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臺灣臺北野外蚊蟲中犬心絲蟲之盛行率

Prevalence of canine heartworm in mosquitoes from Taipei, Taiwan

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中文摘要

犬心絲蟲症 (dirofilariasis) 是由小桿目蟠尾絲蟲科 (Rhabditida: Onchocercidae) 的犬心絲蟲 (*Dirofilaria immitis*) 感染寄生所引起，屬於一種蚊媒性人畜共通傳染病。主要宿主為犬隻，成蟲棲息於犬隻的右心室和肺動脈，臨床症狀包括咳嗽、呼吸困難，甚至死亡。犬心絲蟲也可以感染貓、狼、郊狼和其他哺乳動物。本研究的主要目標在持續監測 2023 年 6 月至 2025 年 5 月臺北地區蚊子感染犬心絲蟲的情形。本研究於臺北市內湖區與木柵區使用以乾冰為誘餌的 CDC 捕蚊燈採集蚊子，每兩週採集一個晚上。蚊子鑒定和分池 (pool) 之後，以巢式聚合酶連鎖反應 (nested PCR) 分析可能參與犬心絲蟲傳播的蚊子種類及其族群動態。本研究共收集 1926 隻，417 池，包含 9 屬 31 種蚊子。以 nested PCR 檢測犬心絲蟲感染，得到 78 池陽性，共計 15 種蚊子感染犬心絲蟲，兩年均有感染。在 *Culex quinquefasciatus*、 *Aedes albopictus* 和 *Cx. pipiens molestus* 物種中發現的陽性池數量最多。臺北市蚊子感染率的偏差校正最大似然估計值 (bias-corrected MLE) 為 7.6% (95% 信賴區間: 6.2% - 9.1%)。此外，本研究也首次發現 *Culex* 屬、*Mansonia* 屬、*Uranotaenia* 屬共 9 種蚊蟲可以被犬心絲蟲感染。本研究所建立之犬心絲蟲病媒蚊族群動態資料將有助於了解犬心絲蟲在臺北地區流行之情形及與病媒蚊之間的交互作用，可提供病媒防治、獸醫及公共衛生領域防疫政策之重要資訊。

關鍵字 (Keywords)：犬心絲蟲症、犬心絲蟲、蚊子、巢式聚合酶連鎖反應、病媒

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Abstract

Canine heartworm, *Dirofilaria immitis* (Rhabditida: Onchocercidae), is a mosquito-borne parasitic nematode that resides in the right ventricle and pulmonary arteries of infected dogs, leading to canine dirofilariasis. Clinical signs of *D. immitis* infection include cough, difficulty breathing, and potentially death. Although dogs are the primary hosts, *D. immitis* can infect cats, wolves, coyotes, and other mammals. To investigate potential mosquito vectors of *D. immitis* in Taipei, Taiwan, adult mosquitoes were collected from June 2023 to May 2025 using CDC light traps baited with dry ice. Detection of *D. immitis* in mosquito samples was performed using nested PCR. A total of 1926 mosquitoes, grouped into 417 pools representing 31 species from 9 genera, were collected. Among these, 78 pools tested positive for *D. immitis*. Infections were identified in 15 mosquito species and occurred throughout two years. The highest number of positive pools were found in species *Culex quinquefasciatus*, *Aedes albopictus*, and *Cx. pipiens molestus*. The bias-corrected maximum likelihood estimation (bias-corrected MLE) of the mosquito infection rate in Taipei was 7.6% (95% confidence interval: 6.2–9.1%). Notably, this study is the first to report natural *D. immitis* infections in 9 mosquito species belonging to the genera *Culex*, *Mansonia*, and *Uranotaenia*. The infection prevalence data presented in this study provide valuable insights into the transmission patterns of *D. immitis* in Taipei and its interactions with diverse mosquito species. These findings provide critical information to enhance vector control strategies and support the development of disease prevention policies in both veterinary and public health contexts.

Keywords: *Dirofilaria immitis*, canine heartworm, mosquito, nested PCR, vector

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1. Introduction

Canine dirofilariasis is a life-threatening disease caused by the mosquito-borne parasitic nematode *Dirofilaria immitis* (Rhabditida: Onchocercidae) (Mullen and Durden, 2019). This disease typically progresses slowly, initially leading to vascular and pulmonary issues and eventually infecting the right ventricle and the pulmonary artery (Simón et al., 2012). As the disease advances, the death of the worms, whether occurring spontaneously or as a result of adulticide medication, can trigger acute complications, such as thromboembolism and severe inflammation (Simón et al., 2012). These complications pose a significant threat to the survival of infected animals (Simón et al., 2012). Clinical symptoms of this disease include cough, dyspnea, anemia, and even possibly death. While dogs are the main hosts, *D. immitis* can also infect various species among carnivores, such as coyotes, foxes, wolves, jackals, black bears, cats, ferrets, sea lions, and snow leopards (Murata et al., 2003; Simón et al., 2012; Moroni et al., 2020; Livingston et al., 2024). *D. immitis* has also been reported in several wild species, such as lemurs (Zohdy et al., 2019) and penguins (Sano et al., 2005; Ledesma and Harrington, 2011). Humans can also serve as hosts for *D. immitis*, leading to a condition known as human dirofilariasis (Simón et al., 2022). Although this condition is usually not life-threatening and often asymptomatic, it can still cause health concerns. The typical characterization of human dirofilariasis is the formation of pulmonary nodules. However, *D. immitis* affects more than just the respiratory system. Published case reports of human dirofilariasis worldwide indicate that infections can occur in the conjunctival tissues, subcutaneous tissues, cranial, hepatic, mesenteric adipose tissues, and testicular arteries (Theis et al., 2001; Theis, 2005; Simón et al., 2012).

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D. immitis impacts both wild and domestic animals, as well as humans in tropical and temperate regions around the world. A systematic review and meta-analysis found a global prevalence of 11% for dirofilariasis in dogs and 12% in Asia (Anvari et al., 2020). In the new world, such as the Americas, epidemiological studies have reported the presence of *D. immitis* in dogs across multiple countries and regions, except for Chile and Uruguay (Simón et al., 2012; Dantas-Torres and Otranto, 2020). The prevalence of *D. immitis* infection varies significantly across the continent. In the old world, recent data on canine dirofilariasis indicate a decreasing or stable trend in Western Europe, including areas that were previously hyperendemic (Genchi et al., 2020). In contrast, prevalence is significantly increasing in Eastern Europe and the Asian regions of Russia, with the condition being reported more frequently in Africa (Genchi et al., 2020). In Asia, documented cases of heartworm infections in dogs are primarily from Japan, South Korea, China, Malaysia, India, Iran, and Thailand (Simón et al., 2012; Genchi et al., 2020).

Notably, in Taiwan, three large-scale studies on the prevalence of canine dirofilariasis have been published over the past three decades. From 1993 to 1997, 837 stray dogs from three cities in northern Taiwan were necropsied for canine dirofilariasis, while 1,228 house dogs from 10 cities, including two offshore islands (Lanyu and Liuchiu), were examined for microfilariae in their peripheral blood, using thick blood smear. The prevalence of adult worms in stray dogs was 57% in northern Taiwan, and in Taipei, it was 67% (Wu and Fan, 2003). Additionally, the prevalence of microfilariae in domestic dogs was 27% (Wu and Fan, 2003). Between 1998 and 1999, the seroprevalence of *D. immitis* infection was investigated in 664 domestic dogs from Taipei and 14 mountainous indigenous areas in eastern Taiwan, using a commercial ELISA kit to detect circulating antigens from female adult

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worms. The seroprevalence was 14% in Taipei and 12% in the mountainous indigenous areas (Fan et al., 2001). In 2015, a study was conducted on 2,064 household dogs from 103 veterinary hospitals across 13 cities in Taiwan (Lu et al., 2017). Circulating antigens from adult female worms were detected using a commercial kit, and microfilariae were identified through a direct blood smear technique. The prevalence of *D. immitis* infection in domestic dogs in Taiwan was 23% and 12% in Taipei (Lu et al., 2017). Other regional studies in Taiwan have also highlighted the severity of the prevalence of canine dirofilariasis (Wang, 1997 and 1998; Yuasa et al., 2012; Chou et al., 2014; Feng et al., 2015)

Dirofilaria immitis is transmitted by mosquitoes from various genera, including *Culex, Aedes,* and *Anopheles,* across different world regions (Perles et al., 2024). According to the American Heartworm Society (2024), the complete life cycle of *D. immitis* involves the invertebrate host (female mosquito) and the vertebrate host (mainly dog). When the mosquito's proboscis punctures the dog's skin, causing the labium to bend sharply. This action causes infective third-stage larvae (L3) of *D. immitis* to rupture through the mosquito's labium, releasing mosquito hemolymph and infective larvae onto the surface of the dog's skin. Shortly after blood feeding, the L3 larvae enter the dog's body through the puncture site. Upon entering the dog's body, the L3 larvae develop into fourth-stage larvae (L4). Between days 50 and 70 after invasion, the larvae undergo a final molt into immature adults while migrating through the dog's body. As early as day 67, they can reach the smallest pulmonary arteries. Sexual maturity is typically reached around day 120 post-infection, with dogs developing patent infections (i.e., circulating microfilariae) as early as six months, but usually between seven and nine months after invasion. Microfilariae circulate in the dog's peripheral blood, enabling the mosquito to ingest

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them during blood feeding. Inside the mosquito, the microfilariae develop within the Malpighian tubules, progressing through the first larval stage (L1), followed by the second stage (L2), and eventually molting into the infective third-stage larvae (L3). The L3 larvae migrate through the hemolymph to the mosquito's head and mouthparts. Transmission of the infective L3 occurs when the infected mosquito takes another blood meal.

