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Nutrition and Energetics in Rodent Longevity Research

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Abstract

The impact of calorie amount on aging has been extensively described; however, variation over time and among laboratories in animal diet, housing condition, and strains complicates discerning the true influence of calories (energy) versus nutrients on lifespan. Within the dietary restriction field, single macronutrient manipulations have historically been researched as a means to reduce calories while maintaining adequate levels of essential nutrients. Recent reports of nutritional geometry, including rodent models, highlight the impact macronutrients have on whole organismal aging outcomes. However, other environmental factors (e.g., ambient temperature) may alter nutrient preferences and requirements revealing context specific outcomes. Herein we highlight factors that influence the energetic and nutrient demands of organisms which often times have underappreciated impacts on clarifying interventional effects on health and longevity in aging studies and subsequent translation to improve the human condition.

Keywords

macronutrients; protein; carbohydrate; longevity; dietary restriction; thermogenesis

1. Introduction

Nutrition in aging research over the last century has largely focused on the influence of the energetic contents of nutrients (i.e., heat energy as measured by joules or calories) in the context of energy balance, disease risk, and even organismal longevity (Weindruch &

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Walford, 1988). This has been predominated by the primary macronutrients – carbohydrates, fats, and proteins – with additional scrutiny placed on the specific types within a given macronutrient class (simple vs. complex carbohydrates, saturated vs. unsaturated fats, particular amino acids, etc.) (Zimmerman *et al.*, 2003). Typical diets in free-living conditions are compositionally diverse with foods most often providing complex mixtures of macronutrients of varying proportions, increasing the complexity and difficulty of assimilating nutrition research findings (laboratory and field based) into the overall study of nutritional energetics. Another layer of complexity arises with the addition of multiple micronutrients and essential factors that provide limited (or no) overall energetic value yet can impact the cell and organism's ability to properly metabolize nutrients containing caloric value (Ames, 2010; Ames, 2005; Ames, 1998). When considered then in the context of organisms' changing energetic demands coincident with fundamental nutrient requirements at different ages, varying levels of physical activities, various body compositions, etc., the desire or quest for identifying an optimized “one size fits all” diet to promote health and longevity seems daunting at best.

Implicit in the suggestion of an “optimal” diet is the notion that energetic and nutrient demands are balanced in the correct proportion for a specified phenotype of interest. Yet optimization often comes with tradeoffs or disadvantages for other outcomes, and may change over time with the development of the organism. For instance, much of the agricultural and livestock nutrition research has focused on the optimization of diets for rapid growth, particularly lean mass accretion, through improved feed efficiency and body composition outcomes (Mehri *et al.*, 2015; Johnson & Chung, 2007; Shurson *et al.*, 2015; Chung & Baker, 1991). In contrast, the founding work of dietary restriction performed in Dr. Clive McCay's research focused on the delayed sexual maturation and physical development of rodents during early life for subsequent late-life health and longevity extension (McCay & Crowell, 1934; McCay *et al.*, 1935). The late-life disease risks associated with rapid and efficient growth may be an acceptable tradeoff to a farmer who has short-term goals for market, but using agriculturally based diet formulations in pre-clinical research of longevity interventions may be in error. Once maturation and development is accomplished, a late-life focus on energetic and nutritional approaches to delay these processes would seem misplaced and/or ultimately insufficient. As such, a single example of the growth axis (i.e., maturation) with multiple inputs from genetic variation to environmental influence might be expected to influence desired phenotypic outcomes across the life course (i.e., aging). Efforts must be made to identify which modifiable inputs (nutrients, energetic demands) provided during specified periods of the life course support effective intervention in the direction of interest (Miller *et al.*, 2000; Miller *et al.*, 2002; Harper *et al.*, 2004; Sadagurski *et al.*, 2014; Sadagurski *et al.*, 2015).

Inherent in the design of most rodent aging or nutrition studies is the choice and use of a single manufactured diet. Although rodent diets may be formed from a complex mixture of ingredients, the ultimate composition is relatively fixed across time in relation to macronutrient proportions, micronutrient composition and caloric amount, with the only variability offered through the modulation of intake (Reeves, 1997; Reeves *et al.*, 1993). Thus, every day, each bite of food for the animal contains roughly the same nutrient and energetic composition. This is in stark contrast to the human condition where choice is an

