

Reproductive biology of the endemic ornamental barb, *Puntius pookodensis* Anna Mercy and Eapen Jacob 2007, from the Western Ghats of India

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ABSTRACT

Puntius pookodensis is endemic to the Pookode Lake, Wayanad, northern Kerala, which is a part of Western Ghats of India, a biodiversity hot spot. One hundred and twelve specimens collected from the Pookode Lake during 2007 were used to study the reproductive biology of the species. Six maturity stages could be identified in the females, based on external morphology and ova diameter distribution, examined microscopically. Size at first maturity was 32.5 mm TL (30-35 mm) in male and 38.5 mm TL (35-40 mm) in female. Ovary showed asynchronous development. Oogenesis was studied using the standard histological procedures. The oocyte development was classified into nine different oogenic stages. *P. pookodensis* was found to be a multiple spawner with protracted spawning. Absolute fecundity ranged from 426-823 in fishes of size range 35-57 mm TL and 0.555 - 2.267 g body weight. The number and size of eggs were found to be directly proportional to the size and age of the fish, with fecundity showing positive linear relationship with the length and weight of the fish as well as ovary weight.

Keywords: Maturity stages, Oogenesis, Ova diameter distribution, *Puntius pookodensis*, Reproductive biology

Introduction

Puntius pookodensis Anna Mercy and Eapen Jacob, 2007 is a newly identified species of *Puntius*, which is an indigenous ornamental fish of the family Cyprinidae (Anna Mercy and Eapen Jacob, 2007). It is endemic to the Western Ghats of India, a biodiversity hot spot and inhabits only the Pookode Lake, a freshwater lake in Wayanad District, Kerala. *P. pookodensis* has all the desirable qualities of an ornamental fish. This fish possesses an iridescent silvery body and yellowish fins with one or two broad black blotches at the caudal peduncle and a shoulder spot. Males develop prominent reddish orange streak at the posterior end during breeding time. Anna Mercy *et al.* (2005a) have described the technology for captive breeding of *P. pookodensis*. The present study was carried out to understand the various aspects of the reproductive biology of this species to help its conservation as well as commercial production.

Materials and methods

A total of 112 specimens (77 females and 35 males) of *P. pookodensis* collected from the Pookode Lake in Wayanad District, northern Kerala during 2007 were used for the present study. Size range of males (Fig. 1) was 20-55 mm TL and females (Fig. 2) was 25 - 60 mm TL. The different aspects of breeding biology *viz.*, size at first

maturity, spawning frequency based on ova-diameter studies and fecundity were studied using standard methods (Nair and Nair, 1984).



Fig. 1. Male *P. pookodensis*

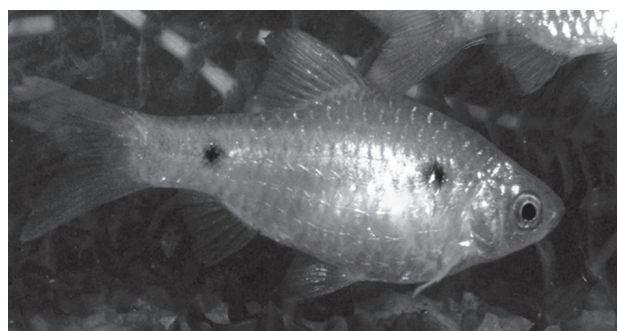


Fig. 2. Female *P. pookodensis*

Classification of maturity stages

Classification of maturity stages was done based on external evaluation of the gonads. Characters used for the purpose were colour and shape of gonad, space occupied by the gonad in the body cavity, size of ova, and texture of ovary and blood supply. Ova diameter measurements were made from the different maturity stages using an ocular micrometer in order to trace the development of ova as maturation progresses. Ovaries belonging to each stage of maturity were preserved in Gilson's fluid (Bagenal and Braun, 1968) for ova diameter measurements. A mixed sub-sample was taken from different parts of each ovary to eliminate the error due to differential distribution of ova stocks in the different parts of the ovary. The ova diameter measurements were done on a monocular microscope with 4×10 magnification, after calibrating the ocular micrometer using a stage micrometer.

