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Investigations into the response of C. elegans to dietary restriction

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Investigations Into the Response of *C. elegans* to Dietary Restriction

by

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DISSERTATION

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This dissertation, and my life,
are affectionately dedicated to
Yongping

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Investigations Into the Response of *C. elegans* to Dietary Restriction

Douglas Crawford

Abstract

Animals have developed complex responses to periodic food deprivation. These include the poorly understood process by which dietary restriction extends lifespan. We investigated whether any of the genes that are known to regulate *C. elegans* lifespan are involved in the response to dietary restriction. The simple answer was no. For at least some animals of every genotype tested, in at least some conditions, it was possible to observe an increase in lifespan when food was restricted. However, lifespan extension (and prolonged reproductive capacity) is not the only strategy available to *C. elegans* adults when food deprived. Offspring can be hatched internally, thus providing the parent's body as food to facilitate the offspring's development. Should the offspring receive enough food, they can develop to an alternative, arrested long-lived form called a dauer larva. The choice made by the parent - whether to lay eggs or retain them for internal hatching - is critical to the long-term survival of the genotype in periodically austere environments. Animals hatch offspring internally at a rate inversely proportional to the concentration of food in the environment. This choice between egg laying and retention of eggs appears to be regulated by the perception of food, as sensory mutations exacerbate the propensity to hatch progeny internally in low food conditions. By contrast, a food-deprived eating-defective mutant with a normal sensory capacity hatches offspring internally at the same low rate as wild type when grown on plates, surrounded by bacteria.

We have shown that weak *daf-2* and sensory mutants can substantially increase lifespan while only weakly affecting reproduction (and not at all in the case of *daf-10(m79)*). This suggests that simple evolutionary models for tradeoffs between lifespan and reproduction are not sufficient. It also supports the view that dietary restriction and the insulin/IGF-1 pathways are different since dietary restriction invariably prolongs reproduction. Corroborating this finding, we showed that *daf-2(RNAi)* treatment of adult animals results in lifespan extension, but does not prolong the reproductive period.

Finally, we have developed a high-throughput screening protocol that successfully identified novel lifespan regulatory genes. Accelerating screening protocols will permit more complex lifespan screens.

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Chapter 1

Introduction

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Abstract: Lifespan extension, the compensatory reward of privation

One challenge of the natural world that seems to resonate with biologists is the struggle to make ends meet. How can we survive through the lean times? For example, evolutionary biologists have focused much of their attention on the relative advantages conferred by traits that improve resource acquisition in times of scarcity (Grant and Grant, 2002). Perhaps this focus on scarcity may stem from the fact that humans can only endure brief periods without food, just a small fraction of their normal lifespan. What a surprise it was to discover that laboratory animals actually live longer when in a perpetual state of hunger (McCay et al., 1935). For lifespan at least, less is more. This result requires us to reconsider our assumptions. Longevity is not the axis upon which evolutionary maximization is occurring. If given the chance, animals appear keen to rush headlong to death, and opt for the longest possible life only in less than ideal conditions. Does this also require us to recalibrate lifespan as a measure of health? In addition to these philosophical musings, these findings cry out for a mechanistic explanation. When deprived of food, animals make physiological adjustments that confer increased survival. It would be very appealing to offer a human therapy that mimics dietary restriction even when food is plentiful. With a population of 6.4 B, it is hard to argue that humans must concern themselves with increasing net population growth rates, but we are (and should be) keenly interested in increasing average and maximal survival. So both to satisfy our curiosity about this bit of fundamental biology, and in hopes of providing therapeutics that will extend human lifespan, understanding the means by which caloric restriction extends lifespan is a vital mission.

Dietary restriction delays development, extends lifespan, and reduces fertility

In seminal work, McCay restricted rats immediately after weaning to a diet equivalent to 50% of the levels they would eat if fed *ad libitum* (McCay, 1939). While these rats failed to measure up to two traditional indices of health, in that they were diminutive and unable to reach reproductive maturity until their diets were increased, they nonetheless exceeded their *ad libitum* cohorts in mean and maximal lifespans. These results have subsequently been extended to include a broad range of organisms including yeast, mice, flies, fish, dogs, and most importantly to this study, *C. elegans (Chapman and Partridge, 1996; Kealy et al., 2002; Klass, 1977; Lin et al., 2000).* The widespread distribution of this response to dietary restriction, or DR, (and the similarities of the secondary phenotypes described below) suggests a common mechanism of lifespan extension may exist.

DR of *C. elegans* causes a substantial lifespan extension when initiated prior to the fifth day of adulthood (Johnson et al., 1984; Klass, 1977). If *C. elegans* is hatched into an environment without any food at all, it arrests as an L1 larva, and can survive for approximately two weeks. If such arrested animals are then provided with food they develop normally to fertile adults, and their adult lifespan is unaffected by the length of time it spent as an arrested larva. This delay of development and net increase in lifespan is similar to the pattern observed by McCay in rats. If *C. elegans* is provided with only small amounts of food from the time of hatching, it can grow to an alternative larval state – the dauer (Riddle, 1977). Dauers are extremely resistant to environmental challenges

and can survive up to six months and yet still recover and grow to normal adulthood if supplied with food. Finally, if a subsistence level of food is provided to adults, they live longer. However, if starved as adults, they hatch their offspring internally, thereby providing the progeny with their own bodies as food (Chen and Caswell-Chen, 2003).

Three distinct protocols have been employed to study DR in *C. elegans*: growth in liquid media with defined concentrations of the bacterium OP50, growth in axenic media made up of yeast extract and bovine hemoglobin (Vanfleteren and Braeckman, 1999), and growth of mutant strains with an impaired capacity to ingest food (Eat mutants) (Lakowski and Hekimi, 1998). Feeding-defective mutations and direct caloric restriction produce a 30-50% lifespan extension, whereas axenic media provides a 150 – 200% extension. There are distinct advantages and liabilities to each experiment that must be weighed carefully when interpreting and comparing the results, and these differences form a substantial potion of the following study.

By contemporary animal husbandry standards, McCay's rats were not particularly healthy in that they were often subject to infectious disease. Consequently, the control lifespans were shorter than those found in later studies were. Nevertheless, the fact that DR helped to prolong the lifespan of these chronically challenged rats presaged the widespread protection conferred by DR. Apparently, no matter what the normal cause of mortality, DR provides protection from that pathology as well as many normal alterations that occur with aging (Koubova and Guarente, 2003). For instance, DR slows the rate at which organisms accumulate oxidative damage (Sohal and Weindruch, 1996), develop cancer (Kritchevsky and Klurfeld, 1986), suffer retinal decay (Obin et al., 2000), or experience immunological decline (Pahlavani, 2004).

In addition to normal aging, DR provides protection for animals challenged with environmental or genetic stressors. Rats fed reduced diets are less likely to develop tumors when exposed to either chemical carcinogens (Kritchevsky et al., 1984) or radiation (Gross and Dreyfuss, 1984); DR rats are more thermotolerant (Hall et al., 2000); and in *C. elegans*, the eating-defective mutants are better able to survive the lifespan-shortening loss-of-function mutations *daf-16(m26)* (Lakowski and Hekimi, 1998) and *hsf-1(RNAi)* (Hsu et al., 2003).

All of these studies have tended to focus researchers' minds on the prospect that caloric restriction may extend the lifespan of higher organisms. In 1987, the National Institute of Aging began funding an assay of the effects of caloric restriction on Rhesus monkeys (Mattison et al., 2003). Given that the mean lifespan of Rhesus monkeys is 25 years, this is an ambitious endeavor. An insufficient number of animals have died to prove the case, but survival rates are consistent with the prediction that DR will extend primate lifespan. Numerous biomarkers such as insulin levels, serum lipid levels, and blood pressure also suggest that the DR animals are healthier.

Primate studies may provide useful information, but the animal we are most curious about is the human. Humans are difficult subjects because they are so longed-lived and their diets are so difficult to control. However, several lines of evidence suggest that even humans may enjoy DR benefits. Several biomarkers significantly affected by caloric restriction in primates are positively correlated with biomarkers identified in the Baltimore Longitudinal study that are held by the longest lived portion of the sample (Roth et al., 2002). Similarly, the residents of Biosphere II spent two years consuming approximately 70% of their ad libitum levels of calories (Walford et al.,

2002). These hungry residents enjoyed beneficial alterations in important biomarkers such as blood pressure, cholesterol, and insulin. Furthermore, a number of people are voluntarily restricting their own food consumption. A recent study of 18 such self-restricted people found healthier levels of serum lipids, insulin sensitivity, and blood pressure (Fontana et al., 2004). There is now even a resort catering (so to speak) to people looking for low calorie vacations (http://www.antiaging-europe.com/cntr_croat.php). The Anti-Aging Center on the Adriatic offers 7 – 28 day programs featuring 500 – 1000 Kcal/day regimens.

Some epidemiological data support the hypothesis that low food yields increased lifespan in humans. Chronic underfeeding in human populations is normally correlated with negative health factors such as poor sanitation, malnutrition due to missing micronutrients, and inadequate medical care. Underfed human populations share a number of characteristics with DR rodents: small stature, delayed sexual maturation, and reduced fertility, but these populations do not generally enjoy lifespan extension. However, Okinawa, Japan provides a promising example of the potential benefits to human health if low food consumption is separated from the usual negative correlates. Okinawans consume fewer calories than do most Japanese, enjoy lower rates of heart disease and cancer, and most importantly enjoy longer lifespans (Mimura et al., 1992; WILLCOX et al., 2002). An independent study showed that women who had been hospitalized for anorexia early in their lives were 50% less likely to develop breast cancer (Michels and Ekbom, 2004); this is consistent with the lower incidence of spontaneous tumors in DR rodents.

Heartened by these preliminary findings and undaunted by the difficulties in controlling human behavior, researchers have begun a multi-center clinical trial to determine whether DR provides benefits to humans. The Comprehensive Assessment of Long-term Effects of Reducing Intake of Energy (CALERIE) is a one-year study of healthy middle-age men and women that compares responses to exercise, calorie reduction, and combinations of the two on a variety of physiological measures (http://calerie.dcri.duke.edu/index.html).

Time of Action

As noted earlier, McCay's original studies restricted food availability following weaning of rats. The lifespan of the restricted rats was extended in part by postponing adulthood. This delay in development was the explanation provided by McCay et al. for the lifespan extension. However, subsequent studies demonstrated that the benefits of caloric restriction in mammals are available for adults as well. Initiating caloric restriction in rats at 6 months of age rather than 6 weeks actually produced slightly lower mortality rates (Yu et al., 1985), and substantial increases in lifespan were still available to mice at the mid-point of their mean lifespan (Weindruch and Walford, 1982). Finally, Klass's DR experiments in *C. elegans* were initiated as young adults (though he was unable to see any benefit for worms older than day 4 of adulthood). These studies demonstrated that adult physiology is still sufficiently plastic to enjoy the benefits of DR. The real extent of this plasticity has been demonstrated in *Drosophila* in which hazard rates were compared in flies shifted to low food regimes at various stages of life (Mair et al., 2003). After a transition period of roughly two days, there was no difference in the

hazard rates for latecomers to DR as compared to those who had been on a low calorie diet their entire lives. If the fly had survived long enough to make the transition, it was no worse off then its control peers. This more than any other finding in the history of aging research, should give hope to those with misspent youths and gathering years (which include the author).

Dietary restriction regulates reproductive activity

Well before DR survival advantages are observed, reductions in fertility occur. Reproduction is expensive and dangerous, and best postponed until sufficient resources exist for both the parents and the offspring. The DR-induced alterations in physiology have been studied carefully in mammals (Wade and Jones, 2003). Every step in the reproductive process from sexual maturation to care for offspring can be delayed and in some cases reversed. Indeed, many starved animals can abort, or even more efficiently resorb, developing fetuses. These delays are reversible, and fecundity is restored upon refeeding, though subsequent fertility is compromised by the delay (Tu and Tatar, 2003).

How does caloric restriction work?

A stimulating explanation for the extended lifespan, and one that is largely consistent with the existing data, is that DR lowers insulin signaling activity to result in elevated stress capacity and ultimately lifespan extension. Three lines of evidence

suggest this model: first, the insulin signaling system maintains bioenergetic homeostasis in organisms ranging from *C. elegans* to humans; second, like DR, the insulin signaling pathway has been shown to regulate lifespan and fertility; and third, there is some direct and some circumstantial evidence linking insulin signaling activity to DR.

In mammals, serum glucose levels must be maintained within a narrow range. This is a challenge since feeding is episodic, and occasionally inadequate, and energy demands can vary substantially. Serum glucose homeostasis is achieved via insulin signaling. Feeding results in elevated serum glucose levels, which stimulates insulin secretion from beta cells in the pancreas. Elevated levels of insulin result in the uptake of glucose by peripheral tissues and stimulate anabolism. However, excessive cycling of the insulin signaling system, as seen with overweight animals and humans, diminishes insulin's effectiveness in maintaining energetic homeostasis and contributes to Type II diabetes in both animals and humans (Must et al., 1999). By contrast, long term food deprivation leads to low steady-state serum glucose and insulin levels in mammals ranging from mice to humans (Dhahbi et al., 2001; Walford et al., 2002). Thus, insulin signaling plays a central role in the response of animals to changes in their intake of food both in the short and long term. It is sensible, therefore, to believe that the lifespan extension conferred by chronic underfeeding would be mediated at least in part by the insulin system.

Strong support for this proposition is supplied by the finding that mutations that reduce the activity of the insulin receptor result in lifespan extension in animals ranging from *C. elegans* (Kenyon et al., 1993) to mice (Bluher et al., 2003). Reduction-of-function mutations in the insulin/IGF-1 like receptor in *C. elegans*, *daf-2*, provide up to a

100% increase in lifespan and stimulate dauer formation. This lifespan extension is dependent upon the forkhead transcription factor *daf-16* which regulates numerous targets including many responsible, metabolism and stress response (Murphy et al., 2003).

Humans and mice (Kitamura et al., 2003) that lack the insulin receptor have shortened lifespans, but a conditional fat cell-specific knockout confers an 18% increase in mouse lifespan (Bluher et al., 2003). Thus for mice as well as worms, insulin receptor hypomorphs enjoy lifespan extension. Interestingly, like DR mice, these mice are lean, though they consume more food per unit mass than their controls (Bluher et al., 2002). This reduction in fat, and only modest lifespan extension led Bluher et al. to suggest that the lifespan extension in mice may not be due to its effects on insulin signaling, but may instead be a consequence of reduced adiposity.

Not only do both DR and insulin signaling mutations result in lifespan extension, but relatively strong *daf-2* mutations also diminish fertility and prolong the reproductive period (Gems et al., 1998; Kenyon et al., 1993). For instance, at 20° C wild-type *C. elegans* have an average of 278 offspring, whereas the *daf-2(e1370)* has 212 offspring (Kenyon et al., 1993). The reproductive system itself also regulates the activity of *daf-2* and *daf-16* to control lifespan (Hsin and Kenyon, 1999). Removal of the germ cells, either via laser ablation of the primordial germ cells in L1 larva or via mutations that abrogate germ cell development, results in a substantial lifespan extension. This extension of lifespan requires the presence of *daf-16*. By contrast, removal of the entire somatic gonad results in a lifespan equal to that of wild-type animals. This data is consistent with a model in which germ cells produce a lifespan shortening signal and the

somatic gonad produces a lifespan extending signal. The two signals appear to provide equal and opposite inputs to lifespan since lifespan remains unchanged when both tissues are removed. That DR effects fertility and that reproductive tissues regulate lifespan, suggests that the DR lifespan response may be achieved by modulating reproductive signaling.

Data from the fly has also provided evidence of a relationship between DR and insulin signaling, though of an unusual sort. If deprived of food altogether, flies can survive approximately six days (Clancy et al., 2001). The susceptibility to starvation is enhanced by a mutation in the Drosophila insulin receptor substrate, *chico* (Clancy et al., 2002). Their interpretation was that the *chico* mutant had a pre-existing physiological switch to the caloric restriction response. This leaves *chico* mutants vulnerable to starvation when food is lowered past a critical point (in their experiments this occurred at food supply 20% below the normal concentration of food). At each datum below 80% of the normal food concentration, the *chico* survival was significantly shorter than that of wild-type. Between 150% - 80% of normal food levels, both the *chico* and wild-type animals lived progressively longer as the food supply was reduced. At these higher levels of food, the *chico* survival response paralleled that of wild-type. It may not be surprising to find that animals with a mutation in a gene responsible for the caloric restriction response can be further extended by direct caloric restriction, but I am surprised to find that the slope of that response is the same at high concentrations. One possibility is that moving from 150% to 100% of normal food levels extends lifespan in a different way then changing from 100% to 80%. What is required is another measure of health besides lifespan, the most potent of which would have been reproductive output.

If the animals grown on 150% of the normal food concentration had had fewer offspring than those at 100%, we could conclude that this increase in lifespan exhibited by both *chico* and wild-type animals was due to relief of toxicity rather than caloric restriction. Finally, it should be noted that the lifespan extension generated by the *chico* mutation in this study was approximately 10%; this is considerably smaller than the 48% reported in an earlier paper (Clancy et al. 2001). Even if this study provides only weak evidence for a role of insulin signaling in the response of flies to low food, it does provide an important suggestion. Perhaps by considering the interaction of genotype and environmental changes, or by considering other phenotypes besides aging alone, we may yet be able to demonstrate a relationship between the insulin signaling system and caloric restriction.

Another interesting component of the insulin signaling system is the histone deacetylase *sir-2.1* (the *C. elegans* homolog of *SIR2* in yeast). In both *C. elegans* and yeast, increased expression of this histone deactylase results in lifespan extension (Kaeberlein et al., 1999; Tissenbaum and Guarente, 2001). In yeast *SIR2* activity is regulated by NAD, which may allow it to act as an energy censor (Imai et al., 2000). Indeed, *SIR2* is required for DR replicative lifespan extension in DR yeast (Lin et al., 2000). Furthermore, the mammalian homolog SIRT1 expression is elevated in cells drawn from DR rats, and its expression can be suppressed with exogenous insulin and IGF-1 (Cohen et al., 2004). However, it has also been shown that in yeast, NAD levels fall, and the ratio of NAD to NADH remains unchanged in calorically restricted yeast (Anderson et al., 2003). *SIR2* is also strongly regulated by the product of the reaction it catalyzes, nicotinimide (Anderson et al., 2003). Thus, NAD may not be the stimulus that

activates *SIR2* in low food. Nonetheless, the data indicate that *SIR2* plays an important role in regulating lifespan in response to environmental perturbations such as caloric restriction, though it remains to be seen whether this is true of *C. elegans*.

The strongest contrary evidence for a requirement of insulin signaling is provided by *C. elegans*. The forkhead transcription factor *daf-16* is required for the lifespan extension generated by mutations in *daf-2* and *sir2.1*, but not for the lifespan extension offered by caloric restriction, as produced by an eating-defective mutant (Lakowski and Hekimi, 1998), by direct caloric restriction, or by growth in axenic media (Houthoofd et al., 2003). Moreover, all three treatments are capable of further extending the lifespan of *daf-2* mutants. A complex contrary result is also offered by DR of the *Prop*^{df} dwarf mouse (Bartke et al., 2001). Mutations that result in developmental defects in the pituitary gland are small, have lower insulin and IGF-1 levels, and are long lived. Direct caloric restriction was capable of further extending the lifespan of the *Prop*^{df} mouse. One cannot conclude though that this means that DR is independent of the insulin signaling system in mice for two reasons. Firstly, *Prop*^{df} is not a complete loss of function of the insulin signaling pathway, so even if they are both members of a linear pathway then the effects could be additive. Secondly, mice lacking a pituitary gland may not be living long only because of changes in insulin/IGF-1 signaling.

The strongest experimental approach taken so far is the caloric restriction of the *daf-16* null mutant in *C. elegans*. This experiment has the double advantage of involving at least one null mutation and of contrasting opposite phenotypes, since DR extends whereas loss-of-function of *daf-16* reduces the wild-type lifespan by approximately 20%. As indicated, these experiments indicate that at least in worms, *daf-16* is not required for

the DR lifespan regulation. It should be noted, though, that these experiments were qualitative and did not explore the possibility of a partial suppression or diminished response to DR. Experiments described in this thesis seek to address this possibility.

Another reasonable explanation for DR's benefits is that DR lowers metabolic flux, and this reduced metabolic flux reduces the generation of reactive oxygen species (ROS). Sohal and Weindruch proposed that caloric restriction reduces ROS in a manner equivalent to lower temperature or activity in poikilothermic animals. Indeed, reduction of mitochondrial electron transport results in lifespan extension, reduced fertility, and slow rates of development (Dillin et al., 2002; Lakowski and Hekimi, 1996; Lee et al., 2003). As the mitochondrial electron transport chain plays a central role in the conversion of food to energy, we should consider the model that DR achieves its beneficial effects upon lifespan by reducing mitochondrial electron transport.

What evolutionary advantage does the dietary restriction response offer?

This seems the easiest question yet to answer – if times are tough it must be better to have a set of tools that permit one to hunker down and wait for times to improve. Survival during these trying times could require additional stress resistance, resources for which could be borrowed from reproduction, and as discussed earlier, these are exactly the characteristics of DR (Holliday, 1989). This is a nice application of the Disposable Soma theory promoted by Kirkwood (Kirkwood and Rose, 1991), which is itself an application of the antagonistic pleiotropy model (Kirkwood and Rose, 1991). In each

model, the assumption is that changes that increase lifespan (or the reproductive period) must be linked with consequences that reduce early fitness. Indeed, DR results in small less fertile animals that certainly appear to be sacrificing early advantages for their increase in lifespan and reproductive period.

Support for the Disposable Soma model is provided by exceptions to the caloric restriction rule. Both Mediterranean fruit flies(Carey et al., 2002) and some rotifers(Kirk, 2001) fail to live longer when their food is restricted. Interestingly, these species also fail to modulate their reproductive activity when food levels are reduced. Thus, lifespan and reproductive regulation seem to move in lock step, in a manner that permits survival through periodic episodes of food deprivation. These data also gainsay the notion that DR works non-specifically, as is suggested by the rate of living and ROS hypotheses.

There are two problems with the Disposable Soma model. The first is that in fully fed conditions it is not clear why trade-offs between lifespan and reproduction must be made (Mitteldorf, 2001). Why not both repair DNA damage and produce offspring, particularly in animals that consume significant amounts of energy producing heat? Why engage in futile mitochondrial cycles to generate heat – when protein turnover and scavenging ROS would also produce heat?

