The *Caenorhabditis elegans* Dauer Larva: Developmental Effects of Pheromone, Food, and Temperature

JAMES W. GOLDEN[1] AND DONALD L. RIDDLE[2]

Division of Biological Sciences, University of Missouri, Columbia, Missouri 65211

Three environmental cues influence both the entry into and exit from the developmentally arrested dispersal stage called the dauer larva: a dauer-inducing pheromone, food, and temperature. The pheromone, which is a measure of population density, induces dauer larva formation at the second (L2) molt and inhibits recovery in a dose-dependent manner. Food acts competitively to reduce the frequency of dauer larva formation and to enhance recovery. The pheromone causes a specific extension of the second larval stage, coupled with a transient decrease in the growth rate of the L2. Second-stage larvae grown in the presence of added pheromone are morphologically distinguishable from L2 larvae grown without pheromone. We have named the pre-dauer L2 larva the L2d. Commitment to dauer larva formation can occur at the L2d molt. When L2d larvae are shifted out of pheromone to a lawn of *E. coli* just before the L2d molt, a few worms complete development into dauer larvae. In contrast, worms are essentially committed to the non-dauer life cycle by the first larval molt if the L1 larvae are not grown in appropriately high levels of pheromone. In the presence of pheromone, the percentage of dauer larva formation is enhanced at higher temperatures within the normal growth range. Temperature down-shifts induce dauer larva recovery. Temperature-shift experiments show that the enhancement of dauer larva formation requires exposure to the higher temperature around the L1 molt. Two sensory mutants defective in thermotaxis are altered in their sensitivity to the dauer-inducing pheromone, but their pheromone response retains temperature dependence. Response of dauer larvae to environmental cues is highly age dependent, with older dauer larvae exhibiting an increased tendency to recover.

INTRODUCTION

Under favorable growth conditions, the free-living soil nematode *Caenorhabditis elegans* hatches from the egg, and in about 3 days passes through four larval stages (L1 through L4) before reaching reproductive maturity (Byerly *et al.*, 1976). In conditions of high population density and a limited food supply, a developmentally arrested nonfeeding dispersal stage, called the dauer larva, is formed at the second molt (Cassada and Russell, 1975). Dauer larvae have a unique cuticle structure and their mouth is tightly closed, providing them with enhanced resistance to desiccation and harsh chemical treatments such as exposure to detergents. They are relatively thin and dense due to radial shrinkage of the body, and their behavior is adapted to enhance survival. Pharyngeal pumping and grazing motions of the head are suppressed. They often remain motionless but are capable of rapid movement in response to mechanical stimulation, and unlike other larval stages, they may stand on their tails and wave their bodies in the air. When dauer larvae are placed into fresh medium they begin feeding and eventually molt into L4 larvae (Cas-

sada, 1975). Dauer larvae have been referred to as "non-aging" forms because they are capable of surviving for several months, and then recover to form adults with a normal post-dauer life span (Klass and Hirsh, 1976).

Specific environmental cues provide sensory information controlling dauer larva formation and recovery. The concentration of a fatty acid-like pheromone apparently serves as a measure of population density. Higher concentrations induce dauer larva formation and prevent recovery (Golden and Riddle, 1982). The *Caenorhabditis*-specific, dauer-inducing pheromone can be detected by bioassay at more than a 200-fold dilution from crowded nematode culture medium. A food-signal, found in yeast extract and bacterial cultures, has effects antagonistic to the pheromone, and induces dauer larvae to recover. Worms apparently integrate these two chemical cues to maximize their survival in the soil by developing to the adult only when sufficient food is available to support reproduction. Although soil nematodes of other genera do not produce a pheromone active on *C. elegans*, the biological activities of the pheromone produced by wild-type strains of *C. elegans* and *C. briggsae* are indistinguishable from each other (Fodor *et al.*, 1983; Golden and Riddle, 1984). The wild-type *Caenorhabditis* strains exhibit a temperature-dependent response to the combination of pheromone and food present in the environment, such that higher temperatures

[1] Present address: Department of Biophysics and Theoretical Biology, The University of Chicago, Chicago, Ill. 60637.

[2] To whom all correspondence should be addressed.

within the normal growth range increase the fraction of the population entering the dauer stage.

Genetic analysis of dauer larva formation has utilized two classes of mutants (Riddle, 1977; Riddle *et al.*, 1981): (1) "constitutive" mutants, which form dauer larvae independently of environmental stimuli, were selected by their formation of dauer larvae in low-density populations on a lawn of *E. coli*, and (2) "defective" mutants which were identified as cloned populations unable to form dauer larvae after the food supply on the petri plate was outgrown. Some dauer-defective mutants have behavioral and ultrastructural abnormalities indicating they are affected in chemosensory function (Albert *et al*, 1981). Along with abnormal dauer larva formation, these mutants are defective in chemotaxis, osmotaxis, and male mating behavior. Ultrastructural analysis revealed that some mutants have morphological abnormalities in afferent sensory nerve endings. With the use of temperature-sensitive dauer-constitutive mutants, it was shown that the same genetic defects that block entry into the dauer stage also prevent recovery of dauer larvae.

In the work reported here, we have used partially purified dauer-inducing pheromone, a controlled food supply, and the newly identified effects of temperature to study the developmental sequences of dauer larva formation and recovery in wild-type *C. elegans*. The developmental process leading to dauer larva formation begins at least as early as the first molt, and the second larval stage progressing toward the dauer larva can be morphologically distinguished from the L2 stage grown in the absence of dauer-inducing conditions.

MATERIALS AND METHODS

Nematode growth and synchronization. Unless otherwise indicated, experiments were done with the wild-type *C. elegans* strain N2. Stocks were maintained at 20°C on NG agar plates streaked with *E. coli* strain OP50 (Brenner, 1974). The OP50 strain was used as the bacterial food in all experiments. Three methods were used to obtain synchronized populations of worms. When a large number of worms were needed and a high degree of synchrony was not necessary, eggs were purified using alkaline hypochlorite (Emmons *et al.*, 1979) and then immediately used for starting cultures. When a higher degree of synchrony was needed, the eggs, isolated as above, were resuspended in test tubes with M9 buffer in the absence of food, and incubated for 18 to 24 hr on a shaker at 20°C (Swanson and Riddle, 1981). This procedure provided synchronous L1 larvae, but for unknown reasons it often decreased the percentage of a population induced to form dauer larvae as compared to eggs laid

directly onto a pheromone-containing plate. Experiments which required a high degree of synchrony and a high percentage of dauer larvae used eggs from young gravid adults which were laid directly onto the plate during a one hour time period. After 1 hr of egg laying, adults were removed from the plates by aspiration.

