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Vector Competence of Aedes sierrensis and Culex pipiens complex (Diptera: Culicidae) for Dirofilaria immitis (Spirurida: Onchocercidae) in Northern California

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VECTOR COMPETENCE OF *Aedes sierrensis* AND *Culex pipiens* COMPLEX (DIPTERA: CULICIDAE) FOR *Dirofilaria immitis* (SPIRURIDA: ONCHOCERCIDAE) IN NORTHERN CALIFORNIA

by

Jeffrey Allan Kurosaka

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Jeffrey Allan Kurosaka
DEDICATION

This thesis is dedicated to my friends and family, without whom none of this would have been possible. To my best friend, Tommy Bolton, our friendship is something I could not do without. You believed in me when even I had my doubts. Someday, I will find a way to repay you for all that you have done for me. To my parents, Donald and Marilyn Kurosaka, you were my teachers before I even knew the definition of the word, before I even took my first breath. You taught me right from wrong and made me who I am today. No matter what I do in life, it will never be enough to show my gratitude for all that you both have sacrificed. As such, know that my accomplishments are your accomplishments. Knowing that you are both proud of me is all the satisfaction that I will ever need. To my older brother and sister, Douglas and Lisa Kurosaka, you have protected and watched over me since I was born. I want you to know that I appreciate you both not only for who you are, but for everything you have done for me whether I knew about it or not. Life has thrown many curveballs my way. This document is a testament to the difference all five of you have made in my life.
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Vector Competence of *Aedes sierrensis* and *Culex pipiens* complex (Diptera: Culicidae) for *Dirofilaria immitis* (Spirurida: Onchocercidae) in Northern California

Abstract

By Jeffrey A. Kurosaka

University of the Pacific
2017

*Dirofilaria immitis* Leidy (dog heartworm) is a life-threatening parasite transmitted by mosquitoes to domestic dogs. Endemic in the eastern United States, cases have become more prevalent over the last few decades. While prevalence in California is generally low, Lake and San Joaquin Counties have reported rates comparable to the East Coast at 3.73% and 0.71% (CAPC 2017), respectively. *Aedes sierrensis* is thought to be responsible for transmission in California, but in some cases, it exists in inadequate quantities and temporal ranges to explain parasite activity. Based on Huang et al. (2013) and Tran (2016), bloodfeeding patterns, and other vector criteria, *Culex pipiens complex* and *Culiseta incidens* were chosen to evaluate for vector competence. Female field-caught mosquitoes were reared, infected (2.5-5 mff/μl), and decapitated at 15, 18, or 21 days post infection (dpi). *Cs. incidens* was reluctant to feed using an artificial feeding system and will require additional trials. On the contrary, trials on *Ae. sierrensis* and *Cx. pipiens* complex were both completed successfully. Both species were determined to be competent vectors of *D. immitis*. Based on our findings, more than half of *Ae. sierrensis*
females produced emerging L3s by 21 dpi, while *Cx. pipiens* complex never produced L3s in more than 5% of females. In conjunction with other factors such as the detection of *D. immitis* in wild mosquitoes, host-seeking preferences for domestic dogs, and appropriate temporal overlap, this suggests that both *Ae. sierrensis* and *Cx. pipiens* complex may play central roles in Lake or San Joaquin Counties, CA when abundant. Targeted control efforts are necessary to reduce the incidence of canine heartworm in these areas. While Lake and San Joaquin Counties, CA were the focus of this study, our results may be applicable to the western United States when these species are relevant.
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Chapter 1: Background Information

Canine Heartworm

Canine heartworm, otherwise known as *D. immitis*, is a parasitic nematode that derives its name from the primary definitive host (domestic and wild canids) and organ (heart) in which reproduction occurs (Cullens 2008). Belonging to the superfamily *Filarioidea*, heartworms are often described as filarial, or thread-like, in nature and cause a disease known as filariasis (Anderson 2000; Lok, Walker, Scoles 2000). *Dirofilaria immitis* is one of many filarial nematodes known to affect public health. Others include *Wuchereria bancrofti* Cobbold and *Brugia Malayi* Brug, *Loa loa* Cobbold, and *Mansonella* Manson, which are responsible for lymphatic, subcutaneous, and serous cavity filariases, respectively (CDC 2013; CDC 2015; CDC 2016).

Morphologically, heartworms are slender, white, and vary in size depending on their sex and stage in development, with adults reaching lengths of approximately one foot (Taylor 1960). Completion of a heartworm’s life cycle can take between 6 and 9 months in the canine host, with infections remaining patent up to 7.5 years (Abraham 1988; Anderson 2000; Newton 1968).

Heartworm disease, or dirofilariasis, is the manifestation of chronic symptoms that result from the physiological burden that heartworms place cardiovascular system of the definitive host. If left untreated, the infection can be fatal (Calvert, Rawlings, McCall 1999). Heartworm disease remains one of the most serious parasitic diseases...
affecting domestic dogs in North America and perhaps the world (Bowman and Atkins 2009; Simón et al. 2012).

**Life Cycle**

Heartworm transmission occurs when a susceptible female mosquito ingests an infected bloodmeal containing microfilariae (early stage larvae, mf) from a definitive host, supports development of the parasite to its infective stage, and transmits it to another receptive, definitive host. Transmission requires a vector (carrier of transmission for the parasite) to take at least two separate and appropriately timed bloodmeals. The first must contain the microfilarial infection, while the second must be staggered sufficiently to allow infective stage larval development (Cancrini and Gabrielli 2007). If a female mosquito cannot accomplish this, then the parasite will not be transmitted (Simón et al. 2012).

Development in the intermediate host. It is a common misconception that all mosquitoes bite. In fact, both male and female mosquitoes can survive on water and sucrose alone. Blood is required only by the female as a means of acquiring the protein necessary to develop their eggs. Exceptions to this include autogenous species, which utilize protein reserves accumulated during their larval stages to produce their first batch of eggs. By comparison to anautogenous species that can require several blood meals for egg-laying, autogenous species are considered relatively less important as vectors as their chances of larval uptake and transmission is reduced (Cancrini and Kramer 2001; Cancrini and Gabrielli 2007).