More than 70 mosquito species are suitable arthropod hosts for *D. immitis* (Azari-Hamidian et al., 2009), but only a few are proven to be significant vectors in nature (Morchón et al., 2012; Dantas-Torres and Otranto, 2020). In the US, 24 mosquito species have been known to be naturally infected with L3 *D. immitis*, including *Aedes albopictus, Ae. canadensis, Ae. sierrensis, Ae. trivittatus, Ae. vexans, Anopheles crucians, An. punctipennis, An. quadrimaculatus, Culex. quinquefasciatus.* These nine species have been recognized as key vectors in the US, and data on their presence can serve as one of the risk factors in developing the forecasting model for *D. immitis* prevalence (Ledesma and Harrington, 2011; Brown et al., 2012; Wang et al., 2014). In Europe, various studies have indicated that some *Aedes, Anopheles, Coquilletidia, and Culex* mosquitoes support the development of L3 *D. immitis*, such as *Ae. albopictus, Ae. caspius, Ae. geniculatus, Ae. vexans, Cx. pipiens, Cx. theileri, An. maculipennis,* and *Cq. richiardii* (Morchón et al., 2012; Shaikevich et al., 2019). The current systematic review and meta-analysis of published studies up to 2019 found that the overall prevalence of *D. immitis* in field-caught mosquitoes was 21.9‰ for infected (L1-L2) stages and 2.6‰ for infective (L3) stages (Riahi et al., 2021). However, research on this topic in Taiwan is limited and outdated. Previous research determined that *Cx. quinquefasciatus, Cx. tritaeniorhynchus, Cx. alis, Ae. albopictus* and *Ae. togoi* were susceptible to *D.*

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immitis (Wu et al., 1988b). In a field survey conducted from 1993 to 1995 in northern Taiwan, the prevalence of *D. immitis* was 4.3% in *Cx. quinquefasciatus* and 1.3% in *Ae. albopictus*. Specifically, all *D. immitis* larvae found in *Cx. quinquefasciatus* were L3 (Wu et al., 1997). These findings indicated that *Cx. quinquefasciatus* was the primary vector of *D. immitis* in northern Taiwan. Between 1997 and 1998, the prevalence of *D. immitis* in central Taiwan was 4.3% in *Cx. quinquefasciatus* and 3.7% in *Ae. albopictus* (Lai et al., 2001). However, no natural infections of *D. immitis* were detected in other mosquitoes in both studies. Additionally, a further study on the vector competence of *Ae. albopictus* and *Cx. quinquefasciatus* suggested that *Cx. quinquefasciatus* may play a significant role as a vector in transmitting *D. immitis* in central Taiwan (Lai et al., 2000).

Since 2000, no further epidemiological studies on the vectors of *D. immitis* have been conducted in Taiwan. Therefore, information about the transmitting mosquito species was still scarce in Taiwan. Given the emergence of drug resistance in *D. immitis* (Bourguinat et al., 2011), the potential impact of climate change, which may promote the development of *D. immitis* larvae in mosquitoes during colder months, extend mosquito activity periods, prolong transmission seasons (Ledesma, 2011; Morchón, 2012; Simón, 2012; Riahi, 2021), and introduce newly invasive mosquito species, such as *Cx. pipiens molestus* (Lien et al., 1996). These factors may affect the infection rate of *D. immitis* in mosquitoes in Taiwan, potentially increasing the risk of transmission to canines or humans. Moreover, using different detection methods for mosquitoes infected with filarial species can also impact the accuracy of the infection rate. In tradition, mosquito dissection and morphological identification of filarial species under a microscope have been regarded as the gold standard for detecting infections of filarial species in mosquitoes. While it is labor-

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intensive and costly when screening large numbers of mosquitoes, making it impractical for large-scale surveillance programs (Scoles and Kambhampati, 1995; Watts et al., 1999; Chambers et al., 2009; Licitra et al., 2010; Vezzani et al., 2011; Ledesma and Harrington, 2011; Tahir et al., 2017; Aung et al., 2022). To date, the ongoing development and refinement of polymerase chain reaction (PCR)-based molecular diagnostic techniques, which provide enhanced sensitivity and specificity, have facilitated more accurate identification of filarial species in mosquitoes, allowing for the rapid screening of large numbers of mosquitoes, whether pooled or individually categorized.

The objectives of this study were to estimate *D. immitis* infection rates in mosquito populations in Taipei, assess transmission risk through heartworm development units (HDUs), and evaluate the significance of mosquito vectors using the vector index (VI). The findings will provide crucial data for improving vector control strategies and informing disease prevention policies for both animal and public health.

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2. Materials and methods

2.1 Study sites

Three study areas are located within Taipei City in northern Taiwan, which has a subtropical climate. During 2023-2025, the average monthly temperature ranges from 15.5 to 30.7°C, with rainfall exceeding 2,000 mm and relative humidity ranging from 72% to 85%, according to the Climate Observation Data Inquiry System (CODIS) of the Central Weather Administration, Taiwan. Other meteorological data, such as rainfall (mm) and relative humidity (%) for the study period, are also presented in Appendix 1. Mosquito collections were conducted at the Taipei Animal Shelter (TAS), Cho Mama Dog Shelter (CMDS), and Taipei Zoo (ZOO). TAS and CMDS are located in the Neihu District, within the basins of the Keelung River, while ZOO is situated in the Wenshan District along the Jingmei River.

i) Taipei Animal Shelter (25°03'38.2" N 121°36'12.1" E) (Figure 1):

According to the Pet Registration Information System of the Ministry of Agriculture, the TAS is the only facility in Taipei dedicated to containing stray dogs and cats. TAS is currently housing 745 dogs, with a maximum capacity of 840. The shelter ensures a hygienic environment by improving ventilation and addressing odor issues, reducing overcrowding in dog areas, minimizing disease transmission, and implementing well-planned zoned management strategies. Additionally, regarding the health of shelter dogs, a 4-in-1 test kit including *D. immitis, Anaplasma* spp., *Babesia* spp., and *Ehrlichia canis* detection (Bioguard, Taiwan) is used for screening during adoption. If test results are positive, antibiotics are continuously administered to eliminate microfilariae, and, in some cases, adult worms are also treated, depending on

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available funding. All dogs receive monthly ivermectin for *D. immitis* prevention.

ii) Cho Mama Dog Shelter (25°03'46.8" N 121°36'28.9" E) (Figure 2):
The CMDS, 650 m away from TAS, is located on a rugged hillside and currently houses 11 stray dogs. Unlike TAS, it has suboptimal sanitary conditions, and the environment is relatively untidy and chaotic, with rodents and insects thriving in the area. Despite these challenges, the caretaker ensures adequate food, water, and supplies and administers regular preventive medications to mitigate the risk of *D. immitis* infection and its transmission.

iii) Taipei Zoo: Formosan animal area (24°59'53.0" N 121°34'49.6" E) (Figure 3):
According to the Department of Information and Tourism, Taipei City Government, Taipei Zoo is the largest public zoo in Southeast Asia. Its 2023 annual report stated that the zoo currently houses 339 species of animals, totaling around 2,242 animals (excluding insects, certain fish species, and rescued animals). The animal housing environment of Taipei Zoo aims to imitate the natural habitats of the animals. Nevertheless, the zoo administration ensures a hygienic environment by conducting regular cleaning and maintenance. Additionally, they provide regular preventive treatments and screenings for the animals in their care to reduce the risk of infectious diseases and their transmission within the zoo.

2.2 Mosquito collection and identification

Mosquitoes were collected at each site biweekly from June 2023 to May 2025 using CDC light traps (John W. Hock Company, USA) baited with UV light and 1 kg dry ice (Figure 4). The traps were set at approximately 1.5 m above the

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ground and operated from 6:00 p.m. to 10:00 a.m. the following day. Non-mosquito species and male mosquitoes were discarded, and the female mosquitoes were identified to species level based on morphological characteristics according to the key (Lien, 2004) using a stereomicroscope (Leica MZ 125). Once species were identified, the wings and legs of female mosquitoes were removed using fine forceps and pooled in groups of up to 25 individuals based on species, blood-fed, collection site, and collection date. The pools were then stored at -20°C before DNA extraction.

2.3 Genomic DNA extraction from mosquito pools

Genomic DNA was extracted from each mosquito pool using the gSYNC™ DNA Extraction Kit (Geneaid, Taiwan) with minor modifications as follows. 100, 200, 300, 400, or 500 μL of GST buffer was used for grinding 1–5, 6–10, 11–15, 16–20, or 21–25 mosquitoes, respectively, and 100 μL of macerated solution was used for subsequent extraction steps. Each pooled sample's DNA concentrations and purity (A260/280 and A260/230 ratios) were measured using a NanoDrop™ 2000 spectrophotometer (Thermo Fisher Scientific, USA). Samples with concentrations exceeding 100 ng/μL were diluted to 100 ng/μL and stored at -20 °C until PCR analysis.

2.4 *Dirofilaria immitis* positive control

Genomic DNA from *D. immitis*, provided by Professor Wei-Hsiang Huang from the Department of Veterinary Medicine at National Taiwan University, was extracted using the gSYNC™ DNA Extraction Kit according to the manufacturer's instructions and was subsequently confirmed as *D. immitis* following the procedures outlined in sections 2.7 and 2.8.

