UNDERSTANDING THE PATHOLOGIC AND IMMUNOLOGIC RELATIONSHIP BETWEEN ELASMOBRANCHS AND THE MARINE LEECH *BRANCHELLION TORPEDINIS*

by

DAVID PAUL MARANCIK

(Under the Direction of Alvin Camus)

ABSTRACT

Abstract: *Branchellion torpedinis* is a marine leech that exclusively parasitizes elasmobranchs. The purpose of this project was to explore the pathogenic relationship between this relatively unknown leech species and its elasmobranch host, and to elucidate functional elements of the elasmobranch immune response, induced by parasitism.

To examine pathogenesis and extent of disease caused by *B. torpedinis*, 12 yellow stingrays *Urobatis jamaicensis* were infected with one or three leeches for 14 days. Leeches induced extensive cutaneous ulceration and were associated with anorexia, decrease in host packed cell volume and serum total solids, and mortality in three rays. Decreases in packed cell volume positively correlated with parasite:host ratios and ulcer size. Decreases in total solids also positively correlated with ulcer size. Host erythrocytes observed microscopically within leech intestine confirmed blood feeding.

The salivary gland transcriptome of *B. torpedinis* was evaluated by extracting mRNA from leech salivary tissue and preparing cDNA for 454 pyrosequencing. Genetic sequences with significant homology to bioactive proteins belonging to the ADAMTS

(disintegrin, metalloproteinase and thrombospondin motif) superfamily of proteins, anticoagulants, and immunomodulators, were identified. Putative protein activities correlate with gross and microscopic lesions observed in elasmobranchs at leech feeding sites.

Serum IgM responses to leech salivary gland extract were examined retrospectively in captive zebra sharks *Stegostoma fasciatum* by ELISA and Western blot assays in 20 serum samples from six zebra sharks with a 5 year history of leech infection, and 18 serum samples from 8 captive bred zebra sharks with no history of leech exposure. ELISAs demonstrated significantly higher serum IgM titers to salivary gland extract in exposed zebra sharks compared to the non-exposed population. One-dimensional and two-dimensional Western blot assays revealed IgM targeted specific salivary gland proteins within the 40, 55 and 70 kD range. Antigenic proteins identified by liquid chromatography–mass spectrometry and *de nov*o peptide sequencing include an ADAMTS family protein, tubulin, aldehyde dehydrogenase, and two unknown proteins.

Results from this study provide much needed information on the pathologic potential and relevance of *B. torpedinis* in captive elasmobranchs and enhances our understanding of this host-parasite relationship, to facilitate better management of infected animals.

INDEX WORDS: aquarium; *Branchellion torpedinis;* elasmobranch; infection; infestation; leech parasitism; *Stegostoma fasciatum; Urobatis jamaicensis;* yellow stingrays; zebra sharks

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DAVID PAUL MARANCIK

B.A., Assumption College, 2001

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|---|---|
| Major Professor: | Alvin Camus |
| Committee: | Donald Champagne |
| | Alistair Dove |
| | Mark Fast |

Electronic Version Approved:

Maureen Grasso
Dean of the Graduate School
The University of Georgia
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TABLE OF CONTENTS

# CHAPTER 1

# INTRODUCTION

Parasitism by the marine piscicolid leech *Branchellion torpedinis* is known to cause mortalities in captive elasmobranchs and is difficult to control when inadvertently introduced into public aquaria. Infestations often consist of high numbers of leeches, resulting in ulceration and tissue destruction at attachment sites. Heavy infestations of the skin, oropharynx, gill slits, and cloaca have been observed to cause anemia, anorexia, lethargy, and death.

Management of *B. torpedinis* has proven challenging at the Georgia Aquarium due to its high reproductive capability. Population control methods have included vacuuming of exhibit substrate to remove eggs and larvae. However, encased in "gravel cocoons," separation and filtration of eggs from the substrate is difficult. Few chemotherapeutants are described for treatment of leeches in animals, and controlled studies have not been performed in elasmobranchs. The organophosphate trichlorfon (Dylox®) has been used effectively against *B. torpedinis* (Burreson 2006). However, the efficacy and safety of trichlorfon in elasmobranchs has not been determined in controlled studies. Additional safety concerns exist when dealing with exhibits housing mixed species of animals of unknown sensitivity and the logistics of treating large volumes of water in aquariums.

In the absence of safe and effective treatment options, some facilities rely on repeated capture and restraint of parasitized animals for manual removal of leeches. As *B. torpedinis* often attaches deep within the pharynx, removal necessitates anesthesia and endoscopy. These procedures are stressful to the fish, pose risks to aquarium personnel, and are labor intensive.

Despite the significance of *B. torpedinis* infestations in captive elasmobranchs, little is known about the biology of the leech or this host-parasite relationship. This research project was designed to explore several different, but interconnected, facets relating to the pathogenic potential of *B. torpedinis* and the immune response of the elasmobranch host. Areas of investigation include infectivity trials using yellow stingrays *Urobatis jamaicensis* as hosts, transcriptome analysis of the *B. torpedinis* salivary gland, and investigation of humoral responses of zebra sharks *Stegostoma fasciatum* chronically exposed to *B. torpedinis* salivary antigens through parasitism..

Twelve yellow stingrays were infected with one or three leeches to monitor *B. torpedinis* induced disease. Behavioral, biochemical and hematologic changes were monitored for 14 days and gross and microscopic lesion development was characterized at leech attachment and feeding sites. Data were correlated with cutaneous damage and parasite:host weight ratios. Investigating these processes helps to understand infection pathogenesis and risk factors for clinical disease, as well as local and systemic host inflammatory responses.

The microscopic anatomy and biochemical composition of the leech salivary gland was explored using histologic and genetic sequencing techniques. Microscopic examination of leeches revealed ingestion of host blood and serum. It is hypothesized

that secreted salivary gland proteins contribute to the feeding activity of *B. torpedinis*. Through isolation of salivary gland mRNA, cDNA library construction and 454 pyrosequencing, bioactive proteins were identified that may play a role in proboscis penetration and leech feeding through anti-hemostatic, proteolytic and immunomodulatory actions. These findings suggest mechanisms of tissue destruction at leech feeding sites and the ability of leeches to locally alter host physiologic processes. As *B. torpedinis* infections in elasmobranchs most likely represent millenia of host-parasite adaptation, identifying anti-hemostatic factors and immunomodulators indirectly suggests a platform to explore elasmobranch physiologic processes.

Host responses to leech infections present a unique model to investigate humoral immune responses in elasmobranchs. Systemic IgM responses to leech salivary gland extract was examined in zebra sharks chronically exposed to leeches. Antibody titers assessed by ELISA were compared to non-exposed zebra sharks. One-dimensional and two-dimensional Western blot assays were used to identify antibody targeting of specific salivary gland antigens. These results shed light on elasmobranch humoral responses to leech infection and suggest possible roles for vaccination to control parasite burdens.

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# CHAPTER 2

# LITERATURE REVIEW

## *Leech Phylogenetics*

Leeches are clitellate annelids found throughout terrestrial, freshwater and marine environments. Grouped within the Class Clitellata (Sawyer 1986), leeches are recognized as being closely related to oligochaetes based on embryology, the presence of a specialized cocoon secreting clitellum, hermaphrodism, and a direct life cycle (Siddall 1998). Although there is little debate in classifying leeches as annelids, phylogenetic relationships within the class Clitellata are disputed. It is suggested that current family groups are not all-inclusive, as they are based more on traditional morphology and life history, rather than genomic sequence data (Apakupakul 1998).

Difficulties in phylogenetically characterizing leeches based on life history and anatomy is reflected in the number of traits shared with other non-related species. Leeches inhabit similar environments as monogeneans. Both are hermaphroditic and utilize attachment and reattachment of the anterior and posterior suckers for locomotion (Kearn 2004). These similarities are believed to be more reflective of convergent evolution rather than a common genetic lineage, and they complicate correct taxonomic placement. The presence of a haemocoelom, compound eyes, a digestive system, and a tendency towards terrestrialism reflects characteristics of arthropods. The significance of

this is unknown, but it has been hypothesized this may reflect some degree of relationship between clitellates and arthropods (Sawyer 1986).

Siddall (1988) argued that leeches should comprise their own class distinct from oligochaetes based on morphologic differences, including described coelomic reduction and the lack of true body segmentation in leeches. Unlike oligochaetes, that possess true internal and external segmentation and are able to regenerate lost or damaged segments, leeches are described as having modified body segments. This feature is believed to accommodate a continuous gut and anterior and posterior suckers, which unlike oligochaetes, makes them unable to regenerate lost or damaged segments (Kearn 2004).

More recently, molecular studies suggest leeches are most closely related to the oligochaete Family Lumbriculidae and should comprise a combined order within the Class Oligochaeta (Siddall 2001). As genomic data are collected from more leech species, taxonomic groupings traditionally based on morphology will most likely continue to be revised and reorganized.

***Leech Taxonomy***

As described by Sawyer (1986), leeches are currently separated into four, independently evolved orders: Branchiobdellida, Acanthobdellida, Arhynchobdellida and Rhynchobdellida. Each displays significant variation in anatomy, physiology, life span, and behavior, and use specific strategies to parasitize various terrestrial, freshwater and saltwater mammals, fish, crustaceans, and other invertebrates (Sawyer 1986). Branchiobdellids are small ectosymbionts of crayfish that feed on detritus and external microflora on the host shell. Acanthobdellidans largely parasitize salmonids in cold-water habitats of Alaska and Scandinavia (Sawyer 1986). Historically, Arhynchobdellida

and Rhynchobdellida comprise the larger and more studied of these orders. Both groups contain blood feeders, as well as predatory leeches that feed on invertebrates (Kearn 2004).

The arhynchobdellidans have been traditionally grouped into four suborders: the blood feeding Irudiniformae, the non-sanguivorous Erpobdelliformae, the terrestrial Haemadipsidae, and the predaceous Haemopidae (Apakupakul 1998). Generally, arhynchobdellidans are leeches found in or near water, although significant diversity in life history and geographic distribution exists. The Irudiniformes, which include the medicinal leech family, Hirudinae, have long been of medical interest and have received attention for human pharmaceutical purposes. These jawed leeches aid feeding and digestion by injecting salivary gland proteins into bite sites. Bioactive proteins, including anticoagulants (hirudin), trypsin-inhibitors, and fibrinolytic metalloproteinases have demonstrated properties that modulate host physiologic and immunologic responses and promote local tissue regeneration (Chalisova 2003, Baskova 2004, Baskova 2008).

***Taxonomy and Description of B. torpedinis***

*B. torpedinis* belongs to the Order Rhynchobdellidae. These jawless leeches use a straw-like proboscis for feeding that is believed to be facilitated by secreted salivary gland proteins released into the tip of the proboscis (Sawyer 1986, Sawyer 1991, Moser 1995). The *Rhynchobdellidae*, comprised of the families *Glossiphonidae* and *Piscicolidae*, are parasites of freshwater and saltwater fish. Glossiphinid and piscicolid leeches may have evolved from a single ancestor, although it is not clear whether this progenitor was a true blood feeder or predator that acquired sanguivory later in its evolutionary development (Borda 2003).

Detailed morphology of *B. torpedinis* was described by Sukatschoff in 1912 and has since been summarized for taxonomic purposes (Sawyer 1975, Kearn 2004). *B. torpedinis* is distinct from other rhynchobdellidans in having 33 pairs of foliaceous, gill-like branchial structures that may be involved in respiration (Kearn 2004). The nearly identical *B. ravenelii* has only 31 pairs of branchial structures (Kearn 2004). Like other rhynchobdellidans, *B. torpedinis* possess two suckers. The small oral sucker is distinct from the neck and houses the proboscis-salivary gland complex, while the caudal attachment sucker is relatively larger and distinct from the body (Sawyer 1975). *B. torpedinis* has a prominent clitellum through which the fertilized egg and cocoon are deposited into the environment (Sawyer 1975). The gut is continuous and ends in a terminal anus (Sawyer 1975).

***Salivary Gland Complex Anatomy and Secretory Capabilities***

Histologic and ultrastructural characterization of the salivary gland-proboscis complex has been described for a number of rhynchobdellidan species, with genus-specific morphology described for multiple species, including *Placobdella ornata, P. parasitica* and *Desserobodella picta* (Moser 1995). The salivary gland of *Placobdella* spp. is composed of compact pairs of anterior and posterior salivary glands. Salivary cells produce a single mucinous secretion and two types of proteinaceous secretions. Salivary glands of *D. picta* are more diffusely arranged and contain two mucous and two proteinacious secretions within salivary cells. In all species, ductules leaving salivary gland cells enter the base of the proboscis and extend anteriorly to pores located along the tip, body and luminal wall of the proboscis.

The salivary complex of jawless leeches has been found to produce a number of components believed to modulate host physiological mechanisms and play a role in leech feeding and digestion. Both cementum, which is secreted into oral sucker attachment sites (Min 2010), and orgelase, a mucolytic hyaluronidase that may increase permeability of other salivary gland secretions through host skin (Linker 1960), are believed to aid in leech attachment. Other proteins are involved in anti-hemostasis, such as anti-platelet protein (Ricci-Silva 2005) that inhibits platelet aggregation, and ghilianten (Condra 1989) and antistasin (Nutt 1988), that inhibit factor Xa in the coagulation cascade. Additional products include the thrombin inhibitor theromin (Salzet 2000) and the fibrinolytic protein hementin (Sawdesh 1990).

Leech salivary gland secretions also contain various proteins with immunomodulatory activities, although evidence for functional immunosuppression is circumstantial. Eglin a,b, and c, isolated from the salivary gland of medicinal leeches, inhibit granulocyte proteases *in vitro* and may act as antibiotics to prevent putrefaction of blood stored within the crop and gut (Baskova 2008). Bdellin blocks the pro-inflammatory activities of plasmin and trypsin and is found in salivary gland secretions of rhynchobdellidans and archynchobdellidans (Baskova 2008, Min 2010).

Localized immunomodulation may also influence hematophagous parasites as vectors of disease (Burreson 2007). Trypanosomes and trypanoplasmas are documented pathogens of elasmobranchs that can be transmitted by leeches (Yeld 2006, Burreson 2007). Transmission may be further facilitated by the ability of protozans to survive for months and reproduce within the leech gut (Nehili 1994). Additionally, bite sites from medicinal leeches are associated with an increased incidence of secondary bacterial

infections (Tiene 2007). In other hematophagous parasites, including the sand fly, salivary immunomodulatory and anti-inflammatory proteins have been positively correlated with transmission of leishmaniasis in humans (Kamhawi 2000). Similarly, components of tick saliva are believed to favor transmission of *Borrelia borgderferi,*the agent of Lyme disease (Bowman 1997).

***Leech Life History***

Although the life history of *B. torpedinis* has not been fully elucidated, insight into the biology of the leech can be deduced from observations made in aquaria and through comparisons to known life-histories of other leech species. Leeches are generally categorized as temporary or semi-permanent depending on the time spent on their host prior to detachment (Burreson 2006). Temporary parasites leave the host soon after taking a blood meal to deposit cocoons within a sheltered location. Semi-permanent leeches take multiple blood meals prior to leaving the host for cocoon deposition. Most aquatic leeches that leave their host to release cocoons utilize a solid substrate and attach cocoons onto vegetation, rocks, or the shells of crustaceans (Sawyer 1986). Certain species attach cocoons upon the host or may attach eggs to their ventral body surface (Burreson 2006). Observations of *B. torpedinis* suggest a more permanent life cycle, with leeches remaining attached to the elasmobranch host for the duration of their lives, while laying cocoons that fall to the bottom and become encased in gravel.

Cocoons for most leeches contain anywhere from 1-5 embryos (Khan 1976) that develop and hatch in a temperature dependent fashion (Reynolds 1998). However, this is described as variable for many species and can depend on habitat, geography and host biology (Khan 1976). Reproductive cycles and egg hatching for *Piscicola salmositica,* a

freshwater parasite of Pacific salmon, is more closely associated with salmon migration patterns than through factors related to *P. salmositica* habitat (Becker 1965, Bower 1987). In a few leech species, that may include *B. torpedinis*, egg laying is continual with the production of successive generations by a single adult (Burreson 1977).

Reproductive cycles of *B. torpedinis* are difficult to elucidate further in closed aquarium systems where environmental conditions, such as temperature, are relatively constant. Six years of observation at a large public aquarium have revealed variation in population numbers, although fluctuations have not directly coincided with seasonal changes. Instead, population spikes may be more closely tied to host availability, in particular, the presence of cownose stingrays (*Rhinoptera bonasus*). Leech numbers fluctuate as the cownose ray population increases or decreases in the exhibit, suggesting cownose rays may serve as a primary host for *B. torpedinis*.

Host specificity varies between piscicolid leech species. Some are exclusively associated with certain species of fish (Rhode 1984), while others exhibit less specificity and will parasitize multiple fish species or families (Sawyer 1975). Factors governing host specificity in leeches are unknown, although observations suggest they may be attracted to host skin mucous (Rhode 1984). Similar interactions have been described for monogeneans that utilize mechanical and chemical cues to recognize hosts, including mucous glycoproteins (Buchmann 2002, Ohashi 2007).

*B. torpedinis* has been reported predominantly from rays and skates off the Atlantic coast of the United States, with a few descriptions off the west coast of Africa and the Mediterranean Sea (Sawyer 1975). A certain amount of host specificity has been observed for *B. torpedinis* in one aquarium setting. Generally, demersal elasmobranchs

that remain in close contact with bottom substrate, where leeches are present, are affected more often than pelagic species that remain higher in the water column. However, *B. torpedinis* does exhibit some degree of selectivity within the demersal species. While observations indicate zebra sharks (*Stegostoma fasciatum*) and sawfish (family *Pristidae*) are often parasitized, in contrast, blotched fantail rays (*Taeniura meyeni*) and spotted wobbegongs (*Orectolobus maculates*) in the same exhibit are rarely observed with leeches. While largely pelagic, cownose rays are bottom feeders and appear to consistently harbor the most severe parasite burdens, suggesting their role as primary hosts.

***Leech Parasitism of Fish***

Parasitism of wild elasmobranchs by leeches has been reported only sporadically in the literature (Burreson 2000, Soto 2000, Soto 2003). Interactions in teleosts, as well as their physiologic and pathologic effects are better described. Many report short-term, seasonal infestations associated with unusual environmental factors hypothesized to support leech reproduction while simultaneously acting as stressors to lower host resistance. An epizootic involving *Calliobdella carolinensis* in Atlantic menhaden was described in two estuarine streams in South Carolina during the winter of 1970. Upwards of 200 leeches were observed in the oral cavity of menhaden. Increased water turbidity and colder than normal water temperatures, may have contributed to higher infection densities (Sawyer 1973). A report of *Macrobdella decor*a and *Haemopis grandis* infecting brook trout *Salvelinus fontinalis* in Maine was attributed to warm water temperatures causing trout to congregate around cool underwater springs. Increased host

numbers in small spaces provided leeches close proximity to higher host concentrations (Rupp 1954).

The most commonly described physical effect of leeches in fish is ulceration at attachment sites (Thompson 1927, Sawyer 1973, Noga 1990) with localized petechial hemorrhages resulting from feeding activity (Jones 1990). Although histologic findings are not particularly detailed, microscopic descriptions of attachment sites portray variable loss or hyperplasia of epidermal and dermal layers, accompanied by hemorrhage, and inflammatory infiltrates. Skin ulcerations have also been suggested as possible routes for infection by secondary pathogens, including bacteria and fungi (Burreson 2006).

Host morbidity and mortality from leech infections varies between published reports. In general, leeches are rarely implicated as significant parasites of fish and only when large numbers are present (Burreson 2006). The previously described *C. carolinensis* epizootic in the oral cavity of Atlantic menhaden describes no host mortality (Sawyer 1973). Severe oral ulcerations in largemouth bass infested with *Myzobdella lugubris* in North Carolina appeared to have little overall affects on fish health (Noga 1990). Similarly, heavy infestations of *Piscicola punctata* in bigmouth buffalo *Ictiobus cyprinellus* in Illinois resulted in localized ulcers, but no reported host mortality (Thompson 1927). A reported *Zeylanicobdella arugamensis* infection in tank-reared orange-spotted grouper *Epinephelus coioides* in the Phillipines describes hundreds of leeches per fish but no mortality (Cruz-Lacierda 2000).

Conversely, a few reports describe disease and significant mortality resulting from leech infections. Infestation of pink salmon *Oncorhynchus gorbuascha* fry by *Pisicola salmonicitica* in hatchery trays resulted in high host mortality attributed to blood

loss (Earp 1954). In Australian sand whiting *Sillago ciliata, Austrobdella translucens* infestation in stocked saltwater ponds resulted in the death of dozens of fish during successive years. Fish harbored as many as 100 leeches resulting in large ulcerations and presumably exsanguination (Badham 1916). Similar anemia and death were described in the previously mentioned report of *M. decor*a and *H. grandis* infection of brook trout in Maine (Rupp 1954).

Specific differences contributing to overall mortality are not clear, but may be related to factors such as host size, parasite burden, and host fitness. The ability of the fish to physiologically compensate for leech feeding may also play a role in disease resistance. Ectoparasites can represent stressors for fish and have been associated with decreased foraging (Milinski 1984), impaired anti-predator behavior (Milinski 1985), and reduced fecundity and growth (Iwama 1999). Energetics in shorthorn sculpin *Myxocephalus scorpius* infected with the leech *Malmiana brunnea* was significantly lower than uninfected sculpin. Energy deficiencies attributed to leech feeding, tissue damage, and loss of metabolites, accounted for approximately 750 cal/g of leech per week energy loss to host metabolism (Mace 1972).

***Interactions at the Host:Parasite Interface***

In general, host-parasite relationships are complex and thought to represent generations of coevolution and adaptation (Woolhouse 2002). Interactions at the host-parasite tissue interface involve strategies by the parasite to exploit host environments for feeding and propagation, while avoiding immune detection and response, such as infection of immunopriviledged sites or areas of the body not prone to significant immune responses. For example, *Diplostomum* spp. appear to preferentially infect the

eye lens of fish (Owen 1993) and monogeneans are often reported to preferentially attach to fish fins and spines, which are believed to be areas of low immunoreactivity (Whittington 1996).

Although the most successful parasites minimize their effects on the host, physical damage by ectoparasites often triggers local and systemic changes in the host. Monogeneans infecting skin and gills of teleosts have been associated with hypersecretion of mucus (Milinski 1985) and *Ichthyophthirius multifilis* is often associated with epithelial cell hyperplasia (Ventura 1985). In an attempt to repel infection and limit collateral tissue damage, hosts also recognize and respond immunologically to parasites (Buchmann 2002). Studies examining *Lepeophtheirus salmonis* infection in salmonids show lower parasite loads in salmonid species capable of producing strong innate immune responses to the parasite at attachment and feeding sites (Johnson 1992). Japanese flounder *Paralichthys olivaceus* induce a rapid and severe granulocytic and histiocytic inflammatory reaction against the monogenean *Neoheterobothrium hirame* (Nakayasu 2005).

