

Morphology, Histology and Serotonin Immunoreactivity on Salivary Glands of Stick Insect, *Phobaeticus serratipes* (Phasmida: Phasmatidae)

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Abstract

The salivary gland plays a significant role in physiological processes in insects including food lubrication, extra-oral digestion and enzyme secretion. This study was conducted to describe the morphology and histology of salivary glands of stick insect, *Phobaeticus serratipes* (Phasmida: Phasmatidae). The observation on gross morphology of salivary glands was photographed by using DSLR Canon EOS 6D camera attached to a stereo microscope. The histological study of the salivary glands involves special staining procedures of periodic Schiff's acid reagent and Alcian blue method. The immunohistochemical study of the biogenic amine serotonin distribution was observed under fluorescence microscope Zeiss AxiocamMRm Apotome.2. Results showed that the salivary glands were the acinar type that consists of two cells, parietal cell and zymogenic cell. The serotonin immunoreactivity of the salivary glands which located on the nerve fibers and might act as a neurotransmitter.

Keywords: Morphology, histology, serotonin, *Phobaeticus serratipes*, salivary gland

1. Introduction

Phasmatodea insects are herbivores that use their bodies' similarities to twigs, leaves, branches and lichen as an advantage for camouflaging themselves with vegetation (Bedford, 1978). Phasmatodea consists of 3000 species that are divided into 3 Families and 500 Genera (Whiting *et al.*, 2003) including the largest insect, *Phobaeticus chani* Bragg, with the length of the female can reach up to 567 mm (Hennemen and Conle, 2008). They belong to the monophyletic group within the Orthopteroidea that is similar with Orthoptera, Blattaria, Dermaptera, Dictyoptera, Grylloblattodea and Mantophasmatodea (Flook and Rowel, 1998). The earliest described stick insects from West Malaysia were *Marmessoidea rosea* (Fabricius) in 1793 and *Heteropteryx dilatata* (Parkinson) in 1798 (Seow-Choen, 2005). To date, only five families of stick insects were recorded in Malaysia. They are Heteronemiidae, Phasmatidae, Aschiphasmatidae, Bacillidae and Phylliidae.

The salivary gland of insects is the gland associated with the nutrients intake where secretion is usually involved in the digestion and lubrication of food (Ali, 1997). There are two main types of the salivary glands of insects which are acinar as in the locust and cockroaches and tubular as in blowfly and Lepidoptera (Ali, 1997). The general function of salivary secretions is digestion but they may perform additional functions in some insects. For example, the saliva of locusts and cockroaches contains

digestive enzymes (Gardiner, 1972; Kendall, 1969), the saliva of mosquitoes contains anticoagulants and irritants (Gardiner, 1972; Ribeiro, 1992) and labial on Lepidoptera can produce silk (Kafatos, 1968).

Salivation can be controlled either by direct nervous innervation or via neurohormone. Biogenic amines are one of the mediators involved in the control of insect salivation. They also act as neurotransmitters, neuromodulators or neurohormones in the nervous system and various peripheral organs of vertebrates or invertebrates (Baumann *et al.*, 2003; Blenau and Baumann, 2001; Evans, 1980; Roeder, 1994). Serotonin, dopamine, octopamine and tyrosine hydroxylase are some of the biogenic amines. Salivary glands can be innervated from several sources. Most insects have salivary nerve which projects from suboesophageal ganglion but some species like *Periplaneta americana* and *Rhodnius prolixus* are also equipped with a salivary nerve that projects from stomatogastric nervous system (Baptist, 1941; Davis, 1985). Dopamine and serotonin emulate an important role in the control of salivary glands in most insects (Baines and Tyrer, 1989; Berridge, 1970; House, 1973). Salivary nerves of stick insects receive axonal projection from salivary nerve 1 (SN1) and salivary nerve 2 (SN2) in the subesophageal ganglion (Ali and Orchard, 1996). Immunohistochemistry shows that dopamine presents in SN1 whereas serotonin presents in SN2.

Not much research has been done on the salivary glands of stick insects. In this study, we report on the morphology and histology of the salivary gland of

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Phobaeticus serratipes. We present a detailed description of the salivary gland using stereo microscope and light microscope. We also present results of the location of serotonin in the salivary gland. Stick insects are hypothesized to have an acinar type of salivary glands based on their feeding behaviour with chewing mouthpart. Salivary glands of insects have at least two different types of secretion cells such as parietal cells and zymogenic cells (Beams and King, 1932). The serotonin might be present on the nerve of the salivary gland as it is known as a monoamine neurotransmitter.

