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Calories or protein? The effect of dietary restriction on lifespan in rodents is explained by calories alone

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ABSTRACT

Almost exactly 100 years ago Osborne and colleagues demonstrated that restricting the food intake of a small number of female rats extended their lifespan. In the 1930s experiments on the impact of diet on lifespan were extended by Slonaker, and subsequently McCay. Slonaker concluded that there was a strong impact of protein intake on lifespan, while McCay concluded that calories are the main factor causing differences in lifespan when animals are restricted (Calorie restriction or CR). Hence from the very beginning the question of whether food restriction acts on lifespan *via* reduced calorie intake or reduced protein intake was disputed. Subsequent work supported the idea that calories were the dominant factor. More recently, however, this role has again been questioned, particularly in studies of insects. Here we review the data regarding previous studies of protein and calorie restriction in rodents. We show that increasing CR (with simultaneous protein restriction: PR) increases lifespan, and that CR with no PR generates an identical effect. None of the residual variation in the impact of CR (with PR) on lifespan could be traced to variation in macronutrient content of the diet. Other studies show that low protein content in the diet does increase median lifespan, but the effect is smaller than the CR effect. We conclude that CR is a valid phenomenon in rodents that cannot be explained by changes in protein intake, but that there is a separate phenomenon linking protein intake to lifespan, which acts over a different range of protein intakes than is typical in CR studies. This suggests there may be a fundamental difference in the responses of insects and rodents to CR. This may be traced to differences in the physiology of these groups, or reflect a major methodological difference between 'restriction' studies performed on rodents and insects. We suggest that studies where the diet is supplied *ad libitum*, but diluted with inert components, should perhaps be called dietary or caloric dilution, rather than dietary or caloric restriction, to distinguish these potentially important methodological differences.

Contents

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1. Background: early studies on caloric and protein restriction (PR)

The first paper suggesting an impact of food restriction on lifespan was published almost exactly 100 years ago (history of CR reviewed by Masoro, 2010). In 1917 Osborne and colleagues published a short paper showing that restricting the food intake of four female rats in their early life (between 1 and 17 months of age) retarded their growth rate, but had the benefit that their reproductive capacities in later life, and their total lifespan, were extended. The impact of the restriction of food on rats was presumed at the time to be due to the very obvious retarded growth of the food restricted animals. However, the evidence was confused. Some years earlier it had been shown (Slonaker, 1912) that feeding rats either a strictly vegetarian diet, or one with occasional meat supplements, had a profound effect on growth (the vegetarians grew much less), but the growth retarded individuals lived shorter lives. During the 1920s several groups also showed that growth can be impacted by altering the percentage protein in the diet (Osborne and Mendel, 1926; Hoagland and Snider, 1927): hence the question arose as to whether the impact on lifespan due to food restriction was specifically due to variation in the level of protein intake, or other dietary components, like the energy content. Supporting a role for protein as the mediating factor in the lifespan effect, restricting the growth of brook trout, by restricting their protein intake, extended their lifespan (McCay et al., 1929). In the 1930s, Slonaker made a systematic study of the impact of protein content of the diet on growth and other metabolic aspects of rats, culminating in a series of papers, one of which addressed the impact of protein content of the diet (varying between 10 and 26%) on lifespan (Slonaker, 1931). These papers suggested that growth was negatively, and lifespan was positively, impacted by lowered dietary protein contents. Hence the overall view at this point was that PR was the primary driver of the life extension effects of food restriction. However, it is worth noting that the diets with the lowest protein contents used by Slonaker also had the lowest fat contents (Slonaker, 1931).

Some years later McCay investigated the effects of food restriction on lifespan in rats confirming in a much larger sample than used by Osborne that reducing the intake of food extends both the median and maximum longevity (McCay et al., 1935). Given the effect of protein intake on lifespan of rats (Slonaker, 1931), and their own earlier work on brook trout (McCay et al., 1929), McCay's group was interested in what components of the food might underpin the lifespan enhancing effect of food restriction, in particular whether the impact was due to lowered protein intake. Following a set of further experiments they argued that the food restriction effect was unlikely to be due to lowered protein since the same extension of lifespan could be found when the diet contained 40% protein and hence under restricted conditions the animals still obtained more protein than when feeding on standard chow (McCay et al., 1939). These studies strongly suggested that the nutritional manipulation responsible for the life extending effect of food restriction was caloric intake, and thereafter food restriction studies became generally termed 'caloric restriction', and this term widely replaced 'food restriction' or 'dietary restriction' to describe the phenomenon (Speakman and Mitchell, 2011). Indeed McCay's study in 1935 is widely cited as the origin of the whole field of 'caloric restriction' (Masoro, 2010).

Studies of dietary impacts on growth and longevity proliferated in the 1940s and 1950s, but studies of CR were more rare, and focussed more into the effects of CR on specific diseases, such as cancer, rather than varying the macronutrient contents of the diets. Contrasting earlier work, additional studies of dietary composition suggested that different levels of protein intake had no impact on longevity (Kao et al., 1941). In addition, a study repeating the earlier work on vegetarian diets found no difference between vegetarian and omnivorous diets on longevity of rats (Carlson and Hoelzel, 1948), although this might have been because in this case the percent protein in the diet for even the vegetarian option was 30% (Carlson and Hoelzel, 1948). In addition to protein, interest was also directed towards fat contents of diets, with some landmark studies showing that there was a negative effect on lifespan of rats feeding on a high fat diet, compared with rats fed a high carbohydrate diet that actually supplied greater calorie intake (French et al., 1953). Hence, the extensive work of Slonaker (1931) implicating lowered protein intake as a driver for extended life, could in fact have been an effect of lowered fat intake (Slonaker, 1931).

In the 1960s there was renewed interest in the potential roles that might be played by different macronutrients in the 'CR' effect. This included the extensive studies of Ross (1959, 1961) and Ross and Bras (1971) on the impacts of protein to carbohydrate ratios, which indicated that in an unrestricted condition diets low in protein and high in carbohydrate resulted in increased lifespan. Animals fed diets with a low Protein:Carbohydrate ratio, however, spontaneously ate about 25% less food than mice fed diets high in protein, with variable carbohydrate contents, or with both low protein and carbohydrate together. So attributing this effect to altered macronutrient composition is difficult. When intakes were restricted the advantage of the low protein-high carbohydrate diet was eliminated, indicating that the magnitude of the benefit of CR for longevity was dependent not only on the level of calories that were fed, but also the composition. However, this conclusion is also confused by the fact that different levels of restriction were applied when the rats were fed the different diets. It was shown that commencing restriction slightly later in life, so as not to impact growth, had similar life enhancing effects, effectively dismissing the suggestion that the impact of CR was *via* growth retardation (Berg and Simms, 1960). Nakagawa and Masana (1971) varied the protein content of the diets of rats between 8 and 27% (Nakagawa and Masana, 1971) and found no impact on lifespan. Importantly in this experiment the total food intakes were identical across the different groups thereby avoiding any confounding effects of total energy intake. Hence this work further supported the notion that the effect of CR is independent of PR. Masoro and colleagues extended these studies throughout the 1980s (Yu et al., 1982, 1985; Masoro et al., 1989; Iwasaki et al., 1988). These comprehensive investigations showed a clear impact of 40% food restriction on lifespan, but that this life enhancing effect was almost identical if the protein content of the diet was altered so that protein intake was not simultaneously reduced. In addition, Davis et al. (1983) fed mice on three unrestricted diets containing 12, 20 or 28% casein, or restricted diets (by 66%) containing 18, 30 or 42% casein (Davis et al., 1983). Hence the mice on restriction had equal levels of protein in their intake as mice in the unrestricted groups. Using this design it was shown that caloric restriction extended lifespan, independent of the level of protein in the diet. Similarly, Horakova et al. (1988) showed that Fischer 344 rats with identical protein intakes, but different (restricted v non-restricted) calorie intakes, lived for different durations. By the end of the 1980s it was pretty much accepted that the CR effect on lifespan was mediated only *via* a reduction in energy intake, and that the contribution of different macronutrients, particularly protein, was negligible (summarised in Masoro, 1990; Shimokawa et al., 1996). Similar experiments also demonstrated that fat is probably also not a mediating nutrient of the energy effect (Klurfeld et al., 1987; Iwasaki et al., 1988).

