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Laboratory maintenance of the bacterial endosymbiont, *Neorickettsia* sp., through the life cycle of a digenean, *Plagiorchis elegans*

Stephen E. Greiman[1,*], **Maksym Tkach**[1], **Jefferson A. Vaughan**[1], and **Vasyl V. Tkach**[1]

[1]Department of Biology, University of North Dakota, Grand Forks, North Dakota 58202, U.S.A

Abstract

The Digenea (Platyhelminthes: Trematoda) are a diverse and complex group of internal metazoan parasites. These parasites can serve as hosts to obligate intracellular bacteria belonging to the genus *Neorickettsia* (Family: Anaplasmataceae). Neorickettsiae persist within all stages of the fluke life cycle and thus are maintained through vertical transmission. However, the low prevalence of *Neorickettsia* in nature limits study of their transmission biology at different steps of digenean life cycles. To resolve this dilemma, we have developed for the first time a laboratory model allowing to maintain *Neorickettsia* sp. through the whole life cycle of a digenean, *Plagiorchis elegans*. 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). This paper focuses on the development of the laboratory life cycle, as well as outlines its potential uses in studying the transmission biology of *Neorickettsia* and its evolutionary relationship within its digenean host.

Keywords

Digenean; Life cycle; *Neorickettsia*; *Plagiorchis elegans*

1. Introduction

The Digenea (Platyhelminthes: Trematoda) are the largest group of parasitic flatworms with roughly 18,000 nominal species (Cribb et al, 2001). They have complex life cycles, using mollusks (primarily gastropods) as first intermediate hosts, several phyla of animals (such as mollusks, arthropods or vertebrates) as second intermediate hosts and all major vertebrate groups as definitive hosts. Due to their highly complex life cycles, cultivation of digeneans in the laboratory also requires cultivation of gastropods and other appropriate host species, and thus is more labor-intensive than that of more typical model organisms. Nevertheless,

Corresponding author: Stephen E. Greiman, Department of Biology, University of North Dakota, Grand Forks, ND 58202, U.S.A, Phone: 847 809-6311, FAX: 701 777-2623, stephen.greiman@my.und.edu.

laboratory cultivation of digeneans allows for major contributions in immunological studies and vaccine development against digenean infection, diagnostics and taxonomic studies, and biochemical or physiological studies (Smyth and Halton, 1984; Ndiaye et al., 2012; Greani et al. 2014; Mwangi et al., 2014).

Since the 1950s, it has been known that some digeneans harbor intracellular bacteria. These endosymbiotic alpha-proteobacteria belong to the genus *Neorickettsia* (Order Rickettsiales, family Anaplasmataceae) and are transmitted vertically from one digenean life cycle stage to the next throughout the life cycle (Vaughan et al., 2012). In some cases, *Neorickettsia* bacteria can also be transmitted horizontally from the digenean to the vertebrate definitive host. When this happens, the bacteria may infect vertebrate leucocytes and cause disease. In the USA, Philip et al. (1953, 1955) discovered that a lethal disease of dogs, known locally as 'salmon dog poisoning' because of its association with salmon spawning along the Pacific Northwest, was actually caused by the bacterial endosymbionts of an intestinal digenean species, *Nanophyetus salmincola*. This digenean species uses salmonid fish as its second intermediate host and piscivorous mammals (e.g., raccoons, bears, dogs, etc.) as definitive hosts. Philip (1955) erected a new genus and species, *Neorickettsia helminthoeca*, to accommodate the digenean-borne bacteria responsible for salmon dog poisoning. At least 3 other species of *Neorickettsia* have since been described that can cause vertebrate disease (i.*e., N. risticii, N. elokominica, N. sennetsu*). Recently, an increasing number of other, genetically distinct species level lineages of *Neorickettisia* of uncertain pathogenicity have been discovered (Tkach et al., 2012; Greiman et al., 2014). The most geographically widespread species of *Neorickettsia* is *N. risticii*, which can infect horses (=Potomac horse fever) causing septicemia, severe diarrhea, foundering, even death. *Neorickettsia risticii* is also the most widespread in terms of the taxonomic diversity of digeneans in which it has been found, including members of several digenean families such as lecithodendriids *Acanthatrium* sp., *Lecithodendrium* sp., dicrocoeliid *Conspicuum icteridorum*, echinostomatid *Echinoparyphium rubrum*, heronimid *Heronimus mollis*, and macroderoidid *Alloglossidium corti* (Pusterla et al., 2003; Gibson et al., 2005; Gibson and Rikihisa, 2008, Tkach et al., 2012).

