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Temperature and food mediate long-term thermotactic behavioral plasticity by association-independent mechanisms in *C. elegans*

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Summary

Thermotactic behavior in the nematode *Caenorhabditis elegans* exhibits long-term plasticity. On a spatial thermal gradient, *C. elegans* tracks isotherms near a remembered set-point (T_S) corresponding to its previous cultivation temperature. When navigating at temperatures above its set-point ($T>T_S$), *C. elegans* crawls down spatial thermal gradients towards the T_S in what is called cryophilic movement. The T_S retains plasticity in the adult stage and is reset by ~4 h of sustained exposure to a new temperature. Long-term plasticity in *C. elegans* thermotactic behavior has been proposed to represent an associative learning of specific temperatures conditioned in the presence or absence of bacterial food. Here, we use quantitative behavioral assays to define the temperature and food-dependent determinants of long-term plasticity in the different modes of thermotactic behavior. Under our experimental conditions, we find that starvation at a specific temperature neither disrupts T_S resetting toward the starvation temperature nor induces learned avoidance of the starvation temperature. We find that prolonged starvation suppresses the cryophilic mode of thermotactic behavior. The *hen-1* and *tax-6* genes have been reported to affect associative learning between temperature and food-dependent cues. Under our experimental conditions, mutation in the *hen-1* gene, which encodes a secreted protein with an LDL receptor motif, does not significantly affect thermotactic behavior or long-term plasticity. Mutation in the *tax-6* calcineurin gene abolishes thermotactic behavior altogether. In summary, we do not find evidence that long-term plasticity requires association between temperature and the presence or absence of bacterial food.

Supplementary material available online at
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Key words: *C. elegans*, behavior, thermotaxis, navigation, long-term plasticity.

Introduction

Animals adapt to their environment by adjusting behavioral strategies in response to experience. The nematode *Caenorhabditis elegans* exhibits several forms of behavioral plasticity in response to food-dependent cues. For example, *C. elegans* moves more slowly in the presence of bacterial food than in its absence, and the degree of slowing depends on prior starvation (Sawin et al., 2000). Starvation causes a variety of changes in *C. elegans*' behavior that may improve its likelihood of finding food. For example, when *C. elegans* is removed from food, its initial behavioral strategy is area-restricted search of its immediate vicinity. After several minutes of prolonged starvation, *C. elegans* changes its strategy to long-range dispersal, which is characterized by extended periods of uninterrupted forward movement (Hills et al., 2004; Tsalik and Hobert, 2003; Wakabayashi et al., 2004; Gray et al., 2005). Rhythmic pumping of the pharyngeal muscles enables *C. elegans* to consume food, and starvation increases the pumping rate, presumably increasing the likelihood of ingesting food by chance (Avery and Horvitz, 1990). Such behavioral alterations are triggered directly by starvation and do not require integration of different sensory inputs. Food also modulates behavior in ways that imply associative links between different sensory inputs. For example, starving or feeding animals in the presence of salt or certain volatile chemicals modulates chemotaxis with respect to those chemicals (Saeki et al., 2001; Nuttley et al., 2002; Torayama et al., 2007).

Thermotaxis is one of the best-studied forms of long-term experience-dependent behavior in *C. elegans* (Bargmann and Mori, 1997). Hedgecock and Russell (Hedgecock and Russell, 1975) discovered that, when placed on a spatial thermal gradient, *C. elegans* is able to track isotherms and accumulate near the temperature of its previous cultivation, as well as avoid temperatures at which they were starved. An attractive interpretation of thermotactic behavior is that it helps *C. elegans* to find food, either by driving worms towards their previous

cultivation temperature or away from starvation temperatures. Along this vein, mechanisms for long-term plasticity in *C. elegans* thermotaxis have been postulated to involve direct association between temperature and food-dependent cues.

C. elegans exhibits different modes of thermotactic behavior in different temperature ranges. When *C. elegans* is placed on a spatial thermal gradient within 2–3°C of its previous cultivation temperature, it tends to track isotherms, exhibiting a stored memory that we call the thermotactic set-point (T_S). Luo et al. analyzed the strength of isothermal tracking behavior and found that the frequency or duration of isothermal tracks exhibited by worms navigating near their T_S was not significantly affected by starvation at their T_S for as long as 4 h (Luo et al., 2006). They also found that worms exhibit isothermal tracking behavior on spatial thermal gradients both in the presence and absence of bacterial food (Luo et al., 2006). Thus, isothermal tracking behavior may not be a mechanism by which *C. elegans* locates food, but simply a mechanism for staying near an acclimated temperature, irrespective of bacterial food.

The T_S can be reset by cultivation of animals at a new temperature (Hedgecock and Russell, 1975). Recently, we studied the determinants of T_S resetting by analyzing isothermal tracking behavior (Biron et al., 2006). We grew worms overnight at 15°C or 25°C, shifted the worms for defined periods of time to new temperatures, and quantified the temperature range of the isothermal tracking behavior on steep linear thermal gradients. We found that worms gradually reset their T_S from 15 to 25°C or from 25 to 15°C over about 4 h with exponential time courses, and these time constants for T_S resetting were unaffected by the presence or absence of bacterial food at the new temperature. These observations suggested that T_S resetting occurs *via* integration of temperature history over time and does not require an association between temperature and food cues.

When *C. elegans* is placed more than 2°C above its previous cultivation temperature ($T>T_S$), it crawls towards lower temperatures in what is called cryophilic movement (Hedgecock and Russell, 1975). The behavioral strategy that underlies cryophilic movement is the biased random walk (Ryu and Samuel, 2002; Clark et al., 2007). *C. elegans* motility in isotropic environments is a random walk that is characterized by successive periods of forward movement (runs) interrupted by spontaneous reorientation maneuvers (turns and reversals). During cryophilic movement, *C. elegans* biases its random walk towards lower temperatures by prolonging or shortening each run in response to decreasing and increasing temperatures, respectively.

C. elegans has also been reported to exhibit thermophilic movement, in which worms crawl towards their cultivation temperature from lower temperatures or in which thermophilic mutants migrate to the warmest point on a spatial thermal gradient (Hedgecock and Russell, 1975; Mori and Ohshima, 1995). However, Ryu and Samuel (Ryu and Samuel, 2002) found that *C. elegans* exhibits an unbiased random walk when it navigates at $T<T_S$. Yamada and Ohshima (Yamada and Ohshima, 2003) showed that, whereas *C. elegans* exhibits active cryophilic movement towards its cultivation temperature from higher temperatures, it is atactic at temperatures below its cultivation temperature. One possibility is that *C. elegans* exhibits active thermophilic behavior within a narrower range of experimental parameters than it does for isothermal tracking behavior or cryophilic behavior. Such an argument may reconcile reports of thermophilic behavior (e.g. Hedgecock and Russell, 1975; Mori and Ohshima, 1995) with other reports that worms do not actively move up thermal gradients at $T<T_S$ (e.g. Ryu and Samuel, 2002; Yamada and Ohshima, 2003).