Acquired immune responses to parasites have been demonstrated in mammals and teleosts. Most notably, bioactive compounds identified in saliva of arthropods play an important role in stimulating humoral immunity (Brown 1984). The antigenic properties of tick salivary components have been exploited in the production of vaccines shown to reduce fecundity and decrease parasite burdens in multiple mammalian species (Mulenga 1999). In fish, serum antibody responses have been described in Atlantic salmon following natural infection by *L. salmonis* (Grayson 1991). Although the functional significance of this response remains to be validated, current studies are examining the

utility of vaccines produced from copepod homogenates as preventative therapy (Raynard 2002). To date, the ability of elasmobranchs to respond to leech salivary gland antigens has not been examined.

***Elasmobranch Immunology and Inflammatory Responses***

Like teleost fishes, elasmobranchs are likely dependent upon innate and adaptive immune mechanisms to protect against invading pathogens. Compared to higher vertebrates, however, morphologic and functional characterization of the cells and mediators involved remain poorly understood. As such, elasmobranch responses to disease have not been well described and few functional trials have been conducted. Elucidation of these mechanisms is further complicated by species-specific differences among elasmobranch groups.

A defining characteristic of elasmobranchs is the presence of an evolved immune system composed of true lymphoid organs comparable to higher invertebrates. A characteristic structure unique to elasmobanchs is the epigonal organ, comprised of hematopoetic tissue attached to testicle or ovary (Zapata 1996). The paraesophageal Leydig organ has been described as the predominant lymphomyeloid tissue in a limited number of elasmobranch species (Fänge 1983), although it is lacking in most (Zapata 1996). In addition to thymus and gut-associated lymphoid tissue, lymphomyeloid tissue has also been observed in the meninges and kidneys, although this may be restricted to specific species and developmental stages (Torroba 1995). The spleen has been proposed as the main site for antibody production in elasmobranchs (Fänge 1983). However, splenectomized sharks fail to exhibit diminished antibody levels, demonstrating the ability to produce antibodies within alternate lymphoid tissue (Zapata 1996).

***Elasmobranch Innate Immunity***

Classification of granulocytes is problematic in elasmobranchs due to species-specific variation in granule morphology and staining characteristics and a lack of studies correlating microscopic structures with function. Granule-containing cells have been described as heterophils (Luer 2004), granulocytes (Hyder 1983), fine and course eosinophilic granulocytes (Ferreira 2010), eosinophils (Hine 1987), neutrophils (Van Rijn 2010), and mast cells (Luer 2004), with little distinction between cell-types. These discrepancies make it difficult to characterize the role of inflammatory cells in the elasmobranch immune response and provide generalizations regarding elasmobranch immunity and inflammatory responses.

Phagocytic activity has been demonstrated for granulocytes *in vitro*, although it is not completely understood which cell types are represented (Hyder 1983). Fc receptors have been recognized in what were described as elasmobranch heterophils by Secombes (1996) and may contribute to opsonization and phagocytosis. Phagocytic activity has been demonstrated in elasmobranch monocytes, macrophages, and thrombocytes (Secombes 1996, Hamlett 1999). However, unlike elasmobranch heterophils and mammalian mononuclear phagocytes, Fc receptors have not been described on elasmobranch macrophages (Haynes 1991). Phagocytosis appears to occur without opsonization, which suggests it occurs from direct phagocyte recognition of amino acid and glycoprotein groups on the target cell. Although phagocytic activity has been demonstrated in elasmobranch thrombocytes, a lack of intracellular digestion precludes their current classification as an immune cell (Secombes 1996).

The complement cascade may represent an important component of innate immunity in elasmobranchs. Six functional proteins have been described in the nurse shark complement pathway: C1n, C2n, C3n, C4n, C8n, C9n (Jensen 1981). The membrane attack complex (MAC) has not been demonstrated, but evidence of C8 and C9, the last two factors needed for construction of MAC, suggest its existence (Ross 1973). Genetic analysis has shown significant homology between elasmobranch and mammalian complement proteins with shark C1, C3, and C4 reported to have two chains bearing approximately 50% similarity to their mammalian counterparts (Nonaka 2000). Studies in nurse sharks *Ginglymostoma cirratum* have demonstrated complement factors opsonize and hemolyse foreign red blood cells (Jensen 1981) and attract granulocytes and macrophages (Obernauf 1992). These results suggest a role of complement in the acute phase response of elasmobranchs.

Other innate immune components identified in elasmobranchs include nitric oxide (Walsh 2006), C-reactive protein (Karsten 2004) and amyloid P (Robey 1983), although characterization has generally been through genomic and other molecular techniques that do not describe functional roles in disease responses.

***Elasmobranch Adaptive Immunity***

Elasmobranchs genetically diverged from other chordates approximately 470 million years ago, and are considered the earliest vertebrate with an immunoglobulin driven adaptive immune system (Leonard 1977). Lymphocytes are the most abundant leukocyte in elasmobrach peripheral blood, consisting of approximately 40-60% of circulating white blood cells (Luer 2004). B- and T-lymphocytes have been characterized in multiple shark species, although functionality of these subtypes is largely

unknown. Maturation of B-cells into plasma cells has been observed in nurse sharks (Hamlett 1999), but data is lacking for most elasmobranch species. Genetic homology within the coding regions for T-cells, T-cell antigen receptors and major histocompatibility gene complexes suggests similarity to higher vertebrate functions (Miracle 2001).

Molecular and serologic studies indicate fundamental differences between humoral immunity in elasmobranchs and bony fish and mammals. Most notably, three classes of immunoglobulins (Ig) have been identified in elasmobranchs: IgM, IgNAR (novel antigen receptor) and IgW (IgX). IgM is expressed in monomeric and pentameric forms and is the most prevalent immunoglobulin constituting approximately half of the total serum protein in adult sharks (Luer 2004). IgNAR is found in low amounts in serum, yet may primarily drive the antibody response by giving rise to higher affinity antibodies (Dooley 2006). It is secreted as a homodimer with single variable regions, but with no associated light chains (Dooley 2005). The role and relative importance of IgW is largely unknown, as low serum levels and high sensitivity to proteolysis have made isolation difficult. It is thought to exist as either a seven-domain form or a three-domain form (Dooley 2005).

The clustered organization of Ig genes is different from all other vertebrates and allows for plasticity in the number and types of Ig present in elasmobranchs. This organization precludes class switching (e.g. IgM to IgG), limiting elasmobranchs to expression of a single Ig isotype/cell (Stavenezer 2004). Serologic studies of IgM levels, and in part IgNAR, have shown that sharks take months to years to reach significant antigen-specific titers and that somatic hypermutation is responsible for subsequent

affinity maturation (Stavenezer 2004, Dooley 2005). The prolonged time frames required for protective antibody titers to develop prompt questions as to the role of adaptive humoral immunity in elasmobranchs and the functions of different Ig isotypes.

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# CHAPTER 3

# EXPERIMENTAL INFECTION OF YELLOW STINGRAYS *UROBATIS JAMAICENSIS* WITH THE MARINE LEECH *BRANCHELLION TORPEDINIS*

Marancik, D., Dove, A., Camus, A.
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***Abstract***

Infestations of elasmobranchs by the marine leech *Branchellion torpedinis* can be problematic in aquaria and negatively affect host health. To better characterize the extent and pathogenesis of disease, twelve yellow stingrays *Urobatis jamaicensis* were infected with one or three leeches for 14 days. *B. torpedinis* parasitism was associated with anorexia, extensive cutaneous ulceration, decreased host packed cell volume and serum total solids, and mortality in three rays. Ulcer size positively correlated with leech number (Mann-Whitney test, p-value 0.037). Average decrease in host packed cell volumes positively correlated with ulcer size (Pearson's coefficient 0.731, p-value 0.007) and parasite:host ratio (Pearson's coefficient 0.370, p-value 0.026). Average decrease in host serum total solids positively correlated with parasite:host ratio (Pearson's coefficient 0.592, p-value 0.042). Blood chemistry and total white blood cell counts revealed no significant trends. Additional necropsy findings included gill and splenic pallor (8/12), pericardial edema (5/12), perirenal edema (3/12), and low hepatocellular lipid deposits (4/12). Microscopic evaluation of leeches demonstrated host erythrocytes and proteinacious fluid within parasite intestines. Elucidation of this host-parasite interaction will lead to better understanding of the pathogenic potential of *B. torpedinis* and facilitate care of parasitized elasmobranchs.

***Introduction***

Infestations of fish by leeches have been sporadically reported in the literature. Epizootics involving wild (Noga 1990, Rupp 1954, Sawyer 1973) and cultured fish (Cruz-Lacierda 2000) describe large numbers of leeches attached to host skin and within the buccal cavity. Variable amounts of ulceration, hemorrhage, and inflammation were associated with leech attachment sites. Host mortality, when present, (Badham 1916, Cruz-Lacierda 2000, Rupp 1954) has been attributed to blood loss. Infestations in wild fish were seasonal and it was hypothesized that certain environmental conditions, such as water turbidity and temperature may have allowed parasite populations to increase and simultaneously acted as stressors to host fish.

The negative effect of leeches on host physiology has been demonstrated in shorthorn sculpin (*Myxocephalus scorpius*) parasitized by *Malmiana brunnea*, which showed differences between growth rates in parasitized and non-parasitized fish. Leech feeding and physiologic strain accounted for 750 cal/g/leech/week energy loss (Mace 1972).

Leeches also play a role as vectors of disease in fish. Transmission of hematozoa, including *Trypanosoma* spp. and *Trypanosplasma* spp., has been demonstrated in elasmobranchs and teleosts (Burreson 1972, Khan 1976). There is evidence implicating leeches as mechanical vectors of viruses and bacteria in teleosts (Ahne 1985, Bragg 1989, Mulcahy 1990, Faisal 2009) and speculation that attachment site ulcers may predispose hosts to bacterial infection (Faisal 2011).

*Branchellion torpedinis* is a marine leech that exclusively parasitizes elasmobranchs. *B. torpedinis* belongs to the Order Rhynchobdellida, which is comprised

of the families *Glossiphonidae* and *Piscicolidae* that parasitize both freshwater and saltwater fish. These jawless leeches have a large caudal sucker for attachment and feed on host fluids through a straw-like proboscis inserted from a smaller, cranial sucker (Kearn 2004). Feeding is believed to be aided by salivary gland enzymes released into the tip of the proboscis, which enable penetration of host tissue and prevent hemostasis (Moser 1995, Sawyer 1991). Inadvertent introduction of *B. torpedinis* into closed aquarium settings is problematic, as parasites thrive with low environmental stress and high host availability. High leech fecundity and a lack of adequately studied, safe and effective chemotherapeutics often make management difficult.

There is little information published on *B. torpedinis* beyond its ecology and anatomy (Kearn 2004). We have been able to gain insight into its life history by observing the leech in aquaria under constant, 21°C environmental conditions. Unlike many semi-permanent leech species that dislodge from hosts after taking a bloodmeal to deposit cocoons on vegetation and bottom substrate (Kearn 2004), *B. torpedinis* appear to remain attached to the host for the duration of its life. This prolonged, close contact with elasmobranch hosts may result in more extensive disease compared to leeches that act as temporary parasites (Burreson 2006). Cocoons are shed from the host into the bottom substrate and have been observed to hatch after approximately 30 days (personal observation). When removed from the host, *B. torpedinis* are short-lived in laboratory settings with survival lasting less than five days (personal observation). Clinical observations indicate that *B. torpedinis* negatively impact host health in aquaria. Parasitism of a number of demersal and pelagic elasmobranch species has resulted in

ulceration of the skin, oropharynx, gill slits, and cloaca. Severe infestations have been associated with lethargy, anorexia, and death of the host.

To better understand the pathogenic potential of *B. torpedinis* and to characterize risk factors for disease, yellow stingrays *Urobatis jamaicensis* were used as an infection model. Behavioral, biochemical and hematologic changes were monitored for 14 days following controlled infection. Rays were necropsied on day 14 and gross and microscopic lesion development examined at attachment and feeding sites. Data were correlated with cutaneous damage and parasite:host weight ratios. Investigating these processes will enhance understanding of this host-parasite interaction and elasmobranch responses during disease states.

***Materials and Methods***

*Experimental System*

Twelve wild caught yellow stingrays were maintained individually in partitioned sections of a 3000 L aquarium with sand substrate and recirculating artificial sea water (Instant Ocean® Sea Salt, Aquarium Systems, Inc., Mentor, OH) maintained at 21° C and 30 parts per thousand salinity. The rays received alternating 12 hour periods of light and dark and were fed a daily, rotating diet of shrimp, fish, and clam *ad libitum.* Amounts consumed were recorded. Physical exam and three repetitive skin scrapes prior to experimental trials revealed no ectoparasite infections.

Rays were assigned to two experimental groups and allowed a minimum of 3 weeks to acclimate. The L1 group consisted of six rays (2 males/4 females, weight range 380-550 gm), each infected with one leech (weight range 632-763 mg). The L3 group consisted of six rays (3 males/3 females, weight range 315-475 gm), each infected with

three leeches (combined weight range 1.831-2.145 mg). Leeches collected from captive elasmobranchs were transported directly to the laboratory for infection trials and manually placed on the dorsum of the rays. When infected with three leeches, each leech was placed approximately 8 cm apart.

*Sampling regimen*

Blood was initially sampled from three randomly selected rays at time 0, then at 3, 7, and 14 days to mimic the experimental trial and to ensure that repeated blood collection had no affect on hematology and chemistry values. Parameters measured at time 0 and days 7 and 14 include serum chemistry, total white blood cell count, leukocyte differential, packed cell volume (PCV) and total solids (TS). Additional blood was collected on day 3 for PCV and TS only. After a thirty day recovery period, the three rays were assigned to experimental groups.

Blood collection was performed via intracardiac puncture using a 22 G x 1" needle and 3-ml syringe (Kendall Monoject, Tyco Healthcare Group LP, Mansfield, MA) under general anesthesia by immersion in 75 mg/L MS-222 buffered 1:1 by weight with sodium bicarbonate. Four-hundred µl of blood was divided between serum separator tubes (BD Microtainer® Gold Tubes, Franklin, NJ) for blood chemistry, blood collection tubes containing 1.1 mg/ml lithium heparin (BD Microtainer® Green Tubes, Franklin, NJ) for total white blood cell (WBC) counts, and duplicate 40 mm glass microcapillary tubes containing ammonium heparin (Statspin® Microhematocrit Tubes, Westwood, MA) for PCV and TS. Blood smears were made immediately for each animal using whole blood and blood preserved in lithium heparin. All samples were processed within 1 hour of collection.

*Serum Chemistry*

Whole blood was allowed to clot and serum separator tubes were centrifuged for 10 minutes at 3,000 x gravity (g). Serum was pipetted off and analyzed on a Roche/Hitachi P 800 Modular Analytics System (Roche Diagnostics Corporation, Indianapolis, IN) to measure total protein, urea nitrogen, albumin, alkaline phosphatase (ALP), alanine aminotransferase (ALT), aspartate aminotransferase (AST), glucose, creatine kinase (CK), cholesterol, triglycerides (TG), total bilirubin, uric acid, sodium, potassium, chloride, bicarbonate, calcium, phosphorous, and magnesium. Serum osmolality was calculated using the equation 2 Na (mEq/L) + (Urea [mg/dL])/2.8 + (Glucose [mg/dL])/18 (Carlson 1997) as sufficient blood volumes for measured osmolarity could not be collected.

*Leukocyte Count*

Twenty µl of heparinized whole blood was added to 1980 µl of Natt-Herrick stain (ENG Scientific, Inc, Clifton, NJ) adjusted for use in elasmobranchs by adding 0.3 gms NaCl and 0.2 gms urea to 10 ml of filtered Natt-Herrick's stock. Total numbers of granulocytes and mononuclear cells were determined manually using an improved Neubauer hemocytometer chamber and previously described methods (Arnold 2005). Thrombocytes were not included in leukocyte counts (Arnold 2005). An automated slide stainer (7120 Aerospray slide stainer®, Wescor Inc., Logan, UT) was used to stain blood smears for differential counts.

*Packed Cell Volume and Total Solids*

Microcapillary tubes were centrifuged for 3 minutes at 3,000 x g in a CritSpin® microhematocrit centrifuge (Statspin Inc, Norwood MA 02062). PCVs were interpreted

by measuring the erythrocyte-plasma interface on a capillary tube reader (Statspin®, Iris Sample Processing, Westwood, MA 02090). TS were measured using a hand-held refractometer (Reichert®, Reichert Analytical Instruments, Seefeld, Germany).

*Necropsy*

Three rays died prior to day 14 and were necropsied immediately. Based on scheduled monitoring of animals, the interval between death and necropsy ranged from 1-5 hours. Rays remaining at day 14 were euthanized by immersion in 250 mg/L MS-222 (Finquel, Argent Chemical Laboratories Inc., Redmond, WA) and complete necropsies were performed. Skin scrapes and gill clips were evaluated by light microscopy. Leeches, leech attachment sites, normal skin, and representative samples of brain, gill, thyroid gland, heart, liver, spleen, kidney, pancreas, gastrointestinal tract, epigonal organ, and rectal gland were examined. Tissues were fixed in 10% neutral-buffered formalin and routinely processed for microscopic examination. Sections 4 µm were cut and stained with hematoxylin and eosin (H&E) for microscopic evaluation. Sections of ulcerated skin were additionally stained with Lillie-Twort tissue Gram's stain. Rays were weighed on a digital scale (Ohaus Corp., Pine Brook, NJ) on day 0 and at necropsy.

*Statistical Analyses*

Statistical analysis was performed using Minitab version 15.0 (Minitab Inc., State College, PA). A two-sample Wilcoxon rank sum (Mann-Whitney) test was used to compare ulcer size with leech number. A Pearson correlation coefficient (Pearson) was applied to the correlation of ulcer area, beginning leech:host weight ratio, PCV decrease, and TS decrease. A p-value less than 0.05 was considered significant.

***Results***

Leeches attached within seconds and remained on the dorsum of rays throughout the 14 day challenge. Four of 12 rays were observed rubbing on substrate for up to 24 hours following attachment. Anorexia was a common clinical sign. Average total feed intake in the L1 group decreased 25% by day 7 and 50% by day 14. Weight loss of 2 and 4 gms was observed in two rays. Feed intake in the L3 group decreased 75% by day 7 and 100% by day 14. Weight loss of 2-7 gms was observed in four rays with an average of 5 gms.

*Fish Pathology and Mortality*

Variably sized cutaneous ulcers associated with the leech caudal sucker were observed in all rays (Figure 1A). Peripherally surrounding attachment sites were variable numbers of 1 mm in diameter hemorrhages presumed to be leech feeding sites (Figure 1A). Cumulative ulcer size averaged 2.54 $cm^2$ (range 1.6 -5.25 $cm^2$) and 5.83 $cm^2$ (3.1-12 $cm^2$) in the L1 and L3 groups, respectively. The three rays that died prior to day 14 exhibited the largest areas of skin ulceration. One L1 ray that died on day eleven had an ulcer area of 5.25 $cm^2$. Two rays from L3 that died on days five and seven had ulcer areas of 12 $cm^2$ and 6 $cm^2$, respectively. Cumulative ulcer size correlated with leech number (Mann-Whitney test, p-value 0.037), but did not significantly correlate with leech weight (Pearson -0.539, p-value 0.070.)

Microscopically, attachment site ulcers began as discrete, well-demarcated foci of epithelial erosion bordered by a narrow margin of hyperplasia. Lesions progressed to full thickness necrosis of epithelium and superficial dermis, overlaid by a thick layer of cellular and acellular debris (Figure 1B). Hemorrhage and intensely eosinophilic fluid

were widespread within epithelial, dermal and hypodermal tissue, with variable infiltration by scattered to moderate numbers of fine-eosinophilic granulocytes, fewer coarse-eosinophilic granulocytes, and scattered lymphocytes, plasma cells, and macrophages (Figure 1C). Mixed populations of Gram-negative and Gram-positive rods and cocci colonized the surface of ulcers in 4/12 rays, with dissemination into dermal tissue of one animal. Although bacterial cultures were not performed, no bacteria, inflammatory infiltrates, or necrosis were present in internal organs to indicate systemic infection. In 5/12 rays, there was multifocal skeletal muscle degeneration and necrosis. Affected areas were characterized by pale, shrunken muscle bundles containing individual fibers exhibiting loss of cross striations, as well as sarcoplasmic coagulation, vacuolation, and fragmentation (Figure 1C). Nuclei were pyknotic to absent.

Presumed feeding site lesions consisted of well-demarcated, 1 mm in diameter, oblique tracks extending through the epidermis and dermis (Figure 1D). Tracks were filled with erythrocytes, proteinacious fluid, variable numbers of fine-eosinophilic granulocytes, and a few coarse-eosinophilic granulocytes. Although inflammatory infiltrates confined to proboscis tracks were occasionally intense, inflammation and necrosis in the surrounding tissue was minimal.

Additional gross necropsy findings included approximately 5-30 ml of clear, yellow-brown fluid within the pericardial (Figure 1E) and coelomic cavity of 5 rays. Gill and splenic pallor was present in 8 animals. The livers of 4 rays were small, dark, and firm. Internal microscopic changes included decreased hepatocellular lipid vacuolation and decreased prominence of splenic red pulp, in 4 and 6 rays, respectively.

The intestinal tract of leeches contained ingested host erythrocytes, proteinacious fluid, and granulocytes (Figure 6F).

*Hematology*

Average PCVs for the three rays subjected to the pre-challenge bleeding regimen remained between 30 and 32% on days 0, 3, 7, and 14 (Figure 2A). Rays in both experimental groups exhibited progressively decreasing PCVs at all consecutive time points (Figure 2A). Decrease in PCVs between days 0 and 14, or last recorded value prior to mortality, averaged 18% for the L1 group and 25% for the L3 group. The three rays that died prior to day 14 exhibited marked decreases in PCV. The L1 ray that died on day 11 had a PCV of 26% on day 0 and a last recorded value of 7% on day 7. The two L3 rays that died on days 5 and 7 had baseline PCVs of 30% and 33%, respectively. Both had last recorded values of 6%. Declining PCV levels for the L1 and L3 groups positively correlated with leech:host weight ratios (Pearson 0.731, p-value 0.007) (Figure 3A) and ulcer areas (Pearson 0.370, p-value 0.026) (Figure 3B).

