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Distribution and movement of *Caenorhabditis elegans* on a thermal gradient

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Summary

To analyze thermal responses of *Caenorhabditis elegans* in detail, distribution of a worm population and movement of individual worms were examined on a linear, reproducible and broad temperature gradient. Assay methods were improved compared with those reported previously to ensure good motility and dispersion of worms. Well-fed, wild-type worms distributed over a wide temperature range of up to 10°C, and, within this range, worms migrated in both directions of the gradient at similar frequencies without any specific response to the growth temperature in most cases. By contrast, worms migrated down the gradient if put in a region warmer than the warm boundary of distribution. The distribution range changed depending on the growth temperature and starvation, but active avoidance of a starvation temperature was not detected. These findings contradict previous hypotheses of taxis or migration to the growth temperature in association with food and instead indicate avoidance of a warm temperature. Our results favor a model for thermal response of *C. elegans* that postulates a single drive based on warm sensation rather than downward and upward drives in the physiological temperature range. Mutants in *ttx-3*, *tax-2*, *tax-4* or *egl-4* genes showed abnormal thermal responses, suggesting that these genes are involved in warm avoidance. Laser ablation and gene expression studies suggest that AFD neurons are not important, and *tax-4* expression in neurons other than AFD is required, for warm avoidance.

Key words: *Caenorhabditis elegans*, thermosensation, behavior, behavioral plasticity, neural integration, thermal gradient.

Introduction

Behavior of *Caenorhabditis elegans* on a thermal gradient has been studied as one of several plastic responses of the organism to environmental signals. *C. elegans* is suitable for studying neuronal mechanisms underlying behavioral control, since it has a compact nervous system whose connectivity has been mapped almost completely (White et al., 1986) and it moves in a relatively simple way of forward and backward movements with spontaneous turns (Croll, 1975).

It was previously reported that *C. elegans* migrates towards a feeding temperature and stays there by moving isothermally (Hedgecock and Russell, 1975). Balancing of opposing neural pathways that drive worms up or down the temperature gradient was proposed to regulate such movement (Hedgecock and Russell, 1975; Mori and Ohshima, 1995), and genes or neurons involved in the pathways have been studied (for reviews, see Bargmann and Mori, 1997; Mori and Ohshima, 1997). When starved, worms are said to disperse from that temperature (Hedgecock and Russell, 1975). These results led to an intriguing hypothesis that worms memorize a culture temperature in association with food condition and regulate their behavior in reference to the memorized temperature. However, as described below, such understanding of *C. elegans* behavior is not always based on very reliable results, and therefore the basic concept of so-called ‘thermotaxis’ and the resulting neural model are disputable.

Previous studies on the thermal behavior (or thermotaxis) of *C. elegans* were mainly performed either by observing tracks of one or a few worms on a 9 cm agar plate carrying a radial temperature gradient or by measuring the distribution of a worm population on a linear temperature gradient (Hedgecock and Russell, 1975; Mori and Ohshima, 1995; Komatsu et al., 1996; Hobert et al., 1997; Cassata et al., 2000a). We noticed several problems in both types of assay. First, ‘isothermal movement’ of a worm indicated by a circular track on a radial gradient occurs, on average, only in a small fraction of an assay period, although good examples have been provided. Worms more often migrate in other temperature regions, but such behavior has not been analysed thoroughly and understanding of the whole behavior is lacking. Second, the radial temperature gradient changes over time, even during a 1 h assay period, and is quite sensitive to room temperature. Therefore, even with a good thermal sensor, precise estimation of the actual temperature of an isothermal track at the time it is drawn is difficult. This means that although an isothermal track was assumed to provide good evidence for memory of the growth temperature, the relationship between the actual temperature at which it is drawn and growth

temperature is ambiguous. Third, in distribution assays of a worm population on a linear temperature gradient, other problems exist, mainly concerning the movement of worms. Worm motility is largely influenced by ambient temperature (Dusenbery and Barr, 1980), which might bias the distribution. Detailed observation of movement is required to assess the effect of motility as well as to determine whether *C. elegans* really exhibits thermotaxis, but such studies have not been performed until quite recently (Ryu and Samuel, 2002). In addition, inefficient motility of worms was often observed when worms were handled in a buffer solution before an assay or when they ran through a mold of Sephadex slurry, as used previously (Hedgecock and Russell, 1975; Komatsu et al., 1996; Hobert et al., 1997; Cassata et al., 2000a). Under severe conditions, worms did not disperse well even at constant temperature, raising the question of whether the observed accumulation on the gradient reflects the full range of response to a temperature gradient *per se*. Finally, linear temperature gradients that were used previously (Komatsu et al., 1996; Hobert et al., 1997) were approximately 0.8 deg. cm^{-1}, and the temperature range covered by an assay was less than 12°C. Such gradients may not have permitted the full range of movement of worms within the usual assay period of 60 min even when they were fully motile. A gradient that covers a wider temperature range should be used. The best assay period seems to be approximately 1 h, as used before, since a longer period may lead to adaptation to the new temperature, and a shorter period such as 30 min is clearly too short for movement over a wide range. Given these limitations, the gradient should also be steeper.

In the present study, we improved on previous assay methods to maintain the motility of worms and examined the distribution and movement of wild-type worms on a linear, reproducible, broader and steeper thermal gradient. The results show that fed worms dispersed over a wide temperature range. The limits of distribution were modulated by feeding temperature or by starvation. However, inconsistent with previous proposals, no specific response to either feeding or starvation temperature was detected. Together with our analysis of responses in several mutants, these results suggest a fundamental change to the basic concept of thermal behavior in *C. elegans*.

Materials and methods

Culturing worms

The following *Caenorhabditis elegans* L. strains were used; wild-type N2, FK134 *ttx-3(ks5)*, FK293 *ttx-3(tm268)*, FK100 *tax-2(ks10)*, PR678 *tax-4(p678)*, FK102 *tax-4(ks11)*, PR675 *tax-6(p675)*, DA572 *eat-4(ad572)*, MT6308 *eat-4(ky5)*, MT633 *lin-11(n389);him-5(e1467)* and FK315 *egl-4(ks61)*. We used two N2 lines of different culture history, one freshly obtained from the *Caenorhabditis* Genetics Center (CGC) and the other maintained in our laboratory. *ttx-3(tm268)* has a 2.9 kb deletion in the *ttx-3* gene, which covers the first exon and an upstream region, and was backcrossed four times to N2. Worms were grown on NGM (0.3% NaCl, 0.25% peptone, 5 μg ml^{-1} cholesterol, 1 mmol l^{-1} $CaCl_2$, 1 mmol l^{-1} $MgSO_4$, 1.7% agar in 25 mmol l^{-1} potassium phosphate buffer, pH 6.0) agar plates with *Escherichia coli* OP-50 at the indicated temperature (Brenner, 1974).

Temperature gradients and preparation of thin ttx-agar plates

A reproducible and linear temperature gradient of a broad range was produced on an aluminum slab (20 cm long × 10 cm wide × 2 mm thick) by circulating water through 3 cm-wide chambers under each end of the slab from two water baths regulated at 4°C and 33°C (Fig. 1A). The room temperature was 24–26°C. A 1.25 mm-thick ttx-agar plate containing 2% Difco Bacto agar (Becton Dickinson, Franklin Lakes, NJ, USA) and ttx-buffer (0.3% NaCl, 25 mmol l^{-1} potassium phosphate buffer, pH 6.0) was prepared. An appropriately sized piece of the ttx-agar plate (slightly larger than the frame wall described below) was cut out and transferred onto a thin plastic sheet (we used a piece of OHP sheet) and was then walled with a frame to keep worms on the plate. A frame 4 cm wide and 15 cm long was used for assays of population distribution, while a frame 4 cm wide and 6 cm long was used for movement analysis of individual worms. After placing the worms to be tested on the plate, as described below, the plate was set on the aluminum slab with a temperature gradient. Thus, the full area of the bottom of the ttx-agar plate contacted the thin plastic sheet, which was placed on the aluminum slab. The surface temperature of the aluminum slab and an agar plate put on the slab for behavior tests (see below) was monitored using a thermister probe attached to a thermometer (Anritsu 357E and HFT-58; Anritsu, Tokyo, Japan). The temperature of the agar surface reached equilibrium within 1 min (for example, agar surface temperatures at position 7 were 18.6±0.2°C), and a stable gradient of approximately 1.4 deg. cm^{-1} in the range of approximately 9–29°C was obtained (Fig. 1B).

