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## Periodicity of *Dirofilaria immitis* microfilariae in canine and murine hosts

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### Summary

Microfilariae of *Dirofilaria immitis* were recovered from an experimentally infected dog and inoculated intravenously into adult female BALB/c mice. Each mouse received  $1 \times 10^5$  microfilariae. In one experiment mice were sublethally irradiated with gamma radiation 24 h prior to microfilarial inoculation; microfilariae were counted at 11.00 and 23.00 h 7, 14, 21, 28, 35 and 42 days after inoculation. A similar experiment was performed in nonirradiated mice to ensure that sublethal irradiation had not caused microfilarial periodicity. In a third experiment microfilaria numbers were determined in sublethally irradiated mice at 4-h intervals for 48 h beginning 14 days after microfilarial inoculation; microfilaria numbers at each bleeding were compared to microfilarial density at the same time in the donor dog. In a fourth experiment 2 groups of mice were irradiated, inoculated and bled as before; one group was acclimated to light from 07.00 h to 19.00 h, whereas the other group was acclimated to light from 19.00 h to 07.00 h. The microfilaremia was subperiodic, varying regularly with time in both the dog and mice. Microfilariae in the dog reached maximum numbers during light hours. In each experiment, regardless of actual time, microfilariae in mice reached maximum numbers during dark hours.

**Key words:** *Dirofilaria immitis*; microfilaria; periodicity.

### Introduction

Microfilarial periodicity is observed in many filarial infections. Periodicity is defined as a cyclical rise and fall in microfilaria numbers over time, following the pattern of a simple harmonic wave (Sasa and Tanaka, 1972). It can be fur-

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ther classified as nocturnal or diurnal depending upon what time of day maximum numbers of microfilariae (mff) are present in peripheral blood. *D. immitis* mff exhibit a subperiodicity, where the wave pattern is present but mff are never completely absent from peripheral blood. *D. immitis* microfilaremiias have been described as subperiodic without clear nocturnal or diurnal peaks (Church et al., 1976). The physiologic basis for periodicity is unknown; however, work of several investigators [e. g. Hawking (1967), Eberhard and Rabalais (1976), and Zielke (1980)] suggests that one or a variety of host factors influence the periodic trend of some microfilaremiias.

In the present study, *D. immitis* mff were transferred from an infected dog to sublethally irradiated or nonirradiated BALB/c mice to study the microfilaremia in an alternate mammalian host. Microfilaremiias in both the mice and dog were subperiodic; however, in different host species mff reached maximum numbers at different times of the day. The subperiodicity observed in mice was independent of sublethal irradiation. Further, the periodicity could be altered in mice by changing their light cycle.

### Materials and Methods

Blood was collected by venipuncture into a heparinized syringe from an experimentally infected dog containing approximately  $1 \times 10^5$  *D. immitis* mff/ml blood. The dog was housed in an individual cage in a temperature- and humidity-controlled environment on a 07.00 h to 19.00 h light cycle and provided with daily laboratory ration and water ad libitum. Microfilariae were isolated from blood by a modification of the method of Sawyer and Weinstein (1963). Microfilaremic blood was diluted (1:11) in 0.2% Saponin with 0.85% NaCl in distilled water. This mixture was incubated at 37°C for 15 min and centrifuged at  $900 \times g$  for 5 min. The resulting pellet was washed twice with 0.01 M phosphate buffered saline (PBS) pH 7.2 and resuspended in 5 ml 0.04% DNase (Sigma DN-100, Sigma Chemicals Co., St. Louis, MO, USA) in PBS and gently agitated until a homogenous preparation of mff was observed. DNase was removed by centrifugation and the pellet was washed once with PBS.

In the first experiment 6 female BALB/c mice (Charles River Breeding Laboratories, Wilmington, MA, USA) aged 6 to 8 weeks were irradiated with 550 R  $\gamma$ -radiation from a Cesium-137 source 24 h prior to microfilarial inoculation. Individual mice were inoculated intravenously with  $1 \times 10^5$  mff in 0.3 ml PBS. The mice were maintained on a 07.00 h to 19.00 h light cycle and provided with laboratory ration and water ad libitum. Blood was collected at 11.00 h and 23.00 h from the retro-orbital plexus into heparinized capillary tubes 7, 14, 21, 28, 35 and 42 days after inoculation. Individual blood samples were mixed with 1.0 ml 2% formalin and centrifuged at  $6000 \times g$  for 1 min. The resultant pellet was stained with methylene blue and mff were enumerated.

