University of North Dakota
UND Scholarly Commons

Theses and Dissertations | Theses, Dissertations, and Senior Projects

January 2015

Parasites Within Parasites: Transmission And Evolution Of Neorickettsia In Digeneans

Stephen Edward Greiman

Follow this and additional works at: https://commons.und.edu/theses

Recommended Citation
Greiman, Stephen Edward, "Parasites Within Parasites: Transmission And Evolution Of Neorickettsia In Digeneans" (2015). *Theses and Dissertations*. 1777.
https://commons.und.edu/theses/1777

This Dissertation is brought to you for free and open access by the Theses, Dissertations, and Senior Projects at UND Scholarly Commons. It has been accepted for inclusion in Theses and Dissertations by an authorized administrator of UND Scholarly Commons. For more information, please contact zeineb.yousif@library.und.edu.

PARASITES WITHIN PARASITES: TRANSMISSION AND EVOLUTION OF *NEORICKETTSIA* IN DIGENEANS

by

Stephen Edward Greiman
Bachelor of Science, University of North Dakota, 2011

A Dissertation

Submitted to the Graduate Faculty

of the

University of North Dakota

in partial fulfillment of the requirements

for the degree of

Doctor of Philosophy

Grand Forks, North Dakota
May
2015

Copyright 2015 Stephen Greiman

This dissertation, submitted by Stephen Edward Greiman in partial fulfillment of the requirements for the Degree of Doctor of Philosophy from the University of North Dakota, has been read by the Faculty Advisory Committee under whom the work has been done and is hereby approved.

Vasyl Tkach

Jefferson Vaughan

Rebecca Simmons

Robert Newman

Joseph Hartman

This dissertation is being submitted by the appointed advisory committee as having met all of the requirements of the School of Graduate Studies at the University of North Dakota and is hereby approved.

Wayne Swisher
Dean of the School of Graduate Studies

April 29, 2015
Date

PERMISSION

Title PARASITES WITHIN PARASITES: TRANSMISSION AND EVOLUTION OF *NEORICKETTSIA* IN DIGENEANS

Department Biology

Degree Doctor of Philosophy

In presenting this dissertation in partial fulfillment of the requirements for a graduate degree from the University of North Dakota, I agree that the library of this University shall make it freely available for inspection. I further agree that permission for extensive copying for scholarly purposes may be granted by the professor who supervised my dissertation work or, in his absence, by the chairperson of the department or the dean of the School of Graduate Studies. It is understood that any copying or publication or other use of this dissertation or part thereof for financial gain shall not be allowed without my written permission. It is also understood that due recognition shall be given to me and to the University of North Dakota in any scholarly use which may be made of any material in my dissertation.

Stephen Edward Greiman
April-24-2015

TABLE OF CONTENTS

LIST OF FIGURES

Figure Page

List of Tables

ACKNOWLEDGMENTS

Thanks go out to all who helped with this study. My committee: Dr. Vasyl Tkach, Dr. Jefferson Vaughan, Dr. Robert Newman, Dr. Rebecca Simmons, and Dr. Joseph Hartman. Lab assistance from: Dr. Jacob Cain, Max Tkach, Kayla Steffes, Roy Nelson, Chris Bothun, and Devon Peightal. Specimen/sample donations from: Dr. Michael Kent, Dr.Yasuko Rikihisa, Dr. John Madigan, Dr. Sabine Dittrich, Dr. Xuejuan Ding, Dr. Eric Pulis, Dr. Stephen Curran, Dr. Amal I. Khalil, and Thomas Fayton. Funding from: the National Science Foundation, the National Institutes of Health, the University of North Dakota Biology Department, the University of North Dakota School of Graduate Studies, and the University of North Dakota division of Research Development & Compliance. Special thanks to Dr. Ha Binh and Tran Thi Binh for their help with logistics and sample collections in Vietnam. Special thanks to Dr. Poom Adisakwattana for his help with logistics and sample collections in Thailand. Special thanks to Dr. Paul Newton for his help with logistics and sample collections in Laos.

ABSTRACT

Digeneans are endoparasitic flatworms with complex life cycles that involve two or more different animals as definitive and intermediate hosts. Some digenean species harbor bacterial endosymbionts belonging to the genus *Neorickettsia* (Order: Rickettsiales, Family: Anaplasmataceae). *Neorickettsia* occur in all life cycle stages of digeneans and are maintained by vertical transmission. Far from benign however, *Neorickettsia* may also be transmitted horizontally by digenean parasites to their vertebrate definitive hosts. Once inside, *Neorickettsia* can infect macrophages and other cell types. For some vertebrate species (e.g. dogs, horses and humans), neorickettsial infections cause severe disease. With a few exceptions, studies of *Neorickettsia* have been traditionally carried out by bacteriologists, medical, and veterinary researchers, while helminthologists have rarely participated in these research endeavors. Despite the in-depth research published on different aspects of molecular biology, immunology, diagnostics and treatment of neorickettsiae and neorickettsial diseases, the quantitative aspects of transmission of these bacteria and their ecological and evolutionary interrelationships with their invertebrate and vertebrate hosts have received little attention. Recent progress in molecular techniques, particularly the polymerase chain reaction (PCR) and DNA sequencing has made possible the efficient and reliable detection of *Neorickettsia* at every step of their circulation, whether in the digenean host of the neorickettsiae or in the invertebrate and vertebrate hosts of the digenean. The same technology also allows for reliable identification of the digeneans. Taken from a mostly parasitological,

perspective, this study focused on the modes and quantitative aspects of *Neorickettsia* transmission and co-evolution with their digenean hosts through the use of molecular techniques.

To understand the biology and evolution of *Neorickettsia* within the digenean host we focused on four specific aims: **1) screen for *Neorickettsia* DNA a large collection(s) of diverse Digenea taxa from a wide variety of hosts and a broad geographic range using molecular methods and conduct molecular phylogenetic analyses of neorickettsiae in order to estimate interrelationships among all available genotypes; 2) develop and maintain a laboratory life cycle of a digenean, *Plagiorchis elegans*, harboring *Neorickettsia* sp.; 3) assess and quantify *Neorickettsia* vertical transmission efficiency through all stages of a digenean life cycle; 4) localize the bacterial endosymbiont within all stages of the digenean life cycle using immunofluorescent microscopy.**

In this study (specific aim 1) we screened more than 3,000 digenean samples for *Neorickettsia* collected from various vertebrates and invertebrates in terrestrial, freshwater, brackish and marine habitats from multiple countries and continents. Neorickettsiae were detected using a real-time PCR protocol targeting the GroEL gene and verified with nested PCR and sequencing of a 1371 bp long region of 16S rRNA. Twenty isolates of *Neorickettsia* have been obtained. Bayesian phylogenetic analyses were conducted to estimate interrelationships among all known species/genotypes of *Neorickettsia.* We identified 14 new genotypes of *Neorickettsia*, more than doubling the number of known species level lineages. Additionally, we identified 14 new digenean species and 7 digenean families as hosts of *Neorickettsia*. Our findings suggest that further surveys from broader geographic regions and wider

selection of digenean taxa are likely to reveal new *Neorickettsia* lineages as well as new digenean host associations and geographic records.

To accomplish specific aims 3 and 4 we for the first time, have maintained *Neorickettsia* sp. through multiple generations in the laboratory life cycle of a digenean*, Plagiorchis elegans* (aim 2). The laboratory life cycle of *P. elegans* consists of a snail first intermediate host, *Lymnaea stagnalis*, an aquatic arthropod second intermediate host, *Culex pipiens* (mosquito larva), and a vertebrate definitive host, *Mesocricetus auratus* (Syrian hamster). Using the newly developed laboratory life cycle we were able to quantify the number of bacteria within individual parasites at all stages of the digenean life cycle. To accomplish this we developed a quantitative real-time PCR assay targeting a 152 bp fragment of the heat shock protein coding gene, GroEL, using a g-block synthetic quantitative positive control. Furthermore, the laboratory life cycle has allowed us to localize the bacterial endosymbiont within eggs, sporocysts, cercariae, metacercariae, and adults of the digenean *P. elegans*, using immunoflourescent microscopy. Interestingly, unlike other genera of bacteria within the family Anaplasmataceae, *Neorickettsia* is not localized within the ovarian cells. The bacteria, is instead maintained from one generation of the digenean to another by infecting the vitellarium.

CHAPTER I

LITERATURE REVIEW

Introduction

Today, biologists, healthcare professionals and veterinarians are well aware that bloodsucking arthropods can serve as biological vectors of disease. Fewer realize that endoparasitic helminths can also play a role in transmission of animal and human infectious diseases. Three of the earliest known examples (before the discovery of *Neorickettsia*) include turkey black-head disease caused by a flagellate protist *Histomonas meleagridis* transmitted, among other ways, by a nematode *Heterakis gallinarum* (Graybill and Smith, 1920; Tyzzer, 1934; Ruff et al., 1970); swine influenza caused by a virus that can also be transmitted by a parasitic nematode, *Metastrongylus apri* (Lee 1971; Shope 1941, 1943); and "salmon dog poisoning" disease caused by a bacterium transmitted by a digenean *Nanophyetus salmincola* (Lee 1971; Philip 1955).

Transmission of bacterial diseases by digenean parasites was almost simultaneously and independently discovered on opposite sides of the globe during the mid-1950s. In North America, Philip et al. (1953, 1955) found that a lethal dog disease, previously well known as "salmon dog poisoning," is caused by a rickettsial agent transmitted by an intestinal fluke *N. salmincola*. Philip et al. (1953) erected a new genus, *Neorickettsia*, for this new bacterium species. Meanwhile, Japanese researchers (Fukuda et al. 1954; Misao and Kobayashi 1954) discovered that

a previously known human disease of unknown etiology, the Sennetsu fever, is also caused by a new rickettsial agent associated with flukes.

The genus *Neorickettsia* (Family Anaplasmataceae) comprises a small group of obligate intracellular bacteria normally endosymbiotic within digeneans. Digenean life cycles are complex and typically involve several stages parasitizing different host species. Neorickettsiae persist within all stages of the fluke and thus are maintained through vertical transmission. Under certain circumstances, adult flukes may pass their neorickettsial endosymbionts to the vertebrate definitive hosts. Once inside, neorickettsiae invade and multiply within the cells of the vertebrate. In some vertebrates, neorickettsial infections of macrophages, monocytes and other cells types lead to severe, sometimes fatal, disease. Before the beginning of our studies there were at least seven species/genotypes of *Neorickettsia* and four distinct diseases attributed to *Neorickettsia*.

With a few exceptions, studies of *Neorickettsia* have been traditionally carried out by bacteriologists, medical and veterinary researchers while helminthologists have rarely participated in these research endeavors. While a broad array of research has been published on different aspects of molecular biology, immunology, diagnostics and treatment of neorickettsiae and neorickettsial diseases, the quantitative aspects of transmission of these bacteria and their ecological and evolutionary interrelationships with their invertebrate and vertebrate hosts received much less attention. *Neorickettsia* transmission systems are inextricably intertwined with complex multihost life cycles of digeneans. Thus full elucidation of the transmission patterns of these neglected, but potentially widespread diseases of animals and man will require equal participation of helminthologists, rickettsiologists, epidemiologists and vector biologists.

Recent progress in molecular techniques, particularly the polymerase chain reaction (PCR) and DNA sequencing has made possible the efficient and reliable detection of *Neorickettsia* at every step of their circulation, whether in the digenean host of the neorickettsiae, or in the invertebrate and vertebrate hosts of the digenean. The same technology also allows for reliable identification of the digeneans, regardless of the stage of their development. Increasing number of *Neorickettsia* DNA sequences in the GenBank and other internationally accessible public databases has facilitated rapid and accurate comparisons of genotypes discovered in different parts of the world. Phylogenetic reconstructions of *Neorickettsia* have revealed new lineages, in some cases potentially new species. Recent publication of complete genomes of two *Neorickettsia* species opened new possibilities for studies of their evolution and phylogeny, metabolism and treatment of diseases caused by these bacteria.

Prior to this study, several significant reviews have been published on different aspects of *Neorickettsia* and neorickettsial diseases. These reviews usually covered *Neorickettsia* as a part of a larger discussion of diseases caused by various groups within the Anaplasmataceae (Rikihisa 1991, 2003, 2010; Walker and Dumler 1996; Rikihisa et al. 2005) or focused on particular species of *Neorickettsia* of veterinary importance (Mulville 1991; Palmer 1993; Madigan and Pusterla 2000; Headley et al. 2011).

Taxonomy and Phylogeny of *Neorickettsia*

Systematic Position of *Neorickettsia*

The classification of rickettsiae and other intracellular bacteria was traditionally based on biological, morphological or immunological characteristics. Advent of DNA sequencing has dramatically changed the field. Among other

findings, molecular phylogenies have led to a reorganization of the order Rickettsiales into two families, Rickettsiaceae and Anaplasmataceae (Dumler et al. 2001). This revision was based primarily on phylogenetic analysis of 16S rRNA and *groESL* gene sequences. Secondary characters included outer membrane protein sequences (amino acid or gene sequences) and biological characteristics, such as morphology, host cell tropism, disease ecology, and clinical presentation. *Neorickettsia* is one of four generally accepted genera belonging to the family Anaplasmataceae, namely *Anaplasma*, *Ehrlichia*, *Wolbachia*, and *Neorickettsia* (Dumler at al. 2001; Taillardat-Bisch et al. 2003; Rikihisa 2010; Figure 1). Recent publications (Seng et al. 2009; Rikihisa 2010) suggest the existence of three to five additional genera in the family, some of which are not formally established yet.

All Anaplasmataceae are obligate intracellular bacteria that grow within membrane-bound vacuoles of host cell origin (=morulae). Members of the genera *Anaplasma* and *Ehrlichia* are exclusively tick-borne and include species of medical or veterinary importance. *Aegyptianella* is also considered by some as another valid genus of the Anaplasmataceae (e.g., Rikihisa 2010), although in our analysis based on partial ribosomal 16S sequences, *Aegyptianella* clustered together with members of *Anaplasma* (Figure 1). Both are transmitted horizontally from tick to vertebrate to tick and vertical transmission plays little role in their natural cycle. Representatives of *Wolbachia* infect the ovaries of many species of arthropods and filarial nematodes; infection is transmitted exclusively through vertical transmission. There are no known vertebrate hosts of *Wolbachia*.

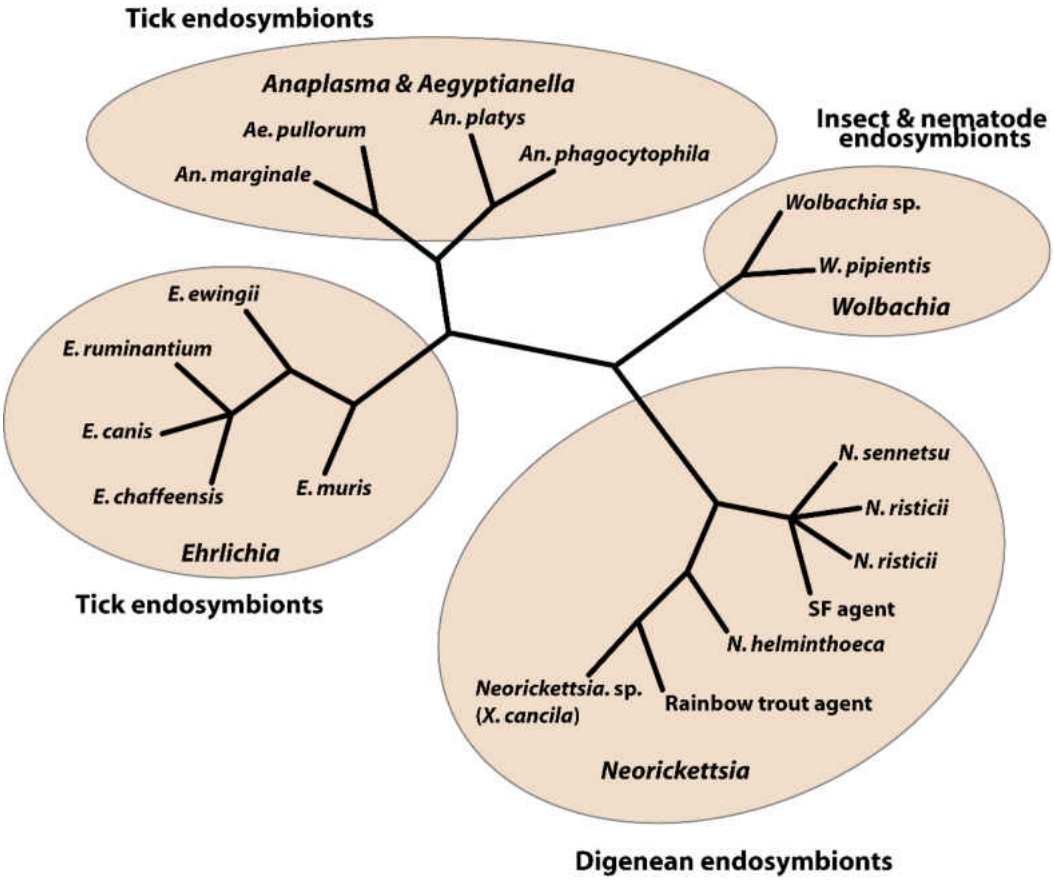

Figure 1. Phylogenetic interrelationships among Anaplasmataceae based on Bayesian analysis of partial 16S rRNA sequences

The most genetically divergent genus is *Neorickettsia*; all its species are endosymbionts of digenean flukes. *Neorickettsia* are unique among the Anaplasmataceae in that both vertical transmission and horizontal transmission (at least from fluke to vertebrate) have been documented (Gibson et al. 2005; Cordy and Gorham 1950). *Neorickettsia* can be grown *in vitro* within certain cell types (*e.g.*, macrophages and monocytes) of various vertebrate species (e.g., mouse, dog, human).

Taxonomy and Phylogenetic Interrelationships Among Species of *Neorickettsia*

Before this study, there were three published named species and seven genotypes of *Neorickettsia*, four of which cause distinct diseases, including a human disease (Table 1). The separation between species in literature is based primarily on levels of 16S rRNA sequence divergence (Dumler et al. 2001; Stackebrandt et al. 2002). Species of *Neorickettsia* differ in their ability to produce clinical illness within

certain vertebrate species but not in others. At the same time, different species demonstrate antigenic relatedness, as evidenced by immunological cross-protection between different neorickettsial species. For example, *N. sennetsu* causes clinical illness in humans but not horses. However, when horses are inoculated with *N. sennetsu*, they become immune against challenge with *N. risticii*, a species normally pathogenic to horses (Rikihisa et al. 1988). Likewise, *N. elokominica* is pathogenic to black bears but only mildly so to dogs. However, when dogs are inoculated with *N. elokominica*, they become immune against challenge with *N. helminthoeca*, which is highly virulent to naïve dogs (Farrell et al. 1973).

Currently, the taxonomy and principles of species differentiation among *Neorickettsia* species are far from being stable and universally agreed upon. For instance, multiple neorickettsial infections in digeneans from Korea identified as *N. risticii* (Chae et al. 2003; Park et al. 2003) may not belong to this species. Based on the levels of sequence homology of 16S gene provided by these authors, their isolates are more closely related to the "rainbow trout" genotype of *Neorickettsia* reported from North America and thus may belong to a phylogenetic lineage different from that of *N. risticii*. Although Chae et al. (2003) did not present a phylogenetic tree and did not submit sequences to a publicly accessible database, their work demonstrate the potential of finding additional forms in eastern and southeastern Asia.

Ecology and Transmission of Neorickettsiae

There are four distinct diseases described in the medical/veterinary literature attributable to *Neorickettsia*; salmon poisoning disease, Elokomin fluke fever, Sennetsu fever and Potomac horse fever (Table 1). The causative agents for these diseases are generally regarded as separate species based on differing pathologies,

Table 1. *Neorickettsia* species/genotypes described in literature and diseases caused by them.

***Neorickettsia* species/forms**	**Disease**	**Vertebrates affected by disease**	***Neorickettsia* localization**	**Vertebrate cell type infected**	**Geographic Distribution**	**First described**
N. sennetsu	Sennetsu fever	Humans rodents (exp.)	lymph nodes	Monocytes, endothelium	Japan, Malaysia, Laos, Thailand	Fukuda 1954
SF agent	Not known	mice (exp.)and dogs (exp.)	Not known	macrophages	Japan	Fukuda et al. 1973
N. helminthoeca	Salmon poisoning	canids	lymph nodes	macrophages	North America (Pacific slope of Cascade Mountains), Brazil	Philip et al. 1953
EEF agent	Elokomin Fluke Fever	Bears, dogs (exp.)	lymph nodes	macrophages	Northwestern USA	Farrel et al. 1973
N. risticii	Potomac Horse Fever	horses	Large colon	glandular epithelia, macrophages, monocytes	USA, Brazil & South Korea	Holland et al. 1985
Rainbow trout agent	Not known	Not known	Not known	Not known	USA – northern CA	Pusterla et al. 2000
Neorickettsia sp. from needlefish	Not known	Not known	Intestine	Not known	Cambodia	Seng et al. 2009
Neorickettsia sp. from notothenioid	Not known	Not known	Intestine	Not known	Antarctica	Ward et al. 2009
Catfish agent 1	Not known	Not known	Not known	Not known	USA-North Dakota	Tkach et al. 2012
Catfish agent 2	Not known	Not known	Not known	Not known	USA-Mississippi	Tkach et al. 2012

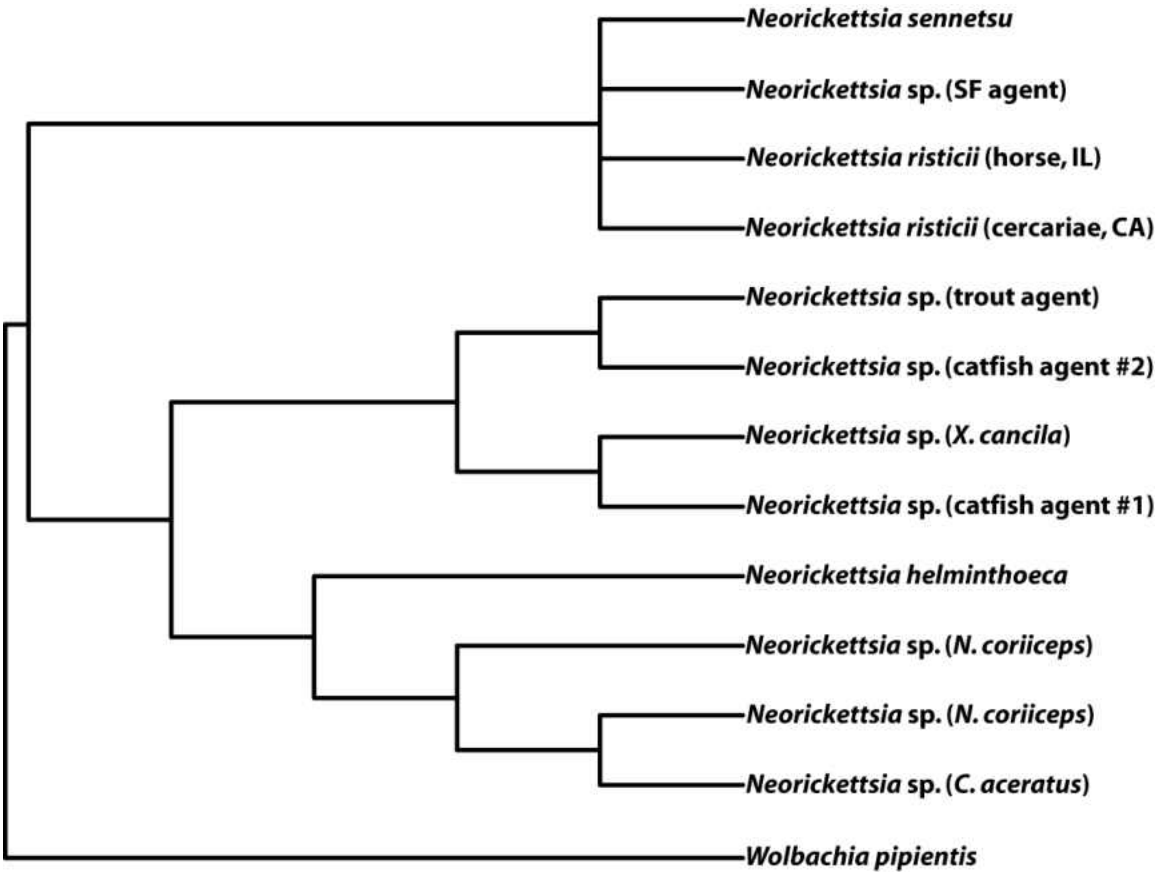

Figure 2. Phylogenetic tree of known species and genotypes of *Neorickettsia* before our study resulting from Bayesian analysis of partial 16s rRNA sequences.

serology, antigenic profiles and DNA sequence analyses. In addition, there are at least five other species/genotypes of neorickettsial endosymbionts whose pathogenicity to humans or other vertebrates is not known. The discovery of non-pathogenic *Neorickettsia* genotypes resulted largely from ecological studies targeting the pathogenic species. For example, studies on Sennetsu fever discovered the SF (abbreviated from *Stellantchasmus falcatus*) agent and ecological studies on *Neorickettsia risticii* discovered the neorickettsiae provisionally designated as rainbow trout agent, catfish agent 1, catfish agent 2 and undefined *N. risticii*-like agents.

Neorickettsia helminthoeca and Salmon Poisoning Disease

It was common knowledge among native Americans and white settlers along the Pacific coast of northern California, Oregon and Washington that if dogs were allowed to eat the dead or dying "spawned out" salmon, that the dogs were likely to become very ill and die. It was assumed that the fish contained a toxin that was somehow poisonous to dogs, foxes and coyotes but not to other fish-eating carnivores such as raccoons or mink. Thus the affliction got its name "*salmon poisoning of dogs*." Symptoms include rapid onset of fever and weight loss, accompanied later by vomiting and black, bloody diarrhea. If left untreated, death is almost certain (90% mortality rate) within two weeks after onset. Because of the extreme severity of this disease, concern about salmon poisoning disease (SPD) among veterinarians and dog owners in the Pacific Northwest persists to the present day.

As early as in 1911, Pernot (1911) demonstrated that SPD was not due to a toxin but instead was caused by an infectious agent. Blood from sick dogs was injected into healthy dogs. After an incubation period of 2 to 4 days, injected dogs developed symptoms characteristic of salmon poisoning. Serial injections of blood from these dogs into naïve dogs also produced disease with similar symptoms and incubation periods. Furthermore, the few dogs that managed to survive their illnesses were immune to the effects of subsequent injections. However, the exact etiology of the infection remained unknown until Donham (1925) made a key observation. He reported that autopsied dogs that died of SPD all harbored minute adult digenean flukes (later identified as *Nanophyetus salmincola* by Chapin, 1926). Donham correctly surmised that the afflicted dogs had acquired their adult flukes by ingesting fluke cysts (=metacercariae) found in

the flesh and internal organs of local salmonid fish. Furthermore, he speculated that presence of the fluke was a necessary component of contracting the disease. Simms and colleagues (Simms et al. 1931a, b, 1932) demonstrated conclusively that metacercariae and adult flukes of locally collected *Nanophyetus salmincola* caused SPD when fed or injected into dogs. They suspected that the infection was rickettsial or haemosporidian in origin. Their hypothesis was confirmed when Cordy and Gorham (1950) described intracytoplasmic rickettsial-like organisms in reticuloendothelial cells from Giemsa-stained lymph node impression smears taken from a dog that died of salmon poisoning. Philip et al. (1953) named the agent *Neorickettsia helminthoeca* in recognition that it was a new type of rickettsia and that digeneans were essential to their transmission.

During the 1950s and 1960s, two main groups of researchers studied the disease. Researchers at Oregon State University (Millemann et al. 1964; Millemann and Knapp 1970; Gebhardt et al. 1966; Baldwin et al. 1967; Nyberg et al. 1967 Schlegel et al. 1968) conducted ecological studies on the digenean parasite and its intermediate and definitive hosts. At the NIH Rocky Mountain Laboratory in Hamilton MT, C.B. Philip and his colleagues performed studies on the transmission and pathology of the agent (Philip at al. 1954a, b; Philip 1955). From the work of these researchers and others, the disease agent, *N. helminthoec* is now known to be found in all life stages of the digenean parasite, *Nanophyetus salmincola* (see Figure 4 for fluke life cycle; Bennington and Pratt 1960).

In nature, dogs acquire infections by consuming fish containing *N. salmincola*-infected metacercariae. When dogs eat infected fish, the ingested fluke metacercariae mature to adults deep within the mucosa of the upper part of the small intestine.

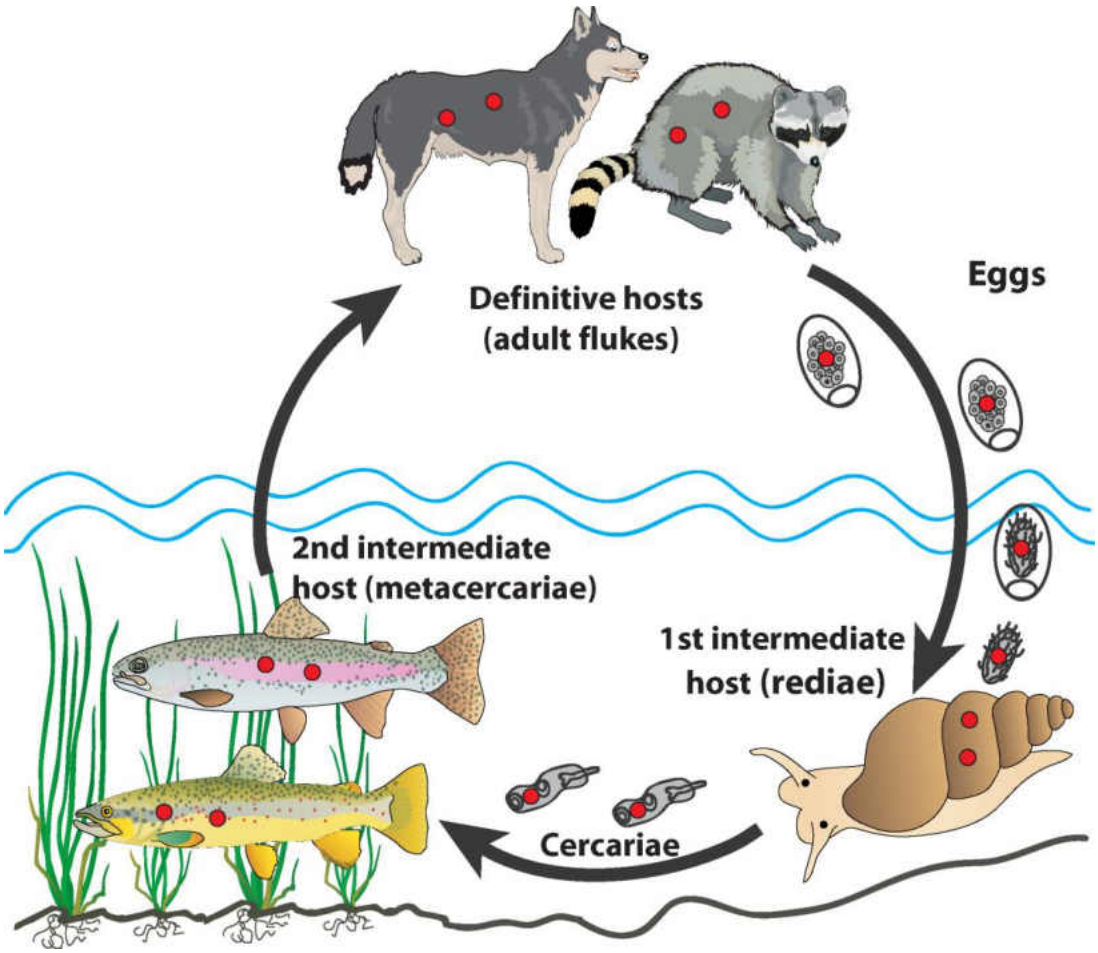

Figure 3. Circulation of *Neorickettsia helminthoeca* (red dots) through the life cycle of its digenean host, *Nanophyetus salmincola*.

Adult worms begin producing eggs in 5 to 10 days, which corresponds closely to the normal pre-patent period of SPD symptoms in dogs. The mechanism by which neorickettsial organisms are transferred from the developing fluke to intestinal tissues and into macrophages of the dog is unknown. If left untreated, most dogs die within 6 to 10 days after onset of clinical symptoms. During the rapid course of disease, gross pathology is largely restricted to the visceral lymph nodes, which present as enlarged and fleshy, often with areas of hemorrhage and necrosis. The relatively mild histopathological changes induced by SPD belies its severe and lethal consequences, suggesting that *N.*

helminthoeca possesses a highly potent virulence factor(s) (Philip et al. 1954a). Yet, experimental infections with SPD fail to produce more than a transient fever in other non-canid mammals, including raccoons, bears, mink, bobcats, domestic cats, guinea pigs, rats, mice or hamsters (Cordy and Gorham 1950, Simms et al. 1931b; Simms et al. 1932). Humans can be infected with the fluke (Eastburn et al. 1987; Harrell and Deardorff 1990) but are apparently refractory to the disease. Philip (1955) infected himself by eating raw metacercarial-infected trout, samples of which produced a fatal infection when fed to a dog. He suffered no ill effects, but some parasites did attain maturity as evidenced by the appearance of eggs in his stool 10 days after ingestion of the raw fish.

Indeed, because of the unique pathogenicity of SPD against canids, wildlife biologists have investigated the feasibility of using fish from SPD endemic regions as a possible alternative to traditional poisoned baits to control coyotes, *Canis latrans* (Foreyt 1982, 1987; Green et al. 1987; Foreyt and Gorham 1988). Dogs, foxes and coyotes that recover from salmon poisoning disease, either naturally or via antibiotic therapy, are immune to subsequent infection (Simms et al. 1931a). However, the flukes in their intestines continue to harbor infectious neorickettsiae and convalescent dogs may continue to shed neorickettsiae-infected fluke eggs in their feces (Philip et al. 1954b). In the laboratory, SPD has been transmitted experimentally from dog-to-dog by intra-peritoneal injection of blood or lymph node homogenates prepared from sick dogs (prepatent period = 2 to 4 days, Philip et al. 1954b). During experiments to determine if SPD could be transmitted among dogs by direct contact, Bosman et al. (1970) successfully infected dogs via aerosolization and enemas of lymph node suspensions and rectal mucosa homogenates. This indicated that in theory, SPD could be communicable.

However, contagious infection within a natural setting is probably rare because Sims (1932) reported that over the course of seven years, over 100 susceptible dogs were maintained in kennels together with dogs infected with SPD and in no instance was SPD transmitted to susceptible dogs by such contact. This is in spite of the fact that SPD-stricken dogs produce vomit and diarrhea.

Salmon poisoning disease has also been transmitted experimentally from fluke-to-dog by intraperitoneal injection of whole and macerated adult flukes (prepatent period = 6 to 7 days), and of snail tissue containing rediae (pre-patent period of 28 – 33 days, Philip et al. 1954a). The disease was also successfully transmitted by injecting dogs with fluke eggs homogenized in glass tissue grinder, but not with intact eggs, indicating that infectious neorickettsiae are contained within the interior of the unembryonated *N. salmincola* eggs, but not on the exterior surfaces of the egg shells (Nyberg et al. 1967). This was conclusive proof that *N. helminthoeca* is transmitted transovarially to successive generations of digeneans.

Until recently, SPD was considered to be restricted exclusively to the western United States and Canada, ranging from the Pacific coast westward to the Cascade Mountains and longitudinally from southern Vancouver Island (Booth et al. 1984) to the Sacramento River of northwest California (Sykes et al. 2010). However, *N. helminthoeca* has also been confirmed recently in south-central Brazil using immunological and molecular techniques (Headley et al. 2004, 2009). This indicates that *N. helminthoeca* may use at least one additional digenean species and that it is more widespread than previously appreciated. The fluke species responsible for SPD transmission in Brazil remains unknown. In North America, SPD is only transmitted by *N. salmincola*. Thus the

geographic distribution of SPD is constrained by the distribution of *N. salmincola* which is in turn determined solely by the distribution of the fluke's first intermediate host, *Oxytrema silicula* (Family: Pleuroceridae). This snail species is common in streams and rivers of the Pacific Northwest, but is found nowhere else (Bennington and Pratt 1960). Although *O. silicula* serves as a host to several other digenean species (including *Acanthatrium oregonense*, which harbors a different *Neorickettsia* species - see *N. risticii* below), the asexual development and production of cercariae by *N. salmincola* can only occur in this snail species (Millemann and Knapp 1970).

Interestingly, a close relative of *N. salmincola*, namely *N. schikhobalowi*, infects fish-eating mammals, including humans, living alongside the Amur and Ussuri Rivers of southeastern Siberia. The ecology of the fluke is similar to its sister species in the Pacific Northwest, yet SPD is not known in the region and the local dogs eat raw fish and carry adult flukes in their intestines (Filimonova 1963). This suggests that Asian species of the *Nanophyetus* fluke do not harbor neorickettsiae or, if they do, they harbor a different species of *Neorickettsia* that is nonpathogenic to dogs.

Elokomin Fluke Fever Agent

In 1973, Farrell and colleagues published a series of papers describing what they designated as a second neorickettsial agent transmitted by the same fluke vector, *Nanophyetus salmincola* (Farrell et al. 1973; Sakawa et al. 1973; Kitao et al. 1973). Their original purpose was to determine if black bears, *Ursus americanus* (proven earlier to be refractory to SPD) could act as the natural reservoirs of SPD. Metacercarial-infected trout were fed to 6 captive bears, 4 of which developed fever, anorexia and 'lassitude'. Upon autopsy, the sick bears had swollen mesenteric lymph nodes suggestive of salmon

poisoning. Three of the bears had detectable rickettsial bodies in lymph node impression smears. Upon injection of individual lymph suspensions from 5 autopsied bears into groups of 4 to 6 dogs each, 72% of the dogs developed low-grade fever and diarrhea for 4 to 12 days, but did not die. Serial passage of blood during the febrile state into fresh dogs produced similar, mild symptoms. When convalescent dogs were then fed metacercaria-infested trout 3 months later, 87% died of SPD. Because the clinical symptoms differed and there was lack of conferred immunity between the 2 diseases, Farrell et al. named the disease "*Elokomin fluke fever"* (EFF) because the source of the infective trout was the Elokomin River. Later studies confirmed that EFF agent was immunologically distinguishable from both *N. helminthoeca* and *N. sennetsu* (see below) by complement fixation (Sakawa et al. 1973), immunofluorescent antibody tests and live animal challenges (Kitao et al. 1973). Farrell et al. (1973) considered EFF and *Neorickettsia helminthoeca* to be a disease complex and indeed the EFF agent has been provisionally designated as *Neorickettsia elokominica* in the Merck Veterinary Manual.

No further work has been published on the EFF agent. Recently, however, Gai et al. (2008) reported salmon dog poisoning in two captive sun bears (*Ursus malayanus*) that developed disease upon eating fresh trout wild caught in Northern California. Both bears developed symptoms (vomiting, diarrhea, anorexia and lethargy) consistent with those observed in dogs sick with SPD. Fecal samples from sick bears contained eggs of *Nanophyetus salmincola*, the fluke responsible for transmission of SPD. The authors concluded that the disease was SPD. It can be the case considering that sun bears as a species presumably have not had exposure to *N. helminthoeca* throughout the course of their evolution and may not have well pronounced immunity to this disease agent. At the

same time, as mentioned above, it has been experimentally proven that black bears native to the region, do not develop such severe illness disease upon exposure to *N. helminthoeca*. The symptoms described by Gai et al. (2008) are also strongly reminiscent of the symptoms described by Farrell et al. (1973) for the Elokomin fluke fever. Thus, it cannot be excluded that the disease described by Gai et al. (2008) could have actually been EFF. Since no DNA sequences were obtained in either case, it is difficult at present to judge on the identity of these diseases and their respective agents. Regardless, feeding raw salmonid fish from SPD-endemic areas to various species of bears (e.g., sun bears and polar bears) remains an important veterinary concern for zoos that maintain captive bears (Bourne et al. 2010).

Neorickettsia sennetsu and Sennetsu Fever

About the same time that early studies on SPD were conducted in the USA, Japanese scientists were investigating the cause of an acute, incapacitating rickettsial mononucleosis of humans that had a variety of local names Sennetsu, Hyuga or Kagami fevers, endemic to Miyazaki Prefecture in western Kyushu, Japan (Fukuda et al. 1954; Misao and Kobayashi 1954, 1955; Misao and Katsuta 1956). Symptoms include high fever, enlarged postauricular and posterior cervical lymph nodes, malaise, anorexia and peripheral blood mononucleosis. Hepatosplenomegaly occurs in some patients. Disease lasts up to 2 weeks; fatalities are unknown (Misao and Katsuta 1956; Tachibana 1986). Epidemiological studies suggested that the disease is acquired by eating raw grey mullet fish (*Mugil cephalus*) infested with metacercariae (Fukuda 1958). When 96 human volunteers ate metacercaria-infested raw mullet captured from the Oyodo River, five of the volunteers developed clinical symptoms of the disease (Fukuda et al. 1962). The

agent was named *Neorickettsia* (formerly *Ehrlichia*) *sennetsu*. *Neorickettsia sennetsu* was isolated from patients and later from fish, but it was never isolated from metacercariae, nor was the fluke host ever identified. Later, the agent of this disease was reported from patients in Malaysia (Holland et al. 1985b; Ristic 1990; Weiss et al. 1990). Monkeys, rodents and dogs can be successfully infected experimentally and develop multiple symptoms and enlarged lymph nodes (Fukuda et al. 1954; Ohtaki and Shishido 1965; Holland et al. 1985). Interestingly, the disease has not been reported from Japan for a long time. However, *N. sennetsu* was successfully propagated in primary canine blood monocyte cultures which allowed to develop an immunological test that revealed numerous *N. sennetsu* infections among patients with fevers of unknown origin in Malaysia (Holland et al. 1985b).

A recent study (Newton et al. 2009) revealed that a high seroprevalence of *N. sennetsu* (17% of 1,132 patients) occurs in Vientiane municipality and Savannakhet Provinces in Laos. Newton et al. (2009) concluded that Sennetsu neorickettsiosis is a common infection in Laos. The same study showed a 4% seroprevalence in patients in Thailand. It should be noted that only one of 91 buffy coat samples from patients with non-malarious undifferentiated fever was PCR-positive for *N. sennetsu*. Sequences of PCR products of three different genes obtained from the human patient sample were 100% identical to previously published sequences of *N. sennetsu* strain Miyayama. Newton et al. (2009) considered it possible that at least some of the seropositive samples found in their survey resulted from exposure to organisms closely related to *N. sennetsu*, but not *N. sennetsu*.

Laos is a country where raw fish is commonly consumed and fish-borne intestinal and hepatic digenean infections are prevalent (Chai et al. 2005b, 2007; 2009a, b; Hortle 2007; Sayasone et al. 2009a, b; Andrews et al. 2008; Rim et al. 2008; Table 2). Therefore, Newton et al. (2009) screened local fish for *Neorickettsia* using standard and real-time PCR (RT-PCR) targeting the 16S rRNA gene. After screening 238 samples and an impressive 88 fish species including 10 fish species most commonly consumed in and around the Laos capital Vientiane, Newton et al. (2009) discovered *Neorickettsia* infections in three fish species, namely the dwarf snakehead fish (*Channa gachua*), the croaking gourami (*Trichopsis vittata*) and the climbing perch (*Anabas testudineus*). While the sequences from the first two fishes were significantly different from the sequence of *N. sennetsu* (92.2% and 95.8% homology), the sequence from *A. testudineus* showed 99.1% homology with the previously published sequence of *N. sennetsu* strain Miyayama. The results were confirmed with RT-PCR using two additional genes frequently used in *Neorickettsia* systematics, namely *glt*A and *Omp*85. Newton et al. (2009) presented a phylogenetic analysis showing that the samples from the human patient and *A. testudineus* clustered together with *N. sennetsu*. Sequences from the other two fish species (*C. gachua* and *T. vittata*) were basal to all *Neorickettsia*. These recent findings of up to four new forms of Anaplasmataceae in fish from the same region by Newton et al. (2009) and Seng et al. (2009) clearly indicate that there is yet much to be learned about these bacteria, their diversity and diseases caused by them.

Coincidentally with the study of Newton et al. (2009), scientists in the Korean-Laos Cooperation Project on Parasite Control in Lao PDR conducted extensive parasitological surveys along the Mekong River, including four villages in Vientiane and

four villages in Savannakhet Province (Chai et al. 2005a,b, 2007). Their survey revealed that 62% of 1,580 examined people had active digenean infections. Combining the anti-*Neorickettsia* serology and the digenean prevalence data suggests that 27% of the digenean infections that occurred in Vientiane and Savannakhet Provinces during 2001 to 2003 could have resulted in neorickettsioses - i.e., 17% overall anti-Sennetsu seropositivity (Newton et al. 2009) divided by 62% overall digenean parasitism (Chai et al. 2005b; 2007). It means that over a quarter of all food-borne digenean infections in these 2 Laotian provinces could have resulted in neorickettsioses.

The Korean-Laotian collaborative study found at least 7 digenean species present and 75% of infected people were parasitized by more than one digenean species (Table 2). Most infected people harbored a few dozen flukes while some others harbored hundreds, even thousands. Although the relative contribution of species varied by location, three species dominated in all locations – a liver fluke, *Opisthorchis viverrini* (Opisthorchiidae), and two intestinal flukes, *Haplorchis taichui* (Heterophyidae) and *Prosthodendrium molenkampi* (Lecithodendriidae). The first two are acquired from eating raw fish (Chai et al. 2009b). The third species, *Pr. molenkampi*, uses dragonfly nymphs as 2nd intermediate host (Manning and Lertprasert 1973). Taking into account that insects are known to host other species/genotypes of *Neorickettsia*, it cannot be excluded that arthropods may represent another potential source of human neorickettsial infections. Considering the extremely wide-spread consumption of raw or undercooked fish products in the region, as well as common use of various invertebrates (crustaceans, insects, mollusks) as food (Chai et al. 2005a, b; Hortle 2007), human neorickettsioses are probably more common in this part of the world than currently known.

Table 2. Species diversity and worm burden of digenean-infected people living within two known *N. sennetsu* endemic regions of Laos, 2002-2004 (data from Chai et al., 2005b, 2007).

Species Composition	**Prevalence of infection (%), mean intensity of infection**		
	Vientiane (n=18)	Savannakhet (n=29)	TOTAL (n=47)
Opisthorchis viverrini	100%, 26	76%, 35	70%, 30
Haplorchis taichui	89%, 10	69%, 11	63%, 11
Haplorchis pumilio	6%, 1	10%, 18	7%, 9
Haplorchis yokogawai	11%, 31	7%, 1	7%, 7
Centrocestus caninus	17%, 1	0%,0	5%, 1
Echinostomatidae spp.	0%, 0	10%, 23	5%, 23
Prosthodendrium molenkampi	28%, 2	41%, 10	30%, 6
Phaneropsolus bonnei	0%, 0	10%, 8	5%, 8
Dual infections (2 digenean species)	44%	34%	38%
Polyparasitism (3 or more digenean spp.)	44%	31%	36%

Nearly nothing is known about distribution of *N. sennetsu* among mammals other than humans, with the exception of a single report from rodents in Japan (Fukuda et al. 1962). Taking into account the known pathogenicity of *N. sennetsu* in some non-human mammals resulting from experimental infections (see above), it cannot be excluded that this agent may cause disease in wild animals as well. Unfortunately, our knowledge of the ecology of *N. sennetsu* and other related genotypes is rather marginal. The thorough study done by Newton et al. (2009) that resulted in multiple records of *Neorickettsia* in fish, did not incriminate any digenean species. Considering the extremely diverse traditional diets in the region, more *Neorickettsia* transmission pathways are possible than merely through fish. The case of *N. risticii* which can infect a variety of digeneans having diverse life cycles, from entirely aquatic to entirely terrestrial, provides a clue that the circulation of *Neorickettsia* in nature may be more flexible than we currently appreciate.

