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Pestycydy, 2007, (3-4), 11-16.

ISSN 0208-8703

## **Peptidergic innervation of the excurrent ostia of two Orthopteroid insects**

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**Abstract:** The dorsal vessels of two Orthopteroid insects, the African migratory locust, *Locusta migratoria*, and the Vietnamese stick insect, *Baculum extradentatum*, were examined for their morphology. The dorsal vessels of both of these species consist of a tubular heart and an aorta that extends anteriorly into the head. Alary muscles, associated with the hearts, are anchored to the body wall with attachments to the dorsal diaphragm. Alary muscle contraction draws haemolymph into the heart through incurrent ostia. Excurrent ostia are present in both species with 7 pairs in the locust and 3 pairs in the stick insect. Each excurrent ostium is made up of a mass of cells that produce openings or slits that allow the haemolymph to exit the heart and enter the perivisceral cavity directly without traveling through the aorta. Muscle fibers are associated with the excurrent ostia and these receive innervation from nerve processes containing proctolin-like immunoreactivity. These data indicate that the excurrent ostia are most likely under modulation from neuropeptides that might result in microcirculatory changes in these insects.

**Keywords:** Scanning Electron Microscopy, proctolin, heart, immunohistochemistry, ostia

### **INTRODUCTION**

The morphology and function of the dorsal vessel has been examined in a variety of insects [1, 2]. Typically, in Orthopteroid insects the dorsal vessel is composed of a chambered heart within the abdomen and an unchambered aorta in the thorax and head. During diastole, haemolymph is drawn through lateral

incurrent ostial valves, facilitated by contraction of alary muscles, and during systole, contractions of the cardiac muscle close the ostia and force haemolymph anteriorly towards the head capsule. In a limited number of species, excurrent ostia are also present [3], allowing haemolymph to pass out of the dorsal vessel into the main body cavity in the anterior abdominal/thoracic region. In the hemimetabolous insect, *Periplaneta americana*, paired lateral cardiac nerve cords provide two physiologically distinct nervous components [2]; cardiac neurons that give rise to motor axons that innervate the heart, and neurosecretory cells. The segmental nerves from the ventral nerve cord join the lateral cardiac nerve cords. Neurosecretory axons within the segmental nerves, together with the cardiac neurons and intrinsic neurosecretory cells, comprise the cardio-regulatory system.

The insect heart has often been used as a model for examining the influence of neurotransmitters and neurohormones, and a variety of these neuroactive chemicals have been shown to modulate heart beat [1, 2]. In particular, the neuropeptide proctolin, the first insect neuropeptide to be sequenced and synthesized [4], is a potent stimulator of insect visceral muscle and has also been shown to be a potent stimulator of heart rate [5, 6].

This short communication examines the morphology of the excurrent ostia of two Orthopteroid insects, the African migratory locust, *Locusta migratoria*, and the Vietnamese stick insect, *Baculum extradentatum*, using scanning electron microscopy and immunohistochemical techniques.

## MATERIALS AND METHODS

**Animals:** Insects from colonies of the Vietnamese stick insect, *Baculum extradentatum*, and the African migratory locust, *Locusta migratoria*, were reared at high relative humidity in incubators kept at 25 °C and 30 °C, respectively.

**Scanning Electron Microscopy:** Locusts and stick insects were dissected with an antero-posterior lateral incision. The alimentary canal, along with the reproductive tissues, was removed and the heart, with associated structures and central nervous system, was left intact. The surface of the specimens were then washed with the use of phosphate buffered saline (PBS; 10 mM sodium phosphate, pH 7.2, containing 0.9% NaCl), and then placed in 2.5% glutaraldehyde (in 0.1 M phosphate buffer, pH 7.4) overnight at 4 °C. The specimens were then washed 3 times using PBS and then dehydrated with 50%, 70%, 95%, 100% ethanol series, followed by 100% acetone. Specimens then underwent critical point drying with liquid carbon dioxide, followed by mounting on aluminum stubs and then gold