Average TS for pre-challenge animals remained between 5.4 g/dL and 5.6 g/dL throughout the study, but declined progressively in both experimental groups (Figure 2B). An average decrease in TS of 1.4 g/dL occurred in the L1 group, while an average decrease of 2.9 g/dL was observed in L3 between days 0 and 14, or the last recorded timepoint. TS decrease was positively correlated with decrease in PCV (Pearson 0.587, p-value 0.045), leech:host weight ratio (Pearson 0.592, p-value 0.042) (Figure 3C), and ulcer area (Pearson 0.415, p-value 0.180) (Figure 3D), although the latter was not statistically significant.

Total white blood cell counts for the pre-challenge, L1 and L3 groups showed no significant trends during the study. Observed average WBC counts for the pre-challenge group was 18,260 WBC/µl on day 0, 19,030 WBC/µl on day 7, and 18,920 WBC/µl on day 14. The average WBC count for the L1 group was 18,040 WBC/µl, 18,070 WBC/µl, and 17,600 WBC/µl on days 0, 7 and 14, respectively. The average WBC count for L3 was 18,736 WBC/µl on day 0, 17,182 WBC/µl on day 7, and 19,470 WBC/µl on day 14.

Significant lysis of white and red blood cells was present on blood smears, therefore, differential leukocyte counts could not be reliably interpreted for a statistically useful number of samples and are not included in these results.

*Serum Chemistry*

Serum urea nitrogen, albumin, ALP, ALT, AST, glucose, creatine kinase, cholesterol, triglycerides, LDH, total bilirubin, uric acid, sodium, potassium, chloride, bicarbonate, calcium, phosphorous, magnesium, and calculated osmolality showed no significant trends between days 0, 7 and 14. The mean, median and range of fifteen pre-challenge and experimental day 0 chemistry values are reported in Table 1.

***Discussion***

Infection by 1-3 *Branchellion torpedinis* leeches led to a number of pathologic changes in yellow stingrays and was associated with host mortality in less than 14 days. Large areas of skin ulceration began as button-shaped areas of necrosis that conformed to the caudal sucker, suggesting lesions resulted from pressure induced ischemia. Hydrostatic pressures of -90 cm $H_20$ have been recorded from the caudal sucker of *Placobdella parasitica* (Gradwell 1972) and it is hypothesized that contact with host skin may be enhanced by secretion of hydrolytic enzymes that dissolve the epithelial mucous

layer (Appy 1982). Attachment of *B. torpedinis* typically resulted in skin sloughing within 7 days, followed by relocation of leeches to an adjacent area of intact epithelium. Overall tissue damage and inflammatory changes associated with caudal sucker attachment sites were significantly more pronounced compared to feeding sites, indicating pressure necrosis caused by the caudal sucker is highly traumatic to elasmobranch tissue, compared to the more restrained proboscis insertion.

Ulceration area was determined by measurements of gross epithelial loss. Pinpoint hemorrhages at presumed feeding sites were not included in calculating lesion size, but may have contributed to the physiologic effects of ulceration, including blood loss. Ulcers ranged from 1.6-12 $cm^2$ and positively correlated with leech number, but were not dependent on leech weight. Although larger leeches were anecdotally observed producing larger attachment site ulcers, this lack of correlation may have been affected by other factors, including leech behavior. In 5/6 animals from the L3 group, leeches congregated, resulting in larger coalescing ulcers. Similar to what has been suggested for other parasites, leech congregation may support reproductive strategies or represent shared or preferential feeding sites (Soler-Jiménez 2012). Leeches also exhibited mobility that contributed to larger ulcer size as described above. The largest ulcer, at 12 $cm^2$, was produced by three leeches with the lowest combined weight as they migrated down the wing of the ray.

Inflammation at attachment and feeding sites consisted predominantly of fine eosinophilic granulocytes. Although there is no complete agreement regarding classification of elasmobranch leukocytes, there is evidence to suggest their granulocytes have secretory (Hine 1987) and phagocytic (Hyder 1983) roles in inflammation. Fine-

eosinophilic granulocytes may play a role in host attempts to eliminate parasites (Dezfuli 2000). Degranulation and release of phosphatases and esterases (Hine 1987) from granulocytes may contribute to necrosis observed in host dermal and subdermal tissue.

Superficial bacteria were evident in ulcers of four rays with extension of bacteria into deeper dermal tissues of one. Skin trauma is often hypothesized as an infection route for pathogens including bacteria, viral, and fungal agents (Burreson 2006). Although, there were no gross or microscopic changes to suggest bacterial sepsis during the course of the study, ulceration must be considered a potential risk factor for invasion by secondary pathogens.

PCV, TS, total white blood cell counts, and serum chemistry in pre-challenge animals showed minimal variability from experimental day 0 values, suggesting blood sampling had minimal effect on bloodwork results. However, upon leech infection, compared to 31% average PCV levels at day 0 (n=12), ten rays had PCV levels less than 15% by day 14 and six of those rays had a PCV less than 7%. Rapid decline in PCV was observed in the three rays that died prior to day 14, with the PCV in one ray decreasing from 30% to 6% in three days. Rapid blood loss may be more poorly tolerated than slow declines that permit time for physiologic adaptation (Caroll 1984). Despite a lack of published reference ranges for yellow stingrays, the dramatic drop in PCV, coupled with pale gills and viscera, are consistent with anemia.

As anticipated, decreases in PCVs correlated with ulcer size and parasite:host weight ratios. Ulcers were associated with significant dermal hemorrhage and edema suggesting blood loss from lesion sites. Three rays that died prior to day 14 had the largest areas of skin ulceration, indicating ulcer size is a significant contributing factor to

mortality. Correlation of decreasing PCV with parasite:host weight ratios suggest parasite burden as a risk factor for disease. Furthermore, host erythrocytes within the leech gut confirms blood feeding by *B. torpedinis*.

Progressive decreases in TS were evident in all rays at all timepoints. Pericardial and coelomic fluid was associated with TS measuring less than 3.4 g/dL, suggesting low serum oncotic pressure may have been associated with effusion. The free, dark fluid was interpreted as abnormal, suggesting the presence of hemolyzed erythrocytes, and more abundant than that intermittently encountered during elasmobranch necropsies (personal observation).

Variation was present between TS measured by refractometry and total protein recorded by the Roche analyzer. In mammals, TS are used as an estimate of total protein, although TS are expected to be higher in all species (George 2001). Yellow stingray TS measured by refractometry averaged 5.6 g/dL on day 0 and 3.6 g/dL for the last recorded value. Total proteins measured on the Roche analyzer averaged 2.9 g/dL on day 0 and 1.4 g/dL as a last recorded value. This dissimilarity is consistent with a previous study in cownose stingrays (Ferreira 2010) and supports the premise that total solids do not adequately correlate with total protein in elasmobranchs. There are indications elasmobranchs produce little or no serum albumin (Metcalf 2005), which may account for the disparity. In addition to protein, refractometry results may be affected by the high concentrations of blood urea and trimethylamine oxide (TMAO) (Olson 1999) present in elasmobranchs.

The cause of decreased TS is likely multifactorial. Proteinacious fluid present in leech intestine and correlation between TS decrease and parasite:host ratios, suggest

leech feeding contributed to loss of serum analytes. Loss of blood and tissue fluid at ulcer sites may also have contributed, although statistical correlation with ulcer size was not significant. Extracellular fluid shifts to vascular spaces have been described in elasmobranchs secondary to hemorrhage and acute hypovolemia (Caroll 1984). The impact of similar homeostatic mechanisms on TS, secondary to blood loss in these parasitized rays, is unknown. Anorexia may have also influenced TS levels. Protein-deficient diets and anorexia of greater than 14 days negatively affects urea production and osmoregulation in elasmobranchs (Armour 1993, Leech 1979, Wood 2010). Food intake was reduced 50% in L1 rays and 100% in L3 rays by day 14. Negative energy balance was supported by weight loss in six rays and loss of hepatocellular lipid deposits typically present in elasmobranchs (Pike 1993) in four rays.

Serum chemistries traditionally measure the serum components comprising TS. Serum chemistries were not significantly altered in this study, despite significant decreases in TS in all rays. This discrepancy suggests the refractive index was affected by one or more analytes not accounted for by the Roche analyzer. This highlights the difficulty in assessing metabolic trends in elasmobranchs using traditional analytical methods. Past studies indicate elasmobranch metabolism is fundamentally different from mammals and bony fish. Ketone bodies are described as important metabolites for elasmobranchs, with less utilization of amino acids and carbohydrates, and negligible metabolism of lipids (Ballantyne 1997). Traditional serum chemistry panels may not include metabolically active elements more indicative of elasmobranch physiology.

Assessing serum osmolality was of particular interest in this study. Disruption of skin epithelium is often hypothesized as causing osmoregulatory disturbances in fish.

Marine elasmobranchs maintain extracellular fluids that are hyperosmotic to seawater due to high concentrations of urea and TMAO (Olson 1999). Plasma sodium and chloride levels are lower than that found in seawater (Shuttleworth 1988). Theoretically, skin ulceration could result in unabated fluid shifts, specifically, water and electrolyte influx and urea and TMAO efflux. No changes in electrolytes or calculated osmolality were appreciated in this study. This implies adequate, although potentially physiologically stressful, compensation through normal osmoregulatory mechanisms, or that no significant changes in osmotic homeostasis occurred.

Although inflammation was present at feeding and attachment sites, *B. torpedinis* infection did not elicit a systemic inflammatory response, as indicated by a lack of significant changes in total WBC values throughout the experiment. However, it is difficult to interpret changes without differential white blood cell percentages. Despite using multiple techniques, distortion and lysis of leukocytes precluded critical interpretation of blood smears. Similar difficulties have been reported by Arnold (2005).

Findings in this study indicate *B. torpedinis* has the potential to be an important parasite of certain elasmobranch species. In yellow stingrays, relatively low numbers of leeches produced significant morphologic and physiologic changes, including skin ulceration, anemia and decreased total solids, as well as death in less than 14 days. Progression of skin lesions and the trends in bloodwork suggest infections extending past day 14 would result in further host pathology and mortality. Extended trials would help assess host physiologic, immunologic, and regenerative host responses. Larger elasmobranch species that may be more resistant to the pathogenic effects of leeches may serve as more suitable models for chronic infection.

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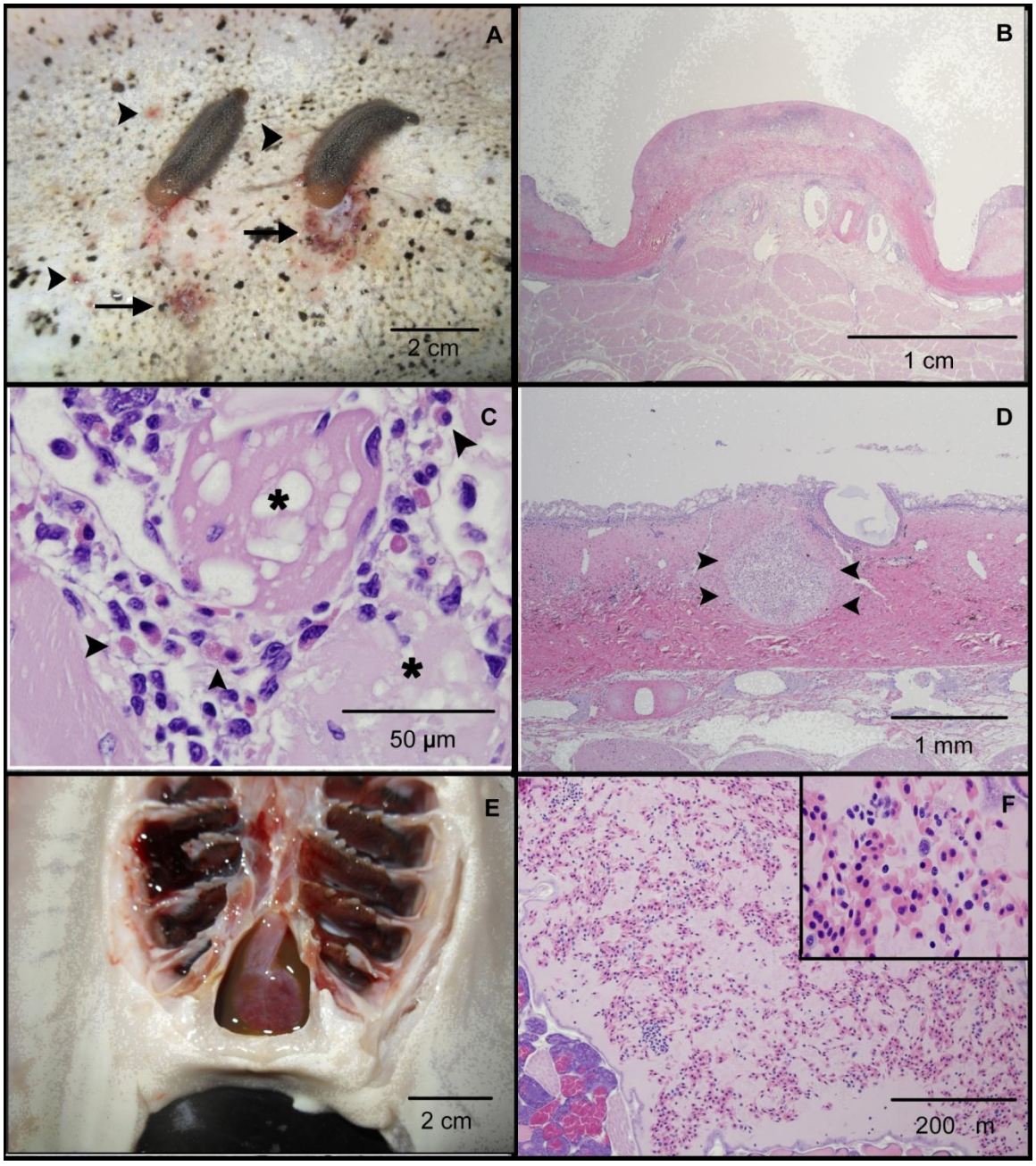


Figure 1. Gross and microscopic necropsy findings. A. Two leeches attached to the dorsum of a yellow stingray. Caudal suckers are associated with epidermal ulceration (arrows). Leeches are surrounded by multiple, pinpoint hemorrhages presumed to be feeding sites (arrowheads). B. Typical microscopic appearance of coalescing, button-shaped ulcerations associated with caudal sucker attachment sites. H&E. C. Inflammatory infiltrates of fine-eosinophilic granulocytes (arrow heads) associated with skeletal muscle degeneration and necrosis (asterix). H&E. D. Microscopic appearance of subepidermal tracks produced by the proboscis at feeding sites (arrowheads). Areas are well demarcated, filled with debris, erythrocytes and proteinacious fluid. Note minimal inflammatory reaction in the surrounding dermis. H&E. E. Pericardial fluid

accumulation associated with decreased packed cell volume and serum total solids. F. Section of leech intestine filled with abundant red blood cells and granulocytes admixed with protenacious fluid. H&E. Insert: 100X view depicting erythrocytes and proteinacious fluid within leech intestine. H&E.

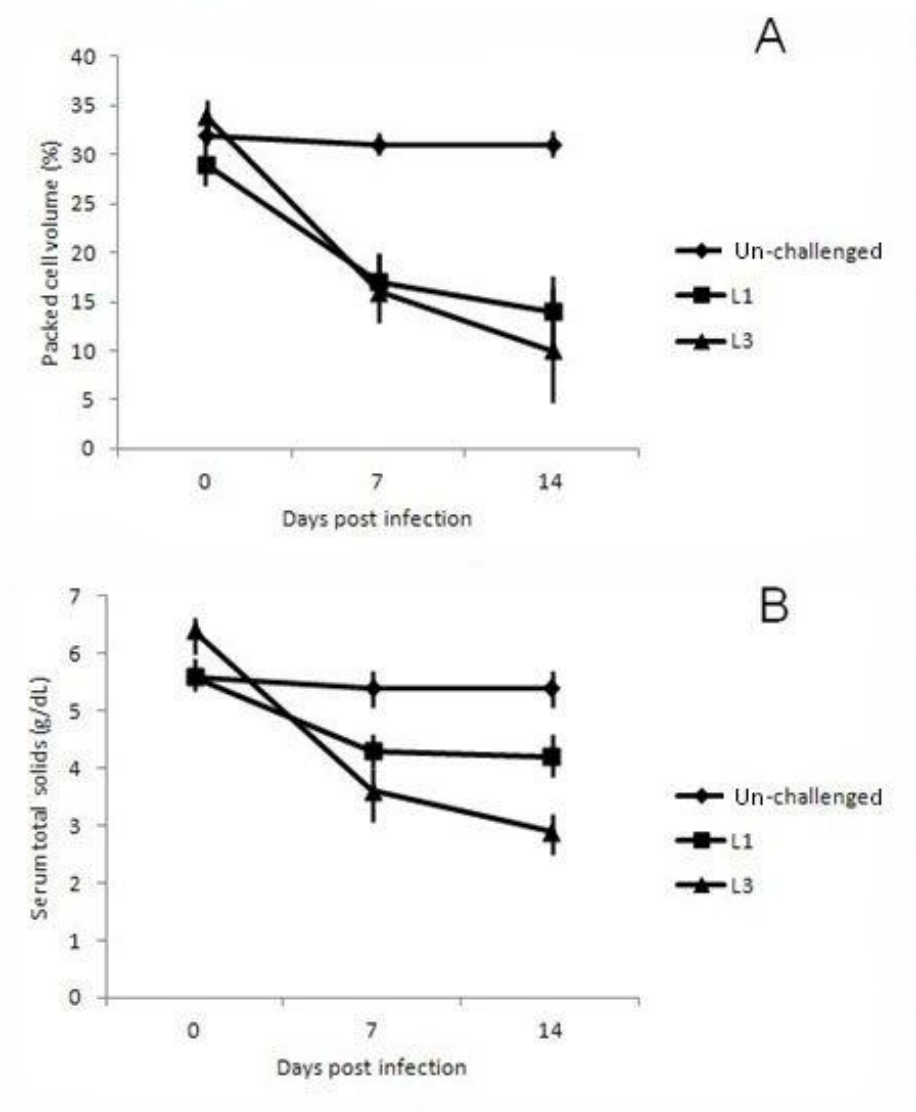


Figure 2: Hematologic trends during the pre-challenge and infection trials. Change in packed cell volume (A) and serum total solids (B) for the pre-challenge group (n=3), L1 group infected with one leech (n=6), and L3 group infected with 3 leeches (n=6). Day 3 values are not represented to allow consistency between x-axis intervals.

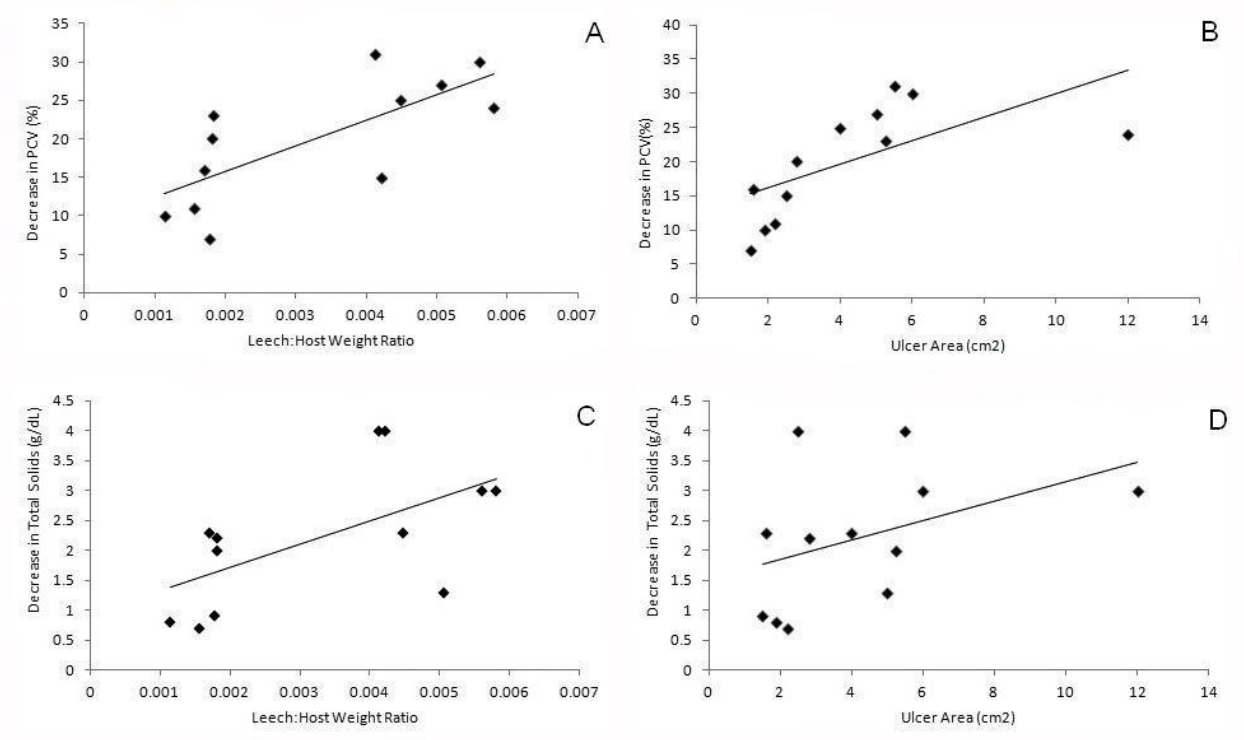


Figure 3. Scatter plots with linear trendline depicting: Decreased packed cell volume in relation to (A) increasing leech:host weight ratios (Pearson 0.731, p-value 0.007) and (B) increasing ulcer size (Pearson 0.370, p-value 0.026). Decreased serum total solids in relation to (C) increasing leech:host weight ratio (Pearson 0.592, p-value 0.042) and (D) increasing ulcer area (Pearson 0.415, p-value 0.180).

| | Mean | Median | Range |
|---|---|---|---|
| Total Protein (g/dL) | 2.9 | 2.2 | 1.3-3.8 |
| BUN (mg/dL) | 1,028 | 1,038 | 834-1367 |
| Albumin* (g/dL) | - | - | < 0.2-0.5 |
| ALP (U/L) | 12 | 10 | 5-25 |
| ALT* (U/L) | < 0.4 | - | <0.4-13 |
| Glucose (mg/dL) | 30 | 32 | 18-38 |
| Sodium (mmol/L) | 265 | 266 | 247-277 |
| Potassium (mmol/L) | 5.1 | 4.7 | 3.9-8.5 |
| Chloride (mmol/L) | 275 | 277 | 253-296 |
| Bicarbonate (mmol/L | 3.5 | 4 | 3-4 |
| Calcium (mg/dL) | 16.6 | 17.3 | 12.7-18.8 |
| Phosphorus (mg/dL) | 3.4 | 3.4 | 2.8-4.7 |
| Magnesium (mg/dL) | 3.8 | 3 | 1.7-10 |
| AST (U/L) | 51 | 32 | 8-113 |
| CK (U/L) | 953 | 979 | 232-2368 |
| Cholesterol (mg/dL) | 145 | 253 | 50-282 |
| TG (mg/dL) | 241 | 153 | 84-546 |
| Total Bilirubin (mg/dL) | 0 | 0 | 0 |
| Uric Acid* (mg/dL) | - | - | <0.2 |
| Osmolality (Osm/kg) | 901 | 926.9 | 803.2-1028.1 |

Table 1. Mean, median, and ranges for the baseline serum chemistry values of captive yellow stingrays (n=15) prior to challenge with *B. torpedinis*. Female and male results are pooled as they did not differ statistically. Analytes notated with an asterix (*) fell below instrument measurement ranges and mean and median values could not be calculated.

