CALORIES AND FASTING – A NEW PERSPECTIVE INTO DIETARY RESTRICTION

By

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ABSTRACT

Calorie restriction (CR), reduction in caloric intake without malnutrition, is a gold standard for geroprotective interventions, not only extending lifespan and healthspan, but also preventing or delaying many age-associated diseases. While effective in rodents and other mammalian species, an abstemious CR diet is difficult for most people to maintain. Understanding the mechanisms by which CR promotes healthspan and developing effective CR-mimicking dietary regimens or pharmaceuticals is therefore essential to harnessing the benefits of CR.

Recently, it has become apparent that when we eat may be just as important as what and how much we eat. Research into feeding paradigms have found that time-restricted feeding and intermittent fasting has metabolic benefits, protecting mice from the negative metabolic effects of a high-fat, high-sucrose “Western” diet. Similarly, meal-fed mice, which are fed an *ad libitum* portion of food but consume it over ~12-15 hours, live longer than truly *ad libitum* fed animals. A largely overlooked question is whether the metabolic health benefits of CR arise solely from the reduction in calories, or if this enforced period of daily fasting is also required as daily fed CR animals consume their food in ~2 hours and are subjected to fasting for the remaining 22 hours per day.

To distinguish fasting dependent and independent effects, I designed multiple feeding paradigms to determine if the metabolic effects of CR are mediated by reduced caloric intake, or also require prolonged fasting. I found that only mice on a traditional CR regimen, fed once daily, had improved sensitivity to insulin, a phenotype of CR that has been suggested to mediate many of CR’s beneficial effects on longevity. I also found distinct metabolomic signature associated with once-a-day CR compared to the *ad libitum*

control. However, the metabolomic signature of the diluted diet group resembled closely that of the *ad libitum* control. This was also true with the acetylation profiles of histones, which suggest fasting, not reduced calorie intake, mediates the metabolomic and epigenetic profile typically shown in calorie restriction studies.

Finally, I show that fasting alone without a reduction in calorie intake recapitulates the metabolic phenotypes and transcriptional effects of a CR diet. Additionally, fasting is required for the beneficial effects of CR in aging C57BL/6J male mice, including improvements in insulin sensitivity, reduction in frailty, and extension of lifespan. Therefore, while many of the metabolic benefits of a CR diet are mediated by reduced caloric intake, the prolonged fasting induced in CR mice fed once a day is required for systemic insulin sensitivity, as well as the transcriptomic, metabolomic and epigenetic profiles observed in calorie restriction studies.

Current prevailing model of CR is reduced nutrient responsive pathways – insulin signaling and the activity of mTOR Complex 1 (mTORC1), a protein kinase that is a key regulator of metabolism. Genetic and pharmaceutical inhibition of the mTORC1 signaling pathway extends lifespan, and studies in model organisms suggest an epistatic interaction, suggesting a key role for mTORC1 in the response to CR. However, studies have found that CR and mTORC1 inhibition have distinct effects on the transcriptome and metabolome of tissues, and the interaction of CR and reduced mTORC1 signaling on metabolism, health, and longevity have not been formally investigated in mammals. In fact, due to their very different feeding patterns, it is not even clear that CR-fed animals have a reduction in mTORC1 signaling relative to *ad libitum* (AL) controls. Therefore, I

comprehensively determined the physiological and molecular response to fasting every 4 hrs during a 24 hr cycle in ad libitum-fed and CR mice.

I discovered that the most widely conserved effect of CR in mammals – increased insulin sensitivity – is only observed at certain times relative to the last feeding. CR animals have increased insulin sensitivity only after 20 hrs following their last meal, but they exhibited insulin resistance relative to *ad libitum* (AL) control mice when tested 4-16 hrs after their last meal. Interestingly, insulin levels appeared to be dependent on the time of day suggesting that insulin secretion and/or clearance is heavily dependent on peripheral circadian controls.

mTORC1 activity is a critical regulator of insulin sensitivity through S6K1 mediated feedback inhibition of insulin receptor substrate. Therefore, I hypothesized that insulin sensitivity may be the result of negative feedback regulation of mTORC1 on insulin action. However, when examining mTORC1 activity, I found that hepatic mTORC1 activity was decreased to the same degree in both AL and CR. This suggest that mTORC1 activity is highly dependent on feeding and fasting conditions.

In this dissertation, I address how calories and length of a fast impact the outcome of dietary restriction and the importance of feeding and fasting conditions when examining for metabolic phenotypes and understanding and identifying the molecular processes that drive the effects of a CR diet.

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CHAPTER 1: INTRODUCTION

Aging is defined as a progressive physiological decline, leading to impaired function and increased mortality [1]. Characteristics of aging and metabolism is the development of metabolic syndrome, which is defined as collective disorders including glucose intolerance, decrease in metabolic flexibility and energy utilization, obesity, dyslipidemia, and hypertension [2]. Metabolic syndrome is often associated with brain health and vice versa. For example, those affected by dementia and Alzheimer's disease have a higher risk of developing diabetes and cancer [3-5]. Other biomarkers, as well as precursors to age-related metabolic decline, include increased oxidative damage, altered endocrine and neuroendocrine function – in particular growth hormones, thyroid, reproductive and adrenal systems [1, 6]. There is also growing evidence in the role of intestinal health in regulating nutrient absorption as well as its interaction with the microbiome and immune cells for the progression of diseased states [7-9]. Finally, recent research have unveiled a central role for a rhythmic regulation in glucose, fatty acid, and cholesterol pathways, and age-related damping of these biological rhythms have been closely linked to age-related decline [10].

Calorie restriction (CR), defined as a reduction in caloric intake without malnutrition, is a gold standard intervention for extending health and lifespan of diverse array of species [11]. CR falls under the realm of dietary restriction (DR) which includes all diets that reduces particular or total nutrient intake without causing malnutrition including protein restriction (PR) and fasting regimens [12]. This chapter will focus on the evolution of dietary restriction research and the mechanisms that promote health and longevity, specifically understanding the different implementations of dietary restrictions

and the role of calories, proteins, and fasting, with the goal of providing a comprehensive introduction to the following dissertation chapters.

History of calorie restriction

Lifespan extension with DR has a rich history with the first documented work being published over 100 years ago. In 1917 Osborne et al. showed that restricting food extended lifespan and reproductive capabilities in female rats, and this effect was believed to be due to stunted growth in restricted animals [13]. Then in 1920 Robertson and Ray found correlation between the rate of growth with lifespan validating Osborne's study [14]. Finally, in the late 1920's to early 1930's, McCay and colleagues as well as Slonaker postulated that protein restriction, and not energy restriction per se, was the primary driver of lifespan effect via growth retardation [15, 16]. However, prior to these studies, in 1912 Slonaker showed that rats fed a vegetarian diet were blunted in growth and lived shorter lives [17]; therefore there were some flaws in this reasoning. It was not until 1935 when McCay and colleagues investigated the effects of overall food restriction on lifespan in rats [18], and a follow-up study in 1939, which revealed that even under conditions where food was rich in protein (40% protein), the same lifespan extension could be observed when energy intake was restricted [19]. Therefore energy restriction became popularized and many within the field would consider these studies to be the origin of calorie restriction (CR) [20]. However, it is of note that McCay was still an advocate of the growth restriction hypothesis.

During the 1940-50's, research on CR were largely centered for its effect on delaying age-associated diseases, such as cancer [21-24]. While studies on the impact of macronutrient compositions were declining, there were two notable studies during this time. In 1946, Carlson and Hoetzel showed that rats could live longer if they fasted one day out of every four days [25], which became the primary protocol we know today as every other day feeding (EOD). Then in 1953, French and colleagues were one of the first to uncover that a high fat diet had negative effect on lifespan of rats compared to rats fed a high carbohydrate diet [26]. Later, Berg and Simms challenged the growth restriction hypothesis and postulated that the reduction of body fat from a CR regimen was the property that extended life [27]. While many dismissed this hypothesis and were proponents of McCay's growth restriction hypothesis, these studies were the start of associating obesity with health risk in humans [20]. By 1960's there was a renewed interest in the role of different macronutrient composition to elicit the CR effect. Most notable were the studies done by Morris Ross who showed that rats that were restricted in both calories and protein had the longest lifespan [28]. While much of these findings were a validation of previous works, the thorough experimental design made this a landmark study in the CR field.

There was great interest in the metabolic effects of CR and aging in the 1970's. Arthur Everitt who showed that hypophysectomy decreases the aging process in rats and proposed that the CR effect is due to a decrease in secretion of aging factors by the pituitary [29, 30]. This work became the basis for the role of Insulin/Growth Hormone-IGF-1 axis in the life extension properties of CR. Around the same time Walford and colleagues showed the effects of CR in immune function in mice [31], and in the

following years Fernandes and colleagues showed the beneficial effects of CR in mice with autoimmune diseases [32]. Towards the end of the 1970's, Sacher proposed that the decrease in metabolic rate per body weight extends lifespan when animals are placed under CR [33]. Although not formally tested, this hypothesis gained favor over McCay's growth restriction hypothesis [20].

1980's was an important decade in CR research as many of the original hypotheses surrounding CR were challenged. Berg and Simms's theory on decrease in adiposity in extending lifespan were questioned by two studies – Bertrand *et al*. and Harrison *et al*. showed that while the overall adipose volume is decreased in food restricted animals, the number of adipocytes were not decreased, concluding that adipose mass is not involved in lifespan [34, 35]. Satcher's hypothesis on decrease in metabolic rate was challenged by Masoro's group where they showed that rats fed a CR regimen, when adjusting for food intake per gram of body mass, ate more compared to their ad libitum counterparts [36]. Weindruch and Walford demonstrated that CR initiated later in life when fully grown (12 months of age) significantly extended the life of mice [37] and this was followed up with Masoro's group where they showed that rats have the same lifespan extension whether initiated at 6 weeks of age or 6 months of age [38], thereby ending the era of the growth restriction hypothesis. Furthermore, several comprehensive studies showed that restricting individual macronutrient and micronutrient intake did not underlie the life extension properties of calorie restriction [38-44], and the acceptance of global food restriction mediating lifespan extension.

Transitioning into the 1990's several new hypotheses emerged regarding the life extension properties of CR. One of which was that CR extends lifespan by decreasing

accumulation of damaged cells by increasing apoptotic activity [45]. Others have speculated that CR attenuates oxidative damage [46, 47], and while many adopted this hypothesis, there have been no conclusive evidence [48]. Some have proposed that the reduction in body temperature, a byproduct of CR, extends life [49, 50]. A more recently accepted hypothesis of CR was hormesis, defined by a constant exposure to low grade stress [51]. Finally, continuing the initial findings from Arthur Everitt, William Sontag and colleagues suggested for an endocrine compensatory mechanism in mediating lifespan by examining the effects of growth hormone and IGF-1 [52]. Of course, the most notable studies in the 90's were that of CR effect on non-human primates that were done at the University of Wisconsin, Madison and NIA, which will be discussed in the next section.

The 21st Century could be described as a resurgence in diet and metabolism studies with the emphasis in understanding potential pathways mediating many of the effects observed in dietary restrictions. A challenging aspect of disentangling some of these effects is the inconsistencies of the conditions placed on animals in these studies (e.g. level of restriction, food composition, fasting duration, etc.), as well as the complex interaction between major pathways. In this review, I will highlight key works which addresses these challenging aspects of diet research, and our current understanding of how dietary restriction mediates health and longevity.

Non-human primate studies

Translatability of CR to humans became a major interest in the late 1980's. Two independent longitudinal studies on calorie restriction were conducted in rhesus monkeys. One by the National Institute on Aging (NIA) involving 121 rhesus monkeys

and the other by the University of Wisconsin-Madison (UW) involving 76 monkeys [53, 54]. The UW study showed a beneficial effect of CR including decrease risk factors that link to age-associated diseases – muscle function, glucose homeostasis and incidence of neoplasia, cardiovascular disease and reduced age-associated brain atrophy – as well as extension of lifespan [53, 54]. In contrast, the NIA study showed no significant differences between the control and CR group [55]. Due to these conflicting findings from the two studies, detailed analysis on the implementation of CR between the two studies were examined [56]. A major difference between the two studies was that while UW initiated the study only in adult monkeys that were born and raised in the UW facility, NIA initiated in both young and old-onset groups collected from various origins. There were three differences in feeding conditions as well. First, NIA did not allow for free feeding conditions for the control monkeys. Second, the diet compositions were different as NIA accounted for micronutrient differences to account for seasonal variations, in contrast UW used a semi-purified that was consistent throughout the study. Additionally, NIA diet had lower fat and sucrose and higher protein and fiber content. Finally, the NIA monkeys were fed two meals each with access to food at night if any food was remaining, while UW monkeys were fed once a day with any remaining food removed prior to dark cycle. Nonetheless both studies have provided valuable insights into human aging and health.

Re-evaluation of protein restriction as the mediator of lifespan in dietary restriction models

Although it has long been accepted that energy restriction was the primary mediator of lifespan extension, there were still ongoing studies on protein restriction (PR) [57-59]. It was not until mid-2000's PR studies regained immense attention when Linda Partridge's group suggested that the impact of dietary restriction on lifespan of Drosophila melanogaster was not due to calories but restriction in proteins [60, 61]. These studies showed that reduction in dietary protein (using yeast) had a much stronger effect than reduction of calories (using sugar) [61], and adding back amino acids to calorically restricted flies abolished life extension [62]. The overall conclusion, also corroborated by other groups working with Drosophila [63-67], was that reduction of calories does not extend lifespan, at least in insect species, and the CR effect is actually mediated by the altered protein to non-protein ratio in diets [68, 69]. More recently, restriction of specific amino acids, such as BCAAs (leucine, valine, and isoleucine) have been shown to increase lifespan and delay age-related frailty in mice [70-72].

While simple in concept, dietary restriction studies have proven to be more complex than originally considered. A particular challenge in this field is dissociating caloric intake from the intake of macronutrients. To address these challenges Simpson and Raubenheimer developed an approach called Geometric Framework or Nutritional Geometry, which models three components: 1) the interaction of the animal and the environment 2) interactions under multiple dietary components, and 3) relative consequences (e.g. health outcomes or lifespan) based on the animal-environment interaction [68, 73-76]. This approach was verified with a massive study involving 25 different diets that varied in the protein to non-protein ratios, carried out under *ad libitum*

conditions, and following the lifespan of mice [77]. Their findings suggested that the key factor influencing health and longevity was due to the protein to non-protein ratio in the diet and no distinct effect of caloric intake [77], as observed in flies [63-67]. In a subsequent study, involving three diets that varied in protein to carbohydrate ratio under ad libitum or calorically restricted conditions, they showed that a low protein high carbohydrate diet mimicked the metabolic effect of CR, and combining these interventions do not produce any additive effects [78]. These studies provide additional support for the notion that part of the effect on CR on rodent lifespan is due to specific amino acid restriction, such as methionine [79-81].

While these studies provide convincing evidence on the role of protein restriction on health and lifespan, there are important caveats that need to be considered in understanding these results. First, the absolute lifespan of mice conducted in these studies [77] were shorter than what is generally reported within the same strain under ad libitum conditions [82-85]. Furthermore, the group with the longest median lifespan had high levels of protein (42% Protein, 29% Carbohydrate, 29% Fat) [77, 86]. Nonetheless, it appears that there is a disparate effect of dietary protein restriction compared to global calorie restriction. Speakman and colleagues demonstrated this effect with a detailed meta-analysis comparing the effects of dietary interventions on lifespan of rodents [87]. They concluded that while protein reduction has an impact on lifespan, it functions over a different level of restriction. Lifespan extension of 30% was observed when protein was restricted between 10-65% in calorie restricted model; however protein restriction required about an 50-85% reduction in dietary protein, and the effect was substantially reduced (15% extension) compared to previous reports with

CR [87]. Finally, an important distinction, and perhaps the primary driver in the discrepant results is the method used to restrict the animals. To restrict energy intake Solon-Biet et al. used a diet that replaced the bulk of the diet with indigestible cellulose, this type of dietary dilution is frequently used in insect studies to study the effect of dietary restriction. However, our group recently demonstrated that the continuous consumption of food from diluted diets actually decreases lifespan [83], this will be discussed in more detail later in the chapter and in Chapter 3.

The Advent of Fasting Regimens

Fasting has been practiced over a millennia and the best studied model is Ramadan fasting [88]. More recently, fasting has become popularized with diets such as intermittent fasting (IF) and commercialized with diets such as fasting mimicking diet (FMD). Researchers studying the effect of fasting have often regarded this type of feeding regimen distinct from calorie restriction (CR) [89]. Perhaps this is due to the method of restriction, rather than restricting a specific component within a diet, fasting restricts time. However, many within the dietary restriction field would argue that fasting to a certain degree is a CR feeding regimen as restricting time limits the amount of calories that could be consumed within a timeframe [86]. We recently dissociated the effects of calories and fasting in mice and found that fasting is sufficient and necessary to have the health and lifespan extension properties of calorie restriction, and in order to see these effects prolonged daily fasting is required [83].

Intermittent Fasting (IF) is a loosely defined term that applies to fasting duration ranging from few hours to 48hrs followed by a normal feeding pattern [90]. This encompasses many variations, one of which that is most common is alternate day fasting (ADF), which requires fasting every other day with ad libitum access to food between fasting [91], and the 5:2 diet which provides 500-700 calories for 2 days per week [92, 93]. ADF has been shown to induce ketogenesis and improve metabolic health, stress resistance, and markers of inflammation [94]. A shorter fasting regimen, time-restricted feeding (TRF), is a variant of IF and defined as restriction of daily caloric intake within 6-8 hr window with fasting the remainder of the day [95]. Metabolic benefits of TRF were initially demonstrated in mouse model of diet-induced obesity (DIO) and were shown to protect from high-fat diet-induced obesity and associated metabolic diseases [95, 96]. Isocaloric TRF studies, at least in rodents, have shown to improve glucose and insulin homeostasis and liver pathology, as well as maintenance of intrinsic circadian rhythm [95-98]. Research into TRF is relatively new and its effect on health and lifespan extension is limited. Only one study has looked into this in male mice which TRF (13hr feeding window) or meal-fed mice showed an extension of lifespan compared to ad libitum fed mice[99]. Nonetheless, TRF has gained enough attention to move forward to clinical studies due to its feasibility in adherence to the diet, which will be described in more detail later in this chapter.

Metabolic and Molecular effects of dietary restriction

CR delays or prevents the onset of age-associated pathophysiological changes, including reduced metabolic rate and oxidative damage [100], enhanced cellular

turnover and protein homeostasis [101] and decrease incidences of obesity, insulin resistance, dyslipidemia and hypertension [102]. Many of these benefits have often been associated with an overall lean body composition [103]; however, a minimum level of adiposity must be preserved in the second year of life to see the full benefit of CR [82, 104]. In rodents, it has been reported that CR could extend lifespan by up to 50% [105], but this was dependent on genetic background, sex and age [82], and this may be true for longer-lived organisms such as non-human primates [106]. Reports in other forms of dietary restriction is limited compared to CR, but recent studies show promising results in the lifespan extension of other forms of dietary restriction. IF has been shown lifespan extension in both vertebrate and invertebrate model organisms [89, 103, 107-109]. Similarly in flies and rodents, both total protein and specific amino acid restrictions improves health and increases lifespan [57, 61, 71, 72, 78]. However, there is a lack of comprehensive understanding for the mechanism responsible for lifespan extension by these dietary restriction interventions.

Current understanding of how dietary restriction mediates health and lifespan is its effect on nutrient sensing pathways and stress-responsive pathways [110]. For example, at least for CR and fasting regimens, ketone bodies and adiponectin are increased, while insulin, insulin-like growth factor (IGF-1) and leptin levels are decreased [1, 110]. It has also been suggested that protein or essential amino acid restriction may extend lifespan through inhibition of mTOR signaling (this will be discussed in more detail in the next section) and increased secretion of fibroblast growth factor 21 (FGF21) [111-113]. Other pathways have also been closely linked to the mechanism of aging including forkhead box protein O (FOXO), sirtuins and nuclear

factor erythroid 2-related factor 2 (NRF2) [1, 114], and these have already been extensively covered in other reviews and will not be discussed in this chapter.

Nutrient sensitive pathways mediating lifespan

One of the main pathways that has been reported to extend lifespan in the rodent models is the downregulation of the somatotropic axis [115], which encompasses growth hormone (GH), upstream hypothalamic hormones, and insulin-like growth factors (IGFs) [116-118]. Downstream of this signaling axis is the FOXO transcription factor which works inversely to the GH/IIS signals and promote the expression of genes involved in cell death, cell cycle arrest, DNA repair, stress resistance and detoxification [119]. This metabolic switch from somatic growth to maintenance have been recognized to promote longevity [120]. Because CR downregulates GH and insulin signaling, it has generally accepted that CR extends lifespan by downregulating the somatotropic axis [121] and subsequently activating FOXO transcription factors [122]. While considerable amount of research has been dedicated to uncovering the relationship between Insulin/IGF-1 and FOXO in mediating the lifespan extension in CR, this has not been completely resolved.

The best example is the long-lived Ames dwarf mice, which has a mutation of Prop-1 and disrupting pituitary gland development thereby reducing the levels of circulating GH and IGF-1 [116, 123]. Similarly, mice with mutations in the growth hormone receptor (GHRKO) are also long lived [116, 124]. Interestingly, primary difference between these two strains is that Ames dwarf mice will respond to CR and ADF while GHRKO male mice have no further extension of overall longevity when placed on a 30% CR diet [125,

126], suggesting that lifespan extension via GH suppression may occur through a dissociative mechanism from that of CR. This could possibly be explained by the fact that CR and EOD increases insulin sensitivity in multiple tissues of Ames dwarf mice, in contrast, GHRKO mice only showed improved insulin sensitivity in the muscle [127]. It does appear that FOXO signaling is required for the full benefit of CR-mediated lifespan extension as CR fails to extend the lifespan of both heterozygous and homozygous mutant mice for FOXO3 [128]. However, observations in lower organisms suggest that FOXO signaling may not be the main driver of CR [129-131], and worms with DAF-16 mutation, the sole ortholog of FOXO transcription factors, showed a differential response to CR depending on the type of CR [132]. Understanding how these pathways are mediated by fasting and protein restriction is not as well worked out; however, similar changes attributed to lifespan extension such as insulin sensitivity, stress resistance and immune function may suggest that many of the same pathways are in play.

Downstream of insulin signaling is the mechanistic target of rapamycin (mTOR), a master regulator of metabolism which mediates growth and proliferation in response to nutrient signals [133, 134]. mTOR is a serine/threonine kinase with two complexes of mTOR – mTORC1 and mTORC2 – which both have distinct functions, but essential for viability[135, 136]. mTORC1 is activated by amino acids, in particular branched-chain amino acids, which regulates protein synthesis and degradation for growth and proliferation, while mTORC2 is activated in response to growth factors, such as insulin and IGF-1, and mediates stress response for cell survival[133]. I will not discuss the comprehensive mechanism of action of mTOR, as this has already been extensively

reviewed by Kenney and Lamming [133]. In brief, mTORC2 mediates the crosstalk between insulin signaling pathways and TOR signaling by phosphorylating AKT, in turn modulating mTORC1 function [137, 138].

As a major nutrient sensor, mTORC1 is thought to mediate the longevity and health benefits of calorie restriction and it has since been assumed that CR in higher organisms is likewise mediated at least in part by reduced mTOR signaling [139, 140]. Genetic or pharmaceutical inhibition of mTOR Complex 1 (mTORC1) extends life in organisms ranging from yeast [141-146], worms [107, 147-159], flies [160, 161], and mice [162-165] as does genetic inhibition of genes and processes downstream of mTORC1, including S6K1 and protein translation [150, 166-169]. Indeed, organisms genetically modified of components of mTOR pathway fail to extend life by CR [141-146, 149, 153, 158, 161, 169], and at least in yeast and worms, there is an epistatic relationship between CR and TOR signaling [150, 170]. In fruit flies, 4E-BP1/eukaryotic translation initiation factor 4E binding protein (eIF4E), is required for lifespan extension with dietary restriction [141-146, 171]. Conversely, mTORC1 signaling is increased in many age-related diseases, such as cancer [172]. While CR has been associated with decreased mTOR activity, very few studies have looked into characterizing mTOR activity in a dietary restriction model in mammals. However, extensive “omics” studies suggest that rapamycin, an inhibitor of mTORC1, and CR have distinct largely non-overlapping effects [173-177].

Since mTOR regulates many different metabolic processes, mTOR itself is highly regulated by other components. One of which is via inhibition by AMP-dependent protein kinase (AMPK), which is an evolutionarily conserved energy sensor in

eukaryotes [178, 179]. AMPK plays a central role in detecting AMP:ATP ratio and is stimulated in instances where ATP synthesis is compromised, such as low nutrient availability during the fasting state or exercise, and stressed conditions such as hypoxia and ischemia [178, 180]. Therefore, AMPK has been implicated in playing a central role in mediating the CR response. Initial studies with yeast and worms show that upregulating the AMPK pathway does indeed extend life [153, 181-183], and genetic perturbation studies show that worms with a mutation for aak-2, one of the catalytic subunits of AMPK, abrogates the lifespan extension by CR [153]. However, interestingly, AMPK was not required to extend lifespan when worms were kept in a diluted bacteria in liquid culture [132]. This suggests that a fasting-related mechanisms, independent of calorie restriction alone, may drive this response. Indeed, when these worms were given mild nutritional stress through feeding 2-deoxy-D-glucose (2-DG) or complete food deprivation (mimicking fasting), AMPK signaling was required for the extension of lifespan [184, 185]. In mammalian studies, there are some discrepancies on the activation of AMPK [180]. Acute starvation have been shown to activate AMPK in the liver [186] as well as long-term dietary restriction in the skeletal muscle [187]; however in some cases, chronic CR failed to activate AMPK [188]. This discrepancy may be due to the tissue observed, type of CR implemented and how this was measured. Supporting this idea, we recently show that, at least in the liver, both *ad libitum* and once-daily fed CR mice have the same mTOR and AMPK activity at multiple timepoints as long as the feeding the fasting conditions were exactly matched (discussed in Chapter 4).

One of the major challenges in connecting all the information between studies is the variation in dietary restriction protocols (e.g., dietary dilution vs. once-daily fed vs. ADF) as well as the conditions for tissue collection to examine molecular pathways (e.g., fasted vs. fed). Additionally, at least in the context of mammalian mTOR activity, tissue-specific function dictates the outcome of health and longevity. For example, downregulation of mTORC1 signaling in the pancreas inhibits beta cell mass and proliferation; however elevated mTORC1 signaling in the heart have been associated with pathological hypertrophy and insulin resistance in the heart [189, 190]. It is also not clear that a CR diet reduces mTORC1 signaling *in vivo*, as investigation of the effects of a CR diet on mTORC1 signaling is substantially complicated by time-of-feeding effects of CR on longevity. In Chapters 3 and 4, we address some of the complications of conducting dietary restriction experiments.

Humans Trials on Dietary Restriction

Initial studies in humans have suggested that CR may potentially postpone age-related diseases by reducing risk factors associated with such diseases. In one study CR reduced the risk factors for cardiovascular disease where participants on CR, with or without exercise, had significant decrease in ratio of total cholesterol to HDL cholesterol and systolic blood pressure [191]. Another study showed that CR was able to improve memory verbal memory score as well as decrease in fasting plasma levels of insulin and c-peptide[192]. Finally, a study looking into endurance training and CR found that CR significantly lower fasting insulin levels and higher insulin sensitivity as well as

decreased fasting glucose levels with high serum levels of adiponectin and free fatty acids and lower serum inflammatory mediators, similar to rodent studies [193].

Although these preliminary studies in humans suggest similar benefits can be attained as other model organisms under CR, implementing long-term CR is a major challenge for humans and compliance to this diet decrease with time. Withdrawal rates from a 12-month study were 40% for CR and 30% for periodic fasting participants [194]. In the 2-year CALERIE clinical trial, the compliance rate was further reduced to 82% with only a 12% restriction, half the targeted reduction [191]. To date, the only successful long-term study has been the CRONies study in which the CR group ate close to half of the calories compared to the AL subjects; however, this relied upon strong will-power from the participants [195]. Additionally, there may be some side effects associated with some DR protocols. One study showed that prolonged period of daily fasting (>15 hours) has been associated with mortality and disease [196, 197]; however long-lived populations practice 12-13 hr TRF with no adverse effects [102]. This discrepancy could possibly be explained by the timing of food intake, as breakfast skipping have been associated with decreased metabolic health outcomes [196, 198]. Also, while rodent studies of TRF were promising, human studies only showed mild improvement with detrimental effects on glucose homeostasis [199-201]. However, like animal studies, these studies were not controlled for circadian variations and future studies should carefully consider all the various experimental conditions to understanding diet and aging in humans.

Conclusion

One of the main goals for DR studies is to discover molecular mechanisms that mediates the beneficial health and lifespan extension observed in model organisms of DR, ultimately translating it to a human setting. Despite over a century of research, the mechanism of DR still remains elusive. Work on nutrient responsive pathway, such as Insulin/IGF-1 pathway and mTOR have been extensively studied; however, these studies are limited to testing and identifying only single genes and pathways. Considering the intertwined network of metabolic processes, it is unlikely that a single gene or pathway is mediating the CR response. Furthermore, different implementation of DR protocols further convolutes the interpretation between studies as fasting durations and time of feeding are often disregarded. In this dissertation, I address how calories and fasting duration impact the outcome of dietary restriction and the importance of feeding and fasting conditions when examining some of these metabolic pathways.

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CHAPTER 2: THE METABOLIC RESPONSE TO A LOW AMINO ACID DIET IS INDEPENDENT OF DIET-INDUCED SHIFTS IN THE COMPOSITION OF THE GUT MICROBIOME

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AUTHOR CONTRIBUTIONS

Experiments were performed in the Lamming Lab at UW-Madison. Heidi Pak, Nicole Cummings and Dudley Lamming contributed in the study conception and design. All authors participated in the performance of the experiments. Heidi Pak and Dudley Lamming prepared the manuscript with edits from Nicole Cummings, Cara Green and Irene Ong

ABSTRACT

Obesity and type 2 diabetes are increasing in prevalence around the world, and there is a clear need for new and effective strategies to promote metabolic health. A low protein (LP) diet improves metabolic health in both rodents and humans, but the mechanisms that underlie this effect remain unknown. The gut microbiome has recently emerged as a potent regulator of host metabolism and the response to diet. Here, we demonstrate that a LP diet significantly alters the taxonomic composition of the gut microbiome at the phylum level, altering the relative abundance of Actinobacteria, Bacteroidetes, and Firmicutes. Transcriptional profiling suggested that any impact of the microbiome on liver metabolism was likely independent of the microbiome-farnesoid X receptor (FXR) axis. We therefore tested the ability of a LP diet to improve metabolic health following antibiotic ablation of the gut microbiota. We found that a LP diet promotes leanness, increases energy expenditure, and improves glycemic control equally well in mice treated with antibiotics as in untreated control animals. Our results demonstrate that the beneficial effects of a LP diet on glucose homeostasis, energy balance, and body composition are unlikely to be mediated by diet-induced changes in the taxonomic composition of the gut microbiome.

INTRODUCTION

Around the world, approximately 425 million people have diabetes, and that number is expected to grow by 50% over the next three decades [1]. Beyond the direct effects of diabetes on mortality, its impact is amplified by its association with other causes of morbidity and mortality, such as cardiovascular disease [2], cancer [3], and Alzheimer's disease [4]. Type 2 diabetes, which is associated with diet and obesity, accounts for the vast majority of diabetes cases, and the epidemic rise in obesity has fueled the development of this health crisis.