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2.5 PCR detection of the *COI* gene-based barcoding of mosquitoes

Rare mosquito species, particularly *Culex* species that are difficult to identify morphologically, were amplified using *cytochrome oxidase subunit I* (*COI*) primers MCB-F and MCB-R (Table 1; Kumar et al., 2007) by PCR. PCR reactions were performed in a final volume of 10 μL, containing 5 μL of 2× Taq PCR Mix-RED (Bioman, Taiwan), 0.3 μL of each forward and reverse primer (10 μM), 1 μL of template DNA, and 3.4 μL of ddH_2O. The PCR conditions were as follows: initial denaturation at 95°C for 5 min, followed by 5 cycles of denaturation at 94°C for 30 s, annealing at 45°C for 30 s, extension at 72°C for 1 min, and 35 cycles of denaturation at 94°C for 30 s, annealing at 51°C for 30 s, extension at 72°C for 1 min and a final extension at 72°C for 10 min. DNA extracted from laboratory-reared female *Aedes albopictus* served as the positive control, while ddH_2O was used as the negative control in each PCR run. The reactions were carried out using an Applied Biosystems™ Veriti™ 96-Well Thermal Cycler (Thermo Fisher Scientific, USA). The amplification products (5 μL) were analyzed by electrophoresis on a 1% agarose gel stained with SYBR™ Safe DNA Gel Stain (Thermo Fisher Scientific, USA), and then the stained gel was visualized under UV light. The positive samples were purified with GenepHlow™ PCR Cleanup Kit (Geneaid, Taiwan) and subsequently sent for sequencing. The primers used for sequencing were the same as those employed in the PCR. The obtained nucleotide sequences were analyzed using BLASTn to find the closest matching sequences available in the GenBank database.

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2.6 PCR detection of the *actin-1* gene of mosquitoes

To prevent false-negative results caused by potential PCR inhibitors in extracted DNA, the *actin-1* gene of mosquitoes was used as an internal control. The gene was amplified by the primers Act-2F and Act-8R (Table 1, Staley et al., 2010) by PCR. PCR preparation was described above. The PCR conditions were as follows: initial denaturation at 95°C for 5 min, followed by 40 cycles of denaturation at 95°C for 30 s, annealing at 56°C for 30 s, extension at 72°C for 30 s, and a final extension at 72°C for 5 min. DNA extracted from laboratory-reared female *Ae. albopictus* served as the positive control, while ddH_2O was used as the negative control in each PCR run. The reactions were carried out using an Applied Biosystems™ Veriti™ 96-Well Thermal Cycler (Thermo Fisher Scientific, USA). The amplification products (5 μL) were analyzed by electrophoresis on a 1% agarose gel stained with SYBR™ Safe DNA Gel Stain (Thermo Fisher Scientific, USA), and then the stained gel was visualized under UV light.

2.7 Nested PCR detection of the *COI* gene of *D. immitis* in mosquitoes

The first PCR run

Dirofilaria immitis in each mosquito pool was first amplified by *cytochrome oxidase subunit I* (*COI*) primers DI_COI-F and DI_COI-R (Table 1, Rishniw et al., 2006) by PCR. PCR preparation and cycling conditions were as described above, conducted with 30 cycles.

The second PCR run

The first run product (1 μL) was used as a DNA template for the second PCR run. The product from the first run was amplified by the primers DI_COI-Fin and DI_COI-Rin (Table 1) by PCR. PCR preparation and cycling conditions were as

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described above, conducted with 30 cycles. In each PCR run, *D. immitis* DNA was used as the positive control, while DNA from laboratory-rearing female *Ae. albopictus* and ddH_2O served as the negative controls. The amplification product (5 μL) was analyzed by electrophoresis on 2% agarose gel stained with SYBR™ safe DNA gel stain and then visualized under UV light.

2.8 *Dirofilaria immitis* confirmation

For *D. immitis*-positive samples amplified from *Ae. albopictus*, *Cx. pipiens molestus*, and *Cx. quinquefasciatus,* 20% of the amplicons were sequenced to confirm *D. immitis* identity, while all positive samples amplified from less abundant species were sequenced. The positive samples were purified with GenepHlow™ PCR Cleanup Kit (Geneaid, Taiwan) and subsequently sent for sequencing. The primers used for sequencing were the same as those employed in the second reaction of the nested PCR. The obtained nucleotide sequences were analyzed using BLASTn to find the closest matching sequences available in the GenBank database.

2.9 Infection rate of *D. immitis* in mosquitoes

Infection rates (IRs) were determined using the minimum infection rate (MIR) and bias-corrected maximum likelihood estimate (MLE) per 100 mosquitoes with 95% confidence intervals (CI) by using the PooledInfRate v4.0 Excel Add-In (Biggerstaff, 2009).

2.10 Vector index

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Vector Index (VI) estimates the presence of infected mosquitoes in a geographic area by combining mosquito density and infection rate data into a single measure. It was calculated using the formula:

$$VI = \sum_{i=species} \bar{N}i \times \hat{P}i$$

Mosquito density ($\bar{N}$) refers to the mean number of female mosquitoes collected per trap night. It is calculated by dividing the total number of female mosquitoes collected in a specific geographic area by the total number of trap nights during the study period. The infection rate ($\hat{P}$) of *D. immitis* in mosquitoes was determined using the bias-corrected MLE described in section 2.9. A combined Vector Index (VI) is the total number of infected mosquitoes collected per trap night in a geographic area, calculated by summing the vector indices (VIs) of each species (CDC, 2024).

2.11 Heartworm Development Unit

A prediction for the start and end of the heartworm transmission season in Taipei was made using Heartworm Development Units (HDUs). HDUs are calculated as degree days, based on the average daily temperature exceeding the minimum threshold of 14°C required for *D. immitis* development (Ledesma and Harrington, 2015). HDUs were computed using mean daily temperature data. The sum of HDUs in each month during the study period was determined using the formula: HDUs $= \sum \text{mean daily temperature} - 14$. The mean daily temperature data for 2023 and 2024 from two climate stations (i.e., Neihu: 25°04'45.8" N 121°34'31.8" E, Wenshan: 25°00'08.3" N 121°34'32.5" E) were obtained from CODIS of the Central Weather Administration, Taiwan.

3. Results

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3.1 Mosquito abundance

The mosquito collection data are illustrated in Table 2. A total of 1,926 female mosquitoes, grouped into 417 pools, representing 31 species across 9 genera (*Aedes, Anopheles, Armigeres, Culex, Malaya, Mansonia, Mimomyia, Ochlerotatus,* and *Uranotaenia*), were collected in Taipei from June 2023 to May 2025. The most commonly trapped species were *Cx. quinquefasciatus* (n = 1,048; 54%), *Cx. pipiens molestus* (n = 369; 19%), *Ae. albopictus* (n = 201; 10%), and *Cx. tritaeniorhynchus* (n = 158; 8%) comprised over 90% of all specimens. Other trapped species included *Ar. subalbatus* (n = 31; 1.6%), *Cx. mimulus* (n = 25; 1.3%), *Cx. sitiens* (n = 24; 1.2%), and others (n = 70; 3.6%).

Among the three study sites, CMDS recorded the highest number of mosquitoes captured (n = 1,431, 74%), followed by TAS (n = 281, 15%) and ZOO (n = 214, 11%). The relative frequencies of mosquito species captured at each site are shown in Figure 5. Six species (*Ae. albopictus, Ar. subalbatus, Cx. pipiens molestus, Cx. quinquefasciatus, Cx. sitiens,* and *Cx. tritaeniorhynchus*) were captured at all sites. *Cx. quinquefasciatus* was the most abundant in CMDS and TAS, followed by *Cx. pipiens molestus*, while *Ae. Albopictus* was the most frequently captured species at ZOO.

3.2 Detection of the mosquito *actin-1* gene

The *actin-1* gene was successfully amplified from the mosquito pools using established detection methods (Staley et al. 2010). Of the 417 mosquito pools, 353 (85%) tested positive for *actin-1*, comprising 1,321 mosquitoes from 9 genera (*Aedes, Anopheles, Armigeres, Culex, Malaya, Mansonia, Mimomyia, Ochlerotatus,* and *Uranotaenia*). To avoid detecting *D. immitis* DNA from blood meals, blood-fed

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females with undigested blood in the abdomen were excluded. After this exclusion, 303 mosquito pools remained, comprising 1,136 mosquitoes from the same 9 genera (Table 3).

3.3 Prevalence of *D. immitis* in mosquitoes

Dirofilaria immitis infection rates of field-caught mosquitoes are presented in Table 3. *Dirofilaria immitis* DNA was detected in 78 mosquito pools (26%), representing 15 species from 6 genera (*Aedes*, *Anopheles, Armigeres, Culex, Mansonia,* and *Uranotaenia*). The top three positive pools included *Cx. quinquefasciatus* (n = 23; 29%), *Ae. albopictus* (n = 15; 19%), *Cx. pipiens molestus* (n = 11; 14%). DNA sequencing confirmed that all randomly selected DNA products belonged to *D. immitis*, with sequence similarities of 97–100% compared to reference sequences in the GenBank database (NC_005305.1).

