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Original Article

Periodicity of *Dirofilaria immitis* in Longterm Infections

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Abstract

The concentration of *Dirofilaria immitis* microfilariae in peripheral blood samples was measured in four experimentally infected dogs. Samples were collected at hourly intervals from 6.30h to 17.30h from all dogs at 11, 22, and 27 months post-infection, and at 39 months post-infection for two dogs only. Microfilarial periodicity follows the form of a simple harmonic wave over a 24h period, and concentration data was fit to sine wave for each sample date to characterize changes in periodicity over time. We found the periodicity index (i.e. wave amplitude) to decrease with time ($p = 0.016$, $R^2 = 0.97$) dropping from 74.57 (95% CI, 63.79 to 85.34) at 11 months post-infection to 5.55 (95% CI, 0 to 14.82) at 39 months post-infection. The time of peak microfilaremia was calculated to be 17.36 h (95% CI, 17.01h to 18.08 h) at 11 months post-infection and did not change significantly with time ($p = 0.17$, $R^2 = 0.70$). No significant trend was observed in total microfilarial count for individual dogs ($p > 0.10$). The data presented here indicate a gradual but significant loss of periodicity over the two-year study period despite maintenance of overall microfilarial levels.

Introduction

The canine heartworm (*Dirofilaria immitis*) is a parasite significant in veterinary medicine for its impact on the health of domestic dogs and cats. Microfilariae (mf) are released by adult female worms in the pulmonary arteries and enter circulation where they become available to the mosquito vector during blood meals. While mf can be found in the blood at all times of the day, periodicity is also observed in which mf concentration rises and falls in a circadian cycle. This phenomenon is well-documented in related filarioid parasites *Wuchereria bancrofti* and *Brugia malayi*, and is noted to coincide with the peak feeding hours of key vector species (Hawking 1967, Abe et al. 2003). Microfilarial periodicity has also been reported in *Dirofilaria repens* naturally infected dogs as well as numerous heartworm infections (Church et al. 1976, Rhee et al. 1998, Ranjbar-Bahadori et al. 2011, Di Cesare et al. 2013), but to our knowledge, no longitudinal studies have been conducted in the canine host. In this study, we measured the microfilaremia of four dogs experimentally infected with *D. immitis* with the aim of characterizing changes in mf periodicity over a two-year period.

Materials and Methods

Study animals and blood sample collection

Four male beagle dogs 16 to 17 weeks of age were infected with *D. immitis* L3 (2005 MO strain) provided by the Filariasis Research Reagent Resource Center (FR3; Athens, GA, USA). Each dog received a subcutaneous injection of 50 L3 in the right medial thigh. Nineteen days post-infection, dogs were transferred to Bayer Animal Health GmbH (Monheim, Germany) where blood samples were collected from all dogs at 11, 22, and 27 months post-infection, and at 39 months post-infection for two dogs only. Blood was collected hourly from 6.30 h to 17.30 h local time. This corresponds with the period during which blood samples are likely to be collected for research or clinical purposes. For each sample, mf were quantified by 10 thick smears of 10 µl each by Giemsa staining and microscopic examination. Thick smears were prepared by mixing 40 µl deionized water and 20 µl heparinized blood on a glass microscope slide, allowing to air dry for at least 24 h, and staining with Giemsa stain (mixed in a 1:9 ratio with TAE buffer) for 30 min. Microfilaria can be morphologically identified as *D. immitis* by their overall length (approximately 300 µm) and width (approximately 6 µm) which distinguishes them from other filarial species including *Dirofilaria repens* (approx. 370 µm x 9 µm), *Acanthocheilonema dracunculoides* (approx. 260 µm x 5 µm), and *Acanthocheilonema reconditum* (approx. 265 µm x 5 µm) (Magnis et al. 2013). Figure 1 shows a representative *D. immitis* mf stained in this manner.

Data analysis

Analyses were performed on corrected mf ratios derived from raw concentrations to reduce the effect of variation in microfilaremia between animals (Sasa and Tanaka 1972). Mean mf concentration for each dog at each date post-infection was calculated. The concentrations from each of the 12 sampling times were then divided by mean mf concentration and multiplied by 100 to obtain mf ratios for each sampling time.

Microfilarial periodicity is assumed to follow a simple harmonic wave pattern as observed by Sasa and Tanaka (1972, 1974), so for each date post-infection mean mf ratios across all sampled dogs were fit to a sine wave equation using GraphPad Prism version 6.01 (GraphPad Software, La Jolla, CA, USA). We constrained the baseline variable to 100, which is the average mf ratio across all sampling times, and constrained the period to 24 h. We used the resulting phase shift value to calculate the time of peak microfilaremia for each sample date. Wave amplitude is equivalent to periodicity index. Linear regression analysis in Prism was performed to assess the change in peak hour and periodicity index over time with a Bonferroni

correction applied to analyses on mean mf concentrations in individual animals.

