A Study of Syngnathid Diseases and Investigation of Ulcerative Dermatitis

by

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ABSTRACT

A STUDY OF SYNGNATHID DISEASES AND INVESTIGATION OF ULCERATIVE DERMATITIS

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University of Guelph, 2012

Advisor: Dr. John S. Lumsden

A 12-year retrospective study of 172 deceased captive syngnathids (Hippampus kuda, H. abdominalis, and Phyllopteryx teaniolatus) from the Toronto Zoo was performed. The most common cause of mortality was an ulcerative dermatitis, occurring mainly in H. kuda. The dermatitis often presented clinically as ‘red-tail’, or hyperaemia of the ventral aspect of the tail caudal to the vent, or as multifocal epidermal ulcerations occurring anywhere. Light microscopy often demonstrated filamentous bacteria associated with these lesions, and it was hypothesized that the filamentous bacteria were from the Flavobacteriaceae family. Bacteria cultured from ulcerative lesions and DNA extracted from ulcerated tissues were examined using universal bacterial 16S rRNA gene primers. A filamentous bacterial isolate and DNA sequences with high sequence identity to Cellulophaga fucicola were obtained from ulcerated tissues. Additionally, in situ hybridization using species-specific RNA probes labeled filamentous bacteria invading musculature at ulcerative skin lesions.
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ATCC</td>
<td>American type culture collection</td>
</tr>
<tr>
<td>BA</td>
<td>blood agar</td>
</tr>
<tr>
<td>BLAST</td>
<td>basic local alignment search tool</td>
</tr>
<tr>
<td>CA</td>
<td>cytophaga agar</td>
</tr>
<tr>
<td>CITES</td>
<td>Convention on International Trade in Endangered Species of Wild Fauna and Flora</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>FFPE</td>
<td>formalin-fixed paraffin-embedded</td>
</tr>
<tr>
<td>GBD</td>
<td>gas bubble disease</td>
</tr>
<tr>
<td>ISH</td>
<td><em>in situ</em> hybridization</td>
</tr>
<tr>
<td>LCM</td>
<td>laser capture microdissection</td>
</tr>
<tr>
<td>MCA</td>
<td>MacConkey’s agar</td>
</tr>
<tr>
<td>min</td>
<td>minutes</td>
</tr>
<tr>
<td>MSA</td>
<td>mannitol salt agar</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PBS-T</td>
<td>phosphate buffered saline with tween</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PEA</td>
<td>phenylethyl alcohol agar</td>
</tr>
<tr>
<td>PVK</td>
<td>Pierce van der Kamp</td>
</tr>
<tr>
<td>rDNA</td>
<td>ribosomal deoxyribonucleic acid</td>
</tr>
<tr>
<td>RNA</td>
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<td>rRNA</td>
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</tr>
<tr>
<td>sec</td>
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</tr>
<tr>
<td>TCBS</td>
<td>thiosulfate-citrate-bile-sucrose</td>
</tr>
<tr>
<td>TSA</td>
<td>trypticase soya agar</td>
</tr>
</tbody>
</table>
DECLARATION OF WORK PERFORMED

All work reported in this thesis was performed by me, with the exception of the following:

Duplicates of PCR using universal 16S rRNA gene primers of bacterial isolates was performed by Elena Contador.

Necropsies prior to 2006 and processing for histology were performed by fish pathology laboratory staff and zoo staff: Dr. Christopher J. Dutton, Dr. Graham Crawshaw, Dr. Jean Paré, Dr. Maya Kummrow, Dr. David McLelland, Dr. Charlene Berkvens, Spencer Russell, Karrie Young, and Lowia Al-Hussinee. Initial necropsies at the Toronto Zoo were also performed later by Dr. Iga Stasiak and Dr. Pauline Delnatte.
INTRODUCTION

The family syngnathidae includes seahorses, seadragons, pipefish and pipehorses. The population status of many species is unknown; however, many are considered globally threatened, with declining populations (Project Seahorse 2003). Syngnathid habitat degradation worsens with encroaching human populations and exploitation of the wild populations continues for the aquarium trade, traditional Chinese medicine and curiosities (Vincent 1996). Over the past two decades, there has been an increase in publications on syngnathids, which has corresponded with their increased popularity as display fish and their iconic role in fisheries and habitat conservation.

The review of the literature summarizes the present literature about syngnathid physiology, conservation, husbandry, breeding, nutrition and disease.
CHAPTER 1. REVIEW OF SYNGNATHID LITERATURE

This chapter corresponds to the following manuscript:


INTRODUCTION TO SYNGNATHIDS

The family Syngnathidae includes over 295 species (Froese & Pauly 2011) of morphologically diverse fish commonly known as seahorses, seadragons, pipefish and pipehorses. The word syngnathus is derived from Greek, roughly translating to “fused jaw” (Wallis 2004; Koldewey 2005). While the mandible and maxilla are not actually fused together, the epithelium and muscle covering the mouth forms an enclosed tube with a small opening at the rostral end used for siphoning prey.

Syngnathids do not have scales like most other fish. Instead, their body is covered by a series of bony segments, each having raised centres or edges. Together, these elevations form rings and ridges that run the length of the entire body. Where rings and ridges cross, the junctions normally form tubercles. These may become large in some species and form foliage-like or spiny extensions while others remain short and blunt (Kuiter 2001). The absence, presence, size or shape of tubercles on the head and body, are often identifying features, but these may vary between different life stages or between sexes. In general, juveniles are spinier than adults and females are spinier than males (Kuiter 2001).
All syngnathids share multiple features, one of which is their ability to evade predators by crypsis. This remarkable ability to remain unseen using camouflage has resulted in many species remaining undiscovered by humans for decades. In the last ten years alone, six new species have been described, including one pipefish, one pygmy pipehorse and four pigmy seahorses (Kuiter 2000; Kuiter 2001; Lourie & Randall 2003; Browne & Smith 2007; Lourie & Kuiter 2008). Syngnathids occur in a range of habitats, depths and locations across the globe but generally inhabit shallow inshore waters, including eel-grass beds, estuaries and coral reefs.

There are over 35 species of seahorses that range greatly in size. The largest, *Hippocampus abdominalis* can attain heights of over 30 cm while the smallest recorded, *Hippocampus satomiae*, measures less than 1.2 cm in height (Lourie & Kuiter 2008). *H. satomiae* is part of a group of diminutive species of seahorses, collectively known as ‘pygmy seahorses’, found in Australia and Asia. Pygmy seahorses measure less than 5 cm in length. Pipefish are the largest group of syngnathids, including greater than two hundred species. Their morphology resembles that of a straight-bodied seahorse, often swimming horizontally in the water column rather than vertically like its relatives, the seahorse and seadragon (differences in broodpouch morphologies are discussed later in this chapter). Pipefish and pipehorses have a very similar morphology with the exception of the tail; pipefish have a non-prehensile tail with a caudal fin while pipehorse tails are prehensile without a caudal fin, like the seahorse (Kuiter 2000). Pygmy pipehorses have also been discovered (Kuiter 2004). In contrast to the diversity exemplified by seahorses and pipefish, there are only two genera of seadragons with one species in each genus: the weedy (*Phyllopteryx taeniolatus*) and leafy (*Phycodurus eques*) seadragons. Seadragons
are generally shaped like seahorses; however, they are highly ornate with leaf or weed-like extensions of their tubercles. They overlap in size with only the largest of seahorse species. Seadragons are endemic to Australian coastal waters (Kuiter 2001).

**PHYSIOLOGY**

**Reproduction**

The unique adaptation of males to incubate and give birth to live young has rightfully resulted in a large proportion of syngnathid research focused on their reproductive physiology. Three major groupings are described based on the morphological adaptations for egg attachment and incubation in male syngnathids: ventral gluing without coverage, two pouch flaps, and a completely enclosed and sealed sac (Herald 1959; Kornienko 2001; Carcupino et al., 2002). The first type occurs when the eggs adhere to a specialized patch of tissue over the ventral tail or abdomen, leaving the eggs completely exposed. Syngnathids with this type of brooding adaptation include seadragons and pipehorses of the genus *Phyllopteryx, Nerophis, Phycodurus, Solegnathus* and *Syngnathoides* (Herald 1959; Kornienko 2001). The second adaptation involves attachment of eggs on a similarly located patch of tissue; however, there are two flaps that close over the eggs and seal either completely or partially over the midline when brooding. This adaptation is found in pipefish of the genus *Syngnathus* and *Corythoichthys* (Herald 1959; Kornienko 2001). The final adaptation occurs when eggs are enclosed in an abdominal pouch with a pore-like opening to the outside, which is found in *Hippocampus* species (Herald 1959; Kornienko 2001).
These specialized brood sites are commonly referred to as brood patches and brood pouches, respectively. The female deposits eggs over the male’s brooding surface or brood pouch opening, where the eggs are fertilized externally and are taken into the brood pouch or attached to the brood patch. The eggs remain along the brood surface or within the brood pouch until free-swimming fry are released (Ripley & Foran 2009; Woods 2000a). It was previously thought that seahorses fertilized eggs within their brood pouch; however, current research demonstrate sperm duct openings to the exterior, suggesting release of spermatozoa to the external marine environment (Van Look et al., 2007). This supports the hypothesis that fertilization occurs externally, making this feature common to all three types of brooding systems. Despite external fertilization, it has been shown that there is little to no sperm competition in some syngnathids species such as the straight-nose pipefish (Ah-King et al., 2006). Syngnathid spermatozoa have been shown to be activated by ovarian fluid and not by water; sperm are not shed into the water but need to be released near the eggs, which is suspected to limit sperm competition (Ah-King et al., 2006). As may be expected with a brooding organ, recent findings suggest an osmoregulatory and nutrient exchange role from the paternal circulation to the embryos (Carpucino et al., 2002; Partridge et al., 2007; Ripley & Foran 2009). However, the extent to which the various brood patches and pouches protect, aerate, osmotically buffer and nourish the embryos is still not well described.

**Locomotion**

Seahorses, some pipefish (including pipehorses) and likely also seadragons are fish that swim exclusively by undulating their dorsal and pectoral fins. Locomotion by fin undulation is associated with relatively low speeds and high precision for mating,
predation, and predator avoidance within complex, obstacle-strewn habitats such as coral reefs (Consi et al., 2001; Ashley-Ross 2002). This pattern of fin movement is characterized by a traveling wave passed along the fin that is caused by the phased oscillation of the fin rays (Consi et al., 2001). The very high frequencies and low amplitude at which syngnathids undulate their dorsal fins results in very slow swimming speeds (Ashley-Ross 2002). Seahorse dorsal fins beat at 30-42 Hz, while pipefish dorsal fins oscillate at 13-26 Hz (Ashley-Ross 2002). Because of the low speeds at which these fish swim, one might expect the muscles powering the dorsal fins to be weak. However, considering that these muscles must work against significant resistance in the water, the opposite is true. In fact, the work performed by the dorsal fin musculature of syngnathids has been compared to that of the pectoral muscles of hummingbirds; while hummingbird wings flap at speeds of 30-78 Hz, the medium they work in is air, offering significantly less resistance than water (Ashley-Ross 2002). Fin undulation also enables the syngnathids to slowly and stealthily stalk their prey, which mostly consists of small crustaceans. Their adaptations for remaining undetected also includes kairomone suppression, making them chemically inconspicuous to their prey (Cohen & Ritz 2003). Finally, their unique fin undulations also enable them to perform complex courtship manoeuvres such as those documented in *Hippocampus whitei*, *Hippocampus zosterae* and *Nerophis lumbriciformis* (Vincent & Sadler 1995; Masonjones & Lewis 1996; Monteiro et al., 2002).

**Prey capture mechanism**

Syngnathids capture their prey with a very rapid striking motion of the head and neck towards their target as they suction prey into their buccal cavity. Common to many bony
fishes, syngnathids exhibit a generalized kinematic pattern of prey capture including almost simultaneous head elevation, hyoid depression and mouth opening; their unique morphology results in a very characteristic feeding behaviour often called a “snick” (Bergert & Wainwright 1997). The maneuver begins with a preparatory phase consisting of a slow ventral head flexion followed by a rapid elevation of the head and snout as the prey is drawn into the mouth by suction (Bergert & Wainwright 1997). The majority of the work involved in this mechanism appears to be through the movement of the head and neck, generated by the epaxial and hypaxial muscles, not suction as was previously thought (Wassenbergh & Aerts 2008; Wassenbergh et al., 2008; Flammang et al., 2009; Roos et al., 2009). Morphology and kinematics of syngnathid prey capture demonstrate that this movement is too rapid to be generated solely by muscle. Recent studies on pipefish prey capture suggest an elastic recoil mechanism involving elastic fibers within the tendons of the epaxial and hypaxial muscles (Wassenbergh & Aerts 2008). The epaxial and hypaxial muscles and associated tendons therefore play a crucial role in syngnathid feeding biomechanics.

Seahorse skin

The seahorse epidermis has a specialized surface cell type called flame cells or flame cone cells (Bereiter-Hahn et al., 1980). These cells are found on all epidermal surfaces except over fins and eyes and are found in greater numbers along the ventral side of the tail. The density of flame cells along the ventral tail is greater than double the density of flame cells found over the epidermis of the head (Bereiter-Hahn et al., 1980). They are mucous-producing cells; in the mature cell, mucous is excreted out of pores at the apical border of the cell which gives the cell its flame-like appearance under light microscopy.
The mucous caps are associated with epiphytic organisms. In *Hippocampus kuda*, threads formed by algae and bacteria have been observed to interconnect several mucous caps forming a confluous layer of epiphytes along the ventral aspect of the tail. It has been hypothesized that the mucous caps can dislodge if overgrown with bacteria or other organisms (Bereiter-Hahn et al., 1980).

**CONSERVATION**

In the last two decades, syngnathids have gained attention because of growing concern about their ability to survive over the next few decades (Foster & Vincent 2004; Martin-Smith & Vincent 2006; Shokri et al., 2009). Threats to syngnathid populations include habitat loss and degradation, and over-exploitation. Syngnathids are generally strongly site-associated, slow-moving species, commonly found in near-shore habitats. These characteristics increase their vulnerability to population decline from site-specific threats such as coastal development, trawling and dredging. Millions of syngnathids are collected from the wild each year and are sold for the aquarium trade, traditional medicines and as curiosities (Vincent 1996). They are sourced from trawl by-caught and manual collection in some developing countries such as Indonesia, Malaysia and the Philippines (Vincent 1996; Martin-Smith et al., 2006; Meewig et al., 2006). In 2006 it was estimated that approximately 98% of marine fish for the aquarium trade are wild caught versus only 2% in freshwater varieties. This data is also representative of the syngnathid trade (Foster & Vincent 2005; Job et al., 2006).
As new data continues to demonstrate the unsustainable practices of wild syngnathid collection, this group of fish has become a “flagship species” for their ecosystems across the globe, gaining public attention through their charisma and enigmatic nature. They have provided a focus for efforts to push towards sustainable use of marine resources and international trade controls implemented in May 2004 by the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) Appendix II (Martin-Smith & Vincent 2006). Seahorses, whale sharks and basking sharks were the first marine fish of commercial importance to be brought under CITES trade controls. All subsequent trade in these species among parties to CITES must demonstrate that the trade does not threaten wild populations (Martin-Smith & Vincent 2006). Research efforts currently in place to decrease pressures on wild populations include population surveillance, minimum size-catch limits, improvement of survival in captivity and syngnathid aquaculture (Foster & Vincent 2005). The effectiveness of these strategies has yet to be determined.

HUSBANDRY AND CAPTIVE BREEDING

Over the last two decades the freshwater aquarium trade has made significant progress with breeding and increasing survival rates of fish in captivity, but the marine aquarium trade has lagged behind for many species including syngnathids (Job et al., 2006). This may simply be a reflection of experience; the majority of freshwater fish are supplied by aquaculture compared to a very small fraction of marine ornamentals. What results, therefore, is a greater collective knowledge, efficiency and success in the culture of freshwater species compared with marine species (Koldewey & Martin-Smith 2010).
Aquaculture has been proposed as one of the solutions to address sustainable trade of syngnathids for traditional medicine, aquarium fishes and curios. It is therefore important to evaluate the feasibility of syngnathid aquaculture as part of a solution to relieve pressures on wild populations. Questions that must first be answered are: what problems are encountered with these species in captivity; what are their minimum requirements; can they be bred in captivity; and, is this a profitable industry?

At least thirteen species of seahorses are currently in commercial culture or under research for their potential culture (Koldewey & Martin-Smith 2010). All species of syngnathids require or can tolerate a range of salinities, temperatures, tank substrates and population densities. As with other fish, acute changes in water quality parameters such as salinity, temperature and ammonia and nitrite levels can be lethal for syngnathids, while gradual changes are generally tolerated to a critical point (Adams et al., 2001; Hilomen-Garcia et al., 2003).