Pheromone extract. Pheromone was partially purified as follows, with recovery of greater than 90% of initial biological activity. One liter of exhausted liquid culture medium (Golden and Riddle, 1982) was reduced in volume by drying under a stream of air at 100°C, and then centrifuged at 10,000*g* for 10 minutes. The supernatant was completely dried in a vacuum oven at 60°C. This residue was extracted four to six times with 95% ethanol until the extract was only slightly colored. The extracts were combined and dried under a stream of air at 60°C. The resulting oily residue was then back extracted into 10 ml distilled water, filtered through Whatman 3 mm paper, sterilized by autoclaving, and stored at 2°C. A pheromone bioassay based on the inhibition of dauer larva recovery is only semiquantitative (Golden and Riddle, 1982). Therefore, each batch of pheromone extract was assayed for activity to determine the amount which induced approximately 75% N2 dauer larvae at 25.4°C. Typically, 20 to 50 μl pheromone stock solution were needed per 2 ml agar plate.

Induction of dauer larva formation on pheromone plates. Indicated amounts of pheromone extract were added to NG agar prepared without peptone, and 2-ml aliquots were placed into 35 × 10 mm petri dishes. After the agar solidified, bacteria suspended in S medium (Sulston and Brenner, 1974) containing streptomycin (50 μg/ml final concentration) were spotted onto the surface (see figure legends for amount of food added). Each experiment was performed utilizing a single preparation of pheromone extract and bacteria to provide internal consistency. After 12 hr the "pheromone plates" were inoculated with worms, then incubated in sealed containers (to minimize evaporation) at the appropriate temperature until the worms which did not form dauer larvae had become adults. Plates were visually scored for the percentage of the population that formed dauer larvae. With the exception of experiments in Figs. 9 and 10, data shown were obtained from one of several essentially identical experiments which yielded qualitatively similar results. Quantitative variability which occurred between experimental trials was apparently due to slight differences in the pheromone extract and bacterial food supply.

Molting cycle and body length measurements. Development was monitored by scoring pharyngeal pumping (Cassada and Russell, 1975). Molts are identified by a period of lethargus, when movement and feeding are

suppressed. Length measurements were made with an eyepiece graticule, using 1% phenoxypropanol anesthesia to straighten and immobilize the worms (Cassada and Russell, 1975). Lengths given are the average of eight worms.

Pheromone shifts. Larvae were shifted into dauer-inducing conditions at various times during development to determine when commitment to the L3 (non-dauer development) occurred. L1 larvae, synchronized by incubation in M9 buffer, were placed onto standard NG agar plates at zero time. At each interval, approximately 50 larvae were transferred on the tip of a 0.2-mm-diameter platinum wire to medium which induced dauer larva formation (petri plates or liquid cultures containing pheromone). Incubation was continued until the culture could be scored for the percentage of dauer larvae.

The time of commitment to dauer larva formation was determined by shifts out of dauer-inducing conditions. Tested worms were obtained from pheromone plates started with either freshly laid eggs or synchronous L1 larvae. Larvae were transferred at various times from dauer-inducing conditions to standard NG agar plates seeded with bacteria. Behavior and development were monitored before and after each shift.

Temperature effects. Temperature dependence and shift experiments used pheromone plates for the induction of dauer larvae. Temperature dependence experiments were started with purified eggs. Temperature-shift experiments (15 to 25.4°C up-shifts, or 25.4 to 15°C down-shifts) were started with L1 larvae synchronized in M9 buffer. The developmental rate was monitored by observing the molting cycle at the starting temperature for each experiment.

Dauer larvae. Synchronously induced dauer larvae were prepared for testing the effects of temperature and dauer larva age on recovery, and for determining when dauer larvae become committed to recovery. Purified eggs were grown at 25.4°C on a shaker in S medium (Sulston and Brenner, 1974) containing 0.25% (w/w) bacteria and pheromone extract suitable for inducing 100% dauer larvae. A dilution series of each batch of pheromone extract was tested for the concentration needed to produce 100% dauer larvae. The density of worms can be varied widely, up to 5000/ml. The experiments described here used 2000 worms per ml.

Temperature downpulse induction of dauer larva recovery. Approximately 75, 48-hr-old, synchronously formed dauer larvae were transferred to pheromone plates containing bacteria and incubated at 25.4°C for 12 hr. The plates were checked to confirm the absence of recovered dauer larvae, shifted to 15°C for the times shown in Fig. 7, then returned to 25.4°C for at least 12

hr. The plates were then scored visually for the percentage of the dauer larvae which had been induced to recover.

Dauer larva commitment to recovery. Dauer larvae, prepared as for the pheromone bioassay (Golden and Riddle, 1982) or induced synchronously, were placed into recovery conditions at room temperature (23°C) for various periods of time, then returned to pheromone to determine if recovery could be inhibited. Dauer larvae were quickly (5 min) washed three times with water and transferred to an NG agar plate containing a lawn of bacteria. At each time point, 10 to 20 dauer larvae were transferred to a microtiter well containing 50 μl of M9 buffer and pheromone. The concentration of pheromone was the same used for the induction of 100% dauer larvae (approximately a 100-fold excess over the amount needed to prevent recovery in the absence of food). The dauer larvae were scored for recovery after incubation for an additional 4 hr.

Temporal changes in dauer larva response. Dauer larvae were synchronously induced with pheromone as described above. Dauer larvae formed on the second day of incubation, referred to as Day 0 for dauer larva age. On the third day, they were transferred to M9 buffer containing pheromone to prevent recovery, and parallel cultures were incubated at 20 and 25.4°C in test tubes on a rotary shaker. On each day indicated in Fig. 10, a sample of dauer larvae was obtained from cultures incubated at each temperature for use in the pheromone bioassay (Golden and Riddle, 1982). The dauer larvae were washed three times with water by low-speed centrifugation and resuspended in 0.05% Bacto yeast extract (food-signal source) in M9 buffer. Ten to 20 dauer larvae in 30 μl of buffer were added to each microtiter well containing a 30-μl pheromone sample. The pheromone source was filter-sterilized, starved liquid culture medium and each dauer larva sample was used to assay a 7-step, fourfold pheromone dilution series in M9 buffer (from undiluted to a 1/4096 dilution). The bioassay was incubated at 23°C for 4 hr, then scored visually for the inhibition of dauer larva recovery. Samples of dauer larvae also were tested for resistance to a 30 min exposure to 1% sodium dodecyl sulfate (SDS) after they had incubated for 10, 19, and 28 days at both temperatures.