# CHAPTER 4

# MICROSCOPIC AND TRANSCRIPTOME CHARACTERIZATION OF THE MARINE LEECH *BRANCHELLION TORPEDINIS* SALIVARY GLAND

Marancik, D., Champagne, D., Dove, A., Camus, A.
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***Abstract***

The marine leech *Branchellion torpedinis* is a parasite exclusive to elasmobranchs. Infestations on the skin, gill slits, oropharynx, and cloaca can be severe, resulting in ulceration, anemia, lethargy, anorexia, and death. Secretory proteins of the *B. torpedinis* salivary gland are thought to be directly involved in the feeding mechanism of the leech and contribute to lesion development in elasmobranch tissue. Microscopic evaluation of the salivary gland-proboscis complex revealed abundant salivary gland tissue within the neck region of the leech surrounding the central probosics. Salivary gland somata were filled with secretory product that exhibited variable staining charactersics with hematoxylin-eosin and periodic acid-Schiff stains. Bioactive proteins of the *B. torpedinis* salivary gland were evaluated by extracting messenger RNA from salivary tissue and preparing cDNA for 454 pyrosequencing at the University of Georgia Molecular Genomics Facility. A total of 528,268 reads, with an average length of 258 bp, were recovered. Assembly yielded 4734 unique contigs, each presumably corresponding to a unique cDNA sequence. tBLASTx analysis revealed sequences encoding proteins potentially involved in feeding and lesion development. In particular, significant matches were found to peptidases belonging to the ADAMTS (disintegrin and metalloproteinase with thrombospondin motifs) protein family. Recognized anticoagulants include C-type lectins, destabilase, and homologues of antistasin. Eglin and cystatin, two protease inhibitors with known immunomodulatory activity, were also identified. A novel finding was a homologue of apyrase, an ADP phosphohydrolase with thrombocyte aggregation inhibiting activity known in saliva of blood feeding arthropods, but not previously reported from leech salivary glands. Additionally, peptidase inhibitor

16 was identified, but has unknown roles in blood feeding. Identifying bioactive salivary proteins helps characterize parasite:host interactions occurring at *B. torpedinis* feeding sites. As this is the first sialomic description of a piscicolid leech, comparison to salivary gland factors identified in other leech families contributes to our understanding of sanguivory evolution in *Hirudinea*.

***Introduction***

The salivary gland complex plays an integral role in the feeding mechanism of hematophagous leeches. Bioactive salivary gland proteins secreted into feeding sites are believed to assist in sanguivory by modulating host physiologic and defense mechanisms. Historically, most research into this area has concentrated on polypeptides from salivary glands of the jawed arhynchobdellidans, including medicinal leeches. Various proteins identified have roles in anti-hemostasis, such as hirudin (Haycraft 1884) and decorsin (Seymour 1990), preventing blood coagulation at feeding sites and within the leech gut. Proteins with putative immunomodulatory activities have also been identified in leech saliva, although evidence for functional immunosuppression is circumstantial. These include the proteinase inhibitor hirustasin, that inhibits kallikrein, trypsin, chymotrypsin and neutrophil cathepsin G (Söllner 1994), and eglin, with activities against granulocyte proteases (Baskova 2008). Many proteins are believed to have cross-functional activity. Bdellin blocks both the proaggretory affect of plasmin on platelets (Min 2010) and the pro-inflammatory activity of plasmin and trypsin (Baskova 2008).

Similar physiologic functions are described for salivary gland proteins from the jawless rhynchobdellidans, a diverse order of leeches known to parasitize freshwater and saltwater fish (Kearn 2004). These leeches feed through a straw-like proboscis that is inserted through the host skin (Sawyer 1986) and hematophagy is believed to be facilitated by salivary gland proteins released into the proboscis (Moser 1995). Histologic and ultrastructural characterization of the salivary gland-proboscis complex has been described for a number of rhynchobdellidans, revealing genus-specific morphology (Walz 1988, Moser 1995).

Between the rhynchobdellid families *Glossiphonidae* and *Piscicolidae*, salivary components have only been described for leeches belonging to the former. These include collagenolytic proteins that may aid proboscis penetration (Ricci-Silva 2005) and proteolytic and competitive inhibitors of hemostasis and inflammation (Nutt 1988, Condra 1989, Sawdesh 1990, Salzet 2000). Similar bioactive salivary gland proteins are believed to support leech feeding and digestion in piscicolid leeches, but descriptions are lacking in the literature. Piscicolid leeches evolutionarily represent a phylogenetically intermediate position connecting glossiphoniid and jawed leeches (Siddall 1998). Elucidation of bioactive salivary gland proteins of piscicolid leeches may help piece together the evolution of leech blood feeding in *Hirudinea*.

*Branchellion torpedinis* is a marine piscicolid leech that exclusively parasitizes elasmobranchs (Kearn 2004). Inadvertent introduction of *B. torpedinis* into closed aquarium settings is problematic, as parasites thrive with low environmental stress and high host availability. Parasitism of a number of demersal and pelagic elasmobranch species often results in ulceration of skin, oropharynx, gill slits, and cloaca. Severe infestations have been associated with lethargy, anorexia, and death of the host ([1]Marancik 2012, in press).

Microscopic evaluation of leeches collected from elasmobranchs at the Georgia Aquarium reveals host erythrocytes and proteinacious fluid within the leech gut, indicating active blood feeding ([1]Marancik 2012, in press). Leech feeding sites often consist of well-demarcated, 1 mm diameter proboscis tracks through the host epidermis and dermis that are associated with hemorrhage, proteinacious fluid, and variable numbers of inflammatory cells ([1]Marancik 2012, in press). The salivary gland-proboscis

complex is believed to play an integral role in *B. torpedinis* feeding (Sukatschoff 1912, Kearn 2004) and secreted salivary gland proteins may contribute to proboscis penetration, hematophagy, and clinical disease in parasitized elasmobranchs.

The *B. torpedinis* salivary gland was examined by light microscopy and the sialomic transcriptome was characterized by parallel large-scale pyrosequencing to further understand the composition and potential role of salivary proteins in this host-parasite relationship. Elucidation of bioactive salivary gland proteins from *B. torpedinis* help reveal the evolution of blood feeding in leeches, provide a platform for future physiological studies in elasmobranchs, and may lead to options for vaccine production to reduce parasite burdens in captive elasmobranchs.

## *Methods*

### *Salivary Gland Histology*

Leeches collected from captive elasmobranchs were fixed in 10% neutral-buffered formalin, serially sectioned, and routinely processed for microscopic examination. Sections were cut 4 µm thick and stained with hematoxylin and eosin (H&E). Selected sections were stained by the Alcian blue pH 1.0 and 2.5, periodic acid-Schiff (PAS), and Masson's trichrome methods.

### *mRNA isolation and cDNA library construction and sequencing*

Leech segments VI-X, containing the salivary gland complex, were dissected from freshly collected leech specimens and preserved in RNAlater® (Applied Biosystems/Ambion, Carlsbad, CA) at -80°F prior to mRNA extraction. Specimens were frozen in liquid nitrogen and ground to a fine powder. mRNA was isolated from 5 mg of powder using the Dynabeads® mRNA Direct Micro kit (Life Technologies, Carlsbad,

CA), following the manufacturer's protocol. cDNA was synthesized using the SMART kit (Clontech, Mountain View, CA) following the protocol for SMART library construction, with reverse transcription using Superscript III (Invitrogen, Grand Island, NY) and PCR amplification using Platinum Taq DNA Polymerase High Fidelity (Invitrogen, Grand Island, NY). PCR cycles consisted of denaturation at $94^{o}$ for 15 seconds, annealing/extension at $68^{o}$ for 10 minutes, and a 12 minute final extension. After an initial round of 20 cycles, the PCR amplification was monitored every 3-4 cycles by agarose gel electrophoresis, to prevent overamplification and loss of longer cDNA transcripts.

The amplified cDNA was size selected on a Chroma-Spin 400 column (Clontech, Mountain View, CA). Fractions containing cDNA > 250 base pairs (bp) were pooled into < 1 kb and >1 kb allotments to facilitate subsequent re-amplification for pyrosequencing. The small and large fractions were combined in equal proportions prior to sequencing. The cDNA sample was normalized with the Trimmer protocol (Evrogen, Moscow, Russia) prior to shearing into ~650 bp fragments and preparation of the emulsion phase library using Roche reagents. The library was sequenced by 454 pyrosequencing (Roche, Florence, SC) using the ½ plate scale. Normalization, preparation of the emulsion phase PCR library, and 454 pyrosequencing were carried out at the University of Georgia Molecular Genomics Facility (http://dna.uga.edu/).

*Data analysis*

Sequencing reads were assembled into contigs and isotigs using V230 Assembler software (Roche, Florence, SC). Blast2GO (http://www.blast2go.com) was used to compare assembled contigs and isotigs to a non-redundant database comprised of all

sequences in GenBank, NCBI, and Swissprot. For simplicity, both contigs and isotigs will be further referred to as "contigs." Contigs that matched salivary protein families known from leeches or blood-feeding arthropods were further examined for a signal peptide, indicative of secretion, or a signal anchor, using Signal P (http://www.cbs.dtu.dk/services/SignalP/). Contigs that appeared to encode secreted proteins were translated (http://web.expasy.org/translate/), and compared by BLASTp to a nonredundant database of all sequences or to sequences from leeches and arthropods (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Sequences giving high-scoring matches (generally E < 1e-04) were retrieved and aligned with the query contig using Clustal W (http://www.genome.jp/tools/clustalw/) with the default settings. Where appropriate, UPGMA (Unweighted Pair Group Method with Arithmetic Mean) rooted trees were produced using software linked at the same site as Clustal W. Protein mass and isoelectric point were calculated using the Expasy Compute pI/Mw tool (http://web.expasy.org/compute_pi/).

***Results and Discussion***

*Salivary Gland Histology*

The salivary gland-proboscis complex is contained within the neck region of *B. torpedinis*, constituting segments VI-X. Similar to what has been described in *Placobdella* spp. (Moser 1995), glandular tissue is enclosed within layers of outer radial and inner longitudinal striated skeletal muscle embedded within collagenous connective tissue (Figures 1a and 1b). Salivary gland tissue is closely associated with the muscular proboscis enclosed within the central cavity. The proboscis has an outer refractile cuticle and central lumen lined by vacuolated cuboidal cells. The body of the proboscis consists

mainly of ordered arrays of circular and longitudinal striated skeletal muscle with lesser amounts of collagen. Along the periphery of the proboscis are numerous 15-50 µm diameter pores often filled with PAS positive material (Figure 1d). As described in other rhynchobdellidans, pores likely represent the terminal portions of salivary gland ductules leading from salivary somata, where secretory product is presumably released into feeding sites (Walz 1988, Moser 1995).

Salivary gland tissue appears as 30 - 500 µm in diameter soma that are bordered by flattened to cuboidal epithelial cells and separated by interstitial space and longitudinal muscle (Figure 1c). Salivary gland product within somata exhibit variable histochemical staining properties. Soma less than 50 µm in diameter often stain uniformally eosinophilic to amphiphilic with H&E, and magenta with PAS, indicating the presense of polysaccharides. Glandular structures greater than 50 µm in diameter stain deeply basophilic with H&E and often contain a distinct, central area of eosinophilia that stains magenta with PAS. Variation in staining characteristics likely represent differences in secretory product, although microscopic staining has also been proposed to be affected by cellular physiologic state (Walz 1988). Alcian blue pH 1.0 and 2.5 staining was uniformally negative, indicating a minimal to negligible mucin component to salivary gland product within somata.

*Salivary gland transcriptome*

Pyrosequencing yielded a total of 528,268 reads, with an average length of 258 bp. Assembly generated 4,734 unique contigs, each corresponding to a unique cDNA sequence. Contigs clustered within the same isogene by the V230 assembler were found to have completely different, but highly significant, BLAST hits, indicating that unrelated

contigs were assigned to the same isogene. The results were discounted and each contig was analyzed individually.

Of the 4,734 unique contigs, over half gave no significant match to known proteins following analysis by tBLASTx. Others matched to ribosomal RNA, non-secretory structural proteins such as actin, and a vast array of housekeeping enzymes that were considered extraneous and will not be considered further in this paper.

The cDNA library was normalized to limit recovery of non-target housekeeping proteins, often abundant in conventional transcriptome analyses (Min 2010), and maximize recovery of unique sequences. Consequently, information was lost regarding abundant versus rarer transcripts. Contigs were considered as likely to encode secreted proteins based on a significant BLAST match to protein families identified in salivary glands of other hematophagous animals, the presence of a signal peptide (indicating secretion to the exterior of the cell), and absence of transmembrane domains, GPI anchor sequences, or other features that would suggest a membrane-bound protein.

Identified proteins, including ADAMTS metalloproteinases, C-type lectins, 5´-nucleotidase type apyrase, antistasin, destabilase, cystatin, and CRISP families, and a homologue of the non-classical Kazal protease inhibitor eglin, suggest that *B. torpedinis* saliva contains a pharmacopeia of bioactive proteins targeting multiple aspects of host hemostatic and immune defenses.

*ADAMTS family proteins*

Several contigs significantly matched to members of the ADAMTS (a disintegrin and metalloproteinase with thrombospondin domain) superfamily of proteases. These secretory proteins are divided into families numbered 1-20, and impart

diverse biological functions within the extracellular matrix through functional disintegrin, metalloproteinase, and thrombospondin domains (Tang 2001).

Excluding two contigs that matched to non-secreted mitochondrial pitrilysins, two sets of sequences contained a signal peptide. Four related contigs bore significant similarity to ADAMTS proteins, including an ADAMTS-15 precursor from the blood-feeding louse, *Pediculus humanus* (Accession # XP_002428665, E = $9e^{-05}$). Of these four contigs, Contig 00433 encoded a signal peptide and Contigs 00233, 07575, and 07873 were partial sequences that lacked the N-terminal portion, relative to Contig 00433 (Figure 2b). ADAMTS-15 proteins are known to block fibrinogen binding to the endothelial cell vitronectin receptor, integrin αvβ3, inhibiting blood clot formation at feeding sites (Tang 2001). A fibrinolytic 80 kDa metalloproteinase, hementerin, has been partially purified from the leech *Haementeria depressa* and enzymatically characterized (Chudzinski-Tavassi 1998), but the sequence has not been published. It is possible that this enzyme is related to the *B. torpedinis* ADAMTS proteases.

Contig 03368 encoded a 122 amino acid long polypeptide with highly significant identity to ADAMTS-17 proteases known in arthropods, including the ant *Camptonodus floridanus* (Accession # EFN73100, E = $5e^{-13}$) and the mosquito *Culex quinquifaciatus* (Accession # XP 001847379, E = $6e^{-09}$). Contig 03368 appeared to be truncated relative to other ADAMTS-17 proteins (Figure 2a), and included only the N-terminal portion of the protein not extending to the zinc-binding motif. Further work is needed to determine the sequence of the full-length cDNA.

The disintegrin, metalloprotease, and thrombospondin domains of ADAMTs proteins may impart various biologic functions at *B. torpedinis* feeding sites.

Metalloproteinases, including reprolysins secreted in snake venom, are known to break down collagen and connective tissue matrix (Gutierrez 2000, Siigur 2001) and may aid *B. torpedinis* proboscis penetration through host tissue. Cleavage of tumor necrosis factor-α, prior to release from the plasma membrane (Blobel 1997) suggests such metalloproteinases may have immunomodulatory functions at feeding sites (Ren 2011).

Disintegrin and metalloproteinases also have well described anti-hemostatic activities that may aid hematophagy during *B. torpedinis* feeding. The disintegrin motif of ADAMTS proteins consist of non-enzymatic integrin antagonists that bind αIIbβ3 integrin on the surface of platelets. Previously described in snake venom (Sánchez 2006) and saliva of jawed leeches (Min 2010), ticks (Wang 1996), and tabanids (Ma 2009), disintegrins inhibit platelet activation and aggregation and fibrinogen cross-linking, even in the presence of agonists such as ADP (Mans 2002). In ticks, metalloproteases have described fibrinolytic activity, roles in disrupting blood vessel endothelium, and inhibit thrombocyte adhesion to collagen (Francischetti 2010, Moura-da-Silva 2012). Shared characteristics between mammalian platelets and elasmobranch thrombocytes (Pica 1990), suggest secreted ADAMTs proteins likely aid *B. torpedinis* feeding through counteracting host hemostatic mechanisms.

Thrombospondins inhibit maturation, proliferation, and migration of endothelial cells, and antagonize the activity of growth factors leading to angiogenesis (Lawler 2012). Inhibition of blood vessel growth may delay wound healing and help keep lesions "open" and promote exudation at *B. torpedinis* feeding sites. Unlike many leech species that are temporary or semi-permanent parasites, (Kearn 2004), *B. torpedinis* appears to remain attached to the host for the duration of its life, resulting in more prolonged feeding

times (personal observation). Therefore, it may be advantageous for *B. torpedinis* to maintain open wounds for long periods of time.

*C-type lectins*

Eleven contigs containing a signal peptide, matched best to mosquito (*Aedes* or *Anopheles*) or sandfly (*Lutzomyia*) salivary galactose-specific C-type lectins, with E values ranging from $5e^{-06}$ to $6e^{-14}$ (Table 1). Inspection of the UPGMA tree indicates that most of the *B. torpedinis* C-type lectins are closely related to one another, and form a sister clade to the insect salivary lectins (Figure 3). However, Contig 01085 is basal to both clades and is more similar to lectoxin than any of the other *B. torpedinis* C-type lectins. Lectoxins are known from medicinal leeches (Min 2010), and similar to other C-type lectins present in arthropods and snake venom, have described anti-hemostatic mechanisms. In addition to competitive inhibition of platelets, von Willebrand factor, and collagen receptors, C-type lectins inactivate Factor IX, Factor X, and thrombin in the mammalian coagulation cascade (Atoda 1994, Charlab 1999, Lu 2005).

The presence of mammalian clotting factors has not been validated in elasmobranchs. Molecular studies support the existence of a calcium-dependent blood coagulation system, although functionally, elasmobranch plasma appears to take longer to clot and is less senstitive to calcium than that of mammals and boney fish (Doolittle 1962, reviewed by Davidson 2003). Biochemical studies indicate elasmobranch plasma contains prothrombin, thrombin, and fibrinogen, which may imply the presense of Factor Xa (Doolittle 1962). Indirectly, the presence of C-type lectins, and as will be discussed, other factors in *B. torpedinis* salivary gland known to inhibit Factor X, additionally support the presence of a functional clotting cascade in elasmobranchs.

*Apyrase*

Contig 00624 encoded a 20.4 kDa protein with significant similarity to 5´-nucleotidase type apyrases from ixodid ticks such as *Ixodes scapularis* (Accession # XP_002413593, E = $2e^{-22}$) and argasid ticks such as *Orithodoros savyngi* (Accession # ABS30896, E = $2e^{-20}$). Alignment with tick apyrases suggests the sequence is slightly incomplete at the 5´ end, as it lacks the region corresponding to the signal peptide and the first few residues of the mature protein (Figure 4).

Secreted apyrases are ubiquitous and prominent enzymes in the saliva of hematophagous arthropods. 5´-nucleotidase family apyrases are found in mosquitoes, blackflies, triatomine hemipterans, and lice, whereas bedbugs and sand flies have a distinct "Cimex-type" apyrase, and fleas have an apyrase related to vertebrate CD39 (reviewed by Francischetti 2010). Apyrases function by degrading tri- (ATP) and di-phosphonucleotides (ADP) released from damaged endothelium and activated platelets to the inactive monophosphate form (AMP + Pi) (Komoszyński 1996). ADP is an important stimulator of platelet aggregation in mammals and elasmobranchs (Pica 1990, Gachet 2008) while ATP activates neutrophil responses and induces nociception in mammals (Kuroki 1994, Wang 2012). Secretion of salivary apyrase into feeding sites may impede host hemostasis and suppress inflammatory and nociception responses at *B. torpedinis* feeding sites.

*Antistasin*

Contig 00837 and Contig 04418 had significant similarity to members of the antistasin family (pfam02822) of serine protease inhibitors (Figure 5). Antistasins are derived from members of the *Glossiphoniidae* and are known to bind arginine residues

and inhibit Factor Xa in the coagulation cascade (Brankamp 1990, Chopin 2000). High sequence similarity to one another, suggest Contig 00837 and Contig 04418 to be paralogues. Contig 00837 was most similar to ghilanten from the Amazon leech *Haementeria ghilianii* ($E = 1e^{-10}$), although significant similarity to antistasin from the Mexican leech *Haementeria officinalis* ($E=1e^{-9}$) and to therostatin from *Theromyzon tessulatum* ($E=2e^{-04}$) was noted. Contig 04418 matched most closely to antistasin ($E=2e^{-12}$), with high similarity to ghilanten ($E=3e^{-11}$).

Both contigs were markedly less similar to antistasin family-like proteins from jawed leeches, including guamerin from *Hirudo nipponia* (E=.003 and .007 for Contig 00837 and Contig 04418, respectively), hirustatin from the medicinal leech *Hirudo medicinalis* (E=.004 and $5e^{-04}$), and bdellostatin, also from *H. medicinalis* (E=.026 and .001). Guamerin, hirustatin, and bdellostatin lack direct anti-Factor Xa activity and instead impart anti-hemostatic and immunomodulatory capabilities via inhibition of kallikrein, trypsin, plasmin, cathespin G, and elastase (Jung 1995, Söllner 1994, Kim 1998). Collectively, this suggests that the two *B. torpedinis* proteins most likely target the coagulation cascade, specifically by inhibiting Factor Xa.

