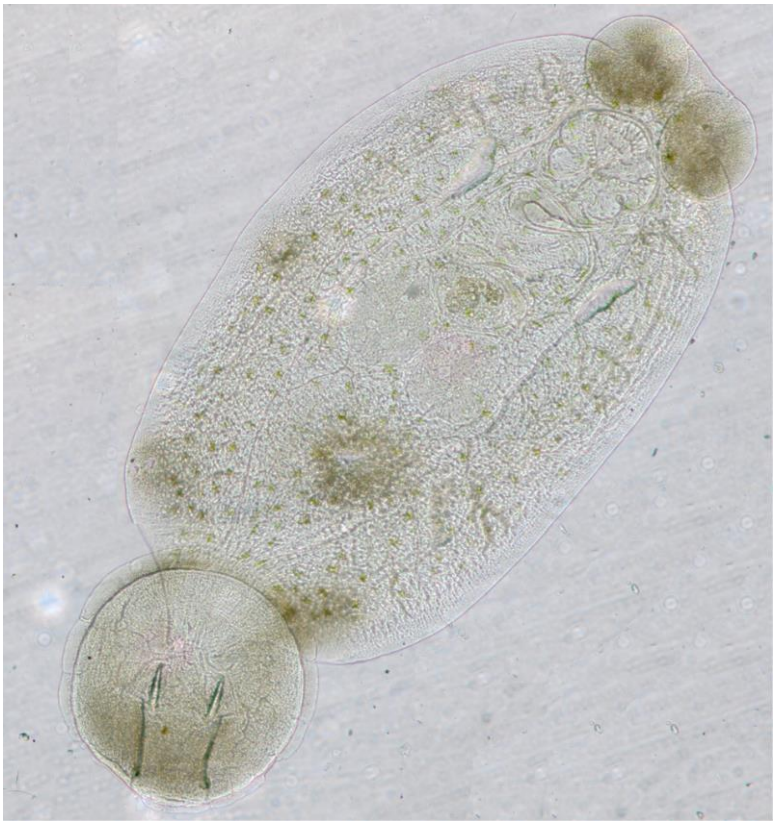
The biology of the cosmopolitan fish parasite *Neobenedenia girellae*



Thesis submitted by

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Statement on the Contribution of Others

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Chapter	Co-author	Contribution
Chapter 2	Terry Bertozzi	Terry Bertozzi provided assistance in genetic analyses and editorial support.
	Terry Miller	Terry Miller provided assistance in genetic analyses and editorial support.
	Ian Whittington	Ian Whittington was instrumental in the development of ideas, the

		provision of samples, and taxonomic identification of specimens.
	Kate Hutson	Kate Hutson provided financial and editorial support and assisted in the development of ideas.
Chapter 3	Richard Saunders	Richard Saunders assisted in developing the aims of this paper, in the statistical analysis of the data, and in editing.
	Terry Miller	Terry Miller assisted in the genetic analysis of samples included in this paper.
	Kate Hutson	Kate Hutson assisted in financial support, editorial assistance, and the development of ideas.
Chapter 4	Kate Hutson	Kate Hutson provided assistance in finances, experiments, statistical analyses, and editing.
Chapter 5	David Francis	David Francis assisted in the biochemical analysis of samples in this study and provided editorial support.
	Kate Hutson	Kate provided assistance in the development of ideas and editing.
	Guy Carton	Guy Carton assisted in the development of ideas and the editing of this paper.
Chapter 6	David Francis	David Francis assisted in the biochemical analysis of samples in this study and provided editorial support.
	Guy Carton	Guy Carton assisted in the development of ideas in this paper.
	Kate Hutson	Kate provided assistance in the development of ideas, financial support, and editing.

Title page image: Adult *Neobenedenia girellae.* Photograph taken by A. Brazenor. Image dimensions = 250 μm × 100 μm.

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This thesis is dedicated to my family, my friends, and to those who took the time in their lives to support me throughout this journey.

Abstract

Monogeneans are a fascinating parasite group to study. They can be harmful pathogens of finfish and display interesting relationships with their hosts making research on these species engaging from both ecological and economic perspectives. *Neobenedenia* (Family Capsalidae) Yamaguti, 1963, is a notable monogenean genus and is notorious for the large number of potential host species that the constituent parasite taxa are able to infect. The distinct lack of research on *Neobenedenia* biology and the diversity of species present in Australian waters presented me with an opportunity to conduct a series of research studies designed to improve understanding of this parasite genus. The aim of this thesis was to provide an in-depth investigation of *Neobenedenia* phylogenetics and detailed biology (including the growth, morphology, reproduction, and biochemistry) of the species in Australia.

The first data chapter (Chapter 2) determined the phylogenetic relationships between 33 *Neobenedenia* isolates by amplifying three genes; two nuclear (*H3* and *28S rDNA*) and one mitochondrial (*cytochrome b*). Isolates were collected from a total of 23 host species and nine countries in both hemispheres and included 16 isolates from 12 host fishes in Australia. Representative samples for *Neobenedenia melleni* MacCallum, 1927, *Neobenedenia pacifica* Bravo-Hollis, 1971, and *Neobenedenia longiprostata* Bravo-Hollis, 1971, formed discrete clades and collectively accounted for seven of the 33 samples used in this study. The remaining 26 isolates formed a single clade genetically distinct from all representative specimens. Morphological observation of these 26 isolates confirmed that they were morphologically indistinguishable from *N. melleni* despite displaying clear genetic differences in the phylogenetic trees. This confirmed the suggestion by the scientific community that *Neobenedenia girellae*

Hargis, 1955, a species that was synonymised with *N. melleni* in 1996, should be reinstated as its own taxon, a recommendation that is encouraged by the authors.

The second data chapter (Chapter 3) focussed on the effect that a number of environmental variables had on the morphology of *N. girellae*. *Neobenedenia girellae* is extremely flexible in the morphology it exhibits. Temperature was identified as the strongest factor affecting the morphology of this species, however, there was an indication that morphology may also be dictated by the host species that the parasite attaches to. Between different host species, parasites exhibited changes in the morphology of the attachment organs which come in direct contact with their host's surface. This is proposed to be a function of maximising the ability of their attachment organs to the scale/mucus interface of heterogeneous host surfaces. The morphology of parasites attached to the same host species at different temperatures differed most in their total body size. Features associated with the attachment of the parasite to the host did not differ which is likely a product of the homogenous host surface available to the parasite. The variation observed in this species explains why *N. melleni* and *N. girellae* have been misidentified, fuelling considerable taxonomic confusion in *Neobenedenia* in the past.

The third data chapter (Chapter 4) focussed on elucidating reproductive and life cycle biology of *N. girellae* in temperatures and salinities typical of tropical regions. *Neobenedenia girellae* completed its life cycle almost twice as fast in warm, high saline conditions compared to cooler temperatures. Hatching and infection success and oncomiracidia longevity was significantly reduced in salinities less than 22 ‰ compared to higher saline conditions (35 and 40 ‰). A total of two strategically timed treatments on stock was recommended in cool to moderate temperatures in salinities of seawater or higher. In warmer conditions (> 30 ºC), a third treatment of stock is

required as parasites reach sexual maturity faster than the time taken for all eggs to hatch in a given period. The development of an accessible and user-friendly strategic treatment timetable informs fish farmers and aquarists alike when to treat their fish to maximise the efficacy of treatments and minimise labour costs and reinfection.

The fourth data chapter investigated the feasibility of collecting sufficient quantities of *N. girellae* eggs for biochemical analysis (Chapter 5). An established laboratory culture of *N. girellae* was optimised to enable the production of larger quantities of eggs over short periods of time for collection and subsequent biochemical analysis. Eggs were found to be composed primarily of water (79.12 %) followed by protein (11.51 %) and lipid (2.50 %). Lipids were composed of approximately equal amounts of saturated, monounsaturated, and polyunsaturated fatty acids (35.43 ± 0.38 %, 29.08 ± 0.38 %, and 35.50 ± 0.53 %, respectively). The predominant lipid classes were phosphatidylcholine (21.90 ± 1.42 %) and triacylglycerols (TAG) (33.82 ± 1.20 %). This is the first study to quantify the biochemical components of marine monogenean eggs but only provided insight into the biochemical contents of *N. girellae* eggs after they had been laid and not throughout development.

Chapter 6 expanded on the topics identified in Chapter 5 and quantified the biochemical contents of eggs throughout embryogenic development and across a range of temperatures. This provided valuable information on metabolic fuels employed by embryos as they developed and in different environments. Additionally, reproductive biology (fecundity, egg-laying period, and egg volume) was investigated to provide a complete picture of *N. girellae* reproductive investment. Adult parasites were significantly larger at cooler temperatures and produced significantly larger eggs towards the end of their reproductive lives. Fecundity of adults was highest at 20 and 30 ºC compared to 25 ºC. The biochemistry of freshly laid eggs was similar to the

results observed in Chapter 5. Proximate composition of eggs did not significantly change over the three temperatures tested, however, warmer temperatures resulted in the significant decrease in a number of mono- and polyunsaturated fatty acids throughout embryogenesis. The most prolific lipid classes were phospholipids and TAGs. The most notable change from the biochemical profile observed in Chapter 5 was that the amount of acetone mobile polar lipids in eggs more than doubled in the present study to compose up to 25% of the lipids in *N. girellae* eggs.

Neobenedenia girellae is a parasite with a turbulent taxonomic history. Now that appropriate genes have been recognised that can accurately identify this species, potentially misidentified samples from previous studies can be clarified and the geographic and host records can be built on a solid foundation. This allows for future research to confidently credit any biological research to this taxon which has important implications for modelling populations on hosts, epidemiology, and management of this species on dozens of host taxa. If, as predicted, parasites and pathogens become a more immediate problem for global food security in a rapidly changing climate, accurately defining and identifying threats and developing methods for management and eradication of these species is paramount. This thesis presents a comprehensive assessment of one of the most cosmopolitan monogenean species in the world and the intimate knowledge generated on its biology can be used to reduce the impact of outbreaks on food fish production.

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Chapter 1: General Introduction

Marine aquaculture or ‘mariculture’ is a form of rearing aquatic organisms for commercial purposes, either in open coastal environments or in marine pond systems (Seng et al., 2006). The industry has grown rapidly in the last decade because of the depletion of wild fishery resources (Pauly 2008) and the strong demand for, and high price of, marine products (Seng 1997). Aquaculture is widely considered to be one of the best prospects for supplying food to a growing global population (Cressey 2009; Guo & Woo 2009).

The use of intensive farming practices is a characteristic of modern aquaculture farms and is driven by a number of factors including the increasing competition faced by the sector for available resources (Tilman et al., 2002; Foley et al., 2005) and the drive for increased productivity per unit area (Tacon & Halwart 2007). Ultimately, this means that high concentrations of organisms are held in aquaculture systems to ensure food security and to promote financial gains (Marra 2005; Duarte et al., 2009; Guo & Woo

2009). Unfortunately, the mass production of any organism is inevitably accompanied by pathogens and parasites which threaten the economic viability and production capability of aquaculture farms (Shinn et al., 2015).

Parasites are ubiquitous components of the environment, occur at all trophic levels, and constitute an important part of biodiversity (Minchella & Scott 1991; Marcogliese 2004; 2005). The most accepted definition of a parasite is an organism which lives in or on another organism, termed the 'host', which it feeds on, and to which some level of morphological adaptation has developed (Poulin 2011). Parasitism is an extremely successful life strategy and has evolved at least 60 times in the evolutionary history of animal life on Earth with almost 50% of documented animal species adopting a parasitic life-style (Price 1980; Windsor 1998; Poulin & Morand 2000). Parasites can regulate the abundance of their host population (Mouritsen & Poulin 2010), influence the composition and structure of animal communities (Mouritsen & Poulin 2002) and affect the functioning of ecosystems (Thomas et al., 2005). This is the case for terrestrial environments, freshwater, and marine ecosystems.

Parasitic disease outbreaks are one of the most serious limiting factors in the aquaculture industry and one of the greatest threats to global food security (Meyer 1991; Seng 1997; Lebel et al., 2002; McDermott & Grace 2011). Typically, parasites only attract attention when they cause significant pathology and disease outbreaks or degrade biological products, thereby reducing production yields and economic benefits (Marcogliese 2004). This can be seen most drastically in food production sectors. In aquaculture, parasite epidemics can result in extreme financial costs (Bauer & Hoffman 1976; Paperna & Overstreet 1981; Seng 1997; Deveney et al., 2001; Whittington et al.,

2004; Seng 2006; Rückert et al., 2008; Costello 2009; Guo & Woo 2009). Poor water quality, high stocking conditions, and handling of stock for grading or health management are all characteristics of intensive aquaculture systems and stress the cultured organisms (Conte 2004). Stress increases susceptibility to infection and allows parasite populations to build to damaging levels (Barton & Iwama 1991; Nowak 2007). High infection intensities can cause mortalities of stock, financial costs from organism deformities, reduced growth rates, decreased consumer confidence, and the increased investment in stock and disease management infrastructure needed to deal with infection events (Minchella & Scott 1991; Ogawa 1996; Marcogliese 2004; Lackenby et al., 2007; Fajer-Ávila et al., 2008).

Parasites with a single host life cycle (such as Platyhelminthes) are of particular concern. They are able to parasitise fish directly and proliferate in culture conditions without the need for multiple species to be present to complete their life cycle (Diamant et al., 1999). The Monogenea is a class of Platyhelminthes, and the constituent species are predominantly parasitic on the skin and gills of freshwater and marine teleosts (Whittington 2004). Monogeneans are of particular threat to aquaculture stock in temperate and tropical regions of the world because they can multiply rapidly in high-density aquaculture environments. Certain oviparous monogeneans can produce large quantities of viable eggs (Ogawa 1996; Dinh Hoai & Hutson 2014) that tangle in sea-cage netting, filters, pipes, algae, and other structures, leading to high retention in the immediate surroundings which results in high re-infection rates amongst farmed fish (Ogawa et al., 2006; Shirakashi & Hirano 2015; Figure 1).

Monogeneans in the family Capsalidae are particularly notorious for their virulence and are recognised as serious pathogens of finfish in sea-cage and semi-open pond aquaculture (Ogawa 1996). Capsalid monogeneans which only require a single host to complete their life cycle are particularly suited to these host-rich settings. In these human-mediated environments which have artificially inflated densities of hosts, these traits lead to rapid growth of parasite populations. When conditions are appropriate, massive losses of aquaculture stock, representing a huge investment in capital and considerable animal welfare issues, can occur (Bauer & Hoffman 1976; Paperna & Overstreet 1981; Deveney et al., 2001; Whittington 2004). The current taxonomic classification of the Capsalidae comprises nine subfamilies, 57 genera, and over 300 species, most of which are ectoparasitic on marine fishes (Whittington 2004, Gibson et al., 2010). Some of the most infamous are species in the genera *Benedenia* and *Neobenedenia* which represent significant threats to the mariculture industry. The genus *Neobenedenia* is currently comprised of six recognised species, namely *Neobenedenia melleni* MacCallum, 1927*, N. muelleri* (Meserve, 1938) Yamaguti, 1963*, N. pacifica* Bravo-Hollis, 1971*, N. longiprostata* Bravo-Hollis, 1971, *N. isabellae* (Meserve, 1938) Yamaguti, 1963, and *N. adenea* (Meserve, 1938) Yamaguti, 1963. This genus was revised in 1996 by Whittington & Horton and forms the basis for our current taxonomic understanding of *Neobenedenia*.This review of *Neobenedenia* controversially synonymised *N. girellae* Hargis, 1955, with *N. melleni*, based on a lack of variation in morphology between the two species. This has subsequently been called into question by some researchers and *N. girellae* is still widely referred to in recent studies (Ogawa & Yokoyama 1998; Koesharyani et al., 1999; Yoshinaga et al., 2000; Ishida et al., 2007; Hirayama et al., 2009; Hirazawa et al., 2011; Maffioli et al., 2014; Shirakashi & Hirano 2015).

Neobenedenia melleni is renowned as a widespread pathogen in aquaria and aquaculture due to its broad geographic range and its ability to infect a multitude of wild and cultured fish species (Whittington 2004; Whittington et al., 2004). *Neobenedenia melleni* was originally described from tropical fish held in the New York Aquarium by MacCallum in 1927 and it was thought to have been introduced from the Caribbean through the ornamental fish trade (Dyer et al., 1992; Whittington & Horton 1996). Since this initial outbreak in 1927, *N. melleni* has been recorded to infect over 100 species in more than 30 families from five orders of captive and wild teleosts (Kaneko et al., 1988; Dyer et al., 1992; Ogawa et al., 1995; Whittington & Horton 1996; Bullard et al., 2000; Deveney et al., 2001; Whittington 2004; Rückert et al., 2008; Kerber et al., 2011). Deveney et al. (2001) were the first to document *Neobenedenia* in Australian waters when they recorded an outbreak of *N. melleni* on farmed barramundi, *Lates calcarifer* Bloch, 1790. This outbreak in Queensland resulted in the mortality of over 200,000 fish equating to a loss of over $AU500,000 and highlights the economic impact a single epizootic can have on one farm. Doubt has recently been cast on the accuracy of this identification and many other identifications of *N. melleni* due to the morphologically conserved nature of *Neobenedenia* isolates (ID Whittington, pers. comm). Recent genetic evidence reinforces this hypothesis and suggests that *N. melleni* is in fact a species complex (Whittington 2004), possibly including the synonymised *N. girellae.*

Problems related to morphologically distinguishing between *Neobenedenia* species and the paucity of genetic research conducted on the genus make it difficult to understand the geographic distribution and host specificity of *Neobenedenia* species and therefore

the biology of each species. Unfortunately, accurately identifying original sources of infection is complicated by the global trade of ornamental and food fishes. Moreover, many studies have not accessioned research specimens in curated museum collections for further study making validation of original identifications impossible. An additional difficulty is that some specimens, particularly valuable type specimens, are not permitted to be lent out by the museum and researchers must visit the collection to view samples.

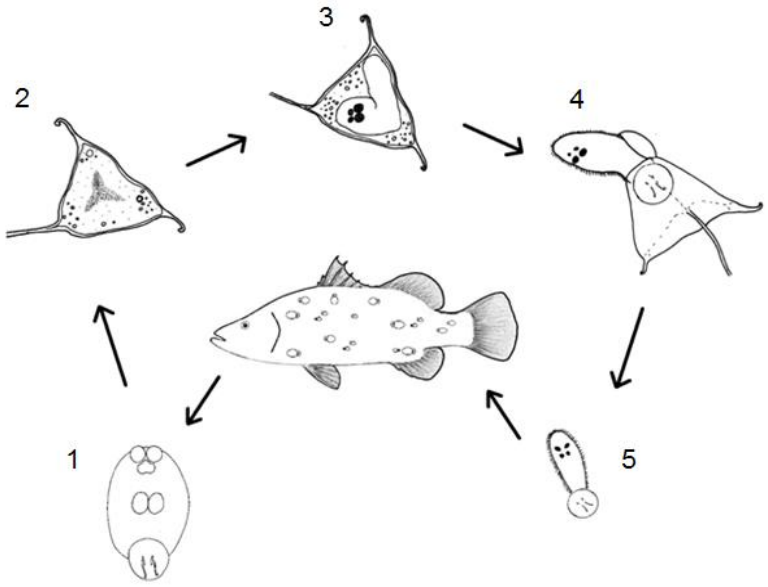

Figure 1: Schematic diagram of the life cycle of *Neobenedenia* spp.. Adult parasites (1) attached to the epithelial surface of host fish and deposit eggs into the environment. Eggs embryonate (2, 3) and hatch (4) into ciliated larvae (oncomiracidia) which re-infect fish (5). Illustration by the author.

There is a paucity of information on the identification and biological characteristics of *Neobenedenia* in Australia. Despite anecdotal evidence that *Neobenedenia* spp. infect several species of commercially important fishes in Australian waters, there have been few formal documentations or studies in Australia since the initial discovery by

Deveney et al. (2001). Recent research has focused on potential treatments, biology, and histopathology (Hutson et al., 2012; Militz et al., 2014; Dinh Hoai & Hutson 2014; Trujillo-González et al., 2015a; 2015b), however, considerable gaps in knowledge still remain. There has been no dedicated research on the diversity of *Neobenedenia* species in Australian waters, the hosts able to be infected, life cycle responses to environmental parameters, or early life biology of Australian *Neobenedenia* spp.. These parasites represent a significant threat to the Australian aquaculture industry (Whittington & Horton 1996; Deveney et al., 2001) and research that relates to accurate identification and life cycle biology can aid to anticipate or mitigate infections of valuable stock.

Accurate morphological and molecular identification of *Neobenedenia* spp. is crucial to assessment of disease risk. This is because closely related cryptic parasite species may exhibit variable traits to one another such as host pathogenicity (Homan & Mank 2001; Skovgaard et al., 2002; Haque et al., 2003), epidemiology (Murrell & Pozio 2000) and environmental tolerance. The accurate identification of *Neobenedenia* isolates is of importance for biosecurity on farms and efficient responses to parasite detection but also for taxonomic accuracy. A crucial part of ensuring accurate identification is the development of robust molecular markers which allow for simple, fine-scale species differentiation within this genus. To supplement genetic identification, recognition of how *Neobenedenia* spp. behave phenotypically to environmental variables such as host species and temperature will assist in reducing incorrect species identification in this genus. Unless research is focused on species identification of *Neobenedenia* spp., taxonomic confusion will persist and make targeted management of these species difficult (Bickford et al., 2006; Olstad et al., 2009; Barcak et al., 2014). Once an infection has been accurately identified on stock, management and treatment of the

infection and infected hosts is required. Precise measurements of the effect of environmental variables (e.g. temperature, salinity) on the biology and life cycle stages is needed to enable the development of strategic management timetables that can effectively break the life cycle and maximize treatment efficiency (Tubbs et al., 2005).

The virulent nature of *Neobenedenia* spp. infections and potential for infection events to seriously impact the aquaculture industry makes research on these parasites an engaging and topical prospect, particularly from the perspective of marine teleost health. The overarching research aim of this thesis was to examine the diversity and life history of *Neobenedenia* to ultimately assist in the application of parasite diagnosis and management methods in aquaculture farms. This broad aim entailed pursuing four discrete research topics that collectively form a coherent progression of ideas that developed whilst conducting the research and are presented as five separate data chapters, each structured as a discrete manuscript (Appendix A).

Although previous research has broached the possibility that the phylogenetics of *Neobenedenia* may need revision, no studies have comprehensively addressed this topic. **Chapter 2** focused on the confusion surrounding the identification of *N. melleni* and *N. girellae* and provides much needed clarification on the phylogeny of this group by sequencing nuclear and mitochondrial genes from 33 *Neobenedenia* isolates from 24 hosts collected in nine countries. This chapter begins the process of building reliable host species and location records for *N. girellae*.

Chapter 3 highlighted some of the reasons why taxonomic confusion has plagued this genus for decades. Parasites are notoriously variable in the morphology they display as a result of the environmental conditions in which they develop and is why *N. melleni* and *N. girellae* were incorrectly synonymised. This chapter

experimentally examined whether morphological variation observed between individuals genetically identified as *N. girellae* was the result of phenotypic plasticity or selection. To test this hypothesis, morphological variation of genetically constrained *N. girellae* individuals was quantified in response to varied host species, temperature, and salinity.

Understanding how parasite populations respond to environmental variables enables the opportunity to apply treatments that will manage infections in commercial systems. **Chapter 4** examined the reproductive biology of *N. girellae* at a range of temperatures and salinities. These data were then modelled to develop an interactive, online strategic treatment plan which can be used by farmers and hobby aquarists to effectively break the parasite's life cycle.

The final chapters examined early life biology and biochemical components of *N. girellae* eggs. Biochemical analysis of parasite eggs is rarely conducted because of the need for large quantities to comprise a sufficiently sized sample for accurate quantification of biochemical components. **Chapter 5** was conducted as a pilot study to verify that the *N. girellae* culture established in the Marine Parasitology Laboratory at James Cook University was able to produce eggs in large enough quantities for biochemical analysis. Building on the confirmation of this hypothesis, **Chapter 6** further investigated reproductive characteristics of *N. girellae* and quantified the effects of temperature on the reproductive biology of *Neobenedenia girellae*. This broad aim was addressed through four discrete studies to determine the effect of temperature on; 1) fecundity of adult parasites; 2) changes in egg volume throughout the life span of adults; 3) energy reserves available to developing embryos, and; 4) how embryos utilised energy reserves during development. In doing so, this final data chapter

provided, for the first time, an indication of how the biochemistry of marine monogenean eggs changed over the development of the embryo.

Each chapter of this thesis can be read as a separate body of work separately containing an Abstract, Introduction, Methods, Results, and Discussion. The final chapter, Chapter 7, is a General Discussion which presents the main findings of the thesis, implications of results, knowledge gaps filled, and avenues for future research.

Chapter 2: DNA profiling reveals *Neobenedenia girellae* as the primary culprit in global fisheries and aquaculture

Alexander K. Brazenor, Terry Bertozzi, Terrence L. Miller, Ian D. Whittington, Kate S. Hutson

This Chapter has been submitted for publication and is currently under review in *Molecular Phylogenetics and Evolution*

2.1 Abstract

Accurate identification of parasite species is crucial to the process of mitigating the risk of epidemics and emerging disease. Species of *Neobenedenia* are harmful monogenean ectoparasites that infect economically important bony fishes in aquaculture worldwide, however, the species boundaries between two of the most notorious taxa, *N. melleni* and *N. girellae*, has been a topic of contention for decades. Historically, identifications of *Neobenedenia* isolates have overwhelmingly been attributed to *N. melleni*, and it has been proposed that *N. girellae* is synonymous with *N. melleni*. Thirty-three *Neobenedenia* isolates were collected from 23 host species spanning nine countries and three genes were amplified including two nuclear (*Histone 3* and *28S rDNA*) and one mitochondrial (*cytochrome b*). Three clades were identified using Maximum Likelihood and Bayesian inference analyses; clades B and D were composed of two isolates each and corresponded to *N. melleni* and *N. pacifica* respectively. All unidentified isolates collected fell into clade A, determined to be *N. girellae* through morphological observation. The results of this study indicate that *N. girellae* is a separate species to *N. melleni*, and that a large proportion

of previous samples identified as *N. melleni* may be erroneous and a revision of identifications is needed. The large diversity of host species that *N. girellae* is able to infect (as determined in this study) and the geographic range in which it is present (between 23.8426° S and 24.1426° N) makes it a globally cosmopolitan species and a threat to aquaculture industries around the world.

Key words: phylogeny, host specificity, Monogenea, Capsalidae, skin fluke, *Neobenedenia melleni*, cryptic

2.2 Introduction

The Monogenea, one of three classes in the phylum Platyhelminthes, predominantly comprises ectoparasitic flukes that infect the gills and skin surfaces of marine and freshwater fishes (Whittington 2004). Monogeneans in the family Capsalidae (Monopisthocotylea) are recognised as virulent pathogens of finfish in sea-cage and semi-open pond aquaculture and are able to multiply rapidly in high-density aquaculture environments as they have a direct, single host life cycle (Jahn & Kuhn 1932, Ogawa 1996). Some species are responsible for considerable epidemics (Bauer & Hoffman 1976; Paperna & Overstreet 1981; Deveney et al., 2001; Whittington et al., 2004). According to current taxonomic classification, the Capsalidae comprises nine subfamilies, 57 genera, and over 300 species, most of which are ectoparasitic on marine fishes (Whittington 2004; Gibson et al., 2010). One of the most notorious of capsalid genera is *Neobenedenia*

Yamaguti, 1963, which have a large host range and have been implicated in causing severe pathology and devastating economic losses in aquaculture worldwide.

Neobenedenia spp. harm fish by mechanical attachment and subsequent grazing on the epithelial surface of their host, which can cause epidermal degradation, dermal erosion, inflammation and allow the ingress of pathogens that may cause secondary infections (Kaneko et al., 1988; Trujillo-González et a., 2015b). The genus *Neobenedenia* is currently comprised of six recognised species, namely *Neobenedenia melleni* MacCallum, 1927*, N. muelleri* Meserve, 1938*, N. pacifica* Bravo-Hollis, 1971*, N. longiprostata* Bravo-Hollis, 1971, *N. isabellae* Meserve, 1938, and *N. adenea* Meserve, 1938. This genus was revised in 1996 by Whittington & Horton and formed the basis for our current taxonomic understanding of *Neobenedenia*. This genus has gained global attention primarily due to the notoriety of the type species, *Neobenedenia melleni* MacCallum, 1927, which was originally described from tropical fishes held in the New York Aquarium (Whittington & Horton 1996). While the origin of the infection is not clear, it is thought that the parasite may have been introduced from the Caribbean through the ornamental fish trade (Dyer et al., 1992; Whittington & Horton 1996). *Neobenedenia melleni* is infamous as a widespread pathogen in aquaria and aquaculture and the list of recorded host taxa spans over 100 species from 30 families and five orders of teleosts Whittington & Horton 1996, Bullard et al., 2000, Whittington 2004, Whittington et al., 2004). The low host specificity reported for *N. melleni* is atypical for monogeneans, where it is considered that approximately 80% of all monogenean species only infect one host species and usually only in a single ocean (Rohde 1979; Byrnes & Rhode 1992; Whittington 1998; Whittington et al., 2000).

Given the nature of a parasitic lifestyle that necessitates the need for conserved morphology, it is not surprising that the morphology of some species is highly conserved and accurate identification of *Neobenedenia* species has proven challenging (Ogawa et al., 1995; Whittington 2004; Whittington et al., 2004). Of particular controversy and confusion has been the delineation of *N. melleni* and *Neobenedenia girellae* Hargis, 1955. *Neobenedenia girellae* was originally described by Hargis (1955) infecting opaleye, *Girella nigricans* Ayres, 1860, from California. Twenty-six additional host species have been subsequently reported from a range of localities (Nigrelli, 1947; Bravo-Hollis, 1958; Bravo-Hollis & Deloya, 1973; Moser & Haldorson 1982; Love et al., 1984; Gaida & Frost 1991; Ogawa et al., 1995). Twenty years ago, Whittington & Horton (1996) synonymised *N. girellae* with *N. melleni* based on morphology and low host specificity. This decision was not unanimously accepted and many authors continue to use '*N. girellae*' in scientific literature (e.g. Ogawa & Yokoyama 1998; Koesharyani et al., 1999; Yoshinaga et al., 2000; Wang et al., 2004; Ogawa et al., 2006; Zhang et al., 2014).

Studies employing molecular techniques in systematics have revealed a large number of morphologically similar parasite species that were previously recognized as a single taxon but are actually genetically distinct (Donald et al., 2004; Miura et al., 2005; Wu et al., 2005; Xiao et al., 2005; Saijuntha et al., 2007; Leung et al., 2009; Blasco-Costa et al., 2010; Sepúlveda & Gonzalez 2014). For example, Sepúlveda & Gonzalez (2014) found that a related capsalid species, *Benedenia seriolae* (Yamaguti, 1934) Meserve, 1938, is in fact a complex of cryptic species and not a single taxon. The first molecular-based evidence suggesting that a species complex may be present among *Neobenedenia* spp. was presented by Whittington et al. (2004) where *28S rDNA* sequences were compared between

two *Neobenedenia* isolates identified as *N. melleni*. This study indicated that there may be distinct taxa present within a complex of morphologically similar individuals. Since this research, very few molecular studies have addressed *Neobenedenia* taxa in order to resolve the confusion. A brief study comparing *N. melleni* and *N. girellae* by Wang et al. (2004) used short sequences of *28S rDNA* and indicated that the synonymy of these species proposed by Whittington and Horton (1996) was supported. Unfortunately, the details of the speciemens used in this study are not known as no reference to an accessioned collection was made. A more comprehensive study by Perkins et al. (2009), addressed the phylogeny of many capsalid species using multiple genes (*28S rDNA, Histone 3,* and *Elongation Factor 1α*), and seems to support the opposite view, that *N. melleni* and *N. girellae* are two separate species based on molecular differences.

Neobenedenia was first documented in Australian waters in 2000. An outbreak of *N. melleni* was reported in farmed barramundi, *Lates calcarifer* Bloch, 1790, and resulted in the death of over 200,000 fish (Deveney et al., 2001). However, it was only in 2011 that research on *Neobenedenia* in Australia began to develop following the collection of *N. girellae* individuals from a fish farm in north Queensland and the subsequent establishment and maintenance of a continuous *in vivo* culture in the Marine Parasitology Laboratory at James Cook University, Australia (Hutson et al., 2012; Hutson et al. in review). Morphological identification of the species of *Neobenedenia* in culture has been problematic and all previous research from this laboratory has referred to the parasite as *Neobenedenia* sp. (e.g. Dinh Hoai & Hutson 2014; Hutson et al., 2012; Trujillo-González et al., 2015a; 2015b). There is a need to accurately identify the species of *Neobenedenia*

currently being used as a parasite model in this laboratory so that research can be ascribed to the correct taxon allowing for more meaningful application.

The aim of this study was to use molecular characterisation methods to generate a robust phylogenetic framework for the identification of *Neobenedenia* spp. to underpin our understanding of the ecology of the taxa implicated in causing damage to wild and aquaculture fish stocks worldwide.

2.3 Materials and Methods

2.3.1 Sample collection

Neobenedenia specimens were collected between 2000 and 2015 from wild and captive fish in nine countries and preserved in 70% analytical grade ethanol. The majority of Australian isolates were collected by the authors while many of the international samples were donated by research colleagues (Table 1). Samples were collected by removing live parasites from the skin of their hosts using a scalpel blade or after bathing the host in freshwater, which kills the parasites. Parasites were fixed and stored in 70% analytical grade ethanol.

A slice of tissue (sliver) was removed from the right-hand side margin of individual parasites, opposite the reproductive organs, taking care to avoid the gut. The remainder of the specimen was washed three times using distilled H_2O and stained with haematoxylin. Parasites were then dehydrated through an alcohol series, cleared in methyl salicylate or cedarwood oil, and mounted on microscope slides in Canada balsam for further study

(Hutson et al., 2012). At least one mounted specimen collected per host by A.K. Brazenor and K.S. Hutson was accessioned into the Queensland Museum helminthology collection. Specimens from the late I.D. Whittington's collection were accessioned to the Australian Helminthological Collection at the South Australian Museum (Table 1).

2.3.2 DNA preparation, PCR amplification, and amplicon sequencing

DNA was extracted from parasite slivers using either the PUREGENE DNA purification system (Gentra Systems) protocol for DNA purification from solid tissue or a QIAgen DNeasy Kit (QIAGEN Inc., Valencia, California, USA) according to the manufacturers' protocols. PCR amplifications of partial *H3*, *28S rDNA*, and *Cytb* sequence were carried out in 25µl reactions using the primers listed in Table 2 and either Amplitaq Gold DNA polymerase, or Phusion high-fidelity polymerase with the following reaction and cycling conditions: Amplitaq Gold – methods documented in Perkins et al. (2009) were followed except that annealing temperature was standardised at 55°C and a maximum of 34 PCR cycles was used. Phusion – a final concentration of 5µL of 5xPhusion® HF buffer, 0.5 µL of 10mM dNTPs, 1.25 µL of each primer (10mM), 0.25 µL of Phusion® Tag DNA polymerase, and 4 µL of DNA template were used with an initial denaturation step of 98 °C for 30 s, followed by 35 cycles of PCR; denaturation at 98 °C for 10 s, annealing 53-62 °C for 20 s, extension at 72 °C for 30 s, with an additional final extension at 72 °C for 7.5 min. The double-stranded amplification products were visualised on 1.5% agarose gels and purified using a Multiscreen – PCR Plate (Millipore Corporation). Purified products were sent to the Australian Genome Research Facility for cycle-

sequencing in both directions using the BigDye Terminator v3.1 cycle-sequencing kit (Applied Biosystems) on an AB3730xl capillary sequencer.

2.3.3 Phylogenetic analyses

Sequence chromatograms were edited with SeqEd 1.0.3 (Applied Biosystems) and aligned using Se-Al2.0a11 (http://tree.bio.ed.ac.uk/software/seal/) using inferred amino acid translations (*H3* and *Cytb*) and the predicted *28S rDNA* secondary structure model for *Gyrodactylus salaris* Malmberg, 1957, (Matejusová & Cunnigham 2004). Subsequently, the *28S rDNA* alignment was trimmed to 411bp to remove sequence that could not be aligned unambiguously and sequences from all three genes concatenated to give a final alignment length of 1409bp. We were unable to amplify all target fragments for some samples and these are coded in the final alignment as "missing data". All gene sequences have been deposited in GenBank. PartitionFinder v1.1.1 (Lanfear et al., 2012) was used to partition the data and select the most optimal model of nucleotide substitution for each partition based on the Akaike Information Criterion.

Bayesian phylogenetic analyses were run using the MPI version of MrBayes 3.2.6 (Ronquist et al., 2012) on a 12-core virtual machine on the NeCTAR research cloud under an Ubuntu 16.04 LTS image using Open MPI version 2.0.1 (https://www.open-mpi.org/). Analyses employed two runs, each with four chains, with ten million steps, sampling every 1000 steps. The first 20% of sampled topologies was discarded as burn-in based on stability of log likelihood values and that sampled topologies were essentially identical across runs, with standard deviation of split frequencies ~0.01 or less. Samples for

numerical parameters were also essentially identical, with variance between versus within runs approaching unity (Ronquist et al., 2012). The majority-rule consensus tree was constructed from the combined post-burn-in samples.

Maximum likelihood (ML) analyses was conducted using the RAxML BlackBox server (http://embnet.vital-it.ch/raxml-bb/) implementing the methods of Stamatakis et al. (2008). Data were partitioned as recommended by PartitionFinder and run using the Gamma model of rate heterogeneity.