A mosquito species is considered a competent vector if it supports development of the parasite to its infective stage. Development occurs in a series of stages. Each stage is
characterized by a molt and a migration, but not necessarily in that order (Kartman 1953a; McCall et al. 2008). Transmission begins when a susceptible female mosquito ingests a bloodmeal containing microfilariae. The microfilariae flow through the pharynx and into the midgut along with the blood (Figure 1.1). Over the course of 24 hours, the microfilariae escape the blood bolus and make their way into the Malpighian tubules (M.T.) via its junction with the midgut. Upon reaching the distal end of the lumen, the microfilariae invade the large, primary cells and transform into “sausage” stage larvae. Although this is often considered the worm’s first larval stage (L1), this is a misnomer as ecdysis (molting) does not occur at this time. By the seventh day, the L1 migrate back into the lumen of the Malpighian tubules to continue developing. Larvae molt into second-stage larvae (L2) around 10 dpi and then third-stage larvae (L3) by day 13. Upon completion of development into L3s, the larvae will perforate the distal ends of the Malpighian tubules and migrate toward the head via the hemocoel (body cavity) (Abraham 1988; Bradley, Sauerman Jr., Nayar 1984; Bradley and Nayar 1987; Cancrini and Kramer 2001; Kartman 1953a; Manfredi, DiCerbo, Genchi 2007; Serrão, Labarthe, Lourenço-de-Oliveira 2001; Taylor 1960). Third-stage larvae are considered “infective” once they have reached the cephalic spaces of the head, the salivary glands (S.G.), or the proboscis (Manfredi, DiCerbo, Genchi 2007; McCall et al. 2008; Montarsi et al. 2015; Taylor 1960). Canine heartworm exhibits positive thermotaxis, which confers a drive to migrate toward higher temperature gradients (Stueben 1954). During the female mosquito’s next bloodmeal, the L3 will be drawn to the warmth of the definitive host and burrow out of the mouthparts of the mosquito. Emergence can occur from various locations on the mosquito’s proboscis (needle-like mouthpart), from the
folded labium (lower lip) to the tip which is called the labellum (Abraham 1988). Third-stage larvae are carried along with the mosquito’s hemolymph and become deposited onto the skin of the definitive host near the feeding wound. Once the mosquito has finished ingesting its bloodmeal and removes its proboscis from the host, the larvae will enter the host through the residual feeding wound (Cancrini and Kramer 2001; Grassi and Noe 1900; McGreevy et al. 1974). Mosquitoes that prohibit development, whether it be through mechanical defenses or innate immunity, are considered refractory (Michalski et al. 2010). Globally, over 60 species of mosquitoes have been shown to be susceptible to infection, 13 of which exist in the United States (Bowman and Atkins 2009; Lok 1988; McCall et al. 2008; Otto and Jachowski Jr 1980). Research is necessary to determine which of these mosquitoes can support development of heartworms to their infective stage.

Figure 1.1. Development of *D. immitis* within the mosquito. Arrows depict the pathway followed by *D. immitis* as it progresses throughout the mosquito. S.G., Salivary Glands. M.T., Malpighian Tubules.
Heartworm development in mosquitoes that can support *D. immitis* is temperature-dependent and proportional to the degree of thermal exposure above the 14°C threshold (Knight and Lok 1998; Lok and Knight 1998; McCall et al. 2008; Stueben 1954). The rate of development described above was based on mosquitoes held at 26-27°C and 80% relative humidity (RH), which require 10-17 days (McCall et al. 2008; Taylor 1960). As climates continue to warm, the seasonal activity and geographic range of the parasite is expected to continue to increase (Genchi et al. 2009; Ledesma and Harrington 2011; Otranto, Capelli, Genchi 2009; Sacks, Chomel, Kasten 2004).

Approximately 130 heartworm development units (HDU) are required to allow the parasite to reach its infective stage within the mosquito (Knight and Lok 1998). Heartworm development units are defined as the accumulation of thermal units within a range of 14°C to 30.5°C, regardless of if they are consecutive (Christensen and Hollander 1978; Knight and Lok 1998; Lok and Knight 1998). Ambient temperatures below the threshold will cause development to cease and may cause larvae to withdraw back into the mosquito (Stueben 1954). The degree-day calculation below factors in the average daily temperature above the developmental threshold for canine heartworm to approximate the number of HDUs accumulated (Ledesma and Harrington 2015).

\[
\text{Accumulated HDUs} = \sum \text{Average Daily Temperature} - (14^\circ\text{C})
\]

Development in the definitive host. Third-stage larvae utilize the residual feeding wound site as a portal of entry into the definitive host (Grassi and Noe 1900; McGreevy et al. 1974). By 3 dpi, the larvae molt into fourth stage larvae (L4) within the surrounding subcutaneous tissue. Between 50 and 70 dpi, larvae molt into fifth-stage immature adults.
(L5) and enter the bloodstream through the surrounding blood vessels. Immature adults can be found in the heart as early as day 70 (Grieve, Lok, Glickman 1983). Complete migration and maturation of all worms is observed by 120 dpi. Mature worms are not only 10 times larger than their previous, immature state, but also they are capable of producing microfilariae via sexual reproduction (Lichtenfels et al. 1985; Lichtenfels, Pilitt, Wergin 1987; McCall et al. 2008). Microfilariae can be detected within definitive host’s blood between 6 to 9 months post infection, producing a patent infection capable of infecting susceptible mosquitoes (Abraham 1988; Grieve, Lok, Glickman 1983; Knight and Lok 1998; Kotani and Powers 1982; Kume and Itagaki 1955; McCall et al. 2008; Orihel 1961). A vertebrate host that is incapable of supporting development or producing a patent infection is considered a dead-end host. Dead-end hosts are not at risk of becoming an infectious reservoir or developing most of the symptoms associated with the parasite (Grieve, Lok, Glickman 1983; Ledesma and Harrington 2011).

Figure 1.2 is a simplified representation of *D. immitis*’s life cycle. Canine heartworm’s life cycle is obligate to both its intermediate host and its definitive host for survival. While many courses of action are available to treat infected definitive hosts, prevention is by far our best option (Nelson, McCall, Carithers 2014).
Figure 1.2: Complete life cycle of *D. immitis*, from microfilarial development in the mosquito (mff-L3) to its infection of the host (Dog/Human/etc.).

**Potential Hosts**

While domestic dogs are the primary reservoir of infection for canine heartworm, several other species are also at risk. Other receptive hosts include, but are not limited to, wild canids such as coyotes, felids, ferrets, raccoons, sea lions, penguins, and humans (Bowman and Atkins 2009; Grieve, Lok, Glickman 1983; Ledesma and Harrington 2011; McCall et al. 2008; Sacks, Chomel, Kasten 2004; Sano et al. 2005; Simón et al. 2009; Theis 2005). Only some of the above hosts are capable of developing patent, communicable infections (Bowman and Atkins 2009).

Hosts infected with canine heartworm can remain asymptomatic for months or even years, with some never becoming symptomatic at all. Factors that may influence the development of symptoms include the species of definitive host, the density of filarial
burden, individual reactivity, and the level of exercise (McCall et al. 2008). In dogs, symptoms range from persistent coughing to lethargy, anorexia, and even mortality (Calvert, Rawlings, McCall 1999; Simón et al. 2009; Simón et al. 2012). Human cases, however, are less severe as they are incapable of supporting adult heartworm development. Symptoms include coughing, hemoptysis, chest pain, fever, and sometimes pleural diffusion (Global Health - Division of Parasitic Diseases 2012; Roy, Chirurgi, Theis 1993; Simón et al. 2009; Simón et al. 2012; Theis 2005).

Although human cases are not fatal, they often present diagnostic complications for physicians. For example, heartworms are recognized on chest radiograms and CT scans as coin lesions, or dense, circular masses with smooth edges (McCall et al. 2008). While the diagnostic differential for a coin lesion varies, a common preliminary interpretation is cancer (Allison et al. 2004; Theis 2005; Toomes et al. 1983; Trunk, Gracey, Byrd 1974). Extensive clinical tests are necessary to determine the etiology of the condition, the cost of which can exceed $80,000 per patient (Theis 2005). Meanwhile, the patient is left to deal the possibility that they may have cancer when in fact the actual cause of their coin lesion was a small pulmonary infarction (tissue death) caused by heartworm-related embolic clots (Gómez-Merino et al. 2002; Rena, Leutner, Casadio 2002; Roy, Chirurgi, Theis 1993; Simón et al. 2012). Although none of the differentials are desirable, treatment for the latter is preferred as it usually only requires a simple procedure to surgical remove any nodules or worms present (Simón et al. 2012).

