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REVIEW

Interpreting interactions between treatments that slow aging

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Summary

A major challenge in current research into aging using model organisms is to establish whether different treatments resulting in slowed aging involve common or distinct mechanisms. Such treatments include gene mutation, dietary restriction (DR), and manipulation of reproduction, gonadal signals and temperature. The principal method used to determine whether these treatments act through common mechanisms is to compare the magnitude of the effect on aging of each treatment separately with that when two are applied simultaneously. In this discussion we identify five types of methodological shortcomings that have marred such studies. These are (1) submaximal lifespan-extension by individual treatments, e.g. as a result of the use of hypomorphic rather than null alleles; (2) effects of a single treatment on survival through more than one mechanism, e.g. pleiotropic effects of lifespan mutants; (3) the difficulty of interpreting the magnitude of increases in lifespan in double treatments, and failure to measure and model age-specific mortality rates; (4) the non-specific effects of life extension suppressors; and (5) the possible occurrence of artefactual mutant interactions. When considered in the light of these problems, the conclusions of a number of recent lifespan interaction studies appear questionable. We suggest six rules for avoiding the pitfalls that can beset interaction studies.

Key words: aging; epistasis; insulin/IGF signalling; dietary restriction; *Caenorhabditis elegans*; biodemography.

Introduction

Aging is a process of intrinsic physiological decline apparent demographically as an increase in mortality and decline in fecundity at later adult ages. Although the mechanisms that determine the rate of aging are unknown, a growing number of experimental interventions have been shown to slow demographic aging, or to increase lifespan, often used as an indication of the rate of aging. Recently, numerous studies have attempted to establish whether different forms of intervention into the aging process act on the same, unidentified lifespan-determining mechanismμr on different ones (Dorman *et al*., 1995; Vanfleteren & De Vreese, 1995; Lakowski & Hekimi, 1996, 1998; Apfeld & Kenyon, 1999; Hsin & Kenyon, 1999; Gems & Riddle, 2000; Bartke *et al*., 2001). A salient example is reduced insulin/IGF signalling (IIS) and dietary restriction (DR). Mutations in IIS genes increase lifespan in the nematode *Caenorhabditis elegans* and the fruitfly *Drosophila melanogaster* (reviewed in Partridge & Gems, 2002). Lifespan is also increased by DR in a number of animal groups, including nematodes (e.g. Klass, 1977; Lakowski & Hekimi, 1998), insects (e.g. Chapman & Partridge, 1996; Nusbaum & Rose, 1999) and mammals (e.g. McCay *et al*., 1935; Masoro *et al*., 1982). It has been suggested that the effect of nutritional status on lifespan in DR is mediated by insulin (Kimura *et al*., 1997), or IGF-I (Gems & Partridge, 2001). This hypothesis has been addressed in *C. elegans* by two studies examining the effects of interactions between DR and IIS mutations on lifespan (Vanfleteren & De Vreese, 1995; Lakowski & Hekimi, 1998). These studies employed life-extending mutations in the IIS genes *age-1* and *daf-2* (Friedman & Johnson, 1988; Kenyon *et al*., 1993; Morris *et al*., 1996; Kimura *et al*., 1997). Based partly on the finding that DR increased the lifespan of IIS mutants, both studies concluded that these two treatments act on different determinants of longevity and aging.

We call this kind of study of the interactions between different treatments affecting lifespan 'lifespan interaction studies'. The following discussion first assesses the way that such studies have been designed and the rationale that underlies their interpretation, then closely examines several such studies involving *C. elegans*, *Drosophila* and the mouse in the light of this assessment. Our analysis leads us to conclude that recent studies of interactions between treatments that retard aging are a morass of confusion and contradictions. We identify five types of problem associated with interaction studies, and propose six rules for designing and interpreting them so as to minimize the difficulties.

Lifespan interaction studies: the simple view

The published studies of interactions between mutations or treatments affecting lifespan employ a logic generally similar to classical genetic epistasis analysis. The more common understanding of the term epistasis is that of Mendelian geneticists; here, when the effects on one locus mask those of another, the

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former gene is said to be epistatic to the latter, which is said to be hypostatic (Avery & Wasserman, 1992; Huang & Sternberg, 1995). However, the term epistasis is also sometimes used to describe any situation where the effects of two loci are non-additive; this more inclusive definition of epistasis is current among quantitative geneticists (for a discussion of the different uses of the term epistasis, see Phillips, 1998). In both conceptions of epistasis, additive effects on the trait are taken to indicate that the interventions act through different mechanisms. Non-additive effects (where the magnitude of the effect of one intervention depends upon whether the other is also present) are taken to indicate that the treatments interact in their determination of trait value. This finding in turn is taken to indicate that the interventions act in the same pathway or process in the determination of trait value.

