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Sawarkar A. B.
Department of Zoology,
BP Arts, SMA Science & KKC Commerce College Chalisgaon,
Dist- Jalgaon (Mah.)– 424101,
India.

Tembhare D. B.
Department of Zoology,
Hislop College, Civil Line,
Nagpur, India.

Correspondence:
Sawarkar A. B.
Department of Zoology,
BP Arts, SMA Science & KKC Commerce College Chalisgaon,
Dist- Jalgaon (Mah.)– 424101,
India, Surguja (C.G.) 497001.

Testis morphology and spermatogenesis in the Indian honeybee, *Apis cerana indica* F. (Hymenoptera: Apidae)

Sawarkar AB, Tembhare DB

Abstract
Testis morphology and spermatogenesis were studied in Indian honeybee, *Apis cerana indica* by using light microscopy. The testes were paired, creamy white, oval-shaped bodies lying at anterior side of the mucus glands and situated in between the 2nd and 3rd abdominal segments. Each testis was internally packed with seven tube-like follicles compactly filled with the cysts. In the pupal stage, the cysts were filled with different stages of spermatogenesis. It was noticed that regulation and termination of spermatogenesis observed in the pupal stages. The sperm bundles were well evident in the newly emerged adult drones. Reduction in the size and weight of adult testis were associated with transfer of sperms in the seminal vesicle. Due to excessive transport of sperms, empty cavities and darkly stained residual bodies in the testis follicles were observed in 12-day old drones.

Keywords: Testis, morphology, spermatogenesis, drone, *Apis cerana indica.*

1. Introduction
The social hymenopterans (ants, bees and wasps) are efficient pollinators and possess considerable ecological and economic importance. In the colony, researchers mainly focus on female (queen and workers) reproductive physiology and their communication behavior which has great significance in the colony organisation. However, the male remains as the neglected gender compared to the queen and workers, even though they play the major role in the reproduction and colony expansion. The male reproductive system, development and process of spermatogenesis has been investigated earlier in several hymenopterans: *Bombus terestrus* [3], *Melipona quadrifasciata* [2], *Camponatus* [4], *Apis mellifera* [1, 5, 6].
In *Apis mellifera*, the aggregation of the structural units of testes is already formed in the third larval instar and germ cell clusters in the fourth larval instar [10]. However, maturation divisions and spermiogenesis arise during the pupal stage, when testes attain their maximal development and spermatogenesis is terminated before the adult drone emerges [1, 7, 8, 10]. The spermatozoa then enter the pre-vesicular portion of the deferent ducts and subsequently reach the seminal vesicles where they are temporarily stored. Following spermatozoa migration to the seminal vesicles, the testes shrink and become flattened show signs of testes degeneration [9].
Most of the information on the structure and development of the testis in bees is confined to *A. mellifera*, there have been few studies in the reproductive biology of Indian honeybee drone, *Apis cerana indica* [11, 12, 13], with little being known about the sexual maturation. In India, *A. c. indica* is widely domesticated and it is a dominant hive-bee of the apiculture industry. The present study was undertaken to investigate the histo-morphological changes of testes and spermatogenesis from pupal to adult stage in this honeybee species.

Material and Methods
Collection of Drone pupae and adult
During the present study, pupae and adult drones of *Apis cerana indica* were collected from the hive established at the premises of the CES College, Chalisgaon, Dist- Jalgaon (India) during the year 2010 to 2013. The pupal stages i.e. early-pupa, mid-pupa and late-pupa were staged according to the color of the compound eyes while adult drones staged by pigmentation of thoracic cuticle.

