OCELLAR PIGMENTATION AND PHOTOTAXIS IN THE NEMATODE *MERMIS NIGRESCENS*: CHANGES DURING DEVELOPMENT

A. H. JAY BURR*, DONNA WAGAR AND PARMJIT SIDHU

Department of Biological Sciences, Simon Fraser University, Burnaby, British Columbia, Canada V5A 1S6

*e-mail: burr@sfu.ca

Summary

After 1 or 2 years of dormancy in the soil, *Mermis nigrescens* females emerge to lay eggs on vegetation where their grasshopper hosts are likely to feed. Females collected at this life stage exhibit a strong positive phototaxis and have a tubular region of pigmentation near the anterior tip consisting of concentrated oxyhaemoglobin. A previous investigation of the scanning motion of the 'head' and orientation of the 'neck' has implicated the shadowing of a photoreceptor inside the tube as the mechanism for identifying the direction of light during phototaxis. Here, we describe the development of the pigment in young adult females and investigate phototaxis in early developmental stages that lack the pigment. The orientation of the neck to a horizontal 420 nm stimulus (intensity 10^{13} photons s^{-1} cm^{-2}) was measured for unpigmented fourth-stage larvae and immature adult females as well as mature females with pigmented ocelli. The orientation of the larvae and immature adults was weakly negative, whereas that of the mature adults was strongly positive. Head and neck movements were otherwise the same in the three stages. Thus, the pigmentation appears to be required for positive phototaxis, and the results provide further support for the shadowing role of ocellar haemoglobin.

Key words: ocellus, phototaxis, orientation, mechanism, transverse phototaxis, development, haemoglobin, molecular opportunism, life cycle, nematode, *Mermis nigrescens*.

Introduction

A pigmented region adjacent to photoreceptive nerve endings provides the mechanism for discriminating the direction of light in a wide variety of small animals (Burr, 1984a). For example, some planarian species have a pair of shallow pigment cups lined with several hundred photoreceptors (Taliaferro, 1920; Carpenter et al., 1974), and *Drosophila* spp. larvae have lateral pits in the black-pigmented pharyngeal skeleton that contain a cluster of 12 photoreceptors (Sawin et al., 1994). In these relatively transparent animals, the structures would restrict the illumination of the photoreceptors to a selected region of visual space, and the nerve signals generated as the animals move would provide the information used to orient relative to the direction of light (Burr, 1984b). *Planaria maculata* seek dark regions of their environment and crawl away from a horizontal light source. *Drosophila* spp. larvae burrow into their food and are negatively phototactic during their feeding phase. However, this changes to positive phototaxis during the third instar, which probably aids in finding a suitable pupation site (Sawin et al., 1994).

Pigmented ocelli are rare in Nematoda, but crop up in widely separate taxa and with such widely disparate morphologies that they have probably evolved independently several times (Coomans, 1979; Wright, 1980; Coomans and De Grisse, 1981; Burr, 1984a). Negative phototaxis has been observed in three free-living aquatic species (*Chromadorina bioculata*,

Oncholaimus vesicarius and *Enoplus anisospiculus*) in which each of the paired lateral ocelli has been shown to consist of a tiny mass of melanin-like pigment lying immediately posterior to the sensory ending of a single neuron (Croll, 1966; Croll et al., 1972; Bollerup and Burr, 1979; Burr and Burr, 1975; Burr, 1984a). This photoreceptor would be shadowed when the anterior is oriented away from the light source, providing an optical mechanism for discriminating the preferred orientation (Burr, 1979).

In contrast, phototaxis is positive in mature females of *Mermis nigrescens* collected as they are crawling through vegetation (Burr et al., 1989). At this life stage, the females have a region of bright red pigmentation near their anterior tip suggestive of an ocellus (Cobb, 1926) and consisting of a hollow cylinder of concentrated oxyhaemoglobin (Ellenby, 1964; Burr et al., 1975). A role as the photosensitive pigment of phototaxis is ruled out since the sensitivity spectrum of phototaxis is unlike the absorption spectrum of oxyhaemoglobin (Burr et al., 1989).

As *Mermis nigrescens* crawls through vegetation or over a horizontal surface, a continual bending motion of the anterior 2 mm (the 'head') swings the anterior tip containing the ocellus horizontally and vertically while the anterior is elevated above the substratum. During phototaxis, this scanning motion, combined with a slower orientational bending of the neck,

swings the pigment cylinder about the direction of the light source. The average azimuth and elevation of the head are adjusted by controlling the orientation of the neck. Locomotion by the body proceeds in the direction of average orientation of the neck (Burr et al., 1990). The same head and neck motions occur in the dark without orientation. As in other nematodes, locomotion is undulant and accomplished with a unique, perpendicular attachment of muscle sarcomeres to the cuticle (Burr and Gans, 1998); however, in *M. nigrescens*, the dorso-ventral flexures are perpendicular to rather than in the plane of the surface (Gans and Burr, 1994).

An investigation of the scanning motion and orientation during phototaxis has indicated a photoreceptor-shadowing role for the haemoglobin pigmentation (Burr et al., 1990; Burr and Babinszki, 1990). When the light beam is experimentally interrupted in phase with the bending motion on swings to the left, the average neck orientation and direction of locomotion deviate to the right, and *vice versa*. This result and the other observations are best explained if the pigment modulates the illumination of a photoreceptor located inside the cylinder (Burr and Babinszki, 1990). The results reported in this paper provide further support for this shadowing role.

M. nigrescens parasites leave the grasshopper host as fourth-stage larvae and burrow deep into the soil. The haemoglobin pigmentation is not present at this stage, and thus it must develop during the 1–2 year dormant period in soil prior to the emergence of the female for egg-laying. It never develops in males, who expire after mating in the soil.

