Prevalence of *Dirofilaria immitis* (Spirurida: Onchocercidae) Infection in *Aedes*, *Culex*, and *Culiseta* Mosquitoes From North San Joaquin Valley, CA

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ABSTRACT Canine heartworm is one of the most serious infections primarily affecting domestic dogs but will also infect cats and wild canids. To evaluate the potential of mosquitoes as vectors of dog heartworm, *Dirofilaria immitis* (Leidy) in San Joaquin County, CA, we collected mosquitoes in 2011 and analyzed for infection with heartworm by using polymerase chain reaction. Of 3,000 mosquito pools (total number of specimens = 36,554), *D. immitis* DNA was detected in 97 pools of seven species, and the overall minimum infection rate (MIR) for all mosquito species was 2.69: *Culex pipiens* L. (*n* = 40; MIR = 3.66), *Culex tarsalis* Coquillett (*n* = 25; MIR = 1.89), *Culiseta incidens* (Thomson) (*n* = 11; MIR = 2.81), *Aedes vexans* (Meigen) (*n* = 7; MIR = 2.18), *Aedes melanimon* Dyar (*n* = 5; MIR = 4.64), *Culex erythrothorax* Dyar (*n* = 5; MIR = 3.96), and *Culiseta inornata* (Williston) (*n* = 4; MIR = 2.65). *Cx. pipiens* and *Cx. tarsalis* had the highest number of *D. immitis* infections and collectively accounted for 67% of all positive pools. *Ae. melanimon*, *Ae. vexans*, and *Cx. erythrothorax* were found to be infected with *D. immitis* only in rural and agriculture areas, whereas infections in other species were identified in rural and agriculture areas, and urban and residential settings. The majority of positive pools were identified from June through November and peaked during August through October. This is the first report of *D. immitis* infection in *Ae. melanimon*, *Cx. erythrothorax*, *Cx. tarsalis*, *Cs. incidens*, and *Cs. inornata*. The frequent detection of *D. immitis* in field-collected *Cx. pipiens* and *Cx. tarsalis* in concert with their seasonal abundance and widespread distribution suggest a central role for these species in dog heartworm transmission. Other species, including *Ae. vexans*, *Ae. melanimon*, *Cs. incidens*, *Cs. inornata*, and *Cx. erythrothorax*, may play a secondary role in transmission.

KEY WORDS *Dirofilaria immitis*; mosquito; North San Joaquin Valley, CA

Canine heartworm is caused by the mosquito-borne parasitic filarial nematode, *Dirofilaria immitis* (Leidy). It is one of the most serious infections primarily affecting domestic dogs but will also infect cats, wild canids (coyotes, foxes, wolves, etc.), ferrets, and raccoons with a worldwide distribution (McCall et al. 2008). *D. immitis* larvae require 6–7 mo to develop into adults after penetrating the host's skin and invading the bloodstream. The adults can live up to 7 yr residing within the right ventricle and pulmonary arteries (Abraham 1988). The physiological burden caused by the aggregation of substantial numbers of adult heartworms leads to a chronic infection and is often fatal.

In humans, *D. immitis* may more rarely cause dirofilariasis, a condition known as "coin lesion," where a pulmonary nodule is formed as a result of parasite aggregation (Acha and Szyfres 2003).

Surveys conducted by the American Heartworm Society (AHS) indicate a considerable increase in the prevalence of heartworm in dogs during the past decade throughout the United States. The highest infection rates (up to 45%) have been observed within 250 km of the Atlantic and Gulf coasts and along the Mississippi River and its major tributaries (AHS 2009). In San Joaquin County, CA, surveys of local veterinarians performed by the San Joaquin County Mosquito and Vector Control District detected a similar increase in heartworm incidence among domestic dogs from 0 reported cases in 2005, to 4 in 2006, 25 in 2007, and 39 in 2008 (unpublished data).

D. immitis is maintained in an enzootic transmission cycle involving canines and various mosquito species depending on geography. Natural infection with *D. immitis* has been reported in >60 mosquito species worldwide and 24 species in the United States (Bow-

man and Atkins 2009, Ledesma and Harrington 2011). In California, *D. immitis* infection has been detected in *Aedes sierrensis* (Ludlow) (Walters and Lavoipierre 1982, Walters 1996), *Aedes vexans* (Meigen) (Walters and Lavoipierre 1982), and *Anopheles freeborni* Aitkin (Walters 1996). Local populations of *Culiseta incidens* (Thompson) from Los Angeles County reared to adulthood from field-collected larvae and egg rafts have also been shown to be highly competent vectors in a laboratory study (Theis et al. 2000).