**Therapy in Canines**

Over the span of approximately 6 to 9 months, large quantities of adult heartworms can obstruct blood flow, form clots, and produce infectious microfilariae
within the vasculature of the definitive host (Anderson 2000; Rawlings et al. 1993). Therapy can be very effective when it comes to preventing infection, averting further damage, and precluding the development of additional symptoms. When it comes to therapy, two types exist: preventative therapy and adulticidal therapy (Bowman and Atkins 2009).

*Preventative therapy.* Preventatives are the best option for uninfected dogs (Nelson, McCall, Carithers 2014). Providing protection with minimal side effects, preventatives can be used in dogs as young as 8 weeks old (Cruthers et al. 2008; McCall et al. 2001). Regimens range from monthly to yearly depending on the formula and dosage. Avermectins and milbemycins are two series of commercially available drugs that rely on a group complex parasiticidal compounds known as macrocyclic lactones (McCall et al. 2008). Macro cyclic lactones kill larvae up to 60 days old (McCall et al. 2001). A single dose has been observed to be 80% to 90% effective (Blagburn, Paul, Newton 2001; Dzimianski et al. 2001; Lok, Knight, Ramadan 1989).

Currently, no other active ingredient other than macrocyclic lactones have been approved as a prophylactic in dogs by the FDA. Caution and strict adherence to proper usage is strongly advised to minimize the risk of resistance (Bowman and Atkins 2009; Prichard 2005). While they have been used experimentally to prevent the development of additional adult heartworms in already infected hosts, this is not recommended as preventatives are no longer a viable option once the larvae have developed into adults (Blair, Williams, Ewanciw 1982; Bowman et al. 1992; Lok, Knight, Ramadan 1989). As a result, macrocyclic lactones are not approved for safe usage in dogs with adult heartworms or in dogs with significant symptoms (Lok, Knight, Ramadan 1989).
Adulticidal therapy. Melarsomine dihydrochloride is the only treatment approved by the FDA to treat adult heartworms (Bowman and Atkins 2009; Nelson, McCall, Carithers 2014). Melarsomine dihydrochloride is an organoarsenic compound that acts as an immicide to kill adult heartworms and prevent further damage to the pulmonary vasculature. Tests have shown that two doses given intramuscularly every 24 hours have an efficacy of at least 96%. Repeated 4 months later, 99% of adult heartworms were killed (Miller et al. 1995). While extremely effective, this method possesses several shortcomings, including, but not limited to, the cost, the ill effects of arsenic exposure, and the threat posed by dead worms. Dead heartworms can lead to inflammation, thromboembolisms, arterial obstruction, and vasoconstriction (Kramer 2006).

Furthermore, melarsomine must be used in conjunction with tranquilizers over the entire course of the regimen to reduce the circulation of the infection. While this does not typically confer added risk, it does add to the expenses of treatment and require continuous maintenance to keep the patient sedated (Miller et al. 1995; Nelson, McCall, Carithers 2014).

Due to the risks involved with chemical methods, invasive surgery is not uncommon. Extracting heartworms from anesthetized dogs using forceps has shown to be 90% effective initially, with some patients dying from heart and renal failure post-treatment. Unfortunately, follow-up treatments still require melarsomines to completely cure the infection (Bowman and Atkins 2009; Morini et al. 1998; Nelson, McCall, Carithers 2014; Sasaki Y, Kitagawa H, Ishihara K. 1989).
Vector Control

Vector control is one option that shows promise to not only reduce the incidence of heartworm disease, but also to limit the spread of all mosquito-transmitted infections. Mosquito species important to transmission can be identified based upon their completion of key vector identification criteria (Ledesma and Harrington 2011). Ranked in order of importance, first the infection must be detected in wild-specimens. Second, the geographic distribution of the mosquito must overlap sufficiently to explain the prevalence of the infection in either wild or domestic animals. Third, the species of mosquito in question must feed on relevant wild or domestic animals in nature. Fourth, the mosquitoes must feed frequently on those same hosts in nature. Lastly, field strains of the mosquitoes must demonstrate competence, or the ability to develop the infection to its infective stage. This final criterion is typically evaluated in a laboratory, where infection variables are easier to control. By completing these criteria, it is possible to identify and target vectors of importance to transmission. Targeting vectors of transmission interferes with the intermediate host’s ability to continue the life cycle of the causative agent. This is especially useful when it comes to canine heartworm since all potential reservoirs for canine heartworm have yet to be determined.
Chapter 2: Vector Competence of *Aedes sierrensis* and *Culex pipiens* complex

(*Diptera: Culicidae*) for *Dirofilaria immitis* (*Spirurida: Onchocercidae*) in Northern California

**Introduction**

Canine heartworm, caused by the filarial parasite *Dirofilaria immitis*, is almost entirely preventable with oral prophylactics (Blagburn, Paul, Newton 2001; Dzimianski et al. 2001; Lok, Knight, Ramadan 1989). Despite this, contracting the infection is practically inevitable over the lifetime of an unprotected domestic dog in areas endemic with the parasite (Nelson, McCall, Carithers 2014). According to the Companion Animal Parasite Council (2017), 1.28% of dogs in the United States tested positive for *D. immitis* in 2016. Unfortunately, this value does not represent the overall prevalence of the parasite amongst domestic dogs as it pertains only to those tested for the parasite, 1 million of which came back positive ((APPA) American Pet Products Association; CAPC 2017). Estimates predict the actual activity of the parasite to be at least three times that as most infections go undiagnosed (Genchi, Kramer, Rivasi 2011; IDEXX Laboratories and ANTECH Diagnostics). Furthermore, domestic dogs are not the only definitive hosts susceptible to being infected. Heartworms have been recovered from coyotes and other wild canids, felids, mustelids, ungulates, marine mammals, and humans.
(Grieve, Lok, Glickman 1983; Ledesma and Harrington 2011; McCall et al. 2008; Sacks, Chomel, Kasten 2004; Sano et al. 2005; Simón et al. 2009; Theis 2005). As a result, prevalence of *D. immitis* worldwide is expected to be even greater than previously estimated (IDEXX Laboratories and ANTECH Diagnostics; McCall et al. 2008).

Failure to address the rising incidence of *D. immitis* has considerable repercussions on public health, veterinary and human alike. Chronic infections can lead to irreparable damage to the heart, lungs, and arteries in domestic dogs, which result in persistent coughing, lethargy, anorexia, and in some cases, death (Calvert, Rawlings, McCall 1999; McCall et al. 2008; Simón et al. 2009; Simón et al. 2012). In addition to these effects in dogs, previous studies suggest that mosquitoes coinfected with other filarial nematodes like *D. immitis* could transmit arboviruses, such as chikungunya virus, at higher rates to humans and other animals (Vaughan and Turell 1996; Zytoon, El-Belbisi, Matsumura 1993; Zytoon, El-Belbasi, Matsumura 1993).

