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Micro-Anatomical and Morpho-Histological studies of Ovary of stone carp *Psilorhynchus pseudecheneis* (Menon & Dutta, 1964) from Trishuli river of Nepal

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Abstract
Stone carp *Psilorhynchus pseudecheneis* were monthly sampled throughout a year and the histological analysis of their ovaries was done to determine the changes occurring in ovarian development. Based on histological examination of the ovaries, the organic process of *P. pseudecheneis* undergoes distinct cyclic and seasonal morphological changes. Five different developmental stages were identified under three major categories: pre-spawning (immature, maturing, mature), spawning (ripe-running) and post-spawning (spent). The peak spawning period of carp was noticed during December to February. The gonadosomatic index (GSI) and ova diameter ranged from 0.79 to 3.61% and 543 to 1123 µm respectively. The highest mean GSI (3.61 ± 0.16) and oocyte diameter (1123 ± 55 µm) were observed in December indicating that during this month the gonadal development reached maturity.

Keywords: *Psilorhynchus pseudecheneis,* gonad, Nepalese minnow and hill stream fishes

1. Introduction

Nepal is a land-locked country possesses a large number of rivers with perennial water supply from melting snows of the Himalayas, considerable number of lakes, a few reservoirs, an increasing number of fish ponds and irrigated fields, as well as marginal swamps. All these are suitable for fisheries development [1]. Nepal is situated between 26º20'-30º10'N latitude and 80º15'-88º19'E longitude. It borders on the Tibetan Autonomous Region of China in the North, and India in the South, East and West [2]. Nepal covers an area of 147,181 square kilometers and is divided into three parallel geographical zones running east to west: the Terai plain in the south, the Hills (which are the foothills of the Himalaya) and the Himalayan Mountains [3]. The Terai plain is an extension of the Gangetic plains of India while the hilly area is located in the middle part of the country varying in altitude from 610 m to 4877 m, with the lower hills up to 2700 m, and upper hills to 4877 m. The Himalayan Mountains are located from above 4877 m, above the tree line. Mountains and hills cover 83% of Nepal while the Terai occupies only 17% [4].

Aquaculture in Nepal is basically small due to no costal area and many fishermen with their families are engaged in capture fisheries, which represents nearly 0.28% of the total population of Nepal. River is the major source of capture fishery covering 395000 ha of the surface of natural water resources. The fish production has increased from 750 t/year in 1981/82 to 10,300 t/year in 1995/96 [5]. Presently, Around 75000 people are engaged in aquaculture with net fish production of 64900 Mt. in the year 2014. About 6,000 rivers flowing from north to south are characterized by low water temperature, high dissolved oxygen, high turbulent fast current in higher mountainous and hilly region [6].

Trishuli river is the most famous fishing and rafting river lies in the bottom of the Ganesh Himal region. Rafting, Kayaking and Fishing are the major activities can be done in Trishuli in the free time especially after monsoon season [7]. The name of Trishuli is related to the god Shiva, where God Shiva drove his trident into the ground to create the three springs. The river start flowing from Tibet and combine with Bhotekoshi [8]. While fishing in Trishuli River you can see the mountain of Langtang region. Betrawati also join the Trishuli and change its flowing capacity [9]. The river Trishuli is big, bouncy and safe among others and has more attracting in fishing. Various kinds of fish can be seen while you will involve in fishing trip in Trishuli [10].