integral component of diet and macronutrient proportions may be significantly different between meals and snacks, over the course of a day, and across the lifespan (Tucker, 2007; Rumpler *et al.*, 2006). Clearly a young, actively growing animal would have different energetic and nutritional requirements than a mature, reproductively active or older (reproductively senescent) organism. To partially address this, rodent diet formulations are sometimes modified for use with designation for high energy demand states in “growth” formulations (higher protein and fat) versus lower states of “maintenance” in post-reproductive adulthood (Reeves, 1997). Nevertheless, the recommended and common practice of using a single dietary composition (or at the most, sequential dual composition for growth and maintenance) for the duration of a longevity study leaves open the question of just how well this models the human condition and what impact dietary rigidity may have on integrated outcomes related to health and longevity. In the following sections, we highlight factors that influence the energetic and nutrient demands of organisms which often times have underappreciated impacts on clarifying interventional effects on health and longevity in aging studies.

2. Macronutrient Balance

McCay *et al.* (1935) set the foundation for dietary manipulation of aging by using rats to examine effects of slowed growth through dietary restriction on longevity. Researchers have since built upon the work to examine what component of the diet (calories from any macronutrient source), when restricted, extends lifespan. To address this question, the relative proportions of protein to carbohydrate have been changed in rodent diets, generally by adjusting available sucrose or dextrin. Riesen *et al.* (1947) used a base diet containing 36% casein and 48% sucrose to compare the effects of additional energy intake from sucrose on lifespan of rats. Increased energy intake from sucrose to an average 72% sucrose in the diet resulted in reduced median lifespan for rats when compared to rats fed only the base diet.

Over time, research has shown that the ratio of protein to carbohydrate in the diet is also an important consideration in regards to lifespan. Ross (1959, 1961) developed two diets that differed in the levels of casein and sucrose while holding the fat level constant. When provided to rats *ad libitum*, the diet containing the lower protein to carbohydrate ratio (P:C of 0.10, 8% protein and 83% carbohydrate) supported three times longer maximum lifespans for rats when compared to rats fed the diet with a higher protein to carbohydrate ratio (P:C 0.49; 30% protein and 61% carbohydrate). Rats fed the 0.10 P:C ratio diet also consumed less food on average than rats fed the 0.49 P:C ratio diet. Yu *et al.* (1985) demonstrated moderate improvements to both median and maximum lifespan for rats fed a diet *ad libitum* with a P:C of 0.19 (12.6% protein and 67.11% carbohydrate) when compared to that of rats fed *ad libitum* a diet with a P:C of 0.36 (21% protein and 58.65% carbohydrate). In their study, the rats consumed similar amounts of food, suggesting the composition and not necessarily total food intake contributed to the differences in reported lifespan.

Studies have shown that the macronutrient composition of a diet directly influences the amount of food ingested by an animal. Improperly balanced diets, especially in regards to protein and non-protein energy, trigger compensatory food intake mechanisms in an effort to

re-balance an organism's nutritional state and ultimately fitness (Simpson & Raubenheimer, 2007). Discerning the appropriate balance of macronutrients in animal (and human) diets remains a significant challenge when using a traditional reductionist experimental approach, in part due to greater emphasis on preventing undernutrition than over-nutrition. The Geometric Framework (GF) protocol, however, allows the investigator to capitalize on evolutionarily conserved mechanisms inherently driving an organism to choose a balance in macronutrients to support an optimized phenotype during a distinct phase in life history (Simpson & Raubenheimer, 2007). Thus, diets can be designed based on the organism's nutrient preferences that best support the phenotype of interest.

Simpson and Raubenheimer (1995, 2012) have demonstrated the usefulness of the GF protocol in nutritional studies for animals ranging from insects to humans and provide a set of guidelines for applying the technique. In brief, animals naturally require a certain balance and amount of nutrients (i.e., nutrient target) to optimally support life processes in specific phases of the life course (e.g., growth, maturation, reproduction). Due to energetic costs of feeding and wastes from inefficient food processing, animals must ingest an amount of food to offset these costs while still meeting the nutrient target, which is termed the intake target. When the intake target for a phenotype is unknown, foods with different ratios of the nutrients of interest (e.g., protein and carbohydrate level) that span the hypothesized nutrient targets can be proffered to groups of animals. The intake of each food is monitored, and the phenotype is measured in response to food intake. The intake target can then be assessed with a three dimensional surface plot by plotting the relationship between nutrient intake (e.g., protein vs. carbohydrate amount ingested) and the measured phenotype from the animal (e.g., weight gain, reproductive output, or lifespan). The highest phenotype point on the graph indicates the appropriate ratio of nutrients needed to optimally support the phenotype. The GF protocol can thus be used to evaluate many nutrient ratios simultaneously to discern the appropriate balance to formulate.