A second experiment was designed to insure that pre-inoculation irradiation had not caused microfilarial periodicity. Six nonirradiated BALB/c mice maintained on a 07.00 h to 19.00 h light cycle were inoculated with mff as before. Blood samples were collected at 11.00 h and 23.00 h on days 7, 14, 21 and 28 after inoculation and mff were counted.

In a third experiment daily fluctuations of microfilaria numbers in sublethally irradiated female BALB/c mice were compared to the microfilaremia in the source dog. The dog and mice were maintained on a 07.00 h to 19.00 h light cycle. Eleven mice were inoculated as before and divided into groups of 5 and 6 mice. Blood collection was alternated between groups every four hours over a 48-h period of time to minimize trauma. In preliminary experiments intragroup variance in microfilarial numbers in mice was minimal two weeks after inoculation (Grieve, unpublished data); therefore, blood collection was initiated 14 days after inoculation. Each time blood was

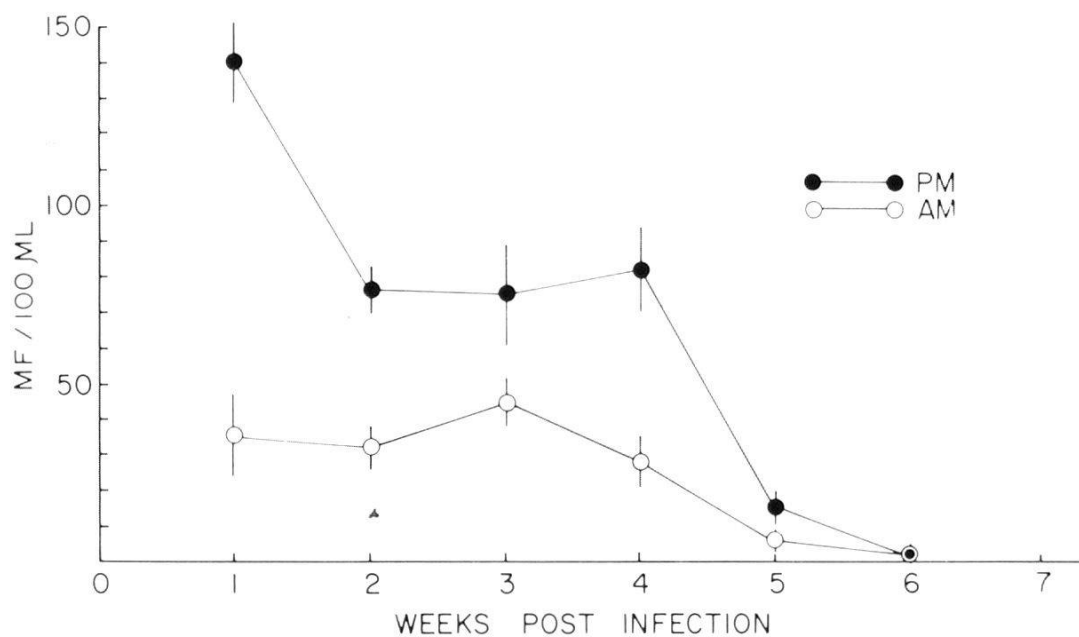


Fig. 1. *D. immitis* microfilaria numbers in sublethally irradiated BALB/c mice. AM and PM values were determined at 11.00 h and 23.00 h, respectively. Vertical bars represent the standard error of the mean.