Histology

For histological studies, fresh material was used. Small pieces of the ovary at different stages of maturity were fixed in Bouin's fluid. Paraffin sections at 6 - 8 μm thickness were stained with Harris's haematoxylin and counter stained with eosin (Weesner, 1960). The sequence of histological changes during the origin, maturation and liberation of the germ cells were studied from these sections.

Spawning frequency

Ova diameter measurements of ripe ova were made using an ocular micrometer. A mixed sub-sample from different parts (anterior, middle and posterior) of a ripe ovary was taken. Ova diameter classes (64 μm interval) and their respective percentage frequencies were plotted to study the spawning frequency.

Size at first maturity

The length at first maturity is the size at which 50% of the population is mature. The total lengths of all the fishes collected for the study were grouped into different length classes of 5 mm class interval. The percentage occurrences of mature fishes (early ripening, late ripening, ripe and partially spent) for the females and males were taken. By plotting the percentage occurrence of mature fish (males and females) against respective length classes, the length at which 50% of the fishes were mature was demarcated (Nair and Nair, 1984).

Fecundity

The ovaries, being small, were kept whole in Gilson's fluid with appropriate labels showing standard lengths, ovary length, and ovary weights. They were then shaken vigorously and left to stand for about 24 h. For the absolute

fecundity, all the ripe/ripening eggs (yolked eggs) in the ovary were counted as per Hickling and Rutenberg (1936).

Relative fecundity was expressed in terms of eggs per unit length and weight of the fish and ovary. The linear relationship between absolute fecundity and (i) total length, (ii) body weight, and (iii) weight of ovary were computed by regression analysis by the method of least squares after \log_{10} transformation and the linear equation fitted was of the form (Zar, 1999):

$$\log Y = \log a + b \log X$$

The gonadosomatic index (GSI) was estimated for ripe fishes using the formula:

$$\text{GSI} = \frac{\text{Weight of the ovary} \times 100}{\text{Weight of the fish}}$$

Results and discussion

A series of scales of ripeness have been worked out for each group of fishes for delimiting the different stages of the sexual cycle, based on a universal scale of six stages proposed by Nikolsky (1963), mainly for temperate species. In the present study, a modified six-stage key based on the reproductive strategy of tropical fishes was used.

(i) Stage-I: Immature virgin

These are the young individuals that have not yet spawned. Ova not visible to naked eye. A cursory examination under the microscope was required to differentiate ovary from the testis. Microscopically, the oocytes were oval/spherical in shape and completely transparent with a large nucleus. Maximum size of the ova was 160 μm (Fig. 3a). Under microscope the testes appeared as undifferentiated tissue.

(ii) Stage-II: Maturing virgin

The length of both the lobes equal. Ova still microscopic oval/spherical in shape with large nucleus and with slight yolk deposition up to yolk vesicle stage. Ova size reached up to 352 μm (Fig. 3b). The testes occupied less than 1/4 of the body cavity and were thin and transparent.

(iii) Stage-III: Early ripening (Spent recovering)

Ovaries generally off white /creamy in colour occupying half to less than three fourth of the body cavity. Ova quite visible to the naked eye. The maximum size of ova was about 640 μm (Fig. 3c). The testes were transparent occupying about 1/4 of the body cavity.

(iv) Stage-IV: Late ripening

Ovaries pale yellow in colour, occupying three-fourth or more of the body cavity. The maximum size of ova was

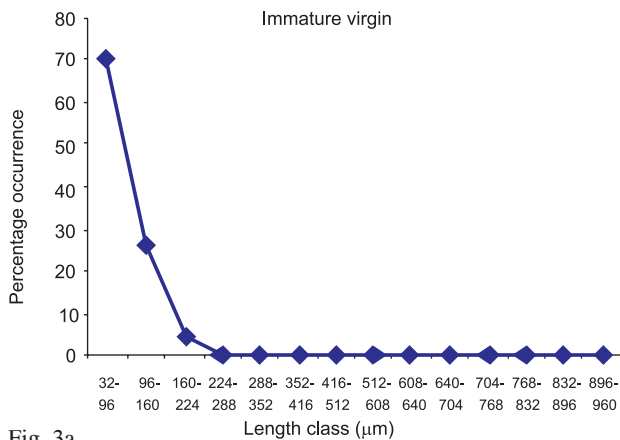


Fig. 3a.

