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JAMES COOK
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# BEHAVIOUR, HISTOPATHOLOGY AND IMMUNOBIOLOGY: INTERACTIONS BETWEEN THE ECTOPARASITE *NEOBENEDENIA* (MONOGENEA: CAPSALIDAE) AND ITS HOST, *LATES CALCARIFER* (PERCIFORMES: LATIDAE)



Thesis submitted by

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In partial fulfilment of the requirements for the
Degree of Masters of Philosophy (Science)
In the College of Marine and Environmental Sciences
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step of my thesis. They remain great and irreplaceable friends. Jared Huk and Jarod Guppy volunteered in all chapters of my thesis and proved to be excellent helpers.

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*This thesis is dedicated to my family, my friends, and to those who come and go, but who took the time in their lives to support me throughout this endeavour.*

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# MPHIL ACHIEVEMENTS

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| | 2015 | **Trujillo-González A.,** Constantinoiu C.C., Rowe R. & Hutson K.S. (2015). Tracking transparent monogenean parasites on fish from infection to sexual maturity. *International Journal of Parasitology: Parasites and Wildlife* **4**, 316-322. |
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| | 2013 | Best poster, Postgraduate student conference, School of Marine & Tropical Biology, James Cook University, Townsville Australia, June 12, 2013. |
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| | 2013 | **Trujillo-González A.,** Constantinoiu C.C., Johnson L.K. & Hutson K.S. Differences in epithelial pathology of fish microhabitats infected with the ectoparasitic monogenean *Neobenedenia* sp.. Australasian Scientific Conference on Aquatic Animal Health. Cairns, Australia, July 4, 2013. |
| | 2012 | **Trujillo-González A.,** Perez-Pacheco R. & Realpe E. The effect of habitat complexity over the parasitic efficiency of *Romanomermis iyengari* (Nematoda: Mermithidae) on *Culex quinquefasciatus* (Diptera: Culicidae). Australian Society for Parasitology conference . Launceston, Tasmania, July 3, 2012. |
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# ABSTRACT

Capsalid monogeneans are harmful skin ectoparasites of ornamental and farmed fishes in tropical and subtropical marine environments. Within this group, *Neobenedenia* includes of particularly virulent species that exhibit low host specificity, a direct life cycle, high fecundity, environmentally resilient eggs, and have been associated with mass mortalities in aquaculture. There is a paucity of information on the interaction between *Neobenedenia* spp. and their fish hosts. Examination of *Neobenedenia* spp. infection and invasion behaviour, associated pathology and the effect on host immune responses can enable a deeper understanding of the potential impact of parasites on fish health and the complexity of parasite-host interactions. This study examined the interaction between *Neobenedenia* sp. and barramundi, *Lates calcarifer*, an important finfish species in commercial fisheries and aquaculture.

*Neobenedenia* spp. are cryptic in nature, which makes infection success and invasion routes challenging to elucidate. Larval recruitment and microhabitat preference was examined through time (Chapter 2) by using *Neobenedenia* sp. oncomiracidia (larvae) labelled with a fluorescent marker. Parasites were tracked on the body surface of the host with an epifluorescence stereomicroscope at 10 time intervals post exposure (15, 30, 60, 120 min, 24, 48 h, four, eight, 12, and 16 days). Parasites retained the fluorescent signal throughout the experiment. *Neobenedenia* sp. larvae settled opportunistically on the fish and then migrated to preferred microhabitats. Once recruitment had ceased (48 h), preferred microhabitats included the eyes, fins, and dorsal and ventral extremities on the main body. Reproduction could be an important factor for *Neobenedenia* sp. distribution, indicated by parasites aggregating on the fins within 24 h of attaining sexual maturity. Interestingly, some parasites attached beneath the scales of host fish, which may enable the parasite to be almost entirely secluded from the environment and could reduce the efficiency of current parasite management methods (e.g. chemical and freshwater bathing) in aquaculture.

High infection intensities of *Neobenedenia* species are well-known to cause pathology, however, the damage associated with mechanical attachment of the main attachment organ, the haptor, has not been examined. The pathology associated with haptor attachment of *Neobenedenia* sp. to *L. calcarifer* was examined through prepared histopathology sections at the haptor-host interface (Chapter 3). Fish were infected with *Neobenedenia* sp., and skin samples with attached parasites were collected from the eyes, mandible, operculum, middle body, ventral body and caudal fins 20 days post-infection. Histological slides were prepared by embedding, sectioning and staining tissue samples from the site of parasite attachment to the skin of host fish. Epithelial thickness and mucous cell abundance were measured in samples from uninfected and infected fish. Infected fish had lower mucous cell abundance, and the middle and ventral body surfaces had thinner epidermis compared to uninfected fish. Infected fish presented signs of dermal inflammation, epithelial loss, loss of intraepithelial attachment, and vacuolated epidermis compared to uninfected (control) fish.

The antibody response and acquired resistance of *L. calcarifer* to *Neobenedenia* sp. infections was examined following consecutive experimental infections (Chapter 4). Twenty fish were infected with *Neobenedenia* sp. oncomiracidia for 10 days with recovery periods (two weeks) between four consecutive exposure events. Before and after each exposure event, each fish was weighed, measured, and blood and mucous samples were collected for ELISA. After each infection the parasites were collected from each fish to analyse infection success, parasite size and reproductive status. Results showed that infected fish had significantly lower feed conversion efficiency than uninfected fish, parasites were significantly smaller on previously exposed fish and *Neobenedenia* infection success was significantly lower following three exposure events. There was no difference in infection success between the first, second and fourth exposure events. No differences in blood and mucous IgM levels between uninfected and infected fish could be detected by ELISA

This thesis provided an innovative and rigorous approach to standard scientific methodologies to gain new information on the interactions between harmful monogenean parasites and host fish. Fluorescent labelling enabled rapid

assessment of infection success and invasion routes of *Neobenedenia* sp. and revealed intriguing parasite behaviours that could aid parasite survival and reproductive success. Careful precision with histopathology at the haptor-host interface showed morphological differences on the epithelium of *L. calcarifer* when infected with *Neobenedenia* sp.. Nevertheless, parasite attachment did not stimulate an immune response to consecutive infections.

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**Chapter 1**

**General Introduction**

*Marine parasites in finfish aquaculture*

Marine aquaculture involves rearing aquatic organisms for commercial purposes (Seng et al., 2006). This industry has developed rapidly because of declining wild fishery resources (Pauly 2008) and the strong demand for marine products (Lem et al., 2014; Seng 1997). Indeed, aquaculture is the fastest growing primary industry that supplies food to the growing global human population (Lem et al., 2014; Cressey 2009; Guo & Woo 2009). The increasing competition faced by the agriculture sector for available resources (Foley et al., 2005; Tilman et al., 2002), the need for economies to scale-up, and the drive for increased productivity per unit area has caused the aquaculture industry to use intensive farming systems (Tacon & Halwart 2007). A wide range of finfish species are cultured around the world with more than 41.6 billion tonnes of fish produced in 2011, followed by molluscs and crustaceans at 14.4 and 5.9 billion tonnes, respectively (Lem et al., 2014).

In intensive aquaculture, fish are reared in confined areas and farmers control production factors, such as farm size, stocking and feeding of fish. Nonetheless, intensive aquaculture production can favour proliferation of pathogens and parasites that threaten economic viability and food production (Owens, 2012; Rückert et al., 2009; Rohde, 2005). Parasitic outbreaks in aquaculture have been linked to overstocking, stress of the stock, fluctuations in water temperature, and decreased water quality (Villanueva et al., 2013; Owens, 2012; Rohde, 2005). These altered parameters cause immunosuppression in the host species (Seng et al., 2006), which increases finfish susceptibility to pathogenic infection, morbidity and mortality (Denevey et al., 2001; Ogawa, 1996; Kaneko et al., 1988). Parasite outbreaks have been associated with significant losses of farmed species in marine aquaculture (Marcogliese, 2004; Deveney et al., 2001; Kaneko et al., 1988), creating a critical

need to understand how to strategically manage harmful parasites to ensure appropriate animal health.

Marine parasites affect the morphology, behaviour, appearance, energetic demands, fecundity and growth of hosts (Marcogliese, 2004; Minchella & Scott, 1991). Moreover, some parasites are known to influence the composition and structure of animal communities by regulating the abundance of their host population (Mouritsen & Poulin, 2010; Mouritsen & Poulin, 2002). For example, Infection of echinostome trematodes in the New Zealand cockle, *Austrovenus stutchburyi*, reduces the abundance of the host (an important primary consumer) and causes a cascading effect in the functioning of the ecosystem (Mouritsen & Poulin, 2010; Thomas et al., 2005). Most marine parasite-host interactions have been predominantly studied in wild populations, aiming to increase the current knowledge of their ecology and behaviour (Rohde, 2005). Remarkably, these interactions remain relatively poorly studied in farmed and commercial species, creating significant gaps of knowledge to manage parasite outbreaks and improve aquatic animal health.

*Ectoparasitic monogeneans*

Monogeneans are considered serious pathogens of teleost fish, and a growing threat to marine finfish aquaculture (Ogawa, 2005; Deveney et al., 2001). Monogenean parasites attach to the external surfaces and gills of freshwater and marine fishes (Whittington, 2004) and feed on the epidermal, mucous and blood cells of the host; causing severe skin lesions, abrasions and haemorrhages that directly affect the health of the fish and can increase the risk of secondary infections (Buchmann & Lindenstrøm, 2002; Leong & Colorni, 2002; Thoney & Hargis, 1991; Kaneko et al., 1988).

Within the Monogenea, the Capsalidae comprises approximately 200 species, of which the majority are ectoparasites on marine fishes (Whittington 2004). In this group, *Neobenedenia* includes particularly virulent species that exhibit low host-specificity, a direct life cycle, high fecundity and environmentally resilient eggs (Ogawa et al., 2006; Bullard et al., 2000; Whittington, 2004). *Neobenedenia* eggs

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bear filamentous strings that entangle with each other and on submerged structures (Fig. 1a), leading to high re-infection rates amongst captive fish (Ogawa et al., 2006). Within each egg, a ciliated larva (oncomiracidium) develops (Fig. 1b). Emerging oncomiracidia can swim and locate a suitable host by detecting chemical substances in fish epithelium using cilia-based sensilla (Yoshinaga et al., 2000; Whittington et al., 1999). *Neobenedenia* spp. use two attachment organs located anteriorly and one larger posterior attachment organ (haptor) to attach to the body surface of the host, where they graze on epidermal and mucous cells (Whittington, 1996). Adult *Neobenedenia* (Fig. 1c) can reproduce sexually (Whittington & Kearn, 1993), in isolation (Dinh Hoai & Hutson, 2014), and can share spermatophores between individuals (Ogawa et al., 2014).
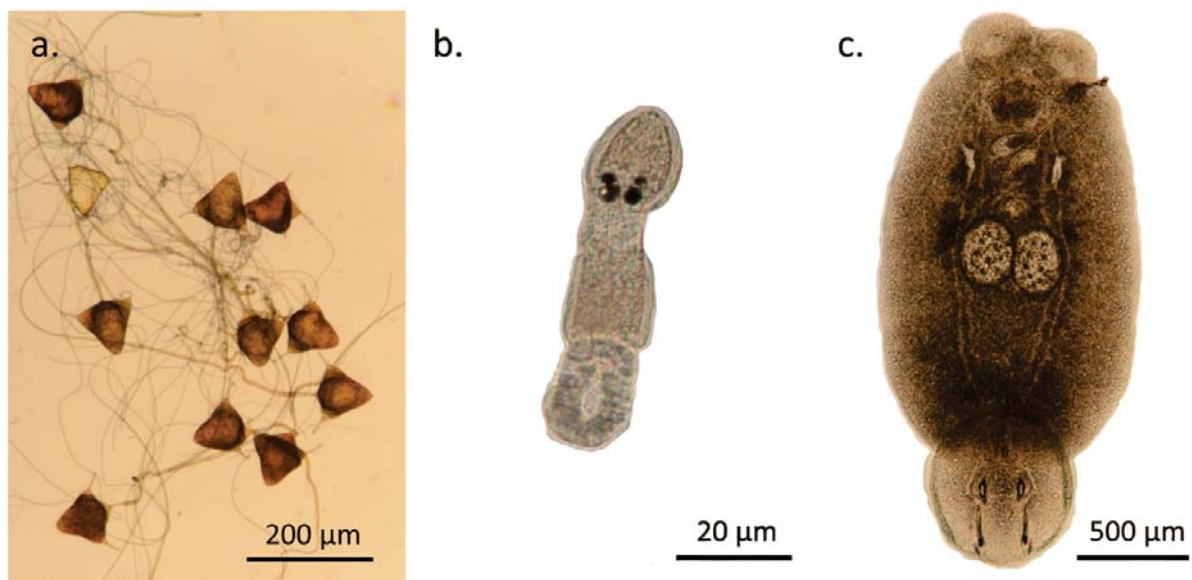


**Fig. 1.** *Neobenedenia* sp. life stages. Eggs (a), oncomiracidia (< 3h old) (b) and adult (10 days) (c).

*Neobenedenia* species have been reported to infect over 100 fish species and high infection intensities have been associated with mass mortalities (Whittington, 2012; Rückert et al., 2009; Deveney et al., 2001; Whittington & Horton, 1996; Ogawa et al., 1995). Current treatments to control *Neobenedenia* involve repetitive acute bathing of infected stock in either formalin or freshwater solutions (Hirazawa et al., 2010; Thoney & Hargis 1991; Kaneko et al., 1988). Although these treatments kill

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attached *Neobenedenia*, eggs are highly resilient and generally remain viable after the treatment with high reinfection rates (Ogawa et al., 2006; Ellis & Watanabe, 1993; Müeller et al., 1992). Therefore, it is imperative to understand how *Neobenedenia* species affect the host's well-being in order to effectively manage parasitic outbreaks; however, several aspects of how parasites interact with their hosts remain poorly studied.

*Monogenean habitat selection on teleost fish*

Parasite habitat selection is influenced by multiple factors of parasite ecology. Initially, parasites attempt to establish on the host, which resists the infection through its defense mechanisms and immune system (Sitjà-Bobadilla, 2008). Following initial attachment, parasite distribution and site-specificity have been associated with particular feeding guilds and diets (Karvonen et al., 2007; Marcogliese, 2002), mate finding habits (Whittington & Ernst, 2002; Chisholm et al., 1997), evasion of the host immune and defensive systems (Sitjà-Bobadilla 2008; Buchmann & Lindenstrøm 2002), camouflage and evasion of predators (Whittington, 1996), or avoidance of intra and interspecific competition (Rohde et al., 1995). To understand how parasites are distributed on the body surface of teleost fish, these multiple factors must be considered as potential explanations for the exhibited parasite life-style. Parasites also have different requirements during their life-span after initial attachment of the larvae, and the importance of feeding resources, host immune response avoidance, or reproduction, may change accordingly.