However, the more serious question regards the paucity of experiments that the wealth of evolutionary musings has generated. With only a couple of exceptions, few experiments that have led to mechanistic insights have been suggested by the evolutionary theory of aging (such as those by Carey or Kirk). What has been more productive in the recent history of aging research is to focus on the how aging occurs. Following this lead, the following study seeks to glean from the worm's complex and

often beautiful life history insights into how at least this animal responds to caloric restriction. The worm apparently is an expert since they have so many responses: larval arrest, dauer, lifespan extension, and even internal hatching of offspring. It turns out that by focusing only on lifespan we have missed many of the insights regarding gene action and caloric restriction that come in these related responses.

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Chapter 2

A role for sensory neurons and insulin/IGF-1 signaling in the complex response of C. elegans to dietary restriction.

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Abstract

In *C. elegans*, dietary restriction (DR) results in lifespan extension, thermotolerance, and prolongation of the reproductive period. All of these phenotypes can be generated, to some degree, by mutations in lifespan regulatory genes. This correlation suggests that at least some of the caloric restriction response might be mediated by these life span regulatory pathways. We provide evidence here that indeed caloric restriction involves complex responses that are partially mediated by sensory neurons and *daf-2*. Specifically, sensory neurons and the DAF-2 insulin/IGF-1 receptor play a central role in regulating egg laying in low food conditions. In low food, internal hatching of offspring (bagging) permits offspring to develop to the stress-resistant dauer larva stage, and is the natural response of wild-type animals to adult starvation. This response is strongly enhanced by loss of function mutations that inactivate sensory neurons, as well as mutations in the insulin/IGF-1 receptor *daf-2*, but does not require the forkhead transcription factor *daf-16* that is required for the dauer formation or lifespan extension produced by *daf-2* mutations. Intriguingly, the propensity to hatch offspring internally seems to be driven primarily by the perception of food, rather than food consumption, as an eating defective mutant (*eat-2(ad1116)*) has normal bagging rates on plates but bags at wild-type rates when food is limiting. DR's effect on reproduction and bagging suggested that the DR lifespan extension may be a consequence of perturbations in reproductive signaling, but removal of the gonad does not suppress the DR lifespan increase. Similarly, we suspected that inhibition of respiration may extend lifespan in a manner equivalent to DR, but growth of an eating defective mutation on respiratory-chain

RNAi did not prolong reproduction, and could result in a substantial positive synergy between the two effects. Thus, the phenotypes induced by caloric restriction in the worm may not be triggered simply by a reduction in energy supplies. In addition, our findings suggest that sensory perception plays a significant role in the response to DR. When an animal perceives that the environment is no longer propitious for growth it faces a choice to either bag, facilitating dauer formation, or not to bag, allowing an underlying mechanism to prolong lifespan and reproduction in hopes of postponing reproduction until better times.

Introduction

Dietary restriction (DR) has been shown to extend the lifespans of a broad range of organisms. Caloric restriction in mammals results in delayed maturation, prolonged lifespan, reduced body size, thermotolerance, diminished brood sizes, and prolonged fecundity (Weindruch and Walford, 1988). Many of these phenotypes are recapitulated in *C. elegans* grown in liquid with defined levels of food (Houthoofd et al., 2003; Klass, 1977). Eating defective mutants also exhibit the diminutive size, late progeny, and lifespan extension associated with DR (Lakowski and Hekimi, 1998).

In the last decade, it has been shown that reductions in the function of a number of genes results in lifespan extension in *C. elegans* (Guarente, 2000; Hekimi and Guarente, 2003). These genes can generally be placed in one of three broad, but not necessarily independent, pathways: insulin/IGF-1 like signaling, reproductive signaling, and respiration-dependent lifespan influences.

Insulin signaling in mammals modulates physiological responses due to changes in food intake (Saltiel and Kahn, 2001). In organisms ranging from *C. elegans* to mice it has been shown that mutations that reduce the activity of the insulin pathway result in lifespan extension (Bluher et al., 2003; Kenyon, 2001). In *C. elegans*, strong hypomorphic mutations in the insulin/IGF-1 receptor homolog *daf-2* result in lifespan extension, fat accumulation, delayed maturation, late reproduction, and thermotolerance (Gems et al., 1998; Kenyon et al., 1993). The similarity in phenotypes and the physiological role of the insulin pathway have led some to suggest that caloric restriction may achieve its effects by down-regulating the insulin signaling pathway. Evidence in support of this model comes from the reduced lifespan extension and accelerated starvation witnessed in *chico* mutant flies (Clancy et al., 2002). Fly lifespan can be extended when grown on diminished concentration of food. However, at very low levels of food lifespan is shortened presumably via starvation. *chico* mutants have a similar response to that of wild-type flies, but the dose response curves are shifted to higher levels of food, suggesting that *chico* mutants have already engaged in the DR response. In *Saccharomyces cerevisiae*, lower glucose levels result in increased reproductive lifespan (Lin et al., 2000). This increase in reproduction requires the presence of the NAD-dependent histone deacetylase Sir2p. In *C. elegans*, overexpression of the SIR2 homolog (*sir-2.1*) results in a *daf-16* dependent lifespan extension (Tissenbaum and Guarente, 2001). Finally, DR elevates steady state expression levels of the mammalian homolog SIRT1 in adult rats. *In vitro* this elevation of SIRT1 suppressed apoptosis and was itself suppressed by insulin and IGF-1 (Cohen et al., 2004).

By contrast, in *C. elegans* there are suggestions that DR does not extend lifespan by down-regulating the *daf-2* insulin/IGF-1 signaling pathway. A double mutant containing a mutation that models DR by inhibiting eating and a mutation in the insulin/IGF-1 receptor [*eat-2(ad465); daf-2(e1370)*], has a longer lifespan than either single mutant (Lakowski and Hekimi, 1998). Moreover, while *daf-16* is required for the lifespan extension of hypomorphic mutations of *daf-2*, *eat-2(ad1116); daf-16(m26)* double mutants lived longer than did *daf-16(m26)* alone. Houthoofd et al. employed direct caloric restriction experiments of *C. elegans* treated with FUDR to prevent reproduction. They showed that *daf-16* was not required to mediate the DR lifespan extension, indeed *daf-2(e1370)* exhibited synergistic lifespan benefits when grown in axenic media. These results in *C. elegans* contrast with those reported in flies and yeast, and this difference is a significant puzzle.

Another possibility is that DR prolongs aging by somehow perturbing reproductive signaling. As noted, one hallmark of caloric restriction is diminished fertility and prolonged fecundity. In *C. elegans*, laser or genetic ablation of germ-cell results in lifespan extension, whereas removal of the entire gonad produces a wild-type lifespan (Hsin and Kenyon, 1999). These and other findings suggest the operation of an antagonistic signaling system in which the germ-cells produce a life shortening signal and the somatic gonad produces a lifespan prolonging signal. It could be the case that caloric restriction operates by perturbing the balance of this reproductive signaling system.

Treatments that reduce mitochondrial electron transport capacity in *C. elegans* result in lifespan extension, delayed maturation, and smaller size (Dillin et al., 2002;

Lakowski and Hekimi, 1996). These phenotypes are similar to those seen in DR animals. Perhaps DR extends lifespan by reducing mitochondrial electron transport activity.

It should be noted, though, that *C. elegans* enjoys alternative life histories unavailable to the other model organisms that have been used to investigate DR. Specifically *C. elegans* larvae are capable of becoming dauers, which exhibit extraordinary environmental resistance and lifespan extension. However, adults that find themselves without food can no longer revert to dauer development, and their progeny born into a world without food do not have sufficient resources to make it to dauer. *C. elegans* can pursue an alternative in which progeny are hatched internally and their own bodies can on occasion provide the resources needed to reach dauer ((McCulloch and Gems, 2003) (Chen and Caswell-Chen, 2003), and data presented here). In this study, we show that this decision is strongly influenced by the perception of food rather than its consumption, and is strongly influenced by sensory neurons and *daf-2*. These differences in potential life strategies may help explain the differences observed between the *C. elegans* and other organisms under caloric restriction conditions.

Results and Discussion

Dietary restriction does not extend lifespan by reducing mitochondrial electron transport

Like rodents subjected to caloric restriction, one of the fastest and most profound effects of DR in *C. elegans* is to reduce fertility. When young adult animals are shifted

from fully fed conditions to low food, in the following 24 hours they produce only 1/3 the number of offspring of their fully fed cohorts (Figure 2 – 1G). The offsetting virtue of the diminished reproductive output is protracted fecundity. DR animals continue to produce offspring for nearly twice the interval of animals grown in high food (Figure 2 – 1 G). Despite the protracted fertility, DR animals produce substantially fewer offspring (data not shown). Eat mutants also exhibit the diminished brood size and protracted reproductive period characteristic of caloric restriction ((Davis et al., 1995).

RNAi treatments that reduce mitochondrial function and increase life span also result in diminished brood sizes (Dillin et al., 2002). These phenotypes parallel that of DR, and suggest that DR's effects may be due to reduction in mitochondrial electron transport. If this were the case we hypothesized that reduction of mitochondrial electron transport would, like DR, prolong the reproductive period. However, despite being slow (Clk) in so many aspects of their physiology, and of having a profound effect upon fertility, we found no evidence for late progeny in *cco-1(RNAi)* and *nuo-2(RNAi)* (Figure 2 – 1 A – D). The other mitochondrial RNAi treatments tested (*atp-3* and *cyc-1)* resulted in very few progeny, and those that did occur all were laid within the first 3 days of adulthood. Similarly, *clk-1(qm30)* and *clk-1(e2519)* both delay the onset of reproduction and diminish fertility, but neither allele extends the reproductive period, as described by Chen et al. for *clk-1(qm30)* ((Chen et al., 2001) and discussed in Chapter 3).

To further test a possible association between DR and reduction of mitochondrial respiration, we tested whether simultaneous treatment would have an additional benefit on lifespan. We found that not only is it possible to extend the lifespan of *eat-2(ad1116)* by reducing mitochondrial function, but RNAi of three genes required for efficient

electron transport (*cco-1*, *cyc-1* and *nuo-2*) produced significant synergistic effects upon lifespan (Figure 2 - 2). *eat-2(ad1116)* grown on *atp-3* RNAi bacteria had a biphasic survival distribution that despite the fact that the 75% percentile survival was 19 days greater than the vector-only treated worms, was not statistically significantly using the logrank test ($p = 0.09$). It is possible that the ATP synthase is part of the response to DR, which seems unlikely since it did not have a DR-like reproductive; alternatively, it is possible that *atp-3(RNAi)*e animals are simply unable to live any longer for some reason. Because the other three RNAi treatments produced synergistic lifespan extension with DR, we feel it is most likely that the two treatments do not exist in a single pathway, though they may have common effectors.

The lifespan extension caused by dietary restriction does not require reproductive signaling

The strong association between DR and the regulation of reproduction suggested that DR could extend lifespan by modulating reproductive signaling. To test this hypothesis we ablated the gonad (specifically the somatic gonad precursors Z1 and Z4) of both *eat-2(ad1116)* and wild-type animals that were subsequently calorically restricted. In both cases, gonad ablation did not suppress the lifespan extension conferred by DR. In fact, *eat-2(ad1116)* animals lived 32% longer without a gonad ($p = 0.0114$), as did the DR animals at each concentration (Figure 2 – 3, Table 2 – 2)). Similarly, ablating the germ cell precursors (Z2 and Z3) did not prevent DR lifespan extension (Figure 2 – 3 C). Interestingly though, in this preliminary study, removing the germ cells from animals

grown in relatively high concentrations of food did not result in an extension in lifepan as compared to the intact controls. Further investigation is required to determine whether specific environments prevent germ cell signaling from extending lifespan.

In the wild type, ablation of the whole gonad completely suppresses the lifespan extension produced by germ-line ablation. Genetic experiments suggest that somatic gonad ablation shortens lifespan by dis-inhibiting the DAF-2 insulin/IGF-1 pathway (Hsin and Kenyon, 1999). In strong *daf-2* mutants, ablation of the whole gonad, like germ-line ablation, lengthens lifespan. Since DR has the same effect, this raises the possibility that DR may inhibit *daf-2* pathway activity in some way, so that it is unavailable to shorten lifespan in response to somatic gonad ablation (see below).

Dietary restriction enhances internal hatching of offspring

When fertile wild-type adults are shifted to low-food conditions, internal hatching of offspring occurs at a rate inversely proportional to the bacterial concentration (Figure 2 - 4). The animals that hatch internally consume their parent, which in some cases provides sufficient resources to achieve dauer development. To confirm that it was indeed their own parent that had provided the resources, we identified a number of dauers that were still encased in their parent's cuticle (Figure 2 - 5). Dauers are capable of surviving roughly 12 times longer than the normal adult lifespan and are capable of enduring harsher environments; and yet once food is restored, dauers can fully recover to have normal fertile adulthoods. Thus, the capacity to make it to dauer in low food must provide a strong advantage, making internal hatching an adaptive response to low food.

Why do these animals bag? Interestingly, we observed that the eating defective mutant *eat-2(ad1116)* grown on plates bags at a rate comparable to that of wild-type (2/241 vs. 2/355 respectively). The lifespan and reproductive behavior of *eat-2(ad1116)* grown on plates is comparable to that of wild-type worms grown in liquid culture containing approximately 10^8 bacteria/ml. Yet only approximately 1% of the *eat-2(ad1116)* worms bag, whereas 18-70% of the DR wild-type worms bagged (Figure 2 – 4 A). We suspected that one difference between the two circumstances might be the perception of food availability. Whereas *eat-2* mutants consume less food, they are exposed, nonetheless, to an environment containing an abundant food supply. In contrast, even at high bacterial concentrations in liquid culture, the animals are in an environment that contains much less food than is present on plates. Perhaps then, the propensity to hatch offspring internally is caused by the perception of less food in the environment rather then reduced consumption. To test this hypothesis, we grew *eat-2(ad1116)* in a low food environment (liquid culture with defined bacterial concentrations) and assayed the bagging rate. Consistent with the hypothesis, we found that *eat-2(ad1116)* bagged at a rate equivalent to that of WT (Figure 2 – 4 G) when grown in low food. If amount of food consumed influenced the decision to bag then the combination of diminished eating efficiency with a low food environment could have elevated bagging rates, but at each concentration, *eat-2(ad1116)* bagged at the wild-type rate. This finding suggests that worms bag in response to perception, not consumption of food.

To further test the model that food perception may influence bagging rates we tested whether mutations that impair sensory neurons would affect bagging rates when

grown in a low food environment. We found that *osm-5(p813)* and *tax-4(p678)* had substantially elevated rates of bagging in liquid culture (Figure 2 – 4 A, B, and C). Sensory mutant and wild type bagging rates tended to converge at very low levels of food. One interpretation is that the perception of food restriction causes bagging, and that very low real food concentrations elicit bagging as effectively as sensory mutations. This finding is consistent with our findings with *eat-2(ad1116)* mutants, in that perception of food availability appears to influence the decision as to hatch offspring internally.

As sensory neurons regulate dauer formation, and bagging in low food facilitates dauer formation, perhaps the decision to bag is an adult dauer pathway. If this is the case, we anticipated that bagging, like dauer formation, would be enhanced by hypomorphic mutations of *daf-2* and dependent upon *daf-16*. Indeed, *daf-2(e1370)* also had higher rates of bagging in a low food environment (Figure 2 – 4 D). However, unlike dauer formation, the ability to hatch offspring internally does not require *daf-16*, as a *daf-16* null allele bagged at a wild-type rate (Figure 2 – 4 E). Furthermore, *daf-16* was unable to suppress the elevated bagging rates of *osm-5(p813)* and a null mutation of *daf-2* (*daf-2(m65)*) (Figure 2 – 4 F and G). These results suggest that the sensory neurons and the DAF-2 insulin/IGF-1 homolog receptor homolog monitor the presence of food in the environment and act to suppress bagging in a *daf-16* independent manner.

If low food stimulates bagging, and this effect is strongly associated with the function of sensory neurons, we wondered whether the longevity response to DR would also be influenced by sensory neuron function. We found that, qualitatively, each of the mutants tested (*tax-4(p678)*, *osm-5(p813)*, and daf-*16(mu86); osm-5(p813)*), exhibited at

least some evidence of lifespan extension when grown in diminishing levels of bacteria (Figure 2 – 5 and 6). However, the degree of benefit and the reliability of the response varied between genotypes. For *osm-5(p813)* even the qualitative evidence was weak, since only the step from 7.5 x10^9 to 1.4 x 10^9 bacteria/ml yielded an increase in lifespan. In the range at which the most reliable results normally occur (10^9 to 10^8) a decrease in mean lifespan occurred (Figure 2 – 6 E). However, these results require some caution in their interpretation since a large portion of the initial sample was censored due to bagging. Nonetheless, these results suggest that if offered an opportunity to hatch offspring internally, *osm-5* and *tax-4* mutants enjoy smaller and less reliable lifespan benefits because of DR.

As environments containing low levels of food stimulate bagging in a *daf-16* independent manner, we were curious as to whether DR lifespan may be regulated by *daf-2*, but independently from *daf-16*. To test this hypothesis we used a null mutation of *daf-2* (*daf-2(m65)*, and found it that was still possible to observed a lifespan extension in diminishing levels of food even without *daf-2* (2 – 6 F).

Discussion

Food supplies are a key regulator of *C. elegans* biology. Diverse life history options are available to worms deprived of food at various stages of the development, including lifespan extension and internal hatching of offspring. Our studies indicate that life history effects depend upon the method by which food or energy supplies are limited.

Sensory neurons and *daf-2* play a role in regulating these life history choices, but they do so independently of their *daf-16* dependent lifespan regulatory role. Moreover, while a primary variant in the life history choice is when and how many offspring to produce, the reproductive system is not required for DR lifespan extension.

DR does not control lifespan or reproductive behavior by reducing mitochondrial respiration since the phenotypes and time of action are distinct between the two treatments. We found that unlike DR, inhibition of mitochondrial respiration with RNAi did not extend the reproductive period. *cco-1(RNAi)* and *nuo-2(RNAi)* both substantially reduced total fertility, but neither exhibited a tendency to prolong the reproductive period, whereas DR both reduces fertility and profoundly prolongs the reproductive period. This is an interesting difference, since it suggests that fertility and reproductive timing are separable traits that help distinguish these two treatments. In addition, both treatments result in lifespan extension, however the effect of inhibiting mitochondrial respiration of an eating defective mutant *eat-2(ad1116)* is much greater than the sum of either effect. On its own this finding is difficult to interpret since neither treatment is necessarily produces the maximum possible effect, but it is nonetheless consistent a model suggesting that the two treatments have independent effects upon lifespan. Why then did *atp-3(RNAi)* not exhibit an additive effect? Two possibilities are plausible. The combined treatment may have gone too far in their reduction of available energy supplies. These animals were extremely small, sterile, and moved very little. Alternatively, *atp-3*, which is a complex V component, may act in a different way then the other complexes. Finally, Dillin et al observed that all-of-life treatment with *cco-1(RNAi)* was capable of extending lifespan. DR, on the other hand, can effectively extend lifespan and

reproduction when initiated in the adult, again consistent with the two treatments extending lifespan in distinct ways.

In low food environments, *C. elegans* often chooses to hatch offspring internally in hopes of providing enough resources for the offspring to become a long-lived dauer larva. It appears as if this decision is regulated by sensory neurons and *daf-2*, since loss-of-function mutations for either result in elevated bagging rates in low food environments, whereas an eating defective mutant had a wild type bagging rate. The acceleration of the bagging response to low food is also true of a mutant in the insulin/IGF-1 signaling pathway, *daf-2(e1370)*. Taken together these data suggest that adult animals have a dauer decision capacity akin to that observed in L1. While this may indeed be the case, the adult bagging decision does not require the presence of *daf-16*, and so this bagging appears to lie in a novel pathway.

Materials and Methods

Strains: The following strains were used in this study N2, *eat-2(ad1116)*, *daf-16(mu86)*, *tax-4(p678)*, *osm-5(p813)*, *daf-16(mu86); osm-5(p813)*, *daf-16(mgDf50); daf-2(m65)*, *daf-2(e1370)*,

Lifespan Assay: Dietary restriction assays were performed at 20° C as described by Klass (Klass, 1977). Briefly, OP50 bacteria were grown for approximately 8 hours in TB media, and then washed 3 times with S-Basal media. Using this bacterial stock and S-Basal, defined dilutions were created by direct assay of bacterial concentration using a Petroff-Hausser bacterial counting chamber (Electron Microscopy Sciences, Hatfield,

PA). Experiments were conducted in 6-well plates in which each well contained 1 ml of the defined bacterial solutions in S-basal. Lifespans were initiated by transferring fully-fed young-adult animals from plates to the liquid media at a sample density of five worms per well. They were then transferred to fresh plates every other day until the completion of the reproductive period, and approximately every 4 days thereafter. Animals were scored as dead if they were unresponsive to a light touch; or were censored if they were contaminated, lost, exploded, or died due to internal hatching (bagging).

Progeny Production: Worms were treated as in the lifespan analysis, except that they were grown individually in six well plates. Each day for 10 days the worms were transferred to fresh wells and 2 days later the progeny were counted.

Ablations were performed as described by Kenyon et al. 1993.

RNAi Lifespan and Progeny Production. RNAi experiments were performed as described elsewhere (Dillin et al., 2002; Dillin et al., 2002; Fraser et al., 2000) except that they were conducted at 20° C. Progeny production assays were synchronized as young-adults.

Statistical Analysis: Data were assembled and analyzed with Stata Statistical Software: Release 8.2. (Stata Corporation, College Station, TX: StataCorp LP), using Cox Proportional Hazards.

