ADDRESSING ANGIOSTRONGYLIASIS ON HAWAIʻI ISLAND WITH RESEARCH, EDUCATION OUTREACH, AND HOST CONTROL

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Abstract

Hawaii Island has the highest incidence of rat lungworm disease (RLWD) of all the Hawaiian Islands and the mainland United States. The relatively recent introduction of the semi-slug *Parmarion martensi*, an effective intermediate host, and the wide-spread use of rainwater catchment systems may play a role. Studies were designed to investigate the ability of drowned gastropods to shed larvae, the location in a water column where larvae would most likely be found, the potential for larval passage through a 20µm filter, and the ability of the larvae to survive outside the slug/snail host. Whole *P. martensi* shed many, viable *A. cantonensis* larvae with >90% of larvae found in samples taken from the bottom of the water column, suggesting they may settle near the bottom of a catchment tank. Larvae that were able to pass through a 20µm sieve could not survive acid, were active for at least 56 days outside the slug host, and tested positive for RLW by qPCR. Larvae that could not pass through a 20µm sieve were able to survive HCl-pepsin, were active for at least 21 days, and tested positive for RLW. First stage larvae can survive gut acid when swallowed after migration from the lungs but cannot withstand acid immersion again until they become third stage larvae. The study results merit further investigation into the potential link between poorly maintained rainwater catchment systems and the high incidence of RLWD on Hawaii Island, and the studies clearly demonstrate the need for control of hosts of *Angiostrongylus cantonensis.*

Hawaii's remote location makes food security an important issue. State-wide efforts to promote the Grow Local, Eat Local movement are reflected in the growing number of residential gardens, small farms, farmers' markets, school and youth garden projects, and the recent passage of the Farm to School Bill. However, efforts to educate farmers, food handlers, and consumers about rat lungworm disease and the need for disease prevention and host control has not been

similarly supported. In collaboration with five partner schools on Hawaii Island, the University of Hawaii, Daniel K. Inouye College of Pharmacy's Hawaii Island Rat Lungworm Working Group worked with students and teachers to develop an integrated pest management plan for school garden projects. Integrated pest management allows for the careful consideration of applications available to control a pest event and chooses those practices that are least harmful to human and environmental health. These best practices include preventative cultural practices, monitoring, mechanical control, biological control, and the responsible use of pesticides. Students were intensively educated about RLWD, the parasite's life cycle, and prevention measures. Using best management practices, we set up traps and collected data on gastropod species abundance, and shelter-type capture rate. Integrating STEM curriculum makes the project attractive to schools as it supports student academic success. Adoption of this management project by the many school and youth garden projects in areas of RLWD can exponentially increase community awareness, encourage control efforts, and potentially map disease risk.

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CHAPTER 1

Angiostrongylus cantonensis, introduced gastropods, and rainwater catchment use; evidence and implications for the high incidence of angiostrongyliasis on Hawaii Island.

Introduction

The negative impact of invasive species on the unique flora and fauna of the Hawaiian Island archipelago is well recorded, and evidence can be found in the number of extinction events and threatened and endangered status listings of many of Hawaii's endemic and indigenous species (Vitousek 1988). Some invasive species bring disease to Hawaii as well. A prime example is the mosquito *Culex quinquefasciatus*, introduced in 1827, which is the vector of avian malaria *Plasmodium relictum,* and avian pox Avipoxvirus (Hardy 1960; Atkinson et al. 2000; van Riper, van Riper 2002). More recently, the introduction of the fungus *Ceratocystis fimibiatoa*, is having a devastating effect on Hawaii's most widespread native tree *Metrosideros polymorpha*, and causing rapid ohia death (Mortenson et al. 2016). The spread of these diseases has, and will continue to have, a devastating impact, especially on remaining endemic Hawaiian bird populations. The arrival of rats and non-native terrestrial land snails has also impacted Hawaii's biodiversity, bringing with them another important but perhaps lesser-known disease-causing organism, the parasitic nematode *Angiostrongylus cantonensis* the rat lungworm.

First identified in Hawaii in the 1950's, *A. cantonensis* is globally one of the most common infectious causes of eosinophilic meningitis with an estimated total of 2800 cases worldwide. This is very likely an underestimate as it is agreed within the medical community that difficulty with diagnosis and lack of familiarity makes the disease under-reported (Alicata 1964; Wang 2008; Ramirez-Avila et al. 2009; Hochberg et al. 2011). First cases of human

infection of angiostrongyliasis were reported on Oahu in 1961, which spurred *A. cantonensis* research in Hawaii for about a decade (Alicata 1964; Wallace & Rosen 1969). The early cases involved two Filipino men, one aged 50 and the other 70, both of which died and both had autopsies performed on their brains. Both men had been admitted to the same State mental hospital due to abnormal behavior. The 50-year old male died about 13 months after admission, and the autopsy showed well-preserved worm segments that indicated the worms were alive at the time of the victim's death. The second patient died approximately 7 months after he was admitted, and while it could not be proven that the 70-year old patient was also infected with *A. cantonensis,* the findings of foreign material in the brain and cellular reactions very similar to those of the first patient led doctors to presume the case to also be caused by *A. cantonensis* infection (Rosen et al. 1962).

I can find no published accounts of cases of angiostrongyliasis in Hawaii, after these first reports, until after the turn of the millennium. In 2001 clusters of cases, many serious, began to surface in the Puna District on Hawaii Island, and the trend continues to this day (Hochberg et al. 2007; Hochberg et al. 2011; Howe 2013; Kwon et al. 2013). Although *A. cantonensis* infection has been found in slug and snail populations on all of the major Hawaiian Islands with the exception of Lanai, currently more than 90% of cases originate on Hawaii Island (Hochberg et al. 2007; Kim et al. 2014). The link explaining the reason for the occurrence of a greater number of cases on Hawaii Island has yet to be found.

Identified by Chen (1935) in Canton China, *A. cantonensis* is endemic to Southeast Asia. It was spread throughout the Pacific Islands and Hawaii during the World War II era when the increase in trade and shipping in the Pacific assisted the spread of common definitive hosts of *A. cantonensis,* including rat species such as *Rattus rattus, Rattus noviculus,* and *Rattus exulans,* as

well as the movement of intermediate terrestrial and aquatic gastropod hosts. These included the giant African snail *Achatina fulica,* and apple snails in the family *Ampulleriidae*, which were intentionally introduced, primarily as food sources (Mead 1961; Hochberg et al. 2011; Wang et al. 2008). Today *Angiostrongylus cantonensis* is found in approximately 30 countries including China, Thailand, Taiwan, Okinawa and mainland Japan, Papua New Guinea, American Samoa, the Philippines, Australia, Sri Lanka, Indonesia, Malaysia, India Egypt, South Africa, the Ivory Coast, Madagascar, Cuba, Jamaica, Puerto Rico, Haiti, Dominican Republic, Ecuador, Brazil, and the Canary Islands (Cowie 2013; Wang et al. 2008). First reported in the continental United States in 1987, besides Hawaii it is established in Florida, Louisiana, and Texas (Kim et al. 2002; Hochberg et al. 2011; Teem et al. 2013; Stockdale-Walden et al. 2015) and has been detected in Oklahoma (York et al. 2016). The parasite's range is expected to expand with climate change and angiostrongyliasis is considered to be an emerging tropical disease (Prociv, Spratt, & Carlisle 2000; Cowie, 2013).

Lifecycle and transmission of *Angiostrongylus cantonensis* in permissive and non-permissive hosts

The life cycle of *A. cantonensis* is complex and requires two distinctly different hosts. The adult lungworms reproduce in the heart and pulmonary arteries of the rat where the female worm lays eggs, which are flushed to the lungs and embryonate. First stage larvae emerge, make their way up the bronchial tree, are swallowed, surviving the acidic environment of the rat's gut, and are eliminated in feces. Intermediate hosts, slugs and snails, ingest the infected feces. Once in the gastropod host, the worm develops to a second-stage, and then to third-stage larva, remaining coiled and relatively immobile in muscle tissue and retaining both molt skins during

this period. When rats ingest the snail, stomach acids dissolve the molt skins, activating the larva, and the larva then burrows through the intestine, enters the blood stream, and makes its way to the central nervous system (CNS) and brain. There it will develop to a fourth stage larva, go through one molt, become a 5th stage young adult, and then force its way out of the CNS and move into the heart and pulmonary arteries where it will mature and reproduce, completing they cycle (Mackerras & Sandars 1955).

There are other organisms, termed paratenic hosts, which can harbor *A. cantonensis* and can serve as pathways of infection. These include shrimp, prawns, land crabs, frogs, water monitor lizards, and predacious flatworms (Wang et al. 2008; Cowie 2011). In Asia, human infection is usually caused by consumption of raw or undercooked snails. In Tahiti, a high incidence of eosinophilic meningitis correlated with the consumption of raw prawns (Kliks et al. 1982; Alicata & Brown 1962). In Hawaii, most infections are generally thought to be the result of accidental consumption of a slug or snail on fresh, raw produce, such as salad greens (Alicata 1964; Wang et al. 2008; Hochberg et al. 2011). While most adult mollusks are large enough to be easily seen, juvenile slugs and snails, which can be infected, can be quite small and may be overlooked if fruits and vegetables are not carefully washed (Hollingsworth et al. 2007; Yeung et al. 2013).

Humans are accidental, or non-permissive hosts; the parasite can infect but cannot reach sexual maturity and reproduce. Accidental hosts include birds, bats, dogs, horses, and primates among others (Reddacliff et al. 1999; Prociv et al. 2000; Kim et al. 2002; Duffy et al. 2004; Monks et al. 2005; Wang et al. 2008; Stockdale-Walden et al. 2015). Infection in an accidental host proceeds as in the rat host with the worm traversing the gut and migrating to the CNS. Once in the brain, tissue damage occurs from the larvae's mechanical actions (migration, feeding,

excretion, molting) as well as from the body's immune system response (eosinophils) causing inflammation.

Mouse studies, which can show immune responses and pathological changes in the brain similar to those in humans, also show an increase in reactive oxygen species (ROS) and oxidation of cellular proteins resulting from *A. cantonensis* infection, which potentially contribute to meningitis-related brain damage (Chung et al. 2010). While in the non-permissive host the larvae generally are believed to stay in the brain until death, there are some fatal cases in young children in Australia and Fiji where, upon autopsy, mature, non-gravid larvae have been found in the lungs (Prociv et al. 2000). Because of ensuing neurological damage, angiostrongyliasis, or rat lungworm disease (RLWD), is recognized as a serious threat to human health and can result in a clinical manifestation of eosinophilic meningitis, eosinophilic encephalitis, and ocular angiostrongyliasis (Sawanyawisuth & Sawanyawisuth 2008; Wang et al. 2008). Cases in the South Pacific and Hawaii, as in other countries with RLW, have resulted in permanent disability, coma, and death (Beaver & Rosen, 1964; Kliks et al. 1982; Prociv et al. 2000; Wang et al. 2008; Sawanyawisuth et al. 2010; Howe 2013; Kwon et al. 2013).

Introduction of non-native slug and snails to Hawaii and their environmental impacts

The latest study of slug and snail populations on the Hawaiian Islands showed the potential for a wide range of species to be capable intermediate hosts of *A. cantonensis* (Kim et al. 2014). Of 37 non-native snail species screened in the study, 16 tested positive for *A. cantonensis* and infection was found in two of seven native snail species. Non-indigenous slug and snail species are the main route of infection for rats and accidental hosts such as humans, and therefore it is worthwhile to understand how they arrived in Hawaii.

The tropical Pacific Islands, including Hawaii, have been impacted by both the intentional and accidental introduction of non-native gastropod species, arriving as stowaways with horticultural and other shipments, introduced as food sources, or released as bio-control agents (Cowie 1998; Cowie 2001; Cowie 2002; Cowie et al. 2008). The introduction of these snails has led to the decline of endemic, native tree-snail populations through competition and predation, which has led to major extinctions. The International Union for Conservation of Nature (IUCN) Red List of Threatened Species (2015) lists land-snails more than any other invertebrate group. Among these are the achatinelline tree-snails of the Hawaiian Islands and the partulid tree-snails from other Pacific Islands (Curry et al. 2016).

One of the earliest observed non-native gastropod arrivals was the giant African snail *Achatina fulica,* first recorded in Hawaii in 1936 (Mead 1961; Cowie et al. 2008). This large-bodied snail was introduced throughout the South Pacific as a food source. The snail populations grew so dense that in the 1950's the Hawaii Board of Agriculture and Forestry (now Department of Agriculture) began to examine the potential for bio-control efforts with predacious snail species. Due to public pressure to reduce the population of this large snail, a thorough study was not carried out to determine if there were any potential detrimental effects of bio-control. Between 1952 and 1957, three predacious snail species were released: *Gonaxis kibweziensis, Gonaxis quadrilateralis, and Euglandina rosea* (Mead 1961; Cowie 2001). These three species became established and began to prey on the estimated 750 species of endemic terrestrial snails, decimating these populations. Most native species are now extinct, with estimates of only 10-35% of original native species remaining (Cowie 2001).

None of these introduced predacious snails were effective in controlling *A. fulica*, instead they have added to the problem of disease as *E. rosea* has been identified as an

intermediate host of *A. cantonensis* (Wallace & Rosen 1969). The predacious flatworm *Platydemus manokwari* was also introduced to control *A. fulica,* and it has been identified as a carrier of *A. cantonensis* in Hawaii and a paratenic host and known source of human infection in Japan (Asato et al. 2004: Hollingsworth et al. 2013; Qvarnstrom et al. 2013). Additionally, *P. manokwari,* native to New Guinea, is considered to be one of the most serious predators of and threats to native endemic snails on oceanic islands (Regnier et al. 2009).

Invasive slugs also negatively affect native forest regeneration. The study by Joe and Daehler (2008) tracked the survival rates of out-planted seedlings in replicate plots in a mesic forest on Oahu that provides habitat for twelve endangered plant and two endangered animal species. The forest is protected from ungulates with fencing, and from rats with snap traps. The four slug species present were *Deroceras laeve, Limacus flavus, Limax maximus,* and *Meghimatium striatum.* The study found slugs decreased the survival of seedlings of the critically endangered plant species *Cyanea superba* and *Schidea obovata* by 51% on average. What was particularly concerning to the authors was that the out-planted seedlings were fairly large (~28-42mm in height). In comparison, the impact of slugs to invasive species *Clidemia hirta* and *Psidium cattleianum* and non-endangered, endemic *Nestegis sandwicensis* were not significant. The authors concluded that introduced slugs are an under-appreciated, direct cause of rare plant endangerment.

Invasive gastropod rat lungworm parasite-burden surveys conducted in Hawaii

Wallace and Rosen (1969) conducted early studies in Hawaii on gastropod intermediate hosts after the first human cases of eosinophilic meningitis caused by *A. cantonensis* were confirmed on Oahu. All of the gastropod species infected with the parasite were non-native,

some of which had been introduced as bio-control efforts to control *Achatina fulica*. The study collected terrestrial mollusks from the islands of Oahu, Hawaii; Tahiti, Society Islands; Rarotonga, Cook Islands; and Majuro, Marshall Islands to determine which species served as intermediate hosts for *A. cantonensis*. The study found that of the species collected on Oahu, *A. fulica had a* 69% infection rate with one individual's burden estimated at 90,800 larvae, the rosy wolfsnail *Euglandina rosea* had an infection rate of 78%, followed by leatherleaf, or leatherback slug, *Laevicaulis alte* at 45%. Other infected terrestrial species included the small, conical, yellow land-snail *Subulina octona* (4%), the Asian trampsnail *Bradybaena similaris* (4%), the marsh slug *Deroceras laeve* (1%), and *Prosopeas javanicume* (3%). The aquatic species *Pomacea paludosa*, which is cultivated for human consumption, was also infected (27%). It was noted in study areas that the average number of larvae increased from August to January and then dropped from April to July. It was also determined that the percentage of infected mollusk hosts was lower in drier areas as were the quantities of larvae in the hosts.

The first survey of snail hosts of *A. cantonensis* on Hawaii Island was conducted in 2005 after three residents from the Puna District, on the eastern windward side of the island, became sick with symptoms indicative of RLWD after eating homegrown lettuce. The lettuce was reported to be contaminated with juvenile semi-slugs *Parmarion martensi*, an invasive species from Southeast Asia that was first found on Oahu in 1996, and then in Paradise Park in the Puna District of Hawaii Island in 2005 (Hollingsworth et al. 2007). Twenty-six specimens of semi-slugs were collected from the property where the illness was reported and sent to the Center for Disease Control for analysis to determine if they were infected with the rat lungworm. All samples tested positive for *A. cantonensis* and worm-burden levels in many individuals were extremely high. The semi-slugs were found in close proximity to human habitation and

exhibited behaviors such as high speed of movement (relative to other snail species), propensity to climb, and attraction to human food including beverages. This, in addition to its high infection level, gave reason to believe it could be an important vector of RLWD. The semi-slug was found to have a much higher infection rate (77.5%) than *Veronicella cubensis*, the Cuban slug (24.3%), with larger individuals having higher parasite counts than smaller individuals (Hollingsworth et al. 2007).

The most recent islands-wide survey of *A. cantonensis* infection in non-native snails by Kim et al. (2014) shows some changes in infection levels when compared with those from Wallace and Rosen's 1969 study. In 2013 *Achatina fulica* infection level was at 11% (vs. 69%), *Euglandina rosea* at 21% (vs. 78%), *Laevicaulis alte* at 30% (vs. 45%), *Subulina octona* at 4% (same), *Bradybaena similaris* at 0% (vs. 4%), *Deroceras laeve* at 0% (vs. 1%). New species included in the 2013 study that tested positive include *Veronicella cubensis* (4%), *Pomacea canaliculata* (2%), *Ovachlamys fulgens* (10%), *Lehmannia valentiana* (9%), and *Limax maximus* (27%). This same study showed Parmarion *martensi* to continue to test high at 68% compared with 77.5% in a 2004 study (Hollingsworth et al.), pointing to its continued importance as an effective intermediate host in Hawaii

Incidence and causes of angiostrongyliasis

Cases of angiostrongyliasis are often undiagnosed and therefore it is generally accepted by health agencies and researchers that the disease is under-reported. This is likely due to lack of awareness of the parasite and disease by the medical community, and the lack of a good diagnostic (Wang et al. 2012). Currently there is no readily available blood test in Hawaii and diagnosis requires a spinal tap, an invasive procedure with risk. Cerebral spinal fluid (CSF) is

drawn and a visual examination by microscopy may find evidence of nematodes, however they are rarely observed, and so an analysis of the CSF is used to determine eosinophil levels (Punyagupta et al. 1975). The CSF can also be analyzed using a polymerase chain reaction (PCR) or quantitative PCR (qPCR), which may provide DNA evidence of *A. cantonensis* infection. More frequently in Hawaii, diagnosis is based on patient's exposure and clinical symptoms as well as laboratory findings of eosinophils, a white blood cell, in the CSF (Hawaii State Dept. of Health 2016). While the first human case of angiostrongyliasis in Hawaii was reported in 1959, more than 70 cases have been reported in Hawaii since 2001, the majority of which are from the windward side of Hawaii Island (Alicata 1964; Hochberg et al. 2007; Wallace 2013; Kim et al. 2014; Hawaii Dept. of Health 2016; Fujimori 2016).

Infection is generally accepted to result from the intentional or accidental ingestion of a raw or undercooked, infected slug, snail, or paratenic host (e.g. crab, shrimp, prawn, frog), or ingestion of a piece of slug, snail, or flatworm containing the infective stage larvae on undercooked or raw food (Center for Disease Control, 2016; Hawaii State Dept. of Health, 2016). Slime may contain larvae although it is believed that the numbers of larvae that could be found in slime would be low (Hollingsworth et al. 2013). A study conducted by the Center for Disease Control (CDC) on Hawaii Island in 2007 analyzed slime with microscopy, which revealed one and four motile larvae in two samples, one of which was positive for *A. cantonensis* by PCR, and of 25 slugs that were naturally infected only three shed larvae in their mucus (Qvarnstrom et al. 2007). Highly infected slugs may be able to shed more larvae in slime. Jarvi et al. (2012) tested midsection, tail, back, head, and slime of naturally infected *P. martensi* and found a correlation between a higher level of infectivity and higher numbers of larvae in slime, using qPCR. Slugs with tail sections showing larvae/mg of tissue by qPCR of 41.34, 6.42, and

2.85 had slime with 0.83, 0.11, and 0.10 larvae/mg respectively. More testing may be helpful to determine the potential for slime to contain higher burdens of *A. cantonensis*. The potential for infection through skin was revealed in a mouse study which showed successful transmission through lacerated and unabraded skin, and anal, vaginal, conjunctival mucosa, and foot pad but not through the tail (Wang, Chao, & Chen, 1991). We have spoken with several RLWD victims in Hawaii who believe that they were infected through skin contact.

In my personal conversations with victims, few have visually seen or were aware of biting or tasting a slug or snail on food and could not pinpoint how they were infected, which makes the question regarding pathways of transmission troubling. Some victims have expressed a belief that consumption of catchment water may have been the cause of their infection. While consumption of water with infected larvae is not listed as a transmission source by the Hawaii Department of Health (DOH) or the CDC, there are papers that express and support the potential for infection from water contaminated with *A. cantonensis* (Cheng & Alicata 1964; Crook, Fulton & Supanwang 1967; Richards & Merritt, 1971; Cowie 2013). When one understands the issues with water availability in Hawaii, we can see this may be an important, and possibly overlooked, factor in the rise of incidences of RLWD.

The usage of rainwater catchment on Hawaii Island

The use of private rainwater catchment systems is widespread on Hawaii Island. In the 1950's and 60's, before zoning laws were introduced, Hawaii County approved the development of many large subdivisions in the Puna and Kau Districts with little or no infrastructure (Cooper & Daws 1990). Today, many residents in these subdivisions lack the infrastructure to access county or private water companies, and in some cases power companies, and therefore rely on

rainwater catchment and solar power and/or generators for these utilities (Hawaii County Department of Planning 2015). The College of Tropical Agriculture and Human Resources (CTHAR) publication, "Guidelines on Rainwater Catchment Systems for Hawaii," estimated 30,000-60,000 people in the state of Hawaii rely on catchment water for household water requirements, with the majority of users being on Hawaii Island and the greatest percentage from the Puna, Kau, and Hamakua Districts (Macomber 2010).

Prior to 2014 the Hawaii State DOH did not approve rainwater catchment as safe for domestic uses. Because of this policy, military veterans were not able to buy affordable homes in these subdivisions if they had rainwater catchment. However, in February 2014, because of the high percentage of homes in the State of Hawaii with rainwater catchment, the Veterans Administration (VA) released Circular-26-14-4, Clarification of the Department of Veterans Affairs (VA) Acceptance of Water Catchment Systems in the State of Hawaii, to provide clarification of the VA policy for individual properties with rainwater catchment systems that serve as collateral for a VA loan in the State of Hawaii. The State of Hawaii DOH determined that rainwater catchment systems could be made safe for domestic use if certain guidelines were followed. Lenders were required to provide a printed copy of the CTHAR publication mentioned above and the veteran purchaser must sign an affidavit acknowledging they received the publication. However, in Hawaii, the DOH maintains it does not regulate individual rainwater catchment systems. To be considered safe for household use the system should be properly designed and maintained so that potable water is available at all taps. The cost of such a system can be substantial and includes proper roofing material, gutters, tank and tank cover, pumps, and filters including ultraviolet (UV) light to kill bacteria. Catchment maintenance

includes keeping overhanging vegetation from roofs and regularly cleaning gutters and tanks, changing filters, and replacing UV bulbs.

We can get an idea of the growth and economic status of the Puna District, where there is a high percentage of rainwater catchment and a greater number of cases of RLWD, by examining State and County statistics. Affordable property makes Puna one of the fastest growing districts in the state. The district contains nearly 45% of Hawaii County's total subdivided lots and from 2000-2010 the population increased by 66%. Puna has the 3rd highest rate of unemployment and the 3rd lowest per capita income of all districts in the state; in 2000 the per-capita income was 40% less than the state average. Approximately 85% of Puna residents have Section 8 low income-housing certificates, and 55% of families rely on food stamps. (U.S. Census Bureau 2000; Pahoa Community Development Plan, UH Center on the Family 2003; Puna Community Development Plan, 2007; Carter 2013).

Considering these statistics, the cost of properly constructing and maintaining a rainwater catchment system may place an economic burden on some residents beyond their financial means, which may result in a system that is providing water that would not meet the safe drinking water standards required of public water systems. Access to the County spigots requires a vehicle to transport water and the individual's ability to lift and haul heavy containers, which may challenge those without vehicles, or the elderly or disabled. Although residents have access to potable water at County water spigots, which many residents use, they still may not have potable water at all sink and shower outlets, instead the County spigot water is only used for drinking.

In addition to the economic costs associated with rainwater catchment systems, natural disasters, such as hurricanes, lava flows, and droughts can affect resident access to potable water

if using a rainwater catchment system. When Hurricane Iselle made landfall in Puna in 2014 it left some residents without power for a month because of downed power lines, and lack of power impacts pumping and filtration of catchment water. Six months after the hurricane a lava flow came very close to severing the main road that connects the lower Puna district to the rest of the island. Had the flow proceeded many services would have been cut off, including power, again impacting the pumping and filtration of water. Dry seasons and droughts, which are predicted to increase with climate change, also challenge residents using rainwater catchment (U.S. Fish and Wildlife Service 2016). During dry times many residents must resort to having water hauled in to refill their catchment tanks. During dry conditions slugs and snails may be drawn to water sources. Residents, tank cleaners, and the author herself have reported seeing slugs and snails on and inside tanks and in the water, and it is reasonable to suspect that this behavior may increase under dry conditions.

There is ample evidence that natural disasters could negatively impact basic necessities for this rural district, including access to whole-house potable water. Rainwater catchment may be a variable of interest when considering the factors that may be causing higher disease incidence. It must be kept in mind that rainwater catchment use is not only confined to residential use, it is also used for agriculture on Hawaii Island. There is no information available as to the extent of agricultural rainwater catchment in use on Hawaii Island, nor can we know how well farm catchment systems are designed or maintained, or if they have any type of filtration systems installed.

Early evidence supporting the ability of infective larvae to survive outside the slug or snail host

The CTAHR publication, "Guidelines on Rainwater Catchment Systems for Hawaii," states that it is not known whether the rat lungworm parasite could survive outside of a slug or snail host, however if it could, the infectious larvae would be stopped by a 20-micron filter. The publication also states that infection reports could be traced back to eating vegetables that were not cleaned and/or cooked, and that as of the time of writing of the manual human infections had not been linked to drinking water (Macomber 2010). However, in a recent occurrence of angiostrongyliasis infection the person became ill several days after siphoning water from his catchment tank because his power source had failed. He was diagnosed for angiostrongyliasis with a spinal tap and hospitalized (personal communication 2015). There are others who also think this may have been the source of their disease transmission. One victim of RLWD expresses her concerns regarding catchment in Beyond the Fear: A Documentary About Rat Lungworm Disease (Mariful Films 2016). While transmission of RLWD by catchment water in Hawaii has not been shown, the literature is helpful in determining the potential for transmission in water.

In the ten-year period following the death of the two patients on Oahu in 1959-1960 from *A. cantonensis* infection, and as cases of infection spread through the Pacific Islands, research expanded the understanding of the parasite, its definitive and intermediate hosts, the disease itself, and sources of disease transmission. Some of these studies provide supportive evidence verifying the ability of slugs and snails to shed *A. cantonensis* larvae when immersed in water. These studies are important if we are to try to understand the variables that may be causing an increase in the incidence of RLWD on Hawaii Island, particularly when residents and

catchment tank cleaners report findings of slugs and snails climbing on the inside of tanks and deceased in the water.

Cheng and Alicata (1964) conducted experiments to determine if water could serve as a source of *A. cantonensis* infection by partially submerging infected *Achatina fulica* (n=15), *Subulina octona* (n=2), and *Veronicella alte* (n=1) in tap water. Five of the *A. fulica* used were intentionally injured. After 22-26 hours, two to ten third- stage *A. cantonensis* larvae were found in water containing the uninjured snails, and 55 larvae in water associated with the injured snails. The uninjured *S. octona* and *V. cubensis* also shed low numbers (2, 4, and 1 respectively) and larvae were observed to survive for up to 72 hours. Escaped larvae were fed to rats and young *A. cantonensis* adults were recovered in the brain of one of the rats after 17 days. The authors concluded that torrential rainfall events common in the Pacific tropics could lead to mollusk death by drowning and larvae could be released, especially by injured ones, making it theoretically possible to be infected by drinking water.

Several years later Richards and Merritt (1967) conducted a very thorough study that substantiated not only the potential for larvae to be shed by dead gastropods and potentially transmittable in water, but also the durability of the larvae and its ability to survive in fresh water, salt water, and dead snail hosts. Additionally, they provided evidence that expanded the potential list of water-snail species that can serve as intermediate hosts. Of 26 species of freshwater gastropods exposed to first-stage larvae, 23 became infected, and larvae grew to third-stage in 20 species. Third-stage larvae were observed to survive for up to 12 months in the snail with no apparent ill-effects to the host.

The survival of first-stage larvae was tested by immersion of rat fecal pellets in both fresh and salt water. Rat feces containing *A. cantonensis* larvae were added to two, 1-gallon jars

filled with de-chlorinated tap water, and the aquatic snail *Biomphilaria glabrata* were added, 20 to each jar. All 40 snails became infected. Numerous active larvae were observed in samples from the bottom of the jar for 12 days after which activity decreased, and larvae with slight activity were observed for up to 21 days. Eight rat fecal pellets containing first-stage larvae were added to three, 1-gallon jars filled with chlorinated tap water. Ten snails were added to one jar after 14 days, and all ten snails become infected. Five rats were infected with larvae from these snails and rats also became infected by drinking the water containing third-stage larvae released from snails, demonstrating the feasibility of *A. cantonensis* infection by contaminated water. Active larvae were recovered from snails that were dead for four days, but not from snails that had dried out.

Sea water tests also gave surprising results. Rat fecal pellets with first-stage larvae were added to two jars of seawater. Larvae were only observed to be active for two days, but larvae taken in samples up to 14 days became active when transferred to tap water. Sea water was removed after seven days from one of the jars, and tap water and ten snails were added. All 10 snails became infected; three snails yielded counts of 280, 218, and 317 third-stage larvae, and five rats were infected with larvae from the snails. Digestion of snails after four days in seawater produced active larvae that were fed to mice, and larvae were later recovered from the brains of exposed mice.

Richards and Merritt (1967) confirmed the outcomes of Cheng and Alicata. They suggested that naturally decomposing snails probably release few active larvae, but that "the release into the water by mechanical tearing of the tissues or from the digestive tract of animals feeding on the snails may be of epidemiologic significance." They found that larvae released

from snails into fresh water were alive and active for at least 7 days and rats became infected after drinking water containing third-stage larvae.

A third study by Crook, Fulton, & Supanwong (1971) was based on the association of snails and public water sources in Thailand, where numbers of the giant African land snails *Achatina fulica* crawling and falling into wells and congregating around water jars, and well-water contaminated with *A. cantonensis* were reported. Snails were collected from around one well, and examination for infection showed three of 82 snails to be infected with larvae that were fed to rats and recovered as fifth-stage larvae 21 days later. They then conducted a study in which 30 *A. fulica* were drowned in sedimentation funnels; 18 of these proved to be infected and larvae were found in the water and digestion sediment of all infected samples after 60 hours. Larvae fed to white *R. norvegicus* successfully infected them in all cases.

The authors went on to conduct a test to determine the effects of chlorine and iodine on larvae. Experiments showed no appreciable effect of chlorine at 1.0 ppm. One quart of water was treated with 16.0 mg iodine and 100-300 larvae were added. After 30 minutes the water was put into sedimentation filters and the descending larvae were collected and fed to rats. Numerous living larvae were recovered this way over a total of seven experiments and four of the rat feedings from these larvae developed into brain infections. The authors concluded *A. cantonensis* infections could occur from contaminated water and the observation is "particularly applicable in regions where private and public culinary waters sources are often subject to snail contamination." This is precisely the situation currently occurring on Hawaii Island, where catchment use is widespread, the design and maintenance of catchment systems may be substandard, and the systems are subject to slug and snail contamination.

Observations and discrepancies

The most recent islands-wide study of *A. cantonensis* infection in intermediate hosts confirmed infection in sites on all of the main Hawaiian Islands with the exception of Lanai. The study reports Kauai and Hawaii to have the highest percentage of sites with infection at 34% and 33% respectively (Kim 2013). While the number of sites with infection in Kauai were very similar to that on Hawaii Island, Kauai has had very few cases of human angiostrongyliasis. Kauai, like the rest of the Hawaiian Islands, also has very little catchment use (Patricia Macomber, personal communication), although rainwater harvesting is being promoted throughout the state (University of Hawaii, Sea Grant). While water is not often listed as one of the routes of transmission of angiostrongyliasis, from these studies we can see there is good evidence for its potential. If drowned slugs or snails can shed *A. cantonensis* larvae, and if these liberated larvae can live for some days outside the deceased host, could rainwater catchment tanks become a reservoir for the larvae and might this be a factor in the discrepancy between RLWD incidences through the island chain?

Another observed factor that should be considered and may play a role in helping to explain the higher incidence of RLWD on Hawaii Island are the populations of the semi-slug *P. martensi,* which currently are only established on Oahu and Hawaii Island (Hollingsworth et al., 2007, Hochberg et al. 2011). This slug from Southeast Asia is well-adapted to Hawaii's warm, wet, and vegetated conditions, like those found on the east side of Hawaii Island. It has been shown to be an effective intermediate host with a high rate of infection, it exhibits a propensity for climbing, and it is often found near human dwellings. However, while this important intermediate host can be found on both islands, angiostrongyliasis case numbers are low on Oahu. And like Kauai, Oahu has very little use of residential rainwater catchment.

