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Original Article

Reproductive biology of anemone fish, *Amphiprion sebae* (Bleeker, 1853) (Pomacentridae) from the Gulf of Mannar, India

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Abstract

Different aspects of the reproductive biology of the anemone fish, *Amphiprion sebae* (Pomacentridae) from the Gulf of Mannar, India were studied between October 2002 and March 2004. The ovarian and testes development were analyzed by using histological techniques. The reproductive cycle was determines and quantitatively analysed. Five stages of gonadal developments were established namely resting, developing, ripe, spawning and spent. The results of monthly changes of GSI values show that *A. sebae* has one major spawning peak (February – March) and a minor spawning peak (December – January). The reproductive cycle of *Amphiprion sebae* shows a clear seasonally related with the water temperature and Gonado Somatic Index (GSI).
***Key words*:** *Amphiprion sebae*, GSI, gonad, reproduction

Introduction

Reproductive strategies of coral reef fishes are diverse and not well known in India. They tend to be highly fecund species which produce eggs that vary greatly in number from tens to hundreds to thousands at a time, on a daily, fortnightly, monthly or less frequent schedules (Sale, 1991). He further recognized that reef fish are also flexible in how they determine sex. In addition to the conventional gonochoristic species in which the sex of individuals is fixed, there are numerous hermaphroditic species. Some of these are simultaneous hermaphrodites, most of them sequential hermaphrodites, and in being so, a majority of the species are protogynous (female first, then male) while few are protandrous. Most reef fishes lay pelagic eggs although some lay demersal eggs with parental care, some are oral or mouth brooders and some are viviparous.

The knowledge on the reproductive biology of fishes is of utmost importance, not only to fill up the lacunae in our present day academic knowledge, but also in the utility of that knowledge for effective advancement of fishery entrepreneurs for evolving judicious pisciculture management. Perusal of available literature reveals that most of the studies in anemone fishes have concentrated on symbiotic association, sex-reversal and behaviour except for few studies by Allen (1975a and 1991) and Fautin and Allen (1992) on reproductive biology. No published information is however, available on any aspect of its reproductive and gonad development cycles, and except for one publication relating to captive spawning by Ignatious *et al.*, (2001) and biology of clown fish, *A. sebae* by Sathish Gold (2002). To fill up this big gap in the knowledge, the present study was undertaken to document all the facets of reproductive and gonad cycles under the captive and collected conditions.

The annual reproductive cycle and spawning season of an anemone fish could be ascertained by analyzing a number of reproductive and body indices such as gonadosomatic index (GSI), condition factor (K), fecundity, size at first maturity, hepatosomatic index (HSI) and length and weight frequency distribution. So, in the present study, an attempt had been made to provide some definite and dependable information on the gonadal cycles of *A. sebae* by observing seasonal changes in ripening and microanatomical changes in gonads during development and relates these to changes in gonad-body weight ratio and various reproductive traits such as gonadosomatic index (GSI), condition factor, fecundity and length and weight frequency distribution of the

marine ornamental fish of *A. sebae* was applied to ascertain the reproductive cycle and strategies.

Materials and Methods

Sample Collection

Fortnightly the samples of anemone fish, *A. sebae* were collected for a period of 18 months (October 2002 to March 2004) from Van and Kaswari tivu (Thoothukudi group of islands) Gulf of Mannar, India. Each sample consisted of about 100 specimens and was utilized to record data on total length, standard length, body weight, stages of sexual maturity, liver weight and length and weight of the gonads. The length measurements were taken to the nearest centimeter and weight was determined in grams. Sex (male or female or immature or maturing) was determined by macroscopic observation of the gonads.

Histological Technique

The dissected ovaries used for histological studies were fixed in Bouin's fluid for 24 h and transferred to 70% alcohol. After embedding in paraffin wax, sections were cut at 6µm and stained with Haemotoxilin and Eosin using routine procedure as described by Marcus *et al.,* 1984.

Gonadosomatic Index (GSI)

The ovaries and the testes of each fish were dissected out from the body cavity and weighed to the accuracy of 0.1mg by using an electronic balance. Temporal patterns in gonadal development were described by using the gonadosomatic index (GSI) for each male and female of *A. sebae* according to the formula (Pen *et al.,* 1993).

$$GSI = (W_1 / W_2)\ 100$$

Where, W_1 is the wet weight of the gonad in grams and W_2, the wet weight of fish in grams. Changes in GSI values plotted against the months of collection were used to determine the spawning season for each sex (Ha and Kinzie, 1996).

Results

A. sebae is a hermaphrodite fish, during the male phase, the gonad is an ovatestis in which the male zone is peripheral (Plate-1;2), while the female part is more centrally located (Plate-1;3). In the cranial region, the gonads of both sides are found as tube like structures, which are closed at their ends and in this portion of the gonad the germinal tissues are surrounded by a lumen (Plate-1; 1), which looks like an ovarian cavity which is known for most teleost ovaries. Posteriorly, the gonadal tubes of both sides open and assume the shape of thickened bands, which maintain a topographical segregation of the heterologous elements.

In the male region, each step of spermatogenesis from spermatogonia to spermatozoa is discernible. The testicular organization is classic, with seminiferous tubules consisting of spermatogenic cysts (Plate-2;1) delimited by a single layer of sertoli cells. In the ovarian part, oogonia and previtellogenic oocytes can be seen (Plate-2: 5&6). Since, the separation between ovarian and testicular tissues is less sharp in *Amphiprion* than in other ambisexual species, attention was focused on the contact between male and female germ cells.

