21′38.67″ N;88° 6′16.60″ E), Padmapur (22° 20′52.58″ N;88° 6′ 9.85″E), Kantakhali (22° 19′ 45.13″ N;88° 6′ 6.59″ E), and Falta (22° 17′19.28″ N;88° 6′ 9.86″ E) of Godakhali, South 24 Parganas (Fig. 1) district of West Bengal. Live hilsa was placed on a plane surface to collect the milt. The length and weight of each fish were measured before the milt collection. The fish count from which milt was procured was narrowed down from 31 to 8 which were fully matured with oozing out semen. This also assured contamination free milt samples. The genital papilla was cleaned and dried with paper towels. After that, the abdominal part was gently massaged in the anteroposterior way to eject the milt. The total

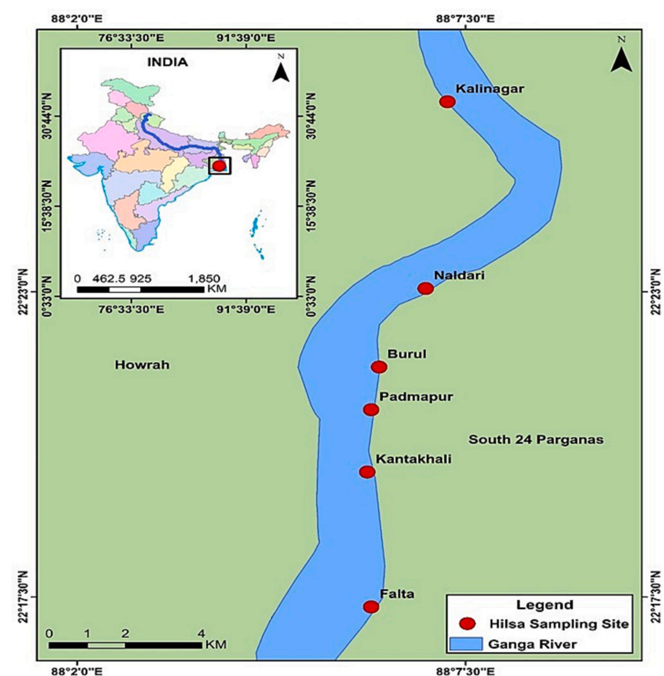

Fig. 1. GIS map of hilsa sampling sites.

milt samples collected from 8 fish was approximately 1.08 ml with 135 μl of milt collected from each of eight fish. The semen sample was collected in 2 ml cryo chill vial and divided into 5 subsamples each having approximately 27 μl of semen (total volume of one sample is approximately 135 μl). A cryopreservation like technique was used for sample stabilization within 1 h of the milt sample collection with an extender solution made in double distilled water (100 ml) containing chlorides of sodium (750 mg), potassium (70 mg), calcium (20 mg), sodium bicarbonate (20 mg), and glucose (10 mg) with DMSO and glycerol as a cryoprotectant reagent (Routray et al., 2007). The cryopreservation solution contained the extender and cryoprotectant ratio at 13:1.5:1.5 *v*/v. Each milt sample was diluted 3 times using the cryopreservation solution. Thereafter, all the vials were kept in liquid nitrogen for antioxidant, fatty acid, and amino acid studies. After milt collection, the fish were euthanized with MS-222 (3-aminobenzoic acid ethyl ester methane sulfonate, Sigma-Aldrich) (Ganguly et al., 2020), dissected and testis was preserved in Bouin's solution for the histological study. The length-weight measurements of the fish gonad were noted and the gonado-somatic index was calculated using the formula; GSI = (GW/W) × 100, where W represents the fresh body weight of the fish and GW, the gonad weight, both in gm (Wootton, 1998). The relative condition 'Kn' were calculated following the formula Kn = W/W1, where W = recorded body weight and W1 = calculated weight for the observed length (LeCren, 1951).

2.2. Determination of milt antioxidant

The hilsa milt antioxidant potential sampled from each weight group was evaluated by radical scavenging potential using commercial free radicals viz.; DPPH, ABTS, and FRAP. The results were compared with standard antioxidants and expressed in mean ± SE.

2.2.1. Free radical scavenging assay using 2, 2-diphenyl-1-picrylhydrazyl (DPPH)

The scavenging property of DPPH• π-radical by the hilsa milt samples was evaluated following the procedure mentioned by González-Palma et al. (2016) with a few modifications. In brief, the sample reaction mixture contained 10 μl EtOH, 280 μl of freshly prepared DPPH

solution (0.1 mM) and 10 μl hilsa milt sample. The control was prepared with 20 μl methanol and DPPH solution. The absorbance was determined at 517 nm using 96-well microplate, following 30 mins incubation in dark. The results were expressed in DPPH radical scavenging (%) and compared with that of butylated hydroxy toluene (BHT) (Sigma Aldrich).

2.2.2. Antioxidant assay using 2, 2′-azinobis-[3-ethyl-benzothiazoline-6-sulfonic acid] (ABTS)

The $ABTS^{+}$ antioxidant capacity was evaluated as per the protocol mentioned by Arnao et al., 2001. In brief, the reaction mix consisted of 7 mM ABTS solution (Sigma Aldrich) and 2.4 mM of potassium persulfate (Sigma Aldrich) in equal quantities. The diluted solution was added to the sample and control vials. The sample contained 10 μl methanol, 280 μl ABTS solution, and 10 μl of hilsa milt sample. The control was prepared with 20 μl methanol and 280 μl ABTS solution. $ABTS^{\bullet+}$ generated from ABTS in the presence of antioxidants were monitored by absorbance at 734 nm. ABTS inhibition (%) of the samples was compared with that of BHT.