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Figure 2-1

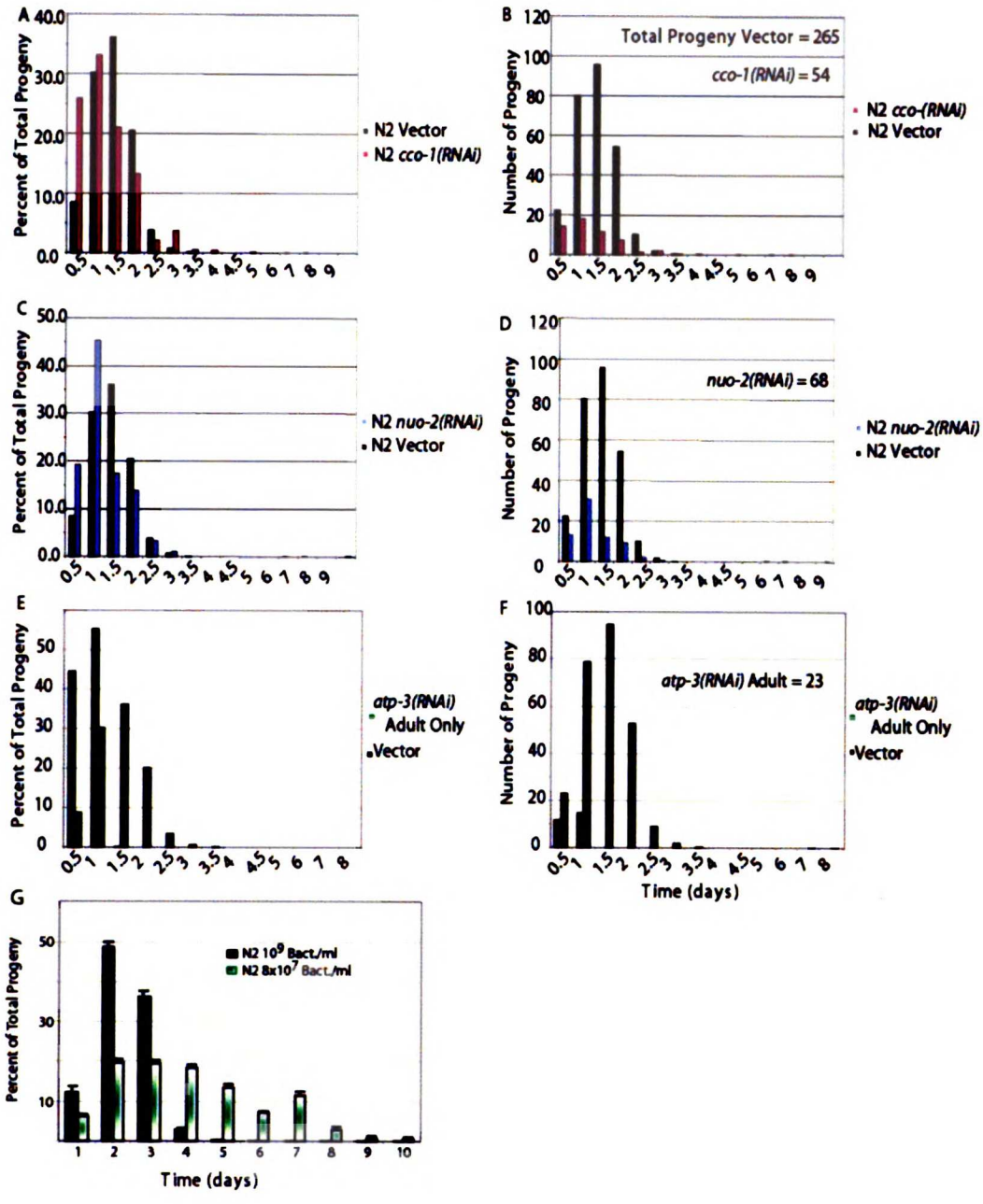

Figure 2 – 1. RNAi treatments that reduce mitochondrial respiration reduce fertility, but do not prolong the reproductive period. (A, C, & E) Percent of total progeny occurring in each time interval (each 12 hours in days 1 – 4 and each 24 hours thereafter). (B, D, & E) Number of progeny produced in each time interval. (A – B) *cco-1(RNAi)*; (C – D) *nuo-2(RNAi)*; (E – F) Animals grown bacteria containing vector-only were shifted to *atp-3(RNAi)* as young adults. (G) Animals grown at either 10^9 bacteria/ml (solid green bars) or 10^8 bacteria/ml (light green bars).

Figure 2-2

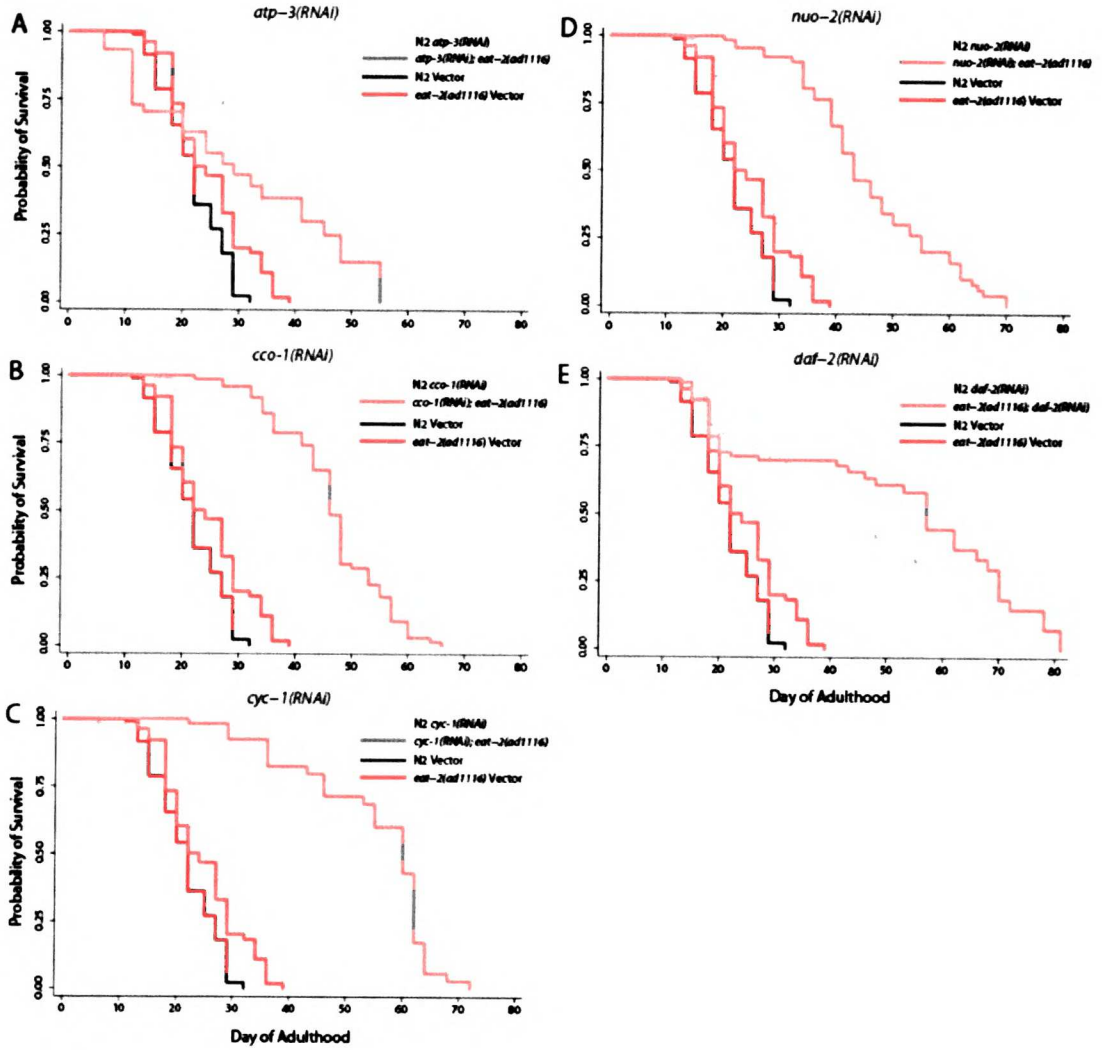

Figure 2 – 2. Survival curves for N2 and *eat-2(ad1116)* treated with RNAi that reduces mitochondrial respiration. Each graph shows the effect of a different RNAi clone that reduces mitochondrial respiration, as well as the same vector-only treated N2 (black) and *eat-2(ad1116)* (red) in each figure. Treatments are: (A) *atp-3(RNAi)* (B) *cco-1(RNAi)* (C) *cco-1(RNAi)* (D) *nuo-2(RNAi)* (E) *daf-2(RNAi).*

Table 2 – 1. Lifespan summary *eat-2(ad1116)* treated with RNAi clones that impair mitochondrial function.

Table 2-1

Genotype	RNAi Treatment	n	Dead	Mean Lifespan (sem (days))	p	% Vector	Censor	% Bag
N2	Vector	99	90	21.6 (0.56)			9	0
N2	*atp-3*	90	61	32.4 (0.93)	<0.0000	150%	29	0
N2	*cco-1*	99	75	31.0 (0.91)	<0.0000	144%	24	3%
N2	*cyc-1*	101	76	32.9 (0.91)	<0.0000	152%	25	4%
N2	*daf-2*	102	61	44.3 (2.46)	<0.0000	205%	41	12%
N2	*nuo-2*	100	69	26.6 (0.78)	<0.0000	123%	31	3%
eat-2(ad1116)	Vector	101	60	24.7 (0.87)	0.0011	114%	41	0%
eat-2(ad1116)	*atp-3*	90	32	30.2 (2.80)	0.0905	122%	58	0%
eat-2(ad1116)	*cco-1*	99	67	46.4 (1.11)	<0.0000	188%	32	0%
eat-2(ad1116)	*cyc-1*	100	37	54.4 (1.84)	<0.0000	221%	63	0%
eat-2(ad1116)	*daf-2*	99	43	50.6 (2.97)	<0.0000	205%	56	10%
eat-2(ad1116)	*nuo-2*	100	51	45.7 (1.66)	<0.0000	185%	49	0%

p values and percent of vector are for comparisons within strain except for *eat-2(ad1116)* vector, which is compared to the N2 grown on Vector-only bacteria. p values are derived from the logrank test.

Figure 2-3

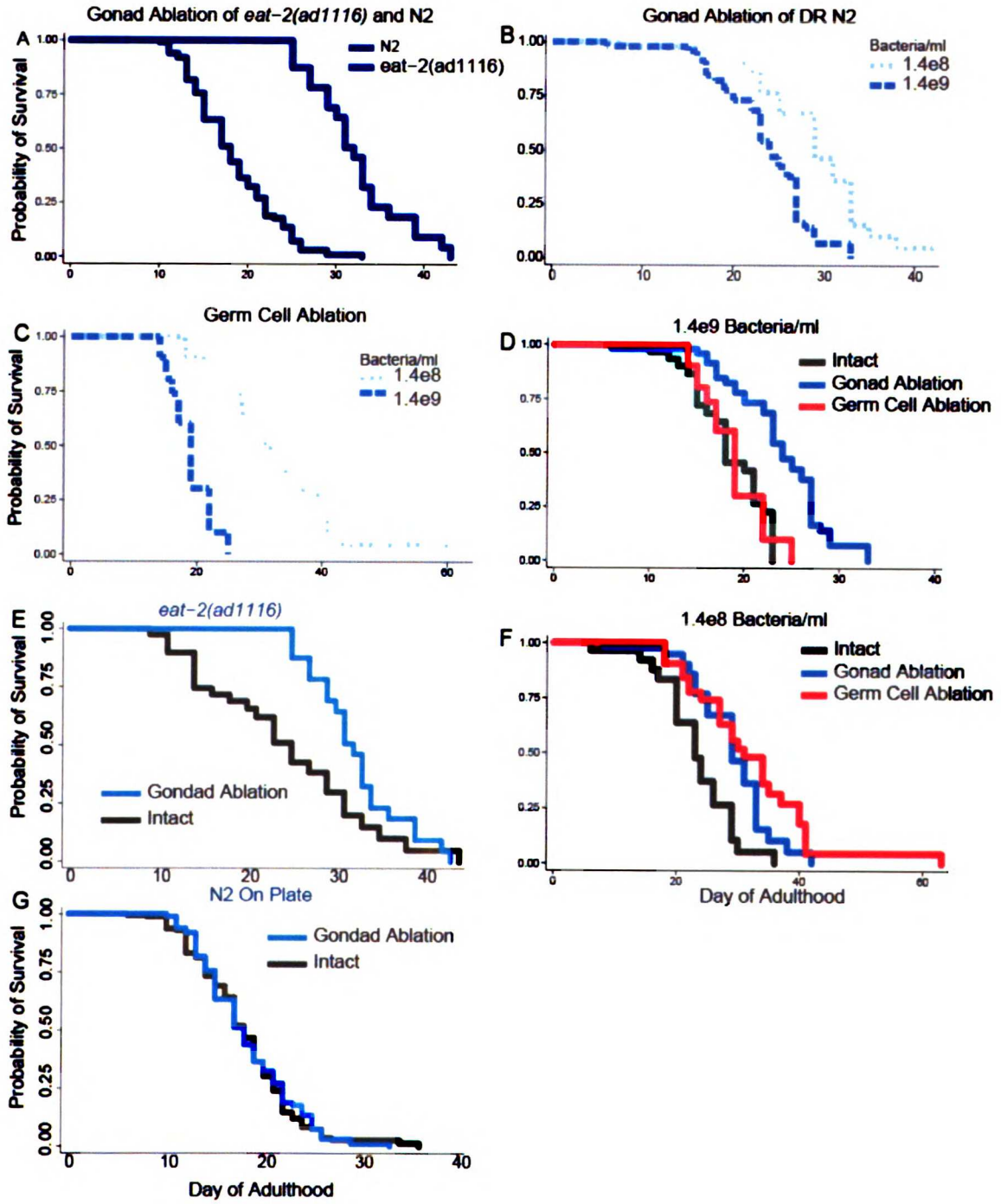

Figure 2 – 3. Reproductive signaling is not required for dietary restriction to extend lifespan. (A) Lifespans of gonad ablated N2 (navy) and eating defective *eat-2(ad1116)* grown on plates. (B) Gonad ablated animals grown in liquid in higher food concentrations (1.4e9, navy) versus those grown in low food concentration (1.4e8, light blue). (C) Germ cell ablation of N2 at 1.4e9 (dark blue) and 1.4e9 bacteria/ml (light blue). (D, E, and F) Gonad ablation (light blue) results in lifespan extension in each low food treatment when compared with intact animals (navy). (D) 1.4e9 bacteria/ml; (E) *eat-2(ad1116)* grown on plates; (F) 1.4e8 bacteria/ml. (G) N2 intact (navy) have the same lifespan as gonad ablated animals (light blue) when grown on plates.

Figure 2-4

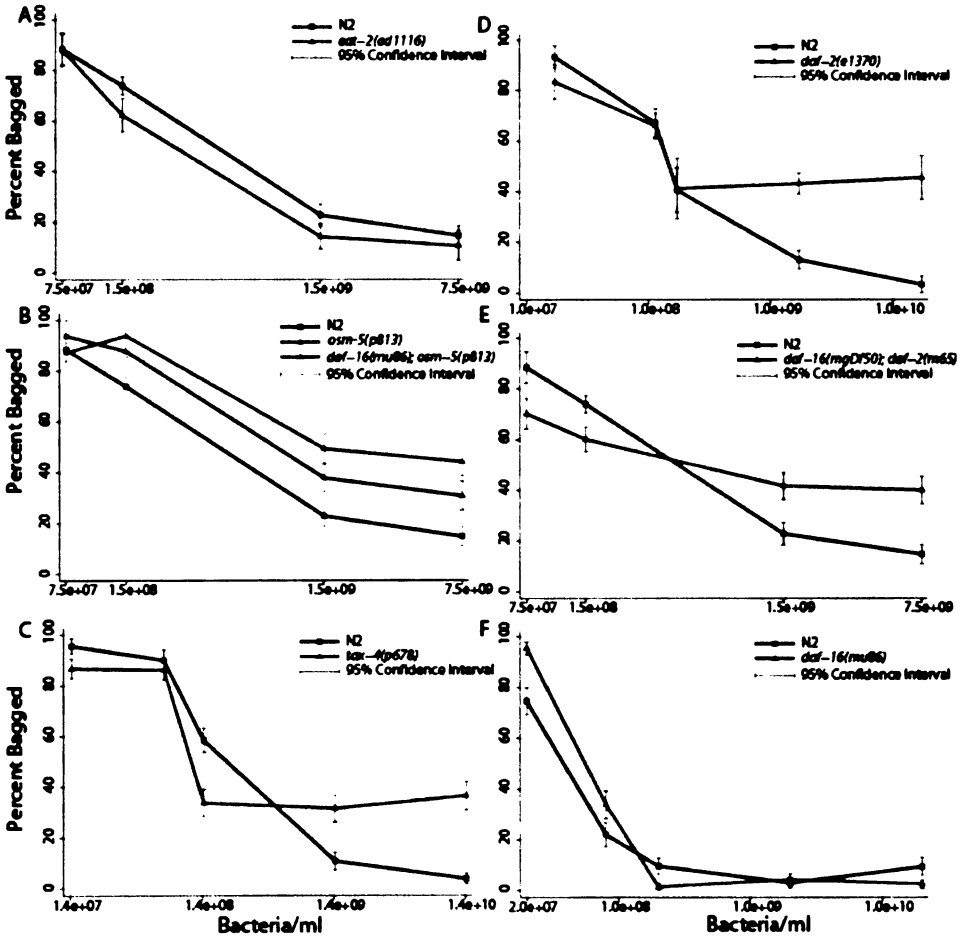

Figure 2 – 4. Low food and loss-of-function mutations of sensory neurons and *daf*-2 result in elevated bagging rates. Each figure contrasts the bagging rate of a mutant (red) with the simultaneous N2 control (dark blue). Figures A, B, and E employ the same N2 data. Animals were censored if they did not die or bag. Vertical bars represent the 95% confidence interval.

Table 2 – 2. Gonad ablation of calorically restricted worms.

Table 2-2

Strain	Bacteria/ml	Ablation	n	Dead	Total Censor	Mean LS (sem (days))	p	% Intact
Liquid DR:								
N2	1.4e8	Intact	29	20	9	23.3(1.3)		
	1.4e8	Z1/Z4	44	21	23	29.1(.13)	0.0025	125%
	2.8e8	Intact	30	27	3	21.7(1.0)		
	2.8e8	Z1/Z4	61	28	33	26.2(1.0)	0.0023	121%
	1.4e9	Intact	32	27	5	18.5(0.7)		
	1.4e9	Z1/Z4	50	43	7	23.7(0.8)	<0.0000	128%
On Plates:								
N2		Intact	200	137	59	18.1(0.43)		
		Z1/Z4	101	97	4	18.3(0.49)	0.8409	101%
eat-2(ad1116)		Intact	41	28	13	24.4(1.6)		
		Z1/Z4	28	22	6	32.3(1.1)	0.0114	132%

p values and percentages were calculated against the simultaneous intact animals of the same genotype or concentration. p values are for logrank test against intact animals receiving the same treatment.

Figure 2-5

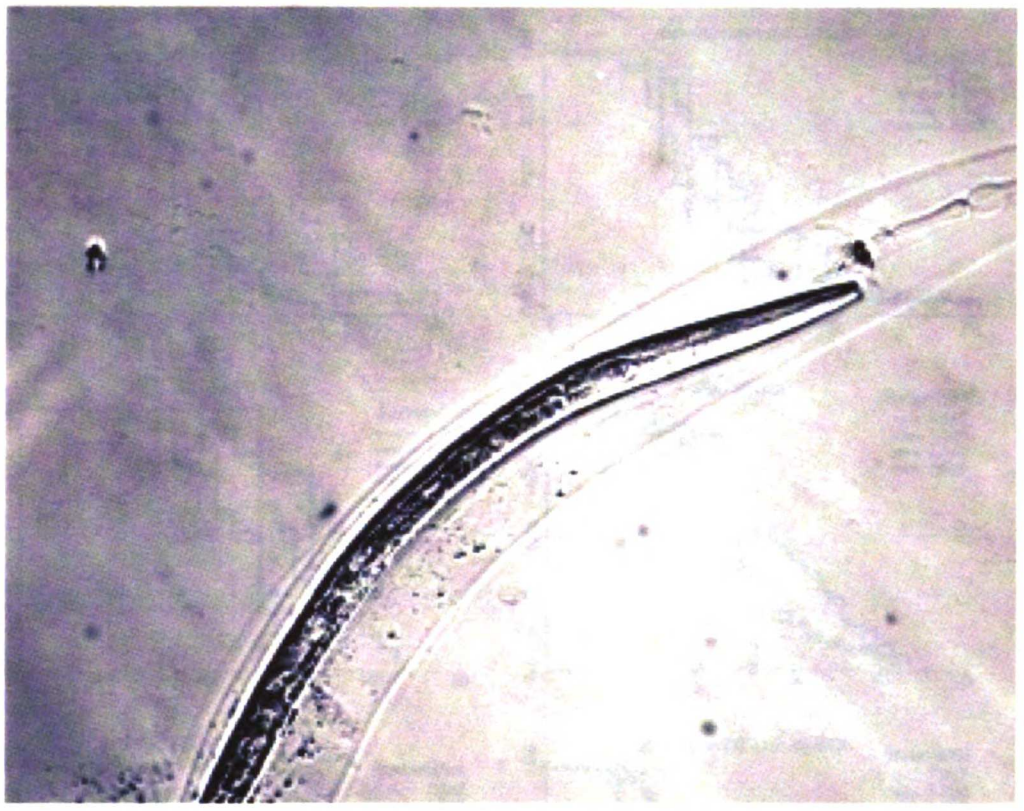

Figure 2 – 5. A dauer still trapped within its parent. The adult has provided sufficient food for its offspring to reach dauer when placed in media lacking food.

