THE RAT LUNGWORM, *ANGIOSTRONGYLUS CANTONENSIS*: INTERMEDIATE

HOSTS AND DISTRIBUTION IN HAWAI'I

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Abstract

Eosinophilic meningitis caused by the parasitic nematode *Angiostrongylus cantonensis* is an emerging infectious disease in tropical/subtropical locations. Humans contract it through ingestion of infected gastropods, the intermediate hosts of *A. cantonensis*, rats being the definitive hosts. The goal of this thesis is to better understand the host range and geographic distribution of the parasite in Hawaii by: 1) identifying the gastropod species that act as hosts and assessing their parasite loads; 2) ascertaining the distribution of *A. cantonensis*, modeling the environmental factors influencing it, and predicting habitat suitability throughout Hawaii. Numerous gastropod species (16 of 37 screened) tested positive for *A. cantonensis*, with a large range of parasite load among and within species. The parasite occurs on five of the six largest islands (not Lanai). A habitat suitability model using mean annual temperature and rainfall predicted that windward areas (higher rainfall) and lower elevations (higher temperatures) were most suitable.

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Preface

The research presented in this thesis has resulted from the efforts of various people. The two main chapters (Chapters 2 and 3), which will be published as peer-reviewed papers and are therefore formatted as such, will both be multi-authored, with myself as the lead author. This preface explains the rationale for the authorship of these two papers.

Robert H. Cowie, my advisor, is an author on both papers, and has provided valuable advice and guidance in writing all chapters. Additional co-authors on both papers are Norine W. Yeung and Kenneth A. Hayes who helped with project design as well as survey and laboratory work. Patrick A. Curry will be a co-author on the paper derived from Chapter 3 because of the extensive advice and help he provided for data analysis and interpretation. For both projects, I conducted the majority of the research and wrote both manuscripts with input from all co-authors.

Chapter 1. Introduction: a study of *Angiostrongylus cantonensis* hosts and distribution in the Hawaiian Islands

Alien species and emerging infectious diseases

The transport and dispersal of alien species is a worldwide problem, becoming more serious with increasing globalization and human activity (Bryan 1996; Jenkins 1996; Perrings et al. 2002; Pimentel 2002; Pimentel et al. 2005). The significant environmental change brought about by humans and the rising frequency at which alien species are invading both contribute to biotic homogenization (Vitousek et al. 1996; McKinney and Lockwood 1999). This replacement of numerous, often highly localized native species with a few species introduced widely by people could potentially reduce biodiversity and has become an issue of great concern (Vitousek et al. 1996; Myers 1997; Baskin 1998). Invasive species are the main reason that 42 % of the species listed as Threatened or Endangered under the U.S. Endangered Species Act are at risk (Wilcove et al. 1998). The annual cost of damage and loss in the United States due to invasive alien species has been estimated at $120 billion (Pimentel et al. 2005).

More than $47 billion of this annual total spent in the United States is related to alien plants and to livestock and human diseases (Pimentel et al. 2005). Although these figures are very rough estimates, they show how detrimental invasive species can be. The rapid movement of alien organisms due to human activity and expansion into new areas appears to be causing the emergence of infectious diseases (Fidler 1996; Pimentel et al. 2005). The predicted spread of infectious diseases is thought to be influenced by a number of factors including changes in climate, population density, habitat alteration, dispersal barriers, human intervention, biotic interactions and disease control efforts (Lafferty 2009). With increasing numbers and diversity of carriers arriving in an area, there may be greater chances for infection of these new or growing populations of potential hosts (Despommier et al. 2007). Changes made to the landscape for anthropogenic purposes are also thought to make additional resources available for vectors or pathogens to expand their ranges into these areas (Despommier et al. 2007).

The volume, speed and reach of travel have increased, creating more opportunities for the transport of pathogenic microbes between continents (Wilson 1996). Alien organisms that can carry these pathogens, including insects, rats and snails, can often be hidden in or on cargo (Andow 2003). These organisms have the potential to cause the emergence or reemergence of an infectious disease (Wilson 1996).

Alien snail and slug species in the Hawaiian Islands

Small landmasses, such as islands, appear to be more susceptible to invasions by alien species (Vitousek et al. 1987). The Hawaiian archipelago is one of the most isolated island groups in the world, located in the Pacific Ocean over 3,000 km from the nearest continent (Loope and Mueller-Dombois 1989). Alien snails and slugs, in particular, are serious problems in the Hawaiian Islands (Cowie 1997, 1998). Lowe et al. (2000) listed two land snails, *Achatina fulica* and *Euglandina rosea*, both present in Hawaii, as among 100 of the world's worst invasive alien species. Once consisting of over 750 species (Cowie 1995), the native Hawaiian land snail fauna is in decline and being replaced by a number of these alien snails and slugs (Cowie 1998). The deliberate introduction of snails, notably the predatory snail *Euglandina rosea*, for use as biocontrol agents in ill-conceived efforts to control the giant African snail (*Achatina fulica*) has had a devastating effect on native snails not only in the Hawaiian Islands but also in many other islands across the Pacific, such as the Society Islands (Murray et al. 1988; Hadfield et al. 1993; Cowie 1998, 2001).

Likewise, the horticultural industry has played a major role in the accidental introduction of non-indigenous snails and slugs (Cowie et al. 2008). The spread of these invasive species has a significant impact on native ecosystems, and human commerce and health (Hadfield et al. 1993; Hollingsworth et al. 2007; Cowie et al. 2008; Joe and Daehler 2008). Of the many invasive snail and slug species found in the Hawaiian Islands, the semislug *Parmarion martensi*, almost certainly introduced inadvertently by the horticultural trade, has been the most frequently implicated vector of a nematode parasite, *Angiostrongylus cantonensis*, the rat lungworm (Hollingsworth et al. 2007).

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***Angiostrongylus cantonensis* and eosinophilic meningitis**

Angiostrongylus cantonensis is a parasitic nematode of many animals, and one of the major causes of eosinophilic meningitis in humans (Wallace and Rosen 1969a; Weller and Liu 1993; Cowie 2013a). Eosinophilic meningitis is characterized in mammals by an increase in eosinophils (specialized white blood cells) in the cerebrospinal fluid or peripheral blood (Koo et al. 1988). This immune reaction is a response to nematodes in the central nervous system tissue (Kliks et al. 1982).

Various rat species are the definitive hosts of *A. cantonensis* and become infected by ingesting contaminated material or intermediate hosts (snails and slugs) containing third stage larvae (Wallace and Rosen 1965, 1969b; Cross and Chen 2007; Chen et al. 2011a) (Figure 1). The third stage larvae enter the bloodstream through the intestine, and then move into the central nervous system (CNS), notably the brain and spinal cord, where they mature to the fifth stage. These fifth stage larvae then re-enter the bloodstream and mature fully in the heart and pulmonary artery, where they reproduce. Females lay eggs, which travel through the bloodstream to the alveoli of the lungs. The eggs hatch into first stage larvae, penetrate the alveoli and move up the airway, to be swallowed and excreted in the feces. Snails and slugs act as intermediate hosts, ingesting rat feces containing the larvae. The ingested first stage larvae go through two molts to become third stage larvae in the intermediate hosts, which are then consumed by the definitive host and the cycle repeats (Prociv et al. 2000; Cowie 2013b).

Intermediate and paratenic hosts of *A. cantonensis*

Although the definitive and intermediate hosts are obligatory hosts in the development of *A. cantonensis*, paratenic hosts are animals that do not support development of *A. cantonensis*, but can accumulate and carry multiple stages of the worm as a result of predation and scavenging (Kliks and Palumbo 1992; Gutiérrez 2000). Paratenic hosts of *A. cantonensis* include freshwater prawns (Alicata and Brown 1962), land planarians (Ash 1976), frogs (Ash 1968), amphibious and land crabs (Alicata 1965a) and monitor lizards (Radomyos et al. 1964).

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While many naturally infected intermediate and paratenic hosts have been identified, studies have also been done to test the susceptibility to infection of a number of other animals. Wallace and Rosen (1967) experimentally infected two fish species, *Trachurops crumenophthalmus*, a commonly eaten fish in Hawaii, and *Tilapia mossambica*, a freshwater cichlid, and concluded that both could be infected by *A. cantonensis*. Wallace and Rosen (1966) and Ash (1968) experimentally infected shrimp and the sea snake *Laticauda colubrina*. A number of land and freshwater snail and slug species are susceptible to experimental infection (Appendix), as well as a few marine mollusc species (Cheng and Burton 1965; Morley 2010).

Accidental hosts of *A. cantonensis*

The wide range of intermediate and paratenic hosts that *A. cantonensis* is able to take advantage of allows for multiple pathways of infection of accidental hosts, which include humans and other mammals and birds (Wright et al. 1991; Reddacliff et al. 1999; Duffy et al. 2004; Monks et al. 2005; Lunn et al. 2012). Very often, the infected are those who purposefully eat raw or undercooked intermediate or paratenic hosts carrying the parasite (Alicata and Brown 1962; New et al. 1995; Marsh 1998; Panackel et al. 2006; Cowie 2013c). Other cases involve inadvertently eating an infected intermediate or paratenic host unseen, for example, on produce (Slom et al. 2002; Hollyer et al. 2010; Cowie 2013c).

In these accidental hosts, the third stage larvae travel to the central nervous system, as in rats (Figure 2). However, these larvae die in the central nervous system, primarily the brain, before complete maturation, which can lead to eosinophilic meningitis (Graeff-Teixeira et al. 2009). Dead larvae are thought to be more antigenic than live ones, causing a stronger immune reaction (Alto 2001). Depending on the number of larvae in the infected snail ingested, the symptoms can range from mild, such as nausea and headache, to coma and occasionally death in the most severe cases (Wang et al. 2008, 2010).

Spread of *A. cantonensis*

First recorded in China, *A. cantonensis* has spread around the world, with the majority of recorded infections occurring in Southeast Asia and the Pacific Basin (Kliks and Palumbo 1992). It was first recorded in rats (Chen 1935) and later (1945) discovered to infect humans (Beaver and Rosen 1964). It has since been recorded from other countries in Asia, and has also been recorded in North America, Australia, Africa and South America. Its presence on many islands and archipelagos has also been documented (Wang et al. 2008; Cowie 2013b).

The parasite was first observed in Hawaii in 1960 (Ash 1962), and the first human case of disease was reported there in 1961 by Horio and Alicata (1961). More recently there has been an increase in recorded cases, notably on the island of Hawaii, probably caused by accidental consumption of infected snails and slugs (Hochberg et al. 2007; Hollyer et al. 2010; Howe 2013). About 60 human cases of eosinophilic meningitis caused by *A. cantonensis* have been recorded in the main Hawaiian Islands since 2001 (Hochberg et al. 2007, State of Hawaii Department of Health, 2013, S. Y. Park, Hawaii Department of Health, personal communication, December 2013), with all six of the largest islands seeing cases (Char and Rosen 1967; Kliks and Palumbo 1992; Hochberg et al. 2007).

Thesis goals

Many drugs have been tested, but the treatment of eosinophilic meningitis caused by *A. cantonensis* is not well defined, although in general current therapy includes the use of anthelmintics and corticosteroids, used separately or in combination (Wang et al. 2012, Wang et al. 2013; Murphy and Johnson 2013). Because eosinophilic meningitis is a serious human health problem, it is important to be able to understand and prevent the spread of this parasite in the Hawaiian Islands.