The complexity of *C. elegans* thermotactic behavior and the discrepancies between different studies underscore the importance of quantifying thermotactic behavior during performance of the navigational task and under well-defined and reproducible experimental conditions. Standard behavioral assays define thermotactic phenotypes by allowing worms to navigate thermal gradients and then measuring the temperature at which they accumulate (Ito et al., 2006). However, it is difficult to dissect thermotactic behavior using snapshots of worm accumulation after thermotactic behavior has already taken place. It is more informative to track and quantify the movements of individual worms as they actively respond to well-defined thermal gradients (Clark et al., 2007; Luo et al., 2006).

In the present study, we continue our analysis of associativity in long-term thermotactic plasticity since recent results suggest that associative learning may not be required. In particular, Yamada and Ohshima (Yamada and Ohshima, 2003) found that worms do not specifically avoid temperatures at which they were starved, and Biron et al. (Biron et al., 2006) showed that T_S resetting is not affected by the presence or absence of bacterial food. Here, we quantify the thermotactic movements of *C. elegans* on spatial thermal gradients after sustained exposure to specific temperatures with and without bacterial food. After confirming that unstarved worms exhibit cryophilic behavior at $T>T_S$ and isothermal tracking behavior near the T_S under our experimental conditions, we went on to demonstrate that T_S resetting towards ambient temperature is unaffected by food and, more specifically, that *C. elegans* does not actively avoid temperatures at which it has been starved. While starvation did abolish cryophilic behavior, it did not induce behavior that was correlated to the starvation temperature. We found no evidence that long-term thermotactic behavioral plasticity requires associative learning between temperature and food-dependent cues.

Materials and methods

Strains

Caenorhabditis elegans wild-type Bristol N2 were cultivated using standard methods (Brenner, 1974). The wild-type strain (var. Bristol N2) and *hen-1(tm501)*, *ttx-3(ks5)* and *tax-6(p675)* mutant strains were obtained from the *C. elegans* Genetics Center (http://www.cbs.umn.edu/CGC/). Worms were grown on bacterial lawns of the *Escherichia coli* strain OP50. In cases that worms were starved for defined periods of time, they were removed from overnight cultivation with bacterial food, rinsed in NGM buffer (Sulstan and Hodgkin, 1988) and placed on clean agar plates without bacterial food.

Quantitative analysis of thermotactic behavior at temperatures above or below the cultivation or starvation temperature

Young adult worms that were cultivated overnight and/or starved at 15°C or 25°C were transferred to the surface of a

9 cm-diameter plate with a linear spatial thermal gradient spanning 18–22°C without bacterial food. The plate was allowed to thermally equilibrate for 5 min and then the positions of individual worms were recorded every 2 s for 60 min. A ring of glycerol was distributed around the rim of the Petri plate to prevent worms from crawling up the agar meniscus and the plastic walls of the Petri plate, allowing us to monitor a population of worms for long periods of time without losing sight of individual worms. Individual worm trajectories were analyzed using MATLAB (The Mathworks, Natick, MA, USA) using custom-written software for population-wide behavioral quantification as described in Clark et al. (Clark et al., 2007). The position of each worm over time allowed us to calculate the distribution of positions on the gradient and the mean migration over time. In addition, the trajectories of individual worms were broken down into alternating periods of runs and reorientations. Run duration was calculated by flagging the reorientation at the start and end of each run as any change in the direction of worm movement by >45° in 4 s, a procedure that is insensitive to small-angle turns. Run orientation was calculated with respect to gradient direction. The statistics of run duration as a function of run orientation were calculated using MATLAB.

Quantitative analysis of isothermal tracking behavior

Young adult worms were cultivated overnight at 15°C or 25°C and then transferred as young adults to 25°C or 15°C, respectively, for fixed intervals of time. Individual worms were then transferred onto a 9 cm-diameter plate with a linear spatial thermal gradient of 0.5 deg. cm^{-1} without bacterial food. The movements of individual animals were recorded for 25 min. Isothermal tracks were defined as straight vertical trajectories significantly longer (>2 cm) than the vertical trajectories exhibited by worms at temperatures far from the T_S. The temperature corresponding to each isothermal track was scored manually. The T_S for each set of conditions was measured as an average of tracking temperatures for each population of animals.

Results

Prolonged starvation suppresses cryophilic movement at $T>T_S$

To evoke cryophilic movement at $T>T_S$, we cultivated worms overnight at 15°C, and placed populations of worms near the middle of a linear thermal gradient spanning 18–22°C, such that the mean temperature of the population started near 20°C. This temperature range was selected to avoid evoking isothermal tracking behavior, which occurs within 2–3°C of T_S (Hedgecock and Russell, 1975; Luo et al., 2006). Over the course of observation, worms actively migrated towards the cold side of the plate, and the mean temperature of the population decreased over time (Fig. 1A; Movie 1 in supplementary material). By contrast, when worms cultivated at 25°C were placed on the same spatial thermal gradients, again to avoid evoking the isothermal tracking behavior, they moved randomly and distributed themselves uniformly between 18 and 22°C (Fig. 1B; Movie 2 in supplementary material). Thus, under these experimental conditions, well-fed *C. elegans* exhibits an active mechanism for cryophilic movement at $T>T_S$ and is atactic at $T<T_S$.

Next, we asked whether starvation affects cryophilic movement. In this case, we analyzed worms that had been cultivated overnight at 15°C but then placed on agar plates without food at 15°C for several hours before being tested on the spatial thermal gradient (Fig. 2). In order to quantify and compare the movements of worm populations, we tracked the temperature position of individual worms within the population as they navigated the linear thermal gradients over time. In each experiment, worms were again placed near the middle of the 18–22°C thermal gradient, so that the mean temperature of the population started at 20°C. We found that cryophilic movement becomes suppressed after worms are deprived of food. After 2 h of starvation at 15°C, *C. elegans* is able to move towards the cold side of the plate as quickly as well-fed worms. After 4 or more hours of starvation, the mechanism for cryophilic movement becomes weaker, and worms change their behavior to more-random dispersal on the spatial thermal gradient, and the mean temperature of the population stays at 20°C (Fig. 2). In this experimental set-up, random dispersal may be inferred from constancy in the mean temperature position; as worms distribute themselves uniformly on the 18–22°C thermal gradient over time, the mean temperature of the population stays near 20°C. However, *C. elegans* does not appear to invert its thermotactic behavior after prolonged starvation; worms do not actively migrate to and accumulate near the warm side of the plate after being starved at cold temperatures (Fig. 2).

Quantifying the net migration of worm populations up or down spatial thermal gradients is a macroscopic measure of thermotactic behavior. When interpreting the macroscopic behavior of worms, it is important to know whether changes in macroscopic behavior are, in fact, due to changes in the underlying navigational strategy that affects the detailed movements of individual worms. To execute cryophilic movement, *C. elegans* extends (or shortens) periods of forward movement that are directed towards colder (or warmer) temperatures (Clark et al., 2007). Thus, by analyzing the trajectories of individual worms navigating spatial thermal gradients and quantifying the amount of bias in the random walk, we are able to obtain a microscopic measure of navigational strategy.

