MINOR RESEARCH REPORT

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Study on Digestive Physiology and Reproductive Physiology of Lepidophagus Fish, *Neotropius khavalchor* Kulkarni, from Western Ghats India.

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INTRODUCTION

"Research is about engaging in a conversation with a brand."

-Matthew Rhodes

Introduction

Fishes are grouped as herbivores, carnivores and omnivores on the basis of their food habit (Chakrabarti et al. 1995). Carnivore fishes consumed different body parts among which scales are probably the most common (Sazima 1983). Till date five freshwater families and seven marine families contain lepidophagous (scale eating) species (Peterson & Winemiller 1997). Scales eating fishes are known to have special oral morphology which supports their lepidophagous habit (Peterson & Winemiller 1997). Although lepidophagous fishes share no universal set of morphological attributes, most possess some specialization in dentition and jaw structure, especially those species that are obligate scale-eaters. Most of the information on lepidophagus fish is only restricted to the oral morphology and scale eating behavioral adaptation but information regarding the digestive physiology of these fishes is poorly understood.

Scales are chemically made up of chitin, which is second most abundant polysaccharide in nature and resistance to chemical degradation (Gutowska et al. 2004; Gooday 1990). But it is assumed that chitin is rapidly degraded by bacteria in the marine environment (Gooday 1990). In fishes, chitinolytic enzyme activities are vary greatly between fish species with various roles including disruption of

chitinous exoskeleton (Lindsay 1984, Gutowska et al. 2004). Chitinase have also been found in the intestine where they help in removal of fragment blockage (Lindsay 1984). Unlike other, gut microflora of the scale eating fishes might plays significant role in digestion of the chitin rich scales. However, very little information is available concerning the chitinase producing bacterial population in the gut of lepidophagous fish.

Tropical freshwater lepidophagous fish, *Neotropius khavalchor* of the family Schilbeidae is described by Kulkarni in 1952 from Panchaganga River (Kulkarni, C. V. 1952). This species is described in the genus *Neotropius* but currently it belongs to the genus *Pachypterus* (Ferraris, 2007). (Note: Further in this report it is treated as *Pachypterus khavalchor*). It is endemic to Western Ghats and currently assessed as data deficient in IUCN Red List (Dahanukar et al., 2012). The species name "*khavalchor*" of this fish was derived from its scale eating habit. In Marathi, Khaval = scales, Chor = thief (Dahanukar et al., 2012).Therefore, lepidophagy in *N. khavalchor* raises the possibility of presence of some kind of the specialized scale digestion mechanism probably in the form of bacterial gut flora to digest the chitin rich scales of prey animals.

Fishes are known to have a special morphological evolution in the form of asymmetry in mouth structures. One striking example, scale-eating cichlid fishes

from Lake Tanganyika (Stewart and Albertson, 2010). Members of the Perissodini tribe of cichlid fishes have evolved dental and craniofacial asymmetries in order to more effectively remove scales from the left or right flanks of prey. Their mouths bend to one side of the head, which allows them to strike from a more posterior orientation that makes them less visible to intended victims. Mouths bend to the right in some individuals and to the left in others (Palmer, 2010). Tooth position, tooth morphology, and the length of the jaws are also known to provide some insight into attack methods. Currently no information is available about the attack behavior of the *N. khavalchor*, since it is the data deficient species. Therefore, head of *N. khavalchor* will be studied for its symmetry and also for its feeding strategy.

Neotropius khavalchor is endemic to the Krishna River system (Menon 1999; Jayaram 2010). It is a very rare species and has been considered as threatened by Menon (2004) by suggesting that small changes in water quality is likely to have adverse effects and may result in the loss of this species. This species is also recorded from Kamshet, Lonavala (Dahanukar et al., 2012). The species is also known from Panchaganga River near Kolhapur (Kulkarni, 1952; Kalawar and Kelkar, 1956), Krishna River near Islampur (Kulkarni, 1952), Koyna River near Patan (Jadhav et al. 2011), Krishna River near Sangli (Jayaram, 1995), Krishna River at Jamkhandi (Jayaram, 1995), Tunga-Bhadra River (Shahnawaz and Venkateshwarlu, 2009) and in the Eastern Ghats of Andhra Pradesh (Devi and

Indra, 2003). However, since there is little information about the population status, life history and ecology of this species, *N. khavalchor* is assessed as Data Deficient in the current IUCN Red List (Dahanukar, 2010).

Due to the Data Deficient status of this species (Dahanukar, 2010), we wish to undertake the study of Physiological aspects like reproductive physiology of this fish. Reproductive physiology study related to the sex ratio, spawning season, Gonadosomatic index (GSI), fecundity are extremely important for conservation program of the fish. Information on the reproductive physiology of this fish is believed to be important due to its threatened status suggested by the Menon (2004). Similarly, Length-Width relationship (LWR), Sexual dimorphism, asymmetry in head structure of lepidophagous fishes study would aid in better understanding of other aspect of fish related to the growth. Length–weight relationships for fish have been used extensively to provide information on the condition of fish, their isometric or allometric growth, in the analysis of ontogenic changes, to compare life histories of fish species between regions as well as other aspects of fish population dynamics (Verdiell-Cubedo et al. 2006).

Current study has been conducted in order to understand the specialized oral structures in *P. khavalchor* to feed on scales of other fishes. Role of its gut micro symbionts and their role in digestion of the scales eaten by *P. khavalchor*.

OBJECTIVES

Life is like riding a bicycle, in order to keep your balance, you must keep moving.
-Albert Einstein

Objectives of the current study

1. To study the Length-Weight relationship (LWR) and Sexual dimorphism in *N. khavalchor*.

2. To study sex ratio, spawning season, Gonadosomatic index (GSI), Hepatosomatic index (HSI) and fecundity.

3. To study the asymmetry in head structure and feeding behavior of *N. khavalchor*

4. Study of oral morphology and dental arrangement

5. Histological and histochemical study of digestive system

6. Study of gut microbiota and its role in lepidophagy

MATERIALS AND METHODS

"Research is creating new knowledge."

-Neil Armstrong

Materials and methods

Study site and sampling of fishes

Literature survey was carried out in order to determine the potential collection sites. Based on the previous literature survey *Pachypterus khavalchor* is known to occur in various rivers. However its availability is very rear hence fishes were collected from the local fish market situated at the Karad, Maharashtra, India. The details of the collection sites are as follows: latitude 17.288°N; longitude 74.183°E. *P. khavalchor* were collected monthly from June 2015 to November 2015. The fishing method employed in this area by local fishermen mainly includes the use of different mesh sized nets, such as gill net and cast net. Fish were preserved in 10% formalin and brought to the laboratory.

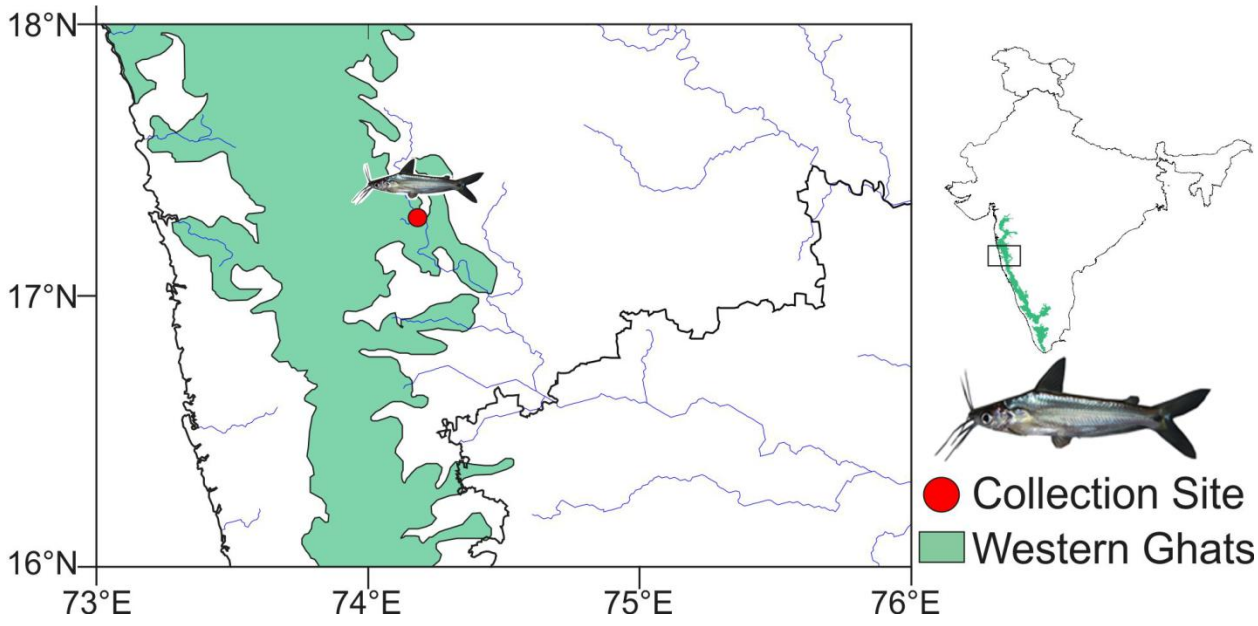


Fig. Collection site of Pachypterus khavalchor

Measurements and data collection

For length-weight relationship study, standard length (SL) and total length (TL) was measured for each specimen to the nearest 0.01 mm using a digital caliper (Mitutoyo, Japan). Weight (W) was measured to the closest 0.01 g using digital weighing balance (Contech, India).

Fig. *Digital caliper used for morphometry*

Length-Weight Relationship (LWR) and Length-Length Relationship (LLR)

The LWR and LLR were determined. The LWR was determined using the allometric equation W = aSLb (Pauly, 1984) and logarithmically transformed into log (W) = log (a) + b log (SL) where, W is the weight of the fish in gram and SL is the length standard length of the fish measured in millimeter. The parameters 'a' (proportionality constant) and 'b' (exponent) of the LWR were estimated by least square regression (Zar, 1999). The coefficient of determination (R2) was indicated as the goodness of the regression. To test whether the power b = 3, calculated as a

slope of the log-log plot for weight and length, was different for males, females and combine data, two tailed t-test was used (Zar, 1999). The LLR was estimated as $TL = aSL^b$ and its log-log form log (TL) = log (a) + b log (SL). The coefficient of determination (R2) was indicated as the goodness of the regression. To test whether the power b = 1, calculated as a slope of the log-log plot for standard length and total length, was different for males, females and combine data, two tailed t-test was used (Zar, 1999).

Sexual dimorphism and sex ratio

Formalin-preserved specimens of various size ranges were visually examined in order to find out morphological character indicative of sexual dimorphism. Results of visual examination were further confirmed by observing gonadal morphology after dissection. Data obtained based on the sexual dimorphism was used to estimate sex ratio. Data was analyzed using chi-square (χ^2) test to test the null hypothesis that the male to female sex ratio is 1:1.

Fecundity parameters (F)

Fecundity was estimated using 30 gravid females. After morphometric measurements of SL, TL, and W, ovaries were dissected and then preserved in 5% formalin solution for 24 h so that the eggs would swell up for easy calculations. The fecundity of the fish was calculated using the gravimetric method due to its

high accuracy (Simpson 1959). In this method, a subsection of the ovaries was weighed (0.1 g) and the number of eggs was counted. The total number of eggs in the ovary was calculated as a proportion. Absolute fecundity (F) was finally calculated using following formula:

$$F = n \times W$$

Where W= total weight of ovary in grams and n = number of eggs per 0.1 g of subsection ovary.

Relative fecundity was determined by the ratio of the total number of eggs per unit weight or length of fish. A relationship between absolute fecundity and standard length (SL), body weight (Wt), ovary length (OL) and ovary weight (OW) was determined using following equation:

$$\text{Log } F = \log (a) + b \log (SL)$$

$$\text{Log } F = \log (a) + b \log (W)$$

$$\text{Log } F = \log (a) + b \log (OL)$$

$$\text{Log } F = \log (a) + b \log (OW)$$

Where, F = fecundity, SL = standard length (mm), W = body weight (g), OL = ovary length, OW = ovary weight, and '*a*' and '*b*' are regression constants.

Gonadosomatic Index (GSI) and Hepatosomatic Index (HSI)

To understand the sexual cycle and to determine the spawning period, GSI was calculated based on the monthly collection using following formula (Dadzie and Wangila 1980):

$$GSI = GW/BW * 100$$

Where GSI = Gonadosomatic index, GW = Gonad weight (g), BW = body weight (g)

To understand status of energy reserve in an animal the Hepatosomatic index was calculated based on the monthly collection using following formula (Wootton et al. 1978)

$$\textbf{HSI= Liv wt/TW* 100}$$

Where HSI = Hepatosomatic index, **Liv wt** = Liver weight (g), TW = Total body weight (g)

Statistical analyses

The data obtained from the morphometric and reproductive parameters were subjected to statistical analysis using Microsoft Excel 2010 and PAST version 3.18 (Hammer, Harper and Ryan 2001). For all statistical analyses, α was set to 0.05.