If these slugs do crawl into tanks and drown, can they shed *A. cantonensis* larvae? If they can shed larvae, and if those larvae are able to survive outside the slug host, where in the water column would they most likely be found; the top, middle, or bottom? This question is important because the recommendation from the CTHAR rainwater catchment manual is to situate the intake pipe for a rainwater catchment system near the bottom of the tank, not too low, to prevent uptake of bottom sludge, but close enough to the bottom to maximize water availability. This observation led to my first hypothesis and the subsequent study designed to provide answers to the question.

Study 1:

Location of *A. cantonensis* in a water column: 10-day study

When we digest snails to collect live *A. cantonensis* larvae for our studies we use a modified Baermann filter apparatus and always observe the larvae moving though a paper Kimwipe used for a filter (Kimtech Science Brand, Rosewell, GA) and down into a collection tube. Can *P. martensi* shed *A. cantonensis* larvae, and if so, would they also migrate to the bottom of a water column?

Hypothesis 1: If *Parmarion martensi* can shed *A. cantonensis* larvae in rainwater the larvae would be found near the bottom of the water column.

Methods

Rainwater for the study was collected from a residence in Hilo, Hawaii in a clean, food-grade, five-gallon bucket placed below the drip edge of a metal roof with no overhanging trees or gutters and therefore mostly clean and debris-free. Water was transferred into three clean, two-

liter glass jars (2) and transported to Dr. Susan Jarvi's laboratory at the University of Hawaii at Hilo, Daniel K. Inouye College of Pharmacy. The rainwater used for the study was examined daily by microscopy using a Leica EZ4 dissecting microscope for evidence of organic matter, organisms or *A. cantonensis* larvae. The water was used for the duration of the study.

Ten *P. martensi* were collected from a residence in Koae in the Puna District where we can consistently find *A. cantonensis* infection in this species. Captured specimens were held in individual collection tubes to avoid cross-contamination. Each specimen was given an RLW log number and information on the species, weight, and condition (whole vs. chopped) was recorded. With single-use safety blades, two tissue snips of <25mg each were taken from the tail section of each specimen for qPCR analysis of DNA to confirm infection by *A. cantonensis* (Jarvi et al. 2012). The tissue samples extracted from the tails were weighed and placed in100µL of lysis buffer, a solution used to break open the cells when analyzing cellular compounds, and stored in a minus 20˚ freezer. Digestion of tissue samples was done adding 20µL of Proteinase K and incubating at 55˚ C overnight with gentle rocking.

Extractions were done according to standard protocol, with elution in 200µL AE repeated twice for a total volume of 400µL of genomic DNA. DNA was quantified using a Bio-Spec Nano (Shimadzu). Extracted DNA was subjected to qPCR using species-specific primers for the internal transcribed spacer (ITS) region (Qvarnstrom et al. 2010) The assay used for qPCR was a Custom TaqMan Gene Expression Assay (Life Technologies, Grand Island, NY, assay ID: A139RIC) and carried out as described in Jarvi et al. (2012). Cycling conditions were 94˚C for 30 seconds 65˚C for 30 seconds and 72˚C for 1 min with 45 cycles. Each sample was run at least twice using two different volumes. The C_T values for these samples were then plotted on a standard curve, which had been generated by Jarvi et al (2012) using multiple volumes of

undiluted DNA as well as dilutions of DNA to create a range of template equivalent to a known number of larvae per reaction (0.71-63.9). Using the C_T values and standard curve we can estimate larvae numbers from tissue samples of organisms of interest. (All subsequent studies followed the same procedures for tissue digestion, DNA extraction, and qPCR).

Five slugs were left whole and five slugs were coarsely macerated with a safety blade. The slugs were immersed in the 50mL Falcon tubes filled with rainwater until drowned. After about 4 hours, when the slugs remained motionless and were assumed deceased, the first 5mL samples were drawn at spaced intervals (about 15 minutes) from the top, middle, and bottom of the water column with care taken to minimize disturbance. Samples were drawn with disposable 5mL pipettes that were changed between each sample draw. Each 5mL sample was placed into a 6 cm, non-gridded petri dish, and 15mL of the collected rainwater was added to each dish so as to cover the bottom. A similar volume of water was added back to the 50mL Falcon tube containing the deceased slug to maintain volume. Samples in the petri dishes were scanned with a Leica EZ4 dissecting microscope and larvae seen were recorded. A total of 30 samples were drawn daily in this manner for a total of five days. On the sixth day, due to the time constraints limiting the number of dishes that could be examined, the slugs and vials of water were discarded and only the petri dishes containing the samples drawn on the first four to five days were followed.

Results

Examination of the rainwater used in the study using a Leica EZ4 dissecting microscope showed the water to be free of living organisms at all time. The qPCR results from the tissue samples showed all ten of the *P. martensi* used in this study to be infected (Table 1.1).

Table 1.1. Study 1 sample data. Study date 03/19/14. *Parmarion martensi* from Koae, Puna District, Hawaii Island (100% infection in random collection). Estimates of larval numbers uses C_T values and standard curve developed by Jarvi et al (2012). Discrepancies in replicate values are likely due to pipetting errors. W=whole, M=macerated.

Slug #	RLW#	Condition (W/M)	Slug Weight (g)	Tissue Weight (mg)	qPCR result	Estimated # larvae/mg tissue (replicates)
1	1507	W	1.34	3.8/25.3	positive	155.7/54.1
2	1508	M	1.58	14.5/11.7	positive	45.0/21.6
3	1509	M	1.85	7.5/14.1	positive	52.9/24.8
4	1510	W	1.73	11.4/9.6	positive	75.2/35.0
5	1511	W	1.54	9.6/9.1	positive	77.0/51.2
6	1512	M	1.95	9.3/11.0	positive	93.2/57.0
7	1513	W	1.85	10.1/6.7	positive	50.4/21.7
8	1514	M	1.47	17.6/13.3	positive	34.8/13.6
9	1515	M	2.39	7.9/8.7	positive	126.5/52.9
10	1516	W	2.17	13.6/16.5	positive	44.1/26.9

Visual examination of the 5mL samples by microscopy showed active larvae (2) within about 2 hours of slug mortality from one of the 50mL Falcon tubes containing a drowned slug. On day one, three swimming L3 larvae were found in the sample collected from the bottom of the water column. On day two, a total of five larvae were seen, and on day three, a total of about 410 larvae, all coiled and none moving, were collected from all ten of the slugs. On day five coiled larvae were found in samples taken from all of the 50mL tubes containing slugs. Due to time constraints and high numbers, larval counts were estimated from all but three of the tubes that had low counts. A conservative estimate put larvae numbers at <400 (Table 1.2, Fig. 1.1).

Table 1.2. Numbers of larvae found in samples taken in the first 144-hours after the slugs were placed into the 50mL falcon tubes. W=whole, M=macerated.

Slug #	Condition	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5
1	W	0	3	0	170	139	>50
2	M	0	0	0	1	23	>50
3	M	0	0	0	4	1	<50
4	W	0	0	1	60	0	<50
5	W	0	0	0	10	28	>50
6	M	2	0	0	1	0	8
7	W	0	0	0	139	0	>50
8	M	0	0	4	0	0	11
9	M	0	0	0	16	2	18
10	W	0	0	0	10	13	>50

Of the larvae released from the slugs from days 0-5, the greatest number were found in the 5mL samples taken from the bottom of the 50mL Falcon tubes and accounted for 93.5% of all larvae shed (Fig. 1.1). A Kruskal-Wallis, non-parametric test shows the bottom location is significantly greater in median number of larvae with a P-value of < 0.0001 (Fig. 1.2)

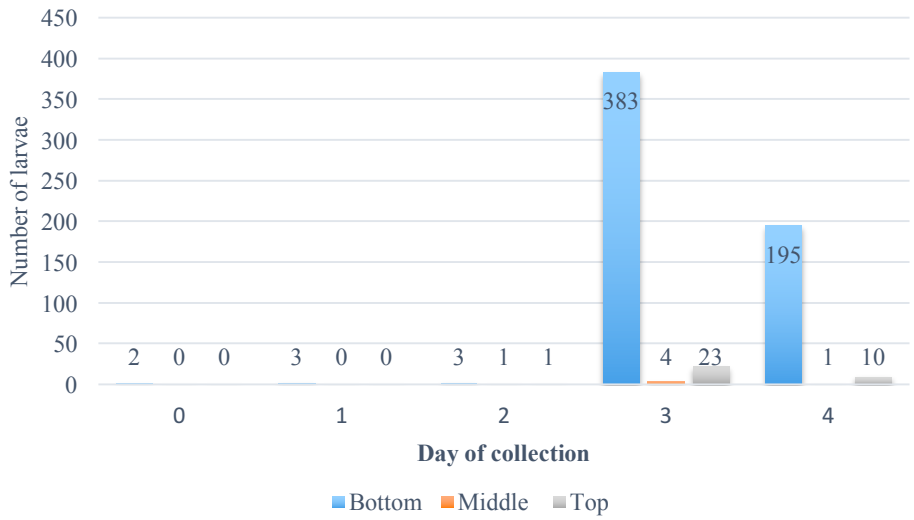

Fig. 1.1. Larvae found in 5mL samples taken from the bottom, middle, and top of the 50mL falcon tubes over first 120 hours of the 10-day study.

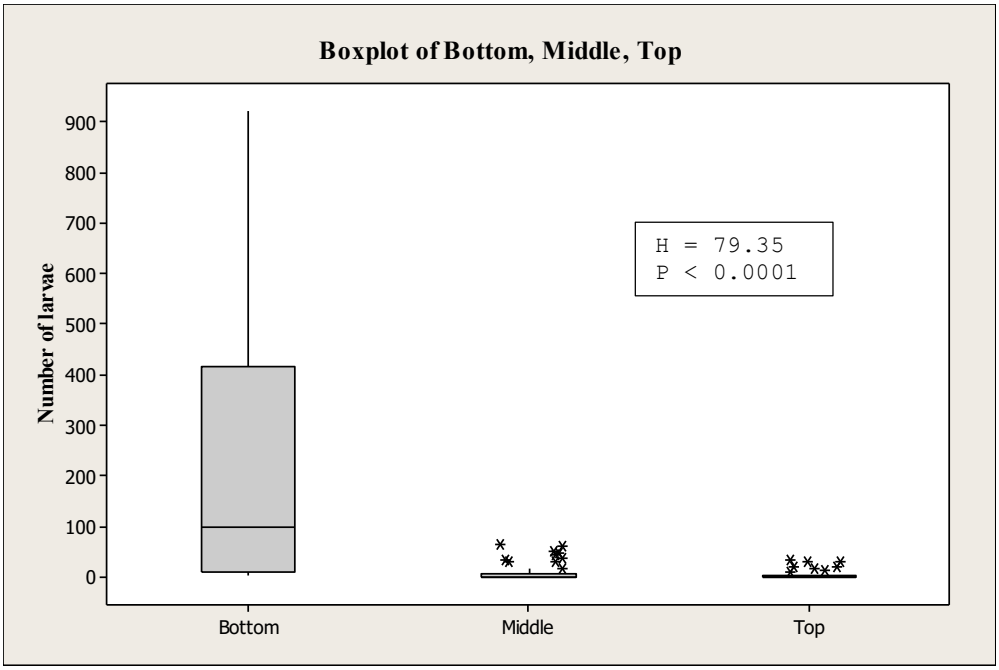

Fig. 1.2 Kruskal-Wallis Test: Parasite count vs. location. The location bottom is significantly higher in larvae.

The greatest numbers of larvae were shed from whole slugs as opposed to mechanically macerated slugs (Fig. 1.3). A Mann-Whitney test shows significance in numbers of larvae shed from whole slugs versus macerated slugs (P= 0.014) (Fig. 1.4).

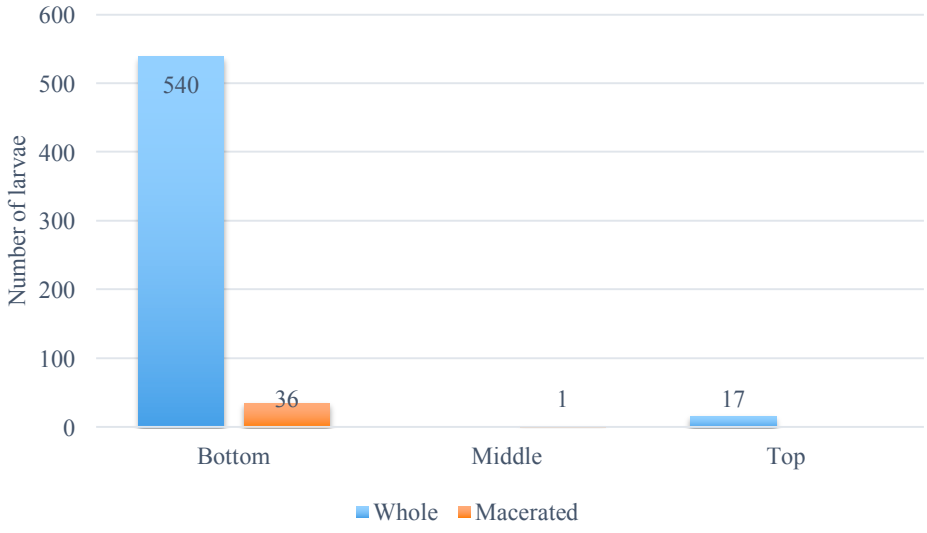

Fig 1.3. Location in the 50mL column of water where larvae were found. The greatest numbers were found in the bottom of the water column and from whole versus mechanically macerated slugs.

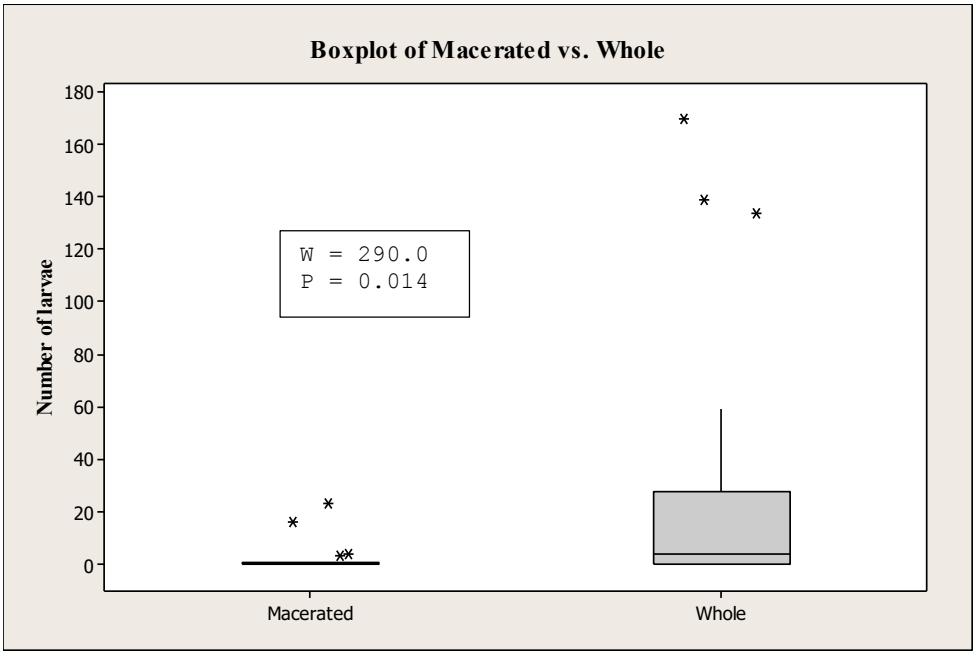

Fig. 1.4. Mann-Whitney test of numbers of larvae shed from whole versus macerated slugs showing that more slugs yield significantly more larvae (P = 0.014).

Due to the high volume of larvae dropped and time constraints the 50mL Falcon tubes with slugs were disposed of on day five and the petri dishes containing larvae from slugs found from days three to five were kept for daily observation and recording of larval movement. The shape of the larvae found is best described as coiled or spiral, and comma or c-shaped. The c-shaped larvae were dull in color whereas the coiled and spiral-shaped larvae were shiny and translucent. The c-shaped larvae were never observed to show movement or emerge into active larvae and would fit the descriptions of late 1st stage/early 2nd stage larvae found in the literature (LV 2009; Zeng et al. 2013).

The motionless coiled and c-shaped larvae found in the 5mL samples on days three and four were retained for observation. Over the remaining five days of the 10-day study, many of the coiled larvae were observed to emerge from their coiled state and become vigorously

swimming larvae, while the c-shaped larvae exhibited no movement or emergence. At the end of the 10-day study actively swimming larvae were still observed (Fig. 1.5).

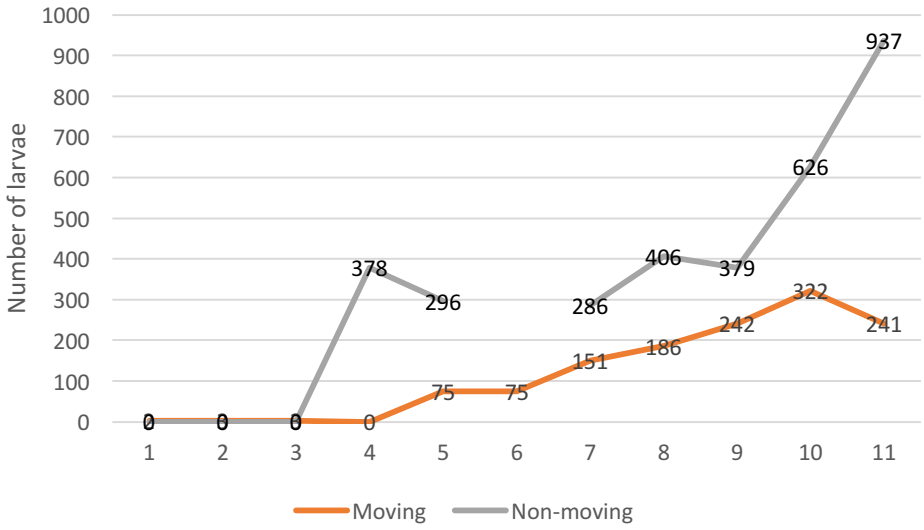

Fig. 1.5. Counts of moving vs. non-moving larvae held in petri dishes over 10-day study. Note the increase in numbers of larvae on days 3-5.

This study suggests that catchment water could be a potential source of infection and worthy of further research in the efforts to prevent human infection by *A. cantonensis* in Hawaii. Factors that made accurate counts difficult included high numbers of larvae, bits of tissue that obscured larvae, and where the larvae were in the water. The microscope focus, if set for one depth (e.g. bottom of petri dish), did not allow for clear observation of larvae in the middle or top of the water, as was sometimes observed, however the greatest number of larvae were always seen at the bottom of the dish. The larvae swam in a snake-like fashion, often coiling and uncoiling in a q-shape. Vigorous larvae were quite active and many congregated along the sides of the dish, although dishes with large numbers had coverage throughout the medium. At the conclusion of the 10-day study there were about 250 active larvae remaining (Fig. 1.6).

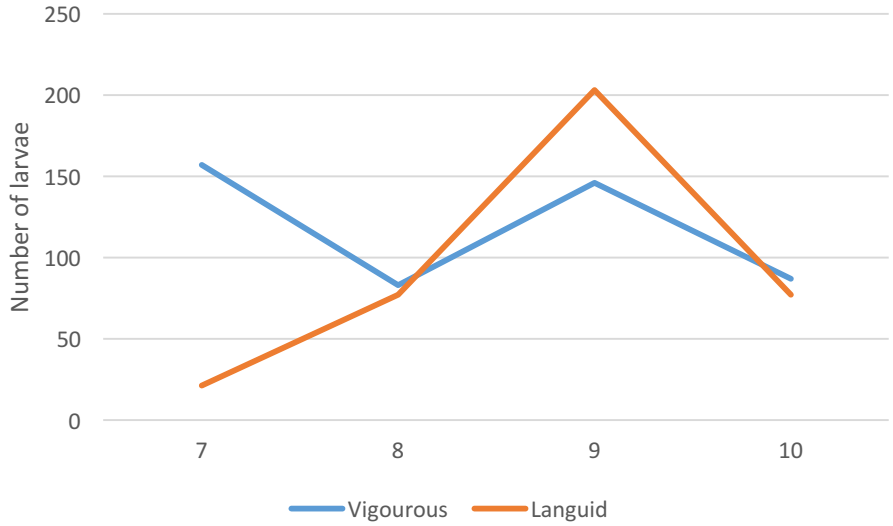

Fig. 1.6. Larval vitality from Day 3-5 samples. Many larvae emerged from coiled state into swimming state. This graph shows larval vigor over days 7-10 of the 10-day study.

Discussion

Low numbers of emerged (swimming) larvae were observed within the first 24-72 hours, after which large numbers of coiled larvae were observed in 5mL samples. The greatest numbers of coiled larvae were found in the samples drawn from the bottom of the water column (> 93%), confirming the null hypothesis. While qPCR was not run on the larvae themselves, the qPCR results from the tail tissue samples from each of the ten *P. martensi* used in the study confirmed *A. cantonensis* infection in all of the specimens (100% infection). A study by Ash (1970) described the morphology of third stage larvae of four metastrongyloid lungworms, *Angiostrongylus cantonensis, Angiostrongylus vasorum, Aelurostrongylus abstrusus,* and *Anafilaroides rostratus* and states that the termination of the tail is a consistent and reliable characteristic, and in *A. cantonensis* the posterior end always terminates as a fine point. While not definitive, this characteristic was consistent in the larvae found and observed in this study.

The study also showed that larval release is not dependent on the slug being digested or

macerated, and that whole *P. martensi* slugs can release of parasites. This is a very different observation than that found in the earlier studies where the macerated mollusks shed the greater numbers of larvae; however, *P. martensi* were not used in earlier studies as it was not yet introduced to Hawaii. The results of this study substantiates that this soft-bodied, very effective intermediate host may be problematic in areas where rainwater catchment is used in Hawaii as it does shed larvae when drowned.

The larvae dropped from the slugs and held for observation were seen slowly moving as they began to emerge from their inactive, coiled state, and then develop into vigorously swimming larvae. Numbers of viable larvae increased from days 6-8, and then began to show a downward trend. While there were still active and vigorous larvae at day 10, many lay motionless on the bottom of the petri dish. There remained a number of c-shaped larvae that were presumed to be late 1st or 2nd stage larvae incapable of emergence. The study by Ash (1970) examined 35 third stage larvae of *A. cantonensis* and found the maximum width to be an average of 26μ with a range of 23-34μ. This size is quite close to the 20-micron filter size recommended by the "Guidelines on Rainwater Catchment Systems for Hawaii" and it would be advisable to develop a follow-up study to determine if a 20-micron filter is sufficient to prevent larval passage in a rainwater catchment system.

Study 2:

Diversify mollusk species, extend timeframe, and verify larvae location (20-days)

A second study was designed to confirm results of the first study, and to diversify species to better understand the larval shedding action of whole versus mechanically macerated mollusks. The second study also included one paratenic host, the flatworm *Platydemous*

manokwari. Time constraints in the first study limited the observation of larval shedding to the first five days of the study and this study extended that timeframe to a full, 20 days. As in the first study, 5mL samples drawn from the bottom, middle, and top of the water column for the entire duration of the study to (1) confirm the location of shed parasites in a column of water and (2) to better understand how long a drowned mollusk is capable of shedding larvae.

Hypothesis 1: *All drowned whole mollusks regardless of species can shed A. cantonensis larvae.*

Hypothesis 2: *Drowned mollusks can shed larvae for > 5 days.*

Methods

Thirteen specimens were used for this trial, twelve mollusks and one *Platydemus manokwari* planarian of a species known to be a paratenic host of *A. cantonensis*. Species used were *Achatina fulica* (2 adult, 2 juvenile), *Laevicaulis alte* (2 adult), two *Veronicella cubensis* (2 adult), *Platydemus manokwari* (1), and *Parmarion martensi* (3 adults) collected from the Waa Waa area of the Puna District, and one (1 adult) *Parmarion martensi* from the Koae area of the Puna District for use in this trial (Fig. 1.7). The Koae site has been used repeatedly for the capture of slugs for RLW studies by the Jarvi Lab because of the high rate of infected slugs and snails at this site. Distance between the two collection sites is approximately a three mile, straight-line distance, the entire area is heavily vegetated, has a relatively dense tree canopy, and is in the vicinity where cases of angiostrongyliasis have been reported. Specimens used were placed in individual collection vials to avoid cross-contamination and transferred to the Jarvi laboratory.

Fig. 1.7. Specimens used in study two came from (pinned) locations near the eastern-most point of Hawaii Island in the Koae and Waa Waa areas.

Rainwater used in this trial was collected from the same residence and in the same manner as before, in a clean, food-grade quality, 5-gallon plastic bucket placed directly under the drip edge of a roof with no overhanging vegetation and no gutters, and transferred to the lab in a clean, half-gallon glass Mason jar. The rainwater was inspected on a daily basis by microscopy to confirm the presence or absence of RLW larvae. All mollusk specimens were weighed and measured. Care was taken to prevent cross-contamination of samples. Tail snips of < 25 mg were taken from all specimens for analysis of infection of *A. cantonensis* by qPCR as in the first study. Two tail snips were taken from each of the 13 specimens using sterile, single use safety blades. Tissue samples weights ranged from .003 to .025g and were placed in180µL of lysis buffer and stored in a minus 20˚ freezer for molecular testing. Tissue digestion, DNA extraction, and qPCR was done in the same manner as described in study 1. Specimens were divided into species, and each species group was divided into individuals to be left whole or be macerated. Maceration was done with a safety blade. Mollusk specimens were placed in a 50 mL Falcon

tube filled with rainwater. Tubes containing whole mollusks were inverted until no movement was observed.

Samples were taken as described in the first study. The first 5mL samples were drawn approximately 14 hours after cessation of mollusk movement from the bottom, middle, and top of each of the 50 mL Falcon tubes. Each sample was placed in a 6cm, non-gridded petri dish and 15 mL of rainwater was added to each sample to cover the bottom of the dish and to prevent drying. Dishes were examined for RLW larvae with a Leica EZ4 dissecting microscope. These same sampling procedures were carried out for 20 days and larvae presence/absence and location in the water column was recorded. Because of the great number of petri dish samples that would be generated over the course of the 20-day study, only petri dishes containing larvae were retained for observation, examined daily, and larvae counts were recorded. Water samples from the rainwater used in the study were examined on a daily basis for observation of larval presence.

Larvae were analyzed for confirmation as *A. cantonensis* either individually or in small batches using the digestion, extraction, a qPCR methods described in study 1, and some larvae were transported to the US Pacific Basin Agricultural Research Center in Hilo, HI for analysis by USDA nematologists and sent to the mainland for a blast search. PCR amplification was conducted in 35 µL reactions containing 17.5 µL GoTaq MasterMix, 0.4 µM of each primer (1.4 uL each of a 10uM stock), and the 14.7 µL macerated nematode solution. The macerated nematode solution was prepared by putting a drop of water on a slide with the nematode and dissecting the nematode in half with microsurgical tools. For species identification, PCR was performed on single IJ specimens using general nematode primers and sequenced. Nematodes were tentatively identified using morphological methods and prepared for PCR in 35 µL reactions (Cabos et al. 2013). Primers ITS-F (5'-TGTAGGTGAACCTGCTGCTGGATC-3')

and ITS-R (5'-CCTATTTAGTTTCTTTTCCTCCGC-3') (Saeki et al. 2003) were used to amplify the ITS region. The PCR conditions were 95° C for 2 minutes, 40 cycles of 95° C for 45 sec, 56° C for 30 sec, 72° C for 20 sec, followed by 5 min at 72° C. A phiX174 DNA/HaeIII marker and 10 µL of each PCR product were separated on a 1.2% agarose gel, stained with GelRed in 1xTAE, and visualized under UV light. For samples with visible bands, the remaining 25 µL of the PCR product was purified and sequenced by Eurofins MWG Operon (Huntsville, AL).

On day twenty an estimated 400 live larvae were remaining in petri dish samples from RLW 1524 (*L. alte*), RLW 1530 (*P. martensi*) and RLW 1532 (*P. martensi*). Larvae were removed from petri dishes by pipette and placed in respective 1.5mL Eppendorf tubes, which were centrifuged with a Thermo Scientific – Sorvall Legend Micro 21R at 21.1 g's for five minutes to pelletize the contents. The supernatant was removed by pipette and tubes were stored in labeled 1.5 mL Eppendorf tubes at -20^{o} C. Photographs were taken of some larvae from each sample before centrifuging. The pelletized samples were digested, and DNA extraction and qPCR was done on the pelletized samples as described in study 1.

Results

Of the thirteen slug/snail/flatworm specimens used, six were positive for nematode larvae by microscopy examination of the 5mL sample of fluid drawn from the bottom of the 50mL Falcon tube, and five tested-positive by qPCR analysis although one of these was a very low positive. Agreement of infection by visual inspection and by qPCR strongly coincided in two specimens (*L. alte, P. martensi*). There was a weak positive by microscopy and strong positive by qPCR in one specimen (*L. alte*), a negative by microscopy (juvenile *A. fulica*) but positive by

qPCR, and a strong positive by microscopy (*P. martensi*) but negative by qPCR, and a weak positive by microscopy and negative by qPCR (adult *A. fulica*) (Table 1.3).

Table 1.3. Study date 07/07/2014. Samples collected in Waa Waa and Koae in the Puna District, Hawaii Island for the second study, increasing species diversity and study time and preliminary filter trial. W=whole, M=macerated.

Species	**RLW#**	**Condition (W/M)**	**Mollusk Weight (g)**	**Tissue Weight (mg)**	**Location collected**	**qPCR result**	**Estimated # larvae/mg tissue (replicates)**
***A. fulica* (juvenile)**	1520	W	0.803	9.0/7.1	Waa Waa	Positive	2.612/1.057
***A. fulica* (juvenile)**	1521	M	0.896	7.6/11.4	Waa Waa	Negative	None detected
A. fulica	1522	M	16.50	17.0/10.2	Waa Waa	Negative	0/ none detected
A. fulica	1523	W	7.51	6.9/13.9	Waa Waa	Negative	None detected
L. alte	1524	M	8.17	9.5/3.7	Waa Waa	High positive	361.231/328.197
L. alte	1525	W	9.46	7.2/4.3	Waa Waa	Positive	18.675/16.84
V. cubensis	1526	M	5.42	5.9/5.5	Waa Waa	Negative	None detected
V. cubensis	1527	W	3.57	6.3/3.2	Waa Waa	Negative	None detected
P. manokwari	1528	W	0.178	7.0/7.0	Waa Waa	Likely negative	0/0.002
P. martensi	1529	M	1.57	7.0/6.0	Waa Waa	Negative	None detected
P. martensi	1530	W	1.88	15.0/11.0	Koae	Negative	None detected
P. martensi	1531	M	2.36	14.0/13.0	Waa Waa	Negative	None detected
P. martensi	1532	W	1.39	16.0/25/0	Waa Waa	Positive	6.833/3.429

Daily water samples were held and each day were observed with the dissecting microscope for any sign of organic life or larval emergence, however all samples remained clean and free of life. A sample of the rainwater used in the study was also analyzed by qPCR and was negative for *A. cantonensis*.

Location of larvae in the water column

As in the ten-day study, the majority of larvae (95%) were found in the 5mL samples taken from the bottom of the water column (Fig. 1.8). Samples were taken with care, creating

the least disturbance possible in the column, however, there were times where the specimen came into suction contact with the pipette. The location of the deceased specimen varied between the top and bottom of the 50mL Falcon tube and would sometimes change as decomposition occurred.

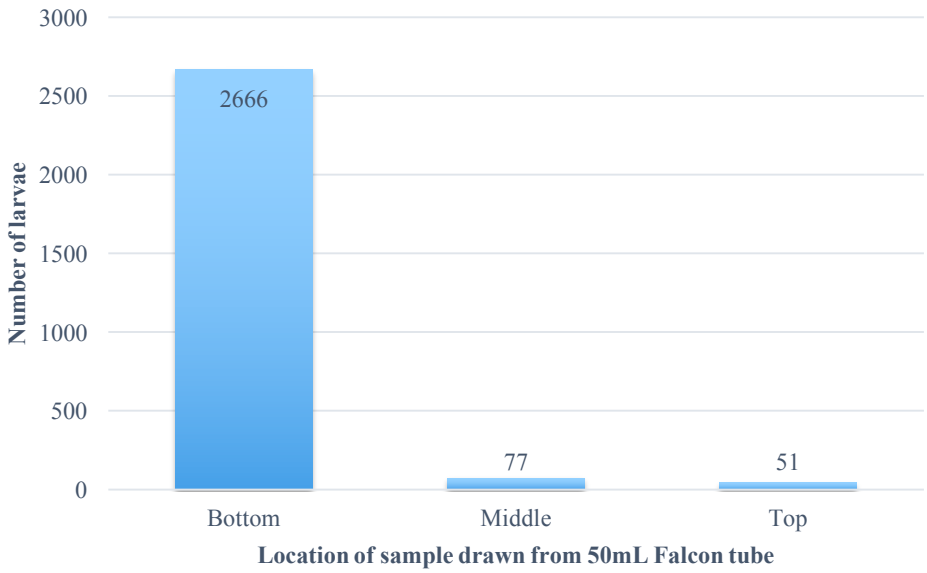

Fig. 1.8. Location of larvae in the water column from the 20-day study, confirming the results from the 10-day study that the bottom samples contained the most larvae.

