



Infrared light photobiostimulation mediates periodicity in *Dirofilaria immitis* microfilariae

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ABSTRACT. Microfilariae (Mfs) of filarial nematode parasites exhibit nocturnal periodicity, with their numbers in peripheral blood peaking at night and decreasing during the day. However, the reason for their appearance at night remains unknown. In this study, *in vitro* photobiostimulation experiments showed that Mfs exhibited positive phototaxis toward infrared light with lower photon flux densities of infrared light at wavelengths of 890 and 700 nm, in particular, mediating paradoxically higher velocity than intense ones. Microarray analysis revealed that infrared light stimulation influenced gene expression in Mfs and induced significant upregulation of genes, with phosphorylation- and neurogenesis-related genes being highly enriched. Weaker natural infrared beams from the atmosphere only at midnight may induce microfilaria periodicity, and the nature of the periodic pattern is innate and plastic, as demonstrated by artificially changing the light-dark cycle. This is the first report of positive phototaxis toward infrared light in *Dirofilaria immitis* Mfs. The notable finding is that they moved in union despite the lack of a fluid current inside the container, indicating that infrared light appears to control nocturnal periodicity in *D. immitis* Mfs. The newly developed culture medium and the adoption of charge-coupled device (CCD) camera and time-lapse VHS videocassette recorder used in this study made possible to be a long observation.

KEY WORDS: *Dirofilaria immitis*, infrared light, microfilaria, nocturnal periodicity, phototaxis

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Microfilariae (Mfs), as the first-stage larva of filarial nematode parasites, have periodicity as a circadian rhythm. Filarial nematodes, such as *Wuchereria bancrofti*, which causes human elephantiasis, and *Dirofilaria immitis*, which causes canine heartworm disease, invade the lymphatic vessels and pulmonary arteries of the host and reproduce, after which a high number of Mfs enter the host's blood circulatory system and exhibit nocturnal periodicity, peaking at night and decreasing during the day.

Several hypotheses have been retrospectively proposed to explain the mechanism underlying this periodicity, including control by the host's metabolic rhythm; control by periodic production of Mfs by females; immunological destruction of Mfs by the reticulo-endothelial system of the host; and synchronization of Mf movement with the blood-feeding behavior of mosquitoes, which becomes active at night. These hypotheses have been noted [9], but none have been proved. There is little published information on the nature of periodicity.

The purpose of this study was to establish an *in vitro* cultivation manner and monitor for a long period the phototactic behavior of Mfs with high viability; to record their phototactic behavior against photobiostimulation with certain wavelengths among visible and infrared beams, with additional support from microarray analysis to gain a better understanding of the mechanism involved; and to test whether the periodicity pattern is reversible by artificially changing the light-dark cycle.

MATERIALS AND METHODS

Mf periodicity in the peripheral circulation of a dog

Peripheral blood samples (20 μ l) were obtained from a dog with microfilaremia that had been naturally infected with *D. immitis* (a 5-year-old male German pointer) every hour for 1 week. Each sample was stained with methylene blue, and the number of Mfs was microscopically counted. This study was approved by the institutional animal research committee at Yamaguchi University.

Permeability of the dog's skin to light waves

A spectroradiometer (URE-30, USHIO Electric Co., Ltd., Tokyo, Japan) was used to measure the radiant illumination passing through the dog's skin. The skin-piece used in this experiment was obtained from thoracic part of short-haired mixed breed dog

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about 20 kg body weight and tested after shaving. Wavelengths exceeding 1,000 nm were omitted from the analysis because heat-taxis by heat rays was associated with phototaxis.

Recovery of Mfs

Mfs were isolated from a dog with microfilaremia (an approximately 6-year-old male mongrel weighing 21 kg) that had been naturally infected with *D. immitis* using a previously documented procedure [5]. Briefly, the sediment as an isolated Mf mass was suspended in a sterilized disposable plastic container (5.3 × 3.5 × 2.2 cm; 44 ml in volume) containing culture medium comprising 36 ml of 8.5% saline, 4 ml of normal dog serum, and 4 ml of phosphate buffered saline (PBS; pH 7.2). Then, the container was tightly sealed under sterile conditions using Parafilm to avoid contamination with bubbles and leakage of the medium. Mfs (approximately 7.5×10^4) that were suspended in the culture medium could survive for at least 1 month when incubated at 20–21°C in the dark (viability=100%); however, they died within 1 week at 25°C and within a few days at 37°C.

Phototactic behavior of Mfs

Just before the test, the container was gently shaken once to equalize the suspension and was then placed under an inverted microscope in a dark room and exposed to light. In Experiment 1, a 90-W white light bulb (silicon white bulb; HITACHI Co., Ltd., Tokyo, Japan) was placed 50 cm and at a 45° angle in incidence from the upper left side of the container to provide 345-lux illumination (LX-1330 digital lux meter; GUSTON, Tokyo, Japan); in Experiment 2, a 90-W white light bulb was used at a similar setting and in the same position as that used in Experiment 1, and after finishing the white light test, immediately switched on a 740 nm infrared fluorescent lamp also positioned 30 cm and at a 45° angle in incidence from the upper right side of the container, although the lux output of this was not determined. Experiments 1 and 2 were conducted with a different container of Mf suspension arranged in the same preparation manner. The movements of the Mfs were recorded using a bio-microscope attached to a charge-coupled device (CCD) camera and time-lapse VHS videocassette recorder.

