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Structure of the female reproductive system of the dragonfly *Orthetrum sabina sabina* (Drury 1770) (Anisoptera: Libellulidae)

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Abstract
The female reproductive system in *Orthetrum sabina sabina* consists of a pair of ovaries and a post ovarian genital complex (POGC). The ovaries are long, thin panoistic type germinal tissues occupying the first to the sixth abdominal segment. The mature ovariole consist of the terminal filament, germarium, vitellarium and pedicel. The POGC is composed of the sperm storage organ and vagina. The sperm storage organ is formed of a small bursa copulatrix and a pair of spermathecae with long tubular duct. The vagina is a short laterally folded tube covered by thick muscle bands. On the mid-dorsal region, the POGC bears a highly complex sclerotized cuticular collar formed of three pairs of cuticular plates. The POGC is ectodermal in origin and is composed of outer muscle, middle epithelial and inner cuticular layer. These layers exhibit site specific modifications with respect to the functional significance of the components.

Keywords: Bursa copulatrix, post ovarian genital complex, sperm storage organs, spermatheca, vagina

Introduction
In Odonata, the first documented study on the female reproductive system was undertaken by Rathke [1] in his memoirs "*De Libellarum Partibus Genitalibus*". A comprehensive account of the odonate female reproductive system was put forth by Marshall [2] and Tillyard [3]. Later, Hogben [4] studied the involvement of nucleolus in oocyte maturation in the dragonfly *Libellula depressa* and George [5] studied the development of the genitalia and genital ducts in the Zygoptera, *Agrion.* Although the female reproductive system has been studied in the following dragonflies- (*Epiophlebia superstes* [6], *Pantala flavescens* [7], *Sympetrum danae* [8], *Orthetrum chrysis* [9], *Somatochlora arctica* [10], *Orthetrum chrysis* [11], *Orthetrum chrysostigma* [12], *Copera marginipes* [13], *Ischnura rufostigmata* [14] and *Tramea virginia* [15,16]), because of the species specific variation in the post ovarian genital complex, a great lacuna still exist in understanding the various morphological and physiological aspects of this system especially with respect to the phenomena of fertilization and sperm competition. The present work is therefore undertaken to study in details the female reproductive system of the dragonfly, *O. s. sabina* with special impetus on the post ovarian genital complex.

Material & Methods
Female *Orthetrum s. sabina* were collected from the local water bodies (during the post monsoon period of 2013-14) and brought in laboratory. The reproductive organ were dissected, fixed in Bouin's fluid for 12-18 hours, dehydrated, cleared in xylene and embedded in paraffin at 60-62 ºC. Serial sections of 4-6µm thickness were cut and stained with Delafield's Haematoxylin– Eosine and Heidenhain's Iron-Haematoxylin-Orange G. for scanning electron microscopy (SEM), the genital complex was dissected in phosphate buffer and fixed in 3% Glutaraldehyde (6.8 pH) and preserved in phosphate buffer. After fixation, the samples were dehydrated in a series of 70%, 90% and absolute ethyl alcohol for 20 min. in each and treated in carbolxylene for 2-4 hr. The tissue was transferred to absolute ethyl alcohol for 10 min. and then to Hexamethyldisilazane (chemical dryer) for 2-3 min. The dried tissue was placed on a double stick tape fixed in copper grid and coated with 24 nm palladium in a sputter coating machine. The tissues were observed under Zeiss EVOMA10 and JEOL 6380A scanning electron microscope at 20 kV/EHT and at 3.20 to 18.60 kx. The measurements were carried out by Smart Tiff V1.0.0.12 software at VNIT, Nagpur and IARI, Pusa, New Delhi. Histological measurements were undertaken using "Catcam 130" the microscope eyepiece digital camera.

Observation

The female reproductive system in *O. s. sabina* consists of the ovaries and the post ovarian genital complex connected by the tubular oviducts.

