Morphological and Functional Aspects of Feeding in The Freshwater Fish Louse *Argulus foliaceus* (Linnaeus, 1758)

A Thesis Submitted for the Degree of

Doctor of Philosophy

By

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August 2017

To

My supportive husband Rashid

My mother Aina and father Ahmed

To the spirit of my niece Aisha Al Alawi

To my kids Nujood, Al Wajih, Wail and Tareem, to my big family and to my lovely country the Sultanate of Oman.

Declaration

This thesis has been composed in its entirety by the candidate. Except where specifically acknowledged, the work described in this thesis has been conducted independently and has not been submitted for any other degree. All images presented in this thesis are original, unless otherwise stated.

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Acknowledgments

I thank almighty God for his loving kindness, creating an opportunity for me to undertake this study and granting me the ability to complete it successfully. This is a great milestone in my career development.

I am glad that my PhD has led to interesting results, for which I would like to express my sincere gratitude to my supervisors. Firstly, I would like to express my sincere gratitude to my principal advisor Professor James Bron for his continuous support of my PhD study and related research, for his patience, motivation, and immense knowledge. His guidance helped me throughout my research and the writing of this thesis. My sincere thanks also goes to my second supervisor Dr John Taggart who gave me an opportunity to be one of his students and who provided support and guidance for all aspects of my molecular research. I thank Dr. Andy Shinn, my external supervisor, for helping me in structuring this thesis before he left and for giving me the contacts I needed to get my samples. Thank you for being such enthusiastic and committed supervisors.

This thesis also was produced with the help, support and contributions of a network of people who deserve recognition for their different roles in making it possible. Without their precious support, it would not have been possible to conduct this research.

Besides my advisors, I would like to thank Dr. Stefanie Wehner and Dr. Michaël Bekaert, the bioinformaticians at the Institute of Aquaculture, who both contributed and worked hard to help me obtain and analyse the transcriptomic data, in order to achieve the exciting results, I obtained. My thanks also to Dr. Monica Betancor who kindly helped me in the gene expression analysis. Thanks to Carol McNair who helped get

me started in my research and whose excellent work on sea lice provided some framework for aspects of this study. My thanks also goes to Dr. Michael Leaver for helping in finding the reactive centre loops (RCL) of serpin.

My sincere thanks also go to Dr. Sean Monaghan for his guidance and encouragement, even though there were many challenges, through the preparation of material for proteomics, immunohistochemistry, lectin labelling and western blot analysis and for offering to read and correct the first draft of the thesis, which without doubt ensured that this PhD study was a success. Many thanks also go to Jacque Ireland for training me in the molecular techniques of RNA extraction, *in situ* hybridisation and gene expression analysis and continuing to offer assistance whenever I needed it. Thanks to Debbie for her help and for sharing expertise in histological preparation and to Hillary McEwan and Karen Sneddon for their generous help and support in the immunology laboratory. Many thanks also to Melanie Cruickshank for helping me to sort out the many financial issues associated with this project.

Many thanks to Jimmy Poole at Loch Fad fisheries for his enormous assistance in providing *Argulus* samples throughout the duration of this thesis and to Chris Williams and Amy Reading at the Environmental Agency in Brampton who also provided *Argulus* samples. Without your help, there would have been nothing to study.

I would like also to thank the team of the electron microscopy department in College of Medicine, Sultan Qaboos University, Oman Mrs Kawthar Al Adawi, Mr. Muhammad Al Kindi and Mr. Abdul Al Rahman Al Nabhani for their generous help and support.

I must also thank my colleagues Taslima Khanam, Jamila Rizgalla, Sulieman Isa and his wife Sumaya, Khalid Shahin, Benjamin Clokie, Michael Clarkson, Lucas Torati and Margaret Aanyu from the Institute of Aquaculture for motivating and encouraging me, especially during the most challenging times. I sincerely value your contribution to my PhD. Thank you, Margaret for advising me to apply for the Schlumberger Foundation scholarship.

I sincerely thank my husband Rashid Al Kindi for enabling me to undertake this study while he took care of the family especially the last year of my study. Thank you so much for the support, love and encouragement you have given me throughout my life with you and throughout the preparation of this study. Special thanks also goes to my parents especially my mother who follows me with her care, love and prayers, to my kids for their great patience during the completion of these studies and to my brothers and sisters. Many thanks also to Salim Al Kindi who provided much-needed support and advice throughout my studies that helped me to keep going when things were difficult.

With great pleasure, I would also like to thank Sultan Qaboos University, Oman, and the Schlumberger Foundation for co-funding my PhD study and giving me the time to complete my studies successfully.

Abstract

Argulus foliaceus (Linnaeus, 1758) is a member of the branchiuran family Argulidae and has a worldwide distribution, causing major economic impacts for freshwater aquacultured fish species worldwide. In the UK, it has economic impacts for both aquaculture and sports fishing industries. Previous studies observed haemorrhagic and inflammatory responses after *Argulus* infection, which has been taken to support the idea that the parasite secretes chemicals during the feeding process to assist with the ingestion of blood and epithelial tissue. The present study suggests that the blood-feeding ectoparasite of fish*, A. foliaceus*, may use similar mechanisms for evading host immune responses to those used by sea lice and other haematophagous arthropods. No previous studies have directly investigated the nature of the bioactive compounds / proteins, assumed to be released from these ectoparasites, and which are considered to contribute to feeding processes and host-parasite interactions during infection. Thus, the work described in this thesis was undertaken with the objective of identifying, describing and characterising the secretory components that have previously been suggested to be secreted from glandular cells associated with the feeding appendages of *Argulus foliaceus*. The current study applied transcriptomic and proteomic techniques in conjunction with *in situ* methods to investigate known immunomodulatory genes that may serve a function in parasite-host interactions. Overall, the findings of this project have generated considerable additional knowledge concerning the biology of *Argulus* spp. and have provided a list of proteins that may be used by the parasite to facilitate feeding processes by secreting these active molecules into the host and hence modulating their immune defence mechanisms. This information can be used as a baseline for developing freshwater lice control strategies to help prevent the spread of Argulosis in aquaculture by applying vaccination as means of control using the candidate antigens described in this study to specifically target *Argulus* spp. Knowledge generated by the work described in this thesis can also contribute to the development of drugs for controlling Argulus or functional components of feed that may serve to protect fish against this parasite. Furthermore, data from this thesis enhances the knowledge of the distribution of toxin/venom or venom-like substances in crustaceans and arthropods in general.

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Chapter 1. Life-cycle, biology and impact of argulid branchiurans

1.1 Branchiura

The crustacean subclass Branchiura Thorell, 1864 is parent to the order Arguloida Yamaguti, 1963, which in turn is parent to the family Argulidae Yamaguti, 1963 (see **Table 1.1**). The Argulidae is currently the only recognised family within this order and includes ~175 species, belonging to four recognised genera: *Argulus* O.F. Müller, 1785, *Dolops* Audouin, 1837, *Chonopeltis* Thiele, 1900 and *Dipteropeltis* Calman, 1912. These four genera are mostly identified as freshwater fish ectoparasites, except for *Argulus,* which can also be found in the marine environment (Boxshall 2005) and for which genus almost 25% of species are marine (Kabata, 1988).

Chonopeltis has 14 valid species and is endemic to Africa (Poly 2008; van As *et a*l., 2017). This species shows specific preference for particular fish families and individual fish species (van As *et al.*, 2017). *Dolops* has 14 valid species (http://www.marinespecies.org), with most of the species being found in South-America. *D. ranarum* (Stuhlmann, 1892) is found in sub-Saharan Africa and *D. tasmanianus* Fryer, 1969 is found in Tasmania (Møller, 2009). *Dipteropeltis* was described for a long time as the only monotypic genus of the Branchiura as it contained only one species *Dipteropeltis hirundo* Calman, 1912 (Poly 2008; Møller 2009; Neethling *et al.*, 2014). This genus is found only in to South America (Poly 2008; Neethling *et al.*, 2014). However, more recently a new species *Dipteropeltis campanaformis* Neethling, Oliveira, Malta & Avenant-Oldewage, 2014 was described

(Neethling *et al.*, 2014) and added to the genus *Dipteropeltis*. The genus *Argulus*, being the topic of this thesis, is discussed in more detail below.

1.1.1 ***Argulus***

Argulus is one of the most common crustacean fish ectoparasite genera encountered worldwide (Fryer 1968; Kabata 1970; Byrnes 1985), being frequently referred to as "fish lice". *Argulus* is the largest and most diverse genus of the family Argulidae (see **Table 1.1**) (Møller, 2009) and to date, 145 species have been identified and described (Walter and Boxshall 2014). Members of this genus are responsible for the condition argulosis, a parasitic disease affecting both wild and cultured fish populations (Rahman 1995; Sahoo *et al.*, 2013). Amphibia are also infected by *Argulus*, which can cause death of larval amphibia (Ippen and Zwart, 1996).

Table 1.1 Taxonomic classification of the genus Argulus (After Boxshall, G.; Walter, T. Chad (2009), http://www.marinespecies.org).

Taxonomic Level	**Taxa**
Phylum	Arthropoda
Subphylum	Crustacea Brünnich, 1772
Class	Maxillopoda
Subclass	Branchiura Thorell, 1864
Order	Arguloida Yamaguti, 1963
Family	Argulidae Leach, 1819
Genus	*Argulus* O.F. Müller, 1785

The translocation of *Argulus* spp., via imported ornamental fish, has been reported from a number of different countries. *Argulus foliaceus* was Introduced to Pakistan

from Thailand along with its host (Iqbal *et al.*, 2013) and it was also recorded in Iran to infect goldfish and koi imported from Southeast Asian countries (Mirzaei & Khovand 2013; Noaman *et al.* 2010). In the Netherlands, until 2010, *A. foliaceus* was the only species reported, however, *A. japonicus* was recorded in four locations for the first time from that date (Soes *et al.*, 2010). Recently, *Argulus quadristriatus* Devaraj and Ameer Hamsa, 1977, was reported to infect three new fish hosts in Japan (Uyeno *et al.*, 2017). Transportation of live fish, due to the increase in aquaculture production and recreational fisheries of carp *e.g.* koi carp and ornamental carp breeding, led to the wide distribution of *Argulus foliaceus* and *A. japonicus* (Rushton-Mellor 1992; Northcott *et al.*, 1997; Catalano & Hutson 2010; Møller 2011). Such translocations can lead to argulosis in wild fish populations if the parasite transfers to natural ecosystems and native fish populations (Mirzaei & Khovand 2013). In addition to the freshwater *Argulus* spp., one marine species, *A. arcassonensis* Cuénot, 1912, has been recorded in UK coastal waters (Rushton-Mellor 1992). Alsarakibi *et al.* (2012) found that in China, *Argulus* spp. were able to acclimate to three locations with different ecosystems, fish farms, rivers and reservoirs, with the lowest number of *Argulus* recorded in the river and the heaviest intensity recorded at the site of fish farms, which was suggested to indicate that *Argulus* prevalence could be subject to stability of the aquatic environment. *Argulus japonicus* has been recorded in Africa, the Far East, Europe and North America suggesting broad tolerance across different environments. Similarly the wide distribution of *A. foliaceus* across Europe, Central Asia, and North America indicates that this species can successfully adapt to a range of different habitats (Alaş *et al.* 2010; Møller 2011). In terms of tolerance, *Argulus* spp. can also tolerate polluted ecosystems, as described by Alsarakibi *et al.* (2012).

1.1.1.1 ***Argulus* in the UK**

Memebers of the genus *Argulus* are the only members of the Argulidae found in the UK. The UK freshwater *Argulus* species comprise *A. foliaceus* (Linnaeus, 1758), *A. coregoni* Thorell, 1865 and *A. japonicus* Thiele, 1900, with *A. foliaceus* and *A. coregoni* considered to be endemic species in the UK (Bower-Shore 1940; Taylor *et a*l., 2005 Campbell 1971; Rushton-Mellor 1992; Northcott *et al*., 1997; Evans 2000; Taylor *et al*., 2005), while *A. japonicus* is a non-native species, possibly introduced through the ornamental trade from the Far East (Rushton-Mellor, 1992; Northcott and Walker 1997). Although *A. foliaceus* and *A. coregoni* can be found in mixed populations, as described by Mikheev *et al.* (2001), each of these species is considered to have a particular favoured environment. *A. foliaceus* has been recorded in mesotrophic and eutrophic lakes (Gurney 1948; Campbell 1971), but also has the ability to tolerate more saline waters up to 8-12 ppt at 25°C (Möller, 1978) and can reach epizootic levels in brackish water as mentioned by Pasternak *et al.* (2000). Rivers, streams and cool oligotrophic lakes are considered to be habitats that better suit *A. coregoni* (Gurney 1948; Campbell 1971; Rushton-Mellor 1992).

Argulus have been recorded in the UK and are reported to be one of the most problematic freshwater fish parasites for trout fisheries and farms (Taylor *et al*., 2005). Campbell (1971) recorded the presence of *Argulus* spp. in southern areas of the central highlands of Scotland and believed the presence of this parasite to be restricted to this area, however, *A. foliaceus* is widely distributed across the UK.

Argulus mostly affects still water trout fisheries and can lead to massive mortalities. This can lead to adverse impacts for the trout industry and for anglers. As reported from a Countryside Alliance report (2001) cited by Taylor *et al*. (2005), UK angling provides extensive economic benefits, supporting more than 5000 full-time jobs in Britain's countryside. The ~430,000 trout anglers in England and Wales spend ~£300 million on game fishing (Taylor *et al*., 2005). The UK trout industry produced 4235.4 tonnes of trout in Scotland and Northern Ireland and 8690.5 tonnes in England and Wales in 2012, with a total production value of ~£29 million (Ellis *et al*., 2012). *Argulus* spp. in the UK, can have a considerable impact on managed, recreational, sports fisheries, by causing fish morbidity leading to reduction in feeding, which in turn lowers angling effort leading to closure or reduction of trout fisheries resulting in economic loss (Taylor *et al*., 2006). *Argulus* species affect not only rainbow trout fisheries but are also a problem for carp farms (Nolan *et al*., 2000). Taylor *et al*. (2006) conducted an ecological cross-sectional study from May to December in 2001 on 77 UK still water trout fisheries to determine the percentage experiencing *Argulus* spp infection in three regions, central and southern England, and southern Wales and the related factors that might help explain this incidence. Three main factors were suggested to be associated with *Argulus* infections in UK fisheries, as mentioned by Taylor *et al*. (2006). These comprised a negative relationship to levels of algal blooms / low water clarity, a positive relationship to slow rates of stock turnover and a negative relationship to water levels dropping > 30 cm. **Table 1.2** shows the proportion of UK still water trout fisheries experiencing *Argulus* spp. infections in 2000 (Taylor *et al*., 2006).

Table 1.2 The proportion of UK still water trout fisheries surveyed with experience of *Argulus* spp. infections, according to region (Taylor, Sommerville and Wootten, 2006).

	Northern (%)	Central (%)	Southern (%)	Total (%)
Argulus spp. present in 2000				
Present	2 (10)	14 (56)	5 (22)	21 (30)
Absent	19 (90)	11 (44)	18 (78)	48 (70)
Problem *Argulus* spp. infection in 2000				
Present	0 (0)	13 (52)	4 (17)	17 (25)
Absent	21 (100)	12 (48)	19 (83)	52 (75)

Disease is listed as the principal challenge and constraint for rainbow trout farming and production in the UK (Bassett, 2010). Because of the direct impact of *Argulus* on the trout fishing industry, it is important to focus on this parasite. Mortality caused by epizootics of *A. foliaceus* on rainbow trout (*Oncorhynchus mykiss* (Walbaum, 1792)) and brown trout (*Salmo trutta* Linnaeus, 1758) were also recorded by Northcott *et al.* in Scotland (1997). While *Argulus* spp. in the UK have severe impacts on stocked fisheries and some fish farms, they may be of less concern in the wild, although the distinction becomes more complex in extensive fisheries holding both stocked and wild fish. More than 25% of the recreational trout fisheries studied by Taylor *et al.* (2006) were found to be infected by *Argulus* spp., with *A. foliaceus* being the most dominant species present.

Kennedy (1974) listed the fish hosts of *A. foliaceus* in the UK and Ireland from previous records and publications and found it to be reported from the following fish hosts: brown trout (*Salmo trutta*), rainbow trout (*Oncorhynchus mykiss*), salmon *Salmo salar* Linnaeus, 1758, powan *Coregonus clupeoides* (Lacépède, 1803), vendace *Coregonus vandesius* (Richardson, 1836), pike *Esox lucius* Linnaeus, 1758, perch *Perca fluviatilis* Linnaeus, 1758, common bream *Abramis brama* (Linnaeus, 1758), common carp *Cyprinus carpio* Linnaeus, 1758, crucian carp *Carassius carassius*

(Linnaeus, 1758), gudgeon *Gobio gobio* (Linnaeus, 1758), roach *Rutilus rutilus* (Linnaeus, 1758), rudd *Scardinius erythrophthalmus* (Linnaeus, 1758), tench *Tinca tinca* (Linnaeus, 1758) and three-spined stickleback *Gasterosteus aculeatus* Linnaeus, 1758 (Kennedy, 1974). Given the range of hosts observed, this list is unlikely to be exhaustive.

1.2 **Morphological taxonomy of *Argulus* spp.**

Crustacean parasites are frequently quite large, especially the adults and can often be seen with the naked eye. They share a number of common morphological features as all they have a hard exoskeleton, and largely display identifiable antennae, mandibles and primary and secondary maxillae with five pairs of appendages in the head / cephalosome region (Hoole *et al.*, 2001). Macroscopically *Argulus* spp. appear as green-brown in colour with semi-translucent, ovoid, dorso-ventrally flattened carapaces. The carapace both provides protection and serves to improve the parasite's hydrodynamic efficiency and reduce drag when attached to the fish (Taylor 2004; Hoole *et al.*, 2001).

The body of *Argulus* spp. is divided into three parts: the cephalothorax, comprising the cephalon and head region, the thorax and the abdomen. The head is equipped with paired compound and one nauplius eye; paired first and second antennae; mandibles within the mouth tube; first and second maxillae (Møller, 2011). The first antennae are located anterior to the compound eyes and take the form of curved hooks, which serve as attachment organs, particularly for younger stages (Alaş *et al.*, 2010; Møller 2011).

The first maxillae, sometimes termed attachment suckers or discs, are a feature of the genus and help in host attachment and in providing mobility over the surface of the host. The suction discs originate from the cross-boundary area between the first and second podomeres of the first maxillae (Kaji *et al*., 2011). The first maxillae of *Argulus* spp. larval stages are equipped with hooks, with the suction discs starting to appear at stage 5 when the first (/second) segment of the hook becomes more disc-shaped (Møller *et al*., 2008). The second maxillae, having 5 segments, are located posteriorly to the suckers (Alaş *et al*., 2010). A posteriorly oriented mouth tube and anteriorly oriented pre-oral spine are located in the midline between the maxillae. The thorax, which is the second part of the *Argulus* body, has four separate appendages or segments. Each segment consists of a pair of swimming legs or thoracopods. The first segment is partially merged into the cephalic region. The abdomen is small, bilobed, unsegmented and without appendages (Hoffman 1977; Hoole *et al*. 2001; Kearn 2004). From the shape of the abdominal lobes it is possible to identify sexes and differentiate between some *Argulus* species (Kearn 2004). Suggested “respiratory” areas are found on the dorsal surface of the carapace (Hoffman 1977), however ultrastructural study of these areas suggests that they may function in ion-exchange or osmotic regulation (Haase, 1975). Alaş *et al*. (2010) stated that the posterior respiratory areas are larger than the anterior ones.

Size varies between *Argulus* species and sex where females bigger than males. *Argulus foliaceus* and *A. japonicus* adults are almost the same size, 6-8mm in length (Shafir and Van 1986; Rushton-Mellor 1994), while *A. coregoni*, is larger, reaching up to 12 mm in length (Gurney 1948).

1.2.1 **Morphology of *A. foliaceus***

Rahman (1995) described the body of *A. foliaceus* as having an oval-shape, flattened dorso-ventrally and being convex dorsally (**Fig 1.1**). The adult carries four pairs of thoracic legs, and a first antenna and a second antenna located anterior to the compound eyes. *A. foliaceus* like all *Argulus* spp. has pair of first and second maxillae, these are appendages being spinous on the basal segments and having small claws at the tip. In later stages, the first maxillae are modified into muscular suckers to help in host attachment. Cephalic appendages, first and second antennae, and maxillae contain hooks, spines or claws to assist the parasite in attaching to hosts (Gresty *et al.*, 1993).

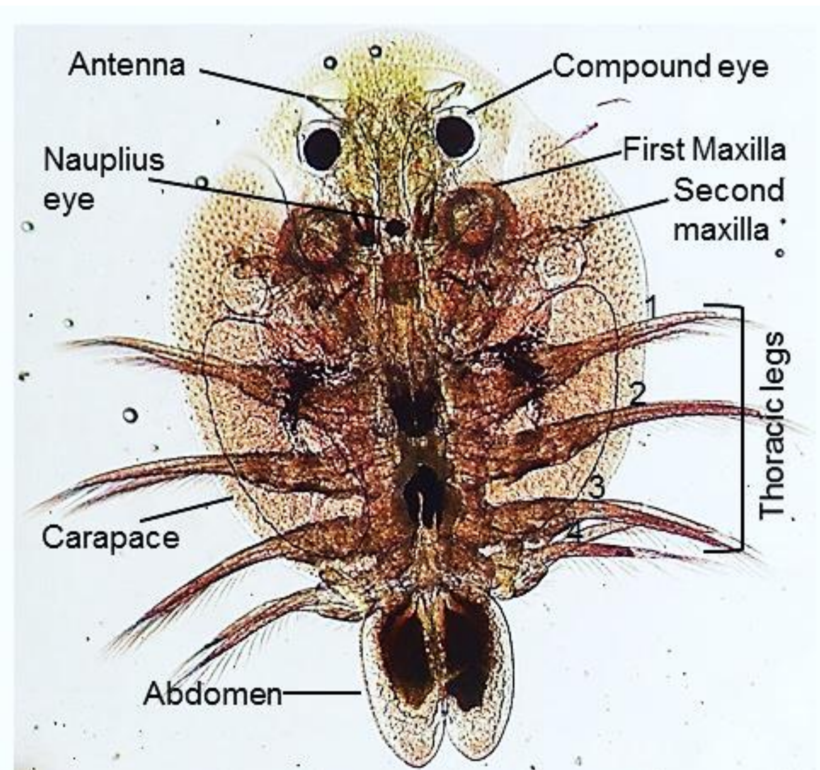


Figure 1.1. Dorsal aspect of *Argulus foliaceus* male adult showing different body parts.

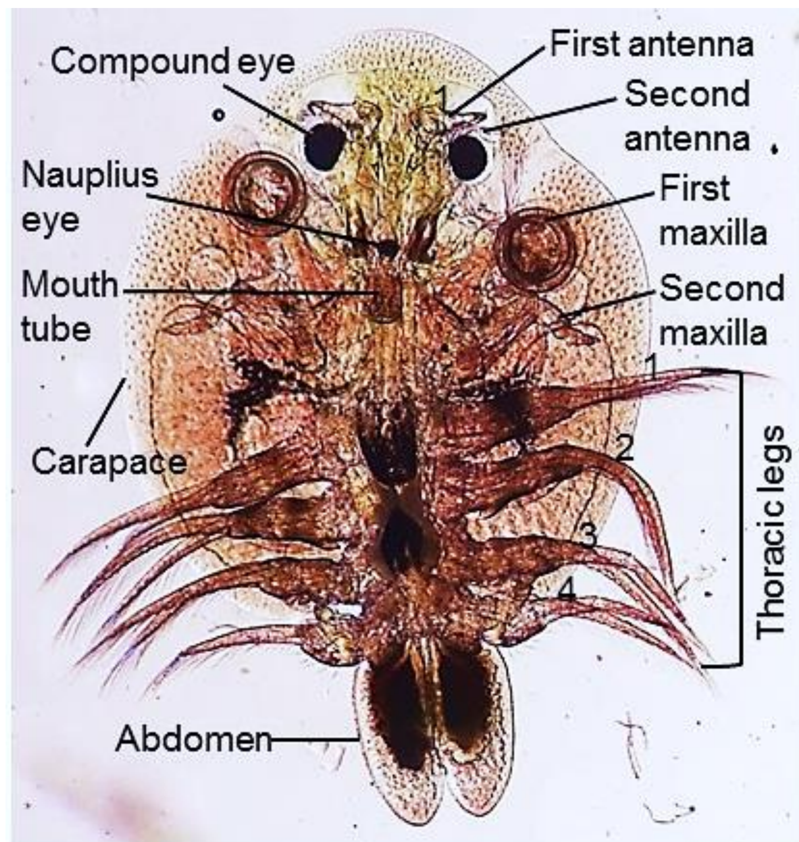


Figure 1.2. Ventral aspect of *Argulus foliaceus* male adult showing different body parts.

The carapace provides physical protection and also provides the principal zone for respiratory exchange in *Argulus* spp. with specialised areas, named incorrectly as ‘‘respiratory areas’’, located ventrally, and likely involved in ionic or osmotic regulation. The parasite has highly branched gut caecae (Haase 1975 as reported by Boxshall 2005, Møller 2011). The abdomen contains paired male testes or the seminal receptacles; spermatothecae, in the female, where sperm are stored until needed to fertilise eggs.

The pre-oral spine lies in the mid-ventral line of the cephalon, situated between the two suckers. The stylet is a unique feature of the branchiuran genera *Argulus* and *Dipteropeltis* (Gresty *et al.*, 1993), however Neethling *et al.* (2014) described the pre-oral structure of *D. hirundo* as being tiny and triangular in shape without an apparent

duct or spine. The mouth tube is located posterior to the pre-oral spine (Gurney 1948; Shafir and Van 1986; Gresty *et al.,* 1993; Rahman 1995; Boxshall 2005).The mouth opening, with toothed mandibles, is situated at the tip of the mouth cone which is fused entirely into a tube. The mouth tube comprises a lower labium and upper labrum. The anatomy of the mouth tube and pre-oral spine are discussed in more detail in section 1.4.1.3.

Numerous mucous glands were found at the base of the suckers of *A. coregoni*. While the main function of the mucous glands was not identified, it may help in sealing the sucker to the substratum. The suckers are regarded as the primary attachment organ, being connected to two types of muscles, suction muscles and cup muscles, which serve to generate suction and control movement and orientation of the sucker (Gresty *et al*., 1993).

1.3 ***Argulus* Biology**

1.3.1 **Life cycle**

As *Argulus* spp. are obligate parasites in later stages, their life-cycle and development are intimately related to host finding and attachment (**Fig 1.3**) and are therefore strongly linked to the biology of the fish host. Free-swimming larval stages were suggested to survive less than 2 days and adults almost 1 week without a host (Kollatsch 1959; Mikheev *et al*., 2000). In experimental studies with *A. japonicus*, Shafir *et al*. (1992) observed that *A. japonicus* rapidly moves between fish hosts and

can be off the host for 3 to 4 days, while in the wild they cannot stay separate from the host for more than 2 days. According to Walker *et al.* (2011) the factors which may affect the maximal off-host period include: last meal ingestion time, activity level and temperature. Kollatsch (1959) observed that *A. foliaceus* females needed to be host-associated for copulation and then detached from the host to find a suitable firm surface upon which to lay eggs *e.g.* rocks, plant stems *etc*. Later, Gault *et al.* (2002) observed that *A. foliaceus* in still water tanks laid their eggs on the tank concrete walls, plastic containers, rocks and tree stems. The same was found by Taylor *et al.* (2005) from a research study on different still water fisheries in UK. For *A. coregoni* females were also found to move from fish host to pond walls to lay a single layer of eggs (Shimura 1983), Shimura and Egusa (1980) suggested that *Argulus coregoni* prefers rough surfaces to smooth ones. *Argulus coregoni* was found to prefer deep water to lay their eggs whereas *A. foliaceus* lays eggs in shallow water (Mikheev *et al.*, 2001).

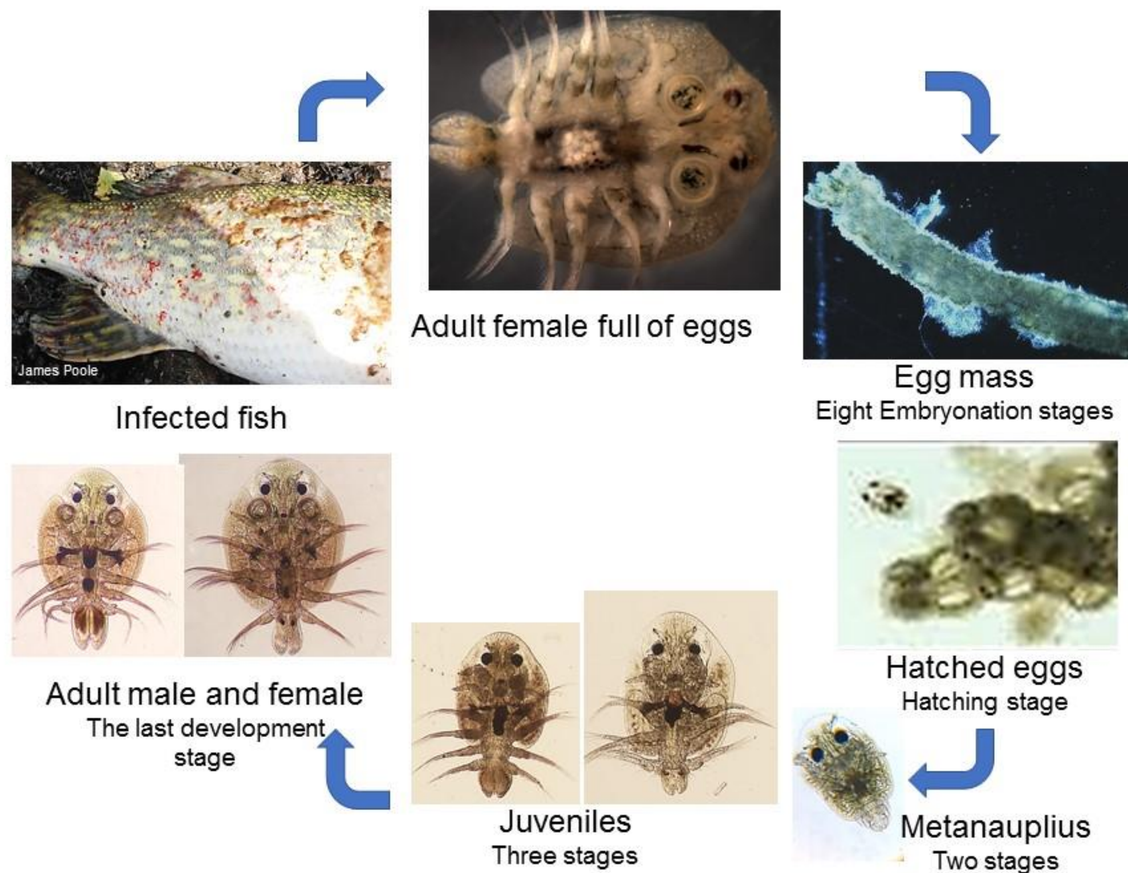


Figure 1.3. Life cycle of *Argulus foliaceus.* Rahman (1995) described it to comprise nine embryonation stages and six developmental stages from copepodid to adults.

Hatching of overwintering eggs in spring, when water temperature rises above 10° C, was found to initiate the principal annual cycle of *A. coregoni* (Mikheev *et al*. 2001; Hakalahti and Valtonen 2003). *Argulus foliaceus* eggs and a restricted number of adults on fish hosts, were found to provide overwintering stages capable of relaunching populations with increasing spring temperatures (Pasternak *et al.*, 2000; Gault *et al.*, 2002; Harrison *et al.*, 2006; Taylor *et al.*, 2009). By studying the life cycle of *A. foliaceus*, Claus (1875) found it to have 2 to 3 generations per year (as cited in Shimura 1983). Later, Stammer (1959) recorded the same result. Gault *et al.* (2002)

showed similar findings, with around two generations every year and some parasite stages overwintering. Pasternak *et al.* (2000) also reported two overwintering strategies for *A. foliaceus*, comprising adults and resting stages. Shimura (1983) found that *A. coregoni* can have two generations per year. The growth stage length of *A. foliaceus* is strongly influenced by temperature, with growth rates increasing with higher temperatures Schluter (1979). Hoffman (1977) and Boxshall (2005) believed that *Argulus* development time is dependent on species and temperature. Rizvi (1969) observed that within only two months during summer, two new generations of *Argulus foliaceus* appeared and that reproduction stopped if the temperature was below 14°C, while Gault *et al.* (2002); Harrison *et al.* (2006) and Taylor *et al.* (2009) observed that egg laying occurred at water temperatures >10° C. In contrast, Möller (1978) observed that *A. foliaceus* showed lowest activities between 0˚C and 5˚C and at 10˚C females started to lay their eggs. Hoffman (1977) thought the life cycle of *Argulus* spp. needed a minimum of 40 days to complete, with this extending to 100 days. They also noted that while *Argulus* males usually survive for less than one year, females could survive for up to 18 months (Hoffman 1977). Walker *et al.* (2011) observed that *A. japonicus* and *A. foliaceus* reach maturity in 25 days at 23 ˚C and this result is clearly at odds with the Hoffman (1977) report. Rahman (1995) found that the *A. foliaceus* life cycle needed 55 days to be completed at 20˚C and he described the different developmental stages: nine stages observed during the embryonation period and five stages from copepodid to adults. Branchiurans start to be parasitic from the second larval stage, however, as they develop they start to leave the host to find new hosts (Boxshall 2005). Ikuta & Makioka (1997) recorded the swimming and cleaning behaviours of stage 1 and stage 2 of *A. foliaceus* and concluded that stage 1, which is the hatching stage, is

sufficiently advanced to be described as a metanauplius stage. It was also found that the stage 2 larval stage, was as efficient a swimmer as the adult.

1.3.2 **Reproduction**

In *A. japonicus*, copulation was reported by (Shafir and Van 1986) to occur both on fish during parasitic and off fish during free-swimming periods, with gravid females present all year round. Rahman (1995) observed that copulation of *A. foliaceus* took place on the host surface. Martin (1932) described the position of the oocytes in the female ovary of the *A. foliaceus* as being found on the ventral aspect (Martin, 1932) and Ikuta & Makioka (1997), for *A. japonicus,* noted that the oocytes leave the germarium and migrate down to the outer surface of the ovarian epithelium. In terms of fertilisation, *A. coregoni* males were observed to transfer sperm from the semen capsule in the third leg, using the fourth leg, to the spermathecae of the female (Everts, 2010), from whence females obtain stored sperm for fertilisation.

Following fertilisation *A. japonicus* eggs were observed to be deposited on hard substrates, with 4 developmental stages recognised and with hatching-time being temperature dependent. As temperature increases, egg hatching-time decreases; at 35˚C hatch takes 10 days whereas it takes 61 days if the temperature is 15˚C. From following the reproductive cycle of *A. japonicus*, the fastest growth of the parasite was observed during summer (Shafir and Van 1986). The total number of eggs produced by *Argulus* species in each batch was suggested to vary between 60-200 eggs/batch (Fryer 1968; Hoffman 1977).

For *A. foliaceus*, Bower-Shore (1940) noted that after seven days, egg deposition begins, with gravid females laying eggs for 30 minutes per batch on hard substrates, in this case the bottom and wall of an aquarium. This can be continuous, with two or three batches being laid over 24 hours. Deposited eggs were arranged in two or three rows with the number of deposited eggs varying from 40 to 200, depending on the size of females. Bower-Shore (1940) recorded that the development of *A. foliaceus* eggs needed 28-36 days, however, the temperature was not recorded in that study. Hoffman (1977) stated that *Argulus* species may lay 20 to 300 eggs at one time, potentially undergoing more than three generations per year. An *Argulus* spp. egg is oval in shape, 0.3-0.6 mm in length and the egg batch is covered in a gelatinous capsule, which allows the eggs to be firmly attached to the substrate and may provide some protection against predation.

Argulus coregoni eggs need 30 days at 18-22° C to hatch (Shimura 1983; Hakalahti *et al.*, 2004) . It was observed that high quality eggs, showing good hatching success, tended to be produced by females displaying higher fecundity. Female fecundity was also found to be correlated positively with the length of the egg hatching period for individual *A. coregoni*. From the observation of different treatments on *A. coregoni*, it was concluded that this parasite uses the bet-hedging strategy through use of an extended hatching pattern, (Hakalahti *et al.*, 2004), with eggs capable of hatching after two years submerged in the sediment (Mikheev *et al.*, 2001). Hakalahti *et al.* (2004) recorded differences between *A. coregoni* and *A. foliaceus* life cycles, finding that females of *A. foliaceus* leave the host several times to lay their eggs (Kollatsch 1959; Pasternak *et al.*, 2000) while for *A. coregoni*, only 25 % of the females returned to the fish host. According to the findings of Hakalahti & Valtonen (2003) there is a high

probability of *A. coregoni* females dying after laying their eggs, with males therefore found to be numerically dominant during the egg lying season.

The egg hatching cycle of *Argulus* spp. has been found to be affected by egg-laying time (Shimura 1983), illumination (Mikheev *et al*. 2001) and water temperature (Shafir and Van 1986). Hoffman (1977) indicated that hatching of eggs may be delayed up to 30 days in the dark, while light stimulates hatching. Shimura and Egusa (1980) concluded that egg-laying of *A. coregoni* was also influenced by the absence of light, with 98% of egg-laying occurring at night.

Rahman (1995) divided the development of *A. foliaceus* into three main phases: embryonation, copepodid "metanauplius" and adult (**Fig 1.3**). The first nine stages described belonged to the embryonation period, with five stages from copepodid to adult. The first stage includes freshly laid eggs which measure 0.33 in length and 1.2 in width. By stage seven larval shapes started to appear and after 15 to 16 days, termed stage 8, larval appendages were obvious with presence of a clear black eye. Transparent, hatched copepodids, stages 10 and 11, were 0.6-0.8mm long with two pairs of large branched antennae. At this stage the pre-oral spine, proboscis and alimentary canal were distinguishable while the suckers were not yet developed. Metanauplius also termed copepodites, needed to attach to the fish host within 2-3 days otherwise they died. Thoracic legs, abdomen and raptorial claws were well-developed and the antennae reduced by stage 12. Sexes can be distinguished from stage 13. After 20-30 days from the hatching, stage 15 according to Rahman, 2.28-4.21 mm adults with modified suckers can be seen. The first attaching stage of *A. foliaceus* was found to attach itself while still reliant on its yolk, with no blood being observed in the gut, which means that the first infective stage may not have started to

feed on blood yet (Tam and Avenant-Oldewage 2006), although it could nevertheless be ingesting epithelial tissue / mucus.

1.3.3 **Population dynamics**

From a study by Shafir and Oldewage (1992), it was observed that a stable age distribution of *A. japonicus* existed within particular study periods, suggesting that the physical environment has a major impact on reproduction, sex ratio and growth patterns for the parasite. New generations (cohorts) were observed in summer and spring months only. Although *A. japonicus* is suggested to be a continuously reproducing population throughout the year, due to the presence of gravid females in all months, the population size was found to increase in summer. *A. japonicus* is considered to overwinter using the same mechanisms as *A. foliaceus*. Sex-ratios recorded in this study showed that males were favoured over females throughout the year, which may suggest a genetic sex bias. Overall this species shows rapid population growth that is affected by water temperature (Shafir and Oldewage 1992). The growth and activity of *A. foliaceus* was also found to be affected by water temperature (Möller 1978; Schluter 1979). The same results were recorded by Koyun (2011), where *A. foliaceus* intensity of infection increased with increasing temperatures. The growth rate of *A. coregoni* males was found to be faster than that of females until they reach ~3 mm in length, after which point females grow faster (Pasternak *et al.*, 2004).

From a study by Shimura (1983) on *A. coregoni*, maximum infestation levels of the parasite were recorded during summer in Japan, 142 parasites /fish, and then dropped

to no parasites during winter season; January to March. Overwintered eggs hatched in April. The sex ratio of all sizes was almost 1:1, but changed for sizes >7.5mm length, with females being more prevalent amongst large individuals (Shimura 1983).

For *Argulus foliaceus,* females' numbers were found to be higher than males (Bower-Shore 1940). Alsarakibi *et al*. (2012) showed the same results, with ratios of females to male of 2.3:1. This was considered to reflect the fact that females are more obligate parasites than males, with males thought to spend most of their time searching for mates in the water column. Shimura (1983), in contrast, believed that the sex ratio of *A. foliaceus*, *A. coregoni* and *A. japonicus* was almost 1:1. Stammer (1959) observed that males of *A. foliaceus* were more predominant than females, with a sex ratio of approximately 2:1, this also being observed by Nagasawa *et al*. (2010) in Japan, where populations were male-biased in Amur catfish infestations.

Argulus population growth during winter is known to be delayed by cessation of egg-hatching, thus no new generations are observed during the winter months and distribution of *Argulus* spp. may also be affected by water quality (Abowei and Ezekiel 2011).

1.3.4 **Host searching**

During their life cycle, *A. foliaceus* needs to find a fish host at least once in order to survive. If the parasite is removed from the fish for any reason, such as searching for a mate, laying eggs or following the death of the host *etc*. (Kollatsch, 1959; Stammer, 1959), it must search for another fish host. As an adult, it attaches to its host largely

through means of the sucking discs (modefied first maxillae) and the second maxillae, which act to produce a form of frictional attachment (Evans 2000). The first maxillae play a major role in attachment to the host, for both parasitic and free-swimming situations, for the larval stages and adults *A. foliaceus* and in the cleaning of the naupliar swimming appendages (Møller *et al.*, 2007).

The host-switching strategy of *Argulus* was suggested by Bandilla *et al.* (2007) to be associated with the anti-parasitic behaviour of the fish host, the need to locate a more suitable host or the activity of a given host's immune system against the parasite (Bandilla *et al.*, 2007). Competition between parasites on the same host may also prompt the parasite to search for another host (Dawson *et al.* 2000; Fredensborg and Poulin 2005); and mate searching could also lead to switching hosts (Jaworski and Holm 1992; Pasternak *et al.* 2004). Bandilla *et al.* (2007) suggested that *Argulus* spp. females are more stationary than males, which are found to more active swimmers when searching for either a suitable host or an appropriate mate. This strategy employed by *Argulus* spp. suggests that females spend their energy for growth and fecundity while males use their energy to find an appropriate host or suitable mate.

Some researchers such as Bohn (1910), as reported by Mikheev *et al.* (1998), believed that *A. foliaceus* employed active movements prompted by the host's shadow, to intercept and infect fish. Others like Herter (1927), as reported by Mikheev *et al.* (1998), considered that parasites searched for hosts randomly. Later experiments by Mikheev *et al.* (1998) determined that swimming behaviour and infection rate are affected by visual conditions and object reflectivity. *A. foliaceus* can be attracted rapidly to any reflective object from 10-15 cm distance. Fast movement of the parasites towards any sudden illumination change was also observed by

Kollatsch (1959). This may explain why newly attached parasites can be found on the most reflective parts of the fish's body. The rate of infection is reduced with very highly reflective objects, which may confuse the parasites (Mikheev *et al.*, 1998). *A. foliaceus* was also found to attach more easily to motionless fish rather than actively swimming ones. Bandilla *et al.* (2005) has reported that rainbow trout did not show any resistance following challenge experiments with *A. coregoni*. They believed that fish behaviour, particularly slow swimming, was the main factor increasing success of parasite attachment. The infection rate is thus suggested to be negatively correlated with hosts' swimming speed. Moreover, *A. foliaceus* was found to search for hosts at night or under dark conditions and to swim more quickly when starved. *Argulus* were found to swim 3-4 times faster in the dark than under the light conditions. Host odour was also found to speed up the movement of the parasites. In conclusion, there are two determinants for host searching behaviour, this being controlled either endogenously (*e.g.* starvation state) or exogenously (*e.g.* light and host odour) (Mikheev *et al.*, 1998; Mikheev *et al.*, 1998).

As for many aquatic ectoparasites, infection levels for *Argulus* are positively correlated with host size. For example, Amghass *et al.* (2013) recorded *A. foliaceus* infection intensity of rainbow trout to be positively correlated with host size and this was suggested to be due to increase in total surface area of the host skin. The same results have been recorded previously for a wide range of researchers for different skin ectoparasites. Grutter (1994) found that the total number of parasites correlated positively with the standard length of coral reef fish in Australia and Poulin and Morand (2000) also concluded that the prevalence and intensity of copepod ectoparasites of fish were correlated with their host body size (Poulin and Morand, 2000). It should,

however, be noted that correlation with host body size does not necessarily reflect host surface area but may also result from differences in habitat use / behaviour at different host ages or differences in immuno-competence in *e.g.* fry or mature adults.

1.3.5 **Host specificity**

Argulus spp., including *A. foliaceus*, *A. coregoni* and *A. japonicus*, have been found to infect most fish species in the UK, which mean that it has wide host tolerance (Bower-Shore 1940; Fryer 1968; Kabata 1970). Similarly, Alaş *et al.* (2010) noted that *A. foliaceus* in Turkey, considering records made from 1976 to 2006, had been found to parasitize 19 different hosts from several freshwater regions. On the other hand, for some African species of *Argulus* described by Fryer (1968), higher host specificity is recorded. Bower-Shore (1940) also observed that some fish species were more susceptible than others, giving brown trout as an example, and noting them to be more infected than the brighter rainbow trout, and ascribing this difference as being due to their colour. In a separate study, the attachment of *A. japonicus* to goldfish and fathead minnows separately were reported as showing no significant difference in attachment of *A. japonicus* (LaMarre and Cochran 1992).

Although *A. foliaceus* is known to have low host specificity (Kollatsch, 1959; Petrushevski, 1970; Shulman, 1970 as reported by Mikheev *et al* 2000), some authors have suggested that *A. foliaceus* nevertheless shows partial host specificity or preferences for some fish species. As an example, *A. foliaceus* in Finland was found to have higher preferences for perch *Perca fluviatilis* Linnaeus, 1758 than roach *Rutilus rutilus (Linnaeus, 1758)* (Valtonen *et al.*, 1997). However, Mikheev *et al.* (1998)

observed the reverse behaviour from their experiments using black-lined aquaria, with *A. foliaceus* attaching more to roach than perch. The same observation is recorded by Pasternak *et al.* (2000) for hosts captured in the wild. Mikheev *et al.* (1998) also found that *A. foliaceus* switches its host-preferences between light and dark conditions. It prefers to attach to perch juveniles in the dark and move to the roach juveniles in the light. Mikheev *et al.* (2007) also recorded that newly hatched *A. coregoni* metanauplii preferred roach over rainbow trout, with host preference shifting to rainbow trout as the parasite matures. The same authors observed that once the parasite reaches 2mm it shows greater response to visual and olfactory stimulation (Mikheev *et al.*, 2004). These results suggest that this ontogenetic shift is genetically programmed, being innate not acquired. However, in contrast *A. foliaceus* did not show any ontogenetic change in host preference through its life cycle (Mikheev *et al.*, 2007). By following the growth / development of *A. coregoni* metanauplii in both roach and rainbow trout, it was found that roach can provide a temporary host for *A. coregoni* in the absence or scarcity of rainbow trout (Pasternak *et al.*, 2004).

1.3.5.1 **Host attachment preferences**

Shimura (1983) studied the attachment site preferences for different sizes of *A. coregoni* and concluded that parasites <1.5 mm attached to all skin surfaces including fins. In this study 96% of the larvae were found to attach to the body surface while the remaining 4% were found in the oral and gill cavities. He concluded that as the parasites increased in size, they altered their attachment position from the body surface and fins to the pectoral and pelvic fin area, while *A. foliaceus* was found to be

scattered all over the host skin. Shimura (1983) considered that there was a correlation between the site of attachment and host behaviour with carp being relatively inactive swimmers living in still water. Bower-Shore (1940) also recorded *A. foliaceus* scattered in different positions over the host surface without moving for several days. He also observed that parasites could continue attaching to dead hosts for about 8 days, which has important implications for removal of dead fish from infected fisheries. Overall, *A. foliaceus* demonstrates its success in host searching and finding by infecting different types of fish during the day and night (Mikheev *et al.*, 1998). Its swimming speed increased significantly with fish presence or any chemical or mechanical stimuli (Mikheev *et al.*, 2000). Later Mikheev *et al.* (2004) studied the difference between metanapulii, juveniles and adults in responding to light and fish odour stimulation. Younger argulids were found to be attracted by non-visual stimuli but were not stimulated by olfactory cues while juveniles were attracted by both stimuli. This supports the contention that sensory modalities develop at later ontogenetic stages (Mikheev *et al.*, 2004) and that vision is the main sense used by both the juvenile and adult *Argulus* (Mikheev *et al.*, 2004; Bandilla *et al.*, 2007).

Bandilla *et al.* (2007) also reported that *A. coregoni* males were more attracted to light and fish odour than to female odour which suggests the priority for *Argulus* males is to find a suitable host then search for a female.

Del-claro and Takemoto (2003) observed *Argulus elongatus*, *Argulus juparanensis Lemos de Castro, 1950*, *Dolops carvalhoi* Lemos de Castro, 1949 and *Dipteropeltis hirundo* Calman, 1912 to be more common in river fish hosts than in pond fish hosts. Water currents may affect the swimming ability of the parasite, thus it may not be easy for parasites to detach and reattach in particular environments. In ponds, it is much

easier for the parasites to switch between hosts and leave hosts for mating and reproduction. Northcott *et al.* (1997) and Evans (2000), observed in Scotland and Ireland respectively, that high fish intensities in small lake volumes were correlated to high infection levels.

Early stages of *A. coregoni* and *A. foliaceus* were found not to show discrimination for specific fish hosts but to prefer more highly reflective fish. During later stages of the life cycle, *A. foliaceus* was found to show low host specificity whereas *A. coregoni* was observed to change its host preference from roach to rainbow trout at the beginning of the reproduction season (Mikheev *et al.*, 1998; Mikheev *et al.*, 2004). The residence time, the period between the attachment and the first detachment of *A. coregoni* on the rainbow trout was greater than for roach (Pasternak *et al.*, 2004).

It was found that off-host time of adult and larval stages of *A. coregoni* and *A. foliaceus* were affected by temperature and that adults can survive off the host, in water, for a longer time than larvae and juveniles. The latter may reflect quantities of stored resources *e.g.* lipids in these different stages. *A. japonicus* was found to be more resistant to starvation at higher temperatures under controlled off-host conditions and *A. foliaceus* was found to be more resistant to starvation at lower temperatures (Walker *et al.*, 2011), possibly reflecting different temperature optima for these species.

1.4 Feeding

1.4.1 Feeding mechanisms

Argulus foliaceus is suggested to feed principally or entirely on blood and can last only a few days off the host, depending on the parasite's size and ambient water temperatures (Kollatsch 1959). From previous observations of feeding in *Argulus* spp. it has been suggested that feeding involves rapid, repetitive piercing of the host skin by thepre-oral spine, which is believed to inject active components, accompanied by blood feeding through the mouth tube / proboscis (Gresty *et al*., 1993; Boxshall 2005).

Gresty *et al*. (1993) suggested that the parasite feeds in the same location for some time and that, during the feeding process, the pre-oral spine is lunged into the fish's skin. It was suggested that this mechanical action, with the help of secreted fluids, causes ruptures and cracks in the host's epithelium. Then, the parasite was suggested to move forward to place its mouth opening on the lesion. According to Gresty *et al* (1993), the spine is able to pierce the fish epidermis to different depths, with the main function of the spine being to damage the host epithelium to assist in the feeding process. Kabata (1970) believed that during feeding, the spine caused the main damage to the fish host, due to the repeated lunge of the spine and its lytic secretions. The secretions may function as lytic agents or anticoagulants or both.

The *Argulus foliaceus* mouth-tube was suggested to have great flexibility, allowing active movement around the host integument (Møller and Olesen 2010). Boxshall

(2005) thought that the labial mandibles, located in the opening of the mouth-tube, might introduce the same digestive enzymes as thepre-oral spine.

1.4.1.1 **Blood ingestion**

Although *Argulus* has been largely described as a blood-feeder (Wilson, 1902; Van Duijin 1956; Bauer, 1959; Walker *et al*., 2011), some researchers were unable to find any blood cells in the parasite gut from their observations (Minchin, 1909; Kabata, 1970). Bower-Shore (1940) recorded blood clots in the gut of large female *A. foliaceus*, which could be evidence that only the adults feed on blood.

Argulus may have the capacity to feed on both host blood and epidermis (Kearn, 2004). *A. foliaceus* larvae were observed by Bower- Shore (1940) to gather around the higher mucus areas on the fish skin. The glandular secretions delivered by the pre-oral spine might stimulate vasodilation of the dermal blood vessels, whereas the mandibles may assist removal of the superficial epidermis. This may help us to understand *Argulus* feeding behaviour, which involves both mobile and stationary phases (Kearn, 2004). Walker *et al*. (2011) observed that larval *A. japonicus* did not feed on red blood cell, while adult guts contained erythrocytes. The size of blood cells was found to be larger than the size of the aperture opening of the *A. japonicus* larval parasite's buccal cavity (Tam and Avenant-Oldewage, 2006). All previous studies observed haemorrhagic and inflammatory responses after *Argulus* infection, which may support the idea that the parasite secretes chemicals during the feeding process to assist the ingestion of blood and epithelial tissue (Shimura & Inoue 1984; Forlenza *et al*., 2008; Walker *et al*., 2011).

1.4.1.2 **Digestive system**

Tam (2005) found that *A. japonicus* adults and larvae have similar digestive systems comprising oesophagus, oesophageal funnel, anterior midgut, midgut enteral diverticula, posterior midgut and hindgut. From a study on the cell types of the digestive system of *A. japonicus*, Tam & Avenant-Oldewage (2009) found that microvillar R cells were dominant in the anterior midgut while enteral diverticula had two types of cells, microvillar R cells mostly in the proximal diverticula and microvillar F cells in the distal diverticula. Papilliform vacuolar B cells and microvillar R cells were found in the posterior midgut, with B cells considered to be responsible for intracellular digestion. The morphology of the digestive tract of *A. japonicus* is similar to that of the adult but the epithelial cells lining the midgut of newly hatched larvae contained large amounts of yolk (Tam & Avenant-Oldewage 2006).

1.4.1.3 **The pre-oral spine and the proboscis (mouth tube)**

From previous studies, the two main structures involved during the feeding process are the mouth-tube and the pre-oral spine (**Fig.1.4-5**).

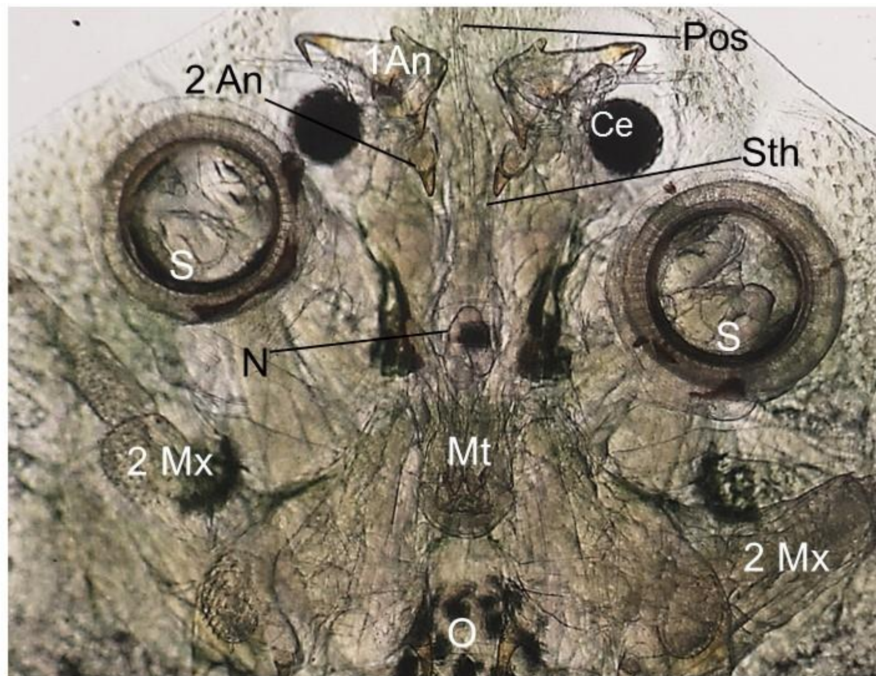


Figure 1.4. Ventral aspect of adult female *A. foliaceus* showing the position of compound (Ce) and nauplius (N) eyes and cephalothorax and feeding associated appendages including pre-oral spine (Pos) within the spinal sheath, mouth tube (MT), first and antennae 1 and 2 An), suckers (S) and second maxillae (2 Mx). (O) ovary.

Mouth Tube

The mouth-tube comprises a tubular structure with the mouth located at the distal end of the tube. *Argulus japonicus* larvae and juveniles appear to have the same mouthparts as adults, *i.e*. mandibles, labrum and labium, with differences in the overall size of the mouthparts, with the aperture of the buccal cavity being considerably larger in adult lice (~20 µm) than in larval lice (~9 µm) (Walker *et al*. 2011). The mouth is enclosed by an upper labrum and a lower labium which arise from the ventral cephalic surface (Gresty *et al*., 1993). The large labium (**Fig.1.5**), which is equipped with a pair of tubular labial spines and possesses paired labial muscles, is enclosed by the tip of the labrum.

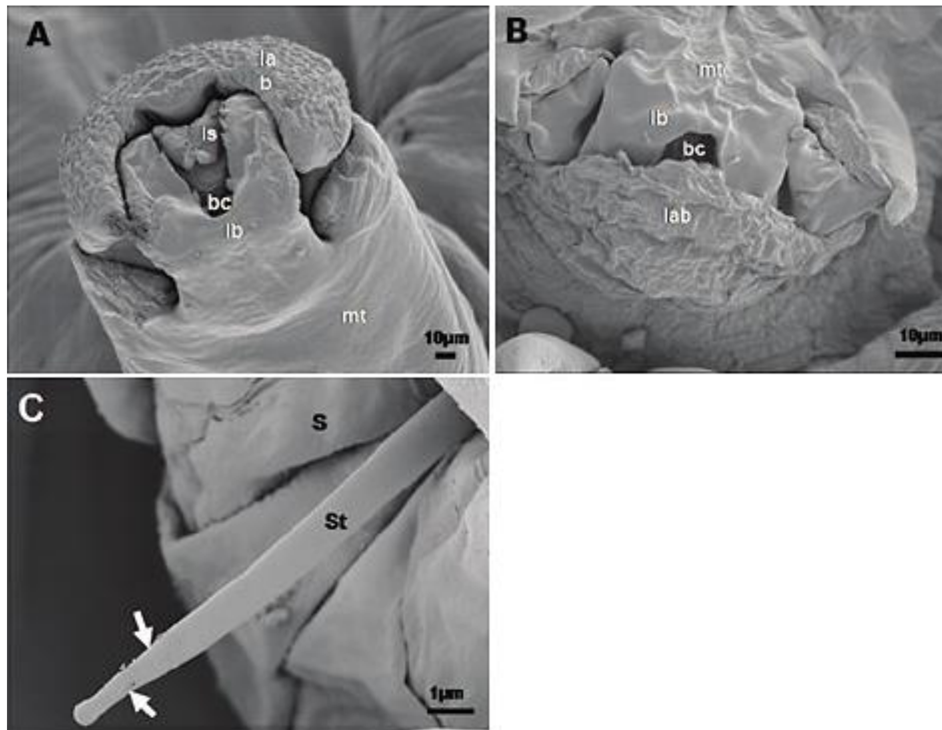


Figure 1.5. Scanning electron micrographs of the mouth parts of an adult (A) and larval (B) *Argulus japonicus* showing the mouth tube (mt), labium (lab), labrum (lb), labial spine (ls) and buccal cavity (bc). The pre-oral spin of an adult (C) takes the form of a hollow spine (St) possessing two openings near to its tip (white arrows) and is contained within a sheath (S) into which it can be retracted when not in use (Walker, 2008).

The elongated structure of the mouth tube is reflected by the mandibular musculature. The mouth-tube is supported internally by internal mandibular apodemes, which extend from its tip to the head carapace interior on either side of the cerebrum, and externally by the buccal bars in the external wall of the mouth tube. The mandibular coxal processes are held within the buccal cavity, however, during feeding they have the ability to rotate and slit and cut / abrade host tissue (Møller, 2011). Kearn (2004) suggested that the paired mandibles are used during feeding for scraping tissues rather than serving as part of a piercing mechanism. At the proximal end of the mandible, rigid mandibular apodemes form the attachment for 3 pairs of muscles, *i.e.*

the abductor muscles and the two sets of adductors. The first set of muscles originates from the ventral cephalic tendon and lies parallel to the oesophagus. The other set of mandibular adductors originates at the dorsal cephalic wall anterior to the mouth tube.

A narrow oesophagus runs back from the buccal opening along the length of the mouth tube to the lumen of the anterior midgut. The part of the oesophagus lying within the mouth tube opening is lined with fine spinules (Gresty *et al.*, 1993), which might aid in the feeding process *e.g.* allowing maceration in order to mediate the primary digestion step of the host tissue before passing through the oesophagus to the anterior midgut.

Pre-oral spine

Gresty *et al* (1993) described the anatomy of the pre-oral spine of *A. foliaceus* using light and electron microscopy (SEM and TEM). The pre-oral spine is located anteriorly to the mouth-tube and comprises an eversible sheath proximally and a tapering distal spine. The tip of the pre-oral spine has a diameter 1.5-2.0 μm and has a tiny pore and a large central duct which extends inside the spine. The pore on the dorsal side was suggested by Shimura (1983) to be the opening of a chemoreceptive structure and he suggested that TEM examination of the dendrites of sensory cells would prove this hypothesis. The middle of the pre-oral spine has an oval cross-section measuring 10.35 μm in diameter (Gresty *et al.*, 1993), while at its proximal end the diameter is approximately 13.8 μm. The central duct of the pre-oral spine is hollow and is surrounded by two layers, a thick cuticular outer layer and a thin cuticle forming the central duct. Osmophilic granules found in the outer layer may correspond to deposits of materials which enhance the cuticle hardness or strength, as suggested by Gresty *et al* (1993). Large, elongated nuclei with dense nucleoli are found around the lumen

of the spine and beneath the sheath cuticle's epithelium. These cells are considered to be involved in protein production due to the abundance of ribosomes and the presence of large nucleoli close to the nuclear membrane. The central duct is believed to be connected to the spine glands in the body, and TEM sections through the base of thepre-oral spine, indicate the presence of glandular tissue containing abundant secretory vesicles and rough endoplasmic reticulum (Gresty *et al.*, 1993).

The pre-oral spine was considered by some earlier researchers to act like the mosquito proboscis, being used to pierce the skin and suck blood or lymph (Van Duijin, 1956 and Kabata, 1970). This derived from a hypothesis that it was connected to the oesophagus (Ivanfi 1926; Stammer 1959), however, more recent studies have demonstrated that the pre-oral spine is not directly connected to the oesophagus (Martin 1932; Cressey 1983; Madsen 1964; Gresty *et al.*, 1993). It has therefore been suggested that the pre-oral spine is used by the parasite to inject glandular secretion(s) through the central duct, with these being released via a terminal pore into the host's skin. In this respect, Gresty *et al* (1993) have suggested that *Argulus* uses the pre-oral spine to inject lytic secretion(s) or vasodilatory or anticoagulant components from the associated glands, as their study confirmed that pre-oral spine was not connected to the oesophagus but to these gland cells. Bauer (1959) suggested that the main function of the pre-oral spine secretion was to paralyse or kill the fish, while others considered that the pre-oral spine was used to inject toxins into the host to break down blood cells (Bower-Shore 1940). Cressey (1983) stated that a wound is produced by the pre-oral spine due to the injection of digestive secretions into the host surface. It was suggested that the mouth-tube then serves for ingestion of the blood cells and fluids so-formed. From experiments done by Shimura & Inoue

(1984) on rainbow trout, where they injected fish muscle with extracts from *A. coregoni* mouth-tube, pre-oral spine and associated glands, a haemorrhagic response was found to be caused by the extract, with no observed haemolytic or cytotoxic effects. These authors suggested that such haemorrhagic responses would aid in blood-feeding by the parasite.

Although the pre-oral spine has been suggested by previous researchers to deliver products which may help to break down the epithelial cells of the host to create a localised inflammatory response or encourage haemorrhage, its exact function remains entirely unclear. Likewise, the function of the labial spines is so far unknown (Walker *et al*., 2011).

1.4.1.4 **Glands associated with the feeding appendages**

Saha *et al*. (2011) found that two types of glands, the spinal gland and the proboscis glands, were associated with the feeding apparatus in *Argulus siamensis*. The spinal gland was observed to be situated at the base of the pre-oral spine and comprised four polygonal cells with no connection to the buccal cavity. The proboscis glands were located parallel to the spinal sheath, one on each side, and opened directly into the mouth tube. Each gland was reported to comprise two giant cells. All the gland cells were found to contain numerous secretory vesicles. Saha *et al*. (2011) believed that the secretion of the spinal gland did not play any role in cytotoxic or haemorrhagic responses but might function as anaesthetic or vasodilator while the secretion of the proboscis glands could have anticoagulant properties. Some differences exist with respect to the reported location and terminology for the feeding-associated glands in

different *Argulus* spp. studied *e.g. A. japonicus* and *A. siamensis* (Swanepoel & Avenant-Oldewage 1992; Gresty *et al*. 1993; Saha *et al*. 2011). A further gland, termed the ‘dorsal carapace gland’ was reported in *A. japonicus* by Swanepoel & Avenant-Oldewage (1992). The secretion(s) of this gland were suggested to pass into different ducts feeding to the gut and buccal cavity.

1.5 **Effect of parasites on fish behaviour / infection**

Poulin and FitzGerald (1989) found that stickleback infected with *Argulus canadensis* attracted more parasites than uninfected individuals, with no difference in the infection rate between males and females. Bandilla *et al*. (2005), pre-exposed fish to *Argulus* infection and demonstrated that this did not lead to an acquired resistance, with no indication of susceptibility differences among the examined fish. However, Mikheev *et al*. (1998; 2000) observed *A. foliaceus* males were 1.6 times faster than females in terms of host attachment behaviour and Pasternak *et al*. (2000) observed that males switched between fish host more than females. Bandilla *et al*. (2007) recorded no differences in host searching with respect to female age group when examining juveniles, sub-adults and adults, while for males infectivity increased as the parasite got older. In another experiment, the same researchers found males to be more active swimmers than females under three different conditions: with mating partners absent, with mating partners on the same host and with mating partners on a different host. Again, the results showed that female host searching strategy did not change under these conditions.

1.5.1 **Impacts of parasitism**

Argulus species can cause direct mortality on fish farms, both directly through feeding and indirectly, by increasing susceptibility to secondary invaders *e.g.* viruses, bacteria and fungi (Benz *et al.*, 1995). It is suggested to be define vector pleaser for spring viraemia of carp virus (SVCV) (Ahne, 1985) and also acts as an intermediate host to nematodes of the family *Skrjabillanidae* (Molnar and Székely, 1998). Kabata (1970) also noted that severe damage to fish skin caused by heavy *Argulus* infestations might ends in death of hosts. Mortality of marine farmed salmonids has also been reported as a consequence of infection by *Argulus*, which indicate that *Argulus* spp. such as *A. arcassonensis* Cuenot (Yamaguti, 1963) and *A. vittatus* (Ra¢nesque-Schmaltz) (= *A. purpureus* Risso) (Schram *et al.*, 2005) can also impact marine aquaculture (Boxshall 2005).

Argulus is considered to be highly pathogenic as a consequence of a number of aspects of the feeding process: 1) feeding lesions may provide a site for secondary bacterial or fungal infection (Kabata 1970; Kabata 1985; Hoffman 1977); 2) haemorrhages caused by feeding itself or by secretions possibly delivered through the mouth tube or pre-oral spine (Shimura and Inoue 1984).

The secretions produced by *Argulus*, although not thus far confirmed or characterised, are considered by some authors to be responsible for the observation of a severe inflammatory response as a consequence of infection (Becker, 1941 (as cited by Kabata, 1970), Gresty *et al.*, 1993; Saha *et al.*, 2011; Boxshall 2005; Møller 2011; Walker *et al.*, 2011). Some previous researchers believed that withdrawal of host blood

and introduction of an anti-coagulant by *Argulus* spp. caused the weakening of the fish host which could lead to death if combined with the impacts of a secondary infection (Bandilla *et al*. 2005). Infection of salmonids by *A. coregoni*, for instance, has been suggested to have allowed or potentiated infection by *Aeromonas salmonicida,* causing furunculosis in *salmonids Oncorhynchus masou (Brevoort, 1856)* (Shimura *et al*., 1983).

Hoffman (1977) suggested that *Argulus,* in puncturing the skin, injects a cytolytic toxin through the pre-oral spine and believed that a single wound from *A. giordani* Brian, 1959, was sufficient to kill a larval eel. Feeding sites have been also reported to become ulcerated and haemorrhagic, providing a portal of entry for secondary pathogens. As noted above, *Argulus* has also been suggested to serve as a vector and has been proposed to transmit spring viremia, a viral disease of carp, with Ahne (1985) mentioning that the virus was transmitted between hosts as a result of the parasite's discontinuous feeding behaviour.

Bower-Shore (1940) noted damage to host pigment cells by *A. foliaceus* and the parasite can also do major damage to fins, tail and to the mucus layer of the host, which may facilitate secondary fungal infection (Bower-Shore, 1940). Bandilla *et al*. (2006) recorded the mortality of rainbow trout after exposure to both *A. coregoni* and *Flavobacterium columnare*, a bacterium often associated with "fin-rot". The loss of rainbow trout to *F. columnare* alone was 21% while 46% of fish died if infected with the combination of *A. coregoni* and *F. columnare*. In addition, the results showed that clinical symptoms were apparent earlier with the mixed infection than with *F. columnare only*. This suggests that *Argulus* spp. increase fish vulnerability to microbial infections. Other studies have shown that fish immune systems are suppressed after

they become infected by *Argulus* spp., this similarly making them more susceptible to secondary infection (Ruane *et al.*, 1999; Van der Salm *et al.*, 2000).