Neorickettsia risticii and Potomac Horse Fever

In the late 1970's, a mysterious, summertime illness of horses appeared in the rural counties of Maryland and Virginia surrounding Washington D.C. Clinical symptoms varied, but usually included fever (often biphasic), depression, anorexia, and colitis (inflammation of large colon), accompanied by acute diarrhea (Holland et al. 1985c; Rikihisa 1991). In severe cases, horses exhibited laminitis. Pregnant mares often aborted (Coffman et al. 2008; Long et al. 1995). If left untreated, overall mortality approached 30% (Cordes et al. 1986). The clinical term for this condition is equine monocytic ehrlichiosis but because the initial cases occurred in horses pastured close to the Potomac River, the disease became known as "*Potomac horse fever*" (PHF). Stools from diarrheic horses contained the infectious agent and, when fed to susceptible horses,

could produce PHF (Biswas et al. 1994; Palmer and Benson 1994). However, the highly seasonal occurrence of the disease, coupled with the fact that susceptible horses stabled with sick horses rarely acquired PHF, suggested that PHF was not a communicable disease but instead was a vector-borne disease.

The causative organism was initially placed in the genus *Ehrlichia* (Holland et al. 1985a, b, c; Rikihisa and Perry 1985). This led to some early confusion regarding the transmission of PHF because *Ehrlichia* are known to be tick-borne. However, vector competence studies at the time failed to incriminate local tick species in the transmission of the PHF (Hahn et al. 1990; Levine et al. 1990).

The source of PHF remained enigmatic for years, until advances in molecular biology transformed bacterial systematics in the early 1990's. When Yasuko Rikihisa and her team at Ohio State University applied molecular methodology to the study of ehrlichial diseases, they not only accelerated the development of accurate diagnostic tools for PHF (Messick and Rikihisa 1992a, b; Barlough et al. 1997; Mott et al. 1997; Pusterla et al. 2006) but importantly, their efforts as well as others, allowed for the phylogenetic reconstruction of the Rickettsiales (Pretzman et al. 1995; Dumler et al. 2001; Inokuma et al. 2001). As a result of this landmark accomplishment, it became evident that the PHF agent was phylogenetically closer to the fluke-borne agents of SPD, Sennetsu fever and the SF agent than it was to tick-borne ehrlichiae. This proved to be a textbook example of the utility of systematics and phylogenetics in resolving pressing practical questions related to a new emerging disease and it provided the clue that enabled the University of California Davis veterinarian team of John Madigan and colleagues to begin to put the pieces of the PHF puzzle together (Dumler 2000).

Using PCR technology, the UC Davis team isolated *N. risticii* DNA from small pleurocercid snails (*Juga* spp.) collected in the streams of an endemic area (Barlough et al. 1998; Reubel et al. 1998). When cercariae and sporocysts from fluke-infected snails were inoculated into horses, the horses became ill with PHF (or Shasta River crud, as it is known locally; Madigan et al. 1997). *Neorickettsia risticii* were recovered from buffy coats of the sick horses (Pusterla et al. 2000 a, c), satisfying Koch's postulates. Further studies elucidated the ecology of PHF in northern California (Pusterla et al. 2000a, c, 2003; Chae et al. 2000, 2002) and in central Pennsylvania (Mott et al. 2002; Gibson et al. 2005; Gibson and Rikihisa 2008). Transmission cycles in these areas involve several fluke species that use river snails (e.g., *Juga*) as the 1st intermediate host, aquatic insects (*e.g*., caddisflies, mayflies) as the 2nd intermediate host, and insectivorous birds and/or bats as the definitive hosts (Figure 4). Within PHF-endemic areas, rates of *N. risticii* infection in intermediate hosts of digeneans were high. In northern California, up to 26% of the snails harboring larval digeneans tested PCR positive for *N. risticii* DNA (Pusterla et al. 2000a, c). Likewise in central Pennsylvania, 5 of 42 pools (12%) of cercariae and sporocyts collected from snails tested PCR positive for *N. risticii* DNA, as did 2 of 10 pools (20%) of adult mayflies and 3 of 8 pools (38%) of adult caddisflies (Mott et al. 2002).

To determine if metacercaria-infected insects harbored viable *N. risticii*, two horses were fed pools of field-collected caddisflies that had tested positive for *N. risticii*. Six to 11 days later, both horses became neorickettsemic and clinically ill with PHF (Mott et al. 2002). Therefore, transmission in these areas supposedly occurs when horses somehow swallow insects containing metacercariae. This could occur in a variety of

ways either through consuming insects while grazing, eating insect-contaminated hay, or by drinking insects that have been attracted at night to lights over watering troughs and fell in the water (Farren 2007).

At least 2 different genera of adult flukes (*Acanthatrium* and *Lecithodendrium*) parasitizing bats and swallows were found to harbor *N. risticii* DNA (Pusterla et al. 2003; Gibson et al. 2005; Gibson and Rikihisa 2008). Importantly, unparasitized tissues (liver and spleen) from the definitive hosts also contained *N. risticii* DNA (Pusterla et al. 2003), suggesting that insectivorous birds and bats may act as natural vertebrate reservoirs of *N. risticii*. However, several reports indicate that enzootic transmission cycles of *N. risticii* are not necessarily restricted to lotic (i.e., riverine) ecosystems or to a single type of digenean life cycle. For example, Barlough et al. (1998) reported detecting 16S rRNA sequence of *N. risticii* from a pool of *Stagnicola* (Lymnaeidae) snails in Oregon. *Stagnicola* are common inhabitants of ponds and lakes throughout North America and are hosts to representatives of many digenean families (Schell 1985). On the shores of Lake Merin, Uruguay, Dutra et al. (2001) found a significant clustering of cases of PHF (=churrido equino) in horses that were regularly pastured in the low marshy fields bordering the lake (36 per 1,000 horse-years) compared to the horses pastured in rice plain fields (3 per 1,000 horse-years). Tkach et al. (2012) isolated *N. risticii* DNA from two different species of cercariae shed from *Helisoma trivolis* snails (Planorbidae) collected at Lake Itasca, Minnesota, USA. Taken together, these observations indicate that natural transmission of *N. risticii* may also involve lentic (=lake and pond) ecosystems.

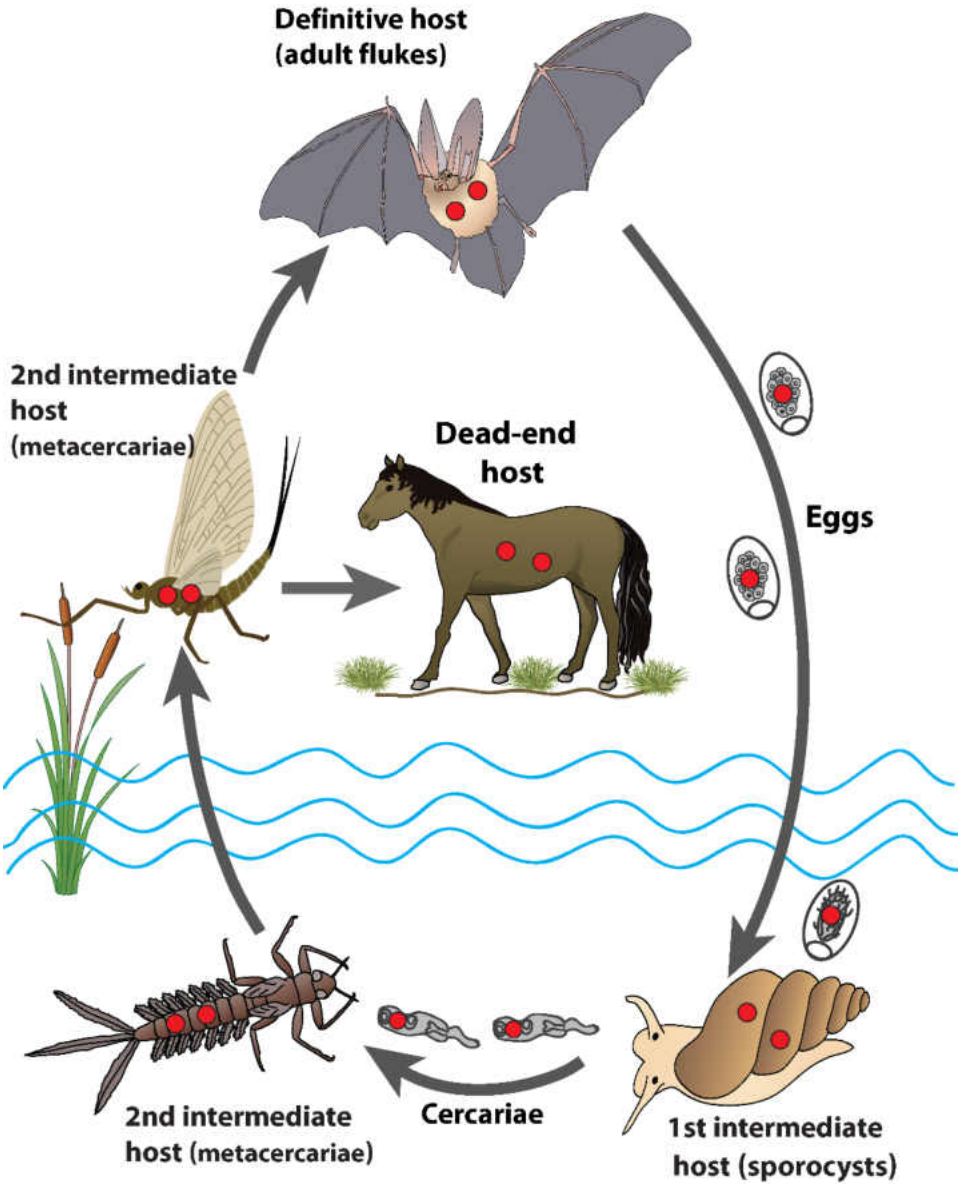

Figure 4. Circulation of *Neorickettsia risticii* (red dots) involving lecithodendriid digeneans and horses as a dead-end host.

Moreover, *N. risticii* DNA has been recovered from an adult dicrocoeliid fluke parasitizing a small passerine bird in North Dakota, USA (Tkach et al. 2012). This is significant because all dicrocoeliid flukes utilize land snails or slugs as their 1st intermediate hosts and terrestrial invertebrates (usually arthropods) as 2nd intermediate hosts (Yamaguti 1975; Schell 1985). In addition, Tkach et al. (2012) detected by PCR and sequenced *N. risticii* in digeneans with fully aquatic life cycles, namely

Alloglossidium corti (parasite of catfishes) and *Heronimus mollis* (parasite of freshwater turtles). The former uses arthropods as second intermediate hosts while the latter does not have a second intermediate host and metacercariae remain within the snail until eaten by a turtle. Thus, *N. risticii* is present in various digenean groups having all of the main types of life cycles typical of freshwater/terrestrial ecosystems (Figure 6). Thus far, *N. risticii* has not been reported from marine or estuarine environment. These findings demonstrate the great plasticity of *N. risticii* circulation pathways in nature. All this indicates that enzootic transmission cycles of PHF occur in many ecosystems, even fully terrestrial ecosystems, which explains how horses contract PHF in pastures far removed from rivers, streams, lakes or ponds. Thus, the risk of horses contracting PHF may be more widespread than previously recognized.

We are not aware of any studies demonstrating the presence of adult flukes in horses that have contracted PHF. Indeed, the digenean fauna of horses is generally very poor and so far, none of the digenean species from which *N. risticii* DNA has been recovered belonged to groups that can develop to the adult stages in horses. Hence, it appears that digenean metacercariae do not need to complete development to the adult stage in order to pass the infection to horses. Unlike the situation with SPD and dogs, horses can truly be considered "dead-end hosts" for PHF transmission. It remains unknown whether all species of *N. risticii*-infected metacercariae ingested by a horse can lead to PHF or whether only certain species or groups of digeneans can successfully transmit their neorickettsial endosymbionts before being destroyed in the horse's digestive system or expelled with the droppings.

SF Agent

During the search for the agent of Sennetsu fever, another neorickettsial agent was discovered in *Stellantchasmus falcatus* metacercariae infesting grey mullet and has been consistently found during at least 17 years of regular studies (Fukuda et al. 1973; Fukuda and Yamamoto 1981). The agent has become known as "*SF agent*" (short for "*Stellantchasmus falcatus* agent") and differs from *N. sennetsu* in western blot profiles probed with anti-SF sera, clinical and pathologic responses of mice, and 16S rRNA sequence comparisons (Wen et al. 1996). The SF agent is not known to cause disease in humans or in experimentally infected monkeys. It produces splenomegaly and lymphadenopathy when injected into mice, but produces only mild clinical symptoms in dogs (Shishido et al. 1965; Hirai 1966; Fukuda and Yamamoto 1981; Tachibana 1986; Rikihisa 1991; Wen et al. 1996). This genotype of *Neorickettsia* can be maintained by mouse passages in laboratory (Fukuda and Yamamoto 1981). Its digenean host, *Stellantchasmus falcatus* has a snail / fish / mammal type of life cycle associated with lotic/estuarine habitats. When ingested by humans, *S. falcatus* metacercariae are able to mature to the adult stage in the intestine (Katsuta 1931; Seo et al. 1984). Infection with this small heterophyid fluke is of minor medical concern, causing only mild intestinal discomfort and occasional diarrhea (Seo et al. 1984). Despite the wide geographic range of this fluke species (Japan, Korea, Southeast Asia, Hawaii, Australia, Egypt, Palestine and Israel according to Katsuta 1931; Pearson 1964; Chai and Sohn 1988), SF agent has only been reported from Japan and the US (Oregon) (Fukuda et al. 1973; Wen et al. 1996; Rikihisa et al. 2004). It is not known if it may inhabit other digenean species or other vertebrate animals.

Rainbow Trout Agent

During the studies to elucidate the ecology of PHF in California, thirty-five rainbow trout were collected from the Shasta River in a PHF-endemic area. Neorickettsial DNA was recovered from 3 species of adult digenean parasites collected from the gall bladder and intestine of the trout (*Crepidostomum*, *Creptotrema*, *Deropegus* spp.) and from the eggs of a blood fluke (*Sanguinicola* sp.) recovered from gill capillaries (Pusterla et al. 2000b). Neorickettsial DNA was also recovered from unparasitized fish tissues, which may have represented either neorickettsial infection of host blood/tissue or infection by undetected blood fluke eggs. Sequences of the amplicons of 16S rRNA gene obtained from fish tissue and flukes were identical to each other and very close to the sequences of members of *Neorickettsia*. At the same time, they were distinct enough from *N. risticii*, *N. sennetsu,* SF agent and *N. helminthoeca* (95-96% sequence homology) to suggest that the "rainbow trout agent" is a previously unrecognized genotype of *Neorickettsia*. Nothing is known about its pathogenicity in fish or in vertebrates that eat fish.

***Neorickettsia* sp. From Needlefish in Cambodia**

Seng et al (2009) studied frozen fish and fish-based ingredients imported from Thailand, Vietnam and Cambodia to Asian markets in Marseille, France. They examined fish digestive tracts for parasitic worms and subsequently extracted DNA from digestive tracts. For detection of Anaplasmataceae they used PCR with primers specific for the 16S rRNA and citrate synthase genes of this group of bacteria. Three different forms of Anaplasmataceae were determined in the fish digestive tracts. Based on the level of the sequence homology, the authors suggested that two of them represented new genera

within the Anaplasmataceae while the third represented a new species of *Neorickettsia*. To define specific and generic levels of sequence divergence in the Anaplasmataceae, the authors chose a threshold of 97% identity among homologous 16S rRNA gene sequences generated from each isolate. In a phylogenetic tree of the Anaplasmataceae, the three genotypes all clustered together with *Neorickettsia*. The two new genera were basal to all *Neorickettsia* while the third belonged to the clade containing the "rainbow trout agent" (Figure 2). The new *Neorickettsia* sp. was found in a freshwater needlefish *Xenentodon cancila* from Cambodia. No digeneans were found in the needlefish, thus, the potential circulatory pathway of these neorickettsiae remain unknown.

Neorickettsia sp. From Antarctic Notothenioid Fishes

Ward et al. (2009) studied the intestinal microbiota of two Antarctic notothenioid fish species. They analyzed bacterial 16S rRNA gene sequences obtained from the intestinal tract of *Notothenia coriiceps* and *Chaenocephalus aseratus*, which differ in their pelagic distribution and feeding strategies. Interestingly, both samples revealed two novel genotypes of *Neorickettsia*. Ward et al. (2009) found that their sequences were only 93% similar to “Rainbow trout agent” and within their phylogeny (based on neighbor joining) the sequences formed a monophyletic cluster within the dendrogram of other *Ehrlichia*/*Neorickettsia* spp., leading them to suggest that they may represent organisms unique to Antarctic notothenioid fish. No digeneans were found/looked for in the notothenioid fishes, thus, the potential circulatory pathway of these neorickettsiae remain unknown.

Catfish Agents

Tkach et al. (2012) isolated two genotypes of *Neorickettsia* from different digeneans parasitic in catfishes in the USA. These genotypes showed high sequence homology (95-98%) with members of *Neorickettsia* and belonged to two different clades within the genus (Figure 2). One of them was obtained from a gorgoderid digenean, *Phyllodistomum lacustri* found in the bladder of a stonecat catfish (*Noturus flavus*) and tentatively called catfish agent 1. The other was obtained from an allocreadiid digenean, *Megalogonia ictaluri* found in the intestine of channel catfish (*Ictalurus punctatus*) and tentatively called catfish agent 2. The former genotype seems closest to *Neorickettsia helminthoeca* while the latter clustered with the published sequence of the "rainbow trout agent" (Figure 2). These findings, combined with discoveries of the "Elokomin fluke fever agent" and "rainbow trout agent" suggest that a relatively diverse *Neorickettsia* species/genotype complex exists in digenean parasites of North American freshwater fish.

These forms deserve more detailed studies, especially in view of the fact that the catfish agent 2 was recovered from a digenean species parasitic in the channel catfish — an important fish species that is raised commercially and packaged for sale and consumption throughout the United States and elsewhere. Worldwide, the channel catfish production has increased steadily and has reached well over 450 thousand tons per year according to FAO fishery statistics http://www.fao.org/fishery/culturedspecies/Ictalurus_punctatus/en). Therefore, while the public health importance of this and other poorly characterized *Neorickettsia* genotypes is

unclear, these discoveries nevertheless indicate that, even in the United States, neorickettsiae occur dangerously close to the human food chain.

Geographic Distribution of *Neorickettsia*

There are reports of confirmed or probable neorickettsial infections from all continents, with *N. risticii* being by far the most widely distributed and most frequently reported species of *Neorickettsia* (Dumler et al. 2005). However, the majority of these reports including all information available from Europe, Africa, Australia, as well as some Asian (e.g., India) and South American (e.g. Venezuela) countries is based exclusively on serological testing of horses without further evidence from either PCR/sequencing or culturing the bacteria. In most cases the vaccination or relocation histories of serologically positive horses were not known or reported. Clearly, serological tests have limitations and depend on previous exposure whether from natural infections or vaccinations. Madigan et al. (1995) provided convincing evidence of high rate of false-positive results when the most common serological diagnostic tool, the indirect fluorescent antibody (IFA) test, was used to diagnose PHF in California. Later, Mott et al. (1997) compared relative sensitivity and usability of IFA versus nested PCR while concurrently culturing bacteria the majority of samples. At the time, horses with serologic evidence of prior exposure to PHF were found throughout the U.S., Canada and Europe, and seroepidemiological studies reported high seroprevalences of horses that lacked clinical symptoms of PHF. However, culture of *N. risticii* was rarely done and most of diagnoses were based on IFA testing. Mott at al. (1997) emphasized that IFA testing at a single time point is useless as a surveillance tool for detecting PHF in vaccinated horses and expressed the same concern as Madigan et al. (1995) regarding the reliability of

reports based on serological evidence alone. Mott et al. (1997) concluded that the PCR test was as accurate and sensitive as culturing and has an advantage over the IFA test because it is independent of the past vaccination history of a horse. Therefore, in this section and on the map on the Figure 5, we consider only data resulting from application of PCR/sequencing techniques or culturing of the bacteria. Exceptionally, we consider a few cases when sufficient vaccination/relocation histories were reported for individual serologically positive horses.

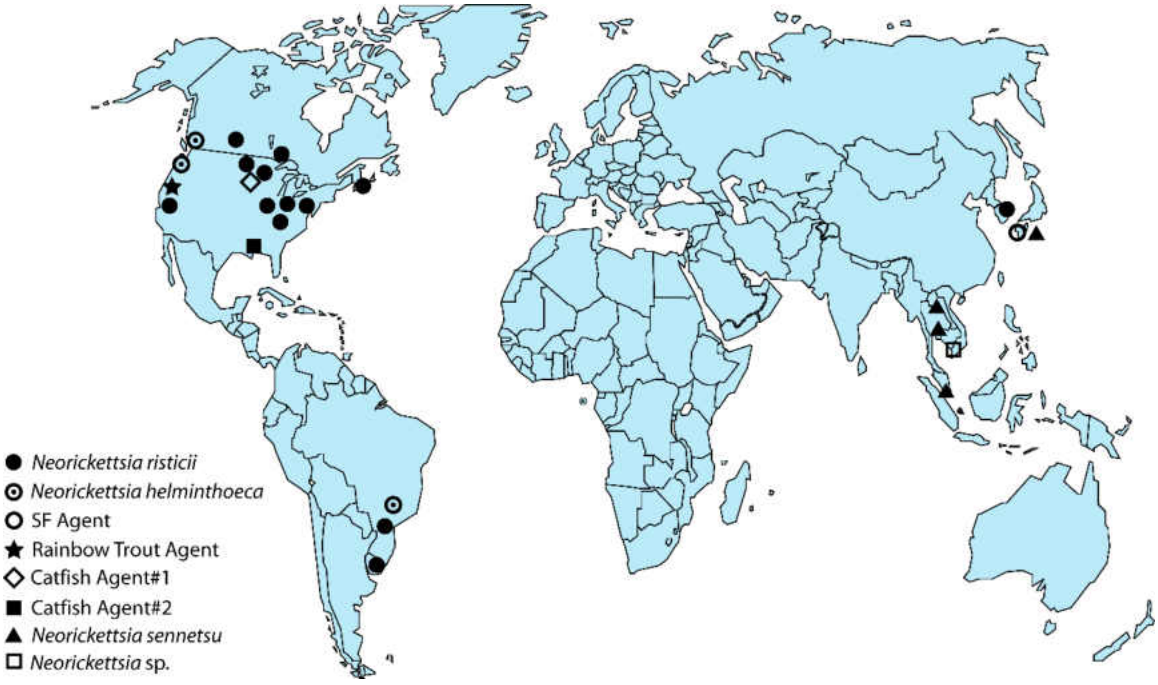

Figure 5. Geographic distribution of known species and genotypes of *Neorickettsia*. Records based solely on serological diagnostics are not included.

Even after applying the stricter criteria outlined above, *N. risticii* remains the most widely distributed species/genotype of *Neorickettsia* (Figure 5). It is broadly distributed throughout North America and was recently reported from Brazil, and Uruguay (Figure 5). Korean researchers (Chae et al. 2003; Park et al. 2003) also reported *N. risticii* from larval stages of several digenean species in the Republic of Korea. However, the

relatively high level of 16S sequence divergence between Korean isolates and those from the United States suggests that the species identity of the Korean genotype should be considered with some caution (see discussion in section 2.2 of this review). In any case, one or another *Neorickettsia* species is certainly distributed in the Republic of Korea.

Another species of *Neorickettsia* that seems to be rather broadly distributed is *N. helminthoeca* that was long known to cause dog disease in the Pacific northwest of the USA, but then was found in other parts of the western coast of North America, from British Columbia to California (Booth et al. 1984; Sykes et al. 2010) as well as in Brazil (Headley et al. 2004, 2009). The third named *Neorickettsia* species, *N. sennetsu*, is the most widely distributed member of the genus in Asia and was found so far in Japan, Malaysia, Laos and Thailand. SF agent has been isolated only from digeneans in Japan, however, it was also isolated from the spleen of a dog fed with wild caught trout in Oregon. All remaining 4 *Neorickettsia* genotypes have very limited distribution and have been found in a single geographical area each (Figure 5), either in North America, Japan or Southeast Asia. Invariably these genotypes have been discovered as a by-product of studies targeting pathogenic species.

Thus, the known confirmed distribution of *Neorickettsia* is very uneven from the geographical viewpoint. If we use the conservative approach outlined above, well documented information on *Neorickettsia* is lacking from Africa, Australia, Europe, most of South America, most of Asia and nearly all island countries. In our opinion, this patchy distribution reflects a lack of studies and insufficient knowledge rather than the true absence of *Neorickettsia* from most regions of the planet. As far as we are aware there were no studies focusing on finding *Neorickettsia* anywhere in Africa or Australia.

At the same time, already proven *Neorickettsia* symbiosis in representatives of all major phylogenetic lineages of the Digenea suggests that these bacteria may likely be widely distributed geographically. It is probably not accidental that the species with widest distribution are those of significant veterinary or medical importance. It reflects the fact that much more resources have been put into studies of these species. An additional important factor might be the high mobility of humans and high frequency of relocation of domestic animals that could facilitate the spread of some of the *Neorickettsia* genotypes. Available evidence suggests that PHF was almost certainly historically absent from North America. Considering the vital importance of horses to the economy and culture of early settlement of North America, it seems strange that PHF had never before been diagnosed as a recognizable disease prior to 1970. It would be difficult to expect that a disease with visible clinical symptoms would have gone completely unnoticed for centuries. As it turns out, *N. risticii* has been identified as the causative agent for "churrido equino" – a diarrheic disease of horses known for over 100 years in the Lake Merin region of Uruguay and Brazil (Dutra et al. 2001; Coimbra et al. 2005). We hypothesize that PHF might have been introduced to North America from South America relatively recently. Comparative phylogenetic studies of a greater number of samples from as broad geographic area as possible, optimally using sequences of several genes, may provide some insight into this interesting question and either confirm or refute this hypothesis.

Obviously, there are natural causes of differences in the distribution of various *Neorickettsia* species/genotypes. For instance, the most widely spread species, *N. risticii*, is the only one found so far in flying migratory vertebrate animals (birds and bats) which

may have an impact on the distribution of their digeneans and digenean endosymbionts. The most obvious mechanism limiting the geographic distribution of the majority of *Neorickettsia* genotypes is related to the specificities of the bacteria to their fluke hosts on the one hand, and of flukes to their intermediate and definitive hosts, on the other. Most digeneans are rather tightly ecologically and evolutionarily associated with their mollusk hosts as well as other host categories (Yamaguti 1975; Cribb et al. 2001, 2003). It means that even if one of the hosts in the digenean complex life cycle has a limited distribution, it will limit the distribution of the fluke species. *Nanophyetus salmincola* is a good example of such dependency due to its high specificity to a mollusk host with a limited geographic distribution.

As studies of neorickettsiae are undertaken in new regions, we anticipate that the known geographic distribution of these endosymbionts will expand significantly.

Phylogenetic Associations Between *Neorickettsia* and Digenea

Neorickettsiae are widely distributed among digeneans. In fact, one or another species or genotype of these endosymbionts is found in representatives of all major lineages of the Digenea (Figure 6). Some *Neorickettsia* species seem to have a narrow host range, restricted to within a single digenean family (*e.g*., salmon poisoning, EEF agent and SF agent, see Figure 6). Other species of *Neorickettsia* have been isolated from phylogenetically distant digenean families (*e.g*., rainbow trout agent, see Figure 6). *Neorickettsia risticii* displays the broadest digenean host utilization, with its DNA having been isolated from an astonishing diversity of digeneans including a range of families from one of the most basal digenean lineages (Schistosomatidae and Strigeidae) to the

most advanced lineages (Lecithodendriidae and Microphallidae). This indicates that neorickettsiae have had a long evolutionary history with Digenea and suggests that

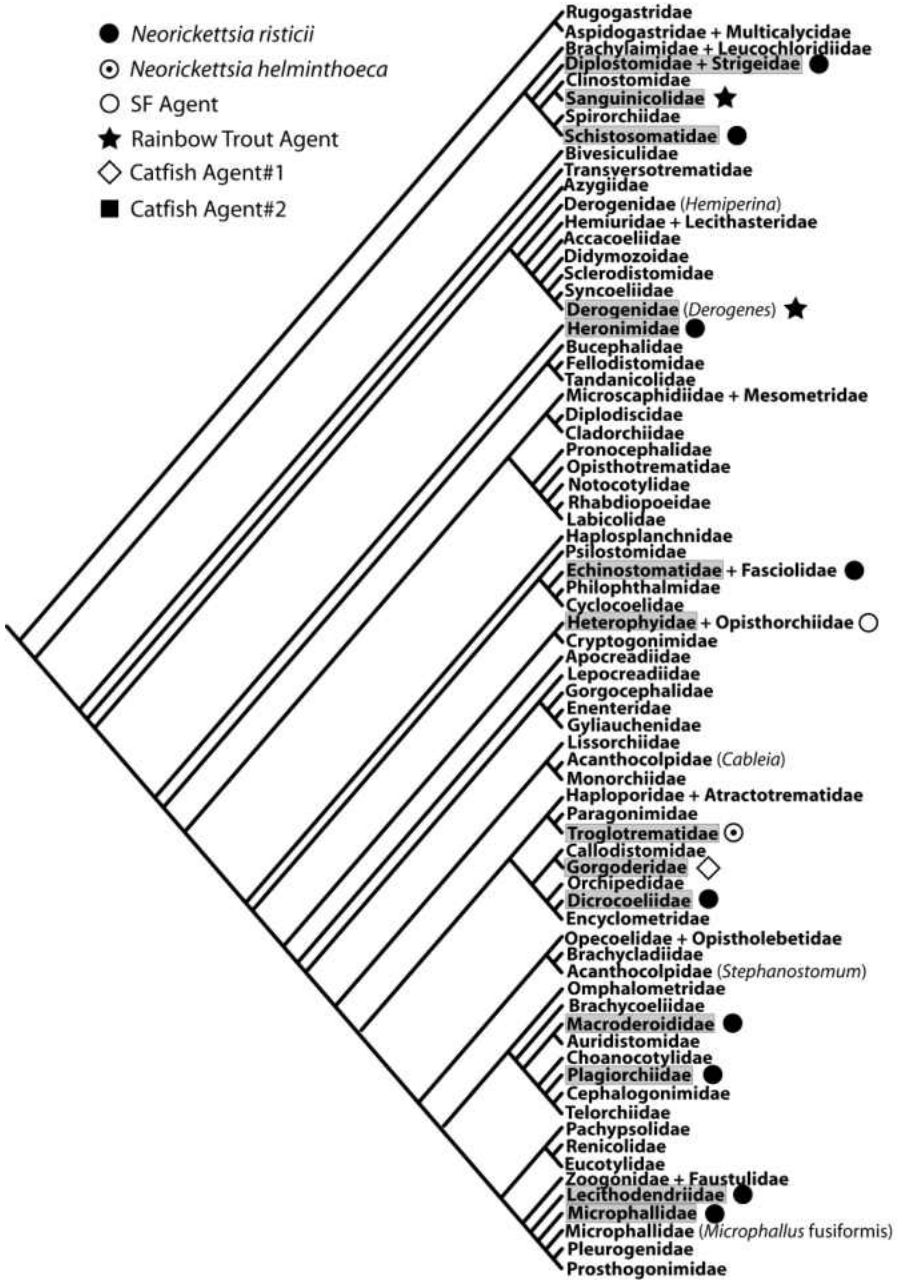

Figure 6. *Neorickettsia* species/genotype records mapped on the phylogenetic tree of the digenean families from Olson et al. (2003).

neorickettsiae may be potentially found in many additional groups of Digenea. At the same time, there is no evidence of clear co-evolutionary relationship between certain

digenean lineages and their bacterial endosymbionts. For example, identical sequences of 16S rRNA have been obtained from widely divergent digenean taxa, however, it is important to note that these are relatively short sequences in a conserved region of the 16S rRNA. Conversely, different genotypes producing different pathologies in dogs and bears, i.e., *N. helminthoeca* and EFF agent, share the same digenean host species, *N. salmincola*.

The exact nature of the endosymbiotic relationship among neorickettsiae and their digenean hosts at physiological level is currently unclear. It is likely not mutualistic because there is no clear dependency of digeneans on these endosymbionts. Some individuals within the same digenean species may be infected with neorickettsiae whereas other individuals are not. This situation is different, for instance, from the mutualistic relationships among *Wolbachia* and certain species of filariid nematodes that demonstrate a clear dependency on their endosymbionts (Taylor et al. 2005; Casiraghi et al. 2005; Ferri et al. 2011; Fischer et al. 2011).

Study Objectives and Gaps in Current Knowledge

Objective 1: Screen for *Neorickettsia* DNA a large collection(s) of diverse Digenea taxa from a wide variety of hosts and a broad geographic range using molecular methods and conduct molecular phylogenetic analyses of neorickettsiae in order to estimate interrelationships among all available genotypes.

It has been more than 50 years since the discovery that digeneans harbor bacterial endosymbionts which cause disease in humans and dogs. And yet, it is safe to say that even now the studies of neorickettsial diversity are in their infancy. As demonstrated above, only a small fraction of digenean taxa has ever been examined for the presence of

Neorickettsia. Screening a broad diversity of digeneans around the world will likely reveal numerous new *Neorickettsia*-digenean host associations and very probably new species of *Neorickettsia*. Numerous, in some cases very extensive, collections of digenean DNA obtained for systematic, ecological, genomic and diagnostic purposes around the world represent a great underutilized resource for studies of *Neorickettsia*. These collections can be screened for presence of neorickettsial DNA, dramatically enhance our knowledge of these endosymbionts and provide new insights into evolutionary relationships between neorickettsiae and their digenean and vertebrate hosts.

It is also certain that the current knowledge of *Neorickettsia* geographic distribution is highly incomplete and does not accurately portray the real picture. Lack of information on *Neorickettsia* from Africa, Australia, most of Eurasia and majority of large islands reflects insufficient sampling rather than true absence of *Neorickettsia* in these continents and geographical regions.

Objective 2: Develop and maintain a laboratory life cycle of a digenean, *Plagiorchis elegans*, harboring *Neorickettsia* sp..

The Digenea (Platyhelminthes: Trematoda) are an overwhelmingly diverse group of internal metazoan parasites, with roughly 18,000 nominal species (Cribb et al. 2001). They have complex life cycles, using gastropods and other mollusk groups as first intermediate hosts, several phyla (mostly Arthropoda, but also Annelida, Vertebrata, etc.) as second intermediate hosts and all major vertebrate groups as definitive hosts. Due mainly to their highly complex life cycles, the cultivation of digeneans in the laboratory is difficult. At the same time, laboratory life cycles of digeneans have many uses, and in

case of *Neorickettsia* the laboratory life cycle can help answer questions about the transmission biology of this unique genus of bacteria.

Objective 3: Assess and quantify *Neorickettsia* vertical transmission efficiency through all stages of a digenean life cycle.

Probably the largest gap in our knowledge of *Neorickettsia* biology is the very limited information available regarding the quantitative aspects of their perpetuation during the complex digenean life cycles. To persist, the endosymbionts must survive several developmental processes in which their digenean hosts undergo during a single life cycle. To proliferate and prosper, it seems reasonable to assume that the endosymbiont must undergo replication during those times at which its digenean host is also undergoing reproduction, asexual in the mollusk and sexual in the vertebrate definitive host. But the efficiency to which bacterial reproduction keeps pace with that of its host is not known. Determining the prevalence of *Neorickettsia* in different stages of digenean life cycles, from eggs to cercariae, metacercariae and adult worms is a logical starting point for understanding this aspect of *Neorickettsia* biology.

Objective 4: Localize the bacterial endosymbiont within all stages of the digenean life cycle using immunofluorescent microscopy.

The localization of *Neorickettsia* in the vertebrate definitive hosts tissue is well known. A number of studies (Rikihisa et al. 1985 and 1988; Wen et al. 1996; Pusterla et al. 2000; Chae et al. 2002; Headley et al. 2009) have used techniques such as in-situ hybridization, immunofluorescent microscopy and transmission electron microscopy to localize *Neorickettsia* in macrophages and other tissues such as the spleen, intestine and lymph nodes. However, there has been no study identifying the location of the bacteria

within a digenean host. As mentioned above, *N. helminthoeca* was successfully transmitted by injecting dogs with fluke eggs homogenized in glass tissue grinder, but not with intact eggs, indicating that infectious neorickettsiae are contained within the interior of the unembryonated *N. salmincola* eggs but not on the exterior surfaces of the egg shells (Nyberg et al. 1967). This lead to the conclusion that *N. helminthoeca* is transmitted transovarially to successive generations of digeneans. However, there could be other explanations for how the bacteria infiltrates the egg that need to be explored. At the same time, we do not know where the bacteria could be in other life cycle stages or even in the adults. Therefore, to fully understand the transmission and biology of *Neorickettsia* in its digenean host, the localization of the bacteria is an important step.

CHAPTER II

LABORATORY LIFE CYCLE AND MATERIALS AND METHODS

Introduction

Bacteria in the genus *Neorickettsia* are intracellular endosymbionts of digeneans. The Digenea (Platyhelminthes: Trematoda) are an overwhelmingly diverse group of internal metazoan parasites, with roughly 18,000 nominal species (Cribb et al. 2001). They have complex life cycles, using gastropods and other mollusk classes as first intermediate hosts, several phyla (mainly arthropods) as second intermediate hosts and all major vertebrate groups as definitive hosts. Because of their highly complex life cycles, cultivation of digeneans in the laboratory also requires cultivation of gastropods and other appropriate host species which creates additional difficulties (Smyth and Halton 1983). Nevertheless, laboratory cultivation of digeneans allowed for major contributions in our knowledge of these parasites, especially species of medical and veterinary importance (Smyth and Halton 1983; Ndiaye et al. 2013; Greani et al. 2014; Mwangi et al. 2014). They proved to be particularly important immunological, biochemical, developmental and physiological studies (Smyth and Halton 1983).

Symbiosis of *Neorickettsia* with digeneans provides an avenue for yet another use for the digenean laboratory life cycle. Laboratory life cycles of digeneans infected with *Neorickettsia* could provide a controlled environment to study the transmission biology

of the bacterial endosymbiont with its digenean host, something that is difficult to do using only natural infections. Currently, the majority of research on *Neorickettsia* has focused on the molecular biology, immunology, diagnostics and treatment of neorickettsiae and neorickettsial diseases, while fundamental aspects of the transmission biology of neorickettsiae within their digenean hosts has been little studied. This project established and maintained for the first time *in vivo* culture of *Neorickettsia* throughout all stages of a digenean, *Plagiorchis elegans*, lifecycle in the laboratory. The laboratory setup and the techniques employed are given below.

In 2012, neorickettsial DNA was detected in field isolates of *Plagiorchis elegans*, a plagiorchiid digenean that infects local pond snails (Lymnaeidae). Originally reported to be *N. risticii* (Greiman et al. 2013) and doubtless very closely related to that species, the neorickettsial strain within *P. elegans* has since been characterized as genetically unique from *N. risticii* based on phylogenetic analysis of a 1400 bp fragment of the 16s rRNA gene (Chapter 3). *Plagiorchis elegans* is a generalist parasite, capable of utilizing multiple groups of aquatic arthropods as second intermediate hosts (*e.g*., *Chironomus* sp., *Gammarus pulex*, *Asellus aquaticus*, *Culex* spp.)(Gorman 1980)-and a wide range of vertebrate species as definitive hosts (*e.g*., LACA mice, rats, hamsters, gerbils, chicks, ducklings and pigeons) (Gorman 1980). This makes *P. elegans* a highly suitable candidate for establishing a laboratory life cycle.

In addition to the laboratory life cycle of a digenean harboring *Neorickettsia*, new DNA extraction methods, PCR protocols and primers, real-time PCR protocols and primers, and quantitative real-time PCR (qPCR) protocols were developed to study the transmission biology of this complex genus of endosymbiotic bacteria. This chapter

focuses on materials and methods used throughout my dissertation work including field collections (snail and definitive host collections), molecular techniques, immuno-histochemistry, and the development of a laboratory life cycle of a digenean, *P. elegans*, harboring *Neorickettsia* sp.

Materials and Methods

General Materials and Methods

Snail collection. In order to study the transmission biology of *Neorickettsia* within the digenean host a local source of *Neorickettsia* infected digeneans had to be identified. To accomplish this a survey of small freshwater ponds in Nelson County and Towner County, North Dakota, was undertaken during the summers and falls of 2011–2014. Approximately 40 ponds were surveyed for aquatic snails.

Snails were collected by hand, or by net, and placed into a container for transport to the lab. Snails were rinsed with water and placed individually into glass jars filled with aged tap water treated with commercial aquarium conditioner to remove chlorine/chloramines. Snails were identified using Burch's "North American Freshwater Snails" (1989).

Screening of snails for digenean infections. Glass jars containing snails were kept for several hours under fluorescent lamps followed by several hours without light. Afterwards, the water in jars was examined for the presence of cercariae using a stereomicroscope. Snails shedding cercariae were maintained individually in labeled containers (both snail # and type of cercariae) and fed with lettuce. Cercariae were identified to type using Schell's "Handbook of Trematodes of North America North of Mexico" (1985).

Collection of larval digeneans for DNA extraction. Using a stereomicroscope, cercariae were obtained with sterile pipettes. Pools of 40–50 cercariae were collected and placed into 1.75ml microcentrifuge tubes. Microcentrifuge tubes with cercariae were centrifuged at 13,000 rpm for 5 minutes. Supernatant was removed and 60 µl of ultrapure water added.

For the majority of snails shedding cercariae, a subsample of cercariae (approximately 300–500) was added to a 2ml vial with 90% EtOH. This was done to ensure we had a source of DNA if the original DNA extract failed, the snail died prematurely, or the cercariae were positive for *Neorickettsia*.

If a snail died prematurely, it was placed in a Petri dish and dissected under a stereomicroscope to collect sporocysts. Sporocysts were fixed in either 80% EtOH or 4% buffered formalin.

DNA extraction by direct sonication. Cercariae were disrupted by direct sonication using a UP100H compact ultrasonic processor (Hielscher USA, Inc., Ringwood, New Jersey) at an amplitude of 90% for 20 seconds, and immediately placed on ice to prevent DNA degradation due to enzymatic activity. Sonicates were used directly as a template for PCR amplification procedures. DNA was extracted from the remainder of each homogenate using the guanidine thiocyanate method (Tkach and Pawlowski 1999).

Molecular screening for *Neorickettsia*. *DNA* extracts were first tested for the presence of *Neorickettsia* using a real-time PCR protocol designed and extensively tested by myself. Five microliters of each DNA extract were used. The real-time PCR amplified a 152-bp portion of the 3' end of the heat shock protein coding gene, GroEL. The primer

pair used is listed in Table 3. Samples that tested positive with real-time PCR were verified using a substantially modified nested PCR protocol initially described by Barlough et al. (1997). Five microliters of each DNA extract were used for the first PCR reaction and 1 µl of the first PCR product was used for the nested PCR. A 1470 bp long fragment of the 16S rRNA gene was first amplified using the primer pair listed in Table 3. The nested PCR step amplified a 1371-bp fragment using internal primers also listed in Table 3. The same nested PCR primers were used in sequencing reactions along with internal forward and internal reverse primers (Table 3).

The real-time PCR reactions were run on a Bio-Rad CFX96 Touch real-time PCR detection system (Bio-Rad Laboratories, Hercules, California) using iTaq universal SYBR Green supermix (Bio-Rad Laboratories, Hercules, California) according to the manufacturer's instructions. A two-step program was used with a denaturation temperature of 95°C for 3 seconds, followed by an annealing and extension temperature of 64°C for 25 seconds and 36 cycles. In addition a melt curve was run starting from 72°C and increasing at 0.2°C increments every 5 seconds until reaching 87°C. The nested PCR reactions were run on EP Gradient thermocycler (Eppendorf, Hauppauge, New York) using Quick load OneTaq mastermix (New England Biolabs, Ipswich, Massachusetts) according to the manufacturer's instructions. Annealing temperature of 58^{o}C and 40 cycles were used in both first and nested PCRs. DNA of *N. sennetsu* used as a positive control was graciously provided by Dr. Sabine Dittrich (Lao Oxford Mahosot Welcome Trust Research Unit). Pure water was used for negative controls in both real-time and nested PCRs.

Nested PCR product visualization. PCR products were visualized using 1.5% agarose gels. Two microliters of loading dye was mixed with 5 µl of PCR product, and loaded into a 1.5% agarose gel. The gel was run at 96v for 50–60 minutes, and then stained with ethidium bromide for 10 minutes. The gel was then visualized on a UV light table, and photographed.

Table 3. PCR Primers used in the study

Reaction type	**Primer**	**Sequence (5'–3')**
Real-time	groel-1500F	ATAGATCCAGCKAAGGTAGTGCGTGT
	groel-1620R	TTCCACCCATGCCACCACCAGGCATCATTG
1st round PCR (*Neorickettsia*)	n16S-25F	TCAGAACGAACGCTAGCGGT
	n16S-1500R	AAAGGAGGTAATCCAGCCGCAGGTTCAC
Nested PCR/sequencing (*Neorickettsia*)	n16s-50F	TAGGCTTAACACATGCAAGTCGAACG
	n16S-1400R	CGGTTAGCTCACTAGCTTCGAGTAA
Sequencing (*Neorickettsia*)	16S-n900F	GACTCGCACAAGCGGTGGAGTAT
	16S-n900R	ATACTCCACCGCTTGTGCGAGTC
PCR/sequencing (digenean)	digl2	AAGCATATCACTAAGCGG
	1500R	GCTATCCTGAGGGAAACTTCG
Sequencing (digenean)	300F	CAAGTACCGTGAGGGAAAGTTG
	900F	CCGTCTTGAAACACGGACCAAG
	300R	CAACTTTCCCTCACGGTACTTG
	ECD2	CTTGGTCCGTGTTTCAAGACGGG

Identification of digenean hosts. Digenean cercariae were identified to the lowest possible taxonomic level using partial sequences of the nuclear large ribosomal subunit gene (28S). Digenean DNA was amplified from the extracted DNA by PCR using the primer pair listed in (Table 3). The same PCR primers and additional internal primers were used for sequencing (Table 3).

DNA sequencing. PCR amplicons of both *Neorickettsia* and digeneans were purified using the Zymo DNA Clean & Concentrator™ -5(Zymo Research, Irvine, California) or ExoSap PCR clean-up enzymatic kit from Affimetrix (Santa Clara, California) according to the manufacturer's instructions. The PCR products were cycle-sequenced using ABI BigDye™ chemistry, ethanol precipitated, and run on an ABI Prism 3100™ automated capillary sequencer. Contiguous sequences of *Neorickettsia* and digeneans were assembled using Sequencher™ ver. 4.2 (GeneCodes Corp., Ann Arbor, Michigan) and submitted to GenBank.

Laboratory Life Cycle of a *Neorickettsia* Infected Digenean, *Plagiorchis elegans*

Ethics statement. The use of vertebrate animals was approved by the University of North Dakota Institutional Animal Care and Use Committee (protocol 1011–1c).

Snail collection. Aquatic snails, *L. stagnalis*, infected with *Neorickettsia* positive digeneans, *P. elegans*, were collected during the summer of 2011 from Pond 1 (see results). Assessment of *Neorickettsia* infection was done using the above mentioned screening methods outlined in the "General Materials and Methods" section.

Snail intermediate host cultures. Twenty to thirty uninfected locally collected *Lymnaea stagnalis* were used to establish a laboratory colony of snail first intermediate hosts. Adult snails were placed in large plastic containers with three to four inches of aged tap water conditioned with commercial aquarium conditioner to remove chlorine/chloramines. Egg sacs were periodically removed and placed into smaller containers for easier care when hatched. Once hatched, young snails were allowed to develop to approximately 1.5–2 cm in length before being used for infection with *P. elegans* eggs. For general rearing, snails were maintained on leaf lettuce, feces were

removed daily, and every several days ca. 1/3 of the water was removed and replaced with fresh water.

Mosquito intermediate host cultures. *Culex pipiens* mosquito stocks originated from wild populations captured in Larimer County, Colorado. Larvae were reared in flat polyurethane pens containing ca. 500 ml of aged tap water supplied daily with a small amount of commercial fish food (TetraPond®) ground to a fine powder using a coffee grinder. Upon pupation, the pupae were transferred to aluminum screen cages to emerge as adults. One to two weeks after eclosion, female mosquitoes were allowed to blood feed on an anesthetized rodent. Two to three days later, cages were supplied with a container of aged tap water for gravid females to oviposit. Egg rafts were transferred to larval rearing pens to begin the cycle anew.

Establishment of laboratory life cycle of *P. elegans* infected with *Neorickettsia* sp. The life cycle of *P. elegans* harboring *N.* sp. was initially established as follows (Figure 7). Approximately 20–40 *Culex* larvae (3rd and 4th instar) were exposed for 2 to 3 hours to approximately 500 *Neorickettsia* sp. positive *P. elegans* cercariae shed from a naturally infected *Lymnaea stagnalis* snail. Cerceriae penetrated and encysted in the *Culex* larvae forming metacercariae. To ensure that metacercariae had formed within the mosquito host, one to three *Culex* larvae were flattened under a cover slip and visualized for metacercariae under a compound microscope.

