ARTICLES

TEMPERATURE REQUIREMENTS FOR THE DEVELOPMENT OF *DIROFILARIA IMMITIS* IN *AEDES TRISERIATUS* AND *AE. VEXANS*

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ABSTRACT. Development of *Dirofilaria immitis* to the 3rd infective stage in the mouthparts of *Aedes triseriatus* was first noted at constant temperatures of 30, 26, 22 and 18°C after 8, 11, 17 and 29 days post-infection respectively, and in *Ae. vexans* after 12 days at 26°C. In infected *Ae. triseriatus* held at 16, 14 and 12°C only partial or no development occurred. In infected *Ae. triseriatus* held at 16°C for 12 or 22 days development of *D. immitis* to the infective stage occurred when mosquitoes were transferred to 26°C. Pigmental encapsulation of *D. immitis* was observed more frequently in *Ae. vexans* than in *Ae. triseriatus*. Mortality in infected mosquitoes was apparently higher than in unaffected mosquitoes at all temperatures investigated.

INTRODUCTION

Dirofilaria immitis Leidy, the mosquito-borne filarial parasite of dogs has been reported with increasing frequency in dogs in Canada (Slocombe 1978, Slocombe and McMillan 1978). Many of these dogs had not left Canada and the disease is considered enzootic in southwestern Ontario and in the area of Winnipeg, Manitoba.

At least 22 species of mosquitoes reported by Ludlam et al. (1970) as potential vectors of *D. immitis* exist in Ontario (Wood et al. 1979). *Aedes triseriatus* (Say) and *Ae. vexans* (Meigen) which are widespread in southwestern Ontario (Shipp 1976, unpublished data, Wood et al. 1979) are considered capable of supporting development of *D. immitis* to the infective stage (Hu 1931, Yen 1938, Phillips 1939, Bemrick and Sandholm 1966, Intermill 1973, Bickley et al. 1976, Todaro et al. 1977, Arnott and Edman 1978). However the effects of various temperatures on the development of *D. immitis* in *Ae. triseriatus* or the suitability of a Canadian strain of *Ae. vexans* have not been investigated, and we report now some data from such studies.

MATERIALS AND METHODS

Eggs from an Indiana strain (WALTON) of *Ae. triseriatus* were obtained from Dr. George Craig, University of Notre Dame. The eggs were hatched and maintained in nutrient medium as described by Hayes and Morlan (1957). Larvae were maintained at 26 ± 1°C and 80 ± 5% RH with a 12 hr photoperiod. Staple fish food, Tetramin®, was added as food for larvae when required. Adult mosquitoes, upon emergence, were transferred to plexiglass cages (30.5 × 30.5 × 30.5 cm), supplied with distilled water on saturated wicks of paper towelling and 10% sucrose solution on cotton batting. A restrained mouse provided the bloodmeal for the stock colony of *Ae. triseriatus*.

Immediately before exposure of mosquitoes to a dog naturally infected in Ontario with heartworm, 1 ml of blood was withdrawn from the cephalic vein and numbers of microfilariae (mf)/ml was estimated as described by Church et al.

(1976). Mosquitoes 4 to 13 days old were deprived of sucrose for 24 hr and placed in a plexiglass cylinder (7.6 cm diam, 2.4 cm high) which was covered at both ends with cheesecloth. The plexiglass cylinder was held against the shaved side of the dog and removed after the majority of mosquitoes had taken a blood meal (ca. 20 min). All mf counts and mosquito feedings took place between 16:00 and 18:00 hr.

Blood-fed females were returned to the plexiglass cages and supplied with water and 10% sucrose as described previously. The cage was placed in an incubator at predetermined temperatures. For *Ae. triseriatus* these were: 1) constant temperature of 30+1, 26±1, 22±1, 18±2, 16±1, 14±1 or 12±1°C and 2) 16±1°C for 12 or 22 days followed by 26±1°C for 14 and 11 days respectively. *Aedes vexans* were exposed only to a constant temperature of 26°C. *Aedes triseriatus* fed on a dog not infected with *D. immitis* were placed at 30, 26, 22, 18, 14 and 12°C. In all experiments the photoperiod was 12 hr and RH 80±5%.