Differences in host gene expression profiles have been recorded with respect to larval and juvenile infective stages of *A. japonicus* (Forlenza *et al.*, 2008) infecting common carp *Cyprinus carpio* L. The results showed that the fish immune response to *A. japonicus* larval stages was limited to the site of infection for about 2 days, while after day 6, having reached the juvenile stage, the response was found to be distributed more widely across the skin. From the experimental observations, larval stage parasites started to move over the host skin from day 5 thus extending the area of impact more widely across the skin. Transcriptional up-regulation of chemokine CXCa was noted to be a good indicator of parasite induced skin damage (Forlenza *et al.*, 2008). Kar *et al.* (2013) studied the effect of *A. siamensis* infection on the immune system of rohu, *Labeo rohita* and recorded that once the parasite, even as early life stage metanauplii, attached to the host, it had the ability to regulate both innate and specific elements of the immune system. They also found that as the parasite developed, the impact of the infection established by *A. siamensis* was spread to multiple sites.

A range of abnormal behaviours have been recorded for argulosis-affected fish, such as discolouration, irritation, weakness and appetite loss. Hypothesised cytolytic toxins injected through the pre-oral spine and the consequences of feeding have been proposed as the main causes of these mortalities (LaMarre & Cochran 1992). From another study conducted to assess the effects of *A. funduli* on the chloride cells of the gills, it was concluded that *Argulus* lesions damage ion transport in affected epithelia,

reducing chloride-secretion rate as a consequence of the density of chloride cells approaching near zero Marshall e*t al.* (2008).

The mode of attachment and crawling have been suggested by Abowei and Ezekiel (2011) to lead to irritation and abrasions, and these can result in proliferation, desquamation and erosion respectively. Changing attachment site has the advantage of alleviating previous damage, however, with a heavy infestation this will not reduce overall impact. As a result, the infected fish loses appetite and growth (Abowei and Ezekiel 2011).

Nolan *et al*. (2000) conducted a study to examine the effects of feeding cortisol, a stress hormone, on the mucous cell population of the head skin of rainbow trout *Oncorhynchus mykiss*, examining the effects on the parasite-host interaction, including the abundance of mucous cells, during *A. foliaceus* infection. It was observed that cortisol-fed fish stimulated vesicle synthesis in the pavement and filament cells of the upper epidermal at 24 h post feeding which was suggested to explain the attachment of a lower number of parasites. *A. foliaceus* infection did not increase the plasma cortisol level or the total numbers of mucous cells at 48 h post-infection with the parasite but this may be due to the low number of *Argulus* used for the experiment, being only 6 parasites per fish.

1.6 **Control of *Argulus* spp. in aquaculture**

As noted earlier, *Argulus* spp. can have a major impact on finfish aquaculture, particularly in freshwater environments. As a consequence of their direct impacts and

since *Argulus* spp. have been confirmed by different researchers to be blood-feeders and therefore able to spread other diseases, Kabata (1985) suggested that aquaculture producers should attempt to control infections immediately.

A broad range of different methods have been used to eliminate *Argulus* spp. in order to stop their harmful effects on the fish. Control methods should, however, consider all the different parasite stages in the life cycle, not just attached stages. In the past, fish farmers have tended to use chemical baths to kill fish ectoparasites, this being one of the most common methods still employed to control *Argulus*, although it has not proven to be totally effective (Fenton *et al.*, 2006). In-feed drugs and use of egg-laying plates have been suggested to be the most common argulid control methods used in recent years (Fenton *et al.*, 2006), although it has been noted that no chemical drugs against *Argulus* spp. are currently licensed for use in the UK (Bark, 2000 as reported by Taylor 2004). Similarly, according to Steckler and Yanong (2012), no food drugs were approved by FDA in USA to eliminate *Argulus* spp. and this is due to the potential side effects of chemicals on the fish and surrounding environment. In addition, parasites may develop resistance to these chemicals after repetitive use of the same chemical (Hemaprasanth *et al.*, 2012).

1.6.1 **Chemicals**

Different methods have been suggested and have been used as bath or in-feed treatments to control, treat and prevent *Argulus* infection (Walker 2004; Møller 2011; Steckler & Yanong 2012). The selection of particular drugs / medicines depend on the life cycle of the parasite, and the treatment should ideally target all life stages, including

eggs, juveniles, and adults, both on the fish and in the environment (Steckler and Yanong, 2012), although this is seldom if ever possible.

1.6.1.1 **Salt water**

Although salt water can be used to control many freshwater ectoparasites, this method is not generally effective for *Argulus* spp. and neither is the use of formalin (Steckler & Yanong 2012). However, Marshall *et al.* (2008) mentioned that for *Argulus funduli* Krøyer, 1863 infections could be treated successfully by hypersaline exposure, with detachment and death of adult *A. funduli* observed after a hypersaline challenge (twice seawater ~70‰). Bath treatment of sturgeon fry infected with *A. foliaceus*, in 20g L^{-1} NaCl for 15 minutes, was also found to be effective (Vasilean *et al.*, 2012). As a result of the failure of more generic treatments, fish farmers have employed a range of other chemotherapeutants to assist control.

1.6.1.2 **Organophosphates**

Synthetic pesticides such as organophosphates can kill *Argulus* spp., but as Jones *et al.* (1992) mentioned, they may not be 100% efficacious, which means the parasite may infect the fish again. Organophosphates act on the nervous system of the parasite leading to death. This class of drug targets the enzyme acetylcholinesterase (AChE) by cleaving and thus inactivating the neurotransmitter acetylcholine at cholinergic synapses. This lead to acetylcholine accumulation and is followed by extreme stimulus of acetylcholine receptors at the postsynaptic membrane and consequent toxicity

(Haya *et al.*, 2005; Walsh *et al.*, 2007; Carmichael 2013). Tokşen (2006) observed good treatment of an Oscar, *Astronotus ocellatus* (Agassiz, 1831) infected by *A. foliaceus* following bath treatment with 1 ppm of DDVP (dichlorvos, 0,0-dimethyl-0-2,2-dichloro vinyl phosphate) administered to 250 L tanks for 1 hour at 26°C. Trichlorfon, one of the most effective organophosphate pesticides, was found to give good results if used on a weekly basis at 0.25-0.50 mg L^{-1} for one month. Benz *et al.* (1995) stated that trichlorphon in seawater was found to be effective in killing *Argulus melanostictus* Wilson, 1935 within 24 h but it has adverse effects on the environment and *Argulus* spp. may become resistant to this chemical after use in the long term (Benz *et al.*, 1995). Tavares-dias *et al.* (1999), tested the effect of triclorphon at 0.4 mg 500 L^{-1} water by immersing the infected fish twice within a two-day interval (Tavares-dias *et al.*, 1999). Pyrethroids, synthetic analogues of plant-derived pyrethrum compounds, also affect the parasite's nervous system by activating the Na+-ion channel and causing consequent synaptic hyperactivity and were found to be effective at 20-200 ppm (Piasecki & Avenant-Oldewage 2008; Møller 2011).

Although treatment with organophosphate pesticides have proved to be effective against *Argulus* infections (Walker 2004; Piasecki and Avenant-Oldewage 2008) these pesticides may, however, create short and long-term effects on fish, users and the environment, hence the use of these chemicals is limited (Costello *et al.*, 2001; Hakalahti-Sirén *et al.*, 2008; Møller 2011). Use of this class of chemicals in arthropods, including copepods such as *L. salmonis*, has a long history of drug resistance development, which quickly renders the drug inefficacious with continuous repeated treatments.

1.6.1.3 **Insect growth regulators**

Insect growth regulators (IGRs) inhibit chitin synthesis and thereby interfere with the parasite's moulting ("shedding") of the cuticle ("exoskeleton") during growth and development. Diflubenzuron and lufenuron (13 mg/L) are suggested to be effective against both adult and larval stages but are not recommended, as this treatment required prolonged immersion of the infected fish in these chemicals which results in adverse effects of these chemicals on the fish food and humans consuming treated fish (Steckler & Yanong 2012). Unlike sea lice (e.g. *L. salmonis*), these treatments may prove effective against adults as Argulus do not undergo a terminal moult but continue to moult as they grow. In sea lice, this class of chemicals, including diflubenzuron, teflubenzuron and more recently lufenuron, have been used as in-feed treatments, though under current plans the latter will only be administered to salmon in freshwater prior to seawater transfer. Mayer *et al.* (2013) showed that lufenuron, used once a week for 5 weeks to treat fish infected by *Argulus* spp. could affect the life cycle of *Argulus* resulting in the elimination of the parasite. Although lufenuron and other IGRs have been found to be effective, national regulatory bodies including those in UK (*e.g.* Scottish Environment Protection Agency, SEPA) are reluctant to sanction their use in the aquatic environment due to potential for impacts on non-target organisms and scope for eliciting sensitisation allergies in humans.

1.6.1.4 **Macrocyclic lactones**

Hakalahti *et al.* (2004) noted the effective use of emamectin benzoate (Slice, Schering-Plough Animal Health, Saffron Walden, UK), in killing argulids attached to rainbow trout, *O. mykiss*, as an in-feed medication at 50 µg kg^{-1} of emamectin benzoate daily for a period of 7 days providing protection over a subsequent period of 72 d. Emamectin benzoate is a macrocyclic lactone, a class of chemicals that is believed to act on invertebrate neurons by increasing the permeability of binding to glutamate-gated chloride channels of invertebrate nerves, thus causing paralysis and death of the parasite (Hakalahti *et al.*, 2004; Hanson and Hill 2011). Slice has a high efficacy in killing crustacean parasites such as sea lice in salmonids, freshwater copepod gill parasites such as *Salmincola edwardsii* (Olsson 1869) and argulids such as *A. coregoni*. Treatment of goldfish with emamectin benzoate 50µg Kg^{-1} of fish body weight daily for one week was found to be effective in controlling *Argulus* infestation (Hanson and Hill 2011). Although, this medication was found to be effective in controlling *Argulus*, more studies are needed to investigate the long-term effect on the fish and environment and with frequent use, parasites may become resistant to it, as has been the case for sea lice (Carmichael, 2013). Recently, the use of ivermectin and doramectin against *Argulus siamensis*-infected carp in India was studied by Hemaprasanth *et al.* (2012) in order to check the efficacy of both drugs in freshwater fish. Ivermectin is used to treat a wide range of parasites, including both internal and ectoparasites and has been found, by different researchers, to have high efficacy in sea lice and some other parasitic copepods (Hemaprasanth *et al.*, 2012). Doramectin has also been used to control roundworms and external parasites of terrestrial

animals. By applying these two drugs in freshwater aquatic systems, Hemaprasanth *et al*. (2012) found that both of these drugs were effective in killing the parasites either as oral dose or through intramuscular administration. Ivermectin was observed to be more toxic, even at low concentrations of a single dose of 150 µg kg-1 body weight, and could cause fish mortality, while doramectin was found to be safer but was only effective at higher concentrations.

1.6.2 **Natural products**

Recently, a number of researchers have focused on the use of natural, largely plant-derived, products in order to develop control methods for *Argulus* that have lower environmental impacts. Different plant products have been used by different researchers to target *Argulus bengalensis* Ramakrishna (1951), Rotenone, azadirachtin, piperine and tobacco leaf extracts; nicotine, are examples of plant products tested. Nicotine in tobacco leaf dust (TLD) at a concentration of 8ppt, was found to be effective in killing 100% of adult *A. bengalensis* within 48 h. This resulted from nicotine being absorbed across the parasite body surface, preventing nerve ganglia from functioning normally and thus causing death of the parasite. Conversely, nicotine molecules cannot penetrate the egg's gelatinous coat, which means that treatment with nicotine should be applied following egg hatch (Banerjee and Saha 2013). Kumar *et al.* (2012) found 15 mg L^{-1} of azadirachtin could be used to remove *Argulus* spp. from infected fish. More research urgently needs to be done in this area.

1.6.3 **Physical**

A range of physical control approaches have also been employed to help manage *Argulus* infections. Eggs may be removed by physically cleaning, disinfecting and drying aquaria / tanks to make sure all the eggs are dead (Steckler & Yanong 2012), however, such activities are more difficult in larger farms or open fisheries. Mikheev *et al*. (2001) and Hakalahti *et al*. (2004) also postulated that eggs submerged in sediments might form an “egg bank” where eggs could survive for several years and hence infection outbreaks could reoccur despite efforts to control them. In larger water bodies, synthetic spawning substrates, where gravid females can lay their eggs, have been employed in various places for control, with substrates being removed and dried to kill eggs following egg laying (Hoffman 1977; Gault *et al*., 2002). Hoffman (1977) indicated that desiccation can kill eggs, larvae and adults within 24 hours. Gault *et al*. (2002) recorded success after applying floating egg-laying boards in a 12.9 ha rainbow trout *Oncorhynchus mykiss* fishery, which was highly infested by *A. foliaceus*. Within one year the egg laying activity had decreased by 145-fold, from 228000 to 1566 eggs harvested, *Argulus* spp. infection prevalence was reduced 9-fold and intensity of infection 6-fold. This method has also been recommended for elimination of *A. coregoni* from trout farms, by other researchers including Hakalahtiet *et al*. (2004); Harrison *et al*. 2006. Good evidence, involving suitable controls and durations for assessing effectiveness, are still latgely lacking, and this approach therefore needs further research to prove its effectiveness at a wider scale (Taylor *et al*., 2005; Walker 2008).

Desiccation or freezing of substrates *e.g*. by air exposure of emptied ponds, is another approach to the elimination of infection that operates through eradication of *Argulus* eggs and overwintering adults (Stammer 1959; Kabata 1970; Northcott *et al*. 1997) and liming of empty ponds has also been employed to control infections from season to season (Hoffman 1977; Gault *et al*., 2002).

It has also been suggested that introduction of different fish species to farms infected with *Argulus* spp. might also serve as a biological control method, presumably acting through visual predation of free-swimming individuals. However, this method has the potential to have an adverse effect on the introduced fish *i.e*. parasites may infect the fish before being eaten (as reported by Taylor 2004) and may hence provide an additional reservoir of infection and even lead to exacerbation of the original problem.

The use of only physical removal and prevention methods has been suggested to be insufficient to fully prevent infections as 'bet-hedging' strategies by the parasite can make them refractory to such strategies (Møller, 2011). Thus, Møller (2011) suggested the use of both chemical and physical approaches to increase the efficiency of control and management strategies.

1.7 **Management approaches**

The most important factors which result from bad management and can exacerbate infections in aquaculture systems have been suggested to be high stocking density, poor husbandry, occurrence of host vectors and sources of increased host stress (Kumar *et al*., 2012). Del-Claro & Takemoto (2003) believed that high temperature,

reduced water levels and poor water quality were key in stimulating higher infection by branchiuran ectoparasites. Hoffman (1977) considered that high fish density, high temperatures, low dissolved oxygen and standing water were the main factors helping to precipitate epizootic outbreaks. Bad fish stocking practices and high water temperatures, combined with high organic matter, have similarly been suggested to increase levels of *A. foliaceus* (Mahdy *et al.*, 2013). Knowing the principal factors involved, may help fishery managers to reduce or prevent *Argulus* infections.

Considering the worldwide distribution of *Argulus* spp. and the high economic losses caused by *Argulus* outbreaks, stringent efforts need to be made in order to improve management and control. Improving the management of aquaculture fisheries can provide measurable reductions in the impacts of this parasite (Møller, 2011). Good biosecurity is a key component of management that needs to be applied when introducing any new fish to a fishery, which involves establishment of the source of the fish and the routine quarantining of new fish (Steckler & Yanong 2012). Fenton *et al.* (2006) stated that controlling *Argulus* spp. eggs and juvenile stages will have a high impact in suppressing the argulid population, thus stopping infections. Walker *et al.* (2011) found A. *foliaceus* and *A. coregoni* adults to be capable of surviving for up to 14 days off the host, which means that transportation of water without disinfection before disposal may lead to introduction of *Argulus* species.

According to Steckler & Yanong (2012) the successful management of *Argulus* epizootics should reduce biological and economic impacts. This requires accurate parasite identification and treatment of the infected fish and environment with drugs showing the least environmental impact or through application of other best practices. As recorded by Taylor (2004), the literature suggests a number of prevention practices

which may reduce spread of *Argulus* spp. and help to cure the affected area. These suggestions include: sterilisation and drying of all fishing equipment before and after angling, prevention of live bait use and ensuring that fish, weed or boats are not transferred from infected areas, quarantining of fish prior to stocking and filtering of incoming water.

Because the use of chemicals on fish farms generates considerable public concern due of the possibility of tissue residues in food fish and potential for environmental impacts, actions should be taken before disease problems surface. Therefore, working staff in the field (fish farmers) should be trained in fish husbandry, hygiene and disease recognition and treatment so that they are able to institute good management practices to keep farms disease free (Costello *et al.*, 2001). Li *et al.* (2002) in China developed a Fish-Experts web-based program that can be employed to help educate farmers and assist them in diagnosing fish disease and such systems have the capacity to play an important role in controlling disease.

McPherson *et al.* (2012) listed three different control approaches that were used in an attempt to eliminate *Argulus* from infected UK trout fisheries. The first approach was deployed before stocking, with fish receiving emamectin benzoate (Slice®, Schering-Plough Animal Health) as a prophylactic. For ponds already containing the parasite, artificial substrates can be used to collect and remove parasite eggs. The third approach is only applied for very heavy infections. This involves draining and drying the pond and adding lime (CaO) to kill all the eggs. Once there are no eggs or parasites, the fishery is refilled and stocked with uninfected fish (McPherson *et al.* 2012).

Taylor *et al*. (2006) postulated that stocking management in trout fisheries could play a key role in controlling *A. foliaceus* infection. Consequently, McPherson *et al*. (2012) supported this by applying mathematical models to real world data that suggested that increasing the capture rate led to an increase in parasite removal (McPherson *et al.*, 2012).

Study objectives

The work described in this thesis was undertaken with the aim of identifying, describing and characterising the secretory components that have previously been suggested to be secreted from glandular cells associated with the feeding appendages of *Argulus foliaceus*.

The key hypothesis tested is that argulids produce secretions with active components that assist feeding by directly pre-processing host tissue or by immuno-modulating or otherwise impacting key elements of host biology / physiology.

No previous study has thus far confirmed the presence of immunomodulatory or other active products in the glandular secretions of argulids, which mirror those reported for other parasitic Crustacea, particularly *L. salmonis*. At the inception of this work it was proposed that identification and characterisation of these components might improve understanding of the host-parasite relationship for argulids and assist in the development of novel control methods.

The study can be divided into a number of defined research foci as follows:

I. An initial description of key glands present in *A. foliaceus* and their relationships to the principal appendages employed during feeding. This investigation

includes elucidation of the morphology, localisation, and types of tissues associated with the pre-oral spine and mouth tube. The principal techniques involved in this study were light and fluorescence microscopy and transmission and scanning electron microscopy.

II. The application of transcriptomic approaches to *A. foliaceus* and *A. coregoni* to create databases of expressed transcripts. This approach can provide important tools for understanding parasite biology and creates the necessary resources to look for potential protein targets that might relate to host-parasite interactions and feeding-associated activities. This approach employs high-throughput sequencing and the use of a range of bioinformatics tools and methodologies.

III. Earlier provision of transcriptomic resources allows for the subsequent identification of secretory excretory products (SEPs) using proteomics analysis tools including mass spectrometry and specific antibody / lectin labelling.

IV. Localisation of expression of transcripts / genes considered to play roles in host immuno-modulation by other ecdysozoan parasites, such as *L. salmonis*, can be achieved following successful outcomes of transcriptomic / proteomic studies. Such localization allows the glands related to SEP production to be identified using *in situ* labelling methods including *in situ* expression hybridisation, immunohistochemistry and fluorescent lectin labelling.

Chapter 2. Morphological characterisation of glands associated with feeding in *Argulus foliaceus*

2.1 Introduction

Previous studies on *Argulus* spp have focused on the anatomical and morphological aspects of this parasite. Only a few have examined the glands associated with the pre-oral spine “stylet” and mouth tube, with even fewer having characterised the composition of the secretions (Shimura, 1983; Shimura & Inoue, 1984; Swanepoel & Avenant-Oldewage, 1992; Gresty *et al.,* 1993; Saha *et al.*, 2011). Detailed study of the classes and biological properties of secretory products produced and released by the pre-oral spine and proboscis glands are notably lacking in the literature.

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 (Bandilla *et al.*, 2006; Walker, 2008).

Although a few published studies have provided information focused on glands related to the pre-oral spine and mouth tube (Shimura 1983; Shimura & Inoue, 1984; Swanepoel & Avenant-Oldewage 1992; Gresty *et al.*, 1993; Saha *et al.*, 2011), the available information is currently limited and no conclusive data are available regarding the host-parasite interactions involving these glands.

While previous studies have focused on *A. coregoni* Thorell, 1865 and *A. japonicus* Thiele, 1900, the current study was focused on *A. foliaceus* (Linnaeus, 1758), which is one of the most prevalent and problematic *Argulus* species in the UK.

Immunomodulation by aquatic arthropods

To date, the presence of immunomodulatory products, previously reported for other parasitic Crustacea, such as the presence of prostaglandin, trypsin or peroxidases in *Lepeophtheirus salmonis* (Krøyer, 1837) has not been confirmed in *A. foliaceus* glandular secretions (Buchmann and Woo, 2012). Fundamental biological aspects of *A. foliaceus* feeding need to be investigated before applying more advanced techniques to determine the classes of substances secreted by the glands of *Argulus* spp. These processes include elucidation of the morphology, localisation, and types of tissues associated with the pre-oral pre-oral spine and mouth tube, and the relationships between the spinal and proboscis glands. This chapter deals with the description of these glands and their relationships to the principal appendages employed during feeding.

Although 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 (Walker *et al*., 2011). Claus (1875), Wilson (1902), Martin (1932) and Madsen (1964) have all hypothesised that the pre-oral spine injects poisonous substances into the host. Leydig (1886) suggested that it worked as a tactile organ while Ivanf (1926) supposed that the pre-oral spine facilitated sucking during feeding (noted in Swanepoel and Avenant-Oldewage, 1992).

The most detailed description of the *A. japonicus* feeding apparatus was conducted by Gresty *et al*. (1993). Prior to that, a histological study on the morphology of the pre-

oral spine and associated structures of *A. japonicus* was described by Swanepoel and Avenant-Oldewage (1992). The attempts of Swanepoel and Avenant-Oldewage (1992) to trace the ducts of the glands lead to them to describe the "posterolateral" glands as the spinal gland which is contrary to the findings of Saha *et al.* (2011). Saha *et al.* (2011) confirmed the position of the spinal gland and the proboscis gland in *A. siamensis;* Wilson C.B. 1926, but the relationships of the posterolateral glands to the feeding apparatus were not studied in this *Argulus* species. More recently, von Reumont *et al.* (2014) 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.

As described in chapter one, the pre-oral spine consists of a tapering spine enclosed by the spinal sheath and located in the midline of the louse anterior to the mouth tube. The description of the pre-oral spine and related tissues by Gresty *et al.* (1993) suggested that the spine duct originated from secretory glandular tissue, which meant that the pre-oral spine functioned to secrete substances, playing a possible role in host immuno-modulation during feeding. The position of the spinal gland, however, was not determined in this account, nor an answer to the question of whether the glandular tissue at the basal rim of the spinal sheath belonged to the proboscis gland as defined by Gresty *et al.* (1993). Gresty and co-workers named the four glandular cells at the base of the pre-oral spine as the proboscis gland (Gresty *et al.*, 1993). In addition to that description, no more details are specified about the other types of glands associated in the feeding process or the secretory products of those proboscis glands that may be involved during feeding (von Reumont *et al.,* 2014).

The proposed gland at the base of the pre-oral spine and the mouth tube, comprising four large cells, is described later by Saha *et al.* (2011) as the spinal gland following the hypotheses of Wilson (1902), Martin (1932) and Madsen (1964). One key limitation to the histological analysis conducted by Saha *et al.* (2013) on the spinal gland of *A. siamensis* was the exclusive use of longitudinal sections.

The principal aims of the study described in this chapter 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. Lectin labelling were applied to characterise the glands of *A. foliaceus*. Lectins which are used as biochemical tools in different types of research are proteins / glycoproteins recognise and bind to specific sugar moieties or certain glycosidic linkages of polysaccharides, glycoproteins and glycolipids (Sharon and Lis, 1989).

The knowledge generated from these studies provides essential underpinning for the biomolecular, proteomic and transcriptomic studies of *A. foliaceus* feeding mechanisms described in later chapters.

2.2 Materials and Methods

2.2.1 Sample collection

Initial investigations made use of archived specimens of *A. foliaceus* which were collected from trout (*Onchorhynchus mykiss* (Walbaum, 1792)) by Dr. Nicholas Taylor (CEFAS, UK) during a survey of an ecological, cross-sectional study of the UK still water trout fisheries in 2001 (Taylor, 2004). The samples were fixed in 70% ethanol and were still in an appropriate condition to be used for light microscopy. Fresh samples of *A. foliaceus* were received from two sources - from Loch Fad, an extensive stocked fishery, which is located on the Isle of Bute, and from the Environmental Agency (EA) in Brampton, where samples were collected from a number of fisheries affected by *Argulus*. The samples were transported either by overnight courier or by personal delivery. These samples were recovered from pike (*Esox lucius* L.), rainbow trout (*O. mykiss*) and common carp (*Cyprinus carpio* L.) according to the personnel collecting the samples.

2.2.2 Light Microscopy

2.2.2.1 Fixation and paraffin wax embedding

Parasites were fixed in 10% (v/v) neutral buffered formalin (NBF) for 48 hours prior to processing for paraffin wax (SAKURA-TEKII wax, polymer added) embedding and

routine histology. Both the fresh samples, which were fixed in 10% NBF, and the archived specimens fixed in 70% ethanol, underwent the same processing method except for the fact that 70% ethanol fixed samples were directly changed through the first stage of alcohol dehydration. Fixed tissue was processed using a Shandon Citadel 2000 tissue processor (Thermo Fisher Scientific Inc., UK) for 16 hours (overnight Tissue was processed using a standardised laboratory protocol (**Appendix 1**) including dehydration thorough a graduated alcohol series, clearing with xylene and infiltration with paraffin wax (SAKURA-TEKII wax, polymer added). Specimens were embedded using an embedding machine (*Leica,* HistoEmbedder). Blocks were then 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 hour prior to staining.

2.2.2.2 **Haematoxylin and eosin staining of paraffin sections**

Haematoxylin and eosin (H&E), is one of the most common stains employed in histology, and was used to stain 3µm thick paraffin wax sections using a standard laboratory protocol from the Institute of Aquaculture histopathology laboratory. Briefly, sections were deparaffinised with xylene (BDH, U.K.), brought to water, and stained in Mayer's haematoxylin and eosin, dehydrated in an ethanol series, cleared in xylene and coverslipped with Pertex (Cellpath; see **Appendix 2** for details).

2.2.2.3 **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 minutes followed by a running tap water wash and a distilled wash respectively. Then, they were stained in 1% (Aq) periodic acid for 5 minutes and washed in two changes of distilled water. Sections were then stained in Schiff's reagent for 15 minutes, washed with tap water and stained in Mayer's haematoxylin for 5 minutes. After a final wash in tap water, sections were dehydrated in alcohol and xylene and mounted with Pertex (**Appendix 3**).

2.2.2.4 **Periodic acid-Schiff with tartrazine counterstain and haematoxylin**

This stain is used for for detecting the prescence of carbohydrates in various tissues. Three micron thick longitudinal, sagittal and tangential serial sections of 10% BNF 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 by three changes of absolute alcohol, cleared in xylene then coverslipped with Pertex (**Appendix 4**).

2.2.2.5 **Technovit® 7100 glycol methacrylate resin embedding**

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 minutes. Then, the specimens were pre-infiltrated in 1:1 100% ethanol: base Technovit 7100 for 2 hours. The specimens were kept in the infiltration solution (1 g of hardener I in 100 mL base liquid and mixed for 10 minutes) 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 minute). Moulds containing specimens were each covered by a microtome block holder and left for 2 hours 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.

2.2.2.6 **Haematoxylin and eosin staining of glycol methacrylate sections**

Serial sections were stained in Mayer’s haematoxylin (Mayer, 1903) for 15 minutes. They were then washed in running tap water for 10 minutes. They were differentiated in 1% acid alcohol and washed in running tap water followed by a wash in distilled water. Two minutes’ wash in Scott’s tap water for blueing was followed by washing in 2 changes of distilled water. Sections were stained in eosin (one part of Putt’s eosin: eight parts of eosin Y stain 1%) for 5 minutes. Sections were then washed in running

tap water, and then dehydrated in 2 changes of absolute alcohol, cleared in xylene then mounted in Pertex.

2.2.3 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.

2.2.4 Scanning electron microscopy of *A. foliaceus*

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 minutes followed by fixation in 2.5% glutaradehyde (v/v) for 2 hours. Samples were then washed twice with 0.1M Sodium cacodylate buffer and post-fixed in 1% osmium tetroxide for 1 hour. 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 protocol was applied for these samples.

2.2.5 **Transmission electron microscopy of *A. foliaceus***

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 hour. 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 Ultra cut 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 uranyl acetate super saturated (Agar Scientific, UK) for 30 minutes and Reynold's lead citrate (Agar Scientific, UK) for 30 minutes. Sections were observed using a JEOL JEM 1230 TEM at 80 KV.

2.2.6 **Fluorescent lectin labelling of *A. foliaceus* glands**

Argulus foliaceus parasites were collected from Loch Fad; 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 1) hydration with 6 changes of absolute ethanol and two changes of isopropanol

2) clearing with two changes of xylene for 30 minutes in each change and 3) infiltration in two changes of paraffin wax (SAKURA-TEK II wax; polymer added) for 1 hour each. Then samples were 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: 1) section of spinal and proboscis glands and 2) deeper section through the specimen at the level of the nauplius eye incorporating other labial glands.

Lectin wash buffer (LWB; 50mM Tris, 150mM NaCl, 2mM $MgCl_2$, and 1mM $CaCl_2$ pH 7.4) was prepared prior to starting the labelling. Slides were taken from -20°C to 55°C oven and kept for approximately 30 minutes and were labelled using 19 different lectins (**Table 2.1**) obtained from fluorescein lectin kits I-III (Vector Laboratories, Burlingame, CA, U.S.A.). Lectins were diluted in lectin wash buffer (LWB) to 5µg mL^{-1}; the recommended concentration range for use is 5-20µg mL^{-1}. Sections were then dewaxed manually into two changes of xylene for 3 minutes each, followed by a dehydration step using 100% then 70% ethanol for 2 minutes in each step. After washing in distilled water for 1 minute, 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 hours. Then the sections were washed in LWB three times for 5 minutes. 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 hours 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.

Table 2.1 Lectin names, concentrations used and binding preferences and specificities for each lectin. Binding preferences of each lectin according to EY Labs and Vector Laboratories.

Fluorescein lectins and binding preferences	Scientific / Latin name	Conc (mg L^{-1})	Binding specificity
D-Mannose / D-Glucose / N-acetylglucosamine			
Con A	Concanavalin A	2	Man, Glc
PSA	*Pisum sativum agglutinin*	2	Man, Glc
LCA	*Lens culinaris agglutinin*	2	Man, Glc
Chitin-binding lectins			
N-acetylglucosamine / N-acetyllactosamine			
WGA	Wheat germ agglutinin	2	GlcNAc, NeuAc
sWGA	Succinylated-wheat germ agglutinin	2	(GlcNAc) n
STL	*Solanum tuberosum lectin*	1	(GlcNAc) 2–4
DSL	*Datura stramonium lectin*	1	GalNAc, GlcNAc
LEL	*Lycopersicon esculentum lectin*	1	(GlcNAc) 2–4
N-acetylgalactosamine			
DBA	*Dolichos biflorus agglutinin*	2	Gal, GalNAc
VVA	*Vicia villosa agglutinin*	1	Gal, GalNAc
Galactose / N-acetylgalactosamine			
JAC	*Artocarpus integrifolia*	1	Gal
PNA	Peanut agglutinin	2	Gal, GalNAc
SBA	Soybean agglutinin	2	Gal, GalNAc
GLS-I	*Griffonia simplicifolia lectin*	2	α- GlcNAc, α- Gal
N-acetylglucosamine			
GLS-II	*Griffonia simplicifolia lectin*	1	α, β-GlcNAc, Glycogen
N-acetyllactosamine / N-acetylgalactosamine			
ECL	*Erythrina cristagalli lectin*	1	Gal, GalNAc
Galactose / N-acetylglucosamine / Mannose			
PHA-E	*Phaseolus vulgaris Erythroagglutinin*	2	Oligosaccharide
PHA-L	*Phaseolus vulgaris Leucoagglutinin*	2	Oligosaccharide
Fucose			
UEA-I	*Ulex europaeus agglutinin-I*	2	α-L Fucose

Fuc, fucose; Gal, galactose; GalNAc, N-acetylgalactosamine; Glc, glucose; GlcNAc, N-acetylglucosamine; Man, mannose; NeuAc, N-acetylneuraminic acid.

2.3 Results

2.3.1 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 **(Fig. 2.1)**.

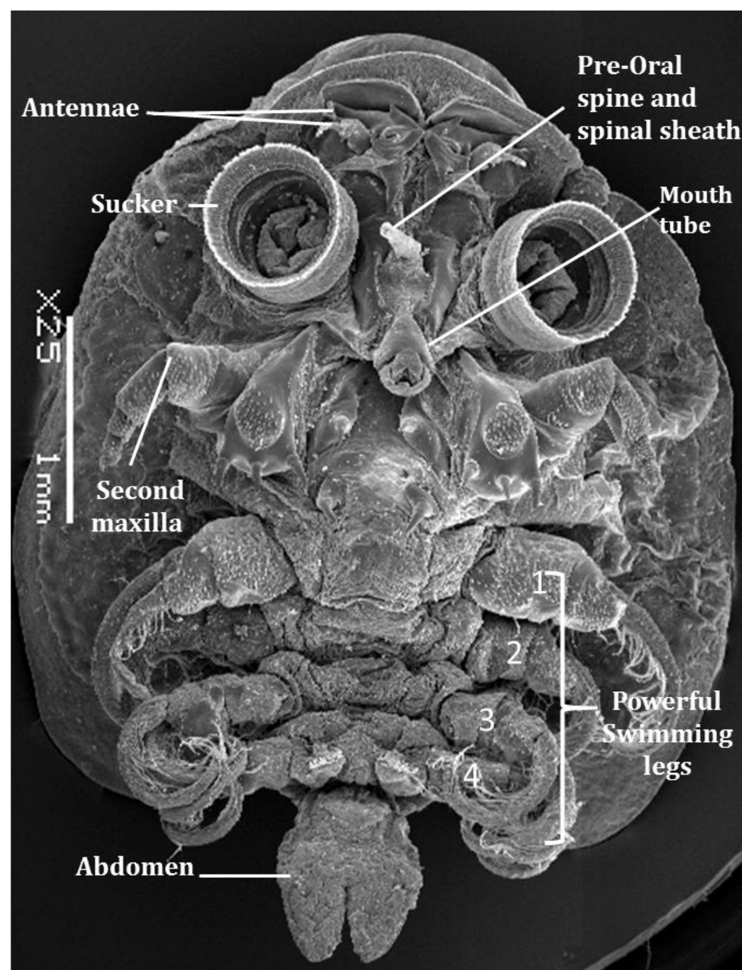


Figure 2.1. SEM micrograph of the ventral aspect of adult *A. foliaceus* showing the pre-oral spine (stylet) position relative to other appendages / tagmata.

The two key structures considered to be directly involved in feeding processes are the mouth tube and the pre-oral spine (**Figs. 2.1 and 2.2**).

The pre-oral spine is located anterior to the mouth tube. An eversible spinal sheath surrounds the spine. The distal part of the spine is narrow with a swollen tip (**Fig. 2.14**).

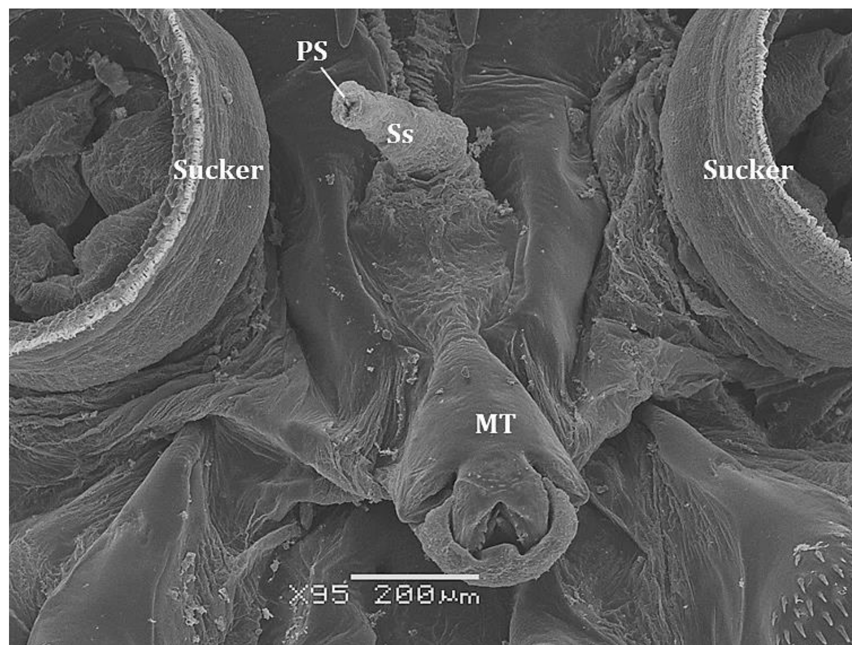


Figure 2.2. SEM micrograph of *A. foliaceus* pre-oral spine. Ventral view of the mouth tube (MT) and the pre-oral pre-oral spine (PS) protruding from the spinal sheath (Ss).

As is shown in **Fig. 2.3** 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.

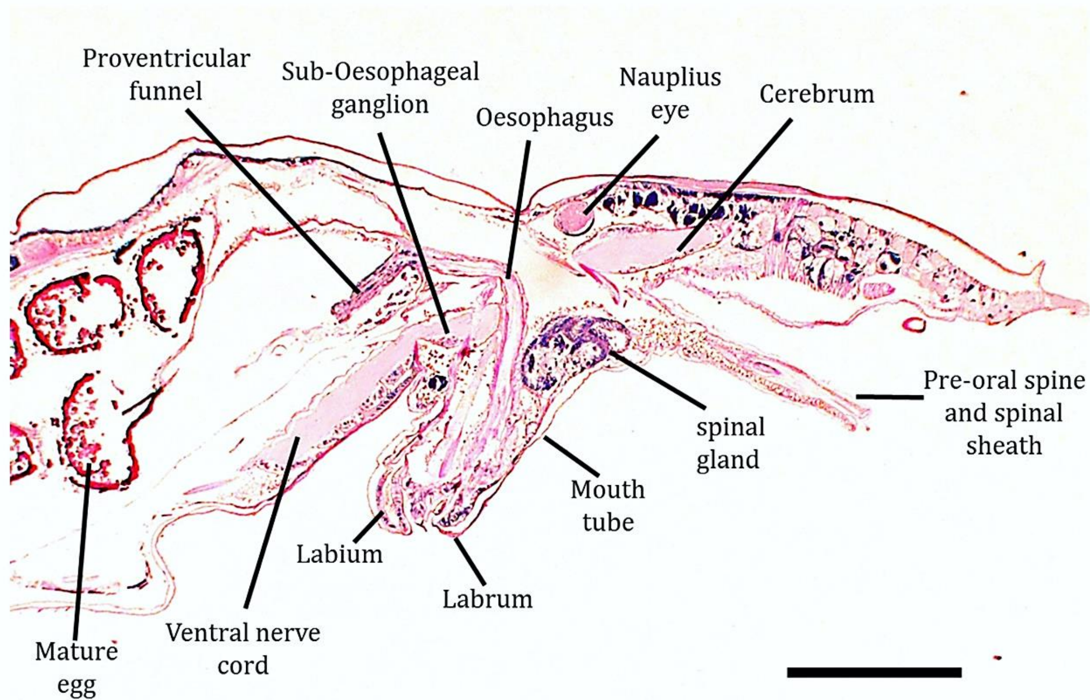


Figure 2.3. Light micrograph of sagittal section through the oral region of female *A. foliaceus* showing the position of the pre-oral spine (stylet) and spinal gland to the mouth tube. The spinal gland is located at the base of both the mouth tube and the spinal sheath. H&E. Scale bar = 500 µm.

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. 2.4, 2.5b-2.6a**) and a long discharge duct opens into the buccal cavity. Spinal and proboscis glands cells stained intensely with PAS (**Figs 2.4b, 2.5b**), suggesting the production of neutral carbohydrates.

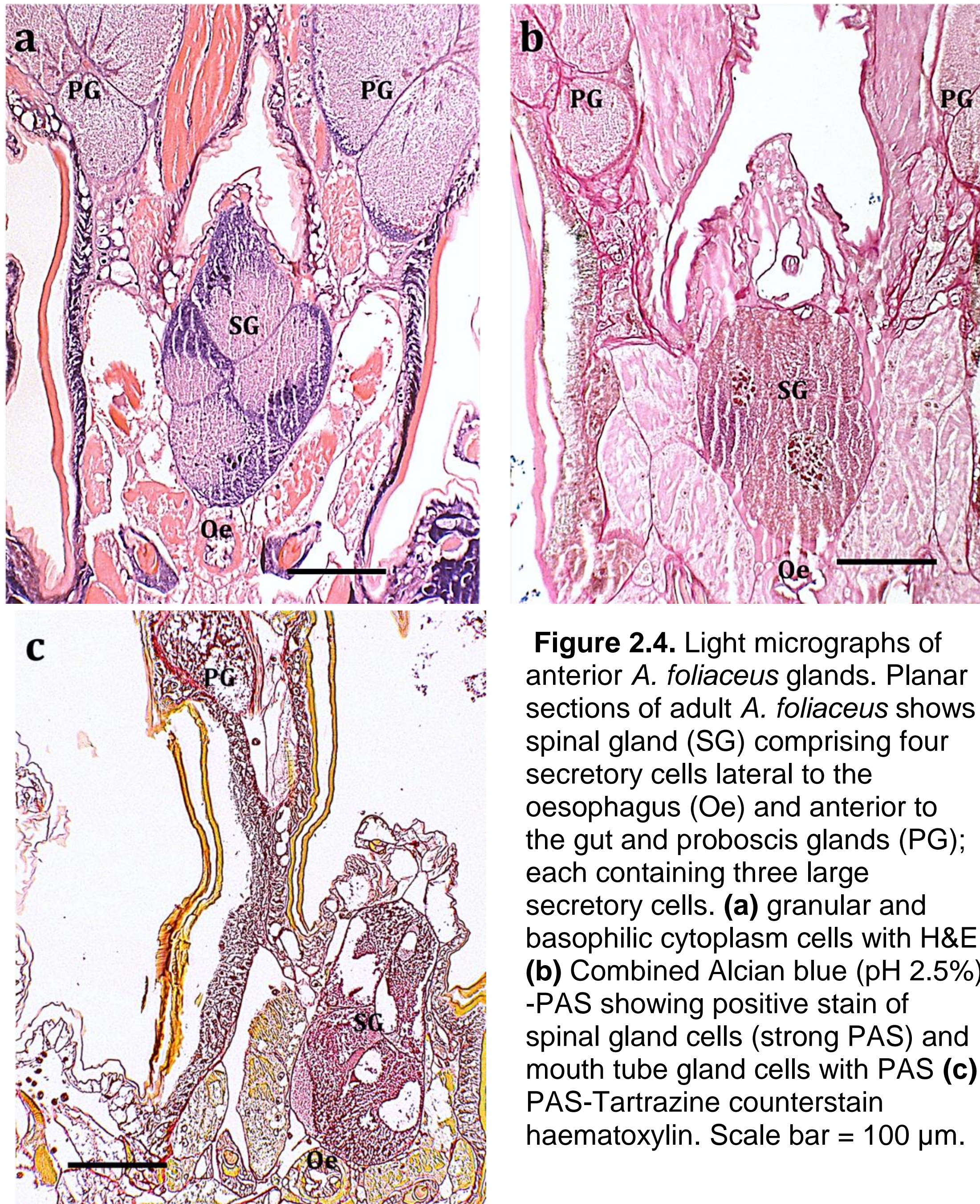


Figure 2.4. Light micrographs of anterior *A. foliaceus* glands. Planar sections of adult *A. foliaceus* shows spinal gland (SG) comprising four secretory cells lateral to the oesophagus (Oe) and anterior to the gut and proboscis glands (PG); each containing three large secretory cells. **(a)** granular and basophilic cytoplasm cells with H&E **(b)** Combined Alcian blue (pH 2.5%) -PAS showing positive stain of spinal gland cells (strong PAS) and mouth tube gland cells with PAS **(c)** PAS-Tartrazine counterstain haematoxylin. Scale bar = 100 μm.

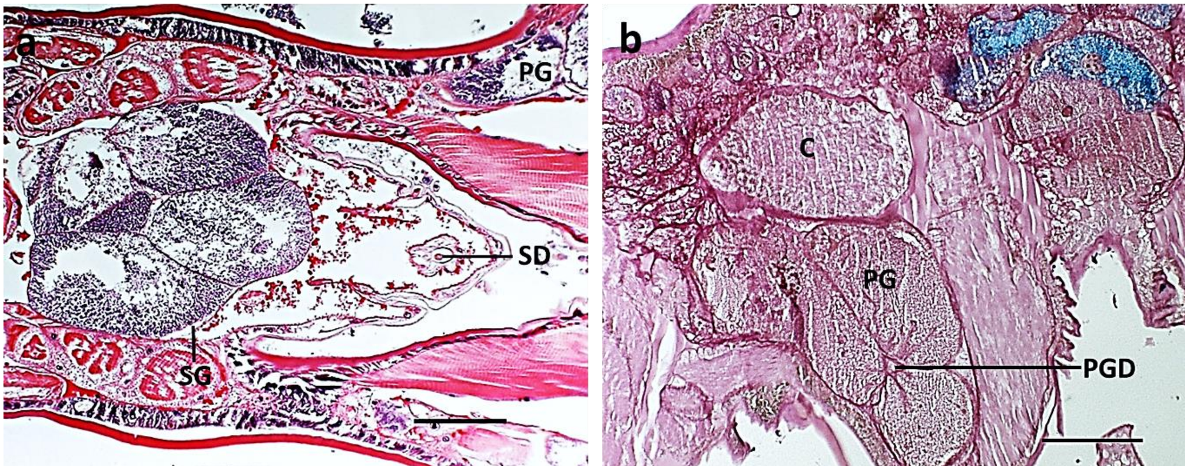


Figure 2.5. (a) Light micrograph of planar section of *A. foliaceus* spinal gland (SG) and pre-oral spine (stylet) duct (SD). PG-proboscis gland cell. H&E. Scale bar = 50 µm. **(b)** Light micrograph of section of one proboscis gland (PG) which comprises 3 large PAS-+ve secretory cells located ventral to the cerebrum, Combined Alcian blue (pH 2.5%)-PAS. Collecting proboscis gland duct (PGD) in the centre of the trio of gland cells. Note ramifying collecting ducts within cells. Combined Alcian blue-PAS. Scale bar = 100 µm.

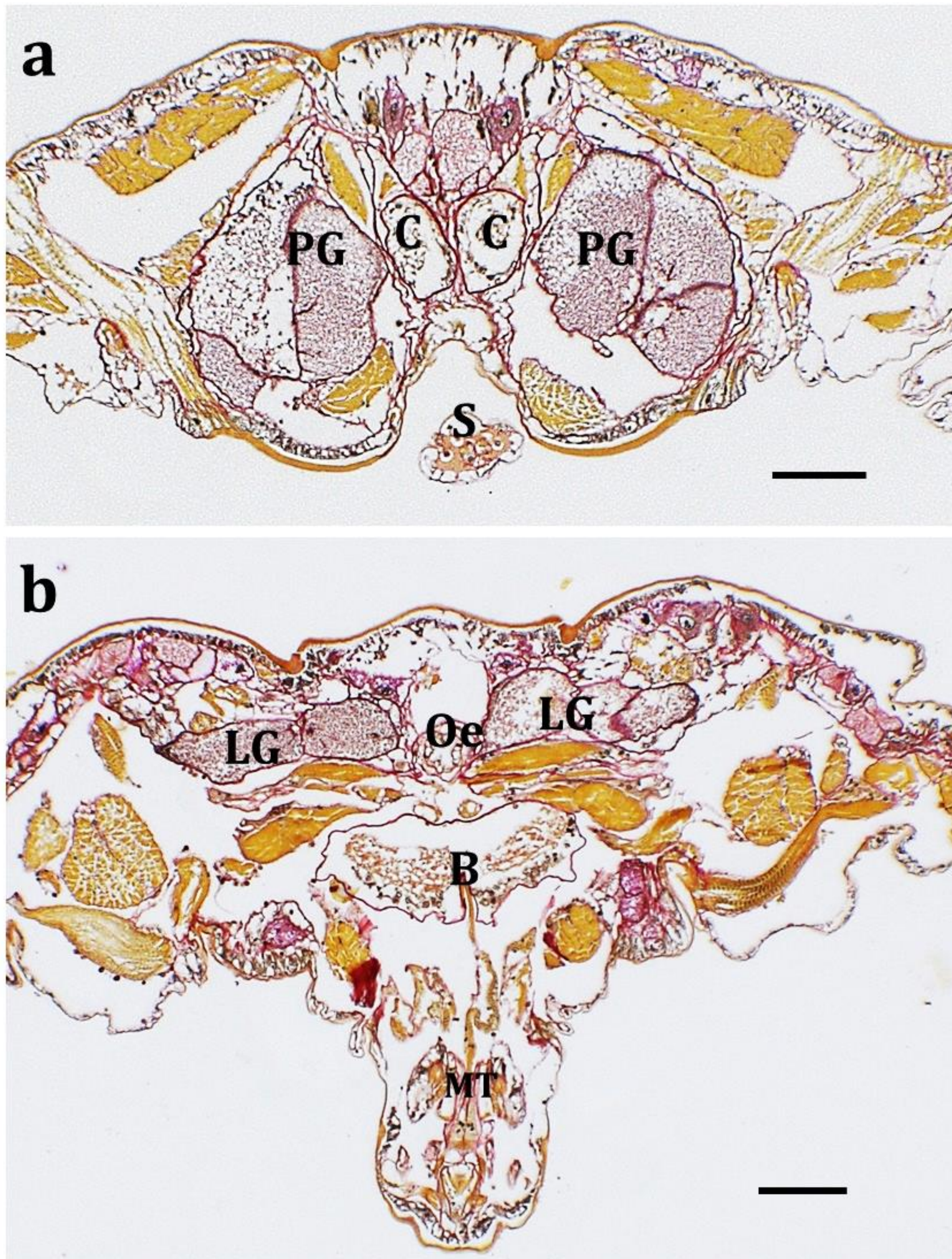


Figure 2.6. **(a)** Light micrograph of cross section through the head of *A. foliaceus* showing the position of proboscis glands PG. C- cerebrum; S- pre-oral spine. Scale bar = 50 μm **(b)** Light micrograph of cross section of *A. foliaceus* showing the pair of labial glands (LG) adjacent to the oesophagus and the mouth tube (MT), and brain (cerebrum) (B). PAS-Tartrazine counterstain haematoxylin. Scale bar = 100 μm.

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 encircled by the labium,

is smooth externally with 3 pairs of sensory papillae and sharp denticulate margins. The labium is bigger and is covered by tiny spinules and extends a pair of tubular labial spines with apical pores (**Figs. 2.7-2.8**).

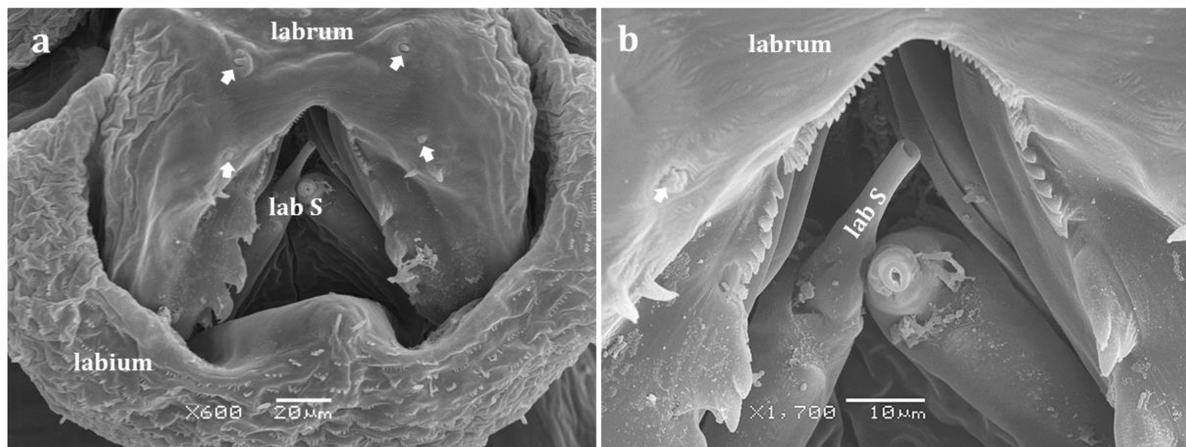


Figure 2.7. SEM micrograph showing ventral view of the proboscis (mouth tube) of *A. foliaceus* showing **(a)** labrum enclosed by labium and 2 pairs of sensory papillae indicated by arrows **(b)** enlarged view of the tubular labial spine (lab S) with the denticulate margins of the labrum.

A pair of labial tubes known as labial spines arise within the buccal (labial) aperture. Each contains an apical pore (**Fig. 2.7b**) 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 is termed in this study the “labial glands”. Serial cross-sections of the mouth tube show the ducts supplying these labial spines (**Figs. 2.8**).

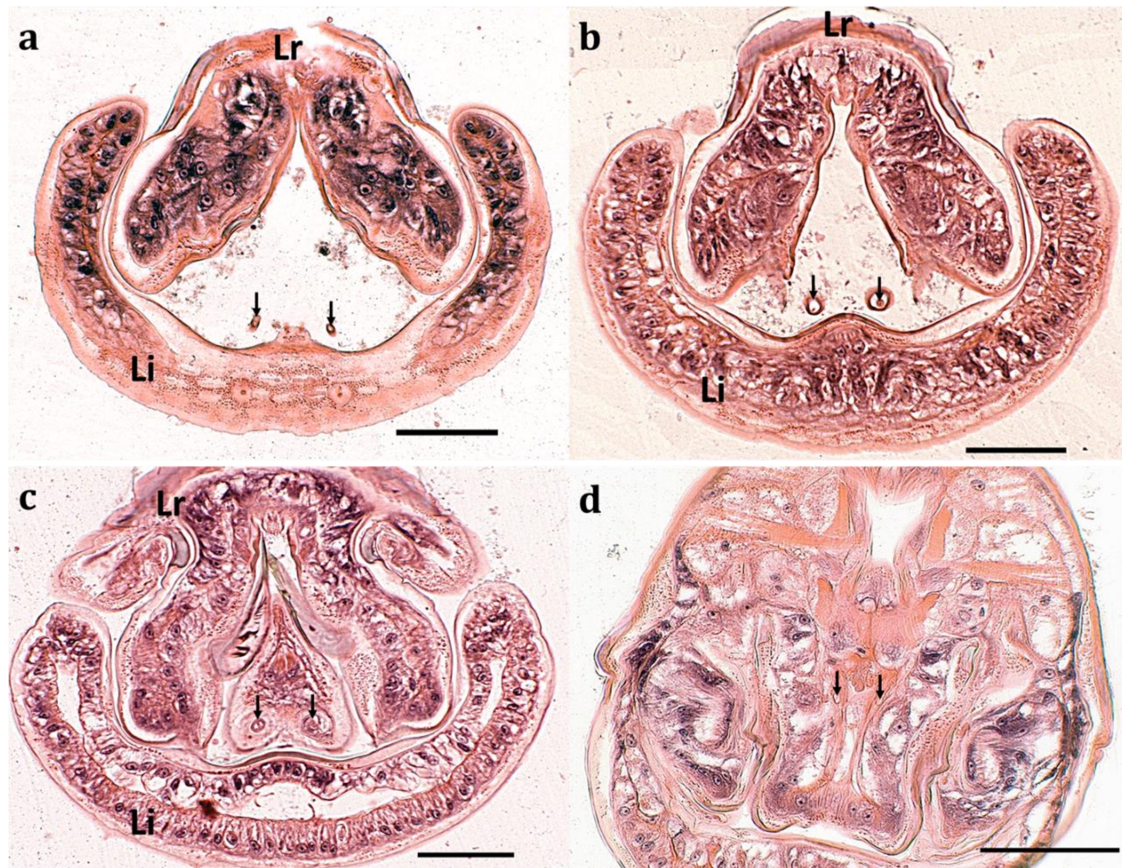


Figure 2.8. Light micrograph of transverse serial sections (a-d: representing apical end – basal side) through the mouth tube of *A. foliaceus* following the ducts of the tubular labial spine as highlighted by arrows. Li-labium and Lr- labrum. H&E; Technovit 7100. Scale bar = 50 µm.

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. 2.9-2.10**).

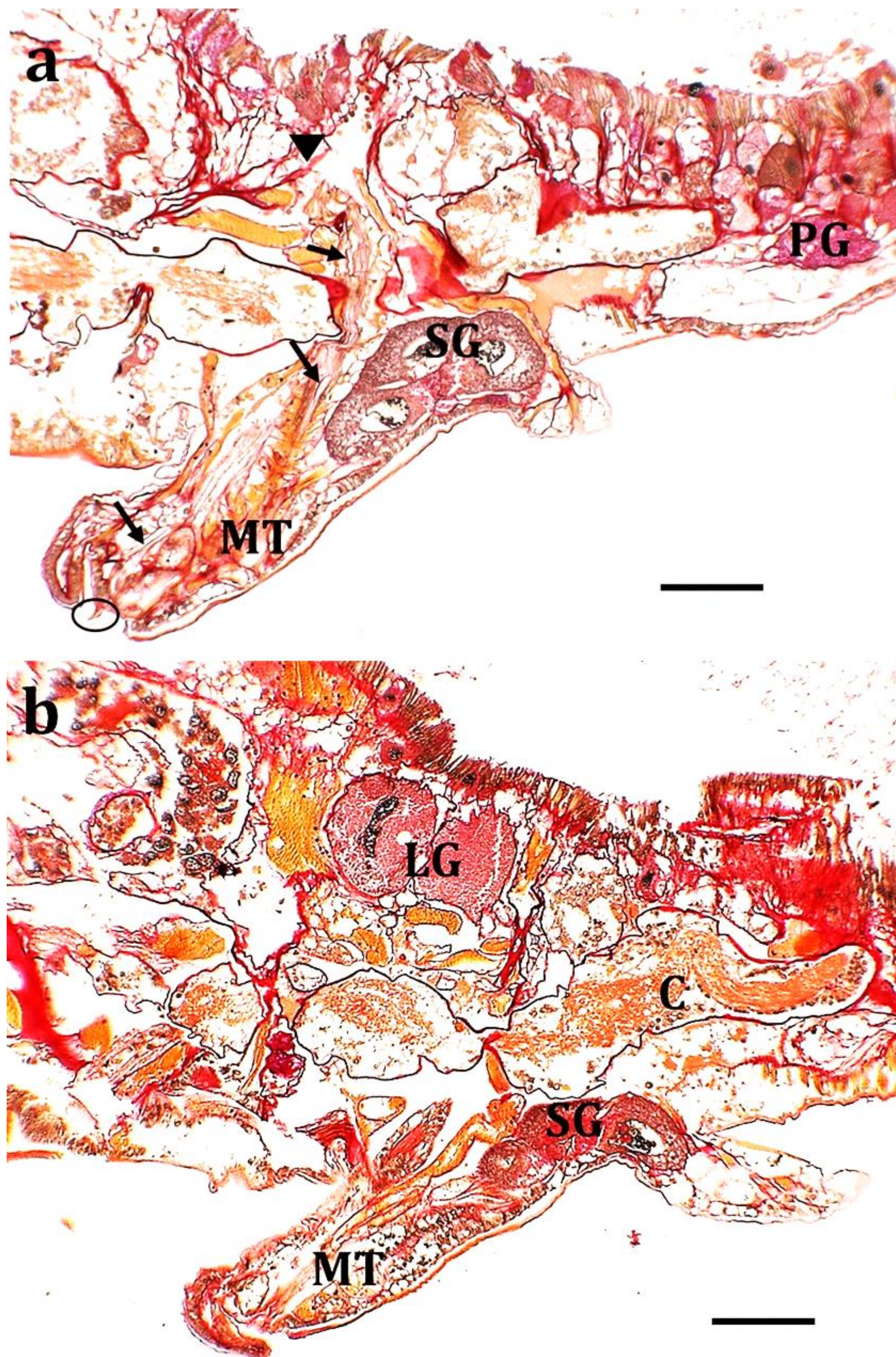


Figure 2.9. Light micrograph of sagittal sections through the oral region of *A. foliaceus* following labial gland ducts (arrowed) extending from the tubular labial spine (O) in the mouth tube **(a)** micrograph showing the origin of the labial gland duct in the vicinity of the labial gland (LG) indicated by an arrowhead and following the labial gland ducts to reach the tubular labial spine (▶) in the mouth tube (MT) **(b)** micrograph showing labial gland position. C = cerebrum; SG = spinal gland; PG = proboscis gland. PAS-tartrazine counterstain haematoxylin. Scale bar = 100 µm.

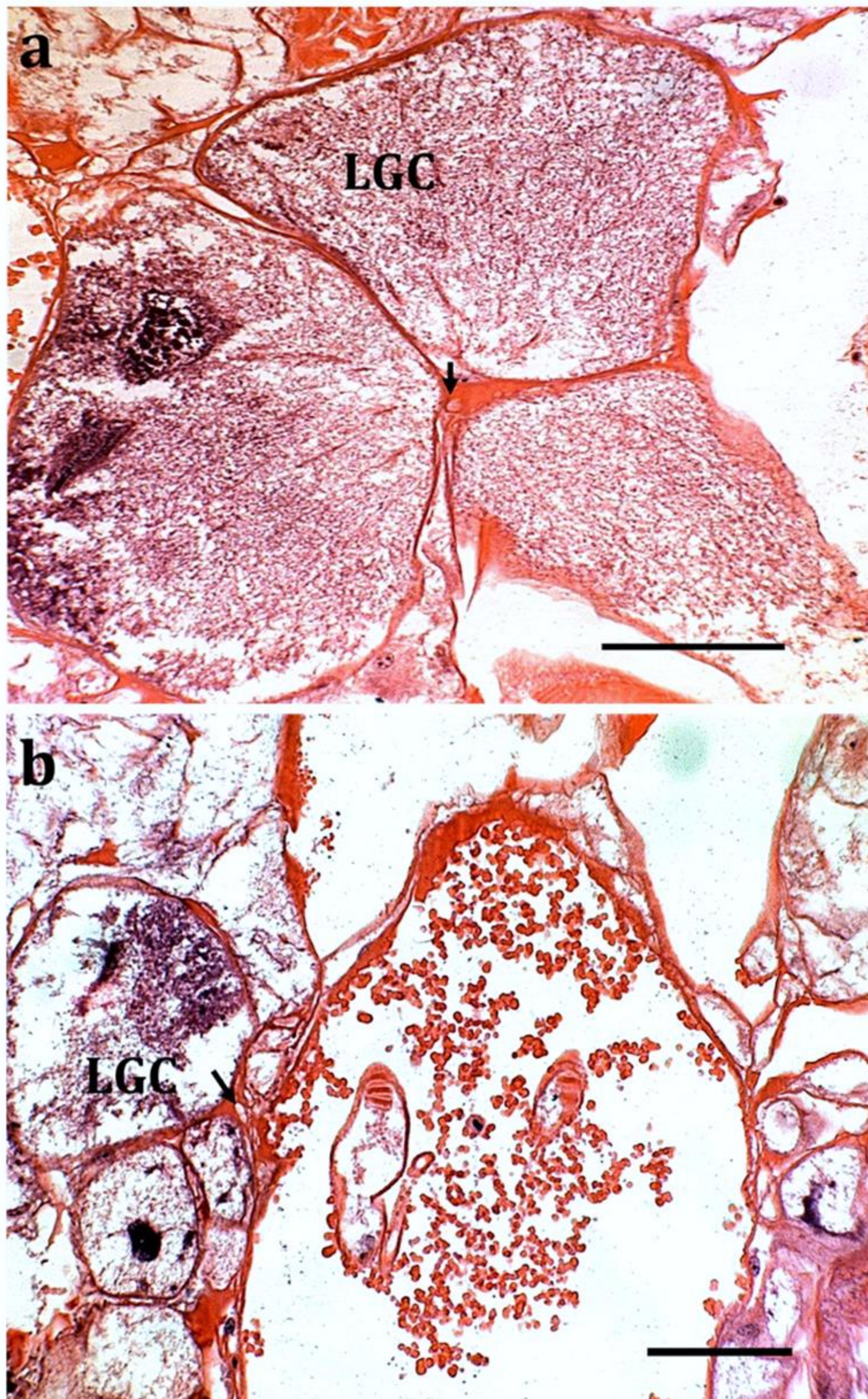


Figure 2.10. Light micrograph of **(a)** *A. foliaceus* labial gland cells (LGC) and their collecting duct (arrowhead) **(b)** showing the collecting duct draining into the oral cavity (planar section). H&E. Scale bar = 20 µm.

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 louse body; each containing five large gland cells (**Fig. 2.11**).

The pre-oral spine is situated anterior to the mouth tube (**Fig. 2.1**) and consists of a proximal cylinder-shaped sheath encasing the distal spine. The spine is narrow distally and has a tapered and pointed tip (**Fig. 2.12**) and an oval spine duct running down the core of the spine **(Figs. 2.13-2.14c)** with this duct being compressed in the spinal sheath (**(Figs. 2.14e, f, 2.16a, b**).

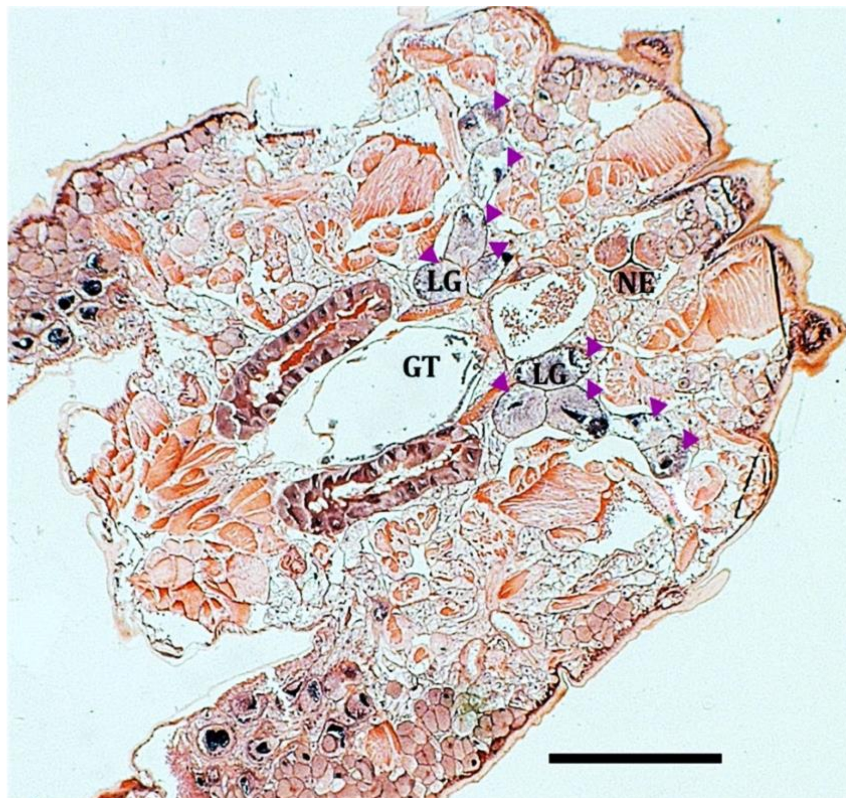


Figure 2.11. Light micrograph of planar section of *A. foliaceus* cephalothorax showing the position of the labial glands (LG) anterior to the gut (GT) and between the oral cavity and on the same level of nauplius eye (NE). Five gland cells are evident on each side as indicated by arrowheads; H&E. Technovit 7100. Scale bar = 500 µm.

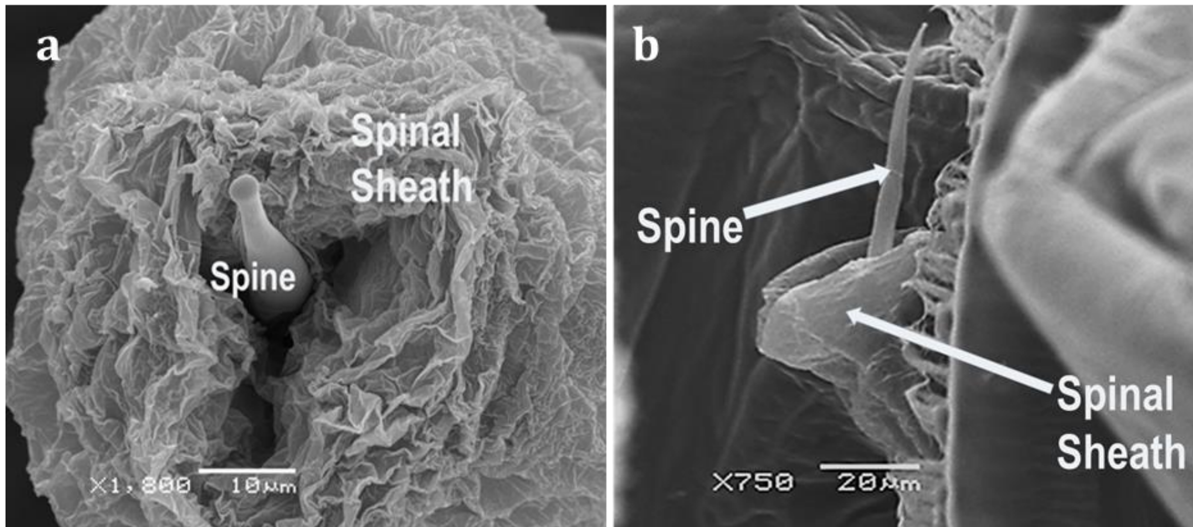


Figure 2.12. SEM micrograph of *A. foliaceus* pre-oral spine. **(a)** An enlargement view of the pre-oral spine (stylet) showing the spine protruding from the spinal sheath **(b)** tip and side view of the pre-oral spine.