2. Material and Methods

2.1. Samples preparation

Samples were collected from Langkawi Island, Kedah, Fraser's Hill, Pahang and Gunung Ledang, Johor. Overall, 30 fresh samples were used in this study.

2.2. Gross Morphology of Salivary Glands

All stick insects were weighed before dissection. Solutions of methylene blue were injected at the jointed segment between the legs and abdomen and also between the head and thorax of the stick insects. Samples were left for an hour at room temperature. Dissections were done in phosphate-buffered saline (PBS) and images of salivary glands in-situ and ex-situ were captured using a DSLR Canon EOS 6D camera attached to a stereo microscope.

2.3. Tissue sections

The salivary glands were fixed in the formalin solution for 2-4 hours. Then, the formalin solution was removed by washing in 70% ethanol. Next, the glands were dehydrated through a series of ethanol (50%, 90%, 100%) for an hour each. Tissue was left in sub-Xylene for an hour, infiltrated with wax (3x at 58°C) and embedded. Tissue was sectioned (3-5µm) using Leica RM2245 microtome. The slides containing tissues were stained using Alcian blue staining followed by periodic acid-Schiff's reagent (PAS). Images of the stained sections were observed under the light microscope (Zeiss Axio Scope) with iSolutionLite software.

2.4. Tissue sections immunofluorescence

The serotonin detection was performed in the tissue sections and whole mounts salivary gland tissue (Wan Nurul 'Ain and Nurul Wahida, 2015). Briefly, slides of tissue sections were rehydrated through a series of solutions (xylene and ethanol (2x100%, 95%, 70%). Then, the slides were further rehydrated with phosphate-buffered saline (PBS) for 10 minutes. The excess wash buffer was drained. Slides were partially dried after removal from PBS except for the tissue sections. After that, 2 drops of pre-blocking agent PBT (PBS of 50ml +0.2% bovine serum albumin, 25ul +0.1% TritonX-100, 5ul) was used to cover tissue sections and left for 20 minutes and then tapped off and wiped away. Tissue sections were covered with diluted primary antibody (anti serotonin) or negative control (PBT). The primary antibody was diluted 1 in 1000(1µl in 1ml of PBT + 1% normal goat serum, 100ul) (PBT+N). Slides were incubated overnight at 4°C. Slides were rinsed with PBS to wash off excess serum and drained. Tissues were then covered with PBT plus normal goat serum (PBT+N). Then, the tissues were incubated

overnight (4 °C) with secondary antibody conjugated to Dylight (dilution 1:300). The antibody was washed with PBT (30 min, 4x) before a complete inversion in PBS and further washing with series of ethanol (100%, 95%, 70%, 50%) to rehydrate it. The slides were mounted in a mixture of 50% glycerol and PBS and covered with a coverslip. The slides were then dried out on a slide warmer overnight and observed under a fluorescence microscope (Zeiss AxiocamMRm Apotome.2) with ZenPro2012 software and Olympus FSX100 microscope.

2.5. Whole mount tissue immunofluorescence

Fresh salivary glands were fixed in 4% paraformaldehyde in PBS (18 hours at 4°C). After washing it with PBS, the tissue was permeabilized by exposing it to methanol (5 min, 70% MeOH in PBS, 60min, 100% MeOH and 5min, 70% MeOH in PBS) before washing with PBS (5min, 2x). The tissues were incubated for 30min in 100 mL of PBT+N (PBT + 5% normal goat serum) before being processed with 100 mL of diluted (1:1000) primary antibody serotonin and incubated overnight at 4°C. The antibodies were washed off by multiple rinses with PBS (5min, 3x) and PBT (45min, 2x). Then, secondary antibody Dylight (1:300) was added to each vial, and the tissues were incubated at 4°C overnight. The tissues were washed in PBT (5min, 3x) before a complete inversion in PBT for 2 hours. The tissues were cleared in a mixture of 50% glycerol and 50% PBS overnight before mounting on slides. The control samples were processed with similar procedure but with absence of the primary antibody. The distribution of serotonin was observed under fluorescence microscope (Zeiss Axiocam MRm Apotome.2) with ZenPro2012 software and Olympus FSX100 microscope.

3. Result and Discussion

3.1. General morphology of salivary glands

The salivary glands of *Phobaeticus serratipes* consist of cluster of small globular types of acini. This acinar type of salivary glands had also been reported in other species of stick insect such as *Carausius morosus* (Asimakopoulos and Orchard, 1998) and other solid feeder insects such as grasshopper, *Gastrimargus musicus* (Nurul-Wahida and Cooper, 2014) and cockroach, *Periplaneta americana* (Just and Walz, 1996). Based on the gross morphology of the salivary glands that had been studied, the salivary glands of *P. serratipes* are paired glands that can be found at both sides of the lateral prothorax and extended to the metathorax (Figure1).