At various days post-infection (PI) mosquitoes were removed from the incubators and dissected on a glass slide in physiological saline. The gut, Malpighian tubules, thorax, head and mouthparts were examined separately. Nematode larvae found were identified as to stage, using the taxonomic criteria of Taylor (1960) and Iyengar (1957); location in the mosquito and their lengths and widths were measured at 400X. If the nematodes were extremely active, their motility was slowed by gentle heat to facilitate accurate measurement. A linear regression was determined (Snedecor and Cochran 1972) for the relationship between the developmental rate (1/days) of first appearance of infective larvae (y) and temperature (x).

RESULTS

In any one experiment not all 5 tubules were invaded, but in infected tubules the cytoplasm and cell membranes were destroyed and the tubules appeared sac-like containing a few floating nuclei (Fig. 1).

DEVELOPMENT AT 12±1°C AND 14±1°C. In one experiment at 12°C and extending up to day 16 postinfection (PI) approximately 30% of the mf were alive in the midgut and without apparent change in length and width. No larvae were found in the tubules. Mortality in 102 uninfected and 76 infected mosquitoes after 16 days was 95.0 and 97.3% respectively.

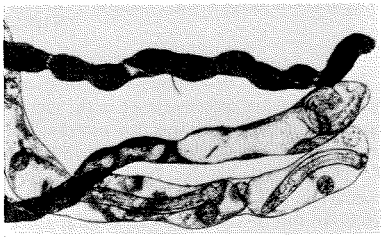

Fig. 1. Malpighian tubules of *Aedes triseriatus* infected with *Dirofilaria immitis*. The tubule at the top of the photograph is uninfected. A first stage larvae is present at the distal end of the middle tubule and the bottom tubule contains second stage larvae. 126 X.

At 14°C in one experiment extending up to day 20 PI the majority of mf remained in the midgut, and at the end of the experiment 50% of the mf were alive. On day 2 PI some L_1s (first stage larvae) were found in the tubules. However, in only one L_1 found on day 20 PI was there some slight thickening. This nematode was 255.8 μ long and 9.8 μ wide. Mortality in 151 uninfected and 78 infected mosquitoes after 20 days was 81.5 and 84.6% respectively.

DEVELOPMENT AT 16±1°C. In one experiment extending up to day 43 PI, 31 L_1s were recovered from 15 of 35 mosquitoes dissected. Details of linear measurements for the L_1s are presented in Fig. 2. First stage larvae were first observed in the tubules on day 2 PI. A slight thickening was observed on day 12 PI but by day 43 no molt had occurred, although some L_1s were in the early sausage stage. Ranges for length and width for L_1s were 135.2–285.4 μ and 6.2–27.1 μ respectively.

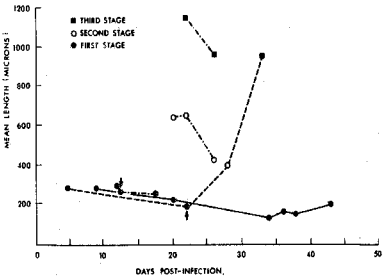

Fig. 2. Length of developmental stages of *Dirofilaria immitis* in *Aedes triseriatus* at 16±1°C and subsequently transferred to 26±1°C. Arrows indicate time of transfer (—.— transfer at Day 12 PI;—transfer at day 22 PI;—no transfer).

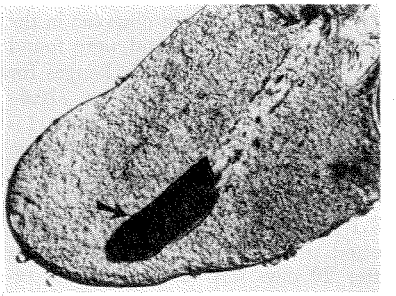

Fig. 4. Pigmental encapsulation at anterior and posterior ends (see arrows) of a *Dirofilaria immitis* 1st stage larva in a Malpighian tubule of *Aedes triseriatus* found on day 28 post-infection at 18°C. 410 X.

DEVELOPMENT AT 18±2°C. In 3 experiments and up to day 45 PI, 269 nematodes were recovered from 89 to 129 mosquitoes. Details of linear measurements of the larval stages are present in Fig. 3. L_1s were first observed in the tubules on day 1 PI, started to shorten and thicken by day 7 PI and were seen in the tubules as late as day 33 PI. Live mf-like L_1s were seen there as late as day 31 PI. Encapsulation of larvae was observed on day 28 PI, and the cuticle of the sausage stage L_1 was coated with a dark brown substance (Fig. 4). Ranges for length and width for L_1s were 152.5–327.2 μ and 6.2–34.4 μ respectively. L_2s were first observed on day 20 PI and were found up to day 45 PI. Ranges for lengths and widths for L_2s were 270.6–787.2 μ and 22.1–41.8 μ respectively. L_3s were observed in the mouthparts from days 29 to 45 PI. Ranges for lengths and widths for L_3s were 708.5–1215.2 μ and 19.7–34.4 μ respectively. Mortality in 94 infected and 62 uninfected mosquitoes after 32 days was 84.6 and 77.4% respectively.