Because of the orientation of the parasites during the Scanning EM procedures the duct was not visible in the SEM micrographs but following the serial sections of the pre-oral spine of *A. foliaceus* a central spine duct was detected (**Figs. 2.13-2.15**).

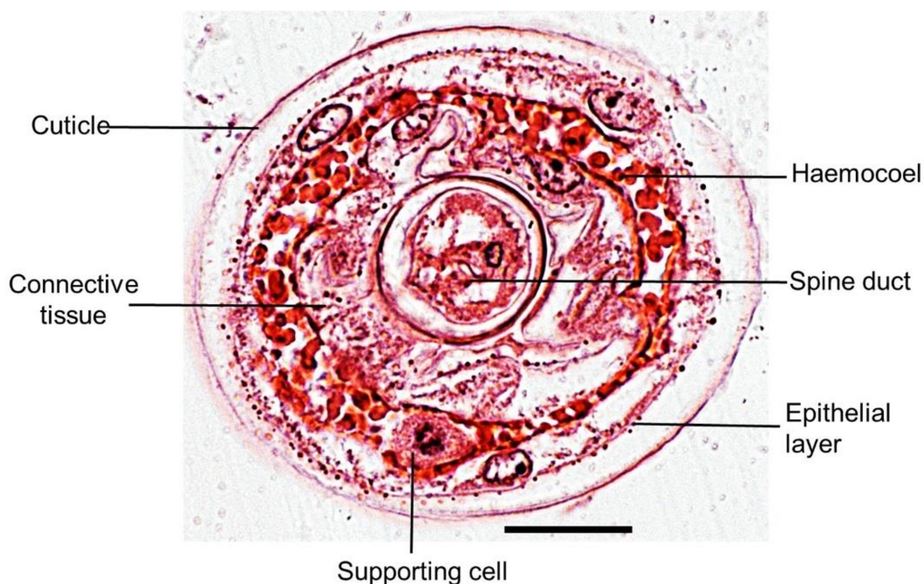


Figure 2.13 Light micrograph of a cross section through the pre-oral spine of *A. foliaceus* showing annotated regions. H&E; Technovit 70. Scale bar = 50 µm.

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. 2.3-2.5a, 2.9). The spine consists of an outer cuticle and thin epithelial layers surrounding the duct with the supporting cell (Figs. 2,13-2.14a, 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. 2.14d, e).

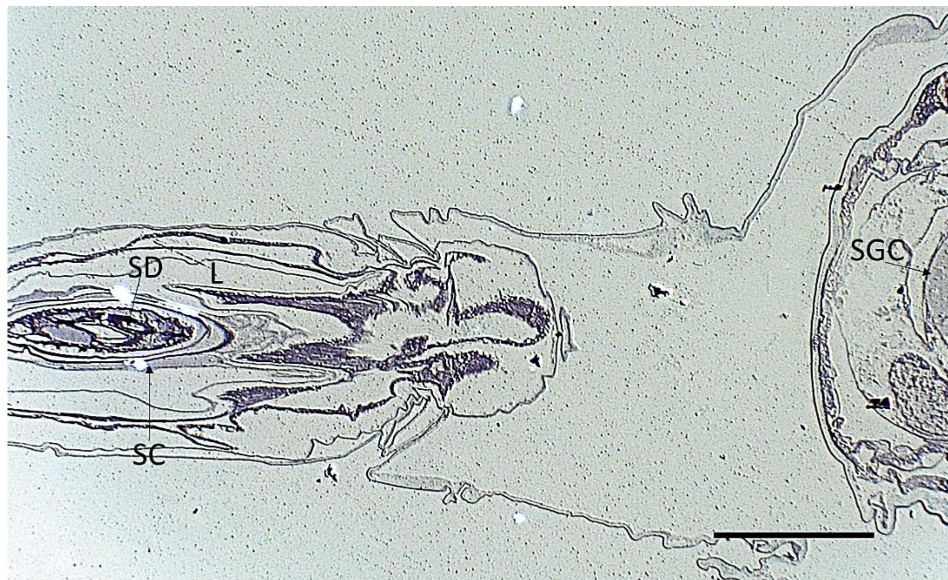


Figure 2.14. Semi-thin section of *A. foliaceus* pre-oral spine duct (SD) and the spinal gland cells (SGC) lying at the base of the pre-oral spine. (SC) spine cuticle, (L) lumen. 0.5% methylene blue. Scale bar = 20μm.

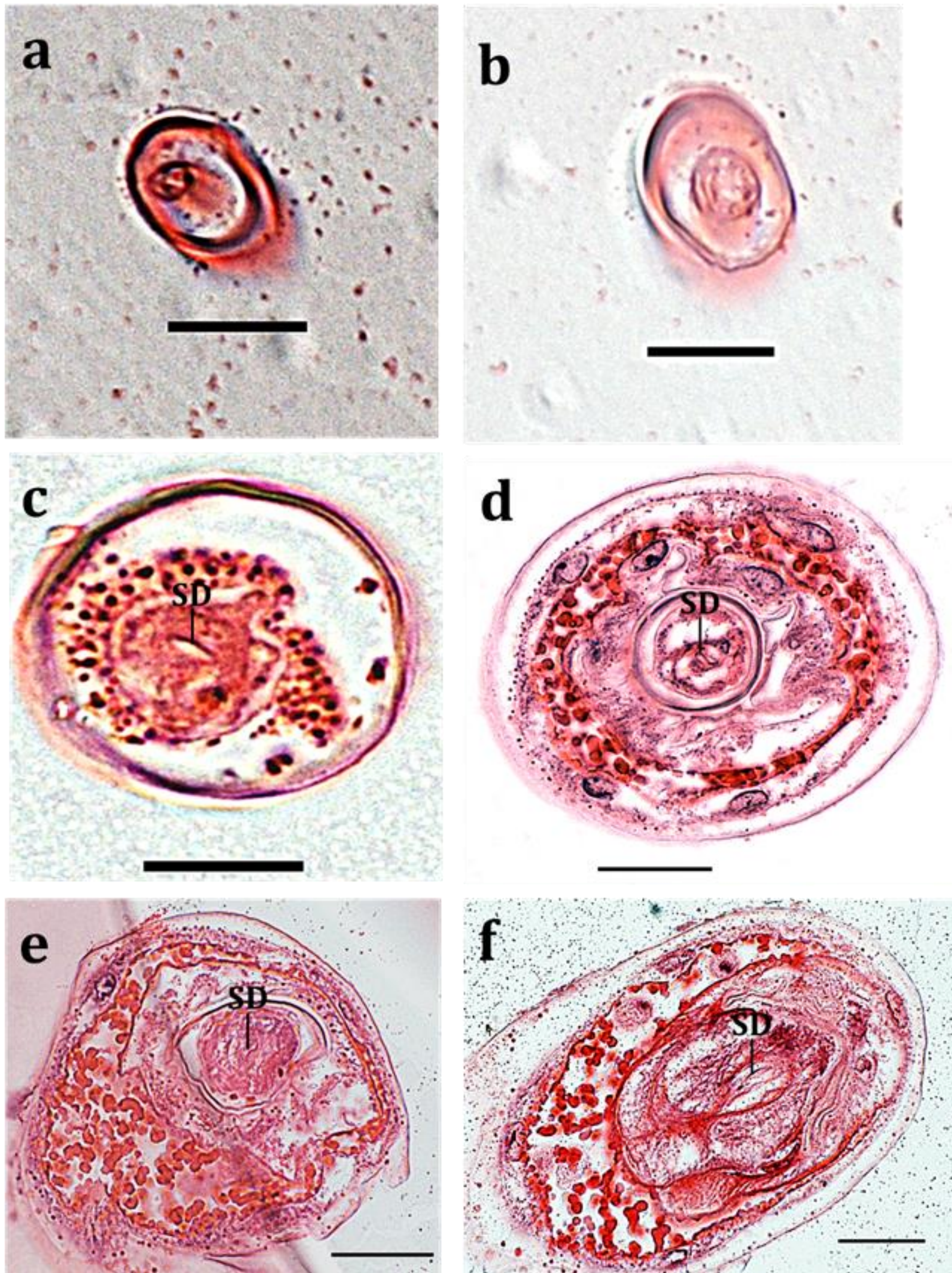


Figure 2.15. Light micrograph of cross sections through the pre-oral spine of *A. foliaceus* at different sites along its length. **(a)** and **(b)** sections through the spine and **(c-e)** are sections through the spinal sheath. **(f)** shows that the spine duct (SD) passes into the glandular tissue at the base of the spinal sheath. See Figure 2-13 for detailed annotations. H&E; Technovit. Scale bar (a, b & c = 10 µm) (d, e & f = 20 µm).

The central duct of the pre-oral spine is empty with double layers of cuticle surrounding it. The epithelium at 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. 2.16a, b)**.

Large elongated nuclei (NE) (**Fig. 2.16a, c**) are present within the epithelial layer lining the sheath cuticle (ShC), one of these cell nuclei showing a large nucleolus (N) (**Fig. 2.16a**). The epithelial tissue (E) around the spine duct (SD) and the cytoplasm of these large nucleolus-containing cells, contains extensive rough endoplasmic reticulum (rER) showing a tubular structure and the presence of free ribosomes (Rb) (**Fig. 2.16c, d**).

At the base of the spine are an abundance of widely distributed rough endoplasmic reticulum, ribosomes, and secretory vesicles (**Fig. 2.17a**). A pair of muscles, the pre-oral spine 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. 2.19b**).

The pre-oral spine duct originates from a spinal gland at the base of the spinal sheath (**Fig. 2.15-2.16a**). This gland is made of four polygonal cells with granular and basophilic cytoplasm as described earlier (**Figs. 2.3-2.5a, 2.9**). 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. 2.17-2.19a**).

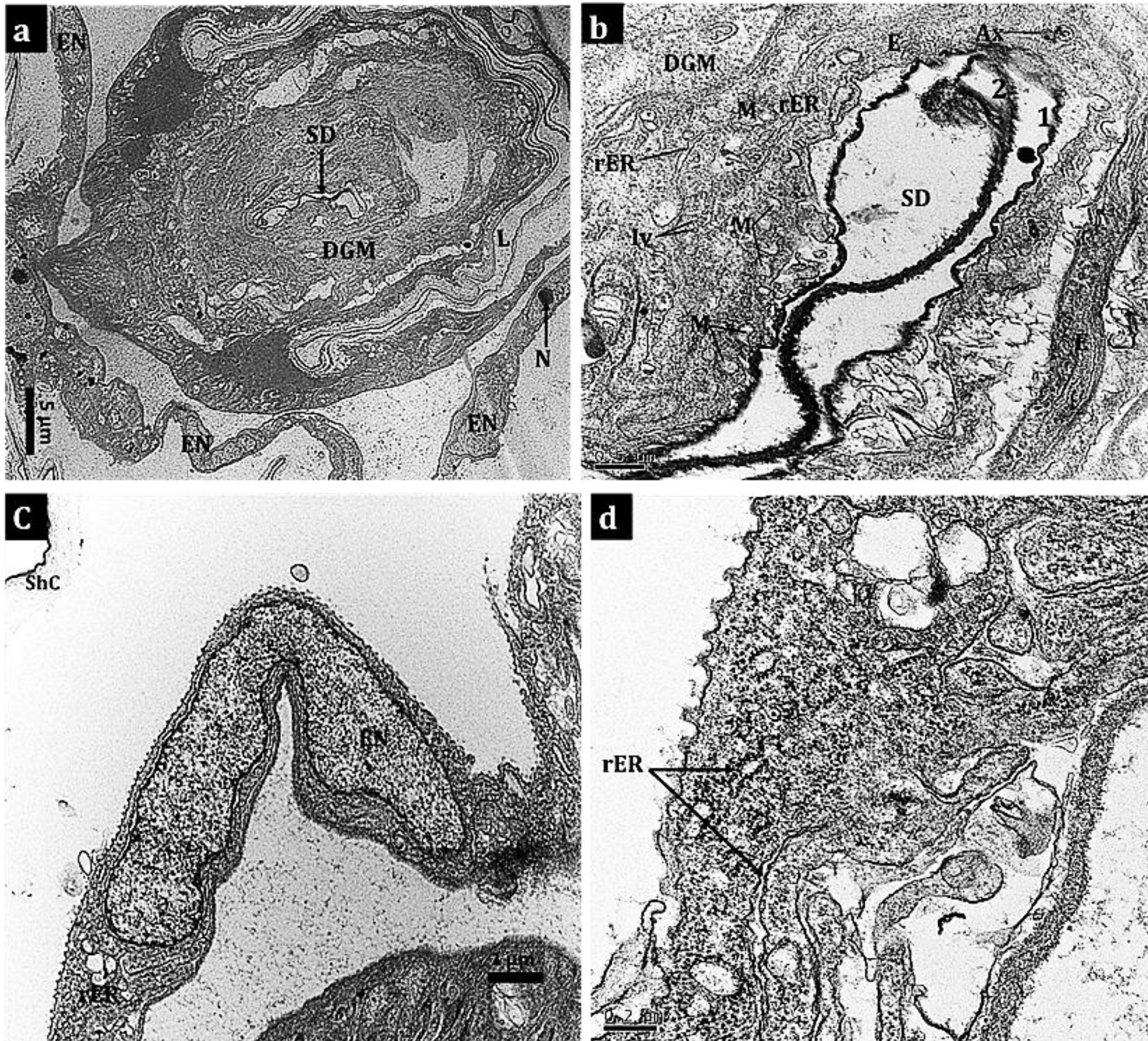


Figure 2.16. TEM micrographs of *A. foliaceus* **(a)** showing the pre-oral spine within the lumen and the folding spine duct (SD), large elongate nucleus cells (EN) within the epithelium of spinal sheath; in this section, only one cell shows the nucleolus (N); **(b)** shows spine duct is lined with two layers of cuticle1 & 2, intermediate vesicles (Iv) and dense granular matrix (DGM) extends from the spine duct to the epithelium (E) beneath the spinal sheath cuticle and two axons (Ax) **(c)** Detailed section of large elongate nucleus cells (EN). **(d)** Detail of the large elongate nucleus cell cytoplasm extensive rough endoplasmic reticulum (rER) and free ribosomes.

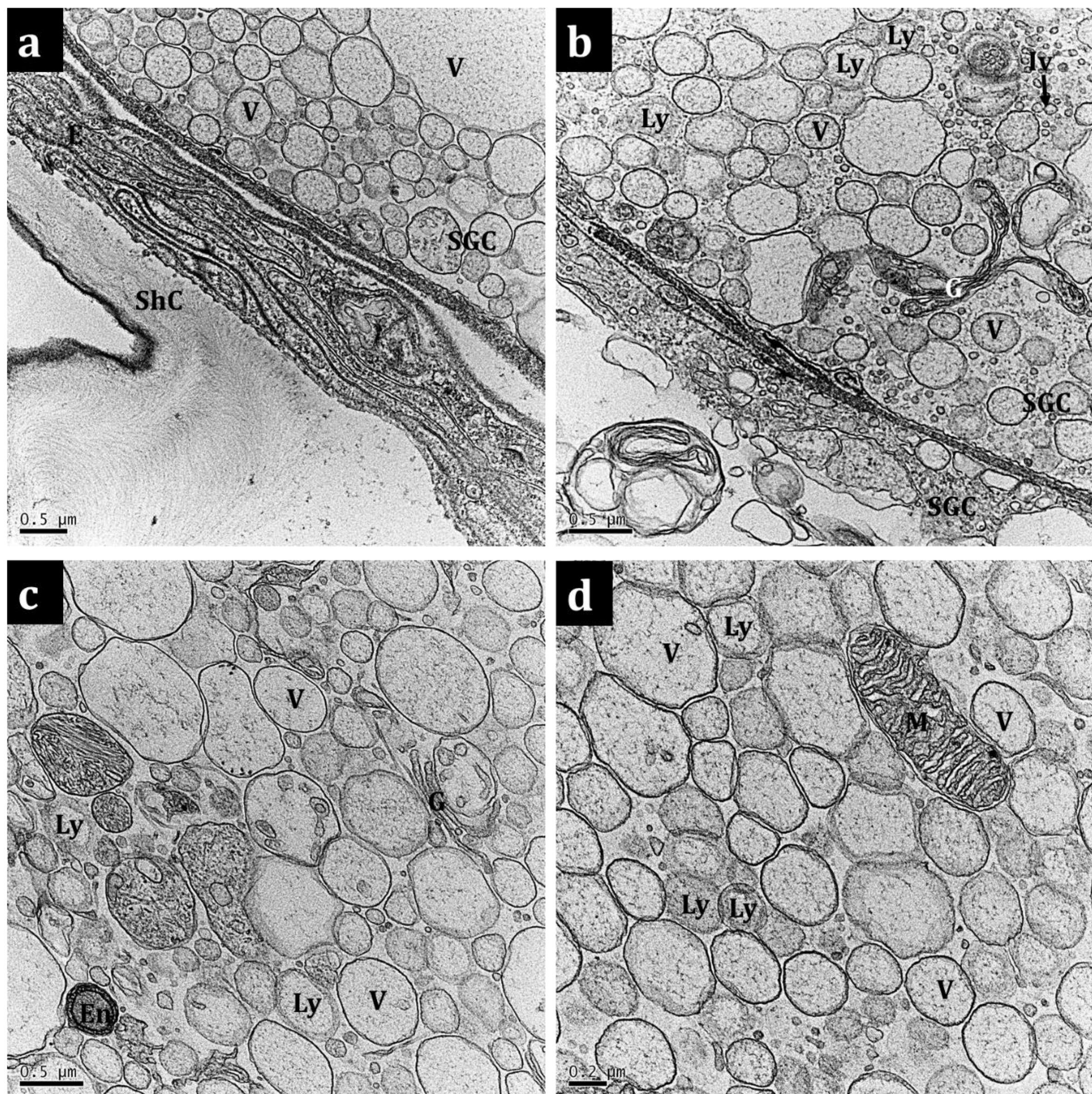


Figure 2.17. TEM micrographs of the *A. foliaceus* spinal gland cell organelles **(a)** showing part of the spinal gland cell (SGC) with numerous secretory vesicles (V) at the base of the epithelial tissue (E) of the spinal sheath cuticle (ShC); **(b)** two adjacent spinal gland secretory cells containing Golgi bodies (G), secretory vesicles of different sizes (V) and intermediate vesicles (Iv), lysosomes (Ly) and endosomes (En) at the base of the cell next to another SGC; **(c)** part of the SGC where endoplasmic reticulum with secretory vesicles (V) are seen together; **(d)** mitochondrion (M) shown within the cytoplasm of SG.

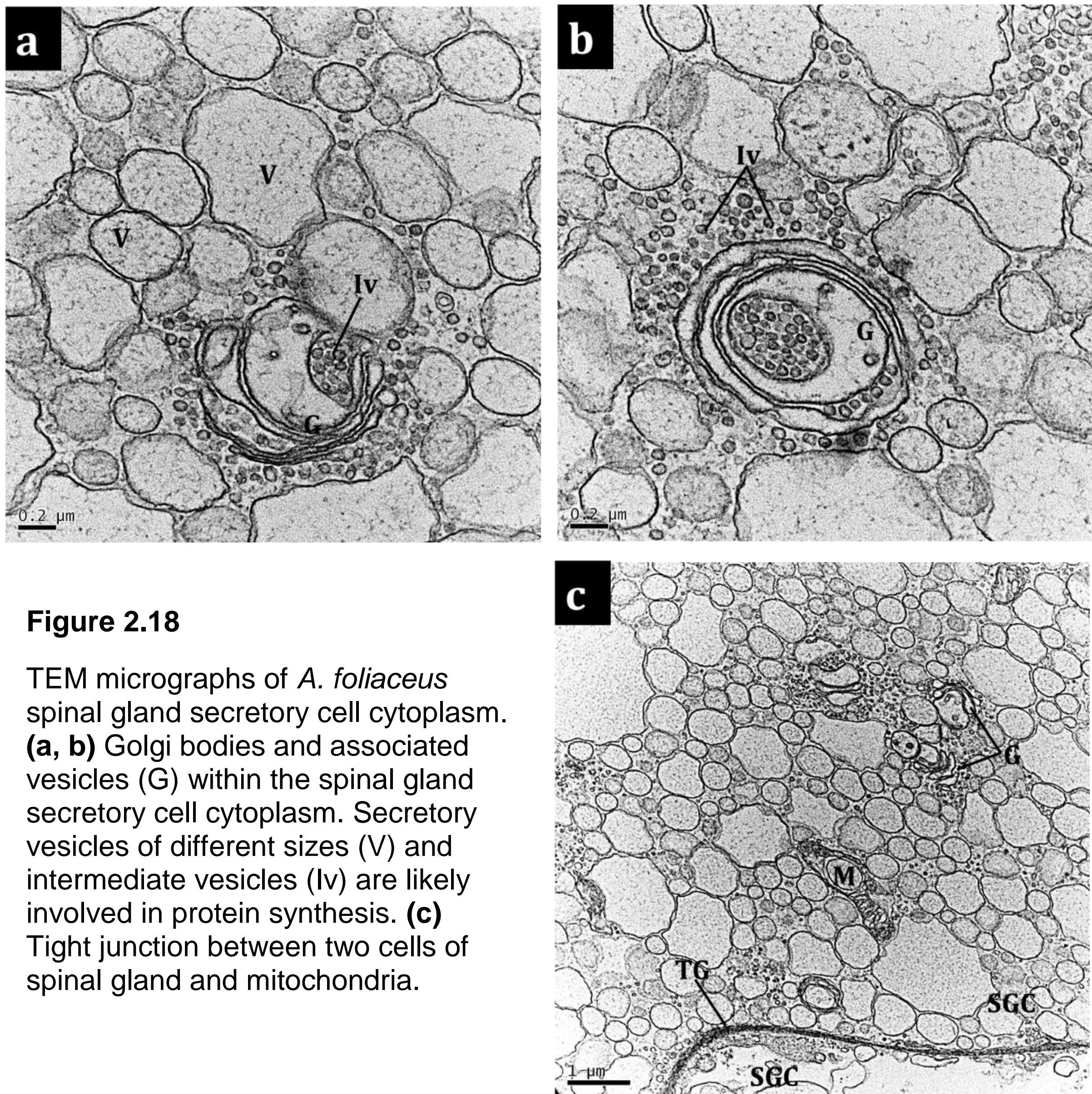


Figure 2.18

TEM micrographs of *A. foliaceus* spinal gland secretory cell cytoplasm. **(a, b)** Golgi bodies and associated vesicles (G) within the spinal gland secretory cell cytoplasm. Secretory vesicles of different sizes (V) and intermediate vesicles (Iv) are likely involved in protein synthesis. **(c)** Tight junction between two cells of spinal gland and mitochondria.

From the histology, transverse and sagittal sections, and TEM micrographs it was apparent that only one duct along the pre-oral spine is connected to the spinal gland (4 gland cells at the base of the spine). Although in some of the sections it appears to show as two ducts as illustrated in **Fig. 2.16a**, this was due only to collapsed ducts

within the spinal sheath, as was demonstrated by 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 two cuticle layers. It is hollow with no haemolymph as in the spinal sheath. The duct is surrounded by double layers of cuticle as indicated from the semi-thin (**Fig. 2.15)** and TEM micrographs (**Fig. 2.16a-b)**. There are large elongated nucleated cells within the spinal sheath epithelial layer. These cells contain numerous rER and free ribosomes (**Fig. 2.16d**).

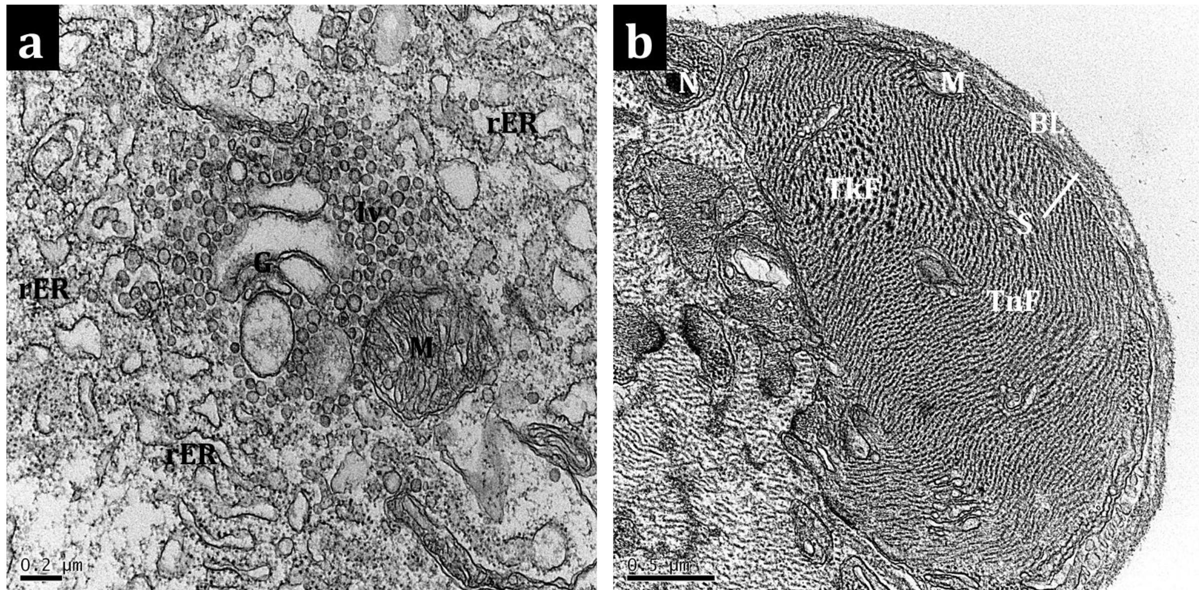


Figure 2.19. TEM micrographs of *A. foliaceus* secretory cells **(a)** part of secretory cells cytoplasm showing typical organelles involved in protein synthesis: abundant intermediate vesicles (Iv) and rough endoplasmic reticulum (rER), Golgi (G), mitochondria (M) and free ribosomes **(b)** Detail of pre-oral spine muscle involved in antero-posterior movement with thick (TkF) and thin (TfL) filaments, (s) sarcolemma, (M) mitochondria and peripheral nucleus (N).

From structural and ultrastructural microscopic studies, three types of glands were found to be associated with the feeding apparatus; the pre-oral spine and mouth tube, in different locations and at different depths. Hence, these glands were suggested to be related to feeding activities. Two of these glands were thought to be related to the

mouth tube and one connected directly to the pre-oral spine. The spinal gland consists of four large secretory cells, with one duct observed by light microscopy, from longitudinal and sagittal sections, and TEM micrographs. The spinal duct opens from the base of the spinal sheath above the gland cells to the tip of the large elongated nuclei epithelial cell in the spinal sheath containing an abundance of ribosomes and large nucleoli near the nuclear membrane. The labial glands and the proboscis glands were found to secrete their products via the mouth tube.

2.3.2 **Characterisation of lectin-binding properties**

To characterise 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 these lectins (**Table 2.2**). Four lectins were not observed to bind to any cells in the sections used, giving the same background fluorescence as the negative control: lectin wash buffer (LWB) (**Fig. 2.20A**), a subjective description was used to estimate the flourence labelling intensity. These lectins were PNA, ECL, UEA-I (**Fig. 2.21A**) and PHA-L. Out of the 19 lectins screened, 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 the secretory cells around the brain (**Table 2.2**).

As presented in **Table 2.2**, *A. foliaceus* spinal gland cells showed weak binding intensity with WGA, sWGA, SBA and LCA while DSL, JAC, Con A 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.

Table 2.2 Lectin binding pattern 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 flourecense 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.

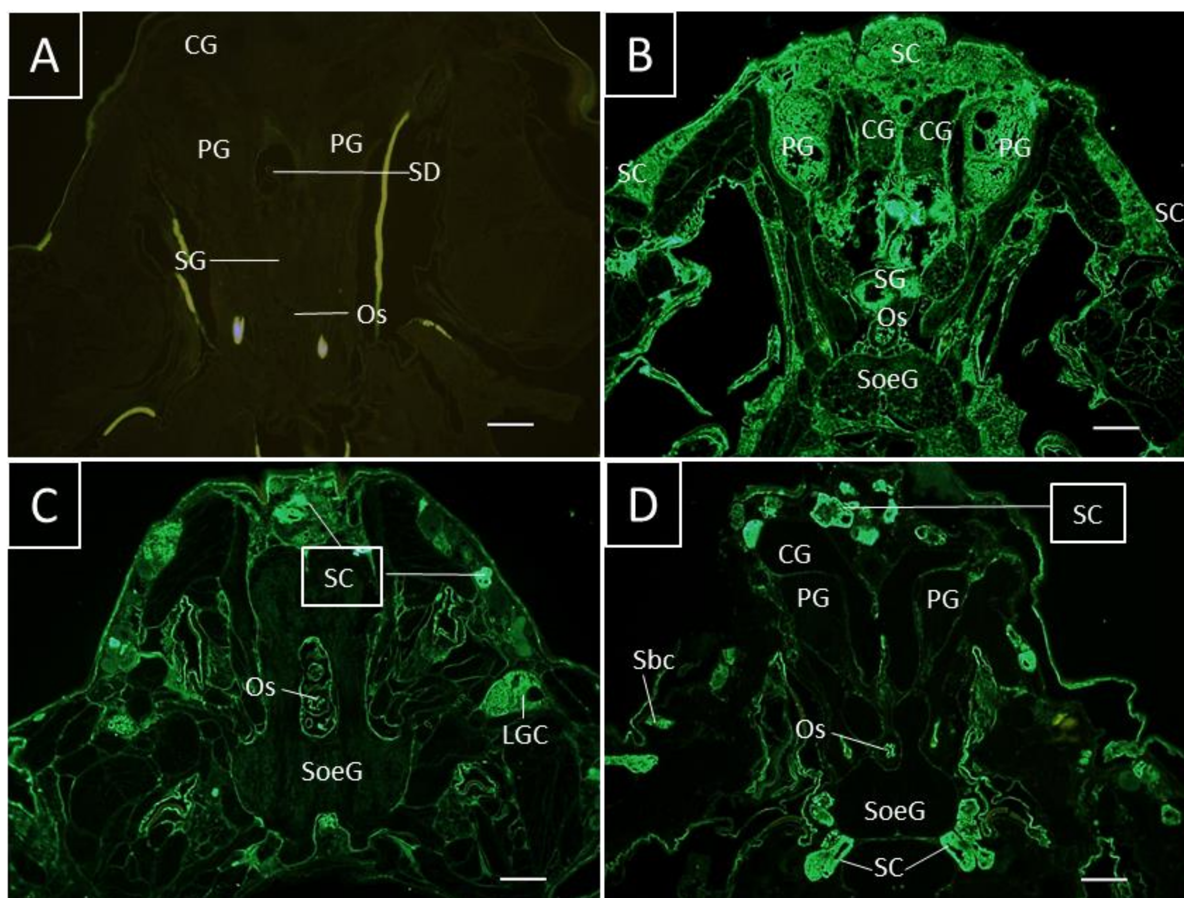


Figure 2.20. Planar section of *A. foliaceus* labelled with different fluorescent lectins **(A)** *A. foliaceus* cephalic region shown as a negative control; sections treated by LWB **(B)** Con A binds intensely to spinal (SG) and proboscis gland (PG) cells and other secretory cells (SC) all around the cuticle and brain **(C)** 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 **(D)** shows GSL-I lectin has strong binding affinity to sub-cuticular cells (Sbc) and secretory cells (SC) around the brain. (Os) oesophagus (CG) cerebral ganglion, (SoeG) sub-oesophageal ganglion. Note background staining of cuticle is a consistent artefact observed with fluorescent microscopy and lectin staining.

Strong binding of WGA and LCA were detected in the gut and moderate, weak binding with sWGA and DSL, respectively. Only JAC lectin binds strongly to labial gland cells while WGA and Con A shows weak binding to the same cells. The cerebral ganglion was labelled weakly by sWGA and intermediate binding occurred with WGA. Thirteen lectins were found to label both subcuticular secretory cells and secretory cells

scattered around the brain; cerebral ganglion and sub-oesophageal ganglion. These were WGA, sWGA, SBA, JAC, Con A, SBA GSL-I and GSL-II. With the exception of SBA, which weakly bound to sub-cuticular secretory cells, all bound strongly. In addition to the previous lectins (WGA, sWGA, SBA, JAC, Con A, SBA GSL-I and GSL-II); LEL, STL, DSL, DBA and VVA also exhibited a moderate to strong binding intensity to secretory cells scattered around the brain (**Table 2.2**).

STL, sWGA and VVA showed faint binding to oocytes, either to their outer membrane or cytoplasm or both, whereas WGA, LEL, PSA and LCA, all showed moderate binding. SBA and GSL-I bound strongly to sperm cells either in the spermatothecae or in the testes and WGA, sWGA and JAC bound weakly to the sperm cells.

WGA, sWGA, Con A (**Fig. 2.20B**), JAC (**Fig. 2.20C**) and LCA all have generic labelling properties whereby they were able to bind to most of the tissues screened (**Table 2.2**). 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.2.21B**) bound specifically to the cytoplasmic granules of glandular cells distributed around the cerebral ganglion and sub-oesophageal ganglion. These cells showed to stain with acidic alcian blue (pH 2.5 %) indicating the presence of acidic mucopolysaccharides. 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. 2.21D**); within the spermatothecae in female sections, like GSL-I (**Fig. 2.20D**). 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.

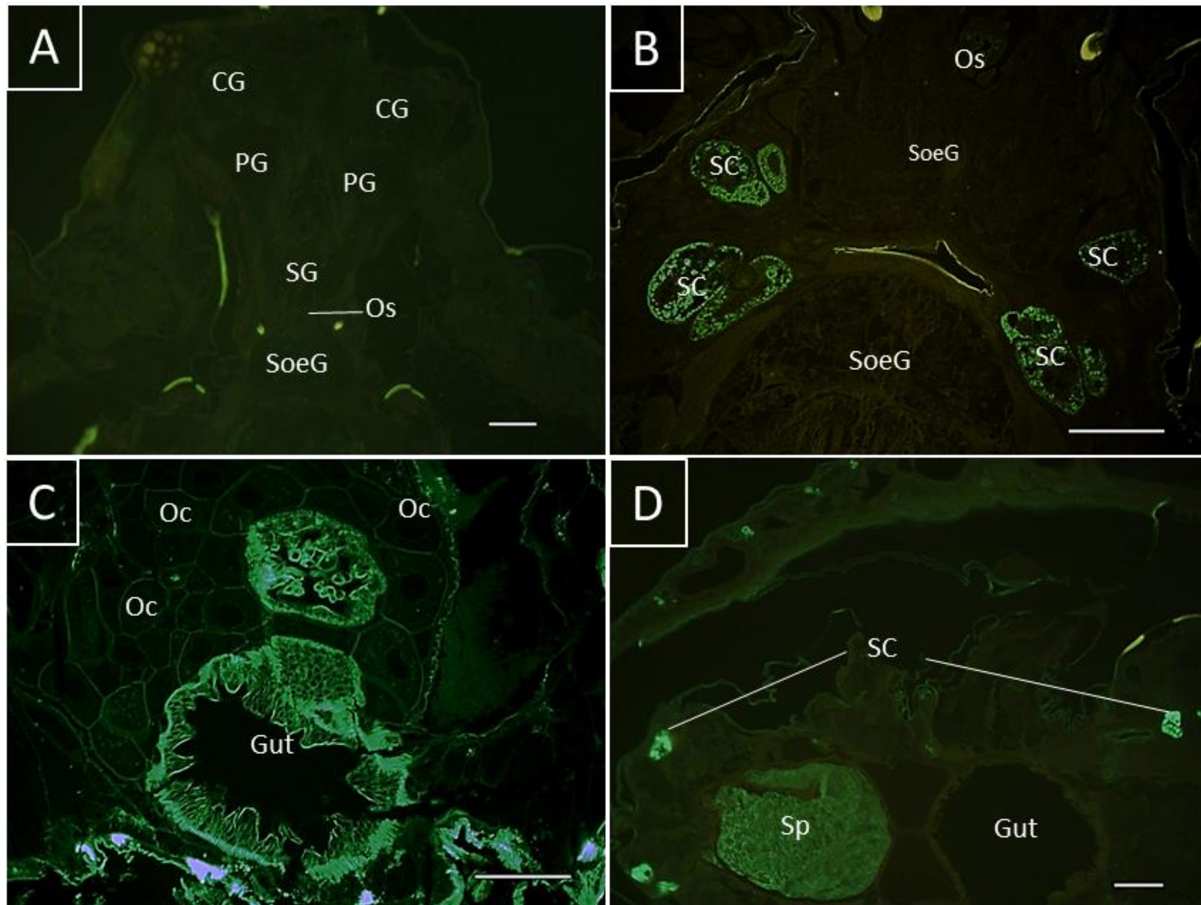


Figure 2.21. Planar section of *A. foliaceus* labelled with different fluorescently labelled lectins **(A)** no staining of UEA-I lectin associated with specific labelling of any tissues **(B)** DBA lectin binding specifically to cytoplasmic granules of secretory cells (SC) around the brain. These cells are found around cerebral ganglion (CG) and sub-oesophageal ganglion (SoeG) **(c)** 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 **(D)** SBA lectin had a strong and specific binding to secretory cells might be associated with 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.

Proboscis gland cell ducts were strongly labelled with LCA; and moderately by WGA, sWGA and PSA, while the cytoplasmic granules of the same cells were stained strongly by JAC and Con A (**Fig. 2.20B**). Faint labelling occurred with WGA, sWGA and LCA. WGA and LCA (**Fig. 2.21C**) also bound strongly to the gut tissue.

2.4 **Discussion**

In order to examine the potential role of *Argulus* glandular secretions during feeding on host skin, and gain some perspective on the associated impacts, the work reported in this chapter was intended to provide a detailed description of the secretory glands of the parasite and to determine the morphology and distribution of these glands within *A. foliaceus*. 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 (1992) considered that the pre-oral spine of *A. japonicus* has two ducts which run to the glands in the dorsal region of the cephalosome. While the proboscis glands of *A. japonicus* was suggested to contain 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 (Swanepoel and Avenant-Oldewage, 1992). 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 identifying with the spinal gland cells described earlier by Claus (1875), Wilson (1902), Madsen (1964) and Saha *et al.* (2011). The results of this study concur with findings of Martin (1932), Wilson (1902), and Saha *et al.* (2011) 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.* (2011) described the spinal gland cells in *A. siamensis*; which is the same gland defined in the current study for *A. foliaceus* as the spinal gland, Saha *et al.* (2011) 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.* (1993) were able to describe the spine pores; spine duct and subterminal pore, which is not connected to the dorsal tube, previously assumed by Shimura (1983) to be a chemosensory pore. Gresty *et al*. (1993) 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 support the results of Gresty *et al*. (1993) and hypothesis of Shimura (1983) 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*. (1993) with clear dense nucleoli, but were rarely seen in the current study whereby only one cell was observed with a nucleolus, 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 these cells of large nucleoli is an indication, however, that these cells are involved in protein synthesis. Furthermore, the presence of numerous secretory vesicles, intermediate vesicles, rough endoplasmic reticulum, Golgi bodies, and lysosomes in the spinal gland cell cytoplasm is a likely indication of extensive protein synthesis. Intermediate vesicles

are known to act as carriers of newly synthesized proteins from the rough endoplasmic reticulum to the Golgi.

The 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 (Gresty *et al.*, 1993). 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.* (1993) 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* (Saha *et al.,* 2011), 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* (Gresty *et al*., 1993). Regarding the location of the proboscis gland, the current study corroborates the findings of Swanepoel and Avenant-Oldewage (1992) and Saha *et al.* (2011) that it is located ventral to the optic tracts. However, Saha *et al.* (2011) 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 (1992) for *A. japonicus*. The assumptions that 1) either *A. siamensis* differs from other *Argulus* spp, even in the number of the 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 (1932) and later by Madsen (1964) 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 (Swanepoel & Avenant-Oldewage, 1992). Walker (2004) and Gresty *et al*. (1993) revealed that previous studies; conducted by Madsen (1964) and Shimura (1983), suggested that these tubular labile spines secrete enzymes and thus have a secretory role during feeding. In this study, the labial ducts were found to originate from two glands; each with 5 glandular cells, based on the postlateral side of the parasite. The ducts of the labial glands were mentioned briefly by Swanepoel and Avenant-Oldewage (1992), but in this study the ducts were followed histologically following serial sectioning and found to originate from a site comprising five gland cells located posterolaterally to the nauplius eye. These labial spines have muscles which were described by Gresty *et al*. (1993) to originate on the underside of the ventral cephalic tendon and pass through the sub-oesophageal ganglion. Debaisieux (1953) and Swanepoel and Avenant-Oldewage (1992) concluded that the two glands comprising five large cells, positioned in the posterolateral cephalosome, are the spinal glands. 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”. Comparing to other crustaceans the number and location

of *Argulus* glands connected to the feeding apparatus were relatively well known. Eichner *et al. (*2015) reported, for example, that sea lice *L. salmonis* salivary gland roles were not well known. However, recently, Øvergård *et al.* (2016) found that *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 extended ventrally into the mouth tube with openings in the oral cavity on the ventral surface of the labium and empty near the mandible teeth (Øvergård *et al.*, 2016). Caligid copepods such as *Caligodes laciniatus* (Krøyer, 1863) also have an intrabuccal pre-oral spineon the inner surface of the labrum (Dojiri and Ho, 2013) 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 (Bron *et al.*, 1993). This suggested that labial glands correspondents to salivary glands roles as the production site of biologically active components that are produced during feeding to enhance the feeding process (Thorp & Alan 2009 and Nishida & Ohtsuka 1996). The number of labral gland cells is variable between the copepod species (Thorp & Alan 2009) as shown in *L. salmonis* (Øvergård *et al.*, 2016) and other copepods such as *Derocheilocaris typica* (Pennak & Zinn, 1943) which have two or three secretory cells (Elofsson and Hessler, 2005) whereas pelagic copepod *Heterorhabdus* spp. (Nishida and Ohtsuka, 1996) 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*. (2014) that the *A. foliaceus* host-parasite interaction is facilitated by the spinal gland, the proboscis glands and the labial glands. The present study highlighted the 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 staining of three types of *A. foliaceus* glands, involved during feeding, with Alcian blue / PAS, with the spinal gland showing stronger staining, suggests the production of neutral glycoproteins by these glands. The anterior gland complex and circum-oral glands of *Lernaeocera branchialis* (Linnaeus, 1767) showed similar features (Barker, 2009).

The current study also suggested that the pre-oral spine has a secretory function, which may aid in immunomodulation, 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, *e.g*. proteases and anticoagulants as described for other copepods such as *Heterorhabdus* spp. which inject the host with venom and anaesthetic via hypodermic-needle-like teeth (Nishida and Ohtsuka, 1996) (see immunomodulation, **Chapter 4**).

Haematophagous (blood feeding) parasites often modify the host immune response during feeding by secreting compounds with immunomodulatory properties such as serine proteases in parasitic helminths (Yang *et al.*, 2015), aspartic proteases, as found to be expressed in the salivary glands of *Haemaphylasis longicornis*, Neumann,

1901 (Boldbaatar *et al.*, 2006), cathepsin-like genes such as those from the parasitic stage of salmon louse *Caligus rogercresseyi*, Boxshall & Bravo, 2000 (Maldonado-Aguayo *et al.*, 2015), PGES as in *L. salmonis* (Fast *et al.* 2004, 2005) and different secreted proteins from the salivary glands of the sand fly *Lutzomyia longipalpis*, Lutz and Neiva 1912, the latter having been suggested targets for vaccine development to control Leishmania chagasi Nicolle, 1908 infection (Valenzuela *et al.*, 2004). Thus, it is vital to study *Argulus* glands, which are similarly believed to be associated with these types of secretions. The information obtained on the exact position of the glands involved with such secretions during feeding, provides information for the application of other advanced tools in order to characterise the secretory products of these glands, and determine more specifically their roles during feeding. Lectins are proteins or glycoproteins of non-immune origin that recognise and bind to specific carbohydrate moieties (Sharon & Lis, 1989). Following from these properties, lectin histochemistry provides an inexpensive, powerful and relatively efficient technique to characterise carbohydrate moieties within the parasite's glands cells and other tissues, and provides baseline information concerning some of the secretory molecules from these glands by determining the type of carbohydrate groups bound by specific lectins. Identifying immunomodulatory secretion molecules can facilitate the development of vaccines for control of arthropod parasites (Nisbet *et al.,* 2013) and thus has potential for assisting control of *Argulus* spp.

This is belived to be the first lectin-labelling screening study conducted on *Argulus* spp. to identify cells of glands and other tissues with particular carbohydrate characteristics. The presence and distribution of binding sites for 19 fluorescent lectins were analysed here on adult *A. foliaceus* sections to search for specific binding to

glycoproteins associated with the spinal, proboscis and labial glands. As carbohydrate moieties are often immunologically relevant to virulence factors of arthropod parasitic sections, detection of lectin-binding molecules can help in understanding the host-parasite interactions of *Argulus* spp, and thus facilitate future studies in identifying novel control methods. It is known that lectins in parasites play a role in the recognition systems involved in host specificity and the processes of invasion (Knaus and El-Matbouli, 2005). Lectins are mainly specific to the terminal carbohydrates of sugar chains and because of that, they are widely used histologically to differentiate between cells according to their glycoconjugate contents (Ibrahim *et al.*, 2014). Xu *et al.* (2001) concluded that treatment with LCA, UEA-I and WGA lectins reduced the invasion of excised fins of channel catfish *Ictalurus punctatus* by *Ichthyophthirius multifiliis* theronts *in vitro*. The presence of carbohydrates in different parasite surfaces provides some support for their involvement in recognition of the host and attachment to host cells (Xu *et al.*, 2001). For example the presence of N-acetyl glucosamine residues in mosquito midgut glycoproteins suggests a possible role as recognition sites for malaria parasites (Ramasamy *et al.*, 1997).

Although the focus of this study was to screen the binding of different types of fluorescently labelled lectins in the spinal, proboscis, and labial glandular cells, and thus identify the types of glycoproteins produced from these glands and determine their potential association in feeding processes of *A. foliaceus*, other cells were also found to have an affinity to some of these lectins, and some of these were tissue-specific. The binding properties of lectins, particularly their specificity for particular moieties, means that they are widely employed across a range of research areas and represent an important tool for biotechnology (Zatta & Cummings 1992 and Liener

1986). In this respect, for example, it was demonstrated that lectins had a great ability to identify and separate glycosylated antigens from the proteins of the parasitic nematode *Haemonchus contortus,* (Rudolphi, 1803) Cobb, 1898, extracts (Smith *et al.*, 1994). Understanding the lectin binding specificity on *Argulus* spp. is a useful tool for biochemical and immunochemical studies. Furthermore, as any secretory components produced from these glands during feeding, they may expose to the host immune system, and could play important roles in immunomodulation and immune evasion.

Two sections from different depths were used with each lectin labelling procedure without specifying the sex or life stage. However, many key tissues of the parasite sections were included on the two selected sections. Thus, the result of this study provided information of the distribution of carbohydrates, not only within the glands of interest, but throughout the parasite. The novel findings arising from this work may thus form the basis for interested researchers to investigate aspects of the parasite physiology further, possibly with different sex and life stages of *A. foliaceus*, and provides information for the first time on some aspects of the presence of carbohydrates in parasite / host interaction either in terms of the secretory products from the studied glands; spinal, proboscis and labial glands, or from the other cells, which showed specific binding by lectins such as the gut, sub-cuticular glands and apparent glandular cells around the brain.

From the labelling characteristics observed, 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 presence of galactose and mannose residues in these glands. The strong binding with Con A, PSA and LCA suggests the presence of at least one of these groups D-Mannose, D-Glucose and N-acetylglucosamine in the labelled tissues. The lectins which had binding preference to chitin-binding lectins, N-acetylglucosamine, and N-acetyllactosamine were WGA, sWGA, STL, DSL and LEL. All DBA, VVA, JAC, SBA, GLS-I and GLS-II bound to N-acetylgalactosamine. In addition, JAC, SBA and GLS-I binds to galactose whereas GLS-II binds to glycogen groups.

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 play an important role in host-parasite interaction, as noted by Hammerschmidt & Kurtz (2005), Burton *et al.* (1999) and Knowles *et al.* (1991). Inhibition of adhesion of *Entamoeba histolytica* Schaudinn, 1903, which caused amoebiasis, to human colonic epithelial cells was blocked by galactose or N-acetylgalactosamine. Saffer & Petri (1991) and Petri *et al.* (1987) observed that animals treated by galactose lectin were able to combat amoebiasis. 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 elements of the composition 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 the parasitic invasion is reviewed by Basseri *et al.* (2002). Previous studies have also demonstrated the presence of GlcNAc moieties in the triatomine bug *Rhodnius prolixus* (Stål, 1859) (Basseri *et al.* 2002) and in the mosquito *A. stephensi* (Rudin and Hecker, 1989), 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.

In the present study, lectins with greater affinity in the gut bound to galactose and mannose residues; WGA, sWGA, DSL and LCA. Galactose residues were also detected previously in the midgut of the mosquito *Anopheles tessellatus*, Theobald, 1901 (Ramasamy *et al.*, 1997) and were suggested to be the recognition sites for malarial parasite invasion. The detection of specific carbohydrate moieties at the parasite surface and host-parasite boundary of the myxozoans *Tetracapsuloides bryosalmonae* (= *T. bryosalmonae*, Canning *et al.*, 1999) (Morris and Adams, 2004), *Myxobolus cerebralis* Hofer, 1903 (Knaus and El-Matbouli, 2005) and Enteromyxum

scophthalmi (Redondo *et al.*, 2008) by lectins, similarly suggests a role in host-parasite interaction (as reviewed by Alvarez-Pellitero 2008). From the present study, these compounds are assumed to have a role in interacting with host tissue during invasion and feeding. As stated by Alvarez-Pellitero (2008), the interaction between soluble or transmembrane lectins and sugar residues such mannose and galactose in different fish parasites such as *I. multifiliis* (Xu *et al.*, 2001), *Glugea plecoglossi* Strickland, 1911 (Kim, Ogawa and Wakabayashi, 1999) and *Gyrodactylus derjavivni* Mikailov, 1975 (Buchmann, 2001) has revealed their central roles in host-parasite interaction and invasion and host immune evasion (Alvarez-Pellitero, 2008).

The binding properties of lectins to specific carbohydrate moieties may allow the development of new insecticides / parasiticides, as was recently demonstrated with PF lectin; from *Lonely tesota* seeds, which had insecticidal activity against *Zabrotes subfasciatus* (Boheman, 1833) larvae. This activity was suggested to be a result of PF2 recognition by glycoproteins within the insect gut, known to have biological roles in growth and development (Lagarda-Diaz *et al.*, 2016).

Endocrine glandular cells with cytoplasmic granules, scattered around the *Argulus* cerebrum, showed a diversity of lectin labelling, with 11 of the tested lectins binding strongly or moderately. This suggests that different carbohydrate moieties; galactose, glucose, and mannose, exist in these cells and is indicative of the presence of surface associated sugars which may play key roles for *Argulus*. However, only DBA, which has a binding preference for N-acetylgalactosamine, bound specifically to these cells and this might indicate the role of this glycoprotein in mediating specific biological functions in this type of cell.

With variations in binding strength, sub-cuticular gland cells, connective tissue, cuticle epithelial cells and oocyte outer membranes, all had galactose, glucose, and mannose residues. Some of the lectins were found to have identical sugar specificities but different binding affinities such as LCA and PSA. This was also noted by Jacobson & Doyle (1996) where lectins showing identical sugar specificities may bind to complex glycoconjugates with different binding patterns and result in different conformations. Furthermore, lectin reactivity may also be affected by the nearest attached residues (Jacobson and Doyle, 1996). Marlowe *et al*. (1994) also stated that different lectins with similar binding preferences showed different labelling intensity depending on the binding sites within the molecules (Marlowe *et al*., 1994). Although JAC and PNA lectins 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 (Marlowe *et al*., 1994), thus the suggestion that is 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 *T. bryosalmonae*, where UEA-1 lectin was also the only one not binding to the parasite surface (Morris and Adams, 2004).

The lectin-binding patterns of *A. foliaceus* tissues tested with assayed lectins were summarised in **Table 2.2**. Galactose and mannose/glucose residues were abundant within the parasite's body as demonstrated by the lectin binding specificity with GlcNac, GalNac and D-mannose groups. It has previously also been noted that glucose/mannose residues exist in *Enteromyxum scophthalmi* (Palenzuela, Redondo & Alvarez-Pellitero, 2002) stages and other myxosporeans (Redondo *et al*., 2008)

such as *T. bryosalmonae* (Morris & Adams, 2004). Carbohydrate terminals with N-acetyl-galactosamine (GalNAc)/galactose moieties are known to play a key role in parasite-host interactions (Redondo *et al*., 2008), particularly in attachment/invasion by parasites and pathogens to/into host cells / tissues and are involved in interactions with vector tissues (Basseri *et al*., 2002) allowing evasion of host immune responses. Previous studies have also identified interactions between the parasites' carbohydrate terminals; such as those of *Ichthyophthirius multifilis* (Fouquet, 1876) (Xu *et al*., 2001) and *Gyrodactylus derjavini* (Mikailov, 1975) (Buchmann 2001), and fish host immunity. Buchmann (1998) earlier found that Con A bound to the cephalic glands opening of *G. derjavini* and complement factor C from rainbow trout also bound specifically to the cephalic glands ducts. Therefore, it was suggested that mannose residues in this region activated the complement pathway of this parasite during infection (Buchmann 1998). Initiation of fish innate immune responses involved complement as one of the key components involved in this process (Alvarez-Pellitero 2008). *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 (Saurabh *et al*., 2010). Lectin labelling investigations can thus provide useful information relevant to determining the composition of secretory substances during feeding, which may in turn relate to mechanisms used by *Argulus* spp. to counteract host immunity and conversely, potential targets utilised by host immune pathways. It should be noted that the current work has focused on relatively few stages and has not examined sex or stage differences in the parasite, such that future studies needs to employ a more granular approach.

In 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, enabled characterisation of the carbohydrate moieties associated with these glandular cells, revealing important biomolecular aspects of these, and other, *A. foliaceus* tissues. The studies reported in the following chapters (**chapter 4 and 5**) will serve to better characterise the secretions of the identified glands.

Chapter 3. Developing transcriptomic tools for studies of argulid biology

3.1 Introduction

High-throughput RNA sequencing (RNA-seq) is a powerful tool for transcriptome analysis and uses deep-sequencing technologies to produce millions of short cDNA reads (Shaina *et al.*, 2016). It is a method that can be used for both mapping and quantifying transcriptomes (Wang *et al.*, 2009). The most common high-throughput RNA sequencing machines platforms available nowadays are Illumina, Pacific Biosciences Single-molecule real-time (SMRT) sequencing and Oxford nanopore technologies. Illumina technology is characterised by low error rate, high numbers of short reads and low cost. The current sequencers from Illumina are MiSeq, NextSeq 500, and the HiSeq series where MiSeq and HiSeqs are the most well-known platforms. The MiSeq is designed as a fast, personal benchtop sequencer, with run times as low as 4 hr and outputs intended for targeted sequencing and sequencing of small genomes. The HiSeq 2500, on the other hand, is engineered for high-throughput applications, yielding current outputs of 1 Tb in 6 days (Reuter *et al.*, 2015). The use of this analytical platform is increasing and becoming a common method in biology (Shaina *et al.*, 2016). Pacific Biosciences and Oxford Nanopore technologies produce a smaller number of reads which are characterised by extremely long mean read lengths (kilobases) but higher error rates. To date, Illumina technologies in particular, have been widely applied to organisms lacking sequenced genomes, helping researchers to create databases of transcripts expressed in whole organisms or tissue

through a combination of *de novo* assembly (where necessary) and transcript annotation. Decreasing costs, coupled with the advantages associated with high-throughput, rapid turnover and high-sensitivity of current RNA-seq technologies have served to facilitate a sharp increase in the number of transcriptomic studies conducted in different organisms in recent years (Han *et al.*, 2015). Havird and Santos (2016) have, for instance, demonstrated an exponential increase in crustacean transcriptomic publications over a five year period as determined from NCBI database searches (Havird and Santos, 2016). RNA-seq is a technique whereby extracted RNA, following construction of a cDNA library, may be subject to high-throughput transcriptome sequencing. Following annotation / allocation of transcripts and further downstream bioinformatics analysis, the heterogeneity and representational profile of the sequenced transcriptome may established (Havird and Santos, 2016). Assessment of transcript expression and examination of the patterns of regulation and functional classes involved, can provide important tools for understanding parasite biology (Cantacessi *et al.*, 2012).

Relatively poor genomic resources exist for the Crustacea, particularly when compared to other ecdysozoan groups such as the Hexapoda (insects) and Nematoda. Aside from purely phylogenetic questions, three principal objectives have motivated much of the development and generation of existing crustacean genomic resources, as summarised by Carmichael (2013): (a) the commercial aspects of a particular species (b) provision of species models to study *e.g.* environmental and evolutionary physiology and (c) use of crustaceans to study the effects of toxic chemicals on biological organisms (Carmichael 2013).

RNA-seq has been used to study transcriptomic expression in a range of different crustacean species lacking the fully sequenced genomes (Mykles *et al.*, 2016). The Crustacea comprise a very large group of arthropods and one of the most diverse groups of metazoans, comprising of more than 70,000 species across a range of sizes (Havird & Santos 2016) and have limited genomic resources. Compared to other, well studied, groups such as insects and vertebrates, the Crustacea have limited genomic resources and therefore there has been a need to develop a well-characterised model crustacean system resembling *Drosophila* in insects (Havird & Santos 2016). Although, the cladoceran microcrustacean *Daphnia pulex* Leydig, 1860 (Colbourne *et al.*, 2011) has a well-studied genome, it is not of itself adequate to be used as a model for the rest of this diverse group, especially as this genus is not closely related to most of the crustaceans of more general interest. The lack of relevance of *Daphnia* to other groups is underlined by the fact that more than a third of recognised *Daphnia* genes have no recognised counterpart in the proteomes of other organisms (Havird and Santos, 2016). Recently, Mykles and Hui (2015) suggested cherry shrimp, *Neocaridina denticulata* (De Haan, 1844), as a decapod crustacean model, however, this species still needs considerably more investigation to prove a useful model (Mykles and Hui, 2015). Bron *et al.* 2011 reported that copepod genomic and transcriptomic analysis expands knowledge of the evolution of specializations for particular host-associations, parasite development and adaptation, help in understanding the changes between different stages by allowing measurement of gene expression variation and support the development of new therapeutants, as well as helping to identify novel compounds, such as immunomodulators, produced by parasitic copepods (Bron *et al.*, 2011). Genomic studies of a range of parasitic

copepod species, including *Pacific Lepeophtheirus salmonis oncorhynchi* Skern-Mauritzen, Torrissen & Glover, 2014 and *Atlantic Lepeophtheirus salmonis salmonis* (Krøyer, 1837)*, Caligus clemensi* (Parker & Margolis, 1964) and *Caligus rogercresseyi* (Boxshall & Bravo, 2000)*,* which all cause huge economic losses for the salmon farming industry globally have proven a considerable addition to crustacean genomic resources (Yasuike *et al.*, 2012). Although genomic and transcriptomic analyses of free living copepods are limited to a small number in comparison compared to the number of copepod species identified, over 12,000 species (Bron *et al.*, 2011), genomic techniques have been applied to some species such as *Tigriopus californicus* (Baker, 1912) (Barreto *et al.*, 2011), *Eurytemora affinis* (Poppe, 1880) (Legrand *et al.*, 2016) and *Calanus sinicus* Brodsky, 1962 (Ning *et al.*, 2013), which have provided the foundation for future genetic and genomic studies of related species and copepods in general.

Branchiura, Thorell, 1864 is an ectoparasitic crustacean group, which includes 4 genera including *Argulus*, Müller (1785), *Dolops* Audouin (1837), *Chonopelti*s, Thiele (1900) and *Dipteropeltis*, Calman (1912) (Tam, 2005). While the Branchiura were initially classified as a class of Maxillopoda, more recent molecular phylogenomic studies have placed them as a sister group to the endo-parasitic Pentastomida. Ostracoda, Mystacocarida, Branchiura and Pentastomida together have been suggested to form the superclass Oligostraca (Regier *et al.*, 2010). Phylotranscriptomics comprises the identification of homologous and orthologous genes from sequenced transcriptomes using a phylogeny-based approach. A study conducted by Oakley *et al.* (2013) on ostracods proposes that the Oligostraca form a sister group to the rest of the Pancrustacea and, based on the phylogenetic results,

Oakley *et al.* suggested the inclusion of the Oligostraca in wider phylogenetic arthropod investigations, particularly the Branchiura and Ostracoda due to ease in collection (Oakley *et al.*, 2012).

Ostracods have been used as model organisms for evolutionary research. Detailed transcriptomic studies of this crustacean group are rarely conducted and genomic techniques employed to date have been largely employed for phylogenetic studies (Schön and Martens, 2015). The number of studies conducted on Ostracods using next generation sequencing (NGS) are limited and most of the studies used classical genomic methods. Oakley *et al.* (2013) conducted the first study on ostracods using the NGS technology in investigating the phylogenetic position study of ostracods among the pancrustaceans. However, the number of identified genes arising from this study is limited (Schön & Martens 2015). From a spermatological comparative study conducted between a pentastomid, *Raillietiella hemidactyli* Hett, 1934, and a branchiuran crustacean, *Argulus foliaceus* (Linnaeus, 1758), Wingstrand (1972) found that they form a sister group (Wingstrand 1972).

Although branchiurans affect aquaculture and fisheries globally, few studies have examined the mechanisms underlying physiological and behavioural aspects of the parasite (Singhal *et al.*, 1990). To date, control methods employed have targeted few of the biological aspects of the parasite and have not helped in preventing argulosis, and those controls that do exist, all have critical limitations in their use (Sahoo *et al.*, 2013). Hence new strategies for control are still urgently needed. One avenue for control is the potential for developing vaccines or other immunoprophylactic approaches such as use of immunostimulants / functional feeds. To achieve this,

better knowledge of the host response to the parasite and the parasite's mechanisms for combating host immunity are required.

An initial attempt to examine the immune responses of hosts to *Argulus* was conducted by Ruane e*t al.* (1995) in a study on rainbow trout, which showed that fish immunised with an *A. foliaceus* antigen extract showed a humoral response, which suggested further invesigations for vaccine against crustracean ectoparasites. More recently, Saurabh *et al.* (2012) identified two immunodominant polypeptides in an *A. siamensis* extract. It was suggested that these could be potential candidate antigens for use in immunoprophylaxis against *Argulus* infection (Saurabh *et al.*, 2012).

Argulus japonicus and *Argulus coregoni* Thorell, 1864 were described as causing a haemorrhagic response in carp and rainbow trout respectively (Walker *et al.,* 2011, Forlenza *et al*., 2008 and Shimura & Inoue 1984) and it was suggested that *Argulus* secretes substances during feeding to impair host immunity or to facilitate blood feeding (Walker *et al.*, 2011). In this respect, *A. siamensis* was suggested to suppress serum α-2M, serum alternate complement activities and ceruloplasmin levels through modulation of the innate immune system of the freshwater fish rohu; *L. rohita* (Saurabh *et al.*, 2010). A general lack of knowledge in this area led von Reumont *et al.* (2014) to call for studies looking into the composition of the expected active secretions originating from branchiuran exocrine glands associated with feeding.

Techniques allowing examination of the parasite's transcriptome, including sequencing techniques, can provide a greater understanding of the biology of the parasite and of key genes involved in particular activities with such genes and the products they code for providing potential targets for use in control of argulosis (Sahoo

et al., 2013). The first transcriptome analysis for argulids was conducted by Sahoo *et al.* (2013) using *A. siamensis,* which has a severe impact on the aquaculture industry in India. An Illumina HiSeq 2000 platform used for the sequence analysis, generating 75,126,957 reads and 46,352 transcripts. In total, 19,290 CDS including 184 novel CDS and 59,019 ORFs were identified. Using this same transcriptome shotgun assembly (TSA), Christie (2014) was subsequently able to identify 27 transcripts encoding putative neuropeptide precursors, these peptides being known to play a role in physiological/behavioral control across species. This was the first finding of neuropeptide encoding sequences in any member of the subclass Branchiura (Christie 2014).

Recently, Pinnow *et al.* (2016) provided a new source of *A. foliaceus* transcriptome data (NCBI sequence read archive (SRA) under the Bio-Project number PRJNA293150. 52,725,850) using Illumina HiSeq 2500 genome sequencer. The assembly resulted in 8,424 contigs. These transcriptome results were only used to identify and characterise hemocyanin subunits in *A. foliaceus* and *A. siamensis* and provided evidence that hemocyanin was the principal respiratory protein in the stem lineage of the Pancrustacea (Pinnow *et al.*, 2016).

Despite the above, genomic resources for *Argulus* spp. and for the Branchiura more widely, remain extremely impoverished providing a major constraint to further research in this area. The work reported in this chapter sought to provide new transcriptomic resources for *A. foliaceus* and *A. coregoni* (Thorell, 1864), the two native *Argulus* species in the UK and to combine the new sequences with the previous two transcriptome libraries established for *A. siamensis* (Sahoo *et al.*, 2013) and *A. foliaceus* (Pinnow *et al.*, 2016). Understanding key biological processes in *Argulus*

spp. will help in searching for candidate immunomodulatory targets, which may help researchers to develop vaccines as an alternative control method. This study also highlights the fact that *Argulus* spp. might prove productive as an ectoparasite model. The results of this study will help fill the gap in genomic knowledge for this genus and will provide a useful addition to crustacean genomic resources, particularly for Branchiura and will provide the first list of potential host immunomodulators for these three *Argulus* species.

3.2 **Materials and methods**

3.2.1 **Sample Collection:**

For this work, two different species of *Argulus* were used, *Argulus coregoni* and *Argulus foliaceus*. Adult male and female samples of *A. coregoni* were collected from common carp by Environment Agency staff in Brampton in England, UK and sent to the Institute of Aquaculture in RNA*later*™ (Invitrogen, Thermo Fisher Scientific, Waltham, Massachusetts, USA). Samples of *A. foliaceus* were collected from Loch Fad in Isle of Bute in Scotland, UK and fixed in RNA*later* and kept at 4°C for 24h. Both groups of samples were then drained of RNAlater according to manufacturer’s guidelines and stored at -70°C until required. Four different samples of *A. foliaceus* were used (each has an approximate weight of 0.2 g); one male adult, one female adult, a pool of juvenile stages and a pool of 6 dissected pieces from adult parasites comprising the expected position of the pre-oral spine and proboscis glands as established from histological sections (**Chapter 2**).

3.2.2 **RNA extraction and purification**

Total RNA was extracted from *Argulus* samples using TRI Reagent® (Sigma-Aldrich, UK) according to the manufacturer's protocol. In brief, parasite samples were homogenised by adding 0.5 mL of TRI Reagent (Sigma Aldrich Company Ltd, UK) in 1.5 mL screw cap tubes and then incubating on ice for 30 min. Then, samples were homogenised in a mini-bead beater-24 (BioSpec Products Inc, USA) for 45 s. The homogenised samples were incubated at room temperature for 5 min. Then, 500 µL of chloroform was added to each sample and samples were shaken vigorously by hand for 15 s and then incubated at room temperature for 15 min. The samples were centrifuged at 20,000 × g for 15 min at 4 ºC. The aqueous (upper) layer that developed at the surface of each sample was removed slowly with a pipette and transferred to a new 1.5 mL tube. After phase separation, RNA was precipitated from the aqueous phase by addition of ½ volume of RNA precipitation solution (1.2M NaCl and 0.8M sodium citrate sesquihydrate) to ½ volume 100% isopropanol; propan-2-ol, isopropy alcohol and 2-propanol (Fluka, 59304). The samples were gently inverted 4-6 times, incubated for 10 min at room temperature and centrifuged at 20,000 × g for 10 min at 4 °C. The RNA precipitate formed a gel like pellet on the side/bottom of the tube. The supernatant was removed by pipetting leaving behind the pellet, which was washed for 1 h at room temperature with 1 mL of 75 % ethanol in water (v/v). This was accomplished by flicking and inverting the tube a few times to lift the pellet from the bottom and ensure the entire surface of the pellet and tube were washed. The samples were centrifuged at 20,000 x g for 5 min at room temperature. Thereafter each supernatant was removed using a separate pipette. The samples were spun for 3-5 s

and any remaining ethanol was removed with a pipette. The RNA pellets were then air dried at room temperature for 3-5 min until all visible traces of ethanol were gone and the pellets were re-suspended in an appropriate amount of RNase free water. The tubes were flicked to ensure the pellets completely dissolve in the RNase free water. For each sample, the RNA concentration and purity were measured at absorbance wavelength ratios of 260/280 nm and 260/230 nm, respectively, by spectrophotometry using a NanoDrop ND 1000 (Thermo Scientific, Wilmington, USA). The integrity of the RNA was confirmed by agarose gel electrophoresis using 1 % agarose gel in 0.5 x TAE buffer containing 0.3 - 0.5 -1 µL ethidium bromide (10 mg mL^{-1}) and Ultra-Violet fluorescent detection (INGENIUS, Syngene Bio imaging, Cambridge, UK).

The total RNA was re-suspended in nuclease-free water (Ultra-pure water). RNA concentrations were measured using UV spectroscopy (NanoDrop ND-1000, Thermo Scientific, USA) and RNA integrity evaluated by agarose gel electrophoresis and 1.5% ethidium bromide staining prior to storage at -70°C for later use.

3.2.3 **Primer design**

Since little genomic data were available for branchiuran parasites at the inception of this work, candidate transcripts for immunomodulatory peptides and associated targets were identified from other crustacean groups and employed to design specific or degenerate primers for *Argulus*. The closest relevant group having good genomic resources was the Copepoda, and transcripts from this group, particularly from the Caligidae, were employed to search for homologous targets in *A. siamensis* data.

