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Review

Deceptively simple but simply deceptive – *Caenorhabditis elegans* lifespan studies: Considerations for aging and antioxidant effects

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ABSTRACT

The nematode worm *Caenorhabditis elegans* (*C. elegans*) is increasingly popular as a model organism for aging studies as well as for testing antioxidants and other compounds for effects on longevity. However, results in the literature are sometimes confusing and contradictory [1–4]. This review introduces *C. elegans* as a model organism, discusses aspects that make it attractive for aging and antioxidant research, and addresses some problems and potential artifacts.

1. Introduction

1.1. Caenorhabditis elegans as a model organism

The soil-dwelling nematode (roundworm) *Caenorhabditis elegans* (*C. elegans*) is a small (adult size about 1 mm length), free-living, bacteriovorus vermiform with a mean lifespan of around 20 days under laboratory conditions. Under these conditions, *C. elegans* are predominantly (>99%) self-fertilizing hermaphrodites and populations can be maintained effectively isogenic, since each individual hermaphrodite produces approximately 300 clonal progeny during the first week of adult life [5]. *C. elegans* hermaphrodite adults contain 959 somatic cells, 302 of which are neurons. The complete cell lineage is known, including those cells that undergo apoptosis during development [6]. The *C. elegans* research community has generated a library of mutant strains as well as an extensive toolkit for the genetic manipulation of this organism. *C. elegans* are transparent at all developmental stages and a large number of green fluorescent protein (GFP) reporter-gene strains have been generated, making it possible to visualize anatomy, developmental processes and many signalling pathways in real time. Strains can be cryogenically preserved at −196 °C [7] and many mutant strains can easily be obtained from the *C. elegans* Genetics Center (http://biosci.umn.edu/CGC/).

The *C. elegans* genome has been completely sequenced and contains approximately 18 000 genes, many of which have human homologs [8]. Double-stranded RNA (dsRNA) interference can be accomplished relatively easily, i.e. by feeding worms with bacteria expressing dsRNA for the gene of interest, thereby causing a loss of function phenotype [9]. When *C. elegans* was introduced as a model organism by Brenner et al. over 30 years ago, it was intended mainly as a convenient model for investigations into developmental pathways and neurobiology [7]. Since then it has become a widely accepted model and discoveries made in *C. elegans* have been awarded with Nobel Prizes in 2002 for work on the genetics of organ development and programmed cell death, in 2006 for RNA interference and in 2008 for work on GFP, some of it in *C. elegans*.

1.2. Worms and aging

Nematodes were soon appreciated as convenient model organisms for aging research, in part due to their short lifespan, morphological simplicity, ease of maintenance and of genetic manipulation [5,10,11]. While some aspects of human aging, such as increased cancer risk or loss of stem cell function, are not easily modelled in *C. elegans*, there is evidence that some key mechanisms of aging may be "public", i.e., likely to be conserved across large

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evolutionary time-spans [12]. One such putative public mechanism of aging is free radical-mediated macromolecular damage and mitochondrial decline, central mechanisms of aging proposed by the free radical theory of aging (FRTA) [13,14]. Detailed review of the FRTA is beyond the scope of this article, but can be found elsewhere [15–18]. However, below we outline some of the key *C. elegans* results in the context of the FRTA (for a more extensive review, see [19]).

1.3. C. elegans and the free radical theory of aging

The FRTA states that endogenous reactive oxygen species (ROS) cause aging. If this is true, then modulation of endogenous antioxidant defences or supplementation with antioxidants ought to be promising strategies for delaying aging.

Some evidence from mutations affecting *C. elegans* lifespan supports the FRTA. For instance *mev-1* mutants, which carry a mutation that affects mitochondrial complex II and increases ROS production, are hypersensitive to oxygen, show elevated levels of oxidative damage, accumulate abnormal mitochondria and are short-lived [20].

Conversely, many long-lived aging mutants show increased resistance to oxidative damage [15,21,22]. The *daf-2* mutants, carrying mutations in the *C. elegans* insulin/insulin-like growth factor (IGF) receptor, are long-lived and express elevated levels of many stress response and antioxidant genes, including at least one of the two mitochondrial superoxide dismutase (SOD) genes (*sod-3*), responsible for dismutation of superoxide into oxygen and hydrogen peroxide [23–26]. Expression of some of these antioxidant genes, including *sod-3*, appears to be required for the full lifespan extension associated with *daf-2* mutations [24]. Another gene whose expression is elevated in *daf-2* mutants is *gst-10,* which encodes a protein involved in the detoxification of the lipid peroxidation product 4-hydroxy-2-nonenal (HNE). While overexpression of *gst-10* increases oxidative stress resistance and extends lifespan in wild-type (WT) worms, RNAi silencing of *gst-10* shortens lifespan and increases HNE toxicity in both WT and *daf-2* animals [27,28].

On the other hand, it has recently been reported that genetic inactivation of any of the five forms of superoxide dismutase (SOD) in *C. elegans*, does not lead to significant shortening of WT lifespan [29,30]. In fact, inactivation of the *sod-4* gene encoding a cytosolic form of SOD even causes lifespan extension in *daf-2* mutants, possibly suggesting a signalling role for superoxide in this setting [29,30], although compensation by changes in other SOD isoforms has also been shown [31], and so these observations need to be interpreted carefully.

Overall, these results support the concept that antioxidants and oxidative stress are relevant in aging, although, whether lifespan extension depends on modulation of oxidative damage remains to be proven. Loss of function mutations in the *clk-1* gene, which is necessary for the synthesis of coenzyme Q (CoQ), have also been found to extend lifespan [32]. CoQ has antioxidant properties (at least *in vitro*) but is also required for proper functioning of the mitochondrial electron transfer chain (ETC) in *C. elegans* (see also Section 3.1). The lifespan-extending effect of the *clk-1* mutation is therefore consistent with the large number of mitochondrial ETC genes whose inactivation leads to lifespan extension as revealed by RNAi screens [33]. However, these results are difficult to interpret. While reduction in membrane potential, for instance, can reduce ROS production from the ETC [34], metabolic and compensatory effects are difficult to separate from these "indirect" antioxidant mechanisms. Using an RNAi dilution strategy to modulate mitochondrial ETC gene expression, Rea et al. [35] showed that decreased expression of several ETC genes during L3/L4 larva stages results in complex target-gene and dose-dependent lifespan effects. Reduction of expression beyond a gene-specific threshold level (typically to 40–60% of WT levels) resulted in lifespan extension of up to 70%. While some tradeoffs consistent with lowered metabolism (smaller size, delayed development, lower fecundity) were reported, no clear correlation of lifespan extension with oxidative protein damage, nor modulation of lifespan extension by the *C. elegans* oxidative stress response transcription factor (SKN-1) was detected [35]. However, mitochondrial function, mitochondrial metabolism and mitochondrial ROS production were not directly determined.