2. The modern revival of protein restriction as the potential underlying mechanism for life extension

During the 1990s it was discovered that reducing the level of a single essential amino acid (methionine) in the diet can also increase lifespan (Orentreich et al., 1993; Richie et al., 1994). This was pre-shadowed by demonstrations that reductions of dietary tryptophan intake may also extend lifespan in both rats and mice (Ooka et al., 1988). Moreover, in the Mediterranean fruit fly (*Ceratitis capitata*) it was shown that there was an abrupt change in the mortality trajectory of female flies (but not males) when yeast was made available in the diet (Carey et al., 1998). In spite of the convincing evidence about the roles of energy and protein accumulated over the several preceding decades, these

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studies served to reopen the debate about the contribution of these individual amino acids, and protein in general to the CR effect. This impetus was given considerable momentum by a series of studies by Linda Partridge and colleagues on *Drosophila melanogaster* (Piper and Partridge, 2007; Mair et al., 2005) in which it was argued that the impact of dietary restriction on lifespan in *Drosophila* specifically IS NOT due to calorie deficit and implicated that the impact is due to variation in protein intake. This conclusion was based on studies that showed the extension of lifespan in *Drosophila* is strongly dependent on the composition of the diet, with reductions in dietary yeast content (hence protein) having a much stronger effect per calorie than the effect of reduced sugar (Mair et al., 2005). This effect has been subsequently demonstrated in several other insect species: Queensland fruit flies (Fanson et al., 2009), field crickets (Maklakov et al., 2008) and independently by other groups working on *Drosophila* (Min and Tatar, 2006; Lee et al., 2008; Skorupa et al., 2008; William et al., 2009; Bruce et al., 2013). Perhaps the most striking demonstration of the role of protein on lifespan in *Drosophila* was the demonstration that adding back individual amino acids to food restricted, and hence life extended flies, could abolish the restriction effect (Grandison et al., 2009). The overriding conclusion of these studies is that reduction in calories is neither sufficient nor necessary to extend lifespan in these insect species, and that the impression of an effect of reduced energy intake (*i.e.* CR) is brought about by a strong effect of altered ratios of protein to non-protein (normally expressed as carbohydrate) components in the diets (Raubenheimer et al., 2009; Piper et al., 2011). It has been suggested that this impression comes about by compressing the complex variation in nutritional manipulations into a single axis. An approach called the 'Geometric framework' or 'Nutritional geometry' (Simpson and Raubenheimer, 1993, 2007; Raubenheimer et al., 2012) provides a framework to dissect out the contrasting impacts of nutrition on multiple output variables, including lifespan (Piper et al., 2011; Raubenheimer et al., 2012). Reanalysing the insect data using this framework supported the idea that CR in these animals is not a response to calories alone.

These studies raise the interesting question of whether there is something fundamentally different happening in insects and rodents with respect to how food restriction mediates its impacts (Taormina and Mirisola, 2014). One potential difference is that many of the studies of insects have been performed on females. The contrasting responses of male and female Medflies to the addition of protein to their diet seems to reflect the fact that females respond to increased protein in the diet by initiating egg production (Piper and Partridge, 2007). In contrast, most studies of rodents are performed on individually housed animals (or small same sex groups) that are prevented from investing in reproduction. Can applying nutritional geometry shed light on the question of whether there is a fundamental difference between insects and rodents? Recent work on mice has involved varying the protein to carbohydrate ratio in *ad libitum* fed mice and then plotting their responses including lifespan in the context of a two-dimensional framework defined by the balance of intake of protein to non-protein components (Solon-Biet et al., 2014). This massive experiment involved feeding mice 25 different diets that varied in their protein to non-protein ratios, and following cohorts of them until they died. The data clearly indicated that the key factor influencing longevity, as was observed in insects, was the protein to non-protein (carbohydrate) ratio in the diet. As the protein to carbohydrate ratio declined, lifespan increased, recapitulating the same finding detailed above (Ross, 1961). In contrast there was no clear effect of calorie intake: moderate reductions increased lifespan but large reductions decreased it. This result indicated that perhaps rodents are actually no different to insects, and this impression is reinforced by the statement that "*It should be noted that previous caloric restriction experiments that reduce total food availability and observe longevity extension are unable to conclusively determine whether this effect was due to restriction of total calories or one or more specific nutrients in the diet* (*such as protein*)" (p. 421, Solon-Biet et al., 2014). The implication is that in rodents the impact on lifespan is probably due to changes in other nutrients (like protein). However, while the above statement is correct, it only pertains to situations where a single diet has been utilised and the effects reported. As detailed above, however, unlike the situation in insects prior to 1995, there has been a large body of work in rodents where multiple diets have been used, and where the impacts of different nutrients/energy can be elucidated. Nevertheless, to further emphasise that the previous suggested impacts of CR may in fact reflect changing dietary protein levels, an additional experiment was performed which involved manipulating mice for 8 weeks by exposing them to 3 diets that varied in their protein to carbohydrate ratio under *ad libitum* or restricted conditions (Solon-Biet et al., 2015). In this situation the metabolic impact of CR on for example glucose homeostasis, was mimicked by the *ad libitum* low protein high carbohydrate diet, and combining these interventions did not produce any additive effects. Further demonstrations of the impact of methionine restriction in rodents throughout the 2000s (Miller et al., 2005; Malloy et al., 2006; Sun et al., 2009) lend weight to the notion that at least part of the effect of CR on rodent lifespan is due to reduced protein intake (*via* restriction of specific amino acids).

3. Does protein restriction or energy restriction mediate the lifespan impact in mammals?

Since the discovery a century ago of an effect of reduced food intake on lifespan it has been disputed whether this effect stems from a reduction in calorie intake or a reduction in protein intake. At various times the question seems to have been resolved only for it to be raised again a couple of decades later. At present there seems to be a strong consensus that in studies of insects the restriction effect is NOT due to calories, and probably more to do with PR (reviewed in Piper et al., 2011). In rodents recent work is pointing in a similar direction, but this work is at odds with a long history of demonstrations in rodents that calories seem to be more important than protein in mediating the effect (reviewed above). In this paper we aimed to review the literature on CR and PR in rodents to come to some conclusion about whether it is restricted calories or restricted protein (or other macronutrients) that mediate the effect on lifespan, and if both contribute, their relative importance.

4. Data extraction procedure

To assess the roles of restricted calorie and protein intake on lifespan of rodents we have extracted data from the literature dating back to the 1930s. In particular we have collected data from 3 types of experiment where lifespan modulation was the primary outcome: A) where food was restricted relative to an unrestricted group, and hence both protein and calories were reduced in parallel, called 'CR with PR' studies, B) where food was restricted, but its protein content was modified relative to the baseline condition, so that only calories were reduced and the protein intake was not changed, called 'CR without PR' studies, and C) where protein content of the diet was varied and animals were permitted to consume it *ad libitum*.