2.2.3. Antioxidant assay using ferric reducing antioxidant potential (FRAP)

The antioxidant property using (FRAP) protocol (Sigma Aldrich) works on the principle of the reduction of Fe^{3+} (ferric) to Fe^{2+} (ferrous) by antioxidants present in the sample. The assay was done following the instructions given by Risso et al., 2021. FRAP assay is done at low pH based on an electron transfer mechanism with the formation of aryloxy radical.

The working reagent was prepared with sodium acetate buffer (300 mmol/L), 2,4,6-Tris(2-pyridyl)-*s*-triazine (TPTZ) (10 mM) solution prepared by dissolving 0.31 g of TPTZ in 100 ml of 40 mM HCl and 20 mM Iron (III) chloride hexahydrate ($FeCl_3.6H_2O$) dissolved in 10 ml millipore water. Since the sperm sample had a turbid color, the working solution was prepared both with and without TPTZ solution. The standard curve was prepared with 2000 μM Iron (II) sulfate heptahydrate ($FeSO_4.7H_2O$) from 0 ppm to 2000 ppm. The blank was prepared with 10 μl of millipore water and 290 μl working solution with TPTZ; the sample blank with 10 μl of millipore water and 290 μl working solution without TPTZ and sample solution was prepared with 10 μl of sample and 290 μl working solution with TPTZ. The absorbance was taken at 37 °C for 15 mins followed by 5 mins at room temperature. The absorbance of the Fe^{2++} TPTZ complex was read at 593 nm.

$$\text{Absorbance of } FeSO_4.7H_2O = \text{Absorbance of } FeSO_4.7H_2O \text{ recorded} - \text{Blank absorbance}$$

$$\text{Sample absorbance} = \text{Sample absorbance recorded} - \text{Sample blank absorbance}$$

When Y is replaced with sample absorbance, the calculated X value of the standard curve equation represents the Fe^{2+} in μM/L in the analyzed sample. Finally, the Fe^{2+}/ml of the sample were calculated and the results are expressed as equivalent μmoles of $Fe^{2+/}$ml of the sample.

2.3. Estimation of amino acid

The hilsa milt amino acid content was performed following Ishida et al. (1981). Briefly, the protein present in the sample was hydrolyzed using 6 N HCL under anaerobic environment for 12h at 110 °C. The hydrolyzed milt samples were then neutralized by NaOH (6 N). Thereafter, the samples were derivatized with a fluorescence kit (AccQ-Fluor Reagent, WAT052880, Waters). The HPLC (1525, Waters) analysis of the samples were done using a C-18 reverse phase column attached with a fluorescence detector (2475, Waters). The amino acids present in the sample were subsequently identified and quantified on comparing with respective amino acid standards (WAT088122, Waters). The sum of all data has been reported as mean ± SE ($n = 6$).

2.4. Estimation of fatty acid

The fatty acid composition were analyzed by gas chromatography and mass spectrometry (GC/MS, ITQ-900). Initial total lipid extraction was done in accordance with Folch et al. (1957). Briefly, using a motor pestle and chloroform: methanol (2:1) organic solvent mixture, samples were homogenised and then filtered in vacuum condition. The total lipids present in the chloroform fractions were obtained and made 10 ml concentrate using a vacuum rotary evaporator. Following Metcalfe et al. (1966), 150 mg of comparable fat was obtained from the total fat extract for transforming it to methyl esters of fatty acid (FAME). GC/MS consists of a GC (Trace GC Ultra, Thermo Scientific) with a TR-FAME column (30 m × 0.25 mm i.d × 0.25 μm) and MS detector (ITQ 900, Thermo Scientific). The respective fatty acids were separated at 50 °C oven temperature kept for 1 min initially, then raised to 150 °C from 50 °C at 20 °C/min and was held at 150 °C for 15 min. Lastly, there was an increase in temperature from 150 to 240 °C with an increment of 20 °C per min and hold for 3 min. Helium was employed as the carrier gas with 1 ml/min column flow. The MS parameters were: ionisation voltage of 70 eV, 45–600 *m/z* mass range, and scan time is equivalent to GC run time. The identification and quantification of the fatty acids was done on comparing to known standards (Supelco 37 Component FAME Mix, CRM47885) assayed using the above stated protocol and confirmed using NIST Library search (Version 2.2, 2014).

2.5. Gonado-somatic index

The calculation of the gonado-somatic index (GSI) is given as the gonad mass as a percentage of the total fresh body mass. The parameter draws light on the fish breeding biology. The weight of each individual fish was recorded, and the male and female fish gonads were carefully removed and weighed in an electronic balance after excess moisture was removed with blotting paper. Using the formula, gonado-somatic index, the method of Lowerre-Barbieri et al. (2011) and Calagui (2020) were used to calculate the percentage gonad weight or gonado-somatic index.

2.6. Age determination

Hilsa age-related to the length was interpreted based on the earlier study (De, 1980) and the relationship was studied by regression analysis.

2.7. Histology

The testis of mature hilsa was collected and preserved in Bouin's solution for histological study and processed through graded alcohol series. Thereafter, the samples were rinsed with chloroform, and finally entrenched in paraffin wax. The sections were sliced in a thickness of 5-micron using Leica RM2125RTS microtome, stained with hematoxylin and eosin, and mounted in D.P.X. The image of the prepared sections was taken at 1000× magnification under the Axiostar Carl Zeiss microscope.