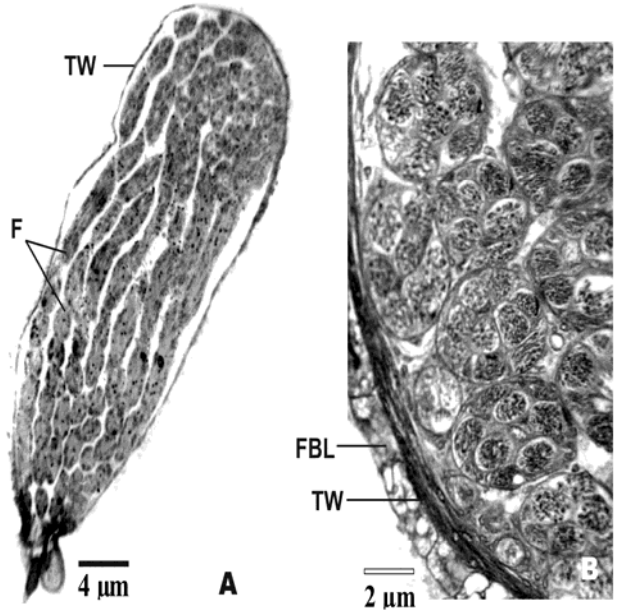
2.2 Histological Methods

The testis of the drone honeybees (staged pupal and adult stages) were dissected in the insect Ringer solution. Weight, length and diameter of about 10 testes from different stages were measured. Some testes immediately fixed in Bouin's or Carnoy's fixative for 18-24 h, dehydrated in ethanol, cleared in xylene and embedded in paraffin wax at 58-60 º C. The sections were cut at 4-6 µm thickness. The Bouin's fixed sections were stained with Ehrlich's Haematoxylin Eosin (HE) and Heidenhain's Iron haematoxylin-orange G (Fe-H) histological techniques [14].

3. Results

3.1 Histology

In *Apis cerana inndica*, the testes were elongated, oval-shaped, creamy white structures, situated in between 2nd and 3rd abdominal segments on either side of the mucus glands. Each testis was externally covered with fat bodies and richly supplied with tracheae. Each testis follicle had opened posteriorly into the vas eferens. All vasa eferentia unite together and formed a tubular vas deferens. Histologically, each testis was internally packed with seven tube-like follicles and externally covered with two peritoneal sheaths and fat body layer. The outer peritoneal sheath forms the outermost covering of the testis while the inner sheath was folded and extended internally forming the double-walled inter-follicular septa. The septa was separated the adjacent follicles from one another (Fig. 1A, B).



Fis. 1 A: Longitudinal section of testis,
B: Anterior region of testis showing FBL- Fat body layer, PS- Peritoneal sheath, F- Follicles.

3.2 Histomorphological Changes

The testis had shown gradual increase in length, diameter and weight in early-pupa (EP), mid-pupa (MP) and late-pupa (LP) while decrease in newly-emerged adult (NEA), immature adult (IMA) and mature adult (MA) stages (Fig. 2-3). Consecutively, the weight of paired testis were gradually increased in their size from 6.490±0.1464 to 9.570±0.1938 mg/pair in EP and MP respectively and then decreased to 7.82±0.2265 mg/pair in LP. It again decreased from 4.530±0.1585 to 1.282±0.0525 mg/pair in the adult drones (Fig. 4).