Figure 2-6

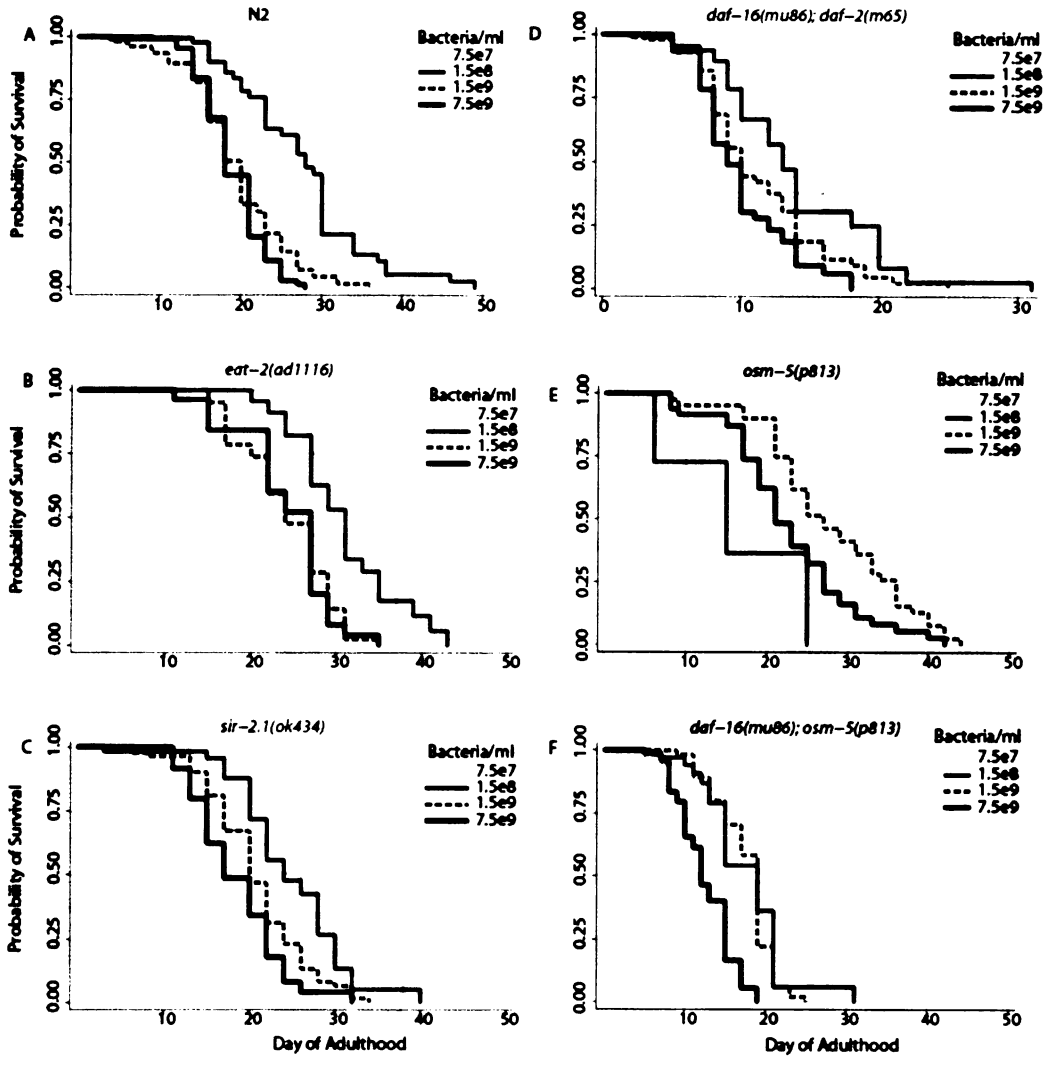

Figure 2 – 6. Dietary restriction of various lifespan regulatory mutants. Survival curves of animals shifted as young-adults to S-Basal with defined concentrations of bacteria. The darker the line the higher the bacterial concentration: black = 7.5 x 10^9, navy-dashed = 1.5 x 10^9, blue = 1.5 x 10^8, light blue = 7.5 x 10^7 bacteria/ml. (A) N2, (B) *eat-2(ad1116)*, (C) *sir-2(ok434)*, (D) *daf-16(mgDf50); daf-2(m65)*, (E) *osm-5(p813)*, (F) *daf-16(mu86); osm-5(p813)*.

Figure 2-7

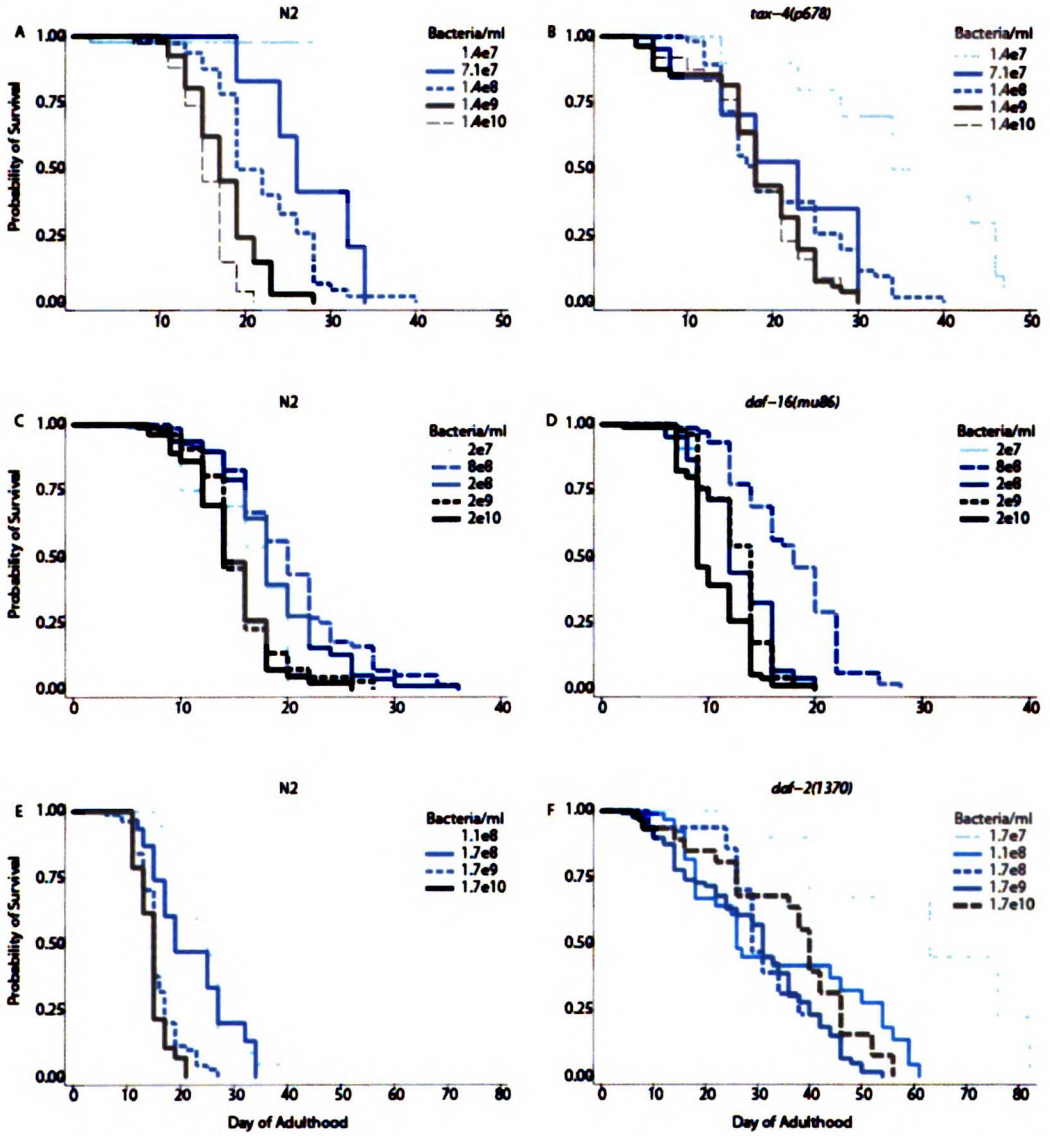

Figure 2 – 7. Dietary restriction of various lifespan regulatory mutants. Survival curves of animals shifted as young-adults to S-Basal with defined concentrations of bacteria. The darker the line the higher the bacterial concentration, with concentrations in figure legends. (A, C, & E) N2, (B) *tax-4(p678)*, (D) *daf-16(mu86)*, (F) *daf-2(e1370)*.

Chapter 3

Uncoupling Aging and Reproduction in Caenorhabditis elegans

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Abstract

The availability of long-lived mutants provides an opportunity to investigate the relationships between aging, reproduction, and specific gene activities. Many mutations affecting the insulin/IGF-1 pathway have been shown to extend both the lifespan and the reproductive period of *C. elegans*[1,2], and the same is true of low temperature[3] and food limitation. However, we find that mutations in many genes extend lifespan without substantially altering the timing of reproduction. Surprisingly, one long-lived mutant, *daf-10(m79),* has completely normal reproductive timing and fecundity. Because lifespan and reproductive behavior can be uncoupled, our findings suggest that the widely accepted evolutionary theory that lifespan extension entails a cost of reproduction[4,5] need not necessarily hold. Instead, aging and reproduction can be coupled because certain regulatory systems, such as the insulin/IGF-1 pathway, co-coordinately control both processes.

Results and Discussion:

To analyze the relationship between aging and reproduction, we first examined the effects of several different environmental conditions on both processes. We found that both food limitation and low temperature prolong lifespan and reduce the rate of reproduction (Figure 1)[3]. Animals grown at 20°C lived 37% longer than did animals raised at 25°C (Klass and Figure 1E and legend). In addition, the lower temperature substantially prolonged the reproductive period, and enhanced fecundity (Figure 1A; Table 1). Thus, temperature co-coordinately influences reproduction and lifespan

Rodents subjected to caloric restriction produce fewer progeny than animals fed ad libitum, and these progeny are produced over a longer time period[6]. Limiting food availability also extends lifespan and reduces brood size (Figure 1F; Table 1)[3]. We found that food limitation profoundly alters the reproductive profile, reducing fecundity and extending the reproductive period (Table 1; Figure 1B). Thus again, we observed a correlation between lifespan and reproductive timing. When we transferred relatively old animals from media containing low levels of food to media containing high levels of food, the worms produced a burst of progeny (Figure 1D). At this age, well-fed control animals were post-reproductive (Figure 1B). Thus in *C. elegans,* as in mammals[7], reproduction can be postponed when food is scarce until conditions improve.

Lifespan is shortened by agents that cause oxidative damage, such as paraquat[8,9]. Because this agent damages cellular components, we expected that it might decrease, and/or delay reproduction. Surprisingly, we found that animals treated with low doses of paraquat (0.2 M for one hour) had a burst of progeny production before exhibiting the

expected reproductive decrease and delay. In the first twelve hours of the reproductive period following this acute exposure to paraquat, the animals produced approximately 50% more progeny than normal (48.6 ± 2.7 s.e.m. (n = 8); control 32.1 ± 1.9 (n = 20); P <0.0001). Thus, this transient acceleration of reproduction appears to be an acute response to oxidative shock. Because it requires substantial, complex biosynthesis, accelerated reproduction seems unlikely to be a passive consequence of oxidative damage. Instead, the animals appear to have a regulatory system that interprets oxidative stress as a signal to accelerate reproduction. In principle, this system could have survival value by ensuring that animals in danger of dying from oxidative stress are able to reproduce before it is too late. In addition, this response to oxidative stress could be the mechanism by which the restoration of food following dietary restriction accelerates reproduction. Because food increases metabolic flux, it might produce a burst of reactive oxygen species.

Like temperature and food availability, the *C. elegans* insulin/IGF-1 pathway is known to regulate both lifespan and reproduction[1,2,10]. Mutations in the insulin/IGF-1-like receptor *daf-2* extend adult lifespan dramatically, and some of these mutations allow the animals to produce an occasional offspring very late in life[1,2]. We asked whether the reproductive profiles of these mutants earlier in life might resemble that caused by food limitation. We found that *daf-2(e1370)* mutants, which can produce progeny at very old ages, also have a protracted reproductive period as young adults, although the magnitude of the effect was smaller than that produced by food limitation (Figure 2a). The lifespan extension of *daf-2* mutants requires the transcription factor DAF-16[10-12], and we found

that the protracted reproductive schedule of *daf-2(e1370)* required DAF-16 activity as well (Figure 2a).

Not all *daf-2* mutations cause animals to produce progeny at very late ages[1,2]. We asked whether one such mutant, *daf-2(e1368),* would nevertheless exhibit protracted reproduction at earlier stages. We found that *daf-2(e1368)* animals had progeny production profiles similar to that of wild type, in spite of the fact that these animals had longer lifespans than those of food-limited animals (Figure 3a)[2]. This finding suggested that lifespan extension does not require changes in the timing of reproduction.

Overexpression of the *C. elegans sir-2.1* gene, which encodes a putative NAD-dependent histone deacetylase, also extends the lifespan of *C. elegans*[13,14]. *sir-2.1* is thought to function in the *daf-2* pathway to regulate lifespan because its lifespan extension is *daf-16* dependent and its overexpression appears to promote dauer formation via the *daf-2* pathway. We found that overexpression of *sir-2.1* had little or no effect on the timing of reproduction (Figure 3b). Thus, like *daf-2(e1368)*, the overexpression of *sir-2.1* uncoupled aging from reproductive timing.

Many mutations that inhibit sensory perception extend lifespan[15]. We asked whether three sensory mutations, the cilium structure mutations *daf-10*[16] and *osm-3*[17], and the cyclic-GMP-gated calcium channel mutation *tax-4*[18], changed the timing of reproduction. We found that they did not (Figure 3C). The progeny profiles of *osm-3* and *tax-4* mutants were normal in spite of the fact that they lived twice as long as wild type[15], and much longer than calorically-restricted animals (ref [3]; Figure 1). *daf-10* mutants not only had normal reproductive timing but also had wild-type fecundity (ref [15]; Table 1). This is the first time that such a mutation has been described, and it indicates

that neither the timing nor the magnitude of reproduction must change in long-lived animals.

Mutations in the gene *clk-1*, which is required for ubiquinone biosynthesis[19], extend lifespan approximately 40% and also slow the rates of development and behavior (refs[20,21] and data not shown). We found that *clk-1* mutants reached adulthood 1 day later than did wild type. However, their reproductive profiles were then identical to those of wild type (Figure 3D). Thus *clk-1* mutants, which live roughly as long as food-limited animals, do not exhibit the protracted period of reproduction characteristic of food limitation.

Finally, we also examined the reproductive schedules of *eat* mutants[22]. These mutants, which have defective pharyngeal contractions, ingest less food than wild-type animals. *eat* mutants have been postulated to live long because they are calorically restricted[23]; however, this interpretation is problematic because not all *eat* mutants are long lived and *eat* mutations could conceivably affect other processes in addition to food consumption. We found that *eat-2* mutants had protracted reproductive profiles similar to those of food-limited animals (Figure 2B). This finding supports the interpretation that *eat* mutants are calorically restricted. Surprisingly, we found that *eat-10(ad606),* which has a normal lifespan[23], also had a protracted reproductive profile. Thus protracted reproduction need not be associated with longevity, just as longevity need not be associated with protracted reproduction.

The *clk-1* pathway, as well as the *sir-2*/insulin-IGF-1 pathway, have both been postulated to mediate the response to caloric restriction in *C. elegans*[14,23,24]. However, our findings suggest that this is not the case for either pathway. Food limitation

dramatically extends the reproductive period, whereas neither *clk-1* mutations, nor *daf-2(e1368)* mutations, nor *sir-2* overexpression has this effect. These findings do not rule out the possibility that these pathways mediate the effects that caloric restriction has on lifespan but not reproduction. However, neither can be part of the mechanism that converts food limitation into an internal signal, such as a change in mitochondrial activity, a drop in insulin levels, or a rise in NAD levels, that initiates the overall response to food limitation. We decided to test the hypothesis that the *sir-2*/insulin/IGF-1 pathway mediates the response to dietary restriction in a different way as well. If food limitation extends lifespan by down-regulating an insulin-like *daf-2* ligand, and/or by activating SIR-2, then its ability to extend lifespan should require *daf-16* activity. However, we found that food limitation extended the lifespan and the reproductive period of *daf-16* null mutants (Figure 1c and g). Thus, the *sir-2/insulin/IGF-1* pathway cannot mediate the effects that food limitation has on either longevity or reproduction.

These findings are consistent with those of several previous studies. First, culturing nematodes in axenic media extends their lifespan in a *daf-16*-independent fashion, although this lifespan extension may be caused by a qualitative, rather than quantitative change in diet[25]. Second, *eat-2* mutations can extend the lifespans of *daf-16* mutants as well as wild-type animals[23].

Previously we showed that sensory mutations extend the lifespan of *C. elegans,* and that this lifespan extension depends, at least in part, on the insulin/IGF-1 pathway[15]. Thus it is possible that the sensation of food (or a correlate of food availability, such as crowding) rather than the consumption of food, may regulate the activity of the insulin/IGF-1 pathway in this organism.

In summary, in this study, we have shown that whereas some mutations that extend lifespan also delay reproduction, whereas others do not. In fact, one mutation does not seem to have any effect on reproduction at all. These findings are not predicted by the most widely accepted evolutionary theories of aging[5,26]. One theory, the disposable-soma theory, is based on the idea that in order for an animal to have a longer lifespan, it must devote an increased portion of its resources to cellular maintenance and repair. The theory proposes that these resources will be diverted from reproduction; thus longevity will be accompanied by a reproductive trade-off, also known as a cost of reproduction. A related theory, known as antagonistic pleiotropy, states that animals age because selection has favored genes activities that increase reproductive fitness early in life, and at least some of these same activities have detrimental effects later, when the animals are post-reproductive. This theory also predicts that perturbations that extend lifespan will do so at the expense of reproduction.

In this study, we have shown that many mutations, including mutations affecting insulin/IGF-1 signaling, sensory perception, and mitochondrial activity extend lifespan without changing the profile of progeny production. Thus, the association between the timing of reproduction and aging in response to temperature and food availability does not reflect a mandatory coupling of these two processes. In addition, our surprising discovery that the *daf-10(m79)* mutation can extend lifespan without a trade-off in either the timing or the extent of reproduction cannot be reconciled with evolutionary theory. The existence of this mutant indicates that the resources necessary to maintain the integrity of long-lived animals need not come at the expense of reproduction, as would be predicted by the disposable-soma theory. In addition, *daf-10* cannot be a gene that

promotes fertility at the expense of lifespan, as would be predicted by the antagonistic-pleiotropy theory. In addition, it is unlikely that *C. elegans* contains other such genes that act over this period to shorten lifespan; if there were such genes, then *daf-10* mutants would not be able to have such long lifespans. Instead, the phenotype of *daf-10* mutants indicates that reproduction and longevity must be distinct molecular processes. The fact that these two processes do change together in response to certain perturbations suggests that they can be regulated coordinately.

Some *daf-2* mutations extend the reproductive period as well as lifespan, indicating that the insulin/IGF-1 pathway is one regulatory system that links aging with reproduction. Insulin/IGF-1-like pathways appear to regulate aging and reproduction in flies and yeast as well as worms[27]. Thus, it is possible that mutations affecting this system have allowed lifespan and reproduction to change coordinately during evolution. Interestingly, in the accompanying paper, Dillin *et al.* show that *daf-2* operates at different times to regulate reproduction and lifespan; such a system may allow considerable evolutionary flexibility.

The insulin/IGF-1 pathway is not the only pathway that may link aging and reproduction. Previously we showed that the germ-line regulates the lifespan of *C. elegans*, possibly by regulating the production of a steroid hormone[28]. It is possible that changes in the activity of this system have also allowed aging and reproduction to evolve coordinately.

It was significant that an *eat-10* mutant had protracted progeny production but not extended lifespans. In nature, this type of mutation would probably be under strong negative selection, because it reduces early fecundity. Nonetheless, *eat-10* indicates that

protracted reproduction is not a sufficient predictor of lifespan. In selective breeding experiments, enrichment for fruit flies that produce progeny late in life has yielded long-lived populations[29]. This finding has lent support to the evolutionary theories of aging that predict a trade-off between longevity and reproduction. However, our findings indicate that these selections could also have yielded animals that are not long-lived.

In a previous study, when animals carrying mutations in the *age-1* PI 3-kinase, which functions in the *C. elegans* insulin/IGF-1 pathway, were cultured together with wild type, the frequency of the mutation did not decline over time[30]. This finding also indicates that a trade-off between longevity and reproduction is not necessary[5]. When the mixed population was subjected to periodic starvation, the frequency of the mutant allele did decline. This has suggested that a cost of reproduction may not manifest itself in all environments, but may prevent the emergence of long-lived mutants in nature. However, we note that, in principle, an animal need only compete successfully in one stable ecological niche in order to be favored by natural selection. Thus for any given condition, one may be able to find mutations that provide lifespan extension with no cost of reproduction. Interestingly, *age-1* mutations are resistant to warm temperatures[31]. Wild-type animals cannot survive incubation at 35°C (95°F) for 10 hours, whereas *age-1* mutants are essentially unaffected. Thus, mutations that extend lifespan may well out-compete wild-type worms in certain environments.

Methods

Progeny production assay

All animals were raised at 20°C unless otherwise indicated, and were cultivated as described[32,33], except for food-limitation assays which were performed in liquid with defined *E. coli* OP50 concentrations as described[3]. For liquid assays, bacterial concentration was determined by counting using a Petroff-Hauser counting chamber. Worms were placed as young adults on individual plates (or in 1 ml of media in 6-well plates for liquid assays) and transferred at the times indicated. The plates from which they were transferred were kept at 20°C for 2 days and then moved to 4°C until progeny were counted. At this point, the offspring were L4 larvae or young adults. The progeny data excludes offspring from animals that did not survive the fertility period (though we note that inclusion of these animals would not have changed our conclusions, see Table 1 legend). Individual-animal total fecundities were then employed to calculate percent of total progeny/time interval. Statistical analysis was performed employing Statview 5.01.

Lifespan assays

Lifespan assays were performed at 20°C as described[3,10]. Animals were censored if they crawled off the plates, exploded, or died because of internal hatching. Internal hatching occurred at a higher frequency in low concentrations of food, but at an equal frequency in N2 and *daf-16(mu86)* animals. The data was analyzed using Statview 5.01.

Paraquat dichloride assay

L4 larvae were placed in 1 ml of 0.2 M Paraquat dichloride (Sigma-Aldrich #M2254) for 1 hour and then transferred to an NG plate seeded with OP50 bacteria. After an additional hour, the worms were transferred to fresh plates to ensure that no residual Paraquat dichloride remained. Progeny production was then assayed as described above.