Larval drop

As in the first study there were low numbers of active swimming larvae in the samples taken on day one. The daily draws from RLW 1524 (*L. alte*) produced numerous larvae up to day seven, the majority of which were coiled and c-shaped larvae as had been observed in the 10-day study. As in the 10-day study, many of the coiled larvae emerged into swimming worms while the c-shaped larvae remained motionless. Whole *P. martensi* were again observed to shed larvae, however, while both of the *L. alte* used in this study tested positive for *A. cantonensis* by qPCR, only the chopped *L. alte* released larvae. The semi-slug *P. martensi* is a much softer-

bodied slug, whereas *L. alte* has a dry, tough outer skin, hence the common name leatherback or leatherleaf slug.

Low numbers of larvae were observed in samples drawn on day one (five from RLW 1524; 20 from RLW 1530), and day two (30 from RLW 1524; two from 1532). Larvae were found in bottom samples taken from the 50mL Falcon tube up to day 17 from RLW 1524, with the greatest numbers collected between days two through day seven, however no larvae were found in samples taken from RLW 1530 and 1532 after day one. These petri dishes containing the samples were kept and held for daily observation. Again, all microscopy was done using a Leica EZ4 dissecting microscope.

The daily observations by microscopy showed a steady increase in the numbers of active larvae, peaking at days six to ten at approximately 850-950 larvae per dish. It could not be observed where the larvae were emerging from but it was assumed they were in small particles of tissue from the slugs, one of which was mechanically macerated *(L. alte*) and one was whole (*P. martensi*). Discrepancies in larval counts can be attributed to the fact that it was difficult to count large numbers of moving nematodes and, while the larvae were mostly concentrated at the bottom of the dish, some were in the middle and top of the medium and could be missed depending on where the microscope focus was set. Some larvae were removed from the petri dish to be used for identification by PCR, there was larval die-off (motionless, emerged larvae were visible) and there was probably new emergence. Finally, some larvae were embedded in bits of tissue and were not clearly visible, however their movement was noticeable.

Larval characteristics: morphological and qPCR

As opposed to the larvae that emerged in the 10-day study, these larvae were a range of sizes, which were categorized as small, medium, and large. The small and medium-sized worms

swam in an s-like motion, often coiling and uncoiling into a Q-shape, as we have seen with acid-digested *A. cantonensis.* This movement has been described as distinctive to *A. cantonensis* and not observed in other free-living nematodes in a publication from China (Lv et al. 2009). This study also described refractive granules that begin to emerge in the larval body at the later L1 stage, which can even obscure the gut, and a clear line that divides the anterior section of the larval body with few refractive granules, and a posterior section dense with granules. Many of the larvae found in samples were observed to have these features (Fig. 1.9). By day 18 all small larvae observed had partially shed larval molt sheaths (Fig. 1.10). The considerably larger worms swam in a back-and-forth, thrashing motion, had dark interiors, and the morphology did not fit that of *A. cantonensis* (Fig. 1.11).

Fig. 1.9. Larvae from RLW 1524 with granules in posterior section and distinct division between anterior and posterior sections (40x).

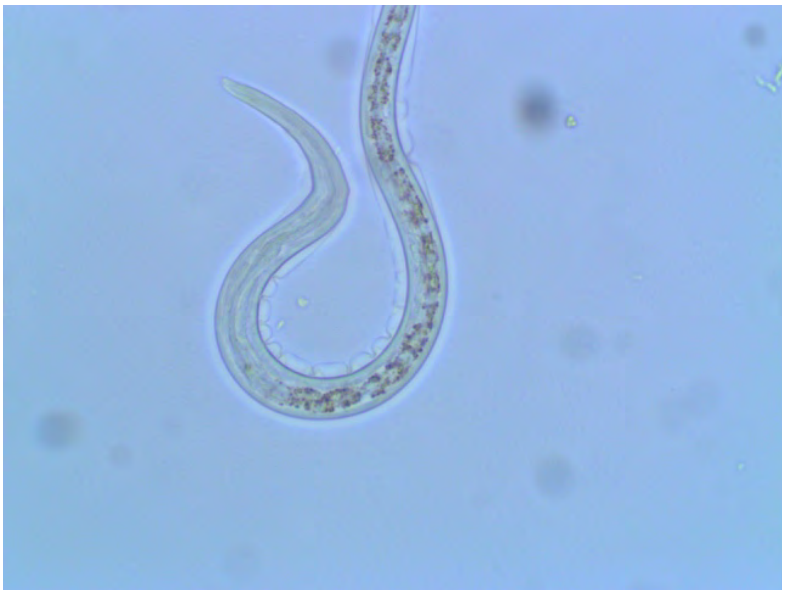

Fig. 1.10. Folds in retained molt sheath are clearly visible (40x).

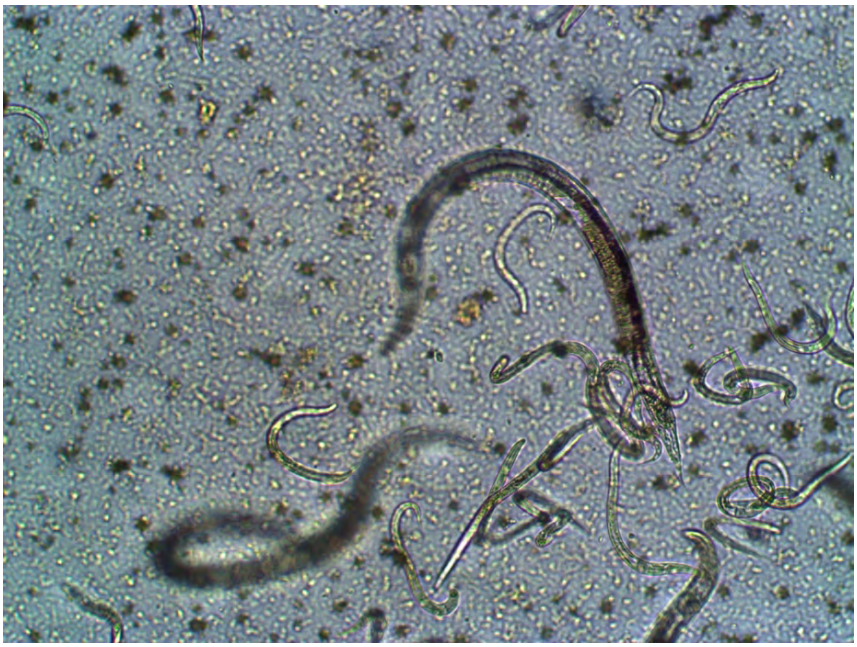

Fig. 1.11. The sizes of larvae varied considerably, the smaller larvae moved in a S-like manner, often coiling into a Q, an action which is stated to be distinctive characteristic of *A. cantonensis.* The large larvae were considerably larger, had dark interiors, and moved in a thrashing manner, although some displayed the coiling, Q movement as well (10x).

Because of the significant size variation of larvae, considerable effort was made to determine if the small and mid-sized larvae were *A. cantonensis*. On day three, larvae found in

the 5mL sample from RLW 1530 (*P. martensi*) were pipetted from the petri dish for qPCR analysis for *A. cantonensis.* Two large larvae and five small larvae were taken from RLW 1530. The two large larvae were placed into individual 0.5mL Eppendorf tubes, three small larvae were placed into another, and the two small larvae were put into the fourth tube for qPCR. Rainwater (20 µL) used in the study was also put into a separate Eppendorf tube. Lysis buffer (50 µL) was added and the larvae and water sample were stored in a -20º freezer until digestion and DNA extraction could be completed. The DNA yields extracted from the larvae samples were low, and when analyzed by qPCR the results were negative for *A. cantonensis*.

On day fifteen 11 large larvae, and 16 dead plus 2 live small larvae were removed from the day one petri dish sample (*L. alte* RLW 1524) by pipette for DNA analysis by qPCR. DNA concentrations from the samples were low (0.73 and 0.07 respectively), and qPCR results were negative for *A. cantonensis.* On day seventeen larvae were from one sample were transported to the US Pacific Basin Agricultural Research Center in Hilo, HI, for analysis by USDA nematologists. The larger larvae were identified as possible bacterial feeders based on the morphology of the basal bulb. A few larvae were extracted at this facility and sent to the mainland for a Basic Local Alignment Search Tool (BLAST) query to determine if the larvae were *A. cantonensis*. The remaining larvae were returned taken back to the lab and observed each day for the remainder of the trial. The results from the USDA blast search came back with low results; 52%, of the query aligned with a hit for *A. cantonensis*.

The qPCR of tail snips from these three individual slugs, which had a large release of larvae that looked alike and moved in similar motions showed a positive confirmation for *A. cantonensis* for the slug specimens RLW 1524 and RLW 1532 but not for RLW 1530. On day twenty all remaining live worms from petri dish samples from RLW 1524 (*L. alte*), RLW 1530

(*P. martensi*) and RLW 1532 (*P. martensi*) were removed from petri dishes by pipette and placed in respective 1.5mL Eppendorf tubes. Results were negative for samples from RLW 1530. Two samples from RLW 1524 were positive for *A. cantonensis* in both replicates run, and two samples had positive confirmation with one replicate but not the other. The sample from RLW 1532 was positive. The second tail tissue sample from RLW 1530 was digested, extracted, and analyzed by qPCR, but the results were again negative for *A. cantonensis.* When the contents of the petri dishes were pelletized and analyzed by qPCR the results were positive for petri dish samples from RLW 1524 and RLW 1532, but again, not for RLW 1530, and yet the larvae shed from RLW 1530 emerged in a similar timeframe as the others and looked and moved in a similar manner as the larvae from RLW 1524 and 1532. That the larvae from these three slug specimens had similar emergence and morphology, yet conflicting qPCR results, caused some consternation in the attempt to correctly identify them. There was also a question as to the state in which the larvae were emerging from the slug, as they initially were not visible by microscopy when the samples were drawn from the 50mL Falcon tubes.

Molt sheaths and tail structure

Larvae from RLW 1524, 1530, and 1532 were photographed using an Olympus CX31 compound microscope and LW Scientific (865 Marathon Parkway Lawrenceville GA 30046) MiniVID USB 5MP Digital Eyepiece Camera and ToupView software. The shedding molt skins could be seen on all of the small larvae by day eighteen (Figs. 1.12a, b). Comparisons of larvae from the three slugs show similarity of the head and tail structure and the molting larval sheath (Figs.1.13a, b).

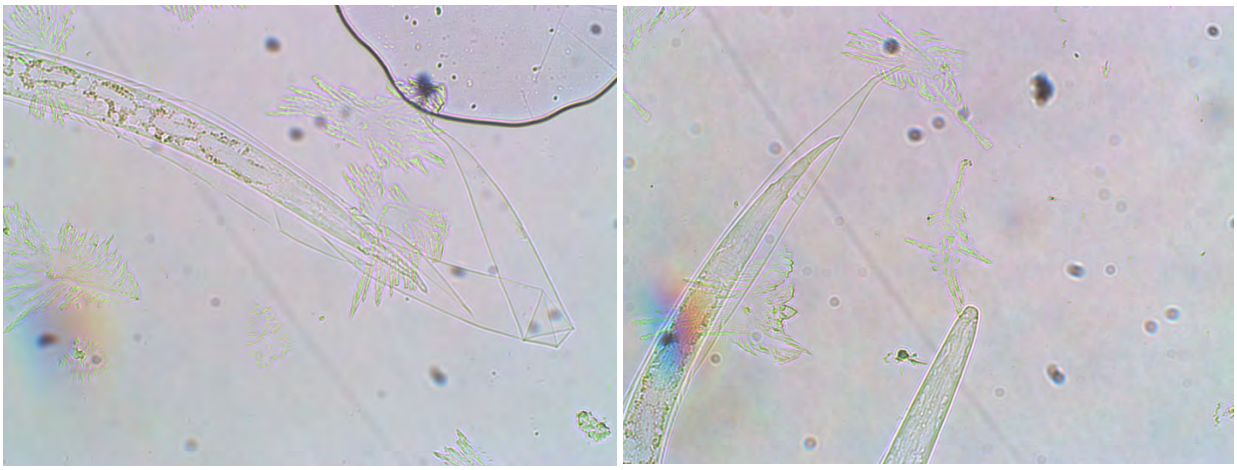

Fig. 1.12 (a, b). Partially cast larval molt sheath visible, larvae from RLW 1524 (40x).

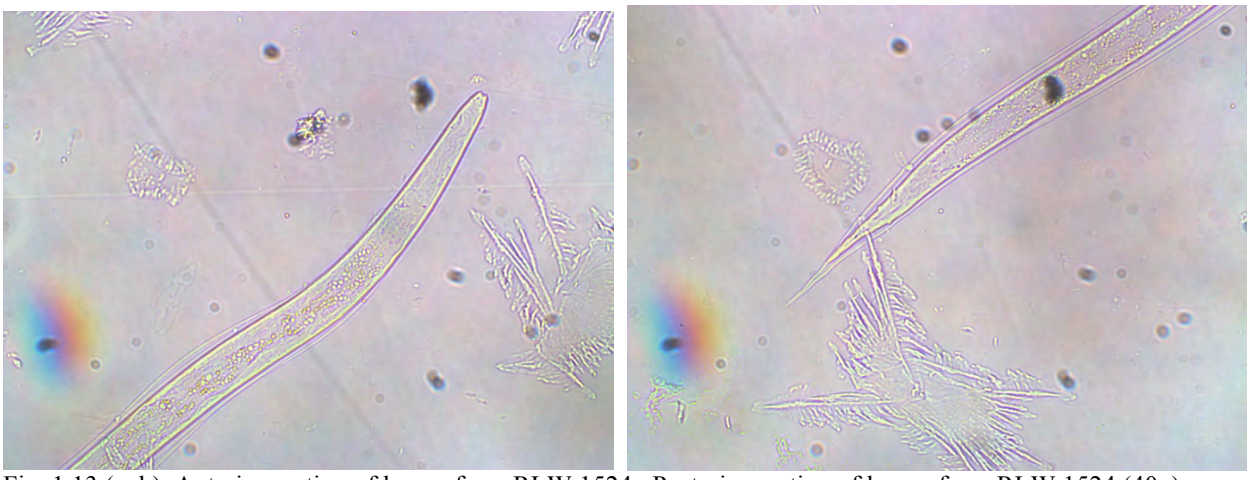

Fig. 1.13 (a, b). Anterior section of larvae from RLW 1524. Posterior section of larvae from RLW 1524 (40x)

The termination of the posterior end in a fine point is claimed to be a consistent and reliable character of *A. cantonensis* (Ash 1970) and the mouth of the L1 is pursed (Lv et al. 2009), both features were seen in the larvae in this study (Figs. 1.14a, b).

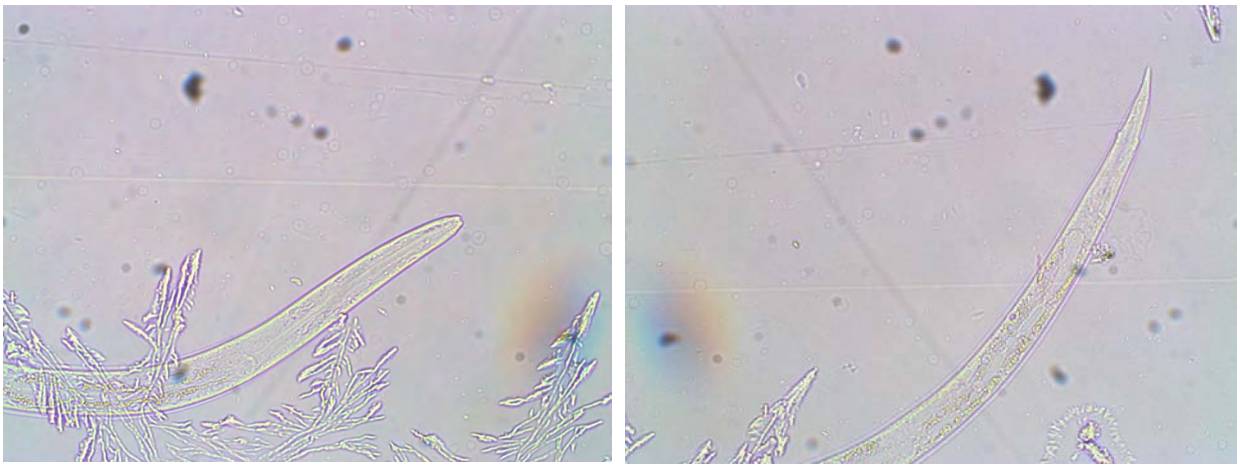

Fig. 1.14 (a, b). Anterior section of larva from RLW 1530. Posterior section of larva from RLW 1530 (40x).

Similar features can be seen in some of the large larvae. While substantially larger in size, the tails of some of these larvae also ended in fine points and the head structure was fairly similar (Fig. 1.15a, b).

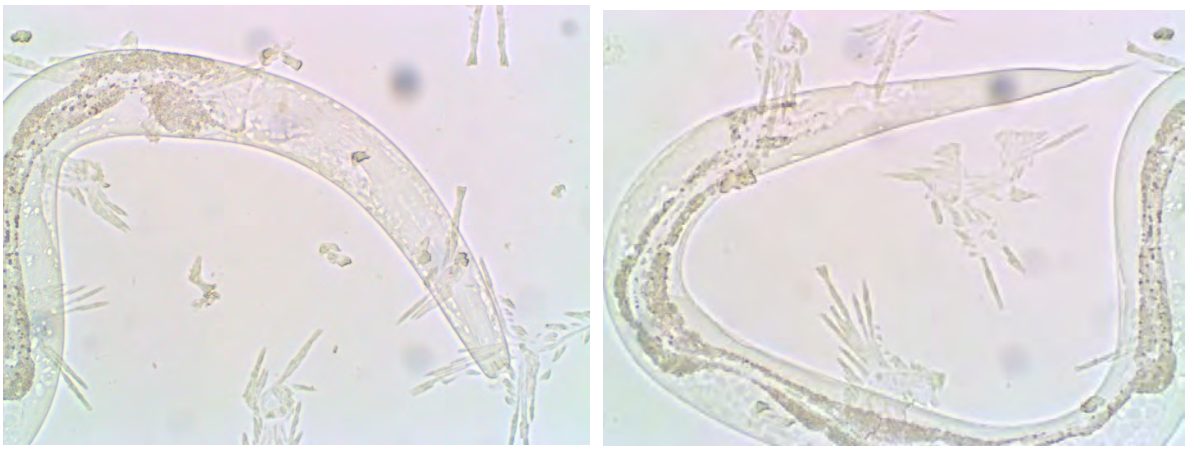

Fig. 1.15 (a, b). Anterior section of large worm RLW 1524. Posterior section of a large worm (40x).

Artifacts were also found along with the larvae that resembled eggs (Fig. 1.16a, b).

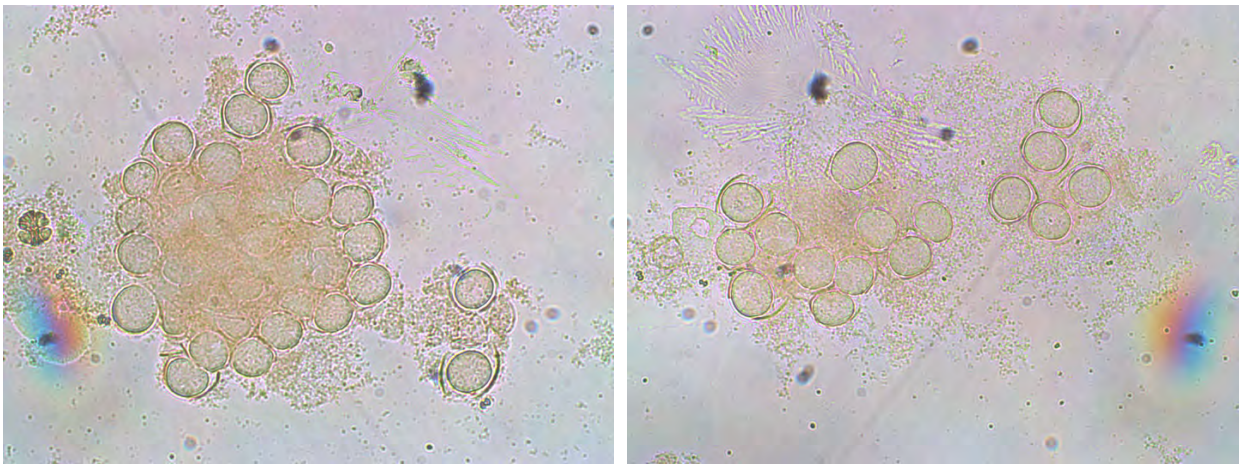

Fig. 1.16 (a, b). Egg-like artifacts found in sample from RLW 1524 (40x).

Discussion

This second study confirmed the first study; whole (*P. martensi*)) can release nematode larvae when drowned in water and the greatest number of larvae will be found in the bottom of the water column. This may not be the case with other species of slugs and snails, as both *L. alte* specimens used in the study had confirmed *A. cantonensis* infection by qPCR of tail tissue, however only the macerated slug had a larval release. A new phenomenon not observed in the 10-day study occurred in the petri dishes that held samples containing larvae from days one and two from one macerated *L. alte* and two whole *P. martensi*. Some samples taken on these days contained very few swimming larvae, but over the course of days the numbers increased until hundreds could be found in some petri dishes, but their origin of emergence could not be defined, perhaps emergence was from tissue or from eggs. However, the literature states that L1 *A. cantonensis* larvae hatch in the lungs of the rat host, migrate up the bronchial tree, are swallowed and shed in feces, and emergence from eggs not align with the literature. This over-time emergence of large quantities of larvae was perhaps one of the most surprising and mystifying

results of this second study. Another result of the study was the substantiation of larval longevity; larvae shed were viable for at least 20 days.

Identification of the larvae that emerged from the two *P. martensi* (RLW 1530 & 1532) and one *L. alte* (RLW 1524) proved to be difficult and qPCR results were confounding. Analysis of individual larvae removed by pipette and combined for qPCR was negative for *A. cantonensis* as was a BLAST search done on small numbers of larvae by plant pathologist Roxana Meyer at the Agricultural Research Service, USDA (Hilo). The qPCR of tail snips from these three individual slugs, which had a large release of larvae that looked and behaved in similar fashion, showed a positive confirmation for *A. cantonensis* for two of the slugs but not for the third. These results were especially confounding as this particular specimen was captured in an area where we generally collect slugs if we are looking for infection for RLW studies. Indeed, for the first study all of the *P. martensi* used were collected from this site and 100% were confirmed positive by *A. cantonensis* infection by qPCR

The driving question of this study became an exercise in trying to identify the large quantities of multiple-sized larvae emerging from the day one and two samples. I was advised by Dr. Alder Dillman, a nematologist at Stanford University, that nematode identification by morphology is difficult if not impossible and to rely on qPCR for species confirmation, looking at the 18S and 28S markers. However, the 18S RNA gene is too conserved among nematode species to determine species-specific detection, but the first internal transcribed spacer (ITS1) is much more effective at differentiating closely related species, and the TaqMan assay used in the qPCR reactions for these studies targets ITS1 (Qvarnstrom et al., 2010).

There are few photographs available of first and second stage *A. cantonensis* larvae, and photographs of third-stage larvae are generally from acid-digested intermediate hosts, which

have lost their molt sheaths. The retention of the molt sheath from L1-L2, and L2-L3, with third stage larvae remaining encased in two sheaths, has been well described (Mackerras & Sanders 1955; Richards and Merritt 1967; Hata & Kojima 1990; Lv et al. 2009). Larvae recovered from RLW 1524, 1530, and 1532 all showed evidence of retained molt sheaths when examined by microscopy. There was some discussion as to whether the nematodes could be soil nematodes, however of the thirteen specimens used in this study all but one was captured within a 35 ft. radius of each other and the remaining specimen came from a farm about three miles away. If these were soil nematodes it would seem that more of the specimens that were collected in close proximity would have evidence of nematodes.

The larvae released from the three slugs all showed similarity in their movement. The large larvae in this second study were substantially longer and had darker interiors and also had vigorous movement. The smaller larvae were more translucent and swam in a manner similar to that which we have seen in acid digested larvae, exhibiting the Q-like motion stated to be specific to *A. cantonensis*. The large larvae all perished before the end of the study, whereas the smaller larvae showed much greater vitality and longevity, and many were alive and swimming vigorously at the end of the twenty-day study. When examined by microscopy the smaller larvae had similar head and tail shapes and all had molt sheaths that were in the process of being shed.

There are very few studies that have looked at *A. cantonensis* nematode larvae from what could be refer to as "water-digested" slugs and snails; most studies have been done on the L3 larvae, which were gathered from acid digestions, and acid will destroy the L1 and L2 stages. Using water as a digesting/decomposing medium preserves these stages and, keeping in mind that these slugs and snails were not laboratory raised, it is highly possible that they may have harbored multiple or even all three stages of larvae. It is very likely that various stages of larval

development were observed in this study, possibly even long-retained third stage larvae, as the Richards and Merritt paper (1967) states L3 larvae were able to survive for up to 12 months after infection without an apparent effect on the snail. As the slugs and snails used in this study were infected in the wild it is also very possible that some of the larvae observed in this study may not have been *A. cantonensis*, however the great number of larvae that emerged from the slugs is in keeping with estimates of slugs harboring hundreds, thousands, or even tens to hundreds of thousands of *A. cantonensis* nematodes as reported in other studies. Given that some samples had positive qPCR results, we can be fairly certain that some of them were indeed *A. cantonensis* (Wallace & Rosen 1969; Kim et al. 2014).

Study 3a:

Filter study: preliminary study to determine ability of larvae to traverse filters

The first two studies confirmed the ability drowned slugs to shed larvae, some of which may be *A. cantonensis* larvae and can survive as free-living organisms for some time outside the intermediate gastropod host, and that the majority of the nematode larvae will be found at the bottom of the water column. The studies support the implications that rainwater catchment may harbor *A. cantonensis,* especially if the system is poorly designed or maintained and slugs are allowed to access and die in the tank. If the situation were to occur, the filtration system would then be critical to prevent passage of larvae into the household water supply. The Guidelines on Hawaii Rainwater Catchment Systems manual state a 20µm filter should be sufficient to prevent passage of the larvae if they could survive outside the gastropod host. A study by Ash (1970) examined the morphology of 35 third-stage *A. cantonensis* larvae and determined the mean width to be 26µm with a range of 23-34µm. Based on this information it was determined a preliminary

filter trial should be run, and this was the focus of Study 3 as well as continuing the effort to identify the larvae that emerge from drowned slugs.

Hypothesis 1: *A. cantonensis nematode larvae shed by drowned slugs will not be able to traverse a 20µm filter.*

Hypothesis 2: *Some of the larvae shed from drowned slugs are A. cantonensis larvae.*

Methods

Rainwater used in this study was collected in the same manner as described in the previous studies. The pH of the rainwater was recorded as well. Samples were examined on a daily basis. A 15mL sample was taken every day and placed into a 6cm petri dish and examined by microscopy with the Leica EZ4 dissecting scope used throughout the course of all of the studies. Each dish was labeled with the date the sample was taken, and the dishes were observed daily for the emergence of any larvae or organic life.

Slugs used in the study were first prepared for the trial filter study and four days later another grouping was prepared for similar trials. The first group consisted of two *P. martensi* (RLW1536, 1537) and two *L. alte* specimens (RLW1538, 1539), which were prepared in similar fashion to those in the ten and twenty day studies except after weighing all individuals were washed by placing in a 50mL Falcon tube filled with tap water. The tubes with the specimen were inverted for 3 minutes and the slugs were patted dry with a paper towel. The washing was done in an attempt to remove any exterior soil nematodes that may potentially have been on the slug's exterior. Tail snips were taken from all specimens for qPCR analysis for *A. cantonensis* in a manner consistent with those used throughout all studies described. In these filter studies all slugs used were chopped and 5mL samples were only drawn from the bottom of the 50mL

Falcon tube. Three samples were drawn each day and put into 5cm petri dishes with 10mL of rainwater. The 50mL Falcon tubes holding the slugs, and the petri dish samples, were kept in an incubator at room temperature. Rainwater was occasionally added to the petri dishes to prevent drying. On day three, the Falcon tubes containing the four slugs were inverted after the third sample was taken in an attempt to dislodge larvae in tissue and another sample was taken from each of the tubes.

Many sizes of larvae were seen from some of the samples and larval size was described as small, medium, large, and very large. On day twelve, a total of 600µL of liquid was taken from a petri dish with a large number of larvae from the day two sample from RLW 1539 and 300µL was put into each of two, 1.5mL Eppendorf tubes for qPCR. The tubes were centrifuged at 5000 x g for five minutes in an attempt to concentrate the larvae, however the larvae did not pelletize, and so the tubes were centrifuged at 10,000 x g for five minutes. There were still larvae in the supernatant, indicating that the larvae do not respond to centrifugation, and 100µL of fluid was drawn off and put into a new Eppendorf tube for digestion, extraction, and qPCR, using methods already described.

The second batch of slugs consisted of two *P. martensi* (RLW 1540, 1541) and two *L. alte* (RLW 1542, 1543). These specimens were prepared for a comparison of larvae exposed to acid and those that emerge from drowned slugs. The specimens were weighed and washed in tap water as described above and tail snips were taken for qPCR confirmation for *A. cantonensis.* The specimens were dissected longitudinally with the right half placed in a 0.5% Hall/pepsin mix and digested in an incubator set at 37° C and the contents run through a Baermann filter. The left half of the organism was chopped and placed in the rainwater that had been collected for the

study. Larvae were recovered from both the rainwater and acid and photographed for morphological comparison.

As this was a preliminary trial, and it was beyond the scope of funding allowed for the study to use the filters, pumps, pressure tanks or other apparatus found in household catchment systems, it was determined to use a 20-micron stainless steel sieve (Hogentogler & Co., 9591 Gerwig Lane, Columbia MD 21046), and 8-10 and 20-25µm Whatman filter paper (GE Healthcare Life Sciences Whatman, cat. No. 10312209 (8-10µm) and cat. No. 1004-055 (20-25µm)) to challenge the larvae. Larvae that emerged from the slugs used in this study were trialed on these different filters. The 20µm stainless sieve was placed into a 1000mL beaker with 150mL of rainwater to just cover the sieve. Larvae were carefully added to the top of the sieve and the beaker was covered with aluminum foil and left overnight. The sieve was carefully removed and the water in the beaker below the sieve was examined by microscopy for larvae. The larvae found below the sieve were transferred onto two, 8-10µm Whatman filters situated in glass funnels that were placed on vacuum flasks, and the water containing the larvae was carefully added. The filters were trimmed and placed into 1.5mL Eppendorf tubes for digestion with 600µL of buffer ATL (Qiagen) and 40µL of proteinase K was added to each tube. The samples were incubated at 39.9°, however the filters did not digest well and so the fluid was pipetted off and saved, and the filter remnants were removed from the Eppendorf tube and put into a new, 1.5mL tube with a bit of the filter paper protruding from the top so when the top was snapped in place it suspended the filter above the bottom of the tube. The two filters in the tubes were then centrifuged (Thermo Scientific Sorvall-Legend XTR) at 3600 RPM for three minutes to remove as much liquid as possible from the filters. The fluid from the Eppendorf tubes were then extracted in the manner as described in the previous studies and prepared for qPCR.

On the last day of the study all surviving larvae were challenged to a sieve trial. Two 20µm sieves were placed in 1000mL beakers filled with tap water to cover the top of the sieve and live larvae from RLW 1540, 1541, and 1543 were carefully added to the top of the sieve. The beakers were covered with foil and left to stand for four hours, after which the sieve was carefully removed and the liquid below the sieve was checked by microscopy for larvae. Larvae that migrated through sieve/filter material were held for observation to ascertain larvae vitality and longevity. Sieves were cleaned between uses by soaking in a 15% saltwater solution for 20 minutes, which we have shown will kill *A. cantonensis* larvae. The sieves were then washed with Dawn dishwashing soap, rinsed thoroughly, and dried in an incubator at ~50º C, after which they were placed in Clean Spot (Coy Laboratory Products, 14500 Coy Dr. Grass Lake, MI 49240) for 15 minutes on each side, and then into a UV crosslinker (UVP CX-2000, Upland, CA USA) at 2000 Jules to destroy any DNA on the sieves.

To test the ability of the larvae to traverse the 8-10 and 20-25µm Whatman filters, the filters were first trimmed and placed into separate 50mL Falcon tubes so the filter protruded slightly above the top of the tube. Rainwater was added to each tube so that the water just slightly covered the bottom of the filter. Larvae shed from the slugs were pipetted onto the top of each tube with a 5mL disposable pipette. The lids were fastened on to the Falcon tubes so that the filter paper was caught by the lid and kept the filter paper from falling into the tube. The tubes were left for two days and the contents after which the filters were removed and the contents of the Falcon tube were examined by microscopy for the presence of larvae.

Larvae that migrated through stainless sieve and paper filters were either held for observation or sacrificed for qPCR, as were all larvae remaining in petri dishes. Saved larvae were occasionally observed to test for larval longevity. Some larvae that were sacrificed for

qPCR were pipetted into 1.5mL Eppendorf tubes and centrifuged first at 5000 x g for five minutes and then 10,000 x g for five minutes (Thermo Scientific Sorvall-Legend XTR) and the supernatant was removed. The larvae were held in a -20º freezer until prepared for digestion, the DNA extracted and quantified, and analyzed by qPCR for presence in the manner described in study 1. A second method adopted later in the study to improve DNA yields was to pipette larvae into a 1.5mL Eppendorf tube, transfer the contents into a glass tissue grinder with 5mL tap water, and grind the contents for about five minutes. The solution was pipetted out of the tissue grinder and into a new Eppendorf tube, and the tissue grinder was rinsed with lysis buffer that was also pipetted into the tube. Larvae were held in a -20º freezer for later digestion, extraction, and qPCR analysis. The glass tissue grinder was washed with a 20% bleach solution.

