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Lifespan Extension Via Dietary Restriction: Time to Reconsider the Evolutionary Mechanisms?

Joshua P. Moatt,* Eevi Savola, Jennifer C. Regan, Daniel H. Nussey, and Craig A. Walling*

Dietary restriction (DR) is the most consistent environmental manipulation to extend lifespan. Originally thought to be caused by a reduction in caloric intake, recent evidence suggests that macronutrient intake underpins the effect of DR. The prevailing evolutionary explanations for the DR response are conceptualized under the caloric restriction paradigm, necessitating reconsideration of how or whether these evolutionary explanations fit this macronutrient perspective. In the authors' opinion, none of the current evolutionary explanations of DR adequately explain the intricacies of observed results; instead a context-dependent combination of these theories is suggested which is likely to reflect reality. In reviewing the field, it is proposed that the ability to track the destination of different macronutrients within the body will be key to establishing the relative roles of the competing theories. Understanding the evolution of the DR response and its ecological relevance is critical to understanding variation in DR responses and their relevance outside laboratory environments.

that the underlying physiological mechanisms of DR are evolutionarily conserved and thus DR has been widely used to study the causes and consequences of variation in lifespan and ageing. Despite this attention, both the evolutionary and physiological mechanisms underpinning DR responses remain poorly understood. Here, we provide a concise description of the current evolutionary explanations of the lifespan response to DR. We then provide a synthesis of how these theories could and are being applied to emerging evidence on the importance of macronutrients rather than calories. We describe how the current empirical evidence fits with this macronutrient-focused consideration of each theory and discuss how each of these theories falls short of explaining how and why the DR response evolved. We highlight additional work that is needed to test these theories in relation to macronutrient intakes. Finally,

we discuss how a macronutrient perspective could fit with an integrated ecological and physiological framework that has recently been proposed.

1. Introduction

Dietary restriction (DR), a moderate reduction in food intake whilst avoiding malnutrition, is the most consistent environmental manipulation to extend lifespan and delay ageing.^[1–3] First described in rats,^[4] DR has since been shown to extend lifespan in wide range of taxa: from model lab species such as *Drosophila melanogaster*^[5,6] and mice,^[7,8] to non-model species such as sticklebacks,^[9] crickets,^[10] and non-human primates^[11,12] (but see ref. [13]). Owing to this taxonomic diversity, it is presumed

2. Dietary Restriction


2.1. What is Dietary Restriction?

Since its inception in 1935,^[4] DR has become an all-encompassing description for multiple forms of dietary interventions. The most widely studied form of DR is calorie restriction (CR), a reduction in overall calorie intake whilst avoiding malnutrition. Common forms of CR include providing a restricted food portion, dilution of the diet, or restricting food availability temporally.^[11,14] Positive effects of CR on lifespan are well supported^[15,16] (but see ref. [17,18]). Initial explorations of the role of specific dietary components, such as protein content, found that the effects were largely driven by caloric intake.^[19] Consequently, until recently DR and CR were largely interpreted as synonymous terms. Owing to this focus on CR, the predominant evolutionary explanations of the DR effect were developed to explain responses to CR and not macronutrient availability.

More recently, there has been a resurgence of interest in the idea that intake of specific nutrients and not calories may be underpinning the DR response. This has largely been driven by the development of the geometric framework of nutrition

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(GFN);^[20,21] an integrative framework where diets are presented as n-dimensional nutrient spaces, where each dimension represents a dietary parameter of interest. These could be macronutrients (protein, carbohydrate, or lipid) or micronutrients (e.g., amino acids and vitamins). Variation in traits of interest can then be easily visualized against variation in dietary parameters to create a form of trait/fitness landscape. The GFN differs from previous approaches because it allows multiple dietary components to be varied simultaneously rather than taking a “one variable at a time” approach.^[3] Critically, by making fine scale adjustments of specific dietary components, the GFN allows a simultaneous test of the role of calories and macronutrients, the identification of key dietary components involved in the DR response and an increased comparability between studies and species.

2.2. What is the Effect of Macronutrients in Insects?

The GFN has been widely applied in insects, the overwhelming majority of results suggesting that dietary macronutrients, not calories, drive the DR effect. For example, when *D. melanogaster* flies were fed diets varying in both protein:carbohydrate (P:C) ratio and calorie density, lifespan was maximized on a P:C ratio of 1:16, reproductive rate at 1:2 and fitness (lifetime egg production) at an intermediate ratio of 1:4.^[6] Similar patterns have been observed repeatedly in *D. melanogaster*^[22–24] and in a number of other insect species, including: the field cricket *Teleogryllus commodus*,^[10,25] tephritid fruit flies *Anastrepha ludens*,^[26] and the Queensland fruitfly *Bactrocera tryoni*.^[27,28] However, many of these studies are from flies, and often manipulate feeding through a capillary feeder (CAFE assay).^[29] The CAFE assay results in unnaturally short lifespans, which may reflect that individuals are actually being starved, possibly due to difficulty in accessing food.^[6,23] However, the patterns from CAFE assay studies have been replicated where capillary feeding has not been used^[10,22,24] (although these are often restricted to fewer diets than those used in CAFE assays^[22]). Therefore, these findings appear to be generalizable, limitations with the CAFE assay notwithstanding, though more work is needed to clarify this. A key pattern repeatedly observed is that lifespan does not increase with decreasing calorie content,^[6,10,26] suggesting no overall effect of caloric intake and even some instances of lower lifespan on calorie-diluted diets.^[6,23] Consequently, there is a growing acceptance that variation in macronutrients, not calories, are driving DR responses in insects.^[3,19]

2.3. What is the Effect of Macronutrients in Vertebrates?

This pattern is less clear in vertebrate species. In mice, it has been suggested that P:C ratio, rather than calories, is driving the effect of DR on lifespan^[8] and reproduction,^[30] the observed patterns being similar to those seen in insect studies. However, a study comparing calorie to protein restriction in mice from a different laboratory contradicted the findings, showing that protein restriction could not recapture the effects of CR for a suite of health-related measures.^[31–33] It has been suggested that methodologi-

cal differences in the mode of restriction cause the discrepancies between these studies.^[19] Typically, studies reporting significant effects of CR use a classical restriction method, where caloric intake is reduced through limiting the availability or size of the food portion. Whereas studies finding a greater effect of macronutrients typically restrict caloric intake by diluting the diet. Some have suggested that dilution and restriction may trigger different responses.^[19] However, a recent study in a vertebrate species, the three-spine stickleback (*Gasterosteus aculeatus*), combined varying macronutrient content with a classical restriction technique and still found the effect of macronutrients to be greater than that of calories: the effect of macronutrients largely mirrored the findings from insect studies.^[9] This lends support to the suggestion that the DR response is evolutionarily conserved and driven by macronutrient content of the diet and not caloric intake.