In summary, genetic support for the causative role of ROS in aging is suggestive but somewhat ambiguous in *C. elegans*. Nevertheless, there is reasonable evidence for age-dependent accumulation of ROS-mediated damage products [36–38], mitochondrial abnormalities and mitochondrial dysfunction as well as decreased metabolic capacity [37,39–42] in *C. elegans*. Excellent tools exist for the characterization of some of these age-dependent changes [43].

Despite these issues, antioxidants are still often considered as a promising strategy for modulating aging and extending lifespan. However, antioxidant studies in mammals give ambiguous results and are expensive (reviewed in [16,17]), hence the interest in identifying antioxidants with lifespan-extending efficacy in *C. elegans.* For this approach to succeed, methodological challenges must be addressed. First, studies need to provide robust data on lifespan effects that can be reproduced in other laboratories and are free from artifact. Second, the observed effects need to be attributable unambiguously to antioxidant efficacy, which requires measurement of oxidative damage in parallel with effects on lifespan. Some of these challenges are discussed below.

Genetic differences can exist in independent isolates of "N2" worms and significant genetic drift, affecting, amongst other traits, nematode lifespan, can occur when animals are maintained over many generations under laboratory conditions [44]. It is therefore important to regularly refresh lab cultures from frozen stocks, derived after minimal passage from the CGC reference strains and to backcross newly generated mutant strains into the CGC N2 male stock [44]. More information on methodological considerations, including advice on genetic inhomogeneity and drift can be found in [45,46] online (www.wormbook.org), (http://biosci.umn.edu/CGC) and (www.wormbase.org) as well as in print [47].

2. Lifespan studies: simple or deceptively simple?

The relative ease and apparent simplicity with which lifespan data can be obtained has led to an increasing number of investigators to use *C. elegans*. However, this apparent simplicity can belie some of the complexities that exist. There is evidence from the literature as well as our own experiences that ensuring the robustness of lifespan experiments can be challenging. For instance, Melov et al. [1] reported that compounds designed as synthetic superoxide dismutase/catalase mimetics (SCMs) could extend mean nematode lifespan by more than 40% in liquid culture, supporting the FRTA [1]. However, attempts at independent replication of these data, both using liquid culture and solid nematode growth medium (NGM), have failed [3,4], although lifespan extension under the original culture conditions was later confirmed by some of the original investigators [2]. These compounds did not extend lifespan in other invertebrate systems [48,49].

The reasons for this discrepancy are unclear. One suggestion is that the original culture conditions might have exposed animals to elevated oxidative stress [46]. However, the lifespan of control worms in the original report does not appear abnormally low and treated animals appear long-lived compared to the typical lifespan under standard conditions (see Section 2.1.3). The SCMs, given their complex chemistry, may have complex biological effects and might more accurately be called "redox modulatory

compounds" [16]. Since recent evidence suggests that superoxide dismutase might generally not be as important a determinant of lifespan as previously believed [30], the difference in outcome does suggest that culture conditions might indeed have been unusual in some way or that the observed effects might have been independent of the supposed SOD mimetic efficacy.

In fact, all laboratory culture conditions are far from "normal" for *C. elegans* [50]. The general assumption, nevertheless, is that aging mechanisms might be universal enough that they can still be investigated as long as conditions are standardized. However, the above example illustrates that even well established *C. elegans* researchers might not easily come to a consensus regarding lifespan effects of compounds. Even when "standard" conditions are used, a number of issues and challenges exist, some of which are discussed below.

2.1. Specific points on lifespan studies

2.1.1. Generating and maintaining synchronized cultures

Synchronization of cohorts is vital for lifespan studies. Two methods are commonly used. One is by hypochlorite treatment, essentially dissolving gravid adults in bleach solution to leave only eggs that then give rise to a synchronized cohort (for detailed protocols see [51]).

Smaller numbers of synchronized worms can be obtained by placing a few gravid adults on a hatching plate, allowing them to lay eggs for only a short period of time [52]. Apart from the smaller numbers of offspring obtained, a potential issue with this "egg lay" method is that great care needs to be taken to remove all of the adults (by counting the number of worms placed/removed) and that offspring derive from only a small number of individual adults. Whichever approach is taken, synchronized offspring should subsequently be randomly distributed to treatment plates, to avoid systematic differences in bleach or egg lay batches. In a variation on this method, eggs can be allowed to hatch in liquid culture in the absence of food, leading to L1 arrested larvae. These arrested larvae develop into highly synchronized adult cohorts upon exposure to food [53,54]. While this approach can be used when a high degree of synchronization is required (e.g. for investigations into development), it should be appreciated that the same signalling pathways that modulate stress resistance, dauer formation and aging in *C. elegans* also govern this L1 arrest [55]. Even though there is evidence that lifespan of starved L1 larvae (post re-feeding) is conserved [56], the possibility cannot be excluded that early starvation might affect some aspects of aging and stress resistance later in life.

Once generated, synchronized cultures need to be maintained, that is, offspring have to be prevented from overcrowding the aging cohort. Three approaches are commonly utilized. The conceptually easiest approach is to move adults, ideally daily, to fresh plates until reproduction ceases [57]. The advantages are that no drugs or mutant strains are required and the risk of confounding factors is thereby reduced. One disadvantage, apart from a higher workload, is that it is possible to accidentally transfer second-generation larvae along with parents. If not detected, this could cause a dramatic artifact, particularly affecting maximum lifespan as the second-generation worms will typically be between three and seven days younger than the parent cohort. Every transfer carries some risk of injury, death or loss of worms. If large batches of worms have to be grown, transfer of individual worms is generally not a practical option.