3.2.3.1 **Design primers using sea lice immunomodulatory targets**

Two candidate targets were initially selected to allow optimisation of protocols. These targets comprised aspartic protease (Accession: BT120884) and cathepsin L (Accession: BT120520), from the salmon louse (*Lepeophtheirus salmonis*) for which sequences were obtained from NCBI online data. These are believed to have an immunomodulatory role during the feeding process. Aspartic proteases are involved in blood digestion and are responsible for initiating host haemoglobin degradation (Maldonado-Aguayo *et al*. 2015; Walton 2008) while cathepsin L is thought to be involved in feeding and immune evasion and in reproduction (Maldonado-Aguayo *et al*. 2015; Dalton *et al*. 2003; Rhoads & Fetterer 1995). BLASTn for dissimilar sequences was employed against the Transcriptome Shotgun Assembly (TSA) database for *A. siamensis* in order to find regions of similarity between *L. salmonis* and *A. siamensis*, for each target homologue. Following this process, BLASTn was used to look for other crustacean homologues, looking for those with closest taxonomic affinity to *A. siamensis*, in order to help design degenerate primers if necessary.

3.2.3.2 **Polymerase chain reaction (PCR)**

Aspartic protease and cathepsin-L primers were checked against two *A. foliaceus*; male and female, RNA template samples (100 ng/µL). For RT-PCR reactions, the Bioline MyTaq™ One-Step RT-PCR kit (BIO-65048) was employed to prepare a master mix. PCR product was checked in 1 % agarose gel ethidium bromide staining.

To make 100 ng / µL from each template, 1 µL from the adult female RNA template (RNA con. 1842.67 ng / µL) was added to 19µL of nuclease free water to make a total of 20 µL and from the adult male (RNA con. 283.14 ng / µL) RNA template 6.6 µL was added to 13.4 µL of H_2O to make total of 20 µL with 100 ng / µL. Five microlitres from each RNA template was mixed with 25 µL of 2x 1 step Bioline MyTaq mix followed by addition of 2 µL forward and reverse primers (10 µM). Then, 1 µL RNase inhibitor was added followed by the addition of 0.5 µL of the reverse transcriptase. The PCR reaction was run at different temperatures 54 - 61°C to optimize the reactions for 40x cycles.

Different troubleshooting steps were used to optimize the methodology, (1) cDNA templates were prepared using Tetro cDNA synthesis kit, Bioline (BIO-65042) followed by two RT- PCR reactions with different annealing temperatures 61°C at 40 x and 62.5°C at 30x (2) use of only a female template run in a PCR reaction with gradient temperatures of 60.5, 61, 61.5 and 62°C at 35x (3) use of a further PCR reaction run between 59-62°C for 34x using a different type of master mix MyTaq™ Mix, Bioline (BIO-), with aspartic primers only (4) use of a fast extraction method to extract DNA samples from *A. foliaceus* collected from loch Fad in 100% ethanol to check for the presence of the selected proteins; Aspartic and Cathepsin-L. 34x cycles RT- PCR reaction was run using DNA template at gradient temperature 55-64 °C with Aspartic and Cathepsin-L primers.

3.2.4 *De novo* transcriptome sequencing and assembly for *A. coregoni* and *A. foliaceus*

3.2.4.1 Library construction and sequencing of *A. coregoni* using MiSeq V2

Extracted RNA samples from *A. coregoni* were prepared following the requirements of Edinburgh Genomics, the core genomics and bioinformatics facility of University of Edinburgh. Turbo DNase treatment and removal reagents (Ambion® TURBO DNA-free™ kit; Life Technologies, AM1907) were used as per manufacturer's instructions, to remove any contaminating DNA and divalent cations such as magnesium and calcium, which can catalyse RNA degradation when RNA is heated with the sample. The DNase-treated RNA samples were purified using RNeasy Mini columns as per manufacturer's instructions (Qiagen, 74106). The RNA was finally eluted in 50 µL of pre-warmed (55°C) nuclease-free dH2O. RNA quantification was measured using (i) Nanodrop (the GenePool literature states that the purified RNA ratios must to be >1.8) and (ii) Qubit, the Qubit RNA HS kit was used to prepare the samples (Life Technologies, Q32852) as per manufacturer's instructions. The RNA templates were frozen at -70°C and later diluted to ca. 100ng μL^{-1}. To check RNA integrity for each RNA sample, samples were run on a 1% agarose gel containing EtBr (final concn. = 0.05mg mL^{-1}) before being sent for sequencing. cDNA library construction and sequencing for *A. coregoni* adult males and females, was carried out by Edinburgh Genomics. mRNA was isolated using oligo (dT) and fragmented using fragmentation buffer. To perform second strand-synthesis, reverse transcriptase and random primers were used, with ends repaired for ligation of the primers. This was followed by

purification and enrichment of the product with a PCR generating clusters with the final cDNA library. The constructed cDNA library was sequenced using paired-end (2x150) sequencing on a MiSeq v2 (Illumina, Inc.).

3.2.4.2 **Library construction and sequencing of *A. foliaceus* using HiSeq 2500**

RNA-Seq libraries for *A. foliaceus* samples were generated by Theragen BioInstitute, Republic of Korea. The Theragen recommendation was to check the total RNA integrity following isolation using an Agilent Technologies 2100 Bioanalyzer and requiring an RNA Integrity Number (RIN) value greater than or equal to 8. RNA that has DNA contamination will result in an underestimation of the amount of RNA used. Theragen recommends including a DNase treatment step with the RNA isolation method.

RNA concentrations and integrity were checked by Nanodrop (LabTech) and a 1.5% agarose gel correspondingly. Purification was conducted using the RNeasy Mini Kit (Qiagen; 74106) as described by the manufacturer (Qiagen). The quality was rechecked by Nanodrop and 1.5 % agarose (+EtBr) gel using 0.5X TAE buffer. Purified RNA samples were aliquoted and stored at -70°C until required. As recommended by Theragen, the best preparation of RNA for storage and transport is ethanol precipitation. For this, 0.1 volume of 3M sodium acetate (NaAce, pH7-8) was added to the RNA solution and mixed gently followed by 2 volumes of 100% ethanol (molecular grade, Sigma) and similarly mixed. Following this, samples were shipped on dry ice to the laboratory. cDNA library construction and sequencing of *A. foliaceus* was conducted by Theragen BioInstitute. The quality of the constructed cDNA libraries

was evaluated by Theragen BioInstitute using an Agilent 2100 BioAnalyzer (Agilent, CA, USA). They were quantified with the KAPA library quantification kit (Kapa Biosystems, MA, USA) according to the manufacturer's library quantification protocol. Following cluster amplification of denatured templates, sequencing was progressed as paired-end (2×100bp) using an Illumina HiSeq2500 (Illumina, CA, USA).

3.2.4.3 Reference data used

To assist assembly, two existing transcriptome datasets *Argulus* were used. These comprised a *A. siamesis* dataset (P. K. Sahoo *et al.*, 2013) and an *A. foliaceus* dataset (Pinnow *et al.*, 2016) logged under the accession numbers Bioproject Accession: PRJNA293150 and Bioproject Accession: PRJNA167720 respectively.

3.2.5 Sequence data processing and transcriptome assembly for *A. foliaceus* and *A. coregoni*

The transcriptome reads obtained for *A. coregoni* and *A. foliaceus* were assembled using the following bioinformatics pipeline.

3.2.5.1 Quality Control Processing

The raw data were download as a "Fastq" text file and quality control of these raw data were conducted using FastQC v0.11.4 (Andrews, 2010). The data were then trimmed

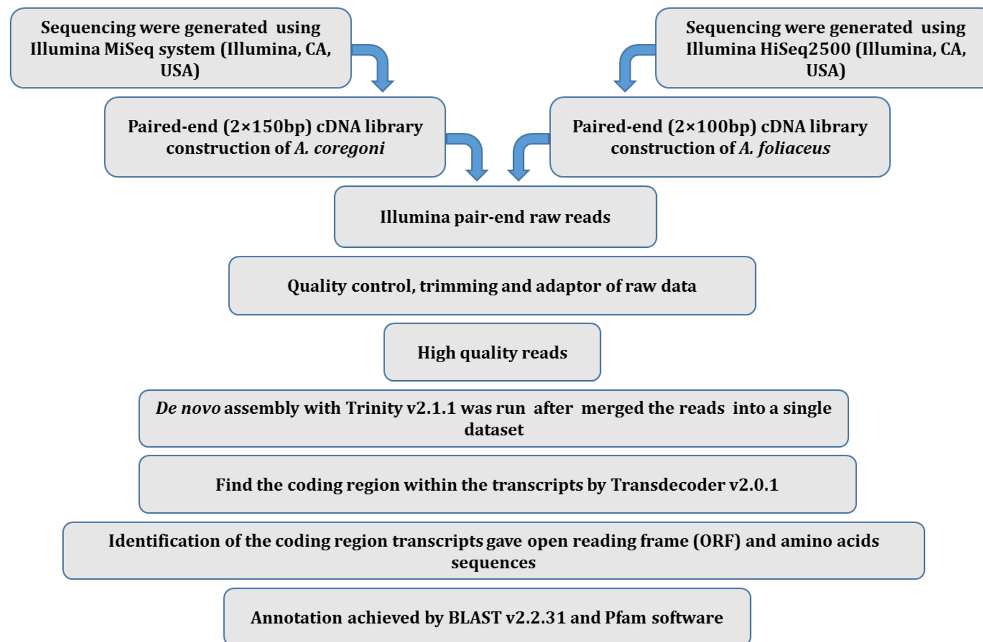
using the Trimmomatics v0.35 program (Bolger *et al.*, 2014). This program was run using the parameters option “ILLUMINACLIP”. Deconseq v0.4.3 program (Schmieder & Edwards, 2011) was used to remove any sequence contamination. For this, reads were mapped against the following databases: *Homo sapiens* (GCF_000001405), *Oreochromis niloticus* (GCF_000188235), green monkey, *Chlorocebus sabaeus* (Linnaeus, 1758) (GCF_000409795.2), *phix,* Atlantic salmon, *S. salar* (GCF_000233375), rainbow trout, *O. mykiss* and viruses.

3.2.5.2 *De novo* assembly and annotation

De novo assembly was performed on the cleaned RNA-seq raw data using Trinity v2.1.1 (Grabherr *et al.*, 2015). The selection of the Trinity tool for final assembly was decided upon following a trial with other assembler software such as Velvet and ABySS where the use of Trinity found to give higher numbers of more consistent reads. All the sample reads were merged into a single dataset for each species and the assembly was run. Then Transdecoder v2.0.1 (Haas *et al.*, 2013) was used to find the coding region within the transcripts. Identification of the coding region transcripts gave open reading frames (ORFs) and amino acid sequences, to prepare the assembled dataset for annotation.

Annotation was achieved by 1) BLAST v2.2.31 software (BLAST Altschul *et al.* 1990, BLAST+ Camacho *et al.* 2008) using the uniprot/trembl-invertebrates database as query (The UniProt Consortium, UniProt 2015) and 2) Annotation HMMER v3.1b2 (Eddy, 1998) using the Pfam A v29.0 as query (Finn *et al.*, 2016).

The summary of *de novo* whole transcriptome analysis workflow of *A. coregoni and A. foliaceus*



3.2.6 **Gene Ontology annotations**

Gene Ontology (GO) annotations were retrieved from the UniProt GO annotation project [http://www.ebi.ac.uk/GOA]. All proteins described in the UniProt (Swiss-Prot and TrEMBL) KnowledgeBase, including the uniprot/trembl-invertebrate's database, and used during the annotation stage, are associated with GO controlled vocabulary. The set of all GO annotations for the proteins in the UniProt KnowledgeBase (UniProtKB) was downloaded [accessed the 2016-11-09] and for each, the uniprot/trembl-invertebrates GO annotation was recovered and added to the transcriptome annotations. GO annotation was conducted for both *A. foliaceus and A. coregoni* transcriptomes as well as for the pre-existing *Argulus* transcriptomic libraries

(*A. siamensis* with Accession: PRJNA167720 and *A. foliaceus* with Accession: PRJNA293150) held by the National Centre for Biotechnology Information (NCBI).

3.2.7 **Identifying shared transcript expression between the three *Argulus* species**

OrthoVenn is a powerful web platform genomics tool used for comparative and visualisation analyses of orthologous protein clusters from different species. The resulting overlap helps in identifying proteins' function and evolution across multiple species (Wang *et al.*, 2015). OrthoVenn was applied in this study to check for shared expressed genes / proteins. The tool was applied to identify gene clusters enriched in four transcriptome libraries for three *Argulus* species comprising *A. foliaceus*, *A. coregoni* and *A. siamensis*. To achieve this, a list of proteins was first prepared for each *Argulus* species from the four transcriptome datasets. Then, the ClusterVenn tool was used for generating Venn diagrams from *Argulus*-defined cluster files by clustering proteins of similar sequence and function, *i.e.* orthologous and paralogous genes, between species. The tool compares between clusters only and ignores singletons where there are no orthologues between species and no paralogues within species. The default OrthoVenn parameters were used i.e. e-value cutoff of 1e-5 for all-to-all protein similarity comparisons and an inflation value (-I) of 1.5 for the generation of orthologous clusters.

In addition to the previous features, OrthoVenn also allows searching for specific proteins using the BLAST feature. In this study, it was used to search for some known immunomodulatory candidates to check if any existed within the annotated shared

clusters and related functions. The selected targets are: trypsin, serpin (serine protease inhibitor), cathepsin-L, aspartic protease, ferritin, cysteine protease, enolase, phospholipase, adenosine deaminase, apyrase, metalloprotease, thrombin inhibitor and venom serine protease. Serine protease inhibitor (Serpin), cysteine, aspartic protease and metalloprotease all belong to the protease family.

3.2.8 **Phylogenetic reconstruction**

The longest coding sequence (CDS) for selected proteins of *A. foliaceus*, *A. coregoni* and *A. siamensis* were translated into amino acid sequences and a BLASTP was performed against all Oligostraca sequences present in the NCBI NR database (accessed the 2016-11-09). A total of 10 species, shared the top 100 shared proteins (**Appendix 5**). Each shared protein sequence was retrieved and aligned individually using ClustalO v1.2.3. Each alignment was concatenated before running RaxML v8.0.0 using partitioned models (one model per protein, based on a GAMMA model of rate heterogeneity with an estimate of proportion of invariable sites and using the BLOSUM62 substitution rates and maximum-likelihood estimate of the base frequencies) with 1000 bootstrap [-# 1000 -m PROTGAMMAIBLOSUM62X].

3.3 Results:

3.3.1 Degenerate primers:

The result of the BLASTn of aspartic protease and cathepsin-L; showed good matches to *L. salmonis*. The general blast also showed good hits for other species but these were phylogenetically distant from Crustacea. Having identified conserved regions, two primers for each target were designed using Primer-Blast in NCBI. Reverse Transcriptase PCR reaction results for the Cath_ASi and Asp_Asi primers initially showed multiple bands. Thus, the work was repeated using different optimising measures *e.g.* (1) higher annealing temperatures, (2) Tetro cDNA synthesis kit, Bioline (BIO-65042), (3) female cDNA only and (4) genomic DNA. No genomic DNA or cDNA templates showed any positive or expected results. Thus, the decision was taken to wait for the species-specific transcriptome data.

3.3.2 De novo transcriptome sequencing of *Argulus coregoni* and *Argulus foliaceus*

3.3.2.1 Quality Control Processing

Extracted RNA quantity and quality were checked before sending RNA samples for sequencing. The quality and concentration were within the expected range (a ratio of

~2.0 is generally accepted as "pure" for RNA 260/280 ratio) for both species (*A. foliaceus* and *A. coregoni*) (see **Table 3.1**).

Table 3.1 Total RNA concentration before and after purification and 260/280 value of

(a) Argulus coregoni

Sample	Before purification		After purification	
	RNA conc. (ng/µL)	260/280	RNA conc. (ng/µL)	260/280
Adult Female (n=1)	1421	2.15	150.3	2.14
Adult Male (n=1)	1146	2.11	161.6	2.15

(b) Argulus foliaceus

Sample	Before purification		After purification	
	RNA conc. (ng/µL)	260/280	RNA conc. (ng/µL)	260/280
Adult Male (n=1)	369	2	121.9	2.19
Adult Female (n=1)	610.6	2.11	63.3	2.17
Larvae (total weight = 2 g)	1476.2	2.11	308	2.17
Gland-rich tissue pool (n=6)	409.7	2.02	145	2.18

3.3.2.2 ***De novo* assembly and annotation**

To create the *A. coregoni* transcriptome sequence resource, total RNA of adult male and adult female samples was subjected to Illumina 150 bp paired end sequencing using the Illumina MiSeq platform (Illumina, Inc.). A total of 11,265,959 raw sequence reads (**Table 3.2**) was generated. Of the original reads, 10,840,092 reads passed quality control and filtering, and were then merged into a single dataset, assembled

by Trinity v2.1.1, and blasted to generate a *de novo* transcriptome assembly comprising 40,954 transcripts (contigs). The maximum transcript contig length was 9,791 bp, the mean contig length was 1,787 and the N50 value was 2,339 bp.

The high throughput RNA-Seq of *A. foliaceus* resulted (**Table 3.2**) in the generation of a total number of 88,255,979 paired end reads of 2×100bp, obtained from four cDNA libraries sequences (adult male, adult female, juveniles and dissected glands from six adult samples) using Illumina HiSeq2500 platform (Illumina, CA, USA). FastQC quality control, and adaptor trimming resulted in 84,256,934 reads of high quality. The high-quality reads were merged into a single dataset, assembled with Trinity v2.1.1, and blasted to get total assembled contiguous sequences (contigs) of 66,940 reads. The maximum transcript contig length was 17,078 bp, the mean transcript contig length was 1,842 bp and the N50 value was 2,573 bp. The *K*-25 default parameter of the Trinity software was used for the assembly.

The existing transcriptome shotgun assembly (TSA) sequences dataset of *A. siamensis* (Accession PRJNA167720) and *A. foliaceus* (Accession: PRJNA293150) were checked and the total number of reads, number of assembled contigs (transcripts) and N50 are summarised in (**Table 3.2**) alongside the results for the libraries sequenced in the present study.

Table 3.2 Summary data generation and statistics for *de novo* transcriptome assembly of *A. coregoni, A. foliaceus* and *A. siamensis*, first two columns comprise sequencing results for the current study.

Data generation	*Argulus coregoni*	*Argulus foliaceus*	*Argulus siamensis**	*Argulus. foliaceus**
Raw reads	11,265,959	88,255,979	77,759,443	52,725,850
Total contigs	73,164,334	123,272,467	50,396,610	16,894,535
Number of contigs	40,954	66,940	46,352	8,424
Largest contig (bp)	9,791	17,078	26,436	16,889
Mean contig (bp)	1,787	1,842	1,211	2,006
N50 value in bp	2,339	2,573	2,302	1,499
GC%	41.3	40.74	38.29	42.05
RNA-Seq Technology	Illumina MiSeq v2	Illumina HiSeq 2500	Illumina HiSeq 2000	Illumina HiSeq 2500
Assembly software	Trinity v2.1.1	Trinity v2.1.1	Velvet/Oases	CLC-Genomics Workbench 7.5.1

*NCBI Reference genomic resources. *A. siamensis* (Accession PRJNA167720) and *A. foliaceus* (Accession: PRJNA293150)

3.3.3 Gene ontology annotation

Gene ontology (GO) analysis facilitates the biological interpretation of the studied organism through fast and reliable functional annotation. It serves to classify gene products and standardise their representation across species (Götz *et al.*, 2008).

GO terms were retrieved for the annotated transcripts, with the longest sequence for each transcript selected. Following the use of BLASTp against the UniProt_Trembl (invertebrate) database, the maximum number of GO terms were found. The GO analysis was applied to the experimental species (*A. foliaceus* and *A. coregoni*) and

the reference datasets from NCBI (*A. siamensis* and *A. foliaceus*). **Table 3.3** summarises the GO distribution of the three *Argulus* species from the four transcriptome datasets.

3.3.3.1 **GO functional annotation of *A. coregoni* transcripts**

GO analysis of *A. coregoni* was conducted using the transcriptome data. Transcriptome analysis resulted in 183,125 GO terms assigned to 1,904 transcripts. Of the three catogeries, 30% (91,041) of the terms were associated with transcripts for molecular function (MF), 50% (55,981) to biological process (BP) and 20% (36,103) to cellular components (CC). The GO category terms were the same as those obtained for *A. foliaceus*.

3.3.3.2 **GO functional annotation of *A. foliaceus* transcripts**

For the *A. foliaceus* transcriptome analysis, 251,456 GO terms were found and assigned to 2,076 transcripts. Of the three catogeries, 50% (125,676) of the terms were associated with molecular function (MF), 31% (77,049) to biological process (BP) and 19% (48,731) to cellular components (CC). In the MF category, binding activity and catalytic activity were the most heavily represented. For BP the most represented terms were “transport” and “metabolic process”. The most represented terms from the ontology categories were “membrane” and “integral component of membrane” for CC.

3.3.3.3 **GO functional annotation of database *A. siamensis* transcripts**

GO analysis of the databased transcriptome assembly of *A. siamensis* (Accession PRJNA167720) resulted in 179,710 terms assigned to 1,931 transcripts. MF comprised 52% (93,791) of the total GO terms while 32% (57,668) were associated with BP and 16% (28,251) with CC.

3.3.3.4 **GO functional annotation of database *A. foliaceus* transcripts**

Transcriptome assembly of databased *A. foliaceus* (Accession: PRJNA293150) resulted in 42,361 GO terms assigned to 1,596 transcripts. These included MF terms 49% (20,952), 31% (13,146) BP and 20% (8,263) CC.

Table 3.3 Summary of GO distribution for the three *Argulus* species.

Species	***A. foliaceus***	***A. coregoni***	***A. siamensis***	***A. foliaceus****
Total GO terms	251,456	183,125	179,710	42,361
Number of assigned transcripts	2,076	1,904	1,931	1,596
Number of cellular component terms	48,731 **(19%)**	36,103 **(20**%)	28,251 **(16%)**	8,263 **(20%)**
Number of molecular function terms	125,676 **(50**%)	91,041 **(50%)**	93,791 **(52%)**	20,952 **(49%)**
Number of biological process terms	77,049 **(31%**)	55,981 **(30%)**	57,668 **(32%)**	13,146 **(31%)**

Overall, the GO distributions for the four transcriptome results of the three species (*A. coregoni*, *A. foliaceus* and *A. siamensis*) were highly similar, as shown in **Table 3.3** and in the GO distribution chart (**Fig. 3.2, 3.3, 3.4, 3.5**) and (**Fig. 3.6)** for *Argulus*

species, which represents the GO distribution of the first 20 clustered annotated proteins from the four transcriptome dataset.

3.3.4 **Identifing shared genes expressed in *Argulus* species**

Venn diagrams (A and B) showing the shared orthologous gene clusters among three *Argulus* species. The transcriptome results of *A. foliaceus* (135,679 transcripts, 57,928 unique CDS and 7,932 clusters) of this study and *A. foliaceus* with Accession: PRJNA293150 (8,424 transcripts, 8,424 unique CDS and 6,567 clusters) (Pinnow *et al.*, 2016) were combined to check for shared proteins/genes. Out of 7955 clusters of both datasets, 6522 orthologous clusters and 6425 single-copy gene clusters were found (Venn Diagram A). OrthoVenn analysis of the three *Argulus* species showed that 13,324 orthologous clusters were formed based on the protein sequences from the three species. The diagram shows that 6,674 gene clusters are shared by all three species. See Venn Diagram B.

Blasting of the selected targets (trypsin, serpin, serine protease, cathepsin-L and aspartic protease, ferritin, cysteine protease, enolase, phospholipase, adenosine, apyrase, metalloprotease, thrombin inhibitor, venom serine protease) against the resulting shared clusters (6,674) gave a number of hits. For trypsin, 25 annotated clusters were identified and classified into six groups according to their functions. Serpin resulted in four clusters described as alaserpin (Swiss-Prot Hit) all having the same GO annotation. Eleven clusters were found for serine protease and grouped in eight GO annotation groups. Only a single cluster each was identified for cathepsin (cathepsin-L), apyrase, aspartic protease (Aspartic protease 6), cysteine protease,

enolase and thrombin inhibitor. Two types of ferritin were found with one being yolk ferritin. Two cluster proteins comprised phospholipase A2, enzymes that hydrolyse phospholipids into fatty acids and other lipophilic substances, combining 11 GO annotation functions. There were 17 clusters of adenosine as named by Swiss-Prot Hit; each one having a different GO annotation function. By checking for matches to metalloprotease, 5 clusters distributed between three GO annotation functions were identified. Finally, searching for matches to venom serine protease resulted in 6 genes (proteins) clusters with 3 different GO annotations. (**Table 3.4**).

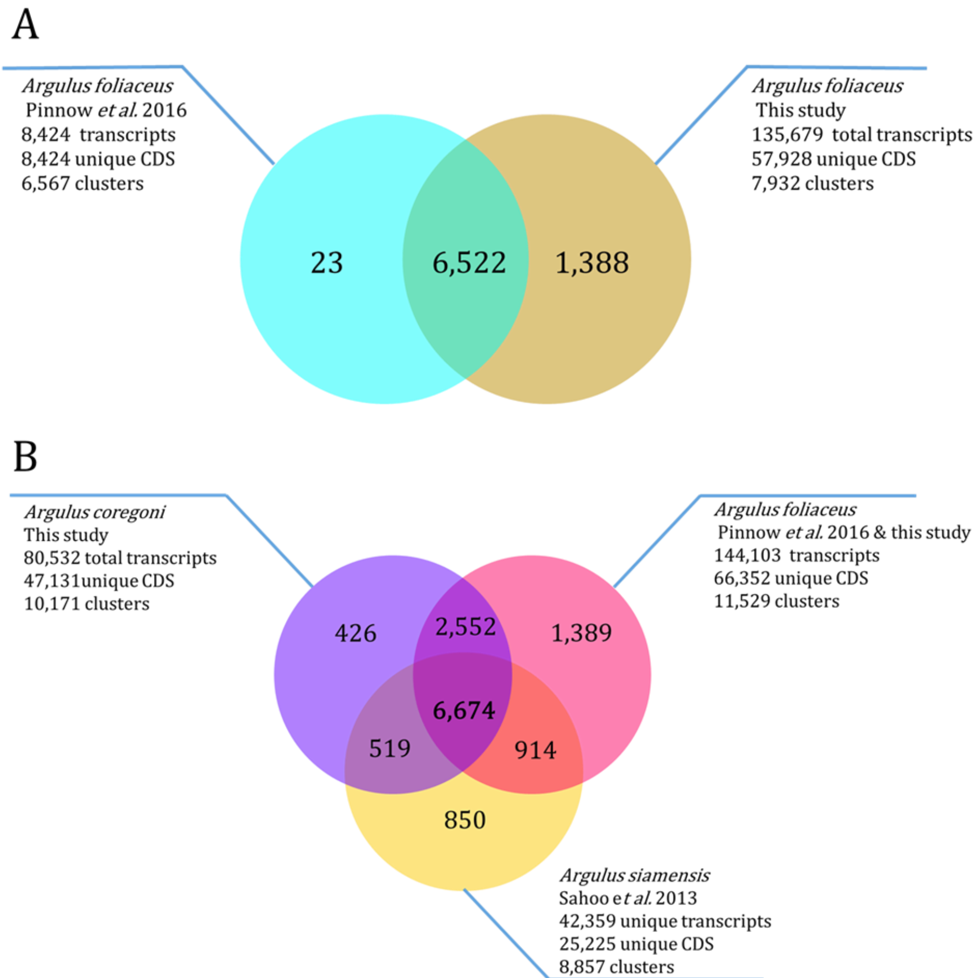


Figure 3.1. Venn diagrams showing the number of orthologous clusters. (A) Two *A. foliaceus* transcriptomes, which shared 6,522 orthologous clusters and (B) transcriptomes from all three targeted species (*A. foliaceus*, *A. coregoni* and *A. siamensis*). The three species display 13,324 clusters with 6,674 orthologous clusters shared between the species.

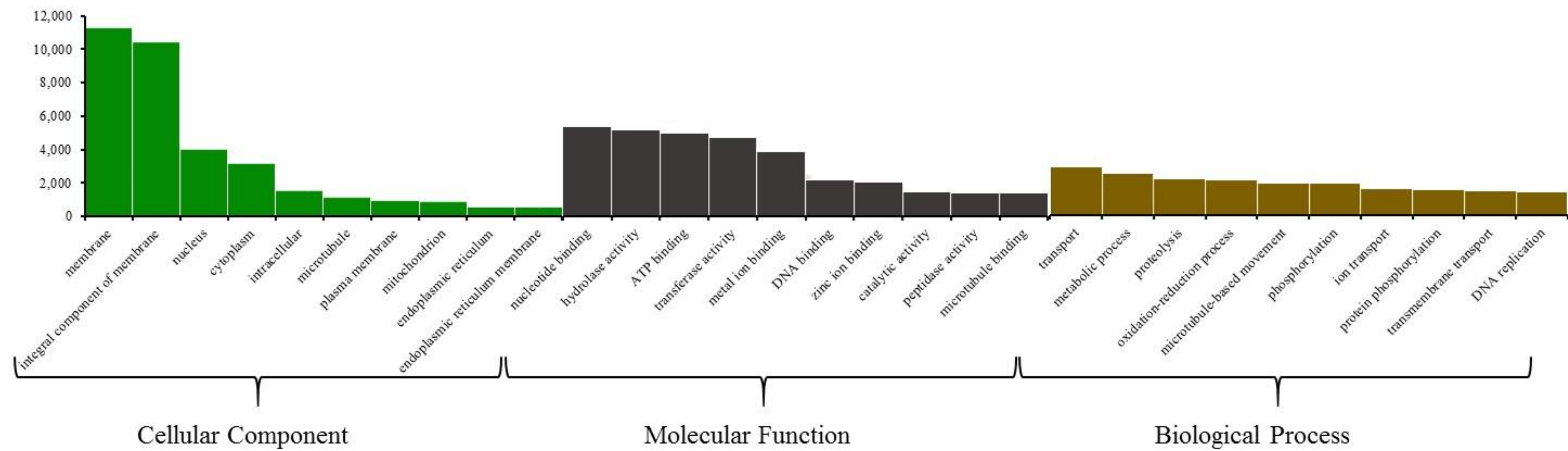


Figure 3.2 Distribution of GO terms from BLASTp hits with the transcriptome of *A. foliaceus* from this study.

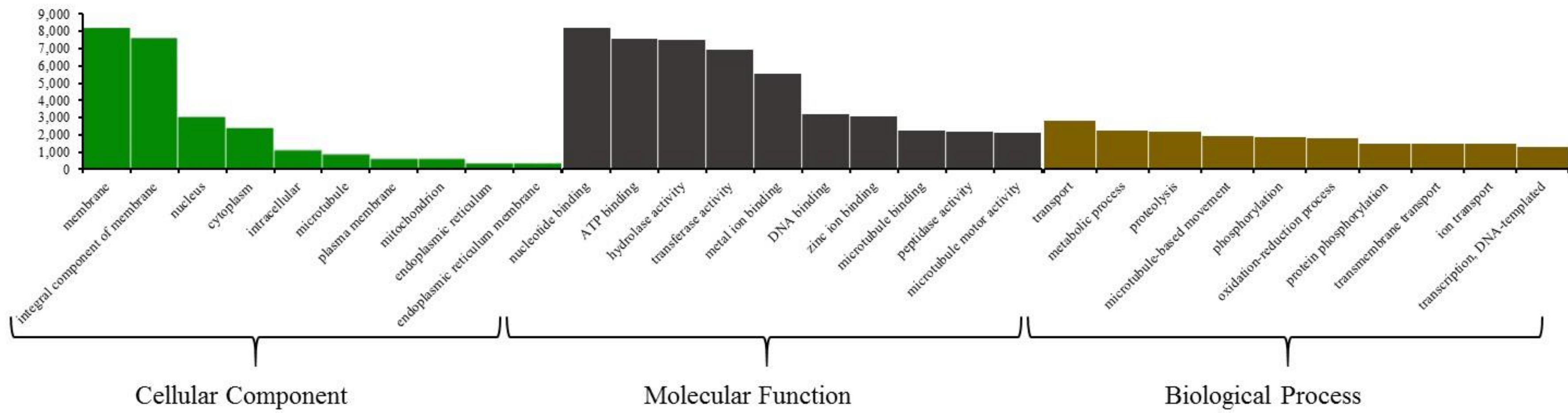


Figure 3.3 Distribution of GO terms from Blastp hits with the transcriptome of *A. coregoni* from this study.

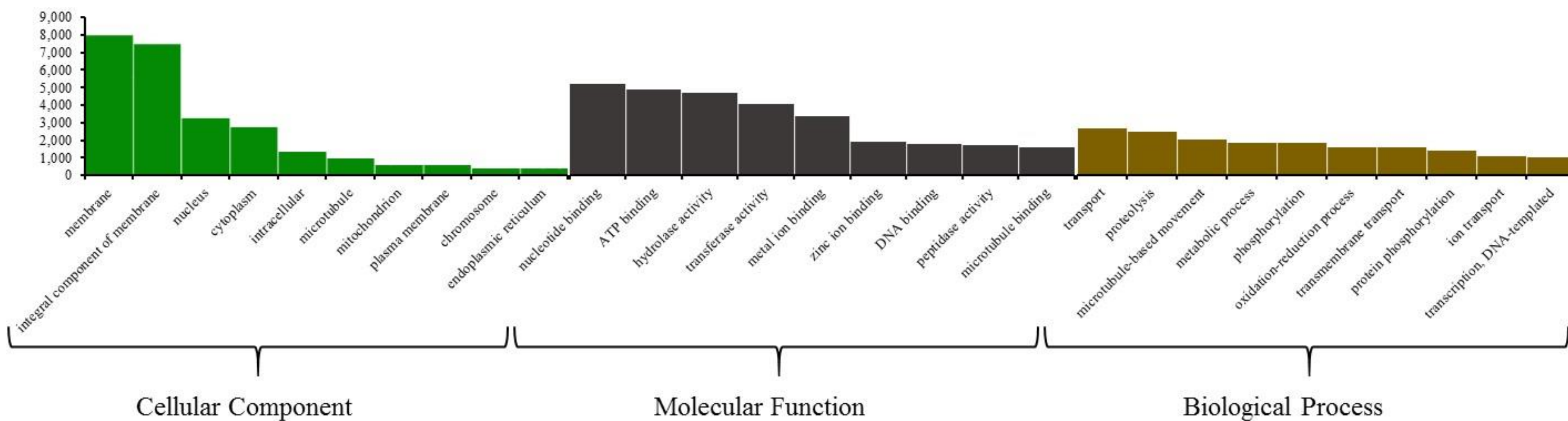


Figure 3.4 Distribution of GO terms from Blastp hits with the transcriptome of *A. siamensis* (Accession PRJNA167720).

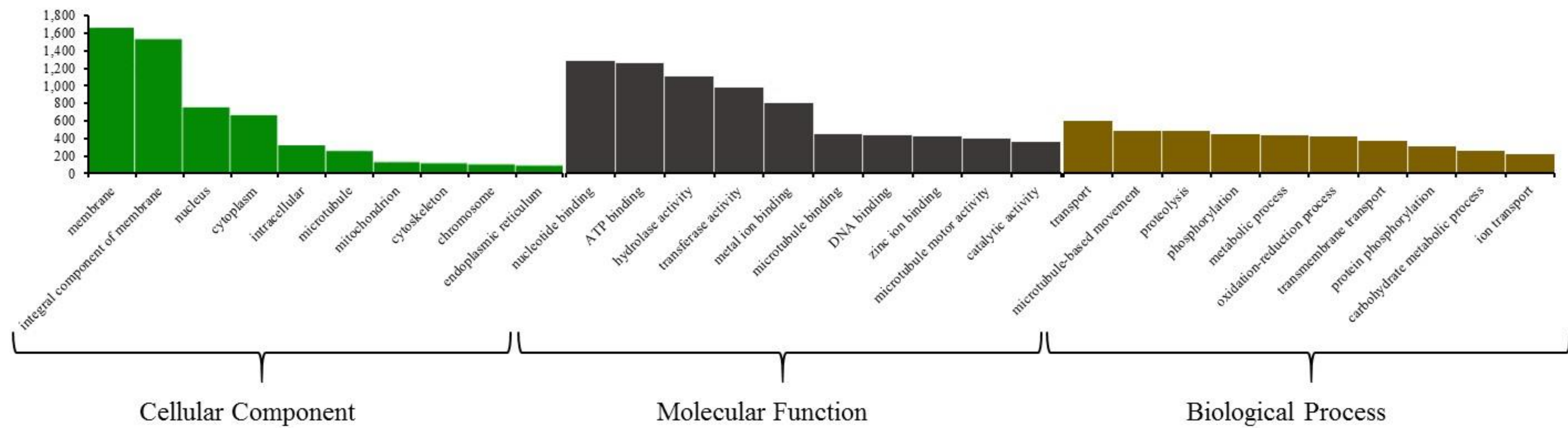


Figure 3.5 Distribution of GO terms from Blastp hits with the transcriptome of *A. foliaceus* (Accession: PRJNA293150).

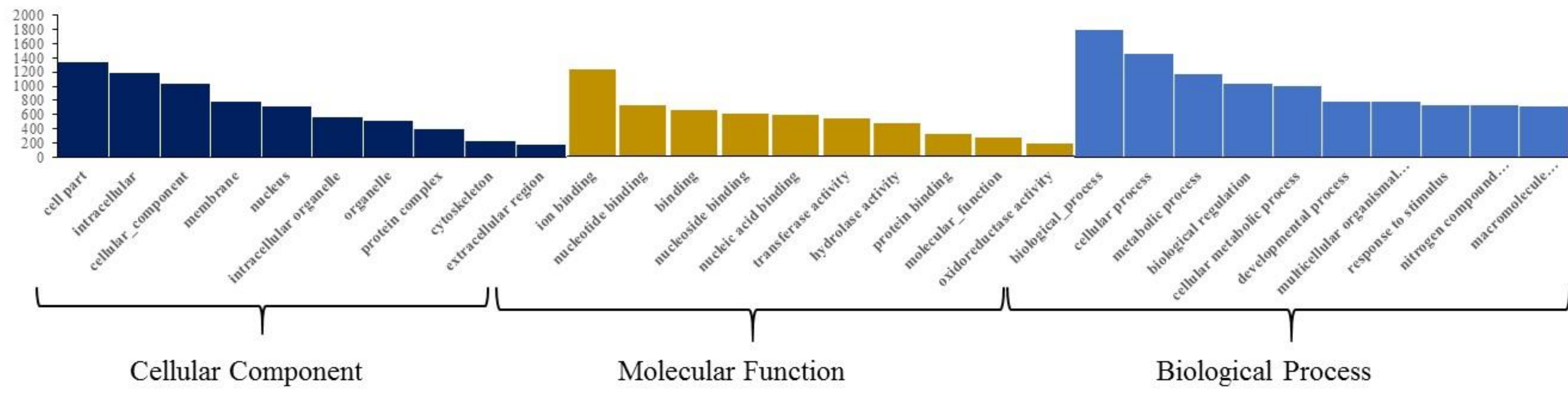
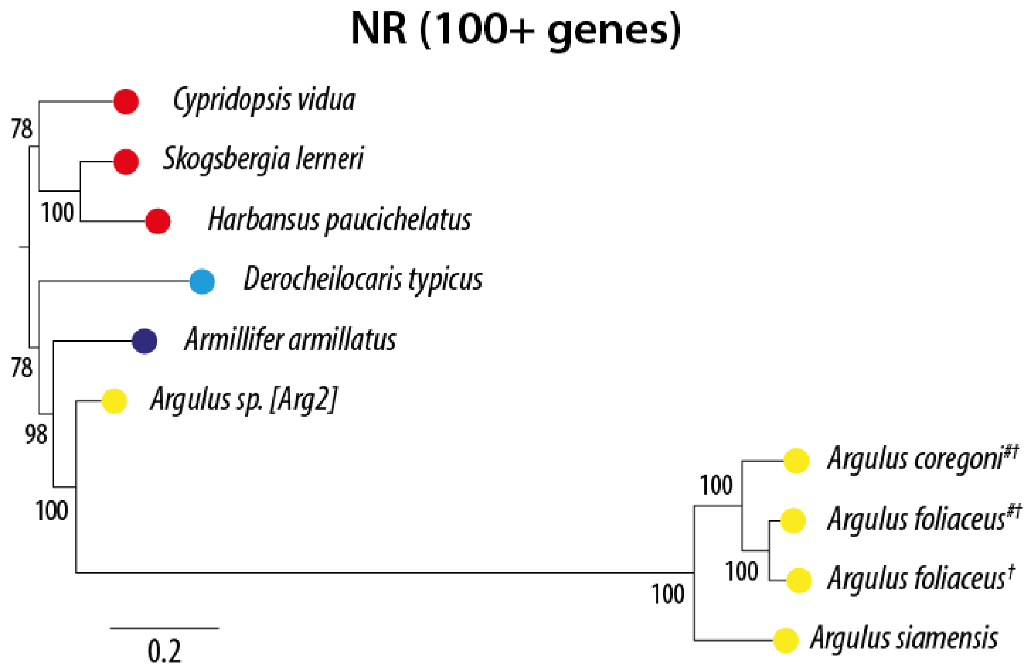


Figure 3.6 Distribution of GO terms of the first 20 clustered annotated proteins from the four transcriptome results of *Argulus* species.

Table 3.4 Identification of targeted immunomodulator candidates from *Argulus* spp.

Protein Class	Protein	# Proteins	Function(s)	References
Serine protease	Trypsin	25	Digestion and anti-haemostatic	Fast *et al.* 2003 and Firth *et al.* 2000
Protease inhibitor	Alaserpin	4	Anti-blood clotting and anti-complement activation	Ribeiro & Francischetti 2003 Mulenga *et al.*, 2013
	Serine protease inhibitor	2	Anti-coagulation Vasodilator Anti-inflammation	von Reumont *et al.* 2014
Protease	Aspartic protease	1	Haemoglobin proteolysis	Maldonado-Aguayo *et al.* 2015
	Cysteine protease	1	Anti-inflammatory Haemoglobin digestion	Kotsyfakis *et al.* 2006 and Yamaji *et al.* 2009
	Venom serine protease	8	Extracellular matrix degradation Anti-clotting, Inflammation	von Reumont *et al.* 2014
	Cathepsin-L	1	Prevention of blood coagulation	Rhoads & Fetterer 1995
	Metalloprotease	4	Anti-hemostatic	Francischetti *et al.* 2003
Glycoprotein	Ferritin	2	Iron storage and transport - involved in homeostasis of iron during feeding	Rawal *et al.* 2016
Metalloenzyme	Enolase	1	Degrades plasminogen Aids host penetration	Chen *et al.* 2012
Phospholipase	Phospholipase A2	2	Hydrolyses phospholipids (deactivate platelet-activating factor)	Pawlowic & Zhang 2012
Purine metabolism enzyme	Adenosine deaminase	2	Vasodilator Antiplatelet	Ribeiro & Francischetti 2003 and Champagne 2004
Diphosphohydrolase	Apyrase	1	Anti-pain Anti-inflammatory Antihaemostatic Platelet aggregation inhibitors	Ribeiro & Francischetti 2003; Champagne 2004 and Chmelar *et al.* 2012
Serine protease inhibitor	Thrombin inhibitor	1	Anticoagulation inhibitor	Ribeiro & Francischetti 2003

3.3.5 **Phylogenetic reconstruction**



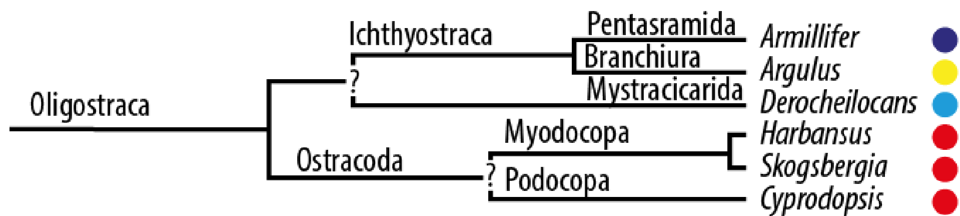


Figure 3.7 Phylogenetic tree showing the position of *Argulus* species from this study transcriptome (*A. foliaceus* and *A. coregoni*) and the previous transcriptome results (*A. foliaceus* and *A. siamensis*) relative to other members of the phylum Oligostraca. Top section: Phylogenetic reconstruction based on the top 100 shared genes, including those generated from this study. Bottom section: Oligostraca subtree, reproduced from Regier *et al.* 2010. Circle colour reflects subphylum. #† Species from this study. † from transcriptome reference (Pinnow *et al.* 2016).

3.4 **Discussion:**

There is currently limited understanding of many aspects of the general biology of argulids, however, use of new technologies can help to fill gaps in knowledge. Next generation RNA sequencing technologies allow rapid sequencing of the entire transcriptome of an organism at low cost compared to the previous technologies such as SAGE and cDNA / oligo microarray (Páez *et al.*, 2015). In this study, sequences identified from different *Argulus* species using RNA-seq technologies (Ilumina HiSeq and MiSeq) were analysed according to two strategies: *de novo* assembly of *A. foliaceus* and *A. coregoni* transcripts from samples collected as part of the current study, and reference transcriptome based assembly of *A. siamensis*; PRJNA167720, and *A. foliaceus;* PRJNA293150 (Sahoo *et al.,* 2013 and Pinnow *et al.,* 2016). *De novo* assembly means that: (1) there is no need for a reference genome and (2) the quality of the assembly depends on the depth of sequencing, rate or error-read and the transcriptome complexity. Although, these types of assemblies are very sensitive to sequencing errors, paired-end reads providing extensive sequence repetition can, given sufficient depth of sequencing, facilitate the construction of the original fragments (Páez *et al.*, 2015). The analysis of RNA-seq data generated by high throughput technologies is complicated and time consuming, thus the approach used for interpretation is critical. This situation is made more difficult by the fact that RNA-seq analysis procedures are developing continuously. As RNA-seq is an open technology, as opposed to *e.g.* microarray, novel transcripts can be detected from RNA-seq data (Oshlack *et al.*, 2010).

The transcriptomic sequencing work was undertaken with the key aim of identifying transcripts for proteins capable of playing a role in host-parasite interactions. In the absence of genomic references, *de novo* transcriptome technology can help in the detection of such transcripts (Oshlack *et al*., 2010).

This study has demonstrated the successful use of high-throughput sequencing strategies to generate sequence resources for the poorly represented species *Argulus* (*A. foliaceus, A. coregoni* and *A. siamensis*). This study, has provided 66,940 transcripts with 57,928 unique CDS from *A. foliaceus,* 40,945 transcripts with 47,131 unique CDS from *A. coregoni* as well as 8,424 transcripts and 8,424 unique CDS from the existing *A. foliaceus* database and 42,359 transcripts with 25,181 unique CDS from *A. siamensis*. This represents a considerable contribution to the resources available for *Argulus* spp. in particular and for the Branchiura more generally.

Although there are two genomic datasets for *Argulus* species in the National Centre for Biotechnology Information (NCBI) database, which were uploaded relatively recently for *A. siamensis* and *A. foliaceus*, these are limited in extent while the transcriptome results for the current study deliver the largest current genomic data set for *Argulus* with 87,954,885 reads assembled into 66,940 contigs and provides also the first sequence for *A. coregoni* with 73,164,334 which assembled to give 40,954 contigs. The number of transcripts produced from *A. foliaceus* shows the efficiency of Illumina HiSeq 2500 in producing higher numbers of transcripts.

Because there is no reference genome for *Argulu*s, the results of the transcriptome of both *A. foliaceus* and *A. core*goni were assembled using Trinity v2.1.1 software. Trinity was found in this study to give a better and deeper assembly than Velvet and ABySS

software. The assembled transcriptome results are important for both genomic and proteomic studies, especially the use of the complete ORF "open reading frame" information for genes that may play important roles in biological processes and can therefore provide potentially suitable vaccine candidates (Sahoo *et al*., 2013).

GO analysis was performed to give an overview of the functional roles of the successfully sequenced transcripts and their respective proteins for the three *Argulus* species of interest. GO application enables the mapping of information from model organisms to non-model species (Blake, 2013). It was reported that 50 % of the crustaceans' transcripts from transcriptomic studies were orthologous genes to a model organism and most of these genes belong to biological processes involving metabolism, development, or regulation of a biological process (as reported by Clark & Greenwood 2016). This concurs with the *Argulus* GO outcomes, as the predominant genes from the biological process analysis were involved in response to metabolic process. This was a predictable result as shown from other organisms, due to the enormous number of GO terms that cover basic processes required to maintain a living organism (Laurino *et al.*, 2016). One of the biological processes that developed very early in multicellular evolution and is shared between invertebrates and vertebrates is the innate immune system (Cooper and Alder, 2006). Knowledge of crustacean immune and stress responses are key in understanding crustacean physiology (Clark and Greenwood, 2016). Although, the annotation method applied in this study is not enough to provide detailed information about the immune pathways, the GO molecular functions associated with the *Argulus* transcripts revealed activities that might be related to immunity such as hydrolase, transferase and metallopeptidase activities which were dominant. Hydrolases, transferase enzymes and metallo-endopeptidase

proteins were described as being among the most abundant components in the venom of the parasitoid wasp *Toxoneuron nigriceps* (Viereck) (Hymenoptera: Braconidae) (Laurino *et al.*, 2016). Hydrolases have been identified from different parasite venoms which are known to include proteases, peptidases and glycosidase enzymes (Asgari & Rivers 2011; Moreau & Guillot 2005). Correlating with the results in this study, the presence of hydrolases might reflect their contribution in host-parasite interactions in *Argulus* species. The identified metalloproteases from different arthropods are assumed to be related to different biological processes such as digestion and host immune suppression (Laurino *et al.*, 2016). In general, the results of the GO annotation of the transcripts from the three-species identified a high representation of genes involved in metabolism, protein binding and nucleotide binding. These are linked with high basal metabolic activity (Bai *et al*. 2011), and although the precise reason is not known, various studies on the effects of parasitim on infected mammals have reported that parasites increased the basal metabolic rate of the host (Morand & Harvey 2000). The patterns of the GO analysis from three *Argulus* species were similar.

The sequences provided by this study are expected to contribute to future investigations of argulid biology. Clark and Greenwood (2016) recently suggested the use of a combined annotation approach, using manual annotation of blastx searches of the NCBInr and Swiss-Prot protein databases, along with a blastx search of the IIID; Insect Innate Immunity Database (Clark and Greenwood, 2016). Applying this type of analysis to the *Argulus* transcripts in the future might provide a better understanding of genes involved in immune pathways. Further characterisation of these transcript functions and their potential contribution to successful parasitism can provide a

valuable addition to *Argulus* biology and physiology. In summary, the presented databases will accelerate investigations of *Argulus*-host interactions.

The construction of a limited phylogenetic tree based on the top 100 shared genes for the four transcriptome datasets from *Argulus* available from this study and from previous results demonstrates the position of the sequenced *Argulus* species in the Oligostraca phylum as expected according to Regier *et al*. (2010) results and suggests that *A. coregoni* is more closely related to *A. foliaceus* than to *A. siamensis*. This probably reflects the fact that *A. foliaceus* and *A. coregoni* are indigenous *Argulus* species in the UK with closer and more recent evolutionary links than invading species from Asia. Long association in similar environments is also reflected by similar host specificities, or lack of them, infecting such disparate species as pike and rainbow trout. This also suggests that they may share similar mechanisms for successful parasite-host interaction.

The sequenced *Argulus* spp. transcriptomes were used to look for genes showing shared expression across the species examined. This was achieved by using the OrthoVenn software, which allowed the identification of orthologous clusters from the different species. Comparative functional annotation of overlapping clusters between the *Argulus* species indicated that transcripts sequenced for these three-species shared 6,674 gene clusters, suggesting continued conservation after speciation. Deeper / more extensive sequencing would no doubt increase this number of clusters. Identification of conserved genes can help in the search for more universal control targets for these species, for other *Argulus* species and perhaps for Branchiuran parasites more widely. Almost all studied blood feeding arthropods' saliva contains immunosuppressive proteins and investigating their roles and mechanisms of action

can provide novel methods for controlling these arthropods and the diseases that they may transmit as specific vectors (Titus *et al.*, 2006) or through phoresis. Specific components of blood-feeding arthropod saliva have active functions in altering the host immune response to help blood sucking (Valenzuela, 2002) and exploiting aspects of these powerful substances can provide an effective way to control disease distribution (Charlab *et al.*, 1999).

This transcriptomic study has revealed, for the first time in *Argulus* and indeed for the *Branchiura* more widely, a range of proteins / genes that have been previously characterised as being important immune mediators for blood-feeding insects and other haematophagous parasites. Trypsin, serpin, serine protease, cathepsin-L, aspartic protease, ferritin, cysteine protease, enolase, phospholipase, adenosine, apyrase, metalloprotease, thrombin inhibitor, and venom serine proteases were all represented in the identified shared transcript clusters for the three *Argulus* species and these results provide potential candidates for investigation of *Argulus* controls the future.

From a review conducted on different types of parasitic helminths; including nematodes, cestodes and trematodes, it is clear that in addition to the role of serine proteases in controlling physiological processes, they also have a major role in host-parasite interaction (Yang *et al.*, 2015). In this respect, proteases are commonly used by parasites in immunomodulation of the host where they can serve to assist parasite development and nutrition, host tissue and cell invasion, anticoagulation, and immune evasion (Yang *et al.*, 2015; Skern-Mauritzen *et al.*, 2009; Dzik 2006). Trypsin is one of the most common proteases employed by different haematophagous arthropods species such mosquitoes *Aedes aegypti* (Linnaeus in Hasselquist, 1762) (Isoe *et al.*,

2009), hard tick *Haemaphysalis longicornis*; Neumann, 1901 (Tirloni *et al.*, 2015), sand fly *Lutzomyia longipalpis* (Diptera : Psychodidae) (Evangelista and Leite, 2002), cattle tick *Rhipicephalus* (*Boophilus*) *microplus* (Tirloni *et al*. 2014), cattle warble fly *Hypoderma lineatum* (Viller, 1789) (Wikel, 1999), nematode *Heligmosomoides polygyrus* (Hewitson *et al.*, 2011), the biting midge *Culicoides sonorensis* (Diptera: Ceratopogonidae) (Campbell *et al.*, 2005), argasid tick *Ornithodoros moubata* (Díaz-Martín *et al.*, 2013) and a number of other different species of helminths, nematodes, cestodes and trematodes (Dzik, 2006).

Transcripts of a trypsin-like protease were detected in *Argulus* in the current study. Similarly, the mucus of Atlantic salmon (*Salmo salar*) infected by sea lice *L. salmonis,* for instance, contains trypsin-like proteases derived from the parasite, which suggests the secretion of this protease during feeding as a mechanism to impair the host immune response (Valenzuela-Miranda & Gallardo-Escárate 2016; Valenzuela-Munoz *et al.*, 2015; Poley *et al.*, 2016; Kvamme *et al.*, 2004; Fast *et al.*, 2003; Johnson *et al.*, 2002; Firth *et al.,* 2000). In another study on the same parasite, seven types of trypsin-like proteases were identified which were related by the authors to digestion and other host-parasite interactions (Johnson *et al.* 2002). Valenzuela-Munoz *et al.* 2015 similarly identified the up-regulation of trypsin-1 gene in *C. rogercresseyi* post-treatment with organophosphate this being reported as a drug response gene and it is one of genes associated with the resistance / susceptibility to organophosphate (Valenzuela-Munoz *et al.*, 2015).

In the current study serine protease inhibitors (serpins) were also discovered among the SEPs of *Argulus* (**Chapter 4**). While they have been identified before in other crustaceans, such as the remipede *Speleonectes tulumensis*; *Xibalbanus tulumensis*

(Yager, 1987); now known as *Xibalbanus tulumensis* (von Reumont *et al*. 2014), forming part of the secretion of “venom serine protease” glands, this is the first record for Branchiurans.

Serpins are also recognised as the largest protein group in tick saliva and were suggested to play an important role during tick feeding by modulating the host (Tirloni *et al*., 2016; Rodriguez-Valle *et al*., 2015; Kim *et al*. 2015; Mulenga *et al.,* 2013; Mulenga *et al*., 2007; Muleng *et al*., 2001). It has been suggested that blood-feeding ticks inject the host with this protein to frustrate the host defence (Tirloni *et al*., 2016). Three dominant protease inhibitors are recognised in tick saliva, these belonging to the family of Kunitz domain-containing proteins, to the serpin superfamily and to the group of thrombin inhibitors (Chmelar *et al*., 2012). They are all considered to include active antihaemostatic molecules that impair the host immune response by modulating the proteolytic balance in the feeding sites to facilitate tick attachment and haematophagy. These molecules target thrombin, FXa, and other proteases from the coagulation cascade (Champagne, 2004). Thrombin inhibitors target thrombin; a vital component for the coagulation cascade that activates platelets and enzymes required for this haemostasis process and other physiological processes including complement activation / inflammation. Factor Xa inhibitors, another important antihaemostatic molecule in ticks, target host FXa, the activator of prothrombin, which has a central role in the coagulation cascade (Chmelar *et al.*, 2012). Tick Kunitz-domain inhibitors are believed to be inhibitors of factor XIIa (FXIIa) and factor XIa (FXIa), which contribute to thrombosis, and kallikrein which is involved in the coagulation cascade (Decrem *et al*., 2009). Examples of some of these ticks are *Ixodes scapularis* (Ibelli *et al*., 2014) *Ixodes ricinus* (Prevot *et al*., 2006 and Chmelar *et al.,* 2011) *Rhipicephalus*

microplus (Rodriguez-Valle *et al*., 2015 and Tirloni *et al*., 2014), *Rhipicephalus haemaphysaloides* (Yu *et al*., 2013), *Dermacentor andersoni* (Mudenda *et al*., 2014) and *H. longicornis* (Tirloni *et al*., 2015). Serpins in ticks were found to have a range of vital roles in tick-host interaction, which help facilitate the feeding process. For example, two serpins were identified from the saliva of *Amblyomma americanum*, (a) serpin 6 which is believed to have an anti-clot and anti-complement activity and is a trypsin-like proteinases inhibitor (Chalaire *et al*., 2011; Mulenga *et al.,* 2007 and Mulenga *et al*., 2013) and (b) serpin 19, which is a trypsin-like-protease inhibitor that has an anti-haemostatic role (Kim *et al*., 2015). Serpin characterised from *Ixodes scapularis* saliva was found to inhibit thrombin and trypsin activity and lower platelet aggregation (Ibelli *et al*., 2014). Serpins are also recognised from insects, such that *e.g.* salivary serpin from *Aedes. aegypti* mosquitoes, serve as an active anti-coagulant due to their ability to inhibit Factor Xa, which is the activated form of the coagulation factor thrombo-kinase (Stark & James 1998). Serpins have been noted to be differentially expressed in salivary glands of the mosquito *Anopheles culicifacies,* suggesting involvement in the blood feeding process (Rawal *et al*., 2016).

Cathepsin-L, a relatively common endopeptidase, is expressed and secreted from many parasitic worms and other parasites and has therefore been suggested to play a key role in some host-parasite interactions (Dalton *et al.,* 2003, *Liu et al*., 2015). Cathepsin L-like proteases have been suggested to have important roles in host invasion and immune defence evasion, feeding and egg shell establishment in the helminth parasites *Fasciola hepatica* (Smooker *et al*., 2000) and *Schistosoma mansoni* (Dvořák *et al*., 2009; Brady *et al*., 2000).

Transcripts for cathepsin-L identified in *Argulus* in the present study may signify a role for this protease in digestion and / or modulation of the host immune system. Cathepsin-L from *Hysterothylacium aduncum* (Rudolphi, 1802) a parasitic anisakid nematode of fish, was proposed to be a vital protein involved in digestion processes (Malagón *et al.*, 2010), and was also identified as a potential anticoagulant in excretory/secretory products of adult *Haemonchus contortus*, a parasitic nematode of sheep and other ruminants (Rhoads and Fetterer, 1995). Cathepsin L from the parasitic ciliate *Ichthyophthirius multifiliis* was suggested to have an invasion role (Jousson *et al.*, 2007). Cathepsin L-like activities were also identified in the gut extracts of the bloodsucking bug *Triatoma infestans*; Klug, 1834 (Kollien *et al.*, 2004) and hard tick *Haemaphysalis longicornis*, Neumann, 1901 (Yamaji *et al.*, 2009; Mulenga *et al.*, 1999) which suggests it has a role in digestion and it is also suggested to have a role in haemoglobin degradation in the tick's gut (Yamaji *et al.*, 2009; Jousson *et al.*, 2007; Renard *et al.*, 2003). Franta *et al.* (2011) assumed that this can be a novel target against ticks and tick-borne pathogens (Franta *et al.*, 2011). HlCPL-A, HlCPL-A, a cathepsin L-like cysteine protease produced by the hard tick *Haemaphysalis longicornis*, was also suggested to have a major role in haemoglobin digestion during feeding (Yamaji *et al.*, 2009). Transcripts for cathepsin-L were reported to be expressed by the copepodid life stages of sea lice; *C. rogercresseyi* (Maldonado-Aguayo *et al.,* 2015; Eichner *et al.*, 2008) and showed activity in the secretory / excretory products of *L. salmonis.* This observation led the hypothesis of a role in modulating the host immune system to establish host attachment and feeding (McCarthy *et al.*, 2012). It has also been suggested to be expressed by the blood feeding copepod; *Phrixocephalus cincinnatus* Wilson C.B. 1908 (Perkins *et al.*, 1997).

Transcripts for aspartic protease 6 were also identified from *Argulus* in this study, this being a protein common to the three species examined. In a number of nematodes *e.g. Ancylostoma* spp., *N. americanus* and *H. contortus*, metalloproteases, cysteine and aspartic proteases are suggested to have a major role in digestion and host invasion (Cantacessi *et al.*, 2012) and, in addition to physiological functions, may play similar roles in haematophagous arthropods. Aspartic protease has been proposed to have a role during feeding of the parasitic copepod *C. rogercresseyi*, where aspartic protease D was highly expressed in the chalimus and adult stages. This enzyme was suggested to play a haemoglobin proteolysis function during feeding on the host (Maldonado-Aguayo *et al.*, 2015). The aspartic protease *Na*-APR-2, obtained from the parasitic nematode *Necator americanus* or *"hookworm"*, was suggested to be a potentially protective antigen in vaccines targeting human hookworm disease, due to the key role played by this enzyme in cleaving human haemoglobin and serum proteins (Williamson *et al.*, 2003).

Studies conducted on haematophagous arthropods to date indicate that these animals contain enormous numbers of novel defensins / host immunomodulating molecules that can potentially be used as vaccine targets and each of these haematophagous arthropods has been suggested to contain at least one each of anticlotting, vasodilatory, and antiplatelet compounds but showing different levels of molecular diversity (Andrade *et al.*, 2005). The salivary glands of blood feeding arthropods frequently contain anticoagulation inhibitors (Ribeiro and Francischetti, 2003) and in this study we able to find the thrombin inhibitor rhodniin in all the three *Argulus* species. This is a thrombin-specific inhibitor identified by Friedrich (1993) and co-authors from *Rhodnius prolixus* Stål, 1859, which exhibits high homology to protease inhibitors

belonging to the Kazal-type family and appears to form 1:1 complexes with thrombin. Its function was reported to be that of preventing blood clotting by inhibiting thrombin (Friedrich *et al.*, 1993) to facilitate blood acquisition.

Apyrase is another immunomodulatory molecule, which was found in *Argulus*; this enzyme is believed to have anti-pain; anti-inflammatory and anti-haemostatic activity, thus potentially playing a role during feeding to prevent blood clotting and host behavioural responses (Ribeiro and Francischetti, 2003). Salivary apyrase of blood feeder arthropods has been indicated to block platelet aggregation by blocking ADP, a platelet aggregation mediator (Valenzuela, 2002). Apyrase has been identified in a sand fly, *Lutzomyia ayacuchensis* Cáceres A., Galati EAB, 1988 salivary gland transcripts (Kato *et al.*, 2013) and was shown to inhibit platelet aggregation (Ribeiro *et al.*, 2003). Ribeiro *et al.* (1990) described the apyrase from *R. prolixus* to act as platelet aggregation inhibitor.

A number of metalloproteases were identified in *Argulus*, these being a common enzyme group in other blood-feeding arthropods as well. Saliva from the tick *Ixodes scapularis* contains a metalloprotease enzyme that is similar to that found in snake venom serine proteases, and which was suggested to act as an anti-haemostatic compound to help sustain the feeding process (Francischetti *et al.*, 2003). The parasitic copepod *P. cincinnatus* was also found to produce it (Perkins *et al.*, 1997). From a *de novo* study on strains of sea lice, *L. salmonis,* treated with organophosphate, up-regulation of metalloprotease associated gene transcripts was observed (Valenzuela-Munoz *et al.*, 2015). Apyrase and metalloprotease found in tick salivary secretions were suggested to affect antiplatelet aggregation and coagulation activity (Chmelar *et al.*, 2012). Transcriptome analysis conjugated with KEGG pathway

analysis conducted by Sahoo *et al*. 2013 on *A. siamensis* resulted in the identification of proteases including serine proteases and metalloproteases which have been described to have antigenic properties in some ectoparasites (Sahoo *et al*. 2013). From this viewpoint, metalloprotease in *Argulus* may have similar functions to those reported in other arthropods in terms of regulating the interactions between the parasites and their host to facilitate blood feeding. These molecules might interfere with the haemostatic system and be responsible for haemorrhage (Radulović *et al.*, 2014).

Adenosine deaminase, which can act as a vasodilator and antiplatelet aggregation factor for arthropod feeding (Ribeiro & Francischetti 2003, Champagne 2004) is also found in *Argulus*. By searching for adenosine deaminase using OrthoVenn BLAST feature, two contigs of adenosine deaminase transcripts resulted from *Argulus* species adenosine deaminase and RNA-specific adenosine deaminase-1. These genes may play a role during *Argulus* feeding and host immune system inhibition as has been suggested for the sand fly *Lutzomyia longipalpis* (Charlab *et al.*, 1999) and the mosquito *Aedes aegypti* (Ribeiro *et al.*, 2001). It is suggested that adenosine deaminase, can assist feeding and modulate host immune responses by removing adenosine; a molecule associated with pain awareness and mast cell degranulation, and through its downstream effects on inosine; an inhibitor of pro-inflammatory cytokines (Valenzuela, 2002). Different genera of arthropods use a range of different proteins as vasodilators. As an example, sand flies of the genus *Phlebotomus* have adenosine in the saliva as the vasodilatory component while 6.5-kDa peptide, maxalidan, serves as the vasodilator peptide in sand flies of the *Lutzomyia* genus (Moro & Lerner 1997; Lerner *et al.*, 1991). This diversity of active molecules also

applies to those associated with anti-clotting and anti-platelet activities (Ribeiro & Francischetti, 2003), so that if the host evolves to escape one compound, others can still serve to provide a similar functional role. In two mosquito genera, *Aedes* and *Anopheles*, there are two different anticlotting saliva constituents. Factor Xa salivary inhibitor (serpin) is found in *Aedes aegypti* (Stark & James 1998), however, *Anopheles albimanus* has a 6.5 kDa peptide named Anophelin , anti-thrombin peptide (Francischetti *et al.*, 1999).

Cysteine protease transcript was found in all three species of *Argulus* in this study. The exact role of these secretory proteins in *Argulus* is not known but it is known that cysteine proteases, enzymatic proteins utilizing a cysteine thiol group to hydrolyse peptide bonds (McKerrow *et al.*, 2006) across a wide range of organisms play an important role in extracellular and intracellular protein degradation and processing. In blood feeding ticks for example they are involved in two important physiological processes as they are described to be the main digestive enzymes used by the tick's gut and have a potential function in yolk protein degradation and embryogenesis. This degradation help larval ticks to survive until they accomplish the first blood meal by providing amino acids for protein catabolism (as reported by Sojka *et al.*, 2011). Secreted cysteine proteases from the nematode *Haemonchus contortus* (Rudolphi, 1803) Cobb, 1898 were suggested to have roles in nutrition and / or host immune evasion (Rhoads and Fetterer, 1995). Due to the proteolytic activity and over expression of cysteine proteases from the trophont parasitic stages of the ciliate, *Ichthyophthirius multifiliis* (Jousson *et al.*, 2007) and blood feeding parasitic copepod *Phrixocephalus cincinnatus* C. B. Wilson, 1908 (Perkins *et al.*, 1997), it was suggested that they have a critical function in the infection process (Jousson *et al.*, 2007; Perkins

et al., 1997). Cysteine proteases have been shown to play important roles in trematode parasites. Their roles include catalysing turnover of parasite peptides and growth factors and regulation of proteins, host tissue penetration and invasion, digestion and modulation of host defence systems (McKerrow *et al*., 2006; Na *et al*., 2006).

Phospholipases were also represented in the shared *Argulus* clusters, with 14 different clusters recognised in Swiss-Prot homology blasting. One of the most notable of these is phospholipase A2 (PLA2), a platelet-activating factor acetyl hydrolase, which known to be used in deactivating platelet-activating factor by *Leishmania* parasites (Pawlowic and Zhang, 2012) and known to facilitate blood feeding (Francischetti *et al*., 2009). PLA2 were reported on the secretions of bees and snake venoms as common and essential components and found to be involved in lysis, inactivation of platelet aggregation and anticoagulant effects as they interact with cells or by the degradation of phospholipid which then generates free arachidonic acid (Kini, 2005). phospholipases A2 (PLA2) are secreted enzymes involved in biological processes such as inflammation and host defence and eicosanoid generation these being a family of powerful, hormone-like compounds that include prostaglandins, prostacyclins, leukotrienes, and thromboxanes (Murakami & Kudo 2004; 2002). Sialotranscriptomic study of the gulf coast tick, *Amblyomma maculatum* revealed the presence of this enzyme and its possible effect in host inflammation and immunity signaling pathways (Karim *et al*., 2011). The presence of these transcripts in *Argulus* species suggest that they play a similar role to these proteins in other parasites, so phospholipase A2 may inhibit platelet aggregation in the lesion site during feeding to facilitate blood sucking process.