Behavior assays

Fed worms were prepared by transferring approximately 30–100 young adult hermaphrodites to an NGM plate with plentiful *E. coli* and incubating at the original temperature for several hours before assay. To starve the worms, approximately 100 L4 larvae were transferred to a fresh plate with *E. coli* and incubated overnight. Resultant adults were harvested from the plate by flushing with ttx-buffer and collected by trapping on a 30 μm mesh screen. Worms were further flushed with ttx-buffer to wash off *E. coli*, transferred with a glass pipette onto an agar plate (2% Difco Bacto agar in ttx-buffer) and incubated at the original temperature. To test the effects of serotonin, worms were incubated for 6–7 h before behavioral assays on a plate containing 2 ml of 40 mmol l^{-1} serotonin creatinine sulfate complex (Sigma, St Louis, MO, USA) in M9 buffer (Brenner, 1974) and 8 ml of 2% agar in ttx-buffer prepared in a 6 cm-diameter dish. A control plate contained the same amount of M9 buffer. A further 200 μl of 5 mmol l^{-1} serotonin solution was poured onto some of the serotonin plates 1 h prior to the behavior assay.

For behavior assays, fed worms were allowed to briefly

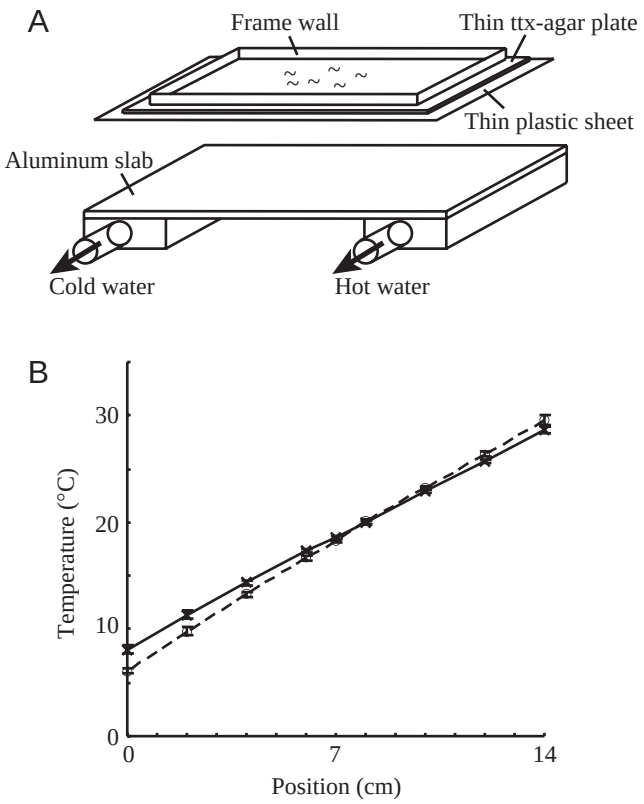


Fig. 1. (A) Illustration of the apparatus for the temperature gradient and set-up of an agar plate for behavior assays. (B) Temperature gradients on an aluminum slab and an agar plate. A temperature gradient was produced on an aluminum slab, and temperatures of surfaces of the slab (open circles, broken line) and an agar plate placed on it (crosses, solid line) were monitored at various positions, as described in the Materials and methods section. The means (± S.D.) of temperatures obtained in 19 experiments are shown.

migrate on an agar plate to remove *E. coli* from their body surfaces and were then transferred to a thin ttx-agar plate prepared as described above. Starved worms were directly placed on a thin ttx-agar plate. All these procedures were performed at room temperature within approximately 10 min. Worms were manipulated by picking them up with a platinum wire to retain motility. To make sure this manipulation did not affect behavior, we also examined the fed worms collected with buffer and handled with a glass pipette. To avoid keeping the worms in a buffer for a long period, they were collected by trapping on a mesh screen instead of settling in a tube. The worms thus collected with a buffer showed good motility, and essentially the same results were obtained as for worms manipulated by picking with a wire (data not shown).

To examine population distribution on the gradient, about 30 worms were used in one experiment. The thin ttx-agar plate with worms was set on the aluminum slab with the temperature gradient and covered with an opaque lid. Preliminary experiments showed that 30 min is sometimes too short for full dispersion, whereas 2 h is too long since starvation during the assay begins to alter the behavior (data not shown); thus, assays were run for 1 h. Worms were killed using chloroform gas, the plate was sectioned into 1 cm-wide zones perpendicular to the gradient and numbers of worms in each zone were counted under a stereomicroscope.

To monitor movement of an individual worm, about five worms were put on a thin ttx-agar plate, which was placed on a thermal gradient. The apparatus was further surrounded by a wall to protect the plate from wind and to keep the temperature constant. Worms in a 2.7 cm-long and 2 cm-wide region of the gradient were observed from above through a macro lens (Nikon Micro NIKKOR 55 mm f/2.8; Nikon, Tokyo, Japan) mounted on a CCD camera (Sony DXC-C1) and recorded on videotape. Subsequent analysis was performed on a Power Macintosh computer using the Scion Image program (Scion Corporation, Frederick, MD, USA). From the recording, 120 frames were captured at roughly 5 s intervals using the built-in digitizer of Power Macintosh G3 with a magnification of 0.1 mm pixel^{-1}. Total duration required for capturing the frames was divided by the number of intervals to obtain a mean duration between frames. *X–Y* positions of the approximate center of a worm (around the vulval position) were determined. Instantaneous velocities and their elements in directions of the thermal gradient were calculated using the displacement of the worm center in successive samples and the mean duration between frames. The values observed for worms in a 5 mm-wide zone perpendicular to the gradient were averaged. Analysis began a few minutes after the agar plate had been set on the gradient. Worms observed in a field for more than a few minutes were analysed.

Ablation of AFD neurons

A pair of AFD neurons from wild-type N2 or N2 worms carrying an extrachromosomal array containing the *gcy-8::gfp* fusion gene (a kind gift from M. Koga) as an AFD marker (Yu et al., 1997) was eliminated by laser irradiation during the L1 stage (Bargmann and Avery, 1995). The behavior of animals grown to the adult stage was examined.

Expression constructs of TAX-4 and generation of transgenic animals

Indicated promoter regions amplified from N2 genomic DNA were fused to the *tax-4* cDNA and inserted into a pPD95.77 green fluorescent protein (GFP) expression vector. The 1.7 kb upstream region of *gcy-8* (Yu et al., 1997), the 4.6 kb upstream region of *nhr-38* (Miyabayashi et al., 1999) and the 4.5 kb upstream region of *gpa-3* (Zwaal et al., 1997) were used. The construct for TAX-4 expression by the *tax-4* promoter contains the genomic region of the 13 kb promoter plus the first three exons of the *tax-4* gene and the *tax-4* cDNA of the remaining part, which is made from the *tax-4::gfp* fusion construct (a gift from I. Mori). Plasmids were injected into gonads of *tax-4(p678)* animals at a concentration of 100 ng μl^{-1} or at a concentration of 70 ng μl^{-1} together with 30 ng μl^{-1} of an injection marker, *kin-8::gfp* (a gift from M. Koga) (Mello et al., 1991).