A second way of maintaining a synchronized culture is the use of 5-fluoro-2′-deoxyuridine (FUdR) [54,58]. FUdR inhibits DNA synthesis and kills dividing cells. Since cells of the adult worm are largely post-mitotic, FUdR treatment kills larvae and prevents progeny development but does not kill adults [11,54,58]. FUdR has been shown to prevent progeny growth in both liquid culture and on solid nematode growth medium (NGM) agar plates and has been used at concentrations between 25 μM (in liquid culture) and as high as 5 mM without evidence for a decrease in lifespan [54,58,59]. In fact, FUdR treatment at 400 μM on monoxenic NGM plates has been claimed to cause a slight (7%) *increase* in lifespan [54].

However, the timing of transfer is critical as the larva stages are highly sensitive to FUdR. Therefore, nematodes should be transferred to FUdR plates only just before they reach maturity, that is, shortly before they start laying eggs. If nematodes are transferred too early, severe abnormalities result [54,58,60]. If the cohort is transferred too late, offspring can hatch despite FUdR. Moreover, even in adult nematodes there is evidence that FUdR does affect some experimental parameters including causing a slight reduction of pharyngeal pumping rates, changes in body size and morphology and even that it increases superoxide dismutase levels [54,58,60,61]. While FUdR therefore appears not without side effects, it has been shown to have only mild lifespan effects even at 5 mM [59]. There are also reports that key aspects of the aging process in FUdR-treated animals are qualitatively unchanged [39,54].

FUdR is today widely utilized on solid NGM at concentrations between 40 and 500 μM (Table 1a). In some of our studies investigating the effect of "antioxidant" plant extracts on lifespan, we found that there were occasionally larvae that seem to escape the action of FUdR at low concentrations (⩽50 μM), possibly due to its interaction with components of certain extracts. Our laboratory therefore currently utilizes FUdR at 200 μM and up to 500 μM for compound screens. It seems advisable to keep FUdR levels to the minimum that will reliably prevent progeny production in a given setting.

A third method for maintaining synchronized cohorts utilizes temperature-sensitive fertility or egg-laying mutant strains. These mutants produce progeny normally at a permissive temperature (typically 15 °C or 20 °C), but are sterile at a higher restrictive temperature, typically 25.5 °C [62]. Use of such strains has the advantage that no drugs are required and that large batches of synchronized worms can be grown relatively easily. However, the mechanism of action of some fertility mutations has not been fully elucidated and it is possible that they cause additional effects on worm physiology. Also, worm lifespan is shortened at elevated

Table 1
Mean lifespan reported for control animals in 12 studies designed to examine lifespan effects of compounds or extracts. Studies were selected because they utilized N2 WT worms and reported standard culture conditions (20 °C, monxenic *E. coli* culture) either on solid NGM (a) or in liquid medium (b). Means of controlling progeny is indicated as either FUdR treatment or transfer of adults to fresh plates.

	Reference	Mode of controlling progeny	Medium of growth	Mean lifespan (days)
(a)				
1	[80]	FUdR (500 μM)	NGM	21.9
2	[128]	FUdR (50 μM)	NGM	14.6
3	[129]	Transfer every 2 days	NGM	18.7
4	[130]	Transfer daily	NGM	17.1
5	[131]	Transfer every 2 days	NGM	18.4
6	[132]	FUdR (40 μM)	NGM	12.8
7	[127]	Transfer daily	NGM	12.4
8	[107]	Transfer every 2 days	NGM	16.7
9	[122]	Transfer daily	NGM	20
			Mean ± S.D.	17 ± 3.2
(b)				
1	[133]	Transfer daily	Liquid culture	21.1
2	[134]	Transfer every 2 days	Liquid culture	19.3
3	[135]	FUdR (120 μM)	Liquid culture	23.3
			Mean ± S.D.	21 ± 2.0

temperatures [59], and contaminating organisms tend to grow faster. It is therefore possible that the higher temperatures required might sometimes affect experimental results (see Sections 2.1.2 and 2.1.4). Furthermore, there have been suggestions that some temperature-sensitive mutants are not entirely sterile at the restrictive temperature but can produce up to 0.1 progeny per adult [62,63]. While this might seem low, it could result in 10% of the aging cohort actually being from the next generation. Since maximum lifespan is often determined as the mean of the longest living 10% of the cohort, this could cause significant artifact [136].

2.1.2. Contamination and the need for strictly monoxenic culture

Lifespan/aging studies using rodents have had a long history and some of the insights gained may be applicable to nematodes. Thus, it has been pointed out that animals used for such research should be housed under pathogen-free conditions to avoid confounding effects due to disease [64] or changes in gut flora. Nematodes are usually grown on monoxenic NGM plates or liquid culture using as a food source the uracil auxotroph *Escherichia coli* (*E. coli*) strain OP50, whose growth is limited on NGM plates [7]. It is known that various pathogenic strains of bacteria can reduce nematode lifespan and may even increase oxidative stress [65,66]. However, treatment with antibiotics as well as inactivation of bacteria can extend nematode lifespan even on OP50 monoxenic NGM plates, possibly due to some residual pathogenicity even of OP50 [67–69]. Also, antibiotics can sometimes exert antioxidant effects, and this must be borne in mind [16,70]. Usually monoxenicity is therefore maintained without the use of antibiotics.

While *C. elegans* can be maintained axenically, development, fecundity and lifespan as well as metabolism and stress resistance are all affected under such conditions, probably due to some nutritional requirement for metabolically active microbes or an unknown heat-labile component [71,72]. Heat- (e.g.: 65 °C for 30 min [73]) or gamma-inactivated OP50 can be utilized to reduce bacterial metabolism, for instance when compounds are tested which might be metabolized by *E. coli*.

While the current consensus in the *C. elegans* community seems to be that monoxenic OP50 *E. coli* culture at 20 °C without antibiotics or antifungal agents constitutes "normal" conditions, this poses demands on maintaining sterility. WT worms can live up to 40 days at 20 °C and any contamination has ample time to grow and affect worm survival, especially in the later phases of lifespan experiments. Even with great care, contaminations can occur [58,62]; one occasionally encountered virulent mould is known as "the orange death" [51]. In liquid culture fungal contamination is a common problem and can occur even with the use of antibiotics [58,62]. In lifespan studies, contaminations can be difficult to control, as old worms are likely to be killed if transferred repeatedly, making it difficult to remove the contaminating organism. This challenge is worsened when mutant strains are utilized at their restrictive temperature, typically 25 °C, where contaminations tend to grow more rapidly. In addition to careful sterile technique and frequent checking of stocks, a dedicated, well-maintained horizontal laminar flow hood for all worm handling and microscopy is essential.

