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Histological and histochemical characterisation of glands associated with the feeding appendages of *Argulus foliaceus* (Linnaeus, 1758)

Aisha AmbuAli[1 & 2*], Sean J Monaghan[1], Kawther Al-Adawi[3], Mohamed Al-Kindi[3], James E Bron[1]

1 Institute of Aquaculture, University of Stirling, Stirling, U.K.

2 Department of Marine Science and Fisheries, College of Agricultural and Marine Sciences, Sultan Qaboos University, Oman

3 Department of Pathology, College of Medicine and Health Sciences, Sultan Qaboos University, Oman

Abstract

Argulus foliaceus (Linnaeus, 1758) is a member of the branchiuran family *Argulidae*, a group comprising parasitic "fish lice". *A. foliaceus* is distributed worldwide and causes major economic impacts for cultured freshwater fish globally. The work described in this study was undertaken with the objective of identifying, describing and characterising glands associated with feeding in *A. foliaceus*. From structural and ultrastructural microscopic studies of *A. foliaceus*, three types of gland were determined to be associated with the pre-oral spine and mouth tube and were suggested to be involved in feeding activities. Two of these glands, the labial glands and the proboscis glands, appeared to secrete their products via the mouth tube and a third, the spinal gland, was connected directly to the pre-oral spine. The current study confirmed that the pre-oral spine delivers active secretions from the spinal gland, which may aid in immunomodulation, while the tubular labial spines and proboscis glands openings within the mouth tube may serve to enhance the feeding process by delivering salivary components to aid pre-digestion and immune-modulate the host. The suggested functions are supported by histological and histochemical staining, coupled with fluorescent lectin-binding assays, which enabled characterisation of the carbohydrate moieties associated with these glandular tissues.

Keywords: *Argulus;* fish lice; secretory; lectin; gland; pre-oral spine; proboscis; labial.

1. Introduction

Argulus is one of the most common crustacean fish ectoparasite genera encountered worldwide [1–3] and is the largest and most diverse genus of the family *Argulidae* [4]. Members of this genus are responsible for the condition argulosis, a parasitic disease affecting both wild and cultured fish populations [5,6]. In the UK, argulosis causes economic impacts for both aquaculture and sports fishing industries. During feeding *Argulus* species have been suggested to cause damage to the host's skin largely as a result of the mechanical movements of the pre-oral spine and through enzymatic action / toxicity of secretory substances produced by the spinal gland. The mandibles of the mouth tube also inflict mechanical damage during the feeding process leading to secondary infections and serious damage [7,8]. Previous studies have also reported haemorrhagic and inflammatory responses after *Argulus* infection [9–11], which have been taken as evidence to support the idea that the parasite secretes substances / enzymes such as proteases or anticoagulants during the feeding process to assist with the ingestion of blood and epithelial tissue.

Although previous published studies have provided information focused on glands related to the pre-oral spine and mouth tube [9,12–15], the available information remains limited and no conclusive data are available regarding the host-parasite interactions involving these glands.

While studies on the feeding mechanism of *Argulus* and the function(s) of the pre-oral spine and related glands have been conducted, many aspects of this area of research are still under

debate [11]. More recently, von Reumont *et al.* [16] highlighted the need for newer studies to focus on particular glands as "venom apparatus" involved during feeding in branchiurans with emphasis on *Argulus* species and the type of substances they secrete. To date, the presence of digestive and immunomodulatory products, previously reported for other parasitic *Crustacea*, such as the expression of prostaglandin, trypsin or peroxidases in salmon lice, *Lepeophtheirus salmonis* (Krøyer, 1837) [17,18], has not been confirmed in *A. foliaceus* glandular secretions [19]. Secretory products such as trypsin have been identified in the salmon louse gut and are thought to facilitate digestion [20] and possibly contribute to inhibition of localised host inflammatory responses [21]. Recent mapping of the glands of *L. salmonis* have highlighted the importance of secretions produced by a group of tegumental glands and labial glands to parasite virulence [22]. The similarity of the mode of parasitism expressed by *Argulus* spp. to that of other crustacean fish ectoparasites prompts the inference that immunomodulatory mechanisms of this parasite might mirror those of other parasitic species.

The principal aims of the work described in this study were, therefore, to determine the sites of secretory production involved in host-parasite interactions and to begin to assess the role of the pre-oral spine in this interaction. This study was conducted by investigating the glands of *A. foliaceus* hypothesised to be associated with feeding, using light and fluorescence microscopy and transmission and scanning electron microscopy. Taking advantage of their properties to recognise and bind to specific sugar moieties or certain glycosidic linkages of polysaccharides, glycoproteins and glycolipids [23], fluorescently labelled lectins were also utilised as biochemical tools to further characterise the glands and feeding apparatus.

2. Materials and methods

2.1. Sample collection

Fresh adult samples of *A. foliaceus* were collected from fisheries in the UK. The specimens were collected from pike (*Esox lucius* Linnaeus, 1758), rainbow trout (*Oncorhynchus mykiss* Walbaum, 1792) and common carp (*Cyprinus carpio* Linnaeus, 1758) and were processed according to downstream analytical requirements.

2.2. Light Microscopy

2.2.1. Fixation and paraffin wax embedding

Parasites were fixed in 10% (v/v) neutral buffered formalin (NBF) for 48 h prior to processing for paraffin wax (SAKURA-TEKII wax, polymer added) embedding and routine histology. Fixed tissue was processed using a Shandon Citadel 2000 tissue processor (Thermo Fisher Scientific Inc., UK) for 16 h. Tissue was processed using a standardised laboratory protocol including dehydration through a graduated alcohol series, clearing with xylene and infiltration with paraffin wax. Specimens were embedded using a Leica HistoEmbedder and blocks were trimmed and sectioned at 3µm using a rotary microtome (*Leica,* Jung Biocut 2035). Sections were dried at 40°C on a hotplate (Raymond Lamb) and slides incubated at 60°C for a minimum of 1 h prior to staining.

2.2.2. Haematoxylin and eosin staining of paraffin sections

Haematoxylin and eosin (H&E) was used to stain 3µm paraffin wax sections using a standard laboratory protocol. Briefly, sections were deparaffinised with xylene (BDH, U.K.), brought to water, and stained in Mayer's haematoxylin and eosin for 5 min each, dehydrated in an ethanol series, cleared in xylene and coverslipped with Pertex (Cellpath).

2.2.3. Combined Alcian blue-PAS stain for acid and neutral mucins (Mowry, 1956)

This stain is used to differentiate between neutral and acidic mucins. Serial sections were stained in Alcian blue (pH 2.5) for 10 min followed by a running tap water wash and a distilled water wash, respectively. Then, they were exposed to 1% (Aq) periodic acid for 5 min and washed in two changes of distilled water. Sections were then stained in Schiff's reagent for 15 min, washed with tap water and stained in Mayer's haematoxylin for 5 min. After a final wash in tap water, sections were dehydrated in alcohol and xylene and mounted with Pertex.

2.2.4. Periodic acid-Schiff with tartrazine counterstain and haematoxylin

This stain is used for detecting the presence of carbohydrates in various tissues. Longitudinal, sagittal and tangential serial sections (3µm) of 10% NBF fixed paraffin wax embedded tissues were cut. The sections were dewaxed with xylene (BDH, U.K.), brought to water, and were stained in freshly prepared 1 % periodic acid, Schiff reagent followed by Mayer's haematoxylin. The sections were washed with running tap water after each step, blueing in Scott's tap water. The counter stain used for this procedure was tartrazine. Sections were dehydrated in three changes of absolute alcohol, cleared in xylene then coverslipped with Pertex.

2.2.5. Technovit® 7100 glycol methacrylate resin embedding and Haematoxylin and eosin staining

To obtain better resolution of finer structures, fixed samples were resin processed and embedded using Technovit®7100 glycol methacrylate. Tissue samples fixed in 70% ethanol were dehydrated in 2 changes of 100% ethanol for 30 min. Specimens were then pre-infiltrated in 1:1 100% ethanol: base Technovit® 7100 for 2 h. The specimens were kept in the infiltration solution (1 g of hardener I in 100 mL base liquid and mixed for 10 min) and were then incubated overnight. Specimens were placed in plastic moulds and embedded in an

embedding solution (15 parts of the infiltration solution were added to one part of the hardener II, mixed for 1 min). Moulds containing specimens were each covered by a microtome block holder and left for 2 h to cure at 21°C. Serial sections of 2µm thickness were prepared using clean glass blades and flattened in an ambient temperature water bath prior to picking up on glass slides. Serial sections were stained with H&E and mounted in Pertex.

2.3. Light photomicrography

Light microscope images were taken using an Olympus BX51TF light microscope with a Zeiss AxioCam MRc colour digital camera. MRGrab version 1.0 (Zeiss) software was used to capture and save images.

2.4. Scanning electron microscopy

For scanning electron microscopy (SEM), samples fixed in 10% neutral buffered formaldehyde were initially used due to a lack of fresh samples. *A. foliaceus* were washed in buffer for 10 min followed by fixation in 2.5% glutaradehyde (v/v) for 2 h. Samples were then washed twice with 0.1M sodium cacodylate buffer and post-fixed in 1% osmium tetroxide for 1 h. Samples were washed in distilled water and then dehydrated through a graded alcohol series. Critical point drying was conducted using an Autosamdri critical point drying machine. Finally, a JEOL JSM-5600V scanning electron microscope was used for scanning and obtaining micrographs. At a later date, fresh samples were received and fixed in 2.5% glutaradehyde for overnight fixation and then transferred to a 0.1M sodium cacodylate buffer solution and kept in the fridge (4°C) until used. The same critical point drying protocol was applied for these samples.