Plagiorchis elegans metacercariae, in some cases, can become infective to the definitive host after 48 hours (Gorman 1980). To ensure that metacercariae were maximally infective we waited 5 days before feeding metacercariae-infected mosquito larvae to the definitive host, a Syrian hamster (*Mesocricetus auratus*). Hamsters were

anesthetized lightly with isoflurane and fed with 15–20 *Culex* larvae. Hamsters were maintained in individual cages on standard rodent diet (Purina LabDiet®) and the bedding was replenished twice a week.

To monitor infection success, wet mounts of hamster feces were periodically examined under a compound microscope for digenean eggs. Eggs were first found within the feces 7 days post-infection (PI) and were still present after 29 days PI. After 11–29 days PI, hamsters were anesthetized with isoflurane and euthanized by cervical dislocation. The small intestines were removed, placed flat in a Petri dish with saline, teased apart with forceps, and examined for adult *P. elegans* under a stereomicroscope.

Adult worms were removed with a glass pipette and placed in Petri dishes containing sterile saline. From each dish, ten adult worms were selected and placed individually in separate Petri dishes containing heat-sterilized aquarium water. Individual worms were then teased apart with fine needles to remove mature eggs from the distal portion of the digenean uterus. To determine if each of these parent "egg donors" actually harbored *Neorickettsia*, the remainder of each worm was then homogenized and screened for *N. risticii* using real-time PCR as described above. The eggs were evenly distributed around the dish using a clean glass pipette, and left to incubate for 7 days at room temperature. To minimize excessive microbial growth during the weeklong incubation, approximately a third of the water was carefully removed every day with a clean pipette and replenished with fresh water. It has been shown previously that an incubation period of 7 days is sufficient to allow *P. elegans* miracidia to develop and become infective to lymnaeid snails (Gorman 1980). To confirm this, periodically a single egg was pipetted onto a slide

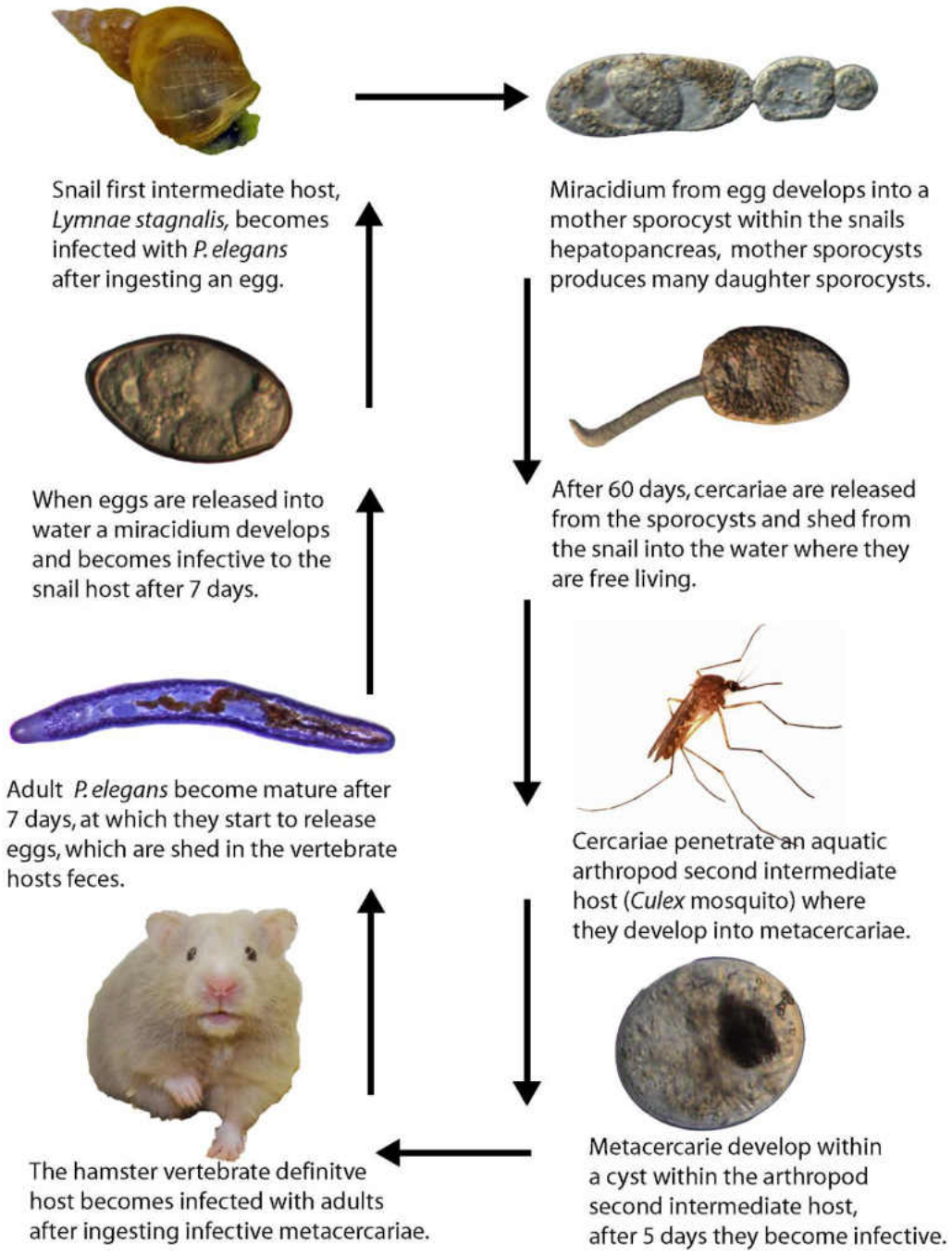

Figure 7. Laboratory life cycle of *Plagiorchis elegans* harboring *Neorickettsia* sp.

with water, cover slip added, and visualized under a compound microscope to monitor the embryonic development of the miracidium. After 7 days, groups of 10 uninfected snails were placed into each of the Petri dishes containing embryonated eggs and allowed

to graze along the bottom for ca. 1 hour. Afterwards, snails were removed, placed in separate labeled containers with aerated water at 20–22°C and maintained as described above. Fifty to 60 days after infection, surviving snails began to release cercariae. Newly shed cercariae were collected and screened for *Neorickettsia* using real-time PCR as described above. Snails shedding *Neorickettsia* positive cercariae were placed into containers with uninfected mosquito larvae in order to produce *Neorickettsia* positive metacercariae and thus repeat the lifecycle.

Chapter 3 Specific Materials and Methods

Ethics statement. Digeneans were collected from marine and freshwater fishes, amphibians, reptiles, birds, mammals, and invertebrates from 2008–2014 from multiple localities in Argentina, Australia, China, Costa Rica, Egypt, Laos, Philippines, Thailand, Vietnam, and several states in the USA (Mississippi, Louisiana, Florida, Oregon, North Dakota, Minnesota). An IACUC protocol 10100105 was issued by the University of Southern Mississippi for collecting and humane euthanasia of wild animals in Mississippi, Florida and Louisiana. Birds were collected by shotgun in accordance to the state and federal permits, raccoons were live trapped and euthanized in the field by firearm (.22 caliber rifle) in accordance to issued permits, and amphibians and reptiles were collected by hand and humanly euthanized by immersion in a solution of chlorobutanol (chloretone) in water in accordance with the issued permits and IACUC protocol. IACUC protocols were not required for invertebrate collecting in Oregon, North Dakota, and Minnesota or for fish (in Argentina, Costa Rica, Australia, Vietnam, Thailand and China. Fish were purchased dead from food markets or caught by cast net (Australia). When fish were caught by cast net they were placed on ice after capture, and

euthanized using of a 250 mg/l bath of Tricaine methane sulfonate (MS-222) in accordance with the issued permits.

Sample collections. All digenean samples were collected over a period of 7 years, as parts of several independent projects dealing with parasite biodiversity and systematics. Thus the source of DNA available for *Neorickettsia* screening was opportunistic, but as inclusive as possible. Required scientific collecting permits were obtained in all cases. The Florida Fish and Wildlife Conservation Commission issued the permits LSSC-11-00074 and FNW-13-05(renewal) that allowed for the collection of invertebrates, amphibians, reptiles, and mammals, as well as freshwater fishes in Florida. The Mississippi Department of Wildlife Fisheries and Parks issued a permit 0123131 for the collection of invertebrates, amphibians, reptiles, mammals, and freshwater and marine fishes in Mississippi, and the Louisiana Wildlife and Fisheries issued a permit LNHP-13-017, also for the collection of the above mentioned animals in Louisiana. Additionally, a federal permit (MB681207-0) was issued by the US Fish and wildlife service for collection of reptiles, mammals, birds and freshwater fish in Mississippi and Louisiana. Three separate permits were issued in Australia for the collection of fish; Western Australia (Government of Western Australia, Department of Fisheries, SPA-01-10); Northern Territory (Northern Territory Government, Department of Resources, S17/2932); and Queensland (Department of Primary Industries and Fisheries 133621). Collections in Laos were done under a permit held by a colleague, Dr. Paul Newton (Lao-Oxford-Mahosot-Hospital-Wellcome Trust Research Unit, Mahosot Hospital, Vientiane, Laos). Collections in Vietnam were done under permits held by a colleague, Tran Thi Binh (Department of Parasitology, Institute of Ecology and Biological Resources,

Vietnam Academy of Science and Technology). Only abundant commercial fish species were purchased from local markets and fishermen in China, Vietnam, Thailand, Laos, Argentina and Costa Rica, and therefore, permits were not required. Additionally, aquatic snails were collected from North Dakota, Minnesota, and Oregon. Snails were identified using Burch's (1989), and also did not require permits. We do not provide a complete list of screened digenean species because in the case of *Neorickettsia* a negative result does not necessarily mean that a certain species of digenean cannot be a host for *Neorickettsia*. As discussed by Tkach et al. (2012) digeneans do not have any known co-dependency with their *Neorickettsia* endosymbionts and therefore, different individuals of the same digenean species may or may not be infected.

Snail crushing. Snails collected in Vietnam, Laos and Thailand were brought back to a lab (appropriate labs in each country) and sorted by species. Either 5 snails (for smaller species) or 2 snails (for larger species) of a species were placed into small dishes with a small amount of filtered/distilled water and left to shed cercariae over night (Figure 8). Dishes with snails were examined under a stereomicroscope for cercariae. If cercariae were found then all snails within the dish were crushed using wire cutters to identify the snail(s) infected with digenean larvae. For snails found to be infected a subsample of sporocysts and cercariae was taken using a 200 µl pipette and sterile tips for each sample, and transferred into a vial with 90% EtOH. Snails collected in the Philippines were brought back to the camp and crushed right away using the above mentioned technique.

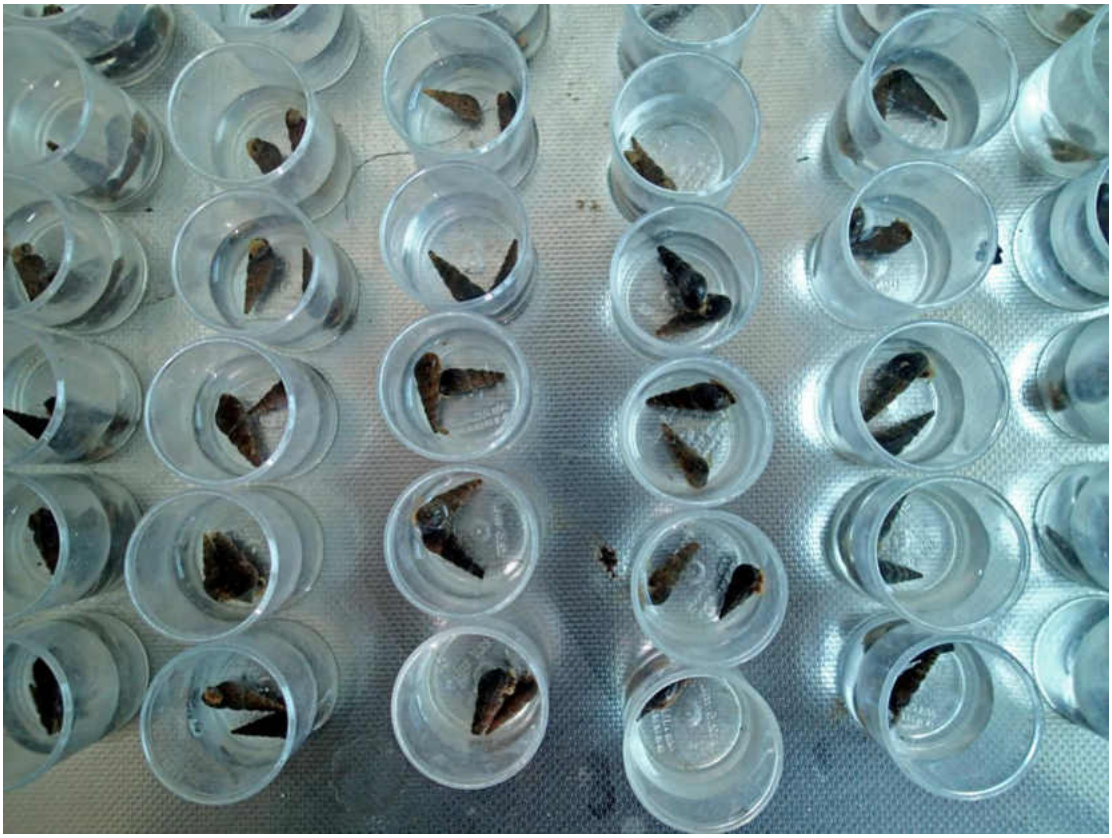

Figure 8. Snails sorted out by species and separated into small dishes with a small amount of water to identify snails shedding digenean cercariae.

Sample processing and molecular screening. All samples were processed and screened as outlined in "General Materials and Methods" section.

16SrRNA amplification and sequencing. All samples that tested positive for *Neorickettsia* using the real time PCR protocol had a 1470 bp long fragment of the 16S rRNA gene amplified and sequenced following the protocol outlined in the "Molecular screening for *Neorickettsia*" subsection, primers listed in Table 3**.**

GroESL amplification and sequencing. A nested PCR amplifying a 1940 bp fragment of the GroESL operon was carried out for members of the "*Neorickettsia risticii*" clade (Figure 16, Chapter 3). Five microliters of each DNA extract were used for

the first PCR reaction and 2 µl of the first PCR product was used for the nested PCR. The primers used for the initial PCR were; hs10F 5'-CTCAAATGAAACAAT-CCGTTTGTTTGTAGC-3' and hs2090R 5'- CATTCCACCCATGCCA-CCACCAGGCAT-3'. The primers used for the nested PCR were hs90F 5'-GTAGGTCTTGAAAAATATCACAGCG-3' and hs2010R 5'-GTAGTCACTA-GAACACTAGCAACAGA-3'. The same nested PCR primers were used for sequencing along with internal forward primers; hs120F 5'- TACGATATTTGATT-CTGTAGGTCATTAG -3'and hs910F 5'- TGGTTCAATTTCTGCTAACGGCAAT-3' and internal reverse primers; hs700R 5' GCTTTTTCATTCGCCTGTGAGGTAGCCT-3' and hs1620R 5'- CTTTAACCTCAACTTCTGTAGCACCAC-3' designed by SEG.

Phylogenetic analysis. Newly obtained sequences (Table 10 and Fig. 16, Chapter 3) and sequences of neorickettsiae from GenBank were used in the phylogenetic analysis. The new sequences and sequences obtained from GenBank were initially aligned with the aid of ClustalW as implemented in the BioEdit program, version 7.0.1 (Hall 1999). The alignments were manually refined in MacClade, version 4 (Maddison and Maddison 2005).

The first phylogenetic analysis was run using a larger dataset including all available *Neorickettsia* 16S sequences and *Wolbachia pipientis* as the outgroup. The analysis was carried out using Bayesian inference (BI) as implemented in the MrBayes program, version 2.01 (Huelsenbeck and Ronquist 2001) with the following nucleotide substitution parameters: lset nst=6, rates=invgamma, ngammacat=4, that correspond to a general time reversible (GTR) model including estimates of the proportion of invariant sites (I) and gamma (G) distributed among-site rate variation. Posterior probabilities were

approximated over 1,500,000 generations for both data-sets, log-likelihood scores plotted and only the final 75% of trees were used to produce the consensus trees by setting the "burnin" parameters at 375,000 generations. The GTR+I+G model was used for the analysis based off of the results obtained from jModelTest, version 0.1.1 (Guindon and Gascuel 2003; Posada 2008).

The second phylogenetic analysis was run using a much smaller dataset based on the partial sequences of the groESL operon, including only species/genotypes of *Neorickettsia* closely related to *Neorickettsia risticii*. *Neorickettsia helminthoeca* was used as the outgroup based on the larger 16s analysis. The analysis was carried out using Bayesian inference (BI) as implemented in the MrBayes program, version 2.01 (Huelsenbeck and Ronquist 2001) with the following nucleotide substitution parameters: lset nst=6, rates=propinv, prset (prior assumptions) pinvarpr=fixed (0.6380), that correspond to a Tamura-Nei (TrN) model including estimates of the proportion of invariant sites (I). Posterior probabilities were approximated over 500,000 generations, log-likelihood scores plotted and only the final 75% of trees were used to produce the consensus trees by setting the "burnin" parameters at 125,000 generations. The TrN+I model was used for the analysis based off of the results obtained from jModelTest, version 0.1.1 (Guindon and Gascuel 2003; Posada 2008).

Chapter 4 Specific Materials and Methods

Snail collection. *Lymnaea stagnalis* snails were collected during the months of May through November of 2011–2012 from three ponds in Nelson County, North Dakota; Ponds 1–3 (Table 6). Snails were rinsed with water and placed individually into glass jars filled with aged tap water treated with commercial aquarium conditioner to

remove chlorine/chloramines. Snails were kept for several hours under fluorescent lamps followed by several hours without light. Afterwards, the water in jars was examined for the presence of cercariae. Snails shedding cercariae were maintained individually in labeled containers and fed with lettuce.

DNA extraction and molecular screening. Initial screening for *Neorickettsia* was done using pooled cercariae. Fifty cercariae from each shedding snail were pipetted into a 1.75 ml microcentrifuge tube and centrifuged at 13,000 rpm for 5 minutes. Supernatant was removed and 60 µl of ultrapure water added. Cercariae were disrupted by direct sonication as described above in the "General Materials and Methods" section.

If *Neorickettsia* was detected in pooled cercariae from an individual snail, 50 or 100 individual cercariae or sporocysts from that snail were tested for *Neorickettsia*. One of the snails with *Neorickettsia*-positive digenean infection was dissected for individual screening of both sporocysts and cercariae. A single cercaria or sporocyst was placed in 50 µl of ultrapure water and disrupted by direct sonication as described above in the "General Materials and Methods" section. We did not detect any PCR inhibition with either pooled cercarial or single cercarial/sporocyst sonicates.

Sonicates were assayed for *N. risticii* using a substantially modified nested PCR protocol initially described by Barlough et al. (1997). Five microliters of each sonicate were used for the first PCR reaction and 1 µl (pooled cercariae) or 3 µl (single cercariae/sporocysts) of the first PCR product were used for the nested PCR. The nested PCR amplified a 527-bp portion of the 5' end of the 16S rRNA gene. The primer pairs (designed by myself using an alignment of different species of *Neorickettsia*, *Ehrlichia*, and *Anaplasma*) used in the first round were n16S-25F (5'-

TCAGAACGAACGCTAGCGGT-3') and n16S-610R (5'-GACGTTCCTCTTGATATCTACG-3'). Primers used in the nested PCR round were n16S-70F (5'- GAATCAGGGCTGCTTGCA-3'; designed by myself) and ER2-R (5'-GTTTTAAATGCAGTTCTTGG-3' from Barlough et al. (1997)).

The PCR reactions were run on an EP Gradient thermocycler (Eppendorf, Hauppauge, NY) using Quick load OneTaq mastermix (New England Biolabs, Ipswich, MA) according to the manufacturer's instructions. Annealing temperature of 54^{o}C and 40 cycles were used in both initial and nested PCRs. DNA extracts of *N. risticii* were used as positive controls (graciously provided by Dr. John Madigan, UC Davis). In negative controls, ultrapure water was used in place of sonicates.

DNA sequencing. PCR amplicons of *Neorickettsia* were purified using either the DNA Clean & Concentrator™ -5 kit (Zymo Research, Irvine, California) or ExoSap PCR clean-up enzymatic kit (Affimetrix, Santa Clara, California) according to the manufacturers' instructions. The PCR products were cycle-sequenced using ABI BigDye™ chemistry, alcohol precipitated and run on an ABI Prism 3100™ automated capillary sequencer. PCR products were sequenced in both directions. For sequencing of *Neorickettsia*, nested PCR primers were used. Contiguous sequences of *Neorickettsia* were assembled and edited using Sequencher™ ver. 4.2 (GeneCodes Corp., Ann Arbor, Michigan) and submitted to GenBank under accession numbers [KF556679].

Chapter 5 Specific Materials and Methods

Ethics statement. The use of vertebrate animals (both mice and hamsters) was approved by UND IACUC (protocol 1011-1c). IACUC protocols were not needed for invertebrate collecting or maintenance in the laboratory.

Snail collection. *Lymnaea stagnalis* snails were collected during the month of November from pond 5 (Table 6) in Towner county, ND.

Initial screening. Initial screening was carried out following the protocol described in the "General Materials and Methods" section above.

Parasite Collection. Three *L. stagnalis* shedding *P. elegans* cercariae infected with *Neorickettsia* sp. were used to begin the study. All stages of the parasite's life cycle were maintained in the same room at room temperature (23°C). The laboratory life cycle was repeated as described above, with minor changes.

Cercariae. From each of the three snails, 50 individual cercaria, were pipetted into separate 1.75 ml microcentrifuge tubes containing 25 µl of ultra pure water.

Metacercariae. Approximately 150 *Culex* mosquito larvae were distributed into three shallow containers housing each of the three snails actively shedding cercariae. Each container had approximately an inch and quarter of aquarium water. Distribution of larvae was spaced a day apart for each snail to ensure proper/sufficient time for screening (Table 4). *Culex* larvae were exposed to cercariae for one hour to ensure that a majority became infected with *P. elegans* metacercariae. After one hour the mosquito larvae were removed from the snail container and placed into separate labeled containers. Metacercariae originating from each of the three snail were left to develop for 24 hours and 6 days before screening (Table 4).

To obtain individual metacercariae, single *Culex* larvae were pipetted onto a glass microscope slide with a small amount of water and placed under a stereomicroscope. A glass cover slip was used to gently smash the mosquito larvae to visualize the metacercariae. If metacercariae were present, then fine forceps were used to carefully

remove the metacercariae from the *Culex* larvae. A total of 50 metacercariae (150 total metacercariae), for each of the three parasite groups (originated from the three snails) and for each of the two time points (1 and 6 days), were removed and placed in a small Petri dish with ultrapure water. While some mosquito larvae were uninfected, others were lightly infected (1–2), or heavily infected (5–10) with metacercariae. Under a stereomicroscope 50 individual metacercaria originating from each of the three snails (parasite groups), were carefully pipetted, one at a time, onto a small square piece of glass cover slip with as little water as possible, and sandwiched under a second small square piece of glass cover slip. The metacercaria sandwiched between two small glass cover slip pieces was then transferred with the aid of fine forceps into a 1.75 ml microcentrifuge tube containing 25 µl of ultra pure water**.**

Juvenile and adult parasites. Of the initial 150 Culex larvae infected with metacercariae for each of the three snails (parasite groups), approximately 50-75 were used to obtain metacercariae. The remaining 75-100 (or less, some died) were used to infect hamsters. For each parasite group two hamsters were infected (i.e., six hamsters total) (Table 5). To infect them, hamsters were lightly anesthetized with isoflurane and force-fed 15-20 metacercaria-infected *Culex* larvae. Hamsters were maintained thereafter on standard rodent diet (Purina LabDiet®) in individual cages that were cleaned twice a week. After 48 hours or 14 days post-infection hamsters were anesthetized with isoflurane and euthanized by cervical dislocation according to the IACUC protocol. The small intestine was removed, placed flat in a Petri dish with saline and teased apart with forceps and examined for either juvenile or adult *P. elegans* under a stereomicroscope.

Table 4. Time line for *Culex* larvae exposures to *P. elegans* cercariae and subsequent dissection of larvae for metacercariae 24 hours and 6 days after infection for the three snails (parasite group).

	Day 1	**Day 2**	**Day 3**	**Day 4**	**Day 6**	**Day 7**	**Day 8**
Snail 1	Larvae infected	24 hr. dissection	_____	_____	6 day dissection	_____	_____
Snail 2	_____	Larvae infected	24 hr. dissection	_____	_____	6 day dissection	_____
Snail 3	_____	_____	Larvae infected	24 hr. dissection	_____	_____	6 day dissection

Juvenile or adult worms were removed from the intestines with a glass pipette and placed in a Petri dish with fresh, sterile, saline. At day 2 post-infection, fifty juveniles worms were obtained from parasite groups 1 and 3, while only 36 were obtained from parasite group 2. At day 14 post-infection, fifty adult worms were obtained from parasite groups 2 and 3, while only 36 were obtained from parasite group 1. Individual worms were pipetted into separate 1.75 ml microcentrifuge tubes containing 50 µl of ultra pure water (juveniles) and 250 µl of ultra pure water (adults).

Eggs. The 14 day dissection of the hamster for parasite group 3 yielded over 100 adult worms. Eggs were harvested from two worms.. Each worm was placed in a separate Petri dish with heat-sterilized aquarium water and teased apart with fine needles to remove mature eggs in the distal portion of the digenean uterus. Under a stereomicroscope 25 eggs were individually pipetted on to small square fragments of glass cover slip, transferring as little water as possible. A second cover slip fragment was gently lowered on top of the isolated egg, crushing it, and the entire 'sandwich' was then transferred with fine forceps into a 1.75 ml microcentrifuge tube containing 20 µl of ultra

pure water. These represented early embryonic parasites. The remaining eggs were incubated for 6 days at room temperature to allow the embryos to grow and develop to miracidial stage. Twenty five of these late-embryo eggs were individually crushed and transferred into 1.75 ml microcentrifuge tubes as described above.

Table 5. Time line of infection of definitive hosts (hamsters) with *P. elegans* metacercariae and subsequent dissection of hamsters for juvenile worms 48 hours post-infection and adult worms 14 days post-infection for the three snails (parasite group).

	Day 7	**Day 8**	**Day 9**	**Day 10**	**Day 11**	**Day 20**	**Day 21**	**Day 22**
Snail 1	Hamsters infected	_____	48hr dissec.	_____	_____	14 day dissec.	_____	_____
Snail 2	_____	Hamsters infected	_____	48hr dissec.	_____	_____	14 day dissec.	_____
Snail 3	_____	_____	Hamsters infected	_____	48hr dissec.	_____	_____	14 day dissec.

DNA extraction/sonication. Sonication was slightly modified from what was explained in the "General Materials and Methods" section. For cercariae, metacercariae, and eggs, the samples were indirectly sonicated at an amplitude of 85% for 2 minutes and 40 seconds using a Model 705 Sonic Dismembrator (Fisher Scientific, Waltham, MA) with a 3 inch Cup Horn attachment (Fisher Scientific, Waltham, MA) (Figure 9). To avoid overheating water within the Cup Horn was changed between samples. For juvenile and adult worms the samples were indirectly sonicated at an amplitude of 85% for 2 minutes 30 seconds, water changed, and then indirectly sonicated for an additional 2 minutes. Immediately following sonication, all samples were placed on ice to avoid DNA degradation.

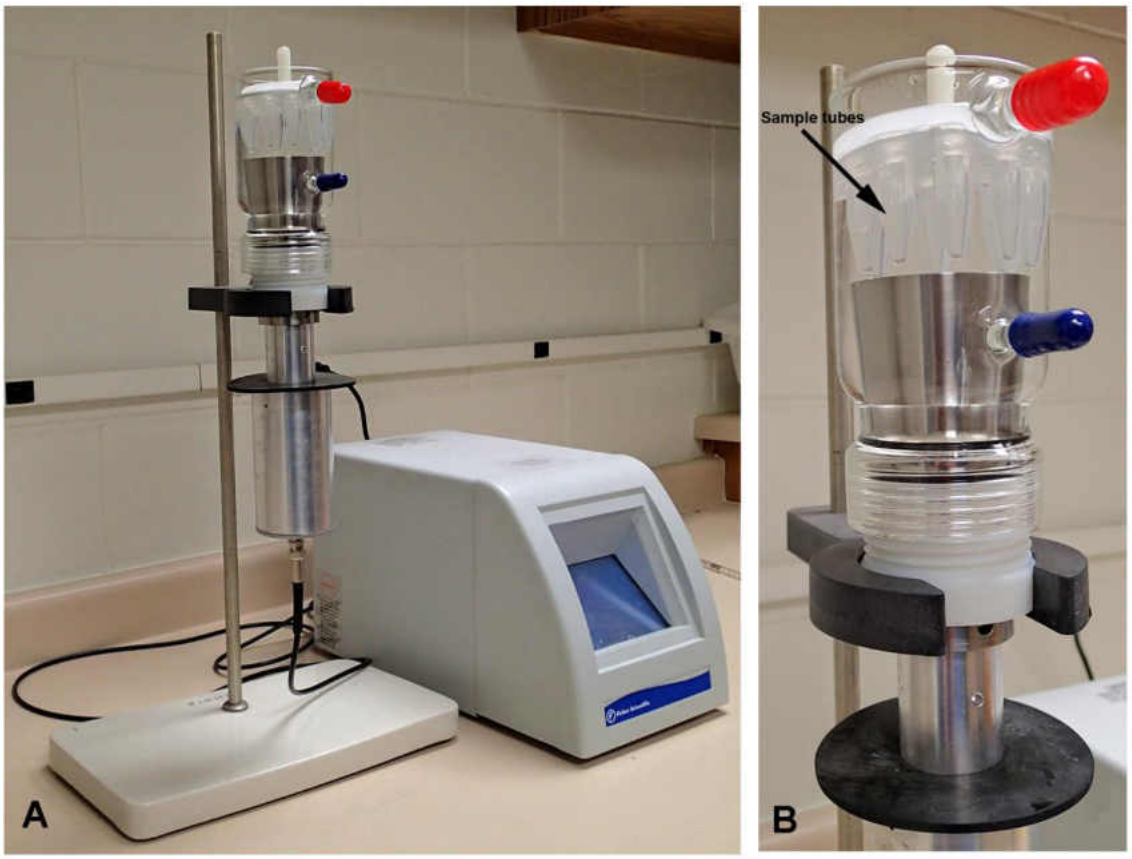

Figure 9. Model 705 Sonic Dismembrator (A) with a 3 inch Cup Horn attachment (B) for simultaneous indirect sonication of 8 samples.

Quantitative real time PCR. The real time PCR protocol for *Neorickettsia* detection previously described in the "General Materials and Methods" section was modified slightly for quantitative real time PCR (qPCR). Instead of 36 cycles, the reaction was run for 40 cycles. In order to generate the standard curves necessary for quantification of *Neorickettsia* spp., a 224 bp laboratory synthesized fragment of the *Neorickettsia risticii* GroEL gene was designed(gBlocks®, Integrated DNA Technologies Inc.). The 224 bp gBlocks® gene fragment of the GroEL gene contains the same 152 bp fragment that is amplified by real-time PCR primer pairs described in Table 3. The gene fragment was delivered at a dry weight of 200 ng. The total copy number of GroEL gene fragments was calculated as follows: number of copies = (200ng of DNA * 6.022×10^{23})/

(length of fragment [i.e., 224bp] * $1x10^9$ *650). This formula assumes that the average weight of a base pair is 650 daltons. Using this formula, the total copy number of GroEL gene fragments was calculated to be 8.27x10^11. The dried gene fragment was suspended in 41.35 µl of sterile TE buffer, resulting in a stock concentration of $2.0x10^{10}$ copies/µl. From this stock solution, eight 10-fold dilutions were made (10 to 10^8 copies) and used to construct standard curves.

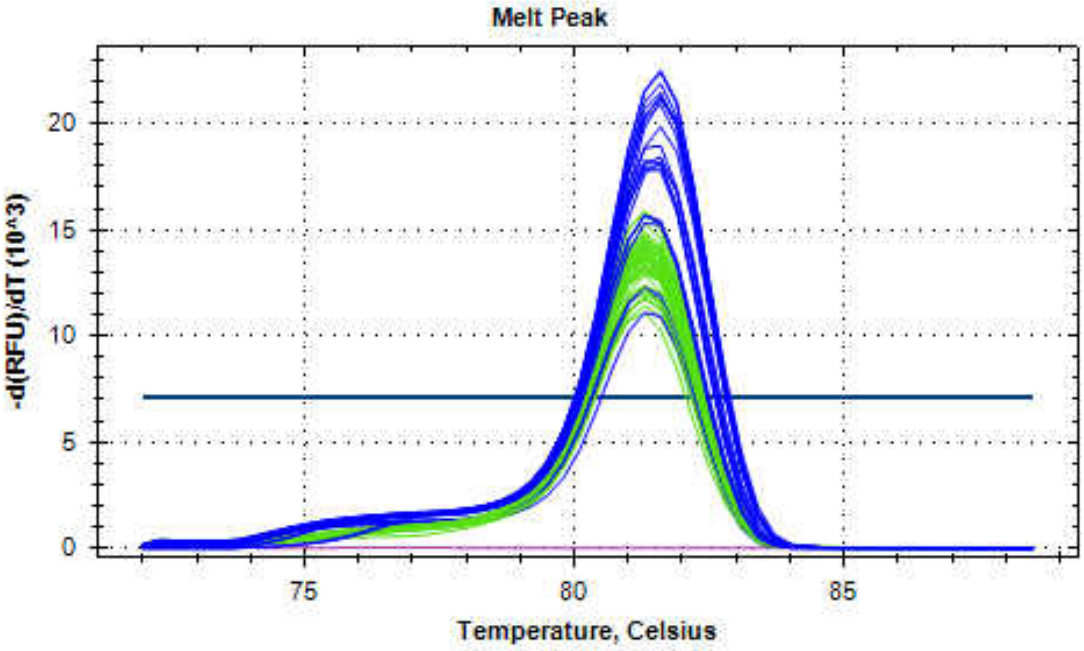

Figure 10. Postamplification melting-curve analysis for 50 individual 6 day-old metacercariae. blue indicates the standards, green indicates the single 6 day metacercariae, and purple indicates the negative control.

Postamplification melting-curve analysis was performed by slowly increasing the temperature from 72°C to 88.5°C (increments of 0.3°C, holding for 4 s), while fluorescence was measured continuously. Thus, the specific PCR product was verified based on the specific melting temperature and distinguished from possible nonspecific products (Figure 10).

Statistical analysis. One-way analyses of variance (ANOVA) were used to o determine if the number of bacteria differed among life cycle stages of the digenean *P. elegans* or between parasite groups (snails 1, 2 and 3). For all life cycle stages (cercariae, 24 hour metacercariae, 6 day metacercariae, 48 hour juveniles, 14 day adults, early embryo eggs, and late embryo eggs) the data were not normally distributed, however, after log transformation all data became normally distributed. Number of bacteria had equal variance between the life cycle stages and between parasite groups. Therefore, we were able to run one-way ANOVA's, followed by Tukey's post hoc tests if the ANOVA's were significant.

Efficiency test of sonicate utility for PCR amplification. To determine whether direct use of sonicates for PCR amplification is as effective as complete DNA extractions (guanidine thiocyanate method) both qPCR and nested PCR reactions were run. Forty *P. elegans* cercariae, taken from a snail shedding *Neorickettsia* positive digeneans, were placed into 3 separate 1.75 ml microcentrifuge tubes with 50 µl of ultrapure water. The tubes were spun down quickly in a microcentrifuge at 13,000 rpm, and sonicated as described above. Five microliters of each sample was used for the initial PCR and qPCR. One microliter of the initial PCR was used for the nested PCR. The remaining 40 microliters of each sonicate was DNA extracted using the guanidine thiocyanate method (Tkach and Pawlowski 1999) and re-suspended in 40 µl of ultra-pure water. Five microliters of each DNA extract was used for the initial PCR and qPCR. One microliter of the initial PCR was used for the nested PCR. The above mentioned nested PCR and qPCR protocols were used.

A One-way ANOVA was run to determine if there was a significant difference in amount of target DNA between sonicates and complete extracts.

Chapter 6 Specific Materials and Methods

Parasite collection. Cercariae, metacercariae, adults, and eggs of *P. elegans* infected with *Neorickettsia* were obtained from the laboratory lifecycle and used for immunohistochemistry.

Sonication, DNA extraction, and screening. *Plagiorchis elegans* cercariae and adults were homogenized by direct sonication following the protocol described in the "General Materials and Methods" section above.

Fixation and cryosectioning. Infected *L. stagnalis* snails were crushed and digestive glands with sporocysts were removed. Sporocysts and snail tissue were fixed in buffered 4% paraformaldehyde at 4℃ for 24 hours. Metacercariae infected *Culex pipiens* larvae and adult worms were also fixed in buffered 4% paraformaldehyde at 4℃ for 24 hours. Following fixation, specimens were equilibrated in 30% sucrose overnight, embedded in Neg^{50} (Fisher Scientific/Thermo Scientific, Pittsburgh, PA), cryosectioned on a Leica HM550 cryostat, and placed on gelatin subbed slides. The type of microscopy dictated the section thickness. Eight or 10 µm sections were cut when visualizing on an Olympus BX51WI fluorescent microscope. Twenty five or 30 µm sections were made when visualizing on a Zeiss LSM-510 meta confocal microscope.

Immunolabeling. Immunolabeling was done as follows. In brief, sections were blocked and permeabilized in 3% donkey serum, 2% goat serum (Vector Laboratories, Burlingame, California), 0.1% Triton X-100, and 1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) overnight at 4℃. Hyperimmune anti-*N. risticii* horse

sera was obtained from Dr. Yasuko Rikihisa (Department of Veterinary Biosciences, College of Veterinary Medicine, The Ohio State University, Columbus, Ohio) (Rikihisa et al. 1988). The primary antibody was diluted to a concentration of 1:500 in the blocking buffer described above. One hundred and fifty microliters of the primary antibody solution was added to each slide and left to incubate in a moist chamber for 3 hours at room temperature (RT) or overnight at 4℃. Negative controls consisted of the blocking buffer minus the anti-N. risticii horse sera as well as non-immune horse sera. After primary antibody application, slides with sections were washed, 3 times, in PBS for 15 minutes. The fluorochrome-coupled secondary antibodies (cy3 goat anti-horse, Jackson ImmunoResearch Laboratories,Inc., West Grove, Pennsylvania) were diluted to a concentration of 1:200 in blocking buffer, 150 µl added to the slides, and left to incubate for 1 hour at RT. Nuclei were labeled with DAPI (Sigma, St. Louis, Missouri) when visualized on the Olympus BX51WI fluorescent microscope or labeled with ToPro3 (Fisher Scientific/Thermo Scientific, Pittsburgh, Pennsylvania), at a concentration of 1:1000, when visualized on the Zeiss LSM-510 meta confocal microscope. Samples were cleared and mounted in VECTASHIELD® HardSet mounting medium (Vector laboratories, Burlingame, California).

Transmission Electron Microscopy. Adult specimens of *P. elegans* were obtained from the above mentioned laboratory life cycle. Living worms were removed from their hosts, rinsed in 0.9 % NaCl and fixed in cold (4°C) 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer at pH 7.2, rinsed in 0.1 M sodium cacodylate buffer at pH 7.2, post-fixed in cold (4°C) 1% osmium tetroxide in the same buffer for 1 h, dehydrated in ethanol and propylene oxide, embedded in Spurr and polymerised at 60°C for 24 h.

Ultrathin sections (60–90 nm thick) were obtained using the ultramicrotome (Power tome PC, RMC Boeckeler ®) with a diamond knife. Sections were placed on 300 and 200-mesh copper and gold grids. Sections on copper grids were stained with uranyl acetate and lead citrate (Zakikhani et al. 2003). Sections on gold grids were stained with periodic acid, thiocarbohydrazide and silver proteinate (Tkach 2008). This technique was employed for the location of glycogen. Sections were examined on a Hitachi H-7650 transmission electron microscope, operating at an accelerating voltage of 80 kV, in the "Service d'Étude et de Recherche en Microscopie Electronique" of the University of Corsica (Corte, France).

Results

Pond Screening

The presence of *Neorickettsia*-infected snails in sampled water bodies varied greatly throughout the study period (2011–2013). Out of the 40 ponds (with snails) sampled during the study period 5 were found to harbor snails shedding *Neorickettsia* positive cercariae (Table 6). Pond 1 had *L. stagnalis* infected with *Neorickettsia* positive digeneans during the spring and summer of 2011, ponds 2 and 3 produced positive snails during the summer of 2012, pond 4 had positive snails during the summer of 2013, and pond 5 had positive snails during the summer of 2013 and fall of 2014 (Table 7).

Table 6. Ponds with snails harboring *Neorickettsia* positive digeneans

Pond #	County	Coordinates
Pond 1	Nelson County ND, USA	48°0'24.18"N, 97°56'37.32"W
Pond 2	Nelson County ND, USA	48°1'43.8"N, 97°59'24.88"W
Pond 3	Nelson County ND, USA	48°10'48.6"N, 98°7'51.29"W
Pond 4	Nelson County ND, USA	47°55'43.95"N, 98°19'58.18"W
Pond 5	Towner County, ND, USA	48°14'1.5"N, 98°8'28.44"W

Table 7. Total number of snails collected, snails infected with digeneans, and snails shedding *Neorickettsia* positive digeneans from 5 surveyed ponds in North Dakota.

Pond #	Total snails collected	Snails infected with digeneans (%)	Snails with *Neorickettsia* positive digeneans (% of digenean infected snails)
1	100	9 (9)	1 (11)
2	187	47 (25)	11 (23)
3	329	184 (56)	6 (3)
4	219	139 (64)	10 (7)
5	306	105 (34)	9 (9)
Total	1141	484 (42)	37 (8)

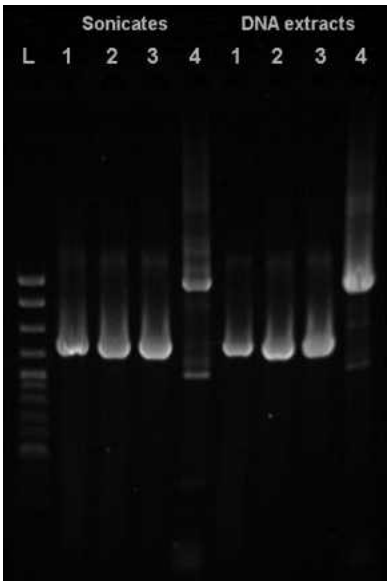

Figure 11. Agarose gel showing efficiency of sonicates used directly for nested PCR compared to complete DNA extracts. Lanes 1,2 and 3 are 28s amplicons of approximately 800bp and lane 4 is an ITS amplicon of approximately 1800 bp in length.

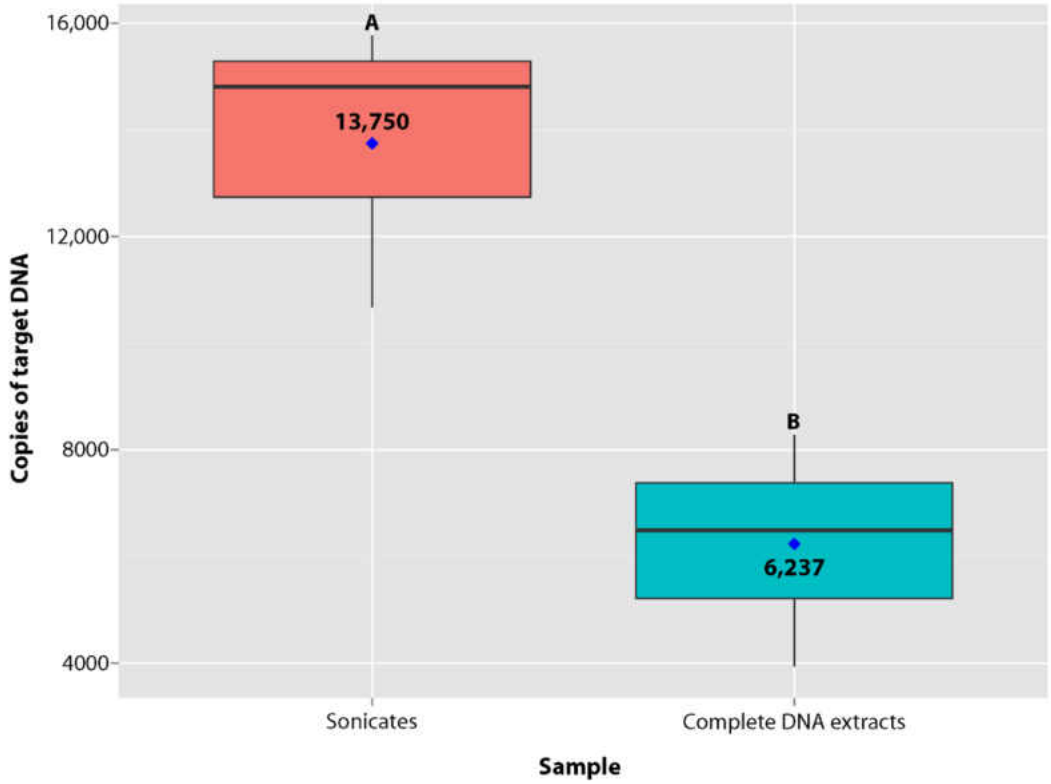

Figure 12. Boxplots comparing the mean number of amplicons generated when using cell sonicates directly versus DNA extraction of sonicates as templates for qPCR of *Neorickettsia* 16s gene in infected *P. elegans* cercariae. Mean DNA copy number indicated by blue diamond.

Extraction Method

DNA amplification by nested PCR from sonicates, without further processing, appears to be just as efficient/sensitive as DNA amplification from DNA extracts completed using the guanidine thiocyanate method (Figure 11). The qPCR verified what the nested PCR revealed, except it provided a more quantitative test. Interestingly, using sonicates directly for PCR resulted in a statistically ($F_{(1,4)}$=10.804, P=0.0303) higher number of DNA copies compared to using complete DNA extracts for PCR (Figure 12).

Laboratory Life Cycle

We have for the first time maintained *Neorickettsia* in the laboratory throughout the entire life cycle of a digenean. (Figure 13). One generation of the laboratory life cycle of *P. elegans* takes approximately 90 days – i.e., 5 days for metacercariae to become infective, 20 days for adults to develop, 7 days for miracidium to become infective within the egg, and 50–60 days for the snail to start shedding cercariae after ingestion of an infective egg.

Neorickettsia sp. infection was maintained in the digenean, *P. elegans*, for three generations. In the first generation, 50 uninfected snails were exposed to and presumably ingested *Neorickettsia* positive *P. elegans* eggs. Of those 50 snails, only 8 (16%) survived the 50–day incubation period to produce cercariae. Five of the 8 surviving snails died soon after shedding cercariae. Although all three remaining snails produced cercariae, only one produced cercariae harboring *Neorickettsia* (*i.e*., *Neorickettsia* infections were lost). That single snail was used to generate a second life-cycle. In the second generation 30 snails were exposed to *Neorickettsia*-infected *P. elegans* eggs. Of those, only six (20%) survived the requisite 50-day incubation period and only three (10%) shed

cercariae, of which only one (5% of total) shed cercariae that were PCR-positive for *Neorickettsia*. Again, a single snail parasitized by *Neorickettsia*-infected *P. elegans* was used to perpetuate the life-cycle.

We attempted to use both hamsters and outbred mice as hosts for *P. elegans*. However, only hamsters were found to be a suitable hosts for *P. elegans* (Table 8).