It is well established that there is a genetic contribution to the response to CR (Harrison and Archer, 1987; Forster et al., 2003; Sohal et al., 2009; Liao et al., 2010). We restricted the review therefore to species and strains of rodent where it is established that there is a beneficial effect of CR on lifespan. This meant including all strains of rats, but excluding studies of DBA2 mice (Swindell, 2012), and the series of ILSXISS strains (Liao et al., 2010). We made this selection on the basis that it is only possible to elucidate if the effect on lifespan is due to calories or protein if there is indeed an effect on lifespan to start with. For the same reason we omitted the study of wild derived mice which also showed no effect of CR on median lifespan (Harper et al., 2006), although it does indicate some positive impacts in other measures. For those species and strains where the impact of 'CR with PR' was negative, then separating whether this effect is due to CR or PR is also an

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interesting question, but we considered it beyond the scope of this review. We also omitted studies of transgenic mice and also studies of mice with specific mutations (*e.g.* ob/ob mice Harrison et al., 1984). In some cases these latter studies generate abnormally large impacts, and in others smaller impacts because the pathway implicated in generating the lifespan effect was disrupted. We only included studies where the restriction continued for the whole life, *i.e.* excluding studies where restriction was terminated at a fixed age and the animals then reverted to *ad libitum* feeding, but included studies where the start of restriction was delayed up until the animals where 1 year old. Studies where restriction commenced later than 1 year old were not included. In total we found 67 experiments of type A 'CR with PR' and 8 experiments of type B 'CR without PR', across a total of 37 publications.

This is not an exhaustive compilation and doubtless there are many additional studies that we missed. We feel however that this is a sufficiently large and representative sample to draw valid conclusions on the question at hand. In all the studies we extracted the following information: date of publication, authors and publication source, species and strain involved, sex, age restriction started, % CR, % increase in median lifespan of the restricted group relative to the control '*ad libitum*' group and dietary composition (% protein, % fat and % sucrose). Insufficient studies reported total carbohydrate to include it in the analysis. Most studies indicated that the % contributions were by weight rather than calories. Some studies did not state and in those cases we assumed the contributions were by weight. We used extension of median (or mean if median unavailable) lifespan, rather than extension of maximal lifespan, because these are strongly correlated (Swindell, 2012), but the median is intrinsically less variable as it depends on the mortality of the whole population rather than a selected few individuals. Moreover, the criteria for 'maximum' lifespan vary between studies, with some using the top 10% and others the absolute oldest individual, but few studies citing both values. We expressed % CR as the reduction in calories provided, *i.e.* if animals were given 60% of the daily *ad libitum* intake of the control group, this was called 40% restriction. Occasionally ($n = 4$ papers) we could not extract data on the diet compositions, either because the full text of the papers were hidden behind pay-walls, or the specialised diet manufacturer appeared to be no longer in business, and the paper only cited the source of the diet rather than its composition. In 8 experiments (2 papers) the exact % CR was unclear because the animals were maintained on an intake that kept their body weight at a specific level, rather than being fed a fixed intake relative to the controls. We rejected one study of C57BL/6 mice (Harrison and Archer, 1987) where the impact of restriction shortened life by 33%, since this result was a clear outlier relative to other studies of this strain (Swindell, 2012). From the Ross study (1961) we omitted the data for group A, where restriction resulted in a 196% increase in lifespan (more than 2× the highest extension reported across the other studies), since this appeared to be a consequence of an abnormally short mean lifespan of the unrestricted group rather than an exceptionally long lifespan of the restricted animals. The extracted data from the type A and B studies are detailed in Table 1 in chronological order. To explore the effect of level of restriction (with or without PR) we performed unweighted no-constant regression analyses on the individual data sets, and on the combined data set used generalised linear modelling, and multiple regression to assess the contribution of different dietary components.

For studies where the protein content of the diet was varied, and lifespan was measured as an outcome, we found a total of 10 publications which included lifespan measurements, on a total of 50 different diets with varying protein composition (Table 2). Twenty five of these diets were from the comprehensive study of C57BL/6 mice (Solon-Biet et al., 2014). Again we do not pretend this is an exhaustive compilation, but it is probably sufficiently large to be representative. Since there is no standard protein content for the reference intake, we took the measured median lifespans for diets in the range 18 to 26% as being the lifespans for the 'reference' diet, and then expressed the median lifespans of rodents kept on other diets relative to this reference. Hence, we only included studies where there were at least 2 diets included in the paper, where the principal factor being manipulated was protein content of the diet — including one (or more) diets within, and one (or more) diets outside, the reference range.

5. The effect of 'CR with PR' on rodent lifespan does not differ from the effect of 'CR without PR'

As we, and others, have observed previously (Weindruch and Walford, 1982; Merry, 2002; Speakman and Hambly, 2007), there was a positive relationship between the extent of CR (with PR) and the percentage increase in lifespan relative to unrestricted individuals (Fig. 1). The no-intercept regression equation %increase in lifespan = 0.758 * %restriction explained 36.6% of the variation in the %increase in lifespan ($F_{1,61} = 317.96$, $p < 0.0005$). The coefficient of this relationship indicated that the impact of a given percentage increase in restriction was to generate about 3/4 of the effect on % lifespan extension. Hence, for example, 40% CR (with PR) generated on average a 30% increase in median lifespan. Inspection of the residuals did not indicate that there was any non-linearity in the relationship over the range considered. Hence, the highest level of restriction (68%) generated lifespan increases in the range 28 to 71%, mean = 51%. Clearly, this trend cannot continue indefinitely, since we know at 100% restriction (*i.e.* no food) mice die with much reduced lifespans. The point at which the curve inflects however is presently uncertain as no one has yet published data on restrictions greater than 68%.

Although there were less data, the relationship between %lifespan extension and the %CR (without PR) was almost identical to the relationship for %CR (with PR). The no-intercept regression equation %increase in lifespan = 0.731 * %restriction explained 42.9% of the variation in the %increase in lifespan ($F_{1,7} = 76.2$, $p < 0.0005$). The coefficient in this fitted equation indicates that increasing application of 'CR without PR' generates an indistinguishable effect on lifespan from 'CR with PR'. When the two data sets were combined into a generalised linear model analysis with the different dietary treatments as a factor, and % restriction as a covariate the effect of %restriction was highly significant ($F_{1,67} = 37.90$, $p < 0.0005$), but there was no significant group effect ($F_{1,67} = 0.04$, $p = 0.841$) and no significant interaction ($F_{1,66} = 0.07$, $p = 0.788$). There was a high level of variation in the responses at any given level of restriction. Hence, the absence of a group effect could be a type 2 error because of the low power to resolve a difference. However, the overlap of the fitted regressions was striking, and it is clear that this is not a situation where there is a trend for an effect that fails to reach significance because of the high variation in the responses within treatments. The conclusion of this comparison is that CR exerts its impact on lifespan independent of whether the protein component of the diet is restricted or not. The effect on lifespan is due to calorie deficit not protein deficit.

We emphasise that this point is not new, and we would not wish to claim to have discovered it. It has been made previously by the authors of the individual studies that contribute to the data where protein levels in the restricted diet were manipulated to ensure CR was imposed without PR (McCay et al., 1939; Weindruch and Walford, 1982; Davis et al., 1983; Weindruch et al., 1986; Horakova et al., 1988; Masoro, 2006). Our analysis simply places these multiple studies within the context of the wider database of studies of CR with PR to emphasise the point. We feel this is required in the light of the recent revival of the idea that the impact of CR may be traced to differences in PR in studies of insects (Partridge et al., 2011) and most recently in mice (Solon-Biet et al., 2014).