Transcripts for two types of ferritin were also found in *Argulus* in the present study, Ferritin-1 heavy chain and yolk ferritin. Recently, ferritin was observed to be up-regulated in the salivary gland of the mosquito *A. culicifaciens*, after blood feeding (Rawal *et al.*, 2016), which results in iron overload and then ferritin plays an essential role in iron homeostasis through iron transport and storage (Zhou *et al.*, 2007). *Cardiodectes medusaeus;* a blood feeding copepod belong to the family Pennellidae, produces and stores ferritin crystals to dispose of excess iron during blood feeding, which would otherwise be toxic to the parasite (Boxshall, 2005). Hajdusek *et al.* (2010), working on *Ixodes ricinus* and *Rhipicephalus annulatus* Say, 1821, illustrated the success of using the secreted form of ferritin 2 protein (FER2) as an antigen in an anti-tick vaccine. The results showed that the vaccine efficiency was between 64% and 72% for *I. ricinus* and 98% overall vaccine efficacy (E) against *R. annulatus* (Hajdusek *et al.*, 2010). Thus, ferritin in *Argulus* spp. are expected to have the same roles in processing and storing iron after blood meals from the host.

Transcripts for enolase B, a glycolytic enzyme (Hewitson *et al.*, 2009) that has been suggested to be involved in blood digestion / feeding and defensive activity in ticks and other blood feeing parasites (Xu *et al.*, 2016), were also found in *Argulus* spp. in the current study as well as in sea lice, *L. salmonis* (Eichner *et al.*, 2008). Similarly, in a study conducted to describe the response of sea lice after treatment, differential transcriptional expression of enolase was also detected (Valenzuela-Munoz *et al.*, 2015). Enolase in *Fasciola hepatica* Linnaeus, 1758, a liver fluke, was also considered as a potential vaccine antigen (Ravida *et al.*, 2016). Enolase is a relatively well conserved protein and for both the cestode *Echinococcus granulosus*, Batsch, 1786 (Gan *et al.*, 2010) and the nematode *Ascaris suum*, (Goeze, 1782) (Chen *et al.*, 2012),

investigations have suggested enolase to be a promising vaccine candidate (Gan *et al.*, 2010; Chen *et al.,* 2012). Enolase act as a profibrinolytic plasminogen receptor in tick *Ornithodoros moubata* and *Schistosoma bovis* (Bilharz, 1852) to keep the fluidity of host blood (Díaz-Martína *et al.*, 2013). Both ferritin and enolase, were highly expressed in the mid- gut of partially engorged female *Haemaphysalis flava* Neumann, 1897, which supports their importance in blood feeding (Xu *et al.*, 2016).

Future studies need to be conducted to further identify and characterise the transcripts recognised from the *Argulus* transcriptome in the current study and to confirm suspected role(s) of the proteins they encode for during feeding and host invasion. Some of these proteins are recognised as secreted or transmembrane proteins, which are considered to have the potential to act as novel potential anti-parasite drug targets or vaccine candidates (Liu *et al.*, 2015). Hence some of the proteins described could provide effective vaccine candidates for *Argulus*, a species for which there is an urgent need to improve control and management. Future work also needs to focus upon the question of whether identified compounds are secreted / expressed from *Argulus* glands believed to be associated with feeding *i.e.* pre-oral spine and proboscis glands. This study provides the largest existing genomic resource for *Argulus* and is the first study to look for shared gene expressed across species and to provide a preliminary list of potential feeding enhancers / immunomodulators / defensins. The presence of conserved proteins across the speciescan potentially provide generic vaccine / drug targets.

3.5 **Conclusion:**

In the absence of a fully annotated *Argulus* genome and in view of the previously limited genomic resources available for argulids and branchiurans more widely, this study has made a key contribution to argulid genomics and shed further light on crustacean fish parasites more widely. High-throughput sequencing technologies have allowed the discovery of a number of conserved proteins across the species studied and has shed light on possible immunomodulator targets. The data produced from this study for *A. foliaceus* and *A. coregoni* adds considerably to that available from previous transcriptome studies.

The current study has provided evidence, for the first time, to our knowledge, of potential effectors of host immunomodulation, potentially providing future studies with targets for *Argulus* drug or vaccine development. There are 145 described species of *Argulus* worldwide (Walter and Boxshall, 2016) and it is therefore useful to find conserved secretory proteins that may be used for control across different *Argulus* species. Thus, a universal vaccine or drug treatment strategy could be applied for different *Argulus* species, as has been previously suggested with respect to the development of a universal vaccine for ticks (Parizi *et al.*, 2012). The first step for developing any new control treatment is to search for potential targets (Yang *et al.,* 2015) and the outcomes of the research described in this chapter provide important initial steps in this direction. The work described in succeeding chapters will serve to better characterise and localise some of the potential control targets identified in this initial work.

Chapter 4. Identification and gene expression of proteins from the *Argulus foliaceus* secretory products

4.1 Introduction

Provision of baseline proteomic data for *A. foliaceus* secreted proteins is an essential aspect of investigating the proteins involved in parasite feeding activities. During blood feeding, haematophagous arthropods inject the host with salivary products rich in proteins and other pharmacologically active molecules that have anti-haemostatic, anti-inflammatory and immunomodulatory properties, which help in counteracting the host immune response and hence facilitate feeding (Rawal *et al.*, 2016; Carvalho-costa *et al.*, 2015; Anstead *et al.*, 2015; Tirloni *et a*l., 2014; Díaz-Martín *et al.*, 2013; Oliveira *et al.*, 2011 Chmelar *et al.*, 2011; Maritz-olivier *et al.*, 2007; Xu *et al.*, 2007; Steen *et al.*, 2006; Wynne Weston & Nuttalla 2002; Horn *et al.*, 2000).
Recently, proteomics-based studies have contributed considerably to the identification of proteins from the salivary glands of a number of haematophagous insects such as *Anopheles stephensi*, *A. campestris-like*, *Aedes aegypti* and *Culex pipiens quinquefasciatus,* these studies providing data relating to their functional roles, and enabling molecular and biological characterisation (Rawal *et al.*, 2016). Transcriptomic and proteomic studies of salivary glands from different species of ticks have also been conducted recently, providing a list of active molecules / proteins that modify the hosts physiology (Ribeiro *et al.*,, 2011; Karim *et al.*, 2011; Francischetti *et al.*, 2009). As suggested by Díaz-Martín *et al.* (2013) concerning *Ornithodoros moubata*, the development of an effective vaccine against ticks may well depend upon the identification of their salivary proteins.

It has been hypothesised that as *Argulus* spp. attach to the host's skin via suction cups; suckrs, a range of active components are secreted from the spinal, proboscis and labial glands through the pre-oral spine and the mouth tube to help facilitate blood feeding by counteracting host defence systems as for other ectoparasitic arthropods. *Argulus* spp. feed on blood (Hoffman 1977, Mikheev *et al.,* 2000 and Walker *et al.,* 2004), mucus (LaMarre & Cochran, 1992) and host skin (Van der Salm *et al.*, 2000), thus the need to mediate host immune defence mechanisms including those associated with pain, haemostasis, inflammation, complement activation and tissue repair via pharmacologically active substances. Proposed secretory products are suggested, like those of other parasitic arthropods, to act as anti-haemostatics, anti-coagulants and immunomodulators (Claus 1875; Shimura and Inoue 1984; Swanepoel & Avenant-Oldewage 1992; Gresty *et al.*, 1993; Møller & Olesen 2010; Saha *et al.*, 2011; Al-Darwesh *et al.*, 2014). However, to date little formal data has been collected on either the composition or function of potential *Argulus* spp. secreted proteins (von Reumont *et al.*, 2014). Thus, identification of the secretory proteins comprising the *Argulus* secretion is a key requirement to establishing the nature of these proteins and characterising potential functional properties, which might be helpful to developing controls for argulosis.

Saurabh (2012) and his colleagues conducted a trial that looked to identify immunodominant peptides present in *A. siamensis* and were able to determine two polypeptides that satisfied their criteria. In a previous study, Ruane *et a*l. (1995) demonstrated an antibody response of immunised rainbow trout, *Oncorhynchus mykiss*, to *Argulus foliaceus* antigens. In addition to this, several antigenic components were also identified by hyper-immune sera of rabbits immunised with *A. foliaceus* antigen on immunoblots probed with whole-body extracts of *A. foliaceus*. Both

Saurabh *et al*. (2012) and Ruane *et al*. (1995) suggested that these were potential antigens of *Argulus siamensis* (Wilson) (Saurabh *et al*., 2012) and *A. foliaceus* (Ruane *et al*., 1995) might lead to the development of an effective vaccine against *Argulus* spp. Ruane *et al*. (1995) found components of the parasitic copepods *L. salmonis* and *C. elongatus* to be recognised by rainbow trout anti-*A. foliaceus* sera suggesting that despite their phylogenetic distance, these ectoparasitic ecdysozoans share antigenic components.

The aim of the work described in this chapter was to identify and characterise the proteins present within the secretions of *A. foliaceus*. This was achieved by integrating the transcriptomic dataset established in **Chapter 3** with a proteomic investigation conducted using GeLC-MS/MS analysis. Expression levels for selected transcripts identified from the transcriptomic analysis (**Chapter 3**) were also investigated. The targeted proteins and genes from this study have also been suggested to have immunomodulatory roles secretions of other blood feeding arthropods, which provides a useful comparison for secreted *A. foliaceus* products.

4.2 Materials and Methods

4.2.1 Preparation of secretions samples for proteomic analysis

Prior to start the experiment two sets of *A. foliaceus* samples were used; each contains 25 adult *A. foliaceus*. Samples were incubated in 0.1 mM dopamine in artificial freshwater while the second set of samples were incubated in artificial freshwater for overnight at 10°C. The results showed no differences in terms of the bands resulted

in SDS-Page analysis. Hence, the decision was to use the artificial freshwater only as incubated medium.

4.2.1.1 **Preparation of artificial freshwater**

To avoid extraneous protein sources or other contaminants, artificial freshwater was prepared following a recipe used to culture zooplankton by Klüttgen *et al*. 1994. In this medium, "AdaM Daphnia medium", two types of stock solutions are used: stock solution A (29.4 g Calcium chloride dihydrate, CaCl2. 2H2O in 250 mL DW), stock solution B (6.3g Sodium bicarbonate, $NaHCO_3$ in 250 mL DW). Parasites were maintained in the sterilised artificial freshwater and incubated overnight at 10°C to collect secretions.

4.2.1.2 **Incubation of the parasites in artificial freshwater**

Adult argulids were collected from Loch Fad; on the Isle of Bute, Scotland, UK were used in this experiment. Between five and nine parasites (depending on the parasite life stage / size) were placed in 1.5 mL sterilised Eppendorf's containing 1 mL of artificial freshwater. A total of 560 parasites were used, divided into 92 tubes. Artificial freshwater without parasites was used as a negative control (50 x 1.5mL sterilised tubes of 1 mL of artificial freshwater). All the tubes were incubated at 10°C overnight for >18 h. The following day, water samples expected to contain secretory products from *A. foliaceus*, were pooled into 2 x 50 mL sterilised Falcon tubes and the negative control pooled into an additional 50 mL sterilised tube. These samples and the control were rapidly chilled to -70°C and maintained frozen until used for sodium dodecyl

sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). The parasites used during incubation were also kept at -70°C in the original tubes.

4.2.1.3 **Preparation of secretions samples for SDS Page analysis**

Secretion samples and the negative control were thawed on ice and decanted into 15 mL centrifuge filter units (Millipore) and centrifuged at 4000xg for 30 min to concentrate proteins >10kDa prior to sample denaturation. The filtrate / eluate was removed from the filter tube and the step was repeated until all the samples were concentrated.

A dilution series of *A. foliaceus* secretions samples for SDS-PAGE analysis were set up for different stains: 2 x gels, one for silver stain and one for Coomassie stain. As 15µL is needed for each well of the gels, 20µL of concentrated sample was combined with 20µL of 2 x SDS sample buffer (SDS reducing buffer) (2.5mL 0.5 M Tris-HCL pH 6.8, 2 mL Glycerol, 4 mL10% SDS, 0.31g Dithiothreitol (DTT), 2 mg Bromophenol blue and DW added to give 10 mL) to make the first dilution (1:1). Then, 40µL of the concentrated sample was added to 40µL of double distilled water (DDW) to make a doubling dilutions series of 1/4 and 1/8 dilutions. Samples were kept on ice during dilution preparation. The samples were denatured at 100°C in boiling water for 5 min then centrifuged (Micsolite, Thermo IEC) for 2 min at 16000xg. Five µL of 2-250 kDa mixed range pre-stained molecular weight markers (Precision Plus Protein™ Standards- BIORAD) were loaded into the first well of two Precast 12-well Polyacrylamide gels (12% Mini-PROTEAN® TGX™ Precast Protein Gels (Bio-Rad)) followed by 15µL of the samples in each well in descending order of sample concentration. The gels were run at 130 Volt for 75 min then, for staining, the gels

were washed with 3 x 100 mL DDW for 5 min rinsed with DDW and placed in a staining tray.

4.2.1.4 **Staining the SDS PAGE gels**

QC Colloidal Coomassie Stain: The gel was subsequently fixed in fixing solution (40% ethanol, 10% acetic acid, 50 % DDW) for 15 min with gentle agitation. After discarding the fixing solution, the gel was washed 3 x 100mL DDW for 5 min then QC colloidal Coomassie stain (Bio-Rad) was added to the gel and incubated for 16 h at room temperature with gentle agitation (Stuart Scientific; Gyro-Rocker). Then the stain was removed and the gel was washed with 4 x 100mL DDW for 3 h with gentle agitation until obvious bands with clear backgrounds were observed. The gel was scanned using a benchtop scanner (EPSON expression 1680 Pro) and kept at 4°C in a sealed polyethylene bag with DDW until sent for gel and liquid chromatography electrospray ionisation tandem mass spectrometry (GeLC-ESI-MS/MS)

The lane of the ½ sample dilution was selected for MS/MS analysis conducted at the Moredun Research Institute, Edinburgh.

Sliver Stain: After electrophoresis, the gel was fixed overnight with 100mL of fixing solution (40 % ethanol and 10 % acetic acid). The fixing solution was discarded and the gel was washed with 100 mL of 30 % ethanol for 10 min with gentle agitation on the shaker. The silver staining kit, ProteoSilver ™- PROTSIL1-1KT, was used for the remainder of the procedure. The gel was washed with 200 mL of DDW for 10 min and then 100mL of Sensitizer solution was added and incubated for 10 min. The gel was then washed 2 x 200 mL DDW for 10 min with agitation. Silver solution was used to

stain the gel for 10 min followed by a wash step with 200 mL DDW for 1-1.5 min. The water was then discarded and 100 mL of developer solution was added. The gel was developed with Proteo™ developer for 5 min until bands appeared with limited background, and the reaction was stopped by adding 5 mL of ProteoSilver™ stop solution. The gel was then stored overnight in 200mL of DDW prior to scanning.

4.2.2 **Western blot**

Western blot was used to visulize the presence of specific target proteins among the secretory products of *A. foliaceus*, obtained from infected rainbow trout. The targeted screening explored the presence of antigens from *A. foliaceus* secretions using anti-serpin D1 (Invitrogen™, heparin cofactor II polyclonal antibody (1mg mL^{-1}) Prod# PA5-29105) and Heparan Sulfate 6-O-Sulfotransferase 3 (HS6ST3 polyclonal antibody (0.2mg/ml) Prod# PA5-47733) and to proteins bound by *Phaseolus Vulgaris* Erythroagglutinin (PHA-E), and Jacalin biotinylated-labelled plant lectins (Vector Laboratories). SDS-PAGE was carried out using the method described in section 4.2.1.2 with some modifications. An equal volume of *A. foliaceus* secretions to 2 x SDS loading buffer (Laemmli, 1970), was boiled at 100°C for 10 min. After centrifugation at 16000 x g for 2 min, 15 µL from each soluble protein samples were run on a 12% polyacrylamide resolving gel and a 15-well 4–15% Mini-PROTEAN® TGX™ Precast Protein Gel (Bio-Rad) with 5 µL of marker and 15 µL of 0.1% of BSA in ultra-pure water used as a non-specific protein negative control. Proteins were separated by running the gels on a Mini- PROTEAN® II electrophoresis cell (Bio-Rad Laboratories) at 115 Voltage for 90 min with running buffer (25 mM Tris, 250 mM glycine, 0.1% w/v SDS, pH 8.3). One of the gels was then fixed in fixing solution for 1 min followed by rinsing

with DDW and stained with QC Colloidal Coomassie stain overnight with gentle agitation. Then gels were then washed with 3 x DDW for 3 h.

The proteins from the other gels were transferred from the gel to PROTRAN nitrocellulose transfer membrane (Amersham™Hybond ECL, GE Healthcare Lifescience). A semi-dry blotting apparatus (Thermo Scientific™ Pierce™ G2 Fast Blotter) was used to apply 25V for 7 min to transfer proteins from the gel to the membrane. The membrane was then washed 3 x with Tween Tris buffered saline (TTBS; 150 mM NaCl, 10 mM Tris-HCl, 0.05% v/v Tween20, pH 7.5) for 5 min each and then blocked by 3% skimmed milk powder (casein, Marvel) in TBS overnight at 4°C followed by 3 x 5 min washes with TTBS.

For detection of Serpin D1; goat anti-rabbit IgG polyclonal antibody and HS6ST3 polyclonal, rabbit-anti-sheep FITC, IgG antibody were diluted TBS to 4 µg mL^{-1} and 0.8 µg mL^{-1}, respectively. Biotinylated-PHA-E and biotinylated-Jacalin lectins were diluted to working concentrations of 10 µg mL^{-1} in TBS. TBS buffer was used as negative control, anti-sheep rabbit biotinylated antibody (10µg mL^{-1}) was the nonspecific control.

The membranes were incubated with the primary antibodies or biotinylated lectins and controls for 1 h at room temperature with gentle agitation. Membranes were again washed 3 x 5 min with TTBS.

The lectin stained membranes were then incubated with 0.2 µg ml^{-1} streptavidin conjugated with horseradish peroxidase (HRP; Sigma Chemical). HCII, TBS and nonspecific controls were incubated with goat, anti-rabbit HRP antibody (Sigma) and HSPG antibody was incubated with rabbit, anti-sheep-FITC. The incubation was for 1 h at room temperature with gentle agitation followed by washing steps with TTBS as before. Then the membranes were washed a final time with TBS for 1 min. To develop

the bands from each membrane, DAB (ImmPACT™ DAB, Vector) was used with reactions stopped by the addition of DDW and continuous quick washing with DDW. The membranes then scanned using an EPSON scanner.

4.2.3 Gel and Liquid chromatography electrospray ionisation tandem mass spectrometry (GeLC-ESI-MS/MS)

At Moredun Research Institute, the selected gel lane (1:1 dilution of secretions), was excised and sliced horizontally from top to bottom to yield a series of 25 equal gel slices of 2.5 mm deep. Each of the resulting gel slices was then subjected to standard in-gel destaining, reduction, alkylation and trypsinolysis procedures (Shevchenko *et al.* 1996). Digests were transferred to low-protein-binding HPLC sample vials immediately prior to GeLC-MS/MS analysis. Liquid chromatography was performed using an Ultimate 3000 Nano-HPLC system (Dionex) comprising a WPS-3000 well-plate micro auto sampler, an FLM-3000 flow manager and column compartment, a UVD-3000 UV detector, an LPG-3600 dual-gradient micropump and an SRD-3600 solvent rack controlled by Chromeleon™ chromatography software (Dionex). A micro-pump flow rate of 246 μL min^{-1} was used in combination with a cap-flow splitter cartridge, affording a 1/82 flow split and a final flow rate of 3 μL min^{-1} through a 5cm x 200 μm ID monolithic reversed phase column (Dionex) maintained at 50°C. Samples of 4 μL were applied to the column by direct injection. Peptides were eluted by the application of 15 min linear gradient from 8-45% solvent B (80% acetonitrile, 0.1% (v/v) formic acid) and directed through a 3 nL UV detector flow cell. LC was interfaced directly with a 3-D high capacity ion trap mass spectrometer (amaZon-ETD, Bruker Daltonics) via a low-volume (50 μL min^{-1} maximum) stainless steel nebuliser (Agilent,

cat. no. G1946-20260) and ESI. Parameters for tandem MS analysis were based on those described previously (Batycka *et al.* 2006).

4.2.3.1 **Database mining**

The MS/MS data, formatted as Mascot Generic Format (mgf), was imported into ProteinScape™ V3.1 (Bruker Daltonics) proteomics data analysis software for downstream mining of a custom *Argulus* database. This custom database was constructed from the transcriptome databases (in the absence of a full *Argulus* genome) and comprised 60257 sequences in total.

Database searches were conducted utilising the Mascot™ V2.4.1 (Matrix Science) search engine. Mascot search parameters were set in accordance with published guidelines (Taylor and Goodlett, 2005) and to this end, fixed (carbamidomethyl "C") and variable (oxidation "M" and deamidation "N, Q") modifications were selected along with peptide (MS) and secondary fragmentation (MS/MS) mass tolerance values of 0.5 Da whilst allowing for a single 13C isotope. Protein identifications obtained from each of the 25 individual gel slices per lane were compiled using the "protein list compilation" feature within ProteinScape. From the compiled protein lists individual protein identifications were inspected manually and considered significant only if a) two peptides were matched for each protein, b) peptides were represented by a sequence coverage of > 5% and c) each matched peptide contained an unbroken "b" or "y" ion series represented by a minimum of four contiguous amino acid residues.

4.2.3.2 **Analysis of proteomics results**

Identity and function of the predicted proteins was checked using the Pfam 31.0 tool applying the amino acid sequence search. Then, all the proteins giving a hit with Pfam, and also those with no Pfam match hits, were blasted in NCBI BLAST P against 'Metazoan' to give an indication of the characteristics of the identified protein sequences. As these proteins were obtained from the *A. foliaceus* secretory products, Signal P 4.1 (http://www.cbs.dtu.dk/services/SignalP/) server was used to predict the presence and location of signal peptide cleavage sites in amino acid sequences which, could inform of any associated secretory property of the protein. A FASTA file containing all the predicted protein sequences was submitted to the Signal P tool where the program default settings were used to search against Eukaryotic organisms. In addition, the same FASTA file was uploaded to NetNGlyc 1.0 Server to predict the N-glycosylation sites.

Predictions as to the inhibitory or non-inhibitory function of the sequenced serpin were based on the reactive centre loop (RCL) consensus sequence. To obtain this, the serpin sequence obtained from the *A. foliaceus* secretion was searched against the human reference protein database using BLASTp, followed by multiple sequence alignment using the Constraint-based Multiple Alignment Tool (COBLAT). The inhibitory RCL region was then identified for the purposes of predicting inhibitory properties.

4.2.4 **Measuring mRNA expression of selected targets from RNA seq data set by quantitative polymerase chain reaction (qPCR)**

4.2.4.1 **RNA extraction and complementary DNA (cDNA) synthesis**

Fed and starved *A. foliaceus* were collected from Loch Fad. Ten fed adult parasites (5 males and 5 females) were removed directly from their host fish rainbow trout and transferred immediately to RNA*later* while ten additional parasites were kept alive in fresh water from the loch. The live parasites were maintained starved, without hosts, for 48 h at 10°C. The 5-live adult *A. foliaceus* males and females were then preserved in RNA*later* overnight. Total RNA was extracted from all 20 samples of *A. foliaceus;* including fed and starved males and females, using TRI Reagent® (Sigma-Aldrich, UK) following a protocol for organic extraction as described in **3.2.2** (**Chapter 3**).
RNA concentrations were measured using UV spectroscopy (NanoDrop ND-1000, Thermo Scientific, USA) and RNA integrity evaluated by agarose gel electrophoresis and ethidium bromide staining prior to storage at -70°C for later use. The High capacity cDNA reverse transcription kit (AB Applied Biosystems, UK) was used in two step reactions to synthesize first strand complementary DNA (cDNA) by reverse transcription. Briefly, 2 µg of total RNA from each sample were made up to a volume of 14 µL with nuclease-free water in 0.2 mL tubes. To prepare the first master mix 5µL of 5 x Random Primers dNTPs was added to each RNA sample to give a total of 19 µl. The samples were mixed gently and pulse centrifuged followed by heating on a Biometra TOptical Thermocycler (Analytik Jena, Goettingen, Germany) at 75°C for 3 min and kept on ice until the second step reaction. The RT master mix was prepared with the following: 55 µL 10 x RT Buffer, 7.6 µL dNTP Mix (100mM), 22 µL

MultiScribe™ Reverse Transcriptase and 15.4 µL nuclease-free water. Five microliters were added to the 19µL previously heated reactions to give a total of 24 µL. Template RNA was reverse transcribed in a Biometra TOptical thermocycler with the cycle conditions shown in **Table 4. 1**. A standard PCR reaction was run with the 10 primers at three different temperatures with cDNA and DNA templates to check for the best annealing temperatures. The cDNA stocks were stored at -20°C until further analysis.

Table 4.1 High-Capacity cDNA Reverse Transcription program used in Biometra TOptical thermocycler for whole specimen *A. foliaceus* cDNA synthesis

Step No.	Temperature °C	Time
1.	25	10 Minutes
2.	37	120 Minutes
3.	85	5 Minutes
4.	20	1 Second

4.2.4.2 **Primer design and standard polymerase chain reaction (PCR)**

Using the *A. foliaceus* transcriptomic data for some target genes known to have an immunomodulatory function in other arthropods, 8 genes were selected for closer study, with two housekeeping genes, β-actin and elongation factor 1α. The nucleotide sequences were checked in NCBI and resulted in hits with good homology to equivalent genes from other organisms. Primers were subsequently designed using PrimerSelect software (**Table 4.2)**. The designed primers (Eurofins Genomics) were tested at three different temperatures by standard PCR reactions prior to qPCR using 1:10 diluted adult female *A. foliaceus* cDNA (5nM).

4.2.4.3 **Quantitative polymerase chain reaction (qPCR)**

The expression of genes was determined by real-time quantitative polymerase chain reaction (qPCR) using a Biometra TOptical thermocycler (Analytik Jena, Goettingen, Germany). Elongation factor-1α (elf1α) and β-actin were used as reference genes (**Table 4.2**).

The efficiency of primers was tested at seven different serial dilutions (1/10, 1/20, 1/50, 1/100, 1/200, 1/400 and 1/800 in triplicate) of cDNA pooled from the samples to ensure that efficiency was between 0.8 - 1.10. The standard curves were established by plotting the Ct values against (-1/slope) the log10 of the seven dilutions of cDNA by applying the equation: E = 10 (-1/slope).

qPCR cycling was performed in 96-well plates in duplicate 20 µL reaction volumes that contained 5 µL of 50-fold (1/50) diluted cDNA and 15 µL of qPCR master mix comprising 3.8 µL nuclease-free water, 0.6 µl (10 µM) each for the forward and reverse PCR primer, and 10 µl of 2X Master Mix (Luminaris Color HiGreen qPCR Mix; Thermo Scientific, Hemel Hempstead, UK), with the exception of the reference genes, which were determined using 2 µL of cDNA. Standard amplification parameters consisted of pre-heating samples at 50 °C for 2 min, initial denaturing at 95 °C for 10 min, denaturation at 95 °C for 15 s, and two annealing temperatures were used depending on the primer tested, 58 and 61 °C for 30 s, and an extension at 72 °C for 30 s. The Ct (cycle threshold) value is defined as the number of cycles required for the fluorescent signal to cross the significant threshold (i.e. exceed background level). In addition, triplicate dilutions of pooled cDNA from all the samples (1/10, 1/20, 1/50, 1/100, 1/200, 1/400 and 1/800), pooled samples of reverse transcription RT minus were prepared from 6 samples; 3 from each group; fed and starved, but without adding

the enzyme MutiScribe™ Reverse Transcriptase, and a no-template control (NTC) containing no cDNA were included in each run as a calibrator between plates and to control for any genomic DNA contamination.

4.2.5 **Statistical analysis**

All statistical analyses were performed using SPSS Statistical Software System version 15.0 (SPSS Inc., Chicago, USA). Gene expression results were analysed using the relative expression method with efficiency correction (Pfaffl 2001) to determine the statistical significance of expression ratios (gene expression fold changes) between treatments using the geometric mean expression of the references genes. Data were tested for normality, and homogeneity of variances with Levene's test prior to one-way analysis of variance (ANOVA) followed by a Tukey-Kramer HSD multiple comparison of means post-hoc test. Additionally, two-way ANOVA, which examined the explanatory power of the variables "treatment" and "sex" as well as "treatment x sex" interaction was used. Differences were regarded as significant when $p<0.05$.

Table 4.2 Primers used for quantitative real-time PCR analysis of targeted *A. foliaceus* genes and their optimal annealing temperatures.

Target name	Gene symbol	Oligonucleotide sequences (5'- 3')	Size (bp)	T (°C)	Target sequence identity
Trypsin-like serine proteinase	*try*	F: GACTCTGGTGGGCCGTTGATTTA	100	61	TRINITY_DN42709_c0_g1_i1
		R: CCCCTGGATAGTCTGGATGAGCA			
Prostaglandin E 2 synthase	*pge2s*	F: ACGGAGTACTTAACGCCATAGA	156	61	TRINITY_DN47068_c2_g1_i1
		R: ATTTACCACATCACATTGCCTTCA			
Serpin	*ser*	F: TCGCGTCGGGAAGATGATG	158	61	TRINITY_DN47712_c1_g1_i1
		R: ACAATACCACCGAAGCAAGATGAC			
Aspartic protease	*asp*	F: ACGGCGTTCAAATTCAACAAGGAA	174	61	TRINITY_DN1668_c0_g1_i1
		R: TGGCATCGAAGTTCACTCCCATAA			
Cysteine protease	*cys*	F: GCACAATTCCGGAGGGTTCCT	145	61	TRINITY_DN52629_c1_g1_i1
		R: CGCCGAGTTCGAGCGTGTC			
Ferritin	*fer*	F: TTCGCGATAAATATCAGACTTGTTC	106	58	TRINITY_DN31540_c0_g1_i1
		R: TTTGATCTCCACGAAATCTTGTCAT			
Thrombin inhibitor	*thr*	F: ACTCGGACCCGATGTACTTTGATT	187	58	TRINITY_DN51603_c0_g1_i3
		R: TTTGTCGCAGACGATTACATTCATT			
Venom serine protease	*vser*	F: GAACAACAGGGTGCGACCTCA R: TTGTGCCGCACTTCACCACTCA	172		TRINITY_DN47823_c0_g1_i2
Housekeeping genes					
B- actin	*B-actin*	F: ATGGCGGGGCAGTTGAAGGTCT	177	58	TRINITY_DN16154_c0_g1_i1
		R: CCGTGCAGCCATGTAAGGGAGTG			
Elongation factor-1A	*elfa-1A*	F: GGAAGACCACTGAGGAGCAC R: CTCTGACGGCAAACCTTCCT	153	61	TRINITY_DN54457_c0_g3_i1

4.3 **Results:**

4.3.1 **SDS PAGE gels**

SDS-PAGE analysis showed 10 intense bands of proteins with molecular masses in the range of 3-45 kDa. The use of three different dilutions of the secretions indicated a dilution effect in the intensity and number of bands obtained. The lack of bands observed from the sterilised artificial water (negative control) confirmed that the protein bands resulted from *A. foliaceus* parasites, either as secretions or excretory products, *e.g.* minimal environmental contamination (**Fig. 4.1**). Interestingly, there were 4 intense bands even in the most diluted sample;1/8, with approximate molecular weights of 5, 25, 28 and 46 kDa, and an intense band was seen in the more dilute samples at around 100 kDa compared to the1/2 diluted sample (**Fig. 4.1B**).

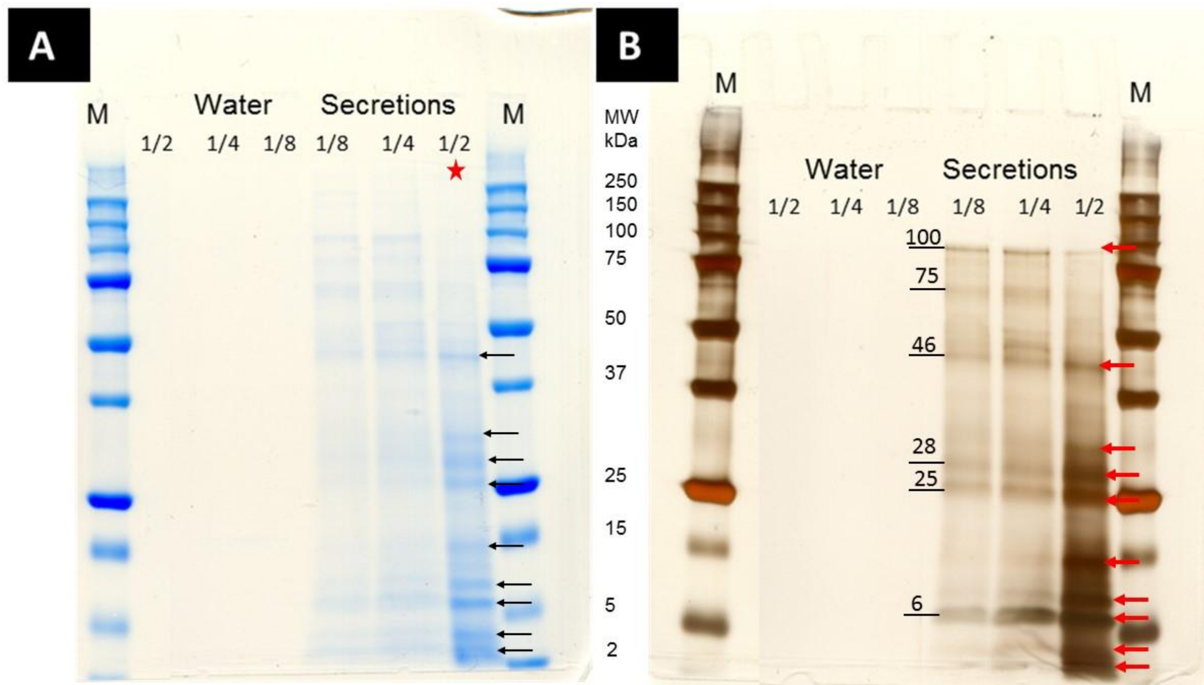


Figure 4.1. SDS-PAGE of *A. foliaceus* secretions. Secretions were collected and proteins separated on 12% SDS-PAGE gels and stained with (A) QC colloidal Coomassie stain and (B) silver stain to visualise the protein bands. Numbers on the left indicate the approximate molecular mass of the proteins within the most diluted sample. Ten distinct bands (arrows) were observed after staining the gels. Molecular mass (2-250 kDa) marker is shown in the middle between the two gels. Asterisk indicates the lane from the Coomassie stained gel that was selected for GeLC-MS/MS analysis. No bands were seen in the water control used.

4.3.2 **Western blot**

Almost 12 bands resulted from western blot screening using the biotinylated lectin Jacalin at the range of molecular mass proteins between 13-130 kDa and the most intense bands were approximately 15, 25, 46, 80 and 85 kDa (**Fig. 4.2 C**). TBS and nonspecific controls, which were used during the investigation to detect any contamination, resulted in no obvious bands (**Fig. 4.2 A-B**). Although the secretions sample used in this investigation was not diluted, the Coomassie stain produced similar bands to those observed in the previous SDS-PAGE gel, but with more intense bands (**Fig 4.2 D**).

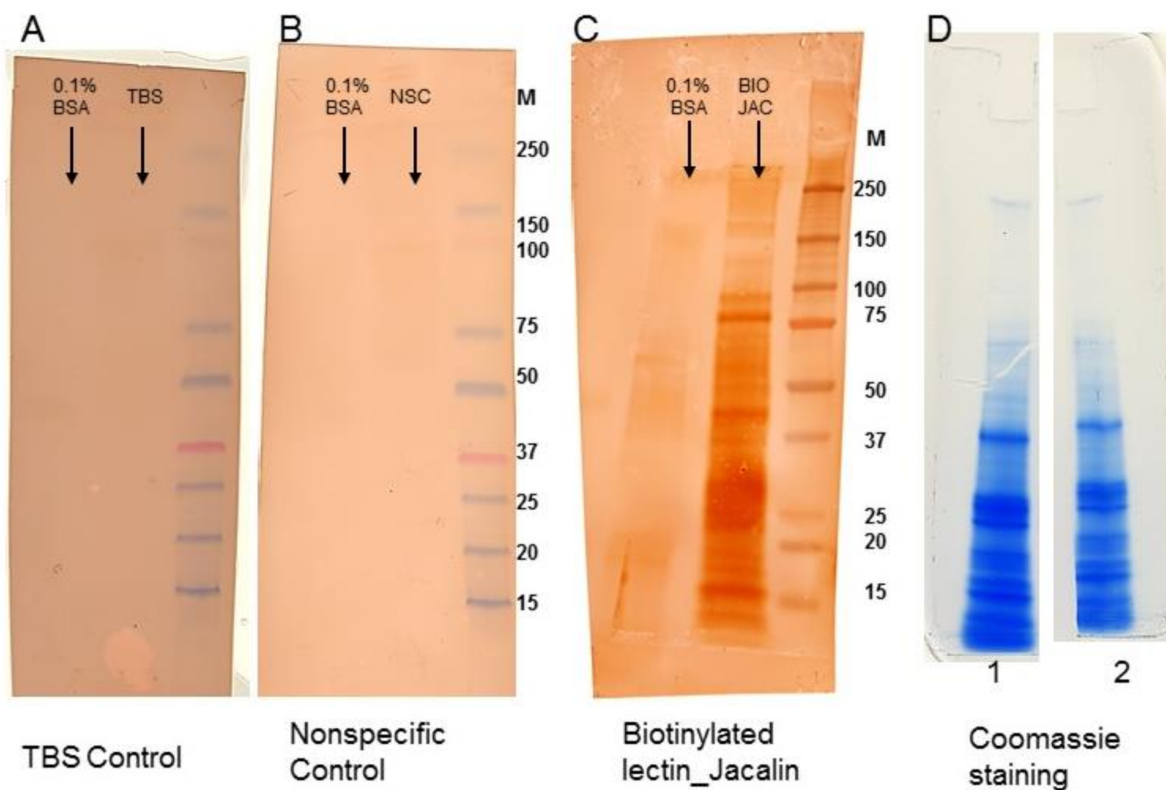


Figure 4.2. Western blot for identification of secreted proteins from *A. foliaceus* secretion. No bands detected using (A) Negative control, TSB and (B) Nonspecific control; anti-sheep rabbit biotinylated antibody (C) Reaction of the biotinylated-Jacalin lectin revealed the detection of multiple bands of a range of different molecular weight (kDa) proteins (D) 12% and 4-15% SDS-PAGE gels of *A. foliaceus* concentrated secretion samples stained by QC colloidal Coomassie stain run alongside the immunoblots.

A band of 25 kDa was detected in the three western blot membranes of the biotinylated lectin PHA-E, Serpin D1 and heparan sulphate proteoglycans antibody; HS6ST3 (**Fig. 4.3**). From the biotinylated lectin PHA-E almost 13 bands were observed within the range of 13-130 kDa with the more intense bands approximately 28, 46 and 54 kDa (**Fig. 4.3**).

Serpin D1 showed only one band at the range of 25 kDa. Several bands resulted from heparan sulphate proteoglycans antibody labelling; HS6ST3, from 15-130 kDa but,

there were 3 intense bands of around 25, 46 and 85 kDa. Some background staining was observed in control blots (**Fig. 4.3).**

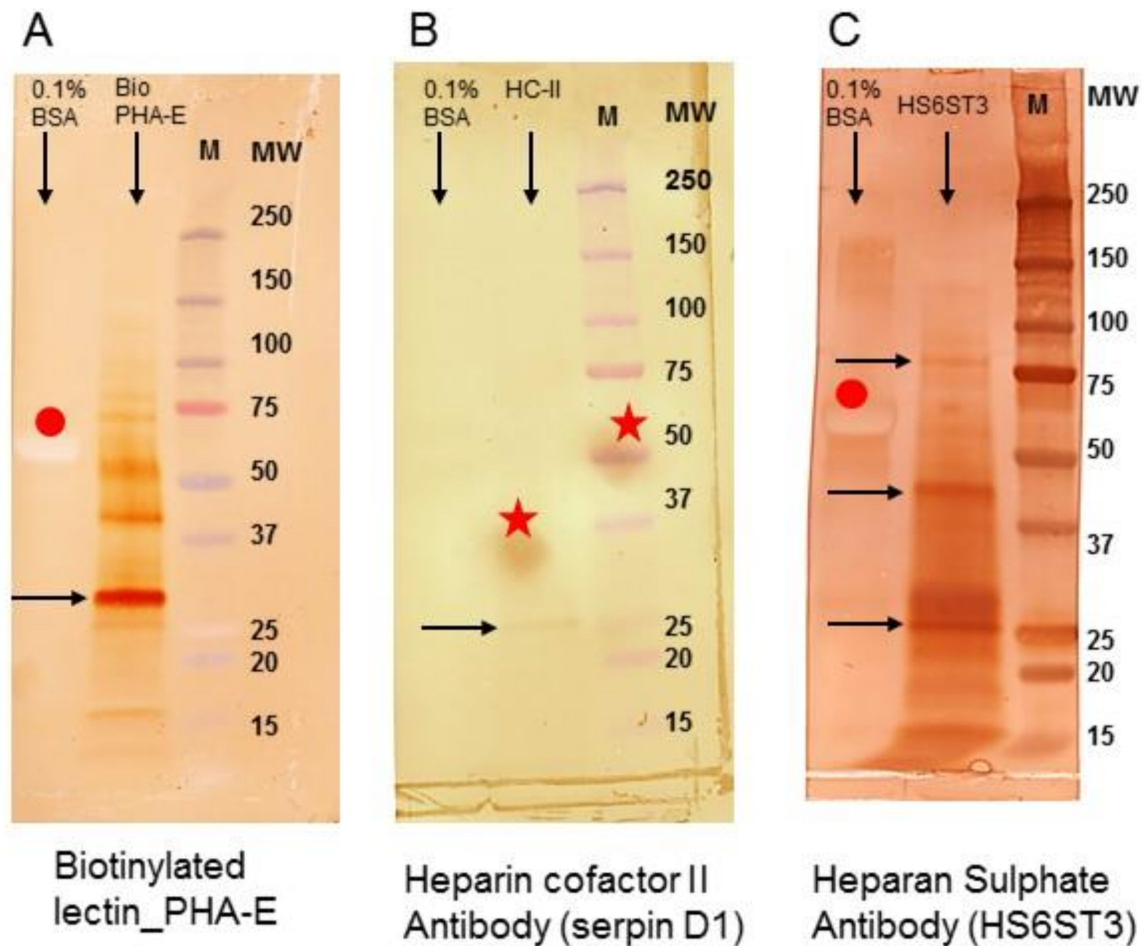


Figure 4.3. Western blot analysis of Immuno-detection of secreted proteins from *A. foliaceus* secretions. (A) Biotinylated PHA-E lectin detecting several proteins of different sized proteins ranging from approximately 13-130 kDa, with the most intense band detected around a molecular mass of 28 kDa (B) Serpin D1; antibody detecting a protein at a molecular weight of 25 kDa (C) Heparan sulphate proteoglycans antibody detecting different bands with three intense bands at 25, 46 and 85 kDa. ● = digested protein bands of BSA, ★ = background artefacts, ⟶ = intense bands.

4.3.3 **GeLC-MS/MS analysis**

Secretions were collected from 560 live *A. foliaceus* parasites for GeLC-MS/MS analysis and following identification of proteins which was confirmed by positive

matches with transcript sequences. The number of peptide fragments returned from the sample of pooled *A. foliaceus* secretions was 1182, however, only 45 of these passed the specified "stringent" quality criteria and were associated with 26 annotated proteins (**Table 4.3**; **Table 4.5**). To confirm the functional identity of these proteins, the amino acid sequences were scanned against Pfam database and in order to find homologous proteins in Metazoa, the NCBI BLASTP program was used (**Table 4.3**; **Table 4.4**) and the homology of the proteins with the best e-values were selected. The identified proteins by Pfam were vitellogenin-N, haemocyanin-N, haemocyanin-M, haemocyanin-C, astacin, serpin, fasciclin, trypsin, animal haem peroxidase, peptidase-M14, CUB, pro-isomerase, VIT/VWA; Vault protein inter-alpha-trypsin domain and von Willebrand factor type A domain.

Of these predicted protein sequences 13 did not show amino acid identities to previously annotated proteins in Pfam and only 6 out of these 13 showed hits when blasted against Metazoa in NCBI BLASTP with a higher e-value **(Table 4.5)**.

In **Table 4.3**, the other thirteen predicted proteins; which showed Pfam functional descriptions, were listed with the suggested functions from other arthropod studies and signal peptide predictions. Number of peptides, Signal P, N-glycosylation, molecular weight of proteins and presentage of sequence coverage all listed in the same table.

Thirteen protein sequences were predicted, but not found to have any significant hit in Pfam. When running a blast search of these sequences to metazoan in NCBI BLASTP only 6 of them resulted in homology with high e-values (**Table 4.5**).

Signal P showed that out of these 26 predicted proteins 14 were found to have a signal peptide sequence. Putative N-glycosylation sites were predicted in 19 of the predicted proteins using the NetNGly1.0 server **(Table 4.3; Table 4.5).**

The identified proteins included zinc carboxypeptidase enzyme; M-14 peptidase, Vitellogenin-N, Haemocyanin-N, Haemocyanin-M, Haemocyanin-C and Astacin, Proteinase inhibitor; Serpin, Fasciclin, Trypsin, An-peroxidase, Peptidase_M14, CUB, Pro-isomerase, VIT/VWA.

Table 4.3 List of predicted proteins identified by GeLC-MS/MS analysis of *A. foliaceus* secretions showing hits with Pfam database. Pfam functional description suggested functions reported in different species of arthropods, Signal P and N-glycosylation: N-glyco, + symbol indicates the strength of glycosylation in at least one site. MW: Molecular weight of protein SC %: Sequence coverage (%).

Protein family	Description	No. of peptides	Sequence length	MW [kDa]	SC [%]	Signal P	N-glyco	Function	References	Organism	Tissue
Vitellogenin-N	Lipoprotein amino terminal region	44	1583	183.8	30.1	No	Yes/++	Transporters Osmolality Immunity Clotting	(Díaz-Martín *et al.* 2013) (Kristoffersen *et al.* 2009) (Liu *et al.* 2009) (Hall *et al.* 1999)	*Ornithodorosmoubata* *Pelagic* *Cyprinus carpio* *Crayfish*	Saliva Eggs Female carp Hepatopancreas
Haemocyanin-N	Haemocyanin, all-alpha domain	20	291	34	45.7	Yes	No	Respiratory Protein storage	Pinnow *et al.* (2016)	*Argulus*	Haemolymph
Haemocyanin-M	Haemocyanin, copper containing domain	18	690	80.3	26.6	Yes	Yes/++	Respiratory Protein storage	Depledge and Bjerregaard 1989	Decapods	Haemolymph
Haemocyanin-C	Haemocyanin, ig-like domain	8	314	36.1	30.6	No	Yes/++	Immunity; antifungal activity	Destoumieux-Garzon *et al.* 2001	Shrimp	Haemolymph
Astacin	Astacin (Peptidase family M12A)	12	688	76.3	17.3	No	Yes/+	food digestion host penetration, immune evasion or activation	Lun *et al.*, 2003 Gallego *et al.*, 2005 Park *et al.*, 2010 Baska *et al.*, 2013	*Trichinella spiralis* *Strongyloides stercoralis* *Caenorhabditis elegans* *Ancylostoma ceylanicum*	E/S products whole parasites whole parasites whole worm
Serpin	Serpin (serine protease inhibitor)	7	419	46.7	22	Yes	Yes/++	anticoagulation activity, modulate host immune response regulation of host inflammation, antihaemostatic effects, and platelet aggregation	Yu *et al.* 2013 Chalaire *et al.* 2011 Mulenga *et al.*, 2001 Imamura *et al.*, 2005; Prevot *et al.*, 2006; Ibelli *et al.*, 2014	*Rhipicephalus haemaphysaloides* *Amblyomma americanum* *Ixodes ricinus, Haemaphysalis longicornis, Ixodes scapularis* *Rhipicephalus appendiculatus*	Salivary gland Saliva
Fasciclin	Fasciclin domain	5	327	36.4	23.6	Yes	Yes/++	mediate cell adhesion	(Gobert *et al.* 2010) (Sharma *et al.* 2015)	*Schistosoma mansoni* *mosquito Anopheles culicifacies*	Whole parasites Salivary gland

Trypsin	Trypsin	2	448	44.9	25.5	Yes	No	digestion, maintain host-parasite relationship	(Johnson *et al.*, 2002)	*Lepeophtheirus salmonis*	Whole preadult parasite
An-peroxidase	Animal haem peroxidase	8	239	26.6	42.9	No	Yes/+++	vasodilatory activity oxidase / peroxidase activity PGE2 synthesis	(Ribeiro & Nussenzveig 1993) Champagne (1994) Tootle and Spradling (2008	*Anopheles albimanus* *A malaria vector, Anopheles albimanus* *Drosophila*	Salivary glands Salivary glands
Peptidase_M14	Zinc carboxypeptidase	5	425	48.3	16.7	No	Yes/++	proteolytic-enzyme	Braden *et al.* (2017)	*Lepeophtheirus salmonis*	Whole parasites
CUB	CUB domain	4	386	11.7	17.1	No	Yes/+++	a component of metalloprotease which involved in invasion process	Gallego *et al.*, (2005)	*Strongyloides stercoralis*	
Pro-isomerase	Cyclophilin type peptidyl-prolyl cis-tra	3	237	22.4	19.3	Yes	Yes/++	immunogenic enzymes, proline isomerisation and folding protein	Lewis *et al.*, (2015)	*Ixodes scapularis*	Saliva
VIT/VWA	Vault protein inter-alpha-trypsin domain and von Willebrand factor type A domain	4	870	96.7	7.5	Yes	Yes/++	VWA is inhibitors of primary haemostasis	(Modica *et al.* 2015)	*vampire snail, Colubraria reticulata*	Salivary gland

Table 4.4 BLASTP analysis for predicted proteins that were identified by Pfam from *A. foliaceus* secretions against the NCBI, Metazoan.

Family	Protein hit name (NCBI BLASTP)	Hit organism	E-value	Accession No.
Vitellogenin-N	vitellogenin-like	*Parasteatoda tepidariorum*	3.00E-14	XP_015915706
Haemocyanin-N	haemocyanin 1	*Argulus foliaceus*	1.00E-109	CUH82791
Haemocyanin-M	haemocyanin 1	*Argulus foliaceus*	<1.00E-109	CUH82791
Haemocyanin-C	haemocyanin 2	*Argulus foliaceus*	<1.00E-109	CUH82792
Astacin	protein SpAN-like	*Saccoglossus kowalevskii*	2.00E-47	XP_018024452
Serpin	serine protease inhibitor (Serpin-5)	*Daphnia magna*	7.00E-69	JAN34275
Fasciclin	Periostin	*Orchesella cincta*	2.00E-22	ODN00130
Trypsin	trypsin-3	*Bombus terrestris*	4.00E-88	XP_020722765
An-peroxidase	peroxinectin	*Panulirus longipe*	3.00E-56	AGO05992
Peptidase-M14	mast cell carboxypeptidase A	*Cebus capucinus imitator*	4.00E-89	XP_017379291
CUB	PREDICTED: cubilin homolog isoform X2	*Drosophila miranda*	0.004	XP_017135474
Pro-isomerase	peptidylprolyl isomerase B precursor	*Bombyx mori*	1.00E-93	NP_001040479
VIT/VWA	Inter-alpha-trypsin inhibitor heavy chain h4 precursor	*Daphnia magna*	4.00E-157	JAN60290

Table 4.5 Predicted proteins from *A. foliaceus* secretions by mass spectrometric analysis that showed no hits in Pfam and were blasted against Metazoan in NCBI BLASTP, signal P and N-glycosylation: N-glyco, + symbol indicates the strength of glycosylation in at least one site. MW: Molecular weight of protein, SC %: Sequence coverage (%).

Protein	Sequence length	MW [kDa]	SC [%]	Protein name	Hit organism	E-value	NCBI BLASTP	Signal P	N-glyco
TRINITY_DN54782_c3_g1_i1	193	20.7	40.1	No significant hit				No	No
TRINITY_DN48022_c0_g1	140	14.7	42.4	uncharacterized protein Dmel_CG42255	*Drosophila melanogaster*	0.022	NP_729748	Yes	Yes/++
TRINITY_DN55212_c4_g6_i1	463	50.7	18.4	PREDICTED: high choriolytic enzyme 1	*Galendromus occidentalis*	1.00E-15	XP_003745942	No	Yes/++
TRINITY_DN46775_c0_g1_i1	199	22.1	49	No significant hit				Yes	Yes/+
TRINITY_DN41468_c0_g2_i1	399	43.6	16.6	PREDICTED: tolloid-like protein 2	*Branchiostoma belcheri*	2	XP_019626246	Yes	Yes/++
TRINITY_DN55212_c4_g3_i2	332	36	11.5	No significant hit				No	No
TRINITY_DN52747_c2_g1_i2	234	26.5	15.5	PREDICTED: adhesion G-protein coupled receptor F3 isoform X10	*Macaca mulatta*	9.1	XP_014967371	Yes	Yes/++
TRINITY_DN51399_c0_g1_i1	200	22	18.1	No significant hit				Yes	No
TRINITY_DN46775_c0_g2_i1	230	25.7	13	No significant hit				No	No
TRINITY_DN44739_c0_g1_i1	383	43.4	10.6	No significant hit				Yes	Yes/++
TRINITY_DN34222_c0_g1_i1	393	42.9	6.1	blastula protease 10-like isoform X2	*Parasteatoda tepidariorum*	0.25	XP_015905755	Yes	No
TRINITY_DN51502_c3_g2_i1	263	30.7	10.7	Gamma-crystallin-4	*Orchesella cincta*	4.00E-13	ODM96300	No	Yes/++
TRINITY_DN52386_c2_g1_i1	134	15.1	19.5	No significant hit				No	Yes/++

Table 4.6 Sequence alignment of the *A. foliaceus* serpin detected by GeLC- MS/MS analysis. The predicted inhibitory function of this serpin is based on the consensus sequence for the reactive centre loops (RCL) after running multiple alignment against 11 human serpins from the human reference protein database. RCL highlighted as bold / underlined text.

Protein name	Protein Sequence	Accession Number	Genbank Index
Argulus foliaceus Serpin	K**GSELAAATIAT**--NT**RV**RVGKM-**MRVNR**PFLFFIRDNYSGLPVFWARIVKPQDKEGPSA		
serpin B10	QGTEAAAGSGSE-IDIRIRVPSIEFNANHPFLFFIRHNKTNTILFYGRLCSP--------	NP_005015.	gi|4826902
PREDICTED: serpin B10 isoform X2	EGTEAAAATGDS-IAVKSLPMRAQFKANHPFLFFIRHTHTNTILFCGKLASP--------	NP_005015	gi|156071456
serpin A12 precursor	RGTEGAAGTGAQ---TLPMETPLVVKIDKPYLLLIYSEKIPSVLFLGKIVNPIGK-----	NP_776249	gi|27777657
serpin A12 precursor	EGTEAAAATGIG-FTVTSAPGHENVHCNHPFLFFIRHNESNSILFFGRFSSP--------	NP_776249	gi|807201021
serpin B13 isoform 1	KGTEAAGAM---FLEAIPMSIPPEVKFNKPFVFLMIEQNTKSPLFMGKVVNPTQK-----	NP_001294852	gi|50363217
alpha-1-antitrypsin precursor	SGTRAAAATGTIFTFRSARLNSQRLVFNRPFLMFIVDN---NILFLGKVNRP--------	NP_000286	gi|194018472
plasma serine protease inhibitor preproprotein	EGVEAAAATAVVVVELSSPSTNEEFCCNHPFLFFIRQNKTNSILFYGRFSSP--------	NP_000615	gi|28076869
serpin B4 isoform 1	EGAEAAAATAVVGFGSSPTSTNEEFHCNHPFLFFIRQNKTNSILFYGRFSSP--------	NP_002965	gi|5902072
serpin B3	NGTQAAAATGAV-VSERSLRSWVEFNANHPFLFFIRHNKTQTILFYGRVCSP--------	NP_008850	gi|17998551
serpin B4 isoform 2	EGVEAAAATAVVVVELSSPSTNEEFCCNHPFLFFIRQNKTNSILFYGRFSSP--------	NP_778206	gi|669250853
PREDICTED: antithrombin-III isoform X1	EGSEAAASTAVVIAGRSLNPNRVTFKANRPFLVFIREVPLNTIIFMGRVANPCVK-----	XP_005245255	gi|530364787
serpin B9	EGTEAAAASSCFVVAECCMESGPRFCADHPFLFFIRHNRANSILFCGRFSSP--------	NP_004146	gi|4758906
	* * * * *		

4.3.4 **Total RNA extraction and gene expression analysis**

In order to check the expression of selected target proteins from the transcriptome dataset results from earlier in this study, especially during feeding, total RNA from 10 fed and 10 starved *A. foliaceus* samples; 5 males and 5 females from each group, were prepared and the concentration and quality (280/260 nm ratio) were measured and recorded (**Table 4.6**). RNA integrity was also evaluated by agarose gel electrophoresis and ethidium bromide staining (**Fig. 4.4**). These transcripts: trypsin, PGE2S, serpin, aspartic protease, cysteine protease, venom serine protease, ferritin and thrombin inhibitor may play a major role in host-parasite interactions in-line with homologues in other haematophagous arthropods. The housekeeping genes used were β-actin and elongation factor-1α. The result of the PCR reactions using RNA samples from adult female *A. foliaceus* showed the expected product size for each transcript selected (**Table 4.2**; **Fig 4.5**).

Table 4.7 Concentration (ng/µl) of extracted total RNA for whole fed and starved *A. foliaceus* samples

Sample	Con	280/260	Sample	Con (ng/µl)	280/260
Female Fed-1	857.6	2.08	Female Starved-1	576.1	2.01
Female Fed-2	621	2.03	Female Starved-2	479.5	2.01
Female Fed-3	547.5	2.04	Female Starved-3	470.9	2
Female Fed-4	523.4	2.02	Female Starved-4	403.6	2.02
Female Fed-5	467.2	2	Female Starved-5	335.1	2
Male Fed-1	437.9	2	Male Starved-1	411.5	2.01
Male Fed-2	375.6	2	Male Starved-2	363.1	2.01
Male Fed-3	343.9	2.05	Male Starved-3	291.3	2.01
Male Fed-4	280.5	2.01	Male Starved-4	219.7	2.01
Male Fed-5	251	2.01	Male Starved-5	140.5	2.02

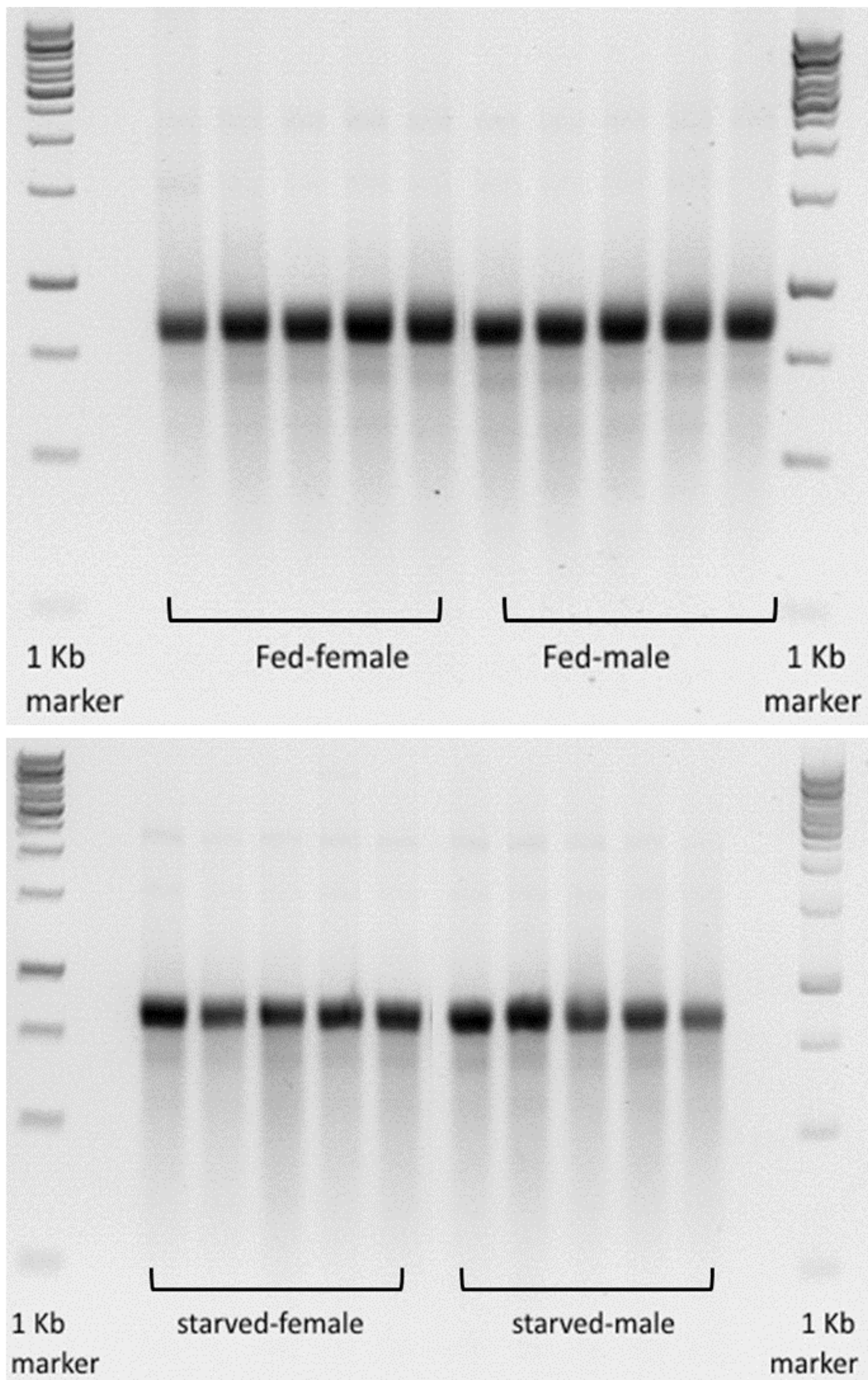


Figure 4.4. Total RNA Isolation for two groups of adults *A. foliaceus*, fed and starved males and females, using Tri Reagent.

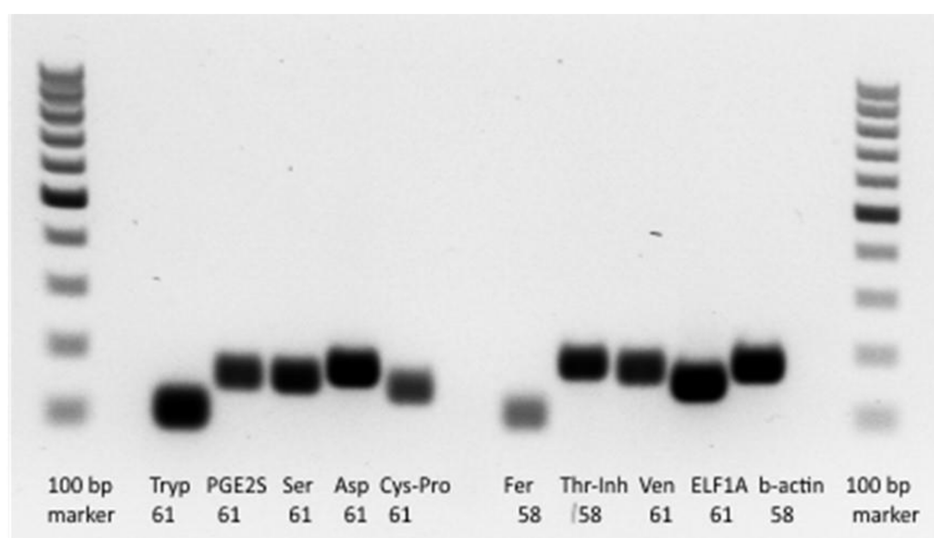


Figure 4.5. PCR reaction for qPCR targets using RNA samples from adult female *A. foliaceus* at 61°C for trypsin, PGE2S, Serpin, Aspartic protease, cysteine protease, venom serine protease and elongation factor-1α and 58°C with ferritin, thrombin inhibitor and β-actin for 3h.

A summary of the gene expression results by qPCR is shown in **Table 4.7** and **Fig. 4.6.** There were no indications of RNA degradation as the results were consistent with strong correlations observed. The two-way ANOVA revealed that no significant interactions were evident between the factors "treatment" and "sex" with "sex" differences being significant for five genes out of eight and fed state having no significant effect (**Table 4.7** and see **appendix 6**).

Venom serine protease; *vser*, was significantly differentially expressed between females and males (p = 3.55E-06), being up-regulated in females (**Fig. 4.6).** The expression in females was almost the same for both conditions, but the fed males had lower expression levels than starved ones, albeit not significantly different.

Thrombin inhibitor, *thr*, expression was also significantly differentially regulated by sex with females exhibiting higher relative expression levels than males ($p < 0.01$, **Fig. 4.6**).

Cysteine protease; *cys*, was significantly up-regulated in males (p = 0.026, **Fig. 4.6**). Starved males exhibited higher expression levels, although expression was not different between fed males and females, whereas starved females showed significantly lower expression levels than starved males.

No significant differences were observed in the relative expression levels of aspartic protease; *asp* in terms of “sex” (p = 0.187) or “treatment” (p = 0.333).

PGE2S; *pge2s*, was highly expressed in males compared to females (p =0.004) and starved females had the lowest expression levels although expression was not significantly different to fed females (**Fig. 4.6**).

Ferritin; *fer* expression was significantly different between fed and starved female lice (p = 0.008 **Fig.4.6**), but not male lice. Expression levels in fed groups were approximately the same, but were higher in starved males than starved females albeit not significantly (**Fig.4.6**).

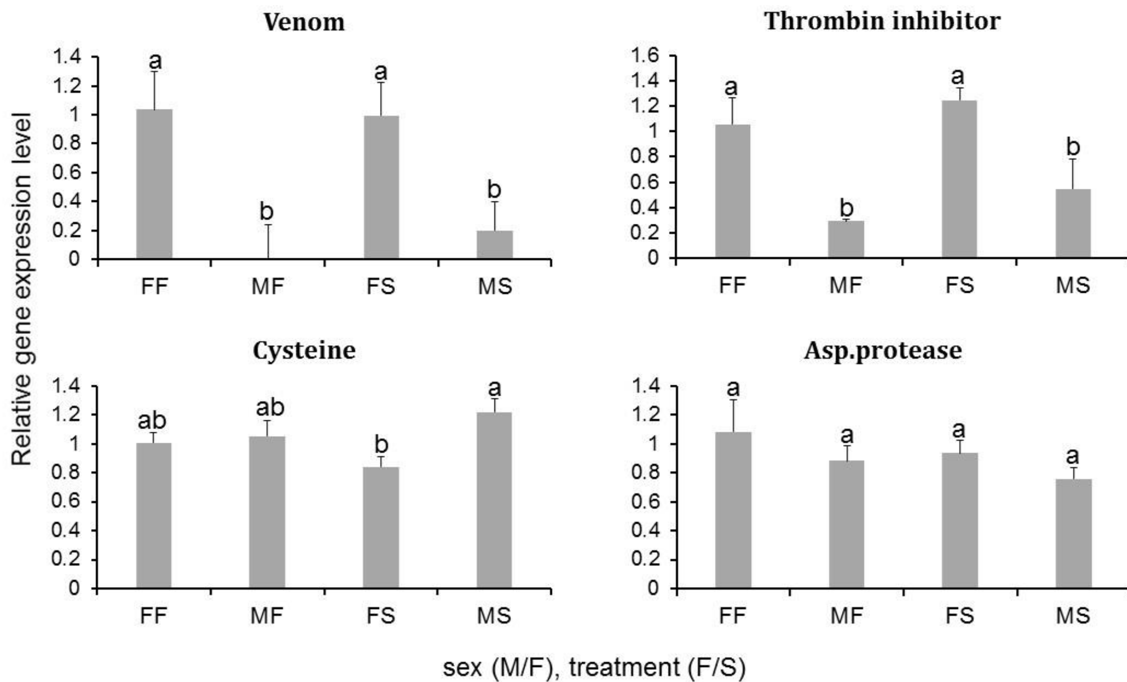
Trypsin expression (**Fig. 4.6**) did not show significant differences in terms of “treatment” (p = 0.604) and “sex” (p = 0.090) like aspartic protease.

Serpin, *ser*, was expressed at significantly higher levels in male lice than females (p = 0.008) and starved females had the lowest expression of serpin although the expression levels were not significantly different to those from fed females (**Fig. 4.6**).

Table 4.8 *p* values for the effect "treatment", "sex" and their interactions with transcript expression.