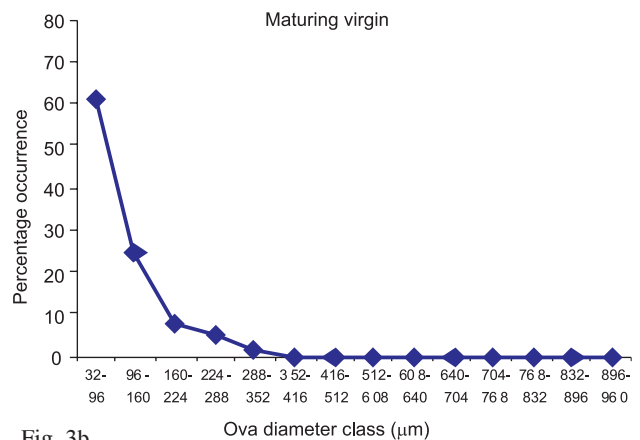


Fig. 3b.

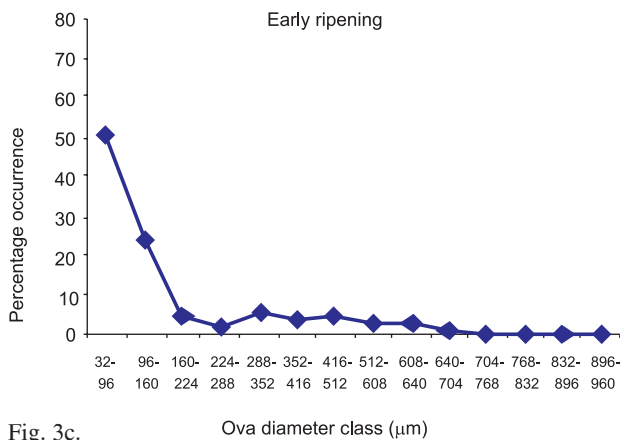


Fig. 3c.

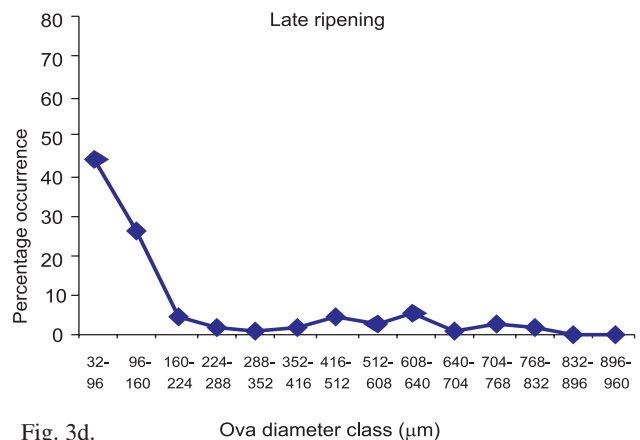


Fig. 3d.

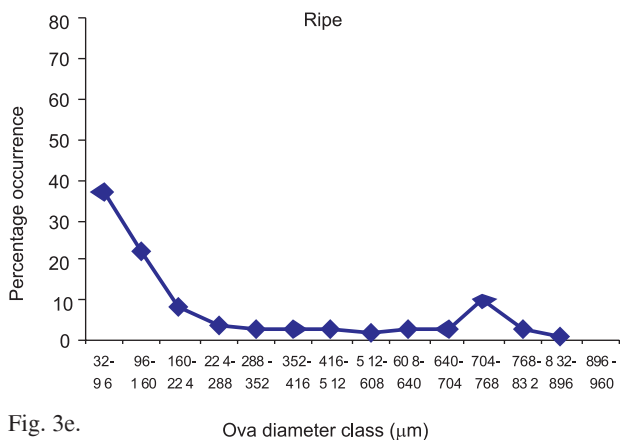


Fig. 3e.