In this paper, we report an investigation of the development of the haemoglobin pigmentation during maturation of female *M. nigrescens*, and the phototactic orientation of larval and immature-adult females before pigmentation appears. Are these unpigmented females light-sensitive? If so, do they orient in the same manner as mature females? If the eye haemoglobin plays a significant shadowing role in the mature female, the phototaxis would be expected to differ. If females are phototactic in the absence of shadowing pigment, what other optical mechanism could enable orientation relative to light direction?

Materials and methods

Cultivation and collection of worms

Female *Mermis nigrescens* (Dujardin, 1842; syn. *Mermis subnigrescens* Cobb, 1926) were raised in desert locusts, *Schistocerca gregaria*, maintained at approximately 30 °C. For series 1 experiments, high-density infections were initiated by feeding 50–80 eggs. After 3.5–4 weeks, the abdomen of the locust was slit to allow the fourth-stage larvae to emerge and burrow into moist autoclaved soil in 15 cm diameter clay flowerpots at 21–23 °C. For series 2 experiments, 15–25 eggs were fed (low infection level), and the larvae were released after 5 weeks as they take longer to reach the larger full size (length >100 mm, diameter approximately 0.4 mm). Two to four weeks after emergence, the just-moulted, virgin immature adult females were separated from males and kept in moist

autoclaved soil in plastic bags at 21–23 °C. Mature females were picked off vegetation on a rainy day in May in Rosedale, British Columbia, Canada, and stored in a refrigerator at approximately 8 °C in plastic bags of soil.

For the series 1 experiments, emergent larval females were tested within 2 days of emergence, before moulting had begun. Immature females were tested 16 weeks post-emergence. Mature females, probably 1–2 years old, were maintained for at least 7 days at 21–23 °C prior to testing. For the series 2 experiments, immature females were tested 11–12 weeks post-emergence.

Arena and recording arrangement

Test runs were performed under conditions similar to those in a previous study with mature females (Burr et al., 1989). A 29 cm wide by 60 cm long piece of dampened black felt covered the bottom of a glass chamber. The felt was dense enough to prevent burrowing and provided a fibrous surface on which the worm could propel itself. The black colour minimized light scatter and provided a contrasting background for videotaping the worms. To block stray light and to ensure humid conditions, the sides and back of the chamber were lined with black felt and sprayed periodically with a fine mist of distilled water at room temperature, and the chamber was covered with a glass top. The arena temperature was the same as the storage temperature of the worms, 22±1 °C.

A black-and-white video camera (Cohu CCD, 4910 series) provided adequate near-infrared sensitivity. Positioned 75 cm above the arena and fitted with a 75 mm zoom telephoto lens and 5 mm extension tube, the focal length was adjusted to provide a 34 cm×38 cm field of view for the series 2 observations. For series 1, a 2× tele-extender and 5 mm extension tube were added to provide a 2.6 cm×3.0 cm field of view at 75 mm focal length. To keep the anterior of the worm in the field of view, the experimenter slid the chamber along orthogonal tracks while viewing the video image on a monitor. A time/date generator (Panasonic WJ 810) imprinted the time on the image recorded by a video cassette recorder (Mitsubishi HS-U62C).

Motion was observed under continuous near-infrared light (730 nm to approximately 990 nm, approximately 2 mW cm^{-2} or approximately 8×10^{15} photons s^{-1} cm^{-2}) provided by a microscope illuminator projected through a Corning 2-64 glass filter, a 47B Wratten filter and 2 cm of water. In the presence of the observation source alone, sampled orientations were not significantly different from a uniformly distributed population (dark controls, see Tables 2–3). Mature females were previously observed to be not significantly sensitive to horizontal stimuli of this wavelength range or to horizontal monochromatic stimuli at wavelengths longer than 560 nm (Burr et al., 1989).

The stimuli

Monochromatic test stimuli at 420 and 500 nm were isolated by interference filters (Ealing). Intensities covering four orders of magnitude were controlled using neutral-density filters that

provided optical densities of 3, 2, 1 and 0 (no filter) corresponding to transmissions of 0.1%, 1%, 10% and 100%, respectively. Optical density 0 corresponds to 1.33×10^{13} photons s^{-1} cm^{-2} (6.3 μW cm^{-2}) at 420 nm and 5.7×10^{13} photons s^{-1} cm^{-2} (23 μW cm^{-2}) at 500 nm. A broad-band test stimulus (approximately 380–610 nm, 860 μW cm^{-2}) was defined by the emission spectrum of the 500 W projector lamp at short wavelengths and Corning 4-69 and 1-69 blocking filters at long wavelengths. Heat radiation was removed from the stimulus beam, successively, by the projector's built-in heat filter, a Corning 1-69 infrared-absorbing filter (to prevent cracking of the 4-76 filter), a Corning 4-76 filter (to block far red and near-infrared light), the Ealing interference filter with its blocking filter and finally 6 cm of water, which blocks all far-infrared wavelengths. This combination effectively eliminates all radiation capable of significantly heating the nematode or the arena substratum (Burr, 1985). Baffles and masks were placed to prevent any stray radiation from reaching the arena. Light intensities were measured using a radiometer (International Light, Inc., model IL500) and converted to photon flux densities.

Experimental protocols

Two series of experiments were performed under slightly different conditions. In series 1, one worm at a time was observed in a 2.6 cm×3.0 cm field of view, and all three age groups were investigated. In series 2, five worms were videotaped simultaneously in a 34 cm×38 cm wide field of view, and only immature females were investigated. Prior to any experiment, approximately 10 washed females were preconditioned in a humid chamber illuminated horizontally by broadband light. Approximately 1–2 h was required for worms to become active. Worms that either coiled repeatedly or remained still were not used.