RESULTS

Induction of Dauer Larva Formation

The competitive influences of pheromone and food on the induction of dauer larva recovery (Golden and Riddle, 1982) also were found in dauer larva formation. Addition of pheromone extract to growth medium con-
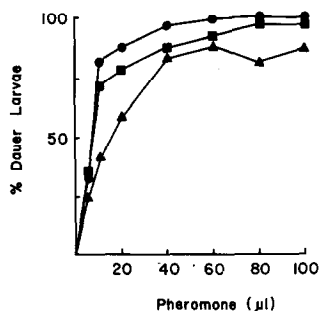
FIG. 1. Pheromone induction of dauer larva formation. NG agar medium without peptone (2 ml) containing increasing amounts of pheromone extract, was spotted with 5 (●), 10 (■), or 20 (▲) μl of 4% (w/w) *E. coli* OP50 treated with streptomycin. The plates were started with approximately 100 isolated eggs, incubated at 25°C for 48 hr, and scored visually for percent dauer larvae.

taining controlled amounts of bacteria induced dauer larva formation in a dose-dependent manner. Figure 1 shows the effect of exogenously added pheromone on dauer larva formation in populations of the wild-type strain N2, when they were grown on petri plates in three different amounts of bacteria. Greater concentrations of pheromone induced a greater fraction of the population to form dauer larvae. The response was not linear, but "saturated" at a maximum level dependent on the amount of bacterial food. Omission of streptomycin from the medium resulted in an 80 to 90% decrease in the percentage of dauer larvae induced by pheromone. Thus, increasing amounts of bacteria had two effects: (1) inhibition of dauer larva formation at a given concentration of pheromone, dependent upon the amount of food present, and (2) depression of the maximum percentage of a population that could be induced to form dauer larvae. At each level of bacterial food, the worms were exposed to a lawn of bacterial cells sufficient for growth to the adult. The differing levels of pheromone response show that the animals can discriminate between the total amounts of bacteria in the different lawns.

The results in Fig. 1 show that it is necessary to control both the level of pheromone and the food supply in order to induce dauer larva formation. It is assumed that the food supply is sensed by the worms via one or more chemical signals. To decrease the production of the food-signal, *C. elegans* was grown on bacteria treated with streptomycin. Streptomycin at 50 μg/ml does not affect the growth or development of *C. elegans*, but it does allow a degree of control over bacterial metabolism and its inhibitory effects on dauer larva formation. We could not grow *C. elegans* on bacteria killed by autoclaving or ultraviolet irradiation. The use of streptomycin-treated bacteria on pheromone plates without a carbon source provides better reproducibility and a significantly higher

percentage induction of dauer larvae than is obtained without streptomycin (data not shown).

Developmental Effects

The effect of the dauer-inducing pheromone on the rate of development was determined by monitoring the lethargus periods which accompany each larval molt, and by measuring the rate of increase in body-length. Figure 2a shows the fraction of feeding animals during development for a synchronously growing population of strain N2 on NG agar plates. The molts are marked by a drop in the percentage of animals exhibiting pharyngeal pumping (Cassada and Russell, 1975). The times of the four larval molts at 25°C were 11, 18, 24, and 32 hr after the synchronized L1 larvae were transferred to food, and egg laying began at 42 hr. In the presence of pheromone sufficient to induce 10% of the population to form dauer larvae (Fig. 2b) there was a very slight delay of the first molt and a significant delay of the second molt. The animals that did form dauer larvae ceased pharyngeal pumping at the second-molt and completed radial shrinkage of the body at 34 hr. Even the worms that did not form dauer larvae showed delayed development. The times of the four larval molts were 11.5, 24, 29, and 39 hr, and the onset of egg laying was at 48
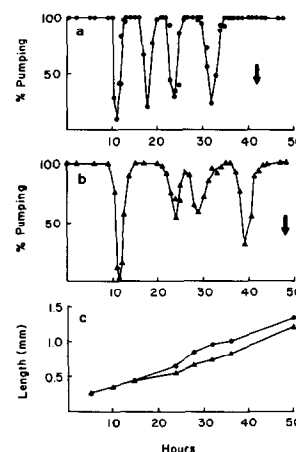

FIG. 2. Molting cycle and growth in the presence and absence of exogenous pheromone. (a) NG agar plates seeded with *E. coli* OP50 were started at time = 0 with synchronized L1 larvae, and incubated at 25°C. Pharyngeal pumping (feeding) was scored at the times shown. Larval molts are marked by suppression of pharyngeal pumping. The onset of egg laying is indicated by the arrow. (b) As in (a) except pheromone extract sufficient for the induction of 10% dauer larvae was incorporated into NG agar medium and the percentage of the population pumping was calculated for only non-dauer larvae. (c) Growth rate in the presence and absence of pheromone. The length of anesthetized worms was measured in the absence (●) and presence (▲) of pheromone. Growth conditions were as in (a) and (b), respectively.

hr. Although the length of the L3 and L4 intermolt period, and the timing of the onset of egg laying after the L4 molt, are comparable to the results obtained in the absence of pheromone, the absolute times of these events were delayed approximately 6 hr in the presence of pheromone, as a result of the prolongation of the L2 intermolt period. Thus, the effects of pheromone on growth and development seem to be restricted to the second and dauer larval stages under our experimental conditions.

Worms grown in the presence of pheromone did not increase in length at the normal rate during the second larval stage. The delay in growth was similar to the delay in the molting cycle, about 6 hr. Thus, the length of the worms at the time of the onset of egg laying was the same in the presence or absence of pheromone.