To mitigate these downstream effects and reduce the overall activity of *D. immitis*, preventative and immiticidal therapy are both effective options (Bowman and Atkins 2009; Cruthers et al. 2008; Hampshire 2005; Sasaki Y, Kitagawa H, Ishihara K. 1989; Vezzoni, Genchi, Raynaud 1992). Access to these options, however, is not always possible. Mosquito vector control is an effective alternative capable of avoiding many of the complications associated with *D. immitis* therapy or the lack thereof (Vezzoni, Genchi, Raynaud 1992). By interfering in the parasite’s development within the mosquito, it is possible to reduce the incidence of cases among all susceptible definitive hosts, not just domestic dogs. While research has been completed in the United States, much of it is not directly applicable as it pertains to the East Coast (Butts 1979; Lewandowski Jr, Hooper,
Newson 1980; Licitra et al. 2010; Lindsey 1961; Lowrie 1991; Parker 1986; Sauerman Jr. and Nayar 1983; Thrasher, Ash, Little 1963; Thrasher 1968; Thrasher et al. 1968; Tolbert and Johnson Jr. 1982; Villavaso and Steelman 1970; Wallenstein and Tibola 1960; Watts et al. 2001). Mosquito biology varies from species to species as well as between different localities (Knight and Lok 1998; Ledesma and Harrington 2011; Nayar, Knight, Bradley 1988; Tiawsirisup and Kaewthamasorn 2007). Research specific to the West Coast is necessary to identify mosquito species important for parasite transmission in Northern California.

Lake County and San Joaquin County were chosen as the sites of this study due to their higher than average levels of parasite activity at 3.73% and 0.71%, respectively, in 2016 (CAPC 2017). Previously, Aedes, Culex, and Culiseta mosquitoes were tested for the presence of D. immitis in San Joaquin County, CA (Huang et al. 2013). Seven of the fifteen mosquito species tested positive for this parasite. Culex pipiens complex and Culiseta incidens were among them and have been chosen for further investigation based on availability and their ability to fulfill key vector criteria (Ledesma and Harrington 2011). Culex pipiens complex (total number of specimen, n=40; minimum infection rate estimates infection rates per 1,000 mosquitoes, MIR=3.66) was selected because it tested positive for D. immitis in San Joaquin County, displays significant ecological overlap with the parasite, possesses appropriate seasonality, habitually feeds on dogs, and possesses a geographical distribution that could help explain activity in San Joaquin County (Huang et al. 2013; Thiemann et al. 2012). Similarly, Culiseta incidens (n=11; MIR=2.81) tested positive in San Joaquin County, exists in urban/residential settings, and exhibits a strong tendency to feed on domestic dogs, which is imperative for parasite
transmission (Huang et al. 2013; Theis et al. 2000; Thiemann). *Aedes sierrensis* (n=1; MIR=6.7) was chosen as a positive control for this study since it has been shown to support development in previous studies (Theis et al. 2000; Tran, Nelms, Thiemann 2016; Walters and Lavoipierre 1982; Walters 1995). While *Ae. sierrensis* has been considered the primary vector of *D. immitis* in northern California since 1974, ecological data suggests that other mosquito species may be contributing to the observed activity due to the limited potential of *Ae. sierrensis* in some areas (Huang et al. 2013; Weinmann and García 1974).

The current study explored the potential of *Ae. sierrensis*, *Cx. pipiens* complex, and *Cs. incidens* as vectors for *D. immitis* in Northern California by examining *D. immitis* prevalence, mosquito abundance, and temperature trends (CAPC 2017; Lake County Mosquito and Vector Control District 2005-2015; NOAA 2017). These mosquito species of suspected importance were then evaluated based on their ability to support *D. immitis* development to its infective L3 stage. Identifying vectors of potential importance to *D. immitis* transmission should help vector control districts better target competent mosquito species, reducing the overall incidence of canine heartworm disease.

**Methodology**

**Mosquito abundance.** Species-specific mosquito abundance data were obtained for Lake County, CA from the Lake County Vector Control District and can be accessed using CalSurv Gateway, a California Vector-borne Disease Surveillance System that stores reported surveillance data from the California Department of Public Health, the Mosquito and Vector Control Association of California, and the University of California (CalSurv Gateway, UC Davis Center for Vector-borne Diseases 2017; Lake
County Mosquito and Vector Control District 2005-2015). Abundance data for each species was compiled into 11-year (2005-2015) average daily collections per trap per night to approximate species seasonality and relative abundance.

Mosquitoes were collected using the following methods: carbon dioxide (CO$_2$) traps, New Jersey light traps (NJLT), and resting collections. CO$_2$ traps, which rely on carbon dioxide to attract host-seeking mosquitoes, were only deployed between February and October by comparison to the other methods which were conducted year-round. New Jersey light traps use light to attract insects, including mosquitoes (Mulhern 1942). Resting collections were actively collected using aspirators and then placed into collection containers.

**Temperature data.** U.S. Climate Normals, which are three-decade averages (1981-2010), were obtained from the National Oceanic and Atmospheric Administration (NOAA) to estimate the average daily temperature in Lake County, CA throughout the year (NOAA 2017). Temperature readings originated from a single station southeast of Clearlake, CA. Although temperatures may vary throughout the county, Climate Normals were chosen due to inconsistencies in data collection at other stations over time. Heartworm development units (HDUs) were determined by calculating the 30 day (average life-span of a mosquito) differential between the average daily temperature and the development threshold for the *D. immitis* (14 °C) (Christensen and Hollander 1978; Huang et al. 2013; Knight and Lok 1998; Ledesma and Harrington 2015; Lok and Knight 1998).

Accumulated HDUs = $\Sigma$ Average Daily Temperature – (14 °C)
Mosquito collection/rearing. Mosquitoes were collected as egg rafts or larvae from Lake (Ae. sierrensis and Cs. incidens) and San Joaquin Counties (Cx. pipiens complex), California (Figure 2.1). Larvae were reared and maintained on tropical fish food until emergence in an insectary. Adults were maintained on 10% sucrose solution at 26°C with a 14:10 (L:D) photoperiod.

Microfilaremic blood handling. Microfilaremic blood was obtained from the National Institute of Health/National Institute of Allergy and Infectious Diseases (NIH/NIAID) Filariasis Research Reagent Resource (FR3) Center (Missouri strain – Ae. sierrensis, Cx. pipiens complex, & Cs. incidens) and from TRS Lab Inc. (Wildcat strain – Ae. sierrensis). The Missouri isolate originated from an animal pound in Missouri in 2000, while the Wildcat isolate originated from Stanwood, MI and has been maintained since August 2012.

To determine the initial titer of microfilariae present, the blood was first diluted (1:10) and observed in 20µl dual-chambered plastic cellometer slides (Nexcelom Bioscience, Lawrence, MA). Depending on the initial titer, the infected blood was either diluted with rabbit blood or concentrated via centrifugation. Centrifugation of the blood occurred in 15 ml centrifuge tubes at 4°C for 10 minutes at 1000g with a gradual acceleration and deceleration, consistent with FR3 guidelines. The above steps were repeated as necessary to achieve the desired 2.5 mff/µl low titer and 5 mff/µl high titer. Microfilarial infections range within domestic dogs from 0.1 mff/µl to 50 mff/µl, with most infections being around 10 mff/µl. The microfilarial range chosen for this experiment was based on research by Lai, whom concluded that lower microfilarial
Figure 2.1A

Figure 2.1B

Figure 2.1. Geographic distribution of collection sites in Northern California between 2016-2017. (A) Lake County, CA (B) San Joaquin County, CA
densities may play a major role in transmission between dogs and mosquito vectors (Lai et al. 2000).