Genes tested	Symbol	Treatment		Sex		TXS
Venom serine protease	***vser***	0.56	ns	3.55E-06	**	0.379
Thrombin inhibitor	***thr***	0.194	ns	< 0.001	**	0.836
Cysteine protease	***cys***	0.991	ns	0.026	*	0.069
Aspartic protease	***asp***	0.333	ns	0.187	Ns	0.936
prostaglandinE2 synthase	***pge2s***	0.795	ns	0.004	**	0.356
Ferritin	***fer***	0.008	**	0.856	Ns	0.48
Trypsin	***try***	0.604	ns	0.09	Ns	0.114
Serpin	***ser***	0.061	ns	0.008	**	0.388

Asterisks indicate significant differences as $^{**}P \leqslant 0.01$, $^{*}P \leqslant 0.05$. ns indicates no significant differences



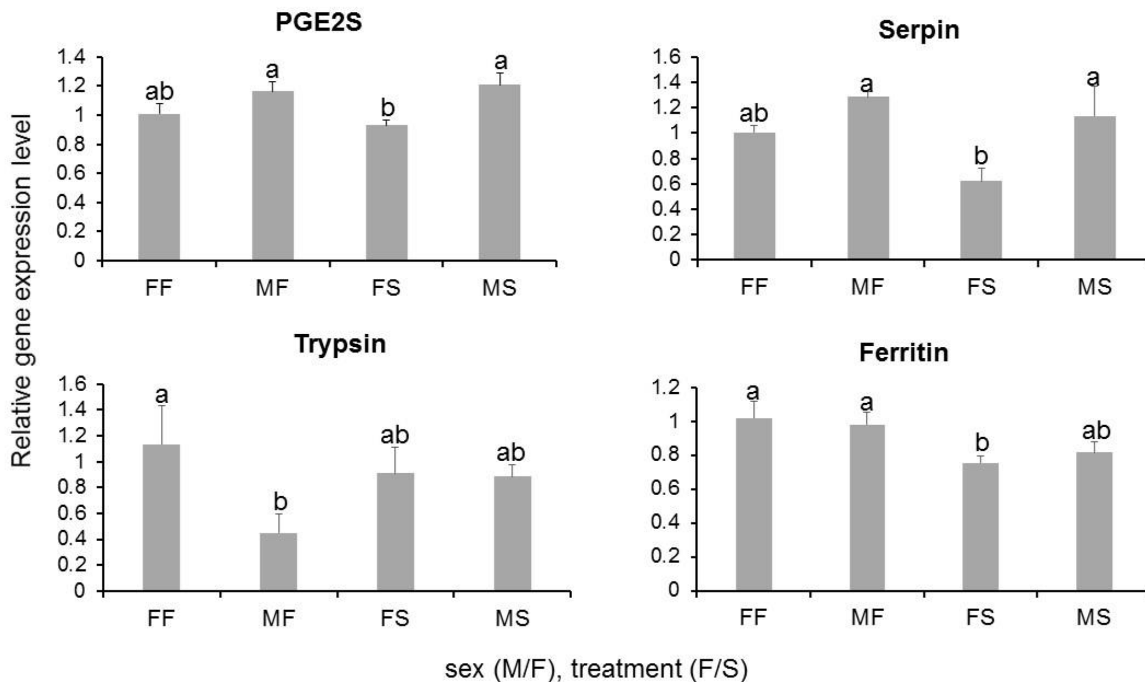


Figure 4.6. Expression levels of venom serine protease, thrombin inhibitor, cysteine protease, aspartic protease, PGE2S, ferritin, trypsin and serpin transcripts of *A. foliaceus*. Significance was identified by two-way ANOVA ($p < 0.05$) followed by post-hoc Tukey-Kramer HSD test to determine pairwise significance. Different superscript letters denote significant differences between sex (M/F), treatment (F/S). FF: fed female, MF: fed male; FS: starved female; MS: starved male.

4.4 Discussion

Studies of the secretory products of *A. foliaceus* are important for determining how the parasite establishes host attachment and facilitates blood feeding, as the components of ectoparasite secretions are known to play functional roles in such interactions (Salát *et al.*, 2010; Champagne *et al.*, 2004; Gillespie *et al.*, 2000). In this regard, the secretions of *Argulus* spp. resemble those in saliva of ticks and other haematophagous arthropods, containing bioactive molecules released to maintain a successful feeding site. Using an GeLC-MS/MS methodology proteins such as serpin, trypsin and fascilin

have been previously associated with this role in tick saliva such as that of male *Ornithodoros moubata* (Díaz-Martín *et al.*, 2013). Sea lice trypsins, vitellogenin-like proteins and proteins with immunomodulatory functions and host adhesion properties have similarly been studied in this aspect and have been proposed as potential vaccine antigen candidates (as reported by Carpio *et al.*, 2011). In order to complete feeding *Argulus* spp. needs to modulate host immune defence mechanisms (haemostasis and immunity) and inhibit host tissue repair responses as do other haematophagous arthropods such as ticks (Radulović *et al.*, 2014; Mulenga *et al.*, 2013; Chmelar *et al.*, 2012; Chmelar *et al.*, 2011; Ribeiro 1987).

4.4.1 **SDS-PAGE and western blot**

SDS-PAGE of the secretory components of *A. foliaceus* identified a range of different polypeptide bands between 3 - 100 kDa. Ruane *et al.* (1995) showed similar protein profile ranges from whole *A. foliaceus* homogenates, with proteins of molecular weights between 15 - 100 kDa (Ruane *et al.*, 1995). From *A. siamensis* homogenates (Saurabh *et al.*, 2012), polypeptide bands between 16.22 - 130.55 kDa were detected by SDS-PAGE. Analysis in the current study was limited to components of secretions and also run in non-native conditions. The immunolabelling using polyclonal antibodies for Serpin D1 (anti-HC II antibody), and Heparan Sulfate 6-O-Sulfotransferase 3; (antibody HS6ST3), for secreted proteins of *A. foliaceus* by western blotting revealed that some of the detected proteins are recognised by these antibodies. The anti-serpinD1 antibody in the current study recognised a protein at 25 kDa. This result suggests that the secretions contain serpin D1. Serpins from arthropods have been

characterised previously to have functional roles in host-parasite interactions such as anticoagulation activity, immunomodulation, host inflammatory regulation and anti-haemostatic properties (Ribeiro 1987; Muleng *et al*., 2001; Leboulle *et al.,* 2002; Chmelar *et al.,* 2011; Ibelli *et al*., 2014).

Immunoblots with heparan sulphate antibody detected proteins of molecular mass range between15-130 kDa with 3 intensive bands at ~ 25, 46 and 85 kDa MW. Multiple bands were labelled by the biotinylated-PHA-E and biotinylated-Jacalin lectins. This is because these lectins bind to glycosylated regions, and not to a specific antigen, and thus one lectin can bind to multiple proteins containing the same recognised glycosylated residues. Nonetheless, a dominant band of approximately 25 kDa was observed when the separated secreted proteins were screened with biotinylated PHA-E and Jacalin lectins, which was also the MW of the immunodominant bands recognised by the anti-serpin D1 antibody and the heparan sulphate antibody (HS6ST3). This cross recognition of this 25kDa antigen in these different immunoblots may be the result of the serpin protein containing N-acetylglucosamine oligomers residues that are recognised by the lectins, whilst the simultaneous complexing of the protein to heparan sulphate may account for its recognition by the HS6ST3 antibody at the same MW. Previous studies have demonstrated that most identified serpins are glycosylated (Tirloni *et al.,* 2014), this designating glycosylation as a general feature of serpins, which play important roles in biological activity *i.e*. in thrombosis, haemostasis and fibrinolysis (Rau *et al*., 2007). For instance, the production of serpins in ticks' saliva were postulated to play a role in interruption of the host homeostatic balance in order to facilitate parasitism via effects on inflammation, complement activation and blood coagulation pathways (Muleng *et al*., 2001; Mulenga *et al*., 2009).

Tollefsen, (1997) described the structure of serpin D1 (HCII) to have three potential asparagine residue-linked glycosylation sites and to contain 10% carbohydrate by weight, and this may explain the presence of the intensive band at 25 kDa MW in all the lectins and HSPG (HS6ST3) antibody immunoblots. Indeed, the identified serpin by spectrometric analysis of the *Argulus* secretory products in the current study was predicted to be glycosylated as showed by NetNGlyc 1.0 Server (**Table 4.3**). Although this might be a different type of secreted serpin, as the MW of the identified serpin from the *A. foliaceus* secretions by GeLC-MS/MS is 46.6 kDa. Heparan sulphate is known to complex with serpin, which is thought to instigate effector functions by conformational changes in the protein (Tollefsen 1997; Function *et al.,* 2006; Koide 2008). Glycosaminoglycans are proteoglycans where the polysaccharide chains are covalently linked to a core protein (Sampaio *et al*., 2006). In order for serpins to inhibit thrombin, glycosaminoglycans such as dermatan sulphate, heparin or heparan sulphate are required to activate those serpins *e.g*. serpin D1 and antithrombin, to become efficient inhibitors of coagulation proteinases (Koide, 2008). Horn *et al*. (2000) suggested that saliva serpin of ticks was responsible for the anticoagulant properties of tick saliva.

A band of 15 kDa was detected in the immunoblot of the lectins and HSPG antibody as showed in the results of western blot. A 15 kDa *Ixodes scapularis* salivary protein was described to function as an immunomodulator (Anguita *et al*., 2002; Das *et al*., 2001) named as serpin Salp15 and was reported to be associated with the immunosuppressive activity of tick saliva by binding to the leukocyte cell membrane and modulating the expression of cytokines at the transcription level (Anguita *et al*.,

2002). Further investigations need to be done to look out these immunodetected proteins.

The overall results from SDS-PAGE confirmed the presence of secreted proteins and western blot confirmed the presence of important proteins involved in host-parasite interactions, *e.g.* serpins. Considerable further investigations are needed, however, to confirm the presence of and characterise these proteins.

4.4.2 **GeLC-MS/MS**

No proteomic studies of any *Argulus* spp. secretions had previously been conducted prior to this study. In order to identify the major proteins of *A. foliaceus* secretions, an integrated transcriptomic and proteomic approach was used. The tandem-mass spectrometric (GeLC-MS/MS) data combined with the *A. foliaceus* transcriptome, used as a reference database, resulted in the identification of a number of different proteins nominally secreted by the parasite. As an initial caveat it should, however, be recognised that not all of these proteins may be associated with host-associated external secretions by the parasite. Despite the collection technique it is possible that individual proteins may be present in the sample through *e.g.* presence in faeces or release through accidental damage of parasites. In particular, proteins with well-recognised physiological roles such as haemocyanin and vitellogenin could fall into this category. In situ labelling (*e.g.* IHC) and other tools may shed light on such questions in further studies.

Overall, the analysis of the secretions sample retrieved 26 predicted protein sequences where only 13 were recognised by Pfam and the other 13 protein

sequences could not be recognised because they did not match any known sequence in the Pfam database, and the ones which matched with NCBI revealing a high range e-value. From these there were interesting targets that have previously been identified as playing a role in host infection in other haematophagous arthropods. These include vitellogenin-N, haemocyanin-N, haemocyanin-M, haemocyanin-C, metallopeptidases as Peptidase_M14 and Astacin (Peptidase family M12A), proteases such as trypsin; and serpin, peroxidase; animal haem-dependent peroxidases and other protein domains such fasciclin, CUB, pro-isomerase; cyclophilin type peptidyl-prolyl cis-tra and VIT/vWA; Vault protein inter-alpha-trypsin domain and von Willebrand factor type A domain. Of the 26 identified proteins, 14 proteins carried a predicted signal sequence suggesting them to be extracellular proteins and discharged within the secretions of the parasites. Signal peptide predicts a short peptide (usually 16-30 amino acids long) present at the N-terminus of the majority of newly synthesized proteins that are destined towards the secretory pathway (Kapp *et al.*, 2013). Extracellular proteins are synthesised in the endoplasmic reticulum-ribosomes which then leads to the Golgi apparatus and from there to lysosomes, or secretory vesicles, and to the extracellular environment (Ojefua, 2009). Signal peptide targets a protein for translocation across the endoplasmic reticulum (ER) membrane in eukaryotes (von Heijne, 1986). Those that were not predicted to have a signal peptide, even when found in the secretions, may occur there be due to the fact that while they were secreted proteins, only partial protein sequences were recognised, which were insufficient for prediction by Signal P. However, further studies need to confirm this. Out of these 26 proteins 19 were found to be glycosylated as NetNGlyc 1.0 Server

predicted them. The presence of these glycosylated proteins was also showed from the detection of a range of bands in lectin labelling of blots.

This study has provided the first outline of novel proteins (*i.e.*, with no similarity in metazoan databases) identified from *A. foliaceus* secretions and hence provides opportunity for further investigation to understand their role in parasite-host interactions. Although the biological functions of these secreted proteins are unknown, they may have properties controlling physiological functions during *Argulus* attachment. As some of these proteins were not characterised in Pfam and the NCBI database, this suggests that *Argulus* has unique proteins compared to other well characterised haematophagous ecdysozoa, such as insects, ticks and nematodes, for potentially modulating or evading their host immune system. Some of the proteins analysed with functional identification in this study have also been previously described in other haematophagous arthropods such as for tick salivary proteomes (Tirloni *et al.*, 2015; Tirloni *et al.*, 2014; Maritz-olivier *et al.*, 2007). This suggests the role of these proteins from *A. foliaceus* secretions during host-parasite interactions may be similar to other ectoparasites and supports their importance for *A. foliaceus* in evading host immune defences. The proteins identified are discussed below according to their hypothesised function and / or family.

4.4.2.1 **Transporters**

Vitellogenin is identified in this study and found to be glycosylated protein. It is a lipoprotein generally related to reproduction in arthropods, however, it has been shown that the production of this protein can be positively associated with the size of blood

meals, as in ticks vitellogenin binds to the derivative haem from the host to initiate the reproduction cycle (Galay *et al*., 2013; Donohue *et al*., 2009; Rosell-Davis and Coons 1989). This was supported by the findings of Galay *et al*. (2013) that silencing of the secretory ferritin gene of the hard tick *Haemaphysalis longicornis* affected two vitellogenin genes (Galay *et al*., 2013). Moreover, Rosell-Davis and Coons (1989) showed that onset of feeding initiates vitellogenin production (Rosell-Davis and Coons 1989).

Attardo *et al*. (2005) demonstrated that vitellogenesis was activated by the amino acids provided from the blood meal. Dalvin *et al*. (2011) examining *L. salmonis* did not observe any transcription of this protein in the ovary but they did observe the presence of these proteins in the haemolymph (Dalvin *et al*., 2011). Vitellogenins and vilellogenin-like proteins; lipoproteins, have been suggested to be involved in other developmental processes, such as regulation of osmolarity in pelagic fish eggs (Kristoffersen *et al*., 2009), in carp *C. carpio* innate immunity (Liu *et al*., 2009) and clotting (Hall *et al*., 1999). Since vitellogenins and clotting proteins have been found be similar (Hall *et al*., 1999), it could be useful to collect secretions from both sexes separately to determine if this lipoprotein were expressed in both *A. foliaceus* males and females as this might support it being a separate but closely related lipoprotein type and provide information on the involvement of this protein during feeding. Further studies to localise this protein in *Argulus* spp. need to be done to give an indication of any additional functions of this protein in addition to its reproductive role. Nevertheless, the results in this study could not determine the role of vitellogenin in the secretions.

Three haemocyanin protein domains were detected from nominal *A. foliaceus* secretions: haemocyanin-N, haemocyanin-M and haemocyanin-C. Haemocyanin

proteins in arthropods have been characterised as the main oxygen transporter in the haemolymph of many species (Pinnow *et al.*, 2016) but in addition to their respiratory role, haemocyanin proteins are involved in a range of other physiological processes including osmoregulation, protein storage and enzymatic activities (Paul & Pirow 1997; Depledge & Bjerregaard 1989). Destoumieux-Garzón (2001) and co-authors revealed the importance of this protein to crustacean immunity in terms of the production of antifungal (poly) peptides (Destoumieux-Garzón *et al*., 2001). Recently, Pinnow *et al*. (2016) identified two haemocyanin subunits from *A. foliaceus,* which were confirmed in this study by the proteomic analysis. Using the NCBI database, BlastP showed that haemocyanin-N and haemocyanin-M were homologues of haemocyanin 1 described by Pinnow *et al*. (2016) while haemocyanin-C was a homologue of haemocyanin 2. The functions of the haemocyanins in *A. foliaceus* were described by Pinnow *et al*. (2016) as being related to respiration and protein storage. However, C-terminal sequences of haemocyanin from shrimp were described to have roles in immunity as observed by Destoumieux-Garzón *et al*. (2001). Although, the production of haemocyanins is normal for respiration and Pinnow *et al*. (2016) described haemocyanin 2 as a storage protein, any additional importance of this protein in *Argulus* secretions has yet to be determined. Therefore, the potential secretory roles of vitellogenin and haemocyanin proteins in *Argulus*, should they indeed be secreted, needs further investigation.

4.4.2.2 **Peroxidases**

Animal-haem peroxidase enzyme was identified from the secretion of *A. foliaceus*. Tick peroxidase enzymes that are secreted from the salivary glands during feeding are suggested to play a role in host immunomodulation at the site of the feeding lesion and in defence of the gut after blood ingestion (Øvergård *et al*., 2016; Steen *et al*., 2006). Animal-haem peroxidase has been detected from other blood feeding arthropods such as *Anopheles albimanus* mosquito salivary glands and has been suggested to have a role in inactivation of vasoconstrictor substances released during the haemostatic process during feeding (Ribeiro and Nussenzveig 1993). This enzyme was also reported to have oxidase / peroxidase and vasodilation activity (Champagne 1994; Ribeiro and Nussenzveig 1993). Secreted animal-haem peroxidase in *A. foliaceus* identified in the current study might interfere with host immune mechanisms through involvement in vasodilation.

4.4.2.3 **Metalloprotease**

Two metalloproteases found to be secreted by *A. foliaceus* were astacin and peptidase M14, which were also found to be secreted from the salivary glands of the haematophagous Mediterranean colubrariid snail *Colubraria reticulate;* feeding on the blood of fishes (Modica *et al*., 2015) and were similarly overexpressed in Atlantic fed *L. salmonis* (Braden *et al*., 2017). Members of the astacin family have been considered to maintain blood flow to the lesion site through hydrolysis of fibrinogen and fibronectin

(Da Silveira *et al.*, 2007; Da Silveira *et al.*, 2002) leading to local haemorrhage (Trevisan-Silva *et al.*, 2010). It has been hypothesised that astacins have digestive and anticoagulation roles, also inactivating prey/host vasoactive peptides (Modica *et al.*, 2015; Lun *et al.*, 2003) to maintain host-parasite relationships (Baska *et al.*, 2013; Park *et al.*, 2010; Kim & Kim 2008; Gallego *et al.*, 2005). The presence of these proteases in the secretions may therefore be indicative of their functional roles in haematophagy of *Argulus*. Along with serine-proteases (see below) metalloproteases may also have further key roles in counteracting the host immune system, platelet aggregation, inflammation and vasoconstriction.

4.4.2.4 **Proteases**

Trypsin and zinc carboxypeptidase (pro-isomerase), are proteases that were also identified from *A. foliaceus* secretions. Trypsins are secretory endopeptidases within the serine protease superfamily, known to facilitate food digestion, host penetration and to help in maintaining the host-parasite relationship (Kim and Kim, 2008). They can also act as an anticoagulating protein (Santiago *et al.*, 2017; Modica *et al.*, 2015; Fast *et al.*, 2007; Kvamme *et al.*, 2004).

Although, proteases have diverse biological functions within different tissues of blood feeding arthropods (Santiago *et al.*, 2017), the detection of transcripts of putative secreted trypsins in the proteome, verified by signal peptides, confirmed that these trypsins have a vital role in *A. foliaceus* feeding processes and might play a role in parasite-host interactions. Trypsin-like serine proteases found to be secreted in the salmon louse gut act as a general digestive protease (Kvamme *et al.*, 2004; Johnson

et al., 2002). Trypsin-like proteases have been detected both in the sea lice *Caligus rogercresseyi* and *L. salmonis* and in their secretory/excretory products (Braden *et al.*, 2017; Valenzuela-Miranda & Gallardo-Escárate 2016; Fast *et al.*, 2007; Fast *et al.*, 2003), as well as in the mucus of Atlantic salmon infected by *L. salmonis*, where it has been suggested to facilitate feeding and evasion of the host immune response (Firth *et al.*, 2000).

Pro-isomerase, peptidyl-prolyl cis-trans isomerase B, cyclophilin B, or PPIB (Pyott *et al.*, 2011), has a role in the folding of peptide chains (Hasel *et al.*, 1991; Freskgard *et al.*, 1992). PPIB is a cellular enzyme responsible for proline-containing peptide bond conversion from a cis to a trans configuration (Fischer *et al.*, 1989). Proteins belonging to the cyclophilin family, have also been reported to function as chaperonin mediators of intra- and intercellular communications (Bell *et al.*, 2006). PPIB has also been identified in the *Toxoneuron nigriceps* venom. Although the function was not specified, it may play a role, with other proteins that were identified, in modulating the host immune system during invasion (Laurino *et al.*, 2016) and was one of the immunogenic enzymes identified in *I. scapularis* tick saliva (Lewis *et al.*, 2015). Transcripts for this enzyme were found to be highly expressed in the salivary glands of the hard tick *Haemaphysalis longicornis,* which suggests that is active as a secretory protein in host-tick interactions (Boldbaatar *et al.*, 2008), and it is similarly overexpressed in Atlantic salmon-fed *L. salmonis* (Braden *et al.*, 2017) and *Toxoplasma gondii* (High *et al.* 1994). Although the physiological function of trypsin is not fully determined, it has been assumed to have a role in mediating transport and/or function of associated proteins (Boldbaatar *et al.*, 2008) and might interact with host cell proteins (High *et al.*, 1994).

4.4.2.5 **Serine protease inhibitors**

The identification of serpin, a serine proteinase inhibitor, by mass spectrometric analysis supports the presence of this serine protease inhibitor enzyme in the *A. foliaceus* secretions. From matches in the NetNGlyc database, this serpin was found to be N-glycosylated and from BLASTp against human serpin sequences, followed by multiple alignment and identification of the RCL, this serpin was predicted to be inhibitory. Serpins have previously been found to be secreted in arthropod saliva released into the feeding site in order to facilitate blood meal acquisition through counteracting host defence mechanisms (Tirloni *et al.*, 2015). For example, serpins from saliva derived from different species of tick were reported to play important roles in modulating host immune response. This is achieved by impairing the hosts homeostatic and inflammatory responses, platelet aggregation and anticoagulation activity (Tirloni *et al.*, 2014; Ibelli *et al.*, 2014; Mudenda *et al.*, 2014; Mulenga *et al.*, 2013; Yu *et al.*, 2013; Chmelar *et al.*, 2011; Prevot *et al.*, 2009; Prevot *et al.*, 2006; Steen *et al.*, 2006; Leboulle *et al.*, 2002; Muleng *et al.*, 2001 and Horn *et al.*, 2000). In this respect, serpin has been identified as an immunomodulator in the cattle tick *Rhipicephalus (Boophilus) microplus* ticks (Tirloni *et al.*, 2014; Mudenda *et al.*, 2014). Tick salivary gland transcriptomes revealed that Kunitz-domain serine protease inhibitor transcripts are one of the most abundant protein families (Schwarz *et al.*, 2014). Ticks such as *Haemaphysalis longicornis, Rhipicephalus appendiculatus, Rhipicephalus microplus* and *Ixodes ricinus* fed on hosts immunised with tick recombinant serpins showed mortality and reduced feeding efficiency of the fed ticks (Jittapalapong *et al.*, 2010; Prevot *et al.*, 2007; Imamura *et al.*, 2006; Imamura *et al.*,

2005 and Sugino *et al*., 2003). *Amblyomma americanum* serpin 6 was demonstrated from the tick salivary gland and has been shown to have an inhibitory role in blood clotting and complement activation (Mulenga *et al*., 2013), and *Ixodes scapularis* salivary serpin was found to inhibit the action of thrombin, platelet aggregation and trypsin at the tick-host interface (Ibelli *et al*., 2014). Serpins are likely to be inhibitors of pro-inflammatory and pro-coagulant proteases (Tirloni *et al*., 2016) such as *Iris2* where serpins in *Ixodes ricinus* have been shown to inhibit inflammation by inhibiting cathepsin G and chymase (Chmelar *et al*., 2011). Kim *et al*. (2015) concluded that serpin was involved in host defence mechanisms during feeding by the inhibition of host proteases trypsin and trypsin-like proteases including plasmin and blood clotting factors Xa and Xia and other blood clotting enzymes including fXIIa, fIIa (thrombin) and fIXa, as well as chymotrypsin and tryptase (Kim *et al*., 2015).

The postulated role of serpins in ticks in the modulation of host responses make them appropriate targets for tick vaccines (Tirloni *et al*., 2014; Muleng *et al*., 2001). Observation of serpin in *A. foliaceus* secretions suggests that they play the same role in facilitating parasitism and modulating host immune responses in argulids. Observation of serpins by MS are supported by SDS-PAGE, where an intense band was observed at 46.6 kDa and furthermore, western blot indicated that the targeted serpin D1 (or recognised homologue) was detectable in secretions, albeit at a lower MW of around 25kDa. Similar to other blood-sucking arthropods *Argulus* spp. need to evade the host defence mechanisms for ingestion of a successful blood meal, and since serpin was identified from the secretions, it is suggested to be one of the major components involved in regulating host immune responses.

4.4.2.6 **Secreted proteins domains**

CUB protein was detected in *A. foliaceus* secretions, this previously having been reported as being present in proteins that act in haemostasis and immunity (Gaboriaud *et al.*, 2011). This protein domain from the nematode *Strongyloides stercoralis* was identified as a component of a metalloprotease involved in the infection process (Gallego *et al.*, 2005). Since this protein was associated with other secreted proteins known to be involved in host- parasite- interactions, it is suggested to be associated with one or more of the proteins that facilitate feeding of *A. foliaceus*.

Fasciclin protein domain, which is also known as transforming growth factor-beta-induced protein (Kim and Kim, 2008), was also identified from the *A. foliaceus* secretions with a revealed signal peptide. This protein has also been identified from the saliva of the argasid tick *Ornithodoros moubata* with signal peptides supporting a secretory nature (Díaz-Martína *et al.*, 2013), salivary glands in the mosquito *Anopheles culicifacies* (Sharma *et al.*, 2015) and *L. salmonis* (Braden *et al.*, 2017). The fasciclin protein function was predicted to be associated with mediation of cell adhesion and signaling (Sharma *et al.* 2015; Gobert *et al.* 2010), though its role in haematophagous arthropods remains unclear.

VIT/vWA; Vault protein inter-alpha-trypsin protein domain and von Willebrand factor type A protein domain, were detected in the secretions of *A. foliaceus* and are associated with a signal peptide. Interestingly, this protein has been identified recently from the salivary subset of vampire snail *C. reticulata* (Mollusca, Gastropoda)*,* feeding on fish blood, and was reported as the most overexpressed salivary transcript of the feeding-related proteins in the salivary glands (Modica *et al.*, 2015). Modica (2015)

and co-authors described the anti-haemostatic action of *Colubraria* vWA, which competes with the prey vWF, von Willebrand factor, in binding host thrombocytes to prevent their interaction with collagen and thus, inhibiting thrombocyte aggregation (Modica *et al.*, 2015). In this sense, since *A. foliaceus* vWA was detected in the secretions and possessed a signal peptide, this supports its secretory nature and thus it is likely to act as an anti-haemostatic protein. This represents the first evidence of the presence of this anti-haemostatic protein in *Argulus* secretions.

The identified proteins with unknown functions from *A. foliaceus*, which were not detected on Pfam and had no similarities in the NCBI database, are interesting targets to investigate, which may lead to deeper insights into the proteins associated in *Argulus* feeding processes.

4.4.3 **Gene expression**

It is proposed that feeding state and sex might affect the associated transcription of key proteins within the glands of *A. foliaceus* therefore gene expression analysis was performed for eight selected transcripts in fed and starved states for males and females. As a *caveat*, while the chosen transcripts were selected according to recognised functions in other haematophagous arthropods, their functions in *Argulus* and their presence / absence in secretions are for the most part uncertain as the same protein can be involved in a range of different biological functions depending on the site and context of expression.

The expression levels of the selected transcripts; trypsin, PGE2S, serpin, aspartic protease, cysteine protease, venom serine protease, ferritin and thrombin inhibitor, were significantly associated with sex, which may suggest that the role of these transcripts in physiology may be, at least in part, sex-biased. Poley *et al.* (2016) identified sex-biased genes from *L. salmonis* where male-biased transcripts showed higher degrees of sex-bias (35 - 43 %) and associated mainly with reproduction while female biased transcripts related to reproduction as well as morphology, feeding, detoxification and immunity. Only ferritin was significantly associated with the treatment, *i.e.* expressed in the fed *Argulus*, which is not perhaps surprising, as it is associated in other haematophagous invertebrates, with iron sequestration during blood acquisition. This transcript was up-regulated in feeding *L. salmonis* and the protein is recognised as being related to oxidative stress and iron homeostasis (Braden *et al.*, 2017). Up-regulation of ferritin in *Anopheles culicifacies* mosquitos was suggested to be due to the need to cope with excess levels of iron related to the ingested blood meal (Rawal *et al.*, 2016) and was identified in *Haemaphysalis longicornis* saliva, where is was suggested to serve for haem / iron metabolic processes (Tirloni *et al.*, 2015). Ferritin identified in *Ixodes scapularis* saliva was similarly reported to be important for iron storage and protective functions for successful blood feeding and reproduction (Lewis *et al.*, 2015). Silencing of ferritin genes affected *I. ricinus* and *Haemaphysalis longicornis* ticks' feeding and reproductive capacity and caused high mortality after a blood meal, which underlines its importance to the parasite (Galay *et al.*, 2014; Galay *et al.*, 2013). Hajdusek (2010) and co-authors reported reduction in *Ixodes ricinus* tick infestations after immunisation with recombinant tick FER2; ferritin, providing evidence for FER2 as a suitable antigen

for vertebrate vaccination and protection against ticks (Hajdusek *et al*., 2010). In accordance with Hajdusek *et al*. (2016) who suggested that target antigens associated with tick iron and haem metabolism might lead to new vaccine development, *Argulus* ferritin could therefore be considered as a potential vaccine target.

Thrombin inhibitor; *thr*, and venom serine protease; vser, genes were found to be more highly expressed in females than males. This is contrast to PGE2S and serpin; *ser*, which were more highly expressed in males. As adult females repeatedly leave hosts to lay eggs, it is possible that in the latter case, this reflects longer-term occupation of feeding sites by males and therefore a need to provide more sustained suppression / modulation of host immunity by males.

Cysteine protease, *cys,* was found to be highly expressed in *Argulus* males and has similarly been found to be secreted in other arthropods and to be involved during feeding. Cysteine proteases have been suggested to act as potential digestive enzymes, secreted into the host to aid in haemoglobin digestion (Tirloni *et al*., 2015; Sojka *et al*., 2013) and have been described as being important for digestion of host haemoglobin by *Schistosoma mansoni; Sambon, 1907* (Wasilewski *et al*., 1996), *Plasmodium falciparum*, *Haemonchus contortus* (Knox *et al*., 1993) and *Necator americanus* (Brown *et al*., 1995). The potential roles of serpins in feeding were described throughout the proteomic analysis and the importance of PGE2S were similarly discussed in **Chapter 3**.

Thrombin inhibitor was one of the proteins identified from the transcriptome dataset and is known to delay clotting time and inhibit both thrombin-induced fibrinogen clotting and thrombin-induced platelet aggregation (Tirloni *et al*., 2015). Various thrombin inhibitors have been identified from haematophagous ticks, where they counteract the

host immune system by overcoming host thrombin, factor X (FX) and platelet aggregation and adhesion (Maritz-olivier *et al.*, 2007). In *H. longicornis* ticks, haemalin is a salivary protein that is described as a thrombin inhibitor, and has an anticoagulant function (Tirloni *et al.*, 2015). *Colubraria* also exhibited highly overexpressed transcripts with similarities to the hard tick thrombin inhibitors, which suggested anti-clotting action for effective feeding and digestion (Modica *et al.*, 2015).

In the current study, except for ferritin, the findings suggest a role for these proteins in biological and physiological functions that show sex biases. Some of these transcripts might reflect reproductive pressures and could be related to differences in blood feeding processes between *Argulus* females and males due to the behavioural nature of each sex and their requirements during feeding. Although the physiological meaning of these differences in expression is not yet clear, these data nevertheless contribute new knowledge concerning the biology of *Argulus* and can be indicative of different functional roles of these transcripts in male and female physiology. As for other topics tackled in the reported work, these findings require considerable follow-up research.

The relative expression levels of aspartic protease, *asp*, and trypsin, *try*, transcripts of *A. foliaceus* were not affected by sex or treatment. Haematophagous arthropods release anti-haemostatic proteases within the saliva during blood feeding to modulate the haemostatic pathways, and in the midgut to facilitate the process of blood meal digestion such as trypsin and aspartic protease (as by reviewed Santiago *et al.,* 2017; Tirloni *et al.*, 2015; Sojka *et al.*, 2013). The aspartic proteases secreted in the saliva of blood feeding parasites are suggested to be involved in initiating host haemoglobin degradation (Williamson *et al.*, 2003; Banerjee *et al.*, 2002; Brindley *et al.*, 2001). The importance of this sex-biased result can contribute in control strategies for the

parasite, as suggested by Poley *et al.* (2016). However, it is still unknown whether the transcripts selected for gene expression from the transcriptome dataset are secreted or play other biological roles in *A. foliaceus*. Further investigations using the transcriptome data of both *A. foliaceus* and *A. coregoni* in **Chapter 3** can be used to look for genes transcribed in male and females separately and integrated with qPCR and in situ hybridisation to check for expression sites.

4.5 **Conclusions**

The studies described in this chapter represent the first proteomic analysis of *Argulus foliaceus* secretions, and the proteins identified can provide useful information relating to basic and applied aspects of the host-parasite interaction. The baseline list of identified secretory proteins achieved through this study can help to identify and characterise roles for these proteins in *Argulus* secretions. This enhanced knowledge of the *A. foliaceus* secretory products may also lead to new candidate antigens for vaccines or targets for drugs for the purpose of *Argulus* control. This study has contributed new knowledge concerning *A. foliaceus* secretory proteins that are likely to facilitate infection, immune modulation of the host and processing of host blood following feeding. Although, many of these proteins have been characterised and demonstrated to be important components of the saliva of other haematophagous parasites, more investigations are needed to shed light on the identified secreted proteins from *A. foliaceus* and define their roles in modulation of the fish host immune response.

Chapter 5. Investigation and characterisation of the *Argulus foliaceus* spinal and proboscis glands and their role in feeding

5.1 Introduction

Argulus is known to feed on blood (Hoffman 1977; Mikheev *et al.,* 2000; Walker *et al.,* 2004), mucus (LaMarre & Cochran, 1992) and host skin (Van der Salm *et al.*, 2000) and in addition to mechanical feeding processes, secretory components are hypothesised to facilitate blood digestion, anti-coagulation and immunomodulation (Claus 1875; Shimura and Inoue 1984; Swanepoel & Avenant-Oldewage 1992; Gresty *et al.*, 1993; Møller & Olesen 2010; Saha *et al.*, 2011; Al-Darwesh *et al.*, 2014).

Secretions released from haematophagous arthropod parasite salivary glands or similar organs during feeding, their components and their role in host-parasite interactions have been discussed extensively, particularly with regards to species serving as disease vectors. Because of the effects of these secretions in terms of modulating the host immune system, salivary glands of different arthropod species have been previously investigated by a number of authors (Ribeiro 1987; 1995; Andrade *et al.,* 2005; Chagas *et al.,* 2010). Study of the products secreted during feeding allows description of their immunomodulatory and pharmacological activities and in turn this can elucidate how components suppress the host immune response (Gillespie *et al.*, 2000). Studies of parasitic arthropod saliva and their glands have demonstrated the presence of anti-haemostatic molecules as well as vasodilators, platelet aggregation inhibitors, and anti-coagulants (Champagne, 2004). Molecules

secreted from the saliva and salivary glands of ticks, *e.g.* ixodids, modulate host cytokines (Wikel *et al.*, 1994). Prevot *et al.* (2006) similarly identified and characterised a range of different bioactive proteins with anti-haemostatic and immunosuppressive properties from haematophagous soft and hard ticks.

Other ectoparasites similarly modulate their host's immune system by delivering a mixture of biologically active components comprising proteases and other constituents involved during infection (von Reumont *et al.*, 2014). Claus (1875) hypothesised that some product (a venom?) was delivered through the *A. foliaceus* pre-oral spine during infection, a hypothesis which has more recently been strengthened in studies by Gresty *et al.* (1993) who also considered that the pre-oral spine/stylet functions to inject the host with secretory products through the sub-terminal pore during feeding. Saha *et al.* (2011), Gresty *et al.* (1993) and Swanepoel *et al.* (1992) described ducts leading from the proboscis glands that were hypothesised to deliver secretions externally during feeding. Although to date the role of secretory glands associated with the feeding apparatus is not yet known, it has been postulated that the spinal gland, proboscis glands and labial gland secretions may modulate the host immune defence mechanisms (von Reumont *et al.*, 2014).

In addition to improving knowledge of the glands and delivery apparatus related to secretions that facilitate feeding, von Reumont *et al.* (2014) proposed investigations into the nature and composition of the branchiurans' supposed venom, this remaining entirely unknown (von Reumont *et al.*, 2014).

In order for haematophagous arthropods to acquire a blood meal, they evade or modulate the host defense immune system (Gillespie *et al.,* 2000) via

pharmacologically active components within the saliva such as prostaglandins and proteases (Wikel *et al.,* 1994; Andrade *et al.,* 2005). Studies of salivary constituents of different arthropods reveal that they share factors active in immunomodulation such as inflammation inhibitors, cytokine mediators and anti-coagulants (Gillespie *et al.*, 2000). The presence of anti-inflammatory and immunosuppressive factors in hard tick saliva facilitate the blood sucking process as well as pathogen transmission through the saliva (Randolph & Nuttall 1994; Gillespie *et al.,* 2000). Bioactive compounds in tick saliva have been reported to obstruct host haemostatic process mechanisms including platelet aggregation and the coagulation cascade involved in vasoconstriction (Bowman *et al.*, 1997). Platelet aggregation inhibitors and vasodilators have also been reported from mosquito saliva (Stark & James 1998). The identification of bioactive constituents from haematophagous arthropod's saliva can provide possible targets for vaccine development (Andrade *et al.*, 2005). Prostaglandin E2 (PGE2) and trypsin like proteases have been identified from sea lice; *L. salmonis* secretions, and their role in host immune response and immune-modulation during feeding predicted (Fast *et al.*, 2007). Trypsin-like proteases are suggested to assist *L. salmonis* attachment to the host through host tissue invasion and host immune response modulation, while PGE2 is assumed to act as a vasodilator, anti-coagulant and regulator of T-lymphocytes, which enable successful parasitism and feeding (Fast *et al.*, 2007). Trypsin-like transcripts have been identified recently in *Caligus rogercresseyi* and have been suggested to play a role in metabolizing delousing drugs after exposure to the delousing compounds deltamethrin and azamethiphos (Valenzuela-Miranda and Gallardo-Escárate, 2016). Lewis *et al.* (2014) also found that

L. salmonis secretory products modulate the cellular innate immunity, particularly macrophage function, of salmon.

Argulus have well defined secretory glands which appear to be associated with feeding processes (described in detail in **chapter 2**), thus it is hypothesised that these glands may similarly produce specific bioactive compounds that play key roles in evading the host immune system and facilitating blood feeding. Investigating the type of secretory and expressed components released from these glands, for example the spinal and proboscis glands associated with *Argulus* feeding, may provide potential targets for the development of methods of parasite control. In this respect identification of proteases and protease inhibitors in *Argulus* has already been considered as potential route for providing control targets for *Argulus* (Sahoo *et al.*, 2013).

Relatively few vaccines have been successfully developed to target protistan and metazoan parasites. One successful example was the development of TickGARD and TickGARD Plus, a vaccine against the cattle tick *Boophilus microplus* based on the discovery of Bm86 tick gut antigen (Willadsen *et al.*, 1989), which was the first commercialised anti-arthropod vaccine (Willadsen, 1997).

In examining the effect of secretions produced from *Argulus* mouthparts on the fish host, Shimura and Inoue (1984) found that an extract of the *A. coregoni* mouth parts had a haemorrhagic effect; but not haemolytic or cytotoxic effects. Later, Saha *et al.* (2011) worked with *A. siamensis* and showed that spinal gland secretion had a vasodilatory function whereas the proboscis gland secretion was anticoagulant in nature. Although the functions of spinal gland secretions have been described both as lytic and vasodilatory in different published research (Claus 1875; Shimura and Inoue

1984; Swanepoel & Avenant-Oldewage 1992; Gresty *et al.*, 1993; Møller & Olesen 2010; Saha *et al.*, 2011) no study has thus far applied transcriptomic or proteomic analyses to investigate the specific expressed genes or proteins from the glands, which relate to the feeding process and no attempts have been made to study the components of each gland, spinal and proboscis, separately (von Reumont *et al.*, 2014). The two transcriptomic studies that were conducted recently on *Argulus*, one on *A. siamensis* conducted by Sahoo *et al.* (2013), showed the presence of proteases; which were proposed to have antigenic activity, however, no further investigations were performed to localise the expression site of any of these proteases. The second transcriptome study, conducted on *A. foliaceus,* focused on the identification of haemocyanin (Pinnow *et al.,* 2016).

To date, no proteins have been identified directly from the spinal or proboscis glands, which may have an active role at the feeding site and may facilitate the feeding process. Thus, the objective of this study was to look for potential immuno-modulatory compounds. The current study applied transcriptomic data in conjunction with expression *in situ* hybridisation (ISH), immunohistochemistry (IHC) and fluorescent lectin labelling to investigate known immunomodulatory genes, which have been recognised in other arthropods such as mosquitoes, ticks and sea lice that may serve a function in parasite-host interactions.

Although a considerable amount of information is available on the biology, physiology and ecology of *Argulus* (Christie 2014; Forlenza *et al.* 2008) more data is required with regards to their effects on the host (Forlenza *et al.*, 2008). Determining the physiological/behavioral control mechanism (Christie, 2014) and clarifying the modulation process used by *Argulus* secretions (von Reumont *et al.,* 2014) are major

goals for elucidating the host-parasite relationship. The host skin damage resulting from *Argulus* infections is due to the attachment by suckers and feeding apparatus, mouth tube and pre-oral spine (Forlenza *et al.*, 2008), which are hypothesised to release toxins/digestive enzymes (Shimura and Inoue, 1984) through the spinal duct and tubular labial spines.

The transcripts of proteins suggested to be involved in host-parasite interactions in *A. foliaceus*, such as protease inhibitors, were shown to be present in *A. foliaceus* through a combination of data mining of existing databases (Pinnow *et al.*, 2016), *de novo* transcriptome sequencing performed on an Illumina HiSeq 2500 platform (**Chapter 3**) and from the results of proteomic analysis studies of *A. foliaceus* secretions (**Chapter 4**). The transcriptome data generated for *A. siamensis* by Sahoo (2013) and colleagues also resulted in the identification of protease inhibitor transcripts such as serine proteases and metalloproteases, which were detected in salivary glands and saliva of other ectoparasites. These serine proteases were suggested to be involved during host infection and may be promising targets for vaccine development (Sahoo *et al.*, 2013). Recently, Christie (2014) identified 27 transcripts encoding putative neuropeptide precursors from the *A. siamensis* transcriptomic dataset prepared by Sahoo *et al.* (2013) in an attempt to understand the physiological/behavioral control of crustacean ectoparasites (Christie, 2014).

The different techniques employed in the following study all aimed to identify and characterise the proteins that may secreted from spinal and proboscis glands to enhance feeding and impair host immune defence. *In situ* hybridisation can detect mRNA of selected proteins, immunohistochemistry can localise the site of protein synthesis and release and lectin-labelling provides clues on the type of glycoprotein

residues secreted by the glandular cells associated with the feeding apparatus during host invasion and blood-feeding processes.

5.2 Materials and methods

5.2.1 Sample collection and fixation

All samples used in this work were collected from rainbow trout from Loch Fad, Isle of Bute, Scotland, fixed in 4% paraformaldehyde overnight then transferred into 70% ethanol and stored at -20°C until ready for use. All glassware was baked at 180°C to ensure it was nuclease-free before the samples were processed manually to protect nucleic acids. Samples went through six changes of absolute ethanol and two changes of isopropanol for the dehydration step followed by clearing with two changes of xylene and infiltration of two changes in paraffin wax at 60°C. Parasite samples were then embedded in paraffin wax, sectioned at 5 µm and mounted on treated glass slides (Superfrost® Plus glass slides, Thermo Scientific, Epsom, Surrey, UK), briefly dried on a hotplate and stored at -20°C until ready to be used. To limit any contamination during sectioning, the water bath was RNAse Zap (ThermoScientific, p/n 7002) treated and filled with MilliQ (nuclease-free) water. RNAse Zap was sprayed on all the surfaces prior to sectioning for the preparation of *in situ* hybridisation sections.

5.2.2 *In situ* hybridisation

In situ hybridization (ISH) was used in this study to localise transcript expression in the *A. foliaceus* spinal and proboscis glands. The transcripts selected were *PGE2S*, *trypsin*, *DPP IV*; *venom dipeptidyl peptidase* and *serpin* as these have been identified by other authors as potentially being associated with immunomodulatory processes.

5.2.2.1 Target selection, RNA extraction and reverse transcription

To identify immunomodulatory targets for *in situ* expression hybridisation, online transcriptome reference data for *A. foliaceus* were used (Pinnow *et al*. 2016). Prostaglandin E synthase 2, *PGE2S* (contig_875), trypsin-like serine proteinase, *trypsin* (contig_3517) and *venom peptidase* (contig_10455) genes were selected. Another source of targets was the list of proteins resulting from the proteomic MS/MS analysis of *A. foliaceus* secretions (**Chapter 4**) from which *serpin* (contig_1658|m.1805) was chosen. These targets were selected because they had previously been found to play a role in host immunomodulation in some studied arthropods. This was confirmed after BLAST searches of the selected protein sequences of *PGE2*, *trypsin* and *venom peptidase* in NCBI. BLASTx and BLASTp were used to characterize *serpin* amino acid sequences, which predicted the presence of these proteins within other arthropods, while the Pfam database was used to look for the classification of protein families and domains.

PrimerSelect software was then employed to design suitable primers for the selected transcripts and oligonucleotide primers were ordered from Eurofins Genomics. Prior to application, primers were diluted in sterile 1X TE buffer to 100pmol/µl. After mixing and brief centrifugation, the primers were then kept at room temperature for 30 min to ensure solubilisation. Then 20µl of the forward and 20µl of the reverse primers for each gene were diluted in 160µL MilliQ water (1:10) which was then mixed and divided into 4 x 1.5 mL labelled Eppendorf tubes and stored at -20°C until use.

Total RNA was extracted from *Argulus* samples; one adult male and one adult female, using TRI Reagent® (Sigma-Aldrich, UK) according to the manufacturer's protocol as described in **3.2.2** (**Chapter 3**).

The total RNA was re-suspended in nuclease-free water (Ultra-pure water). RNA concentrations were measured using UV spectroscopy (NanoDrop ND-1000, Thermo Scientific, USA) and RNA integrity evaluated by agarose gel electrophoresis and 1.5% ethidium bromide staining prior to storage at -70°C for later use.

5.2.2.2 ***In situ*** **probe production**

To produce sense and antisense probe sets for the selected targets for *in situ* expression hybridisation (ISH), the protocol for the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems PN 4368813) was followed for reverse transcription of total *A. foliaceus* RNA to single-stranded cDNA templates. In brief, 2x reverse transcription (RT) master mix was prepared, then 2µg of total RNA was added to give a 1x mix solution before RT was performed in a thermocycler for 2 h according

to the manufacturer’s protocol. RT-PCR reactions of *A. foliaceus* cDNA templates were carried out, using 2x MyTaq™ HS Mix (BioLine) for hot-start PCR as per the manufacturer's instructions, at 62°C annealing temperature for 32 cycles with each PCR product run on a 1.5% agarose gel (+ EtBr) to confirm the size of the product. PCR products were purified using the QIAquick PCR Purification kit (Qiagen, p/n 28104) as per manufacturer’s instructions except that the cDNA was eluted in 30 µl nuclease-free water preheated to 55°C. The samples were quantified using the Nanodrop and then around 40-60ng was run on a 1.5% agarose gel. Purified probes were sent to GATC Biotech for sequencing and validation.

The methods applied for the expression *in situ* hybridisation in this study followed the protocol employed by Kvamme *et al.* (2004) with some modifications. Digoxigenin (DIG)-labelled RNA probes from the selected genes were prepared with T7 promotor (TAATACGACTCACTATAGGG) using DIG RNA Labelling kit (Roche, p/n 11175 025 910; 10x NTP labelling mixture, 10x Transcription buffer, Protector RNase Inhibitor and RNA polymerase T7) and incubated at 37°C for 2 h. The resulting probes were quantified using Nanodrop and then evaluated using gel electrophoresis; 50ng of the unlabeled samples (*i.e.* original PCR products) and 0.5µL of the labelled samples were run on a 1.5% agarose gel (containing EtBr), to verify the size of the transcripts obtained and estimate the labelling efficiency (**Fig 4.1**), while dot-blot analysis was carried out to determine the yield of the DIG-labelled RNA probes (**Fig 4.2**). For dot blot, a series of dilutions of the tested RNA probes were applied to a positively-charged nylon membrane (Roche, p/n 11 209 299001) along with several known dilutions of the kit control RNA. The membrane was incubated at 120ºC for 30 min to fix the nucleic acid. The DIG Nucleic Acid Detection Kit (Roche, p/n 11 175 041 910) was used, as

per manufacturer's instruction, to determine if the probes had been successfully labelled. For washing/blocking of the membrane, the DIG Wash and Block Buffer set (Roche, p/n 11 585 762 001) was used. The membrane was incubated overnight at room temperature in the dark and then the reaction was stopped by washing the membrane with nuclease free water. After colorimetric detection, membranes were photographed using the Syngene Transilluminator using a white light source. According to the protocol, if the most dilute spot visible on the membrane was observed at the fifth RNA probe spot then the probe contains the expected amount of labelled RNA probe *i.e.* 416ng/µl, and if the fourth spot is the most dilute visible, then there is an adequate concentration and the levels were suitable for use in the ISH.

Hybridisation and target detection was performed on 5 µm paraffin embedded parasite sections; three slides were used for each target transcript to include antisense, sense and control probes, preceded by baking of the sections for 30 min at 60°C and two 10 min dewaxing steps in xylene (Fisher Scientific, 10784001) at room temperature. Dewaxing was followed by rehydration with 95%, 70% and 50% ethanol/water solution, respectively (Fisher Scientific, 10437341) made using nuclease-free water for one min in each concentration. Then, sections were then washed in 2x SSC; Saline–Sodium Citrate buffer (prepared from 20X SSC – Fisher Scientific, BP1325-1) for one min. Afterwards, the slides were dried and an ImmEdge pen (Vector Labs, p/n H-4000) was used to encircle the sections, to prevent solution leakages, and warm Proteinase K mix (10µl/ mL Proteinase K in 100mM Tris/50M EDTA buffer) at 37°C was added to each slide; to facilitate digestion of tissue to allow probes to infiltrate the tissue. The proteinase K was incubated at 37°C for 5 min in a humidified box containing the tissue soaked in 5x SSC. This was followed by a 5 min post-digestion

fixation step with freshly prepared ice cold 4% paraformaldehyde, washed twice with 1x PBS for 2 min, dried and the sections placed in a GeneFrame (Fisher Scientific, p/n AB-0578). Prior to hybridisation, pre-hybridisation solution (20x SSC, formamide and nuclease-free dH_2O) was added to each section and incubated for 10 min at 37°C. The hybridisation mix was then prepared by mixing first 10% dextran sulphate, 5x SSC, 50% formamide, 250 μg/mL^{-1} and 5x Denhardt's solution. Then, 10% dextran sulphate was added gradually, whilst incubating (shaking) at ~ 30°C to help it dissolve. Once dissolved, tRNA, 500 μg mL^{-1} and 1% blocking solution (blocking solution is from the DIG Wash and Block Buffer set (Roche, p/n 11 585 762 001)) were added. Then, herring sperm DNA denatured at 95°C for 5 min, was added to the master mix. Once the master mix was prepared, 150 μL of the hybridisation mix was added to the labelled probe tubes. Then, antisense and, sense probes were added, with the negative control containing only hybridisation mix, to give a final concentration of 0.3-0.8 ng μL^{-1}.

The probe containing hybridisation mix was heated at 80°C for 5 min and chilled on ice. For hybridisation, the hybridisation mix was added to tissue sections and sections were incubated overnight at 60°C. Then, slides were rinsed at room temperature with 2x SSC twice for 30 min, incubated at 65°C for 30 min with 50% formamide/ 2x SSC followed by two washes with 2x SSC for 10 min each at 37°C. Before the detection step (Roche DIG Nucleic Acid Detection Kit (p/n11 175 041 910) and DIG Wash and Block Buffer Set (p/n 11 585 762 001) were used), sections were washed with 1x Wash Buffer for 5 min at room temperature, then incubated for 30 min at room temperature with 1x blocking solution with shaking and 2 h at room temperature with anti-digoxigenin antibody-AP diluted 1: 5,000 in 1x blocking solution without shaking.

Excess antibody was removed by washing at room temperature for 15 min twice with 1x washing buffer with shaking, then tissue sections were equilibrated for 5 min in 1x Detection Buffer. Hybridisation of antisense probes localised the specific transcripts whereas hybridisation with sense probes detected the non-specific staining. The Fast-red method was used for chromogenic detection; using Tris and Fast Red TR/Napthol AS-MX tablets (Sigma, F4648) and the staining was monitored to avoid over development and high background. Labeled probes were then checked using a fluorescent microscope (Arcturus^{XT} Laser Capture Microdissection System, Applied Biosystems) and micrographs were captured with an attached digital camera.

5.2.3 PHA-E and DSL Lectin labelling of spinal and proboscis glands

Lectin-labelling was used in this study to identify possible protein carbohydrate interactions in the *Argulus* secretory glands.

Lectin wash buffer (LWB; 50mM Tris, 150mM NaCl, 2mM $MgCl_2$ and 1mM $CaCl_2$) was prepared prior to the start of labelling. Sections were taken from -20°C, heated in a 55°C oven for approximately 20 min then labelled with 2 types of lectins; PHA-E and DSL (Vector Laboratories, Burlingame, CA, U.S.A.). Lectins were diluted in LWB to 5µg mL^{-1}; the recommended concentration range for use is 5-20µg mL^{-1} (**Table 5.1**). The sections were then dewaxed manually in two changes of xylene for 3 min each, following by a dehydration step using 100% then 70% ethanol for 2 min at each step. The sections were washed in distilled water for 1 min and sections were kept in water to prevent drying until labelled by diluted lectins. A circle was drawn around each section using an ImmEdge pen (Vector labs, p/n H-4000) to prevent leakage of the

lectin/buffer from the section. From each lectin solution, 200µl was pipetted on to the sections and incubated in a dark chamber at room temperature for 2 h. Then the sections were washed by LWB three times for 5 min. A negative control was used treated in the same way whereby LWB only was used during incubation. Slides were mounted with DAPI (Vectorshield, mounting medium for fluorescence with DAPI from Vector Laboratories, Burlingame, CA, U.S.A.), coverslipped and sealed with nail varnish for prolonged storage. The slides were kept in the dark for 2 h then moved to 4°C until viewed, with checking carried out within one week. The slides were checked using a fluorescent microscope (ArcturusXT Laser Capture Microdissection System, Applied Biosystems) and micrographs were captured with an attached digital camera.

Table 5.1 Lectins name, concentration used and binding preferences and specificities of DSL and PHA-E lectins. Binding preferences of each lectin according to EY Labs and Vector Laboratories. Gal, galactose; GalNAc, N-acetylgalactosamine

Fluorescein Lectins and binding preferences	Scientific / Latin name	Conc (mg/l)	Binding specificity
Chitin-binding lectins / N-acetylglucosamine / N-acetyllactosamine			
DSL	*Datura stramonium lectin*	1	Gal, GalNAc
Galactose / N-acetylglucosamine / Mannose			
PHA-E	*Phaseolus vulgaris Erythroagglutinin*	2	Oligosaccharide

5.2.4 Immunohistochemistry of spinal and proboscis glands

The results obtained from the lectin labelling directed the immunohistochemistry (IHC) study of the spinal and proboscis glands for further investigation of the suggested immunomodulatory targets. The spinal gland cells showed lectin binding, particularly

with PHA-E; *Phaseolus vulgaris agglutinin-erythroagglutinin*. This lectin binds specifically to terminal galactose, N-acetylglucosamine and mannose residues. Two antibodies were selected for this investigation that are known to be involved with blood coagulation. Serpin D1 is also known as heparin cofactor II (HCII) and is a coagulation factor that inhibits thrombin. This inhibition is accelerated in the presence of glycosaminoglycans such as heparin, dermatan sulphate (DS) or heparan sulphate proteoglycans (HSPG's). To determine if there was any match between the serpin identified from the proteomic data (see **Chapter 4**) and other serpins from arthropods and especially with HCII, the amino acid sequence of serpin protein (contig_1658|m.1805) resulting from the proteomic analysis of *A. foliaceus* secretions was blasted against the database using NCBI BLASTp. The highest identities, 35 %, were obtained for the serine protease inhibitor of *Daphnia magna*. Another BLASTp search was conducted for this contig against Serpin D1 ''gi|123055|sp|P05546.3|HEP2_HUMAN'' and the homology match revealed a 27% identity. The BLAST step was conducted to confirm there were homologies between the *A. foliaceus* serpin and other serine protease inhibitors from other arthropods and resulted in partial hits to serpin D1 in other species.

Thus, serpin D1; Invitrogen™ heparin cofactor II polyclonal antibody (1mg mL^{-1}) Prod# PA5-29105 and Heparan Sulfate 6-O-Sulfotransferase 3; HS6ST3 polyclonal antibody (0.2mg mL^{-1}) Prod# PA5-47733 were ordered from Thermo Scientific to investigate the production of these proteins in the spinal and proboscis glands (**Table 5.2**).

The IHC protocol was optimised with different parameters: (1) various dilutions of both antibodies, (2) two washing buffers applied including PBS; phosphate buffered saline, and TPBS; Tween20 phosphate buffered saline and (3) two blocking solutions applied

5 % casein and 0.1 % BSA in TPBS. In addition to these blockers 3 % H_2O_2 in methanol was also applied.

Immunogenic detection was performed on 5 μm paraffin embedded tissue sections. In this protocol, for each antibody tested (HCII and HS6ST3; HSPG's) in this protocol duplicate slides were used and a single slide for each negative control; nonspecific isotype matched antibody and PBS controls. After heating for 30 min at 55°C followed by dewaxing in 2 x 5 min changes in xylene, rehydration was completed by 2 changes of 100% ethanol for 5 min and 70% for 3 min. After washing in 2 changes of distilled water for 2 min, a wax circle was drawn around each section using an ImmEdge pen (Vector labs, p/n H-4000) and slides were kept hydrated in TPBS before the blocking step. Slides of both controls (PBS; negative control, and reagent (antibody) controls including nonspecific irrelevant sheep IgG 1:200 and irrelevant rabbit IgG 1:200 antibodies) and specific target antibodies, were incubated first in 3% H_2O_2 in methanol for 10 min in a dark chamber at room temperature. Three changes of TPBS were used to rinse the blocking solutions for 3 min each and the primary antibodies; Invitrogen™ heparin cofactor II antibody and heparan sulphate, HS6ST3 antibody, prepared in TPBS, were added to the sections and incubated at room temperature for 60 min in the dark followed by 3 x TPBS rinse for 3 min each. Immunohistochemistry procedures were repeated using different dilutions, see (**Table 5.2**) for both primary antibodies to optimise the methodology; HSPG's (1:40, 1:80, 1:100, 1:250 and 1:500) and heparin cofactor II (1:100, and 1:300, 1:600 and 1:1000). To prevent non specific labelling, sections were incubated in 10% goat serum for 20 min at room temperature and then rinsed in 3 x TPBS for 3 min each. Then, secondary antibodies were added to the sections and incubated for 1 h in the dark at room temperature; anti-rabbit horseradish

peroxidase (HRP) (goat IgG) 1:200 for HC II and anti-sheep-FITC (rabbit IgG) 1:500 was added to HS. Sections were rinsed with 3 changes of TPBS for 3 min each and then HS sections were incubated for 1 h with a tertiary antibody; anti-rabbit HRP (goat IgG) 1:500, and followed by a TPBS rinse as previously described. Peroxidase activity sites were visualised with DAB Kit (ImmPACT™ DAB Vector, Burlingme, CA SK-14105). Tissues were developed for 30 sec before being washed with distilled water to stop the reaction. Sections were counter stained with hematoxylin for 4 min, rinsed with distilled water and dehydrated through on ethanol series of 70% ethanol for 3 min and 100% ethanol 5 min then xylene for 2 x 5 min. Finally, sections were coverslipped with Pertex and allowed to dry in the fume hood overnight. Sections were examined and light microscope images taken using a bright-field microscope; Olympus BX51TF light microscope with a Zeiss AxioCam MRc colour digital camera.

Table 5.2 Working antibody dilutions for IHC methodology; heparin cofactor II (HCII) and heparan sulphate proteoglycans (HS6S6T3) and nonspecific control

Test	Primary Antibody / Test Target	Blocking	Secondary Antibody	Tertiary Antibody
1	HS6S6T3 (1:500) in TPBS	H_2O_2/ 0.1 BSA	Rabbit Anti Sheep FITC 1:500	Goat anti-rabbit 1:500
2	Heparin Cofactor II (1:1000) in TPBS	H_2O_2/ 0.1 BSA	Goat Anti Rabbit HRP 1:200	
3	HS6S6T3-Irrelevant nonspecific antibody control for (Sheep IgG) (1:500)	0.1 BSA	Rabbit Anti Sheep FITC 1:500	Goat anti-rabbit 1:500
4	HCII-Irrelevant nonspecific antibody control (Rabbit IgG) (1:200)	0.1 BSA	Goat Anti Rabbit HRP 1:200	
5	Negative Control	0.1 BSA	Goat Anti Rabbit HRP 1:200	

5.3 **Results**

5.3.1 **Transcripts selection and primer design**

One of the important initial steps was to find suitable putative immunomodulatory genes from the transcriptome and secretory proteome and design primers to the best targets (**Table 5.2)**.

Molecular "BLAST" (www.ncbi.nlm.nih.gov) analysis of the selected genes revealed reasonable homologies and similarities with other arthropod species. For *PGE2S* the highest degree of similarity of the selected gene was to the predicted prostaglandin E synthase 2 from a beetle species (*Anoplophora glabripennis*) and for *trypsin* the transcript was more identical to the trypsin-like serine protease of other crustaceans such as *Fenneropenaeus chinensis*, *Panulirus argus*, *Priapulus caudatus* and *Marsupenaeus japonicus*. For *venom peptidase,* the similarity was greatest with dipeptidyl peptidase protein from crustaceans e.g. *Daphnia magna* and beetles *e.g. Aethina tumida*. The *serpin* matches in NCBI BLASTp showed amino acid sequence similarities with the serine protease inhibitors of different arthropod species but the highest degree of identity was with crustaceans *Daphnia pulex* and *Daphnia magna*.

The NCBI blast tool also allowed the conserved domains of *PGE2S*, *trypsin*, *venom peptidase* and *serpin* transcripts to be determined (**Table 5.3)**. Although similarities and identities between these transcripts and those of other arthropods were limited $\leqslant$ 56 identities, this confirmed that these proteins do exist within other arthropods and that the differences indicate divergence across taxa.

Table 5.3 Conserved domains within selected transcripts; *PGE2S*, *trypsin*, *DPP IV, venom dipeptidyl dipeptides IV* and *serpin* as resolved by NCBI BLAST tool.

Transcript	Domain Name	Accession	Description	Interval	E-value
PGE2S	GST_C_mPGES2	cd03197	C-terminal, alpha helical domain of microsomal Prostaglandin E Synthase Type 2	86-529	2.01E-44
	GST_N_mPGES2	cd03040	GST_N family; microsomal Prostaglandin E Synthase Type 2 (mPGES2) subfamily	683-910	6.75E-27
	Glutaredoxin	pfam00462	Glutaredoxin	758-907	5.21E-05
Trypsin	Tryp_SPc	cd00190	Trypsin-like serine protease	29-718	1.24E-54
	Tryp_SPc	smart00020	Trypsin-like serine protease	35-721	2.73E-52
	Trypsin	pfam00089	Trypsin	35-718	1.19E-47
	COG5640	COG5640	Secreted trypsin-like serine protease	17-730	2.00E-01
DPP IV	DPPIV_N	pfam00930	Dipeptidyl peptidase IV (DPP IV) N-terminal region	999-2111	2.59E-42
	Peptidase_S9	pfam00326	Prolyl oligopeptidase family	177-725	9.25E-24
	DAP2	COG1506	Dipeptidyl aminopeptidase/acylaminoacyl peptidase	180-977	3.55E-17
Serpin	Serpin	cd00172	SERine Proteinase INhibitors (serpins)	30-397	1.86E-112
	Serpin	pfam00079	Serpin (serine protease inhibitor	33-400	1.87E-109
	Serpin	smart00093	SERine Proteinase INhibitors;	50-400	1.26E-95
	Serpin	COG4826	Serine protease inhibitor	34-401	8.88E-58
	PHA02660	PHA02660	serpin-like protein	51-400	3.09E-14

The homologous hits for *PGE2S*, *trypsin*, *DPP IV,* and *serpin* within the arthropod taxa predicted the selected targets as suitable for advancing to the next step for localising these genes within *A. foliaceus* sections using ISH. The amplicons obtained from the selected genes ranged between 509 – 647 bp (**Table 5.4**).

Table 5.4 Forward; sense, and reverse; anti-sense, primers for the targeted transcripts by ISH.

Contig No.	Protein	Gene target	Primer seq	Product size
contig_875	Prostaglandin E Synthase 2	*PGES2_F*	CTCCCACTCTCCTACCTCTGAAA	647
		PGES2_R	GAGCCTATGGATACTACTTGGTGA	
contig_3517	Trypsin-like serine proteinase	*Trypsin_F*	ACGATTAACGGACCCCAAAAAG	509
		Trypsin_R	AAGCCGCAGCATAATCGTGAGT	
contig_10455	Venom peptidase	*V-peptidase_F*	ACCCCAGCCCCTCCTTCC	536
		V-peptidase_R	CCCATAACTCCACCCCCAGAT	
contig_1658|m.1805	Serpin	*Serpin_F*	GAGCCCGCCGCCAGAAG	580
		Serpin_R	CGCGATGTAGTGCCGTGTCAA	

5.3.2 ***In situ* hybridisation**

To identify where the selected transcripts; *PGE2S*, *trypsin*, *venom peptidase* and *serpin*, are located or expressed in adult *A. foliaceus,* RNA probes for *in situ* hybridisation were used. Adult *A. foliaceus* samples were selected randomly for each test irrespective of sex. This was because the main objective was to check if any of these genes are transcribed in the spinal and/or proboscis glands. The quantity (ng/µl) and quality (260/280 ratio) of RNA isolated from adult males and females was determined using a Nanodrop (**Table 5.5)** and by agarose gel electrophoresis.

Table 5.5 RNA concentration of the samples used to detect the transcripts in ISH

Sample	RNA conc. (ng/µl)	260/280
Female	299.5	2.04
Male	286.4	2.02

Prior to sequencing the ISH probe PCR products, they were purified using the QIAquick PCR Purification kit and then the concentration (ng/µl) and 260/280 were measured by Nanodrop (**Table 5.6**). The trypsin probe produced the lowest concentration followed by V-peptidase then PGE2. Serpin probes produced the greatest concentration and the PCR product sizes were as expected (**Fig 5.1**; **Table 5.4**).

Table 5.6 Purified PCR products for ISH probes

Probe set	PCR product conc.(ng/µl)	260/280
PGE2S	114.8	1.90
Trypsin	32.6	1.72
V-peptidase	115.3	1.89
Serpin	237.1	1.81

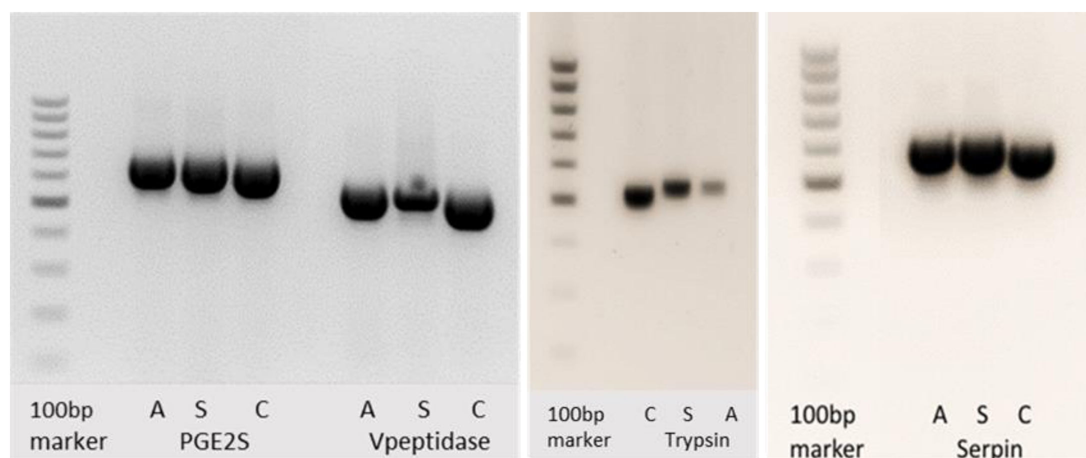


Figure 5.1 Agarose gel after electrophoresis of PCR purified products of *PGE2S*, *DPP N* (Vpeptidase), and *trypsin* at 62°C and *serpin* at 63°C for 32x; control (C): using ordinary sense and antisense primers (i.e. without the T7 promotor) to act as a negative control, Sense (S) and Antisense (A).

The sequencing results for the purified PCR products provided by GATC Biotech confirmed the expected sequences for each target and consequently *in situ*

hybridisation analysis was conducted. The DIG RNA labelling kit was used to transcribe DNA into single standard RNA probes using T7 polymerase (**Fig 5.1**). This verified the size of the transcripts and estimated the labelling efficiency. The DIG labelling was successful for all the transcripts, but the *trypsin* antisense (Try+7r) was not as expected. This may have been due to the lower concentration of the *trypsin* PCR product (**Table 5.6**)

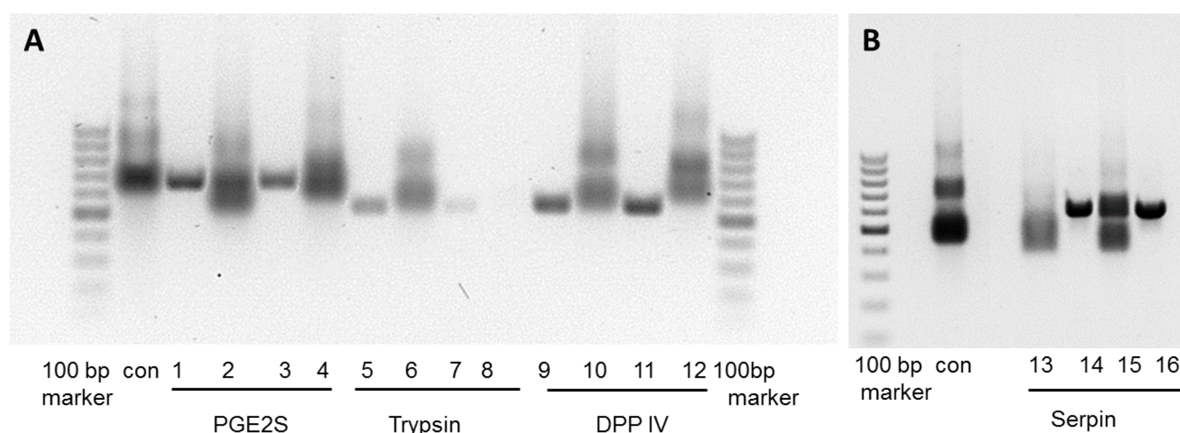


Figure 5.2 Agarose gel after electrophoresis of PCR and DIG labelled products of (A) *PGE2S*, *trypsin* and *venom peptidase* and (B) *serpin* ISH probes. con= kit DNA control, 1, 5, 9, 16 = sense PCR product of PGE2S, trypsin, DPP IV and serpin respectively, 2, 6, 10, 15 = sense + DIG of PGE2S, trypsin, DPP IV and serpin correspondingly, 3, 7, 11, 14 = antisense PCR product of PGE2S, trypsin, DPP IV and serpin correspondingly and 4, 8, 12 p, 13 = antisense + DIG of PGE2S, trypsin, DPP IV and serpin respectively.