Results

Rainwater

The rainwater used in the study had pH of 6.5. Daily rainwater samples (15mL) that were observed by microscopy showed no evidence of visible, organic life forms. Rainwater samples taken for qPCR were negative for *A. cantonensis.*

Laval drop

By day three, coiled and swimming larvae were observed emerging from all 5mL samples taken from day two from RLW 1538 and 1539. There were no larvae seen in any samples from day two for RLW 1536 and 1537, however samples drawn from these on day three yielded a few coiled larvae, as did samples from RLW 1538 and 1539. The tubes were inverted in an attempt to dislodge more larvae that might still be in the tissue and another sample taken. These samples yielded many coiled larvae from RLW 1538 and 1539, and a few were found in

samples from RLW 1536 and 1537. Samples taken from all specimens on day three had coiled larvae. Of the three 5mL samples taken from RLW 1539 on day two, an increase in larvae was progressively seen and by days seven and eight it was estimated that there were at least 1,000 larvae of various sizes in the petri dish. This was similar to what was witnessed in study 2, and again, the source of emergence was difficult to define but was assumed the larvae were lodged in bits of tissue. A daily sample of the rainwater was placed into a petri dish, examined by microscopy, and held for observations. No organic or inorganic matter was observed in the rainwater sample, and no larvae emergence was witnessed over time.

Larval morphology and vitality: acid versus water

All four of the slugs (RLW 1540, 1541, 1542, 1543) that were randomly collected for this trial had confirmed *A. cantonensis* infection (Table 1.4). Larvae were released from all of the longitudinally cut, half acid/ half water digested slugs.

Table 1.4. The qPCR results of specimens used in study 3, filter trials (100% infection in randomly selected slugs).

Species	RLW#	Tissue Weight (mg)	Location collected	qPCR result	Estimated # larvae/mg tissue (replicates)
P. martensi	1536	25.0/24.0	Koae	positive	2.68/1.51
P. martensi	1537	28.0/17.0	Koae	positive	1.40/0.91
L. alte	1538	23.0/33.0	Waa Waa	positive	0.0006/3.16
L. alte	1539	9.0/18.0	Waa Waa	positive	2.35/1.30
L. alte	1540	23.0/11.0	Waa Waa	positive	1.43/0.95
L. alte	1541	13.0/16.0	Waa Waa	positive	4.67/2.86
P. martensi	1542	20.0/14.0	Koae	positive	6.74/3.99
P. martensi	1543	22.0/18.0	Kale	positive	6.86/3.85

Of the three, 5mL samples that were drawn from the 50mL Falcon tube containing half a chopped slug in rainwater, all three samples from each of the tubes yielded larvae, some of which were photographed. Likewise, the sections from each of the four slugs that were acid digested also yielded larvae and some of these were photographed as well. Photographs taken of the water versus acid larvae showed both had similar features (Fig. 1.17a, b). The day two larvae

released by these slugs placed in water could best be described as glassy, very shiny and silvery, and very active. On day four there was a drop of coiled larvae, some of which could be seen emerging from the coiled state. The larvae resulting from an acid digestion survived for 24 hours but succumbed within 48 hours of digestion, however the larvae resulting from slugs that were placed in water were still very active 19 days later, and live larvae numbers were estimated at >1,000.

Fig 1.17 (a, b). Larvae from RLW 1542 water digest (a) and larvae from RLW 1542 acid digest (b) have similar structures with knob-like tips (KT) and a rod-like structure (RS) as described by Lv et al. (2009). The excretory pore (EP) and esophagus bulbous (EP) as well as the anus (A) are clearly seen in the larvae on the left (40x).

Larval qPCR results

The sample from RLW 1539 day two sample had generated approximately 1000 larvae by day eight, and on day twelve some liquid with larvae was taken from the petri dish and analyzed by qPCR for *A. cantonensis*; the results were positive. Larvae from the same sample were used in the sieve trial and held for observation for 30 days, at which time approximately 126 larvae were taken for qPCR and the results were a weak positive for *A. cantonensis*. These larvae were not ground in a glass tissue grinder prior to preparation for qPCR analysis. The remaining larvae that were pooled from the sieve test and harvested at 53 days had a strong positive qPCR result. These larvae were ground in a glass tissue grinder.

Filter trials

Microscopy revealed that the larvae that were visually smaller that had emerged from the RLW 1539 day two sample and placed on the 20µm stainless sieve overnight were able to migrate through the sieve but the large larvae were not. The second 20µm sieve that was tested had identical results when larvae were placed on it. The qPCR results of the 8-10 Whatman paper filter used to capture the larvae that had migrated through the sieve was positive for *A. cantonensis*. Of the larvae that were placed onto 8-10 and 20-25µm Whatman filter paper and left for 48 hours, a great number of both large and small larvae were found below the intact filters, indicating they were able to migrate through the paper barrier. The pooled, surviving larvae from RLW 1540, 1541, and 1543 that were transferred onto one of two, 20µm sieves again showed the smaller larvae were capable of migrating through the sieve (Fig. 1.18). When the sieves were carefully removed after four hours and liquid below was examined by microscopy, it was revealed that many larvae that had successfully traversed the sieve (Fig. 1.18). The use of tap water in the sieve trials did not appear to have an adverse effect on the larvae.

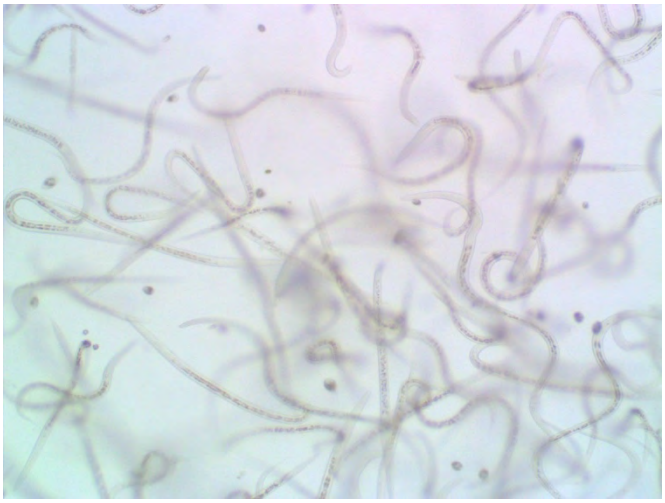

Fig. 1.18. Larvae found below sieve in water were able to survive for >50 days and qPCR results were positive for *A. cantonensis* (10x).

Larval longevity

The larvae were held in petri dishes in an unpowered incubator at room temperature. Rainwater was occasionally added to the petri dishes to prevent drying. When the dishes were brought out for observation the larvae were often initially sluggish and then appeared to become more active when exposed to light. At 53 days, live larvae from RLW 1540 1541, and 1543 that had traversed the sieve and had been held for observation were sacrificed. Approximately 270 larvae were ground in a tissue grinder and prepared for qPCR analysis as in all previous studies. The qPCR results were positive for *A. cantonensis.*

Discussion

Of the various larvae shed by the slugs, only the smaller larvae were able to pass through a 20µm stainless sieve, and those that did traverse the sieve were surprisingly long-lived. When these sieve larvae were collected for qPCR analysis, results were weak for larvae collected at 30 days and strong for larvae collected at 53 days. The use of a glass tissue grinder when preparing larvae for qPCR analysis may have been a factor in the strong positive qPCR result of the 53 day larvae. This study confirmed that some *A. cantonensis* larvae are able to pass through a 20µm stainless sieve and therefore the alternative hypothesis must be accepted. Larvae of all sizes, both large and small were able to migrate through the 8-10 and 20-25µm Whatman filter paper. Confirmation that some of the larvae shed by the drowned slugs are *A. cantonensis* was supported by the positive qPCR results from the filter paper containing larvae, and from individual and pooled larvae released from the dead slugs.

While there were instances of positive qPCR confirmation for *A. cantonensis* in this study there were still questions regarding the identity of the larvae that were highly varied in size that gradually emerge from the day one and two samples, versus those coiled larvae that are very clearly visible from samples taken on day three and four. Because larvae were able to traverse both the stainless metal sieve and the 8-10 and 20-25µm filters, it was important to re-confirm that emerged larvae and larvae that are able to migrate through the sieve are *A. cantonensis*.

Study 3b

Methods

Seven *Laevicaulis alte* were collected from the Waa Waa area in the Puna District, an area of known *A. cantonensis* infection (RLW 1568, 1569, 1570, 1571, 1572, 1573, 1574). The slugs were weighed, washed in tap water, blotted dry, and tail snips were taken for qPCR, and then chopped and placed into 50mL Falcon tubes filled with rainwater. Three 5mL samples were taken from the bottom of each tube at 24, 48, and 72 hours after the slugs were immersed in water and samples were held for observation for larval emergence. Larvae that emerged from the drowned slugs were challenged to traverse the 20µm stainless sieve, however this time the sieve was placed in a 1000mL beaker filled with deionized water just to cover the screen. The sieve was carefully removed after one hour and the water in the beaker was transferred to three 50mL Falcon tubes for observation by microscopy. Larvae that successfully traversed the sieve were held for observation. Fifty-six days after emergence from the slugs 413 larvae that traversed the sieve were first transferred by pipette into a 1.5mL Eppendorf tube and then into a glass tissue grinder with 100µL of lysis buffer and ground for five minutes. The contents were

then digested, extracted, and analyzed by qPCR using protocols previously described in all earlier studies.

To gain a better understanding of where larvae may be located in the slug, two whole *L. alte* were dissected into anterior, middle, and posterior sections and subjected to digestion by HCl/pepsin and Baermann filtering as described previously to observe for presence of nematode larvae. The two slugs were cut into three and four sections respectively (tail, mid, head; tail, mid-tail, mid-head, head). Attempts were made to section the slug as evenly as possible. No tail snips were taken from these two individuals. Any larvae resulting from the acid digest were considered L3 larvae, and these were challenged to the 20µm sieve, which was placed into a 1000mL beaker with rainwater just to cover top of sieve. One hundred of the acid-digested larvae were added to the top of the sieve and left for 24 hours, after which the water under the sieve was examined for presence of larvae. The sieve was then inverted in another 1000mL beaker and rinsed with deionized water to rinse any larvae that may not have been able to traverse the sieve. This water was then observed by microscopy for presence of larvae.

Results

Larval presence; qPCR and microscopy (sieve trial)

Of the seven *L. alte* used for the sieve trial in this sub-study, five showed *A. cantonensis* infection with qPCR analysis, two of which were strong positives, one was a weaker positive, two were low positives, and two were negative. Within 24 hours of the slug drowning, many coiled and swimming larvae were observed by microscopy from RLW 1568, and 72 hours after drowning larvae were observed in the 5mL samples from RLW 1568, 1570, and 1572, with the heaviest infection showing in RLW 1568, which corresponded to the qPCR results from the tail

tissue taken from these specimens. On day seven the 5mL samples taken at 24 hours of slug drowning and held for observation in petri dishes showed considerable larval presence from RLW 1568, 1569, 1571, and 1574. Of these four *L. alte,* the qPCR results were negative for RLW 1569 and 1574, and a low positive for 1571, and yet the samples taken from these slugs within the first 24 hours produced copious numbers of larvae (Table 1.5).

Table 1.5: qPCR results for *L. alte* used in study 3b to confirm results of Study 3, sieve trail. Slugs which yielded large quantities of larvae observed by microscopy are labeled high positive, and those which yield few are labeled low positive. Results by microscopy and qPCR do not always correlate, which could be due to tissue sampling location.

Species	**RLW#**	**Tissue Weight (mg)**	**Location collected**	**Visual result**	**QPCR result**	**Estimated # larvae/mg tissue (replicates)**
l. Alte	1568	19/11	Waa	Positive (high)	Positive	2.06/1.88
l. Alte	1569	8/5	Waa Waa	Positive (high)	Negative	0/0
l. Alte	1570	22/12	Waa Waa	Positive (low)	Positive	1.82/1.70
l. Alte	1571	10/7	Waa Waa	Positive	Low Positive	0.002/0.002
l. Alte	1572	19/20	Waa Waa	Positive (low)	Positive	0.32/0.24
l. Alte	1573	26/23	Waa Waa	Negative	Low Positive	0.001/0.001
l. Alte	1574	8/16	Waa Waa	Positive (high)	Negative	0/0

Sieve trials (water)

Larvae collected from RLW 1568, 1569, 1571, and 1574 that were transferred to the top of a 20µm stainless sieve were able to traverse the barrier within one hour. Deionized water had no noticeable negative effect on larvae.

Larval vitality (sieve trial)

The qPCR analysis of the 56 day old larvae was positive for *A. cantonensis.* These results were similar to that of the 53 day old larvae, which were also confirmed positive for *A. cantonensis* (Table 1.6).

Table 1.6. The qPCR results for *A. cantonensis* larvae tested for longevity at 12, 53, and 56 days outside the slug host.

Species	RLW # origin	Longevity	qPCR result	Estimated # larvae/mg sample (replicates)
A. cantonensis	1539	12 days	Positive	0.281/0.128
A. cantonensis	1540, 1541, 1543	53 days	Positive	0.041/0.023
A. cantonensis	1568, 1569, 1571, 1574	56 days	Positive	0/0.113
A. cantonensis	1568, 1569, 1571, 1574	56 days	Positive	0.092/0.078

Larval presence (acid digest)

None of the sections (head, mid, tail) of the first individual yielded larvae, which could either indicate no infection, or if there was infection, the larval stages were younger L1 or L2 and were destroyed by the acid. The tail, mid-tail, and head sections of the second yielded larvae that were both coiled and actively swimming.

Sieve trial (acid digest larvae)

After an elapse of 24 hours, there was no evidence of larvae that had been exposed to an acid digest in the water under the 20µm sieve. After the sieve was turned over and rinsed with deionized water, approximately 20 larvae, both coiled and open, were found in the rinse water.

Discussion

The results of study 3a and 3b confirmed hypothesis two; a drowned slug *P. martensi* can shed *A. cantonensis* larvae. However, that qPCR results for some of the larvae found below the sieve were positive for *A. cantonensis* indicate that the alternative for hypothesis one should be accepted; some *A. cantonensis* larvae can pass through a 20µm sieve. The study also showed that larvae that can traverse a 20µm stainless sieve can survive outside the intermediate host for more than 50 days. The qPCR results for the larvae that were able to survive the

0.5%HCl/pepsin acid indicate they are probably infective third stage *A. cantonensis* larvae and trials indicate that these larvae are unable to traverse a 20µm sieve. The smaller larvae that can traverse the barrier might then be first stage larvae as qPCR results are for these larvae are positive for *A. cantonensis*. Another study should be done to confirm this. The use of deionized water in this study confirmed deionized water has no noticeable effect on viability of larvae.

The glass tissue grinder improved DNA yield for analysis of the larvae, however qPCR results for the larvae were not always consistent with the qPCR results for the slug tail tissue. This may suggest that either the larvae that emerge in great quantities from some slugs, such as RLW 1530 in Study 2, are not *A. cantonensis*, or the sampling location for tissue collection was not optimal. The sectioning and acid digestion of two *L. alte* confirmed that the head section can also contain third stage larvae but it did not help in identifying where the first or second stage larvae might be sequestered. If these larval stages are not in the tail tissue, then they would be missed by the qPCR analysis. A recent rat lungworm study in Florida found that to decrease the number of false qPCR negatives, multiple tissue samples must be extracted from muscle tissue. The study reports that of 18 positive snails collected, only five were positive in both extractions. The authors concluded that additional research is needed to find the optimal sampling location when testing for *A. cantonensis* (Iwanowicz et al. 2015).

This study verified that there can be a release of larvae within the first 48 hours of drowning but the larvae are not initially visible by microscopy with a dissecting microscope. However, the larvae are present and will begin to emerge and become visible in the sample, reaching maximum presence within 144-168 hours of slug death. The study also verified the clearly visible drop of coiled larvae within 72-96 hours of slug death. Morphological, the larvae

released in this and previous studies are similar in appearance and many displayed the coiling and Q-shaped movement described as specific to *A. cantonensis* (Lv et al. 2009).

Study 4: Validation of larvae shed as first versus third stage larvae (21-day study)

The previous studies provided supporting evidence that viable larvae could be shed from both whole and damaged slugs, and larvae appeared at different times after the slug's death. Some larvae will be shed within 24-48 hours of drowning, and these may not be visible initially if using a dissecting microscope but will emerge as visible, swimming larvae of various sizes with maximum emergence at around 144-168 hours after slug mortality. Other larvae will be shed generally 72-96 hours after mortality and are visible either as coiled larvae or as swimming larvae that are similar in size. The majority of the larvae that emerge under either of these scenarios display vigorous movement, swimming in a snake-like manner and coiling and uncoiling in the Q-shape that has been described as definitive for *A. cantonensis*. Larvae that have maximum emergence at 144-168 hours after mortality have been observed to traverse a 20µm stainless sieve as well as 8-10 and 10-20µm filter paper, and some of these larvae survived at least 56 days and tested positive for *A. cantonensis* by qPCR. Larvae that survived 0.5% HCL/pepsin were not able to pass through the 20µm stainless sieve. This compilation of evidence led me to question if the coiled larvae are the third stage larvae and the larvae than can traverse the 20µm stainless steel sieve are first stage larvae. Generally, a rat study would be done to determine if the larvae were infective stage *A. cantonensis*, however, the funding needed for a rat trial is substantial and beyond the scope of this study. Therefore, it was determined to verify whether larvae were infective stage larvae by subjecting them to an acid mixture of 0.5% HCl/pepsin.

Hypothesis 1*: The larvae that emerge within the first 24-48 hours after slug mortality are first stage A. cantonensis larvae that can pass through a 20µm stainless steel sieve and will not survive acid, and the larvae that emerge 72-96 hours after slug mortality are third stage larvae that will not pass through a 20µm stainless steel sieve and will survive acid.*

Additionally, I want to verify that a whole *P. martensi* is able to shed nematode larvae. In previous studies tail snips were always taken to verify infection of the slug by qPCR, and while the slug was generally intact, it was certainly damaged. Can entirely whole *P. martensi* shed *A. cantonensis* larvae?

Hypothesis 2: *Whole drowned P. martensi will shed A. cantonensis larvae.*

A final question to be answered is if there is any noticeable effect of municipal city tap water versus rainwater on the vitality of nematode larvae shed from a drowned slug?

Hypothesis 3: *Tap water will have an adverse effect on the larvae shed from a drowned slug.*

Through the course of this study I will also attempt to determine the smallest number of larvae needed to achieve a positive qPCR reaction

.

Methods

The study was conducted in a 21-day time period during which a number of trials will be conducted and observations will be recorded. In all earlier studies the rainwater was collected off of a clean roof with no gutters and the rainwater was checked daily for any signs of organic life by microscopy. Additionally, the rainwater was tested for *A. cantonensis* by qPCR and results were negative. For this study, in an effort to avoid any possible contamination issues, rainwater was collected in a glass, half-gallon Mason jar with a funnel placed in the mouth and set in an open area and filled directly from falling rain. Rainwater was checked by microscopy

for any evidence of larvae. All larval counts were done using a laboratory counter (Clay Adams, Division of Becton, Dickenson, & Co., Parsippany, N.J. 07054).

Sixteen *P. martensi* were collected from the same location in Koae where previous collections in the earlier studies had been done and where *A. cantonensis* infection is high. All slugs were assigned an RLW number, the weight recorded, and the slugs were washed in deionized water. Ten of the slugs (RLW 1713-1722) would have tissue samples taken from the tail for qPCR and for a tissue squash. Tissue squashes were done to determine if the method is effective for determining RLW infection. Five of the slugs were placed in a 50mL Falcon tube filled with rainwater (pH 6.11) and five were placed in similar tubes filled with municipal tap water (pH 9.05). Tissue squashes consisted of a small (5-10 mg) piece of tissue cut from the tail section of the slug, which was then squashed between two glass microscope slides and the tissue was observed under a compound microscope (10x and 40x). Other than the tissue taken for qPCR and tissue squashes the slugs were left whole. Six other *P. martensi* were left entirely whole, three were placed in 50mL Falcon tubes filled with rain water and three were placed in tubes filled with municipal tap water. These slugs were labeled #1 whole through # 6 whole.

Three 5mL samples were taken from the ten slugs (RLW 1713-1722) within an hour after the demise of the slug, and additional samples were taken 24, 48, 72, and 96 hours after the slug had drowned. Rainwater (15mL) or tap water (15mL) was assigned and added to each 5mL sample to cover the dish and prevent drying of the sample. The number of samples taken at 72 and 96 hours was reduced to two 5mL samples to aid with time constraints and limited space. Previous studies showed the first two 15mL samples taken contained the greatest number of larvae. No samples were taken after 96 hours as earlier studies have shown that optimal larval shed occurs within the first 96 hours of a *P. martensi* drowning. All *P. martensi* used in the

study were disposed of after 96 hours and only the larvae were held for observation over the remaining 17 days of the study. The slides were viewed under a compound microscope and photographs taken of items of interest.

To quantify how many larvae were needed to get a positive qPCR reaction larvae were sorted to size (large, medium, and small) and were harvested in batches of 10, 25, 50, 75, and 100. All larvae were ground for five minutes in a glass tissue grinder with the water they were suspended in (~200µL) after which they were transferred to 0.5mL Eppendorf tubes and 100µL of lysis buffer added to each. The tubes were stored in a -20º freezer until ready for digestion, extraction, and qPCR.

Acid trials were conducted on live larvae up until the end of the 21-day study Some of the shed larvae that appear from samples taken within the first 48 hours (days zero, one, and two), and larvae that appear as coiled larvae and emerge 72-96 hours (days three and four) after drowning were exposed to a 0.5%HCl/pepsin mixture to mimic the stomach acid of a rat. The time that larvae are able to survive acid emersion, displayed by active swimming, was recorded. These acid tests were conducted from day three through day 21 of the study. Any larvae that survived the acid were considered infective third stage larvae and were analyzed by qPCR to determine if they were *A. cantonensis*. Those that did not survive were considered to potentially be first stage *A. cantonensis* larvae, which would be determined by qPCR.

On day three the samples from RLW 1721 and 1717 yielded many coiled and some slow moving larvae. Some of the larvae, most of which were coiled and a few slow moving, were removed by pipette from each of the sample and transferred to an individual 1.5 mL Eppendorf tube with ~400µL of tap water in the bottom. When the larvae had been collected in the tubes a 200µL sample was transferred from each tube to individual petri dishes marked with the RLW

number and the liquid was checked by microscopy to affirm it contained larvae and the state of the larvae (mostly motionless). Acid (15mL of 0.5%HCL/pepsin) was added to each dish and the time the acid was added was noted. The effect of acid on the larvae was observed with a dissecting microscope one and two hours after the acid was added.

A day two sample from whole slug #1 yielded a large number of larvae and about 50 were removed for a similar trial by acid. A similar situation occurred on day three with whole slug #1 and #5 when large numbers of coiled larvae were observed in both samples from #1 (399 and 231 respectively), some of which were transferred in the manner previously described to the acid mixture. Photographs were taken of larvae from whole slug #1 that were in acid on a slide with a micrometer for measurements. Approximately 50 of the larvae that had survived acid for 4.5 hours were transferred into a 1.5 mL Eppendorf tube with ~400μL tap water and then pipetted into a tissue grinder and ground for five minutes, then put into 100 μL lysis buffer and prepared for qPCR. Approximately 50 larvae from the same slug (#1 whole) that were not subjected to acid were also prepared for qPCR in the same manner.

On day six days a sample taken on day one from RLW 1713 still contained about 145 swimming worms of all sizes. Twelve very large larvae were pipetted out, ground, and prepared for qPCR. Larvae were also pipetted out and trialed with acid as described above. A similar test was prepared for four larvae that emerged from a day two sample from RLW 1716. A sample from RLW 1718 day two had a total of 12 larvae, two of which were swimming and ten of which were coiled. Acid (10mL) was added to the petri dish and the larvae response was observed.

On days seven and eight one of the samples from whole slug # 5 day two contained >150 coiled and moving larvae. Larvae (6 swimming, 13 coiled) were collected from this sample and 15 mL of acid was added to the petri dish and the dish was observed by microscopy to see the

larval reaction to the acid. The day one samples from RLW 1722 contained about 62 larvae, most of which were not moving. Acid was added (10mL) to the dish and the dish was observed for larval reaction to the acid.

On day eleven the day three sample taken from whole slug # 1 contained twelve swimming and many motionless coiled larvae. Twenty coiled larvae were pipetted from the dish and put into 15mL acid, the time was recorded, and the dish was observed to determine the effect of acid on the condition of the larvae.

Seventeen days into the study, larvae from RLW 1719 day one were removed for both quantification of optimal larval numbers to use for qPCR analysis and for an acid trial. One group of 25 larvae and another group of 50 larvae were removed by pipette and prepared for qPCR. Another 30-50 larvae from the same sample were removed and placed into 15mL of acid, the time recorded, and observations were made on the larval reaction to the acid. An additional acid test was run on a sample from #1 whole, day three, in which three or four coiled larvae were observed. Acid (20mL) was added to the dish and emerged larvae were removed by pipette, combined with 11 larvae from another #1 sample taken on the same day, and all larvae were prepared for qPCR analysis.

Eighteen days into the study a day three sample from RLW 1717 still contained many larvae and 40 of these larvae were removed for grinding and prepared for qPCR. Two samples were taken from RLW 1719 day one for qPCR analysis, one for quantification of optimal larval numbers needed for qPCR and the other for an acid test. One hundred active larvae were removed and ground and prepared for qPCR and after the larvae were removed 10mL of acid were added to the dish and observations were made to determine the effect of the acid on the

larvae. Twenty-three minutes after the acid was added 42 swimming larvae were removed, ground, and prepared for qPCR.

Similar tests were carried out the following day (day 19). A day two sample from RLW 1719 contained one swimming larva, one moving larva, and a number of motionless larvae. Acid was added (10 mL) and its effect on the larvae was observed. After 30 minutes 22 actively swimming or moving larvae were taken for qPCR. A day two sample from RLW 1718 was observed to have only one active larva. Acid (10mL) was added and observed at ten and 45 minutes, at which time 14 active larvae were removed for qPCR.

On the final day of this 21-day study, a sample from #5 (whole slug) day two was observed by to contain two swimming, one moving, and 88 motionless (not coiled) larvae. Acid (10mL) was added to the dish, the time was recorded, and the larvae were observed by microscopy to determine the effect of the acid on the larvae. Thirty-five minutes after acid was added to the sample, 80 active larvae were removed by pipette, ground, and prepared for qPCR.

Sieve trial/acid trial

On day nineteen of this study a sieve trial was conducted with the many (>1000) remaining larvae from #2, #4, RLW1717, and RLW 1722. All larvae were combined into one petri dish and 20 mL of the liquid with larvae was pipetted onto the top of a 20μm stainless sieve set into a 1000 mL beaker filled with tap water just to cover the sieve material. The beaker was covered with foil and left for 48 hours in the dark at room temperature, after which the beaker was removed and the liquid below the sieve was transferred to a petri dish and observed by microscopy for evidence of larvae. Sixty-seven active worms were pipetted from the dish and transferred to a 1.5 mL Eppendorf tube. The contents were then transferred to a glass tissue

grinder and ground for about five minutes. Lysis buffer (100µL) was added to the grinder and the solution was transferred by pipette into a 0.5mL Eppendorf tube and stored in a -20º freezer until digestion, extraction, and qPCR analysis in keeping with all other studies could be done. An acid trial was performed on the remaining larvae that were able to traverse the sieve. Some of the larvae were pipetted into a small drop of water in a petri dish and 5mL of 0.5%HCl/pepsin acid was added to the drop of water and the effect of the acid on the larvae was observed by microscopy with a dissecting microscope (Lesica EZ4). All of the remaining larvae from the combined batch of larvae were then trialed by adding acid (10 mL) to the dish and the effect of the acid on the larvae was observed.

Frozen larvae/acid test

Larvae (6 swimming and 29 coiled) were removed by pipette from the petri dish holding a day four sample from RLW 1721 and transferred into a 1.5 mL Eppendorf tube with ~200µm of water, and placed into a freezer set at a temperature (-18º C; similar to that of a normal household freezer). After 25.5 hours the tube was removed from the freezer and thawed and the contents were pipetted into a dish and observed by microscopy with a dissecting microscope (Lesica EZ4). Acid (5mL) was added to the larvae and they were observed for signs of motion.

Quantification of optimal larval numbers for qPCR results

Larvae were sorted as to large, medium, and small, and one sample of ten extra-large larvae was also collected. The larvae from each sample were ground and prepared for qPCR in the manner described throughout the studies. Two samples containing ten larvae each, and one sample containing 30 larvae were prepared from #6 whole to determine what quantity of larvae were necessary to achieve positive qPCR results. Two samples were taken from RLW 1719 day one, one containing 25 larvae and one containing 50 larvae, and a third sample of 30 larvae was

also taken. Two samples of 25 and 50 larvae were taken from RLW 1720 day one. Twelve larvae, combined from #1 and #5 whole slugs that had survived acid for two hours were tested, and one very large larvae from #2 (day two) was also tested. The very large larva was also photographed with a micrometer to estimate size.

Results

Water samples

No larvae or any other motile organisms were observed in the rainwater samples through the duration of the study. Water samples were checked by microscopy on a daily basis. No effect on larval presence or vitality was noticed between tap water and rainwater and, in fact, higher numbers were observed in samples from slugs immersed in tap water.

Tissue squashes and qPCR results

Analysis of the tail tissue by qPCR confirmed *A. cantonensis* infection in all ten of the *P. martensi* used in this study (Table 1.7).

Table 1.7: qPCR results of slugs used in Study 5; 100% infection in all *P. martensi* collected from Koae.

Species	**RLW#**	**Water type**	**Mollusk weight (g)**	**Tissue weight (mg)**	**Location collected**	**qPCR result**	**Estimated # larvae/mg tissue (replicates)**
P. martensi	1713	rain	1.31	0.8/0.8	Koae	Positive	1.734/1.753
P. martensi	1714	rain	1.27	19.0/22.0	Koae	Positive	2.154/2.13
P. martensi	1715	rain	1.80	14.0/14.0	Koae	Positive	2.318/1.553
P. martensi	1716	rain	1.54	18.0/16.0	Koae	Positive	4.149/3.858
P. martensi	1717	rain	1.96	0.7/0.8	Koae	Positive	5.949/5.916
P. martensi	1718	tap	1.15	18.0/18.0	Koae	Positive	7.152/7.543
P. martensi	1719	tap	0.96	12.0/12.0	Koae	Positive	1.656/1.908
P. martensi	1720	tap	2.68	1.0/0.5	Koae	Positive	0.923/0.919
P. martensi	1721	tap	1.98	15.0/11.0	Koae	Positive	12.111/10.68
P. martensi	1722	tap	0.96	0.9/0.9	Koae	Positive	14.126/12.31

All tissue squashes from the 10 *P. martensi* that had tail snips taken had evidence of nematode larvae. Larvae were both coiled and C-shaped, and sizes seemed to vary (Fig 1.19). Some of the larvae had a clear distinction between the esophagus-intestine line, due to heterogeneous distribution of refractive granules, and were identified as late first stage and second stage larvae (Fig. 1.20). Three of the slugs showed heavy infection by tissue squash (RLW 1715, 1721, 1722) and two of these correlated to qPCR results (RLW 1721, 1722).

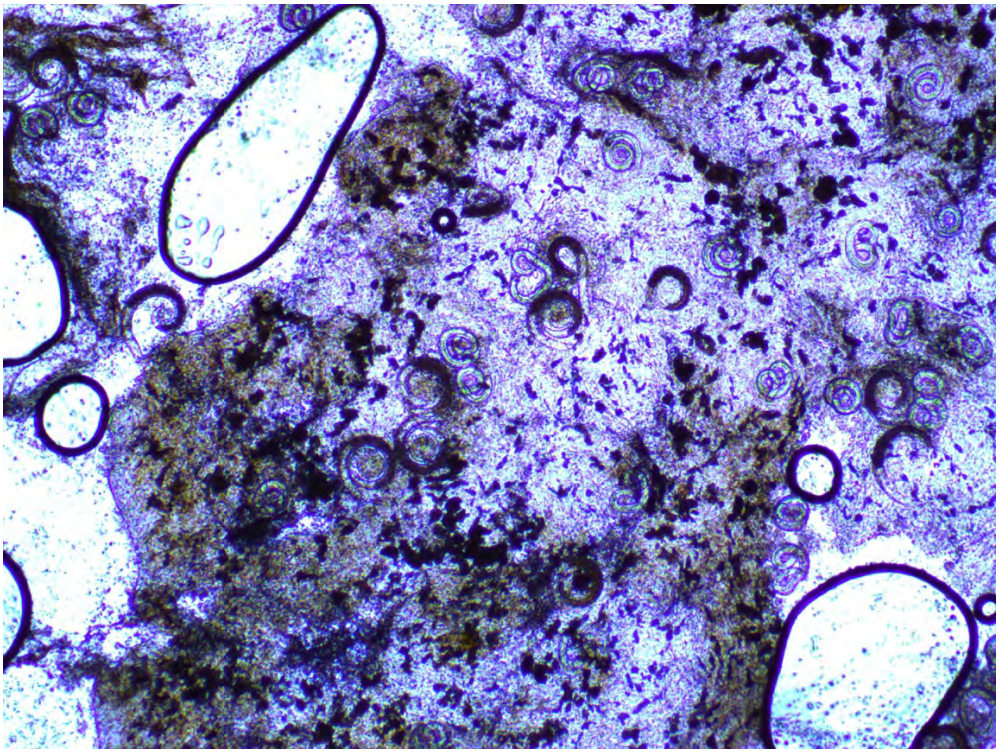

Fig. 1.19. Tissue squash from RLW 1722 showing the size variation and density of larvae (4x).