The Ovaries: The first five abdominal segments bear paired long ovaries which lie along the lateral margin of the gut. Each ovary is composed of numerous strings of diagonally placed panoistic ovarioles. The apical end of each ovariole forms a cord of terminal filament along its inner border while the base of each ovariole opens into the lateral duct which runs along the outer border of the ovary. Each ovariole is distinctly divided into the apical end, a small germanium and a large tubular vitellarium following by the pedicel which is a short stalk connecting the vitellarium to the lateral oviduct. The wall of pedicel is composed of elongated epithelial cells. The germarium is a small region present in-between the terminal filament and the vitellarium. It mostly contains the pre-follicular cells, oogonia and very few primary oocytes. The pre-follicular cells have indistinct cell boundaries, a single nucleolus and coarse chromatin material. They lie toward the periphery and are also found scattered among the oogonia. The oogonia are small spherical cells occupying the anterior region of the germarium. Oogonia contain a large quantity of cytoplasm and a nucleus with one or two nucleoli. The posterior region of the germarium contains some primary oocytes (Figs.1-4). The vitellarium is the largest part of the ovariole and in this region the oocyte undergoes successive stages of vitellogenesis. The mature vitellarium can be divided into three growth zones, the anterior is the zones of pre-vitellogenic oocytes, the middle of vitellogenic oocytes and the posterior of matured oocytes (Fig.4). Each oocyte is encircled by a layer of follicular cells. As the oocyte grows, the follicular epithelium also changes in shape and size. The entire process of vitellogenesis is divided into five developmental stages- pre-vitellogenic, early vitellogenic mid-vitellogenic, late vitellogenic and maturation. At the pre-vitellogenic stage, the oocytes are oval measuring, 112±26 µm in diameter and the nuclei lies at the centre. The oocyte at the early vitellogenic stage is cylindrical measuring 250±33 x 150±28 µm while at mid-vitellogenic stage it measures 410±30 x 380±25 µm. The late vitellogenic stage oocyte enlarges to 450±30 µm x 400±25 µm while at maturation stage it measures 510±5 µm x 430±18 µm (Figs. 5-8). The antero-posteriorly linear development of the egg can be clearly demarcated in the ovarioles. The lateral oviducts arise from the outer edge of the ovaries and descend in the sixth and seventh abdominal segment around the midgut. They join at the base of the seventh segment to form a short median oviduct. In gravid females, the oviducts are filled with two to three tiers of eggs descending down into the vagina (Figs. 15-16).

The Post Ovarian Genital Complex: It is an oval pouch - 1.5±0.03 mm long and 0.8±0.02 mm broad present on the eighth abdominal sternum. Morphologically it can be distinctly divided into the sperm storage organ and vagina. The sperm storage organ is in the form of a small mid-apically place bursa copulatrix and a pair of spermathecae with long tubular ducts (Figs.9, 10). The bursa copulatrix is a small spherical chamber embedded in the muscles of the POGC. It is 280±25 µm long and 150±18 µm broad and cannot be clearly differentiated in dissected specimens. The length of the spermatheca including the duct is 0.34±0.08 mm while the diameter of the spermatheca is 190±12 µm which narrows down to 130±15 µm along the duct (Figs.11, 12). The vagina is a short muscular sac-like laterally folded tube covered by thick layer of muscle bands along the lateral sides. The median oviduct opens into the vagina anteriorly and the vagina posteriorly communicates with the sub genital plate of the ninth segment through the female gonopore.

The Cuticular Collar-Complex: The cuticular collar-complex is an elaborate set of three pairs of cuticular plates embedded in the mid-dorsal surface of the POGC (Figs. 11-14).

The First Pair of Cuticular Plates (CP-1): These are present at the junction of spermathecal duct and bursa copulatrix and composed of a pair of pear shaped plates with their apical ends firmly fixed in the dorsal muscles of the vagina. Each plate is about 90±15 µm long and 40±8 µm broad. A small cuticular plug is present along the underside of their junction. KOH preparation of the post ovarian genital complex reveals that these plates are internally connected to the bursa communis and the lateral vaginal plates.