At the start of an experiment, the one worm or the five worms were placed 20 cm from the near end of the arena. In series 1 experiments, mature and larval females were tested with the following sequence of stimuli: (a) 30 min at 420 nm, optical density 0, (b) 5 min dark, (c) 30 min at 420 nm, optical density 1, (d) 30 min at 420 nm, optical density 0, and then (e) 30 min dark. Immature females were tested with: (a) 30 min at 420 nm, optical density 0, (b) 5 min dark, (c) 30 min at 420 nm, optical density 0, and (d) 60 min dark. In series 2 experiments, 10 min treatments were presented to immature females in the following sequence: (a) broad-band light, (b) dark, (c) reference 1 (420 nm, optical density 0), (d) dark, (e) 420 nm, optical density 3, (f) 420 nm, optical density 2, (g) 420 nm, optical density 1, (h) 420 nm, optical density 0, also serving as reference 2, (i) dark, (j) 500 nm, optical density 3, (k) 500 nm, optical density 2, (l) 500 nm, optical density 1, (m) 500 nm, optical density 0, (n) dark, and finally (o) reference 3 (420 nm, optical density 0).

Measurement and analysis

The angular orientation of a 3 mm region beginning 2 mm from the anterior tip was measured from the monitor screen during playback of the video recordings. This recorded the 'neck' orientation, avoiding the scanning motion of the 'head'. Measurements in both series 1 and series 2 were made at 30 s intervals to the nearest 5°. To create independent measurement periods for averaging and to avoid any possible influence of the preceding treatment, the orientations during the initial 4 min of the 30 min treatment periods of series 1 were not measured. Similarly, the initial 2 min of the 10 min periods of series 2 were ignored, and with the approximately 1 min period required for moving the worms back to the start position, a 3 min buffer period was provided. Also, the repositioning ensured that the measurement periods were independent.

Once a worm had reached the side or end wall of the chamber, measurement of that worm was discontinued until the following measurement period, the worm having been placed back at the start position. Orientations were not measured during conflicting activity such as repeated coiling, poking into the felt substratum, reversal bouts or nictating (swinging the anterior in a circle to increase the chance of encountering a vertical object to crawl onto). Any measurement period with less than six observations was ignored.

Statistical analyses

Circular statistics are described in Batschelet (1981) and Mardia (1972). A sample of n orientations a_i is represented by vectors with tails at the centre of a circle, with tips pointing in the direction a_i and with lengths of 1. Orientations during a measurement period were averaged by calculating the mean vector, **a**. The direction of the mean vector is the mean direction, a, and its length is the directedness, r. The latter varies between 0 (perfectly uniform distribution) and 1 (all orientations in the mean direction). The projection of r on the direction towards the source, v, is a measure of phototactic efficiency (Burr, 1979; Batschelet, 1981; Burr et al., 1989). A negative value of v indicates net movement away from the source (negative phototaxis). The distribution of mean vectors $\mathbf{a_j}$ from a given treatment was illustrated graphically. The Hotelling's test for uniformity of the distribution of $\mathbf{a_j}$ (Batschelet, 1981) provided a useful test for non-uniformity of a distribution of mean vectors. Watson's U^2-test for uniformity of the distribution of mean orientations (Watson, 1961) is not affected by multimodality, but the length of the mean vectors is ignored. The V-test was used to test whether the mean orientations tend towards 0° or 180° (positive or negative phototaxis). Circular statistics were computed from the measured orientations using tested APL-language routines.

Results

Development of haemoglobin pigmentation

The development of *M. nigrescens* was followed during cultivation at room temperature (21–23 °C) over a period of a year. After 4–6 weeks in the host, the larval females have grown to the full 100 mm length and approximately 0.4 mm diameter, and their approximately 20 mg mass consists mostly of food stored in preparation for the nonfeeding period outside

Fig. 1. The anterior of adult *Mermis nigrescens* females: two immature females at different times after the adult moult and a mature female at 12 months. Haemoglobin accumulates gradually in the ocellus during maturation. The yellowish content of the trophosome (food-storage body) appears posterior to a region where cerebral ganglia and the nerve ring are located. Body wall muscle lies between these tissues and the cuticle. Nerve tracts extend through the pseudocoelom to sensory structures in the anterior tip, passing through the hollow cylinder of pigmentation in the mature females. Whole mounts, illumination incident from the sides.

the grasshopper. This is an unusually large size for nematodes. Prior to emergence, the thin porous cuticle of the growing parasite is replaced in the fourth-stage larva by a thick cuticle suitable for coping with and crawling in the terrestrial environment. Most of the fourth-stage larvae burrow into the soil within a few hours of emergence. Females and males can be distinguished at this stage by body size and the appearance of the gonadal precursor tissue. The adult moult occurs in the soil at variable times during the first 1–3 weeks after emergence from the host. A collar of thickened cuticle 200–400 μm behind the tip (Fig. 1) distinguishes adult from larval cuticle in females. After moulting, adult females remain tightly coiled at the bottom of the flowerpot or bag of soil. If mated, the females slowly convert most of the food stored in the trophosome to approximately 10 000 eggs. When collected, the mature, gravid females were migrating over soil, climbing

through vegetation or laying eggs on vegetation. When kept in moist (not wet) soil at 8 °C in the dark, they remain dormant for years.

The haemoglobin pigmentation appears in the immature adult female during the dormant period in the soil whether or not mating has occurred. Reverse transcription followed by polymerase chain reaction (RT-PCR) results indicate that the expression of eye globin starts 3–5 weeks post-emergence, after moulting has been completed (Burr et al., 2000). The haemoglobin accumulates gradually, with a faint colour first visible 3–4 months after the moult (3.5–4.5 months post emergence) as a yellowish hue characteristic of dilute haemoglobin (Fig. 1). Mature levels of pigmentation are not observed until at least 9 months. Mature females collected in the field in May are probably 1–2 years old.