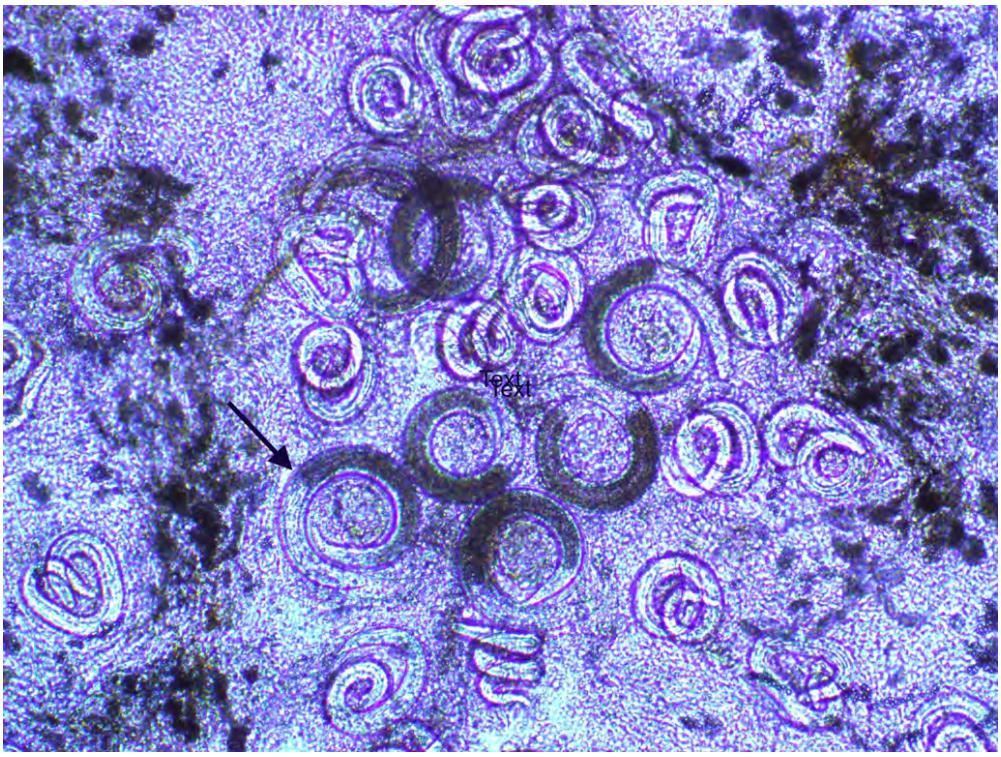

Fig. 1.20. Tissue squash from RLW 1722 (10x). The division (arrow) between the esophagus (clear) and the darker intestine is apparent as is size variation between larval stages (10x).

Larval drop from ten P. martensi

No larvae were observed in the water samples taken on day zero but were observed in samples taken at 24, 48, 72, and 96 hours (Table 1.8). All ten of the *P. martensi* that had tail snips taken had larvae present in the samples taken on day one and were observed as swimming, coiled, and C-shaped. Samples taken on day two showed larval presence from nine of the *P. martensi*. Two of the nine had low numbers of larvae and five had high numbers of larvae, most larvae were swimming but some were coiled. Of the samples taken on day three, larval presence was observed from six of the *P. martensi,* the majority of the larvae were coiled, some were moving, and only a very few were swimming or C-shaped. The larvae numbers in samples taken on day four were lower, however larvae were still found in eight of the ten *P. martensi,* almost all of which were coiled or C-shaped and only a very few were swimming. Larvae shed over the

four days were used for acid and qPCR trials. Increases and decreases in larval counts from samples taken on day one and day two were observed by microscopy (Fig. 1.21, 1.22).

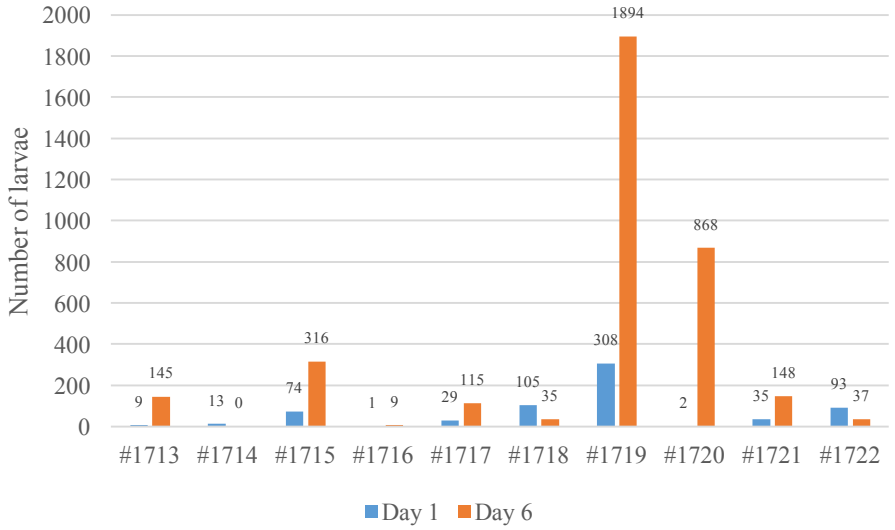

Fig.1.21. Changes in larval numbers observed from day one to day six from samples taken on day 1 from ten *P. martensi.* Increases were seen in samples taken from seven of the ten slugs. Larvae from sample RLW 1719 taken on day one that spiked on day six contained larvae of all sizes, small, medium, large, and very large.

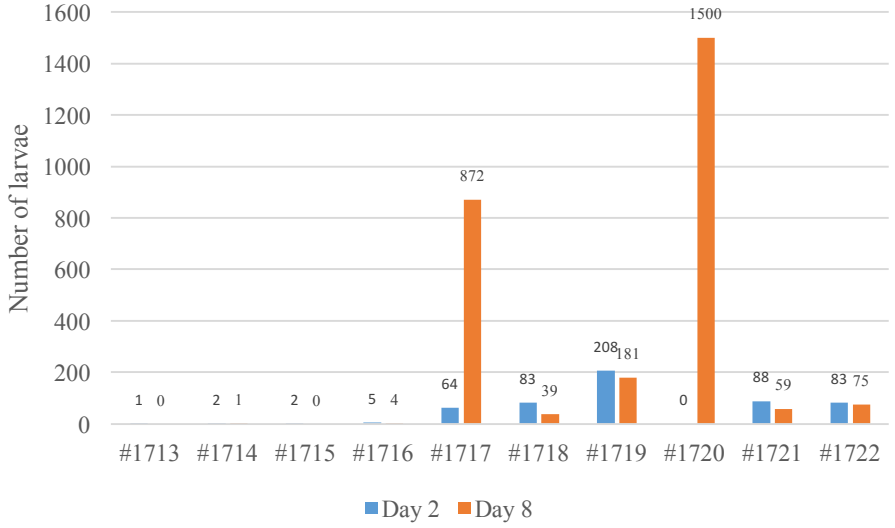

Fig. 1.22. Changes in larval numbers observed from day two to day eight from samples taken on day two from ten *P. martensi.* Increases were seen in samples taken from two of the ten slugs.

Larval shed from whole P. martensi

No sample was taken from the whole *P. martensi* on day zero. Samples from five of the six, whole slugs had larval presence on day one. Three of slugs were in tap water, and two of these (#5 and #6) had high larval presence (91 and 217 respectively). Two of the slugs in rainwater had larvae present (#1 and #2), one low and one high (14 and 179 respectively). When drawing the first sample from the bottom of the 50mL Falcon tube containing whole slug #1, a large, very clear and mucilaginous blob of slime-like matter was sucked into the pipette. While I had encountered slime-like matter before, particularly in the tubes with drowned *L. alte,* the material was always much darker in color and only encountered a number of days after the slugs' demise, when decomposition has set in. I elected to keep this clear, slime-like sample and placed it into a petri dish and examined it under the dissecting microscope and discovered six, swimming larvae in the material. Of all of the larvae seen in samples from all whole slugs taken on day one, most were swimming.

Samples from whole slugs # 1, 2, and 5 had high larval counts on day two (472, 345, and 477 respectively). Lower numbers were found in samples from # 6 (70) and very low or none were found in #3 and 4 (1 and 0 respectively). The slime-like sample showed eight swimming larvae when observed on day two. On day three samples from #1 had high numbers of larvae in both of the samples taken (245 and 146 respectively) the majority of which were coiled and motionless (382) and only a very few were swimming (9). The slime-like sample contained 328 larvae of all sizes five days after it was first collected.

Observation by microscopy showed the change in the number of larvae from samples taken from the whole *P. martensi* on day one over time, and it reflected what was seen in earlier studies where larvae numbers often continued to dramatically increase in some samples whereas

there was a decrease in others. In this case there was an increase in 50% of samples taken on day one from the whole *P. martensi* (Fig. 1.23). Samples taken on day two only showed a substantial increase in samples from one *P. martensi,* a small increase in a second, and a decrease in the other four *P. martensi* (Fig.1. 24).

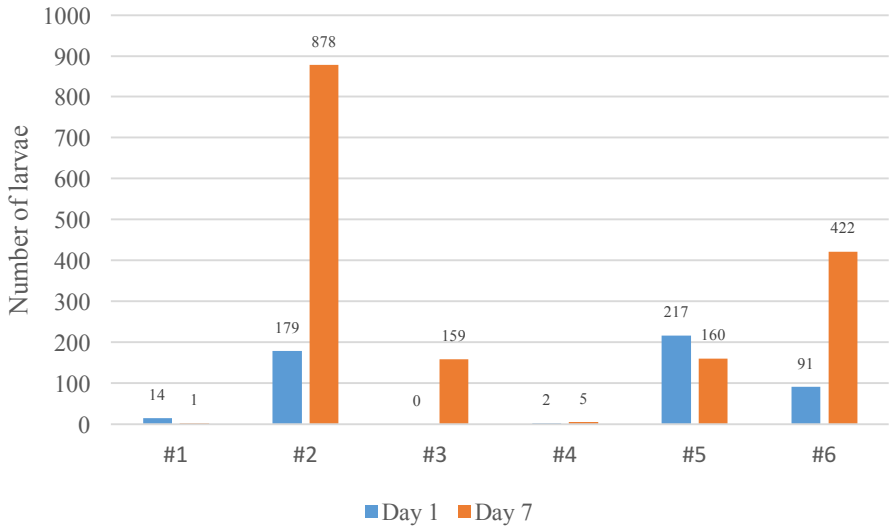

Fig. 1.23. Changes in larval counts over time. By day 7 larval numbers had increased in 50% of samples taken from whole *P. martensi* on day 1.

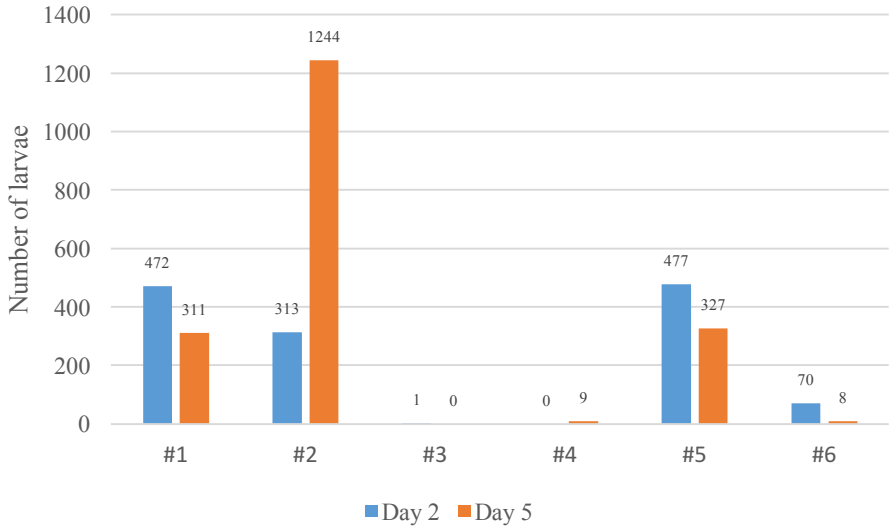

Fig. 1.24. Observation by microscopy show changes in larvae numbers in samples taken on day 2 from whole *P. martensi.* Samples from #2 show a marked increase, there was a slight increase in #4, and a decrease in larval numbers from the other four slugs.

Larval trials

Three days after slug death

On day three the samples from RLW 1721 and 1717 yielded many coiled and some slow moving larvae some of which were removed for an acid test. When acid was added there was an increase in movement with coiled larvae beginning to uncoil and moving larvae becoming actively swimming larvae. All larvae had uncoiled and were actively swimming after one hour, and the same larval condition was observed at two hours after acid was added, showing that these larvae would most likely survive the gut of a rat or vertebrate organism.

The larvae removed from a day two sample taken from whole slug #1 were trialed by acid and larvae were observed transitioning from tightly coiled to vigorously swimming over a one-hour observation period. A similar observation was made with larvae recovered from a day three sample from whole slug #1 and #5. Again, larvae from both slugs were observed to go from tightly coiled to actively coiling/uncoiling and then swimming. Larvae were observed for 4.5 hours and activity was vigorous. Photographs that were taken of larvae from whole slug #1 that were in acid were consistent with Ash's measurements for third-stage, *A. cantonensis* larvae (Ash, 1970) (Fig. 1.25). The qPCR results of the approximately 50 larvae that survived acid for 4.5 hours were positive for *A. cantonensis* as were the qPCR results of the approximately 50 larvae from the same slug (#1 whole) that were not subjected to acid. The replicate values of the samples from the larvae that had survived acid were higher (1.798/2.097) than the replicate values of samples from the same slug but were retained in water (0.783/0.792) and not subjected to acid.

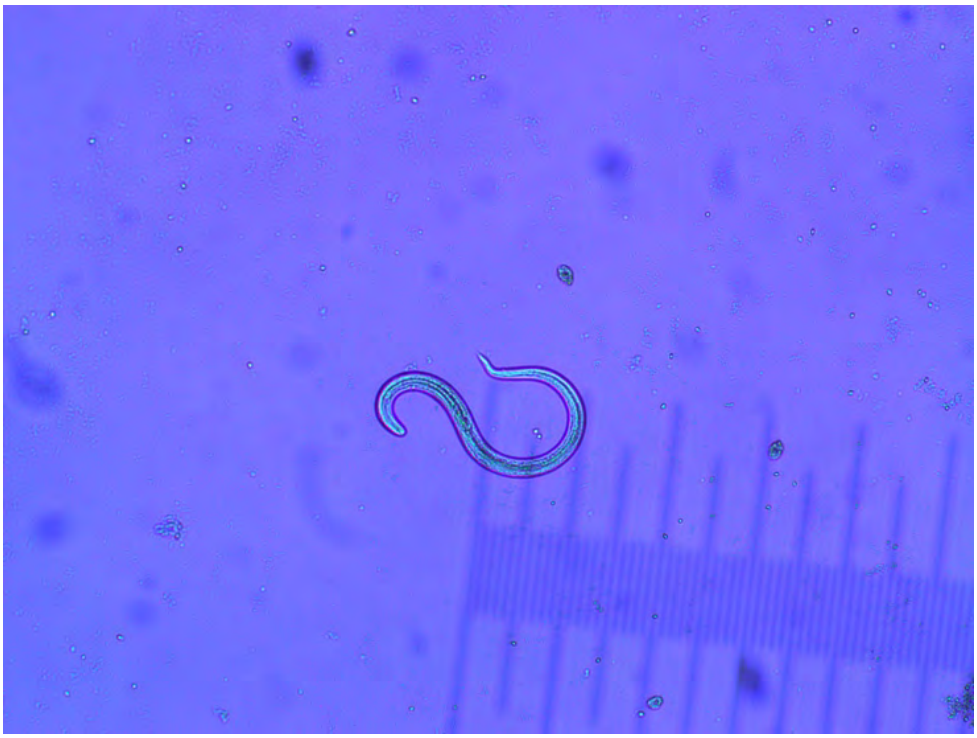

Fig. 1.25. Larvae from a day 3 sample from #1 whole *P. martensi,* subjected to 0.5% HCL/pepsin acid mixture and photographed. Each small tic mark equals 10µm. This larva fits the description of a third stage larva as described by Ash (1970). (Photo at 10x, 0.01mm micrometer).

Six days after slug death

One of the petri dishes with a sample taken on day one from RLW 1713 still contained about 145 swimming worms of all sized six days after the sample was taken. Twelve very large larvae were analyzed by qPCR and results were negative for *A. cantonensis.* Similar sized larvae were trialed with acid and all larvae were destroyed and dissolved. Four larvae that emerged from a day two sample from RLW 1716 succumbed in a similar fashion almost immediately when exposed to acid. The 12 larvae (two swimming and ten coiled) from the day two sample from RLW 1713 began to uncoil and emerge when 10mL of acid were added to the petri dish.

Seven and eight days after slug death

Larvae (6 swimming, 13 coiled) from whole slug # 5 day two subjected to an acid test. Less than five minutes after adding 15 mL of acid to the petri dish 16 larvae were actively

moving or swimming and three remained coiled; after 2.5 hours, eight swimming larvae were observed. Acid was added (10mL) to the day one samples from RLW 1722 that contained about 62 larvae, most of which were not moving. Within several minutes about 75 larvae were observed to be actively swimming, and one hour after adding acid 31 moving/swimming larvae remained.

Eleven days after slug death

Observations on the twenty coiled larvae that were pipetted from the day three sample taken from whole slug # 1 and put into 15mL acid showed seven larvae to be actively swimming. in less than thirty minutes. The number of swimming larvae increased to a maximum of 11 very active worms and then began to decline, and after two hours and fifteen minutes had decreased to two swimming larvae.

Seventeen days after slug death

The qPCR results for the 25 larvae and 50 larvae batches taken from RLW 1719 day one to quantify optimal numbers of larvae to use for qPCR were both positive for *A. cantonensis*. When 30-50 larvae were removed from the same sample and were placed into 15mL of acid, all but two disappeared In less than one minute, and within three minutes all were dissolved. An acid test was conducted on a sample from #1 whole (day three) that had three or four coiled larvae. Thirty minutes after 20mL of acid was added to the dish, 25 swimming larvae were seen. These larvae were removed from the acid and combined with another sample from # 1 taken on the same day, and were the qPCR results were positive for *A. cantonensis*.

Eighteen and nineteen days after slug death

The qPCR results for the 40 larvae removed from a day three sample from RLW 1717 for were positive for *A. cantonensis.* Two samples were taken from RLW 1719 day one for qPCR

analysis, one consisted of 100 larvae taken for quantification of optimal larval numbers needed for qPCR and the other consisted of 42 active larvae and was used for an acid test. The results were positive for the quantification sample of 100 larvae. The qPCR result for the 42 active larvae that were pipetted out of the petri dish twenty-three minutes after the addition of acid (10mL) was also positive for *A. cantonensis*. Similar acid tests that were carried out the following day also showed the stimulating effect of acid on the larvae. Ten minutes after acid was added to a day two sample from RLW 1719, which initially was observed to contain one swimming larva, one moving larva, and a number of motionless larvae, 39 actively swimming larvae were observed. The number of swimming larvae dropped to 22 after thirty minutes, and the qPCR result for the 22 larvae taken for qPCR was positive for *A. cantonensis*. This stimulating effect of acid on larvae was repeated when acid was added to a day two sample from RLW 171. Initially only one active larva was seen in the dish, however ten minutes after acid was added 21 swimming larvae were observed, and after 45 minutes 14 larvae were still active, which were removed for qPCR and the result was positive for *A. cantonensis*.

Twenty-one days after slug death

On the final day of this 21-day study, a sample from #5 (whole slug) day two was observed by to contain two swimming, one moving, and 88 motionless (not coiled) larvae. Acid (10mL) was added to the dish and almost immediately the motionless larvae were seen to begin to move. Eight minutes after the acid was added 80 larvae were observed to be swimming again, and after 13 minutes 110 swimming larvae were counted. Thirty-five minutes after acid was added to the sample 80 active larvae were removed by pipette, ground, and prepared for qPCR, and the result was positive for *A. cantonensis.*

Sieve trial/acid trial

On the nineteenth day of this study a sieve trial was conducted with the many (>1000). remaining larvae from #2, #4, RLW1717, and RLW 1722. A great number of larvae that had been pipetted onto the top of a 20µm stainless sieve set into a 1000 mL beaker filled with tap water and left for 48 hours were found below the sieve when it was removed, and the qPCR result for the 67 active larvae that were pipetted out of these larvae was positive for *A. cantonensis*. Some of the larvae that were able to traverse the sieve were placed into a small drop of water in a petri dish and acid was added (5mL). Within three minutes all larvae had dissolved with the exception of a few that had ceased moving. The remaining larvae were trialed by adding acid (10 mL) to the dish and within eight minutes almost all of the larvae were dead or had dissolved with the exception of a few that were similar in size. After 19 minutes had elapsed only eight larvae were observed moving, three of which appeared translucent and were very actively coiling and uncoiling in the Q-movement described as common to *A. cantonensis.*

Frozen larvae/acid test

The larvae (6 swimming and 29 coiled) that were placed into a freezer (-18º C) for 25.5 hours and then thawed were observed to be motionless. After acid (5 mL) was added no movement was observed and the larvae began to look as if they were breaking down, becoming less glassy and translucent in appearance and the refractive granules were quite distinctive.

Quantification of optimal larval numbers for qPCR results

The results of the two samples containing ten larvae each, and one sample of ten extra-large larvae that were prepared from #6 whole, were negative for *A. cantonensis.* Of the two sample taken from RLW 1719 (day one) the result for the sample containing 25 larvae was positive for *A. cantonensis,* and result for the sample containing 50 larvae was low and late

(likely negative). The qPCR result for the 30 larvae taken from this same sample was positive. The qPCR results for the two samples taken from RLW 1720 (day one) were negative for 25 larvae and low and late (likely negative) for 50 larvae. A sample of 30 larvae from #6 (day one) yielded positive qPCR results, and one very large larvae from #2 (day two) was low and late (likely negative). The lowest number of larvae for which a positive qPCR resulted was for 12 larvae that were combined from #1 and #5 whole slugs and had survived acid for two hours. Larvae were photographed with a micrometer to show the variation in size between large larvae and the more abundant, smaller-sized larvae. The large larvae appear to be about 3.0µm in diameter, slightly larger than the diameter of a third stage *A. cantonensis* larvae, and the small larvae appear to be about 1.5-2.0µm in diameter (Fig. 1.26, 1.27). The large larvae were considerably longer than the smaller larvae and were easily distinguishable.

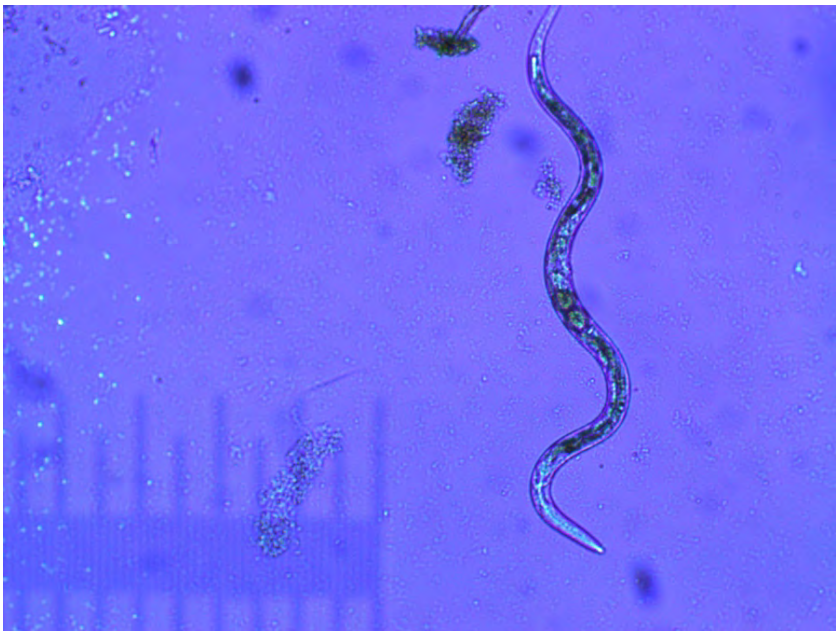

Fig. 1.26. Results from qPCR analysis indicate the large larvae viewed are not *A. cantonensis*. Large larvae usually appeared in samples taken on day 1 or day 2 from infected slugs. (Photo at 10x, 0.01mm micrometer).

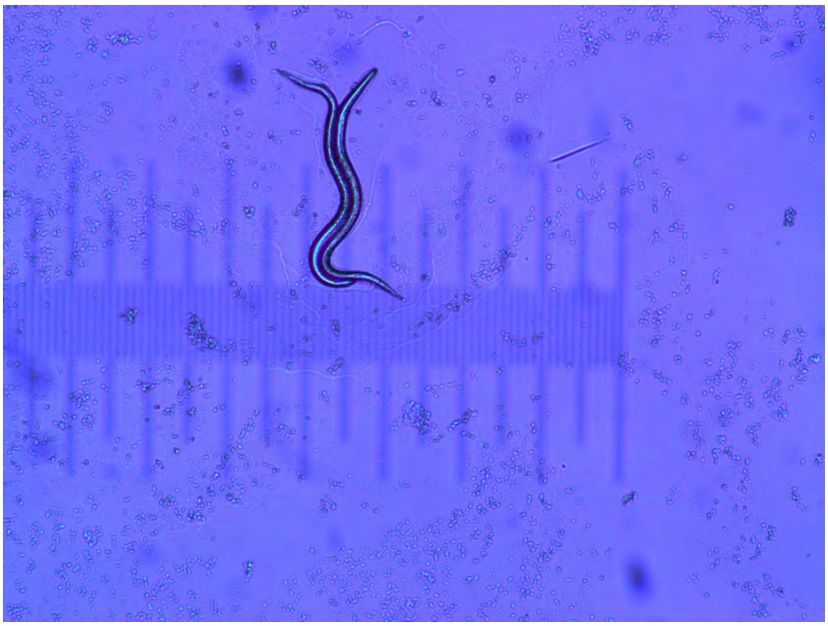

Fig. 1.27. Two larvae from RLW 1720 day 1. There were many, active larvae remaining in this sample 18 days after the slug's demise. The qPCR results for the larvae from this sample indicate that these are not *A. cantonensis* larvae. (Photo at 10x, 0.01mm micrometer).

A concise record of the results of various trials is shown in Table 1.8.

Table 1.8. Results of trials with larvae included exposure to water/acid, 20µm sieve, and counts (quantity) to determine the minimum number of larvae needed for qPCR. There are no qPCR results for tissue samples from whole slugs 1-6.

RLW #	Origin (RLW or slug #)	Slug qPCR result	Water type	Date slug death	Larvae harvest date	# larvae	Larval trial	qPCR result	qPCR larva/mg sample quantity
1771A	#1	NA	Rain	1/12/16	1/15/16	50	acid	positive	1.7978/2.0965
1772A	#1	NA	Rain	1/12/16	1/15/16	50	water	positive	0.7382/0.7915
1773A	1713	1.74/1.75	Rain	1/11/16	1/18/16	12	RLW confirm	negative	undetermined
1774A	#1, #5	NA	Rain/ Tap	1/12/16	1/22/16	12	acid	positive	0.5117/0.5641 `
1774B	#6	NA	Tap	1/12/16	1/25/16	10	quantity	negative	undetermined
1775A	#6	NA	Tap	1/12/16	1/25/16	10	quantity	negative	0.0003/0.0003
1775B	#6	NA	Tap	1/12/16	1/25/16	10	quantity	negative	0.0003/0.0004
1776A	#6	NA	Tap	1/12/16	1/25/16	10	quantity	negative	undetermined
1776B	#6	NA	Tap	1/12/16	1/25/16	10	quantity	negative	undetermined
1777A	#6	NA	Tap	1/12/16	1/25/16	10	quantity	negative	0.0003/0.001
1777B	#6	NA	Tap	1/12/16	1/25/16	10	quantity	negative	0.0002/0.0005
1778A	#6	NA	Tap	1/12/16	1/25/16	10	quantity	negative	0.0006/0.0008
1778B	1720	0.92/0.92	Tap	1/11/16	1/29/16	25	quantity	negative	undetermined
1782A	1720	0.92/0.92	Tap	1/11/16	1/29/16	50	quantity	negative	0.0003/0.0005
1782B	1719	1.66/1.91	Tap	1/11/16	1/29/16	25	quantity	positive	0.0307/0.0328
1783A	1719	1.66/1.91	Tap	1/11/16	1/29/16	50	quantity	negative	0.0005/0.0006
1783B	1719	1.66/1.91	Tap	1/11/16	1/29/16	30	quantity	positive	0.2015/0.009
1784A	#1		Rain	1/12/16	2/1/16	33	acid	positive	1.4427/1.3738
1784B	1717	5.95/5.92	Rain	1/11/16	2/1/16	40	water	positive	0.0523/0.0575
1785A	1719	1.66/1.91	Tap	1/11/16	2/1/16	75	before acid	positive	0.2694/0.2295
1785B	1719	1.66/1.91	Tap	1/11/16	2/1/16	42	after acid	positive	1.3164/1.2525
1786A	1719	1.66/1.91	Tap	1/11/16	2/1/16	100	before acid	positive	0.5571/0.2148
1786B	1717	5.2/5.916	Rain	1/11/16	2/2/16	21	after acid	positive	0.4069/0.3249
1787A	1719	1.1/1.908	Tap	1/11/16	2/2/16	22	after acid	positive	0.99751.0693
1787B	1718	7.1/7.543	Tap	1/11/16	2/2/16	14	after acid	positive	0.5579/0.4966
1788A	#6	NA	Tap	1/12/16	2/2/16	30	Confirm as RLW	positive	0.0044/0.0042
1788B	#2	NA	Rain	1/12/16	2/2/16	1	Confirm as RLW	Likely negative	0.0005/0.0015
1789A	#5	NA	Tap	1/12/16	2/4/16	80	acid	positive	2.5193/2.0217
1789B	Com-bined*	NA	Rain/ Tap	1/11 &1/12/16	2/4/16	67	sieve/ acid	positive	0.0029/0.0029

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*Combined larvae from #2, #4, RLW 1717, RLW 1720

Discussion

Study four confirmed that some of the larvae that emerge within the first 24-48 hours after slug mortality are first stage *A. cantonensis* larvae that can pass through a 20µm stainless steel sieve and will not survive acid. Some of the larvae that emerge 72-96 hours after slug mortality are third stage *A. cantonensis* larvae as they will survive acid but they are not able to pass through a 20µm stainless steel sieve. The qPCR results of larvae that were subjected to both sieve and acid tests confirmed Hypothesis 1. This study also confirmed hypothesis 2; whole drowned P. martensi can shed A. cantonensis larvae, the slug does not have to be damaged for larvae to be shed. The study did not support hypothesis 3, municipal tap water has no adverse effect on larval vitality and therefore the alternative hypothesis must be accepted.

The opportunity for a freeze trial presented itself during this study and it is good to note that freezing does kill the larvae. In regards to the minimum number of larvae needed to get a positive qPCR result, as low as 14 larvae were capable of giving as strong or stronger of a qPCR result as 100 larvae (0.56/0.50 vs. 0.56/0.22). It was surprising that even 21 days after the slugs' demise larvae maintained vitality enough that when acid was added larvae that were motionless and appeared "dead" began actively swimming again. It is not known however if these larvae are viable enough to become reproductive adults, a rat trial would be necessary to confirm this. While a rat study would be valuable to confirm that the larvae shed by slugs that have been identified as infective, third stage larvae by an acid test and qPCR analysis are able to become reproductive adults, a rat study would not be able to confirm that the larvae that can pass through a 20µm sieve are *A. cantonensis* as the first stage larvae would not be able to survive the acid in the gut of the rat. Therefore, qPCR analysis is the only method for confirmation of these larvae as *A. cantonensis.*

The large numbers of larvae of various sizes that can emerge from samples taken 24-48 hours after a slug drowns is still perplexing. Some of the larvae are quite large and yet are not initially visible with a dissecting microscope, however the dissecting microscope easily reveals the coiled larvae that drop about 72-96 hours after the slug drowns. That the larvae from these samples are not visible leads one to wonder if they are in egg form when the sample is taken and emerge from the egg over time. However, as previously mentioned, all of the literature speaks of *A. cantonensis* hatching in the lungs of the rat and the first stage larvae migrating up the bronchial tree, being swallowed, and expelled in feces. Could it be that we are missing something in the life cycle, and can we turn to histology for any answers?

Study 5: Histological location of nematode larvae in *Parmarion martensi*

The previous studies described in this paper enlisted the use of techniques that are helpful for identification of larvae as *A. cantonensis*; qPCR, acid digestion, tissue squashes, and the characteristic Q-movement of larvae. Another tool that may provide answers to some of the questions brought up by the studies to the location of larvae in the mollusk host is histology. Contradictions encountered in both the visual and qPCR results still left many questions. Larvae that appeared from different *P. martensi* emerged in similar timelines, looked similar, and moved in the same manner and yet there were cases where both the tail snips and larvae tested positive for *A. cantonensis* and some did not. Could the location chosen for a tissue sample miss the infection, and where might the larvae that emerge in such great numbers be located in a slug?