2.8. Statistical analysis

The statistical programme PAST 4.03 was employed for performing the principal component analysis (PCA) to evaluate the significant factors influencing fish sperm quality (Caldeira et al., 2018) by using the covariant matrix features. The PCA analysis predicts the correlation of samples and identifies the considerable variable influencing the total variance in the multi-dimensional sample set. The dataset consisting of % antioxidant activity of triplicate hilsa milt samples done by DPPH, ABTS, and FRAP assay and studied its association with the fish average weight and length which were reduced to two principal components; Component 1 and Component 2 while maintaining the total variance in the data. The correlation coffecients of the components were implied to map the biplot. The mirror image correlation matrix was performed at

an alpha value <0.05. The percent data for antioxidant was tested for goodness-of-fit and homogeneity.

We have tested the normality of the percent antioxidant data using Jarque-Bera goodness-of-fit test and found the p-value for all the three processes of antioxidant evaluation [(DPPH; $p = 0.382652$), (ABTS; $p = 0.404345$) and (FRAP; $p = 0.602814$)] to be higher than the considered alpha = 0.05. Also, we performed the chi square test of homogeneity and found the alpha < p – value (0.187798689), hence we could conclude that the data was devoid of skewness and kurtosis and follows homogeneity (Supplementary Fig. S1).

3. Results

3.1. Antioxidant assay of milt

Eight different fish milt samples of different weight groups were assessed for their antioxidant activity using three different free radical scavenging methods. The sample codes with their respective weight and length are tabulated (Table. 1).

3.1.1. 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay

The antioxidant assay's findings showed that the effectiveness in scavenging DPPH radicals of the hilsa milt sample M3 (72.13% ± 0.60) was closer to synthetic antioxidant butylated hydroxyl toluene (BHT) (84.9% ± 0.2) and showed a substantial ability to reduce and a great capability to block free radical. The least was found for sample M12 (8.34% ± 3.5) (Fig. 2).

3.1.2. 2, 2′-azinobis-[3-ethyl-benzothiazoline-6-sulfonic acid] (ABTS) antioxidant assay

The ABTS antioxidant activity of the milt sample was colloquial with the DPPH scavenging activity with the highest being recorded in M14 (84.5% ± 0.48) followed by M3 (84.2% ± 1.4) as compared with BHT (95.39% ± 0.99). The lowest was reported in M12 (27.53% ± 3.5) (Fig. 2).

3.1.3. Ferric reducing antioxidant potential (FRAP) antioxidant assay

The ferric-reducing ability of the hilsa milt was assessed as a marker of oxidative protein damage. An interesting correlation emerged in all three methods. The highest ferric-reducing ability was found in M3 (75.27% ± 0.25) followed by M12 (65.77 ± 1.5) as compared to ascorbic acid used as a standard antioxidant (85.23% ± 0.06).

The results are found to be consistent in all three methods. The highest antioxidant activity was found in fish milt samples M3 and M14 and the lowest in M12. The scavenging value also follows a fish weight-dependent gradient (Fig. 2).

Table 1
Length and weight of male hilsa.

Sl No.	Fish Sample Code	Weight (gms)	Length (cms)	Relative condition (Kn)*
1	M3	358.6 ± 0.75	33.1 ± 0.1	1.53
2	M6	196.06 ± 0.11	26.6 ± 0.03	1.50
3	M7	195 ± 0.17	26.7 ± 0.11	1.50
4.	M12	168.35 ± 0.12	25.1 ± 0.17	1.45
5	M14	272.61 ± 0.28	30.5 ± 0.01	1.45
6	M23	186.87 ± 0.03	27 ± 0.03	1.28
7	M26	203.91 ± 0.06	26.5 ± 0.01	1.58
8	M32	204.45 ± 0.08	27.7 ± 0.02	1.41

Values are expressed as mean ± standard error (SE).
* The relative condition Kn is calculated following (LeCren, 1951).

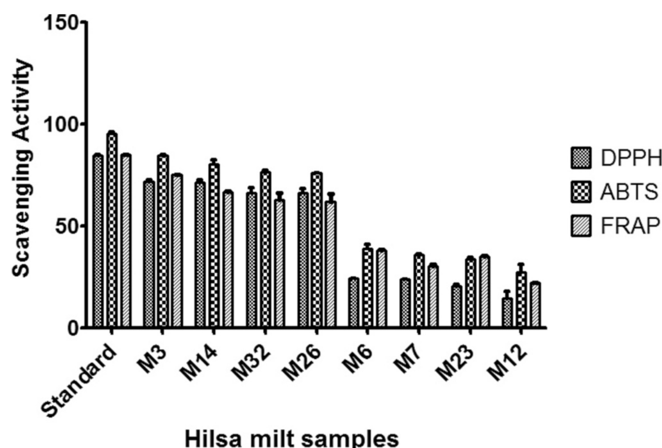

Fig. 2. Antioxidant activity of hilsa fish milt samples (M3 – M32) using DPPH, ABTS and FRAP assay and compared with synthetic standards.

3.2. Amino acid profile

The amino acid composition of hilsa milt showed that, among all the amino acids, arginine (0.70 g/100 g) was present in the highest quantity followed by glutamic acid (0.14 g/100 g) and proline (0.14 g/100 g) (Table 2).

3.3. Fatty acid composition

The composition of fatty acid present in hilsa milt showed that among the saturated fatty acids (SFA), C16:0 (palmitic acid) was found to be high (20.98 ± 1.12%) followed by C18:0 (stearic acid). Among the monounsaturated fatty acids (MUFA), C18:1 (oleic acid) was found to be the highest (4.90 ± 0.45%). EPA (C20:5) and DHA (C22:6) were the predominant PUFA present i.e. 25.09 ± 1.45% and 37.22 ± 2.23%, respectively (Table 3).

3.4. Gonadosomatic index

The weight range of matured male hilsa during the spawning season ranges from 113.8 to 432 g. The GSI of those male testis ranges from 0.71 to 1.82. The average body weight of the 31species was 231.52 ± 29.06 g.

Table 2
Amino acid composition of hilsa milt (gm/100 g).