2.4. Is It Protein, or the Ratio of Protein:Non-Protein?

With the plethora of macronutrient DR studies being published over the last decade, the question remains, what is driving the DR response: protein content, non-protein content, or the ratio of protein:non-protein (P:NP) in the diet? The most commonly discussed finding across macronutrient DR studies is that of protein intake: low protein intakes maximize lifespan and high protein intakes maximize reproduction (Table 1),^[6,10,23,27,34] leading to the suggestion that macronutrient DR is the result of protein restriction. However, in both mice and male sticklebacks, the non-protein component of the diet (carbohydrate and lipid, respectively), rather than protein content per se, underpinned changes in lifespan.^[8,9] Furthermore, many of the studies reporting significant effects of protein intake on lifespan, also report significant effects of non-protein components on lifespan (Table 1). Given the number of studies reporting significant effects of both protein and non-protein dietary components, we feel the focus on protein alone is unwarranted, and the effect of DR should be discussed in terms of the ratio of P:NP in the diet.

For the remainder of this review, we focus on the role of macronutrients and refer to diets in terms of the P:NP ratio, unless explicitly discussing the effect of a specific macronutrient. We do not discuss the growing evidence of specific amino acid effects in DR responses, this is beyond the scope of this review (see Box 1). By taking this macronutrient-orientated view, many of the current evolutionary explanations for the DR response must be reconsidered as they were developed to explain a response to CR and not to manipulation of macronutrient ratio. In addition, those theories that do take a more macronutrient orientated approach to understand the evolution of DR, typically focus on protein, and do not consider the effects of a wider range of macro- and micro-nutrients. Given the extensive interest in using DR or DR mimetics as a potential ageing intervention for humans,^[2] a more informed hypothesis of how and why the DR response evolved is critical to understanding the significant variation in the DR response^[35–37] and how the effect of DR will change when taken outside the benign laboratory environment. For the remainder of this review we will discuss the current evolutionary explanations of DR, attempt to reconcile each of them with the recent evidence from GFN studies and highlight where

Table 1. Effects of macronutrient intake on lifespan in GFN studies.

	Species	Sex	P	P ²	NP	NP ²	PxNP
Lee et al. 2008 ^[6]	<i>D. melanogaster</i>	Females	–	+	+		–
Skorupa et al. 2008 ^[38]	<i>D. melanogaster</i> (Yw Strain)	Females	+	–	+	–	+
	<i>D. melanogaster</i> (Conton-S)	Females		–	+	–	+
Jensen et al. 2015 ^[23]	<i>D. melanogaster</i>	Males	–		+	–	
		Females	–	+	+	–	
Carey et al. 2008 ^[26]	<i>A. ludens</i>	Males	+	–	+	–	–
		Females	+	–	+	–	+
Fanson et al. 2009 ^[27]	<i>B. tryoni</i>	Females	–	+	+	–	
Fanson et al. 2012 ^[28]	<i>B. tryoni</i>	Males	–				
		Females ^{a)}	–		+		
Fanson et al. 2012 ^[39]	<i>B. tryoni</i>	Females	–		+		
Malod et al. 2017 ^[40]	<i>C. cosyra</i>	Males	–		+	–	+
		Females	–		+	–	
Maklakov et al. 2008 ^{[10]b)}	<i>T. commodus</i>	Males	✓	✓	✓	✓	✓
		Females	✓	✓	✓	✓	✓
Harrison et al. 2014 ^[41]	<i>G. veletis</i>	Males			+	–	NA
		Females			+		NA
Solon-Biet et al. 2015 ^{[30]c)}	<i>M. musculus</i>	Males		NA	+	NA	
		Females		NA	+	NA	
Moatt et al. 2019 ^[9]	<i>G. aculeatus</i>	Males			+	–	
		Females ^{d)}		+/-			

^{a)} Fanson et al.^[28] reports effects for 3 female treatments (mated, virgin, and sterilized) however the effect of macronutrient intake was consistent across all three groups; ^{b)} Maklakov et al.^[10] do not report the direction of the macronutrient effects in the main text or supplement and the raw data is no longer available. It is not possible to estimate the direction of the effects from the figures, therefore we have used ✓ to indicate a significant effect for comparison purposes; ^{c)} Solon-Biet et al.^[30] do not report the direction of macronutrient effects, however the significance is given in Table S4, Supporting Information, and directions can be assumed from Figure 4; ^{d)} In Moatt et al.^[9] the effect of protein intake on female lifespan varies temporally, we have used +/- to represent this variation. Summary of the main effects of protein (P) and non-protein (NP; carbohydrate or lipid) on lifespan in GF studies. P/NP represent linear effects, + denoting a positive effect and – a negative effect. P²/NP² indicate non-linear effects, + indicating a convex curve (trough) and – a concave curve (peak). PxNP represents interaction effects where the effect of one macronutrient is dependent on the other (+ indicating positive interaction and – a negative interaction). No study reports the interaction between P²xNP². NA indicates an effect that was not reported. Only studies that used a full GF approach (i.e., varied P:C ratio and calorie density simultaneously) and measured lifespan or survival were included, and only significant effects were reported. For consistency, where studies report mortality risk^[9] we have interpreted a negative effect of macronutrients (i.e., reducing mortality risk) as a beneficial effect and used the corresponding + sign.

these theories and the empirical evidence do not agree. In discussing the empirical evidence we focus on studies using females as observations from studies in males are rarer and seem to be more complex (see **Box 2**).

3. The Resource Reallocation Hypothesis

3.1. What is the Resource Reallocation Hypothesis?

The most widely accepted evolutionary explanation of DR is a trade-off model based around Kirkwood's disposable soma theory of ageing.^[47] First described by Holliday^[48] and later tested through simulation models,^[49] this theory has been known by various names, including the Y-model of DR^[28] and the adaptive resource reallocation model.^[50] Here we use the term resource reallocation hypothesis (RRH; as in ref. [51]), as we feel this highlights the key distinction between this and the other theories discussed below, as the reallocation of resources is the central theme of this hypothesis.

The disposable soma theory suggests that a trade-off exists between reproduction and somatic maintenance (lifespan).^[47] The RRH^[48,49] proposes that during periods of famine (e.g., CR), natural selection should favor a switch in allocation, in which context organisms reallocate energy almost exclusively to somatic maintenance and not to reproduction. By investing heavily in somatic maintenance, organisms will improve their chances of surviving the period of famine, when it is likely that the cost of reproduction is high and offspring survival low, resulting in lower fitness returns. Once conditions improve, investment in reproduction can resume, and that should result in higher fitness. Critically, the reinvestment strategy described in the RRH will only lead to higher fitness if conditions improve. Owing to the trade-off, the RRH predicts that under DR conditions in the lab, there should be an increase in lifespan accompanied by a corresponding decrease in reproduction.