There are a number of biological characteristics that may impact the quality of life and survival of syngnathids in captivity. While some syngnathids, such as *Hippocampus whitei*, are truly monogamous, others exhibit social promiscuity (Vincent & Sadler 1995). However, despite promiscuous courtship behavior in some species, these syngnathids demonstrate genetic monogamy (Wilson & Martin-Smith 2007). This aspect may have greater implications when attempting to breed or close the life cycle of syngnathids in captivity. Due to the effect of pair bonding, separating a pair may cause significant stress to each animal, predisposing it to disease and also delaying breeding for a prolonged period of time. In some cases, this separation may lead to permanent cessation of breeding.
Environmental enrichment has had much less emphasis in fish husbandry than for mammals. Enrichment to meet behavioral needs is crucial when considering syngnathids in aquaculture conditions. Enrichment opportunities include providing various perching devices or plants, as many syngnathids have prehensile tails. They become distressed when floating at the surface and will often grasp other individuals continuously in attempts to anchor themselves. Additionally, plants and other tank “ornaments” can divide the tank space into small territories and provide camouflage from other tank mates as well as the public, thereby further reducing stress.

In addition to the constraints described above, there are a number of technical problems involving disease, breeding, and management of several species of syngnathids in captivity (Lockyear et al., 1997; Wilson & Vincent 1998; Woods 2000b; Job et al., 2006; Hora & Joyeux 2009). Disease problems are recognized in syngnathid culture, but most reports are anecdotal and are not in the scientific literature. On the other hand, syngnathid breeding and husbandry problems are extensively documented (Woods 2000b; Job et al., 2002; Takahashi et al., 2003; Ah-King et al. 2006; Ortega-Salas & Reyes-Bustamante 2006; Garcia & Hilomen-Garcia 2009). A major issue with captive culture of syngnathids has been closing the life cycle due to poor survival of initial stages. Syngnathid fry pose many challenges, the main one being the delivery of adequate nutrition due to their small size (Woods 2000a). Finding nutritionally complete and appropriately-sized prey items for these fish is very difficult.

Current evidence suggests that syngnathid aquaculture is not presently economically viable since it cannot compete with the pricing of wild-caught animals (Koldewey & Martin-Smith 2010). While the knowledge base for reproduction and management of
marine fish is rapidly expanding, further improvement is required before syngnathid aquaculture is considered a sustainable and successful industry. Nevertheless, research continues in hopes of establishing syngnathid aquaculture to supply animals for the traditional medicine market and aquarium trade and as an alternative livelihood project in developing countries.

NUTRITION

Providing optimal nutrition for animals in captivity can be very complex. Designing an appropriate diet is often by trial and error with very little knowledge of the animal’s diet and eating patterns in nature. In tropical and subtropical species, natural diets often include a large variety of exotic food items, making it almost impossible to mimic in captivity. However, knowledge of wild food items and their nutritional composition, as well as the nutritional composition and bioavailability of cost-effective feedstuffs available in captivity is required.

The diet of more than twenty species of wild syngnathids studied consists largely of crustaceans, ranging from seventy to one hundred percent of the entire diet in some syngnathid species (Tipton & Bell 1988; Frazoi et al. 1993; Teixeira & Musick 2001; Woods 2002; Lyons & Dunne 2004; Garcia et al., 2005; Kendrick & Hyndes 2005). The remainder of their diet commonly includes nematodes, small molluscs, fish, insects and eggs (Kendrick & Hyndes 2005; Castro et al., 2008).

The intestinal microbiota of an animal influences the host in many ways, including the metabolism of several nutrients. In seahorses, the predominant microbes belong to the
genera *Vibrio, Enterovibrio,* and *Bacillus* (Tanu et al., 2011). Enzymatic studies of these bacteria indicate the ability to degrade lipids, cellulose, xylan, starch and proteins. The majority of the intestinal isolates possess lipase activity, providing seahorses with the ability to utilize lipid-rich foods (Tanu et al., 2011). The importance of lipids and the bacteria which digest them is further emphasized by the minimum dietary requirement for n-3 highly unsaturated fatty acids (HUFA) of some species of syngnathids (Payne et al., 1998; Chang & Southgate 2001).

In captivity, syngnathid diets are composed mainly of live *Artemia,* copepod nauplii and frozen mysids, as these stimulate higher strike rates and provide the best feed conversion ratios when compared to pelleted mysid shrimp or commercial diets (Payne & Rippingale 2000; Chang & Southgate 2001; Wilson et al., 2006; Woods 2003b). While there have been some attempts to develop better pelleted feeds for syngnathids, to date these have had poor growth performance, which would negatively impact long-term survival (Wilson et al., 2006). Evidence suggests that the ideal diet for syngnathids is live enriched feed, whereby the prey item consumes the enrichment product (called ‘gut-loading’) (Payne & Rippingale 2000; Woods 2003a; Woods & Valentino 2003; Wilson et al., 2006). When the fish consumes the enriched *Artemia,* for example, it receives both the nutrition of the crustacean as well as that of the enrichment product. Unfortunately, live feed poses a challenge for many aquaria. If live feed is purchased, it must be from a source known to be disease-free. It can be expensive when compared to the cost of frozen feeds, both as a direct cost and for the holding costs until they are used. The alternative is to culture the feed, which is very labour intensive and is one of the main reasons many aquaria and aquaculture facilities have resorted to other feed types. While some facilities
have had success with frozen feed, it is questionable as to whether or not adequate nutrition can be delivered through a single type of food item, especially over the long-term. Frozen mysid can be soaked in an enrichment product such as Fishvits®; however, the percentage of nutrients lost in the water before it is consumed is unknown. Many products are available on the market to enrich live feed; Selco® and Algamac products are popular choices in aquaria (LePage, personal observation). It would be useful to compare the nutrient composition of wild diets versus the common food items and enrichment products fed to syngnathids in captivity. Their digestibility would also need to be assessed to determine the adequacy of captive diets for individual species.

DISEASE

Relatively little literature investigates disease in syngnathids when compared to other teleosts and there are, in fact, only about twenty peer-reviewed scientific papers reporting diseases in syngnathids in over thirty years of publications. While bacteria and parasites are commonly identified pathogens of syngnathids, their significance in clinical disease is often not well understood. Other causes of morbidity and/or mortality in syngnathids include fungi, virus, neoplasia, gas bubble disease and congenital defects.

Bacteria

Bacteria of clinical significance that have been described to affect syngnathids are mostly limited to Mycobacterium spp. and Vibrio spp. (Alcaide et al., 2001; Bombardini et al., 2006; Balcázar et al., 2010; Martins et al., 2010; Balcázar et al., 2011). However, Aeromonas spp., Tenacibaculum maritimum and Tsukamurella paurometabola have also
been associated with morbidity and mortality in these fish (Bombardini et al., 2006).

Mycobacteriosis in syngnathids is usually caused by atypical or non-tuberculous species; *Mycobacterium chelonae, M. fortuitum* and *M. marinum* (Koldewey 2005). Gross lesions are mainly restricted to skin ulcerations, which can be mild or very severe with ulcers extending to bone. Histologically, mycobacterial lesions are observed as granulomatous inflammation within the central nervous system, kidneys and other organs leading to neurological deficits and associated organ failure (Bombardini et al., 2006). Vibriosis can be observed clinically as hemorrhagic to necrotic skin lesions in seahorses and pipefish. The main etiological agents are *Vibrio harveyi* and *V. alginolyticus* and less often *V. splendidus* (Alcaide et al., 2001; Tendencia 2004; Bombardini et al., 2006; Balcázar et al., 2010; Martins et al., 2010). Bacterial branchitis in syngnathids has been reported and suspected to be associated with *Flavobacteriaceae* due to the filamentous morphology of bacteria within necrotic gill lesions (Bombardini et al., 2006). There are two published reports of epitheliocystis in syngnathids; one in a weedy seadragon (Langdon et al., 1991), which was associated with a Chlamydia-like bacterium by virtue of the analysis of sequences amplified from formalin-fixed tissues (Meijer et al., 2006), and the second in a greater pipefish (Longshaw et al., 2004). All cases of epitheliocystis identified to date have been interpreted to be without clinical significance.

**Parasites**

There are six phyla of parasites known to infect syngnathids; Ciliophora, Apicomplexa, Platyhelminthes, Acanthocephala, Nematoda, and Cnidaria.
**Ciliophora**

Scuticocilliates, primarily *Uronema marinum*, and to a lesser extent *Philasterides dicentrarchi*, are noted parasites infecting syngnathids and have been identified in seahorses (Cheung et al., 1980), seadragons and pipefish (Umehara et al., 2003; Bombardini et al., 2006; Rossteuscher et al., 2008) These agents have been associated with erosive/ulcerative lesions of the skin and gills and are often invasive, causing inflammation in deeper tissues such as muscle, connective tissue, blood vessels, viscera, spinal canal and the coelomic cavity (Cheung et al., 1980; Umehara et al., 2003; Bombardini et al., 2006; Rossteuscher et al., 2008; Stidworthy 2008). Peritrichous ciliates, such as *Trichodina* sp., have also been noted in the gills of pipefish and seahorses (Longshaw et al., 2004; Bombardini et al., 2006). Lesions associated with these protists were limited to mild epithelial hyperplasia and the organisms were considered opportunistic and of limited consequence (Bombardini et al., 2006; Longshaw et al., 2004).

**Apicomplexa**

Two species of coccidians have been documented in syngnathids; *Eimeria syngnathi* in *Syngnathus abaster*, formerly *Syngnathus nigrolineatus* and *Eimeria phillopterycis* in *Phyllopteryx taeniolatus* (Yakimoff & Gousseff 1936; Upton et al., 2000). Clinical signs noted were anorexia and stunted growth (Upton et al., 2000). Lesions were limited to the small intestine with various stages of the parasite encysted primarily within columnar epithelial cells but also within the lamina propria; there was a mild inflammatory response (Upton et al., 2000). While the associated tissue damage was not extensive, a heavy burden of *Eimeria* could cause severe malabsorption.
Platyhelminthes

Gyrodactylids and digenic trematodes are the only platyhelminthes documented to infect syngnathids. To date, six species of gyrodactylids have been identified in syngnathids and they have only been documented in pipefish; *Gyrodactylus syngnathi* (Appleby 1996), *G. corleonis* (Paladini et al., 2010), *G. neretum* (Paladini et al., 2010), *G. epipayipi* (Vaughan et al., 2010), *G. shorti* (Holliman 1963), and *G. pisculentus* (Bombardini et al., 2006; Williams et al., 2008). In many of these publications, the morphology of gyrodactylid species is well described but less emphasis is placed on the significance of these agents in disease. These parasites were found on the epithelium of the skin, fins and gills and two species, *G. eyipayipi* and *G. shorti*, were also found inside the broodpouch, and *G. shorti* was found exclusively at that location (Holliman 1963; Vaughan et al., 2010). The primary cause of mortality in these cases was usually attributed to another disease process such as bacterial disease and concurrent stress from acclimation and water quality (Paladini et al., 2006; Williams et al., 2008).

Cnidaria

Cnidarians, specifically myxosporeans of the genera *Sinuolinea* (Garner et al., 2008), *Kudoa* (Longshaw et al., 2004), *Sphaeromyxa* (Vincent & Clifton-Hadley 1989), and *Myxidium* have been documented in pipefish (Longshaw et al., 2004), seahorses and seadragons. *Kudoa cf. quadratum* was noted within the skeletal muscle of *Syngnathus acus* with minimal host response, which is generally characteristic of infections with *Kudoa* spp. (Longshaw et al., 2004). *Myxidium cf. incurvatum* in the same species was found within the gall bladder, eliciting an extensive host response, including epithelial hyperplasia and necrosis, with mild multifocal atrophy of epithelial cells (Longshaw et
al., 2004). Sinuolinea phyllopteryxa was identified to cause mild renal tubular dilation, renal tubular cell hypertrophy and marked dilation of central veins draining kidneys in wild-caught Phyllopteryx taeniolatus (Garner et al., 2008). The authors describing these infections considered that morbidity was attributable to the myxosporean infections; however, they were not the primary causes of mortality.

A variety of other parasites have been noted as incidental findings in syngnathids: the acanthocephalan Corynosoma australe (Braicovich et al., 2005); digenean metacercarea of Cryptocotyle lingua encysted in the muscle (Longshaw et al., 2004); an unidentified digenean in the stomach (stage not noted) (Longshaw et al., 2004); an unidentified nematode larva within the ovary (Vincent & Clifton-Hadley 1989); the larval nematode Anisakis simplex in the viscera; unidentified cestode plerocercoids within the intestine (Longshaw et al., 2004); and finally Botryllus schlosseri, an ascidian tunicate adhered to the skin (Kayiş 2011).

Fungi
While fungi are often considered secondary invaders in fish, microsporeans are a notable exception in syngnathids. The only reports of mycoses causing mortalities in seahorses have been caused by microsporidians. Microsporidiosis caused by Glugea heraldi has been well described in wild-caught Hippocampus erectus (Blasiola 1979; Vincent & Clifton-Hadley 1989; Bombardini et al., 2006). This microsporidian causes multifocal cysts in the skin along the body and tail that eventually coalesce and rupture, predisposing to secondary bacterial infections. G. heraldi has been documented to cause over 95% mortality within 6 months in wild-caught H. erectus (Vincent & Clifton-Hadley 1989). Two species of Exophiala species have been documented to cause a disseminated
infection in seadragons (Nyaoke et al., 2009). Lesions consisted of parenchymal and vascular necrosis with fungal invasion of gill, kidney and swim bladder but occasionally the heart, liver, spleen, intestine, mesentery and spinal cord. Of 20 seadragon cases, 65% had epithelial ulcerations with associated mats of fungal hyphae invading dermis, hypodermis, fascia and skeletal muscle (Nyaoke et al., 2009). A variety of gram-negative bacteria were also cultured from skin lesions of these animals; however, no species of bacteria was consistently isolated. While the vascular invasion and visceral lesions were fungal, it is not unknown if the inciting lesion was bacterial, fungal or traumatic (Nyaoke et al., 2009). Stress of captivity and artificial systems may contribute to immunosuppression and predispose fish to opportunistic infections.

**Viruses**

No peer-reviewed publication has identified viral infections in syngnathids to date.

**Neoplasia**

Neoplasia has been described in syngnathids (LePage et al., 2012; Lourie & Kuiter 2008). Many of these fish had clinical signs expected with neoplasia in animals: anorexia, emaciation, general poor-doing and some with buoyancy disturbances. Organs involved in primary tumors are the broodpouch (Willens et al., 2004), kidneys, heart, pancreas and intestines (LePage et al., 2012). While there appears to be a predisposition of adult males for developing neoplasms, too few have been reported to date to draw any definite conclusions.
Gas bubble disease

Gas bubble disease (GBD) is not well described in the literature. Anecdotally, it is a well-known syndrome of syngnathids in captivity. The condition can present in a number of ways. The first presentation involves a large focus or pocket of gas in the coelomic cavity, gastrointestinal tract or brood pouch lumen of males or over-inflation of the swimbladder (LePage, personal observation). The condition can also present as subcutaneous emphysema anywhere along the body or emphysema within well-vascularized tissues such as the choroid rete and brood pouch walls. It has been suggested that subcutaneous emphysema of the tail is restricted to males (Greenwell 2002). While not proven in experimental trials, it is generally accepted that gas accumulation is either due to nitrogen supersaturation of tank water or due to bacterial infections (LePage, personal observation).

The effects of varying light intensity, stocking density and temperature on GBD have been studied in *Hippocampus erectus* (Lin et al., 2010). Lower temperature appears to be the only factor having a significant effect on the development of GBD. Fish held at 23°C versus 26°C and 29°C had a higher incidence of gas bubble disease. The group of seahorses held 26°C had the lowest incidence of GBD and the highest survival rate after the 4 week study (Lin et al., 2010). In captivity, *H. erectus* has been held at temperatures between 13°C and 28°C, with the average temperature being approximately 23°C (Koldewey 2005). Although other factors may come into play over a long-term trial, this may be a good reason to reconsider the preferred optimal temperature range in this syngnathid species, shifting the average to 26°C.
**Congenital defects**

A single case of a congenital malformation has been documented in syngnathids - an underdeveloped or absent swim bladder in a weedy seadragon (Garland et al., 2002). While this has only been documented once, anecdotally, many aquaria in North America have described the same condition in seadragons. Affected fish are usually described as ‘poor-doers’ or ‘runts’. Upon necropsy of these animals, the swim bladders are usually underdeveloped (LePage, personal observation). Considering the fact that there is one main supplier of these fish for aquaria and that it is difficult to close the life cycle in captivity, one explanation may be a narrowing of the genetic pool leading to increased incidence of this congenital defect.
RATIONALE, HYPOTHESES & OBJECTIVES

Syngnathids are a unique family of fish, which pose numerous challenges in terms of conservation in the wild, and husbandry and disease management in captivity. While much of the work to date has focused on conservation efforts and some unique physiological features, there is relatively little published about diseases in these animals despite their increasing popularity in aquaria and elevated mortality rates in captivity.

1. A first objective of this study was therefore to describe and summarize diseases of captive syngnathids from the Toronto Zoo from 1998 until 2010.