Early studies at the Jarvi Lab had examined optimal locations for tissue sampling when testing for *A. cantonensis* with qPCR and determined the tail section to be the best location to find larvae in *P. martensi* (Jarvi et al. 2012). The study by Lv et al. (2009) which used the snail

Pomacea canaliculata, showed the larvae can be located in the lung of this species, but the Richards and Merritt (1967) paper gives the most detailed account for a timeline and location of a larval infection in the intermediate host. The aquatic snail *Biomphalaria glabrata* was used for the study. Four hours after infection numerous larvae were in the esophagus, stomach and intestine, penetrating the mucosa of the intestine, where some coiled and become inactive, and some penetrating to the hemocoel, the primary body cavity containing circulatory fluid. Six hours after infection larvae were observed in the circulatory fluid of the tentacles and mantle collar, and after 24 hours many coiled larvae were seen in the intestinal folds, rectal walls and rectal fold, the kidneys and kidney fold, and the mantle lining and mantle collar.

If a slug was newly infected with *A. cantonensis* first stage larvae, a qPCR of a tail snip would most likely miss the infection. There is also the possibility that a slug or snail might be infected with more than one stage of larvae as it is unknown how often they consume rat feces; they may have third stage larvae in tail tissue but may also have first stage larvae in the circulating fluid or organs. Could this have been the case with RLW 1530 in study two, which shed large numbers of larvae in a similar timeframe to other slugs that had positive qPCR results, and the larvae were similar in appearance to those other larvae but did not test positive for *A. cantonensis*? At the time of study two the use of a tissue grinder to extract a larger amount of DNA, which contributed to more successful extraction in later studies, had not been employed and that may have affected the qPCR results. Another question raised by the previous studies is the route through which the larvae exit the deceased slug host. It is a natural occurrence, once the host dies, for the parasite to exit the host (Dr. Paul Prociv, personal communication), but is the method of escape through mucus or tissue decomposition?

Hypothesis 1: *Histology can be used to show the location of A. cantonensis larvae in the intermediate slug host.*

Hypothesis 2: *Histology can aid in identification of the routes of larval exit from the slug host.*

Methods

Two *P. martensi* were collected from a residential area in Hilo Hawaii. The specimens were not initially held in individual containers and because there was a potential for cross-contamination, the tail snips taken from both slugs were combined for qPCR and the sample was confirmed positive, however it was unclear as to whether only one or both slugs were infected due to the combined samples. Traditional histological preparation followed. The shells were first removed from the slugs which were then immersed in glacial acetic acid for 24 hours to dissolve any remaining shell fragments. The specimens were fixed in 10% formalin for 48 hours and then transferred to 70% ethanol, after which they were cut laterally into three sections (head, middle, tail). The sections were processed in a tissue processor, blocked in wax, cut in 7µm sections which were placed on glass slides, and stained with traditional hematoxylin and eosin. Best attempts were made to cut ten sections, skip ten sections, and cut ten sections. The slides were then observed by microscopy.

Results

Histological sectioning provided useful information regarding tissue differentiation, organ structure, larval location, and potential routes of exit. Larvae were only found in one of the two slugs. In the slug in which larvae were found they were primarily in the head and tail

sections, often near the foot, some in the mantle area, and many were located very close to the body wall (Fig. 1.28, 1.29, 1.30, 1.31, 1.32, 1.33).

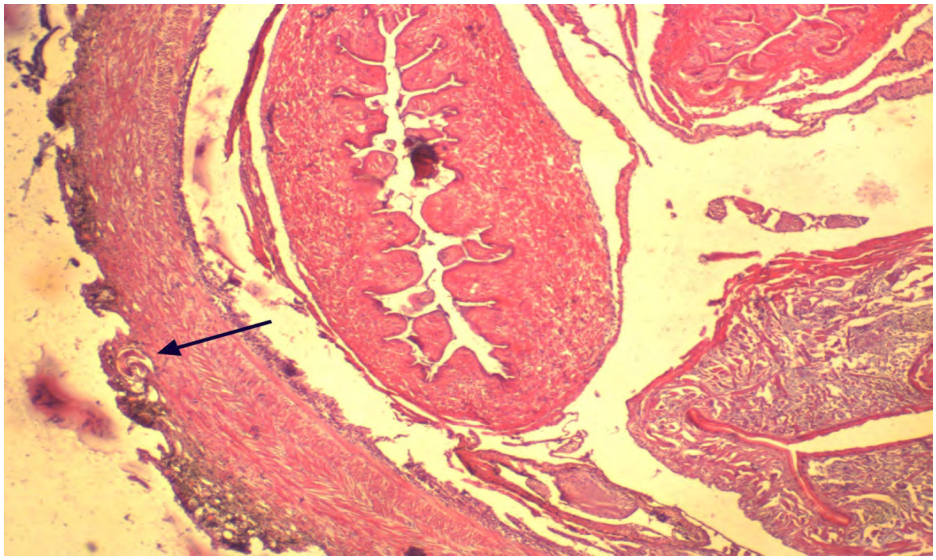


Fig. 1.28. Larva in mantle area close to body wall. Location is closer to mid-section of the slug as evident by body organs (4x).

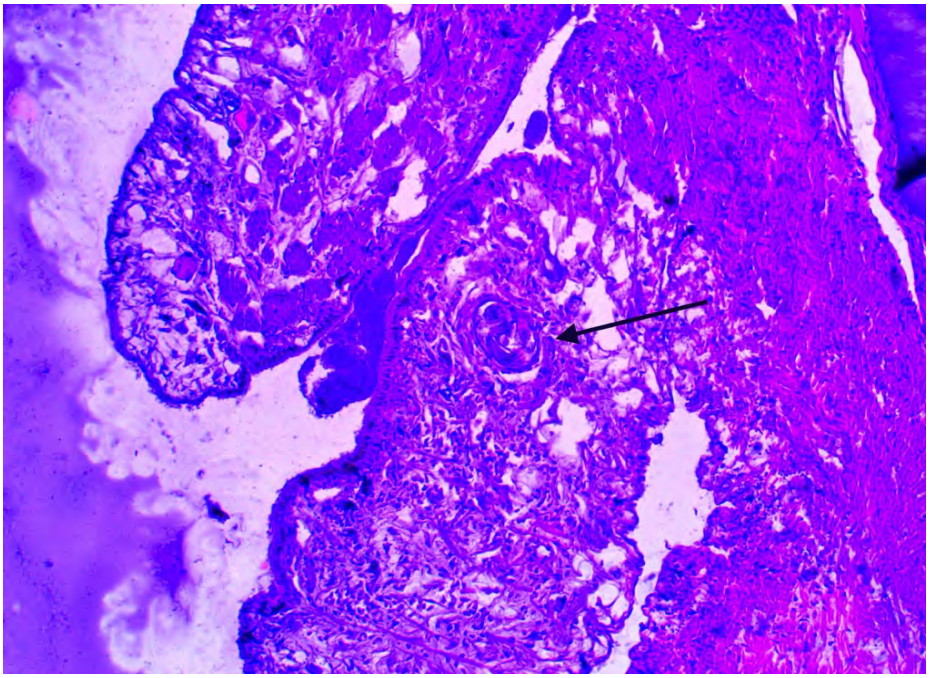


Fig. 1.29. Coiled larva visible in muscle tissue near body wall. The mantle fold is visible in the upper left-hand side of the photograph (10x).

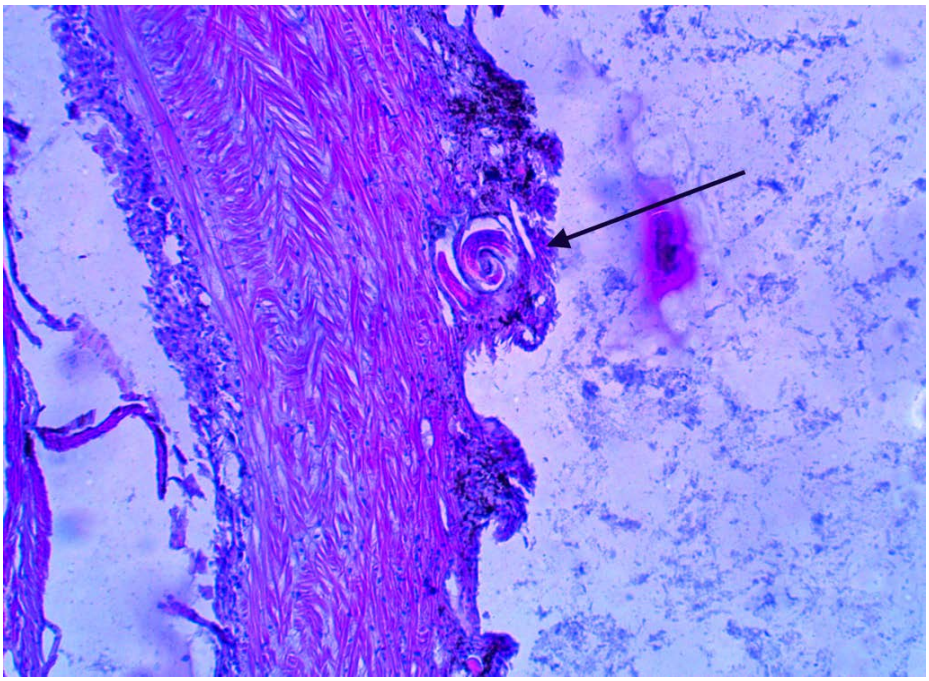


Fig. 1.30. A coiled larva in muscle tissue and almost exiting the slug, very close to the exterior of the body wall (10x).

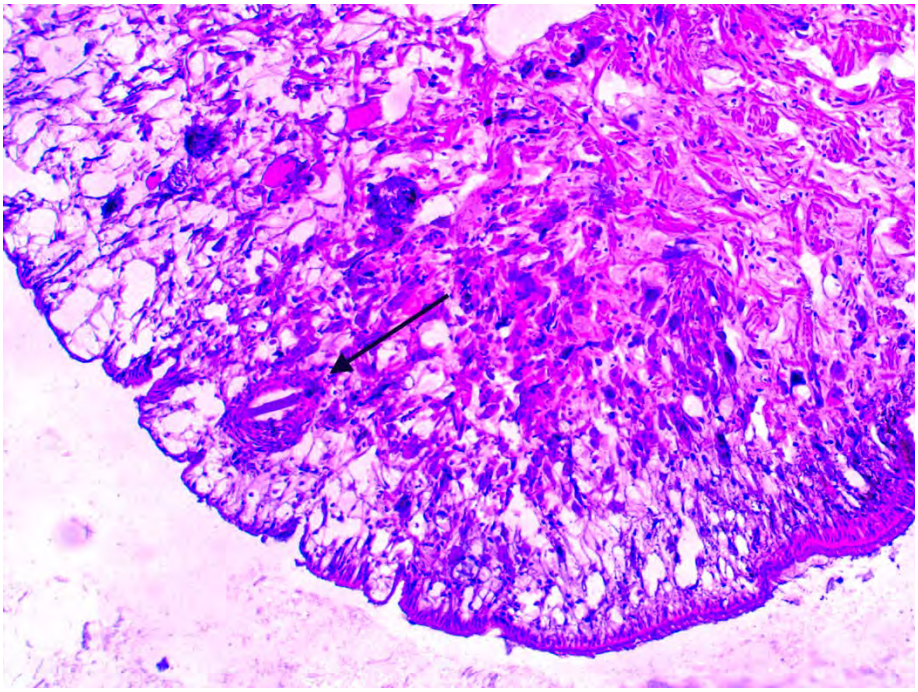


Fig. 1.31. Larva in foot of slug close to body wall (10x).

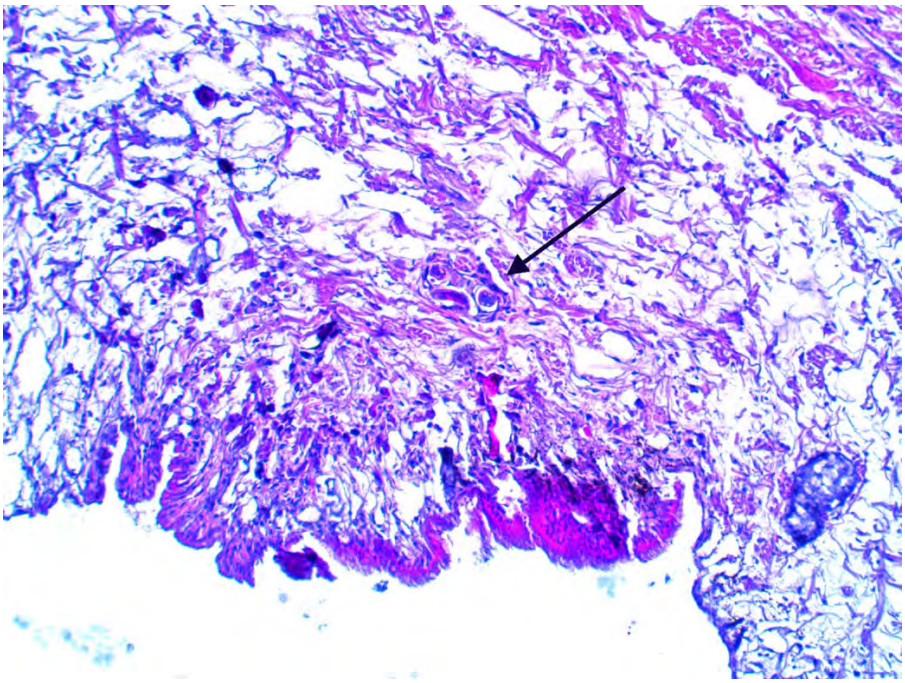


Fig. 1.32. Cross-section of a coiled larva in tissue, note the deep mucus glands *P. martensi* (10x).

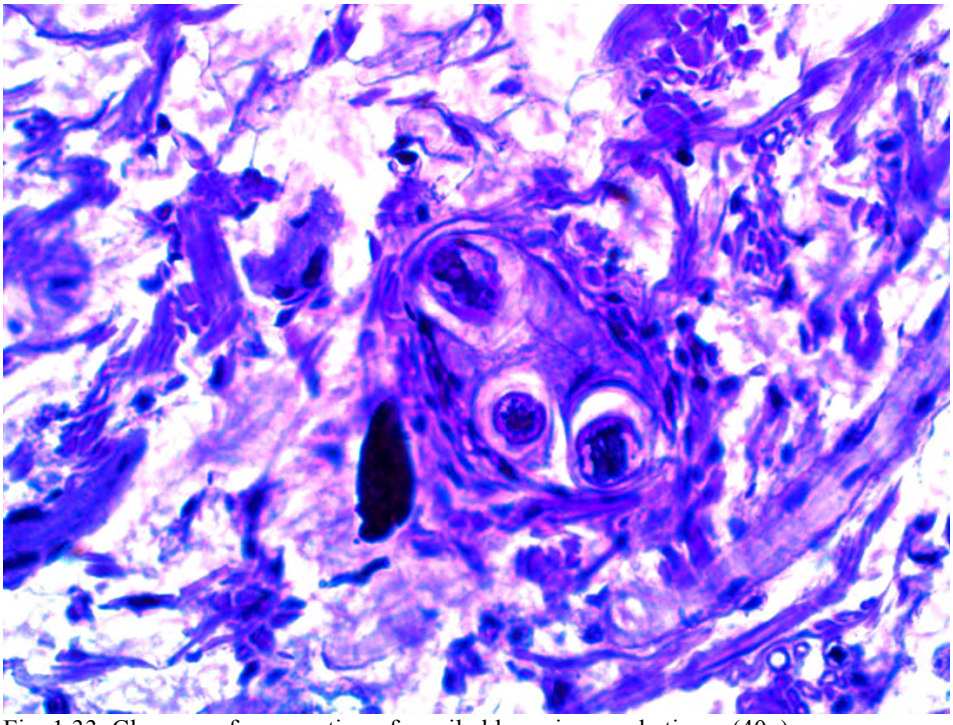


Fig. 1.33. Close-up of ross section of a coiled larva in muscle tissue (40x).

The mid-section of the body, which contained most of the viscera, was difficult to section and much of the visceral mass was lost. Histology provided an opportunity to view *P. martensi* from

a different angle, the inside. The eyestalk, tentacle, and food of a juvenile *P. martensi* were clearly defined, as was the shape of the foot in relation to the rest of the body (Fig. 1.34a, b).

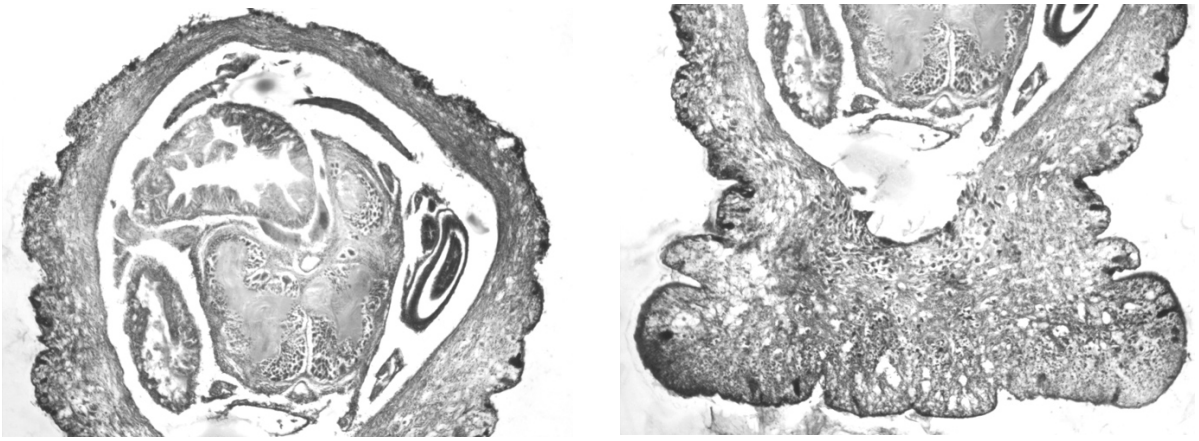


Fig. 1.34 (a, b). Juvenile *P. martensi* head with eye stalk and tentacle visible (left). Foot of juvenile *P. martensi* (right) (4x).

Cellular differentiation and organ structure of *P. martensi* was also apparent with histological sectioning. Muscle tissue was very evident in the tail section, while tissue located in the mantle had a very different appearance (Fig. 1.35a, b).

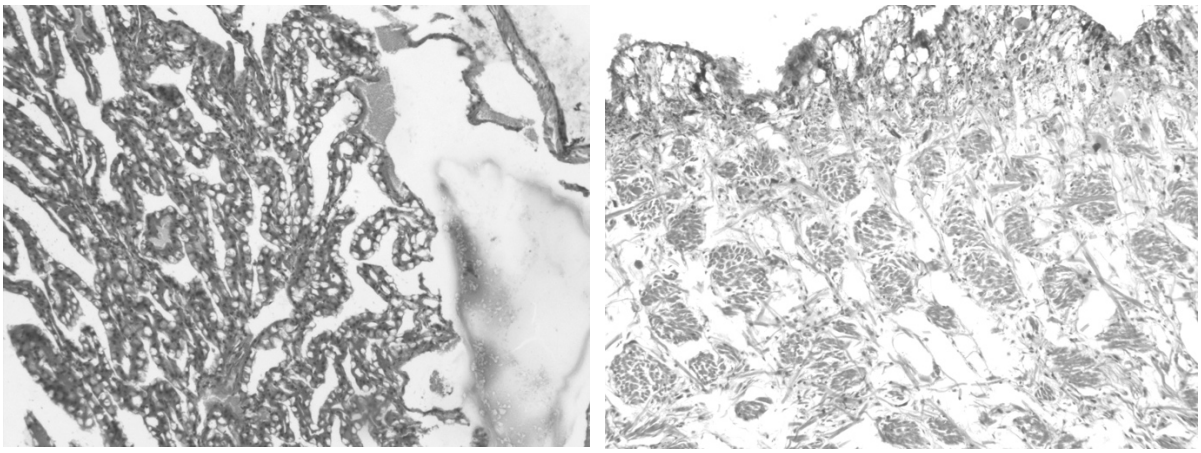


Fig. 1.35 (a, b). Tissue in mantle area (left) has a much different appearance than muscle tissue (right) (10x).

Parmarion martensi will produce copious amounts of slime when disturbed, which will often cover the whole body and is whitish in color. It is not surprising that histology showed this species possessed deep epithelial mucus glands (Fig. 1.36) and larvae found in the slug showed that larvae can be positioned extremely close to the body wall. It may be very likely that a very

highly infected slug, when drowned, could shed larvae in slime and this may be an exit route for the larvae. While some slime studies have been conducted the consensus among the scientific community is that only very low numbers of larvae are found in slime, however those studies have been done with living slugs and snails. More studies relating to the potential for slime to contain RLW parasites should be conducted, especially with highly infected individuals, to truly understand the relationship between *P. martensi,* slime, and *A. cantonensis* shedding.

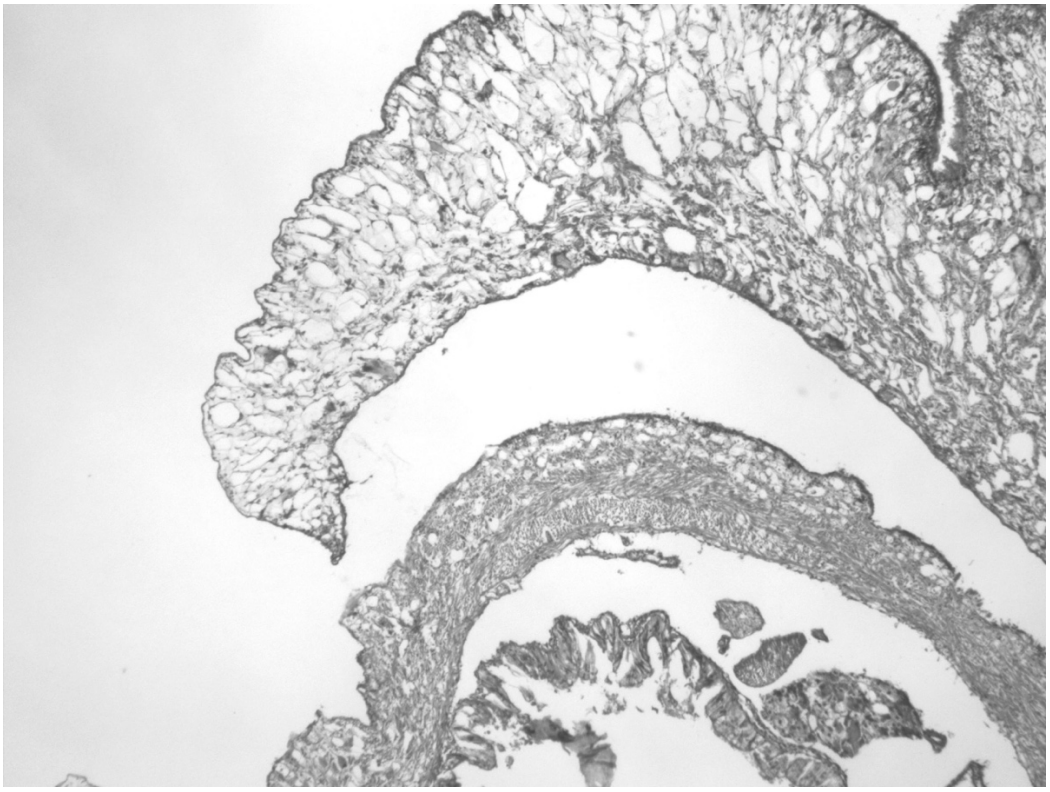


Fig. 1.36. Mantle of a juvenile *P. martensi* shows the deep mucus ducts of this slug (10x).

One of the short-comings of histology is that it cannot provide proof that larvae observed are *A. cantonensis*, nor can it clarify the larval stage. Many juvenile stages of the Metastrongyloidea (Order: Strongylida) have an association with mollusks as intermediate hosts (Grewal et al. 2003) and knowing that the larva you are seeing are the target organism you are looking for is challenging. While the morphology of *A. cantonensis* has been described by Ash

(1970), morphological identification is difficult and analysis using DNA is preferred (Dr. Alder Dillman, personal conversation). Also, larvae that could recently have been ingested and may be in the digestive tract or some other organ and could be missed with histology.

A third method that I found useful in determining nematode infection in an intermediate or paratenic host is the tissue press, where a small amount of tissue from the host is squashed between two glass microscope slides. This method is described by Ash (1968) when examining frogs, a paratenic host of *A. cantonensis.* I found this method to be extremely useful as a quick method to identify infection in soft bodied gastropod hosts, such as *P. martensi*, or a planarian such as *Platydemous manokwari*. The first tissue squashes were performed with the latter from a flatworm captured in a school garden plot, not far from the Koae area in the Puna District, where many of the slugs used throughout these studies were collected. The tissue of this planarian is very soft and is easily squashed on the slide. This technique revealed many live larvae, some that were mobile and some that retained their coiled shape (Fig. 1.37, 1.38). In my final study I took tail snips from ten *P. martensi,* part of the which were reserved for qPCR, and a small section of which was squashed and observed by microscopy. The squash very easily revealed larvae in various stages of development. Those without dark interiors were capable of movement, coiling and uncoiling, and moving in a snake-like or "Q" movement, the latter of which has been defined as characteristic and distinctive for *A. cantonensis* (Lv et al. 2009).

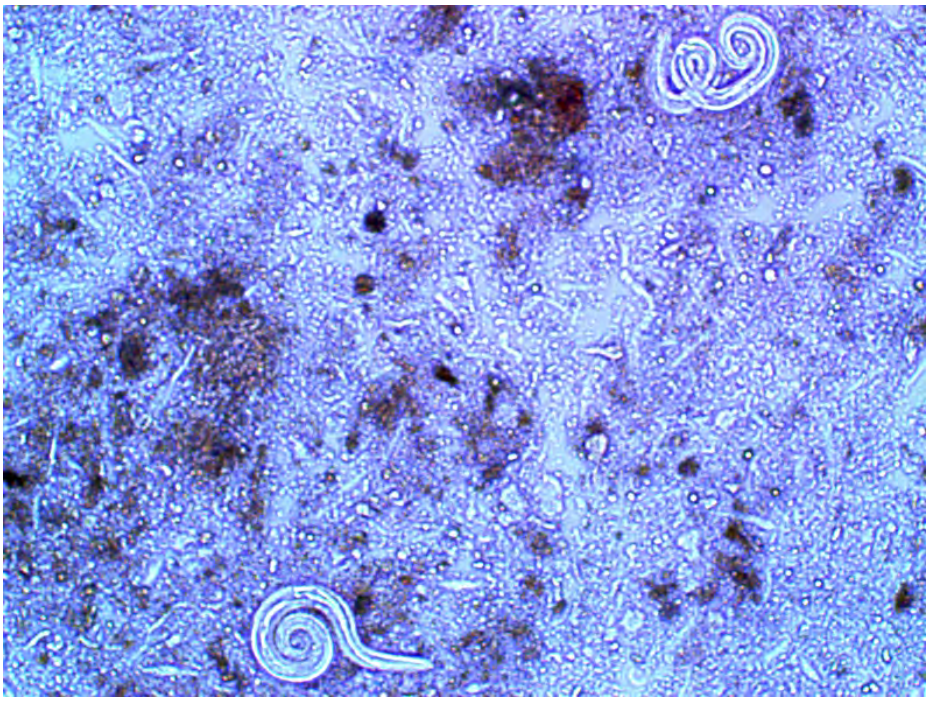


Fig. 1.37. Coiled larvae are clearly visible in this tissue squash of a *Platydemous manokwari* the New Guinea flatworm, a paratenic host of *A. cantonensis* (10x).

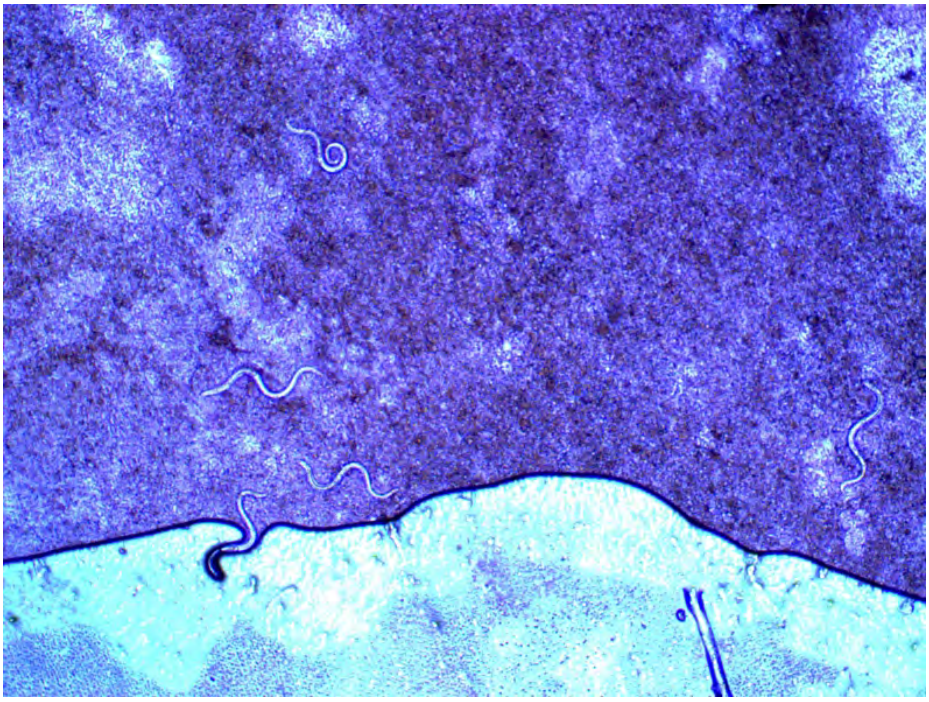


Fig. 1.38. Some of the coiled larvae emerged into moving larvae. The tissue squash allows viewing of live larvae in the host or paratenic host tissue, such as in the tissue of this *P. manokwari* the New Guinea flatworm (4x).

Conclusion

These studies are the most recent in over forty years to examine the potential for drowned terrestrial gastropods to shed *Angiostrongylus cantonensis* larvae, and the first to examine the capacity for parasite shedding by the very effective, newly introduced semi-slug *Parmarion martensi.* The studies presented here demonstrate that whole, uninjured *P. martensi* can shed larvae if the slug drowns, and the shed larvae will include 1st, 2nd, and infective 3rd stage larvae. Shed larvae that can pass through a 20mμm sieve cannot survive acid, can survive for up to 56 days, and qPCR analysis for *A. cantonensis* from preparations of the larvae are positive, indicating that these are 1st stage larvae. Those shed larvae that cannot pass through the sieve can survive acid and also test positive by qPCR for the parasite, suggesting these are 3rd stage larvae. Larvae that have been outside a gastropod host for up to 21 days are stimulated by acid and will regain vitality and begin swimming movements. A rat study would need to be done to determine the maximum number of days outside the slug host a larva will have sufficient vigor to be infective and grow into a reproducing adult worm. All sizes of larvae are able to traverse an 8-10 and 20-25μm Whatman paper filter. Whether the larva perforates a gut by mechanical or chemical methods might have some bearing on whether or not they might be able to pass through a commercial-grade, fiber filter. Trials of commercial filters are needed in order to recommend an effective filter size for rainwater catchment users.

The high numbers of coiled larvae shed in the first study were unexpected and led to subsequent studies, each of which was designed based on results of the prior studies. Wild-harvested slugs, snails, and flatworms were chosen for the studies rather than laboratory raised and infected specimens, as the they best represent existing environmental conditions that may be impacting residents. Water-borne infection is not often mentioned when discussing transmission

routes of angiostrongyliasis, however at the 4th International Rat Lungworm Workshop held in Brisbane Australia in September 2016, a representative from the U.S. CDC spoke of three recent cases of human angiostrongyliasis that occurred in Texas in 2015 after a flood event; it was alluded that infection was water-related (Qvarnstrom personal conversation 2016). There is a real need for studies that examine the potential for transmission of angiostrongyliasis by water.

While these studies do not confirm that the rise in cases is due to the high use of rainwater catchment they do implicate that catchment may play a role. It would be of value for further studies to be conducted and investigations made into the use and conditions of rainwater catchment systems and to examine biological communities found in tanks. The sampling of catchment tanks was not a part of these studies, however one such tank sampling was done during the course of these studies for a Puna resident who was diagnosed with angiostrongyliasis and succumbed. Observation showed the victim's tank and filter system were not well-maintained. Two, one-liter samples were drawn from the tank with a hand-held suction pump for laboratory analysis at the Jarvi lab. A 20µm sample of the water was pipetted from one of the samples and examined with a compound microscope. Two nematode larvae were seen; one was C-shaped as is common to a 2nd stage *A. cantonensis* larvae, and the other was a swimming larvae bearing a resemblance to the 1st and 3rd stage larvae that were seen in these studies. A number of other live, microscopic organisms were also observed. Residents must be alerted to the importance of system maintenance if using rainwater catchment.