All *Neobenedenia Cytb* and *28S rRNA* sequences on GenBank were downloaded and aligned to our data. We also included the *Cytb* sequence of the only full mitochondrial genome of this genus (JQ038228). A neighbour-joining analysis of the aligned data for each locus was conducted in MEGA 6.06 (Tamura et al., 2013), using uncorrected *P* distance, in an attempt to evaluate current identification.

2.4 Results

2.4.1 DNA sequence characteristics

Amplified *Cytb* and *H3* sequences did not contain any premature stop codons or frameshift mutations, contributing 704 and 292 characters respectively to the overall concatenated alignment. Even though we were able to amplify over 800bp of sequence for *28S rDNA*, the sequence spans a highly variable region which we were unable to unambiguously align, even when using the secondary structure of the *28S rDNA* sequence for *G. salaris* as a guide. Thus, we were forced to trim our *28S rDNA* sequences to 411

characters. Some *28S rDNA* and *H3* sequence chromatograms contained overlapping peaks indicative of heterozygous alleles and these sites were scored with IUPAC ambiguity codes for dimorphic sites. All individuals included in the analyses are represented by sequences of at least two of the genes (*Cytb* and *H3* or *28S rDNA*).

2.4.2 Phylogenetic analyses

Analysis of the concatenated dataset in PartitionFinder v1.1.1 suggested that three partitions (*Cytb* codon position 3; *Cytb* codon positions 1 and 2; *H3* codon positions 1, 2 and 3 and *28S rDNA*) were optimal for the data. PartitionFinder selected the General Time Reversible (GTR) model with a gamma distribution for rates across sites (GTR + G), GTR + G incorporating a proportion of invariable sites (GTR + I + G) and the Symmetrical model with a gamma distribution for rates across sites (SYM + G) for the partitions respectively.

Bayesian (Fig. 1) and Maximum Likelihood (ML) (Fig. 2) analyses yielded essentially identical topologies, with *Neobenedenia* samples forming a monophyletic clade to the exclusion of the outgroups and separated into four major clades (A-D). The *Neobenedenia* isolate recovered from *Lutjanus argentiventris* Peters, 1869, was the exception and was recovered as part of clade A in the Bayesian analysis (Fig. 1) but as sister to clade A in the ML analysis (Fig. 2; labelled A* for clarity) albeit with low bootstrap support (BS 24%).

Support for clades A-D was also not consistent between the Bayesian and ML analyses, with ML bootstrap far lower than expected from the Bayesian analysis (Fig. 1).

Samples that comprised clades B and D have been morphologically identified by the co-author IDW as *Neobenedenia pacifica* Bravo-Hollis, 1971, and *Neobenedenia longiprostata* Bravo-Hollis, 1971, respectively, while samples from both A and C are morphologically '*N. melleni*'. In Figure 2, Clade C contains a single specimen collected from *Sphoeroides annulatus*, while clade A contains 28 of the 33 in-group samples (excluding the sample from *L. argentiventris*) included in the study. The isolate currently in culture at the Marine Parasitology Laboratory at James Cook University, Townsville, Australia also fell into clade A (*Lates calcarifer*; Bowen, Queensland)).

The neighbour-joining tree constructed from all available *Cytb* sequences is presented in Figure 3. Given the proportions of informative sites, it is not surprising that the topology is reflective of the Bayesian and ML analyses. Furthermore, the sample collected from *L. argentiventris* is recovered as the sister of the clade A in congruence with the ML analysis (Fig. 2; clade A*). All but one of the sequences added from Genbank fall into clade A. The only sequence in another part of the tree is HMM222533 collected from *S. annulatus* and included in the study conducted by Perkins et al. (2009), which falls into clade B. Similarly, the neighbour-joining analysis from all available *28S rRNA* sequences also recovered all previously identified clades. The specimen collected from *L. argentiventris* was once again recovered as a separate lineage but clusters with another specimen collected from *Sebastes rubrivinctus* Jordan & Gilbert, 1880, that was part of clade A in all other analyses. Several sequences fell into clade B but all were sampled from *S. annulatus*.

Table 1: Host fish species from which *Neobenedenia* were sampled and used for sequencing from Australia and overseas.

Field code and host species	Family	Origin	Location	Country	Collector	Museum Accession Code
Lates calcarifer	Latidae	Wild	Gladstone, Queensland	Australia	Roger Chong	Code forthcoming
Lates calcarifer	Latidae	Captive-Farm	Bowen, Queensland	Australia	Alexander Brazenor	Code forthcoming
Lates calcarifer	Latidae	Captive-Farm	Hinchinbrook, Queensland (2011)	Australia	Kate Hutson	Code forthcoming
Lates calcarifer	Latidae	Captive-Research	MARF, Townsville, Queensland	Australia	Kate Hutson	Code forthcoming
Lates calcarifer	Latidae	Captive-Farm	-	Singapore	Erik Vis	Code forthcoming
Lates calcarifer	Latidae	Captive-Farm	Hinchinbrook, Queensland (2000)	Australia	Ian Whittington	IDW personal collection
Seriola lalandi	Carangidae	Captive-Farm	Western Australia	Australia	Ian Whittington	IDW personal collection
Trachinotus kennedeyi	Carangidae	Wild	La Paz	Mexico	Ian Whittington	IDW personal collection
Oligoplites altus	Carangidae	Wild	La Paz	Mexico	Ian Whittington	IDW personal collection
Gnathanodon speciosus	Carangidae	Wild	La Paz	Mexico	Ian Whittington	IDW personal collection
Gnathanodon speciosus	Carangidae	Captive-Aquarium	Durban	South Africa	David Vaughan	IDW personal collection
Seriola rivoliana	Carangidae	Captive-Farm	Hawaii	United States of America	Federico Notman	IDW personal collection
Rachycentron canadum	Rachycentridae	Captive-Research	MARF, Townsville, Queensland	Australia	Kate Hutson	Code forthcoming
Rachycentron canadum	Rachycentridae	Captive	-	Panama	Ian Whittington	IDW personal collection
Rachycentron canadum	Rachycentridae	Captive	-	Italy	Ian Whittington	IDW personal collection
Mycteroperca rosacea	Serranidae	Wild	La Paz	Mexico	Ian Whittington	IDW personal collection
Epinephelus coioides	Serranidae	Captive-Research	Cairns, Queensland	Australia	Terry Miller	Code forthcoming
Arothron caercaeruleopunctatus	Tetraodontidae	Captive-Ornamental	Townsville, Queensland	Australia	Thane Militz	Code forthcoming
Canthigaster bennetti	Tetraodontidae	Captive-Ornamental	Townsville, Queensland	Australia	Thane Militz	Code forthcoming
Sphoeroides annulatus	Tetraodontidae	Wild	La Paz	Mexico	Ian Whittington	IDW personal collection
Coryphaena hippurus	Coryphaenidae	Captive	Western Australia	Australia	Mark Hilder	IDW personal collection
Coryphaena hippurus	Coryphaenidae	Captive-Aquarium	Durban	South Africa	David Vaughan	IDW personal collection
Mugil curema	Mugilidae	Wild	La Paz	Mexico	Ian Whittington	IDW personal collection
Mugil curema	Mugilidae	Wild	La Paz	Mexico	Ian Whittington	IDW personal collection
Nemateleotris decora	Microdesmidae	Captive-Ornamental	Townsville, Queensland	Australia	Thane Militz	Code forthcoming
Pseudocheilinus hexataenia	Labridae	Captive-Ornamental	Townsville, Queensland (June 2012)	Australia	Thane Militz	Code forthcoming
Pseudocheilinus hexataenia	Labridae	Captive-Ornamental	Townsville, Queensland (December 2012)	Australia	Thane Militz	Code forthcoming
Pseudochromis fridmani	Pseudochromidae	Captive-Ornamental	Townsville, Queensland	Australia	Thane Militz	Code forthcoming
Verasper variegatus	Pleuronectidae	Captive-Farm	-	Japan	Noritaka Hirazawa	IDW personal collection
Sebastes rubrivinctus	Sebastidae	Captive-Farm	-	United States of America	Ian Whittington	IDW personal collection
Neocirrhites armatus	Cirrhitidae	Captive-Ornamental	Townsville, Queensland	Australia	Kate Hutson	Code forthcoming
Echeneis naucrates	Echeneidae	Captive-Ornamental	Woods Hole, Massachusetts	United States of America	Roger Williams	IDW personal collection
Sparus aurata	Sparidae	-	Eilat	Israel	Ariel Diamant	IDW personal collection
Lutjanus argentiventris	Lutjanidae	Wild	La Paz	Mexico	Ian Whittington	IDW personal collection

MARFU = Marine Aquaculture Research Facility Unit, James Cook University, Townsville.

Table 2: Primers used for PCR amplification of *Neobenedenia* spp. isolates.

Gene	Primer ID	Sequence (‘5-‘3)	Forward/Reverse	Source
H3	G926	GACCGCYCGYAAAAGYAC	F	[a]
	G927	AGCRTGRATDGCRCACAA	R	[a]
	H3aF	ATGGCTCGTACCAAGCAGACVGC	F	[b]
	H3R2	ATRTCCTTGGGCATGATTGTTAC	R	[b]
28S rDNA	C1	ACCCGCTGAATTTAAGCAT	F	[c]
	D1	TGGTCCGTGTTTCAAGAC	R	[c]
	EC-D2	CCTTGGTCCGTGTTTCAAGACGGG	R	[d]
Cytb	M1676	TGAGTTATTATTGATGTAGAGG	F	[e]
	M1677	AAAATATCAKTCAGGCTTWA	R	[e]

[a] Perkins et al. (2009); [b] Colgan et al. (1998); [c] Chisholm et al. (2001); [d] Littlewood et al. (1997); [e] present study.

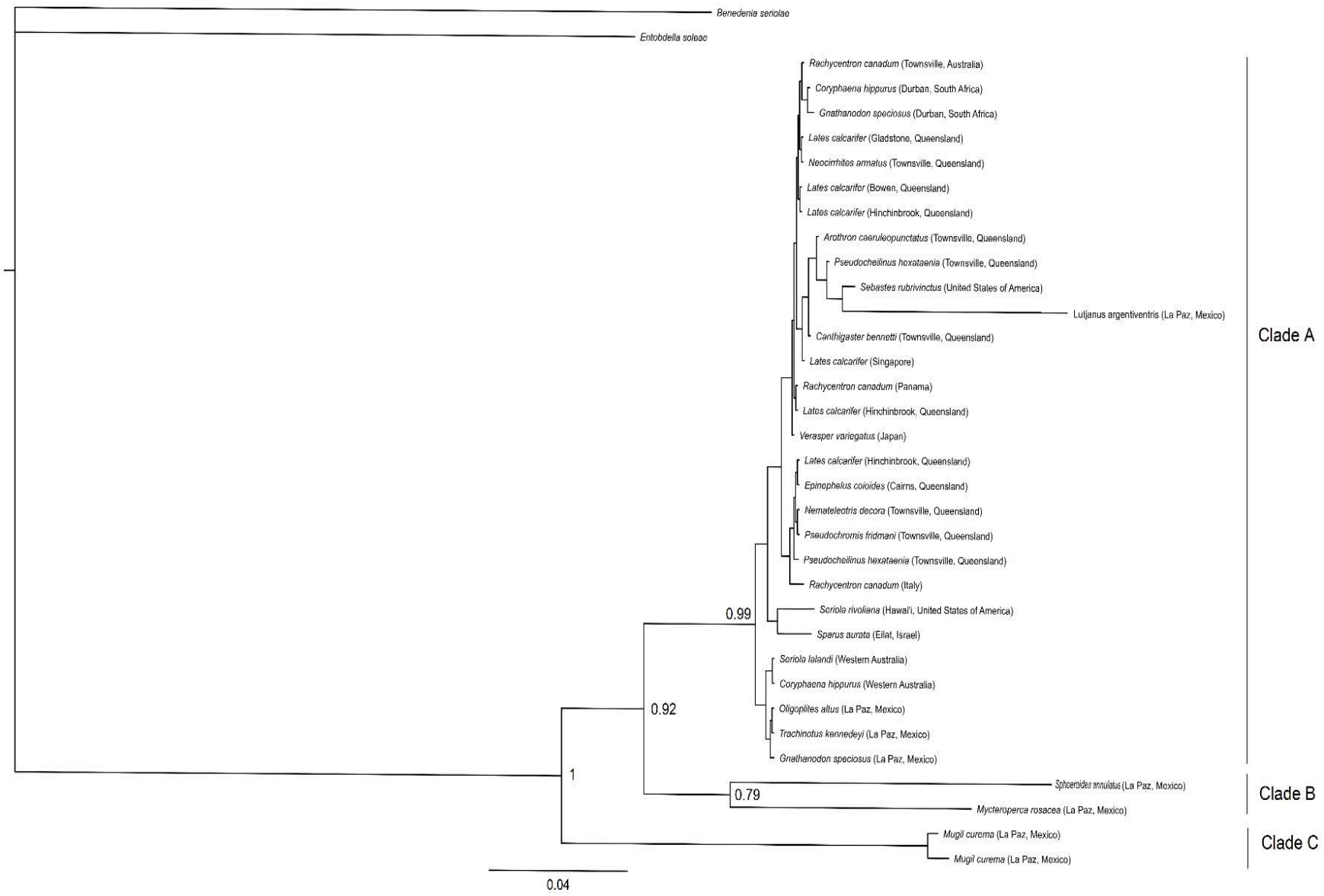
Benedenia seriolae
Entobdella soleae
Rachycentron canadum (Townsville, Australia)
Coryphaena hippurus (Durban, South Africa)
Gnathanodon speciosus (Durban, South Africa)
Lates calcarifer (Gladstone, Queensland)
Neocirrhites armatus (Townsville, Queensland)
Lates calcarifer (Bowen, Queensland)
Lates calcarifer (Hinchinbrook, Queensland)
Arothron caeruleopunctatus (Townsville, Queensland)
Pseudocheilinus hexataenia (Townsville, Queensland)
Sebastes rubrivinctus (United States of America)
Lutjanus argentiventris (La Paz, Mexico)
Canthigaster bennetti (Townsville, Queensland)
Lates calcarifer (Singapore)
Rachycentron canadum (Panama)
Lates calcarifer (Hinchinbrook, Queensland)
Verasper variegatus (Japan)
Lates calcarifer (Hinchinbrook, Queensland)
Epinephelus coioides (Cairns, Queensland)
Nemateleotris decora (Townsville, Queensland)
Pseudochromis fridmani (Townsville, Queensland)
Pseudocheilinus hexataenia (Townsville, Queensland)
Rachycentron canadum (Italy)
Seriola rivoliana (Hawai'i, United States of America)
Sparus aurata (Eilat, Israel)
Seriola lalandi (Western Australia)
Coryphaena hippurus (Western Australia)
Oligoplites altus (La Paz, Mexico)
Trachinotus kennedeyi (La Paz, Mexico)
Gnathanodon speciosus (La Paz, Mexico)
Sphoeroides annulatus (La Paz, Mexico)
Mycteroperca rosacea (La Paz, Mexico)
Mugil curema (La Paz, Mexico)
Mugil curema (La Paz, Mexico)
0.99
0.92
1
0.79
0.04
Clade A
Clade B
Clade C

Figure 1: Relationships of species of *Neobenedenia* isolates collected from wild and captive host fish based on Bayesian inference and maximum likelihood analyses of the *H3*, *28S rDNA*, and *cytochrome b* concatenated dataset. Posterior probability values are next to major nodes. *Benedenia seriolae* (HM222526.1 (*Seriola hippos* – South Australia, Australia), AY033941.1 (*Seriola quinqueradiata* – Japan), and FJ972088.1 (*Seriola hippos* – South Australia, Australia)) and *Entobdella soleae* (FJ972108.1 (*Solea solea* – United Kingdom), AY486152.1 (*Solea solea* – United Kingdom), HQ684799.1 (unknown host – China)) sequences obtained from Genbank were included as outgroups.

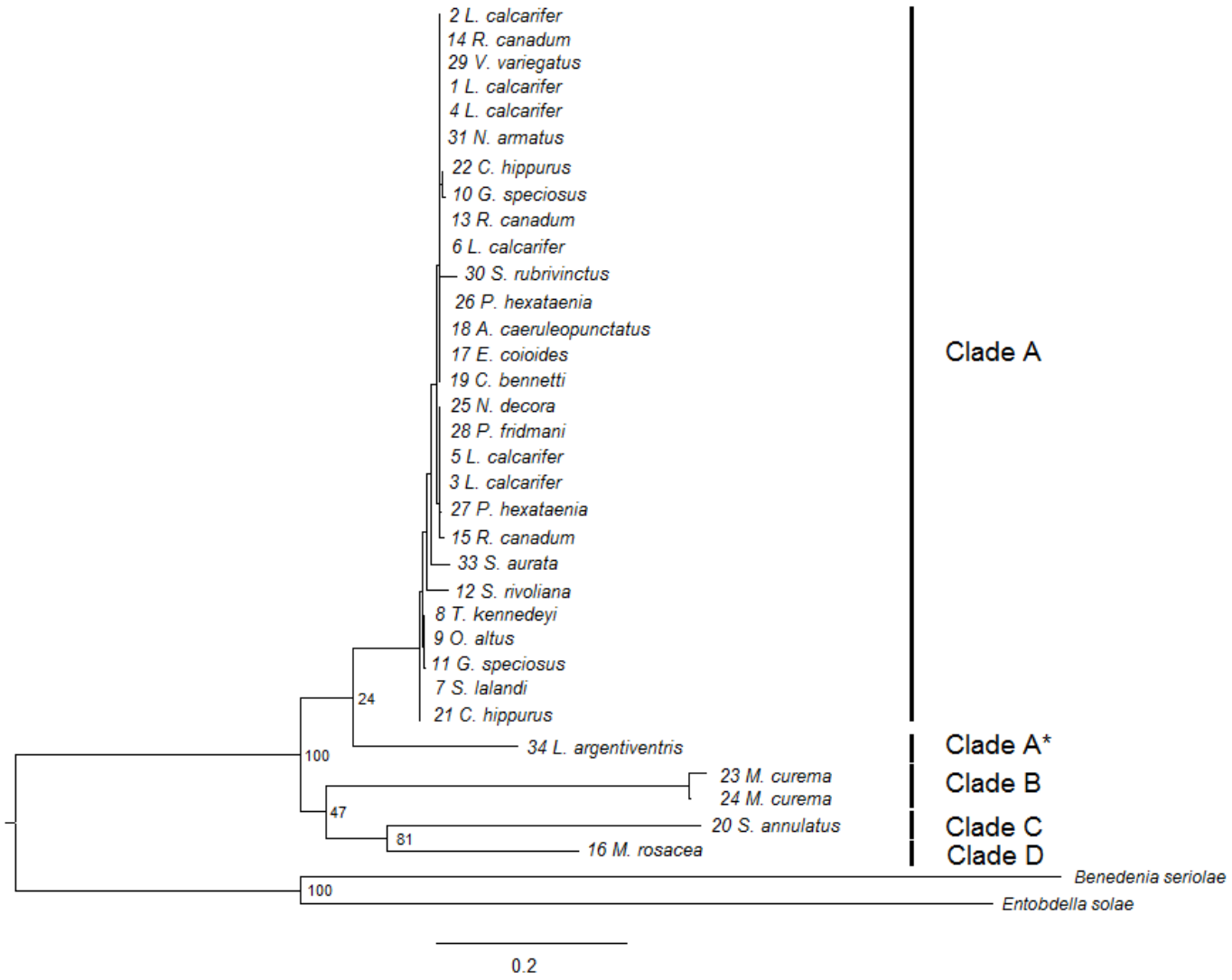
2 L. calcarifer
14 R. canadum
29 V. variegatus
1 L. calcarifer
4 L. calcarifer
31 N. armatus
22 C. hippurus
10 G. speciosus
13 R. canadum
6 L. calcarifer
30 S. rubrivinctus
26 P. hexataenia
18 A. caeruleopunctatus
17 E. coioides
19 C. bennetti
25 N. decora
28 P. fridmani
5 L. calcarifer
3 L. calcarifer
27 P. hexataenia
15 R. canadum
33 S. aurata
12 S. rivoliana
8 T. kennedeyi
9 O. altus
11 G. speciosus
7 S. lalandi
21 C. hippurus
34 L. argentiventris
23 M. curema
24 M. curema
20 S. annulatus
16 M. rosacea
Benedenia seriolae
Entobdella solae
Clade A
Clade A*
Clade B
Clade C
Clade D
24
100
47
81
100
0.2

Figure 2: Relationships of species of *Neobenedenia* isolates collected from wild and captive host fish based on Maximum likelihood analysis of the *H3*, *28S rDNA*, and *cytochrome b* concatenated dataset. Maximum likelihood bootstrap proportions are next to major nodes. *Benedenia seriolae* (HM222526.1 (*Seriola hippos* – South Australia, Australia), AY033941.1 (*Seriola quinqueradiata* – Japan), and FJ972088.1 (*Seriola hippos* – South Australia, Australia)) and *Entobdella soleae* (FJ972108.1 (*Solea solea* – United Kingdom), AY486152.1 (*Solea solea* – United Kingdom), HQ684799.1 (unknown host – China)) sequences obtained from Genbank were included as outgroups.

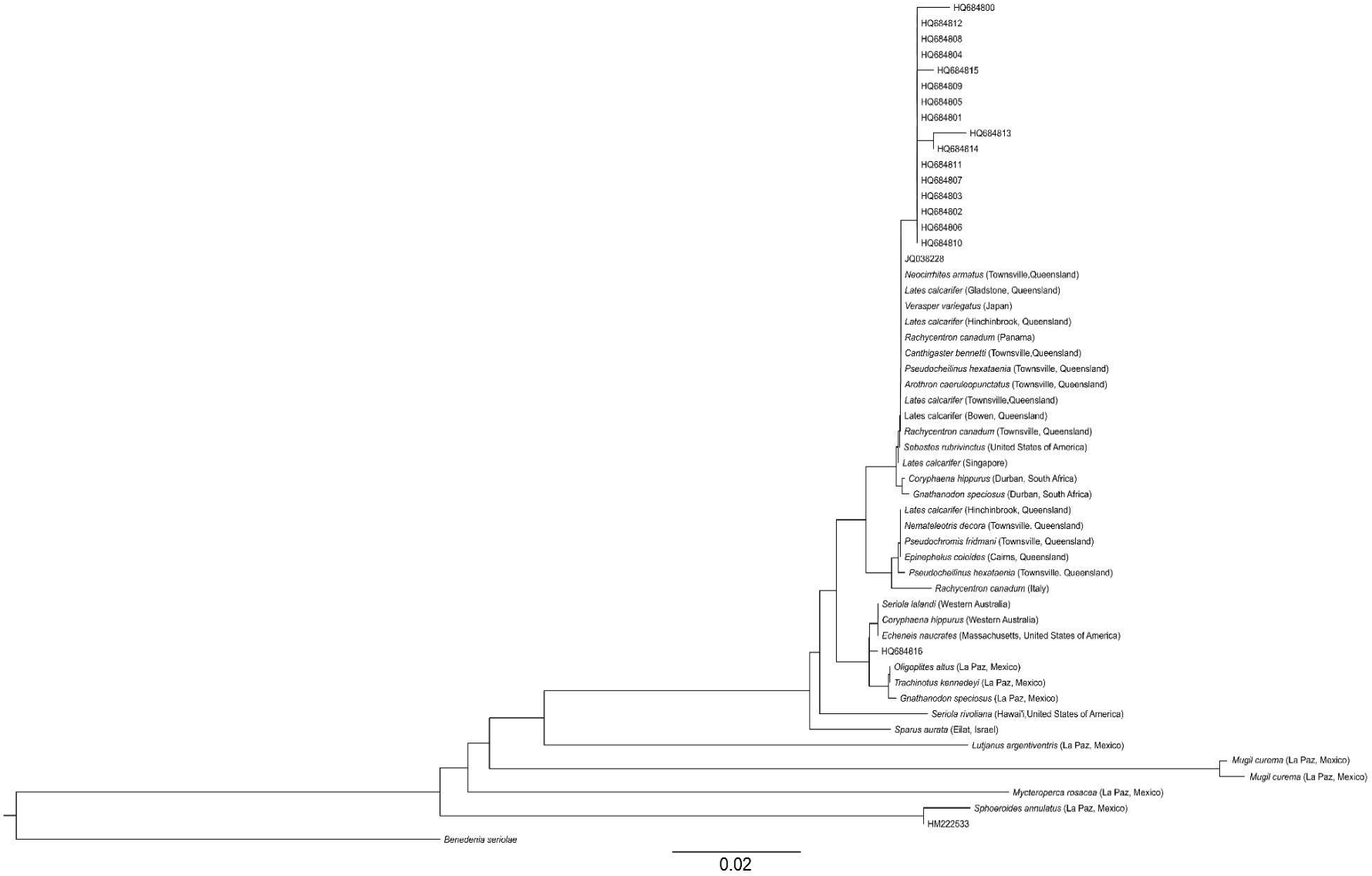
HQ684800
HQ684812
HQ684808
HQ684804
HQ684815
HQ684809
HQ684805
HQ684801
HQ684813
HQ684814
HQ684811
HQ684807
HQ684803
HQ684802
HQ684806
HQ684810
JQ038228
Neocirrhites armatus (Townsville,Queensland)
Lates calcarifer (Gladstone, Queensland)
Verasper variegatus (Japan)
Lates calcarifer (Hinchinbrook, Queensland)
Rachycentron canadum (Panama)
Canthigaster bennetti (Townsville,Queensland)
Pseudocheilinus hexataenia (Townsville, Queensland)
Arothron caeruleopunctatus (Townsville, Queensland)
Lates calcarifer (Townsville,Queensland)
Lates calcarifer (Bowen, Queensland)
Rachycentron canadum (Townsville, Queensland)
Sebastes rubrivinctus (United States of America)
Lates calcarifer (Singapore)
Coryphaena hippurus (Durban, South Africa)
Gnathanodon speciosus (Durban, South Africa)
Lates calcarifer (Hinchinbrook, Queensland)
Nemateleotris decora (Townsville, Queensland)
Pseudochromis fridmani (Townsville, Queensland)
Epinephelus coioides (Cairns, Queensland)
Pseudocheilinus hexataenia (Townsville, Queensland)
Rachycentron canadum (Italy)
Seriola lalandi (Western Australia)
Coryphaena hippurus (Western Australia)
Echeneis naucrates (Massachusetts, United States of America)
HQ684816
Oligoplites altus (La Paz, Mexico)
Trachinotus kennedyi (La Paz, Mexico)
Gnathanodon speciosus (La Paz, Mexico)
Seriola rivoliana (Hawai'i,United States of America)
Sparus aurata (Eilat, Israel)
Lutjanus argentiventris (La Paz, Mexico)
Mugil curema (La Paz, Mexico)
Mugil curema (La Paz, Mexico)
Mycteroperca rosacea (La Paz, Mexico)
Sphoeroides annulatus (La Paz, Mexico)
HM222533
Benedenia seriolae
0.02

Figure 3: Relationships of species of *Neobenedenia* isolates collected from wild and captive host fish based on a Neighbour-joining analysis of partial *cytochrome b* sequences collected from Genbank and the present study. Genbank samples included in this analysis are titled using their Genbank identification code and were collected from a variety of host species. *Benedenia seriolae* (FJ972088.1 (*Seriola hippos* – South Australia, Australia)) sequence obtained from Genbank was included as an outgroup.

2.5 Discussion

The correct identification of species underpins all biological study and, for pathogens, is critical in understanding infection dynamics, predicting outbreaks, and determining effective control strategies. In the genus *Neobenedenia*, however, morphological variation, attributed to host induced morphological variation (Whittington & Horton 1996), failure to accession specimens (including wet and mounted material) to curated collections (e.g. Jahn & Kuhn 1932; Nigrelli & Breder 1934; Nigrelli 1935; 1937; 1947; Müeller et al., 1992; Robinson et al., 1992; Cowell et al., 1993; Ellis & Watanabe 1993; Koesharyani et al., 1999; Zhang et al., 2014) and the lack of a robust genetic framework to aid in identification, has hampered these efforts. This study presents the most comprehensive phylogenetic investigation of *Neobenedenia* spp. to date, incorporating 33 isolates spanning 23 host fish species and nine countries and used both mitochondrial and nuclear gene markers.

Our results clearly show that there is a single species of *Neobenedenia* (Fig 1-3; clade A) that is widespread geographically, able to infect a large number of different host fishes, and can be found on wild, farmed, and captive fish. Most importantly, clade A does not contain the *Neobenedenia* specimen from *Sphoeroides annulatus*, which is likely to be the type host of *N. melleni* according to the description by McCallum (1927) and analysis by Whittington & Horton (1996). The sample from *S. annulatus* (Fig 1-3; clade B), which was collected from wild fish in southern Mexico, is well differentiated from clade A and is the sister species to *N. longiprostata* in our analyses. Furthermore, except for HM222533, which was sampled from *S. annulatus* (Perkins et al., 2009) and clusters with our sample in clade B, all other *CytB* sequences obtained from Genbank which are named both *N. melleni*

and *N. girellae* all fall in clade A, including the *CytB* sequence from the full mitochondrial genome (JQ038228), which is supposedly *N. melleni* (Zhang et al., 2014). Unfortunately, metadata associated with these sequences are brief and do not describe the host species or accurate locality data from which parasites were collected. Similarly, all Genbank *28S rRNA* sequences fall into clade A unless they were sampled from *S. annulatus*, where they clustered with clade B. Thus, the results of the present study support the findings by Whittington et al. (2004) and Perkins et al. (2009) that *N. melleni* and *N. girellae* are cryptic species in that they are morphologically very similar but genetically distinct. In an effort to stabilise the naming of *Neobenedenia* species, it seems prudent to ascribe *N. melleni* to individuals in clade B and retain *N. girellae* for those individuals in clade A. The authors recommend reinstating *N. girellae* as a member of *Neobenedenia*.

It is likely that clade A* (Fig 2) represents another species of *Neobenedenia,* however, at this stage it is unclear whether this sample is a previously recorded *Neobenedenia* species or if it is novel to science. Morphologically, this specimen was classified as '*N. melleni*' by IDW. The single specimen represented in this clade is the only one collected from a lutjanid host (*Lutjanus argentiventris* collected from La Paz, Mexico) and the assessment of this family of hosts for potential *Neobenedenia* species is an important avenue of research to pursue. The Bayesian placement of this specimen into clade A (Fig 2) appears incorrect given the long branch length and ML and neighbor-joining analyses clearly differentiate it from other samples in this study. Further morphological and molecular work is underway to assess that status of this individual.

The findings of this study suggest that, historically, many of the parasites identified as *N. melleni* may in fact be *N. girellae* given how few isolates were identified using

molecular methods as *N. melleni* in this study (e.g. Bullard et al., 2000; Deveney et al., 2001; Wang et al., 2004; Kerber et al., 2011; Landos, 2012; Zhang et al., 2014). Accurate identification of *Neobenedenia* species may be hampered by intraspecific morphological plasticity thereby resulting in false identifications. Despite the recognised difficulty in differentiating *N. melleni* and *N. girellae*, the majority of studies focussing on *Neobenedenia* spp. have not stated that they have accessioned samples to museum or private collections. This makes clarification of previous identifications almost impossible and accurate identification of historical host species and geographic locations for each of these two taxa unachievable. Given the number of isolates that were included in this study and the varied host species and locations they were collected from, it is likely that the number of hosts and locations that *N. melleni* has been credited infecting and inhabiting has been overestimated. To facilitate morphological and genetic comparison of individuals in the future, it is recommended to accession reference material (both mounted and fixed specimens).

Using our genetic framework, it will be possible to reassess the morphological differences between *N. girellae* and *N. melleni a posteriori*. The *CytB* neighbour-joining analysis shows significant substructuring in *N. girellae*, which may account for the variable morphology noted by Whittington & Horton (1996). Morphological variation can be due to epigenetic factors which influence the phenotype expressed depending on the particular environment being experienced or may simply be due to the normal growth of the parasite during its life (Via et al., 1995; Agrawal 2001; Lackenby et al., 2007; Olstad et al., 2009; Mati et al., 2014; Chapter 3). The flexibility displayed by these parasites makes the identification of species solely through morphological means considerably challenging

(Barcak et al., 2014; Bickford et al., 2006). As closely related cryptic parasite species may exhibit variable traits to one another such as host pathogenicity (Haque et al., 2003; Homan & Mank 2001; Skovgaard et al., 2002), and epidemiology (Murrell & Pozio 2000), accurate identification is crucial to understand the risk posed by the presence of a particular species in a system.

Neobenedenia girellae infects a large number of fish species of economic importance including those from commercial fisheries, aquaculture, and the ornamental trade (Ogawa et al., 1995; 2006). *Neobenedenia girellae* isolates were collected from several fish species that support major aquaculture production or commercial fisheries including *Lates calcarifer* (barramundi or Asian sea bass), *Epinephelus coioides* (gold-spot grouper) Hamilton, 1822, *Coryphaena hippurus* (mahi mahi or dolphinfish) Linnaeus, 1758, *Plectropomus leopardus* (coral trout) Lacepède, 1802, *Rachycentron canadum* (cobia) Linnaeus, 1766, *Seriola lalandi* (yellowtail kingfish) Valenciennes, 1833, and *Seriola rivoliana* (almaco jack) Valenciennes, 1873 (Fig. 1). Parasites infecting eight species of popular ornamental fish species from seven families were among the isolates included in this study. All represent new host records for *N. girellae* and show the diversity of host species this parasite can infect (Table 1; see *3.1.*). Ornamental fish support a huge, multi-national industry that involves hundreds of fish species (and their associated pathogen communities) being transported all around the globe (Bruckner, 2005). This provides an excellent opportunity for the dispersal of *N. girellae*, further encouraged by its lack of host specificity.

Isolates collected from two outbreaks on *L. calcarifer* which occurred in Queensland Australia, the first on a fish farm in Hinchinbrook in 2000 and the second in

Gladstone in 2014 were included in this study (Table 1) and were determined to be a part of the *N. girellae* clade (Figure 1-2). The parasites of concern were initially identified as *N. melleni* by Deveney et al. (2001) and Landos (2012), however, our study suggests that these were erroneous identifications and the species associated with these mortality events was in fact *N. girellae*. In addition, an unidentified population of *Neobenedenia* cultured at the Marine Parasitology Laboratory at James Cook University, Townsville has also been identified as *N. girellae* using this analysis. As such, all previous research on this species from the Marine Parasitology Laboratory is now ascribed to *Neobenedenia girellae* (i.e. Hutson et al., 2012; Dinh Hoai & Hutson 2014; Militz et al., 2014; Brazenor & Hutson 2015; Militz & Hutson 2015; Trujillo-Gonzalez et al., 2015a; 2015b).

The large diversity of host species that *N. girellae* is able to infect (23 host species determined from the present study) and the geographic range in which it is present (found between latitudes 23.8489° S and 24.1422° N (wild isolates) and 23.8426° S and 24.1426° N (all isolates) from samples included in this study) makes it a globally cosmopolitan species and a threat to aquaculture industries around the world.

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Chapter 3: Morphological variation in the cosmopolitan fish parasite Neobenedenia girellae (Capsalidae: Monogenea)

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3.1 Abstract

Intra-species morphological variation presents a considerable problem for species identification and can result in taxonomic confusion. This is particularly pertinent for species of *Neobenedenia* which are harmful agents in captive fish populations and have historically been identified almost entirely based on morphological characters. This study aimed to understand how the morphology of *Neobenedenia girellae* varies with host fish species and the environment. Standard morphological features of genetically indistinct parasites from various host fish species were measured under controlled temperatures and salinities. An initial field-based investigation found that parasite morphology significantly

differed between genetically indistinct parasites infecting various host fish species. The majority of the morphological variation observed (60%) was attributed to features that assist in parasite attachment to the host (i.e. the posterior and anterior attachment organs and their accessory hooks) which are important characters in monogenean taxonomy. We then experimentally examined the effects of the interaction between host fish species and environmental factors (temperature and salinity) on the morphology of isogenic parasites derived from a single, isolated hermaphroditic *N. girellae* infecting barramundi, *Lates calcarifer*. Experimental infection of *L. calcarifer* and cobia, *Rachycentron canadum*, under controlled laboratory conditions did not confer host-mediated phenotypic plasticity in *N. girellae*, suggesting that measured morphological differences could be adaptive and only occur over multiple parasite generations. Subsequent experimental infection of a single host species, *L. calcarifer*, at various temperatures (22, 30 and 32 ºC) and salinities (35 and 40‰) showed that in the cooler environments (22 ºC) *N. girellae* body proportions were significantly smaller compared with warmer temperatures (30 and 32 ºC; $P < 0.0001$), whereas salinity had no effect. This is evidence that temperature can drive phenotypic plasticity in key taxonomic characters of *N. girellae* under certain environmental conditions.

Keywords: Platyhelminthes; Skin fluke; Phenotypic plasticity; Natural selection; Aquaculture; Cryptic species

3.2 Introduction

Phenotypic plasticity is the ability of an organism to change its phenotype, including morphology and physiology, in response to changes in the environment (Kelly et al., 2012; Padilla and Savedo, 2013). In aquatic systems, there is increasing interest in the role of phenotypic plasticity in species interactions (Gimenez, 2004; Badets et al., 2009; Padilla and Savedo, 2013). Examples of this include the development of defensive structures (e.g. spiky projections from the carapace, enlarged false eye spots) by prey in response to the presence of predators (Harvell, 1986; Harvell and Padilla, 1990; Lönnstedt et al., 2013). These changes can be crucial for the survival of the organism and individuals which show high phenotypic plasticity have higher fitness than those that do not (Price et al., 2003). Changes can be due to epigenetic factors which influence the phenotype expressed and depend on the environment (e.g. temperature, nutrients) (Via et al., 1995; Olstad et al., 2009; Padilla and Savedo, 2013; Mati et al., 2014).