It is important to note that despite often being referenced to explain macronutrient DR, the RRH was proposed to explain the effects of CR and a reduction in energy intake/change in energy allocation, not changes in dietary P:N ratio. The merits of the

Box 1. Amino Acids

There is growing evidence for a role of micronutrients in DR responses, particularly amino acids.^[36,42,43] It has been suggested that dietary protein sources often do not contain the optimal ratio of amino acids and when this occurs, organisms overconsume total protein to maximize intake of limiting essential amino acids—such as methionine in *D. melanogaster*.^[42] However, when overconsuming total protein, organisms also ingest a greater quantity of all amino acids, and one or more of these is suggested to have negative effects on lifespan.^[36,42] By achieving the optimal balance of amino acids, it is possible to maximize both lifespan and reproduction simultaneously. For example, exome-matched diets in *D. melanogaster* have been shown to be more satiating, being associated with enhanced growth and higher reproduction, yet with no negative effects on lifespan.^[43] The role of amino acids in DR responses is likely to be a central topic for consideration in the future. However, we feel there is likely to be a great deal of species specificity in amino acid requirements and the generality of these effects across species have not been fully demonstrated, although early indications suggest exome-matching is also possible in mice.^[43] A full discussion of the role for specific amino acids is beyond the scope of this review, so we restrict our discussion to the role of protein as a whole.

Box 2. The Male Problem

Throughout this review, we highlight the recurring pattern from macronutrient DR studies in females, where in general, lifespan is maximized on lower P:NP intakes and reproduction on higher P:NP intakes, suggesting that protein is essential for female reproductive activities.^[6,9,10,30] This pattern has also been observed in males.^[9,30,34] However, it has also been observed that carbohydrate intake, not protein intake, is key for male reproductive activities,^[10,23,25,44] and that males are able to maximize both lifespan and reproduction simultaneously on low P:NP intakes.^[10,23] This contradictory evidence in males is likely caused by the effects of P:NP ratio being dependent on the male reproductive trait of interest.^[9,30,45] Whether a single reproductive trait can act as a proxy for male reproductive investment (e.g., courtship^[34]) or whether a more complex array of traits^[9,30,45] is more relevant is likely to be highly species specific. Discussion surrounding this topic is further hampered by a lack of studies utilizing males in DR studies.^[46] Given these contradictory findings, the complexity in their interpretation and an overall shortage of empirical evidence in males, we only use observations from females when discussing the potential evolutionary explanations for the DR response.

RRH in terms of CR have been discussed elsewhere.^[50,51] Here, we will consider whether the RRH can be reconciled with the emerging view that macronutrients, not calories, are driving the DR response.

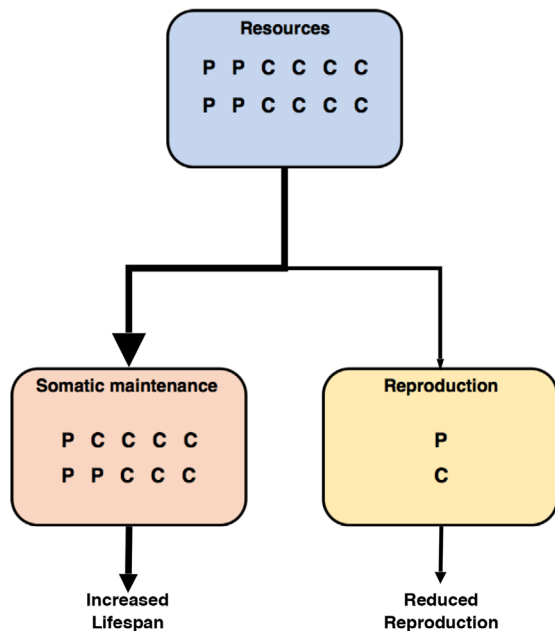
3.2. Can the RRH be Applied to Macronutrient DR?

Results from macronutrient DR studies are often interpreted in the light of the RRH despite its foundation in results from CR studies. Broadly, results from macronutrient DR studies suggest that lifespan and reproduction are maximized at different points of the nutrient landscape: lifespan is highest on low P:NP intakes and reproduction is maximized at higher P:NP intakes. This pattern has strong support in females, as seen in a wide range of species including: a variety of fruit fly species,^[6,22,23,26,27,40] crickets,^[10,25,34] sticklebacks^[9] and mice.^[8,30] Generally, these results have been interpreted as being indicative of a diet-mediated trade-off between lifespan and reproduction, where lifespan and reproduction cannot be maximized on the same P:NP intake (but see ref. [43] and Box 1). Therefore, organisms must trade off ingesting diets that maximize lifespan (low P:NP) against those that maximize reproduction (high P:NP)—fitness (lifetime reproductive success) is often maximized on diets intermediate between these two optima.^[6]

The central theme of the RRH is that resources are reallocated from reproduction to somatic maintenance under periods of nutrient limitation. However, the diet-mediated trade-off described above does not necessarily involve any reallocation of resources. For the RRH to explain the findings of macronutrient DR studies, we must consider how resource reallocation may be involved. Increasingly evident from studies using the GFN is the importance of protein for reproductive activities and of non-protein dietary components for somatic maintenance and lifespan. It has therefore been suggested that on low P:NP diets, organisms cannot ingest sufficient protein for reproduction and this triggers the DR response described in the RRH, involving higher investment in somatic maintenance at the expense of reproduction.^[28,52] This occurs by reallocating the limited protein that is available, from reproduction to somatic maintenance while also investing less of the available non-protein resources into reproduction (Figure 1). On very high P:NP diets, there is ample protein for reproduction but there is limited non-protein resources. This results in the available non-protein resources being invested in reproduction, rather than somatic maintenance, leading to high reproductive output but reduced survival (Figure 1). This is our attempt, based on previous suggestions,^[28,52] to propose how the RRH and the idea of a reallocation of resources could be applied to general patterns emerging from macronutrient DR studies.

However, as mentioned above, the observed diet mediated trade-off does not necessarily require a reallocation of resources. The most parsimonious explanation is provided by the concept that organisms are constrained by the P:NP ratio of the diet and are simply not able to maximize both lifespan and reproduction at the same intake. Many studies report direct negative effects of protein on lifespan^[10,28] (but see ref. [9]), which is not necessarily indicative of two processes competing for available resources. Rather, this suggests a direct physiological cost of protein metabolism, which results in reduced lifespan.^[28,53] The increase in lifespan on lower P:NP diets could be explained equally well as a reduction in the physiological costs of protein ingestion due to being maintained on a low protein diet. The idea of a direct physiological cost of protein will be discussed in more detail below (see Section 5), but evidence of a direct physiological cost