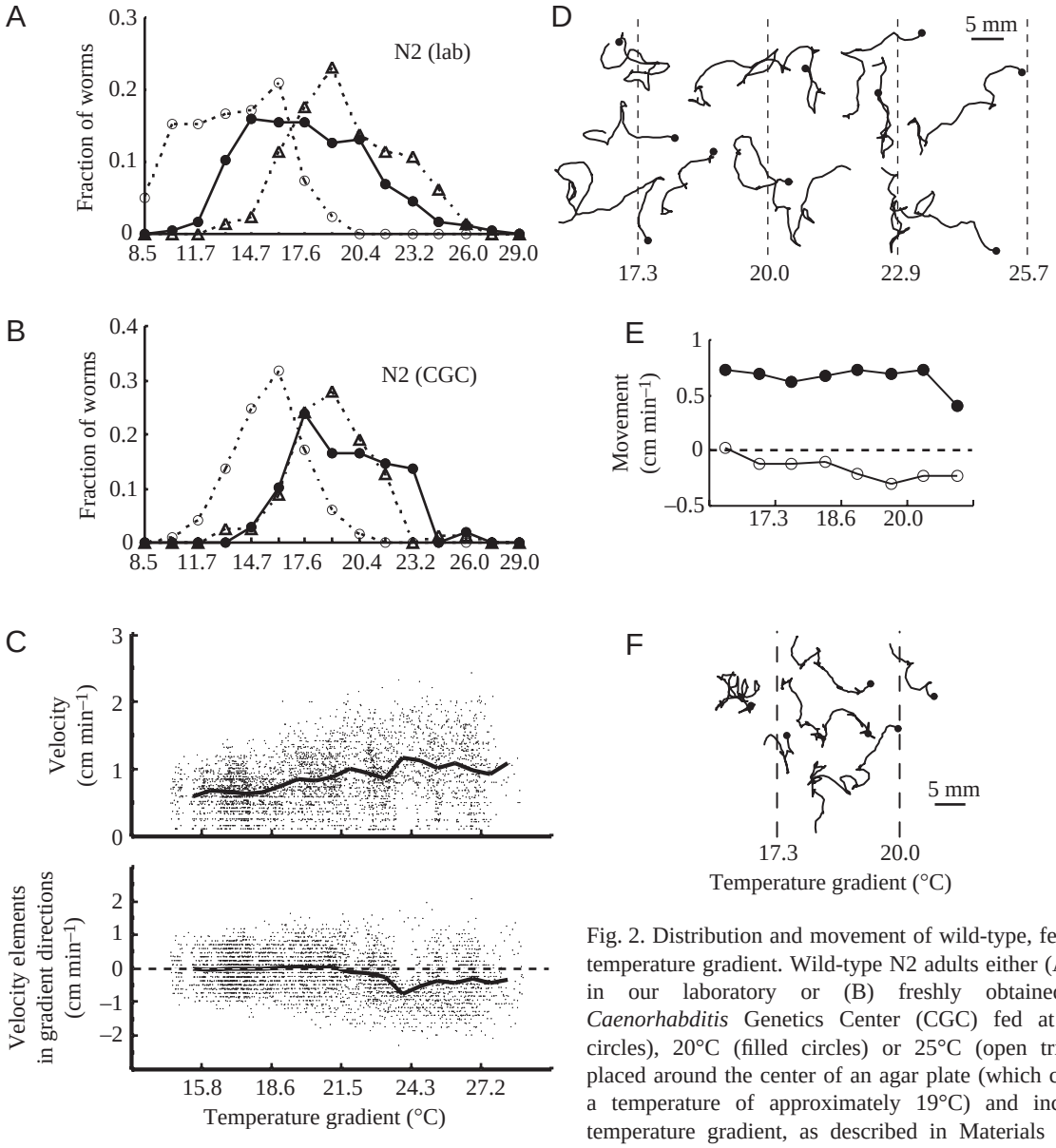


Fig. 2. Distribution and movement of wild-type, fed worms on a temperature gradient. Wild-type N2 adults either (A) maintained in our laboratory or (B) freshly obtained from the *Caenorhabditis* Genetics Center (CGC) fed at 15°C (open circles), 20°C (filled circles) or 25°C (open triangles) were placed around the center of an agar plate (which corresponds to a temperature of approximately 19°C) and incubated on a temperature gradient, as described in Materials and methods. After 1 h of incubation, distribution of worms along the gradient was determined. Approximately 30 worms were tested in one experiment and the results of several experiments were combined. Total numbers of worms (*N*) and experiments (in parentheses) were as follows: A: 25°C, *N*=130 (5); 20°C, *N*=175 (6); 15°C, *N*=163 (6); B: 25°C, *N*=79 (3); 20°C, *N*=109 (4); 15°C, *N*=117 (4). (C) N2 worms (CGC line) fed at 20°C were put on a temperature gradient and movement was observed for approximately 10 min. Instantaneous velocities (upper panel) and their elements in directions of the gradient (lower panels) are shown (dots) with their means in each area of the gradient (line). Positive values of velocity elements indicate movement up the gradient, and negative values indicate movement down the gradient. Results of 3743 points are shown. (D) Examples of traces observed in C are shown with starting positions (closed circles). Broken vertical lines show positions on the temperature gradient. (E) Movement of N2 (CGC) worms fed at 15°C was observed for approximately 10 min on a temperature gradient. Means of instantaneous velocities (filled circles) and their elements in directions of the gradient (open circles; positive values indicate upward movement; negative values indicate downwards movement) were obtained from 1141 intervals in total. (F) Examples of traces observed in E are shown with starting points (filled circles) and positions on the temperature gradient (broken lines).

Results

Well-fed worms dispersed in a wide temperature range

To ensure good motility during a behavior assay, an agar slab was used as a substrate and worms were manipulated mainly by picking them up with a wire. Worms thus transferred onto an agar plate and left for 1 h without a temperature gradient readily dispersed throughout the plate (data not shown). We first examined the distribution of wild-type worms on a linear temperature gradient. A gradient of approximately 1.4 deg. cm^{-1} was established (Fig. 1), which was steeper than those used in previous studies, expecting that it helps efficient dispersion. When wild-type (N2) worms that had been

maintained in our laboratory and cultured with *E. coli* at 20°C were left on the gradient, they scattered in a region that spanned a range of approximately 10°C (Fig. 2A). The cold limit of the distribution reached almost 13°C, and a considerable fraction of the worms was observed up to about 23°C. When wild-type N2 strain freshly obtained from the CGC was used (Fig. 2B), similar results were obtained but with a narrower distribution range of about 8°C (from 15°C to 23°C). The distribution range was not affected by the starting position of the worms; starting from places close to the distribution boundary did not expand the distribution area (data not shown). Neither was the distribution range altered as a result of different ways of manipulating the worms or different assay substrates: worms collected quickly in buffer by trapping on a screen mesh gave similar results. We also performed assays on Sephadex slurry that was thin (2–3 mm deep) and loose enough to allow efficient dispersion; essentially the same wide distribution on the gradient was observed. Assays on Sephadex slurry also suggested that the difference in humidity did not affect distribution range. From all these results, we concluded that the observed range of distribution represented a native response of the worms to the temperature itself.

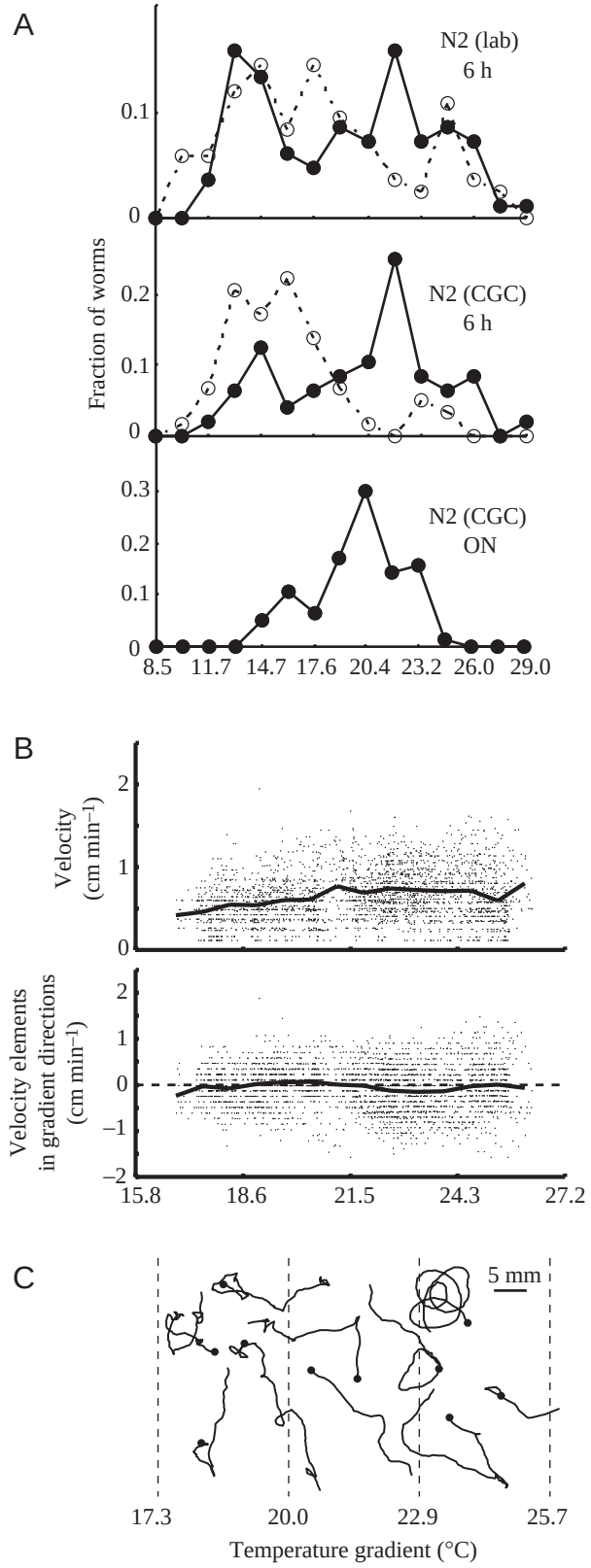
The distribution was relatively uniform instead of peaking around the feeding temperature (20°C), which implies that worms dispersed regardless of the temperature gradient in a moderate temperature range but avoided warm or cold areas. To examine the mechanisms for determining the distribution of a worm population, we observed the movement of individual worms of the wild-type strain freshly obtained from the CGC (all the movement analyses presented in this paper are those of the CGC N2 strain). When worms were put inside the distribution area, they moved both up and down the gradient (Fig. 2C,D). The mean velocity elements in directions of the gradient were nearly zero, indicating that both upward and downward movement occurred at similar frequencies without strict preference to the feeding temperature. By contrast, if worms were initially placed at a temperature higher than the limit of the distribution (approximately 23°C), almost all worms migrated down the temperature gradient (Fig. 2C,D). Once these worms reached the distribution zone, they started to move in different directions of the gradient. Within the distribution area close to the warm limit, worms maintained fast movement but did not enter the warmer region, suggesting that they controlled directions of movement near the boundary to stay inside the distribution region. We noticed some isothermal traces near the warm boundary (Fig. 2D), which may represent the same phenomena as those reported previously as isothermal movement (Hedgecock and Russell, 1975). They may occur when a worm near the boundary migrates almost straight to prevent overrunning the boundary.