The present analysis is most disparate with the study by Solon-Biet et al. (2014) which concluded that the main driver of lifespan is protein to carbohydrate ratio (strongly correlated to protein % in the diet) and specifically NOT calorie intake (Solon-Biet et al., 2014). Given this contrasting conclusion it is worth exploring the reasons that might underpin this discrepancy in more detail. Solon-Biet et al. (2014) exposed

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Table 1
The effect of calorie restriction (CR) on lifespan in rodents. Type A studies involve CR with simultaneous protein restriction (PR). Type B studies involve CR with no PR. Studies are ordered by date of publication. Details of the different diet compositions are included. In type B studies two dietary compositions are provided which detail the composition of the diets used for the control animals and underneath the diets of the individuals on restriction with protein levels compensated. %LS = lifespan % increase relative to *ad libitum* fed controls. Day start is age in days at commencement of restriction. M = male, F = female. CHO = carbohydrate.

Date	% CR	% LS increase	% Protein	% Fat	% Sucrose	% CHO	Species	Strain	Day start	Sex	Reference
Type A: CR with PR											
1935	–	69.8	40	15	10	–	Rat	Albino	21	M	McCay et al. (1935)
1935	–	−3.2	40	15	10	–	Rat	Albino	21	F	McCay et al. (1935)
1935	–	85.1	40	15	10	–	Rat	Albino	35	M	McCay et al. (1935)
1935	–	3.1	40	15	10	–	Rat	Albino	35	F	McCay et al. (1935)
1939	–	26.5	40	15	10	–	Rat	Albino	21	M	McCay et al. (1939)
1939	–	78.8	40	15	10	–	Rat	Albino	21	F	McCay et al. (1939)
1961	40	57	49	8.5	33.9	33.9	Rat	Sprague Dawley	21	M	Ross (1961)
1961	10	−2	7.8	5	83	83	Rat	Sprague Dawley	21	M	Ross (1961)
1961	63	55	20.4	13.5	54	54	Rat	Sprague Dawley	21	M	Ross (1961)
1960	46	39.1	24.4	4	–	54.2	Rat	Sprague Dawley	21	F	Berg and Simms (1960)
1960	46	25.3	24.3	4	–	54.2	Rat	Sprague Dawley	21	M	Berg and Simms (1960)
1966	–	24	–	–	–	–	Rat	Holtzman	–	M	Kibler and Johnson (1966)
1971	–	57	–	–	–	–	Rat	Chas River	–	M/F	Ross and Bras (1971)
1972	68	71	22	6	58	58	Rat	CD	30	M	Ross (1972)
1972	21	17	19	12	70	70	Rat	Sprague Dawley	68	M	Leveille (1972)
1972	20	21.2	23	18.5	–	–	Rat	Albino	21	M	Nolen (1972)
1972	20	15.3	23	18.5	–	–	Rat	Albino	21	F	Nolen (1972)
1972	40	30.9	23	18.5	–	–	Rat	Albino	21	M	Nolen (1972)
1972	40	15.3	23	18.5	–	–	Rat	Albino	21	F	Nolen (1972)
1973	68	28.1	10	13.5	70.5	–	Rat	Sprague Dawley	21	M	Ross and Bras (1973)
1973	68	43.2	22	13.5	58.5	–	Rat	Sprague Dawley	21	M	Ross and Bras (1973)
1973	68	52.1	51	13.5	29.5	–	Rat	Sprague Dawley	21	M	Ross and Bras (1973)
1976	10	24	26	4	49	49	Mouse	C57BL/6J	30	M	Leto et al. (1976)
1976	40	21.7	–	–	–	–	Rat	Albino		M	Drori and Folman (1976)
1976	50	81.7	22	5	–	66	Mouse	B/W (NZB × NZW) F1	56	M	Fernandes et al. (1976a)
1976	50	30.3	6	5	–	82	Mouse	B/W	56	M	Fernandes et al. (1976a)
1976	50	30.2	22	20	–	51	Mouse	B/W	56	M	Fernandes et al. (1976a)
1976	50	46.7	6	20	–	67	Mouse	B/W	56	M	Fernandes et al. (1976a)
1976	50	70.1	22	5	–	66	Mouse	B/W	56	F	Fernandes et al. (1976a)
1976	50	28.8	6	5	–	82	Mouse	B/W	56	F	Fernandes et al. (1976a)
1976	50	39	22	20	–	51	Mouse	B/W	56	F	Fernandes et al. (1976a)
1976	50	57.6	6	20	–	67	Mouse	B/W	56	F	Fernandes et al. (1976a)
1981	50	42.4	–	–	–	–	Rat	Sprague Dawley	21	M	Merry and Holehan (1981)
1982	40	40.6	21	10	15	58.6	Rat	F344	42	M	Yu et al. (1982) (18)
1982	33	7.3	20.5	–	–	–	Rat	Sprague Dawley	90	F	Zamenhof and van Marthens, 1982
1983	57	20.8	21.6	13.5	39.1	54.1	Mouse	B10C3F1	24	F	Cheney et al. (1983)
1984	33	0	22	7	0	50	Mouse	B6	28	F	Harrison et al. (1984)
1985	40	34.2	21	10	15	58.6	Rat	F344	42	M	Yu et al. (1985)
1985	40	50.8	21	10	15	58.6	Rat	F344	42	M	Yu et al. (1985)
1986	22.7	19.3	20	13.5	26.1	52.2	Mouse	C3B10RF	21	F	Weindruch et al. (1986)
1986	55	44.9	23	13.5	26.1	52.2	Mouse	C3B10RF	21	F	Weindruch et al. (1986)
1986	64	64.6	23	13.5	26.1	52.2	Mouse	C3B10RF	21	F	Weindruch et al. (1986)
1986	25	19.8	23.9	5.7	3.7	48.7	Mouse	CD2/F1	42	F	Nelson and Halberg (1986)
1987	33	−33	22	7	–	50	Mouse	B6	28	M	Harrison and Archer (1987)
1987	33	20.9	22	7	–	50	Mouse	B6CBAFI	28	M	Harrison and Archer (1987)
1988	40	52.7	21	10	15	–	Rat	F344	42	M	Horakova et al. (1988)
1988	40	45.9	12.6	10	15	–	Rat	F344	180	M	Horakova et al. (1988)
1990	40	47.1	21	10	15	–	Rat	F344	–	–	Masoro (1990)
1993	40	31.8	21	10	15	58.6	Rat	F344	42	M	Shimokawa et al. (1993)
1993	40	32.7	21	10	15	58.6	Rat	F344	42	M	Shimokawa et al. (1993)
1994	40	25	18	4	–	63	Rat	F344	28	M	Thurman et al. (1994)
1994	40	11.9	18	4	–	63	Rat	F344	28	F	Thurman et al. (1994)
1995	40	35	14	10	66	–	Rat	F344	133	M	Murtagh-Mark et al. (1995)
1995	40	0.83	14	10	–	66	Rat	F344	133	M	Murtagh-Mark et al. (1995)
1995	40	15.4	18	4	–	63	Mouse	C57BL/6	28	M	Blackwell et al. (1995)
1995	40	24.5	18	4	–	63	Mouse	C57BL/6	28	F	Blackwell et al. (1995)
1997	40	40.0	21	10	15	43.7	Rat	F344	42	M	McCarter et al. (1997)
1997	40	33.3	21	10	15	43.7	Rat	F344	42	M	McCarter et al. (1997)
1997	40	34.1	18	4	–	63	Rat	F344 × BNR	98	F	Fernandes et al. (1997)
2003	30	8.7	18.2	4.8	–	57.9	Rat	F344	42	M	Shimokawa et al. (2003)
2003	40	24	18.4	4.5	–	–	Mouse	C57BL/6	120	M	Forster et al. (2003)
2003	40	26.3	18.4	4.5	–	–	Mouse	B6D2F	120	M	Forster et al. (2003)
2006	40	28.2	21	10	15	–	Rat	F344	–	–	Masoro (2006)
2008	45	13.1	19	4	–	62	Rat	Brown Norway	60	M	Merry et al. (2008)
2008	45	11.3	19	4	–	62	Rat	Brown Norway	365	M	Merry et al. (2008)
2008	30	10	18.2	4.8	–	57.9	Rat	Wistar	21	M	Zha et al. (2008)
2015	40	31.7	20.3	15.9	10	63.8	Mouse	C57BL/6J	28	M	Lopez-Dominguez et al. (2015)
2015	10	15.3	21	10	–	58.9	Rat	F344	42	M	Richardson et al. (2015)
2015	40	19.0	21	10	–	58.9	Rat	F344	42	M	Richardson et al. (2015)