Parasite distribution affects how hosts react to infection. Microhabitats that exhibit greater parasite loads, could be more susceptible to epidermal damage and subsequent secondary infection (Buchmann & Lindenstrøm 2002; Leong & Colorni, 2002; Thoney & Hargis, 1991; Kaneko et al., 1988). Some parasites are known to actively migrate after initial attachment to a wide variety of microhabitats (Hirazawa et al., 2011; Whittington, 1996; Kearn, 1984), while others remain attached to a single location throughout their life-span (Glennon et al., 2007; Karvonen et al.,

2007; Morand et al., 1999). For example, larvae and post-larvae *Branchotenthes octohamatus* (Monogenea: Hexabothriidae) are highly site specific and attach only to the gills of the southern fiddler ray, *Trygonorrhina fasciata* (Glennon et al., 2007). Other species, including *Entobdella soleae* and *Benedenia lutjani* (Monogenea: Capsalidae), migrate to different microhabitats following initial attachment (Whittington & Ernst, 2002; Kearn, 1984). *Neobenedenia* spp. have been observed attached to all external surfaces of the host including the nostrils, eyes, inner mouth cavity and fins (Trujillo-González et al., 2014; Hirazawa et al., 2011; Whittington, 1996), and are believed to migrate over the body surface of the host (Ogawa et al., 2006; Hirayama et al., 2009; Hirazawa et al., 2011). However, *Neobenedenia* spp. are transparent and cryptic in nature, making live parasites extremely difficult to observe (Whittington 1996). This has limited research efforts and examination of the post larval ecology of *Neobenedenia*. The possibility of overlooking parasites due to their cryptic nature needs to be considered in relation to previous studies describing the distribution of Neobenedenia spp. on their hosts (Chigazaki et al., 2000; Glennon et al., 2007). Understanding *Neobenedenia* spp. patterns of migration across the body surface of the host can indicate which microhabitats are selected for attachment and subsequent development, and which microhabitats may be more susceptible to infection.

*Effect of monogenean ectoparasites on teleost immune system*

The immune system detects, protects, and distinguishes an organism's own tissue from a wide range of external microbes, viruses and parasites. All living organisms have either a rudimentary immune system (in the form of enzymes which protect against bacteriophage infections present in bacteria) or a more complex immune system comprised of innate, adaptive and humoral responses (in higher vertebrates) (Rauta et al., 2012; Van Muiswinkel & Van Der Wal, 2006). All vertebrate gnathostomes for instance, share an essential immune structure characterised by: (i) a highly conserved innate system, (ii) consistent development of a combined immune system, and (iii) bilateral communication between components of the innate and adaptive immunity (Rauta et al., 2012).

Teleost fish are the earliest vertebrates known to develop an adaptive immune system (Rauta et al., 2012; Whyte 2007). Adaptive immune responses in teleost fish are generally characterised by immunoglobulins, T-cell receptors, cytokines, and major histocompatibility complex molecules (Watts et al., 2001). However, compared to higher vertebrates, teleost fish immune responses are less efficient, less complex, and have a limited repertoire of antibodies involved in innate and adaptive immune responses (Rauta et al., 2012; Salinas et al., 2011; Brandtzaeg 2009). As such, teleost fish rely on their skin and mucosal surfaces as external protective barriers against a continuous flow of microbes and stressors (Whyte 2007; Delamare-Deboutteville et al., 2006). If a pathogen breaches these barriers, the innate immune system provides an immediate, non-specific response (Jones 2001).

Fish immune responses do not necessarily result in adverse effects on marine ectoparasites (Buchmann & Lindenstrøm 2002). This is because most ectoparasites develop adaptive strategies to avoid the effect and effectors of the host immune response (Sitjà-Bobadilla, 2008). Parasites can have one or more immune evasive mechanisms to cope with the effectors of the immune response (Buchmann & Lindenstrøm 2002). For example, the eel monogenean, *Pseudodactylogyrus bini*, exploits parasite-induced tissue reactions (i.e. embedding within the host tissue) to improve attachment on the surface of the host (Buchmann 1997). Such tissue reactions have been reported in other gill-dwelling monogeneans, such as *Linguadactyla molvae* infecting blue ling*, Molva dipterygia* (see Bychowsky & Evseevich 1962), and *Cleidodiscus robustus* infecting bluegill, *Lepomis macrochirus* (see Thune & Rogers, 1981). Other avoidance mechanisms reported in parasites are based on antigenic variation, antigen mimicry, and modification of the host immune system (Salzet et al., 2000). Antigenic variation as described in various blood-dwelling parasites (e.g. *Plasmodium* and *Trypanosoma* species) has not been reported for ectoparasitic monogeneans (Buchmann & Lindenstrøm 2002). However, previous studies have suggested that passive acquisition of host molecules and their incorporation into the parasite's surface linings to be used as an immunological disguise could be possible for monogeneans (Buchmann & Lindenstrøm 2002; Salzet et al., 2000).

The likelihood of disease resulting from *Neobenedenia* infection varies amongst teleost hosts (Ohno et al., 2009; Rubio-Godoy et al., 2011). Some teleost fish exposed to *Neobenedenia* have been reported to develop immunity (Hatanaka et al., 2005) and morphological changes that could convey acquired protection to subsequent infections (Hirazawa et al., 2011; Hirayama et al., 2009; Ohno et al., 2008; Bondad-Reantaso et al., 1995). Continuous exposure to *Neobenedenia* could stimulate the development of an acquired immunity associated with increased anti-parasitic mucus and serum antibodies in the fish host (Jones, 2001; Magnadóttir, 2006). Nonetheless, there is currently no evaluation of skin and blood antibody response of *L. calcarifer* when fish are exposed to successive infections of *Neobenedenia*.

*Study Species*

This research used a *Neobenedenia* sp. – *Lates calcarifer* parasite-host experimental model. The species of *Neobenedenia* investigated in this study was collected from private land (barramundi aquaculture industry) in North Queensland, Australia, and maintained in controlled conditions (25 °C, 35 ppt, ammonia, nitrite and nitrate concentrations <3 mg/L) at the Marine Parasitology Laboratory in James Cook University (Townsville, Queensland). The *Neobenedenia* sp. used for this research is currently unidentified because of absent diagnostic criteria to differentiate between geographical/host isolates and species (Whittington, 2012; Whittington, 2004). For the purposes of this thesis, the species is presented as *Neobenedenia* sp..

Barramundi or Asian sea bass, *Lates calcarifer,* Bloch 1790, is a catadromous teleost that inhabits freshwater rivers, lagoons, mangrove swamps and coastal bays (Milton et al., 2008; Cappo et al., 2005; Russell & Garrett, 1988). It is a species of important economic value commercially and recreationally in Australia and Southeast Asia (Katersky & Carter, 2005; Boonyaratpalin, 1997; Barlow et al., 1992), and for aquaculture within Australia and throughout the Indo-Pacific (Katersky & Carter, 2005). Global production is approximately 26,000 tonnes of which Australia contributes 4,000 tonnes (De Silva & Phillips, 2007). *Neobenedenia* is a well-known

parasite of barramundi, and has been reported to cause significant economic losses to the mariculture industry (Deveney et al., 2001).

*Thesis objectives and aims*

The aim of this thesis was to address three poorly documented aspects of the interaction between *Neobenedenia* sp. and *Lates calcarifer*. Firstly, the infection route and subsequent distribution of *Neobenedenia* sp. was evaluated over time in *Lates calcarifer*. Secondly, the pathology and epidermal differences associated with the attachment of *Neobenedenia* sp. on *L. calcarifer* was examined in different microhabitats. Finally, the development of the protective immunity in *L. calcarifer* was evaluated when repeatedly exposed to *Neobenedenia* sp. over time. Each of these questions represents one of three data chapters presented in this thesis. The chapters were prepared as three separate manuscripts to be submitted for publication, but each sequentially builds on the knowledge developed in each preceding chapter. A publication statement is made prior to each chapter to indicate current publication status. To finalise the thesis, the findings of each chapter are discussed in a General Discussion (see Chapter 5) with respect to the importance of parasite-host interactions and the links between parasite behaviour, histopathology and host immunobiology.

## CHAPTER 2 PUBLICATION STATEMENT

Chapter 2 was submitted for publication on 7th April 2015 to the *International Journal of Parasitology: Parasites and Wildlife*. Referees comments were received immediately prior to submission of this thesis (18th May 2015) and the manuscript was accepted for publication on the 3rd of July 2015. The manuscript was published as:

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# CHAPTER 2

**Tracking transparent monogenean parasites on fish from infection to sexual maturity**

## ABSTRACT

The infection dynamics and distribution of the ectoparasitic fish monogenean *Neobenedenia* sp. (Monogenea: Capsalidae) throughout its development was examined on barramundi, *Lates calcarifer* (Bloch) (Latidae), by labelling transparent, ciliated larvae (oncomiracidia) with a fluorescent dye. Replicate fish were each exposed to approximately 50 fluorescent oncomiracidia and then examined for parasites using an epifluorescence stereomicroscope at 10 time intervals post-exposure (15, 30, 60, 120 min, 24, 48 h, four, eight, 12, and 16 days). Fluorescent labelling revealed that parasites attached underneath and on the surface of the scales of host fish. Parasite infection success was 20 % within 15 minutes, and peaked at 93 % two days post-exposure, before gradually declining between four and sixteen days. Differences in parasite distribution on *Lates calcarifer* over time provided strong evidence that *Neobenedenia* sp. larvae settled opportunistically and then migrated to specific microhabitats. Parasites initially attached (< 24 h) in greater mean numbers on the body surface (13 ± 1.5) compared to the fins (4 ± 0.42) and head region (2 ± 0.41).  Once larvae recruitment had ceased (48 h), there were significantly higher mean post-larvae counts on the head (5 ± 3.4) and fins (12 ± 3) compared to previous time intervals.  *Neobenedenia* sp. aggregated on the eyes, fins, and dorsal and ventral extremities on the main body. As parasites neared sexual maturity, there was a marked aggregation on the fins (22 ± 2.35) compared to the head (4 ± 0.97) and body (9 ± 1.33), indicating that *Neobenedenia* sp. may form mating aggregations.

*Keywords*: Monogenea, *Neobenedenia*, Capsalidae, development, migration, microhabitat, fluorescent labelling

# 1. Introduction

The distribution of ectoparasites on their hosts is linked to adaptive strategies and life traits inherent to their evolution (Rohde, 2005). Parasite distribution and site-specificity have been associated with particular feeding guilds and diets (Marcogliese, 2002; Karvonen et al., 2007), mate finding habits (Chisholm et al., 1997; Chigazaki et al., 2000; Whittington and Ernst, 2002), evasion of the host immune system and toxic compounds (Buchmann and Lindenstrøm, 2002; Sitjà-Bobadilla, 2008), camouflage and evasion of predators (Whittington, 1996), and avoidance of intra and interspecific competition (Rohde et al., 1995). Many ectoparasitic monogeneans are able to migrate over the body surface of their host and gain access to select microhabitats which are subsequently colonised (Cone and Burt, 1981; Whittington and Ernst, 2002) and where sexual maturity is reached (Kearn, 1984; Kearn and Whittington, 1992; Whittington and Ernst, 2002). Consequently, some host microhabitats exhibit greater ectoparasite loads and have increased susceptibility to epidermal damage and subsequent secondary infection (Kaneko et al., 1988; Thoney and Hargis, 1991; Buchmann and Lindenstrøm, 2002; Leong and Colorni, 2002).

Capsalid monogeneans are harmful ectoparasites of ornamental and farmed fishes in tropical and subtropical marine environments (Thoney and Hargis, 1991; Deveney et al., 2001; Hirazawa et al., 2011; Hutson et al., 2012; Whittington, 2012). Within this group, *Neobenedenia* is comprised of particularly virulent species that exhibit low host specificity, a direct life cycle, high fecundity and environmentally resilient eggs (Bullard et al., 2000; Whittington, 2004; Ogawa et al., 2006; Militz et al., 2013; Dinh Hoai and Hutson, 2014). *Neobenedenia* spp. have been observed attached to all external surfaces of the host including the nostrils, eyes, mouth cavity and fins (Whittington, 1996; Ogawa et al., 2006; Hirazawa et al., 2011; Trujillo-González et al., 2014). The invasion route and site-selection of *Neobenedenia girellae* (Hargis)(see Whittington and Horton (1996) for an account of its likely synonymy with *N. melleni*) has been previously described on Japanese flounder, *Paralichthys olivaceus* (Temminck and Schlegel)(see Bondad-Reantaso et al., 1995) and quantified on amberjack, *Seriola dumerili* (Risso)(see Hirayama et al., 2009). In both

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studies, post-larvae were found attached to the fins, while older parasites were found on the dorsal and ventral body surfaces. These studies used skin scrapings (Bondad-Reantaso et al., 1995) and stereomicroscopy (Hirayama et al., 2009) to detect live parasites.

The cryptic nature of *Neobenedenia* spp. makes live parasites extremely difficult to observe. Juveniles are small in size and may be transparent or have pigments that serve as camouflage when attached to the host (Whittington, 1996). Fluorescent labelling is a useful tool to examine the infection biology of parasites and has been previously used to describe the invasion route and site-selection of monogeneans (i.e. *Branchotenthes octohamatus* (Glennon, Chisholm and Whittington) on elasmobranchs (Glennon at al., 2007) and *Heterobothrium okamotoi* (Ogawa) on tiger puffer fish, *Takifugu rupribes* (Temminck and Schlegel)(see Chigazaki et al., 2000)) and actinospores in salmonid and cyprinid species (Yokoyama and Urawa, 1997). The aim of this study was to examine *Neobenedenia* sp. patterns of recruitment and parasite aggregation over a spatial-temporal scale on the body surface of barramundi, *Lates calcarifer* (Bloch). We used fluorescent labelling to examine monogenean distribution patterns over a prolonged period of time to account for potential differences in post-larval, juvenile and adult parasite distribution.

## 2. Materials and methods

### 2.1. Source of fish and Neobenedenia *sp.*

Fifty hatchery reared *Lates calcarifer* (150 ±30 $L_T$ mm) were maintained in 100 L fresh water aquaria at the Marine Parasitology Laboratory, James Cook University. Fish had not been previously exposed to *Neobenedenia.* Fish were acclimated to sea water 24 h prior to experiments by increasing salinity to 10, 20, 30 and 35 ppt over 2 h intervals. Fish were fed until satiation every two days (~one gram per fish) with pellets formulated for *L. calcarifer* (Ridley Aqua-Feed™). Parasite eggs were sourced from an experimental infection in the laboratory, which was established using methods previously described (Militz et al., 2013). *Neobenedenia* sp.

investigated in this study is presently unidentified given the absence of diagnostic criteria to differentiate between geographical/host isolates and species (Whittington, 2004; Whittington, 2012). Phylogenetic analysis of approximately 12 *Neobenedenia* spp. isolates collected from multiple fish hosts in northern Australia is ongoing and may provide species level-clarification (Brazenor, unpublished data). Meanwhile, representative specimens mounted on slides were accessioned in the South Australian Museum, Australia (SAMA) in the Australian Helminth Collection (AHC); SAMA AHC 35461 (see Hutson et al., 2012). Parasite eggs were collected daily and held in Petri dishes with fresh sea water. Newly hatched oncomiracidia (<3 h old) were gently aspirated with a pipette and used in the experiments described below.