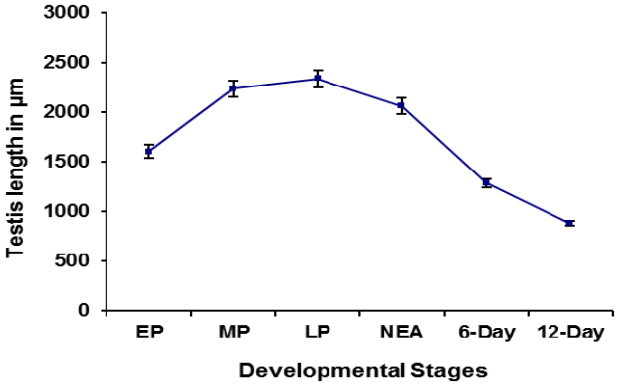


Fig 2: Length of Testis

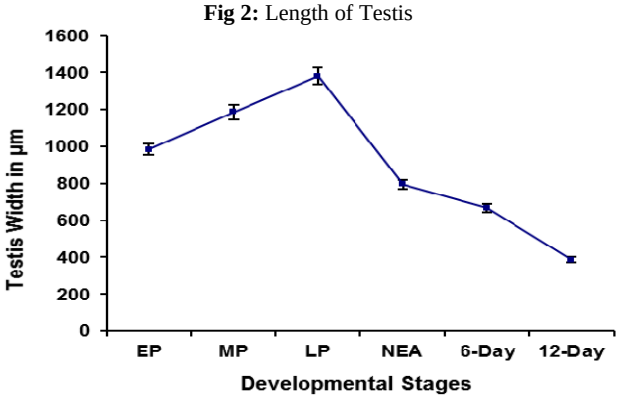


Fig 3: Width of Testis

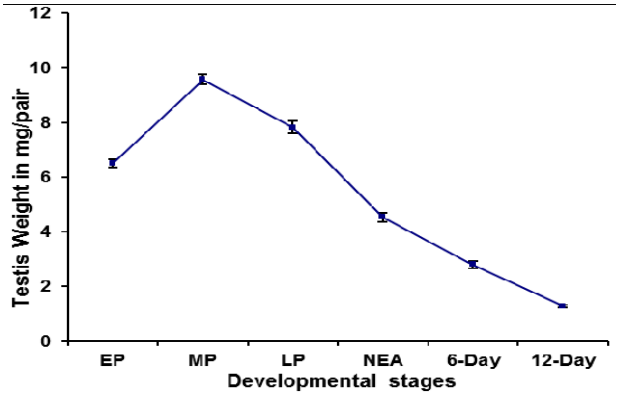


Fig 4: Weight of Testis

3.3 Spermatogenesis

In the early pupa, loosely arranged numerous primary spermatogonia (PSG) and few secondary spermatogonia (SSG) were observed in the cysts of each follicle. They had spherical shape with prominent nuclei at the center. About 80% cysts filled with PSG were observed at the end of early pupal stage (4-day old pupae) (Fig. 5A-B). In the midpupa, initially, the majority of the cysts contained SSG and remaining were filled with spermatocytes. Later on, the spermatocyte cysts became more in number in comparison to the spermatogonial cysts. In some cysts the spermatocytes were developed into spermatids. In the late-pupa, the nuclei of spermatids were initially spherical but later on undergo elongation and formed spindle-shaped nuclei of the spermatozoa. The nucleated spermatozoa then transform into the tailed spermatozoa (Fig. 7). In the newly-emerged adult drones, most of the follicles were filled with spermatocytes, spermatids and spermatozoa. The sperm bundles occupied middle and posterior region of the follicles of the testis (Fig. 5C-D).

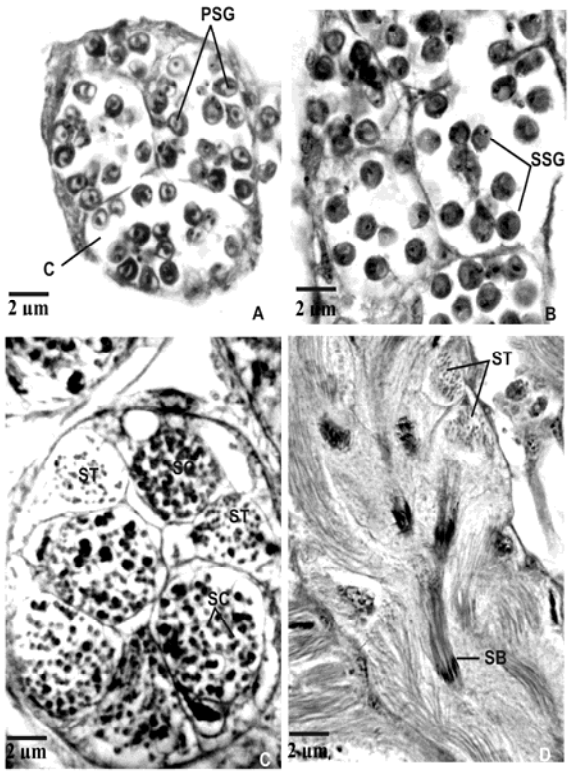


Fig. 5 A: Follicle showing PSG in early pupal stage, B: Follicle showing SSG in mid pupal stage, C: Cysts showing SC and ST, D: Testis of newly emerged drone showing SB.

Abbr. PSG- Primary spermatogonia, SSG - Secondary spermatogonia, SB- Sperm bundle, SC- Spermatocytes,ST - Spermatids, SZ-Spermatozoa

The sperm bundles migrate from the cysts and pass through the vasa eferentia into the vas deferens and finally were stored in the seminal vesicle. The secretory droplets and large residual bodies were observed amongst the spermatids and spermatozoa in the 6-day old drones (Fig. 6A). In the 12-day old drones, the cysts were shrinking greatly. They became empty or consist of very few numbers of spermatozoa, due to migration of sperm bundles to the seminal vesicle. Due to excessive transport of sperm, empty cavities and darkly stained residual bodies were observed in the testis follicles (Fig. 6B).