Amino Acids	Hilsa milt
ASP	0.09 ± 0.01
SER	0.08 ± 0.01
GLU	0.14 ± 0.02
GLY	0.10 ± 0.01
HIS	0.03 ± 0.00
ARG	0.70 ± 0.02
THR	0.11 ± 0.02
ALA	0.04 ± 0.01
PRO	0.14 ± 0.01
CYS	–
TYR	0.04 ± 0.01
VAL	0.09 ± 0.01
MET	–
LYS	0.08 ± 0.01
ILE	0.05 ± 0.01
LEU	0.10 ± 0.00
PHE	0.07 ± 0.01

Values are expressed as mean ± standard error (SE).

Table 3
Fatty acid composition of hilsa milt (% of total area of fatty acids).

Fatty acid	Hilsa milt
C14:0	0.15 ± 0.01
C15:0	0.52 ± 0.02
C16:0	20.98 ± 1.12
C17:0	0.45 ± 0.08
C18:0	5.05 ± 0.56
C20:0	0.44 ± 0.01
C16:1	1.22 ± 0.62
C18:1	4.90 ± 0.45
C18:2	0.31 ± 0.01
C18:3	0.19 ± 0.01
C20:4	3.37 ± 0.89
C20:5	25.09 ± 1.45
C22:6	37.22 ± 2.23

Values are expressed as mean ± standard error (SE).

3.5. Age of hilsa

The collected mature hilsa male fish were mostly found in the size range of 266 mm to 331 mm which comes under 2 + years. However, a few fish were found to belong to the age group of 1+ year (211 mm - 255 mm). The regression analysis of the log-transformed length and age variables showed a good linear fit with $R^2 = 0.893$ following a regression equation of $y = 2.047\times + (-2.591)$. The model was found to be statistically significant with $p < 0.05$.

3.6. Histological observations

Spermatogonium of type A (SG-A) germ cells were present in immature and dormant gonad (Fig. 3). SG-A starts making significant divisions before maturation even begins. There are cysts in the gonads that contain type B spermatogonia (Fig. 4). Type B spermatogonia turn into primary spermatocytes. The cysts reveal the presence of spermatids that go through spermiogenesis following the completion of the meiotic divisions. The first spermatozoa can be seen in the most advanced cysts (Fig. 5). The Sertoli cells and spermatozoa are no longer connected. As more and more cysts mature, more spermatozoa are released (Fig. 6). The dark stained by heamotoxylin of spermatozoa was conspicuous in both Fig. 5 and Fig. 6, showing the gonad at advance stages of the maturity. The gonad at the commencement of this stage is filled with cysts made up of cells from all the preceding stages of spermatogenesis, including spermatids, type B spermatogonia, primary and secondary spermatocytes. Spermatozoa are transferred to the efferent duct as their number in the lobule lumen rises. The lobules occasionally include a single cyst with spermatids or even spermatocytes when a few cysts have delayed development. The mature spermatozoa were expelled from the gonads after spawning, although some were present in the testis (Fig. 7).

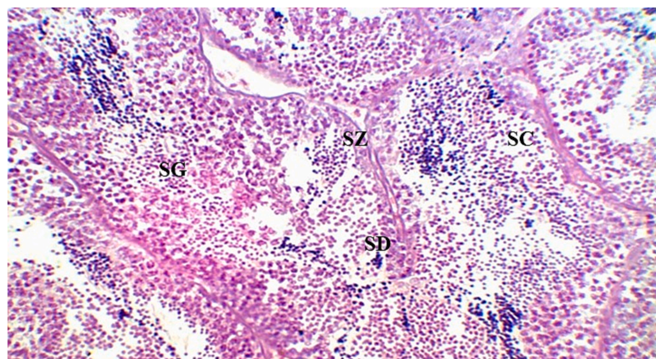

Fig. 3. Early spermatogenic stage: All spermatogenetic cells in hilsa testicular lobules: type A spermatogonia (SG); spermatocytes (SC), spermatids (SD); and spermatozoa (SZ) released into the lobule lumen.

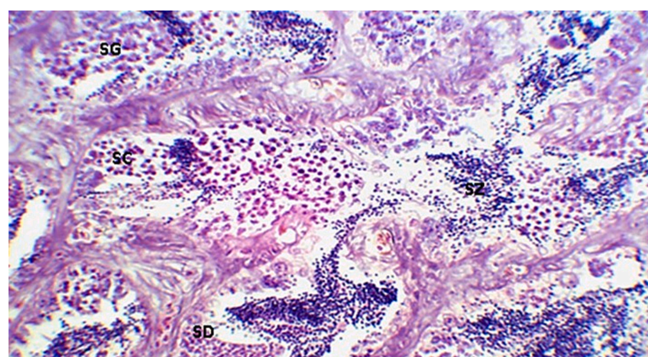

Fig. 4. Late spermatogenic: spermatogonia (SG); spermatocytes (SC), spermatids (SD); and spermatozoa (SZ) released into the lobule lumen.

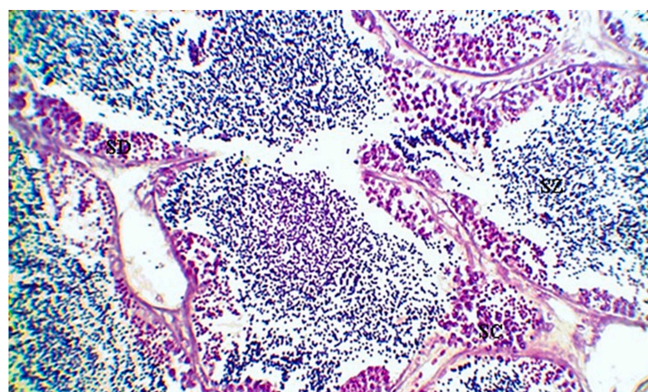

Fig. 5. Early spermiating: showing a large number of spermatozoa nests (SZ) and spermatid nests (SD), and a small number of spermatocytes (SC). (H & E) x 400.