Parasites can exhibit extensive morphological variation and numerous environmental factors influence their morphology (Dmitrieva and Dimitriov, 2002; Huyse and Volkaert, 2002; Davidova et al., 2005; Olstad et al., 2007, 2009). The specific host species that is infected has considerable implications for the morphology of terrestrial and aquatic parasites (Waller and Thomas, 1978; Ponce de Leon, 1995; Mouhaid et al., 1997; Mladineo et al., 2013). One example of this is the smaller size of sclerotised features of *Gyrodactylus salaris*, Malmberg, 1957, when infecting preferred host species compared with taxa that are less appropriate for infection (Mo, 1991a; Olstad et al., 2009). This is hypothesised to be a consequence of increased reproductive rate and accelerated

embryogenesis on the preferred host species (Olstad et al., 2009). Similarly, camouflage pigment in the parasite's body tissue (e.g. spots of melanin) can vary between individuals of the same species depending on which microhabitat is exploited (Roubal and Quartararo, 1992) or which host fish species is infected (Whittington, 1996). Furthermore, nutrient deprivation has been reported to distort development of embryos and the sclerotised structures on the attachment organ, the haptor, in the monogenean, *Gyrodactylus gasterostei* Gläser, 1974 (see Cable et al., 2002). The extent of morphological variation exhibited within a single parasite species underpins our understanding of biodiversity, biogeography, taxonomy, and the management of animal health (Barber et al., 2000; MacKenzie, 2002; Young et al., 2007). Inaccurate assessment of morphological plasticity can compound taxonomic confusion and result in inaccurate species-level diagnoses.

Monogenean parasites are excellent model organisms to examine the environmental drivers of morphological variation because they possess a small number of distinct morphological features used in their taxonomy (Perkins et al., 2009). The limited number of features by which species definition can be made morphologically has been implicated as one of the main reason for why taxonomic confusion has occurred in this group of parasites (see Perkins et al., 2009; Sepúlveda and González, 2014). Many monogeneans are also able to self-reproduce (Dinh Hoai and Hutson, 2014; Kearn and Whittington, 2015) and isogenic populations can be established that remove genetically mediated morphological differences between individuals (Olstad et al., 2009). Morphological identification of species is largely based on body size and the sizes and shapes of sclerites and hamuli (Jahn and Kuhn, 1932; Hargis, 1955). Intraspecific variability in morphology presents a considerable problem for morphology-based taxonomy when informative

characters are affected and this issue must be addressed if further taxonomic confusion is to be avoided (Bickford et al., 2006; Olstad et al., 2009; Barcak et al., 2014).

Most monogenean species, including species in the genus *Neobenedenia*, are delineated and diagnosed based on their morphology and understanding the range of morphological variation is key to accurate identification (Vossbrinck and Debrunner-Vossbrinck, 2005; Barcak et al., 2014; Bazsalovicsova et al., 2014). The aim of this study was to determine whether host species, temperature and salinity elicit morphological variation in the monogenean ectoparasite *Neobenedenia girellae*.

3.3 Materials and Methods

3.3.1 Morphological variation of Neobenedenia girellae *isolates from wild and farmed fishes*

Host-induced morphological variation was investigated for *N. girellae* infecting six fish species. Wild-caught, farmed or captive perciform fish were sampled for parasites including *Lates calcarifer* Bloch, 1790 (Latidae), *Epinephelus coioides* Hamilton, 1822 (Serranidae), *Rachycentron canadum* Linnaeus, 1766 (Rachycentridae), *Plectropomus leopardus* Lacepède, 1802 (Serranidae), *Neocirrhites armatus* Castelnau, 1873 (Cirrhitidae), and *Atrosalarias fuscus* Rüppell, 1838 (Blenniidae). The origin and collection localities for each fish species examined are summarised in Table 1. Parasites were collected by bathing fish in dechlorinated freshwater for 5 min which kills and detaches monogeneans (see Militz et al., 2013) but causes little stress to marine fish given

the short period of bathing (see Evans, 2008). Parasites were preserved in 70% ethanol (EtOH) for genetic analysis, staining, mounting and measuring.

Morphological measurements were made for ~30 individual, randomly selected parasites from each host species (Table 1). Parasites fixed in 70% EtOH were washed three times using distilled H_2O and stained with Mayer's haematoxylin. Parasites were then dehydrated through an alcohol series, cleared in cedarwood oil and individually mounted on microscope slides in Canada balsam under a coverslip. Parasite total length, total width, diameter of testes, haptor diameter, anterior attachment organ diameter, pharynx diameter, anterior hamuli and accessory sclerites were measured using a micrometer and ImageJ 1.44p (Java 1.6.0_20; Fig. 1). These features were chosen as body size (body length and width) and sclerotised features (e.g. hamuli and sclerites) are considered to be taxonomically important characters for *N. girellae*, Hargis, 1955 (see Hargis, 1955; Whittington and Horton, 1996). Measurements are given as: mean (minimum – maximum range). Measurements of the total length, total width, anterior hamuli, and accessory sclerites follow Lackenby et al. (2007). All other measurements were taken at the longest or widest point (Fig. 1). Representative parasite specimens (10 individuals per host fish species) were accessioned in the South Australian Museum, Australia (SAMA) Australian Helminth Collection (AHC) (SAMA AHC codes: 36338-36387).

3.3.2 Genetic analysis of Neobenedenia girellae *isolates from wild and farmed individuals*

Representative *Neobenedenia* specimens from all six fish species were sequenced to provide molecular evidence that only a single species of parasite was recovered. Two individual parasites were sequenced for each fish species by slicing a thin strip of tissue from the parasite's right margin with a scalpel blade. Verification that parasites were the same species was made by amplifying three genes; two nuclear (H3 and 28S rDNA) and one mitochondrial (*cytochrome b* (*Cytb*)).

In brief, DNA was extracted from parasite slivers using either the PUREGENE DNA purification system (Gentra Systems, United States of America) protocol for DNA purification from solid tissue or a QIAgen DNeasy Kit (QIAGEN Inc., Valencia, California, USA) according to the manufacturers' protocols. PCR amplifications of partial H3, 28S rDNA, and *Cytb* sequence were carried out in 25 µl reactions using the primers listed in Table 2 and either Amplitaq Gold DNA polymerase, or Phusion high-fidelity polymerase with the following reaction and cycling conditions: Amplitaq Gold –following Perkins et al. (2009) except that the annealing temperature at 55°C was standardised and a maximum of 34 PCR cycles was used; Phusion - a final concentration of 5 µL of 5xPhusion® High-Fidelity buffer, 0.5 µL of 10mM dNTPs, 1.25 µL of each primer (10 mM), 0.25 µL of Phusion® Tag DNA polymerase, and 4 µL of DNA template was used with an initial denaturation step of 98 °C for 30 s, followed by 35 cycles of PCR; denaturation at 98 °C for 10 s, annealing 53-62 °C for 20 s, extension at 72 °C for 30 s, with an additional final extension at 72 °C for 7.5 min. The double-stranded amplification products were visualised on 1.5% agarose gels and purified using a Multiscreen –PCR Plate (Millipore Corporation, United States of America). Purified products were sent to the Australian Genome Research Facility for cycle sequencing in both directions using the

BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, United States of America) on an AB3730xl capillary sequencer. All gene sequences have been deposited in GenBank (MG193660-MG193675).

3.3.3 Experimental assessment of morphological variation in genetically constrained Neobenedenia girellae *isolates on three host species under controlled conditions*

To determine if host has an effect on parasite morphology, an experiment was performed to control potential confounding environmental variables including infection source, parasite population genetics, light periods, temperature, salinity and host nutrition. Three captive fish species (*L. calcarifer*, *R. canadum* and *Epinephelus lanceolatus* Bloch, 1790) were challenged with parasite larvae (oncomiracidia) hatched from eggs laid by a single, isolated hermaphroditic *N. girellae*. *Neobenedenia girellae* is capable of reproducing in isolation and produces embryonated eggs that hatch into viable, infective oncomiracidia (Dinh Hoai and Hutson, 2014). Parasites were sourced from an established monoculture maintained on barramundi, *L. calcarifer* (see Hutson et al., 2012; James Cook University, Australia, ethics approval: A1989). A single *L. calcarifer* host fish (~140 mm total length) was infected with a single oncomiracidium at ~23 ºC. Monitoring for egg production was performed each day (as per Section 2.4.) and once eggs were detected, those were collected and incubated in 35 ‰ seawater at 24 ºC for use in the experiment. Confirmation that all parasites used and collected in this experiment were genetically identical was made by comparing *Cytb* sequences from three individual parasites per host fish species sampled as per previous protocols (see Section 2.1.). *Cytb* was chosen for

amplification because it is effective for determining intraspecific differences in *Neobenedenia* spp. (Brazenor et al., unpublished data). DNA was extracted using the methods stated above (see Section 2.2.) and PCR amplification of a partial *Cytb* sequence was carried out using previously developed primers (Brazenor et al., unpublished data). Cycling parameters were as described above (see Section 2.2.).

Ten replicate fish from three species (*L. calcarifer, E. lanceolatus* and *R. canadum*; mean 141 total length (TL) (116–265 mm), 105 TL (90–127 mm) and 191 TL (175–220 mm), respectively) were individually infected with 50 *N. girellae* oncomiracidia. Experimental fish were sourced from fish hatcheries in Queensland, Australia. Each individual fish was held separately in a 10 L recirculating aquarium. The aquarium system comprised two 300 L saltwater (35 ‰) recirculating systems (i.e. 15 aquaria per system) on a 12:12 light:dark cycle. Individual fish were randomly allocated to aquaria using the random number generator function in Excel 2010. The flow rate in each aquarium was 0.6 L/min and each aquarium was aerated with an airstone. Each aquarium was provided with a hide (12 cm long, 4.5 cm diameter, white, plastic pipe). Water in the system was continuously filtered through a 100 μm filter.

The temperature was maintained at 22.4 ± 2.8 °C. Dissolved nutrients including ammonia, nitrite and nitrate were monitored daily and were maintained below 0.5, 1 and 20 ppm, respectively. Salinity was monitored daily using a refractometer and maintained at 35 ‰ throughout the duration of the study. A partial water change (~20 % of the total volume) was conducted every 4 days and all fish were fed 1 g of extruded fish pellets (2 mm diameter Ridley fish-meal pellets) every 2 days.

Fish were sampled for juvenile parasites (8 days post infection (p.i.); $n = 3$ individual fish per species) and adult parasites (14 days p.i.; $n = 7$ individual fish per species). To sample parasites, fish were individually bathed (fully immersed) twice in dechlorinated freshwater for 5 min to kill *N. girellae*. The epithelial surface of the fish was gently rubbed in order to dislodge any remaining attached parasites (see Militz et al., 2013). Parasites were collected by filtering the bath solution through a 60 µm mesh and then counted under a stereomicroscope at 30x magnification. Infection success was determined as the proportion of parasites collected divided by the number of oncomiracidia initially introduced. Each individual fish was numbered and their corresponding parasites were fixed in separate vials in 70 % EtOH. *Neobenedenia girellae* were stained, dehydrated, mounted and measured as above (see Section 2.1.). Representative parasite specimens (10 individuals from each host fish) were accessioned in the SAMA AHC (SAMA AHC codes: 36388-36399).

3.3.4 Effect of temperature and salinity on morphological variation of genetically constrained Neobenedenia girellae *infecting* Lates calcarifer in vivo

The effects of temperature and salinity on the morphological variation of *N. girellae* were examined by measuring parasites which developed in one of three temperatures (22, 30, or 32 ºC) at two salinities (35 and 40 ‰) in vivo on a single host species, *L. calcarifer.* Temperature and salinity were chosen as variables to investigate as those are recognised as being crucial to the development of *N. girellae* (Brazenor and Hutson, 2015). The effect of salinity on morphology of parasites was measured by

separating the same individuals above by the salinity they developed in either 35 or 40 ‰. Fish (mean 180 TL (150–200 mm)) were maintained individually in 10 L aquaria with individual air-stones with five replicate aquaria for each temperature and salinity. The aquaria were placed into an incubator (Sanyo: ML-351 Versatile Environmental Incubation Chamber) with fluorescent lighting for a 12:12 light:dark cycle and aerated using battery powered aerators. Oncomiracidia sourced from a genetically constrained laboratory culture and used to infect fish in this study were collected by incubating eggs in each temperature and salinity treatment combination. Immediately following hatching, 10 vigorously swimming oncomiracidia were gently aspirated with a glass pipette and slowly ejected from the pipette into each aquarium. Five replicates were made for each temperature/salinity treatment. Pieces of large gauge (1 mm) netting (20×10 cm) were placed into each of the aquaria and inspected under a stereomicroscope at 2× magnification daily at 10:00 h for tangled eggs, indicating the presence of at least one sexually mature parasite. A pilot study showed that eggs always entangled on the netting on the first day that they were laid (A.K. Brazenor unpublished data). Accordingly, time to sexual maturity was considered to be the day that eggs were first observed on netting. Fish were bathed twice in dechlorinated freshwater for 5 min the day following first detection of eggs. The epithelial surface of each fish was gently rubbed in order to dislodge attached parasites (see Militz et al., 2013). The solution was filtered through a 60 μm mesh to collect dislodged individuals which were counted under a stereomicroscope. Parasites were then collected and mounted and measured as per the methods described above (see Sections 2.1. and 2.3.). Representative specimens (five individual parasites from each temperature and salinity) were accessioned in the SAMA AHC (SAMA AHC codes: 36400-36405).

All procedures used in this research were approved by the Animal Ethics Committee of James Cook University, approval code: A1989.

3.3.5 Statistical analysis

Morphological variation in *N. girellae* was examined using measurements of standard morphological features (Fig. 1) from parasites with 100% sequence homology (for H3, 28S rDNA, and *Cytb*) from various host fish species captured in the field (see Section 2.1.) and under controlled conditions in the laboratory (see Section 2.3.). *Neobenedenia girellae* grows continuously until death (A.K. Brazenor, unpublished data), a phenomenon known to occur in *Benedenia seriolae,* Yamaguti, 1934 (Lackenby et al., 2007) and other monogenean species (Kearn, 1963). To discount the potential for age and size related trends, measurements are presented as morphometric ratios (Sepúlveda and González, 2014). Ratios included body length:body width, haptor width:anterior attachment organ width, pharynx width:body width, testes width:body width, anterior attachment organ width:body width, sclerite length:body length, hamulus length:body length, haptor width:body width. With the exception of body length:body width, ratios comprised measurements of morphological features made in the same plane on the organism (i.e. lateral or longitudinal measurements of paired morphological features; Fig. 1).

Morphometric ratios were initially fourth root transformed prior to analysis and then used to create Bray-Curtis Similarity Matrices (Clarke, 1993). Multivariate data were visualised as a non-metric multidimensional scaling ordination and a permutational

analysis of variance (PERMANOVA) was conducted in PRIMER 7.0 (PRIMER, Plymouth, UK) followed by pairwise comparisons to determine significant differences between morphometric proportions among host species, temperature, and salinity. The relative contributions of each ratio to the variation observed was determined using similarity percentage (SIMPER) analysis. Differences in parasite morphometric ratios in each of the three experiments (see Sections 2.1. – 2.4.) were investigated independently.

A parametric analysis of variance (ANOVA) was used to assess parasite infection success at days 8 and 14 (see Section 2.2.). Data satisfied normality and homogeneity of variance assumptions. Analyses were performed using the S-Plus 8.0 software package from Spotfire®. Post hoc comparisons of group means were performed using Tukey's honest significance difference test. Significance was accepted at $P < 0.01$.

3.4 Results

3.4.1 Morphology of Neobenedenia girellae *isolates from wild and farmed fishes*

Parasites collected from six wild and captive fish species were genetically similar and determined to represent a single species, *Neobenedenia girellae* (Brazenor et al., unpublished data). Through observation of these specimens, it was noted that some were significantly dissimilar in morphology. Host species significantly influenced the morphology of parasites (PERMANOVA, Pseudo-$F_{5, 172} = 42.9$, $P < 0.0001$). The morphology of every parasite population was significantly different from one another ($P \leq 0.0006$) with the exception of parasites collected from *Lates calcarifer* and *Atrosalarias*

fuscus ($P = 0.0119$) (Fig. 2). The SIMPER analysis indicated that the majority of the variation observed in the data was driven by morphological characters associated with attachment of the parasite to its host including haptor width, anterior attachment organ width, sclerite length and hamulus length (Table 3).

3.4.2 Experimental assessment of morphology in genetically constrained Neobenedenia girellae *isolates in captive fishes*

Analysis of a partial gene sequence of *Cytb* extracted from representative parasites, hatched from an individual, isolated parent, showed that all parasites had identical partial *Cytb* sequences. Infection success of *N. girellae* was significantly higher on *L. calcarifer* compared with *R. canadum* on days 8 and 14 following initial infection (ANOVA, $F_{2, 8} = 14.68$, $P = 0.00128$ and ANOVA, $F_{2, 15} = 18.86$, $P < 0.0001$, respectively). No infection of *E. lanceolatus* was observed (Fig. 3). Parasites infecting *L. calcarifer* and *R. canadum* did not exhibit significant morphological variation from one another on days 8 or day 14 following infection under the same controlled conditions (PERMANOVA, $F_{1, 55} = 2.351$, $P = 0.0483$; Fig. 4A and PERMANOVA, $F_{1, 70} = 0.969$, $P = 0.438$; Fig. 4B, respectively).

3.4.3 Experimental assessment on the effects of temperature and salinity on morphological variation in Neobenedenia girellae *infecting* Lates calcarifer *in vivo*

Parasites that developed on a single host species, *Lates calcarifer*, exhibited significant temperature-mediated morphological variation at cool (22 ºC) compared with

warm (30 and 32 ºC) temperatures. Although temperature significantly influenced the morphology of parasites (PERMANOVA, Pseudo-$F_{2, 114} = 20.308$, $P < 0.0001$), this effect was not observed between parasites in 30 and 32 ºC treatments ($P = 0.116$) (Fig. 5A). Salinity (35 and 40 ‰) did not significantly impact parasite morphology (PERMANOVA, Pseudo-$F_{1, 111} = 0.616$, P=0.601) (Fig. 5B). Parasite populations reached sexual maturity (first indication of egg production in aquaria and the day sampled for morphological measurements) at different rates and, as such, developed for different periods of time. Parasites infecting fish in the 22 ºC treatment reached sexual maturity 12 days p.i. whereas those from 30 and 32 ºC took 6 days. As such, measurements were obtained for parasites of different ages.

Parasite body proportions (body length:body width and haptor width:body width) drove the majority of variation observed between parasites at different temperatures. Similarity percentage analysis confirmed that parasite populations which developed at 22 and 30 ºC, and 22 and 32 ºC, accounted for 36.51% and 37.62% of the morphological variation, respectively (Table 4). Ratios related to sclerotised features (sclerite length:body length and hamulus length:body length) accounted for a considerably smaller proportion of the overall variation observed compared with the previous controlled experiments examining the influence of host species (Tables 4, 5).

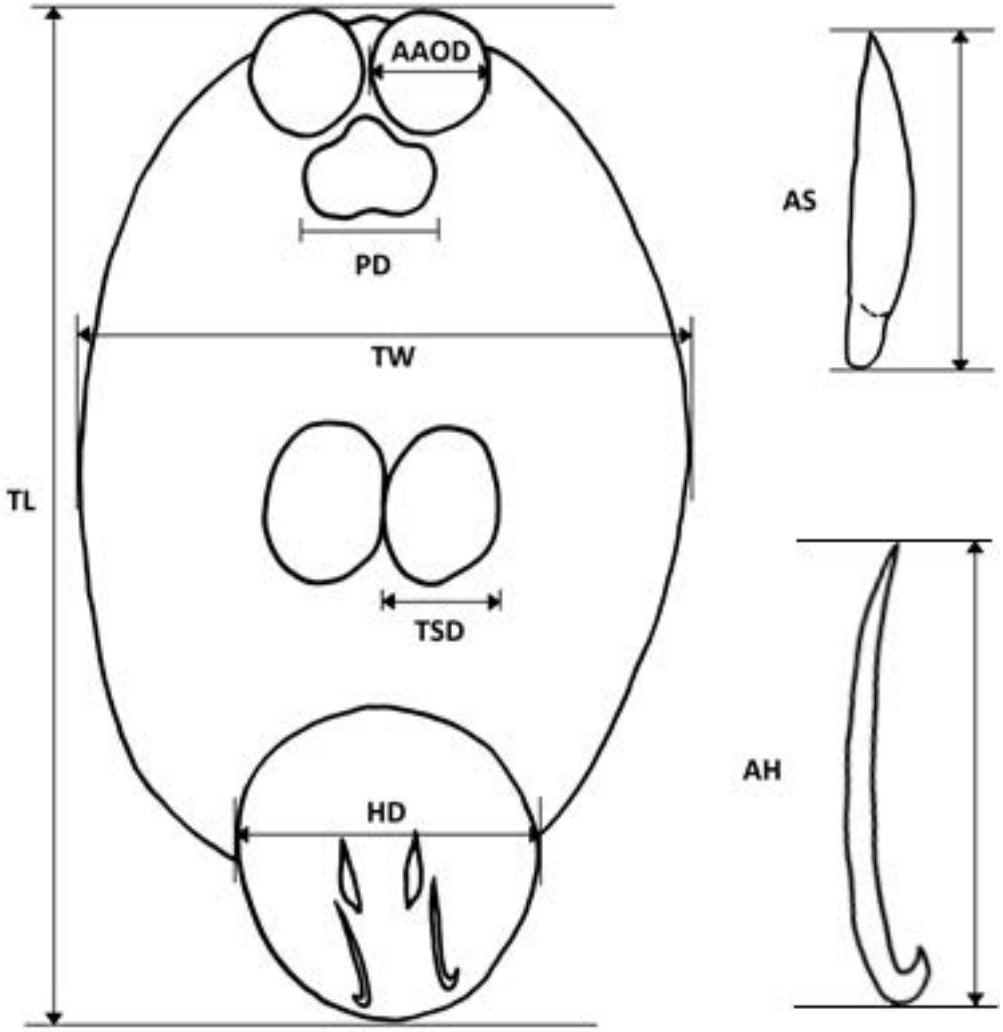

Figure 1: *Neobenedenia girellae* morphometric parameters: AAOD = Anterior attachment organ diameter: AH = Anterior hamulus length: AS = Anterior sclerite length: HD = Haptor diameter: PD = Pharynx diameter: TL = Total Body length (including haptor): TSD = Testis diameter: TW = Total Body width.

Table 1: Wild and captive host fish species for *Neobenedenia girellae* measured for morphology. MARFU = Marine and Aquaculture Research Facility Unit.

Host species (Family)	No. of fish examined	No. of parasites examined	Collection location	Origin of fish	Accession #
Lates calcarifer (Latidae)	3	30	Marine fish farm, Bowen, Queensland	Good Fortune Bay, Kelso, Townsville, Queensland	Codes forthcoming
Rachycentron canadum (Rachycentridae)	1	30	MARFU facility, Townsville, Queensland	Marine fish farm, Bowen, Queensland	Codes forthcoming
Plectropomus leopardus (Serranidae)	1	30	MARFU facility, Townsville, Queensland	Wild caught between Bowen and Hinchinbrook, Queensland 3-6 months prior to infection	Codes forthcoming
Epinephelus coioides (Serranidae)	3	30	James Cook University aquarium facility, Cairns, Queensland	Marine fish farm, Innisfail, Queensland	Codes forthcoming
Atrosalarias fuscus (Blennidae)	1	26	Ornamental supplier, Townsville, Queensland	Wild collection	Codes forthcoming
Neocirrhites armatus (Cirrhitidae)	1	30	Personal aquarium, Townsville, Queensland	MARFU facility, Townsville, Queensland	Codes forthcoming

Table 2: Proportion of variation (%) in field-based data explained by morphological ratios between six species of fish as determined using similarity percentages (SIMPER) (PRIMER 7). The five ratios which explained the most variation between each species pair are shown. LC = *Lates calcarifer*, PL = *Plectropomus leopardus*, RC = *Rachycentron canadum*, NA = *Neocirrhites armatus*, EC = *Epinephelus coioides*, AF = *Atrosalarias fuscus*, AAO = anterior attachment organ. Only statistically significant comparisons are presented as determined by permutational analysis of variance (PERMANOVA).

Species compared	Haptor:AAO	Sclerite:Body length	Length:width	Pharynx:width	Hamulus:length	AAO:width	Haptor:width	Testes:width
LC/PL	18.94	15.26	15.21	13.70	11.16	-	-	-
LC/RC	17.16	16.23	-	-	12.69	16.79	12.79	-
LC/NA	10.80	11.15	-	-	12.71	18.94	20.48	-
LC/EC	15.81	17.70	16.61	-	-	-	12.02	10.44
RC/PL	11.64	20.94	12.42	-	15.66	-	11.21	-
RC/NA	-	15.59	-	-	14.98	21.30	19.52	-
RC/EC	16.62	-	12.51	-	13.43	16.66	14.64	-
RC/AF	18.71	12.69	10.70	-	-	19.79	14.94	-
NA/PL	16.88	-	12.11	-	-	20.52	18.20	9.89
NA/EC	11.10	16.76	-	-	12.47	18.80	19.19	-
NA/AF	-	11.48	14.66	-	14.41	13.38	17.23	-
EC/PL	15.93	22.59	14.80	10.50	11.12	-	-	-
EC/AF	15.32	12.76	17.27	-	-	14.40	14.76	-
AF/PL	18.56	12.10	18.19	-	-	12.43	11.76	-

Table 3: Proportion of variation (%) in controlled experimental data explained by morphological ratios between two species of fish held under the same conditions as determined using similarity percentages (SIMPER) (PRIMER 7). The factors which explained the most variation are shown. LC = *Lates calcarifer*, RC = *Rachycentron canadum*, AAO = anterior attachment organ.

Species compared	Haptor:AAO	Sclerite:body length	AAO:width	Haptor:width	Testes:width
LC/RC	21.70	14.72	11.68	10.77	11.85

Table 4: Proportion of variation (%) in data explained by morphological ratios between parasites raised in three temperatures, 22 ºC, 30 ºC, and 32 ºC on a single host species; *Lates calcarifer* determined using similarity percentages (SIMPER) (PRIMER 7). The five ratios which explained the most variation between each temperature pair are shown. Only statistically significant comparisons are presented as determined by permutational analysis of variance (PERMANOVA). AAO = anterior attachment organ.

Temperatures compared	Haptor:AAO	Length:width	Pharynx:width	AAO:width	Haptor:width
22 ºC / 30 ºC	13.03	19.24	14.84	15.18	17.27
22 ºC / 32 ºC	13.17	18.36	14.80	15.62	19.26

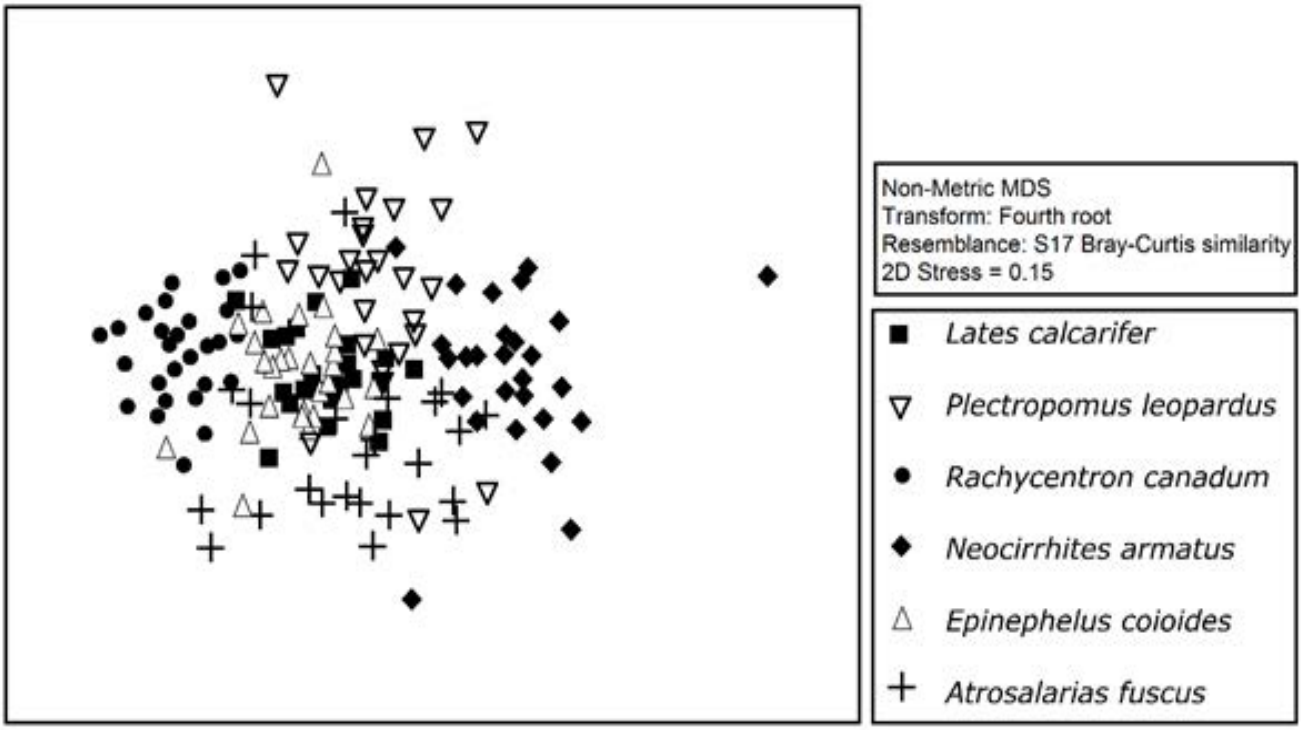

Figure 2: Non-metric multidimensional scaling of proportional morphometrics for *Neobenedenia girellae* infecting six fish species; LC = *Lates calcarifer*, PL = *Plectropomus leopardus*, RC = *Rachycentron canadum*, NA = *Neocirrhites armatus*, EC = *Epinephelus coioides*, AF = *Atrosalarias fuscus*. Stress = 0.15. Symbols distinguish between parasites collected from different fish species.

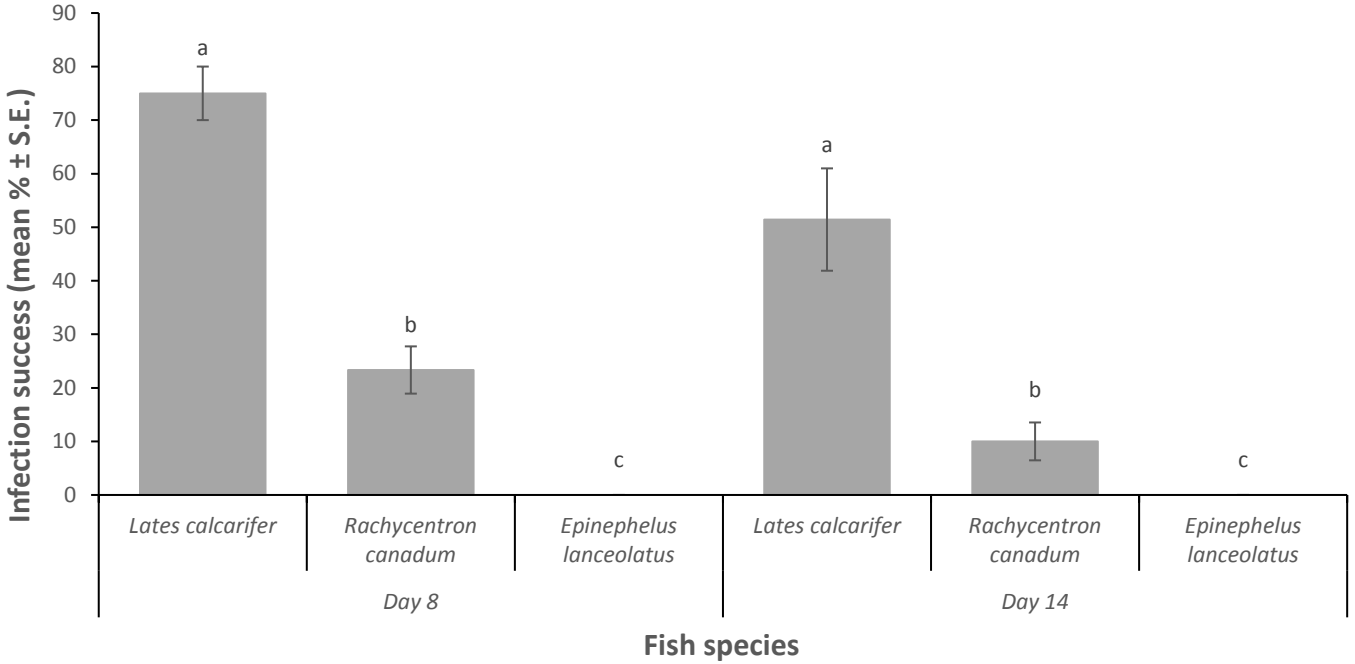

Figure 3: Infection success of *Neobenedenia girellae* infecting three fish species; *Lates calcarifer*, *Rachycentron canadum*, and *Epinephelus lanceolatus* at day eight and 14 post-infection. Pairwise comparisons for infection success at day eightwere performed individually from day 14 data. ‘a’, ‘b’ and ‘c’ indicate differences between pairs of means determined using Tukey’s HSD pairwise comparison test, $p < 0.05$.

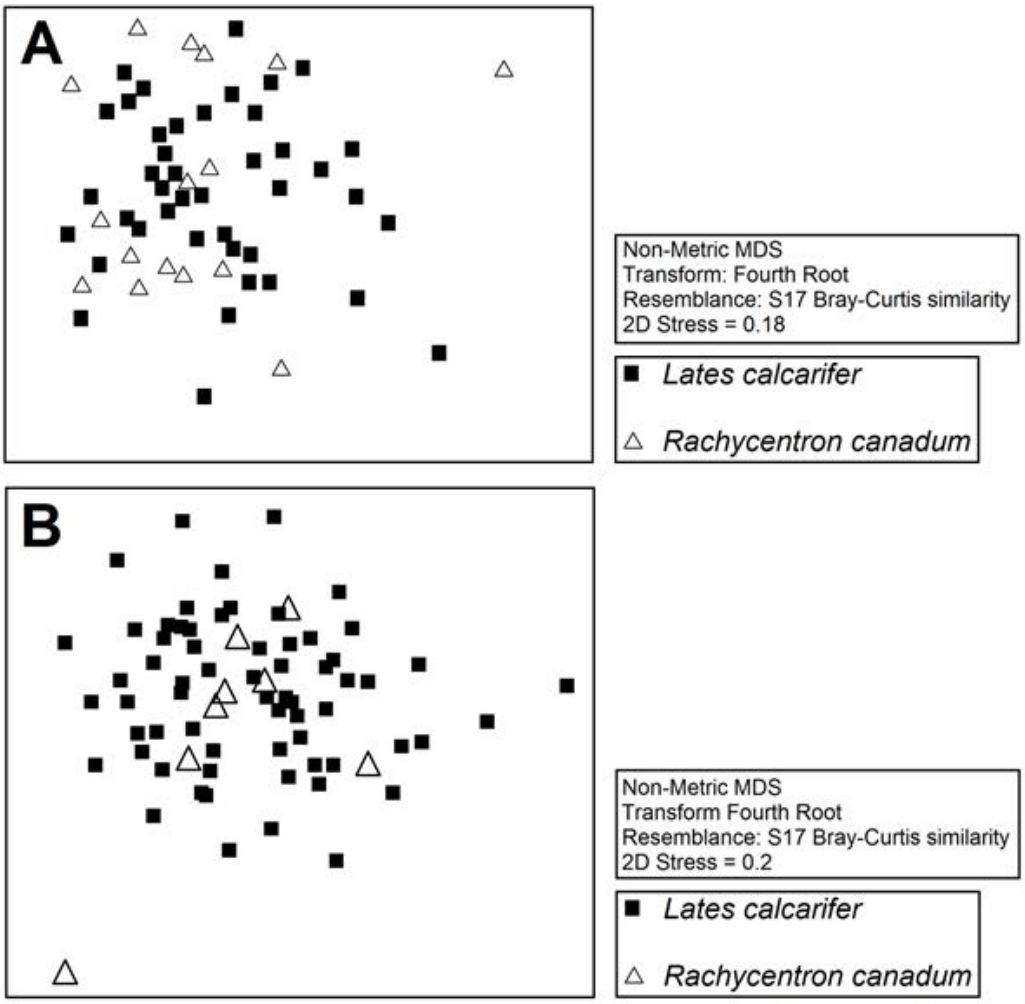

Figure 4: A) Non-metric multidimensional scaling of proportional *Neobenedenia girellae* morphometrics infecting two fish species after eight days of development; *Lates calcarifer* and *Rachycentron canadum* in identical laboratory conditions. Stress = 0.18. Symbols distinguish between parasites collected from different fish species. B) Non-metric multidimensional scaling of proportional *Neobenedenia girellae* morphometrics infecting two fish species following 14 days of development; LC = *Lates calcarifer* and RC = *Rachycentron canadum*. Stress = 0.2. Symbols distinguish between parasites collected from different fish species.