The logic of interaction studies involving lifespan may be illustrated by three hypothetical genes affecting lifespan, *a*, *b* and *c*. Suppose that mutations in either *a*, *b* or *c* increase lifespan. The following double mutants may be constructed: (*a* + *b*), (*a* + *c*) and (*b* + *c*), and lifespans measured (Fig. 1a). The result in Fig. 1(a) may be interpreted as follows: because (*a* + *b*) lives no longer than *a* or *b* alone, *a* and *b* are involved in the same mechanism of lifespan determination. Furthermore, this mechanism is different from that mediating the effect of *c*, because the addition of *c* to *a* or *b* further increases lifespan. (A less parsimonious interpretation is that *a* and *b* extend lifespan via different mechanisms, but a pleiotropic deleterious effect of *a* blocks any further extension of lifespan by *b*, and/or vice versa).

Examples of the (*a* + *b*) case in *C. elegans* are the interaction between *eat-2(ad465)* (which causes DR due to an eating defect) and *clk-1(e2519)* (Lakowski & Hekimi, 1998), and *age-1(hx546)* and *daf-2(e1370)* (Dorman *et al*., 1995). There is no increase in lifespan in the double mutant compared with either mutant alone. Examples of the (*a* + *c*) case are interactions between *daf-2(e1370)* and *clk-1(e2519)* (Lakowski & Hekimi, 1996), *daf-2(e1370)* and *eat-2(ad465)* (i.e. DR) (Lakowski & Hekimi, 1998), *daf-2(e1370)* or *age-1(hx546)* and DR due to culture under axenic conditions (Vanfleteren & De Vreese, 1995), *daf-2* (several alleles) and ablation of the germ line (Hsin & Kenyon, 1999), and *daf-2* (several alleles) and maleness (Gems & Riddle, 2000). A recent (*a* + *c*) case in mammals involved DR and the Ames dwarf mutation in mice (Bartke *et al*., 2001).

A second form of lifespan interaction study involves epistasis analysis using suppressors of the life-extension trait, exemplified by *C. elegans daf-16(–)* (Kenyon *et al*., 1993). Suppose that life extension resulting from *a* is suppressed by mutation of gene *d*. One may then examine lifespan in double mutants (*b* + *d*) and (*c* + *d*) (Fig. 1b). We see that *b*, like *a*, is suppressed by *d*, but *c* is not. This might suggest that *c* involves a distinct mechanism of life extension to that of *a* and *b*.

Examples of (*a* + *d*) or (*b* + *d*) interactions are those of *daf-16(–)* (*d*) with either *daf-2(e1370)* (Kenyon *et al*., 1993), germline ablation (Hsin & Kenyon, 1999) or maleness (Gems & Riddle, 2000). Examples of (*c* + *d*) are *daf-16(m26)* with *clk-1* or *eat-2* (Lakowski & Hekimi, 1996, 1998), although these findings have been contested (Murakami & Johnson, 1996; Braeckman *et al*., 1999, 2000).

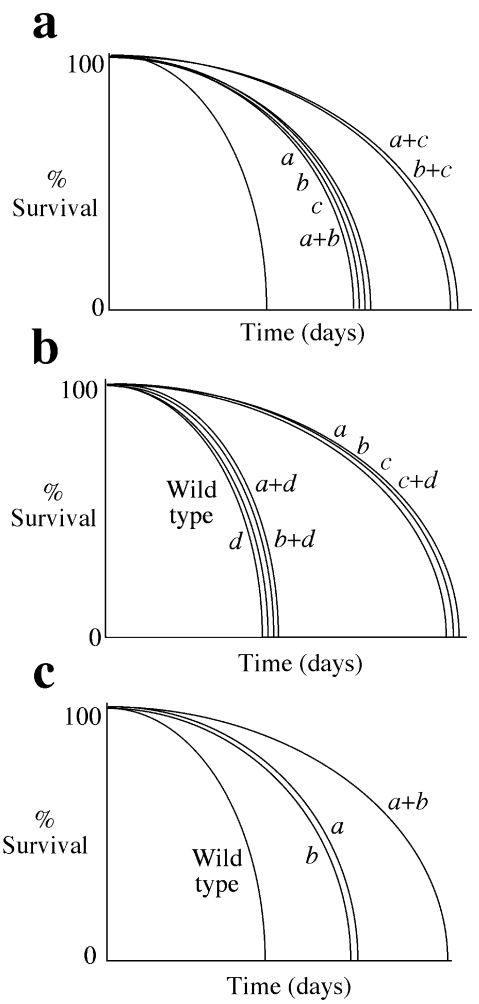

Fig. 1 (a) Interaction studies of three mutations that extend lifespan. In this hypothetical case, there is either complete *qualitative* interaction (*a* + *b*) or no interaction at all (*a* + *c*, *b* + *c*). In reality, complex *quantitative* effects may occur, and must be interpreted. (b) Epistasis analysis involving a suppressor of life extension, *d*. (c) An interaction study between two mutations that extend lifespan.

Studies such as these have given rise to the slightly shaky current consensus that, in *C. elegans*, DR and the clock gene mutations extend lifespan by a common mechanism which is distinct from that of insulin/IGF pathway mutations (reviewed in Gems, 1999; Vanfleteren & Braeckman, 1999).