The Second Pair of Cuticular Plates (CP-2): These are composed of a pair of smaller pear shaped plates 70±15 µm long and 20±8 µm broad, located just below the first pair of plates and firmly embedded in the dorsal walls of the vagina. These are attached along their inner margin and placed at an angle to form a tent like elevated tunnel. These plates are internally integrated with the fertilization pore.

The Third Pair of Cuticular Plates (CP-3): These plates are located under the second cuticular plates just above the mid-vagina region. CP3 is composed of a pair of small cuticular structures which are anteriorly connected to CP-2 and are firmly embedded in the lateral walls of vagina. The plates of CP-3 are comparatively smaller (55±12 µm long and 25±5 µm broad) than those of CP-1 and CP-2.

Histology

The post ovarian genital complex is ectodermal in origin and composed of the outer muscle, middle epithelial and inner cuticular layers. The epithelium rests on a thin basement membrane. The outer muscle layer is mostly intrinsic around the sperm storage organ but the vagina is enveloped by thick prominent muscle bands and firmly attached to the sternum. The cuticular intima displays diverse structural modification along the lumen of the complex.

The Spermatheca: The spermathecae are tiny tubular structure (globular when filled with male ejaculate) attached to the terminal end of thin, long and narrow spermathecal duct. Except for the dilation in the lumen, it is very difficult to differentiate between the spermatheca and its duct. Each spermatheca is externally enveloped by fat body followed by a thin muscle layer mostly composed of longitudinal muscles and a few oblique muscle strands. The epithelium is prominently placed in a single tier on a thin basement membrane. The epithelial cell is 32±5 µm long and 10±3 µm broad (Fig. 18, 19). The nucleus is centrally placed and found to be in different phases and stain in only some of the cell where the chromatin material is condensed while in other cells the chromatin material is found in diffused condition. The cuticular intima is thin (16±4 µm) lightly wavy to almost smooth (Fig. 20). The muscles of spermathecae are 28±6 µm thick. The spermathecal duct has similar histology but the muscle layer is much thicker (40±6 µm) and the cuticle forms prominent ridges and furrows. In copulated females,

the spermathecae and their ducts are packed with sperm material. In some females, male ejaculate of different density is clearly visible in the spermathecal ducts but such condition is not found either in the spermathecae or in the bursa copulatrix.

The Bursa Copulatrix: In *O. s. sabina,* the bursa copulatrix is a small spherical chamber with a tiny circular lumen and is located just above the vagina and behind the terminal abdominal ganglion (Figs. 12, 17, 22-25). The lumen of the bursa copulatrix is initially circular but later on changes to horse-shoe shape with the enfolding of its ventral wall. The cuticular intima is thin and unsculptured (Fig. 21). The bursa copulatrix is composed of outer thin (26±5 µm) layer of intrinsic muscles along its dorsal side while the lateral and ventral sides are firmly embedded in the dorsal muscle bands of the vagina. The epithelial layer is composed of small cells (14±4 µm long and 11±2 µm broad) and is distinct only along the dorsal curve of the bursa copulatrix.

The Cuticular Canaculi: The cuticular intima of the postero-ventral region of bursa copulatrix and antero-dorsal vagina undergoes profound modification to form a connecting link, of a complex cuticular canaculi which encompasses the bursa communis and the fertilization pore.

The Bursa Communis: The cuticle of canaculi thickens and curves upward forming a tunnel which is inlayed with cuticular denticles. The roof of the tunnel splits up to form a cuticular passage between the bursa copulatrix and vagina. This region is lined with denticles, pegs and thick spines and forms a valve like structure with arms rising towards the lumen of the bursa copulatrix, this duct forms the bursa communis. The bursa communis is a "toothed passage' about 170±18 µm long and lying between the bursa copulatrix and vagina. The bursa communis extends down and comes to lie below and between the cuticular plates of CP-2. At this junction, the plate internally fuses with the bursa communis to form a second cuticular passage, the fertilization pore (Figs. 22-25).