In order to show that changes in thermotactic behavior for the data shown in Figs 1 and 2 can be attributed to changes in navigational strategy, we quantified run duration as a function of run orientation for the detailed trajectories of individual worms in the manner described by Clark et al. (Clark et al., 2007). For well-fed wild-type animals at $T>T_S$, mean run duration is a smoothly varying function of the angle between the run and gradient directions: runs pointed down (or up) the gradient are extended (or shortened) (Fig. 3A,B). For well-fed animals at $T<T_S$, run duration is not modulated by the gradient direction (Fig. 3A,B). Using the average duration of all runs pointed down the gradient (τ_{dn}) (within 36° of down) and the average duration of all runs pointed up the gradient (τ_{up}) (within 36° of up), we may compute an index for cryophilic bias:

$$\text{Cryophilic bias} = (\tau_{dn} - \tau_{up}) / (\tau_{dn} + \tau_{up}) . \quad (1)$$

Thus, a positive cryophilic bias should drive worms towards colder temperatures on a spatial temperature gradient; zero cryophilic bias should lead to random dispersal; negative cryophilic bias would drive worms towards higher temperatures in what would be thermophilic migration.

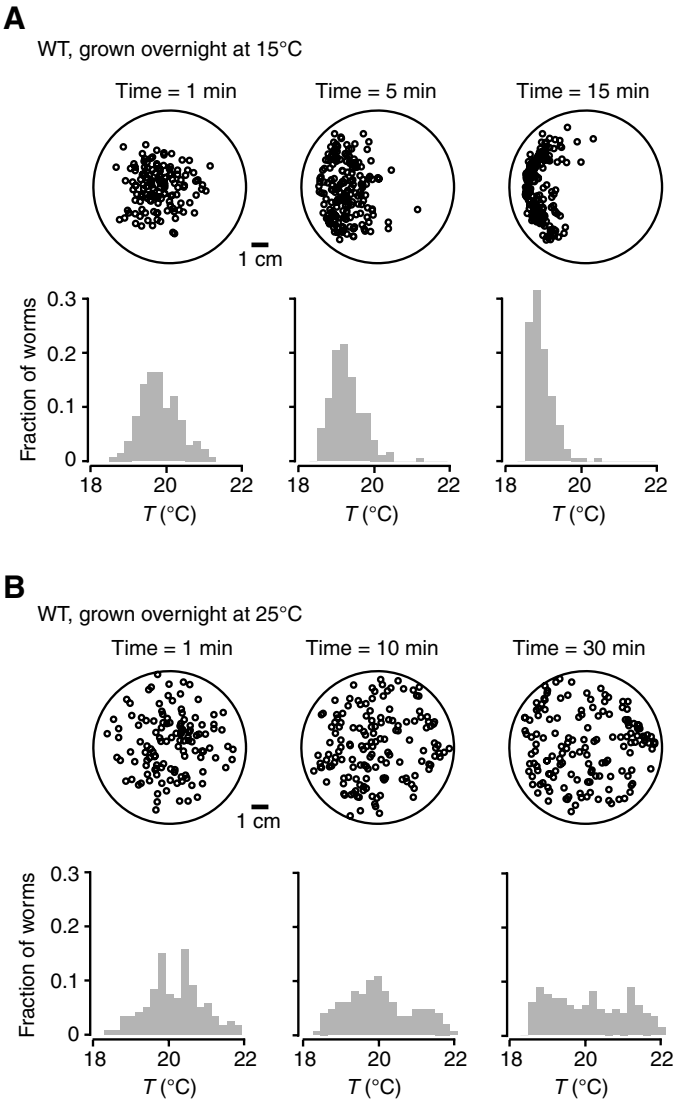

Fig. 1. *C. elegans* crawls towards its set-point (T_S) from higher temperatures but not from lower temperatures. (A) Wild-type (WT) worms that were grown overnight at 15°C were initially placed near the middle of a linear thermal gradient spanning 18–22°C across a 9 cm plate, and their instantaneous positions over time were recorded by video microscopy. Each upper panel shows overlaid snapshots of instantaneous worm positions from six independent plates containing ~100 worms in total, with the position of each worm indicated by an open circle. Lower panels show the corresponding histograms of worm positions. The evolution of the worm distributions over time indicates that these worms migrate towards the previous cultivation temperature in what is called cryophilic movement. (B) When wild-type worms that were grown overnight at 25°C were initially placed near the middle of the linear thermal gradient, they exhibit random dispersal.

A simple way to quantify macroscopic cryophilic migration in the experiments shown in Figs 1 and 2 is to quantify the net change in the mean temperature of the population of worms after 15 min of navigation. Indeed, as prolonged starvation reduced the macroscopic measure of cryophilic migration, the microscopic measure of cryophilic bias was correspondingly reduced (Fig. 3C). Thus, the effect of prolonged starvation on cryophilic movement is directly linked to changes in the biased random walk strategy.

Long-term plasticity does not require associative learning between temperature and food

Biron et al. showed that worms cultivated overnight at 15°C (or 25°C) and then shifted as adults to 25°C (or 15°C) will shift their T_S within roughly 4 h as measured by the temperature range of isothermal tracking behavior (Biron et al., 2006). Since we find that prolonged starvation suppresses cryophilic movement, we can make specific predictions regarding the movement of worms on spatial thermal gradients after growing worms at one temperature and then shifting them to a new temperature in the presence or absence of food.

We grew worms overnight at 15°C or 25°C, shifted them as adults with or without food to 25°C or 15°C for 4 h and then analyzed their movements on spatial thermal gradients spanning 18–22°C (Fig. 4A,B). We chose the 4 h interval for starvation as it allows time for readjustment of T_S (Biron et al., 2006) without completely eliminating cryophilic movement (Fig. 2B). As expected, unstarved worms that had been grown overnight at 15°C exhibited robust cryophilic movement on the spatial thermal gradients (Fig. 4B). When worms were grown overnight at 15°C and starved at 15°C for an additional 4 h, they exhibited weakened cryophilic movement (Fig. 4B). However, when worms were grown overnight at 15°C and starved at 25°C for 4 h, they exhibited atactic movement, consistent with the T_S having shifted to higher temperatures. In this case, worms distributed themselves uniformly across the thermal gradient, and we did not observe avoidance of the 25°C starvation temperature (Fig. 4A). Similar results were obtained with worms grown overnight at 15°C and shifted to 25°C for 4 h in the presence of food (data not shown). Unstarved worms that had been grown overnight at 25°C exhibited random dispersal on the 18–22°C spatial thermal gradients since their $T<T_S$. When worms were grown overnight at 25°C and starved at 25°C for an additional 4 h, they continued to exhibit random dispersal. However, when worms were grown overnight at 25°C and starved at 15°C, cryophilic movement is restored, consistent with the T_S having shifted to lower temperatures (Fig. 4B).