The most common cause of mortality in this population of yellow seahorses, *Hippocampus kuda*, was an ulcerative dermatitis associated with filamentous bacteria under light microscopy. This disease was investigated in more depth due its impact on this population of seahorses and because skin ulceration is a common clinical presentation in aquaria. It was hypothesized that the bacteria observed within epidermal ulcerative lesions under light microscopy were bacteria of the Flavobacteriaceae family, due to their filamentous morphology and propensity to cause ulcers in other teleosts.

Additional objectives of this thesis were therefore:

2. To culture bacteria from ulcerative lesions and identify using 16S rRNA gene amplification and sequencing, and phenotypic testing,

3. To extract 16S rDNA from bacteria within ulcerative lesions preserved in formalin-fixed paraffin-embedded tissues using laser capture microdissection and compare the amplified sequences to those of isolates cultured from lesions.
4. To design probes for *in situ* hybridization based on 16S rRNA genetic sequences of candidate bacteria from the preceeding objectives and use these probes to characterize and localize bacteria within ulcerative lesions under light microscopy.
CHAPTER 2. A STUDY OF DISEASES OF CAPTIVE SYNGNATHIDS AT THE TORONTO ZOO

This chapter corresponds to the following manuscript:


ABSTRACT

Seahorses, pipefish and seadragons are fish of the Family Syngnathidae. From 1998 to 2010, 172 syngnathid cases from the Toronto Zoo were submitted for post-mortem diagnostics. Among the submitted species were yellow seahorses (*Hippocampus kuda*; n = 133), pot-bellied seahorses (*Hippocampus abdominalis*; n = 35) and weedy seadragons (*Phyllopteryx taeniolatus*; n = 4). The three most common causes of morbidity and mortality in this population were bacterial dermatitis, bilaterally symmetrical myopathy and mycobacteriosis, accounting for 24, 17 and 15% of cases, respectively. Inflammatory processes were the most common diagnoses, present in 117 cases. Seven neoplasms were diagnosed, environmental etiologies were identified in 46 cases and 2 congenital defects were identified.
INTRODUCTION

Seahorses, pipefish and seadragons are fish of the Family Syngnathidae, which includes over 295 species (Froese & Pauly 2011). Syngnathids have attracted attention for decades due to their unique morphology, remarkable camouflage ability and the distinctive phenomenon whereby males give birth to live young. For many years, syngnathids have been collected from the wild for curiosities, exhibition in aquaria and traditional medicine. There is increasing data demonstrating that the current practice of wild syngnathid collection is unsustainable (Martin-Smith & Vincent 2006). Due to their charismatic nature and their vulnerability, syngnathids have become a flagship species for their respective ecosystems across the globe (Martin-Smith & Vincent 2006, Shokri et al., 2009). They have provided a focus for efforts towards sustainable use of marine resources, with international trade controls implemented in 2004; the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) Appendix II (Martin-Smith & Vincent 2006).

A suggested alternative to wild-caught animals is captive culture or syngnathid aquaculture. While this has been successful for some species of syngnathids, it has not been shown to be cost effective (Koldewey & Martin-Smith 2010). Captive breeding and rearing of many species of syngnathids remains a challenge due to disease from various pathogens, inappropriate nutrition and environmental conditions. There is very little literature published on syngnathid disease when compared to other teleosts bred in captivity or even compared to other areas of syngnathid research.
This report describes the causes of morbidity and mortality of three species of captive syngnathids held at the Toronto Zoo, Ontario, Canada from 1998 until 2010: the yellow seahorse *Hippocampus kuda*, pot-bellied seahorse *Hippocampus abdominalis* and weedy seadragon *Phyllopteryx taeniolatus*. A better understanding of the types of disease that affect this characteristic group of fish will improve future diagnostic procedures. This precedes the implementation of husbandry modifications, disease prevention, palliative care and treatment with the ultimate benefit of improved survival and welfare in captivity.
MATERIALS AND METHODS

From 1998 to 2010, 172 syngnathids from the Toronto Zoo were submitted for post-mortem diagnostics. Among the submitted species were yellow seahorses 
(*Hippocampus kuda*; n = 133), pot-bellied seahorses (*Hippocampus abdominalis*; n = 35) and weedy seadragons (*Phyllopteryx taeniolatus*; n = 4). These fish were held in a variety of marine enclosures (salinity 22-26 ppt) at the Toronto Zoo in Scarborough, Ontario, Canada. All tanks had biofiltration systems (Hagen Aquaclear and Hagen Bio Foam, Hagen Inc., Montreal, Québec, H9X 0A2, Canada). Water temperatures ranged from 18 - 20°C for the pot-bellied seahorses and 24 - 28°C for the weedy seadragons and yellow seahorses (Thermal Compact Heaters, Hagen Inc., Montreal, Québec, H9X 0A2, Canada), and all fish were kept on a 13 h day, 11 h night cycle. Tank substrates varied from coarse sand to bare tanks, some housing live plants, while others had plastic plants and perches. Males and females were approximately equally represented in the populations of each species.

All syngnathid mortalities were routinely investigated. Cases included fish found dead, and moribund fish euthanized by an overdose of tricaine methanesulphonate (Aqualife TMS, Syndel Laboratories Ltd, Qualicum Beach, British Columbia, V9K 1V5, Canada) by bath immersion. The coelomic cavity was opened and whole fish immersed in 10% neutral-buffered formalin (ACP Chemicals Inc., Saint-Léonard, Québec, H1R 1A5, Canada) for 24 to 48 h.

Formalin-fixed fish were transferred to decalcifying solution (Surgipath Decalcifier II, Surgipath Medical Industries Inc., Richmond, Illinois, 60071, USA) for 2 h. Entire fish
were then sectioned transversely in segments approximately 3 to 4 mm in width using a scalpel blade. Segments were then processed routinely for paraffin embedment and histologic sections; 4 µm sections were stained with hematoxylin and eosin (Animal Health Laboratory, Guelph, Ontario, N1G 2W1, Canada). Additional stains were used on selected sections as required included Brown-Hopps, Brown-Brenn, Masson’s trichrome, periodic acid-schiff, Pierce Van der Kamp, Ziehl-Neelsen and modified Ziehl-Neelsen (Animal Health Laboratory, Guelph, Ontario, N1G 2W1, Canada).

It should be noted that the cases evaluated represent only cases submitted to the fish pathology diagnostic service: a number of fish were not submitted by the Toronto Zoo because they were a portion of a mortality event or due to the degree of autolysis. The diseases noted here are those identified via gross examination, light microscopy and where warranted and possible, limited microbiological investigations. The diagnoses ascribed to cases were those considered to be significant contributors to the morbidity and mortality of the fish.
RESULTS

Of 172 syngnathid cases, the three most common causes of morbidity and mortality were bacterial dermatitis, bilaterally symmetrical myopathy and mycobacteriosis, accounting for 24, 17 and 15 percent of cases, respectively (Table 2.1). The temporal trend of the five most common causes of mortality over 12 years of this study demonstrate that the mortalities were not due to any single mortality event but rather mortality trends over time which vary for each etiology and each year (Figure 2.1). Diagnoses are discussed in broad categories: inflammatory, neoplastic, environmental and congenital (Table 2.2). Inflammatory processes were most commonly diagnosed and were present in 117 cases, while environmental etiologies were implicated in 46 cases, and finally seven neoplasms and two congenital defects were identified.

**Inflammatory**

**Bacterial**

Diseases caused by bacteria were the leading cause of morbidity and mortality, found in 95 of 172 cases. Bacterial dermatitis was the most common cause of morbidity and mortality in this population of fish (41/172). This dermatitis often presented clinically as ‘red-tail’, a common term used to describe the hyperaemic ventral aspect of the entire length of the tail caudal to the vent (Figure 2.2a). A second clinical presentation or possible progression of ‘red-tail’ was multifocal skin ulcerations occurring anywhere along the body. Under light microscopy these lesions were characterized by little to no inflammatory infiltrate, degeneration and necrosis of the epidermis leading to erosion and/or ulceration, and extensive edema within the underlying dermis and musculature. In the majority of cases (28/41), large numbers of filamentous bacteria were adhered to the
necrotic epithelium and infiltrated the underlying dermis (Figure 2.2b). When present, the inflammatory reaction was composed of small numbers of debris-filled macrophages that were scattered within the dermis, but less commonly they were also present between and within underlying myofibres. Other infectious agents were often present in combination with filamentous bacteria, such as ciliated protozoa and fungal hyphae (Figure 2.2c).

Mycobacteriosis was identified in 15% cases (26/172). Gross lesions included granulomas within organs and/or ulcerative skin lesions. Under light microscopy, the granulomatous lesions were found most commonly within the kidney, liver and gastrointestinal tract however some fish were found to have granulomatous inflammation within the choroid rete, central nervous system, muscle and reproductive tract. Acid-fast staining demonstrated rod-shaped bacteria within granulomas and ulcerative lesions.

Non acid-fast bacteremia occurred in 10% of cases (17/172) and in most instances (greater than 70%) progressed from a severe ulcerative bacterial dermatitis. The organs most commonly affected were liver, kidney, gastrointestinal tract and in one case the epicardium. Lesions consisted mainly of necrosis centered on blood vessels of these organs with an intense collar of large numbers of macrophages filled with necrotic debris, fewer lymphocytes and intralesional bacteria.

Fifteen fish had branchitis associated with bacteria; however, three of these had a mixed infection that also involved ciliated protozoans. The most common presentation involved extensive necrosis of lamellae and entire filaments, which were surrounded by extensive mats of filamentous but also morphologically-mixed bacteria (Figure 2.3a). In affected
gills, there were small to moderate numbers of macrophages filled with debris within the central venous sinus and lamellar vascular spaces and interstitium. Autolysis of the gills was a common finding.

There were two cases of epitheliocystis, both of which occurred in weedy seadragons. Multiple (greater than 5 per gill arch) circular to ovoid intracytoplasmic bacterial microcolonies (approximately 25-100 μm diameter) that were acid-fast negative but stained positively with Pierce Van der Kamp stain were present within the gill lamellar epithelium (Figure 2.3b). These microcolonies were also found within epithelial cells lining the nasal pit, as well as the branchial and oral cavities.

Large numbers of morphologically-mixed bacteria within the lumen of the gastrointestinal tract was a common finding due to excessive bacterial growth on or within ingested food. In one case, a bacterial enteritis, presumably an extension of the bacterial overgrowth described above, was identified (1/172) (Figure 2.4). A population of short bacterial rods was noted that invaded and elevated the intestinal epithelium from the basement membrane, this lesion was associate with epithelial degeneration and necrosis. These bacteria were also seen within the lamina propria and underlying submucosa and there was mild to moderate expansion of both layers by edema. In cases of bacterial overgrowth, large numbers of morphologically-mixed bacteria were restricted to the intestinal lumen and the epithelium was either intact or autolysed. There were also typically copious food items in the intestinal lumen that contained these same bacterial populations.
Parasitic

Seven fish were diagnosed with cryptosporidial enteritis. In these cases, the intestinal lumen was dilated and there was diffuse epithelial necrosis and attenuation with moderate numbers of both epicellular (∼5 µm basophilic blebs) and intraepithelial (∼1 µm eosinophilic and basophilic forms of apicomplexans consistent with Cryptosporidium sp. (Figure 2.5). The connective tissue and blood vessels of the lamina propria and submucosa were expanded by edema. Within all layers of the intestine and surrounding foci of necrosis, there were small numbers of inflammatory cells, debris-filled macrophages and fewer lymphocytes.

While the presence of protozoa was common in this population of syngnathids, protozoal dermatitis was found to be a significant contributor to morbidity and/or mortality in 13 fish. Of these cases, 9 protozoan infestations occurred in combination with a bacterial dermatitis. The organisms were teardrop-shaped ciliated protozoa, averaging 8 by 15 µm. Mild disease involved epithelial erosion, mild necrosis with ciliates along the affected epithelial surface. In severe infections, bacteria and occasionally fungi were also involved with ulceration of the epithelium and superficial dermis and with invasion of ciliates into the underlying musculature (Figure 2.6). Occasionally, in cases involving multiple newborn seahorse mortalities, which occurred as epizootics, protozoa were the only infectious agent present. The shape and size of these ciliated protozoa suggested a Uronema-like species.

Another type of protozoa was present in 2 cases involving mortality of multiple seahorse fry with a mixed bacterial and parasitic dermatitis (also including Uronema-like protozoa). The protozoa were eosinophilic circular bodies, 2-3 µm in diameter containing
small basophilic nuclei. They were observed within dermal musculature and along damaged surface of the epidermis (Figure 2.7). Inflammation associated with these unidentified parasites was sporadic; however, areas with inflammatory infiltrate consisted of small numbers of macrophages with occasional phagocytosis of the whole parasite.

**Viral**

Viral enteritis was suspected in adults and juvenile syngnathids. The disease was thought to be worst in younger fish; lesions were more severe with a higher mortality rate that occurred acutely with minimal clinical signs. The suspect viral enteritis was a problem for several months in one year and these lesions have not appeared since. Under light microscopy, the lesions considered typical of a viral infection were a markedly dilated intestinal lumen and a very attenuated epithelium with the absence of a visible etiological agent. Mild necrosis of epithelial cells and submucosal edema were also present (Figure 2.8). Electron microscopy is currently being performed to rule in or out a virus; to date, there are no other disease agents present or other known causes for this enteritis.

**idiopathic**

Idiopathic inflammation was the final diagnosis when inflammatory lesions were present without evidence of etiological agents and lesions were not characteristic of a particular disease agent pattern (7/172 cases). Organs most commonly affected were kidney and liver. The nephritis was usually mild and characterized mainly by macrophages and small numbers of lymphocytes within the renal interstitium. The hepatitis had similar cellular infiltrates, but inflammation was centered on blood vessels.
Neoplastic

Seven separate neoplasms and two neoplastic-like lesions were identified in eight individuals consisting of one *P. taeniolatus*, one *H. abdominalis* and six *H. kuda* (LePage et al., 2012; expanded and published separately). The overall prevalence of neoplasia in syngnathids at the Toronto Zoo was 4.1%. Types of neoplasms included: cardiac rhabdomyosarcoma, renal adenoma, renal adenocarcinoma, renal round cell tumor, exocrine pancreatic carcinoma and intestinal carcinoma. The most commonly diagnosed neoplasm was a renal round cell tumor, likely lymphoma (2/7). Furthermore, the kidney was the likely site of origin for over half of the neoplasms (4/7). Males more commonly had neoplasms (6/7) and all affected fish were adults. However, due to the low prevalence of neoplasia in this population, no definite conclusions can be drawn regarding the predisposition of adult males to develop neoplasia.

Environmental

Gas bubble disease

Gas bubble disease (GBD) was characterized by the gross observation of excess gas accumulation in tissues. In syngnathids at the Toronto Zoo, gas was found to accumulate most commonly in subcutaneous tissues anywhere along the body and within the broodpouch. The basis for diagnosis of all these cases was gross post-mortem findings and case history. Light microscopy was not always a useful aid in the diagnosis of GBD as artifactual lifting of the epidermis and separation along tissue planes commonly confounded interpretations. Over 85% of cases (13/15) with gas bubble disease also had a concurrent disease. These fish presented with buoyancy problems and were often distressed and floating at the surface or had lost their ability to right themselves. The
most common concurrent disease for those accumulating gas subcutaneously was a bacterial dermatitis, occurring as a sequella to rupture of the bullae. Gas accumulation within tissues can cause excessive buoyancy. Subcutaneous bullae may rupture during a struggle to remain within the water column, rubbing against tank walls and trying to grasp objects.

**Bilaterally symmetrical myopathy**

A myopathy was observed in *H. kuda* only (29/172 cases). Clinical signs noted by aquarists previous to death in those animals found later to have myopathy were muscle weakness, as evidenced by decreased strength of prey striking or complete anorexia, inability to remain upright in the water column and lethargy. These fish were often found laterally recumbent at the bottom of the tank or with an abnormally bent neck and were euthanized due to their inability to eat. Tube feeding was attempted with some of these fish; however, the stress of frequent handling was counterproductive to the positive effects of this treatment. Under light microscopy the myopathy was bilaterally symmetrical and was present in the axial musculature, mainly the epaxial muscles of the nuchal region. Significant inflammatory infiltrates were not always present in the affected muscle tissue and when present, consisted of macrophages filled with debris. Three stages of the myopathy were noted: an early myopathy, presenting as hypereosinophilia with loss of striation but without detectable inflammation; an active “myopathy” with large numbers of macrophages; and a chronic or resolving myopathy with small condensed myofibres and myocyte sheath remnants but little to no detectable inflammatory cells in muscle tissues (Figure 2.8). Lesions in all stages included varying degrees of condensed hypereosinophilic myofibres and centralized nuclei; however,
rowing of nuclei was not prominent. Variation in myofibre diameter, likely compounded by atrophy due to starvation, was prominent. While infectious agents were not directly associated with these lesions, concurrent disease was very common. Twenty-two of twenty-nine cases with bilaterally symmetrical myopathy also had a dermatitis (bacterial and/or parasitic), but some fish also were affected with cryptosporidiosis, mycobacteriosis, non-acid-fast bacteremia, GBD and neoplasia.