The Fertilization Pore: The fertilization pore of *O. s. sabina* is a short (120±15 µm) tunnel formed by the cuticular plates of the bursa communis embedded in the dorsal muscles of the vagina. The fertilization pore has a spiny tunnel which extends and hangs along the roof of the vagina. The tunnel is composed of a mid-dorsal plate attached to two lateral plates. The inner lining of the tunnel bears sharp, short and long spines (17-40 µm). A pair of epithelial horned folds is found on the fertilization pore. On either side of the fertilization pore, the vaginal intima modifies into thick bands of heavily sclerotized lateral cuticular vaginal plates (Figs. 26-29).

The Vagina: The vagina is in the form of a long (0.18±0.03 mm), narrow, pouch extending from the terminal tip of the post ovarian genital complex to the gonopore. The sac-like vagina is greatly modified in *O. s. sabina* (Figs. 22, 24, 25). It is composed of outer, thick, highly developed muscle bands, a prominent middle epithelial layer exhibiting similar peculiarities as found in the spermathecae and a highly complicated and modified cuticular intima. Initially, just behind the median oviduct, the vagina exhibits a very simple structure with a thin folded cuticular intima and tri-radiate branching lumen with two prominent lateral arms and a median arm which divide at the tip to form sub-branches. The median arm moves upward and connects to the lumen of the bursa copulatrix. Concomitantly, the ventral region of the vagina integrates with the underlying median oviduct so that the median oviduct opens directly into the vagina. This opening is guarded by a cuticular valve secreted by the epithelium of the vagina. The cuticular valve continues down as a pair of membranous cuticular folds upto the gonopore. The lateral region of the vagina on either side of the fertilization pore forms thick sclerotized cuticular plugs which posteriorly continue into the lateral wall of the vagina to form the lateral vaginal plates. These plates around the fertilization pore contain 6-8 mechanoreceptors arranged in rows. The lumen of the vagina is modified greatly and is initial 'V' shaped (158-170 µm broad) in transverse section just below the bursa copulatrix. But later, it modifies into an 'I' shaped chamber (72-75 µm long) with a pair of dorsal and ventral arms extending in the muscle layers. It also extends a pair of antero-lateral and postero-lateral branches so as to increase the volume of the lumen during copulation and fertilization (Figs. 30-32). The cuticular intima of the vagina is serrated along the inner dorso-lateral sides. The vagina is initially divided into two chambers by a horizontal cuticular septum. The lower chamber separates the passage for the egg from the oviduct while the upper chamber houses the bursa communis. The cuticular septum disappears half way just below the fertilization pore for the smooth transport of egg.