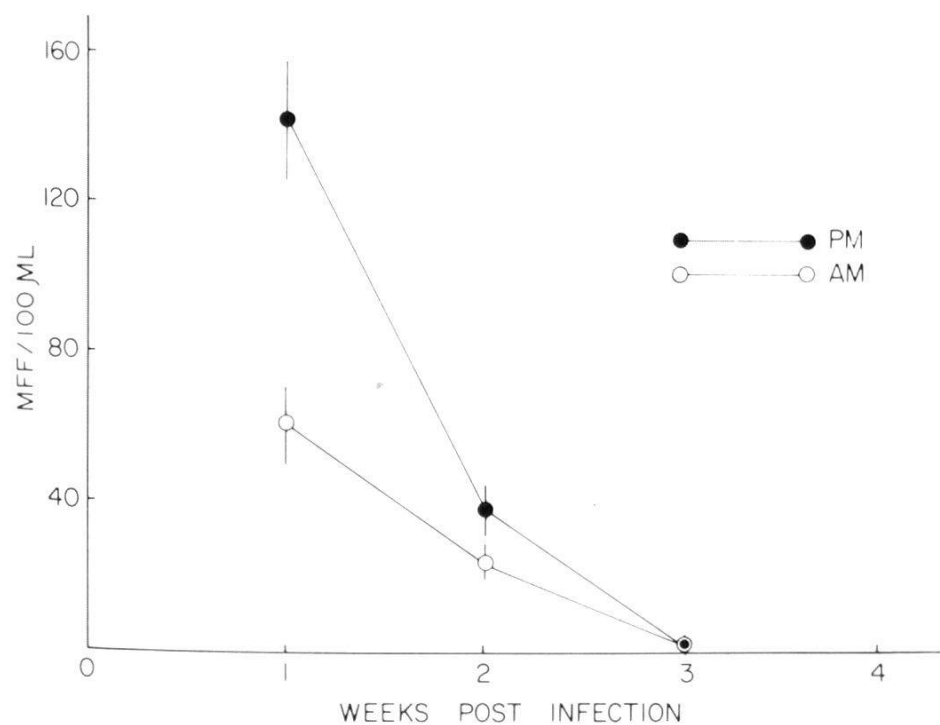


Fig. 2. *D. immitis* microfilaria numbers in nonirradiated BALB/c mice. AM and PM values were determined at 11.00 h and 23.00 h, respectively. Vertical bars represent the standard error of the mean.