Mutations in the *ttx-3* gene, which encodes a LIM-homeobox gene that is expressed in the AIY interneuron, cause worms to exhibit cryophilic movement at all temperatures, irrespective of the T_S (Hobert et al., 1997). Upon starvation, similar to wild-type worms, the cryophilic movement of *ttx-3* mutants is also decreased (Fig. 4C).

If worms actively avoid the specific temperatures at which they had been starved, then, if placed on a spatial thermal gradient that spans the starvation temperature, they might seek to evacuate themselves from the starvation temperature. To study this possibility, we placed worms that had been starved at 20°C on spatial thermal gradients that span 20°C. However, worms simply crawled randomly to all temperatures on such spatial thermal gradients, spreading themselves uniformly over time on 18–22°C gradients and on 15–25°C gradients (Fig. 5A,B).

Our observations suggest that *C. elegans* changes its T_S based on sustained exposure to specific temperatures, but not in association with the presence or absence of food. Since

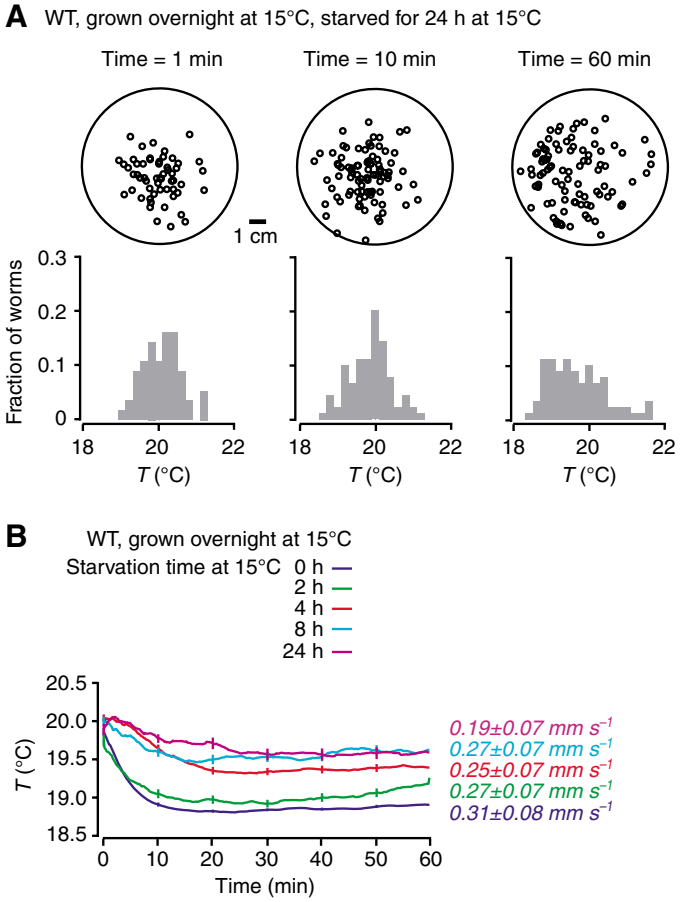

Fig. 2. Starvation inactivates the mechanism for cryophilic movement. (A) When wild-type (WT) worms that were grown overnight at 15°C then starved for 24 h at 15°C are placed near the middle of the linear thermal gradient, they exhibit nearly random dispersal. (B) From snapshots of the temperature positions of individual worms on the spatial thermal gradients, we calculated the mean temperature of the population, and we plotted the mean temperature over time for worms that had been starved for different durations. Worms were initially placed near 20°C, and a subsequent decrease in the mean temperature indicates cryophilic movement. The speed of instantaneous forward-crawling movements exhibited by individual worms in each experiment is indicated in italics (mean ± s.d.), showing that the atactic behavior caused by starvation is not simply due to lack of mobility.

starvation weakens the cryophilic movement towards the stored T_S, the overall navigational behavior of starved animals is altered, but not in a way that requires worms to learn temperatures at which they were starved.

Thermotactic behavior in putative associative learning mutants

We next examined worms carrying mutations in genes previously suggested to alter associative learning between temperature and food cues. Mutations in the *hen-1* gene, which encodes a secretory protein with an LDL receptor motif, have been suggested to affect associative learning in thermotaxis between temperature and the absence of food. Worms carrying the *hen-1(tm501)* mutation have been claimed to be defective in avoiding temperatures at which they have been starved (Ishihara et al., 2002). One interpretation is that *hen-1* affects integrative behavior, as it is expressed in interneurons that are

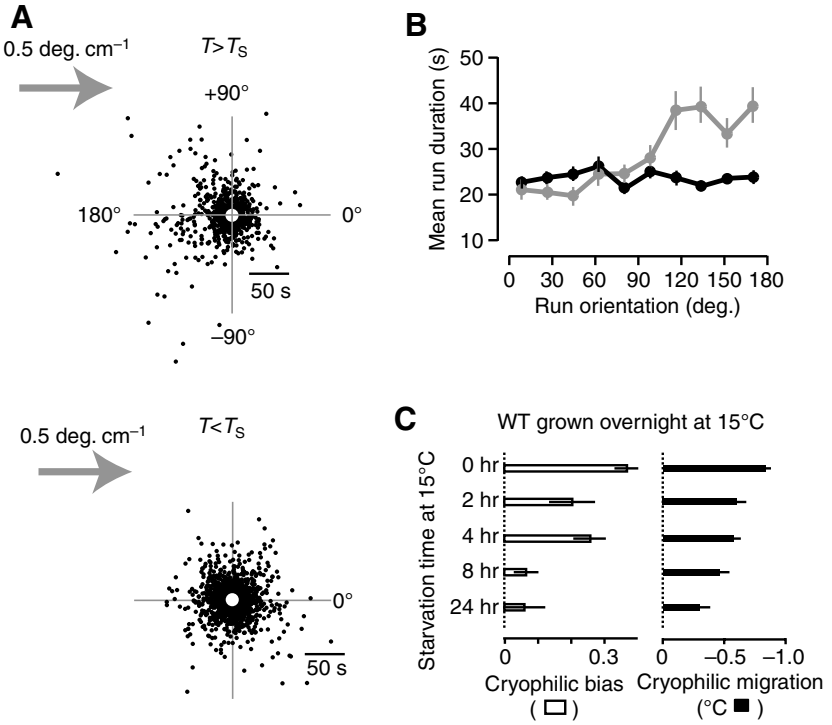

Fig. 3. Changes in cryophilic movement are attributed to changes in cryophilic bias. (A) Scatter plots show the correlation between run orientation and run duration of the detailed crawling trajectories exhibited by individual well-fed wild-type (WT) worms navigating a linear spatial thermal gradient at $T>T_S$ (upper plot) and at $T<T_S$ (lower plot). The starting point of all runs is set to the origin. Each black dot denotes the relative end-point of each run; duration is indicated by distance from the origin (see scale bar) and run orientation is indicated by the angle with respect to the thermal gradient (defined to be 0° for worms crawling up the spatial gradient, shown by the arrow). For wild-type worms tested at $T>T_S$ (cultivated at 15°C and allowed to navigate on a gradient between 18–22°C), runs oriented down the gradient are extended, and runs oriented up the gradient are shortened. By contrast, for wild-type worms tested at $T<T_S$ (raised at 25°C and allowed to navigate on a gradient between 18–22°C), there is no significant correlation between run orientation and duration. Each scatter plot represents run statistics collected from ~1000 runs exhibited by ~100 worms. (B) Plots of mean run duration as a function of run orientation of wild-type animals, corresponding to the scatter plots in A. Error bars represent 1 s.e.m. Cryophilic bias at $T>T_S$ is represented as prolonged runs pointed down the gradient (grey data points, fit to a constant with $P<10^{-5}$). The weak or undetectable thermotactic response at $T<T_S$ is represented as invariance of run duration with run orientation (black data points, fit to a constant with $P>0.1$). (C) Two measures of cryophilic behavior – the cryophilic bias, calculated using Eqn 1, and the mean of cryophilic migration after 15 min – are plotted for wild-type worms that had been starved for different durations. Errors bars are 1 s.e.m.