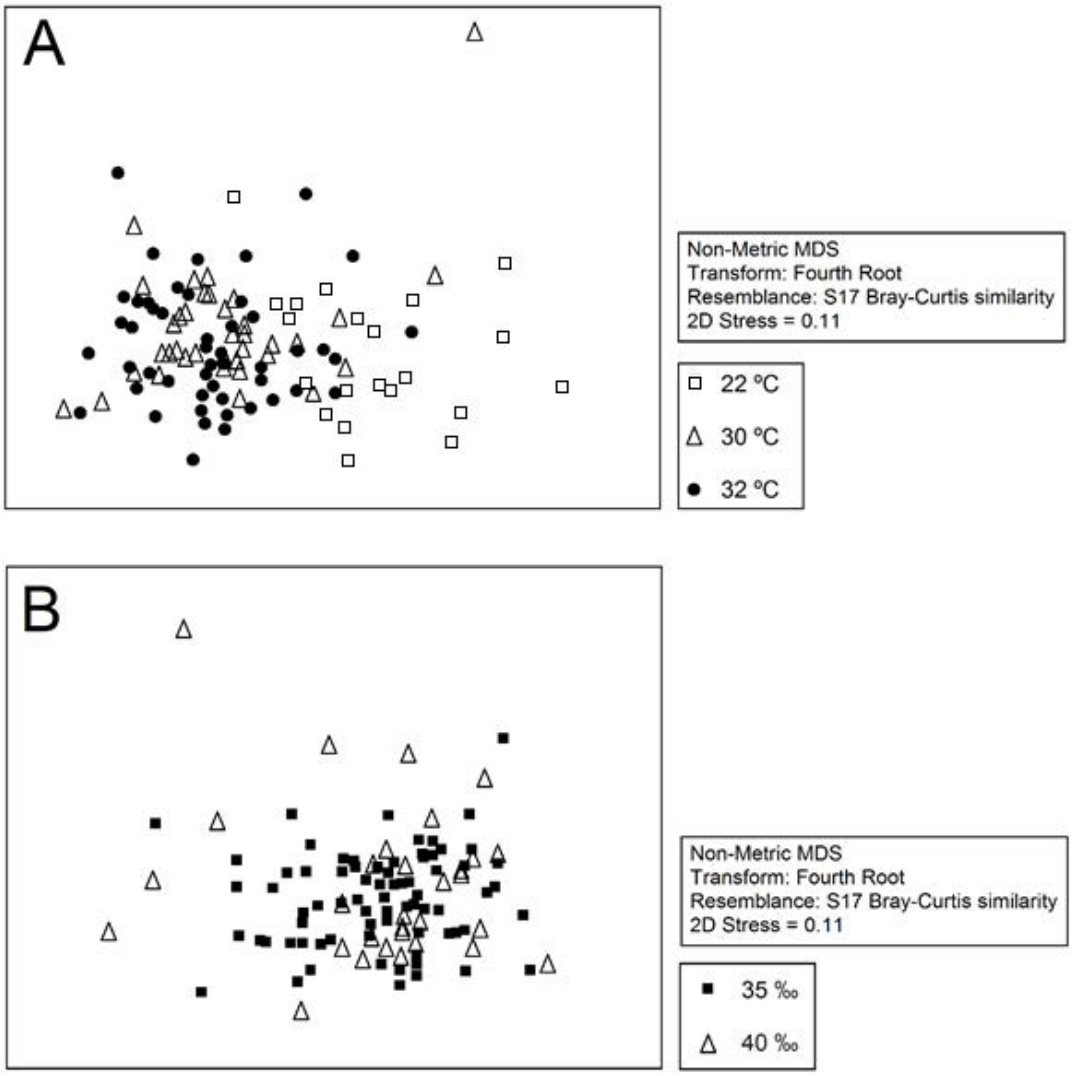

Figure 5: A) Non-metric multidimensional scaling of proportional morphometrics of *Neobenedenia girellae* infecting *Lates calcarifer* in three separate temperatures. Stress = 0.11. Symbols distinguish between the temperatures used to maintain infected fish. N = 19, 34, and 49 parasites measured for 22, 30, and 32 °C, respectively B) Non-metric multidimensional scaling of proportional morphometrics of *Neobenedenia girellae* infecting *Lates calcarifer* in two separate salinities; 35 = 35 ‰ seawater (n = 58) and 40 = 40 ‰ solution (n = 30). Stress = 0.11. Symbols distinguish the salinity of the solution used to maintain infected fish.

3.5 Discussion

An analysis of the morphology of genetically indistinguishable parasites collected from six host fish species in the field suggested that host species might influence the morphology of their parasites' attachment organs. A controlled experiment utilising two of the original six host species (*L. calcarifer* and *R. canadum*) and an isogenic population of *N. girellae* was designed to exclude any variation due to environmental conditions experienced by the fish hosts and parasite populations in the field-based study. The influence of host species was unable to be experimentally verified as a factor that affected parasite morphology. The parasites recovered in the field study and those derived from the parasite culture (from a single individual hermaphroditic parasite) were genetically identical for H3, 28S rDNA, and *Cytb* gene regions, respectively. Although there remains a possibility that variation in other genetic markers exists, the choice to include *Cytb*, a variable genetic marker among *Neobenedenia* spp. (Brazenor et al., unpublished data), gives the authors confidence that there was no significant genetic variation among parasite populations studied. Therefore, the morphological variation observed in the field study was not the result of inherent differences in the phenotypes of genetically distinct parasites. To investigate alternate drivers of morphological variation, controlled laboratory experiments were designed to deduct the relative influence of external environmental factors of temperature and salinity. In these studies, temperature, not salinity, was a significant driver of morphological variation of *N. girellae* related to the proportional size of the parasite. The ability to respond flexibly to the range of temperatures experienced suggests that *N. girellae* possesses a predisposition for phenotypic plasticity as a response to specific environmental conditions.

The majority of the variation observed between parasite populations on different host fish species in the initial field-based investigation related to morphological features involved in attachment to the surface of their host species (haptor, sclerites, hamuli and anterior attachment organs; Table 3). These differences could enable more efficient attachment to accommodate the variability in the microhabitats on host fishes, such as scale morphology. The scales of the host fishes examined in the field-based study are different from one another in shape and in relative size (Barlow et al., 1991; Allen and Erdmann, 2012) and *Atrosalarias fuscus*, like many blenniid species, does not possess scales at all. The authors speculate that the variability of scale morphology presents different surfaces to which parasites can attach, potentially influencing the proportional morphology of sclerotised structures and attachment organs used for anchoring the parasite to the host. The sizes of scales may also have played a significant role in dictating the morphology of attached parasites. Trujillo-González et al. (2015) observed that *N. girellae* initially attach to the surface of the fish under the scales of *L. calcarifer*. *Rachycentron canadum* and *A. fuscus* possess comparatively very small scales and no scales, respectively, which would not allow this behavior to occur.

Differences in microhabitat or attachment site exploited by parasites could also contribute to morphological variability. For example, populations within the polyopisthocotylean monogenean species *Kuhnia scombri*, Kuhn, 1829, *Kuhnia sprostonae,* Price, 1961, and *Pseudokuhnia minor*, Goto, 1984, each exhibit different intraspecific attachment organ morphology depending on which microhabitat the population infects (Rohde and Watson, 1985a, 1985b). This is thought to have differentially driven the development of attachment organs that could perform best in each microhabitat, a phenomenon also observed in other monogeneans (Chisholm and Whittington, 1998). While *N. girellae* is known to inhabit a wide range of microhabitats

on a single host fish species (Trujillo-González et al., 2015), it may exploit different microhabitats on separate host fish species. For example, *N. girellae* individuals have been observed to preferentially aggregate around the head and eyes of *R. canadum* (see Ogawa et al., 2006; Hirazawa et al., 2010). It has been hypothesized that a reduced immune response can occur by the host in particular microhabitats such as the eyes (Jones, 2001; Trujillo-González et al., 2015). In this study, there were no detectable host-mediated morphological differences between parasites infecting experimental *R. canadum* and *L. calcarifer*. Future research could evaluate the potential for host-mediated morphological changes in the parasites following several generations on separate host species.

Parasite origin could also influence the significant morphological differences in *N. girellae* observed in the initial field study. Through natural selective pressures, *N. girellae* could rapidly adapt to new environmental conditions including new host environments. This species exhibits extraordinary reproductive capability as they are fecund (Dinh Hoai and Hutson, 2014), exhibit a rapid generation time (Brazenor and Hutson, 2015) and can reproduce in isolation (Dinh Hoai and Hutson, 2014). Structures that improve parasite attachment to the host confer an evolutionary advantage to individuals that survive to sexual maturity. Over subsequent generations, heritability of these traits could explain the significant differences observed between parasite attachment structures in the field-based study as they become more suited to attach to that particular host species (see Agrawal, 2001). A number of studies have documented the fitness trade-off of adopting optimal morphology in specific habitats for a wide range of taxa (Ehlinger, 1990; Schluter, 1993; Robinson et al., 1996; Robinson, 2000; Svanbäck and Eklöv, 2004). The genetic reinforcement of specific morphological traits over generations can allow the colonization of new habitats or species as seen in plants

and arthropods (Pandey and Nagar, 2002). Additionally, many of the collections of *N. girellae* were made from fish monocultures and so it is likely that the parasite populations sampled were derived from previous generations on the same host species. This implies that the effect of selection of morphological features that confer an advantage to living on a particular host (e.g. specific attachment organ morphology) may be intrinsic in affecting *N. girellae* morphology when populations infect a single host species over time (Table 5).

Verification of the results observed in the initial field trial, where parasite morphology was significantly affected by host species, was unable to be replicated in a controlled experiment (Table 5; Fig. 4A,B). An isogenic lineage of *N. girellae* was utilized to reduce genetically mediated morphological variation inherent in parasites with some level of genetic heterogeneity (Olstad et al., 2009). A lack of morphological response supports the suggestion that selection of morphological traits in a population of parasites that infects the same host species over multiple generations is the strongest driver for parasite morphology under controlled environmental conditions. Consistent infection of *L. calcarifer* by the population of *N. girellae* cultured in the James Cook University Marine Parasitology Laboratory, Australia may have selected for features which conferred an advantage to attachment to barramundi skin and scales. Therefore, a single exposure to a different host species was insufficient to elicit significant morphological variation to separate the two parasite populations.

Temperature had a significant effect on the morphometrics of *N. girellae* while salinity did not (Fig. 5A,B). Temperature is an exceptionally strong factor in determining morphology and almost every physiological trait of organisms (Haynes, 1964). Ectotherms are particularly prone to strong biological changes under different temperature regimes and, typically, development at low temperature results in slower

growth and delayed maturation at a large size in many organisms (Atkinson, 1994, 1995; Gillooly et al., 2002; Angilletta et al., 2004). Body length:width and haptor width:body width explained the majority of the variation in different temperature treatments. Ratios related to sclerotised features for attachment to the host surface were not among the top five contributing ratios towards the morphological variation observed at different temperatures (Table 4). Instead, the variation in morphology of parasites at different temperatures was driven by ratios relating to body size (body length:width, haptor width:body width, and pharynx width:body width). An inverse relationship between temperature and mean body size can be observed for a wide range of species including plants (Jorgensen, 1968; Latala et al., 1991), insects (Partridge et al., 1995; Crill et al., 1996; Forster et al., 2012), Crustacea (Lee et al., 2003), and Platyhelminthes (Bondad-Reantaso et al., 1995; Hirazawa et al., 2010; Brazenor and Hutson, 2015). As a single species of host was chosen for the study (*L. calcarifer*), the host scale surface and microhabitats that parasites attach to were consistent across all treatments. Therefore, it is expected that changes in morphology would be observed in the body size proportions compared with those relating to attachment to host surfaces.

Morphological variation due to environmental variables is a well-known phenomenon among monogeneans, particularly for sclerotised structures related to attachment to the host (Malmberg, 1970; Cable and Harris, 2002; Brazenor and Hutson, 2015). Body size (length and width), hamuli and sclerites are all considered to be taxonomically important features for *N. girellae* (Hargis, 1955; Whittington and Horton, 1996) and, as a result, within species variability in these characters hampers the use of morphology-based identification and the use of diagnostic keys (e.g. Malmberg, 1970; Kulemina, 1977; Mo, 1991a, b; Dmitrieva and Dimitriov, 2002; Davidova et al., 2005). All of these features were found to explain considerable amounts of

morphological variation between parasite populations on different host species and at different temperatures. As such, this study shows that the environment (host and temperature) could be considered a driver of morphological variation when examining monogenean parasites either through selection of features over long time periods or through phenotypic plasticity on the part of the parasite as a response to the experienced conditions. Further work on the variables that influence morphology in this and other parasite genera will help to reduce misidentifications in the future (Dmitrieva and Dimitriov, 2002). The flexible morphological nature exhibited by *N. girellae* in this study serves to highlight how important robust genetic markers are to determine species identities not only in *Neobenedenia* but in many parasite genera in which species complexes are present. As the identities of several *Neobenedenia* spp. are challenging to corroborate without the use of such molecular techniques, it is strongly advised that these methods be employed in the future.

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Chapter 4: Effects of temperature and salinity on the life cycle of *Neobenedenia girellae* (Monogenea: Capsalidae) infecting farmed barramundi (*Lates calcarifer*)

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4.1 Abstract

In this study, the effects of temperature (2 °C increments from 22 to 34 °C) and salinity (0, 11, 22, 35, 40 ‰) on the life cycle parameters (embryonation period, hatching success, oncomiracidia (larvae) longevity, infection success, and time to sexual maturity) were examined for *Neobenedenia girellae* (Monogenea: Capsalidae), a harmful ectoparasite of farmed marine fishes. Experiments were conducted in controlled conditions in the laboratory. The life cycle was faster in warm, high saline conditions compared to cooler conditions (10–13 days between 26–32 °C, 40 ‰; 15–16 days between 22–24 °C, 40 ‰). Warm seawater and high saline conditions (24–32 °C, 35–40 ‰) improved egg hatching success, reduced time to sexual maturity, and resulted in parasites reaching sexual maturity at a larger size (at 30–32 °C) compared to cooler conditions (22 °C). In contrast, cool, hypersaline conditions (22 °C, 40 ‰) increased oncomiracidia longevity and infection success. Linear and quantile regression models were used to construct an interactive, online parasite management interface to enable strategic treatment of parasites in aquaculture corresponding to observed

temperature and salinity variation on farms in the tropics. It was recommended that farmers treat their stock more frequently during monsoonal summers (27–31 °C) when parasites can complete their life cycle more quickly. Nevertheless, farmers should be aware of the potential for increased *N. girellae* infections during winter months (21–26 °C) as a result of increased infection success.

Keywords: Aquaculture, Treatment, Asian sea bass, Monogenea, *Neobenedenia*, Egg hatching

4.2 Introduction

Integrated parasite management of farmed stock requires comprehensive and accurate knowledge of parasite life cycles and the influence of environmental parameters to be effective. In agriculture, parasite management may include a combination of chemical treatments, resistant stock breeds, and grazing pasture management practices such as fallowing or alternative species grazing (Barger & Southcott 1978; Baker 1996; Waller 1997; Stromberg & Averbeck 1999). The aquaculture industry is, comparatively, in its infancy and knowledge of parasite/fish biology is not always sufficient to enable efficient and effective treatments. One method of managing ectoparasites in aquaculture is to administer treatments at times that disrupt or break the life cycle of the parasite. The life cycle is interrupted by initially killing parasites on the host. Successive treatments are temporally coordinated to occur after all eggs have hatched, but before new parasite recruits reach sexual maturity and contribute new eggs into the system (Tubbs et al., 2005). Essential to this method is the accurate determination of embryonation periods and time to sexual

maturity at a range of environmental parameters, allowing for treatments to be tailored to particular localities and farms (Hirayama et al., 2009). Effective administration of this method results in lower re-infection rates, ultimately necessitating fewer treatments (Tubbs et al., 2005). Coordinated treatments designed to improve the efficiency of parasite management are recommended and have been successful in controlling parasites in agriculture including cow and sheep nematodes (Bumgarner et al., 1986; Stuedemann et al., 1989; Kelly et al., 2010) and sea lice outbreaks on salmon farms (Costello 2004), whereas issues such as a lack of life cycle understanding and improperly coordinated treatments have been implicated in reduced management efficiency (Bravo et al., 2008; Bravo et al., 2013). Management strategies are not routinely implemented for monogenean infections due to a lack of knowledge of parasite life cycle parameters.

Monogeneans in the family Capsalidae are a threat to the aquaculture industry as some species have been responsible for epizootic events (Bauer & Hoffman 1976; Paperna & Overstreet 1981; Deveney et al., 2001; Whittington 2004). Species of the genus *Neobenedenia* Yamaguti, 1963, (Monogenea: Capsalidae) are known to be harmful ectoparasites and have been recorded from over 100 teleost species, many of which are important tropical and subtropical fin fish species in marine aquaria and aquaculture (Whittington & Horton 1996; Ogawa & Yokoyama 1998; Hirazawa et al., 2004; Ogawa et al., 2006; Whittington 2012). Their direct life cycle, short generation time and filamentous eggs, which entangle on structure, cause difficulties in managing infections and large numbers of parasites can become present in a system over a short period of time (Jahn & Kuhn 1932; Ogawa et al., 1995; Ogawa et al., 2006; Dinh Hoai & Hutson 2014). *Neobenedenia* spp. graze on the epithelial surface of their host and

feeding habit on host fish causes inflammation, dermal ulceration, and allows the ingress of pathogens that may cause secondary infections (Kaneko et al., 1988).

Current management methods for capsalid monogeneans involve immersing ('bathing') infected fish in hydrogen peroxide, formalin, or freshwater (Ernst et al., 2002; Ogawa et al., 2006), which only temporarily relieves stock of infection by removing attached parasite stages (Whittington 2012). These treatment methods are ineffective in killing embryos within eggs (Sharp et al., 2004; Militz et al., 2014) and re-infection can occur immediately following treatment of stock. Bathing continues to be the most commonly used management practice for monogenean infections and is widely used in the treatment of *Gyrodactylus* spp. epizootics (Santamarina et al., 1991; Buchmann & Bresciani 2001; Buchmann & Kristensson 2003; Schelkle et al., 2011). Although promising results have been recorded from the administration of praziquantel through intubation, the oral administration of anthelminthics has been hindered by palatability issues (Williams et al., 2007; Hardy-Smith et al., 2012; Forwood et al., 2013) and variable efficacy between species (Hirazawa et al., 2013).

Data on parasite life cycle parameters in a variety of temperature and salinity conditions enables more accurate bathing regimes and can improve the efficacy of current farm practice. The aim of this study was to investigate the effects of temperature and salinity on the life cycle parameters (embryonation period, hatching success, oncomiracidia longevity, infection success, and time to sexual maturity) of *N. girellae* Hargis, 1995, in order to develop an interactive strategic management tool for the treatment of farmed barramundi (*Lates calcarifer* Bloch, 1790) (Perciformes: Latidae). This enabled strategically timed management recommendations that can be tailored to temperature and salinity conditions on aquaculture farms.

4.3 Materials and Methods

4.3.1 Parasite collection, temperature and salinity treatments

A laboratory infection of *Neobenedenia girellae* sourced from farmed marine *L. calcarifer* was established and maintained to ensure a continuous source of parasites for experimentation (see Militz et al., 2014). Experimental *L. calcarifer* were sourced from a freshwater hatchery and acclimated to 35 ‰ seawater for 24 h prior to experimentation. Fish were not previously exposed to *N. girellae* infection. The species of *Neobenedenia* sp. investigated in this study was identified as *N. girellae* following phylogenetic analysis (Chapter 2). 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). Temperature (22, 24, 26, 28, 30, 32, and 34 °C) and salinity (0, 11, 22, 35, and 40 ‰) treatments were selected to represent seasonal temperature and salinity variation experienced in tropical monsoonal climates in Queensland, Australia. Two degree increments were chosen from the minimum average winter sea surface temperature (22 °C) to the average summer ocean temperature (34 °C) predicted by the year 2050 at Lucinda, Queensland (18°31'41.271"S: 146º19'53.04"E; Australian Institute of Marine Science (AIMS) 2008) under the Intergovernmental Panel on Climate Change (IPCC) (2007a; 2007b) scenario of ‘business as usual’ emission levels. Selected increments included the current average summer ocean temperature (30 °C) and maximum summer ocean temperature (32 °C) (AIMS 2008). Temperatures were described as ‘cool’

(22 °C), ‘mild’ (24–28 °C), ‘warm’ (30–32 °C), and ‘hot’ (34 °C) (see Results below). A range of salinities including freshwater (0 ‰), hyposaline (11 and 22 ‰), seawater (35 ‰), and hypersaline solutions (40 ‰) were chosen to represent extreme salinity fluctuations observed in open, semi-closed, and closed aquaculture systems used to farm *L. calcarifer* in tropical climates. Hyposaline solutions (11 and 22 ‰) were prepared by mixing distilled water and filtered seawater to the desired concentration in a sterile container. The hypersaline solution was prepared through the addition of Mermaid Marine salt to 35 ‰ filtered seawater. Salinities were determined using a refractometer.

4.3.2 Embryonation period and hatching success

The effect of temperature and salinity on the embryonation period and hatching success of *N. girellae* eggs was examined in the laboratory. *Neobenedenia girellae* eggs were collected from infected laboratory fish as per Militz et al. (2014). Eggs were divided into groups of ten using fine-tipped forceps and placed into individual glass cavity blocks (40 mm^2). Six replicates were made for each temperature/salinity combination. The blocks were filled to the brim and a glass cover placed on top in order to reduce the occurrence of oncomiracidia lysing or becoming trapped in the surface tension (Ernst & Whittington 1996). Eggs were incubated (Sanyo: ML-351 Versatile Environmental Incubation Chamber) with fluorescent lighting for a 12:12 LD cycle. One third of the solution was changed each day with minimal disturbance to the eggs. Blocks were monitored every 24 h at 1100. When hatching occurred, oncomiracidia were removed with a pipette and the day of hatching was recorded. Experiments were continued until 48 h passed without hatching in any treatment, at which time any

unhatched eggs were not considered viable. Embryonation period was defined as the time taken to first and last hatching of eggs. Hatching success of *N. girellae* eggs was measured as the number of oncomiracidia removed from each cavity block divided by the total number of eggs.

4.3.3 Oncomiracidia longevity

The effect of temperature and salinity on the life span of *N. girellae* oncomiracidia was examined in the laboratory. *Neobenedenia girellae* eggs did not hatch in 0 and 11 ‰ treatments and consequently these salinities could not be investigated in any further experiments. Approximately 100 eggs were incubated in a Petri dish in each salinity (22, 35, and 40 ‰) at each temperature (22, 24, 26, 28, 30, 32, and 34 °C) (Model UP150 refrigerated incubator) with fluorescent lighting for a 12:12 LD cycle. One third of the solution in each Petri dish was changed every 24 h. In order to obtain newly hatched oncomiracidia, each egg mass was placed into fresh solution on the day hatching was first observed. Oncomiracidia that hatched over the next 2 h were used for experimentation. Individual oncomiracidia were gently removed from Petri dishes using a plastic pipette and placed into separate glass cavity blocks in the salinity in which they were incubated. Eggs incubated in 22 ‰ solution at 24, 26, and 28 °C did not yield enough oncomiracidia for sufficient experimental replication. Consequently, oncomiracidia used in 22 ‰ treatments were sourced from 35 ‰ at the corresponding temperature. The solution in each cavity block was filled to the brim. Water was not changed throughout the experiment in order to minimise disturbance to oncomiracidia. Six replicates, containing a single individual oncomiracidium per replicate, were made for each temperature/salinity treatment. Each oncomiracidium was

monitored every 2 h to assess survival. Oncomiracidia were considered dead once they showed no signs of motion and failed to respond to a gentle stream of water from a plastic pipette. Once determined to be dead, oncomiracidia were examined in the subsequent monitoring periods to confirm death. Oncomiracidia longevity was expressed as the elapsed time from initial immersion in the treatment to death.

4.3.5 Infection success, time to sexual maturity, and size at sexual maturity

The effect of temperature and salinity on *N. girellae* oncomiracidia infection success, time to sexual maturity, and size at sexual maturity was examined *in vivo*. Approximately 200 eggs were incubated in each temperature (22, 24, 26, 28, 30, 32, and 34 °C) and salinity (22, 35, and 40 ‰) treatment to provide a source of oncomiracidia for the infection experiments. Eggs incubated at 32 °C and 34 °C and in 22 ‰ solution did not yield enough oncomiracidia for sufficient replication. Oncomiracidia used in 22 ‰ treatments were sourced from 35 ‰ and oncomiracidia used in 32 and 34 °C treatments were sourced from 30 °C.

Immediately following hatching, ten vigorously swimming oncomiracidia were gently aspirated with a glass pipette and slowly ejected from the pipette into a 10 L aquarium containing in 6 L of UV filtered seawater and an individual *L. calcarifer* (mean 180 L_T (150–200 mm)). Five replicates were made for each temperature/salinity treatment. The aquaria were placed into an incubator (Sanyo: ML-351 Versatile Environmental Incubation Chamber) with fluorescent lighting for a 12:12 LD cycle and aerated using battery powered aerators. Pieces of large gauge (1 mm) netting (20×10 cm) were placed into each of the aquaria and inspected under a stereo-microscope at 2× magnification daily at 10 am for tangled eggs, indicating the presence of at least one

sexually mature parasite. A pilot study showed that eggs always entangled on the netting on the first day that they were laid (AKB, unpublished data). Accordingly, time to sexual maturity was considered to be the day that eggs were first observed on netting.

The following day, fish infected with sexually mature parasites were bathed twice in dechlorinated freshwater for 5 min and the epithelial surface gently rubbed in order to dislodge attached parasites (see Militz et al., 2014). The solution was filtered through a 60 µm mesh to collect dislodged individuals which were counted under a stereomicroscope. Infection success was determined as the proportion of parasites collected divided by the number of oncomiracidia introduced to the fish. *Neobenedenia girellae* were fixed in 70 % EtOH and then stained with haematoxylin, dehydrated through an alcohol series, and mounted on microscope slides in Canada balsam. Total length, total width, anterior hamuli, and accessory sclerites were measured using a micrometer and ImageJ 1.44p (Java 1.6.0_20) (Table 2). Measurements are given as: mean (minimum – maximum range) and follow Lackenby et al. (2007).

4.3.6 Life cycle

The life cycle, or minimum time to reinfection by sexually mature parasites, was calculated as the sum of time taken for eggs to begin hatching and minimum time to sexual maturity (see above). Oncomiracidia were considered to be able to infect fish immediately following hatching (Trujillo-González et al., 2015a).

4.3.7 Theoretical strategic management tool

A theoretical model was developed (from data obtained in above paragraphs) to determine when fish should be bathed in order to maximise treatment efficiency in varied temperature and salinity scenarios. The time between the initial and subsequent treatment(s) was determined to occur within a period that allowed eggs deposited by adult parasites pre-treatment to hatch, but before new parasite recruits matured on fish. Assumptions were: 1) an initial bath treatment kills 100 % of attached parasite stages on stock on day one; 2) that oncomiracidia can reinfect fish immediately following treatment and; 3) that oncomiracidia survive no longer than 24 h more than the observed life span. The assumptions were made given that the efficacy of freshwater bath treatments against *Neobenedenia melleni* MacCallum, 1927, is 100 % with treatments as short as 2 minutes (Kaneko et al., 1988), while oncomiracidia can infect fish within 15 minutes in laboratory conditions (A.T. Gonzalez, unpublished data). To account for variable oncomiracidia longevity observed between studies (Militz et al., 2014) and to convert longevity into whole days for use in a calendar-day program, longevity values were rounded up to the nearest day (Fig. 1B; Table 1).

The interactive strategic treatment tool can be accessed via internet connection (including smart phone), which provides farmers easy and flexible access to the program. The model was developed using the linear and quantile regression equations (see above). Equations and decision functions were entered manually into JavaScript 1.8.5. The program was then exported to http://marineparasites.com/paratreatmentcal.html and hosted on this URL. The program can be accessed using either Firefox or Google Chrome web browsers. The website interface presents hatching success, infection success, and recommended timed treatments for each whole degree Celsius and salinity (‰) increment between 22–34 °C

and 22–40 ‰. This range encompasses temperatures and salinities experienced throughout a year in the tropics.

All procedures used in this research were approved by the Animal Ethics Committee of James Cook University, approval code: A1989.

4.3.8. Statistical analyses

Hatching success, oncomiracidia longevity, and infection success data did not fulfil the conditions of normality and homogeneity of variance and were analysed by permutational analysis of variance in the PERMANOVA function of PRIMER 6.0. PERMANOVA compares the observed value of a test statistic (F-ratio) against a recalculated test statistic generated from random permutation of the data. PERMANOVAs with 9999 permutations based on Euclidean distance were used to statistically evaluate experimental treatments (hatching success, oncomiracidia longevity, and infection success), followed by pairwise comparisons. Data satisified the assumptions of PERMANOVA. Significance was accepted at $p < 0.05$.

A two-way analysis of variance (ANOVA) was used to determine whether temperature and/or salinity had significant effects on parasite size. A one-way analysis of variance (ANOVA) was used to assess the effects of temperature on parasite size (see above; as salinity was found to have no significant effect). Data satisfied ANOVA assumptions. Analyses were performed using the S-Plus 8.0 software package from Spotfire®. Significance was accepted at $p < 0.05$. *Post hoc* comparisons of group means were performed using Tukey's HSD test.

Linear models were used to describe the relationship between treatment (temperature and salinity) and response variables (hatching and infection success, time

to last hatch, and time to sexual maturity). Quantile regression models were created for 'time to last hatch' (tau = 0.95) and 'time to earliest sexual maturity' (tau = 0.05) data. Equations used to generate each relationship are outlined in Table 3. Linear and quantile regression models were created using the statistical program R 3.0.0. using the 'quantreg' package (Koenker 2013).

4.4 Results

4.4.1 Embryonation period and hatching success

Egg embryonation periods were shorter in mild temperatures and in seawater and high salinity (4–5 days at 24–28 °C, 35–40 ‰) while the embryonation period was longer at the temperature extremes (6–8 days at 22 °C, 22 ‰; 4–9 days at 30–34 °C, 22–40 ‰; Fig. 2; Table 1). Time to first hatch was shorter between 24–32 °C, with eggs hatching on day 4, whilst cool, hypersaline conditions (22 °C, 40 ‰; Fig. 2; Table 1) delayed hatching to day 7.

Mild to warm, hypersaline water provided optimal conditions for *N. girellae* egg hatching success. More than 80 % of eggs hatched between 26–32 °C, 40 ‰ (Fig. 1A, 3A). No *N. girellae* eggs hatched in low salinities (0–11 ‰). Hatching success was low in 22 ‰ saline (<40 % hatching success) and in hot, hypersaline conditions (<5 % hatching success at 34 °C, 35–40 ‰; Fig. 1A). Egg hatching success was significantly influenced by temperature (PERMANOVA, Pseudo-$F_{6, 175}$ = 39.08, p <0.0001) and salinity (PERMANOVA, Pseudo-$F_{4, 175}$ = 424.87, p <0.0001) (Fig. 1A, 3A). There was a significant interaction between temperature and salinity on egg hatching success (PERMANOVA, Pseudo-$F_{24, 175}$ = 15.92, p <0.0001).

4.4.2. Oncomiracidia longevity

Oncomiracidia survived for longer periods of time in cool, seawater and hypersaline water. Oncomiracidia longevity was significantly reduced in warm and hot water (8 ± 0.8 h at 30–34 °C) and in low saline (5 ± 0.5 h in 22 ‰) conditions. Conversely, cool, hypersaline water (22 °C, 40 ‰) prolonged the longevity of larvae (49 ± 1.5 h; Fig. 1B; Table 1). Longevity was significantly influenced by temperature (PERMANOVA, Pseudo-$F_{6, 105}$ = 186.24, p <0.0001) and salinity (PERMANOVA, Pseudo-$F_{2, 105}$ = 391.35, p <0.0001) (Fig. 1B). The interaction between temperature and salinity on the longevity of *N. girellae* oncomiracidia was also significant (PERMANOVA, Pseudo-$F_{12, 105}$ = 53.57, p <0.0001).

4.4.3 Infection success, time to sexual maturity, and size at sexual maturity

Cool, hypersaline water provided optimal conditions for *N. girellae* infection success (>81 % success at 22 °C, 40 ‰; Fig. 1C, 3B). Infection success significantly decreased with increasing temperature (<15 % success at 34 °C) and was reduced in low saline solutions (<10 % at 22 ‰; Fig. 1C, 3B). Infection success was significantly influenced by temperature (PERMANOVA, Pseudo-$F_{6, 64}$ = 4.61, p=0.0006) and salinity (PERMANOVA, Pseudo-$F_{2, 64}$ = 41.34, p <0.0001). There was no significant interaction between temperature and salinity on *N. girellae* infection success (PERMANOVA, Pseudo-$F_{6, 64}$ = 1.66, p=0.103).

Neobenedenia girellae reached sexual maturity rapidly in mild to warm seawater (6 days at 26, 30, and 32 °C, at 35 ‰; Table 1). Parasites took twice the amount of time to reach sexual maturity in cool temperatures (12 days at 22 °C, 35 ‰; Fig. 3D). Time taken to reach sexual maturity was longer in hyposaline conditions (12 days at 26 °C, 22 ‰ and 8 days at 30 °C, 22 ‰) compared to high salinities at the same temperatures (7 days at 26 °C, 40 ‰ and 6 days at 30 °C, 40 ‰).

Parasites were significantly larger at sexual maturity in warm water. Sexually mature parasites exhibited a total body length of 1.8 ± 0.6 and 2.0 ± 0.5 mm at 30 and 32 °C, respectively, while parasites in cool conditions (22 °C) were 1.0 ± 0.3 mm in length (Fig. 4; Table 2). All morphological features were proportional in their measurements with respect to temperature and showed the same trend of generally increasing in size with increasing temperature. Temperature had a significant effect on all morphological characters measured including total length (ANOVA, $F_{5, 199} = 5.119$, $p < 0.0001$), total width (ANOVA, $F_{5, 199} = 21.091$, $p < 0.0001$), anterior hamulus length (ANOVA, $F_{5, 199} = 18.358$, $p < 0.0001$) and accessory sclerite length (ANOVA, $F_{5, 199} = 35.847$, $p < 0.0001$) (Fig. 3A, B). Salinity did not significantly influence size at maturity. There was no significant interaction between temperature and salinity on morphological measurements.

4.4.4 Life cycle

Parasites completed their life cycle faster in mild to warm, high saline conditions. Parasites took 10–12 days to complete the life cycle in mild and warm (26, 30, and 32 °C), high saline (35–40 ‰) conditions, compared to 18 days in cool seawater (22 °C, 35 ‰; Table 1). In cool to mild conditions (22–24 °C) and in seawater and high

salinity (35–40 ‰), a second generation of *N. girellae* oncomiracidia emerged between 15–18 days, compared to 10–14 days at warm temperatures (26–34 °C) in comparable salinities. In warm, high saline conditions, *N. girellae* has the capacity to produce three consecutive generations within one month (30 days), whereas only two consecutive generations could be achieved within one month in cooler conditions (Fig. 3C, D; Table 1).

4.4.5 Theoretical strategic management tool

The strategic management tool showed that treatments should occur earlier and more often in mild to warm seawater and hypersaline environments (26–34 °C, 35–40 ‰) compared to cooler conditions (22–24 °C, 35–40 ‰). The online interface permits the user to determine the appropriate time for subsequent treatments, following an initial treatment of stock (see http://marineparasites.com/paratreatmentcal.html). Generally only a single subsequent treatment (occurring between days 7–14) is needed to break the parasite's life cycle in cool conditions in all salinities (22–24 °C). In contrast, warm to hot, high saline conditions (26–34 °C) require two subsequent treatments between days 5–12 (http://marineparasites.com/paratreatmentcal.html).