In 2012, we detected neorickettisal DNA in field isolates of *Plagiorchis elegans*, a plagiorchiid digenean that is commonly found in local pond snails (Lymnaeidae) in North Dakota. Originally reported to be *N. risticii* (Greiman et al., 2013) and doubtless very closely related, the neorickettsial strain within *P. elegans* has since been characterized as genetically divergent from *N. risticii* based on Bayesian phylogenetic analysis of a 1400 bp fragment of the 16s rRNA gene (Greiman et al., 2014). *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 wild vertebrates as definitive hosts. Importantly, it can also use as definitive hosts several species of animals such as LACA mice, rats, hamsters, gerbils, chicks, ducklings and pigeons (Gorman, 1980). This makes *P. elegans* a prime candidate for establishing a laboratory life cycle.

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 *Neorickettsia* within the digenean host have been little studied (Vaughan et al., 2012). Establishing laboratory life cycles of digeneans infected with *Neorickettsia* provides a controlled environment in which to study the transmission biology of *Neorickettsia*, something that is difficult to do in the field. We describe here for the first time the *in vivo* culture and maintenance of *Neorickettsia* throughout all stages of a digenean life cycle in the laboratory.

2. Materials and Methods

2.1 Ethics statement

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

2.2 Snail Collection

Aquatic snails, *Lymnaea stagnalis*, were collected during the summer of 2011 from a pond in Nelson County, North Dakota (Lat: 48° 1′43.80″N and Long: 97°59′24.88″W). Snails were rinsed with water and placed into glass jars filled with aged tap water conditioned with commercial aquarium conditioner to remove chlorine/chloramines. To promote cercarial shedding, snails were kept for several hours under fluorescent lamps followed by several hours without light. Afterwards, the water in jars was examined at low magnification (5 to 20x) with transillumination for the presence of xiphidiocercariae. Snails shedding xiphidiocercariae were given a small section of fresh leaf lettuce and maintained singly in labeled jars until the xiphidiocercariae were assayed for the presence of *Neorickettsia*. Snails not shedding cercariae were placed together in large trays with 3–4 inches of treated water and maintained for breeding stock.

2.3 DNA extraction

Initial screening for snails shedding *Neorickettsia* positive *P. elegans cercariae* was done using homogenates from pooled cercariae. Approximately 20–30 cercariae from each shedding snail were pipetted into a 1.75 ml microcentrifuge tube and centrifuged at 13,000 rpm for five minutes. Supernatant was removed and 75 μl of ultrapure water added. Cercariae were homogenized by direct sonication using a UP100H compact ultrasonic processor (Hielscher USA, Inc., Ringwood, NJ) at an amplitude of 80–90% for fifteen seconds, and immediately placed on ice. A subsample (5 μl) of each homogenates was used directly as template for real-time PCR amplification procedures. The remainder of each homogenate was extracted for DNA using the guanidine thiocyanate method as described by Tkach and Pawlowski (1999).

2.4 Molecular detection and identification

Homogenates were first tested for the presence of *Neorickettsia* using a real-time PCR protocol designed by Greiman et al. (2014). Five microliters of each homogenate 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 was: groel-1500F (5′-ATAGATCCAGCKAAGGTAGTGCGTGT-3′) and groel-1620R (5′-TTCCACCCATGCCACCACCAGGCATCATTG-3′). The real-time PCR reactions were

run on a Bio-Rad CFX96 Touch Real-Time PCR detection system (Bio-Rad Laboratories, Hercules, CA) using iTaq universal SYBR Green supermix (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's instructions. A two-step program was used with a denaturation temperature of 95°C for 3 seconds, a annealing and extension temp of 64°C for 25 seconds and 36 cycles. A melting curve was run starting from 72°C and moving at 0.2°C increments every 5 seconds until reaching 95°C.