Osteology

Since structure of the head, asymmetry is known to involve in shaping the lepidophagous behavior, osteological investigation was carried out. Osteology of the specimen was carried out using the protocol by Potthoff (1984).

Behavioral experiments

Experiment 1 was performed to see whether P. khavalchor is obligate scale-eater or not. During trials, one P. khavalchor was released undisturbed in a large glass aquarium (92 x 61 x 61 cm). After 1 hr of acclimatization period, fish food pellets were offered and fish was allowed to feed on it. A similar experiment was repeated using the frozen worms. Total ten trials were performed. The total observation period was 96 hrs.

Experiment 2 was performed to understand whether *P. khavalchor* attack and eat scales from dead fish. *Rasbora daniconius* was used as prey species. During trials, one *P. khavalchor* was released undisturbed in a large glass aquarium (92 x 61 x 61 cm) and was allowed to feed on the scales of dead prey species after 1 hr of acclimatization period. After every 24 hrs, new dead fish was added and previously added fish was removed and observed for any denuded spot on both left and right flank sides.

Experiment 3 was performed to study the scale-eating behavior of *P. khavalchor* in laboratory conditions. Different live prey fish species mentioned above (section 2.1) with all intact scales were used as a prey. During trials, in a large glass aquarium (92 x 61 x 61 cm) pair of P. khavalchor were released undisturbed along with prey fish species and allow to acclimatize for 1 hr. Using digital camera (Canon 600D) scale-eating behavior was recorded. After 24 hrs, prey fish were taken out and observed for any denuded spot on both left and right flank sides of prey fish. Total ten observations were carried out. Photographs of prey fish were captured before and after the experiment.

Study Oral morphology and dental arrangement

Scanning electron microscopy (SEM) analysis of oral cavity of P. khavalchor was carried out following the protocol by Fishelson et al., (2014). Samples were examined and photographed using JOEL analytical scanning electron microscope (JOEL JSM– 6360A) at an accelerating voltage of 10 kV.

Histology and histochemistry

Tissue fragments of the esophagus, stomach (cardiac, fundic and pyloric), intestine (anterior, middle and posterior) and liver were obtained from the dissected specimens and were subjected to routine histological investigation using

hematoxylin-eosin (H and E) method. Formalin fixed tissues were washed properly in distilled water, dehydrated in alcohol, cleared in xylene and were embedded in paraffin. Tissues were sectioned at 6 µm using a Leica RM 2125 RTS rotary microtome (Leica Ltd, Wetzlar, Germany). To understand the basic histologically arrangement in various tissue, the sections were stained with routine haematoxylin-eosin (HE) method. In order to understand the chemical characteristics of mucous secreted by esophagus, stomach and intestine some sections were processed for carbohydrate and protein analysis. In order to identify neutral glycoconjugates periodic acid Schiff (PAS) staining was used. Carboxylated and sulphated glycoconjugate were identified using Alcian blue (AB) staining at pH 2.5 and 1.0 respectively. Interpretation of the results was based on the staining intensity, as determined by Díaz et al. (2003). Staining intensity ranged from - (no staining) to + + + (intense staining). All the protocols for histology and histochemistry were as per Bancroft and Gamble. Appropriate controls were used in each case. All stained slides were mounted permanently in DPX and were observed and photographed under a Zeiss light microscope (Zeiss Axioscope A1) mounted with a digital camera (Jenoptik).

Isolation and molecular characterization of the gut microbiota:

Fish (72 hrs starved; *n* = 3) were anesthetized using 0.03% Tricaine methanesulphonate (MS–222), washed with distilled water thoroughly to remove the traces of MS–222, and the ventral surface of each fish was thoroughly scrubbed with the absolute alcohol for surface decontamination. Fish were dissected aseptically in laminar airflow and stomach was removed for the isolation of the adherent bacteria. Stomach was cleaned, cut into pieces slit open by a longitudinal incision, and flushed carefully 3 times with 0.89% sterile sodium chloride (NaCl) solution to remove the non-adherent microflora (Ghosh et al., 2010). Stomach segments from 3 fish specimens were pooled together and a homogenate solution was made using 0.89% sterile NaCl solution (10:1; v/w) (Das and Tripathi, 1991). Pooled samples were used in order to avoid erroneous conclusions due to individual variation in gut microflora, as already described (Ghosh et al., 2010). The stomach homogenate was used after 10 serial 1:10 dilutions, by mixing the homogenate solution with 0.89% sterile NaCl solution using vortex mixer (Beveridge et al., 1991). The inoculum was spread on Nutrient Agar (NA) medium and incubated at 37°C for 24 hrs. After incubation, colonies with apparently different morphological appearance were streaked separately on NA medium and sub-cultured to obtained pure isolates. Pure isolates were maintained on NA medium slants for further studies. Morphological characterization of the colony

was done using a compound microscope with colony color, shape, size, margin, elevation, opacity, consistency, motility, and gram staining as standard characters. Total 9 isolates were obtained which were further screened for extracellular chitinase production. Pure colonies were streaked on colloidal chitin agar plates (1%), incubated at 37°C for 24 hrs and then selected based on their ability to hydrolyze and grow on chitin agar medium. The appearance of the zone of clearance indicates the chitinase production (Uma et al., 2009). Only chitinase positive isolates were identified by sequencing partial 16S rRNA gene following the protocol mentioned in Shah and Dahanukar (2012). Sequences were analyzed using Basic Local Alignment Search Tool (BLAST) (Altschul et al., 1990) to find the best match with known sequences in NCBI database (www.ncbi.nlm.nih.gov/). Sequences are submitted to GenBank under the accession numbers KU499849 and KU499850. Pure isolates of the bacterial isolates are deposited in the Microbial Cell Culture (MCC), Pune, Maharashtra, India, under the accession numbers MCC 2886 and MCC 2830.

In vitro bacterial scale degradation assay

The purpose of this experiment was to see if the chitin using isolates from the gut of *P. khavalchor* would actually degrade fish scales. For the assay, 0.5 g of air dried, sterilized scales were added in a flask with 50 ml Basal salt media (BSM; composition as per Shah and Dahanukar, 2012) and a loopful of the pure bacterial isolate (chitin utilizers). Flask was incubated at 37°C for 72 hrs in shaker incubator operated at 30 rpm. A control flask without inoculum was also incubated in same conditions. After 72 hrs, media was checked for growth and filtered to remove the remaining scales, if any. Remaining scales were then air dried and weigh. The assay was replicated thrice and the mean value of the dry weight of scales before and after degradation was calculated. The weight of remaining scales (before and after) was converted into percent scale degradation. One-way ANOVA followed by Tukey's pairwise comparisons were performed to check whether the remaining weight of scales differed between control and the two test organisms (chitin utilizers). The statistical analysis was carried out using the PAST 3.13 (Hammer et al., 2001). The level of significance (α) was set to 0.05.

RESULTS AND DISCUSSION

Evolution is the fundamental idea in all of life science - in all of biology.
-Ray Comfort

Results and discussion

Length-Weight relationship (LWR) and Sexual dimorphism in N. khavalchor

Details of minimum and maximum values of SL, TL, and BW are given in Table 1. New maximum TL (170.54 mm; female; Table 1) was recorded for *P. khavalchor* (Froese and Pauly 2018). Maximum total length reported previously was 150 mm without providing the details of gender (Talwar and Jhingran 1991). Though, many times extremely small sized *P. khavalchor* (up to 72.03 mm) was also observed in the market indicating that *P. khavalchor* is fished out at very small size probably before they get mature and breed. Small sized fishes have less reproductive potential compared to larger ones and thus cropping of small-sized individuals would negatively affect the reproductive outcome (Mudjirahayu et al. 2017). This could be one possible reason for the rapid decline and rear occurrence of this species as suggested earlier (Dahanukar et al. 2012). Table 2 shows results of LWRs, LLR's and related statistics for male, female and pooled data. Scattered plot for *P. khavalchor* LWR and LLR are given as Figure 1 (A–F). Based on LWR analysis, regression coefficient (*b*) values were 3.63 (r^2 = 0.95; positive allometry; Table 2; Fig. 1A) for males, 3.13 (r^2 = 0.93; isometric; Table 2; Fig. 1B) for females and 3.30 (r^2 = 0.93; positive allometry; Table 2; Fig. 1C) for combine sexes. Similarly, regression coefficient (*b*) for length-length relationship (LLR) were 1.01 (r^2 = 0.99; Isometric; Table 2; Fig. 1D) for males, 0.97 (r^2 = 0.98;

isometric; Table 2; Fig. 1E) for females and 0.98 (r^2 = 0.98; Isometric; Table 2; Fig. 1F) for combine sexes. No LWRs information was available in FishBase for *P. khavalchor* (Froese and Pauly 2018) and hence the results cannot be compared. However, the estimated *b* value for *P. khavalchor* male, female and combine sexes was found to be higher than *Pachypterus atherinoides* study carried out by Hossain and Afroze (1991), Buragohain (2018) and Ahamed et al. (2018) in Bangladesh (River not specified), India (Lachia River) and southern Bangladesh (Payra River) respectively. According to Hanif et al. (2017) fish with ideal growth shows the coefficient of determination (r^2) between 0.9 and <1. The coefficient of determination (r^2) in present study was found to be always greater than 0.9, explains the quality and reliability of the LWR model or linear regression. The value of the parameter '*b*' obtained in the present study was found to be within the expected range of 2.5–3.5 for female and combine sexes (Froese, 2006). However, the exponent of '*b*' value of LWR in the male range between 3.52–3.75 indicates that males exhibited wider size range than the 95% confidence limits of Bayesian hierarchical approach LWR prediction value (2.81–3.27) in FishBase (Froese, Thorson, and Reyes 2014). Such variation in the '*b*' value may be attributed to various factors such as length range used, sampling site, season, stomach fullness, gonadal maturity, sex, diet, condition factor, sampling gear, mesh size, frequency of sampling (Froese 2006; Hanif et al. 2017; Mahadevan, Ravi, and Murugesan

2017; Rahman et al. 2018). Moreover, types of growth pattern are known to be affected by feeding behavior of the species, abundance of food or prey items, low or absence of competitors and predators (Ogunola, Onada, and Falaye 2018). The observed growth pattern in this study could be linked to feeding behavior (lepidophagy) of the species. *Pachypterus khavalchor* is known for its voracious scale eating habit (Gosavi et al. 2018). Due to such unique feeding habit chances of competitors for food could be least. As a feeding tactics, *P. khavalchor* is known to dislodge the scales without much harm to host fish and thus do not kill the host fish (Gosavi et al. 2018). As fishes can regrow the lost scales, chances of depletion of the food resources for *P. khavalchor* are negligible. It is well established fact that, fish increases their weight when food resources are abundant and/or constant, competition for food is less and utilization of food items is maximum (Kamaruddin et al. 2011). Thus, the presence of positive allometric growth could be due to less competition, maximum and constant food supply in the form of scales and maximum utilization of the food items available for growth.

Table 1: Descriptive statistics for *Pachypterus khavalchor* sampled during 2015-2016 from the River Panchaganga (tributary of Krishna River System, India).

		SL (mm)		**TL (mm)**		**BW (g)**	
Organism	***N***	**Min**	**Max**	**Min**	**Max**	**Min**	**Max**
Pachypterus khavalchor	417	57.43	142.28	72.03	**170.54**	2.43	46.46

N: sample number; SL, Standard length; TL, Total length; BW, Body weight; min, minimum; max, maximum; bold = new maximum total length (no reference on LWR in FishBase; first world report).

Table 2: Summary of estimated parameters of LWR and LLR of *Pachypterus khavalchor*

Regression Parameters										
	Sex	***n***	***a***	***b***	**SE(*b*)**	**95% CI (*b*)**	**r^2**	***t***	***p***	**GT**
LWR	Male	210	0.0000009	3.63	0.10	3.52 - 3.75	0.95	5.89	0.0001*	PA
	Female	217	0.000008	3.13	0.11	3.03 - 3.25	0.93	1.22	0.9341	I
	Pooled (M+F)	427	0.000004	3.30	0.08	3.22 - 3.39	0.93	3.74	0.0002*	PA
LLR	Male	210	1.18	1.01	0.01	1.00 - 1.02	0.99	1.10	0.2696	I
	Female	217	1.37	0.97	0.01	0.95 - 0.99	0.98	-1.39	0.1641	I
	Pooled (M+F)	427	1.35	0.98	0.01	0.97 - 0.99	0.98	-1.59	0.1111	I

N, number of individuals; a, intercept; b, slope; CI, confidence interval; r^2, coefficient of determination; t, test for isometry; P, level of significance; GT, Growth type; PA: positive allometric; I: isometric, * indicate significant difference.