The western treehole mosquito, *Ae. sierrensis*, is considered the principal vector of *D. immitis* in many areas of northern California (Weinmann and Garcia 1974, Walters and Lavoipierre 1982, Walters 1996). However, development of microfilaria to the infective L3 stage requires an average of 130 heartworm development units (HDU), which is defined as the minimum accumulated degree-days that heartworm larvae are subjected to temperature above the threshold for development (14°C) (Knight and Lok 1998). In San Joaquin County, the period with 130 or higher HDU approximately corresponds to mid-March to November, but *Ae. sierrensis* populations are only found from March to May, with peak abundance in mid-April to mid-May. Furthermore, *Ae. sierrensis* populations are very low in San Joaquin County with an average abundance of only 0.17 per trap night (unpublished data). Therefore, the vector potential of *Ae. sierrensis* for *D. immitis* would appear to be limited because of its comparatively low abundance and short seasonal activity.

The major mosquito species present from mid-March to November in San Joaquin County include *Ae. vexans*, *Culex pipiens* L., *Culex tarsalis* Coquillett, and *Cs. incidens.* During the 2005–2007 reports of the dog heartworm activity in the region, our mosquito collection data indicated a surge in the abundance of *Cx. pipiens* and *Cx. tarsalis*, while other mosquito species exhibited a population decline. In an attempt to gain a better understanding of the local epidemiology of *D. immitis*, we hypothesized that: *Cx. pipiens* and *Cx. tarsalis* might be significant vectors of *D. immitis* in addition to *Ae. vexans* and *Cs. incidens.* Accordingly, mosquitoes were collected as part of an ongoing mosquito and West Nile virus (WNV) surveillance operation in 2011 and examined for the presence of L3 *D. immitis* using a polymerase chain reaction (PCR)-based molecular method. In mosquitoes, microfilaria (the youngest stage of *D. immitis*) develop into infective larva (L3), which migrates to the head or mouthpart and is transmitted to a new host in subsequent blood-feeding. The presence of L3 *D. immitis* larvae in heads and thoraces of field-collected mosquitoes is the most important criterion for evaluating vectorial capacity. This indicates that a mosquito feeds on infected hosts, acquires the infection, and supports microfilaria development to the L3 stage.

Materials and Methods

Study Area. The study was conducted in San Joaquin County, CA, which is located in the northern most part of the San Joaquin Valley, south of Sacramento, and east of the San Francisco Bay area. The county covers >3,693 km^2 consisting mostly of rural and agricultural lands, along with highly populated urban areas that include the city of Stockton. The county is home to 685,990 residents. An abundance of waterways in the county, coupled with many agricultural water sources in rural areas and unkept swimming pools in foreclosed homes in urban areas, create prime habitats for several mosquito species including *Cx. pipiens*, *Cx. tarsalis*, *Cs. incidens*, and *Ae. vexans.*

Mosquito Collection. Mosquitoes were collected once a week from May through October 2011 as part of the San Joaquin County mosquito and WNV surveillance program using 61 CO$_2$-baited encephalitis vector surveillance (EVS) traps (Rohe and Fall 1979) and eight hay infusion-baited gravid box traps (Cummings 1992) from a county-wide network of 69 permanent trapping sites consisting of 47 sites in rural and agricultural habitats such as rice fields, wetlands, and pastures and 22 sites at urban and residential settings. From March to June, 13 of the weekly EVS traps in urban and residential sites were replaced with Fay–Prince traps to collect *Ae. sierrensis* mosquitoes weekly. After the peak season of WNV surveillance, trapping was reduced to biweekly collections at one-third of the sites, and gravid traps were not deployed. Mosquito trapping was not carried out from mid-December to mid-January. Mosquito abundance was calculated as the number of mosquitoes collected per trap and per night.

Mosquitoes were transported to the laboratory in coolers containing dry ice, identified to species based on morphological characters (Darsie and Ward 2005), and pooled by species, location, and collection date. The heads and thoraces were removed and pooled (up to a maximum of 10 mosquitoes per pool) for each species of *Aedes*, *Anopheles*, and *Culiseta* and tested for L3 *D. immitis* by PCR as described in "PCR Detection of *D. immitis*" below. Specimens of *Culex erythrothorax* Dyar, *Cx. pipiens*, and *Cx. tarsalis* were initially tested for WNV by reverse transcriptase-PCR, and thus it was not feasible to dissect the heads and thoraces from these individuals. PCR diagnosis of *D. immitis* on these females was performed on pools of whole mosquitoes (10–50 mosquitoes per pool).

Genomic DNA Extraction from Mosquito Pools. Each mosquito pool was homogenized in a 2-ml vial containing 1 ml of phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na$_2$HPO$_4$, and 1.47 mM KH$_2$PO$_4$), and a copper BB pellet. Homogenization was carried out using Qiagen Tissuelyser II (QIAGEN, Valencia, CA). Genomic DNA was then extracted using GeneJET Genomic DNA Purification Kit (Fermentas, Waltham, MA) according to the manufacturer's instructions. DNA was eluted in 100 μl of elution buffer (10 mM Tris-Cl, pH 9.0, and 0.5 mM EDTA), and stored at −80°C for further analyses.