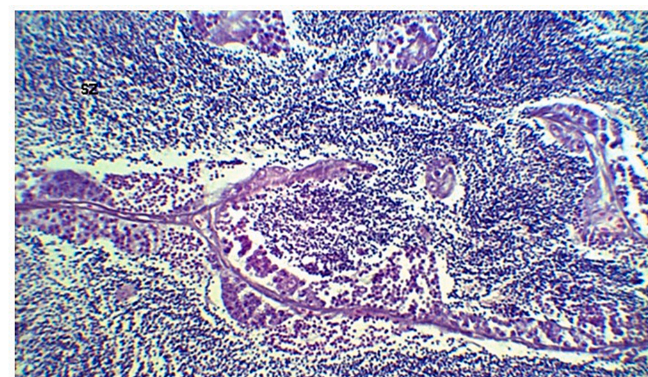

Fig. 6. Mature hilsa testis: The lumen of all the lobules is filled with free spermatozoa (SZ).

3.7. Principal component analysis

In this study, 5 components were considered for the analysis. Component 1 separates the fishes with higher weight (> 200 g) and contains the antioxidant variables (DPPH, ABTS FRAP). The lower-weight fishes lie in component 2 which also consists of the independent variable; viz.: weight and length of fish samples. The PC1 has an eigenvalue >1 with a combined variance of 99.9%. In the first component (PC1: variance = 86.047%). All the variables showed a positive

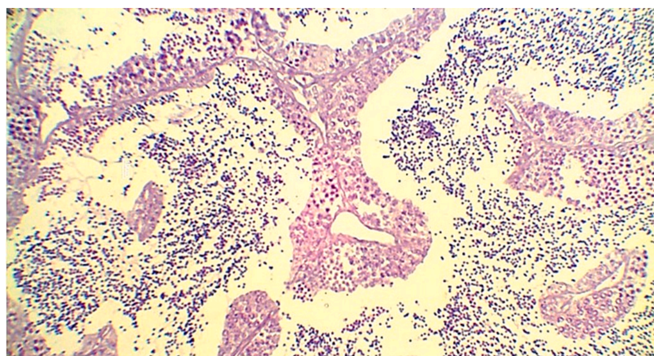

Fig. 7. Spent testis of hilsa: showing some spermatozoa (SZ) remaining in the lobule. Multiplying spermatogonia (SG) are visible by the lobule walls. (H &E) X 400.

loading. The scree plot (Fig. 8) shows component one (PC1) is the most prolific followed by component two (PC2). The factor loading plot (Fig. 9) shows that all five variables have a positive correlation with each other and contribute to the component one constitution. For components 2 and 4, weight and length (loading >0.5) are the significant components. However, the antioxidant values are moderately and negatively correlated to component 2 (loading <0.5). For components 3 and 5, the data generated by DPPH procedure is positively correlated and that of FRAP (loading = −0.81867) and ABTS (loading = −0.75003) are strongly and negatively correlated with components 3 and 5, respectively. Weight (loading = −0.66834) is negatively correlated with PC4 whereas length (loading = 0.67766) is positively correlated, both of the same strength.

The mirror image correlation matrix ($p < 0.05$) (Fig. 10) shows that length is strongly related to weight (r = 0.98) followed by FRAP (r = 0.76) and ABTS (r = 0.71). A similar trend of correlation is seen with length and antioxidant variables. The data generated from DPPH antioxidant assay shared a strong correlation with ABTS (r = 0.98) and FRAP (r = 0.97).

A second PCA analysis was carried out among amino acid, fatty acid and antioxidant profiles of hilsa milt, generating two principal components (PCs) with eigenvalue >1. The PCA's first component (PC1) with Eigen value 21.12 contributed to 70.40% of the total variance. The key factors with significant loading values were Asp, Ser, Glu, Gly, Arg, Thr, Pro, Tyr, Lys, Ile, C14:0, C20:0, C16:1, C18:2, C18:1, C20:4, C18:3, C22:6, C20:5, were grouped in PC1 (Fig. 11). The second component (PC2) with eigenvalue 8.87 accounted for 29.59% of the total variance and was influenced by DPPH, ABTS, His, Ala, Val, Leu, Phe, C16:0, C17:0, C18:0. The analysis suggested Gly with the highest loading to be a significant factor. The PCA analysis were done separately in order to select the best number of components briefing all the relevant features.

4. Discussion

The nutritional value and palatability of hilsa fish is unprecedented. Nevertheless, being a migratory fish, its sustained stock can only be assured by captive breeding. In this context, the cryopreservation like measures poses a major challenge towards the potentiality of hilsa milt. Hence, research has persisted and will do so in the future in an effort to develop better and efficient hilsa milt collection techniques.