Among the most highly infected mosquito species, *Cx. mimulus* had the highest *D. immitis* infection rate (MIR: 26.3%; bias-corrected MLE: 27.7%), followed by *Cx. pallidothorax* (MIR: 23.1%; bias-corrected MLE: 23.0%), *Cx. sitiens* (MIR: 11.1%; bias-corrected MLE: 10.7%), *Ae. albopictus* (MIR: 8.2%; bias-corrected MLE: 8.4%), *Cx. pipiens molestus* (MIR: 7.3%; bias-corrected MLE: 7.6%), *Cx. tritaeniorhynchus* (MIR: 6.5%; bias-corrected MLE: 6.8%), *Cx. quinquefasciatus* (MIR: 3.9%; bias-corrected MLE: 4.5%), *Ar. subalbatus* (MIR: 3.4%; bias-corrected MLE: 3.4%), and others (MIR: 27.9%; bias-corrected MLE: 28.3%) (Table 3). The overall *D. immitis* infection rate across all mosquito species was estimated at 6.9% (MIR) and 7.6% (bias-corrected MLE).

3.4 Geospatial patterns of *D. immitis* infection in mosquitoes

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Dirofilaria immitis infection rates of field-caught mosquitoes at three study sites are presented in Table 4. Of the 78 *D. immitis*-positive pools, 39 (50%) were identified from mosquitoes collected at CMDS, 21 (27%) at ZOO, and 18 (23%) at TAS. Positive pools of *Cx. quinquefasciatus, Cx. pipiens molestus, Ae. albopictus, and Cx. sitiens* were detected at all three sites. Positive pools of *Cx. tritaeniorhynchus* was found at CMDS and TAS, while a positive pool of *Ar. subalbatus* were only found at ZOO.

ZOO revealed the highest overall *D. immitis* infection rate (MIR: 13.4%; bias-corrected MLE: 13.6%), followed by TAS (MIR: 9.1%; bias-corrected MLE: 10.0%) and CMDS (MIR: 5.0%; bias-corrected MLE: 5.5%) (Table 6).

3.5 Temporal variation in mosquito population dynamics and *D. immitis* infection

Temporal dynamics of field-caught female mosquitoes are illustrated in Figure 6. There were no clear seasonal trends in the mosquito population. *Cx. quinquefasciatus* was the most prevalent mosquito species throughout the two-year study, showing no clear seasonal trends (Figure 7A). Population of *Cx. pipiens molestus* peaked from December to April (Figure 7B). *Ae. albopictus* was primarily collected between June and October (Figure 7C). *Cx. tritaeniorhynchus* was collected between March and November, with its presence peaking in August and September (Figure 7D).

Except for July, October 2024, January, March, and May 2025, *D. immitis*-positive mosquito pools were detected every month from June 2023 to May 2025 (Figure 6). Most *D. immitis*-positive pools (n = 71; 91%) were identified from June 2023 to May 2024, with infections primarily found in *Cx. quinquefasciatus, Ae.*

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albopictus and *Cx. pipiens molestus*. The highest number of *D. immitis*-positive pools occurred in June and September 2023 (n = 12; 15%). The second-highest number occurred in August 2023 (n = 10; 13%). The earliest detection of *D. immitis* occurred in January and February each year, with positive pools of *Cx. quinquefasciatus, Cx. pipiens molestus, Ae. albopictus, Cx. mimulus,* and *Ur. novobscura.* Temporal dynamics of *D. immitis* infection in four major mosquito species are illustrated in Figure 7. Starting from February 2024, *D. immitis*-positive pools were primarily found in *Cx. quinquefasciatus* and *Cx. pipiens molestus* (Figure 7A, B).

3.6 Estimation of the transmission risk of *D. immitis* by mosquitoes

The HDU values at the three study sites indicated that weather conditions were favorable for *D. immitis* development in all months except December (96 HDUs in 2024), January (89 in 2024; 63 in 2025), February (106 in 2024; 64 in 2025) during which values fell below the required threshold of 130 HDUs (Figure 8). However, *D. immitis*-positive pools were still detected during these months (Figure 7).

The vector index of primary *D. immitis*-infected mosquito species was 0.77 (Table 5). Among these, *Cx. quinquefasciatus* had the highest vector index (VI = 0.31), followed by *Cx. pipiens molestus* (VI = 0.18) and *Ae. albopictus* (VI = 0.11).

4. Discussion

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4.1 Mosquito abundance

Culex quinquefasciatus was the most prevalent mosquito species throughout the two-year study, showing no clear seasonal trends. The second most abundant species was *Cx. pipiens molestus*, an invasive species first reported in Taipei in 1996 (Lien, 1996), with its presence peaking from December to April. It is well-adapted to the low temperatures of winter in Taiwan (Tsai, 1998). *Ae. albopictus* ranked third, with collections primarily occurring between June and October, consistent with earlier observations from Taichung (Lai et al., 2001). *Cx. tritaeniorhynchus* was the fourth most abundant species, peaking in August and September, similar to previous findings from Taishan, New Taipei City (Lin and Lu, 1995). Its population density is strongly correlated with rice cultivation, with larval abundance reaching its highest levels in rice fields, particularly when the plants are tall and densely grown, as these conditions provide an optimal environment for development (Becker et al., 2010). In Taiwan, the climate allows for two rice harvests annually, with the second cropping season occurring from July to November. This seasonal overlap further suggests that the population dynamics of *Cx. tritaeniorhynchus* are closely linked to the rice-growing cycle. Beyond these four dominant species, the study also collected a total of 27 species across 8 genera, including *Anopheles, Armigeres, Culex, Malaya, Mansonia, Mimomyia, Ochlerotatus,* and *Uranotaenia*. However, their sample sizes were too small for meaningful seasonal analysis.

Geospatial analysis revealed higher mosquito catches at CMDS than TAS and ZOO. This disparity may stem from the poorer sanitary conditions and general disarray observed at CMDS, creating more favorable mosquito breeding sites. *Culex quinquefasciatus* is generally associated with habitats such as artificial containers, catch basins, and wastewater effluent (Ledesma and Harrington, 2011), and CMDS

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and TAS are close to urban residential areas, offering breeding sites for the species. Likewise, *Cx. pipiens molestus*, known for breeding in underground water storage and septic tanks (Tsai, 1998), was the second most abundant species at CMDS and TAS. Conversely, *Ae. albopictus*, though ranking third in overall collection, was the most frequently caught species at ZOO. This species was originally found in forested areas, where it bred in natural containers such as tree holes, bamboo internodes, and bromeliads, and was therefore traditionally regarded as a rural vector (Bonizzoni et al., 2013). However, it has adapted to suburban and urban settings, breeding in artificial containers such as discarded tires and water storage vessels (Bonizzoni, 2013). At ZOO, the diverse environment offers a wide range of oviposition sites, enabling this species to utilize both artificial containers and natural outdoor habitats for egg-laying. It's important to note that the study's reliance on CDC light traps, which are most effective for crepuscular and nocturnal mosquitoes, probably led to an underestimation of *Ae. albopictus* and other species are active during the day. For example, *Ae. japonicus* (formerly *Ochlerotatus japonicus*) has also been confirmed as a competent vector of *D. immitis* through experimental studies (Silaghi et al., 2017). In this research, only two *Ae. japonicus* specimens were collected at ZOO, and no *D. immitis* infection was detected. The absence of infection in this species may be attributed to the extremely limited sample size (n = 1). Additionally, *Ae. vexans*, a globally recognized vector of *D. immitis* (Holderman et al., 2021; Huang et al., 2013; McKay et al., 2013; Paras et al., 2014; Shaikevich et al., 2018; Watts et al., 2001; Yildirim et al., 2011), which is typically active from early evening to midnight (Ledesma and Harrington, 2011), was not collected at any sampling site. Further studies targeting these species and their association with *D. immitis* are needed to assess their potential as heartworm vectors in Taipei, Taiwan. To enhance

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sampling efforts, different trapping methods, such as BG-Sentinel traps, gravid traps, and human landing collections, as well as longer operation periods, should be employed, primarily to target species that are active during the day.

4.2 *Dirofilaria immitis* infection prevalence in mosquitoes

Natural infections of *Cx. quinquefasciatus* with *D. immitis* have been documented in various regions globally (Riahi et al., 2021). Numerous studies have identified this mosquito species as a competent and effective vector for *D. immitis* transmission (Lai et al., 2001; Tiawsirisup and Nithiuthai, 2006). In the present study, *Cx. quinquefasciatus* accounted for the greatest number of *D. immitis*-positive detections. However, the infection rate was relatively low (bias-corrected MLE: 4.5%). Its infection rate is similar to previously reported infection rates for this species in Taiwan, 4.3% in Taipei and Taichung (Wu et al., 1997; Lai et al., 2001). Under similar altitude and climate conditions, the *D. immitis* infection rates for *Cx. quinquefasciatus* in Gainesville, Florida, USA (MIR: 1.11%) (Holderman et al., 2021) and Tabasco, Mexico (MIR: 0.29%) (Torres-Chable et al., 2018) were lower than the rate observed in the present study.