In all stages, the cornea-like anterior tip (Fig. 1) would be expected to concentrate light near the midline when pointed towards the light, and the cylindrical body would concentrate light inside when oriented perpendicular to the light source. In the mature female, the walls of the cylinder of pigment would block perpendicular illumination from entering and illuminating the putative photoreceptor. The fraction absorbed by the haemoglobin is maximal at approximately 420 nm (99 %) and near a minimum at 500 nm (25 %), and would be higher for oblique illumination. Phototactic efficiency remains high from 350 to 520 nm and drops to non-significance by 580 nm, even though the haemoglobin absorptance remains above 25 % up to 590 nm. The long-wavelength limit of phototaxis we attribute to a drop in photoreceptor sensitivity (Burr et al., 1989).

The photoreceptor would remain exposed in the emergent larvae and 3-month-old immature adult females. Note the greater amount of scattered light in immature females under laterally incident illumination (Fig. 1). This is also present in the emergent larvae (not shown).

In addition to being anatomically similar except for the pigmentation, the emergent larvae, immature adult and mature adult females all display the same locomotion patterns on a damp felt surface. A lacing motion of the anterior, which pushes the tip under surface fibers, alternates with a period of head elevation above the substratum. Propulsive forces are applied against surface fibres of the felt as the body glides by. In particular, during the periods of head elevation, all developmental stages exhibit the scanning motion of the tip caused by cyclical bending of the 'head' (the anterior 2 mm) and the orientational bending of the 'neck' behind the head. Thus, the primary feature distinguishing the younger stages from the mature adult is the ocellar pigmentation.

Series 1 experiments

In these experiments, the phototaxis of emergent larvae, approximately 3.7-month-old immature adult and mature adult females was investigated with 420 nm monochromatic light at optical densities of 0 and 1 and in the dark (see Table 1 for absolute intensities). The immature adults were unmated and eggless. Descriptive statistics for the three developmental
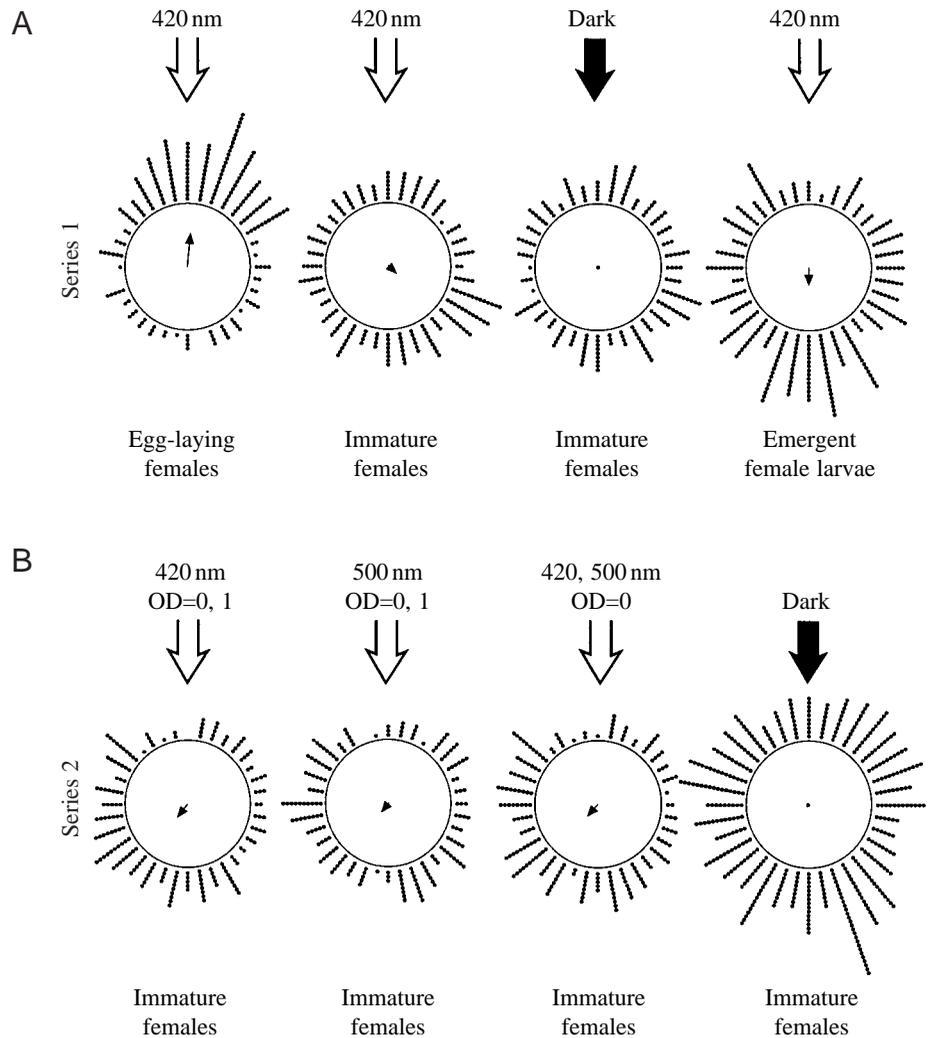
Fig. 2. Distribution of neck orientations measured at 30 s intervals relative to the direction of monochromatic light. Mean vectors plotted within each circular histogram point in the mean direction, and their lengths indicate the concentration of observations in that direction (radius of circle 1.0). For corresponding statistical analyses, see Table 1. (A) Series 1 results. All treatments illustrated are at 420 nm and optical density (OD)=0. (B) Series 2 results. Different wavelength and intensity treatments are pooled in various combinations. See footnote of Table 1 for intensities corresponding to optical densities of 1 and 0 at each wavelength.

stages are compared in Fig. 2A and Table 1. The mean direction *a*, magnitude of *r* (concentration of observations in the mean direction) and sign and magnitude of *v* (phototactic efficiency) suggest (i) that mature females may be positively phototactic, (ii) that larvae may be negatively phototactic and (iii) that immature females may not be phototactic.