**Congenital**

Two congenital defects were observed in this population of syngnathids. The first was a blood-filled cyst in the ventricular myocardium. A 2-yr-old *H. kuda* had an expansive 200-250 μm mass that effaced the myocardium at the apex of the ventricle and extended from the ventricular lumen to the epicardium (LePage et al., 2012; expanded and published separtetely). The second congenital defect was observed in an adult female *H. abdominalis*. On gross examination there appeared to be a duplication of the ovipositor (Figure 2.9a). On light microscopy there were two gravid ovaries, which were equal in size. Each ovarian duct appears to exit through its own ovipositor rather than merging to a single one along midline. The ovipositors were located on each side lateral to the expected location (Figure 2.10). This finding was incidental and did not appear associated with morbidity or mortality.
Table 2.1. Summary of the top ten causes of morbidity and mortality diagnosed in syngnathids at the Toronto Zoo from 1998 to 2010.

<table>
<thead>
<tr>
<th>Disease</th>
<th>Individuals affected</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacterial dermatitis</td>
<td>41</td>
</tr>
<tr>
<td>Myopathy</td>
<td>29</td>
</tr>
<tr>
<td>Mycobacteriosis</td>
<td>26</td>
</tr>
<tr>
<td>Non acid-fast bacteremia</td>
<td>17</td>
</tr>
<tr>
<td>Gas bubble disease</td>
<td>15</td>
</tr>
<tr>
<td>Bacterial branchitis</td>
<td>15</td>
</tr>
<tr>
<td>Parasitic dermatitis</td>
<td>13</td>
</tr>
<tr>
<td>Neoplasia</td>
<td>7</td>
</tr>
<tr>
<td>Cryptosporidiosis</td>
<td>7</td>
</tr>
<tr>
<td>Idiopathic enteritis, hepatitis, nephritis</td>
<td>7</td>
</tr>
</tbody>
</table>
Table 2.2. Summary of disease processes diagnosed in syngnathids at the Toronto Zoo from 1998 to 2010. Note that there can be more than one disease process per case.

<table>
<thead>
<tr>
<th>Disease</th>
<th>Cases affected</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inflammatory</td>
<td>123</td>
</tr>
<tr>
<td>Bacterial</td>
<td>95</td>
</tr>
<tr>
<td>Parasitic</td>
<td>13</td>
</tr>
<tr>
<td>Viral$^1$</td>
<td>2</td>
</tr>
<tr>
<td>Mycotic</td>
<td>2</td>
</tr>
<tr>
<td>Idiopathic</td>
<td>7</td>
</tr>
<tr>
<td>Neoplastic</td>
<td>7</td>
</tr>
<tr>
<td>Environmental</td>
<td>46</td>
</tr>
<tr>
<td>Gas bubble disease</td>
<td>15</td>
</tr>
<tr>
<td>Suspect nutritional myopathy</td>
<td>29</td>
</tr>
<tr>
<td>Congenital</td>
<td>2</td>
</tr>
</tbody>
</table>
| Open             | 30$^2$         

$^1$Cases suspected to be of viral etiology based on histological lesions.

$^2$Open cases are those that were most often autolysed fish (21/30) or fish in good condition for which no histological diagnosis was found (9/30).
Figure 2.1. Yearly trend of the five most common causes of mortality in syngnathids at the Toronto Zoo as number of cases per year.
Figure 2.2. a. Female *Hippocampus kuda* with a hyperemic tail or ‘red-tail’. b. Severe ulcerative skin lesion with adherent and invasive filamentous bacteria (H&E). c. Severe ulcerative skin lesion with morphologically-mixed bacteria and protozoa invading the dermis (H&E). Inset: Higher magnification of ciliated protozoans (H&E).
**Figure 2.** a. Female *Hippocampus kuda* with a hyperemic tail or ‘red-tail’. b. Severe ulcerative skin lesion with adherent and invasive filamentous bacteria (H&E). c. Severe ulcerative skin lesion with morphologically-mixed bacteria and protozoa invading the dermis (H&E). Inset: Higher magnification of ciliated protozoans (H&E).
Figure 2.2. a. Female *Hippocampus kuda* with a hyperemic tail or ‘red-tail’. b. Severe ulcerative skin lesion with adherent and invasive filamentous bacteria (H&E). c. Severe ulcerative skin lesion with morphologically-mixed bacteria and protozoa invading the dermis (H&E). Inset: Higher magnification of ciliated protozoans (H&E).
Figure 2.3. **a.** Necrotic branchial filaments and lamellae are surrounded by mats of filamentous bacteria mixed with bacteria of varied morphology (H&E). Arrows point to a mat of bacteria. Inset: Higher magnification of bacterial mats. **b.** Numerous intracytoplasmic bacterial microcolonies within branchial lamellar epithelial cells that expand and distort lamelllar structure (PVK).
Figure 2.3. a. Necrotic branchial filaments and lamellae are surrounded by mats of filamentous bacteria mixed with bacteria of varied morphology (H&E). Arrows point to a mat of bacteria. Inset: Higher magnification of bacterial mats. b. Numerous intracytoplasmic bacterial microcolonies within branchial lamellar epithelial cells that expand and distort lamellar structure (PVK).
Figure 2.4. a. Enteritis with bacteria underrunning the intestinal mucosa (H&E). b. Intestinal luminal bacterial overgrowth from contaminated food (H&E). Arrows point to mats of bacteria in both images.
Figure 2.4. a. Enteritis with bacteria underrunning the intestinal mucosa (H&E). b.

Intestinal luminal bacterial overgrowth from contaminated food (H&E). Arrows point to mats of bacteria in both images.
**Figure 2.5.** Intracellular and epicellular forms of suspected cryptosporidial parasites associated with an attenuated and flattened intestinal epithelium with mild necrosis (H&E). Arrows point to cryptosporidial parasites.
Figure 2.6. Ulcerative dermatitis with *Uronema*-like ciliated protozoa invading deeply within the dermis of a juvenile seahorse (H&E). Arrows point to protozoa.
Figure 2.7. Ulcerative dermatitis with numerous unidentified protozoa invading deeply within the dermis. Mats of filamentous bacteria and a *Uronema*-like parasite are also present within this lesion. Arrows point to the unidentified protozoa.
**Figure 2.8.** Intestinal cross-section with marked dilation of the intestinal lumen and thinning of the epithelium. Inset: Higher magnification of the thinned epithelium with mild necrosis and the absence of an etiological agent (H&E).
Figure 2.9. Skeletal muscle (cervical region of epaxial muscles) affected by a suspect nutritional myopathy with marked myofibre diameter variation and hypereosinophilia. Inset: Typical bilaterally symmetrical pattern of lesions (H&E).
Figure 2.10. Ovipositor duplication in *H. abdominalis*, showing left and right lateral views. Each space on the bottom ruler represents one millimeter. Arrows indicate the right and left ovipositors respectively.
DISCUSSION

Inflammatory

Bacterial

Bacterial diseases are very common in teleosts and have been described in syngnathids (Alcaide et al., 2001; Bombardini et al., 2006; Balcázar et al., 2010; Martins et al., 2010). Clinically significant bacterial diseases described in syngnathids are mostly limited to mycobacteriosis (Bombardini et al., 2006) and vibriosis (Alcaide et al., 2001; Tendencia 2004; Bombardini et al., 2006; Balcázar et al., 2010; Martins et al., 2010). However, Aeromonas spp, Tenacibaculum maritimum and Tsukamurella paurometabola have also been associated with morbidity and mortality in these fish (Bombardini et al., 2006). Bacteria that have been isolated from syngnathids are mainly from wild caught clinically healthy animals or from captive animals with clinical signs of disease.

In teleosts, including syngnathids, common bacteria associated with ulcerative skin lesions include Aeromonas (Bombardini et al., 2006; Roberts et al., 2009), Tenacibaculum (Ostland et al., 1999; Avendano-Herrera et al., 2006; Bombardini et al., 2006), Vibrio (Alcaide et al., 2001; Tendencia 2004; Bombardini et al., 2006; Roberts et al., 2009), and Mycobacterium species (Bombardini et al., 2006; Roberts et al. 2009).

Vibriosis can be observed clinically as hemorrhagic to necrotic skin lesions in seahorses and pipefish. The main etiological agents are Vibrio harveyi and V. alginolyticus and less often V. splendidus (Alcaide et al., 2001; Tendencia 2004; Bombardini et al., 2006; Balcázar et al., 2010). As in other teleosts, mycobacteriosis in syngnathids is typically caused by atypical or nontuberculous species, e.g. Mycobacterium chelonae, M. fortuitum and M. marinum (Koldewey 2005). Lesions in previous study range from skin ulcers to
systemic disease manifested as granulomatous inflammation within the kidney, liver, gill, muscles and occasionally the central nervous system. These findings are consistent with other cases of mycobacteriosis in syngnathids (Bombardini et al., 2006).

Non acid-fast bacterial dermatitis was the most common cause of morbidity and mortality in this population of fish, accounting for almost 25% of cases. Lesions were often associated with a filamentous bacterium observed within these erosive to ulcerative lesions under light microscopy. The consequences of ulcerative skin lesions in fish are more significant than in mammals as the epidermis and dermis not only acts as a barrier against pathogens but also limit significant shifts of water from the fish to the external environment in the case of marine fish. When skin ulcerations cover too large a surface area for the fish to compensate, they can quickly lead to mortality (Ferguson 2006). These lesions and the agents associated with them are the subject of Chapter 3 of this thesis.

In the syngnathid population examined here, there was one instance of bacterial enteritis that was interpreted to be clinically significant. Bacteria within these lesions were not characterized; however, gastroenteritis and necrotizing enteritis has been associated with species of Vibrio in other teleosts (Lee et al., 2002), for example, V. carchariae in summer flounder (Soffientino et al., 1999) and groupers (Yii et al., 1997). In healthy captive H. kuda, the predominant bacterium was found to belong to the genus Vibrio (Tanu et al., 2011). This may indicate that gastroenteritis caused by Vibrio, at least in syngnathids, could be an opportunistic infection that is precipitated by other stressors or gastric mucosal damage.
Bacterial branchitis in syngnathids has been reported and was suspected to be associated with *Flavobacteriaceae*, due to the filamentous morphology of bacteria within necrotic gill lesions (Bombardini et al., 2006). This is not unlike gill disease in freshwater teleosts caused by *Flavobacterium columnare* (Bernadet 1989; Decostere et al., 1998). In the present study, there were seven cases with significant gill lesions that involved moderate multifocal branchial epithelial cell necrosis with intralesional mixed populations of bacteria, some of which were filamentous. There are two published reports of epitheliocystis in syngnathid gills; one in a weedy seadragon (Langdon et al., 1991) that was later associated with a chlamydial agent (Meijer et al., 2006) and the second in a greater pipefish (Longshaw et al., 2004). Two cases of moderate to severe epitheliocystis, as determined by the relative size and number of bacterial inclusions, were observed in this population of syngnathids and these were both in weedy seadragons. All cases identified previously were incidental findings and by the low numbers of inclusions, most likely did not have clinical significance (Langdon et al., 1991; Longshaw et al., 2004). It is possible that seadragons have a propensity for development of epitheliocystis or a susceptibility to chlamydia-like organisms, but firm conclusions cannot be drawn from such low case numbers.

Bacterial epicarditis is not common but has been reported in fish, involving *Vagococcus salmoninarum* (Michel et al., 1997), *Renibacterium salmoninarum* in salmonids (Fryer & Sanders 1981) and *Streptococcus agalactiae* in Nile tilapia (Filho et al., 2009). A single case of bacterial epicarditis was found in this syngnathid population; however, the etiological agent was not characterized.
Parasitic

Parasites are the most extensively documented group of infectious agents in syngnathids. More specifically, metazoans noted to infect syngnathids include monogeneans (Holliman 1963; Appleby 1996; Williams et al., 2008; Paladini et al., 2010; Vaughan et al., 2010), acanthocephalans (Braicovich et al., 2005), nematodes (Longshaw et al., 2004), trematodes (Longshaw et al., 2004) and myxosporeans (Vincent & Clifton-Hadley 1989; Longshaw et al., 2004; Garner et al. 2008); protozoans reported to infect syngnathids include ciliophorans such as scuticociliates (Cheung et al., 1980; Umehara et al., 2003; Rossteuscher et al. 2008), peritrichs (Longshaw et al., 2004); and apicomplexans (Upton et al., 2000). The parasites observed in this population of syngnathids is consistent with the literature; Uronema-like ciliates, cryptosporidians, trematodes and nematodes. Overall, the clinical significance of parasitic diseases of syngnathids in this study was likely minimal with the exception of cryptosporidiosis.

Scuticociliates, primarily Uronema marinum, and to a lesser extent Philasterides dicentrarchi, commonly parasitize syngnathids and have been identified in seahorses (Cheung et al., 1980), seadragons (Umehara et al., 2003; Rossteuscher et al., 2008) and pipefish (Bombardini et al., 2006). These agents have been associated with erosive lesions of the skin and gill and have often been observed invading and causing mild inflammation in deeper tissues such as muscle, connective tissue, blood vessels, viscera, spinal canal and coelomic cavity (Cheung et al., 1980; Umehara et al., 2003; Bombardini et al., 2006; Rossteuscher et al., 2008; Stidworthy 2008). This is in agreement with the infection of Uronema-like organisms found in the Toronto zoo syngnathids, in which deep invasion by uronema was observed on occasion; however, this was usually
associated with widespread dermal ulceration and concurrent bacterial dermatitis.

Peritrich ciliates, such as trichodina, have also been noted in gills of pipefish (Longshaw et al., 2004; Bombardini et al., 2006) and seahorses (Bombardini et al., 2006); however, the infections were of minimal significance (Longshaw et al., 2004; Bombardini et al., 2006).

Cryptosporidiosis was a more significant pathogen in this population of syngnathids, causing regionally extensive lesions in the intestinal mucosa of otherwise healthy animals, that is, animals with no other evidence of concurrent disease on gross and histopathologic observation. Two species of coccidians (also apicomplexan parasites like cryptosporidia) have been documented in syngnathids; *Eimeria syngnathi* in *Syngnathus abaster*, formerly *Syngnathus nigrolineatus* (Yamimoff & Gousseff 1936) and *Eimeria phillopterycis* in *Phyllopteryx taeniolatus* (Upton et al., 2000). The clinical signs noted were anorexia and stunted growth (Upton et al., 2000). Lesions were limited to the gastrointestinal tract with various stages of the parasite encysted within the gastrointestinal mucosa and submucosa with a mild inflammatory response (Upton et al., 2000). While tissue damage in these cases was not extensive, a heavy burden of eimeria could cause severe malabsorption and discomfort leading to the clinical signs noted above. Cryptosporidia may have similar pathogenicity; however, they have been found to be largely incidental in some species of fish such as tilapia, and more often pathogenic in young fish (Lumsden 2006; Ryan 2010). Due to the young age and severity of lesions noted in affected seahorses, cryptosporidia are likely not an incidental finding. That is, these parasites were, at least in part, responsible for morbidity and mortality of these individuals. Regardless of pathogenicity, in fish they are always restricted to the intestine.
and/or stomach (Ryan 2010). Brine shrimp *Artemia* have been implicated as the mode of transmission of *Cryptosporidium* in cultured fish (Méndez-Hermida et al., 2007) and this may have been the case for the small outbreak of cryptosporidiosis in this population of seahorses.

**Mycotic**

While fungi are generally considered secondary invaders in fish (Roberts et al., 2009), there have been reports of mycoses causing mortalities in seahorses. Microsporidiosis caused by *Glugea heraldi* has been well described in wild-caught *Hippocampus erectus* (Blasiola 1979; Vincent & Clifton-Hadley 1989; Bombardini et al., 2006). This microsporidian causes multifocal cysts that eventually coalesce and rupture, predisposing to secondary bacterial infections. *G. heraldi* has been documented to cause over 95% mortality within 6 months in wild-caught *H. erectus* (Vincent & Clifton-Hadley 1989). Two *Exophiala* species have been documented to cause a disseminated infection in seadragons (Nyaoke et al., 2010). Lesions consisted of parenchymal and vascular necrosis with fungal invasion of gill, kidney and swim bladder but occasionally also heart, liver, spleen, intestine, mesentery and spinal cord. Of these cases, 65% had epithelial ulcerations with associated mats of fungal hyphae invading the dermis, hypodermis, fascia and skeletal muscle (Nyaoke et al., 2010). A variety of gram-negative bacteria were also cultured from the skin lesions of these animals; however, no species of bacteria was consistently isolated. The vascular invasion and visceral lesions were associated with only *Exophila* sp.; however, it is unknown if the inciting lesion was bacterial, fungal or traumatic (Nyaoke et al., 2010). In the present study, mycotic infections were restricted to the skin and were only found in syngnathids with other
causes of disease. The stress of captivity, artificial systems and concurrent infections may contribute to immunosuppression and predispose captive syngnathids to opportunistic infections (Nyaoke et al., 2010).