Dietary interventions to control or prevent type 2 diabetes could be highly effective and affordable, but long-term reduced calorie diets have not proven to be sustainable for most people. Diets that alter the level of specific macronutrients without a decrease in caloric consumption may be more sustainable [5]; one variety of such diets are high protein, low carbohydrate diets such as the Atkins diet, which promise rapid weight loss without restricting calories [6]. Some clinical trials have observed that high protein diets can promote weight loss [7-9], at least in highly compliant subjects [10]. However, long-term prospective cohort studies have observed that high protein consumption is associated with increased insulin resistance, diabetes, cancer, and cardiovascular disease, and an overall increase in mortality [11-13].

In agreement with these findings, recent long-term studies in *Drosophila* and mice, as well as a short-term randomized control trial conducted in humans, find that low protein (LP) diets are associated with improvements in health, survival, and insulin sensitivity [13-18]. Reducing dietary protein largely blocks the effect of a high-fat diet on glucose tolerance [18], and we recently showed that in a mouse model of pre-existing diet-induced

obesity, reducing dietary protein rapidly restored metabolic health, dramatically reduced adiposity, and improved glucose tolerance and insulin sensitivity [19]. While some of these phenotypes are mediated in part by the insulin-sensitizing and energy expenditure promoting hormone fibroblast growth factor 21 (FGF21), it is likely that other mechanisms are also involved [18-24].

Over the last decade, numerous studies have found that the composition of the gut microbiome plays an important role in regulating the metabolic health of both rodents and humans [25, 26] by mediating the response to drugs, diet, and aging [27-33]. One major pathway by which the gut microbiota regulates glycemic control is by altering bile acid metabolism and activating the farnesoid X receptor (FXR) – FGF15 signaling axis [34, 35]. Recent work suggests that at least in rodents, the major dietary factors that regulate the taxonomic composition of the gut microbiome are protein and carbohydrate intake [36]. However, the source of dietary protein – e.g. red meat, white meat, dairy, or plant protein – also has an important effect on the taxonomic composition of the gut microbiome [37]. It remains unknown if the effect of a LP diet on the composition and function of the gut microbiome plays a role in its beneficial metabolic effects.

In this study, we determined that an amino acid defined LP diet, which has similar metabolic benefits to a LP diet containing natural protein [22], alters the taxonomic and functional composition of the gut microbiome. We found that a LP diet significantly alters the hepatic transcriptome, possibly reducing FXR-FGF15 signaling. Finally, we found that ablation of the gut microbiome with antibiotics does not significantly alter the metabolic response to a LP diet. Our data suggests that while dietary protein plays an important role in shaping the taxonomic and functional composition of the gut microbiome, these

diet-induced changes do not mediate the beneficial metabolic effects of a LP diet in young, lean mice.

MATERIALS AND METHODS

Animals and Treatments

For all experiments, male C57BL/6J mice were purchased from The Jackson Laboratory and group housed in static microisolator cages in a specific-pathogen free animal facility. For experiments investigating the composition of the gut microbiome and transcriptional profiling of the liver, mice were purchased at 8 weeks of age, and diet changes occurred at 9 weeks of age. Approximately 4 months later, cecal contents and livers were collected from mice sacrificed in the morning following an overnight, approximately ~16 hr fast. For experiments in which the gut microbiome was ablated with antibiotics, mice were purchased at 5 weeks of age; starting at 6 weeks of age, mice were randomized at the cage level to receive water or water containing antibiotics as described below. Diet changes occurred at 9 weeks of age, and antibiotic treatment was continued for the duration of the experiment. All procedures involving animals were approved by the Institutional Animal Care and Use Committee of the William S. Middleton Memorial Veterans Hospital (Madison, WI), and all experiments were performed in accordance with relevant guidelines and regulations.

Diets

Prior to 9 weeks of age, animals were fed the standard facility chow (Purina 5001; Purina Mills, Richmond, IN, USA). Amino acid defined animal diets (non-irradiated) were obtained from Envigo (formerly Harlan Laboratories). At 9 weeks of age, animals were

switched to either a Control amino acid defined diet (TD.140711; 22.0% of calories derived from amino acids; 59.4% from carbohydrate; 18.6% from fat) or a Low Protein amino acid defined diet (TD.140712; 7.1% of calories derived from amino acids; 74.4% from carbohydrates; 18.5% from fat). The complete composition of these diets has been previously described [22].

Antibiotic Treatment

The gut microbiome was ablated using an antibiotic treatment protocol previously described to efficiently ablate the gut microbiome of mice [38]. Briefly, mice were provided with free access to autoclaved water containing 1 g/L ampicillin, 500 mg/L vancomycin, and 1 g/L neomycin; however, in contrast to the protocol followed in [38], aspartame was omitted due to its negative effects on glucose homeostasis and body composition in mice [39]. The mice and the water bottles were weighed and changed biweekly to monitor water intake. Control mice were provided with autoclaved water not containing antibiotics. To verify the efficacy of the antibiotic treatment, fecal pellets were collected and total DNA was extracted using a modification of a previously described protocol [40]. Briefly, fecal pellets (~30-50 mg) were resuspended in a solution containing 500 µL of extraction buffer [200 mM Tris (pH 8.0), 200 mM NaCl, 20 mM EDTA], 210 µL of 20% SDS, 500 µL phenol:chloroform:isoamyl alcohol (pH 7.9, 25:24:1) and 500 µL of Fisher Scientific 1.4mm diameter ceramic beads (Cat# 15340159). Following mechanical disruption using a FastPrep 24 (M.P. Biomedicals), the solution was centrifuged at 8,000 rpm at 4°C for three minutes. The aqueous layer was then sequentially precipitated using sodium acetate/isopropanol and sodium acetate/ethanol. DNA samples were then quantified using a Nanodrop 2000c.

Mouse metabolic phenotyping

Glucose and alanine tolerance tests (GTT and ATT) were performed by fasting the mice overnight for 16 hr and then injecting either glucose (1 g/kg) or alanine (2 g/kg) intraperitoneally as previously described [41, 42]. Glucose measurements were taken using a Bayer Contour blood glucose meter and test strips. Mouse body composition was determined using an EchoMRI 3-in-1 Body Composition Analyzer. For assay of multiple metabolic parameters (O_2, CO_2, food consumption) and activity tracking, mice were acclimated to housing in a Columbus Instruments Oxymax/CLAMS metabolic chamber system for ~24 hr, and data from a continuous 24-hr period was then recorded and analyzed.

Gut microbial community DNA preparation

Approximately 20-50 mg of cecal matter was added to an autoclaved Sarstedt 2m micro screw-cap tube (Ref# 72.693.005) containing approximately 500 µL of BioSpec Zirconia/Silica beads (Cat# 11079101z) and one large Bio Spec bead (Cat# 11079132ss). To this, 500 µL of 200 mM Tris-HCl, pH 8.0/200 mM NaCl/20 mM EDTA was added, as well as 210 µL 20% SDS. 500 µL of Phenol/Chloroform/isoamyl alcohol, pH 7.9, 25:24:1, was added before bead beating using a FastPrep 24 (M.P. Biomedicals) until sample was fully homogenized in solution. Tubes were centrifuged at 8,000 rpm at 4°C for three minutes. The aqueous layer, approximately 500 µL, was transferred to a new microcentrifuge tube (Axygen). 60 µL of 3M NaAcetate was added, then 600 µL of isopropanol, then inverted to mix. The samples were placed on ice for one hour before centrifuging at 13,000 rpm at 4°C for 20 minutes. Samples were decanted, and pellet was rinsed with 200 µL of 100% EtOH, then decanted and briefly dried. The pellet was

dissolved in 100-200 µL of TE buffer. 100 µL of DNA was cleaned using the Macherey-Nagel PCR Clean-up kit, using 2 NT3 washes and eluting with 50-100 µL of elution buffer.

Construction and Sequencing of v3-v4 16S Metagenomic libraries

Purified genomic DNA was submitted to the University of Wisconsin-Madison Biotechnology Center. DNA concentration was verified fluorometrically using either the Qubit® dsDNA HS Assay Kit or Quant-iT™ PicoGreen® dsDNA Assay Kit (ThermoFisher Scientific, Waltham, MA, USA). Samples were prepared in a similar process to the one described in Illumina's 16S Metagenomic Sequencing Library Preparation Protocol, Part # 15044223 Rev. B (Illumina Inc., San Diego, California, USA) with the following modifications: The 16S rRNA gene V3/V4 variable region was amplified with fusion primers (forward primer 341f: 5'-ACACTCTTTCCCTACACGACGCTCTTCCGATCT$(N)_{3/6}$CCTACGGGNGGCWGCAG-3', reverse primer 805r: 5'-GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT$(N)_{3/6}$GACTACHVGGGTATCTAATCC-3'). Region specific primers were previously described ([43]; underlined sequences above), and were modified to add 3-6 random nucleotides ($(N)_{3/6}$) and Illumina adapter overhang nucleotide sequences 5' of the gene-specific sequences. Following initial amplification, reactions were cleaned using a 0.7x volume of AxyPrep Mag PCR clean-up beads (Axygen Biosciences, Union City, CA). In a subsequent PCR, Illumina dual indexes and Sequencing adapters were added using the following primers (Forward primer: 5'-AATGATACGGCGACCACCGAGATCTACAC[55555555]ACACTCTTTCCCTACACGACGCTCTTCCGATCT-3', Reverse Primer: 5'-

CAAGCAGAAGACGGCATACGAGAT[77777777]GTGACTGGAGTTCAGACGTGTGCT CTTCCGATCT -3', where bracketed sequences are equivalent to the Illumina Dual Index adapters D501-D508 and D701-D712,N716,N718-N724,N726-N729). Following PCR, reactions were cleaned using a 0.7x volume of AxyPrep Mag PCR clean-up beads (Axygen Biosciences). Quality and quantity of the finished libraries were assessed using an Agilent DNA 1000 kit (Agilent Technologies, Santa Clara, CA) and Qubit® dsDNA HS Assay Kit (ThermoFisher Scientific), respectively. Libraries were pooled in an equimolar fashion and appropriately diluted prior to sequencing. Paired end, 300 bp sequencing was performed using the Illumina MiSeq Sequencer and a MiSeq 600 bp (v3) sequencing cartridge. Images were analyzed using the standard Illumina Pipeline, version 1.8.2. OTU assignments and diversity plots were created using QIIME analysis pipeline [44].

Microbiota analysis using QIIME

Microbiota analysis to obtain OTU assignments and diversity plots were performed using Quantitative Insights Into Microbial Ecology (QIIME) [44] version 1.9.1. Illumina sequencing reads were adapter and quality trimmed using the Skewer [45] trimming program to remove low quality (<Q25) bases and sequencing adapters. Reads shorter than 100 nucleotides after trimming were discarded. Flash [46] was used to merge paired end reads into amplicon sequences using a minimum overlap of 10 nucleotides. Amplicons were then PCR primer trimmed and quality filtered. Sequences were then clustered in OTUs using an open-reference OTU picking protocol based on 97% identity using UCLUST [46] against the Greengenes reference database [47]. Representative sequences (most abundant sequence in OTUs) were picked, aligned to GreenGenes

[47] Core reference alignment using PyNAST [48]. Taxonomic assignments were associated with OTUs based on the taxonomy associated with the Greengenes reference sequence defining each OTU. UniFrac distances between samples were calculated using the Greengenes reference tree (ftp://greengenes.microbio.me/greengenes_release/gg_13_5/gg_13_8_otus.tar.gz). The resulting biom-formatted OTU table was filtered to remove singletons and OTUs that could not be aligned using PyNAST. Alpha rarefaction curves were calculated for all samples with a rarefaction upper limit of (median depth/sample count). Samples were removed from further characterization if they did not contain sufficient reads at a depth where the Good's coverage value for most samples was greater than 0.9. Beta diversity was calculated using weighted and unweighted unifrac on OTU data leveled according to the lowest sample depth. An alternative normalization by CSS [49] is also provided for additional downstream analysis.

Liver mRNA preparation and transcriptional profiling

Total liver RNA was extracted with Trireagent (Sigma) as previously described [19]. Concentration and purity of RNA was initially determined using a Nanodrop 2000c, and then submitted to the University of Wisconsin- Madison Biotechnology Center Gene Expression Center & DNA Sequencing Facility. RNA quality was then assayed using an Agilent RNA NanoChip, and stranded mRNA libraries with polyA enrichment were prepared as described in the Illumina TruSeq Stranded mRNA Sample Preparation Guide Rev. E. DNA sequencing was performed using an Illumina HiSeq 2500 1x100 (TruSeq v3) full flowcell. Pathway enrichment was performed using the “edgeR” [50] and “org.Mm.eg.db” [51] packages in R version 3.4.4 [52].

Gene expression (qPCR)

Total RNA was isolated from liver with Tri-Reagent, and cDNA was generated as previously described [24]. Oligo dT primers and primers for real-time PCR were obtained from Integrated DNA Technologies (Coralville, IA,USA). Primer sequences used for qPCR were as follows: *Cyp8b1*: F: GTTTCTGGGTCCTCTTATTCCTG, R: TGGGAGTGAAAGTGAACGAC; *Zfp36l1*: F: CACACCAGATCCTAGTCCTTG, R: CTGGGAGTGCTGTAGTTGAG; *Cyp7a1*: F: AACGATACACTCTCCACCTTT, R: CTGCTTTCATTGCTTCAGGG; *Ppp2cb*: F: ATGGAAGGATATAACTGGTGCC, R: AGGTGCTGGGTCAAACTG; *Map2k1*: F: CGTACATCGTGGGCTTCTAC, R: CAGAACTTGATCCAAGGACCC; *Shp*: F: CTACCCTCAAGAACATTCCAGG, R: CACCAGACTCCATTCCACG; *Abcb11:* F: CCTCATACGGAAACCCAAGATC, R: CTGACTGTTGATAGGCGATGG; *Actb*: F: ACCTTCTACAATGAGCTGCG, R: CTGGATGGCTACGTACATGG. Reactions were run on an Applied Biosystems StepOnePlus Real-Time PCR System (Thermo Fisher Scientific) with Invitrogen SYBR Green PCR Master Mix (ThermoFisher Scientific). Actin (*Actb*) was used to normalize the results from gene-specific reactions.

Statistics

Statistics were carried out in Prism 7 (Graphpad Prism). For each measured parameter, we conducted a two-factor ANOVA which included an effect of drug treatment (vehicle or antibiotics), an effect of diet (Control or Low AA), and an interaction between diet and treatment. A Sidak's post-test was performed to determine the significance of factors identified as significant in the two-factor ANOVA. PCA analysis was performed using Clustvis [53].

Data Availability

Liver transcriptional profiling data has been deposited at GEO, accession number GSE115683. All other datasets generated during the current study are available from the corresponding author on reasonable request.

RESULTS

A Low Amino Acid Diet Alters the Taxonomic Composition of the Gut Microbiome

To determine if the taxonomic composition of the microbiome is altered by consuming reduced dietary protein, we fed mice amino acid (AA) defined diets modeled on the AA profiles of naturally sourced control and low protein diets. The Control diet is modeled on a naturally sourced 21% protein diet, while the Low AA diet is based on a naturally sourced 7% protein diet, and we have previously shown that low protein and Low AA diets are comparable in their effect on glycemic control and body composition [22]. As shown in Figure 1, mice fed a Low AA diet for four months have improved glucose tolerance (**Fig. 1A**) and reduced weight and fat mass gain relative to mice fed the Control diet (**Fig. 1B**).

We sacrificed mice fed the Control and Low AA diets after four months, collecting cecal contents and the liver. We prepared DNA from the contents of the cecum, and performed 16S ribosomal RNA (rRNA) sequencing in order to identify alterations in the microbial composition of the gut microbiome. We utilized QIIME to determine relative taxonomic composition of each sample at the phylum and family levels (**Fig. 1C, 2A**). While the alpha diversity did not significantly differ between Control and Low AA diet-fed mice (**Fig. 2B**), we observed a major shift in the taxonomic composition of the gut

microbiome. Utilizing principal component analysis, we determined that the first two principle components explained a majority of the variability in the taxonomic composition of the gut microbiome, and individuals clustered by diet (**Fig. 1D**). We observed major differences at the phylum level, with an increase in the relative abundance of Firmicutes (**Fig. 1E**), and a decrease in the relative abundance of Bacteroidetes and Actinobacteria (**Fig. 1F**). At the family level, we found that the increased relative abundance of Firmicutes was primarily driven by an increase in the order *Clostridiales* (**Fig. 2**); we also observed a decrease in the abundance of the Bacteroidetes families *S24-7* and *Odoribacteraceae*, and the Actinobacteria families *Bifidobacteriaceae* and *Coriobacteriaceae* (**Fig. 2D**).

A Low Amino Acid Diet Alters the Hepatic Transcriptome, but does not activate FXR-FGF15 signaling

A low protein diet improves glucose tolerance in part by improving hepatic insulin sensitivity [22, 54]. While induction of the insulin-sensitizing hormone FGF21 has been shown to play a role in this response [20, 21], possibly via inhibition of hepatic mTORC1 (mechanistic Target Of Rapamycin Complex 1) [55], we recently observed that dietary methionine restriction, which mimics many of the effects of a low protein diet, can improve glucose tolerance independently of changes in FGF21 and hepatic mTORC1 [24].

Over the past decade, it has become clear that the composition and function of the gut microbiome can regulate host metabolism, including glycemic control [56, 57]. One recently characterized pathway by which the microbiome can regulate host glucose metabolism is through bile acids; alterations in the amount and type of secondary bile

acids can regulate glycemic control by activating or repressing the FXR – FGF15 signaling axis [34, 35].

We used RNA-Seq to identify gene expression changes induced by a low protein diet. We identified several differentially expressed metabolic pathways when mice were fed a Low AA diet (**Fig. 3A, 3B**). In particular, we observed altered expression of many genes involved in xenobiotic or drug metabolism as well as steroid hormone biosynthesis. However, our analysis did not identify bile acid signaling as significantly altered, and we did not observe significantly altered expression of *Shp*, *Cyp7a1*, or several other genes that have been shown to be regulated by the FXR-FGF15 signaling axis (**Fig. 3C**) [58-60]. Targeted qPCR analysis of mRNA from a larger, additional cohort of mice confirmed that a Low AA diet did not significantly alter the expression of any of one of these genes (**Fig. 4**). However, we observed an overall effect of diet consistent with reduced FXR-FGF15 signaling (**Fig. 4**).

The metabolic effects of protein restriction are not mediated by the gut microbiota

In order to directly assess if the altered taxonomic composition of the gut microbiome contributes to the metabolic effects of a low protein diets, we pretreated mice with either vehicle or antibiotics (ABX) for three weeks; mice were then randomized to either Control or Low AA diets. Antibiotic-treated mice continued to receive antibiotics throughout the course of the experiment (**Fig. 5A**). As expected, mice treated with antibiotics had significantly reduced fecal DNA content (**Fig. 5B**). Over the course of the experiment, we tracked weight and body composition of mice in each group (**Fig. 5C-F**).

As expected based on our previous studies, mice fed the Low AA diet gained less weight, less fat mass, and less lean mass than mice fed a Control diet. While antibiotic administration increased weight gain and lean mass gain compared to vehicle treated mice, the Low AA diet had similar effects on weight and body composition in the presence and absence of antibiotics.

As we determined previously, mice fed a Low AA diet had improved glucose tolerance compared to mice fed a Control diet (**Fig. 6A**). We also specifically assessed gluconeogenesis in the liver by performing an alanine tolerance test; as in our previous studies utilizing pyruvate, we observed a decrease in the area under the curve (AUC) in mice fed a Low AA diet, indicating improved suppression of gluconeogenesis (**Fig. 6B**). We saw equivalent reductions in AUC in response to a Low AA diet in both vehicle and antibiotic fed mice; and we did not observe any differences in AUC resulting from antibiotic treatment.

Rodents fed a low protein diet have increased food consumption and increased energy expenditure [15, 18, 20-22, 61]. We examined the effect of antibiotics on these phenotypes by placing mice in metabolic chambers and assessing food consumption, spontaneous activity, respiratory exchange ratio (RER), and energy expenditure (**Fig. 7A-F**). In agreement with our previous results, we observed increased food consumption (**Fig. 7A, 7B**) and energy expenditure (**Fig. 7E, 7F**) in mice fed a Low AA diet, with equivalent effects in both vehicle and antibiotic treated mice. Surprisingly, while a Low AA diet increased both the daytime and nighttime RER of vehicle-treated mice, mice treated with antibiotics did not have increased daytime RER when fed a Low AA diet, and had a reduced increase in nighttime RER (**Fig. 7C)**. The effects of diet on spontaneous

activity were relatively small, with increased activity in Low AA fed mice during the day (**Fig. 7D**).

DISCUSSION

Understanding the mechanisms by which dietary choices impact metabolic health is an area of significant research interest due to the increasing prevalence of diabetes and obesity in the population. Recently, we and others have shown that reducing dietary protein can promote metabolic health in both humans and rodents, but the molecular mechanisms that mediate these effects are unclear.

Here, we examine the effect of a low protein diet on the gut microbiome. In general agreement with the results of Holmes and colleagues [36], we find that reducing dietary amino acid levels (and increasing dietary carbohydrates) results in an increased Firmicutes-to-Bacteroidetes ratio. These findings demonstrate the robustness of this intervention on the gut microbiome, as the same effect can be observed in two different laboratories on different continents, using either naturally sourced or defined diets. These results are somewhat surprising, as some previous studies have suggested a link between obesity and an increased prevalence of Firmicutes [62]. However, they are consistent with the model of Holmes and colleagues that increased caloric intake promotes Firmicutes abundance [36].

Since the gut microbiome has proven to be a potent regulator of metabolic health, obesity, and glycemic control, we tested the hypothesis that diet-induced changes in the gut microbiome mediate the beneficial effects of a low protein diet on metabolism. We first used a targeted approach, examining the response of the liver, which is responsive

to microbiome-mediated alterations in the amount and type of secondary bile acids via the FXR-FGF15 signaling axis [34, 35], at the transcriptional level. While we observed altered expression of many genes in response to reduced dietary protein, we found no evidence of altered bile acid signaling and no evidence of increased signaling by the FXR-FGF15 signaling axis. However, a follow-up qPCR analysis of a targeted panel of genes regulated by FXR-FGF15 signaling in a second cohort of mice suggested that a low protein diet might reduce hepatic FXR-FGF15 signaling.

To directly test the role of gut microbiome, we then took an unbiased approach, testing the requirement for an intact gut microbiome in the metabolic response to reduced dietary protein. Following three weeks of high-dose antibiotic treatment, a regimen previously shown to ablate the gut microbiome and dramatically reduce fecal DNA content, we placed mice on either Control or Low AA diets. As anticipated based on previous studies in mice and many other mammals [63-66], antibiotics had an overall positive effect on growth and lean mass. However, we observed that protein restriction had very similar effects on weight, body composition, glucose tolerance, and energy expenditure in both the presence and absence of antibiotics. The one major difference we observed was that antibiotic treated, Low AA-fed mice had a lower RER relative to their vehicle treated counterparts, suggesting increased utilization of lipids and decreased utilization of carbohydrates as a fuel source.

A significant caveat of our studies is that they were conducted in young, lean C57BL/6J mice, which have relatively low intestinal permeability; this may limit the ability of the gut microbiome composition to affect host metabolism. The microbiome could play a larger role in the metabolic response of obese or older animals, which have increased

gut permeability [67, 68]; therefore, investigating the role of the gut microbiome in response to reduced dietary protein in aged or obese animals might be an important area for future study. In addition, we did not examine the taxonomic composition of the gut microbiome of antibiotic treated mice, which could help clarify if there are any antibiotic-resistant microbes which might mediate the metabolic effects of a low protein diet. Finally, we did not examine the metabolic effects of protein restriction in germ-free mice; examination in these animals, which completely lack all bacteria, could conceivably reveal subtle effects of the gut microbiome on the response to dietary protein that were not detectable using our antibiotic-ablation model.

Our study also examined only a limited number of metabolic phenotypes associated with a low protein diet, over a relatively short period of time; other phenotypes associated with reduced protein consumption, including increased longevity and healthspan [69], may be more directly linked to composition of the gut microbiota. A more detailed investigation of the transcriptional response to protein restriction in antibiotic-treated or germ-free mice may provide valuable clues to identify specific phenotypes that are dependent upon changes in the gut microbiome. There is also growing understanding that the specific amino acid composition of the diet mediates metabolic health [70-72], and there may be a role for the microbiome in the metabolic response to other diets with unusual amino acid profiles or from particular dietary sources.

Our findings highlight that dietary macronutrient composition plays an important role in determining the taxonomic composition of the gut microbiome. Yet, while the effects of a low protein diet on the gut microbiome are dramatic, at least in the short term, an intact gut microbiome is not required to realize the metabolic benefits of a low protein

diet on glucose homeostasis, body composition, or energy balance. Identifying the physiological and molecular mechanisms by which reducing dietary protein can promote metabolic health remains critical to developing drugs which can take advantage of these pathways to combat obesity and diabetes.

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COMPETING INTERESTS

The authors declare no competing interests.

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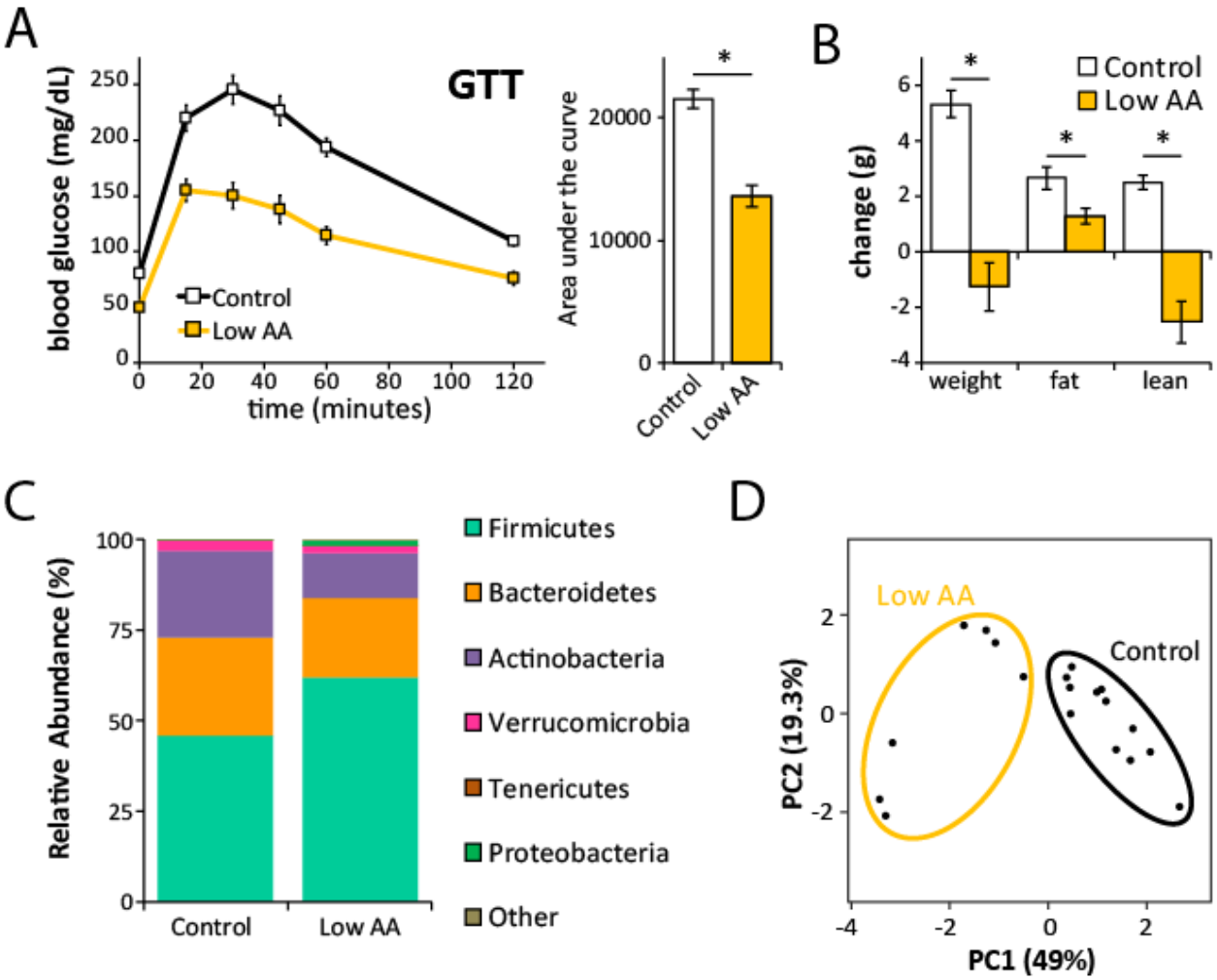
A
GTT
blood glucose (mg/dL)
time (minutes)
Control
Low AA
Area under the curve
B
change (g)
weight
fat
lean
C
Relative Abundance (%)
Firmicutes
Bacteroidetes
Actinobacteria
Verrucomicrobia
Tenericutes
Proteobacteria
Other
D
Low AA
Control
PC2 (19.3%)
PC1 (49%)
E
Phyla more abundant in Low AA-fed mice
Firmicutes
Relative Abundance (%)
F
Phyla less abundant in Low AA-fed mice
Bacteroidetes
Actinobacteria

Figure 1. A low protein diet promotes metabolic health and alters the taxonomic composition of the cecal microbiome. A) Glucose tolerance test on male C57BL/6J mice fed a Control (22% of calories from amino acids) or Low AA (7% of calories from amino acids) diet for 4 months (n = 8-10/group; * $p < 0.05$, t-test). B) Weight and body composition were measured immediately prior to diet start and after 10 weeks on the indicated diets (n = 8-10/group; * $p < 0.05$, = t-test). C) Bar plot of average relative abundance at the phylum taxonomic level. Top 6 phyla are shown. D) Principle component analysis of demonstrating the effect of diet on taxonomic composition. E-F) Bacterial phyla differentially represented in cecal contents from mice fed the specified diets for 4 months (n= 7-12/group; Sidak's test following ANOVA, * = $p < 0.05$). Error bars represent SEM.