The successive neck orientations measured at 30 s intervals might be serially correlated, and, if so, inference tests such as the *V*-test cannot be used on the pooled raw orientations. This possibility was examined by measuring the duration of runs of successive orientations falling within a 100° range. In a total of 429 neck orientations, 152 runs of two or more successive measurements were identified. These frequently lasted 1–3 min, covering 2–6 measurements (Fig. 3).

Independent samples were constructed by second-order analysis (Batschelet, 1981), a method pioneered by Wallraff (1978a,b) for bird migration studies. Mean vectors were calculated for orientations sampled during each 26 min measurement period (the first 4 min of each treatment period was ignored). Mean vectors from sufficiently separated periods and for different worms should be independent. For example, in the series 1 protocol, each worm was tested with the 420 nm,

optical density 0 stimulus in two 30 min periods. Since the measurement periods are separated by 39 min for the six
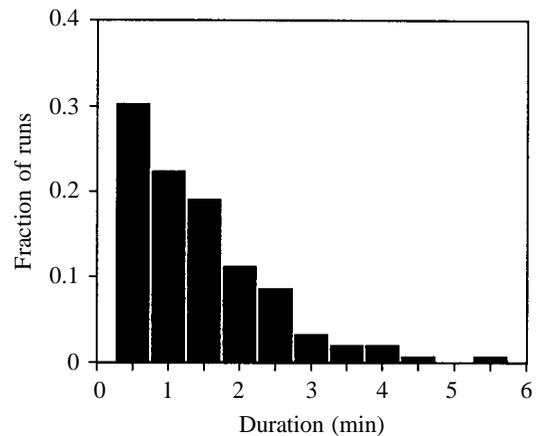

Fig. 3. Frequency distribution of run durations in the dark. The duration of runs of successive neck orientations falling within a 100° range was determined for 10 immature worms over 8 min measurement periods. 152 runs were identified among 429 neck orientations measured.

Table 1. *Descriptive statistics of pooled neck orientations (see Fig. 2)*

Treatment	n	Means a	Means r	Means v
Series 1 experiments				
Mature females				
420 nm, OD=0	210	10	0.48	0.47
420 nm, OD=1	101	−14	0.15	0.15
Dark	79	−155	0.18	−0.16
Emergent larvae				
420 nm, OD=0	298	−180	0.23	−0.23
420 nm, OD=1	151	100	0.12	−0.02
Dark	129	134	0.17	−0.12
Immature females				
420 nm, OD=0	207	134	0.13	−0.09
Dark	186	100	0.10	−0.02
Series 2 experiments:				
Immature females				
420 nm, OD=0	99	−134	0.19	−0.13
420 nm, OD=1	75	−134	0.30	−0.21
420 nm, OD=1, 0	174	−134	0.24	−0.17
500 nm, OD=0	92	−132	0.27	−0.18
500 nm, OD=1	80	88	0.05	0.00
500 nm, OD=1, 0	172	−139	0.13	−0.10
420, 500 nm, OD=0	191	−133	0.23	−0.16
Dark	418	−177	0.06	−0.06

Neck orientations measured at 30 s intervals have been pooled over each treatment or combination of treatments.

Intensities corresponding to optical densities (OD) of 0 and 1 are 100 % and 10 %, respectively, of 1.33×10^{13} photons s^{-1} cm^{-2} at 420 nm and 5.7×10^{13} photons s^{-1} cm^{-2} at 500 nm.

n, number of neck orientations; a, mean direction; r, length of mean vector; v, phototactic efficiency.
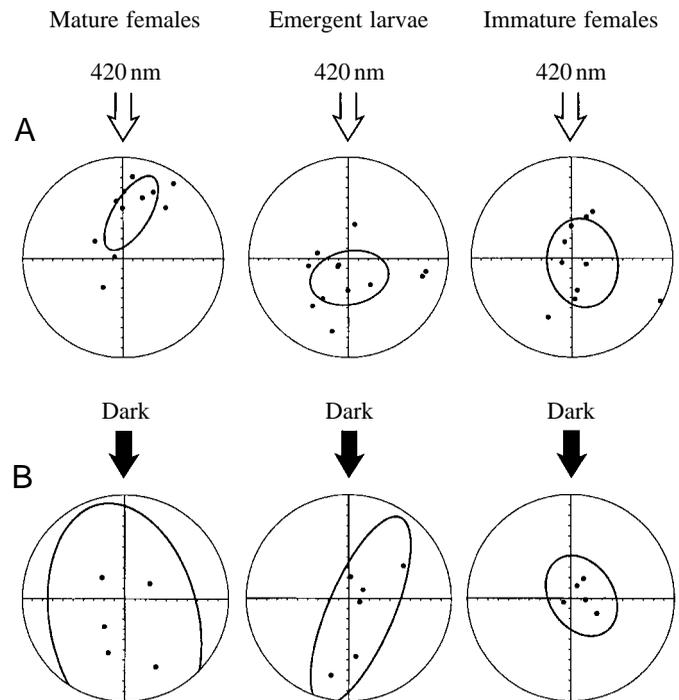

Fig. 4. Average orientations of females at the three stages of development tested in series 1 experiments. (A) Stimuli at 420 nm and optical density (OD)=0 (for corresponding intensity, see Table 1); (B) dark controls. Filled circles mark the tips of mean vectors with roots at the axis origin. Each mean vector is calculated from neck orientations measured during the last 26 min of 30 min treatment periods. The maximum possible vector length is 1.0, the radius of the circle. The Hotelling's confidence ellipse encloses the 95 % probable location of the population mean vector tip. For corresponding statistical analyses, see Table 2.

mature females and six larvae or by 9 min for the five immature females, the mean vectors of orientations measured during the 26 min periods were treated as independent.