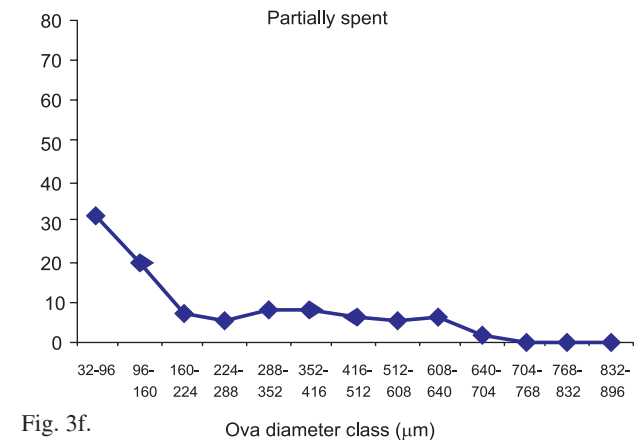


Fig. 3f.

Fig. 3a-f. Oocyte size frequency distribution in *Puntius pookodensis* at different maturity stages of ovary

about 800 μ (Fig. 3d). The testes occupied less than $\frac{1}{2}$ of the body cavity.

(v) *Stage-V: Ripe*

Ovaries yellow in colour, very turgid and occupied more than $\frac{3}{4}$ of the body cavity. Ovarian lobes quite stout and equal in length. Ova size reached a maximum of

896 μ (Fig. 3e). The testes occupied about half of the body cavity and the lobes were white and fleshy.

(vi) *Stage-VI: Partially spent*

Ovaries at this stage were slightly flaccid, but still retaining a number of residual ripe ova/atretic ova after the spawning. The largest ova that could be found in this stage

was 704 μ (Fig. 3f). This stage enters the maturation cycle at early ripening stage. In this stage, testes looked flabby and occupied about $\frac{1}{4}$ of the body cavity.

Histology

Based on the morphological changes taking place in the ovum during the course of maturation, nine stages were discernible (Table 1; Fig. 5 - 10). These were usually based on the size, amount and distribution of various cell inclusions like nucleus, nucleolus, and other cytoplasmic inclusions like yolk nucleus, yolk vesicles, yolk granules and lipid globules.

Table 1. Oogenic stages during oocyte development in *P. pookodensis*

Chromatin nucleolus stage: (Fig. 7)	Measuring about 32 μ in diameter, and the youngest encountered in the different maturity stages of the ovary. Spherical nucleus with a distinct nuclear membrane enclosing centrally placed large nucleolus and the chromatin material scattered in the nucleoplasm.
Early perinucleolus stage: (Fig. 7)	Oocytes appear bigger, more spherical due to accumulation of more cytoplasm. The centrally placed nucleus had also grown into germinal vesicle acquiring a spherical shape, followed by formation of the nucleoli, while the existing nucleolus, has moved to periphery almost retaining earlier size and structure.
Perinucleolus stage: (Fig. 7)	The oogonium remained spherical shape but further increased in size. The chromatin nucleolar and perinucleolar stages are sometimes referred to as 'primary growth phase' (Wallace and Selman, 1981) or first growth phase (Forberg, 1982).
Yolk vesicles stage: (Fig. 7)	The follicular layer got closely pressed on to the outer surface of the oocyte, getting squeezed between the enlarged oocytes and not as distinct as in the previous stage. The oocytes much enlarged. The vitelline membrane distinct in this stage as a compact membrane of dense, homogenous cytoplasm surrounding the oocyte. Empty yolk vesicles appeared peripherally.
Primary yolk stage: (Fig. 8)	The appearance of clusters of minute granules, yolk granules in the periphery of the cytoplasm and the complete proliferation of yolk vesicles were distinguishing characters of this stage. The follicular layer was thin. All the nucleolar bits were seen migrated to the nuclear periphery, closely aligned to the inner surface of the nuclear membrane and showed clumping tendency.
Secondary yolk stage: (Fig. 8)	The yolk vesicles were found to clump together, fusing to form larger vesicles. The yolk formation in this stage began with the accumulation of yolk globules in the periphery of the oocytes below the vitelline membrane.
Tertiary yolk stage: (Fig. 8)	The follicular layer and vitelline membrane remained prominent as before and were almost equally thick. The yolk globules completely filled the cytoplasm.
Migratory nucleus stage: (Sub-peripheral nucleus stage) (Fig. 9)	The distinguishing features of this stage are the peripheral migration of the germinal vesicle and the liquefaction of the nucleolar material, which exude into the cytoplasm. The follicular layer very conspicuous, the individual cells being more distinguishable. The yolk globules almost completely fill the cytoplasm.
Mature oocyte: (Fig. 8)	The vitelline membrane developed into a well recognizable membrane. The yolk vesicles are few and scattered with the interspaces filled with yolk globules and granules. Yet another development at this stage is the formation of the micropyle.
Partially spent: (Fig. 10)	In the partially spent and spent recovering ovary, the few unspawned eggs undergo degeneration and there were the empty follicles of the spawned ova. These empty pockets easily get pushed out by the fast developing immature oocytes.

granules and globules are characteristic of vitellogenic oocytes (primary, secondary and tertiary yolk stages) as per Yamamoto (1956), Yamazaki (1965) and Davis (1977).