In lieu of these studies, the use of catchment water for agriculture is a concern as it is unknown to what degree agricultural-use catchment water is filtered if at all. During the course of these studies an infected *Platydemous manokwari* flatworm was examined in the Jarvi lab. When the planarian died after several days it rapidly decomposed into a puddle of dirty-looking

water which still contained live, infective-stage *A. cantonensis* larvae. The remnants of infected tissue from a decomposed slug, snail, or flatworm could contaminate leafy greens and the microscopic parasites would be missed even if the produce was examined for evidence of the host organism. Knowing the ability of the larvae to survive outside the dead slug, snail, or flatworm host in wet conditions, environmental exposure to the larvae in soils, puddles, etc. may be possible sources of disease transmission.

Some questions still remain, most particularly puzzling is the appearance of large numbers of larvae over time from samples that initially do not appear to have larvae. It may be that the larvae are tightly coiled or in egg form, although the literature always mentions that the larvae migrate up the bronchial tree and does not mention of an exit in the form of eggs. More examination of samples using a higher powered microscope will eventually help solve this mystery. The route of exit is also questionable. These "invisible" larvae exit the drowned slug on different days than the visible coiled larvae, and it may be that they are located in different areas of the body of the intermediate host, possibly in the lung region or digestive tract rather than in the muscle tissue, which is where 2nd and 3rd stage (c-shaped and coiled) larvae are usually found. The exit from the deceased host may be via the mucus ducts, pneumostome, or anus, and an osmotic pressure difference may be a causative agent.

Another question raised pertains to the effect, if any, of entry of the 1st stage larvae in a human or accidental host through a laceration or conjunctival tissue. This may be a question of importance as work progresses in the U.S. for diagnosis for angiostrongyliasis with a 31kD antibody-based blood test, such as that already in use in Thailand. Why would a person have a positive antibody response but have only experienced very mild or no symptoms from a RLW infection, as is mentioned on the CDC's website (CDC.gov)? While it is thought that the number

of larvae ingested may determine the severity of the illness, could a case with very mild or no symptoms be the result of an infection by 1st stage larvae through a route other than oral, such as through a laceration or through conjunctival tissue? A mouse study would be useful to look at the antibody response of this type of infection.

Annual cases of rat lungworm disease continue to be reported and serious cases have resulted in death and permanent disability in Hawaii. There is an urgent need for more research to aid in better understanding the possible disease transmission pathways so infections be prevented. Host control of both rats, and slugs and snails is key if we are to reduce parasite populations in areas of high infection. Continued island(s)-wide sampling should be done to determine where the "hot-spots" of infection are and where control efforts should be targeted. An education campaign is key to encourage residents to control hosts around their homes. An open and honest discussion about the issues pertaining to rat lungworm can help agencies and educational entities collaborate to develop innovative solutions for this important, community health problem.

CHAPTER TWO

Partnering with Hawaii Island school garden projects to develop an integrated pest management plan for intermediate hosts of *Angiostrongylus cantonensis* and supporting educational activities to promote food security and expand community education for rat lungworm disease prevention.

Introduction

At the 59th annual meeting of the American Society of Tropical Medicine and Hygiene in Atlanta, Georgia, information was presented on the results of the Center for Disease Control (CDC), Hawaii Department of Health (DOH), and the United States Department of Agriculture (USDA)/Pacific Basin Agriculture Research Center (PBARC) study on Hawaii Island in 2005-2007 (Hochberg et al. 2007; Hollingsworth et al. 2007; Bandea, Warashina, Bucholz, Qvarnstrom, & da Silva 2010, Qvarnstrom, Bishop, deSilva 2013). Investigators showed the infection rate in mollusks (slugs and snails) ranged from 24 to 78%, depending on species analyzed, and they confirmed that 57% (n=37) of the rats were positive by examination, having active infection with worms found in the heart and lungs, and 100% were positive by examination of lung tissue sample using realtime PCR, providing DNA evidence of current or past infection. Of the rats tested by PCR, 32% (n=9) had very high infection levels, which corresponded to more than 100 larvae in ~25mg of tissue. The presenters remarked at the conference that "the severe cases of eosinophilic meningitis being reported could be due to overexposure of humans to highly infected mollusks: there are no measures in place in Hawaii to control the spread of *A. cantonensis."* The authors concluded "angiostrongyliasis may be a more serious public health issue in Hawaii than currently estimated and measures to control its spread in mollusks and rodents may be warranted" (Bandea et al. 2010).

To this day, some nine years after the 2007 CDC and DOH study was conducted in Hawaii, there has been no concerted effort made by any county, state or federal agency to control the spread of *A. cantonensis* in mollusks and rats, nor has there been a public education campaign to encourage residents to control these hosts. It is important to remember that the hosts of *A. cantonensis* are also agricultural pests, some of which are listed among the top 100 worst invasive species in the world and include the rat (*Rattus rattus*) the flatworm, *Platydemous manokwari*, and the snails *Achatina fulica* and *Euglandina rosea* (Mead 1961; Lowe, Browne, Boudjelas & De Poorter 2000). Without control efforts in place, the rat lungworm parasite and its hosts could pose a direct threat to agricultural production, food safety, and food security in Hawaii.

The State of Hawaii estimates it imports 85-90% of its food, making it vulnerable to natural disasters and world events should there be a disruption in the shipping and the food supply. In 2009, under the direction of then-Governor Linda Lingle, the *Increased Food Security and Food Self-sufficiency Strategy* was prepared by state agencies with the objective of increasing the amount of locally grown food consumed by Hawaii's residents (http://files.hawaii.gov/dbedt/op/spb/INCREASED_FOOD_SECURITY_AND_FOOD_SELF_SUFFICIENCY_STRATEGY.pdf). The public has shown avid interest in supporting local food production and proof of support can be seen in the growing number of farmers' markets, grocery stores, and restaurants selling locally grown produce and products. As the interest in the local food movement has grown so has the support for school garden projects. Each of the main Hawaiian Islands now has a school garden hui (group) and there are an estimated 200 school and youth gardens in the State of Hawaii, with almost 70 of these located on Hawaii Island (Nancy Redfeather, personal communication). To further promote healthy diets and local agriculture, in

2015 the Hawaii Farm to School Bill, a program intended to introduce students to healthier eating habits by introducing them to new vegetables and fruits that are procured from local farmers, was signed into law.

While public support of the local food movement has grown, efforts to control the spread of the rat lungworm parasite and its hosts, and to educate the general public of risks and disease prevention has not followed a similar trend. It has been stated that a media campaign to inform residents and visitors about what some consider a "relatively small" risk could "have a significant economic impact on local produce growers and statistically speaking, consumers in Hawaii are much more likely… to get hit by a car while walking across a street than to be infected by the rat lungworm" (Hollyer 2013). Indeed, the Hawaii DOH 10-year summary for cases of reported cases of notifiable diseases from 2005-2014 only show 39 cases of angiostrongyliasis, 35 of which were reported for Hawaii County, three for Maui County, and one for Oahu County (health.hawaii.gov). There is no report of cases for 2005 and 2006 as angiostrongyliasis was not a reportable disease at that time. However, the DOH epidemiologist S. Y. Park does state that there have been approximately 60 reported cases from 2001-2012 (Kim et al. 2014) and because many cases go undiagnosed or unconfirmed it can be assumed that the number of human cases of angiostrongyliasis in Hawaii is actually much higher.

Dr. Jon Martell, a clinician at Hilo Medical Center in Hilo Hawaii who has treated many cases of RLWD, now reports that this is a mainstream disease and we should all be concerned (personal communication). People who are aware of the situation with RLW and take precautions to prevent the disease are still contracting the illness, and therefore it is essential to continue to define potential transmission routes and share this information with the public. Rat lungworm disease is a foodborne disease, can be a waterborne disease, and transmission via skin

could be possible. A mouse study by Wang, Chao, and Chen (1991) provides evidence that this may be possible. Mice were exposed to 3rd stage *A. cantonensis* larvae by various routes including oral, intraperitoneal, sub-cutaneous, mucosa (anal, vaginal, conjunctival), lacerated skin, unabraded skin, foot pad and tail penetration. More worms were recovered from the mice infected by intraperitoneal and subcutaneous inoculation than by stomach intubation. The results showed worm recovery ratio efficiency from lacerated skin infection (40%) was higher than those from the unabraded skin (6%) or the foot pad infections (4%). Anal infection had higher worm recovery (51%) than vaginal (28%) or conjunctival (21%) infections. Mice infected by subcutaneous inoculation had a significantly higher worm burden that those infected orally ($P<0.05$). The study concluded that skin or mucosa contact with 3rd stage larvae may cause angiostrongyliasis.

The simple act of bathing or washing in inadequately filtered catchment water could theoretically be a source of exposure, although currently there has been no evidence for infection in this manner (Cowie, 2013). However, at the 4th International Rat Lungworm Workshop, held in Brisbane Australia in September 2016, the United States Center for Disease Control (CDC) attributed three human cases of RLWD to water transmission during a flood event in Houston Texas in 2015 (Qvaranstrom, presentation). While it was not described as to how they believe the victims were infected, it does acknowledge that health agencies agree that infection via water does occur. When examining the evidence for potential routes of transmission that may be responsible for the high rates of RLWD in Hawaii, and keeping in mind the high rate of rainwater catchment usage and the disproportionate number of cases originating from Hawaii Island, it is clear that immediate steps need to be taken to reduce infection. Weak political and commercial sector support, complicated by geographic distances and socio-cultural and

economic differences provide some of the reasons why control measures and public education regarding rat lungworm disease and its prevention have been inadequate in the past. However, in lieu of the continued number of cases occurring annually, an effective host control and public education campaign is critical for Hawaii.

Pilot project:

At the 2011 International Rat Lungworm Workshop in Honolulu, Hawaii, a prioritized list of objectives and top needs was developed by workshop participants from six countries. It was agreed that public education was a priority, and one pathway for providing education for hard-to-reach, rural communities was to involve children ages 7-14 in the effort by developing rat lungworm, STEM (science, technology, engineering, math) curriculum (Cowie, 2013). The Hawaii Island Rat Lungworm Working Group, herein referred to as the RLW Working Group, housed at the University of Hawaii (UHH) Daniel K. Inouye College of Pharmacy (DKICP), was formed in 2012 to address gaps in research and education outreach relating to RLWD in Hawaii. In 2013 the RLW Working Group published an educational activity book titled "The Mystery of Rat Lungworm Disease," which provides basic information on the rat lungworm parasite's lifecycle and steps to prevent disease. The book is based on the Hawaii Science Standards for the 2nd grade level and was developed in collaboration with teachers from five schools; 10,000 copies were printed and all have been distributed to school children, educators, and medical clinics in Hawaii, with continued requests for more.

To build on these education efforts, the RLW Working Group addressed the deficiency of advocacy for control programs for hosts of the RLW parasite through a collaborative project with the UHH Tropical Conservation Biology and Environmental Science (TCBES) Masters of

Science Program, the DKICP, and five public K-12 schools on Hawaii Island. The project's objectives are to develop an Integrated Pest Management Plan for the control of non-native slugs and snails for Hawaii School Garden Projects, and educational STEAM (science, technology, engineering, art, math) activities related to RLWD and prevention. The project engaged students as community educators and use citizen science to assist researchers in gathering information. The lack of information about RLWD and control efforts for the parasites' intermediate hosts could jeopardize student health and the school garden projects themselves. Can school garden projects provide an avenue to initiate control measures and education within their ranks and aid in educating the public about RLWD and the need for control of intermediate hosts?

Objective 1: *We can teach students to safely remove invasive slugs and snails from school garden projects in Hawaii.*

Objective 2: *We can educate rural populations and the general public by educating K-12 students who are involved with school garden projects.*

Methods

The graduate student and author of this paper met with each partner school on a weekly basis for the first half of the Fall 2015 semester, and then bi-weekly until the end of the semester. In the Spring 2016 semester the project leader made monthly visits to each of the partner schools. The project incorporated place-based education, curriculum integration, and interdisciplinary, hands-on, student centered learning (Leiberman & Hoody, 1998). Place-based education supports pedagogies embraced by educators that are shown to be successful in student achievement. It is an educational practice supported in Hawaii and is included in the state's

efforts to improve education through the integration of *Every Student Succeeds Act* of 2015. Analysis of non-native terrestrial slugs or snails brought from the partner schools for of *A. cantonensis* infection was done at the Jarvi Lab at the University of Hawaii, Daniel K. Inouye College of Pharmacy.

Partner schools

Five partner schools were specifically chosen from the east side of Hawaii Island (Fig. 37). All partner schools are public charter schools (PCS) that have a school garden and are connected with the Hawaii School Garden Network (HISGN), which is coordinated by the Kohala Center, a non-profit organization located in Waimea, on Hawaii Island. The schools chosen promote experiential, hands-on, place-based, project-based activities and were receptive of participating in the project. The school locations were chosen to aid in evaluation of non-native terrestrial slug and snail species and population compositions as they respond to location and elevation on Hawaii Island. There are 43 established species of non-native terrestrial slugs and snails in the State of Hawaii (Kim et al. 2014). Monitoring at each of the partner school's locations was done to provide a better understanding of where the different species of these agricultural pests reside.

Kua o ka La Public Charter School (KOKL), located near the eastern-most point of Hawaii Island and in the vicinity of the original outbreak of RLWD clusters in the Puna District, was the lowest elevation school at about 5.2m above sea level. This area has many diversified farms growing vegetables and tropical fruits, and there is large acreage of land in papaya production in this area. Na Wai Ola (NAWA), also in the Puna District, is located near the small, rural town of Mountain View at an elevation of about 457.2m and is also an important agriculture area for vegetables and tropical fruits. Volcano School of Arts and Science (VSAS)

is located on the border of the Puna and Kau Districts just outside of Volcanoes National Park and is the highest elevation school at about 1158.2m. This area is known for production of cool weather crops, including blueberries and tea. Some of Hawaii's native snails can still be found in high elevation refugia in this area, such as that provided by the national park and nearby Waiakea Forest Reserve. Laupahoehoe (LAUP), at an elevation of about 365.8m is located in the Hamakua District. Formerly an important area for sugar cane production, fallow land in this area is being converted to diversified farming. The northernmost school, Kanu o ka Aina (KANU), is located in the Kohala District at an elevation of about 838.2m and is one of Hawaii's most important agricultural areas for the production of cool weather crops, such as lettuce and brassicas.

Fig. 2.1. Map of the partner school locations on Hawaii Island. Partner schools are shown with yellow flags, the University of Hawaii at Hilo is depicted with the white building. The black and blue dots show the locations of other public and private K-12 schools on Hawaii Island.

Adoption of Integrated Pest Management practices

Integrated pest management (IPM) practices were applied in the control plan for the partner school garden projects and targeted the intermediate hosts, slugs and snails, and flatworms (paratenic hosts). We encouraged the schools to include rat control as a part of their maintenance programs but this project did not include rat control. Integrated pest management practices received wide support when the ecology movement of the 1970's pressed for the integration of methods for pest control that lessened the negative effect of pesticide, herbicides, and fungicides on ecosystems. Integrated pest management incorporates the least toxic methods for control of pests and includes the careful consideration of available pest control methods including biological, chemical, and cultural practices. These management practices are dynamic and involve monitoring systems to continuously evaluate population levels for decision making and adaptive management (Flint & van den Bosch 1981). The IPM plan used at each of the schools was adopted from the University of California Statewide Integrated Pest Management Program guidelines for snails and slugs, which recommends the use of cultural practices (removing materials where they can hide), traps (providing specific shelters for them to hide under), hand picking, barriers (copper, Bordeaux mixture, ashes or other abrasives), natural enemies (predacious snails and domesticated fowl such as ducks, chickens), and baits.

For this project shelters (traps) were used as it provided the opportunity to identify, monitor, and remove populations and was not intrusive nor involved the use of chemicals, an important factor to consider in school gardens where children are present. Thirty days before the start of the Fall 2015 school year, two sets of five shelters each were placed in each school garden area, with each set located at opposite ends of the garden or the determined observation area. All shelters were $2ft.^2$ and consisted of the following materials: a wooden board raised off

the ground by 2x2inch wooden rails; heavy-duty cardboard; black plastic; weed-cloth mesh; and an insulated shelter consisting of a black plastic bottom, ¼ inch fiberfill insulation middle, and a reflective tarp top, all fastened together with duct tape. The ground below the shelters was cleared of vegetative material and the moistened with water to provide conditions favorable for an attractive sheltering place for invasive slugs and snails. Shelter-type capture rate was evaluated as was as species/shelter preference. Shelters were checked by the project leader with the students at each school on a weekly basis for the first half of the fall semester, and then on a bi-weekly basis for the second half of the semester. Shelter checking and recording was to be the responsibility of the school garden teacher and the students during the spring semester. In addition to trapping with the shelters, hand collection was also used as part of control methods and non-toxic baits could be used if needed.

Education: Understand basic gastropod biology

Students were educated about basic gastropod biology. Gastropods, more commonly known as slugs and snails, are a toxemic class within the phylum Mollusca, the third most successful animal group after arthropods and vertebrates. Gastropods have generalized characteristics that include a distinct head with tentacles and eyes, a broad flattened foot, and a dorsal mass that is at least partially covered by a mantle and sometimes covered by a calcareous shell. Most all terrestrial gastropods are pulmonates and have lungs or lung-like organs in the mantle cavity, which opens externally at the pneumostome. Slugs and snails are similar in structure and biology, snails have a spiral shell and slugs do not, and semi-slugs, such as *Parmarion martensi,* have a vestigial shell. The absence or reduction of a shell in slugs lessens the need for calcium salts and provides great range of habitat than that required by snails. It is a contributing reason as to why terrestrial slugs are one of the most successful of all mollusk

groups. Lack of a shell and a worm-like body allows slugs to access crevices and squeeze through small openings (South 1992).

Slugs and snails are hermaphrodites, each individual is capable of producing eggs, and any two, sexually mature individuals are able to be mutually receptive in cross fertilization. Mating generally for both slugs and snails occurs at night and can last for several hours, and breeding cycles typically occur under the wettest, warmest conditions. Snails will usually deposit partially or heavily calcified eggs, which provides the developing embryo with the calcium it needs to make a shell. Eggs will either have a jelly-like layer, which helps prevent drying, or they absorb their moisture from the soil. Eggs are usually laid in holes or crevices in the soil, or on the surface of the soil but covered by stones, wood, or other organic debris. Often fecal pellets are deposited near the eggs. Some snails and slugs lay their eggs clumped together, and some, like the Cuban slug, lay their eggs in a spiral held together by a strong strand of mucus with the eggs strung to it like pearls. The number of eggs varies widely between species (South 1992).

Snails and slugs are generally active at dusk, through the night time hours and into the early morning hours when conditions are wet and cool. The timing of activity can vary depending on species, temperature, and season. Slugs and snails are omnivorous and will consume a wide range of food including fungus, mosses, and lichen, however they can show a high degree of selection in their feeding behavior. Some species are coprophagus and will feed on feces, and some species are carnivorous and will feed on carrion and living invertebrates including other slugs and snails, earthworms, and insects. When conditions become hot and dry snails will aestivate, they will draw up into their shells and secrete very thin, parchment-like covering over the shell opening called an epiphragm. This will prevent the snail from drying out,

and the snail can survive even dry, desert conditions. Slugs are relatively tolerant to water loss and will often group together to minimize water loss. In dry conditions some slugs will burrow into the soil and create a crevice with mucus to wall themselves in, awaiting the rain again. Others will burrow and congregate together on the underside of a rock. Slugs and snails will often follow each other's slime trails and will often form attachments to particular shelters, especially during hot or dry weather (South 1992).

Education: Learn to identify common, non-native gastropod species

A set of laminated identification cards consisting of common species found on Hawaii Island were given to each school. These cards were used by students to help identify slugs and snails found under the traps. Identification of some species can be difficult. Identification requires dissection and examination of reproductive organs by microscopy, which is beyond the realm of this project. Experts were sought out for help with species identification by properly photographing the species in question. The unidentified specimen was photographed from the right side, showing the position of the pneumostome in relation to the body, and the length of the mantle covering the body. The specimen was photographed from above, to identify if the specimen has a keel, a ridge-like protrusion such as that on the semi-slug *Parmarion martensi.* An object of recognizable size, such as a coin, pencil, ruler, etc. was placed in the photo view as a reference to the specimen's size.

Education: Invasive terrestrial gastropods as pests of native species

Students learned the negative impact the introduction of non-native snails, slugs and predacious flatworms has had on the extinction and endangerment of Hawaii's endemic snail species. Students also understood the negative impact non-native gastropods have on native plant species and forest regeneration.

Education: Incorporate safety practices

Students were educated in the proper handling of slugs and snails found under shelters or when conducting searches. Flatworms were also collected as they can be paratenic hosts of the RLW. Gloves were worn at all times and tongs or chopsticks were used when collecting. For shelled species or those found in locations that are difficult to access with tongs or chopsticks, the use of non-permeable gloves was required for handling. Slugs and snails were deposited into a "slug jug," a one gallon, wide-mouth plastic jar filled with ½ gallon of tap water and one and 1/8 cups of table salt. A high-salinity (15%) mixture has been shown to be effective at killing the slug and the rat lungworm parasite (Jarvi lab, unpublished). The jugs, salt, tongs, chopsticks, and gloves were supplied. When targeted organisms were added to the slug jug students were instructed to avoid looking directly into the jug and to avoid any splashing. Bleach was occasionally added (1/4 cup) to the slug jug to help dispel unpleasant smells. When sufficiently full, the jug was emptied on a gravel roadway or in an area where weeds or plants are not desired to grow. A time of 5-7 days was allowed to pass between the addition of the last organism and emptying the contents of the jug to ensure the death of any parasite that might have been harbored in the target organisms.

Education: Understand the rat lungworm lifecycle

The lifecycle of the rat lungworm is complex and has been described in chapter one. It is important that the general public understand the lifecycle, be able to identify where the infective stage larvae could be harbored, and understand why control of hosts is crucial to prevention. If students are to be educators for their families, they must have enough familiarity with the lifecycle so that they can explain it to others. Students at all of the partner schools worked in groups to model the rat lungworm lifecycle using modeling clay. Students used the information

to explain the lifecycle to individuals and classes not involved with the project (Fig 2.14 a). Students used art to compare and contrast the lifecycle of rats, and slugs and snails.

Education: Paratenic and accidental hosts

Paratenic hosts, organisms that can also harbor the rat lungworm parasite, are discussed in chapter one. Students should understand that there are paratenic hosts that can harbor the rat lungworm and be able to name some of these organisms. Particular emphasis was placed on flatworms, in particular *Platydemous manokwari.* Flatworms found under shelters were included as organisms targeted for control and those found were recorded on data sheets.

Students should understand that humans and other animals can be accidental hosts if they are exposed to infective stage rat lungworm larvae. Accidental hosts can include dogs, horses, primates, monkeys, and other species including birds, and this information was discussed with students as well as measures they can take to protect their pets.

Education: Rat Lungworm Disease transmission, symptoms and treatment, and prevention

Students were informed of the various known routes of infection; ingestion on raw or undercooked food, paratenic hosts, water, as well as potential transmission through skin, eyes, and mucosal tissue. As rainwater catchment is common in many communities served by the partner schools, catchment design and maintenance was discussed as were other diseases that can be transmitted through water in catchment tanks such as mosquito, lizard, rat, and bird-borne diseases. Students understood disease symptoms through discussion and communication with individuals who have experienced the disease. A community member who had contracted the disease in Fall 2014 was invited as a guest to each of the partner schools to relate his story and experience with rat lungworm disease. Disease prevention topics included food preparation demonstrations, which showed how to check and clean food with special attention paid to

bunching or heading vegetables, such as celery and lettuces. Discussions included the effects of cooking and freezing on the parasite.

Students (KANU) from the 9th grade, cellular biology compared rat lungworm disease with other degenerative brain diseases (Parkinson's, Alzheimer's) and acquired brain injuries. Students researched symptoms and treatments for these other diseases and were able to compare and contrast diseases and treatments. Students understood both western and alternative treatments, including acupuncture and the use of supplements, for different types of brain injury, and addiction that medications used might cause. Students researched the cellular biology of the central nervous system. Student teams made PowerPoint presentations of their findings to the class.

Data recording

Data collection occurred on a regular basis during the Fall 2015 semester but not during the Spring 2016 semester. At each school involved in the project, data sheets were distributed to the teacher who distributed them to students who were responsible for helping record data, and teaches rotated the student helpers. The project leader also collected data and assisted the student helpers. Data were collected for later analysis of species variation and frequencies, shelter-type capture rate, and hand-collection capture rate. A rain gauge was provided to each school and temporal data were collected to determine the correlation, if any, with population levels. When examining shelters and recording data, slugs, snails, and flatworms were identified by their scientific name using a 3 letter abbreviation format (e.g. *Parmarion martensi* = PAR MAR). Students from the 7th and 8th grades at one of the schools (KOKL) graphed data collected as part of a math class using Sheets, a program similar to Excel

that is available to student computers in Google Drive. Students made bar graphs and pie charts to represent data they collected.

Analysis of slugs, snails, flatworms by qPCR

Slugs, snails, and flatworms found under shelters during the first school visit in the Fall 2015 semester were collected and taken to the Jarvi lab for qPCR to determine infection by *A. cantonensis*. Tail snips were taken from the specimens and tissue digestion, DNA extraction, and qPCR was the same as that described used in all studies in chapter one.

Reporting

Photography was used as one method for reporting on various IPM and educational activities. The project leader provided a camera to a student during each school visit, and that student was responsible for photographing the events of that visit. The photographs taken were used as part of a story map or for educational purposes at the school. The project also used the ArcGIS online, story map application. The ESRI, ArcGIS software is available free of charge to all K-12 schools in the State of Hawaii and allows users to create and share maps, scenes data, and applications, which can be edited and published. Partner schools were encouraged to apply for the technology so that each school could create a story about their experience that could be shared with others online. At the completion of the Spring 2016 semester, a workshop was given, and the project leader and teachers and students from the five partner schools shared their experiences and project results with teachers, school garden personnel, and the general public.

Results

School visits

A total of 85 school visits were made from July 2015 through May 2016. There were a total of 11 classes from the five partner schools involved with the project. This included three 5th grade classes, three 6th grade classes, two 7th grade classes, one 8th grade class, one 9th grade class, and one 12th grade class. Approximately 175 students and six teachers participated in the project. As this was a pilot study teacher involvement was optional, and teachers involved included school garden teachers as well as classroom subject teachers for science, math, technology, biology, and environmental science.

A total of 49 school visits were conducted while students were in session in the Fall 2015 semester, with a range of 8-12 visits per school over the 4.5-month semester. School breaks, holidays, teacher availability, and class events account for variation in the numbers of visits between schools. Shelters were checked at each school visit for the Fall 2015 semester. All schools were visited at least once a month in the spring semester, with the exception of Kua o ka La PCS in Kapoho, which requested weekly or bi-weekly visits. This school is located in what has been known to be one of the most infected areas where a number of cases have originated, and an alumnus of this school was infected with RLWD in spring 2015 after syphoning from his rainwater catchment tank due to power loss. The extra visits allowed for students and their teacher to engage in data analysis and use of the ArcGIS online software for reporting outcomes.

Integrated pest management

The partner schools welcomed the introduction to and integration of integrated pest management as part of their school garden projects. There were a total of 50 shelters placed, ten at each school with five each in two locations in or near the garden area. The shelters were

labeled with informative signage (Figs. 2.2a, b). The use of shelters and searching for slugs, snails, and flatworms were the only methods of control used at four of the five schools. Because of the heavy infection rate of *Cornu aspersum* at the school in Waimea (KANU), a sulfur-based slug/snail control bait (Bug-Geta™) was applied during the three-week winter break. This was done in an effort to control snails that would be emerging from the many eggs deposited in the soil by the large population of adult snails collected from the campus.

Fig. 2.2 (a, b): Shelters made from five different materials were placed in two locations at each school site (a). Signage defining the project and IPM was included at each shelter site for the education of all (b).

Safety measures

Students gained an understanding of why it is important not to touch slugs, snails, or flatworms with bare hands and to use tongs or gloves when collecting them. With the aid of their teacher, students made slug jugs from one gallon, largemouth jars with lids, adding 1 and 1/8 cups of salt to ½ gallon of water, and labeling the jug with waterproof marker as a slug jug and a safety warning to inform other students of its use and to leave alone. All slugs, snails, and flatworms were dispatched into the jug when found (Fig. 2.3 a). The one exception was at one school (KOKL), which is a traditional Hawaiian place of refuge (Puuhonua) and no animal is allowed to have its life taken at that place. Any invasive gastropod or flatworm found was taken off the school grounds to another location where it was placed into a slug jug.

Students also learned that some slugs, snails, and flatworms are predacious and will eat other living or dead slugs, snails and are organisms that could be infected by the rat lungworm parasite. This is the reason why it is best to put the slugs, snails, and flatworms in a slug jug rather than squashing them on the ground (Fig 2.3 b). Student also learned that the parasite can live outside of the dead host for some time in wet conditions and can contaminate soil or other materials, another reason why it is best to use a slug jug. Some students made informative posters (VSAS) and tee-shirts (NAWA) with slogans about the need to control invasive slugs and snails. Some students also made slug jugs with instructions for use, and sold them at a school fair that was attended by parents and the community (Fig. 2.4a, b).

Fig. 2.3: (a) Directions given to students: Wear gloves and use a tongs of chopsticks to place slugs, snails, flatworms in the slug jug. (b) Don't squash slugs or snails as that will not kill the rat lungworm parasite.

Fig. 2.4: (a) Students at NAWA, with their teacher's assistance designed and silkscreened T-shirts with the message to control invasive gastropods by jugging your slugs. (b) Students (NAWA) decorated slug jugs and provided educational material for parents and community, such as the 2nd grade activity book, The Mystery of Rat Lungworm Disease® (UHH DKICP, 2012).

Slugs, snails, flatworms removed from all schools

A total of 4126 non-native terrestrial slugs, snails, and flatworms were recorded and safely removed from the campuses of the five partner schools in the Fall 2015 semester (Table 2.1). The greatest numbers were removed from the Kanu o ka Aina (KANU) campus in Waimea. This school had an infestation of *Cornu aspersum*, more commonly known as the European garden snail, and these snails were easily visible throughout the campus, on buildings, rain barrels, ornamental native plantings, on pavement, and also under the shelters. Of the total number of slugs, snails, and flatworms collected at the partner schools, 3415 were *C. aspersum* that were collected at the Kanu o ka Aina PCS campus. This was the only school where we did whole-campus searches, and this was due to the intensity of the population, the potential risk to students, and the damage the snails were causing to the native plantings on the schools' campus. It should be noted that Kanu o ka Aina is a Hawaiian culture-based school and the plants on the school ground, such as ti (*Cordyline fruticosa*) and mamaki (*Pipturus albidus*), are used for ceremonial, medicinal, and/or culinary purposes. Collection of this species outside of the school

garden area was conducted by the 12th grade, Environmental Science class, who collected 2720 just during the Fall 2015 semester. Another 296 were collected by 6th grade students from the school garden area. The total number of slugs, snails, and flatworms removed excluding *C. aspersum* was 1020 from all five of the partner school garden areas during the Fall 2105 semester (Table 2.1). The most common species found included *Veronicella cubensis* (34%), *Derocersas* sp. (26%), and *Subulina octona* (23%). The greatest number of target organisms found under one trap at one time was 52 at Kanu o ka Aina (KANU).

Table 2.1: List of species and number of each found at the five partner schools. Flatworms are also included as they can be found in the gardens and can be paratenic hosts of the rat lungworm parasite. *Cornu aspersum* comprised 76% of the total population collected. VSAS (Volcano School of Arts and Science PCS); KOKL (Kua o ka La PCS); NAWA (Na Wai Ola PCS); LAUP (Laupahoehoe PCS); KANU (Kanu o ka Aina PCS). ACH FUL (*Achatina fulica*), COR ASP (*Cornu aspersum*), DER sp. (*Deroceras* sp.), LAE ALT (*Laevicaulus alte*), LIM MAX (*Limax maximus*), MEG sp. (*Meghimatium* sp.), PAL sp. (*Pallifera* sp.), PAR MAR (*Parmarion martensi*), SUB OCT (*Subulina octona*), VER CUB (*Veronicella cubensis*), PLA MAN (*Platydemous manokwari*), F.W. (shovelhead flatworm), Snail (unidentified small snail).

School	ACH FUL	COR ASP	DER sp.	LAE ALT	LIM MAX	MEG sp.	PAL sp.	PAR MAR	SUB OCT	VER CUB	PLA MAN	F. W.	Snail
NAWA	0	0	1	1	2	2	11	0	213	237	1	3	0
VSAS	0	0	7	0	9	3	0		0	0	0	10	0
KOKL	1	0	0	0	0	0	0	13	0	67	6	3	0
LAUP	0	0	0	30	1	0	0	15	19	36	4	15	0
KANU	0	3106	251	0	0	1	1	0	0	1	34	4	8
Total	**1**	**3106**	**259**	**31**	**12**	**6**	**12**	**28**	**232**	**341**	**45**	**35**	**8**

Identification

Some of the slugs and snails found at the schools were not represented in the pack of cards. Therefore, photographs were taken of the common species found at each partner school to be used for identification guides relative to each school. Species differed from high elevation (Fig. 2.5, 2.6) to mid-elevation (Fig. 2.7, 2.8) to low-elevation (Fig. 2.9), although there was some similarity between elevations and locations.