DEVELOPMENT AT 22±1°C. In one experiment extending up to day 20 PI, 140 nematodes were recovered from 37 of 49 mosquitoes. Details of linear measurements for the larval stages are portrayed in Fig. 5. L_1s were first observed in the tubules on day 2 PI and were found there as late as day 12 PI. Ranges for length and width for L_1s were 140.2–307.2 μ and 6.9–30.8 μ re-

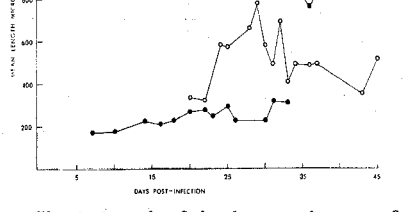

Fig. 3. Length of developmental stages of *Dirofilaris immitis* in *Aedes triseriatus* at 18±2°C.

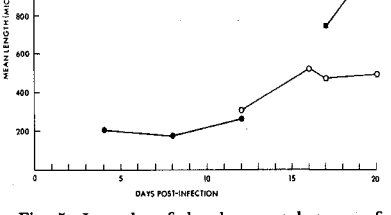

Fig. 5. Lengths of developmental stages of *Dirofilaria immitis* in *Aedes triseriatus* at 22±1°C.

spectively. L_2s were first observed in the tubules on day 12 PI, and were found there as late as day 20 PI. Ranges for length and width for L_2s were 270.6–688.8 μ and 29.5–38.1 μ respectively. L_3s were first observed in the proboscis on day 17 PI. Ranges for length and width for L_3s were 742.9–1195.6 μ and 18.5–27.1 μ respectively. Mortality in 83 infected and 91 uninfected mosquitoes after 20 days was 49.4 and 38.3% respectively.

DEVELOPMENT AT 26±1°C. In 2 replicates and up to day 46 PI, 151 nematodes were recovered from 48 of 61 mosquitoes dissected. Details of linear measurements for the larval stages are presented in Fig. 6. L_1s were observed in the tubules by day 1 PI, had shortened and thickened to the sausage stage by day 3 PI and were observed in the distal end of the tubules up to day 8 PI. Ranges for length and width for L_1s were 147.6–295.2 μ and 9.8–36.1 μ respectively. L_2s were first observed in the tubules on day 8 PI and were found there up to day 18. Ranges for length and width for L_2s were 268.1–735.5 μ and 22.1–36.9 μ respectively. L_3s were observed in the haemocoel, head and mouthparts from day 11 to day 46 PI. No more than 4 nematodes were found in the proboscis at any one time. Ranges for length and width for L_3s were 750.3–1259.5 μ and 18.5–29.5 μ respectively. Mortality in 39 infected and 34 uninfected mosquitoes after 10 days was 51.2% and 26.4% respectively.

DEVELOPMENT AT 30±1°C. In one experiment extending up to day 10 PI, 53 nematodes were recovered from 24 of 37 mosquitoes dissected. Details of linear measurement for the larval stages are presented in Fig. 7. L_1s were observed in the tubules by day 1 PI, and were found in the tubules as late as day 7 PI. Ranges for length and width for L_1s were 159.9–332.1 μ and 5.9–36.9 μ respectively. L_2s were first observed in the tubules on day 7 PI and were found in both the tubules and proboscis from days 8 to 10 PI. Ranges for length and width for L_3s were 750.3–1016.0 μ and 20.9–34.4 μ respectively. Mortality in 59 infected and 41 uninfected mosquitoes after 10 days were 60.7 and 46.3% respectively.

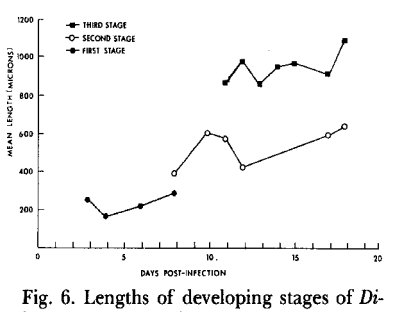

Fig. 6. Lengths of developing stages of *Dirofilaria immitis* in *Aedes triseriatus* at 26±1°C.

