Two Pleiotropic Classes of *daf-2* Mutation Affect Larval Arrest, Adult Behavior, Reproduction and Longevity in *Caenorhabditis elegans*

David Gems,*,[1] Amy J. Sutton,* Mark L. Sundermeyer,* Patrice S. Albert,* Kevin V. King,* Mark L. Edgley,* Pamela L. Larsen*,† and Donald L. Riddle*

**Molecular Biology Program and Division of Biological Sciences, University of Missouri, Columbia, Missouri 65211 and †Division of Neurogerontology and Molecular Biology Program, University of Southern California, Los Angeles, California 90089*

Manuscript received April 23, 1997
Accepted for publication June 11, 1998

ABSTRACT

The nematode *Caenorhabditis elegans* responds to overcrowding and scarcity of food by arresting development as a dauer larva, a nonfeeding, long-lived, stress-resistant, alternative third-larval stage. Previous work has shown that mutations in the genes *daf-2* (encoding a member of the insulin receptor family) and *age-1* (encoding a PI 3-kinase) result in constitutive formation of dauer larvae (Daf-c), increased adult longevity (Age), and increased intrinsic thermotolerance (Itt). Some *daf-2* mutants have additional developmental, behavioral, and reproductive defects. We have characterized in detail 15 temperature-sensitive and 1 nonconditional *daf-2* allele to investigate the extent of *daf-2* mutant defects and to examine whether specific mutant traits correlate with each other. The greatest longevity seen in *daf-2* mutant adults was approximately three times that of wild type. The temperature-sensitive *daf-2* mutants fell into two overlapping classes, including eight class 1 mutants, which are Daf-c, Age, and Itt, and exhibit low levels of L1 arrest at 25.5°. Seven class 2 mutants also exhibit the class 1 defects as well as some or all of the following: reduced adult motility, abnormal adult body and gonad morphology, high levels of embryonic and L1 arrest, production of progeny late in life, and reduced brood size. The strengths of the Daf-c, Age, and Itt phenotypes largely correlated with each other but not with the strength of class 2-specific defects. This suggests that the DAF-2 receptor is bifunctional. Examination of the null phenotype revealed a maternally rescued egg, L1 lethal component, and a nonconditional Daf-c component. With respect to the Daf-c phenotype, the dauer-defective (Daf-d) mutation *daf-12(m20)* was epistatic to *daf-2* class 1 alleles but not the severe class 2 alleles tested. All *daf-2* mutant defects were suppressed by the *daf-d* mutation *daf-16(m26)*. Our findings suggest a new model for *daf-2*, *age-1*, *daf-12*, and *daf-16* interactions.

THUS far, *C. elegans* is the only metazoan organism in which a number of single gene mutations causing large increases in life span have been identified. These genes include *daf-2* (Kenyon *et al.* 1993) and *age-1* (Friedman and Johnson 1988), formerly also known as *daf-23* (Malone *et al.* 1996; Morris *et al.* 1996; Tissenbaum and Ruvkun 1998). These mutations result in mean life spans of up to 250 and 300% of wild type, respectively (Larsen *et al.* 1995). Both genes also control dauer larva formation (Riddle 1988; Gottlieb and Ruvkun 1994).

Dauer larvae are nonfeeding, developmentally arrested, alternative third-stage larvae, which form in response to crowding and reduced food supply (Cassada and Russell 1975; Golden and Riddle 1984a). A constitutively released dauer-inducing pheromone serves as a measure of population density. A low pheromone:food ratio and low temperature promote continuous development through four larval stages (L1–L4) to the adult, but high pheromone levels and higher temperatures promote dauer formation and inhibit exit from the dauer state. Under the latter conditions, L1 larvae molt to a predauer (d) L2d stage, which lasts longer than the L2 (Golden and Riddle 1984a). L2ds retain the developmental potential to molt to the L3 should conditions improve, but if they do not, they molt into the dauer stage, shrink radially, and become resistant to detergent treatment and other environmental insults.

Dauer larvae are long lived relative to the adult, with maximum life spans of around 70 days (Klass and Hirsh 1976) and 30 days (Johnson and Wood 1982), respectively, in liquid culture. Dauer larvae are considered nonaging because the length of time spent in the dauer stage has no effect on postdauer life span (Klass and Hirsh 1976). The basis for the enhanced longevity of dauer larvae is unknown. However, evidence suggests that a reduction in metabolic activity occurs, consistent with long-term survival in the absence of food (O'Riordan and Burnell 1989; Wadsworth and Riddle 1989), and although capable of rapid movement, dauer larvae are largely inactive. Dauer longevity may also be enhanced by increased resistance to stress. Dauer larvae

Corresponding author: Donald L. Riddle, Molecular Biology Program, 311 Tucker Hall, University of Missouri, Columbia, MO 65211. E-mail: riddled@missouri.edu

[1] *Present address*: The Galton Laboratory, Department of Biology, University College London NW1 2HE, UK.

show enhanced resistance to thermal injury (Anderson 1978) and to oxidative damage-inducing chemicals (Larsen 1993). They also show increased activities of superoxide dismutase (Anderson 1982; Larsen 1993) and catalase (Vanfleteren and De Vreese 1995) relative to adults.

Over 30 genes controlling dauer larva formation have been identified. Mutations in these *daf* genes result in either the inability to form dauer larvae in response to crowding and starvation (dauer-defective, or Daf-d), or the constitutive formation of dauer larvae in the presence of abundant food (dauer-constitutive, or Daf-c). Studies of the phenotypes resulting from combinations of *daf-c* and *daf-d* mutations have allowed the *daf* genes to be ordered into complex, branched pathways (reviewed by Riddle and Albert 1997). Mutations in one branch of the pathway (*age-1*, *daf-2*, *daf-16*, and *daf-18*) affect both dauer larva formation and adult life span (Kenyon *et al.* 1993; Larsen *et al.* 1995; Dorman *et al.* 1995). All *daf-2* and most *age-1* mutants are Daf-c and may be involved in transduction of environmental information via the nervous system or by some other route. The *age-1* gene encodes a putative phosphatidylinositol 3-OH kinase catalytic subunit (Morris *et al.* 1996). PI 3-kinases typically transmit signals from cell-surface receptor tyrosine kinases into the cell (Kapeller and Cantley 1994). The *daf-2* gene encodes a receptor tyrosine kinase similar to the vertebrate and Drosophila insulin receptors (Kimura *et al.* 1997).

Adult expression of functions normally expressed in the dauer stage may account for the increased longevity (Age) of *daf-2* (Kenyon *et al.* 1993) and *age-1* adults. The Age phenotype requires *daf-16* activity because a *daf-d* mutation in this gene suppresses the enhanced longevity resulting from mutations in *daf-2* (Kenyon *et al.* 1993) or *age-1* (Larsen *et al.* 1995; Dorman *et al.* 1995). The *daf-16* gene encodes a Fork head-related transcription factor (Ogg *et al.* 1997; Lin *et al.* 1997). The establishment of a causal link between misexpression of a particular dauer trait in the adult and extension of adult life span would be illuminating with respect to the nature of the biological determinants of life span.

Previous comparisons between the canonical allele, *daf-2(e1370)*, and other *daf-2* alleles have revealed considerable variation in the *daf-2* mutant phenotype. For example, the phenotypes of *daf-2(e1370)* and *daf-2(m41)* differ with respect to temperature dependence of the Age phenotype, effects on fecundity, and interactions with mutations in the *daf-d* gene *daf-12* (Larsen *et al.* 1995). The *daf-2(e979)* mutation results in embryonic and L1 arrest (Vowels and Thomas 1992).

To better understand *daf-2* function, we have conducted a detailed phenotypic study of 16 mutant alleles. We have focused, in particular, upon the range of effects on life span, how the severity of other mutant traits correlates with the degree of life span extension, and how different *daf-2* alleles interact with *daf-12* to affect both dauer larva formation and life span. Our results reveal the existence of two overlapping classes of *daf-2* allele, differing both in their mutant phenotypes and in their interactions with *daf-12*. Class 1 mutants display Daf-c, Age, and increased intrinsic thermotolerance (Itt) phenotypes. Class 2 mutants are more pleiotropic, exhibiting the class 1 defects, in addition to other developmental and behavioral defects, and resembling severe *age-1* mutants with respect to their larval arrest phenotype and interactions with *daf-12* (Gottlieb and Ruvkun 1994; Larsen *et al.* 1995). Defects resulting from both mutant classes are suppressed by *daf-d* mutations in *daf-16*, whereas mutations in *daf-12* suppress the class 1 Daf-c and Age phenotype but not the class 2 mutants we tested. Our findings clarify the relationship between *daf-2*, *age-1*, *daf-12*, and *daf-16* in the genetic pathway controlling dauer formation and life span.

MATERIALS AND METHODS

Culture methods and strains: Animals were maintained monoxenically in 60-mm Petri dishes containing 10 ml NG agar seeded with *Escherichia coli* OP50 as the food source (Brenner 1974). The *daf-2* mutations used in this study were *e979*, *e1365*, *e1368*, *e1369*, *e1370*, *e1371*, *e1391*, *m41*, *m65*, *m120*, *m212*, *m577*, *m579*, *m596*, *m631*, *m632*, *sa193*, and *sa223*. Other mutations used were LG I, *daf-16(m26)*; LG III, *dpy-1(e1)*, *mec-12(e1605)*, *unc-32(e189)*, *unc-93(e1500)*; LG V, *dpy-11(e224)*; and LG X, *daf-12(m20)*. The *daf-2(sa193)* and *daf-2(sa223)* strains were provided by J. H. Thomas. In Vowels and Thomas (1992), *daf-2(e979)* was referred to as *daf-2(e1286)* (J. H. Thomas, personal communication).

All alleles were backcrossed to the Caenorhabditis Genetics Center wild-type (N2) male stock at least three times to remove possible second-site mutations. Because most strains had previously been backcrossed once or twice, it was necessary to perform one or two further backcrosses. The twice backcrossed *sa223* strain was backcrossed two more times only after its detailed characterization, but tests on dauer larva formation and life span indicated that the four-times backcrossed strain was indistinguishable from the strain originally received.

Construction of *daf-2; daf-12* double mutants: In the case of ts *daf-2* alleles, *daf-2; daf-12* double mutants were constructed as previously described (Larsen *et al.* 1995). The *daf-2(m65) III* mutation results in nonconditional dauer larva formation. Consequently, the construction of the double mutant carrying *daf-12(m20) X* used *qC1* [*dpy-19(e1259*ts*) glp-1(q339)*] *III* to balance *m65*. *daf-2(m65)/qC1* males were mated with *daf-12(m20)* hermaphrodites at 20°, and the F_1 males were backcrossed with balanced *daf-2* hermaphrodites. F_1 hermaphrodites were selfed individually at 15° to identify cross progeny of genotype *m65/qC1; m20/+* based on the segregation of dauer larvae *(m65)*, sterile adults defective in germline proliferation (Glp) (*qC1*), and longer animals (some *m20* homozygotes are longer than wild type). Also observed among the segregants were dark-bodied animals that exhibited a novel developmental arrest phenotype (see results). A long segregant was used to establish the *daf-2(m65)/qC1; daf-12(m20)* strain.

Construction of *daf-2(m65) unc-32(e189)/qC1* strain: *dpy-1(e1) unc-32(e189)/++* males were crossed with *daf-2(m65)/qC1* hermaphrodites, and F_1 hermaphrodites were selfed. Two of 12 F_1s segregated dauer larvae and Dpy Unc (dumpy, short body; uncoordinated movement) animals. Ten Unc non-Dpy

F_2s were selfed, seven of which segregated Unc dauer larvae and Dpy Uncs (*i.e.*, were + *daf-2 unc-32*/*dpy-1* + *unc-32*). Unc non-Dpy F_3 hermaphrodites were crossed with N2 males, progeny males were crossed with *daf-2(m65)*/*qC1*, hermaphrodite progeny were selfed, and a *daf-2 unc-32*/*qC1* strain was identified as one segregating Unc dauer and Dpy Glp progeny at 20°.

Construction of heteroallelic strains: To test the possible dominance of *daf-2(e979)*, a *daf-2(e979)*/*daf-2(m577)* strain was constructed as follows. *e979*/+ males were crossed with *m577* hermaphrodites at 22.5°, at which temperature *m577* homozygotes do not form dauer larvae. Dauer progeny were picked and allowed to recover at 15°. Approximately half proved to be male, confirming that such dauer progeny resulted from crossing. For progeny testing at 25.5°, dauer larvae that recovered to adult hermaphrodites were allowed to lay eggs for 24 hr, then removed. Progeny were scored after 72 hr of development (measured from the middle of the 24-hr egg-laying period).

Animals heteroallelic for different combinations of ts *daf-2* alleles and nonconditional *daf-2* alleles were constructed to examine the 25° phenotype of nonconditional segregants in the absence (or severe reduction) of maternal rescue. To construct *daf-2(m65) unc-32(e189)*/*daf-2(m577)* +, *daf-2(m65) unc-32*/*qC1* males were crossed with *daf-2(m577)* hermaphrodites at 22.5°. Since *m577* does not result in dauer formation at 22.5°, dauer progeny were necessarily *daf-2(m65) unc-32*/*daf-2(m577)* +. They were induced to resume development by transfer to 15°.

The phenotypes of *daf-2(m65)*/*daf-2(e979)*, *mDf12*/*daf-2(e979)*, and *mDf12*/*mDf11* at 25.5° were determined by scoring brooded cross plates 2, 3 and, if necessary, 4 days after the parental animals were transferred to fresh plates. *daf-2(m65)qC1* or *mDf12*/*qC1* males were mated to either *daf-2(e979)* or *mDf11*/*qC1* hermaphrodites at 25.5°. The number of self-progeny was minimized by mating 10 adult males with two L4 hermaphrodites on plates with a 1-cm-diameter spot of bacteria.

Dauer formation, predauer arrest, and brood size assays: The effects of *daf-2* mutations on brood size, dauer formation, and early larval arrest were examined at 15°, 20°, 22.5°, and 25°. Gravid adults (10–20) grown at 15° were allowed to lay eggs for 1 hr, then removed. The resulting synchronous population was raised at 15° until the late L4 stage. Ten animals were placed singly on plates and shifted to the assay temperature. These P_0 animals were transferred to new plates every 24 hr until the end of the reproductive period. Each brood plate was examined daily to follow development to a terminal phenotype. Any adults or L4 larvae were counted and removed. The number of dauer progeny was scored 72 hr after the midpoint of egg laying at 25.5°, 80 hr at 22.5°, 96 hr at 20°, and 120 hr at 15°. Samples compromised by fungal or bacterial contaminants were excluded. In the case of *daf-2(sa223)*, maternally rescued adults were picked from among progeny of *daf-2(sa223)*/*qC1* hermaphrodites. Although these *sa223* progeny often arrested development as L3s, L4s, or adults (exhibiting darkness of body, reduced motility, and reduced gonad development), a number of adult *sa223* homozygotes developed gonads, and these were picked for brood-size analysis.

In studies of larval development of *daf-2; daf-12* strains, groups of gravid hermaphrodites raised at 15° were allowed to lay eggs overnight (5–6 animals, 22.5°) or for 4 hr (10–15 animals, 25.5°), then removed. At 22.5°, the appearance of larvae was checked 60 hr after the midpoint of egg laying and scored after 80 hr. At 25.5°, the appearance of larvae was checked after 50 hr. Progeny were intermittently observed and scored between 50 and 100 hr after the midpoint of egg laying.

Life span determination: L4 larvae grown at 15° were placed at 15° and 22.5°, typically at a density of 15–30 animals per plate. These were transferred daily to fresh plates during the egg-laying period and subsequently at approximately weekly intervals. Death was scored as the absence of any movement and failure to move at all after several light pokes with a platinum wire. The zero time point was the time of L4 transfer. Samples compromised by bacterial contaminants were excluded. Life span was assayed at 22.5° rather than the usual nonpermissive temperature of 25.5°, at which a high level of mortality is seen throughout adult life in some *daf-2* mutants due to internal hatching of eggs and other unknown causes that may not be related to senescence. It was expected that at 22.5°, population survival curves would be more rectangular and show less variation between trials, facilitating comparisons between strains.

Intrinsic thermotolerance assays: Young adult hermaphrodites grown at 15° were transferred to prewarmed (35°) 60-mm NG agar plates (not spread with bacteria) and maintained at that temperature. The number of worms dead and alive was recorded at 2-hr intervals until all were dead. Any worms that died as the result of crawling up the wall of the plate were excluded from the analysis.

Intrinsic thermotolerance of selected *daf-2*(class 2)*; daf-12* double mutants was determined in the same manner. However, to test the effect of *daf-12* on certain class 1 alleles, the procedure was modified to increase the thermotolerance of the *daf-2* adults. Synchronous populations were raised to adult at 20°, then transferred to fresh plates at 25° for 2 days before testing, as described above. To compensate for the slower development of the class 1 *m41* mutant and the class 2 *e1391* mutant, populations of strains containing these alleles were started 1 day earlier.

Adult behavior and morphology: During the course of life span assays, behavior and appearance of adult animals were examined at 1- to 3-day intervals through a dissecting microscope. At higher temperature several alleles resulted in some shrinkage of the adult body, clearly discernible at ×25 magnification, and gonadal abnormalities, which were easily visible at ×50 magnification as clear regions against the otherwise dark body characteristic of *daf-2* adults. The onset of an obvious reduction in motility and the appearance of coiling behavior was generally rapid (occurring over a 1- to 2-day period), such that motility was readily classified as normal or reduced. Reproducibility of scoring was confirmed by consistency in classification in blind trails on successive days and by independent classification by two observers.

Pharyngeal pumping rate: Worms were raised at 15°, then transferred singly at the L4 stage to fresh plates at 22.5°. The mean pumping rate did not include nonpumping animals. Pumping was scored over a 15-sec interval or at 30-sec or 1-min intervals where pumping rate was reduced. In graphs of pumping rate as a function of percentage of maximum life span, the latter was calculated by dividing the ages at which pumping rate was measured by maximum life spans (shown in Table 3).