*Destabilase*

The cDNA library yielded one contig encoding a 13.2 kDa protein with a significant match (98% coverage, 52% identity, $E = 1e^{-44}$) to *H. medicinalis* destabilase I (Figure 6). Destabilase activity is comparative to antimicrobial lysozymes in their ability to break isopeptide bonds within bacterial cell walls (Zavalova 2010). Destabilase also has activity in breaking isopeptide linking within fibrin clots and inhibition of platelet aggregation, and may have roles in disintegrating blood clots at leech feeding sites

(Baskova 1991). Contig 02570 also had significant identity with a destabilase from the salivary secretion of the tick *Ixodes scapularis* ($E = 7e^{-20}$), and destabilases/lysozymes from blood-feeding insects such as mosquitoes. In comparison to the *H. medicinalis* destabilase, eleven of fourteen cysteine residues were conserved, implying similar but not identical protein folding and formation of disulfide bonds.

*Eglin*

Contig 03667 encoded a 7.5 kDa highly basic (calculated pI = 9.32) protein with significant identity (coverage = 98%, identity = 45%, $E = 9e^{-10}$) to the potato protease inhibitor I family protein eglin, from *Hirudo medicinalis* (Figure 7). Sequence conservation is especially high in the C-terminal region of the protein, including the reactive site loop (underlined in Figure 7). Eglins isolated from the salivary gland of medicinal leeches represent a number of proteins that inhibit granulocyte proteases *in vitro* and may act as an antibiotic to prevent putrefaction of blood stored within the crop and gut of leeches (Baskova 2008).

*Cystatins*

Contig 00873 has significant identity with Kazal family cysteine protease inhibitor. In particular, high identity with cystatin B (Accession # AAN28679, $E = 9e^{-33}$) from the glossiphoniid leech *Theromyzon tessulatum* was noted (Figure 8). Cystatins are part of a large group of reversible cysteine peptidase inhibitors found in a wide variety of organisms, with broad specificity against endo- and exo-peptidases involved in regulating normal physiologic processes (Nicklin 1984, Müller-Esterl 1985). Cystatin B is thought to have a role in innate defenses in *T. tessulatum*, as expression is upregulated following bacterial challenge (Lefebvre 2004). As components of the tick sialome, cystatins inhibit

a number of cathepsins with roles in dendritic cell antigen processing in mammals (Sa-Nunes 2009). *In vitro* and *in vivo* studies with cystatin demonstrate impaired dendritic cell maturation, decreased MHC II presentation, and reduction in pro-inflammatory cytokine activity (Salát 2010). Similar MHCII genomic organization has been found between nurse sharks and mammals, with the presence of cathepsin L orthologs, suggesting conserved antigen presenting mechanisms in elasmobranchs (Cristcitiello 2012). The resultant decrease in recruitment and activation of mononuclear inflammatory cells may have roles in host immunosuppression.

*Peptidase Inhibitor 16*

Contig 07610 encoded a secreted 19.5 kDa protein with significant ($E = 7e^{-05}$) identity to a family of proteins annotated as "venom allergen-like" and "peptidase inhibitor 16-like", known from a variety of helminthes including strongylids, hookworms, and schistosomes. Alignment of Contig07610 with a venom allergen/peptidase inhibitor 16 protein from the strongylid *Heligmosomoides polygyrusbakeri* (Figure 9) indicates that the leech protein is conserved in the N-terminal region, but it may be significantly truncated in the C-terminal region compared to helminth sequences. Contig07610 encodes a highly basic protein, with a calculated pI of 9.03. Much of the protein (residues 8-126 of the mature polypeptide) consists of a SCP-like extracellular domain ($E = 9.0e^{-09}$), and residues 25-120 give a slightly weaker match ($E = 3.21e^{-07}$) to a cysteine-rich secretory protein family domain. Both domain types are characteristic of CRISP (cysteine-rich secretory proteins), antigen-5 related proteins, and pathogenesis-related 1 proteins, found in a diverse array of organisms. CRISP and antigen-5 proteins are commonly found as components of the salivary secretion of blood-

feeding arthropods, though in most cases their function is obscure (Alves-Silva 2010, Rohoušová 2012).

*Evolutionary and life-history implications of leech salivary gland transcriptome*

Phylogenetic studies suggest leeches developed from a single sanguivorous ancestor that lacked jaws or a proboscis (Borda 2004, Siddall 2006). Later in evolution, leeches diverged into taxonomic families exhibiting various life history characteristics, including host specificity and feeding strategy (Apakupakul 1999). Molecular and morphologic systematics indicate evolution of blood feeding that can be traced through divergence of the *Rhynchobdellida* and then later in evolution, divergence of *Arhynchobdellida* (Borda 2004).

As piscicolid leeches comprise an evolutionary intermediate position between glossophiniid and jawed leeches (Siddall 1998), transcriptome data of *B. torpedinis* suggests some salivary factors may have arisen earlier in leech phylogeny than previously believed (Min 2010). In particular, destabilase, and eglin, formerly considered to be phylogenetically restricted to arhynchobdellids, appear to have origins in piscicolid leeches (Figure 10).

Some factors that are present in glossophiniid and jawed leeches, such as the platelet aggregation inhibitors saratin and decorsin (Min 2010), were not found in the *B. torpedinis* transcriptome. There were also a relatively low number of thrombin inhibitors identified. A more comprehensive understanding of elasmobranch platelet-endothelial interactions and platelet cell receptors may help explain if loss of these proteins resulted from adaptations of *B. torpedinis* to elasmobranch-specific physiology.

A number of proteins novel to leeches were identified in *B. torpedinis* salivary gland, including ADAMTs proteins, apyrase, and cystatin. The collagenolytic and cartilaginolytic mechanism of metalloproteinases and the ability of thrombospondins to delay wound healing, may be directly related to the relatively prolonged feeding period exhibited by *B. torpedinis*, as compared to most leeches. Apyrase and cystatin offer evidence for the presence of functional physiologic and immunologic processes in elasmobranchs that have yet to be described. The role of apyrase in impeding ATP and ADP mediated inflammation and coagulation, and cystatin inhibition of MHC II presentation, offer evidence that these mechanisms exist in elasmobranchs. Similarly, the presence of antistasin and C-type lectins suggest the presence of a functional clotting cascade in elasmobranchs that include factor IX and X. Knowledge of these mechanisms provides a platform for future physiologic and immunologic studies in elasmobranchs, including assays to validate the effect of salivary proteins on host hemostasis and immunologic responses.

An understanding of the *B. torpedinis* sialome may also aid treatment and care of elasmobranchs affected by leeches in aquaria. Bioactive proteins involved in leech feeding likely play a role in lesion development, hemorrhage, and clinical disease in aquarium infested elasmobranchs. Localized immunomodulation may have influences on leeches as vectors of disease, and susceptibility of hosts to secondary infections (Yeld 2006, Tiene 2007). In addition, previous studies have demonstrated that parasitized zebra sharks *Stegostoma fasciatum* respond humorally to *B. torpedinis* salivary gland extract ([2]Marancik 2012, in press). The present characterization of secreted salivary gland

components expand identification of potential antigens, which may aid in reducing parasite burdens in aquaria through vaccine production.

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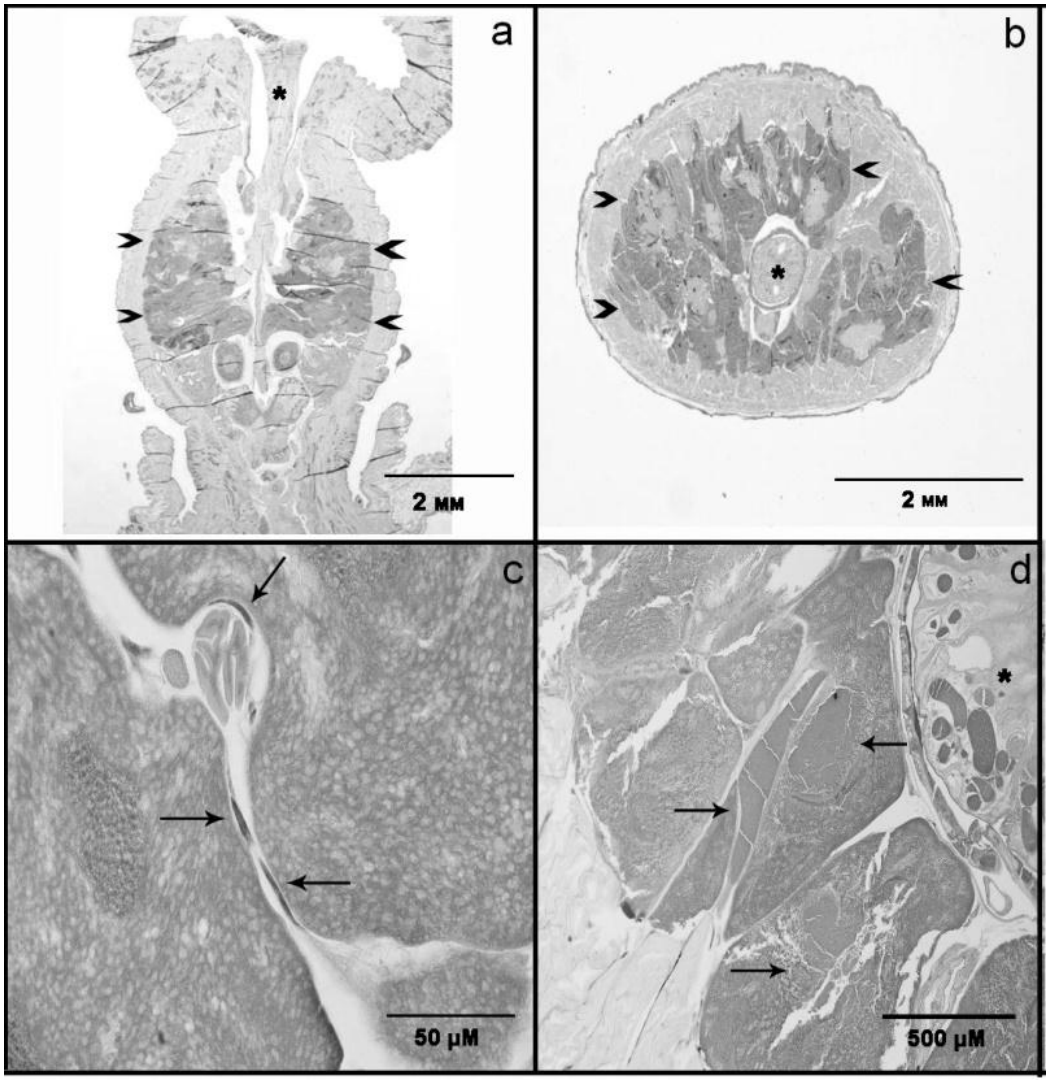


Figure 1. Histologic sections of *Branchellion torpedinis* salivary gland. Longitudinal- (a) and cross-section (b) through segments VI-X showing the salivary gland (arrowheads) enclosed within skeletal muscle and surrounding the proboscis (*). (c) Soma filled with secretory product are surrounded by flattened cells (arrows). (d) Large soma contain secretory product with various density and staining characteristics (arrows). Within the proboscis (*) are numerous pores filled with presumed secretory material.

2A

```
XP_001847379      -MVENNRNRCPSVNLIASAGQLHKNLKHYETLHADQLTHRITKRGAKPSSHPFNTIKEVEE
FN73100           MDKRNALLVYCIFITLQSIHGLHRSLKYYETIHSTHFQHRIVKRGINHSYNPYNKISEIE
isotig03368       ----MRLVLCIFSTILVFAGASLKERLRTFDLFSTHFKHSIGKRHANKQ-----STKHLE
                         :              :.      :.: :: * * **  : .     . ..:*

XP_001847379      FKVLGRNFRLILHPHKDVLHSNFRAYSVDGSGAESIVHLDHDNFLRGRVFGEVHSQVNAH
EFN73100          FYSHGRHFRLILTPRREVIHSNFKAYEVNADGKEKTIHLDHDNFYHGRVFGEIESHAQMH
isotig03368       FNSFGRNFRLNLIRHTSIFTNDFKVIAVDSKGEKEVP--FEPELYHGKLQDEPDSDVKFY
                  *   **:*** *  : .:: .:*:.  *:..* :.     . :: :*:: .* .*..: :

XP_001847379      LED-GILTASVFLPDETYHIEPSWRHLDHLSDRHMIAYKASDIKFSWDQVDAVGGEMGGV
EFN73100          IDN-GILTGSITTLDETYHIEPSWRHLPHLDNQTMIIYKSSDVKLSWEHYKDGEGHTHGA
isotig03368       LDDNGEFTGTIFS----------------------------------------------
                  ::: * :*.::
```

2B

```
contig07873       ----------------------------NVTIRIIFVIDKKLYDRWLKFAGGNSTQADAAL
isotig00433       MAFLNLLPLLLLAAVVALVTCDEKDKYDVTVKVVFVIDNGLYKRWVKFAG--EKKVDAAV
isotig00233       ------------------------------------------------------------
contig07575       ------------------------------------------------------------

contig07873       NYFYSQVFTKARSKLQAETKDFANFDLVFADSVVDKTGELLPPELNNDDPVIFGKPSEFR
isotig00433       NYFYSQVFTKARSKLQAETKDFANFDLVFADSVVDKTGTKLTGDLNNEETSLFIKPTGFR
isotig00233       ------------------------------------------------------------
contig07575       ------------------------------------------------------------

contig07873       KYLKANKLILKKYTNAVLFTS-QGIPSSFSVETLENSMSTCSVVKEDGLISTVNAVIEEI
isotig00433       EYLKEIHNELNNYTTAILFTSRQDFLPSFSLETLRYSMTTCSVVTENGLIPTVNAVVGEI
isotig00233       ------------------------------------------------------------
contig07575       ------------------------------------------------------------

contig07873       AGMFRNTIAD-CKDRIVSGVDKEGFSKLNPCIKDKMKKLISERYTNQVNNYDDYLVASPL
isotig00433       AKMFRKDANDNCKEGDFKGVDKDGFSNLTRCLKTKMKKIIAEKYVNRASSFDDYLAPSPL
isotig00233       --MFRYKIVD-CKDDVVSRVDEFGFSSLSPCIKDKMRALIYEN---RTTRLLDYLAPSPL
contig07575       --MFRDNENE-CNTKSSNVIDKTGFSNLGRCIKRKMREFLSSEIN-----FDDYLVSSPL
                    ***    : *:    . :*: ***.*  *:* **: :: ..         ***..***

contig07873       NQKLDAFPPSMPPNLSADDQCTLLYGTGSTICKRNE-----MCASLICRKNGVCKTSG-I
isotig00433       DWKLDVFPSSIPPALSATEQCELLHGAGWTICEEDKKGGK-MCATLRCKNNGKCRKST-F
isotig00233       GRKLDSLPPSAPPPLSAHEQCNLLYGPESTSCQTGSR----LCVSLRCKKDGKCRDSR-F
contig07575       SAKLDSYPSAIPFPMNPDVHCAFLYGSLSTSCKTPKIEGKSICAALGCMKGNECKHYKGV
                  . ***  *.: *  :..  :* :*:*.  * *:  .     :*.:* * :.. *:    .

contig07873       KAFEGTNCGGKRVCGASRCVRSRG
isotig00433       KAFEGTKCEDQKVCGASKCVWDRR
isotig00233       KAFEGTSCGGNKVCGASRCVQSRG
contig07575       KAFEGTECEKGKVCGASKCVKA--
                  ******.*   :*****:**
```

Figure 2: Alignments of *Branchellion torpedinis* ADAMTS proteins. a) Alignment of contig 03368 with ADAMTS-17 family proteins from *Camptonodus floridanus* (EFN73100) and Culexquinquifaciatus (XP_001847379). Note that Contig 03368 is incomplete, corresponding only to the 5´-end of the insect proteins. Both insect

sequences are truncated at the 3´-end for space. The signal peptide of Contig 03368 is underlined. b) alignment of ADAMTS-15 related contigs. Only Isotig 00433 is complete at the 5´-end; the signal peptide is underlined.

| Contig/Isotig | Most Relevant tBLASTn Hit | Sequence Coverage | Maximum Identity | Accession Number | E value |
|---|---|---|---|---|---|
| ctg00020 | *Aedes aegypti* galactose-specific C-type lectin | 83% | 33% | XM_001661725.1 | 1e-09 |
| istg00023 | *Aedes aegypti* galactose-specific C-type lectin | 98% | 28% | XM_001661725.1 | 4e-09 |
| istg00058 | *Danio rerio* novel protein containing lectin C-type domains | 80% | 31% | XR_044936.3 | 1e-10 |
| istg00059 | *Aedes aegypti* galactose-specific C-type lectin | 77% | 33% | XM_001657798.1 | 5e-10 |
| ctg00287 | *Aedes aegypti* galactose-specific C-type lectin | 77% | 31% | XM_001657798.1 | 4e-10 |
| istg01085 | *Lutzomyia longipalpis* 16.3 kDa salivary protein | 99% | 25% | AY445934.1 | 4e-08 |
| istg01259 | *Sarcophaga peregrina* CLEM 36 mRNA for C-type lectin | 89% | 33% | AB030307.1 | 3e-10 |
| istg01414 | *Aedes aegypti* galactose-specific C-type lectin | 76% | 33% | XM_001661725.1 | 1e-08 |
| istg02042 | *Salmo salar* serum lectin 2 (ssl-2) | 49% | 37% | NM_001123722.1 | 5e-06 |
| istg02257 | *Anopheles gambiae* str. PEST AGAP005332-PC | 98% | 29% | XM_315346.4 | 6e-08 |
| istg03758 | *Salmo salar* serum lectin isoform 1 | 97% | 31% | NM_001123569.1 | 6e-14 |

Table 1**:** Best or best annotated tBLASTn hits for all *Branchellion torpedinis* contigs encoding C-type lectins.

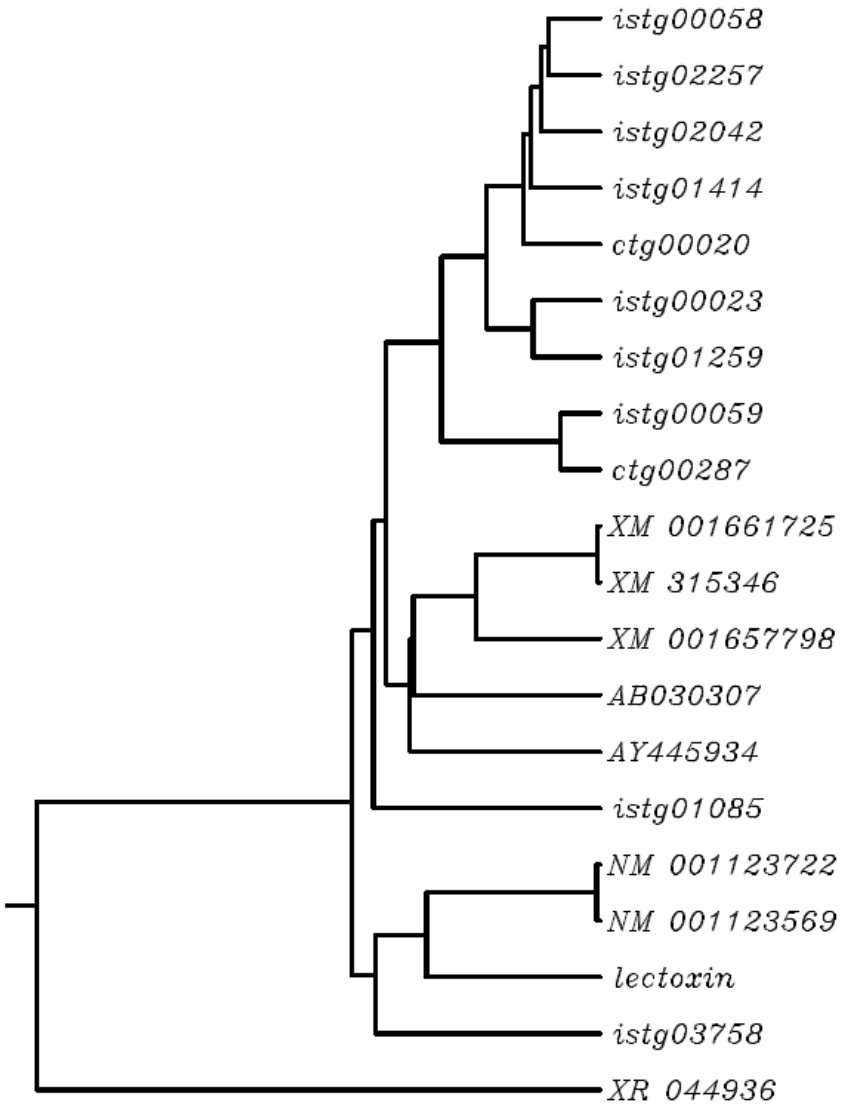


Figure 3. UPGMA tree of *Branchellion torpedinis* and related C-type lectins. Non-*B. torpedinis* sequences are from the medicinal leech *Hirudo medicinalis* (lectoxin), the mosquitoes *Aedes aegypti* (XM 001661725, XM 001657798) and *Anopheles gambiae* (XM 315346), the sandfly *Lutzomyia longipalpus* (AY 445934), the zebrafish *Danio rerio* (XR 044936), and the salmon *Salmo salar* (NM 001123722, NM 001123569).

```
XP_002413593      ---------------MMADAYFAYYADMNTSSPELWSNVNAAIINGGTIRAPLQQGD-IT
ABS30896          ADNKTCRLNECNMLNMVTDSFLAYYADQ-ESPENMWSSVAAAVVNSGFARSSLPKSNSLT
ctg00624          ------------------------------------------------------------


XP_002413593      LGAILTTAPFGQTIIDVTLNGSALRQMFEHSVANFSYKNK-KGEFLQVSGMRVVYNLSLP
ABS30896          MFDIMRALPYESSLVVLTLKGTHLRKMFEHSVAQFTVTADPRGEFLAVSGMKVKYDLARA
ctg00624         GDAISVFPFGNDLVLVTLNGSMLHEVFEHSVAGFSTQDR-AGKYLQVSGVKVEYDFQAK
                     : . *: . :: :**:*: *:::****** *:      *::* ***::* *::

XP_002413593      SSCRVVSLKILCTRCKVPKYEDVEDTKNYTIVTTDFVARGGDGFAKAEIYGQ--SGP--V
ABS30896          PNKRVVSLRILCTQCVVPRYEIVRRNETYRIATTSYIANGGDGFEFDEEVTK--ETKGVV
ctg00624          KRVKSLKVKVDG------KYKKVKDSTDYRIIIPSYLANGGDGYKVFSMVSDTILNIDIL
                     : :.:::        :*: *. .  * *  ..::*.****:   .   .       :

XP_002413593      DFEVLVWYIKTMSPIKTPIEGRIIIEG----------------NVTEPSINSKKLSPQKL
ABS30896          DSEVYLPYIMKMSPLKTPVEGRVLIR-----------------NYPKPVIGSRYDMSWKQ
ctg00624          DVDVFLEYLKDKSPITACIEGRILGMQTFDGWDELFPNFGNNCNGASCKTCSSKVLPMGL
                  * :* : *:   **:.: :***::                   * ..    *    .

XP_002413593      F-----------
ABS30896          EIWV--------
ctg00624          LLVSRFLQKGPW
```