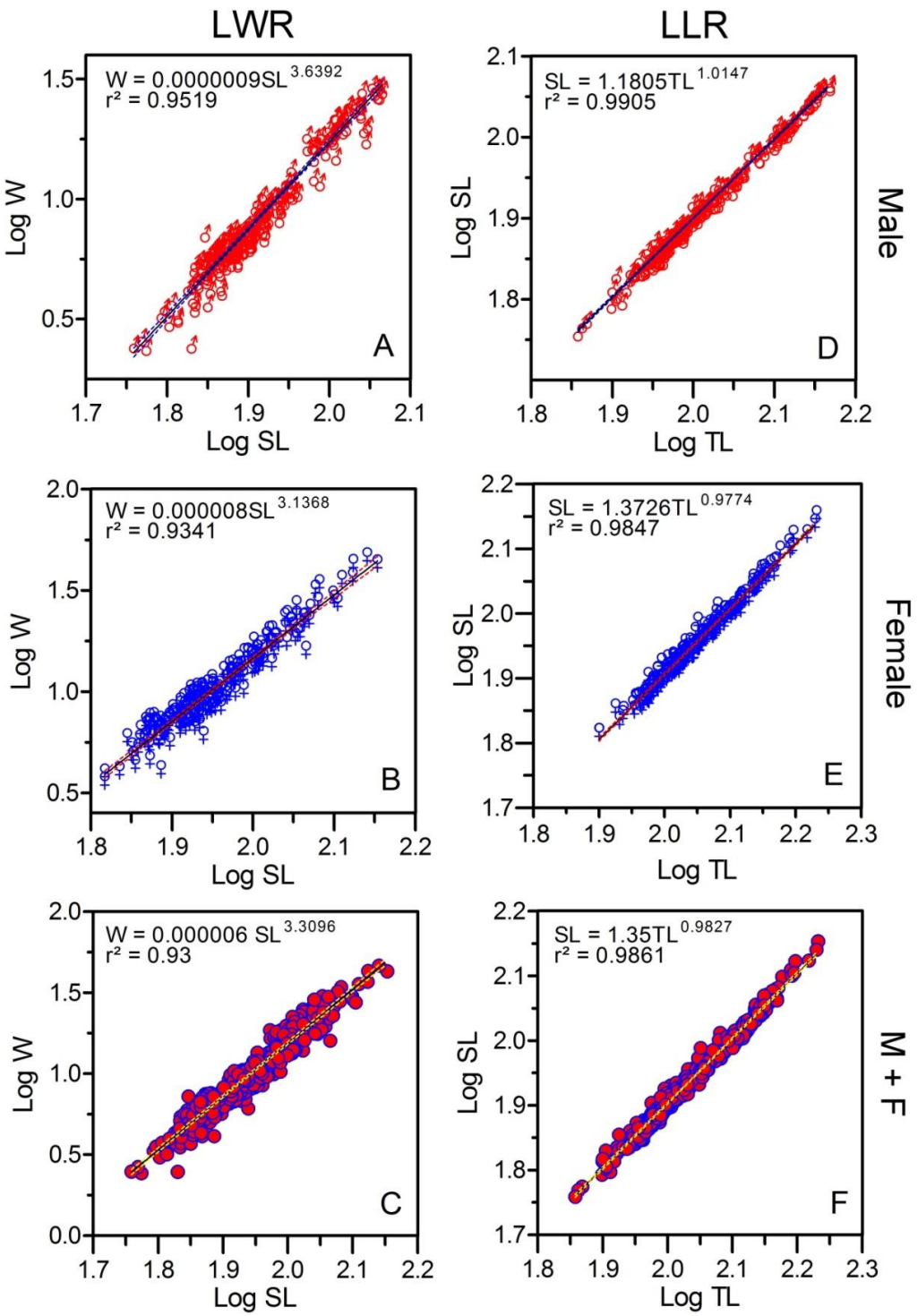


Figure 1: Scattered plots for male (A and D), females (B and E) and combine (C and F) data for LWR and LLR respectively.

Sexual dimorphism and sex ratio

Pachypterus khavalchor is sexually dimorphic fish. Females were found to be significantly larger than males in terms of standard length (t = -6.08; $p < 0.0001$; Figure 2A), total length (t =-5.01; $p < 0.0001$; Figure 2B) and heavier in terms of weight (t =-2.92; p = 0.003; Figure 2C). Out of 427 individuals of *P. khavalchor*, 211 (49.41 %) were males and 216 (50.59 %) were female giving male to female ratio as 1:1.02, which was not significantly different from the expected 1:1 ratio (Chi-square χ^2 = 0.059; p = 0.8088). Males of *P. khavalchor* show sexual dimorphic character in the form cone-like genital papillae. Sexual dimorphism is a common feature found in most animal phyla and is a fairly common characteristic of the many fishes worldwide (Oliveira and Almada 1995; Conway and Britz 2007). Huge variation in sexually dimorphic characters such as color, size and the presence or absence of parts of the body used in courtship displays or fights is observed in fishes. For instance Swordtail (*Xiphophorus helleri*) and gobies (*Gobius sp.*) shows variation in fins, Guppies (*Poecilia reticulata*) and wrasses (*Labrus sp.*) shows variation in color, Eels (*Anguilla sp.*) and mullets (*Mugil cephalus*) shows variation in size, Silurids (*Ompok sp.*) and sculpins (*Myoxocephalus sp.*) shows morphological variations in body proportions (Saurabh et al. 2013). Furthermore, several sexual dimorphic characters can be either temporary (in temporarily dimorphic or dichromic fish) or permanent (permanently

dimorphic or dichromatic fish) depending upon the species. Identification of sexually dimorphic traits is known to be essential in understanding ecology, behavior and life history of the species, biodiversity assessments, reproductive biology, breeding, induced breeding and pheromone biology related studies (Oliveira and Almada 1995; Conway and Britz 2007; Saurabh et al. 2013). Based on our observation, the presence of genital papillae in males of *P. khavalchor* is a permanent character and thus *P. khavalchor* is permanently dimorphic fish.

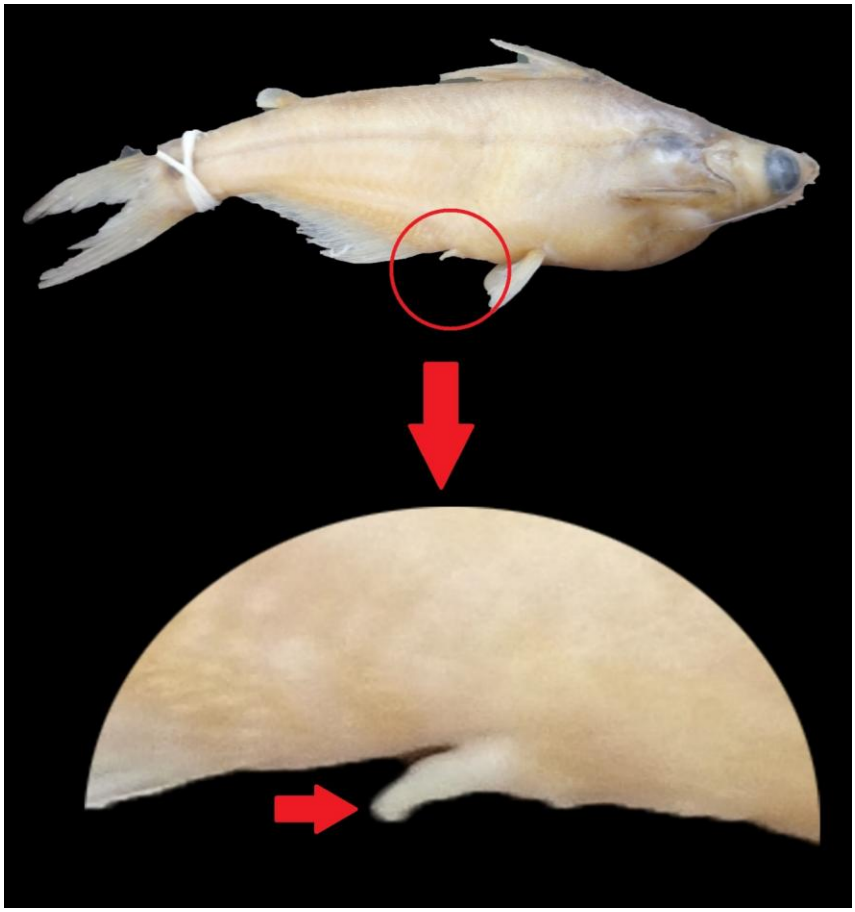
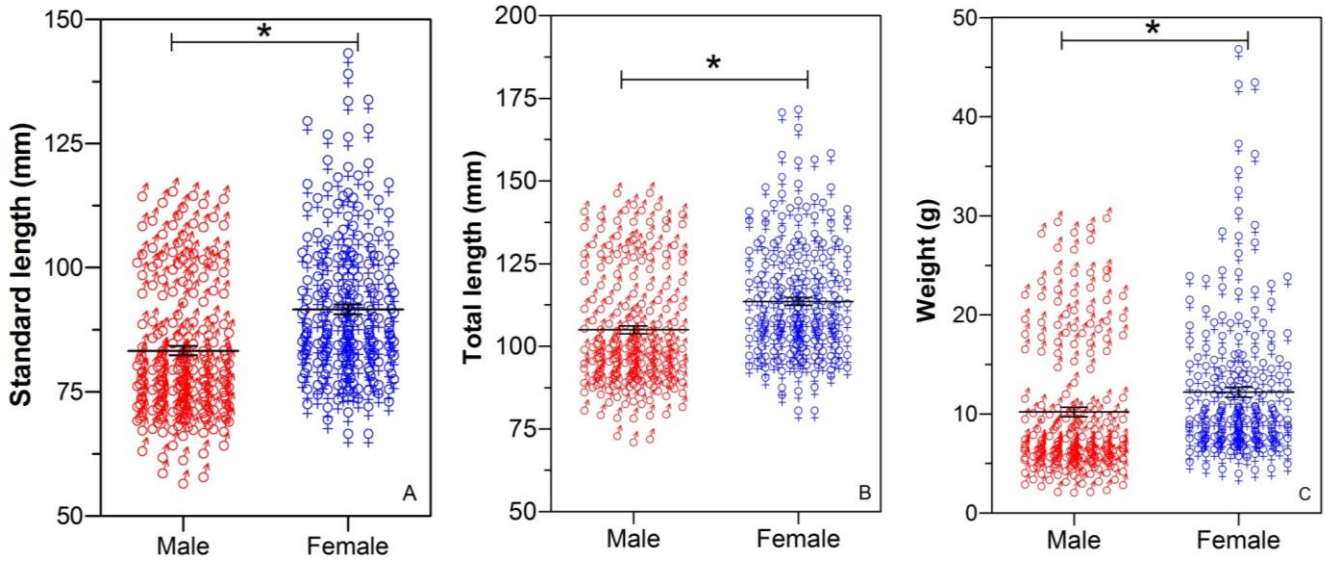


Figure 2: Comparative analysis of standard length (A), total length (B) and total weight (C) between male and female *Pachypterus khavalchor* (* indicate the significant difference between male and female).


Standard length (mm)
150
125
100
75
50
*
A
Male
Female
Total length (mm)
200
175
150
125
100
75
50
*
B
Male
Female
Weight (g)
50
40
30
20
10
0
*
C
Male
Female

Fecundity, Gonadosomatic index (GSI), Spawning season and Hepatosomatic index (HSI)

Fecundity parameters

Based on the analysis of 30 gravid females the details of the minimum, maximum and average values of SL, TL, W, OW, OL, absolute fecundity and relative fecundity are presented in Table 3. Fecundity is the common parameter which has been estimated for many tropical fish species, but not for *P. khavalchor*. Mean absolute and relative fecundity was found to be 7305 oocytes and 432 oocytes respectively (Table 3). Based on the present results it is observed that *P. khavalchor* is highly fecund since the absolute fecundity was found to be 24642 oocytes at 155.42 mm total length. Lack of data on *P. khavalchor* fecundity from other localities restricts our comparisons. However, the Asian Sun catfish, *H. brachysoma*, another member of the family horabagridae shows the highest absolute fecundity 1140 oocytes at the total length of 205 mm and known to be highly fecund (Bindu et al. 2012; Raghavan et al. 2016). It has been stated that fishes which do not show parental care are more fecund (Royce 1972; Bindu et al. 2012). In the same line, *H. brachysoma* does not show any parental care and also known to more fecund (Bindu et al. 2012; Raghavan et al. 2016). Such information on parental care in *P. khavalchor* is not reported till date. However, high fecundity

could be associated with lack of parental care in *P. khavalchor*. Though, this needs to be ascertained with more elaborative in-situ experiments and field observations, since this generalization of high fecundity and lack of parental care apparently does not apply to all species (Schenck and Whiteside 1977; Bindu et al. 2012; Raghavan et al. 2016). For example, *Etheostoma fonticola* is known to provide little or no parental care and also spawn few ova (Schenck and Whiteside 1977). The relationship between absolute fecundity (F) and standard length (SL; Figure 4A), body weight (W; Figure 4B), ovary weight (OW; Figure 4C) and ovary length (OL; Figure 4D) were found highly significant ($p < 0.0001$). Overall, the absolute fecundity showed a strong linear relationship with TL, BW, OL, and OW of the fish. However, among the relationships, the highest relationship was observed between fecundity and ovary weight ($r = 0.97$) whereas lowest relationship was observed between fecundity and ovary length ($r = 0.863$). A positive correlation between fecundity and above-mentioned parameters is common among fishes (Bahuguna and Khatri 2009; Alam and Pathak 2010; Jan, Jan, and Shah 2014; Geremew, Getahun, and Dejen 2015).