2.5. Transmission electron microscopy

For transmission electron microscopy (TEM), fresh specimens were fixed in 2.5% glutaraldehye pH 7.2 in 0.1 M sodium cacodylate buffer for overnight fixation and then transferred to 0.1M Sodium cacodylate buffer solution at 4°C until the samples were processed for TEM. Specimens were rinsed twice in 0.1M cacodylate buffer and post-fixed in 1% osmium tetroxide in distilled water for 1 h. Samples were then rinsed in distilled water and dehydrated through a series of concentrations of acetone: 25%, 75% and 95%. Next, specimens were infiltrated and embedded in epoxy araldite resin (Epoxy Resin Kit, Agar Scientific, UK) at 60°C overnight.

Semi-thin sections were cut using glass blades using a Leica Ultracut UCT ultra-microtome (0.5µm) for light microscopy. Semi-thin sections were then stained in 0.5% methylene blue in water and sodium borate. Ultra-thin sections were cut with a diamond knife using the same microtome at 70nm thickness. Sections were stained in super saturated uranyl acetate (Agar Scientific, UK) for 30 min and Reynold's lead citrate (Agar Scientific, UK) for 30 min. Sections were observed using a JEOL JEM 1230 TEM at 80 KV

2.6. Fluorescent lectin labelling of A. foliaceus glands

A. foliaceus parasites were collected from Isle of Bute, Scotland, and fixed directly in 4% paraformaldehyde overnight then transferred into 70% ethanol and kept at -20°C until ready for processing. Samples were processed manually starting with dehydration in 6 changes of absolute ethanol and two changes of isopropanol, clearing with two changes of xylene for 30 min each and infiltration in two changes of paraffin wax for 1 h each held at 60°C. Samples were then embedded in paraffin wax, serial sectioned at 5µm and mounted on treated glass slides (Superfrost® Plus glass slides, Thermo Scientific, Epsom, Surrey, UK), dried on a hotplate and stored at -20°C until ready to be used. For each lectin 2 sections were employed with sectioning at two different depths: section of spinal and proboscis glands and a deeper

section through the specimen at the level of the nauplius eye, incorporating the labial glands. Slides were taken from -20°C to 55°C oven and kept for approximately 30 min and were labelled using nineteen different lectins obtained from fluorescein lectin kits I-III (Vector Laboratories, Burlingame, CA, U.S.A.). Lectins were diluted in lectin wash buffer (LWB; 50mM Tris, 150mM NaCl, 2mM MgCl2, and 1mM CaCl2 pH 7.4) to 5µg mL^{-1}. Sections were then dewaxed manually into two changes of xylene for 3 min each, followed by a dehydration step using 100% then 70% ethanol for 2 min in each step. After washing in distilled water for 1 min, a wax circle was drawn around each section using an ImmEdge pen (Vector labs, p/n H-4000) to retain the lectin/buffer in place on the tissue section. Two hundred microlitres of lectin solution was pipetted on to the sections and incubated in a dark chamber at room temperature for 2 h. The sections were then washed in LWB three times for 5 min. A negative control was used with each batch of lectins for each labelling investigation and treated in the same way as test lectin sections, except with the use of LWB only. Slides were mounted in 4,6-diamidino-2-phenylindole, dihydrochloride (DAPI) (Vectashield; mounting medium for fluorescence with DAPI from Vector Laboratories, Burlingame, CA, U.S.A.), coverslipped and sealed with nail polish (MUA, Top Clear Coat, # 9607 6972), for prolonged storage. The slides were kept in the dark for 2 h then moved to 4°C and viewed within one week. The slides were analysed using a fluorescent microscope (Arcturus XT Laser Capture Microdissection System, Applied Biosystems) and micrographs were captured with an attached digital camera.

3. Results

1.1. Light, scanning and transmission electron microscopy of A. foliaceus appendages and glands associated with feeding

The feeding appendages of *A. foliaceus* lie between the suckers (first maxillae) in the mid-ventral line of the cephalon. The two key structures considered to be directly involved in feeding processes are the mouth tube and the pre-oral spine (**Figs.1, 2**).

The pre-oral spine is situated anterior to the mouth tube and consists of a cylinder-shaped sheath encasing the spine which can be protruded distally (**Fig. 2a**). The spine is narrow distally and has a tapered and pointed tip (**Fig. 2b, c**) and an oval spine duct running down the core of the spine **(Fig. 3)** with this duct being compressed in the spinal sheath ((**Figs. 3d, e, 5a, b**).

Figure 1b. shows the position of the pre-oral spine (stylet) and spinal gland with respect to the mouth tube. The oesophagus extends from the back of the mouth tube starting from the buccal opening and passing between the sub-oesophageal ganglion and cerebrum and into the lumen of the midgut via the proventricular funnel.

The spinal duct is connected to four secretory glandular cells, forming the spinal gland, and situated at the base of the pre-oral spine and the mouth tube (**Figs. 1b, 4, 7c, d**). The spine consists of an outer cuticle and thin epithelial layers surrounding the duct with the supporting cell (**Figs. 3a, b, c**), while the spinal sheath is made of an outer cuticle layer followed by a thin epithelial layer, both enclosing the spine duct and muscles involved in the spine retraction during feeding. Haemolymph fills the lumen of the sheath (**Fig. 3**).

The central duct of the pre-oral spine appears empty, with cuticle surrounding it, in this case probably doubled as individual is mid-moult. The epithelium at the base of the spine contains extensive rough endoplasmic reticulum (rER) and numerous mitochondria (M). A dense granular matrix (DGM) is situated between the outer layer of the spine and the inner lining of the sheath cuticle (**Figs. 5a, b).**

Large elongated nuclei (NE) (**Fig. 5a**) are present within the epithelial layer lining the sheath cuticle (ShC), with these cell nuclei occasionally containing a large nucleolus (N) (**Fig. 5a**). The epithelial tissue (E) around the spine duct (SD) (**Fig. 5a**) and the cytoplasm of these large nucleolus-containing cells, contains extensive rough endoplasmic reticulum (rER) exhibiting a tubular structure and the presence of free ribosomes (Rb) (**not shown**).

At the base of the spine is an abundance of widely distributed rough endoplasmic reticulum, ribosomes, and secretory vesicles (**Fig. 5b, d**). A pair of muscles, the pre-oral spine levator muscles, which are involved in antero-posterior movement of the spine, are enclosed by a sarcolemma, show abundant mitochondria and display a distinct peripheral nucleus (**Fig. 5d**).

The pre-oral spine duct originates from a spinal gland at the base of the spinal sheath (**Fig. 3e, 5a**). This gland comprises four giant polygonal cells with granular and basophilic cytoplasm as described earlier (**Figs. 1b, 4)**. The TEM micrographs of the spinal gland cell cytoplasm demonstrate numerous secretory vesicles of different sizes (V), intermediate vesicles (Iv), rough endoplasmic reticulum (RER), Golgi bodies (G), lysosomes (Ly) and endosomes (En) (**Figs. 5c, d**).

From transverse and sagittal histological sections and TEM micrographs, it was apparent that only the single duct that runs through the pre-oral spine is connected to the spinal gland. Although in some of the sections it has the appearance of two ducts, as illustrated in **Fig. 5a**, this was due to collapse of the duct within the spinal sheath, as demonstrated from TEM. The

distal part of the spine is rigid, encompassed by a supporting cell, contains a central duct, and is surrounded by a thin epithelial layer and, in this moulting specimen, two cuticle layers (**Fig. 5a-b)**. It is hollow with no visible luminal content. There are large elongated nucleated cells within the spinal sheath epithelial layer. These cells contain numerous rER and free ribosomes (**not shown**).

The buccal opening, which is situated at the distal end of the mouth tube, is bounded by an upper labrum and a lower labium. The labrum which is enfolded by the labium, is smooth externally with 3 pairs of sensory papillae and sharp denticulate margins; the third pair not visible in the micrograph (**Figs. 2d)**. The labium is larger and is covered by tiny spinules (**Figs. 1b, 2d**).

A pair of labial tubes known as labial spines arise within the buccal (labial) aperture. Each presents an apical pore (**Fig. 2d**), which may play a role in secretion of active products during feeding processes and appear to be connected to a third type of glands; which are termed in this study the "labial glands" (**Figs. 7b, d-f)**. Serial cross-sections of the mouth tube show the ducts supplying these labial spines (**Figs. 6**).

The proboscis glands are located ventral to the optic tract and the cerebrum. Each gland contains 3 basophilic giant cells with obvious central collecting ducts (**Figs. 4, 7a**) and a long discharge duct opening into the buccal cavity. Spinal and proboscis glands cells were stained intensely with PAS (**Fig. 4b**), suggesting the production of neutral carbohydrates.

Sagittal and planar serial sections of an adult male *A. foliaceus* indicated that the ducts of the labial glands extend from the mouth tube to the position of these glands (**Fig. 7c, d).**

This suggests that these gland cells have a secretory function with discharge of secretions via the tubular labial spines. There is a pair of the labial glands on both sides of the body, each comprising five giant cells (**Fig. 8**).