Results

Mean mf concentrations measured at hourly intervals are shown in Figure 2A, represented as ratios of the mean concentration, which allows averaging among animals. Sine waves were fitted to the mf ratios to calculate periodicity characteristics for each sample date and are shown in Figure 2B. On the first sample date (11 months post-infection) pronounced mf periodicity is exhibited, with a periodicity index (i.e. amplitude) of 74.57 (95 % CI, 63.79 to 85.34) and peak concentration occurring at 17.36 h (95 % CI, 17.01 h to 18.08 h; Table 1). However, the periodicity index calculated by sine wave fitting was found to decrease with time ($p = 0.016$, $R^2 = 0.97$) dropping to 5.55 (95 % CI, 0 to 14.82) by 39 months post-infection. Peak hour did not appear to change significantly with time ($p = 0.17$, $R^2 = 0.70$). While measured mf concentrations were found to vary between individual animals, no significant trend in these levels was observed over the study period ($p > 0.10$; Table 1).

Discussion

An understanding of the periodicity of *D. immitis* mf is useful for the timing of diagnostic sample collection and potentially for the management of transmission in the environment. In the present study, we collected microfilaremia data from experimentally infected dogs at four time intervals over a period of 28 months to characterize the dynamics of longterm infections. Over this period, we observed a significant negative trend in periodicity index with the infection apparently reaching an aperiodic state at the last time point investigated. This trend did not appear to be influenced by changes in total mf counts averaged over each 12 h sample period; while we observed an expectedly high variability between dogs, there was no consistent tendency for total mf levels to rise or fall over the course of this study (Table 1). Furthermore, peak hour did not change significantly with time. To our knowledge, the data from this longitudinal study represent previously unreported findings in *D. immitis* periodicity.

Canine heartworm has long been known to exhibit a circadian periodicity, with peak microfilaremia usually occurring between 16.00 h and 24.00 h,

Table 1 Microfilarial periodicity characteristics at each sample date

Months post-infection		11	22	27	39	p-value	R2
Mean concentration (mf/ml)	Dog 1	21950	45957	62930	51848	0.25	0.56
	Dog 2	6757	14443	46899	44229	0.15	0.72
	Dog 3	405	198	29	ND	0.10	0.98
	Dog 4	4978	3869	14478	ND	0.53	0.45
Peak hour		17.36 h	17.36 h	17.34 h	17.05 h	0.17	0.70
(95% CI)		(17.01 to 18.08 h)	(16.59 to 18.12 h)	(14.52 to 20.20 h)	(10.42 to 23.28 h)		
Periodicity index		74.57	53.00	27.43	5.55	0.016	0.97
(95% CI)		(63.79 to 85.34)	(44.52 to 61.48)	(7.71 to 47.14)	(0.00 to 14.82)		

ND = not done. p-value and R2 represent non-zero slope significance and goodness of fit, respectively, of linear regression across sample dates.

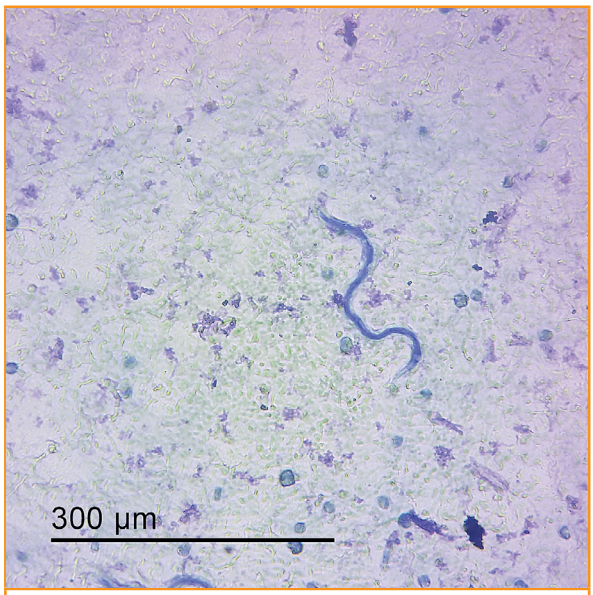

Fig. 1 Giemsa-stained Dirofilaria immitis microfilaria (400X).

seeming to vary with geographic location (Webber and Hawking 1955, Church et al. 1976, Angus 1981, Grieve and Lauria 1983, Rhee et al. 1998, Ranjbar-Bahadori et al. 2011). Because *D. immitis* mf are present in the peripheral blood throughout the day they can be characterized as subperiodic, but due to the variation in peak hour Church et al. (1976) argue that *D. immitis* cannot be accurately categorized as nocturnal or diurnal. A seasonal periodicity has also been observed with peak levels occurring in the summer months (Kume 1975, Sawyer 1975).

While it has long been observed that mf migrate from the peripheral circulation to the capillaries of the lungs, thus accounting for the observed changes in concentration, the mechanisms of periodicity remain poorly understood. The findings of basic research into the mechanisms of filarial periodicity have been extensively reviewed and predominantly indicate that one or multiple host factors are responsible for this phenomenon (Hawking 1967, Masuya 1976, Aoki et al. 2011). The timing of peak mf levels appears to coincide with peak feeding hours of the primary vector species and is most likely an adaptation to maximize uptake and transmission by the intermediate host (Hawking 1967, Tolbert and Johnson 1982, Konishi 1989).