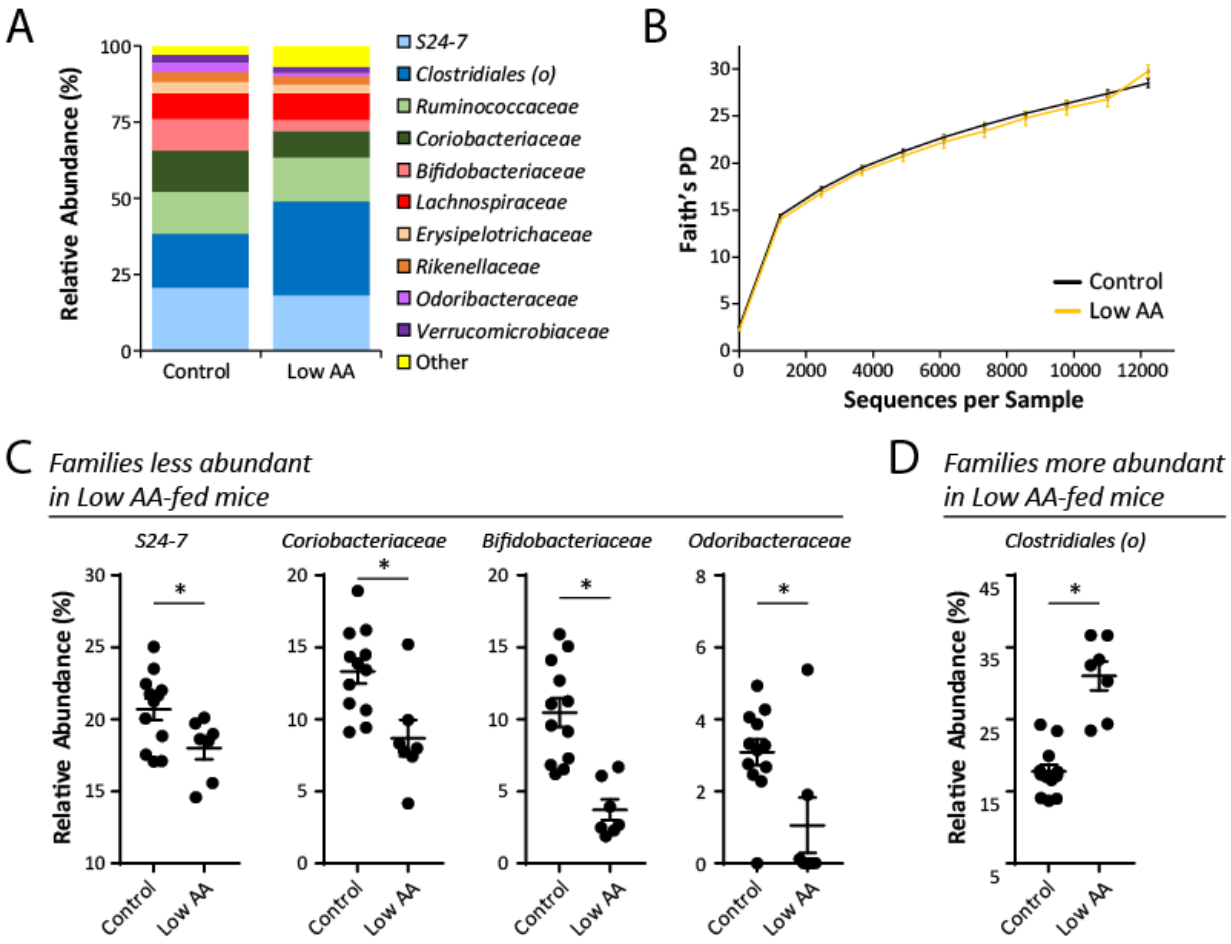

Figure 2. A) Bar plot of average relative abundance at the family taxonomic level. Top 10 phyla are shown. **B)** Bacterial alpha rarefaction curves calculated according to Faith's phylogenetic diversity (PD) index at the phylum taxonomic level. **C-D)** Bacterial families differentially represented in cecal contents from mice fed the specified diets for 4 months (n= 7-12/group; $q < 0.05$, FDR and * = $p < 0.05$, t-test). Error bars represent SEM.

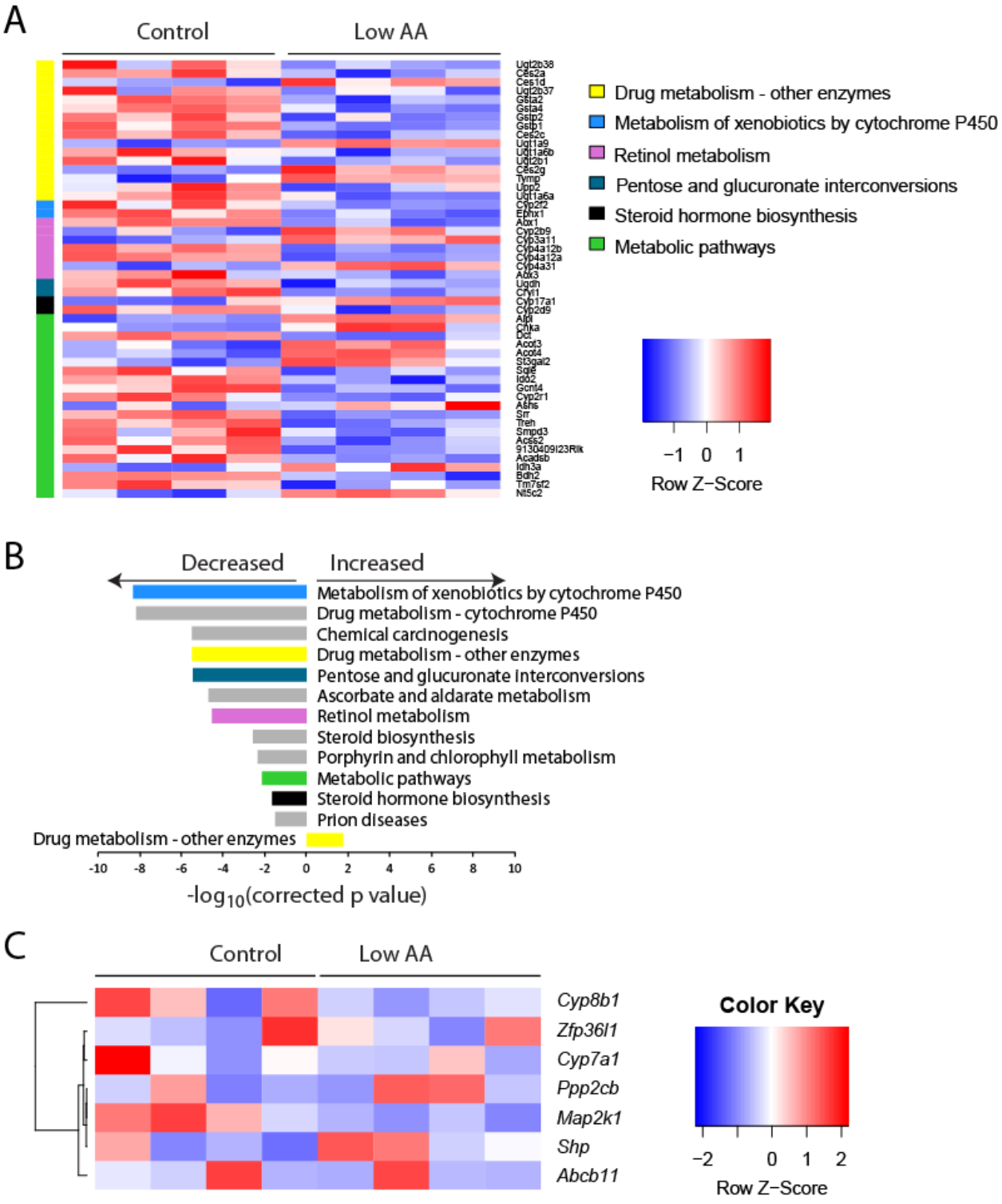
A
Control
Low AA
Drug metabolism - other enzymes
Metabolism of xenobiotics by cytochrome P450
Retinol metabolism
Pentose and glucuronate interconversions
Steroid hormone biosynthesis
Metabolic pathways
−1 0 1
Row Z−Score
B
Decreased
Increased
Metabolism of xenobiotics by cytochrome P450
Drug metabolism - cytochrome P450
Chemical carcinogenesis
Drug metabolism - other enzymes
Pentose and glucuronate interconversions
Ascorbate and aldarate metabolism
Retinol metabolism
Steroid biosynthesis
Porphyrin and chlorophyll metabolism
Metabolic pathways
Steroid hormone biosynthesis
Prion diseases
Drug metabolism - other enzymes
-10 -8 -6 -4 -2 0 2 4 6 8 10
-log10(corrected p value)
C
Control
Low AA
Cyp8b1
Zfp36l1
Cyp7a1
Ppp2cb
Map2k1
Shp
Abcb11
Color Key
−2 0 1 2
Row Z−Score

Figure 3: A low protein diet alters the hepatic transcriptome and shows distinct changes in biological pathways. A-C) RNA-Seq was performed on the livers for mice fed a Control diet or a Low AA for four months. **A)** A heatmap indicating the relative expression of genes involved in the most significantly enriched biological KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways based on genes differentially expressed in the livers of Control and Low AA fed mice ($q < 0.05$, FDR). Genes in more than one significantly enriched KEGG pathway are listed only once, and assigned to the most significantly affected pathway. **B)** Pathway enrichment analysis was performed using g:Profiler (g:GOSt) [73], and the p-values of KEGG pathways significantly up- and downregulated by Low AA diet feeding were determined. Colors are matched to that of pathways in Figure 2A. **C)** Heatmap representing the relative expression of liver genes known to be altered by FXR-FGF15 bile acid signaling.

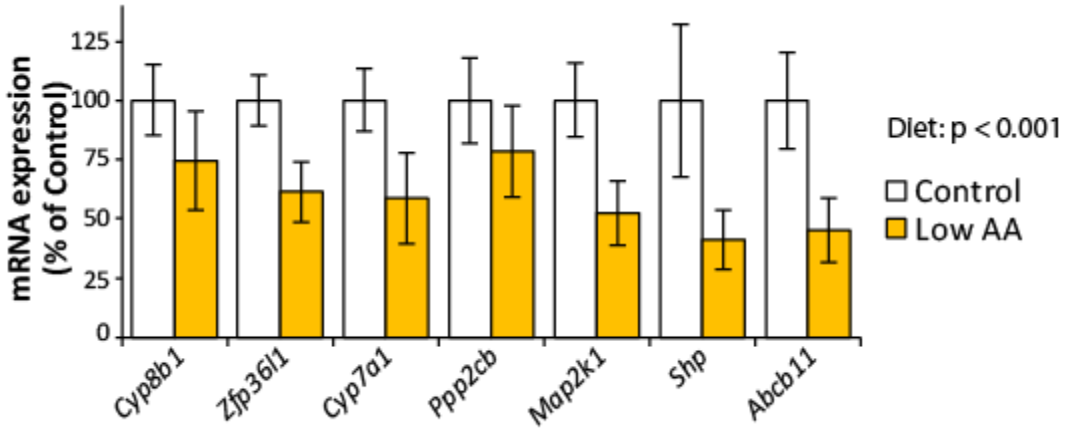

Figure 4: qPCR analysis of FXR-FGF15 controlled genes. mRNA expression of the indicated genes in the livers of mice fed Control or Low AA diets was assessed by qPCR (n=7-8/group; statistics for the overall effects of gene, diet and the interaction represent the p-value from a two-way ANOVA; * = $p < 0.05$ from a Sidak's post-test examining the effect of parameters identified as significant in the two-way ANOVA). Error bars represent SEM.

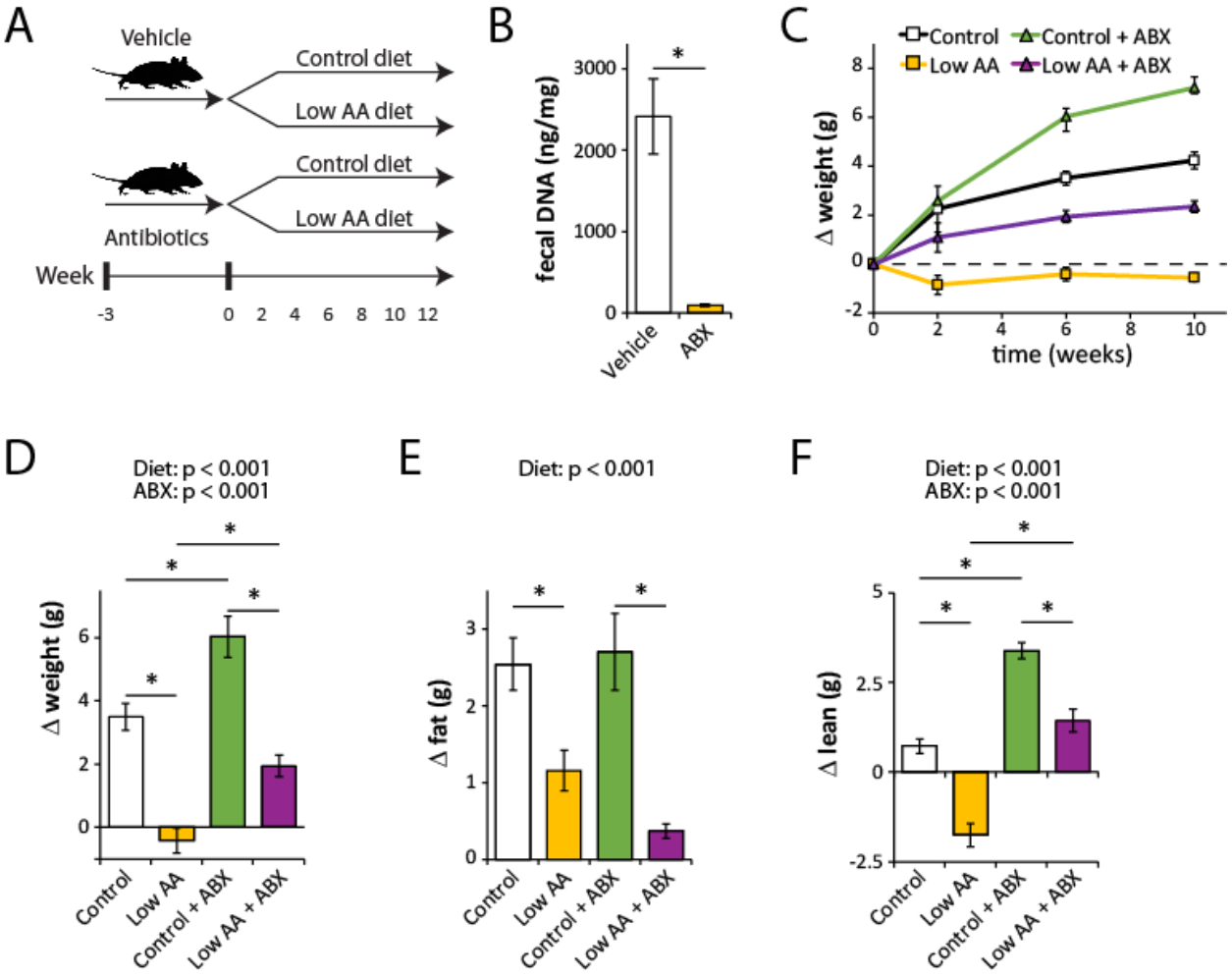
A
Vehicle
Control diet
Low AA diet
Antibiotics
Control diet
Low AA diet
Week
-3
0 2 4 6 8 10 12
B
fecal DNA (ng/mg)
3000
2000
1000
0
*
Vehicle
ABX
C
Control
Control + ABX
Low AA
Low AA + ABX
Δ weight (g)
time (weeks)
D
Diet: p < 0.001
ABX: p < 0.001
Δ weight (g)
Control
Low AA
Control + ABX
Low AA + ABX
E
Diet: p < 0.001
Δ fat (g)
F
Diet: p < 0.001
ABX: p < 0.001
Δ lean (g)

Figure 5: A low protein diet alters body composition similarly in vehicle and antibiotic-treated mice. A) Schematic representation of the experimental plan; mice were pretreated with antibiotics or vehicle for three weeks, and then randomized to either a Control or Low AA diet. **B)** Fecal DNA content was determined following 3 weeks of antibiotic treatment (n= 8/group; * = $p < 0.05$, t-test). **C)** Weight of the mice in each group was tracked following randomization to each diet. **D-F)** Weight and body composition were determined immediately prior to diet start and after 6 weeks on the indicated diets, and the change in **D)** weight, **E)** fat mass, and **F)** lean mass was determined (n = 12/group; statistics for the overall effects of diet, antibiotic (ABX) treatment, and the interaction represent the p-value from a two-way ANOVA; * = $p < 0.05$ from a Sidak's post-test examining the effect of parameters identified as significant in the two-way ANOVA). Error bars represent SEM.

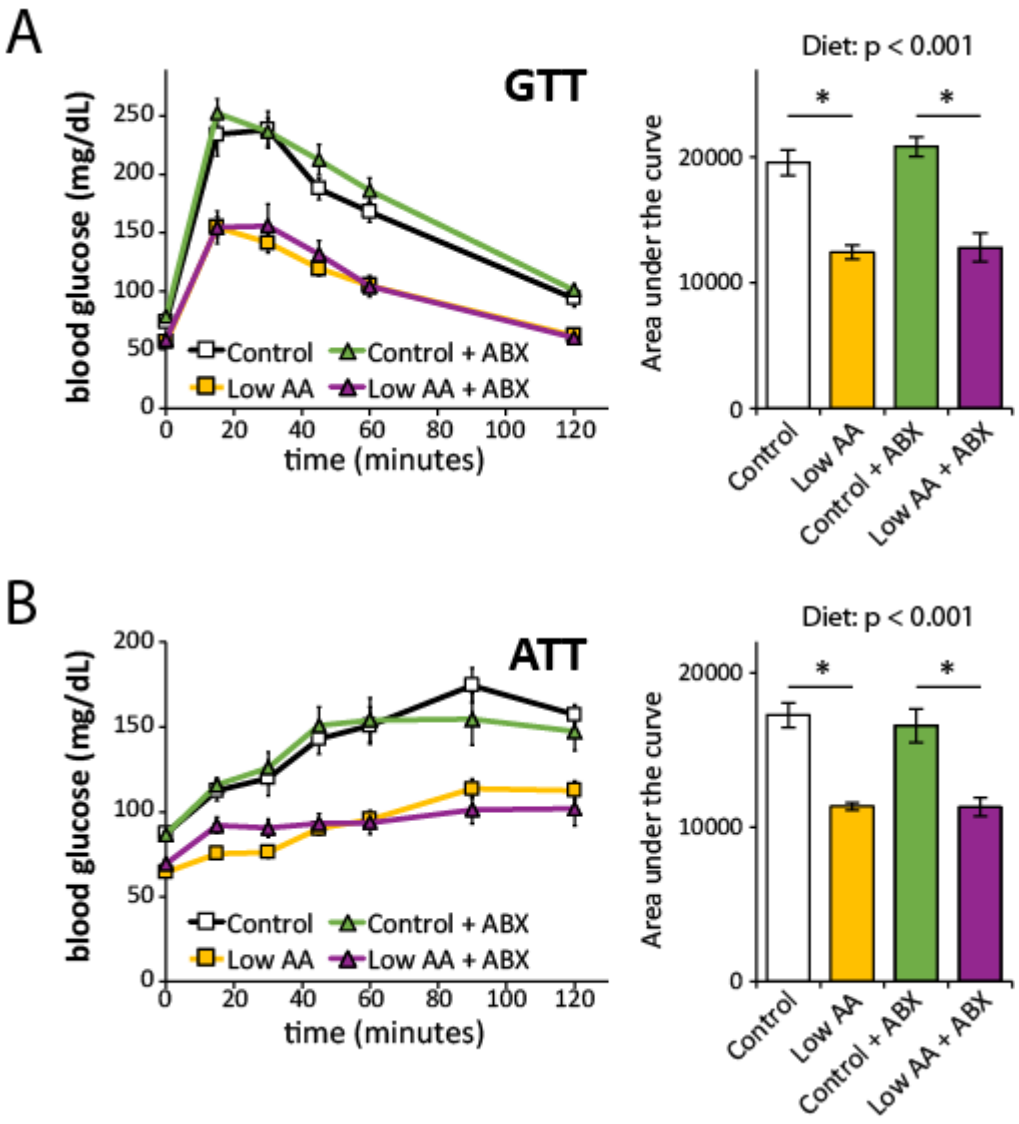

Figure 6: A low protein diet improves glucose homeostasis similarly in vehicle and antibiotic-treated mice. **A)** Glucose and **B)** alanine tolerance tests were conducted in mice fed the indicated diets for 8 weeks and 4 weeks, respectively (n=12/group; statistics for the overall effects of diet, antibiotic (ABX) treatment, and the interaction represent the p-value from a two-way ANOVA; * = $p < 0.05$ from a Sidak's post-test examining the effect of parameters identified as significant in the two-way ANOVA). Error bars represent SEM.

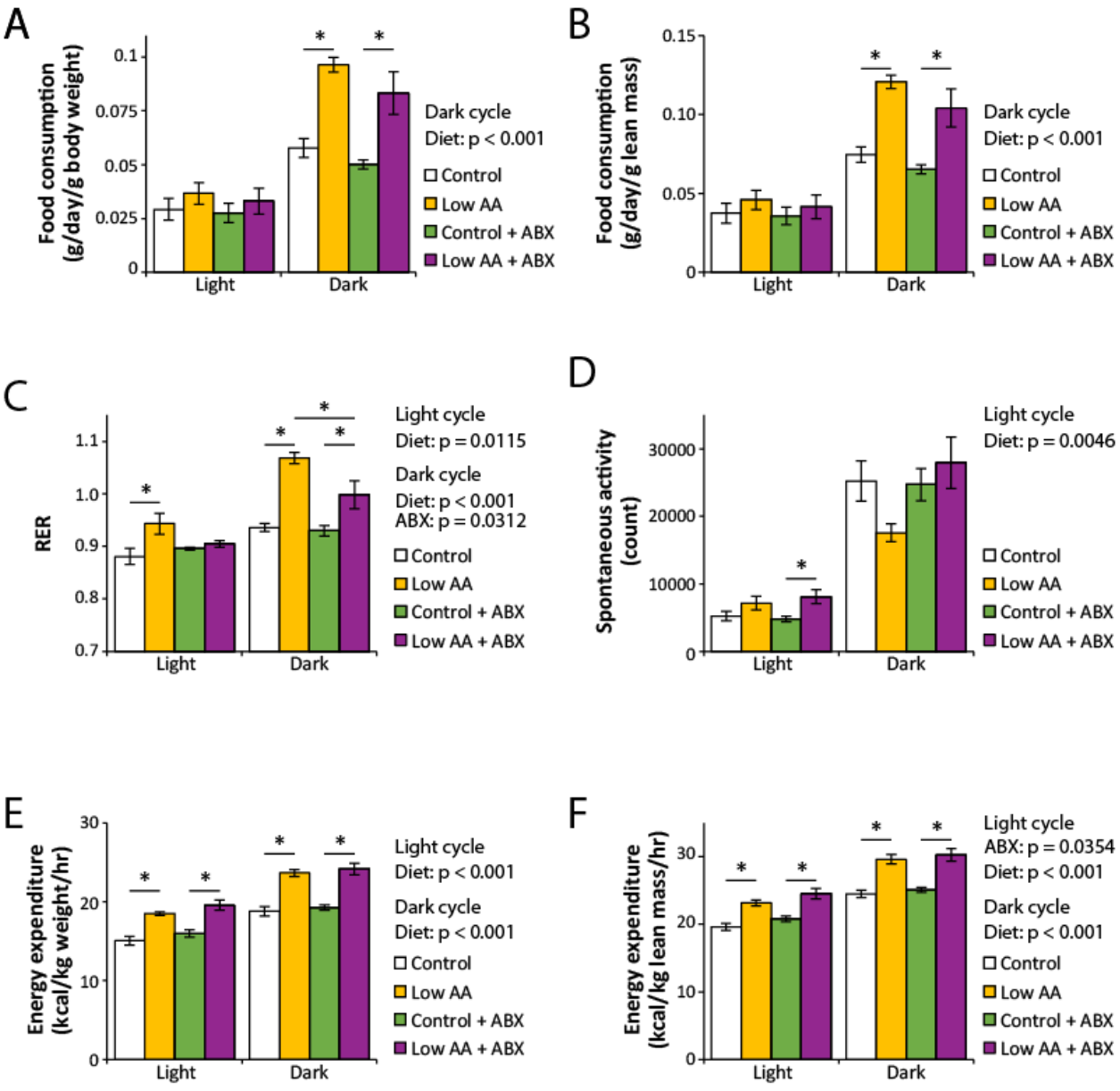
A
Food consumption (g/day/g body weight)
0.1
0.075
0.05
0.025
0
Light
Dark
Dark cycle
Diet: p < 0.001
Control
Low AA
Control + ABX
Low AA + ABX
B
Food consumption (g/day/g lean mass)
0.15
0.10
0.05
0
Light
Dark
Dark cycle
Diet: p < 0.001
Control
Low AA
Control + ABX
Low AA + ABX
C
RER
1.1
1.0
0.9
0.8
0.7
Light
Dark
Light cycle
Diet: p = 0.0115
Dark cycle
Diet: p < 0.001
ABX: p = 0.0312
Control
Low AA
Control + ABX
Low AA + ABX
D
Spontaneous activity (count)
30000
20000
10000
0
Light
Dark
Light cycle
Diet: p = 0.0046
Control
Low AA
Control + ABX
Low AA + ABX
E
Energy expenditure (kcal/kg weight/hr)
30
20
10
0
Light
Dark
Light cycle
Diet: p < 0.001
Dark cycle
Diet: p < 0.001
Control
Low AA
Control + ABX
Low AA + ABX
F
Energy expenditure (kcal/kg lean mass/hr)
30
20
10
0
Light
Dark
Light cycle
ABX: p = 0.0354
Diet: p < 0.001
Dark cycle
Diet: p < 0.001
Control
Low AA
Control + ABX
Low AA + ABX

Figure 7: A low protein diet increases food consumption and energy expenditure similarly in vehicle and antibiotic-treated mice. A-F) Metabolic chambers were used to assess **A-B)** food consumption, **C)** spontaneous activity, **D)** respiratory exchange ratio (RER), and **E-F)** energy expenditure in mice fed the indicated diets for approximately two months. (n=5-7/group; statistics for the overall effects of diet, antibiotic (ABX) treatment, and the interaction represent the p-value from a two-way ANOVA; * = $p < 0.05$ from a Sidak's post-test examining the effect of parameters identified as significant in the two-way ANOVA). Error bars represent SEM.

KEGG Pathway	Gene list
Metabolism of xenobiotics by cytochrome P450	GSTA4,UGT2B1,ENSMUSG00000038155,EPHX1,CYP2F2,UGT1A6A,UGT2B37,GSTA2,ENSMUSG00000060803,UGT2B38,UGT1A6B
Drug metabolism - cytochrome P450	GSTA4,UGT2B1,ENSMUSG00000038155,UGT1A6A,UGT2B37,GSTA2,ENSMUSG00000060803,UGT2B38,AOX1,AOX3,UGT1A6B
Chemical carcinogenesis	GSTA4,UGT2B1,ENSMUSG00000038155,EPHX1,UGT1A6A,UGT2B37,GSTA2,ENSMUSG00000060803,UGT2B38,UGT1A6B
Drug metabolism - other enzymes	UPP2,UGT2B1,UGT1A6A,CES2A,UGT2B37,CES2C,UGT2B38,UGT1A6B
Pentose and glucuronate interconversions	CRYL1,UGDH,UGT2B1,UGT1A6A,UGT2B37,UGT2B38,UGT1A6B
Ascorbate and aldarate metabolism	UGDH,UGT2B1,UGT1A6A,UGT2B37,UGT2B38,UGT1A6B
Retinol metabolism	UGT2B1,UGT1A6A,UGT2B37,UGT2B38,AOX1,AOX3,CYP4A12A,CYP4A12B,UGT1A6B
Steroid biosynthesis	SQLE,TM7SF2,CYP2R1,MSMO1
Porphyrin and chlorophyll metabolism	UGT2B1,UGT1A6A,UGT2B37,UGT2B38,UGT1A6B
Metabolic pathways	SRR,ETNPPL,CRYL1,DCT,SQLE,TM7SF2,UPP2,ACSS2,BDH2,UGDH,CYP2R1,ACADSB,IDO2,MSMO1,SMPD3,TREH,TKFC,UGT2B1,9130409I23RIK,UGT1A6A,UGT2B37,UGT2B38,AOX1,AOX3,CYP4A12A,CYP4A12B,UGT1A6B,GCNT4
Steroid hormone biosynthesis	UGT2B1,UGT1A6A,UGT2B37,UGT2B38,CYP2D9,UGT1A6B
Prion diseases	C9,C6,NCAM2,PRNP
Drug metabolism - other enzymes	TYMP,CES2G,CES1D,UGT1A9

Table 1: List of differentially expressed genes contributing to the identification of KEGG Pathways in Figure 2.

CHAPTER 3: FASTING DRIVES THE METABOLIC, MOLECULAR, AND GEROPROTECTIVE EFFECTS OF A CALORIE RESTRICTED DIET IN MICE

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Keywords: calorie restriction, fasting, dietary restriction, time-restricted feeding, lifespan, healthspan

AUTHOR CONTRIBUTIONS

Experiments were performed in the Lamming, Denu, and Yen laboratories at UW-Madison and in the UAB Nathan Shock Center Mitometabolism Core. Heidi Pak and Dudley Lamming contributed in the study conception and design. All authors participated in the performance of the experiments: Spencer Haws and John Denu performed and analyzed liver metabolomics data; Michelle Johnson, Stephen Barnes, Victor Darley-Usmar and Jianhua Zhang performed and analyzed muscle metabolomics data; and Mitchell Lavarias and Eric Yen provided reagents and analyzed data for gut integrity. Heidi Pak and Dudley Lamming prepared the manuscript with edits from Spencer Haws, John Denu, Jianhua Zhang, Mitchell Lavarias and Eric Yen.

ABSTRACT

Calorie restriction (CR) promotes healthy aging in diverse species. Recently, it has been shown that fasting for a portion of each day has metabolic benefits and promotes lifespan. These findings complicate the interpretation of rodent CR studies, in which animals typically eat only once per day and rapidly consume their food, which collaterally imposes fasting. Here, we show that a prolonged fast is necessary for key metabolic, molecular and geroprotective effects of a CR diet. Using a series of feeding regimens, we dissect the effects of calories and fasting, and proceed to demonstrate that fasting alone recapitulates many of the physiological and molecular effects of CR. Our results shed new light on how both when and how much we eat regulate metabolic health and longevity, and demonstrate that daily prolonged fasting, and not solely reduced calorie intake, is likely responsible for the metabolic and geroprotective benefits of a CR diet.

INTRODUCTION

Calorie restriction (CR) is the gold standard for geroprotective interventions, extending lifespan and healthspan in diverse organisms and preventing or delaying many age-associated diseases [1-6]. In rodents, CR improves metabolic health and glucose homeostasis [1, 7]. As long-term adherence to a reduced calorie diet is difficult for many people, there is significant interest in identifying the physiological and molecular mechanisms that mediate the beneficial effects of a CR diet.

Traditionally, the beneficial effects of a CR diet were believed to be the result of reduced caloric intake, although recent studies suggest that reduction of specific macronutrients may also play a role [8-14]. It has recently been realized that CR regimens, as typically implemented in the laboratory, not only restrict calories, but also impose a prolonged daily fast, as CR animals rapidly consume their entire daily meal within ~2 hours, and then fast for ~22 hours until their next meal [15-17]. Interventions such as time-restricted or meal feeding that involve a fasting period have metabolic benefits and extend the lifespan of mice [18-20]. These findings complicate the interpretation of CR studies, as it is unclear which effects of CR result from reduced caloric intake, and which instead are attributable to the collaterally imposed fast.

We developed a series of diets and feeding regimens enabling us to dissect the contribution of fasting from energy restriction alone in a CR diet. As different strains and sexes have differential responses to a CR diet [21, 22], we examined the metabolic responses of both male and female C57BL/6J and DBA/2J mice. We also tested whether prolonged daily fasting without energy restriction could recapitulate the metabolic and

transcriptional effects of a CR diet. Finally, we tested if fasting is required for the beneficial effects of a CR diet on healthspan and longevity.

We find that fasting is required for CR-induced changes in insulin sensitivity and fuel selection. Additionally, fasting alone without reduced energy intake is sufficient to recapitulate the metabolic phenotypes and transcriptional signature of a CR diet. Finally, we show that fasting is required for CR-induced improvements in glucose metabolism, frailty, and lifespan in C57BL/6J male mice. Our results overturn the long-held belief that the beneficial effects of a CR diet in mammals are mediated solely by the reduction of caloric intake, and highlight fasting as an important component of the metabolic and geroprotective effects of CR.