*2.2. Fluorescent labelling of* Neobenedenia *sp. oncomiracidia*

*Neobenedenia sp. oncomiracidia were labelled with a fluorescent marker to identify individual parasites on the fish body surface. A 10 mM stock solution of the fluorescent dye 5(6)-carboxyfluorescein diacetate N-succinimidyl ester (CFSE; Sigma-Aldrich, Castle Hill, NSW, Australia) was made by resuspending CFSE lyophilised powder in 100 % dimethyl sulphoxide (DMSO), and stored at 4 $^o$C in dark conditions until use. The stock solution was diluted with filtered sea water (35 ppt) to produce a 30 nM working solution of CFSE for labelling (modified from Glennon et al., 2007). Approximately 400 Neobenedenia sp. oncomiracidia were held for 15 min in dark conditions in a 50 mL beaker with 25 mL of sea water (35 ppt) and 5 mL of 30 nM CFSE working solution. Only swimming oncomiracidia were selected for the experiments.*

*2.3.* Neobenedenia *infection of* Lates calcarifer *over time*

Fish were infected with fluorescent oncomiracidia and examined at 10 different time intervals to determine parasite distribution on the host body surface over its development. Fifty *L. calcarifer* were each infected with 50 ± 3 CFSE-labelled oncomiracidia, and held in individual aquaria (20x15x15cm) in sea water (35 ppt; 25 ± 2.5 $^o$C). A pilot study showed that parasite sampling and detection on the fish body surface took an average of 30 min for each individual fish. Thus, to enable precisely

timed sampling, fish were infected over the course of five days, with ten randomly selected fish infected with labelled oncomiracidia each day. Each of the ten fish corresponded to one of ten time periods (15, 30, 60, 120 min, 24, 48, 96 h, eight, 12 and 16 d post-infection). Five replicates were made for each time period. Each fish was euthanised with a dose of Aqui-S aquatic anaesthetic (25 mL L$^{-1}$ for 15 min), which does not cause parasite detachment (Sharp et al., 2004; Trujillo-González et al., 2014). Immediately following euthanasia, each fish was placed under an epifluorescence stereomicroscope (Olympus BX51) and both sides of the body surface (alternating left hand side first) were carefully examined for live parasites (Fig. 1A). The gills, buccal folds, buccal cavity and nasal chamber were not examined. Parasite location was recorded using an XY coordinate system based on a gridded translucent sheet of plastic (25 dots/cm$^2$) placed over the fish. The tip of the mandible of each fish was placed on a marked location on the translucent grid to maintain a consistent coordinate origin. Scaled photographs were taken of each fish and of representative parasites attached to fish in each time period.

*2.4. Statistical analysis*

Infection success (total number of parasites on the host divided by the initial number of larvae introduced; Bush et al., 1997) was reported as a percentage and differences between time intervals were examined using a one-way ANOVA and a Tukey's HSD test in S-Plus 8.2. Parasite distribution on *L. calcarifer* was examined for complete spatial randomness (CSR) using R 3.1.0 for Windows. Parasites found on both sides of the fish were combined and parasites found underneath the pectoral fins (n = 3) were excluded for two dimensional parasite distribution analyses (Fig. 1A). Two different functions were used to test for spatial randomness including 1) origin to point neighbour distances (Ghat) and 2) point to point neighbour distances (Fhat). A complete spatial randomness simulation was then created based on a Monte Carlo test (Dhat), where Dhat = Ghat - Fhat. These three functions are used to test the assumptions of CSR (Diggle, 1983; Rowlingson and Diggle, 1993; Venables and Ripley, 2002). CSR was analysed using the "splancs" library in R 3.1.0 and Dhat values where ranked within 99 simulations of randomly distributed points. Complete spatial randomness was rejected when Dhat > 90 (modified from

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Rowlingson and Diggle, 1993). A contour plot was created to illustrate *Neobenedenia* sp. distribution on the body surface of the host using a kernel density analysis with ARCGIS 10.1. Terminology used to describe the fish body surface microhabitats is defined in Fig. 1A.
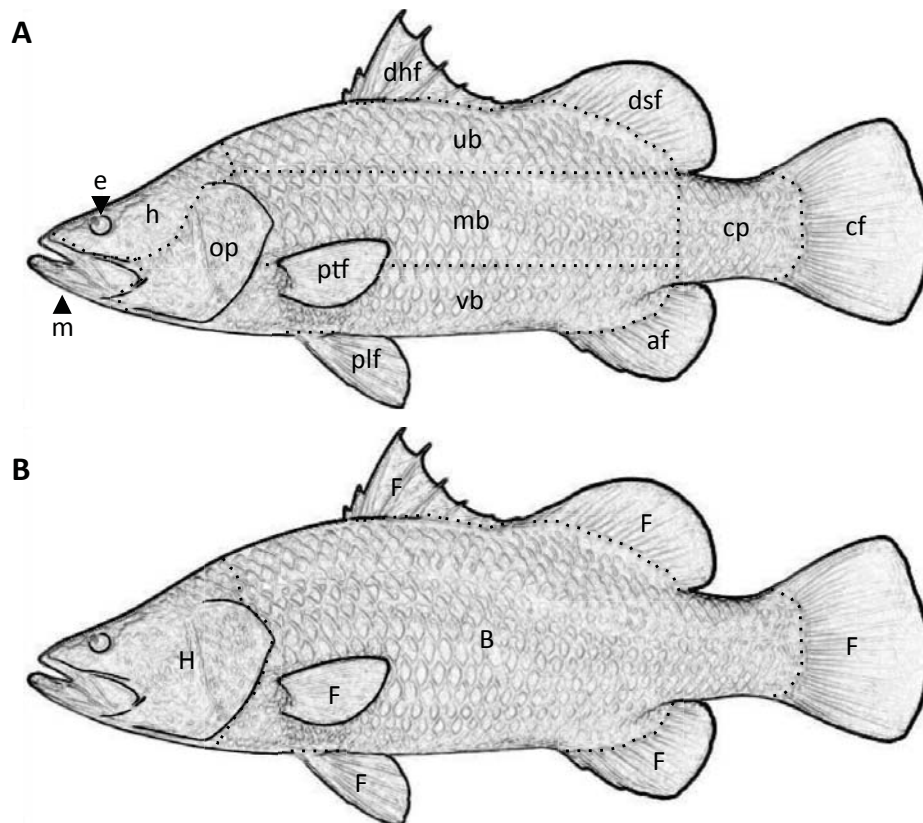


**Fig. 2.** *Lates calcarifer* microhabitat terminology (A) and body surface regions (B) used for statistical analysis. af= anal fin; cf=caudal fin; cp= caudal peduncle; dhf=dorsal hard fin; dsf=dorsal soft fin; e=eye; h=head; m= mandible; mb= middle body; op= operculum; plf=pelvic fin; ptf= pectoral fin; ub=upper body; vb=ventral body. B= body; F=fins; H= head. Terminology is based on Helfman et al.,, (2009) and Roberts & Ellis (2012).

Despite random allocation of fish to treatments, mean total length was higher in fish held in the eight and 12 day time periods (one-way ANOVA, $F_{9,38}=10.01$ p < 0.05). To account for fish size, parasite density was analysed with a kernel spatial point analysis, using parasite coordinates to compare mean number of parasites per standardised unit of measure$^2$. Parasite counts collected from fish at each time period (five fish per time period), were compared between three discrete regions on the fish: the head, the body and the fins (Fig. 1B). The number of parasites in each

region was obtained by pooling parasite counts obtained from the coordinate data following the boundaries shown in Fig. 1B. In order to meet the assumptions of ANOVA a square root transformation was performed. Transformed mean parasite counts were compared between fish regions using one-way ANOVAs within each time period, and a two-way ANOVA to compare parasite counts between time periods with S-Plus 8.2.

## 3. Results

The fluorescent marker enabled rapid and accurate inspection of the host body surface for the presence of small, newly settled post-larvae, including instances where parasites had lodged underneath fish scales (Fig. 2). The CFSE fluorescent signal emitted by the parasite was maintained throughout development, although the signal became weaker over time (Fig. 3). Parasites attached to the host using the haptor as an anchor point with the anterior end "tapping" the host's surface in the proximity of the parasite's total length. Parasites were occasionally observed to crawl over the body surface (as per Yoshinaga et al., 2000; Kearn, 2004).
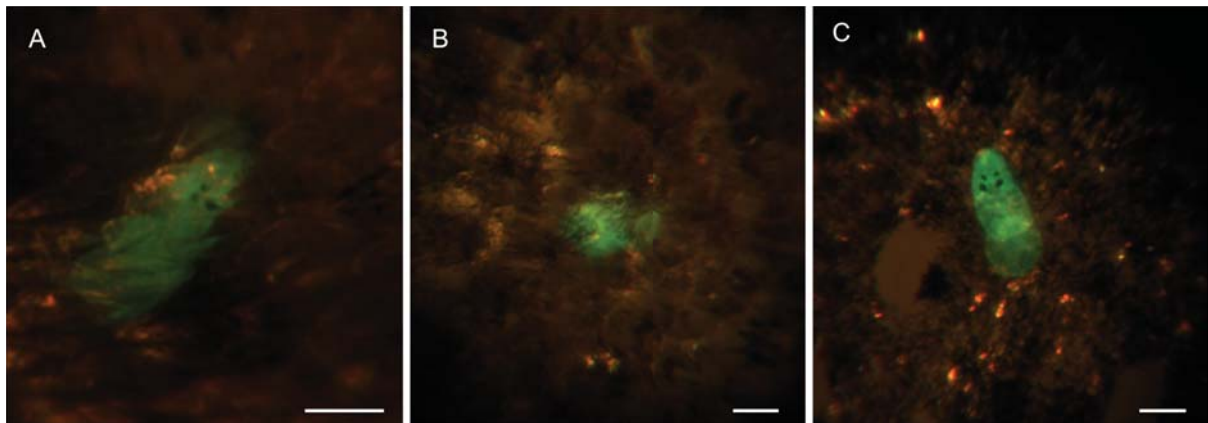


**Fig. 3.** Live fluorescent *Neobenedenia* sp. juveniles attached beneath the scales of *Lates calcarifer* (A, B) and attached to the surface of the fish scales (C). Parasites are 1 h old (A, B) and 2 h old (C). Scale bar = 100 μm.
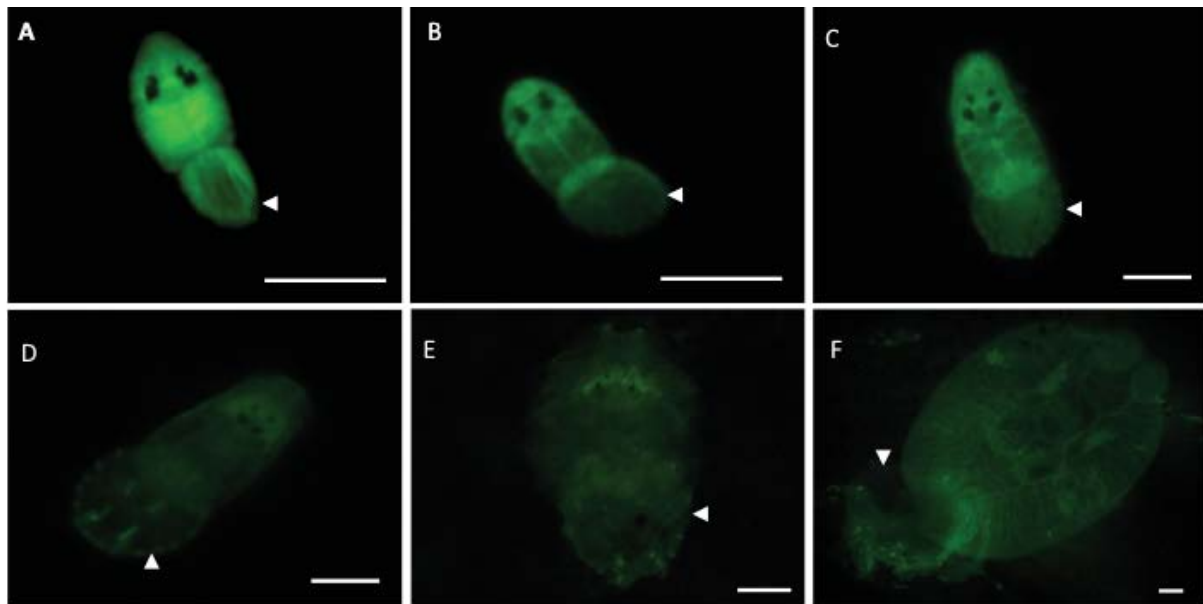
**Fig. 4.** Live fluorescent *Neobenedenia* sp. attached to *Lates calcarifer* over time. Parasites observed attached to fish following 15 min (A), 30 min (B), 2 h (C), 48 h (D), 96 h (E) and 16 d (F) post-infection. Arrow shows the haptor of *Neobenedenia* sp.. A slightly higher exposure was used when photographing parasites at 16 days post-infection to account for faded fluorescence. Scale bar = 100 μm.

*Neobenedenia* sp. infection success increased through time, before gradually decreasing between day four and day 16. *Neobenedenia* sp. oncomiracidia used in this study live for an average of 37 ± 3 h in the absence of a host (at 35 ppt, 25°C; Militz et al., 2013; Brazenor and Hutson, 2015). This indicates that the majority of viable oncomiracidia had successfully recruited to the host in the first 48 h of this study as shown by the peak in infection success (Fig. 4). Twenty ± 2.5 % of oncomiracidia had attached to the host within 15 mins, and 32 ± 5 %, 45 ± 3 %, 45 ± 9 % and 52 ± 9 % attached by 30 mins 1, 2 and 24 h, respectively (Fig. 4). Infection success peaked at 93 %, two days post-exposure, before gradually decreasing in subsequent time intervals.
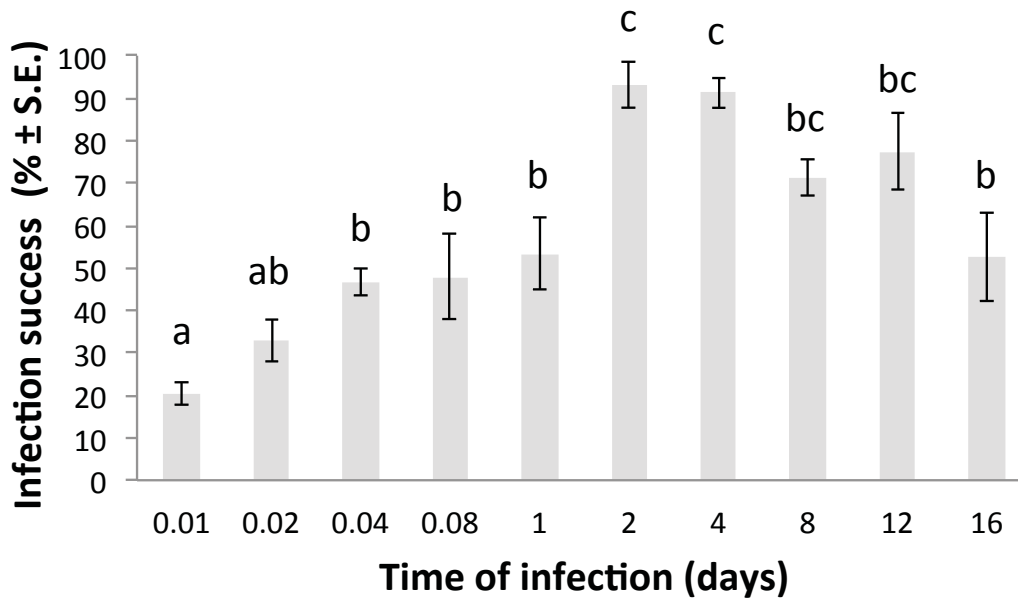
**Fig. 5.** *Neobenedenia* sp. mean infection success on *Lates calcarifer* over time. 'a', 'b' and 'c' = differences between pairs of means determined using Tukey's HSD test, p <0.05.