The sperm quality estimation includes a deeper comprehension of the biological and physical difficulties that frozen sperm must overcome to survive. Among the many efforts undertaken for quality assessment of the sperm, antioxidants and fatty acids estimation are vital for the determination of the defense mechanism against oxidative stress produced during freezing and maintaining the plasma membrane stability. Fish milt is reported to be vulnerable to oxidative stress due to the PUFA present in the spermatozoa membrane (Bozkurt and Yavas, 2021) and also the degradation of antioxidant compounds owing to intensive farming conditions (Sandoval-Vargas et al., 2021). Earlier literature has reported on the antioxidant activity of seminal plasma of terrestrial animals and their correlation with sperm kinetics and protease inhibitor activity (Cecchini Gualandi et al., 2021). Also, our earlier studies on the antioxidant activity of hilsa serum showed very promising results comparable to standards (unpublished work). This is the first evidence that the hilsa fish weight and maturity, as determined by histological investigations, are closely correlated with the free radical scavenging capacity of the hilsa milt. In our work, the total antioxidant capacity (TAC) of the hilsa milt was sampled from natural habitats, which could be used as a standard for other farmed fish studies. Our study revealed that the TAC of the fish milt followed the fish weight-dependant gradient. The highest TAC was found to be highest (72%–84%) corresponding to the highest weighing fish (357.8 g) followed by fish (272.61 g) with TAC of milt (65% - 84%). Nevertheless, FRAP has a significant quantitative relationship with sperm motility parameters (Aparnak and

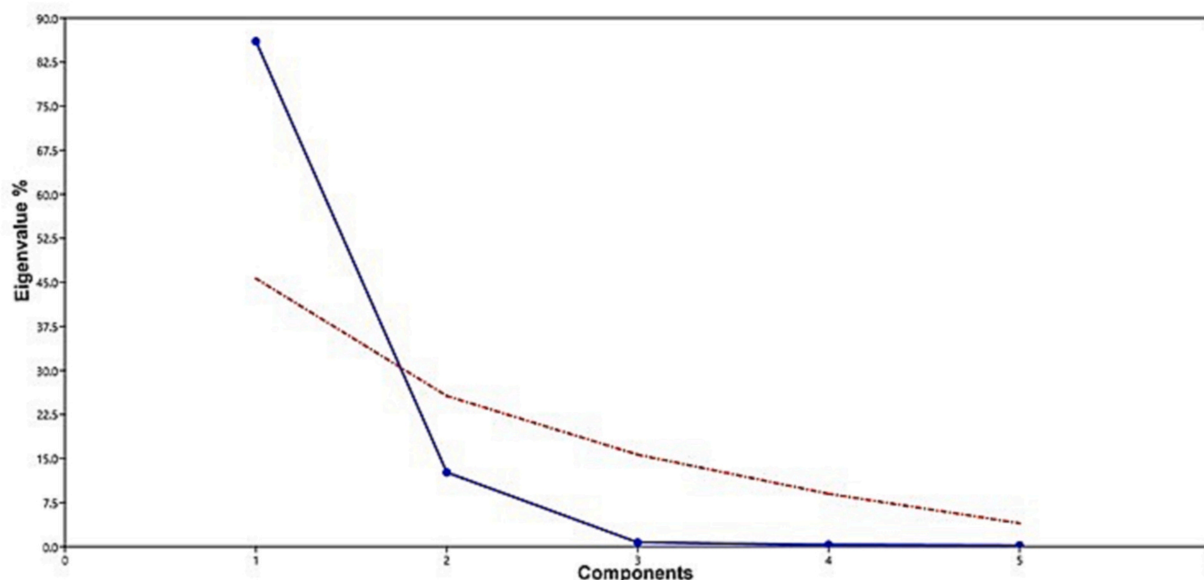

Fig. 8. The Scree plot of the PCA analysis of the five components plotted with eigenvalue%.

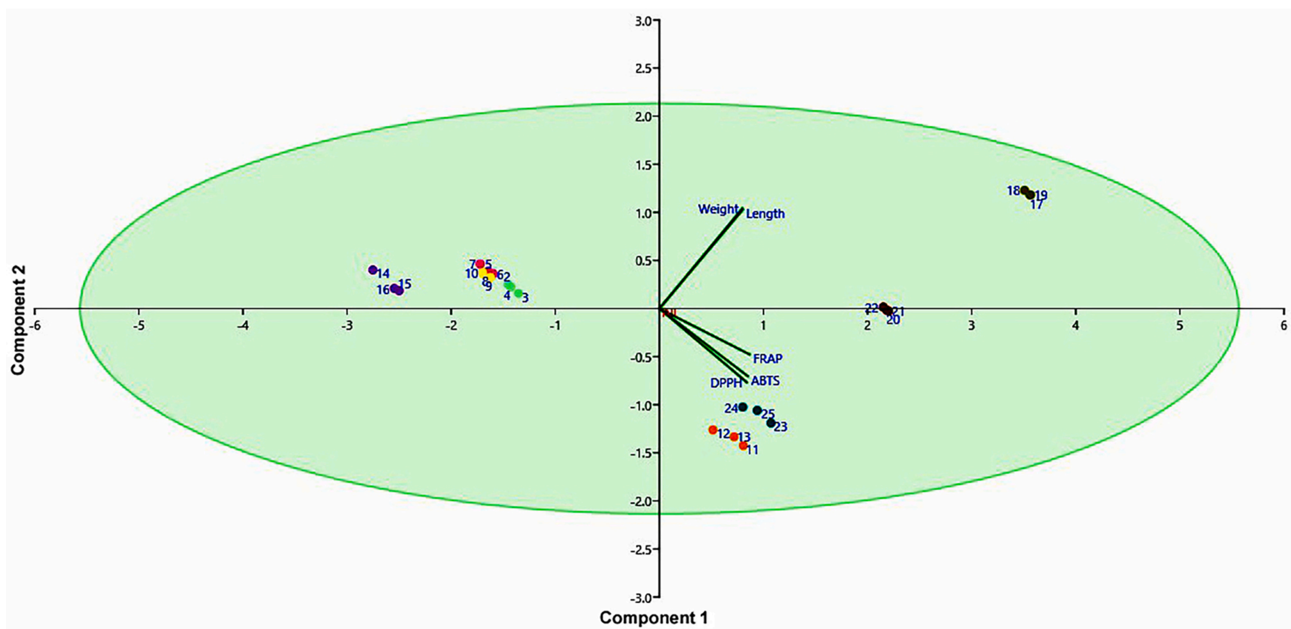

Fig. 9. The PCA bi-plot of the five experimental variables including weight and length of hilsa fish samples and total antioxidant content (TAC) of fish milt samples. The different milt samples corresponding to weight are shown in different colors.