MATERIALS AND METHODS

Animals, Diets, and Feeding Regimens

All procedures were performed in conformance with institutional guidelines and were approved by the Institutional Animal Care and Use Committee of the William S. Middleton Memorial Veterans Hospital (Assurance ID: D16-00403) (Madison, WI, USA). Male and female C57BL/6J and DBA/2J mice (stock numbers 000664 and 000671) were purchased from the Jackson Laboratory (Bar Harbor, ME, USA) at 8 weeks of age and acclimated to the animal research facility for at least one week before entering studies. All animals were housed in a specific pathogen free (SPF) mouse facility with a 12:12 hour light/dark cycle maintained at 20°-22°C. All animals were placed on 2018 Teklad Global 18% Protein Rodent Diet for one week before randomization. At 10 weeks of age, mice were randomized to one of 5 diet groups: 1) AL, *ad libitum* diet: 2) CR, animals in which calories

were restricted by 30%, and animals were fed once per day during the start of the light period; 3) MF.cr, animals in which calories were restricted 30%, and animals were fed three equal meals during the course of the 12 hour dark period using an automated feeder (F14 Aquarium Fish Feeder, Fish Mate) [75]; 4) Diluted AL, animals provided with *ad libitum* access to a low energy diet diluted with indigestible cellulose, which reduced calorie intake by ~30%; and 5) TR.al, a feeding paradigm where mice were entrained to rapidly consume an *ad libitum* portion of food. Animals fed an AL, CR, MF.cr and TR.al diet were fed 2018 Teklad Global 18% Protein Rodent Diet, Envigo Teklad. Animals fed a Diluted AL diet were fed Teklad 2018 with 50% Cellulose (TD.170950). A stepwise reduction in food intake by increments of 10% per week, starting at 20% was carried out for mice in the CR group. In this study we highlight CR regimen as a fasting model; therefore, we decided to feed these animals in the morning as it aligns best with the last feeding time point from the *ad libitum* fed animals and MF.cr. This feeding schedule is also widely used (e.g. by the NIA Aging Rodent Colony [23]), and the time-of-day at which feeding occurs has been shown to not affect the ability of CR to extend lifespan [24]. Mice in the TR.al group were entrained to eat comparably to the AL-fed group within a 3-hour period during the first two weeks, and food was always removed 3 hours after the start of feeding. Body weight and food intake were monitored weekly. Due to shredding behavior of Diluted AL, we performed a comprehensive food consumption where we measured average shredded food and food left in the hopper. We found an average of 23% of the food were shredded on the bed of the cage. We accounted for this value to calculate for Diluted AL food consumption presented in this manuscript. CR and TR.al mice were fed daily at approximately 7:00 a.m. Animal rooms were maintained at 20ºC – 22ºC with 30%-

70% relative humidity and a 12-hr light/dark cycle. The caloric intake of the mice in the AL group was calculated weekly to determine the appropriate number of calories to feed the mice in the CR and MF.cr groups. The caloric intake of the mice in TR.al were calculated daily to monitor food intake.

Metabolic Phenotyping

Glucose and insulin tolerance tests (GTT and ITT) were performed by fasting all mice overnight and then injecting either glucose (1 g/kg) or insulin (0.5U/kg or 0.75U/kg) intraperitoneally [7]. Glucose measurements were taken using a Bayer Contour blood glucose meter and test strips. Mouse body composition was determined using an EchoMRI Body Composition Analyzer. For assay of multiple metabolic parameters (O_2, CO_2, food consumption and activity tracking), mice were acclimated to housing in a Columbus Instruments Oxymax/CLAMS metabolic chamber system for ~24 hour, and data from a continuous 24-hr period was then recorded and analyzed. AL-fed and Diluted AL-fed mice had ad libitum access to their respective diets; MF.cr and CR-fed mice were fed once per day at the beginning of the light cycle as indicated in each figure.

Sacrifice and Collection of Tissues

Mice were sacrificed in either in the fasted or fed state after 16 weeks on diet. Mice sacrificed in the fasted were fasted overnight starting at 11 am and then sacrificed between 9 and 11 am the next morning. Mice sacrificed in the fed state were fed starting at 7 am and sacrificed 3 hours later. Following blood collection via submandibular bleeding, mice were euthanized by cervical dislocation and tissues (liver,

muscle, iWAT, eWAT, BAT, and cecum) were rapidly collected, weighed, and then snap frozen in liquid nitrogen.

Transcriptional profiling and analysis

RNA was extracted from liver or iWAT using Trireagent according to the manufacturer's protocol (Sigma-Aldrich). The concentration and purity of RNA were determined by absorbance at 260/280 nm using Nanodrop (Thermo Fisher Scientific) and sent to UW Biotechnology Center for sequencing and data annotation. Heatmaps were created using Prism 8.0 with log2 transformed data. Networks were constructed using NetworkAnalyst (Version 2) by protein-protein interaction [39-43] and pathways were identified with functional enrichment analysis using gene sets from Kegg.

Liver Tissue Metabolite Extraction

M. musculus liver tissue was powdered in liquid nitrogen using a mortar and pestle. Powdered tissue was transferred to an individual 1.5 ml microcentrifuge Eppendorf tube and incubated with 1 ml -80^{o}C 80:20 MeOH:H_2O extraction solvent on dry ice for 5 minutes post-vortexing. Tissue homogenate was centrifugated at maximum speed for 5 minutes at 4^{o}C. Supernatant was transferred to a 15 ml tube after which the remaining pellet was resuspended in 0.8 ml -20^{o}C 40:40:20 ACN:MeOH:H_2O extraction solvent and incubated on ice for 5 minutes. Tissue homogenate was again centrifugated at maximum speed for 5 minutes at 4^{o}C after which the supernatant was pooled with the previously isolated metabolite fraction. The 40:40:20 ACN:MeOH:H_2O extraction was then repeated. Next, equal volumes of pooled metabolite extract for each sample was transferred to a 1.5 ml microcentrifuge Eppendorf tube and completely dried using a Thermo Fisher Savant ISS110 SpeedVac. Dried metabolite extracts were resuspended in 85% ACN

following microcentrifugation for 5 minutes at maximum speed at 4°C to pellet any remaining insoluble debris. Supernatant was then transferred to a glass vial for LC-MS analysis.

Skeletal Muscle Metabolite Extraction

Frozen skeletal muscle was pulverized using liquid nitrogen. Pulverized tissues (50 mg) were then homogenized in 1 mL cold (-20°C) aqueous HPLC-grade methanol with a single 5 mm metal bead using the Qiagen TissueLyser II. Disruption was carried out at 25 Hz for 30s. Homogenates were clarified by centrifugation, spiked with 13c-succinate, and submitted to the Mass Spec facility for targeted amino acid and TCA metabolite analysis.

Targeted LC-MS Metabolite Analyses

Each prepared metabolite sample was injected onto a Thermo Fisher Scientific Vanquish UHPLC using two distinct column/buffer combinations to maximize metabolome coverage. One combination utilized a Waters XBridge BEH Amide column (100 mm x 2.1 mm, 3.5 μm) coupled to a Thermo Fisher Q-Exactive mass spectrometer. For the Waters XBridge BEH Amide column, mobile phase (A) consisted of 97% H_2O, 3% ACN, 20 mM ammonium acetate, and 15 mM ammonium hydroxide pH 9.6. Organic phase (B) consisted of 100% ACN. Metabolites were resolved using the following linear gradient: 0 min, 85% B, 0.15 ml/min; 1.5 min, 85% B, 0.15 ml/min; 5.5 min, 40% B, 0.15 ml/min; 10 min, 40% B, 0.15 ml/min; 10.5 min, 40% B, 0.3 ml/min; 14.5 min, 40% B, 0.3 ml/min; 15 min, 85% B, 0.15 ml/min; 20 min, 85% B, 0.15 ml/min. The mass spectrometer was operated in positive ionization mode with a MS1 scan at resolution = 70,000, automatic

gain control target = 3 x 10^6, and scan range = 60-186 m/z and 187-900 m/z. The remaining combination utilized a Waters Acquity UPLC BEH C18 column (100 mm x 2.1 mm x 1.7 μm). For the Waters Acquity UPLC BEH C18 column, mobile phase (A) consisted of 97% H_2O, 3% MeOH, 10mM TBA, 9mM acetate with a final pH = 8.1 – 8.4. Organic phase (B) consisted of 100% MeOH. Metabolites were resolved using the following linear gradient: 0 min, 95% B, 0.2 ml/min; 2.5 min, 95% B, 0.2 ml/min; 17 min, 5% B, 0.2 ml/min; 19.5 min, 5% B, 0.2 ml/min; 20 min, 95% B, 0.2 ml/min; 25 min, 95% B, 0.2 ml/min. The mass spectrometer was operated in negative ionization mode with a MS1 scan at resolution = 70,000, automatic gain control target = 1 x 10^6, and scan range = 85-1275 m/z. Individual metabolite data were called from data files using MAVEN version 2011.6.17 [76, 77] with retention times empirically determined in-house. Peak Area Top values were analyzed to determine metabolite expression. Heatmaps were created using Spyder Anaconda 3.

Lifespan and frailty assessment

8-week-old male C57BL/6J mice were obtained from The Jackson Laboratory and maintained on 2018 Teklad Global (18% Protein Rodent Diet, Envigo Teklad) diet until 4 months of age. The animals were then randomized into three groups (AL, Diluted AL or CR) of equivalent body weight. Mice on the Diluted AL diet were fed a formulation of Teklad 2018 with 50% Cellulose (TD.170950), resulting in an approximate 30% decrease in calorie intake. The caloric intake and of the mice in the AL group was calculated weekly to determine the appropriate number of calories to feed the mice in the CR and MF.cr group. CR was implemented with gradual stepwise reduction in food intake by increments of 10% per week, starting at 20%, was carried out for CR mice. CR mice were fed daily

at approximately 7:00 a.m.. Body weight and food intake were monitored every week until food intake was stabilized then monitored every other week. Mice were housed in a specific pathogen free (SPF) mouse facility with a 12:12 hour light/dark cycle maintained at 20°-22°C. Mice were euthanized for humane reasons if moribund, if the mice developed other specified problems (e.g. excessive tumor burden), or upon the recommendation of the facility veterinarian. Mice that developed rectal prolapse were treated with Anusol Plus daily; mice euthanized due to rectal prolapse without other health abnormalities were censored as of the date of euthanasia. Mice were censored as of the date of death if death was associated with a procedure.

Frailty was assessed using a 25-item frailty index based on the procedures defined by Whitehead et al. [78]. The items scored included alopecia, loss of fur color, dermatitis, loss of whiskers, coat condition, tumors, distended abdomen, kyphosis, tail stiffening, gait disorders, tremor, body condition score, vestibular disturbance, cataracts, corneal opacity, eye discharge/swelling, microphthalmia, nasal discharge, malocclusions, rectal prolapse, vaginal/uterine/penile prolapse, diarrhea, breathing rate/depth, mouse grimace score, and piloerection.

Bomb Calorimetry

We determined the caloric content of diet and feces of 19-month-old C57BL/6J male mice (diet implementation at 4-month-old) with an adiabatic bomb calorimeter (6200ea Oxygen Bomb Calorimeter; Parr Instrument Company). Bombs were calibrated using benzoic acid before use.

Intestinal barrier integrity

20-month-old C57BL/6J mice (diet implementation at 4-month-old) were fasted from food for 4 hours prior to and both food and water 4 hours after an oral gavage of 200 uL (50 mg/mL) FITC-Dextran (4kDa; Sigma-Aldrich). Blood was collected at 0, 30, 60, 120, and 240 minutes and fluorescence intensity were measured using an excitation wavelength of 493 nm and an emission wavelength of 517 nm.

Radar Charts

Values for radar chart were calculated for percent change from AL within specific strain and sex. The distance from the center represents the effect of each restricted diet vs. AL-fed mice (no difference = 100%). Data are presented as average of diet group per sex per strain.

Statistics

Data are presented as mean ± SEM unless otherwise specified. For box-plots, center line represents the median; box limits indicate the upper and lower 25th to 75th percentile, and whiskers extend to the smallest and largest data values. Analyses were performed using Excel (2010 and 2016, Microsoft) or Prism 8 (GraphPad Software). Statistical analyses were performed using one or two-way ANOVA followed by Tukey-Kramer post hoc test specified in the figure legends. Other statistical details can also be found in the figure legends; in all figures, n represents the number of biologically independent animals. Sample sizes for longevity studies were determined in consultation with previously published power tables[79]. Sample sizes for metabolic studies were determined based on our previously published experimental results with the effects of dietary interventions[9], with the goal of having > 90% power to detect a change in area under the curve during a GTT ($p<0.05$). Data distribution was assumed

to be normal, but this was not formally tested. Sample size for molecular analyses were chosen in consultation with core facility staff and experienced laboratory personnel.

Blinding Investigators were blinded to diet groups during data collection whenever feasible, but this was not usually possible or feasible as cages were clearly marked to indicate the diet provided, mistakes, and the size and body composition of the mice was altered by strain and diet. However, blinding is not relevant to the majority of the studies conducted here, as the data are collected in numeric form, which is not readily subject to bias due to the need for subjective interpretation. Investigators were not blinded during necropsies, as the size and body composition of the mice was altered by strain and diet and group identity was therefore readily apparent.

Randomization All studies were performed on animals or tissues collected from animals. Animals of each sex and strain were randomized into groups of equivalent weight prior to the beginning of the in vivo studies.

Data availability The data that support the plots and other findings of this study are provided as a Source Data file or are available from the corresponding author upon reasonable request. RNA-sequencing data have been deposited with the Gene Expression Omnibus and are accessible through accession number GSE168262.

RESULTS

The response of C57BL/6J males to multiple feeding paradigms

We acclimated 9-week old C57BL/6J male (B6M) mice for one week to a chow based diet (Envigo Global 2018)[21]. The animals were then weighed and randomized to one of four dietary regimens (**Fig. 1A**):

1) AL, mice given *ad libitum* access to a normal rodent diet (Envigo Global 2018, **Table 1**);
2) Diluted AL, mice provided with *ad libitum* access to Envigo Global 2018 diluted 50% with indigestible cellulose (TD.170950, **Table 1**); equivalent to 30% restriction of calories without imposed fasting[8];
3) MF.cr, mice were fed 30% less food than AL-fed mice, using an automatic feeder to release food in three equal portions during the 12-hour dark period; meal feeding (MF) reduces fasting period and binging behavior;
4) CR, mice fed once per day in the morning; with 30% restriction relative to the AL group and prolonged inter-meal fasting.

The time of feeding for the CR and MF.cr diets were carefully considered. Feeding CR mice once-per-day in the morning has been widely utilized for CR studies, including by the NIA Aging Rodent Colony [23]; and is well established to extend lifespan. Furthermore, the time-of-day at which the feeding of CR mice occurs does not affect the ability of CR to extend lifespan [24]. Feeding MF.cr mice overnight during the dark cycle aligns the completion-of-feeding time of the CR and MF.cr groups; and this MF.cr regimen is similar to a regimen previously shown to extend the lifespan of mice[24].

Some of the most well-established effects of a CR diet include reduced weight gain and adiposity [7, 25, 26]. Tracking the mice on each diet regimen, we found that all three dietary restriction regimens reduced weight gain, fat mass, and adiposity (**Figs. 1B-E,**

Table 2A). Despite all three diets reducing calorie intake by 30% (**Fig. 2A**), there were clear differences between the effects of the diets. CR-fed mice gained more weight than mice fed the Diluted AL diet or the MF.cr diet (**Fig. 1B, Table 2A**). Additionally, all three groups initially lost lean (fat-free) mass to differing degrees, although lean mass eventually rebounded in CR-fed mice (**Fig. 1C, Table 2B**).

Fasting is required for CR-induced insulin sensitivity

Improved regulation of blood glucose is a conserved mammalian response to CR[1]. We examined the contribution of fasting and energy restriction on glucose homeostasis by performing glucose and insulin tolerance tests (**Figs. 1F-G, Figs. 2B-C**), timing the assays such that mice in all groups were fasted for similar lengths of time. We found that all three restricted diets improved glucose tolerance after 9 weeks on diet (**Fig. 1F**) and similarly after 13 weeks on diet (**Fig. 2B**). Surprisingly, we observed that insulin sensitivity as assessed by intraperitoneal (IP) insulin tolerance test was significantly improved only in mice fed the classic CR diet, and not in Diluted AL-fed or MF.cr-fed mice (**Fig. 1G, Fig. 2C**). Improved glucose tolerance was not due to increased insulin secretion, and calculated HOMA-IR did not differ significantly between groups (**Figs. 2D-F**). These results suggest that fasting, and not a reduction in energy intake, mediates the improved insulin sensitivity of CR-fed animals.

Distinct fuel source utilization in CR is driven by fasting

CR-fed mice engage in rapid lipogenesis following refeeding, then sustain themselves via the utilization of these stored lipids[7, 16]. We placed mice in metabolic chambers, allowing us to determine substrate utilization by examining the respiratory exchange ratio (RER). RER is calculated from the ratio of O_2 consumed and CO_2 produced; a value close to 1.0 indicates that carbohydrates are primarily utilized for energy production, while a value approaching 0.7 indicates that lipids are the predominant energy source [27]. An RER >1.0 reflects the utilization of carbohydrates for active *de novo* lipogenesis [16]. AL and Diluted AL-fed mice had *ad libitum* access to food while in the metabolic chambers; CR and MF.cr mice were fed at the same time at the beginning of the light cycle while in the chambers, as technical limitations made feeding the MF.cr mice multiple times during the dark cycle impractical. We observed that RER rapidly rose above 1.0 following refeeding, and then fell below 0.8 during the dark cycle, a fuel-source utilization pattern clearly distinct from that of AL-fed mice (**Fig. 1H).** Calculating fatty acid and carbohydrate/protein oxidation[16], we find that CR and MF.cr diets, but not the Diluted AL diet, increased fatty acid oxidation (**Fig. 1I**).

We examined the contribution of fasting and calories to energy expenditure (**Fig. 3A**), correcting for differences in lean mass (**Fig. 1J**) and body weight (**Fig. 3B**) using analysis of covariance (ANCOVA). As expected, mice consuming fewer calories had decreased energy expenditure relative to AL-fed mice; this effect was dependent on feeding regimen and not body weight. CR-fed and MF.cr-fed mice were more active immediately preceding and following feeding (**Figs. 3C-E**); however, as activity accounts for only about 10% of mouse energy expenditure [28], the decreased energy intake of these mice is likely the primary factor in the reduced energy expenditure. These results

suggest that fasting, and not a reduction in calories, is responsible for the altered fuel utilization of CR-fed animals, while the reduction in calories results in an overall decrease in exergy expenditure.

Fasting produces the distinct molecular signature of CR

We performed metabolomics on the livers of AL, CR, and Diluted AL-fed mice (**Extended Fig. 4**), targeting 59 metabolites in three broad groups based on their chemical structures – Amino Acids, Carbohydrates and Nucleoside/Nucleotides. We analyzed CR-fed mice under two feeding conditions. First, we collected tissues from CR mice that were fed 22 hours previously (CR-Fasted), while AL and Diluted AL groups were fed *ad libitum* overnight, and food was removed in the morning four hours prior to sacrifice, similar conditions as done in previous literature [21]. We also collected tissues from CR mice fed at the start of the light cycle for 3 hours prior to sacrifice (CR-Fed).

We identified 18 metabolites significantly altered in classical CR-Fasted mice as compared to compared to AL-fed mice; in contrast, we observed no differences between Diluted AL-fed mice and AL-fed mice (**Fig. 4A, Table 3**). The metabolomic signature of CR-Fasted mice was distinct from both AL and Diluted AL-fed mice, while the latter two groups overlapped (**Fig. 4B**). The metabolomic signatures of CR-Fed mice were metabolically distinct from AL-fed mice (**Figs. 4A, 4C**), suggesting that CR-fed animals have distinct liver metabolomic signatures from AL-fed mice regardless of feeding state. Nucleotides/nucleosides and metabolites involved in carbohydrate metabolism were more significantly altered in fasted than fed state, with the exception of alpha-

ketoglutarate (**Figs. 5A-B**). We found broad changes in amino acid metabolism, with leucine, ornithine and proline significantly elevated in both CR-Fasted and CR-Fed groups relative to AL-fed controls (**Fig. 5C**). Additionally, three amino acids – lysine, isoleucine and tryptophan – trended upwards in CR-Fasted mice and were significantly elevated in CR-Fed mice (**Fig. 5C**).

Intriguingly, levels of methionine and S-adenosylhomocysteine (SAH), a metabolite of SAM consuming methyltransferase reactions, were elevated in the livers of CR mice (**Fig. 4D**). Changes in levels of methionine and methionine metabolites can result in epigenetic changes, altering global histone post-translational modifications (PTM) [29, 30]. We observed changes in global histone PTMs in the livers of both Diluted AL and CR-fed mice, with the CR diet resulting in a greater number of significantly altered histone PTMs (**Fig. 6, Table 4**). CR-fed mice possessed a distinct histone PTM profile compared to both AL-fed and Diluted AL-fed mice, which were more similar to each other (**Fig. 6A-B**). Restriction of calories non-specifically increased H3 acetylation and non-specifically decreased H4 acetylation (**Fig. 6C**). Tri-methylation of H3 Lys 27 (H3K27), a modification strongly associated with chromatin silencing [31], increased in CR-fed mice; di-methylation of H3K9 decreased while acetylation of H3K9/K14 increased. Our results suggest that prolonged fasting, not simply a reduction of calorie intake, is required for the epigenetic effects of a CR diet.

We also conducted targeted metabolomics in skeletal muscle [32-37] (**Fig. 7, Table 5**). We found many metabolites were altered in both CR-fed mice and Diluted-AL-fed mice as compared to AL-fed controls (**Fig. 7A**). CR-fed mice had a distinct metabolomic signature from AL-fed mice; however, unlike in the liver, Diluted AL-fed mice

had a distinct signature from AL-fed mice (**Fig. 7B**). CR and Diluted AL-fed mice had similar changes in multiple TCA-cycle metabolites with the exception of Malate and 2-OHG. Although prolonged fasting is required to produce the distinct metabolomic signature of CR in both the liver and skeletal muscle, reduction of calories also affects the skeletal muscle metabolome.

Sex and strain response to fasting and calories

Sex and genetic background impact the response to CR in mice [21, 22, 26]; to determine if the distinct roles of calorie and fasting we observed in B6M was conserved across different sexes and strains, we examined female C57BL/6J (B6F) mice, and male and female DBA/2J (D2M and D2F) mice (**Extended Figs. 8-10**). These strains have different metabolic responses to once-daily CR, but CR extends the lifespan of both strains and sexes [21].

All three dietary regimens had similar effects on the body composition of B6M and D2M mice (**Figs. 1B-E and Figs. 9B-E**). While female B6F and female D2F mice gained less weight on all of the reduced calorie regimens, Diluted AL-fed female mice did not gain fat mass nor adiposity (**Figs. 8A-D, 10A-D**), suggesting there may be a sexually dimorphic effect of fasting (or the lack thereof) on fat storage. Glucose tolerance was improved by the reduced calorie regimens in all sexes and strains, with the exception of B6F fed the classical CR diet (**Fig. 1F, Figs. 8F, 9F, 10F and Fig. 11A**). While the non-responsiveness of B6F was somewhat surprising, these findings were in line with our

previous observation that the effect of CR on glucose tolerance is stronger in young males than in young female mice [7]. This was not due to increased insulin secretion (**Fig. 12**).

Insulin sensitivity as assessed by insulin tolerance test using 0.5U/kg insulin was significantly improved only in B6M fed the classic CR diet, and B6F fed either the classic CR diet or the MF.cr diet (**Fig. 1G, Fig. 8G and Fig. 11B).** We did not observe improved insulin sensitivity in response to any of the restricted diets for DBA/2J mice of either sex under these conditions (**Figs. 9G and 10G**), exemplifying the genetic variation in response to insulin between strains [38]. As these mice were less responsive to insulin, after 14 weeks on diet, we challenged both male and female DBA/2J with a higher dose of insulin (0.75U/kg) in order to uncover differences between the diet regimens. We observed improved insulin sensitivity in D2F mice fed the classic CR diet, but not in D2M mice (**Fig. 11B**). In all sexes and strains, mice fed the Diluted AL diet did not have significantly improved insulin sensitivity, despite the reduction in caloric intake. Interestingly, Diluted AL group had the smallest HOMA-IR value for B6F and D2M (**Fig. 12**). These data demonstrate that sex and strain impact the effect of different CR regiments on glucose homeostasis.

Using metabolic chambers, we determined the RER and energy expenditure and calculated substrate utilization of all sexes, strains, and diet groups. All mice fed a classic CR diet had similar RER patterns to that of B6M, rapidly rising above 1.0 following refeeding and then falling to ~0.7-0.8 during the dark cycle (**Fig. 1H and Extended Figs. 8H, 9H, 10H**). While all mice fed the MF.cr diet had similar RER curves to classic CR-fed mice, all mice fed the Diluted AL diet had RER curves that overlapped those of the AL group (**Extended Figs. 8H, 9H, 10H**). As in B6M, CR and MF.cr diets increased fatty acid

oxidation in D2M and D2F mice; B6F fed either one of these diets had FA oxidation indistinguishable from AL-fed controls (**Fig. 1I, Extended Figs. 8I, 9I, 10I**), suggesting that the strain and sex specific responses to CR regimens extends to fat metabolism. In contrast, fatty acid oxidation was not increased in Diluted AL-fed mice of any sex or strain.

All mice consuming fewer calories had decreased energy expenditure relative to their AL-fed counterparts; this effect was independent of both fat-free (lean) mass and body weight (**Fig. 1J, Extended Figs 8J, 9J, 10J, and Fig. 13**).While all restricted groups of C57BL/6J and DBA/2J males had similar energy expenditure, female mice of both strains fed the Diluted diet had lower energy expenditure compared to both CR and MF.cr-fed mice (**Extended Figs. 8J, 9J**). These results suggest that fasting is responsible for the altered fuel utilization of CR-fed animals, while the reduction in calories is responsible for the decreased exergy expenditure.

The physiological responses to these restricted paradigms were differentially regulated by strain and sex. To visualize the overall response, we plotted 17 phenotypes calculated relative to the AL Control. We first plotted how each individual sex/strain of mice responded to the three dietary regimens (**Fig. 14A-D**); secondly, we plotted how all four strains responded to each individual diet **(Fig. 14E-G**). To identify statistically significant interactions of sex, strain and diet, we performed multivariate statistical analyses on the 17 phenotypes. We used two-way ANOVA corrected for multiple comparisons by false discovery rate (FDR) using two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli; the q values (FDR) and individual P values are shown in **Table 6**. The majority of the phenotypic outcomes were both strain and sex dependent. In particular, D2F exhibited higher insulin sensitivity than D2M, and B6F exhibited higher

insulin sensitivity than D2F. For fatty acid oxidation there was a sex-diet interaction for DBA/2J mice, and a strain-diet interaction for both sexes. When total energy expenditure was evaluated against each strain and sex with ANCOVA, there were only strain differences for AL, Diluted AL, and MF.cr-fed mice. However, there were both strain and sex interactions for CR-fed mice.

Overall, there was little difference with strain or sex when only calories were restricted (**Fig. 14H**). As fasting duration increased, we observed an increasing number of sex and strain differences (**Figs. 14I-J**). Our data support the idea that each sex and strain respond to a CR diet uniquely as a result of sex and strain-specific responses to prolonged fasting.

Fasting recapitulates the metabolic effects of a CR diet

We next wanted to examine the possibility that fasting is sufficient to recapitulate the metabolic and molecular effects of a classical once-per-day CR diet. We designed a fifth feeding paradigm, TR.al, in which B6M mice were entrained to consume approximately the same quantity of food as *ad libitum* fed mice within 3 hours (**Fig. 3A, Extended Fig. 9A**), following which they were fasted for the remaining 21 hours of each day.

We found that both classical CR-fed mice and TR.al-fed mice had reduced overall weight and fat mass gain during the 16 weeks of the study, despite the similar calorie intake of AL and TR.al-fed mice (**Fig. 15B, Figs. 16A-E, and Table 7**). Additionally, TR.al-fed mice had improved glucose tolerance and insulin sensitivity after 9-10 weeks (**Figs.**

15C-D). These early improvements in glucose homeostasis were independent of significant difference in weight and adiposity, which only diverged after 12 weeks on diet (**Figs. 16B-E**). Glucose tolerance and insulin sensitivity were similarly improved after 13-14 weeks on the diets (**Figs. 16F-G**), and was not the result of increased insulin secretion (**Figs. 16H-J**). These results demonstrate that prolonged fasting is sufficient to improve body composition, glucose tolerance and insulin sensitivity.

We utilized metabolic chambers to determine substrate utilization and energy expenditure. As previously shown, we found that classic CR-fed mice exhibited two distinct phases of fuel selection following feeding, with high lipogenesis (RER > 1.0) following feeding, and dependence upon lipid oxidation (RER < 0.8) during the dark cycle, and an overall increase in FA oxidation (**Figs. 15E-F**). TR.al mice were essentially indistinguishable from classic CR-fed mice, with overlapping RER curves and similar increases in FA oxidation (**Figs. 15E-F**). The CR and TR.al groups both demonstrated decreased total energy expenditure relative to AL-fed mice that was independent of lean (fat-free) mass and body weight (**Fig. 17**).

Almost all of the metabolic phenotypes of CR-fed mice are also observed in TR.al-fed mice (**Fig. 15G**). Additionally, when comparing all the feeding paradigms examined for B6M mice, we observed that only mice subject to prolonged fasting, the CR and TR.al groups, showed improved insulin sensitivity, while any length of fasting was sufficient to increase FA oxidation (**Fig. 15H**). Together, these results demonstrate that fasting recapitulates the physiological response to CR, and is both necessary and sufficient for the metabolic response to CR.

Fasting recapitulates the molecular effects of a CR diet

We next performed transcriptomic profiling of liver and inguinal white adipose tissue (iWAT) of mice fed either an AL, CR, or TR.al diet. We identified a total of 2,700 genes in the liver and more than 1,800 genes in iWAT that were differentially expressed ($q < 0.05$) in either CR or TR.al-fed mice relative to AL-fed mice (**Fig. 18A-C, Tables 8-9**). A large fraction of these genes was altered in the same direction by both CR and TR.al-feeding (**Fig. 18B**); and over 90% of the differentially expressed genes (DEGs) were the same in iWAT and liver of CR and TR.al animals (**Fig. 18C**).

We next took an unbiased approach in identifying functionally enriched pathways by constructing gene-set networks with NetworkAnalyst[39-43]. DEGs were assigned as "seed" proteins and pathways were constructed with the known interactions of the seed protein with other proteins curated from a large protein-protein interaction (PPI) database (**Figs. 18D-E**). From this we identified likely candidate pathways mediating these responses by limiting pathway hits with enrichment p-values of <0.05 which were calculated with the hypergeometric test – enrichment analysis test that calculates for significant overlap between genes and ranked by their p-value.

More pathways were altered in iWAT than in the liver. We identified 25 upregulated pathways in iWAT that were functionally enriched in tissues from both CR and TR.al-fed mice (**Fig. 18F**). In liver, we identified 5 upregulated and 5 downregulated pathways that were functionally enriched by both diets (**Fig. 18G**). Additionally, some pathways were enriched in tissues for both CR and TR.al-fed mice, but reached statistical significance only for one diet, such as mTOR signaling pathway in iWAT (**Fig. 18F**) or PPAR signaling in liver (**Fig. 18G**). Lastly, we examined the similarities of enriched pathways between

iWAT and liver. We identified six pathways upregulated in both tissues by both CR and TR.al diets – “Insulin signaling pathway”, “PPAR signaling pathway”, “Fatty acid biosynthesis”, “Circadian rhythm”, “Metabolic pathways”, and “Fatty acid metabolism” (**Fig. 18F-G**). We also identified two additional pathways that were upregulated in both tissues by TR.al only – “TGFβ signaling pathway” and “Longevity Regulating Pathway” (**Fig. 18F-G**). Finally, we found two pathways that were regulated in opposite directions in liver and iWAT by a TR.al diet – “Signaling pathways regulating pluripotency of stem cells” and “MAPK signaling pathway” (**Fig. 18F-G**).