Aedes albopictus has been documented as a vector of *D. immitis* in Taiwan (Lai et al, 2000-01; Wu et al., 1997). *Ae. albopictus* had the second highest number of *D. immitis*-positive pools; infection rate was 8.4% (bias-corrected MLE) in this study. Compared to previously reported infection rates for this species in Taiwan, with 1.3% in Taipei (Wu et al., 1997) and 3.7% in Taichung (Lai et al., 2001), the current rate was considerably higher. There was no catch of *Ae. albopictus* infected with *D. immitis* after February 2024. *Ae. albopictus* has already been implicated as a vector in different regions of the world. The *D. immitis* infection rates for *Ae.*

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albopictus are mostly 0.06–3.8% (Cancrini et al., 2003-07; Masetti et al., 2008; Giangaspero et al., 2013; Paras et al., 2014; Shaikevich et al., 2019; Panarese et al., 2020; Spence Beaulieu et al., 2020; Holderman et al., 2021; Younes et al., 2021). Infection rates for this species are relatively higher in Connecticut, USA (MIR: 5.4–6.5%, Bagger et al., 2025)

Culex pipiens molestus had the third highest number of *D. immitis*-positive pools; infection rate was 7.6% (bias-corrected MLE) in this study. This is the first known study to report natural infection of *Cx. pipiens molestus* with *D. immitis* in Taiwan. The risk of vector transmission of *D. immitis* by this species needs to be evaluated. Global records of *D. immitis* infection in this species are limited, primarily because most studies classify it under the *Cx. pipiens* complex. To date, *D. immitis* infection in *Cx. pipiens molestus* was only reported in Setúbal, Portugal, where a single positive pool was detected (Ferreira et al. 2015).

Culex tritaeniorhynchus had the fourth highest number of *D. immitis*-positive pools; infection rate was 6.8% (bias-corrected MLE) in this study. Notably, this is the first documented case of *D. immitis* natural infection in this species in Taiwan. Although *Cx. tritaeniorhynchus* has been well studied as a vector of the Japanese encephalitis virus, evidence of its role in *D. immitis* transmission remains limited. Studies have demonstrated its susceptibility to *D. immitis* in Taiwan and Japan (Wu et al., 1988b; Konishi, 1989b), and it has also been identified as a natural vector in Miki, Japan, with an infection rate of 0.06–0.35% (MIR) (Konishi,1989a). While these findings indicate that *Cx. tritaeniorhynchus* could act as a vector of *D. immitis*, more indigenous data in Taiwan are required to substantiate its involvement in transmission.

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Culex mimulus had the fifth highest number of *D. immitis*-positive pools; infection rate was 27.7% (bias-corrected MLE) in this study. Global records of *D. immitis* infection in this species are lacking. This is the first report that *D. immitis* naturally infects *Cx. mimulus*.

In addition to the five major infected mosquito species, *D. immitis*-positive pools were also found in samples from the genera *Armigeres*, *Anopheles*, *Culex*, *Mansonia*, and *Uranotaenia*, comprising 10 species across five genera. Among these, natural infections of *An. sinensis* and *Ar. subalbatus* have previously been reported in Gyeonggi and Gangwon Provinces, Korea, and in Kuala Lumpur, Malaysia, respectively (Lee et al., 2007; Cheong et al., 1982). After excluding these two species, this study identified 8 unreported potential vector species for *D. immitis* worldwide. However, the sample sizes of these newly identified infected mosquito species were small. As a result, the estimated infection rates may not accurately reflect the true infection levels in natural populations. The precision of such estimates, particularly those based on pooled sample analysis, is influenced by factors including the number of pools tested and the size of each pool (Gu et al., 2003). Increasing the number of sampled individuals and pools will improve the accuracy and reliability of infection rate estimates and help confirm the vector competence of these species.

The overall infection rate among mosquito species in this study was relatively high (MIR: 6.9%; bias-corrected MLE: 7.6%) compared to the global infection rate of 2.19% (MIR) (Riahi et al., 2021). In Taiwan, the higher infection rate observed in both specific mosquito species and across the overall mosquito population may result from a combination of factors, such as vector and host abundance (especially microfilaremic hosts), vector diversity, vector behavior and

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competence (different genetic strains of mosquitoes), environmental and urbanization influences, methodological differences, and seasonal variation in sampling periods (Ledesma and Harrington, 2011; Brown et al., 2012; Simón et al., 2012; Morchón et al., 2012; Spence Beaulieu et al., 2020).

4.3 Geospatial patterns of *D. immitis* infection in mosquitoes

Because the number of mosquitoes at CMDS was 5–7 times higher than that of TAS and ZOO, although CMDS recorded the highest number of positive pools among the three study sites, CMDS had the lowest overall *D. immitis* infection rate (bias-corrected MLE: 5.5%). Some dogs reared within CMDS and stray dogs nearby were infected with *D. immitis*, which likely served as microfilaremic hosts. TAS had the lowest number of positive pools, possibly due to its well-maintained hygienic environment and the regular administration of preventive medication. Additionally, as TAS is located near CMDS, it is possible that the positive mosquito pools detected in TAS originated from mosquitoes that had taken blood meals from microfilaremic dogs in CMDS. ZOO had the second-highest number of positive pools and the highest infection rate (bias-corrected MLE: 13.6%). Although Taipei Zoo implements regular preventive treatment and health screening for the animals under its care, stray dogs with unknown preventive care are frequently observed in the surrounding areas. Moreover, a previous report published in the *Taipei Zoo Bulletin* indicated that 22 stray dogs within ZOO were examined for *D. immitis* infection, and some tested positive (Lin et al., 1998). Therefore, a more recent investigation is needed to confirm whether stray dogs are still present within ZOO premises and/or adjacent areas and whether they continue to pose a risk as potential reservoirs of *D. immitis*.

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4.4 Temporal variation in mosquito population dynamics and *D. immitis* infection

The number of *D. immitis*-positive pools from June 2023 to May 2024 (n = 71) was substantially higher than that from May 2024 to April 2025 (n = 7). The most likely reason for this marked decline between the two years is a reduction in the number of microfilaremic hosts. According to the caretaker at CMDS, there were some dogs within CMDS and stray dogs nearby infected with *D. immitis* that gradually died throughout 2024, suggesting that the reduction in infected dog hosts may have significantly lowered the transmission potential to mosquitoes. Understandably, the number of positive pools in TAS, which is located near CMDS, also declined during this period. Considering other possible factors, such as environmental conditions including rainfall, relative humidity, and temperature, these remained relatively stable between the two years (Appendix 1). In addition, the population dynamics and abundance of the major infected mosquito species were also consistent, with no significant shifts observed across the two periods. This suggests that the decrease in positive pools is not because of a decline in the abundance of dominant infected mosquito species. On the contrary, an increase in abundance was observed in *Ae. albopictus* and *Cx. tritaeniorhynchus* during the second year, while the collected number of *Cx. pipiens molestus* and *Cx. quinquefasciatus* showed only slight decreases. Hence, the exclusion of these two factors further reinforces the key role of microfilaremic host availability as the primary driver of the observed decline in the number of *D. immitis*-positive pools. However, there is insufficient evidence to explain the sharp decline in the number of positive pools from ZOO in the second year. The most likely explanation for this observation is a decrease in the number of microfilaremic hosts.

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4.5 Estimation of the transmission risk of *D. immitis* by mosquitoes

Temperature and the extrinsic incubation period are key parameters in models predicting the risk and seasonality of vector-borne disease transmission (Ledesma and Harrington, 2015). For *D. immitis*, development within mosquito vectors is commonly estimated using Heartworm Development Units (HDUs), which track the accumulation of daily temperatures above the 14°C threshold required for larval development (Fortin and Slocombe, 1981). Research suggests that approximately 130 HDUs are needed for ingested microfilariae to mature into infective L3 and migrate to the mosquito's head and mouthparts (Fortin and Slocombe, 1981). More models based on HDUs have since been developed. For example, the model uses monthly temperature data to support large-scale regional applications (Cuervo et al., 2015). The other applies finer temporal resolutions by using hourly temperature data (Ledesma and Harrington, 2015). HDU-based models have been widely applied in numerous studies to predict heartworm transmission risk and seasonality across diverse geographic regions (Vezanni, 2006; Huang et al., 2013; Ferreira et al., 2015; Șulești et al., 2016; Younes et al., 2021). This study used HDUs to evaluate the risk and seasonal dynamics of *D. immitis* transmission in Taipei, Taiwan. Calculations of the HDUs at the three study sites from 2023 to 2025 showed that weather conditions in Taipei were suitable for *D. immitis* development from March to November, when the values were above the threshold of 130 HDUs. However, *D. immitis*-positive pools were still detected from December to February. This finding supports the American Heartworm Society's recommendation for year-round administration of preventive medications to reduce the risk of heartworm infection.

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Vector indices and related metrics that integrate both vector abundance and infection rates have been widely used to estimate the transmission risk of various vector-borne diseases. These include tick-borne pathogens such as *Borrelia burgdorferi*, as well as arboviruses like western equine encephalitis virus, St. Louis encephalitis virus, and West Nile virus (Bolling et al., 2009). A vector index (VI) is recognized as a more reliable predictor of disease transmission risk than either vector abundance or infection rate alone (CDC, 2024). This study used the vector index to express the transmission risk of *D. immitis* in Taipei, Taiwan. Vector indices were calculated for each mosquito species collected at three sites, demonstrating the potential risk of exposure to *D. immitis*-infected mosquitoes for both humans and pets. Among the species examined, *Cx. quinquefasciatus* had the highest vector index, followed by *Cx. pipiens molestus* and *Ae. albopictus*. These quantifiable results support the conclusions of previous studies conducted in northern and central Taiwan (Wu et al., 1997; Lai et al., 2000-01), which identified *Cx. quinquefasciatus* and *Ae. albopictus* as two primary natural vectors of *D. immitis*. In particular, *Cx. pipiens molestus*, which had the second highest vector index, warrants further investigation to confirm its vector competence and to assess its potential risk to both humans and pets.