These observations indicate that wild-type worms grown at 20°C migrate independently of the temperature gradient in a moderate temperature range between approximately 23°C and 13°C or 15°C but that they sense warm temperatures above 23°C and avoid them. Worms did not go into a region colder than the distribution limit either, and therefore they may sense and avoid cold temperatures as well. Alternatively, the cold boundary may be determined by frequent turning and/or reduction of motility in a cold area: although worms near the cold boundary continued to move, their dispersion was slowed by frequent turning. We could not determine if worms placed outside the cold boundary show taxis up a temperature gradient, since they lost motility in a very short time.

When wild-type worms maintained in our laboratory and fed at 15°C or 25°C were examined, both cold and warm limits of distribution shifted by about 4°C or 2°C down or up, respectively, compared with those grown at 20°C (Fig. 2A). Wild-type worms that had been freshly obtained from the CGC and cultured at 15°C or 25°C exhibited the distribution patterns shown in Fig. 2B. The distribution limits of worms fed at 15°C shifted to lower temperatures by 3–4°C but those of worms fed at 25°C were similar to those fed at 20°C, thus showing essentially no dependence on culture temperature (Fig. 2B). The ranges of distribution of worms fed at 15°C or 25°C were 7–8°C, which were similar to those of worms fed at 20°C and narrower than those of worms maintained in our laboratory (Fig. 2A). Observation of movement of worms freshly obtained from the CGC and fed at 15°C (Fig. 2E,F) confirmed that in a region warmer than their distribution range they migrated down the temperature gradient.

Behavior of starved worms

We next examined the behavior of starved worms. In contrast to fed worms, worms starved for several hours at either 20°C or 15°C dispersed in a wider range of temperatures: they went into warmer temperatures and also into colder temperatures after starved at 20°C (Fig. 3A, upper and middle panels). Worms starved at 25°C did not move very well hence we could not determine the distribution of these worms. Therefore, it is likely that starvation for several hours modulated the warm avoidance so that the worms became less sensitive to warm temperatures. In fact, in movement analysis with worms starved at 20°C, mean velocity elements in gradient directions obtained were nearly zero in a warm area above 23°C (Fig. 3B). The traces of worms placed in a warm area did not indicate migration down a temperature gradient either (Fig. 3C). Since these analyses were performed during a short period of about 10 min just after transfer from starvation culture, the results support the idea that worms starved for several hours failed to avoid warm temperatures. If worms were starved overnight at 20°C, the distribution range became narrow, between approximately 17°C and 23°C (Fig. 3A, lower panel). The more important conclusion is that distribution of worms starved either for 6 h or overnight did not indicate avoidance of starving temperatures. Although the results with worms starved at 20°C for several hours may imply a gap around 20°C, this was not observed with worms starved at 15°C or at 20°C for a longer period. We confirmed this lack of avoidance of starving temperatures by observing movement of worms starved for several hours at 20°C (Fig. 3B,C).



Worms placed at around 20°C migrated in both directions of the temperature gradient without any characteristic response around 20°C as judged from elements of velocities in directions of the gradient and worm tracks.

Fig. 3. Distribution and movement of starved animals on a temperature gradient. (A) N2 worms were starved for 6 h (upper and middle panels) or overnight (lower panel) and examined for distribution on the temperature gradient. Upper panel, N2 (lab line) starved at 20°C (filled circles; *N*=83 worms, 3 experiments) or 15°C (open circles; *N*=84 worms, 3 experiments); middle panel, N2 (CGC line) starved at 20°C (filled circles; *N*=48 worms, 2 experiments) or 15°C (open circles; *N*=58 worms, 2 experiments); lower panel, N2 (CGC line) starved at 20°C (*N*=77 worms, 3 experiments). (B) N2 worms (CGC line) starved at 20°C for 6 h were put on a temperature gradient and movement was observed for about 10 min. Instantaneous velocities and their elements in gradient directions (dots; positive values indicate upward movement; negative values indicate downwards movement) are plotted with their means in each area of the gradient (lines). Results of 2759 points are shown. (C) Examples of traces observed in B are shown with starting points (filled circles), and positions on the temperature gradient (broken lines).

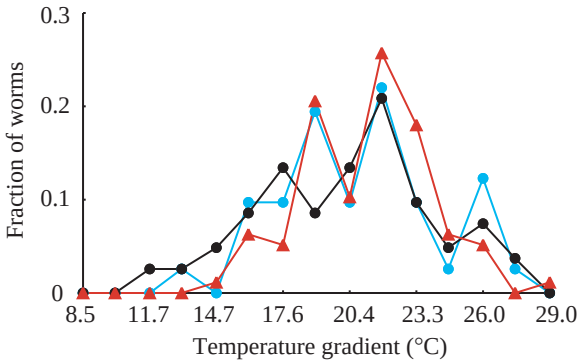


Fig. 4. Effects of serotonin treatment on starving worms. N2 adults were starved on an agar plate with or without serotonin for 6–7 h at 20°C and were then tested for distribution on a temperature gradient. Blue, without serotonin; black, with 8 mmol l^{-1} serotonin; red, 8 mmol l^{-1} serotonin with 5 mmol l^{-1} serotonin added 1 h before the test. Results of two experiments were combined.

Exogenous serotonin is known to modulate several behaviors of *C. elegans* in a manner similar to that observed with change in food levels (Horvitz et al., 1982; Avery and Horvitz, 1990). Therefore, we tested whether serotonergic signaling is involved in the modulation of warm avoidance. Treatment of starving wild-type worms with serotonin did not restore the behavior exhibited by fed worms (Fig. 4). Well-fed worms of the *bas-1* mutant (Loer and Kenyon, 1993), which has a reduced level of serotonin, avoided warm temperatures in the same way as did wild-type worms, as shown by their distribution (Fig. 5A) and movement (data not shown). Another mutant with a reduced level of serotonin, *tph-1* (Sze et al., 2000), also avoided warm temperatures in movement analysis (data not shown), but limits of distribution could not be determined due to slow movement. These results indicate that serotonin is neither required for limiting dispersal into a warm region nor sufficient to mediate food signals for modulation of warm avoidance.