Velocity of phototactic movements in response to different light sources

Velocities of phototactic movements of Mfs in response to light stimulation at wavelengths ranging from 1,200 to 380 nm were compared using a shell-type light-emitting diode (LED). The light source circuit comprised an LED, a dry-cell battery (1.5 or 3 V), and current regulative diode resistors. A quantum meter combined with a silicon diode sensor (QTM-101, Monotech Inc. Saitama, Japan) was used to measure the photon flux density of the LED. Phototaxis of the Mfs was assessed at three photon flux densities per wavelength, representing levels of 100%, 10% and 1% in transparency, using decrement filters to achieve the latter two levels and these substantial level of $\mu\text{mol}/\text{m}^2/\text{sec}$ in reduced photon flux density of the LED were determined with quantum meter. One test container with the suspension of Mfs was used within 1 week because of higher viability, and in the next week, a container newly prepared using the same method was used in next determination. The Mfs were usually recovered from the same dog with microfilaremia described above.

In each test, movements were monitored until the Mfs had moved from one side of the container to the other along the major axis. Each test was generally performed in triplicate; however, the number of tests ranged from one to four depending on the accuracy of data. In all of the tests, background lighting was provided by a 535 nm LED because visible light is required for recording data onto videocassette tapes, and it has been previously confirmed that Mfs are not sensitive to light at this wavelength, meaning that it would have no substantial effect on their phototactic behavior (data not shown).

Infrared light-induced gene expression in Mfs

Mfs were purified using the method described previously [5] and divided into two groups. One group was exposed overnight to 940 nm infrared light ($226.7 \mu\text{mol}/\text{m}^2/\text{sec}$ supplied by three LEDs in a parallel circuit), whereas the other was incubated in the dark as a control. Then, all of the samples were frozen in liquid nitrogen and sent to FILGEN Inc. (Division Bio-Science, Nagoya, Japan, <http://www.geneontology.org/>) for total RNA isolation and expression analysis using microarray technology. A microarray based on the model organism *Caenorhabditis elegans*, which is currently used for genetic analyses of *D. immitis*, was used to provide evidence of responsiveness to infrared light. Functional analyses were also performed via gene annotation using MicroArray Data Analysis Tool Ver. 3.2 with GO to better understand the biological meaning of the microarray results, i.e., the genes that were upregulated by infrared light.

Natural light waves from the atmosphere

Natural light waves passing through an open window in the range of 1,000–300 nm were monitored every 30 min over a 48-hr period using Sun Spectroradiometer S-2440 (HIDAMARI Mini), SOMA OPTICS Ltd., Tokyo, Japan) combined with analytic software.

Artificial changes to the periodic pattern

A dog with microfilaremia, the same one used for recovery of Mfs, who had *D. immitis* infection was reared in an experimental animal room with open windows and was given access to commercial food once per day and tap water *ad libitum* for >3 years. The number of Mfs was counted in 20 μl of peripheral blood every hour for a 24-hr period to check the periodic pattern under normal (control) conditions before starting the Experiment (day 0). Then, the cycle of light and dark exposure was reversed for 7 days by completely covering the windows of the animal room to block out sunlight and using an automatic timer to activate the

room lighting, which comprised day-color fluorescent lights (40 W ×two tubes), from 18:00 to 7:00. The periodic pattern of Mfs in the peripheral blood was then monitored on day 8 for a 24-hr period. Then, the animal was exposed to total darkness for 48 hr, following which the periodic pattern was again monitored on day 11 for a 24-hr period.

RESULTS

Periodicity of Mfs

There was nocturnal periodicity in the activity of *D. immitis* Mfs in the peripheral blood of a dog with microfilaremia with numbers peaking at approximately 3:00 and reaching a minimum at approximately 15:00 each day (Fig. 1).

Light transmittance through the dog's skin

Light at wavelengths ranging from approximately 1,000 to 450 nm was transmitted through the dog's skin at rates of 0.1–1%. Infrared beams are more transmittable quantitatively than visual beams (Fig. 2).

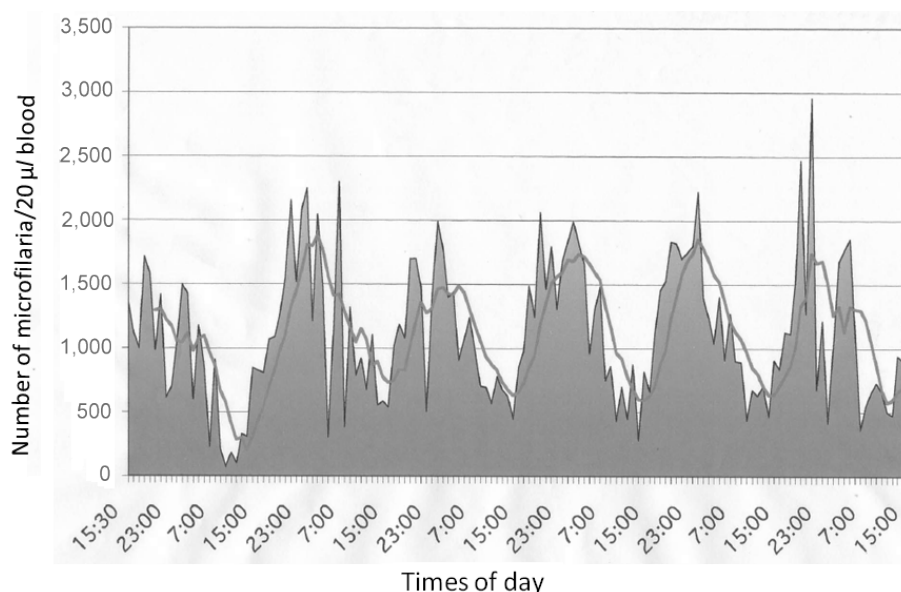


Fig. 1. Periodicity of *Dirofilaria immitis* microfilariae in the peripheral blood circulation of a dog with microfilaremia over 1 week. The change of Mf's number in 20 µl peripheral blood is shown by change of line graph with dark area under the line, and by change of lined approximation curve.