Using the GF protocol, Solon-Biet *et al.* (2014) identified three diet formulations that when fed *ad libitum* to male C57BL/6 mice showed similar or improved measures of lifespan when compared to a previous study conducted at Jackson Labs using a mouse chow containing 22%, 16%, and 62% of the calories from protein, fat, and carbohydrate, respectively (P:C of 0.35) (Yuan *et al.* Mouse Phenome Database 2016). The diet containing 42% protein, 29% carbohydrate, and 29% fat (P:C of 1.45) at an energy density of 4.06 kcal/g improved median lifespan by approximately 10 weeks (8%) and supported a similar maximum lifespan of approximately 150 weeks (assessed as mean of longest lived 10% of mice) when compared to results by Yuan *et al.* (Mouse Phenome Database 2016). The diet containing 5% protein, 75% carbohydrate, and 20% fat (P:C 0.07) at an energy density of 3.11 kcal/g improved maximum lifespan by roughly 7 weeks (5%) and supported a similar median lifespan of 129 weeks as that reported by Yuan *et al.* (Mouse Phenome Database 2016). The diet containing 14% protein, 57% carbohydrate, and 29% fat (P:C 0.25) also at an energy density of 3.11 kcal/g supported similar median and maximum lifespans as that reported by Yuan *et al.* (Mouse Phenome Database 2016). Thus, the macronutrient combinations identified in the GF study, fed to mice from young adulthood to old age, indicate defining the best formulation for healthy aging may depend upon what metric of

health or lifespan (e.g., overall population based on ‘median survival’ or select individuals based on ‘maximal survival’) one desires to improve.

3.0 Defining Longevity Effect

What is the proper comparison and determination of longevity effect – true “aging” deceleration where mean, median and maximal longevity are all improved versus remediation of a negative effect of shortened lifespan back to normative levels? One way to address this is by comparing diets of different formulation or composition within a given species and strain to determine the genetic potential for health and longevity outcomes. This has been done with a number of diet composition comparisons, feeding paradigms and specific rodent strains, with newer iterations of feeding approaches holding promise for disease prevention or delay in rodent aging models (Reeves *et al.*, 1993; Reeves, 1997; Duffy *et al.*, 2002; Christian *et al.*, 1998). However, even under what might be considered optimal conditions for reproducibility – namely the same strain of rodent, same research facility, same dietary formulation, and equivalent husbandry factors, variability in longevity outcomes may exist. Two interesting examples from the literature include comparisons of longevity outcomes with laboratory rats and the other a more contemporary example of multiple studies assessing the same strain of mice (Ghirardi *et al.*, 1995; Strong *et al.*, 2008; Harrison *et al.*, 2009; Flurkey *et al.*, 2010; Miller *et al.*, 2011; Miller *et al.*, 2014; Harrison *et al.*, 2014).

In rats, within and between strain variability in longevity outcomes was assessed by an Italian research group using 26 birth cohorts of males rats (Fischer 344: n=16 cohorts, n=2,646 total rats; and Sprague-Dawley: n=10 cohorts, n=1,156 total rats) over approximately 5 years. Using the same environmental conditions – *ad libitum* feeding of a standard rodent diet (4RF18, 16% protein, 2.5% fat and 7.5% max fiber), with group housing (n=3/cage), 12:12 light:dark cycle and 22±1°C ambient temperature – survival parameters were calculated. Fischer 344 rats median lifespan among cohorts ranged from 23.1 to 29.6 months (28% difference) while Sprague-Dawley median lifespans of cohorts ranged from 22.7 to 28.9 months (27% difference), with statistically significant differences in early versus late-life mortality risks between strains (Ghirardi *et al.*, 1995).

In mice, the Interventions Testing Program uses carefully considered, standardized conditions (e.g., light:dark, diet) and a genetically heterogeneous strain to promote healthy aging for their testing protocols (Nadon *et al.*, 2008). Yet, among the three sites, differences between the shortest and longest facilities’ median lifespan with a single contemporary cohort has reached up to 31% (e.g. males: 704, 807, 925 days) (Strong *et al.*, 2008; Harrison *et al.*, 2009; Miller *et al.*, 2011; Strong *et al.*, 2013; Miller *et al.*, 2014; Harrison *et al.*, 2014). The practice of combined and site specific statistical analyses of interventions provides confidence in findings from the studies, but a question persists as to how many longevity “extending” interventions are truly slowing aging and increasing lifespan, rather than remediating a negative condition of premature aging and shortened lifespan. One informal method that is sometimes utilized is the consideration of historical, normative longevity data for a particular rodent strain and diet. However, which longevity profile is best to use for

comparison – the longest lived published results assuming this represents the genetic longevity potential of the strain under the lowest stressed conditions?