Post-larvae randomly attached on the body surface of the host in the first 24 h (Fig. 5; Dhat<51). Parasites were aggregated between 48 h and 8 d post-exposure (Fig. 5; Dhat=100), and exhibited a random distribution after 12 d post-exposure (Fig. 5; Dhat<51). Between 24 h and 8 days post-exposure there were fewer parasites on the middle body surface, and more on the fins, eyes, operculum, and on the peripheral region of the upper and ventral body surfaces of the host (Fig. 5). Between 12 and 16 days, parasites were concentrated on the head, ventral body surface and fins of the host (Fig. 5). Overall, higher numbers of parasites were observed on the eyes, fins and peripheral areas of the upper and ventral body surface compared to the head and middle body of *L. calcarifer* (Fig. 5, all periods).
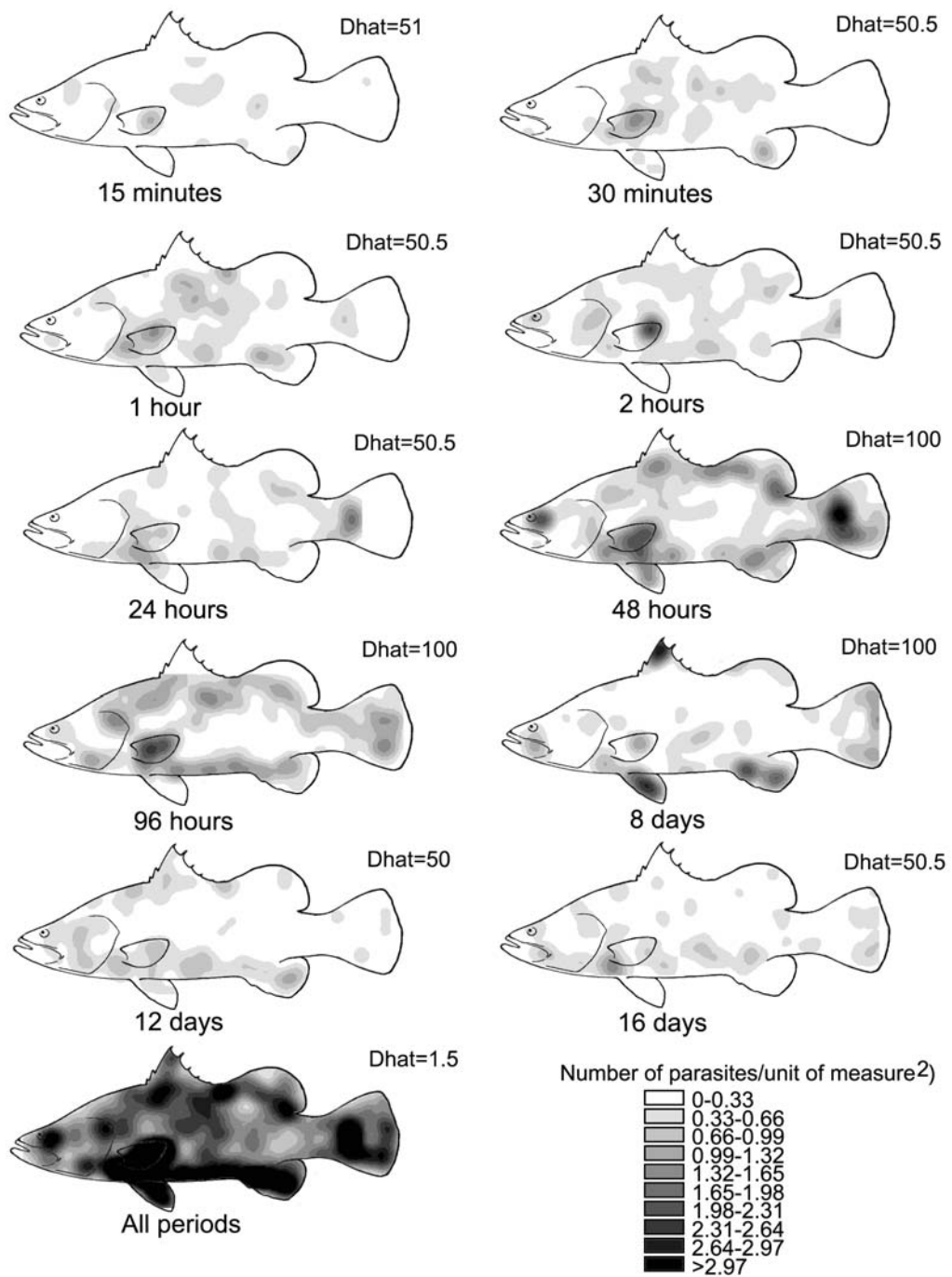
**Fig. 6.** *Neobenedenia* sp. distribution on the body surface of *Lates calcarifer* over time. A kernel spatial point analysis was used to estimate the number of parasites/unit of measure$^2$. Dhat values show the rank of the data within 99 simulations of randomly distributed points. Complete spatial randomness is rejected with values between 90 and 100.

Mean parasite counts were significantly higher on the body region compared to the head and fins of *L. calcarifer* in all time periods except day eight, where mean parasite counts were significantly higher on the fins (Fig. 6, one-way ANOVA, $F_{2,12}$= 34.29, p<0.01 ). Mean parasite counts on the head and fins remained low over the first 24h (Fig. 6A, C, two-way ANOVA, $F_{18,114}$= 10.02, p<0.01), and gradually increased on the body of the host over the first 2h of exposure (Fig. 6B). There was no significant difference in mean parasite counts within regions between 48h and 96h (Fig. 6). Parasite counts were significantly higher on the fins on day 8 (compared to all other time periods) and significantly lower on the body (compared to the five previous time periods) (Fig. 6, two-way ANOVA, $F_{18,114}$= 10.02, p<0.01). Between day 12 and 16 mean parasite counts decreased in all regions (Fig. 6, two-way ANOVA, $F_{18,114}$= 10.02, p<0.01).
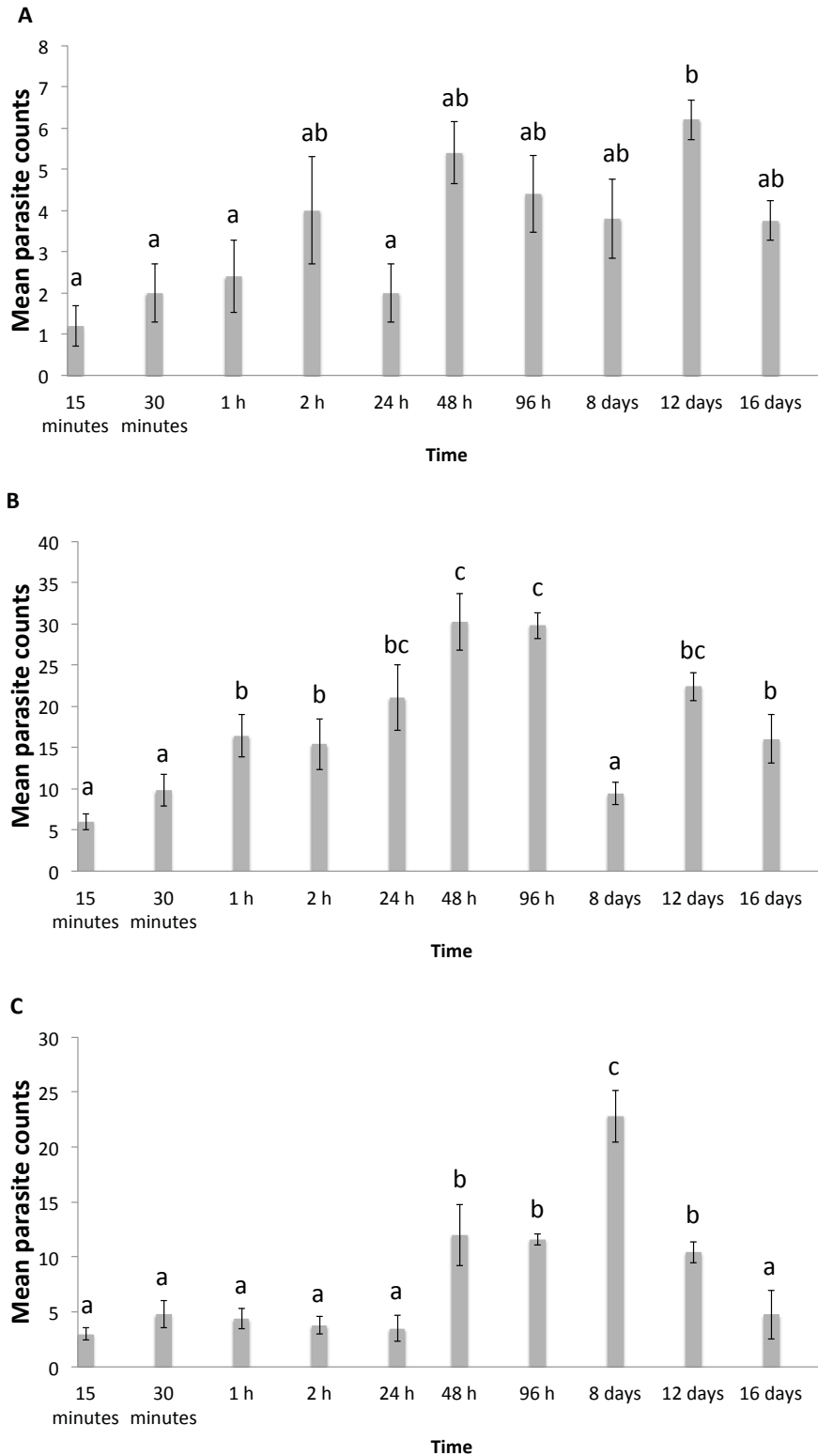
**Fig. 7.** Mean parasite counts of *Neobenedenia* sp. infecting the head (A), body (B) and fins (C) of *Lates calcarifer* over time. 'a', 'b' and 'c' = differences between pairs of means determined using Tukey's HSD test.

## 4. Discussion

*Neobenedenia* sp. settled opportunistically before migrating to preferred microhabitats. In the first 24 h of infection, *Neobenedenia* sp. larvae exhibited a random distribution on the body surface of the host (<24 h, Fig. 5) which indicates that oncomiracidia may not be especially selective of their microhabitat during recruitment, but could be influenced by the need to find a host and ensure transmission (Kearn and Whittington, 1992; Whittington and Ernst, 2002). Considerable aggregation of parasites between 48 h and 8 days indicates that the majority of parasites migrated to specific microhabitats on the host following attachment (Fig. 5). No differences in mean parasite counts within regions between 48 and 96 h indicates that there was no considerable movement of parasites during this time (Fig. 5, 6).

Random attachment of oncomiracidia, followed by migration of post-larvae to specific microhabitats, has been previously observed in monogeneans. Post-larvae of the gill parasite *Urocleidus adspectus* (Mueller) attach randomly on the body of yellow perch, *Perca flavescens* (Mitchill), prior to migration to the gills (Cone and Burt, 1981). *Entobdella soleae* (Lamarck) oncomiracidia attach on the upper surface of the common sole, *Solea solea* (Linnaeus), and migrate to the lower surface and posterior regions over time (Kearn, 1984). In the same manner, *Benedenia lutjani* (Whittington and Kearn) post-larvae attached to the body surface of the host and migrated to the pelvic fins (Whittington and Ernst, 2002). The random attachment of *Neobenedenia* sp. observed in this study differs to that previously observed for *N. girellae* (see Whittington and Horton (1996) for an account of its likely synonymy with *N. melleni*) where oncomiracidia settled predominantly on the fins of host fish species (i.e. *P. olivaceus* and *S. dumerili*) and then migrated to the main body surface as they grew (Bondad-Reantaso et al., 1995; Hirayama et al., 2009).

The fluorescent marker revealed that *Neobenedenia* sp. can attach underneath fish scales. This is a well-known microhabitat for transversotrematid trematodes (Cribb et al., 2002) but is a relatively rare occurrence, or is poorly documented, for monogeneans. Monogenean post-larvae of *U. adspectus* and juveniles and adults of *Entobdella soleae* (Capsalidae) have been observed attached beneath the scales of their hosts (Cone and Burt, 1981; Kearn, 2004). In both

studies, parasites attached to the underside of the scales with the haptor, with the anterior region, including the eye spots, uncovered (Cone and Burt, 1981; Kearn, 2004). The ability of *Neobenedenia* sp. to attach beneath the scales (Fig. 2) may have evolved in response to predation by cleaner organisms. Furthermore, this microhabitat may enable the parasite to be almost entirely secluded from the environment and could reduce the efficiency of current parasite management methods (e.g. chemical and freshwater bathing) in aquaculture.

*Neobenedenia* sp. was found in multiple microhabitats but parasites were more frequently found on the eyes, fins, dorsal and ventral body surface. This observation is in accordance with Hirazawa et al. (2011) who observed higher numbers of *N. girellae* (see Whittington and Horton, 1996) on the pelvic fins and body surface compared to the head of *S. dumerili*. Other monogeneans display high microhabitat specificity. For instance, some benedeniines live exclusively on specific fins or microhabitats on the head region such as lip folds and branchiostegal membranes (Whittington, 1996). Preference for the eyes, pelvic fins, dorsal and ventral body surfaces could confer adaptive benefits including avoidance of predation, competition and localised immune responses of the host (Whittington, 1996; Jones, 2001; Whittington and Ernst, 2002). The fins of the fish for example, could increase protection against predators and provide distinct feeding grounds or spatial resources for each developmental cohort (Whittington, 1996; Whittington and Ernst, 2002).