2.1.3. Food density

Caloric restriction (CR) is defined as significant reduction of calorie intake relative to an "ad libitum" diet while maintaining adequate intake of vital nutrients. CR has been shown to extend mean and maximum lifespan across different animal species, to decrease oxidative damage [74,75], and to delay the onset and progression of age-dependent diseases, including diabetes, autoimmune diseases, sarcopenia and cancer [74–76]. In nematodes, CR in its strictest sense is not easily achieved as reduction of *E. coli* is likely to reduce not only calories but also key nutrients. Restriction of food/specific nutrients is referred to more generally as "dietary restriction" (DR). Various forms of moderate DR, including restricting some individual vital nutrients, have been shown to extend lifespan in *C. elegans*. DR has been achieved using methods such as bacterial dilution of OP50 food stock on NGM plates or in liquid culture, genetic reduction of food intake by pharyngeal pumping-deficient "eat" mutants, control of bacterial growth on NGM plates as well as control of available nutrients in axenic culture [5,71,77,78]. While there is general agreement that DR can extend lifespan in *C. elegans* by 20–80%, there is still uncertainty regarding the role of different signalling pathways [79]. An attempt has been made to define optimized protocols for CR experiments [79].

While DR experiments are an important tool for research into aging, unintended variations in food availability have the potential to cause artifacts in terms of lifespan, markers of oxidative damage, stress resistance as well as developmental schedule and fecundity. Since *C. elegans* do not compensate for low food density by increasing pharyngeal pumping, nutritional intake is proportional to bacterial cell density in the medium [79]. One approach to control cell density directly is to grow OP50 *E. coli* overnight in liquid Luria–Bertani medium at 37 °C and to determine bacterial cell density by spectrophotometer. Stocks can then be concentrated to the desired cell density, aliquoted and frozen at −80 °C until needed. This is necessary if inactivated bacteria or antibiotics are to be used, as overnight growth on NGM plates is not possible in this case. OP50 cell number density considered "ad libitum" on NGM plates varies between labs [5,77,80]. We have found OP50 stock concentrated to 10^{10} bacterial cells per ml to work well, because it is well above DR levels (see below) but low enough not to interfere with microscopic observation. Others have considered "ad libitum" levels that are lower (10^9 bacterial cells per ml) or higher (10^{11} bacterial cells per ml) but significant lifespan extension by DR was detected below 10^8 cells per ml with starvation setting in at 10^7 bacterial cells per ml [5,81]. Bacterial cell density should be consistent across plates and between experiments.

After preparation, plates should be sealed and, especially when intended for lifespan experiments, kept at 4 °C for at most 3 weeks (ideally less). *C. elegans* will consume and redistribute food on NGM plates, necessitating the occasional transfer of worms onto fresh plates, especially during the earlier phases of lifespan studies. This should be done well before worms finish most of the available food and become restricted. Ideally the number of worms should be consistent between plates and plates should be from the same batch to avoid systematic differences in food availability and/or in the number of transfers required.

2.1.4. Hormesis

Exposure to low level stressors, including oxidative stress, can up-regulate stress response mechanisms, compensating (sometimes even overcompensating) for the stress originally encountered. This phenomenon, known as "hormesis", can result in increased stress resistance, enhanced damage repair and sometimes lifespan extension in diverse species in response to a range of stimuli [82]. In *C. elegans*, hormetic lifespan extension has been shown in response to hyperoxia, increased ROS production, UV and ionizing radiation and heat shock [83–88].

While hormesis, like DR, is an exciting avenue for research into mechanisms of aging [82] unintended heat or oxidative stress can lead to artifact. Lifespan extension and significant changes in the transcription of stress response genes have been shown after as little as 2 h at 30 °C [85]. Sometimes, especially when beginning work on nematodes, no dedicated 20 °C incubator is available and improvised or "spare" (often old) incubators are used – even wine coolers have been suggested for this purpose. Care must be taken to ensure that temperatures are correct, do not fluctuate

when environmental temperatures change and are spatially homogeneous inside the incubator. Simple thermometers are often inaccurate to within 1 °C. Also be careful to avoid accidental heat shock. The stage of a stereomicroscope can reach 30 °C or above if a "cold" light source is not used. Plates should never be placed (even temporarily) on light-source boxes, close to monitors or computer cases or any other device prone to heating up. Finally, a Perspex box, laminar flow hood or Petri dish in direct sunlight can easily heat up to temperatures above safe levels. The opposite problem can occur when NGM plates are stored at 4 °C prior to use. Do allow equilibration of plates to 20 °C before transferring worms.

For oxidative stress, a different hormetic effect is of concern. Pre-exposure to conditions inducing elevated oxidative stress can cause up-regulation of endogenous ROS defence systems with subsequent increased resistance to further oxidative challenge and to other stressors. In some cases pro-oxidant treatments have been shown to be associated with extended lifespan. For example exposure of nematodes to the ROS-generator juglone [16] can lead to increased levels of reduced glutathione (GSH) and extension of lifespan [86]. In *C. elegans*, a major transcription factor mediating the response to acute oxidative challenge is SKN-1, the *C. elegans* ortholog of the mammalian Nrf2 transcription factor [89]. GFP-reporter-gene constructs have been generated that can be utilized to detect, for example SKN-1 mediated antioxidant responses to putative pro-oxidant compounds [90].

In summary, nematodes do not maintain their body temperature and respond sensitively to changes in their environmental temperature or elevated stress. Since elevated stress can be protective against endogenous damage and can cause lifespan extension, great care needs to be taken to avoid artifacts associated with these responses, especially when screening antioxidants and other putative lifespan-modulating compounds (see Section 3).

2.1.5. Operator bias

Human expectation affects subjective experience, judgment and perception, mostly without the individual being aware of it. This matters, for instance, in clinical trials where even the color of the medication given can affect outcome, presumably through affecting patient expectations [91].