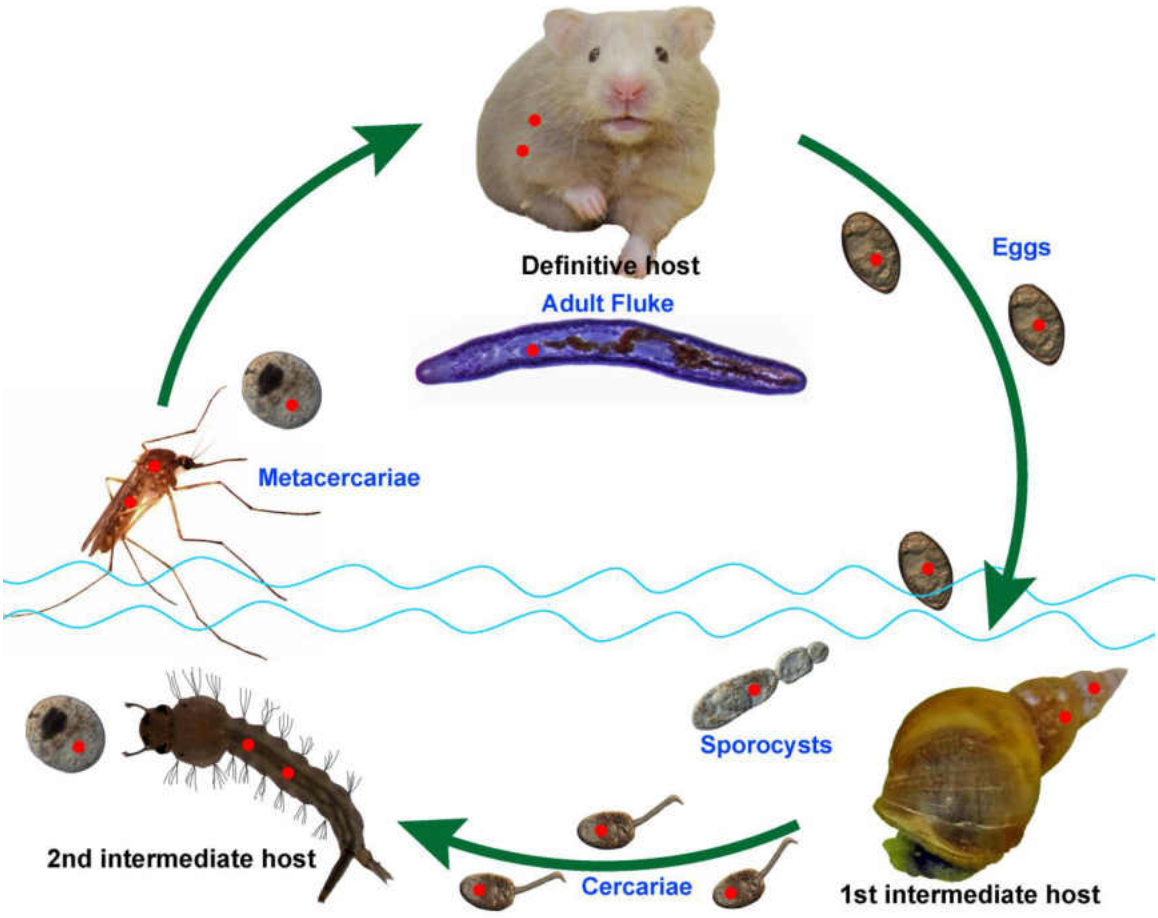

Figure 13. Laboratory life cycle of *Plagiorchis elegans*. Red dots indicate infection with *Neorickettsia* sp..

Table 8. Experimental animals (Mice or Hamsters) infected with *Plagiorchis elegans* metacercariae (mtc.) either from mosquito larvae or in snail tissue with *P. elegans* sporocysts infected with mtc. Days post infection (PI) before dissection of experimental animal and number of adult *P. elegans* in intestine provided.

Experimental animal	**Infected with (mosquitoes/snail)**	**Days PI before dissection**	**Number of adult *P. elegans***
Mouse #1	mosquito larva with *P. elegans* mtc.	11	1 (underdeveloped)
Mouse #2	mosquito larva with *P. elegans* mtc.	17	0
Mouse #3	mosquito larva with *P. elegans* mtc.	17	0
Mouse #4	mosquito larva with *P. elegans* mtc.	16	0
Mouse #5	mosquito larva with *P. elegans* mtc.	25	0
Mouse #6	mosquito larva with *P. elegans* mtc.	7	0
Mouse #7	mosquito larva with *P. elegans* mtc.	7	7 (underdeveloped)
Hamster #1	mosquito larva with *P. elegans* mtc.	11	25
Hamster #2	mosquito larva with *P. elegans* mtc.	14	33
Hamster #3	mosquito larva with *P. elegans* mtc.	14	14
Hamster #4	mosquito larva with *P. elegans* mtc.	17	42
Hamster #5	mosquito larva with *P. elegans* mtc.	12	100
Hamster #6	mosquito larva with *P. elegans* mtc.	27	36
Hamster #7	mosquito larva with *P. elegans* mtc.	28	2
Hamster #8	mosquito larva with *P. elegans* mtc.	22	21
Hamster #9	mosquito larva with *P. elegans* mtc.	27	40
Hamster #10	mosquito larva with *P. elegans* mtc.	29	35
Hamster #11	Snail tissue with *P. elegans* sporocysts infected with mtc.	20	300 +
Hamster #12	Snail tissue with *P. elegans* sporocysts infected with mtc.	16	250 +

Discussion

Fluctuation in Positive Ponds

Prevalences of *Neorickettsia* varied greatly from pond to pond, with a majority of sampled ponds containing snails shedding *Neorickettsia* negative cercariae. Ponds with snails shedding *Neorickettsia* infected digeneans one year did not always have snails shedding *Neorickettsia* infected digeneans the next year. We hypothesize that this could be a result of abiotic factors, such as drought or length of winter, that affected the population and growth of the snail first intermediate hosts.

Benefit of Sonication Extraction Method

The use of sonicates directly for PCR amplification has greatly reduced the amount of time necessary to identify *Neorickettsia* positive samples. When dealing with live organisms, such as snails, there is always the chance that the organism could die prematurely before having the opportunity to complete all necessary experiments. Therefore, limiting the time between collection and screening is critical. Once a snail is identified as *Neorickettsia* positive or negative the appropriate steps, either disposal or greater attention to husbandry, can be taken. It takes approximately 2 hours to sonicate 50 samples and screen for *Neorickettsia* using real-time PCR, whereas for complete DNA extraction and screening it takes approximately 12 hours and is incomparably more labor intensive.

Benefits of Indirect Sonication

For general screening of samples for neorickettsial DNA the direct sonication (when the sonication probe is placed directly into the sample) is fully sufficient.

However, when quantification of bacteria is necessary, a more precise method is required. Indirect sonication, with the use of a Cup Horn attachment, allows us to process sealed tubes. This provides us with 4 major benefits: 1) it eliminates aerosols and the chance of cross contamination, 2) it prevents the loss of sample, either from it sticking to the probe or through aerosolization of the sample during direct sonication, 3) it enables multiple tubes to be processed at one time, and 4) it allows us to place our specimens in less water, which is important for smaller samples, such as single eggs.

Benefits of Real Time PCR

Screening methods for *Neorickettsia* were progressively improved throughout the study. Initially a nested PCR protocol was used to screen all samples for *Neorickettsia*. However, this required a significant amount of time, disposables, and reagents. Additionally, it resulted in a higher likelihood of contamination, due to the high concentration of amplicons resulting from the nested PCR step. To eliminate a majority of these problems, but still maintain the high sensitivity of our screening protocol, we developed a real-time PCR protocol for initial screening of DNA samples of neorickettsial DNA.

The newly developed real-time PCR protocol has provided for a quick and sensitive way of screening large collections of digenean DNA extracts for *Neorickettsia*. Removing the necessity of running a nested PCR and screening the results with gel electrophoreses for every extract has greatly reduced the amount of time required to screen large collections of DNA extracts. The elimination of the use of post-PCR handling steps in real-time PCR also reduces the likelihood of false positive results caused by contamination. Although real-time PCR using TaqMan probes has been used

in the detection of *N. risticii* from snails and horses (Pusterla et al. 2000), the technique was not used for a broad screening of digeneans for *Neorickettsia* and there was no published protocol of "regular" real-time PCR using SYBR Green or another dye as an alternative to the TaqMan probes. Another advantage of the real-time PCR protocol is the ability to run melting curve analysis, which is particularly useful for differentiating potential non-specific binding of primers. It was of great importance for our study since we used relatively generic primers targeting all *Neorickettsia* species and genotypes. The combination of real-time PCR, nested PCR, and sequencing allows for a very robust and accurate screening.

To avoid the potential for contamination during screening we separated all screening steps to different rooms. This included sonication, DNA extraction, real-time PCR, nested PCR, gel electrophoresis, and DNA sequencing. Master mix preparation for real-time PCR and nested PCR was completed in a sterile PCR fume hood with pipettes that have never come in contact with target DNA.

Quantitative Real Time PCR

Most bacteria, including neorickettsiae (based on whole genomes of *N. risticii* and *N. sennetsu*), possess a single copy of the GroEL gene (Goyal et al. 2006). GroEL, one of the best-studied chaperonins, belongs to the 60-kDa group I chaperonin class. GroEL is required for the proper folding of many proteins in vitro (Goyal et al. 2006; Houry et al. 1999). The GroEL gene in *Neorickettsia* is a perfect candidate for qPCR because it is a single copy gene. To our knowledge there has been no study on the quantitative aspects of *Neorickettsia* transmission either in the digenean host or the vertebrate definitive host of the digenean.

Laboratory Life Cycle

The digenean, *Plagiorchis elegans,* was used for our laboratory life cycle for two reasons. First, *P. elegans* is found to be naturally infected with *N. risticii* in North Dakota (Tkach et al. 2012). From three ponds in eastern North Dakota, 240 *L. stagnalis* were found infected with *P. elegans* and out of those 240 cercarial infections, 18 proved to be *Neorickettsia*-positive (7.5%). Second, *P. elegans* is a generalist parasite, utilizing multiple groups of aquatic arthropods as second intermediate hosts (*Chironomus* sp., *Gammarus pulex*, *Asellus aquaticus*, *Culex* sp.) (Gorman 1980), as well as, infecting a wide range of vertebrate definitive hosts (LACA mice, rats, hamsters, gerbils, chicks, ducklings and pigeons) (Gorman 1980).

There are several critically important points in the life cycle that deserve special attention. The first of them is the ability of cercariae to kill overexposed mosquito larvae. Gorman (1980) found that by exposing 6 chironomid larvae to approximately 120 cercariae in the confines of a small Petri dish (5 cm diam.), the insect larvae became infected with 10 to 30 metacercarial cysts. Larger numbers of cercariae per larvae resulted in the death of some chironomid larvae. We exposed 20–30 *Culex* mosquito larvae to approximately 500 cercariae for 2 to 3 hours. We did not count the average number of metacercariae per insect larvae, however, we did observe larval death when exposed to approximately 1000 or more cercariae. Smaller, younger larvae (i.e., 1st and 2nd instar) were less tolerant of multiple cercarial penetrations and mortality was more frequent than with older 3rd and 4th instar larvae. The second very important element is to ensure that heat sterilized water is used for egg development. If the water is not boiled before use, ciliated protozoans may ingest the eggs (Figure 13) and fungi will envelope

developing eggs. The third is to pay close attention to the husbandry of snails after they become infected with digeneans (i.e, after exposure to digenean eggs). It has long been known that larval digeneans, including *P. elegans* (Zakikhani and Rau 1999), can deplete energy reserves and increase mortality of their molluscan hosts (Cheng and Snyder 1962, Pinheiro and Amato 1994). For example, the majority of our parasitized snails died from what we now believe was excessive water removal and replenishment. To prevent the holding water from becoming anoxic and foul, we initially replenished half of the holding water with fresh water on a daily basis. However, when parasitized snails began to die, we reduced the volume of water removal-replenishment to a third of the holding volume and the survival of parasitized snails improved. Excessive water removal may have inhibited the growth of algae that the snails were feeding on, leading to premature snail death. In addition, we used only 30–50 snails for infection due to small snail stock numbers. It would be more preferable to use at least 100 snails for infection during each generation, to ensure continuation of the life cycle.

One large gap in our knowledge of *Neorickettsia* biology that laboratory life cycles can help address is the lack of understanding of the quantitative aspects of *Neorickettsia* perpetuation through the complex digenean life cycle (Vaughan et al. 2012). Like other types of vertically transmitted microorganisms (*e.g*., *Rickettsia*, *Wolbachia*), *Neorickettsia* must coordinate its replication with the reproduction of its invertebrate host. For digeneans, this includes both sexual and asexual reproduction. With the aid of the laboratory life cycle we can determine the vertical transmission efficiency of *Neorickettsia* within all stages of the digenean life cycle.

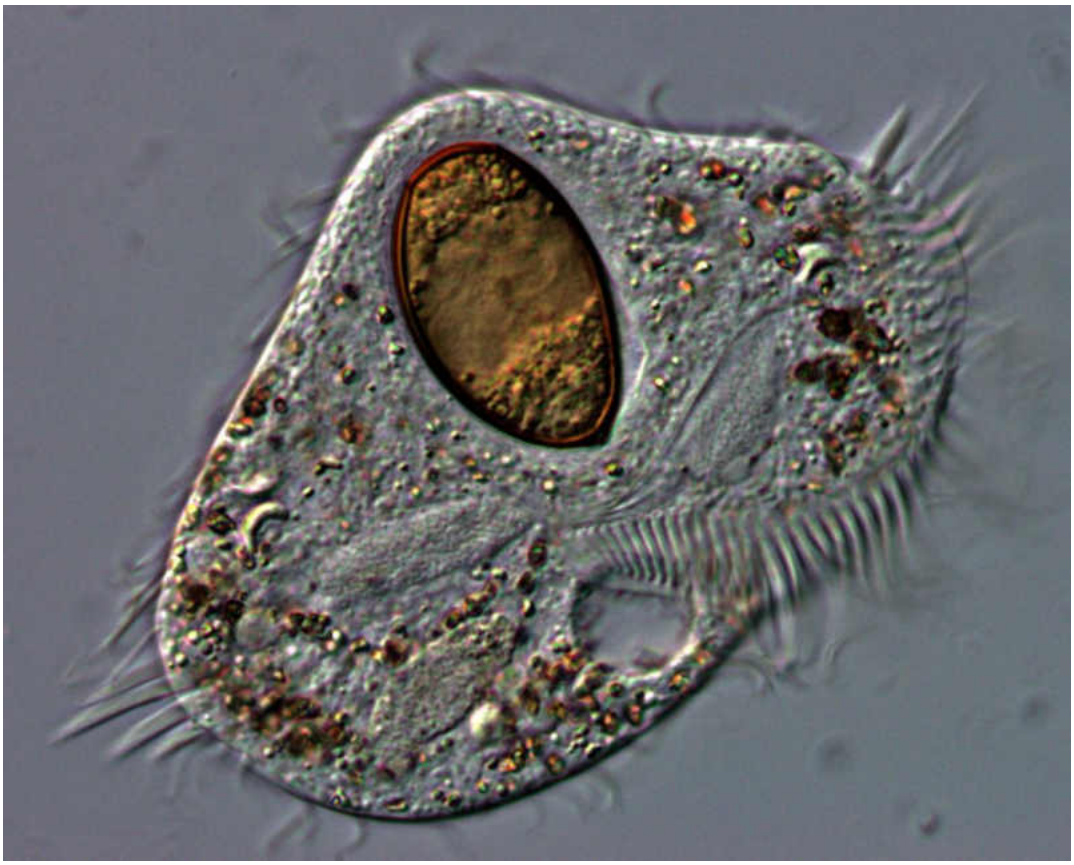

Figure 14. Ciliated protozoan after ingesting a operculate egg of *Plagiorchis elegans* during the laboratory life cycle.

Another important knowledge gap encompasses the transmission biology of *Neorickettsia*. Although horizontal transmission of neorickettsiae from digenean to vertebrate host occurs and can result in vertebrate infection, it is not known whether horizontal transmission of neorickettsiae can occur in the opposite direction from vertebrates to digeneans. It is also unknown whether neorickettsiae can be transmitted from *Neorickettsia*-infected digeneans to uninfected digeneans within a parasitized snail or a vertebrate (i.e., through co-feeding transmission). Such alternate transmission pathways could play a vital part in the circulation and evolution of *Neorickettsia*. To fully elucidate the probability of such events occurring, laboratory life cycles of digeneans harboring *Neorickettsia* are essential.

Conclusion

We have maintained neorickettsiae in the laboratory throughout multiple life cycles of the digenean *P. elegans*. The maintenance of *Neorickettsia* in the laboratory will make it possible to conduct controlled studies and thereby gain a more thorough understanding of the biology and transmission of these enigmatic bacterial endosymbionts capable of causing disease in humans and other vertebrates.

CHAPTER III

LARGE SCALE SCREENING OF DIGENEANS FOR *NEORICKETTSIA* ENDOSYMBIONTS USING REAL-TIME PCR REVEALS NEW NEORICKETTSIA GENOTYPES, HOST ASSOCIATIONS AND GEOGRAPHIC RECORDS

Abstract

In this study we report the results of screening 3,250 digenean samples for *Neorickettsia* collected from various vertebrates in terrestrial, freshwater, brackish and marine habitats in the United States, Argentina, Australia, Canada, China, Costa Rica, Egypt, Japan, Laos, Philippines, Thailand, Vietnam, and Ukraine. *Neorickettsia* were detected using a newly designed real-time PCR protocol targeting a 152 bp fragment of the heat shock protein coding gene, GroEL, and verified with nested PCR and sequencing of a 1371 bp long region of 16S rRNA. Twenty isolates of *Neorickettsia* have been obtained. Sequence comparison and phylogenetic analysis demonstrated that 14 of these isolates, provisionally named *Neorickettsia* sp. 1–14 represent new genotypes. Three isolates were identical to a published sequence of *Neorickettsia* known as SF agent, one was identical to *Neorickettsia risticii*, and one was identical to *Neorickettsia helminthoeca*. Fourteen digenean species that carried *Neorickettsia* represent new host records. Four of the 7 digenean families (Diplostomidae, Haploporidae, Pleurogenidae, and Faustulidae) are also reported for the first time as hosts of *Neorickettsia*. We have

detected *Neorickettsia* in digeneans from Australia, China, Egypt, Philippines, Vietnam, and Thailand for the first time based on PCR and sequencing evidence. Our findings suggest that further surveys from broader geographic regions and wider selection of digenean taxa are likely to reveal new *Neorickettsia* lineages as well as new digenean host associations.

Results

A total of 3,250 digenean samples were screened for *Neorickettsia* from the following states; Florida, Georgia, Illinois, Kentucky, Louisiana, Massachusetts, Minnesota, Mississippi, North Carolina, Pennsylvania, Tennessee, Virginia, and Wisconsin, and the following countries; Argentina, Australia, Canada, China, Costa Rica, Egypt, Japan, Laos, Philippines, Thailand, Vietnam, and Ukraine. However, not all collection sites had digenean samples infected with *Neorickettsia*, therefore only the positive sites are listed in Table 9. A list of digenean taxa screened is not given, since a negative result does not necessarily indicate that a given species/genus of digenean cannot harbor the bacteria. Screening revealed 20 samples positive for *Neorickettsia* (representing 17 genetic lineages of the bacteria) in 16 digenean species belonging to 10 different families (Tables 10 and 11).

Neorickettsia infections were detected in Florida, Mississippi, North Dakota, Oregon, Australia, China, Egypt, Philippines, and Thailand. Samples from Mississippi, Florida, China and Australia were provided by multiple colleagues, Dr. Eric Pulis, Dr. Stephen S. Curran, and Thomas J. Fayton, from the University of Southern Mississippi. DNA samples from Egypt were provided by Dr. Amal I. Khalil, from Tanta University, Tanta Egypt.

Table 9. Geographic origin of *Neorickettsia* positive digeneans.

Country	Collections site	Coordinates	Collector
USA	Pond 1, Nelson County North Dakota,	48°0'24.18"N, 97°56'37.32"W	Stephen Greiman, Vasyl Tkach, and Max Tkach
USA	Pond 2, Nelson County North Dakota,	48°1'43.8"N, 97°59'24.88"W	Stephen Greiman
USA	Pond 3, Nelson County North Dakota,	48°10'48.6"N, 98°7'51.29"W	Stephen Greiman
USA	Pond 4, Nelson County North Dakota,	47°55'43.95"N, 98°19'58.18"W	Stephen Greiman
USA	Pond 5, Towner County, North Dakota,	48°14'1.5"N, 98°8'28.44"W	Stephen Greiman
USA	Pearl River, Mississippi,	30°20'36"N, 89°38'03"W	Vasyl Tkach and Stephen Curran
USA	Cedar Key, Florida,	29°08'13"N, 83°02'36"W	Vasyl Tkach and Stephen Curran
USA	Site 1, Williford Springs, Florida,	30°26'20.40"N, 85°32'50.77"W	Thomas Fayton
USA	Site 2, Williford Springs Florida,	30°26'21.95"N, 85°32'42.26"W	Thomas Fayton
USA	Dunn Forest, Corvallis, Oregon,	44°42'28.48"N, 123°17'55.54"W	Stephen Greiman and Vasyl Tkach
USA	Salt Springs, Florida,	29°21'29.98"N, 81°44'41.93"W	Thomas Fayton
China	Daya Bay, Guangdong	22°43'N, 114°32'E	Eric Pulis
Australia	Eli Creek, Queensland,	25°15'45"S, 152°48'28"E	Eric Pulis
Vietnam	Cat-Ba Island,	20°43'27.86"N, 107°2'58.61"E	Stephen Greiman
Egypt	--------	--------	Amal I. Khalil
Philippines	Sablyayan Municipality, Mindoro Island	12°47'14.99"N, 120°54'57.96"E	Stephen Greiman
Thailand	Talay Thai Seafood market, Sumut Sakhon,	13°32'56.29"N, 100°15'26.11"E	Stephen Greiman, Vasyl Tkach, Jefferson Vaughan
Argentina	Buenos Aires,	34°47'47.61"S, 58°33'35.67"E	Gabriel L. Cicuttin

In addition, a colleague, Dr. Gabriel L. Cicuttin (Sección Serología y Pruebas Biológicas, División Inmunología y Diagnóstico, Instituto de Zoonosis Luis Pasteur, Buenos Aires, Argentina) provided *Neorickettsia* positive DNA extracts from spleens of Brazilian free-tailed bats (*Tadarida brasiliensis*) collected in Argentina, that represent *Neorickettsia risticii* (Cicuttin et al. 2013).

We have detected *Neorickettsia* from digeneans for the first time from the Australian continent, China, Egypt (African continent as a whole), Vietnam, Thailand, Philippines, Oregon and Florida (Figure 15). Three of the forms discovered in our study were identical to the SF agent (16S rRNA sequences), however, their GroESL sequences varied slightly. One of the forms was identical to *N. helminthoeca*. Sequences of 14 other forms clearly differed from all other known forms of *Neorickettsia*, therefore, potentially representing new species. Two samples, one collected in Thailand and one collected in China had identical 16S sequences (Tables 10 and 11).

The larger 16s phylogenetic analysis was run using a dataset including 30 in-group taxa and *Wolbachia pipientis* (a member of Anaplasmataceae endosymbiotic in insects and filariid nematodes) as an outgroup. The alignment included a total of 1,267 sites, of which 1,258 could be aligned unambiguously. Positions that could not be aligned unambiguously were excluded from the analysis. Bayesian analysis produced a tree where all *Neorickettsia* sequences clearly fall into a well-defined clade, with 100% support (Figures 16 and 17).

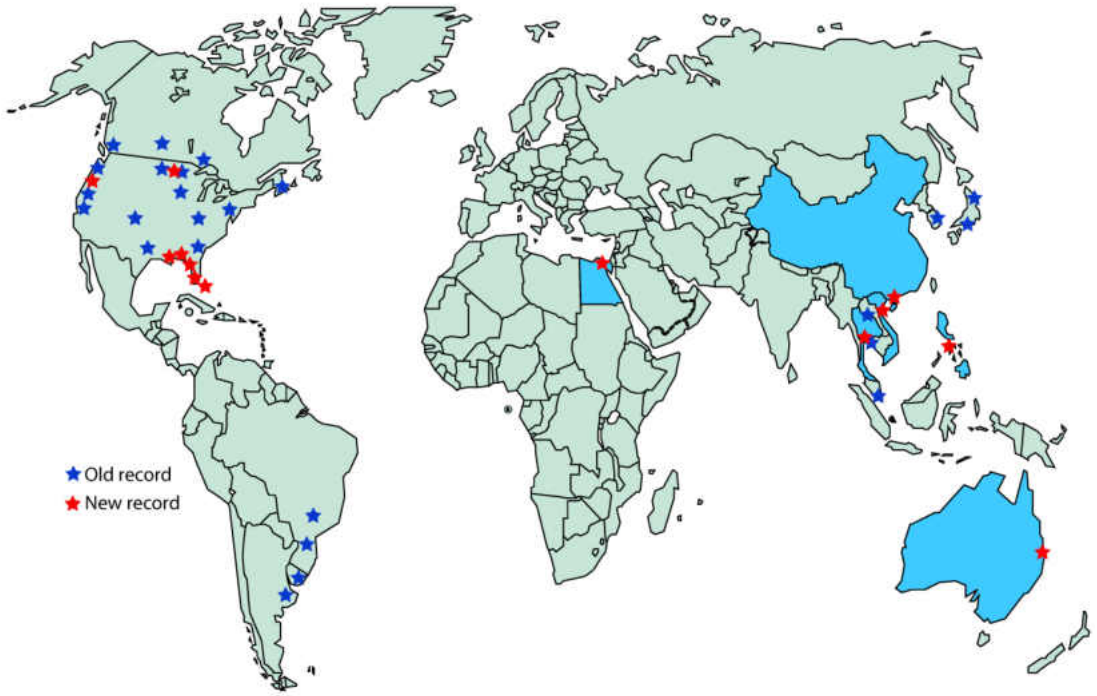

Figure 15. Geographic distribution of *Neorickettsia* spp., countries shaded in blue represent first report(s) of *Neorickettsia* from that country.

Table 10. *Neorickettsia* species/genotypes, natural hosts, and life cycle stage screened.

***Neorickettsia* species**	**Digenean family**	**Digenean genus and species**	**Life cycle stage**	**Host (this study)**
Neorickettsia helminthoeca	Troglotrematidae	*Nanophyetus salmincola*	Dog blood	Dog (*Canis lupus familiaris*)
Neorickettsia sp. 1	Allocreadiidae	*Crepidostomum affine*	Adult	Mooneye (*Hiodon tergisus*)
Neorickettsia sp. 2	Haploporidae	*Saccocoelioides beauforti*	Adult	Striped mullet (*Mugil cephalus*)
Neorickettsia sp. 3	Pleurogenidae	Unknown	Metacercariae	Crayfish (*Procambarus* sp.)
SF agent	Heterophyidae	*Metagonimoides oregonensis*	Adult	Raccoon (*Procyon lotor*)
SF agent	Heterophyidae	*Stellantchasmus falcatus*	Metacercariae	Striped mullet (*Mugil cephalus*)
SF agent	Troglotrematidae	*Nanophyetus salmincola*	Metacercariae	Salmon (*Oncorhynchus* sp.)

Table 10. cont.

***Neorickettsia* species**	**Digenean family**	**Digenean genus and species**	**Life cycle stage**	**Host (this study)**
Neorickettsia sp. 4	Haploporidae	*Saccocoelioides lizae*	Adult	Striped mullet (*Mugil cephalus*)
Neorickettsia sp. 4	Haploporidae	*Saccocoelioides lizae*	Adult	Striped mullet (*Mugil cephalus*)
Neorickettsia sp. 5	Faustulidae	*Bacciger sprenti*	Adult	Spotbanded scat (*Selenotoca multifasciata*)
Neorickettsia sp. 6	Lecithodendriidae	*Prosthodendrium* sp.	Cercariae	Stream snail (*Juga yrekaensis*)
Neorickettsia sp. 7	Derogenidae	*Deropegus aspina*	Cercariae	Stream snail (*Juga yrekaensis*)
Neorickettsia sp. 8	Plagiorchiidae	*Plagiorchis elegans*	Cercariae	Great pond snail *(Lymnaea stagnalis)*
Neorickettsia sp. 9	Diplostomidae	Unknown	Cercariae	Great pond snail *(Lymnaea stagnalis)*
Neorickettsia sp. 10	Lecithodendriidae	*Lecithodendrium sp.*	Adult	Egypt
Neorickettsia sp. 11	Haploporidae	_____	Adult	Striped mullet (*Mugil cephalus*)
Neorickettsia sp. 12	Lecithodendriidae	*Paralecithodendrium sp.*	Adult	Horsfield's bat (*Myotis horsfieldii*)
Neorickettsia sp. 13	Lecithodendriidae	*Paralecithodendrium* n. sp.	Adult	Large-eared horseshoe bat (*Rhinolophus philippinensis*)
Neorickettsia sp. 14	Haploporidae	*Dicrogaster* n.sp.	Adult	Striped mullet (*Mugil cephalus*)
Neorickettsia risticii	Unknown	Unknown	Bat tissue	Free-tailed bats (*Tadarida brasiliensis*)

Table 11. *Neorickettsia* species/genotypes, digenean intermediate and definitive hosts, type of life cycle, and geographic origins.

***Neorickettsia* species**	**Definitive host**	**1st/2nd intermediate hosts**	**Life cycle**	**Locality**
Neorickettsia helminthoeca	Mammals	Aquatic snail/fishes	aquatic/ terrestrial (freshwater)	Corvallis, Oregon
Neorickettsia sp. 1	Fishes	Unknown / aquatic arthropod	aquatic (freshwater)	Pearl River, Mississippi
Neorickettsia sp. 2	Fishes	unknown	aquatic (brackish/ marine)	Cedar Key, Florida
Neorickettsia sp. 3	Mammals and amphibians	Aquatic snail/crustacean	aquatic/ terrestrial (freshwater)	Williford Springs Florida
SF agent	Mammals	Aquatic snail/amphibian	aquatic/ terrestrial (freshwater)	Williford Springs Florida
SF agent	Mammals	Aquatic snail/fishes	aquatic/ terrestrial (brackish/ freshwater)	Cat-Ba Island, Vietnam
SF agent	Mammals	Aquatic snail/fishes	aquatic/ terrestrial (Freshwater)	Corvallis, Oregon
Neorickettsia sp. 4	Fishes	Unknown	aquatic (brackish/ marine)	Daya Bay, Guangdong, China
Neorickettsia sp. 4	Fishes	Unknown	aquatic (brackish/ marine)	Talay Thai Sumut Sakhon, Thailand
Neorickettsia sp. 5	Fishes	Unknown	aquatic (brackish/ marine)	Eli Creek, Queensland, Australia
Neorickettsia sp. 6	Bats and birds	Aquatic snail / aquatic arthropod	aquatic/ terrestrial (freshwater)	Dunn Forest, Corvallis, Oregon
Neorickettsia sp. 7	Fishes and amphibians	Aquatic snail / aquatic arthropod	aquatic/ terrestrial (freshwater)	Dunn Forest, Corvallis, Oregon
Neorickettsia sp. 8	Birds and mammals	Aquatic snail / aquatic arthropod	aquatic/ terrestrial (freshwater)	Nelson county, North Dakota
Neorickettsia sp. 9	Birds	Aquatic snail / fishes	aquatic/ terrestrial (freshwater)	Nelson county, North Dakota

Table 11. cont.

Neorickettsia species	Definitive host	1st/2nd intermediate hosts	Life cycle	Locality
Neorickettsia sp. 10	Bats	Aquatic snail / aquatic arthropod	aquatic/ terrestrial (freshwater)	Egypt
Neorickettsia sp. 11	Fishes	Unknown	aquatic (brackish/ marine)	Cat-Ba Island, Vietnam
Neorickettsia sp. 12	Bats	Aquatic snail / aquatic arthropod	aquatic/ terrestrial (freshwater)	Sablyayan Municipality, Mindoro Island, Philippines
Neorickettsia sp. 13	Bats	Aquatic snail / aquatic arthropod	aquatic/ terrestrial (freshwater)	Sablyayan Municipality, Mindoro Island, Philippines
Neorickettsia sp. 14	Fishes	Unknown	aquatic (brackish/ freshwater)	Salt Springs, Florida, USA
Neorickettsia risticii	Bats	Unknown	Unknown	Buenos Aires, Argentina

The *Neorickettsia* sorted into three major, well supported clades (Figures 16 and 17). Clade I is represented by a single sequence from *Bacciger sprenti* (Faustulidae) collected in eastern Australia. Clade II includes 7 sequences, all of them representing yet unnamed lineages/species of *Neorickettsia* found either within various digenean families that use fish as definitive hosts or in fish tissues (EU780451). Three of these forms were newly sequenced genotypes, *Neorickettsia* sp. 1 (from *Crepidostomum affine* [Allocreadidae] in Mississippi), *Neorickettsia* sp. 2 (from *Saccocoeliodes beauforti* [Haploporidae] from Florida) and *Neorickettsia* sp. 14 (from *Dicrogaster* n. sp. [Haploporidae] from Florida). All internal sub-clades of clade II are well resolved and strongly supported. Six lineages of this group were collected from North America while one (EU780451) was from Southeast Asia.

Most *Neorickettsia* species belonged to Clade III including all three currently recognized named neorickettsial species *N. helminthoeca*, *N. risticii* and *N. sennetsu* (Figures 16 and 17). This large clade segregated into three sub-clades indicated as A, B, C on Figures 16 and 17. Sub-clade A contained a diverse assemblage of genotypes that included *N. helminthoeca* and *Neorickettsia* sp. 9 from a *Diplostomum* sp. (Diplostomatidae) (Tkach et al. 2012) as separate branches, and a well resolved cluster containing one form from Southeast Asia (EU780452) and three genotypes from the Antarctic (FJ456799, FJ456797, FJ456595) (Ward et al. 2009). All digenean hosts of the members of this sub-clade are parasitic in fish at least at some phase of their life cycle. Sub-clade B is 100% supported and contained the largest number (i.e., 14) of neorickettsial genotypes. However, internal interrelationships of its constituent taxa were less resolved than the topologies elsewhere in the tree. This sub-clade incorporates 6 *Neorickettsia* forms discovered in this study. The basal taxon in this sub-clade is *Neorickettsia* sp. 8 from *Plagiorchis elegans*. The closest derived taxon to it is *N. sennetsu*, the agent causing human disease in Southeast Asia. The remaining lineages of the sub-clade B (*N. risticii* clade) form a polytomy that includes several lineages of *N. risticii* and the SF agent. Sub-clade C is comprised exclusively of newly sequenced forms from Haploporid digeneans from China and Thailand (species 4) and from Vietnam (species 11). All, except one, species/genotypes of *Neorickettsia* in this clade are associated with digeneans who use mammals and/or birds as their definitive hosts.

In order to sort out the polytomy of sub-clade B (*N. risticii* clade) in the 16S phylogenic analysis, a second phylogenetic analysis was run based on GroESL gene sequences of the 13 *Neorickettsia* genotypes within the polytomy and *Neorickettsia*

helminthoeca as an outgroup (Figures 18 and 19). The alignment included a total of 1,788 sites, of which all could be aligned unambiguously. Bayesian analysis produced a well-structured consensus tree with strong support (Figure 18 and 19). Based on this analysis, *Neorickettsia* sp. 10 from Egypt and *Neorickettsia* spp. 12 and 13 from the Philippines (Clade II) formed a clade sister to the well supported clade including *N. sennetsu*, *N. risticii*, *Neorickettsia* sp. from *P. elegans*, and SF agent (Clade I). Clade I includes two subclades (A, B). Subclade A includes *N. sennetsu* and all genotypes of *N. risticii*. Subclade B includes SF agent and *Neorickettsia* sp. from *P. elegans*.

Discussion

Screening revealed 20 samples positive for *Neorickettsia* (representing 17 genetic lineages of the bacteria): *N. helminthoeca* from Oregon (dog blood), a new genotype 1 from Mississippi, new genotypes 2, 3, and SF agent from Florida, a new genotype 4 from China and Thailand, new genotype 5 from Australia, new genotypes 6 and 7, and SF agent from Oregon, new genotypes 8 and 9 from North Dakota, new genotype 10 from Egypt, new genotype 11 and SF agent from Vietnam, new genotype 12 and 13 from the Philippines, new genotype 14 from Florida, and *N. risticii* from Argentina (Table 10 and 11). Our findings represent the first records of *Neorickettsia* in digeneans in Australia, China, Egypt, Philippines, Thailand, Vietnam, and one state (Florida) in the USA. Fourteen digenean species detected as hosts of *Neorickettsia* in this study (Table 10) represent new host associations for *Neorickettsia*. Pusterla et al. (2000b) reported *Neorickettsia* from *Deropegus* sp. from California, however neither the species of *Deropegus* or *Neorickettsia* were identified or sequenced. Members of the families

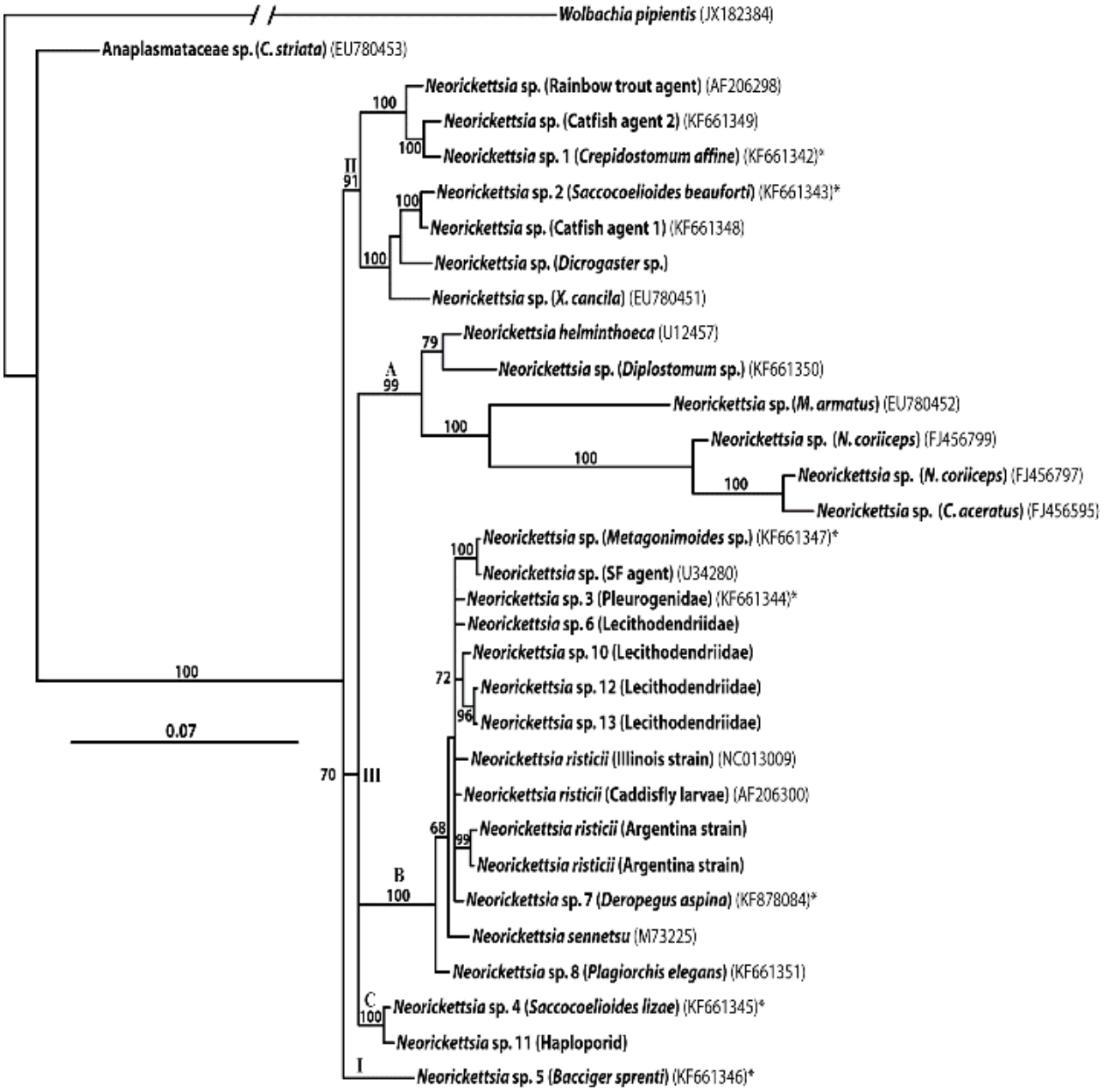

Figure 16. Phylogenetic relationships among 30 taxa of bacterial endosymbionts in the family Anaplasmataceae resulting from Bayesian analysis of partial sequences of 16S rDNA. Roman numerals (I,II,III) represent the different clades within the "*Neorickettsia* clade" and letters (A,B,C) correspond to the subclades within clade III.

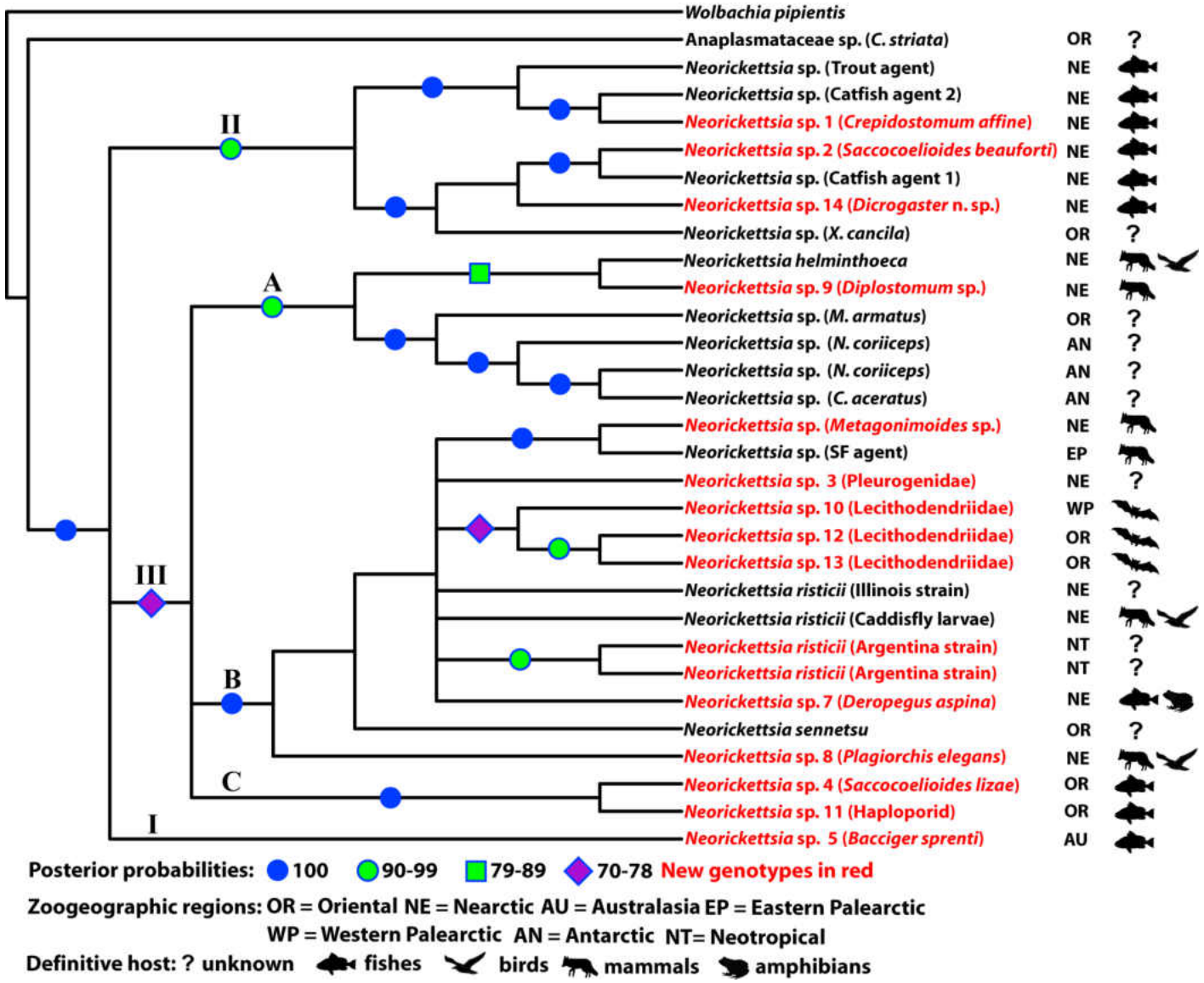

Figure 17. Phylogenetic tree resulting from Bayesian analysis of partial sequences of 16S rDNA.

Diplostomidae, Haploporidae, Pleurogenidae, and Faustulidae have not been previously reported as hosts of *Neorickettsia*.

Sampling of digenean DNA extracts for *Neorickettsia* was mostly opportunistic. Digeneans were collected for various projects prior to our study and only afterwards were used for detecting *Neorickettsia*. This explains the unevenness in the distribution of screened samples among digenean families. Digeneans were not targeted based on their likelihood of harboring the bacterial endosymbionts because very little is currently known about the evolutionary associations among *Neorickettsia* and their digenean hosts. Therefore, we screened as many samples and as diverse digenean taxa as possible**.** Our study is the first geographically and taxonomically broad screening of digeneans for the presence of *Neorickettsia*. Its purpose is to provide baseline data for future more focused studies within certain digenean families or those parasitic in certain hosts.

Sampling effort from Laos, Thailand, and Vietnam was more focused and aimed at finding the digenean host of *N. sennetsu* (the causative agent of Sennetsu fever in humans). As mentioned in Chapter 1, a recent study (Newton et al. 2009) revealed that a high seroprevalence of *N. sennetsu* (17% of 1,132 patients) in Vientiane municipality and Savannakhet Provinces in Laos. Newton et al. (2009) concluded that Sennetsu neorickettsiosis is a common infection in Laos. The same study showed a 4% seroprevalence in patients in Thailand. However, we did not find *Neorickettsia* within any of our digenean samples from Laos. This was likely due to the relatively small number of digenean samples collected from Laos, resulting from over hunting and fishing of natural vertebrate hosts of digeneans in the sampled regions of the country. Therefore, we decided to move our collecting to Vietnam and Thailand, both countries bordering

Laos (Figure 20) where animal conservation efforts are stronger, leading to a higher diversity of digeneans. In Northern Vietnam, during the fall of 2013, I collected a total of 2,402 snails from two sites, of which 137 were infected with digeneans (Table 12 and 13). Unfortunately, all snail samples from those two sites were negative for *Neorickettsia*. I also dissected a total of 75 fishes, representing at least 6 fish species.

From those dissections, a total of 30 fishes were infected with either larval or adult digeneans. I did identify two species/genotypes of *Neorickettsia* from these fish samples (Table 10), however, neither were *N. sennetsu*. During a collecting trip in southern Vietnam and Thailand in the winter of 2014, we collected a total of 1669 snails, and dissected a total of 114 fishes. From those dissections, a total of 75 snails and roughly 50 fishes were infected with digeneans. Although I have only screened a small number of those samples, I did identify one species/genotype of *Neorickettsia* within one of the adult fish digeneans. This genotype was not *N. sennetsu*, however, further screening efforts of digeneans from Vietnam and Thailand has a high potential of identifying the digenean host of *N. sennetsu*, as well as, discovering further *Neorickettsia* diversity.