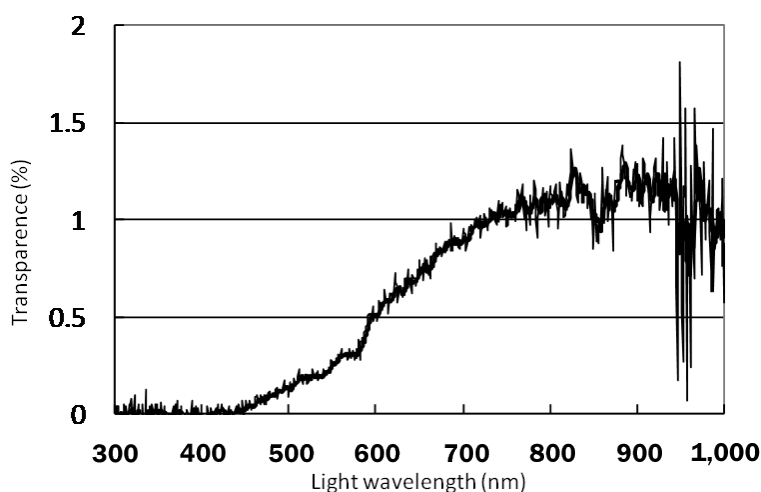


Fig. 2. Light transmittance through the dog's skin measured by spectroradiometer. It showed that a large amount of infrared beams, such as 1,000 to 700 nm, for example, readily passed through the skin than visual beams with a lesser transmittance.