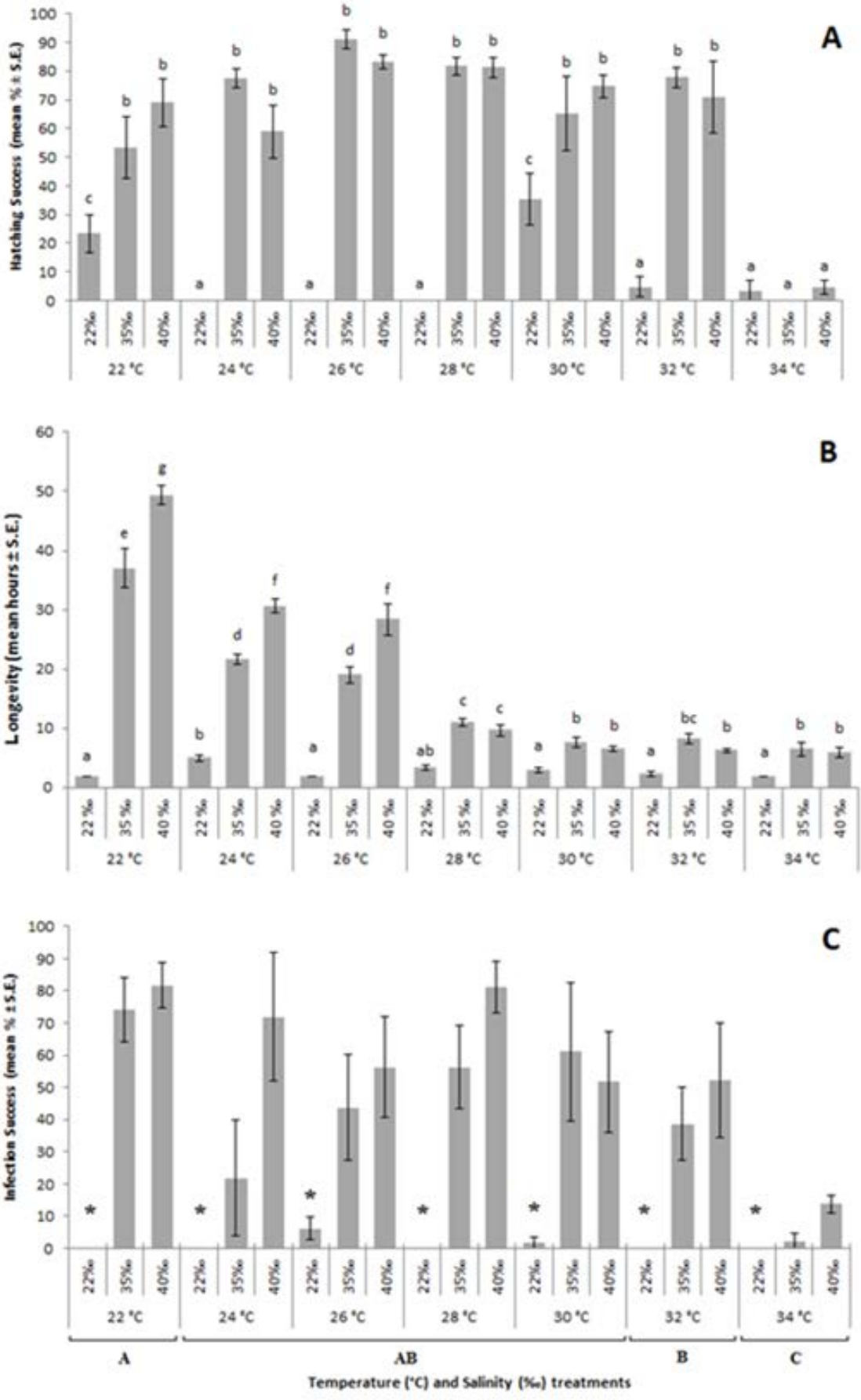
A
Hatching Success (mean % ± S.E.)
22‰
35‰
40‰
22 °C
24 °C
26 °C
28 °C
30 °C
32 °C
34 °C
B
Longevity (mean hours ± S.E.)
C
Infection Success (mean % ± S.E.)
A
AB
B
C
Temperature (°C) and Salinity (‰) treatments

Fig. 1 (A) *Neobenedenia girellae* hatching success in temperature and salinity treatments (0 and 11‰ not shown; no hatching observed). 'a', 'b', and 'c' = differences between pairs of means determined using PERMANOVA pairwise comparison test, $p < 0.05$. (B) *Neobenedenia girellae* oncomiracidia longevity (in hours) in temperature and salinity treatments. Letters above columns = differences between pairs of means determined using PERMANOVA pairwise comparison test, $p < 0.05$. (C) *Neobenedenia girellae* infection success in temperature and salinity treatments; 'A', 'AB', 'B' and 'C' = differences between temperatures. '*' = differences between pairs of means for salinity determined using PERMANOVA pairwise comparison test, $p < 0.05$

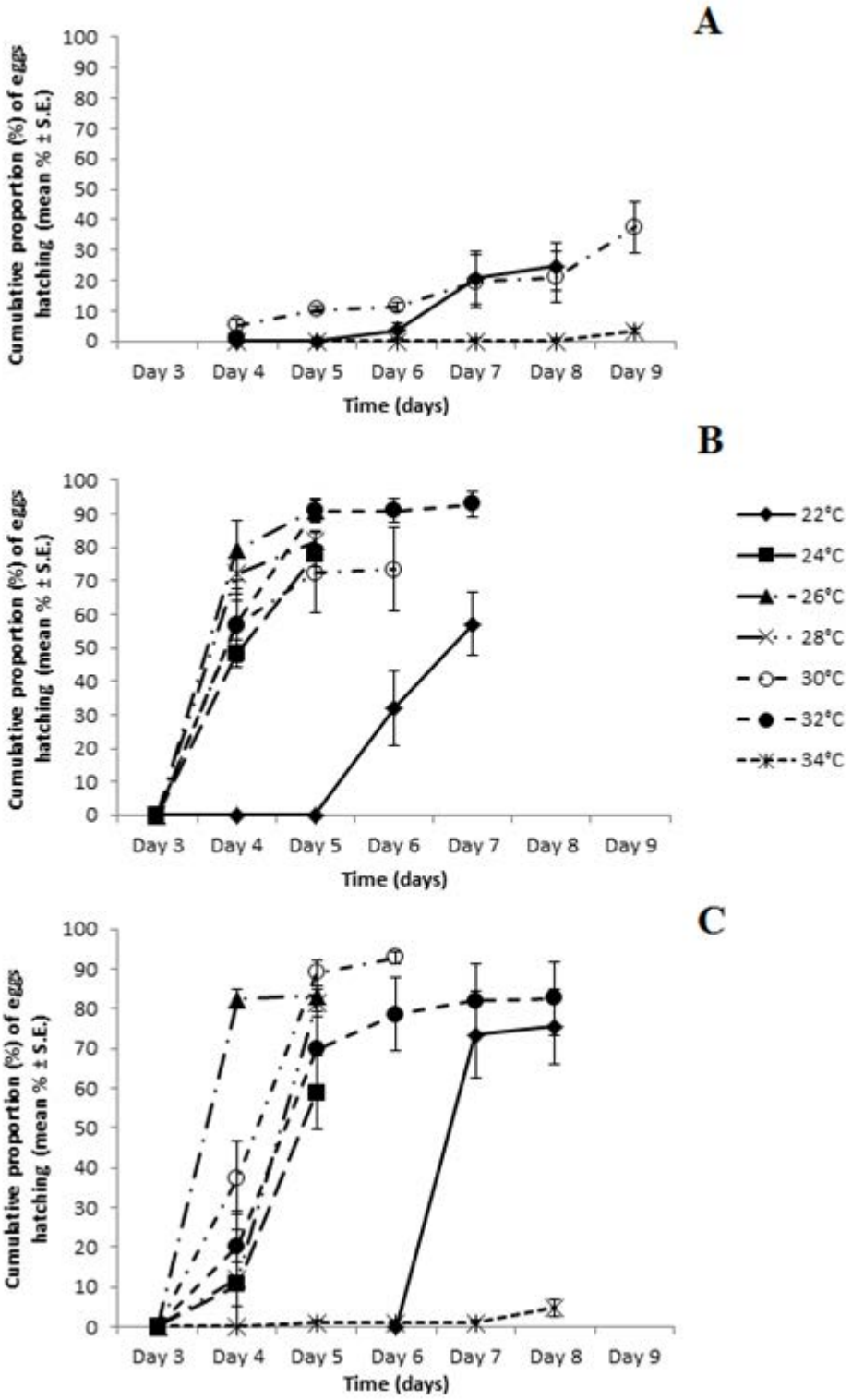

Fig. 2 Cumulative proportion of hatched *Neobenedenia girellae* at 22‰ (A), 35‰ (B), and 40‰ (C). Day of last hatch is indicated where data points finish. Error bars indicate SE

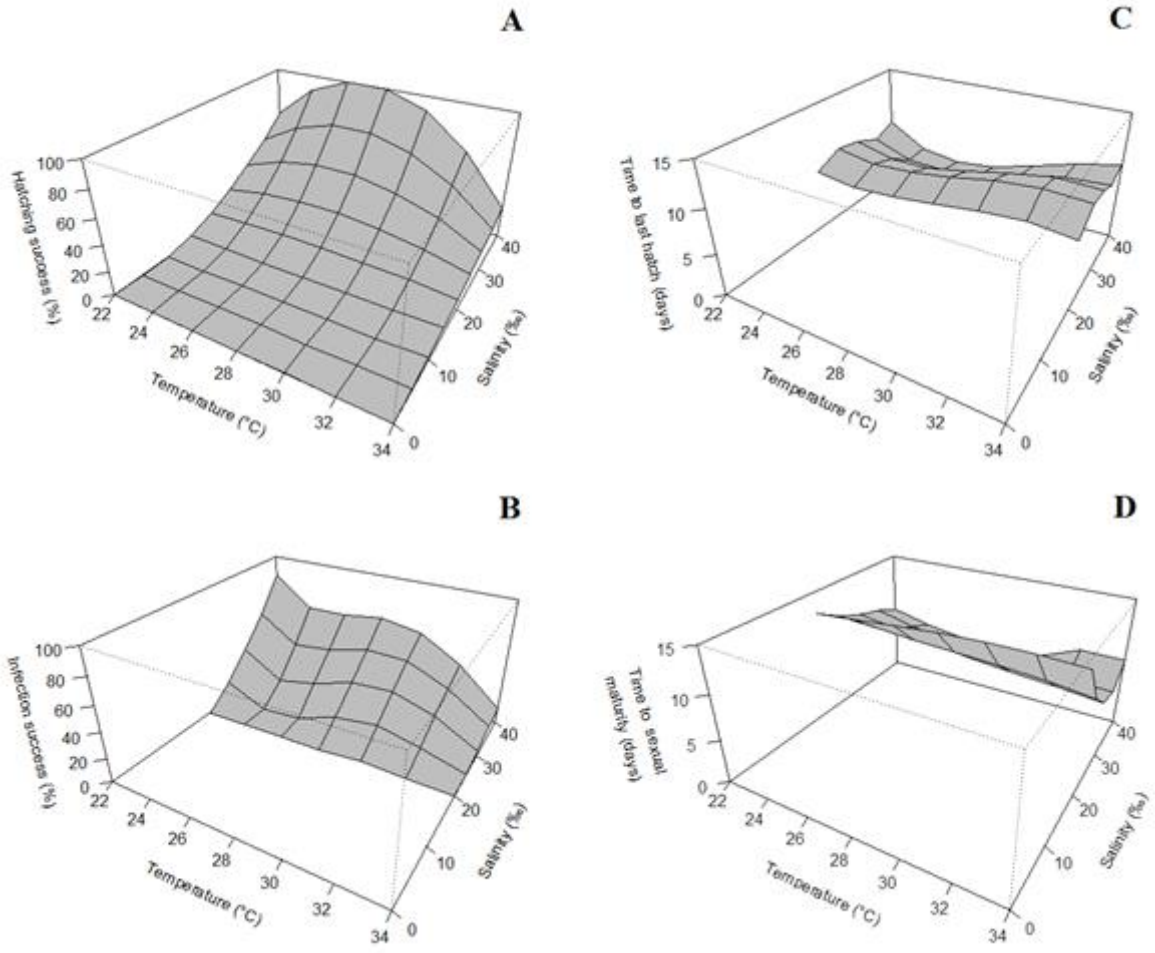

Fig. 3 General linear models describing the relationship between temperature, salinity and (A) hatching success, (B) infection success, (C) time to last hatch, and (D) time to sexual maturity of *Neobenedenia girellae.*

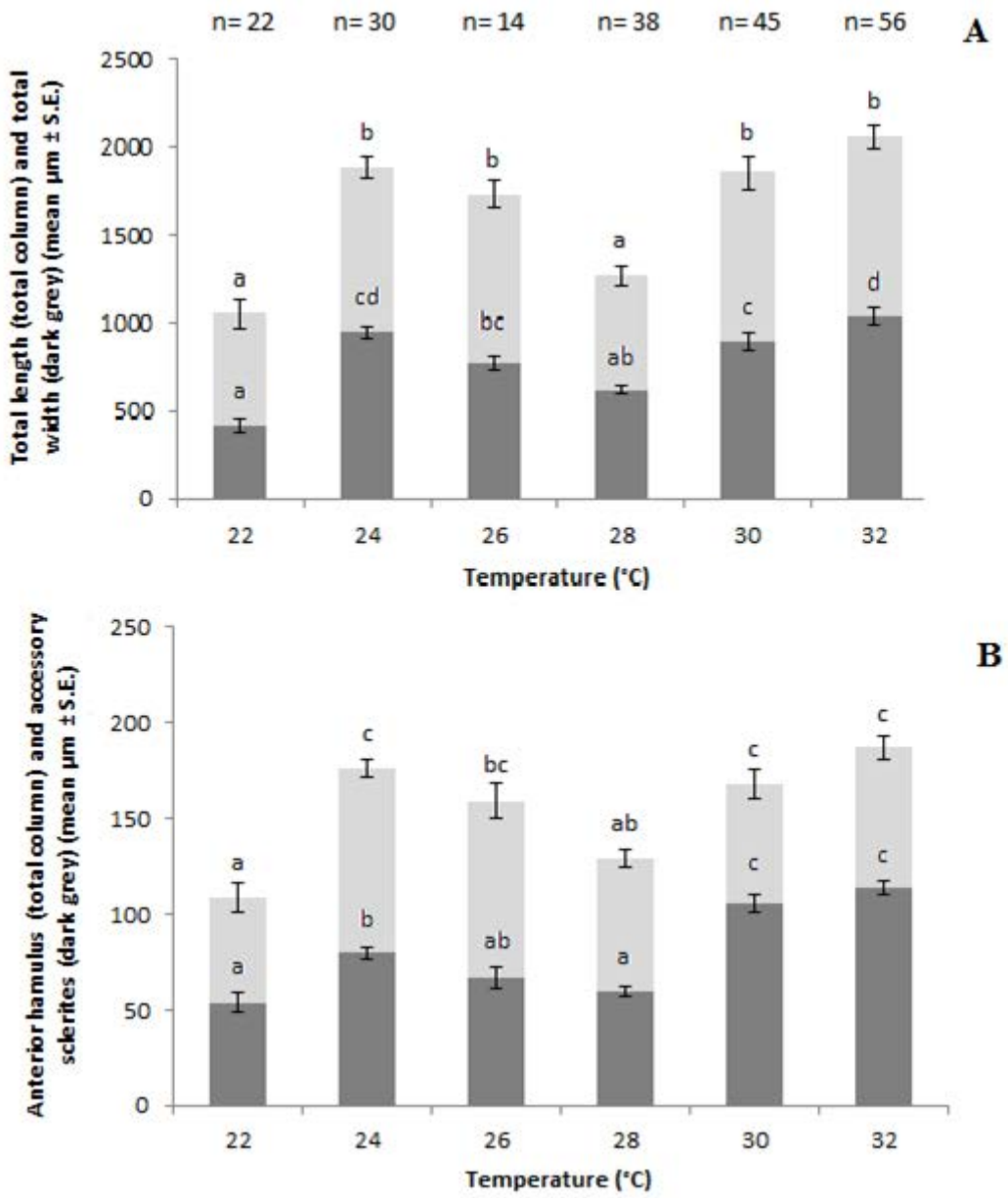

Fig. 4 (A) *Neobenedenia girellae* total length (total column height) and total width (dark grey column) and (B) anterior hamulus (total column height) and accessory sclerite length (dark grey column) with respect to temperature. 'a', 'b' and 'c' = differences between pairs of means determined using Tukey's HSD test, $p < 0.05$. Each parameter was independently statistically analysed

Table 1

Life cycle parameters of *Neobenedenia girellae* infecting *Lates calcarifer* in various temperature/salinity combinations. Time to first and last hatch (F/LH); average oncomiracidia longevity ± SE (OL); minimum time to sexual maturity (SM); minimum time to completion of life cycle (LC).

Temperature (°C)	Salinity (‰)	F/LH (days)	OL (hours)	SM (days)	LC (days)
	22	6 – 8	2 ± 0.0	-	-
22	35	6 – 7	37 ± 3.3	12	18
	40	7 – 8	49 ± 1.5	9	16
	22	-	5 ± 0.5	-	-
24	35	4 – 5	22 ± 0.8	11	15
	40	4 – 5	31 ± 1.1	12	15
	22	-	2 ± 0.0	12	-
26	35	4 – 5	19 ± 1.4	7	11
	40	4 – 5	28 ± 2.6	6	10
	22	-	3 ± 0.5	-	-
28	35	4 – 5	11 ± 0.7	9	13
	40	4 – 5	10 ± 1.0	9	13
	22	4 – 9	3 ± 0.5	8	12
30	35	4 – 6	8 ± 0.8	6	10
	40	4 – 6	7 ± 0.4	6	10
	22	4	2 ± 0.3	-	-
32	35	4 – 8	8 ± 0.8	6	10
	40	4 – 8	6 ± 0.3	8	12
	22	9	2 ± 0.0	-	-
34	35	-	7 ± 1.2	12	-
	40	6 – 9	6 ± 0.9	8	14

Table 2

Comparative measurements of *Neobenedenia girellae* infecting *Lates calcarifer.* Measurements in micrometres (µm); mean followed by range in parentheses; '-' indicates no measurement given as no infecting parasites were observed.

Temperature (°C)	Salinity (‰)	Sample size (n)	Length (µm)	Width (µm)	Anterior hamulus length (µm)	Accessory sclerite length (µm)
22	22	-	-	-	-	-
	35	6	834 (464-2056)	312 (133-964)	95 (55-198)	49 (23-136)
	40	17	1081 (699-1788)	425 (187-754)	113 (69-167)	56 (41-98)
24	22	-	-	-	-	-
	35	6	1834 (1087-2099)	905 (502-1082)	185 (130-234)	78 (45-97)
	40	23	1899 (1358-2586)	956 (605-1445)	174 (119-233)	81 (53-114)
26	22	2	2804 (2413-3194)	1224 (1188-1260)	181 (155-206)	112 (100-124)
	35	6	1461 (1228-1676)	647 (490-847)	133 (128-142)	65 (47-84)
	40	13	1739 (1278-2170)	777 (553-1028)	162 (118-217)	67 (40-102)
28	22	-	-	-	-	-
	35	9	1591 (1228-1850)	707 (558-847)	147 (126-189)	69 (47-101)
	40	26	1167 (611-1794)	592 (258-876)	122 (53-194)	59 (19-94)
30	22	4	2952 (2793-3277)	1738 (1622-1836)	249 (219-264)	139 (125-160)
	35	18	1715 (1498-2858)	855 (170-1545)	161 (65-266)	101 (39-177)
	40	25	1956 (1065-2625)	917 (397-1587)	174 (115-258)	110 (69-156)
32	22	-	-	-	-	-
	35	16	2013 (999-2672)	1049 (427-1631)	187 (111-254)	116 (46-163)
	40	38	2078 (1079-3124)	1029 (424-2347)	188 (111-340)	114 (59-184)
34	22	-	-	-	-	-
	35	1	1865	914	157	107
	40	1	2147	948	180	126

Table 3

Equation values for linear models: ‘hatching success’, ‘infection success’ and quantile regression models: ‘time to last hatch’ and ‘time to earliest sexual maturity data’. ‘-‘ denotes no value, ‘temperature’ data is represented by the term ‘a’ and ‘salinity’ data is represented by the term ‘b’. Values are multiplied together to form equations used to create linear model surfaces.

Terms	Hatching success (%)	Infection success (%)	Time to last hatch (days)	Time to sexual maturity (days)
Intercept	-5.016	$1.660e^{4}$	$3.218e^{2}$	$1.348e^{2}$
a	$2.891e^{-1}$	$-2.292e^{3}$	$-4.574e^{1}$	-6.014
b	$2.120e^{1}$	$-1.184e^{1}$	$1.839e^{1}$	-5.167
ab	-1.633	$5.388e^{-1}$	-1.632	$2.859e^{-2}$
a^2	$-4.862e^{-3}$	$1.172e^{2}$	2.403	-
b^2	-1.207	$1.796e^{-1}$	$-2.120e^{-1}$	-
ab^2	$9.572e^{-2}$	-	$1.073e^{-2}$	-
a^2b	$2.983e^{-2}$	-	$3.975e^{-2}$	-
a^2b^2	$-1.761e^{-3}$	$-2.258e^{-4}$	-	$-9.287e^{-5}$
a^3	-	-2.637	$-5.527e^{-2}$	-
b^3	-	-	$2.823e^{-3}$	-
a^3b^2	-	-	$-2.436e^{-6}$	-
a^2b^3	-	-	$-4.647e^{-6}$	-
a^4	-	$2.198e^{-2}$	$4.702e^{-4}$	$2.522e^{-5}$
b^4	-	-	$-4.473e^{-5}$	$1.118e^{-5}$
a^4b	-	-	$-6.875e^{-6}$	-
ab^4	-	-	$1.173e^{-6}$	-
a^4b^4	-	-	$2.965e^{-11}$	-
a^b	-	-	-	$2.120e^{-62}$
b^a	-	-	-	$-1.136e^{-53}$

4.5 Discussion

Warm, high saline conditions enabled rapid completion of the life cycle of the ectoparasitic monogenean, *N. girellae*. Short life cycles make strategic parasite management more challenging, as a greater number of treatments are required to effectively break the life cycle and minimise reinfection. Parasites took only 10 days to complete the life cycle in mild to warm conditions, compared to 18 days in cool conditions (Table 1). Rapid life cycle completion at warmer temperatures results in a greater frequency of treatments being required in order to manage infections (http://marineparasites.com/paratreatmentcal.html).

Several capsalid monogenean species exhibit short life cycles in warm temperatures (Roubal & Diggles 1993; Bondad-Reantaso et al., 1995; Ernst & Whittington, 1996; Yoshinaga et al., 2000; Tubbs et al., 2005; Lackenby et al., 2007). Accelerated life cycles can be attributed to the increased metabolic and development rate associated with warm conditions (Poulin et al., 1989; Müeller et al., 1992; Conley & Curtis 1993; Ellis & Watanabe 1993). Hirazawa et al. (2010) and Bondad-Reantaso et al. (1995) observed similar life cycle completion times to this study for *N. girellae*. The life cycle of *N. girellae* took only 10 days to complete at 30 °C as opposed to 20 days at 20 °C in seawater (33 ‰) (Hirazawa et al., 2010) and 15 days at 25 °C (Bondad-Reantaso et al., 1995). Time to sexual maturity was influenced by temperature, taking longer at cool temperatures (Table. 1; Bondad-Reantaso et al., 1995; Hirazawa et al., 2010). Although maturation times were longer, sexual maturity by *N. girellae* was reached at a smaller size at cool temperatures compared to warm conditions (Fig. 4A, B; Table 2; Hirazawa et al., 2010). Larger monogenean individuals

lay more eggs than those of a smaller size (Roubal & Diggles 1993), indicating that more eggs per parasite are contributed to a system in warm conditions.

Smaller size at sexual maturity in cool conditions has also been recorded for *B. seriolae* infecting *Seriola lalandi* Valenciennes, 1833 (Tubbs et al., 2005; Lackenby et al., 2007). The time *B. seriolae* took to reach sexual maturity, however, was considerably longer than recorded for *N. girellae* at all temperatures. This finding, combined with the observation that *B. seriolae* has markedly lower infection success than that of *N. girellae* (Hirazawa et al., 2013), indicates that populations of *Neobenedenia* spp. can build more rapidly than those of *B. seriolae* on aquaculture farms at similar temperatures. *Benedenia seriolae* and *N. girellae* co-infect farmed *Seriola quinqueradiata* Temminck & Schlegel, 1845, and *Seriola dumerili* Risso, 1810, in Japan and differences in population growth and proliferation is crucial to understanding the threat that each species poses to aquaculture stock. *Neobenedenia* spp. are capable of producing three generations per month in warm, high saline conditions which are typical of coastal marine aquaculture facilities in the tropics (Table 1). Presently the cause for the unpredictable and sporadic nature of *N. girellae* infection events is unknown and more vigilant and frequent stock monitoring may be required in warm, high saline conditions as infections can build more rapidly (Marcogliese, 2001).

Although warmer temperatures result in an accelerated life cycle, cooler temperatures may facilitate the increase of *N. girellae* infection on farmed fish. Oncomiracidia longevity and infection success were inversely correlated to increasing water temperature (Fig. 1B, C). Hirazawa et al. (2010) also observed that infections of *N. girellae* on *S. dumerili* were significantly reduced at 30 °C compared to cooler temperatures (20 and 25 °C) in seawater. Furthermore, conditions which are cooler than

optimal can compromise the immune system of fish (see Bly & Clem 1992 for review), inhibiting their capacity to withstand parasite infection (Jackson & Tinsely 2002). Deveney et al. (2001) hypothesised that a prolonged period of unseasonably low water temperatures was the cause for a *N. melleni* outbreak on sea caged *L. calcarifer* in Queensland, Australia, during winter. Although the causes behind epizootics of *Neobenedenia* spp. are unknown, increased oncomiracidia longevity and infection success observed at cool temperatures as demonstrated in this study (Fig. 1B, C) may, together with immune-suppressed hosts, contribute to epidemics on farms during cooler months.

Neobenedenia girellae cannot complete its life cycle in low salinity environments. *Neobenedenia girellae* eggs did not hatch in 0 and 11 ‰ and hatching success was significantly reduced in 22 ‰ (Fig. 1A). Accordingly, Müeller et al. (1992) observed that the hatching success of *N. melleni* eggs was <12 % when incubated at salinities of ≤18 ‰ for 4 days. Ellis & Watanabe (1993) found hatching of *N. melleni* was inhibited entirely when eggs were exposed to salinities ≤18 ‰ for 7 days. Oncomiracidia longevity (Fig. 1B) and infection success (Fig. 1C; Ellis & Watanabe 1993) similarly decreased in hyposaline conditions. Ellis & Watanabe (1993) observed a 100 % reduction in *N. melleni* infection after a four day treatment of *Oreochromis* sp. in a range of hyposaline solutions (≤18 ‰). The use of freshwater to treat marine ectoparasites is not a new concept for the mariculture industry and findings from this study and previous studies confirm that both acute and long-term decreases in salinity represent viable treatment options (Fig. 1A–C; Müeller et al., 1992; Ellis & Watanabe 1993).

Freshwater and low salinity solutions represent effective therapeutant options when managing *N. girellae* infections on fish. Many marine fish can tolerate short-term

immersion in freshwater and baths of 2–3 minutes are effective in killing adult monogeneans (Hoshina 1968; Kaneko et al., 1988; Seng 1997; Ohno et al., 2009). These treatments cause little stress to euryhaline fish and are harmless to the culturist, environment, and consumer (Hoshina 1968; Kaneko et al., 1988; Müeller et al., 1992). Prolonged exposure to hyposaline solutions could be used to reduce egg hatching of *N. girellae* on farms (Fig 1A; Müeller et al., 1992; Ellis & Watanabe 1993; Umeda & Hirazawa 2004). This management strategy is highly suited to hatcheries or sea cage farms that can be towed into estuarine environments. However, long-term treatment of stenohaline stock with hyposaline water may be impractical in some situations.

The strategic treatment tool presented in this study is unique in that it offers a method to help break the life cycle of a harmful aquatic parasite while accounting for environmental variables that influence parasite life cycle parameters. The tool enables aquaculturists to adapt timed treatments to specific environmental variables (temperature and salinity) experienced in aquaria or on a farm. Similar to this model, Tubbs et al. (2005) developed treatment timetables for *B. seriolae* and *Zeuxapta seriolae* Meserve, 1938, infecting yellowtail kingfish, *S. lalandi* that account for parasite development time for three separate temperatures. In contrast to *B. seriolae* and *Z. seriolae* which infect a limited number of *Seriola* spp., *Neobenedenia* spp. are known to have exceptionally low host specificity and have been recorded from more than 100 ornamental and aquaculture host fishes (Ogawa et al., 1995; Whittington & Horton 1996). Nevertheless, the proposed tool needs to be tested in the field in order to determine its applicability to multiple *Neobenedenia* isolates, populations and/or species. Accurate treatment timetables reduce the cost incurred by parasite management including labour, stress to stock and cost of chemicals/treatment apparatus, by minimising the number of subsequent treatments required on farm. The proposed

treatment tool can be used for *N. girellae* infections in tropical commercial operations where similar temperatures and salinities ranges are experienced.

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Chapter 5: Biochemical composition of marine monogenean parasite eggs

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5.1 Abstract

Quantifying the biochemical components of marine invertebrate eggs reveals energy substrates available to developing embryos. There is a paucity of research on the biochemical components of marine parasite eggs and their relative importance during embryonic development. This study on the eggs of the tropical monogenean, *Neobenedenia girellae*, presents the first detailed quantitative biochemical information of the eggs of a marine monogenean. Moisture and protein composed the majority of the contents of freshly laid eggs (79.12 ± 0.82 and 11.51 ± 0.49 % respectively) followed by lipid (2.50 ± 0.15 %). Protein contributed the most to energy density of eggs (3.71 J μg^{-1}). Lipids were composed of approximately equal amounts of saturated, monounsaturated, and polyunsaturated fatty acids (35.43 ± 0.39 %, 29.08 ± 0.39 %, and 35.50 ± 0.54 %, respectively). The predominant lipid classes were phosphatidylcholine (45.79 ± 2.17 %) and triacylglycerol (25.09 ± 1.12 %). This study represents a fundamental step towards a better understanding of the early life biology of a parasite species that has had a large impact on global aquaculture industries.

Key words: Platyhelminthes, Biochemistry, Protein, Lipid, Parasites, Reproductive biology

5.2 Introduction

The eggs of marine invertebrates that are laid freely into the environment must contain all of the materials and components necessary to facilitate embryogenesis without further maternal investment (Moore & Manahan 2007). This includes the incorporation of nutritional reserves that satisfy the energetic needs of the developing embryo and hatched larva (Moran et al., 2013). Lipids, proteins, carbohydrates, and free amino acids are the primary components of most marine invertebrate eggs and are accumulated in large quantities during vitellogenesis (Sibert et al., 2004). These are vital as a source of energy during embryogenesis, hatching, and the location of suitable settlement substrate (Sibert et al., 2004). The relative proportions of the energetic reserves present in an egg undergo considerable change during development. Quantifying these changes allows for an estimation of the metabolic energy available for the development of the embryo (Bryan, 2004). The majority of research determining and quantifying the composition of embryonic energy reserves has largely been directed towards free-living echinoid, crustacean, and molluscan species (Figueiredo et al., 2008; Prowse et al., 2008; Reppond et al., 2008). Marine parasite taxa have, in comparison, been neglected despite their recognised importance in the functioning of ecosystems (Marcogliese 2005).

Capsalid monogeneans are prevalent parasites in the marine environment and infect the outer surfaces of bony fishes (Whittington 2012). One of the most notorious genera is *Neobenedenia* Yamaguti, 1963, which is currently composed of six taxa.

Neobenedenia species feed on mucus and epithelial cells of their host, causing epidermal degradation, dermal erosion, and inflammation (Trujillo-González et al., 2015b) and some species have been responsible for disease epidemics (Deveney et al., 2001; Whittington 2004). Collectively, *Neobenedenia* spp. have been recorded to infect over 100 teleost species, many of which are commercially important tropical and subtropical finfish (Whittington & Horton 1996; Whittington 2012).

The small number of biochemical studies on marine parasite taxa have only addressed the composition of adult helminths (Furlong et al., 1995; Sato et al., 2008) and parasitic copepods (Lee 1975; Tocher et al., 2010). One of the principal reasons for this may be the extreme difficulty in collecting small and dispersed eggs that adult monogeneans release into the water column. Consequently, it is challenging to collect sufficient material to ensure accurate biochemical composition data. Recently, Hutson et al. (2012) established a laboratory culture of *Neobenedenia girellae* Hargis, 1955, (Chapter 2) which is capable of supplying a continuous source of eggs. This provided a unique opportunity to study the early life history of this capsalid monogenean and investigate the biochemical composition of new, freshly laid eggs.

The aims of this study were to; 1) quantify the proximate, fatty acid and lipid class composition of newly laid *N. girellae* eggs, and 2) identify potential energy substrates available for use by developing embryos by quantifying the dominant lipid classes and specific fatty acids present in the egg. This study is a crucial first step to improve our understanding of the early life biology of monogenean species; in particular, the occurrence and abundance of specific biochemical substrates in freshly laid monogenean eggs.

5.3 Materials and methods

5.3.1 Parasite egg collection for analysis

A laboratory culture of *N. girellae* was established on barramundi, *Lates calcarifer* Bloch, 1790, to ensure a continuous source of parasite eggs for sampling (see Hutson et al., 2012). All procedures used in this research were approved by the Animal Ethics Committee of James Cook University, approval code: A1989. *Neobenedenia girellae* was identified by sequencing representative specimens from the culture (Chapter 2).

Eight barramundi (mean 140 $L_T \pm 8$ mm) were individually acclimated to 35 ‰ seawater in 10 L aquaria over a period of 24 h prior to the beginning of the study. These fish were individually infected with 80-100 *N. girellae* oncomiracidia (see Brazenor and Hutson, 2015), to collect sufficient quantities of eggs (over 14 days) which were used for analysis. After 24 h had elapsed post-infection, fish were held at a density of three fish per 80 L aquarium in 60 L of filtered, aerated, 35 ‰ seawater at 25 °C (± 1.1 °C). Fish were provided with fluorescent lighting (12 light:12 dark). Thirty litres of seawater were exchanged in each aquarium every 24 h.

Freshly laid egg masses (Fig. 1A-B) were removed from aquaria with fine-tipped forceps and cleaned by placing them on 60 µm mesh and washing gently with distilled water to removed extraneous organic matter and saltwater. Excess water was removed from egg masses by gently blotting each mass on lint-free Kimtech Kimwipes before each mass was individually stored in 2 mL Eppendorf tubes at -80 °C until analysis was performed. Seven replicate egg masses (0.215 g (range: 0.138 – 0.397) wet weight) were collected by pooling eggs collected from multiple fish. This was

performed to ensure sufficient quantities of eggs were available for analysis. Eggs were analysed for their proximate composition, total lipid, lipid classes, fatty acids, and total protein content using the methods described by Conlan et al. (2014). Analysis was performed at the Australian Institute of Marine Science (AIMS), Queensland, Australia. Energy density was determined by converting the protein and lipid masses to energy equivalents using the combustion enthalpy coefficients of Blaxter (1989); 23.6 kJ g^{-1} protein and 39.5 kJ g^{-1} lipid.

5.4 Results

Neobenedenia girellae eggs were predominantly composed of water (79.19 ± 0.82 %), with protein and lipid comprising 11.51 ± 0.49 % and 2.50 ± 0.15 % of the total wet weight, respectively. Energy density was 3.71 J μg^{-1} egg with protein comprising the majority of the energy present (Table 1).

Saturated (SFA), monounsaturated (MUFA), and polyunsaturated (PUFA) fatty acids were present in similar proportions in freshly laid *Neobenedenia girellae* eggs accounting for 35.43 ± 0.39 %, 29.08 ± 0.39 %, and 35.50 ± 0.54 % of total lipid respectively (Table 2). Lipid class composition data for freshly laid *Neobenedenia girellae* eggs is presented in Table 3. Triacylglycerols (TAGs) composed 33. 82 ± 1.20 % of total lipid, the single largest contributor of all lipid classes analysed. Acetone mobile polar lipids (AMPL) together with other polar lipid classes (phosphatidylcholine, phosphatidylethanolamine, and phosphatidylserine/inositol) cumulatively comprised a larger proportion of total lipids (~40 %; Table 3).

Palmitic (16:0) and stearic acids (18:0) accounted for the majority of SFA (116.01 ± 5.27 and 45.51 ± 1.81 mg g^{-1} respectively). MUFA present in eggs were

primarily composed of palmitoleic (16:1n-7) and oleic (18:1n-9) acids (22.86 ± 2.69 and 98.41 ± 3.06 mg g^{-1}, respectively). The primary PUFAs were linoleic (18:2n-6) and docosahexaenoic (22:6n-3; DHA) acids, accounting for 32.69 ± 1.53 and 91.15 ± 2.93 mg g^{-1}.

Table 1

Proximate composition of freshly laid *Neobenedenia girellae* eggs expressed in mg g^{-1} egg and mg g^{-1} lipid ± standard error, respectively, (n = 7 egg masses). 'NFE' = nitrogen-free extract.

Proximate analysis	% ± SE
Wet weight	
Moisture	79.191 ± 0.822
Protein	11.512 ± 0.491
Lipid	2.500 ± 0.145
Ash	1.328 ± 0.084
NFE	5.505 ± 0.227
Energy density (J μg^{-1})	3.705
Dry weight	
Protein	55.215 ± 0.058
Lipid	11.976 ± 0.039
Ash	6.390 ± 0.035
NFE	26.419 ± 0.039

Table 2

Fatty acid composition of freshly laid *Neobenedenia girellae* eggs expressed in mg g^{-1} egg and mg g^{-1} lipid ± standard error, respectively, (n = 7 egg masses). Fatty acids accounting for < 1 mg g^{-1} were omitted from report. 'SFA' = saturated fatty acids, 'MUFA' = monounsaturated fatty acids, 'PUFA' = polyunsaturated fatty acids, 'LC' = long chain.