Figure 4. Alignment of the *Brancehllion torpedinis* putative apyrase with salivary apyrases from the blood-feeding ticks *Ixodes scapularis* (XP_002413593) and *Ornithodoros savignyi* (ABS30896). The *O. savignyi* apyrase has an N-terminal extension relative to apyrase from ixodid ticks and *B. torpedinis*; this extension is truncated here.

a

```
guamerin          MTMTKVDENAEDTHG-LCGEKTCSPAQVC--LNNECV-CTAIRCMIFCPNGFKVDENGCE 56
hirustatin        ------------TQGNTCGGETCSAAQVC--LKGKCV-CNEVHCRIRCKYGLKKDENGCE 45
bdellostatin      -----FDVN---SHTTPCGPVTCSGAQMC--EVDKCV-CSDLHCKVKCEHGFKKDDNGCE 49
therostatin       ----DCENT---ECPRACPGEYEFDEDGC--NTCLCKGCNDAQCRIYCPLGFTTDANGCE 51
ctg00837          ---YKIVVLPEMKCRLGYDLFDCELGTVCNNIHFNCS-CDEVPCRMYCSKGFKYGPDGCQ 56
ctg04418          --------GAGLQCRLGHDLFDCEEGTTCNSIHFNCS-CSQVRCRMHCRFGFRHGPDGCQ 51
ghilanten         ----------QGPFGPGCEEAGCPEGSACNIITDRCT-CPEVRCRVYCSHGFQRSRYGCE 49
antistasin        ----------QGPFGPGCEEAGCPEGSACNIITDRCT-CSGVRCREHCPHGFQRSRYGCE 49
                                                 *     *  *   *   *  *:  .  **:

guamerin          YPCTCAD-----PLESTCSMQACA------------------------------------ 75
hirustatin        YPCSCAK-----ASQ---------------------------------------------- 55
bdellostatin      YACICAD-----APQ---------------------------------------------- 59
therostatin       SFCTCNT-----RET-VCQNVVCSGKRVCNPRSGRCE----------------------- 82
ctg00837          H-CECSSEAYPKVPYEGCPDLRCSLDCPYQYKVNREGCEICSCEKPP------TTPTTTT 109
ctg04418          E-CHCAS--VPAASIEGCPEVRCSLKCRYRYKLDQYGCEICGCDDTPPITTTTTAPTTEA 108
ghilanten         V-CRCRT----EPMKATCDISECPEGMMCSRLTNKCDCKID------------------- 85
antistasin        F-CKCIL----EPMKATCDISECPEGMMCSRLTNKCDCKID------------------- 85
                    * *

guamerin          ------------------------------------------------------------
hirustatin        ------------------------------------------------------------
bdellostatin      ------------------------------------------------------------
therostatin       ------------------------------------------------------------
ctg00837          TTA-----------------------MPKKTRELLDKCLKLSKKNKFTKWFKGYKHCKG 145
ctg04418          TTTKATTPKATTIKAAPTTKKAHPSLFLPIKIRERLEHCRKLKKKFKFLKYIKGFSYCRN 168
ghilanten         ---------------------------------INCRKTCPNGLKRDKLGCEYCECKPKRK 113
antistasin        ---------------------------------INCRKTCPNGLKRDKLGCEYCECRPKRK 113


guamerin          ----------
hirustatin        ----------
bdellostatin      ----------
therostatin       ----------
ctg00837          ILTKYGYAY- 154
ctg04418          LLLKYSYLNL 178
ghilanten         LVPRLS---- 119
antistasin        LIPRLS---- 119
```

**b**

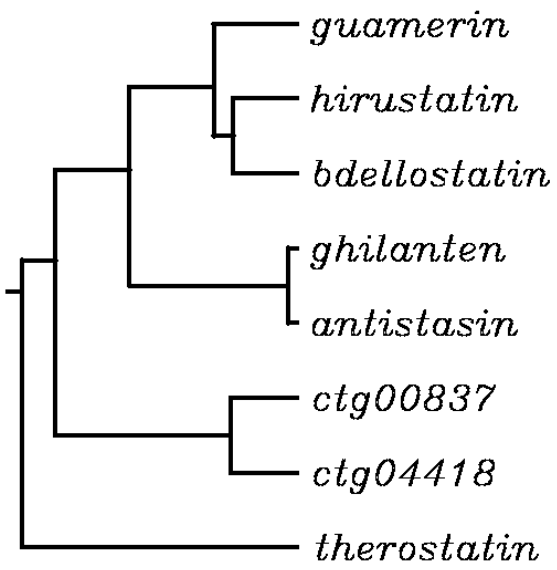


Figure 5. Alignment and UPGMA tree for *Branchellion torpedinis* antistasins. a) Alignment of contigs 00837 and 04418 with guamerin, hirustatin, and bdellostatin from jawed leeches, and with ghilanten, antistasin, and therostatin from proboscis leeches. The two antistasin domains in ghilanten are underlined. b) UPGMA tree showing relative similarity of *B. torpedinis* antistasins with antistasin family proteins from jawed and proboscis leeches.

```
H_med_destab       -----MIIAIYVSLALLIASVEVNSQFTDSCLRCICKVEG-CDSQIGKCGMDVGSLSCGP
contig02570        ------MKTFLFLSVLAAASFLVQSRVPSNCLRAICEVEG-CDRLIGRCNPDGGSDSCGP
I_scap_destab      MHRSTTAACLVLCTVLGAAVAQTVDDVTRQCLDCICQASTKCNTKLACTNAGPNSYYCGP
                           : .  .*  *   . . .. .** .**:..  *:  :.  . . .*  ***

H_med_destab       YQIKKPYWIDCGKPGGG--YESCTKNKACSETCVRAYMKRYGTFCTGGRTPTCQDYARIH
contig02570        YQIKNAYWIDCWRPGNS--WRECAKQKECSERCVHSYMKRYLRGCDITTTNICEKYARLH
I_scap_destab      YQISYAYWVDAGKPGDYPHFEGCLKDKRCSEATVVNYMNKWGTDCDGDGVVTCYDYARMH
                   ***. .**:*. :**.   :. * *:* ***  *  **:::   *    .  * .***:*

H_med_destab       NGGPRGC--KSSATVGYWNKVQKCLR----------------
contig02570        NGGPNGC--RWSSTNVYWQRVKAR------------------
I_scap_destab      KAGRTGCPATWVDSTDYWDLFEQCMGGNQAGGLDARRSKPKT
                   :.*  **      :  **: .:
```

Figure 6. Alignment of *Branchellion torpedinis* Contig 02570 with destabilase I from the medicinal leech *Hirudo medicinalis* (Genbank AAA96144) and with a salivary destabilase from the tick *Ixodes scapularis* (Genbank XP_002410814). The first residue of the mature protein (after cleavage of the signal peptide) is shown in blue, and cysteine residues conserved between the *Hirudo* and *Branchellion* sequences are shown in red. The destabilase domain (pfam05497) is underlined.

```
ctg03667        ---DSEGAFFEKLVGKHKNEAKELIKGRFPDHHVVILPERSAVTLDYRTDRIRVFVNP-Q
Eglin           TEFGSELKSFPEVVGKTVDQAREYFTLHYPQYDVYFLPEGSPVTLDLRYNRVRVFYNPGT
                   .**   * ::***  ::*:* :. ::*::.* :*** *.**** * :*:*** **

ctg03667        QKVIAVPHIG
Eglin           NVVNHVPHVG
                : *  ***:*
```

Figure 7. Alignment of *Branchellion torpedinis* Contig 03667 with eglin from *H. medicinalis* (Genbank CAA25380). The alignment shows the mature peptide, with signal peptide removed. The reactive site loop, necessary for protease inhibition activity, is underlined.

```
AAN28679          MPLCGGTSDVKQADGKTQEIVDKVKHHVESKINKSFKEFKAIQFSSQVVAGTNYFVKVHV
isotig00873       MPLVGGLGSVKEADDFVQQLCEKLKEAVETKTGQKYSIFKAVKYASQIVCGTNYFIKVHV
                  *** ** ..**:**. .*:: :*:*. **:* .:.:. ***:::::**:*.*****:****

AAN28679          GEEEYLHLRVFAPLPSSGGQPELHGIEYPKTKHDELKYFNPN--
isotig00873       GGEDYLHLRVHHPLPCYGNTPQLDGLLKGQRLTDEIKHFECKSE
                  * *:******. ***. *. *:*.*:   :   **:*:*: :
```

Figure 8. Alignment of *Branchellion torpedinis* Isotig 00873 with cystatin B (AAN28679) from *Theromyzon tessulatum*.

```
ctg07610        --MAFKSLFIF---------FALVVLTDQELLTEKERTEFVKILNYNR------------
pep16           MRLVFLALLTITWTSHSDAHRGKRAVCDNSKMSDTIREQILTFHNDARRSVAKGVEPNKS
                  :.* :*: :          .  .: *:. :::. * :::.: *  *

ctg07610        GMLEKAQRLKKLQWSKDLEQMALDRLDTCIYKSSIKEGSLSGEWVAYKQFYP-------A
pep16           GLLNPAKNMYKLEWDCNMEQQAQNDITGCKGKEPVPNMGKNIMWWTGTSFNNPAGTINST
                *:*: *:.: **:*. ::** * : :  *  *..: : . .  * : ..*         :

ctg07610        ITNWWRQRSKLAIEKQQCRLETGCVDYINWMCNMTSKVGCAYKRCNADISHKKFCKDR--
pep16           LSNWWGTAKKVGVDSENRYTDSGLYYFSNMVFYKTTKIGCAYEVCDEELTFTCLYDQAGY
                ::***   .*:.::.::   ::*   : * :   *:*:****: *: :::.. : .:

ctg07610        --------------------------KKCDNTDCEYG----------------------
pep16           FTNAMMWETGEACSADSDCTTFEPRAHSTCDNGLCIKGPDIPEPNEMCPENEGMSDWARE
                                          ..***  *  *

ctg07610        ---------------GCASKGNG-------------------------AFLCLFDNNCYQ
pep16           KYLDLHNSYRSSVARGLEPDGLGGYAPKAAKMQKMVYDCALEATALRQAKKCVYEHSKIR
                              *  ..* *                         *  *::::.  :

ctg07610        K------------------------GSKW--------------------------------
pep16           PGFGENIFANSWLNLDKIEVAKQSSGTWWRELKKHGVGPENILKRDMVSKIGHYSQMAWD
                                       *: *

ctg07610        ------------------------------------------------------------
pep16           ATYRLGCGVAHCPTMTFAVCQYGPAGNYPDSPIYTIGEPCSGCSRSDACSATEGLCIAA
```

Figure 9**.** Alignment of the putative venom allergen/peptidase inhibitor 16 Contig 07610 from *Branchellion torpedinis* with a homologue (Genbank AEP82925) from the strongylid *Heligmosomoides polygyrusbakeri*. The signal peptide is underlined.

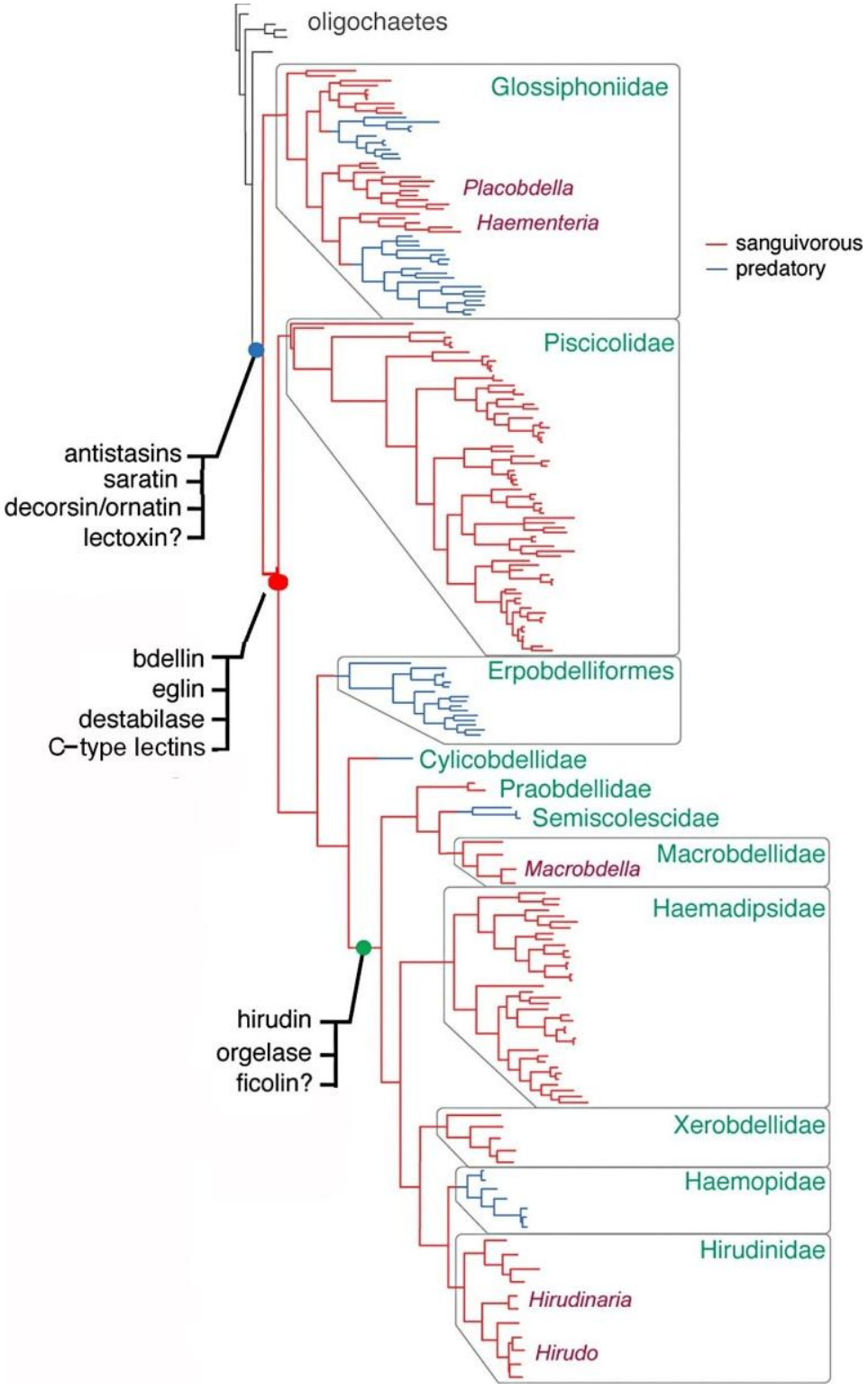


Figure 10. As adapted from Min (2012), phylogenetic relationships of leeches with inferred positions for the origins of various bioactive salivary proteins at the divergence of glossophiniid (blue dot), piscicolid (red dot) and arhynchobdellid lineages (green dot).

# CHAPTER 5

# HUMORAL RESPONSE OF CAPTIVE ZEBRA SHARKS TO SALIVARY GLAND PROTEINS OF THE LEECH *BRANCHELLION TORPEDINIS*

Marancik, D., Leary, J., Fast, M., Flajnik, M., Camus, A.
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***Abstract***

Parasitism by the marine leech *Branchellion torpedinis* is known to cause disease and mortality in captive elasmobranchs and is difficult to control when inadvertently introduced into public aquaria. Preliminary characterization of the salivary gland transcriptome of *B. torpedinis* has identified anticoagulants, proteases, and immunomodulators that may be secreted into host tissues to aid leech feeding. This retrospective study examined serum IgM responses in captive zebra sharks *Stegostoma fasciatum*, to leech salivary gland extract. Antibody response was examined by ELISA and Western blot assays in 20 serum samples from six zebra sharks, with a 5-year history of leech infection, and 18 serum samples from 8 captive bred zebra sharks, with no history of leech exposure. ELISAs demonstrated significantly higher serum IgM titers to salivary gland extract in exposed zebra sharks compared to the non-exposed population. No obvious trends in antibody titers were appreciated in exposed zebra sharks over a 4-year period. One-dimensional and two-dimensional Western blot assays revealed IgM targeted specific salivary gland proteins within the 40, 55, 70 and 90 kD range. Antigenic proteins identified by liquid chromatography-tandem mass spectrometry and *de novo* peptide sequencing include a secreted disintegrin, metalloproteinase and thrombospondin motif containing proteins (ADAMTS), tubulin, aldehyde dehydrogenase and two unknown proteins. Humoral immune responses to leech salivary gland proteins warrants further investigation as there may be options to exploit immune mechanisms to reduce parasite burdens in aquaria.

***Introduction***

Leeches are frequently encountered parasites of sharks and rays (Sawyer 1986, Burreson 2000, Soto 2000, Soto 2003) that represent potential disease risks for the elasmobranch host. High leech infestations of *Branchellion torpedinis* in aquaria have been associated with anemia, hypoproteinemia, anorexia, lethargy and death of demersal and pelagic elasmobranchs that periodically contact the bottom sand substrate (personal observation). Leeches can serve as vectors for hematozoans including trypanosomes and trypanoplasmas (Burreson 2007, Khan 1976) and as described in teleosts, may pose a risk for the transmission of viruses (Faisal 2009), bacteria (Bragg 1989, Faisal 2011), and fungi (Benz 2004).

Knowledge of these host-parasite relationships are limited, including how elasmobranchs respond to leech infections immunologically. Host immune responses to hematophagous ectoparasites have been more extensively studied in mammals and involve a variety of innate and adaptive immune mechanisms that induce measurable control of parasite burdens (Nelson 1972, Brown 1984, Baron 1985, Grayson 1991, Rechav 1991, Mulenga 1999). For example, salivary gland proteins secreted into feeding sites by hematophagous arthropods are known to stimulate host immunity resulting in acquired resistance (Rechav 1991, Mulenga 1999, Tsai 2005, Waitayakul 2006, Narasimhan 2007).

*Branchellion torpedinis* is a species of marine leech that exclusively parasitizes elasmobranchs. These jawless leeches utilize a straw-like proboscis for feeding (Kearn 2004) that is believed to be aided by bioactive salivary gland proteins secreted into feeding sites (Sawyer 1991, Moser 1995). Preliminary characterization of the salivary

gland transcriptome of *B. torpedinis* has identified anticoagulants, proteases, and immunomodulatory compounds (manuscript in progress). Similar compounds are believed to alter host physiology and promote lesion development in mammals (Baskova 2004) and their presence in *B. torpedinis* suggests a similar role in elasmobranch hosts. Unlike many leech species that are temporary or semi-permanent parasites, (Kearn 2004), *B. torpedinis* appears to remain attached to the host for the duration of its life. Protracted contact with the elasmobranch host provides ample opportunity for interaction between salivary gland secretions and host immune cells.

This retrospective study examined serum IgM production in captive zebra sharks *Stegostoma fasciatum* to leech salivary gland extract. Antibody responses in six zebra sharks with a 5-year history of leech infections were characterized by ELISA and Western blot assays, and compared to serum responses from captive bred zebra sharks with no clinical history of leech infection. Results help elucidate this host-parasite interaction and improve our understanding of the elasmobranch immune response.

## ***Materials and Methods***

### *Zebra Shark Serum*

Six adult, zebra sharks (3 males/3 females) regularly documented with infections of 1-40 *B. torpedinis* leeches from March 2006 through December 2011, served as the exposed population. Twenty serum samples were collected from the group between January 2007 and August 2011 during routine examination procedures. The non-exposed population consisted of eight adult zebra sharks (5 males/3 females) captive born in 2006 and maintained in an isolated aquarium system with no history of leech infection. Both populations of zebra sharks were housed in systems with recirculating artificial sea water

(Instant Ocean® Sea Salt, Aquarium Systems, Inc., Mentor, OH) maintained at 21° C and 30 parts per thousand salinity. Eighteen serum samples were collected from the non-exposed population between January 2008 and January 2010. All serum samples had been archived in a -80°F freezer.

*Leech salivary gland extract preparation*

Leeches were collected from captive elasmobranchs and immediately dissected or frozen at -80 °F until dissection could take place. Leeches were dipped in 70% ethanol and briefly rinsed in sterile water. Salivary glands were sharply excised and transferred into 1.75 mL microcentrifuge tubes (Eppendorf®, Sigma-Aldrich Co, St. Louis, MO) containing 1 ml phosphate buffered saline (pH 7.4) and 50 µl of protease inhibitor cocktail (Sigma-Aldrich Co., St. Louis, MO). Tissue was ground using Kontes Disposable Pellet Pestle® (Sigma -Aldrich Co., St. Louis, MO) and unbroken cells and debris were removed by centrifugation at 1,000 g for 15 minutes. The protein concentration was estimated by the Bradford method.

*ELISA*

ELISAs were optimized for antigen concentration and antibody dilutions by checkerboard titrations based on conventional indirect ELISA procedures (Hornbeck 2001) with suitable controls. Polystyrene ELISA wells (Immulon® 2 HB, ThermoLabsystems, Franklin, MA) were coated with 1 µg of leech salivary gland extract in 50 µl PBS/Azide (0.1% $NaN_3$) and incubated for 24 hours at 4°C. Plates were washed 3 times in deionized water and blocked with blocking buffer (0.25% bovine serum albumin in borate buffered saline, pH 8.5, with 0.05% Tween 20, 1 mM EDTA, and 0.05% sodium azide) overnight at 4°C. All incubations were run at room temperature

(20°C) for 1 hour and plates were washed three times in deionized water between steps. Exposed and non-exposed zebra shark serum was serially diluted from 1:500 to 1:64,000 in blocking buffer and 50 µl of sample loaded into triplicates wells. Secondary antibody consisted of mouse monoclonal anti-shark IgM tissue culture supernatant that targets the lambda-kappa chain (clone LK14, courtesy of Martin Flajnik, University of Maryland School of Medicine, Baltimore, MD) diluted 1:100 in blocking buffer. Tertiary antibody was an anti-mouse IgG HRP-conjugate (Thermo-Fisher Scientific Inc., Rockford, IL USA) diluted 1:10,000 in blocking buffer. After incubations and washing, 50 µl of 1-Step™ Ultra TMB-ELISA (Thermo-Scientific, Rockford, IL) was added to each well as substrate. The plate was incubated for 30 minutes in the dark and the reaction was stopped with 25 µl of 8N $H_2SO_4$. Plates were scanned at 450 nm on Biotrak II plate reader (GE Healthcare Biosciences, Pittsburgh, PA).