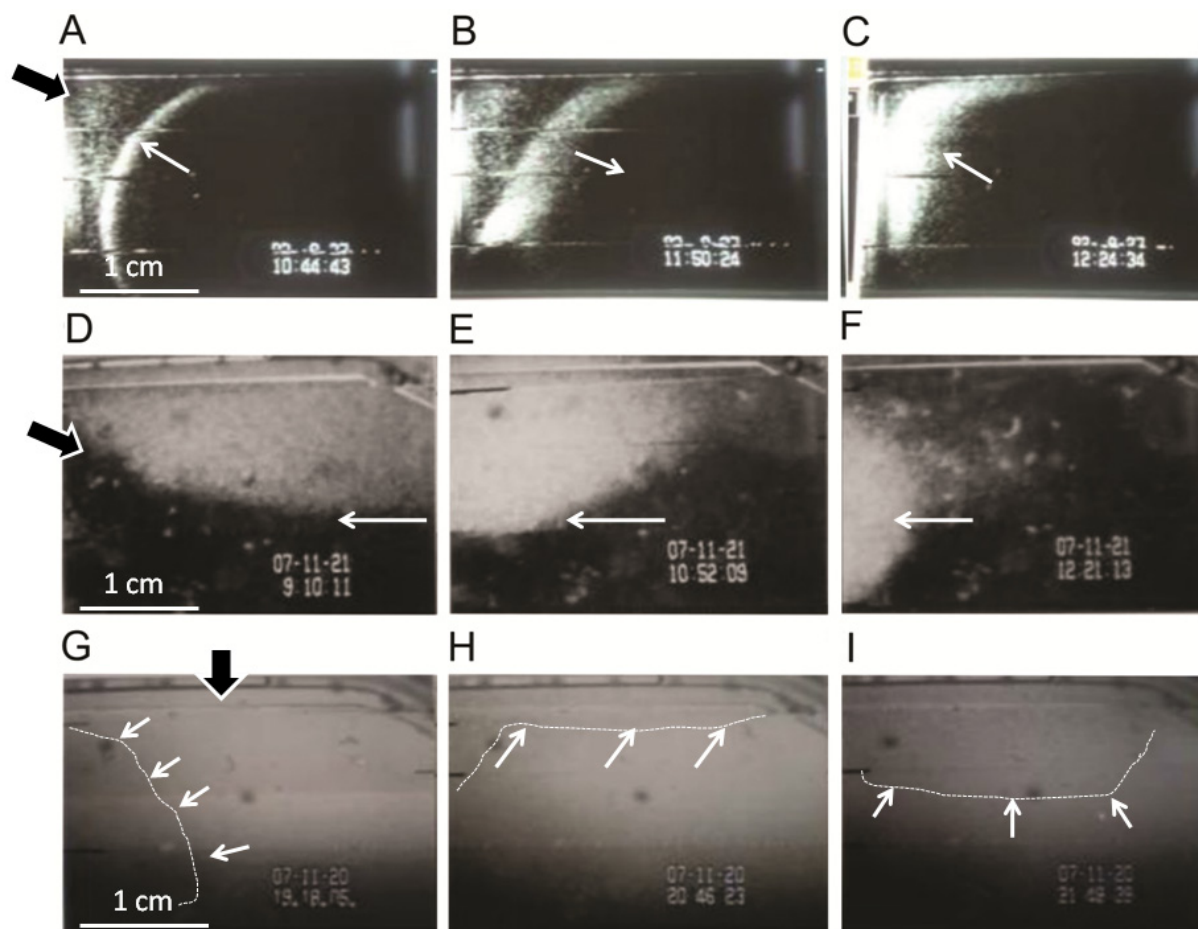


Fig. 3. Phototactic behavior of microfilariae (Mfs) *in vitro* following photobiostimulation. (A–C) Phototactic behavior in response to white light in Experiment 1 as a typical case showing the initial thronging phase (A), the romping phase after 1.1 hr (B), and the final phase when the Mfs became flattened against the wall adjacent to the light source after 1.7 hr (C). The black arrow in the photo A means a direction of incidence of white light and the white arrows indicate Mfs' moving. (D–F) Phototactic behavior in response to white light in Experiment 2 as a typical case showing the initial thronging phase (D), the Mfs starting to move toward the light source after 1.7 hr (E), and the pass-over phase after 3.2 hr (F). The black arrow in the photo D means a direction of incidence of white light and the white arrows indicate Mfs' moving. (G–I) Phototactic behavior in response to a 740 nm infrared fluorescent lamp in Experiment 2 as a typical case showing the initial thronging phase along the left side (G), the Mfs starting to move toward the infrared fluorescent lamp setting at the upper side of the container after 1.5 hr (H), and the Mfs reaching the upper side after 2.5 hr (I) although Mfs was viewed a dim through in the photograph because CCD camera slightly sense a 740 nm wave. The black arrow in the photo G means a direction of incidence of an infrared fluorescent lamp and the white arrows indicate Mfs' gathering and/or locating area. Dotted line showed an edge of Mfs' mass (G–I). Each bar in photos A, D and G indicates 1 cm long.

Phototactic behavior of Mfs

To assess the phototactic behavior of *D. immitis* Mfs *in vitro*, the Mfs were suspended in medium and viewed under an inverted microscope. In Experiment 1, when exposed to white light, the Mfs exhibited increased activity, showing thronging and romping behaviors such as sometimes bending like a whip moving and sometimes forming a semilunar pattern during an observation period of approximately 3 hr (Fig. 3A–C). They moved in unison despite the lack of a fluid current inside the container and probably moved of their own accord.

In Experiment 2, the Mfs were exposed to white light on the upper left side of the container and followed by infrared light (740 nm) on the upper right side of the container. Following exposure to white light, the thronging Mfs moved like schooling fish and gravitated in a stepwise manner toward the white light source (Fig. 3D–F). Immediately after they reached the wall that was adjacent to the white light source, the light source was switched to the infrared fluorescent lamp, shortly after which the thronging Mfs moved toward this light source instead (Fig. 3G–I).

Velocity of Mfs in response to different light sources

The velocity of Mfs during phototaxis measured at three photon flux densities under various wavelength (1,200 to 380 nm) was compared. As a result, the monitoring time spent at each wavelength was between 3.5 and 24.5 hr, depending on the time until Mfs moved to the length along the major axis of the container.