The genus *Psilorhynchus* currently includes 18 species, all but one of which are tentatively divided between the *P. balitora, P. gracilis* and *P. homaloptera* species groups [11]. Four species are currently included within the *Psilorhynchus homaloptera* species group (viz. *P. arunachalensis, P. homaloptera, P. microphthalmus and P. pseudecheneis*), which is diagnosed by a combination of lateral line scale and pectoral finrays counts. Members of the *P. homaloptera* group occur at higher altitudes than other members of the genus [12]. Nepalese torrent minnow *Psilorhynchus pseudecheneis* also known as stone carp was first reported by Menon and Datta in 1964 for the first time from the river of Koshi of Eastern Nepal along 52 other fish species [13]. *Psilorhynchus pseudecheneis* is an apparently rare species found across central, western and eastern Nepal at an altitude range of 200 to 3,000 m. currently, the species is known from only a few localities, but this could be a reflection of sampling effort and the ecology of the species [14]. Since it migrates upstream for reproduction, the population may be impacted by dams. There have been modifications to its habitats; it requires higher levels of oxygen and it could be impacted by pollution. However, the species is a habitat specialist, found in fast flowing, oxygenated hills streams, and it is inferred to be impacted by habitat changes due to dams, and the degradation of its aquatic environment [15]. Further research is required and the species should be reassessed when its distribution, population and impact of threats are better understood.

For proper management of aquaculture practices, a detailed study of gonad maturation is important, since such studies are aimed in understanding the annual changes of the population [16]. The reproductive cycles in teleost occur during a particular phase; some breed once in a year as annual breeders and others as monsoon breeders [17]. Freshwater carps being seasonal breeders exhibit clear changes in the gonads during breeding season [18]. Cyclic gonadal changes have been studied in a few fish species viz. *Channa marulius* [19], *C. punctatus* [20] and *C. striatus* [21]. Histological studies on fish reproduction to determine peak period of spawning and to understand effective methods for increasing efficiency of broodstock and ultimately increasing the fish production are prerequisite. The detailed information on changes in the ovaries of the stone carp during the reproductive cycle is important to provide useful data for the management of this species. Hence, this study was performed to examine the ovarian developmental stages of Nepalese Endemic Torrent Minnow *Psilorhynchus pseudecheneis* during its annual maturation cycle under captive conditions.

2. Materials and Methods

2.1. Sample collection

Fig 1: Stone carp *P. pseudecheneis* collected from Trishuli River

Pisciculture has now become an important adjust to agriculture. For best and successful fish farming and fish conservation, the knowledge of their spawning habit has become indispensable for fishery science and its development [22]. Generally the study of spawning habits of the fishes should be undertaken in the natural habitat of the fishes but being aquatic, fishes possess several difficulties to an observer engaged in the study of its spawning habits. So the spawning habits are mostly studied on the basis of morphological study of the fish gonads i.e. testes and ovaries. Stone carp *Psilorhynchus pseudecheneis* is a rare species of the Genus *Psilorhynchus* and it is first recorded in Nepal by Men on and Dutta [23]. In Nepal this hill stream fish is locally called as Titae Kavre, thus, the experimental stone carps (12-20 cm in total length, 15-50 g in weight) were collected from Trishuli River (26o20'-30o10'N latitude and 80o15'-88o19'E longitude) by a cast net during the months of December to February. The fishes were acclimatized to captive conditions by maintaining them in earthen ponds (3x3x1 m) filled with chlorine free tap water (dissolved oxygen 5.2 mg/l, temperature 28±2 °C, and pH 6.4-7.1) at Central Department of Zoology, T. U., Kirtipur and partially in Department of Zoology of Tri-Chandra Campus, Tribhuvan University, Kathmandu, Nepal. The pond was provided with plenty of aquatic plants (*Hydrilla verticillata*). The fishes were fed on formulated diets (55% fish meal powder, 18% Wheat flour, 15% rice bran, 5% ground nut oil cake, 5% cod liver oil and 2% vitamin and mineral mixtures) following Haniffa *et al.* [24] with slight modification, twice daily (morning and evening) until satiation.