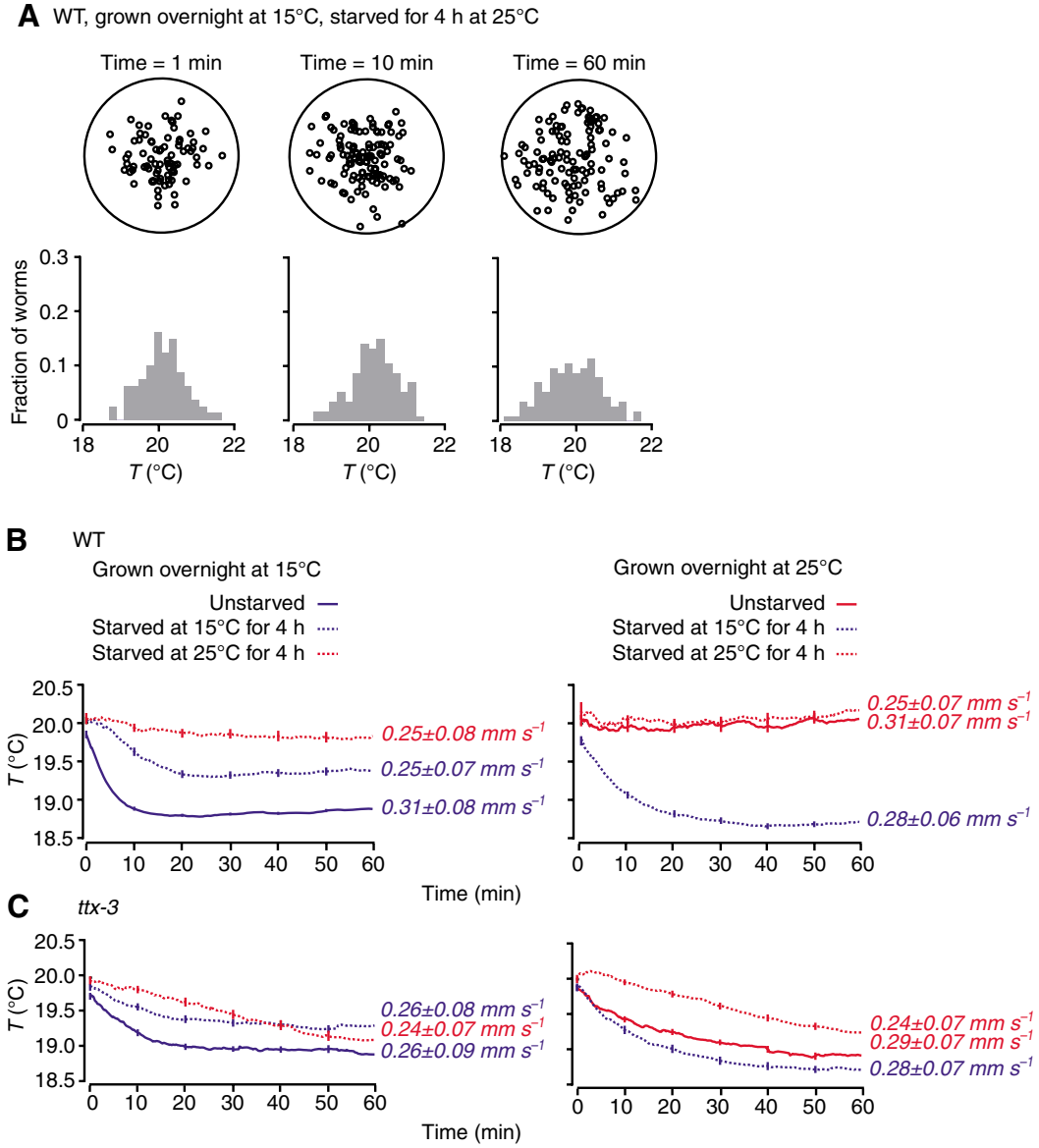

Fig. 4. Starvation and sustained exposure to new temperatures have separate long-term effects on thermotactic behavior. (A) When wild-type (WT) worms that were grown overnight at 15°C and then starved for 4 h at 25°C are placed near the middle of the linear thermal gradient, they exhibit random dispersal. The movements of (B) wild-type worms and (C) *ttx-3(ks5)* mutant worms up or down spatial thermal gradients spanning 18–22°C were quantified after they had been grown at 15°C (left-hand panels) or grown at 25°C (right-hand panels). Line colors indicate the temperature of the worms during the 4 h preceding each experiment; blue represents 15°C and red represents 25°C. Solid lines indicate experiments using unstarved worms. Dotted lines indicate experiments in which worms were starved at 15°C or 25°C before each experiment. In each data trace, error bars (±1 s.e.m.) are shown at 10 min intervals. The speed of instantaneous forward-crawling movements exhibited by individual worms in each experiment is indicated in italics, showing that atactic behavior cannot simply be attributed to lack of mobility (mean ± s.d.).

downstream of the AFD thermosensory neuron, as well as other chemosensory neurons potentially required for food sensation. We grew *hen-1(tm501)* worms overnight at 15°C or 25°C, shifted them as adults without food to 25°C or 15°C for 4 h and then analyzed their movements on spatial thermal gradients at intermediate temperatures. In all of these cases, we found that the behavior of *hen-1(tm501)* worms was indistinguishable from that of wild-type worms (Fig. 6A). Thus, we found no evidence that the *hen-1* gene affects behavior related to associative learning under our experimental conditions.