The goal of this thesis is to better understand the host range and geographic distribution of the parasite in Hawaii by: 1) identifying the gastropod species, both native and introduced, that act as hosts and assessing their parasite loads; 2) ascertaining the

distribution of *A. cantonensis*, modeling the environmental factors influencing it, and predicting habitat suitability throughout Hawaii. This knowledge will help in identifying areas with the potential to support high frequencies of *A. cantonensis* in intermediate hosts, which will help to guide management strategies.
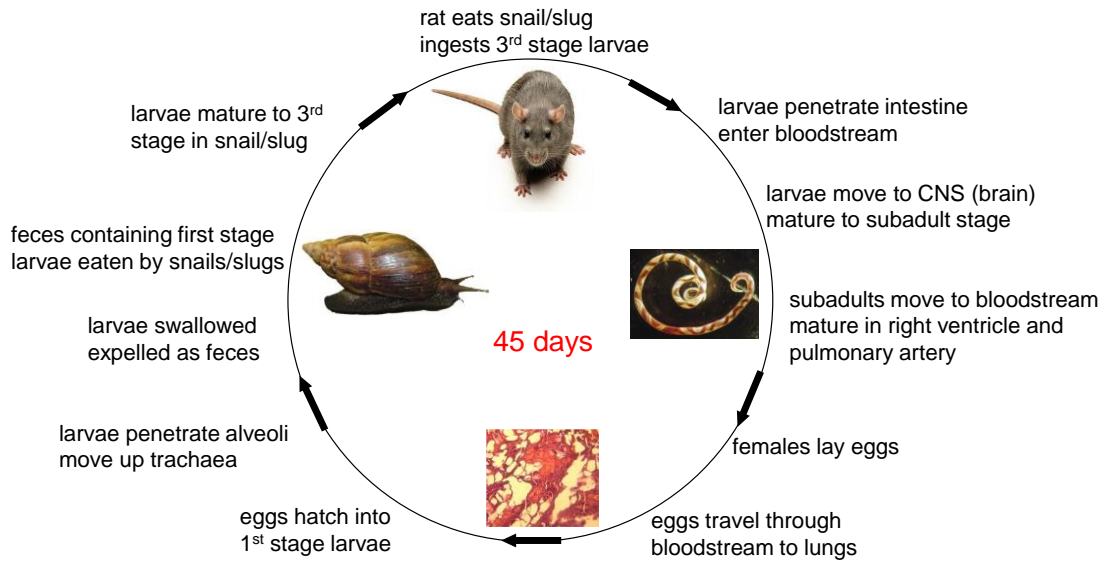
Figure 1.1. The life cycle of *Angiostrongylus cantonensis* in its definitive (various rat species) and intermediate hosts (various snail and slug species). Photos: *Achatina fulica* (D. Preston), adult female worm and histological section (C. Graeff-Teixeira)
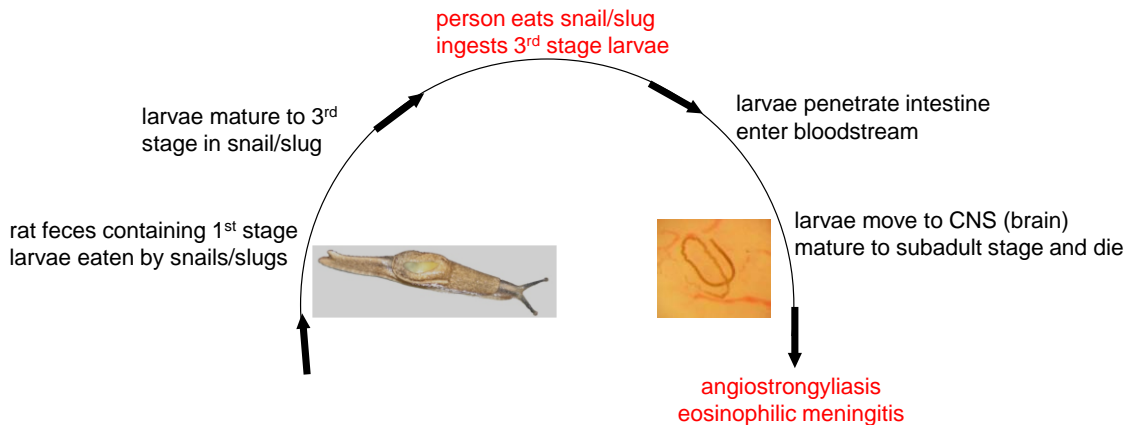
Figure 1.2. The life cycle of *Angiostrongylus cantonensis* in an accidental host. Photos: *Parmarion martensi* (K. Hayes), subadult worm (A. Morassutti)

Chapter 2. Diverse gastropod hosts of *Angiostrongylus cantonensis*

Abstract

Eosinophilic meningitis caused by the parasitic nematode *Angiostrongylus cantonensis* is an emerging infectious disease with recent outbreaks primarily in tropical and subtropical locations around the world, including Hawaii. Humans contract the disease through ingestion of infected gastropods, the intermediate hosts of *Angiostrongylus cantonensis*. Effective prevention of the disease and control of the spread of the parasite require a thorough understanding of the parasite's hosts, including their distributions, as well as the human and environmental factors that contribute to transmission. The aim of this study was to screen a large cross section of gastropod species throughout the main Hawaiian Islands to determine which act as hosts of *A. cantonensis* and to assess the parasite loads in these species. Molecular screening of 7 native and 30 non-native gastropod species revealed the presence of the parasite in 16 species (14 non-native, 2 native). Four of the species tested are newly recorded hosts, including the two native species. Those species testing positive were from a wide diversity of heterobranch taxa as well as two distantly related caenogastropod taxa. Review of the literature showed that many additional gastropod species can also act as hosts. There was a wide range of parasite loads among and within species, with an estimated maximum of 2.8 million larvae in one individual of *Laevicaulis alte*. This knowledge of the intermediate host range of *Angiostrongylus cantonensis* and the range of parasite loads will permit more focused efforts to detect, monitor and control the most important hosts, thereby improving disease prevention in Hawaii as well as globally.

Introduction

Angiostrongylus cantonensis is a parasitic nematode and one of the major causes of eosinophilic meningitis, a potentially fatal disease in humans and other mammals (Chen 1935; Wallace and Rosen 1969a; Koo et al. 1988; Weller and Liu 1993). Additional causes of eosinophilic meningitis include other parasitic, bacterial, viral and

fungal infections, as well as intracranial malignancies or medical devices and allergic reactions to drugs (Diaz 2010). *Angiostrongylus cantonensis* has been recorded on all continents except Europe and Antarctica and over 2,800 human cases of eosinophilic meningitis caused by it have been reported from about 30 countries (Wang et al. 2008; Cowie 2013b). Most records of the disease, also known as angiostrongyliasis or rat lungworm disease, have been from tropical and subtropical areas in Southeast Asia and the Pacific Basin. However, cases have also been sporadically reported in other regions, including places where *A. cantonensis* is not present, when people return from regions where it occurs (Aguiar et al. 1981; Kliks and Palumbo 1992; New et al. 1995; Brown et al. 1996; Wang et al. 2008; Cowie 2013b).

Definitive hosts of *A. cantonensis* include various rat species, mainly in the genus *Rattus*, which become infected by ingesting intermediate hosts (gastropods) containing third stage *A. cantonensis* larvae (Wallace and Rosen 1965, 1969b; Cross and Chen 2007; Chen et al. 2011a; Yong and Eamsobhana 2013). When the parasites reach the rat's small intestine, they penetrate the intestinal wall and enter the blood stream. They travel passively in the blood stream, eventually entering the central nervous system and reaching the brain, where they mature to the fifth stage (subadult). They then return to the blood stream and travel to the pulmonary artery, where they mature fully and reproduce. The eggs move in the blood stream to the lungs, where they hatch into first stage larvae. These larvae then break through the walls of the bronchioles and alveoli, move up the trachea in respiratory secretions and are swallowed, to be released in the rat's feces (Prociv et al. 2000; Cowie 2013b), which may then be ingested by the gastropod intermediate hosts. The ingested first stage larvae go through two molts to become third stage larvae while in the intermediate host, which is then consumed by the definitive host and the cycle repeats (Prociv et al. 2000).

Numerous birds and mammals, including humans, are accidental hosts and are infected in the same manner as rats (Duffy et al. 2004; Monks et al. 2005). However, in these accidental hosts the larvae die when they reach the central nervous system, primarily in the brain, which can lead to eosinophilic meningitis (Graeff-Teixeira et al. 2009). The resulting symptoms include nausea and headache, and in more severe cases, neurologic dysfunction, coma, and death (Wang et al. 2008, 2010). The severity of the

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symptoms depends on the parasite load of the infected gastropod ingested, which can vary within and among snail species (Wallace and Rosen 1969b; Punyagupta et al. 1975; Ibrahim 2007; Tsai et al. 2001; Tesana et al. 2009; Thiengo et al. 2010; Wang et al. 2010). Ingestion of infective larvae can be as a result of either deliberate or accidental ingestion of infected intermediate hosts (Slom et al. 2002; Hollyer et al. 2010; Cowie 2013c).

The spread of *A. cantonensis* has been driven by human activity, through dispersal of definitive and intermediate hosts. Definitive hosts have long been associated with human travel and trade and if infected provide a source of *A. cantonensis* in areas where snails occur (Jin et al. 2005; Wang et al. 2008). Snail intermediate hosts are also easily dispersed by human activities, and are transported around the world both intentionally and accidentally by various pathways, notably the agricultural and horticultural industries (Cowie and Robinson 2003; Cowie et al. 2008). As a result of the increased movement of these hosts around the world, eosinophilic meningitis caused by *A. cantonensis* is an emerging infectious disease, increasing in incidence and expanding in geographical range (Diaz 2010; Chen et al. 2011b). With global climate change, suitable habitat for intermediate hosts may increase and regions with appropriate conditions for parasite transfer to occur could expand. Thus, *A. cantonensis* may expand from being only a tropical concern to a more global one (Lafferty 2009; Lv et al. 2011).

Since the first reported cases in the Hawaiian Islands in 1960 (Horio and Alicata 1961), human infection by *A. cantonensis* has become increasingly prevalent there. From 2001 to 2012 there have been approximately 60 reported cases (Hochberg et al. 2007, 2011; Hawaii Department of Health, 2013; S. Y. Park, personal communication, November 2013). Most cases were probably caused by infection following accidental consumption of live slugs and the consumption of produce containing infected gastropods (Hollyer et al. 2010).

In Hawaii, there are more than 750 recognized native land snail species, a similar number to the fauna of the continental United States and Canada combined. However a high proportion of these native species are now extinct (Pilsbry 1948; Solem 1984; Cowie 1995, 1998; Holland 2009). The number of non-native gastropod species in the Hawaiian Islands is also the highest among the islands of the Pacific (Cowie 2001; Cowie

et al. 2008). Because snails are the intermediate hosts of *A. cantonensis*, determining which snail species carry the parasite is important for understanding the geographical spread of the disease, both on a global scale and locally from the perspective of public health management in the Hawaiian Islands. Therefore, the aim of this study was to screen a large cross section of these native and non-native gastropod species as possible throughout the Hawaiian Islands to determine which of them act as intermediate hosts of *A. cantonensis* and to assess the parasite loads in these species.

Materials and methods

Sampling and specimen selection

      Between 2004 and 2012, surveys were conducted on the six largest Hawaiian Islands to determine the distributions of non-native and native gastropods (Hayes et al. 2007, 2012; Cowie et al. 2008; and unpublished work). Over 8,000 live specimens (50+ species) were collected during these surveys and preserved in 75% and 95% ethanol for morphological and molecular work, respectively. Non-native (n = 1,062) and native (n = 209) gastropod specimens were selected from these collections for screening to provide a broad coverage of species (37 species, 30 of them native and 7 non-native) and locations (182 sites), including in particular species previously recorded as carriers of *A. cantonensis* and those found in high abundances throughout the main Hawaiian Islands (Cowie et al. 2008).

Molecular detection of *A. cantonensis*

      The larvae of *A. cantonensis* are distributed throughout the host gastropod's body, although differentially among the various organs (Brockelman et al. 1976; Jarvi et al. 2012), and encyst in the host tissue (Richards and Merritt 1967). To extract nematode DNA, total genomic DNA was isolated from *ca*. 5 mg (depending on snail size) of foot tissue from preserved gastropod specimens using the IDPure™ Spin Column Plant Genomic DNA Isolation Kit, modified for molluscs (i.e., 250 µl instead of 150 µl of PCL solution). The tissue was then incubated overnight. Before applying the clear lysate to the spin column, 250 µl PB buffer and 150 µl 100% EtOH were added and incubated for 3

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minutes at room temperature with occasional mixing. To elute DNA from the column, 40 µl of a 1:1 mixture of elution buffer to water was applied to the spin column and spun down; this was then repeated a second time for a total volume of 80 µl.

The 18S rDNA gene (*ca*. 1,134 base pairs) was amplified using primers specific to the superfamily to which *A. cantonensis* belongs (Metastrongyloidea), i.e., AngioF1 and AngioR1 (Qvarnstrom et al. 2007, 2010). Amplifications were performed in 25 µl reactions with a final concentration of 1X reaction buffer (ID Labs, London, ON, Canada), 0.2 mM of each dNTP, 2 mM $MgCl_2$, 1.25 U of IDPROOF™ DNA polymerase (ID Labs, London, ON, Canada), 0.16 µM of each primer, 0.4 µg/µl of BSA, 0.5% DMSO and 2 µl of template DNA. A touchdown protocol was used to promote specific amplification of *A. cantonensis* DNA from total gastropod DNA extracts. Amplification parameters were 95 °C for 5 min, 7 cycles of 95 °C for 20 s, 65 °C for 20 s with a 1°C decrease per cycle, and elongation at 72 °C for 45 s followed by 35 cycles of 95 °C for 20 s, 59 °C for 20 s, and 72 °C for 45 s, and a final elongation at 72 °C for 10 min.