4.6 Applications and future work

In conclusion, this study highlights three key findings. First, it reports the first identification of new potential vector species naturally infected with *D. immitis* in Taipei. Second, the detection of *D. immitis* in mosquitoes from Taipei suggests the presence of a local transmission cycle, marked by low but persistent prevalence. Third, the mosquito population dynamics data provide valuable insights into the

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epidemiology of canine heartworm disease and the ecological interactions between the parasite and its mosquito vectors in Taipei. These findings are essential for designing effective, targeted interventions that reduce the risk of zoonotic disease transmission. Although *D. immitis* is primarily a veterinary concern, human dirofilariasis cases have been increasingly reported worldwide. Between 2000 and 2019, cases were documented in 15 countries across four continents, excluding Africa (Simón et al., 2022). Importantly, six cases have been reported in Taiwan (Tsung and Liu, 2003; Lee et al., 2010; Chang et al., 2011; Hsiao et al., 2014). However, the reported number of cases likely underestimates the true incidence, as human dirofilariasis is often asymptomatic or misdiagnosed as other serious conditions. These trends highlight the blurred boundaries between animal and human health. With global fertility rates declining and an increasing number of households adopting companion animals as family members, the physical and emotional bonds between humans and animals are intensifying. This societal shift emphasizes the growing importance of animal health in safeguarding human health. Furthermore, environmental changes continue to reshape vector and pathogen dynamics, reinforcing the urgency of integrated One Health approaches that address the complex interconnections between human, animal, and environmental health.

Future studies should focus on five parts: first, investigating the vector competence of most of these infected mosquito species in the present study to better understand their roles in *D. immitis* transmission. In fact, several field studies worldwide have analyzed captured mosquito heads and abdomens separately to distinguish between infective and merely infected specimens. However, these studies generally did not find significant differences that would alter the classification of a species as a vector or non-vector for *Dirofilaria* spp. DNA

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(Șulești et al., 2016). Therefore, definitive assessment of vector competence requires controlled infection experiments. To date, only a limited number of mosquito species have been confirmed to be competent vectors through such studies (Konishi, 1989b; Russell and Geary, 1992, 1996; Theis et al., 2000; Lai et al., 2000-01; Tiawsirisup and Nithiuthai, 2006; Montarsi et al., 2015; Silaghi et al., 2017; Spence Beaulieu and Reiskind, 2020). Second, climate change has contributed to the increasing prevalence of *D. immitis*, primarily through the expansion of mosquito vector ranges and accelerated vector and parasite survival and development, and extending transmission seasons (Ledesma, 2011; Morchón, 2012; Simón, 2012; Riahi, 2021). However, the impacts of climate change are complex and, in some cases, may even lead to reductions in transmission (Ledesma and Harrington, 2011). So, predictive models for *D. immitis* prevalence, including those using Heartworm Development Units (HDUs) and analyses correlating meteorological data with heartworm prevalence, need further refinement and validation to improve their accuracy in estimating transmission risk. Third, mosquito diversity has been found to be positively correlated with host heartworm prevalence (Spence Beaulieu et al.,2020). But, in disease ecology, there has been ongoing debate about whether biodiversity reduces infection prevalence, known as the dilution effect, or increases it, referred to as the amplification effect (Luis et al., 2018). Accordingly, understanding how biodiversity influences the dynamics of zoonotic pathogens in wildlife is essential. Fourth, although *D. repens* infection in mosquito species has not yet been investigated in Taiwan, future studies employing molecular techniques to examine *Dirofilaria* spp. vectors should include *D. repens* in their analyses. Finally, DNA of *D. immitis* and *D. repens* has been detected in biting midges, *Culicoides paolae*, and blackflies, *Simulium turgaicum* complex (Khanzadeh et al.,

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2020; Napoli et al., 2022). Consequently, assessing the epidemiological role of blood-feeding arthropods beyond mosquitoes remains important and deserves further investigation to enhance the control of *Dirofilaria* spp. transmission to both human and animal hosts. In summary, addressing these research priorities will deepen our understanding of *Dirofilaria* spp. transmission dynamics and support the development of more effective strategies for surveillance, prevention, and control within both veterinary and public health frameworks.

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Fig. 1. Site 1: Taipei Animal Shelter (TAS)



Fig. 2. Site 2: Cho Mama Dog Shelter (CMDS)



Fig. 3. Site 3: Taipei Zoo (ZOO): Formosan animal area

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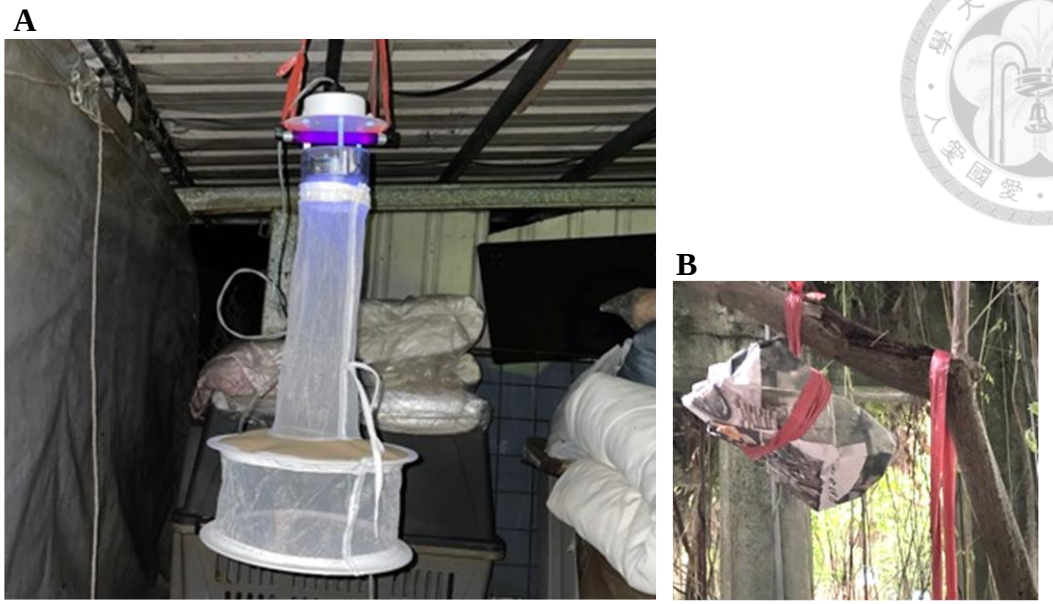


Fig. 4. The mosquito collection tool: (A) CDC trap (UV light + fan), baited with (B) dry ice (CO_2)

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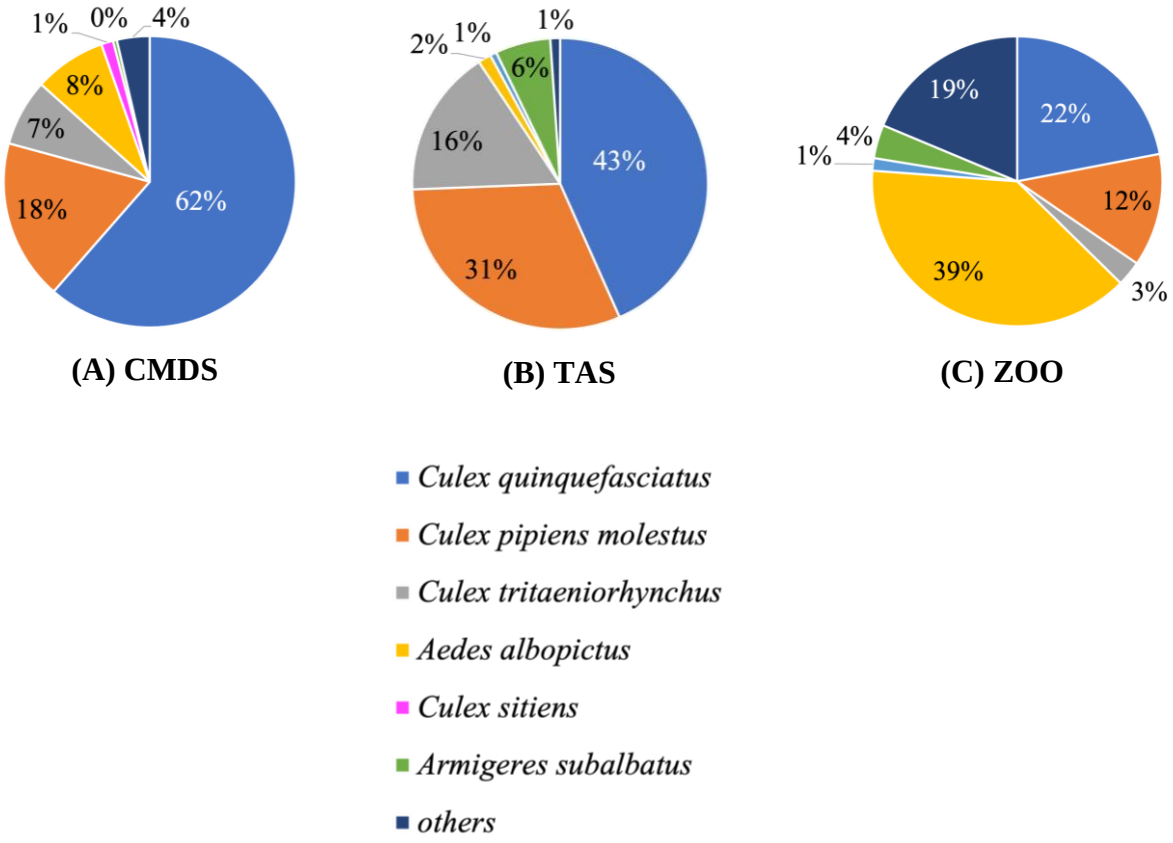