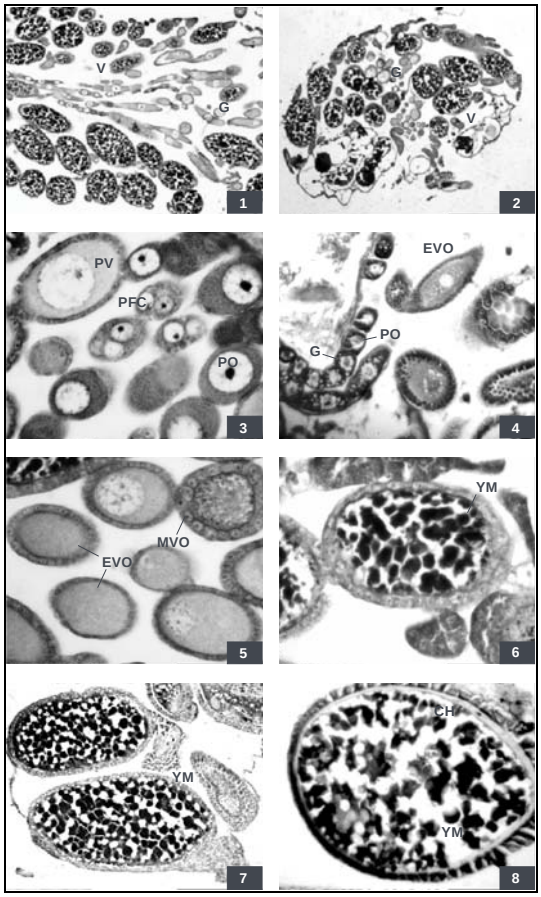

Fig 1-8: Section of the ovary showing panoistic ovarioles and development of the oocyte and vitellogenesis in the dragonfly *Orthetrum sabina sabina.* Figs. 1-2. Vitellarium (V) and Germanium (G) (HE x40). Figs. 3-4. Pre Vitellogenic Stage (PV), Early Vitellogenic Stage (EVO), Primary Oocyte (PO), Pre Follicular Cell (PFC) and Germanium (G) (HE x250). Fig. 5. Early Vitellogenic Stage (EVO) and MVO- Mid-Vitellogenic Stage (HE x250). Figs. 6-8. Yolk Material (YM) and formation of chorion (CH) (HE x300).

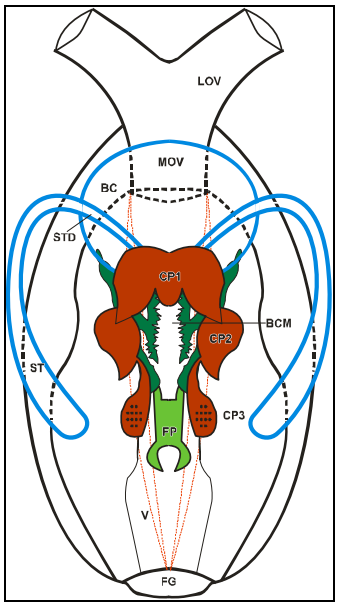

Fig 9: Diagrammatic representation of the post ovarian genital complex of the libellulid dragonfly *Orthetrum sabina sabina* (with cuticular collar separated).[BC- Bursa Copulatrix, BCM-Bursa Communis, CP1- Cuticular Plate1, CP2- Collar Plate 2, CP3- Collar Plate 3, CM- Collar Membrane, FG- Female Gonopore, FP- Fertilization Pore, LOV- Lateral Oviduct, MOV-Median Oviduct, ST- Spermathecae, STD- Spermathecal Duct, VG-Vagina.]

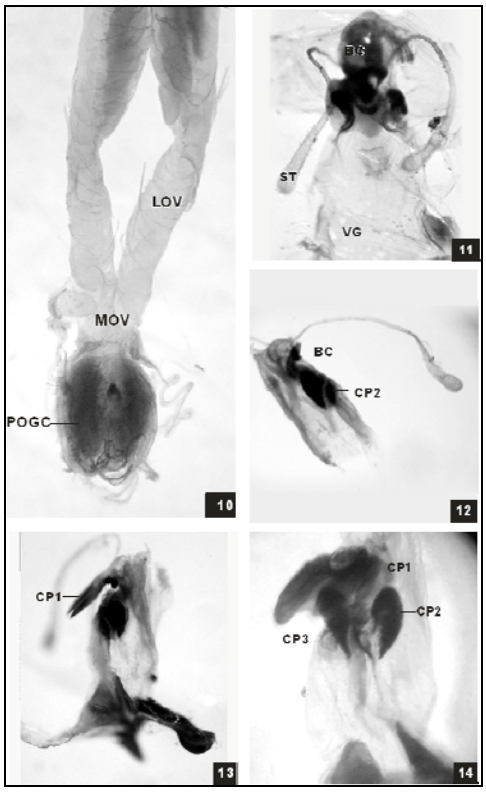

Fig 10-14: *Orthetrum sabina sabina*:- Position and structure of the first (CP1), second (CP2) and third (CP3) cuticular collar plate on the post ovarian genital complex. Fig.10. The eggs descending from the ovary into the lateral oviduct (LOV) and median oviduct (MOV) just above the post ovarian genital complex (POGC) (x20). Figs.11-13. Arrangement of the cuticular collar complex below the bursa copulatrix (BC). Also note the spermathecae (ST) and tubular vagina (VG) (KOH x20). Fig.14. Magnified view of the same showing the second (CP2) and third cuticular collar (CP3) (KOH x40).