Complications and pitfalls in lifespan interaction studies

Closer examination of the assumptions involved in data interpretation, and of the data themselves, raises doubts about the validity of the interpretation of interaction experiments in a number of individual instances. The sources of difficulty may be summarized as follows: (1) The treatment may produce a

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submaximal effect on its target, e.g. mutant alleles employed may be hypomorphic (partial loss of function) rather than null or, when null, may fail to achieve the maximum extension of lifespan possible by the underlying mechanism. (2) The alleles/treatments involved affect survival through more than one mechanism. (3) When two lifespan-extension treatments result in lifespans greater than the single treatments, the results can only be interpreted by analysis of age-specific mortality rates, which is in general not performed. (4) Life extension suppressors may reduce lifespan via deleterious effects unrelated to normal aging. (5) There may be artefactual mutant interactions that do not correspond to interactions between wild-type processes.

Interactions where loss of function is incomplete

If two interventions extend lifespan by the same mechanism, but in combination act additively because they do not jointly reach a ceiling effect (as in Fig. 1c), then it will be incorrectly assumed that they act via different mechanisms. This may occur either because mutant alleles are hypomorphic rather than null, or because a given treatment results in a partial alteration in a particular pathway or mechanism. The data in Fig. 1(c) are therefore consistent with *a* and *b* acting via the same or via different mechanisms. Consequently, in the absence of other evidence, observation of interactions of type (*a* + *b*) are not interpretable in terms of common or distinct life-span determining mechanisms. For example, *clk-1(qm30)* and *clk-3(qm38)* extend mean lifespan (at 18 °C) by 33% and 37%, respectively, whereas the lifespan of the *clk-3*; *clk-1* double mutant is increased by 192% relative to wild type (Lakowski & Hekimi, 1996). Taken alone, and by conventional inference, this result might be taken to mean that these two mutations extend lifespan by different mechanisms. However, since both exhibit the same maternal effect clock (Clk) phenotype, affecting the rate of development, feeding, defecation, etc., it has been assumed that these mutations affect related processes (Lakowski & Hekimi, 1996; Hekimi *et al*., 2001). In this light, the additive effect on lifespan indicates either than these mutations are hypomorphic or alone produce submaximal effects on the mechanism involved. The importance of careful characterization of single mutant phenotypes to the correct interpretation of mutant interaction data is discussed elsewhere (Huang & Sternberg, 1995).

Effects on survival through more than one mechanism

Gene mutation and other treatments may have multiple effects on the biology of an organism. Work on *C. elegans* has made it clear that many different genes can increase or decrease lifespan. It is therefore possible that multiple pleiotropic effects of individual mutations or treatments may affect lifespan in a complex manner. An example of this is the *C. elegans* insulin/IGF receptor gene *daf-2*. Many lifespan interaction studies have employed the canonical allele of *daf-2*, which is temperature sensitive (ts), and bears the allele number *e1370*. Null alleles of *daf-2* are not used since they result in embryonic or early larval lethality. Hypomorphic *daf-2* alleles do not vary across a single range of mutant phenotypes, but rather fall into two distinct allelic series. Class 1 alleles are long lived (Age). Class 2 alleles exhibit this trait plus a complex suite of defects, including reduced feeding, movement and fertility (Gems *et al*., 1998). Class 2 allele-specific pleiotropic traits are likely to affect lifespan over and above any extension of lifespan by the Age phenotype. Given that *daf-2(e1370)* is a class 2 allele, interaction studies with *clk-1*, *eat-2*, dietary restriction, maleness or germline ablation may potentially be confused by the presence of some of the many pleiotropic defects. For example, the reduction of feeding by *daf-2(e1370)* adults at higher temperatures may confound studies of interactions between *daf-2* and dietary restriction (Vanfleteren & De Vreese, 1995; Lakowski & Hekimi, 1998), or metabolic rate (Van Voorhies & Ward, 1999; Vanfleteren & De Vreese, 1995). Moreover, studies of interactions between *daf-12* (which mediates TGF-β signalling) and different alleles of *daf-2* have demonstrated that the results of interaction studies may depend upon whether a class 1 or a class 2 *daf-2* allele is present (Larsen *et al*., 1995; Gems *et al*., 1998). Thus, more interpretable information on interactions between *daf-2(e1370)* and, say, *clk-1* (Lakowski & Hekimi, 1996) or *eat-2* (Lakowski & Hekimi, 1998) would be obtained by using class 1 alleles of *daf-2*.

In higher organisms, pleiotropic mutations may well be the rule rather than the exception (Hodgkin, 1998). Unless the pleiotropic effects of a mutation are well characterized, there is a danger that, as in the study of development (Huang & Sternberg, 1995), lifespan interaction studies will lead to erroneous conclusions. Recent studies demonstrating neuroendocrine regulation of aging suggest the existence of distinct upstream and downstream determinants of aging. Thus, treatments may affect distinct upstream or downstream mechanisms (Fig. 2). Interpretation of interaction studies may be difficult if there are distinct upstream pathways regulating common downstream mechanisms (Fig. 2b).