Fig. 5. Relative abundances of mosquito species among individuals caught at three study sites in Taipei, Taiwan: (A) CMDS, (B) TAS, and (C) ZOO

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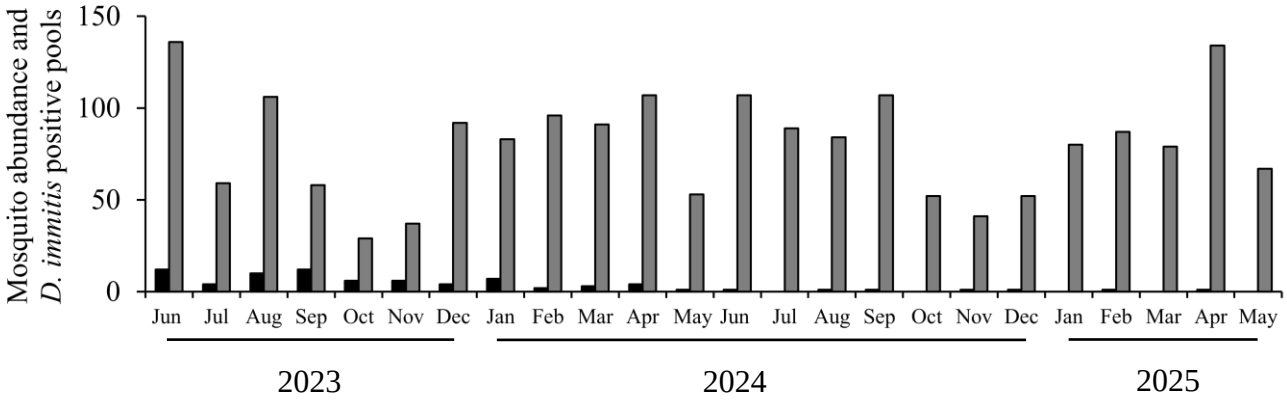


Fig. 6. Temporal dynamics of field-caught female mosquito populations and *Dirofilaria immitis*-positive pools in Taipei, Taiwan. Black columns indicate the number of *D. immitis*-positive mosquito pools, while gray columns indicate the number of mosquitoes collected.

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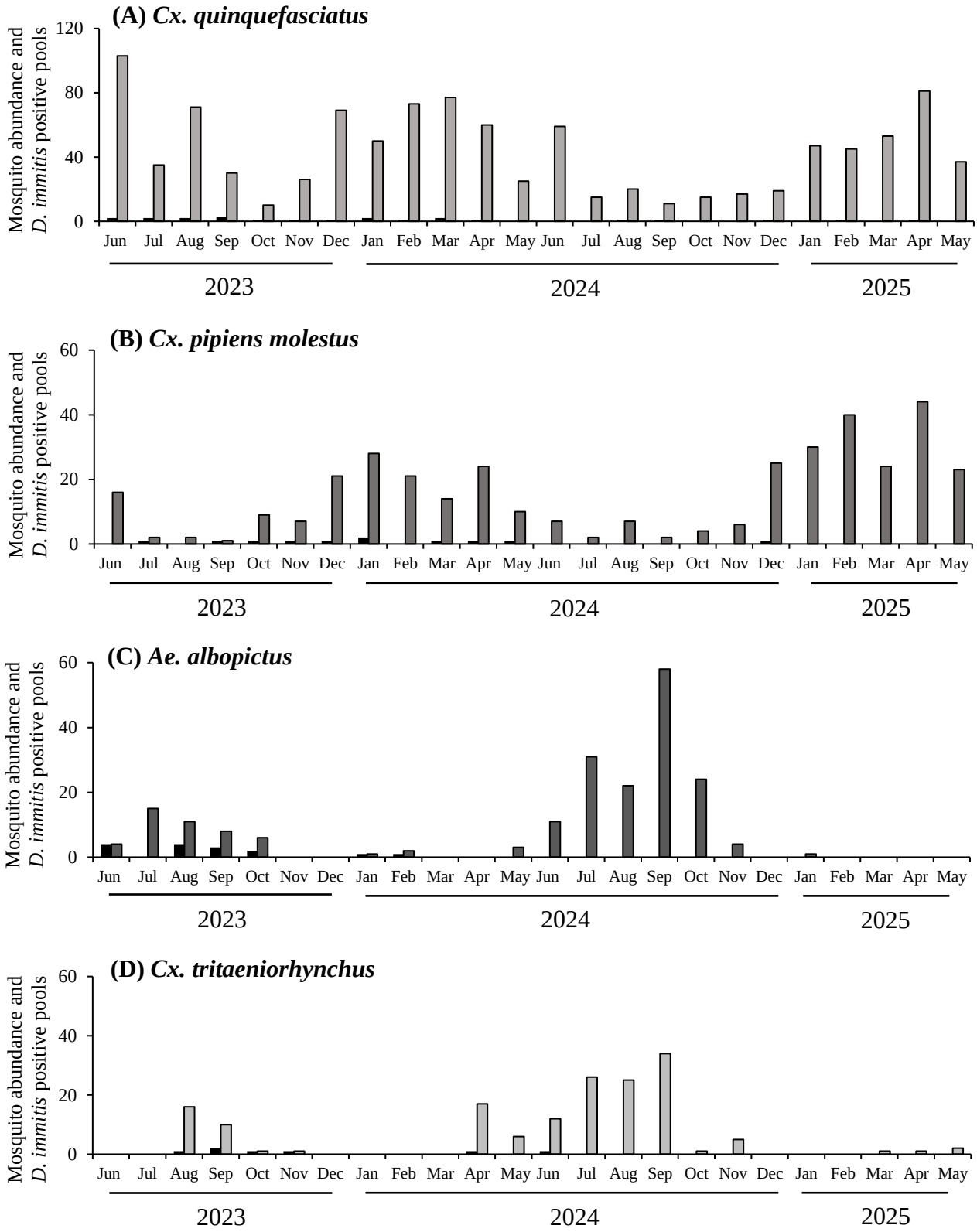


Fig. 7. Temporal dynamics of four most abundant mosquito species and *Dirofilaria immitis*-positive pools in Taipei, Taiwan: (A) *Aedes albopictus*, (B) *Culex pipiens molestus*, (C) *Culex quinquefasciatus*, (D) *Culex tritaeniorhynchus.* Black columns indicate the number of *D. immitis*-positive mosquito pools, while gray columns indicate the number of mosquitoes collected.

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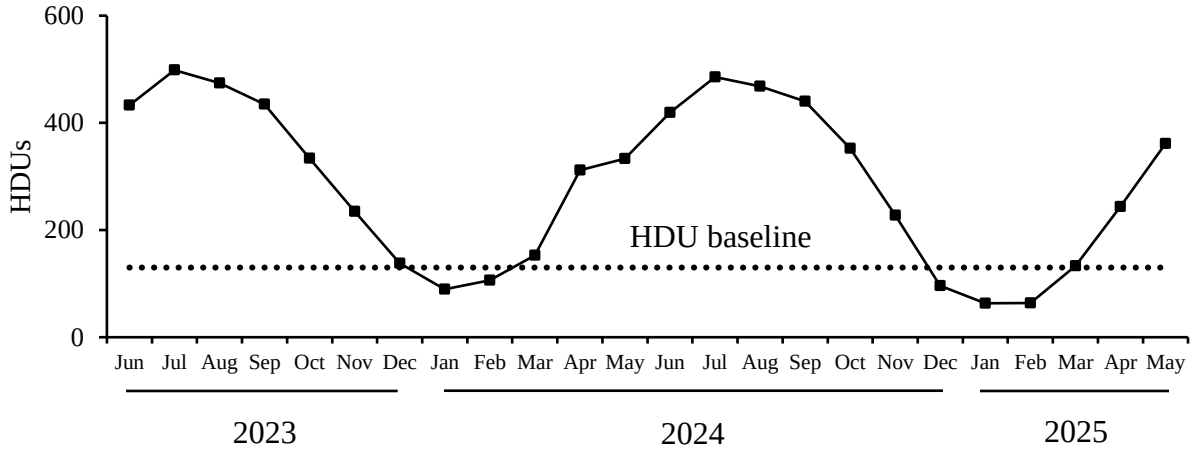


Fig. 8. Heartworm development units (HDUs) in Taipei, Taiwan, during the study period.