*Neobenedenia* sp. aggregated on the fins within 24 h of sexual maturity. Parasites exhibited a random distribution 12 d post-infection on the body of the host. *Neobenedenia* sp. reach sexual maturity (i.e. begin to lay eggs) on day nine post-infection in the described experimental conditions (i.e. 25 °C, 35 ppt; Brazenor and Hutson, 2015). Aggregation on the fins observed on day eight may be a result of parasites seeking other individuals for mating (Fig. 5 day 8; Fig. 6C). Migration to preferred microhabitats at the onset of mating has been observed for the monogenean, *B. lutjani*, where development of the reproductive organs corresponded with migratory movements on the host (Whittington and Ernst, 2002). Although *Neobenedenia* sp. can reproduce in isolation and do not necessarily need to cross-fertilise in order to produce viable offspring (Dinh Hoai and Hutson, 2014), migration to the fins at the onset of sexual maturity as a mating strategy could

provide *Neobenedenia* sp. increased success of cross-insemination (Whittington and Kearn, 1993) or shared spermatophores between individuals (Ogawa et al., 2014). Aggregation on the fins may therefore confer advantages to find suitable mates and a disaggregated distribution after mating could be associated to *Neobenedenia* sp. egg production, its need to forage for resources (Whittington and Ernst, 2002), or a suitable location to disperse eggs (Whittington, 1996).

This study provides compelling evidence that ciliated *Neobenedenia* sp. larvae settled opportunistically and then migrated in search of specific microhabitats. Selected microhabitats included the eyes, fins, upper body and ventral body surfaces of the host. Reproduction could be an important factor determining *Neobenedenia* sp. distribution, indicated by parasites aggregating on the fins within 24 h of attaining sexual maturity. The fluorescent signal used in this study revealed that *Neobenedenia* sp. can attach underneath the scales of fish which could impact treatment efficacy in aquaculture.

# CHAPTER 3 PUBLICATION STATEMENT

Chapter 3 was published in the *Journal of Fish Diseases* in 2014 as follows:

**Trujillo-González A**., Constantinoiu C.C., Johnson L.K. & Hutson K.S. (2014) Histopathology associated with haptor attachment of the ectoparasitic monogenean *Neobenedenia* sp. (Capsalidae) to Asian sea bass, *Lates calcarifer* (Bloch). *Journal of Fish Diseases* doi:10.1111/jfd.12320.

**Histopathology associated with haptor attachment of the ectoparasitic monogenean *Neobenedenia* sp. (Capsalidae) to barramundi, *Lates calcarifer* (Bloch)**

**Abstract**

Epithelial histopathology of farmed barramundi, *Lates calcarifer* (Bloch) infected with the monogenean ectoparasite, *Neobenedenia* sp. (Yamaguti), was examined by light microscopy in six regions. Tissue samples were collected from the eyes, mandible, operculum, middle body, ventral body and caudal fins at 20 days post-infection. Histopathology analysis was restricted to light microscopy slides depicting a physical interface between the haptor and the host. Epidermal thickness and the number of mucous cells were quantified beneath the haptor, epidermis adjacent to the haptor (internal control) and on equivalent areas of uninfected fish. Dermal inflammation and epithelial loss was observed in the majority of infected regions. The haptor caused considerable mechanical compression of the epidermis and epidermal damage including detachment of the basal layer and rupture of epidermal cells. Rarely was the haptor directly associated with haemorrhage or epidermal inflammation and there was no evidence of ulceration in infected fish. Infected fish exhibited significantly lower epidermal thickness ($p<0.001$) on the mandible, operculum, mid-body and ventral body compared to uninfected fish. In all regions, fewer mucous cells ($p<0.001$) were present in infected fish. These data suggest that the haptor of the parasite is associated with mechanical damage to fish epidermis.

**Introduction**

Capsalid monogeneans are harmful ectoparasites of ornamental and farmed fishes in tropical/subtropical marine environments (Thoney & Hargis 1991; Hirazawa et al., 2011; Whittington 2012). *Neobenedenia* spp. have low host specificity, a direct life cycle, high fecundity, and robust eggs, which contribute to their ability to inflict mass mortalities in aquaculture (Ogawa et al.,1995; Deveney et al., 2001; Rückert et al., 2008; Whittington 2012).

*Neobenedenia* spp. attach to external surfaces of their host using two attachment organs located anteriorly, and one larger posterior attachment organ called the haptor. The haptor is believed to act as the principal anchoring organ of the parasite to the host (Whittington 2012). This organ has chitinous structures that provide mechanical attachment including paired anterior hamuli, accessory sclerites, posterior hamuli and peripheral hooklets (Whittington & Horton 1996). A marginal valve on the haptor allows the organ to create suction on the host. The anterior attachment organs lack accessory chitinous structures and are located directly above the pharynx, which is used to graze on epidermal and mucous cells of the fish (Whittington 2012).

The strong adhesion of the haptor, mechanical attachment of the hamuli and sclerites (Ogawa, Miyamoto, Wang, Lo & Kou 2006), as well as the adhesion of the anterior attachment organs when grazing, can damage the host's epidermis (Kaneko et al.,1988; Ogawa et al., 2006; Hirazawa et al., 2010), increasing the likelihood of secondary infections (Thoney & Hargis 1991; Leong & Colorni 2002). Lesion-level histopathology sections that exhibit the haptor-host interface are rare and examinations of the change in epidermal morphology associated with infected fish are limited. In this study, we examined epidermal damage associated with the site of haptor attachment in six separate regions of farmed barramundi. Characteristics evaluated in *Neobenedenia* sp. infected fish included changes in epidermal thickness and in the number of mucous cells compared to similar areas in uninfected controls.

Forty hatchery reared freshwater *L. calcarifer* (mean 125 ± 25 $L_T$ mm) were acclimated to sea water for 24h prior to 20 fish each being infected with 20 *Neobenedenia* sp. oncomiracidia each (see Hutson et al., 2012; James Cook University ethics approval no. A1857). The remaining 20 fish were not infected. The *Neobenedenia* sp. used in this study is presently unidentified given the absence of diagnostic criteria to differentiate between geographical/host isolates and species (Whittington 2004; Whittington 2012). Parasites were accessioned in the Australian Helminth Collection (AHC), South Australian Museum Australia (SAMA: AHC 35461). Twenty-one days post-infection all 40 fish were killed with an overdose of

Aqui-S aquatic anaesthetic in sea water, which did not kill parasites or cause them to detach from the host (see Sharp et al., 2004). Each fish was then immersed in a shallow tray containing sea water (35 gL-1), and tissue samples bearing an individual live parasite were collected from infected fish using a surgical scalpel blade. Tissue samples were collected from the mandible, operculum, middle body, ventral body and caudal fins. Samples were approximately 1cm$^2$ and consistently collected from equivalent locations within regions on uninfected and infected fishes. Eyes were collected whole. Samples were fixed in 1% Bouin's solution for 48 h prior to being routinely processed, paraffin embedded, and 4 µm sections stained with haematoxylin and eosin  (Gibson-Kueh et al., 2012). Sections were made so that the interface between the haptor and the host body surface could be observed by light microscopy. Some parasites detached during the fixation and embedding process, which limited the total number of samples available for analysis (Table 1). Parasites detached from all eye preparations and were not included in the analyses.

**Table 1.** Total number of sections examined for histopathology, epithelial thickness and mucous cell counts for each region.

| Region | Condition | No.  fish | No. skin sections examined |
|--------|-----------|-----------|----------------------------|
| Mandible | Uninfected | 6 | 6 |
| | Infected | 4 | 5 |
| Operculum | Uninfected | 4 | 5 |
| | Infected | 6 | 6 |
| Middle body | Uninfected | 7 | 8 |
| | Infected | 5 | 5 |
| Ventral body | Uninfected | 5 | 5 |
| | Infected | 6 | 6 |
| Caudal fin | Uninfected | 2 | 2 |
| | Infected | 6 | 6 |

Epidermal thickness and the number of mucous cells were quantified beneath the haptor, the epidermis adjacent to the haptor (internal control) and on equivalent epidermis from uninfected fish (Table 1). Epidermal thickness was measured from the basal epithelial layer to the external or apical layer and mucous cells were

counted in four selected microscopic fields along the epidermal layer in each tissue sample at 400x magnification. In the case of infected fish, microscopic fields were selected within the haptor-host interface, and haphazardly on epidermis adjacent to the haptor (internal control). Statistical analysis was performed using S-plus 8 from Spotfire®. One-way ANOVA's were used to analyse differences in epidermal morphology between uninfected and infected fish within each region. Differences between pairs of means were determined using a Tukey's HSD test. A Chi$^2$ contingency test was used to examine differences in the number of mucous cells. Significance was accepted at $p<0.05$.

The haptor of *Neobenedenia* sp. caused direct mechanical damage to the fish epidermis and triggered epidermal morphological changes. Dermal inflammation (Fig. 8D) and epidermal loss (Fig. 8C, D) was observed in the majority of infected regions but was absent in uninfected controls (Fig. 8A, B). The marginal valve of the haptor caused mechanical compression of the epidermis (Fig. 9A, B, D) and may have contributed to detachment of the basal layer (Fig. 9C). Accessory sclerites ruptured the epidermal layer (Fig. 9B, inset). Rarely was the haptor directly associated with haemorrhage or epidermal inflammation (Fig. 8D, Fig. 9D, inset) and there was no evidence of ulceration in infected fish.
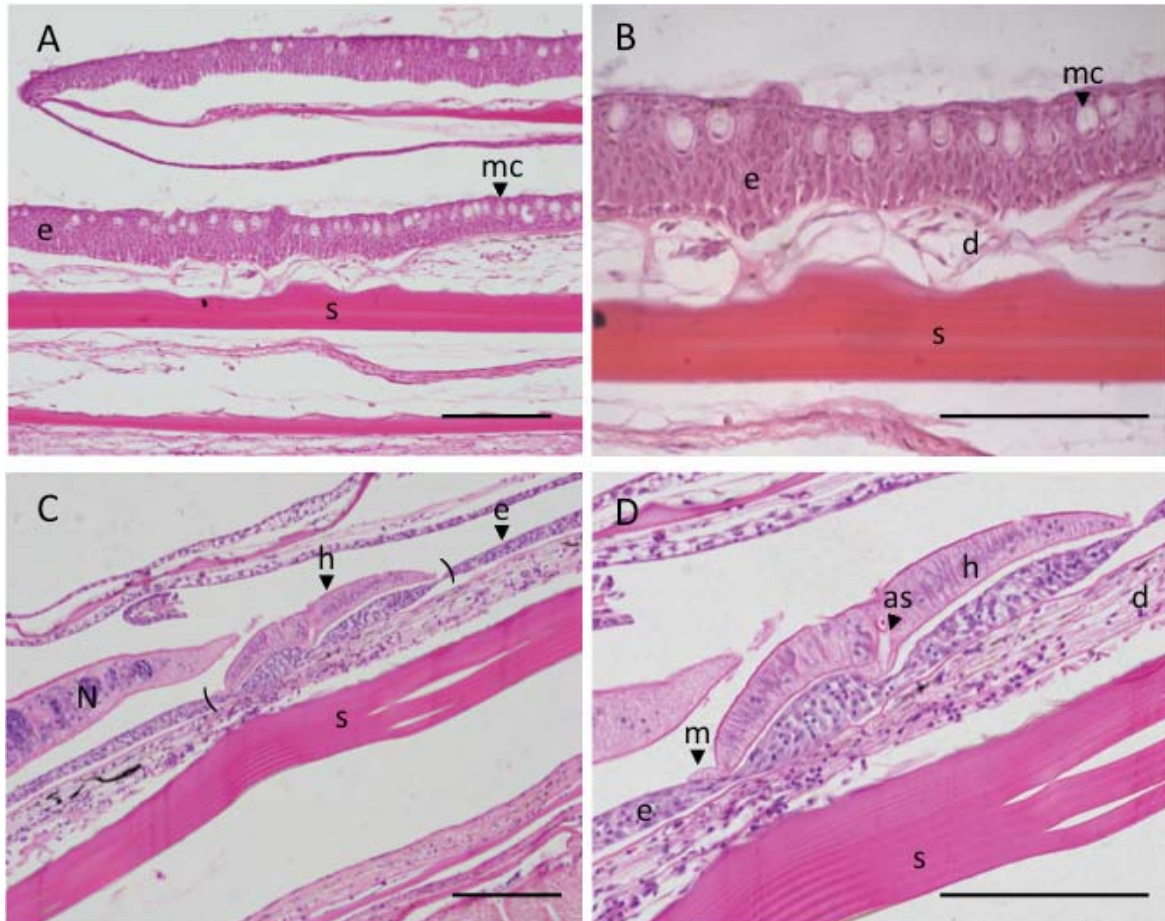
**Fig. 8.** Histopathology of the ventral body surface in uninfected (A, B) and infected *Lates calcarifer* with *Neobenedenia* sp. (C, D). Uninfected fish had numerous mucous cells in the epidermis (A, B). The parasite's haptor compressed the epidermal layer of the host (C, brackets). Infected fish presented thinner epidermis and mucous cells were rare at the haptor/host interface (C, D). N = *Neobenedenia*; as = accessory sclerites; d = dermis; e = stratified squamous epithelial cells; h = haptor; m = marginal valve; mc = mucous cells; s = scale. Morphological terms follow Whittington & Horton (1996) and epidermal morphology follows Takashima & Hibiya (1995). (H&E stain, A, C = x200; B,C = x400), scale bars = 100 μm.
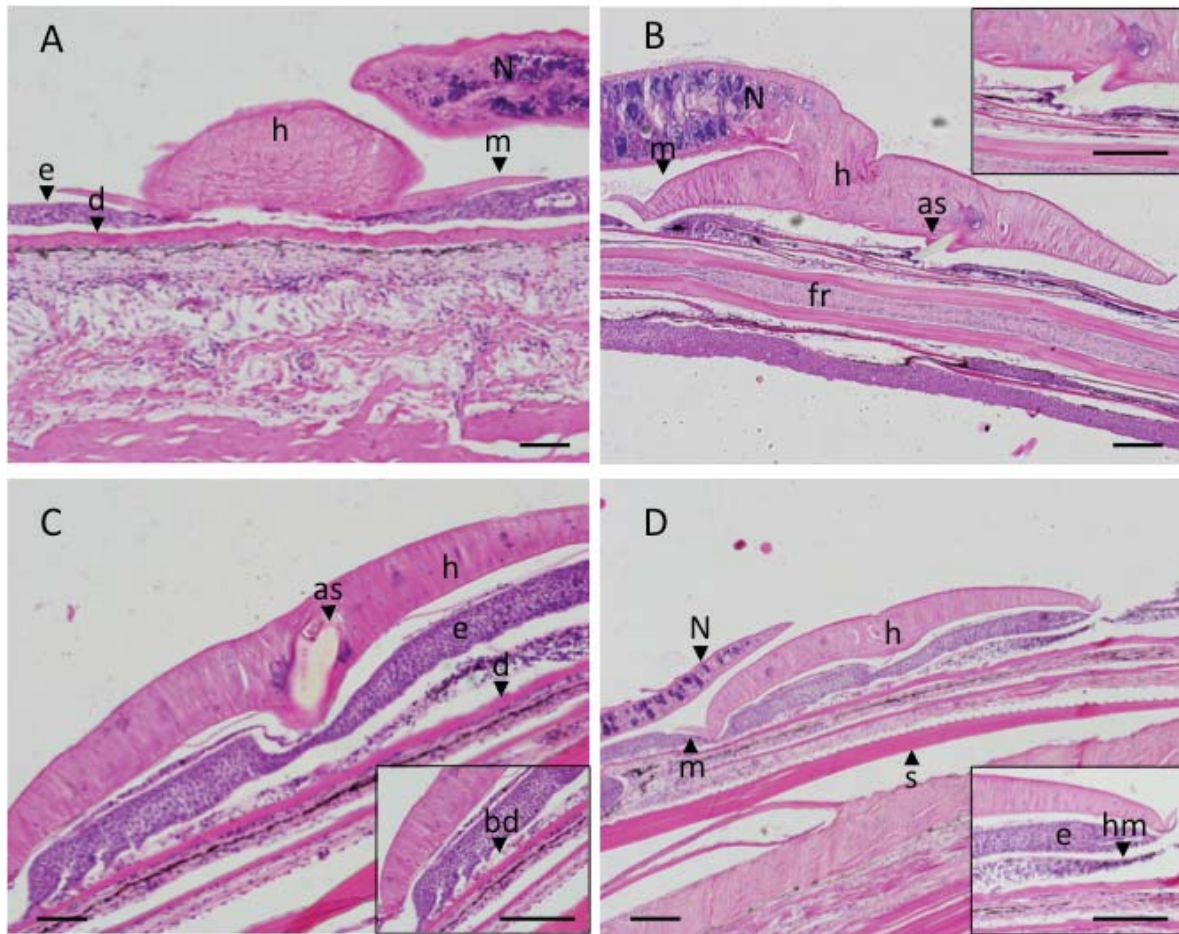
**Fig. 9.** Histopathology associated with haptor attachment of *Neobenedenia* sp. to *Lates calcarifer* on the middle body (A, D), caudal fin (B) and ventral body surface (C) of infected fish. Epithelial loss was observed in the majority of samples at the haptor/host interface (AD). Accessory sclerites caused epidermal damage (B, B inset, C) and the marginal valve compressed the epidermis of the host (A, B, D). N= *Neobenedenia*; as = accessory sclerite; bd = epithelial basal layer (detached); d= dermis; e = stratified squamous epithelial cells; fr = fin ray; h = haptor; hm = haemorrhage; m = marginal valve; s = scale. Parasite terminology follows Whittington & Horton (1996) and epidermal morphology follows Takashima & Hibiya (1995). (H&E stain, x100, insets = x400), scale bars= 100 μm.