Consider one of the most common interventions for longevity extension – dietary restriction. Multiple reviews have highlighted the positive relationship between the amount and duration of the restriction relative to the longevity benefit (Merry, 2002; Swindell, 2012; Speakman & Hambly, 2007). Furthermore, strain specific differences in response to dietary restriction have gained recent attention (Swindell, 2012). Nevertheless, even some of the most highly cited mouse (C57BL/6) and rat (Fischer 344, Sprague-Dawley, Wistar) strains reported to benefit from dietary restriction offer an example of comparison choice dilemmas across studies. For instance, what appears to be a consistent increase in inbred B6 mouse lifespan with dietary restriction in both males and females is less convincing when using the best reported control for each sex in an “interstudy” comparison. Particularly for female B6 mice, the benefit of DR is attenuated with none of the referenced studies reporting longevity extension by DR compared with the longest lived control cohort (see Merry, 2002; Speakman & Hambly, 2007; Swindell, 2012 reviews and included references). In both sexes, the comparison to the longest lived “interstudy” control reduced the extension of median lifespan across studies (females: intrastudy [mean DR effect within study vs. control]: ~3–4%↑, interstudy [mean across study DR effect relative to longest lived reported control] ~10–11%↓; males: intrastudy ~9%↑, interstudy ~2–4%↓) (see Merry, 2002; Speakman & Hambly, 2007; Swindell, 2012 reviews and included references). Identifying the source of the variability of inbred strains of mice within these well-controlled studies may uncover mediating factors influencing individual aging differences (e.g. epigenetic imprinting from maternal influences, litter size, pregnancy conditions, etc). In the published rat longevity data, inbred Fischer 344 exhibit a DR benefit consistently across studies and compared with the interstudy best, while the benefit of DR in outbred Sprague-Dawley (intrastudy 37%↑, interstudy 4%↑) and Wistar (intrastudy 24%↑, interstudy 4%↓) rats are attenuated when comparing to interstudy best controls (see Merry, 2002; Speakman & Hambly, 2007; Swindell, 2012 reviews and included references). In addition to such attempts to consider the “optimal” reports of health and longevity within a given pre-clinical model for comparison, initiatives to improve study designs and reporting to increase rigor and reproducibility may help refine (or temper) the enthusiasm related to the significance of singular findings using laboratory research models.

4. Factors Influencing Energetics

4.1 Ambient Temperature

One ubiquitous environmental factor which influences nutrition and metabolism research in sometimes underappreciated ways is the environmental temperature of laboratory conditions. Homeothermic (endothermic) or warm-blooded organisms must maintain a relatively stable body temperature for normal function. The basic chemical reactions of metabolism (catabolic and anabolic) suffer from thermodynamic imperfection, resulting in entropic energy loss as heat (Mills, 1945). Core body temperature (T_b) of mammals, including humans, is ~35–37°C. However, environmental or ambient temperature (T_a) is variable and frequently below T_b , significantly impacting the metabolic requirements

necessary to maintain a stable T_b (Gordon *et al.*, 1998). When the T_a rises near or above T_b , the ability to dissipate excess heat is reduced, resulting in a thermal stress, triggering adaptive responses to cool the body (Gordon *et al.*, 2014). Conversely, when T_a drops significantly below the T_b , additional heat production is needed to counter the thermal loss of T_b to the environment, accompanied by an inverse, proportional increase in metabolism and caloric intake (Cannon & Nedergaard, 2004). Thus, homeotherms have an optimal range of temperature where thermal demands are low, resulting in reduced metabolic requirements and caloric intake, called the thermoneutral zone (TNZ) [range of temperatures at which a resting organism expends a minimal amount of energy in thermal regulation - i.e., limited heat generation (bounding the lower critical temperature) or dissipation (upper critical temperature) – for a more comprehensive review see references] (Erikson *et al.*, 1956; Scholander *et al.*, 1950; Gordon, 1990; Cannon & Nedergaard, 2004; Cannon & Nedergaard, 2011). Multiple factors influence the TNZ, even within the same species, including body size, age, body composition, acclimatization, environmental exposures, etc. With rodents, and mice in particular, the TNZ has been measured many times with a general overlapping consensus range of ~30–34°C (Herrington, 1940; Cannon & Nedergaard, 2004). Like other homeotherms, humans maintain a T_b within a narrow range, with the mean traditionally defined as ~37°C (Mackowiak *et al.*, 1992). Multiple investigators have measured the energetic response to various T_a in humans (Mills, 1945), with a TNZ in nude, healthy, male subjects ranging from 24°–35°C or more narrowly for the comfort zone from 28–30°C (Erikson *et al.*, 1956; Davis, 1964; Wilkerson *et al.*, 1972; Hardy & Du Bois, 1940). However, the addition of clothing with its insulative properties lowers the TNZ range to 18–24°C depending on conditions (Davis, 1964). Modern society has effectively limited the exposure of humans to T_a outside the TNZ, with the use of central heat and air conditioning, advanced fabrics/clothing, insulated housing, etc. (see Keith *et al.*, 2006; Johnson *et al.*, 2011 and included references). To more accurately model human metabolism, research conditions that result in approximately equivalent metabolic states (i.e., TNZ housing) should be considered, particularly when performing translational research focused on nutrition and metabolism. However, most homeothermic animals, and particularly small rodents, in modern research facilities are chronically cold stressed (are forced to live below thermoneutrality).