Blood was then loaded (1-2 ml) into the reservoir of the Hemotek® feeder (Discovery Workshops, Accrington, UK), which was covered with pig intestine and sealed with a rubber o-ring. Assembled reservoirs were loaded onto the heating unit immediately prior to the infection.

**Dog heartworm infection.** Adult mosquitoes were transported to the Marin-Sonoma Mosquito & Vector Control District (BSL-2 facility) for infection. Approximately 1000 3-4 day old females were used in each infection. Mosquitoes were starved of both water and sucrose solution 24-36 hours prior to bloodfeeding. Using a Hemotek® artificial feeding system, starved mosquitoes were fed blood containing either a low (1-3.5 mff/μl) or high titer (~5 mff/μl) of *D. immitis* microfilariae. After 1 hour, engorged females were anesthetized (CO₂ and ice) and separated into smaller, disposable chambers to be maintained on water and rehydrated craisins in an incubator at 24.5°C and 86% RH. Immediately following the infection, midgut smears were analyzed to confirm the presence of live microfilariae. This was completed on both the experimental species and *Ae. sierrensis*, which was tested prior and acted as a control to confirm the success of the infection.

**Decapitation.** At 15, 18 and 21 days post-infection, females were decapitated under a trinocular dissecting microscope using double depression slides filled with warm phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄, and 1.47 mM KH₂PO₄) and an insect pin (Figure 2.2). Decapitated samples were incubated at 37°C for 30 minutes to allow time for L3 emergence. The number of L3 larva was
recorded and whole mosquitoes (head and body) were transferred into individual 2.0 ml safe-lock tubes. Samples were transported in coolers containing ice packs to the University of the Pacific and stored at -80°C for further testing.

![Figure 2.2](image-url)

**Figure 2.2.** Third-stage larval (circled) emergence from a decapitated female mosquito under a dissection microscope.

**Molecular testing.** Mosquito samples were dissected on glass microscope slides using razor blades to separate the abdomen from the thorax. Afterwards, the thorax was placed in the sample’s original tube along with the head, while the abdomen was placed in a new safe-lock tube. DNA was extracted from the separated mosquito head-thoraces and abdomens using DNeasy 96 Blood & Tissue Kit (Qiagen, Valencia, CA). *Dirofilaria immitis*-specific 5s-sp primers (U.S. Patent No.: 6,268,153 Bl, forward sequence: 5’-
CAAGCCATTTTTCGATG CACT-3’, reverse sequence: 5’-CCATTGTACCGCTTAC TACTC-3’) were used to detect *D. immitis* DNA (193-bp)(Huang et al. 2013; Lizotte-Waniewski, Michelle (Northampton, Steven A. (North Hatfield, MA) 2001). *Dirofilaria immitis* DNA extracted from infected blood (positive) and nuclease-free H$_2$O (negative) were tested alongside each set of experimental samples as controls. PCR amplification was carried out in a 25 µ1 reaction mix containing 10x PCR buffer II, 2.5 mM of MgCl$_2$, 0.2 mM dNTP, 20 mg/ml of BSA, 0.2 µM of each primer, 1 U of Hotstart AmpliTaq Gold polymerase, 6 µ1 of DNA, and an appropriate volume of nuclease-free H$_2$O. Similar to Huang, PCR reactions cycled as follows: 10 min of 95°C; 35 cycles of 95°C for 15s, 52 °C for 30s, and 72 °C for 30s; and 10 min of extension at 72 °C. PCR products were run on 1.2% agarose gels stained with GelRed dye (Biotium, Fremont, CA)(Huang et al. 2013).

**Sample size and statistics.** Approximately 1000 mosquitoes were initially included in each trial. This was done under the assumption that as many as half of these mosquitoes would not feed and another half would not survive to the desired time points (15, 18, & 21 dpi). Roughly 10-20 mosquitoes were decapitated at 15 dpi, 25-50 at 18 dpi, and the rest were decapitated at 21 dpi. Results were analyzed using chi-square, as done in previous vector competence studies(Reisen, Fang, Martinez 2005).

**Results**

**Relative mosquito abundance and HDU accumulation.** From 2005-2015, a total of 29,382 female *Ae. sierrensis, Cx. pipiens* complex and *Cs. incidens* were collected using CO$_2$ traps, New Jersey light traps (NJLT), and resting collections in Lake County, CA(CalSurv Gateway, UC Davis Center for Vector-borne Diseases 2017; Lake
County Mosquito and Vector Control District 2005-2015). Combined, CO\textsubscript{2} traps and resting collections accounted for 99.1% of collections (68% and 31%, respectively). To alleviate collection method bias, both CO\textsubscript{2} traps and resting collections were considered separately to determine the relative abundance and seasonality for each species of interest. The number of mosquitoes captured from NJLT (0.87%) were negligible and therefore excluded.

Of the three species under investigation, \textit{Ae. sierrensis} were the most abundant (n=24,338). CO\textsubscript{2} traps accounted for 74.9% of collections for this species. The earliest detection of \textit{Ae. sierrensis} was in early-March. Abundance rapidly increased in mid-April, peaked in a mid-May, and then gradually declined until the end of October. In total, the majority (~98.7%) of female \textit{Ae. sierrensis} were trapped between April and September. This temporal trend was relatively consistent between CO\textsubscript{2} trap collections and resting collections.

\textit{Culex pipiens} complex were the least abundant (n=391). When deployed concurrently, both CO\textsubscript{2} traps and resting collections were equally effective averaging 0.47 and 0.52 mosquitoes per trap per night between May and September. \textit{Culex pipiens} complex were collected inconsistently year-round. Abundance steadily increased in May, peaked in August and November, and declined in December. Most females (~97.7%) were captured between May and December.

Finally, 4,641 female \textit{Cs. incidens} were collected. Again, both CO\textsubscript{2} traps and resting collections had comparable efficacies, catching 2.2 and 2.9 mosquitoes per trap per night between May and September, respectively. Similar to San Joaquin County, \textit{Cs.}
*incidens* were collected consistently year-round, peaking in July (Huang et al. 2013).

Most females (~91.9%) were collected between March and November.

The relative abundance and degree of seasonal overlap with HDU accumulation is shown in Figure 2.3 using CO₂ traps and resting collections from Lake County, CA. In Lake County, CA, 130 or greater HDUs falls between late-June and mid-October (Week 26-41). Ranked in order based on relative abundance, *Ae. sierrensis*, *Cs. incidens*, and *Cx. pipiens* complex are each present during this period. As such, none of these three potential vectors can be ruled out based on temporal overlap alone.
Figure 2.3. Seasonal dynamics of mosquito collection (left Y-axis) and 30-day HDU accumulations (right Y-axis) between 2005-2015 in Lake County, California. Threshold refers to the 130 HDUs required for *D. immitis* development. (A) CO2 traps (B) Resting collections.
Molecular testing of abdomens and head/thoraces. Heads and thoraces were tested together and abdomens were tested separately to determine the extent to which the infection progressed within each species.