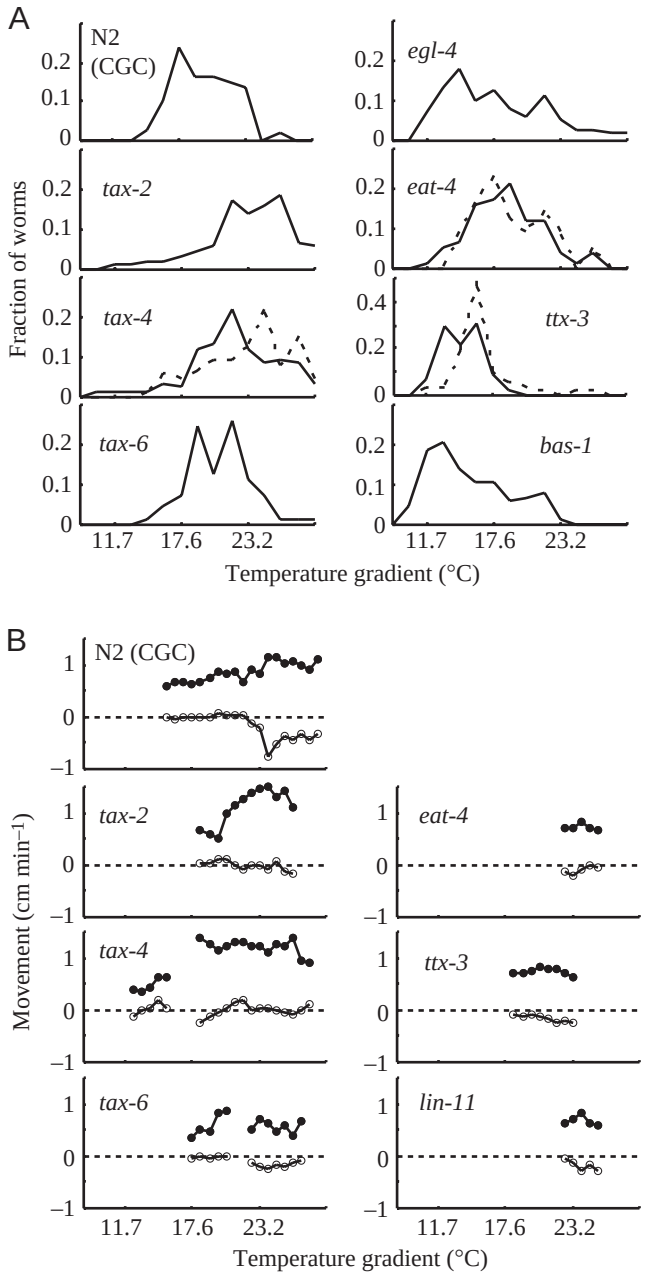


Fig. 5. Behavior of mutant worms on a temperature gradient. Wild-type N2 (CGC) or mutant worms were fed at 20°C and transferred onto a temperature gradient. (A) Population distribution after 1 h or (B) means of instantaneous velocities (filled circles) and their elements in gradient directions (open circles; positive values indicate upward movement; negative values indicate downwards movement) at each position of the gradient observed during 10 min were determined. In A, the following alleles were used, and results of 2–4 experiments were combined: *tax-2(ks10)*, *tax-4(p678)* (solid line); *tax-4(ks11)* (broken line); *egl-4(ks61)*, *eat-4(ad572)* (solid line); *eat-4(ky5)* (dashed line); *ttx-3(ks5)* (solid line); *ttx-3(tm268)* (dashed line); and *tax-6(p675)* (solid line). In B, the total numbers of analysed intervals were as follows: N2, 3743; *tax-2(ks10)*, 723; *tax-4(p678)*, 2607; *eat-4(ad572)*, 984; *ttx-3(ks5)*, 2337; *tax-6(p675)*, 1724; *lin-11(n389)*;*him-5(e1467)*, 731.

Thermal behavior is affected in mutants with defects in some neurons

Previous studies suggested that AFD neurons were thermosensory, based on abnormal behavior of worms whose AFDs were either ablated or had defects (Mori and Ohshima, 1995; Cassata et al., 2000b; Satterlee et al., 2001). Since aberrant phenotypes were observed on a radial gradient that was thought to cover 17–25°C, these phenotypes may suggest that AFDs are sensory neurons for a warm temperature. However, to our surprise, AFD-killed animals clearly avoided warm temperatures above 23°C in the same way as did wild-type worms (Fig. 6B, right panel). Furthermore, these animals were distributed in a temperature range similar to the wild-type worms (Fig. 6A), and movement assays indicated that they migrated both up and down the gradient in a region below 22°C (Fig. 6B, left panel). These results indicate that AFDs are not essential for warm avoidance. To further examine the contribution of AFDs in thermal behavior, we tested population distribution of *ttx-1* mutant animals, which were reported to be cryophilic (Satterlee et al., 2001; Ryu and Samuel, 2002) due to defects in the AFD neurons. The results varied among experiments; in some cases, the *ttx-1* animals were cryophilic and accumulated in a cold area, but in others they showed broad dispersion up to 23–24°C, as the wild-type worms did (data not shown).

Several mutants were reported to be aberrant in thermal behavior on a radial gradient. We examined if they are defective in our thermal behavior assays. Remarkably, *tax-2* and *tax-4* mutants (Hedgecock and Russell, 1975; Coburn and Bargmann, 1996; Komatsu et al., 1996), which are said to be athermotactic, dispersed into regions above 23°C (Fig. 5A), suggesting that they have defects in warm avoidance or its modulation. Analysis of single worm movement of these mutants confirmed that they moved in both directions of the gradient in a warm area at similar frequencies (Fig. 5B). Cold limits of distribution of both the *tax-2* and *tax-4* worms were warmer than that of the wild type. *tax-4* worms placed at around 15°C, a temperature below the observed cold limit of the distribution, did not seem to migrate up the gradient (Fig. 5B); therefore, *tax-4* worms might be altered in motility that resulted in the shift of the cold limit. The *tax-4* gene is expressed in several sensory neurons (Komatsu et al., 1996), among which AFD neurons were presumed to be responsible for defects of *tax-4* mutants in thermal behavior. However, expression of *tax-4* cDNA fused to a *gfp* reporter gene under the control of AFD specific promoter *nhr-38* or *gcy-8* (Yu et al., 1997; Miyabayashi et al., 1999) in *tax-4* worms did not rescue the defects in both population distribution tests and movement analysis (Fig. 7), while worms expressing *tax-4 cDNA::gfp* by *tax-4* promoter were normal in their behavior. We found that *tax-4 cDNA::gfp* expressed under the *gpa-3* promoter (Zwaal et al., 1997) also restored the wild-type behavior. This construct was expressed in several neurons, including pairs of AWB, AWC, ASG, ASK and ADL, but not

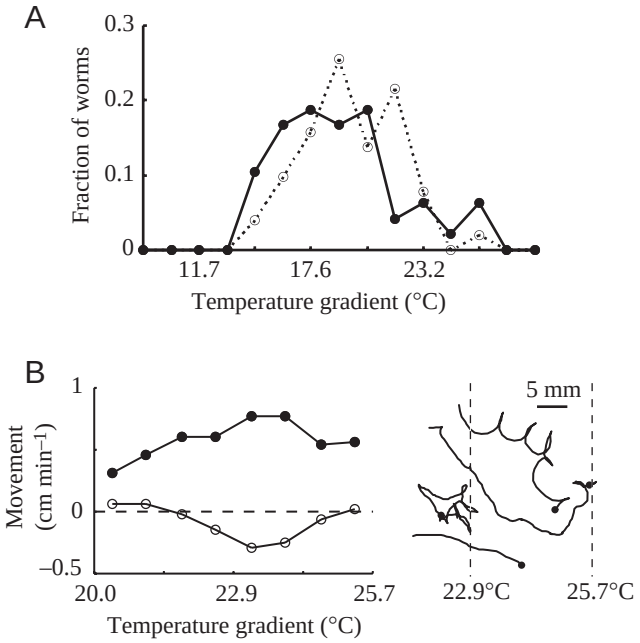


Fig. 6. AFD-killed worms avoid warm temperature. (A) AFD neurons of N2 (CGC) were irradiated during the L1 stage. After worms were grown to adults at 20°C, population distribution on a temperature gradient was examined. Results with 48 AFD-killed worms (combined from four experiments; filled circles) are shown with 51 control N2 animals (simultaneously assayed in three experiments; open circles). (B) AFD neurons of N2 carrying an AFD marker, the *gcy-8::gfp* fusion gene, were irradiated in the L1 stage and, after the worms had grown to adults, movement on a temperature gradient was observed. Means of instantaneous velocities (filled circles) and their elements in gradient directions (open circles; positive values indicate upward movement; negative values indicate downwards movement) are shown in the left-hand panel (N=1178 intervals). Examples of traces are shown in the right-hand panel.

in AFD, as judged by GFP fluorescence (data not shown). These results indicate that expression of TAX-4 in neurons other than AFD is important for warm avoidance.

With *ttx-3* mutants (Hobert et al., 1997), which were classified as cryophilic, the warm limit of the distribution became much colder than those of wild-type worms but the cold limit did not differ significantly (Fig. 5A). When *ttx-3* worms were placed at around 23°C they typically migrated down the gradient (Fig. 5B), suggesting that they avoided moderate temperatures that wild-type worms did not avoid. Interestingly, distribution of *ttx-3* mutant worms showed dependency on growth temperatures, while they were not significantly affected by starvation (Fig. 8). Such was the case with the two putative null alleles, *ks5* (Fig. 8) and *tm268* (data not shown).