2.2 Gonadosomatic index (GSI)

Ovary samples were obtained from 5 female specimens captured using drag net from January to December. Fish (5.7 to 6.8 cm and 1.9 to 2.8 g) were dissected to remove the ovaries. Ovarian samples were weighed (0.035 to 0.065 g) and fixed in 10% buffered formalin prior to histological analysis. The volume of the fishes was also measured. An incision was made along the mid- ventral line and the gonads were removed, placed in aqueous Bouin's fluid for 48 hrs. The gonads were measured and weighed after fixation. The volume of the gonads was also taken. For the histological study small pieces from anterior, middle, and posterior region of the ovaries were drawn and separately fixed in Bouin's fluid. However, the alcoholic Bouin's fluid gave the best results. The dehydration, cleaning and embedding of the material was carried out according to standard procedures. Paraffin wax having melting point 58-62 °C was used for embedding purposes. The sections were cut in 5 to 8 µm and stained in Iron Haematoxylin using eosin as a counter stain and in Mallory triple stain. Gonadosomatic index (GSI) was calculated [25] using the following formula:

GSI = (Weight of Ovary / Weight of Fish) X 100

The diameter of intra-ovarian eggs was measured. All the oocytes in a section of the ovary were symmetrical due to the action of the fixatives and the collateral pressure exerted by the developing oocytes. Therefore, to minimize the error, only round and oval oocytes were measured using ocular micrometer. From each section, 3-5 smaller oocytes and large ripped eggs were measured.

2.3 Histology

Gonad sections were collected from the mid-part of the ovary monthly for histological analysis [26]. These sections were fixed in 10% buffered formalin for further laboratory analysis. After washing in running water, the samples were dehydrated in an ascending series of ethanol (70%, 90% and absolute ethanol) and clarified with xylene. The samples embedded in paraffin blocks were trimmed and 5 mm thick sections taken were stained using haematoxylin and eosin followed by periodic acid Schiff (PAS) reaction. The maturity stages of ovary of stone carps were determined using the modified gonad maturity scale developed by West [27]. The histological sections were examined under the light microscope (Nikon microscope - U III E-400 Eclipse). Diameter of eggs was measured using Magnus Pro Microscope Software with accuracy level of 0.01 mm. One-way analysis of variance (ANOVA) was used to analyze the data, followed by comparison of means using Duncan's multiple range tests [27]. Statistically significant differences were accepted at $p < 0.05$.

3. Results and discussion

Psilorhynchus pseudecheneis was described by Men on and Dutta [23] based on specimens collected during the Indian Cho-Oyu expedition to Nepal, in the headwaters of the Koshi River (Ganges Drainage), from a locality high in the Himalaya, around 2,950 m in altitude. Yazdani *et al.* [28] included *P. pseudecheneis* in Psilorhynchoides. Conway and Kottelat [29] returned *P. pseudecheneis* to *Psilorhynchus*. Shrestha [19], Rainboth [22], Talwar and Jhingran [30], Nebeshwar *et al.* [31], Edds [16], Conway and Kottelat [11], Conway and Mayden [32] and Shrestha [17] consider it to be a valid species. *Psilorhynchus pseudecheneis* is an apparently rare species found across central, western and eastern Nepal at an altitude range of 200 to 3,000 m. The species is also recorded from India. [33]

Currently the species is known from only a few localities, but this could be a reflection of sampling effort and the ecology of the species. Since it migrates upstream for reproduction, the population may be impacted by dams. There have been modifications to its habitats; it requires higher levels of oxygen and it could be impacted by pollution (e.g., siltation and organic pollutants). The species is assessed as least concern at present, due to the spread of records across Nepal and reported presence in India. However, the species is a habitat specialist, found in fast flowing, oxygenated hills streams, and it is inferred to be impacted by habitat changes due to dams, and the degradation of its aquatic environment. Further research is required and the species should be reassessed when its distribution, population and impact of threats are better understood. The species is considered rare in Nepal (Ranjan *et al.* 2006).