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Figure 3-1

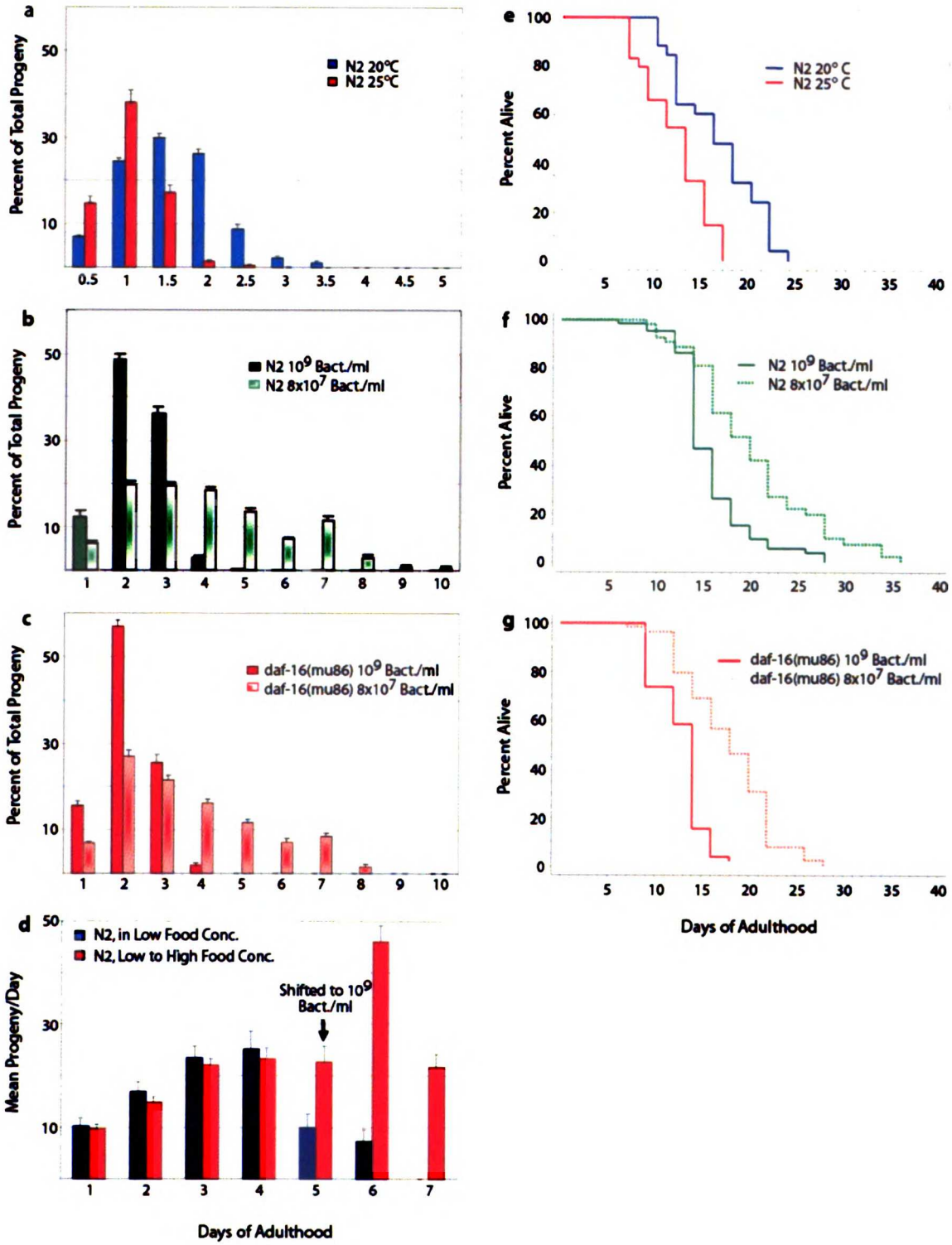
a
Percent of Total Progeny
N2 20°C
N2 25°C
b
N2 10^9 Bact./ml
N2 8x10^7 Bact./ml
c
daf-16(mu86) 10^9 Bact./ml
daf-16(mu86) 8x10^7 Bact./ml
d
Mean Progeny/Day
N2, in Low Food Conc.
N2, Low to High Food Conc.
Shifted to 10^9 Bact./ml
Days of Adulthood
e
Percent Alive
N2 20° C
N2 25° C
f
N2 10^9 Bact./ml
N2 8x10^7 Bact./ml
g
daf-16(mu86) 10^9 Bact./ml
daf-16(mu86) 8x10^7 Bact./ml
Days of Adulthood

Figure 3 - 1. Environmental changes can result in the coordinated extension of progeny production and lifespan. (A – C) Percent of total progeny produced in each time interval. (B) Mean number of progeny produced in time interval. (E – G) The percent of animals remaining alive is plotted against animals' adult age. (E) Mean lifespan (ML) in days for animals at 20°C is 16.4 (n = 25), and at 25°C is 12.0 (n = 28), p=0.0001. (F) ML for N2 in liquid culture with high concentration of bacteria is 15.8 (n = 54), and in low concentration is 20.2 (n = 43), p <0.0001. (G) ML for *daf-16(mu86)* in high concentration is12.8 (n = 53), and in low concentration is 17.9 (n = 40), p <0.0001.

Table 3-1

Treatment	STRAIN	Total Brood Size ± s.e.m.	95% Confidence Interval	P*	Number of animals surviving fertile period/total
Temperature:	N2 at 20°C	314 ± 6.5	300 – 327		30/32
	N2 at 25°C	157 ± 8.0	141 – 173	<0.0001	28/32
Sensory Mutants:	N2 Experiment 1	307 ± 9.9	287 – 328		23/27
	tax-4	272 ± 6.2	259 – 285	0.0040	24/27
	N2 Experiment 2	297 ± 5.5	285 – 309	0.3858‡	21/24
	osm-3(p802)	258 ± 5.7	246 – 270	<0.0001	16/24
	daf-10(m79)	301 ± 3.8	293 – 309	0.5740§	17/24
Insulin/IGF-1 Pathway:	N2	274 ± 11.1	250 – 298		21/23
	daf-2(e1368)	251 ± 10.6	228 – 273	0.1488	19/23
	daf-2(e1370)	229 ± 12.1	204 – 255	0.0123	15/24
	daf-16(mu86); daf-2(e1370)	270 ± 6.3	257 – 284	0.7790	16/22
	N2 + pRF4 [*rol-6(su1066)*]	296 ± 13.7	267 – 325		24/26
	geln3 [*rol-6(su1066), sir-2.1(gf)*)]†	238 ± 6.2	225 – 251	0.0004	23/26
	daf-16(mgDf50); geln3	297 ± 9.4	277 – 316	<0.0001	24/26
Food Limited:	N2 in 10^9 Bact/ml	276 ± 7.4	260 – 292		16/18
	N2 in 8 x 10^7 Bact/ml	236 ± 5.1	225 – 246	0.0019	17/18
	daf-16(mu86) in 10^9 Bact/ml	231 ± 6.4	217 – 245		15/18
	daf-16(mu86) in 8 x 10^7 Bact/ml	170 ± 13.9	140 – 200	<0.0001	16/18
***eat* Mutants:**	N2	298 ± 11.6	272 - 323		12/24
	eat-10(ad606)	198 ± 7.2	183 – 213	<0.0001	17/24
	eat-2(ad453)	221 ± 9.6	200 – 241	<0.0001	18/21
	eat-2(ad1116)	145 ± 5.1	135 – 156	<0.0001	21/24
	eat-18(ad820)	165 ± 6.7	151 - 179	<0.0001	22/24
***clk-1* Mutants:**	N2	293 ± 10.4	271 – 314		21/24
	clk-1(qm30)	181 ± 6.7	167 - 195	<0.0001	23/24
	clk-1(e2519)	201 ± 7.0	186 - 215	<0.0001	20/22

Table 3 – 1. Brood sizes of *daf-2, clk-1*, and sensory mutants; as well as calorically restricted animals.

* Compared with concurrent wild-type control except where noted below.

‡ N2 experiment 2 compared to N2 experiment 1.

§ Including censored animals (see methods) does not influence the conclusion that *daf-10(m79)* and N2 had equivalent fecundity ($P = 0.2383$). Four of the *daf-10(m79)* animals crawled off the plate, two died from internal hatching, and one was destroyed while transferring. Of the concurrent N2 animals, one died of internal hatching, one was infertile, and one plate became contaminated, causing these animals to be censored.

¶ Compared to the same strain at the higher concentration.

† In contrast to previous findings[14] we observed that *sir-2.1* overexpression slightly depressed fecundity in a *daf-16*-dependent manner ($p = 0.0004$).

Figure 3-2

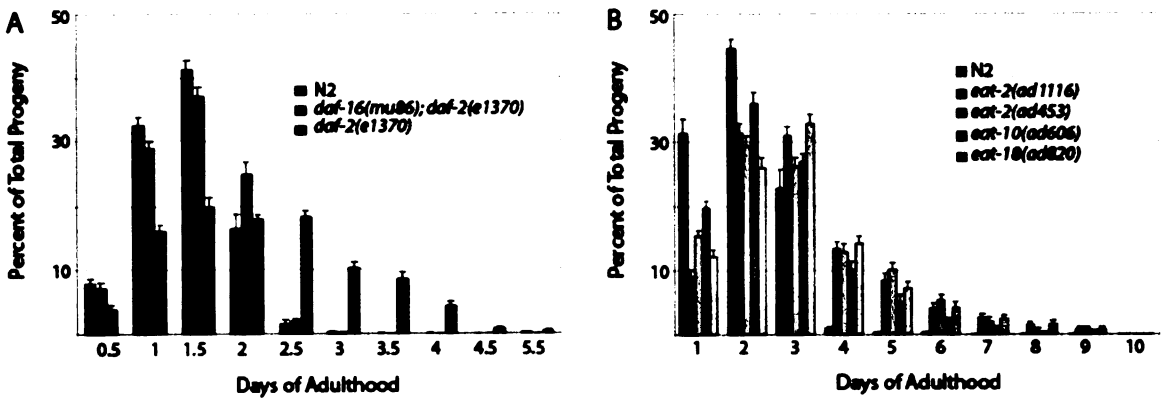
A
Percent of Total Progeny
50
30
10
N2
daf-16(mu86); daf-2(e1370)
daf-2(e1370)
0.5
1
1.5
2
2.5
3
3.5
4
4.5
5.5
Days of Adulthood
B
Percent of Total Progeny
50
30
10
N2
eat-2(ad1116)
eat-2(ad453)
eat-10(ad606)
eat-18(ad820)
1
2
3
4
5
6
7
8
9
10
Days of Adulthood

Figure 3 - 2. Some mutations can result in protracted progeny production. Percent of total progeny is plotted against days of adulthood. *daf-2(e1370)* takes 24 hours longer to reach adulthood[2,10].

Figure 3-3

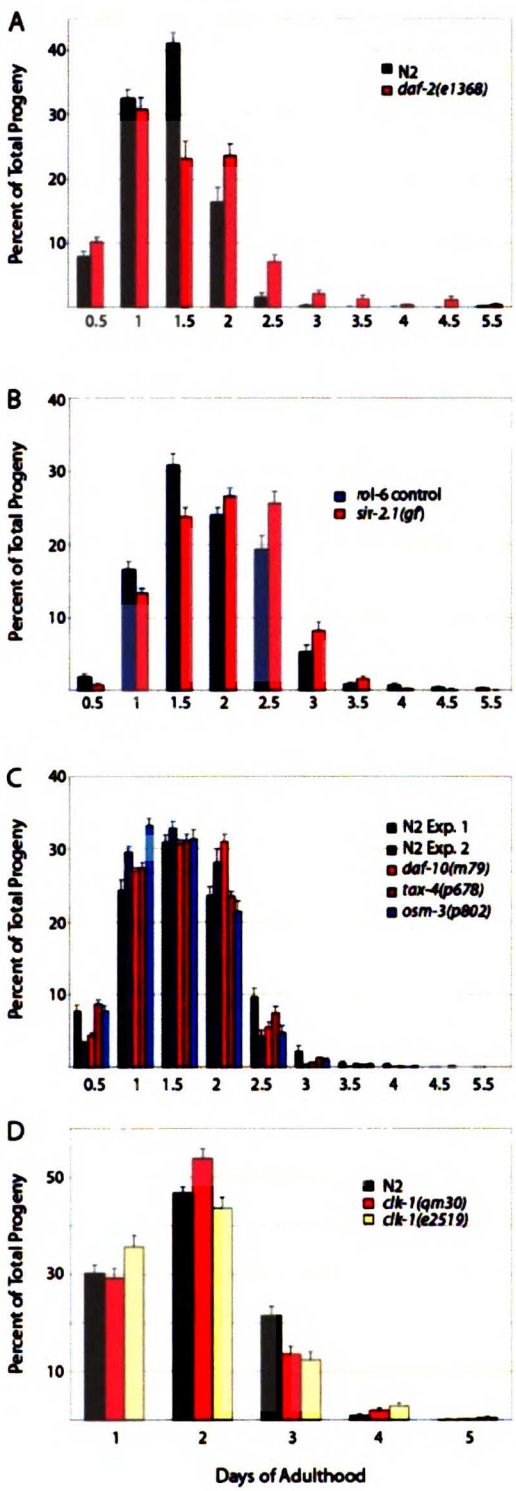
A
Percent of Total Progeny
40
30
20
10
N2
daf-2(e1368)
0.5 1 1.5 2 2.5 3 3.5 4 4.5 5.5
B
Percent of Total Progeny
40
30
20
10
rol-6 control
sir-2.1(gf)
0.5 1 1.5 2 2.5 3 3.5 4 4.5 5.5
C
Percent of Total Progeny
40
30
20
10
N2 Exp. 1
N2 Exp. 2
daf-10(m79)
tax-4(p678)
osm-3(p802)
0.5 1 1.5 2 2.5 3 3.5 4 4.5 5.5
D
Percent of Total Progeny
50
30
10
N2
clk-1(qm30)
clk-1(e2519)
1 2 3 4 5
Days of Adulthood

Figure 3 - 3. Long-lived mutants do not necessarily have protracted progeny production. Percent of total progeny is plotted against days of adulthood. b, A strain carrying an integrated transgenic array (geIn3) that carries additional copies of sir-2.1 as well as the co-injection marker rol-6(1066), was compared to the control strain carrying the rol-6(su1066) co-injection marker alone (N2 + pRF4)14.

Chapter 4 Temporal Regulation of Insulin/IGF-1 Signaling in C. elegans

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Credits: Andrew Dillin and Cynthia Kenyon conceived of the idea of using RNAi to explore the temporal regulation of the insulin/IGF-1 pathway in *C. elegans*. The lion's share of the work described in this paper was performed by Andrew Dillin. My contribution to this project was to extend this thinking to include the reproductive activity of the worms treated with *daf-2(RNAi)*, and to offer my assistance in performing the timing of reproduction assays as well as many of the lifespan assays as well. This work revealed that reproductive performance is indeed susceptible to *daf-2(RNAi)* in a manner similar to strong *daf-2* mutations, but surprisingly, this regulatory activity appears during larval development.

Abstract

Insulin/IGF-1 signaling influences longevity, reproduction, and diapause in many organisms. Because of the fundamental importance of this system in animal physiology, we asked when during the animal's life it is required to regulate these different processes. We find that in C. elegans, the pathway acts during adulthood, to relatively advanced ages, to influence aging. In contrast, it acts during development to regulate diapause. In addition, the pathway controls longevity and reproduction independently of one another. Together our findings show that lifespan regulation can be dissociated temporally from phenotypes that might seem to decrease the quality of life.

Results and Discussion

In *C. elegans,* mutations that decrease the activity of DAF-2, an insulin/IGF-1-like receptor, or downstream PI-3 kinase/PDK-1/AKT signaling components, prolong youthfulness and double the lifespan of the animal. The DAF-2 pathway influences other processes as well. All *daf-2* mutations examined increase resistance to oxidative stress and delay reproduction (some alleles also reduce fertility). Strong *daf-2* mutations cause juvenile animals to enter a state of diapause, called dauer, instead of growing to adulthood. All of these mutant phenotypes require the activity of DAF-16, a forkhead family transcription factor (*1-3*).

Mutations in components of the insulin/IGF-1 pathway also extend lifespan in flies (*4, 5*), and mutations that inhibit pituitary development or growth hormone receptor signaling, which in turn decrease IGF-1 signaling, extend the lifespan of mice (*6*).

Components of insulin/IGF-1 signaling pathways also influence reproduction, stress resistance and entry into diapause-like states in a wide range of organisms (*1-3*).

To investigate when the *C. elegans* insulin/IGF-1 pathway acts to regulate longevity, diapause, reproduction and stress resistance, we used RNAi (RNA interference), which decreases mRNA levels (*7*), to decrease *daf-2* and *daf-16* activity at different times during the life cycle. RT-PCR analysis confirmed that feeding animals bacteria expressing *daf-2* or *daf-16* dsRNA decreased mRNA levels (Fig. 1) (*8*). We found that culturing wild-type animals on bacteria expressing *daf-2* dsRNA from the time of hatching produced Daf-2(-) phenotypes similar to those produced by partial loss-of-function *daf-2* mutations (*9-12*). At 20° the animals grew to adulthood and became long-lived, stress-resistant adults with normal brood sizes but protracted reproductive schedules (Fig. 2A; Tables 1 and 2) (*8*). High temperature is known to induce some dauer formation in wild type (*13*). Our *daf-2* RNAi treatment increased the frequency of dauer formation at high temperatures [27°C, 45% dauers (n = 72) vs. 10% dauers (n = 81) for control animals] (*8*). Strong *daf-2* mutations delay growth to adulthood, and cause uncoordinated movement and some embryonic lethality (*9, 14, 15*). We did not observe these phenotypes (not shown), suggesting that they result from greater reduction of *daf-2* activity. Conversely, feeding *daf-2(1370)* mutants bacteria expressing *daf-16* dsRNA completely suppressed their delayed development (*16*), longevity, and reproductive phenotypes (Fig. 3A). This *daf-2* mutation is temperature sensitive. At 25.5°, the animals become dauers; this phenotype was partially suppressed by *daf-16* RNAi (*17*).

To ask when *daf-2* influences adult longevity, we shifted wild-type animals to bacteria expressing *daf-2* dsRNA at different ages and determined their

lifespans. We found that initiating *daf-2* RNAi treatment in young adults extended lifespan to the same extent as did initiating RNAi at hatching (Fig. 2A vs. 2G, P=0.38, Table 1). Thus, *daf-2* is required during adulthood to regulate adult lifespan.

To determine whether *daf-2* could also function during development to influence adult lifespan, we lowered insulin/IGF-1 signaling specifically during development. First, we initiated *daf-2* RNAi during development and then attempted to turn off the RNAi process during adulthood (*8*). We reasoned that if animals were exposed to dsRNA of a gene required for RNAi to function, such as *dcr-1* (dicer) (*18*), then RNAi activity should remain low. To this end, we shifted animals exposed to *daf-2* RNAi at hatching onto bacteria expressing *dcr-1* dsRNA when they reached adulthood. These animals did not have long lifespans (Fig. 4, Table 1).

We also turned off the effects of a *daf-2* mutation at different stages by shifting *daf-2(e1370)* mutants to bacteria expressing *daf-16* dsRNA. We found that initiating *daf-16* RNAi treatment during young adulthood completely suppressed the long lifespans of *daf-2(e1370)* mutants (Fig. 3A vs. 3G, p=0.19, Table 1). Thus, the presence of DAF-16 activity during development was irrelevant. Therefore, the insulin/IGF-1 pathway appears to operate exclusively during adulthood to influence adult lifespan.

We found that initiating *daf-2* RNAi treatment later in adulthood, throughout the reproductive period, also extended lifespan (Fig. 2, G-J, and Table 1). The magnitude of this extension declined steadily, and became insignificant after about 6 days of adulthood (Fig. 2M, and Table 1), though there was a sharp decline in *daf-2* mRNA at this time (Fig. 1A). Treating *daf-2(e1370)* mutants with *daf-16* RNAi during the reproductive period also extended lifespan (Fig. 3, H-L, Table 1). Again, the magnitude of the effect

declined with age, although we continued to observe effects on lifespan until about day 15 of adulthood (Fig. 3, M-P, and Table 1). In these experiments, *daf-2* and *daf-16* appeared to function for different durations during adulthood; however, wild-type animals (used in the *daf-2* RNAi experiments) age more quickly than *daf-2* mutants (used in the *daf-16* RNAi experiments). In fact, as assayed by tissue morphology, day 6 wild-type animals are approximately the same age as day 15 *daf-2(e1370)* mutants (*19*). Thus, both genes may function at the same time, throughout the reproductive period, to influence aging. We note that the pathway could conceivably function later as well (if, for example, the rate of protein turnover falls in old animals).

We next investigated when *daf-2* and *daf-16* RNAi could affect the timing of reproduction. Surprisingly, treating adults with *daf-2* RNAi had no effect on reproduction (Fig. 2, F-J, and Table 1). Likewise, treating *daf-2* mutants with *daf-16* RNAi during adulthood failed to suppress the *daf-2* reproductive phenotype (Fig. 3, G-L, and Table 1). In contrast, initiating either RNAi treatment at hatching did affect the timing of reproduction (Fig. 2A and Fig. 3A). Thus, *daf-2* appears to control reproduction and longevity independently from one another.

To determine when *daf-2* and *daf-16* might function in reproduction, we subjected the animals to RNAi during development. We found that initiating *daf-2* RNAi treatment of wild-type animals at any time before the last larval stage, L4, delayed reproduction (Fig. 2, A-D), and that initiating *daf-16* RNAi treatment of *daf-2(e1370)* mutants at any time before L4 restored normal reproductive timing (Fig. 3, A-E). Treatment during or after L4 did not affect reproduction (Fig. 2, E-J and Fig. 3, F-L). These findings show that reproductive timing can be specified independently of the dauer decision (which

occurs prior to L3) (*13*), and they suggest that the *daf-2* pathway may function late in development to affect the timing of reproduction. However, we cannot rule out the possibility that the DAF-2 pathway controls reproduction during adulthood, but that initiating RNAi during or after L4 does not lower signaling activity below threshold until after DAF-2 and DAF-16 have completed their roles in reproduction. This seems less likely, because message levels fall sharply within 48 hours of RNAi treatment (Fig. 1), whereas reproduction continues for many days.

The DAF-2 pathway also regulates stress resistance (*9-11, 20*). Because *daf-2* larvae (*21*), and dauers (*13*), are stress resistant, *daf-2* must regulate stress resistance in the larvae. We found that animals treated with *daf-2* dsRNA as adults were resistant to the oxidative-damaging agent paraquat (Table 2). Thus, *daf-2* also acts in the adult to regulate stress resistance. Since the DAF-2 pathway regulates lifespan during adulthood as well, this finding supports the hypothesis that increased resistance to oxidative stress contributes to longevity (*9-11, 20*). Consistent with this, overexpression of the antioxidant superoxide dismutase gene during adulthood can extend the lifespan of *Drosophila* (*22, 23*), as can administering the antioxidant Euk134 to adult worms (*24*).

In summary, in this study, we used RNAi to reduce *daf-2* and *daf-16* activity at specific times during the life cycle. As with any conditional expression system, we cannot be certain when any residual *daf-2* or *daf-16* activity, not removed by RNAi, might function. However, the fact that *daf-16* RNAi completely suppressed the strong longevity and reproductive phenotypes of the *daf-2(e1370)* mutant suggests that the level of residual pathway activity is likely to be minimal, as does the fact that inferences from reciprocal *daf-2* and *daf-16* RNAi experiments agreed.

Our findings suggest that the DAF-2 pathway functions exclusively during adulthood, throughout the reproductive period, to influence adult lifespan. This suggests that the pathway controls downstream gene expression in an ongoing fashion through much of adulthood. Because the dauer is a juvenile form (*25*), the DAF-2 pathway must act in separate regulatory events to control dauer formation and adult lifespan. Previously we proposed that *daf-2* regulates a longevity process that determines the lifespan of adults but that can also be expressed in conjunction with dauer-specific traits to give dauers their long lifespans (*12*). It will be interesting to learn whether the pathway regulates the same genes at two different life stages to influence the lifespans of adults and dauers.

Our findings indicate that the DAF-2 pathway participates in multiple, independent regulatory events to influence aging, reproduction, and diapause. In this regard, the pathway is similar to many growth factor signaling pathways (such as the EGF or TGF-beta pathways), which regulate different aspects of cell growth and differentiation independently of one another. The pleiotropy of insulin/IGF-1 pathway mutations in many organisms, particularly the linkage with diapause-like states, has raised the possibility that longevity achieved through this pathway would invariably be associated with impaired growth or reproduction. Instead, our findings suggest that, in other organisms as well, it may be possible to manipulate insulin/IGF-1 signaling during adulthood to extend youthfulness and lifespan without affecting either of these processes.