Interpreting quantitative interactions between treatments affecting lifespan

Recent studies of interactions between treatments affecting lifespan have employed the following sort of reasoning. If two combined treatments show no additional effect on lifespan over either treatment alone, then they act via the same mechanism. If the extensions of lifespan by each treatment simply add together in the double treatment, they are independent. But what if they are more than additive, i.e. synergistic? It is unclear how to interpret interactions of this type, as has been noted (Hekimi, 2001). According to the quantitative genetic conception of epistasis, such synergistic interaction may imply non-independence (Phillips, 1998). A further problem is the exact meaning of 'additive'. How, precisely, may one distinguish between additive and synergistic and when may interactions be considered less than additive, and how should this be interpreted?

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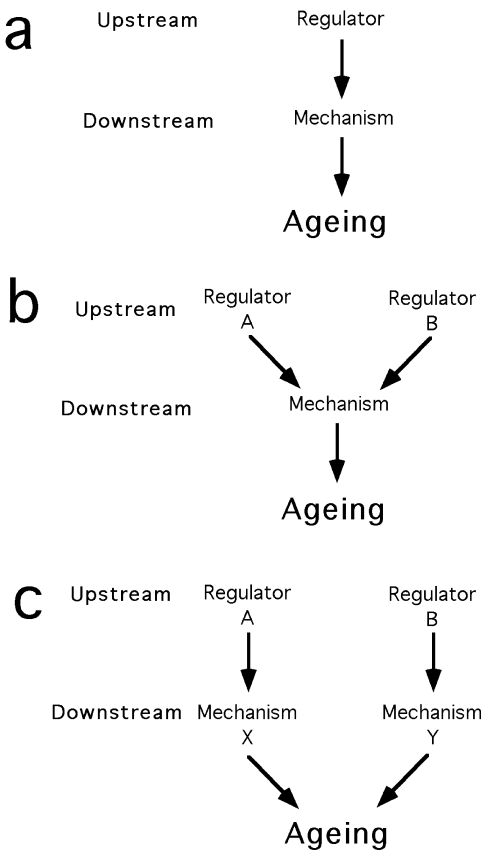

Fig. 2 Interaction studies may involve different elements of hierarchies of upstream and downstream determinants of aging, making the interpretation of results problematic.

As an illustration, consider Fig. 1(a). Let us suppose that *c* acts on lifespan via a mechanism that is fully independent of *a* (forget *b* for now). Let us also suppose that *a* or *c* alone increases mean lifespan from 10 days to 30 days, an increase of 200%. What effect do we expect mutation of *a* to have on the lifespan of a *c* mutant? Mutation of *a* could add 20 more days to the lifespan of the mutant, resulting in a lifespan of 50 days (a straightforward additive interaction); or it might, as has been argued (Hekimi *et al.*, 2001), increase its lifespan by 200%, giving a lifespan of 90 days (a multiplicative interaction). Which is the expected interaction if the interventions act through different pathways? Unfortunately, it is impossible to predict the effect on mean lifespan of simultaneously applied treatments that retard aging by independent mechanisms. However, one may, at least, take the following measures to deal with this problem of interpretation: taking the results of interaction studies involving lifespan, one may calculate from the outcomes of single treatments the predicted outcomes assuming the null hypothesis (no interaction). Statistical tests (e.g. analysis of variance) may then be performed to demonstrate whether subadditive, additive or synergistic interactions have occurred, where 'additive' is taken to be either arithmetic or multiplicative.

A possible solution to the difficulty of defining additive interactions is to turn from survivorship or mean lifespan to age-specific mortality – the proportion of individuals which enter a particular age interval and which die before its conclusion. Inferences about genetic effects on aging using traditional studies of epistasis require a clear definition of the phenotypic measure of aging, plus a scale of measure such that independent genetic effects contribute additively to the phenotype. The problem of choosing an appropriate scale for analysis is a general one within quantitative biology, equally applicable, for example, to the study of growth and body size.

There are several reasons why age-specific mortality, rather than survivorship or mean lifespan, is a relevant measure of aging. First, mortality rates capture age-specific changes. Information on the timing of changes is either lost when information is condensed into mean lifespan, or obscured when presented in terms of survivorship. Second, age-specific patterns of mortality are consistent and reproducible across different genotypes and experimental treatments (Curtsinger *et al.*, 1995). These consistent patterns are often summarized quite well with simple mathematical models (e.g. the Gompertz model). Third, characteristic changes in the dynamics of mortality can indicate different physiological effects. For example, a two-fold increase in lifespan may result from either a delay in the onset of senescence (a delay in the increase in mortality with age, Fig. 3a, left) or from a slowing of the rate of physiological decline after its onset (a slowing of the rate of increase in mortality with age, Fig. 3b, left) (Pletcher *et al.*, 2000). Lastly, a study of the most likely scale for genetic effects on aging suggests that, for segregating genetic variants and spontaneous mutations, genetic effects on log mortality are most likely to be additive (Promislow & Tatar, 1998).