Mutation in the *tax-6* gene, which encodes calcineurin, has been reported to cause a thermophilic phenotype, causing worms to accumulate at the warmest point on a spatial thermal gradient (Kuhara et al., 2002). By rescuing the expression of *tax-6* in subsets of neurons, Kuhara and Mori found that expression of *tax-6* in specific interneurons affects associative learning between temperature and bacterial food (Kuhara and Mori, 2006). However, in our experimental conditions, we found that *tax-6(p675)* mutant worms are simply atactic at all temperatures (Fig. 6B), such that *tax-6(p675)* mutant worms neither move up nor down spatial thermal gradients, irrespective

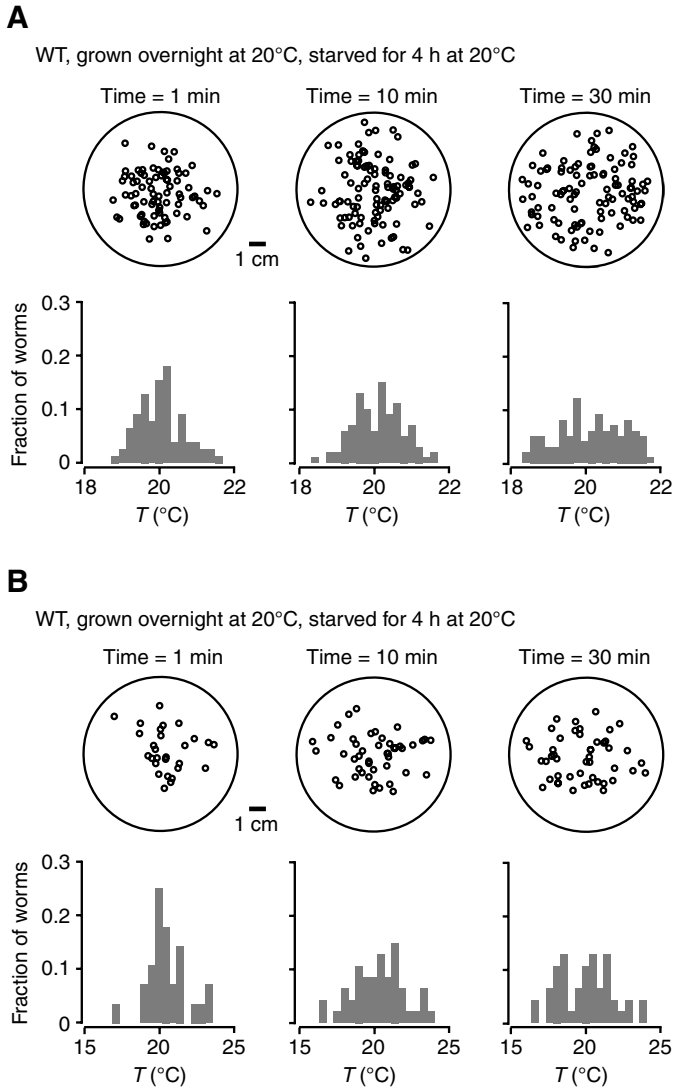

Fig. 5. Worms do not specifically avoid the temperature at which they had been starved. When wild-type worms that were grown overnight at 20°C and then starved for 4 h at 20°C are placed near the middle of linear thermal gradients that span 20°C, they exhibit random dispersal. (A) Data from experiments using a gradient spanning 18–22°C over a 9 cm plate. (B) Data from experiments using a gradient spanning 15–25°C over a 9 cm plate. Upper panels show snapshots of instantaneous worm positions. Lower panels show corresponding histograms.

of feeding state or temperature experience. Since *tax-6(p675)* mutant worms did not exhibit any thermotactic movement in our experimental conditions, we cannot conclude whether *tax-6* affects integrative behavior.

For each of the experiments shown in Figs 4 and 6, we also verified that the observed macroscopic changes in cryophilic migration were correlated with changes in navigational strategy. In every case that worms exhibited rapid cryophilic migration, they exhibited strong cryophilic bias. When worms exhibited slow cryophilic migration, they exhibited weak cryophilic bias (Fig. 7A). Collecting the data from all of our experiments, the macroscopic metric of cryophilic migration and the microscopic metric of cryophilic bias are highly correlated (r^2=0.74) (Fig. 7B). Linear proportionality between these two metrics has been predicted in the theory of biased random walks (De Gennes, 2004; Clark and Grant, 2005).

hen-1 mutant worms exhibit normal time-course for T_S resetting

Since we were unable to detect differences in long-term plasticity between wild-type and *hen-1(tm501)* mutant worms by quantifying behavior above or below the T_S, we next applied the isothermal tracking assay to quantify the rate of T_S resetting in *hen-1* mutants.

First, we verified that *hen-1(tm501)* mutant worms cultivated at specific temperatures are capable of tracking isotherms near

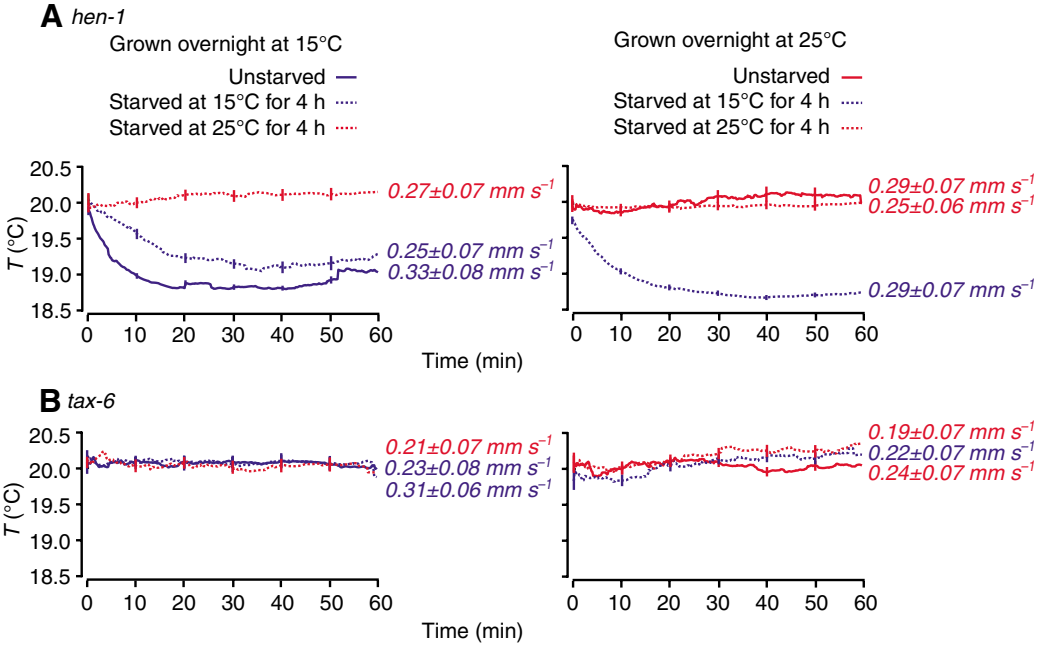

Fig. 6. Analysis of *hen-1* and *tax-6* mutants. The movements of (A) *hen-1(tm501)* mutant worms and (B) *tax-6(p675)* mutant worms after they had been grown at 15°C or 25°C (solid lines in each panel) or starved for 4 h at 15°C or 25°C before each experiment (dotted lines in each panel). The speed of instantaneous forward movements exhibited by individual worms in each experiment is indicated in italics (mean ± s.d.), showing that differences in navigational behavior cannot be simply attributed to differences in mobility.