In this area, it was noticed that the basal lamina is lacking both along the seminiferous tubules and round the previtellogenic oocytes (Plate-2:4). Moreover, the thecal cells, which typically constitute the outer oocyte envelope, are also missing. There are three modes of contact between male and female germ cells: (1) a narrow layer of connective tissue is located between the sertoli cells and follicle cells which surround male and female germ cells, respectively; (2) the sertoli cells are closely applied to follicle cells, respectively (3) the male germ cells are in contact with follicle cells. In the first two types the male germ cells are spermatogonia, spermatocytes and spermatids arranged in cysts (Plate-2: 1). In the third type spermatozoa and sometimes spermatids are observed. Only rarely are any somatic cells detected between the male and female germ cells.

When previtellogenic oocytes are slightly separated from seminiferous tubules, the basal lamina is still lacking, but numerous collagenous fibres are closely applied to both follicle and sertoli cells (Plate-3: 2). Moreover, in the contact between two seminiferous

tubules, a basal lamina can be observed along one tubule while it is missing from the other, which is then surrounded by numerous collagenous fibres. Finally, between two previtellogenic oocytes, the basal lamina may be lacking or may surround each oocyte (Plate-3: 1). Anastomoses from the basal lamina of one oocyte to the lamina of the other are sometimes discernible. In *A. sebae*, the development of male germ cells in the cysts is usually synchronous, but sometimes both primary spermatocytes and spermatids are detected in the same cyst.

In the ovarian part, the previtellogenic oocytes, devoid of thecal cells, are 50-70µm in diameter, but some of them are very small (e.g.14µm) (Plate-3: 6); this reveals cogenetic activity, which is corroborated by the presence of oocytes in meiotic prophase. Previtellogenic oocytes with undulating outlines show nuclear anomalies concerning the nucleoli (Plate-4: 4&5), which are very few in number and the nuclear envelope, which can be disrupted or formed by a single membrane producing finger-like protrusions into a rather empty nucleoplasm. Dense granules are detected in the nucleoplasm and on the nuclear membrane (Plate-4: 4&5). Plasma anomalies are related to the membrane, the mitochondrial envelope, the cristae, which have partially disappeared, and to the presence of some degenerative areas. Similar changes are detected in oocytes in meiotic prophase and in the follicle cells surrounding damaged previtellogenic oocytes, whereas oogonia are never injured. Disturbances in the organization of previtellogenic oocytes can be so great that these cells appear flatterned, electron-dense and devoid of discernible structures.

The gonadal condition was well understood in *A. sebae*. The morphological appearance of the ovaries allows various maturity phases for females were identified. The distinctive features of ovarian developmental stages are given below.

a. Undevelopment phase

The gonad could not be detected in serial cross sections of the whole body (Plate-1: 1).

b. Pre-ripe male phase I

The gonad has a few spermatocyte cysts, spermatids, sperm and perinucleolus oocytes but did not have an epithelium in ovarian cavity, ovigerous lamellae or a complete structure consisting of many spermatocyte cysts (Plate-1: 2).

c. Pre-ripe male phase II

The gonad had a complex structure consisting of many spermatocyte cysts, spermatids and sperm and perinucleolus oocytes and an epithelium but did not have an ovarian cavity, ovigerous lamellae or a complete structure consisting of many spermatocyte cysts.

d. Ripe male phase

The gonad had a complex structure consisting of many spermatocyte cysts at various stages of spermatogenesis, perinucleolus oocytes and an epithelium but did not have any spermatocytes, spermatids or sperm.

e. Ripe female phase I

The gonad had an epithelium, an ovarian cavity and ovigerous lamellae with perinucleolus and cortical alveolus oocytes, but did not have any spermatocytes, spermatids or sperm (Plate-4&5: 1-6).

f. Ripe female phase I

The gonad had an epithelium, an ovarian cavity and ovigerous lamellae with perinucleolus, cortical alveolus and yolk oocytes, but did not have any spermatocytes, spermatids or sperm.

Gonadosomatic Index (GSI)

The gonado-somatic indices of *A. sebae* was obtained from the different seasonal variation by plotting the GSI values against the time of collection (Fig: 1a&b) and was used to determine the spawning season for each sex. The mean GSI of female was 7.285 ±3.147% in early October, 7.285 ±3.147% in late October and 7.285 ±3.147% at the beginning of November just before ovulation and spawning. After spawning, in late November it was 7.285 ±3.147%. Then the GSI declined gradually to its basal level of 7.285 ±3.147% in March. In January, the mean GSI of 7.285 ±3.147% was relatively low because the

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ovaries were in spent and post-spawning condition. The values of female GSI for 18 months are shown in Fig:1a. During April and May, there was a slight increase in GSI, 7.285 ±3.147% and 7.285 ±3.147% respectively marking a minor spawning peak.