In this experiment the body of Titae Kavre fish was a small with a few mm in size. The body was elongated, depressed and flattened with distinct 3 to 5 transverse folds on ventral side. The length of head was 5.55 to 6.4 of caudal 7.38 to 7.69 and height of the body was 7.46 to 8.0 in the total length. A mid dorsal streak was present on just behind occipital region to midway between posterior and of head to origin of dorsal. The snout was spatulate. The length of the snout was 1.66 in head length. The eyes were small, dorsolateral in position. The diameter of the eye was 5.0 to 5.66 and inters orbital space 1.88 to 2.14 in head length. Five tubercles were present on the sides of the snout, around and in between the eye and operculum. Such tubercles often marked on pectoral fins also. Three to five pairs of ventral transverse folds of skin were present along ventral surface in between pectoral and pelvic fins. These folds obliquely situated one on each side. First anterior pair was longer and nearly meets together. Dorsal fin was originating slightly behind ventral. Caudal fin lunate with unequal lobes, lower one slightly longer than upper. Scales were of moderate size. Lateral line system was complete. Nine predorsal scales were present. Scales from chest to vent were absent. The colour of the body was darker with greenish tinge on dorsal side.

Stages of oocyte development similar to those described in most teleost fishes were identified and described [34] in *P. pseudecheneis*. The three maturity stages of oocyte development were determined based on morphological features are described in Table 1. First stage was prespawning stage which was divided into three sub-stages viz, (a) immature, (b) maturing, (c) mature. Second stage was spawning or ripe-running stage and the third was post-spawning or spent stage. Mature ovaries were frequently observed after October and were abundant in December to February signalling the period of spawning. Females with empty ovaries, spent fishes were seen between March and May indicating the recovery period. These different stages corresponded with those described macroscopically for *M. montanus* [35].

Table 1: Ovarian condition of different maturity stages and comparison with histological examination in Stone carp *P. pseudecheneis* collected from Trishuli River, Nepal

Stage and period	Macroscopic appearance	Histological examination
I. Pre-spawning (a) Immature (June-July) (b) Maturing (August-September) (c) Mature (October-November)	Ovary small, thin ribbon like transparent, whitish gray, granular in appearance occupying half of the abdominal body cavity, eggs very minute distinct under microscope.	Monolayer follicle phase in primary oocyte development with stage I present, ovigerous lamellae from the tunica albuginea were evident.
II. Spawning (d) Ripe-running (December-February)	Ovary fully distended and fills the abdominal cavity; oocyte orange yellow and easily shed on application of slight pressure on the ovary	Ovary dominated by tertiary oocytes; few previtellogenic stages begin to grow for the subsequent season;
III. Post-spawning (e) Spent (March-May)	Ovary flaccid and often haemorrhagic if spawning was successful; few oocytes visible giving the ovary a speckled appearance	Post-ovulatory follicles, oocytes undergoing atresia and atretic oocytes

However, microscopic examination of ovary sections reported by Al Mahmud *et al.*[36] revealed four stages of maturity in carp. In this experiment the female fish showed significant ($P<0.05$) weight changes in the ovaries corresponding to the three gametogenic stages (pre-spawning, spawning and post spawning) during different months of the year (Table 2).The GSI value was minimum in July (9.16±0.24%), began to increase in August and reached the stage of complete sexual maturity in November (16.72±0.31%) where the ovaries were ripe and mature (Table 2). Our result showed highest GSI in the months October and November which could be the possible spawning season of stone carps.

Table 2: Showing annual variation in length, weight, volume, and Gonadosomatic index (GSI) of the ovary of Stone carp *P. pseudecheneis* collected from Trishuli River, Nepal