Fatty acid	mg g^{-1} ± SE	% of total lipid ± SE
14:0	4.84 ± 0.319	0.93 ± 0.031
15:0	4.80 ± 0.224	0.94 ± 0.044
16:0	116.01 ± 5.272	22.43 ± 0.331
17:0	11.84 ± 0.527	2.25 ± 0.060
18:0	45.51 ± 1.814	8.81 ± 0.124
16:1n-7	22.86 ± 2.687	4.37 ± 0.388
18:1n-7	11.78 ± 0.727	2.27 ± 0.075
18:1n-9	98.41 ± 3.055	19.09 ± 0.250
20:1n-9	5.76 ± 0.209	1.12 ± 0.017
22:1n-9	1.14 ± 0.104	0.21 ± 0.014
24:1n-9	4.60 ± 0.175	0.89 ± 0.012
22:1n-11	4.94 ± 0.263	0.96 ± 0.025
18:3n-3	1.01 ± 0.065	0.20 ± 0.006
20:3n-3	7.31 ± 0.477	1.42 ± 0.071
20:5n-3	9.75 ± 1.440	1.86 ± 0.225
22:3n-3	2.01 ± 0.227	0.39 ± 0.035
22:5n-3	9.72 ± 0.783	1.87 ± 0.101
22:6n-3	91.15 ± 2.932	17.72 ± 0.466
16:2n-4	1.01 ± 0.289	0.19 ± 0.049
18:2n-6	32.69 ± 1.529	6.37 ± 0.313
18:3n-6	1.48 ± 0.121	0.28 ± 0.016
20:2n-6	1.74 ± 0.051	0.34 ± 0.011
20:3n-6	2.70 ± 0.254	0.53 ± 0.047
20:4n-6	15.14 ± 0.583	2.95 ± 0.134
22:4n-6	1.43 ± 0.038	2.95 ± 0.134
22:5n-6	4.43 ± 0.253	0.87 ± 0.064
SFA	182.677 ± 7.508	35.43 ± 0.385
MUFA	149.928 ± 6.359	29.08 ± 0.387
PUFA	183.765 ± 5.251	35.50 ± 0.539
n-3 PUFA	121.92 ± 4.773	23.62 ± 4.77
n-3 LC PUFA	120.87 ± 4.745	23.41 ± 4.75
n-6 PUFA	59.91 ± 2.448	11.60 ± 2.45
n-6 LC PUFA	23.70 ± 0.957	4.59 ± 0.96

Table 3

Lipid class composition (%) of total lipids present in freshly laid *Neobenedenia girellae* eggs. AMPL = Acetone mobile polar lipids.

Lipid class	% of total lipid ± SE
Sterol ester	8.836 ± 0.474
Triacylglycerol	33.822 ± 1.195
Free fatty acid	1.062 ± 0.076
1,3 distearoylglycerol	3.428 ± 0.417
Sterol ester	6.779 ± 0.188
AMPL	6.98 ± 0.853
Phosphatidylethanolamine	6.089 ± 0.179
Phosphatidylserine/inositol	5.309 ± 0.563
Phosphatidylcholine	21.904 ± 1.423
Lyso-phosphatidylcholine	5.796 ± 0.429
Storage	47.147 ± 0.942
Stuctural	52.854 ± 0.942
Storage:Structural ratio	0.897 ± 0.033

5.5 Discussion

Moisture and protein comprised the majority of the contents of the total wet weight of freshly laid *N. girellae* eggs in tropical north Queensland, Australia. Lipids represented a comparatively small proportion of the total contents (Table 2) and although this may seem unexpected, the eggs of tropical invertebrates are often lipid-poor compared to those in higher latitudes, a trend observed in copepod genera *Calanus* (see Lee & Hirota 1973; Scott et al., 2000), *Euphausia* (see Lee & Hirota 1973; Morris & Hopkins 1983) and *Euchaeta* (see Lee & Hirota 1973). This is thought to be due to the high turnover of biomass and high metabolic rates observed in warmer tropical waters (Lee & Hirota 1973). The total lipid content in these tropical copepod genera are comparable to what is observed in the present study (Morris & Hopkins 1983; Lee et al., 2006). Energy density of *N. girellae* eggs is similar to those found for echinoderm eggs and especially those with lecithotrophic life histories (McEdward & Morgan 2001). Protein composed almost two thirds of this energy and it is currently unknown whether this represents an energy reserve for the developing larva or if it is used primarily in a structural capacity. No parallels between other marine parasites can be made as energy density has, to the authors' knowledge, never been quantified previously.

Triacylglycerols (TAGs) have been identified as crucial sources of energy for a large number of metabolic processes and requirements (Kattner et al., 2003). Triacylglycerols comprised a large proportion of total lipids (~25 %), constituting the majority of neutral lipids, however, the predominant lipid class was phosphatidylcholine (~46 %) which constituted the bulk of polar lipids present in the eggs (Table 2). Traditionally, polar lipids have been viewed as primarily playing a

structural role in bio-membranes (Lee 1975; Kattner et al., 2003). Some studies have suggested that specific phospholipids, particularly phosphatidylcholine, are exploited as energy substrates by some marine fish (Tocher et al., 2008) and crustacean embryos and larvae (Kattner et al., 2003). The observed lower concentration of TAGs in many marine invertebrate eggs has been suggested to be due to a lower concentration of digestive lipases which facilitate their digestion compared to those for phospholipids, as found for marine fish larvae (Zambonino-Infante & Cahu 2001; Villeneuve et al., 2005). In marine species with lecithotrophic embryos and larvae, TAGs have been linked to not only fuelling embryogenesis but also larval hatching, locomotion, and metamorphosis (Byrne et al., 2008; Prowse et al., 2008). Monogenean larvae have very short free-swimming periods where they attempt to locate and attach to a suitable host. If larvae are unable to locate a host within this short window of opportunity (generally <48 h depending on temperature) they expend their energy reserves and die (Whittington et al., 2000a). Brazenor & Hutson (2015) (Chapter 4) reported larval longevity of approximately 19 h at 26 °C for the present study species. If TAGs are exploited as energy substrates for this short larval period, it would provide an explanation for why such a small proportion of these lipids were found in freshly laid eggs. Preferential catabolism of phosphatidylcholine may indicate that the developing embryo is meeting metabolic needs which are not provided by TAGs (Fraser et al., 1988). This could be the provision of specific nutrients such as phosphate and choline which are known to be essential for healthy fish embryo growth and organ formation (Viola et al., 1986). Investigation of the proportion of these lipids in eggs at different temperatures and developmental stages would be essential to determine their roles in embryogenesis.

Lipid profiles similar to those of the present study have been observed for a small number of adult marine parasite species. Lee (1975) and Tucker & Wootten (2000) analysed the lipid profiles of adult *Lepeophtheirus salmonis* (Krøyer) and found that TAGs and phosphatidylcholine comprised the majority of lipids (37 and 30 %, respectively). A comparison of the lipids found in free-living copepods in the surrounding area of the study conducted by Lee (1975) revealed that wax esters were the primary energy source in these non-parasitic copepod species. Beach et al. (1973) observed a similar lipid profile for the elasmobranch cestode *Callibothrium verticillatum* Rudolphi, 1819, with 54 % of total lipids composed of TAGs and 25 % of phosphatidylcholine. Sato et al. (2008) examined the fatty acid content of *N. girellae* adults and the mucus and skin of two of its hosts, *Seriola dumerili* Risso, 1810, and *Verasper variegatus* Temminck & Schlegel, 1846. Minor differences in lipid proportions between *Neobenedenia* adults and eggs reported by Sato et al. (2008) and the present study, respectively, are thought to be due to selective deposition or retention of lipids, *in vivo* bioconversion, or differences in host epithelial chemistry.

This is the first study to investigate the biochemical composition of the eggs of a marine monogenean. Although this study presents a comprehensive analysis of the contents of *Neobenedenia girellae* eggs, it is unknown how egg biochemistry is influenced by environmental variables and how it changes over the development of the embryo. These represent intriguing and novel research topics for future biochemical analysis.

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Chapter 6: Effects of tropical temperatures on *Neobenedenia girellae* (Monogenea: Capsalidae) fecundity, egg volume, and biochemical content

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6.1 Abstract

The reproductive biology and associated biochemical profile changes of the eggs of the marine monogenean parasite *Neobenedenia girellae* were quantified in different temperature conditions (20, 25, and 30 ºC) throughout the adult life span and egg development. Adult parasites grew to a larger total size ($p<0.0001$), produced larger eggs ($p<0.0001$) and laid for a longer duration ($p<0.0001$) at cooler temperatures (20 ºC). Fecundity was highest at 20 and 30 ºC (2,837 ± 10.28 and 2,432 ± 35.70 mean total eggs laid, respectively) compared to 25 ºC (1,698 ± 10.78 mean total eggs laid). Moisture and protein composed the majority of the contents of freshly laid eggs (between 75.45-82.55 % and 9.75-15.53 % respectively) followed by lipid (1.93-3.03 %). Lipids were composed of approximately equal amounts of monounsaturated and polyunsaturated fatty acids (~30 %). Saturated fatty acids were in considerably higher quantities (~40 %). The predominant lipid classes were acetone mobile polar lipids (19.80-25.62 %), phosphatidylcholine (13.37-16.48 %) followed by phosphatidylserine/inositol (11.70-14.15 %). Triacylglycerols comprised a small amount of total lipid, between 8.45 and 13.79 %. Although proximate composition of eggs did not significantly change between the three temperatures analysed, the majority of monounsaturated and polyunsaturated fatty acids were in lower quantities at 30 ºC.

Biochemical substrates were difficult to determine for developing embryos, however, three monounsaturated fatty acids 18:1 n-7, 20:1 isomers, 16:1 n-7, and one polyunsaturated fatty acid 22: 5n-3 were found to significantly decrease in quantity with progressive development of the embryo. A gradual increase in concentration of arachidonic acid, an immune-modulatory compound, suggests that *N. girellae* may preferentially be retaining lipids which assist in attachment to their host after the larvae (oncomiracidia) hatch. Data collected in this study indicate that this parasite adopts a variety of strategies to maximise its performance in environmental conditions. These strategies include changes to its reproductive biology and altering the biochemical contents of its eggs in tropical temperatures.

Key words: Platyhelminth, Capsalidae, lipid, protein, egg volume, reproductive biology.

6.2 Introduction

Eggs produced by marine invertebrates must contain all biochemical material necessary for embryogenesis, hatching and dispersal of the larva (Hoegh-Guldberg & Emlet 1997; Miner et al., 2005; McAlister & Moran 2012). Contents include lipids, proteins, carbohydrates, and free amino acids which are usually accumulated in each egg during vitellogenesis in the parent before eggs are laid (Sibert et al., 2004). Typically, eggs are then either protected by the parent or allowed to disperse in the environment. The use of eggs as a dispersal method is almost ubiquitous amongst parasite species (see Whittington et al., 2000a). Egg dispersal can safeguard against environmental conditions becoming unfavourable in the current location, reduce

inbreeding and the loss of evolutionary adaptability, and reduce the chance of the current host becoming over-infected (Rohde, 2001). As no further maternal influence is possible, the contents of these eggs offers a discrete picture on the nutritional requirements of a particular species during embryogenesis, larval dispersal, and metamorphosis. Biochemical research of marine parasites, however, has been sparse in comparison to free-living echinoid, crustacean, and molluscan species (Figueiredo et al., 2008; Prowse et al., 2008; Reppond et al., 2008). Throughout embryogenesis and larval development, environmental conditions dictate the rate at which metabolic processes occur (Hoegh-Guldberg & Pearse 1995; Rayssac et al., 2010).

The most pivotal abiotic environmental factor is temperature. Temperature is known to strongly influence the metabolic rates of ectothermic organisms. This has direct implications on their activity, growth, development, and reproduction (Magnuson et al., 1997; Marcogliese 2001; Poulin & Mouritsen 2006; Hance et al., 2007). Temperature is one of the most important external environmental factors controlling development and metabolic processes and has been found to be inversely correlated to development times and embryonic energy reserve utilisation (Pechenik 1984; Hoegh-Guldberg & Pearse 1995; Aldridge et al., 1995; Gannicott & Tinsely 1998; Rayssac et al., 2010; Swingle et al., 2013). The fitness and survival of an embryo depends on a number of maternally derived attributes such as egg size and quality (biochemical content) but also temperature related factors such as the metabolic rate at which those contents are consumed during development (Gannicott & Tinsely 1998; Marsh et al., 1999). As such, quantifying temperature effects on fecundity, egg volume, and embryonic energy reserves is crucial to understand patterns of population growth, embryo development, dispersal potential, and life history evolution.

Monogeneans in the family Capsalidae infect the outer surfaces of bony fishes and are a particular threat to the aquaculture industry as some species have been responsible for epizootics (Bauer & Hoffman 1976; Paperna & Overstreet 1981; Deveney et al., 2001; Whittington 2004). Species in the genus *Neobenedenia* Yamaguti, 1963, are particularly harmful and have been recorded from over 100 teleost species, many of which are important tropical and subtropical fin fish species in marine aquaria and aquaculture (Whittington & Horton 1996; Ogawa & Yokoyama 1998; Hirazawa et al., 2004; Ogawa et al., 2006; Whittington 2012; Chapter 2). The embryonation period and time to maturation of *Neobenedenia girellae* Hargis, 1955, a species found infecting a number of important aquaculture and ornamental fish species in northern Queensland (Chapter 2 and 4), has been found to be strongly influenced by temperature (Chapter 3; Chapter 4). Very little data exists, however, for the effects of temperature on early life biology such as fecundity, egg volume and biochemical content, and embryonic utilisation (Chapter 5). One of the primary reasons for this may be that collecting small eggs in the water column is extremely difficult. A reliable and predictable source of eggs is needed to collect sufficient quantities of eggs for biochemical analysis. Recently, a laboratory culture of *N. girellae* established by Hutson et al. (2012) has enabled a continuous and predictable supply of eggs and parasites for research purposes (Chapter 5). This provides an opportunity to investigate the early life biology and biochemistry of eggs of this parasite.

The aim of this study was to measure the effects of temperature on the reproductive biology of *N. girellae*. This broad aim was addressed through four discrete studies to determine the effect of temperature on: 1) fecundity of adult parasites: 2) changes in egg volume throughout the life span of adults: 3) energy reserves available

to developing embryos, and: 4) how embryos utilise energy reserves during development.

6.3 Materials and Methods

6.3.1 Parasite egg collection

A monoculture of *N. girellae* was established on *Lates calcarifer* Bloch, 1790, to ensure a continuous source of parasites for experimentation (see Hutson et al., 2012). Thirty-six barramundi (mean 150 L_T (120–180 mm)) were gradually acclimated to 35‰ seawater from freshwater in one 80 L aquaria over a period of three days prior to the beginning of the study. Twelve replicate fish were held at one of three temperatures; 20 (± 0.7 °C), 25 (± 0.4 °C), or 30 °C (± 0.6 °C) at a density of two fish per 80 L non-recirculating aquarium. Temperature treatments were selected to represent seasonal temperature variation experienced in tropical climates in Queensland, Australia (International Panel for Climate Change, 2007). One hundred and twenty oncomiracidia were added to each container as per published methods (Chapter 4). Fish were maintained in each temperature with fluorescent lighting for a 12:12 LD cycle to collect sufficient quantities of eggs for analysis. Fifty litres of seawater were exchanged in each aquarium every 72 hours.

6.3.2 Collection of eggs for biochemical analysis

Neobenedenia girellae eggs (Fig. 1) were collected from aquaria (methods as per Chapter 5) and analysed for their biochemical composition at specific stages of embryo

development. Egg masses, floating on the water surface, were collected from aquaria using fine-tipped forceps daily. Eggs from each temperature treatment were pooled to ensure that sufficient quantities were available for analyses (as per Barnes 1965). In total ~2 g of hydrated eggs (~500,000 eggs) were collected for each temperature treatment with ~0.5 g of hydrated eggs collected for each of the four stages of development. Egg development stages included; 1) freshly laid eggs, which were collected within 12 hours of being laid; 2) embryonated eggs, which were dark brown and showed evidence of embryonic cellular division; 3) developing eggs, where >50% of embryos possessed visible eye-spots and; 4) imminent hatching, where eggs were collected within 2 hours of egg hatching commencing (Fig. 2). Approximately 0.5 g of hatched eggs (wet weight) was also collected from each temperature to analyse the egg shell and egg filament in the absence of the embryo (Fig. 2E). Eggs were checked every 12 h under a stereomicroscope to determine the stage of development. To ensure eggs did not start hatching before they were collected for the final development stage, eggs were collected in darkness at 6 am on the day of first hatching (see Dinh Hoai & Hutson 2014). Day of first hatch was predicted to be the day after oncomiracidia were first observed moving inside their eggs (Tubbs et al., 2005).

Following collection from aquaria, egg masses were cleaned by placing them on 60 µm mesh and washing them gently with distilled water to remove saltwater and extraneous organic matter. Excess water was removed from egg masses by gently blotting each mass on lint-free Kimtech Kimwipes (see Chapter 5). Each egg mass was then placed into a 2 mL Eppendorf tube, labelled, and stored at -80 °C. Two replicates of approximately equal weight were made for each temperature and development period. Eggs were analysed for ash, moisture, total lipid, lipid classes, fatty acids, and total protein content at the Australian Institute of Marine Science (AIMS), Queensland,

Australia (methods as per Conlan et al., 2014). The only variation from methods described in Chapter 5 was that samples in the present study were homogenised for 3 min using a Biospec Mini-Beadbeater-96 prior to sonication.

6.3.3 Fecundity

To determine the fecundity of *N. girellae* adults at various temperatures, egg production was quantified from a single individual adult attached to host *L. calcarifer* every 24 h until egg production ceased. Fish were sourced from a freshwater barramundi hatchery and then acclimated to saltwater to ensure no prior infection by *N. girellae*, which exclusively occurs in saline water. Each fish was individually infected with one oncomiracidium sourced from the laboratory monoculture (as per Dinh Hoai & Hutson 2014). Ten-litre aquaria were filled with 4 L of filtered, 35‰ seawater and provided with aeration throughout the experiment. To ensure sufficient replicates at each temperature, more fish were initially infected at 30 °C (n=20 fish) than at 25 °C (n=18 fish) or 20 °C (n=16 fish) as high temperatures can decrease infection success (Chapter 4). This resulted in 13, 10, and 7 replicate fish held individually at 20, 25, and 30 °C, respectively. Daily egg production was determined by filtering the seawater in each aquarium through a 60µm filter at 1600 each day. Fish were removed from each aquarium gently by hand prior to filtering and placed into a new aquarium with fresh seawater. Eggs were counted under a stereomicroscope using a hand-held counter to determine egg production/parasite/day. After egg production ceased, fish were bathed for three minutes in dechlorinated freshwater to confirm infection by a single, individual *N. girellae*. Parasites were collected and preserved in 70 % EtOH and then stained with haematoxylin, dehydrated through an alcohol series, and mounted on

microscope slides in Canada balsam. Total length and total width were measured using a micrometer and ImageJ 1.44p (Java 1.6.0_20).

6.3.4 Egg volume

To determine the volume of *N. girellae* eggs laid in tropical temperatures, ten freshly laid eggs were collected every 24 h over the life span of *N. girellae* adults from the experiment described above (see section 6.*3.3*). Eggs were collected every day until egg production ceased. These eggs were individually placed on a microscope slide in seawater media for examination and were photographed using a compound microscope with an attached digital camera (Nikon D7000). Egg compression was avoided by not placing a coverslip over the top of eggs. The edges of each egg were assigned a letter (A, B, C, a, b, or c where (A, a), (B, b), and (C, c) are opposite edge pairs and do not share common vertices; Fig. 3). Egg filaments were not included in length measurements of each edge. The volume of each irregular tetrahedral egg was determined using a Heron-type equation (1) (see section 6.*3.5*).

6.3.5 Statistical analyses

All data were reported as mean ± standard error and were checked for homogeneity of variance and normality prior to statistical analysis. Two-way analyses of variance (two-way ANOVA) were used to assess the effects of temperature on the proportions of moisture, protein, lipid, ash, and nitrogen-free extract (NFE) present in *N. girellae* eggs at each development period. *Post hoc* comparisons of group means

were performed using Tukey's HSD test. Analyses were performed using the S-PLUS stastistical software package. Significance was accepted at $p<0.05$.

The volume of eggs was determined by measuring the edge lengths of each face of the tetrahedral eggs using a micrometer and ImageJ 1.44p (Java 1.6.0_20) (Fig. 3). The edge lengths were then integrated into the Heron-type equation (1) used for determining the volume of irregular tetrahedrons:

(1) Volume of irregular tetrahedral egg

$$= \frac{1}{12} \times \sqrt{(4a^2b^2c^2 - a^2a'^2 - b^2b'^2 - c^2c'^2 + a'b'c')}$$

Where:

$a' = b^2 + c^2 - A^2$

$b' = c^2 + a^2 - B^2$

$c' = a^2 + b^2 - C^2$

Two-way ANOVAs were performed on lipid class and fatty acid datasets to compare biochemistry at different temperatures and development periods of *N. girellae* eggs. *Post hoc* comparisons of biochemistry group means were performed using Tukey's HSD test. Hatched eggs were not included in the analysis of these components but were included in Figures and Tables in the Results section.

Two-way ANOVAs were used to compare fecundity and egg volume over the life span of *N. girellae* adults at each temperature. Fecundity data was square-root transformed prior to statistical analysis as untransformed data did not conform to homogeneity of variance. *Post hoc* comparisons of egg volume group means were performed using Tukey's HSD test. A Fisher's LSD test was used to compare group means of fecundity data.

6.4 Results

6.4.1 Fecundity, time to maturity, and reproductive longevity of Neobenedenia girellae *adults*

Fecundity was highest at the coolest temperature tested, 20 °C (2,837 ± 10.28 eggs laid over parasite lifetime) followed by 30 °C (2,432 ± 35.70) and 25 °C (1,698 ± 10.78) (Table 1). Egg production rate rapidly increased following sexual maturity and peak egg production occurred 9, 8, and 6 days post maturity at 20, 25, and 30 °C respectively (Fig. 4). Egg production rate per parasite was highest at 30 °C (180.2/parasite/day) followed by 20°C (113.5/parasite/day) and 25°C (89.4/parasite/day). Temperature had a significant effect on the fecundity of parasites (ANOVA, $F_{2, 399} = 36.254$, $p<0.0001$). Mean egg production at 20 °C and 30 °C was significantly higher than 25 °C over the lifetime of the parasites with more eggs being produced at the warmest and coolest temperatures tested. Egg production peaked on day 6, 8, and 10 following sexual maturity at 20, 25 and 30 °C respectively, with production two times higher at 30 °C compared to 20 and 25 °C (Fig. 4). The age of the parasite also had a significant effect on fecundity (ANOVA, $F_{24, 399} = 12.656$, $p<0.0001$) with newly sexually mature and senescing parasites producing fewer eggs (Fig. 4). The interaction between temperature and parasite age was also significant (ANOVA, $F_{30, 399} = 5.998$, $p<0.0001$).

Neobenedenia girellae individuals matured considerably faster and began laying eggs earlier at 30 °C following initial infection of the host fish (Table 1). Parasites matured on day 7 at 30 °C, but time to maturity was almost twice as long at 20 °C (day 12). Egg production continued for 13 consecutive days at 30 °C but continued for an

extended period at cooler temperatures; 19 days and 25 days for 25 and 20 °C, respectively (Fig. 4).

6.4.2 Volume of eggs and size of Neobenedenia girellae *adults*

Neobenedenia girellae egg volume was significantly larger in cool temperatures (20 and 25 °C) towards the end of egg production. Egg volume at 30 °C did not significantly change between the first and last days of egg production. Volume significantly increased over the egg laying period of adults at 20 and 25 °C (Fig. 5). Both temperature (ANOVA, $F_{2, 263} = 69.541$, $p < 0.0001$) and when eggs were laid over the life span of the parasite (ANOVA, $F_{21, 263} = 10.882$, $p < 0.0001$) had significant effects on egg volume (Table 1; Fig. 4). The interaction between temperature and parasite age was also significant (ANOVA, $F_{26, 263} = 3.019$, $p < 0.0001$). Mean egg volume was largest at 25 °C ($2.71 \times 10^{-4} \pm 4.42 \times 10^{-6}$) compared to 20 °C ($2.68 \times 10^{-4} \pm 6.65 \times 10^{-6}$) and 30 °C ($2.28 \times 10^{-4} \pm 4.42 \times 10^{-6}$), however, the egg production period at 20 °C was twice as long (Table 1; Fig. 5).

Neobenedenia girellae adults collected at the end of their lives (when egg production had decreased to <50 eggs per day) were significantly larger in cooler conditions (Fig. 6). Temperature significantly influenced the morphology of parasites at the end of their reproductive lives in both total body length (ANOVA, $F_{2, 21} = 14.268$, $p < 0.001$) and width (ANOVA, $F_{2, 21} = 28.306$, $p < 0.0001$). Body length of parasites was not significantly different at 20 and 25 °C (Fig. 6).

6.4.3 Proximate composition of Neobenedenia girellae *eggs*

Irrespective of development stage or temperature, *N. girellae* eggs were predominantly composed of water (min-max; 75.45-82.55%) followed by protein (min-max; 9.75-15.53%), NFE (min-max; 4.13-8.46%), and lipid (min-max; 1.93-3.03%) (Table 2). Dehydrated eggs were composed primarily of protein (min-max; 37.70-69.62%), NFE (min-max; 22.10-36.83%), and lipid (min-max; 9.61-12.52%). Ash content was consistently the lowest component of both hydrated (min-max; 1.12-3.66%) and dehydrated (min-max; 5.94-15.86%) *N. girellae* eggs (Table 2). Hatched eggs showed varying amounts of lipid and protein content across temperatures. Hatched egg values were excluded from analyses and reporting of values in this section.

Development period was not found to significantly influence the proximate composition (moisture, protein, lipid, ash or NFE) of eggs, however, protein in hydrated *N. girellae* eggs was significantly influenced by temperature (ANOVA, $F_{2, 18} = 6.511$, $p = 0.0075$), specifically between eggs produced in 25 and 30 °C where protein was higher in the early stages of egg development at 25 °C. Temperature was not found to be a significant factor influencing levels of moisture (ANOVA, $F_{2, 18} = 2.04$, $p = 0.159$), lipid (ANOVA, $F_{2, 18} = 0.823$, $p = 0.462$), ash (ANOVA, $F_{2, 18}$ 0.559, $p = 0.189$), or NFE (ANOVA, $F_{2, 18} = 1.71$, $p = 0.209$) in hydrated eggs.

Energy density of *N. girellae* eggs closely followed the pattern seen in protein content. Egg energy density was significantly affected by temperature (ANOVA, $F_{2, 12} = 7.32$, $p = 0.0083$) but not by development period (ANOVA, $F_{3, 12} = 0.850$, $p = 0.493$). Eggs produced at 25 °C were significantly richer in energy than those at 30 °C with protein comprising the majority of the energy present at all temperatures and

development periods. Eggs produced at 20 and 30 °C did not differ significantly in their energy density.

A small but consistent amount of lipid and protein was detected in hatched eggs across all temperatures and development stages (Table 2). Photographs taken of eggs immediately following hatching shows remaining material inside egg cases (Fig. 7).

6.4.4 Lipid class composition of Neobenedenia girellae *eggs*

Neobenedenia girellae eggs were predominantly composed of polar lipids irrespective of development period of the embryo or temperature in which they developed (Table 4). Polar lipids comprised between 62.51-70.07 % of total lipids compared to 29.92-37.52 % for neutral lipids. Polar and neutral lipids were not influenced by temperature or development period independently but a significant interaction was observed for both (ANOVA, $F_{6, 12} = 4.88$, $p = 0.0095$). Acetone mobile polar lipids (AMPLs), which include glycolipids and monoacylglycerols, was the predominant lipid class present in *N. girellae* eggs, comprising between 19.80-25.62 % of total lipid across all temperatures and development periods of embryos. *Neobenedenia girellae* eggs which developed at 30 °C had significantly higher AMPL than those at 20 and 25 °C (ANOVA, $F_{2, 12} = 15.12$, $p = 0.0005$). The interaction between temperature and development period (ANOVA, $F_{6, 12} = 11.79$, $p = 0.0002$) was also significant in determining the AMPL content of *N. girellae* eggs. Phospholipids (phosphatidylcholine, phosphatidylserine/inositol, and phosphatidylethanolamine) cumulatively composed over 35 % of the total lipid of *N. girellae* eggs. Phosphatidylcholine was the most prevalent phospholipid (13.37-16.48 % of total lipid) irrespective of temperature and development period followed by

phosphatidylserine/inositol (11.70-14.15 %) and lastly phosphatidylethanolamine (8.99-10.10 %). Phosphatidylcholine and phosphatidylserine/inositol were not significantly influenced by temperature or development period, however, the interaction between the two significantly affected the phosphatidylethanolamine content of eggs (ANOVA, $F_{6, 12} = 3.17$, $p = 0.042$).

Triacylglycerol (TAG) and sterol ester were the most common neutral lipids comprising between 8.45-13.79 % and 9.96-13.85 % of total lipid respectively irrespective of temperature and development period. Each was significantly affected by the interaction between temperature and development period (ANOVA, $F_{6, 12} = 5.41$, $p = 0.0064$) and (ANOVA, $F_{6, 12} = 3.31$, $p = 0.0369$), respectively, but not by each factor independently. Remaining lipid was composed of 1,3 distearoylglycerol (7.51-9.14 %), sterol (5.63-8.82 %), and free fatty acids (1.98-4.22 %). Temperature significantly affected the amount of free fatty acids with significantly lower amounts in eggs which developed at 20 °C compared to 25 °C (ANOVA, $F_{2, 12} = 4.48$, $p = 0.035$). No significant effects of temperature, development period, or the interaction were detected for 1,3 distearoylglycerol or sterol (Table 4).

6.4.5 Fatty acid composition of Neobenedenia girellae *eggs*

Temperature and not development stage of the embryo most strongly influenced the fatty acid content of *N. girellae* eggs and almost exclusively there was less of any particular fatty acid in warmer conditions (Table 3). Monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA) composed approximately 30 % of the fatty acid content of eggs irrespective of development stage of embryos. Monounsaturated fatty acids were significantly lower at 25 ºC compared to 30 ºC whilst

there were significantly more PUFA at 20 and 25 ºC ($p = 0.0004$). The same pattern was followed by n-6 PUFA, n-6 LC PUFA, n-3 PUFA and n-3 LC PUFA, where significantly more was observed at 20 and 25 ºC compared to 30 ºC ($p = 0.0007$). Saturated fatty acids (SFA) composed a larger proportion of total fatty acids (~40 %) and did significantly change over temperature or development period.

Within the SFA, 16:0 and 18:0 were the most prominent individual fatty acids, although neither significantly changed in content between temperatures and development stages of the *N. girellae* embryos. Of the 17 individual fatty acids that were observed changing in content, only a single one was a SFA; 17:0 (comprising approximately 2% of total fatty acid content), which significantly decreased in amount at 30 ºC compared to 20 and 25 ºC ($p < 0.0007$).

The predominant MUFA were 16:1n-7 and 18:1n-9, comprising approximately 5 and 17 % respectively of total lipid in *N. girellae* eggs. 16:1n-7 was significantly higher at the first stage of development (ANOVA, $F_{3, 12} = 7.587$, $p = 0.0017$) and at 20 and 25 ºC (ANOVA, $F_{2, 18} = 15.64$, $p = 0.0001$). Oleic acid did not significantly change in amount over the range of temperatures or development stages tested. Two MUFAs provided results contrary to the majority of other lipids; content of 18:1n-9 trans and 22:1-isomers was significantly higher at 30 ºC compared to the cooler temperatures 20 and 25 ºC ($p \leq 0.02$).

Linoleic (18:2n-6) and docosahexaenoic (22:6n-3; DHA) acids were the PUFA with the highest content in *N. girellae* eggs accounting for approximately 6 and 16 %, respectively. Both of these fatty acids were in significantly lower amounts at 30 ºC compared to 20 and 25 ºC. Development period of the embryo did not significantly influence the amount of these fatty acids. Other PUFA which followed this same trend include 22:3n-3, 22:5n-3, 22:5n-6, 20:4n-6, and 20:3n-6 which were all significantly

reduced at 30 ºC compared to cooler conditions ($p < 0.004$). Eicosapentaenoic acid (20:5n-3; EPA), although only comprising approximately 2 % of total lipid, was at its highest concentration at 25 ºC compared to 20 and 30 ºC ($p = 0.022$).

Development stage of the *N. girellae* embryo was only found to significantly alter the concentration of four fatty acids; palmitoleic (mentioned above), 18:1n-7, 22:5n-3, and 20:1-isomers, where the first stage of development (freshly laid eggs) had higher concentrations than further stages of development of the embryo ($p < 0.018$). With the exception of 22:5n-3, all of these fatty acids are MUFA.

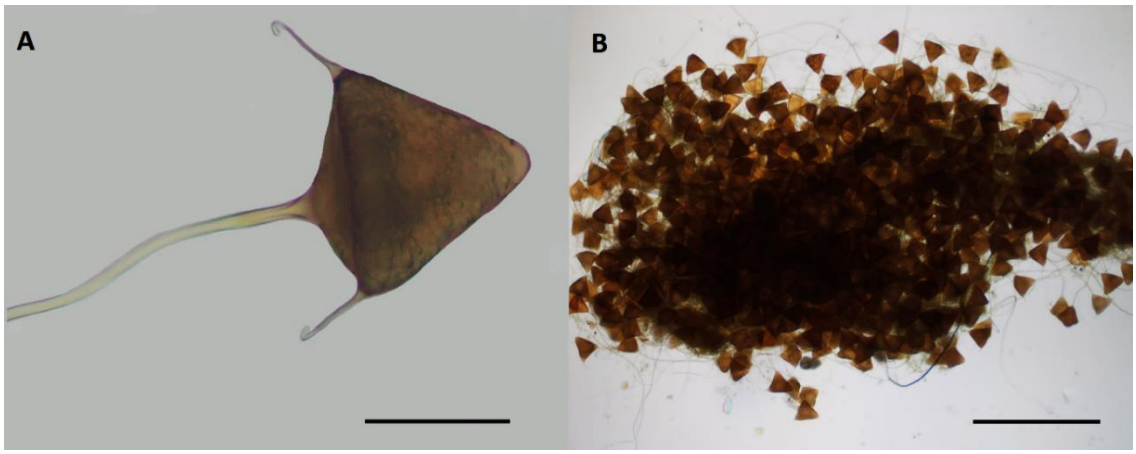

Figure 1: Freshly laid *Neobenedenia girellae* eggs. A = Single egg at high magnification. Scale = 100 µm. B = *Neobenedenia girellae* egg mass. Scale = 600 µm.

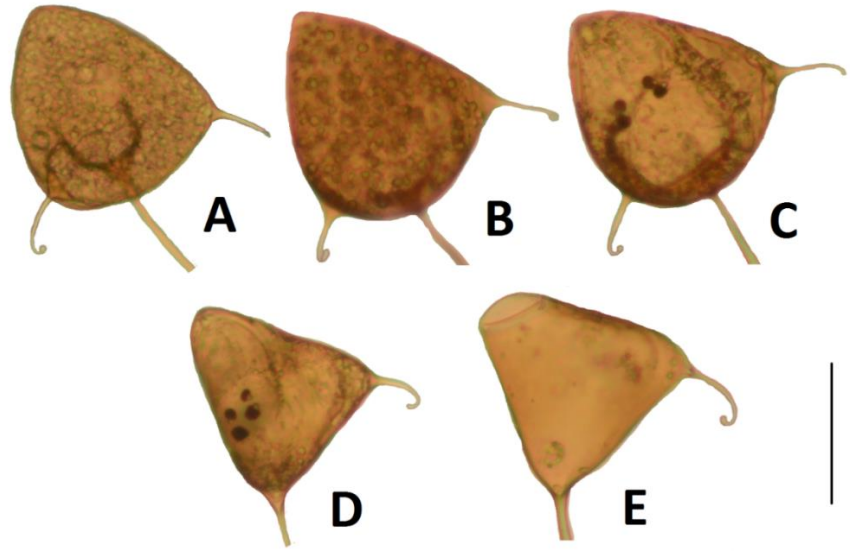

Figure 2: *Neobenedenia girellae* egg development. Egg development was classed into five different stages: stage I = freshly laid (A): stage II = embryonated (B): stage III = developing (C): stage IV = imminent hatching (D): and stage V = hatched (E). Scale bar = 50 µm.

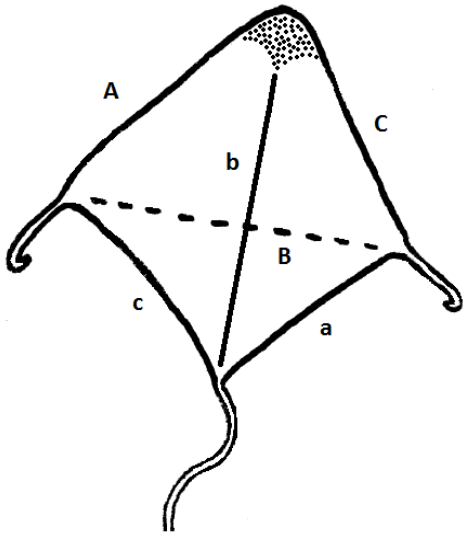

Figure 3: *Neobenedenia girellae* egg with vertices labelled used in the quantification of egg volume (a, b, c, A, B, C).