In general, randomized trials without blinding tend to show larger treatment effects than blinded studies [92]. Hence double-blind, placebo controlled studies are standard in clinical investigations. However, perceptions of scientist–observers in laboratory settings are also influenced by what the observer believes that he/she knows. For example, observers provided with two samples of *Planaria* worms, one of which was described as "low-response-producing" and the other as "high-response-producing", found on average 5 times more head turns and 20 times more contractions in the supposed "high response-producing" sample, despite the fact that both samples contained identical *Planaria* [93]. Is there reason to believe that observer bias might pose a problem for *C. elegans* lifespan studies? Observer bias is particularly problematic if any element of the experimental set-up is based on a subjective assessment (judgment). In *C. elegans* lifespan studies, the observer has to decide at each time-point whether each worm is still alive. Worm survival is typically scored by touching each worm using a platinum wire (worm pick). Worms are scored as alive if this elicits a response or dead if it does not [52]. Old worms, however, become sluggish and are often partially immobilized [94]. Moreover, old worms become more prone to injury and can be killed by rough handling such as vigorous and repeated prodding. Not only does the outcome of survival scoring therefore depend on operator judgment, survival itself can be influenced by subtle differences in the care taken during scoring. Indeed, an additional death criterion (turgor pressure) was considered necessary when lifespan studies were first utilized but has since been largely discontinued [52]. Blinding of lifespan studies, though having been pointed out as highly desirable previously [45], does not seem to be routinely performed in *C. elegans* (recent exceptions: [95,96]). In our own laboratory, compulsory blinding of all *C. elegans* lifespan studies was found to increase consistency between runs and between operators. Randomization and blinding also prevent other potential confounders such as systematic timing effects. Treatment and control plates should always be handled, scored and assayed in parallel. For example, if control plates are always handled before (or after) treated/mutant plates, issues such as rising temperature or decreasing operator alertness might systematically affect outcome.

2.1.6. Some considerations regarding n-numbers

Lifespan studies are time consuming and can be tedious. It has been estimated that an experienced investigator might, under ideal conditions, be able to manually score 10 worms per minute [97]. However, in our experience, when taking into consideration overhead time (unsealing and resealing plates, finding of lost worms) as well as various forms of trouble-shooting (old or buried worms, contamination, transfer if required), this can easily drop to 2–3 worms per minute.

High throughput systems for automated determination of lifespan have been described [97,98]. A well established and commercially available system utilizes liquid worm culture and automatic worm-handling technology (in liquid buffer) and allows determination of fecundity, growth, developmental stage and, utilizing a fluorescence dye (SYTOX green) in conjunction with online fluorescence detection, determination of lifespan [97,98]. While these approaches allow lifespan studies with 100 000s of individuals and open up the possibility of very large scale screens [99] as well as novel insights into aging [100], the use of liquid culture suffers from the drawbacks mentioned in Sections 2.1.2 and 2.1.3.

For manual scoring, there is often a trade-off between the number of animals used and the number of different conditions/strains that can be tested. Some lifespan studies have been published based on only 20–70 animals per repeat (although three repeats are usually reported). Apart from basic consideration of power analysis in terms of largest detectable effect, low *n*-numbers pose additional problems.

For one, maximum lifespan is commonly assessed as the mean lifespan of the longest living 10% of a given worm population. However, maximum lifespan is sensitive to right tail outliers in the survival distribution and even single worms can make a significant difference in sets with small sample size. In such sets, there are simply too few data to detect anything but major deviations from normality [101]. In other words, with small n-numbers, even if normality is formally tested, it is difficult to ensure that there are no outliers that might affect statistical analysis. Any of the artifacts discussed above can cause such deviations from normality. To explore the impact of sample numbers (worm count) on statistical inference, we here utilize experimental data from one of our recent studies. Two worm populations were considered: (a) untreated wild-type (WT) N2 and (b) worms treated with an experimental drug (Fig. 1). This drug has repeatedly been shown in our laboratory to have a small but significant effect in increasing mean and maximum lifespan. Instead of choosing our "best" dataset, we choose this particular dataset (Fig. 1) because it exhibits typical features of real data. In particular, there were some unexplained early deaths in the treatment group. While these were limited to 1 of 20 NGM plates, this unexplained problem reduced statistical significance particularly with respect to the mean lifespan. Despite this problem, the data passes Lilliefors test of normality at a significance level of 5% [101]. Furthermore, Kaplan–Meier statistics show that the two worm populations are distinct ($P < 0.003$) and that the maximum lifespan (de-

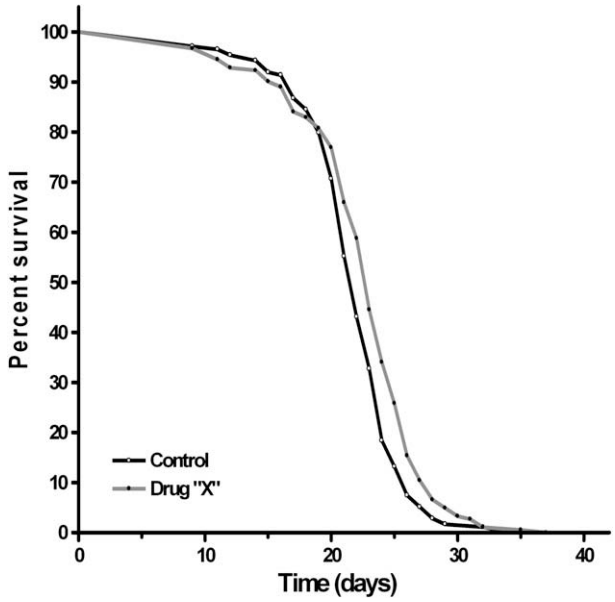

Fig. 1. Lifespan experiment showing the effect of an experimental drug "X" on age-dependent survival of N2 *C. elegans* under "standard" conditions (20 °C, monoxenic live *E. coli* @ 10^{10} cells/ml, solid NGM plates). Lifespan curves show survival of N2 worms treated with the drug "X" ($n = 183$) in comparison to N2 control worms ($n = 174$). The drug treatment caused a significant increase in age-dependent survival (log-rank test, $P = 0.003$). Maximum lifespan increased significantly ($P < 0.03$) from 28.4 to 30.3 days with drug treatment, while mean lifespan was not significantly different between treated (22.5 days) and control (21.6) animals. Note the early deaths in the drug "X"-treated group (for details see Section 2.1.6).

fined as the mean of the longest lived 10% in each group) also is significantly longer in treated worms ($P < 0.03$).