Samples shown to be *Neorickettsia*-positive by real-time PCR, were verified using a substantially modified nested PCR protocol initially described by Barlough et al. (1997). Five microliters of each complete DNA extract were used for the first PCR reaction and 1 μl of the first PCR product was 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 SEG) used in the first round were: n16s-25F (5′-TCAGAACGAACGCTAGCGGT-3′) and n16s-610R (5′-GACGTTCCTCTTGATATCTACG-3′). Primers used in the nested round were: n16s-70F (5′-GAATCAGGGCTGCTTGCA-3′) and ER2-R (5′- GTTTTAAATGCAGTTCTTGG-3′). The nested PCR reactions were run on a Mastercycler EP Gradient thermocycler (Eppendorf, Hauppauge, NY) using OneTaq Quick Load 2X Master Mix with GC Buffer (New England Biolabs, Ipswich, MA) according to the manufacturer's instructions. Annealing temperature of 54°C and 40 cycles were used in both first and nested PCRs. DNA of *N. risticii* used as a positive control was graciously provided by Dr. John Madigan (University of California Davis).

Cercariae were identified using partial sequences of the nuclear large ribosomal subunit gene (28S). Digenean DNA, from the completed DNA extracts of cercarial homogenates, was amplified by PCR using forward primer digl2 (5-AAGCATATCACTAAGCGG-3′) and reverse primer 1500R (5-GCTATCCTGAGGGAAACTTCG-3′).

PCR amplicons of both *Neorickettsia* and digeneans were purified using ExoSAP-IT PCR clean-up enzymatic kit (Affimetrix, Santa Clara, CA) according to the manufacturers' instructions. The PCR products were cycle-sequenced using ABI BigDye™ (Thermo Fisher Scientific Inc, Grand Island, NY) chemistry, alcohol precipitated and run on an ABI Prism 3100™ (Thermo Fisher Scientific Inc, Grand Island, NY) automated capillary sequencer. For sequencing of *Neorickettsia*, nested PCR primers were used. For sequencing of cercariae an internal reverse primer ECD2 (5-CTTGGTCCGTGTTTCAAGACGGG-3) was used. Contiguous sequences of *Neorickettsia* and of cercariae were assembled and edited using Sequencher™ ver. 4.2 (GeneCodes Corp., Ann Arbor, MI) and submitted to GenBank under accession numbers.

2.5 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 6–8 centimeters of aged tap water conditioned with commercial aquarium conditioner to remove chlorine/chloramines. Egg sacs were periodically removed and placed into smaller containers where they hatched. Once hatched, young snails were allowed to develop to approximately 1.5–2 centimeters 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.

2.6 Mosquito intermediate host cultures

Culex pipiens mosquito stocks originated from wild populations captured in Larimer County, Colorado. Larvae were reared in flat polyurethane trays containing ca. 500ml 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 pans to begin the cycle anew.

2.7 Establishment of laboratory lifecycle of *P. elegans* infected with *Neorickettsia* sp

The life cycle of *P. elegans* harboring *Neorickettsia* sp. was initially established as follows (Fig 1). 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 *L. stagnalis* snail. Cercariae 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 squished by a cover slip and examined for metacercariae under a compound microscope.

In some cases *Plagiorchis elegans* metacercariae may become infective to the definitive host after 48 hours (Gorman 1980). To ensure high infectivity of the metacercariae 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 force fed with 15–20 *Culex* larvae. Hamsters were maintained in individual cages on standard rodent diet (Purina LabDiet®) and the bedding was replaced twice a week.

To monitor infection success, wet mounts of hamster feces were periodically examined under a compound microscope for operculate 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 stereo-microscope.

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 “egg donor” digeneans actually harbored *Neorickettsia*, the remainder of each worm was then homogenized and screened for *Neorickettsia* sp. 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 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 placed in water under a cover slip, and visualized under a compound microscope to monitor miracidium development. 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 containing fresh mosquito larvae in order to produce *Neorickettsia* positive metacercariae and repeat the life cycle.

3. Results

3.1 Life cycle

We have for the first time maintained *Neorickettsia* in the laboratory throughout the entire life cycle of a digenean. 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. *Neoricekttsia* sp. infection was maintained in the digenean, *P. elegans*, for three complete generations. In our subsequent experiments we established three lines of *Neorickettsia* originating from three different naturally infected snails.