In the presence of dauer-inducing pheromone, the second-stage larvae in the latter part of the intermolt period become morphologically distinct from L2 larvae grown in the absence of pheromone. We have named these pre-dauer L2 forms L2d larvae. The L2d larvae grow slightly larger than L2 larvae because they continue to feed as the second molt is delayed (Fig. 2c). They appear darker and less transparent when viewed in the stereomicroscope (Fig. 3b), apparently as a result of accumulation of intestinal storage granules. Pheromone concentrations which induce only a few percent dauer larvae are sufficient to induce all second stage larvae to assume L2d morphology. At the second molt, the L2d larvae become either dauer larvae or L3 larvae, in proportions that depend on the relative strengths of the environmental cues. The L2d-L3 molt is similar to other molts, lasting about 1 hr. The resulting L3 larvae are morphologically indistinguishable from L3 larvae formed in the absence of dauer-inducing conditions.

The L2d-dauer molt has been described under conditions different from ours (Singh and Sulston, 1978; Cassada and Russell, 1975; Swanson and Riddle, 1981), and our observations concur with the earlier results. The L2d larvae stop pharyngeal pumping at the beginning of the molt, and for worms which become dauer larvae, pumping remains suppressed. Ten hours later the worm's body shrinks radially (Fig. 3c) and separates from the old cuticle. Approximately an hour after shrinkage the worms have acquired all of the characteristics of dauer larvae including resistance to treatment with 1% SDS.

Timing of Sensitivity to Pheromone

The length of the L2 intermolt period was variable and directly related to the strength of the dauer-inducing conditions and the length of time the worms were ex-
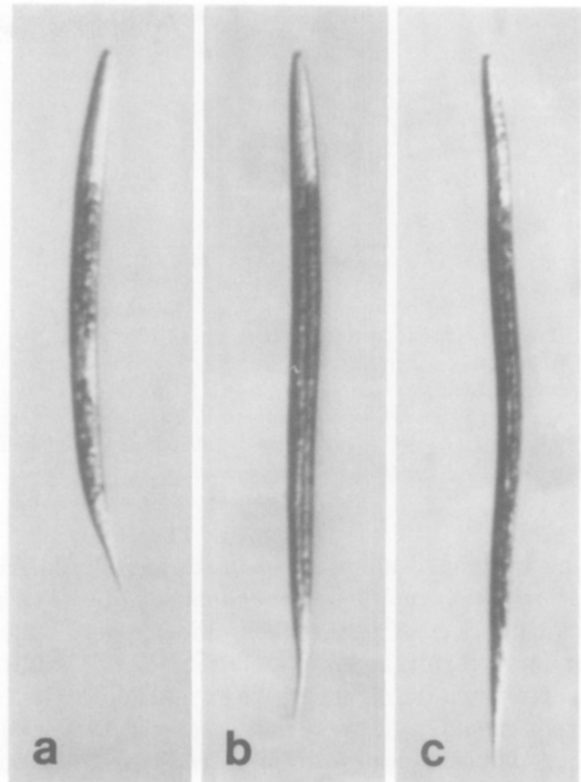

FIG. 3. Photomicrographs of *C. elegans* developmental stages. Larvae were anesthetized with 1% phenoxypropanol and photographed using a Wild M5 stereomicroscope (156× magnification). (a) L2 larva entering second molt, obtained from NG agar plate. (b) L2d larva just prior to second molt, obtained from a pheromone plate containing 50 μl of pheromone extract. (c) Dauer larva approximately 12 hr after radial shrinkage, obtained from a pheromone plate.

posed to them. When L2 larvae that had developed from eggs in dauer-inducing conditions were transferred to standard NG agar, the earlier the worms were shifted, the sooner the second molt occurred. However, even when the shift was made as early as the first larval molt, the L2 intermolt period was significantly lengthened (2 to 3 hr) compared to the period for worms never exposed to dauer-inducing conditions.

Worms must be exposed to pheromone prior to the first larval molt to induce a high percentage of dauer larvae. As shown in Fig. 4, worms shifted into pheromone after the mid-L1 stage were not induced to form dauer larvae. Conditions were used which induced about 75% dauer larvae in a population grown in the conditions from hatching. This experiment suggested that a decision or developmental switch occurred before the first larval molt, which committed worms grown in the absence of pheromone to form L3 larvae at the second molt. However, the commitment is not absolute. In an experiment where the environmental conditions were very stringent (high pheromone and low food concen-
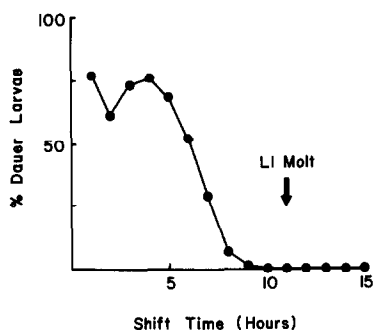
FIG. 4. Developmental commitment to the L3 versus the dauer larva. After incubation for the time shown, approximately 50 synchronized larvae were transferred from standard NG agar to medium containing 50 µl of extracted pheromone. Plates were incubated for a total of 48 hr at 25°C and scored for percentage dauer larvae. The time of the L1 molt is shown.

trations more than sufficient for the induction of 100% dauer larvae if the worms are exposed from the time of hatching), about 10% of the L2 larvae were induced to form dauer larvae if they were shifted into dauer-inducing conditions as late as 1 to 2 hr after the first molt. Later shifts failed to induce any dauer larva formation even under the most stringent conditions tested.

Commitment to complete dauer larva development occurs a few hours before the middle of the L2d molt. L2d larvae were shifted out of dauer-inducing conditions at several times during their development. Shifts to dauer-recovery conditions (NG agar seeded with bacteria) prior to a few hours before the L2d molt always resulted in non-dauer development, but shifts at the beginning of the L2d lethargus produced several worms that had become committed to complete development into the dauer larva. Instead of a 1 hr lethargus followed
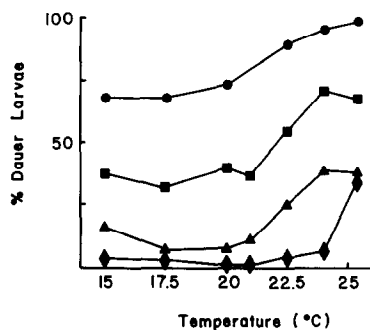
by the L3 stage, these worms continued to suppress pharyngeal pumping and remained lethargic. After 10 hr they shrank, and after a further hour, became resistant to 1% SDS. These dauer larvae then recovered and resumed development during the next few hours.