We began this study with 220–250 worms per group to ensure that even after losses we would obtain data from about 200 animals. To illustrate the impact that a smaller sample size would have had, we randomly choose 10 000 separate subsets, each of 25, 50, 75, 125 and 150 worms from the treated and control groups and repeat the statistical analysis for all pairs of these subsets. Fig. 2 illustrates the result for maximum lifespan. At lower sample numbers there is considerable overlap between the distributions. When only 25, 50 or even 75 worms are considered one is likely to detect no effect at all and it is even possible to obtain an apparent toxic effect (shortening of maximum lifespan). Fig. 3 shows that even when 100 worms are selected, the lifespan curves can only be reliably identified as being significantly different in 50% of the cases.

Effective *n*-numbers depend on the number of worms for which an ultimate fate is known. Worms that crawl off plates, die of injury or suffer internal hatching of larvae are usually excluded (censored). Therefore, in order to maintain statistical power, the starting number of worms should be at least 10–20% higher than the intended final n-number. The number of worms that is appropriate depends on the size of the expected effect as well as the amount of variability, but even for preliminary screening and dose response studies we try to obtain data on at least 100–200 individuals. As with all assays, at least three independent, blinded repeats of any lifespan experiment need to be run before conclusions can be drawn. Therefore, screening a single compound will usually require thousands of worms, each scored daily or every other day over 20–40 days.

2.2. Do these challenges matter?

To explore the question of reproducibility in lifespan studies, we surveyed compound screening studies reported between 1988 and 2009, revealing 12 studies that reported mean lifespan values for control (untreated) N2 WT worms under standard conditions (20 °C, fed on OP50). Nine of these were carried out on solid NGM (Table 1a) and three in liquid culture (Table 1b). The data reveal a high variability under what are supposedly comparable assay conditions. Considering the NGM results, the values for mean lifespan range from 12.4 days to 22 days, average 17 ± 3.2 days (Table 1a). Indeed, the mean difference between labs (2.5 days) is comparable in magnitude to the typical treatment effect reported. Lifespan in the three liquid culture studies was somewhat longer (average 21.2 ± 2.0 days). These data indicate that there is a significant lab-to-lab variability.

Some data from our own laboratory further serve to illustrate some of the challenges discussed above. *C. elegans* work in our laboratory was initially carried out in a shared facility lacking a dedicated horizontal laminar flow hood, cold light microscope and accurately-temperature controlled incubator. After transferring to a dedicated nematode facility, we noticed an increase in mean and maximum lifespan of our N2 controls from 18 and 28 days to 22 and 30 days, respectively, plus a decrease in problems associated with contamination. Moreover, we re-investigated lifespan effects and associated fitness tradeoffs of the polyphenolic phytoalexin resveratrol. Resveratrol was previously shown to extend nematode lifespan [73], whereas others were unable to detect any lifespan extension in *C. elegans* [96]. Using our original facility, we found that resveratrol could extend mean lifespan by almost 50% while showing a fitness trade-off in the form of delayed egg laying [102]. However, in our dedicated nematode facility, the lifespan-extending effect of resveratrol, while still detectable, was significantly smaller, although the effect on egg laying remained unchanged (unpublished observations). Thus confounded data can result from suboptimal conditions.

3. Testing antioxidants and other agents

C. elegans has been utilized for testing the effect of compounds on lifespan for almost as long as it has been used as a model organism [103], and several lifespan-extending compounds have been identified [46,104]. However, it has also been known for a long time that "Drugs do not readily work on *C. elegans*" [105] meaning that drug delivery, bioavailability and timing are often a problem [46,106]. For example, the internal concentration of ethosuximide in worms being treated with 2 mg/ml of this anticonvulsant drug was found to be only 30 μg/ml, or 1.5% of the external concentration [107]. Secondly, *E. coli* are efficient at metabolizing compounds including many polyphenolics of interest with respect to their putative lifespan modulatory efficacy [46,108]. While the latter effect may be ameliorated by utilizing heat-inactivated bacteria, these challenges pose the risk of failing to identify efficacy simply because a compound does not reach the tissue or compartment of interest. It is therefore important to investigate this, especially when interpreting negative effects. If the compound of interest targets a specific pathway or gene this can sometimes be achieved using reporter-gene or knockout strains. For antioxidants, a similar approach can also be utilized by showing lifespan extension in the *mev-1* strain or under exogenous ROS challenge (for more on confirming antioxidant mode of action, see Section 3.1). Of course, physiological effects are possible even if compounds are not detectable in whole worm lysates. For instance the ASI sensory neurons have been shown to be involved in lifespan extension in response to caloric restriction in *C. elegans* [109]. Modulation of sensory neurons by compounds might cause physiological changes without these entering cells.

Drugs sometimes also have other indirect or artifactual effects on lifespan. For example, compounds and extracts can affect the growth of OP50 *E. coli* (if live bacteria are used) or of contaminating,