PCR Detection of *D. immitis*. *D. immitis*-specific 5s-sp primers (U.S. Patent No.: 6,268,153 Bl, forward sequence: 5′–CAAGCCATTTTTCGATGCACT–3′, reverse sequence: 5′–CCATTGTACCGCTTACTA-

CTC–3′) were used to detect *D. immitis* DNA. Both positive and negative controls were included in all PCR reactions. The DNA template used as a positive control was extracted from 20 laboratory *Cx. pipiens* colony mosquitoes spiked with 5–10 *D. immitis* L3s obtained from the National Institute of Health/National Institute of Allergy and Infectious Diseases (NIH/NIAID) Filariasis Research Reagent Resource Center. The negative control was DNA from 20 uninfected laboratory-reared *Cx. pipiens* mosquitoes. PCR amplification was carried out in a 25-μl reaction mix containing 10 mM of Tris-HCl, 50 mM of KCl, 2.5 mM of $MgCl_2$, and 200 μM of each dNTP, 160 μg/ml of BSA, 0.2 μM of each primer, 1 U of AmpliTaq Gold polymerase, 4 μl of DNA, and an appropriate volume of purified H_2O. PCR reactions were performed under the following cycling conditions: 10 min of 95°C; 35 cycles of 95°C for 15 s, 52°C for 30 s, and 72°C for 30 s; and 10 min of extension at 72°C. PCR products were visualized on 1.2% agarose gel stained by GelGreen dye (Biotium, Inc., Hayward, CA). *D. immitis*-positive samples would yield a 193-bp band on agarose gel.

To ensure specificity and stringency, PCR was repeated on all positive samples using 5s-sp primers, and ≈20% of the positive samples were sequenced to confirm *D. immitis* identity. Sequencing data in this study confirmed the specificity of 5s-sp primers. When the sequenced PCR products were blasted against GenBank, the target sequences were invariably of *D. immitis* origin assuming at least 90% sequence identity and at least 60% sequence query coverage.

Estimation of Mosquito Minimum Infection Rates. The maximum likelihood estimates of *D. immitis* infection rates per 1,000 mosquitoes were calculated in software PooledInfRate, Version 4.0 (Biggerstaff 2009).

Results

Mosquito Collection Data. In total, 36,554 female mosquitoes representing 15 species in four genera (*Aedes*, *Anopheles*, *Culex*, and *Culiseta*) were collected and examined for infection with L3 *D. immitis* (Table 1). The most commonly trapped species were *Cx. tarsalis* (*n* = 13,321; 36.4%), *Cx. pipiens* (*n* = 11,223; 30.7%), *Cs. incidens* (*n* = 3,941; 10.8%), and *Ae. vexans* (*n* = 3,229; 8.8%), which collectively accounted for 86.7% of all specimens. Other abundant species included *Culiseta inornata* (Williston) (*n* = 1,509; 4.1%), *Cx. erythrothorax* (*n* = 1,293; 3.5%), and *Aedes melanimon* Dyer (*n* = 1,095; 3.0%). Anopheline mosquitoes were the least frequently captured species. Few mosquitoes were collected in gravid traps: *Cs. incidens* (*n* = 47; 12 pools), *Cx. tarsalis* (*n* = 17; four pools), *Cx. pipiens* (*n* = 14; two pools), *Cs. inornata* (*n* = 2; two pools), and *Anopheles franciscanus* McCraken (*n* = 1; one pool).

D. immitis Infection in Mosquitoes. *D. immitis* DNA was detected in 97 mosquito pools representing seven species: *Cx. pipiens* (*n* = 40; 41.2%), *Cx. tarsalis* (*n* = 25; 25.8%), *Cs. incidens* (*n* = 11; 11.3%), *Ae. vexans* (*n* = 7; 7.2%), *Ae. melanimon* (*n* = 5; 5.2%), *Cx. erythrothorax* (*n* = 5; 5.2%), and *Cs. inornata* (*n* = 4; 4.1%; Table

Table 1. Mosquito collection and infection with *D. immitis* from San Joaquin County, CA, 2011