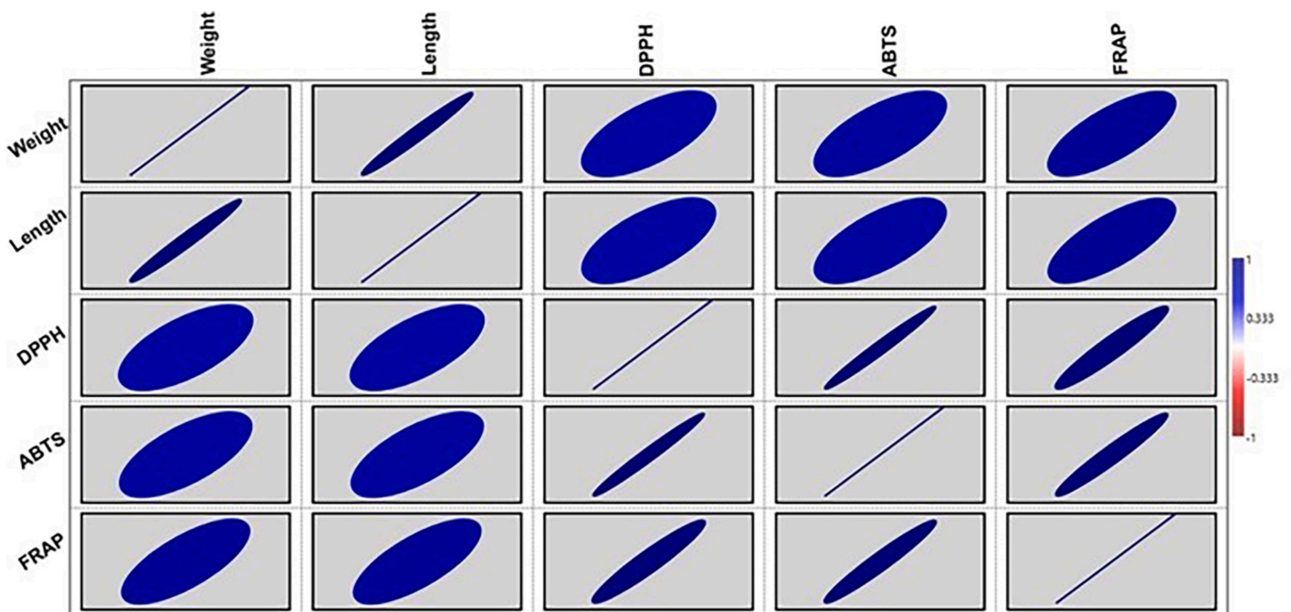

Fig. 10. Mirror image plot of the correlation matrix of hilsa fish weight and length with antioxidant values of the fish milt samples obtained from three different assays (DPPH, ABTS & FRAP). The diameter of the eclipses inversely corresponds to the correlation coefficient value.

Saberivand, 2019). In our study, the highest-weighing fish had 75.27 ± 0.2 equivalent μmols of Fe^{2+} /ml of the sample as compared to the standard ascorbic acid 85.23 ± 0.06 equivalent μmols of Fe^{2+} /ml of the sample. Moreover, in the present work, it was noticed that the age of the male hilsa fish was a determining factor influencing the milt quality. The milt of the largest fish showed the highest antioxidant value comparable to standards. Studies by Liu et al., 2021 showed similar results in the dietary lipid metabolism study of rainbow trout (*Oncorhynchus mykiss*) where a proportionate correlation was noted between weight gain and antioxidant level. The results of PCA analysis were alike, with the significant antioxidant potential of milt samples of hilsa fish weighing >200 g influencing the overall variance of the experiments.

The histological sections of the testis show the changes that occurred during spermatogenesis from immaturity to early maturity and finally to the stage of spawning. The presence of spermatogonia indicates that active spermatogenesis is in its beginning phase. After maturation when mature spermatozoa are discharged from the gonads, few spermatozoa remained to start as a new spermatogenetic cycle, as reported by Dziewulska and Domagala, 2003. Furthermore, the gonadal maturation is a seasonal cycle dependent and particularly in hilsa showed two seasons pre-monsoon and post-monsoon during February and October (De, 2014).

Dietary supplementation with amino acids has been found previously to alleviate the multiple amino acid concentrations in plasma or plasma of seminal fluid in different fish or mammalian species, respectively (Wu et al., 2006; Dong et al., 2016). However, there is scanty