To corroborate the 18S PCR assay results, all positive samples and a random subset (*ca.* 10%) of the negative samples were amplified in an additional assay with the ribosomal internal transcribed spacer one (ITS1) primers (AcanITS1F1 and AcanITSR1), of Qvarnstrom et al. (2010). These ITS1 primers are species-specific and often detect positives that are missed with 18S and vice versa (Qvarnstrom et al. 2010). Amplifications were performed in 25 µl reactions with a final concentration of 10X reaction buffer (ID Labs, London, ON, Canada), 0.2 mM of each dNTP, 2 mM of $MgCl_2$, 1.25 U of IDPROOF™ DNA polymerase (ID Labs, London, ON, Canada), 0.16 µM of each primer, 0.4 µg/µl of BSA, 0.5% DMSO and 3 µl of template DNA. Amplification parameters were 95 °C for 3 min, 45 °C for 1 min, and elongation at 72 °C for 1 min followed by 35 cycles of 95 °C for 20 s, 48 °C for 20 s, and 72 °C for 35 sec with a final elongation at 72 °C for 10 min. Amplified fragments were visualized on an agarose gel to confirm size and quality. All reaction sets included a negative control and positive controls verified by previous amplifications. To evaluate the specificity of amplifications, 24 of the positive amplicons were cleaned using the IDPure Purification Kit (ID Labs, London, ON, Canada) and sequenced with the forward primer (Angio R1) at the Greenwood Molecular Biology Facility (Pacific Biosciences Research Center, University

of Hawaii). The sequences were compared with known *A. cantonensis* sequences (Carreno and Nadler 2003; Tokiwa et al. 2012).

Quantification of parasite load

The parasite loads of all specimens that tested positive for *A. cantonensis* were quantified following the real-time, quantitative PCR (RT-PCR) TaqMan assay of Qvarnstrom et al. (2010) using *A. cantonensis* specific primers to amplify ITS1 from the original total genomic DNA extractions. First, a standard curve was generated from DNA extractions containing a range of estimated numbers of larvae. To do this, nematodes were isolated from snail tissue using the digestion protocol of Wallace and Rosen (1969c) but with a higher pepsin concentration (S. C. Thiengo, personal communication to K. A. Hayes, November 2008). Snails known to be infected were minced, put in a digestion solution of 3 % pepsin – 0.7 % HCL and incubated at room temperature with occasional agitation. The solution was then transferred to a Baermann apparatus and left to filter overnight. Larvae, which remain intact following digestion, were concentrated down into 3 ml of solution from which 100 µl volumes were sampled five times. The number of larvae in each sample was counted under a microscope and an average concentration of parasites was determined. Standard DNA extractions were carried out using a range of volumes of the concentrated larvae solution (1, 5, 10, 100 and 1000 µl) and these samples were diluted 1:5 because inhibitors can often pose problems for PCR and then were run in triplicate. Cycling conditions differed slightly from those of Qvarnstrom et al. (2010), starting with 2 min at 50 °C, 2 min at 90 °C, and finishing with 40 cycles of 15 sec at 95 °C and 1 min at 60 °C. All RT-PCR assays were carried out at the State of Hawaii Department of Health using an Applied Biosystems 7500 Fast Real-time PCR System v1.4.0 and analyzed using Applied Biosystems 7500 Fast System with 21 CFR Part 11 software.

The standard curve was generated by plotting the cycle threshold (C_T) values obtained from the RT-PCR of estimated quantities of parasites against the log number of estimated parasites in those samples. To estimate the number of larvae in each specimen, the preserved snails were patted dry in tissue paper for one minute and weighed. Shells were removed from larger snails, but for small snails the combined shell and body was

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weighed. The average weight of tissue used for DNA extraction of each species was also estimated by sampling a piece of tissue from five similarly sized specimens, patting them dry for one minute and weighing them. The total number of larvae was then estimated for each specimen by extrapolation.

Results

Of the 37 species, 16 tested positive for *A. cantonensis*, with 70 specimens testing positive out of a total of 1,271 (Table 2.1). Among the 30 non-native species, 14 tested positive, two being newly recorded natural hosts of *A. cantonensis* (*Cyclotropis* sp., *Oxychilus alliarius*). Of a total of 1,062 non-native gastropods, 6% were positive for *A. cantonensis*. No specimens of four non-native species (*Bradybaena similaris*, *Deroceras laeve*, *Limax flavus*, *Melanoides tuberculata*) that have been recorded in other studies as hosts of *A. cantonensis* (Appendix) tested positive for the parasite. *Parmarion martensi* had the highest prevalence of infection with 68% of the specimens testing positive for *A. cantonensis* (Figure 2.1).

Of the seven native Hawaiian species screened, two tested positive (one individual of each of *Philonesia* sp. and *Tornatellides* sp.) The proportion of native snails found to carry *A. cantonensis* is significantly smaller than the proportion of non-native snails ($\chi^2 = 9.95$, df = 1, P = 0.002). The proportions of all snails infected were significantly related to their habit ($\chi^2 = 18.5$, df = 3, P = 0.0004): 7 % (67/938) of the individuals of ground-dwelling species tested positive; arboreal and freshwater snails were less susceptible at 2 % (1/62) and 0.7 % (1/138), respectively; and 0.8 % (1/133) of the individuals of species that are both ground-dwelling and arboreal tested positive.

Of 182 sites from which snails were screened, 40 had snails that tested positive for *A. cantonensis* (Table 2.1). The majority of these sites (26 sites) were in windward or northeastern areas, with fewer in leeward or southwestern areas (14). Infected *L. alte* were found at more sites (11) than any other species.

The standard curve data were used to generate the linear equation

y = -5012582x + 36.368099 ($R^2 = 0.90$)

where y is the C_T value and x is the number of larvae in the sample. Using this relationship permitted estimation of the number of parasites in each DNA extraction, which ranged from 2 to 6,427. The real-time PCR data displayed a wide range in parasite load both within and among species (Table 2.1). *Tornatellides* sp. had the highest average concentration of parasites with 4,916 parasites per 5 mg of tissue, followed by *S. octona* and *L. maximus*. However, a *L. alte* specimen had the highest individual parasite concentration at 8,147 parasites per 5 mg of tissue, followed by an *O. alliarius* (7,068) and a *P. martensi* (6,639). The two caenogastropod species, *Pomacea canaliculata* and *Cyclotropis* sp. had the lowest average parasite concentrations, roughly 30 times less than that in *Tornatellides* sp., while an *A. fulica* had the lowest individual parasite concentration at 6 parasites per 5 mg of tissue. Average total parasite loads were highest in *L. maximus*, *L. alte* and *A. fulica* (398,160, 342,971 and 213,515, respectively) and individuals of these species also had the highest individual loads (566,582, 2,801,566 and 870,867, respectively). The lowest average parasite load was in *Cyclotropis* sp. (154 parasites), but an *O. alliarius* specimen had the lowest individual load (63).

Discussion

The diversity of gastropods now known to carry the *Angiostrongylus cantonensis* covers a broad phylogenetic range of both terrestrial and freshwater species (Figure 2.2; Appendix). A single fully marine species (*Discotectonica acutissima*: Architectonicidae) has also been reported as testing positive for *A. cantonensis* (Yang 2012) but this is a sublittoral (50-200 m depth) species (Bieler 1993) and the finding may be incorrect. Species from 58 families have been tested in this and previous studies and all but 12 of these families included species that acted as natural hosts and/or that had been infected experimentally (Figure 2.2). Previous studies had identified species in 31 families as natural hosts of *A. cantonensis*, with the present study adding three more: Achatinellidae, Assimineidae and Oxychilidae. This study found infected individuals in 12 families, not only highly divergent heterobranchs such as *Tornatellides* sp. (Achatinellidae), *V. cubensis* (Veronicellidae) and *P. martensi* (Ariophantidae) but also caenogastropods (i.e., *Cyclotropis* sp. and *P. canaliculata*). Herbivorous species such as *Veronicella cubensis*

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(Hata et al. 1997; Rueda et al. 2002), predatory species like *Euglandina rosea* (Meyer and Cowie 2010), and detritivores like *S. octona* (Juřičková 2006), tested positive despite their dietary differences. Given this extremely broad diversity of gastropods that have been shown to act as hosts of *A. cantonensis*, it seems possible that almost any terrestrial or freshwater gastropod may have the potential to act as a host.

Two families (Achatinidae, Ariophantidae) exhibited high susceptibility, inasmuch as they included species that were reported as natural or experimental hosts in this and all previous studies that screened them for *A. cantonensis*. Species in two families (Orthalicidae and Streptaxidae) tested negative in this and all previous studies, perhaps because they have low susceptibility to infection or the individuals tested came from localities where the frequency of infection of intermediate hosts was low. Nine families were represented by species that were only reported as having been infected experimentally, including the Truncatellidae, which live close to the sea shore where the probability of transmission may be low because wave action washes rat feces away.

Four freshwater species (*Fossaria viridis*, *Melanoides tuberculata*, *Planorbella duryi*, *Physa* sp.) tested negative for *A. cantonensis* in this study and only 1 of 56 (2 %) *P. canaliculata* tested positive. It may be more difficult for snails in freshwater habitats to acquire the parasite as access to rat feces in streams and rivers may be limited (Morley 2010). However, other studies have identified a number of freshwater species able to carry *A. cantonensis*, including *P. canaliculata* and *M. tuberculata* (Appendix). The rate of infection of *M. tuberculata* was often less than 1 %, whereas *P. canaliculata* showed higher rates of up to 40 % (Crook et al. 1968; Hu et al. 2007; Ibrahim 2007; Liu et al. 2007; Zhang et al. 2008, 2009). The low infection rate in *P. canaliculata* in the present study could be due to the majority of these specimens being from irrigated areas such as taro patches, some with flowing water, where incidence of infection may be low because of lower concentrations of parasites in the water than in intact rat feces in terrestrial situations (Yen et al. 1990). In Asia, *P. canaliculata* is commonly identified as the source of infection in human cases of angiostrongyliasis, not necessarily because of the high percentage of infected snails, but because of its popularity as a food source (Nishimura et al. 1986; Lv et al. 2008).

A number of terrestrial snails also did not test positive for *A. cantonensis*. For instance, none of the 65 *Bradybaena similaris* specimens tested positive in this study. It is possible that *B. similaris* is naturally less susceptible to *A. cantonensis* infection than other species, as in a previous study only 8 of 281 (4%) *B. similaris* specimens from Oahu were infected (Wallace and Rosen 1969b). All *Arion intermedius*, *Arion subfuscus* and *Cornu aspersum* specimens tested were from sites higher than 600 m above sea level, where the parasite may not yet be at high densities. No parasites were recovered from several hundred *C. aspersum* in New Caledonia (Ash 1976). Nematode inhibitors that prevent maturation or reproduction have been isolated from *C. aspersum*, possibly explaining this species' low infection rate (Ratanarat-Brockelman 1975, 1977).

Achatina fulica is well known as an intermediate host of *A. cantonensis* (e.g., Alicata 1966; Kliks and Palumbo 1992; Lv et al. 2008; Maldonado et al. 2012). However, the level of infection of *A. fulica* varies widely among localities. In the present study only 7 of 62 (11 %) *A. fulica* tested positive. In one study in Brazil, Neuhauss et al. (2007) found only one infected *A. fulica* out of 244 (0.4 %) screened, while in another Thiengo et al. (2010) found 14 among 33 (42 %) screened. The level of infection at different locations in Guangdong, China, varied widely from zero to 45.4 % (Deng et al. 2012). The most likely explanation for this kind of variability is variability in the presence and abundance of *A. cantonensis* in the environment, for instance related to abiotic factors such as temperature and humidity, but perhaps also to the distribution of infected rats, the species of rats present or differences in the interactions between rats and gastropods.

Among the newly recorded hosts, *Oxychilus alliarius* is a widespread European species. While *A. cantonensis* is primarily a tropical and subtropical parasite, presumably because it is constrained by ambient temperatures (which determine the temperature of its poikilothermic gastropod intermediate hosts), the fact that it can infect temperate gastropod species indicates that global warming trends may allow it to establish more widely in locations where such hosts are already present.