*Gel electrophoresis/Western blots*

Salivary gland extract (14 µg of protein) was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using a 0.75 mm thick, 12.5% separating gel. Proteins bands were visualized with Coomassie blue stain (Thermo-Scientific, Rockford, IL) or further processed for blotting to nitrocellulose by standard techniques. Two-dimensional mini-gel electrophoresis was completed according to the manufacturer's protocol (Mini-Protean® 2-D electrophoresis, Biorad Laboratories, Hercules, CA) using an ampholyte carrier with gradient 5-9 **(**AppliChem Inc., St. Louis, Missouri.) Western blotting was performed using serum from three exposed and three non-exposed zebra sharks with the highest recorded ELISA titers. Filters were blocked with Super Block® Blocking Buffer (Thermo-Fisher Scientific, Inc., Rockford, IL) for 1

hour at room temperature and rinsed three times with Tris-buffered saline and 0.05% Tween-20 (TBST) (Thermo-Scientific, Rockford, IL). Primary, secondary, and tertiary antibodies were diluted in 10% Super Block-TBST. Incubations were run for 1 hour at room temperature and filters were washed three times with TBST. Shark serum was diluted 1:4000, followed by LK14 diluted 1:2000, and monoclonal anti-mouse IgG HRP antibody diluted 1:50,000. Blots were developed with Super Signal® West Pico Chemiluminescent Substrate (Pierce, Rockford, IL) on Kodak Biomax® autoradiography chemiluminescent film (Kodak, Rochester, NY).

Large-format two-dimensional electrophoresis was performed according to published methods (O'Farrell 1975, Burgess-Cassler 1989) by Kendrick Laboratory (Madison, WI). Serum from one exposed zebra shark with the highest ELISA titer was used for Western blotting. Blocking and antibody dilutions were performed with a 2% milk solution in TBST. Shark serum was diluted 1:500, secondary antibody was diluted 1:1000 and tertiary antibody HRP-conjugate was diluted 1:25,000. Incubations and washes were performed as previously described.

*In-Gel Digestion of Proteins*

Five antigenic gel spots were excised and transferred to clean tubes, water was added to completely hydrate gels, and the plastic coating was removed with clean tweezers. Gel spots were prepared for digestion by washing twice with 100 µl 0.05M Tris, pH 8.5/30% acetonitrile for 20 minutes with shaking, then with 100% acetonitrile for 1-2 min. After removing the washes, the gel pieces were dried for 30 minutes in a Speed-Vac concentrator. Gels were digested by adding 0.08 µg modified trypsin (Roche Molecular Biochemicals, Indianapolis, IN) in 13-15 µl 0.025M Tris, pH 8.5. The tubes

were placed in a heating block at 32°C and left overnight. Peptides were extracted with 50 µl 2X 50% acetonitrile/2% TFA. The combined extracts were dried in a Speed-Vac concentrator and redissolved in 20 µL of 0.1% formic acid. A 5-mL aliquot was injected into the LC-MS.

*Liquid chromatography-tandem mass spectrometry and de novo peptide sequencing*

Liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis was done by the Columbia University Protein Core Facility (New York, NY) on a Waters Q-Tof Ultima hybrid quadrupole/time-of-flight mass spectrometer with a nanoelectrospray source. The eluant was introduced into the source through a 15 µm i.d. New Objective PicoTip (New Objective, Inc., Woburn, MA). Capillary voltage was set at 1.8 kV and cone voltage 32V. Collision energy was set according to mass and charge of the ion, from 14eV to 50eV. Chromatography was performed on an LC Packings HPLC with a C18 PepMap column (Dionex, Sunnyvale, CA)using a linear acetonitrile gradient with flow rate of 200 nl/ min. *De novo* sequencing was accomplished by manual inspection of either the raw MS/MS spectra or spectra processed with the MaxEnt 3 algorithm contained in the Waters MassLynx 4.1 software package (Waters Corp, Milford, MA). Search parameters were as follows: Database: NCBInr; Enzyme: trypsin; Allow 2 missed cleavages; Taxonomy: all; Fixed modifications: Propionamide (Cys); Variable modifications: Acetyl (protein N-term), Oxidation (M)[ Peptide tolerance: +/- 1.2 Da; MS/MS tolerance: +/- 0.6 Da. Peptide sequences were submitted against the protein BLAST (pBLAST) search algorithm for non-redundant protein sequences and EST database at NCBI (http://www.ncbi.nlm.nih.gov/BLAST/). Additionally, peptide sequences were compared with 454 pyrosequencing transcriptome data previously

collected from the salivary gland of *B. torpedinis* (unpublished data). Transcriptome sequences with aligned peptides were searched in NCBI, as previously described.

*Statistical analysis*

As multiple samples were available for each individual shark, average optical density (OD) was calculated for each shark for statistical comparison. A two-sample Wilcoxon rank sum (Mann-Whitney) test was performed using Minitab version 15.0 (Minitab, State College, PA) to compare average optical densities. A p-value less than 0.05 was considered significant.

***Results***

*ELISA*

A typical serum titration curve from an exposed and a non-exposed zebra shark is presented in Figure 1. Titration of serum antibody for the exposed zebra sharks demonstrated reactive sigmoidal curves while antibodies from non-exposed zebra sharks displayed a relatively flattened ELISA curve typical of non-reactive antibodies. The cumulative average OD for exposed zebra sharks and non-exposed zebra sharks at 1:4000 dilution was 0.586 (range 0.386 - 0.816) and 0.040 (range 0.026 - 0.058), respectively. Average OD was significantly higher in exposed zebra sharks than non-exposed zebra sharks (Mann-Whitney test, p-value 0.0019) indicating higher serum IgM titers (Figure 2).

IgM titers were examined in serum collected annually from 2007 through 2010 for three exposed sharks. Serum diluted 1:4000 (Figure 3) and a separate assay comparing serial two-fold dilutions from 1:250 to 1:128,000 (data not shown) revealed no appreciable trends in IgM titers over time.

*Gel electrophoresis/Western blot*

SDS-PAGE analysis of *B. torpedinis* salivary gland extract revealed banding patterns for numerous high, mid, and low molecular weight proteins (Figure 4).

One-dimensional immunoblots with sera from 3 exposed zebra sharks revealed antigenic bands within the high to mid molecular weight range, with minor variability between individual sharks (Figure 5). Sera from 3 non-exposed sharks showed no observable reaction (Figure 5). Using the same shark sera, two-dimensional mini-gel immunoblots showed similar consistency between exposed zebra sharks with large banding patterns for high molecular weight proteins (Figure 6B-D) and no appreciable IgM binding for non-exposed sharks (Figure 6A). One serum sample from an exposed zebra shark was used for large-format two-dimensional Western blot analysis. IgM binding was demonstrated for discrete protein spots between 30 and 120 kD and 6-7.5 pH range (including spots 1-4, Figure 7) and a large area of immunoreactivity was present in the 90-120 kD range and pH 6-7 (spot 5, Figure 7).

*Antigenic spot identification*

Five antigenic spots were submitted for identification by LC-MS-MS and *de novo* peptide sequencing. LC-MS-MS followed by an MS/MS ion search of the NCBInr database resulted in identification of spots 1 and 3 as tubulin (Genbank: 135489) and aldehyde dehydrogenase (Genbank: 157103517), respectively (Table 1). *De novo* sequencing of spot 5 based on LC-MS-MS data provided one peptide sequence that matched to an ADAMTS (a disintegrin and metalloproteinase with thrombospondin motifs) family protein (Genbank: XP_003445677.1) through a pBLAST database search (Table 1). Additionally, two *de novo* peptide sequences from spot 5 aligned with two

sequences previously elucidated from the *B. torpedinis* salivary gland transcriptome. Sequences were aligned for consensus and searched against the NCBI database. Resultant matches were similar to ADAMTS family proteins (Table 2). Protein spots 2 and 4 could not reliably be identified through either MS/MS or *de novo* sequencing (Table 1).

***Discussion***

ELISA and Western blot assays present evidence of systemic IgM production in zebra sharks against *B. torpedinis* salivary gland proteins. IgM titers within the exposed zebra shark population were significantly higher than those in the non-exposed population. Western blot results are in agreement with the ELISA data and illustrate comparable antibody binding patterns in exposed zebra sharks, with no observable binding in non-exposed sharks.

Zebra sharks are presumably exposed to antigens during *B. torpedinis* feeding. Similar to what is described for other hematophagous parasites, salivary gland proteins secreted into leech feeding sites likely provide antigenic stimulation (de Castro 1989, Rechav 1991, Mulenga 1999) and promote a subsequent systemic humoral response. Microscopically, leech feeding sites often consist of well-demarcated, 1 mm in diameter proboscis tracks through the host epidermis and dermis that are associated with variable numbers of granulocytes, macrophages, and lymphocytes (personal observation). Exposure of elasmobranch inflammatory infiltrates to foreign antigens may generate antigen processing and presentation mechanisms in an effort to block leech secretory protein activity.

Immunoblots demonstrated antigenicity for a number of leech salivary gland proteins, a few of which were identifiable through molecular characterization. An amino acid peptide homologous to the ADAMTS protein family was identified by *de novo* sequencing from spot 5. Antibody binding exhibited more background in this area than for other protein spots, which is most likely a consequence of using a more concentrated serum dilution. Although the short *de novo* peptide sequence yielded a relatively high e-value, further evidence of an ADAMTS protein is demonstrated by two peptide sequences from spot 5 that aligned with ADAMTS proteins previously elucidated from salivary gland transcriptome sequences. Two-dimensional Coomassie blue and Western blotting staining characteristics suggests spot 5 is comprised of high molecular weight proteins with post-translational modification, of which glycosylation is likely. Similar molecular features have been described for ADAMTS proteins (Tsai 2005).

ADAMTS are secreted disintegrin, metalloproteinase and thrombospondin motif containing proteins that have previously been recognized for having a wide range of biologic activities (reviewed by Apte 2009). As components of tick saliva and snake venom, ADAMTS proteins have demonstrated fibrinolytic and anti-clotting activity, and have been implicated in the destruction of collagen and cartilage, and inhibition of wound healing (Tolsma 1997, Gutierrez 2000, Francishcetti 2005, Decrem 2008, Stanton 2011). Similar mechanisms may accompany leech feeding and contribute to hematophagy and wound development.

Other identified antigens include β-tubulin and aldehyde dehydrogenase, both identified by MS-LC-LC. Tubulin is a structural protein described to be abundant in leech salivary gland cells (Walz 1988) with roles in movement of secretory granules

(Kreis 1989). Antigenicity of parasite-derived tubulin has been previously described in mammals to tick salivary gland secretions (Oleaga 2007) and in goldfish to tubulin from the cell wall of *Trypansoma danilewskyi* (Katzenback 2008). Although neither tubulin nor aldehyde dehydrogenase would be an expected primary component of leech salivary gland secretions, elasmobranchs may be exposed to these proteins when broken or sloughed glandular epithelial cells are washed into the luminal extract.

Two additional proteins could not be identified through conventional MS-LC-LC or *de novo* sequencing. These may represent novel antigens and warrant further exploration, particularly spot 4, which represented a highly abundant protein.

Whether systemic antibody production ultimately protects elasmobranchs against *B. torpedinis* infection at the epithelial surface remains to be seen. Functionality of the elasmobranch humoral response in imparting protection has not been demonstrated against any pathogen. Anecdotally, it has been observed that captive elasmobranchs exposed to leeches for longer periods of time exhibit lower parasite burdens than newly introduced, and presumably naïve, fish. Additionally, not all elasmobranch species appear to be parasitized equally by leeches. Validation of these observations could imply acquired resistance to leeches through immunologic, behavioral, or otherwise unknown parasite-host interactions.

Based on immunization of mammals against tick salivary gland proteins, successful immune responses require high concentrations of the correct isotype specificity to reach antigenic sites and induce damage to parasites. This does not necessarily have to result in direct killing of parasites, but can result in successive reduction of parasite numbers or feeding (Brown 1984). Mucosal immunoglobulins

comparable to IgA in mammals and IgT in boney fish that could react at the host-parasite interface, have not yet been identified in elasmobranchs.

IgM was examined in this study based on availability of anti-shark IgM antibody. Additionally, IgM is described as the most prevalent of the three immunoglobulins described in adult sharks (Luer 2004) and has been shown to respond to antigens in previous studies (Dooley 2005, Karsten 2006). The anti-shark IgM antibody used does not distinguish between monomeric (7S) and pentameric (19S) IgM isoforms. There is evidence the 7S isoform occurs slightly later in the course of immune response with higher specificity than 19S IgM (Dooley 2006), which may represent distinct elements of an antibody response during infection. Additional antibodies that may functionally be involved in the elasmobranch humoral response include IgW and IgNAR. Although LK14 may also bind to light chains of IgW, low serum levels and high sensitivity to proteolysis *in vitro* in nurse sharks suggest its affect on the humoral response is minimal (Dooley 2005). Additional studies in nurse sharks indicate IgNAR is found in lower amounts in serum than IgM, yet may primarily drive the antibody response by giving rise to higher affinity antibodies (Dooley 2006). The role of IgW and IgNAR in elasmobranch humoral response to leech salivary gland antigens should be a focus of future research.

Two exposed zebra sharks exhibited increased antibody titers from 2007 to 2008 but the IgM response was not sustained through the four year study period, despite prolonged exposure to leeches. Questions have been previously raised as to the timeframe in which antibody levels rise in elasmobranchs and how this response is maintained over time. Prior studies in lemon and nurse sharks injected with artificial

antigens describe antibody titers that developed over months to years, with an eventual plateau or decrease in immunoglobulin levels, despite continued antigen exposure (Sigel 1965, Dooley 2006). These zebra sharks had been exposed to leeches for over one year before sample collection began and theoretically, could have already reached this described titer "threshold". The reasoning behind this is unknown, but may be affected by antibody expression at the genetic level. Unlike mammals that exhibit immunoglobulin class switching (e.g. IgM to IgG), plasticity in the number and type of antibodies expressed in elasmobranchs appears to occur through recombination of immunoglobulin gene clusters (Stavenenzer 2004). This raises questions as to the duration and rate at which elasmobranchs produce antibodies. Further elucidation of this process may, in part, help explain these observed antibody expression patterns.

A limitation in this study was the availability of a positive control. Assays were run with the assumption that all animals were immunocompetent. Although mild individual differences in immune function would be expected, efforts were made to minimize variation between exposed and non-exposed groups by using adult zebra sharks known to be in relatively good health. Exhibits were maintained at similar temperature and water quality parameters. The effects of other environmental influences, such as being wild verses captive bred, and whether this could affect humoral immune function, is unknown. Studies comparing immune function in wild and captive bred elasmobranchs are lacking at this time.

***Conclusions***

Findings in this study illustrate a humoral immune response in zebra sharks capable of targeting *B. torpedinis* salivary gland proteins. Exposed zebra sharks

exhibited significantly higher antibody titers to leech salivary gland extract than non-exposed zebra sharks. Serum IgM displayed targeting of specific antigens including a secreted ADAMTS family protein, tubulin, aldehyde dehydrogenase, and a number of unidentified proteins. Further research into this host-parasite interaction is warranted, including the characterization of unidentified salivary gland antigens, exploring the roles of IgNAR and IgW, and examining the functionality of the humoral response. A better understanding of this process may provide options to exploit immune mechanisms and reduce parasite burdens in aquaria.

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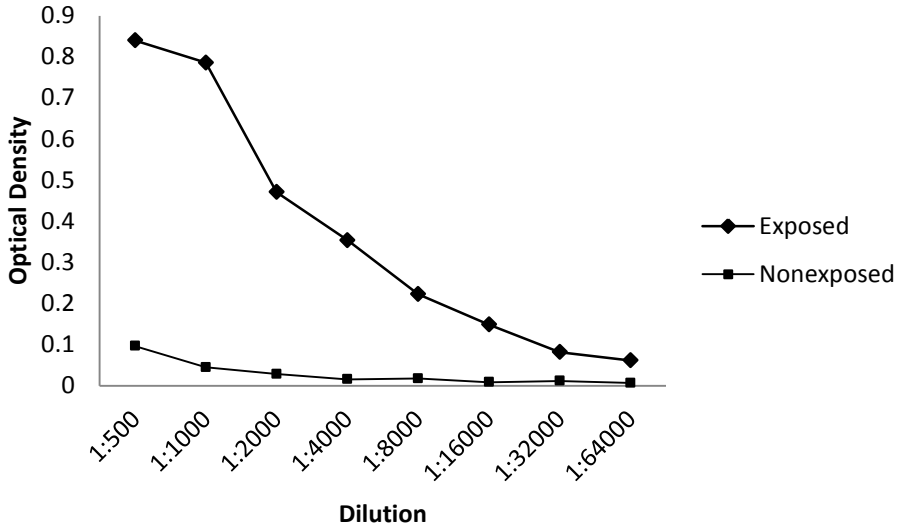



Figure 1: Representative ELISA dilution curve displaying relative binding of zebra shark IgM to *Branchellion torpedinis* salivary gland extract for one exposed and one non-exposed zebra shark. Titration of serum antibody for the exposed zebra sharks demonstrated reactive sigmoidal curves while antibodies from non-exposed zebra sharks displayed a relatively flattened ELISA curve typical of non-reactive antibodies.

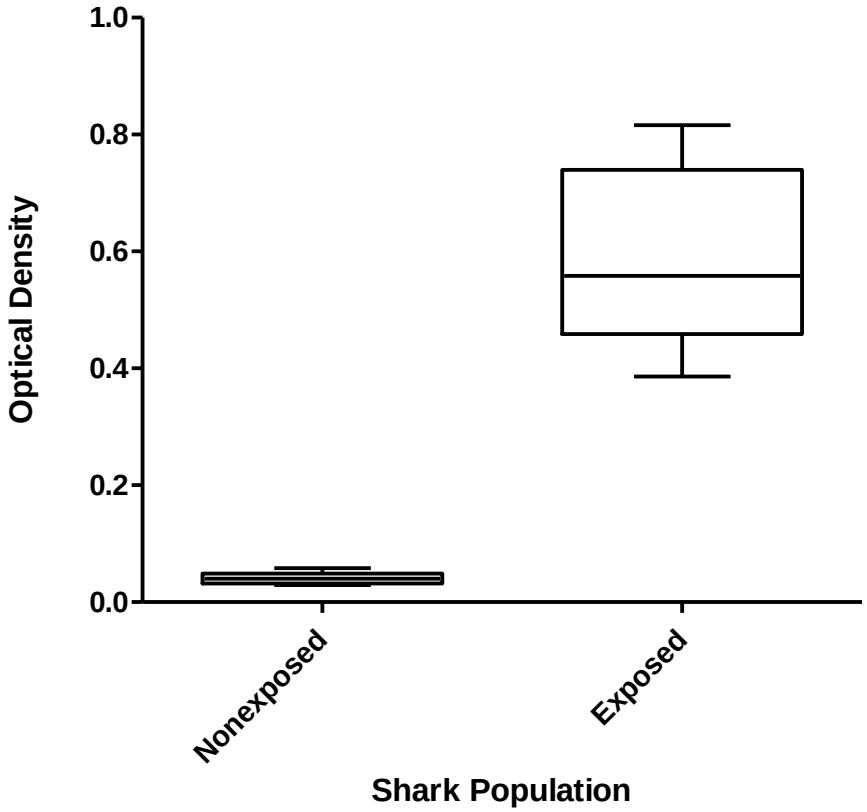



Figure 2: Box plots comparing ELISA IgM binding for non-exposed and exposed zebra sharks at 1:4000 serum dilution. Average optical density for non-exposed zebra sharks was 0.040 with a range of 0.026 to 0.058. Average optical density for exposed zebra sharks was 0.586 with a range of 0.386 to 0.816. Average optical density was significantly higher in exposed zebra sharks than non-exposed zebra sharks (Mann-Whitney test, p=0.0019).

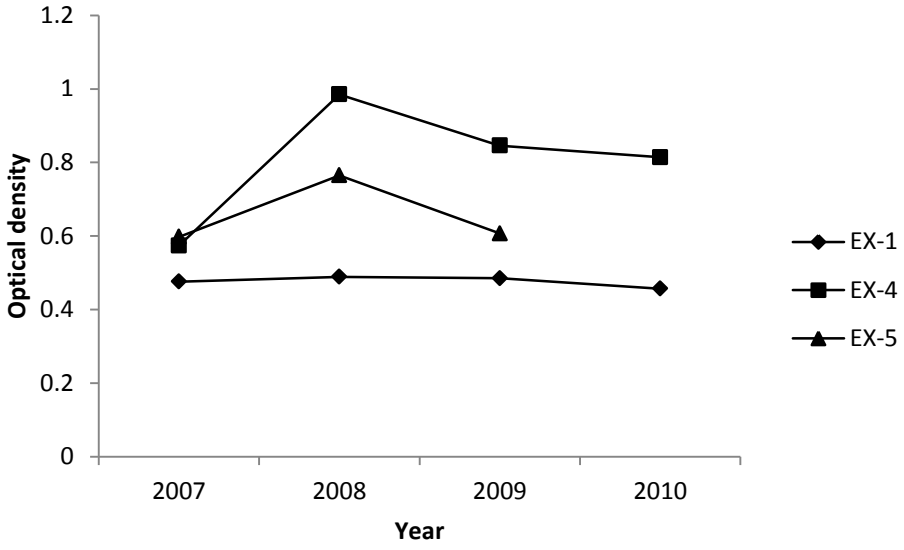



Figure 3. ELISA IgM binding for sera diluted 1:4000 revealed no obvious trends in serum titers for three zebra sharks from 2007 through 2010.

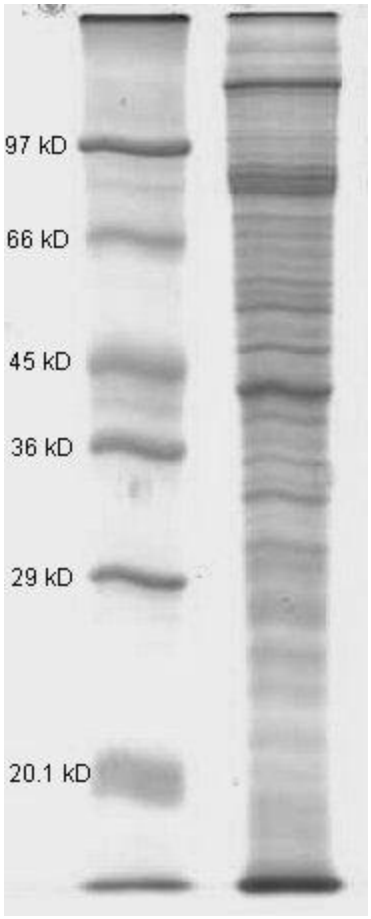



Figure 4: Gel electrophoresis of *Branchellion torpedinis* salivary gland extract and markers exhibit numerous protein bands.