Species	Mosquitoes tested			Pools tested		Positive pools			Min. infection rate (CI)		
	RUR/AG	UR/RES	Total	Total		RUR/AG	UR/RES	Total	RUR/AG	UR/RES	Combined
Aedes dorsalis (Meigen)	3	0	3	1		0	0	0	—	—	—
Ae. melanimon	1,067	28	1,095	172		5	0	5	4.76 (1.77–10.51)	—	4.64 (1.73–10.24)
Aedes nigromaculis (Ludlow)	609	0	609	64		0	0	0	—	—	—
Ae. sierrensis	84	18	102	41		0	0	0	—	—	—
Ae. vexans	3,227	2	3,229	300		7	0	7	2.18 (0.97–4.29)	—	2.18 (0.97–4.29)
Aedes washinoi Lanzaro & Eldridge	35	0	35	5		0	0	0	—	—	—
An. franciscanus	1	2	3	3		0	0	0	—	—	—
An. freeborni	102	39	141	59		0	0	0	—	—	—
Anopheles punctipennis (Say)	13	0	13	7		0	0	0	—	—	—
Cs. incidens	996	2,945	3,941	542		2	9	11	2.01 (0.36–6.54)	3.08 (1.51–5.63)	2.81 (1.49–4.87)
Cs. inornata	1,360	149	1,509	344		3	1	4	2.20 (0.58–5.92)	6.65 (0.39–31.68)	2.65 (0.86–6.32)
Culiseta particeps (Adams)	37	0	37	6		0	0	0	—	—	—
Cx. erythrothorax	1,292	1	1,293	118		5	0	5	3.96 (1.49–8.71)	—	3.96 (1.49–8.70)
Cx. pipiens	9,436	1,787	11,223	458		29	11	40	3.15 (2.16–4.44)	6.42 (3.43–11.08)	3.66 (2.67–4.92)
Cx. tarsalis	11,780	1,541	13,321	880		17	8	25	1.45 (0.88–2.26)	5.28 (2.49–9.94)	1.89 (1.26–2.74)
Total	30,042	6,512	36,554	3,000		68	29	97			
Mean									2.29 (1.80–2.88)	4.55 (3.12–6.43)	2.69 (2.2–3.26)

RUR, rural; AG, agricultural; UR, urban; RES, residential.

1). *Cx. pipiens* and *Cx. tarsalis* collectively accounted for 67% of all positive pools. Of the 21 pools obtained from gravid trap collections, 1 *Cx. pipiens* and 1 *Cx. tarsalis* pools were positive.

The overall minimum infection rate (MIR) for all mosquito species was 2.69 (Table 1). *Ae. melanimon* had the highest MIR (4.64) followed by *Cx. erythrothorax* (3.96) and *Cx. pipiens* (3.66). Although many *Cx. tarsalis* pools were tested positive for *D. immitis*, the high number of collected specimens for this species resulted in the lowest MIR (1.89). Median MIR values among all the positive species were not significantly different ($P > 0.05$).

Spatial Distribution of *D. immitis* Infection in Mosquitoes. Of the 97 *D. immitis*-positive pools, 68 (70.1%) were identified from mosquitoes collected from rural and agricultural areas and 29 (29.9%) from urban and residential sites (Fig. 1; Table 1); 32 of 47 (68.1%) rural and agricultural collection sites and 16 of 22 (72.7%) urban and residential sites produced positive pools. Sites with the highest number of positive pools were located along the Stanislaus River, the east half of the county's southern border line (Fig. 1), and in Tracy and central Stockton, where several dog heartworm cases were reported in 2007. Positive pools of *Cx. pipiens*, *Cx. tarsalis*, *Cs. incidens*, and *Cs. inornata* were detected both from rural and agricultural areas and urban and residential areas, whereas positive pools of *Ae. melanimon*, *Ae. vexans*, and *Cx. erythrothorax* were only found in rural and agricultural areas. The MIR in urban and residential areas (4.55) was significantly higher than that found in rural and agricultural environments (2.29) ($P < 0.05$).

Temporal Dynamics of Mosquito Collection and Infection With *D. immitis*. The majority of *D. immitis*-positive mosquito pools (93.8%) were identified during mid-March to November when the required minimum accumulated degree-days (HDUs) were >130 (Fig. 2A). The earliest detection of *D. immitis* was in February in pools of *Cx. tarsalis*, *Cs. incidens*, and *Cs. inornata.* A gradual increase in mosquito infection with *D. immitis* began in June and peaked in September and October. The majority of *D. immitis* detections were observed from August through October, primarily in *Cx. pipiens* and *Cx. tarsalis* and to a lesser extent in *Ae. vexans*, *Cx. erythrothorax*, *Ae. melanimon*, and *Cs. incidens* (Fig. 2B–E). With the exception of *Cs. inornata* and *Cs. incidens*, the detection of *D. immitis* mostly coincided with the abundance of each species (Fig. 2B–E).

Both *Cx. pipiens* and *Cx. tarsalis* collections increased steadily from May and peaked in September and July, respectively (Fig. 2B and C). They remained moderately abundant through October but declined notably in November. The first detection of *D. immitis* from *Cx. pipiens* was in July ($n = 2$), but majority of the detections were observed in September ($n = 13$) and October ($n = 17$; Fig. 2B). For *Cx. tarsalis*, two early detections were observed in February and in March (Fig. 2C) and again in June ($n = 2$) and July ($n = 1$), but majority of the detections were observed in August ($n = 8$) and September ($n = 7$). *Cx. eryth-*

rothorax was infrequently collected throughout the summer months and exhibited increased abundance in October (Fig. 2C). Detections of *D. immitis* from this species were only observed in September ($n = 1$) and October ($n = 4$).

Cs. incidens was consistently collected throughout the season but this species was most abundant during spring and early summer months, with two discernible collection peaks in April and June (Fig. 2D). Trap collections decreased steadily through July and August, and only a few adults were trapped from September to December. Despite the early season detection of *D. immitis* from *Cs. incidens* (February, $n = 2$; April, $n = 1$), the majority of infected mosquitoes were collected later in the season (August–November, $n = 8$). *Cs. inornata* was collected in relatively small numbers from January to July and October to December, and *D. immitis* infections were only detected in February and November (Fig. 2D).