1.2. Characterisation of lectin-binding properties

To further characterise the function of the glands of *A. foliaceus*, fluorescently labelled lectins were applied to paraffin embedded sections. By screening sections of *A. foliaceus* with 19 lectins different tissues and cells of *A. foliaceus* were found to have differential affinity for binding, this being assessed according to a subjective scoring system, which was used to estimate fluorescence labelling intensity (**Table 1**). Four lectins, PNA, ECL, UEA-I and PHA-L, were not observed to bind to any cells in the sections used, giving the same background fluorescence as the negative control (**Fig. 9a)**. Eight showed an affinity to the spinal gland cells with varying fluorescence intensities, four bound to the proboscis gland cells ducts, gut and oviduct wall, five to the proboscis gland cell cytoplasm and sperm, three to labial gland cells and cuticle epithelial cells, two bound to the cerebral ganglion, seven to sub-cuticular secretory cells and oocytes and six to connective tissue. Twelve of the tested lectins were found to have a moderate to strong binding to cells at the periphery of the cerebrum (**Table 1**).

As presented in **Table 1**, *A. foliaceus* spinal gland cells showed weak binding intensity with WGA, sWGA, SBA and LCA while DSL, JAC (**Fig. 9d)**, Con A (**Fig. 9b)** and PHA-E bind strongly to the spinal gland cells. LCA binds strongly to the proboscis gland cells ducts, however WGA, S-WGA resulted in weak binding intensity and PSA faintly labelled the proboscis gland ducts. In comparison with proboscis gland cell cytoplasm, using subjective visual scoring, JAC

and Con A were found to have a strong binding and WGA, sWGA and LCA bound weakly. WGA and LCA **(Fig. 9e)** also bound strongly to the gut tissue.

LEL, STL and VVA lectins were found to have a specific affinity to the secretory cells around the brain and the outer oocyte membrane. PHA-E and DSL had a strong affinity and specific binding to the spinal gland cells. An exception was DSL, which also bound weakly to the gut and a few small secretory cells around the cuticle. DBA **(Fig.9c)** bound specifically to the cytoplasmic granules of glandular cells distributed around the cerebral ganglion and sub-oesophageal ganglion. These cells stained with Alcian blue (pH 2.5) indicating the presence of acidic mucopolysaccharides, not shown here. Although SBA had a specific affinity to the secretory cells around the brain like DBA, LEL and STL, faint staining was also evident to spinal gland cells and sub-cuticular secretory cells. SBA also bound strongly to sperm **(Fig. 9f)** within the spermatothecae in female sections, as did GSL-I. GSL-I also shared binding characteristics with the GSL-II, *i.e*. strong binding affinity to the secretory cells around the brain and in the cuticle.

4. Discussion

The work reported in this study was conducted to provide a detailed description of the secretory glands of *A. foliaceus* and to determine the morphology and distribution of these glands within the parasite. Although study of the anatomical morphology of *Argulus* species dates back to the early 1900s there has remained an argument as to the location, type and number of glands associated with feeding processes. In the present study, three different types of glands were observed and described for *A. foliaceus*, these being 1) the spinal gland 2) the proboscis glands and 3) the labial glands.

Swanepoel and Avenant-Oldewage [13] considered that the pre-oral spine of *Argulus japonicus* Thiele, 1900, had two ducts which ran to the glands in the dorsal region of the

cephalosome. While the proboscis glands of *A. japonicus* were suggested to consist of a pair of glands, each comprising three giant cells, another gland containing five cells was identified at the base of the pre-oral spine and the mouth tube [13]. The current investigation in *A. foliaceus* confirmed that the gland associated with the pre-oral spine comprises four giant secretory cells at the base of the mouth tube and corresponds with the spinal gland cells described earlier by Claus [15], Wilson [24], Madsen [25] and Saha *et al* [15]. The results of this study concur with findings of Martin [26], Wilson [24], and Saha *et al.* [15] that the base of the pre-oral spine is where the spinal gland is located. These gland cells have features suggestive of a secretory function, including abundant rough endoplasmic reticulum, mitochondria, ribosomes and Golgi bodies.

Although Saha *et al.* [15] described the spinal gland cells in *Argulus siamensis* Wilson C.B., 1926, which is the same gland defined in the current study for *A. foliaceus* as the spinal gland, Saha *et al.* [15]concluded that "the spinal duct leads blindly at the glandular end". In this study, the origin of the duct was found to be integrated with glandular tissue at the base of the spinal sheath. By analysing scanning electron micrographs of the pre-oral spine on the ventral side Gresty *et al.* [14]were able to describe the spine pores; spine duct and subterminal pore, which is not connected to the dorsal tube, previously assumed by Shimura [12] to be a chemosensory pore. Gresty *et al.* [14] also found axon-like structures within the epithelium around the base of the spine duct. In the current study two axon-like (Ax) structures were found within the spine duct epithelium. The presence of these axons supports the results of Gresty *et al.* [14] and hypothesis of Shimura [12] that the other pore at the tip of the pre-oral spine functions as a chemoreceptor. Large elongated nuclei were also observed by Gresty *et al.* [14] with clear dense nucleoli, but these latter were rarely seen in the current study, likely due to the parasite orientation during embedding and the depth of sectioning.

The presence of rough endoplasmic reticulum and ribosomes within the cytoplasm of the cells with large nuclei can be taken as an indication that these cells are involved in protein synthesis. However, as these cells are part of the cuticular epithelium, they are likely to be involved in cuticle reabsorption and construction during moulting and therefor the moult state of the animal at the time of sampling will affect its appearance in sections. The presence of numerous secretory vesicles, intermediate vesicles, rough endoplasmic reticulum, Golgi bodies, and lysosomes in the spinal gland cell cytoplasm is a further likely indication of extensive secretory protein synthesis.

The levator muscles at the base of the spinal sheath have the features of typical arthropod fast muscles and these are responsible for antero-posterior movement of the spine as previously described by Gresty *et al.* [14]. The current study provides new evidence regarding the connection of the pre-oral spine duct to the spinal gland, with the duct being shown to originate from the spinal gland. Although Gresty *et al.* [14] presented detailed information of the pre-oral spine and its duct, the authors of that study named the gland associated with the pre-oral spine as the proboscis gland, whereas in corroboration with previous studies on *A. siamensis* [15], in the current study it is suggested that this gland be referred to as the spinal gland due to its connection to the pre-oral spine.

Each proboscis gland of *A. foliaceus* was found to contain three giant cells as was reported in *A. japonicus* [14]. Regarding the location of the proboscis gland, the current study corroborates the findings of Swanepoel and Avenant-Oldewage [13] and Saha *et al.* [15] that it is located ventral to the optic tracts. However, Saha *et al.* [15] concluded that each gland of *A. siamensis* contains only two giant cells whereas it is evident from the present study on *A. foliaceus* that, in this species at least, there are 3 secretory cells belonging to each gland, as also observed by Swanepoel and Avenant-Oldewage [13] for *A. japonicus*. The assumptions

are that 1) either *A. siamensis* differs from other *Argulus* spp., even in the number of proboscis gland cells or 2) that the section used in the earlier study may not have had enough depth to enable visualisation of the missing gland cell. The latter explanation seems more likely as cells in each gland were observed in both *A. japonicus* and *A. foliaceus*.

Tubular labial spines are located within the mouth tube and were first described by Martin [26] and later by Madsen [25] who suggested that the glands associated with these structures may play more of a role during feeding than the spinal gland due to the greater number of gland cells associated with the tubular labial spine than the pre-oral spine [13]. Walker (2004) and Gresty *et al.* [14] noted that the previous studies conducted by Madsen [25] and Shimura [12] suggested that these tubular labile spines deliver enzyme secretions and thus have a digestive role during feeding. Although the ducts of the labial glands were mentioned briefly by Swanepoel and Avenant-Oldewage [13], in the current study the ducts were followed histologically following serial sectioning and found to originate from a site comprising two glands; each with five gland cells located postero-laterally to the nauplius eye. The labial spines have muscles, which were described by Gresty *et al.* [14] to originate on the underside of the ventral cephalic tendon and pass through the sub-oesophageal ganglion. Debaisieux and Swanepoel and Avenant-Oldewage concluded that the two glands comprising five large cells, positioned in the posterolateral cephalosome, are the spinal glands [13]. In the current study, tubular labial ducts were found to run through the mouth tube from these glands to the tip of the tubular labial spines. These glands, which comprise five large gland cells on each side of the body, were observed to be closely associated with the labium, and thus here it is suggested that these glands be termed the "labial glands". Recently, Øvergård *et al.* [22] found that sea lice, *L. salmonis*, have two labial glands, each made of two large secretory units connected to storage reservoirs accumulating the glandular products before secretion

through the ducts. The *L. salmonis* labial gland ducts extend ventrally into the mouth tube with openings in the oral cavity on the ventral surface of the labium and empty near the zone of activity of the mandibular teeth [22]. Caligid copepods such as *Caligodes laciniatus* (Krøyer, 1863) also have an intrabuccal pre-oral spine on the inner surface of the labrum [28] which may have a similar function to the tubular labial spine of *Argulus* spp. The labial glands of *A. foliaceus* do not appear to have collecting reservoirs and the ducts extending from the labial glands to the tubular labial spines in the mouth tube must secrete through these ducts into the mouth and immediately on to the host surface during feeding. The direct secretion of the *L. salmonis* exocrine glands onto the host tissue was suggested to potentially help in pre-digestion and intake of host tissue [29]. This suggests that the labial glands may correspond to salivary glands in terms of having roles as the production site of biologically active components that are produced during feeding to enhance the feeding process [30,31]. Such activities may include immuno-modulation to aid feeding. The number of labral / labial gland cells is variable between the copepod species [31] as shown in *L. salmonis* [22] and other copepods such as *Derocheilocaris typica*, Pennak & Zinn, 1943, which have two or three secretory cells [32] whereas the pelagic copepod *Heterorhabdus* spp. [30] has two large secretory cells. *Argulus* spp display more labial gland cells than these other species, which may be indicative of their importance for *Argulus* species during feeding and host invasion.