Infected fish had significantly lower mucous cell counts in all regions compared to uninfected fish (p<0.001, Fig. 10A). Infected fish exhibited a trend for lower epidermal thickness in all regions compared to uninfected controls (Fig. 10B), which is congruent with previous studies (Hirayama, Kawano & Hirazawa 2009). Internal controls indicated that morphological changes were not limited to areas under the

haptor, although the greatest impact was quantified at the site of haptor attachment (Fig. 10). This surrounding damage could be a consequence of the feeding activity of the parasite. *Neobenedenia* spp. may use the haptor as a fixed rotation point using the anterior attachment organs to aid feeding within the radius of the total body length. Furthermore, movement of capsalid monogeneans over the body surface of the host may also account for the observed surrounding damage (Whittington & Ernst 2002; Ogawa *et al.,* 2006). *Neobenedenia* spp. are believed to migrate following initial recruitment on the host (Ogawa *et al.,* 2006; Hirayama *et al.,* 2009; Hirazawa *et al.,* 2011), however, it is challenging to track migrations through time due to their small size and cryptic nature (*Neobenedenia* spp. have transparent bodies; Whittington 1996). The extent to which monogeneans remained anchored to a single location on the host is unclear, however, low standard error observed within infected regions (Fig. 10) indicates that consistent damage occurred over the experimental period.
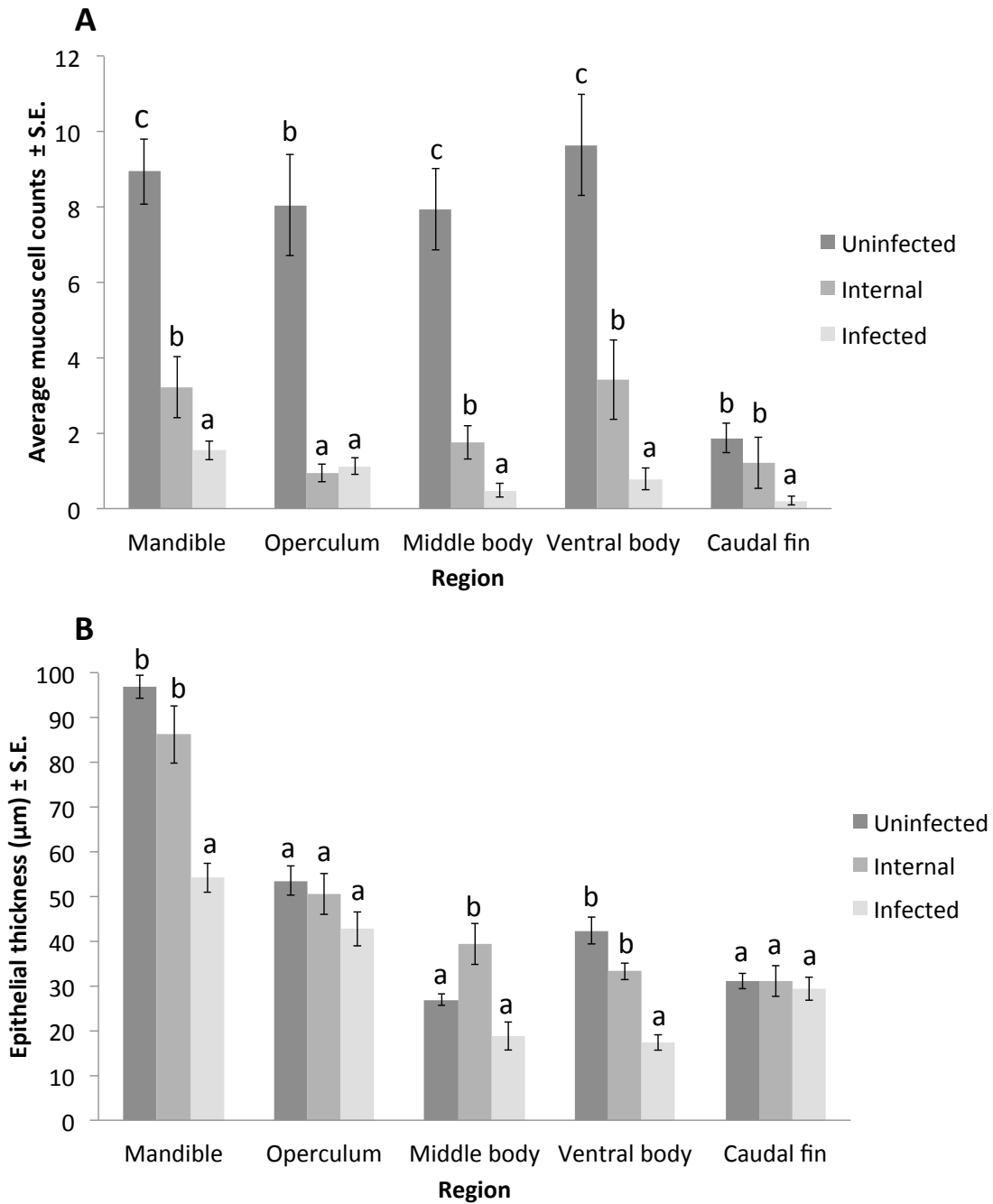
**Fig. 10.** Average mucous cell count (A) and epidermal thickness (B) of sampled regions on infected and uninfected *Lates calcarifer*. Internal controls were taken from comparable locations adjacent to the haptor/host interface. 'a', 'b' and 'c' = differences between pairs of means determined using Tukey's HSD test in (A), and differences in count proportions using a chi-square contingency test in (B).

Epidermal damage can impair the host's immune response to external pathogens. Cutaneous mucus, secreted by mucous cells present in the epidermis, is an important component of teleost immune responses and is considered the first line of defense against infection through skin epidermis (Zhao et al., 2008). A significant drop in the number of cutaneous mucous cells (Fig. 10A) could affect the fish's ability to withstand other opportunistic pathogens (Bonga 1997; Subramanian et al., 2007; Zhao et al., 2008). Teleost epidermis is a metabolically active tissue; significant epidermal damage can affect ion and thermal regulation, sensory perception, and locomotion (Elliott 2000). Reduced epidermal thickness and diminished numbers of mucous cells, associated with parasite attachment could impair the host's metabolic and regulatory processes and expose the host to other opportunistic pathogens.

# CHAPTER 4 PUBLICATION STATEMENT

Chapter 4 is currently in preparation for submission to the *Journal of Fish and Shellfish Immunology* as follows:

**Trujillo-González A**., Constantinoiu C.C. & Hutson K.S. (in prep) Antibody response and acquired immunity of barramundi, *Lates calcarifer* (Bloch) to infection by an ectoparasitic monogenean, *Neobenedenia* sp. intended journal: *Journal of Fish and Shellfish Immunology*

# CHAPTER 4

**Antibody response and acquired immunity of barramundi, *Lates calcarifer* (Bloch) to infection by an ectoparasitic monogenean, *Neobenedenia* sp.**

## Abstract

Acquired resistance of farmed barramundi, *Lates calcarifer* (Bloch) infected with the monogenean, *Neobenedenia* sp., was examined over four consecutive controlled infection events. Fish were infected with approximately 50 parasites for a period of ten days followed by a 14 day resting period. Fish were weighed, measured, and mucus and blood samples were taken before and after each infection event. Parasites were collected from each fish immediately following each infection event, measured and examined for evidence of sexual maturity. Parasite infection success was above 50% for all four infections with the exception of the third infection event, where infection success was halved (24 ±3.88%). Parasites were smaller in size following the first infection ($p < 0.01$), but parasite sexual maturity was not affected by the host acquired resistance. Acquired resistance could have potentially reduced *Neobenedenia* sp. body size, but there was no detected significant difference between the levels of specific Immunoglobulin M between infected and uninfected fish.

Keywords: *Neobenedenia*, Capsalidae, acquired resistance, immunity, Asian sea bass

## Introduction

The immune system detects and protects an organism from a wide range of external microbes, viruses and parasites. All vertebrate gnathostomes for instance, share an essential immune structure characterised by: (i) a highly conserved innate immunity/immune response, (ii) consistent development of a combined immune

system, and (iii) bilateral communication between components of the innate and adaptive immunity (Rauta et al., 2012). Teleost fish are the earliest vertebrates known to develop an adaptive immune response, and are considered the first vertebrates to have developed an adaptive immune system (Whyte 2007; Rauta et al., 2012).

Teleost fish rely on their skin and mucosal surfaces as an external protective barrier against a continuous flow of microbes and stressors (Whyte 2007; Delamare-Deboutteville et al., 2006). However, skin-feeding ectoparasites have developed adaptive strategies to avoid the host immune response and colonize the body surface of the teleost host (Sitjà-Bobadilla, 2008). Monogenean skin flukes are important parasites known to infect a wide range of fishes and cause skin lesions that increase the risk of secondary infections and mortality (Leong & Colorni, 2002). Within the Monogenea, *Neobenedenia* spp. are known to infect a wide range of hosts (Whittington 1996; Bullard et al., 2000), although susceptibility to *Neobenedenia* can vary amongst teleost hosts (Ohno et al., 2009; Rubio-Godoy et al., 2011). Some teleost fish exposed to *Neobenedenia* have been reported to develop immune (Hatanaka et al., 2005) and morphological changes that could convey acquired protection to subsequent infections (Bondad-Reantaso et al., 1995; Ohno et al., 2009; Hirayama et al., 2009; Hirazawa et al., 2011). Continuous exposure to infections by *Neobenedenia* could stimulate the development of protective immunity associated with increased anti-parasitic mucus and serum antibodies in the fish host (Jones 2001; Magnadóttir 2006). Nonetheless, there are no studies evaluating if *L. calcarifer*, an important aquaculture species that is highly susceptible to *Neobenedenia* infections, develops a protective immune response following repetitive exposure to parasites.

Compared to higher vertebrates, teleost fish possess a limited repertoire of antibodies involved in innate and adaptive immune responses (Brandtzaeg 2009; Salinas et al., 2011). Within this repertoire, immunoglobulin M (IgM) has been widely studied and used as an indicator of immune responses across a wide range of species (Salinas et al., 2011; Gautam & Loh 2011) including *L. calcarifer* (Bryant et al., 1999). Specific IgM can be quantified using different serological techniques (e.g. ELISA, immunofluorescence, precipitation) and to accurately analyse antigen-

specific IgM concentration (Kim et al., 2007; Gautam & Loh 2011). The aim of this study was to understand if *Lates calcarifer* acquire resistance when exposed to consecutive infections of *Neobenedenia* sp.. For this purpose, we quantified *Lates calcarifer* (Bloch) IgM specific levels against *Neobenedenia* sp. with ELISA following each of four consecutive infections with *Neobenedenia* sp.. Potential impacts of host acquired immunity on parasite development were assessed by measuring parasite size and examining parasite reproductive status.

**Methodology**

Differences in acquired resistance and antibody response were investigated in fish exposed to *Neobenedenia* sp. in four experimental infection events as detailed below. Before and after each event, infected and uninfected (control) mucus and blood samples were taken from each fish.

*2.1 Baseline parameters prior to infection*

Prior to the first experimental infection all fish (treatment and controls) were sampled to establish a baseline IgM in mucus and sera. A total of forty naïve *L. calcarifer* (total length ($L_T$) 170 ± 14mm) was purchased from a local fish hatchery and acclimated to the laboratory for 10 days. Each fish was individually anaesthetised with a dose of Aqui-S aquatic anaesthetic (25 mL $L^{-1}$) in sea water (35ppt) for five minutes (as per manufacturer's instructions). Following anaesthesia, each fish was weighed, measured and mucus/blood samples were collected. Fish were held in individual, numbered aquaria in sea water (35 ppt; 25 $^{\circ}$C) within two recirculating systems fitted with a biological filter. Fish were then left to recover for a period of five days prior to infection (see 2.2 below). This period of time allowed mucus on the infected fish to replenish and avoid increased stress and mortality (Yamamoto et al. 2011).

*2.2 First experimental infection*

Twenty fish were haphazardly assigned to treatment (infection) and control (uninfected) and individual fish tanks were numbered in order to monitor individual fish throughout the experiment. Infected fish and uninfected fish were maintained in separate recirculating systems to minimise the potential of contamination of control fish with parasite eggs or oncomiracidia. For the first experimental infection, newly hatched oncomiracidia were introduced to separate aquaria holding an individual fish. A total of 20 fish was exposed to ≈50 oncomiracidia (<3 hours old) by gently pipetting the oncomiracidia into each aquarium. Aeration and water flow were turned off for one hour after the introduction of the parasite to facilitate infection (Hirazawa et al., 2010). Water quality was monitored daily, water changes done accordingly, and each fish was offered a ration of 0.5 g of fish pellets every two days (Aquafeed™). At day ten post-infection, fish were transferred to individual buckets containing freshwater for five minutes, which kills and detaches *Neobenedenia* sp. (Militz et al., 2013). Fish were also gently massaged in the water to ensure removal of any remaining attached parasites. Parasites were collected from each infected fish, counted to account for parasite infection success (total number of parasites on the host divided by the initial number of oncomiracidia introduced; Bush et al., 1997) and preserved in 70 % ethanol until further examination (see section 2.5). Ten parasites were collected haphazardly from each infected fish in each infection event at the end of the 10 day infection period for further analysis on parasite size and development (see section 2.6.).