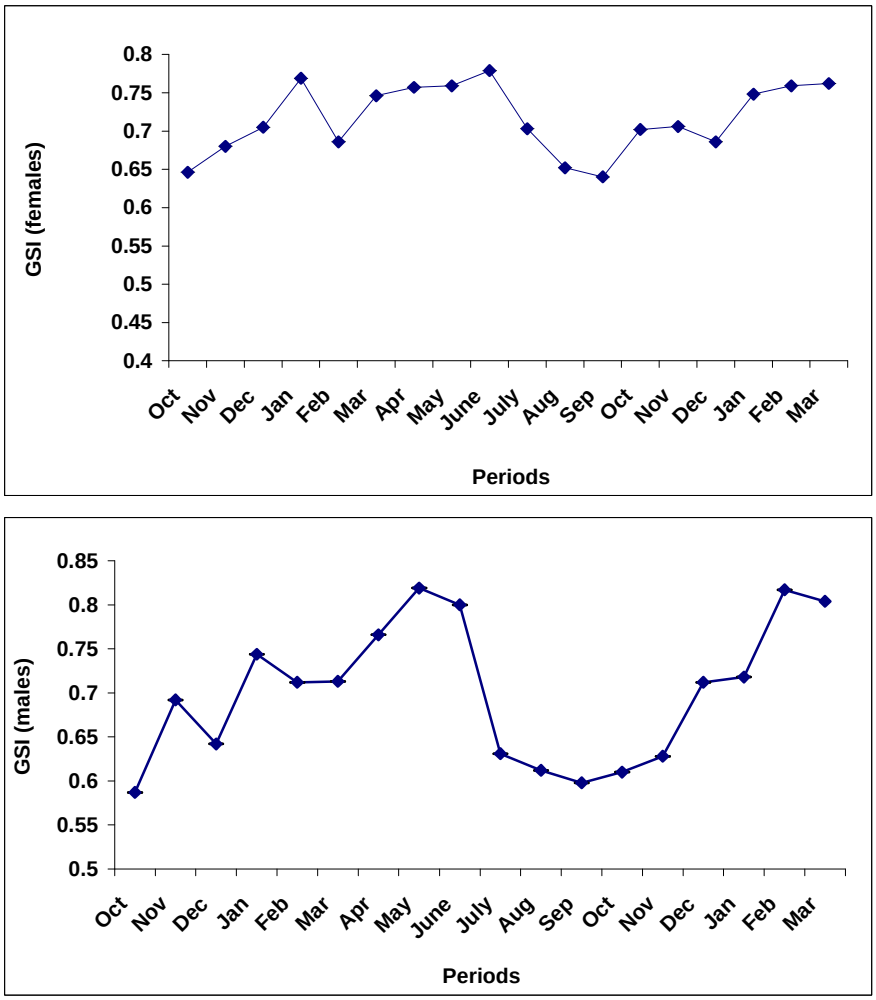

Fig – 1. *A. sebae:* Mean Gonadosomatic index (GSI GSI±SE) of females (a) and males (b) during the annual reproductive cycle.

The ovary of *A. sebae* was very small and immature in June through September. Then GSI increased steadily resulting in a value of 7.285 ±3.147% at the end of September and 7.285 ±3.147% in the early part of October (2003). The maximum GSI of an individual female was 7.285 ±3.147%.

The mean GSI of male during every collection for 18 months is also shown in Fig: 1b. The GSI was 7.285 ±3.147% in October, 7.285 ±3.147% in November and 7.285 ±3.147% in December. Since there was not much difference in the values of samples, the two values in each month are combined. Immediately after the spawning period, the GSI of male was reduced to 7.285 ±3.147% in January. During the non-breeding months of February, March (7.285 ±3.147%) and April, the GSI was very low, indicating that the testes had completely regressed. In late April and May, the GSI of male was slightly higher (7.285 ±3.147% and 7.285 ±3.147% respectively), indicating a short minor breeding season.

Size at first Maturity

By plotting the SL against the GSI of female during the spawning season

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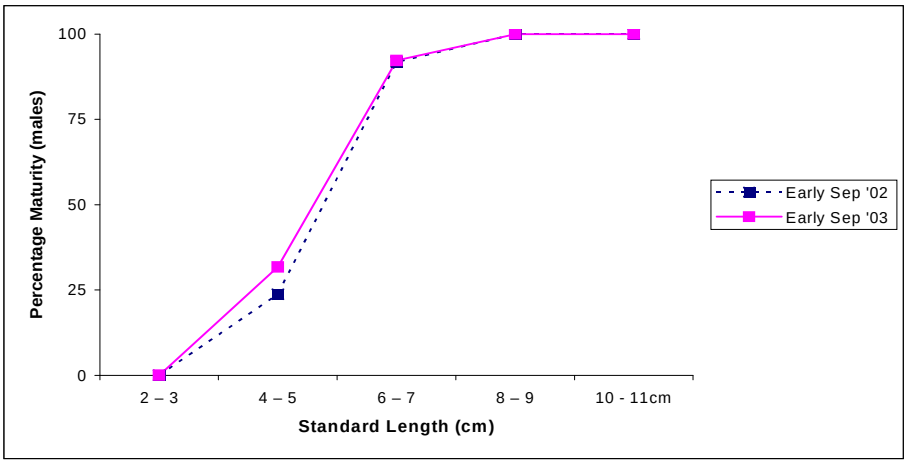

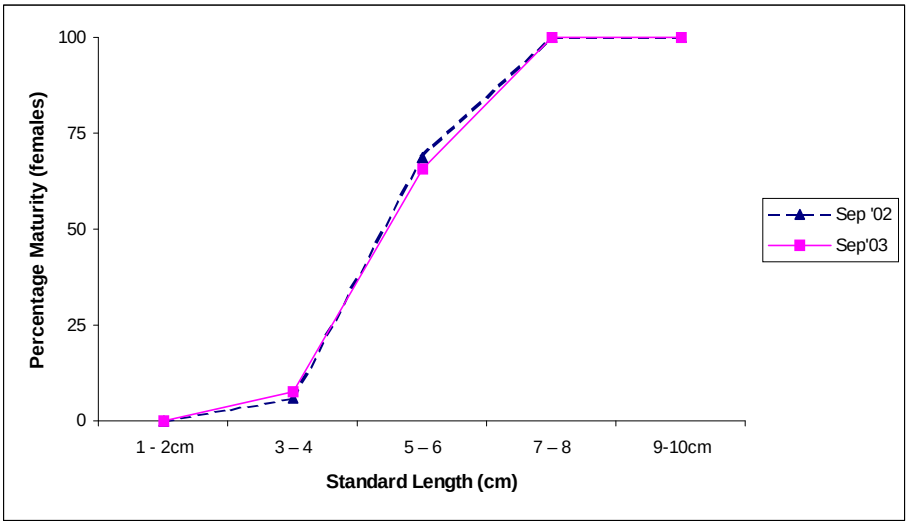

Fig. - 2. *A. sebae*: Percentage of maturity of adult females (a) and males (b) indicating 50% size at first maturity.