This is the first report of native Hawaiian snails carrying *A. cantonensis*. This may have negative implications for the health of the native Hawaiian snail fauna, which is especially vulnerable to additional threats. Once consisting of over 750 species (Cowie 1995), the fauna has declined drastically and is being replaced by a much smaller number

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of alien species (Cowie 1998). The deliberate introduction of predatory snails, notably *Euglandina rosea*, for use as biocontrol agents in ill-conceived efforts to control *Achatina fulica* has had a devastating effect on the native snails (Hadfield et al. 1993; Cowie 1998, 2001). The snail fauna may be facing an additional threat if infection with *A. cantonensis* reduces the snails' fitness. Native snails in Jamaica also carry *A. cantonensis*, but little is known about the effect of the parasite on their fitness (Lindo et al. 2002; Rosenburg and Muratov 2006). Richards and Merritt (1967) recorded a clear tissue reaction to the parasite in *Biomphalaria glabrata*. This type of response to parasitic infection may be costly, using energy usually allocated towards survival and reproduction (Agnew et al. 2000; Rolff and Siva-Jothy 2003). Wallace and Rosen (1969d) showed that mortality was higher in *Physa elliptica* experimentally infected with *A. cantonensis* than in uninfected controls.

Although only 2 out of 210 specimens of native species tested positive for the parasite, this does indicate that the parasite may now have become sufficiently widespread and abundant to begin to infect native snail populations. Most native Hawaiian snails are very sparsely distributed, mostly at high elevations where *A. cantonensis* may not be able to survive in rat feces or develop in the snails. Nonetheless, these high elevation refugia generally support more than one species of native snail, so native species other than those testing positive in this study may yet be found to be susceptible to infection.

Susceptibility to infection may be related to the snails' behavior, location or physiology (Ash 1976). For example, *P. martensi*, a species that is mainly ground-dwelling is highly susceptible, with 68 % of the specimens testing positive in this study, and often heavily infected (Asato et al. 2004; Hollingsworth et al. 2007; Qvarnstrom et al. 2007, 2010; Jarvi et al 2012). However it will readily climb and is often found in trash cans and compost piles, where contact with rats and rat feces is probably a common occurrence (Hollingsworth et al. 2007). These factors may also affect parasite load, which differed greatly within and among species. One *P. martensi* specimen had one of the highest parasite concentrations in its tissue and had on average as many larvae in its entire body as other species four to six times its weight (Table 2.1). Jarvi et al. (2012) also showed that this species can support high levels of *A. cantonensis*. *Parmarion*

martensi is an invasive species found only on the islands of Oahu and Hawaii and is the species most frequently implicated in transmission of the parasite to humans in Hawaii (Hollingsworth et al. 2007).

Although the two caenogastropod species, *P. canaliculata* and *Cyclotropis* sp., had the lowest average parasite concentrations, this was based on only one positive individual for each species. These low parasite loads in *P. canaliculata* may again be due to the lower chance of this species ingesting larvae in a stream or pond than on land. Even though *A. fulica* is a relatively large (heavy) species, it harbored on average as many or fewer parasites than species four to seven times less heavy, and one *A. fulica* had the lowest individual parasite concentration. This could be due to its poorly aerated muscular foot tissue that may be resistant to penetration by *A. cantonensis* (Brockelman et al. 1976). Despite *Tornatellides* sp. having the highest average parasite load per mg of tissue, even if a whole specimen were consumed only *ca.* 2,000 larvae (an overestimate due to the added weight of its shell, which was not removed for these extrapolations) would be ingested, while consuming a whole *L. alte* could result in ingestion of up to a thousand times more larvae.

A diverse assemblage of gastropods can thus serve as hosts of *A. cantonensis* to varying degrees. In the future, monitoring and quarantine efforts should take this into account. In Hawaii there has been a distinct focus on *P. martensi* (Hollingsworth et al. 2007, Qvarnstrom et al. 2007, 2010, Hollyer et al. 2010, Jarvi et al. 2012), and although this species is definitely one of the most important hosts in Hawaii, it is by no means the only one. A number of species commonly found in highly populated areas at lower elevations were positive for *A. cantonensis*. Species such as *A. fulica*, *E. rosea*, *L. alte*, *P. achatinaceum*, *S. octona* and *V. cubensis* are commonly found in Hawaii in nurseries, farms and home gardens, where contact with produce can be a regular occurrence, and inadvertent consumption of infected hosts on produce is thought to be a major pathway of infection (Cowie 2013c). Also it is difficult to remove snails from produce (Yeung et al. 2013).

Both tropical and temperate snails can carry *A. cantonensis*, indicating the potential for future expansion of the parasite's range under climate change and the need for continued concern about angiostrongyliasis as an emerging infectious disease. Since

many species are potential hosts, it is likely that abiotic factors, particularly temperature and perhaps humidity, have a greater influence on infection rates and continued range expansion of the parasite than does the spread of particular host species. Knowledge of the possible vectors of *A. cantonensis* and their parasite loads is important for public health management.
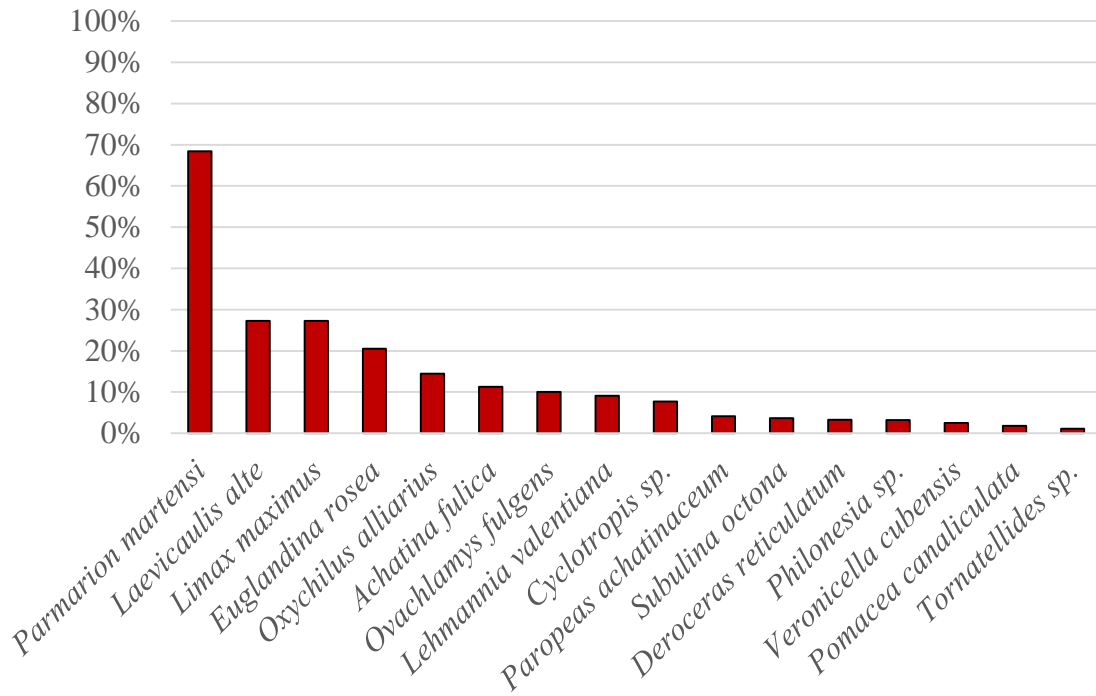
Figure 2.1. Infection rates for species that tested positive in this study.

Table 2.1. Infection rates and average parasite loads (of positive specimens) in the gastropod species screened in this study.

Species	Habit	No. tested	No. (%) positive	No. sites positive	Average C_T 1:5 dilution value (range)	Average no. of parasites per 5 mg of snail tissue	Average no. of parasites in entire specimen (range)
Non-native species							
*A. fulica**	G	62	7(11)	4	25.66 (20.16-31.26)	237	213,515 (14,379-870,868)
Arion intermedius	G	20	0	0	-	-	-
Arion subfuscus	G	8	0	0	-	-	-
*Bradybaena similaris**	G	65	0	0	-	-	-
Bulimulus guadalupensis	G	10	0	0	-	-	-
Cornu aspersum	G	25	0	0	-	-	-
Cyclotropis sp.	G	13	1(8)	1	28.60	133	154
*Deroceras laeve**	G	79	0	0	-	-	-
*Deroceras reticulatum**	G	61	2(3)	1	25.24 (23.54-26.94)	1,564	9,789 (3,500-16,078)
*Euglandina rosea**	G	39	8(21)	5	27.66 (24.37-33.13)	166	43,687 (1,244-113,645)
Fossaria viridis	F	18	0	0	-	-	-
Gonaxis kibweziensis	G	11	0	0	-	-	-
*Laevicaulis alte**	G	44	13(30)	11	24.99 (17.32-31.42)	1,592	342,971 (4,127-2,801,566)
*Lehmannia valentiana**	G	11	1(9)	1	22.19	1,872	24,819
Liardetia doliolum	G	8	0	0	-	-	-
*Limax flavus**	G	8	0	0	-	-	-
*Limax maximus**	G	11	3(27)	2	22.22 (20.36-24.35)	1,960	398,160 (170,067-566,582)
*Melanoides tuberculata**	F	17	0	0	-	-	-
Milax gagates	G	22	0	0	-	-	-
*Ovachlamys fulgens**	G	10	1(10)	1	22.42	1,800	11,118
Oxychilus alliarius	G	69	10(14)	6	25.69 (20.22-34.41)	1,922	13,382 (63-55,807)
*Parmarion martensi**	G	19	13(68)	5	24.23 (17.28-29.43)	912	55,852 (850-341,828)
*Paropeas achatinaceum**	G	73	3(4)	2	27.16 (25.77-29.36)	1,518	11,421 (1,724-21,087)

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Table 2.1. (Continued) Infection rates and average parasite loads (of positive specimens) in the gastropod species screened in this study.

Planorbella duryi	F	20	0	0	-	-	-
*Pomacea canaliculata**	F	56	1(2)	1	23.78	152	68,133
Physa spp.	F	27	0	0	-	-	-
*Subulina octona**	G	54	2(4)	1	24.60 (23.21-25.99)	3,548	39,114 (15,835-62,392)
Succinea tenella	G	25	0	0	-	-	-
*Veronicella cubensis**	G	159	4(3)	3	23.40 (21.35-24.93)	531	116,891 (28,931-253,909)
Zonitoides arboreus	G	18	0	0	-	-	-
Native species							
Auriculella spp.	A	31	0	0	-	-	-
Elasmias spp.	B	18	0	0	-	-	-
Kaala subrutila	G	2	0	0	-	-	-
Lamellidea spp.	B	25	0	0	-	-	-
Philonesia sp.	A	31	1(3)	1	24.57	958	4823
Succinea caduca	G	12	0	0	-	-	-
Tornatellides spp.	B	90	1(1)	1	24.66	4916	2379
Total		**1271**	**71(6)**	**47**			

* Previously recorded as a host in the Hawaiian Islands and/or elsewhere. Habits are ground-dwelling (G), arboreal (A), both ground-dwelling and arboreal (B) and freshwater (F).