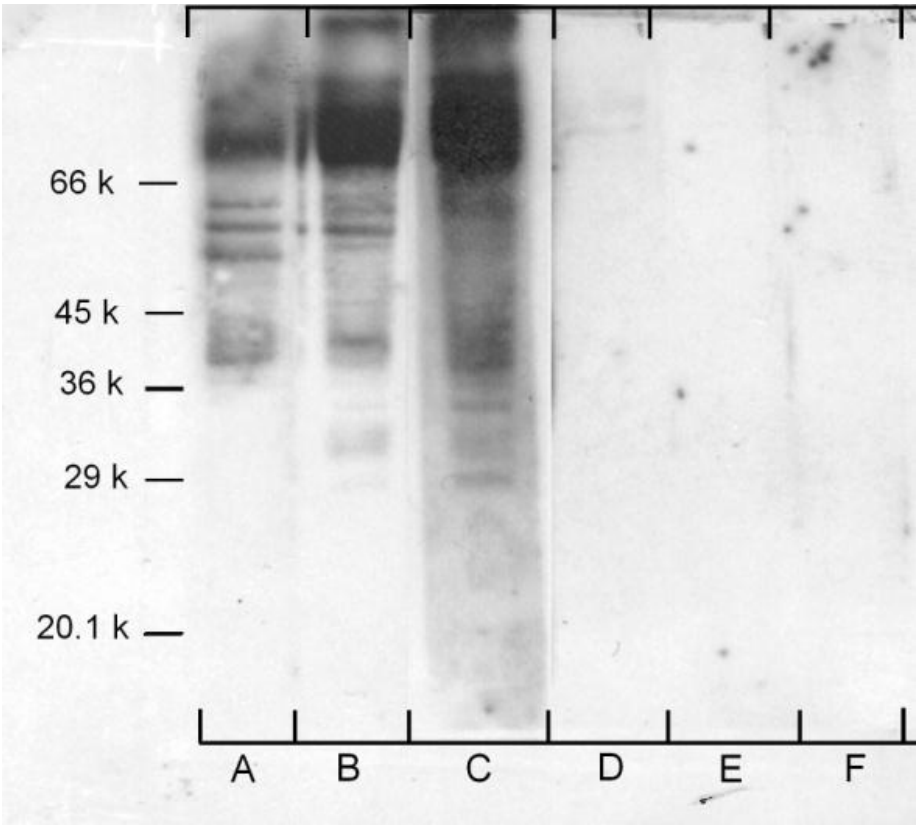



Figure 5. One-dimensional Western blot with sera from three exposed zebra sharks (A,B,C) exhibited consistent immunoreactivity of IgM to *Branchellion torpedinis* salivary gland extract while sera from three non-exposed zebra sharks (D,E,F) showed no reaction.

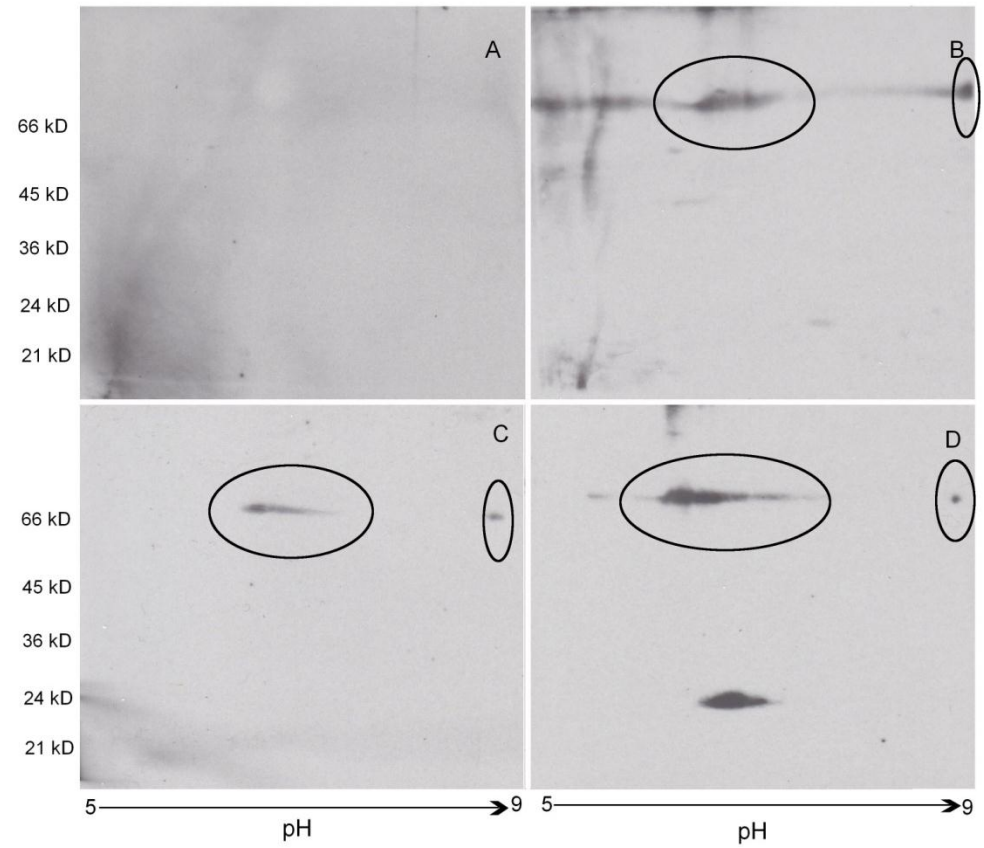



Figure 6. Mini two-dimensional Western blot with sera from a non-exposed zebra shark (A) displays no appreciable IgM reactivity while sera from three exposed zebra sharks (B-D) exhibits distinct IgM binding (circled areas).

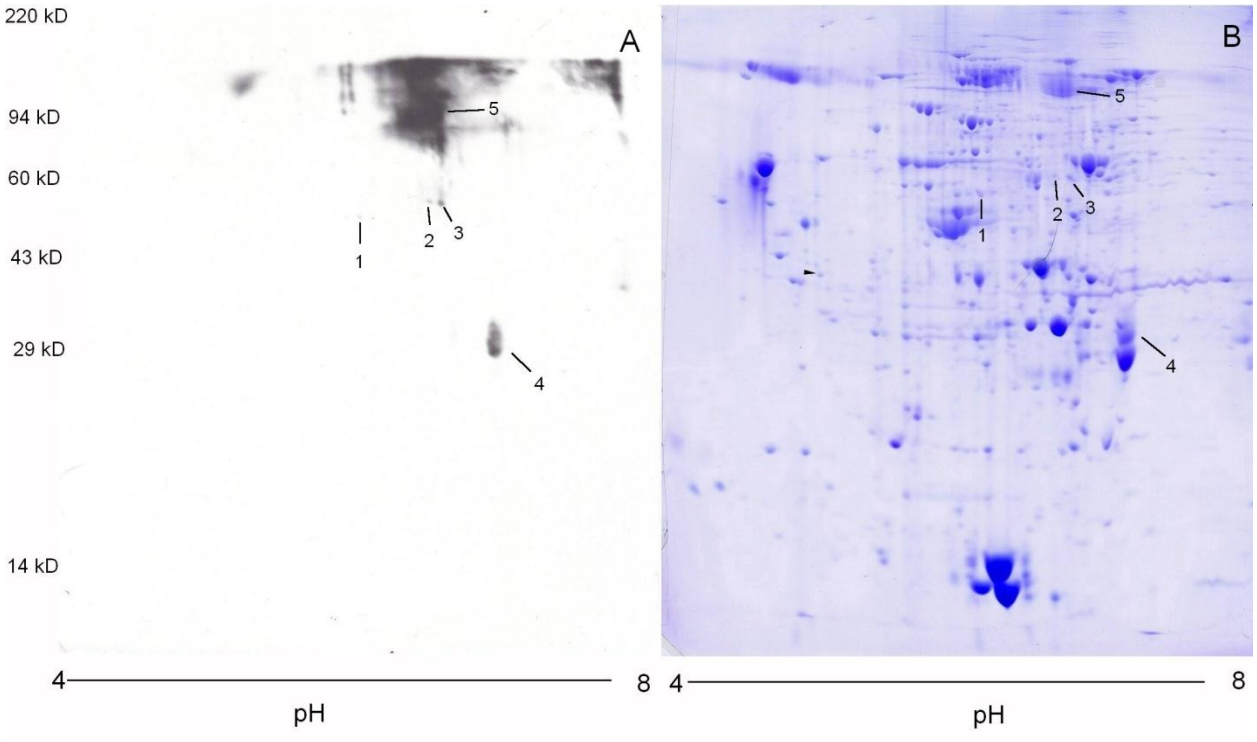
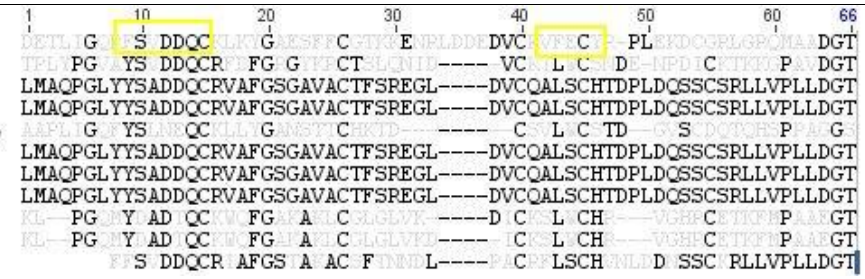



Figure 8. Western blot (A) and corresponding large format 2-dimensional gel spots (B) showing zebra shark serum IgM binding to leech salivary gland proteins. Protein spot numbers indicate immunogenic proteins that could be confidently matched to the stained gel and correspond to proteins in Table 1 submitted for LC-MS/MS analysis and *de novo* sequencing in Table 1.

| | | **LC-MS-MS** | | |
|---|---|---|---|---|
| *Spot #* | *Protein ID* | *Accession Number* | *MASCOT Score* | *# of Peptide Sequences* |
| 1 | Beta-tubulin | gi\|135489 | 415 | 10 |
| 3 | Aldehyde dehydrogenase | gi\|157103517 | 186 | 4 |
| | | ***De novo* sequencing** | | |
| *Spot #* | *Protein ID* | *Accession Number* | *Putative Sequence* | *e-value* |
| 5 | ADAMTS family protein | XP_003445677.1 | FSVDDQCK | 53 |
| | | **Unidentified Proteins** | | |
| *Spot #* | *Protein ID* | *Putative sequences* | | |
| 4 | Unknown | TLDDGG/ | | |
| | | NLTEAQSFK | | |
| | | VTSESAVWK | | |
| | | /EEELDVSR | | |
| | | /TTATSEA/ | | |
| 2 | Unknown | FQVDSDTCKK | | |
| | | NQDLTHTSTTTFNK | | |
| | | FQVDSDTCK | | |
| | | /PVSLSCSK | | |
| | | /(SV)CVQTQSS/ | | |
| | | /LATLSGLTK | | |

Table 1. Immunogenic proteins from *Branchellion torpedinis* salivary gland extract identified by LC-MS-MS and *de novo* sequencing based on LC-MS-MS results. Protein spots correspond with those described in Figure 8.




| Protein ID | Accession Number | Species | e-value |
|---|---|---|---|
| PREDICTED: similar to ADAM metallopeptidase with thrombospondin type 1 motif, 3 proprotein, partial | XP_002120522.1 | *Ciona intestinalis* | 1.2 |
| PREDICTED: A disintegrin and metalloproteinase with thrombospondin | XP_003511772.1 | *Cricetulus griseus* | 3.0 |
| A disintegrin and metalloproteinase with thrombospondin motifs 13 | EGW06892.1 | *Cricetulus griseus* | 3.6 |
| PREDICTED: A disintegrin and metalloproteinase with thrombospondin motifs 9-like | XP_003388992.1 | *Amphimedon queenslandica* | 3.8 |
| A disintegrin and metalloproteinase with thrombospondin motifs 13 precursor | NP_001001322.1 | *Mus musculus* | 4.2 |
| a disintegrin-like and metallopeptidase reprolysin type) with thrombospondin type 1 motif, 13 | EDL08350.1 | *Mus musculus* | 4.4 |
| a disintegrin-like and metalloprotease (reprolysin type) with thrombospondin type 1 motif, 13 | CAM23710.1 | *Mus musculus* | 4.4 |
| ADAM metallopeptidase with thrombospondin type 1 motif, 18 | DAA20322.1 | *Bos taurus* | 8.1 |
| A disintegrin and metalloproteinase with thrombospondin motifs 18 precursor | NP_001179415.1 | *Bos taurus* | 8.2 |
| PREDICTED: A disintegrin and metalloproteinase with thrombospondin motifs 13-like | XP_003445677.1 | *Oreochromis niloticus* | 9.0 |

Table 2. Previously elucidated *B. torpedinis* salivary gland consensus sequence data (1) aligned with two peptide sequences from spot 5 (outlined in yellow) and resultant pBLAST search results matched to ADAMTS family proteins (2-11).

# CHAPTER 6

## SUMMARY AND CONCLUSIONS

The goal of this project was to explore several different, but interconnected, facets relating to the pathogenic potential of *B. torpedinis* and the induced immune response of its elasmobranch host. Areas of investigation included infectivity trials with yellow stingrays, microscopic evaluation and transcriptome analysis of the *B. torpedinis* salivary gland, and investigation of zebra shark humoral responses to leech salivary gland proteins.

Twelve yellow stingrays were infected with one or three leeches to monitor *B. torpedinis* induced disease. The most significant findings in parasitized yellow rays were extensive skin ulceration, severe anemia and hypoproteinemia, and host death in as few as 5 days. The infectivity trial identified several risk factors associated with clinical disease. Parasite burden and cumulative skin ulcer area positively correlated with decreases in host packed cell volumes and total solids, indicating anemia and hypoproteinemia are directly due to blood loss through leech attachment site ulcers and parasite feeding. Additional confirmation of *B. torpedinis* hematophagy was demonstrated through histologic observations of host erythrocytes, granulocytes and proteinacious fluid within leech gut.

Results of this study demonstrate the pathogenic potential of *B. torpedinis* to cause disease in elasmobranchs, and enhance our understanding of the effects this parasite has on host health. The premise that clinical disease can be minimized by maintaining low parasite loads that subsequently limit blood loss through hematophagy and bleeding from leech attachment sites is supported by this infectivity trial. Wound care and systemic antibodioticss may also reduce risk for secondary infections, as mixed colonies of bacteria were often observed in host skin ulcers. Smaller elasmobranchs, such as yellow stingrays, should be monitored carefully for leech infestation, as anemia and hypoproteinemia can occur within days from as few as 1-3 adult leeches. The progression of skin lesions and downward trends in host packed cell volume and total solids implies infections extending past day 14 would likely result in further host pathology and mortality. Extended trials with larger elasmobranch models, potentially more resistant to the effects of parasitism, may help better assess host physiologic, immunologic, and regenerative responses to the typical chronic infections observed in aquaria.

Observations of host inflammatory and immune responses to *B. torpedinis* gave variable results. Localized inflammation at attachment and feeding sites consisted predominately of fine eosinophilic granulocytes, fewer coarse eosinophilic granulocytes, and rare lymphocytes. Although recruitment of fine eosinophilic granulocytes to sites of leech infection was demonstrated, the role this inflammatory cell and other cellular components in innate and acquired immunity in response to parasitism is hampered by a general lack of understanding of immune cell functions and capabilities.

Total white blood cell counts did not display clear reaction patterns to leech infection, even amid obvious clinical disease. This is consistent with previous observations that elasmobranchs respond differently than other vertebrates to infection, and that leukocyte counts may not be an effective method of diagnosing and monitoring disease in these fish.

*B. torpedinis* feeding sites demonstrated discrete proboscis tracks through the host skin filled with host blood and serum. Little inflammation and tissue necrosis was present in adjacent tissue suggesting minimal trauma during proboscis penetration. Along with the hematophagous nature of *B. torpedinis*, this suggests proboscis penetration and feeding may be aided by bioactive salivary gland proteins that help break down host tissue, limit the host inflammatory response, and act to counteract host hemostatic mechanisms.

Microscopic evaluation of leech salivary gland confirmed salivary tissue is abundant within segments VI-X and closely associated with the proboscis. Salivary gland somata exhibited variable staining characteristics in routine hematoxylin and eosin and periodic acid-Schiff stained sections, suggesting the presence of a heterogeneous secretory product.

Over 4,700 unique isotigs were assembled from the salivary gland transcriptome, including sequences homologous to bioactive salivary gland factors previously identified in venomous snakes and sanguivorous parasites. Putative activities of salivary proteins suggest a wide range of capabilities in modulating and counteracting host physiologic responses. In particular, sequences matched to proteins belonging to the ADAMTS (disintegrin and metalloproteinase with thrombospondin motifs) superfamily, with known

roles in anti-hemostasis and the breakdown of collagen and cartilage matrices. Anticoagulants, including C-type lectins and antistasin, were identified, as were the thrombocyte inhibitors, destabilase and apyrase, and immunomodulatory proteins, eglin and cystatin.

These findings correlate with hemorrhage and ulcerative lesions observed at leech feeding sites and provide insight into the intricate and complex interactions occurring at the parasite:host interface. *B. torpedinis* uses a diverse cocktail of salivary compounds to aid feeding, including serine protease inhibitors, peptidases, and competitive antagonists, each likely to target a specific coagulative or hemostatic factors. Similarly, serine and cysteine protease inhibitors antagonize particular host inflammatory and immune responses. Many of these activities represent redundant anti-hemostatic and immunomodulatory activities, directed against diverse host physiologic mechanisms, highlighting the adaptive ability of *B. torpedinis* to ensure successful blood feeding.

As *B. torpedinis* infections in elasmobranchs likely represent millenia of host-parasite adaptation, the presence of salivary factors with known specific inacitivites in other animals, imply that their physiologic targets exist in the host, although they remain to be identified. Apyrase offers evidence for ATP and ADP mediated inflammatory responses and coagulation in elasmobranchs and C-type lectins indicate the presense of a functional clotting cascade that includes Factors IX and X. Cystatin suggests functional MHC II based antigen presentation. Evidence for these mechanisms provides a platform for future physiologic and immunologic studies in elasmobranchs, including functional assays to validate the effect of salivary proteins on host hemostasis and immunologic responses.

This was the first study to characterize salivary gland proteins in a piscicolid leech. Isolation of eglin, destabilase, and a number of C-type lectins from *B. torpedinis* signifies an earlier evolutionary appearance of these salivary factors than previously believed. Differences in sialomic characteristics between rhynchobdellids and arhynchobdellids are likely due to divergence in parasite feeding mechanisms and host selection. Additional sialomic studies that encompass other piscicolid and glossophiniid leeches, as well as the intermediate *Erpobdelliformes*, will help clarify evolutionary and phylogenetic relationships

The demonstrated characteristics of the sialome secretory products and the previously described hematophagous nature of *B. torpedinis* imply chronically parasitized elasmobranchs are exposed to substantial amounts of salivary gland proteins during leech feeding. Additionally, observations in aquaria suggest that captive elasmobranchs exposed to leeches for long periods of time exhibit lower parasite burdens than newly introduced, and presumptively naïve, fish. In an effort to investigate whether elasmobranchs may be acquiring protection through an adaptive immune response to leech salivary gland proteins, the humoral immune response of zebra sharks chronically exposed to *B. torpedinis* was studied. Host responses to *B. torpedinis* presented a unique model to investigate humoral immune activity in elasmobranchs. Although substantial effort has been put into characterizing elasmobranch antibodies on a molecular level, demonstration of functional responses to infection is lacking in the literature.

Serum IgM production in captive zebra sharks chronically exposed to *B. torpedinis* salivary gland proteins was investigated through ELISA and Western blot techniques. Findings illustrate an IgM response in zebra sharks capable of targeting *B.*

*torpedinis* salivary gland proteins. Exposed zebra sharks exhibited significantly higher ELISA IgM titers to leech salivary gland extract than non-exposed zebra sharks. One-dimensional and two-dimensional Western blots illustrated serum IgM targeting of specific antigens including a secreted ADAMTS family protein, tubulin, aldehyde dehydrogenase, and a number of unidentified proteins.

ADAMTS proteins, tubulin, and aldehyde dehydrogenase were also isolated from the *B. torpedinis* salivary gland transcriptome, corroborating their presence as a component of the leech sialome. ADAMTS proteins likely represent an important component in the feeding mechanism of *B. torpedinis*, with known roles in anti-hemostasis and break down of connective tissue and cartilage. Antibodies may target these proteins in an effort to neutralize their bioactive effect at feeding sites. Tubulin and aldehyde dehydrogenase are regarded as structural and housekeeping proteins, and may be secreted into feeding sites from broken or sloughed glandular epithelial cells.

As leech feeding sites observed in the infectivity trial revealed host inflammatory infiltrates within proboscis tracks, it is likely that exposure to antigenic secreted salivary gland proteins occurs at this parasite:host interface and initiates a specific immune response. As salivary transcriptome data indicates *B. torpedinis* has adapted feeding mechanisms specific to elasmobranchs, host antibody production demonstrates the ability of elasmobranchs to respond immunologically, presumably in an effort to repel leech infections.

Similar to previous studies that used innocuous foreign antigens to stimulate immunity, zebra sharks did not demonstrate a continuously increasing antibody titer to leech salivary gland antigens. This indicates that fundamental differences exist between

elasmobranch humoral immune responses and those of other vertebrates. Mechanisms involved in controlling the number and types of immunoglobulins expressed remain to be elucidated.

These results shed light on the ability of this primitive immune system to respond and target antigens during a natural infection. Although protection imparted by antibody responses against B. torpedinis has not been demonstrated, a robust and specific antibody response is induced. To further investigate elasmobranch humoral responses, ELISA and Western blot assays can be applied to study other pathogen:host interactions, including fungi, bacteria, and possibly viruses.

Further research into the adaptive immune response to *B. torpedinis* salivary gland proteins is warranted. Demonstrating the roles of IgNAR and IgW in response to leech infestations will provide a more comprehensive representation of elasmobranch humoral responses. There may also be options to exploit elasmobranch immune mechanisms and reduce parasite burdens in aquaria maintained sharks and rays. Future studies should include vaccination trials using individual antigens identified in this investigation to determine the ability of each to induce a humoral response. If a more robust experimental model can be found, infectivity trials to investigate reduction of parasite burdens through vaccination may help validate functionality of the elasmobranch humoral immune response. At the least, if trials can demonstrate the safety of potential vaccines, it may be beneficial to inoculate newly introduced exhibit animals with salivary gland proteins and observe parasite burdens from a practical level.