When given a choice, mice and rats will voluntarily choose higher temperatures than those used for standard housing (Gordon *et al.*, 1998), with those selections modified by time of day and activity levels (Gordon, 1990; Gordon *et al.*, 1998). Furthermore, housing densities, nesting and enrichment materials and amounts, and other cage items/conditions (e.g. airflow rates) can influence thermal exposures and energetic requirements by creating microenvironments within the cage (Gaskill *et al.*, 2012; Gaskill *et al.*, 2013; Gordon *et al.*, 1998; Nagy *et al.*, 2002; Paigen *et al.*, 2012; Toth *et al.*, 2015). Unfortunately, these methods and items are variable between research sites and studies, with comprehensive details frequently left out of publications. Although typical room T_a of 20–24°C is within the TNZ for a clothed human, it is well below that of a mouse and is in fact a significant cold stress (Gonder & Laber, 2007; Cannon & Nedergaard, 2011). This results in a hypermetabolic state to offset the chronic cold challenge endured by the animal, resulting in elevated food intake, metabolic rate, heart rate, blood pressure, and circulating metabolites (e.g., blood

glucose, lipids) (Swoap *et al.*, 2004; Overton & Williams, 2004). If a mouse living at a thermoneutral (TN) T_a of 30°C is transferred to the typical room temperature (RT) T_a (22°C), an immediate cold response is observed with increases in heat production including shivering, food intake, heart rate, sympathetic nervous system tone, metabolic rate, etc. (Cannon & Nedergaard, 2011). Prolonged exposure to the typical RT (22°C) for 2 weeks or longer results in adaptive changes to meet the increased, chronic, thermal demand (Cannon & Nedergaard, 2004). Therefore, mice in typical animal facilities show few *overt signs* of cold stress unless directly compared to TN housed animals. Even within the ILAR (Institute for Laboratory Animal Research) guidelines, a significant increase in food intake is observed when comparing the lowest and highest recommended T_a (19–26°C personal observation). While this cold-induced hypermetabolism is not unique to rodents and can be observed in humans with a large enough cold stress (Johnson & Kark, 1947) – the chronic nature and magnitude of the T_a exposure of mice in research facilities does not accurately reflect human exposures in modern society. In fact, rats and mice from multiple strain backgrounds show a reduction of caloric intake, VO_2 (ml/min), mean arterial pressure and heart rate when housed at 30°C vs. 23°C (Overton & Williams, 2004; Swoap *et al.*, 2004). As discussed above, metabolism and food intake are inversely related to T_a in mice when housed below the TNZ. As such, a mouse housed at TN (30°C) voluntarily reduces (~40–50%) food intake compared with typical housing T_a (23°C) (Overton & Williams, 2004; Swoap *et al.*, 2004; Cannon & Nedergaard, 2004; Cannon & Nedergaard, 2011), which is near the maximal range of restriction normally practiced in CR/DR studies in rodents (30–40%) (Merry, 2002; Weindrich & Walford, 1988). This raises the possibility that DR suppresses the hypermetabolic state with elevated food intake in typical (cold) housing conditions, returning energy intake to basal levels, and that thermoneutral housing where energy intake is already voluntarily lowered could not be reduced a further 40–50% without inducing malnutrition. Future studies should address this question, particularly in light of the macronutrient requirements for long-term health and longevity in the absence of chronic cold challenges, and the benefit (or detriment) of nutrient restriction under basal intake conditions.