(continued on next page)

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Table 1 (*continued*)

Date	% CR	% LS increase	% Protein	% Fat	% Sucrose	% CHO	Species	Strain	Day start	Sex	Reference
Type B: CR without PR											
1982	44	12	20	13.5	26.1	52.2	Mouse	B10C3F1	365	M	Weindruch and Walford (1982)
			35	13.5	15.8	31.6					
1982	27	20	20	13.5	26.1	52.2	Mouse	C57BL/6J	365	M	Weindruch and Walford (1982)
			35	13.5	15.6	31.6					
1983	33	25.6	12				Rat	Wistar	32	M	Davis et al. (1983)
			18								
1983	33	22.2	20				Rat	Wistar	32	M	Davis et al. (1983)
			30								
1983	33	25.1	28				Rat	Wistar	32	M	Davis et al. (1983)
			42								
1986	55	54.4	23	4.5	15.8	31.6	Mouse	C3B10RF1	21	M	Weindruch et al. (1986)
			35	13.5							
1988	40	30.8	12.6	10	15	67.1	Rat	F344	42	M	Horakova et al. (1988)
			21	10	15	54.6					
2006	40	31.0	21	10	15	57.2	Rat	F344	42	M	Masoro et al. (1989), Masoro (2006)
			35	10	15	43.1					

Table 2
The effect of dietary protein percent on lifespan in *ad libitum* fed rodents. The % Lifespan effect (%LS) was calculated relative to lifespan on reference diet with between 18 and 26% protein. Reference lifespans for each publication are shown in bold.

Date	% Protein	Median LS	% LS	Species	Strain	Sex	Reference
1961	49	596	−0.7	Rat	Sprague Dawley	M	Ross (1961)
1961	7.8	835	38.6	Rat	Sprague Dawley	M	Ross (1961)
1961	**20.4**	**600**		Rat	Sprague Dawley	M	Ross (1961)
1971	10	572	1.4	Rat	Donryu	F	Nakagawa and Masana (1971)
1971	**18**	**564**		Rat	Donryu	F	Nakagawa and Masana (1971)
1971	27	623	10.6	Rat	Donryu	F	Nakagawa and Masana (1971)
1973	10	600	−7.8	Rat	Sprague Dawley	M	Ross and Bras (1973)
1973	**22**	**540**		Rat	Sprague Dawley	M	Ross and Bras (1973)
1973	51	585	4.9	Rat	Sprague Dawley	M	Ross and Bras (1973)
1976	**26**	**685**		Mouse	C57Bl/6	M	Leto et al. (1976)
1976	4	852	24.4	Mouse	C57BL/6	M	Leto et al. (1976)
1976	6	–	0.0	Mouse	NZB	–	Fernandes et al. (1976b)
1976	6	–	3.9	Mouse	NZB	–	Fernandes et al. (1976b)
1976	6	–	34.2	Mouse	NZB	–	Fernandes et al. (1976b)
1976	22	–	–	Mouse	NZB	–	Fernandes et al. (1976b)
1976	**22**	**614**	–	Mouse	NZB	–	Fernandes et al. (1976b)
1978	26	**636**		Mouse	C57BL/6J	–	Goodrick (1978)
1978	4	729	14.6	Mouse	C57BL/6J	–	Goodrick (1978)
1978	26	**642**		Mouse	A/J	–	Goodrick (1978)
1978	4	681	6.1	Mouse	A/J	–	Goodrick (1978)
1978	26	**720**		Mouse	F_1(A/JxC57)	–	Goodrick (1978)
1978	4	843	17.1	Mouse	F_1(A/JxC57)	–	Goodrick (1978)
1985	21			Rat	F344	M	Yu et al. (1985)
1985	12.6		15.5	Rat	F344	M	Yu et al. (1985)
1988	**21**	**715**	–	Rat	F344	M	Horakova et al. (1988)
1988	12.6	835	16.7	Rat	F344	M	Horakova et al. (1988)
2014	5	853	16.7	Mouse	C57BL/6	M/F	Solon-Biet et al. (2014)
2014	5	745	2.0	Mouse	C57BL/6	M/F	Solon-Biet et al. (2014)
2014	5	836	14.4	Mouse	C57BL/6	M/F	Solon-Biet et al. (2014)
2014	5	871	19.2	Mouse	C57BL/6	M/F	Solon-Biet et al. (2014)
2014	14	861	17.8	Mouse	C57BL/6	M/F	Solon-Biet et al. (2014)
2014	14	797	9.1	Mouse	C57BL/6	M/F	Solon-Biet et al. (2014)
2014	14	822	12.5	Mouse	C57BL/6	M/F	Solon-Biet et al. (2014)
2014	14	756	3.5	Mouse	C57BL/6	M/F	Solon-Biet et al. (2014)
2014	14	690	−5.6	Mouse	C57BL/6	M/F	Solon-Biet et al. (2014)
2014	14	621	−15.0	Mouse	C57BL/6	M/F	Solon-Biet et al. (2014)
2014	23	867	18.7	Mouse	C57BL/6	M/F	Solon-Biet et al. (2014)
2014	23	700	−4.2	Mouse	C57BL/6	M/F	Solon-Biet et al. (2014)
2014	23	625	−14.5	Mouse	C57BL/6	M/F	Solon-Biet et al. (2014)
2014	**23 (mean)**	**730**		Mouse	C57BL/6	M/F	Solon-Biet et al. (2014)
2014	33	858	17.4	Mouse	C57BL/6	M/F	Solon-Biet et al. (2014)
2014	33	688	−5.8	Mouse	C57BL/6	M/F	Solon-Biet et al. (2014)
2014	33	750	2.6	Mouse	C57BL/6	M/F	Solon-Biet et al. (2014)
2014	33	886	21.3	Mouse	C57BL/6	M/F	Solon-Biet et al. (2014)
2014	33	746	2.1	Mouse	C57BL/6	M/F	Solon-Biet et al. (2014)
2014	33	550	−24.7	Mouse	C57BL/6	M/F	Solon-Biet et al. (2014)
2014	42	972	33.0	Mouse	C57BL/6	M/F	Solon-Biet et al. (2014)
2014	42	858	17.4	Mouse	C57BL/6	M/F	Solon-Biet et al. (2014)
2014	42	601	−17.8	Mouse	C57BL/6	M/F	Solon-Biet et al. (2014)
2014	60	756	3.5	Mouse	C57BL/6	M/F	Solon-Biet et al. (2014)
2014	60	697	−4.6	Mouse	C57BL/6	M/F	Solon-Biet et al. (2014)
2014	60	590	−19.2	Mouse	C57BL/6	M/F	Solon-Biet et al. (2014)