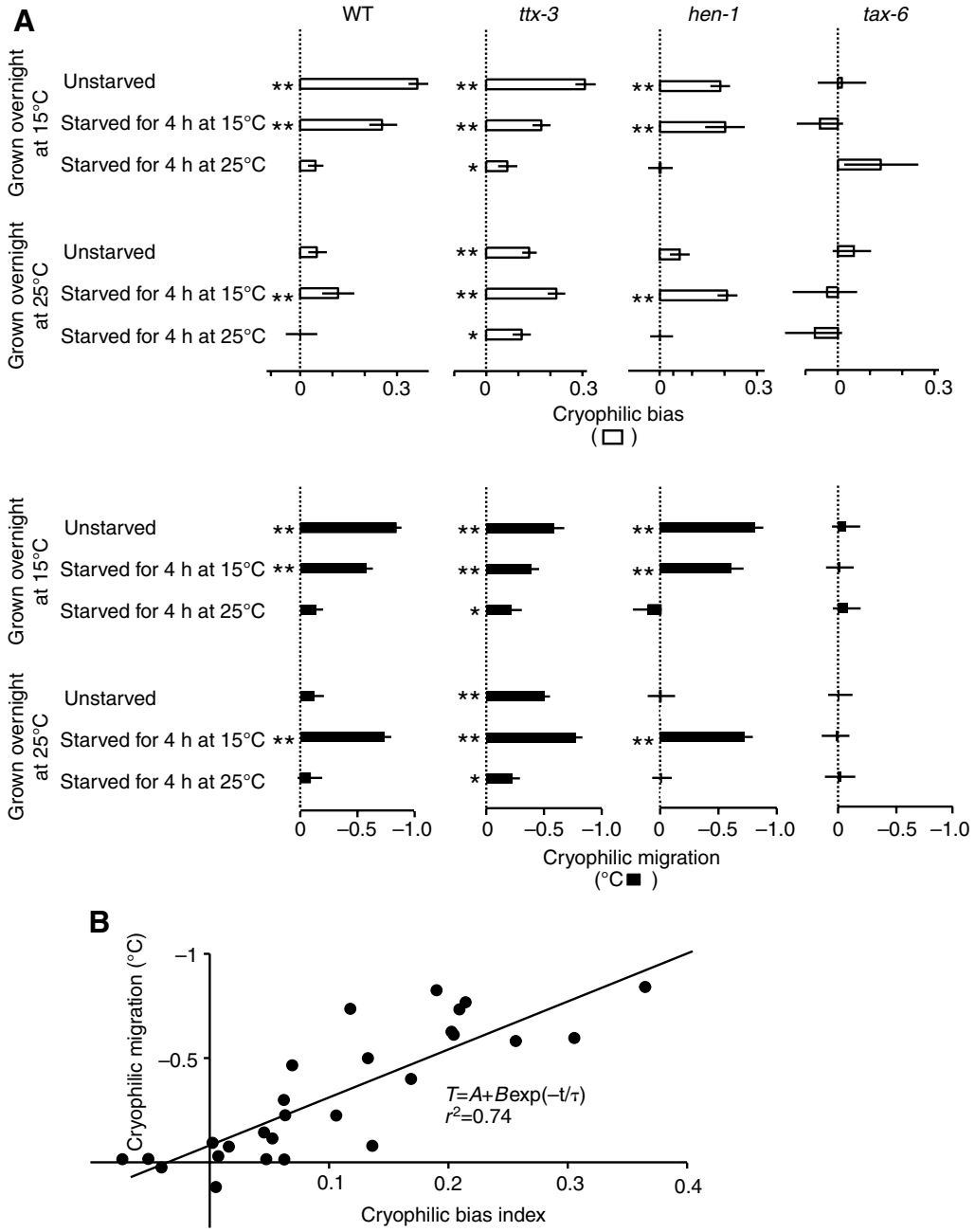

Fig. 7. Comparison of cryophilic movement and cryophilic bias of wild-type (WT) and mutant worms. (A) Cryophilic bias and cryophilic migration were calculated for the experiments using wild-type and mutant worms in Figs 4 and 5, in the manner described in Fig. 3C. Error bars represent 1 s.e.m. Cases in which the cryophilic bias indices or amount of cryophilic migration differ from zero are indicated by * (P<0.05) and ** (P<0.005). (B) Linear correlation between all measurements of cryophilic bias index and cryophilic migration, using all data from Fig. 3C and Fig. 7A.

those temperatures on a spatial thermal gradient (Fig. 8B). We cultivated *hen-1(tm501)* mutant animals at 15°C (and 25°C), then shifted the animals to 25°C (and 15°C), with or without food at the new temperature. At defined times following the temperature shift, we quantified the T_S of these animals by monitoring their isothermal movements on steep spatial thermal gradients spanning the T_S. We found that the *hen-1(tm501)* mutation does not affect the rate of T_S resetting. Like wild-type animals (Fig. 8A), *hen-1(tm501)* mutant worms reset their T_S to the new temperature of the environment after about 4 h. Also like wild-type animals, T_S resetting in *hen-1(tm501)* mutant worms is unaffected by the presence or absence of food (Fig. 8C–F).

Discussion

The sophistication of *C. elegans* thermotactic behavior has complicated the study of long-term plasticity. *C. elegans* thermotaxis involves distinct modes of thermotactic behavior