Month	Length of fish (cm)	Wt of fish (g)	Vol. of fish (ml)	Length of Ovary (cm)	Weight of Ovary (g)	Vol. of Ovary (ml)	GSI (%)
JANUARY	14.73±0.51	12.51±0.15	14.83±0.57	2.77±0.13	1.71±0.25	1.72±0.44	13.91±0.31
FEBRUARY	14.12±0.16	12.28±0.71	14.88±0.59	2.24±0.42	1.83±0.27	1.56±0.47	14.88±0.99
MARCH	13.51±0.26	12.81±0.17	14.92±0.61	2.21±0.46	1.65±0.15	1.41±0.15	12.97±0.47
APRIL	12.91±0.11	12.58±0.36	14.96±0.63	2.71±0.87	1.51±0.36	1.26±0.24	12.27±0.53
MAY	12.30±0.15	12.35±0.70	15.17±0.65	2.53±0.65	1.47±0.71	1.56±0.77	11.33±0.82
JUNE	11.69±0.15	13.51±0.19	15.22±0.67	2.34±0.18	1.33±0.21	1.95±0.42	9.48±0.71
JULY	11.09±0.71	12.91±0.71	15.26±0.69	2.65±0.77	1.24±0.46	1.79±0.19	9.16±0.24
AUGUST	10.48±0.11	12.30±0.21	15.34±0.71	2.52±0.71	1.15±0.63	1.64±0.17	9.53±0.17
SEPTEMBER	9.87±0.14	11.69±0.42	15.38±0.73	2.19±0.73	1.16±0.75	1.49±0.59	9.59±0.89
OCTOBER	9.27±0.11	11.97±0.46	15.43±0.75	2.53±0.95	1.97±0.18	1.33±0.29	16.16±0.29
NOVEMBER	8.66±0.19	11.74±0.44	15.47±0.77	2.14±0.28	1.88±0.42	1.18±0.31	16.72±0.31
DECEMBER	7.50±0.18	12.34±0.76	15.64±0.79	2.54±0.59	1.79±0.13	1.41±0.15	14.89±0.14

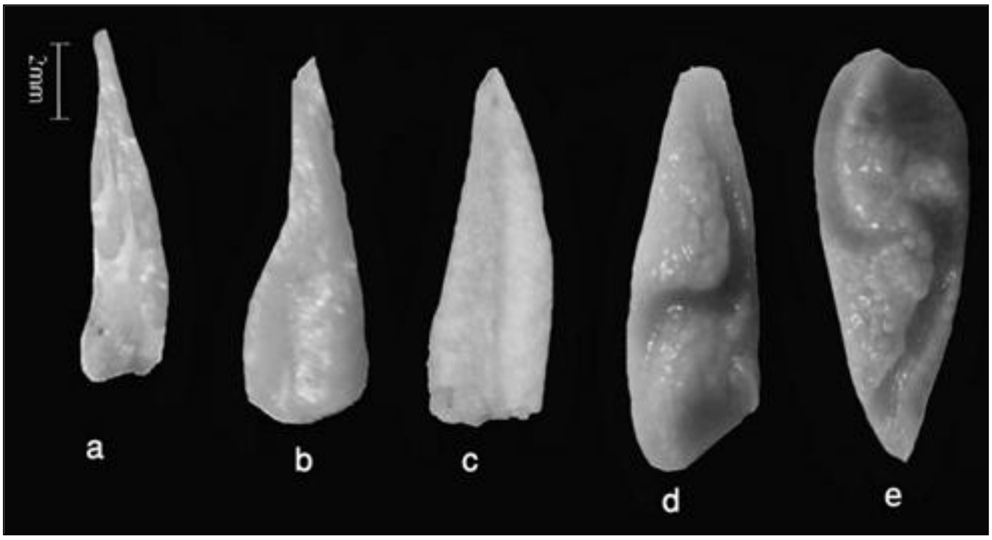

Fig 2: Different stages (a, b, c, d & e) of ovaries collected from stone carp of Trishuli River, Nepal

3.1. Histological features of oocyte developmental stages

Morphology, biochemistry and physiology of gonads have been described by a large number of workers in different groups of fishes. Ovaries are not only responsible to produce eggs but also synthesize and secrete hormones of different kinds that have a far reaching effects on the reproductive biology and behavior of the fish. Oogenesis starts from simple proliferation of oogonia up to the formation of mature oocyte and consequently ovulation after final maturation. During this process, size of oocyte increases many folds and this is mainly due to accumulation of yolk granules which are formed in liver under the influence of a specific steroid hormone, 17β-estradiol [37] and migrate to oocyte through blood [38-39]. Based on the shape, weight, changes in the nuclear and cytoplasmic components of oocytes observed during histological analysis, different oocyte developmental stages were distinguished immature, maturing and mature (Table 1, Fig 1).