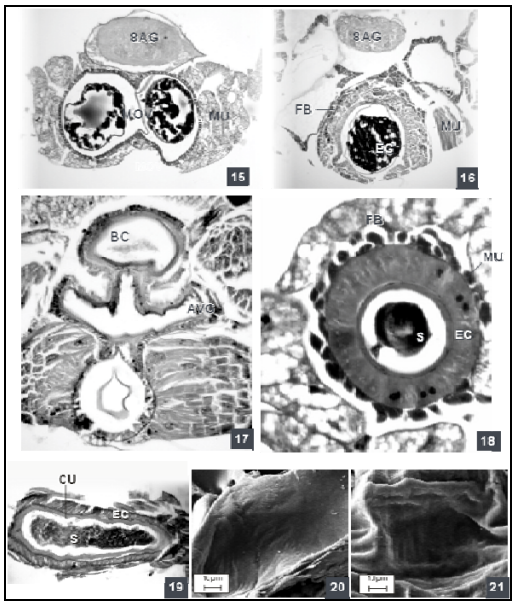

Fig 15-21: *Orthetrum sabina sabina*:- Sections passing through various regions of the post ovarian genital complex. Figs. 15-16. Transverse section through the median oviduct (MOV) located below the eighth abdominal segment (8AG) and covered by thick muscle bands (MU) (HE x120). Fig.17. Section passing through the anterior region of POGC showing the location of the bursa copulatrix (BC), vagina (VG) and the lower region of modifying oviduct (HE x400). Fig. 18. Section of the spermatheca showing lumen filled with sperm (S), epithelial cells showing nucleus in various stages, intrinsic muscle layer (MO) surround by fat body (FB) (HE x 400). Fig. 19. Section passing through the spermathecal duct showing lumen filled with sperm and a uniform wavy cuticular intima (CU) and epithelial cells (EC) (HE x350). Fig. 20. SEM of cuticular intima showing the lightly wavy and smooth intima of spermatheca. Fig. 21. SEM of thin lightly corrugated and folded intima of bursa copulatrix.

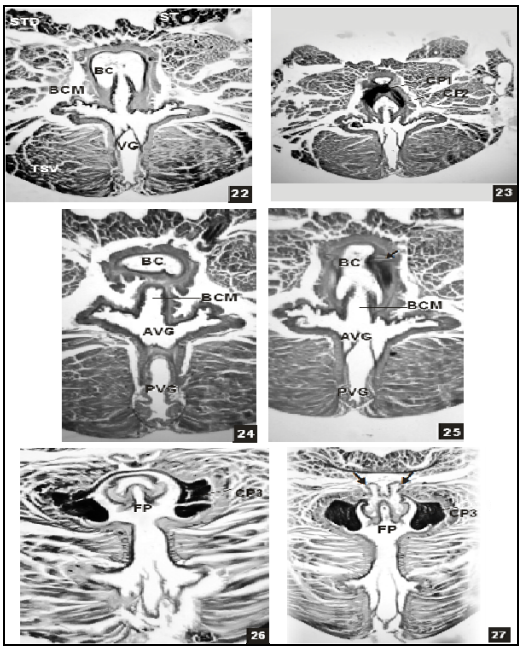

Fig 22-27: *Orthetrum sabina sabina*:- Serial transverse section showing the position of bursa copulatrix (BC), anterior (AVG) and posterior vagina (PVG). Note the modification of cuticular intima to form the bursa communis (BCM) and tubular fertilization pore (FP) around the third cuticular collar plate (CP3). Also note the horned folds (arrows) above the fertilization pore (HE x200; Fig.23 x100].