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Figure 4-1

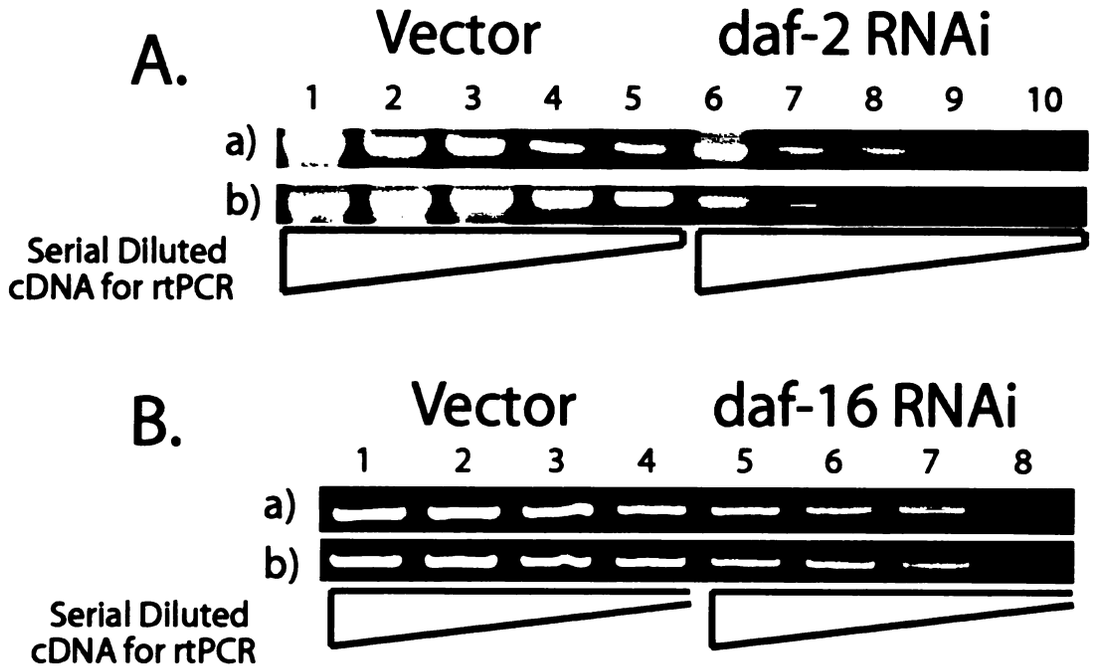
A.
Vector
daf-2 RNAi
1 2 3 4 5 6 7 8 9 10
a)
b)
Serial Diluted
cDNA for rtPCR
B.
Vector
daf-16 RNAi
1 2 3 4 5 6 7 8
a)
b)
Serial Diluted
cDNA for rtPCR

Figure 4 - 1. *daf-2* and *daf-16* bacterial RNAi lower mRNA levels. **(A)** RT-PCR analysis of *daf-2* mRNA following RNAi treatment. Shown are RT-PCR products from serial dilutions of total RNA isolated from control animals grown on bacteria containing vector only (lanes 1-5) or on bacteria expressing *daf-2* dsRNA (lanes 6-10). (a), RNAi was initiated at hatching, and RNA was harvested at L4. (b), RNAi was initiated on day 8 of adulthood and harvested on day 10 of adulthood. **(B)** RT-PCR analysis of *daf-16* mRNA following RNAi treatment. Conditions were the same as in (A), except animals were treated with bacteria expressing *daf-16* dsRNA.

Figure 4-2

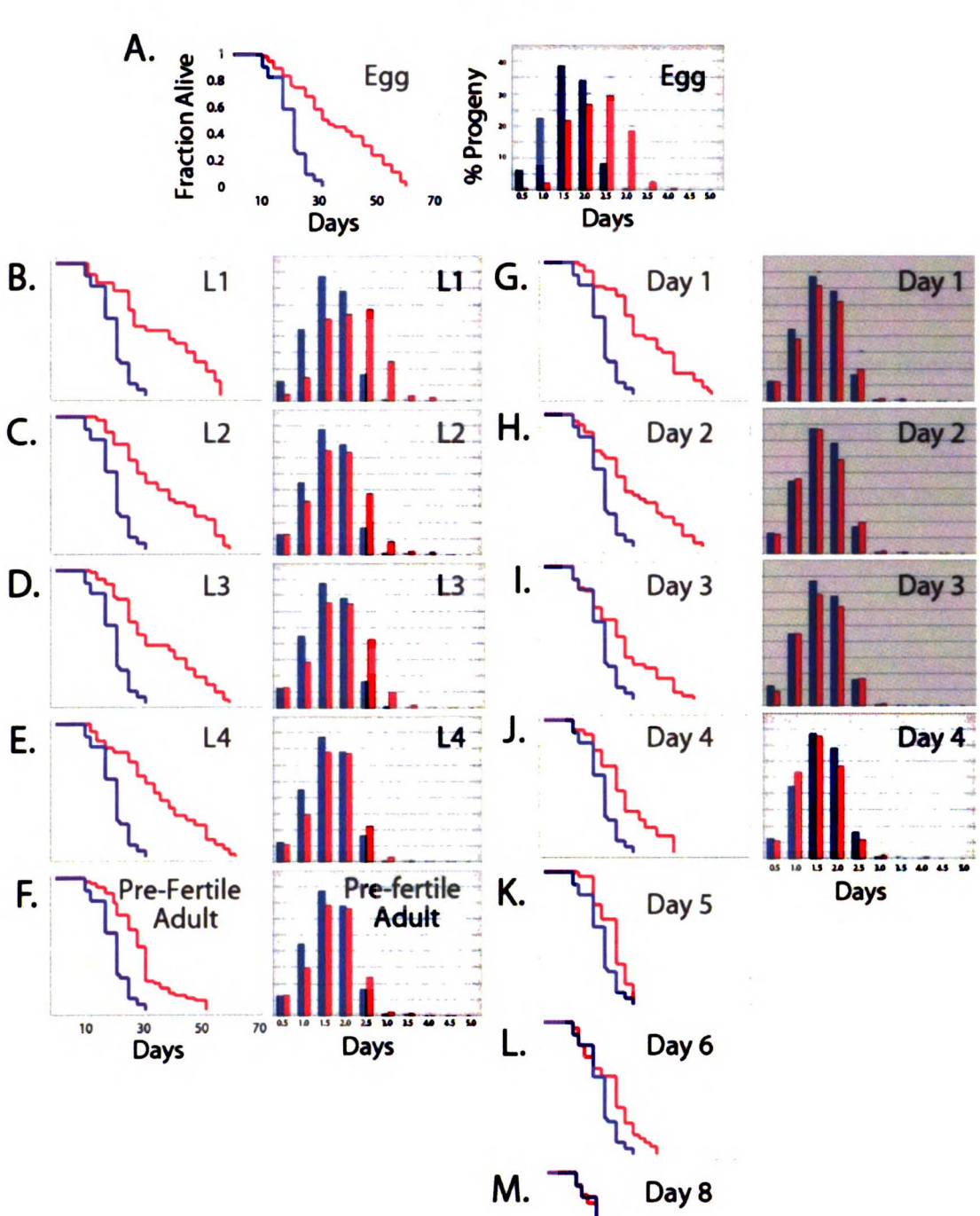
A.
Fraction Alive
Egg
% Progeny
Egg
Days
Days
B.
L1
L1
C.
L2
L2
D.
L3
L3
E.
L4
L4
F.
Pre-Fertile Adult
Pre-fertile Adult
Days
Days
G.
Day 1
Day 1
H.
Day 2
Day 2
I.
Day 3
Day 3
J.
Day 4
Day 4
Days
K.
Day 5
L.
Day 6
M.
Day 8
Days

Figure 4 - 2 . *daf-2* RNAi affects lifespan and reproduction at different times. The time at which animals were transferred onto bacteria expressing *daf-2* dsRNA is shown in the upper right corner of each panel. Blue lines represent the lifespans of animals grown on control bacteria carrying the RNAi vector alone, and red lines represent lifespans of animals grown on bacteria expressing *daf-2* dsRNA. Reproductive profiles of animals in the concurrent lifespan assays are depicted to the right of each lifespan profile. The percent of total progeny produced at each 12-hour time interval is shown. Scales are identical for all graphs. Reproductive profiles of animals treated with bacteria expressing *daf-2* dsRNA after day 4 of adulthood are not shown since these animals were post-reproductive. For statistics, see Table 1.

Figure 4-3

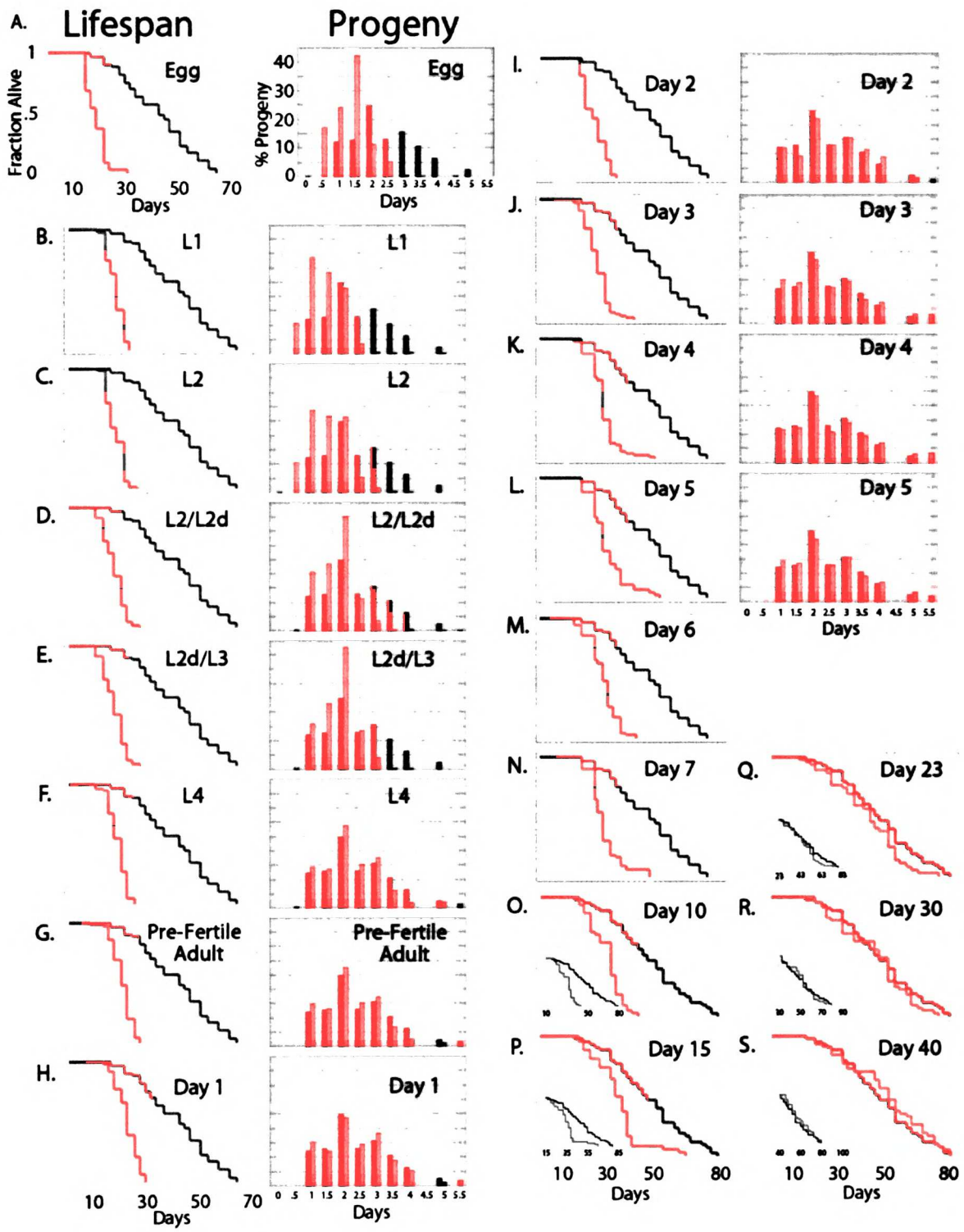
A.
Lifespan
Progeny
Egg
Fraction Alive
% Progeny
Days
B.
L1
C.
L2
D.
L2/L2d
E.
L2d/L3
F.
L4
G.
Pre-Fertile Adult
H.
Day 1
I.
Day 2
J.
Day 3
K.
Day 4
L.
Day 5
M.
Day 6
N.
Day 7
O.
Day 10
P.
Day 15
Q.
Day 23
R.
Day 30
S.
Day 40

Figure 4 - 3. *daf-16* RNAi affects the lifespan and reproduction of *daf-2(e1370)* mutants at different times during the life cycle. The time that *daf-2(e1370)* animals were transferred onto *daf-16* RNAi bacteria is shown in the upper right corner of each panel. Blue lines, lifespans of *daf-2(e1370)* animals grown on bacteria carrying the RNAi vector alone; red lines, lifespans of *daf-2(e1370)* animals grown on daf-16 RNAi bacteria. Note, scales of graphs A-N are identical and O-S are identical. Insets: T_0 is set to the day at which RNAi treatment was initiated in the experimental population. For statistics, see Table 1. Reproductive profiles of animals in the concurrent lifespan assays are depicted to the right of each lifespan profile. The percent of total progeny produced at each 12-hour time interval is shown. Blue, *daf-2(e1370)* mutant animals grown on control bacteria; red, *daf-2(e1370)* mutant animals grown on bacteria expressing daf-16 dsRNA. Reproductive profiles of animals cultured on *daf-16* RNAi bacteria after day 5 of adulthood are not shown since the animals were post-reproductive.

Figure 4-4

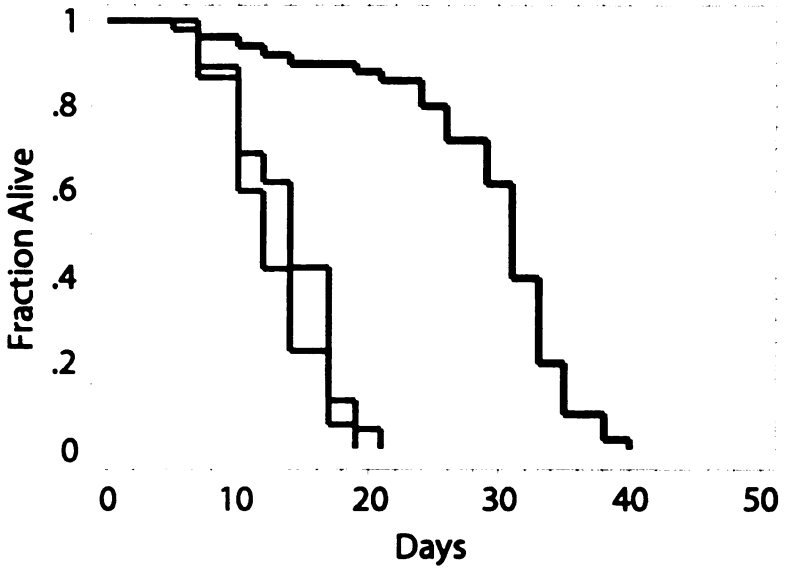

Figure 4 - 4. Loss of *daf-2* function during development does not increase lifespan. Wild-type animals were grown on bacteria expressing *daf-2* dsRNA from hatching until the first day of adulthood and then transferred to bacteria expressing dsRNA of *dcr-1*. Red line, lifespans of wild-type animals grown on *daf-2* RNAi bacteria during development and then shifted during day 1 of adulthood to bacteria expressing *dcr-1* RNAi bacteria. Blue line, lifespan of wild-type animals grown on the control RNAi bacteria during development and then shifted during day 1 of adulthood to *dcr-1* RNAi bacteria. Black line, lifespan of wild-type animals grown of *daf-2* RNAi bacteria during development and adulthood. Lifespans were conducted at 25°C. For statistics, see Table 1.

Table 4 - 1. Effects of *daf-2* RNAi and *daf-16* RNAi on lifespan and brood size.

Table 4-1

Treatment	Mean Lifespan ± s.e.m. (days)	*p*†	75th Percentile* (days)	Average Brood Size ± SD$^{\Delta}$	(Total #Animals Died/Total)§
N2 shifted to daf-2 RNAi as:					
Egg	35.5 ± 1.9	<0.0001 ‡	48	339 ± 41	61/81
L1	35.4 ± 2.6	<0.0001 ‡	52	354 ± 42	33/52
L2	36.2 ± 2.3	<0.0001 ‡	48	373 ± 43	41/48
L3	34.6 ± 1.9	<0.0001 ‡	48	380 ± 44	52/74
L4	35.3 ± 1.9	<0.0001 ‡	48	340 ± 47	56/71

Pre-Fertile Adult	29.1 ± 1.3	<0.0001 ‡	31	344 ± 46	56/71
Day 1 Adult	34.3 ± 2.0	<0.0001 ‡	45	386 ± 88	40/53
Day 2 Adult	29.9 ± 2.3	<0.0001 ‡	41	361 ± 53	36/37
Day 3 Adult	26.6 ± 1.8	0.0006 ‡	35	341 ± 50	42/52
Day 4 Adult	26.9 ± 1.8	0.0001 ‡	33	354 ± 53	33/39
Day 5 Adult	23.7 ± 1.0	0.0051 ‡	28	N.D.	27/35
Day 6 Adult	22.3 ± 1.4	0.0072 ‡	28	N.D.	35/44
Day 8 Adult	19.0 ± 0.8	0.5719 ‡	22	N.D.	49/53
Vector(control)	19.7 ± 0.8		22	371 ± 57	46/53
***daf-2(e1370)* shifted to *daf-16* RNAi as:**					
Egg (α)	17.9 ± 0.6	<0.0001 Ø	21	269 ± 39	35/39
Egg (β)	19.1 ± 0.8	<0.0001¥	25	N.D.	32/47
L1	17.8 ± 0.4	<0.0001 Ø 0.8509#	21	262 ± 37	74/86
L2	17.8 ± 0.4	<0.0001 Ø 0.7903#	21	295 ± 35	67/73
L2/L2d	17.1 ± 0.3	<0.0001 Ø 0.8995#	20	312 ± 42	67/72
L2d/L3	17.2 ± 0.5	<0.0001 Ø 0.5973#	20	315 ± 43	67/69
L4	18.6 ± 0.4	<0.0001 Ø 0.1954#	22	318 ± 41	67/80
Pre-Fertile Adult	20.3 ± 0.4	<0.0001 Ø 0.0006#	22	310 ± 29	71/73
Day 1 Adult	22.2 ± 0.5	<0.0001 Ø <0.0001#	25	278 ± 26	62/67
Day 2 Adult	20.7 ± 0.5	<0.0001 Ø 0.0005#	25	270 ± 23	73/76
Day 3 Adult	21.9 ± 0.5	<0.0001 Ø <0.0001#	25	253 ± 20	75/85
Day 4 Adult	24.3 ±	<0.0001 Ø	27	271 ± 20	76/83

	0.6	<0.0001[#]			
Day 5 Adult	25.6 ± 1.0	<0.0001[Ø] <0.0001[#]	29	255 ± 20	47/63
Day 6 Adult	24.2 ± 0.7	<0.0001[Ø] <0.0001[#]	28	N.D.	69/73
Day 7 Adult	24.5 ± 0.7	<0.0001[Ø] <0.0001[#]	27	N.D.	67/85
Day 10 Adult	28.9 ± 1.0	<0.0001¥ <0.0001[∝] <0.0001Ω	33	N.D.	44/51
Day 15 Adult	33.9 ± 1.6	<0.0001¥ <0.0001[∝] <0.0001Ω	38	N.D.	40/47
Day 23 Adult	41.5 ± 2.3	0.0498¥ <0.0001[∝] 0.0951Ω	51	N.D.	39/55
Day 30 Adult	44.6 ± 2.2	0.2344¥ <0.0001[∝] 0.4830Ω	54	N.D.	45/56
Day 40 Adult	49.6 ± 2.7	0.4305¥ <0.0001[∝] 0.3095Ω	56	N.D.	43/53
Vector (α)	41.2 ± 1.7	<0.0001[#]	50	263 ± 21	59/74
Vector (β)	47.3 ± 2.5	<0.0001[∝]	59	N.D.	43/57
Repression of *daf-2* RNAi by *dcr-1* dsRNA:					
N2 grown on RNAi bacteria during development, then shifted to *dcr-1* RNAi bacteria	12.5 ± 0.5	N.D.	14	N.D.	54/60
N2 grown on *daf-2* RNAi bacteria during development, then shifted to *dcr-1* RNAi bacteria	13.7 ± 0.6	0.0417[ββ]	17	N.D.	45/50
N2 grown on *daf-2* RNAi bacteria during development and adulthood	28.8 ± 1.1	<0.0001[ββ]	33	N.D.	50/50

* The 75[th] percentile is the age when the fraction of animals alive reaches 0.25.

† *P* values were calculated for individual experiments, each consisting of control and experimental animals examined at the same time.

§ The total number of observations equals the number of animals that died plus the number censored. Animals that crawled off the plate, exploded or bagged were censored at the time of the event. Control and experimental animals were cultured in parallel and transferred to fresh plates at the same time. The logrank (Mantel-Cox) test was for statistical analysis.

Δ Average brood size was calculated from the total brood size of at least 15 animals cultured independently in each trial.

‡ Compared with N2 worms grown on HT115 bacteria harboring the RNAi plasmid vector at 20°C.

Compared to *daf-2(e1370)* mutant worms cultured continuously on HT115 bacteria harboring the *daf-16* RNAi plasmid Egg (α), at 20°C, which were analyzed at the same time.

∝ Compared to *daf-2(e1370)* mutant worms cultured continuously on HT115 bacteria harboring the *daf-16* RNAi plasmid, Egg (β), at 20°C, which were analyzed at the same time.

Ø Compared to *daf-2(e1370)* mutant worms cultured continuously on HT115 bacteria harboring the RNAi plasmid, Vector (α), at 20°C, which were analyzed at the same time.

¥ Animals containing vector only (β), at 20°C, which were analyzed at the same time, were compared to *daf-2(e1370)* mutant worms cultured continuously on HT115 bacteria harboring the RNAi plasmid.

Ω *p* value after resetting T_0 of lifespans to time at which RNAi treatment was initiated. For instance; T_0 was set to 10 for assays in which the experimental population was treated with RNAi at day 10. Animals containing vector only (β), at 20°C, were compared to *daf-2(e1370)* mutant worms cultured continuously on HT115 bacteria harboring the RNAi plasmid.