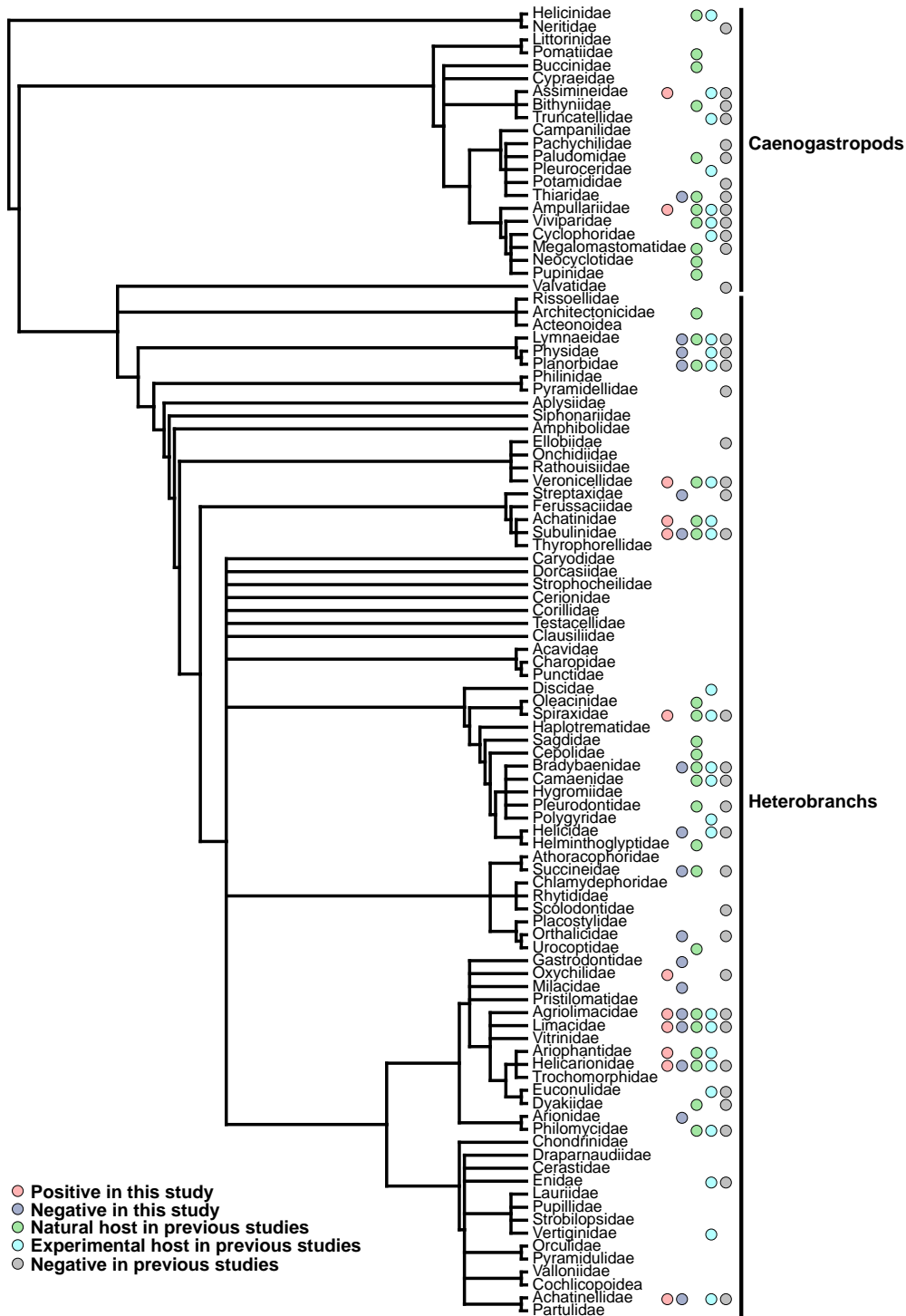Figure 2.2. Phylogeny of mollusc families showing which families have been recorded as intermediate hosts of *Angiostrongylus cantonensis*. Phylogeny constructed using the classifications and phylogenies of Bouchet and Rocroi (2005), Aktipis et al. (2008) and Strong et al. (2008), indicating the diversity of families in which mollusc species have been shown to act as hosts of *Angiostrongylus cantonensis*.

Chapter 3. The geographic distribution of the gastropod hosts of *Angiostrongylus cantonensis* in Hawaii and the environmental factors influencing it

Abstract

Human infection with *Angiostrongylus cantonensis*, a parasitic nematode, can cause eosinophilic meningitis, an emerging infectious disease. This disease has been expanding in range and increasing in incidence throughout the tropics and subtropics. Recently the Hawaiian Islands have experienced an increase in human cases probably because of inadvertent ingestion of infected snails. The goal of this study was to increase understanding of the factors that affect the distribution of *Angiostrongylus cantonensis* and its hosts, which are important in controlling their spread and preventing infection. Specimens from a wide range of snail species and locations throughout Hawaii were screened for the parasite. It was present on five of the largest six Hawaiian Islands (not on Lanai). The data were then used to generate a habitat suitability model for *A. cantonensis*, using temperature, rainfall and combined temperature and rainfall terms, in order to predict the potential for further spread of *A. cantonensis* within the Hawaiian Islands. The best model predicted all of the Hawaiian Islands as suitable habitat with highly suitable habitat in windward areas where rainfall is higher and at lower elevations, which experience higher temperatures. Knowledge of the habitat suitable for *A. cantonensis* and the environmental factors that affect its distribution will help in identifying areas with the potential to support high frequencies of *A. cantonensis* in intermediate hosts, which will help to guide management strategies.

Introduction

Eosinophilic meningitis caused by infection with *Angiostrongylus cantonensis*, the rat lungworm, is an emerging infectious disease, with increasing numbers of cases and recent expansion to new geographic areas (Diaz 2010; Chen et al. 2011b). *Angiostrongylus cantonensis* is a parasitic nematode with a complex life cycle (Alicata 1965a; Wallace and Rosen 1969a; Cowie 2013b). Adult *A. cantonensis* reproduce in the

pulmonary arteries of rats, the definitive hosts. Their eggs are transported in the blood stream to the lungs, where they hatch into first stage larvae. These larvae break through into the lung cavities and are transported in mucous secretions up the trachea, to be swallowed and eventually expelled in the feces. The intermediate hosts are snails and slugs (gastropods), which are infected when they ingest these feces, and in which the larvae develop to the third stage. Rats are infected when they eat the gastropods. The third stage larvae then break through the intestine wall and enter the circulatory system, in which they travel to the brain, where they mature to the fifth subadult stage. Subadults return to the circulatory system, eventually reaching the pulmonary arteries where they mature and reproduce. Various species of rats can act as definitive hosts (Wallace and Rosen 1965, 1969b, Chen et al. 2011a) and numerous gastropod species from widely different taxonomic groups have been recorded as intermediate hosts (Chapter 2).

Accidental hosts such as humans and other mammals are infected in the same manner as the definitive rat hosts. However, in these accidental hosts most larvae remain in the central nervous system, where they eventually die (primarily in the brain), not completing their natural life cycle (Koo et al. 1988; Slom et al. 2002; Hollyer et al. 2010). Humans are usually infected through willful or inadvertent consumption of infected snails on produce or by eating undercooked snails (Cowie 2013c).

The first known human case of eosinophilic meningitis caused by *A. cantonensis* was reported in 1945 in Taiwan (Beaver and Rosen 1964). However, it was not until reports of cases in French Polynesia and Hawaii (Horio and Alicata 1961; Rosen et al. 1961) that the link between the disease and infection by *A. cantonensis* became widely understood. Since then, cases have been reported in about 30 countries, in Asia, Australia, Africa, South America and North America, and including many cases in the islands of the Pacific and the Caribbean (Lindo et al. 2004; Wang et al. 2008; Cowie 2013b). With increasing international trade and travel, the range of *A. cantonensis* may expand, and although it is generally restricted to tropical and subtropical regions, global climate change may permit it to expand into currently more temperate areas (Kliks and Palumbo 1992; Lv et al. 2011; Cowie 2013b).

Angiostrongylus cantonensis was first observed in the Hawaiian Islands in 1960 (Ash 1962). The first definitive case of eosinophilic meningitis caused by *A.*

cantonensis in Hawaii occurred in 1960 (Horio and Alicata 1961). Since then there have been cases on all six of the largest Hawaiian Islands (Kauai, Oahu, Maui, Molokai, Lanai and Hawaii), with a noticeable increase in the number of cases since around 2004 (Char and Rosen 1967; Kliks and Palumbo 1992; Hochberg et al. 2007). Although there have been a number of studies identifying the intermediate hosts of *A. cantonensis* (Chapter 2), little is known about the factors affecting its distribution (e.g., Lv et al. 2011). Determining how factors such as temperature and rainfall affect the distribution of *A. cantonensis* will permit better prediction of its future spread and would allow targeted public health prevention methods.

The purpose of this study, then, was to: 1) document the geographic distribution of *A. cantonensis* across the Hawaiian Islands; and 2) develop a habitat suitability model to predict possible suitable habitat for *A. cantonensis*. We hypothesized that 1) areas with higher rainfall will support higher levels of *A. cantonensis* than will drier areas, as the intermediate gastropod hosts are more abundant and active in such areas; and 2) lower elevation areas will support higher levels of *A. cantonensis* than higher elevation areas, first because globally *A. cantonensis* is restricted to regions with tropical and subtropical climates (Cowie 2013b), and areas at higher elevations in Hawaii would be too cold, and second because of the greater numbers of rats at lower elevations (Amarasekare 1994).

Materials and methods

Over 8,000 live gastropods were collected from the six main Hawaiian Islands and preserved in 75-95% EtOH. Specimens from 182 sites (1,271 individuals) were selected from these collections to provide a broad coverage of species and locations. The sites are spread across all six of the largest islands. Total DNA was extracted from gastropod foot tissue, and presence of *A. cantonensis* was determined using the primers of Qvarnstrom et al. (2007, 2010) and following the protocols of Kim et al. (Chapter 2).

Geographical coordinates were recorded using hand-held global positioning system devices (Rino 520HCx; Garmin International, Olathe, KS, U.S.A) and in cases when the device was not taken into the field, Google Earth was used to estimate the

coordinates. Using ArcGIS, these points were mapped onto mean annual temperature and rainfall raster layers (both at roughly 250×250 m resolution), obtained from Thomas Giambelluca (unpublished) and Giambelluca et al. (2013), respectively. Mean annual temperature and rainfall values were then extracted for each collection site.

A habitat suitability model using a logistic regression framework was generated based on the presence or absence of *A. cantonensis* at the 182 sites, with the explanatory variables being mean annual temperature and annual rainfall. To select the best model I started by running an exhaustive best subsets selection process that created models with all seven combinations of the two variables, including pairwise interactions between the variables, with the glmulti package in the software program R (R Development Core Team 2013; Calcagno 2013). Receiver operator characteristic (ROC) curves were then created to obtain their area under the curve (AUC) scores using the epicalc package in R (Chongsuvivatwong 2012). The package cvAUC was used to perform nine-fold cross validations on each of the seven models (LeDell et al. 2012). This allowed us to test each model's predictive performance and to test for model overfitting. The "set.seed" function was used to set 25 pseudo-random number sets to run the ten-fold cross validations. The models' Akaike Information Criterion (AIC) scores, ROC curves, the averaged cross validated AUC scores and their averaged standard errors were compared to select the best overall predictive model. The selected model was then used to generate a habitat suitability map using ArcGIS (ESRI 2013).

Results

Of the 37 species screened, 16 (71 specimens) tested positive for *A. cantonensis* (Table 3.1). Of the 182 sites, 40 sites, located on five of the six main islands (not Lanai) had infected gastropods (Figure 3.1). Kauai and Hawaii had the highest percentages of sites with *A. cantonensis* (34 % and 33 %, respectively), followed by Molokai, Maui and Lanai combined as "Maui Nui" (18 %) and Oahu (10 %) ($\chi^2 = 10.6$, df = 5, P = 0.014). More windward or northeastern sites (28 %) had *A. cantonensis* than leeward or southwestern sites (16 %) ($\chi^2 = 4.0$, df = 1, P = 0.047).

Specimens that were negative for *A. cantonensis* were from sites with mean annual temperature ranging between 10.3 and 23.9 °C and mean annual rainfall between 224 and 8,615 mm (Figures 3.2 and 3.3). Specimens testing positive were from sites with a narrower range of mean annual temperature (15.2 - 23.9 °C) and mean annual rainfall (291 - 5,960 mm). The average mean annual temperature for sites with *A. cantonensis* was higher (21.3 °C) than for sites where it was absent (20.5 °C). Sites with *A. cantonensis* had higher rainfall (1,816 mm) than sites without it (1,699 mm).

Among the seven habitat suitability models (Table 3.2), which take the form

$$\lambda = \frac{1}{1 + e^{-X}}$$

where λ is habitat suitability and X is the set of model variables and coefficients describing habitat suitability, the best X was

$$X = -1.264974 \times 10^{-2} + (-3.612819 \times 10^{-5} \times R) + (3.653679 \times 10^{-6} \times T \times R)$$

where R is mean annual rainfall and T is mean annual temperature. The AIC score of this model (72.902) was one of the two lowest (only 1.125 higher than Model 4); however, it had the highest AUC score (0.71676) and average cross validated AUC score (0.69240). This model was used to create a map of suitable habitat in the Hawaiian Islands. The range of suitability was not great (highest 56 %, lowest 49 %) but sites at which *A. cantonensis* was present in the survey were clearly located in the more suitable areas (Figure 3.2).

Discussion

Infected snails were found on all six main Hawaiian Islands except for Lanai, although a human case of infection has been recorded on that island (Hochberg et al. 2007). It is possible that produce regularly shipped from Maui was contaminated and the victim was infected through consumption of such produce, or that the victim became infected while on another island.