The histological analysis in the current study supports the proposal of von Reumont *et al.* [16] that the *A. foliaceus* host-parasite interaction is facilitated by the spinal gland, the proboscis glands and the labial glands. The present study has highlighted the potential importance of the labial glands and tubular labial spines during feeding, in addition to the previously reported roles of the pre-oral spine and two types of glands; the spinal and proboscis glands. The positive Alcian blue / PAS staining of three types of *A. foliaceus*

glands involved in feeding activities, with the spinal gland showing stronger staining, suggests the production of neutral carbohydrates and / or glycoproteins by these glands. The anterior gland complex and circum-oral glands of *Lernaeocera branchialis* (Linnaeus, 1767), another fish parasitic crustacean infecting gadoids, showed similar features [33].

The current study also suggested that the pre-oral spine delivers secretory products, which may aid in immune-modulation, while the tubular labial spines within the mouth tube may be structures that enhance the feeding process by secreting saliva components that aid pre-digestion and immune-modulation, *e.g.* proteases and anticoagulants as described for parasitic copepods such as *Heterorhabdus* spp. which inject the host with venom and anaesthetic via hypodermic-needle-like teeth [30].

Following lectin binding assays it seems likely that different parasite tissues may share similar carbohydrate profiles, however, cells in a single tissue may have affinities for different lectins, which may indicate that one tissue type may be made up of differentiated cells / or produce different carbohydrate components. JAC, Con A, PHE-A, DSL, LCA, SBA, WGA and sWGA, which are known to bind to different types of sugars like D-mannose, D-glucose, galactose, N-acetylgalactosamine and N-acetylglucosamine, all bound to the spinal gland. The strong binding of JAC and LCA to the proboscis gland indicated the abundant presence of galactose and mannose residues in these glands.

All three of the principal targeted glands, spinal, proboscis and labial glands, reacted strongly with JAC which indicates that all contained carbohydrate residues of galactose. This suggests that D-Galactose plays an important role in host-parasite interactions, as noted by Hammerschmidt & Kurtz [34], Burton *et al* [35], Knowles *et al.* [36]. Inhibition of adhesion of *Entamoeba histolytica* Schaudinn, 1903, which causes amoebiasis, to human colonic epithelial cells, was blocked by galactose or N-acetylgalactosamine [37,38]. Saffer & Petri

[38] and Petri *et al.* [39] observed that animals treated with monoclonal antibodies targeting parasite galactose lectins were able to decrease cytotoxicity. Con A had a strong affinity to the contents of the spinal and proboscis glands cells, indicative of the presence of mannose residues, which suggests that galactose and mannose are key components of material of the proboscis gland cells. PHA-E and DSL bound to the spinal gland, which suggested that the presence of GlcNAc oligomers. This has also been reported previously from similar studies conducted on salivary glands of different types of blood-feeding insect disease vectors, including those of tsetse fly; *Glossina* spp., Wiedemann, 1830, mosquitoes like *Aedes aegypti* (Linnaeus *in* Hasselquist, 1762), *Anopheles stephensi*, Liston, 1901, and *Anopheles albimanus*, C. R. G. Wiedemann, 1820, and sand flies, *Phlebotomus* spp., Loew 1845, in order to search for carbohydrate moieties within these glands. The assumption that these sugars are involved in vector tissue recognition during parasitic invasion was reviewed by Basseri *et al.* [39]. Previous studies have also demonstrated the presence of GlcNAc moieties in the triatomine bug *Rhodnius prolixus*, Stål, 1859, [39] and in the mosquito *A. stephensi* [40], which were suggested to be associated with interactions with vector tissues. Regardless of the variation of binding strength, in the current study more lectins bound to the spinal gland than to the proboscis and labial glands, which indicates a high level of glycosylation and a diversity in the carbohydrate residues present, suggesting multi-component secretions containing glycoproteins. This is also supported by the strong positive PAS staining of the spinal gland cells in comparison to the proboscis and labial glands.

Although JAC and PNA lectins nominally have the same binding specificity to Gal moieties, it was observed that PNA did not bind to the gland cells like JAC, which exhibited a strong binding to all three glands cells. It is known that PNA lectin does not bind any sialyated sugars [41], thus it could be hypothesised that these glands contain sialyated oligomers *i.e.*

those that have reacted with sialic acid or its derivatives. The lack of binding by UEA-1 suggests an absence of fucose residues in the treated sections of *A. foliaceus*. This result is similar to those obtained for the myxozoan *Tetracapsuloides bryosalmonae*, Canning *et al*., 1999, where UEA-1 lectin was also the only one not binding to the parasite surface [42].

Previous studies have also identified interactions between the parasites' carbohydrate terminals; such as those of *Ichthyophthirius multifilis* Fouquet, 1876, [43] and *Gyrodactylus derjavinoides* Malmberg, Collins, Cunningham & Jalali, 2007 (syn. *Gyrodactylus derjavini* Mikailov, 1975) [44], and fish host immunity. Buchmann (1998) earlier found that ConA bound to the cephalic gland openings of *G. derjavinoides* and complement factor C from rainbow trout also bound specifically to the cephalic gland ducts [45]. Therefore, it was suggested that mannose residues in this region activated the complement pathway of this parasite during infection [45]. Initiation of fish innate immune responses involved complement as one of the key components involved in this process [46]. *A. siamensis* has been shown to modulate rohu *Labeo rohita* (Hamilton, 1822) fish by suppressing serum α-2 macroglobulin (α-2M), the alternative complement pathway and ceruloplasmin levels [47]. Lectin labelling investigations can thus provide useful information relevant to determining the composition of secretory substances during feeding, which may in turn help to elucidate the mechanisms used by *Argulus* spp. to counteract host immunity and conversely, potential targets utilised by host immune pathways.

5. Conclusion

This study provides a detailed description of three important glands; spinal, proboscis and labial glands, in *A. foliaceus* that are likely to play key roles during feeding and possibly

immunomodulation of the host. Extensive fluorescent histological investigations using lectin-binding assays with 19 fluorescently labelled lectins applied to *A. foliaceus* sections, have also enabled characterisation of the carbohydrate moieties associated with these glandular cells, revealing important biomolecular aspects of these tissues.

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Conflict of interest

The authors declare they have no conflict of interest.

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Figure legends

Fig. 1. (a) SEM micrograph of the ventral aspect of adult *A. foliaceus* showing the pre-oral spine (stylet) and mouth tube position relative to other appendages / tagmata. Scale bar = 1 mm **(b)** Light micrograph of sagittal section through the oral region of female *A. foliaceus* showing the position of the pre-oral spine (stylet) and spinal gland to the mouth tube. The spinal gland is located at the base of both the mouth tube and the spinal sheath. H&E. Scale bar = 500 µm.

Fig. 2. SEM micrographs of *A. foliaceus* pre-oral spine and mouth tube (proboscis). (a) Ventral view of the mouth tube (MT) and the pre-oral spine (PS) protruding from the spinal sheath (Ss). (b) A high magnification view of the pre-oral spine (stylet) showing the spine protruding from the spinal sheath (c) lateral view of the pre-oral spine and spinal sheath (d) ventral view of the mouth tube showing labrum enfolded by labium, 2 pairs of sensory papillae indicated by arrows and paired tubular labial spines (lab S) with the denticulate margins of the labrum.

Fig. 3. Light micrograph of cross sections through the pre-oral spine of adult *A. foliaceus* showing **(a)** general structure **(b-d)** sections through the spinal sheath, moving disto-proximally showing spine duct **(e)** shows that the spine duct (SD) passes into the glandular tissue (GT) at the proximal base of the spinal sheath. H&E; Technovit. Scale bars (a = 50 µm; b = 10 µm; c-e = 20 µm).

Fig. 4. Light micrographs of anterior *A. foliaceus* glands. Planar sections of adult *A. foliaceus* shows spinal gland (SG) comprising four secretory cells lying posterior to the oesophagus (Oe) and anterior to the gut (not shown) and proboscis glands, (PG), each comprising three large secretory cells. **(a)** granular and basophilic cytoplasm cells with H&E **(b)** Combined Alcian blue (pH 2.5%) -PAS showing positive staining of spinal gland cells (strong PAS) and proboscis gland cells with PAS **(c)** PAS-Tartrazine counterstain haematoxylin. Scale bar = 100 µm.

Fig. 5. TEM micrographs of adult *A. foliaceus* **(a)** showing the pre-oral spine within the lumen and the compressed spine duct (SD), cells with large elongate nuclei (EN) lie within the epithelium of the spinal sheath; in this section, only one cell shows the nucleolus (N); **(b)** shows spine duct is lined with two layers of cuticle1 & 2 probably due to moulting of individual, intermediate vesicles (Iv) and a dense granular matrix (DGM) extends from the spine duct to the epithelium (E) beneath the spinal sheath cuticle and two axons (Ax); **(c)** mitochondrion (M) shown within the cytoplasm of SG; **(d)** part of secretory cell cytoplasm showing typical organelles involved in protein synthesis: abundant intermediate vesicles (Iv) and rough endoplasmic reticulum (rER), Golgi (G), mitochondria (M) and free ribosomes; **(e)** detail of pre-oral spine levator muscle involved in antero-posterior movement with thick (TkF) and thin (TfL) filaments, (s) sarcolemma, (M) mitochondria, (BL) basal lamina and peripheral nucleus (N).