Dot-blot analysis determined the yield of the DIG-labelled RNA probes. The results of dot-blot analysis revealed that the ‘expected’ amount of labelled RNA probes had been obtained for PGE2S, trypsin and venom peptidase (0.1 pg (4.16 ng/µl)) whilst and ‘adequate’ amount was obtained for serpin (0.3 pg (416 ng/µl ÷3 = 124 ng/µl)) (**Fig. 5.3**) according to the manufacturer’s instructions. The Roche protocol states that if the most dilute spot visible is the fifth then the sample contains the expected; 0.1 pg (4.16

ng/µl), amount of DIG-labelled RNA and if it is the fourth spot then an <u>adequate</u> amount; 0.3 pg (416 ng/µl ÷3 = 124 ng/µl), of labelled RNA probe has been produced. **Fig (5.3**) shows that the DIG-labelled RNA probes of PGE2S, trypsin and venom peptidase contain the expected amount of labelled RNA amount while serpin contains an adequate amount.

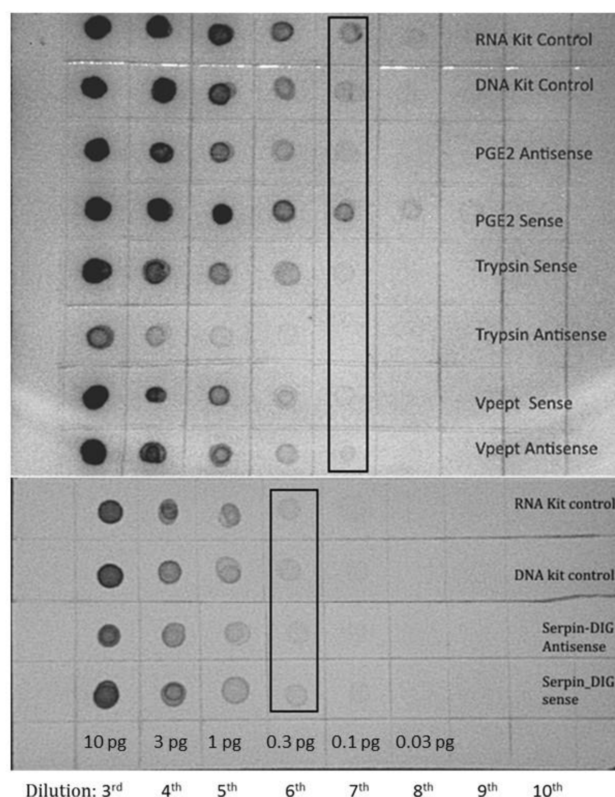


Figure 5.3 Dot- blot of DIG-labelled RNA probes; *PGE2S*, *trypsin*, *DPP IV* (Vpept) and *serpin*. DIG-labelled RNA probe concentrations defined as 'adequate' (124 ng/µl) and 'expected' (416ng/µµl) for use in ISH. *Serpin* labelled probes were obtained up to the fourth whereas *PGE2S*, *trypsin* and *DPP IV* labelled probes were obtained up to the fifth spot.

5.3.2.1 **Localisation of *PGE2S* transcription by *in situ* hybridisation**

PGE2S transcripts were detected in the spinal and proboscis glands, within secretory cells on the head between the cerebral ganglion (**Fig. 5.4A and 5.5A, C**), gut (**Fig.**

5.5B and 5.5E) and testes (**Fig. 5.5C and 5.5F**). The pattern of this expression was scattered in the cells of the spinal and proboscis glands with a stronger signal around the periphery of the cells **(Fig. 5.5A and 5.5D, 5.5C and 5.5F)**. Apart from non-specific signals associated with the cuticle, no signals were observed with the sense probe (**Fig 5.4B, 5.4E**) or negative controls (**Fig. 5.4C, 5.4F**).

5.3.2.2 **Localisation of *trypsin* transcription by *in situ* hybridisation**

I*n situ* hybridisation targeting trypsin detected *trypsin* transcripts in the spinal and proboscis glands **(Fig. 5.6A and 5.6D)**, secretory cells between the cerebral ganglion, epithelial and connective tissue, oocytes and the gut **(5.7 A-F)**. Higher magnification of the midgut shows the expression of trypsin within the epithelial cells lining the gut (**Fig. 5.7C)**. The *trypsin* sense and negative controls verified the results of the *trypsin* anti-sense hybridisation **(Fig 5.6B and 5.6E; 5.6C and 5.6F)**.

5.3.2.3 **Localisation of *venom dipeptidyl peptidase IV (DPP IV)* transcription by *in situ* hybridisation**

DPP IV transcripts were expressed specifically in adult parasite spinal and proboscis glands cells (**Fig. 5.8 A and 5.8D)**. This specific expression was confirmed by the absence of signals in consecutive sections hybridised with sense probes and negative controls **(Fig 5.8B and 5.8E; 5.8C and 5.8F)**.

5.3.2.4 **Localisation of *serpin* transcription by *in situ* hybridisation**

In situ hybridisation revealed expression of *serpin* transcripts in the spinal and proboscis glands (**Fig. 5.9A and 5.9D)**. Focal expression patterns were observed around the cells as distinct pinpoints of intense fluorescence. This gene was also localised to the *A. foliaceus* gut (**Fig. 5.10)**; anterior and posterior midgut. The signals obtained by serpin hybridization were confirmed by negative results for sense probe and negative controls (**Fig. 5-9B and 5.9E; 5-9C and 5-9F)**.

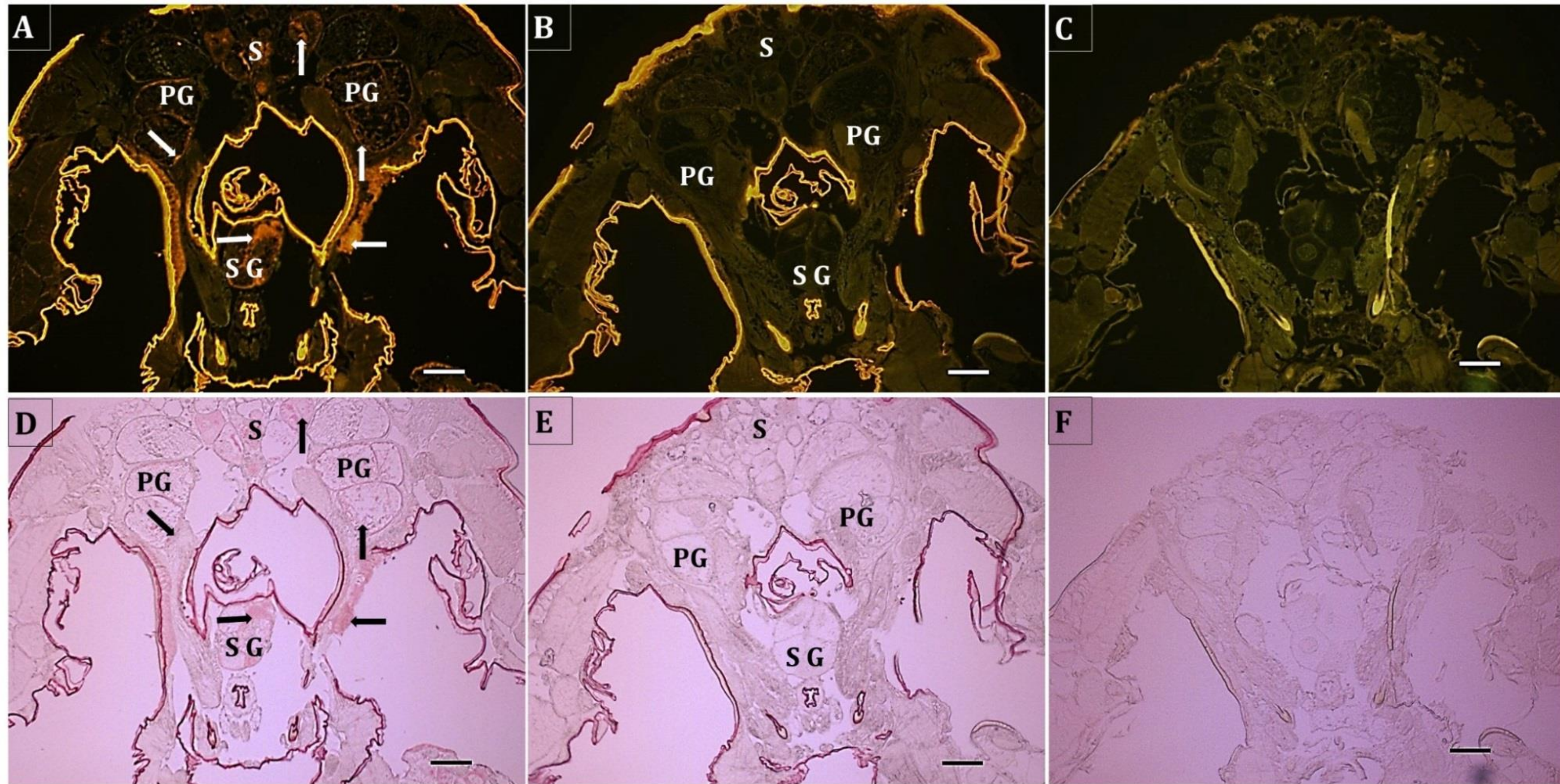


Figure 5.4 Localisation of *Argulus foliaceus PGE2S* transcripts in adult male louse using *in situ* hybridisation. (A) hybridisation using anti-sense probe; positive; (B) sense hybridisation probe control; (C) *in situ* hybridisation negative control (hybridization buffer only) of *PGE2S* transcript. No indication of any expression in (B) and (C). Note non-specific signal is observed around in the cuticle, but no signal within the glands is observed in controls. Spinal gland (SG), Proboscis gland (PG) and (S) secretory cells around the cerebral ganglion. (A), (B) and (C) emitted fluorescent signal; (D), (E) and (F) bright field of (A), (B) and (C). Scale bar=100μm.

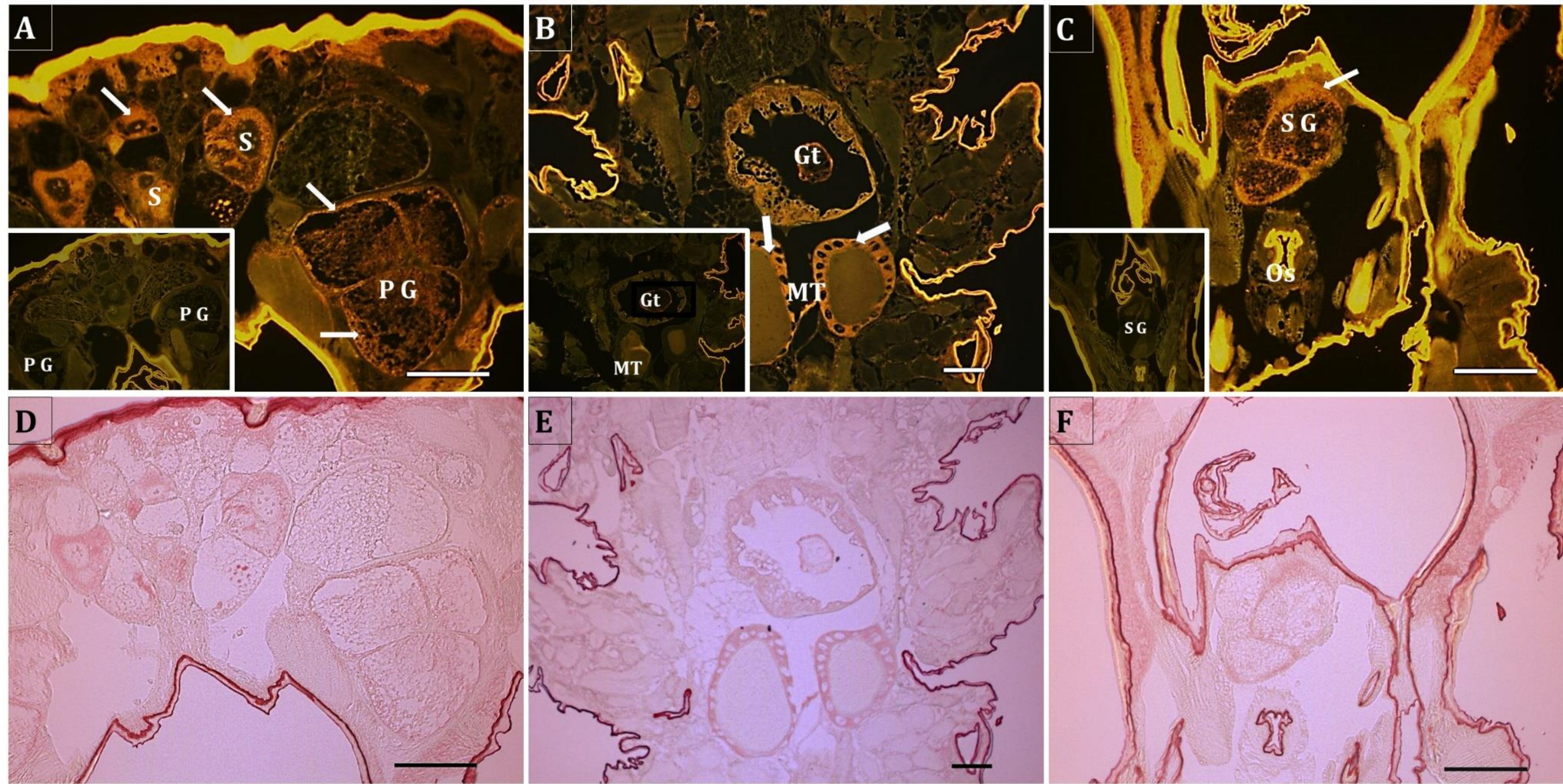


Figure 5.5 Localisation of *PGE2S* transcripts in adult male *A. foliaceus*. (A) proboscis glands (PG) and other secretory cells (S) in the head between the cerebral ganglion (arrows); (B) gut (Gt) and in the outer layer of the male testis (MT) (arrows); (C) spinal gland. (D), (E) and (F) = bright field of (A), (B) and (C). Scale bar=100 μm.

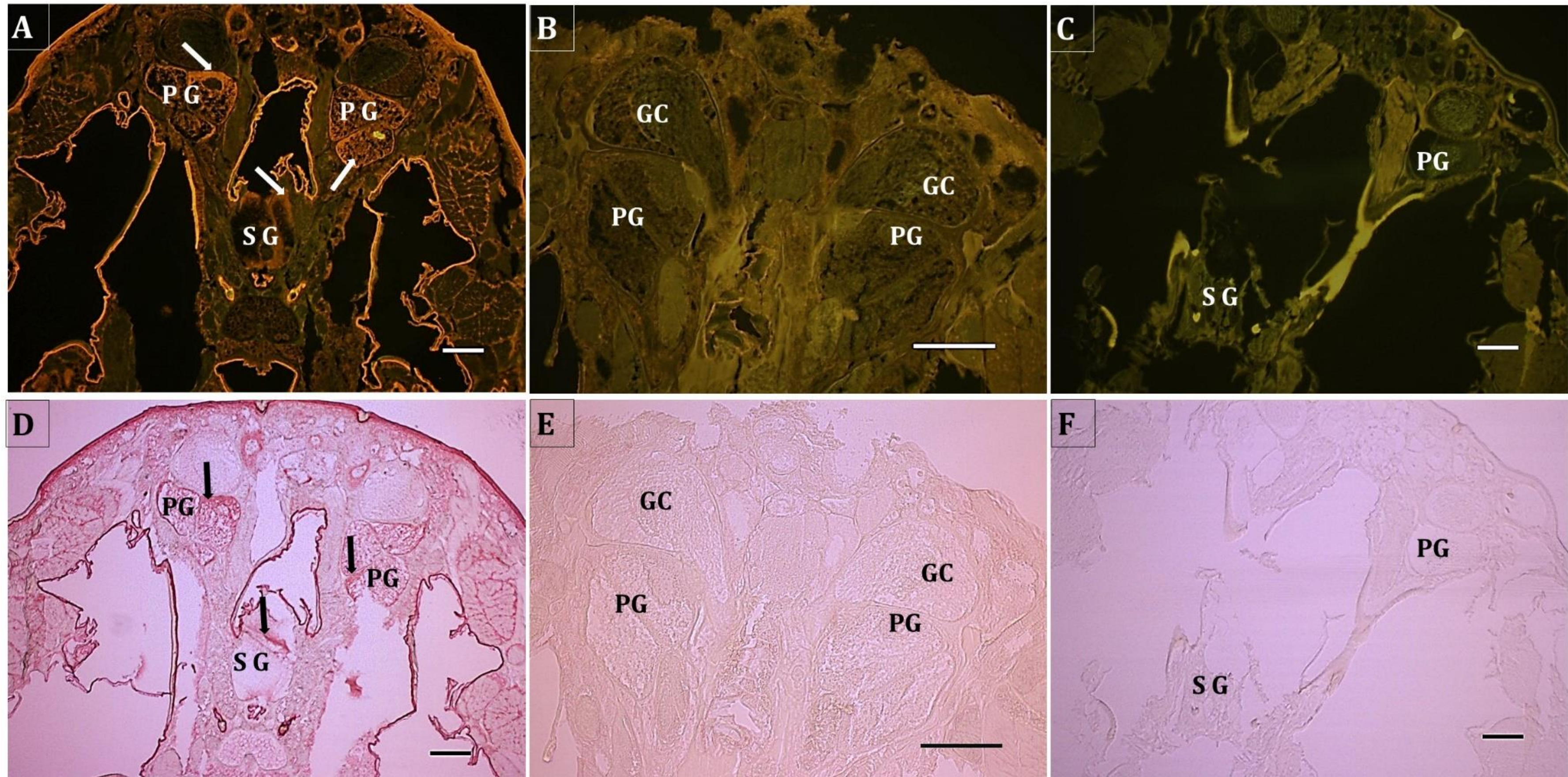


Figure 5.6 Localisation of *trypsin* transcripts in adult *A. foliaceus* (A) hybridisation using *trypsin* anti-sense probe; localisation of trypsin transcript in the spinal and proboscis glands, secretory cells, connective tissue between muscles and cuticle epithelial tissue; (B) *trypsin* sense hybridisation probe used as a control (C) *In situ* hybridization negative control of *trypsin* transcripts (hybridization buffer only). No expression observed in (B) and (C). Spinal gland (SG), Proboscis gland (PG) and (S) secretory cells around the ganglion cerebrum. (D), (E) and (F) = bright field of (A), (B) and (C). Arrows highlight zones of expression. Scale bar=100μm.

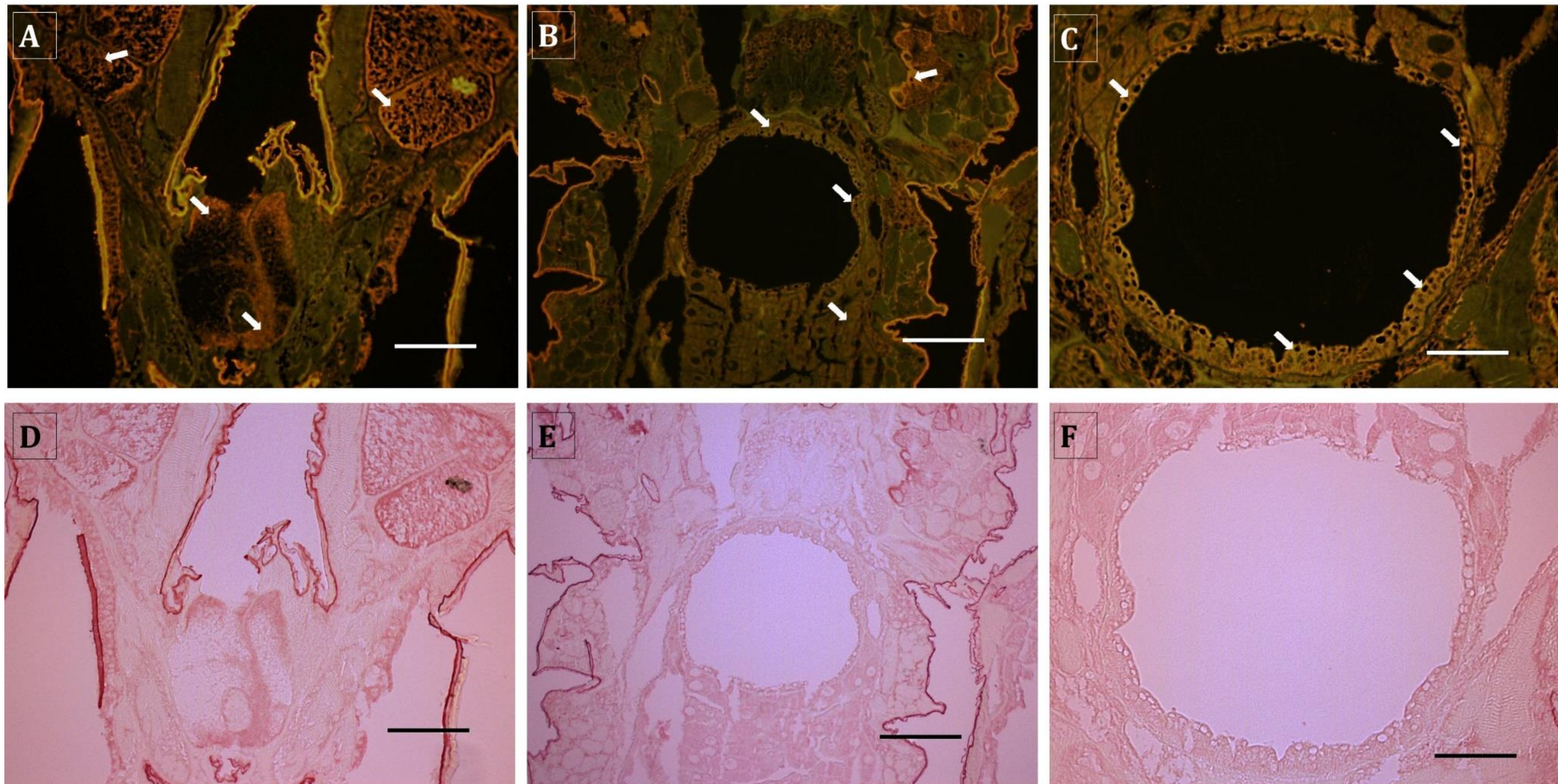


Figure 5.7 Localisation of *trypsin* transcripts in adult *A. foliaceus* using *in situ* hybridization. (A) high magnification showing strong expression of trypsin transcripts in the spinal and proboscis glands; (B) Expression in the midgut, secretory cells, connective between muscles and oocytes indicative of multiple foci of expression of trypsin in *A. foliaceus*; (C) high magnification of the midgut showing the expression of trypsin within the epithelial cells lining the gut. (D), (E) and (F) = bright field of (A), (B) and (C). Arrows highlight the zone of expression. Scale bar, A and B=100µm and C= 50µm.

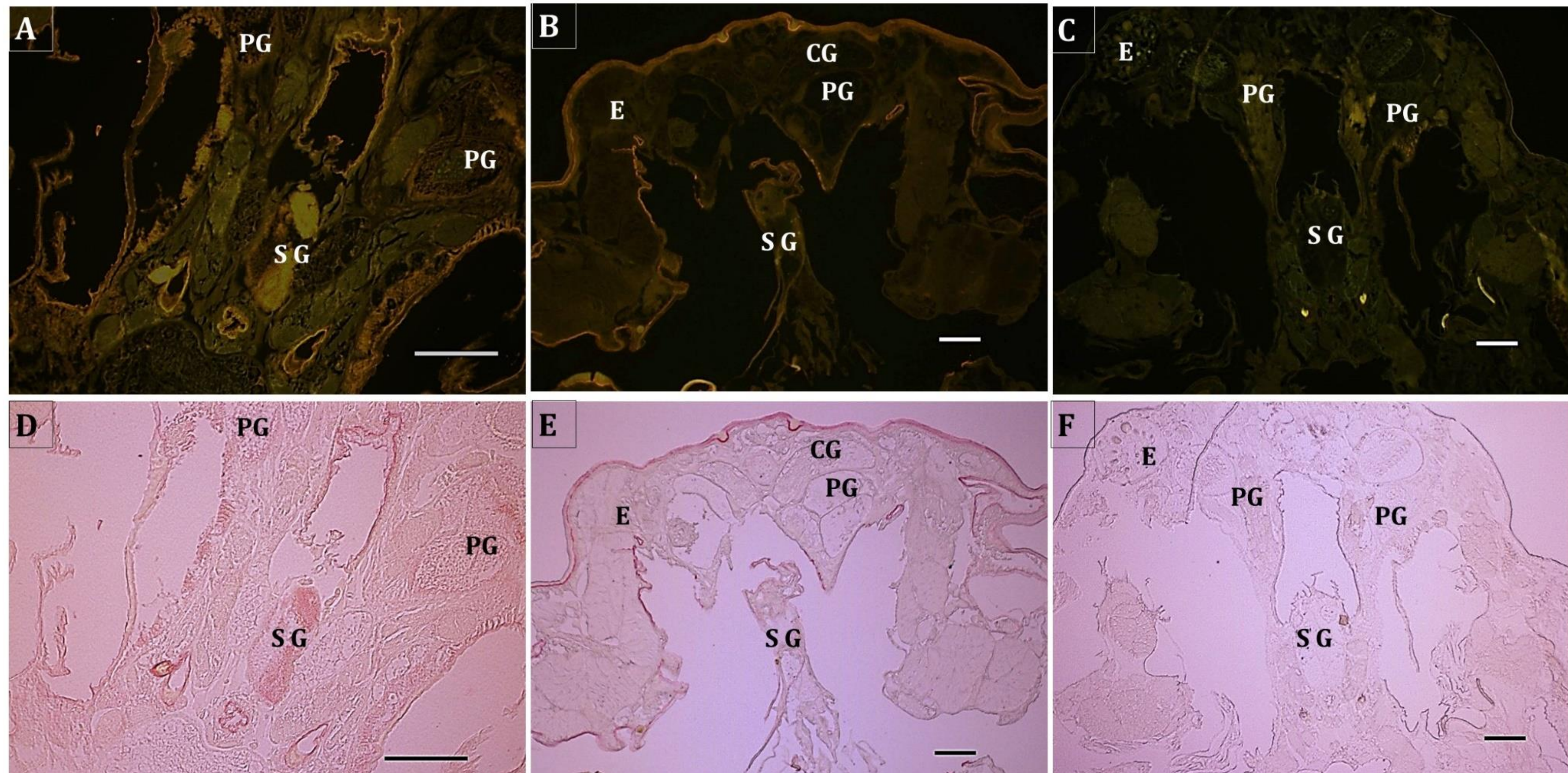


Figure 5.8 Localisation of *A. foliaceus venom DPP IV* transcripts in an adult male louse. (A) Positive hybridisation using anti-sense probe in the spinal gland and proboscis gland; (B) sense hybridisation probe used as hybridization control; (C) *In situ* hybridisation negative control (hybridization buffer) for *V-peptidase* transcripts. No expression was observed in (B) and (C). Spinal gland (SG), Proboscis gland (PG) and (S) secretory cells around the cerebral ganglion. (D), (E) and (F) = bright field of (A), (B) and (C). Scale bar=100µm.

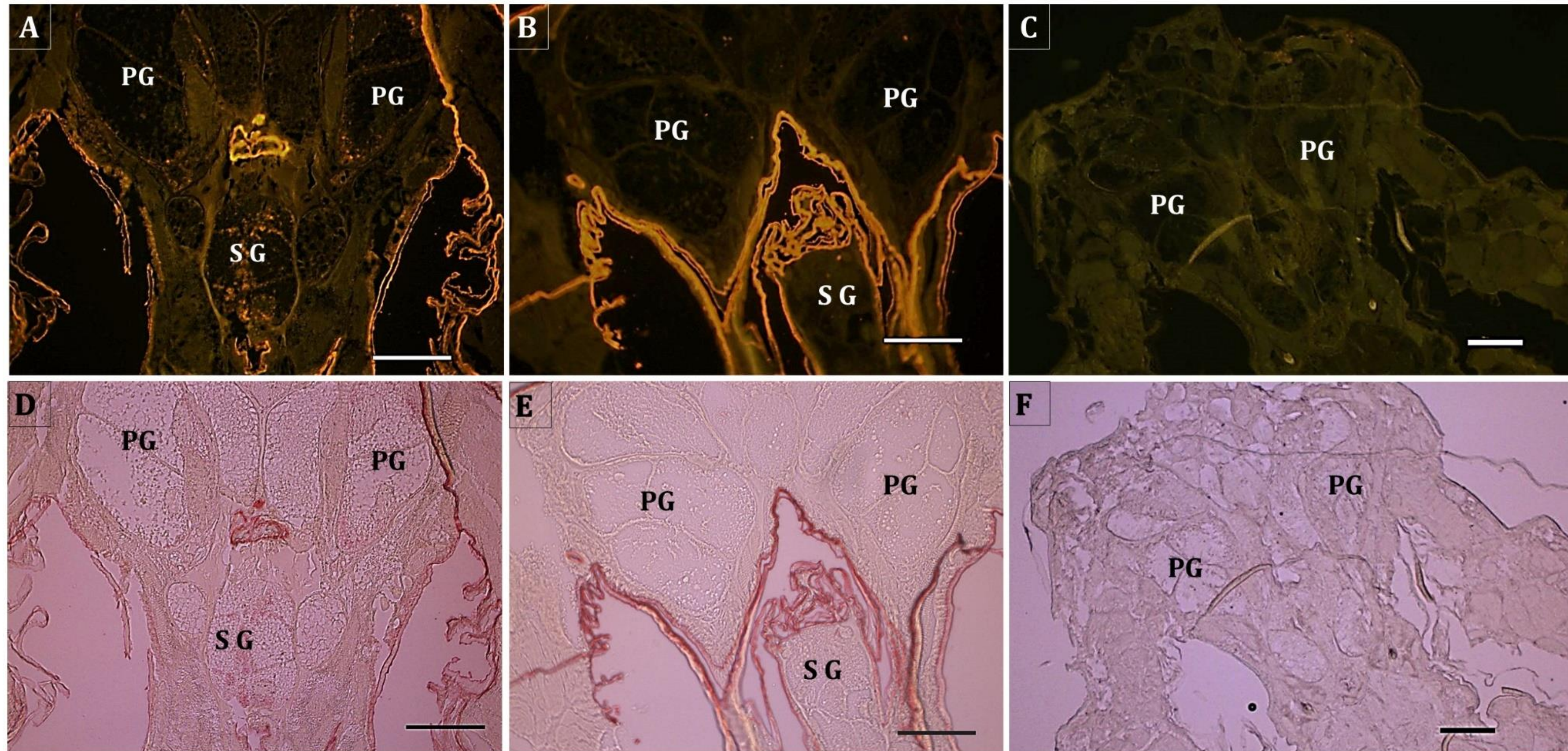


Figure 5.9 Localisation of *A. foliaceus serpin* transcripts in an adult male louse. (A) Positive hybridisation using anti-sense probe in spinal gland and proboscis gland; (B) sense probe was used as a hybridisation control; (C) Negative control (hybridization buffer) used for *serpin in situ* hybridisation. No expression was observed in (B) and (C). Spinal gland (SG), Proboscis gland (PG) and (S) secretory cells around the ganglion cerebrum**.** (D), (E) and (F) = bright field of (A), (B) and (C). Scale bar=100µm.

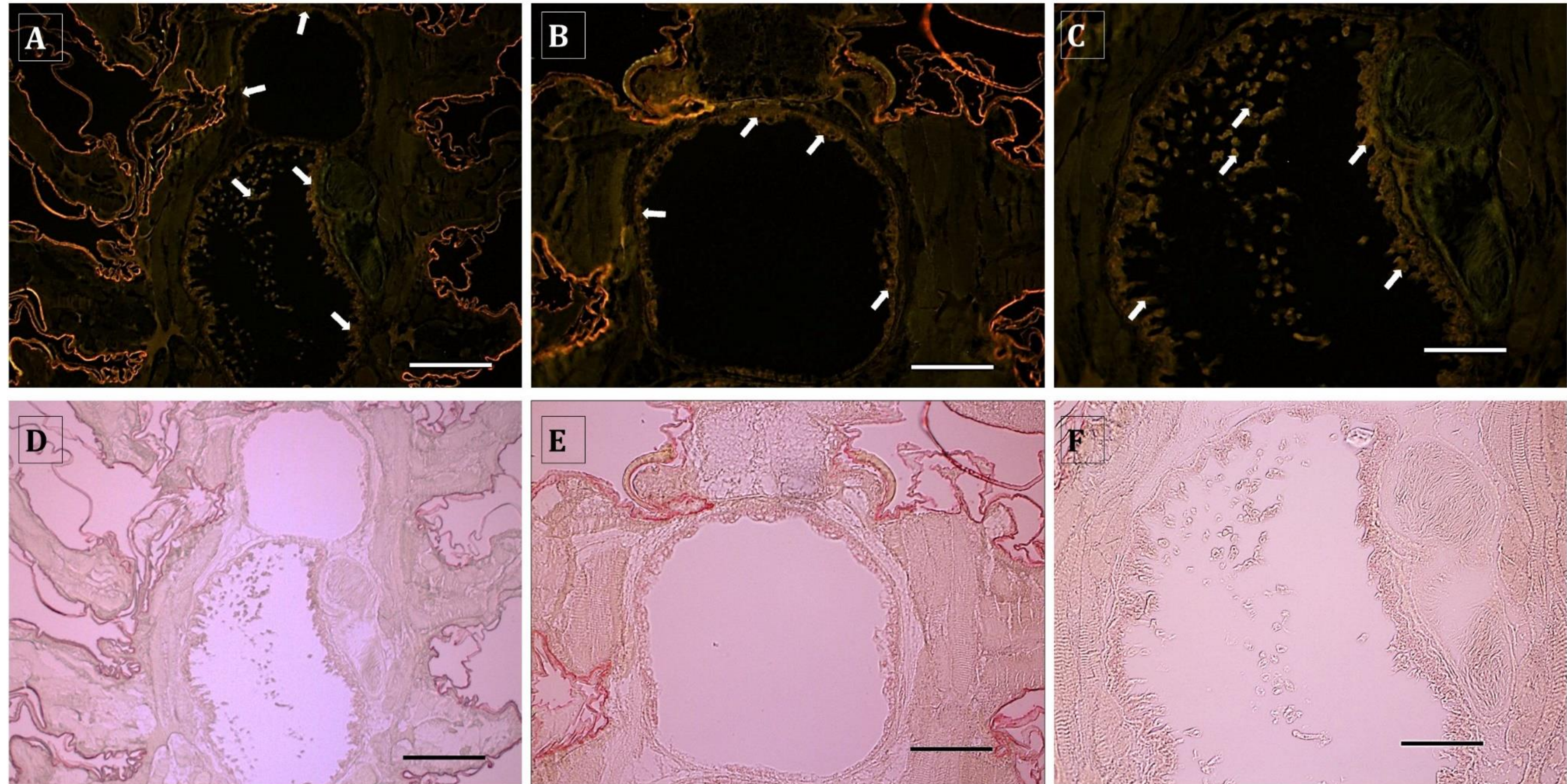


Figure 5.10 Localisation of *A. foliaceus serpin* transcripts in a male louse. (A) Positive hybridisation was observed using anti-sense probe in the gut with moderate expression evident within the gut cells; (B) anterior midgut and (C) posterior midgut. (D), (E) and (F) = bright field of (A), (B) and (C). Arrows highlight the zone of expression. Scale bar, A, B= 100 μm and C= 50μm.

5.3.3 Lectin labelling of spinal and proboscis glands

Fluorescent lectin labelling of adult male *A. foliaceus* sections by PHA-E, *Phaseolus vulgaris Erythroagglutinin*, revealed strong specific binding within the cytoplasm of spinal gland cells only (**Fig 5.11A, B**), which was absent from other *A. foliaceus* glands and tissues such as the testes and gut (**Fig. 4.11 C, D**). Strong binding was observed in the cytoplasm of cells nearest the duct (**Table 4.1)**.

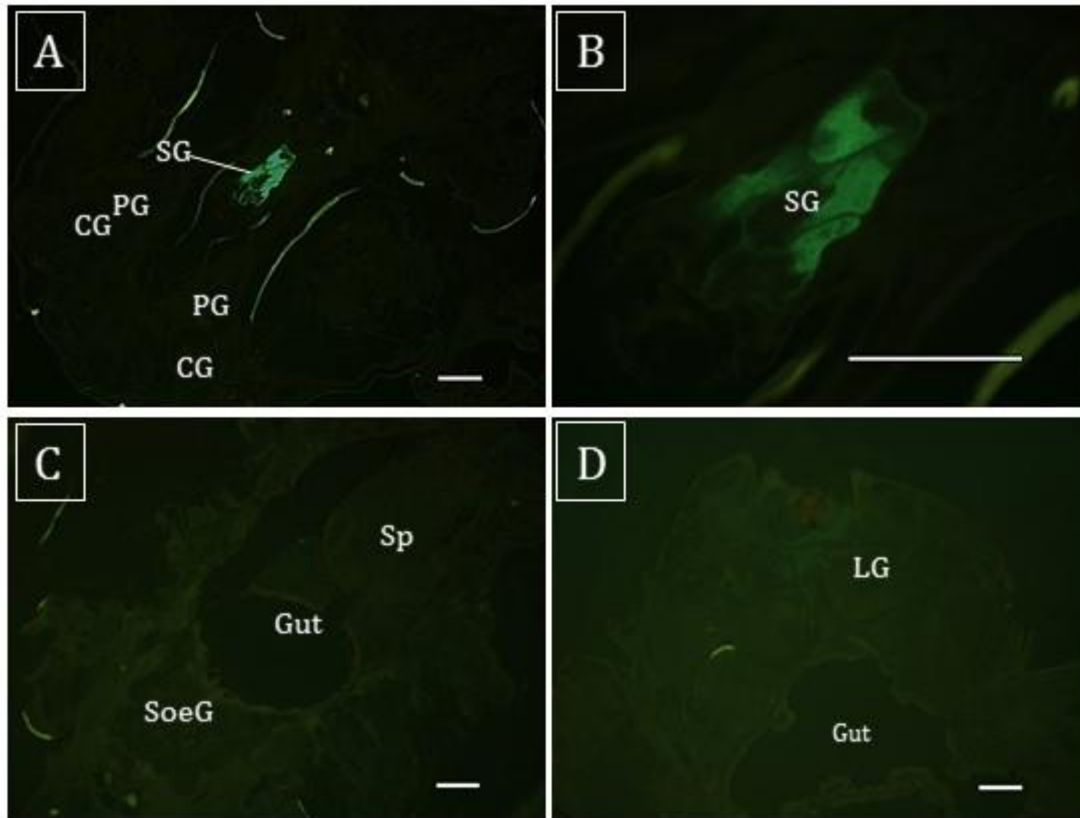


Figure 5.11 Planar section of adult male *A. foliaceus* labelled with PHA-E fluorescent lectin **(A)** and **(B)** PHA-E lectin binding specifically to the spinal gland cells cytoplasm (SG) and not binding to any other tissue or secretory gland cells such as the proboscis gland (PG) or labial gland (LG). Other regions of *A. foliaceus*; were not bound by the PHA-E lectin including the testes **(C)** and gut **(D)**. Cerebral ganglion (CG), suboesophageal ganglion (SoeG), sperm (Sp). Note background staining of cuticle is a non-specific consistent artefact observed with fluorescent microscopy. Scale bar = 100µm.

Datura stramonium lectin, DSL, bound specifically and strongly to the spinal gland cytoplasm cells (**Fig 4-12A, B, C**), moderately to a few small secretory cells around the brain (arrow) (**Fig 4-12A, B**) and weakly to the midgut.

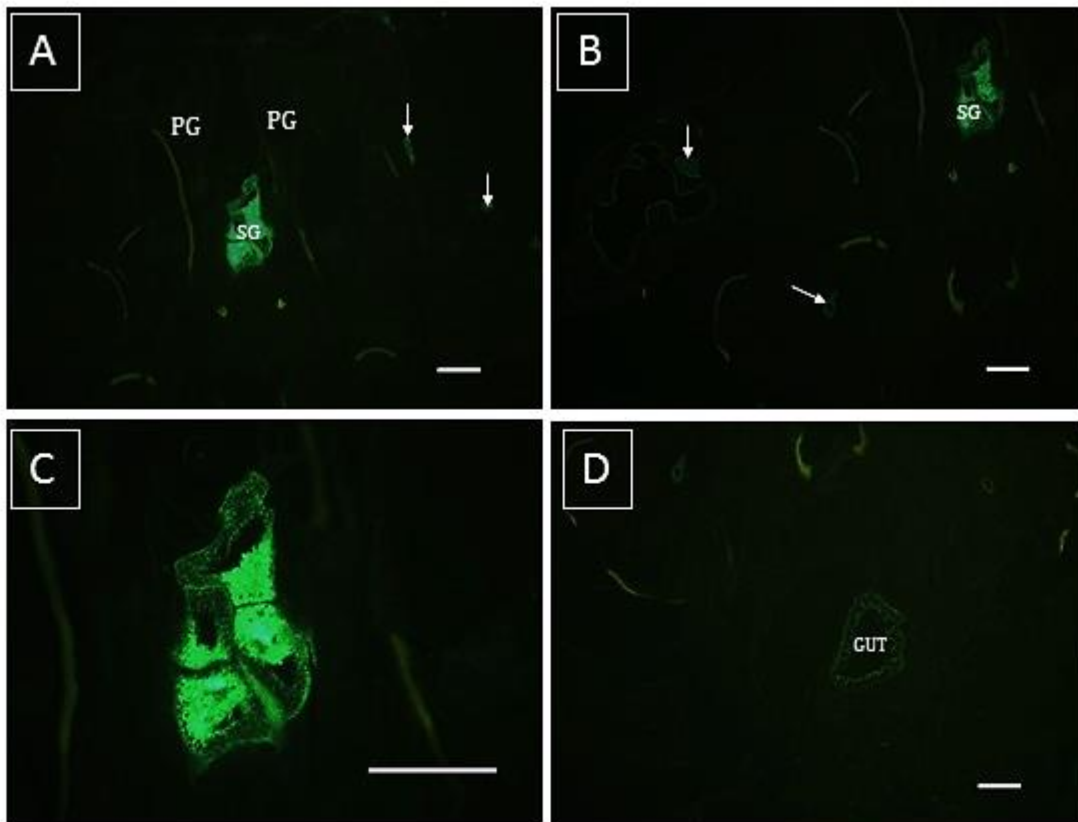
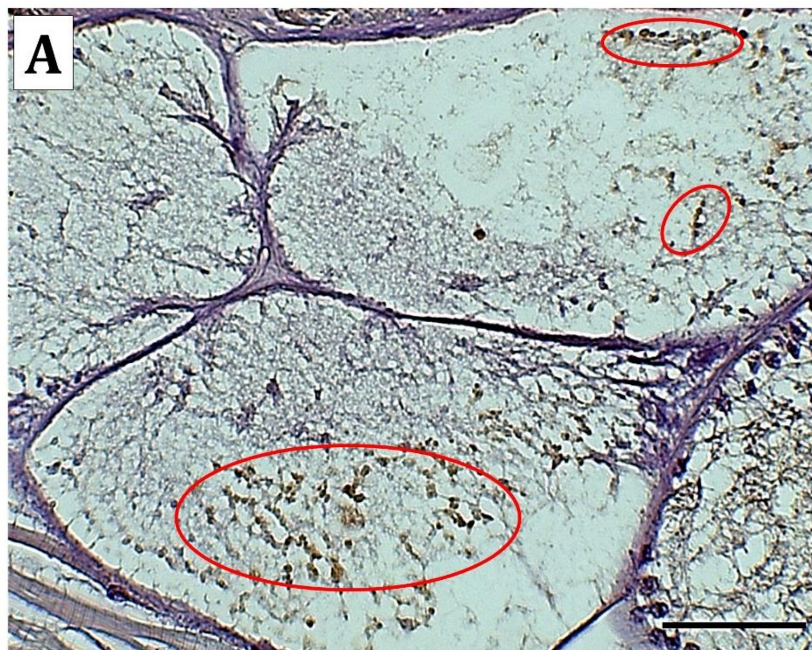


Figure 5.12 Planar section of adult *A. foliaceus* labelled with DSL fluorescent lectin. (A), (B) and (C) DSL lectin shows strong and specific binding to the spinal gland cytoplasm cells (SG); (A) and (B) moderate binding to few small secretory cells (arrow); (D) weak binding to the midgut. DSL did not show any binding affinity to the proboscis glands (PG) or labial glands. Note background staining of cuticle is a consistent non-specific artefact observed with fluorescent microscopy. Scale bar = 100μm.

The pattern of DSL lectin binding to the spinal gland cells was very similar to that of PHA-E lectin, however, it also exhibited faint scattered staining in the cytoplasm of cells further from the duct.

5.3.4 **Immunohistochemical analysis**

Prompted by the positive lectin staining described in 5.2.4, IHC for serpin D1 and heparan sulphate proteoglycans to establish the presence or absence of these proteoglycans in the studied glands. Expression of both serpin D1 and heparan sulphate proteoglycans (HSPG's) were observed in the proboscis gland cells (**Fig 5.13, 5.15)**.



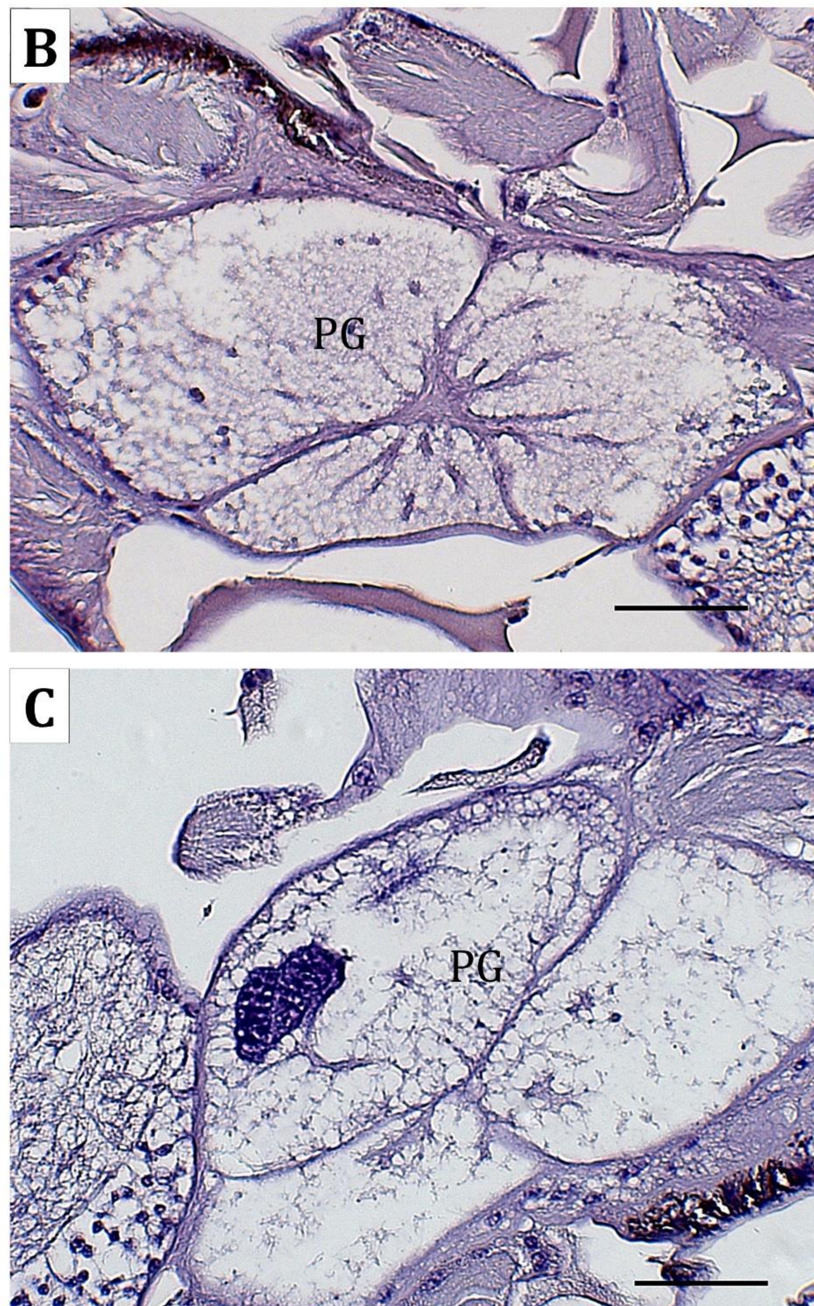
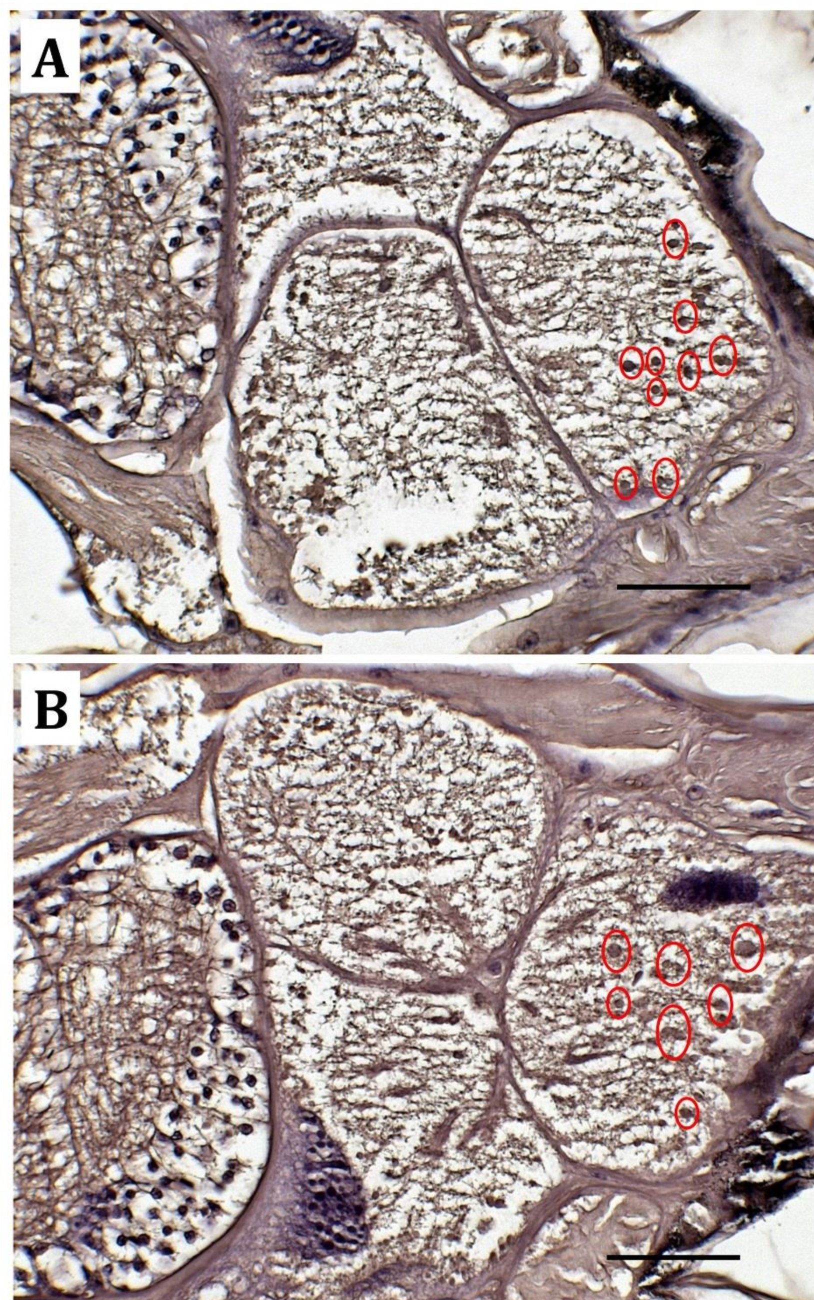


Figure 5.13 Immunohistochemical staining of proboscis glands (PG) in *A. foliaceus* sections using negative controls alongside heparan cofactor II (HCII) antibody (A) Immunohistochemical detection of heparin cofactor II (HCII) antigen in proboscis gland (PG) cells (B) PBS control and (C) non-specific antibody controls. No background staining was observed. Black staining of the epithelial layer lining the inner layer of the cuticle was a commonly observed non-specific artefact. IHC with DAB chromogen and haematoxylin counterstains. Scale bar= 20 µm.

Negative controls; PBS and nonspecific isotype-matched antibody control, used alongside the HCII antibody target, exhibited no expression or nonspecific staining **(Fig 5.13)**, confirming the positive signals obtained for the anti- HCII antibody.



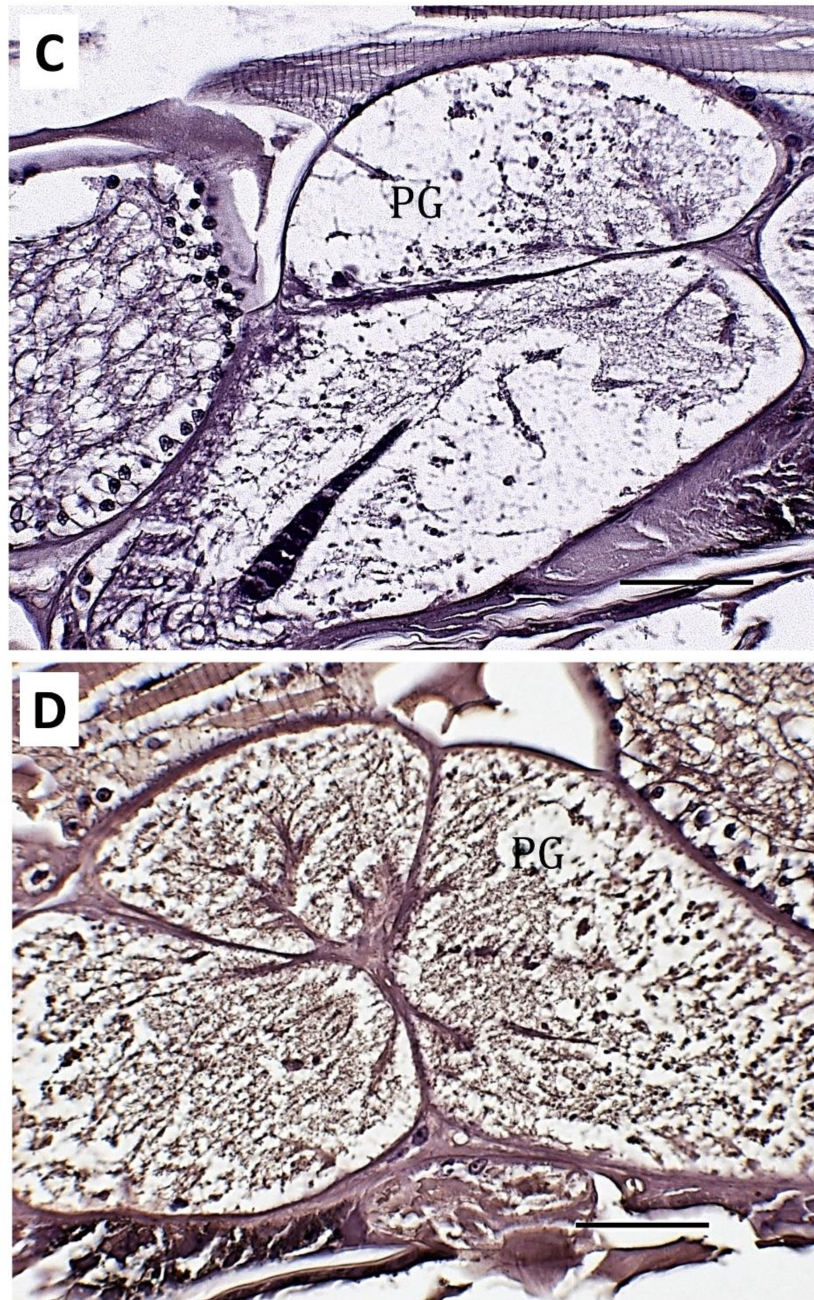


Figure 5.14 Planar sections exhibiting localisation of HS6ST3 antibody (1:500); heparan sulphate proteoglycans, in the proboscis gland (PG) cells. (A) and (B) Expression was characterised by vacuoles (surrounded by red oval shapes). The vacuole staining differed from the brown diffuse background staining that was also observed in control (PBS only and non-specific antibody) section antibody (C) PBS control and (D) non-specific control. IHC with DAB (brown) chromogen and haematoxylin counterstains. Scale bar= 20 µm.

Despite background staining being present in the nonspecific HSPGs antibody control, specific staining was also observed that differed from this only when sections were incubated with the HSPG specific antibody. This was characterised by vacuolar brown staining within the proboscis glands (**Fig 5.14 A-B**).

No expression was observed in the spinal gland cells when incubated with both HCII and HS6S6T3 antibodies (**Fig 5.15; 5.16)**.

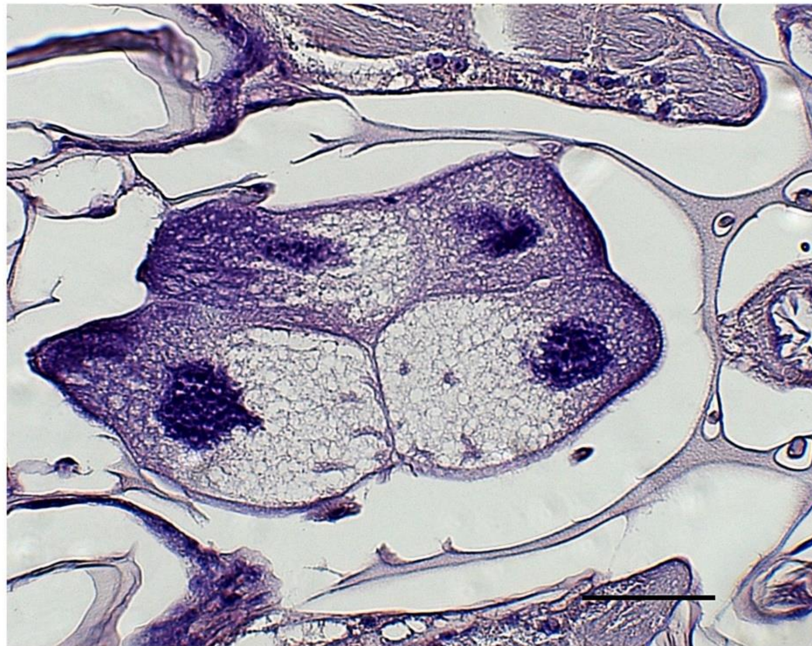


Figure 5.15 Immunohistochemistry on planar section exhibiting no detection of heparin cofactor II antigen in the spinal gland cells of *A. foliaceus***.** IHC with DAB chromogen and hematoxylin counterstains. Scale bar= 20 μm.

Background staining with HS6S6T3 was also obvious within the spinal gland cells **(Fig. 5.16)**.

HCII antigens were localised in the microvilli of *A. foliaceus* gut diverticuli located in the carapace periphery **(Fig 5.17)**. No staining was observed in PBS and non-specific antibody controls **(Fig 5.18)**.

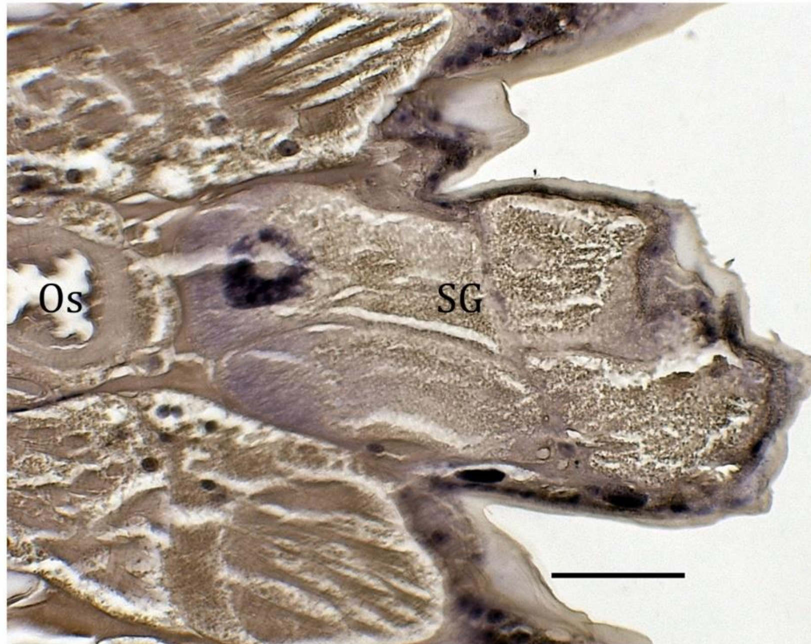


Figure 5.16 Planar section showing no detection of HS6ST3 antibody targeting heparan sulphate proteoglycans (1:500) in the spinal gland cells of *A. foliaceus*. The brownish colour is indicative of background staining. IHC with DAB (brown) chromogen and haematoxylin counterstain. Scale bar= 20 µm.

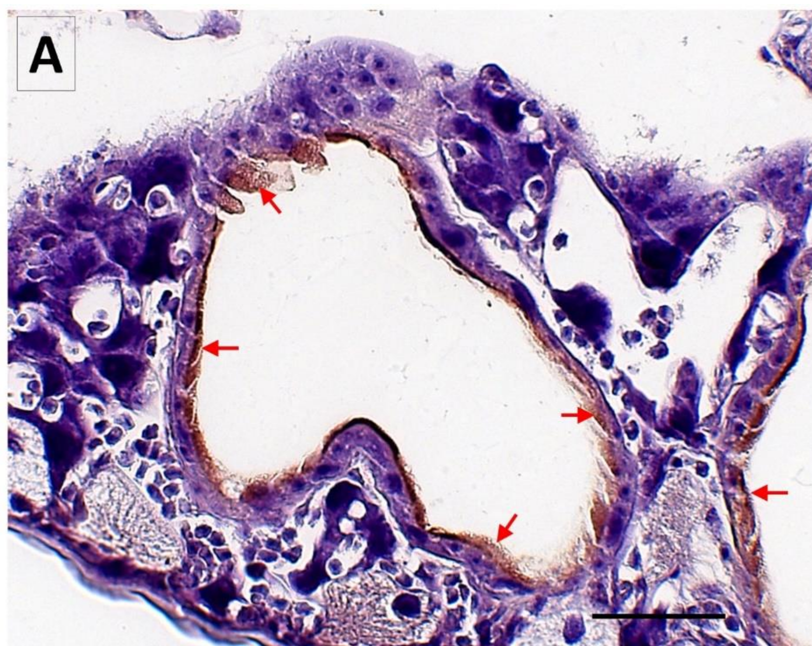


Figure 5.17 Localisation of heparin cofactor II (HCII) in *A. foliaceus* gut diverticulum specifically in the microvilli (arrows) using immunohistochemistry. IHC with DAB (brown) chromogen and hematoxylin counterstains. Scale bar= 20µm.

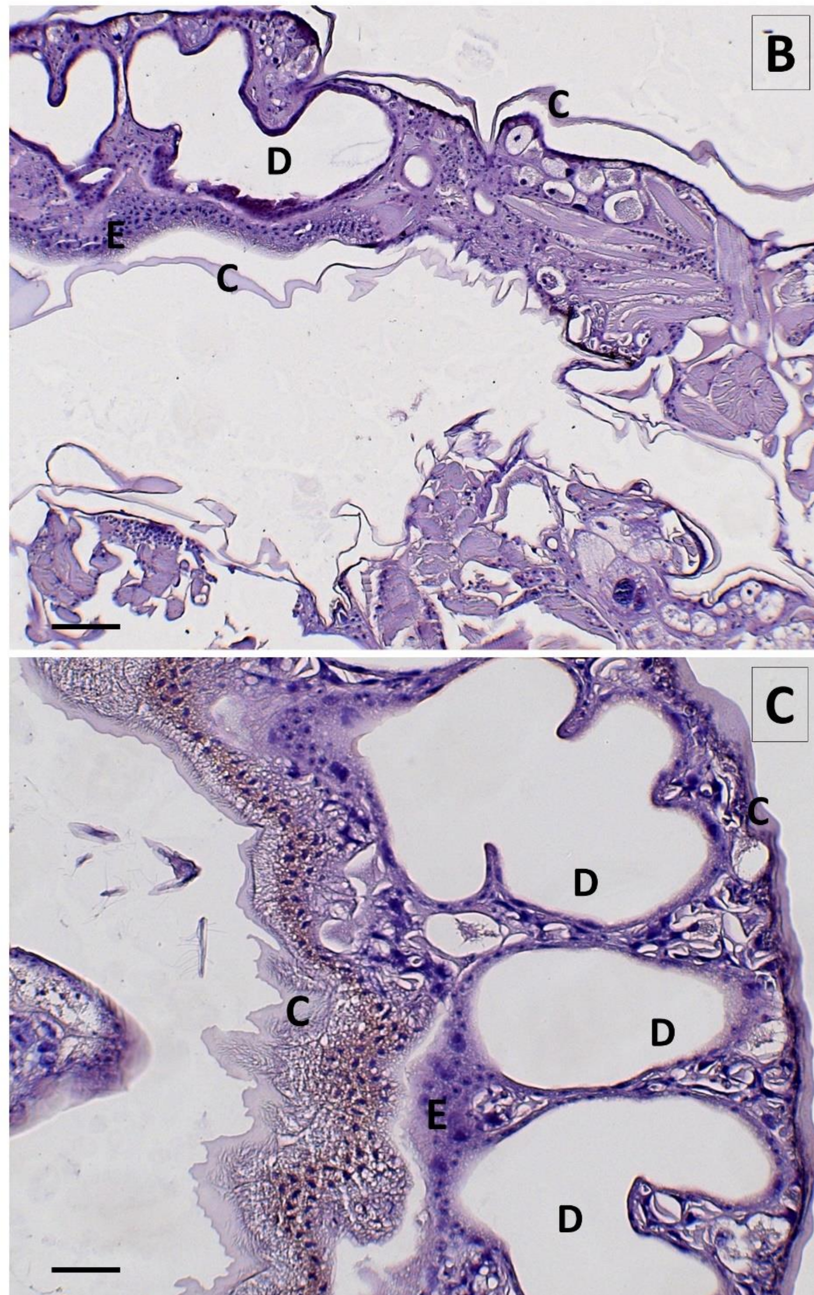


Figure 5.18 Immunohistochemistry of heparin cofactor II in *A. foliaceus* gut diverticuli. (A) PBS negative control and (B) Nonspecific antibody control of HCII antibody. IHC with DAB chromogen and hematoxylin counterstain. Diverticuli (D), epithelial tissue (E), cuticle (C). Scale bar=100 µm.

In the posterior midgut, HSPGs were localised in secretory vacuoles (**Fig 5.19)**.

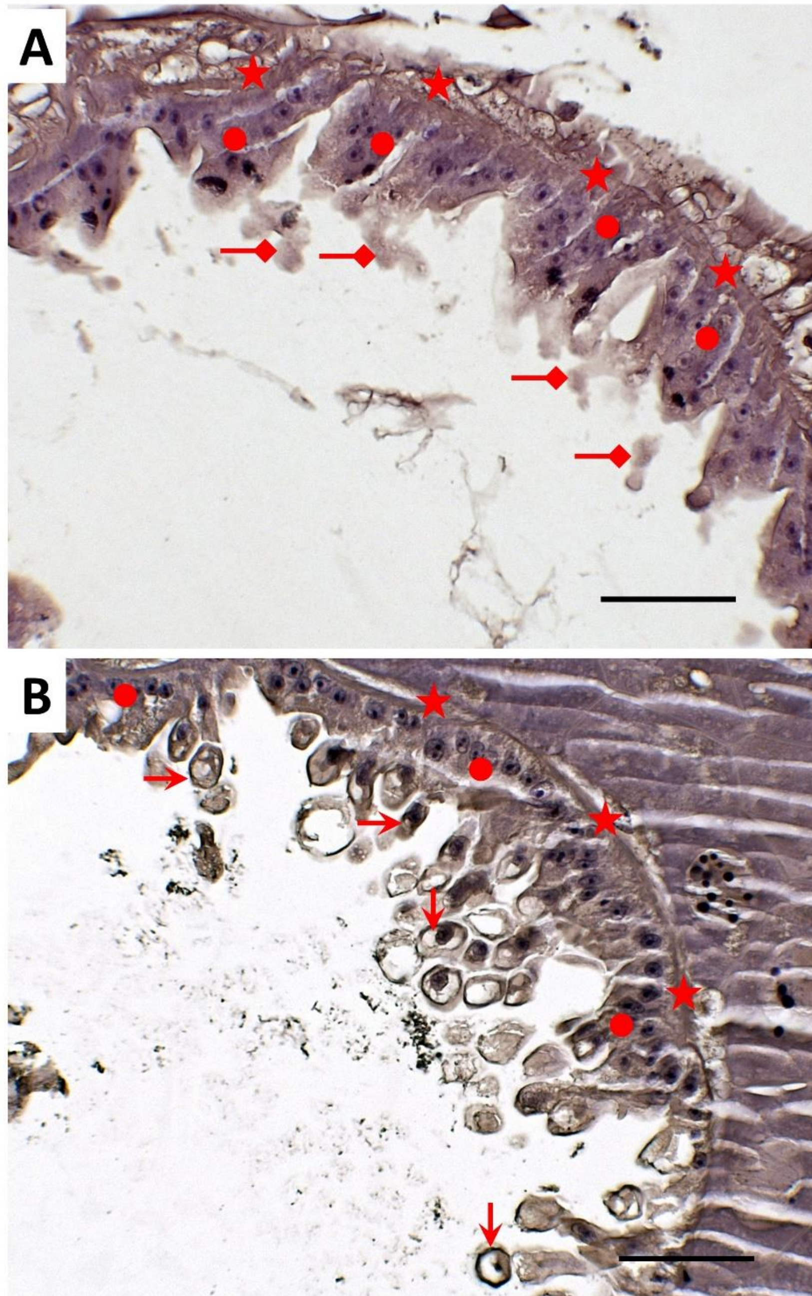


Figure 5.19 Immunohistochemical staining of HS6ST3 antibody detecting heparan sulphate proteoglycans (1:500). (A) planar section of non-specific antibody control; (B) Localisation of HS6ST3 antibody in the *A. foliaceus* posterior midgut indicative of heparan sulphate expression in the vesicles produced by papilliform cells. Epithelial basement membrane (★), epithelial cells (•), secretory product in the gut shows expressed HSPG's (→) compared with the nonspecific staining (—◆). IHC with DAB chromogen and hematoxylin counterstains. Scale bar= 20 µm.