Even more to the point of macronutrient and caloric influences on lifespan, Donhoffer and Vonotzky (1947) reported a choice experiment allowing white mice to modulate both intake amount (calories) and preference (macronutrients) by offering three diets with different macronutrient compositions (Donhoffer & Vonotzky, 1947). Upon housing at typical room temperature for approximately 2–3 weeks, intake stabilized with approximately 2/3 of calories chosen from the fat (lard) diet, with lower amounts of protein (casein) and carbohydrate (cornstarch) diets. Lowering T_a (to 10–11°C) resulted in an increase in caloric intake, albeit this was accounted for primarily from additional carbohydrate consumption with protein intake remaining stable. Furthermore, shifts from low to high T_a (29–33°C) primarily suppressed carbohydrate intake, with again stable intake of protein and fat (Donhoffer & Vonotzky, 1947). These intake responses are in contrast to a “no choice” scenario where lower T_a still induces increased food intake, but proportionally across macronutrients based on the singular diet composition (e.g., to normal ‘low fat’ chow or semi-purified rodent diets), while a macronutrient choice scenario modulates caloric needs based on altered carbohydrate intake (Donhoffer & Vonotzky, 1947; Leshner *et al.*, 1971).

Similar observations have been reported with activity (exercise) preferentially modulating carbohydrate intake in rodents (Collier *et al.*, 1969). Considering most rodent research utilizes sub-thermoneutral housing, including the GF dietary assessments related to longevity discussed above (Solon-Biet *et al.*, 2014), it would be important to know how thermoneutral housing modifies the macronutrient ratio effect on health and longevity outcomes. Similarly, the interaction of exercise or activity on macronutrient ratio optimization could be further explored.

One example of a relatively recent gene by environment interaction in obesity and metabolism is found in the uncoupling protein 1 (*Ucp1*) deletion mice. As mentioned above, the typical housing condition in most rodent facilities requires a significant, adaptive thermogenic response to maintain T_b . Brown adipose tissue (BAT) has been shown to contribute to this thermogenic requirement through uncoupling metabolic substrate utilization from ATP (adenosine triphosphate) production in the mitochondria – producing heat (i.e. a type of energy inefficiency/wasting) (Cannon & Nedergaard, 2004). Thus, it was expected the deletion of the *Ucp1* gene would produce a more “efficient” organism resulting in greater weight gain per calorie consumed. However, early reports of *Ucp1* gene knockout mice demonstrated reduced thermogenic capacity, but with unexpected protection from diet-induced obesity (Enerback *et al.*, 1997; Liu *et al.*, 2003). Follow-up studies with the same *Ucp1* deletion strain performed under housing conditions near the lower end of the TNZ (T_a 29°C) uncovered an obesogenic phenotype of the *Ucp1*^{-/-} mutant, with increased energetic efficiency (significantly greater weight gain despite equivalent energy intake) on both low-fat and high-fat diet protocols (Feldmann *et al.*, 2009). In essence, correcting the thermally-induced, hyper-metabolic response by altering the T_a uncovered a metabolic phenotype in the mutant mouse model which was expected based on the known biochemical and molecular function, but not previously observed. How other diets with altered macronutrient proportions might interact with this *Ucp1*^{-/-} genotype and T_a for metabolic health remain to be fully explored. The number of additional gene by environment (diet and T_a) interactions related to metabolic health and/or longevity outcomes that are concealed in rodent models due to standard operating procedures remains largely untested and unknown, suggesting more research in this area may be warranted.

4.2 Genetics: Dwarf mice

One of the largest determinants of cold stress from sub-thermoneutral housing is body size (Donhoffer, 1986). This is partly due to the surface area to volume (mass) ratio as body surface is two-dimensional (squared) while mass is proportional to the volume which is three dimensional (cubed). Thus, as the volume (mass) become smaller, there is a greater relative surface area to volume ratio and vice versa. Since heat dissipation is lost across the surface area, a relatively greater amount of heat generated (smaller volume/mass) is transferred (larger surface area). As such, animals of significantly different body sizes experience different thermal challenges within the same environment (Chaffee & Roberts, 1971). This is well recognized even with humans where children have a greater thermal challenge than adults (Aherne & Hull, 1964; Aherne & Hull, 1966). Thus, a fundamental aspect of shifting energy balance relies on growth and development. Furthermore, there is a body of evidence indicating alterations in the growth hormone/insulin like growth factor-1