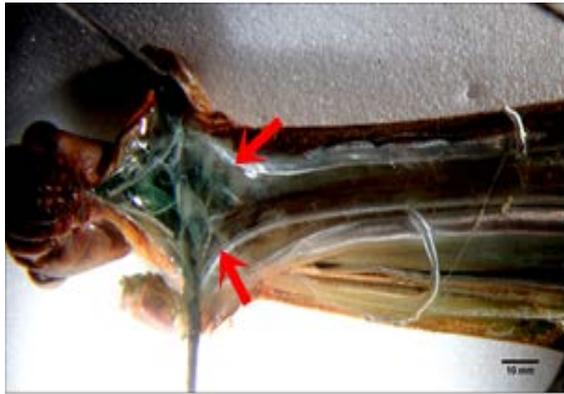


Figure 1. Image of in-situ (red arrows) along the prothorax to metathorax.

The size and distribution of salivary glands are different between the male and female stick insects due to the difference in the size of their body. The size of female is bigger than the male stick insect. The process of saliva secretion occurred at the glands of the globular acinar. The transparent and fine asinus duct canal acts as the connector between all the acini. This canal has an outlet from each of the acinus globules where it channels out the saliva from the acinus to the lower part of the labium of the mouthpart. The saliva will be collected in the collecting ducts before it is secreted. The collecting ducts from both sides of salivary glands will be fused at the head capsule and opened to become a salivary cup at the labium (Kendall, 1969), thus forming the main duct (Figure 2). The saliva is secreted from the main duct.

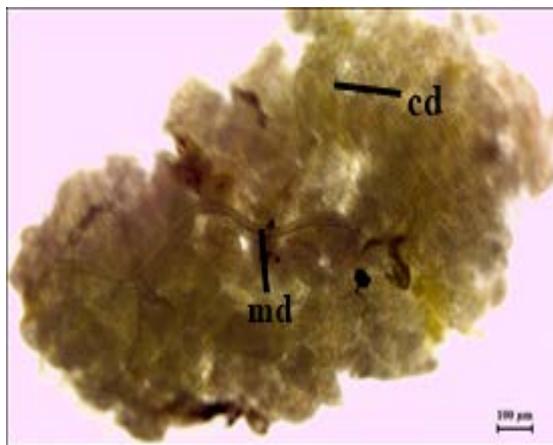


Figure 2. Ex-situ of acinar salivary glands of *P. serratipes*. md (main duct); cd (collecting duct).

Based on *ex-situ* and *in-situ* observation, no secretory gland was detected in *P. serratipes*. This secretory gland can be found in other species such as *Asceles glaber* (Dossey *et al.*, 2012) and *Oreophoetes peruana* (Eisner *et al.*, 1997).

3.2. Histology of salivary glands

The acinar glands of *P. serratipes* consist of two types of cells, parietal and zymogenic (Figure 3). Each acinus cell is covered by a basal membrane on the outside. Basal membrane mould acinus cell a round shape which later forms acinus globule.

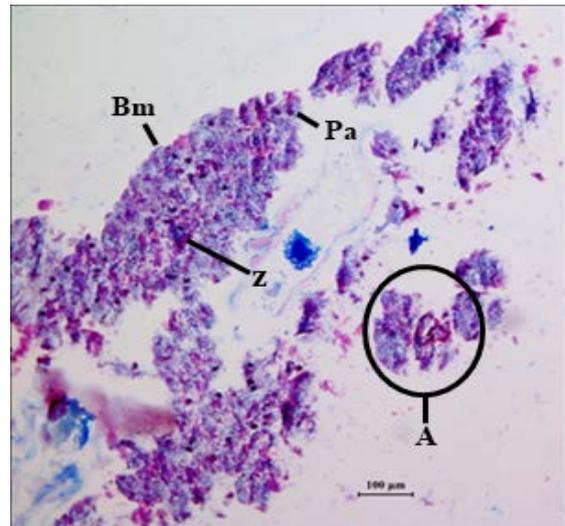


Figure 3. Tissue section of *P. serratipes* salivary gland. Tissue was stained with Alcian blue and PAS reagent. A (Acini); Bm (basal membrane); Pa (parietal cell); z (zymogenic cell).