(Fig:2a&b), it was observed that the smallest female fish with vitellogenic oocytes measured 32mm SL. No female smaller than this had GSI values above 8.24 during the spawning season. Females with ovaries containing mature oocytes had GSI values greater than 10.59. Based on these results, the size at first maturation for female fish was established in this study as 5 - 6cm SL. Similarly, the SL of males was plotted against their respective GSI and the smallest mature male fish had a length of 4 - 5cm.

The results presented in Table 1 show that all female specimens of *A. sebae* below 5.5cm SL are immature. Maturity sets in from the 7 – 8cm stage onwards. The length at which 50% of the specimens attain maturity, considered the length at first maturity lies between 5 and 6 cm SL.

The results presented in Table 2 show that all male specimens below 3 – 5 cm SL are immature. Maturity has started from 5 – 6cm SL onwards. The length at which 50% of the male specimens attain maturity, being the length at first maturity, lies between 4 and 5cm SL.

By another method, the mean size at onset of sexual maturity (Lp50) in female *A. sebae* is estimated at 5 – 6cm SL in October (Fig: 3a) and in male 4 – 5cm SL in October (Fig:3b). The smallest mature female was 6 – 7cm SL and the smallest mature male was 4 –

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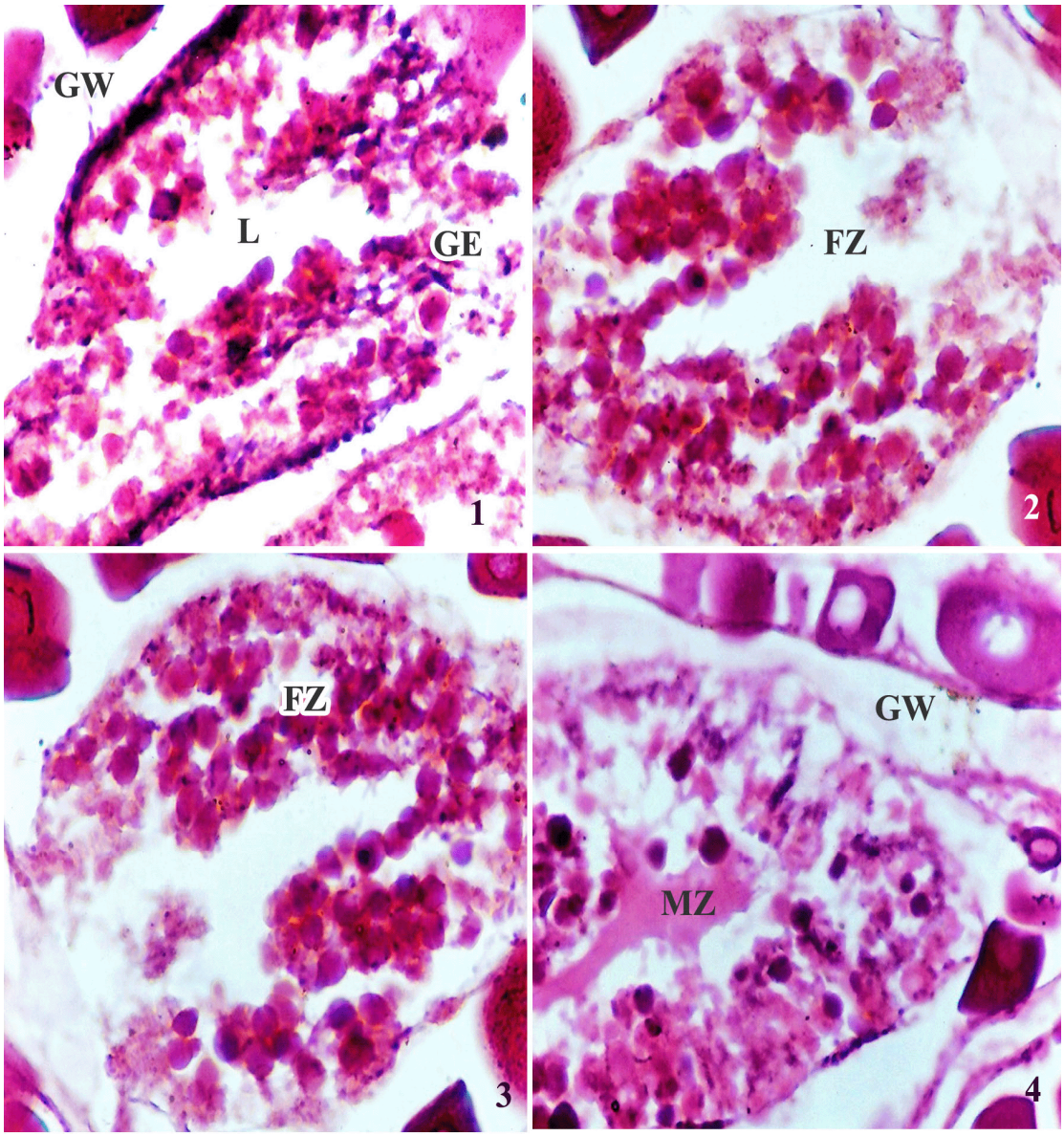
5cm SL. The smallest gravid female was 7 – 8cm in SL and the smallest ripe male was 5 – 6cm in SL. The females were larger than the males.