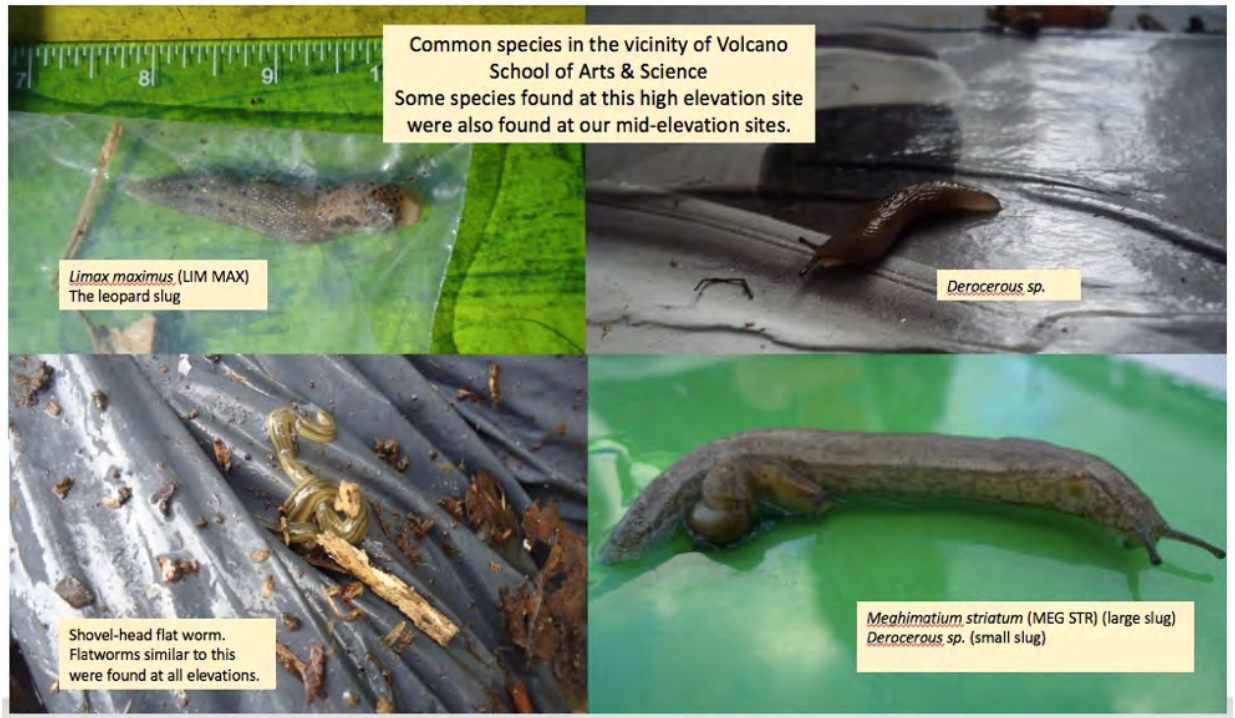


Fig. 2.5: Common high elevation species, Volcano (VSAS).

Fig. 2.6: Common high elevation species, Waimea (KANU).

Fig. 2.7: Common mid-elevation, Laupahoehoe (LAUP).

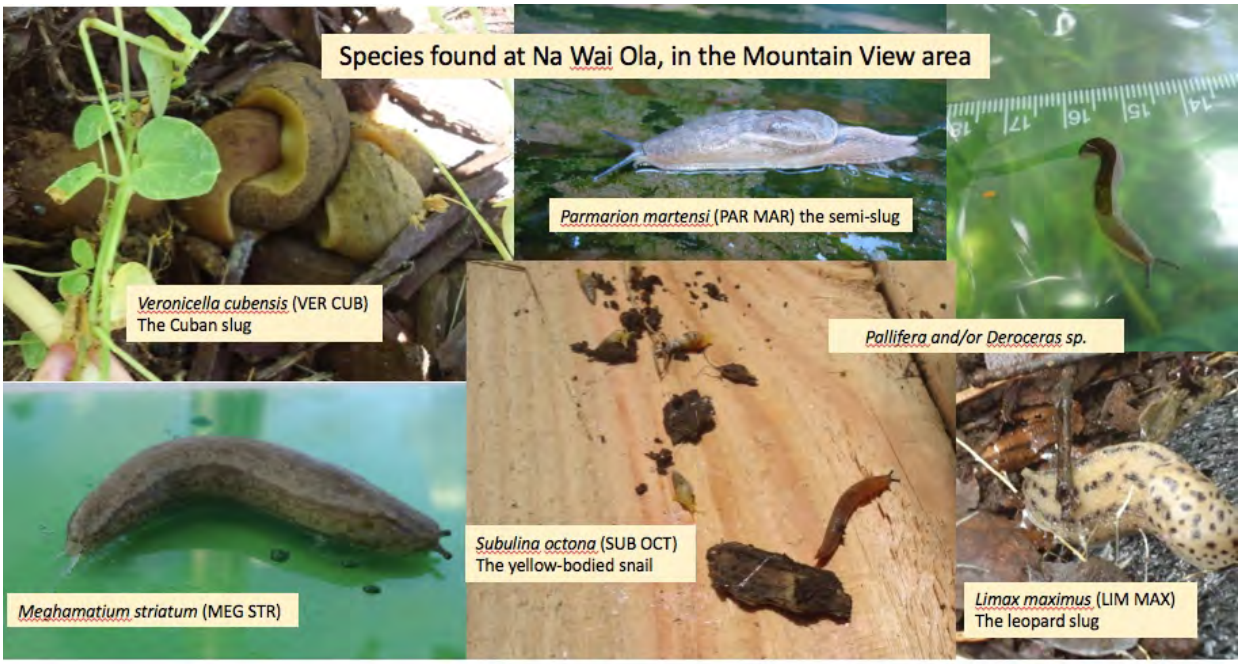


Fig. 2.8: Common mid-elevation, Mountain View (NAWA).

Fig. 2.9: Common low-elevation, Kapoho (KOKL).

Shelter-type capture rate

The capture rate of shelters-types and other methods (not found under shelters or found by searching) were 71% (other), 11% (cardboard), 9% (wood), 5% (reflective), 2% (plastic), and 2% (mesh) (Table 2.2, Fig. 2.10). Cardboard had a highest capture rate of any of the shelters and was the cheapest to make as the material was free. The category "other" refers to locations where slugs, snails, or flatworms were found other than a shelter. Because of the infestation of *Cornu apsersum* at one of the partner schools (KANU) a whole campus search was conducted, which resulted in a large percentage of the total population to be found in locations other than under the shelters. A similar experience occurred at another school (KOKL), which located near an area of recent volcanic activity. The substrate at this school garden was cinder, unlike the soil substrate found at each of the other schools, and the rough, dry substrate did not provide good habitat for slugs, snails, or flatworms, therefore very few individuals were found under the

shelters. However, slugs, snails, and flatworms were found under various materials in and around the garden, such as in or under plastic, flowerpots, wood, buckets, etc. Finding these non-natives gastropods and flatworms under objects other than the shelters did occur at every one of the partner schools, and therefore it must be noted that searching for target organisms is an excellent method for reducing populations. All searches were carried in the daylight during school hours (Fig. 2.11 & 2.12).

Table 2.2: Capture rate of shelter-types and other methods. Searching for slugs even in daylight hours is a very effective method of control.

Shelter/ collection type	**Cardboard**	**Wood**	**Reflective**	**Mesh**	**Plastic**	**Other**
Number collected	411	312	190	58	54	2571

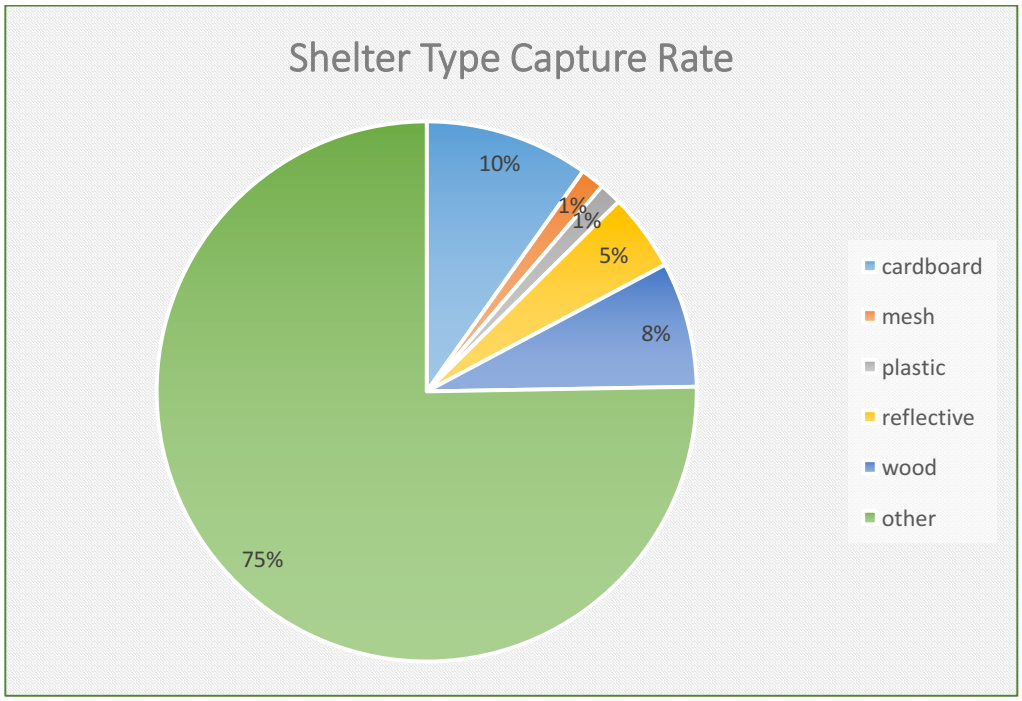


Fig. 2.10: Pie chart of shelter-type capture rate. Collection of target species in areas other than Under shelters was high as the numbers were impacted by the whole-school collection at KANU.

Fig. 2.11: Students from 5th grade check the shelters (VSAS).

Fig 2.12: Searching for snails with 12th grade class (KANU).

Diagnosis of A. cantonensis infection

Funding for this project was limited and therefore only a small percentage (0.6%) of the total population of all schools was tested. A very faint signal for *A. cantonensis* was detected in one *Limax maximus* collected at VSAS. Three *Platydemous manokwari* were positive by qPCR analysis and by microscopy for *A. cantonensis* at KOKL.

Data collection

While data were consistently recorded at all schools during the fall semester, when support was given on a weekly/biweekly basis, consistency in recording declined during the spring semester when recording was to be done independently by teachers and classes. Only three of the five schools collected data and this was not on a regular basis, showing more support for this activity is needed. For this reason, the data from the spring semester was not included in analysis. Data collected on rainfall amounts was also intermittent with only one school, which had its own rain gauge, providing the most accurate and consistent rainfall data. Reasons for the inconsistency at the other schools was related to loss or breakage of the rain gauges. With guidance, students became proficient at identification of the most commonly found, non-native gastropods and flatworms at their schools and were able to effectively record data (Figs. 2.13 (a, b), 2.14).

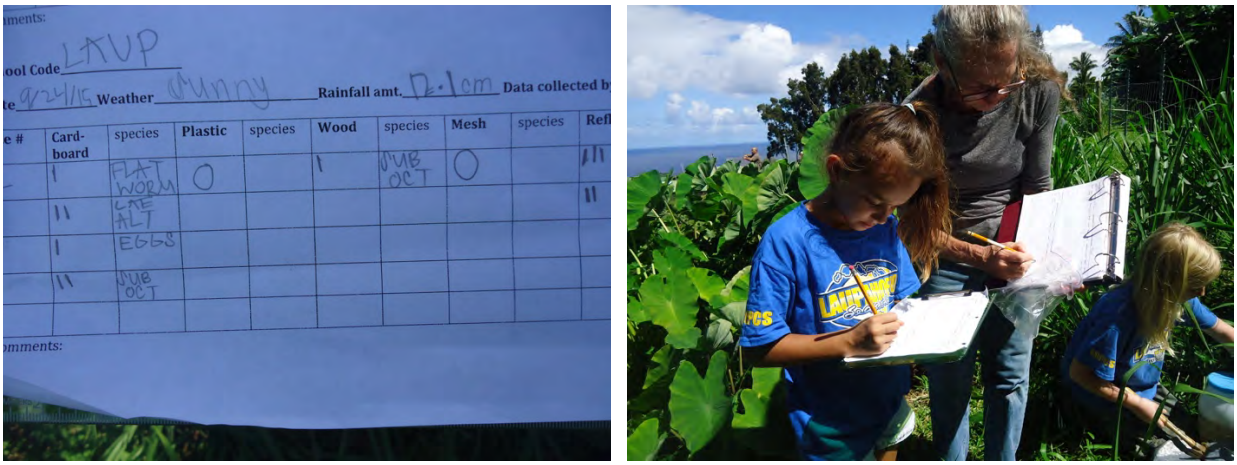


Fig. 2.13: (a)Student records information on data sheet. (b) Student is assisted by project leader in data collection

Fig. 2.14: Students record rainfall information.

Data analysis

Students were able to see how bar graphs and pie charts can be used to represent the data they collected. Students were able to see the value of using graphs and charts as a way to view numbers in a pictorial form that simplifies our understanding of the results (2.15).

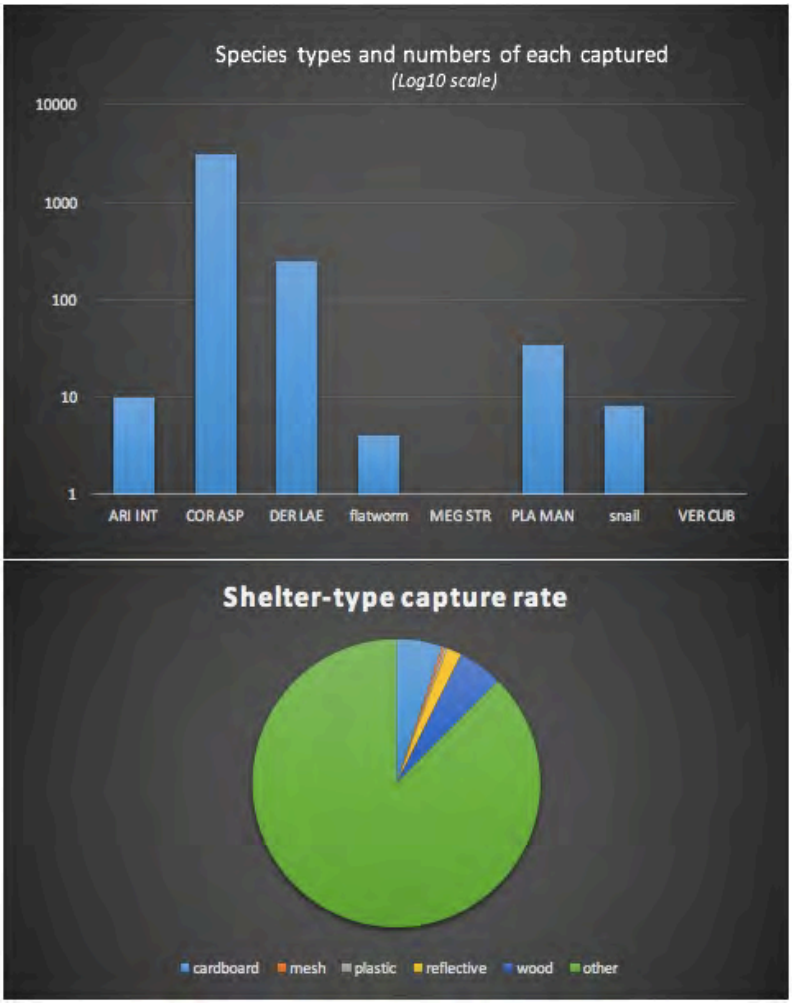


Fig. 2.15: Students used pie charts and bar graphs to display information. This is an example from KANU, where large numbers of *C. aspersum* were collected.

Parasite and host life cycle

Students and teachers found 3D modeling in clay to be an engaging and useful method for students to gain an understanding of the complex lifecycle of the rat lungworm. After the activity students were able to explain the lifecycle to individuals and classes not involved with the project (Fig 2.16 a). Students effectively used art to compare the lifecycle of rats, and slugs and snails and were able to identify differences and similarities in their lifecycles. (Fig. 2.16 b). Most students were able to explain the lifecycle and understood in which host the infective stage of the rat lungworm parasite would be found and why control of those organisms is important.

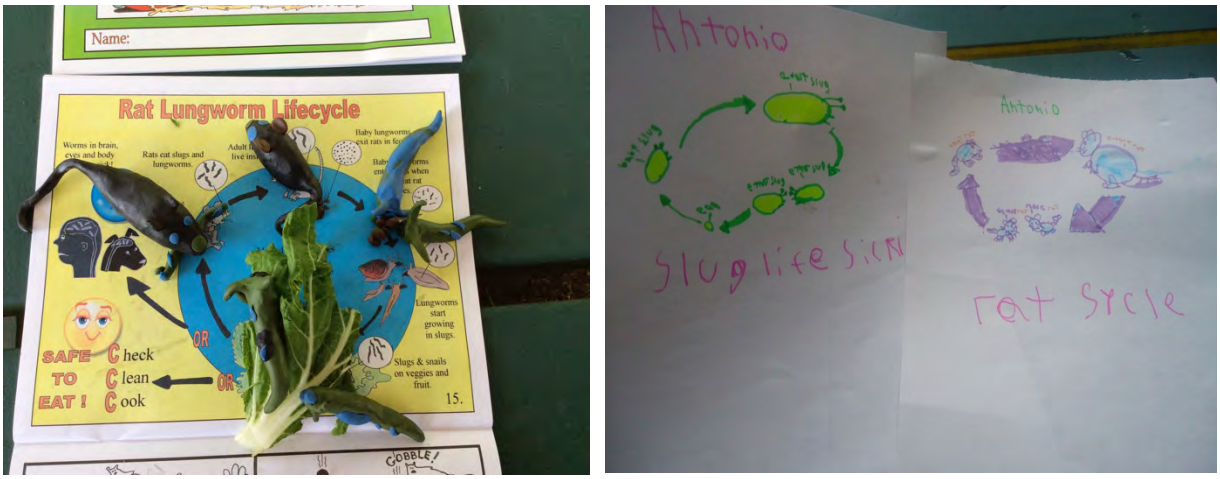


Fig. 2.16 (a, b): Three dimensional modeling of the rat lungworm lifecycle using reference materials produced by the UHH DKICP for guidance in the exercise (a). Students also compared the lifecycles of rats, and slugs/snails (b).

Disease prevention

In addition to learning how to safely handle slugs, snails, and flatworm, students were able to identify organisms that are paratenic hosts (shrimp, prawns, frogs, and lizards), and students understood cooking or freezing any vegetable or paratenic host will kill the rat lungworm parasite if the organism is infected. Students were able to demonstrate proper preparation of leafy greens and that drying will kill the RLW parasite.

Disease symptoms, diagnosis, treatments

Students at three of the five schools (KOKL, NAWA, LAUP) reported that family members, friends, and/or pets that had contracted rat lungworm disease and these students had some prior understanding of the disease symptoms and treatments. The visit by the community member who was a victim of RLWD had a marked impact on both students and teachers, with one garden teacher reporting that she had not been too concerned about the disease prior to his visit, but his visit made such an impact on her that she realized she never would want to contract the disease and would take great efforts for prevention.

Students from the 9th grade Cellular Biology class were able to explain the similarities and differences between rat lungworm disease and other degenerative brain diseases and acquired brain injuries including symptoms and treatments. Students gained an understanding of western and alternative treatments, including acupuncture and the use of supplements, and problems with addiction that medications used might cause, such as the use of opiates for pain. Students gained an understanding of the cellular biology of the central nervous system.

Reporting

An ArcGIS online story map about the project was published and is available for public viewing at the following link (Fig. 2.17).

https://uhh.maps.arcgis.com/apps/MapJournal/index.html?appid=5ccc2fa5cf7b441fa2711c5b60e853b6

Two of the five schools submitted applications for the software through the Hawaii K12 GIS Distribution Center, a Women in Technology partnership with ESRI and the Hawaii Department of Education (http://www.womenintech.com/GISDistributionCenter/). One school (KOKL) acquired the software and students worked together to individually create a page for a story map that told of their school's experience with the project. Their story map was not published as it is still incomplete. Students enjoyed using the software although some issues with the software were noted, particularly that any photographs used had to be uploaded to a public site, such as Facebook, Flickr, or Picasa, and school rules did not allow students to use these sites. It would be beneficial to work with the technology partnership to identify some of the issues encountered and see how they can be addressed.

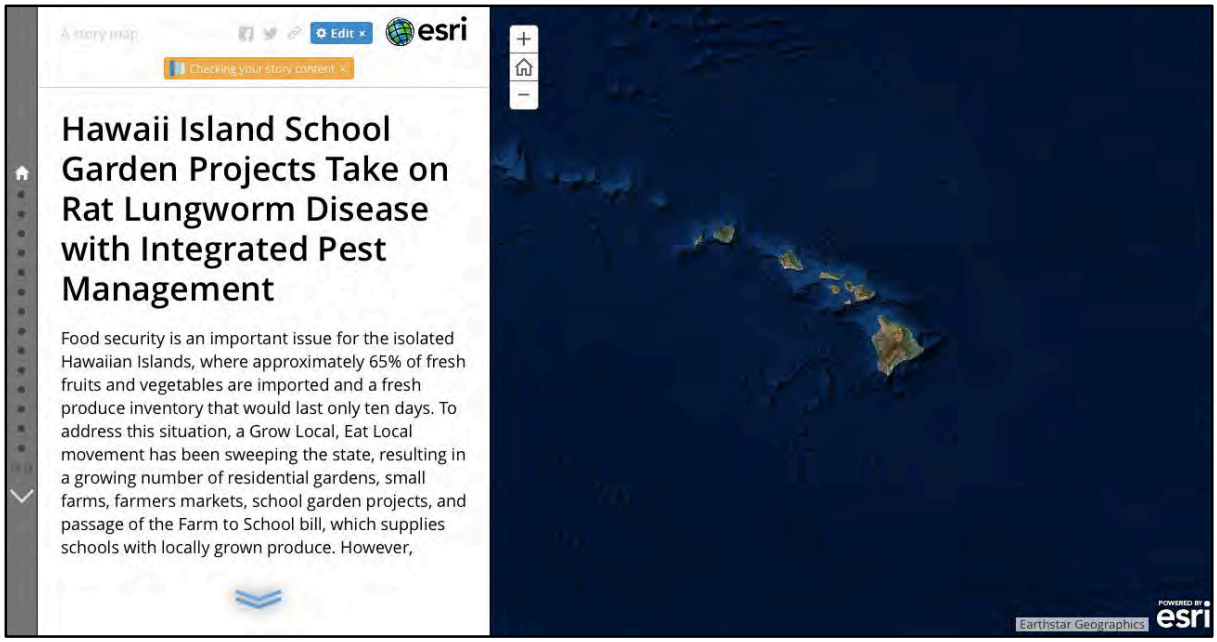


Fig. 2.17: Published ArcGIS online story map reported the results of the project. The map is available to view at https://uhh.maps.arcgis.com/apps/MapJournal/index.html?appid=5ccc2fa5cf7b441fa2711c5b60e853b6&edit

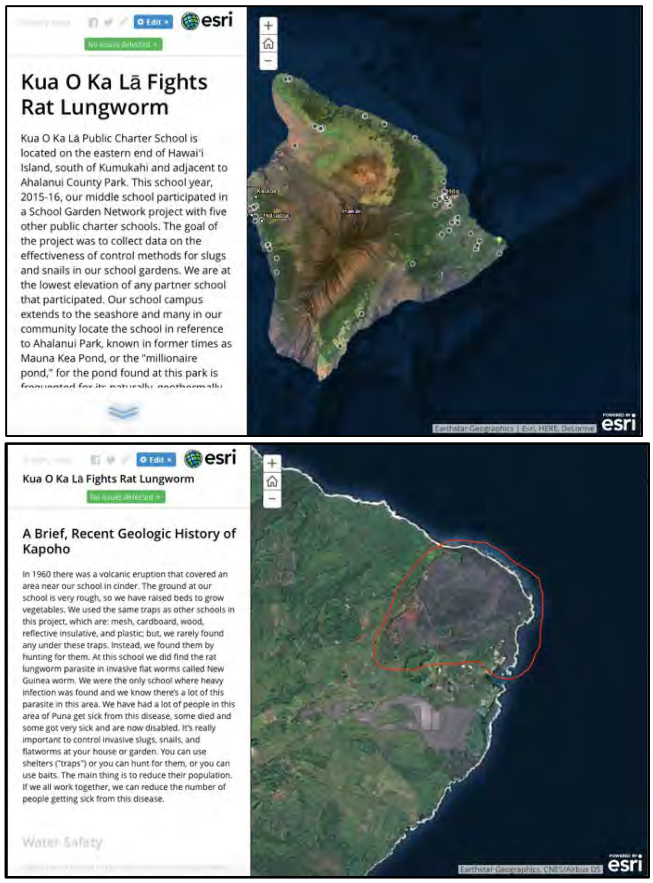
Community educators

Each class from every school involved with the project was required to share the information they had learned about the rat lungworm parasite, the disease it causes, and disease prevention with other students from their schools. In Hawaii this is called a hoike, a final test or exhibition of knowledge. Students shared information by giving speeches, making posters and PowerPoint presentations, skits or puppet shows, and creating t-shirts with informational messages. A workshop sponsored by the Kohala Center and the Hawaii Island School Garden Network was held at UHH at the completion of the Spring 2016 semester. At the workshop students and teachers shared their story and experiences with other educators, politicians, and the general public. The workshop was filmed by Na Leo TV (http://naleo.tv) and can be found in the video stored on their website under "Rat Lungworm Disease Forum." One student independently made a very professional, informational website for community education

purposes using content and videos he found published online, as well as facts he learned from the project (http://rat-lungworm-disease.weebly.com/). All students showed enthusiasm in their roles as educators as they understood the implications the disease has for their communities and way of life. Students and teachers involved in the project expressed the sentiment that they were not afraid of the disease as they were confident in their understanding of how to prevent transmission. Some educators and administrators have expressed the sentiment that school garden projects should be shut down because of the risk of infection, but this action will not prevent a student from getting infected from exposure at their own homes. Education is key to prevention.

Curriculum integration

This project was designed as an example of place-based, integrated curriculum using a framework for education that includes the use of interdisciplinary, hands-on, student centered learning (Lieberman & Hoody, 1998). In addition to the science, technology, engineering, math, and art components the project encompassed, other subject were applied as well. The story map provided an excellent opportunity for students to develop maps, study area geography, and develop paragraphs for pages that fit together to tell a story (Fig. 2.18 a,b). The language arts were also incorporated in the educational speeches students gave to students from other classes that did not participate in the project, and in posters students made that were put up around their school (Fig. 2.19 a,b). Students at some of the schools used the performing arts as a way to share what they learned with other students from their schools (Fig. 2.20). Critical thinking skills were applied as students designed their own slug and snail shelters based on observations and knowledge acquired about slug and snail behavior (Fig. 2.21 a,b).



Figs. 2.18 (a, b) Two of the pages of the ArcGIS online story map developed by students at KOKL. The story map provides a technology component, as well as opportunities for inclusion of language arts and social studies, among others.

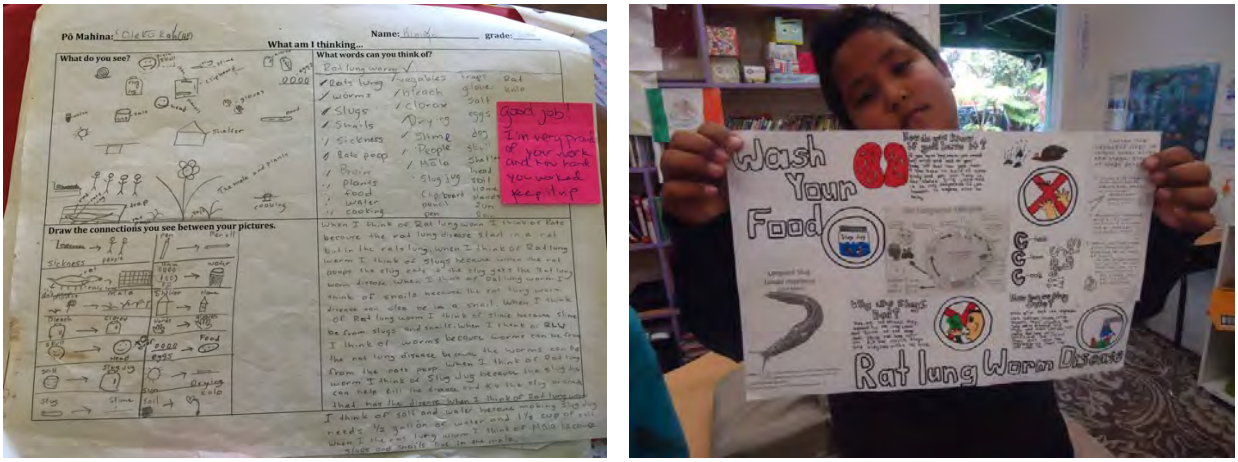


Fig. 2.19. Students develop (a) speeches (NAWA) and (b) posters (VSAS) to help educate other students.

Fig. 2.20. Students perform the story of "The Mystery of Rat Lungworm Disease" for younger students (KOKL).

Fig. 2.21. Students use (a) recycled (VSAS) and (b) natural materials (LAUP) to make slug and snail shelters.

Discussion

During the course of this project we safely removed over 4000 invasive slugs, snails, and flatworms from the five participating schools using low cost, non-toxic methods, in keeping with the practices of integrated pest management. Students and teachers learned and understand how to safely collect and dispatch non-native gastropods and planarians that can host the RLW parasite. The schools, teachers, and students involved became thoroughly educated about the rat lungworm parasite and disease prevention, they understand the importance for control of hosts of the parasite around their schools, gardens, and homes, and they are capable of sharing the information they learned with others including their schoolmates, families, and friends.

One student came from a farming family in an area where slugs and snails were commonly found, and as a result of the project she was able to relate her parents the need for and how to control slugs, snails, and flatworms at their farm. In turn, her parents are able to educate their customers as to how to safely prepare fresh, locally grown produce. Many of the students have rainwater catchment as the source of their water at home, and they were able to relate to their parents and families the need for proper maintenance of the system and the need for control of hosts of the rat lungworm around their home and water tank. At the end of the Spring 2016 semester, a workshop was held at UHH to share the project and its results with other school garden projects and with the general public.

Data recording was consistent during the Fall 2015 semester, when the project leader assisted with the collection. The recording was much more inconsistent for the Spring 2016 semester when teachers were given the responsibility for data collection. This reflects on the need for a written management plan with associated educational activities. While the recording

of data was inconsistent, the level of interest in the project and student and teacher enthusiasm remained high, and the teachers and students were dedicated to providing education outreach for their schools and families. With the exception of one teacher, all teachers from all schools regularly participated in the lessons and activities. Positive feedback was given by principals and teachers at all schools, and schools not participating in this project expressed a desire to participate in the future.

Conclusion

In 2013, a second grade student from one of the schools piloting the development of "The Mystery of Rat Lungworm Disease" activity book said to me "I tell my little brother not to touch the slugs and snails." This statement demonstrates the value and importance students have as community educators for hard-to-reach rural populations. Young children are very likely to notice potentially dangerous actions of their baby brothers' and sisters,' such as handling slugs and snails or even putting them into their mouths. These types of actions have resulted in deaths and permanent disabilities of very young children both in Hawaii and other countries. Decisions to avoid discussion of the rat lungworm and the disease it can cause, so as not to frighten the public, does nothing for prevention and perpetuates the occurrence of the severe cases of rat lungworm disease we are witnessing today in Hawaii. A greater emphasis towards public education and the initiation of and support for a campaign by county and state agencies to control the hosts of *A. cantonensis* is crucial if we are to bring this crisis back to a manageable level in Hawaii.

Presentations on this project have been given at the Hawaii Environmental Education Association Symposium, held in Honolulu, Hawaii in July 2016, and at the 4th International Rat

Lungworm Workshop, held in September 2016 in Brisbane, Australia. Both presentations were well received. After the presentation at the international workshop in Australia it was noted that the Hawaii Island Rat Lungworm Working Group currently leads the world in rat lungworm disease education (Richard Malik, personal communication). Funding to continue this project was recently granted by the Hawaii Invasive Species Council and will support the development of a written IPM plan for control of the intermediate hosts of the RLW parasite and an integrated STEAM curriculum to accompany the management plan. These materials will be made available to all school garden projects in Hawaii and will be a helpful step for school garden and classroom teachers to adopt the activities and curriculum, and will aid in keeping our children and communities healthy. In addition to the management plan and curriculum, assessments will be developed that will track the project's success. This next step of the project will be done in collaboration with teachers involved with the pilot project described in this chapter, as well as other educators and entities that will be identified as important contributors for success. While the materials will focus on RLW and RLWD, there will be opportunities to discuss other diseases that are foodborne, or may be harbored in rainwater catchment tanks such as leptospirosis, salmonella, e-coli, listeria, dengue, zika, and malaria, diseases that are relevant to food production and health for Hawaii.

This project is in keeping with the Food Safety Modernization Act, which was signed into law in 2011, and focuses on preventing contamination and reduction of foodborne illness. The project has the potential to create a large data base to track populations of invasive slugs, snails, and flatworms, and can provide state agencies, such as the Department of Health and the Department of Agriculture, with information crucial to identifying areas of potential RLWD outbreaks and early detection of arrivals of important intermediate hosts, such as *P. martensi*.

Through networking with the Hawaii Island School Garden Project and the Hawaii School Garden Hui, we can raise a generation of producers and consumers who are aware of the parasite's presence in Hawaii, the necessity for control of its hosts, and understand how to prevent disease. The educational materials and practices developed in Hawaii will be a useful template for the development of similar projects for other U.S. mainland states and territories, and for other countries where *A. cantonensis* is found.

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