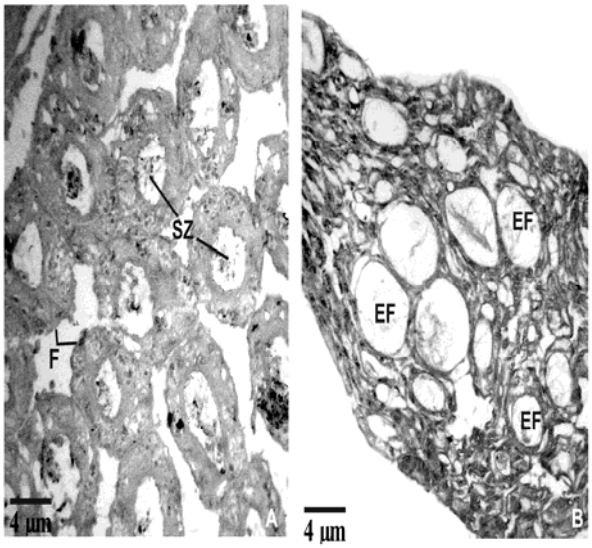


Fig. 6 A) : Testis of 6-day old drone showing few number of sperms (SZ) in follicle.

B) : Testis of 12- day old drone showing empty follicles (EF).

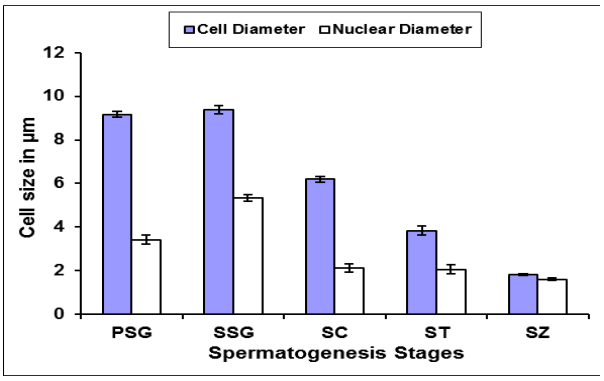


Fig 7: Different stages of Spermatogenesis

Abbr. PSG- Primary spermatogonia, SSG- Secondary spermatogonia, SC- Spermatocytes, ST- Spermatids, SZ- Spermatozoa

4. Discussion

Our understanding on this topic is mainly based on changes in testes development and spermatogenesis during the pupal and adult stages are well observed in *A. mellifera* [1, 6, 9, 15]. In *A. c. indica*, the testes are elongated, oval-shaped, creamy white bodies shown during the pupal stages. The number of testicular follicles in the testes are seven and are variable in number in other hymenopterans [2, 4, 9, 16, 17]. Each follicle is composed of an inner layer of epithelial cells and an outer layer of muscle fibres.

The size of testis increases from early-pupal to late-pupal stage and then it decreases from newly emerge to mature adult drones shows the completion of spermatogenesis process and migration of spermatozoa to the seminal vesicle. In *A. mellifera*, the pupal testes are larger than in mature males and there are changes in the seminal vesicles and mucus glands during sexual maturation [6, 9]. In *A. c. indica*, the testes of mature adult become triangular due to reduction of size upto 1/3 times similar to that in *A. mellifera* [1].

Maturation divisions and spermiogenesis occur during the pupal stage, when testes attain their maximal development and spermatogenesis is terminated before the adult drone emerges shown in *A. c. indica* which is supported by earlier workers [1, 7, 8, 10, 15]. In the early pupa, spherical primary and secondary spermatogonia with prominent nuclei at the center are present in the cysts of each testis follicle. Then the secondary spermatogonia transform into spermatocytes and then spermatids seen more in the mid-pupal stage. The spermatids initially spherical then transform into elongated tailed spermatozoa. The formation of sperm bundles in posterior testis follicles in late-pupal stage is well distinct which then enter and subsequently reach the seminal vesicles via vas deference where they are temporarily stored. According to Snodgrass [9] the sperm bundles are well evident in the lumen of seminal vesicle in newly emerged adult drone of *A. mellifera.*

As the complete migration of spermatozoa to the seminal vesicles, the testes shrink and become flattened. In *A. c. indica*, the size of testis becomes reduced in adult drones due to excessive transport of sperms. In mature adult drones, empty cavities and darkly stained residual bodies are observed in the testis follicles which show evident signs of testes degeneration. It is noticed that size and weight of the testis are associated with the initiation, regulation and completion of spermatogenesis. Sexual maturity is observed in 12-day old drones, as the complete release spermatozoa from the testis, becomes shrink. It may helpful to understand the reproductive physiology of drone in this species and other related Hymenoptera species.

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