(a) Low Protein



(b) High Protein

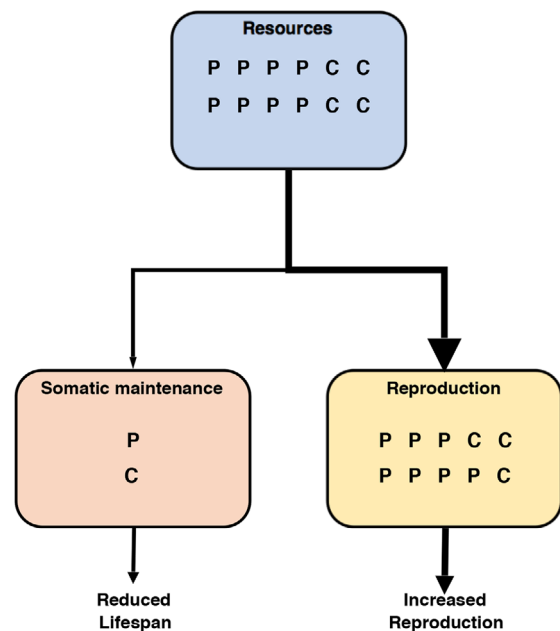


Figure 1. Authors interpretation of how the resource reallocation hypothesis (RRH) could work under macronutrient DR. P = protein and C = carbohydrate. We propose that protein and carbohydrate are not interchangeable and some amount of both macronutrients are required for somatic maintenance/reproduction. Therefore, organisms would face a resource trade-off as described under the RRH. a) Under low protein conditions where protein is limiting, a greater proportion of resources (both carbohydrate and the limited protein available) are allocated to somatic maintenance rather than reproduction, leading to high lifespan and low reproduction. b) Under high protein conditions, where protein is not limiting, a greater proportion of resources are used for reproduction and less for somatic maintenance, therefore reducing lifespan and increasing reproduction.

of protein metabolism contradicts the suggestion of resource reallocation under DR.

The above proposition of how the RRH could be applied to the relationship between macronutrient intake and lifespan (and reproduction) also takes the rather simplistic view that protein is important for reproduction and carbohydrate for lifespan. Under these assumptions, it would be expected that the more carbohydrate an organism ingested, or the lower the P:NP ratio, the greater the lifespan increase. However, this ignores the empirical evidence demonstrating a cost associated with overconsumption of carbohydrate.^[22,38,54] For example, in *D. melanogaster* maximum lifespan peaked on a P:C of 1:4 and medium lifespan on 1:2; lifespan decreased as the carbohydrate content increased.^[22] The same pattern is often observed, many studies finding non-linear effects of carbohydrate (or non-protein) intake on lifespan^[9,23,26] (Table 1). This does not fit with the overly simplistic proposition that only non-protein resources are required for survival.

3.3. The RRH and Macronutrients: Conclusions and Future Directions

In conclusion, it is difficult to assess whether the RRH provides a reasonable evolutionary explanation of the observed relationships between macronutrient variation, lifespan, and reproduc-

tion. It is clear that a modified version of the RRH could explain current patterns, in the context of resources being reallocated between survival and reproduction depending on the macronutrient ratio of the diet. However, as we discuss above, a more parsimonious explanation is a direct constraint effect of diet: survival and reproduction are simply maximized at different macronutrient ratios and reallocation of resources is not required. These diet-mediated trade-offs (or constraints) clearly occur in females and play a role in responses to macronutrient DR. However, the idea of a direct reallocation of resource on low P:NP diets, as expected under the RRH, is not necessarily empirically supported.

The most unequivocal way to distinguish between resource reallocation and a direct effect of diet would be to monitor how ingested protein and carbohydrate is used. For example, the protein component of the diet could be labelled and then monitored to explore how protein is divided between somatic and reproductive tissues and whether this varies across a range of diets varying in P:NP ratio.^[55] If a greater proportion of the labelled protein is integrated in the soma on low P:NP diets compared to high P:NP diets, this would offer direct evidence to support the RRH. Key here is how the proportion of labelled protein changes on different diets, and not the total amount of protein sequestered to the soma and reproductive tissues. If the proportion on somatic tissue increased on low P:NP diets, this would be indicative of a reallocation of resources to promote survival.

4. The Nutrient Recycling Hypothesis

4.1. What is the Nutrient Recycling Hypothesis?

Recently, the RRH has been critiqued,^[50] the argument against it being that adopting a pro-longevity investment strategy is unlikely to increase survival in the wild, where the main sources of mortality are extrinsic (i.e., predation, wounding or infection). Furthermore, Adler and Bonduriansky suggest that any postponement in reproduction should result in a loss of fitness in the wild, as high extrinsic mortality rates make it unlikely that individuals will survive long enough to recommence reproduction.^[50] Instead, they propose an alternative evolutionary explanation that we will term here the nutrient recycling hypothesis (NRH).^[50] As with the RRH, the NRH was proposed to explain an effect of CR, not the more recent suggestion of specific macronutrient effects.

The NRH proposes that rather than sacrificing reproduction to increase longevity, organisms under DR attempt to maintain reproduction as much as possible in the face of reduced energy resources. To achieve this, organisms upregulate the activity of cell recycling mechanisms such as autophagy and apoptosis. This allows better use, and even recycling, of the available energy, which can then be used to maintain reproductive function. The argument here is not that the level of reproduction achieved under DR is greater or even matched to that of a fully fed individual, rather that the loss of reproduction is minimized. An interesting suggestion of the NRH is that the pro-longevity effect of DR is an artefact of benign lab environments. The main sources of mortality in the laboratory are old age pathologies such as cancer, which are ameliorated by upregulation of autophagy and apoptosis.^[50] However, in the wild, cancer and other old-age pathologies are a relatively minor source of mortality, so the protective effect of the DR response may not be observed. Indeed, Adler and Bonduriansky^[50] propose that, as a result of reduced cellular growth and proliferation, upregulation of autophagy and apoptosis in response to DR may actually make individuals more vulnerable to common sources of mortality in the wild (e.g., wounding and infection) and thus reduce survival.

Wider critiques of the logic behind the NRH can be found elsewhere.^[51,56] Here we focus on considering the NRH in light of the importance of specific macronutrients, rather than that of calorie intake, which to our knowledge has not been addressed.

4.2. Can the NRH be Applied to Macronutrient DR?

The central tenant of the NRH is that organisms are attempting to maintain reproductive output at as high a level as their environment will allow.^[50] Therefore, in our opinion, to apply to DR experiments that manipulate macronutrients this theory must be considered mainly in terms of protein availability. As we highlight throughout, protein has been repeatedly shown to be the macronutrient driving changes in reproduction in females.^[9,10,23] We suggest that on high P:NP diets, there are sufficient resources for reproduction to occur, with a plentiful supply of protein. Therefore, organisms can readily invest in reproduction and there would be a low basal level of autophagy, and high levels of translation and cell and tissue growth to support reproductive function (Figure 2). However, on low P:NP diets, protein is