Viral
No virus has been identified from syngnathids to date. In this study, some animals were noted to have lesions consistent with viral-like enteridities using light microscopy. Characteristic blunting of intestinal folds and subsequent flattening of remaining enterocytes resulting in luminal distention in the absence of other pathogens are suggestive of, although not pathognomonic for, viral enteritis. Electron microscopy in addition to molecular studies of tissues in these suspect cases would be required to confirm the cause of this lesion pattern.

Neoplastic
Neoplasia has been described in syngnathids, while reports are not numerous, eight spontaneous neoplasms have been described including a broodpouch fibrosarcoma (Willens et al., 2004), cardiac rhabdomyosarcoma, renal adenoma, renal adenocarcinoma, renal round cell tumor (suspect lymphoma), exocrine pancreatic carcinoma and intestinal carcinoma (LePage et al., 2012). Additionally, one neoplastic-like lesion has also been identified; a renal reticuloendothelial cell hyperplasia (LePage et al., 2012). While neoplasia is not a leading cause of mortality, the types of neoplasms affecting this species should be documented to monitor genetic and environmental predisposing factors.
Environmental

Gas bubble disease

Gas bubble disease (GBD) is defined as the formation of gas emboli and/or bullae formation in tissues or excess gas accumulation in body cavities; areas most commonly affected include blood vessels, eyes, gill, pseudobranch, subcutaneous tissue, coelomic cavity and swim bladder (D’Aoust & Smith 1974; Kulshrestha & Mandal 1982; Speare 1990; Olaf Koppang & Bjerkås 2006). This condition is caused by exposure to supersaturated gases, that is, when the total pressure of dissolved gas, e.g. nitrogen, is higher than that in ambient atmospheric pressure (Bouck 1980). Gas supersaturation can occur in numerous ways including rapid heating of water, high-pressured water source, and heavy macrophyte growth (Noga 2000). GBD rarely causes mortality on its own unless very severe; however, complicating factors such as rupture of emphysematous bullae beneath the epidermis can predispose to secondary bacterial infections. This scenario was observed in the population of syngnathids at the Toronto Zoo. Gas accumulation in documented cases of GBD in syngnathids occurred mainly as subcutaneous emphysema along the tail and in the brood pouch, and less commonly as subcutaneous emphysema along the head region or in the swim bladder (Bombardini et al., 2006; Lin et al., 2010). In this study, one particular episode of GBD was linked to accidental rapid water heating. Gas accumulation occurred mainly in subcutaneous tissues along the tail and brood pouch. Once the supersaturation problem was corrected and stress was minimized, the condition usually resolved spontaneously; however, active removal of gas from brood pouch was occasionally attempted. A second gas accumulation phenomenon occurred in seahorse fry that developed an overinflated swim
bladder and struggled at the surface with excessive buoyancy; this was also often referred to as GBD (LePage, personal observation). Syngnathids are physoclists and the pneumatic duct closes during embryonic development within first 24 hours after birth (Genten et al., 2009). Seahorse fry rise to the surface and gulp their first air bubble to inflate their swim bladder and the pneumatic duct closes shortly thereafter. Some fry remain overly buoyant at the surface, perhaps due to over inflation of the swim bladder; however, the pathogenesis of this condition is unknown. The use of Kreisel tanks or fine mesh at the surface of the water to prevent fry from remaining at the surface after they have taken their first gulp of air have been shown to reduce the number of overly buoyant syngnathid fry. These management interventions are not necessary for long-term holding but can improve survival rates in the first few weeks of life (LePage, personal observation).

**Suspect nutritional myopathy**

Species in which myopathies are recognized most commonly are those involved in intensive rearing such as cattle, poultry, sheep and fish (Scott 1966; Allen 1977; Allen et al., 1986; Ferguson et al., 1986; Van Vleet & Valentine 2007). Myopathies have also been identified in captive non-production or display animals such as geckoes, pelicans, and cats (Campbell & Montali 1980; Vos et al., 1986; Gabor 2005). In animals, the most common causes include nutritional, exertional, congenital, and infectious (Koller & Exon 1986; Wallace et al., 1987; Hartup et al., 1999; Basset & Currie 2003; Turnbull 2006). The most thoroughly investigated cause of myopathy is nutritional muscular dystrophy, linked to vitamin E and selenium deficiency (Koller & Exon 1986). In barramundi,
skeletal myopathy was found to develop in potassium-deficient water (Partridge & Creeper 2004)

Nutritional myopathy presents as muscle weakness, inability to eat and general failure to thrive (Koller & Exon 1986; Wallace et al. 1987; Hartup et al. 1999; Basset & Currie 2003; Turnbull 2006; Van Vleet & Valentine 2007). Light microscopic lesions of nutritional myopathy are bilaterally symmetrical, with minimal inflammation. Myofibres are initially hypercontracted, as evidenced by loss of striation, and are large, rounded and have hypereosinophilic and granular sarcoplasm, described as hyaline changes. Later, macrophages can phagocytose these hypercontracted fiber segments along with other fragmented myofibres that may or may not have undergone mineralization (Van Vleet & Valentine 2007). Typical histological findings in mammals are described as polyphasic and multifocal (Van Vleet & Valentine 2007). Lesions are similar in *H. kuda* with one exception; the lesions found in *H. kuda* have no or minimal mineralization. This is consistent with myopathies described in other teleosts (Holloway & Smith 1982; Turnbull 2006). In this study, the *H. kuda* species appears to be the most susceptible to nutritional myopathy as they were the only syngnathids at the Toronto Zoo to develop the myopathy although all were fed similar diets and kept in environmental conditions deemed appropriate for each species. This could also indicate that a diet suitable for a temperate water species such as *H. abdominalis* may not be adequate for a warmer water species like *H. kuda*. More research is required to determine the nutritional requirements of each syngnathid species in order to tailor diets accordingly.
**Congenital**

Most teleosts produce a large number of offspring, many of which have a high degree of exposure to the external environment during incubation. Both of these factors contribute to an increase in the frequency of congenital defects (Laale & Lerner 1981). Additionally, any defects are more likely to be observed, as the odds of survival are increased in captivity. Ovipositor duplication and the blood-filled cyst within the ventricular myocardium observed in this population of syngnathids are two of the first documented congenital defects in this family of fish. One report describes a weedy seadragon with an absent or malformed swim bladder as observed by CT scan (Garland et al., 2002). While it is quite common to see alterations in gonads, such as ovotestes, in teleosts (Vethaak et al., 2002), to the authors’ knowledge, external openings or structures are rarely reported to be duplicated or altered. The blood-filled cyst within the ventricular myocardium was not dissimilar to the description of blood-filled cysts of calves and humans, which are congenital anomalies that rarely affect function (Marcato et al., 1996; Maxie & Robinson 2007).
CHAPTER 3. INVESTIGATION OF ULCERATIVE DERMATITIS
IN CAPTIVE HIPPOCAMPUS KUDA

This chapter corresponds to the following manuscript:


ABSTRACT

Ulcerative dermatitis was the most common cause of morbidity and mortality in the yellow seahorse *Hippocampus kuda* at the Toronto Zoo from 1998 until 2010 (39/133). Under light microscopy, ulcerative lesions were often associated with filamentous bacteria (22/39). Bacterial cultures were grown from 15 seahorses with ulcerative dermatitis for phenotypic and genotypic characterization. Bacterial cultures were also taken from five seahorses without ulcerative lesions, four water samples and plastic perches from four different tanks. PCR using universal bacterial primers was performed on DNA extracted from bacterial isolates from two of 15 fish with ulcerative dermatitis and yielded amplicons that differed by less than 1% from the ~1.5 kb 16S ribosomal RNA gene sequence of bacteria of the Flavobacteriaceae family. These two isolates were *Cellulophaga fucicola* and *Tenacibaculum mesophilum*. Bacteria of the family Flavobacteriaceae have a filamentous morphology and have also been identified to cause
severe ulcerative lesions in other teleosts. PCR using universal bacterial primers
performed on DNA extracts from formalin-fixed tissues with ulcerative lesions yielded
amplicons that had the greatest nucleotide sequence identity to *Cellulophaga fucicola,*
differing by 12% from its 16S ribosomal RNA gene sequence. In situ hybridization using
~1.5 kb DNA multimeric oligoprobe corresponding to either the *Cellulophaga fucicola*
or *Tenacibaculum mesophilum* 16S rRNA gene sequences confirmed their presence
within yellow seahorse ulcerative lesions. DNA isolated from seahorses without
ulcerative lesions, water samples and plastic perch samples were tested by universal
bacterial PCR and yielded 50 almost complete 16S rRNA gene sequences, none of which
were members of the family Flavobacteriaceae. Results suggest that bacteria of the
Flavobacteriaceae family are associated with ulcerative lesions in the yellow seahorse;
their role in the pathogenesis of ulcerative lesions has yet to be determined.
INTRODUCTION

Common causes of morbidity and mortality in captive syngnathids at the Toronto Zoo from 1998 to 2010 were summarized in Chapter 2. The most common problem was found to be an erosive to ulcerative dermatitis, occurring mainly in the yellow seahorse and accounting for almost 25% of morbidity and mortality. This dermatitis often presented clinically as ‘red tail’, a common term used to describe the hyperaemic ventral aspect of the entire length of the tail caudal to the vent. A second clinical presentation or suspected progression of ‘red tail’ was multifocal to regionally diffuse epithelial ulcerations occurring anywhere along the body but usually involving the tail.

Ulcerative dermatitis is a common cause of morbidity and mortality in captive and intensively cultured fish (Masumura & Wakabayashi 1977; Devesa et al., 1989; Ostland et al., 1999; Decostere et al., 2004; Roberts et al., 2009; van Gelderen et al., 2011). The consequences of ulcerative skin lesions in fish are significant because the epidermis and dermis not only serve an innate immune function as a physical barrier against external pathogens but also act as an osmotic barrier, limiting significant shifts of water from the fish to the external environment in the case of marine fish. Histopathologic assessment of with ulcerative lesions of seahorses often demonstrated filamentous bacteria adhered to the ulcerated edges of skin and invading into the dermis.

While ulcerative lesions are common in syngnathids, they are more usually associated with species of *Mycobacterium* (Bombardini et al., 2006), *Aeromonas* (Bombardini et al., 2006) and *Vibrio* (Alcaide et al., 2001; Tendencia 2004; Bombardini et al., 2006); however, these bacteria are not filamentous. In other fish, filamentous bacteria associated
with erosive to ulcerative lesions include species within the Flavobacteriaceae family such as *Flavobacterium psychrophilum* (Cipriano et al., 1996; Roberts et al., 2009), *F. columnare* (McCarthy 1975; Bullock et al., 1986) and *Tenacibaculum maritimum* (Ostland et al., 1999; Avendaño-Herrera et al., 2006; Roberts et al., 2009). It was therefore hypothesized that the filamentous bacterium observed under light microscopy within ulcerative lesions was a bacterium of the Family Flavobacteriaceae.

The purpose of this study was to culture and identify bacteria associated with ulcerative dermatitis in the yellow seahorse and to localize filamentous bacteria within histological lesions. An understanding of bacteria associated with this disease process and their distribution within lesions may help to elucidate the pathogenesis of the condition, facilitating disease management and treatment.
MATERIALS AND METHODS

Animal data collection

From 1998 to 2010, 172 syngnathids from the Toronto Zoo were submitted for post-mortem diagnostics, including 133 spotted or yellow seahorses (Hippocampus kuda). These fish were from a variety of marine enclosures (salinity 22-26 ppt) at the Toronto Zoo in Scarborough, Ontario, Canada. All tanks had biofiltration systems (Hagen Aquaclear and Hagen Bio Foam, Hagen Inc., Montreal, Québec, H9X 0A2, Canada), water temperatures ranged from 24 - 28°C (Thermal Compact Heaters, Hagen Inc., Montreal, Québec, H9X 0A2, Canada), and all fish were kept on a 13 h day, 11 h night cycle. Tank substrates varied from coarse sand to bare tanks, some housing live plants, while others had plastic plants and perches. Males and females were approximately equally represented in the populations of each species. All syngnathid mortalities were routinely investigated. Data collected for each case included history, gross necropsy report, histopathology and microbiology when warranted. Cases included fish found dead, and moribund fish euthanized by an overdose of tricaine methanesulphonate (Aqualife TMS, Syndel Laboratories Ltd, Qualicum Beach, British Columbia, V9K 1V5, Canada).

Bacterial isolation

From 2007 until 2010, visible skin ulcers and tails of seahorses with a case history of ‘red tail’ were swabbed with sterile CultureSwab™ containing liquid Stuart media (Becton-Dickinson, Franklin Lakes, New Jersey, 07417, USA) for aerobic culture. For ease of discussion, cases with either a history of ‘red tail’ and/or visible ulcers will be
referred to as dermatitis cases or fish with dermatitis. Sterile swabs of lesions from fish with gross evidence of dermatitis (n = 15) were inoculated onto a non-selective medium, Marine Agar 2216 (Becton-Dickinson, Franklin Lakes, New Jersey, 07417, USA), and incubated at room temperature (RT) (20-22°C) for 18-72 h. Control swabs were taken in the same manner from seahorses without visible lesions (n = 5), plastic perches (n = 4) in tanks and tank water (n = 4) samples. Bacterial colonies were subcultured and isolated onto Marine Agar 2216.

For long-term storage of isolated cultures, pure cultures were grown aerobically in glass tubes with 2 ml of Marine Broth 2216 (Becton-Dickinson, Franklin Lakes, New Jersey, 07417, USA) for 24-48 h; culture tubes were then centrifuged at 5000xg for 30 min, bacterial pellets were extracted and placed in 1.5 ml cryotubes with 1 ml of fresh Marine Broth 2216. One ml of 40% glycerol (Fisher Scientific, Fair Lawn, New Jersey, 07410, USA) was added to each tube, vortexed (Benchmixer™, Diamed Lab Supplies Inc., Mississauga, Ontario, L4X 2E2, Canada) for 5 sec to uniformly suspend bacteria in the glycerol solution and immediately frozen at -80°C until processed at a later date. Duplicate storage tubes were created for all isolates. *Escherichia coli* ATCC 25922 and a *Tenacibaculum maritimum* strain (Ostland et al., 1999), were cultured and stored in a similar fashion to serve as various controls indicated below.

**Histopathology**

After samples were collected from seahorses for microbiology, the coelomic cavity was opened and the whole fish was immersed in 10% neutral-buffered formalin (ACP Chemicals Inc., Saint-Léonard, Québec, H1R 1A5, Canada) for 24 to 48 h. Formalin-fixed fish were transferred to decalcifying solution (Surgipath Decalcifier II, Surgipath
Medical Industries Inc., Richmond, Illinois, 60071-7702, USA) for 2 h. Entire fish were then cut transversely in segments of approximately 3 to 4 mm using a scalpel blade. Segments were then processed routinely for paraffin embedding, sectioned at 4 µm, and sections were mounted on glass slides and stained with hematoxylin and eosin (Animal Health Laboratory, Guelph, Ontario, N1G 2W1, Canada). Additional stains on selected histologic sections as required included Masson’s trichrome, periodic acid-Schiff, modified Gimenez or Pierce Van der Kamp, and Ziehl-Neelsen and modified Ziehl-Neelsen staining.

**Biochemical characterization of bacterial isolates**

Cryotubes containing frozen bacterial isolates were taken from storage at -80°C and thawed on ice. Samples were vortexed at high speed (Benchmixer™, Diamed Lab Supplies Inc., Mississauga, Ontario, L4X 2E2, Canada) for 5 sec and plated onto Marine Agar 2216 (MA) using a sterile loop. Colonies were grown for 24-48 h at RT. Each isolate was Gram stained (Becton-Dickinson, Franklin Lakes, New Jersey, 07417, USA), characterized morphologically and tested for biochemical characteristics using API® 20E strips (bioMérieux, Durham, North Carolina, 27712, USA) and other ancillary tests. Isolates were also tested for growth on various media, at 0%, 3% and 5% sodium chloride and at 4°C, 10°C, 15°C and 37°C in addition to RT. Media included blood agar (BA), cytophaga (CA), trypticase soy agar (TSA), MacConkey agar (MCA), thiosulfate-citrate-bile salts-sucrose agar (TCBS), mannitol salt agar (MSA), phenylethyl alcohol agar (PEA), and Lowenstein-Jensen agar with 5% sodium chloride (Becton-Dickinson, Sparks, Maryland, 21152, USA). Additional testing included oxidase (DrySlide™, Becton-Dickinson, Detroit, MI, USA), catalase (Fisher Scientific, Fisher Scientific, Fair Lawn, New Jersey, 07410, USA), flexirubin pigment
production (using potassium hydroxide method; 1 drop KOH 20% w/v added to glass slide with bacterial colony) and hemolysis on BA. Minimum inhibitory concentration was also determined for selected isolates using Sensititre® aquatic plates (Trek Diagnostic Systems, Cleveland, OH, USA), which tests for sensitivity against 10 antimicrobials: trimethoprim/sulfamethoxazole, gentamicin, enrofloxacin, ampicillin, oxytetracycline, erythromycin, florfenicol, flumequine, ormetoprim/sulphadimethoxine, and oxolinic acid. *Escherichia coli* ATCC 25922 was used as a control for all tests.