Each fish was then anaesthetized with a dose of Aqui-S aquatic anaesthetic (25 mL L$^{-1}$) in saltwater (35ppt) for five minutes. Each fish was measured, weighed and placed ventral side up on a foam cradle, and mucus from the skin surface was collected by gently scraping the skin of the fish with the blunt edge of a scalpel. Following, 0.5 mL of blood was drawn from the caudal vein using a 23-gauge needle. Blood was allowed to clot and mucus allowed to settle (2h at room temperature and 24 h at 4 °C), and centrifuged for 40 min at 4 °C with 18 g. Supernatant was collected from mucus and blood samples of each fish, and stored at -20 °C. The same methods described above were carried out for uninfected control fish.

Infected and control fish were held in individual 10 L buckets with freshwater (i.e. not returned to the experimental system) for a period of 14 days to ensure they were not reinfected by *Neobenedenia*, which only occurs in saline environments.

During this time, fish were fed every two days and 80% water changes were done daily. The recirculating marine systems were drained, and each individual aquarium was cleaned with 60 °C water before preparing both systems for the second experimental infection. Following the 14 day rest in freshwater, all fish were individually anaesthetised, measured, weighed and sampled for mucus and blood (see 2.1). Following sampling, fish were returned to their corresponding individual aquaria with saltwater (35 ppt) within the recirculating system. Fish were allowed to recover for a period of five days prior to infection (see 2.3).

*2.3 Second experimental infection*

Fish were infected a second time by exposing all previously infected fish to ≈50 oncomiracidia (<3 hours old) (see 2.2). Fish were sampled for parasites 10 days post infection using the freshwater treatment as previously described (see 2.2). All control fish were handled in the same manner as infected fish. Fish were rested and the experimental system cleaned prior to subsequent infections (see 2.2).

*2.4 Third and fourth experimental infection*

Following the parasite counts on infected fish 10 days post infection of the second infection event, 11 fish had more than 50 parasites attached to them, indicating that there was a reinfection event in the system. The third experimental infection was carried out as per the previous two infections with the following additional precautions: 1. The recirculating marine systems were drained, each individual aquarium was cleaned with 60 °C water, and both systems were left empty until 24h before preparation for the third experimental infection. In order to account for the re-infection event of the second infection event, ten haphazardly chosen, previously uninfected fish were selected for infection, including all other previously exposed fish. Before infection, all previously infected fish and the selected uninfected fish were sampled as described in section 2.2, reinfected with ≈50 oncomiracidia (<3 hours old), and sampled for parasites 10 days post infection using the freshwater treatment and collection method described in section 2.2. All fish were then held in individual 10 L buckets for 14 days, both recirculating systems were cleaned, dried

and prepared for a fourth infection event. After the 14-day resting period, all fish were anaesthetised, sampled as in section 2.2., and previously infected fish were reinfected with ≈50 oncomiracidia (<3 hours old) for a period of ten days. Ten days post infection, all fish were sampled for parasites, blood and mucus as in section 2.2., and euthanized in an ice slurry. After the fourth infection event, nine fish had been infected for four different infection events, and seven were infected for two different infection events.

## 2.5. Neobenedenia *sp. measurements and assessment of reproductive development*

Ten parasites were collected haphazardly from each infected fish following each consecutive infection. Parasites were hydrated with distilled water, cleared in cedar wood oil, stained with haematoxylin, dehydrated cleared and mounted on a microscope slide using Canada balsam. Parasites from fish that survived the four successive infections were photographed and measured with Image J 8.0.2 for their total body length and width, haptor diameter, hamuli length, and accessory sclerites total length (Lackenby et al., 2007). The dehydration process affected the physical integrity of some of the parasites, which were excluded from examination for development. Parasite development was qualitatively examined in each specimen based on anatomical features. Parasites were assigned to one of four developmental categories (modified from Whittington & Ernst, 2002). These were defined as: 1) **immature** (neither male nor female reproductive organs functional); 2) **protandrous** (male reproductive organs fully developed and sperm present within seminal vesicle and vas deferens); 3) **imminent adult** (female reproductive organs regarded as fully developed based on presence of vitelline cells in vitelline reservoir and oocytes in germarium, but no clear evidence of insemination and no signs of egg production); 4) **adult** (male and female reproductive organs fully functional; worms inseminated and or egg production underway).

## 2.7. Antigen preparation and indirect ELISA protocol

*Neobenedenia* sp. IgM specific levels in blood and mucus of uninfected and

successively infected fish were determined by indirect ELISA using crude antigens obtained from adult parasites (Kishimori et al., 2015). All remaining adult parasites from each infection event were collected in 10 mL centrifuge tubes with distilled water and frozen at -80 °C. Parasites were thawed for 5 min in a 37 °C water bath, and frozen again for 5 min at -80 °C. Thawing and freezing was repeated five times. Parasites were sonicated three times, each time for 5 min on ice. Finally, antigen preparations were centrifuged at 18 G for 10 minutes at 4 °C and the supernatant collected. The protein concentration of *Neobenedenia* antigen preparations was determined by reading the absorbance at 280 nm (Nano drop®).

Test wells in microtitre plates (NUNC) were coated with *Neobenedenia* sp. antigens diluted in coating buffer overnight at 4 °C. Wells were then washed (3x 5 min) using a Low Salt Buffer Solution (LSBS), and non-specific binding sites were blocked by incubating test wells with 200 µL of 1 % Bovine serum albumin/Phosphate-buffer solution (BSA/PBS) for two hours at room temperature (RT=25 ±2 °C) (Kim et al., 2007). Following, wells were washed (3x 5min) with a High Salt Buffer Solution (HSBS) and incubated with various dilutions of sera and mucus in 1 % BSA/PBS for 1 hour at RT on a shaker. Wells were then washed with HSBS (3x5min and 1x 5 min on shaker), and incubated with 100 µL rabbit anti-barramundi IgM diluted in BSA/PBS for one hour at RT on a shaker. After incubation with rabbit anti-barramundi IgM, plates were washed with HSBS (3x5min and 1x 5 min on shaker), and incubated with 100 µL of goat anti-rabbit IgG HRP conjugate diluted in 1 % BSA/PBS for one hour at RT on a shaker. Finally, the wells were washed with HSBS (3x5min and 1x 5 min on shaker), and the enzyme reaction developed with 3,3', 5,5'-tetramethylbenzidine (TMB) for 15 minutes in the dark. Lastly, the reaction was stopped by the addition of 100 µL of 1M HCL per well, and the absorbance of each well read at 450 nm with an ELISA Microplate Reader.

## 2.8. Statistical analysis

All data from this study were compared using the statistical analysis programme S-Plus 8.2. Statistical analysis was only performed for fish that survived all four infections events (nine fish in total), and from fish infected twice to account for the re-infection event that occurred after the second infection (seven fish in total). Parasite infection success (total number of parasites on the host divided by the initial

number of oncomiracidia introduced; Bush et al., 1997) was analysed using a one-way ANOVA and a post-hoc Tukey test between infection events. For the specific case of the second infection event, infection success was presented for fish re-infected two times. Parasite measurements were compared between infection events using one-way ANOVA's independently for length, width, hamuli and accessory sclerites lengths. A post-hoc Tukey test was performed for each variable between infection events. The proportion of parasites in each development category in each infection event was compared using a Chi-square contingency test.

**Results**

*Neobenedenia* infection success was consistently over 50% for all experimental infections with the exception of the third infection where infection success was halved. *Neobenedenia* sp. infection success was significantly lower in infection three (24 ± 3.88%) compared to infections one, two and four (58 ± 5.40%, 63 ± 2.8%, 59 ± 4.70% respectively, Fig. 11). There were no significant differences in infection success between infection events one, two and four (Fig. 11).
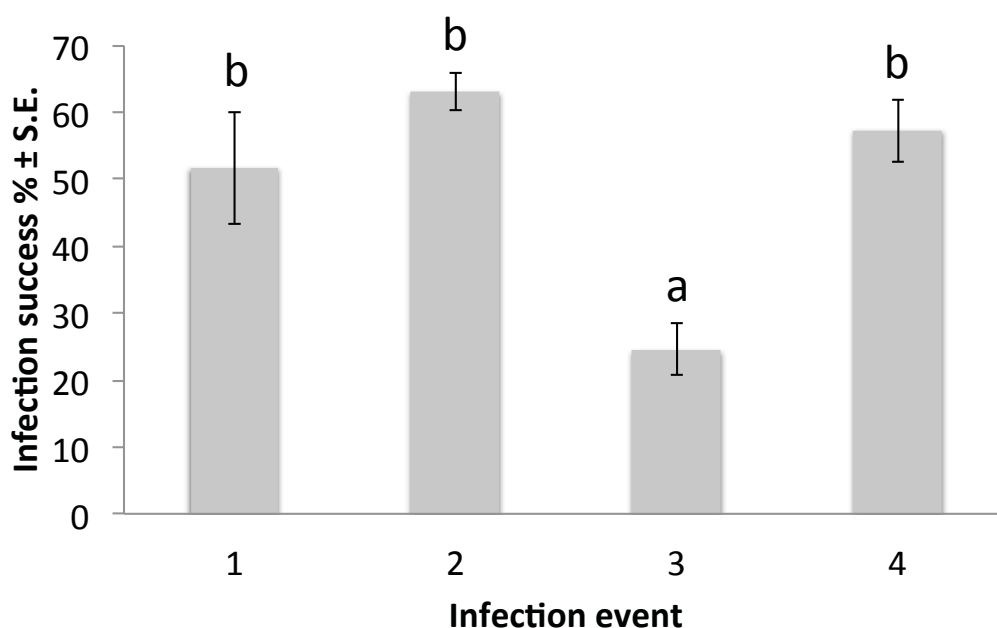
**Fig. 11.** *Neobenedenia* sp. mean infection success in *Lates calcarifer* in four consecutive infections. Fish had significantly lower infection success in the third infection compared to infections one, two and four (one-way ANOVA, $F_{3,43}=9.38$, p<0.01). Post-hoc Tukey test significant differences between groups of infection events are depicted by letters a and b.

Parasites were significantly larger in the first infection event compared to all subsequent infections. Parasites from the first infection were significantly larger in length (body length= 3.49 ± 0.01 mm, one-way ANOVA, $F_{3,439}=575.8943$, P<0.01) and width (body width= 1.85 ± 0.01 mm, one-way ANOVA, $F_{3,439}=580.7569$, P<0.01) compared to parasites from all subsequent infections (Fig. 12). Hamuli and accessory sclerites were significantly longer in parasites from infection one (275 ± 1.8 µm and 122 ± 1.7 µm, respectively) compared to parasites from all other infection events (105 ± 2.0 µm and 250 ± 3.5 µm respectively). There were no significant differences in the proportion of parasites in each development category between infection events and the majority of parasites in all infective periods were either imminent or mature adults (Table 2).
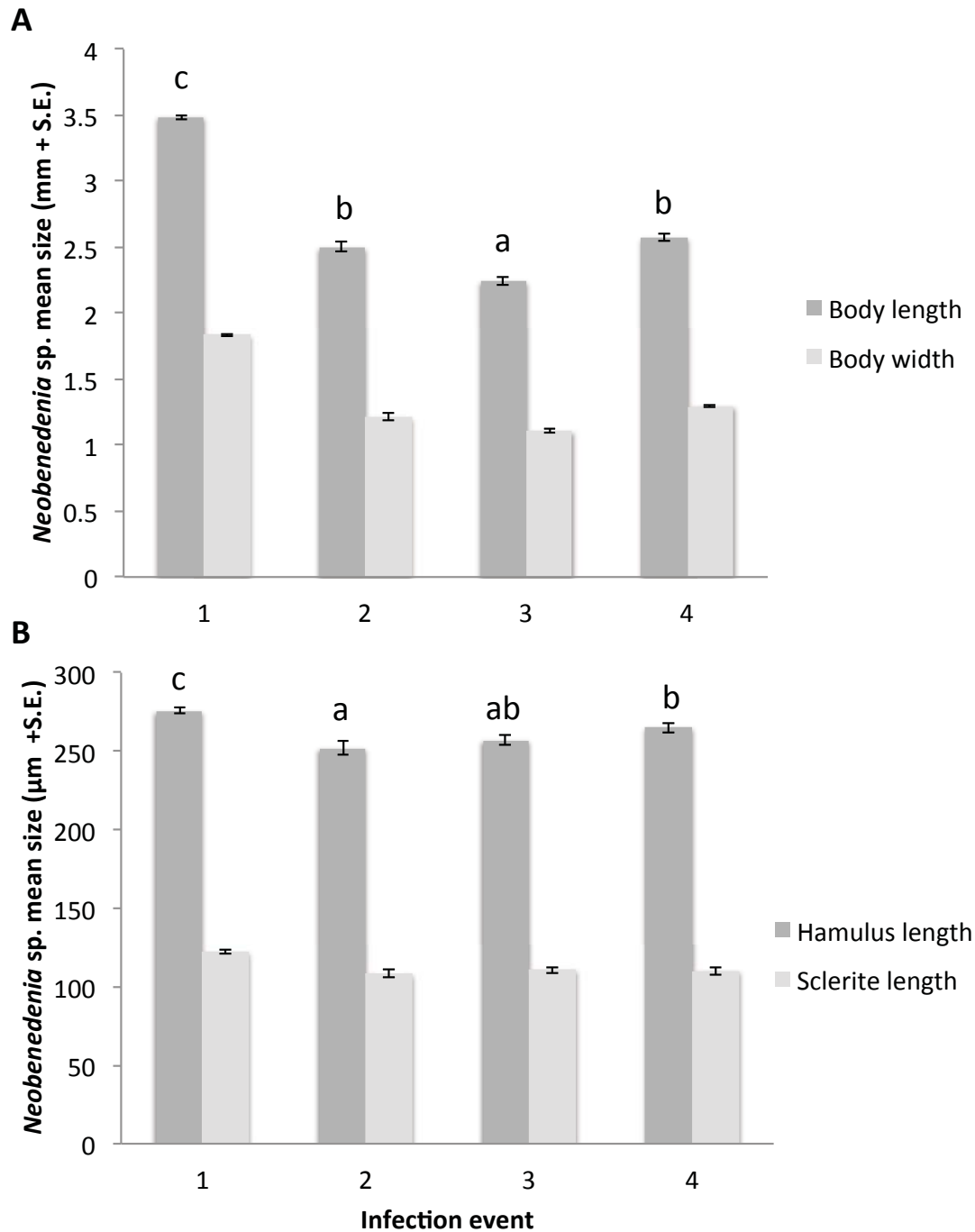
**Fig. 12.** Measurements of *Neobenedenia* sp. infecting *Lates calcarifer* in each infection event. A) Parasite width and length were significantly higher in infection one compared to infection two, three and four (One-way ANOVA, $F_{3,439}=575.8943$, P<0.01 respectively). B) Hamuli and accessory sclerites were significantly longer in parasites from infection one compared to subsequent infections (One-way ANOVA, $F_{3,439}=13.00809$, p<0.01). Post-hoc Tukey test differences between groups of infection events are depicted by letters a, b and c.