Positive bands for *D. immitis* DNA were easily detected in *Ae. sierrensis*. Throughout the various trials, 40-67.85% of abdomens and 42.2-84.2% of head-thoraces tested positive for *D. immitis* DNA. Overall, *D. immitis* DNA could be detected in 71% or more of *Ae. sierrensis* that ingested the infected bloodmeal.

No detectable signs of *D. immitis* DNA were observed at any time point regardless of the body part tested for *Cs. incidens*. Either the microfilariae were never able to become established or any remnants of the parasite were digested and excreted.

Unlike *Ae. sierrensis*, bands for *D. immitis* DNA were difficult to detect in samples extracted from *Cx. pipiens* complex as bands were particularly faint. Between the two titers of the Missouri strain, up to 43.18% of abdomens and 63.5% of head-thoraces tested positive for *D. immitis* DNA. The high titer resulted in significantly fewer ($\chi^2 = 20.77, df = 1, P < 0.001$) PCR positives by the end of each experiment. Overall, *D. immitis* DNA was detected in 77.1% of low titer mosquitoes and 32.6% of high titer mosquitoes.

Vector competence. In total, 1,523 females representing three species, each from different genera (*Aedes, Culex, and Culiseta*), fed on infected blood during this study (Table 1).

*Aedes sierrensis* was infected with both the Missouri and the Wildcat strain of *D. immitis*. Mosquitoes infected with the low titer of the Missouri strain initially displayed no signs of infective stage larvae at 15 dpi. Over the course of the experiment this was
no longer the case as the infective rate increased significantly ($\chi^2 = 11.04$, df = 1, $P < 0.001$) from 0% to 55% by 21 dpi. Of the 38 infective females that survived to be decapitated, an average of 2.7 L3s (range 1-7) emerged. To complement the previous trial, both a low and a high titer of a comparable strain (Wildcat) were completed since the FR3 facility was no longer able to supply adequate titers of the infected blood. Mosquitoes infected with the low titer of the Wildcat strain resulted in more than half of the mosquitoes being infective at any given time point. Of the 43 infective females decapitated, an average of 6.1 L3 (range 1-23) emerged. By comparison, mosquitoes infected with the high titer of the Wildcat strain demonstrated complete infectivity by 18 dpi. A mean of 10.5 L3 (range 2-35) emerged from the 46 decapitated infective mosquitoes. Comparing the two strains at low titer, there was no significant difference in infectivity at 21 dpi. Despite this, the survivorship of mosquitoes infected with the Wildcat strain was significantly lower ($\chi^2 = 16.25$, df = 1, $P < 0.001$). Between the two titers of mosquitoes infected with the Wildcat strain, there was a significant difference ($\chi^2 = 7.42$, df = 1, $P = 0.006$) in the infective rate of mosquitoes at 21 dpi. The increased microfilarial density likely explains the significantly lower ($\chi^2 = 6.85$, df = 1, $P = 0.008$) survivorship of the high titer trial. Regardless, all three infections of Ae. sierrensis produced infective stage larvae.

Unfortunately, Cs. incidens were reluctant to feed using the Hemotek® artificial feeding system. Although 22 females became partially or completely engorged and 11 were eventually decapitated, no L3s emerged. Simultaneously infected Ae. sierrensis confirmed microfilarial uptake and provided some confidence in the results of this trial.
This was the only instance where the desired titer was not successfully achieved as the blood used in this trail contained only 1.16 mff/μl.

Both a low and a high titer trial for the Missouri strain were completed for *Cx. pipiens* complex. Mosquitoes infected with either titer displayed little to no signs of infective stage larvae. Neither time nor varying titers resulted in a significant difference ($\chi^2 < 2.8$, df = 1, $P > 0.09$) in observed infective rates or survivorship. Never more than 2 L3s were observed from infective mosquitoes. A mean 1.2 L3 (range 1-2) and 1 L3 emerged from the low and high titer, respectively. *Aedes sierrensis* confirmed microfilarial uptake for the high titer trial.
Table 2.1. Results of mosquitoes artificially infected with two titers of \textit{D. immitis} (2016-2017). Body parts (Abdomen/Head-Thorax) were tested for the presence of \textit{D. immitis} via PCR. Infective Rates refer specifically to the percentage of samples that produced emerging infective larva(e) in warm PBS. Infected Rates refer to the percentage of samples within each trail where \textit{D. immitis} was detected in either the abdomen, head-thorax, or warm PBS post-decapitation.

<table>
<thead>
<tr>
<th>Strain (Source)</th>
<th>Species</th>
<th>Titer</th>
<th>No. engorged</th>
<th>Survivorship (%)</th>
<th>Dpi</th>
<th>Mosquitoes tested</th>
<th>Abdomens Positive for D.i. (%)</th>
<th>Head-Thoraces Positive for D.i. (%)</th>
<th>Infected Rate (%)</th>
<th>Infective Rate (%)*</th>
<th># of Infective Mosquitoes</th>
<th>Mean Infective L3s ± S.D.</th>
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<tbody>
<tr>
<td>Missouri (FR3)</td>
<td>\textit{Aedes sierrensis}</td>
<td>Low</td>
<td>168</td>
<td>56</td>
<td>15</td>
<td>11</td>
<td>45.4</td>
<td>45.4</td>
<td>72.7</td>
<td>0&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>38</td>
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<td>44.7</td>
<td>71</td>
<td>34.2&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>21</td>
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<td>42.2</td>
<td>80</td>
<td>55&lt;sup&gt;c&lt;/sup&gt;</td>
<td>25</td>
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<tr>
<td></td>
<td>\textit{Culex pipiens} complex</td>
<td>Low</td>
<td>380</td>
<td>64.2</td>
<td>15</td>
<td>20</td>
<td>30</td>
<td>20</td>
<td>35</td>
<td>5&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>\textit{Culex pipiens} complex</td>
<td>High</td>
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<td>72.5</td>
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<td>95</td>
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<td>\textit{Aedes sierrensis}</td>
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<td>34.4</td>
<td>15</td>
<td>19</td>
<td>63.1</td>
<td>84.2</td>
<td>94.7</td>
<td>94.7&lt;sup&gt;e&lt;/sup&gt;</td>
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<td>7.33 ± 3.53</td>
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<td>67.85</td>
<td>57.1</td>
<td>100</td>
<td>100&lt;sup&gt;e&lt;/sup&gt;</td>
<td>28</td>
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D.i., \textit{Dirofilaria immitis}. S.D., Standard Deviation of L3 larva(e) present.

* Percentages with different lowercase letter(s) indicate that they are statistically different by chi-squared test (P ≤ 0.05).
Discussion

Although competence in Ae. sierrensis has been demonstrated previously (Walters and Lavoipierre 1982), Huang et al. (2013) suggested that in regions like San Joaquin County, CA where abundance and seasonal activity may be limited, other mosquito species may be responsible for the observed prevalence in heartworm activity. This study investigated the ability of various mosquito species to develop D. immitis in Northern California to identify key vectors of transmission. Temporally, Ae. sierrensis, Cs. incidens, and Cx. pipiens complex are present to varying degrees during the period in which HDUs exceed 130. In Lake and San Joaquin Counties, CA, this period corresponds with mid-June to mid-October and mid-March to November, respectively (Huang et al. 2013). Combining this evidence with information pertaining to other key vector identification criteria, such as the detection of D. immitis in wild mosquitoes and host-seeking preferences associated with domestic dogs, the potential of these three mosquito species as vectors of D. immitis in northern California cannot be ruled out without information regarding their competence in the laboratory (Huang et al. 2013; Theis et al. 2000; Thiemann et al. 2012; Tran, Nelms, Thiemann 2016; Walters and Lavoipierre 1982; Walters 1995; Weinmann and García 1974).