Table 3: Summary of estimated fecundity parameters based on 30 females of *Pachypterus khavalchor*

	SL (mm)	**TL (mm)**	**W (g)**	**OW (g)**	**OL (mm)**	**Absolute fecundity**	**Relative fecundity**
Average	94.11	115.86	15.47	1.94	26.60	7304.77	432.42
Min	64.41	75.51	3.62	0.21	12.28	932.40	226.18
Max	125.90	155.42	36.99	6.66	43.77	24642.00	723.58

SL, Standard length; TL, Total length; BW, Body weight; OW, Ovary weight; OL, Ovary length; Min, Minimum; Max, Maximum

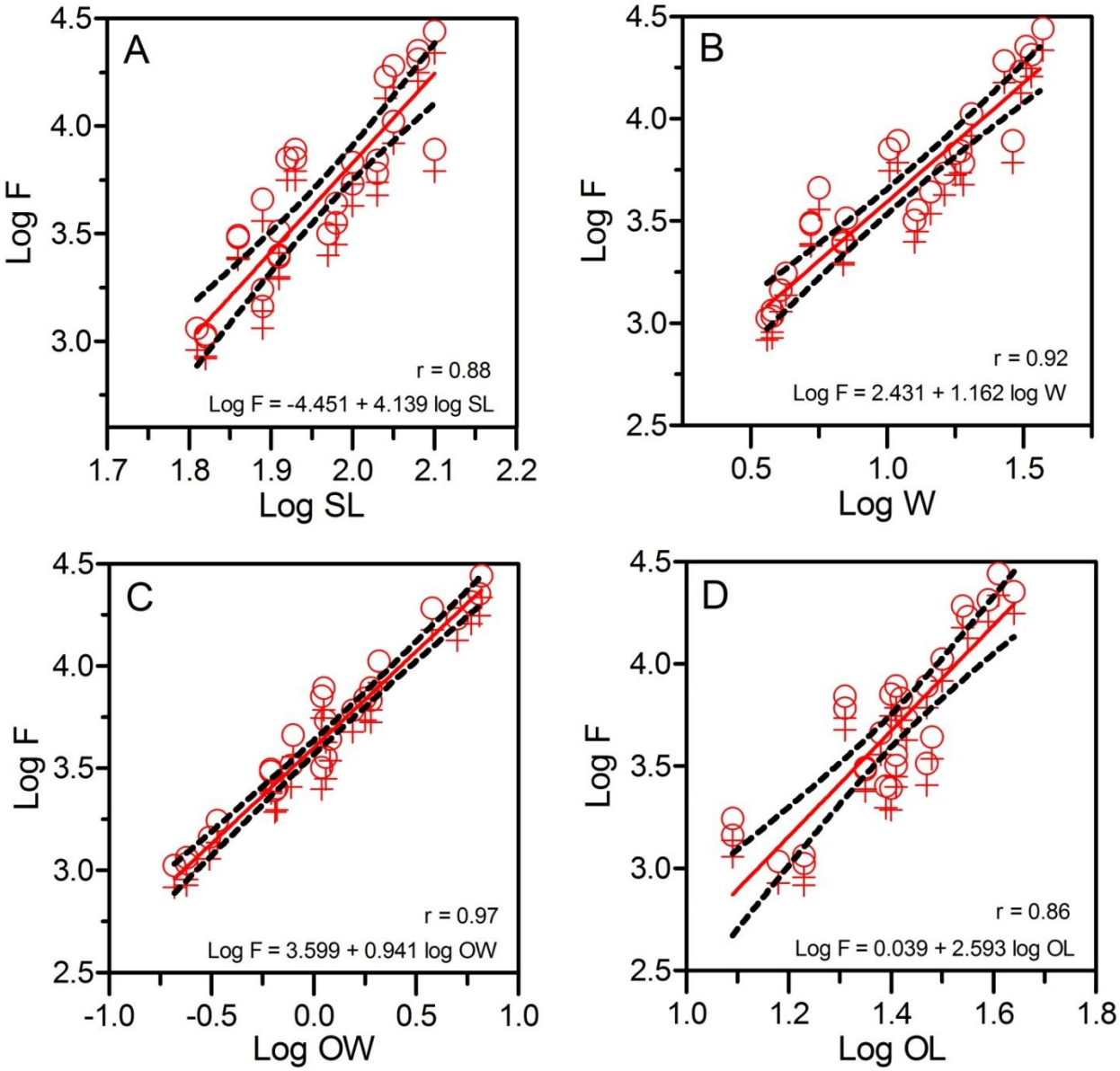


Figure 4: Relationship between absolute fecundity (F) and Standard length (A), Weight (B), Ovary weight (C) and Ovary length (D) for *Pachypterus khavalchor* from River Panchaganga, India.

Gonadosomatic Index (GSI)

GSI is one of the most useful measures to identify the gonadal maturity and spawning season of any fish species (Bindu et al. 2012; Raghavan et al. 2016). GSI of fish is known to increase with maturity and decreases along with depletion of gonadal activity due to spawning (Jan, Jan, and Shah 2014; Geremew, Getahun, and Dejen 2015; Raghavan et al. 2016). The GSI range and mean value calculated for *P. khavalchor* were 0.46–15.74 (6.47%) respectively. The GSI of *P. khavalchor* showed the highest value in April (15.74 ± 0.81) and lowest (0.46 ± 0.03) in December (Figure 5). The steady increase in the GSI from January to March represents the pre-spawning period. Higher values of GSI observed during the month of April to August indicate the period of maturity, spawning and its extension (Spawning period). Thereafter, GSI shows a rapid decline from September to October (Figure 5). Such rapid decline in the GSI values from September to December indicates the post-spawning period. Based on the present investigation it is clear that *P. khavalchor* spawns only once in a year but has prolonged spawning period extending from April to August. Lack of information restricts genus level comparisons. However, *H. brachysoma* is also known to have a prolonged breeding season and is also known to be single spawner (Chandran and Prasad 2014; Jan, Jan, and Shah 2014; Geremew, Getahun, and Dejen 2015; Raghavan et al. 2016). Such extended spawning period could be an evolutionary

adaptation in order to take advantages of both pre-monsoon and monsoon season and also provides the opportunity for the females to release all the eggs in one season (Zamidi et al. 2012). Breeding of tropical species at the beginning of the rainy season is a well-established phenomenon (Chandran and Prasad 2014; Raghavan et al. 2016). Since breeding during the monsoon provides juveniles to take complete advantage of the flooded banks for feeding while protected from predation. However, like *P. khavalchor* many species are also known to breed in pre-monsoon or spring season (Jan, Jan, and Shah 2014; Geremew, Getahun, and Dejen 2015).

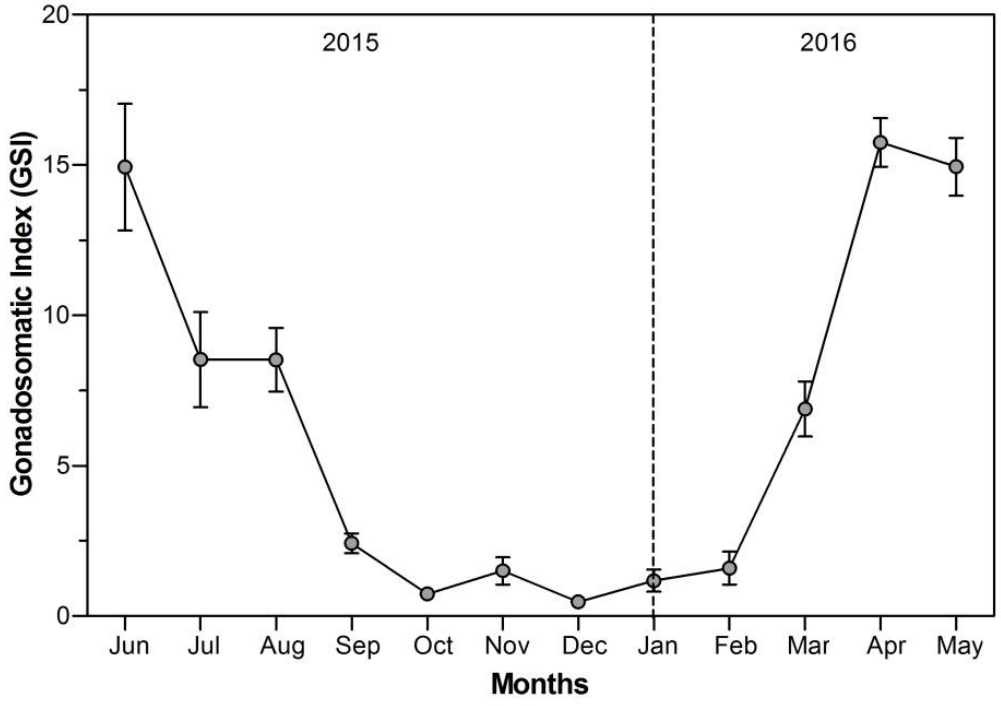
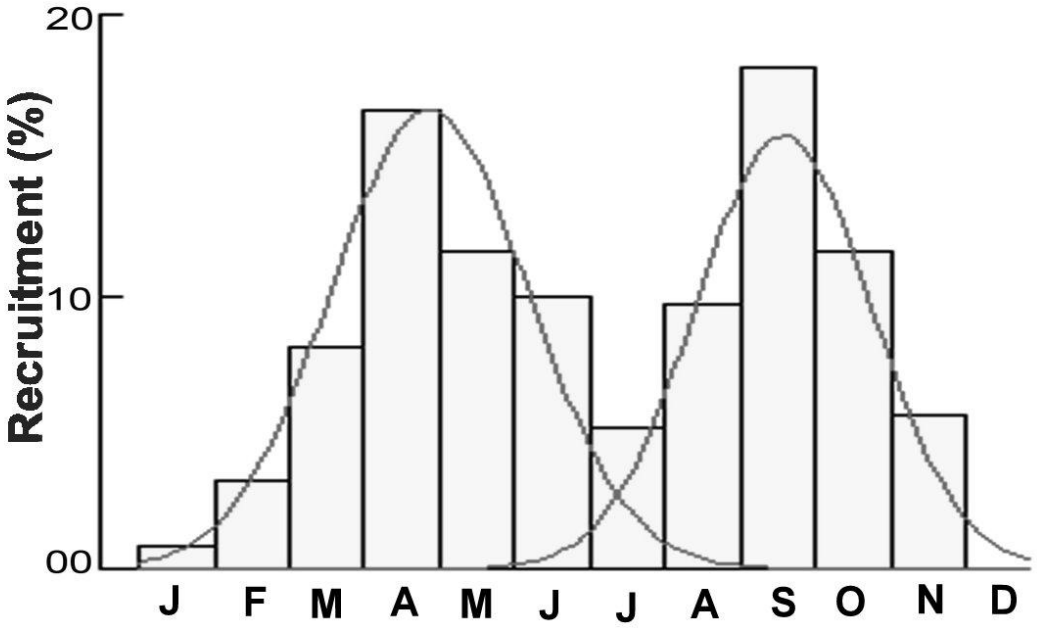


Figure 5: Annual changes in the Gonadosomatic index of *Pachypterus khavalchor* (Females).



Hepatosomatic Index (HSI)

The HSI of females ranged 0.08-2.51 and 0.096-1.41 for males. The trend in the females HSI were low during April to August, meanwhile the trend of HSI males was low in March to August (Figure 3). It was opposite with the trend of GSI (Figure 3). Presence of the opposite trend between the GSI and HSI suggested that the required energy for gametogenesis of *P. khavalchor* may be derived from their liver.