For mutants classified as thermophilic, we tested *tax-6* and *lin-11* mutants (Hedgecock and Russell, 1975; Mori and Ohshima, 1995; Hobert et al., 1998; Gomez et al., 2001). Both mutants were excluded from the warm area in the same way as wild-type worms, and analysis of single worm movement confirmed that both strains preferred to go down the gradient when put in a region above 23°C (Fig. 5A,B). We confirmed previous observations (Mori and Ohshima, 1995) that on agar plates with a radial temperature gradient *tax-6* worms occasionally migrated around peripheral regions (data not shown). Since their traces looked somewhat aberrant, *tax-6* mutants may have defects in movement that results in altered behavior in radial gradient tests where the temperature gradient is not linear and is less steep in peripheral areas of the agar plates.

We also tested several other mutants with behavioral defects. Among those, *eat-4* mutant worms (Lee et al., 1999) showed weak defects in warm avoidance, as judged by movement analysis of individual worms (Fig. 5B). They were almost all excluded from a warm area after 1 h of incubation (Fig. 5A), probably because they were not completely defective in warm avoidance. Distribution of *egl-4* mutant worms (Trent et al., 1983; Daniels et al., 2000) was almost similar to that of wild-type worms, but a fraction of worms was constantly observed in a warm area above 23°C (Fig. 5A). Movement observation showed that when *egl-4* worms were placed at about 25°C, they at first migrated down the temperature gradient until they reached about 23°C, as wild-type worms did. However, after several minutes, some of the worms crossed the boundary and migrated into a warmer region (Fig. 9). Mean velocity elements in gradient directions indicated that warm avoidance became less evident at that time. Therefore, the *egl-4* gene is not an essential component of warm avoidance, but its mutation results in fast loss or modification of the avoidance.

Discussion

Distribution of worms on a temperature gradient

We determined temperature ranges of distribution of a *C. elegans* population on a thermal gradient more accurately than had been reported before, which is one of our major results. For the wild-type worms grown at 15°C, 20°C or 25°C, the distribution ranges were approximately 9–19°C, 13–23°C and 15–25°C, respectively (Fig. 2A). Wild-type N2 freshly obtained from the CGC showed a somewhat narrower (7–8°C) distribution range (Fig. 2B). These ranges are much broader than those that were expected based on previous results. In both cases, migration to a very narrow range such as growth temperature ±1–2°C did not occur. Therefore, the relationship between the growth temperature and the temperature range to which worms migrate is not close in general. The results of wild-type worms that had been freshly obtained from the CGC and cultured at 15°C may differ from those of worms fed at 20°C or 25°C (Fig. 2B) and from those of worms maintained in our laboratory (Fig. 2A). The differences between our present results and those reported previously occur mainly because we handle worms more carefully (to keep their motility intact) and use steeper and broader temperature gradients in the present work to eliminate problems previously

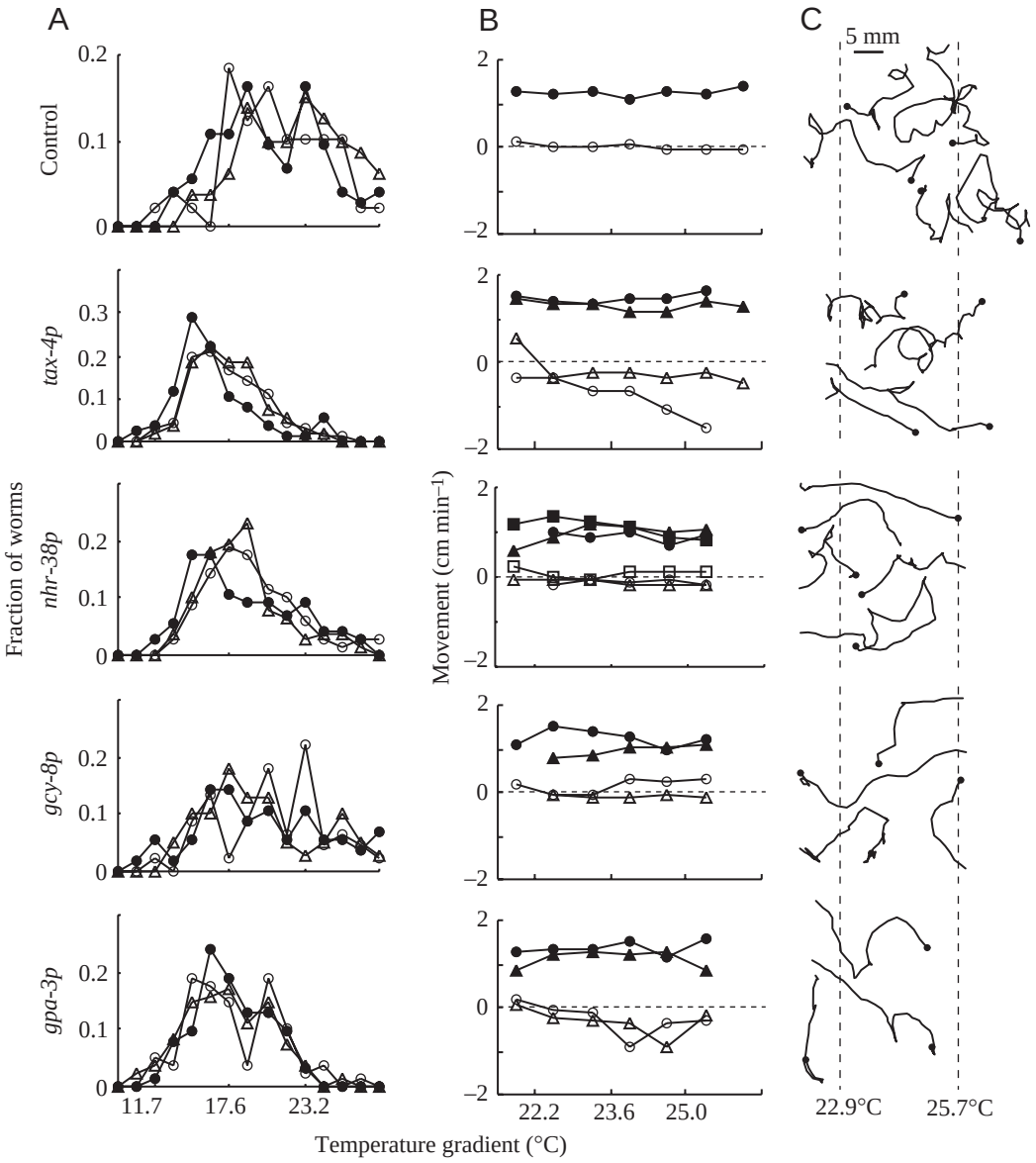


Fig. 7. Rescue of *tax-4* defect by cell-specific expression. The *tax-4(p678)* worms expressing the *tax-4 cDNA::gfp* gene under indicated promoters were examined for their behavior. (A) Population distribution was determined with worms carrying the *tax-4* expression construct and *kin-8::gfp* as an injection marker. Control worms were injected solely with *kin-8::gfp*. Combined results of 2–3 experiments are shown. Total worm numbers analysed and experiment numbers (in parentheses) were as follows: control; 74 (3), 49 (2), 80 (3); *tax-4p*, 76 (3), 71 (3), 54 (2); *nhr-38p*, 74 (3), 69 (3), 78 (3); *gcy-8p*, 56 (2), 45 (2), 39 (2); *gpa-3p*, 63 (3), 80 (3), 82 (3). (B,C) Movement was examined with worms transformed by a *tax-4* cDNA expression construct without an injection marker. Control represents the results of the parent *tax-4(p678)*. (B) Means of instantaneous velocities (open symbols) and their elements in gradient directions (closed symbols; positive values indicate upward movement; negative values indicate downwards movement) are shown. Total numbers of analysed intervals of each line were as follows: no transgene, 1109; *tax-4p*, 249 and 448; *nhr-38p*, 439, 494 and 303; *gcy-8p*, 271 and 754; *gpa-3p*, 575 and 518. In both distribution and movement analysis, two or three lines were tested for each transformation and are shown with different symbols. (C) Examples of traces.

encountered (see Introduction). We have tested different methods of worm manipulation (picking them up with a wire or collecting them very quickly with buffer) or different substrates (an agar plate or thin, dilute Sephadex gel), and a similar wide distribution was observed as long as the methods guaranteed adequate motility of worms. In addition, the limits of distribution on a gradient were not affected by the initial positions of worms. The stage of worms used (young adults), temperature of handling worms before assay and population size of worms are similar to those in previous experiments (Mori and Ohshima, 1995; Komatsu et al., 1996). Remarkably, around the warm boundary of distribution, a distinct change in movement directions was observed as discussed below. From these results, we conclude that the wide distribution represents the natural response of *C. elegans* to the temperature.