Accepting age-specific mortality as the relevant measure of aging, it immediately becomes apparent that the analysis of interactions between treatment effects on mean lifespan can be seriously problematical. As an example, consider two experimental manipulations: one that simply shifts the mortality curve such that the risk of death is proportionately higher or lower throughout life (Fig. 3a), and a second that influences predominantly the rate of aging (Fig. 3b). Examples of such manipulations in *Drosophila* include dietary restriction (Pletcher *et al.*, 2002) and the single-gene mutant *Indy*, respectively (Rogina *et al.*, 2000). If we assume, hypothetically, that these manipulations increase lifespan through entirely independent mechanisms, then what is the expected lifespan from an *Indy* mutant raised under DR? A reasonable expectation is that the double manipulation would both reduce the rate of aging and shift the mortality to a lower level throughout life, and that there would be an additive effect on age-specific mortality (Fig. 3c, left). When considered at the level of survivorship, however, strong synergistic epistasis is suggested (Fig. 2c, right), which taken alone might lead to the conclusion of non-independence. Here, average longevities are: wild-type, 13 days; mutant *a*, 27 days; mutant *b*, 27 days; and double mutant, 71 days. It should be pointed out that one cannot generalize to say that all interactions

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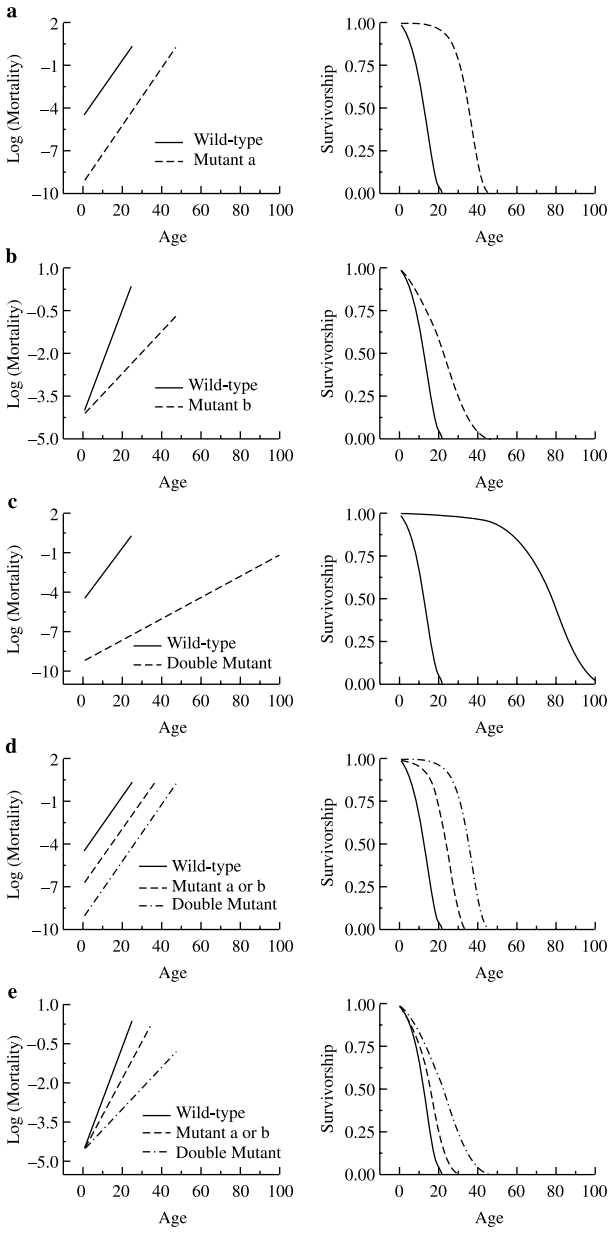

Fig. 3 Measuring mutational effects on aging. (a) Log-mortality (left) and survivorship (right) for a hypothetical wild-type genotype and single longevity enhancing mutant, which shifts the log-mortality rates down by an equal amount at all ages. (b) Log-mortality and survivorship for the same wild-type genotype and single longevity-enhancing mutant, which reduces the rate of increase in mortality with age. (c) Log-mortality and survivorship for the same wild-type genotype and a double mutant in which the effects of mutants 'a' and 'b' act additively and independently on mortality rates (i.e. the double mutant has a reduced mortality at each age and a slower rate of increase in mortality with age). Notice that effects on age-specific mortality that are additive and independent exhibit epistasis (in this case synergistic epistasis) on mean longevity and survivorship. (d) Not all additive interactions on age-specific mortality exhibit epistatic effects on survivorship. Additive genetic effects confined to 'shifts' in the mortality trajectory can exhibit additive changes in survivorship. (e) Genetic effects confined to the increased rate of mortality with age can exhibit large or small amounts of interaction on survivorship depending on details of the wild-type mortality dynamics. In this example, mutational effects on the increased rate of mortality with age are exactly additive – the double mutant exhibits a change in the rate parameter twice that of both single mutants. A small amount of interaction is apparent in relation to survivorship.