Fasting is necessary for CR to improve health and longevity

In order to understand the requirement for fasting in the effects of a CR diet on aging, we placed 20-week-old C57BL/6J male mice on either AL, Diluted AL or CR diets (**Fig. 19A**), and measured weight, body composition, glucose homeostasis, gut integrity, and frailty as the animals aged (**Figs. 19 and Fig. 20**). As we expected, mice fed the classical CR diet ceased to gain weight, fat mass, and lean mass, and maintained a lower adiposity than AL-fed controls (**Fig. 19B**). In contrast, Diluted AL-fed mice had an initial reduction in body weight and fat mass and started to steadily lose lean mass at 9 months of age (**Fig. 19B, Fig. 20A, and Table 10**). To determine if cellulose in the Diluted AL diet impairs the absorption of energy-yielding nutrients, we assessed the gross energy in diet consumed and feces excreted by bomb calorimetry of 19-month old mice. Diluted AL-fed mice ate more and had increased fecal output; when accounting for the amount of cellulose consumed, Diluted AL-fed mice absorbed digestible macronutrients similarly to AL-fed mice (**Fig. 20B**). We additionally checked if gut barrier integrity were affected by

the Diluted AL diet with fluorescein isothiocynate-dextran (FITC-Dextran 4kDa) in 20-month-old mice. While Diluted AL-fed animals had the same level of permeability as the AL, CR-fed animals had improved gut barrier integrity (**Fig. 20C**).

We assessed glucose and insulin tolerance as the mice aged; both diets improved glucose tolerance (**Figs. 19C and 19E**). In contrast, insulin sensitivity was only improved in the classical CR-fed group (**Figs. 19D and 19F**). As in young animals, classical CR-fed mice displayed a distinctive RER curve, with a rapid induction of lipogenesis (RER > 1.0) following refeeding, and a low RER throughout the night (**Fig. 19G**). We calculated that CR-fed mice utilized fatty acids as a fuel source significantly more throughout the day compared to other groups (**Fig. 19H**). CR and Diluted AL groups both demonstrated decreased total energy expenditure relative to AL-fed mice that was independent of non-fat mass or total body weight with no significant differences in activity between groups (**Fig. 21**).

We assessed the frailty of AL, Diluted AL, and CR-fed mice as they aged. As expected [7, 44], frailty was significantly lower in CR-fed mice as they aged (**Fig. 21A**). In contrast, frailty was not reduced in Diluted AL-fed mice (**Fig. 21A**). Intriguingly, the specific deficits of AL-fed and Diluted AL-fed mice that contributed to their equivalent frailty varied, with Diluted AL-fed mice developing kyphosis, and AL mice having declining grip strength and body composition (**Figs. 21B-E**). The quality of the coat condition and fur color were diminished in both AL and Diluted AL mice, while CR mice retained a healthy coat (**Figs. 21F-G**). Additionally, we examined if fasting was required for cognition and memory by testing novel object recognition [45]. Short-term memory was not improved in either Diluted AL-fed or CR-fed mice; however, classic once-per-day CR

feeding, but not a Diluted AL diet, improved long-term memory (**Figs. 21H-I**), suggesting that fasting may play a role in the effects of a CR diet on cognition.

Finally, we analyzed the survival of the mice on the three different diets as they aged. While classic once-per day CR feeding extended lifespan by about 20% (AL vs CR, median lifespan 850 vs 1022, $p < 0.0001$ vs AL, log-rank sum test), consumption of the Diluted AL diet decreased lifespan by 9% (AL vs. Diluted AL, median lifespan 850 vs. 776 days, $p < 0.0001$ vs. AL, log-rank sum test) (**Fig. 21J and Table 11**). Additionally, CR delayed the onset of cancer compared to AL, while the incidence of cancer in Diluted AL-fed mice was low, perhaps due to their shorter lifespan (**Fig. 21K**). In combination, our data suggest that fasting is required for the geroprotective effects of CR on frailty, cognition, and lifespan (**Fig. 21L**).

DISCUSSION

The mechanisms by which CR promotes healthspan and longevity have remained elusive for decades. Most CR studies have overlooked the fact that a traditional once-per-day feeding regimen alters feeding behavior[15, 16]. Meal feeding, which imposes a fasting period, was recently shown to extend lifespan without restricting caloric intake[20, 46], which suggested to us that the fasting period collaterally imposed by a conventional once-per-day CR feeding regimen might be a critical and hitherto overlooked physiological mechanism contributing to the effects of CR.

We tested this hypothesis by using a novel series of feeding paradigms. We found that a reduction of calories without the imposition of a prolonged fast improves glucose

tolerance and body composition. However, prolonged fasting was necessary for CR to improve insulin sensitivity, a key physiological hallmark of the CR response in mammals; alter fuel utilization patterns and increase fatty acid oxidation; and reduce age-related frailty[6, 7, 16, 44, 47, 48]. Of note, we assessed insulin sensitivity via I.P. administration of insulin, which most directly measures insulin-stimulated glucose uptake; future research using clamps may be useful in thoroughly assessing the insulin sensitivity of other tissues including the liver.

C57BL/6J male mice in which calorie intake was reduced without fasting via *ad libitum* feeding of a low-energy diet did not show the anticipated reduction in age-related frailty, and had a reduced lifespan. Our findings align with the results of other labs[8], and with the results of our molecular analysis of the liver, which likewise showed that restriction of calories without fasting had a distinct and muted impact on metabolites and histone PTMs as compared to mice fed CR once-per-day. We observed changes in multiple methionine metabolites, and while it is difficult to directly compare metabolomics data across studies, other groups have seen similar changes [21, 46, 49-52]. In agreement with another study of mice fed the same chow we used [21, 46], we observed that CR increased levels of leucine and isoleucine in the liver.

Our use of a diet diluted with indigestible cellulose (Diluted AL) is a limitation of the present work, as the large bulk of fiber in this diet may have affected the gut microbiome and impacted the metabolic effects we observed. While the diet did not affect macronutrient absorption or gut integrity, absorption of specific micronutrients was not investigated. We examined the effect of a more “normal” CR diet, delivered in multiple meals, (MF.cr), only in our short-term studies. Future studies examining different MF.cr

lengths in longer studies, testing multiple feeding times, and examining macro- and micro-nutrient absorption will permit a more complete understanding of how the length of time between meals contributes to the effects of a CR diet. This may also clarify why some studies report positive effects of cellulose-diluted diets on lifespan [53], and explain why dilution of specific macronutrients extends the lifespan of other model organisms [14, 54-56].

Not only is a fasting period necessary, but the imposition of a prolonged daily fast without reduced calories recapitulates the metabolic benefits and molecular effects of a CR diet in C57BL/6J male mice. Fasting improves glucose tolerance and insulin sensitivity, reduces adiposity, and increases fatty acid oxidation. At the molecular level, fasting and traditional CR had extremely similar and overlapping effects on gene expression, with high similarity in the pathways altered by fasting and CR. Fasting almost completely recapitulates the effects of CR in both liver and iWAT, both in terms of differentially expressed genes and in KEGG pathways. Notably, many of the KEGG pathways identified as altered by both fasting and CR have been previously implicated in the metabolic and geroprotective effects of a CR diet; these include PPAR, insulin, TGFβ and AMPK signaling, as well as multiple metabolic pathways involved in amino acid, carbohydrate, and fatty acid metabolism. Similar changes in amino acid metabolism, PPAR expression, TGFβ and insulin signaling have been found in recent metabolomic and transcriptomic studies of the effects of graded levels of CR [49, 57, 58]. Intriguingly, Circadian rhythm was identified as a significantly altered pathway upregulated by both CR and fasting in iWAT, and by CR in the liver. We speculate that the daily fasting of mice on a classical CR diet may promote health and longevity in part through the circadian

synchronization of metabolic processes [59]. Taken together, our data demonstrates that prolonged fasting is sufficient to recapitulate the majority of the effects of CR at the molecular level in both liver and iWAT. Additional research will be required to determine the role of these various pathways, as well as circadian regulators, in the response to prolonged fasting and CR.

Our study was subject to limitations. First, while we performed our initial sufficiency studies in multiple strains and sexes of mice, our metabolomic and epigenetic analysis was limited to C57BL/6J male mice on just three dietary regimens. As the effects of dietary composition and CR are sex and strain dependent [21, 22, 60], investigating the effects in multiple sexes and strains is critical. Our detailed studies of the effects of fasting alone were confined to C57BL/6J males, as this group showed the greatest metabolic response to a CR diet; and we focused on just a few diets for logistical reasons. Finally, TR.al-fed mice had a small reduction in calorie intake relative to AL-fed mice. We consider it unlikely that this contributes significantly to the effects of a TR.al diet, but follow-up studies should minimize or eliminate this difference. Additional research will be required to determine if fasting is sufficient to recapitulate the metabolic, molecular and geroprotective effects of a CR diet in multiple strains and sexes as well as in genetically heterogeneous mice.

Our results are in broad agreement with a series of studies over the last decade that have demonstrated that a prolonged inter-meal interval has significant health benefits. Restricting feeding to 8-10 hours per day protects mice from diet-induced obesity and insulin resistance without reducing calorie intake, perhaps by promoting regular daily rhythms in fasting and feeding [18, 61]. In pre-diabetic humans, a recent randomized clinical trial found that restricting feeding to 6 hours per day improved insulin sensitivity

and beta cell function without a reduction in calories or weight loss [62]. Several decades ago one study found that the time of delivery of a once-per-day classical CR diet, or delivery of a CR diet in multiple meals limited to the dark period, had similar effects on the lifespan of mice[24]. It was recently shown that single-meal feeding of mice, resulting in an approximately 11-hour fast between meals, extends lifespan and healthspan, although to a lesser extent than a once-per-day classical CR diet [20]. Conversely, mice fed a low-energy density diet *ad libitum* spend more time eating than mice fed higher energy-density diets *ad libitum*, and as we also observed in the present study, have a shorter lifespan[8]. Our results, which demonstrate that prolonged daily fasting without reduced calorie intake is sufficient to recapitulate both the metabolic impact and molecular profile of a CR diet, are in agreement with these results, and clearly demonstrate that a prolonged inter-meal interval can benefit both metabolic health and longevity.

CR extended the lifespan of non-human primates in a study conducted at the University of Wisconsin-Madison (UW)[63], but did not extend lifespan in a study conducted at the National Institute on Aging (NIA)[64]. Notably, NIA control animals were long-lived compared to any data in captivity, possible due to the relatively low caloric intake of these animals. In both studies, when accounting for food intake and weight, animals that ate less and weighed less had increased lifespan. Other differences between the UW and NIA studies includes the age at which CR was initiated and diet composition[65]. There were also differences in feeding behavior, with animals at UW animals fed one main meal in the morning with a smaller snack in the afternoon[66]; as they rapidly consumed both the meal and the snack in under an hour (personal communication, Dr. Ricki Colman), the animals spent the majority of the day in the fasted

state. In contrast, NIA animals were fed twice a day[64]. These conditions may be comparable to the CR and MF.cr groups in our study. While there are improved biomarkers of health in humans subjected to CR [3], humans adhering to a CR diet typically eat multiple meals per day. If our findings apply to humans, sharply limiting the portion of the day during which food is consumed may maximize the healthspan and longevity benefits of CR and may promote healthy aging without requiring a reduction in calorie intake.

Time-restricted feeding studies suggest this may be case[67, 68], although caution is warranted in applying these results to humans. In mice, once-per-day CR feeding performed early in the dark cycle or early in the light cycle has equivalent effects on lifespan[24]. However, in humans, skipping breakfast is associated with an increased risk of atherosclerosis and mortality[69, 70]. In humans eating one isocaloric meal per day, weight declined in a breakfast-only group while weight increased in a dinner-only group[71]. Other studies have observed beneficial effects of once-per-day evening feeding on fat mass, but negative effects on cholesterol, blood pressure, and glucose control in healthy adults [72, 73], and reduced weight and blood glucose control in adults with type 2 diabetes fed in the midafternoon or later [74]. Understanding how when we eat, and not just how much we eat, and its impact on metabolic health and longevity in humans is clearly an important area for future research.

Our work challenges long-standing assumptions about CR, and as summarized in **Figure 23**, we find that collaterally imposed fasting is required for the metabolic, molecular, and geroprotective effects of a CR diet. Our study has important implications for research into the mechanisms which underlie the effect of CR; for example, this may

explain in part why different methods of dietary restriction in worms and flies rely on different molecular pathways [55]. Re-imagining model systems to incorporate periodic fasting may provide valuable new insights into how CR works. Additionally, while restricting calories has long been viewed as unsustainable, fasting is incorporated in many fad diets and religious traditions. If these results apply to humans, fasting alone may be able to recapitulate the benefits of a CR diet without the side effects. In conclusion, our work demonstrates that daily prolonged fasting has powerful health benefits and underlies many benefits of a CR diet in mice, and that while “you are what you eat,” it is equally true that “you are when you eat.”

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COMPETING INTERESTS

D.W.L has received funding from, and is a scientific advisory board member of, Aeovian Pharmaceuticals, which seeks to develop novel, selective mTOR inhibitors for the treatment of various diseases. J.M.D. is a consultant for FORGE Life Sciences and co-founder of Galilei Bio-Sciences. The remaining authors declare no competing interests.

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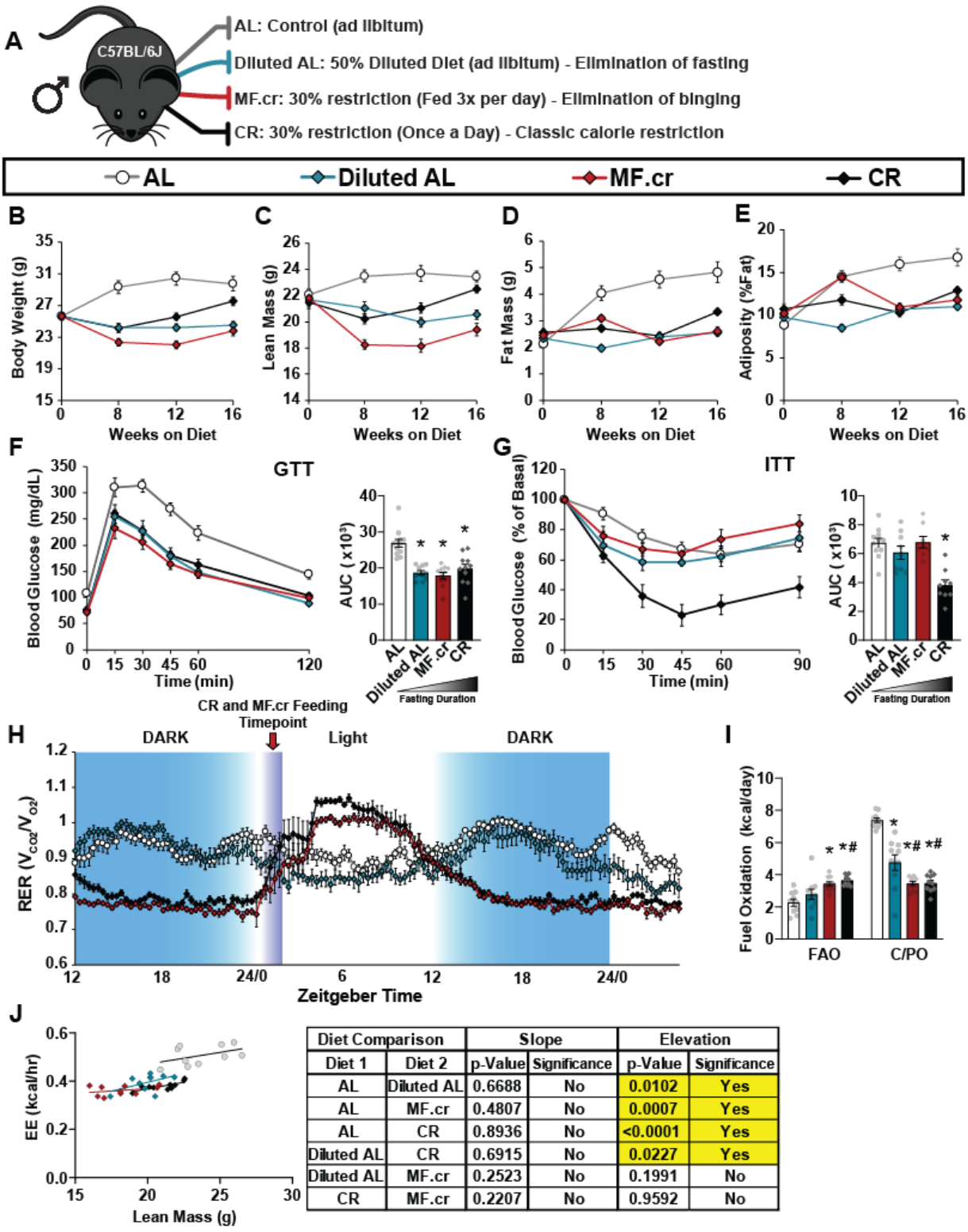


Diet Comparison		Slope		Elevation	
Diet 1	Diet 2	p-Value	Significance	p-Value	Significance
AL	Diluted AL	0.6688	No	0.0102	Yes
AL	MF.cr	0.4807	No	0.0007	Yes
AL	CR	0.8936	No	<0.0001	Yes
Diluted AL	CR	0.6915	No	0.0227	Yes
Diluted AL	MF.cr	0.2523	No	0.1991	No
CR	MF.cr	0.2207	No	0.9592	No

Figure 1: Prolonged fasting is required for the CR-mediated increase in insulin sensitivity and alterations in fuel source selection in male C57BL/6J mice. (A) Outline of feeding regimens: AL, Diluted AL, CR and MF.cr. (B-E) Body composition measurement over 16 weeks on diet (n = 10 biologically independent mice per diet); total body weight (B), lean mass (C), fat mass (D) and adiposity (E). (F-G) Glucose (AL, n = 12; Diluted AL, n = 10; MF.cr, n = 11; CR, n = 11 biologically independent mice) (F) and insulin (AL, n = 11; Diluted AL, n = 9; MF.cr, n = 8; CR, n = 9 biologically independent mice) (G) tolerance tests after 9 or 10 weeks on the indicated diets. * symbol represents a significant difference versus AL-fed mice ($P<0.0001$) based on Tukey's test post one-way ANOVA. (H-J) Metabolic chamber analysis of mice fed the indicated diets. (H) Respiratory exchange ratio vs. time (n = 10 biologically independent mice per diet) (I) Fuel utilization was calculated for the 24-hour period following the indicated (arrow) refeeding time (n = 10 biologically independent mice per diet). * symbol represents a significant difference versus AL (Diluted AL, $p <0.0001$; MF.cr, $p \leq 0.0018$; CR, $p \leq 0.0002$); # symbol represents a significant difference versus Diluted AL (MF.cr, $p = 0.0097$; CR, $p \leq 0.0283$) based on Tukey's test post one-way ANOVA performed separately for FAO and C/PO). (J) Energy expenditure as a function of lean mass was calculated for the 24-hour period following the indicated (arrow) refeeding time (n = 10 mice per diet, data for each individual mouse is plotted; slopes and intercepts were calculated using ANCOVA). All data are represented as mean ± SEM.

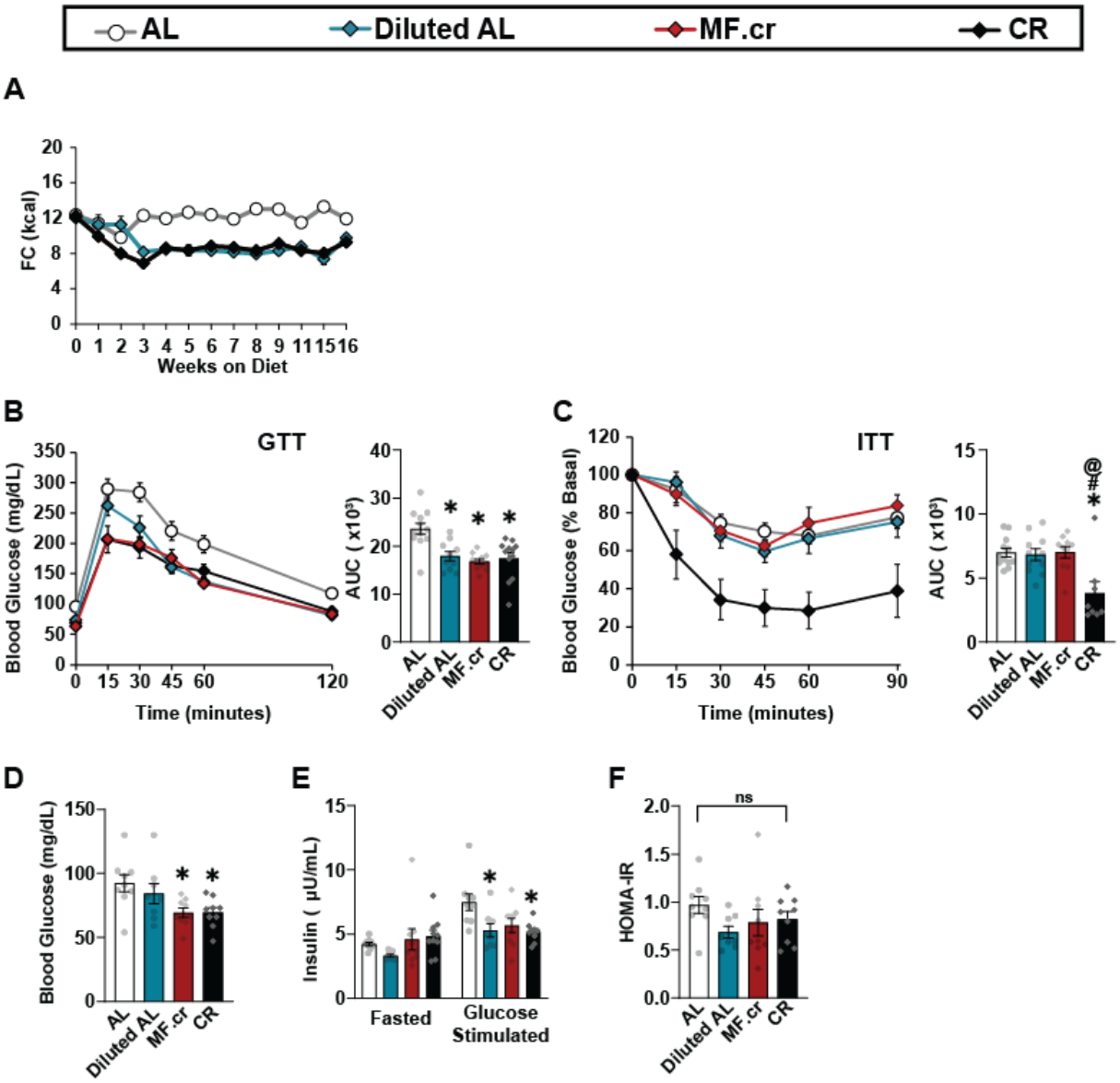
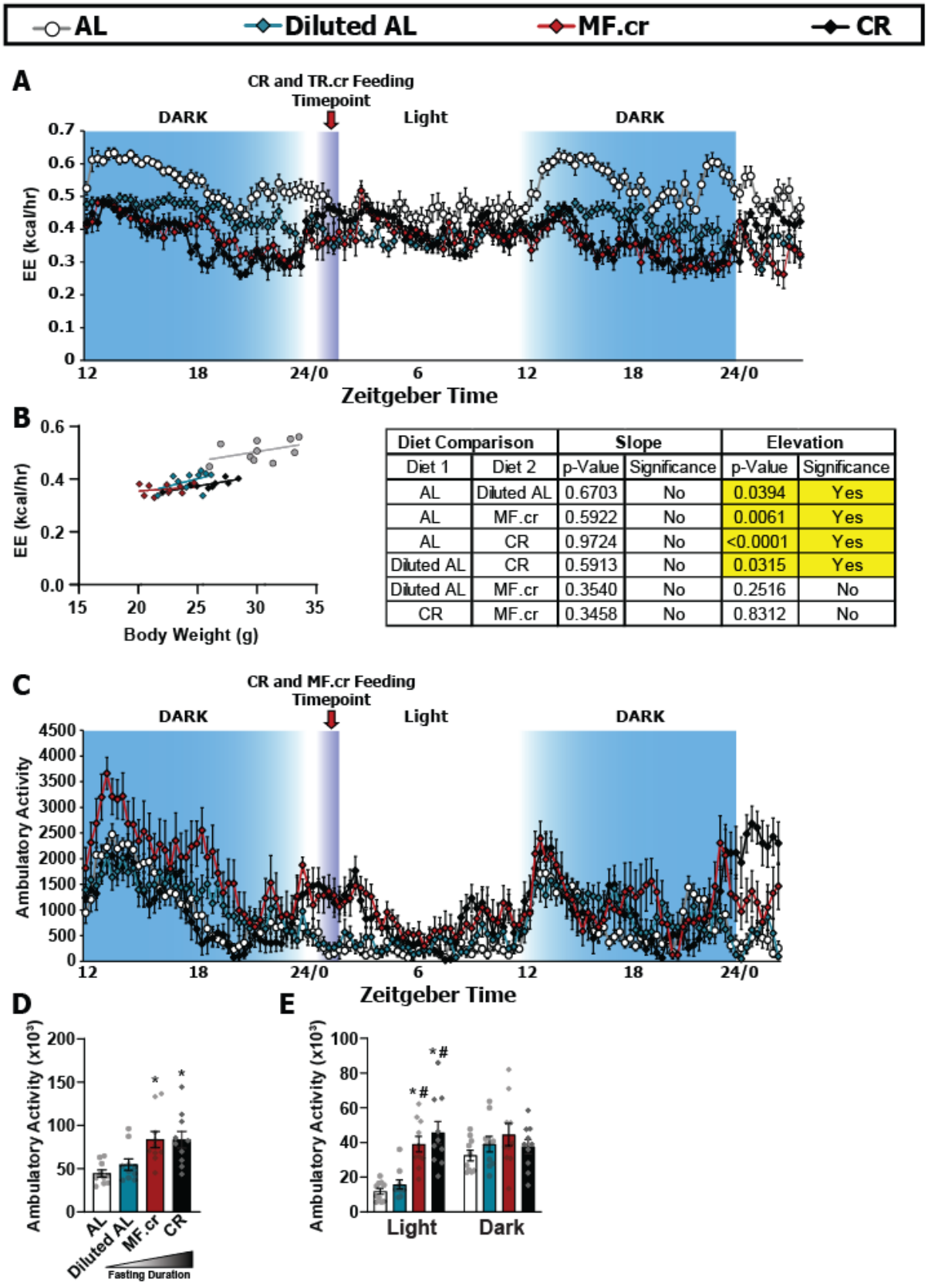

AL
Diluted AL
MF.cr
CR
A
FC (kcal)
Weeks on Diet
B
GTT
Blood Glucose (mg/dL)
Time (minutes)
AUC (x10³)
C
ITT
Blood Glucose (% Basal)
Time (minutes)
AUC (x10³)
D
Blood Glucose (mg/dL)
E
Insulin (µU/mL)
Fasted
Glucose Stimulated
F
HOMA-IR
ns

Figure 2: Additional measures of glucose homeostasis in male C57BL/6J mice.

(A) Food Consumption (B) Glucose (C) and insulin (tolerance tests after 13 or 14 weeks on the indicated diets (n = 10-12 mice per diet; * = $p < 0.05$, Tukey's test post one-way ANOVA). (D-F) Fasting blood glucose (D), fasting and glucose-stimulated insulin secretion (15 minutes) (E), and calculated HOMA-IR (F). (C-E) n = 8-10 mice per group; * = $p < 0.05$ vs. AL, Tukey's test post ANOVA; insulin levels in fasted and glucose-stimulated states were analyzed separately. Data are represented as mean ± SEM.



Diet Comparison		Slope		Elevation	
Diet 1	Diet 2	p-Value	Significance	p-Value	Significance
AL	Diluted AL	0.6703	No	0.0394	Yes
AL	MF.cr	0.5922	No	0.0061	Yes
AL	CR	0.9724	No	<0.0001	Yes
Diluted AL	CR	0.5913	No	0.0315	Yes
Diluted AL	MF.cr	0.3540	No	0.2516	No
CR	MF.cr	0.3458	No	0.8312	No

Figure 3. Additional metabolic chamber data for C57BL/6J male mice. (A-E) Metabolic chamber analysis of mice fed the indicated diets. (A) Energy expenditure vs. time. (B) Energy expenditure as a function of total body mass was calculated for the 24 hour period following the indicated refeeding time (C) Ambulatory activity vs. time (D) Sum of ambulatory activity was calculated for the 24 hour period following the indicated refeeding time (E) Sum of ambulatory activity during the light and dark cycle calculated for the 24 hour period following the indicated refeeding time (n = 10-12 mice per diet, data for each individual mouse is plotted; slopes and intercepts were calculated using ANCOVA). Data are represented as mean ± SEM.

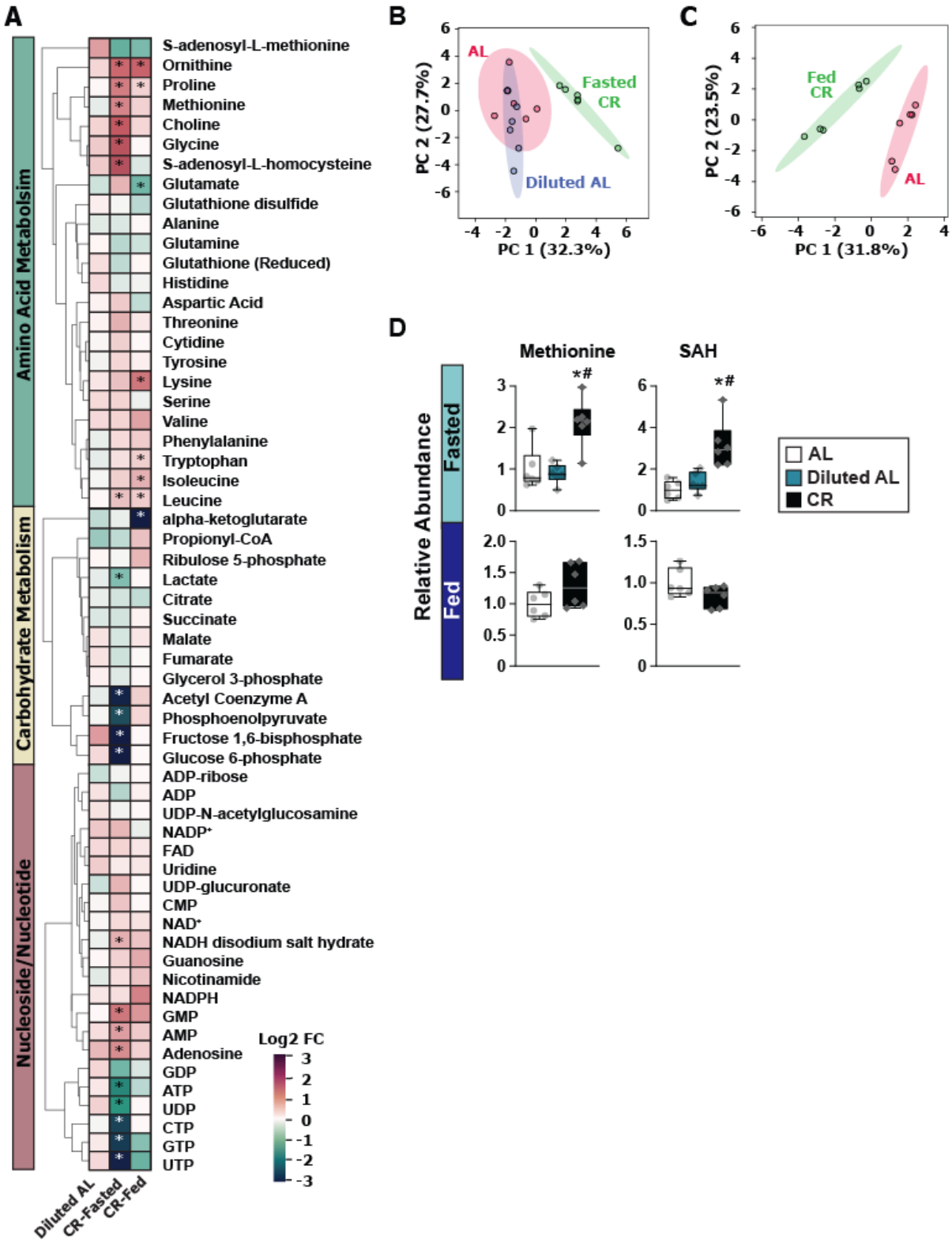

A
Amino Acid Metabolism
Carbohydrate Metabolism
Nucleoside/Nucleotide
S-adenosyl-L-methionine
Ornithine
Proline
Methionine
Choline
Glycine
S-adenosyl-L-homocysteine
Glutamate
Glutathione disulfide
Alanine
Glutamine
Glutathione (Reduced)
Histidine
Aspartic Acid
Threonine
Cytidine
Tyrosine
Lysine
Serine
Valine
Phenylalanine
Tryptophan
Isoleucine
Leucine
alpha-ketoglutarate
Propionyl-CoA
Ribulose 5-phosphate
Lactate
Citrate
Succinate
Malate
Fumarate
Glycerol 3-phosphate
Acetyl Coenzyme A
Phosphoenolpyruvate
Fructose 1,6-bisphosphate
Glucose 6-phosphate
ADP-ribose
ADP
UDP-N-acetylglucosamine
NADP+
FAD
Uridine
UDP-glucuronate
CMP
NAD+
NADH disodium salt hydrate
Guanosine
Nicotinamide
NADPH
GMP
AMP
Adenosine
GDP
ATP
UDP
CTP
GTP
UTP
Log2 FC
3
2
1
0
-1
-2
-3
Diluted AL
CR-Fasted
CR-Fed
B
AL
Fasted CR
Diluted AL
PC 2 (27.7%)
PC 1 (32.3%)
C
Fed CR
AL
PC 2 (23.5%)
PC 1 (31.8%)
D
Relative Abundance
Fasted
Fed
Methionine
SAH
*#
AL
Diluted AL
CR

Figure 4: Fasting is required for CR-mediated reprogramming of the hepatic metabolome. Targeted metabolomics were performed on the livers of male C57BL/6J mice fed AL, Diluted AL and CR diets (n = 6 mice per group). A) Heatmap of 59 targeted metabolites, represented as log_2-fold change vs. AL-fed mice. B) sPLS-DA of liver metabolomics with CR mice sacrificed in the fasted state. C) sPLS-DA of liver metabolomics with CR sacrificed in the fed state. D-E) Relative abundance of D) methionine and its metabolite S-Adenosyl-Homocysteine (* = $p < 0.05$, Tukey's test post one-way ANOVA).