Fig. 6. Light micrograph of transverse serial sections (**a-d**) showing sections moving posteriorly from the distal extremity through the mouth tube of adult *A. foliaceus* and following the ducts of the tubular labial spine (arrowed). Li-labium and Lr- labrum. H&E; Technovit 7100. Scale bar = 50 µm.

Fig. 7. (a & b) Light micrograph of cross sections through the head of adult *A. foliaceus* **(a)** Transverse section showing the position of the proboscis glands PG; C- cerebrum; S- pre-oral spine **(b)** showing the pair of labial glands (LG) adjacent to the oesophagus and the mouth tube (MT), and brain (cerebrum) (B) **(c & d)** Light micrograph of sagittal sections through the oral region of *A. foliaceus* **(c)** following labial gland ducts (arrowed) extending from the tubular labial spine ( ) in the mouth tube (MT) and showing the origin of the labial gland duct in the vicinity of the labial gland (LG) indicated by an arrowhead ( )**(d)** micrograph showing labial gland position **(e)** planar section of *A. foliaceus* labial gland cells (LGC) and their collecting duct (arrowhead) **(f)** showing labial gland cells with the collecting duct (arrowhead) of the labial gland draining into the oral cavity (OC). C = cerebrum; SG = spinal gland; PG = proboscis gland. (a), (b), (c) & (d) PAS with haematoxylin and tartrazine counterstain, scale bars (a = 50 µm, b-d = 100 µm), (e & f) H&E stain scale bars = 20 µm.

Fig. 8. Light micrograph of planar section of adult *A. foliaceus* cephalothorax showing the position of the labial glands (LG) antero-lateral to the gut (GT) and at the same level as the nauplius eye (NE). Five gland cells are evident on each side as indicated by arrowheads; H&E. Technovit 7100. Scale bar = 500 µm.

Fig. 9. Planar section of *A. foliaceus* labelled with different fluorescent lectins **(a)** *A. foliaceus* cephalic region showing negative control (lectin wash buffer only) **(b)** Con A binds intensely to spinal (SG) and proboscis gland (PG) cells and other cells (SC) in the vicinity of the cuticle and cerebrum (**c**) DBA lectin binding specifically to cytoplasmic granules of secretory cells (SC) at the periphery of the cerebrum. These cells are found around the cerebral ganglion (CG) and sub-oesophageal ganglion (SoeG) **(d)** JAC lectin has strong binding affinity to almost all the secretory cell types on the sections (not shown) and also binds to the labial gland cells, secretory cells **(e)** LCA lectin bound partially to the spinal gland cells vesicles, cerebral ganglion; not shown in this micrograph see Table 2.2, oocytes (Oc) outer membrane and secretory cells underneath the nauplius eye (not shown) and had weak binding to (PG) proboscis gland cells cytoplasm (not shown) and strong binding to the gut and proboscis gland collecting ducts (**f**) SBA lectin had a strong and specific binding to secretory cells in the vicinity of the cerebral ganglion and sub-oesophageal ganglion (not shown), small cells around the cuticle and sperm (Sp) in the spermatophore. This lectin bound to the same cell types as GSL-I. Note background staining of cuticle is a consistent artefact observed with fluorescent microscopy and lectin staining.

Figures

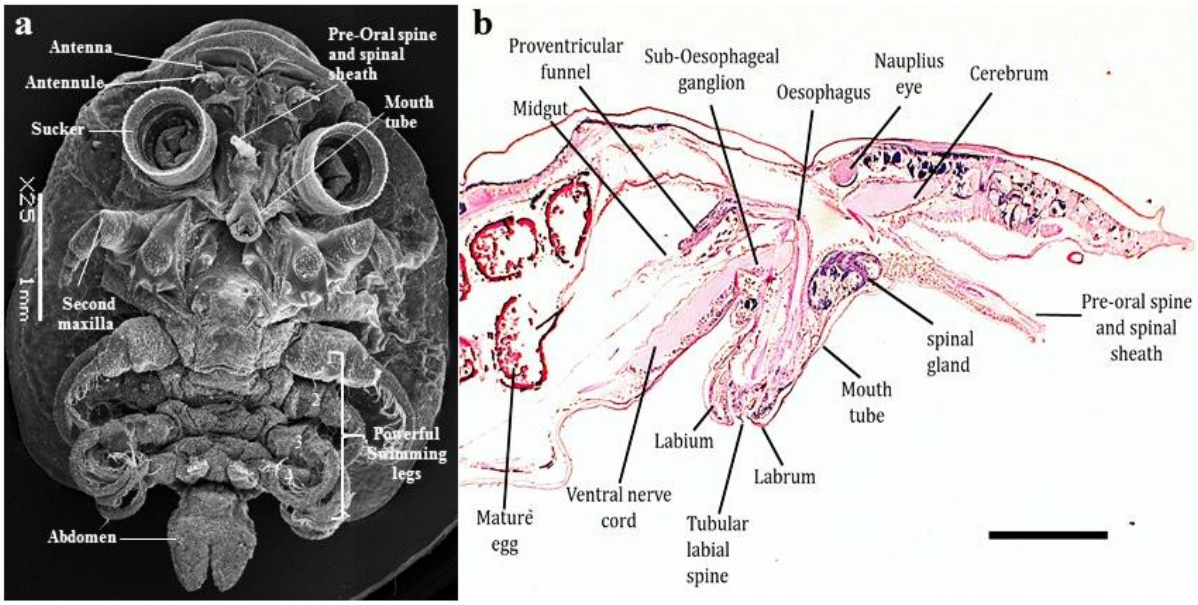


Figure 1

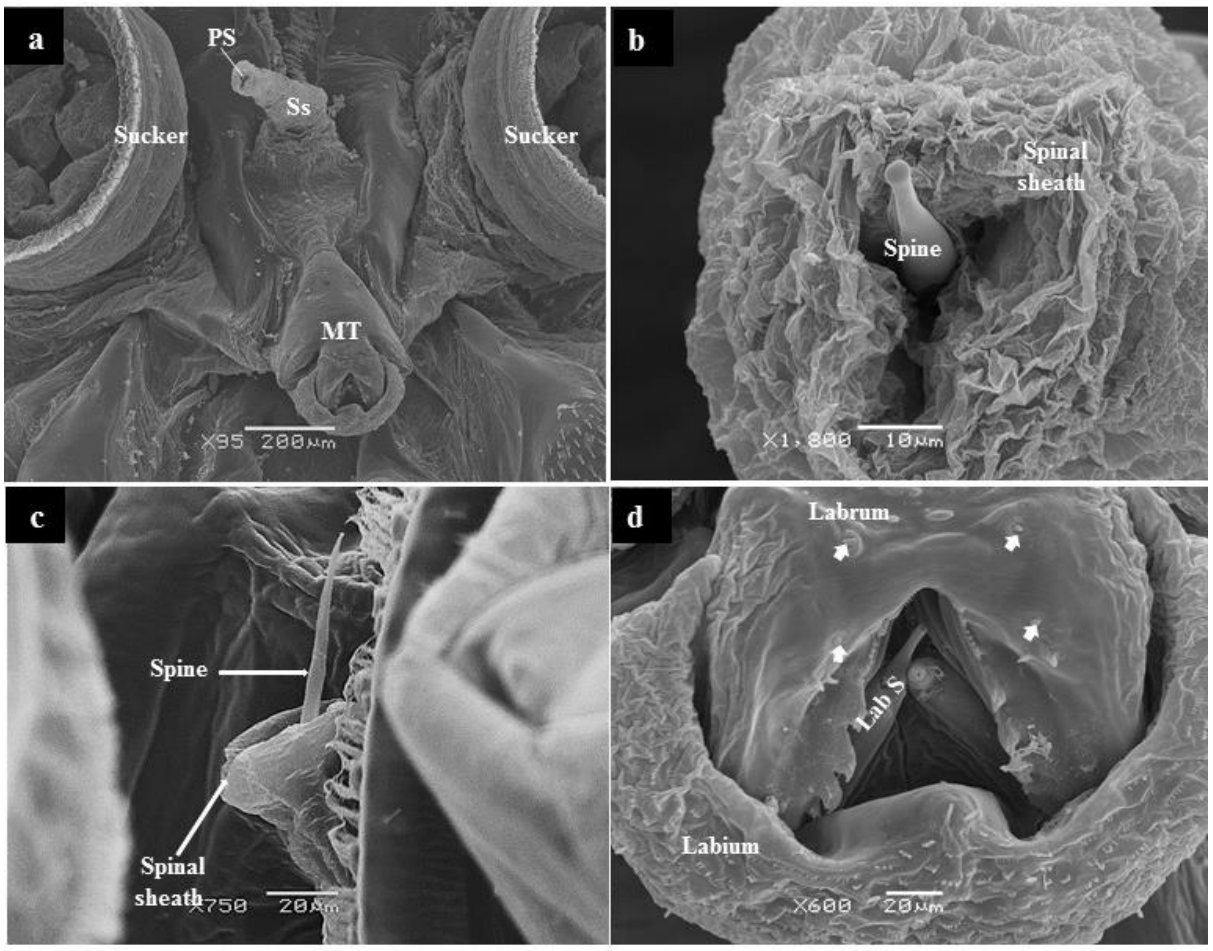
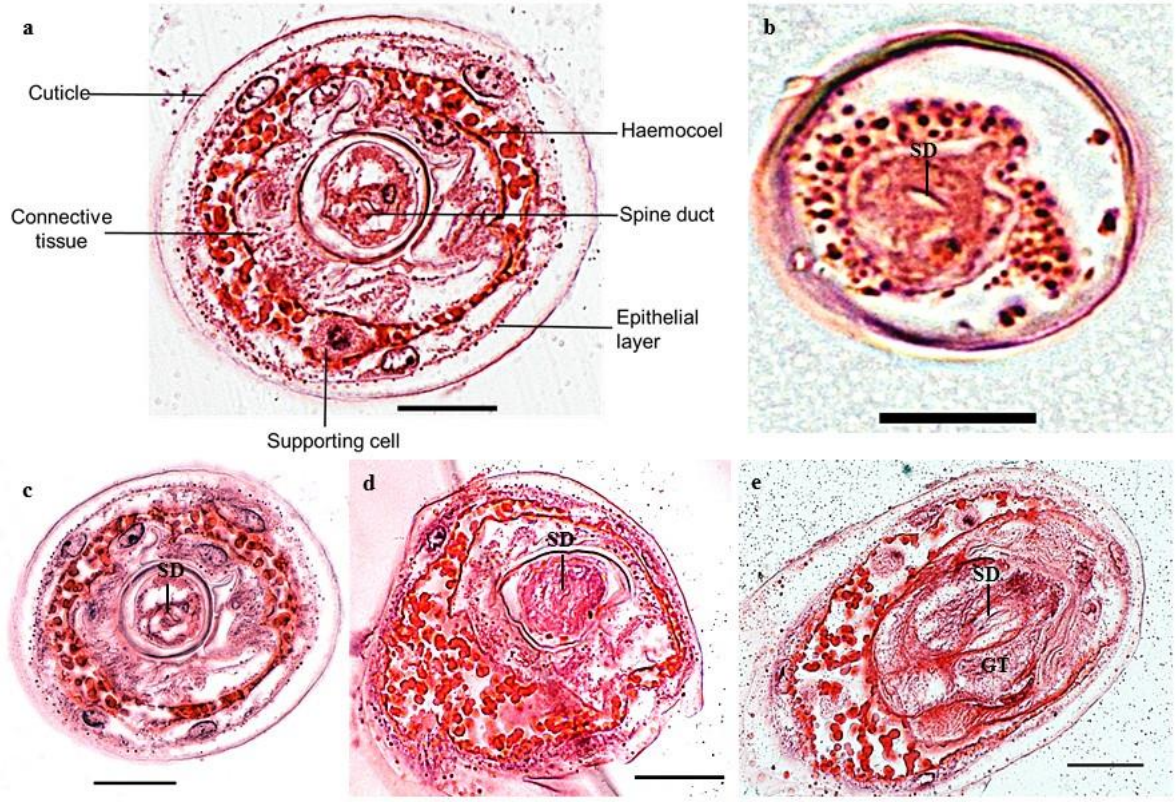