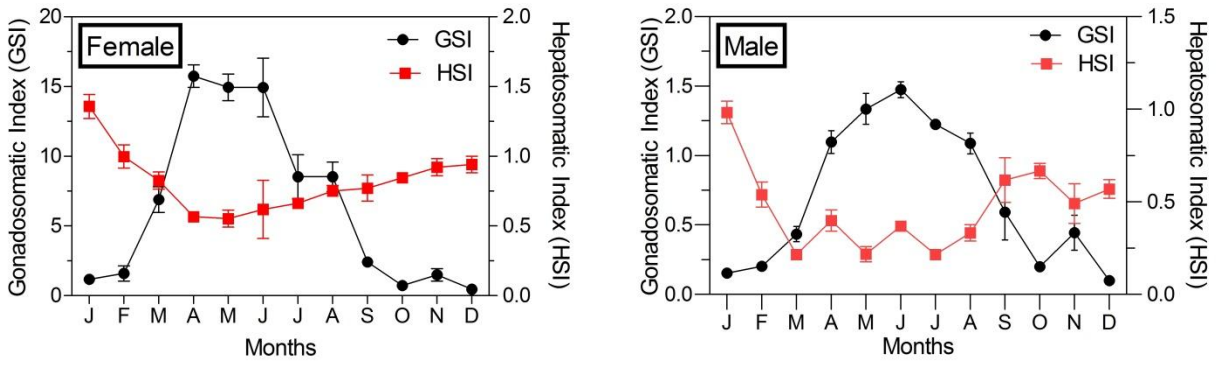


Figure : Hepatosomatic Index (HSI) of females and males and its relationship with GSI

Shape of the head and Feeding behavior of N. khavalchor

Osteology

Clear specimens of the *P. khavalchor* (Fig. 1A) showed no asymmetry in head structures. Both left and right side of the oral structure showed perfect symmetry with each other and no asymmetry was observed from both dorsal (Fig. 1B) and ventral side (Fig. 1C).

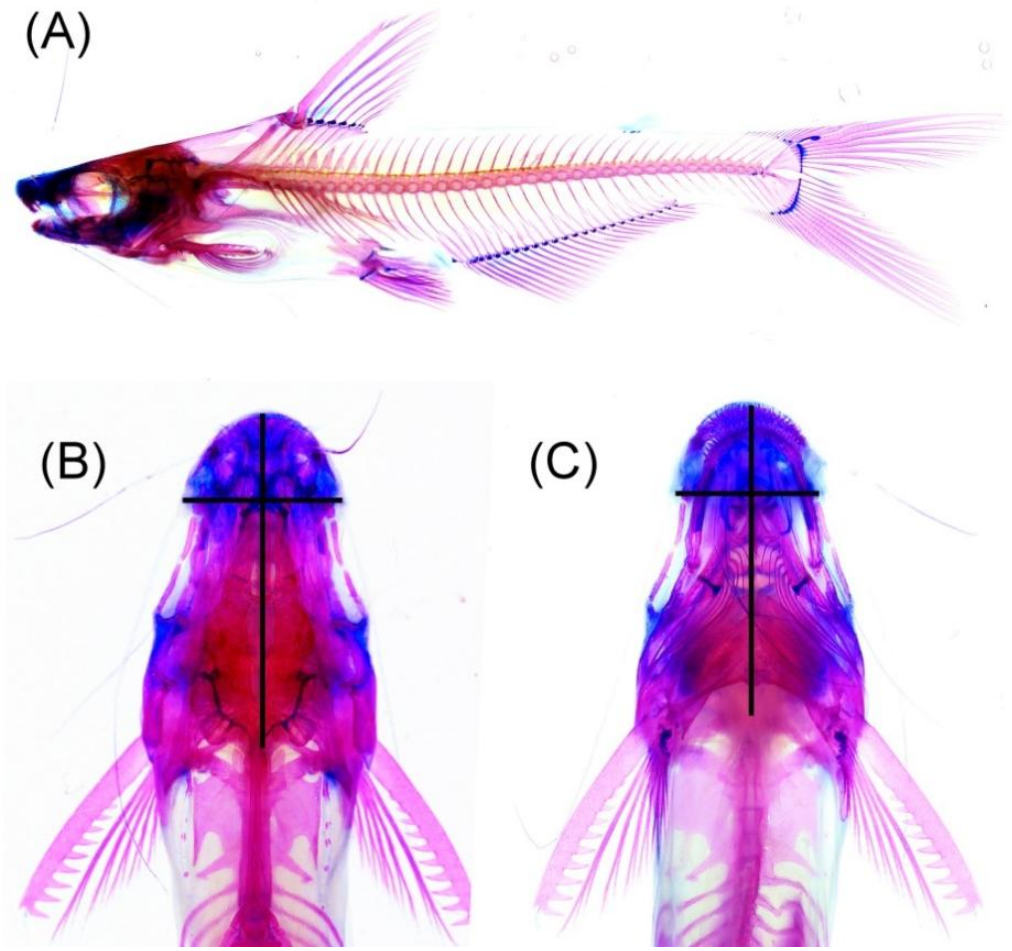


Fig. Osteology of *P. khavalchor*

(A) Clear specimen of *P. khavalchor*; (B): Dorsal view; (C) Ventral view

Behavioral assay

Results of the experiment 1 showed that, even after 96 hrs of starvation, *P. khavalchor* (Fig. 3A) did not touch the fish food pellets or frozen worms. Furthermore, it was evident from the result of experiment 2 that *P. khavalchor* did not eat the scales of dead prey fish. In this experiment even after 96 hrs of

starvation single scale from the left or right flank of dead prey fish was not eaten. On the contrary, results of experiment 3 showed that *P. khavalchor* voraciously fed on scales of live prey fish. In all trials, *P. khavalchor* attacked the prey fish and ate the scales from both right and left flank side. This was evident from the absence of few scales and formation of empty scale patches on right and left flank of the prey fish (Fig. 3B and 3C). *Pachypterus khavalchor* started feeding on scales of prey fish in laboratory conditions after 72 hrs. During the experiment, we also noticed that *P. khavalchor* showed nocturnal predatory behavior.

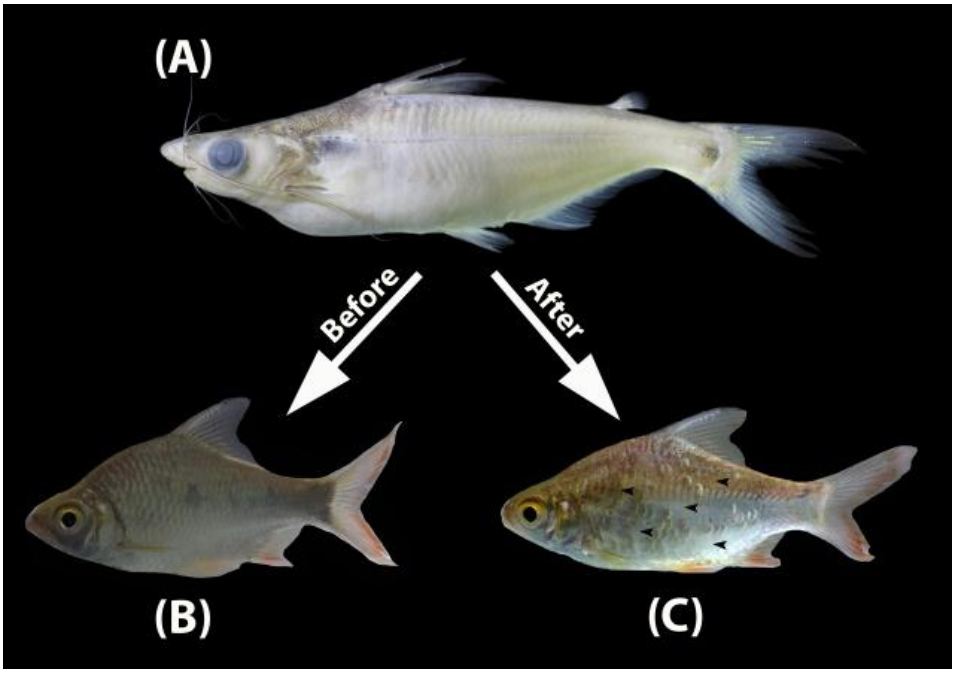


Fig. . Scale-eating behavior of *Pachypterus khavalchor*

(A) Pachypterus khavalchor adult female and (B and C) lateral view of prey (Mystacoleucus sp.) fish before and after behavioral assay respectively. Arrowheads indicate the denuded spots.

Attack behavior showed extensive chasing of prey species (Fig. 4A) followed by a powerful random strike at the flank or near to caudal region in posterior oblique position (Fig. 4B). A single powerful strike (Fig. 4C) dislodged large numbers of scales, leaving a substantially large denuded patch on the flank side of the prey (Fig. 4D). After a strike, *P. khavalchor* did not follow the prey for long distance rather immediately turned back and picked up the falling dislodged scales (Fig. 4D). The dislodged scales (as many as they can) were swallowed immediately during the attack or gathered as they sink. Scales on the substrate were lifted with the help of snout and ingested. Prey fish often escaped without much harm.

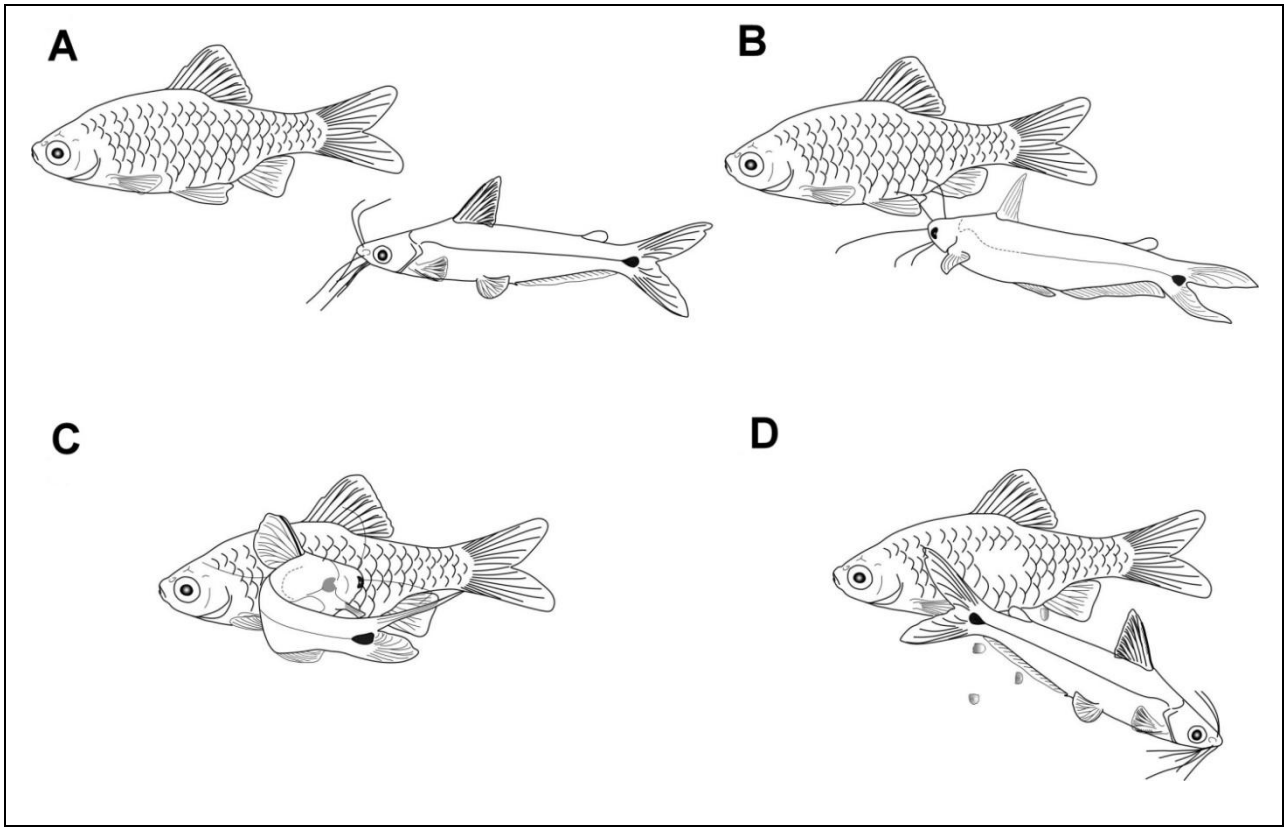


Fig. 4. Scale removing behavior in *P. khavalchor*

(A) Chasing of prey fish; (B) P. khavalchor orienting itself in oblique position and preparation for random attack; (C) Powerful strike to dislodged scales; (D) Prey fish with denuded patch and *P. khavalchor* turned back to collect the dislodged scales.