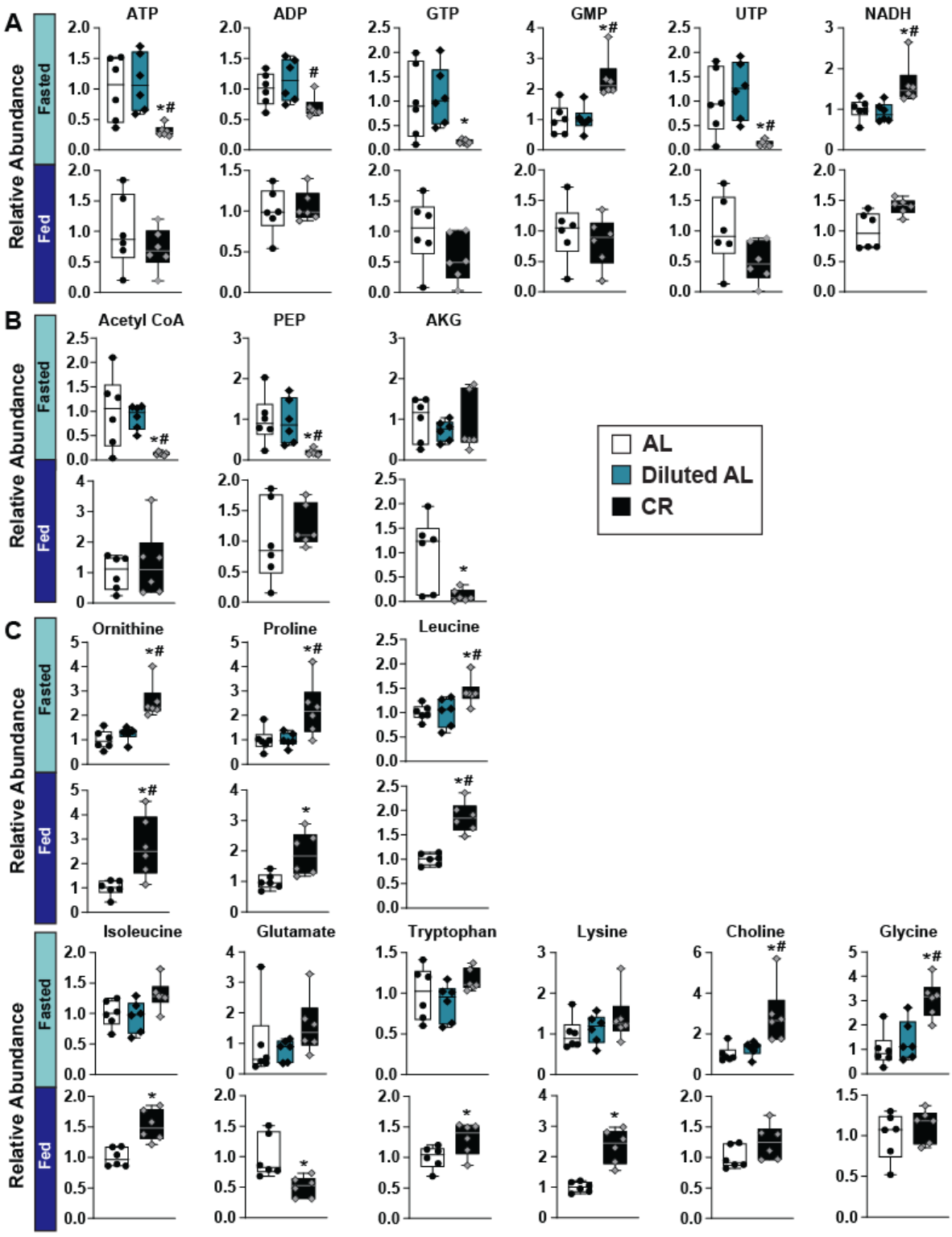

A
B
C
Relative Abundance
Fasted
Fed
ATP
ADP
GTP
GMP
UTP
NADH
Acetyl CoA
PEP
AKG
Ornithine
Proline
Leucine
Isoleucine
Glutamate
Tryptophan
Lysine
Choline
Glycine
AL
Diluted AL
CR

Figure 5: Additional hepatic metabolome data. Hepatic metabolites that showed statistical difference during the fasted or fed state (n = 6 mice per group). A) Relative abundance of nucleotide/nucleoside metabolites B) Relative abundance of TCA cycle metabolites C) Relative abundance of amino acid metabolites (* = $p < 0.05$, Tukey's test post one-way ANOVA).

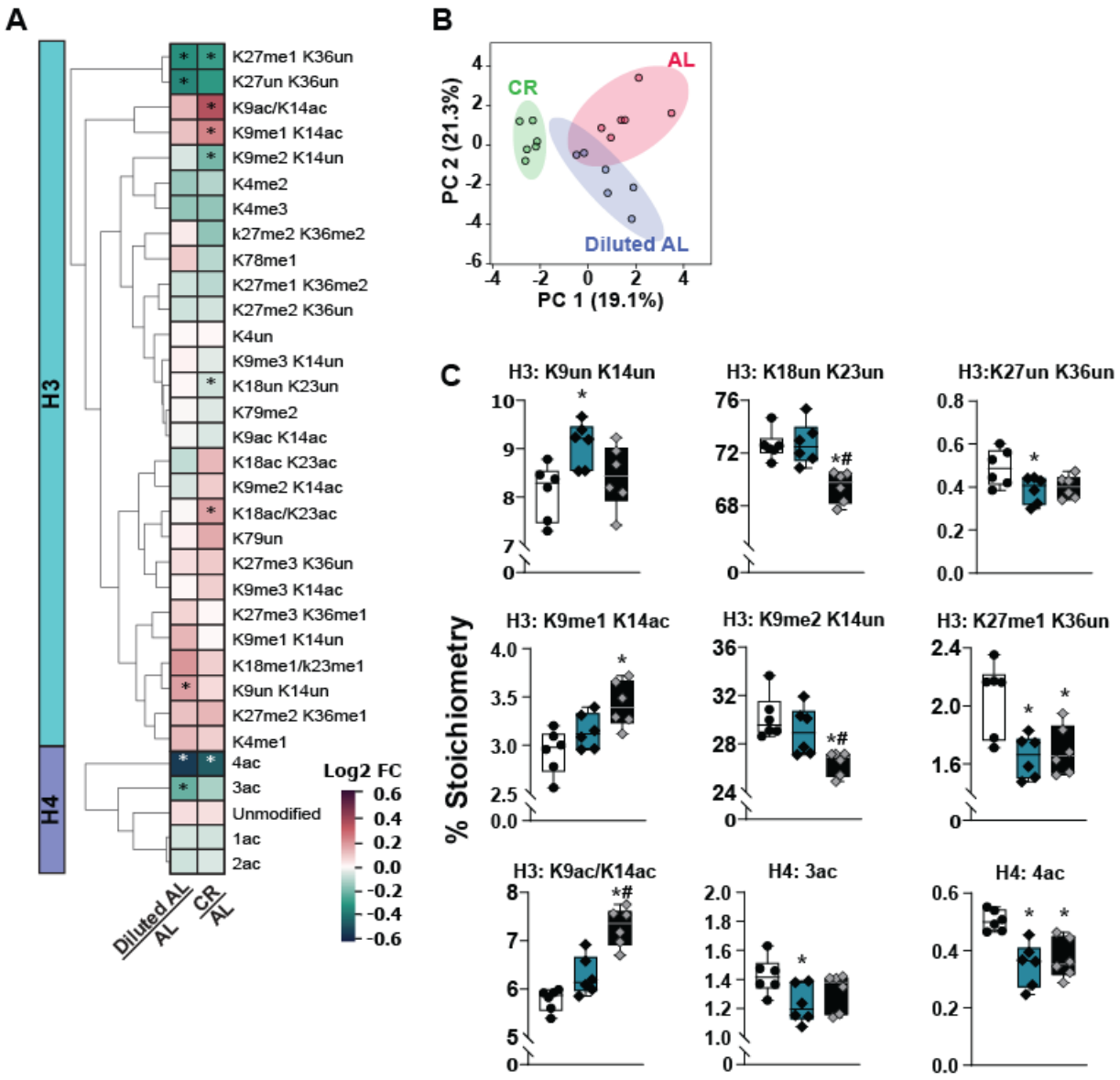


Figure 6: Fasting is required for CR-mediated reprogramming of the hepatic epigenome. Histone proteomics were performed on the livers of male C57BL/6J mice fed AL, Diluted AL and CR diets (n = 6 mice per group). A) Heatmap of histone H3 and H4 peptides represented as log2-fold change from AL. B) sPLS-DA of histone modifications. C) Statistically significant modified histones. Data are represented as mean ± SEM. (* = $p < 0.05$, Tukey's test post one-way ANOVA).

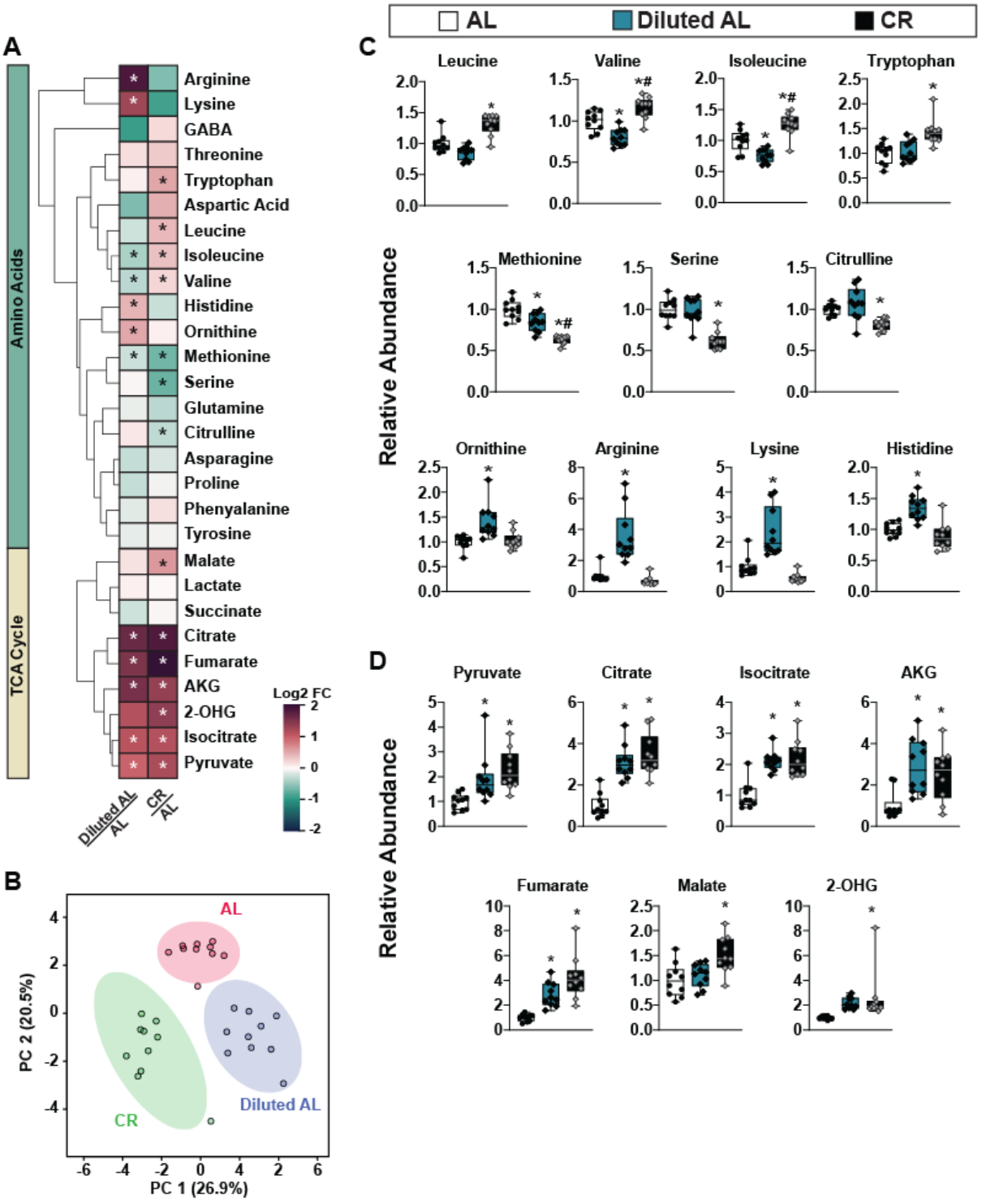
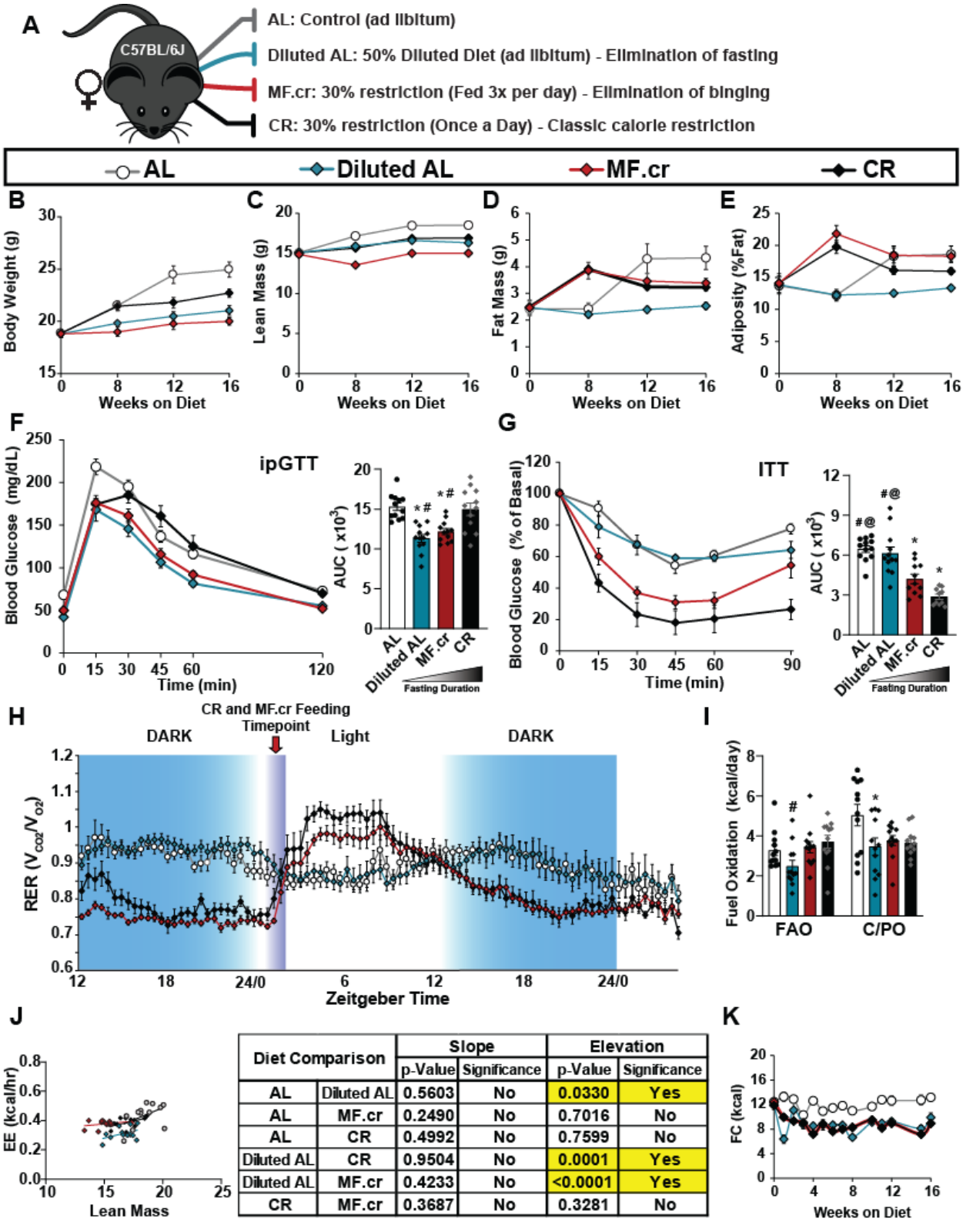
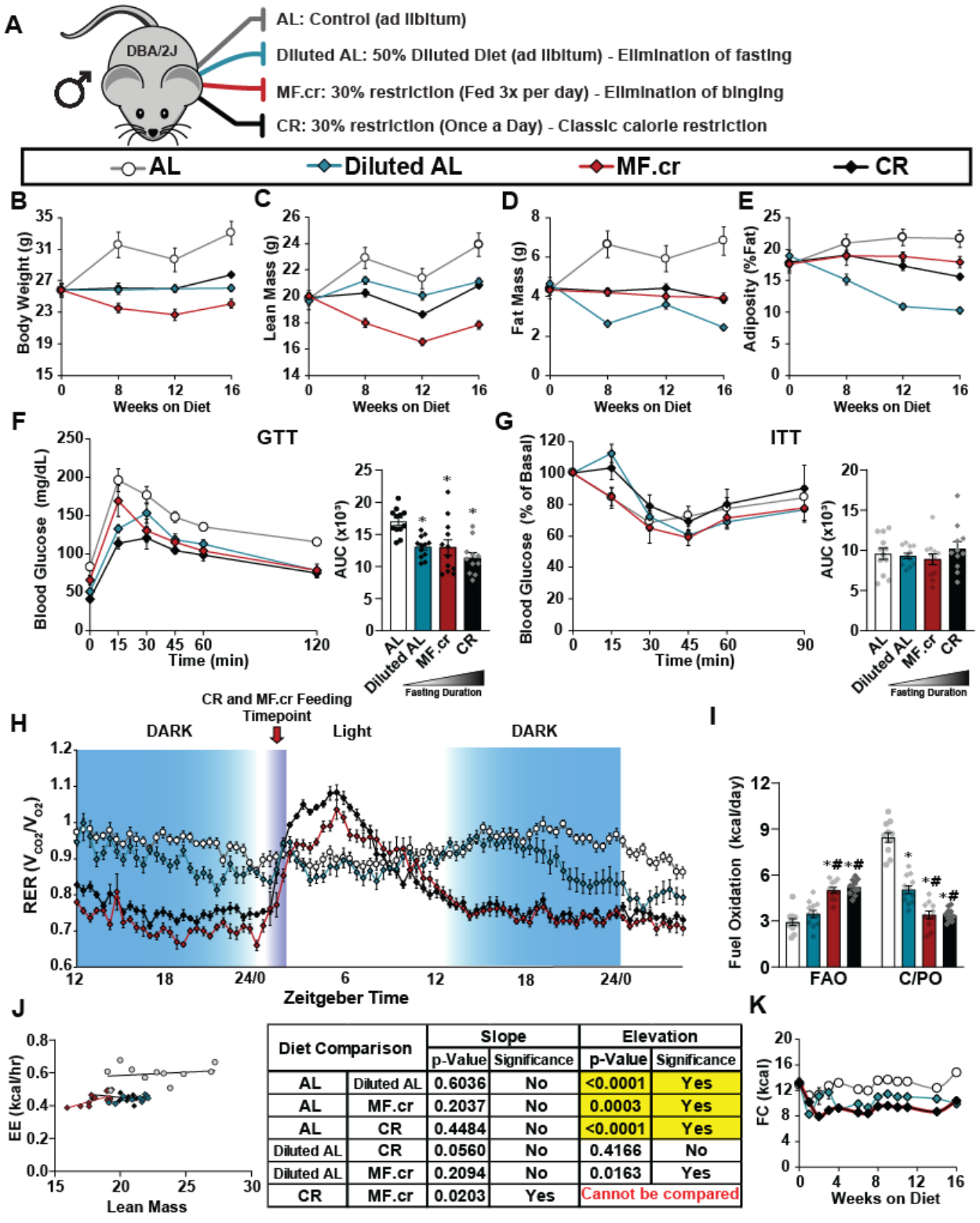

A
Amino Acids
TCA Cycle
Arginine
Lysine
GABA
Threonine
Tryptophan
Aspartic Acid
Leucine
Isoleucine
Valine
Histidine
Ornithine
Methionine
Serine
Glutamine
Citrulline
Asparagine
Proline
Phenyalanine
Tyrosine
Malate
Lactate
Succinate
Citrate
Fumarate
AKG
2-OHG
Isocitrate
Pyruvate
Diluted AL / AL
CR / AL
Log2 FC
2
1
0
-1
-2
B
AL
Diluted AL
CR
PC 2 (20.5%)
PC 1 (26.9%)
C
AL
Diluted AL
CR
Leucine
Valine
Isoleucine
Tryptophan
Methionine
Serine
Citrulline
Ornithine
Arginine
Lysine
Histidine
Relative Abundance
D
Pyruvate
Citrate
Isocitrate
AKG
Fumarate
Malate
2-OHG
Relative Abundance

Figure 7. Metabolomic profile of skeletal muscle from AL, Diluted AL and CR mice. Targeted metabolomics were performed on skeletal muscle from male C57BL/6J mice fed AL, Diluted AL, and CR diets (n =10 per diet group). A) Heatmap of 28 targeted metabolites, represented as log2-fold change vs. AL-fed mice. B) sPLS-DA of skeletal muscle metabolites. C) Relative abundance of amino acid metabolites D) Relative abundance of TCA cycle metabolites (* = $p < 0.05$, Tukey's test post one-way ANOVA).



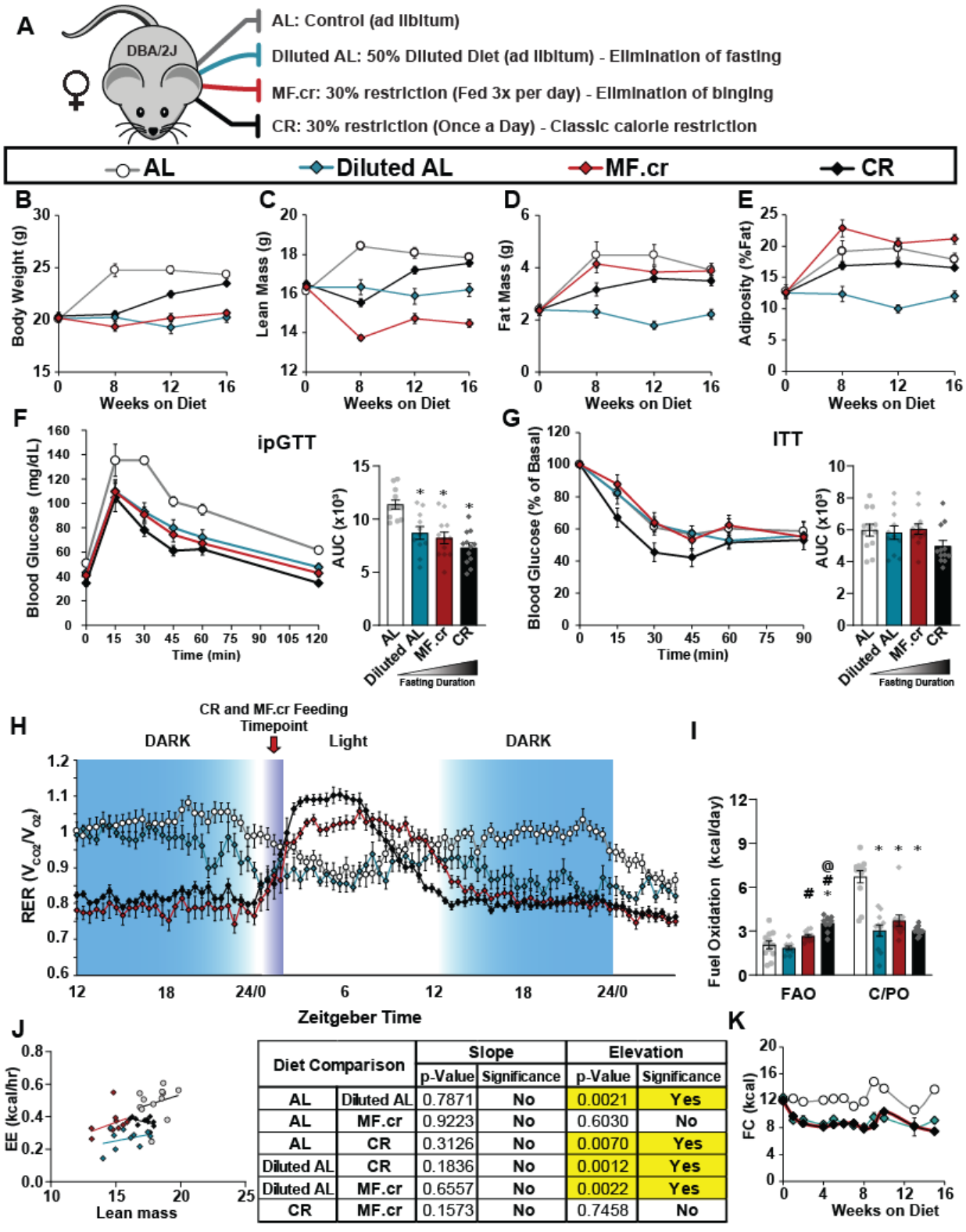
Diet Comparison		Slope		Elevation	
		p-Value	Significance	p-Value	Significance
AL	Diluted AL	0.5603	No	0.0330	Yes
AL	MF.cr	0.2490	No	0.7016	No
AL	CR	0.4992	No	0.7599	No
Diluted AL	CR	0.9504	No	0.0001	Yes
Diluted AL	MF.cr	0.4233	No	<0.0001	Yes
CR	MF.cr	0.3687	No	0.3281	No

Figure 8: The effect of three calorie restriction regimens on female C57BL/6J. (A) Outline of feeding regimens: AL, Diluted AL, CR and TR.cr. (B-E) Body composition measurement over 16 weeks on diet; total body weight (B), lean mass (C), fat mass (D) and adiposity (E). (F-G) Glucose (F) and insulin (G) tolerance tests after 9 or 10 weeks on the indicated diets (n = 10-12 mice per diet; * = $p < 0.05$ vs. AL-fed mice, # = $p < 0.05$ vs. CR, @ = $p < 0.05$ vs. Diluted AL, Tukey's test post ANOVA). (H-J) Metabolic chamber analysis of mice fed the indicated diets. (H) Respiratory exchange ratio vs. time. (I) Fuel utilization was calculated for the 24 hour period following the indicated refeeding time (n = 10-12 mice per diet per sex; * = $p < 0.05$ vs. AL, # = $p < 0.05$ vs. Diluted AL, @ = $p < 0.05$ vs. TR.cr, Tukey's test post one-way ANOVA performed separately for FA and C/P). J) Energy expenditure as a function of body mass was calculated for the 24 hour period following the indicated refeeding time (n = 10-12 mice per diet, data for each individual mouse is plotted; slopes and intercepts were calculated using ANCOVA). Data are represented as mean ± SEM.



Diet Comparison		Slope		Elevation	
		p-Value	Significance	p-Value	Significance
AL	Diluted AL	0.6036	No	<0.0001	Yes
AL	MF.cr	0.2037	No	0.0003	Yes
AL	CR	0.4484	No	<0.0001	Yes
Diluted AL	CR	0.0560	No	0.4166	No
Diluted AL	MF.cr	0.2094	No	0.0163	Yes
CR	MF.cr	0.0203	Yes	Cannot be compared	

Figure 9: The effect of three calorie restriction regimens on male DBA/2J. (A) Outline of feeding regimens: AL, Diluted AL, CR and TR.cr. (B-E) Body composition measurement over 16 weeks on diet; total body weight (B), lean mass (C), fat mass (D) and adiposity (E). (F-G) Glucose (F) and insulin (G) tolerance tests after 9 or 10 weeks on the indicated diets (n = 10-12 mice per diet; * = $p < 0.05$ vs. AL-fed mice, Tukey's test post one-way ANOVA). (H-J) Metabolic chamber analysis of mice fed the indicated diets. (H) Respiratory exchange ratio vs. time. (I) Fuel utilization was calculated for the 24 hour period following the indicated refeeding time (n = 10-12 mice per diet; * = $p < 0.05$ vs. AL, # = $p < 0.05$ vs. Diluted AL, Tukey's test post one-way ANOVA performed separately for FA and C/P). J) Energy expenditure as a function of body mass was calculated for the 24 hour period following the indicated refeeding time (n = 10-12 mice per diet per sex, data for each individual mouse is plotted; slopes and intercepts were calculated using ANCOVA). Data are represented as mean ± SEM.



Diet Comparison		Slope		Elevation	
		p-Value	Significance	p-Value	Significance
AL	Diluted AL	0.7871	No	0.0021	Yes
AL	MF.cr	0.9223	No	0.6030	No
AL	CR	0.3126	No	0.0070	Yes
Diluted AL	CR	0.1836	No	0.0012	Yes
Diluted AL	MF.cr	0.6557	No	0.0022	Yes
CR	MF.cr	0.1573	No	0.7458	No

Figure 10: The effect of three calorie restriction regimens on female DBA/2J. (A) Outline of feeding regimens: AL, Diluted AL, CR and TR.cr. (B-E) Body composition measurement over 16 weeks on diet; total body weight (B), lean mass (C), fat mass (D) and adiposity (E). (F-G) Glucose (F) and insulin (G) tolerance tests after 9 or 10 weeks on the indicated diets (n = 10-12 mice per diet; * = $p < 0.05$ vs. AL-fed mice, Tukey's test post ANOVA). (H-J) Metabolic chamber analysis of mice fed the indicated diets. (H) Respiratory exchange ratio vs. time. (I) Fuel utilization was calculated for the 24 hour period following the indicated refeeding time (n = 10-12 mice per diet per sex; * = $p < 0.05$ vs. AL, # = $p < 0.05$ vs. Diluted AL, @ = $p < 0.05$ vs. TR.cr, Tukey's test post one-way ANOVA performed separately for FA and C/P). J) Energy expenditure as a function of body mass was calculated for the 24 hour period following the indicated refeeding time (n = 10-12 mice per diet, data for each individual mouse is plotted; slopes and intercepts were calculated using ANCOVA). Data are represented as mean ± SEM.