Male mating efficiency: Six *daf-2* alleles were tested for their effects on male fertility at 20° and 25.5°. These alleles (*e1370*, *e1371*, *e1391*, *m41*, *m120*, and *m577*) were selected as a representative sample of the range of severity and variation in mutant phenotypes. Male stocks were established with males obtained by heat shock (Sulston and Hodgkin 1988) or from males occurring spontaneously in hermaphrodite populations maintained at 15°. A standard quantitative mating test was employed (Hodgkin 1983), in which six late L4 *daf-2* males (raised at 15°) and six late L4 *dpy-11(e224)* hermaphrodites were placed together on a 60-mm plate spread with bacteria and incubated for 24 hr at 20°, after which the males were

TABLE 1

Prior phenotypic analysis of *daf-2* alleles

Allele	Features described	Source
e979[a]	Embryonic and L1 arrest, Daf-c	1
e1368	Daf-c, epistasis, temperature-sensitive period	2, 3
e1370	Isolation, Daf-c, epistasis, life span, brood size	1, 2, 4–9
e1391	Daf-c, epistasis, life span, brood size	2, 9
m41	Daf-c, epistasis, life span, brood size	7
m65	Daf-c, epistasis	7
sa193	Isolation, Daf-c, life span	5, 10
sa223	Isolation, Daf-c, adult sterility	10

References: 1, Vowels and Thomas (1992); 2, Riddle *et al.* (1981); 3, Swanson and Riddle (1981); 4, Riddle (1977); 5, Kenyon *et al.* (1993); 6, Gottlieb and Ruvkun (1994); 7, Larsen *et al.* (1995); 8, Dorman *et al.* (1995); 9, Tissenbaum and Ruvkun (1998); and 10, Malone and Thomas (1994), which describes the Daf-c phenotype of a further seven *daf-2* alleles.

[a] *daf-2(e979)* (reference strain CB 1286) is referred to as *daf-2(e1286)* in reference 1.

removed. Total cross-progeny (non-Dpy F_1) were counted. Tests of mating efficiency at 25.5° were performed in a manner similar to those at 20° except that early (rather than late) L4 males were used, such that the entire period of spermatogenesis occurred at the higher temperature.

Isolation and characterization of *daf-2* deficiencies: Two γ-radiation-induced deficiencies were isolated in a noncomplementation screen. Mixed-stage N2 populations containing many males were exposed to 1500 R. Irradiated young adult males were immediately crossed to *dpy-1(e1) daf-2(e1370) unc-32(e189)* hermaphrodites at 20° then shifted to 25° after 24 hr. Three days later the F_1 progeny were screened by visual inspection and SDS selection for the presence of wild-type or Dpy dauer larvae. Four of seven wild-type larvae that failed to recover spontaneously at 25° recovered to the L4 stage after 2 days at 15°, and these four were crossed individually with *daf-2(m65) unc-32(e189)/qC1[dpy-19(e1259) glp-1(q339)]* males at 20°. All four crosses were shifted to 25° after egg laying began (approximately 24 hr). Two of the crosses gave progeny. From each, wild-type nondauer L4 hermaphrodites of putative genotype *mDf[daf-2]/qC1* or *dpy-1 daf-2 unc-32/qC1* were placed individually on plates. Those issuing Dpy Unc progeny were discarded. The remainder for both isolates gave only wild-type and *qC1* progeny, indicating that the new mutations were lethal when homozygous. The two putative deficiencies were named *mDf11* and *mDf12*. Complementation testing showed that neither deficiency deleted *unc-93*; tests against *mec-12* were inconclusive. Thus, the putative deficiency endpoints lie between *dpy-1* and *unc-93*. Progeny counts confirmed that both strains gave approximately 25% embryonic lethal progeny. The *mDf12/qC1* animals, but not *mDf11/qC1*, grow slowly relative to *qC1* homozygotes.

Both deficiencies were shown by PCR analysis to lack *daf-2* sequences encoding portions of the extracellular domain and the tyrosine kinase domain. The sequence of the *daf-2* cDNA was obtained from GenBank (accession no. AF012437) and compared to the *C. elegans* sequence database to identify genomic YAC clones corresponding to *daf-2* (Kimura *et al.* 1997). Two pairs of oligonucleotide primers were designed for PCR, one pair each from genomic sequence encoding the extracellular and intracellular domains of the protein. Primers CTCTCGAACAAAACAGTGCCTATC and AATGAGGGCCA ACTAAAGAAGACC amplified a 659-bp wild-type fragment encoding a portion of the extracellular domain, whereas primers TTCGGACCGTGTGCTATTAAGATT and CTCGGACCT CCACTATGATTCATC amplified a 1082-bp fragment encoding a portion of the kinase domain. Another primer pair (AGCAGCACCAGCAACAGGAGTAAC and TTTCAAACCCC CAACTCATACCTC) from the *lin-31* region of chromosome *II* (cosmid K10G6) was used as an internal positive control to confirm that amplifiable DNA from the deficiency homozygotes was present in the reaction. This primer pair amplified a 523-bp product.

To identify and isolate deficiency homozygotes (dead eggs), newly starved plates of *mDf/qC1* bearing large numbers of unhatched eggs were washed free of gravid adults and most larvae by two gentle rinses with sterile M9 buffer, leaving most eggs still adhering to the agar surface. Washed plates were incubated at 20° for 24 hr and washed again to remove larvae that hatched after the initial rinse. Deficiency homozygotes were identified as eggs that appeared abnormal in shape and remained unhatched after a further 24–48 hr of incubation at 20°. These were picked individually or in groups of up to 15 using a pulled-out 20-μl pipette filled with chitinase solution (Williams *et al.* 1992).

PCR reactions (25 μl final volume) were performed according to Williams *et al.* (1992), except that Taq polymerase (Fisher Scientific) was used at 0.5 unit per reaction, and "master mix" was added to each reaction in 18.5-μl volumes to allow for separate addition of *daf-2* and *lin-31* control primers. Test reactions on N2 DNA using a mix of control and *daf-2* primers resulted in production of both predicted products, whereas parallel reactions with putative *Df* DNA gave only the control product. For each strain and primer pair, assays were performed in duplicate on purified N2 DNA, deionized water blanks, and worm extracts (*Df/qC1* heterozygotes) and in quadruplicate on eggs (*Df* homozygotes). All reactions were brought to 95° rapidly and held for 3 min, cycled 30 or 50 times (95°/30 sec, 58°/30 sec, 72°/60 sec), then held for 7 min at 72°. Amplification products were resolved on 1–2% agarose minigels.

RESULTS

Selection of alleles for study: Most previous studies of *daf-2* used the canonical allele, *e1370*. We selected 15 additional alleles to cover a wide range of severity of the Daf-c phenotype. Fourteen alleles, including *e1370*, were selected from the 40 currently in the Riddle lab collection (of which 28 are conditional and 12 noncon-

TABLE 2

Percentage larval arrest by *daf-2* mutants

	15°		20°		22.5°		25.5°	
Allele[a]	Daf-c[b]	*N*	Daf-c[b]	*N*	Daf-c[b]	*N*	Daf-c[b]	*N*
N2	0	2521	0	2743	0	3133	0	1710
Class 1								
e1365[c]	0	2778	0.1 ± 0.1	2929	0	2641	96 ± 2[d]	1725
m577[c]	0	1988	0	2851	0	2590	96 ± 2[d]	1945
sa193	ND[f]	ND[f]	0	3003	0 (11 ± 2)[g]	2675	85 ± 7[e]	1580
e1371	0	2288	0.1 ± 0.3	2592	0 (58 ± 11)[g]	2810	97 ± 0.9[e]	2070
e1368	0	1516	0.3 ± 0.3	2641	1.5 ± 0.9	2953	89 ± 2[e]	1543
m41	0	2523	14 ± 4	2096	75 ± 9	2296	97 ± 2[d]	1508
m212	28 ± 9	2373	99.5 ± 0.4	3159	99.6 ± 0.5[e]	1738	97 ± 5[e]	1530
e1369	55 ± 6	1637	100 ± 0.3	2489	100	2500	99 ± 0.6[d]	1965
Class 2								
m120	0	2966	0	2387	10 ± 4	2969	99 ± 0.6[e]	1229
e1370	0	1905	0.5 ± 0.7	2641	14 ± 5 (21 ± 8)[h]	2318	97 ± 2[d]	1543
m596	0	1799	0.4 ± 0.4	1926	52 ± 11	2138	95 ± 2[d]	1532
m579	0	1839	0	2155	50 ± 10 (70 ± 10)[h]	2205	96 ± 4[e]	1140
e1391	5 ± 3	1791	66 ± 7	2118	99 ± 0.7[d]	1353	90 ± 3[e]	873
e979	20 ± 3	2134	98 ± 0.8[d]	2357	99 ± 1[d]	1847	0[i]	605
sa223	0[j]	29	0[k]	130	3.3 ± 4[l]	102	95 ± 10[l]	31

[a] Ranked in approximate order of increasing severity with respect to L2d and dauer arrest, within each class.
[b] Values ± standard error, calculated from the percentage dauer larva formation among progeny (complete broods) of 10 individual animals.
[c] Ranking of *e1365* above *m577* based upon suppression of *e1365* Age phenotype by *daf-12(m20)*. See Table 3.
[d] All animals not arrested at the dauer stage arrested as unhatched eggs, L1s, or early L2s.
[e] All animals not arrested at the dauer stage arrested as unhatched eggs, L1s or early L2s, or developed into adults.
[f] Not determined.
[g] Transient dauer larvae; scored at 48 hr.
[h] Arrest at L2d or dauer stage.
[i] 24% embryonic arrest, 76% L1 arrest.
[j] 48% embryonic arrest, 14% L1 or L2 arrest, 38% L2d arrest.
[k] 92% L2d arrest, 1% L1 arrest, 7% embryonic arrest.
[l] All animals that did not arrest at the dauer stage arrested development as L2ds.

ditional). Two other alleles, *sa193* and *sa223*, were provided by J. H. Thomas. All are recessive. Some phenotypic characterization had previously been reported for 8 of the 16 alleles (including 2, *m65* and *sa223*, that are recessive lethal and are maintained in heterozygous stocks) as listed in Table 1. The remaining 8 are previously undescribed, temperature-sensitive (ts) Daf-c mutants and were isolated from mutant screens over the last 25 years in the MRC Laboratory of Molecular Biology, Cambridge, United Kingdom (1973–1975) or subsequently in the Riddle laboratory. All alleles were backcrossed to N2 at least three times to avoid possible phenotypic variation due to differences in genetic background.

Characterization of alleles: We focused on the phenotypic effects of hypomorphic *daf-2* alleles, although analysis of *daf-2(e979)*, nonconditional *daf-2* alleles, and *daf-2* deficiencies suggested that the null [*daf-2(0)*] phenotype has an embryonic and L1 arrest component as well as a nonconditional Daf-c component (see below). By focusing on weaker alleles we have been able to examine the role of *daf-2* in later larval development and in the biology of the adult.

Examination of the phenotypes of 16 *daf-2* mutants revealed two types of *daf-2* allele, class 1 and class 2. The following sections on the phenotypic analysis begin with descriptions of the major traits common to the class 1 and class 2 alleles: Daf-c, Age, and Itt. Next, L1 arrest is described, which results from all *daf-2* alleles but at a greatly elevated frequency in two class 2 alleles. Then follow descriptions of the diverse class 2-specific traits, with an overview and more refined classification of the 16 alleles.

Constitutive dauer larva formation: The percentage of the mutant population that constitutively entered the dauer stage in abundant food was measured at 15°, 20°, 22.5°, and 25.5° for all ts alleles (Table 2). These alleles largely formed a continuous series in severity, from the weakest, *e1365* and *m577*, to the strongest, *e1369*. Although the five weakest alleles with respect to Daf-c are

class 1, some class 1 alleles exhibited a stronger Daf-c phenotype than most class 2 alleles. Differences in the severity of most alleles were revealed at 22.5°; the majority formed no dauer larvae at 15° but formed all dauer larvae at 25.5°.

At 22.5° most alleles resulted in the rapid development of larvae either into L3 or dauer larvae (either transient or persistent). The *sa193* and *e1371* alleles were ranked in severity on the basis of the percentage of dauer larvae that recovered spontaneously within 80 hr after eggs were laid (Table 2). However, in two instances (*e1370* and *m579*) all larvae arrested initially at the predauer L2d stage, followed by slow development either into dauer larvae or into stages apparently intermediate between the L2d and L3 or L4 stages. These dauer-like animals resembled L2ds in their darkness of body yet resembled L3s or L4s in size and gonadal development (Figure 1B).

Two exceptional alleles were *e979*, which at 25.5° arrested as embryos, L1s, or rarely at a stage resembling L2d larvae (as previously reported by Vowels and Thomas 1992), and *sa223*, which formed dauer larvae at 25.5° but at lower temperatures largely arrested development as L2ds (Malone and Thomas 1994). At 25.5°, three of the *daf-2* mutants (*e1368*, *e1371*, and *sa193*) formed dauer larvae that displayed sporadic pharyngeal movement resembling the pumping of feeding animals. Normal dauer larvae do not feed, the buccal cavity is closed, and pharyngeal pumping is not observed.

To examine maintenance of the dauer stage, dauer larvae from all ts Daf-c strains were maintained at 25.5° for 8 days after hatching and examined daily for the presence of adults. A high proportion of *e1371* and *sa193* dauer larvae resumed development. Occasional adults were seen among *e1368* and *m212* dauer larva populations (all population sizes >200). Thus, in the cases of *e1368*, *e1371*, and *sa193*, pharyngeal movement in dauer larvae corresponded with some degree of recovery from developmental arrest.

To determine whether nondauer development was affected by *daf-2* mutations, we tested the effect of 14 *daf-2* alleles on the subsequent development of animals raised at 15° and transferred to 25.5° as early L3s. Only in the case of *e1371* did all the animals grow to gravid adults within 24 hr. In most other cases, the majority of animals developed into gravid adult hermaphrodites, with a minority (5–20%) of pregravid adults. In three cases, *e979*, *e1370*, and *e1391*, abnormal development occurred; 24 hr after upshift, most animals were abnormally dark and thin, with underdeveloped gonads, and in many cases a protruding vulva. After 48 hr, some gonadal development was observed in most of the *e979* and *e1391* adults and in all of the *e1370* adults.

Hermaphrodite life span: The effect of 15 *daf-2* alleles on adult hermaphrodite life span was measured at 15° and 22.5° (Figure 2, Table 3). All mutations increase adult life span. The alleles exhibited a gradient of in-

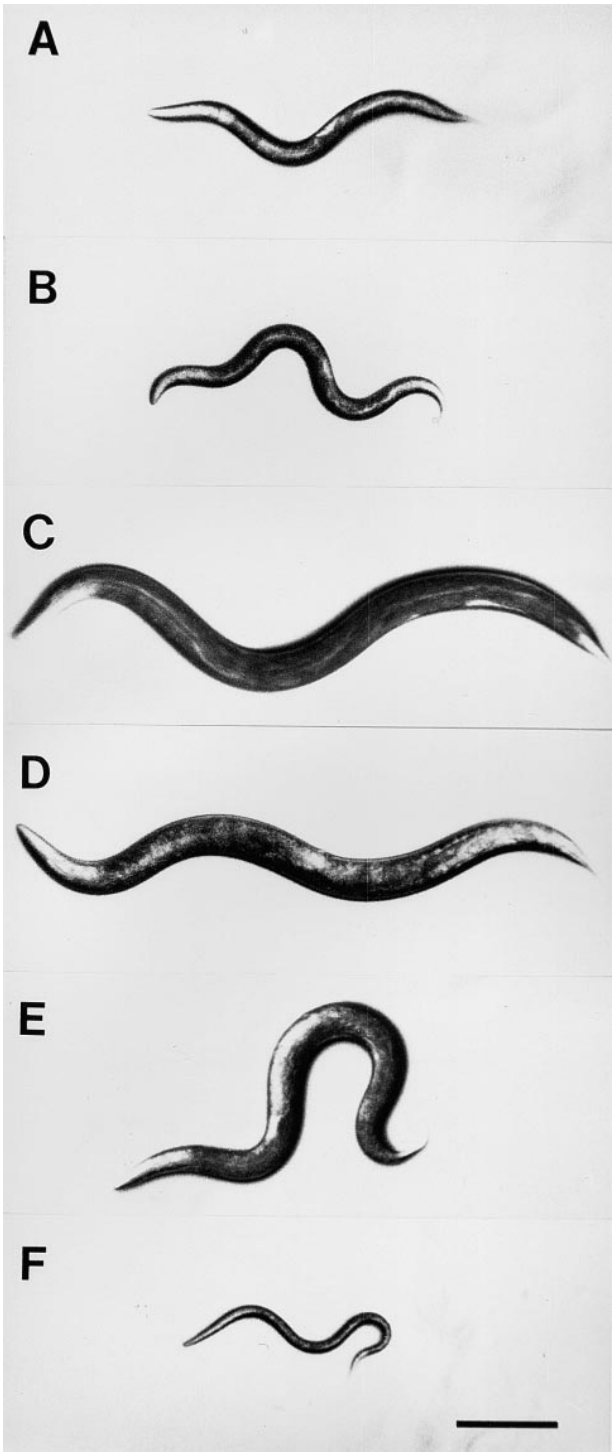

Figure 1.—Morphology of *daf-2* mutant larvae and adults. (A) *daf-2(e1370)* L3, raised in abundant food at 15°; (B) *e1370* dauer-like L3, raised at 22.5°; (C) 5-day-old *e1370* hermaphrodite maintained at 15°; (D) *daf-2(sa193)* hermaphrodite transferred to 25.5° at the L4 stage and incubated for 3 days; (E) *e1370* hermaphrodite transferred to 25.5° at the L4 stage and incubated for 3 days; (F) *e1370* dauer larva raised at 25.5°. (A–F) Scale bar, 0.2 mm.

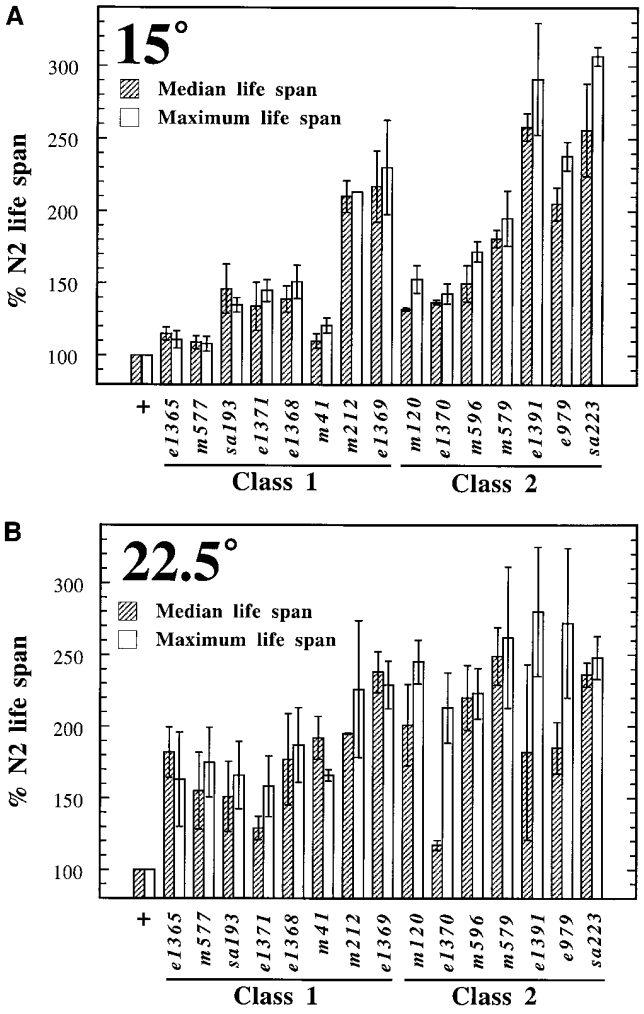

Figure 2.—Effect of *daf-2* mutations on median and maximum hermaphrodite life span. Median and maximum life span are expressed as a percentage of that of the wild-type control. Bars represent standard error. (A) Life span at 15°; (B) life span at 22.5°. Alleles are in order of increasing severity of the L2d and dauer arrest phenotype within each class.

creasing longevity at 15°, but the relative extensions of median and maximum life span were similar in most cases (Figure 2A, Table 3). The greatest increases in maximum life span were approximately 300% of the N2 control. At 15°, increases in life span were only marginal for *e1365*, *m41*, and *m577*.