Table 12. Vietnam site 1: Lat: 21°11' 55.60°N Long: 105°46' 57.98°E (Hanoi gated community)

Snail species	Number	Number shedding	Percent shedding
Thiara scabra	6	0	0%
Tarebia granifera	90	0	0%
Viviparus	16	0	0%
Melanoides tuberculata	452	45	10%
Bithynia longicornis	208	4	1.9%
Parafossarulus striatulus	751	27	3.6%
TOTAL	**1523**	**76**	**5%**

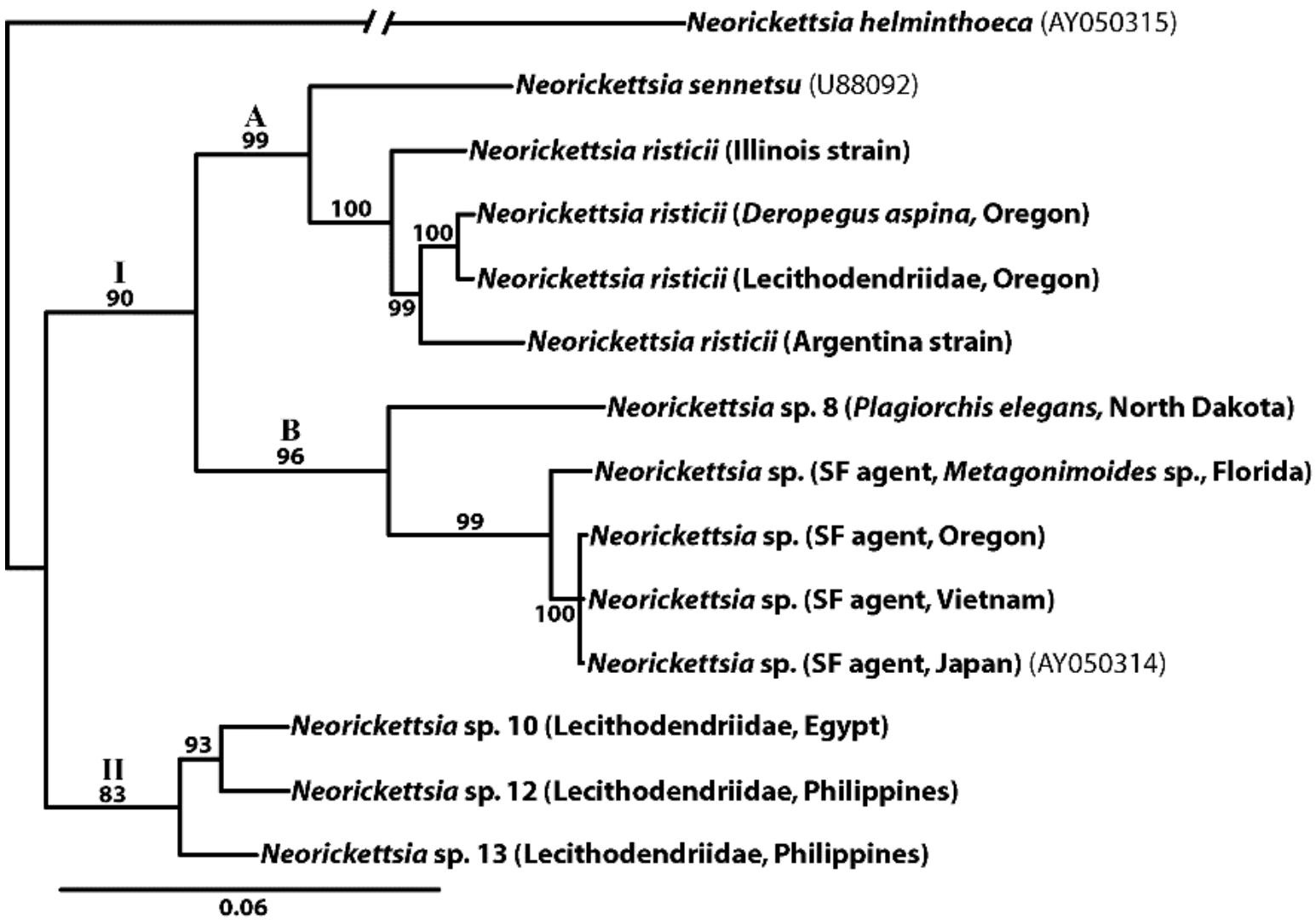

Figure 18. Phylogenetic relationships among 14 taxa of bacterial endosymbionts in the family Anaplasmataceae resulting from Bayesian analysis of partial sequences of the GroESL operon. Roman numerals (I,II) represent the different clades within the "*Neorickettsia risticii* clade" and letters (A,B) correspond to the subclades within clade I.

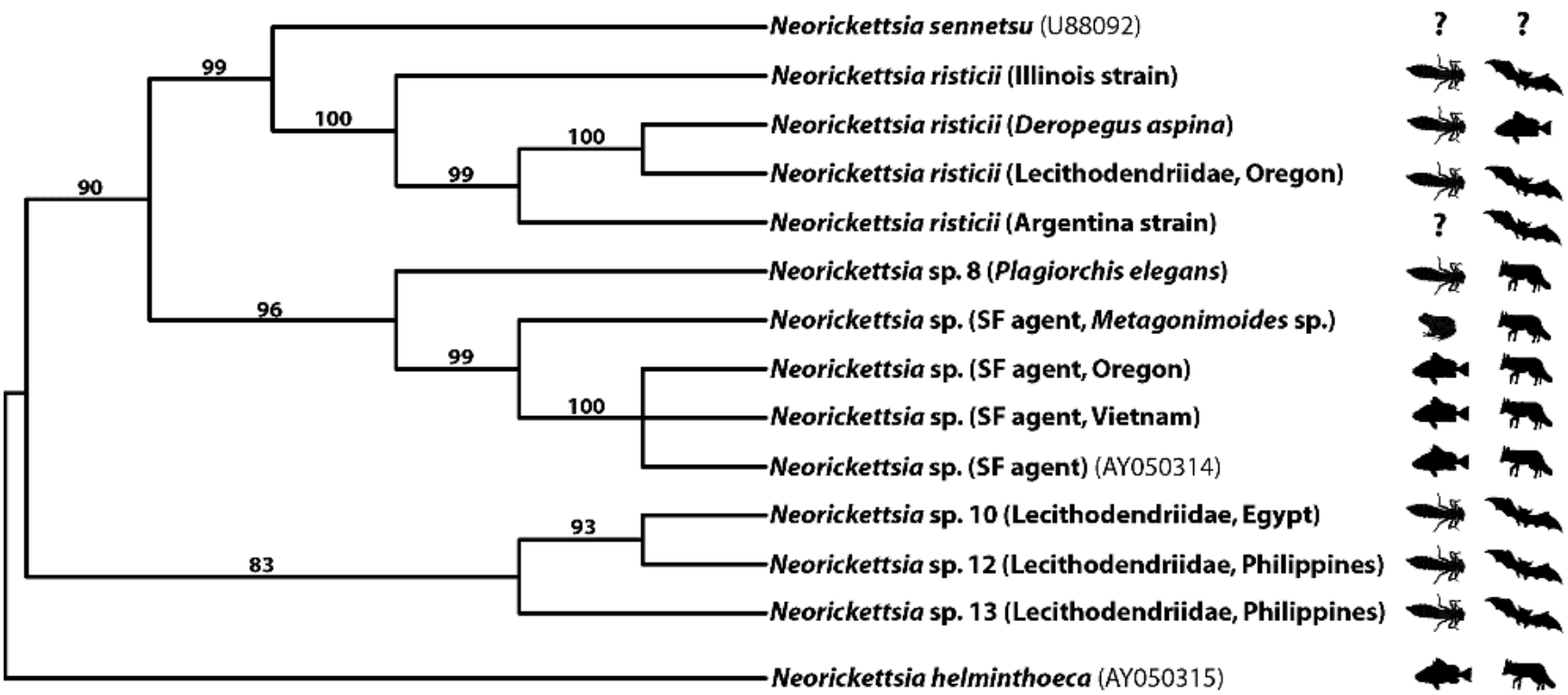

Figure 19. Phylogenetic relationships among 14 taxa of bacterial endosymbionts in the family Anaplasmataceae resulting from Bayesian analysis of partial sequences of the GroESL operon. First row of symbols represents the second intermediate hosts of the digenean hosts and the second row of symbols represents the definitive hosts of the digenean hosts.

Table 13. Vietnam site 2: Lat: 19°46' 23.44°N Long: 105°49' 12.29°E (Jhangh Hoa)

Snail species	Number	Number shedding	Percent shedding
Bithynia fuchsiana	200	7	3.5%
Parafossarulus striatulus	48	12	25%
Melanoides tuberculata	572	37	6.5%
Stenothyca messageri	42	3	7.1%
Lymnaea viridis	17	2	11.8%
TOTAL	**879**	**61**	**6.9%**

Figure 20. Map of Southeast Asia, red stars indicating collections sites in Laos, Philippines, Thailand, and Vietnam (edited from Map of Southeast Asia with Countries by FreeVectorMaps.com).

This study has revealed a new circulation pathway of *Neorickettsia* in the natural environment. Until now, *Neorickettsia* have been found in digeneans with entirely freshwater, freshwater/terrestrial or entirely terrestrial life cycles (Tkach et al. 2012; Vaughan et al. 2012) but not in digeneans with completely marine life cycles. Reports of

Neorickettsia in tissues of notothenioid fishes (*Notothenia coriiceps* and *Chaenocephalus aceratus*) in Antarctica (Ward et al. 2009) and mullet *Mugil cephalus* in the Gulf of Mexico (Larsen et al. 2013) suggested a potentially marine circulation pathway, however, neither of these studies screened any digeneans. We have found three different forms of *Neorickettsia* (genotypes 2, 4 and 5) from three digenean species with completely marine life cycles, namely *Saccocoeliodes beauforti*, *Saccocoelioides lizae* and *Bacciger sprenti*. This suggests that *Neorickettsia* can be encountered in almost every type of environment suitable for digenean life cycles.

According to the 16S phylogeny, *Neorickettsia* sp. 9 from *Diplostomum* sp. collected in North Dakota, is most closely related to *N. helminthoeca*, agent of salmon dog poisoning in the Pacific Northwest. Although it is likely a new species, based on sequence similarity (1298 of 1325 nucleotide (98%) similarity), *Neorickettsia* sp. 9 may have similar pathogenicity in dogs or other piscivorous vertebrates. Unfortunately, we only collected a single snail shedding *Neorickettsia* infected *Diplostomum* cercariae and at the time we did not know its relationship to *N. helminthoeca*. Further screening of snails in ND for *Neorickettsia* infected *Diplostomum* cercariae could provide an opportunity to test the pathogenicity of this species of bacteria through live animal challenges or sequencing of genes related to pathogenicity.

Neorickettsia genotypes obtained from *Metagonimoides oregonensis* in Florida, *Stellantchasmus falcatus* in Vietnam, and *Nanophyetus salmincola* in Oregon fully matched the 16S sequence of SF agent. GroESL sequences showed more variability, compared to SF agent from Japan; *i.e.*, SF agent from Florida had 17 bp differences (out of 1792 bp), SF agent from Vietnam had 2 bp differences, and SF agent from Oregon had

1 bp difference. SF agent was initially found in metacercariae of the heterophyid *Stellantchasmus falcatus* infecting grey mullet in Japan (Fukuda et al. 1973; Fukuda and Yamamoto 1981). Our findings represent the first records of SF agent isolated from a digenean outside Japan. Both *S. falcatus* and *M. oregonensis* belong to the family Heterophyidae, therefore, there is a probability that SF agent could be found in other members of this large digenean family, members of which are parasitic primarily in fish-eating birds and mammals including humans. SF agent causes mild clinical symptoms in experimentally infected mice and dogs, but not monkeys or humans (Hirai 1966; Mott et al. 1997; Rikihisa 1991; Shishido et al. 1965; Tachibana 1986; Wen et al. 1996). Thus, it cannot be excluded that it may cause yet unknown disease in wildlife.

Finding SF agent in the digenean *N. salmincola* is very interesting for several reasons. First, it is the first report of SF agent in a digenean in a family other than Heterophyidae, leading to the possibility that SF agent could be found in a greater diversity of digeneans than other genotypes of *Neorickettsia*. Second, this is the first report of a single digenean species (*i.e., N. salmincola*) serving as the host for more than one *Neorickettsia* species. This implies that the fidelity between endosymbiont and digenean host is not absolute and that host-switching among neorickettsial species can occur. Third and most interesting, *N. salmincola* is the host of *N. helminthoeca*, the causative agent of salmon poisoning disease of dogs. As mentioned in Chapter 1, it is well known that when dogs are infected with *N. helminthoeca* they become immune to re-infection. However, according to veterinarians in Oregon, some dogs who were previously infected with *N. helminthoeca* are returning to their clinics showing symptoms of salmon poisoning disease (Dr. Michael Kent, personal communication). SF agent is

known to cause mild clinical symptoms in experimentally infected dogs, therefore, it could be possible that dogs in Oregon are being infected both with *N. helminthoeca* and SF agent, especially considering they can use the same digenean host, *N. salmincola*. *Nanophyetus salmincola* is known to serve as a vector for an additional species of *Neorickettsia* that causes the disease "*Elokomin fluke fever"* (EFF). As mentioned in Chapter 1, in 1973, Farrell and colleagues published a series of papers describing what they designated as a second neorickettsial agent transmitted by *N. salmincola* (Farrell et al. 1973; Sakawa et al. 1973; Kitao et al. 1973). Trout naturally infected with metacercariae were fed to 6 captive bears, 4 of which developed fever, anorexia and 'lassitude'. Upon autopsy, the sick bears had swollen mesenteric lymph nodes suggestive of salmon poisoning. Three of the bears had detectable rickettsial bodies in lymph node impression smears. Upon injection of individual lymph suspensions from 5 autopsied bears into groups of 4 to 6 dogs each, 72% of the dogs developed low-grade fever and diarrhea for 4 to 12 days, but did not die. Serial passage of blood during the febrile state into fresh dogs produced similar, mild symptoms. When convalescent dogs were then fed metacercariae-infested trout 3 months later, 87% died of SPD. Later studies confirmed that EFF agent was immunologically distinguishable from both *N. helminthoeca* and *N. sennetsu* by complement fixation (Sakawa et al. 1973), immunofluorescent antibody tests and live animal challenges (Kitao et al. 1973). No DNA sequences are available for the *Neorickettsia* species responsible for EFF. We hypothesize that SF agent is the cause of EFF, due mainly to its disease similarity in dogs, and its use of *N. salmincola* as a vector.

Our 16s phylogenetic analysis has produced a tree with strong branch support for most topologies (Figures 16 and 17). Our data corroborate the conclusion by Seng et al.

(2009) who suggested that their sequence obtained from *Channa striata* most likely represents a separate genus of Anaplasmataceae. On the other hand, the Anaplasmataceae sp. from *Mastacembelus armatus* considered by Seng et al. (2009) to be another new genus, clearly falls into the clade II of the "*Neorickettsia* clade"(Figures 16 and 17). *Neorickettsia* sp. 5 genotype is of particular interest because it represents a separate clade of *Neorickettsia* with unresolved affinities to other members of the genus. Denser sampling of digeneans from both marine and freshwater habitats in Australia is necessary to see whether *Neorickettsia* sp. 5 is a unique, divergent form or a member of a larger lineage that includes additional, yet undiscovered, taxa. An additional systematically relevant result of the present phylogenetic analysis is the position of the *Neorickettsia* strain from *Plagiorchis elegans*. This form was initially identified by Tkach et al. (2012) as *N. risticii* based on the comparison of much shorter DNA sequences and a phylogenetic analysis using those shorter sequences and fewer taxa. In the analysis by Tkach et al. (2012) this genotype appeared in a polytomy that included 4 different isolates of *N. risticii* and *N. sennetsu*. The present analysis based on longer sequences and greater number of taxa places "*N. risticii*" from *P. elegans* as the basal lineage in sub-clade B of clade III (Figure 16). It is separated from the remaining taxa in this clade by one of the recognized distinct species, *N. sennetsu*. Thus, this genotype likely represents a different, novel species of *Neorickettsia* which needs to be better characterized and differentiated from formally named taxa.

Some of the clades in the phylogenetic tree show close associations among *Neorickettsia* and the definitive hosts of digeneans in which they were found. For instance, the strongly supported clade II notably comprises neorickettsiae obtained from

digeneans parasitic only in fish, mostly in North America (Figure 17). This clade shows the clearest association with a group of definitive hosts of digeneans. Another group that demonstrates a distinct pattern of associations with definitive hosts of digeneans is the sub-clade B of clade III. Digenean hosts of neorickettsiae in this sub-clade are parasitic in either birds or mammals with the exception of few cases where the vertebrate hosts of digeneans are unknown and *Neorickettsia* sp. 7 found in *Deropegus aspina*, a digenean that uses fish and amphibians as a definitive host (Figure 17). *Neorickettsia sennetsu*, a causative agent of the human disease Sennetsu fever in Southeast Asia, is one of the species with yet unknown digenean hosts. Nevertheless, the phylogenetic tree topology allows us to hypothesize that it should be a digenean that uses either mammals or birds as a definitive host. More importantly, because most genotypes (*N. sennetsu* is unknown) within sub-clade A of clade I (Figures18 and 19) utalize digeneans with arthropods (mostly insects) as second intermediate hosts we can hypothesize that the digenean host of *N. sennetsu* also uses arthropods as a second intermediate host and not fishes.

The least resolved part of the tree is the polytomy in the sub-clade B of clade III that includes several genotypes occupying a derived position in relation to *N. sennetsu* (Figures 16 and 17). The groESL phylogenetic analysis helped resolve the relationships among a majority of these taxa and has produced a tree with strong branch support for all topologies (Figures 18 and 19). The groESL operon was used for the second phylogenetic analysis based on its use by Dummler et al. 2001, where groESL clades strongly supported the 16s rRNA gene analyses for all members of the family Anaplasmataceae. The new genotypes (spp. 10, 12, 13) (from Egypt and the Philippines) identified in this study fall into a clade that is sister to the well supported clade including *N. risticii*, *N.*

sennetsu, *Neorickettsia sp.* (from *P. elegans*) and SF agent. This brings up the potential question regarding the pathogenicity of the new genotype of *Neorickettsia* from Egypt and the Philippines. *Neorickettsia risticii*, *N. sennetsu*, and SF agent are all known to cause potentially debilitating or even fatal diseases in the vertebrate host of the digenean (Vaughan et al. 2012). *Neorickettsia* spp. 10, 12 and 13 from lecithodendriids from Egypt and the Philippines are closely related to these three species of *Neorickettsia* and therefore have the potential of causing disease in domestic animals or wildlife. Further studies with these three genotype need to be done to determine their true pathogenicity.

Patterns of geographic distribution of most of the digenean species harboring *Neorickettsia* in this study are varying. *Crepidostomum affine* was recently described by Tkach et al. (2012) from *Hiodon tergisus* collected from Pearl River and Pascagoula River drainages in Mississippi, USA. Based on a molecular comparison to other species in the genus by Tkach et al. (2012), *C. affine* is likely endemic to these river basins in Mississippi. Therefore, we hypothesize that the distribution of *Neorickettsia* sp. 1 is likely to be limited to that of *C. affine*.

Mugil cephalus (grey mullet), the host of four digenean species harboring *Neorickettsia* in this study (Table 11), is a globally distributed marine fish. However, digeneans *Saccocoelioides beauforti, Saccocoelioides lizae*, *Saccocoelioides* sp., and *Dicrogaster* sp. appear to be much more restricted in their distribution than their fish host. One of them, *Saccocoelioides beauforti* has only been reported from the southeastern United States, namely from North Carolina, Louisiana, Mississippi, and Alabama (Hunter and Thomas 1961; Overstreet 1971). *Saccocoelioides lizae* has only been recorded off the cost of southeastern China (Liu 2002), however, we have now

found it off the northern coast of Thailand. The distribution of *Saccocoelioides* sp. from northern Vietnam is unknown, however, the sequence of *Neorickettsia* sp. 11 differs from *Neorickettsia* sp. 4 from China and Thailand in only 3 bp, likely indicating that its distribution is limited to the marine habitats in the region. The last species, *Dicrogaster* n. sp., is a new species from Salt Springs, Florida. Salt Springs is an artesian spring that discharges brackish water in Lake George. This new haploporid is likely endemic to this region, and therefore the genotype/species of *Neorickettsia* (sp. 14) is likely unique to this area. Based on the combined distribution data of these digeneans and their fish host, it is likely that *Neorickettsia* sp. 2 may be found in other areas along the coast of the southeastern United States and *Neorickettsia* spp. 4 and 11 may be found in other costal countries in Southeast Asia.

Deropegus aspina is only known from the Pacific Northwest of the United States in salmonid fishes, including *Salmo clarki*, *S. gairdneri*, *Oncorhynchis kisutch*, *O. tshawytscha* and a frog, *Rana boyli* (McCauley and Pratt 1961). Therefore, we hypothesize that *Neorickettsia* sp. 7 is limited in its distribution to the Pacific coast of the USA and Canada.

Bacciger sprenti (family Faustulidae) carrying *Neorickettsia* in our study was obtained from fish *Selenotoca multifasciata* (Table 2). This digenean species was originally identified from the intestine of a *Mugil* sp. in Australia (Bray 1982). However, later research by Cribb et al. (1999) only found it in *Selenotoca multifasciata* (13 individuals were infected) and not in 11 individuals of *Mugil cephalus* or 49 individuals of *Mugil georgii*. This prompted Cribb et al. (1999) to speculate that an error was made in the recording of the host fish species in the original description by Bray (1982). This is in

concordance with available data on host associations of other faustulid trematodes, which are not known to be shared between scatophagid and mugilid fishes (Cribb et al. 1999). Currently *Bacciger sprenti* has only been reported from marine fishes in Australian coastal waters, therefore, we can assume *Neorickettsia* sp. 5 is a genotype unique to Australia.

Digeneans in the family Lecithodendriidae parasitic in bats appear to be common hosts of *Neorickettsia*, with a total of 7 species of lecithodendriids and one bat (tissue, parasites not looked for) found to harbor the bacterial endosymbiont. This is a relatively large digenean family comprising at least 12 genera parasitizing bats and occasionally birds, on all continents (Lotz and Font 2008). Currently, lecithodendriids have been found to harbor the bacterial endosymbiont from Egypt, North America (USA), South America (Argentina), and the Philippines.

Currently known distribution of *Neorickettsia* is very geographically uneven and is clearly associated with areas where most studies have taken place. The majority of genotypes were found in the USA and several countries in southeastern and eastern Asia (Vaughan et al. 2012). Even after our discovery of *Neorickettsia* in Australia, China, Egypt and the Philippines, there is no well-documented information supported by PCR/sequence data from Africa (except Egypt), Europe, most of South America, most of Asia, and nearly all island countries (Vaughan et al. 2012). We believe that this situation does not reflect the true distribution of *Neorickettsia* and rather reflects insufficient knowledge due to the lack of broad screening efforts. With more than 18,000 described species of digeneans (Cribb et al. 2001), there is a potential for many more species/genotypes of *Neorickettsia* to be found and characterized.

Chapter IV

TRANSMISSION RATES OF THE BACTERIAL ENDOSYMBIONT, *NEORICKETTSIA* SP., DURING THE ASEXUAL REPRODUCTION PHASE OF ITS DIGENEAN HOST *PLAGIORCHIS ELEGANS*, WITHIN NATURALLY INFECTED LYMNAEID SNAILS

Abstract

Quantitative information on the transmission of neorickettsial endosymbionts throughout the complex life cycles of digeneans is lacking. This study quantified the transmission of *Neorickettsia* during the asexual reproductive phase of a digenean parasite, *Plagiorchis elegans*, developing within naturally parasitized lymnaeid pond snails. *Lymnaea stagnalis* snails were collected from 3 ponds in Nelson County, North Dakota and screened for the presence of digenean cercariae. Cercariae were identified to species by PCR and sequencing of the 28S rRNA gene. *Neorickettsia* infections were initially detected using nested PCR and sequencing of partial 16S rRNA gene of pooled cercariae shed from each parasitized snail. A total of 616 *L. stagnalis* were collected and 240 (39%) shed digenean cercariae. Of these, 18 (8%) were *Neorickettsia*-positive. Six *Neorickettsia* infections were selected to determine the transmission efficiency of *Neorickettsia* from mother to daughter sporocyst and from daughter sporocyst to cercaria. The prevalence of neorickettsiae in cercariae varied from 11 to 91%. The prevalence of neorickettsiae in sporocysts from one snail was 100%. Prevalence of *Neorickettsia* infection in cercariae of *Plagiorchis elegans* was variable and never reached 100%.

Reasons for this are speculative, however, the low prevalence of *Neorickettsia* observed in some of our samples (11 to 52%) is in contrast with the high prevalence of other, related bacterial endosymbionts, e.g. *Wolbachia* in *Wolbachia*-dependent filariid nematode, where the prevalence among progeny is universally 100%. This suggests that, unlike the *Wolbachia*-filaria relationship, the *Neorickettsia*-digenean relationship is not obligatory mutualism. Our study represents the first quantitative estimate of the *Neorickettsia* transmission through the asexual phase of digenean life cycle.

Results

Out of 616 *L. stagnalis* collected in all three ponds, 240 shed digenean cercariae (Table 14). Eighteen (8%) of the 240 cercarial infections were *Neorickettsia*-positive. Prevalences of *Neorickettisia* in single cercariae and sporocysts were only studied from Pond 2. This pond had the highest prevalence of *Neorickettsia* (23%) in digeneans, although not the highest prevalence of digeneans in snails (Table 14). Using DNA sequencing, we were able to identify both the digenean host and the neorickettsial endosymbiont. All *Neorickettsia*-positive infections represented a single digenean species, a plagiorchiid *Plagiorchis elegans*, however, not all *P. elegans* screened were contained *Neorickettsia* DNA. Sequences of cercariae were identical to the sequence of adult *P. elegans* from the same area published by Tkach et al. (2012).

We used 5 *P. elegans* infections for analysis of the efficiency of *Neorickettsia* transmission through the digenean life cycle. All 5 samples of *P. elegans* harbored *Neorickettsia* sp.. The prevalence of individually screened cercariae infected with *Neorickettsia* varied from 11% to 91% among the 5 digenean samples (Table 15). Fifty sporocysts and 50 cercariae were screened from one of the snails. In this case, all

sporocysts assayed were positive for *Neorickettsia* sp., but only 90% of cercariae from the same snail had neorickettsial infection (Table 15; χ^2 with Yates correction=3.37, df=1, p=0.07).

Table 14. Infection prevalences for digeneans in snails (column 2) and *Neorickettsia* in digeneans. All three ponds are in Nelson Co., ND, USA.

	Prevalence of digeneans = % in snails (N)	**Prevalence of *Neorickettsia* = % among snails infected with digeneans (N)**
Pond 1	9% (100)	11% (9)
Pond 2	25% (187)	23.4% (47)
Pond 3	56% (329)	3.3% (184)
Total	39% (616)	7.5% (240)

Table 15. Prevalence of *Neorickettsia* infection among individual cercariae and sporocysts parasitizing field collected snails in Nelson Co., North Dakota, USA

Digenean species	**Life cycle stage**	**% positive (N)**
Plagiorchis elegans	cercariae	11% (100)
Plagiorchis elegans	cercariae	52% (100)
Plagiorchis elegans	cercariae	70% (50)
Plagiorchis elegans	cercariae	91% (100)
Plagiorchis elegans	cercariae*	90% (50)
Plagiorchis elegans	sporocysts*	100% (50)

* sporocysts and cercariae obtained from the same snail individual

Discussion

Transmission of *Neorickettsia* through the complex digenean life cycle does not fit easily into the classical categories of vertical transmission. Due to the presence of both sexual and asexual stages in digenean life cycles it is clear that the vertical transmission of *Neorickettsia* through the digenean life cycle is not wholly dependent on transovarian transmission as in the case of some other bacterial pathogens/symbionts of invertebrates.

Although all three ponds surveyed in the study area produced some snails harboring *Neorickettsia*-infected digeneans (Table 14), the prevalence of *Neorickettsia* infection between ponds differed dramatically between ponds, from only 3.3% in Pond 3 to 23.4% in Pond 2. At the same time, the highest prevalence of digenean infections in snails was in Pond 3 and reached 56% vs 25% in Pond 2 (Table 14). This illustrates the highly heterogenous nature of *Neorickettsia* distribution within a landscape and has certain implications for the epidemiology of the diseases caused by these bacteriae.

It is noteworthy that both low and high prevalences of neorickettsial infections of individual cercaria were found in the same parasite species (*P. elegans*), the same snail species (*L. stagnalis*), and the same body of water (Pond 2; Table 15). Thus, the observed differences in neorickettsial prevalence in single cercariae were not correlated with the digenean host, snail host, or locality. Overall, our data suggest that there is a lot of inherent variability in the efficiency of vertical transmission of *Neorickettsia* during asexual reproduction of digeneans. The reasons for this variability are currently unknown and may depend on a number of factors.

Our data on the prevalence of neorickettsial infection in sporocysts and their cercarial progeny from the same snail (Table 15) indicate that most, but not all, progeny

produced by an infected sporocyst inherit the bacterial endosymbiont (Figure 21). Perhaps, the initial intensity of *Neorickettsia* infection in egg and/or sporocyst may determine the proportion of cercarial progeny inheriting neorickettsial endosymbionts. For example, a sporocyst infected with high bacterial load may transmit *Neorickettsia* to a larger proportion of its cercarial progeny than a sporocyst with fewer bacteriae. This "dosage effect" would be similar to what has been described in the transovarial infection of ticks with *Rickettsia rickettsii*. Female ticks with high intensity of infection transmitted rickettsiae to 100% of their progeny, those with mild infections produced considerably lower percentages of infected eggs (Burgdorfer and Brinton 1975).

Alternatively, the rate of neorickettsial replication may be slower than the rate of development and replication of digenean asexual stages. If true, this asynchrony could result in a lower *Neorickettsia* prevalence in cercariae produced by snails that have only recently begun shedding compared to snails with older digenean infection.

Transmission in the digenean-*Neorickettsia* system is different from that of the other members of the family Anaplasmataceae symbiotic with invertebrates. The Anaplasmataceae includes 3 or 4 (depending on the systematic views of different authors) other genera associated with either ticks and other arthropods (*Ehrlichia*, *Anaplasma*, *Wolbachia*) or symbiotic in filarial nematodes (*Wolbachia*). In the case of *Ehrlichia* and *Anaplasma*, vertical transmission plays little role in their circulation (Dumler et al. 2001; Headly et al. 2011). In contrast, *Wolbachia* infects the ovaries and other tissues of many species of arthropods and filarial nematodes and is transmitted exclusively by vertical transmission (Landmann et al. 2012; Ferri et al. 2011; Werren et al. 1995). Thus, *Neorickettsia* is unique among Anaplasmataceae because it can be transmitted both

vertically and horizontally. In this respect, the patterns of transmission exhibited by *Neorickettsia* are more similar to those observed among pathogenic spotted fever group *Rickettsia* (e.g., *R. rickettsii*, family Rickettsiaceae, order Rickettsiales) (Schriefer and Azad 1994).

Like *Neorickettsia*, *Wolbachia* is maintained through vertical transmission, however, there are key differences between the two genera of bacteria. *Wolbachia* and the filariae have a mutualistic relationship (Dumler 2001; Pusterla et al. 2000). Ferri et al. (2011) supposed that the bacteria may be essential to the biosynthesis of some molecules necessary for filarial host fertility and viability, such as heme, riboflavin or nucleotide synthesis. As a result, filarial species that host *Wolbachia*, are dependent on the presence of these endosymbionts. Landmann et al. (2012) showed that filarial nematodes infected with *Wolbachia* pass it on to 100% of eggs. The exact nature of the interrelationships between *Neorickettsia* and their digenean hosts is not known. Current evidence suggests that digeneans do not have a mutualistic relationship with *Neorickettsia*. In our study, the majority of *P. elegans* did not harbor *Neorickettsia*, which proves that these digeneans are not dependent on neorickettsial endosymbionts. Presently available data are insufficient to categorize the endosymbiotic relationships of *Neorickettsia* with digeneans as either parasitic or commensal.

Conclusions

Our data demonstrate that the transmission efficiency of *Neorickettsia* through asexual stages of *P. elegans* life cycle is lower than 100% (Figure 21). In cercariae from naturally infected snails, the prevalence of *Neorickettsia* ranged from 11 to 91%. Even in a case where 100% of screened daughter sporocysts harbored *Neorickettsia*, only 90% of

the cercarial progeny were infected. These findings are in contrast with the situation in some other bacterial endosymbionts, such as *Wolbachia* in *Wolbachia*-dependent filariid nematodes, where the vertical transmission rates are 100%.

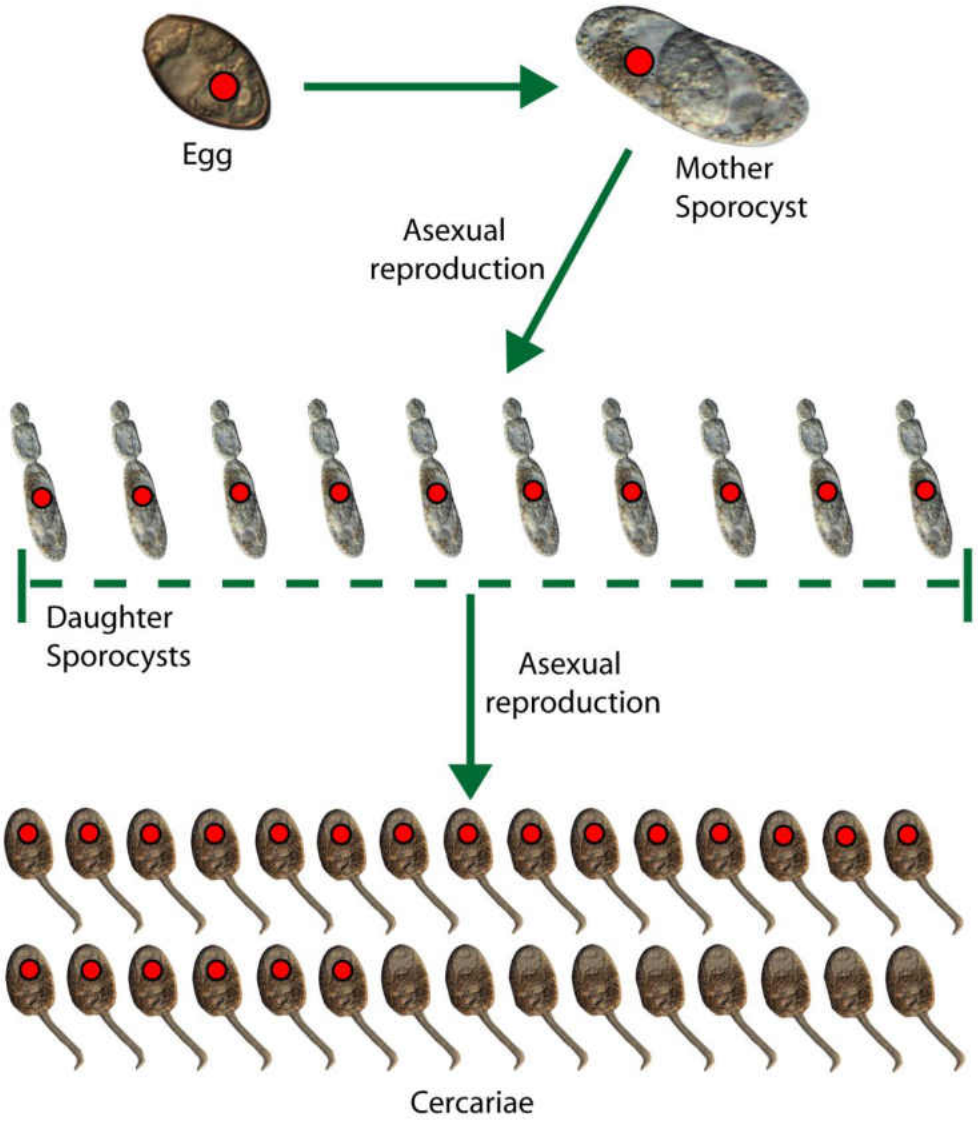

Figure 21. Transmission efficiency of *Neorickettsia* through asexual stages of *P. elegans* life cycle. Infection with *Neorickettsia* is represented by a red dot.

Chapter V

QUANTIFICATION OF THE BACTERIAL ENDOSYMBIONT, *NEORICKETTSIA* SP., WITHIN ALL LIFE CYCLE STAGES OF THE DIGENEAN HOST BY USE OF REAL-TIME qPCR ANALYSIS TARGETING THE GroEL GENE

Abstract

This study quantified the abundance of *Neorickettsia* within all stages of the life cycle of the digenean *Plagiorchis elegans*. *Lymnaea stagnalis* snails were collected from a single pond in Towner County, North Dakota and screened for the presence of digenean cercariae. Cercariae were identified to species by PCR and sequencing of the 28S rRNA gene. *Neorickettsia* infections were initially detected using real-time PCR targeting a 152-bp portion of the 3' end of the heat shock protein coding gene, GroEL. Three *L. stagnalis* were found that were shedding *P. elegans* cercariae infected with *Neorickettsia*. These three snails were used to initiate three separate laboratory life cycles and obtain all life cycle stages for bacterial quantification. A quantitative real time PCR assay targeting the GroEL gene was developed to enumerate *Neorickettsia* sp. within all stages of the digenean life cycle. The number of bacteria significantly increased throughout all stages of the digenean life cycle, from eggs to adults. The two largest increases in number of bacteria occurred from eggs to cercariae and from 6 day metacercariae to 48 hour juvenile worms. This is the first study to quantify *Neorickettsia* within all life cycle stages of a digenean.

Results

Neorickettsia sp. qPCR Assays

For *Neorickettsia* sp. within all stages of the digenean life cycle, 10-fold serial dilutions of qPCR standards were used to generate a standard curve. The standard curves for all assays were highly linear (R^2>0.99), within the range of 100 to 100,000,000 GroEL copies per PCR (Figures 22 and 23). The PCR efficiency (E) was calculated by the slope of the standard curve as follows: $E=10^{(-1/slope)}-1$ (e.g., E=1, or 100%). Efficiency was greater than 95% for all life cycle stages and replicate life cycles.

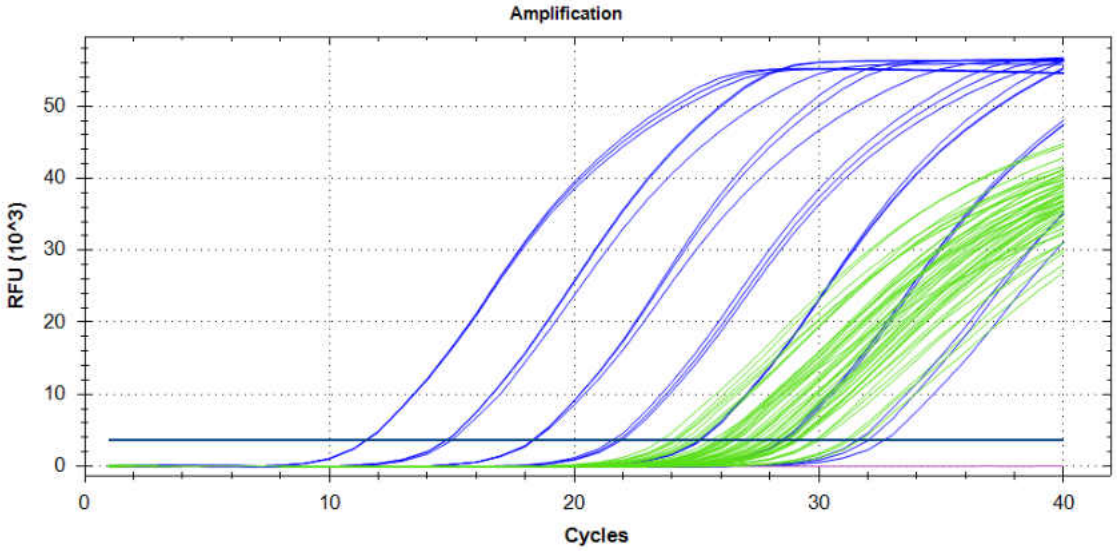

Figure 22. Amplification curve for 50 individual 6 day metacercariae, dark blue indicates the standards, green indicates the individual metacercariae, and purple indicates negative control. The dark purple horizontal line indicates the C_t (threshold cycle).

Quantification of *Neorickettsia* sp. in Cercariae

Fifty complete cercariae from three individual snails each were assayed separately to determine the number of GroEL copies/cercaria. *Neorickettsia* sp. was detected in 149/150 cercariae samples. The number of detected GroEL copies (=bacterial cells) per single cercaria varied from as little as 102 to as many as 36,115 (Figure 24).

Fifty of 50 (100%) cercariae from snail 1 (parasite group 1) were positive for *Neorickettsia*, 49 of 50 (98%) from snail 2 (parasite group 2) were positive, and 50 of 50 (100%) from snail 3 (parasite group 3) were positive. Based on a 1-way ANOVA there was no significant difference in the number of GroEL copies/cercaria between the three snails (parasite groups) ($F_{(2,147)}$=2.49, P=0.0866) (Figure 24).

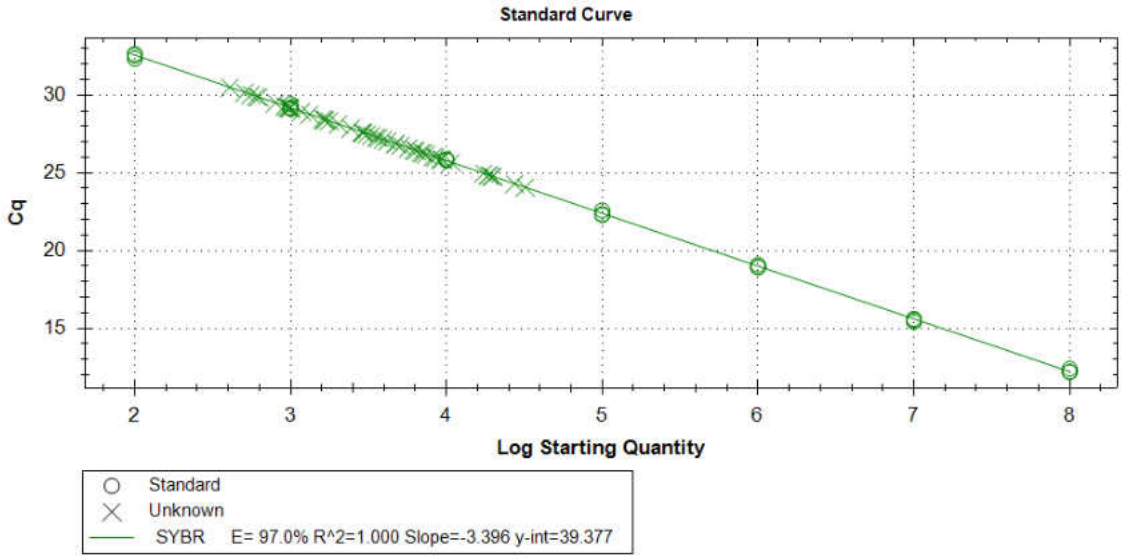

Figure 23. Standard curve for 50 individual 6 day metacercariae, circles represent the 7 standards (in triplicate), X's represent the individual metacercariae, and SYBR indicates the fluorophore used (SYBR Green).

Quantification of *Neorickettsia* sp. in Metacercariae (24 Hour/pre-infective)

Fifty complete pre-infective metacercariae, 24 hours post infection within *Culex* larvae, from the three parasite groups each were assayed separately to determine the number of bacteria per pre-infective metacercaria. *Neorickettsia* sp. was detected in 149/150 24 hour metacercariae samples. The number of detected GroEL copies (=bacterial cells) per a single pre-infective metacercaria varied from as little as 133 to as many as 48,935 (Figure 25). Fifty of 50 (100%) pre-infective-metacercariae from parasite group 1 were positive for *Neorickettsia*, 49 of 50 (98%) from parasite group 2 were

positive, and 50 of 50 (100%) from parasite group 3 were positive. Based on a 1-way ANOVA there was a significant difference in the number of GroEL copies/pre-infective metacercaria among the 3 parasite groups ($F_{(2,147)}$=3.7532, P=0.0257). Based on the results of the ANOVA a Tukey-Kramer test was run. The Tukey-Kramer test showed that number of GroEL copies/metacercaria was significantly different between parasite group 2 and 3 (Figure 25).

Quantification of *Neorickettsia* sp. in Metacercariae (6 Days/infective)

Fifty complete infective metacercariae, 6 days post infection within *Culex* larvae, from the three parasite groups each were assayed separately to determine the number of GroEL copies/metacercaria. *Neorickettsia* sp. was detected in 149/150 infective metacercariae samples. The number of detected bacteria per single infective metacercaria varied from as little as 661 to as many as 157,450 (Figure 26). Fifty of 50 (100%) infective metacercariae from parasite group 1 were positive for *Neorickettsia*, 49 of 50 (98%) from parasite group 2 were positive, and 50 of 50 (100%) from parasite group 3 were positive. Based on a 1-way ANOVA there was a significant difference in the number of GroEL copies/infective-metacercaria among the 3 parasite groups ($F_{(2,147)}$=5.1215, P=0.0071). Based on the results of the ANOVA a Tukey-Kramer test was run. The Tukey-Kramer test showed that number of GroEL copies/metacercaria was significantly different between parasite groups 1 and 3, and 2 and 3 (Figure 26).

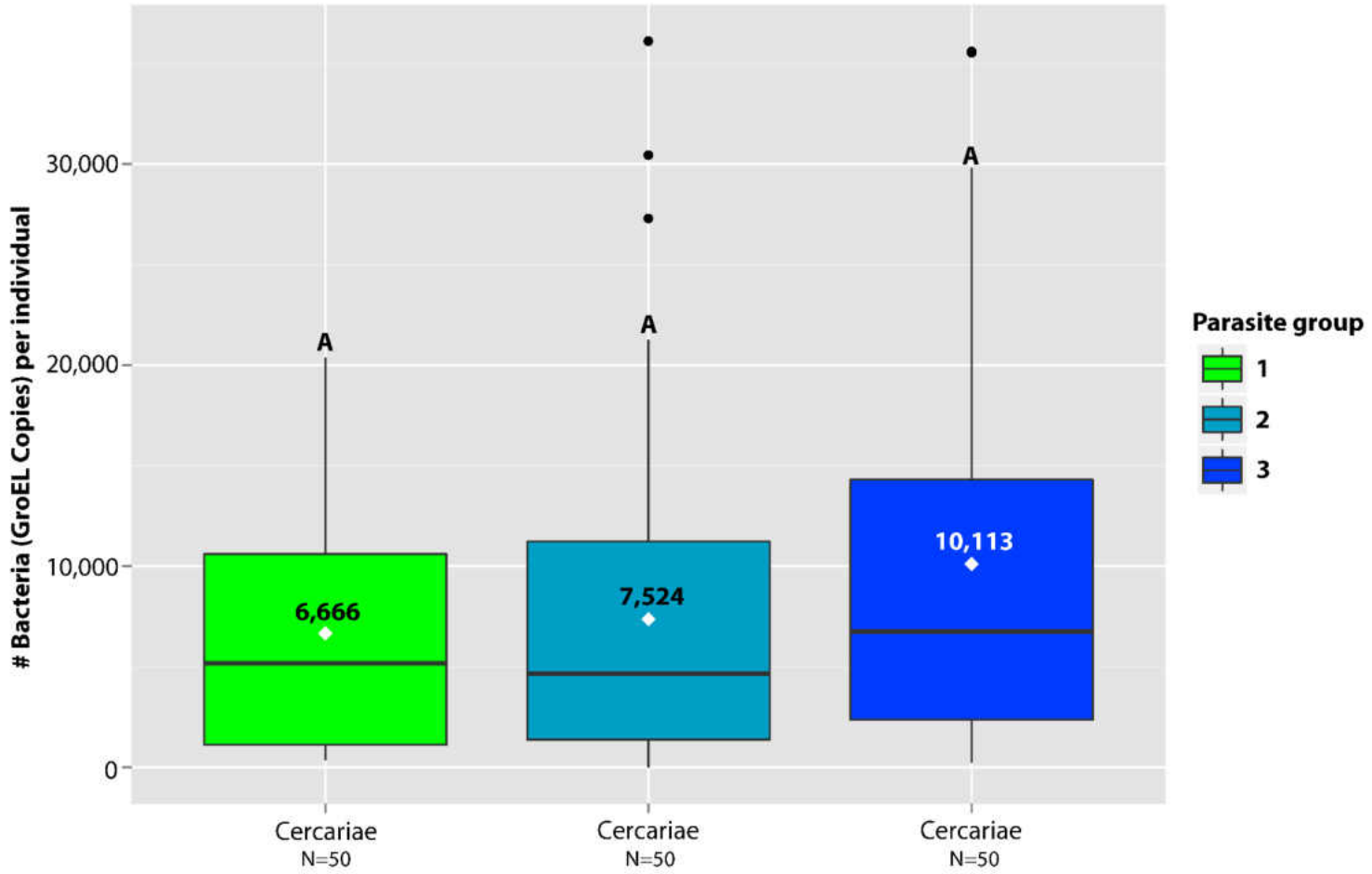

Figure 24. Boxplot showing the number of bacteria (GroEL copies) per cercaria from three separate snails. Diamond represents mean number of bacteria, horizontal bar represents median values, significant differences ($P \leq 0.05$) between parasite groups computed with a Tukey-Kramer HSD test are indicated with different capital letters above the boxplots.