1. *A.sabae*: Cross section of undifferentiated gonad (H&E 60X)
2. *A.sabae*: Pre-ripe male I phase male zone MZ (H&E 60X)
3. *A.sabae*: Pre-ripe Female I phase female zone FZ (H&E 100X)
4. *A.sabae*: Pre-ripe Female II phase female zone FZ (H&E 100X)

Plate-1: *A.sabae* cross section of gonad

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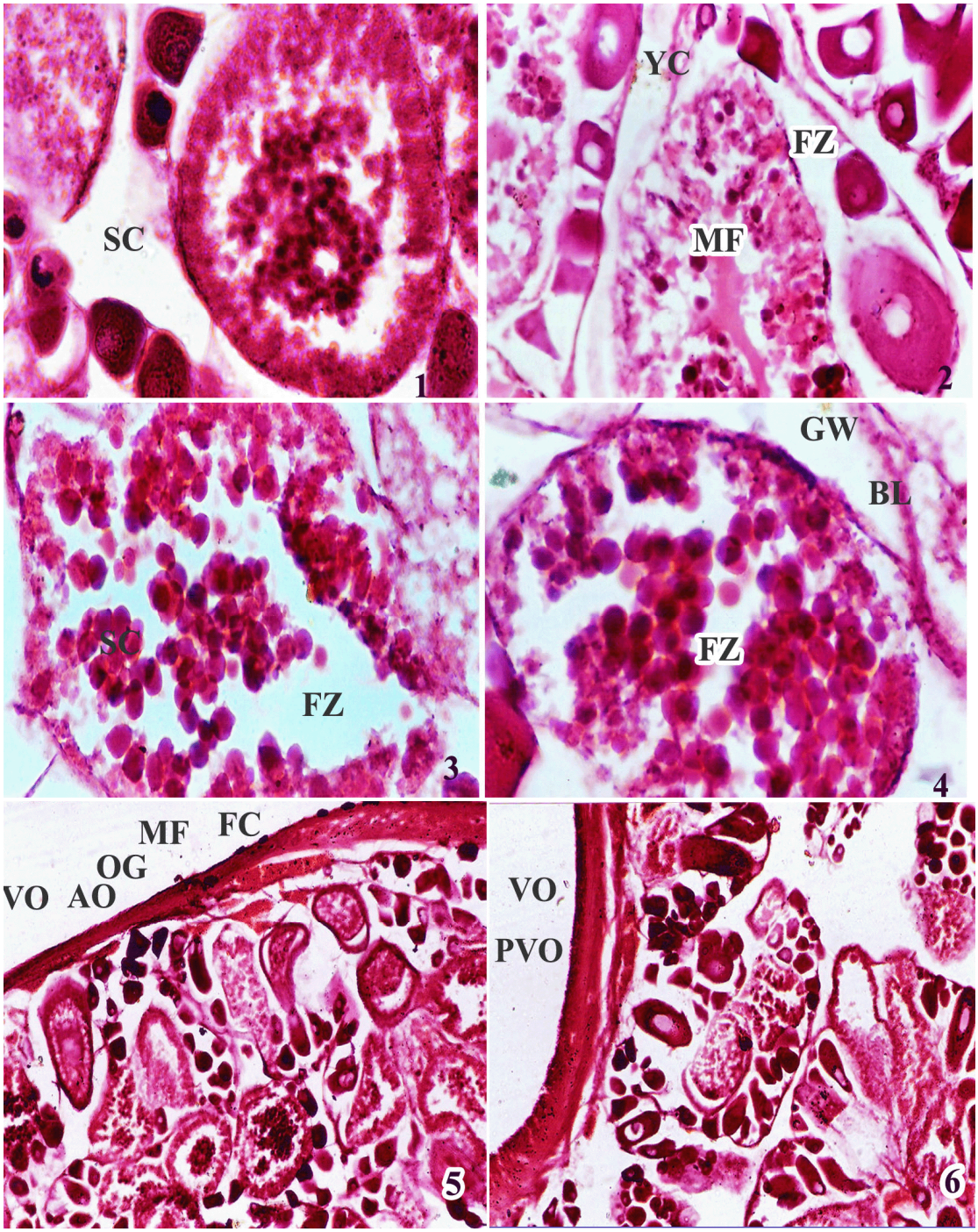

1. *A.sabae*: Cross section view of gonad showing protrandric hermaphoditism (H&E 60X)
2. *A.sabae*: Cross section view of gonad showing male developmental phases (H&E 60X)
3. *A.sabae*: Cross section view of gonad showing male developmental phases (H&E 60X)
4. *A.sabae*: Cross section view of gonad showing female developmental phases (H&E 60X)
5. *A.sabae*: Cross section view of gonad showing female developmental phases (H&E 60X)
6. *A.sabae*: Cross section view of gonad showing female developmental phases (H&E 60X))