The parasite was found predominantly at lower elevation sites with warmer temperatures and higher rainfall, which supports the original hypotheses and predictions of the model. These patterns are reasonable considering the parasite's warmer tropical

and subtropical origins and susceptibility to dry conditions (Richards and Merritt 1967). Higher rainfall may also facilitate transfer of the parasite to hosts by keeping rat feces hydrated and able to support the first stage larvae, as well as providing suitable conditions for the survival of intermediate hosts. It is also probable that suitable temperatures are needed for the development and persistence of the parasite in the intermediate host (Yousif and Lämmler 1975).

Kauai and Hawaii are the two islands with the highest proportions of positive sites. The majority of positive leeward sites on Kauai are clustered around the Kekaha landfill, where there may be higher rat densities that could facilitate higher rates of infection in nearby areas. The island of Hawaii, which is the largest island, has the largest area of highly suitable habitat (Figure 3.2), which is reflected not only in the high proportion of positive sites but also to the high incidence of human infection on that island (Park and Fox 2013). Oahu had the lowest proportion of positive sites, possibly because of the higher proportion of freshwater gastropods sampled from this island. Freshwater snails are less likely to come into contact with rat feces and are less likely to be eaten by rats than terrestrial snails. Also, the proportion of native gastropods, which are generally found at higher elevations than non-native species, sampled from Oahu was the highest, while the proportions of non-native gastropods screened were the highest on Kauai and Hawaii ($\chi^2 = 14.3$, df = 3, P = 0.0025). Freshwater snails have lower infection rates than terrestrial gastropods, as do native compared to non-native gastropods (Chapter 2).

Overall the model predicted higher suitability in windward or northeastern areas where rainfall is greater than in leeward or southwestern areas. On the southeastern side of the island of Hawaii, the Puna district has been the area from which most human infections have been reported (Hollingsworth et al 2007; Dixon 2013). The model predicted that this area is highly suitable for *A. cantonensis*, possibly due primarily to its wetter conditions. Higher elevation areas that experience lower temperatures also seem to be less suitable than lower elevation areas, especially on the windward side of the islands, although rainfall seems to be more important than temperature/elevation. Suitability is uniformly low over the entire island of Lanai, an island that has relatively low rainfall.

The range of habitat suitability falls between 49 and 56 %, indicating that all areas of the main Hawaiian Islands are somewhat suitable for *A. cantonensis*. If the environmental conditions are suitable and the definitive and intermediate hosts are present, the spread of *A. cantonensis* throughout the Hawaiian Islands seems inevitable. However, while the model did not predict any areas of very low suitability, this may reflect the fact that almost none of the collecting sites on which the model was based were at the highest elevations because these very high elevations (e.g., the summits of Mauna Kea and Mauna Loa on the island of Hawaii) are cold, dry and barren and support no intermediate gastropod hosts. Therefore, even though these areas were predicted as having somewhat suitable habitat, the absence of hosts prevents the parasite's spread. It is assumed that such high, cold areas would be unsuitable for *A. cantonensis*, even if the hosts were present. The range of *A. cantonensis* is constrained to be within the range of its hosts. In addition, other factors may be involved in habitat suitability for *A. cantonensis* such as proximity to human settlements or agricultural land. These areas may have higher concentrations of *A. cantonensis* due to higher densities of definitive rat hosts and consistent levels of moisture from artificial irrigation.

Laevicaulis alte exhibits a high frequency of infection and the highest individual parasite load (Chapter 2). It is currently found on all six of the largest Hawaiian Islands. However *P. martensi*, although only recorded on the islands of Oahu and Hawaii (Hollingsworth et al. 2007; Cowie et al. 2008, unpublished data) exhibited the highest frequency of infection as well as a fairly high parasite load (Chapter 1). *Parmarion martensi* was first recorded in the Hawaiian Islands in the 1990s but quickly become widespread and abundant especially in the Puna district of the southeastern part of the island of Hawaii. It is the species thought to be connected with many of the recent cases of human infection due to its high rate of infection and apparent association with human habitation (Hollingsworth et al. 2007). These traits make *P. martensi* an important host in the spread of *A. cantonensis*. The horticulture industry plays a major role in the spread of snails throughout the Hawaiian Islands and globally and may contribute to the continued emergence of this disease (Cowie 2005; Cowie et al. 2008).

Table 3.1. Infection rates in gastropod species screened and distribution of positive
infection among the Hawaiian Islands.

Species	No. tested	No. (%) positive	No. sites positive	Islands with positive specimens**
Non-native species				
*A. fulica**	62	7(11)	4	K, O, M
Arion intermedius	20	0	0	
Arion subfuscus	8	0	0	
*Bradybaena similaris**	65	0	0	
Bulimulus guadalupensis	10	0	0	
Cornu aspersum	25	0	0	
Cyclotropis sp.	13	1(8)	1	H
*Deroceras laeve**	79	0	0	
*Deroceras reticulatum**	61	2(3)	1	Mo
*Euglandina rosea**	39	8(21)	5	K, H
Fossaria viridis	18	0	0	
Gonaxis kibweziensis	11	0	0	
*Laevicaulis alte**	44	13(30)	12	K, O, M, Mo, H
*Lehmannia valentiana**	11	1(9)	1	K
Liardetia doliolum	8	0	0	
*Limax flavus**	8	0	0	
*Limax maximus**	11	3(27)	2	M, H
*Melanoides tuberculata**	17	0	0	
Milax gagates	22	0	0	
*Ovachlamys fulgens**	10	1(10)	1	M
Oxychilus alliarius	69	10(14)	6	K, M, Mo, H
*Parmarion martensi**	19	13(68)	5	O, H
*Paropeas achatinaceum**	73	3(4)	2	K, H
Planorbella duryi	20	0	0	
*Pomacea canaliculata**	56	1(2)	1	K
Physa spp.	27	0	0	
*Subulina octona**	54	2(4)	1	H
Succinea tenella	25	0	0	
*Veronicella cubensis**	159	4(3)	3	M, H
Zonitoides arboreus	18	0	0	
Native species				
Auriculella spp.	31	0	0	
Elasmias spp.	18	0	0	
Kaala subrutila	2	0	0	
Lamellidea spp.	25	0	0	
Philonesia sp.	31	1(3)	1	H
Succinea caduca	12	0	0	
Tornatellides spp.	90	1(1)	1	H
Total	**1271**	**71(6)**	**47**	

* Previously recorded as a host in the Hawaiian Islands and/or elsewhere (Chapter 2).

** K - Kauai, O - Oahu, M - Maui, Mo - Molokai, H - Hawaii

Table 3.2. Fit results for the seven models of *Angiostrongylus cantonensis* habitat suitability.

Model	Model Description	AIC	AUC	Cross-validated AUC	Standard error
1	HS = T	75.642	0.46564	0.27807	0.03234
2	HS = R	73.740	0.70006	0.68041	0.02915
3	HS = T + R	74.695	0.66731	0.62339	0.03084
4	HS = T x R	<u>71.777</u>	0.71355	0.68655	0.02977
5	HS = T + (T x R)	73.454	0.70906	0.68070	0.02837
6	**HS = R + (T x R)**	**72.902**	**<u>0.71676</u>**	**<u>0.69240</u>**	**0.02805**
7	HS = T + R + (T x R)	74.742	0.71484	0.63363	<u>0.02559</u>

HS – habitat suitability; T – mean annual temperature; R – mean annual rainfall. The best model is highlighted in bold. Underlined values are the best scores for each statistic.
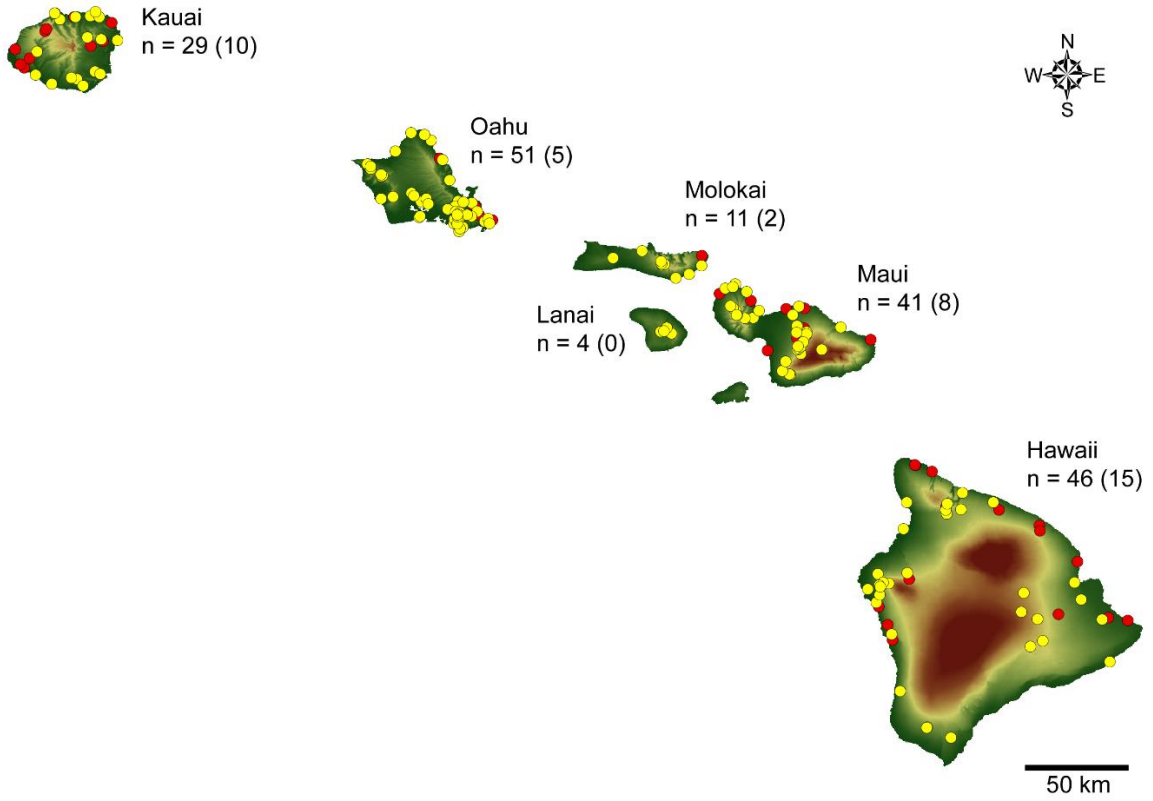
Figure 3.1. Presence (40 sites, numbers in parentheses) and absence (142 sites) of *Angiostrongylus cantonensis* at the study sites. Red – present; yellow – absent; some sites overlap and may not be visible. Green – low elevation; brown – high elevation.
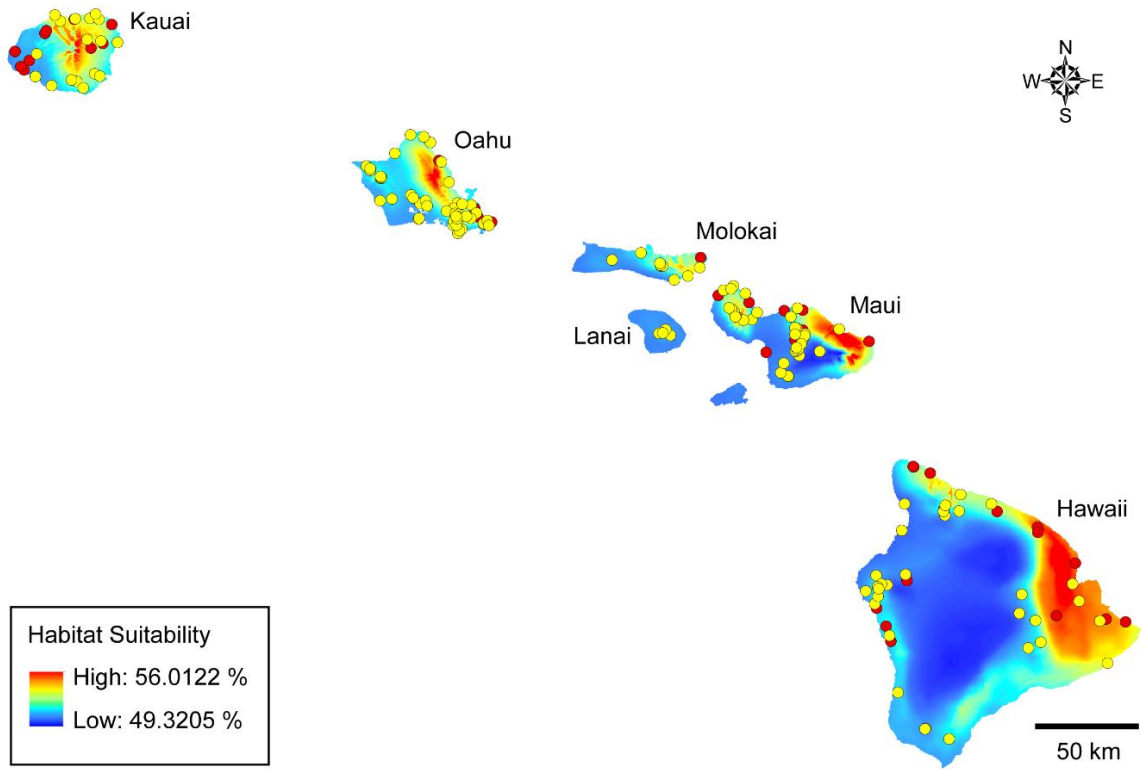
Figure 3.2. *Angiostrongylus cantonensis* habitat suitability and sites at which the parasite was present (red) or absent (yellow).