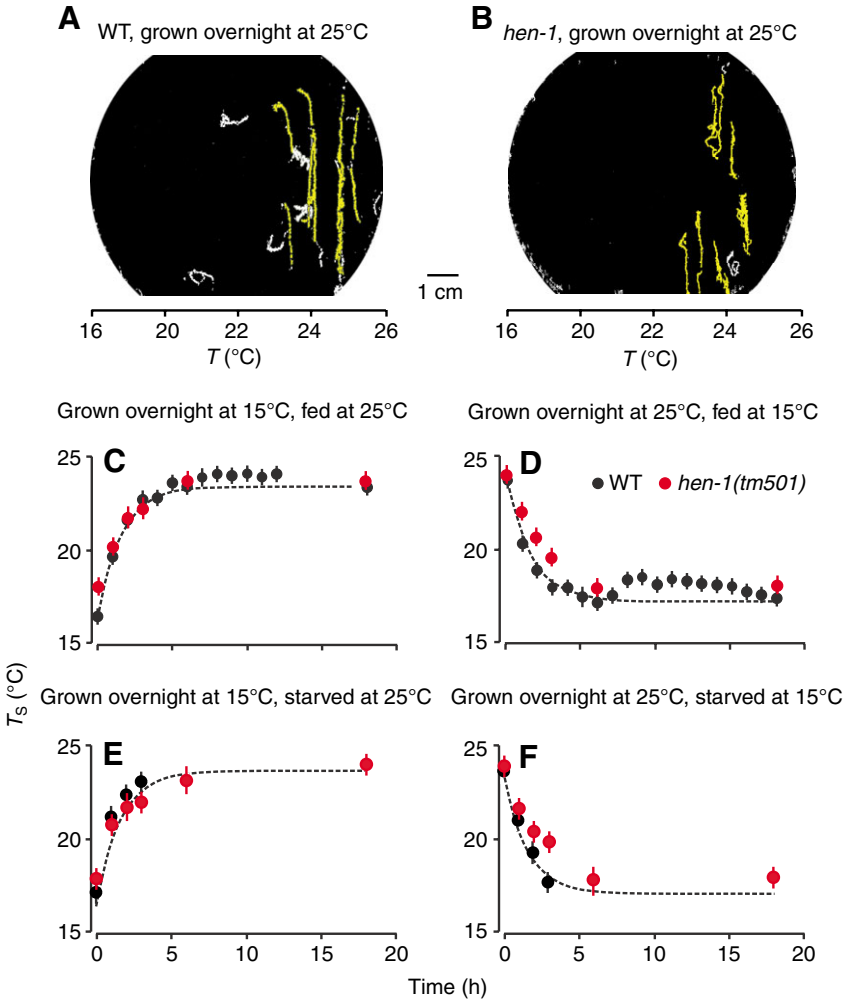

Fig. 8. Rate of T_S resetting measured by quantifying isothermal tracking in wild-type and *hen-1* mutant worms. (A) Isothermal tracks made by wild-type worms grown overnight at 25°C and placed on a steep linear thermal gradient spanning 16–26°C across a 9 cm-diameter plate. Snapshots of the movement of animals on the gradient were digitized and overlaid such that their trajectories were visible. Isothermal tracks, artificially colored yellow, were defined as long vertical trajectories and emerged in a band of temperatures near the previous cultivation temperature. For comparison, a few white trajectories are also shown, representing worms that are not tracking isotherms in the same period of time. The T_S is quantified as the mean temperature of isothermal tracks exhibited by a certain population of worms. (B) Isothermal tracks exhibited by *hen-1(tm501)* worms that were grown overnight at 25°C and placed on steep linear thermal gradients (1°C/cm). (C–F) The time-course of T_S resetting of wild-type animals and *hen-1(tm501)* animals. In C and D, worms were cultivated overnight with bacterial food at 15°C or 25°C, then shifted to a plate containing food at 25°C or 15°C, respectively. In E and F, worms were grown overnight with bacterial food, then shifted to a new plate without food. The circles represent experimental data, and the broken lines depict an exponential fit of the wild-type data. At least two independent plates, 50 worms and 20 isothermal tracks were used for each data point.

that are exhibited in different temperature ranges (Hedgecock and Russel, 1975; Ryu and Samuel, 2002). In addition, although both temperature and food-dependent cues induce long-term changes in *C. elegans* thermotactic behavior, whether long-term plasticity requires direct associations between these cues has not been conclusively verified.

Hedgecock and Russell showed that *C. elegans* tracks isotherms near its previous cultivation temperature (Hedgecock and Russell, 1975). Recently, we showed that adult *C. elegans* reset their thermotactic set-point (T_S) irrespective of the presence or absence of food at new temperatures (Biron et al., 2006). Thus, resetting the T_S does not require associative mechanisms. Hedgecock and Russell found evidence that *C. elegans* disperses from temperatures at which they had been starved (Hedgecock and Russell, 1975). However, Yamada and Ohshima found evidence that *C. elegans* does not actively avoid temperatures correlated with starvation (Yamada and Ohshima, 2003). Several genetic studies have been performed to identify molecules that affect associative learning between temperature and food-dependent cues, but without detailed analyses of the behavioral strategies underlying the presumed associative learning behavior in wild-type worms. The *hen-1* gene has been interpreted as affecting the worm's ability to associate temperature and starvation (Ishihara et al., 2002). Genetic screens have been conducted for *aho* (abnormal hunger orientation) mutants, which are also presumed to be defective in associating temperature and starvation (Mohri et al., 2005). Insulin-like pathways have also been investigated for roles in starvation-induced learning of specific temperatures (Kodama et al., 2006).

We sought additional tests of association between temperature and food-dependent cues. In this study, we used precise and well-defined experimental conditions and analyzed both the macroscopic and microscopic metrics of worm navigation behavior on thermal gradients. Our results indicate that (1) T_S resetting is induced by exposing worms to new temperatures, irrespective of the presence or absence of food at the new temperature; (2) worms do not appear to actively avoid temperatures at which they had been starved; (3) cryophilic movement is suppressed after prolonged starvation. In short, under our experimental conditions, association between temperature and food-dependent cues is not required. Sustained exposure to specific temperatures resets the T_S to those temperatures. Starvation suppresses cryophilic movement, causing worms to randomly disperse on spatial thermal gradients.

Non-associative models for long-term plasticity in thermotactic behavior would be simpler than associative models, as neural circuits would not be required to integrate different sensory inputs. The regulation of thermotactic behavior in *C. elegans* involves the AFD sensory neurons, the AIY interneurons and the AIZ interneurons, which form a synaptically interconnected minicircuit in the nervous system (Mori and Ohshima, 1995). Recent physiological measurements have shown that patterns in the activity of the AFD sensory neurons are correlated with the stored T_S

(Kimura et al., 2004; Clark et al., 2006). In particular, temperature changes evoke Ca^{2+}-dynamics in the AFD neuron and drive AFD synaptic output to the AIY interneuron only at temperatures above a certain threshold temperature, which is near the T_S. Moreover, exposing the animal to new temperatures resets the threshold temperature of AFD neuronal activity in physiological measurements with time courses that correspond to T_S resetting in behavioral measurements (Biron et al., 2006). Mutations in the *dgk-3* gene, which encodes a diacylglycerol kinase expressed in the AFD thermosensory neuron, have parallel effects on the rate of T_S resetting measured at the levels of behavior and AFD physiological activity (Biron et al., 2006). These observations suggest that the physiological operating range of the AFD neuron defines the temperature range of thermotactic behavior. Sustained exposure to new temperatures shifts the sensitivity of the AFD neuron to different temperatures, thereby shifting the set-point of thermotactic behavior to different temperatures.

Our observations suggest that prolonged starvation simply abolishes migration up or down spatial thermal gradients, allowing *C. elegans* to rapidly disperse from any temperature. The mechanisms by which starvation suppresses cryophilic movement are not known. One possibility is that specific pathways in the *C. elegans* nervous system are modified, as in the way that serotonergic circuits are modified to mediate the enhanced slowing response of *C. elegans* when it encounters food after prolonged starvation (Sawin et al., 2000). Another possibility is that starvation induces hormonal changes with broad effects on the nervous system. For example, Tomioka et al. found that an insulin-like signaling pathway may mediate the worm's salt avoidance behavior after starvation in the presence of ordinarily chemoattractive salt (Tomioka et al., 2006).

Our experimental conditions are capable of evoking robust cryophilic behavior, isothermal tracking behavior and long-term plasticity from *C. elegans*. However, based on our quantitative measurements of cryophilic behavior and isothermal tracking behavior under our experimental conditions, we are unable to conclude that *C. elegans* thermotaxis involves associative learning between temperature and food-dependent cues.

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