Plate -2: Cross section view of gonad of *A. sabae*

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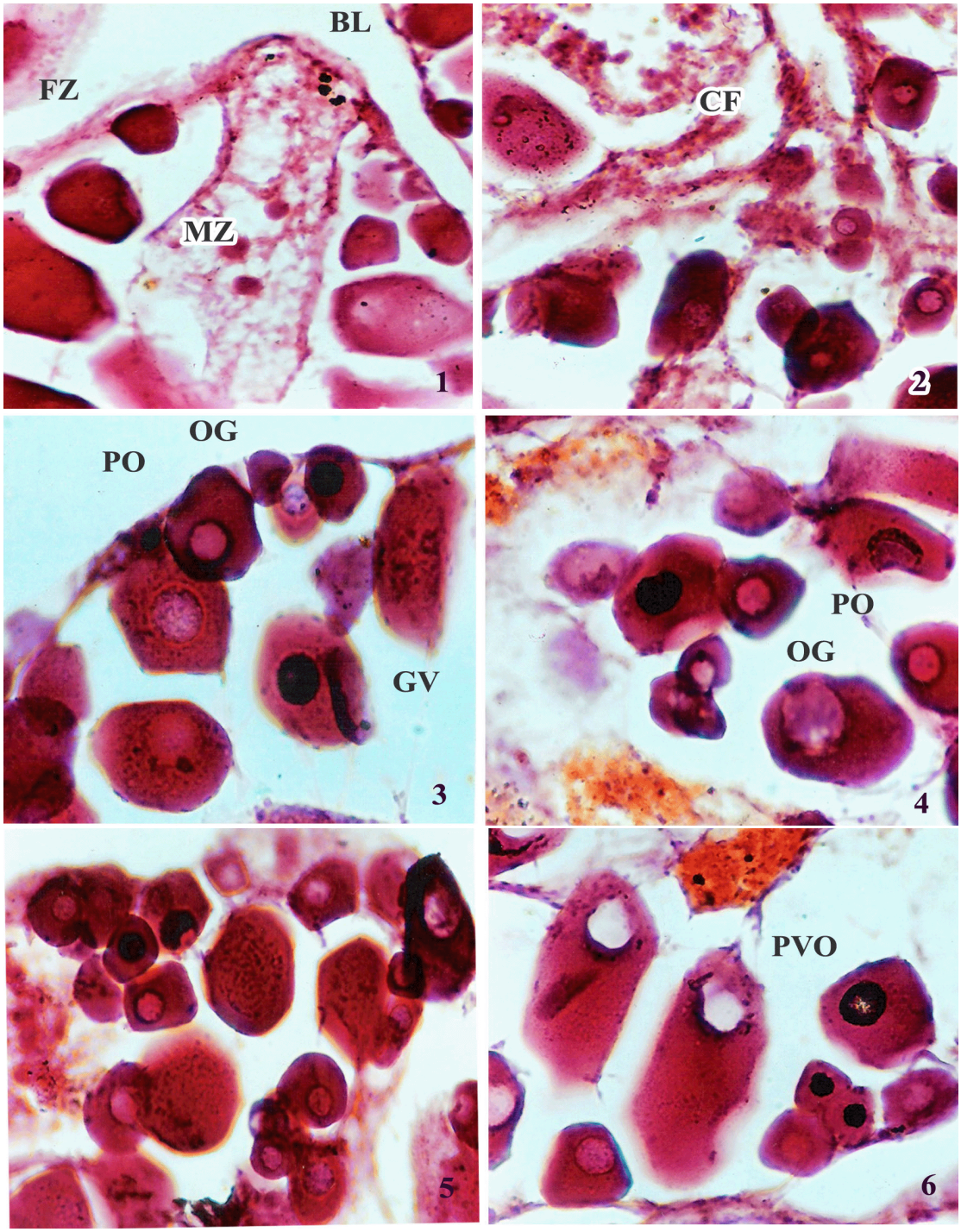

1. *A.sabae*: Cross section of protandric gonad (H&E 60X)
2. *A.sabae*: Focus fn male zone (MZ) (H&E 60X)
3. *A.sabae*: Focus of oogonial cells (OC) and primary oocytes (PO) (H&E 100X)
4. *A.sabae*: Focus of primary oocytes (PO) (H&E 100X)
5. *A.sabae*: Focus of vitelogenic oocytes (VO) (H&E 100X)
6. *A.sabae*: Focus of vitelogenic oocytes (VO) (H&E 100X)

Plate-3: Cross section of ovary of *A.sabae*

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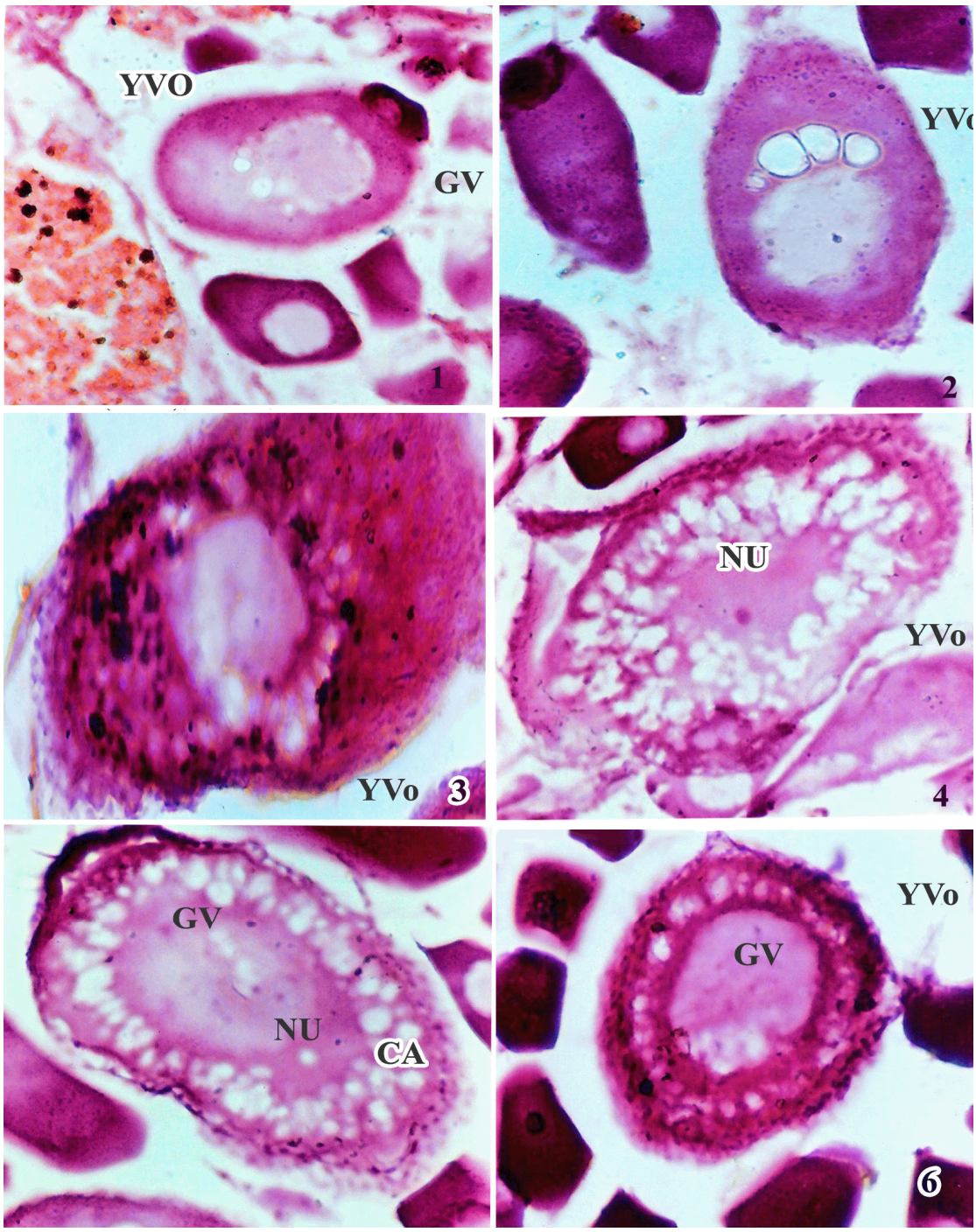