Spawning frequency

The distribution and percentage frequency of ova stocks (based on ova diameter in different stages of ovarian development), are shown in Fig. 3a-f. From the ova diameter frequency distribution of a ripe ovary, it is evident that there are three divisions of eggs; one batch with immature eggs, another batch with ripe eggs and in between

In *Puntius pookodensis*, the yolk vesicles appear at the end of perinucleolar stage. The yolk granules appear first at the primary yolk stage and the yolk globules at the secondary yolk stage. The presence of yolk spheres,

immature and ripe stock there are many irregular batches of ripening eggs (Fig. 3e).

Ova diameter studies reveal that this species comes under the category D of Karekar and Bal's classification

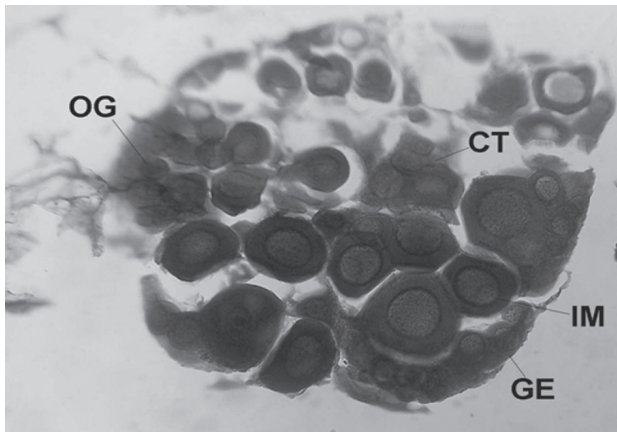


Fig. 5. Transverse section of immature ovary
 IM : Immature oocyte
 OG : Ovigerous lamella; CT: Connective tissue
 GE : Germinal Epithelium, (H&E; x80)

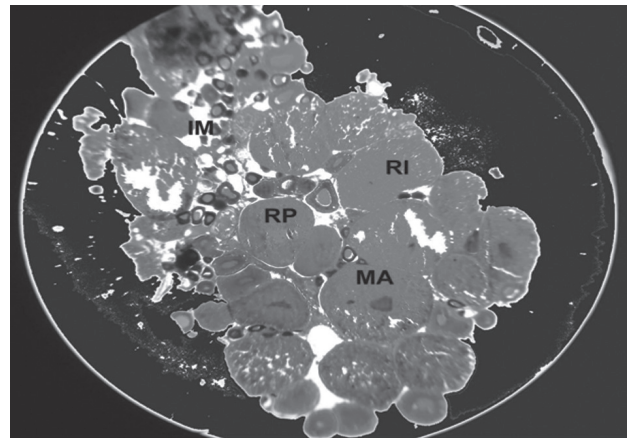


Fig. 6. Transverse section of ripe ovary
 IM : Immature ova; RI: Ripening ova;
 RP : Ripe ova; and MA: Mature oocyte
 (H&E; x80)

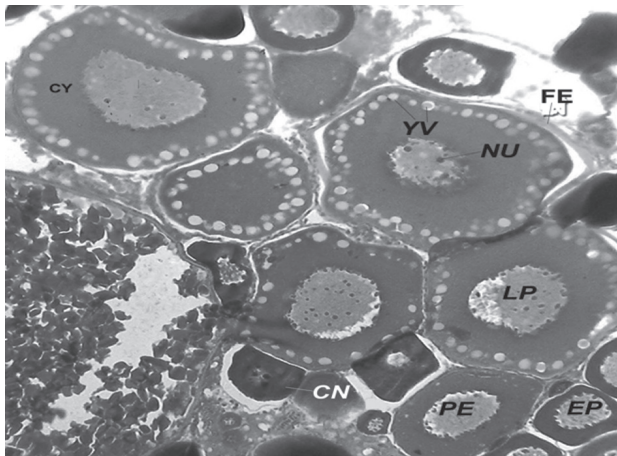


Fig. 7. Part of ovary showing - CN: chromatin nucleus stage
 EP : early perinucleolar stage; PE: perinucleolar stage;
 LP : late perinucleolar stage; YV: yolk vesicle stage;
 NU: nucleoli; FE: follicular envelope;
 CY: cytoplasm, (H&E; x400)