Collections of *Ae. vexans* peaked in June and October (Fig. 2E). The first detection of *D. immitis* was recorded during the first abundance peak in June and continued through September. However, no detection was observed during the second abundance peak in October and thereafter. Specimens of *Ae. melanimon* were primarily collected during late season from October to November, and detections of *D. immitis* were made only in September and November (Fig. 2E).

Discussion

Our study of mosquitoes collected in 2011 and examined for *D. immitis* infection provide new records of mosquito species naturally infected with *D. immitis* in the United States and additional insight into their respective roles as vectors of dog heartworm in San Joaquin County. To the best of our knowledge, this is the first report of *D. immitis* infection in *Ae. melanimon*, *Cx. tarsalis*, *Cx. erythrothorax*, *Cs. incidens*, and *Cs. inornata*. The frequent detection of *D. immitis* in field-collected *Cx. pipiens* and *Cx. tarsalis* in concert with their seasonal abundance and widespread distribution in a relatively small geographic region further suggest a central role for these species. The comparative roles of *Ae. melanimon*, *Ae. vexans*, *Cx. erythrothorax*, *Cs. incidens*, and *Cs. inornata* are less clear but based on their relative abundance, spatiotemporal dynamics, and host feeding behavior, they may play a secondary role in transmission. Contrary to previous assumptions that *Ae. sierrensis* was the main vector of *D. immitis*, we found no evidence of infection in this species. However, it is important to note that we collected and tested very few mosquitoes of this species ($n = 102$; 41 pools).