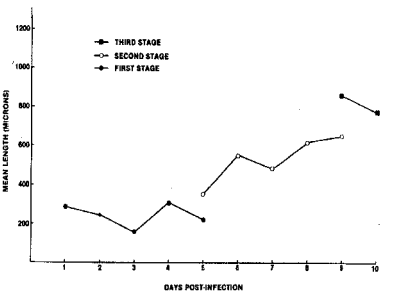

Fig. 7. Lengths of developing stages of *Dirofilaria immitis* in *Aedes triseriatus* at 30±1°C.

STATISTICAL ANALYSIS. The linear regression equation for the relationship between the developmental rate (1/days) of first appearance of L_3s (y) and temperature (x) was $y \neq -0.0078 \times -0.1117$, $r^2 \neq 0.998$ (Fig. 8).

DEVELOPMENT AT 16±1°C FOLLOWED BY 26±1°C. In 2 experiments, 205 nematodes were recovered from 38 of 53 mosquitoes held at 16°C for 12 or 22 days and transferred to 26°C for up to 16 days. Details of linear measurements for the larval stages are presented in Fig. 6. In both experiments some mf had entered the tubules by day 1 PI but remained largely unchanged up to day 12 PI. In mosquitoes held at 16°C for 22 days, 5 1_1s were shortened and thickened. In both

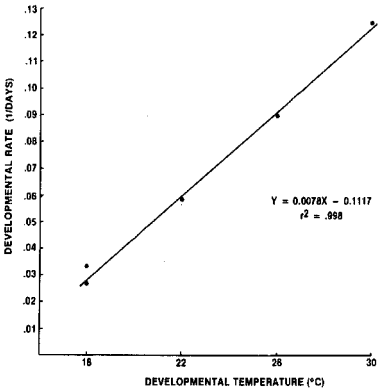

Fig. 8. Rate of development (y) for *Dirofilaria immitis* in *Aedes triseriatus* at different temperatures (x) as determined by first appearance of L_3s in the mouthparts.

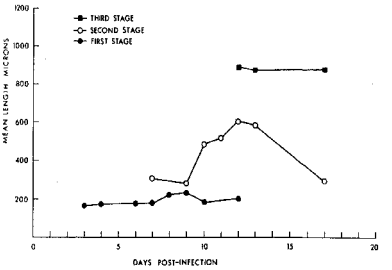

Fig. 9. Lengths of developmental stages of *Dirofilaria immitis* in *Aedes vexans* at 26±1°C.

experiments when mosquitoes were transferred to 26°C, L_1s were found for at least 6 days post-transfer (PT). Ranges for length and width of L_1s from mosquitoes held initially at 16°C for 1) 12 days were 201.7–307.5 μ and 7.4–33.2 μ respectively, 2) 22 days were 152.5–209.1 μ and 9.8–14.8 μ respectively.

L_2s were first found on day 6 and 8 PT for mosquitoes held at 16°C for 22 and 12 days respectively. Ranges for length and width of L_2s from mosquitoes held initially at 16°C for 1) 12 days were 327.2 –725.7 μ and 32.0–40.6 μ respectively, 2) 22 days were 319.8–418.2 μ and 29.0–35.7 μ respectively. L_3s were first observed in the proboscis on day 10 and 11 PT for mosquitoes held at 16°C for 12 and 22 days respectively. Ranges for length and width for L_3s in mosquitoes held initially at 16°C for 1) 12 days were 792.0–1198.9 μ and 19.7–26.6 μ respectively, and 2) 22 days were 846.2–1063.2 μ and 19.7–21.4 μ respectively.

Aedes vexans. DEVELOPMENT AT 26±1°C. In 2 experiments extending up to day 17 PI, 745 nematodes were recovered from 57 of 61 mosquitoes. Details of linear measurements for the larval stages are presented in Figure 9. L_1s were in the tubules by day 1 PI and were found there up to day 12 PI. Encapsulation was common and usually the nematodes were partially encapsulated and active. Ranges in length and width for L_1s were 123.5–279.1 μ and 9.8–41.8 μ respectively. L_2s were first observed on day 7 PI and were found up to day 17 PI. One L_2 was partially encapsulated and active. The encapsulated area was 86 μ in length at the posterior end of the L_2 which was 792.1 μ in length by 32.1 μ in width. Ranges for length and width for L_2s were 246.0–799.5 μ and 17.3–54.1 μ respectively. L_3s were first observed in the mouthparts on day 12 PI and were found up to day 17 PI. Encapsulation of L_3s was not observed. Ranges for length and width were 777.4–993.8 μ and 22.1–54.1 μ respectively.