Continual exposure to dauer-inducing conditions was necessary for induction of a high percent dauer larvae. Synchronized populations of worms were temporarily shifted out of dauer-inducing conditions to NG agar and then returned. A 3 hr exposure to medium without pheromone bracketing the first larval molt resulted in a 74% decrease in the percentage dauer larvae compared to the control population which was not shifted. A five hour "pulse" to NG agar ending at the L1 molt produced a 90% decrease in the percent dauer larvae, and a 5 hr pulse starting at the L1 molt resulted in a 97% decrease in the percentage dauer larvae.

Temperature Dependence

Incubation temperatures above 20°C resulted in an increased percentage of a population induced to form dauer larvae when other environmental parameters were kept constant. Figure 5 shows the temperature dependence of dauer larva formation in four different concentrations of pheromone. The temperature dependence was exhibited at all pheromone concentrations over the fourfold range tested, and the transitions in the curves varied from about 21°C at the highest pheromone concentration, to almost 25°C at the lowest concentration. The shifted transitions observed at the lowest pheromone concentration were reproduced in several experiments. A small enhancement of dauer larva formation at 15°C compared with 17.5°C was frequently observed (Fig. 5 and other experiments not shown).

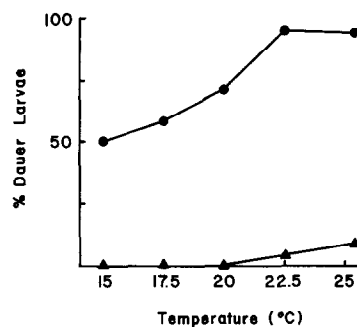The temperature dependence of dauer larva formation

FIG. 5. Temperature dependence of dauer larva formation. Plates were prepared with four concentrations of pheromone extract, 5 (♦), 10 (▲), 15 (■), and 20 (●) µl/ml NG agar without peptone, and incubated at various temperatures between 15 and 25°C. Plates were spotted with 10 µl of 4% (w/w) E. coli OP50 treated with streptomycin, and started with approximately 100 isolated eggs. Duplicate plates were incubated until the non-dauer larvae grew to adults, then were scored for percentage dauer larvae.

FIG. 6. Temperature dependence of dauer larva formation exhibited by thermotaxis-defective mutants PR761 (▲) and PR767 (●). Pheromone plates containing 50 µl pheromone extract were spotted with 5% (w/w) E. coli OP50 treated with streptomycin. Approximately 100 eggs were laid directly onto each plate. Duplicate plates were incubated at the indicated temperatures and scored for percentage dauer larvae.

DEVELOPMENTAL BIOLOGY   VOLUME 102, 1984

may involve any step in the behavioral/developmental sequence. One possibility is that temperatures above 20°C provide a sensory cue for dauer larva formation which is additive with the pheromone. Strain N2 is known to respond to temperature gradients and sensory mutants defective in thermotaxis have been isolated (Hedgecock and Russell, 1975). Two such mutants, PR761 and PR767, were tested for temperature-dependent dauer larva formation, and although their sensitivities to pheromone differ from wild-type (Golden and Riddle, 1984), they both displayed temperature-sensitive dauer larva formation (Fig. 6). Strain PR767 overresponded to pheromone, but showed nearly wild-type temperature dependence. Strain PR761 underresponded to pheromone and only produced dauer larvae at 22.5 and 25°C in these conditions. These results suggest that if higher temperatures provide a sensory stimulus favoring dauer larva formation, the mechanism involved must be at least partially distinct from that affected in these two thermotaxis mutants.

Temperature-shift experiments were performed on synchronous wild-type larvae to determine if exposure to 25°C at specific times during L1 or L2d larval development enhanced dauer larva formation. Temperature upshifts from 15 to 25°C failed to induce an increased percentage of dauer larvae when executed after the first larval molt (Fig. 7). In contrast, upshifts performed just prior to the L1 molt were just as effective as the earliest upshifts performed. Thus, a sharp transition in the temperature-shift curve coincides with the first molt. Larvae must be exposed to the higher temperature no later than the L1 molt in order to influence dauer larva formation.

Downshifts from 25 to 15°C eliminated the enhancement of dauer larva formation if the shifts were per-

FIG. 8. Effect of temperature downshifts during larval development in dauer-inducing conditions. The experiment is as in Fig. 7 except the plates were shifted from 25 to 15°C. The time of the L1 and L2 molts at 25°C are shown.

formed prior to the L1 molt (Fig. 8). Progressively later downshifts during the L1 molt and the early L2d stage resulted in a steadily increased percentage of dauer larvae formed. Paradoxically, downshifts performed after the mid-L2d stage reversed the earlier trend. Shifts executed at these times (16 to 24 hr) produced a progressively lower percentage of dauer larvae, even though the worms had been exposed to the higher temperature for the entire L1 and early L2d larval stages. The initial transition in the curve which occurs at the L1 molt is expected if the animals must be exposed to the higher temperature around the L1 molt to induce high levels of dauer larvae. If that were the only effect of temperature, the percentage dauer larvae should remain at a high level at all downshift times after the initial transition. The return to lower percentage dauer larva formation, which occurs at shift times after the middle of the second larval stage, indicates that the animals enter a new developmental phase after the mid-L2d, a phase in which a temperature downshift in itself favors continued development through the L3 as opposed to formation of a dauer larva.

Exit from the Dauer Stage

Temperature downshifts from 25 to 15°C induce dauer larvae to recover. Figure 9 shows that temperature downpulses of increasing lengths induced progressively greater percentages of a population of dauer larvae to recover and resume development. A pulse length of 10 hr was necessary to induce 50% of the population to recover. The induction of recovery was unique to temperature downshifts; temperature upshifts had no effect on dauer larva recovery. Dauer larvae formed at 15°C were not induced to recover during maintenance at 15°C.