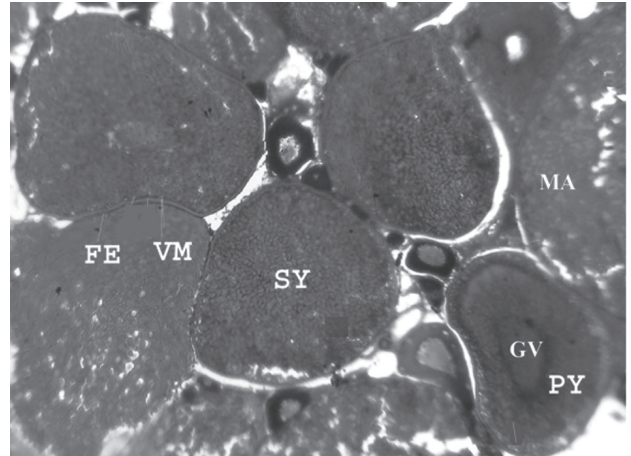


Fig. 8. Part of ovary showing - PY: primary oocyte
 SY : secondary oocyte; GV: germinal vesicle;
 FE : follicular envelope; VM: vitelline membrane;
 MA: mature oocyte, (H&E; x400)

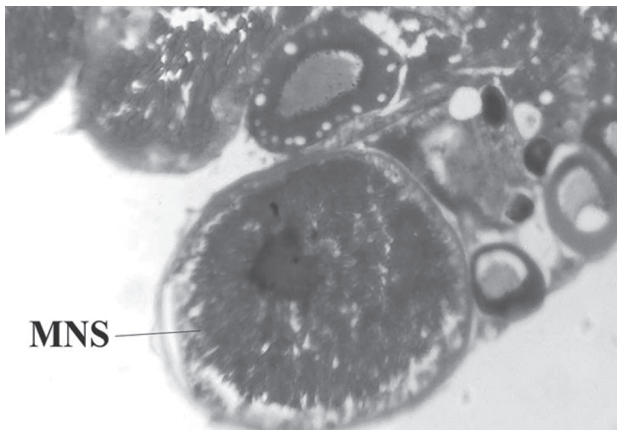


Fig. 9. Part of ovary showing MNS: migratory nucleus stage
 (H&E; x400)

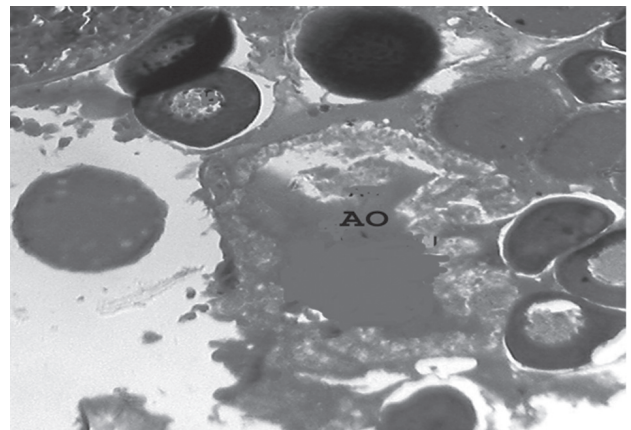


Fig. 10. Section of ovary showing AO: atretic oocyte
 stage with rupturing follicular envelope, (H&E; x400)

(1960), characterized by spawning that is extended over a very long period of almost round the year with individual spawning intermittently. *P. pookodensis* may thus be designated as a multiple spawner with an asynchronous ovarian development. In such tropical fishes, the oocyte size frequency distribution is continuous except in ripe ovaries, where there may be a clear separation between the ripe and yolked oocytes (Wallace and Selman, 1981), a pattern very clearly exhibited in *P. pookodensis*. De Vlaming (1983) also found that most species with asynchronous development have protracted spawning season with multiple spawning. The classic examples for this strategy among cyprinids are the minnows and barbs largely restricted to the lake and river margins and to very shallow riffle areas (Mills, 1991). This condition would be associated with less seasonal environment, early maturation, smaller body size and smaller relative ovary size presumably at the cost of a short life span, typically shown by the small tropical cyprinid barbs.