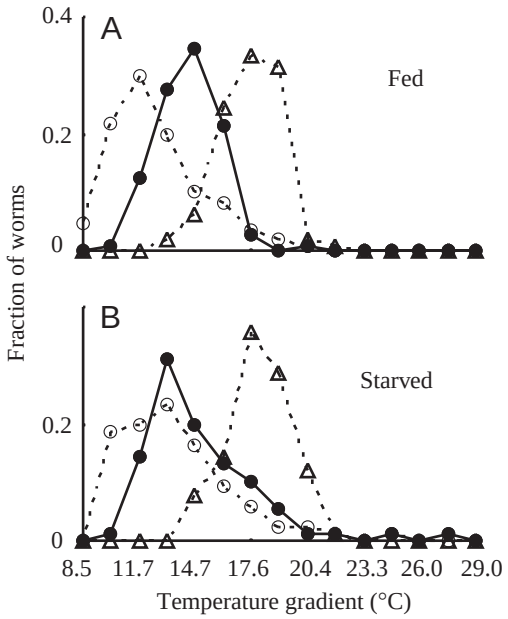


Fig. 8. Modulation of distribution of *ttx-3* mutant worms by growth temperature or starvation. *ttx-3(ks5)* mutant worms were (A) fed or (B) starved for 6 h at 15°C (open circles), 20°C (filled circles) or 25°C (open triangles) and examined for distribution on a temperature gradient. Combined results of two separate experiments are shown.

Kinetic analyses

To examine if the worms really show directional movement to a temperature, we analyzed movement of individual worms on a thermal gradient. Although the initial density of worms in the movement assay to be discussed here (approximately one worm per cm^2) is lower than that in the population distribution assay (several worms per cm^2), the latter is low enough so that interaction among worms seems negligible in both assays. Therefore, we consider that the results obtained in the two assays can be compared as those obtained under similar conditions. Good correlation between both results also supports this notion (see below). The wild-type worms that had been freshly obtained from the CGC and grown at 20°C migrated in both directions of the gradient at similar frequencies in a wide range of moderate temperatures (Fig. 2C). These kinetic analyses, together with the distribution analyses discussed above, showed absence of directional movement, or 'taxis' in a strict sense (Randall et al., 1997), and also absence of kinetic accumulation to the growth temperature, at least for worms cultured at 20°C. For N2 freshly obtained from the CGC and grown at 15°C, it may be possible that directional movement or kinetic accumulation to a temperature near 15°C is responsible for the distribution pattern shown in Fig. 2B. Avoidance of the starvation temperature was not observed in any case (Fig. 3). These findings are important since they are contradictory to the previous hypothesis for thermotaxis. Also, we observed apparent avoidance of a warm temperature (warm avoidance) by the wild type in kinetic analyses (Fig. 2C,D). Namely, worms placed in a position warmer than the distribution limit migrated down the temperature gradient until they reached the distribution area shown in Fig. 2C,D, in which most data points showed negative values of the velocity element in the gradient direction. Also, worms near the warm limit of the distribution moved actively but did not enter a warmer region (Fig. 2D). The temperature of transition from avoidance to non-avoidance in the kinetic assay is well correlated to that of the upper limit of the distribution range or so-called 'warm boundary'. Therefore, it is strongly suggested that the warm boundary of distribution results from this warm avoidance, which should be based on sensation of a warm temperature. On the other hand, mechanisms to limit dispersion into a cold area (<13°C or 15°C for worms fed at 20°C) remain unknown. Worms may sense cold temperatures and avoid them or, alternatively, motility features near the cold boundary, for example frequent turning, might reduce their dispersion. Based on these results, we propose that the major thermal response in the physiological temperature range of approximately 15–30°C, which includes the temperature range previously used for radial temperature gradients, is warm avoidance. We also propose that a single warm-sensing pathway is sufficient for thermal behavior in this temperature range (see below).

Recently, Ryu and Samuel (2002) reported evidence for migration down thermal gradients at temperatures above the cultivation temperature, which was obtained during behavioral analysis of worms subjected to temporal thermal ramps. The ranges of temperature in these ramps are similar to the warm boundaries of distribution shown in the present study, and thus such results are roughly consistent with our finding that worms avoid temperatures above the warm boundary. Our study also revealed that worms disperse into a wide temperature range below the cultivation temperature in a spatial thermal gradient instead of accumulating around the cultivation temperature, which agrees with the result that Ryu and Samuel (2002) did not detect any response to temporal thermal ramps below the cultivation temperature. Their studies are interesting in that they suggested kinetic mechanisms for migration down a spatial gradient and that isothermal tracking was also analyzed. Our studies are complementary to theirs in terms of the experiments performed and include the following points that they did not study: (1) we studied distribution of worms in a spatial gradient in detail; (2) we also analyzed starved worms in order to examine possible avoidance of a starving temperature; (3) we analyzed movement of well-fed and starved worms systematically in an entire physiological temperature range, which is much wider than that in their analysis; and (4) we analyzed many more mutants, eliminated the AFD neurones by laser irradiation and performed experiments to rescue a mutant with cell-specific gene expression. On the whole, our study suggests a fundamental change to the basic concept of thermotaxis, or accumulation of worms around the growth temperature, whereas Ryu and Samuel seem to assume the previous concept. Both our results and theirs are similar in the sense that both are against the previous model in which a balance of upward and downward

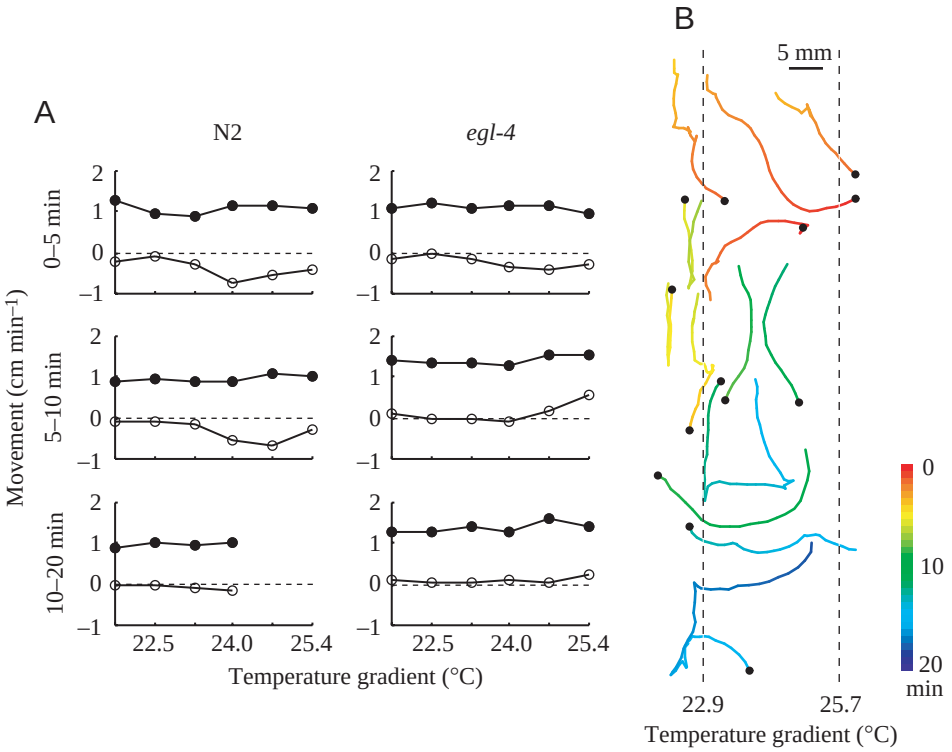


Fig. 9. Movement of *egl-4* mutant worms. N2 and the *egl-4(ks61)* worms were placed in an area at about 25°C on the gradient and observed for movement for 20 min. (A) Instantaneous velocities (filled circles) and their elements in gradient directions (open circles; positive values indicate upward movement; negative values indicate downwards movement) observed during the indicated period after the placement were averaged. After 10 min of the placement, N2 worms were excluded from a warm area above 24°C and no data was obtained above 24°C. Numbers of analysed intervals were as follows: N2: 0–5 min, 994; 5–10 min, 480; 10–20 min, 261; *egl-4*: 0–5 min, 1610; 5–10 min, 1148; 10–20 min, 890. (B) Examples of traces of the *egl-4* worms. Time is indicated by the color scale.

drives leads to aggregation of worms around the growth temperature.