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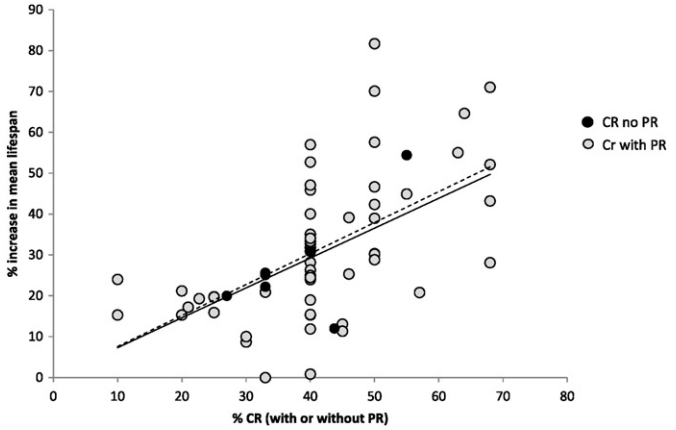

Fig. 1. The relationship between % calorie restriction (CR) either with simultaneous protein restriction (PR) (points) or with no PR (black points), and the % increase in median lifespan relative to *ad libitum* fed animals. The data refer to various rat and mouse strains as detailed in Table 1. The lines show the no intercept fitted regressions for the two data sets (dashed line for CR with no PR). For fitted equation details and statistics see text.

C57BL/6 mice to 25 different diets that varied in their macronutrient composition and imposed these diets at 3 levels of restriction (Solon-Biet et al., 2014). Surprisingly, they found that imposition of 30% restriction actually shortened lifespan, by on average 17%. This is an unusual result for C57BL/6 mice (Tables 1 and 2 and Swindell, 2012), although not unique since Harrison and Archer (1987) also found imposition of CR (with PR) reduced lifespan in the same strain. Nevertheless, given this outcome, which contrasts the majority of other CR studies of this strain (Swindell, 2012) it is not surprising that the conclusion was reached that protein intake (restriction) is more important than calorie intake (restriction) for extending lifespan. Whether this conclusion can be generalised to other studies that showed a positive impact of CR on lifespan is uncertain.

A possible explanation for the unusual response of the mice studied by Solon-Biet et al. (2014) was the manner in which the restriction was applied, which was exceptional among studies of rodents (Solon-Biet et al., 2014). In all previous studies of CR in rodents, the subject animals are given a ration of food that is lower than the intake of an *ad libitum* fed control group. Details of the exact protocols vary, in particular when and how frequently the ration is delivered (reviewed in Speakman and Mitchell, 2011) but they all have in common a shortfall in the quantity (*i.e.* mass) of food eaten, relative to *ad libitum* fed animals. In contrast, Solon-Biet et al. (2014) generated restriction by diluting the diet with indigestible cellulose. Hence, while the mice ingested fewer calories, they did so while ingesting almost twice as much mass of food (Solon-Biet et al., 2014). This difference may be critical, because a potentially key component of the response to CR is a stimulation of the hunger signalling pathways in the brain (Hambly et al., 2007, 2012; Lusseau et al., 2015). When components of these pathways are knocked out, the response to CR is attenuated (*e.g.* NPY null mice). Diluting the diet, rather than restricting the amount available, may potentially generate fundamentally different responses in the neuropeptide pathways that link restriction to its beneficial actions with respect to lifespan (Lusseau et al., 2015). For example, the patterns of response in gut hormones that regulate satiation and satiety, and direct vagal afferents that respond to gut distension, are likely to be very different in mice that are underfed, compared to those that voluntarily overeat a diluted diet. Indeed, the fact the animals fed the diluted diet do not completely compensate for the caloric deficit, in the presence of excess food, suggests that hunger signalling pathways are down- rather than up-regulated.

One potential factor that potentially compromises the interpretation of the CR studies that involve giving the animals less food to eat is that in some protocols the animals may not only be restricted but may also be intermittently fasted (IF) (Simpson et al., 2015). IF, sometimes called 'every other day feeding' protocols involve the deliberate withholding of all food supply for periods in excess of 24 h. It has been shown that such protocols may result in lifespan extension even in the absence of any decrease in overall food intake (Carlson and Hoelzel, 1946; Goodrick et al., 1983; Ansom et al., 2005). In some CR protocols there may be an inadvertent exposure to IF because the animals are fed a large ration on Fridays (3× the normal size) but not refed until Monday. Potentially then the animals may eat all the food on the first day and then be exposed to fasting until the next feed on Monday. The CR protocol would then be confounded by an IF exposure. Unfortunately the exact feeding procedures are not always detailed in papers that describe CR experiments. Moreover, these protocols are generally employed to reduce the staffing costs of feeding animals at the weekend. Consequently there are no data available on how the mice ration their own intake over the weekend days. Maybe they are exposed to IF, or maybe not. In the absence of such information it was not possible to establish if the protocol details were a factor generating the large variation in the response to CR level. However, if the key aspect of the protocols generating the lifespan effect was the presence of IF, as opposed to the level of calories available, then we would not anticipate a progressive impact of the level of restriction in the manner we have reported (Fig. 1). Studies that explicitly employed IF, or every other day feeding, protocols were not included into the present review.

In addition to fasting in excess of 24 h, animals under restriction probably also fast for extended periods within each day. This short term fasting effect may be a confounding factor in protocols that employ only a simple comparison of CR to *ad libitum* fed animals (Mitchell et al., 2015a). Although it seems clear that mice under CR eat all their daily ration within about 4 h of it being provided, it is less certain if there is any difference in this duration between rodents on different levels of restriction, nor if such duration differences can precipitate any lifespan effects, which would be necessary to explain the relationship between the extent of restriction and extent of lifespan extension demonstrated here.

Interestingly, dilution of the diet is also the primary methodology used to restrict the diets of insects like *Drosophila*, where similar conclusions about the roles of calories and protein have recently been drawn. We suggest that to distinguish this potentially important methodological difference, that it may be prudent to replace the terms 'CR' or 'dietary restriction' by 'dietary dilution' or 'caloric dilution', when the method involves supplying *ad libitum* food that has been diluted with nutrient-free components. The terms caloric or dietary restriction might then be reserved for studies where animals are provided with a lower mass of food (as well as fewer calories) compared to controls (although not necessarily of identical composition). We make this suggestion only to distinguish between protocols that appear to generate different effects. There is no intended implication that either protocol is a superior approach to the study of links between diet and ageing.