3.2 Host specificity

We attempted to use both hamsters and outbred mice as definitive hosts for *P. elegans*. However, only hamsters were found to be suitable hosts producing ovigerous *P. elegans* (Table 1).

4. Discussion

The digenean, *Plagiorchis elegans*, was used for our laboratory life cycle for two reasons. First, *P. elegans* is found to be naturally infected with *Neorickettsia* sp. in North Dakota (Tkach et al., 2012; Greiman et al., 2013). From three ponds in eastern North Dakota, 240 *L. stagnalis* were found infected with *P. elegans* and of those, 18 proved to be *Neorickettsia*-positive (7.5%) (Greiman et al., 2013). Second, *P. elegans* is a generalist parasite, utilizing multiple groups of aquatic arthropods as second intermediate hosts, as well as, infecting a wide range of vertebrate definitive hosts (Gorman, 1980). In North Dakota, muskrats are the most common natural definitive host of *P. elegans*. Not surprisingly, hamsters proved to be a highly suitable experimental host for these digeneans because they belong to the same family Cricetidae. Likely, some other vertebrates may be used for this purpose (Gorman, 1980), however, in our experiments mice did not produce adult *P. elegans*.

There are several potential “choke-points” in the lifecycle that deserve special attention. The first is to avoid exposing mosquito larvae to too many cercariae. Gorman (1980) found that by exposing 6 chironomid larvae to approximately 120 cercariae in the confines of a small Petri dish (5 cm in diameter), the insect larvae became infected with 10 to 30 metacercarial cysts. Larger numbers of cercariae per larvae resulted in 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 point of attention is to ensure that heat sterilized water is used for egg development. In non-boiled water, the eggs may be damaged by fungi or ingested by ciliate protozoan. The third important element 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 molluskan hosts (Cheng and Snyder, 1962; Pinheiro and Amato, 1994), especially under stress. In our initial experiments the majority of our parasitized snails died from what we now believe to have been 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. In addition, we recommend to use at least 100 snails for infection during each generation, to ensure continuance of the life cycle.

One large gap in our knowledge of *Neorickettsia* biology that laboratory life cycles can help address is understanding the quantitative aspects of *Neorickettsia* perpetuation during the complex digenean life cycle (Vaughan et al., 2012). Similar to other types of heritable 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. Greiman et al. (2013) were able to determine the vertical transmission efficiency of *N. risticii* within asexual stages (sporocysts and cercariae) of *P. elegans* in naturally infected snails, but not within sexual stages (adults and eggs), something that is now possible using the laboratory model described herein.

Another important gap in our knowledge of *Neorickerttsia* transmission biology is the lack of understanding of their horizontal transmission. Although horizontal transmission of neorickettsiae from digenean to vertebrate host occurs and can cause disease, it is not known whether horizontal transmission of neorickettsiae can go 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 vertebrate (i.e., through co-feeding transmission). Such alternate transmission pathways could play a vital part in both circulation and evolution of *Neorickettsia*. Availability of a laboratory model will help to elucidate the probability of such events.

The maintenance of *Neorickettsia* throughout complete life cycles of digeneans in laboratory makes 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, domestic animals and wildlife.

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Highlights

- Maintenance of *Neorickettsia*, for the first time, through a laboratory digenean life cycle.
- Laboratory life cycle allows for greater study of *Neorickettsia* transmission biology.
- Neorickettsia cause disease in humans, domestic animals, and wildlife.