Size at first maturity

The first mature male was observed in the 25-30 mm length group (16.66%) and the first mature female was observed only in the 30-35 mm length group (13.33%). All males of total length 40 mm and above and all females of total length 45 mm and above were found to be mature. The size at first maturity for males was 32.5 mm TL (30-35 mm) and 38.5 mm TL for females (35-40 mm) (Fig. 4).

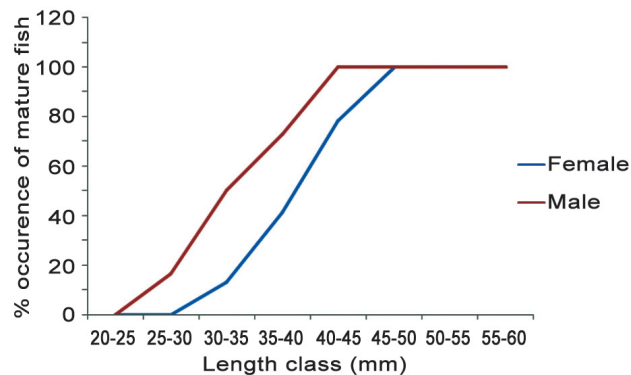


Fig. 4. Size at first maturity in male and female *P. pookodensis*

Fecundity

Absolute fecundity ranged from 426 to 823 in fish of 35-57 mm TL, 0.555 - 2.267 g body weight and 70-310 mg ovary weight. The relative fecundity values ranged from 121.71 - 164.6 per cm body length, 330.6 - 767.57 per g body weight of fish and 2.55 - 7.05 per mg ovary weight. Relative fecundity values decrease with increase in the length and weight parameters.

The absolute fecundity and relative fecundity values derived are given in Table 2 and 3. Fecundity increases

with increase in the length and weight parameters. All the linear relationships are significant at 1% level (Table 4).

Table 2. Absolute fecundity of *P. pookodensis*

Total length (mm)	Body weight (g)	Ovary weight (g)	Fecundity
35	0.555	70	426
41	1.032	135	745
42	1.029	143	602
48	1.171	112	790
49	1.95	285	802
50	1.65	259	823
51	1.83	152	791
55	1.95	182	730
57	2.267	310	792

Table 3. Relative fecundity of *P. pookodensis*

Fecundity	Per cm body length	Per g body weight	Per mg ovary weight
426	121.71	767.57	6.09
745	181.71	721.9	5.52
602	143.33	585.03	4.21
790	164.58	674.64	7.05
802	163.67	411.28	2.81
823	164.6	498.8	3.2
791	155.1	432.24	5.2
730	132.73	374.36	4.01
792	138.95	349.36	2.55

Table 4. Relationship between fecundity and length of fish, weight of fish and ovary weight in *P. pookodensis*

Variant	Equation	Correlation coefficient (R)
Total length (cm)	$\log F = 2.1066 + 1.106 \log TL$	0.807*
Body weight (g)	$\log F = 2.789 + 0.350 \log TL$	0.840*
Ovary weight (g)	$\log F = 2.101 + 0.333 \log TL$	0.975*

*Significant at 1% level

In the present study, the number of eggs released was found to increase with size, ranging from 426 for 35 mm fish to 823 for a fish of size 50 mm. With increase in body size, the relative fecundity per mg ovary weight decreased from 7.05 to 2.55 in *P. pookodensis*. This indicates an increase in size and weight of eggs from the small recruit spawners to the large repeat spawners. Anna Mercy *et al.* (2005b) reported similar findings in *Danio malabaricus*. Fecundity is most significantly correlated with gonadal weight. The correlation coefficient of fecundity with total length is almost similar to that of fecundity with body weight. Similar findings have been reported in six *Barbus* species from Sri Lanka (De Silva *et al.*, 1985).

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