6. Residual variation in the lifespan response to CR is unrelated to dietary composition

Fig. 1 emphasises the large variation in the lifespan response of rodents to imposed CR (with or without PR). If the cause of the 'CR' effect is due to parallel variation in intake of some dietary macronutrient, then we would expect that residual variation in the lifespan response would be correlated to the dietary composition. None of this residual variation was explained by sex ($F_{1,56} = 0.68$, $p = 0.413$), species (mice v rats: $F_{1,60} = 0.34$, $p = 0.560$) or by strain ($F_{12,49} = 1.27$, $p = 0.266$) although in many cases strains were represented by only a single experiment. We therefore investigated the impact of variations in macronutrient content (%protein, %fat and %sucrose contents of the diet) using a multiple regression model, without controlling for these other factors. None of the component macronutrient contents was significantly linked to

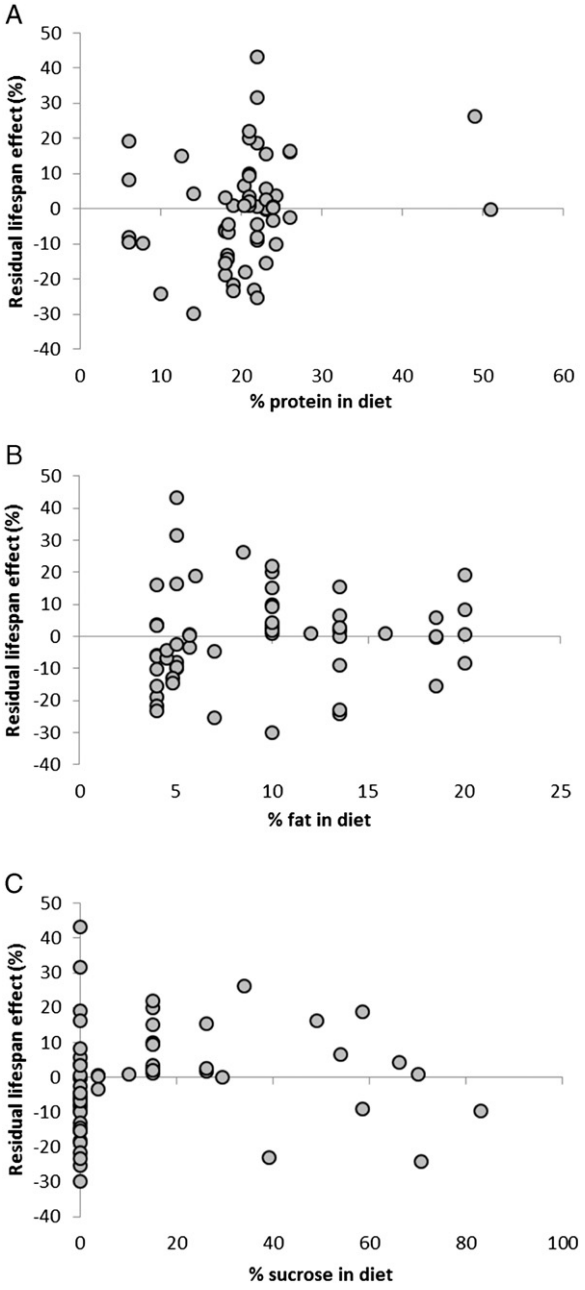

Fig. 2. Residual variation in the relationship between calorie restriction (CR) (with simultaneous protein restriction (PR)) and lifespan as illustrated in Fig. 1, and the macronutrient composition of the diet. A: % protein content, B: % fat content and C: % sucrose content. In all three cases the macronutrients did not significantly contribute to explaining the residual variation in the response.

variation in the response to CR (with PR) (Fig. 2) ($F_{4,48} = 1.27$, p= 0.297, individual T values : %protein T = 1.19, p= 0.242, %fat T = 0.88, p = 0.381, %sucrose T = 0.91, p = 0.367). This was despite the fact that the range of macronutrient contributions was wide (protein: 4 to 60%, fat: 4 to 20% and sucrose: 0 to 80%) and hence it is unlikely that the absence of any effect was because the range of variation in the diets was insufficient, except perhaps for the variation in fat content. This review highlights that more studies are required of the impact of restriction of diets containing >20% fat, although we appreciate that finding an appropriate control intake in these cases may be difficult if obesity is to be avoided in the control animals. The absence of any impact of the different macronutrient contents of the diets on the variation in the restriction response strongly supports the conclusion drawn

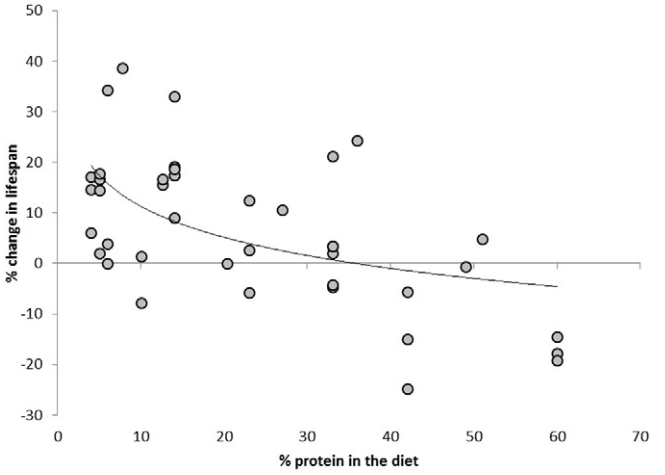

Fig. 3. Effect of percent protein content of the diet on the median lifespan relative to the lifespan for control animals fed diets in the range 18 to 26% protein. The data refer to studies of various mouse and rat strains as detailed in Table 2. The line shows the fitted exponential regression. For fitted equation details and statistics see text.

above that the impact of CR (with PR) on longevity is due to the restriction of calories alone.

Recent work has suggested that the type of particular fatty acids within the fat fraction may have a significant impact on the CR effect (Lopez-Dominguez et al., 2015). It is important to note that this does not imply that the lifespan effect of CR itself is due solely to these particular components rather than calorie intake. Rather the impact of particular fatty acids may be to modulate the magnitude of the CR effect. For example, the presence of high levels of dietary mono- and polyunsaturated fatty acids may become incorporated into tissues, where they provide additional targets for attack by radical oxygen species (ROS), thereby reducing the impact of CR mediated reductions in ROS (Lopez-Dominguez et al., 2015). Considerable further work is required to understand such potential modulatory effects, and their mechanisms, and whether varying the particular composition of protein and carbohydrate also modulates the CR impact on lifespan.

7. Effect of percentage protein composition of the diet on lifespan

Using diets in the range 18 to 26% protein as the reference diets, the % change in lifespan when rodents were exposed to diets that varied in their protein content outside this range was negatively related to the % protein content of the diet (Fig. 3). The relationship was curvilinear, such that between protein contents of 20 to 60% the effect on lifespan was small, but below 20% the effect became more pronounced. The best fit exponential model lifespan (%) = 31.73–8.874(Log_e % protein) explained 28.3% of the variation in the lifespan relative to the reference diets. This finding confirms many previous suggestions that lifespan is extended as protein content of the diet is reduced (but not eliminated) (Slonaker, 1931; Solon-Biet et al., 2014). The lowest protein content in the reviewed studies was 4%, which generated a mean increase in lifespan about 14% higher than the reference diet containing 20% protein. Given intake of a reference diet of 20% protein, a diet containing only 4% protein is equivalent to an 80% restriction.