In this study HS6S6T3 antibody labelling was localised in the free ends of these papilliform cells; which were secretory-like vesicles that were observed during digestion of blood contents.

In the posterior midgut, HSPGs were localised in secretory vacuoles (**Fig 5.19)**

5.4 **Discussion**

Argulus foliaceus, like other arthropod parasites, have been suggested to secrete substances through the feeding apparatus to facilitate feeding and inhibit host immune responses against them (Shimura & Inoue, 1984; Swanepoel & Avenant-Oldewage, 1992; Gresty *et al.*, 1993; Saha *et al*., 2011). Thus, the goal of this study was to investigate possible secretory proteins/genes; within the glands associated with feeding process, that could have immunomodulatory roles in host immune invasion, as proposed in previous studies conducted on other arthropods (Sor-suwan *et al*., 2014; Kato *et al*., 2013; Kotsyfakis *et al*., 2006; Fast *et al*., 2004; Yuan *et al*., 2000; Cappello *et al*., 1998). Candidates for study were selected from transcriptomic and proteomic data obtained earlier in the thesis (**Chapter 3 and 4**, respectively), with a view to determine whether they are expressed in the glands related to *A. foliaceus* feeding (glands as described in **Chapter 2**) in an attempt to postulate the roles of these secretory substances on the fish immune system during feeding. Comparisons were then made with other arthropods to gauge possible similar physiological properties of the transcribed products in *Argulus* spp. A better understanding of *Argulus* spp immunomodulatory molecules and their role in counteracting the host immune

response may facilitate control strategies to prevent spreading of freshwater lice in aquaculture and enhance knowledge of so-called “toxin” / “venom” evolution in crustaceans and arthropods in general (von Reumont *et al.*, 2014). Successful vaccine development against ectoparasites depends on the identification of immunogenic proteins of the pathogen, often targeting those known to inhibit the host immune response, thus many studies conducted on haematophagous arthropods have focused on characterising such secretory molecules in the saliva and salivary glands (Wang & Nuttall 1999; Parizi *et al.*, 2012).

In this study, three methodologies have been used to localise putative secretory molecules associated with immunomodulatory processes in *A. foliaceus* with investigations conducted at the transcript level (*PGE2S*, *trypsin*, *DPP IV*; *venom dipeptidyl peptidase* and *serpin*) by ISH, at the protein level (heparin cofactor II (HCII) and heparan sulphate proteoglycans (HSPGs)) using IHC and at the glycoprotein / peptidoglycan level using fluorescent lectin-labelling.

In situ hybridisation revealed that all four genes of interest, *PGE2S*, *trypsin*, *DPP IV* and *serpin*, were transcribed in both targeted gland tissues, spinal and proboscis, with different degrees of expression. This confirmed the role of these glands in secreting bioactive components that have been identified from other haematophagous arthropods as detailed below. The presence of these transcripts in these glands is in accordance with their suggested role in host-parasite interactions and immunomodulation, as these are regions likely to be involved in the release of active components during blood-feeding. *Serpin* and *DPP IV* were specifically expressed in the spinal and proboscis gland cells. *Serpin* was also transcribed in the anterior and posterior gut indicating a role in digestion or release of gut contents at the site of

feeding. Transcription of *trypsin* was more generally localised and was detected in the spinal and proboscis glands, secretory cells next to the cerebral ganglion, epithelial and connective tissue, oocytes and in the gut. *PGE2S* transcripts were also expressed in the midgut, male testes and secretory cells on the head possibly related to ganglia in the region. This may indicate that *trypsin* and *pge2s* have multiple functions in addition to their role in feeding, which corroborates postulated functions of these genes in arthropods; as described below.

Prostaglandin E 2 synthase

In this study, prostaglandin E 2 synthase, *PGE2S*, an enzyme involved in conversion of prostaglandin H2 (PGH2) to prostaglandin E2 (PGE2) (Kudo and Murakami, 2005), was highly expressed in the spinal and proboscis glands, in secretory cells around the cerebral ganglion, in the gut and in the outer layer of male testis. This suggests that *PGE2S* in *A. foliaceus* may be involved in different biological functions in addition to the anti-coagulation afforded by PGE2. PGE2 in haematophagous arthropods has been shown to inhibit platelet aggregation (Andrade *et al.*, 2005) and might have vasodilatory activity at the feeding site to increase blood flow (Eichner *et al.*, 2015). Fast *et al.* (2004) reported the presence of PGE2 in adult sea lice, *L. salmonis* and suggested that PGE2 in sea lice has an immunomodulatory role (Fast *et al.*, 2007), being postulated to modulate the host immune system by downregulating expression of Atlantic salmon pro-inflammatory genes and increasing blood flow at the feeding site (Fast *et al.*, 2005). *PGE2S* was also noted to contribute to various other biological activities such as vasodilation, cellular proliferation, leucocyte activation and neutrophil chemotaxis and accumulation at inflammatory sites (Fast *et al.*, 2005). Similarly, PGE2 has elsewhere been reported to exhibit various biological functions

related to inflammation, immune response and reproduction (Guo *et al.,* 2015; Eichner *et al.,* 2015). Tick saliva PGE2 has been demonstrated to act on host defense mechanisms by restricting macrophages and dendritic cells (as reviewed by Radulović *et al.,* 2014). Stanley and colleagues also suggested a role of prostaglandins (PGs) in insects is host gene modulation and protein expression (Stanley *et al.*, 2012). Bowman *et al.* (1996) proposed that high levels of prostaglandins in ticks was indicative of an anti-inflammatory role (Bowman *et al.*, 1997).

*Ls*PGES2 of *L. salmonis* was expressed in parasitic and non-parasitic stages of sea lice (Campbell *et al.*, 2009), which indicates the importance of *PGE2S* in different functions and regulation of different biological systems throughout the life cycle of the louse. A study conducted by Kutyrev and colleagues on the tapeworm; *Schistocephalus solidus* and its host, the three-spined stickleback (*Gasterosteus aculeatus*), which has been used as an *in vitro* model system to investigate the role of PGE2 on host-parasite interactions, supports the immunomodulation activity hypothesis of PGE2 on fish hosts (Kutyrev *et al.*, 2014). Moreover, PGE2 has been detected in other tissues in various crustacean species, revealing its role in the development of the ovaries and female reproduction system (Eichner *et al.,* 2015; Nagaraju 2011; Tahara & Yano 2004; Reddy *et al.*, 2004; Spaziani *et al.*, 1995). Ontogenetic expression and *in situ* analysis of *Ls*PGES2 of different life stages of sea lice; *L. salmonis*, indicates the presence of this gene in all life stages with highest expression levels in planktonic stages and reduced levels in parasitic stages (Eichner *et al.*, 2015). The ISH done by Eichner and coauthors showed the detection of *Ls*PGES2 in nauplius and free-living; non-parasitic swimming copepodids, which supports the importance of PGE2 in *e.g.* muscle contraction and the detection of

transcripts in the adult female reproduction system indicated its role, like other crustaceans, in sea lice reproduction (Eichner *et al.*, 2015). Nonetheless, *L. salmonis* parasitic larval stage (chalimus) secretions were found to contain PGE2 (Walton, 2008) as well as the secretions of mature parasitic lice stages (Fast *et al.,* 2004; 2005 and 2007) supporting the hypothesised role of PGE2 in parasite-host interactions. PGE2 in ticks, *Rhipicephalus sanguineus*, exhibited effective immunomodulatory activity (Oliveira *et al.*, 2011). PGE2 has been found in high levels in tick salivary glands and is assumed to stimulate anticoagulant secretions having a direct activity upon the feeding site (as reviewed by Yuan *et al.*, 2000). Gillespie *et al.* (2000) described PGE2 in cattle tick saliva (*Boophilus microplus*), which was suggested to have a physiological effect on cattle (Inokuma *et al.*, 1994) and the high concentration of PGE2 in the saliva of hard ticks is suggested to be indicative of a host immunosuppressive role during feeding (Bowman *et al.,* 1996) and can act as a vasodilatory agent (Bowman 1996; Champagne 1994; Randolph & Nuttall 1994).

Trypsin

Trypsin; a serine protease, was selected as a target gene in the current study due to its identification and characterisation in different haematophagous arthropods. Trypsins are known to play different biological function in arthropods being digestive enzymes playing a main role in digestion (Rawlings and Barrett, 1994). However, the expression of this gene in glands associated in feeding may suggest that its activity includes anticoagulation and/or immunomodulation during blood acquisition. Trypsin inhibitor-like proteins, proteins that inhibit the activity of trypsins from the host, were reported in different haematophagous ticks and insects saliva and salivary glands (Karim *et al.*, 2011). The blood feeding gnathiid juvenile, *Paragnathia formic* (Isopoda:

Gnathiidae), were demonstrated to suppress host haemostatic, inflammatory and immunological responses during feeding by producing trypsin inhibitors and anticoagulants during blood acquisition from fish hosts (Manship *et al.*, 2012).

Although, trypsins are one of the most common proteases found in crustaceans such as shrimps, crabs, crayfish and lobsters (Jones *et al.*, 1997) and have been detected in the Crustacea, for instance the copepoda, their functions and structure are not well known (Johnson *et al.*, 2002). Trypsin in the sea louse *L. salmonis*, have been reported earlier by Jenkins *et al.* (1993) from louse whole-body preparations and later Roper *et al.* (1995) identified trypsin in the gut by using immunocytochemical techniques. The higher protease activity in the skin mucus of Atlantic salmon (*Salmo salar*) after *L. salmonis* infection (Ross *et al.*, 2000) and the detection of trypsin in the midgut of *L. salmonis* (Kvamme *et al.*, 2004; Johnson *et al.,* 2002; Firth e*t a*l., 2000) has led previous authors to suggest that trypsin has a role in modulating host immune responses to facilitate feeding and contribute to host invasion (Johnson *et al.,* 2002; Fast *et al.,* 2007; Wagner *et al.,* 2008). In the current study transcription of this gene; trypsin, was generally observed within different *A. foliaceus* tissues, indicating its diverse biological functions and it was also expressed in the gut, spinal and proboscis glands of the parasite, suggesting that trypsin may also play a role in digestion and immunomodulation in *A. foliaceus*.

In situ hybridisation in the current study revealed that trypsin and *PGE2S* are expressed in the gut, spinal and proboscis glands of *A. foliaceus*, but that *PGE2S* was expressed more intensely in the gut than trypsin.

Venom dipeptidyl-peptidase IV

The putative *venom dipeptidyl-peptidase* IV (DPP IV), identified and localised in the spinal and proboscis gland cells of *A. foliaceus* in the current study, showed homology with *venom dipeptidyl peptidase IV* (*Vespula vulgaris*) with a hit e-value of $1.23E^{-170}$. This enzyme was detected in *Ixodes scapularis* saliva and its role was suggested to be in the termination of the function of bradykinin, a pain-producing peptide involved in the inflammation process, by mediating the pain and causing oedema through increasing capillary permeability (Ribeiro and Mather, 1998). Proteomic analysis of nematode, *Heligmosomoides polygyrus*, secretions also revealed the presence of dipeptidyl peptidase IV with other pharmacologically active compounds (Hewitson *et al.*, 2011). The *venom di-peptidase* produced in *Argulus* glands might thus be involved in regulating immune responses in fish hosts by contributing as an immunomodulatory component. Recently, Braden *et al.* (2017) reported upregulation of dipeptidyl peptidase IV with other protease bioactive components in *L. salmonis oncorhynchi* fed on Atlantic salmon compared to lice fed on Pacific, Coho or Sockeye salmon. Unpublished data (from NCBI tool (BioProject: PRJNA357111)) also indicates that *venom dipeptidyl peptidase 4*-like transcripts were identified in the Asian tiger mosquito, *Aedes albopictus*, by whole genome shotgun sequencing. *Dipeptidyl peptidase* has also been detected in a range of other mosquito species such as the southern house mosquito, *Culex quinquefasciat* (NCBI: XP_001862510), giant mosquito, *Psorophora albipes* (Chagas *et al.*, 2013), encephalitis mosquito, *Culex tarsalis* (NCBI: JAV23620), and also in the human body louse, *Pediculus humanus corporis* (NCBI: XP_002423599) as well as in the Atlantic horseshoe crab *Limulus polyphemus* (NCBI: XP_013794520).

Serpin

The presence of serpin transcripts in the spinal and proboscis glands, and anterior and posterior midgut, detected using ISH and serpin D1, HCII, protein only in the proboscis glands by IHC, suggests the involvement of serpins in *Argulus* host immuno-modulation. The expression of *serpin* mRNA in the proboscis gland and spinal gland cells were focal and peripheral. Serpins in the saliva and salivary glands of ticks are considered to play a major role in suppressing host immune responses (Tirloni *et al.*, 2014). Most serpins have N-glycosylation sites which are vital for their biological functions (Rau *et al.*, 2007). It was proposed that the transcription of serpins in the salivary gland and midgut of the cattle tick *Rhipicephalus (Boophilus) microplus was* associated with anti-clotting activity (Tirloni *et al.*, 2014). Localisation of serpins in these glands support the proteomic identification of serpin in *Argulus* secretions (**Chapter 4**).

A number of studies conducted on salivary glands and saliva of different hematophagous arthropods has revealed the presence of serpins (serine protease inhibitors). For example "*Iris*" is a serpin with immunomodulatory activity in the tick *Ixodes ricinus* saliva (Prevot *et al.*, 2006). *Iris* is expressed in *Ixodes ricinus* tick's salivary glands when ticks are taking a blood meal, and is described as immunosuppressive and a specific elastase inhibitor. This was the first ectoparasite serpin known to modulate both innate and acquired immunity of the host by using different functional domains to restrict coagulation pathways as well as the fibrinolysis process and inhibition of platelets and to modulate T lymphocyte and macrophage responsiveness by inducing a Th2 type response and by inhibiting the production of pro-inflammatory cytokines (Leboulle *et al.,* 2002; Prevot *et al.,* 2006). Proteomic saliva analysis of the tick, *Haemaphysalis longicornis* nymph and adult female life

stages, indicated a 8.99-fold upregulation of serpin in the adult female life stage, thought to be due to differences in the nature of the feeding process between the two stages (Tirloni *et al.*, 2015). Das *et al.* (2001) reported that *Ixodes scapularis* tick salivary glands contain 14 antigenic salivary proteins. Previous investigations conducted on the tick's saliva and salivary glands were able to identify and characterise the presence of serine protease inhibitors of different forms (Prevot *et al.*, 2006). Serine protease inhibitors control essential biological processes such as blood coagulation and inflammation, fibrinolysis and complement activation which are involved in innate immune responses of the arthropods (Tirloni *et al.,* 2014 and Prevot *et al.,* 2006). Many studies on arthropods have indicated the presence of serpins and, considering the importance they play in host-parasite interaction, may represent useful vaccine targets. For example a study of Mulenga *et al*. (2001) showed five serpins in the salivary glands of the tick, *H. longicornis* and four serpins from the tick *Rhipicephalus appendiculatus*, and suggested that serpins are anti-haemostatic agents facilitating blood meal intake (Mulenga *et al*., 2001). The same research group later reported that the tick *Amblyomma americanum* serine protease inhibitor 6 (*Aam*S6), is a cross-class inhibitor of both serine and papain-like cysteine proteases, and showed that it had anti-haemostatic roles during feeding facilitating parasitism (Mulenga *et al*., 2013). Salivary serpin produced by *I. scapularis* during blood sucking showed an active role on thrombin and platelet aggregation (Ibelli *et al*., 2014). Because of the important roles that serpin plays in regulating biological process such as inflammation and coagulation serine protease inhibitors have been considered as potentially important control targets (Fast *et al*., 2007).

Although serpins in ticks have been detected in many studies and were hypothesised to allow evasion of host immune responses, only a few studies have been conducted to confirm the presence of serpins in tick saliva (Kim *et al.*, 2015). In the current study, the presence of serpin within the secretory glands was confirmed by localisation of the transcript in the spinal and proboscis glands using ISH, as well as the presence of serpin D1 protein in the proboscis glands by IHC.

Screening of sections of *A. foliaceus* using fluorescent lectin-labelling was conducted to localise the glycoconjugates in the glands, assuming a relationship between cells that lectins can recognize with feeding processes. Lectins are considered a useful tool for studying cell activity mechanisms (Lis and Sharon, 1998). For example, GS I; *Griffonia simplificola agglutinin I*, lectin was identified in *Tetracapsuloides bryosalmonae* and recognised antigenic structures on the parasite which are important for immunological studies and future vaccine development (Morris and Adams, 2004). The galactose/N-acetylgalactosamine (GalNAc)-binding lectin is expressed on the surface of trophozoites of the parasite *Entamoeba histolytica* (Schaudinn, 1903), the causative agent of amoebiasis, and was found to have a potent role in adhesion to host cells and infection severity (Frederick and Petri, 2005).

The main objective of lectin-labelling in this study was to identify possible protein carbohydrate interactions in the *Argulus* secretory glands, which were postulated to have major functions in modulation of host immunity. Lectin labelling is a fast recognition method whereby precise sugar localisation in the tissue section can be achieved by binding specific carbohydrates of the lectin enabling *in situ* identification of glycoproteins within sections (reviewed by Parillo *et al.,* 2009). Thus, using lectin labelling in this study served to localize the oligosaccharide sequences of

glycoconjugates and the nature of their linkages in the glands associated with the feeding process. Histochemical characterisation of the spinal and proboscis glands was achieved earlier in the thesis by using a panel of different fluorescently labelled lectins alongside negative controls (**Chapter 2**). Of these lectins, PHA-E; *Phaseolus vulgaris agglutinin*-erythroagglutinin, involved primarily in red cell agglutination, and DSL; *Datura Stramonium Lectin*, showed highly specific binding to spinal gland cells. PHA-E lectin recognises and binds specifically to terminal galactose, N-acetylglucosamine and mannose residues of complex glycans with sugar specificity ß-D-Gal-(1-3)-D-GalNAc. DSL are Chitin-binding lectins, N-acetylglucosamine, and N-acetyllactosamine with specificity to Gal, GalNAc residues. PHA-E and DCL share N-acetylglucosamine group binding preference which suggests that the sugar residues labeled in spinal glands are specifically GalNAc. The distribution of binding of these two lectins to the spinal gland cells are the same *i.e*. strong binding in the cytoplasm of cells nearest of the duct which may indicate the site of production of the bioactive GalNAc glycoprotein within the cells of spinal gland and indicate that this gland can synthesise more than one molecule. Results of lectin-labeling from this study in conjunction with the ISH and IHC findings may facilitate understanding of the role that this complex gland plays in immunomodulator / anaesthetic production during blood acquisition. This is the first lectin labeling study conducted using *Argulus* sections and has shown proteins with binding affinity to glycosaminoglycans, GAGs, in the spinal gland cells that might have a role in host invasion. Heparin or heparan sulfate, dermatan sulfate, are known to be efficient inhibitors of red blood cell agglutination, and are examples of effective GAGs (reviewed by Grubhoffer *et al.* 2005) that might be involved in blood feeding. The specific binding of PHA-E lectin to only spinal gland

cells, prompted the IHC analysis performed in this study using HCII and HSPGs antibodies in an attempt to understand more specifically the functions of the spinal gland cells.

Immunohistochemistry performed on *A. foliaceus* sections was conducted to localise possible sites of HCII, Serpin D1, and HSPGs synthesis and expression. Expression of heparin-cofactor II, Serpin D1 (HCII), a serine protease inhibitor, in the *A. foliaceus* proboscis gland cells was detected by IHC. Both serpin D1, HCII, and antithrombin are known to be primary blood coagulation regulators (Koide, 2008). They inhibit thrombin but need a glycosaminoglycan cofactor to accelerate the inhibition rate (Koide, 2008). Thus, heparan sulphate proteoglycans (HSPGs) were also analysed in this study alongside HCII. Immunohistochemistry showed localisation of HCII in the proboscis gland cells plus the gut diverticuli of *A. foliaceus*. This suggests that *A. foliaceus* secretes HCII during feeding to act as an inhibitor of coagulation proteinases. This work is the first report of serpin expression in glandular cells, in the proboscis gland, related to feeding. HSPGs were also localised in the proboscis gland cells. Based on these data, the use of heparin dermatan sulphate (HDS) may be more appropriate for further characterization of these glands, as HDS might be more related to HCII than HSPGs which are more involved with anti-thrombin.

Sarrazin *et al.* (2011) indicated that HSPGs are found within the secretory vesicles of lymphocytes, mast cells and hematopoietic cells, at which stage their major role is suggested to be retaining the protease in an active state. However, after release, HSPGs are involved in different biological activities such as coagulation and host defense regulation (Sarrazin *et al.*, 2011). HSPGs were observed as small vesicles within the proboscis gland cell by IHC. Stark & James (1998) reported that *AFXa*,

directed-anticoagulant factor Xa, isolated from the yellow fever mosquito, *Aedes aegypti* salivary gland secretions, has a similar protein structure to a serpin superfamily of serine protease inhibitors. The tick anticoagulant peptide; a serine protease inhibitor, is the anticoagulant factor in ticks which prevents coagulation of the host blood. Factor Xa and thrombin inhibitors inhibit platelet aggregation and were reported to be present in tick salivary glands and saliva in addition to prostaglandins suggested to act in vasodilation for the ticks (Bowman *et al.*, 1997). Chagas *et al.* 2010 reported anticoagulant activity in salivary gland homogenates of the female black fly, *Thyrsopelma guianense*. In addition to its role as an anti-thrombin activator, heparan sulphate proteoglycan was also found to be responsible for malarial infections by facilitating transmission of *Plasmodium* spp. parasites through the salivary glands of *Anopheles stephensi* mosquito (Sinnis *et al.*, 2007). In addition to the spinal and proboscis glands, both HCII and HSPGs were expressed in the *A. foliaceus* gut, which may indicate roles in ingestion of blood cells at the feeding site and after feeding processes.

The IHC results produced here corroborate with the study conducted on the mosquito *Aedes togoi* (Ha *et al.*, 2014), which also showed the presence of anticoagulants to prevent blood clotting, within the proboscis glands and the gut. However, HCII is the anticoagulant localised in the *A. foliaceus* proboscis glands and gut in contrast to the heparin anticoagulant that was localised in *A. togoi* salivary gland and midgut (Ha *et al.*, 2014). High expression levels of TTI, tsetse thrombin inhibitor, an anticoagulant peptide, were detected in the salivary glands and midguts of adult tsetse flies, which suggested a role for this anticoagulant in both feeding and processing of the blood meal (Cappello *et al.*, 1998). HCII in *A. foliaceus* may also play a role in processing

the acquired blood after feeding as well as at the site of feeding. Ha and coworkers postulated that the presence of heparin in the gut influences infection prevalence by mosquito-borne parasites (Ha *et al*., 2014) and could play a similar role in the *A. foliaceus* gut, as *Argulus* spp. are thought to act as vectors of spring viraemia of carp virus (SVCV) (Bandilla *et al.* 2006; Cusack & Cone, 1986; Ahne, 1985).

IHC localisation of both HCII and HSPGs in the proboscis glands suggest that the HSPGs may play a role in regulating HCII activity or that of other serine protease inhibitors. Furthermore, the localisation of the 4 different hypothesised immunomodulators by ISH in the spinal and proboscis glands supports the suggestion of their role in evading and regulating the host's immune response in addition to other physiological functions. Localisation of most of the transcripts under investigation in different parts of the parasite gut may also suggest a role in digestion. The *Argulus* gut contains different regions with different types of cells involved in various functions including digestive enzyme synthesis and storage of heavy metals and minerals which are used during nutrient absorption, and digestion (Tam & Avenant-Oldewage, 2009).

This study has provided insights into the roles of the glands associated in direct interactions during *A. foliaceus* feeding, including the nature of some of the proteins produced by these glands and their potential roles in modulating the host immune defence. The results of this study indicate that glands associated with feeding processes *i.e.* spinal and proboscis glands, secrete biochemically active molecules that likely modulate host immune responses to facilitate feeding. This is the first reported evidence of expression of *PGE2S*, *trypsin*, *venom dipeptidyl peptidase*, *serpin* and *heparin cofactor II* with anticoagulant activating factor HSPGs, in the spinal

and proboscis glands of parasitic *Argulus* and hence suggests their potential role in host-parasite interactions.

These components may represent novel candidate targets for future drug/vaccine development which may reduce or replace other chemical controls. The idea that proteinases and inhibitors from ectoparasites can be useful vaccine targets/ antigens is evident from previous published works (Willadsen, 2006). This study suggests that the blood-feeding ectoparasite of fish, *A. foliaceus*, may use similar mechanisms for evading host immune responses to other haematophagous arthropods. Although much research has been conducted on sea lice, *L. salmonis* and *Caligus* spp. the role of the salivary glands has not yet been elucidated (Eichner *et al.*, 2015). This study provides further evidence of the possible roles of the *A. foliaceus* spinal and proboscis glands during feeding by localising and characterising some of the secretory proteins. Thus, the results of this study might be considered a baseline for other ectoparasitic crustaceans by highlighting potent secretory molecules, present in the *Argulus* spinal and proboscis glands, that may function to facilitate host immunosuppression for successful blood acquisition. The results of the PHA-E and DSL fluorescent lectin-labelling suggest that N-acetylglucosamine residues in the spinal gland cells function as recognition sites for bioactive synthesis in the gland.

Further investigations with spinal and proboscis glands, in addition to the labial glands which are suggested to be a third gland type contributing to feeding processes by secreting active molecules into the host skin through the tubular labial spine (for more details see **Chapter 2**), are needed to determine the fundamental mechanisms of the expressed transcripts; *PGE2S*, *trypsin*, *DPP IV* and *serpin* in *A. foliaceus* feeding. In conclusion, by using ISH, IHC and fluorescent lectin-labelling localisation of possible

immunomodulators expressed in the *A. foliaceus* glands associated with the feeding apparatus was achieved, which may facilitate vaccine candidate targeting and development of novel drugs for prevention of argulosis. This work also provides potential insights into important molecules involved in host-parasite interactions of other fish ectoparasites.

Chapter 6. General discussion

The potential effects of freshwater fish lice; *Argulus* spp., and their economic impact on different fish species in aquaculture worldwide (Walker *et al*., 2004), has instigated renewed research interest concerning this diverse arthropod group, of which there are ~145 currently recognised species (Walter and Boxshall, 2017). Relatively few of these studies have directly investigated the nature of the bioactive compounds / proteins, assumed to be released from these ectoparasites, and which are considered to contribute to feeding processes and host-parasite interactions during infection. Although previous studies have focused on the morphology of the *Argulus* glands associated with the pre-oral spine and proboscis, the involvement of these glands in secretions and their composition; are largely unknown (von Reumont *et al.,* 2013). While two transcriptomic studies of *A. siamensis* (Sahoo *et al*., 2013) and *A. foliaceus* (Pinnow *et al*., 2016) have been conducted recently, there is still a lack of transcriptomic and / or proteomic information regarding the expressed transcripts and proteins associated with the glandular systems associated with the pre-oral spine and proboscis (von Reumont *et al*., 2014). Thus, in order to gain a greater understanding of the role of these glands and their secretory products, morphological, transcriptomic and proteomic investigations in conjunction with western blot analysis, and alongside different tissue *in situ* methods including immunohistochemistry, *in situ* hybridisation and lectin labelling analysis were conducted throughout this project. The findings may facilitate further research related to developing new drug control strategies and / or for vaccine development, as well as shedding light on general aspects of so-called "venom" and "toxin" evolution in crustaceans and euarthropods (von Reumont *et a*l., 2013).

In an attempt to ameliorate the increasing problem of infection by *Argulus* spp. in still water trout fisheries in the UK, Taylor *et al.* (2006) studied the epidemiology of *Argulus* infections and found that *A. foliaceus* was detected at all of the sampled sites with the exception of one site in which *A. coregoni* was found. This has economic impacts on the aquaculture and sport fishing industries (Taylor *et al.*, 2006). As *A. foliaceus* appears to be more dominant in UK still waters, this study focused its investigations on *A. foliaceus*. One of the sites that has been affected by *A. foliaceus* infections since 2013 is Loch Fad fishery on the Isle of Bute, Scotland. This fishery is regularly stocked with rainbow trout; *Oncorhynchus mykiss*, and has a population of resident species such as pike, *Esox lucius*, perch, *Perca fluviatilis* and roach; *Rutilus rutilus*. With the generous co-operation of the site owner, the samples used in studies throughout this project were collected from this site. Additionally, *A. coregoni* samples that were collected by Environment Agency staff in Brampton in England, UK from common carp were used in **Chapter 3**.

Extensive investigations of haematophagous arthropod salivary glands and saliva contents have been conducted, including those involving ticks and mosquitoes, which have characterised expressed / secreted proteins involved in the modulation / evasion of host immune and haemostatic responses (Rawal *et al.*, 2016; Lewis *et al.*, 2015; Mudenda *et al.*, 2014; Ibelli *et al.*, 2014; Mathias *et al.*, 2011; Francischetti *et al.*, 2009; Prevot *et al.*, 2009; Prevot *et al.*, 2006; Gillespie *et al.*, 2000; Charlab *et al.*, 1999; Ribeiro 1987; Champagne 1994). Among the crustacean parasites affecting teleost hosts, the parasitic copepod *Lepeophtheirus salmonis* has a huge economic impact on Atlantic salmon culture and therefore various studies have also focused on understanding the interactions of *L. salmonis* and its salmonid hosts (Eichner *et al.*,

2015; Lewis *et al*., 2014; Tadiso *et al*., 2011; Easy & Ross 2009; Wagner *et al*., 2008; Fast *et al*., 2007; Fast *et al*., 2005; Fast *et al*., 2004; Kvamme *et al*., 2004; Fast *et al.,* 2003; Johnson *et al*., 2002; Firth *et al*., 2000; Ross *et al*., 2000). Applying similar approaches to elucidating the host-parasite interactions of *Argulu*s spp. was considered to offer the prospect of enhancing management strategies for argulosis of different species around the world and hence contribute to the maintenance of freshwater fish culture in supplying global protein.

In **Chapter 1,** by reviewing the available published studies on *Argulus* spp., gaps in the literature requiring investigation were made evident, such as understanding the role of the *Argulus* pre-oral spine and proboscis and their related glands during feeding, informing knowledge of the relationship between these parasites and their hosts. Although earlier studies have described these glands in attempts to correlate their roles to the feeding appendages (Saha *et al*., 2011; Gresty *et al*., 1993; Swanepoel & Avenant-Oldewage 1992) and trials have been conducted to understand the roles of the secretory products of these parasites by investigating parasite homogenates effects on the host (Saha *et a*l., 2011; Shimura & Inoue 1984; Shimura 1983), the results have been either contradictory, have missed key information or have led to debate (Walker *et al*., 2011).

In **Chapter 2**, in order to provide data on the glands of *A. foliaceus* associated with feeding appendages, the pre-oral spine and proboscis, which have been previously postulated to have a direct secretory role in host-parasite interactions, were subjected to a range of morphological investigations using light and fluorescence microscopy and transmission and scanning electron microscopy. These techniques enabled a description of the morphology and distribution of these glands and their relationships

to the principal appendages employed during feeding. Although, the anatomical morphology of *Argulus* spp. has been studied for more than 100 years the location, type and number of glands associated with feeding processes are still not fully described or known. Thus, **Chapter 2** described three different types of *A. foliaceus* glands that were observed and defined as the spinal gland, the proboscis glands and the labial glands. 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 at the base of the proboscis and the pre-oral spine, comprised of four giant secretory cells. This observation was in line with the findings of Martin (1932), Wilson (1902), Gresty *et al.* (1993) and Saha *et al.* (2011). The suggested secretory function of spinal gland cells was supported by the presence of numerous secretory vesicles, intermediate vesicles, rough endoplasmic reticulum, Golgi bodies, and lysosomes in their cytoplasm; which are a probable indication of extensive protein synthesis. Following the ducts of two different glands showed that these glands are associated with the proboscis. These glands were defined to be the proboscis glands and the labial glands. *Argulus foliaceus* appears to possess two proboscis glands of three giant cells each that are located ventral to the optic tracts. This is the same observation reported previously by Gresty *et al.* 1993 in *A. japonicus*, Swanepoel and Avenant-Oldewage (1992) in *A. coregoni* and Saha *et al.* (2011) in *A. siamensis*. However, Saha *et al.* (2011) observed only two giant cells belonging to each gland. The other glands were believed to have a secretory role and were related to the proboscis, termed the “labial glands”, as the ducts observed originated from a site comprising of five gland cells located posterolaterally to the tip of tubular labial spines. Swanepoel and Avenant-Oldewage (1992) mentioned the presence of these ducts of

the labial glands briefly. It was suggested that tubular labile spines have a secretory role by secreting enzymes (Madsen 1964; Shimura 1983) into the mouth and immediately to the host surface during feeding, which might help in pre-digestion and intake of host tissue. The number of cells of these glands might indicate that labial glands have a greater role than the spinal gland (Madsen, 1964). This chapter highlighted three different glands involved with such secretions during feeding, related to the pre-oral spine and proboscis in *A. foliaceus*. The suggestion here is that the pre-oral spine has a secretory function, which may aid in immunomodulation, while the tubular labial spines within the proboscis may be structures that enhance the feeding process by secreting saliva components that aid pre-digestion. The establishment of this information led to further histological analyses, such as lectin-labelling (**Chapters 2 and 5**), IHC and *in situ* hybridisation (**Chapter 5**), to further characterise the secretory products of these glands, and determine the expected roles during feeding.

Nineteen fluorescent lectins were screened on *A. foliaceus* sections to identify cells of glands and other tissues binding with specific carbohydrate moieties in **Chapter 2**. This is the first study that applied this type of investigation on *Argulus* spp. to date. Identifying the types of glycoproteins bound by specific lectins in the gland cells provided baseline information on the nature of the secretory molecules from these glands. Although the results proved useful in determining the distribution of carbohydrates throughout the whole parasite, the objective was to identify the binding moieties to the feeding associated glands cells. This study provides evidence that all three targeted glands; spinal, proboscis and labial gland, contained carbohydrate residues of galactose. Previous studies have suggested that D-galactose plays an important role in host-parasite interactions (Hammerschmidt & Kurtz 2005; Burton *et*

al., 1999; Knowles *et al.,* 1991). The high affinity of spinal gland cells to different types of sugars like D-mannose, D-glucose, galactose, N-acetylgalactosamine and N-acetylglucosamine indicated high levels of glycosylation and a diversity of carbohydrate residues. However, one of the most interesting finding obtained from this study was that the spinal gland cells were labelled particularly intensively to the PHA-E lectin (**Chapter 2 and 5**), which has affinity to N-acetylglucosamine oligomers. Salivary gland cells from different blood feeding arthropods have previously been reported to contain these sugars and were hypothesised to be involved in mediation of parasite adhesion or invasion of the host cells / tissue (as reviewed by Basseri *et al.* 2002). The main content of the proboscis gland cells were galactose and mannose residues. The interaction between soluble / transmembrane lectins and these sugar residues from fish parasites such as the protozoan *Ichthyophthirius multifiliis* (Xu *et al*., 2001), microsporidian *Glugea plecoglossi* (Kim *et al*., 1999) and monogenean ectoparasite *Gyrodactylus derjavivni* (Buchmann, 2001) suggested they have a vital involvement in host-parasite interaction and invasion as well as host immune evasion (Alvarez-Pellitero, 2008). Redondo *et al.* (2008) suggested that carbohydrates with N-acetyl-galactosamine; (GalNAc)/galactose residues also play a key role in parasite-host interactions by evading host immune response (Basseri *et al*., 2002). Mannose residues were suggested by Buchmann (1998) to activate the complement pathway of rainbow trout *Oncorhynchus mykiss* plasma *in vitro* during infection with the ectoparasitic fluke *Gyrodactylus derjavini*. Modulation of the fish host response by suppressing the alternative complement pathway has been demonstrated in *A. siamensis* infection of rohu *Labeo rohita* activity (Saurabh *et al*., 2010). Applying the

findings of these previous studies to the investigated glands in this study suggests that these molecules may be secreted and function in a similar immunomodulatory way.

For a detailed understanding of *Argulus* spp. biology and the main genes that might influence the processes during feeding, a transcriptomic analysis was applied on whole freshwater lice (**Chapter 3**), especially as previously limited genomic resources have limited investigation of gene expression in this parasite. In this respect RNA-seq has been used to study transcriptomic expression in a range of different crustacean species lacking fully sequenced genomes (Mykles *et al.*, 2016). The findings obtained from **Chapter 3** have considerably enhanced genomic knowledge for this genus and have provided a useful addition to crustacean genomic resources by establishing new transcriptomic resources for *A. foliaceus* and *A.* coregoni; the two native *Argulus* species in the UK, in addition to contributing to an improved understanding of the biology of these parasites. Cantacessi *et al.* (2012) highlighted the importance of transcript expression assessment, examination of regulation patterns, and the functional classes involved, as important tools for understanding parasite biology. The new sequences established in this study were combined with the two previous transcriptome libraries established for *A. siamensis* (Sahoo *et al.*, 2013) and *A. foliaceus* (Pinnow *et al.*, 2016) to provide the first list of potential host immunomodulators for three *Argulus* species; *A. foliaceus*, *A. coregoni* and *A. siamensis*. These immunomodulatory candidates provide rational targets for future investigations into vaccine development as an alternative control method for Argulosis. This study also highlights the potential of *Argulus* spp. for use as an ectoparasite model for host-parasite interaction.

Transcriptomic sequencing was used in this study to identify transcripts of proteins that have previously been suggested, from previous studies on haematophagous arthropods to play a role in host-parasite interactions (Tirloni *et al.*, 2016, 2015; Valdivieso *et al.*, 2015; Liu *et al.*, 2014; Mulenga *et al.*, 2013; Subramanian *et al.*, 2008; Kvamme *et al.*, 2004; Andreotti *et al.*, 2002; Firth *et al.*, 2000). In comparison to the two genomic datasets for *Argulus* species in the NCBI database, transcriptome results for the current study deliver the largest current genomic data set for *Argulus* with 87,954,885 reads assembled into 66,940 contigs for *A. foliaceus*, and also provides the first sequences for *A. coregoni* with 73,164,334, which assembled to give 40,954 contigs. The assembled transcriptome results are important for conducting both genomic and proteomic research (**Chapter 4**). An overview of the functional roles of the successfully sequenced transcripts and their respective proteins for the three *Argulus* species was performed using GO analysis. A phylogenetic tree was also constructed, using the top 100 shared genes for the four-transcriptome datasets available for *Argulus* and *A. coregoni* was subsequently reported as being more closely related to *A. foliaceus* than to *A. siamensis*. The use of OrthoVenn software with the *Argulus* spp. transcriptomes datasets, resulted in 6,674 shared gene clusters from the transcripts sequenced for these three species indicating continued conservation after speciation. The transcriptomic study in this chapter has contributed to identifying, for the first time, a range of proteins / genes in *Argulus*, and more widely for the *Branchiura*, that have been previously characterised as being important immune mediators for blood-feeding arthropods. These included trypsin, serpin, serine protease, cathepsin-L, aspartic protease, ferritin, cysteine protease, enolase, phospholipase, adenosine, apyrase, metalloprotease, thrombin inhibitor, and venom

serine protease. Although, the role of these genes in *Argulus* are not yet known, the functions of these proteins in host-parasite interactions and their association in modulating host responses were compared to those established for other well studied blood-sucking parasites in **Chapter 3**. Key transcripts identified in this chapter were employed for whole louse gene expression analysis (**Chapter 4**), and localised tissue investigations by IHC and *in situ* hybridisation (**Chapter 5**).

The expression levels of some of these transcripts were investigated by qPCR (**Chapter 4**), including eight targeted transcripts and two housekeeping genes, β-actin and elongation factor-1α, with a particular emphasis on expression profiling during *A. foliaceus* feeding (by comparing to starved conditions) in relation to sex. The selected genes were trypsin like serine proteinase, prostaglandin E 2 synthase, serpin, aspartic protease, cysteine protease, ferritin, thrombin inhibitor and venom serine protease. This investigation revealed that with the exception of ferritin, the remaining transcripts appeared to be sex-associated, although the physiological explanation for these observations needs further investigation. However, these findings indicated likely differential functional roles of these transcripts in the physiology of male and female Argulids. This study has made a key contribution to argulid genomics and, more extensively sheds further light on the biology of crustacean fish parasites.

Chapter 4 comprised a proteomic investigation, which was informed by the genomic data generated in **Chapter 3**. In **Chapter 4**, secretions of *A. foliaceus* were explored to identify molecules / proteins produced from the glands associated with the feeding appendages, as described in **Chapter 2**. Of particular interest were biomolecules expected to play vital roles with regards to the impaired host defence system. This was the first study conducted to determine the protein composition of *Argulus* spp.

secretions. Functional proteins from the saliva and salivary glands of different arthropods have been discovered following proteomic investigations in the past such as in ticks (Schwarz *et al*., 2014; Tirloni *et al*., 2014; Mudenda *et al*., 2014), nematode (Hewitson *et al*., 2011), mosquitos (Rawal *et al.* 2016) and wasps (Teng *et al*., 2017). The bioactive products secreted in the saliva of these parasites are suggested to have anti-haemostatic, anti-inflammatory and immunomodulatory properties to assist with counteracting the host immune response and hence facilitating feeding (Anstead *et al*., 2015; Carvalho-costa *et al*., 2015; Tirloni *et al*., 2014; Díaz-Martín *et al*., 2013; Oliveira *et al*., 2011; Chmelar *et al*., 2011; Maritz-olivier *et al*., 2007; Xu *et al*., 2007 Steen *et al*., 2006; Horn *et al*., 2000). Earlier research by Saurabh *et al.* (2012) and Ruane *et al.* (1995) reported antigenic components from *Argulus* spp. crude homogenates using an immunological approach. In this study, a pooled secretion sample was collected from more than 700 *A. foliaceus* parasites. SDS-PAGE identified numerous polypeptide bands of the *A. foliaceus* secretory products between with a molecular mass of 3 -100 kDa. Western blot analysis, using serpin D1 and HS6ST3 antibodies on these proteins, revealed the presence of serpin within *A. foliaceus* secretions. An intense band was detected with a MW of 15 kDa using the biotinylated-PHA-E, biotinylated-Jacalin lectins and HS6ST3 antibody. A 25 kDa molecular weight band was dominant using the biotinylated-PHA-E, serpin D1 and heparan sulphate antibody (HS6ST3), and this was the only band detected in immunoblots screened with serpin D1.

The secretion products of *A. foliaceus* was subsequently subjected to gel and liquid chromatography electrospray ionisation tandem mass spectrometric (GeLC-ESI-MS/MS) analysis (**Chapter 4**). The combined data of GeLC-MS/MS with the

transcriptomic dataset (**Chapter 3**) identified 26 **predicted** protein sequences from the *A. foliaceus* secretory products. Half of these proteins / domains were functionally recognised by Pfam with no matched sequences for the rest of the proteins, which suggests that either *Argulus* has unique proteins to help in modulating their host, or that these proteins contained partial sequences. Nevertheless, these proteins were evidently present in the *A. foliaceus* secretions and might have immunogenic properties during host infection. Signal peptides predicted using Signal P 4.1 software, identified 14 proteins with a signal sequence, although it is possible that other identified proteins may be secreted but were not predicted as they contain only partial protein sequences. Interestingly, *A. foliaceus* proteins / domains that revealed functional identity were described earlier from the salivary glands and saliva of other blood feeding arthropods such as ticks (Tirloni *et al.*, 2015; Tirloni *et al.*, 2014; Maritz-olivier *et al.*, 2007). The findings of this chapter provided baseline knowledge concerning some of the main proteins secreted by *Argulus* for the first time, adding to biological understanding of this parasite genus. The identified proteins have been classified as transporters, peroxidases, metalloproteases, proteases, serine protease inhibitors and secreted domains. Vitellogenin and three hemocyanin protein domains are described within the transporter proteins. The role of vitellogenin as a secretory product of *A. foliaceus* is not yet known, but considering the suggested involvement and multifunction of this lipoprotein, such as in reproduction (Galay *et al.*, 2013), osmoregulatory processes (Kristoffersen *et al.*, 2009), innate immunity (Liu *et al.*, 2009) and clotting (Hall *et al.*, 1999), the protein may play a key role as it was the most abundant protein identified. One suggestion made in **Chapter 4** was to repeat the proteomic analysis for the male *A. foliaceus* secretions sample. This would at least

provide an indication of whether this protein has a role in reproduction. By successful localisation of this protein by *in situ* hybridisation, the production site may be determined and hence a prediction of likely function achieved.

According to Pinnow *et al.* (2016) haemocyanin in *A. foliaceus* is involved in respiration and protein storage, relating to its classification as a transporter protein. An animal-haem peroxidase enzyme was also secreted from *A. foliaceus*. Previous studies have demonstrated the importance of this protein for blood feeding arthropods due to its vasodilation activity of this enzyme (Ribeiro & Nussenzveig 1993; Champagne 1994) involvement in host infection by modulating host defence systems during feeding and after blood acquisition (Øvergård *et al.*, 2016; Steen *et al.*, 2006), and its involvement in PGE2 synthesis (Tootle and Spradling, 2008). Furthermore, it is suggested to inactivate host vasoconstrictor substances during haemostatic processes (Ribeiro and Nussenzveig, 1993).

The two identified metalloproteases secreted from *A. foliaceus* were astacin and peptidase M14. Astacins have been suggested to play a role in maintaining host-parasite relationships (Baska *et al.* 2013; Park *et al.* 2010; Kim & Kim 2008; Gallego *et al.* 2005) as they are assumed to have a digestive and anticoagulation role and inactivate prey/host vasoactive peptides (Modica *et al.*, 2015) (Lun *et al.*, 2003). Two proteases were identified from the *A. foliaceus* proteome: trypsin and pro-isomerase. Transcripts of putative secreted trypsins were detected in the *A. foliaceus* proteome; verified by signal peptide. This suggested the importance of trypsin during *Argulus* spp. host-parasite interactions, as has been suggested earlier for *L. salmonis*, to facilitate feeding and evasion of the host immune responses (Firth *et al.*, 2000). Pro-isomerase detected in the *A. foliaceus* proteome might serve the same or similar

suggested function, as revealed from previous studies where it interacts with host cell proteins in the pathogenic protozoan *Toxoplasma gondii* (High *et al.*, 1994) and functions as chaperonin mediators of associated proteins as in the hard tick *Haemaphysalis longicornis* (Boldbaatar *et al.*, 2008). Serpin was also identified by mass spectrometric analysis and predicted as a signal peptide from *A. foliaceus* secretions. Due to the importance of serpins shown in haematophagous arthropods saliva / salivary glands, it was expected in this study that secreted serpins from *Argulus* play the same roles in counteracting host defence mechanisms for successful acquisition of a blood meal (Tirloni *et al.*, 2015). Serpin has been suggested to contribute to feeding by functioning as an immunomodulator, (Tirloni *et al.*, 2014), as an anti-coagulant, a complement inactivator (Mulenga *et al.*, 2013), and as pro-inflammatory and pro-coagulant proteases inhibitors (Tirloni *et al.*, 2016). Together, serpin and trypsin were suggested to be two of the main secretory proteins of *A. foliaceus* mediating host immune response. This suggestion was supported by localisation of these transcripts in the spinal and proboscis glands by ISH and serpin was also detected by IHC and western blot (**Chapter 5**).

Three proteins domains were detected from the *Argulus* sp. secretions; CUB, fasciclin and VIT/vWA. As they are real domains within the proteome of *A. foliaceus* secretions, it was assumed they were associated with other secretory proteins to facilitate feeding of *A. foliaceus*. vWA was detected recently in the salivary subset of the vampire snail *C. reticulata* and described to have anti-hemostatic activity (Modica *et al.*, 2015). Thus, vWA from *A. foliaceus* secretions was assumed to have the same function; inhibiting thrombocyte aggregation by binding the host thrombocytes to prevent their interaction with collagen (Modica *et al.*, 2015). This study (**Chapter 3**) has elucidated for the first-

time real target proteins / molecules from the *A. foliaceus* secretions that might contribute in developing management strategies for *Argulus* spp.

The findings from **Chapters 2, 3 and 4** directed the work in **Chapter 5** to investigate and characterise the type of secretory and expressed components released from the spinal and proboscis glands. Thus, **Chapter 5** applied both transcriptomic (**Chapter 3**) and proteomic data (**Chapter 4**) in conjunction with *in situ* tissue methods; ISH, IHC and fluorescence lectin labelling, to localise selected secretory proteins/genes that have been postulated to have probable immunomodulatory roles in parasite host immune evasion. *PGE2S*, *trypsin*, *DPP IV* and *serpin* were reported in this chapter to be transcribed in both of the targeted gland cells, spinal and proboscis. This finding confirmed that *A. foliaceus* feeding associated glands (**Chapter 2**) expressed proteins that have been identified from other haematophagous arthropods to serve a function in host-parasite interactions. *PGE2S* contributes to various biological activities such as vasodilation, cellular proliferation, leucocyte activation and neutrophil chemotaxis and accumulation at inflammatory sites (Fast *et al*., 2005). It was observed in this study (**Chapter 5**) that this gene is also transcribed in secretory cells between the cerebral ganglion, gut and the outer layer of male testis, which suggested the involvement of this gene in different biological functions in addition to anti-coagulation by producing PGE2. PGE2 has been reported to exhibit various biological functions related to inflammation, immune response and reproduction (Guo *et al.,* 2015; Eichner *et al.,* 2015). Although trypsin was found to be widely expressed in different tissues of *A. foliaceus*, the high expression of this gene in the glands of interest was an indication of the importance of this protein for *Argulus* during feeding. Previously trypsin has been reported in sea lice to have an involvement in host immune response modulation

to facilitate feeding and contribute to host infection (Johnson *et al.* 2002; Wagner *et al.,* 2008).

The third expressed gene in the glands was *venom di-peptidase DDPV IV* which was assumed, in accordance with previous research on arthropods, to regulate immune responses of the fish hosts (Teng *et al.*, 2017) and may play the same role as proposed for the tick *Ixodes scapularis DDPV IV* by mediating pain and causing oedema through increasing capillary permeability (Ribeiro and Mather, 1998).

The detection of serpins in the *A. foliaceus* spinal and proboscis glands by ISH and the specific detection of serpin D1 in the proboscis glands by IHC support the hypothesised role of serpins in modulating host immune response. This localisation evidence also supported the presence of serpin in the secretion (**Chapter 4).** Serpins from different haematophagous arthropods exhibit anti-haemostatic activity (Mulenga *et al.*, 2013), restrict coagulation pathways as well as the fibrinolysis process and inhibit the activity of platelets (Ibelli *et al.,* 2014; Prevot *et al.,* 2006; Leboulle *et al.,* 2002). Due to the important role of serpins in regulating biological process such as inflammation and coagulation, they have been considered as potentially important control targets (Fast *et al.,* 2007).

Using IHC in **Chapter 5** to localise possible synthesis / expression sites of Serpin D1, and heparan sulphate proteoglycans (HSPGs), it was found that both were localised in proboscis glands as well as in the *A. foliaceus* gut. This suggests their roles in ingestion of blood cells at the feeding site and after feeding processes. PHA-E and DSL lectins showed highly specific binding to spinal gland cells. These two lectins share N-acetylglucosamine group binding preference which suggests that the proteins

with binding affinity to glycosaminoglycans; GAGs, in the spinal gland cells might have a role during host infection.

Towards vaccine approach

Many researchers working with blood feeding parasites are interested in identifying novel vaccine candidates, often targeting secretory products from the parasites. Proteins expressed on cell surfaces also provide promising results as vaccine candidates. The use of advanced molecular biology techniques such proteomics and genomics can provide considerable assistance in identifying these targets (Dalton *et al.,* 2003). Vaccines are generally extremely safe and provide an environmentally friendly and sustainable solution to pathogen challenge. They are useful in prophylaxis while many drugs provide responsive treatment only and can also be susceptible to development of drug resistance in the targeted pathogen.

The results of this study opened up avenues to look into the identified proteins as possible vaccine candidates development against *Argulus* infection and other fish ectoparasites.

Future directions

This work lacks functional studies performed *in vitro* or *in vivo*. Thus, it will be important to apply a range of functional studies such as those already employed to study sea lice:

- Use protease gel zymography to observe and analyse changes in fish mucus after incubation of *Argulus foliaceus* created by the presence of low molecular weight (LMW) protease*s*.
- Production of recombinant protein vaccine candidates using some of the targets identified by GeLC-MS/MS.
- Studies on host response using proliferation assay to measure responses of the host leucocytes cells to the secretions of *Argulus* spp. to assess if there is any proliferation response. Immunoblot with anti-sera collected from a host injected with the *Argulus* spp. can be also conducted in the future to give an indication of the immunogenic antigens size from the parasite secretion.
- Gland sequencing with laser capture microscopy (LCM) will help to specify the secreted transcripts from these glands and hence target them in the future for vaccine development.
- Use the targets produced from the secretory products and investigate by ISH and IHC to check the expression and the production sites of these proteins. IHC can be also be applied for the host response study.

Summary

Overall, the findings of this project have generated considerable additional knowledge concerning the biology of *Argulus* spp. and provide a list of potential proteins that may be used by the parasite to facilitate feeding processes by secreting these active molecules into the host and hence modulating their immune defence mechanisms. This information can be used as a baseline for developing freshwater lice control strategies to help prevent the spread of Argulosis in aquaculture by applying vaccination as means of control where the antigenic components described in this

study specifically target *Argulus* spp. Furthermore, data from this thesis enhances the knowledge of the distribution of toxin/venom or venom-like substances in crustaceans and arthropods in general.

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Appendix 1

Shandon Citadel 2000 tissue processor (Thermo Fisher Scientific Inc., UK)

Container position	Reagent	Programmed Time
1	50% Methylated Spirit	30 min
2	80% Methylated spirit	1.30 h
3	100% Methylated spirit	1.30 h
4	100% Methylated spirit	1.30 h
5	100% Methylated spirit	1.30 h
6	Absolute Ethanol	1.45 h
7	Absolute Ethanol	1.30 h
8	Chloroform	50 min
9	Chloroform	50 min
10	Paraffin wax	1.45 h
11	Paraffin wax	1.30 h
12	Paraffin wax	1.30 h

Appendix 2

Haematoxylin and eosin staining of paraffin sections

<u>Mayer's Haematoxylin (or buy ready prepared Haematoxylin 'Z' from Cellpath)</u>

Haematoxylin 1g

Sodium iodate 0.2g

Potassium alum 50g

Citric acid 1g

Chloral hydrate 50g

Distilled water 1000ml

Allow the haematoxylin, alum and sodium iodate to dissolve overnight in the distilled water (Use a Pyrex flask). Add chloral hydrate and citric acid. Stopper flask with cotton wool. Heat to boiling in a fume cupboard. Boil for 5mins. Cool to room temp. before use.

<u>1% Eosin (or buy ready prepared from Cellpath)</u>

Eosin yellowish 10g

Distilled water 1000ml

Dissolve the eosin in the water. Leave overnight before use.

<u>Putt's Eosin (or buy ready prepared from Cellpath)</u>

Eosin 4g

Potassium dichromate 2g

Sat. Aqueous picric acid 40ml

Absolute alcohol 40ml

Distilled water 320ml

Dissolve the eosin in the distilled water, add the potassium dichromate. Add the absolute alcohol. Then carefully add the picric acid.

<u>1% Acid alcohol</u>

Methylated spirit 1000ml

Hydrochloric acid 10ml

Measure the methylated spirits into a winchester/bottle. Carefully add the hydrochloric acid.

Scott's tap water substitute (or buy ready prepared from Cellpath)

Sodium bicarbonate 3.5g

Magnesium sulphate 20g

Distilled water

Dissolve by heating if necessary, add several crystals of Thymol to preserve .

H & E Staining proceudres:

1. Dewax in two changes of Xylene for 3 min each follwoed by Absolute Alcohol for 2 min and 11/2 min in Methylated spirit.
2. Wash in tap water
3. Stain in Haematoxylin 'Z' for 5 min.
4. Wash in tap water
5. Three quick dip in 1% Acid alcohol
6. Wash in tap water
7. Blueing in Scott's tap water substitute for 1 min
8. Wash in water
9. Stain in Eosin for 5 min
10. Quick wash in tap water
11. Re- dehydrate sections in Methylated spirit for 30 sec follwoed by two changes of Absolute alcohol II for ~2 min
12. Clearing in Xylene for 5 min. Slides should remain in the Xylene (coverslip) as they will dehydrate if removed.
13. Coverslip using Pertex mounting medium

Slides should be left to dry in a fume hood for at least 10-15 mins prior to microscopy.

Appendix 3

Combined Alcian Blue-PAS technique for acid and neutral mucins (Mowry, 1956):

1. Stain in Alcean Blue solution for 10 min.
2. Wash in running tap water, then in distilled water.
3. Stain in 1%(Aq) periodic acid for 5 min.
4. Wash in two changes of distilled water.
5. Stan in Schiff's reagent for 15 min.
6. Wash in running tap water.
7. Stain in Mater's haematoxylin for 5 min.
8. Wash in running tap water.
9. Dehydrate the sections in two changes of absolute alcohol, 10 dips each.
10. Rinse in xylene, 10 dips.
11. Mount with Pertex.

Appendix 4

Periodic acid-Schiff with tartrazine counterstain and haematoxylin

1. Dewax:
- First xylene 3 min
- Second xylene 2 min
2. Dehyrate:
- Absolute Ethanol 2 min
- Methylated spirit 1 min
3. Wash in water 1 min
4. Stain in 1 % Periodic acid 10 min
5. Wash in running tap water 1 min
6. Stain in Schiffs 30 min
7. Wash in running tap water 2 min
8. Stain in Heamatoxylin 5 min
9. Wash in running tap water 1 min
10. 1 % Acid alcohol 3 quick dips
11. Wash in running tap water 1 min
12. Stain in Tertrazine 20 min
13. Absolute ethanol (2x changes) 10 quick dips
14. 70 % ethanol 10 quick dips
15. Clearing in xylene (2x changes) 5 min
16. Put in xylene coverslip until coverslipped in pertex

Appendix 5

Top 100 shared genes from10 species, used in reconstruction of *Argulus* spp phylogenetic tree (Chapter 3) relative to the position to other members of the phylum Oligostraca (Regier *et al*. 2010).

No.	Transcripts sequence identity	No.	Transcripts sequence identity
1	TRINITY_DN52054_c0_g1_i1 [239-2890]	51	TRINITY_DN45721_c0_g1_i2 [1387-4287]
2	TRINITY_DN71251_c0_g2_i1 [3-269]	52	TRINITY_DN44705_c0_g3_i1 [770-4840]
3	TRINITY_DN47265_c1_g1_i1 [95-5941]	53	TRINITY_DN55256_c0_g1_i18 [73-2349]
4	TRINITY_DN51016_c1_g1_i1 [1075-3999]	54	TRINITY_DN52393_c0_g1_i2 [446-1789]
5	TRINITY_DN50550_c0_g3_i1 [2-745]	55	TRINITY_DN54409_c0_g1_i1 [254-1558]
6	TRINITY_DN46290_c0_g1_i1 [787-1443]	56	TRINITY_DN53531_c2_g1_i2 [401-2497]
7	TRINITY_DN51783_c0_g1_i14 [677-3967]	57	TRINITY_DN51105_c0_g1_i1 [481-3834]
8	TRINITY_DN55875_c4_g1_i1 [158-1444]	58	TRINITY_DN5979_c0_g1_i1 [128-1237]
9	TRINITY_DN54182_c0_g1_i1 [32-1105]	59	TRINITY_DN53773_c0_g1_i1 [81-2321]
10	TRINITY_DN52369_c0_g2_i1 [1-1266]	60	TRINITY_DN46463_c0_g1_i1 [221-1549]
11	TRINITY_DN54779_c1_g1_i1 [386-2224]	61	TRINITY_DN36832_c0_g1_i1 [3-2027]
12	TRINITY_DN52480_c0_g1_i4 [121-3282]	62	TRINITY_DN50047_c0_g1_i1 [1888-5364]
13	TRINITY_DN42468_c0_g1_i2 [21-1904]	63	TRINITY_DN52489_c0_g2_i1 [208-2439]
14	TRINITY_DN55874_c4_g1_i1 [2794-7461]	64	TRINITY_DN55950_c0_g1_i1 [252-1424]
15	TRINITY_DN51925_c0_g1_i1 [565-1965]	65	TRINITY_DN71626_c0_g1_i1 [166-1500]
16	TRINITY_DN49293_c0_g1_i8 [77-1909]	66	TRINITY_DN55590_c1_g1_i1 [1022-4360]
17	TRINITY_DN50791_c0_g1_i1 [142-1863]	67	TRINITY_DN39465_c0_g1_i1 [120-692]
18	TRINITY_DN1967_c0_g1_i1 [682-2022]	68	TRINITY_DN3223_c0_g1_i1 [95-1594]
19	TRINITY_DN56682_c0_g1_i1 [63-590]	69	TRINITY_DN49142_c0_g1_i1 [93-1019]
20	TRINITY_DN50671_c0_g2_i1 [120-1292]	70	TRINITY_DN48494_c0_g1_i1 [163-2586]
21	TRINITY_DN47110_c0_g1_i1 [161-1132]	71	TRINITY_DN53028_c0_g2_i14 [367-3360]
22	TRINITY_DN64912_c0_g1_i1 [3-287]	72	TRINITY_DN49520_c1_g2_i7 [402-2243]
23	TRINITY_DN45512_c1_g1_i4 [187-1053]	73	TRINITY_DN56137_c0_g2_i1 [664-6498]
24	TRINITY_DN56367_c2_g1_i3 [495-13769]	74	TRINITY_DN46179_c0_g1_i1 [3-314]
25	TRINITY_DN49940_c1_g1_i2 [183-2141]	75	TRINITY_DN48847_c0_g2_i1 [178-6699]
26	TRINITY_DN40547_c0_g1_i1 [169-2400]	76	TRINITY_DN49938_c0_g1_i1 [367-4320]
27	TRINITY_DN46901_c0_g1_i2 [139-1563]	77	TRINITY_DN50983_c0_g1_i1 [91-2001]
28	TRINITY_DN53404_c1_g1_i1 [273-1082]	78	TRINITY_DN52374_c0_g1_i13 [1910-4003]
29	TRINITY_DN47740_c0_g1_i5 [3-257]	79	TRINITY_DN53726_c1_g2_i1 [5132-6463]
30	TRINITY_DN54176_c0_g1_i2 [134-1600]	80	TRINITY_DN53847_c0_g1_i4 [148-2568]
31	TRINITY_DN30612_c0_g1_i2 [813-2453]	81	TRINITY_DN32016_c0_g2_i1 [1-1185]
32	TRINITY_DN50358_c1_g2_i1 [589-1701]	82	TRINITY_DN41358_c0_g1_i1 [290-1738]

33	TRINITY_DN37386_c0_g1_i1 [1396-3567]	83	TRINITY_DN50071_c0_g1_i2 [22-2310]
34	TRINITY_DN50459_c0_g3_i1 [31-1737]	84	TRINITY_DN48500_c0_g1_i1 [55-2307]
35	TRINITY_DN46401_c0_g1_i1 [951-2402]	85	TRINITY_DN54604_c0_g1_i1 [523-2004]
36	TRINITY_DN54578_c0_g1_i1 [1054-3798]	86	TRINITY_DN48880_c0_g1_i1 [117-1343]
37	TRINITY_DN53135_c1_g1_i1 [1189-3915]	87	TRINITY_DN55506_c3_g1_i1 [168-3701]
38	TRINITY_DN50994_c0_g1_i2 [547-2649]	88	TRINITY_DN50464_c0_g1_i2 [154-2460]
39	TRINITY_DN67066_c0_g1_i1 [213-1844]	89	TRINITY_DN55386_c0_g1_i1 [328-2028]
40	TRINITY_DN56307_c0_g1_i1 [42-3464]	90	TRINITY_DN49998_c1_g1_i1 [687-3188]
41	TRINITY_DN36062_c1_g1_i1 [57-515]	91	TRINITY_DN51117_c1_g1_i3 [3-2153]
42	TRINITY_DN58429_c0_g1_i1 [1-165]	92	TRINITY_DN47007_c0_g1_i3 [463-1536]
43	TRINITY_DN54398_c3_g1_i1 [2215-4107]	93	TRINITY_DN48272_c1_g1_i1 [2542-10239]
44	TRINITY_DN48553_c0_g1_i1 [327-3119]	94	TRINITY_DN52495_c1_g1_i1 [273-2717]
45	TRINITY_DN56339_c0_g1_i1 [256-2319]	95	TRINITY_DN48555_c0_g1_i3 [1848-4169]
46	TRINITY_DN56338_c0_g1_i1 [168-4403]	96	TRINITY_DN50881_c0_g3_i1 [1234-3594]
47	TRINITY_DN55321_c0_g1_i1 [192-2279]	97	TRINITY_DN50775_c0_g2_i1 [166-1488]
48	TRINITY_DN54273_c1_g1_i7 [92-3226]	98	TRINITY_DN44099_c0_g2_i1 [3-2387]
49	TRINITY_DN52152_c2_g4_i5 [2-2428]	99	TRINITY_DN51392_c0_g1_i1 [2-2059]
50	TRINITY_DN56211_c0_g1_i1 [341-1792]	100	TRINITY_DN52773_c0_g1_i2 [134-2437]

Appendix 6

The two-way ANOVA revealed that no significant interactions were evident between the factors “treatment” and “sex” with “sex” differences being significant for five genes out of eight and fed state having no significant effect.

Tests of Between-Subjects Effects

Source	Dependent Variable	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	trypsin	5.647[a]	3	1.882	2.115	.139
	pge2s	.177[b]	3	.059	2.686	.081
	serpin	.825[c]	3	.275	2.860	.070
	aspprot	.307[d]	3	.102	.955	.438
	cysteine	.336[e]	3	.112	3.078	.058
	thrombin	520.365[f]	3	173.455	1.019	.410
	venom	5847.082[g]	3	1949.027	8.199	.002
Intercept	trypsin	65.812	1	65.812	73.928	.000
	pge2s	20.040	1	20.040	910.393	.000
	serpin	17.150	1	17.150	178.436	.000
	aspprot	19.405	1	19.405	180.949	.000
	cysteine	20.544	1	20.544	565.007	.000
	thrombin	198.576	1	198.576	1.167	.296
	venom	8707.799	1	8707.799	36.632	.000
Sex	trypsin	2.918	1	2.918	3.278	.089
	pge2s	.162	1	.162	7.359	**.015**
	serpin	.499	1	.499	5.195	**.037**
	aspprot	.200	1	.200	1.865	.191
	cysteine	.206	1	.206	5.667	**.030**
	thrombin	183.255	1	183.255	1.077	.315
	venom	5594.513	1	5594.513	23.535	**.000**
Treatment	trypsin	.251	1	.251	.282	.603
	pge2s	.003	1	.003	.131	.722
	serpin	.184	1	.184	1.918	.185
	aspprot	.107	1	.107	.994	.334
	cysteine	.000	1	.000	.001	.972
	thrombin	166.349	1	166.349	.978	.337
	venom	246.823	1	246.823	1.038	.323

Source	Dependent Variable	Type III Sum of Squares	df	Mean Square	F	Sig.
Sex * Treatment	trypsin	2.478	1	2.478	2.784	.115
	pge2s	.013	1	.013	.568	.462
	serpin	.141	1	.141	1.468	.243
	aspprot	.001	1	.001	.007	.936
	cysteine	.130	1	.130	3.564	.077
	thrombin	170.762	1	170.762	1.004	.331
	venom	5.746	1	5.746	.024	.878
Error	trypsin	14.243	16	.890		
	pge2s	.352	16	.022		
	serpin	1.538	16	.096		
	aspprot	1.716	16	.107		
	cysteine	.582	16	.036		
	thrombin	2722.343	16	170.146		
	venom	3803.346	16	237.709		
Total	trypsin	85.703	20			
	pge2s	20.570	20			
	serpin	19.512	20			
	aspprot	21.428	20			
	cysteine	21.461	20			
	thrombin	3441.285	20			
	venom	18358.227	20			
Corrected Total	trypsin	19.891	19			
	pge2s	.530	19			
	serpin	2.362	19			
	aspprot	2.023	19			
	cysteine	.917	19			
	thrombin	3242.709	19			
	venom	9650.428	19			

a. R Squared = .284 (Adjusted R Squared = .150)
b. R Squared = .335 (Adjusted R Squared = .210)
c. R Squared = .349 (Adjusted R Squared = .227)
d. R Squared = .152 (Adjusted R Squared = -.007)
e. R Squared = .366 (Adjusted R Squared = .247)
f. R Squared = .160 (Adjusted R Squared = .003)
g. R Squared = .606 (Adjusted R Squared = .532)