æ Compared with N2 worms grown on HT115 bacteria harboring the RNAi plasmid vector during development, then shifted to bacteria expressing *dcr-1* dsRNA at 25°C.

Table 4 - 2. Effect of *daf-2* function on stress resistance.

Table 4-2

	% Alive in 0.4M paraquat after:					
	1 hour	2 hours	3 hours	4 hours	5 hours	6 hours
Control (RNAi vector bacteria)	*52	48	32	20	4	0
	#56	50	34	22	12	4
daf-2 RNAi initiated at hatching	*78	76	74	47	44	41
	#87	83	70	52	40	38
daf-2 RNAi initiated as day-1 adult	*94	94	72	56	53	50
	#90	85	70	58	44	34

The results of two independent trials are shown ($n \geq 25$ trial 1, $n \geq 40$ trial 2).
*Trial 1 is the first row of numbers.

Trail 2 is the second row of numbers.

RT-PCR analysis

Total RNA was extracted from approximately 20000 synchronized, sterile animals using trizol. To avoid mRNA contamination from eggs, which may not be susceptible to bacterial RNAi, we determined the efficacy of our RNAi treatments using a sterile strain, *fer-15(b26), fem-1(hc17)*. Before harvest, animals were exposed to bacteria containing the RNAi vector or containing the *daf-2* RNAi construct from the L1 until the L4 larval stage or from day 8 until day 10 of adulthood. 4 ug of total RNA was used for one round of reverse transcription (RT) using oligo dT primers. Serial dilutions of the RT reaction (1:1 – 1:24) was used for PCR reaction using daf-2 specific primers (5'-GGCACCGGTGCGGGAGCATTGAAACGAACAAAACACATC-3', 5'-TCCAGCACATTTTCATCACCTTATACC-3') to the 3' end of daf-2. RNAi was directed to a non-overlapping 5' end of daf-2. Serial dilutions of the RT reaction (1:1 – 1:20) was used for PCR reaction using daf-16 specific primers (5'-ATCTATGATGATCTAGAATTCCCATCATGGG -3', 5'-CAAATCAAAATGAATATGCTGCCCTCCAGC -3') to the 3' end of daf-16. RNAi was directed to a non-overlapping 5' end of daf-16. 4μl of a 50μl PCR reaction was analyzed on agarose gels using ethidium bromide.

Dauer assays

Wild-type hermaphrodites were allowed to lay eggs onto the control RNAi bacteria or *daf-2* RNAi bacteria at 20°C. The eggs were then shifted to 27°C and the presence of dauer larvae were scored 48 hours later when animals would normally be reproductive adults.

Lifespan, reproduction and stress assays

Lifespan, reproduction and stress assays were conducted at 20oC. We used the pre-fertile period of adulthood as the t=0 for lifespan analysis. Strains were grown at 20oC at for at least two generations before use in lifespan analysis. We used Statview 5.0.1 (SAS) software for statistical analysis and to determine means and percentiles. In all cases, P values were calculated using the logrank (Mantel-Cox) method.

The total number of progeny born to a single worm over time was measured in the following way. Briefly, worms hatched within a 1 hour period were collected and allowed to develop to the L4 stage. Once in the L4 stage, worms were individually placed onto separate plates. In all cases, at least 15 worms were used for each analysis. Worms were transferred to new plates every 12 hours and the resulting progeny were allowed to grow for two days until counted for progeny measurements. The % of total progeny was calculated for each time point by dividing the number of progeny produced on a time point by the total number of progeny produced over the course of the experiment.

For stress resistance assays, wild-type animals were transferred to bacteria expressing *daf-2* dsRNA at the indicated times. After reproduction had ceased, at day 5 of adulthood, worms were submerged in 50 μl of 0.4M paraquat dissolved in S-basal buffer at 20°C. Death was determined on an hourly basis by the lack of movement after prodding with a platinum wire. At least 40 worms were used for each analysis.

RNAi inhibition using dcr-1

To lower *daf-2* activity during the larval stages only, wild-type animals were grown on bacteria expressing *daf-2* dsRNA and then shifted to bacteria expressing dcr-1 dsRNA as day 1 adults. Control animals were grown during development on the RNAi bacteria containing the vector only and then shifted to dcr-1 RNAi bacteria as day 1 adults. Animals were grown at 25oC.

Chapter 5 Use of a Transcriptional Reporter of the Stress-Response Gene sod-3 in a High-Throughput Screen to Identify Lifespan Regulatory Genes

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Abstract

Lifespan has proven to be a very sensitive, but time-consuming phenotype to follow. Extent screens to identify novel genes that regulate lifespan have depended upon labor-intensive efforts. To their credit, each screen has identified useful alleles or RNAi clones, but their results have been far from exhaustive. For instance, a recent genome-wide RNAi screen for clones that increase maximal lifespan did not identify *age-1* though it did produce a significant extension when tested on its own. Furthermore, these screens have not been designed to be capable of finding clones that only benefit mean lifespan, or that have only a small effect. Designs capable of detecting such clones or mutants would be impossible using current methods. Here we describe a novel high-throughput approach that permitted a single person to assay 1.4 million worms in less than two months. This screen successfully identified six RNAi clones that are capable of increasing lifespan by 26 – 79%.

Introduction

One persistent challenge in studying the regulation of aging is the high cost of lifespan assays. Even in short lived organisms like yeast and *C. elegans* measuring lifespan tries the spirits of even the most dedicated researcher (much less the flighty author). *C. elegans* has a mean lifespan of approximately 17 days at 20° C. Determining the average lifespan of a particular allele or RNAi clone requires at lest 3 weeks in which one generally assays the status of every worm in the experiment every other day. An initial sample of approximately 100 animals is required to provide sufficient power and to accommodate censoring. This means that each experiment involves assaying several hundred to a couple of thousand worms every other day. There are two compensatory virtues for all this effort though: as we are interested in lifespan, assaying the phenotype of interest rather than a surrogate is more likely to yield reliable results; secondly, lifespan analysis has proven to be an exquisitely sensitive assay. These virtues have emboldened several members of our lab to attempt screens to identify genes that regulate lifespan.

This first and most ambitious of these efforts was a clonal EMS screen initiated by Javier Apfeld, Honor Hsin, and Bella Albinder, Jennie Dorman, and Bernadine Tsung (Apfeld, 1999). They were quite successful in isolating mutants that reliably exhibited an increase in lifespan, however the subsequent identification of loci involved proved quite difficult. The most interesting mutants were likely to be those that were not Daf-c, or indeed those not having any obvious secondary phenotype. However, to clone these

genes would require following the lifespan phenotype. Each cross would require performing a lifespan assay on numerous candidates to identify those still having the lifespan phenotype, a process that would take at least one month per cross. This makes the cloning process extraordinarily time consuming. To date only one of these mutants has made it to print – a novel allele of *daf-2* (*daf-2(mu150)*) (Garigan et al., 2002). Though I am sure that there are still valuable mutations in the freezer, it is hard to motivate anyone to continue cloning these candidates, particularly since the advent of large scale RNAi feeding screens (Fraser et al., 2000).

RNAi feeding screens have vastly simplified the challenge since this method obviates the requirement for cloning. Once you identify a phenotype of interest all you need to do is identify the library clone that produced it – and you are done (well not quite, but close). Recognizing the promise of this method immediately, Andrew Dillin and other members of the lab initiated an RNAi feeding screen for lifespan regulatory genes. They found that RNAi clones that reduce mitochondrial function produce size, clock (developmental and behavioral retardation), and lifespan phenotypes (Dillin et al., 2002).

Despite the improvements provided by RNAi, this screen still required lifespan assays. In an effort to overcome this limitation, Dillin et al. performed a maximum lifespan screen. In this protocol, a sterile strain was allowed to grow on the initial inoculate of RNAi expressing bacteria for 24 days at which point the plates were inspected; candidate clones were those on which an unusual number of animals were still alive. (They also inspected the plates on day 10 of adulthood to identify loci causing accelerated death, but no effort was made to assess the percent alive for those that were not mostly dead.) This protocol vastly diminishes the power available since it is survival

at a single point that is being tested, and that point is so late in the lifespan that there are likely to be very few animals remaining even if the clone is significant. Furthermore, this assay is prone to contamination since the assay occurs over such a long period. Finally, it would also be interesting to isolate clones that tended to increase mean, but not maximal lifespan.

With these thoughts in mind we have developed a high-throughput lifespan-surrogate screening protocol. The goal has been to use a biomarker associated with lifespan extension whose activity early in life tends to predict lifespan extension. The ideal indicator would provide an easily scored quantitative output. One could then select interesting RNAi treatments by their relative impact on the surrogate. Once such clones had been identified then one would be justified in performing a complete lifespan analysis. Such an approach may be able to find small effectors – the sort of genes that would be very difficult to find in a single point lifespan assay. We employed an *sod-3::GFP* transcriptional reporter constructed by Jennifer Berman (Libina et al., 2003) as our surrogate. Not only is this marker appropriate because of its role in stress response, but it has been shown that its transcription is regulated by the lifespan regulatory gene, *daf*-2 (Honda and Honda, 1999).

Finally, we automated the assay process by using Copas Biosort. The Biosort is capable of high-throughput automated analysis. It can sequentially draw the worms from each well of a 96 well plate and measures their length, light absorption (generally a proxy for area), and most importantly, their fluorescent emission. Thus in brief the protocol involved growing 40 - 60 worms from eggs in 96 well plates using liquid media containing bacteria expressing each clone from the Ahringer RNAi library (Fraser et al.,

2000). On day 2 of adulthood, they were assayed for growth and *sod-3::GFP* emission. This protocol enabled one person to test the activity of approximately 16,000 clones on over 1.4 M worms in less than 2 months.

Clones that significantly increased fluorescent emission, particularly those that permitted normal growth, were then retested (as significant rates of false positives were observed, particularly when sample sizes were small). 106 clones were selected based on their induction of *sod-3::GFP*, then complete lifespan assays were performed. Seven novel lifespan regulatory genes were identified.

Results and Discussion

Validation of the Protocol

Eggs place in 50 μl of MG/carbanycillin/IPTG media with HT115 carrying an empty vector bacteria (vector-only) and incubated at 25° C grow to adulthood and move vigorously, but are thin, clear, and generally have the appearance of calorically-restricted worms. Fertility was not measured as the strain employed (*rrf-3(pk1426)*) is virtually sterile at 25° C even in fully fed conditions (Simmer et al., 2002). Wells did occasionally contain eggs and L1's, but it was not clear whether these were P_0's that failed to reach maturity or F1's. Despite these marginally satisfactory growth conditions, worms grown on bacteria expressing *daf-2* dsRNA had significantly higher fluorescent emission levels than worms grown on either vector only (Figure 5 – 1 A, Table 5 - 1), as has been reported for worms grown on plates (Libina et al., 2003). Indeed the Biosort proved to be an extremely sensitive tool for detecting differences in GFP emissions. Worms grown from egg on *daf-2(RNAi)* are not visibly different from vector when examined with a Kramer dissecting scope until day 3 of adulthood, but the Biosort was able to distinguish *daf-2(RNAi)* treated worms as early as day 1 of adulthood. And by day three, the Biosort data confirmed the visual observations in that the fluorescent emissions were over 4 times greater than that of the vector-only control (Figure 5 – 1 A).

Similarly worms grown on random sample of 96 library clones (those contained in Plate 88 from the Ahringer library), when averaged together, had fluorescent emission values equivalent to, or slightly lower than, that of the vector-only control. Interestingly

though, the worms grown on RNAi Library clones grew faster and larger than those fed either vector-only or the *daf-2(RNAi)* (Figure 5 - 1 B and C). The vector only control has been used in our lab as the control bacteria for a number of RNAi constructs assembled by Andrew Dillin. While the bacterium and vector are meant to be the same as those in the Ahringer library, this finding suggests that a difference exists between the bacterial strain or the vector (Malene Hansen, personal communication).

These results have demonstrated that the Biosort is capable of discerning the difference in *sod-3::GFP* expression caused by growth on *daf-2* RNAi bacteria with greater sensitivity than is possible by dissecting scope microscopy. It should though be noted that much of this precision is the product of the large sample sizes facilitated by the machines high-throughput capacity. The 95% confidence intervals of Figure 5 – 1 (A) are quite narrow in part because each datum represents the average value of 2 - 3 thousand worms (Table 5 - 1). Such a large sample was not feasible for this screen. Under conditions that better reflect the screen, Figure 5 - 2 depicts the well to well variation for both fluorescence and length in the day 2 data displayed in Figure 5 – 1 (A). These values were derived from a single 96 well plate in which 48 wells contained bacteria carrying vector alone and 48 wells contained bacteria expressing *daf-2*-dsRNA. They were in every way treated identically. Yet despite the similarity in treatment mean fluorescent values per well varied from 31 to 91 and from 134 to 296 for vector-only and *daf-2(RNAi)* respectively.

The only significant variable distinguishing the various wells was the number of animals (from 22 to 80). This variation is not surprising given the casual method of distributing the eggs (see Methods). However, for the vector-only control, the number of

worms in the wells was not correlated with the resulting fluorescence ($r = 0.12$, $p = 0.43$ for vector-only). By contrast, *daf-2(RNAi)* treated worms showed a strong negative correlation between *sod-3::GFP* induction and the number of worms per well ($r = -0.6808$, $p<0.0000$). As worms are consuming the finite and unreplenished supply of food, then the number of worms per well may be a predictor of the degree of caloric restriction. If this is the case, then one would anticipate that another classic phenotype of caloric restriction, smaller size, would also be negatively correlated with the number of worms per well. This indeed is the case (Figure 5 – 3 A and B). Both vector-only and *daf-2(RNAi)* treated worms show a strong negative correlation between both length and extinction and the number of worms per well. Surprisingly though, only *daf-2(RNAi)* worms show a negative correlation between the number of worms and the *sod-3::GFP* emission. These results indicate that dietary restriction (DR) does not induce *sod-3::GFP* expression. Furthermore, this finding suggests that the slightly reduced lifespan benefit that *daf-2* mutations enjoy when calorically restricted (Chapter 2), may be due to the requirement of food for *daf-2* mutants to induce the loci that provide these mutants with lifespan extension. Perhaps then, the lack of these gene products would explain the apparent paradox that while *daf*-2 plays a role in the DR response, *daf-16* plays a lesser role – only in the presence of *daf-16* would these additional loci be expressed. A less interesting, but equally plausible possibility is that the *daf-2(RNAi)* effect is proportional to the concentration of the bacteria expressing the dsRNA. A repetition of this experiment using a *daf-2* mutant, rather than RNAi will be required to resolve this issue.

Another possible explanation for the large variation in average fluorescence between wells is capricious expression of the reporter. Indeed, when examining worms

grown of plates with vector-only bacteria, one can easily find worms strongly inducing *sod-3::GFP* (perhaps as many as 1%). Comparing the fluorescence per worm of the day-2 vector-only treatment, and limiting the consideration to only those worms have similar populations per well (48 – 68) and lengths (167 – 207), one finds a huge range in fluorescence (6 – 225, Figure 5 - 2 B). Thus, the estimates of induction resulting from a particular treatment are likely to be accurate in direct proportion to their sample size, suggesting again that high rates of errors are likely in a screen limited to samples of 40 – 60 worms. It would be interesting to compare the variation in expression for a variety of different reporters.

These control experiments have demonstrated the sensitivity of the Copas Biosort to consistently distinguish the elevated *sod-3::GFP* expression generated by *daf-2(RNAi)* treatment. The speed with which a screen of this sort can be executed encouraged me to proceed despite the likely high error rates that small sample sizes are likely to produce. The hope is that threw extensive retesting, interesting candidates will emerge.

Screen results

In 51 days of screening 1.4 M worms were assayed, from 16,125 independent clones from the Ahringer library (Fraser et al., 2000). The results of this screen are summarized in Figure 5 - 4. A number of clones had equivalent or superior capacity to induce *sod-3::GFP*, as the *daf-2(RNAi)* (Figure 5 - 4). As predicted many of the clones that exhibited large increases in *sod-3::GFP* induction during the screen had only normal fluorescent levels when retested.

116 of the clones that positively retested for induction of *sod-3::GFP* were then assayed for lifespan phenotypes. These were selected based on their strength of induction of *sod-3::GFP*. As a result, many of the strongest inducers have yet to have their lifespan assayed. Three clones (107B12, 136E10, and C34F11.5 (when tested after growing the worms on the dsRNA expressing bacteria for two generations)) resulted in larval arrest when animals were grown from eggs on plates. In liquid culture, many of these animals had been able to reach sizes expected only of adults. This difference in growth suggests that, for at least these clones, the liquid regimen had lower expressivity. (This result may bolster the notion that the decline in *daf-2* induction of *sod-3::GFP* with increasing numbers of worms per well results from lower effective dosage of the dsRNA in liquid culture.) Of this set tested for lifespan extension, all three possible classes of lifespan phenotypes were observed: short lived, no difference, and long-lived, and interestingly at roughly equal proportions. Thirty clones produced significantly shorter lifespans than their simultaneous vector-only controls, though none below 11 days (not an unusual control lifespan at 25° C. The absence of short lifespans is surprising, as we thought it would be possible to induce *sod-3::GFP* because of sickness as opposed to stress-free induction as in the case of *daf-2(RNAi)*.

The goal was to find clones with a positive effect upon lifespan, and indeed 30 clones enjoyed lifespans that were significantly longer than the vector-only control (data not shown). The seven best amongst these and provide 25 – 79% increases in control lifespan. One of these was identified in another genome-wide screen (t et al., 2004). Why weren't the other clones observed in the Hansen et al screen? Firstly, these assays were full lifespan tests rather than maximal lifespan screens. Much greater power exists

in a full lifespan than in considering only a single point. Secondly, these tests were performed in an background with enhanced sensitivity to RNAi. Thirdly, an unpleasant fact for RNAi screens is that false negatives are likely. Finally, it should be noted that these lifespans have been conducted only once and therefore some of the clones identified may be false positives.

The clones producing the greatest increase in lifespan contain at least three (and perhaps four) mitochondrial components, an unknown gene, a biosynthetic pathway component, and a protein transporter. Finding these lifespan regulatory clones, has strongly validated our surrogate marker high-throughput screening protocol. However, there are a number of cautionary tales within the data. Notably absent from this list of candidates is any members of the *daf-2/daf-16* insulin/IGF-1-like regulatory pathway. This may not be too surprising, given that *age-1* was not identified in the Hansen et al. on plate maximal lifespan screen; secondly, it is not likely that *sod-3::GFP* induction is more sensitive than lifespan (particularly at day 2 of adulthood); and thirdly, liquid RNAi treatment likely is not likely to be as potent as on plate treatment (as suggested by the larval arrest of *hsp-6* only when grown on plates). However, the screen did identify clones that had not been using a maximal lifespan screen. The speed of the screen suggests that this method should become a frequently employed tool. If screens are easy and fast then researchers may be inclined to try more speculative screens such as multiplexing or enhancer screens.

Materials and Methods

Strains: An *rrf-3(pk1426)*; *sod-3::GFP* double mutant was constructed by crossing *sod-3::GFP*(Libina et al., 2003) males with *rrf-3(pk1426)* hermaphrodites(Simmer et al., 2002). GFP expressing F1's were cloned-out to individual plates (GFP is strongly expressed in neurons in the head and tail even in stress-free wild-type animals). True breeding lines expressing GFP were isolated. The presence of *rrf-3(pk1426)* was then established by shifting larvae from 20° to 25° C and scoring for very low fertility at the higher temperature.

Screen: Replica plates of the library were generated into 96 well plates with 45 μl media composed of a 1:1 ratio of liquid NG and HG (producing 'MG' media) with 1 mM IPTG and 100 uM Carbanyecillin. (I should note that these were made without the necessity of thawing the originals. I would simply scrape the surface of the frozen masters with a "Replicator", and then inoculate the target plates. This method was as efficient as with that of replicating from fully thawed masters and obviated the inevitable decay associated with repeated thawing and freezing of the master plates.) The plates were then incubated overnight at 37° C. Bacterial growth in these conditions was considerably less than a comparable volume of LB, or even HG, would produce. LB has proven to be relatively toxic for worm cultivation however – yielding quite short lifespans (Laura Mitic, personal communication). Moreover, in experiments described below, the worms grown in the HG media were smaller than those grown in NG, despite the larger bacterial growth in the HG media.

Eggs were isolated from well-fed gravid adults by bleaching. After washing 3 times in M9, the eggs were re-suspended in MG media to achieve a final concentration of eggs of approximately 8 eggs per μl. 10 μl of egg suspension was then introduced with a

multi-channel pipette to each of the wells yielding approximately 80 eggs. To keep the eggs in suspension, the mixture was placed on a shaker while aliquating. The eggs were placed a box with numerous wet paper towels to prevent evaporation, and then incubated overnight at 20° C. Then the worms were shifted to 25° C for three days. Not all of the eggs would hatch, so from the initial population of approximately 80 eggs, roughly 50 worms would grow to maturity.

2 day old adults were assayed for length (time of flight, tof), light obstruction (extinction, ext), and fluorescence emission (flu1) using the Copas Biosort. The Copas Biosort runs at a rate of 1 plate per hour (regardless of the number of worms in the wells). 6 to 8 plates were assayed each (one being a control plate carrying 4 rows of vector only as a negative control (pAD12) and 4 rows of *daf-2(RNAi)* as the positive control (pAD48)). Often the control plate was assayed in two stages, doing 2 rows of vector and 2 rows of the *daf-2(RNAi)* in the morning and 2 rows at the end of the day, thus controlling for changes that may occur in the machine or to get an impression of changes that may occur as a consequence of the 10 hours of additional growth.

Statistics: Data were assembled and analyzed with Stata Statistical Software: Release 8.2. (Stata Corporation, College Station, TX: StataCorp LP). The basic strategy was to aggregate the various assays (both primary and retest) into a single file in which each observation was uniquely identified by the plate number, date, and time (this last was particularly relevant during the retest cycle when the assay was performed on the same plate number several times in succession). Following assembly, the data was filtered to eliminate observations prone to errors. This included observations that were flagged by the Biosort as problematic (observations with values other than 5).

Furthermore, if air was introduced into the sample stream the Biosort could be confused into believing that these air bubbles were worms. Thus, wells with very high sample sizes were eliminated. Worms could also be lodged in the tubing or filter and come free during subsequent wells. These worms were thus attributed to the wrong treatment. We performed tests of the frequency with which this occurs and found that a blank well following a well with 60 – 90 worms would register 2 – 5 observations; and a second or third blank well may still encounter an errant observation. A treatment that produced larval lethality may nonetheless produce five adult worms. Therefore, wells were few observations were eliminated. Finally, to remove unhatched eggs, multiple worms passing through the flow cell at once, clumps of bacteria, etc., observations with extremely large or small lengths were deleted.