**DNA extraction**

**Bacterial isolates**

Each isolate was grown on Marine Agar 2216 at RT and resuspended in 1 ml of sterile water in a 1.5 ml microcentrifuge tube. The tube was centrifuged for 1 min at 12,000 rpm and the supernatant was removed (Centrifuge 5424, Eppendorf NA, Inc., Westbury, New York 11590, USA). Bacterial genomic DNA was then extracted from the resulting pellet using the InstaGene™ matrix (Bio-Rad Laboratories, Hercules, California, 94547, USA). Two hundred μl of InstaGene™ matrix was added to the pellet and incubated at 56°C for 20 min in a heat block shaken at 300 rpm (Thermomixer, Eppendorf, Hauppauge, New York, 11788, USA). The tube containing the sample was then vortexed at high speed for 10 sec and placed in 99°C heat block shaken at 300 rpm for 8 min. The tube was vortexed at high speed for 10 sec and spun at 12,000 rpm for 3 min. Extraction was conducted as per manufacturer’s directions. Five μl of the resulting supernatant was either used directly for polymerase chain reaction (PCR) or stored at -20°C until PCR was performed.

*Escherichia coli* ATCC 25922 and laboratory *Tenacibaculum maritimum*, a yellow-
pigmented filamentous marine bacterium isolated from Atlantic salmon with ulcerative dermatitis (Ostland et al., 1999), were used as positive controls for DNA extraction.

**Formalin-Fixed Paraffin-Embedded Tissues**

Genomic DNA was extracted from selected *H. kuda* skin samples with histopathologic evidence of erosive to ulcerative lesions that contained intralesional filamentous bacteria using Laser Capture Microdissection (LCM). A 7 μm thick section was cut from each paraffin block of interest and mounted on Superfrost Plus glass slides (Fisherbrand®, Fisher Scientific, Fair Lawn, New Jersey, 07410, USA) without baking or staining, and the tissue was deparaffinized with xylene (Sigma-Aldrich, St. Louis, MO, USA), then washed with 100% ethanol. LCM was performed to extract the filamentous bacteria from the lesions observed under light microscopy, as per the manufacturer’s recommendation, using a 7.5 μm diameter laser at 75 mW for 700 μs. The resin layer containing tissues was removed from LCM HS caps using sterile forceps and placed in separate 1.5 ml DNAse/RNAse free microcentrifuge tubes. Due to poor DNA yield from LCM, a second method was chosen; sterile excision of a section of ulcerative lesions (~ 1 mm deep by 1 mm wide). The tissue samples were placed in separate 1.5 ml DNAse/RNAse free microcentrifuge tubes. LCM and excised tissues were processed in the same manner from here on. DNA was extracted from the resulting pellet using a DNA extraction kit for formalin-fixed paraffin-embedded (FFPE) tissues following the suggested protocol (QIAamp® DNA FFPE Tissue, Qiagen GmbH, D-40724 Hilden). Tissue was lysed with 180 μl Qiagen ATL tissue lysis buffer and 20 μl proteinase K, vortexed, and incubated at 56°C for 24 h. Samples were heated to 90°C for 1 h, then centrifuged at 6000 g for 2 min.
to allow the lysate to settle. Extraction was conducted as per the manufacturer’s directions. DNA was stored in 1.5 ml microfuge tubes at −20°C for later use.

**PCR**

**Bacterial isolates**

Universal oligonucleotide primers to conserved sites flanking the region of the 16S rRNA gene were used to amplify a ~1500 bp fragment from the the gene (27f, 5’- TTG GAG AGT TTG ATC CTG GCT C -3’, and 1492R, 5’- GGA CTA CCA GGG TAT CTA A -3’) (Lane 1991). Primers for all PCR experiments were synthesized by Sigma-Aldrich and rehydrated to a stock concentration of 100 μM with sterile water. All PCR products were amplified in individual 50 μl reaction mixtures containing 100–300 ng of sample DNA, 25 μl of 2x TopTaq Master Mix (contains TopTaq DNA Polymerase, TopTaq PCR Buffer with 3 mM MgCl₂ and 400 μM each dNTP) (Qiagen), 3 μl of each forward and reverse primer (diluted to 20 μM working solution), 5 μl 10x CoralLoad Concentrate (Qiagen), and 9 μl RNase-free water, using thermal cyclers (Mastercycler personal, Eppendorf). PCR conditions were as follows: 5 min incubation at 95°C, followed by 35 cycles of 30 sec at 94°C, 60 sec at 56°C, and 60 sec at 72°C. A 10 min final step at 72°C was performed to extend any incomplete amplicons. Products were separated by electrophoresis in 1% agarose gels and visualized by SYBR® Safe DNA gel staining (Invitrogen) and UV transillumination. Images were recorded digitally using Gel Doc™ EZ still video image capture system (Bio-Rad Laboratories). All 16S rDNA PCR products were excised and purified from agarose gels (Direct-Gel-Spin™ DNA recovery kit, LAMDA Biotech, St. Louis, MO 63011, USA), and then submitted for direct sequencing (University of Guelph Animal Health Laboratory). *Escherichia coli* ATCC
25922 and *T. maritimum* (Ostland et al., 1999) isolates were used as positive controls and samples with sterile distilled water were used as negative template controls.

**Formalin-Fixed Paraffin-Embedded Tissues**

PCR for FFPE tissues was conducted in the same manner as above for fresh bacterial isolates with the exception that the universal oligonucleotide primers used amplified a ~380 bp fragment of the 16S rRNA gene from the 5’ end of the gene (RW01, 5’- AAC TGG AGG AAG GTG GGG AT -3’, and DG74, 5’- AGG AGG TGA TCC AAC CGC A -3’) (Greisen et al., 1994). Concentrations of DNA yielded were between 10-50 ng/µl.

DNA extracted from *Escherichia coli* ATCC 25922 and *T. maritimum* (Ostland et al., 1999) isolates were used as positive controls for amplification and samples with sterile distilled water were used as negative template controls.

**DNA sequencing and analysis**

Nucleotide sequences of amplicons were generated by oligonucleotide-directed dideoxynucleotide chain termination DNA sequencing reactions using primers 27f and 1492R primers for bacterial isolates and primers RW01 and DG74 for amplicons from FFPE tissues (Guelph Molecular Supercenter, Laboratory Services Division, Guelph, Ontario, Canada). Approximately 3-20 ng of template DNA and the ABI Prism® BigDye® Terminator Cycle Sequencing Ready Reaction kit v3.1 (Applied Biosystems, Foster City, CA) were used for sequencing reactions. Sequencing was performed on a GeneAmp® PCR System 9700 or 2720 Thermal Cycler (Applied Biosystems). The BigDye® Terminator v3.1 Cycle Sequencing Kit Protocol (Applied Biosystems) was followed to set up and conduct the cycle sequencing reactions. Dye terminators were removed from cycle sequencing reactions using Multiscreen-HV plates (Millipore,}

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Mississauga, ON) loaded with Sephadex G-50 superfine (Sigma, Oakville, ON). The clean reactions were electrophoresed on an Applied Biosystems 3730 DNA Analyzer (Applied Biosystems). A minimum read length of 700 bp was generated for each of the reactions. The chromatograms were analyzed using ABI Prism® DNA Sequencing Analysis Software Version 3.7 (Applied Biosystems) to generate quality target sequences within the Software’s clear confidence range. The forward and reverse sequences were aligned using Geneious software (Dummond et al., 2011). Standard nucleotide–nucleotide sequence comparisons were made by conducting basic local alignment search tool (BLAST) searches (Altschul et al., 1990) in GenBank to determine sequences with the highest nucleotide identity to those amplified during the survey. Positive controls were also sequenced and analysed in the same manner.

**In situ hybridization**

*In situ* hybridization (ISH) was accomplished using oligonucleotide multimer probes generated from 1.5 kb 16S rRNA gene sequences amplified from those filamentous bacteria isolated from ulcerative lesions (Wang et al., 2012). Probes were named as follows: FLAVO1 for the probe generated from a *Cellulophaga fucicola*-like isolate; FLAVO2 for the *Tenacibaculum mesophilum*-like isolate; and MYCO for the probe generated from the *Mycobacterium* isolate. Probes were cross-matched against the most common bacterial isolates obtained such species of *Vibrio* and *Bacillus* (Wang et al., 2012). To serve as a control for probe specificity, a ~1.5 kb 16S rRNA gene sequence of an isolate of *Mycobacterium* obtained from a *Hippocampus kuda* from the Toronto Zoo was generated in identical fashion. Signal detection was visualized with standard bright-field microscopy; probes were chromogenically labelled with Fast Red conjugated with
alkaline phosphatase (Wang et al., 2012). Tissue sections stained with HE were examined by light microscopy for ulcerative lesions and distribution of filamentous bacteria, and successive serial tissue sections at 5 µm were mounted unstained on SuperFrost Plus glass slides (Fisher Scientific, Fairlawn, N.J.). Unstained sections were heated to 60°C in a dry incubator for 60 min (Fisher Scientific, Fairlawn, N.J.). Tissues were deparaffinized in two 5-min washes with xylene (Sigma-Aldrich, St. Louis, MO, USA), then two 3-min washes with 100% ethanol, then air dried for 5 min at RT. A hydrophobic barrier was created around the edges of the tissues (ImmEdge Hydrophobic Barrier Pen, Vector Laboratories Inc., Burlingame, CA 94010). Prehybridization steps were modified as described below however hybridization and amplification steps were done according to the manufacturer’s suggestions (RNAscope® 2.0 Red FFPE Assay, Advanced Cell Diagnostics, Hayward, CA 94545). All pre-treatment steps were followed by two 2-min washes with distilled water. Glass slides with tissue samples were flooded with hydrogen peroxide (0.3% v/v) and left to incubate for 10 min at RT. Slides were then immersed in boiling (100-104°C) citrate buffer (10 nmol/L, pH6) for 5 min. Approximately 8 drops of protease (10 µg/mL) was placed on the tissues and left to incubate for 30 min at 40°C in a humidifying tray (Advanced Cell Diagnostics, Hayward, CA 94545). The pre-amplification, amplification and hybridization steps were done using approximately 4 drops of their respective solutions, with incubation in a humidifying tray at 40°C using the following solutions: target probes (2 nmol/L) in hybridization buffer A [6x SSC (1x SSC is 0.15 mol/L NaCl, 0.015 mol/L Na-citrate), 25% formamide, 0.2% lithium dodecyl sulfate, blocking reagents] for 2 hours; preamplifier (2 nmol/L) in hybridization buffer B (20% formamide, 5x SSC, 0.3% lithium dodecyl sulfate, 10%
dextran sulfate, blocking reagents) for 30 minutes; amplifier (2 nmol/L) in hybridization buffer B at 40°C for 15 minutes; and label probe (2 nmol/L) in hybridization buffer C (5x SSC, 0.3% lithium dodecyl sulfate, blocking reagents) for 15 minutes; AMP 3 solution was incubated for 30 min at 40°C; AMP 4 solution was incubated for 15 min at 40°C; then AMP 5-RED solution incubated for 30 min at RT; and the final amplification step was performed using AMP 6-RED solution for 15 min at RT. The colourimetric reaction was obtained by adding 120 μl Fast Red A and B solutions (mixed at a 1:60 ratio) to slides and incubating slides for 10 min at RT. Slides were stained with 50% Gill’s hematoxylin for 2 min, rinsed with dH₂O, followed by five short rinses in 0.01% ammonium hydroxide (Fisher Scientific, Fairlawn, NJ) and a final dH₂O rinse. Slides were then dehydrated with two 5-sec dips in 100% fresh ethanol and one 5-sec dip in xylene (Sigma-Aldrich, St. Louis, MO, USA), then were mounted with xylene-based mounting media (Cytoseal™ XYL, Richard-Allan Scientific, Kalamazoo, MI 49008). Seahorse tissue from H. kuda from the Toronto Zoo without gross or microscopic ulceration or bacterial infection served as negative tissue controls. Tissues that generated the isolates from which the FLAVO1 and MYCO probes were designed were available as positive tissue controls; however, due to limitations of sample collection, positive control tissues were not available for the FLAVO2 probe. The MYCO probe and 0.1 M phosphate buffered saline (PBS) (pH 7.4) served as negative controls.

**Whole-cell agglutination**

Previously developed rabbit antisera for *Flavobacterium aquatile* ATCC 11947-1, ATCC 11947-2, *Flavobacterium psychrophilum* NCMB 1947, *Flavobacterium columnaris* NCMB 2248, B333-97, NB-1 (a&b) marine YPB (Ostland et al., unpublished) were
tested for antigenic specificity against three isolates of the Flavobacteriaceae family (HK 19, HK 87, HK 89). Two adjacent suspensions of the test organism in drops of 0.9% saline were placed on a slide. Once a smooth suspension of the organism was achieved, one drop of antiserum was added to one of the two suspensions and mixed gently using a sterile loop. Slides were examined for agglutination or clumping of the suspension with antiserum and clearing of the saline. A positive result was determined as agglutination of the suspension and a result was called negative when the suspension remained turbid. The positive control was a *F. psychrophilum* strain (FP16) isolated from rainbow trout at an Ontario aquaculture facility and its associated antisera developed previously in the fish pathology laboratory (Hesami et al., 2008). Pre-immune rabbit antiserum served as a negative control.

**Immunohistochemistry**

Tissues from *H. kuda* with ulcerative lesions containing filamentous bacteria were selected for immunohistochemical analysis. Rabbit antisera to an isolate, B333-97, was previously developed in our laboratory (Ostland et al., unpublished). This antisera was chosen because it was readily available and the isolate was similar to those investigated in the present study in that it is a yellow-pigmented filamentous marine bacterium known to cause necrotizing skin lesions in teleosts. It was first isolated in an outbreak of necrotizing stomatitis in Atlantic salmon in New Brunswick, Canada (Ostland et al., unpublished). Tissue sections stained with HE were examined by light microscopy for ulcerative lesions and distribution of filamentous bacteria, and successive serial tissue sections at 5 µm were mounted unstained on SuperFrost Plus glass slides (Fisher Scientific, Fairlawn, N.J.). Tissues were deparaffinized in two 5-min washes with xylene.
(Sigma-Aldrich, St. Louis, MO, USA), followed by two 3-min washes with 100% ethanol and then slides were air dried for 5 min at RT. All steps involving incubation at RT were performed in a humid chamber. Endogenous peroxidase activity was blocked by incubating tissue sections in 0.3% (v/v) hydrogen peroxide in methanol for 5 min followed by washing in PBS with 0.1% (v/v) Tween 20 (Sigma) (PBS-T). Slides were then incubated for 1 h at RT in normal goat serum. After washing three times with PBS-T, rabbit anti-B333-97 serum (1:4000) or pre-immune antiserum (1:1000) diluted in PBS was added and incubated for 1 h. Following three washes with PBS-T, sections were incubated with biotin-conjugated sheep anti-rabbit IgG (1:10,000) for 1 h at RT and washed three times with PBS-T. Slides were then incubated with horseradish peroxidase-conjugated streptavidin (Histostain SP, Invitrogen) for 10 min at RT, followed by a 10 min incubation in AEC chromagen (Histostain SP, Invitrogen) solution before the enzyme reaction was stopped with distilled water and slides were counterstained with CAT hematoxylin for 3 min. Controls included staining with pre-immune rabbit serum replacing rabbit anti- B333-97 as well as omitting the primary antibody step. Positive controls were tissues from the original outbreak from which this bacterium was isolated (case number B333-97).

RESULTS

Bacterial isolation

A total of 103 isolates were cultured and characterized morphologically and biochemically. Fifty-three isolates were grown from ulcerative lesions of 15 seahorses, 22 isolates from the skin of five seahorses with no visible ulcers or clinical signs of disease,
14 isolates from four water samples and 14 isolates from four substrate samples. The majority of isolates were gram-negative rods (Table 3.1).

**Histopathology**

Histologic sections of 133 yellow seahorses were reviewed by light microscopy (Chapter 2). Ulcerative dermatitis was the most common cause of morbidity and mortality in this population of *H. kuda* (39/133). The severity of epithelial necrosis was extremely variable from case-to-case, but in most cases there was complete ulceration of the epidermis. Lesions typically had little to no inflammatory infiltrate, and there was often extensive edema within the underlying dermis and musculature. The inflammatory reaction, when present, was characterized by small numbers of debris-filled macrophages within the connective tissue delineating the muscle and within myofibers. In numerous cases (22/39), large numbers of filamentous bacteria were adhered to the necrotic epithelium and/or dermis and, in fewer cases, also infiltrated the underlying dermis and muscle. Other agents were often present (17/39), such as ciliated protozoa and fungal hyphae. Filamentous bacteria often did not stain differentially with Ziehl-Neelsen or Pierce van der Kamp stain.