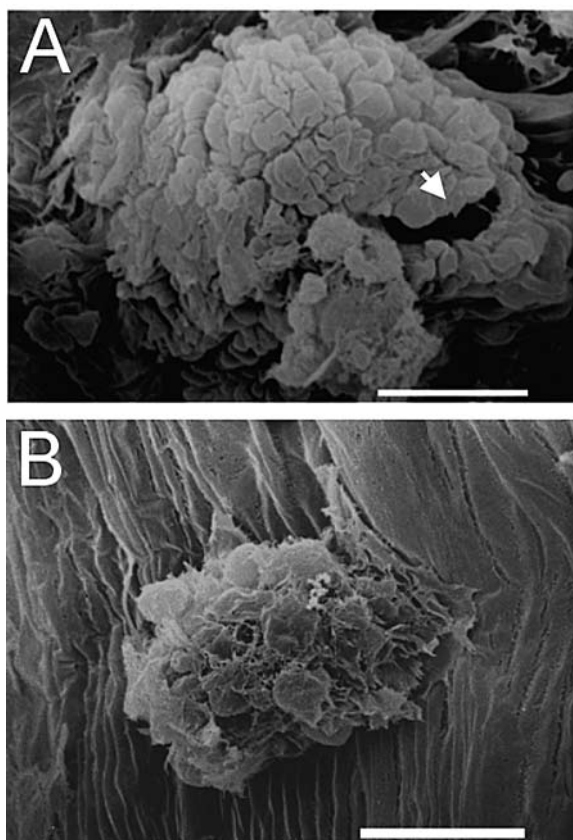
sputter coating (using a SEM PS3 Coating Unit). An Hitachi S-530 scanning electron microscope was utilized for specimen viewing, and pictures were taken using a 35 mm camera.

**Immunohistochemistry:** The heart of the stick insect was processed for immunohistochemistry and phalloidin-Tetramethylrhodamine B isothiocyanate conjugate (phalloidin - TRITC) staining using techniques similar to those previously described [7]. Fixed tissues were incubated in a polyclonal rabbit antiserum generated against proctolin (1:1000; a gift from Dr. Hans-Jürgen Agricola, Friederich-Schiller Universität, Jena, Germany) for 48 h followed by 18 h in Cy3-labelled sheep anti-rabbit immunoglobulin G (1:600).