At 22.5° all 15 mutants had clearly extended life spans (Figure 2B, Table 3). Most weaker alleles showed greater extensions in life span relative to N2 at 22.5° than at 15°. However, the five alleles with the largest increases in median and maximum life span at 15°, *e979*, *e1369*, *e1391*, *m212*, and *sa223*, at 22.5° generally showed similar or reduced extension of median life span and similar extensions in maximum life span (Figure 2, Table 3). The greatest percentage increases in life span at 22.5° did not exceed those seen at 15°, suggesting that the limit of adult life span extension that may result from loss of *daf-2*(+) function has been reached in these cases. At either temperature, this longevity ceiling represents a 2.5-fold increase in median life span and a 3-fold increase in maximum life span relative to wild type.

In some class 2 alleles, the extension of maximum life span at 22.5° greatly exceeded that of median life span (Figure 2B). This suggests either that these mutations have a deleterious effect on adults, resulting in some premature deaths, or conceivably that they cause individuals in the population to age at different rates.

Intrinsic thermotolerance: Mutations in *daf-2* and *age-1* also result in increased tolerance to thermal stress, and it has been suggested that increased Itt, as measured by time of survival at 35°, is a necessary condition for the Age phenotype (Lithgow *et al.* 1995). Because mutant *daf-2* alleles result in a range of Age phenotypes at 15°, the Itt phenotype of populations of young adults raised to the age of onset of egg laying at 15° was tested (Figure 3, Table 4). Increased Itt was observed in all alleles tested except *m41* and *m577*. Seven of the most severely Age alleles at 15° (*e979*, *e1369*, *e1370*, *e1391*, *m212*, *m579*, and *m596*) showed similarly high values for Itt, suggesting that, as in the case of life span, the limit of adult thermotolerance that can result from loss of *daf-2*(+) function has been reached in these cases. The severely Age allele *sa223* was also strongly Itt (data not shown). This suggests that loss of *daf-2* function in mutants that can grow to the adult at 15° may result in, at most, approximately a 75% increase in median survival at 35° (Table 4).

Embryonic and L1 arrest: *daf-2(e979)* exhibits almost 100% embryonic or L1 arrest at 25°. This phenotype was examined in all 15 ts alleles at 15°, 20°, and 25.5° (Table 5). Some embryonic and L1 arrest (mostly the latter), higher than that observed in N2 controls, was observed in all *daf-2* alleles tested at 25.5° (except *sa193*) but in most cases not at 15° or 20° (Table 5). The frequency of such arrest was generally low or undetectable at the temperatures tested (mean, <6%). However, at 25.5° the *e1391* and *e979* mutations resulted in 10.5 ± 2.8 and 100% embryonic and L1 arrest, respectively. A high level of *sa223* embryonic arrest (14/29 total progeny) was seen at 15°.

Brood size: Fecundity of *daf-2* mutant hermaphrodites was assayed at 15°, 20°, 22.5°, and 25.5° (Table 6). In self-fertilizing *Caenorhabditis elegans* hermaphrodites, maximum brood size normally reflects the fixed number of sperm produced before the switch from spermatogenesis to oogenesis. When averaged over all temperatures tested, class 1 mutants have broods ranging from 85 to 100% that of N2, and class 2 mutants (except *sa223*) have broods ranging from 60 to 93% of N2. Two class 1 alleles (*e1368* and *e1369*) were cold sensitive, showing 35–40% reductions in brood size only at 15°. These were the only class 1 alleles where significant brood size reductions ($P < 0.01$) were seen, whereas most class 2 alleles resulted in significant reductions in

TABLE 3

Adult life span

Allele[a]	15° Median	15° Maximum	15° N[b]	22.5° Median	22.5° Maximum	22.5° N[c]
+	24.0 ± 1.6	31.0 ± 3.0	304 (5)	16.3 ± 1.3	22.2 ± 2.4	306 (6)
daf-12(m20)	16.7 ± 0.6	24.8 ± 4.1	80 (4)	10.6 ± 1.4	16.0 ± 1.2	80 (4)
Class 1						
daf-2(e1365)	28.2 ± 2.9	36.5 ± 2.5	224 (2)	29.2 ± 3.0	39.7 ± 8.3	257 (3)
daf-2(m577)	25.8 ± 1.1	34.0 ± 4.3	269 (4)	25.9 ± 3.8	40.3 ± 6.6	246 (4)
daf-2(sa193)	33.5 ± 1.2	40.5 ± 1.5	41 (2)	26.0 ± 2.0	35.0 ± 1.0	39 (2)
daf-2(e1371)	31.8 ± 2.8	45.5 ± 2.2	273 (4)	21.6 ± 0.5	36.3 ± 5.6	247 (4)
daf-2(e1368)	33.2 ± 3.2	47.5 ± 5.3	263 (4)	29.5 ± 4.2	43.3 ± 8.6	223 (4)
daf-2(m41)	27.0 ± 1.3	35.0 ± 1.4	71 (3)	29.4 ± 1.2	35.0 ± 0.0	60 (2)
daf-2(m212)	48.4 ± 1.4	64.0 ± 0.0	40 (2)	34.0 ± 3.0	47.5 ± 4.5	38 (2)
daf-2(e1369)	52.8 ± 2.8	74.5 ± 1.5	213 (2)	38.2 ± 2.4	54.8 ± 3.1	232 (4)
daf-2(e1365); daf-12(m20)	18.2 ± 1.5	29.7 ± 3.8	64 (3)	15.2 ± 2.3	24.0 ± 4.7	77 (4)
daf-2(m577); daf-12(m20)	21.6 ± 3.2	32.7 ± 1.9	61 (3)	18.5 ± 7.5	33.8 ± 1.1	89 (4)
daf-2(sa193); daf-12(m20)	26.7 ± 2.4	42.0 ± 9.0	41 (2)	18.4 ± 2.6	29.5 ± 3.0	91 (4)
daf-2(m41); daf-12(m20)	24.6 ± 3.9	32.8 ± 6.0	89 (4)	17.9 ± 4.8	41.3 ± 4.7	88 (4)
daf-2(m212); daf-12(m20)	25.3 ± 5.6	51.1 ± 7.0	78 (4)	34.0 ± 7.0	52.5 ± 3.6	80 (4)
Class 2						
daf-2(m120)	32.3 ± 1.8	50.0 ± 3.0	252 (2)	31.6 ± 4.4	57.8 ± 7.2	257 (4)
daf-2(e1370)	33.8 ± 0.2	42.0 ± 1.4	66 (3)	20.4 ± 2.4	45.0 ± 0.0	39 (2)
daf-2(m596)	36.8 ± 5.3	56.5 ± 4.5	208 (2)	35.4 ± 3.8	54.3 ± 4.6	257 (3)
daf-2(m579)	44.3 ± 1.3	63.5 ± 1.5	239 (2)	40.0 ± 3.2	64.3 ± 15.0	266 (3)
daf-2(e1391)	63.4 ± 5.0	91.3 ± 10.9	259 (3)	29.2 ± 9.7	68.0 ± 10.0	142 (3)
daf-2(e979)	50.3 ± 2.8	69.0 ± 3.0	105 (4)	28.3 ± 1.7	56 ± 12.0	57 (2)
daf-2(sa223)	58.7 ± 2.7	92.0 ± 2.0	36 (2)	41.0 ± 2.0	53.0 ± 3.0	37 (2)
daf-2(e1370); daf-12(m20)	30.4 ± 2.0	41.7 ± 2.1	65 (3)	21.6 ± 4.6	62.5 ± 1.7	88 (4)
daf-2(e1391); daf-12(m20)	58.7 ± 8.0	81.3 ± 9.6	69 (3)	71.8 ± 2.2	94.0 ± 7.0	94 (4)
daf-2(e979); daf-12(m20)	45.6 ± 4.8	91.5 ± 13.5	37 (2)	43.3 ± 8.7	72.0 ± 2.9	86 (4)

[a] *daf-2* alleles ordered in increasing severity of L2d and dauer arrest phenotype, within each class.
[b] Sample size; number of trials in parentheses.

brood size, which were generally more severe at higher temperatures (Table 6).

The largest reductions in brood size resulted from *sa223*. In this case, brood sizes of maternally rescued animals were measured because progeny of *sa223* homozygotes do not develop to adulthood. Mean *sa223* brood size did not exceed 4% of wild type at any of the four temperatures tested (Table 6). At all temperatures, some *sa223* adults were sterile, even though adults in which some gonad development was evident were selected (see materials and methods).

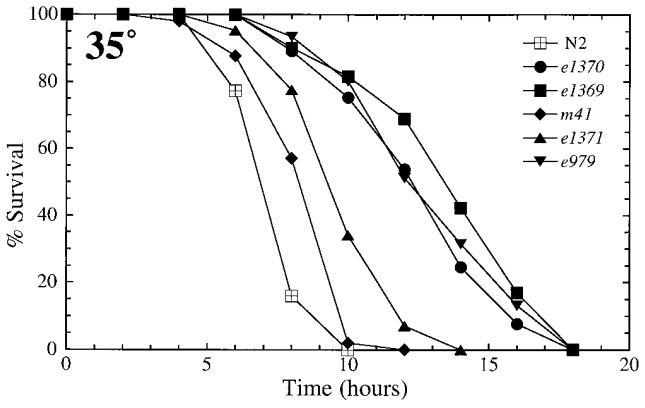

Figure 3.—Intrinsic thermotolerance assays. A representative sample of 35° survival curves for *daf-2* adult hermaphrodite populations, raised at 15°, is shown.

Late progeny: Production of progeny by N2 hermaphrodites at 25.5° ceases after 3–5 days, but *e1370* hermaphrodites continue to produce occasional progeny for at least 50 days (Larsen *et al.* 1995). All 15 ts *daf-2* alleles were examined for late progeny production at 15°, 22.5°, and 25.5° (Table 7). Of these, only the 7 class 2 alleles resulted in this trait.

Because late progeny generally appeared after the transfer of hermaphrodites to fresh plates, and often produced in bursts by a single individual, it is possible that a cue present in fresh bacterial lawns might stimulate late reproduction in these mutants. At least some late progeny were released by their mothers as L1 larvae rather than as eggs. Late eggs were never observed.

Late progeny could result from a delay in production of oocytes or from renewal of spermatogenesis later in life, after depletion of the sperm produced during the L4 stage. To test this latter possibility, the laying of

TABLE 4

Intrinsic thermotolerance of *daf-2* hermaphrodites

Strain[a]	Median survival (hr)	% N2[b]	Maximum survival (hr)	% N2	*N*[c]
N2	7.1 ± 0.5	—	8.6 ± 0.9	—	514 (7)
Class 1					
e1365	8.6 ± 0.3	123 ± 6	11.0 ± 1.0	130 ± 12	299 (4)
m577	8.0 ± 0.8	104 ± 9	10.0 ± 2.0	110 ± 10	151 (2)
sa193	8.1	117	10	125	69 (1)
e1371	9.3 ± 0.4	134 ± 6	13.0 ± 1.7	155 ± 29	275 (2)
e1368	10.0 ± 1.1	144 ± 18	13.0 ± 1.7	154 ± 22	305 (4)
m41	7.9 ± 0.2	109 ± 9	10.5 ± 0.9	118 ± 10	256 (5)
m212	11.6 ± 0.7	166 ± 12	15.0 ± 1.0	188 ± 13	166 (2)
e1369	12.4 ± 1.0	178 ± 17	15.0 ± 1.0	188 ± 13	136 (2)
Class 2					
m120	10.3	166	12	150	57 (1)
e1370	12.2 ± 0.7	172 ± 15	16.4 ± 0.8	197 ± 21	363 (5)
m596	11.2 ± 0.9	163 ± 12	14.5 ± 15.0	172 ± 28	153 (2)
m579	12.1 ± 0.1	178 ± 1	14.0 ± 1.0	156 ± 11	117 (2)
e1391	11.8 ± 0.8	168 ± 9	15.0 ± 1.0	188 ± 13	163 (2)
e979	12.3 ± 0.1	175 ± 3	16.0 ± 0.0	200 ± 0	163 (2)
sa223	ND	ND	ND	ND	ND

ND, not determined.

[a] Alleles ranked in order of increasing severity of L2d and dauer arrest phenotype, within each class.

[b] To calculate these values, mutant median survival at 35° was expressed as a percentage of the wild-type control in each respective trial, and then the mean and standard error were calculated from the set of percentages. For this reason, these values cannot be derived directly from the values in the preceding columns.

[c] Total number of animals; in parentheses, number of trials.

TABLE 5

Embryonic and L1 arrest in *daf-2* mutants

	Embryonic and L1 arrest (%)		
Allele[a]	15°	20°	25.5°
N2	0	0	0.1 ± 0.2
Class 1			
e1365	0	0	4.4 ± 1.7
m577	0.3 ± 0.4	0.03 ± 0.1	3.9 ± 2.0
sa193	ND	0	0.1 ± 0.3
e1371	0	0	2.4 ± 1.1
e1368	0	0	2.9 ± 1.6
m41	0	0	2.7 ± 1.8
m212	0 (9)	0	2.6 ± 4.9 (9)
e1369	0	0	0.7 ± 0.6
Class 2			
m120	0	0	0.7 ± 0.5
e1370	0	0.2 ± 0.3	2.6 ± 1.8
m596	0	0	5.5 ± 1.8
m579	0	0	4.4 ± 3.7
e1391	0	0.8 ± 0.6	10.5 ± 2.8
e979	0.5 ± 0.4 (9)	1.3 ± 0.8	100
sa223	30.4 ± 30.4 (2)[b]	3.6 ± 7.5	5.0 ± 10.0 (5)[c]

Table shows mean and standard error of percentage embryonic or L1 arrest in broods of a set of individual hermaphrodites raised entirely at 15°, or shifted to 20° or 25.5° at the L4 stage. Unless otherwise noted by the number in parentheses, 10 hermaphrodites were tested for each allele. See Table 2 for total progeny scored. ND, not determined.

[a] Ranked in order of increasing severity of the L2d and dauer arrest phenotype, within each class.

[b] of 10 animals tested, only 2 bore progeny.

[c] Of 12 animals tested, only 5 bore progeny.

TABLE 6

Brood sizes of *daf-2* mutants

	Mean brood size[a]				% N2				
Allele[b]	15°	20°	22.5°	25°	15°	20°	22.5°	25°	Mean
N2	252 ± 21	274 ± 37	313 ± 42	171 ± 8	100	100	100	100	100
Class 1									
e1365	278 ± 33	293 ± 33	264 ± 36	173 ± 22	110	107	84	101	101
m577	199 ± 23	285 ± 29	259 ± 78	195 ± 34	79	104	83	114	95
sa193	ND	300 ± 68	268 ± 34	158 ± 60	ND	109	86	92	96
e1371	229 ± 18	260 ± 19	281 ± 91	207 ± 22	91	95	90	121	99
e1368	152 ± 29**	264 ± 26	295 ± 41	154 ± 15	60	96	94	90	85
m41	252 ± 18	210 ± 47	255 ± 66 (9)	151 ± 8*	100	77	81	88	87
m212	264 ± 50 (9)	316 ± 30	191 ± 44 (9)*	170 ± 43	105	115	61	99	95
e1369	164 ± 22**	249 ± 25	250 ± 31	197 ± 10	65	91	80	115	88
Class 2									
m120	297 ± 19	239 ± 15	297 ± 66	123 ± 14**	118	87	95	72	93
e1370	191 ± 44	245 ± 23	231 ± 21	67 ± 13***	76	89	74	39	70
m596	180 ± 16*	193 ± 14*	214 ± 45*	153 ± 13	71	70	68	89	75
m579	184 ± 17*	216 ± 15	221 ± 40	114 ± 17**	73	79	71	67	73
e1391	179 ± 12**	212 ± 25	135 ± 16***	87 ± 18***	71	77	43	51	61
e979	237 ± 20 (9)	250 ± 34	185 ± 37*	60 ± 51*	94	91	59	35	70
sa223[c]	3.2 ± 9 (2)***	10.8 ± 10***	8.5 ± 8 (7)***	2.6 ± 5 (5)***	1.3	3.9	2.7	1.5	2.4

ND, not determined.

* $0.01 < P < 0.05$; ** $0.005 > P < 0.01$; *** $P < 0.005$ (Student's *t* test).

[a] Brood sizes ± standard error of 10 hermaphrodites per allele, unless otherwise stated (in parentheses); sample sizes as in Table 2.

[b] Ranked in order of increasing severity of the L2d and dauer arrest phenotype, within each class.

[c] Maternally rescued hermaphrodites used. At 15°, 20°, 22.5°, and 25.5°, 2/10, 10/12, 7/12, and 5/12 hermaphrodites, respectively, produced progeny.

unfertilized oocytes at the end of the egg-laying period, an indicator of sperm depletion (Ward and Carrel 1979), was monitored at 22.5° (Table 8). All *daf-2* mutant alleles resulted in a reduction in the number of oocytes laid relative to the N2 control, although this reduction was not significant in many class 1 alleles. The seven class 2 mutants, which exhibited late progeny production, also laid the fewest oocytes. Hence, stored sperm may be present in hermaphrodites of the late progeny-producing strains at the end of the normal egg-laying period, and late progeny may result from fertilization of oocytes that are not produced until late in life. That *daf-2(e1370)* hermaphrodites are defective in oocyte production is also suggested by the abnormal appearance of the gonad. Microscopic examination with Nomarski optics of adult *daf-2(e1370)* hermaphrodites 19 hr after shifting from 15° to 25.5° revealed that, instead of the usual 8–10 oocytes stacked in the proximal arm and loop of each gonad arm, only 2–4 oocytes were present (J. McCarter, personal communication).