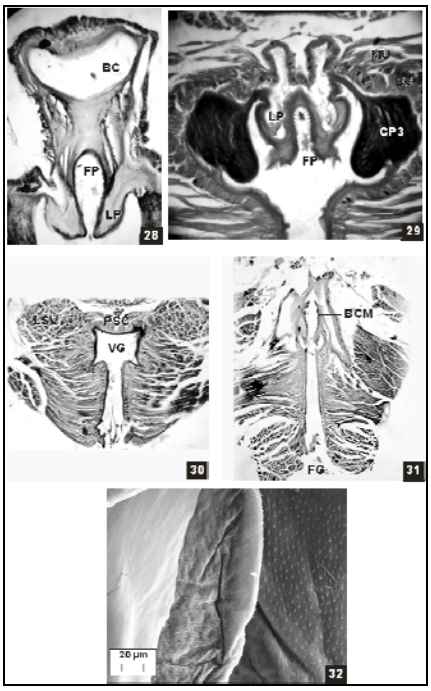

Fig 28-32: *Orthetrum sabina sabina*:- Section passing through the post ovarian genital complex. Fig.28. Magnified view of the spiny fertilization pore (FP) located below the bursa copulatrix (BC). Note the lateral plate (LP) of the fertilization pore (HE x300). Fig. 29. Magnified view exhibiting the close proximity of the fertilization pore (FP), fold/horns (arrow) and third cuticular plate (CP3) (HE x300). Fig. 30. Transverse section of the vagina (VG) showing thick bands of muscles (HE x200). Fig. 31. Longitudinal section of the POGC showing the location of bursa communis (BCM), vagina (VG) and female gonopore (FG) (HE x200). Fig.32. Scanning electron micrograph showing the spiny intima of vagina.

4. Discussion

In Odonata, the female reproductive organs are characterized by the great size and length of the ovaries which extend from the tip of the abdomen down to the seventh segment. In *Pantala flavescens* the ovaries extend from the first to fifth segment [7] in *Orthetrum chrysis*, they are lodged in the first to sixth abdominal segment [9] and from second to sixth abdominal segments in *Tramea virginia* [15]. In *O. s. sabina* they extend from the first to the sixth abdominal segment. In Odonata, the ovarioles are of panoistic type and arranged in an oblique manner [1-17]. Similar oblique arrangement of ovarioles is found in *O. s. sabina*. Ovaries of Odonata consist of numerous separate oocyte strings (ovarioles) and are subdivided into four sections, the terminal end-filament, germanium, vitellarium and pedicel [9, 11, 16]. The entire process of vitellogenesis in the studied anisopteran is divided into five developmental stages- pre-vitellogenic, early vitellogenic mid-vitellogenic, late vitellogenic and maturation, as found in *Orthetrum chrysis* [9] and *Tramea virginia* [16]. Richard & Davies [18] documented that in Odonata the two oviducts are very short and open into a large pouch-like spermathecae in the eighth segment, but it is found that in *O. s. sabina* the two oviducts unite and open into the vagina in the eighth abdominal segment.

In *O. s. sabina* the spermathecae opens through a long spermathecal duct in a small bursa copulatrix. Such condition is rarely found in anisopteran dragonflies. Smaller spermathecae and bursa copulatrix indicate that *O. s. sabina* have to copulate regularly to stock the sperm storage organ. The POGC of Odonata contains cuticular collar(s) which is generally located on the junction of bursa copulatrix and vagina. Although glandular secretory cells have been reported in the spermatheca and its duct [6, 7], such specific glandular cells could not be traced in *O. s. sabina.*