Parietal cells are cone-shaped and have wide basal connected with basal membrane cells. They are located between zymogenic cells and extended towards the centre of each acinus. The nucleus of the parietal cell is big and oval-shaped. It is located at the centre of the cell. In contrast, zymogenic cells have an irregular shape. The basal of the cell is smaller compared to parietal cells. The nucleus is small in size and present on the side of each cell.

3.3. Serotonin distributions on salivary glands

The serotonin on the salivary glands of *P. serratipes* can be seen clearly at the axons along the ducts to the acini globules and the nerve fibers in the acini (Figure 4).

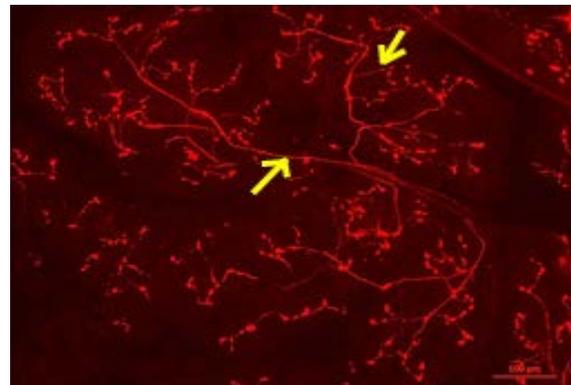
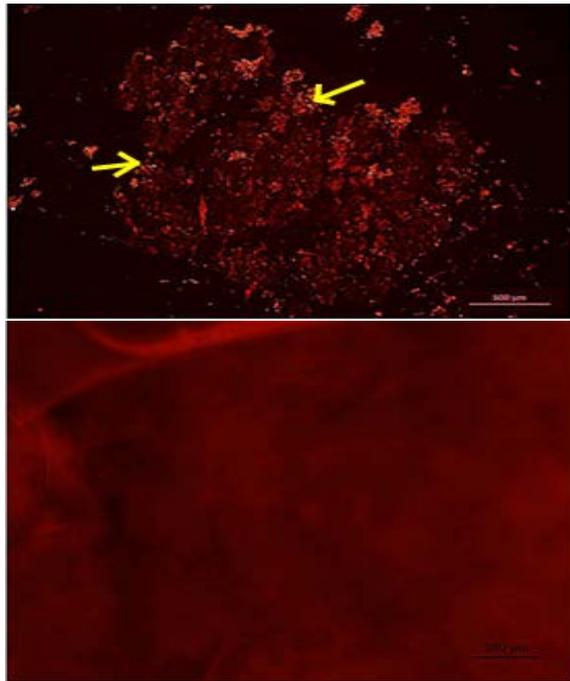


Figure 4. The serotonin distributions on the nerve fibers of *P. serratipes* salivary glands (yellow arrows).

Moreover, for the cross-section of the tissues, the serotonin was distributed on both cells in the salivary glands, parietal cells and zymogenic cells (Figure 5). This result was supported by Nurul-Wahida and Cooper (2014) who reported the presence of serotonin on both parietal and zymogenic cells of yellow-winged grasshopper, *Gastrimargus musicus*. Serotonin is absent on the salivary gland of controlled stick insect (Figure 6).

The presence of serotonin will produce saliva with high protein content (Just and Walz, 1996). Electrical innervation towards the nerves or glands of the salivary ducts that superfusion with dopamine and serotonin will stimulate the secretion of saliva (Just and Walz, 1996).

Liquid secretion rate is controlled by peripheral cell (p-cell) at the base of each acinar globule and involved in the transportation of water and electrolytes as in cockroaches, *Periplaneta americana* (Kessel and Beams, 1963; Sutherland and Chillseyzn, 1968). Central cell, also known as c-cell, will react with serotonergic innervation and supply the proteinaceous components to the saliva (Just and Walz, 1994, 1996; Walz *et al.*, 2006). The parietal cells of stick insects have similar function and morphology as the peripheral cells or p-cells of the cockroaches, whereas the zymogenic cells are similar to the central cells or c-cells.



Figures 5-6. (5) Serotonin distributions on the cross section of salivary glands tissues of *P. serratipes* (yellow arrows). (6) No serotonin-like immunoreactive process on salivary glands of control stick insect.

4. Conclusion

It can be concluded that the serotonin in *P. serratipes* plays a role as a neurotransmitter that is similarly described in the *Periplaneta americana* (Ali, 1997; Ali and Orchard, 1995) due to its presence on the nerve fibers of the salivary glands. Besides, the distribution of serotonin on both parietal and zymogenic cells suggests that the serotonin also innervates the production of proteinaceous and non-proteinaceous saliva for this species.

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