The mean vectors are plotted in Fig. 4, with their tips marked by filled circles and their roots at the axis origins. For mature females, most of the mean vectors were strongly oriented towards light ($r>0.6$). The mean vectors for emergent larvae varied more, with two vectors strongly perpendicular to the light, one pointing strongly away from the light and several pointing weakly away from the light. The immature adult females appear to have little preference relative to light direction.

The Hotelling's confidence ellipse circumscribes the region that has a 95 % certainty of including the tip of the population mean vector (Batschelet, 1981). Where the ellipse excludes the origin, as for mature females, the population mean vector is significantly different from zero at $P<0.05$. Thus, phototactic orientation of mature females is significant. For emergent larvae, the confidence ellipse is shifted in the direction of negative phototaxis, but not significantly so. It is possible that, with a larger sample size, a negative phototaxis could have been detected.

Further statistics given in Table 2 confirm the graphical results. In the Hotelling's one-sample test, an F statistic enables a determination of probability levels (Batschelet, 1981). The Watson's U^2-statistic is used to test whether the distribution of mean orientations, disregarding vector lengths, is non-uniform. By either test, the mature females are shown to orient significantly at an optical density of 0, but not at one-tenth this intensity (optical density 1).

Like the graphical analysis, these tests do not detect phototaxis in emergent larvae or immature adults. However, in the case of emergent larvae, all but one of the 12 vectors point in directions other than towards the light (Fig. 4A). The V-test was used to test whether the mean directions are uniformly distributed, against the alternative that their net tendency is significantly towards or away from light (Burr, 1979; Batschelet, 1981). A significantly negative phototactic orientation is detected at an optical density of 0 (Table 2). This is consistent with the negative shift of the confidence ellipse (Fig. 4A) and the concentration of neck orientations in the −180 ° direction (Fig. 2A; Table 1).

Series 2 experiments

Younger (approximately 2.6-month-old) immature females

Table 2. *Orientation of mature females, immature females and emergent larvae in series 1 experiments (see Fig. 4)*

Treatment	N	n_j	Hotelling's test		Watson's U^2-test		V-test	
			$F_{(2,N-2)}$	P	U^2	P	v	P
Mature females								
420 nm, OD=0	11	6–34	10.85	<0.005**	0.31	<0.005**	0.67	<0.001**
420 nm, OD=1	6	10–28	1.45	NS	0.04	NS	0.19	NS
Dark	5	10–25	0.66	NS	0.05	NS	−0.30	NS
Emergent larvae								
420 nm, OD=0	12	16–34	2.87	NS	0.15	NS	−0.42	<0.05*
420 nm, OD=1	6	21–31	1.19	NS	0.16	>0.05	0.17	NS
Dark	6	8–31	2.54	NS	0.09	NS	−0.04	NS
Immature females								
420 nm, OD=0	10	7–28	0.60	NS	0.05	NS	−0.04	NS
Dark	5	14–56	1.68	NS	0.08	NS	0.03	NS

Mean vectors of neck orientations during each measurement period are treated as independent samples.

N, sample size; n_j, number of orientations averaged for each measurement period (range).

*$P<0.05$; **$P<0.01$; $P>0.05$ marginally not significant; NS, $P>0.1$.

See Table 1 for details of light intensities (OD).

were examined using a protocol that provided a larger sample of mean vectors, testing at both 420 nm and 500 nm and over a wider range of intensities. Previous results indicate that, for mature adults, sensitivity is not different at these two wavelengths and that at an optical density of 0 under the present conditions the intensities are saturating (Burr et al., 1989). Results are presented only for optical densities of 0 and 1; non-significant results were obtained at the lower intensities, optical densities 2 and 3.

Descriptive histograms and statistics of pooled neck orientations (Fig. 2B; Table 1) suggest that there may be a slight tendency to avoid the direction towards light, and a net negative tendency is suggested by the v statistics. Because of probable serial correlation, as discussed above, mean vectors were calculated for orientations sampled during each 8 min measurement period. Those for 420 and 500 nm at an optical density of 0 are plotted in Fig. 5. With fewer orientations

averaged than in series 1 experiments, the mean vectors plotted for each worm are more scattered (compare dark observations for immature females of Figs 4B and 5). Nevertheless, a significant negative displacement of the Hotelling's confidence ellipse is observed at 500 nm, and a non-significant negative shift is seen at 420 nm. The Hotelling's test, Watson's U^2-test and the V-test confirm these observations (Table 3).

Failure to detect a weak phototaxis at 420 nm may be due to an inadequate sample size. Increasing the sample size by pooling the mean vectors from different intensity or wavelength measurement periods appears to be justified, since the optical density 1 and optical density 0 measurement periods at each wavelength were probably independent: between them was a 3 min buffer period during which the worms were moved back to the starting positions. With the mean vectors from both periods pooled, the tests detected a significant phototaxis at 420 nm (Table 3). The 420 nm and 500 nm treatments at optical

Table 3. *Orientation of immature females in series 2 experiments (see Fig. 5)*

Treatment	N	n_j	Hotelling's test		Watson's U^2-test		V-test	
			$F_{(2,N-2)}$	P	U^2	P	v	P
Immature females								
420 nm, OD=0	9	6–16	1.91	NS	0.108	NS	−0.32	>0.05
420 nm, OD=1	8	6–12	1.85	NS	0.121	NS	−0.29	NS
420 nm, OD=1, 0	17	6–16	4.22	>0.05	0.209	≈0.025*	−0.31	<0.05*
500 nm, OD=0	10	7–12	10.07	<0.02*	0.217	≈0.025*	−0.43	<0.05*
500 nm, OD=1	8	6–13	0.05	NS	0.042	NS	−0.10	NS
420, 500 nm, OD=0	19	6–16	8.88	<0.005**	0.289	≈0.005**	−0.38	<0.01**
420, 500 nm, OD=1, 0	35	6–16	5.50	<0.02*	0.295	≈0.005**	−0.30	<0.01**
Dark	33	7–15	0.30	NS	0.046	NS	−0.05	NS

Mean vectors of neck orientations during each measurement period are treated as independent samples.