Dauer larvae become irreversibly committed to recovery within 1 hr after being shifted to fresh medium with food. The first visible sign of dauer larva recovery
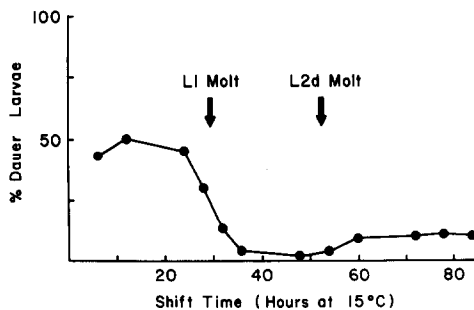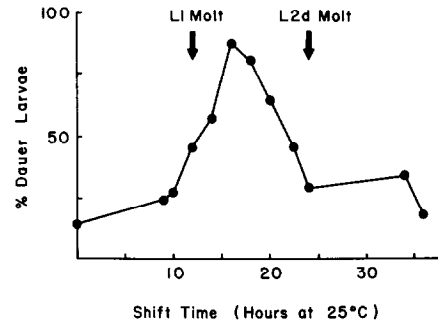

FIG. 7. Temperature upshifts during larval development in dauer-inducing conditions. At time = 0, approximately 100 synchronized L1 larvae were placed onto 24 NG agar plates minus peptone containing 25 μl pheromone extract and spotted with 20 μl 4% E. coli OP50 treated with streptomycin. Duplicate plates were shifted from 15 to 25°C after incubation for the time shown, and scored for percentage dauer larvae after the non-dauer larvae grew to adults. The time of the L1 and L2d molts at 15°C are shown.
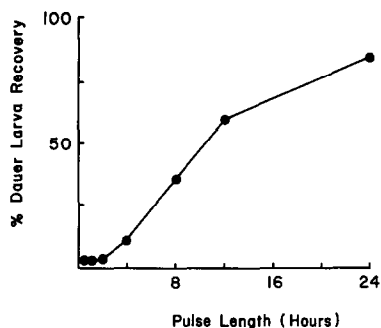
FIG. 9. Temperature downpulse induction of dauer larva recovery. Dauer larvae were placed onto plates containing pheromone (plates as in Fig. 7) and incubated at 25°C. Duplicate plates were shifted to 15°C for the pulse length shown, then returned to 25°C. The plates were scored for percentage recovered dauer larvae after a total incubation period of 48 hr.

is the onset of pharyngeal pumping. Dauer larvae obtained from 1- to 2-week-old liquid cultures, washed and transferred to food, begin feeding in 2 to 3 hr at 23°C. We determined whether dauer larvae become committed to recovery before the onset of feeding by transferring dauer larvae to a bacterial lawn, then returning samples to pheromone with no food at 10-min intervals. Four hours later the samples were scored for percentage recovery. Dauer larvae became committed to recovery between 50 and 60 min after transfer to food in these experiments, 1 hr before they start to show any visible signs of recovery.

Dauer larva recovery is dependent on the age of the dauer larvae and the temperature at which they were maintained. Synchronously induced dauer larvae that had incubated in the presence of pheromone from 1 to 28 days, at either 20 or 25°C, were used in a pheromone bioassay which scores inhibition of dauer larva recovery in the presence of a standardized food-signal (Golden and Riddle, 1982). Pheromone sensitivity was measured by testing a dilution series of a standard pheromone preparation for the greatest dilution which inhibited dauer larva recovery. Figure 10 shows the sensitivity to pheromone of the different dauer larva samples. The dauer larvae did not show obvious visible change over the course of the experiment; they retained viability and resistance to 1% SDS. However, there was a dramatic decrease in the apparent sensitivity of the dauer larvae to pheromone during the early days of incubation. The change was more rapid at 25 than at 20°C. In the pheromone bioassay, it is the ratio of pheromone to food-signal which influences dauer larva recovery, and not the absolute amounts of either stimulus. Thus, we cannot distinguish between a decrease in the animal's sensitivity to pheromone and an increased sensitivity

to food-signal. Regardless of the mechanisms involved, dauer larvae become progressively more predisposed to recovery during the 1- to 2-week period after they are formed.

DISCUSSION

Environmental cues play a crucial role in controlling *C. elegans* larval development. A pheromone induces dauer larva formation in the presence of food, particularly at higher temperatures, whereas an increase in the food supply inhibits dauer larva formation. The combined influence of these three cues determines what fraction of a population will form dauer larvae, while the remainder continue development to the adult. The developmental process leading to dauer larva formation begins no later than the first larval molt (Fig. 11). Dauer-inducing conditions result in characteristic morphological and developmental changes in the second-stage larva which warrant naming the predauer L2 stage the L2d.

The environmental parameters which enhance entry into the dauer larva stage also inhibit exit, and conversely, the conditions which inhibit entry enhance exit. The competitive roles of pheromone and a food-signal in dauer larva recovery have been shown previously (Golden and Riddle, 1982), and a similar competition
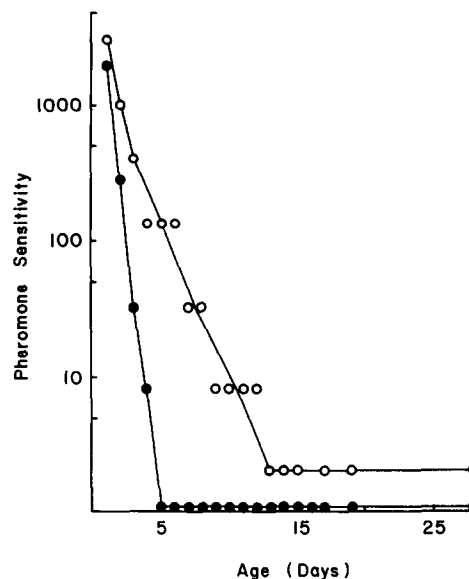

FIG. 10. Age dependence of pheromone sensitivity in the dauer larva recovery bioassay. Synchronously induced dauer larvae were incubated in liquid cultures at 20 (O) and 25°C (●). Dauer larvae were obtained from these cultures for use in the pheromone bioassay after incubation for the indicated number of days (dauer larvae were formed on day = 0). Pheromone sensitivity indicates the reciprocal of the pheromone dilution which produced a borderline response in the bioassay (Golden and Riddle, 1982).
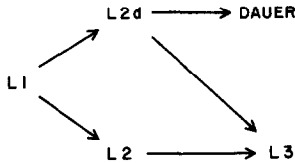
FIG. 11. Developmental branchpoints between dauer and non-dauer life cycles. The L2d larvae retain the potential to form either dauer or L3 larvae, whereas L2 larvae are committed to non-dauer development.

influences dauer larva formation. Higher growth temperatures enhance dauer larva formation, while shifts to lower temperatures enhance recovery. Dauer larva recovery is not only affected by the externally applied cues. The age of dauer larvae also has a dramatic influence on their response to the combination of pheromone and food-signal.