Figure 2



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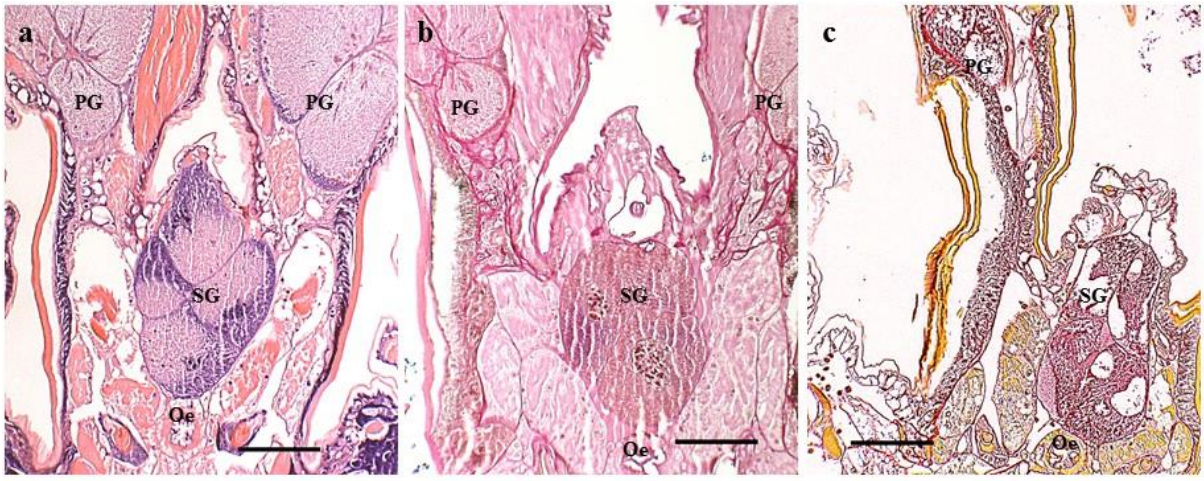

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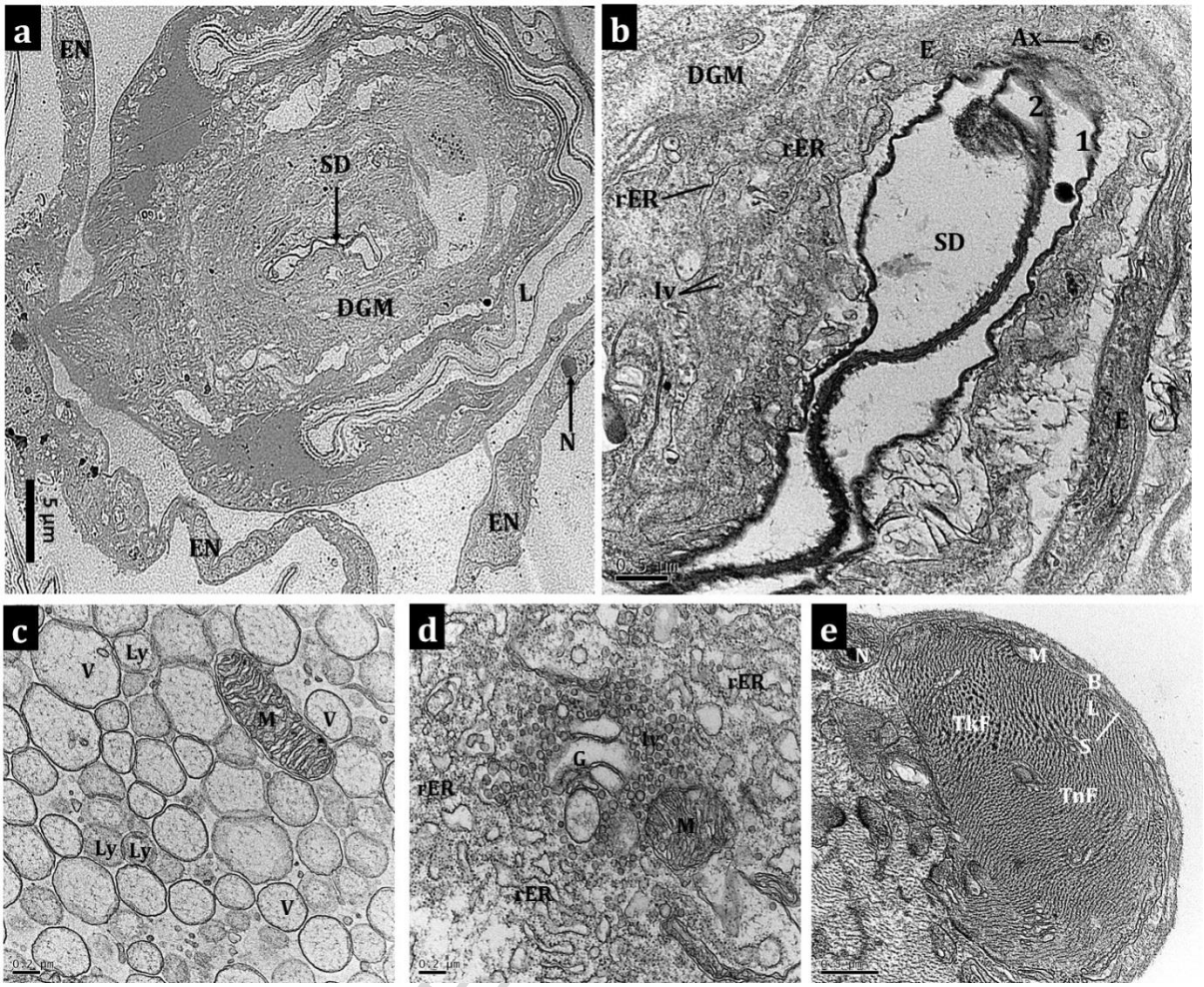