1. *A.sabae*: Early Vitellogenic oocytes (EVO) showing germinal vesicles (CV) and yolk vacuoles (YVO) (H&E 100X)
2. *A.sabae*: Appearance of yolk vesicles in perinuclear region (H&E 100X)
3. *A.sabae*: Multiplication of yolk vesicles (YV) (H&E 100X)
4. *A.sabae*: Multiplication of yolk vesicles (YV) (H&E 100X)
5. *A.sabae*: Appearance of cortical olveoli (CO) in the oocyte cytoplasm (H&E 100X)
6. *A.sabae*: Appearance of cortical olveoli (CO) in the oocyte cytoplasm (H&E 100X)

Plate-4: Cross section of ovary of *A.sabae*

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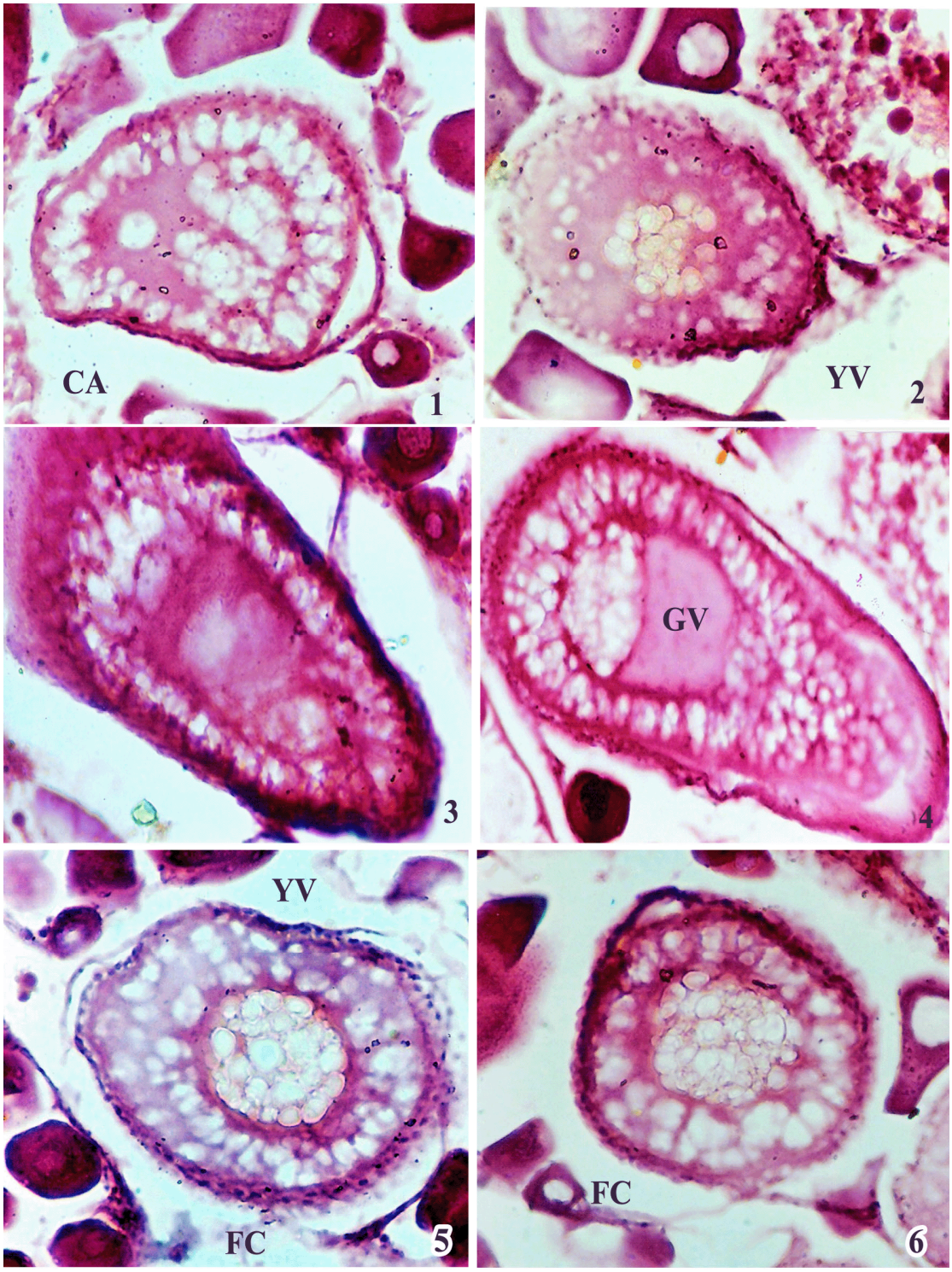