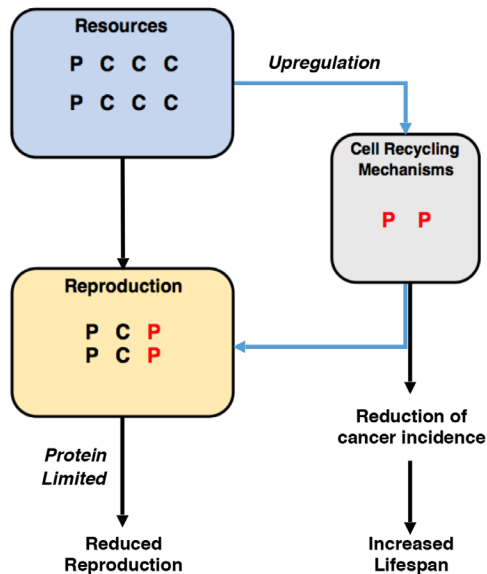
limited and amino acids are not available for reproduction. In these circumstances, organisms may upregulate cell recycling mechanisms to increase the availability of amino acids, which could be used to maintain some reproductive function (Figure 2). Under this scenario, on low P:NP diets where cell recycling mechanisms were upregulated, there would be a corresponding reduction in the incidence of cancer and other old age pathologies and thus an increase in lifespan in the laboratory. Critically, as discussed above, this pattern should only be observed in the lab, and the addition of further environmental stresses, such as infection, should remove the lifespan benefit of low P:NP diets. As with the RRH (Section 3), we are not suggesting that the observed pattern of high lifespans on low P:NP diets conclusively supports this hypothesis, rather that this is our interpretation of how the observed patterns could be consistent with the ideas of the NRH.

Under the predictions of the NRH (discussed above), the lifespan benefit of low-protein diets should only occur in the benign laboratory environment, and would disappear with the addition of environmental stressors (e.g., infection or wounding).^[50] An obvious test of the NRH in relation to macronutrients, therefore, would be to carry out macronutrient manipulations in concert with the addition of environmental stressors, and explore how this changes the effect of macronutrients on lifespan. Although direct tests are lacking (but see below for recent results), a small number of studies provide evidence to suggest that the effect of protein limitation on lifespan changes when accompanied by immune challenge. For example, protein restriction following infection has been shown to reduce immune responses, and individuals often self-select for a higher protein intake following infection, which offers a survival advantage to infected individuals.^[57–61] This would seem to fit with the NRH when applied to macronutrients. Low protein diets trigger an upregulation of autophagy and apoptosis, which generates resources that can be used for reproduction but on these low protein diets, individuals are more susceptible to infection. By extension under more natural conditions, such as exposure to infection risk, the lifespan increase in response to low protein diets may not occur.

However, the suggestion that immune function and survival following infection is improved on high P:NP diets is not clear cut. For example, bacterially challenged *B. tryoni* self-selected a lower P:NP diet that offered survival advantage over flies on more protein rich high P:NP diets.^[62] Similarly, *D. melanogaster* have been shown to have higher survival on low P:NP diets following infection.^[63] These findings would be counter to the predictions of the NRH as laid out above, which would predict lower survival on low P:NP diets when combined with immunological challenge. Furthermore, a recent study in *D. melanogaster* directly testing how the relationship between macronutrient content and lifespan changed with injury and infection found that the overall relationship between protein limitation and survival was the same for infected/wounded flies and control flies (Savola, Vale, Montgomery, Monteith, Waldron & Walling unpublished). Based on the predictions of the NRH, we would have expected the beneficial effect of lower protein intakes on lifespan to be removed by the addition of either wounding or infection.^[50]

Although the above studies provide some evidence against the NRH, the most direct test would be to measure the effect of macronutrient manipulation on lifespan in the wild. As we have already discussed, the NRH would predict no lifespan benefit in

(a) Low Protein



(b) High Protein

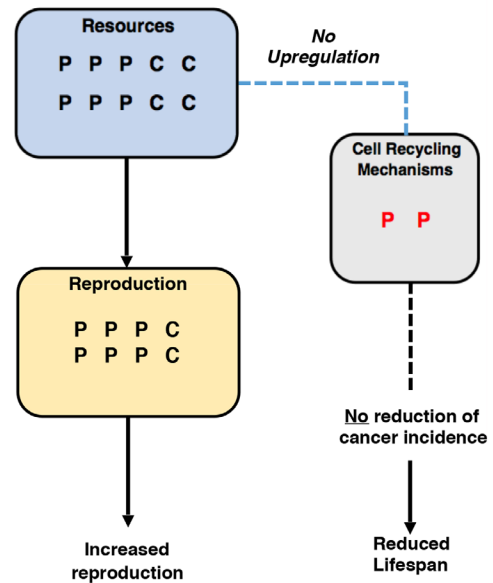


Figure 2. The nutrient recycling hypothesis (NRH) under macronutrient manipulation. P = protein and C = carbohydrate. a) Under low protein conditions, cell recycling mechanisms are upregulated, generating more resources to be used for reproduction and reducing cancer incidence, leading to increased lifespan when housed in the lab. b) Under high protein conditions there is no upregulation of cell recycling mechanisms as there is sufficient protein for reproduction. Consequently, there is no reduction in cancer incidence and no increase in lifespan in the lab. As the upregulation of cell recycling mechanisms only reduces mortality risks associated with the lab, the NRH would predict no effect of macronutrient intake on survival in the wild.

the wild in response to changing P:NP of the diet. A very recent study using antler flies, *Protoperla litigata*, compared the effect of varying macronutrient content on mortality and reproduction in wild and lab individuals.^[64] Interestingly, this study found that the negative effect of increased protein intake (via protein supplementation) on mortality seen in the lab was also seen in the wild,^[64] although this varied between years. Mautz et al.^[64] concluded that the effect of protein on lifespan and mortality was not an artefact of being reared in a benign laboratory environment, but was an ecologically relevant response. Although this work only used a limited number of diets and the results were inconsistent across years, replicating the laboratory protein effect on mortality in the wild is a very difficult finding to reconcile with the NRH.

4.3. The NRH and Macronutrients: Conclusions and Future Directions

In conclusion, by considering the importance of protein for reproduction, it is possible to reconcile some aspects of the NRH with results from macronutrient DR studies. However, despite broad patterns of the relationship between protein content and survival being consistent, key predictions about how infection should alter this relationship are not met, though results are complex and often contradictory. Furthermore, one of the base predictions of the NRH, that the DR effect is a laboratory artefact and will not occur in the wild, is directly contradicted by the recent

finding that the negative effect of protein on lifespan is reproducible in the wild.^[64] In our opinion, the NRH is unlikely to be the main evolutionary mechanism by which the DR response has evolved. We acknowledge that under some instances, maintaining reproductive output during times of nutrient stress could provide a fitness advantage, such as in a temperate herbivore population experiencing spatial variation in habitat quality and food availability during spring.^[51] However, the NRH does not appear to provide a general explanation for the DR phenomenon.