pathways impact aging with mutations in growth signaling resulting in reductions in body size with a concomitant increase in lifespan (Bartke, 2005; Bartke, 2008a; Bartke, 2008b). As might be expected with a reduced body size in mice with impaired growth, the lower bound of the TNZ range is shifted higher than normal sized control mice (Meyer *et al.*, 2004; Meyer *et al.*, 2007). As such, genetic or dietary growth inhibition in mice housed below thermoneutrality may result in greater thermal challenges, such that the mice will be more likely to induce periods of suppressed metabolism and body temperature reduction (i.e. torpor) during period of insufficient energy intake (Bartke & Westbrook, 2012). This physiologic adaption of T_b reduction (including torpor bouts) has been associated with multiple interventions that increase lifespan (Bartke & Westbrook, 2012; Bartke & Brown-Borg, 2004; Brown-Borg & Bartke, 2012). Maintenance of T_b is dependent on the supply and metabolism of sufficient energetic resources, as well as the balance of heat dissipation. How differences in adiposity in some strains of dwarf mice may modify the thermal balance at the upper end of the TNZ requires further investigation (Meyer *et al.*, 2004; Bartke & Westbrook, 2012). Considering these interactions, it will be interesting to understand whether dwarf mice housed under thermoneutral conditions have altered nutrient metabolism, particularly related to suppressed brown adipose tissue utilization of lipid and glucose, which might be expected to reduce insulin sensitivity (particularly with advanced age), negatively impacting the typically observed longevity extension.

5. Longevity Extending Interventions and Energetics

Despite the potential context specific outcomes relating energetics to longevity which have been discussed above, carefully performed and accurately described animal studies may still point to mechanisms underlying health and longevity promotion, albeit verification in multiple models and conditions prior to human translation would be preferred. It is at the critical point of pre-clinical to clinical transition in translational research that the importance of distinct between replication and translational capacity (or generalizability) exist. As an example of this reproducibility distinction, much excitement has surrounded the identification of the Target of Rapamycin (TOR) pathway and its role in aging modulation across species. Both genetic and pharmacological inhibition of the TOR pathway has been shown to increase lifespan in multiple species (Stanfel *et al.*, 2009). Genomic mutation studies deleting components of the TOR pathway in yeast have reported suppressed TOR signaling in both replicative and chronological aging extension, with worm and fly results showing similar benefits (Stanfel *et al.*, 2009). These TOR-dependent longevity extension findings were further supported by chronic treatment with rapamycin (a TOR inhibitor) increasing lifespan, even when started at late-life (past mid-life when calorie restriction normally does not provide much benefit) (Swindell, 2012; Merry, 2002; Speakman & Hambly, 2007), with a stronger effect in females (Harrison *et al.*, 2009). Additional follow-up studies have confirmed these TOR-inhibition longevity benefits with additional strains of mice, dosing regimens, etc. (Neff *et al.*, 2013; Miller *et al.*, 2014; Leontieva *et al.*, 2014). However, one of the more puzzling and contraindicated side effects of rapamycin treatment is the development of insulin resistance which is normally considered counterproductive to aging and disease protection (Blagosklonny, 2011; Bartke & Brown-Borg, 2004). This has led to additional research demonstrating differences between the two primary TOR

complexes (TORC1 and TORC2), wherein TOR interacts with different proteins and targets and the specificity of interactions between rapamycin and analogues appear to mediate the glucose intolerance, along with alternative dosing schedules (Arriola Apelo *et al.*, 2015; Liu *et al.*, 2014). Whether TORC specific inhibitors will recapitulate the benefits of rapamycin and its impressive longevity response, while preserving insulin sensitivity, is yet to be shown.