Chapter 4. Conclusion

Intermediate hosts of *A. cantonensis*

In Hawaii, *Parmarion martensi* has been the snail species most frequently implicated as the host of *Angiostrongylus cantonensis*. However, a wide diversity of gastropod species, both heterobranchs and caenogastropods, were shown to act as intermediate hosts of *Angiostrongylus cantonensis* in the Hawaiian Islands, including 16 of the 37 species tested. A literature review showed that there is an even wider range of intermediate hosts globally. This contrasts dramatically with other parasites that cause human disease. For example, while *Schistosoma mansoni*, the agent causing schistosomiasis, has gastropod hosts belonging to only one genus (*Biomphalaria*) (Negrão-Corrêa et al. 2007), *A. cantonensis* can be can be carried by multiple hosts from a number of families. Tropical as well as temperate gastropod species (e.g., western European *Oxychilus alliarius*) are known hosts of *A. cantonensis*. Thus, available hosts can be found throughout the world, and with global warming, it would be possible for *A. cantonensis* to become established in currently temperate areas (e.g., Lv et al. 2011).

Distribution of *A. cantonensis* and predicted suitable habitat

Infected snails were found on six of the largest Hawaiian Islands excluding Lanai. Sites with infected snails seem to be at lower elevations with warmer temperatures and higher rainfall, agreeing with the original hypotheses. These patterns reflect previous observations that have looked at the parasite's susceptibility to dry conditions and need of suitable temperatures for development of the parasite in the intermediate host (Richards and Merritt 1967; Yousif and Lämmler 1975).

Although cases of infection are mostly confined to the windward side of the island of Hawaii, probably because of the presence of *P. martensi*, which is highly susceptible to infection, and the suitable climate, there are areas that are just as suitable to where the parasite can spread. The best habitat suitability model included both temperature and rainfall as important environmental factors influencing *A. cantonensis*

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distribution. This best model was then used to predict suitable habitat throughout the Hawaiian Islands. The model predicted higher suitability in windward areas where rainfall is greater than in leeward areas. For example, the model predicted suitable habitat on Oahu, where *P. martensi* is also found.

The general public and the medical community need to be more aware of the vast number and taxonomic range as well as the broad geographic distribution of the intermediate hosts of *A. cantonensis*, which will hopefully help prevent infection and improve diagnosis and treatment of the disease. Education on how to avoid infection, including thoroughly washing produce will be important in disease prevention.

Future directions

With the wide range of intermediate hosts now known in Hawaii and globally, the potential for spread is high. Predicting the potential expansion of *A. cantonensis* into areas it has yet to be found in is important for both managing the hosts and for educating the general public and the medical community so that they are aware of the potential for infection. Climate change may result in wider regions of the world becoming suitable for the parasite and its hosts and predicting suitable habitat under various climate change scenarios would help us to understand the additional potential of this parasite to spread. The analysis in this thesis of the environmental factors determining the distribution of *A. cantonensis* in Hawaii only incorporated temperature and rainfall. There may be other factors that influence distribution, such as proximity to human settlements or agriculture, but more analyses are needed to determine this.

Ultimately, development of effective methods to manage hosts (both rats and gastropods) will be important in reducing the chance of infection. As part of this, determining ways to improve agricultural practices and food handling to minimize the chance of contamination with infected gastropod hosts is important. For instance, Yeung et al. (2013) tested the efficacy of various readily available household solutions in washing snails and slugs off lettuce, but found that none of them was better than simply rinsing with tap water. Similar experiments were done to test commercially available produce washing solutions but again, none was better at removing gastropods from

lettuce than simple rinsing with tap water (Yeung, Hayes and Cowie unpublished). Educating the community regarding best practices in food production and handling will be key in limiting the chances of infection (e.g., Hollyer et al. 2010).

Many research needs, not only regarding the potential spread of *A. cantonensis* and the need for education in Hawaii and elsewhere, but also addressing diagnostic protocols and approaches to treatment were developed at an international workshop held in Honolulu in 2011 (Cowie et al. 2012) and revisited in a follow-up workshop in China in 2013. These research priorities set a clear agenda for future research addressing the spread of *A. cantonensis*, management of the hosts, and diagnosis and treatment of the disease. International collaboration among the relatively small number of scientists involved in this research is crucial.

Appendix. Known gastropod hosts of *Angiostrongylus cantonensis*, associated localities and corresponding key references.

Species	Locality	References
Heterobranchia		
Terrestrial		
Achatinellidae		
Lamellidea pusilla (Gould, 1847)		Wallace and Rosen 1969c*
Tornatellides sp.	Hawaii	This study
Achatinidae		
Achatina fulica Bowdich, 1822	Amami Islands (Japan), Brazil, China, Florida, Hawaii, Indonesia, Malaysia, Mariana Islands, Micronesia, Ogasawara Islands (Japan), Okinawa (Japan), Papua New Guinea, Taiwan, Thailand	Alicata 1965b; Lim and Heyneman 1965; Crook et al. 1968; Wallace and Rosen 1969b; Intermill et al. 1972; Margono and Ilahude 1974; Yii et al. 1975; Brockelman et al. 1976*; Sato et al. 1980; Scrimgeour and Welch 1984; Li et al. 2006; Caldeira et al. 2007; Tokiwa et al. 2012; Teem et al. 2013; this study
Agriolimacidae		
Deroceras laeve (Müller, 1774)	China, Cook Islands, Hawaii, New Caledonia, Okinawa (Japan), Tahiti	Alicata and McCarthy 1964; Wallace and Rosen 1969b, c*; Intermill et al. 1972; Ash 1976; Li et al. 2006 [as '*leave*']
Deroceras reticulatum (Müller, 1774)	Hawaii	Weinstein et al. 1963*; this study
Ariophantidae		
Ariophantidae sp.	China	Lv et al. 2008
Cryptosoma imperator (Gould, 1859)		Ko 1991*

Cryptozona bistrialis (Beck, 1837)	Unspecified	Cross and Chen 2007 [as '*bristalis*']
Hemiplecta distincta (Pfeiffer, 1850)	Thailand	Crook et al. 1968
Hemiplecta siamensis (Pfeiffer, 1856)	Thailand	Crook et al. 1968
Macrochlamys loana (Gredler, 1882)	China	Li et al. 2006 (unverified species name)
Macrochlamys nitidissima (Moellendorff, 1883)		Ko 1991*
Macrochlamys resplendens (Philippi, 1864)	Malaysia, Thailand	Liat et al. 1965; Crook et al. 1968 [as '*Sarika*']
Microparmarion malayanus (Collinge, 1903)	Malaysia	Liat et al. 1965
Parmarion martensi Simroth, 1893	Hawaii, Okinawa (Japan)	Asato et al. 2004; Hollingsworth et al. 2007; this study
Bradybaenidae		
Acusta despecta (Sowerby, 1839)	Okinawa (Japan)	Asato et al. 2004
Bradybaena brevispira (H. Adams, 1870)	China	Lv et al. 2008
Bradybaena circulus (Pfeiffer,1846)	Japan, Okinawa (Japan)	Intermill et al. 1972; Noda et al. 1987**
Bradybaena ravida (Benson, 1842)	China	Li et al. 2006 ['*revida*']
Bradybaena similaris (Rang, 1831)	Brazil, China, Cook Islands, Cuba, Hawaii, Mariana Islands, Micronesia, New Caledonia, Tahiti	Alicata 1965b; Wallace and Rosen 1969b, c*; Ash 1976; Li et al. 2006 [as '*similaris similaris*']; Caldeira et al. 2007; Dorta-Contreras et al. 2007
Fruticicola despecta (A. Adams, 1868)	Okinawa (Japan)	Intermill et al. 1972; Noda et al. 1987*

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Appendix. (Continued) Known gastropod hosts of *Angiostrongylus cantonensis*, associated localities and corresponding key references.

Plectotropis applanata (Möellendorff, 1884)	China	Li et al. 2006 ['*appanata*']
Camaenidae		
Camaena cicatricosa (Müller, 1774)	China	Ko 1991 ['*circatoricosa*']*; Lv et al. 2008
Satsuma mercatoria (Pfeiffer, 1845)	Okinawa (Japan)	Intermill et al. 1972
Trichochloritis hungerfordianus (Möllendorff, 1884)	China	Lv et al. 2008
Trichochloritis rufopila (Möllendorff, 1884)	China	Lv et al. 2008
Cepolidae		
Jeanneretia bicincta (Menke, 1830)	Cuba	Dorta-Contreras et al. 2007
Discidae		
Anguispira alternata (Say, 1816)		Campbell and Little 1988*
Dyakiidae		
Quantula striata (Gray, 1834)	Malaysia	Lim and Heyneman 1965
Enidae		
Luchuena reticulata (Reeve, 1849)		Noda et al. 1987*
Euconulidae		
Liardetia sculpta (Möllendorff, 1883)		Wallace and Rosen 1969c*
Helicarionidae		
Girasia peguensis (Theobald, 1864)	Malaysia	Liat et al. 1965
Helicarion sp.		Yong et al. 1981*
Ovachlamys fulgens (Gude, 1900)	Hawaii	Qvarnstrom et al. 2013; this study
Philonesia sp.	Hawaii	This study
Helicidae		
Cornu aspersum (Müller, 1774)		Ash 1976 [as '*Helix aspersa*']*

Appendix. (Continued) Known gastropod hosts of *Angiostrongylus cantonensis*, associated localities and corresponding key references.

Emoda sagraiana (D'Orbigny, 1842)	Cuba	Dorta-Contreras et al. 2007 [as '*sagrayana*']
Helicina adspersa Pfeiffer, 1839	Cuba	Dorta-Contreras et al. 2007
Helicina orbiculata (Say, 1818)		Alicata 1965a*
Viana regina (Morelet, 1849)	Cuba	Dorta-Contreras et al. 2007
Helminthoglyptidae		
Polymita picta (Born, 1780)	Cuba	Dorta-Contreras et al. 2007
Limacidae		
Lehmannia poirieri (Mabille, 1883)		Campbell and Little 1988*
Lehmannia valentiana (Férussac, 1822)	Hawaii, Okinawa (Japan)	Asato et al. 2004 [as '*Limax valentianus*']; this study
Limax flavus Linnaeus, 1758	China, Okinawa (Japan)	Sato et al. 1981; Campbell and Little 1988*; Li et al. 2006 ['*Zimax*']
Limax marginatus (Müller, 1774)	Japan	Tokiwa et al. 2012
Limax maximus Linnaeus, 1758	China, Hawaii	Weinstein et al. 1963*; Yang et al. 2012; this study
Oleacinidae		
Oleacina solidula (Pfeiffer, 1840)	Cuba	Dorta-Contreras et al. 2007
Oxychilidae		
Oxychilus alliarius (Miller, 1822)	Hawaii	this study
Philomycidae		
Meghimatium bilineatum (Benson, 1842)	China, Okinawa (Japan)	Sato et al. 1981 [as '*Incilaria bilineata*']; Li et al. 2006 [as '*bilinestum*']

Pallifera sp.	Hawaii	Qvarnstrom et al. 2013
Philomycus bilineatus (Benson, 1842)	China, Okinawa (Japan)	Intermill et al. 1972; Lv et al. 2008 ['*Phiolomycus*']
Philomycus carolinianus (Bosc, 1801)		Campbell and Little 1988*
Pleurodontidae		
Pleurodonte sp.	Jamaica	Robinson et al. 2013
Thelidomus aspera (Férussac, 1821)	Jamaica	Lindo et al. 2002 [as '*asper*']
Zachrysia auricoma (Férussac, 1821)	Cuba	del Risco Barrios and Diéguez 2004
Polygyridae		
Mesodon thyroidus (Say, 1817)		Campbell and Little 1988*
Polygyra triodontoides (Bland, 1861)*		Campbell and Little 1988*
Sagdidae		
Aquebana belutina (Lamarck, 1822)	Puerto Rico	Andersen et al. 1986
Sagda sp.	Jamaica	Robinson et al. 2013
Spiraxidae		
Euglandina rosea (Férussac, 1821)	Hawaii	Wallace and Rosen 1969b; Campbell and Little 1988*; this study
Subulinidae		
Allopeas gracile (Hutton, 1834)		Wallace and Rosen 1969c* [as '*Lamellaxis oparanum*']
Opeas javanicum	Mariana Islands, Micronesia	Alicata 1965b

Appendix. (Continued) Known gastropod hosts of *Angiostrongylus cantonensis*, associated localities and corresponding key references.