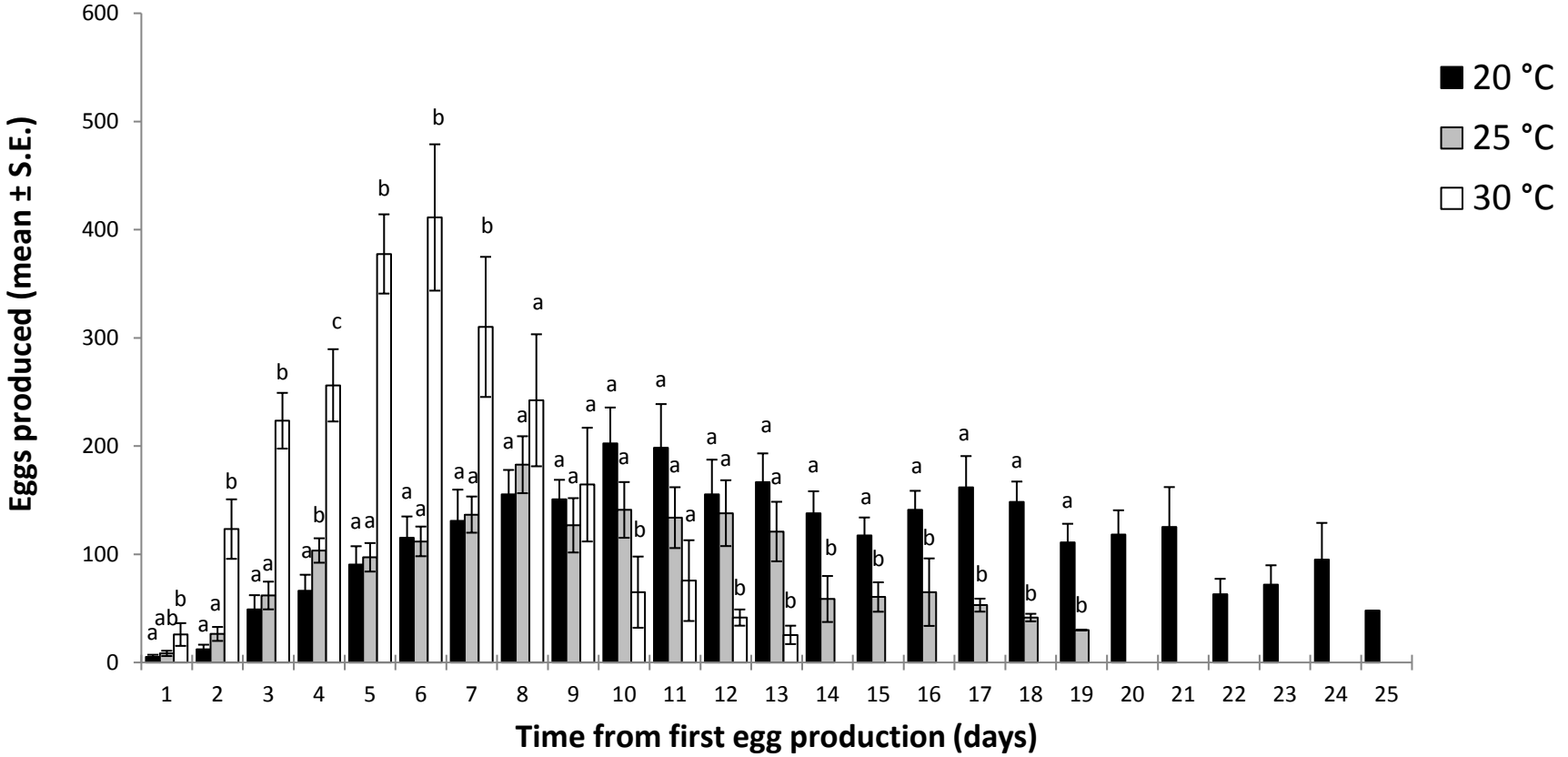

Figure 4: Fecundity of *Neobenedenia girellae* adults over their reproductive periods at a range of temperatures (20, 25 and 30° C). 'a', 'b', and 'c' = significant differences between mean number of eggs produced on each day determined using Tukey's HSD test, $p < 0.05$.

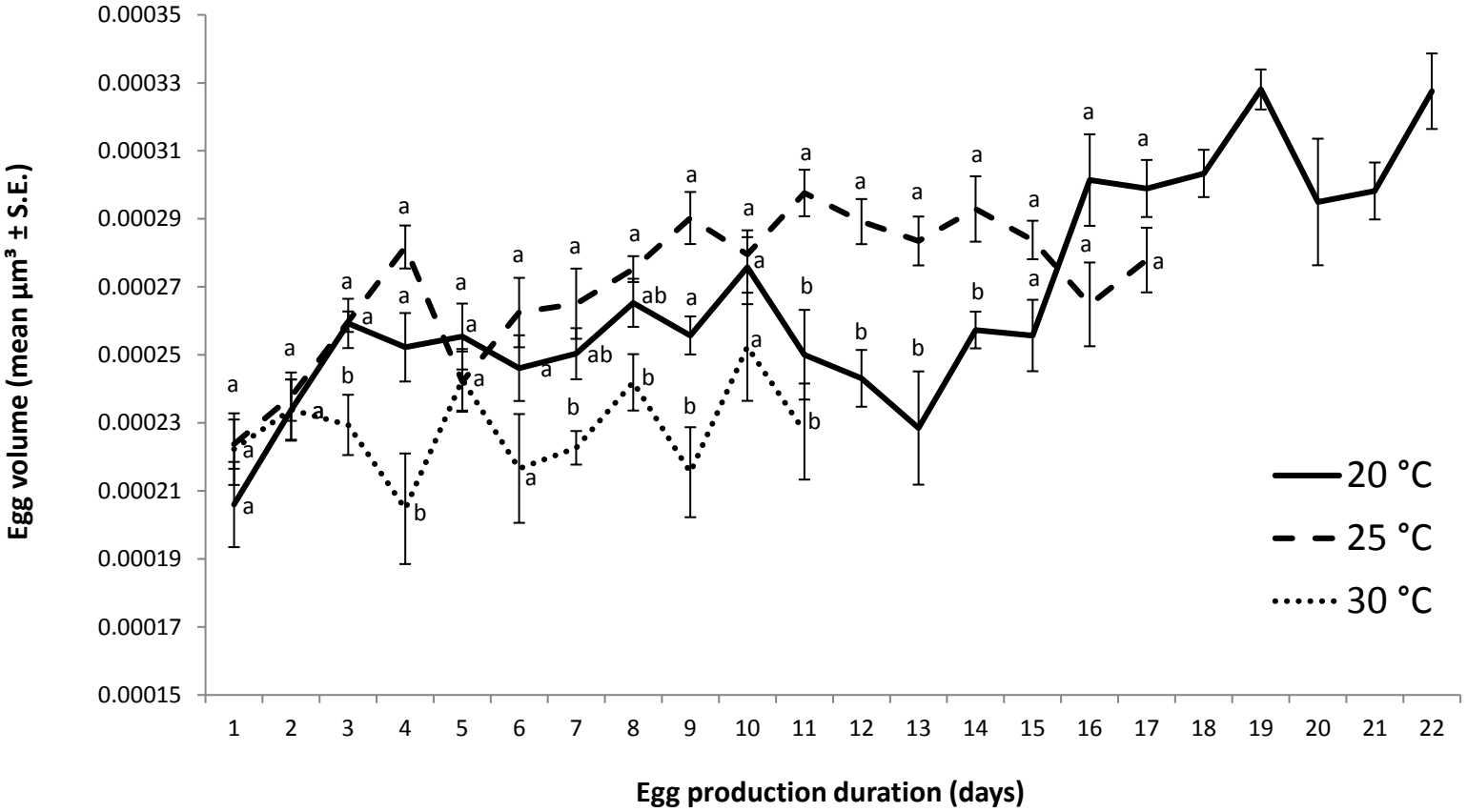

Figure 5: Volume of eggs laid by *Neobenedenia girellae* adults over their reproductive periods at a range of temperatures. 'a', 'b', and 'ab' = significant differences between mean egg volumes at each day of egg production determined using Tukey's HSD test, $p < 0.05$.

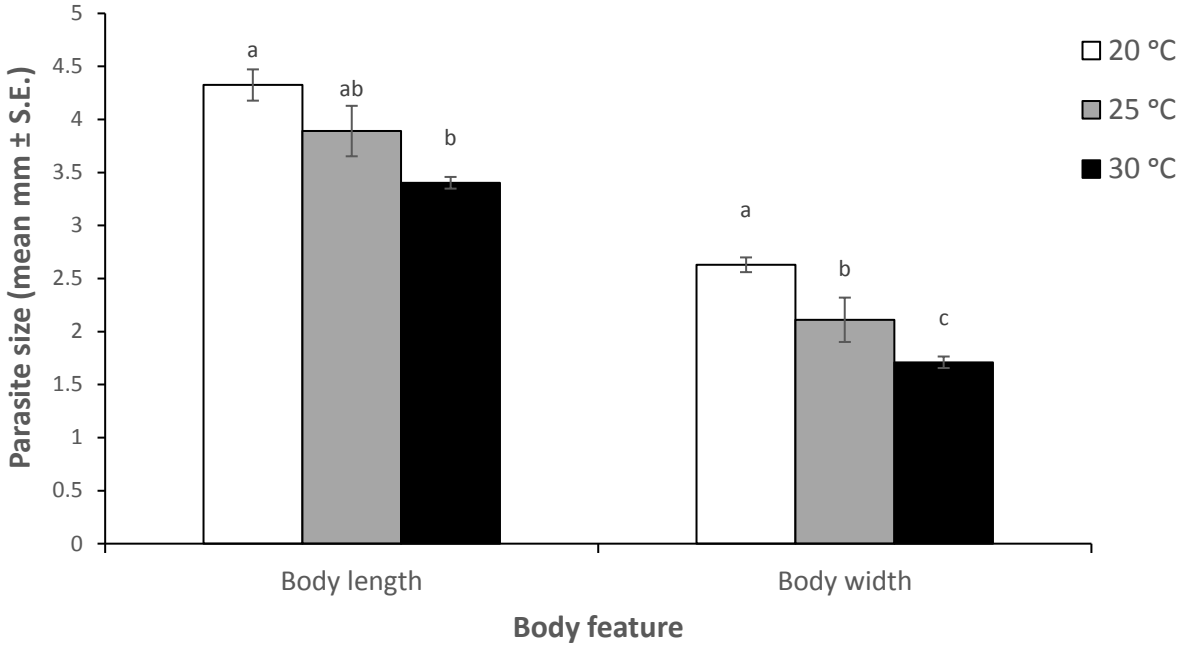

Figure 6: *Neobenedenia girellae* body length and width following collection at the end of the fecundity experiment once parasites lay fewer than 50 eggs per day for two consecutive days. ‘a’, ‘b’, and ‘c’ = significant differences between mean body length and width of parasites as determined using Tukey’s HSD test, $p < 0.05$.

Figure 7: A hatched *Neobenedenia girellae* egg 15 seconds following the emergence of the oncomiracidium. Remaining substance (indicated by white arrow) is suspected to be lipoprotein. Scale = 50 µm.

Table 1

First day of egg production, mean total eggs produced, total length of egg production period, and mean egg volume for *Neobenedenia girellae* at three temperatures; 20, 25, and 30 °C. Mean egg volume was calculated by averaging the egg volume at each of the three temperatures irrespective of age. Values have been adjusted for the size of parasite populations on fish.

	20 °C	**25 °C**	**30 °C**
First day of oviposition following infection	12	9	7
Total egg production	2,837 ± 10.28	1,698 ± 10.78	2,432 ± 35.70
Total length of egg production (days)	25	19	13
Mean egg volume	$2.667 \times 10^{-4} \pm 6.65 \times 10^{-6}$	$2.705 \times 10^{-4} \pm 4.42 \times 10^{-6}$	$2.278 \times 10^{-4} \pm 4.42 \times 10^{-6}$

Table 2

Proximate composition of freshly laid *Neobenedenia girellae* eggs expressed in mg g^{-1} egg and mg g^{-1} lipid ± standard error, respectively; (n = 2 egg masses). NFE = Nitrogen-free extract. I = freshly laid eggs; stage II = embryonated eggs; stage III = developing eggs; stage IV = hatching of eggs imminent; and stage V = hatched eggs.

	20 ºC					25 ºC					30 ºC				
Proximate components	I	II	III	IV	**Hatched**	I	II	III	IV	**Hatched**	I	II	III	IV	**Hatched**
Wet weight															
Moisture	79.97±1.75	80.48±0.019	79.51±1.15	78.56±1.15	91.06±0.58	75.45±1.51	80.45±1.07	80.98±2.77	81.87±0.86	90.8±0.77	82.55±3.07	80.87±2.42	81.39±2.32	81.53±0.29	91.64±1.96
Protein	11.22±1.09	11.85±0.09	12.08±0.62	12.76±0.51	6.52±0.26	15.53±0.43	12.73±1.46	12.17±2.18	11.84±1.27	5.84±0.1	9.75±1.86	8.46±0.41	10.63±1.61	12.11±0.83	5.25±1.81
Lipid	2.36±0.23	2.34±0.23	2.7±0.64	2.64±0.003	0.2±0.01	3.03±0.4	2.28±0.15	2.31±0.67	1.99±0.04	0.12±0.04	1.93±0.66	2.22±0.69	2.52±0.002	2.23±0.27	0.25±0.007
Ash	1.88±1.04	2.09±1.03	1.78±0.09	1.7±0.39	0.43±0.05	1.76±0.34	1.24±0.27	1.78±0.49	1.45±0.48	0.72±0.19	1.12±0.11	3.66±1.1	1.66±0.14	1.34±0.17	0.4±0.1
NFE	5.78±5.78	8.4±8.46	5.45±5.45	4.13±4.14	2.87±2.87	6.45±6.45	5.33±5.33	5.72±5.72	6.03±6.03	2.22±2.22	5.99±5.99	4.54±4.54	4.54±4.54	4.3±4.3	3.25±3.25
Energy density (J μg^{-1})	3.58±0.26	3.72±0.11	3.92±0.59	4.06±0.23	1.62±0.09	4.86±0.34	3.91±.52	3.79±1.03	3.58±0.47	1.42±0.05	3.06±1.12	2.87±0.20	3.51±0.51	3.74±0.55	1.34±0.71
Dry weight															
Protein	51.15±1.67	54.94±1.54	54.4±2.9	55.2±0.74	69.62±0.6	59.04±0.5	61.13±1.53	58.4±0.67	60.35±1.18	59.09±2.33	52.4±0.53	37.7±3.8	52.33±1.15	61.13±2.27	59.12±3.38
Lipid	10.8±0.71	10.8±0.28	12.21±1.77	11.46±0.39	2.11±0.001	11.51±0.5	10.97±0.003	10.98±0.74	10.18±0.24	1.18±0.25	10.22±0.91	9.61±0.77	12.52±0.68	11.25±0.71	2.93±0.38
Ash	8.66±2.52	9.55±2.15	7.98±0.03	7.29±0.59	4.62±0.4	6.73±0.79	5.94±0.46	8.5±0.51	7.36±0.98	7.22±0.61	6.05±0.22	15.86±1.9	8.28±0.79	6.76±0.45	4.52±0.02
NFE	29.39±1.56	24.71±0.89	25.42±4.7	26.05±0.53	23.65±1	22.72±0.79	21.96±1.98	22.13±1.92	22.1±1.92	32.51±1.97	31.33±1.22	36.83±1.84	26.87±0.32	20.86±3.43	33.43±3.01

Table 3

Fatty acid composition of *Neobenedenia girellae* eggs (%). Fatty acids accounting for < 1 mg g^{-1} were omitted from report. 'SFA' = saturated fatty acids, 'MUFA' = monounsaturated fatty acids, 'PUFA' = polyunsaturated fatty acids, 'LC' = long chain, 'NFE' = nitrogen-free extract. (n = 2 eggs masses of approximately 100µg weight each).

	20ºC					25ºC					30ºC				
Fatty acid	I	II	III	IV	**Hatched**	I	II	III	IV	**Hatched**	I	II	III	IV	**Hatched**
14:0	2.49±0.17	2.52±0.08	2.36±0.03	2.74±0.34	16.8±3.1	2.46±0.12	2.02±0.01	2.25±0.17	2.62±0.37	11±3.67	2.76±0	3.05±0.12	2.46±0.13	2.38±0.21	9.11±0.75
15:0	0.94±0.04	0.92±0.02	0.8±0.01	0.82±0.01	0.97±0.11	1.09±0	0.98±0.01	1.01±0.02	1.02±0.1	0.86±0.14	1.18±0.05	1.21±0	1.24±0.03	1.27±0.04	1.14±0.12
16:0	21.87±0.11	21.79±0.47	21.43±0.24	21.73±0.09	36.02±2.02	22.82±0.13	23.16±0.36	23.38±0.47	22.68±0.48	31.38±2.57	26.39±1.05	26.66±0.67	24.83±0.27	25.57±0.87	29.09±1.57
17:0	1.93±0.06	1.91±0.04	2.36±0.18	2.58±0.16	0	1.76±0.01	2.37±0.01	2.27±0.02	2.54±0.03	0.54±0.54	1.88±0.01	1.75±0.07	1.87±0.16	1.74±0.02	0
18:0	9.02±0.02	8.9±0	9.74±0.19	10.31±0.09	21.14±1.47	8.86±0.05	9.03±0.05	9.75±0.05	10.06±0.27	16.73±2.86	10.34±0.13	11.7±0.37	10.52±0.38	10.47±0.51	15.81±0.71
22:0	1.23±0.03	1.05±0.04	1.53±0.03	1.61±0.07	4.42±0.05	1.42±0.05	1.4±0.02	1.59±0.03	1.7±0.05	3.8±1.24	1.62±0.04	1.85±0.27	1.66±0.12	1.59±0.07	3.3±0.17
24:0	0.44±0	0.36±0	0.51±0.01	0.61±0	2.69±0.04	0.5±0	0.5±0.04	0.56±0.06	0.62±0.08	2.04±0.74	0.64±0.03	0.89±0.19	0.66±0.1	0.57±0	1.88±0.14
16:1n-7	5.17±0.27	5.57±0.34	4.47±0.27	4.06±0.7	0.38±0.38	5.29±0.33	3.88±0.3	3.5±1.28	3.5±1.03	1.48±0.44	4.14±0.26	3.5±0.13	2.44±0.03	2.5±0.15	1.92±0
17:1n-7	0.67±0.01	0.64±0.03	0.67±0	0.64±0	0	0.61±0.03	0.56±0	0.54±0.02	0.54±0.02	0	0.41±0.02	0.39±0	0.44±0.01	0.52±0.01	0
18:1n-7	2.24±0	2.25±0.06	1.75±0.06	1.81±0.11	0	2.48±0.05	2.13±0.1	1.9±0.18	1.89±0.17	1.14±0.24	2.18±0.1	1.75±0.06	1.88±0.08	1.94±0.01	1.16±0.03
18:1n-9	17.75±0.45	16.88±0.46	16.42±0.26	15.7±0.81	6.63±0.4	17.03±0.14	18.2±0.05	17.24±0.27	16.35±0.75	11.71±4.35	18.89±0.39	17.59±0.29	19±0.42	17.25±0.05	23.37±0.04
18:1n-9 trans	0.14±0.01	0.14±0.02	0.15±0	0.15±0.01	0	0.14±0.01	0.06±0.06	0.11±0	0.09±0	0	0.26±0.05	0.23±0	0.2±0	0.16±0.02	0.62±0.01
20:1 isomers	0.99±0.02	0.91±0	0.72±0.06	0.68±0	0	1.15±0.03	0.9±0.05	0.83±0	0.78±0.04	0.41±0.41	1.04±0.02	0.82±0.03	0.97±0.02	1.02±0.08	0.52±0.04
22:1 isomers	1.15±0.04	1.21±0.08	1.19±0.01	1.27±0	2.46±0.8	1.32±0	1.12±0.06	1.14±0.08	1.22±0.05	1.86±0.39	1.13±0.03	1.26±0.17	1.15±0.02	1.26±0.06	1.56±0.2
24:1n-9	0.86±0.03	0.87±0	1.05±0.05	1.08±0.04	0	0.92±0	0.91±0	0.89±0.06	0.99±0.09	0.29±0.29	0.82±0.01	0.78±0.04	0.83±0.08	0.9±0.07	0
20:5n-3	0.8±0.02	0.9±0	1.64±0.03	1.02±0.14	0	1.62±0.05	1.24±0.02	1.24±0.08	2.02±0.83	0.47±0.47	0.72±0.12	1.29±0.06	1.5±0.04	1.5±0.14	0.23±0.23
22:3n-3	0.31±0	0.31±0	0.34±0.02	0.37±0.04	0	0.2±0.02	0.26±0.02	0.25±0.04	0.25±0.06	0	0.21±0	0.22±0.01	0.22±0.03	0.18±0.01	0±0
22:5n-3	1.62±0.06	1.75±0.13	1.25±0.08	1.3±0.06	1.56±0.11	1.79±0.05	1.51±0.04	1.43±0.03	1.64±0	1.53±0.19	1.38±0.07	1.74±0.1	1.42±0	1.41±0.22	1.07±0.02
22:6n-3	16.21±0.04	16.75±0.19	15.73±0.38	15.48±0.49	1.64±0.51	16.08±0.35	16.04±0.01	15.37±0.81	15.85±0.74	5.72±3.31	12.79±1.52	13.02±0.98	13.36±0.99	14.67±0.35	2.3±0.48
18:2n-6	6.69±0.02	7.04±0.19	7±0.14	6.69±0.22	1.94±0.27	5.93±0.15	6.3±0	6.63±0.44	5.97±0.7	3.63±1.41	5.19±0.05	4.16±0.14	6.01±0.7	6.55±0.6	2.02±0.05
18:3n-6	0.39±0	0.4±0.01	0.27±0.02	0.29±0.01	0	0.32±0	0.28±0	0.34±0.11	0.18±0.01	0	0.29±0.01	0.17±0.06	0.47±0.01	0.25±0.05	0
20:2n-6	0.31±0.01	0.32±0.02	0.27±0.01	0.27±0	0	0.35±0	0.28±0	0.28±0.02	0.25±0.04	0	0.28±0.01	0.21±0.01	0.32±0.04	0.35±0.04	0
20:3n-6	0.52±0.01	0.59±0.03	0.38±0.02	0.43±0	0	0.41±0.01	0.35±0	0.35±0	0.35±0.06	0	0.39±0.01	0.24±0.04	0.32±0	0.31±0.05	0
20:4n-6	2.68±0.14	2.63±0.2	4.01±0.4	4.4±0.62	0.35±0.35	2.14±0.13	2.89±0.14	3.38±0.66	3.25±0.53	1.5±0.85	1.99±0.26	2.43±0.08	2.75±0.34	2.3±0.12	0.62±0.18
22:5n-6	1.05±0.03	1.04±0	1.17±0.07	1.2±0.07	0	0.6±0.01	0.9±0.04	0.94±0.15	0.87±0.15	0.33±0.33	0.68±0.05	0.65±0.05	0.76±0.05	0.69±0.01	0
Unknown	1.71±0.01	1.5±0	1.83±0.03	0.93±0.84	1.01±0.41	1.51±0.01	1.83±0.01	1.91±0.1	1.78±0.09	1.53±0.43	1.59±0.09	1.64±0.26	1.65±0.11	0.83±0.72	1.82±0.85
SFA	38.14±0.04	37.7±0.47	39±0.74	41.5±0.7	83.52±1.73	39.17±0.41	39.72±0.44	41.05±0.67	41.46±1.2	68.02±11.69	45±1.31	47.36±1.34	43.5±1.22	44.56±0.72	61.7±0.33
MUFA	29.01±0.03	28.5±0.09	26.47±0.62	25.42±0.09	9.48±1.58	28.96±0.26	27.79±0.63	26.18±1.32	25.41±0.53	16.93±5.36	29.1±0.86	26.61±0.11	27.16±0.18	25.66±0.1	29.98±0.24
PUFA	31.13±0.02	32.27±0.37	32.68±0.08	32.12±0.23	5.97±0.56	30.34±0.7	30.63±0.17	30.84±1.88	31.33±1.63	13.51±5.89	24.28±2.08	24.37±1.49	27.67±1.53	28.93±1.55	6.49±0.76
n-3 PUFA	19.3±0.13	20.04±0.34	19.35±0.36	18.59±0.69	3.67±0.06	20.39±0.39	19.45±0.02	18.77±0.65	20.31±0.04	8.04±3.28	15.44±1.69	16.49±1.21	16.88±1.15	18.31±0.85	3.83±0.51
n-3 LC PUFA	19.15±0.13	19.9±0.34	19.19±0.36	18.47±0.69	3.67±0.06	20.04±0.38	19.25±0.02	18.61±0.65	20.11±0.05	8.04±3.28	15.19±1.8	16.41±1.13	16.69±1.09	18.01±0.78	3.83±0.51
n-6 PUFA	11.73±0.16	12.11±0.02	13.21±0.27	13.4±0.93	2.3±0.62	9.86±0.31	11.08±0.19	12.01±1.18	10.96±1.53	5.47±2.6	8.84±0.39	7.87±0.27	10.73±0.33	10.56±0.64	2.65±0.24
n-6 LC PUFA	4.32±0.15	4.34±0.17	5.66±0.45	6.13±0.71	0.35±0.35	3.23±0.15	4.21±0.2	4.75±0.82	4.55±0.76	1.83±1.19	3.07±0.33	3.32±0.18	3.91±0.4	3.4±0.06	0.62±0.18

Table 4

Lipid class composition (%) of total lipids present in freshly laid *Neobenedenia girellae* eggs. (n = 2 eggs masses of approximately 100µg weight each).

	20ºC					25ºC					30ºC				
Fatty acid	I	II	III	IV	Hatched	I	II	III	IV	Hatched	I	II	III	IV	Hatched
Sterol ester	10.34±0.34	10.59±0.22	10.71±1.89	10.98±1.13	10.67±0.5	13.85±0.75	12.83±2.29	11.1±0.61	11.71±0.3	9.55±1.07	11.62±1.1	11.22±1.71	9.96±0.34	12.48±0.41	10.41±0.42
Triacylglycerol	11.69±0.97	16.84±3.62	10.81±0.5	8.45±3.11	6.19±1.16	9.16±4.33	12.84±2.7	10.45±1.35	10.14±0.88	6.53±1.33	8.94±0.33	8.81±0.67	8.38±1.44	13.79±0.61	5.71±0.19
Free fatty acid	2.14±0.03	1.98±0.2	2.14±0.07	2.24±0.24	2.34±0.08	2.69±0.17	2.59±0.4	2.33±0.18	4.22±0.72	2.89±0.2	2.02±0	3.5±0.57	2.66±0.35	2.99±0.56	3.05±0.78
1,3 distearoylglycerol	8.92±0.26	8.06±0.73	8.85±0.51	8.24±0.73	9.8±1.22	9.14±1.44	7.51±0.89	8.94±0.32	8.23±0.29	10.05±1.01	8.62±0.14	9.04±0.75	9.13±0.25	7.57±0	10.19±0.69
Sterol	6.22±0	5.84±0.08	6.34±0.35	8.82±0.49	6.32±0.93	5.63±0.73	6.92±0.59	6.51±0.17	7.09±0.69	6.52±1.18	6.13±0.13	5.85±0.07	6.45±0.1	5.72±0.41	5.25±0.22
Acetone mobile polar lipids	21.76±0.23	20.68±0.42	21.62±0.84	22.74±1.98	25.25±1.54	21.21±0.97	19.8±1.81	21.82±1.02	22.65±0.32	24.42±0.16	25.62±0.07	24.84±0.63	24.7±0.44	21.62±1.17	27.29±1.8
Phosphatidylethanolamine	9.91±0.31	9.3±0.44	10.04±0.09	10.1±0.33	10.53±0.52	9.92±1.05	9.17±1.09	10.04±0.34	9.68±0.24	10.9±0.57	9.58±0.04	9.73±0.58	10.1±0.17	8.99±0.13	10.23±0.58
Phosphatidylserine/inositol	13.65±0.54	12.16±1.15	13.76±0.86	13.13±0.79	14.77±1.6	13.84±1.89	11.83±1.71	13.65±0.42	12.38±0.18	15.06±1.38	12.95±0.26	13.58±1.24	14.15±0.77	11.7±0.17	15.02±0.95
Phosphatidylcholine	15.32±0.08	14.49±0.34	15.69±0.2	15.26±0.64	14.08±0.13	14.53±1.22	16.48±0.5	15.12±0.52	13.85±0.69	14.02±0.65	14.48±1.18	13.37±1.08	14.43±0.02	15.09±0.07	12.8±0.81
Neutral	33.11±1.01	37.48±2.45	32.52±1.96	29.92±3.26	29.01±0.48	34.86±1.96	35.78±4.51	32.83±1.45	34.32±0.75	29.04±1.59	33.11±1.01	37.48±2.45	32.52±1.96	29.92±3.26	29.04±1.59
Polar	66.88±1.01	62.51±2.45	67.47±1.96	70.07±3.26	70.98±0.48	65.13±1.96	64.21±4.51	67.16±1.45	65.67±0.75	70.95±1.59	66.88±1.01	62.51±2.45	67.47±1.96	70.07±3.26	70.95±1.59
Neutral:Polar (proportion)	0.49	0.6	0.48	0.43	0.4	0.53	0.56	0.48	0.52	0.41	0.45	0.48	0.43	0.58	0.41

6.5 Discussion

Temperature significantly affected the early life biology of *N. girellae*, altering the fecundity of adults, and the volume and composition of eggs produced. Parasites maintained in cooler temperatures (20 °C) grew larger, laid larger eggs, and laid more eggs over a longer period compared to those in warmer temperature (25 and 30 °C). The majority of proximate components, lipid classes, and fatty acids did not change over the temperatures or development stages studied with the exception of protein content of eggs which decreased over progressive stages of development at 25 °C. Eggs were predominantly composed of polar lipids, the most common component of which was AMPL which was significantly higher in eggs that developed at 30 °C. Warmer temperatures (30 °C) significantly decreased the amount of a number of MUFAs and PUFAs in *N. girellae* eggs.

Although quantifying the biochemical composition of eggs is important in measuring the energetic requirements and of an organism, assessments of other early-life biology traits (such as fecundity and egg volume) provides a more complete picture of the life history a species has adopted and the investment that is made in their progeny (McEdward & Morgan 2001; Moran & McAlister 2009). Egg production or 'fecundity' of an organism is a measure of how many progeny a parent organism produces. High fecundity increases the likelihood that offspring locate and infect a new host, an important consideration for parasites with disparate and mobile host species (see Whittington et al., 2000a). In helminths, fecundity is often very high, with thousands or hundreds of thousands of eggs produced per day, and egg production is known to be extremely sensitive to environmental conditions (Jackson & Tinsely 1988; Mooney et al., 2008).

Neobenedenia girellae were found to be fecund, producing upwards of 1,698 - 2837 eggs in the lifetime of a single parasite (Fig. 4). Dinh Hoai & Hutson (2014) measured *N. girellae* fecundity at 25 °C and observed a considerably higher egg production rate (3,229 ± 37) per parasite. The reasons for this are not immediately clear, although it has been hypothesised that the immune response of the host may affect attached parasites in diverse ways. Parasites attached to immune-privileged microhabitats, such as the eye (Trujillo-Gonzalez et al., 2015a), could potentially exhibit differences in fecundity (Pritchard et al., 1995). The regularity at which eggs were collected from individual aquaria may have also been a factor. Fish have been observed swallowing parasite egg masses (pers. obs.) and collecting eggs every 24 h in the present study (compared to 3 h by Dinh Hoai & Hutson 2014) may have led to inadvertent egg loss through ingestion by the host.

Temperature is a well-known mediator of fecundity in parasites with strong positive relationships regularly reported from disparate platyhelminth groups (Gannicott & Tinsely 1998). This relationship begins to diminish, however, once the maximum thermal tolerance of the parasites is reached at which point fecundity (and other aspects of biology) begins to deteriorate. For example, egg production decreases in warmer conditions in the monogeneans *Benedenia seriolae* (Yamaguti, 1934) Meserve, 1938, and *Zeuxapta seriolae* Meserve, 1938 (see Tubbs et al., 2005). Maximum thermal tolerance decreased the rate at which sexual maturity is developed and the production of abnormal eggs documented for *Neoheterobothrium hirame* Ogawa, 1999, at 25 °C (Tsutsumi et al., 2002) and egg hatching and infection success of *N. girellae* decreased at temperatures above 32 °C (Brazenor & Hutson 2015; Chapter 4). Cooler temperatures considerably lengthened the reproductive longevity of adult *N. girellae* parasites. Adults laid eggs for almost twice as long in cooler

conditions (20 °C) (Table 1). Similar extensions in longevity has been observed previously for capsalids (Bondad-Reantaso et al., 1995) due to the decreased metabolic rate of the parasites.

Egg size has been demonstrated to be affected by maternal age, size, and nutrition (Moran & McAlister 2009). Maternal age has been reported to influence egg size in a wide range of marine taxa with typically smaller eggs being produced as an organism ages (MacGinitie 1934; Wiklund & Persson 1983; Cavers & Steel 1984; Kane & Cavers 1992; Qian & Chia 1992; Ruohomaki et al., 1993; Braby 1994; Gibson & Chia 1995; Jones et al., 1996; Ito, 1997; Steer et al., 2004). This phenomenon is thought to be due to a progressive decrease in maternal condition and therefore a loss in the resources able to be dedicated to eggs produced (Moran & McAlister 2009). No significant difference in egg volume was observed at 30 °C between the start and the end of egg production; however, at both 20 and 25 °C, progressively larger eggs were produced as parasites aged (Fig. 6). Given the contradictory results in the present study to those reported above it is likely that other maternal effects may influence the results observed.

A more significant factor to consider in relation to the production of larger eggs with increasing reproductive age may be the size of the parasite laying the eggs. Positive relationships have been recorded between maternal size and the size of eggs produced for a range of marine invertebrate taxa (George 1994; Chester 1996; Ito 1997; Marshall et al., 2000; Bingham et al., 2004). Capsalid monogeneans continuously grow as they age (Tubbs et al., 2005; Lackenby et al., 2007) and it would appear that the size of the parasite, rather than age, is a better correlation with egg size. In this study, parasites incubated at 20 and 25 °C had considerably longer to grow than those at 30 °C before the cessation of egg production simply due to the longer reproductive life span

of parasites at those temperatures (Fig. 4-5). This was reflected in the size of parasites once they had been collected from fish at the conclusion of the experiment where parasites in 20 and 25 °C treatments were significantly larger than those from 30 °C (Fig. 6).

Another frequently studied factor affecting the size of marine invertebrate eggs is the temperature in which their parent developed. Temperature has a well-documented negative relationship with egg size in many marine taxa including echinoderms, polychaetes, and molluscs (McLaren et al., 1969; Steele & Steele 1973; Clarke 1983; Levitan 2000; Peck et al., 2007; Moran & McAlister 2009). Similarly, the largest volume *N. girellae* eggs were observed at the two coolest temperatures studied; 20 and 25 °C (Fig. 6). Our results support this general relationship and represent the first time the effect of temperature has been quantified on the egg volume of a capsalid parasite. Although, this pattern is by no means ubiquitous. Studies on the cephalopod *Euprymna tasmanica* Pfeffer, 1884, (Steer et al., 2004) and the sand crab *Emerita analoga* Stimpson, 1857, (Dugan et al., 1991) reported no significant differences between the sizes of eggs produced at a range of temperatures. Despite this, *N. girellae* egg size was influenced by parasite age (and, by proxy, the size of the parasite) and also by the temperature in which the parasites developed with cooler conditions resulting in larger eggs being produced (Fig. 6).

Although egg size has for many years been used as a good indication of egg quality and investment by the parent into offspring, egg content has come into focus (McEdward & Morgan 2001; Moran & McAlister 2009). This has largely been due to the advent of successful biochemical analysis of small quantities of material, allowing for the study of species for which egg collection is difficult. Because eggs used for biochemical analysis in this study were pooled from multiple mothers at a variety of

developmental stages, inferring any differences in maternal investment between small and large eggs and linking this with fecundity is impossible. However, it is known that egg size is not always an accurate predictor of organic content (Gnaiger 1983; McEdward & Carson 1987; McEdward & Coulter 1987; McEdward & Morgan 2001; Moran & McAlister 2009). It is currently unknown whether the larger eggs produced by older parasites represents an increase in maternal investment of biochemical components (see Jaeckle 1995; Moran & McAlister 2009) or whether these eggs are energetically less dense and larger simply because the size of adult monogeneans progressively increases as they age (see Tubbs et al., 2005; Lackenby et al., 2007; Brazenor & Hutson 2015, Chapter 4). Few studies have closely examined the relationship between the size of eggs and their contents most likely due to the lack of analytical sensitivity required to measure biochemical content in a single egg (McAlister & Moran 2012). Presently, pooling material is the only method by which analysis of biochemistry is possible for some species.

Proximate composition of *N. girellae* eggs was comparable to what was observed in a previous study on *N. girellae* egg biochemistry (Brazenor et al., in review, Chapter 5). Moisture and protein composed the majority of total wet weight content of eggs (75.45-82.55 % and 9.75-15.53 %) regardless of development period of the embryo or temperature (Table 2). A small but consistent amount of lipid and protein was detected in hatched eggs and is likely unmetabolised lipoprotein left by the hatched larva (Fig. 7). Photographs taken of eggs immediately following hatching shows remaining material inside egg cases which is possibly lipoprotein (Fig. 7). Eggs were not found to alter in composition based on temperature or development period with the exception of protein content of eggs. Significantly higher protein content was observed in eggs at 25 °C compared to 30 °C. As temperature increases the rate of embryonic

energy utilisation (Hoegh-Guldberg & Pearse 1995; Rayssac et al., 2010), it is understandable why protein content was lower at warmer temperatures. Although not significant, protein content of eggs that developed at 20 °C was also lower than at 25 °C. A possible explanation could be that preferentially higher rates of protein catabolism in cooler conditions occurs in *N. girellae* embryos as is observed in some marine gastropods (Aldridge et al., 1995). Alternatively, eggs may have been provisioned with less protein by the parent parasite at these lower temperatures.