Maternal rescue of larval arrest: To test for maternal rescue of the Daf-c trait, hermaphrodites from the 15 ts *daf-2* strains were crossed with N2 males at 25.5°, and F_1 heterozygotes were selfed at the same temperature. Their progeny were scored after 48 hr. Because at 25.5° all, or almost all, homozygous *daf-2* mutants form dauer larvae, 25% of F_2 animals were expected to form dauer larvae in the absence of maternal rescue. The ratio of nondauer-to-dauer larva progeny was approximately 3:1, except for *m41* heterozygotes, which segregated only 12 ± 8% dauer larvae, indicating that the Daf-c phenotype was maternally rescued in approximately half of the *m41* homozygous progeny (Table 9; data not shown for ts alleles where no maternal rescue was seen). Repeating the *m41* test at 25.5°, + *m41* + / *dpy-1* + *unc-32* parents issued 8% dauer larvae, 66% wild-type L4-adult and 25% DpyUnc progeny (N = 185). The *e979* embryonic and L1 arrest phenotype was found to be fully maternally rescued such that *e979* homozygotes arrested development at the dauer stage or, occasionally, the L2d stage (Table 9).

The *m65* mutation results in nonconditional dauer larva formation, and *sa223* results in formation of L2ds, dauer larvae, and sterile or near-sterile adults. Both these alleles are maintained *in trans* to the balancer chromosome *qC1*[*dpy-19(e1259) glp-1(q339)*]*III*. No maternal rescue of *m65* was detected at 25.5° when assayed by scoring the progeny of *m65*/*qC1* hermaphrodites (Table 9). Homozygous *sa223* progeny of *sa223*/*qC1* either arrested permanently at a stage resembling late L2d or developed to adulthood via dauer-like third and fourth stages. These adults were sometimes thin and dark bodied, and when gonads were present, they often appeared abnormal.

TABLE 7

Late progeny production by *daf-2* hermaphrodites

	15°			22.5°			25.5°		
Allele[a]	Late progeny[b]	Last progeny day	*N*[c]	Late progeny[b]	Last progeny day	*N*[c]	Late progeny[b]	Last progeny day	*N*[c]
N2	0	14	25	0	11	29	0	4	11
Class 1									
e1365	0	ND	217	0	ND	209	0	6	15
m577	0	ND	252	0	ND	231	0	5	14
sa193	0	18	37	0	9	29	0	7	10
e1371	0	ND	234	0	ND	184	0	6	11
e1368	0	ND	255	0	ND	213	0	7	12
m41	0	14	70	0	< 9	26	0	6	13
m212	0	14	40	0	< 9	32	0	6	9
e1369	0	ND	212	0	ND	228	0	9	12
Class 2									
m120	0	ND	251	0.004	15	255	1.6	44	15
e1370	0	14	34	1.0	19	11	3.4	40	12
m596	0	ND	206	0	ND	251	0.1	23	14
m579	0	ND	239	0.2	48	17	0.2	26	16
e1391	0.08	28	256	8.2	54	20	9.2	40	40
e979	0	17	104	0.4	30	59	2.8	40	16
sa223	2.0	84	30	6.6	50	22	0.1	31	16

ND, not determined.
[a] Ranked in order of increasing severity of the L2d and dauer arrest phenotype, within each class.
[b] Progeny produced per animal after 17 days of age at 15°, after 11 days of age at 22.5°, after 10 days of age at 25.5°.
[c] Number of animals in trial on day 18 (15°), day 12 (22.5°), and day 11 (25.5°).

Comparison of progeny of homozygous *sa223* and heterozygous *sa223*/*qC1* hermaphrodites (Tables 2 and 9, respectively) revealed maternal rescue of the L2d-like arrest but no rescue of the Daf-c phenotype. Animals homozygous for the *sa223* allele formed dauer larvae at 25.5° but very few at 22.5°, whether they were progeny of *sa223* homozygotes (Table 2) or of *sa223*/*qC1* (Table 9). These results suggest that, whereas the Daf-c phenotype is not normally maternally rescued (with the exception of *m41*), the L2d arrest phenotype resulting from certain alleles may be.

Death from internal hatching (matricide): Animals that died as the consequence of internal hatching of eggs were excluded from measurements of life span. In most *daf-2* mutants, matricide, resulting in a "bag of worms" phenotype (Trent *et al.* 1983), was observed at a low frequency (<20%), occurring more readily at 22.5° than 15° (data not shown). At 22.5°, a higher frequency of matricide (over 30%) resulted from four class 2 alleles: *e979*, *e1370*, *e1391*, and *sa223*.

Adult morphology and behavior: Certain *daf-2* alleles (*e.g.*, *e1370*) resulted in progressive changes in adult hermaphrodite appearance and behavior during the 3 days after shifting L4s from 15° to 22.5° or 25.5°. Such changes were generally not seen at 15° (Figure 1C), and some alleles (*e.g.*, *sa193*) did not produce such changes at any temperature (Figure 1D). One day after transfer, *e1370* adults were egg-laying defective (Egl), their bodies swollen with retained eggs. Over subsequent days, overall body darkening and shrinkage in diameter were seen. The gonad and intestine, viewed in the stereomicroscope at ×50 magnification, appeared to become squeezed by the contracting body, the intestine becoming reduced to a narrow, longitudinal strip. In such animals, the gonad had an abnormal, clear appearance (Figure 1E). These animals also displayed an uncoordinated phenotype, comprising reduced movement, coiling behavior, and frequent adoption of a kinked posture similar to that seen in dauer larvae (Figures 1E and F).

Appearance and behavior of the 15 ts *daf-2* mutants was monitored during the first 2 weeks of adulthood at 15°, 22.5°, and 25.5°. Morphological and behavioral abnormalities similar to those seen in *e1370* mutants resulted from 11 of the 15 alleles in a temperature-sensitive manner (Table 10), whereas *sa223* exhibited these defects at all temperatures. At 22.5°, only 8 of the 15 alleles resulted in reduced motility. Timing and rate of onset of this Unc trait showed considerable variation among alleles (Table 10). Interestingly, in several alleles adult motility appeared normal for an extended period before becoming reduced.

The *e1370* allele was tested for reversibility of the morphological and behavioral abnormalities. Twenty-six late L4 larvae that were shifted to 25.5° for 3 days became fully Unc. They were then shifted back to 15°. Two days later, wild-type motility and morphology re-

TABLE 8

Production of unfertilized oocytes by *daf-2* hermaphrodites at 22.5°

Allele[a]	Oocytes/animal[b]	% N2	Range	*N*[c]	Late progeny seen at 22.5°
N2	139 ± 111	100	25–331	10	No
Class 1					
e1365	49 ± 40*	35	8–139	10	No
m577	79 ± 57	57	11–196	10	No
s193	23 ± 12***	16	7–48	9	No
e1371	50 ± 29*	36	18–117	10	No
e1368	77 ± 36	56	29–135	9	No
m41	17 ± 13***	12	2–39	9	No
m212	15 ± 10***	11	5–42	13	No
e1369	57 ± 40*	41	1–150	10	No
Class 2					
m120	12 ± 14***	9	0–44	10 (8)	No
e1370	1 ± 2***	1	0–8[d]	10 (2)	Yes
m596	13 ± 11***	9	1–36	9	No
m579	10 ± 5***	7	2–18	9	Yes
e1391	0***	0	—	10 (0)	Yes
e979	4 ± 4***	3	0–11	9 (8)	Yes
sa223	0***	0	0	12 (0)	Yes

* Indicates $0.01 < P < 0.05$; *** indicates $P < 0.005$ (Student's *t* test).
[a] Ranked in order of increasing severity of the L2d and dauer arrest phenotype, within each class.
[b] Oocytes laid at the end of the normal reproductive phase, ± SE.
[c] Number of P_0's examined; number laying oocytes, if different from *N*, in parentheses.
[d] Two animals laid one and eight oocytes, respectively.

TABLE 9

Maternal rescue of developmental arrest

Parent	Temperature	% L4	% Dauer larvae[a]	Other[b]	*N*[c]
m41/+	25.5°	87 ± 9	12 ± 8 (48)	0.6 ± 2	302 (8)
e979/+	25.5°	75 ± 4	25 ± 4 (100)[d]	0	457 (8)
m65/+	25.5°	75 ± 3	23 ± 3 (92)	1.8 ± 0.5	1361 (6)
+/*qC1*	25.5°	97 ± 2	0	3.1 ± 0.2	876 (4)
sa223/qC1	25.5°	73 ± 1	26 ± 1 (100)	1.3 ± 0.6	1561 (7)
sa223/qC1	22.5°	80 ± 2	5 ± 0.8 (20)	15 ± 4[e,f]	816 (2)
sa223/qC1	20°	85	0	16[e,g]	476 (1)
sa223/qC1	15°	76 ± 3	0	24 ± 3[e,h]	493 (2)
m65/qC1	25.5°	69 ± 2	22 ± 2 (88)	9 ± 1	753 (4)
m41	25.5°	0	97 ± 2	3 ± 2	1508
e979	25.5°	0	0	100	605
sa223	25.5°	0	95 ± 10	5 ± 10	31
sa223	22.5°	0	3.3 ± 4	97 ± 4[i]	102
sa223	20°	0	0	100	130
sa223	15°	0	0	100	29

[a] Estimated percentage of the homozygous *daf-2* segregants displaying this phenotype shown in parentheses.
[b] Primarily unhatched eggs, L1s, or clear, unhealthy-looking L2s.
[c] Total number of progeny scored. Number of trials given in parentheses.
[d] Includes a small proportion of late L2ds.
[e] Probably underestimated due to scoring homozygous *sa223* adults with extensive gonad development as wild type.
[f] 0.4 ± 0.2% dead eggs; 2 ± 0.6% L2d; 12 ± 4% dauer-like L3 and L4 forms, and adults without discernible gonad develpment; 1 ± 0.7% adults with some degree of gonad development (underestimated). Scored 80 hr after egg laying.
[g] 0.6% dead eggs; 12% dauer-like L3 and L4 forms, and adults without discernible gonad development; 3% adults with some degree of gonad develpment (underestimated). Scored 96 hr after egg laying.
[h] 0.5 ± 0.1% dead eggs; 4 ± 1% L2ds; 20 ± 1% dauer-like L3 and L4 forms. Scored 120 hr after egg laying.
[i] Developmental arrest at L2d stage.

turned, and >200 progeny were produced. Twelve control hermaphrodites of the same age left at 25.5° produced only two progeny and no unfertilized oocytes. The 24 survivors of the shift were returned to 25.5°, and by day 8 all animals were once again Unc and morphologically abnormal. Twenty survivors were again downshifted to 15°. These animals again recovered wild-type motility and appearance by day 10, at which time they were shifted up to 25.5° for the third time. By day 12, all 15 survivors were Unc and morphologically abnormal. These were shifted to 15°, and by day 13, most animals had again recovered wild-type appearance and behavior. Thus, the behavioral and morphological abnormalities displayed by *e1370* hermaphrodites at higher temperatures are reversible at least three successive times in the same animals. To our knowledge, this is the only case of a reversible ts adult Unc phenotype in *C. elegans*.

Male fertility: The effect of six mutant *daf-2* alleles on male mating efficiency was measured using a standard quantitative mating test (Hodgkin 1983). Males were raised at 15° and tested at 20° and 25.5°. At 20° mating efficiency and fertility were reduced to 31–45% of wild-type controls, except for *e1391*, where it was reduced to 10% of wild-type controls (Table 11). At 25.5°, the class 1 (*e1371*, *m41*, and *m577*) males were as fertile as N2 males or even more so, whereas the class 2 males (*e1370*, *e1391*, and *m120*) males produced no progeny at all. The latter three alleles also resulted in greatly reduced male motility, suggesting that, as in the case of many uncoordinated mutant males (Hodgkin 1983), their failure to sire offspring is the result of their inability to copulate.

Pharyngeal pumping rate: Kenyon *et al.* (1993) compared the rate of pharyngeal pumping, an indicator of the rate of ingestion of food, in wild-type and *daf-2(e1370)* adult hermaphrodites at 20°. They found that although the rate of pumping declines with time at a similar rate in both strains, it is greatly reduced throughout the extended phase of the *e1370* life span. Our examination of pharyngeal pumping in three class 1 and three class 2 mutants indicated that class 2 but not class 1 mutations depress the rate of pharyngeal pumping.

We examined the effect of *daf-2* alleles *e1371* (class 1) and *e1391* (class 2) on the rate of pharyngeal pumping at 22.5° from early adulthood onward. *e1391* mutants exhibited a rapid decline in the rate of pumping with advancing age (Figure 4A), whereas the rate of pumping slowed down more gradually with advancing age in *e1371* animals.

From day 6 onward, the rate of pumping was greater in *e1371* animals than in wild type. Does this signify that *e1371* results in higher rates of pumping in older animals or that *e1371* animals are biologically younger at later chronological ages? To clarify this issue, pumping rate was plotted against age expressed as percentage of maximum life span (Figure 4B). The decline in pumping rate with percentage maximum life span was similar in N2 and *e1371* animals; that is, the decline in pumping rate scaled with life span. This indicates that *e1371* has little effect on the rate of pharyngeal pumping relative to biological or developmental (as opposed to chronological) age and also that, in *e1371* mutants, pharyngeal pumping rate provides a biomarker for developmental age. Conversely, in the case of *e1391*, pumping is a poor indicator of developmental age. This is also true of *e1370* (data not shown).

Animals in which no pumping was observed in a 1-min interval were excluded from pumping rate measurements. In the case of class 2 alleles (*e1370*, *e1391*, and *m596*), the majority of animals exhibited greatly reduced pumping during the first 40% of the life span, but in class 1 (*e1371*, *m41*, and *sa193*) mutants, as in wild-type populations, the majority of animals exhibited greatly reduced pumping only in the second half of the life span (data not shown).

Classification of *daf-2* alleles: On the basis of the above phenotypic descriptions, *daf-2* alleles fell broadly into two types (Table 12). Class 1 alleles resulted in constitutive dauer larva formation (Table 2), increased longevity (Table 3), increased intrinsic thermotolerance (Table 4), a low level of embryonic and L1 arrest (mean, less than 6%) at 25.5° (Table 5), and a reduction in the number of unfertilized oocytes laid (Table 8). Class 2 mutants exhibited all of the above traits plus some or all of the following: a higher level of embryonic or L1 arrest (mean, greater than 6%) at 25.5° (Table 5), formation of dauer-like L3 and dauer-like L4 larvae (Figure 1B), reduced adult motility (Table 10), shrinkage of the adult body and gonad abnormalities at 22.5° and 25.5° (Table 10), reduced brood size (Table 6), increased frequency of internal hatching at 25.5° and production of late progeny (Table 7), greatly reduced (less than 10% that of wild type) number of oocytes laid (Table 8), and a reduction in the extension of median life span as compared to that of maximum life span (Table 3).

The class 1 alleles (except *m41*) can be ranked into an allelic series defined by the severity of Daf-c and Age, where the latter is expressed as maximum life span at 15° (Tables 2 and 3). This allelic series is as follows, in order of increasing severity:

$$\text{N2} < e1365 = m577 < sa193 < e1371 = e1368 < m212 < \text{e1369}.$$

This allelic series also approximately describes the ranking in severity of Itt. The class 1 allele *m41* is among the weakest alleles with respect to Age and Itt when grown at 15° (Tables 3 and 4), whereas with respect to its Daf-c phenotype at higher temperatures it is more severe than *e1368* (Table 2).

The class 1 alleles can be subdivided into four subclasses, 1A–1D (Table 12). Class 1A mutants (*e1368*,

TABLE 10

Reduced movement in adult *daf-2* hermaphrodites

	15°				22.5°				25.5°			
Allele[a]	Unc[b]	Age at onset (partial)[c]	Age at onset (full)	*N*[d]	Unc[b]	Age at onset (partial)[c]	Age at onset (full)	*N*[d]	Unc[b]	Age at onset (partial)[e]	Age at onset (full)[e]	*N*[d]
N2	No	—	—	39	No	—	—	37	No	—	—	24
Class 1												
e1365	No[f]	—	—	—	No	—	—	16	Yes*	12, 9	13, 12	31
m577	No	—	—	44	No	—	—	42	Yes*	11, –	12, 9	30
sa193	No	—	—	41	No	—	—	37	No	—	—	29
e1371	No[f]	—	—	—	No	—	—	39	No	—	—	33
e1368	No	—	—	35	No	—	—	42	No	—	—	29
m41	No	—	—	36	No	—	—	34	No	—	—	27
m212	No	—	—	40	Yes*	—	17	37	Yes*	5, 5	6, 9	28
e1369	No[f]	—	—	—	No	—	—	15	Yes*	11, 7	12, 12	28
Class 2												
m120	No[f]	—	—	—	Yes*	—	11	24	Yes	4, 2	5, 3	25
e1370	No	—	—	38	Yes	11	19	34	Yes	–, –	4, 3	34
m596	No[f]	—	—	—	Yes*	17	19	18	Yes*	11, –	13, 11	28
m579	No[f]	—	—	—	Yes*	17	24	18	Yes*	5, –	6, 4	27
e1391	No	—	—	38	Yes	5	6	43	Yes	–, 1	4, 3	31
e979	No	—	—	33	Yes	8	26	31	Yes	–, 2	4, 3	29
sa223	Yes	14	19	36	Yes	2	24	23	Yes	3, 3	4, 7	19

[a] Ranked in order of increasing severity of the L2d and dauer arrest phenotype, within each class.
[b] Animals that were Unc also developed severe gonad abnomalities, except where marked *.
[c] Days of adulthood. Late L4 stage is day 0. Some or full reduction of motility in some animals, or some reduction of motility in all animals.
[d] Number of animals examined.
[e] Results of two consecutive trials.
[f] Observations made during subculture.

e1371, and *sa193*) do not exhibit any reduced motility at any of the temperatures tested (Table 10) and differ from other class 1 mutants in that dauer larvae display slight pharyngeal movement at 25.5° 72 hr after the egg stage and may eventually recover into adults at 25.5°. The single class 1B allele, *m41*, resembles a class 1A allele, except that no pharyngeal movement or dauer larva recovery occurs at 25.5°. *m41* is also unique in

TABLE 11

Mating efficiency of mutant *daf-2* males[a]

	20°			25.5°		
Allele[b]	Cross progeny/male	% N2	Mating efficency[c]	Cross progeny/male	% N2	Mating efficiency[c]
N2	658	100	4	45	100	4
Class 1						
m577	291	44	4	85	188	4
e1371	295	45	4	70	154	4
m41	230	35	4	50	110	4
Class 2						
m120	203	31	4	0	0	0
e1370	238	36	4	0	0	0
e1391	62	10	2	0	0	0

[a] Six males tested per allele. Males raised at 15° and shifted to 20° at late L4 stage, or to 25.5° at early L4 stage.
[b] Ranked in order of increasing severity of Daf-c phenotype, within each class.
[c] Standard mating efficiency rating, where 4 = 30–100% of wild-type mating efficiency, 3 = 10–30% of wild-type, 2 = 1–10% of wild-type, 1 = less than 1% of wild-type, and 0 = no detectable mating (Hodgkin 1983).