Quantification of *Neorickettsia* sp. in Juvenile Worms (48 Hour)

Fifty complete juvenile worms (48 hours after infection of hamster) from parasite groups 1and 3 and thirty-one complete juvenile worms from parasite group 2 were assayed separately to determine the number of GroEL copies/juvenile. *Neorickettsia* sp. was detected in all 131 juvenile worm samples. The number of detected bacteria per single juvenile worm varied from as little as 92,000 to as many as 2,771,000 (Figure 27). Based on a 1-way ANOVA there was a significant difference in the number of GroEL copies/juvenile among the 3 parasite groups ($F_{(2,128)}$=7.819, P<0.001). Based on the results of the ANOVA a Tukey-Kramer test was run. The Tukey-Kramer test showed that number of GroEL copies/juvenile was significantly different between parasite groups 1 and 3, and 2 and 3 (Figure 27).

Quantification of *Neorickettsia* sp. in Adult Worms (14 Day)

Fifty complete adult worms (14 days after infection of hamster) from parasite groups 2and 3 and thirty-six complete adult worms from parasite group 1 were assayed separately to determine the number of GroEL copies/adult. *Neorickettsia* sp. was detected in all 136 adult worm samples. The number of detected bacteria per single adult worm varied, from 512,500 to 4,939,000 GroEL copies/adult (Figure 28). Based on a 1-way ANOVA there was a significant difference in the number of GroEL copies/adult among the 3 parasite groups ($F_{(2,133)}$=33.549, P<0.0001). Based on the results of the ANOVA a Tukey-Kramer test was run. The Tukey-Kramer test showed that number of GroEL copies/adult was significantly different among all three parasite groups (Figure 28).

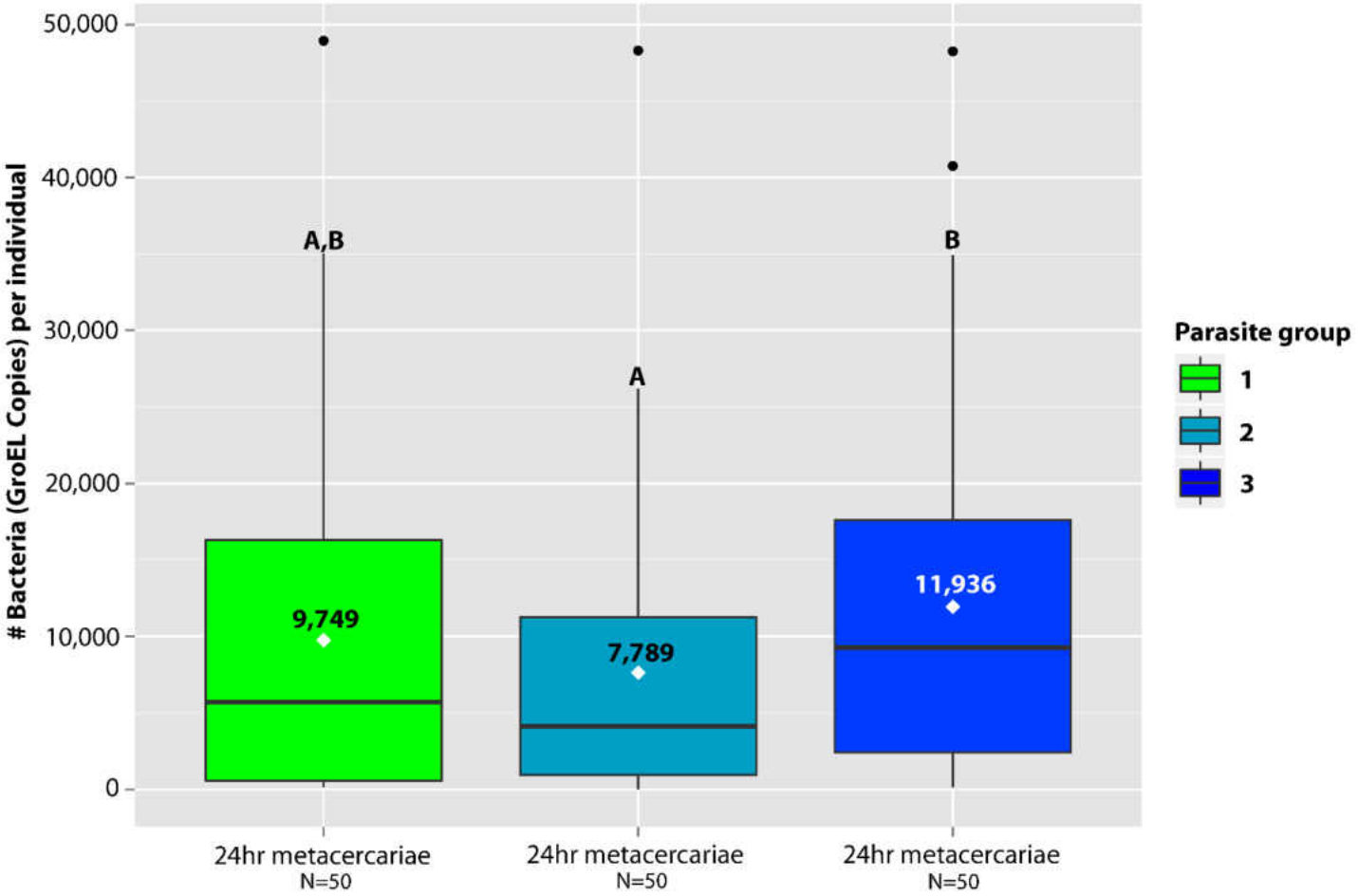

Figure 25. Boxplot showing the number of bacteria (GroEL copies) per 24 hour metacercariae from three parasite groups. Diamond represents mean number of bacteria, horizontal bar represents median values, significant differences ($P \leq 0.05$) between parasite groups computed with a Tukey-Kramer HSD test are indicated with different capital letters above the boxplots.

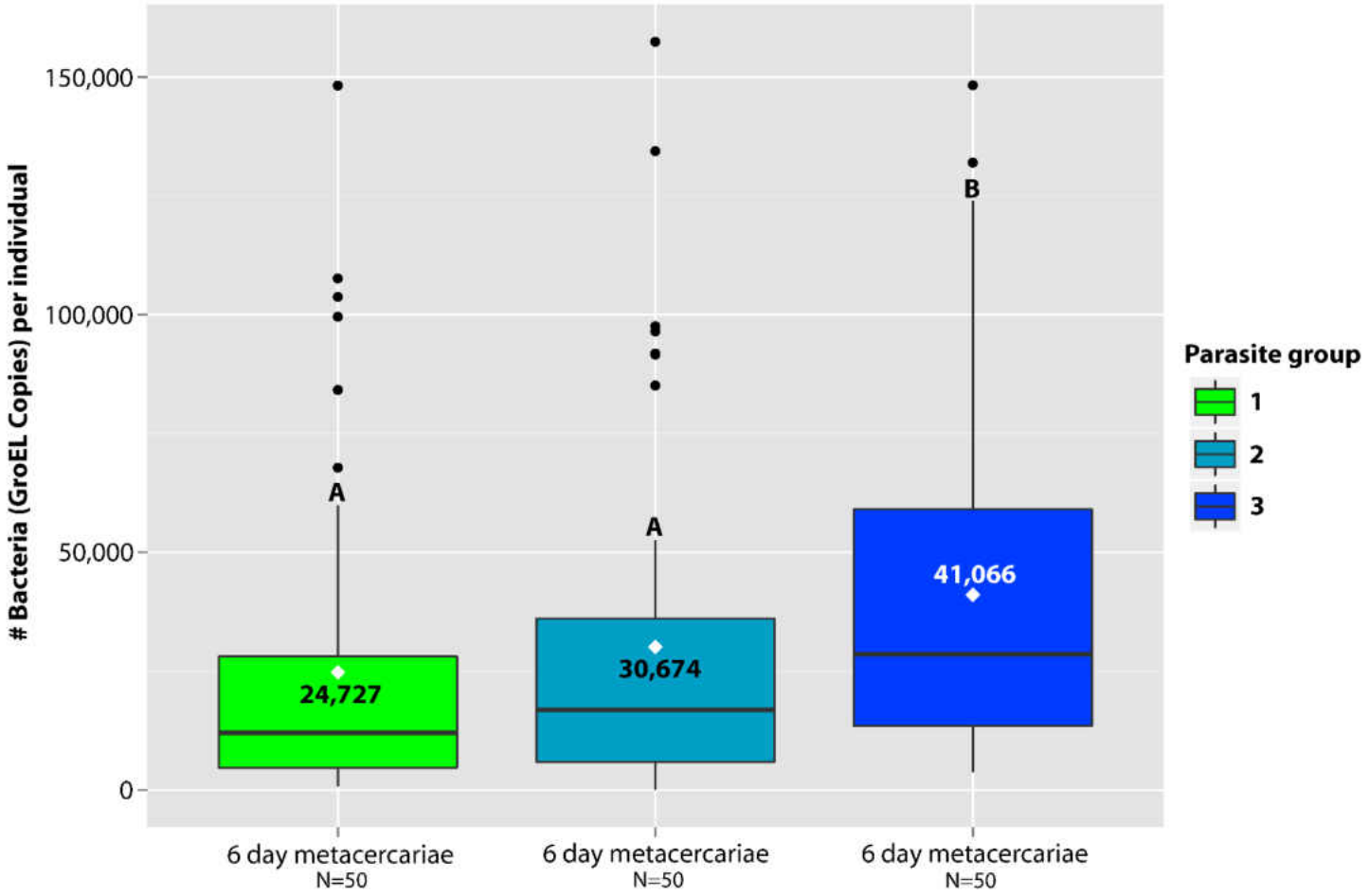

Figure 26. Boxplot showing the number of bacteria (GroEL copies) per 6 day metacercariae from three parasite groups. Diamond represents mean number of bacteria, horizontal bar represents median values, significant differences (P≤0.05) between parasite groups computed with a Tukey-Kramer HSD test are indicated with different capital letters above the boxplots.

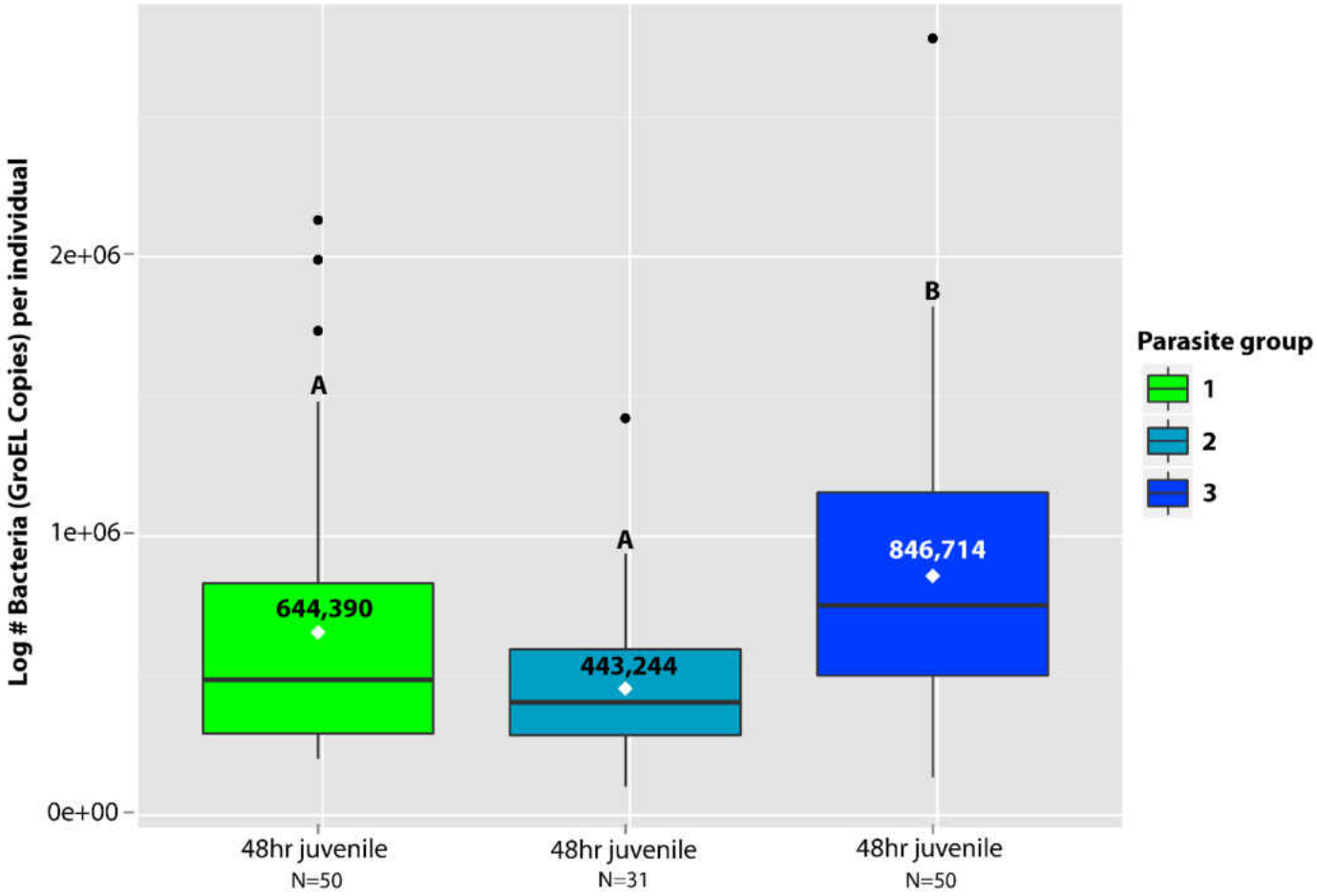

Figure 27. Boxplot showing the number of bacteria (GroEL copies) per 48 hour juvenile worms from three parasite groups. Diamond represents mean number of bacteria, horizontal bar represents median values, significant differences ($P \leq 0.05$) between parasite groups computed with a Tukey-Kramer HSD test are indicated with different capital letters above the boxplots.

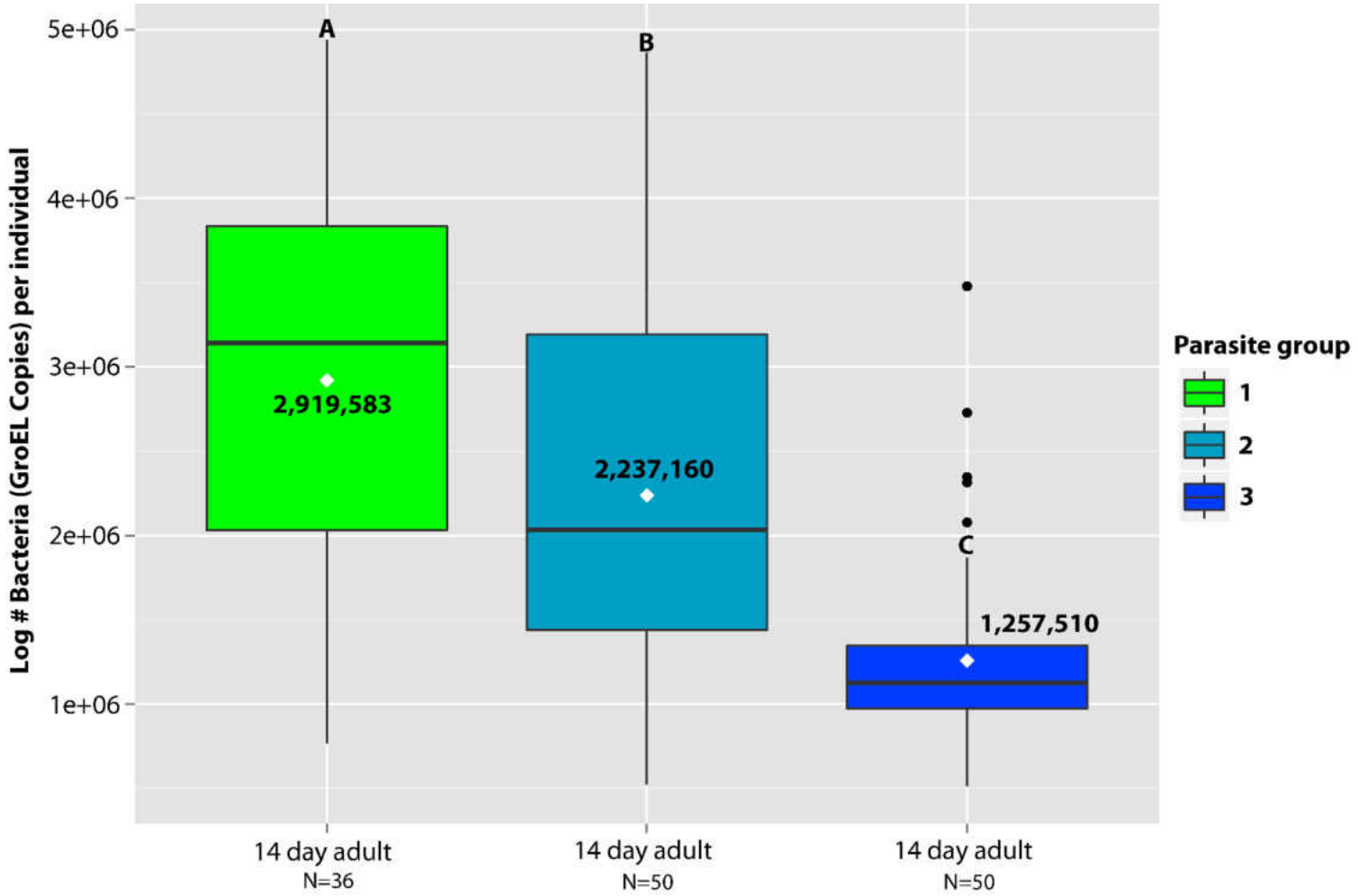

Figure 28. Boxplot showing the number of bacteria (GroEL copies) per 14 day adult worms from three parasite groups. Diamond represents mean number of bacteria, horizontal bar represents median values, significant differences ($P \leq 0.05$) between parasite groups computed with a Tukey-Kramer HSD test are indicated with different capital letters above the boxplots.

Quantification of *Neorickettsia* sp. Within All Life Cycle Stages for Parasite Group 1

Quantitative real-time PCR results for fifty cercariae, fifty 24 hour metacercariae, fifty 6 day metacercariae, fifty 48 hour juveniles, and thirty-six 14 day adult worms from parasite group 1 were compared to determine the difference in bacterial load (GroEL copy number) among the life cycle stages (Figure 29). Based on a 1-way ANOVA there was a significant difference in the number of GroEL copies among life cycle stages ($F_{(4,231)}$=271.31, P<0.0001). Based on the results of the ANOVA a Tukey-Kramer test was run. The Tukey-Kramer test showed that number of GroEL copies was significantly different among all life cycle stages except the transition between free-swimming cercariae and early metacercariae 24 hours after penetration of mosquito larvae (P=0.964) (Figure 29), (Table 16).

Table 16. Results of Tukey-Kramer HSD test showing statistical differences in GroEL copies among different life cycle stages for parasite group 1. P value shown above the diagonal and significance between life cycle stages shown below the diagonal.

Life cycle stage	Cercariae	24 hour mtc.	6 day mtc.	48 hr. juveniles	14 day adults
Cercariae	-------	P=0.964	P<0.0001	P<0.0001	P<0.0001
24 hour mtc.	no	-------	P<0.0001	P<0.0001	P<0.0001
6 day mtc.	yes	yes	-------	P<0.0001	P<0.0001
48 hr. juveniles	yes	yes	yes	-------	P<0.0001
14 day adults	yes	yes	yes	yes	-------

Quantification of *Neorickettsia* sp. Within All Life Cycle Stages for Parasite Group 2

Quantitative real-time PCR results for fifty cercariae, fifty 24 hour metacercariae, fifty 6 day metacercariae, thirty-one 48 hour juveniles, and fifty 14 day adult worms from parasite group 2 were compared to determine the difference in bacterial load (GroEL copy number) between the life cycle stages (Figure 30). Based on a 1-way ANOVA there was a significant difference in the number of GroEL copies between different life cycle

stages ($F_{(4,223)}$=310.27, P<0.0001). Based on the results of the ANOVA a Tukey-Kramer test was run. The Tukey-Kramer test showed that number of GroEL copies was significantly different among all life cycle stages except the transition between free-swimming cercariae and early metacercariae 24 hours after penetration of mosquito larvae (P=0.981) (Figure 30), (Table 17).

Table 17. Results of Tukey-Kramer HSD test showing statistical differences in GroEL copies among different life cycle stages for parasite group 2. P value shown above the diagonal and significance between life cycle stages shown below the diagonal.

Life cycle stage	Cercariae	24 hour mtc.	6 day mtc.	48 hr. juveniles	14 day adults
Cercariae	-------	P=0.981	P<0.0001	P<0.0001	P<0.0001
24 hour mtc.	no	-------	P<0.0001	P<0.0001	P<0.0001
6 day mtc.	yes	yes	-------	P<0.0001	P<0.0001
48 hr. juveniles	yes	yes	yes	-------	P<0.0001
14 day adults	yes	yes	yes	yes	-------

Quantification of *Neorickettsia* sp. Within All Life Cycle Stages for Parasite Group 3

Quantitative real-time PCR results for fifty cercariae, fifty 24 hour metacercariae, fifty 6 day metacercariae, fifty 48 hour juveniles, fifty 14 day adult worms, fifty fully formed fresh eggs (taken from the distal part of uterus of a live worm and processed immediately), and fifty 6 day eggs (taken from the distal part of uterus of a live worm and kept in water for 6 days) from parasite group 3 were compared to determine the difference in bacterial load (GroEL copy number) between the life cycle stages (Figure 31). Not all eggs were infected with *Neorickettsia*. Thirty-seven of 50 fresh eggs (74%) were infected, with a range of 2 to 3,3686 GroEL copies per infected egg. Forty-seven of 50 six-day old eggs (94%) were infected, with a range of 9 to 3,308 GroEL copies per egg. Based on a 1-way ANOVA there was a significant difference in the number of GroEL copies between different life cycle stages ($F_{(6,327)}$=546.8, P<0.0001). Based on the results of the ANOVA a Tukey-Kramer test was run. The Tukey-Kramer test showed that

number of GroEL copies was significantly different among all life cycle stages except the transition between free-swimming cercariae and early metacercariae 24 hours after penetration of mosquito larvae (P=0.994) and the transition between juvenile and sexually-mature adults (P=0.279) (Figure 31), (Table 18).

Table 18. Results of Tukey-Kramer HSD test showing statistical differences in GroEL copies among different life cycle stages for parasite group 3. P value shown above the diagonal and significance between life cycle stages shown below the diagonal.

Life cycle stage	Fresh eggs	6 day eggs	Cercari-ae	24 hour mtc.	6 day mtc.	48 hr. juveniles	14 day adults
Fresh eggs	-------	P<0.003	P<0.001	P<0.001	P<0.001	P<0.001	P<0.001
6 day eggs	yes	-------	P<0.001	P<0.001	P<0.001	P<0.001	P<0.001
Cercariae	yes	yes	-------	P=0.994	P<0.001	P<0.001	P<0.001
24 hour mtc.	yes	yes	no	-------	P<0.001	P<0.001	P<0.001
6 day mtc.	yes	yes	yes	yes	-------	P<0.001	P<0.001
48 hr. juveniles	yes	yes	yes	yes	yes	-------	P=0.279
14 day adults	yes	yes	yes	yes	yes	no	-------

Quantification of *Neorickettsia* sp. in Metacercariae (Encysted Within Snail 2)

Fifty complete metacercariae, encysted within the same snail shedding the cercariae, from parasite group 2 were assayed separately to determine the number of GroEL copies/metacercaria. *Neorickettsia* sp. was detected in all 50 metacercariae samples. The number of detected bacteria per single metacercaria varied from 13,850 to 218,400 (Figure 32). To determine if there was a significant difference in the number of bacteria between metacercariae encysted within snail tissue or *Culex* larva, as well as, between all other life-cycle stages a 1-way ANOVA was run. Based on a 1-way ANOVA there was a significant difference in the number of GroEL copies between different life cycle stages ($F_{(5,272)}$=302.14, P<0.0001). Based on the results of the

ANOVA a Tukey-Kramer test was run. The Tukey-Kramer test showed that number of GroEL copies was significantly different between most life cycle stages, except the transition between free-swimming cercariae and pre-infective metacaercariae 24 hours after penetration of mosquito larvae (P=0.991). (Figure 32), (Table 19).

Table 19. Results of Tukey-Kramer HSD test showing statistical differences in GroEL copies among different life cycle stages for parasite group 2. P value shown above the diagonal and significance between life cycle stages shown below the diagonal.

Life cycle stage	Cercariae	24 hour mtc.	6 day mtc.	Snail mtc.	48 hr. juveniles	14 day adults
Cercariae	-------	P=0.991	P<0.0001	P<0.0001	P<0.0001	P<0.0001
24 hour mtc.	no	-------	P<0.0001	P<0.0001	P<0.0001	P<0.0001
6 day mtc.	yes	yes	-------	P<0.0001	P<0.0001	P<0.0001
Snail mtc.	yes	yes		-------	P<0.0001	P<0.0001
48 hr. juveniles	yes	yes	yes	yes	-------	P<0.0001
14 day adults	yes	yes	yes	yes	yes	-------

Discussion

This chapter and Chapter 4 are very similar, in that they both are focused on the transmission efficiency of *Neorickettsia* through multiple stages of the digenean life cycle. However, due to major limitations, lack of a reliable reference for a standard curve for qPCR and incomplete laboratory digenean life cycle, Chapter 4 focused on prevalence of infection within the asexual stages of the digenean life cycle, and did not provide data on the abundance of bacteria through all stages of the digenean. As stated in Chapter 2 determining the prevalence of *Neorickettsia* in all stages of the digenean life cycle is not feasible using only natural infections because obtaining such data for metacercariae in

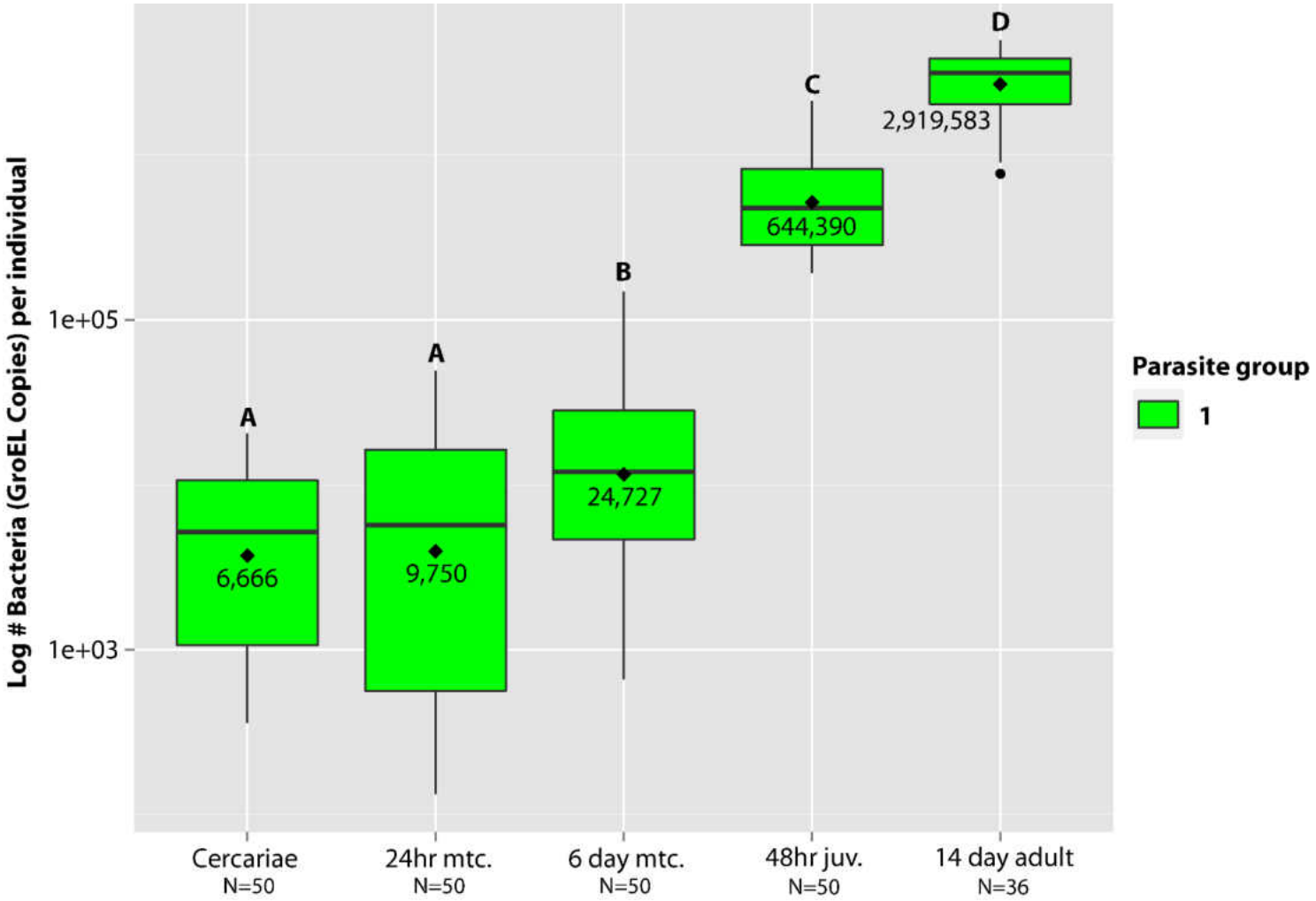

Figure 29. Boxplot showing the number of bacteria (GroEL copies) per individual cercariae, 24hr metacercariae, 6 day metacercariae, 48 hour juveniles, and 14 day adults from parasite group 1. Diamond represents mean number of bacteria, horizontal bar represents median values, significant differences ($P \leq 0.05$) between parasite groups computed with a Tukey-Kramer HSD test are indicated with different capital letters above the boxplots.

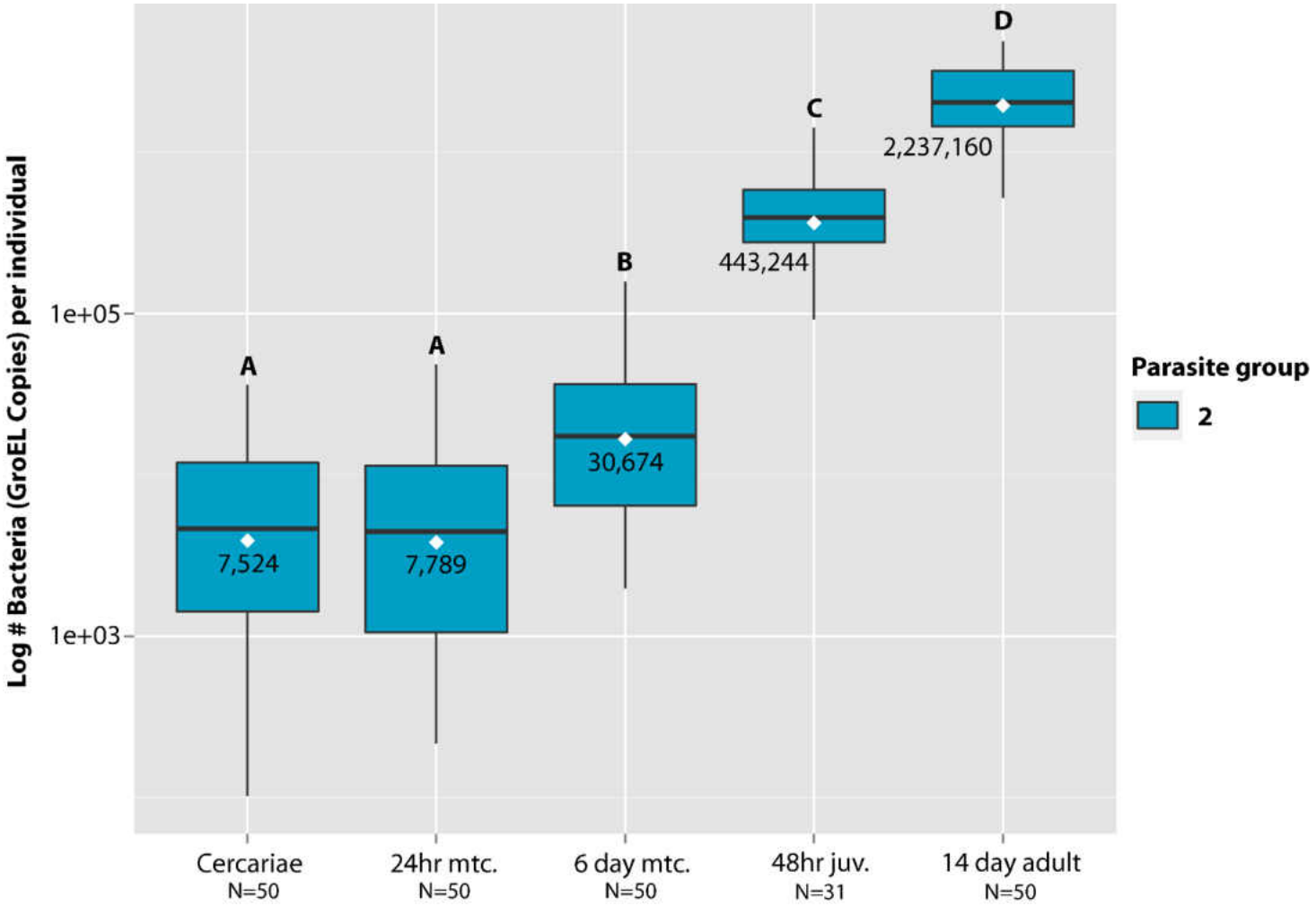

Figure 30. Boxplot showing the number of bacteria (GroEL copies) per individual cercariae, 24hr metacercariae, 6 day metacercariae, 48 hour juveniles, and 14 day adults from parasite group 2. Diamond represents mean number of bacteria, horizontal bar represents median values, significant differences ($P \leq 0.05$) between parasite groups computed with a Tukey-Kramer HSD test are indicated with different capital letters above the boxplots.

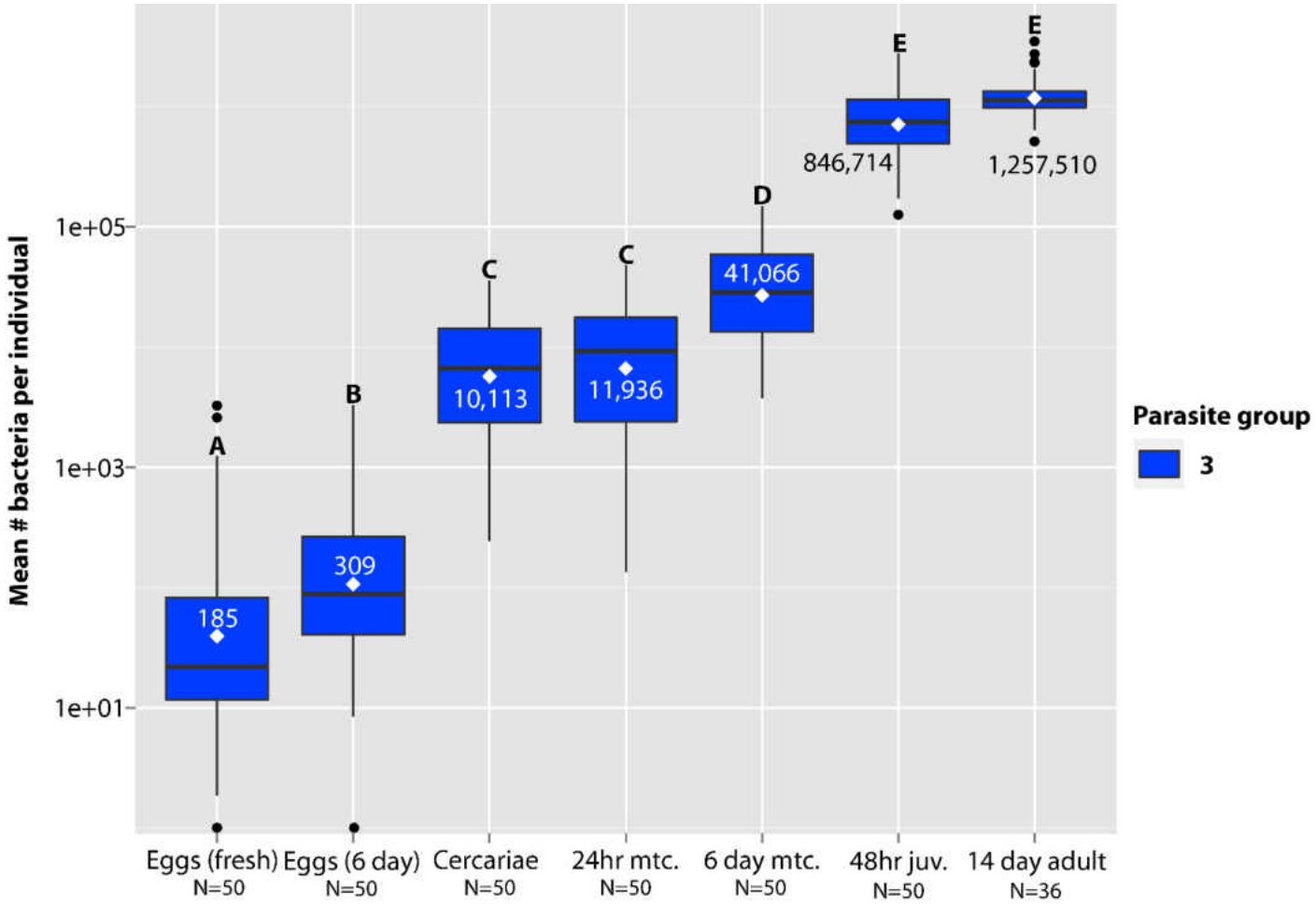

Figure 31. Boxplot showing the number of bacteria (GroEL copies) per individual eggs, cercariae, 24hr metacercariae, 6 day metacercariae, 48 hour juveniles, and 14 day adults from parasite group 3. Diamond represents mean number of bacteria, horizontal bar represents median values, significant differences ($P \leq 0.05$) between parasite groups computed with a Tukey-Kramer HSD test are indicated with different capital letters above the boxplots.

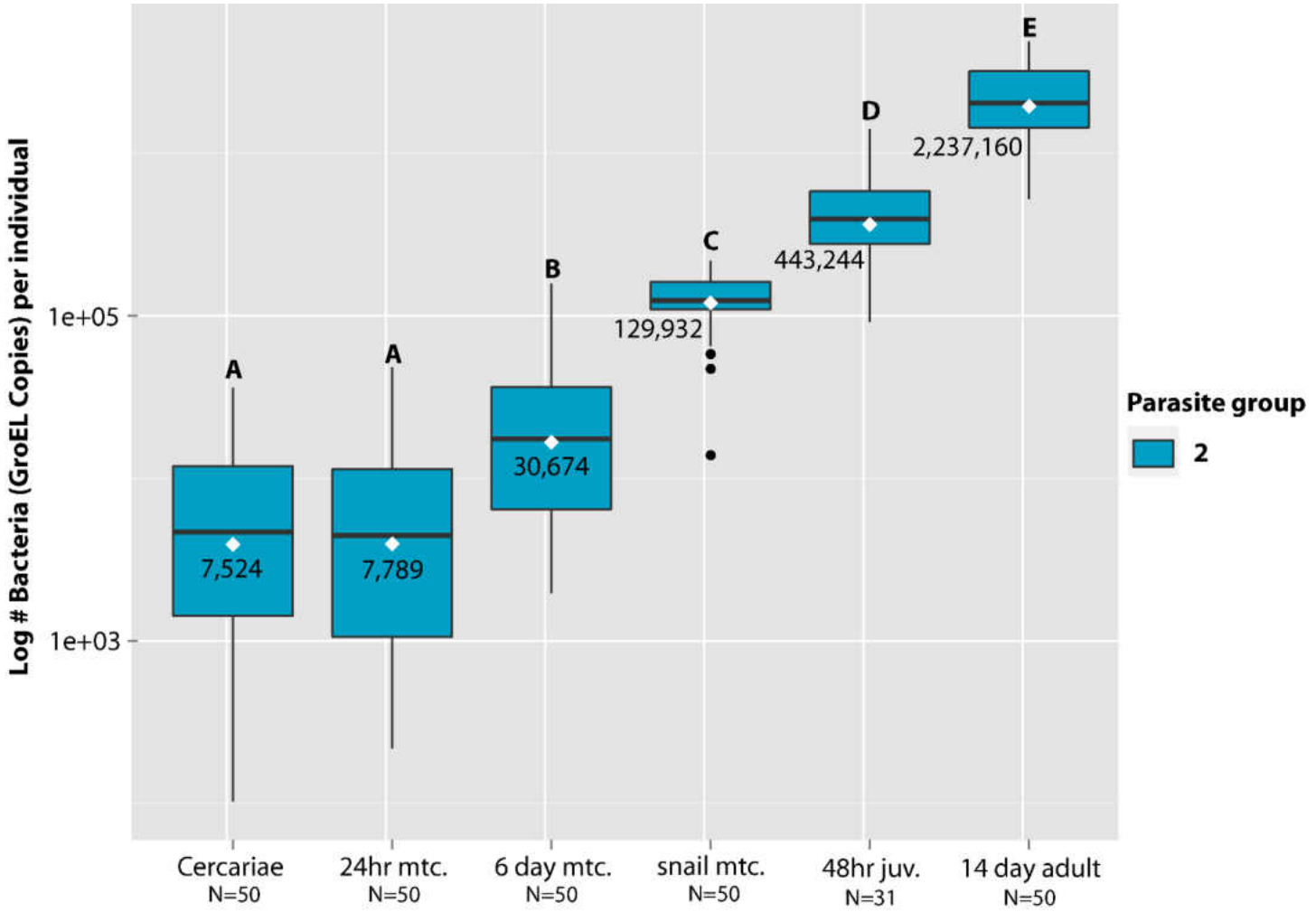

Figure 32. Boxplot showing the number of bacteria (GroEL copies) per individual cercariae, 24hr metacercariae, 6 day metacercariae, metacercariae from the snail, 48 hour juveniles, and 14 day adults from parasite group 2. Diamond represents mean number of bacteria, horizontal bar represents median values, significant differences ($P \leq 0.05$) between parasite groups computed with a Tukey-Kramer HSD test are indicated with different capital letters above the boxplots.

naturally infected arthropod hosts and adults in definitive hosts would be nearly impossible. Development of the laboratory life cycle and availability of long synthetic double stranded DNA (gBlocks® (Integrated DNA Technologies Inc.)) allowed us to determine the prevalence and intensity of bacterial infection within all stages of the digenean life cycle.

As expected there was a significant increase in bacterial abundance from one life cycle stage to the next, with the exception of cercariae to 24 hour metacercariae. The largest increase in abundance occurred from 6 day eggs (infective to first intermediate host) to cercariae, a 33 fold increase in the number of bacteria. This increase is likely a result of availability of nutrients, from a complete lack of exogenous sources of energy (only nutrients within the egg), to large energy supplies (within the snail first intermediate host). Increased metabolic activity and growth of the digenean host with active cell divisions may be another factor promoting bacterial proliferation. Increase at metacercarial stage was more modest. There was a 2.5–4 fold increase in bacterial abundance from 24 hour metacercariae to 6 day metacercariae. This modest rate of bacterial cell divisions may likely be explained by the same reasons, namely the enclosure in the cyst with no additional energy/nutrient supply and lower growth/cell division rate upon achievement of infectivity which usually occurs on 3–4 days after formation of metacercarial cyst. The second largest increase occurred between 6 day metacercariae and 48 hour juvenile worms (14.5–26 fold increase). This large increase corresponds to the switch of the digenean host, from a cold blooded arthropod to a warm blooded mammal. Energetically, the metacercaria in a mosquito is in stasis while the juvenile digenean in the mammalian host is actively feeding and growing. During the

growth and sexual development of juvenile worms to 14 day-old fully mature adult worms there was a 1.5-5 fold increase in abundance of *Neorickettsia*.

In Chapter 4 we found that both low and high prevalences of neorickettsial infections at cercaria were found in the same parasite species (*P. elegans*), the same snail species (*L. stagnalis*), and the same body of water (Table 15). This data suggested that there was a lot of inherent variability in the efficiency of vertical transmission of *Neorickettsia* during asexual reproduction of digeneans. The quantitative data shown in this chapter help to clarify this inherent variability. Almost all cercariae were found to harbor the bacterial endosymbiont, 100% cercariae in parasite groups 1 and 3 were infected, and 98% of cercariae in group 2 were infected with *Neorickettsia*. In addition, all three parasite groups had cercariae with similar intensities of infection ($P>0.05$). This indicates that the efficiency of vertical transmission of *Neorickettsia* from sporocysts to cercariae is highly consistent. The most likely explanation for the variability seen in Chapter 4 is that the rate of neorickettsial replication may be slower than the rate of development and replication of digenean asexual stages. This asynchrony could result in a lower *Neorickettsia* prevalence in cercariae produced by snails that have only recently begun shedding compared to snails with earlier established digenean infection. During the establishment of the laboratory life cycle (Chapter 2), we felt that it was important to monitor the neorickettsial infection of the different digenean life stages. By doing this, we made some preliminary but interesting observations. For example, it appears that during the asexual phase of the digenean life cycle within a single snail, the efficiency of *Neorickettsia* transmission from progenitor (daughter sporocysts) to progeny (cercariae) was initially rather low. On the first day of cercarial shedding by one of the snails, only

one of 10 individual cercariae assayed (10%) was PCR-positive for *Neorickettsia*. Thus, replication of the endosymbiont initially lagged behind the reproductive output of the digenean. However three days later, 2 of 10 (20%) individual cercariae from that same snail tested positive for *Neorickettsia.* Twenty days after the beginning of shedding 47 of 50 individual cercaria (94%) tested positive for *Neorickettsia*. Thus, as asexual reproduction of the digenean proceeded, the endosymbiont seemed to "catch up" and the efficiency of vertical transmission increased.

The three infected snails used in the quantitative study were collected in the late fall and were likely well-established infections, allowing enough time for 100% of released cercariae to be infected with the bacterial endosymbiont. Although vertical transmission efficiency of *Neorickettsia* from daughter sporocysts to cercariae appears to be consistent among the three parasite groups, it becomes more variable as the digenean life cycle progresses. Parasite group 1 showed the largest increase in mean number of bacteria with an average 438 fold increase in bacteria from cercariae to adults, while parasite group 2 demonstrated a 297 fold increase, and parasite group 3 had a 124 fold increase. The reasons for this variability are currently unknown and may depend on a number of factors.

Plagiorchis elegans cercariae are capable of precocious encystment to metacercariae within the daughter sporocysts developing within *L. stagnalis* snails. Metacercariae that result from precocious encystment appear to be fully developed and infective. It also appears that precocious metacercariae are longer lived and contain significantly higher numbers of *Neorickettsia* (mean=129,932) than do cercariae (mean=7,524) or infective metacercariae parasitizing mosquito larvae (mean=30,674). In

North Dakota, one of the natural hosts of *P. elegans* is the muskrat. Because of their diets, muskrats are more likely to become infected with *P. elegans* through the ingestion of precocious metacercariae encysted within snails than ingestion of metacercariae encysted within aquatic arthropods. Therefore, this is one potential mechanism in maintaining *Neorickettsia* infection in *P. elegans* in nature.

Neorickettsia are known to cause diseases in humans, domestic animals, and wildlife. However, it remains unknown how the bacteria are transmitted from the digenean host to the vertebrate, or how much bacteria is needed to initiate a viable infection. With regards to *N. risticii*, the causative agent of Potomac horse fever, horses that were fed pools of field collected caddisflies that had tested positive for *N. risticii* became neorickettsemic and clinically ill after 6 to 11 days (Mott et al., 2002). However, it was unknown how many *Neorickettsia* infected metacercariae were within the caddisflies pools or how long the metacercariae had been encysted in caddisflies. Based on our data, 6 day old *Neorickettsia*-infected metacercariae average between 24,727 and 41,066 bacteria, but it is still unknown whether this number of bacteria is sufficient to establish infection in a vertebrate host. Further research looking at "dosages" needs to be done to understand what level of neorickettsiae infection within the digenean host is necessary for infection and disease within the vertebrate hosts.

Vertical transmission of *Neorickettsia* from adults to eggs is not 100%. Eighty four (84%) out of 100 eggs assayed were positive for *Neorickettsia*. For comparison, *Wolbachia* bacteria that infects the ovaries and other tissues of many species of arthropods and filariid nematodes, are transmitted to 100% of the host progeny. This difference between *Wolbachia* and *Neorickettsia* leads to multiple questions related to the

biology and evolution of *Neorickettsia*. Many of the questions are related to the localization of *Neorickettsia* within the adult worm. In particular, whether the bacteria infect the ovaries of digeneans and if so, why is it not transmitted to 100% of the progeny? Some of these questions will be addressed in the next chapter.