The oogenesis process was classified based on the oocyte size and staining, presence of follicular layer, number of nucleoli and the distribution of cytoplasmic inclusions. In a majority of teleost fishes, five-eight stages of oogenesis have been reported [40-43]. Arockiaraj *et al*. [35] described five stages in the gonad of Mystus montanus. Similarly, Kader *et al*. [44] made similar observations in *Gobioides rubicundu*. Figs. 2 to 5 shows the histological appearances of different stages of ovarian development described in Table 1. A single layer of follicular cells was seen surrounding each developing oocyte. Early perinucleolar oocytes were most immature and polygonal in shape (Fig. 3). However, late perinucleolar oocytes became larger in size with the progress of oocytes development and varied in shape from polygonal to oval (Fig. 2). The yolk vesicles which were first seen at the periphery of the oocyte slowly spread towards the central nucleus (Fig. 4).Yamamoto *et al*. [45] divided the oocyte development of rainbow trout into eight stages which includes chromatin nucleolus stage, perinucleolus stage (subdivided into early and late stage), oil droplet stage, primary yolk stage, secondary yolk stage, tertiary yolk stage, and maturation stages. Shrestha [46] described the ovarian cycle of *Noemacheilus beavani* and divided it into seven distinct phases. Sen *et al*. [47] divided the development stages of *Labeo rohita* (collected from wild rivers of West Bengal, India) into seven different stages as primary growth phase, perinucleolar stage, pre-vitellogenic or yolk vesicle stage, vitellogenic stage post-vitellogenic stage, germinal vesicle break down stage, and spawning stage. Ganias *et al*. [48] studied the pattern of oocyte development in the Mediterranean sardine (*Sardina pilchardus sardina*) and divided it into six stages.

4. Conclusion

The results clearly demonstrated the GSI values of female stone carps showed significant difference between different months in the Trishuli River. The GSI values appeared from January to December with the peak in October, indicating the onset of the reproductive season. The GSI value declined rapidly after spawning. Therefore it was confirmed that the fish spawned once in a year with peak spawning from July to December. It can be concluded that the current study will contribute to better understand the cyclic and seasonal ovarian changes of *P. pseudecheneis* which in turn will help in conservation plans and captive maturation of this valuable species. The current study will also be suitable for selective breeding under captivity and sustainable fishery management of *P. pseudecheneis* in its natural habitats.

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Fig 1: Transverse section through the ovary illustrating oogenesis