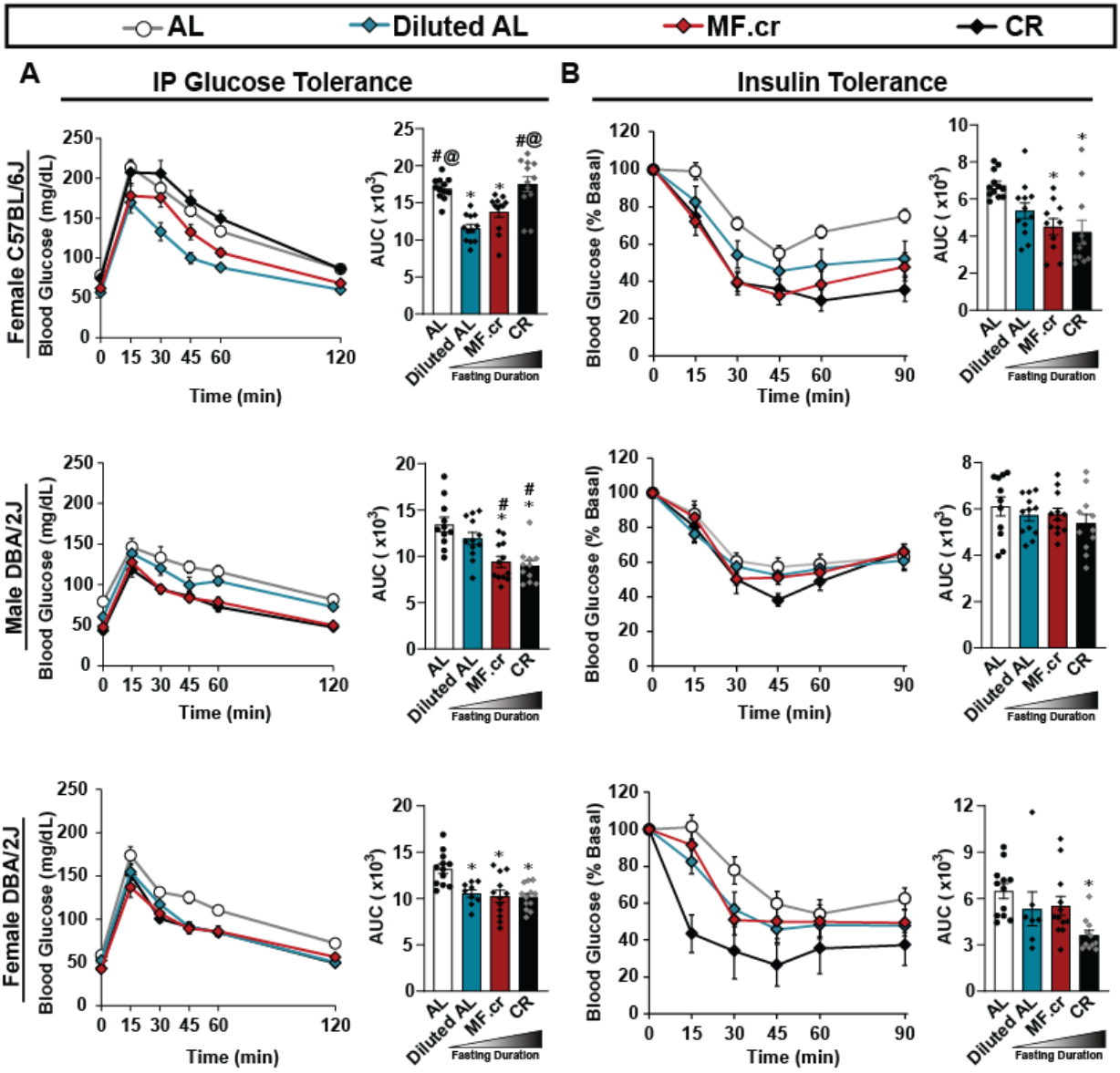

AL
Diluted AL
MF.cr
CR
A
IP Glucose Tolerance
B
Insulin Tolerance
Female C57BL/6J
Male DBA/2J
Female DBA/2J
Blood Glucose (mg/dL)
Blood Glucose (% Basal)
Time (min)
AUC (x10³)
Fasting Duration

Figure 11. Additional measures of glucose homeostasis in female C57BL/6J mice and DBA/2J male and female mice. (A-B) Glucose (A) and insulin (B) tolerance tests were performed on female C57BL/6J mice and DBA/2J mice of both sexes after 13 or 14 weeks, respectively, on the indicated diets. Female C57BL/6J mice were given 0.5U/kg of body weight insulin and DBA/2J male and female mice were given 0.75U/kg of body weight of insulin. n = 10-12 mice per group; * = $p < 0.05$ vs. AL, # = $p < 0.05$ vs. CR, @ = $p < 0.05$ vs. Diluted AL, Tukey test following one-way ANOVA, $p < 0.05$. Data are represented as mean ± SEM.

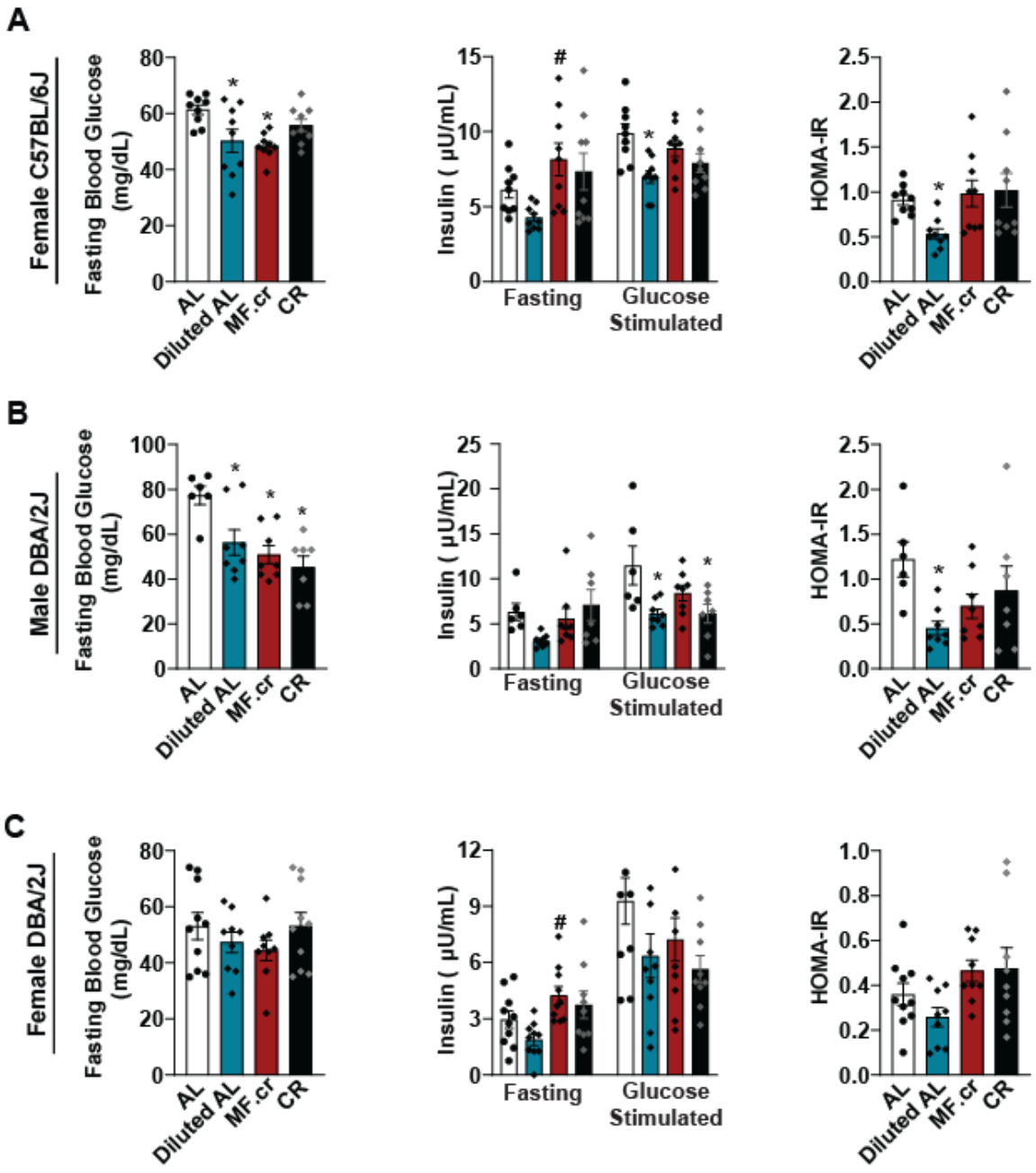
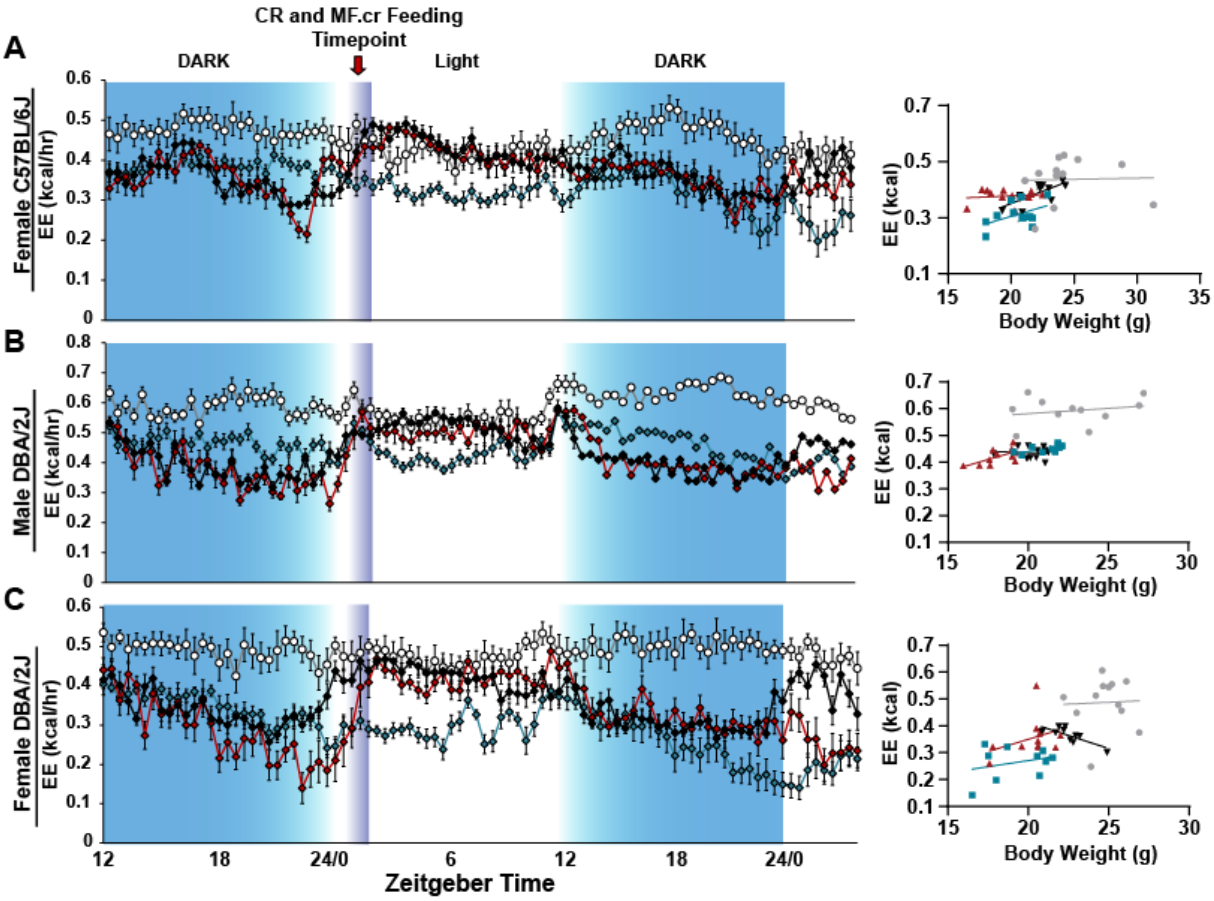

A
Female C57BL/6J
Fasting Blood Glucose (mg/dL)
Insulin (µU/mL)
HOMA-IR
Fasting
Glucose Stimulated
AL
Diluted AL
MF.cr
CR
B
Male DBA/2J
C
Female DBA/2J

Figure 12. Additional measures of glucose and insulin in female C57BL/6J mice and DBA/2J male and female mice. Mice were fasted overnight and blood glucose, fasting insulin, glucose stimulated insulin secretion (after 15 minutes) were determined, and the HOMA2-IR was calculated after 11 weeks on the specified diets for (A) female C57BL/6J, (B) male DBA/2J, and (C) female DBA/2J mice. (A-C) n = 6-11 mice per group; * = $p < 0.05$ vs. AL, @ = $p < 0.05$ vs. TR.cr, Tukey's test post one-way ANOVA; insulin levels in fasted and glucose-stimulated states were analyzed separately. Data are represented as mean ± SEM.



D Female C57BL/6J

Diet Comparison		Slope		Elevation	
		p-Value	Significance	p-Value	Significance
AL	Diluted AL	0.4097	No	0.0060	Yes
AL	MF.cr	0.9333	No	0.1428	No
AL	CR	0.2478	No	0.1889	No
Diluted AL	CR	0.6721	No	0.0033	Yes
Diluted AL	MF.cr	0.2000	No	<0.0001	Yes
CR	MF.cr	0.0267	Yes	Cannot be compared	

E Male DBA/2J

Diet Comparison		Slope		Elevation	
		p-Value	Significance	p-Value	Significance
AL	Diluted AL	0.6826	No	<0.0001	Yes
AL	MF.cr	0.3090	No	<0.0001	Yes
AL	CR	0.6716	No	<0.0001	Yes
Diluted AL	CR	0.171	No	0.1783	No
Diluted AL	MF.cr	0.2663	No	0.4334	No
CR	MF.cr	0.0665	No	0.7964	No

F Female DBA/2J

Diet Comparison		Slope		Elevation	
		p-Value	Significance	p-Value	Significance
AL	Diluted AL	0.8032	No	0.0219	Yes
AL	MF.cr	0.4223	No	0.0062	Yes
AL	CR	0.5978	No	0.2633	No
Diluted AL	CR	0.0687	No	0.0042	Yes
Diluted AL	MF.cr	0.6474	No	0.0190	Yes
CR	MF.cr	0.0634	No	0.8511	No

Figure 13. Additional energy expenditure data for C57BL/6J female and DBA/2J male and female mice. A-C) Energy expenditure vs. time and energy expenditure as a function of total body mass was calculated for the 24 hour period following the indicated refeeding time for A) C57BL/6J female mice B) DBA/2J male mice C) DBA/2J female mice D-F) statistics for A-C (n = 10-12 mice per diet, data for each individual mouse is plotted; slopes and intercepts were calculated using ANCOVA). Data are represented as mean ± SEM.

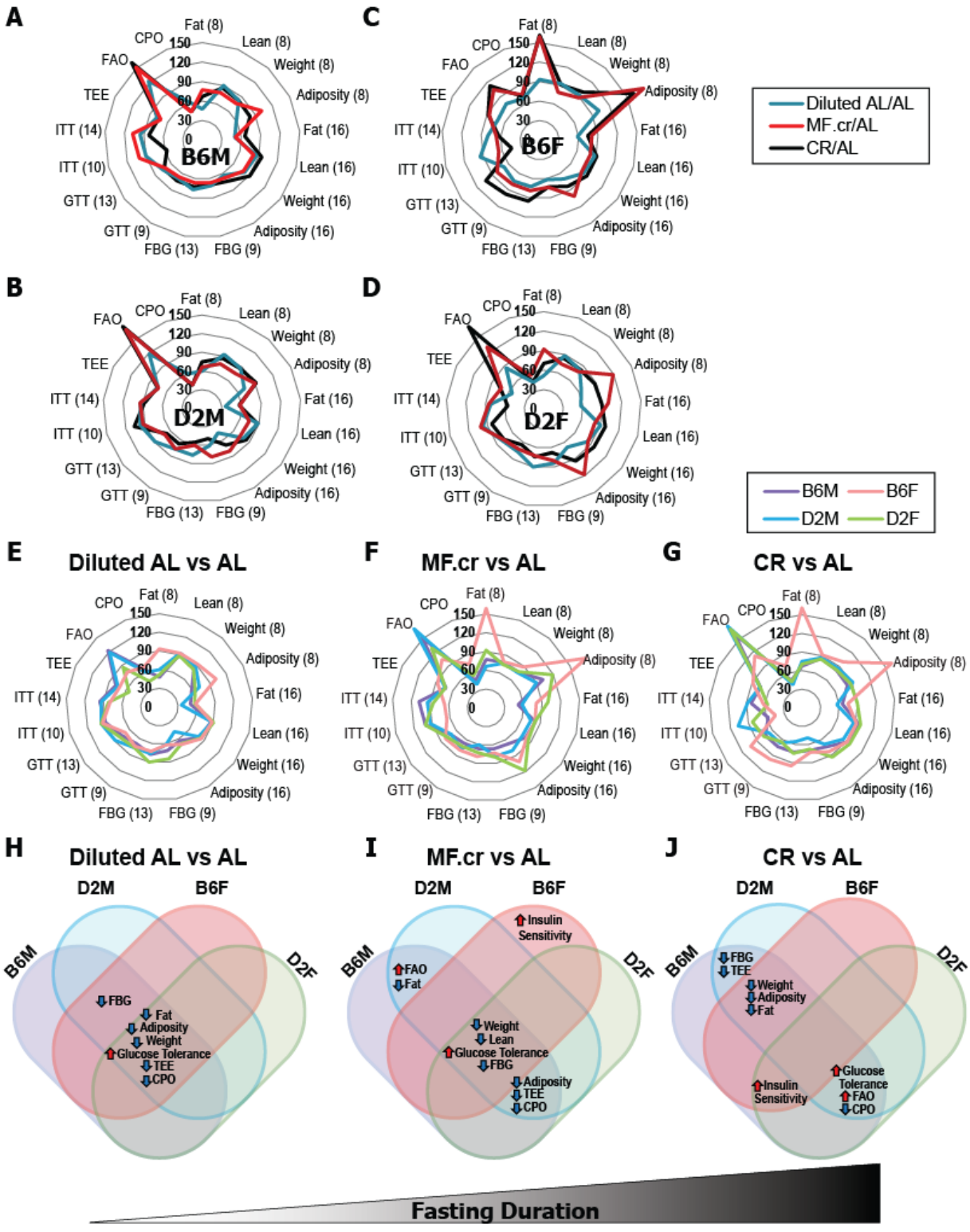

A
B6M
C
B6F
B
D2M
D
D2F
Diluted AL/AL
MF.cr/AL
CR/AL
B6M
B6F
D2M
D2F
E
Diluted AL vs AL
F
MF.cr vs AL
G
CR vs AL
H
Diluted AL vs AL
FBG
Fat
Adiposity
Weight
Glucose Tolerance
TEE
CPO
I
MF.cr vs AL
Insulin Sensitivity
FAO
Fat
Weight
Lean
Glucose Tolerance
FBG
Adiposity
TEE
CPO
J
CR vs AL
FBG
TEE
Weight
Adiposity
Fat
Insulin Sensitivity
Glucose Tolerance
FAO
CPO
Fasting Duration

Figure 14: Distinct physiological responses to diet in different strains and sexes of mice. (A-G) Radar chart visualization of 17 phenotypes measured in C57BL/6J and DBA/2J male and female mice while consuming the indicated diets. (A-D) The distance from the center represents the effect of each restricted diet vs. AL-fed mice (no difference = 100%; Blue – Diluted AL; Red – MF.cr; Black – CR). (E-G) The distance from the center represents the effect of (E) Diluted AL-fed vs. AL, (F) MF.cr-fed vs. AL-fed, and (G) CR vs. AL for the indicated strain and sexes of mice (no difference = 100%). FBG = Fasting blood glucose, GTT and ITT = computed area under the curve for each test; number in parentheses refers to the weeks on the diet each phenotype was determined. (H-J) Summary comparison of diet effect on phenotypes compared to AL for each strain and sex.

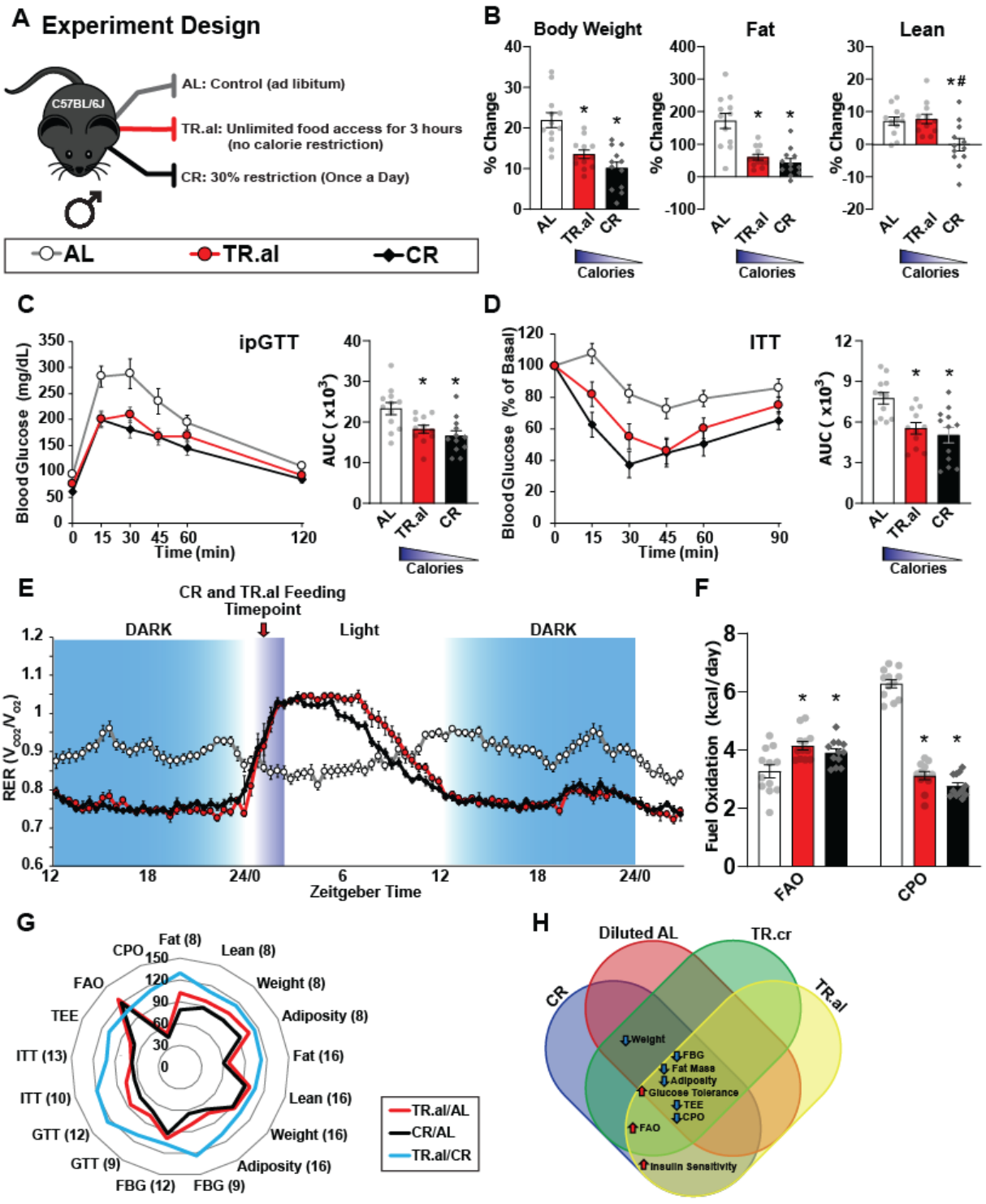

A Experiment Design
C57BL/6J
AL: Control (ad libitum)
TR.al: Unlimited food access for 3 hours (no calorie restriction)
CR: 30% restriction (Once a Day)
AL
TR.al
CR
B
Body Weight
Fat
Lean
% Change
Calories
C
ipGTT
Blood Glucose (mg/dL)
Time (min)
AUC (x10³)
D
ITT
Blood Glucose (% of Basal)
E
CR and TR.al Feeding Timepoint
DARK
Light
RER (VCO2/VO2)
Zeitgeber Time
F
Fuel Oxidation (kcal/day)
FAO
CPO
G
Fat (8)
Lean (8)
Weight (8)
Adiposity (8)
Fat (16)
Lean (16)
Weight (16)
Adiposity (16)
FBG (9)
FBG (12)
GTT (9)
GTT (12)
ITT (10)
ITT (13)
TEE
FAO
CPO
TR.al/AL
CR/AL
TR.al/CR
H
Diluted AL
TR.cr
CR
TR.al
Weight
FBG
Fat Mass
Adiposity
Glucose Tolerance
TEE
CPO
FAO
Insulin Sensitivity

Figure 15: Fasting alone recapitulates the metabolic effects of a CR diet. A) Schematic of feeding paradigms: AL, TR.al, and CR. B) Change in body weight, fat, and lean mass over the course of 16 weeks consuming the indicated diets (n = 12 biologically independent mice per diet). (C-D) Glucose (n = 12 biologically independent mice per diet) * symbol represents a significant difference versus AL-fed mice (TR.al, p = 0.0204; CR, p = 0.0019) based on Tukey's test post one-way ANOVA. (C) and insulin (AL, n = 12; TR.al, n = 11; n = 8; CR, n = 12 biologically independent mice) * symbol represents a significant difference versus AL-fed mice (TR.al, p = 0.0086; CR, p = 0.0010) based on Tukey's test post one-way ANOVA. (D) tolerance tests were conducted after mice were fed the indicated diets for 9 or 10 weeks, respectively. E) Respiratory exchange ratio (RER) vs. time (n = 12 biologically independent mice per diet). F) Fuel utilization was calculated for the 24-hour period following the indicated (arrow) refeeding time (n = 12 biologically independent mice per diet). * symbol represents a significant difference versus AL (TR.al, $p \leq 0.0027$; CR, $p \leq 0.0306$) based on Tukey's test post one-way ANOVA performed separately for FAO and C/PO). G) Radar chart visualization of 17 phenotypes measured in male C57BL/6J while consuming the indicated diets. (A-G) H) Comparison of diet effect on the phenotypes of C57BL/6J male mice for all feeding regimens examined in this manuscript. All data are represented as mean ± SEM.

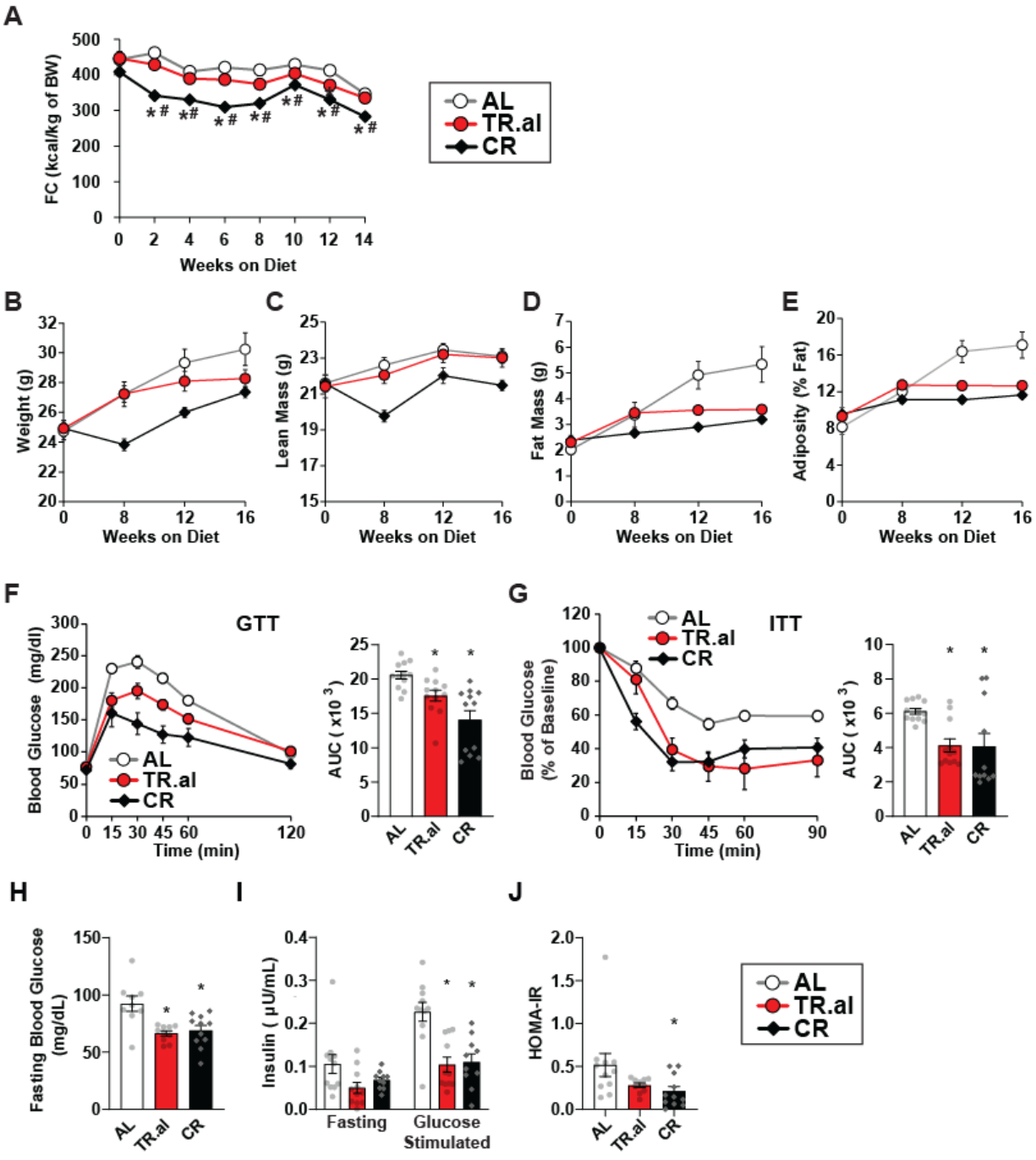
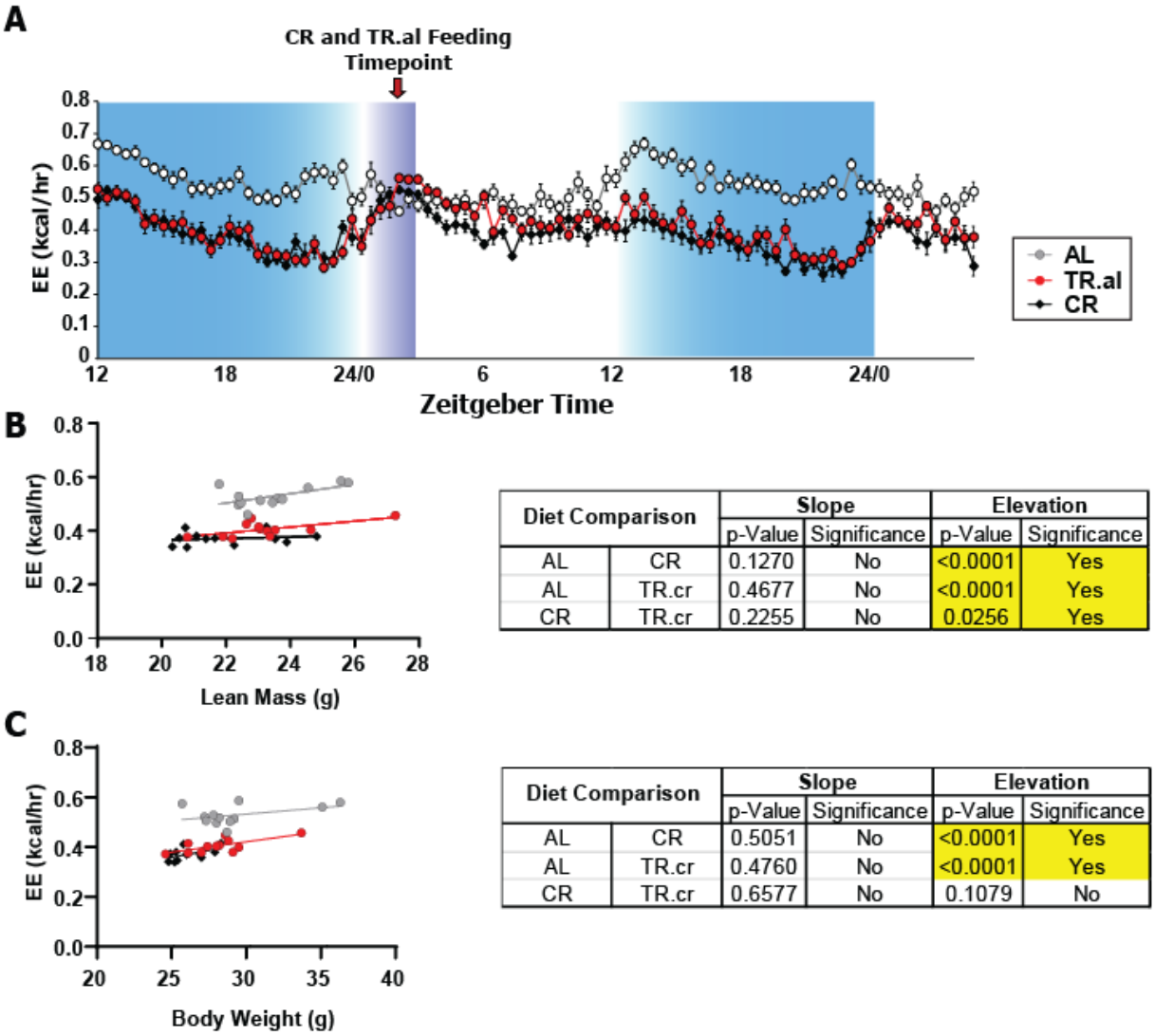

A
FC (kcal/kg of BW)
Weeks on Diet
AL
TR.al
CR
B
Weight (g)
Weeks on Diet
C
Lean Mass (g)
Weeks on Diet
D
Fat Mass (g)
Weeks on Diet
E
Adiposity (% Fat)
Weeks on Diet
F
GTT
Blood Glucose (mg/dl)
Time (min)
AUC (x10 3)
G
ITT
Blood Glucose (% of Baseline)
Time (min)
AUC (x10 3)
H
Fasting Blood Glucose (mg/dL)
I
Insulin (µU/mL)
Fasting
Glucose Stimulated
J
HOMA-IR

Figure 16. Additional data for C57BL/6J male mice fed CR or TR.al diets. A) Food consumption. B-E) Body composition (body weight, lean mass, fat mass and adiposity) of C57BL/6J male mice fed the indicated diets for 4 months**.** F-G) Glucose (F) and insulin (G) tolerance tests were performed after 13-14 weeks, respectively on the indicated diets. (H-J) Fasting blood glucose (H), fasting and glucose-stimulated insulin secretion (15 minutes) (I), and calculated HOMA2-IR (J). (A-G) n = 12 mice per group and (H-J), n = 10-11 mice per group, * = $p < 0.05$ vs. AL, # = $p < 0.05$ vs. TR.al Tukey's test post one-way ANOVA. Values are total average per mouse. Data are represented as mean ± SEM.