DISCUSSION

Several epizootiological considerations are involved when assessing vector suitability of mosquitoes. First is the ability of the vector to allow development of the nematode to the infective stage. Kartman (1954) devised an index of experimental infection to determine the efficiency of various mosquito species as vectors of *D. immitis*. His index involved the proportion of L_3s found in the mouthparts of the mosquito compared to the number of microfilariae (mf) ingested. Using this index, Todaro et al. (1977) found *Ae. triseriatus* and *Ae. vexans*

from central New York to be efficient laboratory hosts. In the present studies *Ae. vexans* and *Ae. triseriatus* appeared to be suitable vectors, the latter allowing infections to be maintained even at low temperatures. Whether or not the results for an Indiana strain of *Ae. triseriatus* could be compared to those for an Ontario strain remains to be ascertained.

Secondly, high populations of mosquitoes are necessary to ensure transmission of disease. *Ae. triseriatus* is often a serious pest in wooded areas adjacent to residential areas (Carpenter and LaCasse 1955), and is widespread throughout southwestern Ontario (Shipp 1976, unpublished data). *Ae. vexans* is found in shady wooded areas and in low herbal canopies in urban areas (Horsfall et al. 1973).

Thirdly, longevity of the mosquito is a factor in vector effectiveness. Horsfall et al. (1973) stated that the average life span of adult *Ae. vexans* in nature is 3 to 6 weeks and given appropriate temperatures, this is ample time for transmission of heartworm disease. In the present study, L_3s were observed in *Ae. triseriatus* up to days 45 and 46 PI at 18°C and 26°C respectively. Thus these mosquitoes may harbor developing larvae of *D. immitis* for long periods of time. Fourthly, a mosquito must take more than one blood meal in order to merit consideration as a potential vector and both *Ae. triseriatus* and *Ae. vexans* take more than one blood meal (Horsfall et al. 1973, Beaty and Thompson 1978).

At 12 and 14°C, development of *D. immitis* in *Ae. triseriatus* appeared to be inhibited after several weeks but many mf were still alive. The majority were observed in the midgut and were probably impeded from advancing to the tubules by the solidity of the undigested blood meal. Bemrick et al. (1965) found that mf of *D. immitis* were alive after blood containing the nematodes was frozen for up to 4 months. Therefore, mf may overwinter in hibernating adult mosquitoes, several of which can be found in Canada (Wood et al. 1979) and which have been proven capable of supporting development of *D. immitis* to the 3rd stage (Ludlam et al. 1970).

At 16°C, development of nematodes was minimal. On day 43 PI, they were in an early sausage stage of development similar to that observed after 3 to 4 days in *Ae. triseriatus* and *Ae. vexans* held at 26°C. The probable length of time to development to the L_3 in nature at a mean temperature of 16°C is not compatible with mosquito survival.

At 18°C, L_3s were first observed on day 29 PI. Jankowski and Bickley (1976) observed the time to first appearance of L_3s in *Ae. vexans* maintained at 18°C to be 27 days, and Christensen and Hollander (1978) working with *Ae. trivittatus* at the same temperature found L_3s initially on day 43 PI. The time required for development of *D. immitis* to the L_3 at this temperature is long and only a few mosquitoes would survive to transmit the disease in nature.

At 22°C, L_3s were first observed on day 17 PI. These results agree with those of Kutz and Dobson (1974) and Christensen and Hollander (1978) working with *Anopheles quadrimaculatus* Say and *Ae. trivittatus* (Coq.) respectively. In nature it is not unreasonable to expect a mosquito to live for 17 days, and complete development of *D. immitis* is probable when temperatures fluctuate about a mean of 22°C.

At 26°C, L_3s were observed in the mouthparts of both *Ae. triseriatus* and *Ae. vexans* on day 12 PI. At this temperature, L_3s have been found in the mouthparts of *An. quadrimaculatus* on day 10 PI (Kutz and Dobson 1974), *Ae. trivittatus* on day 13 PI (Christensen and Hollander 1978) and *Ae. vexans* on day 14 PI (Jankowski and Bickley 1976).