Chapter VI

GERMS WITHIN WORMS: LOCALIZATION OF THE BACTERIAL ENDOSYMBIONT, *NEORICKETTSIA* SP., WITHIN DIFFERENT LIFE CYCLE STAGES OF THE DIGENEAN, *PLAGIORCHIS ELEGANS*

Abstract

One of the largest gaps in our knowledge of *Neorickettsia* biology is the very limited information available regarding the localization of the bacterial endosymbiont within its digenean host. In this study we visualized *Neorickettsia* sp. within several life cycle stages of the digenean *Plagiorchis elegans*. Sporocysts, cercariae, metacercariae, and adults of *P. elegans* infected with *Neorickettsia* were obtained from our laboratory life cycle (outlined in "Chapter 2"). Individuals of each life cycle stage were fixed in buffered 4% paraformaldehyde, equilibrated in 30% sucrose, embedded in Neg^{50} (Fisher Scientific/Thermo Scientific, Pittsburgh, Pennsylvania), cryosectioned (10µm) on a Leica HM550 cryotome, and placed on gelatin subbed slides. Indirect immunofluorescence microscopy was used to study localization of the bacterial endosymbiont within individual life cycle stages. This is the first study to reveal the location of *Neorickettsia* within a digenean host. *Neorickettsia* was found within the tegument of sporocysts, throughout cercarial embryos (germ balls) and fully formed cercariae within the sporocysts, throughout metacercariae, and within the tegument, parenchyma, vitellarium, uterus, testes, cirrus sac, and eggs of adults. Interestingly, neorickettsiae were not found within the ovarian tissue of *P. elegans*, leading us to hypothesize that transmission occurs through infected vitelline cells rather than through

a more common transovarian transmission via infected egg cells.

Results

Investigating the localization of *Neorickettsia* sp. within multiple life cycle stages of the digenean *Plagiorchis elegans* required a laboratory life cycle of a digenean infected with the bacterial endosymbiont. Fortunately, we had developed and maintained a laboratory life cycle at the initiation of this study (Chapter 2). In addition to availability of a laboratory life cycle a reliable technique that could be applied to all life cycle stages was required. Therefore, we chose to use indirect immunofluorescence microscopy on cryosections of different life cycle stages of the digenean host.

The main goal of this study was to identify the mode of vertical transmission from sporocysts to cercariae, and from adults to eggs, by localizing the endosymbiont within these different life cycle stages.

Sporocysts and Cercariae

Ten micron cryosections of *P. elegans* sporocysts were analyzed for the presence of *Neorickettsia* sp. using indirect immunofluorescence microscopy. Immunofluorescence microscopy showed *Neorickettsia* specific antigens within the tegument of the sporocyst and within free floating early cercarial embryos (germ balls) and fully developed cercariae within the brood chambers of the sporocysts (Figures 33 and 34). Interestingly, not all cercarial embryos and developed cercariae within the brood chambers were infected with *Neorickettsia* sp. (lack of specific antigens). *Neorickettsia* within developed cercariae appear to be distributed throughout the whole organism (Figure 34).

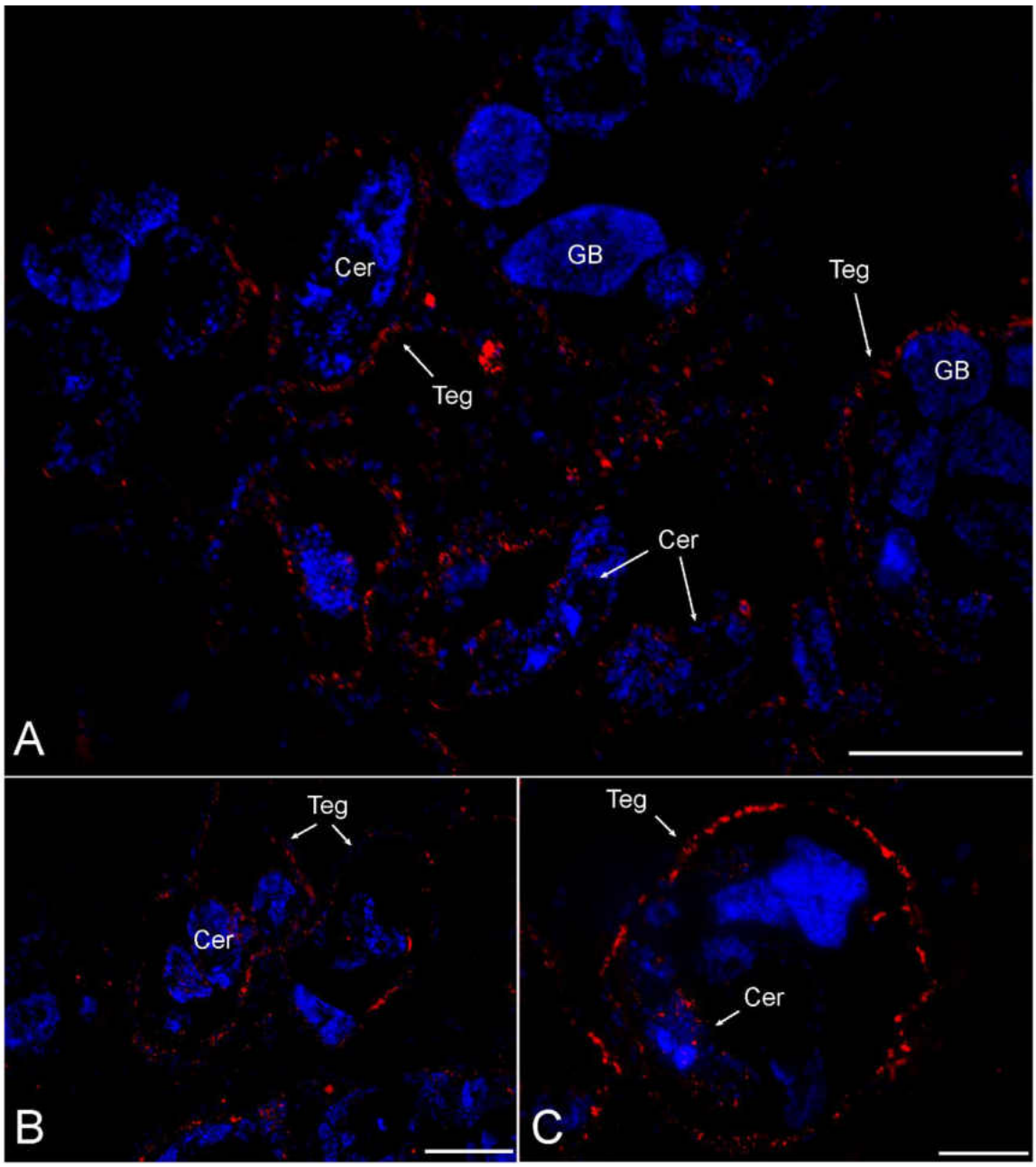

Figure 33. Indirect immunofluorescence microscopy of 10 µm sporocyst cryosections. **A-C**) Multiple sporocysts infected with *Neorickettsia* sp. (red), digenean nuclei stained with DAPI (blue). *Neorickettsia* located within the tegument of the sporocysts and within free floating cercarial embryos (germ balls: GB) and developed cercariae (Cer) within the sporocysts brood chambers. Scale bars 100µm (A, B), 50µm (C).

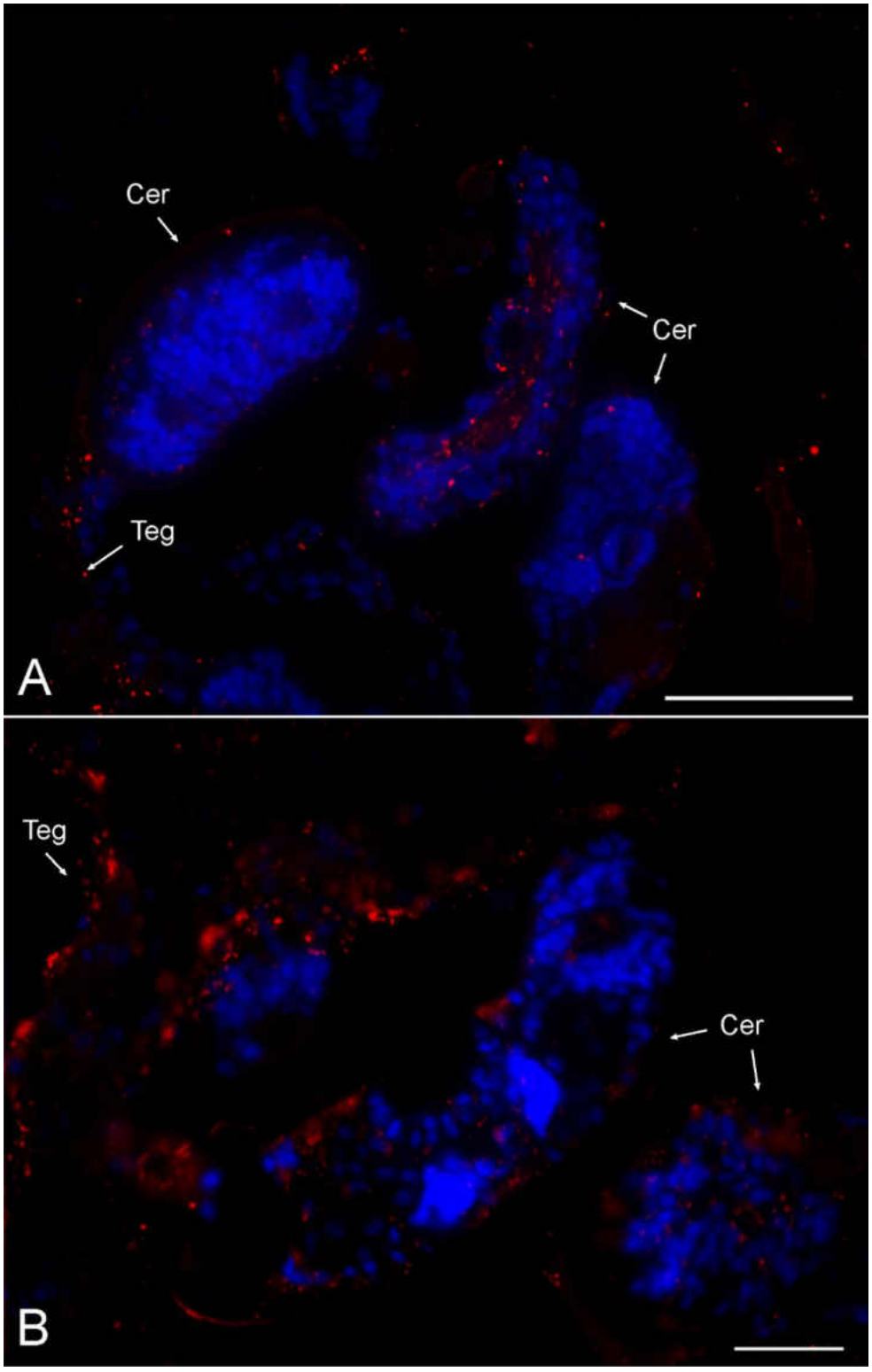

Figure 34. Indirect immunofluorescence microscopy of 10 µm cryosections of sporocyst/cercariae. **A, B**) Cercariae infected with *Neorickettsia* sp. (red) digenean nuclei stained with DAPI (blue). *Neorickettsia* located throughout cercarial embryos (germ balls: GB) and developed cercariae (Cer). Scale bars 50µm (A), 25µm (B).

Metacercariae

Ten micron cryosections of *P. elegans* fully infective metacercariae within the *Culex pipiens* mosquito larva (second intermediate host) were analyzed for the presence of *Neorickettsia* sp. using indirect immunofluorescence microscopy. Immuno-fluorescence microscopy showed *Neorickettsia* sp. specific antigens within metacercariae (Figure 35). Within the same mosquito larva different metacercariae had different intensities of infection with the bacterial endosymbiont. However, the bacteriae within all infected metacercariae appear to be distributed throughout the organism (Figure 35)

Adults

Ten micron cryosections of adult *P. elegans* were analyzed for the presence of *Neorickettsia* sp. using indirect immunofluorescence microscopy. Immuno-fluorescence microscopy showed *Neorickettsia* sp. specific antigens throughout the adult worm. Neorickettsiae was found in abundance within the tegument, parenchyma, vitellarium, uterus, and eggs (Figures 36–38). Not all eggs examined contained *Neorickettsia* sp. (Figure 38). Additionally, the bacterial endosymbiont was found within the male reproductive organs, including the testes and cirrus sac (Figures 37, 39). Interestingly, neorickettsiae were not found within the ovary (Figure 39).

Discussion

This is the first study to demonstrate *in-situ* localization of *Neorickettsia* within successive life cycle stages of the digenean host. Although we were able to demonstrate localization of the bacteria within a majority of the life cycle stages of *P. elegans* (eggs, daughter sporocysts, cercariae, metacercariae, and adults), the two most interesting stages, with regards to vertical transmission, are the sporocysts and adults.

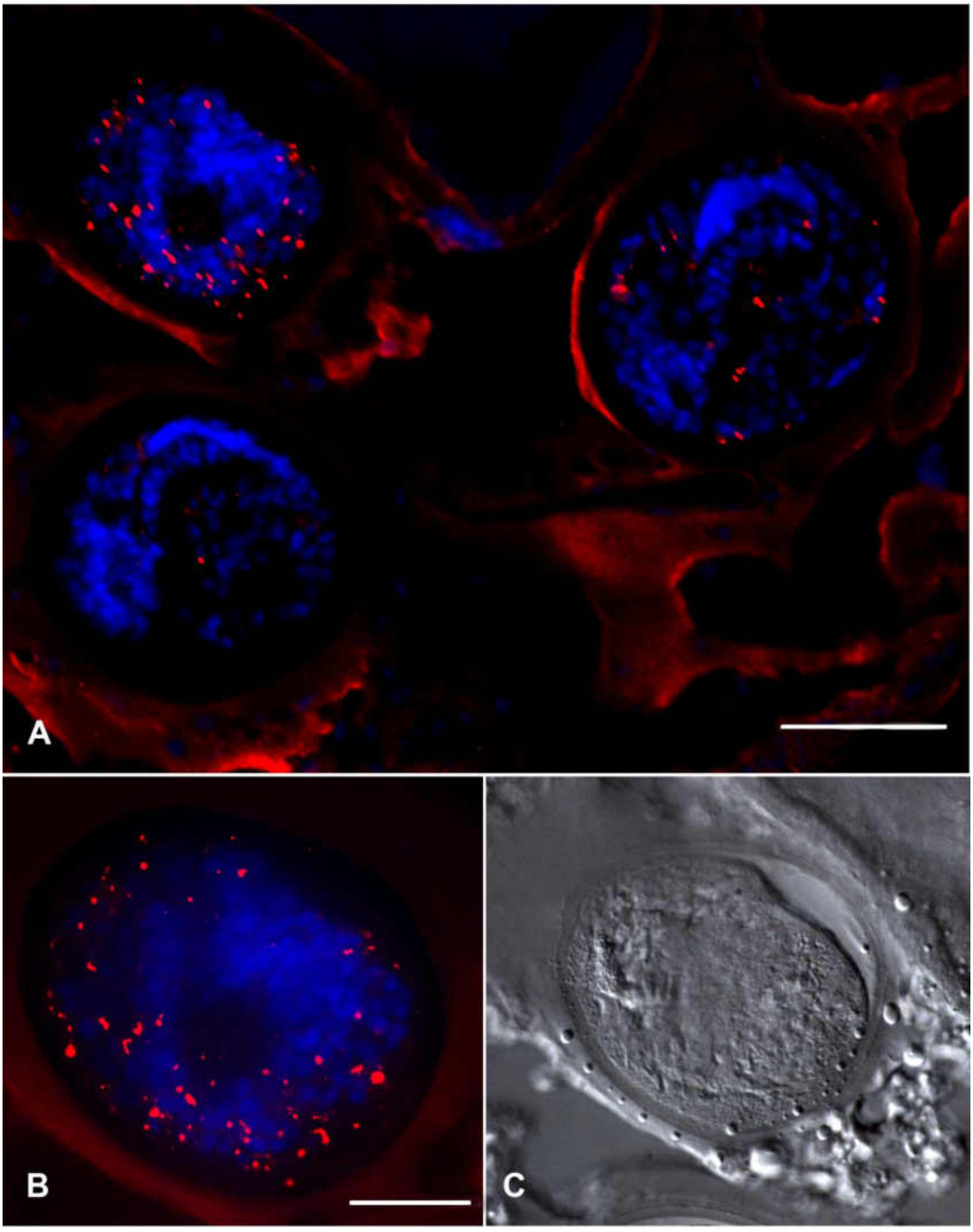

Figure 35. Indirect immunofluorescence microscopy of 10 µm cryosections of *Culex* mosquito larva infected with *P. elegans* metacercariae. **A)** Cross section of *Culex* mosquito larva infected with 3 metacercariae, *Neorickettsia* sp. (red dots) (red hue is autofluorescence) digenean nuclei stained with DAPI (blue). **B, C)** Cross section of individual metacercariae infected with *Neorickettsia* sp. Scale bars 100µm (A), 50µm (B and C).

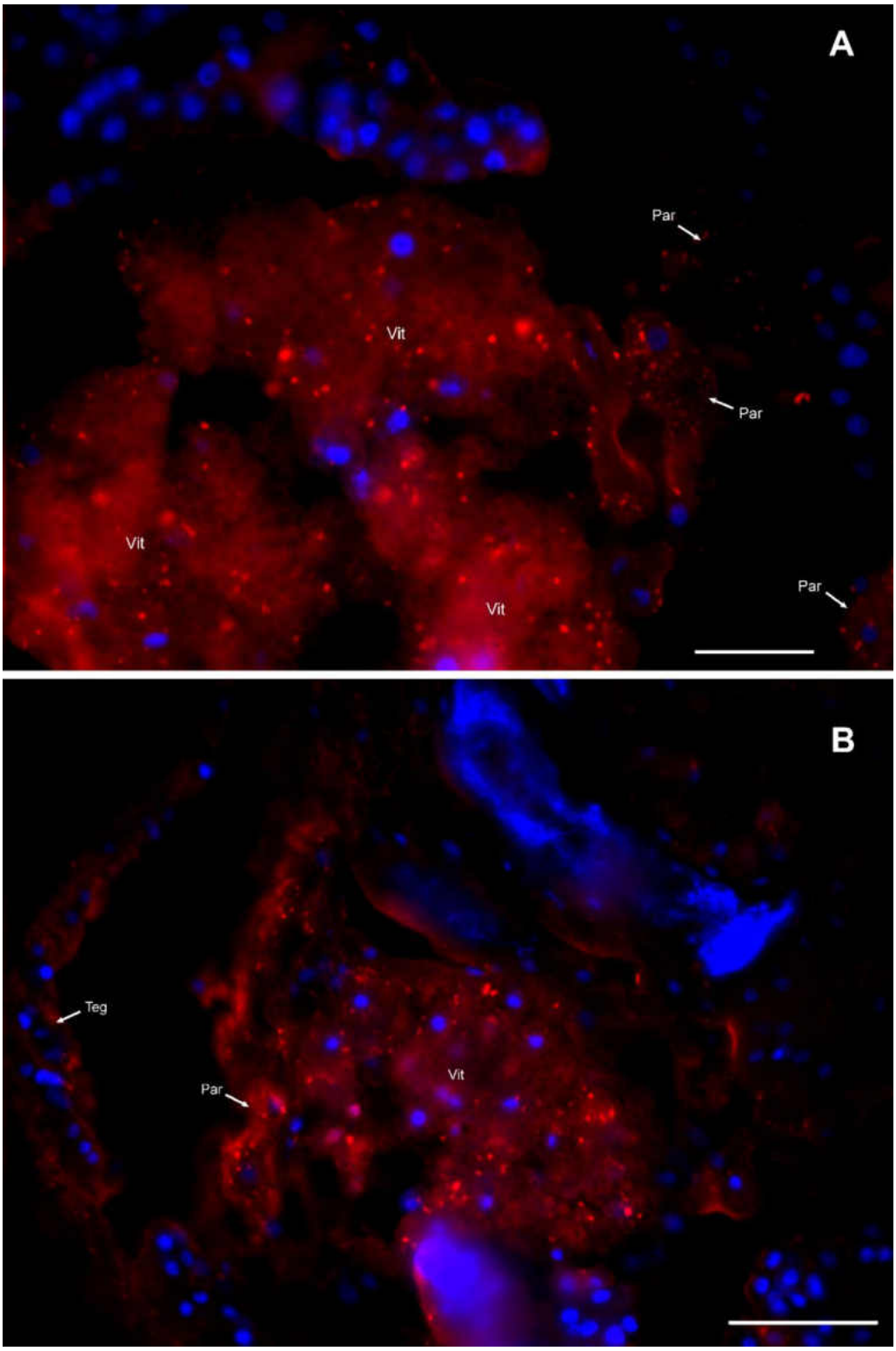

Figure 36. Indirect immunofluorescence microscopy of 10 µm cryosections of adult *P. elegans*. **A, B**) Cross section of adult worm infected with *Neorickettsia* sp. (red dots) (red hue is autofluorescence) digenean nuclei stained with DAPI (blue). *Neorickettsia* located throughout the vitellarium (Vit), tegument (Teg), and parenchyma (Par). Scale bars 25µm (A), 50µm (B).

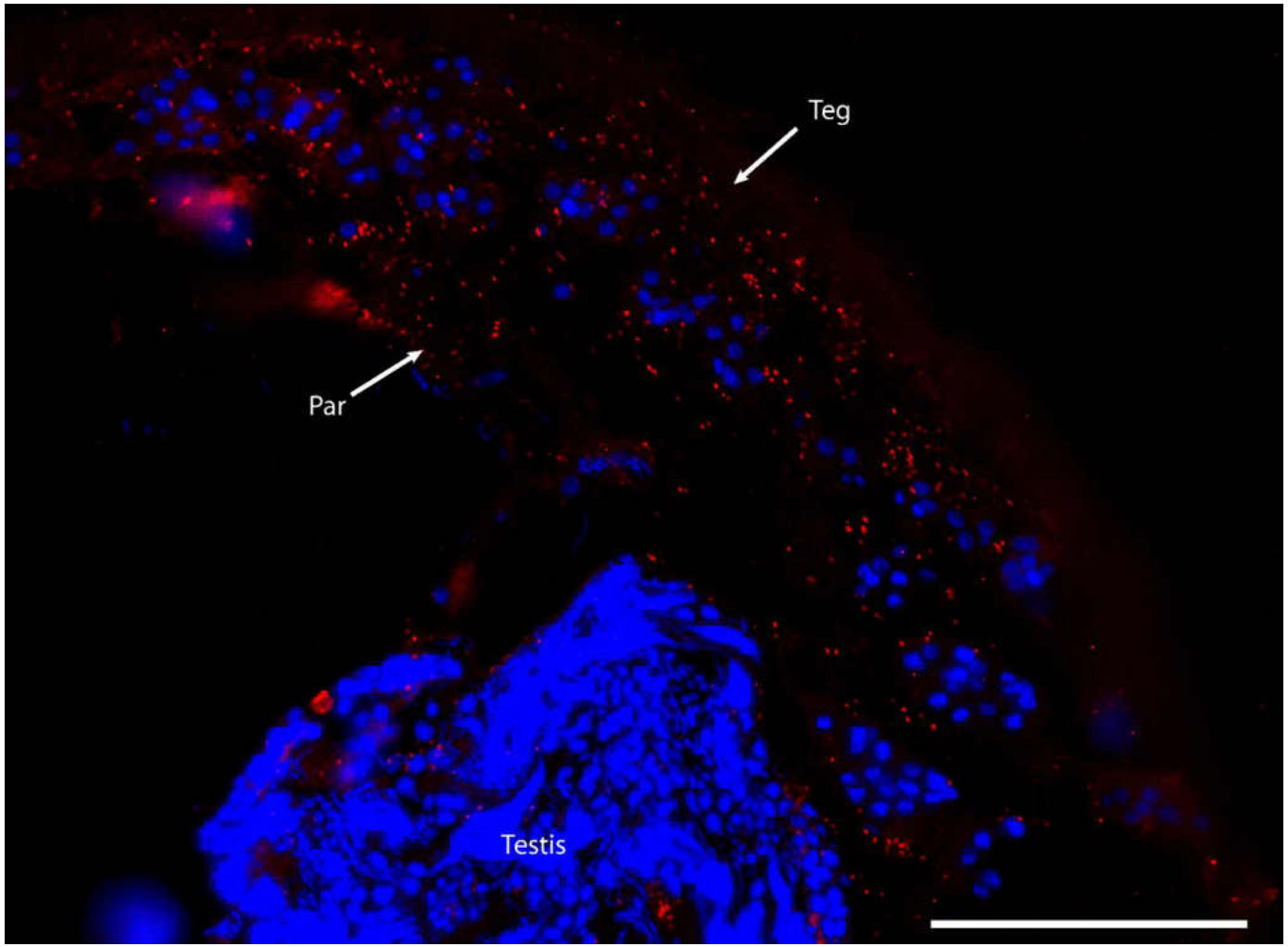

Figure 37. Indirect immunofluorescence microscopy of 10 µm cryosections of adult *P. elegans*. Cross section of adult worm infected with *Neorickettsia* sp. (red) digenean nuclei stained with DAPI (blue). *Neorickettsia* located throughout the tegument (teg), parenchyma (par), and testis. Scale bar 100µm.

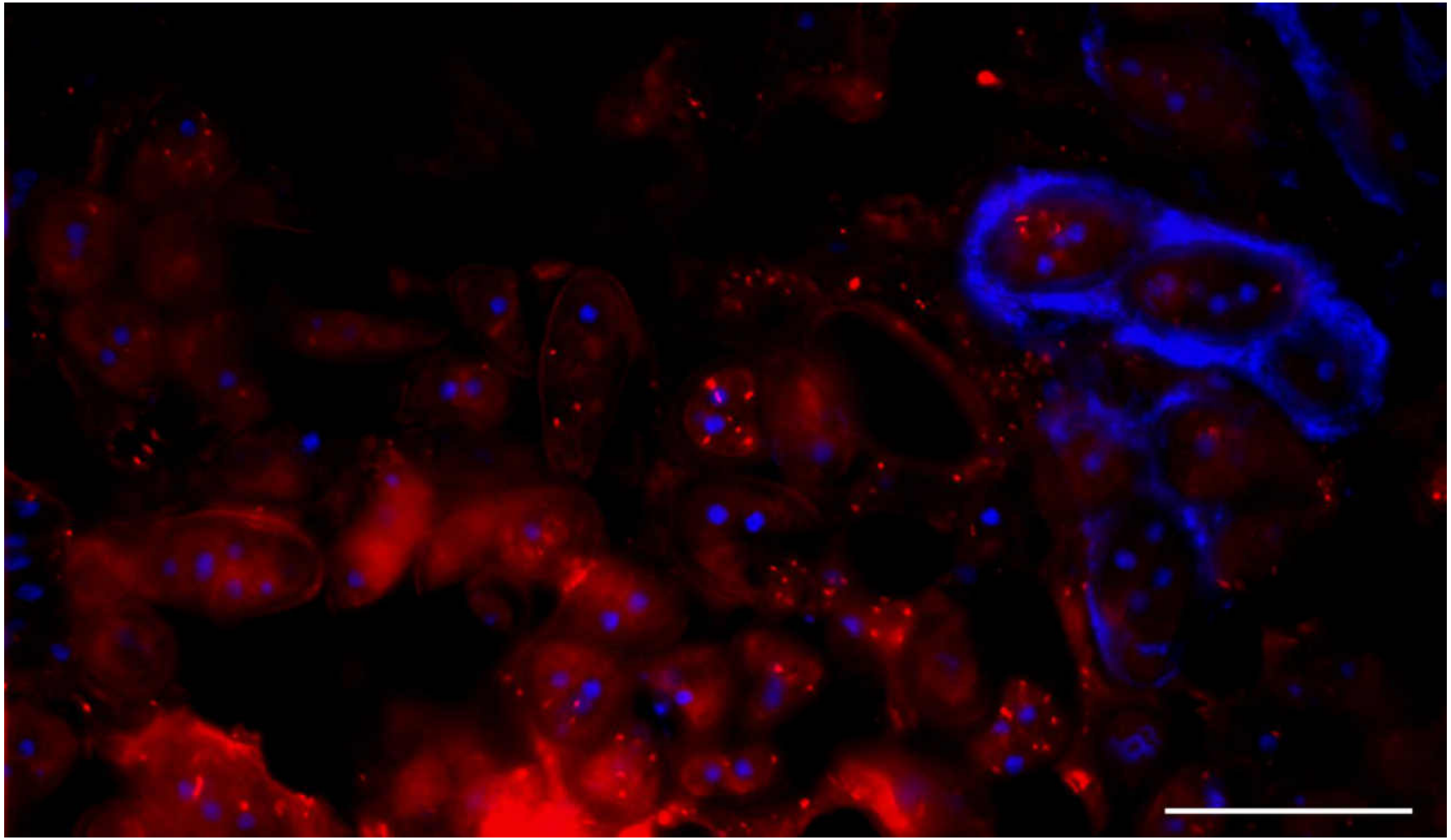

Figure 38. Indirect immunofluorescence microscopy of 10 µm cryosections of the uterus of an adult *P. elegans*. Longitudinal section of adult worm infected with *Neorickettsia* sp. (red dots) (red hue is autofluorescence) digenean nuclei stained with DAPI (blue). *Neorickettsia* located throughout the uterus and within multiple eggs. Scale bar 50µm.

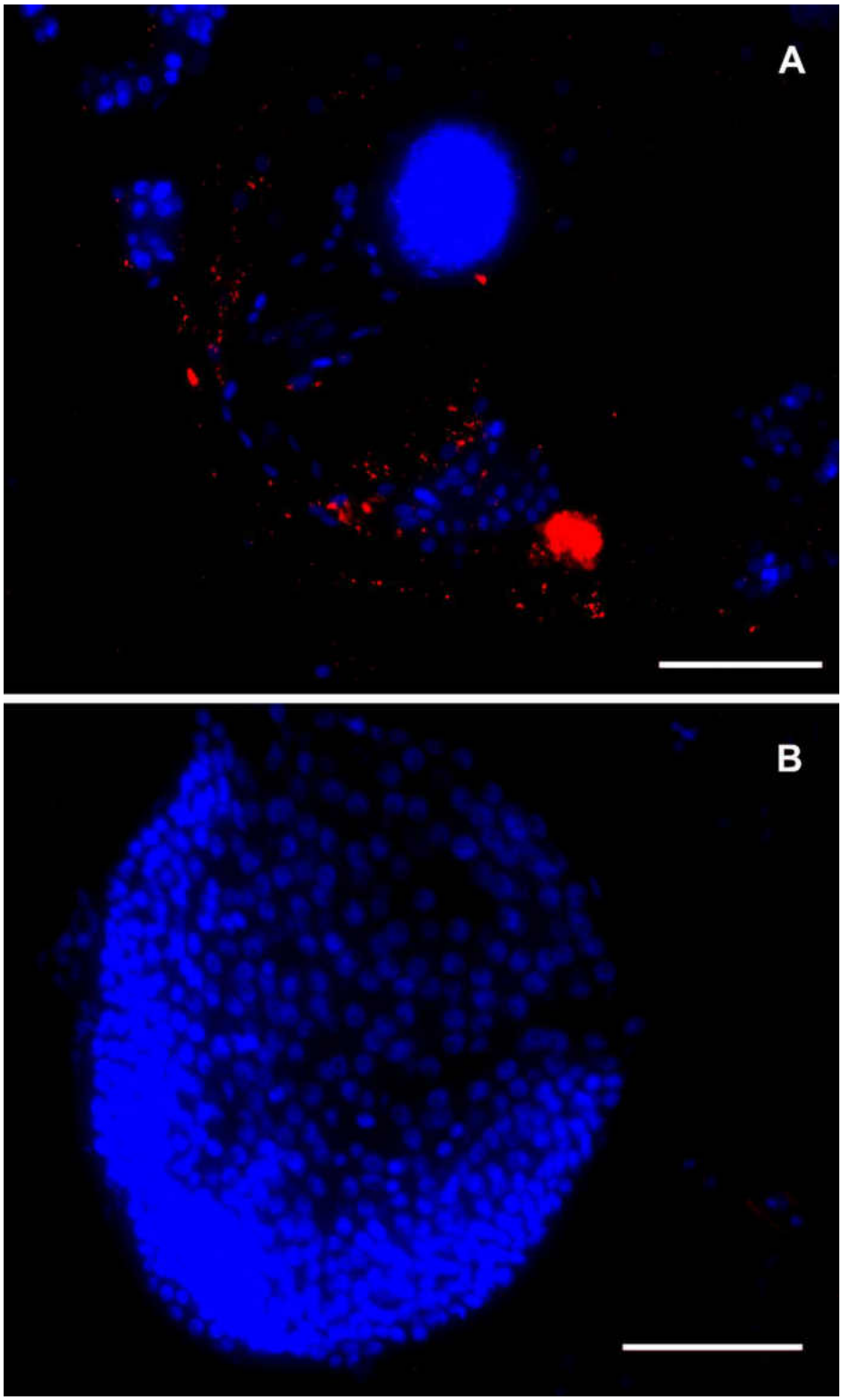

Figure 39. Indirect immunofluorescence microscopy of 10 µm cryosections of adult *P. elegans*. **A)** Cross section of cirrus sac infected with *Neorickettsia* sp. (red) digenean nuclei stained with DAPI (blue). B) Cross section of ovary, *Neorickettsia* sp. not within ovarian tissue. Scale bars 100µm (A), 50µm (B).

These stages are responsible for reproduction and the dramatic increases in number of individuals through either asexual (production of cercariae by sporocysts) or sexual (production of eggs by adults) reproduction. Therefore, this study was focused mainly on determining the location of *Neorickettsia* within sporocysts and adults.

Studies on the early developmental biology of digeneans are practically lacking. The majority of those that were done were focused on parasites of medical importance (i.e. schistosomes and liver flukes) (Galaktionov and Dobrovolskij 2003). However, Cort and Olivier (1943) conducted a study on the development of larval stages of *Plagiorchis muris* in the first intermediate host, which provides insightful information on the potential mechanism of *Neorickettsia* vertical transmission from the mother sporocyst to daughter sporocysts and from daughter sporocysts to cercariae. Although we did not study the localization of neorickettsiae within the mother sporocyst, the fact that all daughter sporocysts screened in our study (Chapter 4) were infected with *Neorickettsia* indicates that mother sporocysts contain the bacteria. According to Cort and Olivier (1943) the mother sporocyst of *P. muris* is round or oval, solid bodied, and packed with approximately 300 to 500 daughter sporocyst embryos. All embryos were at about the same stage of development. Assuming that *P. elegans* has similar development, we hypothesize that every embryo would be infected with *Neorickettsia*, and that the germinal cells within the mother sporocysts responsible for the production of the daughter embryos, would be infected with the bacteria. This is supported by my observations that 100% of 50 daughter sporocysts dissected from a field-collected snail were positive for *Neorickettsia* (Table 15).

In our study, mature daughter sporocysts of *P. elegans* had varying numbers of *Neorickettsia* distributed throughout their tegument (sporocyst wall) (Figure 33). Additionally, free floating cercarial embryos and more developed cercariae within the brood chamber have varying numbers (from none to a substantial number) of neorickettsiae, distributed throughout the organism (Figures 33 and 34). However, it remains unknown how the bacteriae are transmitted vertically from the daughter sporocyst to the cercarial embryos. Cort and Olivier (1943) showed that young daughter sporocysts, still within the brood chamber of the mother sporocyst, consisted of a thin membrane composed of flattened cells surrounding a mass of separate germ cells (germ mass), "which were so closely crowded together that they looked almost like the morula stage of an embryo". As the daughter sporocysts mature, the germ mass becomes smaller, the sporocysts elongate, and cercarial embryos appear (Cort and Olivier 1943) (Figure 40A). Eventually the daughter sporocysts are freed from the mother sporocyst and begin migration throughout the snail host. In one end of the body cavity of an immature daughter sporocyst, usually the more mobile anterior end, , there is a discrete, organized germ mass. Near the germ mass are a few very small cercarial embryos, followed by several larger cercarial embryos (Cort and Olivier 1943) (Figure 40B). Eventually the migrating sporocysts attach to the snails tissue, mature, elongate further, and become filled with embryos and mature cercariae. Mature sporocysts contain a single germ mass at the anterior end as well as numerous small cercarial embryos at different stages of development (Cort and Olivier 1943) (Figure 40C). Based on this description of sporocyst and cercarial development by Cort and Olivier (1943) we can generate a hypothesis on how neorickettsiae are being vertically transmitted from the daughter

sporocysts to the cercarial progeny, and why early in the infection not all cercariae become infected with the bacterial endosymbiont (Chapter 4). We hypothesize that vertical transmission of *Neorickettsia* from daughter sporocysts to cercariae occurs through the germ mass described above. However, because cercarial embryos appear very early in the sporocysts development (while it is still within the mother sporocyst) very few, to none, of the early cercariae shed from the snail will be infected with the bacteria. It may be happening if the multiplication of the bacteria within the daughter sporocyst lags behind the rate of production and development of cercarial embryos. *Neorickettsia* within the tegument and germ mass eventually catch up to the production of cercarial embryos, i.e., every germ cell within the germ mass become infected with *Neorickettsia*, and therefore, every resulting cercarial embryo becomes infected. Whether the germ mass becomes infected early in daughter sporocyst development or whether the germ mass becomes infected from infected tegumental cells is unknown. Although this hypothesis is logical and generally supported by our data and observations, there is a caveat that needs to be addressed. We are assuming that sporocyst development of *P. elegans* is similar to *P. muris*. Therefore, to truly understand vertical transmission of *Neorickettsia* from daughter sporocysts to cercariae within *P. elegans* a study of early *P. elegans* sporocyst development needs to be completed.

Within adult *P. elegans* neorickettsiae, were found within the tegument, parenchyma, vitellarium, testes, cirrus sac, uterus, and eggs. Additionally, it appears that the bacteria is within the gut caeca, however, we did not have a clear publishable image. The two organs/tissues where neorickettsiae were found in high abundance were the parenchyma and vitellarium. The parenchyma is the functional connective tissue of

trematodes, however, far from being a simple packing tissue, the parenchyma is a complex system of cells engaged in carbohydrate metabolism and transport (Smyth and Halton 1983). The parenchymal cells contain large amounts of glycogen, providing an excellent resource for neorickettsial survival and replication. The vitellarium is a group of glands that produce yolk cells (vitelline cells). Vitelline cells accumulate nutritive

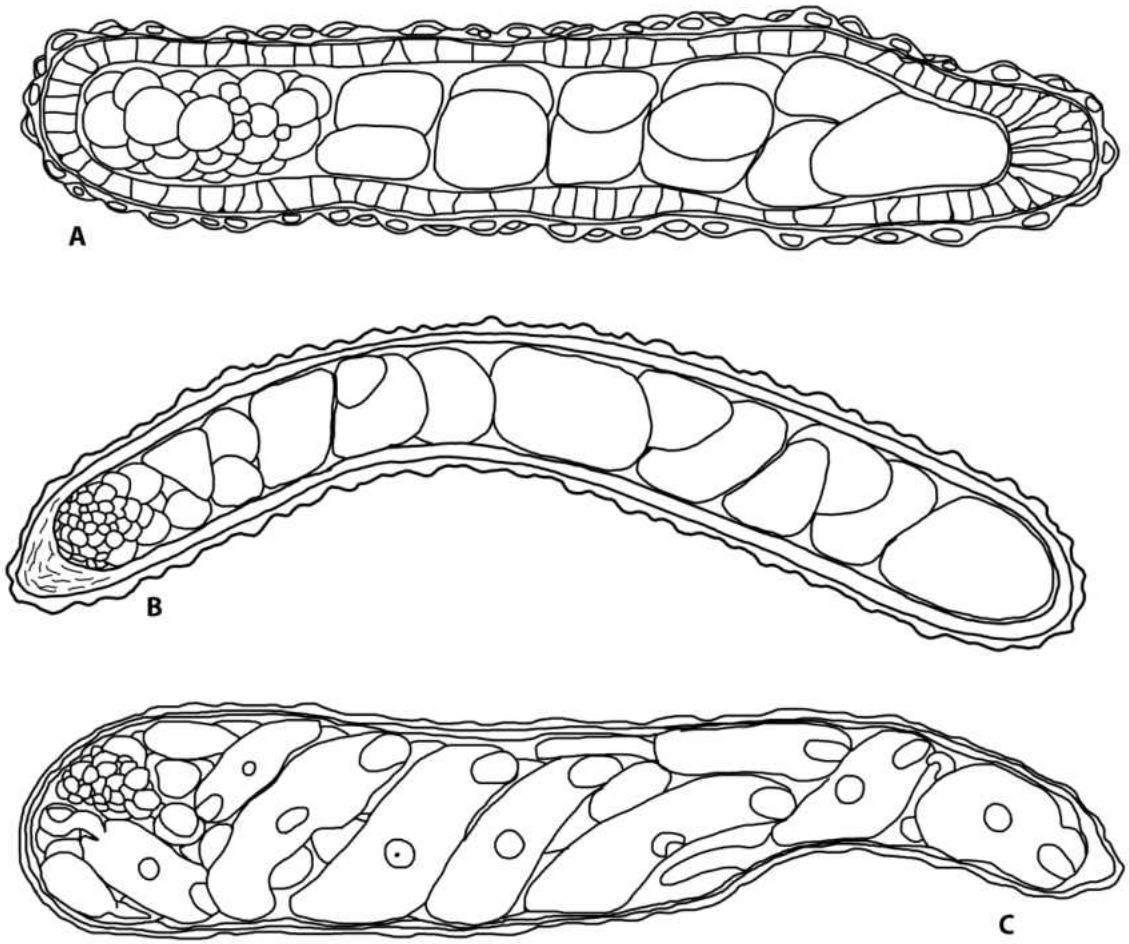

Figure 40. Drawing showing *Plagiorchis muris* daughter sporocysts at different levels of maturity. **A)** Daughter sporocyst embryo about ready to escape from mother sporocyst. Length, 0.2 mm. **B)** Immature daughter sporocyst after leaving the mother sporocyst, at the migrating stage. Length, 0.5 mm. **C)** Mature daughter sporocyst. Length, 1 mm. All drawings redrawn from Cort and Olivier (1943).

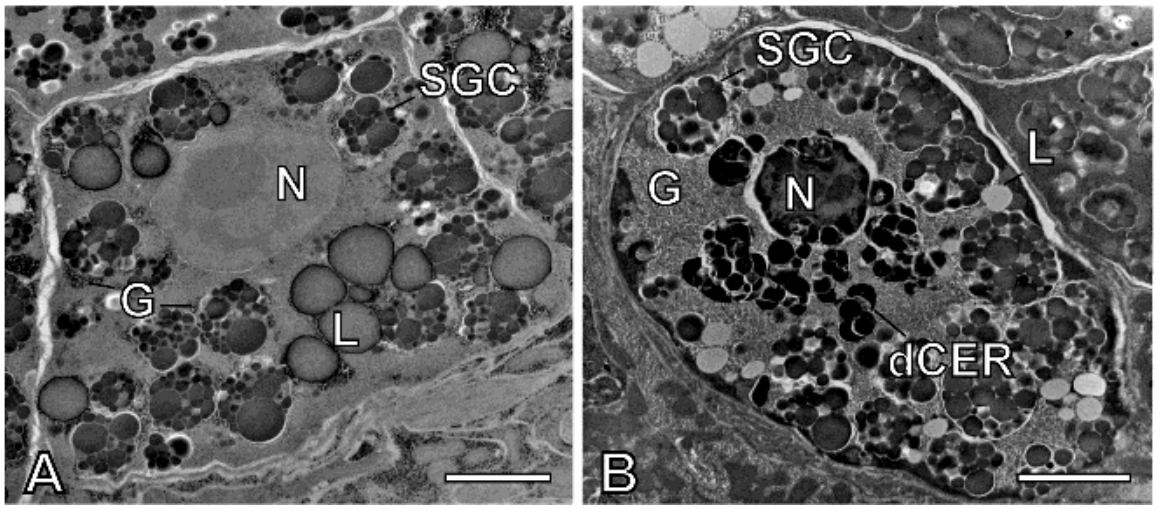

Figure 41. Electron micrograph of the fifth (final) stage of vitelline cell maturation. A) Glycogen granules grouped around lipid droplets and shell globule clusters. B) General view of a vitelline cell at the fifth stage of maturation, characterized by densely coiled sacculi of endoplasmic reticulum near the nucleus. Scale bars 3µm. G: glycogen granules; dCER: dense coiled endoplasmic reticulum saccules; L: saturated lipid droplet; N: nucleus; SGC: shell globule cluster.

reserves for the developing digenean embryo (Adiyodi and Adiyodi 1988; Swiderski and Xylander 2000). Our ultrastructural study of vitellogenesis in *P. elegans* has shown that the whole cytosol of mature vitelline cells is filled with α- and β- glycogen as well as lipids (Greani et al. 2014; Figure 41). Presumably this large supply of nutrients provides the proper environment for neorickettsiae replication.

Prior to our research it had been shown that *Neorickettsia* is transmitted transovarially to successive generations of digeneans. Nyberg et al. (1967) were able to successfully transmit *N. helminthoeca* (the causative agent of salmon poisoning disease) by injecting dogs with fluke eggs homogenized in a glass tissue grinder, but not with intact eggs, indicating that infectious neorickettsiae are contained within the interior of the unembryonated digenean eggs but not on the exterior surfaces. However, what was not known at that time was how the eggs became infected with neorickettsiae. At first, we

hypothesized that *Neorickettsia* sp. infects the ovarian tissue of *P. elegans*. However, based on the immunofluorescence data, the ovary of *P. elegans* is not infected with *Neorickettsia*; instead, neorickettsiae are found in high abundance within the vitellarium (Figure 36). Based on this we hypothesize that the transmission occurs not through infected egg cells, but instead from infected vitelline cells. Trematode egg development is ectolecithal, the egg cell contains little or no yolk, and yolk is contributed by the vitelline cells. We hypothesize that the lack of yolk within the oocytes leads to the inability of neorickettsiae to survive/replicate within these cells. Instead, the bacteria are able to thrive within the nutrient rich vitellarium which ensures their vertical transmission to the next digenean generation with the vitelline cells deposited in the egg.

This study had some significant limitations. The first was the level of resolution obtained with indirect immunofluorescence alone. We were able to identify the location of the bacteriae at the tissue level, particularly in the adults. However, to obtain greater resolution, at the cellular level, transmission electron microscopy would be needed. A second limitation was autofluorescence in thicker sections. We tried multiple methods to try and limit autofluorescence, including different stains (i.e. Sudan Black B and Evans Blue), however they did not appear to help. Therefore, we were limited to thinner sections. A third limitation had to do with the localization of the bacteria within eggs, cercariae, and metacercariae. These very small life cycle stages were difficult to work with, due partly to the inability to use whole mounts with the antibodies and fluorochromes we were using (autofluorescence). Therefore, we were limited to thinner sections of these three life cycle stages, resulting in a difficulty in identifying the exact location of the bacteria. Eggs were especially difficult, because in *P. elegans* the

miracidum does not develop while the egg is within the uterus of the digenean, but instead takes approximately 7 days to develop after being expelled from the digenean (Gorman, 1980).

The study of *Neorickettsia* sp. localization within different life cycle stages of the digenean *P. elegans* has significantly improved our knowledge of the transmission biology of *Neorickettsia*. Similar studies using other species of *Neorickettsia* within other digenean hosts are needed in order to fully clarify details of the vertical transmission of these bacteria through digenean life cycles. Additionally, ultrastructural studies using transmission electron microscopy in conjunction with immunofluorescence microscopy would provide a greater resolution, especially at cellular and subcellular level.

References

Adiyodi, K.G. and Adiyodi, R.G. (1988). Accessory sex glands. Reproductive biology of invertebrates. New York: John Wiley and Sons, 542.

Andrews, R.H., Sithithaworn, P. and Petney, T.N. (2008). *Opisthorchis viverrini*: an underestimated parasite in world health. *Trends In Parasitology* **24**, 497-501.

Baldwin, N.L., Millemann, R.E. and Knapp, S.E. (1967). 'Salmon poisoning' disease. III. Effect of experimental *Nanophyetus salmincola* infection on the host fish. *Journal of Parasitology* **53**, 556-564.