**Biochemical characterization of bacterial isolates**

Three isolates were selected for further characterization and identification, based on colony appearance, bacterial morphology and recovery from ulcerative lesions, i.e. HK19, HK87 and HK89. Isolates Hk87 and Hk89 produced yellow colonies, while isolate Hk19 produced cream coloured colonies. All isolates produced circular and weakly-spreading colonies on MA. All isolates were gram-negative, non-motile, oxidase-positive rods. Isolates did not contain flexirubin-like cell wall pigments. Catalase activity
was present in Hk19, but not in Hk87 or Hk89. The isolates Hk19 and Hk87 grew from 15 to 37°C but not from 4 to 10°C, while Hk89 only grew from 22 to 37°C (Appendix 1). All isolates grew on MCA and BA; none grew on CA (Appendix 2). All isolates hydrolysed gelatin while none produced H₂S, indole or ONPG (Appendix 2).

**DNA sequencing and analysis**

**Bacterial isolates**

All 103 isolates tested with the universal 16S rRNA gene primers yielded 16S rRNA genetic sequence data (Figure 3.1). Three of 103 isolates yielded amplicons with sequences within the family Flavobacteriaceae (Figure 3.1, Table 3.2). All of these amplicons were obtained from swabs taken from *H. kuda* with evidence of skin ulceration or ‘red tail’, and where possible, histological evidence of ulcerative dermatitis. Water samples, tank ornament swabs and swabs from seahorses without ulcerative lesions did not yield amplicons with sequences within family Flavobacteriaceae. The three isolates found to be of the Flavobacteriaceae family were different from one another (Table 3.3), and none of their amplicon sequences were identical to regions of 16S rRNA gene sequences published in GenBank (Table 3.2). These three amplicon sequences were compared to selected Flavobacteriaceae 16S rRNA gene sequences: *Tenacibaculum maritimum* (accession number D14023), *Flavobacterium psychrophilum* (accession number AF090991), *Flavobacterium columaris* ATCC 49513 to illustrate the degree of similarity (Table 3.3). The amplicon sequence from the HK19 isolate had highest sequence identity (99% query coverage, 99% maximum identity) to regions of known sequence classified as ‘*Cellulophaga fucicola*’ in GenBank (accession number NR025287). Hk19 also had 88.8% sequence identity with the *T. maritimum* isolate.
(Ostland et al., 1999). The Hk87 amplicon most closely matched *Tenacibaculum mesophilum* (GenBank accession number NR024736) with 90% query coverage and 99% sequence identity and was 87.7% similar to *Tenacibaculum maritimum* (accession number D14023). The Hk 89 amplicon most closely matched *Gaetbulibacter jejuensis* (accession number FJ490367) with 82% query coverage and 83% sequence identity; however, this sequence identity is not sufficient to characterize this isolate further (Table 3.3).

**Formalin-Fixed Paraffin-Embedded Tissues**

A single amplicon from formalin-fixed tissue yielded sequence with identity to those from bacteria of the Flavobacteriaceae family, e.g. most similar (86% sequence identity, 68% query coverage) to regions of known sequence classified as *Cellulophaga fucicola* (GenBank accession number NR025287) (Table 3.4). This sample was obtained by sterile excision of tissue with a scalpel blade and not by LCM. An amplicon with high sequence identity with a species of *Mycobacterium* was also obtained using this method (AM884326). Using LCM, amplicon sequences most commonly amplified resembled 16S rRNA gene sequence of the following genera in GenBank: *Commomonas* (NR029161 and NR028719) and *Halomonas* (NR025054 and NR044519). None of the amplicons generated from LCM samples had high nucleotide sequence identity to 16S rRNA gene sequences of bacteria of the Flavobacteriaceae family.

**In situ hybridization**

Filamentous bacteria within ulcerative skin lesions were labeled with the ~1.5 kb FLAVO1 and FLAVO2 probes that were generated from the *Cellulophaga fucicola*-like and *Tenacibaculum mesophilum*-like 16S rRNA gene sequences, respectively, thus
identifying these organisms within ulcerative skin lesions of yellow seahorses. The labelling of filamentous bacteria extended into the deep dermis in some cases (Figure 3.2) and was also demonstrated systemically. In this case of systemic infection, labelled filamentous bacteria were present in the liver and within a granuloma (Figure 3.3) occupying greater than 50% of the coelomic cavity and appears to extend from the intestinal wall. Labelling of bacteria within ulcerative skin lesions occured with the FLAVO1 probe in 4 cases while FLAVO2 labelling occured in 1 case. All sections were incubated with the nonhomologous (MYCO) probe, no probe (PBS) or with HS POLAR negative control probe; the filamentous bacteria within ulcerative lesions were not labelled under any of these control conditions (Figure 3.3) however, short rods within the granuloma were labeled with the MYCO probe (Figure 3.4).

**Slide agglutination test**

No agglutination was observed using any combination of flavobacterial antisera available in the laboratory against the three flavobacteria isolates cultured from ulcers in yellow seahorses, thus indicating no significant antigenic cross-reactivity between these isolates and known isolates The control positive was agglutinated by its associated antisera. The pre-immune rabbit serum did not agglutinate any of the reference antigens.

**Immunohistochemistry**

Tissues of the yellow seahorse with ulcerative dermatitis did not contain bacteria that were detected by the antibody to the B333-97 isolate (Ostland et al., unpublished), confirming the slide agglutination results that there was no significant antigenic cross-reactivity between isolates tested.
Table 3.1. Comparison of the number of bacterial isolates from *Hippocampus kuda* with ulcerative dermatitis, *H. kuda* tails without lesions, tank ornaments and tank water samples with morphology and Gram stain characteristics.

<table>
<thead>
<tr>
<th></th>
<th>Ulcerative lesions</th>
<th>Seahorse tail without lesions</th>
<th>Tank ornaments</th>
<th>Tank water samples</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Number of samples</strong></td>
<td>15</td>
<td>5</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td><strong>Gram negative rod</strong></td>
<td>46</td>
<td>19-20*</td>
<td>14</td>
<td>10</td>
</tr>
<tr>
<td><strong>Gram positive rod</strong></td>
<td>5</td>
<td>0-1*</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td><strong>Gram negative cocci</strong></td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><strong>Gram positive cocci</strong></td>
<td>1</td>
<td>2</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td><strong>TOTAL</strong></td>
<td><strong>53</strong></td>
<td><strong>22</strong></td>
<td><strong>14</strong></td>
<td><strong>14</strong></td>
</tr>
</tbody>
</table>

* Gram variable bacterium.
**Figure 3.1.** Bacterial isolates from seahorses with ulcerative dermatitis. Those highlighted in: orange were the most abundant bacterial isolates from all sample types and were also found in control samples, yellow were bacteria of interest or most commonly isolated from ulcerated skin. In the red box were filamentous bacteria of the family Flavobacteriaceae.
Table 3.2. BLAST-nucleotide search results for sequences of amplicons obtained using universal 16S rRNA gene primers.

<table>
<thead>
<tr>
<th>Isolate (Case)</th>
<th>Closest match</th>
<th>Query coverage</th>
<th>E value</th>
<th>Maximum identity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hk19 (B102-08)</td>
<td><em>Cellulophaga fucicola</em></td>
<td>99%</td>
<td>0.0</td>
<td>99%</td>
</tr>
<tr>
<td>Hk87 (B079-03)</td>
<td><em>Tenacibaculum mesophilum</em></td>
<td>90%</td>
<td>0.0</td>
<td>99%</td>
</tr>
<tr>
<td>Hk89 (B079-03)</td>
<td><em>Gaetbulibacter jejuensis</em></td>
<td>82%</td>
<td>0.0</td>
<td>83%</td>
</tr>
<tr>
<td>B179-11</td>
<td><em>Mycobacterium chelonae</em></td>
<td>87%</td>
<td>0.0</td>
<td>89%</td>
</tr>
</tbody>
</table>
Table 3.3. 16S rRNA gene pairwise percentage identity comparisons between seahorse isolates and selected previously published 16S rRNA gene sequences of bacteria of the Flavobacteriaceae family.

<table>
<thead>
<tr>
<th></th>
<th>T.mar</th>
<th>F. psy</th>
<th>F.col</th>
<th>T. mar(O)</th>
<th>Hk19</th>
<th>Hk87</th>
<th>Hk89</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hk19</td>
<td>82.3</td>
<td>82.0</td>
<td>81.8</td>
<td>88.9</td>
<td>100</td>
<td>80.0</td>
<td>73.6</td>
</tr>
<tr>
<td>Hk87</td>
<td>87.7</td>
<td>83.6</td>
<td>81.8</td>
<td>82.0</td>
<td>80.0</td>
<td>100</td>
<td>73.1</td>
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<td>Hk89</td>
<td>73.5</td>
<td>73.1</td>
<td>72.9</td>
<td>76.4</td>
<td>73.6</td>
<td>73.1</td>
<td>100</td>
</tr>
</tbody>
</table>

*T.mar: Tenacibaculum maritimum* (Genbank accesssion number D14023)

*F. psy: Flavobacterium psychrophilum* (Genbank accesssion number AF090991)

*F.col: Flavobacterium columnaris* ATCC 49513

*T. mar(O): Ostland et al., 1999*
Table 3.4. Comparison of 16S rRNA gene sequencing from fresh bacterial isolates, from formalin fixed tissues and *in situ* hybridization.

<table>
<thead>
<tr>
<th>Case number</th>
<th>Bacterial isolates (closest match)</th>
<th>LCM</th>
<th>In situ hybridization</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>FLAVO1</td>
</tr>
<tr>
<td>B102-08</td>
<td><em>Cellulophaga fucicola</em>&lt;sup&gt;a,c&lt;/sup&gt;</td>
<td><em>Halomonas alimentaria</em></td>
<td>(+)</td>
</tr>
<tr>
<td>B073-09</td>
<td><em>Tenacibaculum mesophilum</em>&lt;sup&gt;a&lt;/sup&gt;</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>B179-11</td>
<td><em>Mycobacterium sp.</em>&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td><em>Mycobacterium sp.</em>&lt;sup&gt;**&lt;/sup&gt;</td>
<td>NA</td>
</tr>
<tr>
<td>B239-99</td>
<td>NA</td>
<td>Poor sequence quality</td>
<td>+</td>
</tr>
<tr>
<td>B047-06</td>
<td>NA</td>
<td>Poor sequence quality</td>
<td>+</td>
</tr>
<tr>
<td>B197-06-F1</td>
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<td>-</td>
</tr>
<tr>
<td>B197-06-F2</td>
<td>NA</td>
<td>Poor sequence quality</td>
<td>-</td>
</tr>
<tr>
<td>B197-06-M2</td>
<td>NA</td>
<td>Poor sequence quality</td>
<td>-</td>
</tr>
<tr>
<td>B007-07</td>
<td>NA</td>
<td><em>Pseudorhodoferax soli</em></td>
<td>-</td>
</tr>
<tr>
<td>B107-07</td>
<td>NA</td>
<td><em>Hydrogenophaga defluvi</em></td>
<td>+</td>
</tr>
<tr>
<td>B033-09</td>
<td>NA</td>
<td>Poor sequence quality</td>
<td>-</td>
</tr>
<tr>
<td>B043-10-1</td>
<td>NA</td>
<td>Poor sequence quality</td>
<td>-</td>
</tr>
<tr>
<td>B174-11</td>
<td>NA</td>
<td><em>Cellulophaga fucicola</em>&lt;sup&gt;**&lt;/sup&gt;</td>
<td>NA</td>
</tr>
<tr>
<td>B105-06</td>
<td>NA</td>
<td>Poor sequence quality</td>
<td>+</td>
</tr>
<tr>
<td>B059-09&lt;sup&gt;b&lt;/sup&gt;</td>
<td>NA</td>
<td><em>Mycobacterium sp.</em></td>
<td>-</td>
</tr>
<tr>
<td>B053-03&lt;sup&gt;d&lt;/sup&gt;</td>
<td>NA</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

<sup>a</sup>: Isolate/Sample from which probe was designed.
<sup>b</sup>: Control tissues for mycobacterium probe
<sup>c</sup>: Control tissues for Flavo1 probe
<sup>d</sup>: Negative control tissues (no lesions or bacteria visible under light microscopy)
NA: isolate or tissue not available for testing
**: sample extracted with sterile manual excision of 2mm diameter section of an ulcer edge, not via LCM
(+): staining of bacteria was positive but results questionable due to level of background
**Figure 3.2.** Skin section processed for *in situ* hybridization using the FLAVO1 probe targeting *Cellulophaga fusicola* 16S rDNA within an ulcerative lesion having filamentous bacteria that invade dermis and muscle (60x magnification). Inset: negative control probe (HS POLAR, 100x magnification).
Figure 3.3. Granuloma found in a fish that also had bacterial dermatitis. ISH labeling using the FLAVO1 (a) and MYCO (b) probes showing differential staining of intralesional filamentous and shorter rod-shaped bacteria, respectively. Insets: negative control slide (PBS).
Figure 3.3. Granuloma found in a fish that also had bacterial dermatitis. ISH labeling using the FLAVO1 (a) and MYCO (b) probes showing differential staining of intralesional filamentous and shorter rod-shaped bacteria, respectively. Insets: negative control slide (PBS).
DISCUSSION

In the present study, two bacterial isolates of the family Flavobacteriaceae were found to be associated with ulcerative lesions in the yellow seahorse, i.e. *Cellulophaga fucicola* and *Tenacibaculum mesophilum*. These results demonstrate the involvement of one or both of these bacterial agents in the pathogenesis of ulcerative dermatitis in the captive yellow seahorse at the Toronto Zoo.

The majority of bacterial isolates from this research were gram-negative rods. These findings are consistent with current literature on culturable bacteria from aquatic environments and fish (Roberts et al., 2009). In *H. kuda* it has been shown that relative abundance in descending order of bacterial groups isolated from intestine of clinically healthy seahorses includes *Bacillus, Vibrio, Shewanella, Providencia, Phaecobacter, Enterococcus, Burkholderia, Enterovibrio, Pseudalteromonas* and *Staphylococcus* (Tanu et al., 2011). This is further supported by our findings that *Vibrio* and *Bacillus* species were the predominant genera of bacteria in the environment, as well as the most predominant overall in all our samples including skin (diseased or not), tank water, and tank substrate.

The association of bacteria of the Flavobacteriaceae family with ulcerative dermatitis has been demonstrated extensively in marine and freshwater teleosts. Pathogenic species identified to date are of the *Flavobacterium* and *Tenacibaculum* genera (Avendaño-Herrera et al., 2006; Bernardet & Bowman 2006; Barnes & Brown 2011; van Gelderen et al., 2011). These bacteria have been identified as significant causes of morbidity and mortality and economic losses in intensively reared species. *Tenacibaculum maritimum* in Japanese sea bream was reported to have caused erosive skin lesions and 20 to 30%
mortality among fry a couple of weeks after transfer to inshore net cages (Masamura & Wakabashi, 1977). While the involvement of these bacteria in ulcerative skin lesions has not yet been demonstrated in syngnathids, a *Tenacibaculum* species has been isolated in a case of necrotizing branchitis in a pipefish, *Syngnathus typhle* (Bombardini et al., 2006).

In this study, bacteria of the Flavobacteriaceae family were isolated from ulcerative lesions in 2 of 15 seahorses or 13.3%. It should also be noted that bacteria of the Flavobacteriaceae family tend to be fastidious organisms and are readily outcompeted on agar by other more resilient organisms such as *Vibrio*, *Pseudomonas* and others (Bernadet & Bowman 2006). This could explain why flavobacteria were not isolated from all cases with ulcerative dermatitis. The closest matches to amplicon sequences from the Flavobacteriaceae isolates were *Cellulophaga fucicola* and *Tenacibaculum mesophilum*. These species have not yet been extensively studied and to date have been only been isolated in environmental surveillance studies involving cultivable bacteria on the surface of marine sponges and algae (Johansen et al., 1999; Suzuki et al., 2001). However, marine bacteria causing disease in fish are very often opportunistic pathogens, present in normal seawater. *Flavobacterium succinicans* and *F. hydatis* are good examples of bacteria normally present in the environment with the capacity to become pathogenic (Bernardet & Bowman 2006). The role of these Flavobacteriaceae isolates from this study in the pathogenesis of ulcerative disease in *H. kuda* cannot be discounted due to our lack of knowledge of these species (Hensen & Olafsen 1999).

Other bacteria commonly associated with ulcerative dermatitis in teleost include *Mycobacterium* (Bombardini et al., 2006; Balcázar et al., 2011), *Aeromonas* (Bombardini et al., 2006) and *Vibrio* (Alcaide et al., 2001; Tendencia 2004; Bombardini et al., 2006).
Genotypic analysis of bacteria associated with ulcerative dermatitis yielded amplicon sequences of species of both *Vibrio* and *Mycobacterium*. While these may play a role in the pathogenesis or be the primary pathogen for a subset of these lesions, they are not filamentous in nature and therefore not the predominant bacteria observed within many ulcerative lesions under light microscopy.