In our pheromone dose–response experiments there was a maximum percentage of a population that could be induced to form dauer larvae by exogenous pheromone, depending on the amount of food present. This effect produced the "saturation" curves shown in Fig. 1. This demonstrates that the percentage dauer larva formation is not determined by a simple one-to-one competitive relationship between pheromone and food. Instead, there appears to be a mechanism which permits a small fraction of the population to mature even at high pheromone levels. The more food present, the higher the fraction of animals which appears to be excluded from the dauer larva pathway at high pheromone concentrations. The obvious advantage of this mechanism is that no population will completely abandon reproduction in an environment which can support growth of some of the worms. All tests for pheromone response were performed with a sufficient bacterial lawn to permit growth of the animals to the adult stage. Therefore, the animals must be sensing the concentration of chemical cues which provide information about the size of the total food supply. This "food-signal" may be the same as that which is involved in the control of dauer larva recovery (Golden and Riddle, 1982).

Although it has not been possible to reproducibly induce more than 90% dauer larva formation on a lawn of *E. coli*, growth in liquid culture with limited food and moderate pheromone concentrations provides stringent conditions capable of inducing 100% dauer larvae. This has proven useful in ways not originally foreseen. For example, we have used liquid culture to develop a positive selection for mutants which fail to form dauer larvae in response to the pheromone (Haase *et al.*, 1983).

Worms acquire the full complement of morphological, physical, and behavioral properties characteristic of

dauer larvae at the end of the second larval stage, after a morphogenetic process which takes about 11 or 12 hr at 25°C. The wild-type process of dauer larva formation in the presence of exogenous dauer-inducing pheromone closely resembles that previously observed in the temperature-sensitive (*ts*) dauer-constitutive mutants. Analysis of execution stages for *ts* dauer-constitutive mutants, which produce dauer larvae regardless of the chemical cues, provided evidence that many gene functions were required at or before the first larval molt (Swanson and Riddle, 1981). Similarly, our temperature-shift and pheromone-shift experiments establish that discrimination between formation of a dauer larva and continued growth begins no later than the L1 molt in wild-type animals. The dauer-inducing influences of pheromone and elevated temperatures are effective at this time. A decision to form an L3 larva at the following molt is essentially irreversible, while a decision to prepare for dauer larva formation can be reversed by changing one or more of the three environmental parameters prior to the second molt (Fig. 11).

The temperature downshift experiments demonstrate three phases of predauer development. First, downshifts prior to the L1 molt permit the animals to escape temperature-induced dauer larva formation. This shows that elevated temperature exerts its effect no earlier than the L1 molt. The second developmental phase is represented by the first transition in the downshift curve. It spans the L1 molt and the first third of the L2d stage (Fig. 8). Downshifts performed at these times are too late to prevent temperature-induced dauer larva formation. Comparison of these downshift data with upshift results (Fig. 7) establishes a narrow temperature-sensitive period (TSP) for wild-type dauer larva formation, centered at the L1 molt. Worms growing at 15°C must be shifted to 25°C at or before the L1 molt to enhance dauer larva formation. Therefore, the temperature-dependent process must end early in the second larval stage. All these observations establish a classical TSP at the L1 molt.

In the third phase of predauer development, during the latter two-thirds of the L2d intermolt period, the animals reach a stage where the temperature downshifts themselves antagonize dauer larva formation. Thus, even though the animals have progressed through the TSP entirely at elevated temperature, a downshift reverses the process of dauer larva formation when executed prior to the L2d molt. This downshift effect is marginal at the mid-L2d stage, but is progressively more significant as the shift time approaches the molt. The transition point between the second and third predauer phase occurs at 16 hr after hatching (times at 25°C).

The downshift effect persists into the dauer stage it-

self, in the sense that downshifts performed on dauer larvae trigger recovery. It is the temperature-shift, not the low temperature itself, that is essential for the inhibition of dauer larva formation and the induction of recovery. Downshifts at 16 hr failed to express the inhibitory effect, even though the animals were at the lower temperature during the later half of the L2d and the dauer stages. Dauer larvae formed and maintained at 15°C do not recover spontaneously, nor do dauer larvae upshifted to 25°C.

Temperature downpulse experiments performed on dauer larvae maintained in pheromone, showed that a 10 hr downpulse was necessary to induce 50% of the population to recover. This indicates that developmental commitment to recovery after the temperature downshift is relatively asynchronous and delayed, relative to the 50 to 60 min required for commitment to recovery when dauer larvae were transferred out of pheromone onto a fresh lawn of *E. coli* at 23°C. The temperature downshift apparently represents a relatively weak recovery stimulus. Thus, recovery stimuli of differing magnitude or type produce quantitatively different effects, just as is the case for stimuli enhancing entry into the dauer stage. Both the fraction of the dauer larva population induced to recover and the timing of recovery is dependent on the environmental stimuli applied.

The temperature-dependence of dauer larva formation exhibited by the N2 strain is a true wild-type behavior. A second wild-type isolate of *C. elegans*, DH424, and a wild-type isolate of *C. briggsae*, G16, also exhibit temperature-dependent dauer larva formation in the presence of the *Caenorhabditis* pheromone (Golden and Riddle, 1984). The mechanism of the temperature effect is at least partially distinct from that of the thermotaxis response of *C. elegans* because two tested thermotaxis mutants both show temperature-sensitive dauer larva formation in the presence of exogenous pheromone. The altered response to pheromone of these two mutants indicates a functional overlap between the sensory mechanisms responsible for thermotaxis and dauer larva formation.

The temperature dependence of the wild-type process explains the observed predominance of *ts* dauer-constitutive alleles, at least some of which are null alleles (Golden and Riddle, 1984). The coincidence of the wild-type TSP with that found for five out of six *ts* mutants supports the idea that the mutants reveal, or overexpress, the wild-type temperature-dependent process. The mutant TSPs need not reflect the timing of dauer-constitutive gene functions, but instead may represent the developmental time when assessment of the environment can lead to dauer larva formation. The exceptional

ts dauer-constitutive mutant is *daf-14* (Swanson and Riddle, 1981) which has its major TSP within the first larval stage, although it has a minor TSP around the L1 molt. The *daf-14* mutant may be precocious in its discrimination between the alternate developmental pathways.

The exact time of commitment to the non-dauer life cycle appears to depend on the strength of the environmental cues as well as the timing of exposure. A pronounced decrease in the ability to induce dauer larvae occurs around the first molt. Removal from dauer-inducing conditions at around the first molt for relatively short periods of time results in a significant decrease in the percentage of a population which is induced to form dauer larvae. A developmental switch between the L2 and L2d may occur at this time, with only the L2d still possessing the potential to become a dauer larva. However, an absolute commitment to the L3 does not occur at the first molt because we have been able to force L2 larvae grown at relatively low population densities to form dauer larvae if transferred soon after the first molt to very stringent dauer-inducing conditions.