Natural infection of *Cx. pipiens* with *D. immitis* in field-collected specimens has been reported from several regions (Ledesma and Harrington 2011). This species has also been recognized as a competent and efficient vector of *D. immitis* (Capelli et al. 2013). In the current study, *Cx. pipiens* was the second most abundant species and contained the highest number
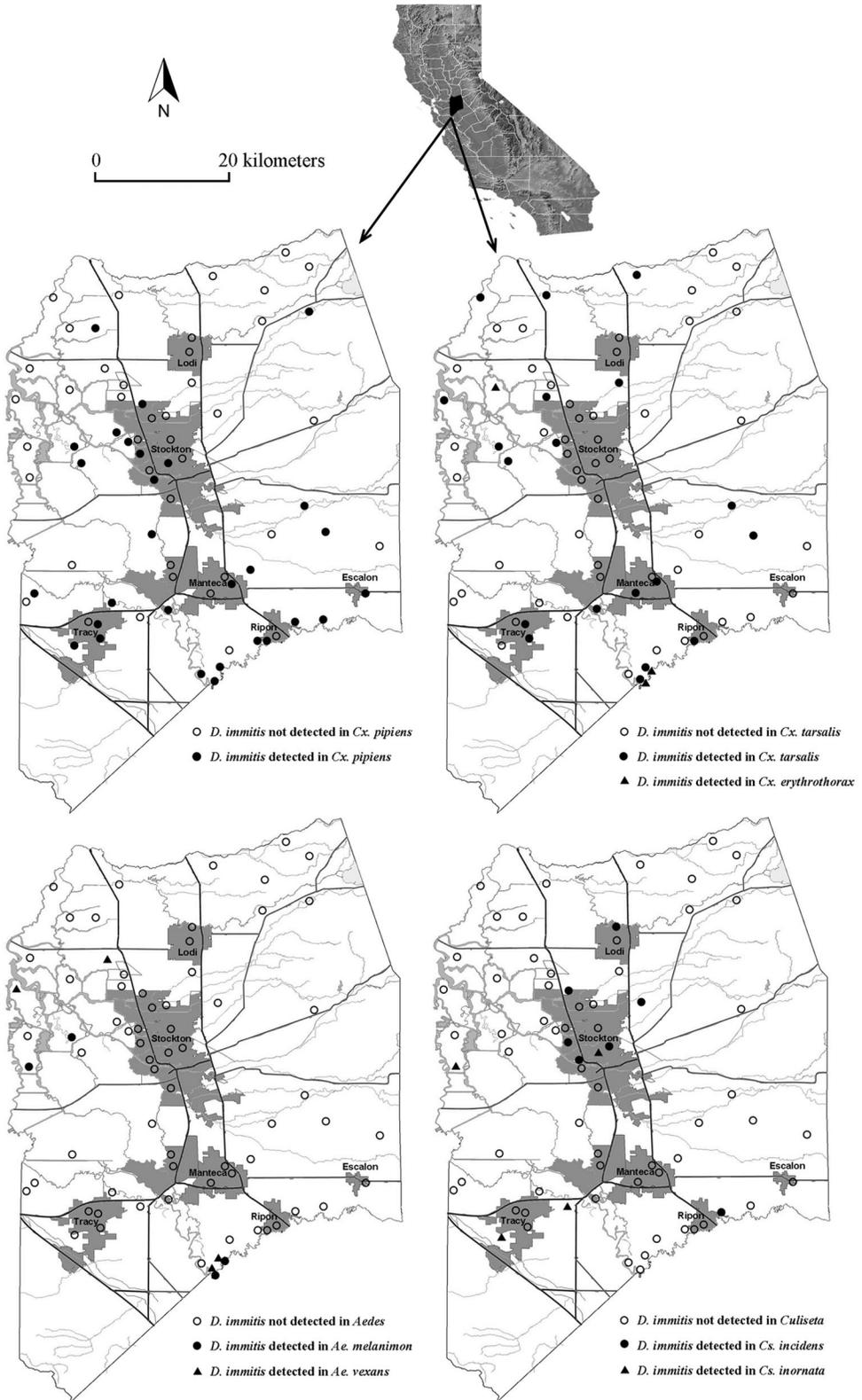
Fig. 1. Geographic distribution of *D. immitis* detection in 2011 in San Joaquin County, CA. Dark gray areas in the county maps depict cities and residential areas.
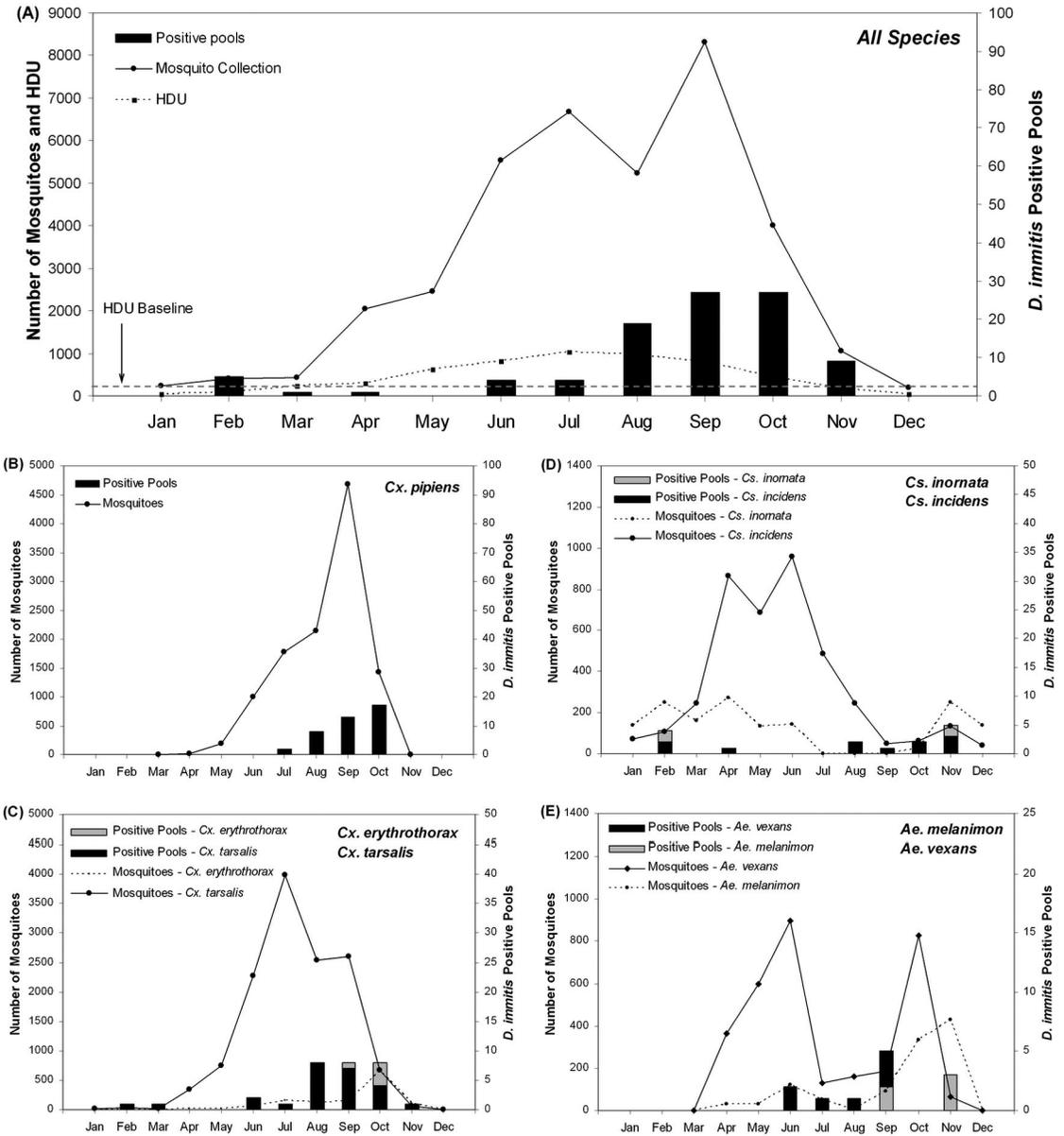
Fig. 2. Seasonal dynamics of mosquito collection and *D. immitis* infection in 2011 in San Joaquin County, California. (A) All species. (B) *Cx. pipiens.* (C) *Cx. erythrothorax* and *Cx. tarsalis.* (D) *Cs. inornata* and *Cs. incidens.* (E) *Ae. melanimon* and *Ae. vexans.*