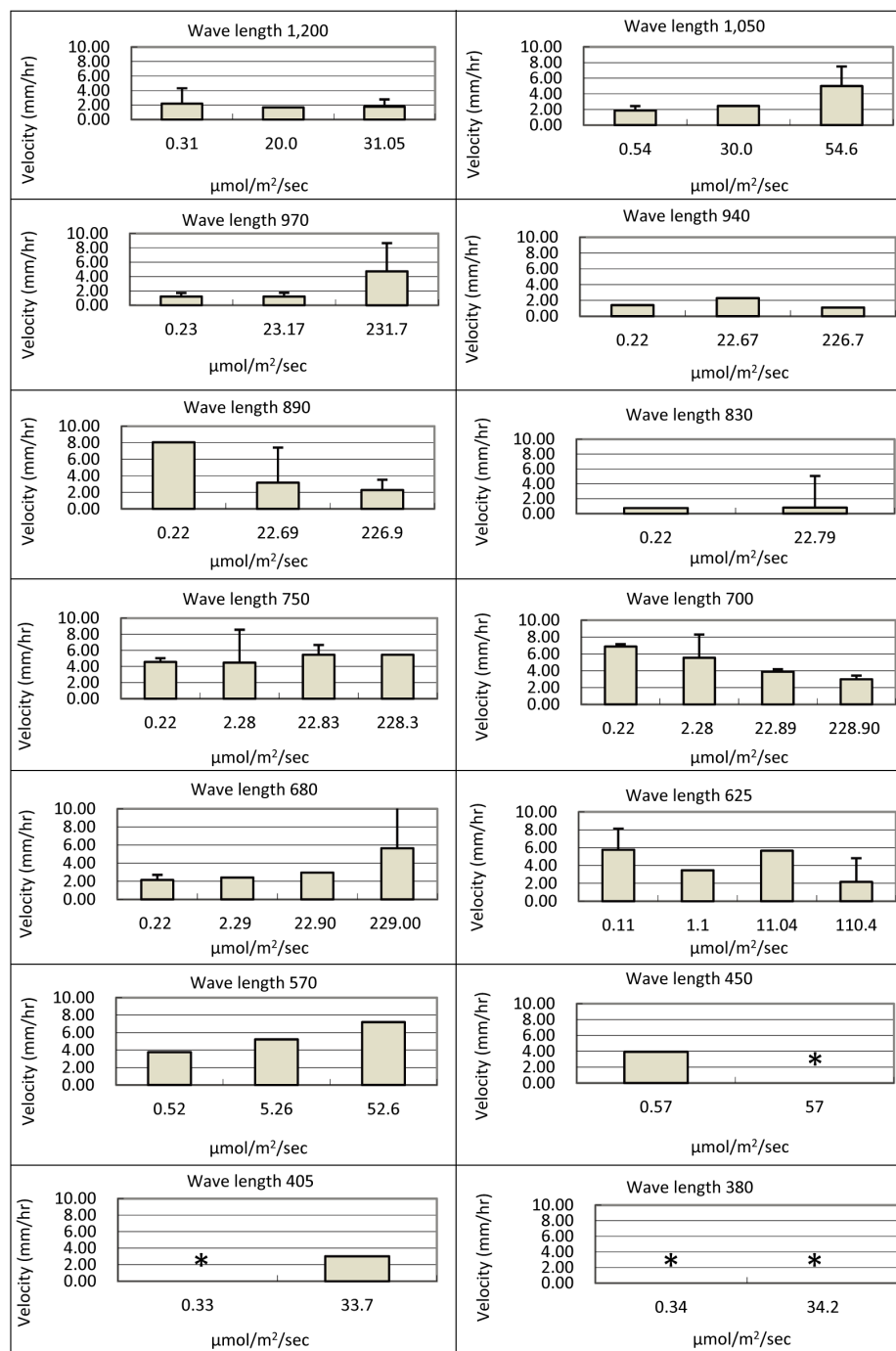


Fig. 4. Velocity of microfilariae in response to various light wavelengths and photon flux densities. Mfs showed slow velocities at 1,200 and 1,050 nm (heat rays) indicating heat-taxis unrelated to Mfs phototaxis, velocities at 970, 940 and 830 nm (longer wave areas of infrared light) were also showed radiation intensity-dependent increase or non-specific tendency with the exposure-response relationship in comparatively lower velocities, shorter wave of at 450, 405 and 380 nm (violet and ultraviolet waves) included some unstable measurements in velocities, therefore these were omitted from Table 1 (see asterisks). Infrared wave of 750 nm (shorter wave of infrared area) and 680, 625 and 570 nm (visual rays) showed radiation intensity-dependent increase or non-specific tendency with the exposure-response relationship in comparatively higher velocities. Contrary to this, 890 and 700 nm waves showed paradoxical response to radiation intensity-dependent decrease in comparatively higher level of velocities.

Paradoxical exposure-response to the intensity of radiation, such as lower photon flux density resulting in higher velocities, was demonstrated in 890 and 700 nm, whereas others varied that usually tended to increase or being incoherent with the exposure-response relationship and the results of the test at 450, 405 and 380 nm which include a few unsteady measurements such as exceptional and/or abnormal slow movement were omitted (Fig. 4).

Microarray analysis

Gene expression profiles of *D. immitis* Mfs were examined using microarray and gene ontology (GO) analyses to identify photobiostimulation-induced genes. Microarray analysis revealed that the expression of genes in Mfs changed following exposure to infrared light (Table 1). The infrared light upregulated 193 genes associated with 72 GO terms, with phosphorylation- and neurogenesis-related genes being highly enriched. This upregulation indicated that stimulation with infrared light induced significant upregulation of several genes, particularly those related to phosphorylation and neurogenesis, further supporting the phototactic behavior of Mfs. In statistics, we performed “Two tailed Fisher’s exact test” for *P*-value calculation by using MicroArray Data Analysis Tool Ver3.2 software.

Natural light waves from the atmosphere

Sun Spectroradiometer showed that a wide range of high-intensity light beams reached the field during the day, including ultraviolet, visible light, and infrared waves. However, at night, only extremely limited amounts of infrared waves reached the field, which represented <1/1,000 of that reaching the field during the day (Fig. 5).

Artificial changes to the periodic pattern

The periodic pattern was reversed by changing the light-dark cycle after a few trial by modifying the light-dark cycle (Fig. 6). By this observation it was suggested that changing the light-dark cycle may be effective to induce reverse-pattern of Mf activity.

Table 1. Infrared light-induced gene expression in *Dirofilaria immitis* microfilariae

Ontology	Gene ontology term	No. of changed genes	<i>P</i> -value
Molecular_function	Phosphoprotein phosphatase activity	8	0.000436
Molecular_function	Phosphatase activity	8	0.00199
Molecular_function	Protein self-association	2	0.00341
Biological_process	Neuron projection development	5	0.00381
Molecular_function	Phosphoric ester hydrolase activity	8	0.00478
Biological_process	Pyrimidine nucleobase metabolic process	2	0.00479
Biological_process	Neuron development	5	0.00553
Biological_process	Regulation of cell growth	2	0.00942
Biological_process	Protein dephosphorylation	5	0.00954
Biological_process	Neuron differentiation	5	0.00986
Biological_process	Dendrite development	2	0.0108
Biological_process	Cell projection organization	5	0.01123
Biological_process	Negative regulation of growth	3	0.01131
Biological_process	Nucleobase metabolic process	2	0.01225
Biological_process	Generation of neurons	5	0.01393
Biological_process	Neurogenesis	5	0.01393
Biological_process	Dephosphorylation	3	0.01477
Biological_process	Execution phase of apoptosis	2	0.01541
Biological_process	Head development	1	0.02124
Biological_process	Head morphogenesis	1	0.02124
Biological_process	Multicellular organismal response to stress	1	0.02124
Biological_process	Peptidyl-aspartic acid modification	1	0.02124
Biological_process	Phagosome-lysosome fusion	1	0.02124
Biological_process	Phagosome-lysosome fusion involved in apoptotic cell clearance	1	0.02124
Biological_process	Protein adenylylation	1	0.02124
Biological_process	Protein nucleotidylation	1	0.02124
Molecular_function	Dihydropyrimidinase activity	1	0.02255
Molecular_function	Direct ligand regulated sequence-specific DNA binding transcription factor activity	1	0.02255
Molecular_function	Ligand-activated sequence-specific DNA binding RNA polymerase II transcription factor activity	1	0.02255
Molecular_function	Malonate-semialdehyde dehydrogenase (acylating) activity	1	0.02255
Molecular_function	Methylmalonate-semialdehyde dehydrogenase (acylating) activity	1	0.02255
Molecular_function	Protein adenylyltransferase activity	1	0.02255
Molecular_function	Protein-L-isoaspartate (D-aspartate) O-methyltransferase activity	1	0.02255
Molecular_function	Spermidine synthase activity	1	0.02255
Molecular_function	Troponin I binding	1	0.02255
Biological_process	Nervous system development	5	0.02336
Molecular_function	Hydrolase activity, acting on ester bonds	10	0.02509
Biological_process	Peptidyl-tyrosine dephosphorylation	4	0.02549