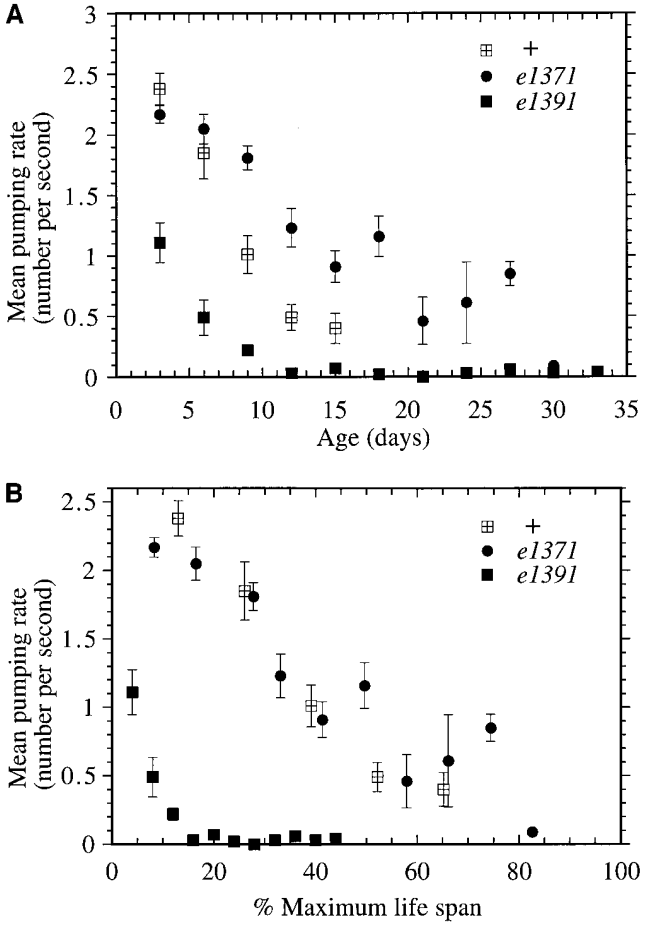

Figure 4.—Variation of *daf-2* mutant pharyngeal pumping with age at 22.5°. (A) Pharyngeal pumping rate plotted against age in days; (B) pumping rate plotted against age expressed as percentage of maximum life span; animals in which pumping was not observed in a 1-min interval were excluded from pumping rate calculations.

that the Daf-c phenotype is maternally rescued, and although moderately severe with respect to Daf-c at 22.5° (Table 2), it is almost entirely ts for Age and Itt (Tables 3 and 4). The class 1C mutants *e1365*, *e1369*, and *m577* exhibit reduced motility after 7 days at 25.5° (Table 10). The single class 1D mutant, *m212*, exhibits reduced motility at both 22.5° and 25.5°. This allele may be considered a borderline class 2 allele.

If both class 1 and class 2 traits are considered, no consistent ranking of allele severity may be achieved. However, on the basis of the severity of the class 2-specific mutant traits alone, the class 2 alleles may be ranked in severity as

$$\text{N2} < m596 < m120 = m579 < e1370 < e979 = e1391 < sa223.$$

The seven class 2 alleles constitute five subclasses, 2A–2E, based on severity and extent of pleiotropy (Table 12). The class 2A allele *m596* results in late progeny production only at 25.5° (Table 7), whereas the class 2B alleles *m120* and *m579* do so at 22.5° as well. *m120* and *m579* also result in slight gonad defects. In addition to these defects, the class 2C allele *e1370* and all the more severe alleles in this series also result in severe gonad defects at 22.5° and 25.5°, a mean brood size of less than 60% that of wild type at 25.5° (Table 6), over 30% mean death due to internal hatching of larvae at 22.5°, and a reduction in unfertilized oocytes laid to less than 5% of wild type at 22.5° (Table 8). Furthermore, *e1370* and some of the more severe alleles in the class 2 series also cause a significant reduction in median life span relative to maximum life span at 22.5° (Figure 2B). The class 2D alleles *e1391* and *e979* resemble *e1370* but additionally result in 11 and 100% mean predauer arrest at 25.5°, respectively (Table 5). *e1391* animals also do not lay unfertilized eggs at 22.5° (Table 8). Almost all of the class 2 defects are most severe in the case of *sa223*, which exhibits gonad defects and reduced motility even at 15° (Table 10), and a brood size reduced to less than 5% of wild type (Table 6). However, with respect to embryonic and L1 arrest, *sa223* is not more severe than *e979*. On the whole, with respect to class 2-specific defects, alleles may be ranked such that all the defects of weaker alleles are present or more severe in stronger alleles. The overall severity ranking of class 2 alleles given above is the most parsimonious, taking all the data into consideration.

The class 2 alleles *e1370*, *e1391*, and *m596* caused a reduction in the proportion of older animals pumping at 22.5°, and in the case of *e1370* and *e1391*, a severe reduction occurred in the rate of pharyngeal pumping (Figure 4, A and B). Conversely, the class 1 alleles *e1371*, *m41*, and *sa193* had only slight effects on pharyngeal pumping. This suggests that suppression of pharyngeal pumping may be a general feature of class 2 but not class 1 alleles. Similarly, the class 2 but not the class 1 alleles tested resulted in males that could not sire progeny at 25.5° (Table 11).

Correlation between mutant traits: Examining correlations between severities of different mutant traits may reveal which *daf-2* pleiotropic effects may be manifestations of the same underlying physiological defect. With the exception of certain alleles, *daf-2* mutant traits fall broadly into two clusters of apparently linked phenomena: first, Age, Daf-c, Itt, and minor traits common to all alleles; second, the class 2-specific defects. The severity of the Age/Daf-c/Itt cluster and the class 2 defects appears largely unconnected. Thus, for example, *e1369* ranks among the four most severe alleles with respect to Age and Daf-c yet displays almost no class 2 defects. Of the six most severe Daf-c alleles, three are in class 1.

We examined further the correlations between Age, Daf-c, and Itt traits. Plotting maximum life span at 15° against dauer larva formation at 22.5° shows a positive correlation in severity among most alleles (Figure 5A). One exception is *m41*, which is short lived (at 15°) relative to the severity of its 22.5° Daf-c phenotype. The

TABLE 12

Phenotypes of ts *daf-2* alleles

Class	Alleles	Phenotype[a]
1 (all)		Dauer constitutive, long-lived, intrinsically thermotolerant, unfertilized oocyte production at 22.5° 11–57% that of N2, up to 6% L1 arrest at 25.5°.
1A	*e1368, e1371, sa193*	25.5°: some pharyngeal movement in dauer larvae, substantial dauer larva, recovery.
1B	*m41*	15° Age and Itt phenotypes reduced relative to severity of 22.5° Daf-c phenotype, maternal rescue of 25.5° Daf-c phenotype.
1C	*e1365, e1369, m577*	Reduced motility at 25.5° only, after 7 days.
1D	*m212*	Reduced motility at 22.5° and 25.5°.
2 (all)		As class 1 (all), except:
2A	*m596*	Reduced adult motility at 22.5°, 25.5°, late progeny production at 25.5°, oocyte production at 22.5° 7–9% that of N2.
2B	*m120, m579*	As class 2A except: slightly abnormal gonads, median life span reduced relative to maximum life span at 22.5°, late progeny production at 22.5° and 25.5°.
2C	*e1370*	As class 2B except: abnormal gonads at 22.5°, 25.5°, oocyte production <4% of N2 at 22.5°. Brood size <60% of wild type at 25.5°, >20% death from internal hatching of larvae at 22.5°.
2D	*e979, e1391*	As class 2C except: 11% (*e1391*) and 100% (*e979*) embryonic and L1 arrest at 25.5°.
2E	*sa223*	As 2C except: L2d arrest, dauer-like L3-adult, abnormal gonad, reduced motility and late progeny at 15°. Greatly reduced brood size at all temperatures.

[a] All percentages are means.

sa223 mutant is long lived relative to dauer formation at 22.5°, but it exhibits an extreme predauer arrest phenotype. Also, *e1391* may be somewhat long lived relative to its Daf-c phenotype (Table 2), suggesting that, in this case, class 2 defects are associated with either an enhancement of Age or a depression of Daf-c.

If Age is plotted against Itt (Figure 5B), a positive correlation is also seen. When the Daf-c (22.5°) and the Itt phenotypes of animals raised at 15° were plotted against one another (Figure 5C), the nine most severe alleles with respect to Daf-c showed a similar level of Itt, with the exception of *m41*. This suggests that in mutants exhibiting greater than 10% dauer formation at 22.5°, Itt in animals raised at 15° is maximally penetrant.

Interactions between *daf-2* and *daf-12*—Dauer formation: Previous attempts to establish the epistasis relationship between *daf-2* and the *daf-d* gene *daf-12* have been complicated by differences between *daf-2* alleles. Whereas *daf-2(m41); daf-12(m20)* animals develop into adults at 25.5°, *daf-2(e1370); daf-12(m20)* animals arrest development either as embryos or L1s, or near the L2 molt (Yeh 1991; Vowels and Thomas 1992; Larsen *et al.* 1995). To test the hypothesis that phenotypes in combination with *daf-12* will be class specific, the phenotypes of six class 1 and four class 2 alleles were examined in combination with *daf-12(m20)*. Comparing developmental phenotypes of the 10 *daf-2; daf-12* strains, two components of the Daf-c phenotype, developmental arrest and dauer larva morphogenesis, may be distinguished. *daf-12(m20)* prevents dauer larva morphogenesis in both class 1 and 2 alleles, and it suppresses developmental arrest in class 1 but not severe class 2 alleles (Table 13). Developmental arrest was suppressed in the case of the weakest class 2 allele, *m596*.

The effect of each *daf-2; daf-12(m20)* combination on dauer formation was assayed (Table 13). In combination with *daf-12(m20)*, all six class 1 alleles behaved similarly. At 22.5°, all animals developed into L4s or adults by 60 hr. At 25.5°, development was slightly retarded such that L3s and L4s were seen instead of adults at 50 hr, but all animals subsequently developed into adults. At 22.5°, the three *daf-2; daf-12* strains containing class 2 alleles behaved similarly to one another. All permanently arrested development at a stage resembling L3 or L4 in size but with an underdeveloped gonad. At 25.5° *daf-2(e1370); daf-12(m20)* resulted in either embryonic or L1 arrest, or arrest at a stage apparently intermediate between an L2 or L3 and an L2d. Almost all *daf-2(e1391); daf-12(m20)* animals arrested as the L2- and L3-like forms seen among *daf-2(e1370); daf-12(m20)* populations. At 25.5°, the *daf-2(e979); daf-12(m20)* strain developed as in the absence of the *daf-12(m20)* mutation (*i.e.*, embryonic lethality and L1 arrest).

The *daf-2(m65); daf-12(m20)* progeny of *daf-2(m65)/qC1*[*dpy-19 glp-1*]*; daf-12(m20)* animals were also examined. The *m65* mutation alone results in nonconditional dauer formation. The *m65; daf-12* homozygotes were developmentally arrested and resembled *daf-2(e1370); daf-12* and *daf-2(e1391); daf-12* arrested larvae. By 3 days at 25.5°, *m65; daf-12* segregants arrested development as dark-bodied, somewhat thin, L3-sized individuals, which comprised 27 ± 3% of the total population ($N = 866$). At 15°, they became larger, arresting development at approximately the size of L4s. No dauer larvae were

seen. These observations suggest that *m65* is a class 2 allele.

Interactions between *daf-2* and *daf-12*—Life span: The effect of eight *daf-2* alleles in combination with *daf-12(m20)* on adult life span was measured (Figure 6, Table 3). As in dauer formation, class 1 alleles behaved similarly to one another with respect to the effects of *daf-12(m20)* on the Age phenotype. Some suppression, but not enhancement, of the Age phenotype by *daf-12(m20)* was observed. In the case of the class 2 alleles, *daf-12(m20)* generally had no effect on the Age phenotype at 15° but enhanced it at 22.5°.

In a *daf-2*(+) genetic background, the *daf-12* mutation reduced median and maximum life span at both temperatures (Table 3). At 15°, the weaker class 1 alleles *e1365*, *m41*, *m577*, and *sa193* resulted in marginal increases in life span, whereas the strong class 1 allele *m212* doubled both median and maximum life span (Figure 6A). At this temperature, the addition of *daf-12(m20)* resulted in a slight reduction in the median life span of the *sa193* strain and a marked reduction in the median life spans of the *e1365* and *m212* strains. Significant shortening of life span was not seen in *m41* and *m577*. At 22.5°, *e1365*, *m41*, *m577*, and *sa193* resulted in large increases in life span, and addition of *daf-12* resulted in some depression of the enhanced longevity (Figure 6B, Table 3). In *daf-2(e1365); daf-12* strains, median and maximum life spans were depressed relative to *e1365*. In the *daf-2(m577); daf-12* and *daf-2(sa193); daf-12* strains, the extension of median life span was marginally reduced, and in the *daf-2(m41); daf-12* strain, clear reduction of the extension of median (but not maximum) life span was seen. Conversely, the *daf-12* mutation did not reduce the *daf-2(m212)* Age phenotype at 22.5°. Thus, *daf-12(m20)* appears to act as a weak suppressor of Age, at least with respect to median life span, more readily suppressing the weaker class 1 *daf-2* alleles.

In the cases of the class 2 alleles *e979*, *e1370*, and *e1391* at 15°, addition of *daf-12(m20)* had no significant effect on life span except for *e979; daf-12*, where maximum life span was enhanced (Figure 6A, Table 3). At 22.5°, addition of *daf-12* generally enhanced median and maximum life spans (Figure 6B, Table 3). Addition of *daf-12* increased *e1391* median and maximum life span to 441% and 453% of N2, respectively, at 22.5°. One *e1391; daf-12* animal lived to the age of 102 days.

Interactions between *daf-2* and *daf-12*—Thermotolerance: The effects of *daf-12(m20)* on thermotolerance did not parallel the effects on larval development and life extension. Although *daf-12(m20)* suppressed the class 1 Daf-c phenotype, and in some cases reduced class 1-enhanced longevity, *daf-2(m41); daf-12* and *daf-2(m577); daf-12* were more thermotolerant than the single mutants (percent increase in median survival 31.5 ± 0.5% and 27.8 ± 6.1%, respectively). Maximum survival was also enhanced (data not shown). Survival of *daf-2(e1365); daf-12* was no different from that of *e1365*.

Class 2 alleles *e1370* and *e1391* interacted with *daf-12(m20)*, resulting in arrested larval development at 25.5°; both double-mutant strains displayed increased maximum life spans at 22.5°. With regard to thermotolerance, the *e1370* double mutants were slightly less tolerant than *e1370*, whereas the *e1391* double mutants were slightly more tolerant (data not shown).

Interactions between *daf-2* and *daf-12*—Internal hatching, late progeny, adult behavior, and morphology: The late progeny and reduced motility traits were slightly enhanced by *daf-12(m20)*. In combination with *e979*, *e1370*, *e1391*, and *m212*, *daf-12(m20)* resulted in a somewhat earlier onset of motility reduction, with full onset beginning 16, 4, 5, and 4 days after upshift of L4s to 22.5°, respectively. Conversely, death by internal hatching was strongly suppressed by *daf-12(m20)* in the class 2 alleles *e1370* and *e1391* but not *e979* (data not shown). Internal hatching in the five class 1 alleles tested was unaffected by the presence of *daf-12(m20)*. In all four cases, the presence of *daf-12(m20)* caused no clear enhancement or suppression of the adult body shrinkage or abnormal gonad phenotypes seen at 22.5° and 25.5°.

Of the eight *daf-2* alleles tested in combination with *daf-12(m20)*, three, *e979*, *e1370*, and *e1391*, resulted in late progeny at 22.5° and 25.5° in a *daf-12*(+) genetic background (Table 7). Addition of *daf-12(m20)* resulted in most cases in a marginal increase in the severity of the phenotype (data not shown). Thus, a very small number of late progeny were produced at 15° (0.15–0.4 late progeny per animal), whereas none were seen in the *daf-2* single mutants at that temperature. An exception was *e1391*, where addition of *daf-12(m20)* reduced late progeny production at 22.5°, from 8.2 to 1.8 late progeny per worm.

The *daf-2* null phenotype: The deficiencies *mDf11* and *mDf12* fail to complement *daf-2* (see materials and methods). Although neither deletes the leftward or rightward markers, *dpy-1* or *unc-93*, both deficiencies were shown by PCR analysis to lack *daf-2* sequences encoding portions of the extracellular and protein kinase domains. Furthermore, *mDf11*/*mDf12* animals exhibit an egg/L1 arrest phenotype, not the maternally rescued *daf-2* null phenotype, indicating that the deficiencies include at least one essential gene in common, in addition to *daf-2*. Crosses of *mDf12*/*qC1* males with *mDf11*/*qC1* hermaphrodites, in which 88% of the adults were cross-progeny, 32% of the F_1 were egg/L1 lethal but there were no dauer progeny.

At 25.5° *m65*/*mDf11* and *m65*/*mDf12* animals formed dauer larvae nonconditionally without detectable embryonic or L1 arrest. However, the latter phenotype could have been maternally rescued. The relative severity of *m65*, *mDf11*, and *mDf12* were compared by examining the Daf-c phenotype of each *in trans* to *e1370* at 20°. At 20°, *e1370* results in less than 1% dauer formation.

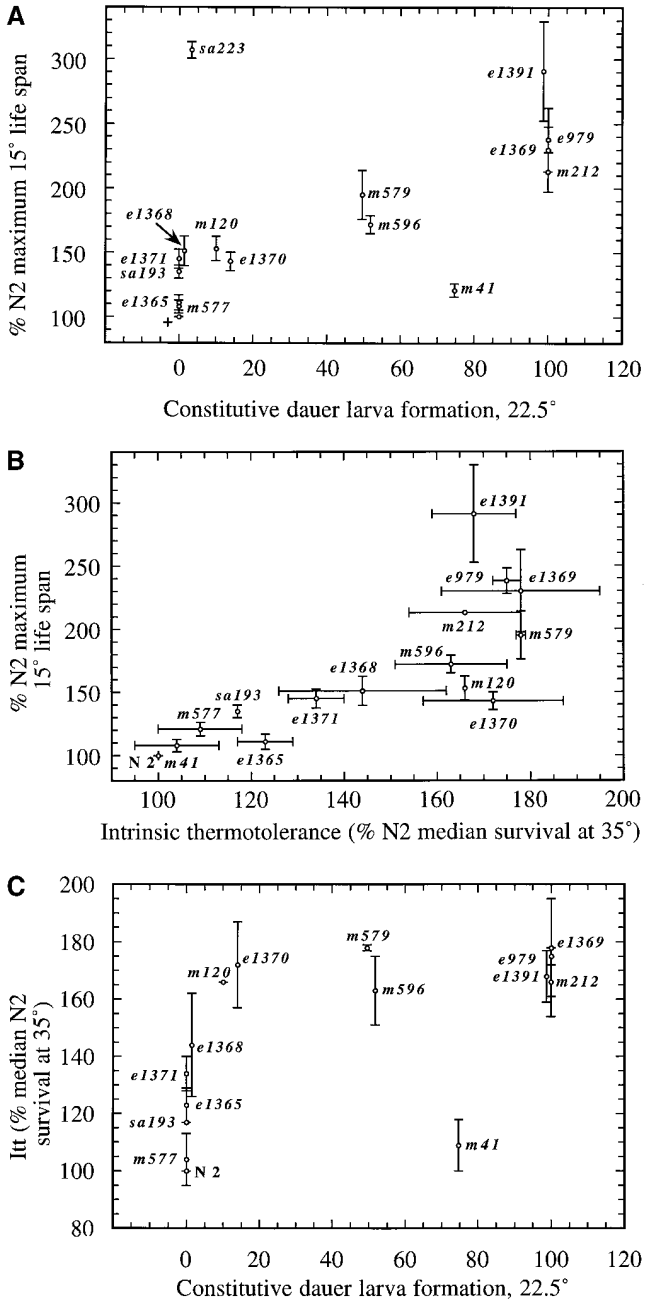

Figure 5.—Plots of Daf-c, Age, and Itt phenotype severities, derived from data displayed in Tables 2, 3, and 4, respectively. Bars represent standard errors. (A) Age versus Daf-c. Note that although *sa223* results in only 3.3% dauer formation at 22.5°, the remaining 96.7% arrest development as L2ds; (B) Age versus Itt; (C) Itt versus Daf-c. Note that Itt was measured for animals grown at 15°, a temperature at which *m41* is wild type.