The antigenic relationship among all isolates was examined by whole-cell agglutination and immunohistochemistry with polyclonal antisera produced in rabbits in previous studies (Ostland et al., 1999; Ostland et al., unpublished data). Within the limitations of our testing, these isolates demonstrated no antigenic relationship to the reference flavobacterial strains available in the Fish Pathology Laboratory.

Of the seven chosen Flavobacteriaceae 16S rRNA gene sequences, the *Cellulophaga* (Hk19) 16S rRNA gene sequence had highest nucleotide identity to the *Tenacibaculum maritimum* laboratory strain previously identified in ulcerative dermatitis in salmonids in British Columbia, Canada (Ostland et al., 1999). The ‘Tenacibaculum’ species was most similar to *Tenacibaculum maritimum*, also well known to cause ulcerative disease in numerous marine teleosts (Bernardet et al., 1990; Cepeda & Santos 2002; Handlinger et al., 1997; Toranzo et al., 2005; Abd El-Galil & Hasheim 2011).

LCM was used to identify bacteria within FFPE sections that contained ulcerative lesions from *H. kuda*. Approximately 380bp of the 16S rRNA gene was amplified from these FFPE tissues; however, amplicon sequences did not match those of bacteria of the Flavobacteriaceae family. Failure to amplify Flavobacteriaceae DNA from these tissues might have been due to a combination of factors, e.g. DNA concentrations relative to
other bacteria within skin lesions, DNA degradation or alteration from prolonged formalin fixation, and additional susceptibility to DNA degradation from environmental exposure due to the peripheral location of lesions. Overall, LCM was not a good technique for this study as either the sensitivity was too low or the additional processing necessary for LCM caused sufficient DNA degradation and loss, thus decreasing our ability to amplify enough DNA. Despite this, ISH demonstrated bacteria of the family Flavobacteriaceae within ulcerative lesions. While ISH was more useful, there were challenges with the preservation of tissue architecture due to the required processing. It was additionally challenging as our target tissues were already fragile necrotic tissues present along the edges of each tissue section and the edges of tissues suffered the greatest degradation from the processing. This technique is likely best suited for target tissues that are away from the edges.

With any research involving bacterial culture and isolation, limitations exist since less than 1% of the total number of bacteria in seawater will grow under laboratory conditions (Colwell et al., 1985; Kjelleberg et al., 1993). In this study, bacteria of the family Flavobacteriaceae were hypothesized to be the etiological agent of ulcerative disease and, therefore, it may have been ideal to culture the initial swab on a marine modification of cytophaga agar. The samples were also biased temporally, that is, the disease took place intermittently over at least 12 year period, but the swabs were taken only taken over a 2 year span for the diseased samples and at 2 sampling times for the control samples. This may have affected our ability to detect the pathogen if sampling was not done at the correct time point by culture and additionally, may have contributed to a low number of cases positive by ISH. If more samples had been taken at regular intervals and for all
ulcerative cases, we may have been able to observe a pathogen more consistently associated with these ulcerative lesions. There are many challenges inherent to a retrospective study. Greater consistency in sampling would have been ideal in this research project. It would have been advantageous for all seahorses with ulcers to have been swabbed, have had tissues taken for histopathologic assessment and therefore had LCM sampling and ISH performed for all samples collected. Additional challenges that affected the quality and consistency of sampling were the distant location of animals and the numerous sample collectors, a result of turn-over of the animal husbandry staff. A larger sample group would have also increased the strength of the current observations.

Probes based on almost full-length 16S rRNA gene sequences from candidate bacteria, C. fucicola-like isolate and T. mesophilum-like isolate, were successfully designed for in situ hybridization. The probes demonstrated these bacteria within ulcerative lesions under light microscopy in 4 and 1 case respectively. The C. fucicola-like isolate was also labelled within granulomatous inflammation (in addition to acid-fast bacilli) and spreading systemically rather than remaining at the surface. Some species of flavobacteria have been known to cause systemic disease, the best known of which is Flavobacterium psychrophilum, a freshwater species (Barnes & Brown 2011). These findings suggest that this Cellulophaga fucicola-like strain may be more invasive than initially thought and/or it may have a synergistic effect with other pathogens such as the mycobacteria. The age of the fish may also play a role, as this individual was a fry, the stage at which F. psychrophilum causes the most serious systemic disease in salmonids (Nematollahi et al., 2003; Barnes & Brown 2011).
In this study, bacteria of the family Flavobacteriaceae were demonstrated within ulcerative lesions in *H. kuda*, supporting our hypothesis. While the species identified to date are currently described as environmental bacteria, many fish pathogens are opportunistic. Flavobacterial isolates were also only isolated in 2 of 15 seahorses with ulcerative dermatitis. But again, most bacteria of the family Flavobacteriaceae are known to be fastidious organisms (Bernardet & Bowman 2006). This suggests that their primary habitat could be limited; for example, that habitat might be limited to the fish tissue environment where they exist as a parasite or saprophyte depending on the immunological state of the fish and virulence of the strain (Bernardet & Bowman 2006).

Some flavobacteriaceae isolates have been shown to survive for long periods of time in water, with enhanced survival times at high sediment-derived nutrient densities (Madetoja et al., 2003). This emphasizes the importance of tank hygiene, which is especially a challenge with syngnathids as they have a very low gastrointestinal transit time. They require multiple feedings in a day or require live feed in tanks throughout the entire day. If feed is not syphoned regularly, this can contribute to high organic loads, which in turn can be an ideal environment for bacterial overgrowth. Additionally, elevated sediment concentrations encourage epiphytic growth along the plants and other surface areas within the tank, potentially creating an optimal environment for *Cellulophaga* and *Tenacibaculum* strains to thrive (Johansen et al., 1999; Suzuki et al., 2001).

An additional complication is that many of the plastic plants and tank objects onto which seahorses grasp with their prehensile tails are abrasive. If their epithelium is continuously abraded, this removes an important innate immune barrier, i.e. their first line of defense,
against agents in the environment. Ulcerative lesions in syngnathids were frequently observed in this population of syngnathids. While these ulcers were found across the entire skin epithelium, there was a larger proportion observed along the tail. The propensity for the tail to develop ulcerative lesions may also be related to the spatial distribution of flame cells across the seahorse skin epithelium. Flame cells are a specialized surface cell type found only in seahorses (Bereiter-Hahn et al., 2006). These cells produce mucous caps which have associated epiphytic organisms along their apical surface. They are hypothesized to play a role in defense against surface pathogens whereby the mucous caps are sloughed if overburdened with bacterial or other growth (Bereiter-Hahn et al., 2006). The hyperemic tail may be representative of a stage in the disease where the flame cells along the tail have sloughed all of their mucous caps.

This work supports the hypothesis that bacteria of the family Flavobacteriaceae are associated with ulcerative dermatitis in *H. kuda*. However, this research does not explore the role of these bacteria in the pathogenesis of ulcerative dermatitis. The distinction between primary and opportunistic pathogens needs to be assessed in future research, although the later is most likely considering what we know of other pathogens within this bacteria family and also because ulcerative dermatitis is most often multifactorial (Law 2001).
GENERAL DISCUSSION AND CONCLUSIONS

A number of diseases were identified in this study, many of which have not yet been described in peer-reviewed publications on syngnathids. The top three causes of morbidity in mortality in this population were; ulcerative dermatitis, suspected nutritional myopathy and mycobacteriosis, accounting for greater than 55% of total morbidity and mortality. Many fish pathogens are opportunistic, which means that changes in the environment and/or host create optimal conditions for the creation of the diseased state. There are many interactions in the epidemiological triad to be taken into account in disease management. Examples of host factors for sygnathids could include genetic predisposition to a disease and immune status of an individual. Environmental factors are numerous in captivity with artificial environments in the form of aquaria; these could include, but are not limited to, water quality, temperature, abrasive tank objects in the case of prehensile species, lighting, and biomass density. Agent factors could include a variety of virulence factors, some of which in turn may be influenced by environmental factors such as temperature. In captive animals a poor quality environment can lead to stress, resulting in an immunocompromised animal with greater susceptibility to disease. For animals held in captivity, optimal husbandry conditions, including proper diet, water quality, and biomass density, are of utmost importance.

For veterinary and husbandry professionals, this emphasizes the importance of understanding each species and their requirements in order to reduce stress and optimize immune status. For pathologists, this thesis provides insight into common diseases in
captive sygnathids, specifically pertaining to their clinical presentation and histopathology.

This thesis gives some insight into the usefulness of techniques that are not as common in the investigation of bacterial diseases as culture and PCR, namely ISH and LCM. In retrospective studies with samples older than one year and with very little target DNA or scattered target DNA, LCM was found not to be very useful due to its low sensitivity and loss of DNA in processing. ISH on the other hand, was found to be useful in a retrospective study as it has higher sensitivity. ISH is also advantageous in that it has high specificity, that is, it labels target RNA of one particular bacteria rather than amplifying DNA from the most bacteria present in highest concentration with a tissue location such as LCM.

While numerous problems were identified, the most common cause of morbidity and mortality in this population was an ulcerative dermatitis associated with filamentous bacteria. These filamentous bacteria were hypothesized to be of the Flavobacteriaceae family due to their morphology and their propensity to cause ulcerative lesions (Cipriano et al., 1996; Ostland et al., 1999; Avendaño-Herrera et al., 2006; Roberts et al., 2009). The etiological agent(s) responsible for the dermatitis was investigated by culture and identification of bacteria from ulcerative lesions using 16S rRNA gene amplification and phenotypic testing. 16S rDNA was extracted from bacteria within ulcerative lesions of formalin-fixed paraffin-embedded tissues using LCM. The sequences obtained were compared to those from cultured isolates and candidate bacterial 16S rDNA was used as a template for in situ hybridization probe design to demonstrate bacteria invading within ulcerative lesions under light microscopy. While the evidence gathered in this research
does support the role of a Flavobacteriaceae isolate in the pathogenesis of ulcerative
disease in *H. kuda*, we do not yet understand all of the elements of the epidemiological
triad relative to this disease process. Identification of the pathogen, however, is a first
step in understanding pathogenesis of a disease, although there are many aspects of agent,
host and environment interactions that remain unknown.

This thesis has provided a direction for future research. From the second chapter, the
suspected nutritional myopathy warrants the most urgent attention as proper nutrition is
critical to any species’ survival. Micronutrients previously associated with nutritional
myopathies in other species are a good starting point for research trials, i.e. investigating
the possible role of vitamin E and selenium in the development of this disease. Trials
investigating the short and long-term effects of varying levels of these micronutrients
with observations of clinical signs and histology of skeletal muscle are required. On the
topic of ulcerative dermatitis, research is needed to fulfill Koch’s postulates. To
conclusively demonstrate the ‘Cellulophaga’ isolate as the etiological agent of ulcerative
dermatitis in *H. kuda*, the disease would need to be reproduced under controlled
conditions and the bacterium re-isolated from diseased fish. Initial infection trials would
use injection of the organism to assess degree of pathogenicity of the isolate and
additional trials would use water-borne or immersion trials with and without abrasion of
the epidermis to reproduce disease.

However, before disease trials or other specific pathogen research takes place, it is
important to know the basic physiological requirements and optimal environmental
conditions of each syngnathid species. Without the ability to maintain healthy
syngnathids in captivity, inadequate environmental parameters become confounding
factors in a research trial. It is currently known that there is significant species variation in temperature, density, and even depth requirements, although the majority of this information is not within peer-reviewed publications (Greenwell, 2002).

The following conclusions can be drawn from:

Chapter 2:

1) The most common causes of mortality in syngnathids at the Toronto Zoo are bacterial dermatitis, bilaterally symmetrical myopathy and mycobacteriosis respectively.

2) Numerous multiple novel causes of mortality were identified: neoplasia, myopathy, crypto, possible viral enteritis.

3) Ulcerative dermatitis was the most common disease entity associated with mortality and was often associated with the presence of filamentous bacteria.

Chapter 3:

1) Bacteria identified from culture of ulcerative lesions using 16S rRNA gene amplification included flavobacterial species: these were most similar to Cellulophaga fucicola and Tenacibaculum mesophilum.

2) 16S rDNA extracted from bacteria within ulcerative lesions preserved in formalin-fixed paraffin-embedded tissues using laser capture microdissection did not match those from isolates cultured from lesions.

3) ISH using probes based on the Cellulophaga fucicola-like and Tenacibaculum mesophilum-like isolates from ulcerative lesions labelled these filamentous
bacteria within ulcerative lesions under light microscopy, demonstrating their association with necrotizing skin disease in *H. kuda*.
REFERENCES


Johansen JE, Nielsen P, Sjøholm C. 1999. Description of *Cellulophaga baltica* gen. nov., sp. nov. and *Cellulophaga fucicola* gen. nov., sp., nov. and reclassification of


**APPENDICES**

**Appendix 1.** Colony colour, Gram staining properties, morphologies, temperature and salinity growth and antimicrobial sensitivity (minimum inhibitory concentration) for isolates of the Flavobacteraceae family.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Color</th>
<th>Gm</th>
<th>morph</th>
<th>temp (10/37)</th>
<th>NaCl* (0/3/5)</th>
<th>SXT</th>
<th>GEN</th>
<th>ENRO</th>
<th>AMP</th>
<th>OXY</th>
<th>ERY</th>
<th>FFN</th>
<th>FLUQ</th>
<th>PRI</th>
<th>OXO</th>
</tr>
</thead>
<tbody>
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<td>-</td>
<td>rod</td>
<td>-/+</td>
<td>0.12/2.3</td>
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<td>0.25</td>
<td>0.03</td>
<td>0.25</td>
<td>4</td>
<td>0.25</td>
<td>0.25</td>
<td>2.38/0.12</td>
<td>0.12</td>
<td></td>
</tr>
<tr>
<td>Hk87</td>
<td>yellow</td>
<td>-</td>
<td>rod</td>
<td>-/+</td>
<td>0.12/2.3</td>
<td>R</td>
<td>0.5</td>
<td>8</td>
<td>1</td>
<td>2</td>
<td>4</td>
<td>1</td>
<td>2.38/0.12</td>
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<td></td>
</tr>
<tr>
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<td>-</td>
<td>rod</td>
<td>-/+</td>
<td>R</td>
<td>R</td>
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<td>4</td>
<td>2</td>
<td>4</td>
<td>2</td>
<td>0.5</td>
<td>76/4</td>
<td>0.5</td>
<td></td>
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</table>

Color = colony color; Gm = gram staining; morph = bacteria morphology; temp = colony growth at 10°C and 37°C; NaCl 0/3 = growth in 0% and 3% sodium chloride agar; SXT = Trimethoprim/sulfamethoxazole; GEN = Gentamicin; ENRO = Enrofloxacin; AMP = Ampicillin; OXY = Oxytetracycline; ERY = Erythromycin; FFN = Florfenicol; FLUQ = Flumequine; PRI = Ormetoprim/sulphadimethoxine; OXO = Oxolinic Acid.

* Media used in this test was too nutrient limiting for these bacteria, these are halopilic bacteria as they grow well in media containing salt such as MA and MCA.
**Appendix 2.** Biochemical test results for isolates of the Flavobactereaceae family.

| Isolate No. | onpg | ODC | LDC | ADH | Cit | H₂S | urea | TDA | Ind | vp | gel | glu | man | ino | sor | rha | sac | mel | amy | ara |
|-------------|------|-----|-----|-----|-----|-----|------|-----|-----|----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| Hk19        | -    | -   | +   | +   | -   | -   | +    | +   | +   | +  | +   | -   | -   | -   | -   | -   | -   | -   | -   | -   |
| Hk87        | -    | -   | -   | -   | -   | -   | -    | +   | -   | -  | -   | -   | -   | -   | -   | -   | -   | -   | -   | -   |
| Hk89        | -    | -   | -   | -   | -   | -   | -    | -   | -   | -  | -   | -   | -   | -   | -   | -   | -   | -   | -   | -   |

<table>
<thead>
<tr>
<th>Isolate No.</th>
<th>ox</th>
<th>cat</th>
<th>BA</th>
<th>βH</th>
<th>MCA</th>
<th>TSA</th>
<th>CA</th>
<th>FXRB</th>
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<tr>
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<td>+</td>
<td>+</td>
<td>+</td>
<td>β</td>
<td>+</td>
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<tr>
<td>Hk87</td>
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<td>-</td>
<td>+</td>
<td>β</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Hk89</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>γ</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

onpg = ortho-nitrophenyl-βD-galactopyranoside (β-galactosidase test); ODC = ornithine decarboxylase; LDC = lysine decarboxylase; ADH = arginine dihydrolase; Cit = citrate; H₂S = hydrogen sulfide; urea = urea; TDA = tryptophane deaminase; Ind = indole; vp = acetoin production (Voges-Proskauer); gel = gelatinase; glu = glucose; man = mannitol; ino = inositol; sor = sorbitol; rha = rhamnose; sac = saccharose; mel = melibiose; amy = amygdalin; arab = arabinose.

ox = cytochrome oxidase; cat = catalase; BA = blood agar; βH = β hemolysis; MCA = MacConkey agar; TSA = trypticase soy agar; CA = cytophaga agar; FXRB = flexirubin pigment production.