Temperature affects almost every aspect of the physiology and biology of an organism and is particularly influential in ectothermic animals (Hochachka & Somero 2002, Fields et al., 2006). As temperature strongly influences a number of biological processes including embryonic energy reserve utilisation (Hoegh-Guldberg & Pearse 1995; Gannicott & Tinsely 1998; Rayssac et al., 2010) it is curious that egg energy density was highest at the intermediate temperature and not in cooler conditions (20 °C). Indeed, the most energy-rich *N. girellae* eggs were produced at 25 °C (Table 2). Although not significantly driven by the development period of the embryo, energy density generally increased with progressing development at 20 and 30 °C. As NFE and ash content decreased over this period of time, it is likely that *N. girellae* embryos accumulate energy-rich reserves throughout development as is observed for echinoderms and mussels prior to hatching and metamorphosis (Gallager et al., 1986; Delaunay et al., 1992; Pernet et al., 2006). In Chapter 5, it is noted that although protein contributed two thirds of the energy content of *N. girellae* eggs, a finding replicated in the present study, it was difficult to determine whether this meant that protein was catabolised as an energy source during embryogenesis. Unfortunately, the results obtained in this study do not aid in clarifying this issue as neither the amount of total protein or lipid changed significantly over the development of the embryo. This is not

necessarily a surprising result as proximate parameters may not change considerably over the short development time exhibited by *N. girellae* embryos. Protein and NFE composed the majority of egg content across development periods and temperatures although no significant differences in content were detected across temperatures or development periods.

Total lipid was a much smaller component of *N. girellae* eggs, comprising between approximately 10 % of the total dry weight egg content across temperatures and development periods. Although lipids represented a comparatively small proportion of the total contents of *N. girellae* eggs compared to protein, they are immensely important to the functioning of parasites both in the production of somatic tissue and fuelling the energetic requirements of the organism (Furlong 1991; Sherman 1998; Mitamura et al., 2000). Tropical invertebrate eggs are also often lipid-poor compared to related taxa at higher latitudes. This trend can be observed in *Calanus*, *Euchaeta*, and *Euphausia* copepods which all exhibit similar total lipid content in their eggs to those observed in the present study (see Lee & Hirota 1973; Morris & Hopkins 1983; Scott et al., 2000). It is suspected that more rapid biomass turnover coupled with higher metabolic rates in warmer, tropical conditions leads to a reduced ability to accumulate lipid in lower latitude species as the continuity of food supplies makes long-term energy supply storage unnecessary (see Lee & Hirota 1973).

Although accumulating lipids is a good indication that these will be utilised as an energy source, it does not necessarily imply that they will be metabolised during embryogenesis. Lecithotrophic invertebrates and fish have been observed preferentially catabolising specific lipids or protein during embryogenesis compared to the larval phase where the organism disperses and does not feed, relying on the energy it was provided by its parent (see Shilling & Manahan 1994). In lecithotrophic marine species,

neutral lipids such as triacylglycerols (TAG) are recognised as common fuels not just during embryogenesis but also in larval hatching, locomotion, and metamorphosis (Kattner et al., 2003; Byrne et al., 2008; Prowse et al., 2008). Although TAGs only comprise a small but relatively consistent proportion of total lipid in *N. girellae* eggs (8.38-13.79 %), there appears to be no clear trend of decreasing amount over the development period of the embryo and it is likely that they are utilised by *N. girellae* oncomiracidia during the larval phase (Table 3). Monogenean oncomiracidia have a limited free-swimming period where they attempt to locate, attach and metamorphose on a suitable host and begin feeding. If oncomiracidia are unable to locate a host within this short window of opportunity (typically <48 h depending on the ambient temperature) they expend their limited energy reserves and die (Gannicott & Tinsely 1998; Whittington et al., 2000a). The longevity of *N. girellae* oncomiracidia has been shown to decrease with increasing temperature (Brazenor & Hutson 2015 Chapter 4). If TAG are exploited as an energy substrate for this brief larval period, particularly in warm conditions (~60 h at 20 °C, ~18 h at 25 °C, and ~ 4 h at 30°C; Chapter 4) it would explain why a comparatively small amount of TAG is present in eggs compared to the eggs of other lecithotrophic mollusc and echinoderm species where TAG is consumed throughout the larval phase (Holland & Walker 1975). The presence of TAGs may also have a role to play in the buoyancy of *N. girellae* eggs. Very small amounts of lipid can have large effects on the buoyancy of eggs and the presence of TAGs (and potentially other lipids) that do not decrease over the development of eggs may indicate that they play a role in this buoyancy regulation (Lee et al., 2006).

Triacylglycerides are also utilised by the parasitic copepod *Lepeophtheirus salmonis* Krøyer, 1837, as the primary energy store despite related free-living taxa showing a preference for wax ester utilisation (Lee 1975; Tucker & Wooten 2000).

Intriguingly, the relatively consistent TAG content in *N. girellae* eggs across temperatures suggests that increased metabolism of larvae after hatching is the cause of the drastic reduction in longevity observed by Brazenor & Hutson (2015; Chapter 4) and not a reduction in TAG apportioning by the parent individual. Collection of sufficient quantities of *N. girellae* larvae would be essential to being able to determine which endogenous energy sources are depleted preferentially during the dispersal and metamorphosis stage of the life cycle. However, neutral lipids are not the only documented source of biochemical energy utilised during embryogenesis, dispersal, and metamorphosis.

Polar lipids comprised the majority of lipids present in *N. girellae* eggs across temperatures and development periods (~65 % of total lipids) (Table 4). Historically, polar lipids have been viewed as playing a primarily structural role in bio-membranes and in the tissues of organisms (Lee 1975; Kattner et al., 2003). Specific polar lipids, particularly phospholipids which comprise the majority of polar lipids in *N. girellae* eggs (Chapter 5; present study), are preferentially metabolised as energy substrates by some marine species (Kattner et al., 2003; Tocher et al., 2008). Recent studies have highlighted that specific phospholipids, especially phosphatidylcholine, are important sources of energy and essential fatty acids during early larval development (Gisbert et al., 2005; Salze et al., 2005) and have storage functions in the larvae of some marine fish (Tocher et al., 2008) and crustacean species, including krill (Hagen et al., 1996; Mayzaud 1997), larval stages of lobster species (Jeffs et al., 2001), and anomuran crabs with lecithotrophic larval development (Kattner et al., 2003). Preferential catabolism of phosphatidylcholine may indicate that the developing embryo is meeting metabolic needs which are not provided by TAG (Fraser et al., 1988). This could be the provision

of specific nutrients such as phosphate and choline which are known to be essential for healthy fish embryo growth and organ formation (Viola et al., 1986).

Phosphatidylcholine comprised between 13.37 and 16.48 % of total lipid in *N. girellae* in the present study but did not show a clear increase or decrease in content with progressive development period, nor did any of the phospholipids quantified in this study. The only four biochemical components which did show a significant trend of decreasing content in *N. girellae* eggs were the monounsaturated fatty acids 18:1n-7, 20:1 isomers, 16:1n-7, and the polyunsaturated fatty acid 22:5n-3. Higher levels of each fatty acid were present in freshly laid eggs than progressive development stages. Although the specific purpose of each fatty acid is difficult to determine, the decrease in content over development period of the embryo suggests that each fatty acid was catabolised to fund the synthesis of tissues and crucial hormonal and/or nervous system components during embryogenesis (Table 4). As such, it is difficult at this stage to determine what, if any, energetic requirements are being met with any other specific lipid classes and fatty acids in *N. girellae* embryos. Despite strong evidence that TAG and phospholipids play important storage and structural roles in a variety of organisms, the lipid class which consistently comprised the largest proportion of lipid in *N. girellae* eggs (Table 3) was acetone mobile polar lipids (AMPLs).

Acetone mobile polar lipids are mostly composed of glycolipids, polar pigments, and small amounts of monoglycerides and are commonly found in the lipid extracts of plants (Napolitano 1994) and natural waters (Parrish 1987). Initially identified by Parrish (1987), it is not commonly measured in lipid class analysis and is the least well-known lipid class in freshwater and marine invertebrates (Ackman 1999). Analysis of this class in plants is comparatively common as many of the glycolipids present in plant tissue are of interest for their biologically active traits (Parrish &

Wangersky 1987; Parrish et al., 1996; Chia et al., 2013). Glycolipids, the most common component of AMPLs, have also been found to possess immunomodulating and antitumor activity in marine sponges (Constantino et al., 1994, 1997, 2001; Natori et al., 1994; Li et al., 1995) and cerebrosides, important nervous system compounds, in helminths (Harrington 1965; Ginger & Fairbairn 1966; Meyer et al., 1966). Additionally, glycolipids are key to membrane structure and fluidity. Organisms which are undergoing thermal stress are known to be able to alter the lipid composition of their cell membranes in order to optimise membrane fluidity and maintain proper cell function in undesirable conditions (Stubbs & Smith 1984). The higher AMPL concentration in *N. girellae* eggs exhibited at 30 ºC is potentially an example of this adjustment in lipid content. Of note is that the quantity of AMPL present in *N. girellae* eggs in the present study is up to four times higher than previously documented (Chapter 5). The reason for why this is the case is unknown and, unfortunately, AMPLs were not explicitly measured by Sato et al. (2008), the only other study on *N. girellae* lipids known to the authors, making comparison with other research impossible.

Some specific differences in biochemistry can be observed between analysis of eggs in Chapter 5 and the present study. Host individuals were fed the same extruded fish pellet diet and held in the same tanks and at the same salinity, however, different host fish were utilised. Temperature and development period at which eggs were collected and specific host fish were the only changed variables. During feeding and development on the fish host, *N. girellae* feed on host derived products such as mucus and epithelial tissue and it is recognised that the fatty acid composition of organisms is most strongly influenced by diet (Kaneko et al., 1988). Similar results were observed in studies of *L. salmonis* with levels of TAG observed in adult parasites mimicking those found in the epithelium of their hosts (Lee 1975; Tocher et al., 2010). Unfortunately, no

comprehensive studies examining the biochemistry of epithelium and skin extracts exist for *L. calcarifer* to the authors' knowledge. It is therefore possible that the lipid profile observed in *N. girellae* eggs is strongly influenced by the lipids present in the mucus and skin of *L. calcarifer.* As no samples of the host were taken, this cannot be verified as a potential factor explaining the difference in AMPL between the two studies. Acetone mobile polar lipids and free fatty acids (FFAs) are breakdown products of phospholipids and TAGs (Parrish 1988; Salvo et al., 2015), potentially accounting for why considerably higher AMPL and lower phospholipid and TAG content was observed in the present study compared to the previous (see Chapter 5). Research investigating AMPL in monogeneans would be useful in shedding light on the discrepancy in AMPL content.

Lipids are, in addition to their roles in energy reserves and cellular structure, also key to a parasite's ability to regulate the immune response of the host. *L. salmonis* has been demonstrated to secrete immune-modulatory products including prostaglandin E_2 (Fast et al., 2004), a metabolite of arachidonic acid (ARA, 20:4n-6), present in *N. girellae* eggs (Table 3). Prostaglandin E_2 is the most commonly found prostanoid in secretions from parasites (Fast et al., 2004). It has a myriad of effects on host cells and tissues including vasodilation (Williams & Higgs 1988) and decreased lymphocyte and cytokine activity; components essential to an effective immune response (Papadogiannakis et al., 1984; Pinge-Filho et al., 1999). Although immune-modulators have never been identified in capsalid monogeneans, there are suggestions that they are employed. A similar lack of host immune response at the site of attachment and feeding can be observed in both *L. salmonis* and *Neobenedenia girellae* infections (Ross et al., 2000; Firth et al., 2000; Fast et al., 2002; 2003; Trujillo-Gonzalez et al., 2015b). Arachidonic acid gradually increases in concentration over the development of the *N.*

girellae embryo at all temperatures (Table 3), suggesting that this lipid could either be synthesised by the embryo prior to hatching and the infection of a host or selectively retained. Changes in the profile of fatty acids of this parasite, both adults and embryos, could potentially positively or negatively influence the interaction between the parasite and its host. This would almost certainly result in changes to infection intensity and/or pathology (Tocher et al., 2010). The results of this study coupled with the observation by Trujillo-Gonzalez et al. (2015b) that *Neobenedenia* sp. appear to preferentially infect immune-deficient sites such as the jaw of the fish justify further investigation towards whether immune-modulators are synthesised and secreted by *Neobenedenia* spp.

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Chapter 7: General Discussion

Above: Juvenile barramundi infested with *Neobenedenia girellae*.

Photograph by Alejandro Trujillo-González.

General Discussion

Parasites are highly specialised organisms and have evolved a range of unique biological traits to facilitate locating, attaching to, feeding, and reproducing on their host. The biology and behaviour of marine parasites reflects an attempt to maximise encounters of patchily distributed appropriate hosts in the environment. In human-mediated environments which have artificially inflated densities of hosts, such as aquaculture facilities, these traits lead to rapid growth of parasite populations. Capsalid monogeneans which only require a single host to complete their life cycle are particularly suited to these host-rich settings. When conditions are appropriate, massive losses of aquaculture stock, representing a huge investment in capital and considerable animal welfare issues, can occur. As a result of the threat that parasites pose to the global aquaculture industry, considerable research has been dedicated to the most common and/or harmful taxa.

Species in the genus *Neobenedenia* Yamaguti, 1963, are particularly notorious among capsalids, known for the extensive list of host species that have been recorded for two *Neobenedenia* taxa in particular. *Neobenedenia melleni* MacCallum, 1927, and *Neobenedenia girellae* Hargis, 1955, have each been implicated in mass mortality events of aquaculture stock but the similar morphology of these two taxa makes distinguishing these species challenging. Following the outbreak of a *Neobenedenia* species on a barramundi farm in Queensland in 2000, and continuous unpublished parasite management issues on aquaculture farms in northern Australia, the need to accurately identify and manage the species present became a priority pursuit (see Deveney et al., 2001 and Whittington et al., 2004).

Additional to the taxonomic confusion surrounding these two taxa, research into their biology was lacking. The ability to explore the fine-scale biology of an organism is only feasible with the development of stable and persistent laboratory cultures. The isolation of *N. girellae* eggs from a barramundi farm in north Queensland in 2011 provided this opportunity and a long-term laboratory culture of *N. girellae* was established in the Marine Parasitology Laboratory at James Cook University (JCU). To the best of the authors' knowledge, this is one of the longest continuously maintained capsalid monogenean cultures (i.e. six years), following Kearn's research spanning over 20 years on *Entobdella soleae* alone.

The aim of this thesis was to examine the diversity and life history of *N. girellae* with a view to assist in parasite diagnosis and management methods in aquaculture farms. This broad aim encompassed investigating phylogeny, morphology, responses to environmental conditions, and early life biology of *N. girellae* thereby establishing a baseline of knowledge for this species. Collectively, the Chapters in this thesis represent a considerable improvement in our understanding of this parasite. The main findings of the thesis, implications of results, knowledge gaps filled, and avenues for future research are discussed below.

Molecular work on *Neobenedenia* has been fleetingly addressed between the initial identification in 2000 by Deveney et al. (2001) and 2009 (e.g. Whittington 2004; Whittington et al., 2004; Perkins et al., 2009). Australian researchers came to the conclusion that the genus warranted more in-depth research (Whittington 2004; Whittington et al., 2004; Perkins et al., 2009). This was due to inconsistencies identified in multiple gene sequences (e.g. *H3*, *28S RNA*, and *Elongation factor 1 α*) that suggested Whittington & Horton (1996) may have been incorrect when they synonymised *N. melleni* and *N. girellae*. Since this publication, there has been debate

among international researchers as to whether *N. girellae* is a taxon separate to *N. melleni*. No definitive evidence had been presented prior to this study (Chapter 2) with analyses either using very few samples or only a single gene (see Zhang et al., 2014; Wang et al., 2004). As such, several clear and pressing topics remained to be addressed at the commencement of this thesis; What is the identity of the isolate found in Australia (and cultured in the Marine Parasitology Laboratory)? is the genus *Neobenedenia* in need of another revision based on the uncertainty present in morphological identification? What is the diversity of *Neobenedenia* species in Australian waters? Answering these questions is recognised as being crucial to appreciate the threat that *Neobenedenia* poses to Australian food and ornamental fish production (Deveney et al., 2001).

Of the 33 samples included in our analyses (collected from 22 host species from nine countries around the world (full list of hosts and localities in Chapter 2)), two were identified as *N. melleni,* two as *Neobenedenia pacifica* Bravo-Hollis, 1971, and one as *Neobenedenia longiprostata* Bravo-Hollis, 1971. The Bayesian and Maximum Likelihood analyses relegated the remaining samples to a distinct taxon, one that is morphologically identical to *N. melleni*. The only *Neobenedenia* species which fits this description is *N. girellae*. This result suggests that the revision and synonymy by Whittington & Horton (1996) was not correct when removing *N. girellae* as a separate taxon in the genus. This has been raised by other researchers (e.g. Ogawa et al., 2006), however, no studies have presented evidence for this case. Our research is the first study to conclusively prove that *N. girellae* is worthy of retaining its status as a separate taxon within *Neobenedenia*. Although this may not be particularly surprising given the discussion surrounding this issue over the past 20 years, what is intriguing is that the vast majority of samples were not identified as *N. melleni* as has been the case

historically. *Neobenedenia melleni* has been credited with over 100 host species records from a large number of localities around the world. The results of our study suggest that many of these records may be a case of mistaken identities given both *N. melleni* and *N. girellae* are morphologically very similar.

Another unexpected result of our analysis was the identification of an isolate collected from *Lutjanus argentiventris* Peters, 1869, which is potentially a taxon novel to science. Cross-referencing this sequence with those of other *Neobenedenia* spp. (e.g. *Neobenedenia adenea* (Meserve, 1938) Yamaguti, 1963, and *Neobenedenia isabellae* (Meserve, 1938) Yamaguti, 1963, is necessary to confirm whether this is the case. Importantly, these two *Neobenedenia* taxa (which could not be included in our analyses) have not been recorded from lutjanids. It is possible that this unidentified isolate has diverged from *N. girellae* (the *Neobenedenia* species it is most closely related to in our analysis) fairly recently as it does not differ from *N. girellae* by as considerable a margin as from *N. melleni*, *N. pacifica*, or *N. longiprostata*.

Historically, *Neobenedenia* spp. were differentiated using their morphology. This practice led to Whittington & Horton (1996) observing that *N. melleni* samples and those identified as *N. girellae* were morphologically identical and therefore led the decision to synonymise the two. This occurred before molecular techniques began to become common practice in supporting morphological parasite taxonomy. This is particularly important for parasite research due to the overwhelming predominance of cryptic species in parasite groups. With the results of Chapter 2 and the determination that *N. girellae* is a separate taxon to *N. melleni*, the morphological confusion that initially caused the taxonomic confusion of the genus can begin to be untangled and morphological variations between the two taxa may be able to be determined.

Preliminary observations on the morphology of Australian *N. girellae* isolates collected for genetic analyses in Chapter 2 indicated potentially distinct morphologies associated with host fish species. A non-metric multi-dimensional scaling (nMDS) analysis of morphological measurements made from parasites infecting six different species showed distinct morphologies according to host fish species, with the majority of the variation observed relating to features associated with parasite attachment. These included haptor, sclerite, hamulus, and anterior attachment organ lengths or diameters (Chapter 3). These features are involved in attachment to the surface of their host species and are important characters used to distinguish species morphologically, yet genetic work from Chapter 2 indicated the parasites were the same species. It is hypothesised that this variation observed was a response by the parasite to maximise their ability to attach to their host surface (e.g. scale morphology) but this requires investigation. Subsequently, the effect of host species, temperature and salinity on phenotypic plasticity was experimentally examined in controlled conditions the laboratory using isogenic *N. girellae*.

In the first controlled study, parasites were raised on one of two hosts; *Lates calcarifer* Bloch, 1790, and *Rachycentron canadum* Linnaeus, 1766. When parasites were collected, ages and environmental conditions were experimentally controlled and no significant difference in morphology was observed between the two parasite populations. This suggests that morphological variation observed between *N. girellae* infecting different host species in the field may be a consequence of natural selection. Specific morphology which confers an advantage to attaching to a specific host fish species will be selected for over long periods of time.

Temperature is one of the most influential environmental conditions to the development of ectoparasites (Atkinson 1994; 1995; Gillooly et al., 2002; Angilletta et

al., 2004). As such, the effect of tropical temperatures on the morphology of *N. girellae* and the potential to elicit a phenotypic response were investigated. The variation in morphology of parasites at different temperatures was driven more by ratios relating to body size (body length:width, haptor width:body width, and pharynx width:body width). This result is to be expected given how organisms respond to different temperatures. An inverse relationship between temperature and mean body size can be observed for a wide range of species (Jorgensen 1968; Latala et al., 1991; Bondad-Reantaso et al., 1995; Crill et al., 1996; Lee et al., 2003; Hirazawa et al., 2010; Forster et al., 2012; Chapter 4).

Studying environmental effects on morphology is important for a number of reasons. Intraspecific variability in morphology presents a considerable problem for morphology-based taxonomy when informative characters are affected (Bickford et al., 2006; Olstad et al., 2009; Barcak et al., 2014). Body size (length and width), hamuli, and sclerites are all considered to be taxonomically important features for *N. girellae* (Hargis 1955; Whittington & Horton 1996) and, as a result, changes in these characters as observed in our study hampers the use of morphology-based identification of individuals and the use of diagnostic keys (e.g. Malmberg 1970; Kulemina 1977; Mo 1991a; 1991b; Dmitrieva & Dimitriov 2002; Davidova et al., 2005). Most monogenean species, including species in the genus *Neobenedenia*, are delineated and diagnosed based on their morphology and understanding the range of morphological plasticity is key to accurate identification (Vossbrinck & Debrunner-Vossbrinck 2005; Barcak et al., 2014; Bazsalovicsova et al., 2014). Studying the effect of environmental variables and how they contribute to morphological variation in *Neobenedenia* spp. is an important part of ensuring that any further taxonomic confusion is avoided.

The identification of *Neobenedenia girellae* in Australian waters and the development of a list of susceptible host species for this taxon led to a series of questions regarding previously unexplored biological features of the species. Specifically, knowledge on how life cycle parameters vary in different environmental conditions can be used to manage the species in fish farms and ornamental aquariums. An established method for controlling ectoparasites in managed systems is the strategic treatment of infected stock at specific stages in the life cycle of the collective parasite population (Chapter 4). The necessity for the development of these sorts of management strategies for parasites lies in the fact that treatment has historically been reactive to infections and are often are performed ineffectively with harsh chemicals. Not taking account of the life cycle of the parasite and the environmental conditions that the stock and parasites are developing in results in a lack of coordination that leads to inefficient treatment, higher labour costs, stress to stock, and increases in cost of chemicals/treatment apparatus.

The first step in developing strategic, timed management strategies is the elucidation of life cycle stages at a range of environmental conditions. Hatching success, hatching period, infection success, and time to sexual maturity were determined at temperatures ranging from 22 to 34 ºC in 2 degree increments and salinities ranging from 0 to 40 ‰ in ~10 ‰ increments. The use of life cycle-specific strategic management of monogeneans has been developed for other taxa. Tubbs et al. (2005) developed rudimentary timetables for the treatment of *Benedenia seriolae* (Yamaguti, 1934) Meserve, 1938, and *Zeuxapta seriola* Meserve, 1938. The theory behind these strategic timetables is based on coordinating the development of all parasites attached to infected fish. An initial treatment of infected stock in a managed system on an assigned ‘Day 1’ removes all attached parasites from fish whether they

are juveniles or sexually mature and producing eggs. Once the treatment ceases, fish are reinfected by eggs hatching in the surrounding environment. A subsequent treatment or treatments (as is sometimes the case in warmer conditions which speeds up the life cycle of the pathogen) once all parasites have hatched from eggs in the environment but before any reach sexual maturity on host fish kills remaining parasites in the system. This is the step that needs careful coordination and appreciation of life cycle stages in a variety of environmental variables.

While timetables for *B. seriolae* and *Z. seriolae* have been developed (Tubbs et al., 2005) for use at three specific temperatures, these offered no flexibility for the environmental conditions in which they can be used (e.g. a range of temperatures). The development of general linear models and equations which describe the trends observed in hatching period and time to sexual maturity at all temperatures and salinities allowed prediction of treatment times at all temperatures and salinities between the minimum and maximum limits tested for each. It was discovered that *N. girellae* has a very rapid life cycle and can complete a single generation in as few as 10 days in warm temperatures compared to 18 days in cooler conditions. In these warm conditions, multiple subsequent treatments are required after the treatment on Day 1. This is due to the life cycle of *N. girellae* being so rapid that attached parasites reach sexual maturity before all eggs in the system are able to hatch. Parasites with such short life cycles make this type of strategic management more challenging as a greater number of treatments are required in order to effectively break the life cycle and minimise reinfection.

Further research on the reproductive biology was conducted in Chapters 5 and 6 including the fecundity of adults, egg volume, and biochemistry of eggs. These aspects of the early life biology have not been previously studied in *N. girellae* and are not

commonly studied in marine parasites. Biochemistry in particular is not common due to the large quantities of material required for accurate analysis of material involving the collection of material over large time periods. Without the development of the laboratory culture of *N. girellae* at James Cook University, this would not have been possible.

Assessments of reproductive biology (e.g. fecundity and egg volume) provides a more complete picture of the life history a species has adopted and the investment that is made in their progeny (McEdward & Morgan 2001; Moran & McAlister 2009; Chapter 5). *Neobenedenia girellae* was found to be fecund, producing 1,698 - 2837 eggs in the lifetime of a single parasite (Chapter 6). Similarly, high fecundity was recorded by Dinh Hoai & Hutson (2014) as was the trend of egg production. Eggs were produced in progressively greater quantities each day from the beginning of egg production in all temperatures with similar egg production totals reached by all three temperatures tested. A peak in egg production was reached before production began to fall occurring 9, 8, and 6 days post maturity at 20, 25, and 30 °C, respectively. The explanation for this lies in the metabolic rate of the parasite at different temperatures. Temperature is a well-known mediator of fecundity in parasites with strong positive relationships regularly reported from disparate platyhelminth groups (Gannicott & Tinsely 1998; Tsutsumi et al., 2002; Tubbs et al., 2005). Parasites developed faster at warmer temperatures but also senesced more quickly. Parasites at 25 and 30 °C produced eggs for a considerably shorter time than at 20 °C. The high fecundity typical of *N. girellae* increases the likelihood that offspring locate and infect a new host (see Whittington et al., 2000a). Faster egg production to a maximum rate at warmer temperatures implies that populations could potentially grow considerably faster in warmer months (see Chapters 4 and 6). The need to monitor stock throughout the year

is therefore recommend as, presently, cooler conditions appear to be when harmful infections occur (Deveney et al., 2001). If infections do become present in warmer conditions, however, the impact on hosts may be considerably higher.

Egg size followed a similar pattern to fecundity with the additional relationship being that the size of *N. girellae* eggs was closely related to the size of parasites (Chapter 6). The size of parasites was inversely correlated to temperature as was the volume of eggs. A positive relationship between adult size and age is not a new concept among capsalids (see Lackenby et al., 2007) and these larger individuals are known to be able to produce more eggs over a given period of time believed to be because larger parasites produce eggs at a faster rate (Bondad-Reantaso et al., 1995; Mooney et al., 2008).

The contents of these eggs were then analysed to provide information on the metabolic fuels available to the developing embryo at tropical temperatures. The biochemistry of *N. girellae* eggs was consistent for both studies. Eggs were predominantly composed of moisture and protein. Lipids only comprised ~10% of total egg contents at all temperatures and irrespective of the stage of development of the embryo or the temperatures in which development took place (Chapters 5 and 6). Lipids are among the most bio-active compounds present in organisms and the types of lipids that are produce and synthesised often relate to the material they have ingested and the metabolic actions they intend to undertake in the future (Furlong 1991; Sherman 1998; Mitamura et al., 2000). One of the most intriguing aspects of parasite biochemistry is the compounds they produce in order to improve their chances of locating and attaching to hosts.

A large proportion of marine invertebrate larvae exploit triacylglycerols (TAGs) as their primary metabolic fuel when dispersing after hatching from eggs (Kattner et al.,

2003; Byrne et al., 2008; Prowse et al., 2008). No consistent decrease in TAG concentration in *N. girellae* eggs over the development of the embryos suggests that it is not utilised during embryogenesis (Chapter 6). The only reliable way of verifying this is to collect large quantities of larvae at the beginning and end of their dispersal period and to compare the relative TAG content between these two stages. Other lipid groups which may serve as embryogenic fuel sources include phospholipids or sterols. A number of studies have demonstrated that phospholipids, particularly phosphatidylcholine, are important sources of energy and essential fatty acids during the development of some species. Although traditionally considered to serve a structural purpose in organisms (cell membranes etc.), they have been shown to have storage functions in a wide diversity of organisms including fish, crabs, lobster, and krill (Hagen et al., 1996; Mayzaud 1997; Jeffs et al., 2001; Kattner et al., 2003; Tocher et al., 2008).

The potential for *N. girellae* to modulate the immune system of their host was suggested by Trujillo-González et al. (2015b) as a result of observing that immunologically deficient areas of *L. calcarifer* were often infected at a higher than average rate. Arachidonic acid, an omega-6 fatty acid, was observed to increase in concentration in *N. girellae* eggs in the present study. This fatty acid has been detected in the harmful salmonid parasite *Lepeophtheirus salmonis* Krøyer, 1837. This parasite has been recorded secreting prostaglandin $E_{2,}$ an immunomodulatory compound and a metabolite of arachidonic acid (Fast et al., 2004). Effects on host cells include vasodilation (Williams & Higgs 1988) and decreased lymphocyte and cytokine activity; components essential to an effective immune response (Papadogiannakis et al., 1984; Pinge-Filho et al., 1999). Although the presence of immune-modulatory compounds has not been confirmed in capsalids, a similar lack of host immune

response at the site of attachment and feeding can be observed in both *L. salmonis* and *N. girellae* infections providing further suggestion that these compounds may be present (Ross et al., 2000; Firth et al., 2000; Fast et al., 2002; 2003; Trujillo-González et al., 2015b).

These studies on *N. girellae* egg biochemistry (Chapters 5 and 6) represent the first investigation of capsalid egg biochemistry to date. There is a strong suggestion that studies on immune-modulatory compounds would be valuable to understanding host-parasite interactions and further research should be pursued.

Future Research Directions

The research conducted in this thesis will meaningfully contribute to the published literature on the phylogeny, biology, and management of *N girellae* infections on a population of hosts. There remain a number of topics yet to be addressed, representing important knowledge that can be gained by further study of this species and other *Neobenedenia* taxa. In particular, future research should consider:

1) Further clarifying phylogenetic relationships between *Neobenedenia* spp. including building reliable host species and geographic records lists for all verified taxa in the genus. This may require the review and analysis of samples from previous studies that have not formally accessioned specimens to museums and collections. Although this may be difficult and, in some cases, impossible, any samples which can be retrospectively identified will considerably assist in clarifying the confusion currently surrounding the taxonomy and host records of this genus.

2) The geographic ranges that each *Neobenedenia* spp. inhabits is currently unknown. Part of this confusion is due to the pedigree of misidentification that is characteristic of this genus. New investigations of range and host species for each *Neobenedenia* species will ensure future identifications are correct provided that dedicated sample collection and genetic analysis and/or accessioning is performed by researchers. Knowing the geographic ranges and potential hosts for species in this genus is important for being able to understand the threat they pose to specific fisheries or aquaculture facilities.

3) Investigating the unidentified *Neobenedenia* sample identified in Chapter 2. This isolate consistently separated from the other defined clades present in the study and it is likely that this sample represents a taxon novel to science. It was the only isolate collected from a lutjanid host (unfortunately removing the option of comparing isolates from related hosts with one another). A thorough investigation of other lutjanids would result in an assessment of the extent to which *Neobenedenia* spp. infect this family of commercially important fish hosts.

4) Assess the feasibility of developing a set of robust morphological characteristics for *N. girellae* and *N. melleni* so that differentiation between these two species can be made purely on a morphological basis. Morphological plasticity makes the identification of species solely through morphological means considerably challenging and is a common trait among parasites. Due to this phenomenon, it is often difficult to determine what normal environmentally-induced morphological variation is and what is a 'true' feature which varies between closely related taxa is. Using the genetic framework established in Chapter 2, it will be possible to reassess the morphological differences between *N. girellae* and *N. melleni a posteriori*. Despite the popularity of molecular methods in

identifying species, particularly morphologically cryptic species, morphological taxonomy is important for the speed of identification that it offers. Additionally, expensive and bulky instrumentation is not required to make a rapid identification if accurate morphological keys are established. Research dedicated to the morphology of *Neobenedenia* spp. will greatly improve the speed and efficiency of identifying isolates on farms where management options are often time sensitive. The genetic assessment of *Neobenedenia* performed in this thesis will not replace the need for morphological observation so much as support its development and validate findings.

5) The strategic management timetable developed in Chapter 4 of this thesis is the most flexible and sensitive of its kind. Using the timing of life cycle events (hatching, infection of host, and reaching sexual maturity) in a range of temperatures and salinities (two of the most common variables on a fish farm) provides sufficient information to know when to target treatments at the weakest life stages of the parasite. This study was conducted in the laboratory and I was not able to validate the results I collected in the field. Field trials of the strategic treatment timetable is necessary to validate whether these findings translate to open systems that are not as closely controlled as in the laboratory. Doing so will enable validation of the timetable is in its current form or whether other variables may need to be considered. One of these possible variables which is known to be important in dictating hatching time of capsalids is dissolved oxygen levels. Low dissolved oxygen is known to delay hatching times of oncomiracidia and fluctuate, sometimes considerably, during a 24-hour period on fish farms. Some interest in the timetable has been expressed from a fish farm in South America indicating that the concept is desirable by farmers. Validating the treatment

times in a range of environmental conditions would be crucial to optimising it for use in a real-world setting.

6) Investigating the extent to which immunomodulatory compounds are produced during embryogenesis would considerably improve our understanding of the chemical methods that this parasite species used to successfully infect hosts. Although the way in which the host's immune system interacts with the attached parasite has been addressed previously (Trujillo-González et al., 2015b), the specific lipids that are involved in the process by which the parasite is able to suppress the immune system of their fish host has rarely been studied for this or any parasite-host relationship. Any findings from this sort of study would be valuable not just for research pertaining to capsalids, but also for the parasite-host relationships of all parasite groups.

Conclusion

The establishment of a reliable, sustainable laboratory culture of *N. girellae* has provided the unique opportunity to elucidate biological, taxonomic, and genetic features of this species relevant to the phylogeny, identification, and management of this species. This research has allowed for more sensitive distinction of species within the *Neobenedenia* genus genetically while improving our understanding of the morphological variability that a single species, *N. girellae*, can have in different environmental conditions. The improved knowledge of life cycle biology has allowed, for the first time, a strategic management option on farms that is no longer reactive and imprecise, but predictive and efficient. The culmination of this research is a greater understanding of an important parasite species which has the potential to pose a threat to global aquaculture production.

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APPENDIX A: Publications arising from this thesis

Brazenor AK, Bertozzi T, Miller TL, Whittington ID, Hutson KS (*in review, submitted 13/08/2017*). DNA profiling reveals *Neobenedenia girellae* as the primary culprit in global fisheries and aquaculture. *Molecular Phylogenetics and Evolution*.

Brazenor AK, Saunders RJ, Miller TL, Hutson KS (*accepted; in press*). Phenotypic plasticity in the cosmopolitan fish parasite *Neobenedenia girellae* (Capsalidae: Monogenea). *International Journal for Parasitology.*

Brazenor AK & Hutson KS. 2015. Effects of temperature and salinity on the life cycle of *Neobenedenia* sp. (Monogenea: Capsalidae) infecting farmed barramundi (*Lates calcarifer*). *Parasitology Research*, 114(5): 1875-1886.

Brazenor AK, Francis DS, Hutson KS & Carton AG (*accepted, in press*). Biochemical composition of marine monogenean parasite eggs. *Molecular and Biochemical Parasitology*. *Molecular and Biochemical Parasitology.*

APPENDIX B: Oral Conference presentations during the tenure of candidature

2015

Brazenor A, Bertozzi T, Miller T, Hutson K. Cryptic capsalids: is *Neobenedenia melleni* really the culprit in global aquaculture? James Cook University College of Marine and Environmental Sciences Post-graduate Conference. Townsville, Queensland, Australia. June 12, 2015.

Brazenor A, Bertozzi T, Miller T, Hutson K. Cryptic capsalids: is *Neobenedenia melleni* (Capsalidae: Monogenea) really the culprit in global aquaculture? 3rd Australasian Scientific Conference on Aquatic Animal Health, Cairns, Queensland, Australia. 6-10th July, 2015.

Hutson K, ***Brazenor A**, Bertozzi T, Miller T, Trujillo-González A, Dinh Hoai T, Militz T, Whittington I. Five intriguing facts about the harmful fish ectoparasite *Neobenedenia* sp. Australian Society for Parasitology conference, June, 2015, Auckland, New Zealand. *Collaboration, presented by Hutson.

2013

Brazenor A & Hutson K. Effects of temperature and salinity on the life cycle of *Neobenedenia* sp. (Monogenea: Capsalidae) infecting farmed barramundi (*Lates calcarifer*). Fisheries Research and Development Corporation (FRDC) Australasian

Scientific Conference on Aquatic Animal Health, 8-12th July 2013, Cairns, Queensland, Australia.

2012

Brazenor A & Hutson, K. The effect of temperature and salinity on the life cycle of *Neobenedenia* sp. infecting barramundi (*Lates calcarifer*). Australian Society for Parasitology conference, 2-5th July, 2012, Launceston, Tasmania, Australia.

APPENDIX C: Grants and awards

2015

Fisheries Society of the British Isles Student Travel Grant ($2,000)

Australian Biological Research Society Travel Grant ($1,500)

2014

James Cook University Graduate Research School Research grant ($1,500)

2013

Researcher exchange, training and travel award funding from the Australian Society for Parasitology ($2,885)

Second prize for Outstanding Student Presentation at the FRDC 2nd Australasian Scientific Conference on Aquatic Animal Health in Cairns, Australia, 2013

2012

Australian Postgraduate Award (PhD stipend for 3 years)

Australian Society for Parasitology Student Travel Grant ($1,000)