between mutations of type 'a' and type 'b' lead to synergistic increases in mean lifespan. However, additive effects on mean lifespan do always occur when two treatments both shift the age-specific mortality curve (Fig. 3d) or when they both result in small changes in the rate of increase of mortality with age (Fig. 3e). In this case, additive means simply additive, and not multiplicative; i.e. returning to the above example, if *a* or *c* alone both increase mean lifespan from 10 to 30 days via a right shift in the mortality curve, an additive interaction in an (*a* + *c*) double mutant would result in a mean lifespan of 50 days. We will present elsewhere a detailed account of the predicted effects of simultaneous application of non-interacting life extending treatments on age-specific mortality and mean lifespan.

Another important possibility is that mutations have independent and age-specific effects. For example, a mutation that reduces instantaneous costs of reproduction might reduce mortality rates only during reproductive ages (Partridge & Andrews, 1985; Partridge, 1999), or prevent a delayed wave of mortality (Sgrò & Partridge, 1999). Let us suppose that such a mutation is combined with a second that acts independently of the first and reduces mortality only at much later ages (say via attenuation of oxidative damage). An example of such a situation is given in Fig. 4. For these strains, average longevity is 19.2, 26.5, 43.1 and 73.1 days for the wild type, mutant line 1, mutant line 2 and double mutant, respectively. Mutant 1 alone causes a 7.3-day (38%) increase in lifespan, while mutant 2 alone results in a 23.9-day (124%) increase in lifespan. Under a naive expectation of independent effects on longevity, the double mutant would be expected to live approximately either 50 days (simple additive), or 59 days (multiplicative). The greater lifespan of the double mutants (at 73 days, an increase of 265% over wild type) might suggest epistatic interactions between the mutations. In reality, however, examination of age-specific mortality would make clear that these mutations act independently and at different times in life.

In conclusion, interpreting relationships based on changes in average lifespan and survivorship will produce results that are likely to be uninterpretable and sometimes misleading. Consider interactions between DR and reduced IIS in *C. elegans*. The mean lifespan of *daf-2(e1370)* mutants fed on *E. coli* is typically around double that of wild type (Kenyon *et al.*, 1993). Culture in defined medium results in DR, which increases wild-type lifespan by up to around 80%; under DR, mean *daf-2(e1370)* lifespan was 191% longer than wild-type, i.e. DR and reduced IIS showed a synergistic interaction in their effects on mean lifespan (Vanfleteren & De Vreese, 1995). However, since age-specific effects were not examined, this result cannot be taken to mean that the two interventions act through either interacting or non-interacting pathways.

Demographic analysis as a tool to identify interventions that do not act through common pathways is potentially powerful, but requires careful application. Use of age-specific mortality rates rather than longevity as the phenotype for analysis will greatly reduce the ambiguities in interpretation.

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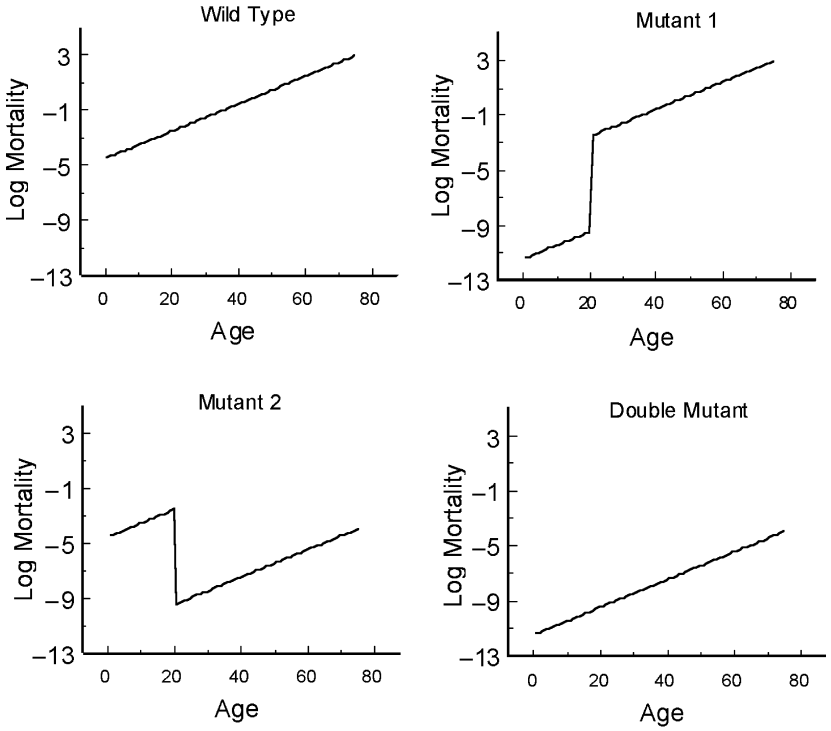

Fig. 4 Cartoon representation of two hypothetical mutations that are independent and have age-specific effects. Strains are assumed to follow the Gompertz mortality model (linear on the log-scale) and mutations are assumed to reduce the level of mortality over a range of ages.