**Table 2.** Developmental stages of *Neobenedenia* sp. specimens recovered from *Lates calcarifer* infected experimentally in four sequential exposure events. Observations are made from ten haphazardly collected parasites from fish that survived all exposure events (total of nine fish). There were no significant differences in the proportion of parasites in each developmental category between infection events (Chi-square$_{Contingency}$= 2.385, d.f.= 6, p=0.881)

| Infection event | Development stage | | | | Number of parasites examined |
| --- | --- | --- | --- | --- | --- |
| | Immature | Protandrous | Imminent adult | Adult | |
| 1 | 0 | 2 | 12 | 65 | 80 |
| 2 | 0 | 2 | 17 | 53 | 74 |
| 3 | 0 | 1 | 14 | 53 | 71 |
| 4 | 0 | 1 | 15 | 61 | 81 |

There was no significant difference in *Lates calcarifer* IgM specific levels against *Neobenedenia* sp. with the combinations of dilutions tested (Table 3). More ELISA's are needed to confirm any differences in IgM levels.

**Table 3.** ELISA Protocols used to test for differences in IgM specific levels of mucous and sera between infected and uninfected *Lates calcarifer* to *Neobenedenia* sp..

| Protocol | Concentration (uL/uL buffer) | | | |
| --- | --- | --- | --- | --- |
| | Antigen | Primary AB | Secondary AB | Tertiary AB |
| 1 | 1/10,1/100,1/1000 | 1/5, 1/50 | 1/33 | 1/500, 1/5000 |
| 2 | 1/1000, 1/10000 | 1/10,1/100,1/1000 | 1/33 | 1/5000 |
| 3 | 1/10,1/100 | 1/10, 1/100 | 1/33 | 1/5000 |
| 4 | 1/100 | 1/10, 1/100 | 1/33 | 1/5000 |
| 5 | 1/10,1/100 | 1/10,1/100 | 1/33 | 1/5000 |

Antigen: *Neobenedenia* sp. Proteins (15.047 mg/mL)

Primary AB: Supernatant collected from mucous and Blood samples of infected and uninfected *Lates calcarifer*.

Secondary AB: mouse anti Asian seabass IgM (Aquatic Diagnostics Ltd., Stirling)

Tertiary AB: Goat anti mouse IgG+ (Life Technologies)

**Discussion**

Teleost fish can develop an acquired resistance against pathogens (Rauta et al., 2012; Sitjà-Bobadilla 2008). However, ELISAs performed in this study did not show any differences in IgM specific levels of *L. calcarifer* against *Neobenedenia* sp.. This contrasts with previous studies where fish have shown marked protective immunity following consecutive monogenean infections. Specifically, *Oreochromis mossambicus* continuously exposed to *Neobenedenia girellae* (see Whittington & Horton, 1996 for likely synonymy with *Neobenedenia melleni*) was reported to display marked protective immunity after four months of continuous infection with increased anti-parasite mucosal antibodies (Kishimori et al., 2015). Moreover, acquired immunity against *Neobenedenia girellae* (see Whittington & Horton, 1996) has been previously suggested in Japanese flounder, *Paralichthys olivaceus* (Temminck & Schlegel) (Bondad-Reantaso et al., 1995), Tilapia, *O. mossambicus* (Peters)(Kishimori et al., 2015; Robinson et al., 2008; Nigrelli 1937) and amberjack, *Seriola dumerili* (Risso) (Hirazawa et al., 2011), and was associated with a marked reduction in infection levels of *N. girellae* in successive infections.

The parasite load (approximately 50 parasites per fish) and the limited number of controlled infections (n = 4) used in this study may have not been sufficient to stimulate a protective immune response. There was no clear trend or reduction in parasite infection success in each successive infection event, although infection success was significantly lower in the third infection (Fig 12). It is possible that *L. calcarifer* requires a longer period of time to develop immunity against *Neobenedenia* sp. (Kishimori et al., 2015). However, more successive infections and more ELISAs are necessary to fully understand how *L. calcarifer* IgM reacts against *Neobenedenia* sp..

Although *L. calcarifer* did not appear to develop a significant specific antibody response against *Neobenedenia* sp. in the experimental period, parasites were significantly smaller in all subsequent infections following the first infection event. Similarly, Hirazawa et al. (2011) observed a reduction in parasite size for *N. girellae* infecting amberjack, *Seriola dumerili*, when parasites infected previously exposed fish (Hirazawa et al., 2011). *Neobenedenia* sp. reach sexual maturity on day nine

post-infection in the described experimental conditions (i.e. 25 °C, 35 ppt; Brazenor & Hutson, 2015). In the present study, where parasites were collected on day 10, the majority of parasites had reached sexual maturity, despite a reduction in parasite size (Table 2; Fig. 12). This indicates that while parasites are smaller in size when infecting previously exposed fish as a result of the acquired resistance on the host, there was no compromise to time to sexual development (Sijtà-Bobadilla, 2008; Buchmann & Lindenstrøm, 2002).

Neobenedenia spp. may have avoidance strategies to cope with the immune response of the host. To avoid any response caused by the attachment or feeding of the parasite, monogeneans can migrate to microhabitats (Chapter 3) with reduced mucous cell densities or reduced immune responses (Sitjà-Bobadilla 2008; Whittington & Ernst 2002), feed on the host (immune) molecules (Salzet et al., 2000), and possibly exhibit antigen masking (Sitjà-Bobadilla 2008; Buchman & Lindenstrøm 2002). It is possible that Neobenedenia sp. uses one (or more) of these avoidance mechanisms to avoid the immune response of the host.

In conclusion, Lates calcarifer did not appear to develop a significant specific antibody response against Neobenedenia sp. in the experimental period, but parasite size was significantly reduced after the first infection. It is unclear if Lates calcarifer developed an acquired immunity against Neobenedenia sp.. Neobenedenia sp. may have avoidance mechanisms that allow it to reduce the immunogenic stimulation of the host, including migration across the body of the host, and feeding from host (immune) molecules for parasite growth.

## ACKNOWLEDGEMENTS

# CHAPTER 5

## General Discussion

Knowledge on parasite-host interactions can provide a framework by which parasites of great ecological, medical or economic importance can be studied to comprehend how they affect the physiology and health of their hosts. This research provided new information on the behaviour, pathology and host response of a potentially harmful marine ectoparasite that infects commercially valued fish. The data chapters presented in this thesis provided experimental evidence on parasite infection and invasion routes, pathology directly associated with parasite attachment, and the impact of repetitive exposure on parasite body size.

Chapter 2 showed that oncomiracidia of the marine fish parasite *Neobenedenia* sp. attached opportunistically to the body of the fish host, *Lates calcarifer*, and migrated to preferred microhabitats over time. Fluorescent labelling allowed for accurate tracking of transparent live parasites and revealed that some parasites attached beneath the scales of the host, and that the majority of parasites aggregated on the fins immediately prior to sexual maturity. Chapter 3 revealed that the main posterior attachment organ of *Neobenedenia* sp., the haptor, caused direct mechanical damage to the fish epidermis. Dermal inflammation and epidermal loss were common in varied microhabitats of infected *L. calcarifer* compared to uninfected fish. Infected fish had significantly lower mucous cell counts in all microhabitats and exhibited a trend for lower epidermal thickness in all regions compared with uninfected fish. Despite no differences in specific antibody levels in the mucus or blood of infected and uninfected *L. calcarifer* after four successive infection events in Chapter 4, parasite body size was significantly smaller in recurrent infections.

The ability of *Neobenedenia* sp. to attach beneath the scales of *L. calcarifer*, as shown in Chapter 2, could provide a secluded and protected environment against predators or sub-optimal environmental conditions (Kearn, 2004; Cribb et al., 2002). This behaviour may provide an ideal environment for *Neobenedenia* sp. juveniles to

cope with stressors (e.g. predators) in the initial stages of infection. As the parasite grows and increase in size, the body size of the parasite may compromise its ability to fit beneath scales (Whittington & Ernst 2002; Whittington & Cribb 2001; Whittington 1996). Furthermore, attachment beneath the scales on the host may be an important factor to consider in mariculture of *L. calcarifer*, as it could reduce the efficiency of current parasite management methods (e.g. chemical and freshwater bathing). The efficacy of freshwater bathing on *Neobenedenia* sp. should be quantitatively examined to account for parasites that may seek refuge beneath scales. This could be achieved through the use of the fluorescent markers to detect surviving individuals following freshwater treatment as outlined in Chapter 2.

*Neobenedenia* sp. may use the body surface of the host according to its requirements at various stages of development. Fish epidermal morphology is different between microhabitats in regards to composition and thickness (e.g. presence of scales, mucous cell abundance; Chapter 3). As such, microhabitats could provide distinct feeding grounds and spatial resources (Whittington, 1996; Whittington & Ernst, 2002) or provide additional protection against host immune responses (Sitjà-Bobadilla, 2008). Microhabitats with low mucous cell density, like the eyes or the fins of the fish, are microhabitats with low immunological activity (Sitjá-Bobadilla, 2008). Lower mucous cell density, as well as movement and ventilation could make the fins of the host a preferred microhabitat for *Neobenedenia* sp. reproduction (Chapter 2). Indeed, numbers of parasites on the fins of fish were significantly higher immediately prior to sexual maturity than previous time periods (Fig. 6, day 8; Chapter 2). Although parasites may be feeding and moving on all microhabitats, microhabitats with specific characteristics (e.g. scales on the body, movement and ventilation on the fins, no mucous cells on the eyes) could be used by the parasite for specific needs throughout its development.

*Neobenedenia* sp. mechanical attachment could potentially avoid the elucidation of host immune responses. In Chapter 3, it was found that *Neobenedenia* sp. haptor attachment caused significant epidermal damage to the skin barrier of the host, but did not cause any noticeable inflammation or ulceration. Fish parasites tend to avoid immune recognition by the host to ensure parasite proliferation and survival, while attempting to prevent host death from the lethal effects induced by

inflammatory responses (Sitjà-Bobadilla 2008). Other monogeneans, like *Dactylogyrus* spp., are known to have a mild impact on the gill epithelium of the host, likely to avoid localised immune responses (Rohde, 2005). As shown in Chapter 3 of this thesis, there was no evidence of inflammatory responses on *L. calcarifer* after 20 days post-infection. It is likely that the attachment of *Neobenedenia* sp. to the skin barrier of the host was not sufficient to cause strong immune reactions against the parasite (e.g. inflammation and subsequent parasite recognition; Watts et al., 2001). This is particularly important for the continuation of *Neobenedenia* sp. life cycle, as the parasite matures and lays eggs after 10 days post-infection in the environmental conditions used by this thesis (25 °C, 35 ppt; Brazenor & Hutson, 2015).

Neobenedenia* sp. migration could also enable the parasite to avoid localised host immune responses. Chapter 2 showed that *Neobenedenia* sp. migrated to preferred locations on the host. Parasite migration is considered a key strategy to mitigate the impact of the host-localised immune responses (Sitjà-Bobadilla, 2008; Whittington & Ernst, 2002; Whittington, 1996). The results of this thesis indicate that previously exposed *L. calcarifer* affected *Neobenedenia* sp. size, but did not affect development. This is a critical aspect to consider when managing *Neobenedenia* sp. outbreaks, as host acquired immunity may not inhibit or delay the parasite's life cycle. It is unclear whether an acquired immunity elicited by *L. calcarifer* can reduce the fecundity of the parasite. Future experiments could evaluate how host protective immunity could affect *Neobenedenia* sp. fecundity, egg size and larval longevity.

This thesis did not find any differences in *L. calcarifer* IgM specific levels against *Neobenedenia* sp.. However it is important to consider that parasite loads used in this study were significantly low compared to those that can occur in farmed conditions. In 2001, 200 000 farmed *Lates calcarifer* were lost during a *Neobenedenia* outbreak near Hinchinbrook Channel in Northern Queensland (Deveney et al., 2001) with individual fish reported to be infected with more than 400 parasites. Furthermore, studies reporting the elucidation of teleost acquired immunity against *Neobenedenia* sp. have experimentally infected teleost fish with a range of 70 to 150 parasites per host (Nigrelli, 1937; Robinson et al., 2008; Hirazawa et al., 2011; Kishimori et al., 2015) compared to approximately 50 parasites per host in this

study (Chapter 4). Nonetheless, more ELISA protocols are needed to further understand if *L. calcarifer* can develop protective immunity against *Neobenedenia* sp..

In conclusion, this thesis presents novel information about the interaction between *Neobenedenia* sp. and its host, *Lates* calcarifer. This is the first study to track *Neobenedenia* sp. movement over a spatial-temporal scale on the body of a teleost host by means of fluorescent labelling, and show that the parasites have an initial random distribution followed by significant aggregation in preferred microhabitats. The scales of the host could provide additional protection against predators and the fins of the fish could be a preferred microhabitat for reproduction. Moreover, it is hypothesised that parasite migration could allow the parasite to avoid localised immune reactions of the host. The ELISA protocols performed in this study were not sufficient to provide evidence of acquired protective immunity on *L. calcarifer* when repetitively infected with *Neobenedenia* sp. over time. Nonetheless, this thesis provided contrasting evidence in regards to the associated pathology of *Neobenedenia* sp. infections with low parasite loads, showing that parasite attachment did not cause the elucidation of strong immune responses on the host, while significantly reducing epithelial thickness and mucous cell abundance across microhabitats of *L. calcarifer*. This was the first study to provide morphological differences on the epithelium of *L. calcarifer* microhabitats when infected with low parasite loads of *Neobenedenia* sp..

To better understand the impact of *Neobenedenia* sp. infection intensity on *L. calcarifer,* other research initiatives must be pursued. No studies have been conducted to examine the impact of parasite load on migration patterns and distribution, or how parasite distribution could change when the host is re-infected by a second generation of parasites. This could provide valuable information of how *Neobenedenia* sp. cohorts distribute themselves on the body surface of the host over time. Pathology associated with high parasite loads has been reported in different fish infected with *Neobenedenia* sp. (Kaneko et al., 1988; Ogawa et al., 2006; Hirazawa et al., 2010), however future studies should examine how the associated pathology to *Neobenedenia* sp. infections may progress over time, and which parasite loads can cause greater pathological changes on the host in shorter periods of time. Future research should also consider differences in parasite microhabitat

selection when infecting previously exposed fish, and how susceptible the host is to secondary infections when exposed to higher loads. The amount of research devoted to marine parasites in general has been remarkably low considering the significant threat these organisms pose to the aquaculture industry and food production. Understanding how parasites interact with their hosts can lead to strategic parasite management in aquaculture.

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