Oral morphology and Dental arrangement

Oral morphology of *P. khavalchor* is characterized by the presence of wide, sub-terminal mouth with protruding snout and without lips (Fig. 2A). Entire snout except for the region near the tip showed the presence of denticulation. Teeth present on the maxilla (undersurface of snout) were molariform, continuous directing outward and formed sub-triangular patch covering the entire undersurface of snout region (Fig. 2B). Most of the teeth present near-tip region were curved and slightly bent pointing outwards (Fig. 2B and C). Remaining maxillary teeth present beneath the snout region were straight and pointing straight. Lower jaw had two different types of the teeth. Teeth present on the lower jaw tip were much stronger and comparatively less pointed/blunt (Fig. 2B). Whereas, teeth present in the oral cavity were curved anteriorly and directed backward (Fig. 2B and 2D). Moreover, oral cavity near pharyngeal region also showed the presence of elongated, sharp villiform pharyngeal teeth on both upper and lower pharyngeal jaw (Fig. 2E).

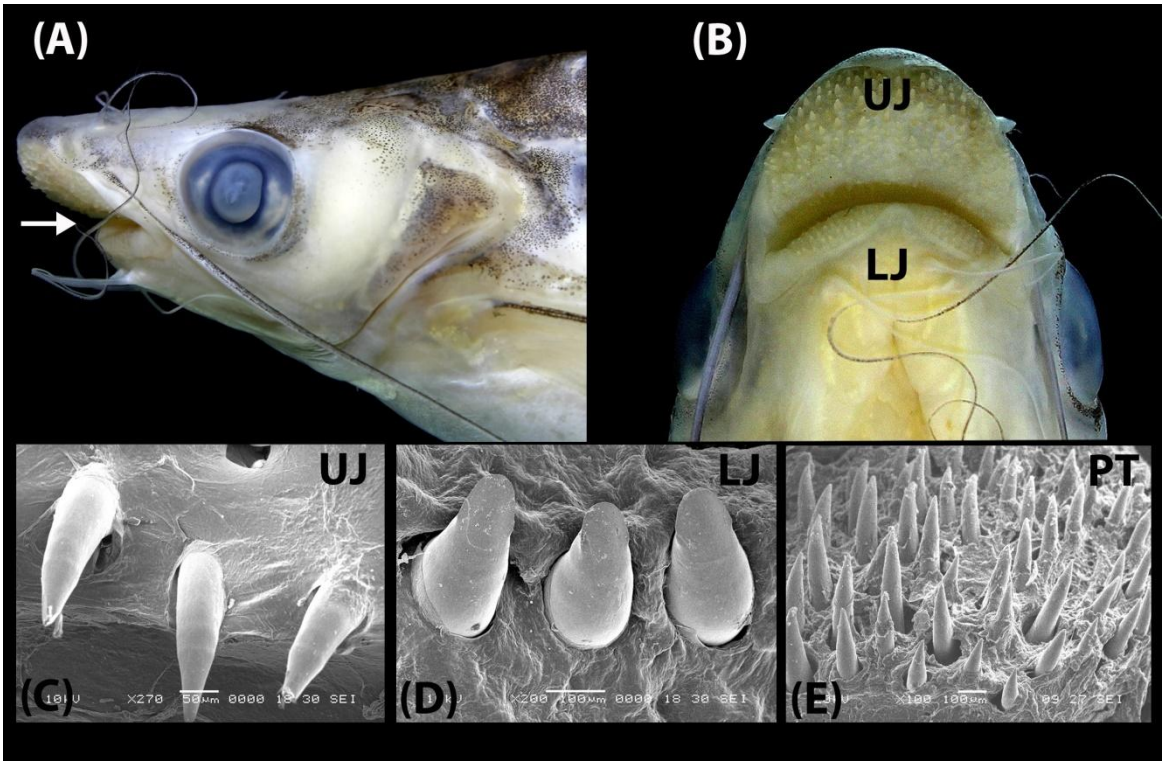


Fig. 2 Oral morphology of P. khavalchor

(A) Lateral view of the head of P. khavalchor showing sub-terminal mouth (arrow); (B) Ventral view of the mouth showing upper jaw (UJ) with large number of blunt molariform teeth and lower jaw (LJ) with sharp and pointed villiform teeth; (C, D, E) SEM of upper jaw (UJ), lower jaw (LJ) and pharyngeal teeth (PT).

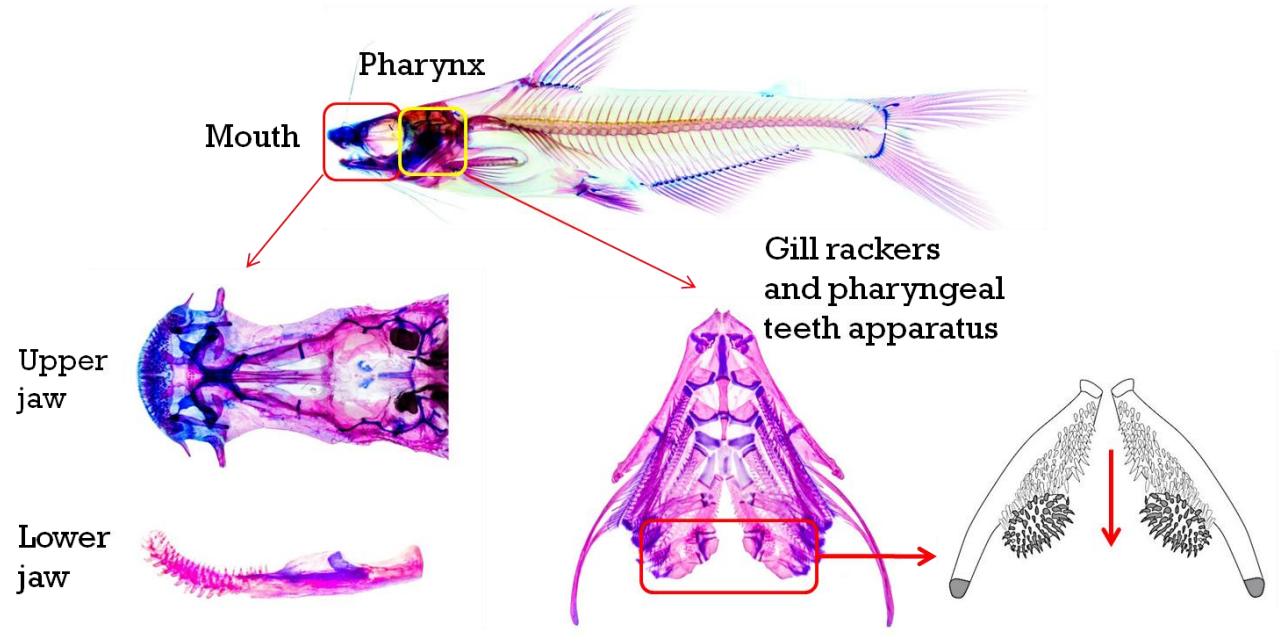


Fig. 2 Skeletal morphology of P. khavalchor

Histological and histochemical study of digestive system

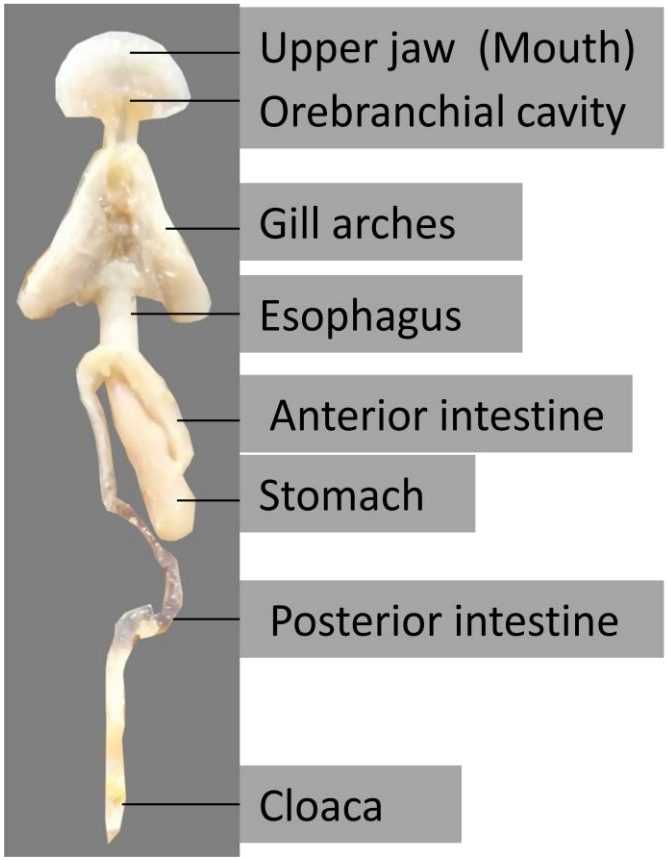


Fig. *Digestive system of P. khavalchor*

Esophagus

The esophageal wall of the *P. khavalchor* consists of three layers: Mucosa (tunica mucosa), the muscular layer (tunica muscularis) and the outer layer (tunica serosa). The mucosa is highly folded, deeply protruded into the esophageal lumen giving rise to roughly triangular shaped villi. The mucosa further consists of mucosal cells placed on the basement membrane forming boundary of the villi.

Basement membrane followed by the lamina propria, which is loose connective tissue layer. Mucosal cells are represented by a squamous epithelium. Squamous epithelium consists of large globular to oval shaped cells with flat, centrally placed nuclei. The distinguishing feature of esophageal epithelium is presence of numerous mucous cells scattered among the squamous epithelial cells. Mucous cells are large, columnar to globular in shaped with basal nuclei. Histochemistry revels that mucous cells of esophageal mucosa is positive for PAS, AB (pH=2.5) and AB (pH=1.0) indicating presence of neutral glycoconjugates, acidic glycoconjugates, and sulphated glycoconjugates. Details of staining intensity are depicted in Table 1. Lamina propria is the connective tissue layer is centrally positioned in mucosal folds and contains blood vessels and muscle cells. The sub-layer of mucosa, lamina muscularis mucosae cannot be clearly distinguished from underlying mucosal layer this was confirm to be absent. The muscular layer (tunica muscularis) was clearly visible with two sub-layers: inner longitudinal layer and outer circular layer of muscle fibres. Both layers are made up of skeletal muscle fibres. Connective tissue separates the bundles from each other. The outermost layer of the esophageal wall is tunica serosa which is thin and consists of the loose connective tissue.