Table 1. Continued

Ontology	Gene ontology term	No. of changed genes	P-value
Biological_process	Cell growth	2	0.02668
Molecular_function	Sequence-specific DNA binding transcription factor activity	10	0.02669
Biological_process	Cell development	7	0.02679
Molecular_function	Nucleic acid binding transcription factor activity	10	0.02709
Biological_process	Pyrimidine-containing compound metabolic process	2	0.03099
Biological_process	DNA catabolic process, exonucleolytic	1	0.0317
Biological_process	Leucyl-tRNA aminoacylation	1	0.0317
Biological_process	Protein localization to endoplasmic reticulum exit site	1	0.0317
Biological_process	Thymine metabolic process	1	0.0317
Biological_process	Valine metabolic process	1	0.0317
Biological_process	Vesicle fusion	1	0.0317
Molecular_function	Protein tyrosine phosphatase activity	4	0.03201
Cellular_component	Endoplasmic reticulum exit site	1	0.03274
Molecular_function	Leucine-tRNA ligase activity	1	0.03364
Molecular_function	Phosphatidylinositol-3,4,5-trisphosphate binding	1	0.03364
Biological_process	tRNA metabolic process	3	0.03573
Molecular_function	Sequence-specific DNA binding	9	0.03799
Biological_process	Carbohydrate homeostasis	1	0.04204
Biological_process	Cellular glucose homeostasis	1	0.04204
Biological_process	Glucose homeostasis	1	0.04204
Biological_process	Intracellular receptor signaling pathway	1	0.04204
Biological_process	Lateral inhibition	1	0.04204
Biological_process	Negative regulation of cell growth	1	0.04204
Biological_process	Protein exit from endoplasmic reticulum	1	0.04204
Biological_process	Retrograde transport, endosome to Golgi	1	0.04204
Cellular_component	Early phagosome	1	0.04341
Biological_process	Regulation of developmental process	5	0.04358
Biological_process	Negative regulation of multicellular organismal process	4	0.04414
Molecular_function	Carboxyl-O-methyltransferase activity	1	0.0446
Molecular_function	Fructokinase activity	1	0.0446
Molecular_function	Glucokinase activity	1	0.0446
Molecular_function	Hexokinase activity	1	0.0446
Molecular_function	Mannokinase activity	1	0.0446
Molecular_function	Protein carboxyl O-methyltransferase activity	1	0.0446

Gene upregulation induced by infrared light exposure was denoted by at least a 2-fold increase in expression. *P* values were determined using a two-tailed Fisher's exact test.

DISCUSSION

Mechanism of periodicity has remained unknown although several unreasonable hypotheses were proposed. I prepared a suitable medium to maintain the viability of microfilaria for a long period even though most commercial artificial media failed. This medium made possible long-term video observation *in vitro* to analyze the phototactic behavior of microfilaria to determine why microfilaria gather in peripheral blood under the skin around midnight. To determine an inducer, I focused attention on beams from the sky at midnight and only infrared rays, and substantially no other beams, were determined to be the inducer.

The present results demonstrated positive phototactic behavior of *D. immitis* Mfs and also its dynamic movement (Fig. 3). Microarray analysis indicated that infrared light supports the phototactic behavior (Table 1), and the plasticity of the nature of microfilaria periodicity which was demonstrated by artificial changes of the day-night cycle was confirmed (Fig. 6).

From these results, it is also conceivable that Mfs may possess an unknown photoreceptor or photosensory transduction mechanism. Moreover, gene expression in response to infrared light exposure examined in *D. immitis* Mfs suggested that light stimulation induced significant upregulation of some genes, with phosphorylation- and neurogenesis-related genes being highly enriched. This may provide evidence to support phototaxis in Mfs. Therefore, these findings indicated that the periodicity may be inherent and possibly plastic based on the changes in the periodic rhythm in response to time-course changes of the day-night cycle. Conversely, *D. immitis* Mfs also exhibit a periodic pattern in cats, classified into abnormal hosts, following experimental infection with infective larvae by the usual manner [6], whereas low and little adaptabilities of parasitism of Mfs to rodents were illustrated in our preliminary trials in which mice showed a periodic pattern over a short term (a few days) and rabbits showed no