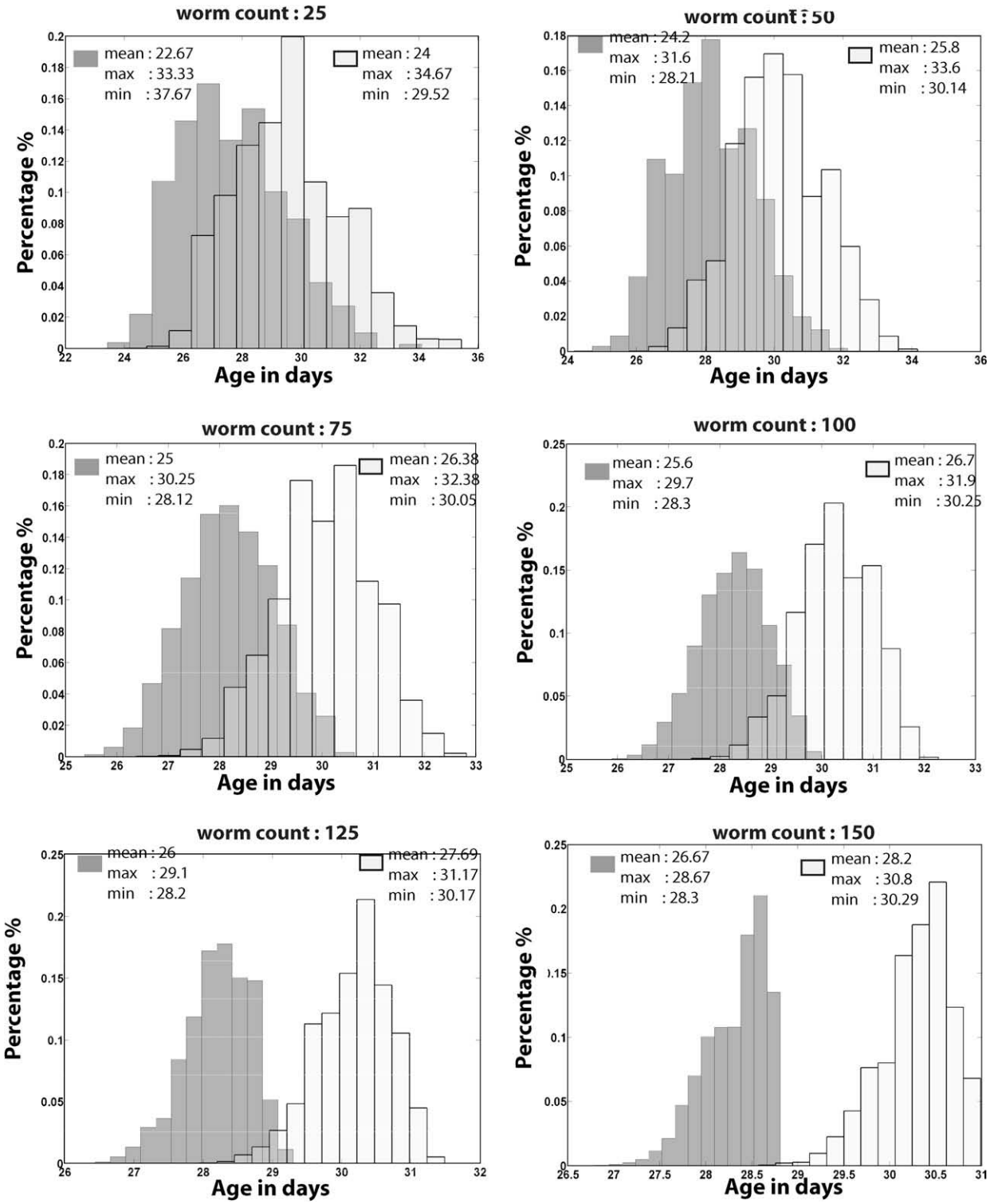

Fig. 2. Effect of sample size on maximum lifespan statistics when sub-sampling the experimental population of worms shown in Fig. 1. Sub-populations of between 25 and 150 animals were drawn from the original treated and untreated populations. The maximum lifespan of each of these sub-populations was determined as the average of the longest surviving 10% in that sub-population and the statistical significance of the treatment effect was determined for each pair of sub-populations (see Fig. 3). This process was repeated in silico 10 000 times for each sub-population size (25, 50, 75, 100, 125 and 150 animals). The histogram with grey bars represents the wild-type control worm populations while the white histogram represents the treated worm populations. Histograms show the distribution of maximum lifespan values within the 10 000 populations generated. The ''mean'', ''max'' and ''min'' values are the average, largest and smallest maximum lifespan values found in the 10 000 sub-samples of the original population. As expected, the degree of separation between maximum lifespan distributions of treated and control groups decreases with decreasing sample size, resulting in increased uncertainty. Also, it is interesting to note that for sample sizes below 75, the degree of overlap between populations is large, illustrating that it is relatively likely to detect an apparent toxic effect (although probably not statistically significant), even though the actual effect is protective.

pathogenic microorganisms. Resveratrol, for instance, has anti-fungal properties in addition to its alleged antioxidant activity [110]. Furthermore, many polyphenolic compounds are unstable in culture media, for example generating hydrogen peroxide

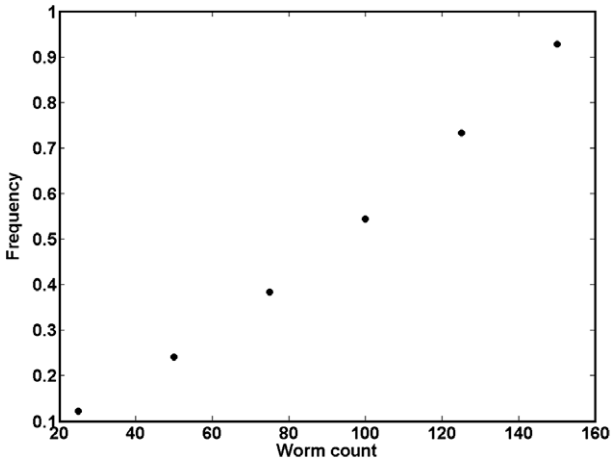

Fig. 3. Frequency of in silico trials that show statistically significant differences in survival as consequence of treatment. The figure illustrates the expected result, that the outcome of hypothesis testing has a strong dependence on sample size. The frequency of statistical significance was obtained for each sample size by comparing each pair (treated vs. control) of the 10 000 sub-populations using Kaplan–Meier analysis for inferring distinct populations at a significance level of 0.05. The analysis confirms the visual impression (Fig. 2) that, for this particular experiment, population sizes <100 would have yielded non-significant results in over 50% of cases.

(H_2O_2) [111,112] and are likely to be so in NGM. Effects attributed to such compounds may therefore be due to their oxidation products or due to hormetic effects (Section 2.1.4), e.g. of H_2O_2. Many other compounds can act as mild stressors thereby inducing a hormetic response, which can affect gene expression patterns, stress resistance, activity levels, fecundity and lifespan [86]. Foul tasting or otherwise unpleasant compounds might affect feeding behavior and reduce food intake, again potentially inducing DR. They may also cause treated worms to crawl off plates, leading to systematic differences between treatment and controls.

It can also be misleading to report effects on lifespan at a single (the "most effective") dose of a compound since there is often complex concentration-dependency [80]. One example of this is an extract of the medicinal herb *Psoralea corylifolia* (PC) [113] investigated by us for its putative antioxidant and lifespan effects in *C. elegans*. PC shows some *in vitro* antioxidant capacity and, at low doses, causes significant lifespan extension. However, PC is also an inhibitor of the proteasome, cell division and mitochondrial function and at higher doses shows clear dose-dependent toxicity and reduction in lifespan [80,113,114]. Dose response studies should always be performed and reported.