of *D. immitis*-positive pools found at 29 different locations. All *D. immitis* detections in *Cx. pipiens* were made from July through October when this species was most abundant and the degree-days temperature was high. Infected *Cx. pipiens* mosquitoes were collected from both rural and urban areas, particularly from locations in central Stockton where a cluster of dog heartworm cases were reported in 2007 and 2008 (unpublished data). Studies have shown geographic variations in blood-feeding of *Cx. pipiens* complex mosquitoes (*Cx. pipiens, Culex quinquefasciatus* Say,

and their hybrids) from mammalian hosts including dogs (Molaei et al. 2012). Although, introgression among members of *Cx. pipiens* complex may lead to a greater propensity in hybrid mosquitoes to readily feed on mammals (Harbach et al. 1984, Spielman 2001, Fonseca et al. 2004, Huang et al. 2009), the impact of hybridization between *Cx. pipiens* (as a predominately ornithophagic species) and *Cx. quinquefasciatus* (as an opportunistic mosquito in blood-feeding) on the feeding behavior of mosquitoes is not entirely clear nor did we make attempts to distinguish between pure or

hybrid individuals of *Cx. pipiens* in this study. Nonetheless, San Joaquin County is located in a highly active hybridization zone (Barr 1957, Urbanelli et al. 1997), where some populations contain >25% hybrids and some mosquitoes do not enter diapause and are continuously collected by CO_2 traps during winter months, November to February (unpublished data). Thus, it is conceivable that *Cx. pipiens* mosquitoes examined in this study contain hybrids that may readily bite mammals including dogs, a characteristic that lends further support to incriminate *Cx. pipiens* as a principal vector of dog heartworm in this region.

Cx. tarsalis was the most abundant mosquito collected throughout the season, and infection with *D. immitis* was detected in populations collected from 19 different trap sites in agricultural and rural areas as well as residential and urban areas. *Cx. tarsalis* abundance and infection with *D. immitis* peaked in July and August, 2 mo earlier than those of *Cx. pipiens*. Although *D. immitis* infection in *Cx. tarsalis* was detected throughout the season from February to November, the majority of the detections were made from August to October, the same period in which the majority of infected *Cx. pipiens* was also collected. Together, *Cx. tarsalis* and *Cx. pipiens* constituted the majority of mosquitoes infected with *D. immitis* from August to October, a period when the numbers of infected mosquitoes among all other mosquitoes species were also high.

Cx. tarsalis has been documented to feed mostly on avian hosts; however, blood feeding on mammals, including dogs, has also been reported from northern California (27.3%; Thiemann et al. 2012), southern California (15.4%; Molaei et al. 2010), and Colorado (24.4%; Kent et al. 2009). These characteristics indicate that *Cx. tarsalis* could be an efficient vector of *D. immitis* in San Joaquin County. It is noteworthy that one infection in *Cx. tarsalis* was identified in February and another in March when degree-days were below 130 HDUs. One plausible explanation is that *Cx. tarsalis* mosquitoes acquired the infection before overwintering and were captured after they emerged from hibernacula as reported earlier (Knight and Lok 1998), suggesting a role for this species in early season transmission. Identification of *D. immitis* infection in *Cx. tarsalis* is very significant as this mosquito is one of the most abundant species in the western United States and is also considered as one of the principle vectors of several diseases-causing pathogens including WNV, St. Louis encephalitis virus, and western equine encephalitis virus. Although the vector competence of *Cx. tarsalis* for transmission of dog heartworm has not been corroborated, this species may have been overlooked as an important vector.

We identified five *D. immitis*-positive *Cx. erythrothorax* pools in three trap locations; however, the relatively low abundance of this species in our collection led to a slightly higher MIR (3.96) than that of other mosquito species, except for *Ae. melanimon*. Earlier studies have shown that *Cx. erythrothorax* is focally abundant in wetland or riparian habitats with dense emergent vegetation (Walton et al. 1998, Gerry et al.

2008), and readily feeds on both birds and mammals including rodents, rabbits, opossums, wild canids, deer, and humans (Walton et al. 1999, Molaei et al. 2010). Therefore, it is logical to infer that this species may be involved in transmission of dog heartworm to wild canids and other competent hosts in these wetland habitats.