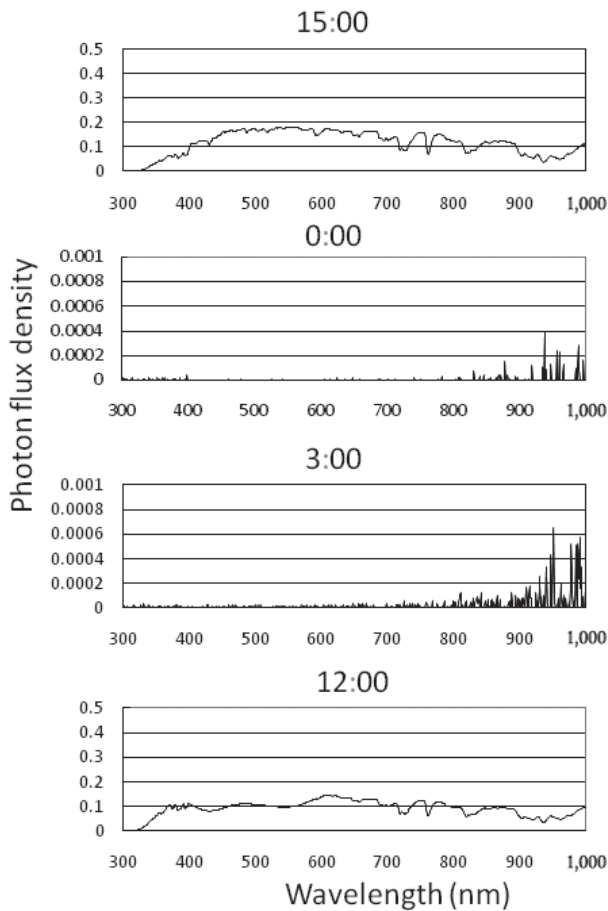


Fig. 5. Patterns in natural light wavelengths in the range of 1,000–300 nm reaching the field from the atmosphere at 15:00, 0:00, 3:00, and 12:00 from August 10 to 11, 2009. Stronger natural beams in wide range reached the field from the atmosphere at 12:00 and 15:00 in day-time, conversely extremely weaker infrared beams only reaches the field in the midnight, which represented <math><1/1,000</math> of photon flux density that reaching the field during the day, although these infrared beams between 1,000–700 nm at 3:00 was demonstrated slightly stronger than that at 0:00.

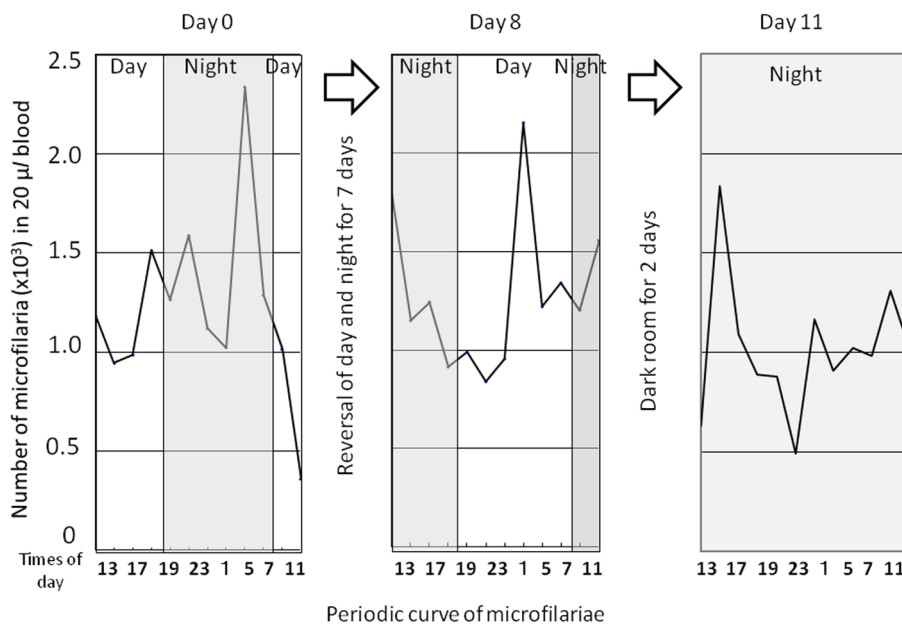


Fig. 6. Effect of experimentally reversing the day-night cycle on the periodicity of *Dirofilaria immitis* Mfs in a dog with microfilaremia. On day 0, periodic curve was showed a normal shift of periodic activity as a typical case, that is microfilaremia number's peaking was at approximately 3:00 and a minimum at approximately 15:00. After changing day-night cycle into reversal situation for 7 consecutive days (day 1–7), then periodic curve in next day (day 8) the peak shifted at approximately 1:00 and reaching a minimum at approximately 22:00, although a 2nd peak at approximately 13:00 appeared. After the animal was kept in dark room in next consecutive 2 days (day 9–10), then the peak shifted at approximately 15:00 and reaching a minimum at approximately 23:00 (day 11), indicating that periodic rhythm was changed into reversal.

periodic pattern when live Mfs (approximately 7×10^4) isolated from a dog were intravenously injected into the tails (mice) or ears (rabbit) (data not shown).