Barlough, J.E., Rikihisa, Y. and Madigan, J.E. (1997). Nested polymerase chain reaction for detection of *Ehrlichia risticii* genomic DNA in infected horses. *Veterinary Parasitology* **68,** 367-373.

Barlough, J.E., Reubel, G.H., Madigan, J.E., Vredevoe, L.K., Miller, P.E. and Rikihisa, Y. (1998). Detection of *Ehrlichia risticii*, the agent of Potomac horse fever, in freshwater stream snails Pleuroceridae *Juga* spp. of Northern California. *Journal of Applied and Environmental Microbiology* **64,** 2888-2893.

Barnewall, R., Ohashi, N. and Rikihisa, Y. (1999). *Ehrlichia chaffeensis* and *E. sennetsu*, but not the Human granulocytic ehrlichiosis agent colocalize with transferrin receptor and up-regulate transferrin receptor mRNA by activating iron-responsive protein 1. *Infection and Immunity* **67,** 2258-2265.

Barnewall, R., Ohashi, N. and Rikihisa, Y. (1999). *Ehrlichia chaffeensis* and *E. sennetsu*, but not the Human granulocytic ehrlichiosis agent colocalize with transferrin receptor and up-regulate transferrin receptor mRNA by activating iron-responsive protein 1. *Infection and Immunity* **67,** 2258-2265.

Bennington, E. and Pratt, I. (1960). The life history of the salmon-poisoning disease fluke, *Nanophyetus salmincola* (Chapin). *Journal of Parasitology* **46**, 91-100.

Biswas, B., Vemulapalli, R. and Dutta, S.K. (1994). Detection of *Ehrlichia risticii* from feces of infected horses by immunomagnetic separation and PCR. *Journal of Clinical Microbiology* **32**, 2147.

Booth, A.J., Stogdale, L. and Grigor, J.A. (1984). Salmon poisoning disease in dogs in southern Vancouver Island. *Canadian Veterinary Journal* **25**, 2-6.

Bordenstein, S.R., Paraskevopoulos, C., Hotopp, J.C.D., Sapountzis, P., Lo, N., Bandi, D., Tettelin, H., Werren, J.H. and Bourtzis, K. (2009). Parasitism and mutualism in *Wolbachia*: what the phylogenomic trees can and cannot say. *Molecular Biology and Evolution* **26**, 231–241.

Bosman, D.D., Farrell, R.K. and Gorham, J.R. (1970). Non-endoparasite transmission of salmon poisoning disease of dogs. *Journal of the American Veterinary Medical Association* **156**, 1907–1910.

Bourne, C.C., Cracknell, J.M. and Bacon, H.J. (2010). Veterinary issues related to bears (Ursidae). *International Zoo Yearbook* **44**, 16-32.

Bray, R.A. (1982). Two new species of *Baccifer* Nicoll, 1914 (Digenea: Fellodistomidae) from mullet in Australia. *Journal of Natural History* **16**, 23-29.

Brouqui, P. and Raoult, D. (1990). In vitro susceptibility of *Ehrlichia sennetsu* to antibiotics. *Antimicrobial Angents and Chemotherapy* **4**, 1593-1596.

Burch, J.B. (1989). North American freshwater snails. Hamburg, Michigan: Malacological Publications. 365 p.

Burgdorfer, W. and Brinton, L.P. (1975). Mechanisms of transovarial infection of spotted fever rickettsiae in ticks. *Annals of the New York Academy of Sciences* **266**, 61–72.

Casiraghi, M., Bordenstein, S.R., Baldo, L., Lo, N., Beninati, T., Wernegreen, J. J., Werren, J. H. and Bandi, C. (2005). Phylogeny of *W. pipientis* pipientis based on gltA, groEL and ftsZ gene sequences: clustering of arthropod and nematode symbionts in the F supergroup, and evidence for further diversity in the *W. pipientis* tree. *Microbiology* **151**, 4015-4022.

Chae, J.S., Pusterla, N., Johnson, E., Derock, E., Lawler, P.E. and Madigan, E.J. (2000). Infection of aquatic insects with trematode metacercariae carrying *Ehrlichia risticii*, the cause of Potomac horse fever. *Journal of Medical Entomology* **37**, 619-625.

Chae, J.S., Kim, M.S. and Madigan, J. (2002). Detection of *Neorickettsia* (*Ehrlichia*) *risticii* in tissues of mice experimentally infected with cercariae of trematodes by in situ hybridization. *Veterinary Microbiology* **88**, 233-243.

Chae, J.S., Kim, E.H., Kim, M.S., Kim, M.J., Cho, Y.H. and Park B.K. (2003). Prevalence and sequence analyses of *Neorickettsia risticii*. *Annals of the New York Academy of Sciences* **990**, 248-256.

Chai, J.Y. and Sohn, W.M. (1988). Identification of *Stellantchasmus falcatus* Metacercariae Encysted in Mullets in Korea. *Korean Journal of Parasitology* **26**, 65-68.

Chai, J.Y., Murrell, K.D. and Lymbery, A.J. (2005a). Fish-borne parasitic zoonoses: status and issues. *International Journal of Parasitology* **35**, 1233-1254.

Chai, J.Y., Park, J.H., Han, E.T., Guk, S.M., Shin, E.H., Lin, A., Kim, J.L., Sohn, W.M., Yong, T.S., Eom, K.S., Min, D.Y., Hwang, E.H., Phommmasack, B., Insisiengmay, B. and Rim, H.J. (2005b). Mixed infections with *Opisthorchis viverrini* and intestinal flukes in residents of Vientiane Municipality and Saravane Province in Laos. *Journal of Helminthology* **79**, 283-289.

Chai, J.Y., Hann, E.T., Gukn, S.M., Shinn, E.H., Sohnn, W.M., Yongn, T.S., Eomn, K.S., Leen, K.H., Jeong, H.G., Ryang, Y.S., Hoang, E.H., Phommasack, B., Insisiengmay, B., Lee, S.H. and Rim, H.J. (2007). High prevalence of liver and intestinal fluke infections among residents of Savannakhet Province in Laos. *Korean Journal of Parasitology* **45**, 213-218.

Chai, J.Y., Han, E.T., Guk, S.M., Shin, E.H., Sohn, W.M., Yong, T.S., Eom, K.S., Min, D.Y., Um, J.Y., Park, M.S., Hoang, E.H., Phommasack, B., Insisiengmay, B., Lee, S.H. and Rim, H.J. (2009a). High prevalence of *Haplorchis taichui*, *Phaneropsolus molenkampi*, and other helminth infections among people in Khammouane Province, Lao PDR. *Korean Journal of Parasitology* **47**, 243-247.

Chai, J.Y., Shin, E.H., Lee, S.H. and Rim, H.J. (2009b). Foodborne intestinal flukes in Southeast Asia. *Korean Journal of Parasitology* **47, supplement**, 69-102.

Chaichanasiriwithaya, W., Rikihisa, Y., Yamamoto, S., Reed, S.M., Perryman, L.E., Crawford, T.B. and Palmer, G. (1994). Antigenic, morphologic, and molecular characterization of 9 new *Ehrlichia risticii* isolates. *Journal of Clinical Microbiology* **38,** 3026-3033.

Chapin, E.A. (1926). A new genus and species of trematode, the probable cause of salmon-poisoning in dogs. *The North American Veterinarian* **7**, 42-43.

Cheng, T.C. and R.W. Snyder. (1962). Studies on the host-parasite relationships between larval trematodes and their hosts. I. Review. II. The utilization of the host's glycogen by the intramolluscan larvae of *Glypythelmins pennsylvaniensis* Cheng, and associated phenomena. *Transactions of the American Microscopical Society* **81**, 209-228.

Cicuttin, G.L., Boeri, E.J., Beltran, F.J. and Gury-Dohmen, F.E. (2013). Molecular detection of *Neorickettsia risticii* in Brazilian free-tailed bats (*Tadarida brasiliensis*) from Buenos Aires, Argentina. *Pesquisa Veterinaria Brasileira* **33**, 648-650.

Coffman, E.A., Abd-Eldaim, M. and Craig, L.E. (2008). Abortion in a horse following *Neorickettsia risticii* infection. *Journal of Veterinary Diagnostic Investigation* **20**, 827-830.

Coimbra, H.S., Schuch, L.F.D., Veitenheimer-Mende, I.L. and Meireles, M.C.A. (2005). *Neorickettsia* (*Ehrlichia*) *risticii* no sul do Brasil: *Heleobia* spp. (Mollusca: Hydrobiidae) e Parapleurolophocecous cercariae (Trematoda: Digenea) como possíveis vetores. *Arquivos do Instituto Biológico*, **72**, 325-329.

Cordes, D.O., Perry, B.D., Rikihisa, Y. and Chickering, W.R. (1986). Enterocolitis caused by *Ehrlichia* sp. in the horse (Potomac horse fever). *Veterinary Parasitology* **23**, 471-477.

Cordy, D.R. and Gorham, J.R. (1950). The pathology and etiology of salmon poisoning disease in the dog and fox. *American Journal of Pathology* **23**, 617-637.

Cort, W.W. and Olivier, L. (1943). The development of the larval stages of Plagirochis *muris* Tanabe, 1922, in the first intermediate host. *Journal of Parasitology* **29**, 81-99.

Cribb, T.H., Anderson, G.R. and Bray, R.A. (1999). Faustulid trematodes (Digenea) from marine fishes of Australia. *Systematic Parasitology* **44**, 119-138.

Cribb, T.H., Bray, R.A. and Littlewood, D.T. (2001). The nature and evolution of the association among digeneans, molluscs and fishes. *International Journal for Parasitology* **31**, 997-1011.

Cribb, T.H., Bray, R.A., Littlewood, D.T.J., Pichelin, S.P. and Herniou, E.A. (2001). The Digenea. In: Interrelationships of the Platyhelminthes. London: Taylor and Francis 168–185.

Cribb, T.H., Bray, R.A., Olson, P.D. and Littlewood, D.T. (2003). Life cycle evolution in the Digenea: a new perspective from phylogeny. *Advances in Parasitology* **54**, 197-254.

Donham, C.R. (1925). So-called salmon poisoning of dogs. *Science* **61**, 341.

Dumler, J.S. (2000). Bugs, snails and horses: expanding the knowledge of infection vectors with new and old technologies. *Equine Veterinary Journal* **32**, 273-274.

Dumler, J.S., Barbet, A.F., Bekker, C.P.J., Dasch, G.A., Palmer, G.H., Ray, S.C., Rikihisa, Y. and Rurangirwa, F.R. (2001). Reorganization of genera in the families Rickettsiaceae and Anaplasmataceae in the order Rickettsiales: unification of some species of *Ehrlichia* with *Anaplasma*, *Cowdria* with *Ehrlichia* and *Ehrlichia* with *Neorickettsia*, descriptions of six new species combinations and designation of *Ehrlichia equi* and 'HE agent' as subjective synonyms of *Ehrlichia phagocytophila*. *International Journal of Systematic and Evolutionary Microbiology* **51**, 2145-2165.

Dumler, J.S., Rikihisa, Y. and Dasch, G.A. (2005). Family II Anaplasmataceae. In: *Bergey's Manual of Systemic Bacteriology* (Garrity, G.M. (Ed.), second ed.). Vol. 2, pp. 117-143. New York: Springer.

Dutra, F., Schuch, L.F., Delucchi, E., Curcio, B.R., Coimbra, H., Raffi, M.B., Dellagostin, O. and Riet-Correa, F. (2001). Equine monocytic ehrlichiosis (Potomac horse fever) in horses in Uruguay and southern Brazil. *Journal of Veterinary Diagnostic Investigation* **13**, 433-437.

Dutta, S.K., Vemulapalli, R. and Biswas, B. (1998). Association of deficiency in antibody response to vaccine and heterogeneity of *Ehrlichia risticii* strains with Potomac horse fever vaccine failure in horses. *Journal of Clinical Microbiology* **36**, 506-512.

Eastburn, R.L., Fritsche, T.F. and Terhune, C.A. (1987). Human intestinal infection with *Nanophyetus salmincola* from salmonid fishes. *American Journal of Tropical Medicine and Hygiene* **36**, 586-591.

Farrell, R.K., Lloyd, M.A. and Earp, B. (1964). Persistence of *Neorickettsia helminthoeca* in an endoparasite of the Pacific salmon. *Science* **145**, 162-163.

Farrell, R.K., Dee, J. F. and Ott, R.L. (1968). Salmon poisoning in a dog fed kippered salmon. *Journal of the American Veterinary Medical Association* **152**, 370-371.

Farrell, R.K., Leader, R.W. and Johnston, S.D. (1973). Differentiation from salmon poisoning disease and Elokomin fluke disease fever: studies with the black bear (*Ursus americanus*). *American Journal of Veterinary Research* **34**, 919-922.

Farrell, R.K., Soave, O.A. and Johnston, S.D. (1974). *Nanophyetus salmincola* infections in kippered salmon. *American Journal of Public Health* **64**, 808-809.

Farren, L. (2007). Potomac horse fever: the final piece of the puzzle. *Equus* **357**, 49-58.

Ferri, E., Bain, O., Barbuto, M., Martin, C., Lo, N., Uni, S., Landmann, F., Baccei, S.G., Guerrero, R., de Souza Lima, S., Bandi, C., Wanji, S., Diagne, M. and Casiraghi, M. (2011). New Insights into the evolution of *Wolbachia* infections in filarial nematodes inferred from a large range of screened species. *Public Library of Science ONE* **6**, 1-17.

Filimonova, L.V. (1963). The biological cycle of the trematode *Nanophyetus schikhobalowi*. *Academy of Sciences of the USSR* **13**, 347-357.

Fischer, K., Beatty, W.L., Jiang, D., Weil, G.J. and Fischer, P.U. (2011). Tissue and stage-specific distribution of *Wolbachia* in *Brugia malayi*. *PLoS Neglected Tropical Diseases* **5**, 1174.

Foreyt, W.J. and Thorson, S. (1982). Experimental salmon poisoning disease in juvenile coyotes (*Canis latrans*). *Journal of Wildlife Diseases* **18**, 159-162.

Foreyt, W.J., Gorham, J.R., Leathers, C.W. and Leamaster, B.R. (1987). Salmon poisoning disease in juvenile coyotes: clinical evaluation and infectivity of metacercariae and *Rickettsiae*. *Journal of Wildlife Diseases* **23**, 412-417.

Foreyt, W.J. and Gorham, J.R. (1988). Evaluation of praziquantel against induced *Nanophyetus salmincola* infections in coyotes and dogs. *American Journal of Veterinary Research* **49**, 563-565.

Fukuda, T. (1958). Rickettsial mononucleosis (synonyms: Hyuganetsu, kagaminetsu). *The Kitasato Archives* of *Experimental Medicine* **31**, 51-56.

Fukuda, T. and Yamamoto, S. (1981). *Neorickettsia*-like organism isolated from metacercaria of a fluke, *Stellantchasmus falcatus*. *Japanese Journal of Medical Science and Biology* **34**, 103-107.

Fukuda, T., Sasahara, T. and Kitao, T. (1962). Studies on the causative agent of "Hyuganetsu" disease. X. Vector. *Journal of the Japanese Association for Infectious Diseases* **36**, 235-241.

Fukuda, T., Sasahara, T. and Kitao, T. (1973). Causative agent of "Hyuganetsu" disease. 11. Characteristics of *rickettsia*-like organisms isolated from metacercaria of *Stellantchasmus falcatus. Kansenshogaku Zasshi* **47**, 474-482.

Fukuda, T., Kitao, T. and Keida, Y. (1954). Studies on the causative agent of "Hyuganetsu" disease. I. Isolation of the agent and its inoculation trial in human beings. *Medical Biology* **32**, 200-209.

Gai, J.J. and Marks, S.L. (2008). Salmon poisoning disease in two Malayan sun bears. *Journal of the American Veterinary Medical Association* **232**, 586-588.

Galaktionov, K.V. and Dobrovolskij, A.A. (2003) The Biology and Evolution of Trematodes. (B. Fried and T.K. Graczyk, eds). Boston: Kluwer Academic Publishers.

Gebhardt, G.A., Millemann, R.E., Knapp, S.E. and Nyberg, P.A. (1966). 'Salmon poisoning' disease. II. Secondary intermediate host susceptibility studies. *Journal of Parasitology* **52**, 54-59

Gibson, D.I., Jones, A. and Bray, R.A. (Eds.) (2002). Keys to the Trematoda, Volume 1. New York: CABI publishing. 544p.

Gibson, D.I., Jones, A. and Bray, R.A. (Eds.) (2005). Keys to the Trematoda, Volume 2. New York: CABI publishing. 745p.

Gibson, D.I., Jones, A. and Bray, R.A. (Eds.) (2008). Keys to the Trematoda, Volume 3. New York: CABI publishing. 848p.

Gibson, K.E., Rikihisa, Y., Zhang, C. and Martin, C. (2005). *Neorickettsia risticii* is vertically transmitted in the trematode *Acanthatrium oregonense* and horizontally transmitted to bats. *Environmental Microbiology* **7,** 203-212.

Gibson, K.E. and Rikihisa, Y. (2008). Molecular link of different stages of the trematode host of *Neorickettsia risticii* to *Acanthatrium oregonense*. *Environmental Microbiology* **10,** 2064-2073.

Gibson, K., Kumagai, Y. and Rikihisa, Y. (2010). Proteomic Analysis of *N. sennetsu* Surface-exposed Proteins and Porin Activity of the Major Surface Protein P51. *J. Bacteriol.* **192,** 5898-5905.

Gibson, K., Pastenkos, G., Moesta, S. and Rikihisa, Y. (2011). *Neorickettsia risticii* surface-exposed proteins: Proteomics identification, recognition by naturally-infected horses and strain variations. *Veterinary Research* **42,** 71.

Gorman, A.M. (1980). Studies on the biology of *Plagiorchis elegans* (Rudolphi, 1802), (Trematodas: Digenea) in its mammalian and molluscan hosts. *The University of Leeds Department of Pure and Applied Zoology*, Dissertation.

Goyal, K., Qamra, R. and Mande, S.C. (2006). Multiple gene duplication and rapid evolution in the groEL gene: functional implications. *Journal of Molecular Evolution* **63**, 781-787.

Graybill, H.W. and Smith, T. (1920). Production of fatal blackhead in turkeys by feeding embryonated eggs of *Heterakis papillosa*. *Journal of Experimental Medicine* **31**, 647-655.

Greani, S., Quilichini, Y., Foata J., Greiman, S.E., Ndiaye, P.I., Tkach, V.V., Marchand B. (2014). Vitellogenesis of the digenean *Plagiorchis elegans* (Rudolphi, 1802) (Plagiorchiodea, Plagiorchiidae). *Parasitology International* 63, 537-543.

Green, J. S., Foreyt, W.J. and Woodruff, R.A. (1986). Salmon poisoning disease: research on a potential method of lethal control for coyotes. *Proceedings of the Twelfth Vertebrate Pest Conference*. University of Nebraska, Lincoln, 312-317.

Greiman, S.E., Tkach V.V. and Vaughan, J.A. (2013). Transmission rates of the bacterial endosymbiont, *Neorickettsia risticii*, during the asexual phase of its digenean host, *Plagiorchis elegans*, within naturally infected lymnaeid snails. *Parasites and Vectors* 6, 303.

Guindon, S. and Gascuel, O. (2003). A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. *Systems Biology* **52**, 696-704.

Hahn, N.E., Fletcher, M., Rice, R.M., Kocan, K.M., Hansen, J.W., Hair, J.A., Barker, R.W. and Perry, B.D. (1990). Attempted transmission of *Ehrlichia risticii*, causative agent of Potomac horse fever, by the ticks *Dermacentor variabilis*, *Rhiphicephalus sanguineus*, *Ixodes scapularis* and *Amblyomma americanum*. *Exp. Appl. Acarol*. **8**, 41-50.

Hall, T.A. (1999). BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series* **41**, 95–98.

Harrell, L.W. and Deardorff, T.L. (1990). Human nanophyetiasis: transmission by handling naturally infected coho salmon (*Oncorhynchus kisutch*). *Journal of Infectious Diseases* **161**, 146-148.

Headley, S.A., Vidotto, O., Scorpio, D., Dumler, J.S. and Mankowski, J. (2004). Suspected cases of *Neorickettsia*-like organisms in Brazilian dogs. *Annals of the New York Academy of Sciences* **1026**, 79-83.

Headley, S.A., Kano, F.S., Scorpio, D.G., Tamekuni, K., Barat, N.C., Bracarense, A.P.F.R.L., Vidotto, O. and Dumler, J.S. (2009). *Neorickettsia helminthoeca* in Brazilian dogs: a cytopathological, histological and immunohistochemical study. *Clinical Microbiology and Infection* **15, Suppl. 2**, 21-23.

Headley, S.A., Scorpio, D.G., Vidotto, O. and Dumler, J.S. (2011). *Neorickettsia helminthoeca* and salmon poisoning disease: a review. *Veterinary Journal* **187**, 165-173.

Hirai, K. (1966). Study of infectious mononucleosis in Kumamoto Prefecture. 1. Isolation of a *Rickettsia sennetsu* -like organism from patients with epidemic glandular fever. *Kumamoto Igakkai Zasshi* **40**, 1159-1173.

Holland, C.J., Ristic, M., Cole, A.I., Johnson, P., Barker, G. and Goetz, T. (1985a). Isolation, experimental transmission, and characterization of causative agent of Potomac horse fever. *Science* **227**, 522-524.

Holland, C.J., Ristic, M., Huxsoll, D.L., Cole, A.I. and Rapmund, G. (1985b). Adaptation of *Ehrlichia sennetsu* to canine blood monocytes: preliminary structural and serological studies with cell culture-derived *Ehrlichia sennetsu*. *Infection and Immunity* **48**, 366-371.

Holland, C.J., Weiss, E., Burgdorfer, W., Cole, A.I. and Kakoma, I. (1985c). *Ehrlichia risticii* sp. nov.: etiological agent of equine monocytic ehrlichiosis (synonym, Potomac horse fever). *International Journal of Systematic Bacteriology* **35**, 524-526.

Hortle, K.G. (2007). Consumption and the yield of fish and other aquatic animals from the Lower Mekong Basin. In: *MRC Technical Report No. 16.* Vientiane: Mekong River Commission.

Hotopp, J.C.D., Lin, M., Madupu, R., Crabtree, J., Angiuoli, S.V., Eisen, J.A., Seshadri, R., Ren, Q., Wu, M., Utterback, T.R., Smith, S., Lewis, M., Khouri, H., Zhang, C., Niu, H., Lin, Q., Ohashi, N., Zhi, N., Nelson, W., Brinkac, L.M., Dodson, R.J., Rosovitz, M.J., Sundaram, J., Daugherty, S.C., Davidsen, T., Durkin, A.S., Gwinn, M., Haft, D.H., Selengut, J.D., Sullivan, S.A., Zafar, N., Zhou, L., Benahmed, F., Forberger, H., Halpin, R., Mulligan, S., Robinson, J., White, O., Rikihisa, Y. and Tettelin, H. (2006). Comparative genomics of emerging human ehrlichiosis agents. *PLoS Genetics* **2**, 21.

Houry, W.A., Frishman, D., Eckerskorn, C., Lottspeich, F. and Hartl, F.U. (1999). Identification of in vivo substrates of the chaperonin GroEL. Nature **402**, 147-154.

Huelsenbeck, J.P. and Ronquist, F. (2001). MRBAYES: Bayesian inference of phylogeny. *Bioinformatics* **17**, 754-755.

Hunter, W.S. and Thomas, L.J. (1961). A new species of *Saccocoelium* (Trematoda, Haploporidae) from Beaufort, N.C.. *Transactions of the American Microscopical Society* **80**, 176-179.

Inokuma, H., Brouqui, P., Drancourt, M. and Raoult, D. (2001). Citrate synthase gene sequence. A new tool for phylogenetic analysis and identification of *Ehrlichia*. *Journal of Clinical Microbiology* **39**, 3031-3039.

Kanter, M., Mott, J., Ohashi, N., Fried, B., Reed, S., Lin, Y. and Rikihisa, Y. (2000). Analysis of 16S rRNA and 51-kDa antigen gene and transmission in mice of *Ehrlichia risticii* in virgulate trematodes from *Elimia livescens* snails in Ohio. *Journal of Clinical Microbiology* **38,** 3349-3358.

Katsuta, I. (1931). Studies on Formosan trematodes whose intermediate hosts are brackish water fishes. I. *Stellantchasmus falcatus* n.sp. with mullet as its vector. *Taiwan Igakkai Zasshi* **30**, 1404-1417.

Kitao, T., Farrell, K. and Fukuda, T. (1973). Differentiation of salmon poisoning disease and Elokomin fluke fever: fluorescent antibody studies with *Rickettsia sennetsu*. *American Journal of Veterinary Research* **34**, 927-928.

Landmann, F., Bain, O., Martin, C., Uni, S., Taylor, M.J. and Sullivan, W. (2012). Both asymetric mitotic segregation and cell-to-cell invasion are required for stable germline transmission of *Wolbachia* in filarial nematodes. *Biology Open* **1**, 536–547.

Larsen, A., Tao, Z., Bullard, S.A. and Arias, C.R. (2013). Diversity of the skin microbiota of fishes: evidence for host species specificity. *FEMS Microbiology Ecology* **85**, 483-494.

Lee, D.L. (1971). Helminths as vectors of microorganisms. In: *Ecology and physiology of parasites* (A.M. Fallis, ed.). pp. 104-122. Toronto: University of Toronto Press.

Levine, J.F., Levy, M.G., Nicholson, W.L. and Gager, R.B. (1990). Attempted *Ehrlichia risticii* transmission with *Dermacentor variabilis* (Acari: Ixodidae). *J. Med. Entomol*. **27**, 931-933.

Lin, M., Zhang, C., Gibson, K. and Rikihisa, Y. (2009). Analysis of Complete Genome Sequence of *Neorickettsia risticii*: Causative Agent of Potomac Horse Fever. *Nucleic Acids Research* **37,** 6076-6091

Liu, S.F. (2002). One new species of *Saccocoelioides* (Digenea: Haploporidae) from *Liza carinatus* in Taiwan Strait. Journal of Oceanography **21**, 37-44.

Long, M.T., Goetz, T.E., Whiteley, H.E., Kakoma, I. and Lock, T.E. (1995). Identification of *Ehrlichia risticii* as the causative agent of two equine abortions following natural maternal infection. *Journal of Veterinary Diagnostic Investigation* **7**, 201-5.

Lotz, J.M. and Font, W.F. (2008). Family Lecithodendriidae Luhe, 1901. In Bray, Gibson, and Jones (eds). Keys to the Trematoda, Volume 3. New York: CABI publishing 527 – 536.

Maddison, D.R. and Maddison, W.P. (2005). MacClade 4: Analysis of phylogeny and character evolution. Version 4.08a. Available: http://macclade.org.

Madigan, J.E. and Pusterla, N. (2000). Ehrlichial diseases. *Veterinary Clinics of North America Equine Practice* **16**, 487-499.

Madigan, J.E., Rikihisa, Y., Palmer, J.E., DeRock, E. and Mott, J. (1995). Evidence for a high rate of false-positive results with the indirect fluorescent antibody test for *Ehrlichia risticii* antibody in horses. *Journal* of the *American Veterinary Medical Association* **207**, 1448-1453.

Madigan, J.E., Barlough, J.E., Rikihisa, Y., Wen, B., Miller, P.E. and Sampson, T.J. (1997). Identification of the Shasta River Crud syndrome as Potomac horse fever Equine monocytic ehrlichiosis. *Journal of Equine Veterinary Science* **17,** 270-272.

Manning, G.S. and Lertprasert, P. (1973). Studies on the life cycle of *Phaneropsolus bonnei* and *Prosthodendrium molenkampi* in Thailand. *Annals of Tropical Medicine and Parasitology* **67**, 361-365.

McCauley, J.E. and Pratt, I. (1961). A new genus *Deropegus* with a redescription of *D. aspina* (Ingles, 1936) nov. comb. *Transactions of the American Microscopical Society* **55**, 373-377.

Messick, J.B. and Rikihisa, Y. (1992). Presence of parasite antigens on the surface of P388D1 cells infected with *Ehrlichia risticii*. *Infection and Immunity* **60,** 3079-3086.

Messick, J.B. and Rikihisa, Y. (1992b). Suppression of I-Ad on P388D1 cells by *Ehrlichia risticii* infection in response to gamma interferon. *Veterinary Immunology and Immunopathology* **32**, 225-241.

Millemann, R.E., Gebhardt, G.A. and Knapp, S.E. (1964). "Salmon poisoning" disease. I. Infection in a dog from marine salmonids. *Journal of Parasitology* **50**, 588-589.

Millemann, R.C. and Knapp, S.E. (1970). Biology of *Nanophyetus salmincola* and 'salmon poisoning' disease. *Advances in Parasitology* **8**, 1-41.

Misao, T. and Katsuta, K. (1956). Epidemiology of infectious mononucleosis. *Japanese Journal of Clinical and Experimental Medicine* **33**, 73-82.

Misao, T. and Kobayashi, Y. (1954). Studies on infectious mononucleosis. I. Isolation of etiologic agent from blood, bone marrow, and lymph node of a patient with infectious mononucleosis by using mice. *Tokyo Iji Shinshi* **71**, 683-686.

Misao, T. and Kobayashi, Y. (1955). Studies on infectious mononucleosis (glandular fever). I. Isolation of the etiological agent from blood, bone marrow and lymph nodes of a patient infected with infectious mononucleosis by using mice. *Kyushu Journal of Medical Science* **6**, 145-152.

Mott, J., Rikihisa, Y., Zhang, Y., Reed, S. M. and Yu, C.Y. (1997a). Comparison of PCR and culture to the indirect fluorescent-antibody test for diagnosis of Potomac horse fever. *Journal of Clinical Microbiology* **35**, 2215.

Mott, J., Rikihisa, Y., Zhang, Y., Reed, S. M. and Yu, C.Y. (1997b). Polymerase chain reaction and Southern blot analysis of *E. risticii* in the blood and feces of horses. *Journal of Clinical Microbiology* **35,** 2215-2219.

Mott, J., Muramatsu, Y., Seaton, E., Martin, C., Reed, S. and Rikihisa, Y. (2002). Molecular Analysis of *Ehrlichia risticii* in Adult aquatic Insects in Pennsylvania, in Horses infected by ingestion of insects, and isolated in cell culture. *Journal of Clinical Microbiology* **40,** 690-693.

Mulville, P. (1991). Equine monocytic ehrlichiosis (Potomac horse fever): a review. *Equine Veterinary Journal* **23**, 400-404.

Mwangi, I.N., Sanchez, M.C., Gerald, M.M., Agola, L.E., Runo, S.M., Cupit, P.M., Cunningham, C. (2014) Praziquantel sensitivity of Kenyan *Schistosoma mansoni* isolates and the generation of a laboratory strain with reduced susceptibility to the drug. *International Journal of Parasitology: Drugs and Drug Resistance* 4, 296-300.

Ndiaye, P.I., Quilichini, Y., Tkach V.V., Greiman S.E., Ba, C.T., Marchand B. (2012) Ultrastructure of the Spermatozoon of the Digenean *Plagiorchis elegans* (Rudolphi, 1802)(Plagiorchioidea, Plagiorchiidae). *Journal of Morphology* 247, 965-972.

Newton, P., Rolain, J.M., Rasachack, B., Mayxay, M., Vathanatham, K., Seng, P., Phetsouvanh, R., Thammavong, T., Zahidi, J., Suputtamongkol, Y., Syhavong, B. and Raoult, D. (2009). Sennetsu neorickettsiosis: a probable fish-borne cause of fever rediscovered in Laos. *American Journal of Tropical Medicine and Hygiene* **81**, 190-194.

Nyberg, P.A., Knapp, S.E. and Millemann, R.E. (1967). 'Salmon poisoning' disease. IV. Transmission of the disease to dogs by *Nanophyetus salmincola* eggs. *Journal of Parasitology* **3**, 694-699.

Olson P.D., Cribb, T.H., Tkach V.V., Bray, R.A. and Littlewood, D.T.J. (2003). Phylogeny and classification of the Digenea (Platyhelminthes: Trematoda). *International Journal of Parasitology* **33**, 733-755.

Ohtaki, S. and Shishido, A. (1965). Studies on infectious mononucleosis induced in the monkey by experimental infection with *Rickettsia sennetsu*. II. Pathological findings. *Japanese Journal of Medical Science & Biology* **18**, 85-99.

Overstreet, R.M. (1971). Some adult digenic trematodes in striped mullet from the northern Gulf of Mexico. *Journal of Parasitology* **57**, 967-974.

Palmer, J.E. (1993). Potomac horse fever. *Veterinary Clinics of North America: Equine Practice* **9**, 399-410.

Palmer, J.E. and Benson, C.B. (1994). Studies on oral transmission of Potomac horse fever. *Journal of Veterinary Medicine* **2**, 87-92.

Park, B.K., Kim, M.J., Kim, E.H., Kim, M.S., Na, D.G. and Chae, J.S. (2003). Identification of trematode cercariae carrying *Neorickettsia risticii* in freshwater stream snails. *Annals of the New York Academy of Sciences* **990**, 239-247.

Pearson, J.C. (1964). A revision of the subfamily Haplorchiinae Loss, 1899 (Heterophyidae). I. The *Haplorchis* group. *Parasitology* **54**, 601-676.

Pernot, E.F. (1911). "Salmoning" of dogs. *Oregon State Board of Health. Bulletin* **5**, 1-21.

Philip, C. B., Hadlow, W. J. and Hughes, L. E. (1953). *Neorickettsia helmintheca*, a new *rickettsia*-like disease agent of dogs in western United States transmitted by a helminth. *International Gongress Microbiol. Rept. Proc*., 6th Gongr., Rome. **Vol. II**, 256-257.

Philip, C.B., Hadlow, W.J. and Hughes, L.E. (1954a). Studies on salmon poisoning disease of canines. I. The rickettsial relationships and pathogenicity of *Neorickettsia helmintheca*. *Experimental Parasitology* **3**, 336-350.

Philip, C.B., Hughes, L.E., Locker, B. and Hadlow, W.J. (1954b). Salmon poisoning disease of canines. II. Further observations on etiologic agent. *Proceedings of the Society for Experimental Biology and Medicine* **87**, 397-400.

Philip, C.B. (1955). There is always something new under the ‘‘parasitological sun” (the unique story of helminth-borne salmon poisoning disease). *Journal of Parasitology* **41**, 125-148.

Pinheiro, J. and Amato, S.B. (1994). *Eurytrema coelomaticum* (Digenea, Dicrocoeliidae): the effect of infection on carbohydrate contents of its intermediate snail host, *Bradybaena similaris* (Gastropoda, Xanthocychidae). *Memorias do Instituto Oswaldo Cruz* **89**, 407-410.

Posada, D. (2008). jModelTest: Phylogenetic Model Averaging. *Molecular Biology and Evolution* **25**, 1253-1256.

Pretzman, C., David, R., Stothard, D.S., Fuerst, P.A. and Rikihisa, Y. (1995). 16S rRNA gene sequence of *Neorickettsia helminthoeca* and its polygenetic relationship with members of the genus *Ehrlichia*. *International Journal of Systematic Bacteriology* **45**, 207-211.

Pusterla, N., Madigan, J.E., Chae, J.S., Derock, E., Jonson, E. and Pusterla, J.B. (2000a). Helminthic transmission and isolation of *Ehrlichia risticii*, the causative agent of Potomac horse fever, by using trematode stages from freshwater stream snails. *Journal of Clinical Microbiology* **38**, 1293-1297.

Pusterla, N., Johnson, E., Chae, J., DeRock, E., Willis, M., Hedrick, R.P. and Madigan, J.E. (2000b). Molecular detection of an *Ehrlichia*-like agent in rainbow trout (*Oncorhynchus mykiss*) from Northern California. *Veterinary Parasitology* **92,** 199-207.

Pusterla, N., Johnson, E. Chae, J.S., Pusterla, J.B., DeRock, E. and Madigan, J.E. (2000c). Infection rate of *Ehrlichia risticii*, the agent of Potomac horse fever, in freshwater stream snails (*Juga yrekaensis*) from northern California. *Veterinary Parasitology* **92**, 151-156

Pusterla, N., Johnson, E.M., Chae, J.S. and Madigan, J.E. (2003). Digenetic trematodes, *Acanthatrium* sp. And *Lecithodendrium* sp., as vectors of *Neorickettsia risticii*, the agent of Potomac horse fever. *Journal of Helminthology* **77**, 335-339.

Pusterla N., Madigan, J.E. and Leutenegger, C.M. (2006). Real-Time Polymerase Chain Reaction: A Novel Molecular Diagnostic Tool for Equine Infectious Diseases. *Journal of Veterinary Internal Medicine* **20**, 3-12

Reubel, G.H., Barlough, J.E. and Madigan, J.E. (1998). Production and Characterization of *Ehrlichia risticii*, the Agent of Potomac Horse Fever, from Snails (Pleuroceridae: *Juga* spp.) in Aquarium Culture and Genetic Comparison to Equine Strains. *Journal of Clinical Microbiology* **36**, 1501-1511.

Rikihisa, Y. (1991). The tribe *Ehrlichia* and ehrlichial diseases. *Clinical Microbiology Review* **4**, 286-308.

Rikihisa, Y. (2003). Mechanisms to Create a Safe Haven by Members of the Family Anaplasmataceae. *Annals of the New York Academy of Sciences* **990**, 548-555.

Rikihisa, Y. (2010). *Anaplasma phagocytophilum* and *Ehrlichia chaffeensis*: subversive manipulators of host cells. *Nature Reviews Microbiology* **8,** 328-339.

Rikihisa, Y. and Jiang, B.M. (1988). In vitro susceptibility of *Ehrlichia risticii* to eight antibiotics. *Antimicrobial Agents and Chemotherapy* **32,** 986-991.

Rikihisa, Y. and Perry, B.D. (1985). Causative ehrlichial organisms in Potomac Horse Fever. *Infection and Immunity* **49,** 513-517.

Rikihisa, Y., Stills, H. and Zimmerman, G. (1991). Isolation and continuous culture of *Neorickettsia helminthoeca* in a macrophage cell line. *Journal of Clinical Microbiology* **29,** 1928-1933

Rikihisa, Y., Zhang, C., Kanter, M., Cheng, Z., Ohashi, N. and Fukuda, T. (2004). Analysis of p51, groESL, and the major antigen P51 in various species of *Neorickettsia*, an obligatory intracellular bacterium that infects trematodes and mammals. *Journal of Clinical Microbiology* **42**, 3823-3826.

Rim, H.J., Sohn, W.M., Yong, T.S., Eom, K.S., Chai, J.Y., Min, D.Y., Lee, S.H., Hoang, E.H., Phommasack, B. and Insisengmay, S. (2008). Fishborne trematode metacercariae detected in freshwater fish from Vientiane Municipality and Savannakhet Province, Lao PDR. *Korean Journal of Parasitology* **46**, 253-260.

Ristic, M. (1990). Current strategies in research on ehrlichiosis, In: *Ehrlichiosis: a vector-borne disease of animals and humans* (J. C. Williams and I. Kakoma, eds). pp. 136-153. Boston: Kluwer Academic Publishers.

Ruff, M.D., McDougald, L.R. and Hanson, M.F. (1970). Isolation of *Histomonas meleagridis* from embryonated eggs of *Heterakis gallinarum*. *Journal of Protozoology* **17**, 10-19.

Sakawa, H., Farrell, R.K. and Mori, M. (1973). Differentiation of salmon poisoning disease and Elokomin fluke fever: complement fixation. *American Journal of Veterinary Research* **34**, 923-925.

Sayasone, S., Vonghajack, Y., Vanmany, M., Rasphone, O., Tesana, S., Utzinger, J., Akkhavong, K. and Odermatt, P. (2009a). Diversity of human intestinal helminthiasis in Lao PDR. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **103**, 247-254.

Sayasone, S., Tesana, S. and Utzinger, J. (2009b). Rare human infection with the trematode *Echinochasmus japonicus* in Lao PDR. *Parasitology International* **58**, 106-109.

Schell, S.C. (1985). *The Handbook of Trematodes of North America North of Mexico*. Moscow, Idaho: University Press of Idaho.

Schlegel, M.W., Knapp, S.E. and Millemann, R.E. (1968). "Salmon poisoning" disease. V. Definitive hosts of the trematode vector, *Nanophyetus salmincola*. *Journal of Parasitology* **54**, 770-774.

Schriefer, M.E. and Azad, A.F. (1994). Ecology and natural history of Rickettsia rickettsii. In: *Ecological Dynamics of Tick-Borne Zoonoses.* Edited by Sonenshine, D.E. and Mather, T.N.. New York, NY: Oxford University Press, 314–326.

Seng, P., Rolain, J.M., Raoult, D. and Brouqui, P. (2009). Detection of new Anaplasmataceae in the digestive tract of fish from southeast Asia. *Clinical Microbiology Infection* **15, Suppl 2**, 88-90.

Seo, B.S., Lee, S.H., Chai, J.Y. and Hong, S.J. (1984). Studies on intestinal trematodes in Korea XII. Two cases of human infection by *Stellantchasmus falcatus*. *Kisaengchunghak Chapchi Korean Journal of Parasitology* **22**, 43-50.

Shishido, A., Honjo, S., Suganuma, M., Ohtaki, S., Hikita, M., Fujiwara, T. and Takasaka, M. (1965). Studies on infectious mononucleosis induced in the monkey by experimental infection with *Rickettsia sennetsu*. 1. Clinical observations and etiological investigations. *Japanese Journal of Medical Science and Biology* **18**, 73-83.

Shope, R.E. (1941). The swine lungworm as a reservoir and intermediate host for swine influenza virus. II. The transmission of swine influenza virus by the swine lungworm. *Journal of Experimental Medicine* **74**, 49-68.

Shope, R.E. (1943). The swine lungworm as a reservoir and intermediate host for swine influenza virus. III. Factors influencing transmission of the virus and the provocation of influenza. *Journal of Experimental Medicine* **77**, 111-126.

Simms, B.T., Donham, C.R. and Shaw, J.N. (1931a). Salmon poisoning. *American Journal of Hygiene* **13**, 363-391.

Simms, B.T., Donham, C.R., Shaw, J.N. and McCapes, A.M. (1931b). Salmon poisoning. *Journal of the American Veterinary Medical Association* **78**, 181-195.

Simms, B.T., McCapes, A.M. and Muth, O.H. (1932). Salmon poisoning: transmission and immunization experiments. *Journal of the American Veterinary Medical Association* **81**, 26-36.

Smyth, J.D. and Halton, D.W. (1983). The physiology of trematodes, 2nd Edn. Cambridge University Press, Cambridge, UK.

Stackebrandt, E., Frederiksen, W., Garrity, G.M., Grimont, P.A., Kämpfer, P., Maiden, M.C., Nesme, X., Rosselló-Mora, R., Swings, J., Trüper, H.G., Vauterin, L., Ward, A.C. and Whitman, W.B. (2002). Report of the ad hoc committee for the re-evaluation of the species definition in bacteriology. *International Journal of Systematic and Evolutionary Microbiology* **52**, 1043-1047.

Swiderski, Z. and Xylander, W.E.R. (2000) Vitellocytes and vitellogenesis in cestodes in relation to embryonic development, egg production and life cycle. *International Journal of Parasitology* **30**, 805-817.

Sykes, J.E., Marks, S.L., Mapes, S., Schultz, R.M., Pollard, R.E., Tokarz, D., Pesavento, P.P., Lindsay, L.A. and Foley, J.E. (2010). Salmon poisoning disease in dogs: 29 cases. *Journal of Veterinary Internal Medicine* **24**, 504-513.

Tachibana, N. (1986). Sennetsu fever: the disease, diagnosis, and treatment. In: *Microbiology-1986* (H. Winkler and M. Ristic, eds). p. 205-208. Washington, D.C.: American Society for Microbiology.

Tallardat-Bisch, A.V., Raoult, D. and Drancourt, M. (2003). RNA polymerase b-subunitbased phylogeny of *Ehrlichia* spp., *Anaplasma* spp., *Neorickettsia* spp. and *Wolbachia pipientis*. *International Journal of Systematic and Evolutionary Microbiology* **53**, 455-458.

Taylor, M.J., Bandi, C. and Hoerauf, A. (2005). Wolbachia bacterial endosymbionts of filarial nematodes. *Advances in Parasitology* **60**, 245-284.

Tkach, V.V. and Pawlowski, J. (1999). A new method of DNA extraction from the ethanol-fixed parasitic worms. *Acta Parasitology* **44**, 147–148.

Tkach, V.V. (2008). Family Plagiorchiidae Luhe, 1901. In Bray, R.A., Gibson, D.I. and Jones, A. (eds.). Keys to the Trematoda. Wallingford: CABI 295-325.

Tkach, V.V., Schroeder, J.A., Greiman, S.E. and Vaughan, J.A. (2012). New genetic lineages, host associations and circulation pathways of *Neorickettsia* endosymbionts of digeneans. *Acta Parasitology* **57**, 285–292.

Tkach, V.V., Curran, S.S., Jeffrey, A.B. and Overstreet, R.M. (2012). A new species of *Crepidostomum* (Digenea: Allocreadiidae) from *Hiodon tergisus* in Mississippi and molecular comparisn with three congeners. *Journal of Parasitology* **99**, 1114-1121.

Tyzzer, E.E. (1934). Studies histomoniasis or ‘blackhead’ infections in the chickens and the turkey. *Proceedings of the American Academy of Arts and Sciences* **69**, 189-264.

Vaughan, J.A., Tkach, V.V. and Greiman, S.E. (2012). Neorickettsial endosymbionts of the Digenea: diversity, transmission and distribution. *Advances in Parasitology* **79**, 253–297.

Vemulapalli, R., Biswas, B. and Dutta, S. K. (1995). Pathogenic, immunologic, and molecular differences between two *Ehrlichia risticii* strains. *Journal of Clinical Microbiology* **33**, 2987-2993.

Walker, D.H. and Dumler, J.S. (1996). Emergence of the Ehrlichioses as human health problems. *Emerging Infectious Diseases* **2**, 18-29.

Ward, N.L., Steven, B., Penn, K., Methe, B.A. and Detrich, W.H. (2009). Characterization of the intestinal microbiota of two Antarctic notothenioid fish species. *Extremophiles* **13**, 679-685.

Weiss, E., Dasch, G.A., Williams, J.C. and Kang, Y.H. (1990). Biological properties of the genus *Ehrlichia*: substrate utilization and energy metabolism. In: *Ehrlichiosis: a vector-borne disease of animals and humans* (J.C. Williams and I. Kakoma, eds). pp. 59-67. Boston: Kluwer Academic Publishers.

Wen, B., Rikihisa, Y., Yamamoto, S., Kawabata, N. and Fuerst, P.A. (1996). Characterization of the SF agent, and *Ehrlichia* sp. isolated from the fluke *Stellantchasmus falcatus*, by 16S rRNA base sequence, serological, and morphological analyses. *International Journal of Systematic Bacteriology* **46**, 149-154.

Werren, J.H., Zhang, W. and Guo, L.R. (1995). Evolution and phylogeny of Wolbachia: reproductive parasites of arthropods. *Proceedings of the Royal Society of Biology.* **261**, 55–63.

Yamaguti, S. (1975). *A Synoptic Review of Life Histories of Digenetic Trematodes of Vertebrates*. Tokyo: Keigaku.

Zakikhani, M. and Rau, M.E. (1999). *Plagiorchis elegans* (Digenea: Plagiorchiidae) infections in *Stagnicola elodes* (Pulmonata: Lymnaeidae): host susceptibility growth, reproduction, mortality and cercarial production. *Journal of Parasitology* **85**, 454-463.

Zakikhani, M., Smith, J.M. and Rau, M.E. (2003). Effects of *Plagiorchis elegans* (Digenea: Plagiorchiidae) infection of *Biomphalaria glabrata* (Pulmonata: Planorbidae) on a challenge infection with *Schistosoma mansoni* (Digenea: Schistosomatidae). *Journal of Parasitology* **89**, 70-75.

Zhang, Y., Ohashi, N., Lee, E. and Rikihisa, Y. (1997). *Ehrlichia sennetsu* groE operon and antigenic properties of the groEL homolog. *FEMS Immunology and Medical Microbiology* **18**, 39-46.

Zhang, Y., Ohashi, N. and Rikihisa, Y. (1998). Cloning of the heat shock protein 70 (HSP70) of *Ehrlichia sennetsu* and differential expression of HSP70 and HSP60 mRNA after temperature upshift. *Infection and Immunity* **66**, 3106-3112.