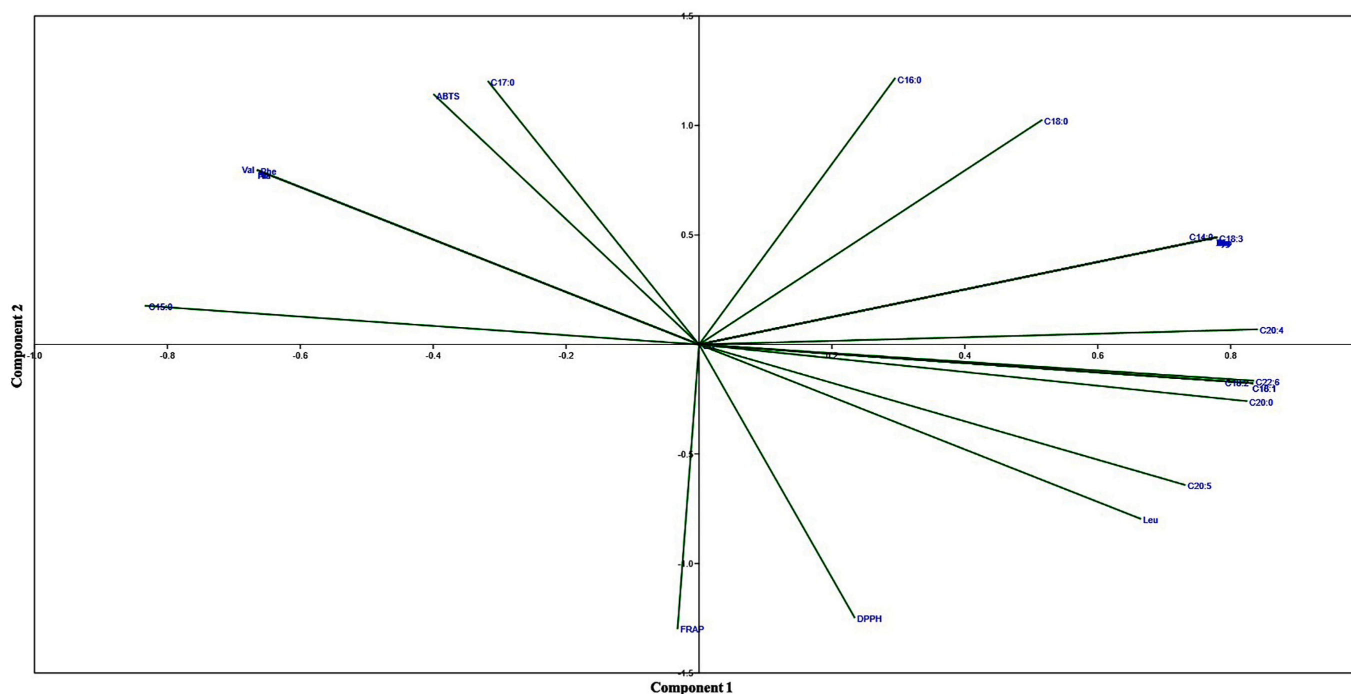

Fig. 11. PCA of amino acid and fatty acids detected in hilsa milt.

literature available on the amino acid content of fish germ cells with their impact on the functionality of sperm. Earlier reports suggest that the amino acid composition of fish gametes directly influences sperm quality as well as the success of the fertilization process (He and Woods, 2003; Kwasek et al., 2014). Therefore, the sperm amino acid profile from different fishes showed that the amino acids play a crucial role in stimulating sperm metabolic activity as well as viability (Lahnsteiner, 2010). In the present study, arginine was found to be in the highest quantity similar to the findings of Ernest Butts et al., 2020 in the sperm of catadromous fish *Anguilla anguilla*. On contrary, the amino acid level in male gonads or in germ cells differs among as well as among species that leads to difficulty in determining a particular pattern of the amino acids present. For example, in the milt of rainbow trout, methionine, arginine, and cysteine were the crucial amino acids whereas, in *Cyprinus carpio*, leucine, arginine, and glutamic acid were mainly present (Lahnsteiner, 2009; Lahnsteiner et al., 2009). Similarly, in perch (*Perca fluviatilis*), the main amino acids were arginine, alanine, and isoleucine whereas leucine, arginine, and methionine were in sea bream (*Sparus aurata*) (Lahnsteiner, 2010). This difference in composition is because of variations in size, age, genetic variations, feeding habits, and dietary sources of protein (Forster and Ogata, 1998) of the respective fish species (Ruchimat et al., 1997). Moreover, there is also evidence that remarkable amino acid content is produced in the duct epithelium of the sperm or by seminal plasma proteolysis (Lahnsteiner et al., 1993, 1994; Ciereszko et al., 1998) that could alter amino acid composition in male gametes.

The amino acid composition in comparison to other fishes showed that arginine present in higher concentrations in sperm is involved in sperm production, motility, and total sperm number (Chen et al., 2018). Glutamic acid and proline are responsible for increasing the number of live sperms, total motility and maintenance of functional membrane, and acrosomal integrity and also help in cryopreservation and decrease lipid peroxidation (Ernest Butts et al., 2020).

5. Conclusion

The hilsa milt was found to be rich in ω-3 fatty acids (EPA and DHA) which are significant for sperm functionality. The antioxidant content of the milt samples was high in higher-weight group fishes. This also indicated broodfish with higher defense against oxidative damage and successful in-situ reproduction for conservation purposes. No previous reports have documented these findings about hilsa fish milt. This research will help promote the maintenance of post-freeze semen of fishes of economic importance and help re-establish their population in adverse environmental conditions naturally or in captivity as well as in hilsa breeding program.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aquaculture.2023.739832.

Animal research declaration

All the animal research in this paper complies with the ARRIVE guidelines.

CRediT authorship contribution statement

Basanta Kumar Das: Conceptualization, Methodology, Writing – review & editing, Project administration. **Debalina Sadhukhan:** Investigation, Formal analysis, Writing – original draft. **Nabanita Chakraborty:** Investigation, Writing – original draft. **Satabdi Ganguly:** Investigation, Writing – original draft. **Amiya Kumar Sahoo:** Writing – review & editing.

Declaration of Competing Interest

The authors declare no conflicts of interest.

Data availability

The data that support the findings of this study are available on request from the corresponding author.

Acknowledgements

The authors are grateful to the Director, ICAR-Central Inland Fisheries Research Institute, Barrackpore, for providing research facilities

and financial support. We are also thankful to all the team members of the National Mission for Clean Ganga project (Project no: NMCG (Phase-II)-120(1)/2020), Ministry of Jal Shakti, Government of India for providing research assistance.

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