Dirofilarial infections begin in the midgut (abdomen), migrate to the Malpighian tubules (abdomen), and become infective within the anterior (head-thorax) (Abraham 1988; Bradley and Nayar 1987; Cancrini and Kramer 2001; Kartman 1953a; Manfredi, DiCerbo, Genchi 2007; McCall et al. 2008; Montarsi et al. 2015; Serrão, Labarthe, Lourenço-de-Oliveira 2001; Taylor 1960). Meanwhile, the mosquito’s biology can digest, degrade, or excrete traces of D. immitis and their DNA over time (Beerntsen,
The current study decapitated laboratory-infected mosquitoes to determine the number of mosquitoes able to produce viable, emerging infective L3s to calculate infective rates. Afterwards, molecular tests on the abdomens and head-thoraces were completed to determine the extent to which the infection developed within females of each species, especially those that were unable to produce viable infective L3s.

**Aedes sierrensis.** Molecular testing on samples of *Aedes sierrensis* elicited consistently detectable, bright bands for *D. immitis* DNA. Although PCR is not a quantitative test, band brightness should correlate with DNA template concentration. Therefore, the bright bands observed likely demonstrate the presence of high levels of DNA, or large quantities of the parasite. Across all three trials, 51.7% of abdomens tested positive for *D. immitis* and 85% of those samples continued to produce infective L3s. The few infective mosquitoes without positive abdomens were likely a result of samples with low quantities of DNA that could not be consistently detected.

*Aedes sierrensis* has been considered the primary vector of *D. immitis* in northern California since Weinmann and Garcia characterized its potential in 1974. By rearing wild larvae from Marin County, CA and allowing the adults to feed on an infected dog, they discovered that all surviving mosquitoes supported infective larvae under laboratory conditions at 20 dpi (Weinmann and García 1974). To determine if this could be replicated under more natural, ambient conditions, Walters and Lavoipierre infected wild mosquitoes using a baited kennel in Tehama County, CA and maintained them at ambient temperatures in an open laboratory (Walters and Lavoipierre 1982). In both rural and residential areas they reported 100% infectivity. Similarly, our findings show that 55 to
100% of female *Ae. sierrensis* supported development of infective larvae by 21 dpi between the three trials completed using the two strains at two different titers. Wild *Ae. sierrensis* have been reported to support *D. immitis* development in the laboratory in other Western states as well. Scoles et al. tested *Ae. sierrensis* in Utah to determine if their arrival triggered the rise in *D. immitis* infections observed since 1987. They found that 85% of mosquitoes supported infective L3 development by 15 dpi (Scoles, Dickson, Blackmore 1993). The results may vary but the trend remains the same; *Aedes sierrensis* demonstrates competence as a vector of *D. immitis* in the laboratory. Biologically, this may be due to the lack of immunological responses or physiological barriers that inhibit the development of the infection in other species (Ahid, Vasconcelos, Lourenço-de-Oliveira 2000; Cancrini and Kramer 2001; Lowrie 1991; Nayar and Sauerman 1975; Poinar and Leutenegger 1971). Additionally, this study found that raising the titer from 2.5 to 5 mff/μl significantly increased both the average number of L3s and the overall infective rate. This builds on research by Lai et al. (2000) since it indicates some species can support significantly more third-stage larvae relative to the microfilarial density. As expected, this was met with a significant increase in mortality as increasing the filarial burden within the mosquito further disrupts the vital excretory functions of the Malpighian tubules (Palmer, Wittrock, Christensen 1986). Increasing the titer beyond this point will likely continue to overwhelm the biology of *Ae. sierrensis* and produce even fewer infective mosquitoes, confirming the hypothesis posed by Lai et al that suggested lower microfilarial densities within definitive hosts likely contribute to a significant portion of *D. immitis* transmission. Also, our data suggests a significant difference ($\chi^2 = 5, df = 1, P = 0.02$) in compatibility during the early stages of infection between the two
strains of *D. immitis* and *Ae. sierrensis* mosquitoes collected from Lake County, CA. The infective rates for the Missouri strain were initially 0% at 15 dpi, while the Wildcat strain presented with 53% at the same time point. By 21 dpi, however, the difference was no longer significant.

In summary, *Ae. sierrensis* frequently produced positives for *D. immitis* DNA in both abdomens and head-thoraces. In conjunction with high infective rates, *Ae. sierrensis* has demonstrated its ability to support development of *D. immitis* to its infective stage and likely plays a primary role in transmission in Lake County, CA, where abundance is high. However, as this is not the case in some regions such as San Joaquin County, CA, *Ae. sierrensis* likely plays a secondary role when abundance is low or its seasonality does not overlap with conditions permissive for *D. immitis* development. This, in part, could explain the 3.02% differential in *D. immitis* prevalence between the two counties.

*Culiseta incidens*. Based on bloodmeal analysis studies, *Cs. incidens* has been shown to feed frequently on domestic dogs. As such, we were very interested in determining the potential of *Cs. incidens* as a vector in the laboratory (Theis et al. 2000). Regrettably, the trial for this species was completed using the Missouri strain of *D. immitis* as the source began to lose its patency. Furthermore, *Cs. incidens* were reluctant to feed using our current methodology. Thus far, our only successful artificial feeding method to date involved a dawn feeding using blood containing <1% sugar after roughly 108 hours of starvation. Results pertaining to this species were inconclusive as none of the few mosquitoes that ingested the infected blood contained any sign of *D. immitis*, whether it be infective larvae or positive molecular tests. Previous studies suggest that
Cs. incidens may be refractory to the development of larvae despite its willingness to feed on domestic dogs, detection of D. immitis in wild-caught mosquitoes, and long seasonality (Huang et al. 2013; Theis et al. 2000; Thiemann et al. 2012; Walters 1995). Walters found that microfilariae failed to develop in 85% of Cs. incidens in Northern California (Walters 1995). On the contrary, other studies from California have reported that it can support D. immitis development and may serve as a vector of transmission. In San Mateo County, CA, Acevedo (1982) reported that 7.14% (n=28) contained L3s either in the Malpighian tubules or the proboscis by 16 and 21 dpi when allowed to feed on an infected dog containing 11 mf/ml (Acevedo 1982). Similarly, Theis et al. infected reared Cs. incidens from Southern California (Los Angeles County) and found that an average of 0.4 L3s emerged from the 18 surviving mosquitoes dissected at 16 dpi (Theis et al. 2000). Additional trials are necessary to determine that status of Cs. incidens as a vector in Lake County, CA.

*Culex pipiens* complex. In San Joaquin County, CA, Cx. pipiens complex is the second most commonly trapped species, comprising 30.7% (n = 11,223) of collections in 2013 and accounting for 41.2% (n = 40/97) of all positive mosquito pools for D. immitis. Seasonally, it is present during mid-March to November, the period in which 130 or greater HDUs can be accumulated (Huang et al. 2013).