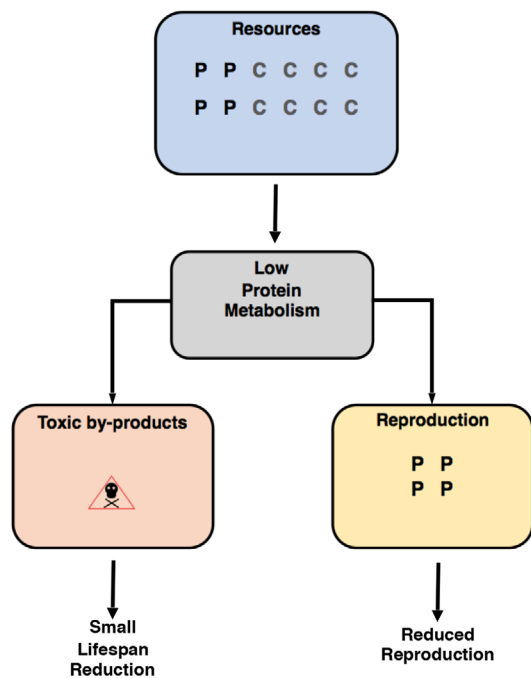
As we highlight, the work of Mautz et al. is the most direct test of the NRH to date.^[64] However, this is a single study in one species, and the results were inconsistent across years. More studies are needed to assess how reproducible the macronutrient DR effect is outside of the benign laboratory environment, using a greater range of species and across longer time scales, to account for the significant variation across years. This is by no means a simple task, however, a small number of recent successful applications of the GFN in wild systems suggest sophisticated data on dietary intake can be collected in the wild.^[65–67]

5. The Toxic Protein Hypothesis

5.1. What is the Toxic Protein Hypothesis?

A more recent hypothesis to be put forward is the toxic protein hypothesis (TPH,^[28] **Figure 3**), which is a constraint-based model rather than an evolutionary theory. Unlike the theories already

(a) Low Protein



(b) High Protein

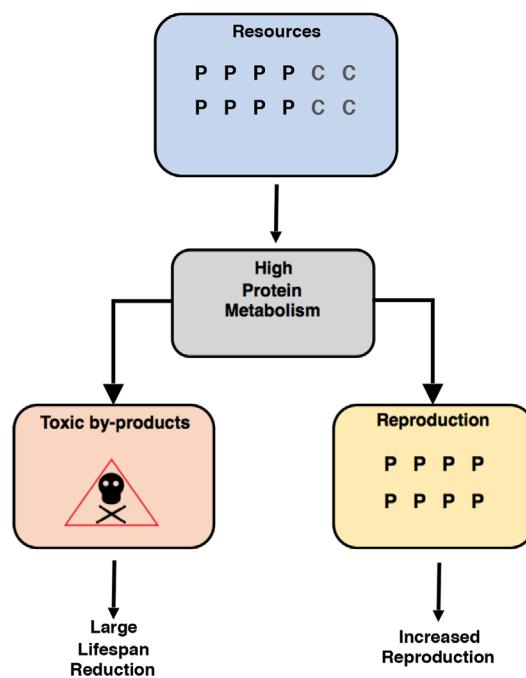


Figure 3. Schematic diagram of the toxic protein hypothesis. P = protein and C = carbohydrate (C's are greyed out as the TPH makes no predictions for a role of carbohydrate intake). a) Low protein diets, have much lower toxicity so reduced pathological effects and increased lifespan, but have much lower reproduction. b) High protein diets lead to increased reproduction, but high protein intake has pathological effects resulting in reduced lifespan.

discussed, the TPH was put forward in light of renewed focus on the role of macronutrients in DR responses. The TPH argues that protein is essential for reproductive function, where increasing protein intake leads to higher reproductive rates.^[28] However, it is proposed that high consumption of protein has direct negative effects on late-life health and lifespan, through increased production of both toxic nitrogenous compounds from protein metabolism and mitochondrial radical oxygen species (Figure 3).^[68,69] Therefore, organisms face a constraint in the amount of protein they can consume, balancing high protein intake to maximize early life reproductive output whilst avoiding overconsumption, which may reduce lifespan and ultimately result in lower fitness. As with the other hypotheses, under the TPH there would be an optimal protein intake that maximizes lifetime reproductive success or fitness. However, the TPH argues that the DR response of increased lifespan is the result of protein restriction reducing the direct physiological costs of protein ingestion (Figure 3).^[28]

5.2. How can the TPH be Applied to Macronutrient DR?

Many of the early results from macronutrient studies report negative effects of protein intake on lifespan and positive effects on reproduction.^[6,10,23,26,34] However, as discussed above (Section 3) this does not distinguish between direct costs and resource reallocation. The first direct test of the TPH used Queensland fruit flies, *B. tryoni*^[28] and compared the effect of changing P:C ratio of

the diet between mated, virgin and sterilized females. This study found no difference in the effect of diet between any of the treatment groups: all treatments showed increasing lifespan as the P:C ratio of the diet decreased. Fanson et al.^[28] suggested that under a resource allocation framework there should be no effect of diet on lifespan in the unmated or sterilized groups, as no resources were needed for reproduction and could be used for somatic maintenance and lifespan. Consequently, the authors interpreted this as a direct toxic effect of protein intake, supporting the TPH.^[28]

However, we suggest that the experiment described above^[28] does not preclude a resource reallocation explanation. Preventing the act of mating or egg production, does not necessarily prevent resources from being partitioned to reproduction.^[70,71] For example, changes in reproductive investment in response to diet are suggested to be mediated by the nutrient sensing pathways, insulin/insulin like signaling pathway (IIS) and mechanistic target of rapamycin (mTOR).^[51,72,73] Sterilizing a female will not change how these nutrient sensing pathways respond to ingested nutrients and the physiological changes triggered. Without disrupting these nutrient sensing pathways, it could be expected that all three groups would respond the same under both the RRH and the TPH. The lack of difference between the mated, unmated, and sterile individuals could be due to there being no difference in their allocation of resources, rather than them experiencing the same pathological effects of protein ingestion. The results of this study, therefore, are not able to distinguish between mechanisms invoked by the RRH and TPH.