Models for thermal responses

We discuss here possible models for thermal responses of *C. elegans* in the physiological temperature range. Models 1 and 2 are based on our present results. In model 1, the warm boundary of distribution is determined by warm avoidance, the cold boundary results from frequent turning or inefficient motility of worms at a cold temperature, and worms have a single temperature sensation, which is for a warm temperature. Model 2 postulates cold avoidance based on sensation of a cold temperature as well as warm avoidance to explain cold and warm boundaries. Logically, model 1 may lead to a distribution pattern in which worms accumulate near the cold boundary, giving a ‘cold trap’. Although clear accumulation of worms near the cold boundary over time was not observed, we consider that both models 1 and 2 are possible at present. The previous model proposed positive migration to the growth temperature and postulated upward and downward drives (Hedgecock and Russell, 1975; Mori and Ohshima, 1995) that may be based on cold and warm senses. This model does not require active avoidance of warm or cold temperatures. Our present results are clearly against the previous model *per se*, in the sense that directional migration to the growth temperature was not observed, However the previous model could be modified based on the present results into model 3, postulating migration to a wide temperature range that is determined in relation to the growth temperature. If model 3 is possible, it should be modified so that the temperature range in which the postulated upward drive works is below approximately 15°C or below the range covered by a radial gradient. Although models 2 and 3 are conceptually different, active migration to a wide temperature range in model 3 and avoidance of warmer and colder temperatures outside of that temperature range in model 2, which may correspond to downward and upward drives in model 3, could give the same distribution pattern of the worms. The ‘original signals’ must be different between the models: they should be warmer and colder temperatures in model 2 and the wide range of medium temperatures in model 3. Even our kinetic studies presented here cannot distinguish between these two models definitively. Identification of the original signal will give the final answer as to which model is true. However, it is much easier to assume avoidance of warm (or cold) temperatures than to assume attraction to a wide range of moderate temperatures, thus suggesting avoidance. As for the mechanism of warm avoidance, we found directional movement as shown in Fig. 2C,D, whereas Ryu and Samuel (2002) found change in the duration of forward movement in response to a temporal temperature gradient as the mechanism down a spatial temperature gradient. As far as the temperature range of their observation fits that of our warm avoidance, change in the duration should work at least as a part of the mechanism of warm avoidance.

An important point is the mechanism for dependence of the thermal response on growth temperature. In general, both warm and cold limits of the distribution depended on a feeding temperature, although such temperature dependence was not observed for CGC N2 worms between 20°C and 25°C (Fig. 2B). However, since distribution ranges were broad, it seems unlikely that worms precisely memorize a feeding

temperature and control movement by assessing the ambient temperature in comparison with the memorized temperature. In model 1, the change of warm boundary depending on the growth temperature is likely to be a change in warm avoidance based on the warm sensation, and the change of cold boundary is due to the change in motility. In model 2, change in the cold boundary results from cold sensation. In the previous model, the change in the distribution range was assumed to be due to the change in the balance between upward and downward drives (Hedgecock and Russell, 1975; Mori and Ohshima, 1995). In this model, both drives are controlled by neural pathways in reference to neural memory of the growth temperature. In models 1 and 2, it is probable that growth temperature affects warm avoidance through neuronal plasticity. However, it could be possible that some metabolic or physiological state, such as fatty acid composition (Tanaka et al., 1996), depending on the culture temperature is the 'memory' of the temperature instead of neural memory since similar thermal avoidance is also observed in organisms that do not have a nervous system (Hennessey and Nelson, 1979; Whitaker and Poff, 1980). In model 1, a single neuronal pathway for thermosensation is required, whereas two pathways are postulated in models 2 and 3.

Genes and neurons involved in the thermal response

In the previous model, athermotactic mutants such as *tax-2* and *tax-4* could most simply be explained by assuming that they are affected in the thermosensor or a thermosensory signaling pathway. Also, cryophilic mutants such as *ttx-3* are expected to be defective in upward (thermophilic) drive or neural pathway, and thermophilic mutants such as *tax-6* are expected to be defective in downward (cryophilic) drive or neural pathway. In our assays, the results with *tax-2* or *tax-4* differed from the above prediction since they behaved as if they were thermophilic; their cold and warm boundaries of distribution both shifted to warmer temperatures (Fig. 5A). The upward shift of the warm boundary is correlated with an equal frequency of downward and upward movement in the kinetic analyses above 23°C (Fig. 5B), suggesting absence of the avoidance of warm temperatures exhibited by wild-type worms. The upward shift of the cold boundary was suggested to be due to alteration of motility (see Results). The results with *tax-2* and *tax-4* mutants are explained in this way based on models 1 or 2.

In the distribution of *ttx-3* mutant worms (Figs 5A, 8), the warm boundary shifted radically to lower temperatures while cold boundaries did not change significantly as compared with those of the parental wild-type strain shown in Fig. 2A. Movement analyses detected overall downward movement in the moderate temperature region of 19–23°C, which wild-type worms do not avoid (Fig. 5B). The results with *ttx-3* can be explained in models 1 or 2 by assuming that the warm sensor or its signal pathway in *ttx-3* is changed to be sensitive to moderate temperatures to which wild-type worms are not sensitive. Furthermore, the dependence of *ttx-3* mutant behavior on culture temperature was clearly shown for the first time in our assay (Fig. 8), which is interesting. As for the modulation by starvation, serotonin did not seem to have an important role, but the *ttx-3* gene seemed to be involved since starvation did not affect distribution of *ttx-3* mutant worms. These results suggest that mechanisms involved in modulation of warm avoidance by growth temperature and those by starvation are different at least in part. Interestingly, the *egl-4* gene is not an essential component for warm sensation, but its mutation led to faster alteration of warm avoidance, probably due to starvation or the ambient temperature during the assay (Fig. 9). Thus, we suggest that *ttx-3*, *tax-2*, *tax-4* and *egl-4* genes are involved in warm avoidance or its modulation.

The neurons involved in thermal sensation and thermal signal transduction are discussed here. Previously, AFD neurons were suggested to be major thermosensory neurons (Mori and Ohshima, 1995). Unexpectedly, AFD-killed animals avoided warm temperatures (Fig. 6) in the same way as did wild type, suggesting that AFD neurons are not essential for warm avoidance. This result may be consistent with the result of Cassata et al. (2000b), which suggested basic thermotaxis without functions of AFD neurons or the *ttx-1* gene. Since AFD-killed animals showed changes in thermal responses in radial gradient assays (Mori and Ohshima, 1995), AFD neurons may have a role in thermal sensation, which was not clearly shown in the present experiments. AFD was recently reported to be involved in isothermal tracking (Ryu and Samuel, 2002), which was not analyzed here in detail. This was because clear and long-distance isothermal tracking is observed only occasionally. *tax-2* and *tax-4* genes have been reported to be expressed in 10 kinds of sensory neurons including AFD (Coburn and Bargmann, 1996; Komatsu et al., 1996). These genes are essential for normal thermal responses including warm avoidance (Fig. 5), which is also supported in the present study by recovery of normal behavior with *tax-4* promoter-driven expression of *tax-4* cDNA in *tax-4* mutants (Fig. 7). However, AFD-specific *tax-4* expression by a promoter for *nhr-38* or *gcy-8* did not rescue the behavioral defects (Fig. 7), but expression in other neurons by the *gpa-3* promoter rescued the defects. Therefore, some of the neurons expressing *tax-4*, except for AFD, must be essential for warm avoidance. Since AIY-killed animals have been reported to be cryophilic (Mori and Ohshima, 1995), AIY neurons may be important in warm sensation or warm avoidance. This possibility is supported by the expression of *ttx-3* in AIY, although not solely in AIY (Hobert et al., 1997), and by our observations showing behavioral changes in *ttx-3* mutants (Figs 5A,B, 8). We could not repeat the thermophilic phenotype of *tax-6* and *lin-11* mutant worms, which were previously proposed to have defects in the second neural pathway that includes AIZ interneurons and opposes the defect involved in the *ttx-3* phenotype.

Role of thermal behavior in C. elegans

C. elegans is reported to avoid a harmful higher temperature such as 33°C (Wittenburg and Baumeister, 1999). The mechanisms of warm avoidance are different from those during escape from such higher temperatures, since *tax-2* and *tax-4* mutants that showed normal responses to a high temperature

(Wittenburg and Baumeister, 1999) were defective in warm avoidance. *osm-5* and *osm-6* mutants, which respond poorly to a very high temperature (Wittenburg and Baumeister, 1999), clearly avoided a warm temperature above 23°C (data not shown).

This work has led to a novel understanding of *C. elegans* behavior on a temperature gradient. Such behavior seems to be suitable for survival of soil microorganisms. When there is plenty of food in soil where the temperature is largely moderate, animals just disperse. Warm avoidance would prevent them from going up to the ground surface where it might be too hot or dry for survival. Worms do not disperse into a cold region either, where they cannot retain motility. However, starvation promotes worms to explore into a wider area in search of food.

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