Some of the natural factors influencing periodicity are absent for laboratory animals. The dogs examined in this study remained in temperature-controlled housing with a constant light/dark cycle, presumably insulating them from the effects of seasonal changes. Furthermore, the *D. immitis* strain we used had been maintained in a laboratory setting for 14 years at the time of infection. It is easy to conceive of how differences between natural and induced heartworm infections factor into parasite behavior, and the pronounced decline in periodicity index (i.e. loss of periodicity) may be a result of these differences in conditions. Further longterm studies, ideally comparing infections with multiple strains, are necessary to gain a clearer perspective on the data presented here and may be useful in identifying key host or parasite factors affecting periodicity.

Ethical approval

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All procedures performed in studies involving animals were in accordance with the ethical standards of the institutions at which the studies were conducted.

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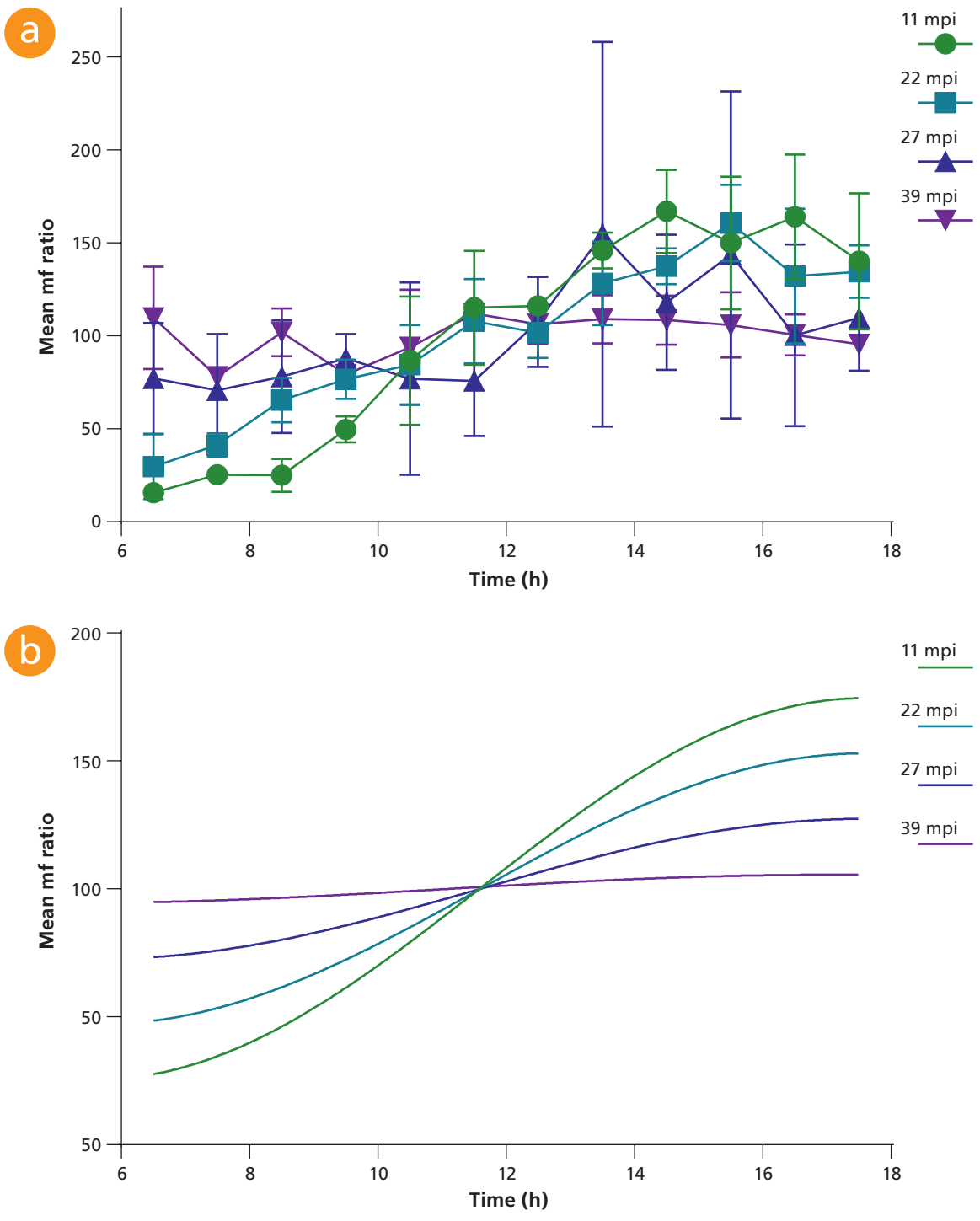

Fig. 2 Observed and calculated microfilarial periodicity at hourly intervals
a: Observed mf ratios averaged between dogs for each time point.
b: Sine waves derived from mf ratios used to calculate periodicity index and peak hour.

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