Problems of life extension suppressor studies

In studies of *C. elegans*, the difficulty of interpreting interactions of type (*a* + *b*) (Fig. 1c) has largely been glossed over by using interaction studies with mutations that suppress life extension (the Age phenotype) to decide the issue. *daf-2* or *age-1* Age is suppressed by mutation of *daf-16* (Kenyon *et al*., 1993; Dorman *et al*., 1995; Larsen *et al*., 1995). According to some reports, *daf-16* fails to suppress Age resulting from mutation of *eat-2* (i.e. DR), or *clk-1* (Lakowski & Hekimi, 1996, 1998). This appears to support the division between Age resulting from reduced insulin/IGF signalling (IIS), which requires *daf-16(+)*, and *clk-1(–)*/DR Age, which does not. However, there are currently two problems associated with *daf-16*/Age suppressor studies: (a) conflicting results; and (b) *daf-16(–)* alone shortens lifespan.

Several reports suggest that *clk-1* life extension requires *daf-16(+)* (Murakami & Johnson, 1996; Braeckman *et al*., 1999, 2000). However, Lakowski & Hekimi (1998) suggested that suppression of the *clk-1* Age phenotype by *daf-16(–)* observed by Murakami & Johnson (1996) was due to the slight reduction of lifespan that *daf-16(–)* causes in any genetic background (Kenyon *et al*., 1993; Larsen *et al*., 1995; Malone *et al*., 1996; Lakowski & Hekimi, 1998). They presented data showing that the slight reduction in lifespan that *daf-16* produces in *eat-2* and *clk-1* mutants is similar in magnitude to that resulting from *daf-16* alone (Lakowski & Hekimi, 1998); by this view, this is an additive (or 'subtractive', Hekimi, 2001) interaction between loci conferring increases and decreases in lifespan.

Table 1 Interaction studies between DR, *daf-2*, *clk-1* and *daf-16*

Trial	Strain	*n*	Mean life span ± SE
1	N2	64	30.9 ± 0.8
1	*clk-1(e2519)*	60	63.4 ± 1.4
1	*daf-16(m27); clk-1(e2519)*	59	35.9 ± 1.0
2	N2	213	26.7 ± 0.4
2	*daf-16(m26)*	209	23.4 ± 0.4
2	*daf-16(m27)*	210	23.9 ± 0.4

Animals were maintained in axenic medium with added autoclaved *E. coli* at 24 °C. N2 is the wild-type strain. Reproduced from Braeckman *et al*. (2000). FUdR was also added to the N2 populations in trial 1, and all strains in trial 2, to prevent egg hatching. FUdR at this concentration is not known to affect life span.

However, this issue remains contentious. Braeckman *et al*. (2000) examined the effect of *daf-16* on *clk-1(e2519)* lifespan in axenic medium (Table 1). This dataset contradicted a key finding of the Lakowski & Hekimi (1998) study. While *daf-16* alone only decreased lifespan by 10–12%, it reduced *clk-1* lifespan by 43%, removing 85% of the *clk-1* extension of mean lifespan. Nonetheless, these data also provide some support for the Lakowski and Hekimi scheme. Firstly, DR increased the effect of *clk-1* on lifespan, so that it produced a 105% increase over DR-ed wild-type animals. Secondly, two mutant alleles of *daf-16* produced only slight reductions in N2 lifespan, suggesting that the increased lifespan resulting from DR does not require *daf-16(+)*. Thus, this data set suggests that *clk-1* but not DR requires *daf-16(+)*, and that these two interventions therefore involve different mechanisms.

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Artefactual mutant interactions

Even where a clear interaction between two mutations is observed, this does not necessarily indicate that the two wild-type processes in which the two genes function interact with one another. An example of a possible artefactual interaction between mutations is the effect of *clk-1(e2519)* and *daf-2(e1370)* on oxidative stress resistance (Oxr). *daf-2* but not *clk-1* adults are Oxr (as measured by resistance to the superoxide generator Paraquat under 98% oxygen), and overexpress *sod-3*, which encodes a dauer-specific manganese superoxide dismutase (Honda & Honda, 1999). Surprisingly, *clk-1* was found to enhance dramatically Oxr and *sod-3* expression when combined with *daf-2*. The authors of this study concluded that *clk-1* has a dual role in determining longevity: in the clock programme itself, and by interacting with insulin/IGF signalling. Given that *clk-1* alone has no effect on Oxr or *sod-3* expression, an alternative possibility is that the role of *clk-1* as an enhancer of Oxr and *sod-3* overexpression is an artefactual mutant interaction that does not correspond to interactions between wild-type *clk-1* and *daf-2* genes, or the processes in which they are involved. Arguably, artefactual mutant interactions are more likely to occur where the genes concerned are central metabolic regulators (e.g. *daf-2* and *clk-1*) than with genes with highly specific and limited roles (say genes encoding collagens or odorant receptors).