At 30°C, development of *D. immitis* proceeded most rapidly and L_3s were observed in the proboscis on day 8 PI. Kutz and Dobson (1974) noted L_3s on day 9 PI in the mouthparts of *An. quadrimaculatus* held at 32.2°C, as did Christensen and Hollander (1978) working with *Ae. trivittatus* held at 30 and 34°C.

At all temperatures under study when L_3s were observed, L_2s were often found concurrently. This asynchrony of development may be important in the field. If an infective mosquito sheds its L_3s while acquiring a blood meal, it will still have the capability to be a vector if its L_2s develop to L_3s prior to the next blood meal.

Although L_1s of *D. immitis* developed only slightly in *Ae. triseriatus* held at 16°C, development to the L_3 occurred when the mosquitoes were transferred to 26°C after being held for 12 and 22 days at 16°C. This suggests an ability of the L_1 to survive long periods in mosquitoes exposed to temperatures of 16°C. However, preliminary work with *D. immitis* in *Ae. triseriatus* at 14°C has suggested that L_1s at the sausage stage level are more susceptible to low temperatures than either ingested mf or L_2s (Fortin and Slocombe 1980).

At all temperatures under study, mortality of infected mosquitoes was higher than the uninfected group in agreement with the results of previous investigations (Kartman 1953, Kershaw et al. 1955; Galliard 1957, Beam 1966, Kutz and Dobson 1974, Christensen 1978). However at 22–30°C a large proportion of infected mosquitoes survived long enough to allow development of infective *D. immitis*. Mosquito survival depends in part upon the magnitude of the parasite burden, and the level of microfilaremia (2789±876 /ml) in the dog used in this study appeared sufficiently low to insure adequate survival of infected mosquitoes.

At 21 and 14°C, mortality was high both in uninfected and infected mosquitoes and was due to low temperatures which inhibit movement and feeding as suggested by Clements (1963). Temperatures of 12 and 14°C are not uncommon in Ontario (Environment Canada 1976–78) and will be a limiting factor in the transmission of the disease. Several days of higher than 37°C may be reached occasionally, but never for prolonged periods (Environment Canada 1976–78). Although such high temperatures may occur, mosquitoes would necessarily rest in some shady place with a lower surrounding ambient temperature.

Pigmental encapsulation of all stages of *D. immitis* was observed more often in *Ae. vexans* than in *Ae. triseriatus*, although not all nematodes in any one mosquito were encapsulated. Salt (1970) suggested that encapsulation and subsequent melanin deposition are effective against alien parasites that threaten the life of the host rather than a habitual parasite such as *D. immitis* that uses the mosquito as an intermediate host. In the present study, encapsulation was more prevalent in those *Ae. vexans* with a high parasite burden. A mechanism for encapsulation may have evolved, therefore, when the parasite burden is detrimental to the life of the mosquito. Completely encapsulated nematodes were dead, and Salt (1970) stated that this may be due to the lack of oxygen within the capsule. Partially encapsulated nematodes were often alive with melanization usually concentrated at either end of the parasite. Oothuman et al. (1974) suggested that stomal or anal secretory products stimulate a defense reaction. In *Ae. triseriatus*, partial encapsulation of nematodes was observed only in those mosquitoes held at 14 and 18°C, and these nematodes were dead.

Christensen and Hollander (1978) predicted rates of development to L_3s in utilizing a regression equation constructed from minimum developmental times to that stage at constant temperatures. The regression equation obtained in the present study for *Ae. triseriatus* was similar, and the developmental rate of *D. immitis* at such temperatures may, therefore, be similar for different species of mosquitoes.

Many other mosquito species are capable of supporting development of *D. immitis* (Ludlam et al. 1970), and several can be found in Ontario (Wood et al. 1979). *Aedes stimulans* (Walker), a spring species, although univoltine, is extremely hardy and may be found late into the summer. *Aedes canadensis* (Theobald) has been used successfully in a dog-to-dog transmission study by Bickley et al. (1977). *Aedes dorsalis*

(Meigen), a multivoltine summer species has a similar breeding habit to *Ae. vexans* and also necessitates consideration as a potential vector.

There is certainly need, therefore, for further study on the development of *D. immitis* in mosquitoes in Canada. It would be of interest to compare survival rates for tropical and temperate strains at low temperatures. It would also be of interest to know the relative importance and potential of available vectors. Only by more rigorous laboratory and field studies will an integrated knowledge evolve concerning the epizootiology of canine dirofilariasis in Canada.

ACKNOWLEDGMENTS

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