To some unquantified extent this relationship may underestimate the role played by protein content on lifespan, because it assumes that the intake of each diet is constant. However, rodents may overconsume low protein diets and under-consume high protein diets in an attempt to reach a protein intake target (the protein leverage hypothesis) (Simpson and Raubenheimer, 2012; Huang et al., 2013). This would have the effect of compressing the %protein in the diet axis with respect to actual protein intake. We could not evaluate the importance of this effect because the actual intakes on each diet were seldom stated, although in at least one study the food intakes followed the

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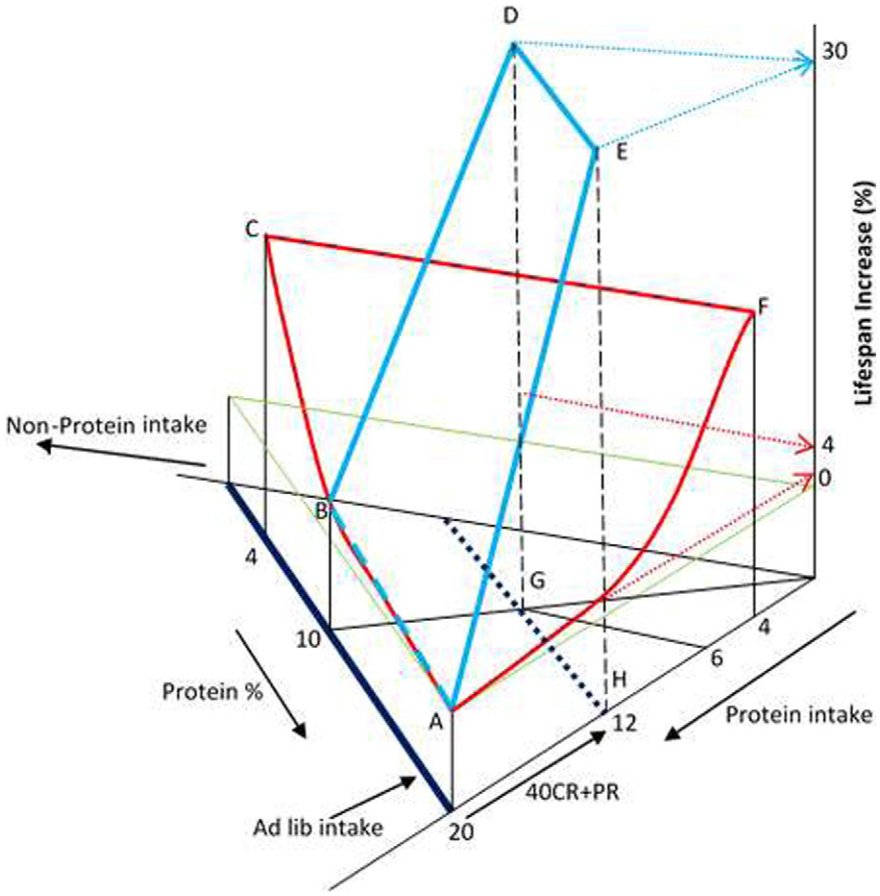

Fig. 4. Nutritional geometric representation of the effects of changing protein intake (red plane) and changing calorie intake (light blue plane) on the median lifespan. For full explanation see text.

converse pattern — *i.e.* the rats ate more of the high protein diet and less of the low protein one (Ross, 1961), and in other studies (Nakagawa and Masana, 1971) the intakes of the different diets were constant, indicating this type of compensation is not universal.

Although the finding of a negative effect of % protein content on lifespan is far from new (Slonaker, 1931), the importance in the current context is the quantification of the impact of protein contents on the lifespan effect. For example, most of the studies of CR (with PR) start with diets in the range 18 to 26% (Table 1 and Fig. 2A). Taking 20% as a typical value, if the supplied diet is restricted by 40%, the effect on protein supply is equivalent to reducing the protein content of an *ad libitum* supplied diet to 12% (assuming no compensation in intake). The fitted regression in Fig. 3 suggests that the expected impact of such a decrease in protein content would be to extend the lifespan by about 4.5% (*i.e.* a predicted 5.1% increase in lifespan at 20% and a 9.6% lifespan increase at 12%). However, as detailed above, the actual increase in lifespan under such a manipulation averages about 6.7× greater (at 30%). This novel analysis further clarifies why the restriction of calories (with PR) effect on lifespan is predominantly, if not exclusively, due to the reduction in calories rather than protein (or sucrose and possibly also fat).

8. Visualising the effects of calorie and protein restriction on rodents in the framework of nutritional geometry

These contrasting effects of protein and calories on lifespan can be usefully visualised by using the nutritional geometric framework approach (Raubenheimer et al., 2012). To achieve this we located the lifespan responses in a three dimensional plot where the basal axes define the level of protein and non-protein calories in the diet (Fig. 4). The Y axis is the lifespan response under different dietary treatments. Different manipulations of the diet are colour coded in different ways. The thin green line is the reference lifespan for rodents eating *ad libitum* a diet providing 18–26% protein. The thick blue line represents *ad libitum* intake. The effect of decreasing the amount of protein in the diet, relative to other components (derived from Fig. 3) is represented by the red plane (ACF). Decreasing protein in the diet may occur because the diet composition changes at *ad libitum* intake levels (moving along the thick blue line) or because the animals eat less food (moving closer to the origin). This plane highlights the curvilinear increase in lifespan as the % protein content falls towards 4%. What happens between 4% and 0% is not known from the reviewed studies. The impact of reducing the amount of ingested food (CR with PR) is represented by the light blue plane (ABDE). This shows lifespan increasing dramatically as food intake is lowered, and at a much greater rate than the effect of reductions in protein intake. A CR (with PR) of 40% is indicated by the dark blue dotted line. The CR (with PR) plane has the same gradient whatever the starting contribution of protein, indicating that protein content of the diet does not modulate the CR effect. Taking two possible diets at 40% restriction (in the *ad libitum* state one at 20% protein and the other at 10% protein) represented by the two vertical dashed black lines (GD and HE), illustrates the observed effect of CR plus PR. Where the dashed lines intersect the light blue plane indicates the observed life extension of about 30% under such diets. Where these same lines intersect the red plane indicates the expected effect due to PR restriction alone (about 4%). The take home message from this figure is that the plane representing the impact of PR on lifespan only intersects with the plane representing the impact of CR plus PR on lifespan at the line of *ad libitum* intake. In other words, the impact of CR plus PR on lifespan is a completely different phenomenon from PR alone. Hence, the effect of reducing food intake on lifespan in rodents acts only *via* the

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restriction of calorie intake. This conclusion is consistent with the fact that the morphological, physiological and behavioural responses of mice to CR with PR (between 0 and 40% restriction) are completely different to the responses of mice to PR alone over the equivalent range (20% down to 12% protein) (Mitchell et al., 2015a,b,c).

9. Summary

Nutritional geometry provides a useful framework for disentangling the impacts of complex nutritional manipulations of the diet on multiple phenotypic outcomes including lifespan. Our analysis of multiple manipulation experiments in rodents over the past 80 years shows that the food restriction effect on lifespan is due to reduced calories and not reduced protein intake (or sucrose intake, and possibly also fat intake), and hence is correctly called 'caloric restriction' or CR. Nevertheless, it is also clear that there is an independent impact of dietary protein reduction on lifespan, but it operates over a different range of restriction (50 to 85%: relative to a reference intake of 18–26% protein in the diet) than that over which CR is effective (10–65% relative to *ad libitum* intake), and has a much smaller impact. Hence, reducing protein levels by 80% (from 20% to 4%) increases median lifespan by about 15%, while reducing calories by half this amount (40%) increases median lifespan by on average twice as much (30%). The present analysis contrasts with recent analyses using the nutritional framework in multiple insect species that point to the effect of food restriction on lifespan in insects being mediated primarily (or exclusively) by altered protein intake. This possibly suggests a fundamentally different longevity response is happening in insects and rodents. This difference could be a consequence of physiological differences between insects and mammals. Alternatively, it may reflect a major methodological difference between studies on insects and rodents. In particular, rodents are generally provided with less food when on restriction, but insects are primarily manipulated by giving them food diluted with indigestible components, and hence, paradoxically, despite being called on 'restriction', they eat a greater mass of food. We suggest future studies where the diet is diluted should perhaps be called dietary or caloric dilution studies to distinguish them from classical dietary or caloric restriction studies. If either difference (physiological or methodological) is indeed the case, it clearly has some profound implications for the suitability dietary dilution studies of insects (including *Drosophila*) as models for understanding the mechanisms underlying what is happening during CR in mammals.

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