A narrow and broad filtering of the data was employed. For the broad analysis the following parameters employed were: $9<n<150$ and $60<tof<1200$; for the narrow analysis: $14<n<120$ and $89<tof<850$. Interesting candidates were identified by establishing the residual value for every worm (except for the positive control, *daf-2(RNAi)*) following a regression analysis employing the following model (utilizing the robust variance covariance matrix):

$$\text{Log(Fluorescence)} = \beta_0 + \beta_1(\text{Log(Extinction)}) + \beta_2(\text{n/well}) + \beta_3(\text{date})$$

The regressions results for the Broad analysis are:

Number of obs	=	960252
F (3, 960248)	=	.
Prob > F	=	0
R-squared	=	0.6118
Root MSE	=	0.55214

Log(flu1)	Coef.	Std.	se	p	95% Confidence Interval

date	-0.0016	0.0000	-146.6700	0.0000	-0.0016	-0.0015
Log(ext)	0.7611	0.0007	1165.2000	0.0000	0.7598	0.7624
n	-0.0004	0.0000	-17.9700	0.0000	-0.0004	-0.0004
_cons	23.4021	0.1692	138.3000	0.0000	23.0705	23.7337

For the narrow analysis:

Number of obs	=	784385
F (3, 784381)	=	.
Prob > F	=	0
R-squared	=	0.4395
Root MSE	=	0.53562

Log(flu1)	Coef.	Std.	se	p	95% Confidence Interval	
date	-0.0016	0.0000	-138.8200	0.0000	-0.0016	-0.0016
Log(ext)	0.7188	0.0012	616.9600	0.0000	0.7165	0.7211
n	-0.0013	0.0000	-43.7400	0.0000	-0.0013	-0.0012
_cons	24.6490	0.1842	133.8200	0.0000	24.2879	25.0100

The mean value per well of the individual residuals then provided a measure of displacement from expectation. Simpler means by well and treatment for fluorescence and length were calculated and contrasted.

Lifespan Analysis: Eggs were isolated as described above and then approximately 12 were distributed to plates containing NGM agar with 1 mM IPTG and 25 μg ml-1 carbenicillinplates seeded with the appropriate RNAi expressing bacterial strains and then grown at 25° C, as described by Fraser et al.(Fraser et al., 2000), scored as described by Apfeld and Kenyon(Apfeld and Kenyon, 1998), and briefly summarized here. On the second, fourth, and, if required, sixth day of adulthood, the animals were transferred away from their progeny onto freshly seeded plates. Survival was assayed every other day until all animals were either dead or censored. Animals were censored if they could not be found, crawled off the plates, exploded, died to internal hatching of

offspring, or if the plate suffered contamination. Only animals in which the bulk of the population was able to reach adulthood were assayed as described in the text. Results were summarized and analyzed employing Stata 8.2 using the Cox Proportional Hazards model employing Efron method for tied observations and robust variance and covariance matrices.

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Figure 5-1

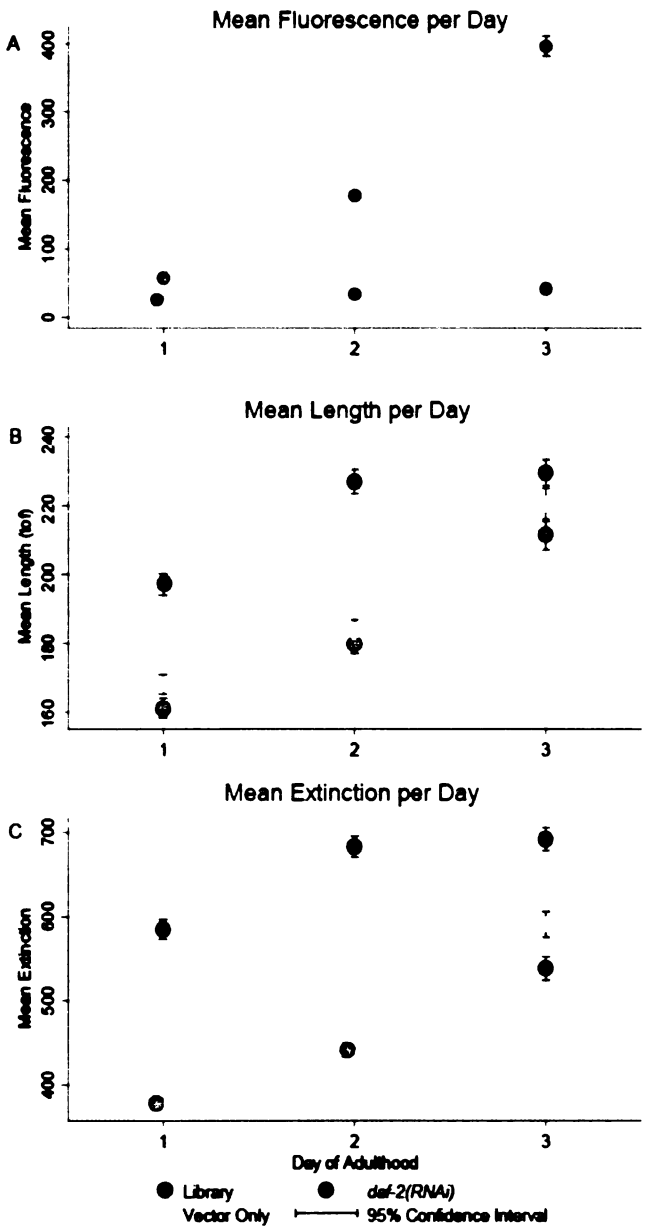
Mean Fluorescence per Day
A
Mean Fluorescence
0
100
200
300
400
1
2
3
Mean Length per Day
B
Mean Length (bp)
160
180
200
220
240
1
2
3
Mean Extinction per Day
C
Mean Extinction
400
500
600
700
1
2
3
Day of Adulthood
Library
Vector Only
daf-2(RNAi)
95% Confidence Interval

Figure 5 – 1. *daf-2* significantly increases *sod-3::GFP* emission and this difference is evident on day 1 of adulthood. Fluorescence, length, and extinction are plotted for animals grown on vector (yellow), *daf-2(RNAi)* (green), or library clones from Plate 88 (blue), on days 1 – 3 of adulthood. Points on graphs represent average values for between 2 and 3 thousand worms grown in samples of 40 – 60 worms per well in 96 well plates. 95% confidence intervals are shown for each datum except where the 95% confidence interval is smaller than the data point.

Table 5 - 1. *daf-2(RNAi)* treatment results in elevated *sod-3::GFP* expression, resulting in significantly increased fluorescent emission as measured by Copas Biosort.

Table 5-1

Day of Adulthood	Treatment	n	Mean Fluorescence (sd)	95% Confidence Interval	Length (sd)	Extinction (sd)
1	Sample of 96 library clones	3508	26(16)	21/31	197(94)	585(343)
	Vector	2186	31(21)	26/37	168(64)	379(166)
	daf-2(RNAi)	2099	58(37)	47/68	161(65)	378(177)
2	Sample of 96 library clones	3699	34(25)	26/42	227(105)	683(377)
	Vector	2395	63(60)	46/79	184(72)	437(206)
	daf-2(RNAi)	2520	178(119)	146/210	180(71)	441(206)
3	Sample of 96 library clones	4172	42(60)	25/60	229(120)	692(445)
	Vector	1498	86(72)	102/220	220(94)	591(292)
	daf-2(RNAi)	1706	396(296)	334/458	211(91)	539(292)

3 sets of plates were initiated and allowed to incubate for 60 to 108 hours (1 to 3 days of adulthood and then assayed with the Copas Biosort on successive days. The sample size (n) represents the number of worms meeting minimum eligibility requirements (status = 5, length >60 and <850)

Figure 5-2

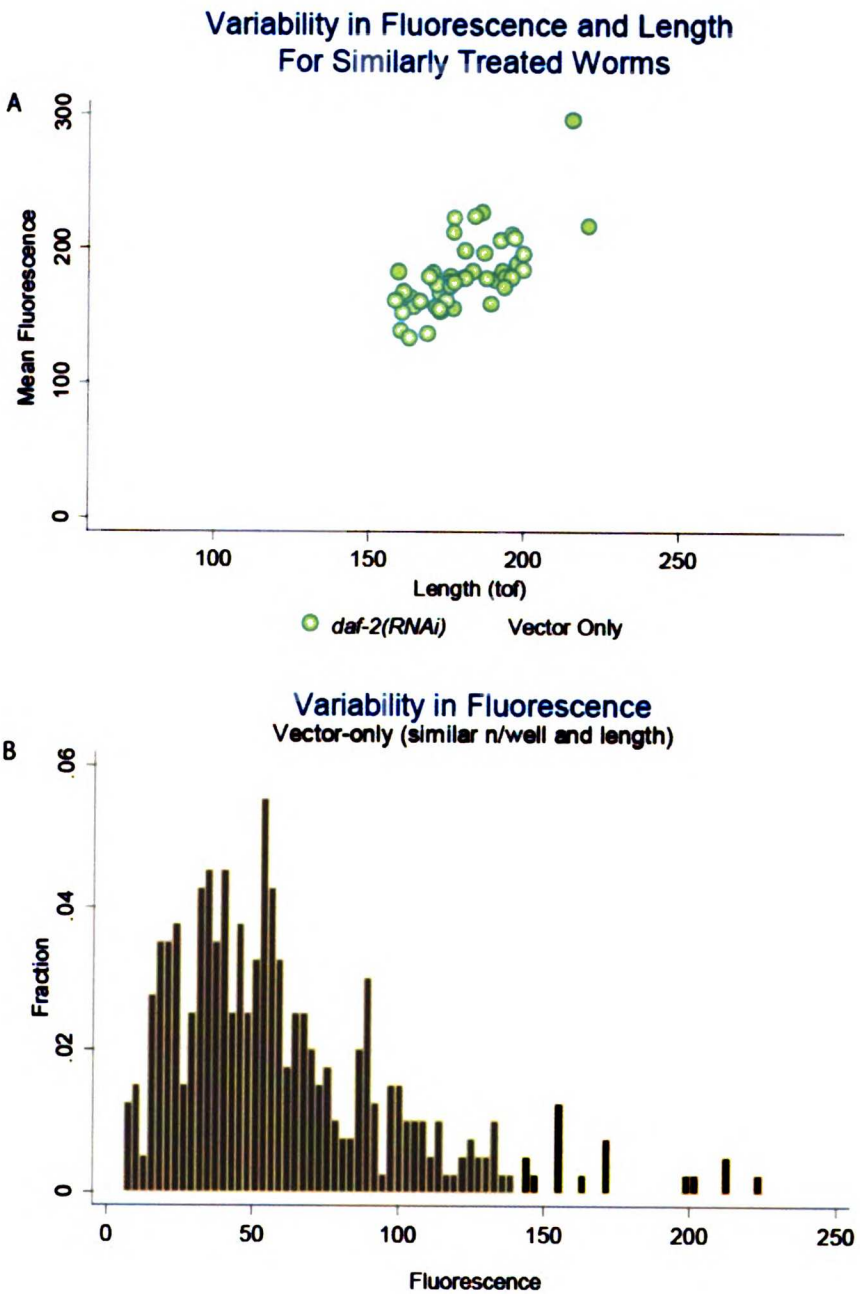
Variability in Fluorescence and Length
For Similarly Treated Worms
A
Mean Fluorescence
0
100
200
300
100
150
200
250
Length (tof)
daf-2(RNAi)
Vector Only
Variability in Fluorescence
Vector-only (similar n/well and length)
B
Fraction
0
.02
.04
.06
0
50
100
150
200
250
Fluorescence

Figure 5 – 2. Significant well-to-well variation in average fluorescence is observed, even for identically treated worms. A - Each point represents the average fluorescence values for the 20 to 80 worms in a well. Green points are for treatment with vector-only, and yellow points are *daf-2(RNAi)* treated. B – Histogram of fluorescence per worm for a sample of vector-only treated worms limited to those of similar size (tof 167 – 207) and from wells with similar numbers of worms per well (48 – 68).

Figure 5-3

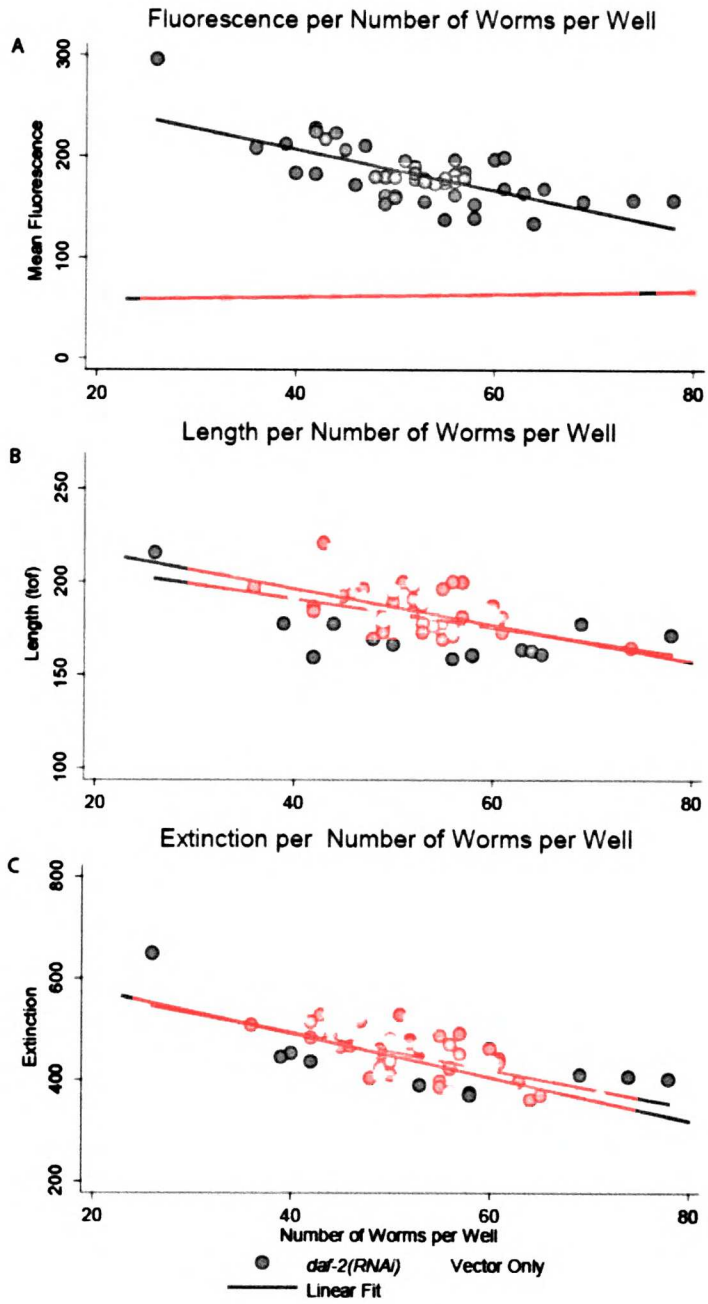
Fluorescence per Number of Worms per Well
A
Mean Fluorescence
0
100
200
300
20
40
60
80
Length per Number of Worms per Well
B
Length (tof)
100
150
200
250
20
40
60
80
Extinction per Number of Worms per Well
C
Extinction
200
400
600
800
20
40
60
80
Number of Worms per Well
daf-2(RNAi)
Vector Only
Linear Fit

Figure 5 – 3. Calorically restricted worms do not induce *sod-3::GFP*, but *daf-2(RNAi)* induction of *sod-3::GFP* is inversely correlated to the number of worms per well. Fluorescence (A), length (B), and Extinction (C) as a function of the number of worms per well for both vector-only (yellow) and *daf-2(RNAi)* (green) treated worms.

Figure 5-4

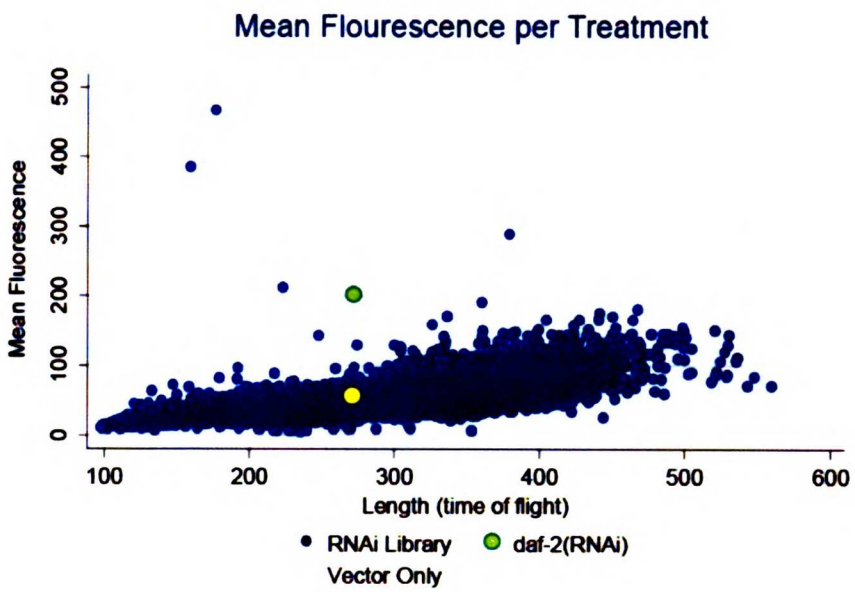
Mean Flourescence per Treatment
Mean Fluorescence
0
100
200
300
400
500
100
200
300
400
500
600
Length (time of flight)
RNAi Library
daf-2(RNAi)
Vector Only

Figure 5 – 4. Summary of average per well for fluorescence and length for more than 16,000 clones from the Ahringer Library. The overall average values for vector-only (yellow) and *daf-2(RNAi)* (green) are indicated as well.

Chapter 6 Conclusions

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Does caloric restriction employ the insulin/IGF-1-like signaling pathway in *C. elegans*?

On a surface, dietary restriction (DR) and the insulin/IGF-1-like system (the *daf-2/daf-16* pathway) share so many traits in common in *C. elegans* that it is hard to let go of the notion that DR employs this signaling system. Both DR and mutations in *daf-2* can prolong lifespan (Kenyon et al., 1993; Klass, 1977), protract reproduction, enhance thermotolerance (Gems et al., 1998; Houthoofd et al., 2003), and increase dauer formation(Riddle, 1997). Furthermore, connecting the mammalian function of the insulin pathway to regulate physiology in the face of variable levels of food, to the capacity of the worms to respond to low food by calling on the homologous system, appeals to biologists' affection for simplicity and conservation.

Surprisingly though, Lakowski and Hekimi (Lakowski and Hekimi, 1998), Houthoofd et al. (Houthoofd et al., 2003), and now the work in this thesis, have all found that while *daf-16* is required for the lifespan extension of *daf-2* mutants, the lifespan extension conferred by DR is not suppressed by *daf-16*. Nor is the thermotolerance (Houthoofd et al.) or the protracted fecundity suppressed by *daf-16* (Chapter 3). Next, we wondered if the DR affects were mediated by *daf-2*, but not by *daf-16*. We found though that the lifespan of the putative *daf-2* null could also be extended by DR (Chapter 2). These are reasonably well constructed epitasis experiments in that the mutant alleles are nulls and have opposite phenotypes than that of DR. Thus, they leave little room for insulin signaling in the DR response in worms. Perhaps the problem is our focus on

lifespan. If we consider the entire life history alternatives the worm enjoys, lifespan modulation is but one, and often the least important.

Direct caloric restriction of *C. elegans* is a challenging experiment. There is no simple way of separating the adult worms away from their progeny; secondly, worms in low levels of bacteria are likely to bag and thus be censored from the lifespan analysis. The solution I took was to transfer the worms by mouth pipette; and to simply endure the irritation of high bagging rates so I could also track reproductive output. A helpful alternative is to use sterile worms – this greatly reduces the need to transfer and eliminates the problem of bagging, then one loses the capacity to measure reproduction. However, suppressing reproduction obscures the central role that internal hatching plays in the response to low food.

What advantage does bagging serve? As Chen and Caswell-Chen (Chen and Caswell-Chen, 2003) and I discovered independently, bagging can facilitate dauer formation in the absence of food. Eggs laid in very low levels of food cannot reach dauer and arrest as L1's instead. They can survive in this fashion for up to three weeks and still proceed to normal development when food is restored (Johnson et al., 1984), but dauers are can survive for six months and can endure much harsher environments than arrested animals. With this in mind, the benefits for self-sacrifice are clear.

Considering this, let me reform the original question: Do sensory neurons and the insulin/IGF-1-like signaling system in the worm affect the normal response to low food? Now the experimental finding is clear – yes, mutations in this pathway elevate the rate of bagging in low food. As both the lifespan and dauer formation phenotypes of sensory neurons and *daf-2* are dependent on *daf-16*; it was surprising to find the bagging response

to low food does not require *daf-16*. This suggests the existence of new targets of *daf-2* that are responsible for the decision to bag. While we have lost the elegance of symmetry with mammalian insulin signaling, we have achieved an internal consistency for the nematode. The same genes that elevate the propensity for bagging in DR-adults also elevate the propensity of animals to form dauers in larvae.

This still leaves open the question of the mechanism by which DR is able to extend lifespan. There do appear to be subtle interactions between the sensory/*daf-2*/*daf-16* pathway and lifespan, in that mutations in this pathway reduce the benefits DR. However, these effects are generally quite small, the notable exception being *osm-5*, which in some cases actually exhibited a decrease in lifespan with diminishing levels of food. These results are difficult to interpret though since the bulk of the sample bagged. This experiment should now be repeated using either a sterile background or FUDR to suppress the potential for internal hatching of offspring. It may be that in the absence of a bagging response one may be able to identify more easily the loci required for the regulation of the DR lifespan extension.

Moderately strong *daf-2* alleles affect, reproduction, but do so in a *daf-16* dependent manner. As bagging does not require *daf-16*, it appears to be the case that the control of bagging is separate from that of the control of reproductive timing. It will be interesting to correlate reproductive output with the propensity to bag. For instance, one could imagine that it would be useful to have a burst of reproductive output prior to executing the decision to hatch offspring internally. In this way, the worm could maximize both its population growth potential, and prepare its offspring in the event that food remains scarce. More directly, it will be interesting to determine the mechanism of

bagging. Trent et al. screened for genes that cause animals to retain eggs (Trent, 1983). Many of these affected neurons or muscles in a way that prevented efficient egg laying in any conditions, but a number of these mutants may be interesting as potential regulators of the bagging response to low food. Secondly, it would be possible to perform a bagging suppression screen, particularly using a GFP marker that was expressed in early larval development. One could then employ a high-throughput approach as described in Chapter 5 to identify RNAi treatments that enhance or suppress the propensity to bag.

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