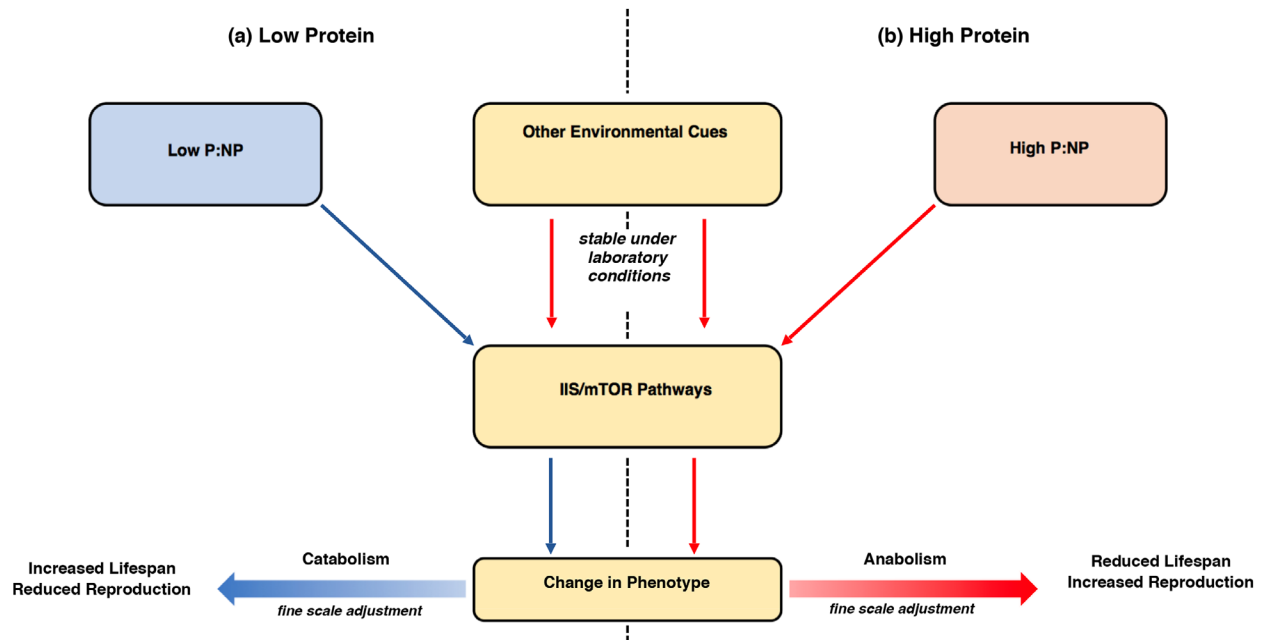


Figure 4. How adaptive plasticity via the nutrient sensing pathways may work in the lab. We suggest that in the wild diet is one cue of overall environmental conditions that can result in shifts along an anabolism–catabolism axis. In the lab, experiments are only carried out under stable laboratory conditions, which are generally more toward the pro-reproduction (anabolic) end of the spectrum of potential environmental conditions (i.e., high temperature, long photoperiod, etc.). Under these stable conditions, changes in dietary P:NP ratio results in more fine scale adjustments in phenotype than might be observed in the wild, where changes in P:NP intake may be accompanied by changes to a wider suite of environmental cues. a) When individuals in the lab are fed low P:NP diets there is a subtle shift toward a more catabolic state leading to increased lifespan and reduced reproduction. b) Conversely, when individuals in the lab are fed high P:NP diets there is a fine scale shift toward a more anabolic state with reduced lifespan and increased reproduction.

In addition, the TPH predicts that consumption of protein poses a direct physiological cost to survival (Figure 4). Consequently, studies on the effect of macronutrient intake should consistently report a negative effect of protein intake on lifespan. Although this is common^[6,10,23,26,34] (Table 1), recent evidence suggests this toxic effect could be due to the presence of certain specific amino acids^[43] (Box 1). Furthermore, there are a number of studies reporting no effect of protein on survival or lifespan. For example, there was no direct effect of protein intake on lifespan in mice (see Table S4, Supporting Information, in both^[8,30] and Table 1), rather changes in lifespan were driven by carbohydrate intake.^[8,30] In addition, a recent study in sticklebacks, reported beneficial effects of protein intake on early life survival in females,^[9] and similar effects are seen in early life survival in *D. melanogaster*^[74] (but see ref. [75]). Although Moatt et al.^[9] do report the expected detrimental effect of protein intake on late life survival in females, we would argue that the TPH does not predict any change with ontogeny in the direct physiological cost of protein consumption.

Finally, we believe the TPH's suggestion of a direct pathological effect of protein on lifespan does not fit with current understanding of the physiological mechanism of DR. As mentioned briefly above, attenuated signaling through the IIS/mTOR pathways has been suggested as the main mechanism through which DR acts.^[72,73] DR responses can be triggered through direct manipulation of both the IIS and mTOR pathways, leading to increased lifespan without the need for macronutrient manipulation.^[52,76,77] Given that differences in lifespan are ob-

served between individuals that differ only in levels of signaling through nutrient signaling pathways and that are consuming the same food,^[73] there is an apparent inconsistency with a direct toxic effect of protein on lifespan described by the TPH. However, there is some evidence that IIS/mTOR manipulations and DR can act additively to increase lifespan^[78] leaving open the potential for a direct toxic effect of protein or specific amino acids (see Box 1).

5.3. The TPH and Macronutrients: Conclusions and Future Directions

In conclusion, whilst there is some evidence for a pathological effect of protein ingestion, particularly at very high protein intakes, we feel the TPH is too simplistic and neglects a number of studies showing no effect, or in some cases beneficial effects, of protein consumption. Furthermore, the suggestion of a direct toxic effect of protein is not supported by recent genetic and pharmacological manipulations of the nutrient sensing pathways. It seems likely that there is some direct negative effect of protein ingestion and metabolism on lifespan, but that there may also be a role for resource reallocation, or a diet mediated trade-off, that would allow for the observed patterns from direct manipulations of the nutrient sensing pathways.

Given the role of the IIS/mTOR network in DR responses, an interesting test of the TPH would be to use individuals with attenuated IIS/mTOR activity, that is, individuals who are

incapable of responding to changes in the nutritional environment, and test how changing P:NP ratio affects lifespan. We suggest that the TPH would still predict increasing lifespan as the ratio of P:NP decreases, as impaired activity of nutrient sensing pathways would not change the pathological effects of protein consumption. Alternatively, as we suggest at the end of Section 3.3, one potential test would be to label the protein components in the diet, then explore how changing P:NP ratio alters the investment of protein into the soma and the germline. We argue that if changing the P:NP ratio did not change the proportion of protein being allocated to these tissues, this would argue against a reallocation of resources and could suggest a role for a direct toxic effect of protein consumption.

6. Future Perspectives: Adaptive Plasticity via “Nutrient Sensing” Pathways

A recently suggested framework with a more integrated ecologically and physiologically considered approach, could offer an interesting future direction for understanding the evolutionary mechanisms underpinning DR responses.^[51] This new perspective proposes that diet is one of a broader suite of environmental cues (such as changes in temperature and photoperiod) that are predictive of current and upcoming environmental conditions. Regan et al.^[51] suggest these cues could be integrated by the IIS/mTOR pathways (often termed the “nutrient sensing” pathways) triggering phenotypic changes to better match an organism’s investment in survival and reproduction to their current or future environment. The range of these phenotypic changes covers fine-scale adjustments (e.g., timing of reproductive investment) to deep physiological remodeling in response to challenging conditions (e.g., hibernating through winter months). It is important to stress that Regan et al.^[51] do not present a new evolutionary theory, rather a shift in perspective, incorporating aspects of the existing evolutionary theories discussed above (Sections 3–5).