1. *A.sabae*: Vitellogenic oocytes (VO) showing yolk vesicles (YV) (H&E 100X)
2. *A.sabae*: Late Vitellogenic oocytes (LVO) more appearance of yolk vesicles (YV) (H&E 100X)
3. *A.sabae*: Mature (ripe) Ova showing crammed yolk vesicles (YV) (H&E 100X)

Plate -5: *A.sabae*: Cross section of ovary

Table–1: Percentage occurrence of females in different stages of maturity at various size groups before the spawning month

Standard Length (cm)	Immature (%)	Maturing (%)	*Mature* (%)
Early Feb '02			
4 – 5	76.24	23.76	-
6 – 7	8.16	64.55	27.29
8 – 9	-	6.78	93.22
Late Feb '02			
4 – 5	68.22	31.78	-
6 – 7	7.76	62.58	29.66
8 – 9	--	--	100.00
Early Feb '03			
4 – 5	81.96	18.04	-
6 – 7	6.28	62.31	31.41
8 – 9	-	8.41	91.59
Late Feb '03			
4 – 5	78.78	21.22	---
6 – 7	3.55	67.88	28.57
8 – 9	---	---	100.00

Table –2: Percentage occurrence of males in different stages of maturity at various size groups before the spawning month

Standard Length (cm)	Immature (%)	Maturing (%)	Mature (%)
Early Feb '02			
3 – 4	100.00	---	---
5 – 6	31.25	68.75	---
7 – 8	---	---	100.00
Late Feb '02			
3 – 4	92.22	7.78	-
5 – 6	34.28	61.57	4.15
7 – 8	--	3.89	96.11
Early Feb '03			
3 – 4	100.00	---	---
5 – 6	30.56	65.11	4.33
7 – 8	-	16.79	83.21
Late Feb '03			
3 – 4	94.26	5.74	---
5 – 6	32.50	63.28	4.22
7 – 8	---	5.78	94.22

The GSI value of females showed a higher peak in October – November (major spawning season) and a minor peak in April – May (minor spawning season). The GSI of males showed the highest peak in October, November and December, but in the other months GSI was at a very low level. GSI value was found to increase with the increase in SL in both females and males.

Discussion

The general pattern of development of the ovaries in *A. sebae* conforms to that of most teleosts (Misra, 1994; Jackson and Sullivan, 1995; Palmer *et al.*, 1995 and Morgan *et al.*, 1995). In most protandric teleosts, the ovarian and testicular areas are distinctly separated by a well-developed thick connective tissue. In anemone fish, however, the heterosexuality of gonads is rather different

since there is no connective tissue between ovarian and testicular regions (Reinboth, 1970).

The ovotestis of *A. frenatus* during the male phase differs from that of sparids and could be compared to the ovotestis of synchronous hermaphrodites such as *Rivulus* (Harrington, 1975) or *Serranus* (Reinborth, 1962) in which ovarian and testicular areas are contiguous. No information about contact between male and female germ cells has been found in the literature on these two genera.

In *A. sebae*, the absence of basal lamina round the previtellogenic oocytes located in the vicinity of male germ cells might be related to degenerating processes similar to those detected on the plasmic membrane of some previtellogenic oocytes or follicle cells.

In the ovarian part of functional males of *A. sebae*, both primary oocytes in meiotic prophase and some very small (young) previtellogenic oocytes are found, besides typical previtellogenic oocytes. This observation reveals that an oogenetic activity (female meiosis) may take place at least in the beginning, without being disturbed by ongoing spermatogenic activity in the ovotestis. According to Reinboth (1988), neoformation of oocytes can take place during the active male phase in other protandric teleosts species.

In protogynous species in which there is no segregation of the two gonadal territories (*Labridae*, *Scardidae*, *Epinephelinae*) the future function as a male is not discernible during the active female phase. However, during sex change, young previtellogenic oocytes can be observed in the newly formed testicular tissue, as has been reported for *A. clarkii* by Bell (1976), and for *Halichoeres poecilopters* by Reinboth (1988). But, in these two protogynous species, as in the protandric *A. sebae*, the oogenitic activity does not proceed beyond the perinucleolar stage. Thus, a gametogenetic activity of the two sexes resembles in its early stages the pattern of synchronous hermaphrodism. Moreover, in *A. sebae*, it is difficult to understand why, in the active male phase, both an early oogenetic activity and degenerative processes of male germ cells are detected.

The results of monthly changes of GSI values show that *A. sebae* has one major spawning peak (February – March) and a minor spawning peak (December – January). Condition factor was positively correlated with GSI values in *Zacco temmincki* in Japan (Katano, 1990). Sexual maturity and spawning have been shown to have remarkable bearing on the condition factor of the fishes (Qayyum and Qasim, 1964a,b). The highest condition factor coincides with the highest GSI values during the spawning season, as observed in *Mystus gulio* (Pandian, 1970). A similar result was also reported in fishes like *Diplodus puntazzo* and *Aplodinotus grunniens* (Palmer *et al.*, 1995).

The relationship between body length, condition factor and GSI value differed greatly between males and females. Body size was positively correlated with the GSI value, as shown for other fish species (Mann and Mills, 1985; Katano, 1990). Male body size has also been reported to show a positive correlation with GSI value in *Anableps dowi* (Mann and Mills, 1985).

The GSI is the highest corresponds to the breeding season of the fish (Stoumbousi *et al.*, 1993). GSI has been employed many times to determine or reduce the spawning season of fish stock (De Silva, 1973). The GSI values correspond closely with the developmental changes of the gonad (Phillip, 1993).

The present study demonstrates a major and minor spawning season for *A. sebae*. This is strongly supported by the trend in monthly GSI, which shows a major peak between February and March and a small peak in December – January. A similar pattern of reproductive cycle was also reported in *A. clarkia* by Bell (1976).

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