D. immitis DNA was frequently detected in *Cs. incidens* with 11 positive pools collected from seven trap sites. As a peridomestic species that aggressively feeds on humans and large animals (Reeves and Hammon 1944, Tempelis 1975), *Cs. incidens* has also been reported as a highly competent vector of *D. immitis* in laboratory conditions (Theis et al. 2000). These characteristics implicate *Cs. incidens* as a potentially important vector of *D. immitis*. However, in the current study, this cold-weather mosquito was most abundant from mid-March to July, and most (10 of 11) of its *D. immitis* detections were observed during August to November when its abundance was very low. Therefore, its vector potential would appear to be limited because of its temporal variations in abundance and infection with *D. immitis*.

Four of *Cs. inornata* pools were tested positive for *D. immitis* in four locations. This species has low abundance in the region, and most collections were observed during December to May. Similar to *Cs. incidens*, its seasonal abundance and the timing of infection with *D. immitis* make *Cs. inornata* an unlikely vector of dog heartworm in the area.

Earlier studies have reported frequent infection of *Ae. vexans* with *D. immitis* (Ledesma and Harrington 2011), and in our study, we obtained seven positive pools but from only 4 of 69 trap locations, all in rural and agricultural areas. The abundance of *Ae. vexans* is closely associated with spring rainfall and agricultural activities, in which spring and fall water overflowing from agricultural lands leads to two abundance peaks in June and October. Temporally, all *D. immitis*-positive pools in *Ae. vexans* were identified during June to September when HDU was the highest, after the first abundance peak in June. *Ae. vexans* is an aggressive biter that feeds almost exclusively on mammals including wild canids (Molaei and Andreadis 2006). Although the abundance of *Ae. vexans* in the current study was relatively low, annual variations exists, especially in agricultural settings. In 2006, >22,000 specimens of *Ae. vexans* were collected in San Joaquin County. This species is a competent vector of *D. immitis* throughout the world and could play a prominent role in the rural and agricultural transmission cycles when its abundance is high.

Testing of *Ae. melanimon* mosquitoes collected from rural and agricultural areas resulted in four *D. immitis*-positive pools in four locations. This mosquito species has limited abundance in the region, and population increases are mostly because of temporary habitats created by the flooding of agricultural fields for waterfowl habitats in fall. Host feeding studies have shown that *Ae. melanimon* is an active biter at dusk that uses mammals including rabbits, dogs, horses, and cattle as the primary source of bloodmeals (Tempelis and

Washino 1967). Therefore, this species could also serve as a secondary vector of dog heartworm in rural and agricultural areas during fall.

The infection rate for all mosquito species in this study was relatively low (MIR = 2.69 or 0.269%) in contrast to some investigations in highly endemic regions such as Louisiana (15%) and Florida (11%; Watts et al. 2001). Other studies have reported lower MIR from a number of mosquito species (Sauerman and Nayar 1983, Parker 1986, Licitra et al. 2010). Although in our study MIR ranged from 1.89 to 4.64 for several mosquito species, differences in MIR among these species were not statistically significant ($P > 0.05$), indicating that multiple mosquito species might concertedly facilitate transmission of dog heartworm in the region rather than a single species. The predominance of *D. immitis*-positive pools in *Cx. pipiens* and *Cx. tarsalis* in conjunction with their abundance during 2005–2007 (data not shown) indicates that *Cx. pipiens* and *Cx. tarsalis* might have been the main underlying force for the surge in the dog heartworm incidence in San Joaquin County.

The California Central Valley including San Joaquin County features urban and residential areas immediately surrounded by agricultural and rural lands. Under such landscape characteristics, mosquitoes commonly disperse from one habitat type to another. The rural and agricultural transmission cycles of canine heartworm are primarily maintained by coyotes as ubiquitous canids in this region (Sacks et al. 1999, Riley et al. 2003). Studies have shown that heartworm infection in coyotes could be widespread (Sacks 1998). The urban and residential transmission cycles, however, are principally maintained by domestic dogs. Interactions between the two transmission cycles could take place in forms of invasion of urban and residential areas by coyotes and exposure of domestic dogs in rural and agricultural habitats in addition to mosquito dispersal from one habitat type to another. In the current study, the abundance and *D. immitis*-positive pools of all mosquito species with the exception of *Cs. incidens* were much greater in rural and agricultural areas than in urban and residential habitats, suggesting that *D. immitis* transmission in rural and agricultural areas is more intense, and the rural and agricultural transmission cycle may enhance the urban and residential transmission cycle.

In summary, our spatiotemporal investigation of the dog heartworm infection in mosquitoes in conjunction with their abundance and host feeding preference indicates that *Cx. pipiens* and *Cx. tarsalis* are probably the most important vectors of *D. immitis* in both agricultural and rural as well as urban and residential transmission cycles. *Ae. vexans*, *Ae. melanimon*, and *Cx. erythrothorax* may play secondary roles in agricultural and rural transmission cycles. In urban and residential transmission cycles, *Cs. incidens* could be viewed as a minor vector of canine heartworm. Finally, in this study, we demonstrated for the first time that *Ae. melanimon*, *Cs. inornata*, *Cx. erythrothorax*, and particularly *Cx. tarsalis* support the development of *D.*

immitis to L3 stages. Further studies are required to confirm vector competency in these species.

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