N, sample size; n_j, number of orientations averaged for each measurement period (range).

*$P<0.05$; **$P<0.01$; $P>0.05$ marginally not significant; NS, $P>0.1$.

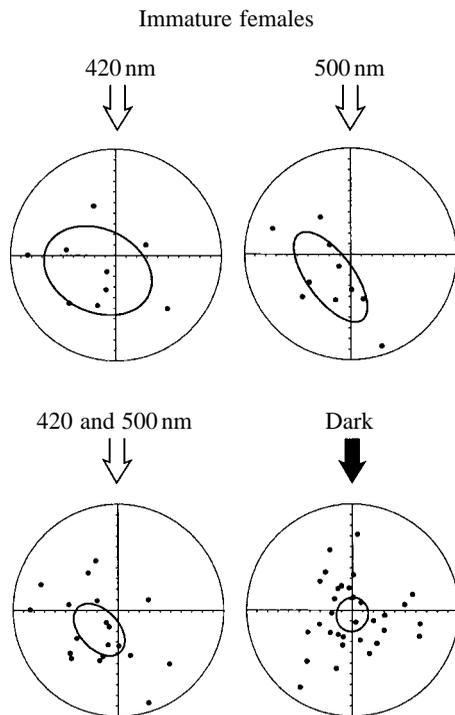See Table 1 for details of light intensities (OD).

Immature females

Fig. 5. Average orientations of immature females treated with 420 and 500 nm stimuli at an optical density of 0 (for corresponding intensity, see Table 1) in series 2 experiments. Mean vectors are pooled in the lower left figure. Each mean vector is calculated from orientations measured during the last 8 min of 10 min treatment periods. For corresponding statistical analyses, see Table 3.

density 0 can also be considered to be independent: they were separated by 40 min, and the worms were repositioned several times. With the two wavelengths combined at optical density 0, a non-uniform distribution of directions is detected at high significance by Watson's U^2-test and a highly significant negative tendency of the directions is detected by the V-test (Table 3).

Discussion

The scanning motion of the head and bending of the neck, involved in the phototaxis of mature females, was also observed in the younger stages. The 2.6-month-old immature females of series 2, but not the 3.7-month-olds of series 1, were weakly negatively phototactic to 420 or 500 nm monochromatic light. For the emergent larvae of series 1, the negative phototaxis was stronger, but still weaker than the positive phototaxis of the mature females.

Mechanism of orientation

For mature females, a strongly positive phototaxis has been observed previously using a different measure of phototaxis: the direction of locomotion relative to the direction of light (Burr et al., 1989). Here, we show that sampling the orientation of the neck yields a similar result. This is understandable according to the shadowing mechanism described previously.

The cylindrical haemoglobin pigment in the anterior tip of the worm would allow maximal illumination of a photoreceptor inside the cylinder when the anterior tip of the worm is pointed at the light source. While crawling towards the source of light, the bending motions of the head and neck swing the tip about the direction of the source, varying the illumination of the photoreceptor. The mature female preferentially orients its neck so that maximal illumination occurs when the bend in the head is zero (Burr et al., 1990; Burr and Babinszki, 1990; Gans and Burr, 1994).

How would the lack of ocellar pigmentation affect their orientation to light? We show here that, for emergent larvae and the 2.6-month-old immature adults of series 2, phototaxis is weakly negative rather than positive. Thus, the pigmentation appears to be required for positive phototaxis, and this provides further support for the shadowing mechanism.

More difficult to explain is the negative phototaxis; we can only speculate as to the mechanism. The photoreceptor is probably positioned, as indicated for the mature females, near the midline and approximately 150 μm behind the tip, where it would later be contained by the pigment cylinder (Burr and Babinszki, 1990). In the absence of the ocellar pigment, light would be able to reach the photoreceptor from all directions except the posterior and be concentrated by the cylindrical optics of the body tube and by the hemispherical anterior tip. Light-scattering by tissues as seen in Fig. 1 would certainly attenuate illumination coming from off-axis posterior directions, where the optical path is large, and scattering may diffuse somewhat the light coming from other directions. Thus, illumination of the photoreceptor is probably high for all orientations except those deviating more than approximately 130° from the source, and a minimum is expected at 180°.

In the presence of the scanning motion, the illumination can be expected to vary cyclically, and the immature females and emergent larvae may orient their neck according to the phase relationship between the photoreceptor illumination and bending, as proposed for mature females (Burr and Babinszki, 1990). Proprioceptive and photoreceptive neural signals would be compared over time. For the phototaxis to be negative, the larval and immature females would have to orient their neck preferentially to minimize, rather than to maximize, photoreceptor illumination when the bend in the head is zero. Perhaps related to this, I have observed mature females to turn away from an intense light beam (A. H. J. Burr, unpublished observation). Without screening pigment, this reaction may occur at lower intensities. However, this does not explain why the negative phototaxis is stronger in the emergent larvae than in the immature females.