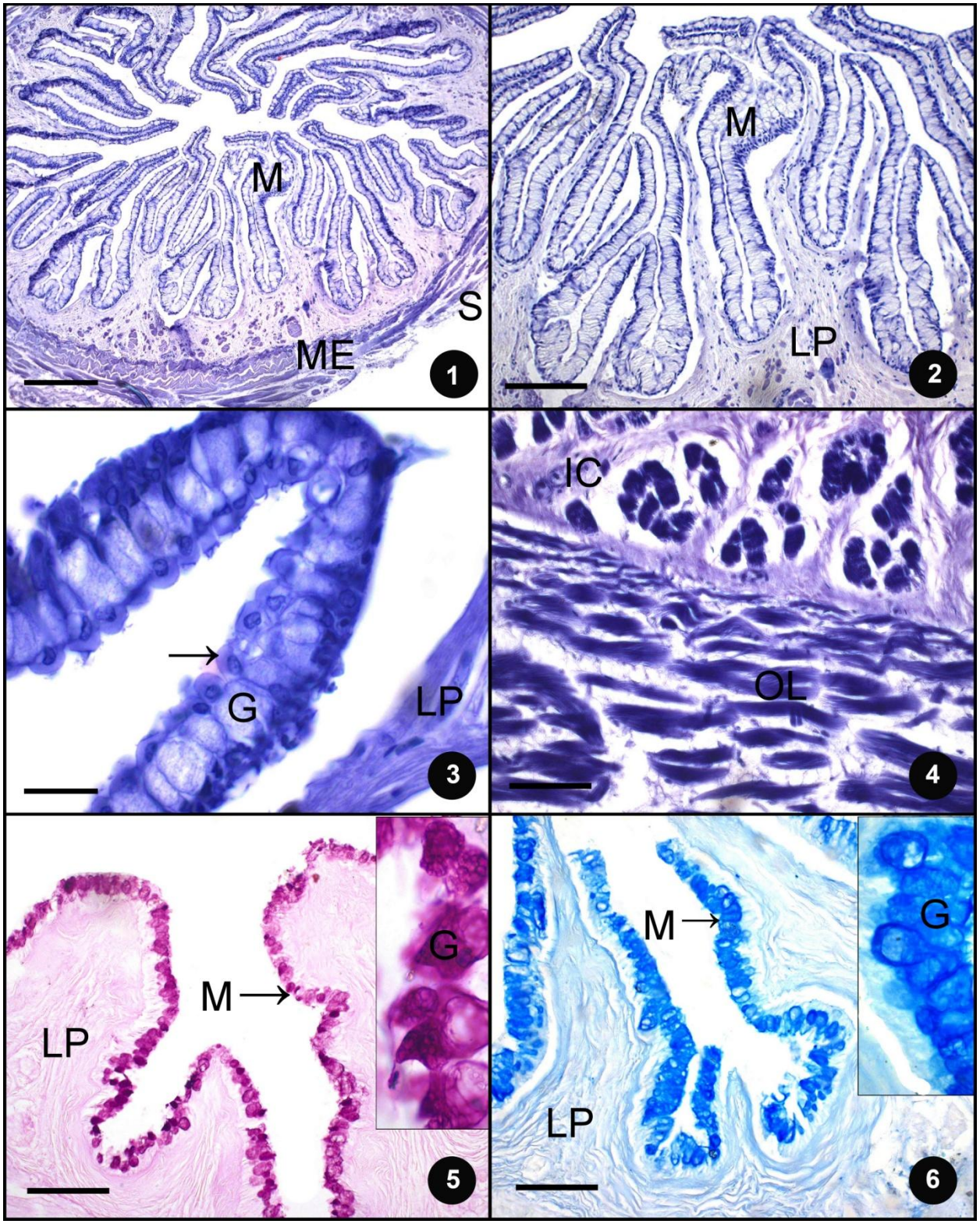


Fig. *Transverse section of esophagus.*

Stomach

The stomach of *P. khavalchor* includes three divisions: cardiac, fundic and pyloric. Histologically not much difference was observed in three divisions. However, few details need attention. Wall of the stomach was found to thick throughout its length and consists of four classical layers: Mucosa (tunica mucosa), submucosa (tunica submucosa) the muscular layer (tunica muscularis) and the outer layer (tunica serosa). The sudden shift in the mucosal lining from squamous epithelium to columnar epithelium and lack of secretory cells is noticeable. Mucosa consists of single layer of epithelial columnar cells (lamina epithelialis), placed o lamina propria followed by lamina muscularis mucosae. The mucosal epithelium along with lamina propria forms well-defined folds in the stomach lumen. Whereas, the lamina muscularis mucosae forms the border of mucosa layer and separates it from underlying submucosa. The epithelial columnar cells show numerous large nuclei at the basal part. Lamina propria consists of many simple tubular gastric glands. The gastric glands consist of large cells with eccentric nuclei and granule filled cytoplasm. When stained with PAS, the columnar epithelium cells were found to strongly positive whereas gastric glands were weekly positive probably because they contained neutral glycoconjugates. Furthermore, when stained using AB (pH=2.5) only gastric glands were found to be weekly positive indicating presence of acidic glycoconjugates. No reactivity

was observed in columnar epithelium cells and gastric glands when stained using AB (pH=1). The mucosa and submucosa are separated from each other by a thin layer of lamina muscularis mucosae, formed of circular strands of smooth muscles. The submucosa (tunica submucosa) is thick layer of the loose connective tissue containing numerous blood vessels, nuclei of fibroblast, nerves and lymph-like vessels. The muscular layer (tunica muscularis) consists of two muscle layers: Inner thick circular layer and outer longitudinal layer. Inner layer was found to be thicker as compared to outer layer. The outermost layer (tunica serosa) consists of thin layer of connective tissue fibres. No histological differences were in the fundic stomach region. The pyloric region of *P. khavalchor* contains no pyloric caeca. The pyloric stomach also shows similar histological arrangement of the four basic layers. The surface epithelium was similar to that of cardiac and fundic region. However, absence of gastric glands was noticeable. The main structural difference of the pyloric stomach is the presence of the pyloric sphincter (a strong circular muscle) at the junction of pyloric end of stomach and anterior intestine. The pyloric sphincter shows extremely thick and strong inner muscular layer of tunica muscularis. The tunica serosa is similar to that of rest of the stomach.

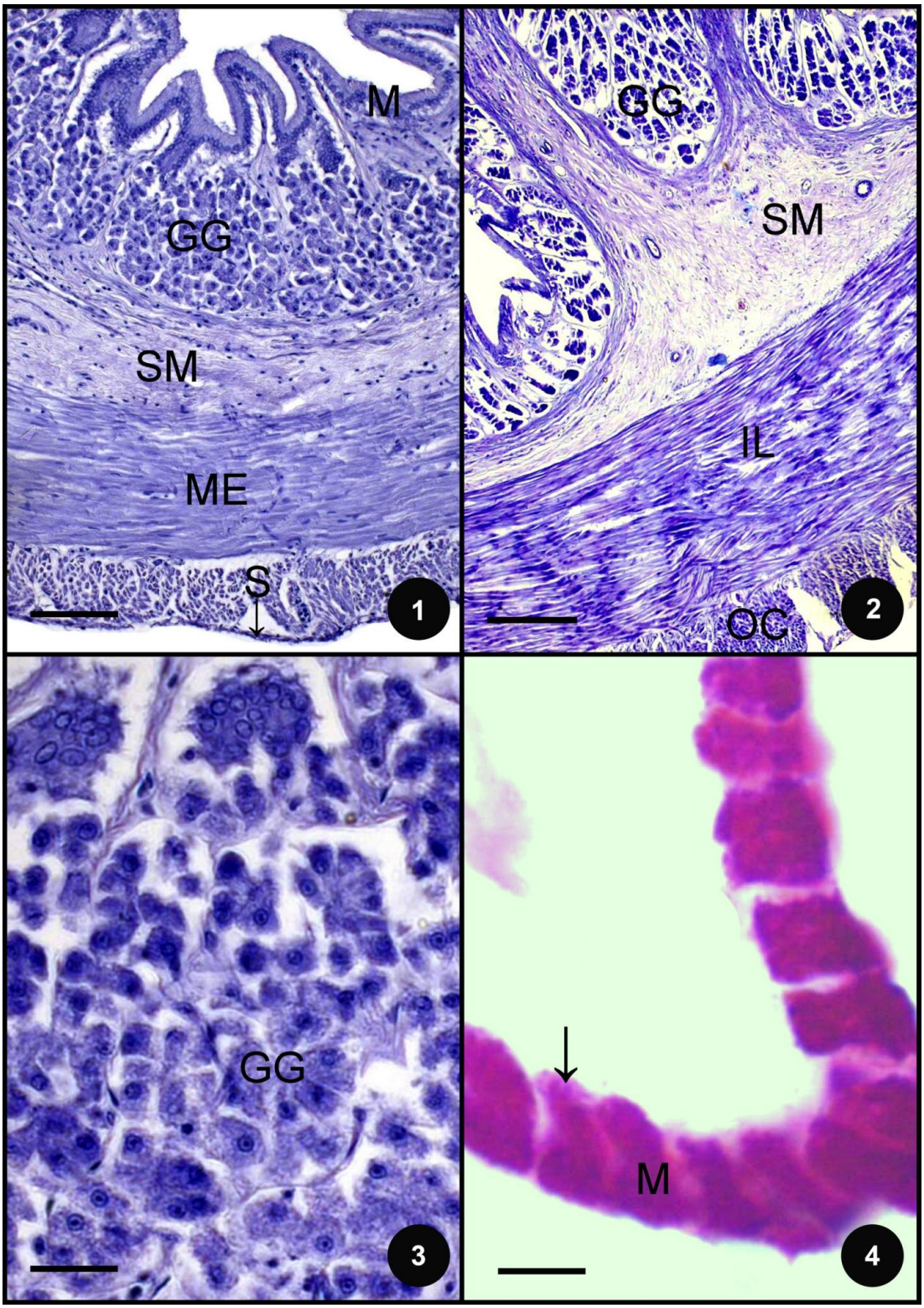


Fig. *Transverse section of stomach*

Intestine

According to the morphological characteristics, the intestine in the *P. khavalchor* could be distinguished into two parts: the anterior and the posterior intestine. Anterior as well as posterior intestine is similar in terms of histology and histochemistry. However, anterior intestine is comparatively larger in diameter than posterior intestine. Wall of the intestine consists of three layers: Mucosa (tunica mucosa), the muscular layer (tunica muscularis) and the outer layer (tunica serosa). The intestinal mucosa is three layered structure comprising epithelium, lamina propria and muscularis mucosae. All the three layers participate in the formation of triangular shaped mucosal folds (villi) into the intestinal lumen. The intestinal epithelium is simple columnar epithelium with numerous microvilli. Columnar epithelium consists of large cells with numerous microvilli at the apical end and basally placed nuclei. The goblet cells are scattered in between the epithelial cells. Each goblet cell is large bubble-like structure, filled with the mucus. Goblet cell opens outside at the base of epithelial microvilli. When stained using PAS, AB (pH=2.5) and AB (pH=1.0), the goblet cells were stained intensely indicating presence of neutral glycoconjugates, acidic glycoconjugates, and sulphated glycoconjugates in large amount. A narrow layer of the connective tissue containing blood vessel enters into each villi thus forming the lamina propria, which is second sub-layer of the intestinal mucosa. Some smooth muscle cells

representing the lamina muscularis mucosae could be seen. However due to absence of the submucosa it is hard to distinguish lamina muscularis mucosae from underlying muscle layer. The muscular layer (tunica muscularis) consists of two muscle layers: Inner thick circular layer and outer longitudinal layer. Tunica serosa which consists of loose connective tissue forms the outermost boundary of the intestinal wall.