Figure 5

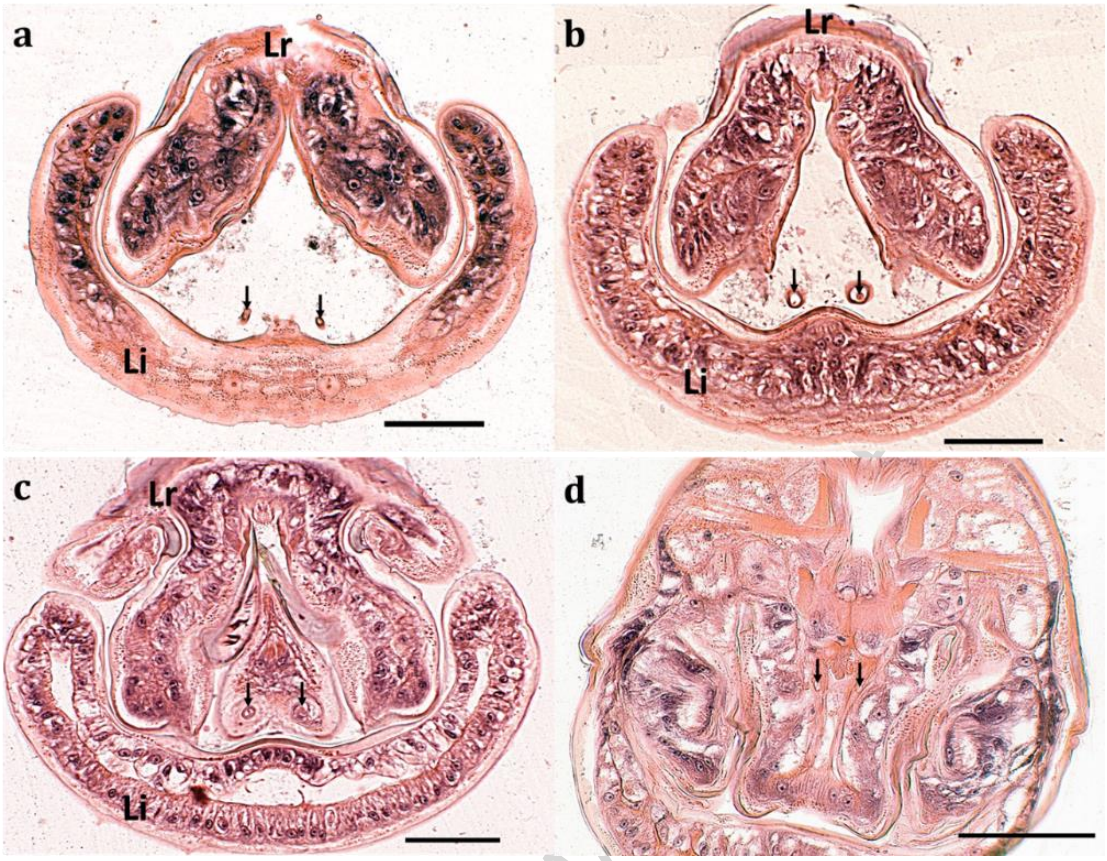


Figure 6

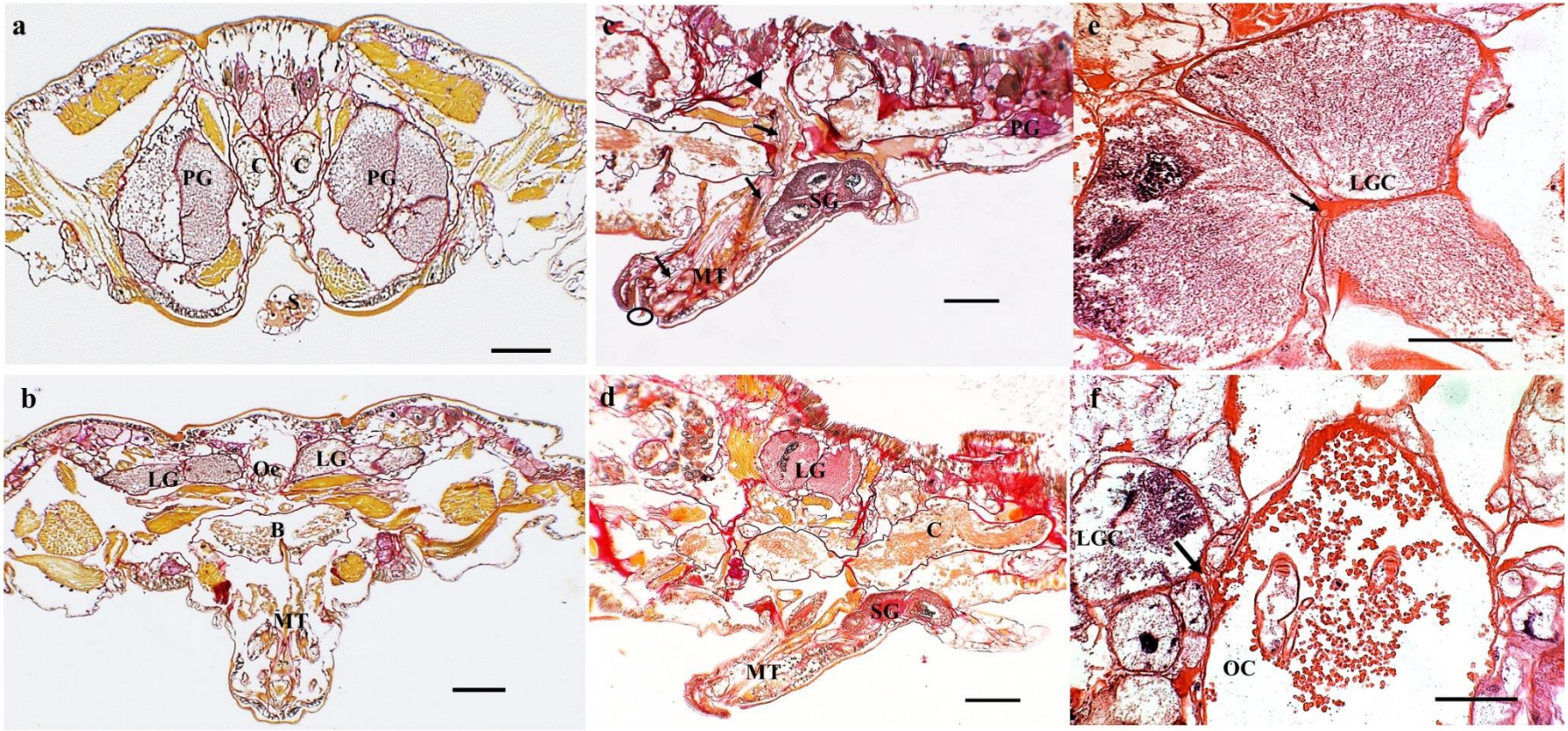


Figure 7

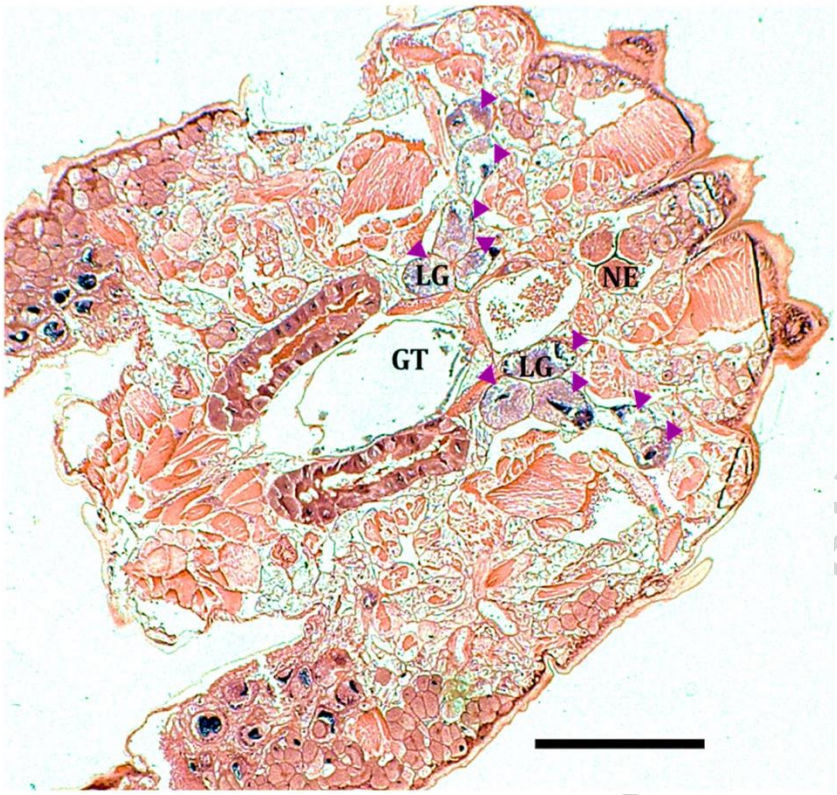


Figure 8

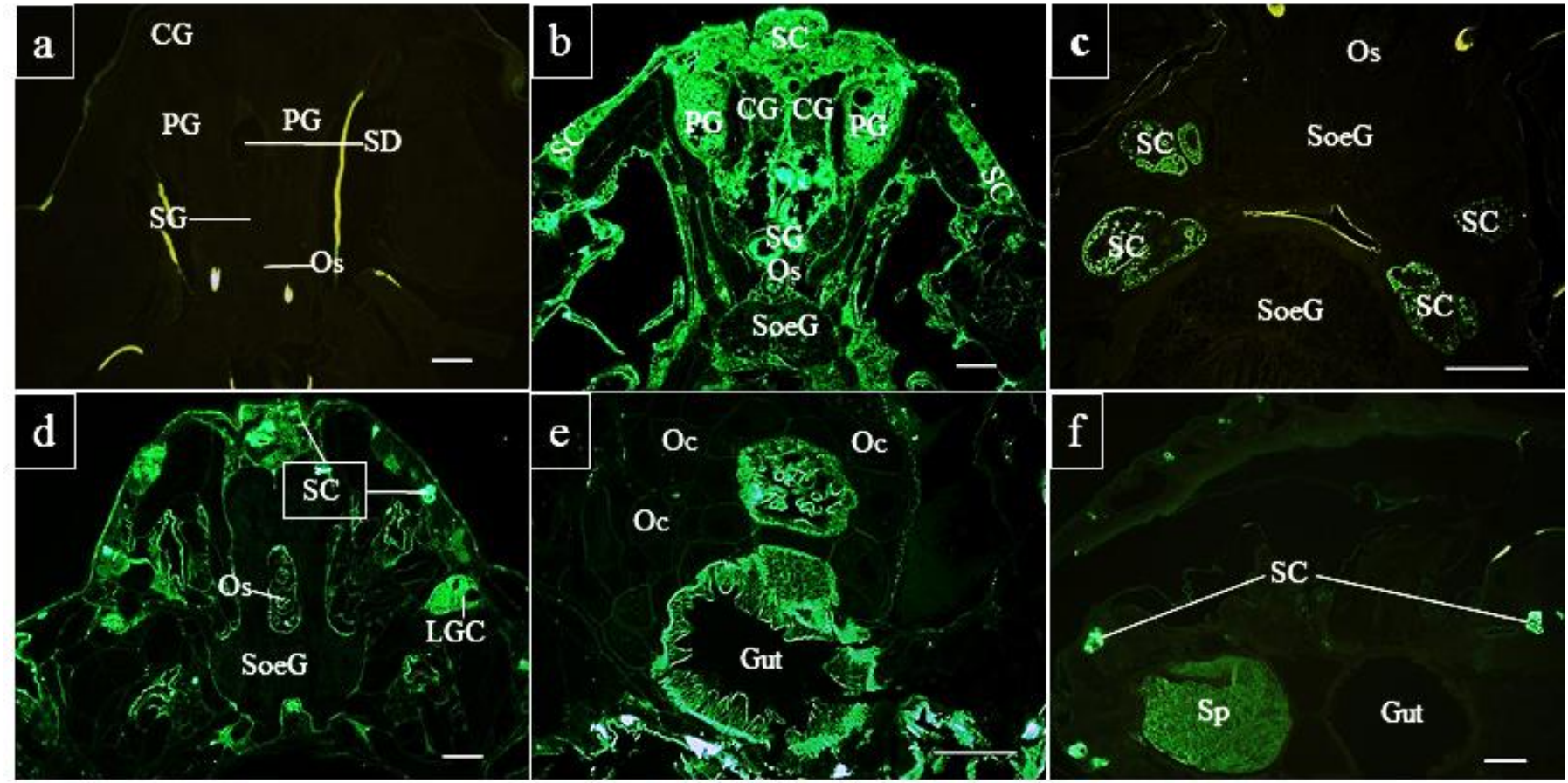
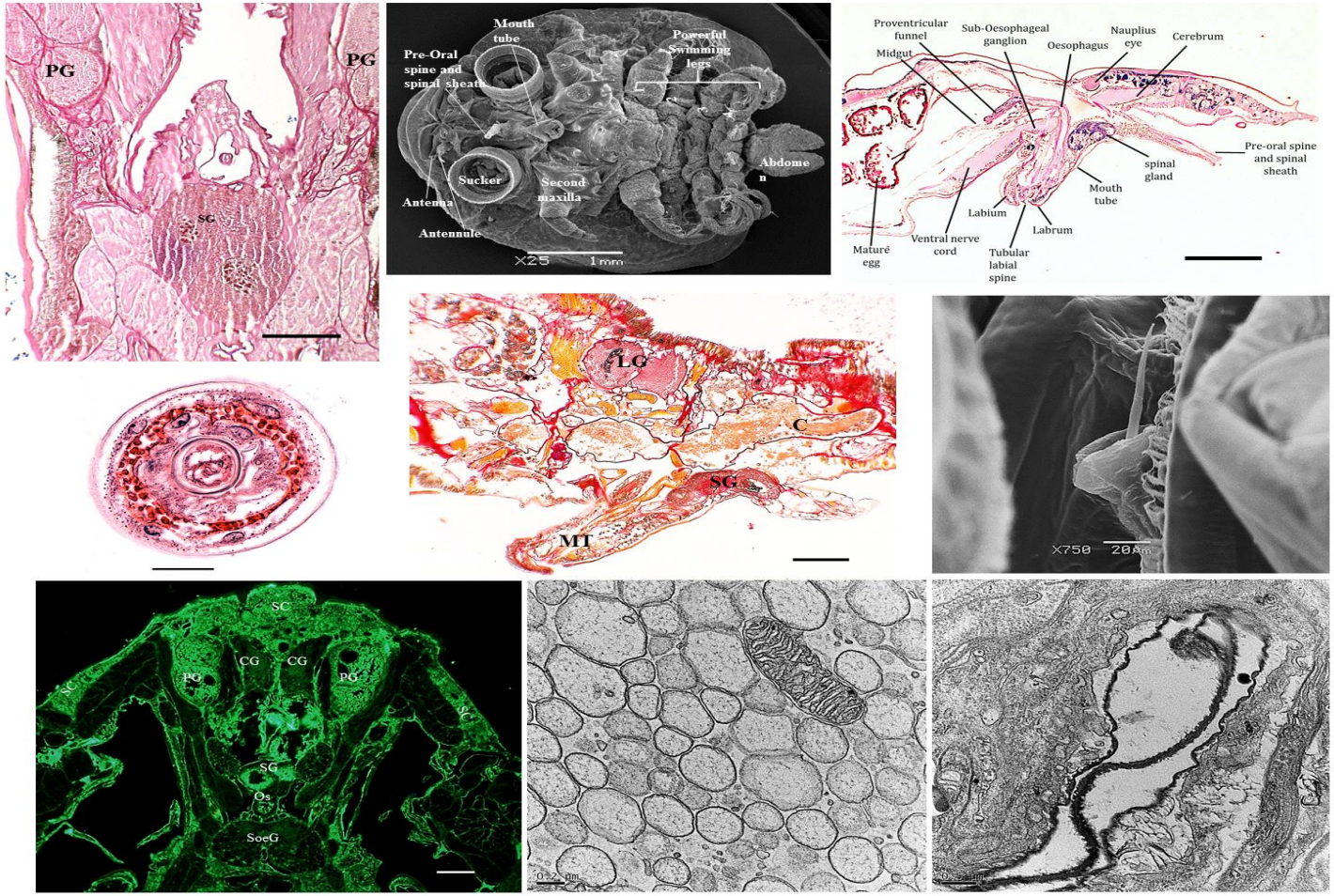


Figure 9

Table 1. Subjective lectin binding affinities to *A. foliaceus* different cells and tissues

Lectin	Spinal gland cells	PG Ducts	PG Cytoplasmic granules	Gut	Labial Gland cells	Cerebrum ganglion	Subcuticle secretory cells	Connective tissue	Cuticle epithelial cells	Secretory cells around the brain	Oocytes outer membrane	Wall of oviduct	Testes	Sperm cells
WGA	+	++	+	+++	+	++	+++	+	+	++	++	---	•	+
sWGA	+	++	+	++	•	+	+++	+	++	++	+	---	•	+
LEL	---	---	---	---	---	---	++	---	---	+++	++	---	•	•
STL	---	---	---	---	---	---	---	---	---	+++	+	---	•	•
DSL	+++	---	---	+	---	---	---	---	---	++	---	---	•	---
DBA	---	---	---	---	•	---	---	---	---	+++	---	---	•	•
SBA	+	---	---	---	•	---	+	---	---	+++	---	---	•	+++
VVA	---	---	---	---	•	---	---	---	---	+++	+*	+++	•	•
JAC	+++	---	+++	---	+++	---	+++	+	---	+++	•	•	•	+
PNA	---	---	---	---	---	---	---	---	---	---	---	---	•	---