Despite the numerous reports verifying the replicability of rapamycin to increase longevity in rodent models, the translational capacity remains to be validated with recent studies revealing notes of caution. For instance, a 2014 study using the same mouse strain (UM-HET3) and rapamycin delivery method (encapsulated in the diet) as the ITP is particularly interesting in light of the insulin resistance phenotype. In contrast with lifespan extension as has been previously reported, mice in this study developed severe insulin resistance and overt glucose intolerance in males (Schindler *et al.*, 2014). Females also developed glucose intolerance to a much lesser extent, with 17beta-estradiol being demonstrated as a protective factor with rapamycin treated ovariectomized female mice (Schindler *et al.*, 2014). Both sexes had partial recovery of glucose tolerance with cessation of rapamycin treatment (Schindler *et al.*, 2014). There are at least two primary differences compared with the previous ITP study. First, the age of rapamycin treatment initiation was earlier in the Schindler report (relative to the ITP studies), and second, of particular interest in light of the energetic interactions, the housing temperature of the mice was at thermoneutrality (30°C) as opposed to typical room temperature (~22°C) in the ITP and other reported studies (Nadon *et al.*, 2008; Harrison *et al.*, 2009). While it is possible that either (or both) of these differences may contribute to the glucose intolerance (and both could be formally tested), it is interesting that a housing condition where the hypermetabolic rate is suppressed to more basal levels, where carbohydrate intake preference is reduced, and where carbohydrate metabolism is suppressed, feeding a fixed macronutrient diet (relatively high in carbohydrate) with a drug with known glucose intolerance side-effects results in severe hyperglycemia (diabetes) that did not fully develop in previous studies despite the observed insulin resistance. Whether other dietary macronutrient compositions (e.g., high protein, low carbohydrate) would be advantageous in combination with rapamycin treatment, particularly given the amino acid nutrient signaling response remains to be determined. However, it would seem that a low protein, high carbohydrate diet recommendation derived from the geometric framework model for longevity extension might in fact be detrimental in the context of rapamycin treatment (Solon-Biet *et al.*, 2014). Alternatively, diets low in protein exhibit reduced TOR signaling suggesting rapamycin benefits might be at best minimal under such nutritional constraints (Solon-Biet *et al.*, 2014). Similarly, combination studies using an anti-diabetic agent like metformin or acarbose in combination with rapamycin should be explored, but given these results, careful consideration should be given to the conditions under which the treatments are tested in translating this to expectations for humans. Long term rapamycin treatment in humans following organ transplant has been shown to increase the incidence of new onset diabetes, as do other transplant rejection suppression medications of similar biochemistry (e.g., macrolides) (Bodziak & Hricik, 2009; Yilmaz *et al.*, 2015; Johnston *et al.*, 2008). Additional studies assessing rapamycin

treatments in healthy human populations may ultimately clarify this potential detrimental side effect.

6. Open Questions, Current Limitations

As discussed in the previous sections, the interaction between T_a and energetic requirements has potential implications for the DR model. Would housing mice or rats in thermoneutral conditions which result in voluntary reduction of caloric intake (~40–50% relative to typical room temperature) recapitulate the beneficial effects of DR? Previous work in mice has shown that low temperature is required for body temperature reductions and the cancer protective effects of DR (Koizumi *et al.*, 1992; Koizumi *et al.*, 1996), suggesting thermoneutral housing could increase cancer risk and development, potentially shortening lifespan. However, studies with rats have suggested the opposite with cold housing (9°C, which is below typical room temperature) having decreased survival relative to warmer housing (28°C) (Kibler & Johnson, 1961; Kibler *et al.*, 1963), and restricted feeding improving longevity at this same temperature (28°C) (Kibler & Johnson, 1966; Kibler & Johnson, 1967).

Related to the question of energetic/caloric effects on longevity under less stressful physiological conditions, what are the nutrient (macro and micro) preferences and requirements under conditions which would translate to humans in modern society? How does activity, particularly the health benefits observed with exercise, modify these nutrient and environmental relationships? Although the GF provides a foundational starting point for such assessments, the impact sub-thermoneutral housing or exercise related activity has on carbohydrate and fat metabolism related to dietary recommendations for optimal health and longevity remain open questions. Similarly, the comparison of multiple dietary patterns may be particularly important for the human translational approach where dietary changes are often accompanied by both energetic and nutrient composition alterations.

Finally, what are the optimal macronutrient requirements for health and longevity with advancing age, and how do compounds that influence nutrient sensing and nutrient metabolism work at older ages? While the addition of growth and maintenance formulations of diets has improved our research practice, further work and expanded nutrition research paradigms (e.g., choice experiments) at and beyond middle-age are still needed. This is a daunting task given the basic logistical difficulties of maintaining a sufficient sample size for robust experimental investigations in the face of age-related mortality. However, our understanding of the role of nutrition in health promotion and maintenance may of necessity require late-life assessment during the critical periods when organisms are at such an increased risk of disease. We share the NIH mission “to seek fundamental knowledge about the nature and behavior of living systems and the application of that knowledge to enhance health, lengthen life and reduce illness and disability” and by refining and further developing these successful pre-clinical models hope to inform the future understanding of nutrition and health.

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Highlights

- Macronutrient balance can influence energy intake, lifespan, and healthspan.
- Energetic demands impact macronutrient preference and utilization in rodents.
- Energetic demands from typical housing influence biomarkers of longevity in mice.
- Macronutrient balance in current rodent diets may not be optimal for longevity.
- Longevity extending interventions must be considered in the context of energetics.