The cuticular collar(s) exhibits a great variation in shape and size. The collar is rod-like in *Trithemis arteriosa, Sympetrum rubicundalum* [20] and *Sympetrum depressiusculum* [19], bilobed in *Crocothemis erythraea*, *Orthetrum cancellatum, Orthetrum chrysostigma* [12], *Brachythemis leucosticta* and *Brachythemis lacustris* [21, 12, 20], forked in *Sympetrum danae* [22], rectangular in *Sympetrum striolatum, Tramea virginia* and *Anax guttatus* [23, 15, 16], 'X' shaped in *Libellula cyanea,* 'A' shaped in *Sympetrum sanguineum* [24]. Two sets of collars are found in *Celithemis elisa* and *Erythemis simpicicollis* [24] and three sets in *Nesciothemis farinose* [12]. In *O. s. sabina* the collar-complex is formed by the amalgamation of three pairs of cuticular plates. Further, the location of the collar between the bursa copulatrix and vagina just above the cuticular canaculi indicate its functional importance during fertilization. The collar forms an anchorage for all the dorsal muscles of the post ovarian genital complex. The collars help to regulate the movement of egg and spermatozoa during fertilization and copulation.

The bursa copulatrix in anisopteran dragonflies differs in shape and size. It may be large as reported in *Trithemis arteriosa* [20], *Crocothemis erythraea* [24], *Sympetrum rubicundulum* [19], *Nesciothemis farinose* [12], *Erythemis simplicicolis* [28], *Sympetrum depressiusculum* [19] and *Tramea virginia* [15] medium size as in *Brachythemis leucosticte* [20], *Brachythemis lacustris* [20] and *Celithemis elisa* [24] or very small as in *Orthetrum cancellatum* [25] and *Orthetrum chrysostigma* [16]. In *O. s. sabina* it is a small spherical chamber with a tiny circular lumen

The vagina is a sac-like tubular organ located on the floor of the eighth abdominal segment. In *Somatochlora arctica*, the median oviduct opens into the vagina through a valved opening encircled with a cuticular rim [10]. The opening of median oviduct inside the vagina is guarded by a pair of thin cuticular membrane which forms a valve so as to control the flow of eggs in the vagina. The variation in the position and shape of the valve is well evident in many libellulid odonates [10-21] and in the present study too a well developed cuticular valve is located at the median oviduct-vagina junction. In *O. s. sabina* the undulating membrane present on the floor of vagina it is very well developed and modified so as to form a thick band with a tiny frill all along its dorsal side. The function of this membrane is two-fold: during copulation it guides the penis towards the sperm storage organ and forms a sliding platform for to and fro movement of the sclerotized penis inside the vagina without damaging the vaginal wall and secondly, it helps to slide the eggs down from the median oviduct into the vagina. It also helps to position the egg so that the micropylar apparatus is perfectly placed under the fertilization pore [15].

In odonates it is of common occurrence (as found in *O. s. sabina*) for the vagina to narrows down and opens distally into the sub-genital plate through the female gonopore. In *O. s. sabina* the vagina is initially divided into two chambers by a horizontal cuticular septum. The lower chamber separates the passage for the egg from the oviduct while the upper chamber houses the bursa communis. The cuticular septum disappears half way just below the fertilization pore for the smooth transport of egg. Siva-Jothy [19] for the first time reported the presence of campaniform sensillae in the vaginal plates of the libellulid dragonfly, *Orthetrum cancellatum*.

Later, these mechanoreceptors were also found in *Crocothemis erythraea* and *Sympetrum depressiusculum* [25, 26, 27]. In *O. s. sabina* the button shaped sensilla are placed equidistant along the lateral vaginal plate. The cuticular intima of the sperm storage organ is smooth and lightly wavy in the spermatheca and unsculptured in bursa copulatrix for the smooth movement of the seminal fluid store in it. The inner lining of the fertilization pore and bursa communis is lined with denticles, pegs and spines for the segregation and movement of individual spermatozoa during fertilization. The serrated and spiny intima of vagina helps to hold the egg and penis during oviposition and copulation, respectively.

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