The Mfs of several types of filarial nematodes, such as *D. immitis* in dogs, *Setaria digitata* in cattle, and *W. bancrofti* and *Brugia malayi* in humans, occur beneath the skin, including peripheral blood, or on the surface of the body, typified by the exudates of ulcerative dermatosis (*Onchocerca volvulus* in humans). Conversely, adult worms always live in deeper regions, such as the pulmonary arteries, abdominal cavity, inguinal lymphatic vessels, and subcutaneous nodules, to avoid light exposure.

Light permeability of the skin showed that the infrared light range was particularly well transmitted, whereas the visible light range was transmitted to a lesser extent (Fig. 2). Thus, infrared beams have the potential to reach the subcutaneous tissues, including peripheral blood vessels. The reason why Mfs is gathered in peripheral blood vessels at the midnight could be explained that the effect of natural infrared light from the sky which may activate Mfs' phototactic movement play an important role under the natural environment.

Paradoxical exposure-response of Mfs to 890 and 700 nm of infrared lights may indicate inducers of the periodicity, because Mfs responded to weaker infrared light (Fig. 4) and they exhibited peaking in number of Mfs during the midnight when a very weak natural infrared beams only being incident from the sky at midnight (Fig. 5). This may provide an evidence to support that the weaker infrared beams could be more optimal for activating nocturnal periodicity, supporting that infrared light at wavelengths of 890 and 700 nm, plays an important role in mediating the nocturnal periodicity.

It was speculated that the peak of Mfs at approximately 13:00, as 2nd peak on day 8 (Fig. 6), might take the place of main peak when the test was still continued under dark situation. Then, the main peak finally shifted into reverse at approximately 15:00 on day 11, as was expected consequence. From this, it was speculated that a slight infrared ray in the midnight play an important role to induce a positive phototaxis, whereas violet and ultraviolet and visual rays and also intense infrared rays in the day-light retrain it, then it mediates periodic movement. However it is still unknown a general mechanism on it, particularly, as in detail case, why more two days of dark situation were necessary till the peak of Mfs shifted into reverse (Fig. 6).

Phototactic behaviors in lower animals often exhibit a positive phototaxis to weaker light; for example, larval mites often appear in illuminated areas in the room during the evening and night, whereas adult mites never appear, indicating a negative phototaxis [1, 2, 7]. In the case of cuttlefish or some fishes, one of the fishing methods involves luring them into a lighted area. This fishing method is particularly effective at night because red or infrared light can penetrate into deeper layers of a dark sea than shorter-wavelength lights; this is likely to be the mechanism of Mf periodicity. It is of interest that the infrared light wave may be a common inducer of phototaxis among lower animals; therefore, it may be necessary to analyze in detail the role of 890 and 700 nm infrared waves henceforth.

Masuya [8] proposed a negative phototaxis hypothesis for the nocturnal periodicity of *D. immitis* Mfs in which he argued that bodies of Mfs contain numerous fluorescent granules that may be irritated by the shorter wavelengths of light in sunlight, causing the Mfs to move to deeper areas of the body during the day in an attempt to avoid light exposure. However, here the dog's skin was found to be impermeable to light of wavelengths shorter than 500 nm, indicating that this cannot explain the observed periodicity.

The free-living nematode *C. elegans* has served as a popular model organism for studying phototransduction. *C. elegans* exhibits negative phototaxis following light stimulation [4, 11]. However, its phototaxis is mediated by sensory neurons that act as photoreceptors, such as LITE-1, or by light-sensitive neurons and channels, and the species must hide in soil for survival [4, 11]. Its phototactic behavior requires cyclic guanosine monophosphate-sensitive cyclic nucleotide-gated channel-dependent phototransduction. Conversely, the negative phototactic behavior of *C. elegans* accelerates its locomotion by responding to shorter-wavelength blue, violet, and ultraviolet light to escape lethal sunlight, i.e., *C. elegans* has a strong response to shorter-wavelength light expressed as a robust acceleration of locomotion [3]. In contrast, no report has uncovered the sensory organ of Mfs of *D. immitis*, indicating that Mfs do not appear to have evolved morphological light sensory organs to mediate phototaxis. However, the finding that *D. immitis* Mfs exhibit positive phototaxis suggests that they may possess an unknown photoreceptor or photosensory transduction mechanism, and signal transduction may also be activated by light stimulation. This may provide evidence to support phototaxis in Mfs.

I first reported here the *in vitro* observation of Mfs' phototactic movement in unison, despite the lack of a fluid current inside the container. It look like that they have something wish to move by themselves, like a school of fish. This observation could be possible to visualize long observation by both of the culture medium which was newly developed and used in this study and the charge-coupled device (CCD) camera and time-lapse VHS videocassette recorder which were adopted in this study.

Furthermore, the examination in changing the light-dark cycle (Fig. 6) introduced changing the shift in periodic rhythm. These findings further illustrate that the periodicity of Mfs is based on the light-dark cycle, therefore the nature of microfilaria periodicity could be plastic, and some photoreceptor mechanism could be responsible for the periodicity.

This study will be useful for analyzing the behavior of parasites, not only filarial species but also other helminths, to understand the nature of their phototaxis of helminth parasites in relation to the mechanism of infection.

In response to the critical question of how or when Mfs obtained periodic rhythm, the possibility of acquire the rhythm by spending some period after release into host's circulation from female adult worm and obtaining a stable disposition could not be ruled out, because direct expose of infrared light not necessarily, possible to exhibit in house-reared dogs and in throughout four seasons, including long period of cloudy days or snowfall days. Moreover, Mfs keep their bodies in the peripheral blood stream, the possibility of hooking to the endothelium of the capillary using the hook on their head [10] also could not be ruled out.

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