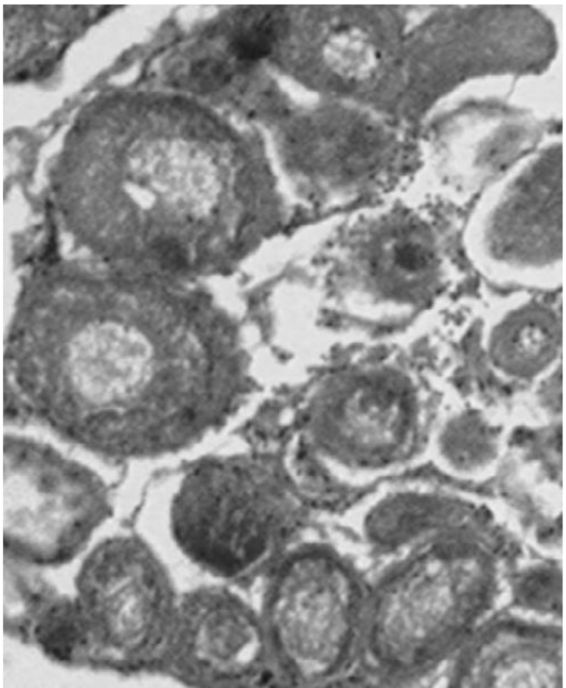

Fig 2: Transverse section through the ovary illustrating peri-nuclear oocytes

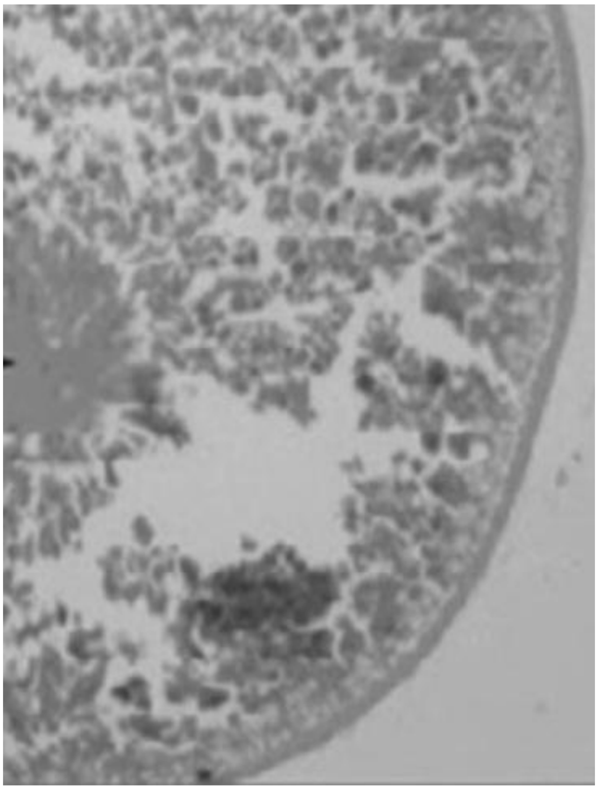

Fig 3: Transverse section through the ovary illustrating different stages of oogonia and chromatin granules

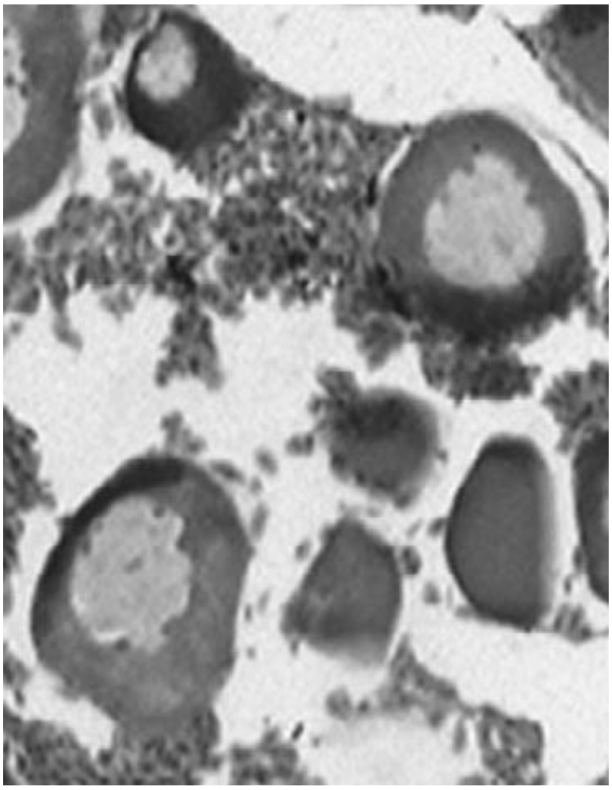

Fig 4: Transverse section through the ovary illustrating different stages of yolk vesicles.

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