Several important developmental events occur near the L1 molt. Divisions of hypodermal seam cells add new nuclei to the hypodermal syncytium (Sulston and Horvitz, 1977). The ventral nerve cord increases from 15 to 55 cells, and some neuronal rewiring occurs in the retrovesicular ganglion near the anterior end of the cord (Sulston, 1976; White *et al.*, 1978). If neural plasticity exists elsewhere in the nervous system at this time, it is possible that chemosensory information could modulate development of neural synapsis in a way which would predispose the animal toward one developmental pathway or the other. Alternatively, a neurosecretory event at the L1 molt may affect programming of the following molt.

Dauer larvae can survive for months (Klass and Hirsh, 1976). However, a dramatic, rapid increase in their propensity to recover occurs during the first few days of incubation. This is a behavioral change which occurs long before energy reserves are exhausted. We could not distinguish between a decrease in sensitivity to pheromone and an increased sensitivity to food-signal because of the competitive influences of these two parameters in the bioassay. The change in pheromone/food sensitivity may be an adaptive response to the stability of the dauer-inducing pheromone (Golden and Riddle, 1982). Dauer larvae may not always be able to disperse from the immediate environment which induced their formation. If they become progressively less sensitive to the pheromone still present, they would be able to recover and develop to reproductive adults after non-dauer larvae died and/or a fresh food supply became

available. The age dependence of the recovery behavior makes it necessary to carefully standardize dauer larva preparations used in the pheromone bioassay. Use of 5-day-old dauer larvae, incubated at 20°C, provides a sensitive bioassay which detects pheromone diluted 200-fold from a crowded culture (Golden and Riddle, 1982).

Results presented here show that the developmental process leading to dauer larva formation in wild-type *C. elegans* is mediated by an integrated behavioral response to three environmental parameters: food, pheromone, and temperature. Discrimination between alternate developmental sequences begins at the L1 molt. Manipulation of specific environmental parameters allows the induction of 100% synchronously formed wild-type dauer larvae. This information will provide us with the tools necessary for further ultrastructural, biochemical, and molecular analysis of wild-type dauer larva formation, and it defines new phases in pre-dauer development useful for the analysis of mutants affected in this developmental pathway.

REFERENCES

ALBERT, P. S., BROWN, S. J., and RIDDLE, D. L. (1981). Sensory control of dauer larva formation in *Caenorhabditis elegans. J. Comp. Neurol.* **198**, 435-451.

BRENNER, S. (1974). The genetics of *Caenorhabditis elegans. Genetics* **77**, 71-94.

BYERLY, L., CASSADA, R. C., and RUSSELL, R. L. (1976). The life cycle of the nematode *Caenorhabditis elegans.* I. Wild type growth and reproduction. *Develop. Biol.* **51**, 23-33.

CASSADA, R. C. (1975). The dauer larva of *C. elegans:* A specific developmental arrest, inducible environmentally and genetically. *In* "Developmental Biology: Pattern Formation and Gene Regulation"
(D. M. McMahon and C. F. Fox, eds.), pp. 539-547. Benjamin, New York.

CASSADA, R. C., and RUSSELL, R. L. (1975). The dauer larva, a postembryonic developmental variant of the nematode *Caenorhabditis elegans. Develop. Biol.* **46**, 326-342.

EMMONS, S. W., KLASS, M. R., and HIRSH, D. (1979). Analysis of the constancy of DNA sequences during development and evolution of the nematode *Caenorhabditis elegans. Proc. Nat. Acad. Sci. USA* **76**, 1333-1337.

FODOR, A., NELSON, F. K., GOLDEN, J. W., and RIDDLE, D. L. (1983). Comparison of a new wild-type *Caenorhabditis briggsae* with laboratory strains of *C. briggsae* and *C. elegans. Nematalogica,* in press.

GOLDEN, J. W., and RIDDLE, D. L. (1982). A pheromone influences larval development in the nematode *Caenorhabditis elegans. Science* **218**, 578-580.

GOLDEN, J. W., and RIDDLE, D. L. (1984). A pheromone induced developmental switch in *Caenorhabditis elegans:* Temperature-sensitive mutants reveal a wild-type temperature-dependent process. *Proc. Nat. Acad. Sci. USA* **81**, in press.

HAASE, C. E., GOLDEN, J. W., and RIDDLE, D. L. (1983). Mutants of *Caenorhabditis elegans* unresponsive to the dauer-inducing pheromone. *Genetics* **104**, s32-33.

HEDGECOCK, E. M., and RUSSELL, R. L. (1975). Normal and mutant thermotaxis in the nematode *C. elegans. Proc. Nat. Acad. Sci. USA* **72**, 4061-4065.

KLASS, M., and HIRSH, D. (1976). Non-aging developmental variant of *Caenorhabditis elegans. Nature (London)* **260**, 523-525.

RIDDLE, D. L. (1977). A genetic pathway for dauer larva formation in *Caenorhabditis elegans. Stadler Genet. Symp.* **9**, 101-120.

RIDDLE, D. L., SWANSON, M. M., and ALBERT, P. S. (1981). Interacting genes in nematode dauer larva formation. *Nature (London)* **290**, 668-671.

SINGH, R. N., and SULSTON, J. E. (1978). Some observation of molting in *C. elegans. Nematologica* **24**, 63-71.

SULSTON, J. E., and BRENNER, S. (1974). The DNA of *Caenorhabditis elegans. Genetics* **77**, 95-105.

SULSTON, J. E. (1976). Post embryonic development in the ventral cord of *C. elegans. Phil. Trans. R. Soc. B.* **275**, 287-297.

SULSTON, J. E., and HORVITZ, H. R. (1977). Postembryonic cell lineages of the nematode *Caenorhabditis elegans. Develop. Biol.* **56**, 110-156.

SWANSON, M. M., and RIDDLE, D. L. (1981). Critical periods in the development of the *Caenorhabditis elegans* dauer larva. *Develop. Biol.* **84**, 27-40.

WHITE, J. G., ALBERTSON, D. G., and ANNESS, M. A. R. (1978). Connectivity changes in a class of motoneurone during the development of a nematode. *Nature (London)* **271**, 764-766.