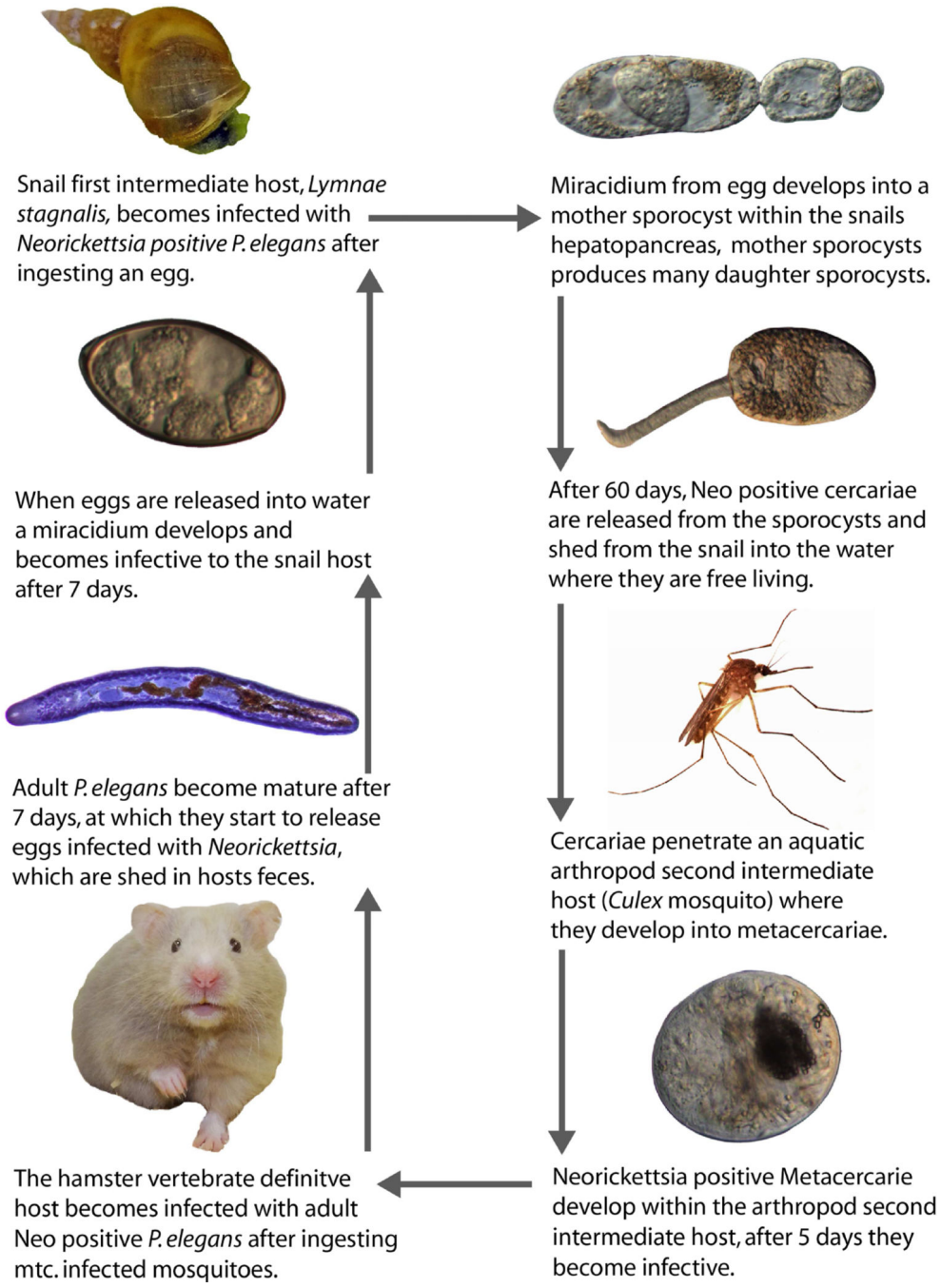

Figure 1.
Laboratory life cycle of *Plagiorchis elegans* harboring *Neorickettsia* sp.

Table 1

Experimental animals infected with *Plagiorchis elegans* metacercariae (mtc.) from mosquito larvae. Days post infection (PI) before dissection of experimental animal and number of adult *P. elegans* in intestine provided.

Experimental animal	Days PI before dissection	Number of adult *P. elegans*
Mouse #1	11	1 (underdeveloped)
Mouse #2	17	0
Mouse #3	17	0
Mouse #4	16	0
Mouse #5	25	0
Mouse #6	7	0
Mouse #7	7	7 (underdeveloped)
Hamster #1	11	25
Hamster #2	14	33
Hamster #3	14	14
Hamster #4	17	42
Hamster #5	12	100
Hamster #6	27	36
Hamster #7	28	2
Hamster #8	22	21
Hamster #9	27	40
Hamster #10	29	35