Six ways to avoid being misled by lifespan interaction studies

Some of the problems identified here are difficult to rectify. However, several steps may be taken to design interaction trials with a greater probability of interpretable results. These are as follows: (1) Where possible, use null alleles, as defined by genetic and molecular analysis, and environmental interventions that have been adjusted to maximize the lifespan obtainable. Unfortunately, for some genes (e.g. *daf-2*), life extension is seen in hypomorphic but not null alleles. (2) Where available, use alleles either without complex pleiotropic effects or, failing that, under conditions that minimize penetrance of pleiotropies. In the case of pleiotropic genes encoding multifunctional proteins it may be possible to select mutant alleles where a single component of the protein, affecting lifespan, is affected. In the case of *daf-2*, class 1 alleles such as *daf-2(m41)* may be employed to avoid the confounding effects of class 2 specific defects (Gems *et al*., 1998; Tissenbaum & Ruvkun, 1998). (3) Use more than one allele of any gene being examined. Even with mutant alleles where no obvious confounding pleiotropic effects are known to occur, a wise precaution against confounding pleiotropic effects is to use several alleles. Of course, they may all have similar confounding effects on lifespan. (4) To establish whether subadditive, additive or synergistic interactions have occurred, calculate the expected value for an additive interaction from the effects of individual treatments on lifespan. Test both possible meanings of additive, i.e. arithmetical or multiplicative.

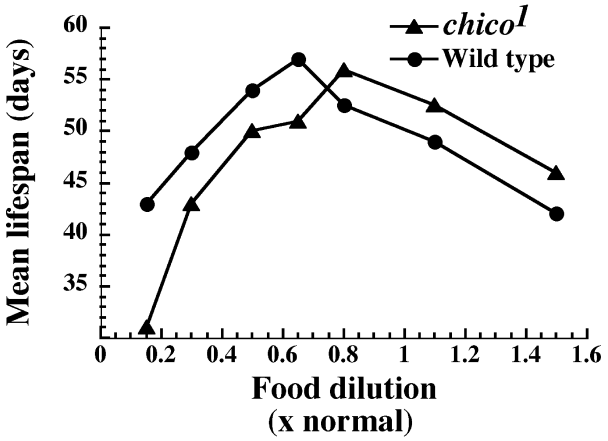

Fig. 5 Interaction between dietary restriction by food dilution and reduced insulin/IGF signalling (*chico*^{1}) in the determination of mean lifespan.

Then perform a statistical comparison of predicted values and actual outcome, to test the hypothesis. (5) Analyse interactions quantitatively using mortality rates (or log-mortality rates) as the phenotypic measure of aging. (6) Perform analyses over a range of severities of one or both of the treatments used to extend lifespan. Given the numerous problems that may confound interaction studies performed under any given condition, a better chance of drawing reliable conclusions about independent or non-independent aging mechanisms may be given by studies of interactions between treatments over a range of severities. An example of this approach is a recent study of interactions between DR and reduced IIS in *Drosophila.* Mutation of the gene *chico*, which encodes an insulin receptor substrate, increases lifespan by up to 48% in female flies under replete nutritional conditions (Clancy *et al*., 2001). Extension of lifespan by DR was found to occur in *chico*^{1} flies (Clancy *et al*., 2002). This finding is consistent with similar studies performed in *C. elegans* (Vanfleteren & De Vreese, 1995; Lakowski & Hekimi, 1998) and, potentially, mice (Bartke, 2001). Yet when *chico*^{1} mutant lifespan was examined over a range of nutritional conditions, it was found to peak at a higher level of nutrition than that of wild-type flies (Clancy *et al*., 2002) (Fig. 5). This meant that *chico*^{1} lifespan was less than wild type at lower levels of nutrition. These results indicate that *chico*^{1} flies are partially DR-ed by their genotype. These results support the opposite conclusion to that drawn from studies of the effect of DR on a single, replete nutritional level: the effects of DR and reduced IIS involve common mechanisms.

Conclusions

Studies of interactions between treatments affecting lifespan carried out by *C. elegans* researchers have been dogged by confusion, which has resulted, at least in part, from the problems identified in this discussion. A particular weakness has been the lack of quantitative analysis. Perhaps such analysis has not been performed because there is no precedent for such analysis

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leading to a clear conclusion about mechanisms of aging. Furthermore, quantitative analysis requires more and larger trials, and more careful control of confounding variables. While all this may make interaction studies more tedious to perform, there is much to be gained from this approach: not only a full understanding of the relationship between the mechanisms underlying, e.g. reduced insulin/IGF signalling, germline signalling and DR, but also the elusive unification of the new genetics of aging with biodemography, and an extension of epistasis analysis to interpret interactions that are less or more than additive.

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