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Table 1. Primers used in this study

Target gene	Primer	Primer sequence (5'-3')	Tm (℃)	Amplicon sizes (bp)	Reference
COI	DI_COI-F	AGTGTAGAGGGTCAGCCTGAGTTA	56	203	Rishniw et al., 2006
	DI_COI-R	ACAGGCACTGACAATACCAAT			
COI	DI_COI-Fin	TCAGCCTGAGTTATCTTTGG	50	173	Designed in this study
	DI_COI-Rin	ATAAAAAAGAAGTTAAATAAGAAGTTC			
actin-1	Act-2F	ATGGTCGGYATGGGNCAGAAGGACTC	56	683	Staley et al., 2010
	Act-8R	GATTCCATACCCAGGAAG-GADGG			
COI	MCB-F	GGATTTGGAAATTGATTAGTTCCTT	45, 51	735	Kumar et al., 2007
	MCB-R	AAAAATTTTAATTCCAGTTGGAACAGC			

Table 2. Female mosquitoes caught in Taipei, Taiwan

Mosquito species	CMDS[1]		ZOO[2]		TAS[3]		Total	
	n	**Pools**	***n***	**Pools**	***n***	**Pools**	***n***	**Pools**
Aedes albopictus	114	25	83	15	4	3	201	43
Anopheles sinensis	5	5	0	0	2	1	7	6
An. ludlowae	0	0	1	1	0	0	1	1
An. tessellatus	0	0	1	1	0	0	1	1
Armigeres baisasi	1	1	2	2	0	0	3	3
Ar. omissus	0	0	1	1	0	0	1	1
Ar. subalbatus	6	5	8	5	17	8	31	18
Culex annulus	1	1	1	1	0	0	2	2
Cx. bicornutus	0	0	1	1	0	0	1	1
Cx. bitaeniorhynchus	0	0	6	6	0	0	6	6
Cx. infantulus	0	0	1	1	0	0	1	1
Cx. macrostylus	3	3	0	0	0	0	3	3
Cx. mimulus	16	12	9	9	0	0	25	21
Cx. nigropunctatus	3	3	0	0	0	0	3	3
Cx. pallidothorax	8	8	7	6	0	0	15	14
Cx. pipiens molestus	255	46	27	18	87	27	369	91
Cx. quinquefasciatus	879	72	47	21	122	31	1048	124
Cx. sitiens	19	10	3	3	2	2	24	15
Cx. tritaeniorhynchus	106	22	6	5	46	14	158	41
Malaya genurostris	7	3	0	0	0	0	7	3
Mansonia uniformis	1	1	0	0	0	0	1	1
*Mimomyia fusc*a	0	0	1	1	0	0	1	1
Mi. luzonensis	0	0	1	1	0	0	1	1
Ochlerotatus dorsalis	1	1	0	0	0	0	1	1
Oc. elsiae vicarious	0	0	0	0	1	1	1	1
Oc. japonicus shintienensis	0	0	2	2	0	0	2	2
Uranotaenia annandalei	2	2	1	1	0	0	3	3
Ur. macferlanei	0	0	2	2	0	0	2	2
Ur. nivipleura	0	0	1	1	0	0	1	1
Ur. novobscura	3	3	2	2	0	0	5	5
Ur. yaeyamana	1	1	0	0	0	0	1	1
Total	**1431**	**224**	**214**	**106**	**281**	**87**	**1926**	**417**

[1] CMDS: Cho Mama Dog Shelter, Neihu district, Taipei
[2] ZOO: Taipei Zoo, Wenshan district, Taipei
[3] TAS: Taipei Animal Shelter, Neihu district, Taipei

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Table 3. *Dirofilaria immitis* infection rates in field-caught female mosquitoes from Taipei, Taiwan

Mosquito species	*n*	Pools	Positive pools	MIR% (95% CI)[1]	Bias-corrected MLE% (95% CI)[2]
Aedes albopictus	182	42	15	8.2 (4.2-12.2)	8.4 (5.5-12.4)
Anopheles sinensis	7	6	3	42.9 (6.2-79.5)	46.5 (14.0-83.0)
Armigeres baisasi	3	3	0	0	0 (0-65.8)
Ar. subalbatus	29	16	1	3.4 (0-10.1)	3.4 (0.2-15.0)
Culex annulus	2	2	0	0	0 (0-65.8)
Cx. bitaeniorhynchus	3	3	1	33.3 (0-86.7)	33.3 (2.1-84.9)
Cx. infantulus	1	1	0	0	0 (0-79.3)
Cx. mimulus	19	15	5	26.3 (6.5-46.1)	27.7 (11.2-51.1)
Cx. macrostylus	3	3	0	0	0 (0-56.1)
Cx. nigropunctatus	3	3	0	0	0 (0-56.1)
Cx. pipiens molestus	151	56	11	7.3 (3.1-11.4)	7.6 (4.2-12.5)
Cx. pallidothorax	13	12	3	23.1 (2-46.0)	23.0 (6.6-49.8)
Cx. quinquefasciatus	583	81	23	3.9 (2.4-5.5)	4.5 (3.0-6.5)
Cx. sitiens	9	6	1	11.1 (0-31.6)	10.7 (7.0-41.2)
Cx. tritaeniorhynchus	107	34	7	6.5 (1.9-11.2)	6.8 (3.3-12.5)
Malaya genurostris	3	2	0	0	0 (0-49.9)
Mansonia uniformis	1	1	1	100	-
Mimomyia fusca	1	1	0	0	0 (0-79.3)
Mi. luzonensis	1	1	0	0	0 (0-79.3)
Ochlerotatus dorsalis	1	1	0	0	0 (0-79.3)
Oc. elsiae vicarius	1	1	0	0	0 (0-79.3)
Oc. japonicus shintienensis	2	2	0	0	0 (0-79.3)
Uranotaenia annandalei	3	3	1	33.3 (0-86.7)	33.3 (2.1-84.9)
Ur. macferlanei	2	2	2	100	-
Ur. nivipleura	1	1	1	100	-
Ur. novobscura	5	5	3	60.0 (17.1-102.9)	-
Total	**1136**	**303**	**78**	**6.9 (5.4-8.3)**	**7.6 (6.2-9.1)**

[1] MIR% (95% CI): the number of positive pools / the total number of mosquitoes tested, under the assumption that each positive pool contains only one infected mosquito

[2] Bias-corrected MLE% (95% CI): the recommended calculation with a skewness-corrected confidence interval in the PooledInfRate add-in. Non-overlapping confidence intervals were taken as evidence suggestive of a statistically significant difference.

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Table 4. *Dirofilaria immitis* infection rates at the three study sites in Taipei, Taiwan

Study sites	*n*	Pools	Positive pools	MIR% (95% CI)[1]	Bias-corrected MLE% (95% CI)[2]
CMDS	782	151	39	5.0 (3.5-6.5)	5.5 (4.1-7.3)
ZOO	157	82	21	13.4 (8.1-18.7)	13.6 (9.4-18.9)
TAS	197	70	18	9.1 (5.1-13.2)	10.0 (6.4-14.7)
Total	**1136**	**303**	**78**	**6.9 (5.4-8.3)**	**7.6 (6.2-9.1)**

[1] MIR% (95% CI): the number of positive pools / the total number of mosquitoes tested, under the assumption that each positive pool contains only one infected mosquito

[2] Bias-corrected MLE% (95% CI): the recommended calculation with a skewness-corrected confidence interval in the PooledInfRate add-in. Non-overlapping confidence intervals were taken as evidence suggestive of a statistically significant difference.

Table 5. Vector indices for field-caught female mosquitoes in Taipei, Taiwan

Mosquito species	Average abundance[1]	Infection rate (%)[2]	Vector index[3]
Ae. albopictus	1.3	8.4	0.11
Ar. subaltatus	0.2	3.4	0.01
Cx. mimulus	0.2	27.7	0.05
Cx. pallidothorax	0.1	23.0	0.02
Cx. pipiens molestus	2.4	7.6	0.18
Cx. quinquefasciatus	6.8	4.5	0.31
Cx. sitiens	0.2	10.7	0.02
Cx. tritaeniorhynchus	0.9	6.8	0.07
Total			**0.77**

[1] Mean abundance of each female mosquito species per trap night = (Total number of each female mosquito species collected) / (Number of trap nights)

[2] Derived from the data presented in Table 2 for the estimated infection rate per 100 female mosquito species calculated by using bias-corrected MLE

[3] Vector Index for each mosquito species = (average abundance of each female mosquito species per trap night) x (Estimate for a proportion of each female mosquito species infected with *D. immitis*)

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Appendix

Appendix 1. Summary of monthly average temperature, rainfall, and relative humidity (RH) in Neihu and Wenshan districts, Taipei, Taiwan

District	Meteorological data	Jun	Jul	Aug	Sep	Oct	Nov	Dec	Jan	Feb	Mar	Apr	May
Neihu	Temperature (℃)	28.8±0.3	30.7±0.4	29.9±0.3	29.2±0.1	25.4±0.2	22.1±0.4	18±0.8	16.5±0.6	17±1.4	18.9±0.4	23.7±1.6	25.9±0.9
	Rainfall (mm)	359±48.1	228.3±129.8	288±72.8	308.3±287.4	275.3±233	39.8±37.1	54.8±44.9	32.8±5.3	58.8±33.6	162.3±72.5	195.3±11.7	228.8±162.3
	RH (%)	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Wenshan	Temperature (℃)	27.5±0.1	29.1±0.3	28.5	28.1±0.4	24.8±0.6	21.3±0.1	17.3±0.8	15.5±0.4	16.3±0.9	18.0±0.4	22.8±1.6	24.6±0.4
	Rainfall (mm)	371.3±18.7	268±182.4	307.5±186	300±252.4	337.8±264.8	74±34.6	75.3±52	87±8.5	98±45.3	199.3±103.6	197±5.7	290±147.1
	RH (%)	82.5±0.7	77±2.8	80.5±0.7	78±1.4	81.5±2.1	79.5±3.5	81.5±3.5	76±4.2	81±1.4	78.5±0.7	80.5±3.5	79.5±0.7

1. Meteorological data were obtained from the CODIS database and represent average values during the study period.
2. Data are expressed as mean ± standard deviation (SD).

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