3.1. Inferring antioxidant mechanisms

Many compounds are antioxidants *in vitro* ("test-tube antioxidants") and the label "antioxidant" is often assigned based simply on such *in vitro* efficacy. However, there are well known challenges in interpreting and extrapolating *in vitro* results to *in vivo* [80,112–118] and many *in vitro* antioxidants show limited or no antioxidant capacity in terms of reduction of oxidative damage *in vivo* [16,115–118]. One must exercise care when inferring *in vivo* antioxidant efficacy or attributing lifespan effects to antioxidant action. In fact, as redox active compounds, test-tube antioxidants have the potential to act as pro-oxidants in certain settings [16,112,118]. Antioxidants may thus cause lifespan modulation by antioxidant-independent mechanisms (including hormesis). In addition, if culture conditions induce elevated oxidative stress, treatment with antioxidants may result in lifespan extension, but this observation does not allow conclusions regarding antioxidant efficacy against endogenous ROS or anti-aging effects under "normal" conditions (Section 2). Therefore, even if treatment with a potent *in vitro* antioxidant causes significant lifespan extension, this does not prove an action by antioxidant mechanisms, nor does it provide support for the FRTA. Several criteria should be met before concluding that a direct antioxidant mechanism is responsible for an observed protective or lifespan-enhancing effect:

(i) Treatment, with the compound at the levels affecting lifespan should result in a dose-dependent reduction of oxidative damage as evaluated by appropriate biomarkers of oxidative damage (for discussion of biomarkers please see: [16,117,119]). For *C. elegans* the best established marker of oxidative damage to date is protein-carbonyl content (PCC) [38], and perhaps lipofuscin [36,37] but others need to be examined and we have yet to elucidate which are the key targets of oxidative damage in this organism.

(ii) Compound treatment should be protective under conditions of elevated oxidative stress, e.g. as induced by pharmacological means (e.g. paraquat challenge), genetic mutation (e.g. *mev-1*) or environmental manipulation (e.g. hyperoxia and radiation).

(iii) Inhibition of the mitochondrial ETC or slowing of metabolism can extend lifespan in *C. elegans* [33,35,120]. Therefore, compound treatment should not disrupt mitochondrial function, reduce metabolism, induce DR or inhibit nematode activity.

(iv) Other antioxidant-independent mechanisms should also be excluded. Compound treatment should not reduce pharyngeal pumping (potentially causing DR), should not delay development or fecundity and should not induce stress response pathways oxidative stress related or otherwise. The latter is testable, for example, using GFP-reporter-gene strains such as SKP1 related GFP strains [90] or the *hsp16.2::gfp* strain CL2070 [115].

Numerous reports suggest antioxidant- mediated lifespan extension, but in light of the discussion above, one may ask how strong the evidence in *C. elegans* for lifespan extension by antioxidants really is. The uncertainty surrounding the question as to whether the superoxide dismutase/catalase mimetics (SCMs) cause antioxidant-based lifespan extension, has been discussed above (Section 2).

3.2. What about other "antioxidants"?

One of the first to be explored was α-tocopherol (vitamin E), now suggested by some [121] to have multiple effects *in vivo* in addition to its antioxidant properties. In 1983, it was reported that treatment with α-tocopherol extends lifespan when begun at larva stage [103]. Although this work was carried out in axenic medium, lifespan effects of α-tocopherol were later confirmed on monoxenic NGM plates [122]. However, lifespan extension was associated with slowed development, suggesting that the effect might, at least, in part be due to developmental delay [122,123]. There is little evidence for *in vivo* antioxidant efficacy of α-tocopherol causing its effect on lifespan. Neither vitamin C alone nor vitamin C plus vitamin E extended lifespan in *C. elegans* [122].

Treatment of WT worms with tocotrienols, compounds structurally related to vitamin E, has been shown to result in lifespan extension and reduction in age-dependent accumulation of oxidatively modified protein, determined as protein carbonyls [124,125]. However, developmental timing or fecundity were not assessed in these animals. Since protein-carbonyl levels increase with age, it is possible that the reduction in protein carbonyls was in fact secondary to any such developmental effects.

The lifespan of *mev-1* mutants is increased by treatment with CoQ_{10} and this treatment decreased superoxide anion production in isolated mitochondria [123]. In the same study CoQ_{10} treatment of WT worms resulted in an 18% increase in lifespan. Thus, it might seem that CoQ has antioxidant efficacy *in vivo* while at the same time extending lifespan. However, paradoxically, *depletion* of some forms of CoQ by dietary intervention or genetic means also extends lifespan [32]. Considering that CoQ is an integral component of the mitochondrial ETC, this suggests that the effects of CoQ on *C. elegans* lifespan involve complex modulation of mitochondrial function in addition to (or perhaps instead of) a direct antioxidant mechanism [126]. They may also depend on the exact form of CoQ tested (for a more detailed review see [19]). Metabolic effects, indirect antioxidant action (modulating ROS *production*) and direct antioxidant action (detoxifying endogenously produced ROS) are difficult to distinguish when a putative chemical antioxidant is also an ETC modulator or mitochondrial nutrient. Similar comments may be made about the reported lifespan-extending effects of α-lipoic acid [127].

As a further illustration, we performed an *in vitro* antioxidant screen of 34 plant extracts and tested the six best to evaluate their *in vivo* antioxidant capacities as well as effects on lifespan in *C. elegans* [80,113]. We found that amongst the highest scoring *in vitro* extracts, *in vitro* antioxidant capacities were a poor predictor of *in vivo* antioxidant capacity [80]. We did, however, identify two compounds with significant effects on lifespan. Only one of these (pine bark extract) showed efficacy consistent with direct antioxidant mechanism in all assays performed, while the other seemed to modulate endogenous stress response pathways, possibly through a hormetic mechanism.

In summary, the question as to whether antioxidants protect against aging in *C. elegans* and, if so, whether they do this by antioxidant and/or other mechanisms, is still open.

4. Conclusion

Many of the principles, challenges and artifacts that we have discussed are well known to researchers familiar with *C. elegans*. However, we hope that others will benefit from this discussion, in particular investigators first using *C. elegans* for antioxidant and lifespan studies. Apart from remaining vigilant towards possible artifacts, trying to control the biological and physical environment as well as possible, blinding all studies and using sufficiently large n-number, one important advice is to record and report the details of as many of the relevant factors as possible. In particular, when interpreting and comparing lifespan results, it is important to know how synchronized cohorts were maintained, the exact culture conditions including the type and density of bacteria used, the number of animals per plate or well, the number of animals censored (and why) as well as the mean and maximum lifespan of controls.

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