Regan et al.^[51] propose that CR induced lifespan extension in the lab, is the result of manipulating a single environmental cue, diet availability, while holding all others cues/conditions constant and at a level that generally indicates favorable conditions for reproduction (high temperature, long photoperiod, etc.). Manipulating energy availability in these lab conditions triggers a phenotypic change toward a state of catabolism, associated with increases in autophagy and cellular recycling, leading to increased longevity and a reduction in reproductive output,^[51] the characteristic laboratory CR response. In the wild this shift toward a more general catabolic state could be an attempt to optimize the timing of reproductive investment during otherwise pro-reproductive conditions and provide a survival advantage during periods of low resource availability, ultimately resulting in higher fitness as suggested by the RRH (see Section 3). Conversely, when energy availability is high and all other environmental variables also favor reproduction, there is a shift toward a more general anabolic state with a greater proportion of resources used for reproduction and fewer resources used for somatic maintenance, resulting in higher reproductive rates and shorter lifespans—as seen in the lab.

We propose that the P:NP ratio of a diet could also act as a cue for immediate/future environmental conditions, rather than overall energy availability. Changes in diet as a result of changes in the availability of food items are common^[79–81] as are seasonal changes in dietary preference.^[82–87] In our opinion these changes in food availability/preference could be a good indication of current/near future environmental conditions. In the same way Regan et al.^[51] propose a reduction in energy availability cues for less favorable current conditions for reproduction, we propose a reduction in P:NP ratio could act in the same way—resulting in a shift to a more general catabolic state with reduced reproduction and increased somatic maintenance. Alternatively, a sudden decrease in the availability of high P:NP food sources in summer could be a signal for unfavorable reproductive conditions, leading to a fine-scale shift toward a lower state of anabolism, decreasing reproductive output in the short term.

We suggest that studies of macronutrient DR in the lab, mirror this natural variation in P:NP intake, but in isolation of wider environmental cues (such as changes in temperature and photoperiod). In the lab, feeding low P:NP diets shifts individuals to a more general catabolic phenotype with lower reproduction, increased somatic maintenance and longer lifespan (Figure 4a). When individuals are maintained on high P:NP diets there is a shift in phenotype to an anabolic state and an increased rate of reproduction (Figure 4b). However, these diet manipulations are generally applied under environmental conditions that favor reproduction (high temperature, long day length etc.). This should result in relatively fine-scale adjustments in investment in survival and reproduction that would allow individuals to deal with short term reductions in resource availability within the laboratory environment and does not result in larger phenotypic changes that may be experienced in the wild. Repeating the studies of macronutrient DR under a wider range of environmental conditions, could result in very different phenotypic responses being observed. Again, overall we feel this interpretation of the effect P:NP ratio in the lab is qualitatively similar to the interpretation of energy availability put forward in Regan et al.^[51]

Our suggestion is a simplified interpretation of how macronutrient DR could fit with the perspective suggested by Regan et al.^[51] There is likely to be some tissue, macro- and micro-nutrient specificity in the response, but a detailed discussion of how macronutrients interact with the IIS/mTOR network is beyond the scope of this review. Our aim is to suggest a general effect that we feel should apply to the general mechanistic explanations for the interplay between resource availability and the IIS/mTOR network already described in Regan et al.^[51] We are far from understanding how studies of macronutrient DR, and the GFN, maps on to the signaling networks and downstream phenotypes, or how the IIS/mTOR pathways cross regulate to incorporate multiple cues from different macronutrients. A great deal of further work is required before definitive conclusions can be drawn.

7. Conclusions

DR is the most consistent environmental manipulation to extend lifespan and healthspan and consequently there is extensive

Table 2. Take home messages and further work.

Theory/Perspective	Take home message	Further work
Resource reallocation hypothesis (RRH)	We propose a scenario where reallocation of resources could be taking place with varying P:NP ratio (Figure 1). However, in our opinion the most parsimonious explanation is that of diet mediated trade-off, in which different P:NP ratios maximize different life-history traits requiring no direct resource reallocation.	Label the protein and carbohydrate in the diet and assess if the proportion used for somatic and reproductive tissues changes across different P:NP diets.
Nutrient recycling hypothesis (NRH)	In our opinion the logic behind the NRH is flawed and empirical support, specifically from macronutrient DR studies, is lacking. We can imagine some circumstances where maintaining reproductive output under challenging conditions leads to improved fitness (Figure 2). However, we feel these conditions are restrictive and thus the NRH is not a general explanation.	Apply the GFN in the wild to explore whether patterns observed in laboratory macronutrient DR studies are reproducible in the wild.
Toxic protein hypothesis (TPH)	A constraint model rather than an evolutionary theory. We see merit in this sort of constraint consideration and a toxic effect of protein intake clearly occurs in some situations (Figure 3). However, we feel the TPH is too simplistic and ignores the strong evidence for an effect of non-protein dietary components in the effect of diet, and the impact of manipulating nutrient signaling pathways under stable diet conditions.	Apply GFN to individuals with attenuated IIS/mTOR activity. Label the protein and carbohydrate in the diet and assess if the proportion used for somatic and reproductive tissues changes across different P:NP diets.
Adaptive plasticity via “nutrient sensing” pathways	A shift in perspective considering the ecological (adaptive plasticity) and mechanistic (IIS/mTOR network) underpinnings of DR responses. We have provided a potential interpretation of how macronutrient intake could work generally within this new perspective (Figure 4). There is likely to be a great deal of complexity in how the nutrient sensing network responds to varying macronutrient intake.	Change environmental conditions within the lab and see how this changes dietary preference in wild type and individuals with attenuated IIS/mTOR activity.

interest in using DR or DR mimetics as a potential intervention in ageing for humans.^[2] The recent advent of the GFN has challenged the long-held view that a reduction in caloric intake underpins DR responses, instead suggesting a pivotal role for macronutrient intake and the ratio of P:NP in the diet. This presents a challenge in understanding the evolution of DR responses, as the majority of evolutionary explanations were put forward under the paradigm of CR. We have discussed how the existing evolutionary theories of DR responses could fit with the recent focus on macronutrients. However, in our opinion none of these theories in isolation can adequately explain the complexity of the results observed so far and thus why the DR response evolved (see **Table 2** for summary). We see some merit in all theories, but would suggest that in many cases a combination of explanations may be most likely: for example, a role for both a direct negative effect of protein on lifespan (TPH) and a reallocation of resources (RRH). One key to understanding the relative importance of alternative explanations will be the ability to track the relative allocation of different macronutrients to different life history processes. An interesting future direction could be the more integrated ecological and physiological framework suggested by Regan et al.,^[51] however as this perspective shift has only recently been proposed, more work is needed before it can be critically considered. Knowledge of the ecological and evolutionary mechanisms underpinning DR will be crucial to understanding the significance of, and variation in, the effect of DR and its generality outside of model systems and benign laboratory environment.

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Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

All authors contributed to the inception and planning of the ideas in this manuscript. J.P.M. and C.A.W. wrote the initial draft and all authors contributed to editing the final manuscript. J.P.M. designed the figures.

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adaptive plasticity, geometric framework of nutrition, nutrient recycling, resource reallocation, toxic protein, trade-off

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