ECL	---	---	---	---	---	---	---	---	---	---	---	---	•	---
UEA-I	---	---	---	---	---	---	---	---	---	---	---	---	•	---
ConA	+++	---	+++	---	+	---	+++	+	+++	+++	•	•	---	---
PSA	---	+	---	---	---	---	---	+	---	---	++**	++	•	---
LCA	+	+++	+	+++	•	---	---	++	---	---	++	+++	•	•
PHA-E	+++	---	---	---	---	---	---	---	---	---	•	•	---	---
PHA-L	---	---	---	---	---	---	---	---	---	---	---	---	•	---
GSL-I	---	---	---	---	---	---	+++	---	---	+++	•	•	•	+++
GSL-II	---	---	---	---	---	---	+++	---	---	+++	---	+++	•	•

---: No staining, +: weak staining, ++: moderate staining, +++: strong staining of fluorescent lectin, •: not shown in the section, +*: labelled oocytes but not the oocyte outer membrane, +**: labelled both the outer membrane and the cytoplasm of the oocytes, PG: Proboscis gland



Graphical abstract

Highlights

- *Argulus foliaceus* was collected from fisheries in the UK.
- Secretions from the spinal gland via pre-oral spine may aid in immunomodulation.
- Proboscis glands and labial glands are associated with the proboscis secretions during feeding that aids pre-digestion.
- Lectin-labelling characterizes the secretory products of these glands