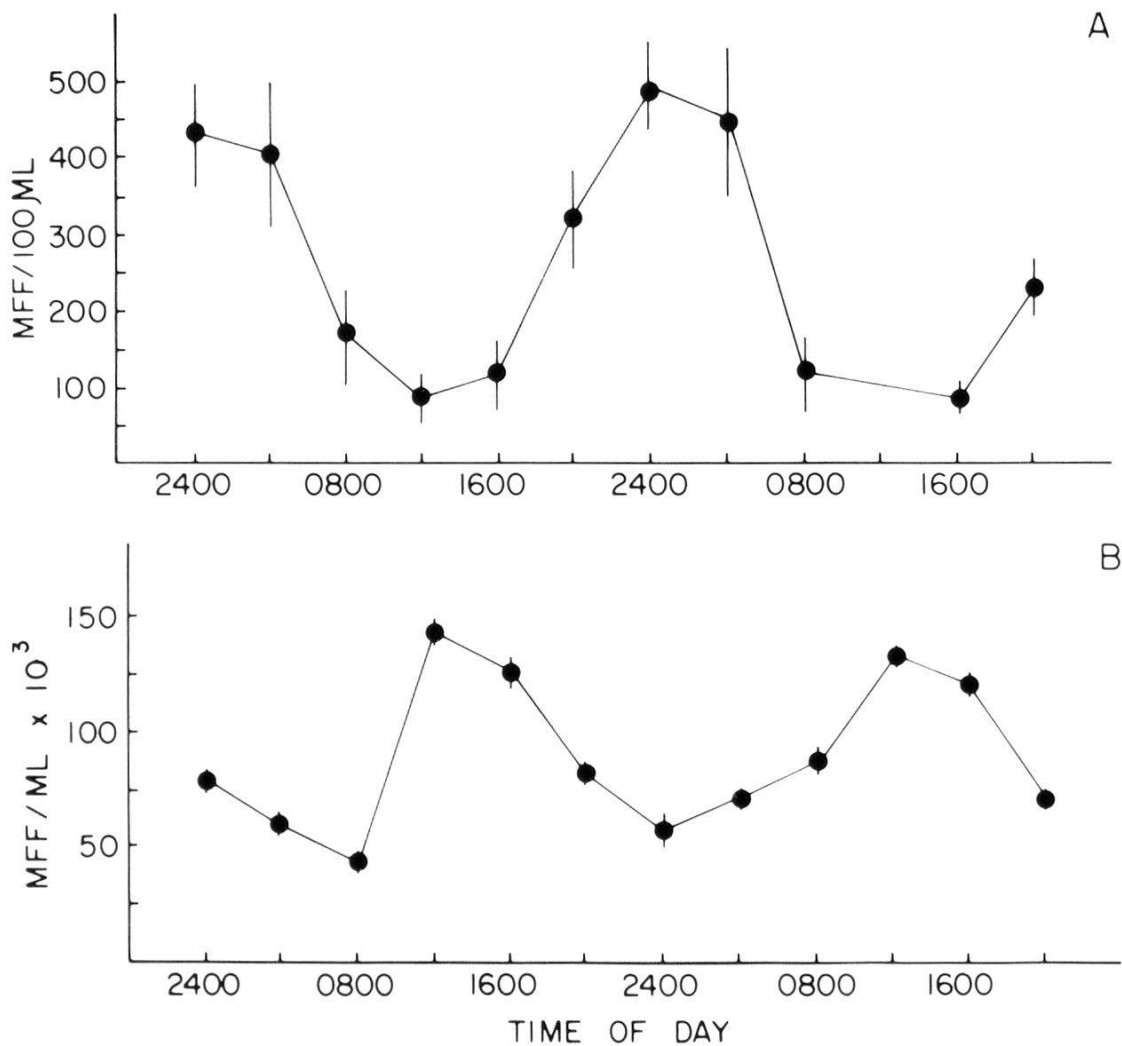


Fig. 3. *D. immitis* microfilaria numbers beginning 14 days after inoculation of microfilariae into mice. A = microfilaria numbers in sublethally irradiated mice. B = microfilaria numbers in the microfilaria donor dog. Vertical bars represent the standard error of the mean.

collected from mice one ml of blood was collected by venipuncture from the jugular vein of the source dog. Canine blood samples were processed by a modified Knott's method (Weiner and Bradley, 1970); three replicate counts were determined from each sample.

In a fourth experiment two groups of BALB/c mice were placed on inverted light cycles. Group A consisted of 7 mice maintained on a 07.00 h to 19.00 h light cycle and Group B consisted of 7 mice on a 19.00 h to 07.00 h light cycle. Mice in Group B were housed in a continuously ventilated light-proof incubator at room temperature. The incubator was equipped with a fluorescent light connected to a timer. Groups A and B were acclimated to their respective environments 14 days prior to inoculation with mff. Blood collection began 14 days after inoculation and mff numbers were determined at 4-h intervals over a 48-h period of time.

## Results

The results of the first experiment are illustrated in Fig. 1. The microfilaraemia was nocturnally subperiodic and lasted 6 weeks. Although the microfilara-

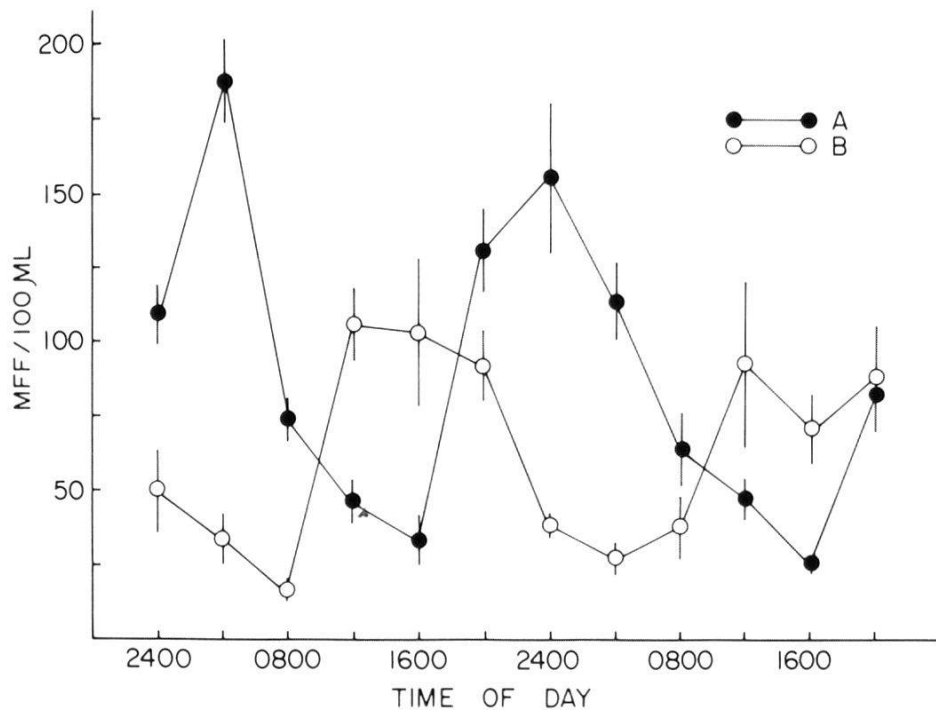


Fig. 4. *D. immitis* microfilaria numbers in sublethally irradiated BALB/c mice beginning 14 days after microfilarial inoculation. A = mice acclimated to light from 07.00 h to 19.00 h. B = mice acclimated to light from 19.00 h to 07.00 h. Vertical bars represent the standard error of the mean.