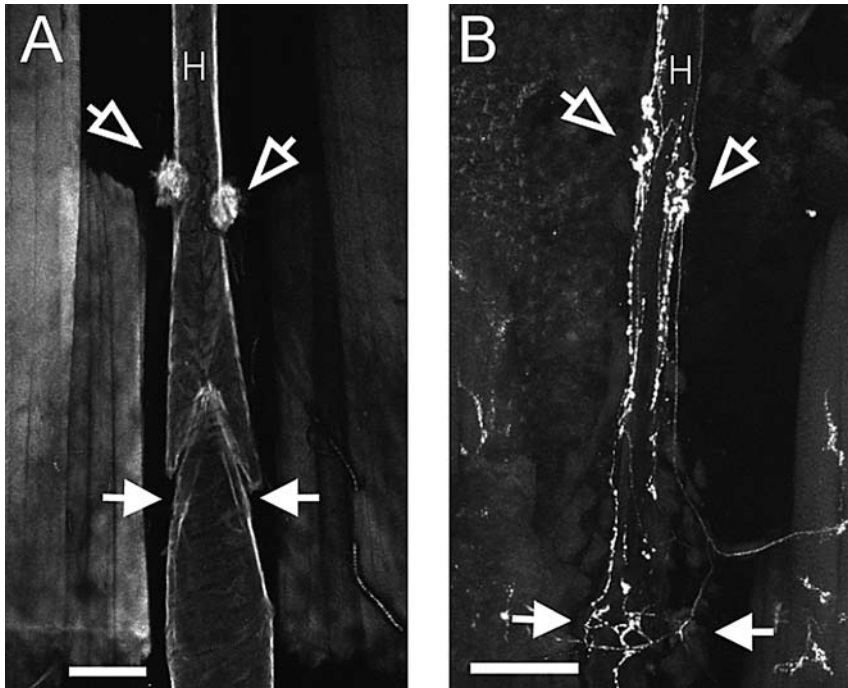
## RESULTS

Using scanning electron microscopy it is possible to get detailed information about the morphology of the excurrent ostia of the locust, *L. migratoria*, and the stick insect, *B. extradentatum*. Seven pairs of excurrent ostia lie on the dorsal vessel of the migratory locust. One pair of these excurrent ostia is located on the mesothoracic ampullae and another pair on the metathoracic ampullae, with the other five pairs located on each of the first five chambers of the heart within the abdominal segments. The excurrent ostia are cellular masses with a diameter of 60-70  $\mu\text{m}$  located on the ventral surface of the dorsal vessel (Figure 1A). It is quite common to see openings or slits between the cells of each excurrent ostium (Figure 1A). Cells of the excurrent ostia stain with phalloidin-TRITC, which stains for the presence of F-actin, indicating the presence of muscle cells associated with these structures (data not shown).

The morphology of the excurrent ostia of the stick insect, *B. extradentatum* is similar to that seen in the locust. They are made up of a mass of cells with an overall diameter of 40-50  $\mu\text{m}$ , ventrally-located, with slits or openings in the cell mass (Figure 1B). Injection of dye in the posterior of the insect reveals a flow of dye anteriorly through the heart and aorta, with some dye leaving through the excurrent ostia. The excurrent ostia also stain with phalloidin-TRITC indicating that muscle cells are associated with these structures (Figure 2A). The stick insect only has three pairs of excurrent ostia, with one pair in the metathoracic segment and the other two pairs in the first and second abdominal segments of the heart. Immunohistochemistry reveals that proctolin-like immunoreactive processes project along the heart with branches over the incurrent and excurrent ostia (Figure 2B).



**Figure 1.** Scanning electron micrographs of a ventral view of an excurrent ostium associated with the heart of the locust, *L. migratoria* (A) and the stick insect, *B. extradentatum* (B). Note the opening or slit formed between the cells of the excurrent ostium (arrow in A). Scale bars: 20  $\mu\text{m}$ .



**Figure 2.** Confocal images of TRITC-labelled phalloidin (A) and proctolin-like immunoreactivity (B) of the heart (H) of the stick insect *B. extradentatum*. The excurrent ostia (open arrows) stain for F-actin and have proctolin-like immunoreactive processes associated with them. The incurrent valves (closed arrows) also receive innervation from proctolin-like immunoreactive processes. Scale bars: 100  $\mu$ m.

## DISCUSSION

Proctolin has not previously been shown to be associated with the insect dorsal vessel although it has been shown to increase heart rate in many insect species [5, 8]. Proctolin-like immunoreactivity is present on stick insect heart, in processes closely following the pattern of alary muscles, with blebs and varicosities indicative of possible release sites [7]. Proctolin-like immunoreactivity is present in processes over all three pairs of excurrent ostia, as well as, over the incurrent ostia. A branch of the segmental nerve projecting to the dorsal vessel contains proctolin-like immunoreactive axons which project along the heart wall to the excurrent ostia. Thus, the excurrent ostia may be both muscular in nature (F-actin

staining), and also be a site of release for proctolin. Very little is known about these unique structures. Nutting [3] has described their presence in Orthopteroid insects (9 orders, 5 suborders, 48 families) and shown that they are ovate openings in the dorsal vessel wall that appear as opaque whitish nodules. While no valves have been described, Nutting [3] believed that excurrent flow of haemolymph from the dorsal vessel is enabled by a papilla of cells surrounding each opening which might expand and contract with each heart beat. Nutting [3] did, however, consider that some excurrent ostia possess muscular tissue that extended a short distance between the basal cells of the valve. The F-actin staining of the excurrent ostia would seem to support their muscular nature, opening up the possibility that they might control the flow of haemolymph into the perivisceral cavity. Indeed, the presence of proctolin-like immunoreactivity over the excurrent and incurrent ostia suggests an important level of neural and peptidergic control involved in the regulation of circulation. In addition, the excurrent ostia might well facilitate the release of proctolin directly into the body cavity, leading to the rapid release and circulation of proctolin as a neurohormone in the insect haemolymph [9]. The excurrent ostia have been an overlooked source of circulation control, with little or no physiology known.

### Acknowledgements

This work was supported by a grant from the Natural Sciences and Engineering Research Council of Canada.

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