Paropeas achatinaceum (Pfeiffer, 1846)	Cook Islands, Hawaii, Tahiti	Wallace and Rosen 1969b, c* [as '*Prosopeas javanicum*']; this study
Rumina decollata (Linnaeus, 1758)	Cuba	Dorta-Contreras et al. 2007
Subulina octona (Bruguière, 1792)	Brazil, Cook Islands, Cuba, Hawaii, Mariana Islands, Micronesia, Puerto Rico, Tahiti	Alicata 1965b; Wallace and Rosen 1969b, c*; Andersen et al. 1986; del Risco Barrios and Diéguez 2004; Caldeira et al. 2007; this study
Succineidae		
Succinea sp.	Cuba	del Risco Barrios and Diéguez 2004
Urocoptidae		
Tetrentodon (*Cilindricoptis*) sp.	Cuba	Dorta-Contreras et al. 2007
Tetrentodon (*Scalaricoptis*) *filiola* (Jaume and Torre, 1972)	Cuba	Dorta-Contreras et al. 2007
Tetrentodon (*Tetrentodon*) *perdidoensis* (Jaume and Torre, 1972)	Cuba	Dorta-Contreras et al. 2007
Veronicellidae		
Laevicaulis alte (Férussac, 1822)	Australia, China, Fiji, Hawaii, India, Indonesia, Malaysia, New Caledonia, Okinawa (Japan), Philippines	Liat et al. 1965; Wallace and Rosen 1969b, c*; Margono and Ilahude 1974; Ash 1976; Mason et al. 1976; Sato et al. 1981; Renapurkar et al. 1982; Uchikawa et al. 1984; Li et al. 2006; Fontanilla and Wade 2008 [as '*altae*']; this study
Sarasinula linguaeformis (Semper, 1885)	Brazil	Espírito-Santo et al. 2013
Sarasinula marginata (Semper, 1885)	Brazil	Caldeira et al. 2007

Appendix. (Continued) Known gastropod hosts of *Angiostrongylus cantonensis*, associated localities and corresponding key references.

Sarasinula plebeia (Fischer, 1868)	Australia, Cook Islands, Fiji, Mariana Islands, Micronesia, New Caledonia, Okinawa (Japan), Tahiti	Alicata 1962, 1965a [as '*Vaginalus plebeius*']; Wallace and Rosen 1969b [as '*Vaginulus plebeius*']; Intermill et al. 1972 [as '*Vaginulus plebeius*']; Mason et al. 1976
Vaginulus ameghini (Gambetta, 1923)		Campbell and Little 1988*
Vaginulus sp.	China	Lv et al. 2008
Vaginulus yuxjsjs	China	Lv et al. 2008 [as 'sp. nov.' but possibly never described]
Veronicella cubensis (Pfeiffer, 1840)	Cuba, Hawaii	Qvarnstrom et al. 2007; Dorta-Contreras et al. 2007; this study
Veronicella siamensis (Martens, 1867)	Thailand	Crook et al. 1968
Veronicellidae spp.	Jamaica	Robinson et al. 2013
Vertiginidae		
Gastrocopta pediculus (Shuttleworth, 1852)		Wallace and Rosen 1969c*
Freshwater		
Lymnaeidae		
Fossaria viridis (Quoy and Gaimard, 1832)		Alicata and Brown 1962 [as '*ollula*']*
Lymnaea columella Say, 1817		Kocan 1972*
Lymnaea japonica (Jay, 1856)		Shiota et al. 1980*
Lymnaea palustris (Müller, 1774)		Kocan 1972*
Lymnaea spp.		el-Shazly et al. 2002
Lymnaea stagnalis (Linnaeus, 1758)		Kocan 1972*
Lymnaea swinhoei H. Adams, 1866		Chang et al. 1968* [as '*swinhoe*']

Lymnaea tomentosa (Pfeiffer, 1855)	Unspecified	Morley 2010
Lymnaea volutata Gould, 1848		Wallace and Rosen 1969c*
Radix natalensis (Krauss, 1848)	Egypt	Ibrahim 2007 [as '*Lymnaea*']
Stagnicola elodes		Morley 2010*
Stagnicola emarginata (Say, 1821)		Kocan 1972*
Physidae		
Physa acuta Draparnaud, 1805		Shiota et al. 1980*
Physa elliptica Lea, 1843		Wallace and Rosen 1969c*
Physa spp.		Richards and Merritt 1967*
Physastra sp.		Yong et al. 1981*
Planorbidae		
Biomphalaria alexandrina (Ehrenberg, 1831)	Egypt	Ibrahim 2007
Biomphalaria glabrata (Say, 1818)		Richards and Merritt 1967*; el-Shazly et al. 2002
Biomphalaria heliophila (Orbigny, 1835)		Richards and Merritt 1967*
Biomphalaria obstructa (Morelet, 1849)		Richards and Merritt 1967*
Biomphalaria pallida (Adams, 1846)		Richards and Merritt 1967*
Biomphalaria pfeifferi (Krauss, 1848)		Richards and Merritt 1967*
Biomphalaria straminea (Dunker, 1848)		Richards and Merritt 1967*
Biomphalaria tenagophila (Orbigny, 1835)		Richards and Merritt 1967*
Bulinus africanus (Krauss, 1848)		Morley 2010*
Bulinus contortus (Michaud, 1829)		Morley 2010*
Bulinus forskalii (Ehrenberg, 1831)		Richards and Merritt 1967*
Bulinus globosus (Morelet, 1866)		Richards and Merritt 1967*
Bulinus senegalensis (Müller, 1781)		Richards and Merritt 1967*

Bulinus tropicus (Krauss, 1848)		Richards and Merritt 1967*
Bulinus truncatus (Audouin, 1827)	Egypt	Richards and Merritt 1967*; el-Shazly et al. 2002
Drepanotrema simmonsi (Ferguson and Gerhardt, 1956)		Richards and Merritt 1967*
Ferrissia tenuis (Bourguignat, 1862)		Richards and Merritt 1967*
Gyraulus hiemantium (Westerlund, 1887)		Shiota et al. 1980*
Helisoma sp.		Richards and Merritt 1967*
Indoplanorbis exustus (Deshayes, 1834)	Malaysia	Liat et al. 1965; Richards and Merritt 1967*
Planorbella duryi Pilsbry, 1934		Wallace and Rosen 1969c* [as '*Helisoma duryi normale*']
Plesiophysa hubendicki Richards and Ferguson, 1962		Richards and Merritt 1967*
Segmentina hemisphaerula (Benson, 1842)		Chang et al. 1968*

Caenogastropoda
Terrestrial
Assimineidae

Assiminea parvula (Pease, 1865)		Wallace and Rosen 1969c [as '*nitida*']*
Cyclotropis sp.	Hawaii	this study
Omphalotropis fragilis Pease, 1860		Wallace and Rosen 1969c [as '*Omphalatropis*']*

Cyclophoridae

Chamalycaeus sinensis (Heude, 1882)		Ko 1991 [as 'Cyclotus']*

Megalomastomatidae

Appendix. (Continued) Known gastropod hosts of *Angiostrongylus cantonensis*, associated localities and corresponding key references.

Farcimen tortum (Wood, 1828)	Cuba	Dorta-Contreras et al. 2007
Neocyclotidae		
Poteria sp.	Jamaica	Robinson et al. 2013
Pomatiidae		
Chondropoma pictum arangoi (Torre and Bartsch, 1938)	Cuba	Dorta-Contreras et al. 2007
Eutudora jimenoi (Arango in Pfeiffer, 1864)	Cuba	Dorta-Contreras et al. 2007
Rhitidopoma sp.	Cuba	Dorta-Contreras et al. 2007
Pupinidae		
Pupina complanata (Pease, 1860)	Micronesia	Alicata 1965b
Truncatellidae		
Truncatella marginata Küster, 1855		Wallace and Rosen 1969c*
Freshwater		
Ampullariidae		
Lanistes carinatus (Olivier, 1804)	Egypt	Yousif and Ibrahim 1978
Marisa cornuarietis (Linnaeus, 1758)*		Richards and Merritt 1967*
Pila ampullacea (Linnaeus, 1758)	Thailand	Punyagupta 1965
Pila angelica (Annandale, 1920)*		Morley 2010*
Pila gracilis (Lea, 1856)	Thailand	Crook et al. 1968
Pila pesmei (Morlet, 1889)	Thailand	Tesana et al. 2009
Pila scutata (Mousson, 1849)	Indonesia, Malaysia, Thailand	Lim and Heyneman 1965; Punyagupta et al. 1970; Margono and Ilahude 1974
Pila turbinis Lea, 1856	Thailand	Crook et al. 1968 [as '*Pila ampullacea turbinis*']

Pila virescens (Deshayes, 1830) [as '*polita*']	China, Thailand	Crook et al. 1968; Tesana et al. 2008*; Cheng et al. 2011
Pomacea canaliculata (Lamarck, 1822)	China, Hawaii, Okinawa (Japan), Taiwan	Nishimura et al. 1986 [as '*Ampullarius canaliculatus*']**; Yen et al. 1990 [as '*Ampullarium canaliculatus*']; Zhang et al. 2008; this study
Pomacea lineata (Spix in Wagner, 1827)	Brazil	Thiengo et al. 2010
Pomacea maculata Perry, 1810	China, Louisiana	Li et al. 2006 [as '*Pila gigas*']; Li et al. 2012 [as '*Ampullarum crossean*']*; Teem et al. 2013
Pomacea paludosa (Say, 1829)	Cuba, Hawaii	Wallace and Rosen 1969b, c*; del Risco Barrios and Diéguez 2004
Bithyniidae		
Bithynia siamensis goniomphalos (Lea, 1856)	Thailand	Tesana et al. 2009
Buccinidae		
Clea helena (Philippi, 1847)	Thailand	Tesana et al. 2009
Paludomidae		
Cleopatra bulimoides (Olivier, 1804)	Egypt	Ibrahim 2007
Cleopatra cyclostomoides (Bourguignat, 1879)	Egypt	Ibrahim 2007
Pleuroceridae		
Goniobasis livescens (Menke, 1830)		Kocan 1972*
Thiaridae		
Melanoides tuberculata (Müller, 1774)	Egypt, Thailand	Crook et al. 1968; Ibrahim 2007
Viviparidae		

Appendix. (Continued) Known gastropod hosts of *Angiostrongylus cantonensis*, associated localities and corresponding key references.

Bellamya aeruginosa (Reeve, 1862)	China	Li et al. 2006
Bellamya chinensis (Gray, 1834)	Taiwan	Chang et al. 1968 [as '*Cipangopaludina*']; Ko 1991 [as '*Cipangopalaudina*']*
Bellamya lithophaga (Heude, 1889)	China	Cheng et al. 2011
Bellamya quadrata (Benson, 1842)	China	Chang et al. 1968 [as '*Sinotaia*']*; Xie and Wu 2013 [as '*Sinotaia*']
Bellamya spp.	China	Li et al. 2006
Cipangopaludina sp.	China	Deng et al. 2012
Filopaludina martensi (Lea, 1856)	Malaysia	Liat et al. 1965 [as '*Bellamya ingallsiana*']
Filopaludina martensi martensi (Frauenfeld, 1865)	Thailand	Tesana et al. 2009
Filopaludina sumartrensis polygramma (Dunker, 1852)	Thailand	Tesana et al. 2009
Sinotaia histrica (Gould, 1859)		Shiota et al. 1980 [as '*Taia*']*
Sinotaia martensiana (Nevill, 1881)	Unspecified	Morley 2010
Marine		
Architectonicidae		
Discotectonica acutissima (Sowerby, 1914)	China	Yang et al. 2012 [doubtful record]
Species of uncertain identity		
P. bilineatus	China	Li et al. 2006

* References reporting only experimental laboratory infection - no locality is given for such studies.

** References reporting both natural and experimental infection.

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