In two trials, 4.7 ± 2.9% (N = 490) and 2.2 ± 0.7% (N = 1351) of *m65*/*e1370* animals formed dauer larvae, whereas *mDf11*/*e1370* and *mDf12*/*e1370* resulted in 5.8 ± 5.8% (N = 579) and 13.0 ± 6.2% (N = 943) dauer formation, respectively. Thus, neither deficiency results in a phenotype significantly more severe than *m65*, supporting the view that *m65* is a null [*daf-2(0)*] allele.

The *daf-2(0)* phenotype is complicated by the fact that the *daf-2* egg-L1 lethal phenotype is maternally rescued, but the Daf-c phenotype is not. At 15°, *daf-2(e979)* results in 20% dauer formation, whereas *daf-2(m65)* results in 100% dauer formation, indicating that *m65* is the more severe allele. Yet at 25.5°, *e979* (but not *m65*) results in 100% embryonic or L1 arrest, a more severe phenotype. Which is the more severe allele? Because the embryonic and L1 arrest phenotype of *e979* is maternally rescued, but the Daf-c phenotype is not (Table 9), we hypothesize that in the absence of maternal rescue, *m65* homozygotes would exhibit 100% embryonic and L1 arrest. Because *m65* homozygotes never develop into adults, it was not possible to test this possibility directly. Instead, we examined the homozygous *m65* progeny of *daf-2(m65)*/*daf-2*ts heteroallelic animals to determine whether reducing the maternal contribution of *daf-2*(+) resulted in an egg-L1 lethal phenotype. Progeny of *daf-2(m65) unc-32(e189)*/*daf-2(m577)* + adults were examined at 25.5°. *m577* is a relatively weak ts allele. The progeny consisted of 71% non-Unc dauer larvae, 17% Unc dauer larvae, 6% unhatched eggs, and 5% L1s (N = 628). Because 25% of these progeny should be homozygous for *daf-2(m65) unc-32*, but only 17% Unc dauer progeny were seen, 32% of *daf-2(m65) unc-32* homozygotes arrest development as embryos or L1s. Less than 2% of *unc-32* progeny arrest development at 25.5°.

Using other ts *daf-2* alleles *in trans* to *m65* or other nonconditional *daf-2* alleles, no greater proportion of embryonic or L1 arrest was seen. However, testing animals carrying strong ts alleles (*e.g.*, *e1369* or *e1391*) *in trans* to *m65* or *m632* was not successful due to very poor recovery of dauer larvae during strain construction or sterility upon testing at 25.5°.

Since we were able to increase the amount of egg-L1 lethality by constructing animals containing ts alleles *in trans* to nonconditional alleles, it is possible that an adult hermaphrodite with no *daf-2*(+) activity would produce only progeny that arrest as embryos or L1 larvae. However, such parents may be sterile, or they may arrest irreversibly in the dauer stage. The above results are consistent with the ts *e979* egg-L1 lethal phenotype representing a complete loss of function at restrictive temperature.

The *daf-2(e979)* embryonic and L1 arrest phenotype was tested for dominance. *daf-2(e979)*/*daf-2(m577)* heteroallelic animals were selfed at 25.5°, and the phenotypes of resulting progeny were scored. A total of 25 ± 6% of progeny were dead eggs or L1s (presumably mostly *e979* homozygotes), and the remainder were dauer larvae (total sample size: 270). From this, it may be inferred that *e979*/*m577* animals largely arrest development as dauer larvae rather than embryos or L1s; that is, *e979* is recessive for the egg-L1 lethal trait. Furthermore, residual *daf-2*(+) activity from the *m577* allele is sufficient to compensate for *e979* loss of function and prevent L1 arrest in *e979*/*m577* animals yet insufficient

TABLE 13

Dauer larva formation in *daf-2* and *daf-12(m20)* strains

	22.5°				25.5°				
	60 hr	80 hr			50 hr	Terminal phenotype[a]			
Allele	Appearance	Larval arrest (%)	Adult (%)	N^b	Appearance	Dauer arrest (%)	Other larval arrest (%)[c]	Adults (%)	$N^{b,d}$
N2	L4, adult	0	100	3133	—	—	—	—	—
Class 1									
daf-2(e1365)	L4, adult	0	100	2641	L2d molt	95.5 ± 0.5	4.5 ± 0.5	0	897
daf-2(m577)	L4, adult	0	100	2590	L2d molt	97.0 ± 1.0	3 ± 1	0	816
daf-2(sa193)	L4, adult	0	100	2675	Post-L2d molt[e]	99.5 ± 0.5[f]	0.5 ± 0.5	0[f]	704
daf-2(m41)	Dauer, L4	72.7	27.3	2296	L2d molt	100	0	0	834
daf-2(m212)	Dauer	99.6	0.5	1738	Post-L2d molt[e]	98.2 ± 0.2	1 ± 0.6	0.8 ± 0.4	933
daf-2(e1369)	—	—	—	—	Post-L2d molt[e]	98.3 ± 0.7	1.7 ± 0.7	0	224
daf-2(m41); daf-12(m20)	L4, adult	0	100	653	L3, L4	0	0.5 ± 0.5	99.5 ± 0.5	794
daf-2(e1365); daf-12(m20)	L4, adult	0	100	536	L3, L4	0	0.4 ± 0.1	99.6 ± 0.1	739
daf-2(m577); daf-12(m20)	L4, adult	0	100	732	L3, L4	0	2 ± 1	98 ± 1	755
daf-2(sa193); daf-12(m20)	L4, adult	0	100	840	L3, L4	0	0	100	589
daf-2(m212); daf-12(m20)	L4, adult	0	100	587	L3, L4	0	0	100	801
daf-2(e1369); daf-12(m20)	—	—	—	—	L4	0	0.7 ± 0.1	99.4 ± 0.1	316
Class 2									
daf-2(m596)	—	—	—	—	L2d molt	95.1 ± 1.0	4.9 ± 1.0	0	431
daf-2(e1370)	Dauer, L4	19.4	80.6	2318	L2d molt	91.6 ± 5.1	8.4 ± 5.1	0	1974[g]
daf-2(e1391)	Dauer	100	0	1353	L2d molt	89.5 ± 0.5	10.5 ± 0.5	0	340
daf-2(e979)	Dauer	100	0	1847	Unhatched egg, L1	0	100[h]	0	339
daf-2(m596); daf-12(m20)	—	—	—	—	L4	0	1.5 ± 0.6	98.6 ± 0.6	368
daf-2(e1370); daf-12(m20)	L3, L4	100[i]	0	70	L1 – L2[j]	0	100[k]	0	284
daf-2(e1391); daf-12(m20)	L3, L4	100[i]	0	83	L2 – L3[l]	0	100[m]	0	542
daf-2(e979); daf-12(m20)	L3, L4	100[i]	0	189	Unhatched egg, L1	0	100[n]	0	325

[a] Measured between 50 and 100 hr.
[b] Sample size.
[c] L1 arrest, except where indicated.
[d] Two simultaneous trials performed.
[e] Nascent dauer larvae undergoing radial shrinkage.
[f] 99.5 ± 0.5% dauer larvae at ~73 hr. By ~95 hr approximately 10% of the dauer larvae were recovering.
[g] Nine trials.
[h] 86.5 ± 1.5% unhatched eggs, 13.5 ± 1.5% L1 arrest.
[i] Animals remained developmentally arrested at a stage resembling L3 and L4 at 100 hr.
[j] L1s, L2ds, or larvae resembling intermediates between L2s and L2ds. Larvae later became Unc.
[k] 43 ± 0% L1 arrest, 57 ± 0% L2- and L3-sized arrest.
[l] Larvae resembled L2s or L3s in size, but were slightly darker, and later became Unc.
[m] 3 ± 0% L1 arrest, 97 ± 0% L2- and L3-sized arrest.
[n] 86 ± 2% unhatched eggs, 14.5 ± 1.5% L1 arrest, plus several L2s.

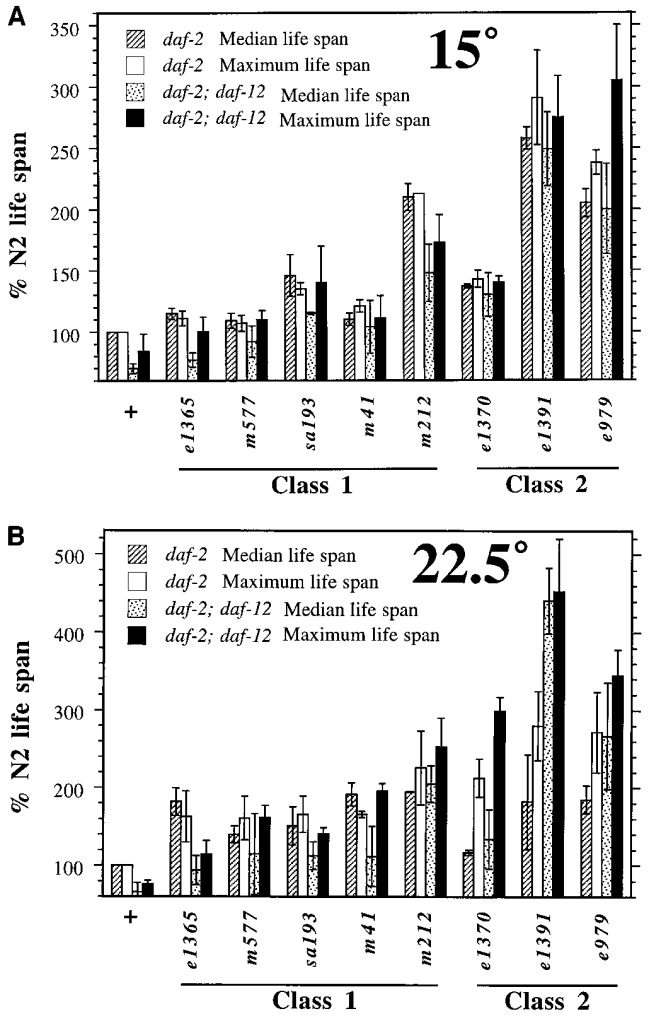

Figure 6.—Effect of *daf-2* and *daf-12(m20)* mutations on median and maximum life span. Median and maximum life span is expressed as a percentage of the respective wild-type controls. Bars represent standard error. (A) Life span at 15°; (B) life span at 22.5°. Alleles are arranged in order of increasing severity of Daf-c, within each *daf-2* class.

to allow maternal rescue of L1 arrest in *e979* homozygous siblings. This implies that in *e979/m577* animals zygotic expression of *m577* prevents L1 arrest. Thus, both maternal and zygotic DAF-2 promote embryonic and L1 development in wild-type animals.

daf-2(e979)/daf-2(m577) adult hermaphrodites were also examined. Median and maximum life spans at 15° were 30 and 35 days, respectively ($N = 18$). This compares to median and maximum life spans of 26 and 34 days, respectively for *m577*, and 50 and 69 days, respectively, for *e979*. Thus, in life span, *e979/m577* adults more closely resemble *m577* than *e979*. Again, *e979* shows no clear dominant effect, consistent with loss of function rather than gain of function.

If *e979*, *m65*, and *mDf12* are all *daf-2(0)* at 25.5°, then the phenotypes of *m65/e979* and *mDf12/e979* animals should exhibit the *e979* lethal phenotype in the absence of maternal rescue. Crosses of *m65/qC1* males with *e979* hermaphrodites at 25.5°, in which virtually all the F_1 were cross-progeny, produced 51 ± 0.4% growing progeny, 36 ± 1.2% dauer progeny, and 13 ± 1.6% egg/L1 arrested progeny. Similarly, crosses of *mDf12/qC1* males with *e979* hermaphrodites produced 53 ± 3% growing progeny, 39 ± 2% dauer progeny, and 8 ± 2% egg/L1 arrested progeny. These results are consistent with the proposition that both *m65* and *mDf12* are *daf-2* nulls. Production of dauer progeny from these crosses is either the result of parental rescue or the reduction of a negative activity of a slightly neomorphic *e979* allele. *e979* hermaphrodites raised to the L4 stage at 15° then shifted to 25.5° do not exhibit such rescue. We conclude that the absence of both parental and zygotic *daf-2* activity results in a phenotype within the range between *e979* and approximately 33% egg/L1 arrest [the result from the most severe heteroallelic animals that could be tested, *daf-2(m212*ts*)/daf-2(m632)*].

Suppression of *daf-2* class 2 defects by *daf-16(m26)*: Mutations in the *daf-d* gene *daf-16* suppress several *daf-2*-associated mutant phenotypes including Daf-c (Riddle *et al.* 1981), Age (Kenyon *et al.* 1993), embryonic and L1 arrest, and reduced brood size (Gottlieb and Ruvkun 1994). We examined the effect of *daf-16(m26)* on the reduced motility, adult body shrinkage, and gonad morphology defects resulting from *daf-2(e1370)* at 25.5°. All three abnormalities were suppressed by *daf-16(m26)*. It was not possible to look for late progeny production by *daf-16(m26); daf-2(e1370)* hermaphrodites because they died of old age before the ages at which late progeny are typically produced by *daf-16(m26)*(+)*; daf-2(e1370)* animals. However, numerous unfertilized oocytes were laid by *daf-16(m26); daf-2(e1370)* hermaphrodites, indicating that sperm had become depleted, as in the wild type. The *daf-16* mutation fully suppresses the Daf-c phenotype of the putative null allele *daf-2(m65)*, but the double-mutant adults are sterile (Larsen *et al.* 1995).

DISCUSSION

Our aims in the present study were (1) to characterize the full range of phenotypes that may result from mutation of the *daf-2* gene; (2) to understand the relationship between different *daf-2* mutant traits, in particular to establish which are linked to the Age phenotype; and (3) to discover the nature of the null [*daf-2(0)*] phenotype. Our results suggest that *daf-2(0)* mutations result in nonconditional dauer larva formation (in the presence of maternal rescue) or embryonic and L1 arrest, as in the case of the ts allele *e979*. To investigate the numerous roles of *daf-2* in later development, ts mutations have been examined, and it is with the analysis of their phenotypes that the greater part of this study is concerned. Fifteen ts *daf-2* alleles, isolated on the basis of their Daf-c phenotype, were characterized with respect to a range of mutant traits.

Two overlapping classes of *daf-2* allele: *daf-2* alleles can be grouped into two overlapping classes based on pleiotropy (Table 12). Although class 1 contains the five weakest Daf-c alleles, it also includes three of the six most severe Daf-c alleles (Table 2). Thus, the severity of Daf-c and the class 2 pleiotropic defects show little correspondence. This suggests that the *daf-2* gene specifies two distinct functions. We designate these *daf-2A* and *daf-2B*, where *daf-2A* affects only those phenotypes common to class 1 and 2 alleles, and *daf-2B* affects the class 2-specific defects. The behavior of *daf-2; daf-12* mutants suggests that *daf-2B* also affects Age and third-stage larval arrest. In this scheme, all 15 ts alleles are defective with respect to *daf-2A* function, whereas only the seven class 2 alleles are markedly defective with respect to *daf-2B* function. The class 2 mutants are *daf-2A*(−)*daf-2B*(−). The two functions may represent a role for the DAF-2 receptor in two different pathways, such that some mutations affect one pathway more than another and/or they may correspond to different functional domains within the DAF-2 protein. Alternatively, *daf-2A* and *daf-2B* could correspond to alternately spliced transcripts containing different numbers and/or combinations of exons, as in the case of *vab-3* (Chisholm and Horvitz 1995) and *mab-18* (Zhang and Emmons 1995).

Alleles of the type *daf-2A*(−)*daf-2B*(+), which display no class 2-specific traits, have been identified, whereas alleles of the type *daf-2A*(+)*daf-2B*(−), displaying class 2-specific traits alone, were not. Such alleles might be expected to display only class 2-specific traits (*i.e.*, reduced adult motility, abnormal gonad) without being Daf-c, Itt, or Age. The absence of *daf-2A*(+)*daf-2B*(−) alleles may be due to the lack of appropriate mutant screens to identify such alleles. Alternatively, all mutations resulting in *daf-2B*(−) may also cause some loss of *daf-2A* function.

Assigning alleles to classes is useful for dealing with the complexity of *daf-2* mutant phenotypes and gene interactions. However, there is overlap between the two classes because a number of class 1 alleles exhibit weak class 2 traits. The class 1C alleles *e1365*, *e1369*, and *m577* result in one marginal class 2 trait: reduced adult motility at 25.5° after 7 days. The class 1D allele *m212* results in reduced motility also at 22.5°. Although the class 1C and 1D alleles might be classified as weak class 2 alleles, we have grouped them together with the class 1 alleles because their adult (class 2) phenotypes are very weak.

The *daf-2* gene encodes a cell-surface tyrosine kinase receptor homolog resembling an insulin receptor (InR; Kimura *et al.* 1997). The nine mutant lesions for which sequence data were reported included four alleles characterized in this study: two class 1 alleles, *e1365* and *e1368*, and two class 2 alleles, *e1370* and *e1391*. The two class 1 mutant lesions mapped to the putative extracellular ligand-binding domain, and the two class 2 alleles mapped to the putative intracellular tyrosine kinase domain. Two further alleles with lesions mapping to the putative ligand-binding domain, *sa187* and *sa229*, were subjected to preliminary phenotypic analysis. *sa229* behaved as a class 1A allele: Adults maintained at 25.5° did not become Unc, and gonadal appearance remained normal (D. Gems, unpublished results). However, *sa187* behaved as a class 2 allele: Adults became Unc and developed abnormal gonadal appearance at 25.5° (D. Gems, unpublished results). In *sa187*, a residue in the Cys-rich region of the InR homolog ligand-binding domain that is conserved between *C. elegans* and humans is substituted.