Molecular tests to detect D. immitis DNA in Cx. pipiens complex revealed significantly fainter bands by comparison to *Ae. sierrensis*. To ascertain whether size or quantity may cause negative molecular tests, samples of individual microfilaria and third stage larvae were tested. While both were determined to be sufficient to elicit consistently positive bands for D. immitis DNA, high densities of DNA other than that of
*D. immitis* could feasibly lower PCR efficiency and hinder detectability. Between the two titers of the Missouri strain, 38% of abdomens tested positive for *D. immitis* and 64% of those samples had a corresponding positive in the head-thorax. Midgut smears were performed on both *Cx. pipiens* complex and *Ae. sierrensis* immediately post-feeding, but only the latter contained microfilariae. The lack of microfilariae in the former was likely biological in nature and specific to *Cx. pipiens* complex.

Of the many potential vectors under suspicion on the West Coast, Huang et al. suggested that *Culex pipiens* complex (*Culex pipiens* and *Culex quinquefasciatus*) is potentially one of the most important, at least in San Joaquin County, CA, due to its spatiotemporal presence, abundance, and host feeding preference (Huang et al. 2013). Lewandowski et al. came to this same conclusion, which prompted him to experimentally infect reared adults from Lansing, Michigan using an infected dog (Lewandowski Jr, Hooper, Newson 1980). Only a single infective larva was found in a single mosquito at 15 dpi. Additionally, as microfilariae were unable to become established in the Malpighian tubules in 87% of mosquitoes, they deduced that *Cx. pipiens* was an inefficient host. In Lake County, CA, the potential of *Cx. pipiens* complex may be limited due to its low relative abundance. In this study, infective rates were never greater than 5%, regardless of the microfilarial titer used. This is consistent with past studies that found *Cx. pipiens* complex to be refractory and prevent the establishment of most worms within the Malpighian tubules (Hu 1931; Kartman 1953b; Lewandowski Jr, Hooper, Newson 1980). Even when prevalence is as high as 37.1%, which was the case in Rio de Janeiro, Brazil, Labarthe et al. found that microfilariae rarely develops into infective L3s in *Cx. pipiens* complex. Of the 865 *Cx.
*quincefasciatus* mosquitoes collected, only 8 contained filariae at all, 3 of which ended up supporting infective L3s (Labarthe et al. 1998). Based on these studies as well as the lack of microfilariae observed in post-infected blood smears, it is possible that the cibarial armature (located within the pharynx) may be responsible for the refractoriness observed in *Cx. pipiens* complex. This explains why very few larvae are ever observed at any individual stage within an individual mosquito since most worms never get a chance to even become established in the first place (Lewandowski Jr, Hooper, Newson 1980). Despite this, many authors agree that worms that can survive passage through the sharp, sclerotized teeth of the cibarial armature find *Cx. pipiens* complex to be a favorable vector (Cancrini and Kramer 2001). This seems to be the case for our study as well. For example, if all the worms were shredded, then the residual *D. immitis* DNA contained within the bloodmeal would have been digested prior to 15 dpi. While bloodmeal digestion is temperature dependent, in most cases it shouldn’t take longer than a few days (Jenkins 2004; MacDonald 1961). This was not the case in our trials, especially considering that some mosquitoes developed infective L3s. While some worms certainly survive the cibarial armature, the fact that most infected mosquitoes do not produce infective larvae eludes to the presence of other biological or defensive mechanisms, such as oxyhemoglobin and melanization (Ahid, Vasconcelos, Lourenço-de-Oliveira 2000; Lowrie 1991; Nayar and Sauerman 1975; Poinar and Leutenegger 1971). Lowrie tested two strains of *Cx. quincefasciatus* (Leogane, Haiti & Convington, LA) at two different titers (5 mf/fu & 20 mf/fu) using an artificial feeding apparatus and found that while up to 27.5% of females supported infective stage larvae, oxyhemoglobin crystals were capable of not only blocking and retaining microfilariae within the midgut, but also
damaging them which likely reduces the viability of the remaining worms that did happen to survive and escape the midgut (Lowrie 1991). Similar to *Ae. sierrensis*, one strain (Haiti) consistently produced more infective larvae. This study is another example of just how much intraspecific diversity exists even amongst seemingly refractory species. Unlike *Ae. sierrensis*, increasing the titer did not result in additional infective mosquitoes or a higher mean number of L3s. One possibility is that centrifugation may have altered the viability of the infected blood by damaging the worms or altering the consistency of the blood. Blood that coagulates too quickly may prevent microfilariae from escaping the blood bolus and reaching the Malpighian tubules before the meal is digested (Cancrini and Kramer 2001). An additional repeat may be necessary to determine if one of these factors may have contributed to the low levels of infectivity observed. Additionally, some literature speculates that in regions where *D. immitis* is hyperendemic, transmission may occur at significantly higher rates (Cancrini and Kramer 2001; Genchi, Kramer, Prieto 2001).

*Culex pipiens* complex is one of the most abundant mosquitoes in San Joaquin County, CA. While *Cx. pipiens* complex possesses all the fundamental traits of a competent vector, such as frequent *D. immitis* field detections, ecological overlap, and appropriate blood feeding patterns, our results suggest that *Cx. pipiens* complex is relatively refractory at low microfilarial densities equal to or below 5 mff/μl (Huang et al. 2013; Thiemann et al. 2012). Furthermore, some members of *Cx. pipiens* complex (*Cx. pipiens*, not *Cx. quinquefasciatus*) are autogenous, meaning that they ingest fewer bloodmeals throughout their lifetime and are less likely to acquire or transmit an infection (Cancrini and Kramer 2001). Regardless, *Cx. pipiens* complex can support
complete development in some instances and should be regarded as a competent vector. Whether *Cx. pipiens* complex is a primary or secondary vector of importance depends on how abundant it may be in any given area.

**Conclusion.** In summary, *Ae. sierrensis*, *Cx. pipiens* complex, and *Cs. incidens* demonstrated that additional information was necessary to evaluate their competence in the laboratory and determine their potential as vectors of *D. immitis*. Based on our findings, only *Ae. sierrensis* and *Cx. pipiens* complex have been confirmed as vectors of *D. immitis* as they were able to support infective L3 development. Both species should be targeted in Northern California to reduce the incidence of new cases of canine heartworm disease. Even a species of low competence such as *Cx. pipiens* complex can result in a noticeable degree of activity if abundance is high, which may be the case in San Joaquin County, CA. Conversely, *Ae. sierrensis*, which has a low abundance and short seasonal activity in San Joaquin County, CA may cause a significant number of cases if practically all infected females can transmit the parasite after being infected. In Lake County, CA, these concerns are heightened as these limitations are not a concern and *Ae. sierrensis* likely plays a primary role in transmission. While *Cx. pipiens* complex is present here as well, less risk of transmission is associated in this case due to competence and relative abundance. In the future, we would like to complete our assessment of *Cs. incidens* as well as study other potential vectors such as *Cx. tarsalis*. Other avenues worth investigating include the effects of coinfection and the clarification of the life cycle of *D. immitis* as many papers gloss over the finer details of development. Although this study serves as a foundation, additional research using both local strains of *D. immitis* and potential mosquito vectors may be necessary to characterize local
transmission due to the diversity that exists within populations of both mosquitoes and *D. immitis* alike.
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