Possible transverse phototaxis was observed during one out of 10 measurement periods in each of the three experiments with immature females, and in two out of 12 during the one experiment with emergent females. The large mean vectors ($r>0.7$) would require an approximately perpendicular orientation to be consistently maintained over the 26 min (series 1) or 8 min (series 2) measurement periods. However, in these experiments, because of the uncertain serial

correlation, it is not possible to test whether the tendencies were statistically significant. The shorter pathlength for light entering perpendicular to the body axis than for other orientations could provide maximal illumination when the tip of the body is oriented perpendicular to the source and thus provide an optical mechanism. Transverse phototaxis has been demonstrated in two other mermithids, which never develop ocellar pigmentation (Robinson et al., 1990). Their small, second-stage larvae actively seek and penetrate their insect host, but the role of the phototaxis is uncertain. Also, we have observed that mature adult female *M. nigrescens* occasionally have a tendency to lock on to a transverse orientation, and this was explained in terms of a secondary maximum of photoreceptor illumination leaked by the pigment cylinder at perpendicular orientations (Burr and Babinszki, 1990).

In mature *M. nigrescens*, the evidence favours photoreceptive nerve endings located near the midline inside the hollow tube of pigmentation. In the experiments reported previously (Burr and Babinszki, 1990), interrupting the light in phase with the scanning motion on swings to one side of the source direction caused neck orientation and phototaxis to deviate from the source direction. The sign of the deviation and morphological considerations indicated that photoreceptor illumination is probably compared over time during several cycles of the scanning motion, and a comparison between two spatially separate photoreceptors was ruled out (Burr and Babinszki, 1990). Even if the unpigmented early female stages had photoreceptors located laterally near the cuticle, it is difficult to conceive of simultaneous comparison giving rise to the negative phototaxis.

Why haemoglobin?

In other nematode ocelli, aggregations of melanin-like granules within extensions of pharygeal cells lie posterior and medial to the adjacent photosensory lamellae or cilia (Burr, 1984a). Casting a shadow from this location provides a good mechanism for the negative phototaxis (Burr, 1979, 1984b). Why was haemoglobin enlisted instead for the shadowing role in *M. nigrescens*?

Mermithidae typically do not have a pharynx: they feed as parasites by absorption through the cuticle. But also important is the probable need for cylindrical pigmentation peripheral to the photoreceptor to provide an optical mechanism for positive phototaxis. Haemoglobin would be suitably located, since in nematodes haemoglobin is found in the hypodermal (epithelial) cells lining the cuticle. Its high absorptivity over much of the visible spectrum could provide the shadow. Normally, however, the cytoplasmic concentration is insufficient, and the optical pathlength through the thin hypodermis is too short to impart colour or to cast a shadow. In female *M. nigrescens*, prior to the need for positive phototaxis, haemoglobin accumulates in the anterior hypodermal cells to such a high level that the cells bulge into and surround the body cavity with a thick layer of haemoglobin crystals (Burr and Harosi, 1985; Burr et al., 2000). Thus, both the very high (approximately $10\,\mathrm{mmol\,l^{-1}}$ haem) concentration

and longer pathlength provide the necessary absorptance, 25–100 % over the 350–560 nm range of phototaxis (Burr et al., 1989).

What is surprising is that the ocellar haemoglobin is normal in many ways. It exchanges oxygen and carbon monoxide reversibly, displaying characteristic spectral changes (Burr and Harosi, 1985), although its oxygen affinity is higher than normally expected for a respiratory pigment (a puzzling property of many nematode haemoglobins). Both eye globin, Mn-GLB-E, and another globin, Mn-GLB-B, are expressed at low levels along the length of the body. However, the mRNA of eye globin is expressed at high levels only in the anterior and only during the accumulation period, and high levels of expression of the other globin occurs during the adult moult. They differ in sequence by only 16 %, and in amino acids of apparently little functional or structural consequence. The secondary and tertiary structures, inferred from the amino acid sequence, are typical of all globins, and certain amino acid substitutions are characteristic of all nematode haemoglobins (Burr et al., 2000).

Thus, eye haemoglobin could be functioning at low levels in an oxygen-bearing role in other tissues, while playing an optical role at high concentration in the ocellus. It would appear that it was recruited because of its physical property of high absorptivity in most of the visible region (350–590 nm), without modification of its biochemistry. Recruitment could have occurred simply by modifying gene regulation so that it accumulates in anterior hypodermal cells to levels high enough to cast a shadow. In this respect, *M. nigrescens* eye haemoglobin is analogous to lens crystallins, which have been recruited to provide the refractive index of vertebrate lenses at high concentration, while maintaining a biochemical role at low concentration in other tissues (Piatigorsky and Wistow, 1991; Piatigorsky, 1998).

Life cycle roles of phototaxis

Mature females are positively phototactic at the time of laying eggs in vegetation. The phototaxis would guide locomotion skywards through vegetation towards regions where the potential grasshopper hosts feed (Burr et al., 1989; Gans and Burr, 1994). In the present study, we report that fourth-stage larvae burrow into the soil within a few hours after emerging from the host and are negatively phototactic at this stage. Negative phototaxis could guide crawling downwards through matted grass from the carcass of the grasshopper.

Most of the female adult stage consists of a 1- to 2-year period of dormancy in the soil during which pigmentation gradually accumulates, becoming just visible approximately 4 months post-emergence and reaching full colour in approximately a year. The negative phototaxis observed in immature females may be a remnant of the larval behaviour, weak in the 2.6-month-olds of series 2 and not detected in the 3.7-month-olds of series 1. It would be interesting to survey phototaxis at intervals under uniform conditions to resolve the transition to mature behaviour. We postulate that a change in the preference for photoreceptor illumination must occur in

addition to the remodelling of optics in the head to switch from negative to positive phototaxis. An analogous rewiring must occur in *Drosophila* spp. larvae when, midway through the third instar, they convert from negative to positive phototaxis (Godoy-Herrera et al., 1992; Sawin et al., 1994). It is possible that the ocellar optics also change: in the third-instar house fly larva, the pigmented pit becomes deeper and more forwardly oriented (Bolwig, 1946).

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