Thus, it is possible that class 1 mutations may, in general, affect the less conserved parts of the ligand-binding domain of the InR-like protein, whereas class 2 mutations affect the conserved region of the ligand-binding domain and the tyrosine kinase domain. A possible interpretation explaining both phenotypic and molecular data is as follows. *daf-2A* and *daf-2B* correspond to two components of signaling into the cell by the InR-like receptor. While *daf-2A* signaling is completely ligand dependent, *daf-2B* signaling is not. Thus, in class 1 alleles, which are *daf-2A*(−)*daf-2B*(+), ligand-dependent signaling is blocked by the defect in the ligand-binding domain, but ligand-independent signaling is not. In class 2 alleles, which are *daf-2A*(−)*daf-2B*(−), both signaling components are blocked. This interpretation suggests that the tyrosine kinase is required for both *daf-2A* and *daf-2B* signaling. Possibly, mutations like *sa187*, which are class 2 and map to the Cys-rich conserved region of the extracellular domain, render the entire protein defective and hence are *daf-2A*(−)*daf-2B*(−).

The model in Figure 7 suggests that *daf-2A* and *daf-2B* activities correspond to signals from the *daf-2* receptor propagated by different signaling intermediates. This is consistent with the nature of vertebrate InRs, which propagate signals via intermediates other than PI 3-kinase (*e.g.*, ras and the MAP kinase cascade). If this is so, how is it that both *daf-2A*(−) and *daf-2B*(−) are suppressed by *daf-16*(−)? The pathway presented includes two *daf-16* activities, *daf-16* (A) and *daf-16* (B), which we suggest may correspond to *daf-16*(+) activity at different times in development or in different cell types, in which the *daf-2* InR signal may be transmitted via different signaling cascades.

The *daf-2(0)* phenotype: Because *daf-2(m65)* results in nonconditional dauer formation at all temperatures, it is maintained in the heterozygous state, usually *in trans* to the balancer chromosome *qC1*. Consequently, the effects of this allele on embryonic and L1 development have previously been masked by the genetic maternal effect. We uncovered the embryonic and L1 arrest component of the *m65* phenotype by examining the progeny of heteroallelic adults in which maternal rescue was reduced. Thus, the *daf-2* gene has a role in embryonic and L1 development, as well as nondauer development,

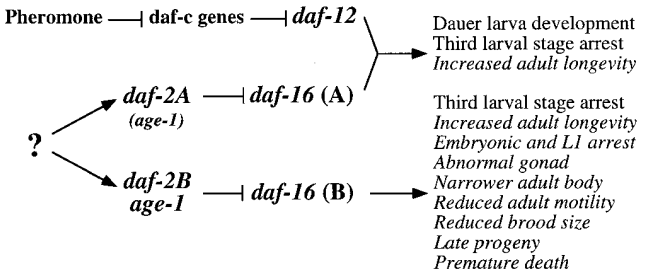

Figure 7.—Genetic interactions of *daf-2*, *daf-12*, *daf-16*, and *age-1* in controlling larval development, life span, and other traits. The pathway is drawn to depict wild-type gene functions that stimulate (arrow) or inhibit (T bar) subsequent activities, or traits. Traits seen only in *daf-2* or *age-1* mutants are shown in italics. They are proposed to result from unmodulated activity of *daf-12* or *daf-16*. Mutations in *daf-2* and *age-1* are Daf-c and Age, whereas those in *daf-12* and *daf-16* are Daf-d. See text for detailed discussion. In this model *daf-12*(+) activity is activated by dauer-inducing pheromone. This activation involves switching off signalling via a number of *daf-c* genes, including *daf-1*, *daf-4*, *daf-7*, *daf-8*, and *daf-14* (reviewed in Riddle and Albert 1997). *daf-2*(+) and *age-1*(+) are also environmentally modulated, but it is not clear by what. As drawn, some physiological factor, *e.g.*, glucose, that is associated with growth-promoting conditions, denoted ?, results in activation of *daf-2*(+) and *age-1*(+). Similarly, the activity of these genes could be inhibited by dauer-inducing conditions. One possible regulator of *daf-2*(+) and *age-1*(+) activities is the nutritional status of the animal. Both *daf-12* and *daf-16* are required for expression of the class 1 traits. Activities that inhibit either *daf-12* or *daf-16* (A) functions prevent expression of these traits. Only *daf-16* is required for expression of class 2 traits. Data clearly indicate that *daf-12*(+) is required for dauer development. In the adult, *daf-12*(+) either weakly enhances or plays no role in the Age phenotype.

and maintenance of adult characteristics. Of the ts alleles, the *e979* egg-L1 lethal is the only candidate for complete loss of function at 25.5°.

In most *daf-2* alleles, the effects of *daf-2B*(−) on early development were maternally rescued, whereas those of *daf-2A*(−) were not. (An exception was *m41*, where the Daf-c phenotype was partially maternally rescued.) This suggests that zygotic expression of *daf-2B* is not required, at least as far as the second molt. However, zygotic expression of *daf-2* is generally required to prevent dauer larva formation.

Embryonic and L1 arrest have also been observed at 25° when *daf-2(e1370)* is combined with mutations in the cilium structure genes *che-3*, *che-11*, *osm-1*, *osm-3*, or *osm-5* (Vowels and Thomas 1992). When wild-type embryos hatch in the absence of food, developmental arrest at the L1 stage occurs, which can last for at least 12 days at 20° without effect on subsequent development (Johnson *et al.* 1984). This L1 arrest is not dependent on chemosensation of food alone because even the most severe cilium structure mutants do not result in significant L1 arrest in the presence of food. The L1 arrest resulting from *daf-2(e1370)* in combination with cilium structure mutants suggests that it may be regulated by two redundant modes of food detection. The first involves chemosensation and is rendered defective by cilium structure mutants. The second is independent of chemosensation and involves *daf-2B*, possibly responding to the nutritional state of the organism. Embryonic and L1 arrest have also been observed in strains combining a severe *age-1* allele, *mg44*, with mutations in the cilium structure genes *che-3* and *daf-10* (Gottlieb and Ruvkun 1994).

Distinctive properties of *m41*: *daf-2(m41)* is the only classically ts allele, and it is the only allele where Daf-c is maternally rescued. The life spans of most alleles at 15° correspond in severity to their Daf-c phenotype at 22.5° (Figure 5A, Tables 2 and 3). However, *m41* life span is only marginally greater than wild type at 15° (Table 3; Larsen *et al.* 1995), despite being the fifth most severe Daf-c allele at 22.5°. Likewise, the Itt phenotype of *m41* animals raised at 15° is also wild type (Figure 5C, Table 4). This suggests that either the synthesis or function of *m41* DAF-2 protein is thermolabile. The other 14 ts *daf-2* alleles are likely to represent hypomorphic mutations, the temperature sensitivity of which reflects the innate temperature sensitivity of wild-type dauer formation (Golden and Riddle 1984b).

Relationship between the class 2 mutant traits: We posit that class 2-specific defects result from differing degrees of defectiveness in one component of *daf-2* gene function (Table 12). Hypothetically, some of the class 2-specific defects may be related to each other as follows. A slight defect in *daf-2B* results in reduced motility and coiling. A slightly more severe defect in *daf-2B* results in late progeny. The latter trait may be due to severely retarded production of oocytes, with fertilization of rare late oocytes occurring at advanced ages. Alternatively, it could result from a failure of egg laying combined with developmental arrest of internally hatched eggs as L1s inside the uterus.

A decrease in class 2 median life span relative to maximum life span at 22.5° (Figure 2B) suggests that many animals may be dying of some deleterious effect of *daf-2B* rather than old age (subsequently referred to as early mortality or premature death). The coincidence of late progeny and increased premature death suggests that these two traits may be related. Although dead animals in which internally hatched larvae were observed were excluded from quantitation of life span, the presence of small numbers of L1 larvae inside the bodies of dead hermaphrodites would have been hard to detect. But given that pharyngeal pumping is greatly reduced in some class 2 mutants, premature death may result from starvation.

In general, later stages in *C. elegans* development are more sensitive to loss of *daf-2*(+). Hence, many alleles result in Age and Itt but not Daf-c phenotypes at 15°. Similarly, the most sensitive indicator of *daf-2B*(−) is reduced adult motility, seen only after 7 days at 25.5° in class A3 alleles (Table 10). More severe alleles in the

class 2 series affect increasingly early stages of development: brood size; later larval development; and, in the most severe alleles, L1 and even embryonic development. Thus, the threshold of *daf-2* activity below which a mutant phenotype results appears to increase with age from the embryo to at least mid-adulthood.

***daf-2(e1370)* ts Unc phenotype:** It has been suggested that the increased longevity of *daf-2* mutants may result from the adult expression of dauer longevity (Kenyon *et al.* 1993). The reduced adult motility, reduced adult pharyngeal pumping, and shrinkage of the adult body seen in *daf-2B* mutants may be interpreted in similar terms: inappropriate expression of dauer-associated behavior and morphology in the adult. The neural basis of dauer-specific behavior (*e.g.*, reduced motility and nictation) is unknown. We have shown that if adult *daf-2(e1370)* hermaphrodites are maintained at 25.5° until reduced motility is seen, then shifted to 15°, they recover wild-type motility. Furthermore, recovered animals may be rendered Unc and non-Unc again by successive rounds of temperature upshift and downshift. This phenotype represents an instance of reversible, temperature-sensitive behavioral plasticity in the *C. elegans* adult that warrants further study.

***daf-2B* defects and *age-1*:** Mutations in *age-1* (formerly also known as *daf-23*) result in Daf-c and Age phenotypes (Friedman and Johnson 1988; Gottlieb and Ruvkun 1994; Malone *et al.* 1996; Morris *et al.* 1996; Tissenbaum and Ruvkun 1998). Gene interaction studies suggest that *age-1* functions at the same point as *daf-2* in the epistasis pathway of genes controlling dauer formation and life span (Gottlieb and Ruvkun 1994; Larsen *et al.* 1995). The phenotype of the severe *age-1* mutants more closely resembles that of class 2 than of class 1 *daf-2* mutants in four respects. First, stronger *age-1* alleles result in arrest of larvae at the predauer L2d stage as well as the dauer stage. For example, at 15° *age-1(mg44)* results largely in L2d arrest and some dark-bodied, sterile adults with protruding vulvas (Gottlieb and Ruvkun 1994). At temperatures below 25.5° *daf-2(sa223)* also results in L2d arrest, and *sa223*/*qC1* hermaphrodites segregate some dark-bodied, sterile *sa223* adults. Shifting *daf-2 (e1370, e979,* and *e1391)* early L3 larvae raised at 15° to 25.5° results in formation of dark-bodied adults with delayed fertility and protruding vulvas. Second, the *age-1* nonconditional L2d or dauer-stage arrest is fully maternally rescued (Gottlieb and Ruvkun 1994; Larsen *et al.* 1995). Whereas dauer formation in *daf-2* mutants is generally not maternally rescued, the L2d arrest seen in progeny of *sa223* homozygotes at temperatures below 25.5° is rescued (Table 9). The L2d arrest resulting from *e1370* at 22.5° shows twice the level of maternal rescue as does dauer arrest. Third, the *age-1* larval arrest phenotype is not suppressed by mutations in *daf-12* (Gottlieb and Ruvkun 1994; Larsen *et al.* 1995). Similarly, *daf-12(m20)* suppresses the *daf-2* Daf-c phenotype but not class 2-specific embryonic and larval defects. Fourth, *age-1(hx546)* did not enhance the *e1370* Daf-c phenotype at 20°, but double mutants were retarded in development, were infertile, and had a reduced rate of pharyngeal pumping (Dorman *et al.* 1995). At 20° *age-1(hx546); daf-2*(+) animals are wild type with respect to larval development, and *daf-2(e1370)* is essentially wild type with respect to class 2 traits. Thus, in the *age-1(hx546); daf-2(e1370)* strain an additive interaction between *age-1(hx546)* and *daf-2B*(−) but not *daf-2A*(−) occurs.

If *age-1* and *daf-2B* act together, then it would be expected that a *daf-2A*(+)*daf-2B(0)* mutation would closely resemble *age-1(0)* mutations. We propose that *daf-2(sa223)* phenotypically resembles severe *age-1* alleles because it may be weakly mutant with respect to *daf-2A* and severely mutant with respect to *daf-2B*.

The *age-1* gene encodes a phosphatidylinositol kinase (Morris *et al.* 1996). The similarity of *daf-2B* and *age-1* mutants suggests the hypothesis that a function of the DAF-2 protein that requires AGE-1 may be defective in class 2 but not class 1 mutants. If *daf-2B* function requires the *age-1* PI 3kinase, and *daf-2A* does not, this suggests that *daf-2A* may act through an alternative signalling pathway.

Reproductive investment and life span: A key element of the evolutionary theory of aging is that evolution will maximize the reproductive success of species even at a cost to longevity (reviewed in Rose 1991). From this, it is expected that mutations that extend life span may also reduce fecundity. In *C. elegans*, mutations extending life span do not consistently reduce brood size (Lithgow *et al.* 1994; Larsen *et al.* 1995). The current study suggests that brood-size reduction, in most cases seen in the *daf-2B* mutants, results from a pleiotropic effect on gonad function that is separable from the Age phenotype (Tables 3 and 6). Furthermore, reduction in the capacity to produce eggs may not be detectable in self-fertilizing, protandrous hermaphrodites, where brood size is limited by the fixed number of self-sperm. In this context, it is intriguing that most *daf-2* alleles result in a significant reduction in the number of unfertilized oocytes laid at 22.5° after the depletion of self-sperm (Table 8). In three class 1 strains examined at 15° (*e1369, m212,* and *sa193*), the number of unfertilized oocytes laid was also reduced (data not shown). This reduction potentially reflects a change in the reproductive physiology of the animal, resulting in a reduction in oocytes produced by the ovary in response to sperm in the spermatheca. Alternatively, the smaller number of unfertilized oocytes laid may reflect a reduction in the laying rather than production of oocytes.

A further consequence of the evolutionary theory of aging is that a selective advantage exists for species with the capacity to respond to reproductive opportunities by facultatively increasing reproductive output, even where this increase shortens life span (Partridge and Harvey 1988). A cost to increased egg production in reduced

life span has been observed in numerous species, *e.g.*, fruit flies (Maynard Smith 1958) and cockroaches (Griffiths and Tauber 1942). Conversely, increasing egg production in *C. elegans* does not shorten life span (Gems and Riddle 1996) nor does decreasing egg production lengthen it (Friedman and Johnson 1988; Kenyon *et al.* 1993). However, this does not exclude the possibility that in *daf-2* hermaphrodites resources are diverted from egg production to the processes underlying longevity. If such resource diversion occurs, it does not reduce egg production in well-fed hermaphrodites.

Epistasis between *daf-2* and *daf-12*: We examined the phenotypes resulting from the combination of *daf-12(m20)* with five class 1 and three class 2 *daf-2* alleles. Our aim was to establish whether or not differences between the phenotypes of *daf-2; daf-12* strains can be related to phenotypic differences between *daf-2* alleles and to clarify the epistasis relationship between *daf-2* and *daf-12*. The effect of *daf-12(m20)* on several *daf-2* mutant traits was examined: Daf-c, Itt, Age, and class 2-specific defects (reduced adult motility, abnormal gonad morphology, embryonic and L1 arrest, and production of late progeny). Only with regard to the class 1 mutant Daf-c phenotype was *daf-12(m20)* clearly epistatic.

Two components of the Daf-c phenotype, dauer larva morphogenesis and developmental arrest, were differentially suppressed by *daf-12(m20)* in a *daf-2* class-dependent manner. When class 1 alleles were present, *daf-12(m20)* resulted in growth, *i.e.*, suppression of dauer larva morphogenesis and developmental arrest at 25.5° (Table 13). Given that class 1 alleles are defective with respect to *daf-2A*, but not *daf-2B*, this indicates that *daf-12(m20)* is epistatic to *daf-2A*(−) during development.

When the severe class 2 allele *e1391* was combined with *daf-12(m20)* at 25.5°, animals arrested as dark L2- or L3-sized larvae (Table 13). *daf-2(e1370); daf-12* resulted in L1- and L2d-like arrest, and *daf-2(e979); daf-12*, like *e979* alone, resulted in embryonic and L1 arrest. At 22.5°, all three double mutants arrested development at a stage resembling slightly dark-bodied L3s or L4s. Thus, while *daf-12(m20)* suppresses dauer larva morphogenesis in both class 1 and class 2 *daf-2* mutants, it suppresses larval arrest in the class 1 but not the severe class 2 mutants. Given that class 2 *daf-2* alleles are defective in both the *daf-2A* and *daf-2B* elements of *daf-2* function, this indicates that *daf-12(m20)* is epistatic to strong *daf-2A*(−) but not to strong *daf-2B*(−) during development.

Revised model for gene interactions: The differences between *daf-2* class 1 and class 2 alleles with respect to their interactions with *daf-12*, *daf-16*, and *age-1* follow from the model represented in Figure 7. The *daf-2* gene specifies two functions, *daf-2A* and *daf-2B*. *daf-2* mutants fall into two groups: class 1, which are *daf-2A*(−)*daf-2B*(+), and class 2, which are *daf-2A*(−)*daf-2B*(−). *daf-2A* but not severe *daf-2B* mutations are suppressed by the *daf-12(m20)* mutation, yet both *daf-2A* and *daf-2B* mutations are suppressed by mutations in *daf-16*. Mutations in *daf-16* suppress Daf-c (Riddle *et al.* 1981), Age (Kenyon *et al.* 1993; Larsen *et al.* 1995), and, to some extent, Itt (K. V. King and D. L. Riddle, unpublished results). In the case of *daf-2B*, *daf-16(m26)* was found to suppress the shrinkage of the adult body, gonad abnormalities, and reduced adult motility resulting from *daf-2(e1370)*. Furthermore, mutations in *daf-16* were previously observed to suppress *daf-2* L1 arrest and reduced brood size (Gottlieb and Ruvkun 1994). Thus, while *daf-12* is epistatic to *daf-2A* only, *daf-16* is epistatic to both *daf-2A* and *daf-2B*; *i.e.*, both *daf-12*(+) and *daf-16*(+) are required for *daf-2A*-mediated mutant effects, whereas *daf-16*(+) but not *daf-12*(+) is required for *daf-2B*-mediated mutant effects (Figure 7).