mia did not persist as long in nonirradiated mice, a nocturnal subperiodicity was again evident (Fig. 2). When blood samples were collected from mice and from the microfilaria-source dog at 4-h intervals the microfilaremia in mice was nocturnally subperiodic with maximum microfilaria numbers appearing between 20.00 h and 04.00 h (Fig. 3). This differed markedly with the microfilaremia in the dog which was diurnally subperiodic with maximum microfilaria numbers between 12.00 h and 16.00 h.

Inverting the light cycle of Group B in the fourth experiment affected the microfilarial periodicity (Fig. 4). Each group of mice displayed the highest numbers of circulating mff during the hours of darkness, regardless of the actual time of day.

## Discussion

The subperiodic *D. immitis* microfilaremia observed in dogs is reproducible in BALB/c mice. Microfilaremias were subperiodic in both sublethally irradiated and nonirradiated mice for at least 4 weeks and 2 weeks following microfilarial infection, respectively (Figs. 1 and 2). When mff had nearly disappeared from peripheral circulation, microfilarial numbers were inadequate to compare results between 11.00 h and 23.00 h blood collections. Although mff and the corresponding subperiodic microfilaremia persisted longer in sublethally irradiated mice, it is clear that subperiodicity was independent of irradiation (Fig. 2).

Microfilaremiias were subperiodic in the source dog and the mice; however, maximum and minimum numbers of mff were observed at different times in the different host species (Fig. 3). The microfilaremia in the source dog was diurnally subperiodic whereas the microfilaremia in mice was nocturnally subperiodic. The diurnal activity of the source dog was in contrast with the nocturnal activity of mice. To determine the effect of host activity on microfilaremia, mice were acclimated to a light cycle which was 12 h opposite the cycle of control mice; the microfilaremia changed accordingly (Fig. 4).

Church et al. (1976) reported that *D. immitis* microfilaremia is subperiodic, but cannot be categorized as diurnal or nocturnal because of the variation in the time of peak microfilarial density. This variation was observed between dogs and within individual dogs maintained under different living conditions. It was suggested that emotional or environmental stresses may affect the hour of maximum microfilaria numbers (Church et al., 1976). These observations are consistent with the contrast in time in peak microfilaria numbers between mice and the source dog. Different circadian rhythms in the two species may account for the differences in the time of peak microfilarial density. The hypothesis of Church et al. (1976) is also supported by the fact that the time of peak microfilarial density in mice could be altered by altering the light cycle.

Zielke (1980) inoculated *D. immitis* mff into multimammate rats, mice and one jird. Microfilariae were present in peripheral blood up to 17 days after infection. In multimammate rats and mice for 3 and 5 days, respectively, mff demonstrated the same periodicity as in the source dog. However, after 5 days the time of peak microfilaria numbers in rodents corresponded to minimum microfilaria numbers in the source dog. The 3- to 5-day period for mff to acclimate to the new host species suggests that mff are directly involved in periodicity. In the present investigation, microfilaria counts at 4-h intervals were not measured until 14 days after inoculation because preliminary experiments revealed a maximum intragroup variance in microfilaria numbers within the first week after inoculation (Grieve, unpublished data). It is likely that this variance is due to the acclimation of mff to the new host species, as reported by Zielke (1980).

The influence of different animal hosts on microfilarial periodicity has also been reported in *Brugia malayi* infections (Laing, 1961). When subperiodic *B. malayi* infections were transmitted from man to cats, the ensuing microfilaremia remained subperiodic. When the same infection was transmitted to monkeys, however, the microfilaremia was nocturnally periodic.

The reproducibility of *D. immitis* subperiodic microfilaremia in inbred mice encourages the use of this model to elucidate the mechanisms of microfilarial periodicity. In addition, this model could be used in studies on immunity to mff and as a primary in vivo screen for microfilaricidal drugs. A similar mouse model for *B. malayi* mff has been developed (Grove et al., 1979) and has been used to demonstrate the role of antibody in microfilarial clearance

(Thompson et al., 1981) and in the in vivo effect of diethylcarbamazine (Neill and Kazura, 1979).

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