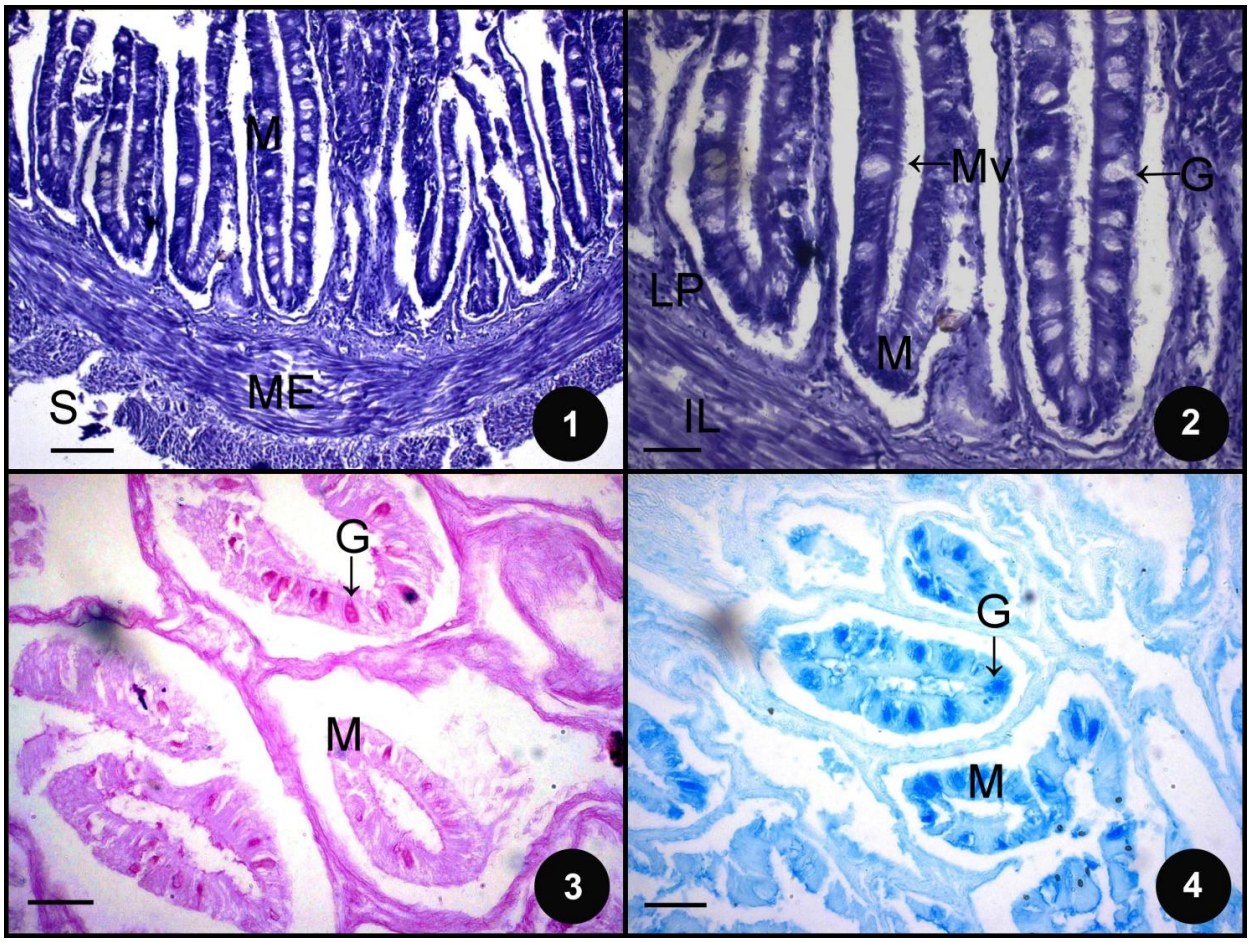


Fig. *Transverse section of anterior intestine*

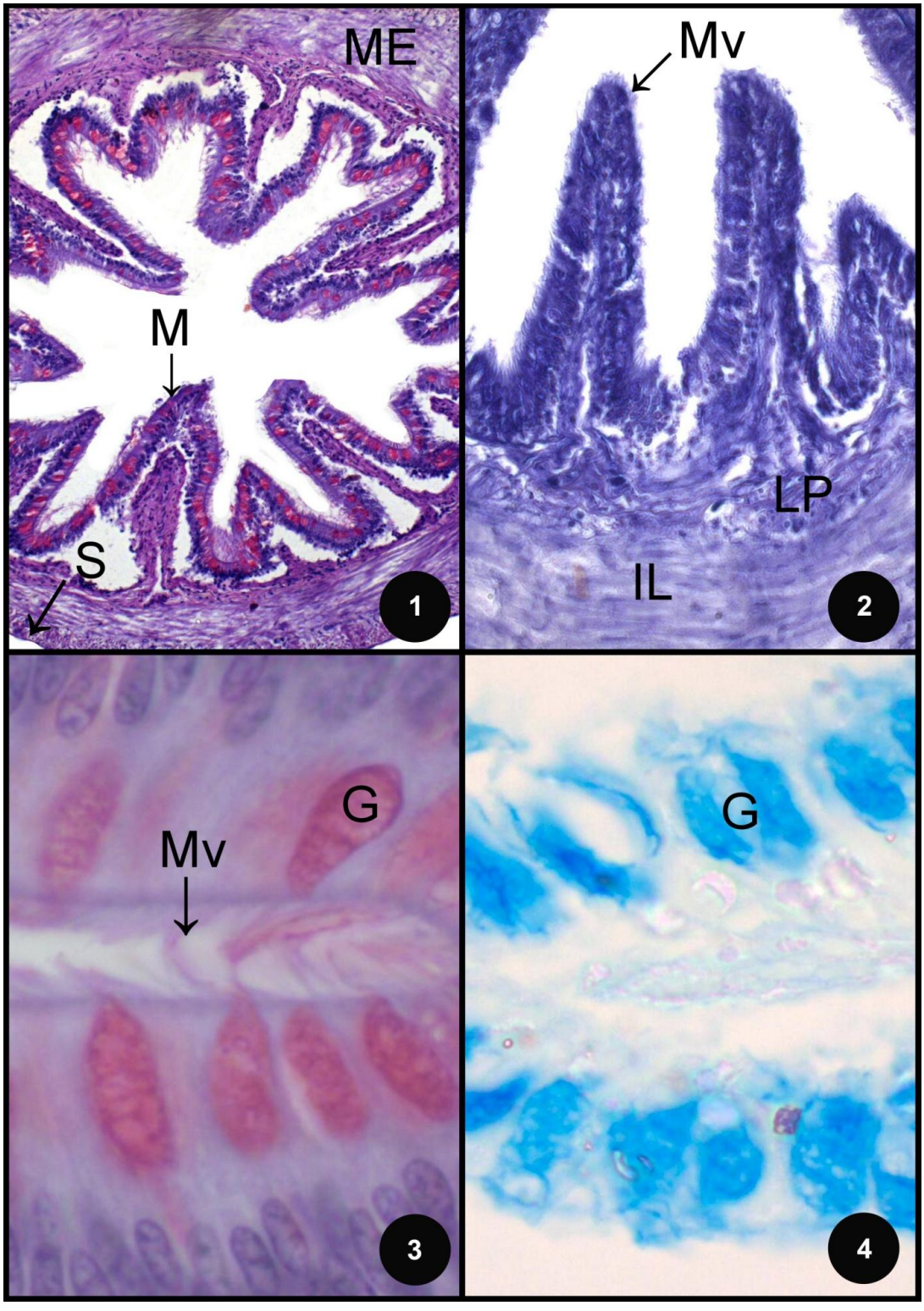


Fig. *Transverse section of posterior intestine.*

Liver

The liver of *P. khavalchor* is bilobed organ, placed above the anterior intestine and surrounds the cardiac portion of the stomach. The liver parenchymal tissue shows presence irregularly arranged hepatocytes separated from each other by hepatic sinusoidal capillaries. Sinusoidal capillaries open together into the central vein. Each hepatocyte is roughly triangular shaped cell with large nucleus and lipid droplets dispersed in the cytoplasm.

Gut microbiota and its role in lepidophagy

Morphological, biochemical and molecular characterization of isolates

Total 9 bacterial isolates were isolated from the stomach. Out of these, only two isolates were chitinase positive (Table 1). Based on the nucleotide homology of 16S rRNA gene sequences, one of the isolates showed 99% nucleotide homology with *Bacillus pumilus* and *Bacillus safensis*. As these two species could not be further differentiated on the basis of our 16S rRNA partial gene sequence, we used biochemical approach suggested by Satomi et al., (2006). Production of acid by utilization of inositol is the characteristic exhibited by *B. safensis* but not by *B. pumilus*. Our isolate failed to produce acid from inositol, hence confirming its

identity as *B. pumilus*. The other isolate was identified as *Bacillus licheniformis.* Details of the colony characteristics for two isolates are provided in Table 1.

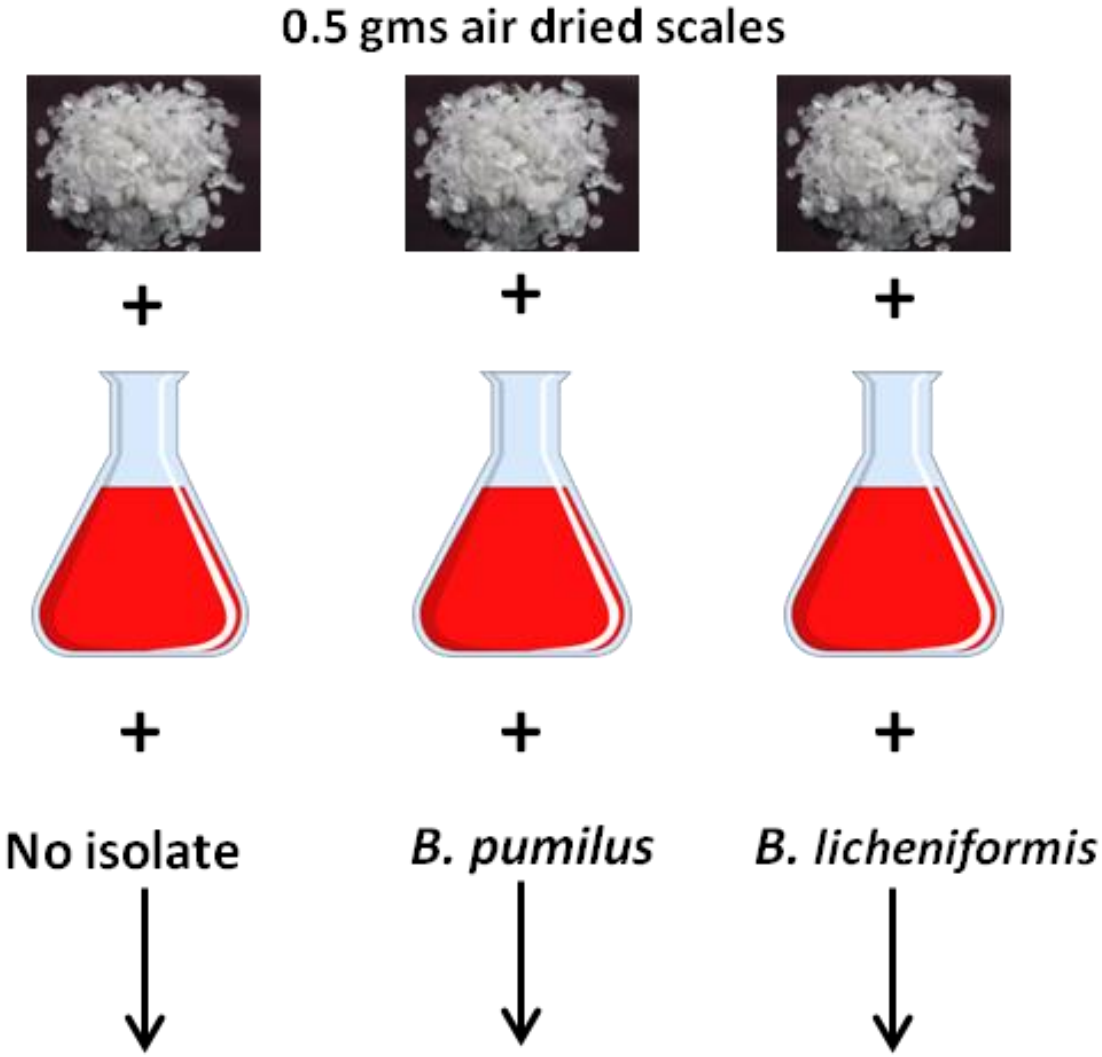
Table . Morphological characteristic of gut microflora and qualitative assay for chitinase production

Character	*Bacillus pumilus*	*Bacillus licheniformis*
GenBank accession number	KU499849	KU499850
MCC depository number	MCC 2886	MCC 2830
Color	Off-white	Off-white
Shape	Circular	Circular
Size	0.1	0.2
Margin	Rough	Rough
Elevation	Slightly convex	Raised
Opacity	Opaque	Opaque
Consistency	Sticky	Sticky
Motility	Motile	Motile
Gram character	Positive	Positive
Chitinase production	Positive	Positive

In vitro fish scale degradation assay

In vitro fish scale degradation assay showed a significant difference in the remaining scales after 72 hrs incubation in control and two different tests ($F_{(2, 6)}$ = 1284; $p < 0.0001$). There was a significant difference between the remaining scales in control and *B. pumilus* (Tukey's pairwise comparison, Q = 63.68*;* p = 0.001, Fig. 1). Similarly, a significant difference was observed in control and *B. licheniformis* (Tukey's pairwise comparison, Q = 60.32*;* p = 0.001, Fig. 1).

However, there was no significant difference between *B. pumilus* and *B. licheniformis* (Tukey's pairwise comparison, $Q = 3.351$; $p = 0.1207$, Fig. 1).



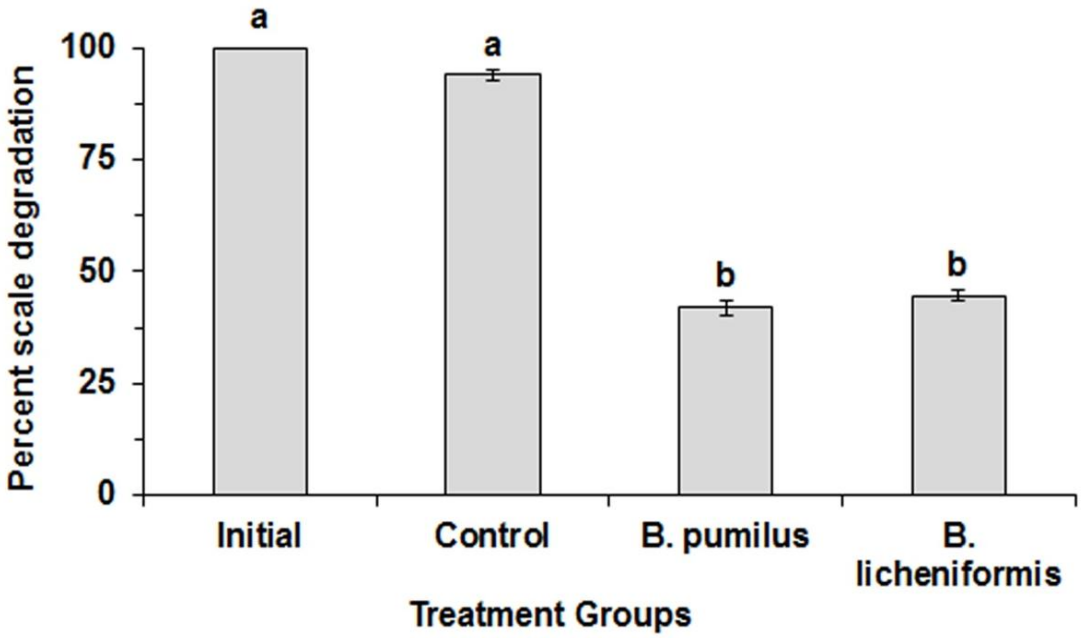


Fig. Chitinase activity Zone of clearance can be seen in media plate due to chitinase activity

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APPENDIX