In the proposed model, the mutant phenotypes result from unmodulated *daf-12*(+) and *daf-16*(+) activity when *daf-2* is mutant. Loss of *daf-2A* function results in active *daf-16*(+). Both *daf-12*(+) and *daf-16*(+) are required to promote dauer larva development and third-stage larval arrest. *daf-16*(+) is also required for increased longevity, as is *daf-12*(+) to some extent. The suppression of *daf-2A*(−) by *daf-12*(−) results from the loss of *daf-12*(+) interaction with *daf-16*(+) (Figure 7). An alternative possibility is that *daf-2A*(+) inhibits *daf-12*(+), in which case the suppression of *daf-2A*(−) defects by *daf-16*(−) would be due to the failure of the latter to interact with *daf-12*(+).

In *daf-2B*(−) mutants, *daf-16*(+) function is active and exerts its effects independently of *daf-12*(+). For the model to work, it must be supposed that the manner in which *daf-16*(+) is activated as the result of *daf-2A*(−) is different from that resulting from *daf-2B*(−). The *daf-16*(+) functions inhibited by *daf-2A*(+) and *daf-2B*(+) are designated *daf-16* (A) and *daf-16* (B), respectively. While it must be assumed that *daf-2A* and *daf-2B* correspond to different elements of DAF-2 function, this is not the case for *daf-16* (A) and *daf-16* (B). *daf-16* (A) and *daf-16* (B) may correspond to *daf-16*(+) activity at different times in development, or in different cell types.

daf-16 (B)(+) activity may require *age-1*(+), given the similarity between *age-1* and *daf-2B* mutant phenotypes (see above), and results in a different set of mutant defects. Some of these (*e.g.*, third-larval stage arrest) are the same as those resulting from *daf-16* (A)(+) and *daf-12*(+) combined, when *daf-2A* is mutant. Others (*e.g.*, adult morphological and behavioral abnormalities and embryonic and L1 arrest) are different. Thus, some overlap exists between the *daf-2A*/*daf-16* (A) and *daf-2B*/*age-1*/*daf-16* (B) pathways. The existence of such overlap explains why, in terms of the model, the severest *age-1* alleles result in dauer larva formation.

The effect of *daf-12(m20)* on the class 2 traits (embryonic and L1 arrest, late progeny and shrunken adult body, defective gonads, and reduced motility) supports the hypothesis that *daf-2B* acts independently of *daf-12*.

In the three class 2 alleles studied (*e979*, *e1370*, and *e1391*), effects of *daf-12(m20)* on these traits were marginal. However, in the case of *e1370* and *e1391*, *daf-12(m20)* significantly reduced the frequency of death from internal hatching.

Epistasis between *daf-2* and *daf-12*—Life span: Among *daf-2* alleles the severity of the Age trait largely correlates with those of the Daf-c and Itt phenotypes (Figure 5) but not the class 2-specific defects. The more severe class 2 defects are, in fact, associated with reduced extension of median life span relative to extension of maximum life span seen at 22.5° (Figure 2B), presumably resulting from early mortality in some animals. In interpreting the effects of *daf-12(m20)* on the life span of *daf-2; daf-12* strains, potential enhancing effects of *daf-2A*(−) and *daf-2B*(−) on longevity must be distinguished from life span-reducing pleiotropic effects of *daf-2B*(−).

Although the epistasis relationship between *daf-2A* and *daf-12(m20)* in dauer formation is clear, in the case of the Age phenotype, the ordering of these two genes is ambiguous. When combined with class 1 *daf-2* alleles, *daf-12(m20)* results in some depression of *daf-2* extended life span (mostly median) but no enhancement (Figure 6, Table 3). To some extent, this conforms with the inference from the *daf-2; daf-12* Daf-c phenotypes that *daf-12(m20)* is epistatic to *daf-2A* (Figure 7). However, *daf-12(m20)* is a poor suppressor of Age compared to *daf-16(m26)*, which fully suppresses the *daf-2* Age phenotype (Larsen *et al.* 1995). Potentially, the failure of *daf-12(m20)* to fully suppress *daf-2* Age reflects the weakness of the *m20* mutation. However, sequence analysis of *m20* shows that it is an amber nonsense mutation (A. Antebi and E. Hedgecock, personal communication), and this allele is fully Daf-d. Taken together, this suggests that the Age phenotype is activated by *daf-16*(+) but that *daf-12*(+) may also have a weak activating role.

An alternative interpretation of the weak Age-suppressing effect of *daf-12(m20)* on *daf-2A* mutations is that loss of *daf-12*(+) is generally deleterious, thereby reducing life span. The life span of *daf-2*(+)*; daf-12(m20)* populations is only about 70% of wild type, supporting this interpretation. However, suppression of Age through deleterious effects of *daf-12(m20)* might have been expected to affect all alleles similarly, and this was not the case.

The fact that *daf-12(m20)* did not suppress the Age phenotype of the class 2 *daf-2* alleles *e979*, *e1370*, and *e1391* (Figure 6, Table 3) both supports the contention that *daf-2B* does not act through *daf-12* (Figure 7) and suggests that *daf-2B*(−) and *daf-2A*(−) result in extended life span. However, the class 2 mutants, which lack both functions, do not live longer than the class 1 mutants.

At 22.5° (but not 15°) addition of *daf-12(m20)* enhanced the longevity of *e979*, *e1370*, and *e1391* (Figure 6, Table 3). This enhancement of the Age phenotype by *daf-12(m20)* could be due either to further retardation of the rate of senescence at 22.5° or to suppression of deleterious effects of *daf-2B*(−). In populations of *e979*, *e1370*, and *e1391* animals, the extension of median life span is generally less than the extension of maximum life span at 22.5° (Figure 6B). In these cases, early mortality may result from deleterious effects rather than senescence itself, and enhancement of the Age phenotype by the *daf-12* mutation (Figure 6B) may result from the suppression of this early mortality. Consistent with this are the similar relative extensions of median and maximum life span seen in *daf-2(e1391); daf-12(m20)* populations (Figure 6B), which would be expected in the absence of an early deleterious effect that depressed the median. Furthermore, *daf-12* has no Age-enhancing effect at 15° when class 2 defects are not expressed. In cases where death is not a reliable measure of senescence, we need other markers of progressive decline in function.

If the enhancement of longevity by *daf-12(m20)* in these cases is due to suppression of premature death, this is likely to be an indirect effect. Mutations in *daf-12* can result in numerous defects in addition to Daf-d. These include reduced brood size (Larsen *et al.* 1995), heterochronic lineage defects in the hypodermis, and failure in migration of the gonadal distal tip cells (Antebi *et al.* 1998). Thus, the suppression of premature death may result from pleiotropic interactions.

The Age phenotype and reduced motility: One interpretation of species differences in life span is that they reflect differences in metabolic rates. The "rate of living" theory postulates that life span depends upon the rate at which a fixed metabolic potential is expended (Pearl 1928). In some species, *e.g.*, the housefly *Musca domestica* (Sohal and Buchan 1981) and fruit fly *D. melanogaster* (Trout and Kaplan 1970), life span is dependent on the level of physical activity. Although evidence suggests that metabolic rate is reduced in dauer larvae (O'Riordan and Burnell 1989; Wadsworth and Riddle 1989), this is not true for *daf-2* adults (Vanfleteren and De Vreese 1995). However, in some respects *daf-2* adults appear dauer-like in their behavior: Both dauer larvae (Cassada and Russell 1975) and older *daf-2* adults (Kenyon *et al.* 1993) show little spontaneous movement but will move away rapidly in response to touch. Likewise, dauer larvae are nonfeeding (Cassada and Russell 1975), and pharyngeal pumping is greatly reduced in older *daf-2* adults (Kenyon *et al.* 1993). However, the current study demonstrates that reduced movement and reduced feeding are not required for life extension because these traits are not seen in some class 1 mutant adults.

In most prior studies of *daf-2* adults, the class 2, canonical allele, *e1370*, has been used. The *daf-2* Age phenotype may be studied in the absence of class 2-specific defects by using class 1A or 1B mutants. We propose *m41* as a class 1 canonical allele. The *m41* allele is a

more convenient allele to work with than the class 1A alleles *e1368*, *e1371*, and *sa193*, as it forms dauer larvae that do not recover at 25.5°.

It remains to be established whether *e1370* adult-expressed dauer-like traits, such as elevated levels of antioxidant and glyoxylate pathway enzymes, are also exhibited by class 1 *daf-2* mutants, and how many traits, such as reduced-pharyngeal movement or adult motility, are class 2-specific characteristics not associated with life extension. For example, it is possible that increased use of the glyoxylate pathway in *e1370* adults occurs in response to starvation resulting from cessation of feeding, a defect not seen in at least some class 1 mutants.

In summary, our results have revealed two overlapping classes of *daf-2* mutant, the properties of which suggest that *daf-2* is a bifunctional gene. These two elements of *daf-2* function regulate different but overlapping sets of developmental events, from embryogenesis to the maintenance of adult characteristics. Our detailed description of the complex mutant phenotypes provides a resource for interpretation of the molecular alterations in the DAF-2 receptor.

We thank A. Antebi, E. Hedgecock, and J. McCarter for communication of unpublished results, S. Botts for technical assistance, and E. A. Malone and J. H. Thomas for providing strains. This work was supported by fellowships from the University of Missouri Molecular Biology Program to D.G., P.L.L., and A.J.S., a grant from the American Federation of Aging Research to K.V.K. and P.L.L., and Department of Health and Human Services grants AG12689 and HD11239 to D.L.R.

LITERATURE CITED

Anderson, G. L., 1978 Responses of dauerlarvae of *Caenorhabditis elegans* (Nematoda: Rhabditidae) to thermal stress and oxygen deprivation. Can. J. Zool. **56:** 1786–1791.

Anderson, G. L., 1982 Superoxide dismutase activity in dauerlarvae of *Caenorhabditis elegans* (Nematoda: Rhabditidae). Can. J. Zool. **60:** 288–291.

Antebi, A., J. G. Culotti and E. M. Hedgecock, 1998 *daf-12* regulates developmental age and the dauer alternative in *Caenorhabditis elegans*. Development **125:** 1191–1205.

Brenner, S., 1974 The genetics of *Caenorhabditis elegans*. Genetics **77:** 71–94.

Cassada, R. C., and R. L. Russell, 1975 The dauerlarva, a post-embryonic developmental variant of the nematode *Caenorhabditis elegans*. Dev. Biol. **46:** 326–342.

Chisholm, A. D., and H. R. Horvitz, 1995 Patterning of the *Caenorhabditis elegans* head region by the *Pax-6* family member *vab-3*. Nature **377:** 52–55.

Dorman, J. B., B. Albinder, T. Shroyer and C. Kenyon, 1995 The *age-1* and *daf-2* genes function in a common pathway to control the lifespan of *Caenorhabditis elegans*. Genetics **141:** 1399–1406.

Friedman, D. B., and T. E. Johnson, 1988 A mutation in the *age-1* gene in *Caenorhabditis elegans* lengthens life and reduces hermaphrodite fertility. Genetics **118:** 75–86.

Gems, D., and Riddle, D. L., 1996 Longevity in *Caenorhabditis elegans* reduced by mating but not gamete production. Nature **379:** 723–725.

Golden, J. W., and D. L. Riddle, 1984a The *C. elegans* dauer larva: developmental effects of pheromone, food and temperature. Dev. Biol. **102:** 368–378.

Golden, J. W., and D. L. Riddle, 1984b A pheromone-induced developmental switch in *Caenorhabditis elegans*: temperature-sensitive mutants reveal a wild-type temperature-dependent process. Proc. Natl. Acad. Sci. USA **81:** 819–823.

Gottlieb, S., and G. Ruvkun, 1994 *daf-2, daf-16* and *daf-23*: genetically interacting genes controlling dauer formation in *Caenorhabditis elegans*. Genetics **137:** 107–120.

Griffiths, J. T., and O. E. Tauber, 1942 Fecundity, longevity, and parthenogenesis of the American roach, *Periplaneta americana* L. Physiol. Zool. **15:** 196–209.

Hodgkin, J., 1983 Male phenotypes and mating efficiency in *Caenorhabditis elegans*. Genetics **103:** 43–64.

Johnson, T. E., and W. B. Wood, 1982 Genetic analysis of life-span in *Caenorhabditis elegans*. Proc. Natl. Acad. Sci. USA **79:** 6603–6607.

Johnson, T. E., D. H. Mitchell, S. Kline, R. Kemal and J. Foy, 1984 Arresting development arrests aging in the nematode *Caenorhabditis elegans*. Mech. Ageing Dev. **28:** 23–40.

Kapeller, R., and L. C. Cantley, 1994 Phosphatidylinositol 3-kinase. BioEssays **16:** 565–576.

Kenyon, C., J. Chang, E. Gensch, A. Rudner and R. Tabtiang, 1993 A *C. elegans* mutant that lives twice as long as wild type. Nature **366:** 461–464.

Kimura, K. D., H. A. Tissenbaum, Y. Liu and G. Ruvkun, 1997 *daf-2*, an insulin receptor-like gene that regulates longevity and diapause in *Caenorhabditis elegans*. Science **277:** 942–946.

Klass, M., and D. Hirsh, 1976 Non-aging developmental variant of *C. elegans*. Nature **260:** 523–525.

Larsen, P. L., 1993 Aging and resistance to oxidative stress in *Caenorhabditis elegans*. Proc. Natl. Acad. Sci. USA **90:** 8905–8909.

Larsen, P. L., P. S. Albert and D. L. Riddle, 1995 Genes that regulate both development and longevity in *Caenorhabditis elegans*. Genetics **139:** 1567–1583.

Lin, K., J. B. Dorman, A. Rodan and C. Kenyon, 1997 *daf-16*: an HNF-3/forkhead family member that can function to double the life-span of *Caenorhabditis elegans*. Nature **278:** 1319–1322.

Lithgow, G. J., T. M. White, D. A. Hinerfield and T. E. Johnson, 1994 Thermotolerance of a long-lived mutant of *Caenorhabditis elegans*. J. Geron. **49:** B270–B276.

Lithgow, G. L., T. M. White, S. Melov and T. E. Johnson, 1995 Thermotolerance and extended life-span conferred by single-gene mutations and induced by thermal stress. Proc. Natl. Acad. Sci. USA **92:** 7540–7544.

Malone, E. A., and J. H. Thomas, 1994 A screen for nonconditional dauer-constitutive mutations in *Caenorhabditis elegans*. Genetics **136:** 879–886.

Malone, E. A., T. Inoue and J. H. Thomas, 1996 Genetic analysis of the roles of *daf-28* and *age-1* in regulating *Caenorhabditis elegans* dauer formation. Genetics **143:** 1193–1205.

Maynard Smith, J., 1958 The effects of temperature and of egg-laying on the longevity of *Drosophila subobscura*. J. Exp. Biol. **35:** 832–843.

Morris, J. Z., H. A. Tissenbaum and G. Ruvkun, 1996 A phosphatidylinositol-3-OH kinase family member regulating longevity and diapause in *Caenorhabditis elegans*. Nature **382:** 536–538.

Ogg, S., S. Paradis, S. Gottlieb, G. I. Patterson, L. Lee *et al.*, 1997 The Fork head transcription factor DAF-16 transduces insulin-like metabolic and longevity signals in *C. elegans*. Nature **389:** 994–999.

O'Riordan, V. B., and A. M. Burnell, 1989 Intermediary metabolism in the dauer larva of the nematode *Caenorhabditis elegans* - 1. Glycolysis, gluconeogenesis, oxidative phosphorylation and the tricarboxylic acid cycle. Compar. Biochem. Physiol. **92B:** 233–238.

Partridge, L., and P. H. Harvey, 1988 The ecological context of life history evolution. Science **241:** 1449–1455.

Pearl, R., 1928 *The Rate of Living*. Knopf, New York.

Riddle, D. L., 1977 A genetic pathway for dauer larva formation in *Caenorhabditis elegans*. Stadler Symp. **9:** 101–120.

Riddle, D. L., 1988 The dauer larva, pp. 393–412 in *The Nematode Caenorhabditis elegans*, edited by W. B. Wood. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Riddle, D. L., and P. S. Albert, 1997 Genetic and environmental regulation of dauer larva development, pp. 739–768 in *C. elegans II*, edited by D. L. Riddle, T. Blumenthal, B. J. Meyer and J. R. Priess. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Riddle, D. L., M. S. Swanson and P. S. Albert, 1981 Interacting genes in nematode dauer larva formation. Nature **290:** 668–671.

Rose, M., 1991 *Evolutionary Biology of Aging.* Oxford University Press, Oxford.

Sohal, R. S., and P. B. Buchan, 1981 Relationship between physical activity and life span in the adult housefly, *Musca domestica.* Exp. Gerontol. **16:** 157–162.

Sulston, J., and J. Hodgkin, 1988 Methods, pp. 587–606 in *The Nematode Caenorhabditis elegans,* edited by W. B. Wood. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Swanson, M. M., and D. L. Riddle, 1981 Critical periods in the development of the *Caenorhabditis elegans* dauer larva. Dev. Biol. **84:** 27–40.

Tissenbaum, H. A., and G. Ruvkun, 1998 An insulin-like signaling pathway affects both longevity and reproduction in *Caenorhabditis elegans.* Genetics **148:** 703–717.

Trent, C., N. Tsung and H. R. Horvitz, 1983 Egg-laying defective mutants of the nematode *Caenorhabditis elegans.* Genetics **104:** 619–647.

Trout, W. E., and W. D. Kaplan, 1970 A relation between longevity, metabolic rate, and activity in Shaker mutants of *Drosophila melanogaster.* Exp. Gerontol. **5:** 83–92.

Vanfleteren, J. R., and A. De Vreese, 1995 The gerontogenes *age-1* and *daf-2* determine metabolic rate potential in aging *Caenorhabditis elegans.* FASEB J. **9:** 1355–1361.

Vowels, J. J., and J. H. Thomas, 1992 Genetic analysis of chemosensory control of dauer formation in *Caenorhabditis elegans.* Genetics **130:** 105–123.

Wadsworth, W. G., and D. L. Riddle, 1989 Developmental regulation of energy metabolism in *Caenorhabditis elegans.* Dev. Biol. **132:** 167–173.

Ward, S., and J. S. Carrel, 1979 Fertilization and sperm competition in the nematode *Caenorhabditis elegans.* Dev. Biol. **73:** 304–321.

Williams, B. D., B. Schrank, C. Huynh, R. Shownkeen and R. H. Waterston, 1992 A genetic mapping system in *Caenorhabditis elegans* based on polymorphic sequence-tagged sites. Genetics **131:** 609–624.

Yeh, W.-Y., 1991 Genes acting late in the signalling pathway for *Caenorhabditis elegans* dauer larval development. Ph.D. Thesis, University of Missouri, Columbia, Missouri.

Zhang, Y., and S. W. Emmons, 1995 Specification of sense-organ identity by a *Caenorhabditis elegans Pax-6* homologue. Nature **377:** 55–59.

Communicating editor: I. Greenwald