Diet Comparison		Slope		Elevation	
		p-Value	Significance	p-Value	Significance
AL	CR	0.1270	No	<0.0001	Yes
AL	TR.cr	0.4677	No	<0.0001	Yes
CR	TR.cr	0.2255	No	0.0256	Yes

Diet Comparison		Slope		Elevation	
		p-Value	Significance	p-Value	Significance
AL	CR	0.5051	No	<0.0001	Yes
AL	TR.cr	0.4760	No	<0.0001	Yes
CR	TR.cr	0.6577	No	0.1079	No

Figure 17. Additional energy expenditure data for C57BL/6J male mice fed CR or TR.al diets. A) Energy expenditure vs. time B) Energy expenditure as a function of lean mass was calculated for the 24 hour period following the indicated refeeding time C) Energy expenditure as a function of total body mass was calculated for the 24 hour period following the indicated refeeding time (n = 10-12 mice per diet, data for each individual mouse is plotted; slopes and intercepts were calculated using ANCOVA). Data are represented as mean ± SEM.

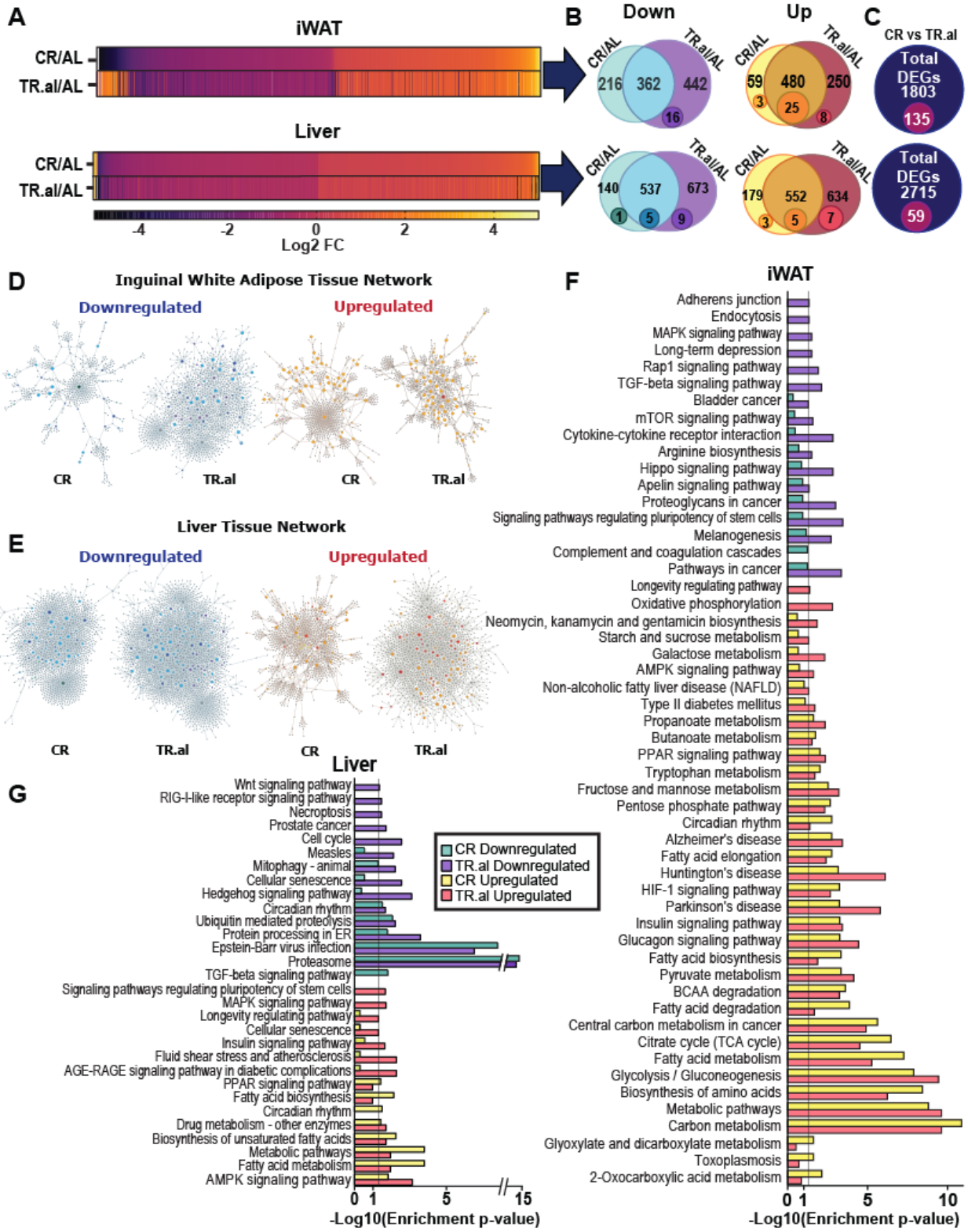

A
iWAT
CR/AL
TR.al/AL
Liver
CR/AL
TR.al/AL
-4
-2
0
2
4
Log2 FC
B
Down
CR/AL
TR.al/AL
216
362
442
16
CR/AL
TR.al/AL
140
537
673
1
5
9
Up
CR/AL
TR.al/AL
59
480
250
3
25
8
CR/AL
TR.al/AL
179
552
634
3
5
7
C
CR vs TR.al
Total DEGs 1803
135
Total DEGs 2715
59
D
Inguinal White Adipose Tissue Network
Downregulated
Upregulated
CR
TR.al
CR
TR.al
E
Liver Tissue Network
Downregulated
Upregulated
CR
TR.al
CR
TR.al
F
iWAT
Adherens junction
Endocytosis
MAPK signaling pathway
Long-term depression
Rap1 signaling pathway
TGF-beta signaling pathway
Bladder cancer
mTOR signaling pathway
Cytokine-cytokine receptor interaction
Arginine biosynthesis
Hippo signaling pathway
Apelin signaling pathway
Proteoglycans in cancer
Signaling pathways regulating pluripotency of stem cells
Melanogenesis
Complement and coagulation cascades
Pathways in cancer
Longevity regulating pathway
Oxidative phosphorylation
Neomycin, kanamycin and gentamicin biosynthesis
Starch and sucrose metabolism
Galactose metabolism
AMPK signaling pathway
Non-alcoholic fatty liver disease (NAFLD)
Type II diabetes mellitus
Propanoate metabolism
Butanoate metabolism
PPAR signaling pathway
Tryptophan metabolism
Fructose and mannose metabolism
Pentose phosphate pathway
Circadian rhythm
Alzheimer's disease
Fatty acid elongation
Huntington's disease
HIF-1 signaling pathway
Parkinson's disease
Insulin signaling pathway
Glucagon signaling pathway
Fatty acid biosynthesis
Pyruvate metabolism
BCAA degradation
Fatty acid degradation
Central carbon metabolism in cancer
Citrate cycle (TCA cycle)
Fatty acid metabolism
Glycolysis / Gluconeogenesis
Biosynthesis of amino acids
Metabolic pathways
Carbon metabolism
Glyoxylate and dicarboxylate metabolism
Toxoplasmosis
2-Oxocarboxylic acid metabolism
0 1 5 10
-Log10(Enrichment p-value)
G
Liver
Wnt signaling pathway
RIG-I-like receptor signaling pathway
Necroptosis
Prostate cancer
Cell cycle
Measles
Mitophagy - animal
Cellular senescence
Hedgehog signaling pathway
Circadian rhythm
Ubiquitin mediated proteolysis
Protein processing in ER
Epstein-Barr virus infection
Proteasome
TGF-beta signaling pathway
Signaling pathways regulating pluripotency of stem cells
MAPK signaling pathway
Longevity regulating pathway
Cellular senescence
Insulin signaling pathway
Fluid shear stress and atherosclerosis
AGE-RAGE signaling pathway in diabetic complications
PPAR signaling pathway
Fatty acid biosynthesis
Circadian rhythm
Drug metabolism - other enzymes
Biosynthesis of unsaturated fatty acids
Metabolic pathways
Fatty acid metabolism
AMPK signaling pathway
CR Downregulated
TR.al Downregulated
CR Upregulated
TR.al Upregulated
0 1 5 15
-Log10(Enrichment p-value)

Figure 18: Fasting and calorie restriction result in highly similar transcriptomic signatures in liver and white adipose tissue. Transcriptional profiling was performed on liver and iWAT from mice fed the AL, CR, or TR.al regimens (n = 6 per diet group). (A) Heatmap of differently expressed genes of CR and TR.al-fed mice compared to AL-fed mice in liver and iWAT. (B-C) Overlap between genes differentially expressed in CR-fed mice and TR.al-fed mice relative to AL-fed mice. Numbers in Venn diagrams represent the number of genes (large circle) and functionally enriched pathways (small circle) identified found from network construction in D-E. (D-E) Network construction of significantly upregulated and downregulated genes of CR and TR.al group in iWAT (D) and liver (E) using NetworkAnalyst. Node size represents number of edges connected to a node and the colors represented by directionality of enrichment. Downregulated: green – CR specific, light blue – CR and TR.al $p<0.05$, dark blue – CR and TR.al with only TR.al $p<0.05$, purple – TR.al specific $p<0.05$, gray – edges and unknown nodes. Upregulated: yellow – CR specific, orange – CR and TR.al $p <0.05$, dark orange – CR and TR.al with only TR.al $p<0.05$, red – TR.al specific $p<0.05$, gray – edges and unknown nodes. (F-G) Significantly upregulated and downregulated pathways identified from network construction in iWAT (F) and liver (G). Bar graphs represents pathways identified enriched in CR and/or TR.al-fed mice with enrichment $p<0.05$ as computed with the hypergeometric test.

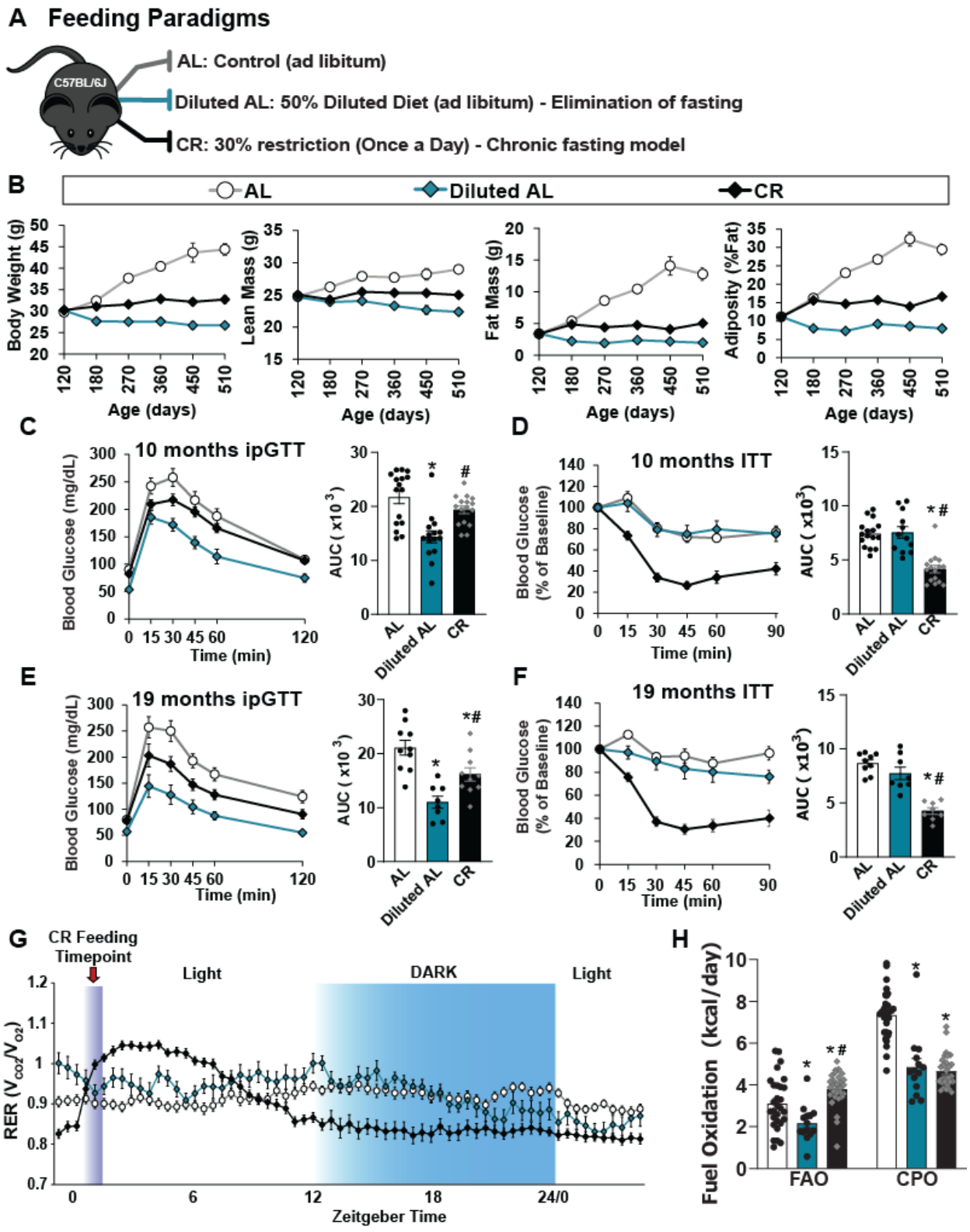

A Feeding Paradigms
C57BL/6J
AL: Control (ad libitum)
Diluted AL: 50% Diluted Diet (ad libitum) - Elimination of fasting
CR: 30% restriction (Once a Day) - Chronic fasting model
B
AL
Diluted AL
CR
Body Weight (g)
Lean Mass (g)
Fat Mass (g)
Adiposity (%Fat)
Age (days)
C
10 months ipGTT
Blood Glucose (mg/dL)
Time (min)
AUC (x10 3)
D
10 months ITT
Blood Glucose (% of Baseline)
AUC (x10 3)
E
19 months ipGTT
F
19 months ITT
G
CR Feeding Timepoint
Light
DARK
Light
RER (VCO2/VO2)
Zeitgeber Time
H
Fuel Oxidation (kcal/day)
FAO
CPO

Figure 19. Prolonged fasting is required for the CR-mediated increase in insulin sensitivity and fuel selection in aged mice. (A) Outline of feeding paradigms: AL, Diluted AL and CR. Diets were fed to C57BL/6J male mice starting at 4 months of age. (B) Change in body composition (body weight, fat mass, lean mass and adiposity) were tracked longitudinally (n varies by days; AL, n= 12-33; Diluted AL, n = 13-33 mice; CR, n = 13-33 biologically independent mice; statistics in **Supplementary Table 10**). (C) Glucose tolerance test performed at 10 months of age (AL, n = 16; Diluted AL, n = 15; CR, n = 16 biologically independent mice). * symbol represents a significant difference versus AL-fed mice (Diluted AL, $p < 0.0001$); # symbol represents a significant difference versus Diluted AL-fed mice (CR, $p = 0.0040$) based on Tukey's test post one-way ANOVA. (D) Insulin tolerance test performed at 10 months of age (AL, n = 16; Diluted AL, n = 12; CR, n = 16 biologically independent mice). * symbol represents a significant difference versus AL-fed mice (CR, $p < 0.0001$); # symbol represents a significant difference versus Diluted AL-fed mice (CR, $p<0.0001$) based on Tukey's test post one-way ANOVA. (E) Glucose tolerance test performed at 19 months of age (AL, n = 10; Diluted AL, n = 8; CR, n = 10 biologically independent mice). * symbol represents a significant difference versus AL-fed mice (Diluted AL, $p < 0.0001$; CR, $p = 0.0237$); # symbol represents a significant difference versus Diluted AL-fed mice (CR, $p = 0.0270$) based on Tukey's test post one-way ANOVA. F) Insulin tolerance test performed at 19 months of age (AL, n = 9; Diluted AL, n = 8; CR, n = 8 biologically independent mice). * symbol represents a significant difference versus AL-fed mice (CR, $p < 0.0001$); # symbol represents a significant difference versus Diluted AL-fed mice (CR, $p < 0.0001$) based on Tukey's test post one-way ANOVA. (G) Respiratory exchange ratio vs. time at ~15 months in age (AL, n = 29;

Diluted AL, n = 13; CR, n = 29 biologically independent mice). (H) Fuel utilization was calculated for the 24-hour period following the indicated (arrow) refeeding time (AL, n = 29; Diluted AL, n = 13; CR, n = 29 biologically independent mice). * symbol represents a significant difference versus AL (Diluted AL, $p = 0.0179$; CR, $p = 0.0352$); # symbol represents a significant difference versus Diluted AL (CR, $p \leq 0.0001$) based on Tukey's test post one-way ANOVA performed separately for FAO and C/PO). All data are represented as mean ± SEM.

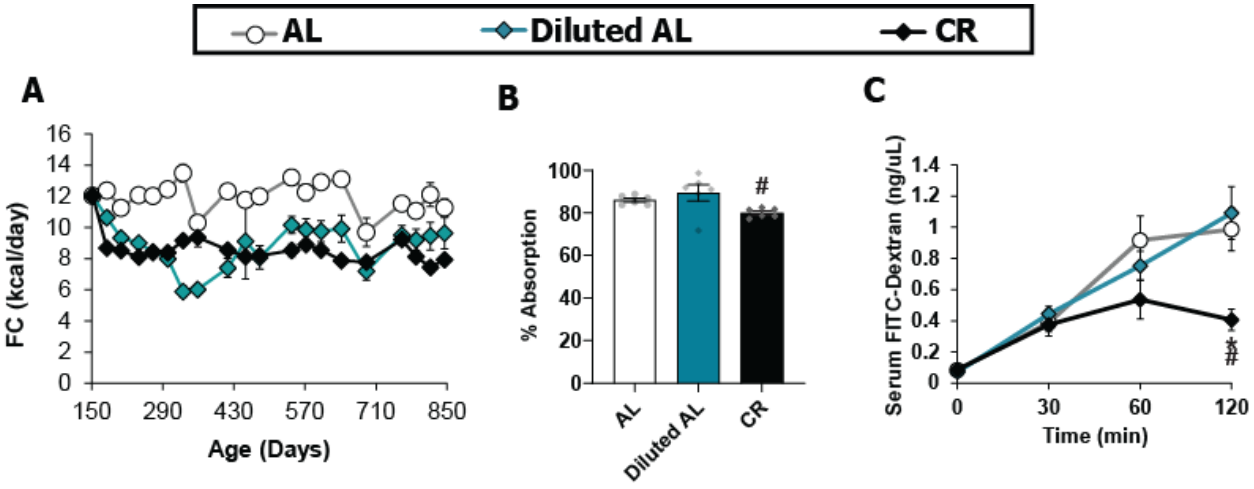


Figure 20. Food consumption, absorption and gut integrity. A) Food consumption B) Food absorption calculation by bomb calorimetry of 19-month-old C57BL/6J male mice fed the indicated diets for 13 months (n=6 mice per diet)**.** C) Gut integrity calculation by FITC-dextran of 20-month-old C57BL/6J male mice (n = 6 mice per group). * = $p < 0.05$ vs. AL Tukey's test post one-way ANOVA. Values are total average per mouse. Data are represented as mean ± SEM.

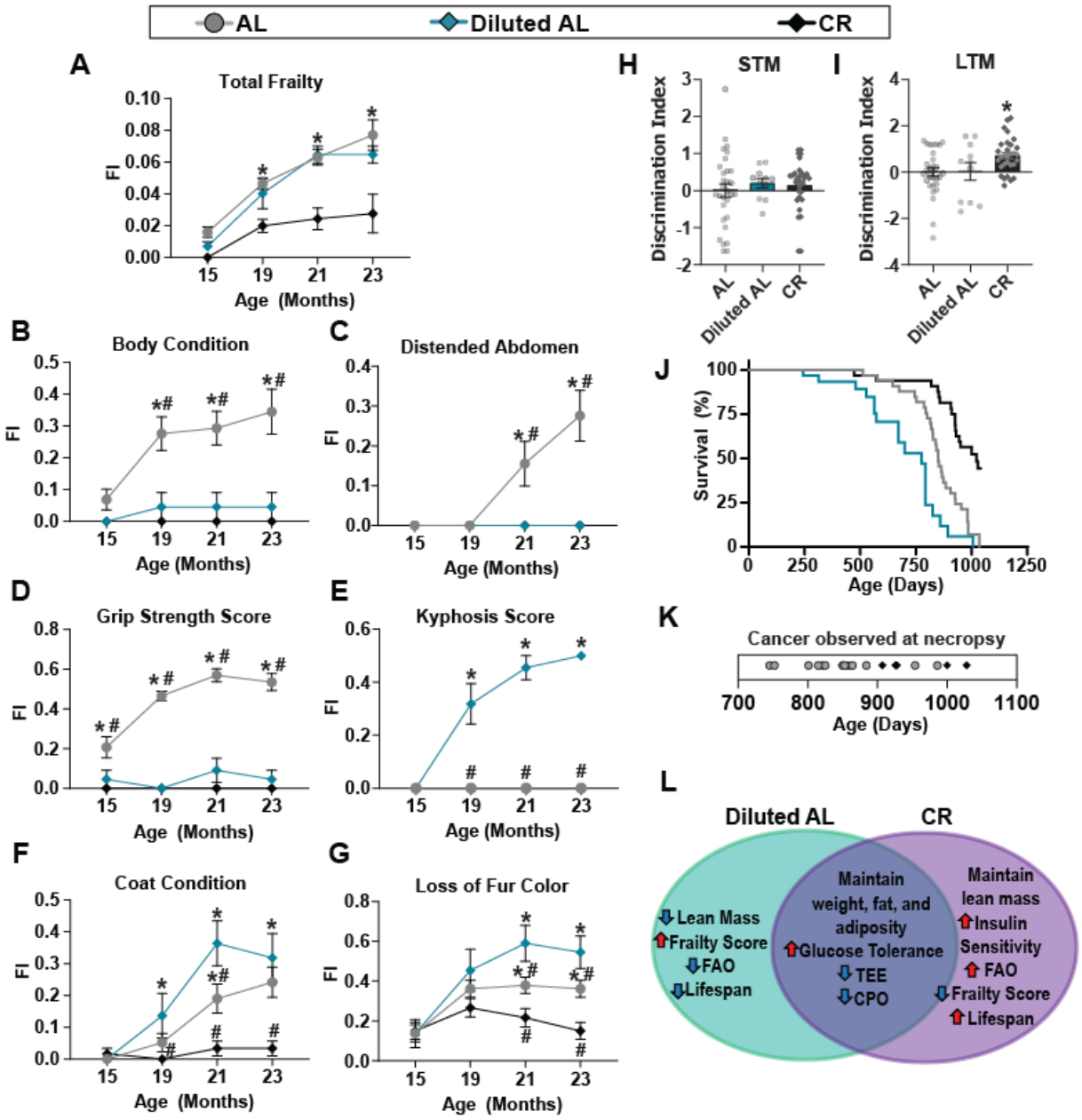
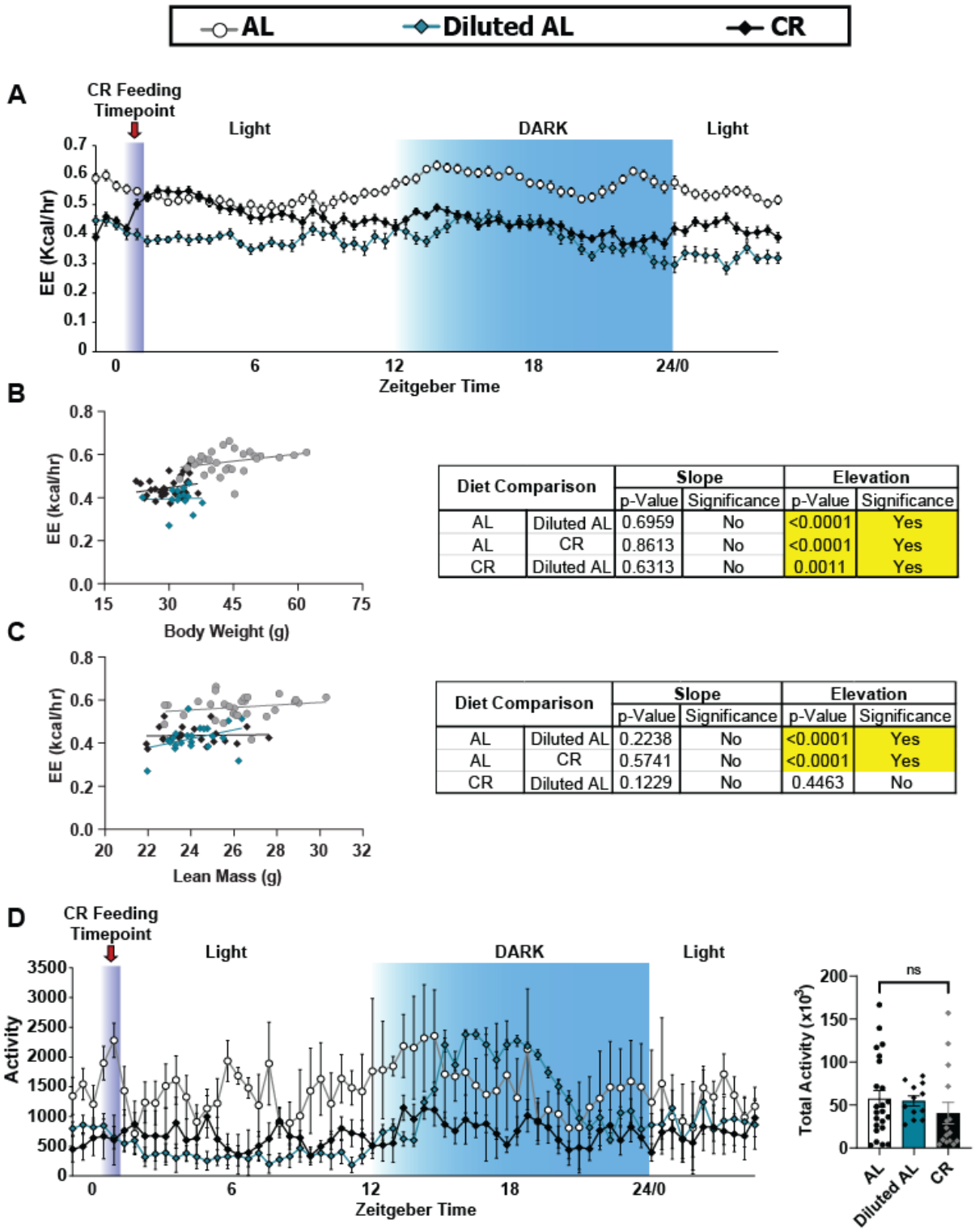

AL
Diluted AL
CR
A
Total Frailty
FI
Age (Months)
B
Body Condition
C
Distended Abdomen
D
Grip Strength Score
E
Kyphosis Score
F
Coat Condition
G
Loss of Fur Color
H
STM
Discrimination Index
I
LTM
J
Survival (%)
Age (Days)
K
Cancer observed at necropsy
Age (Days)
L
Diluted AL
CR
Lean Mass
Frailty Score
FAO
Lifespan
Maintain weight, fat, and adiposity
Glucose Tolerance
TEE
CPO
Maintain lean mass
Insulin Sensitivity
FAO
Frailty Score
Lifespan

Figure 21. Fasting is required for the geroprotective effects of a CR diet. (A-G) Frailty of mice was determined from 15-23 months of age and mice that did not survive up to 23 months of age were excluded. Total Frailty (A); specific deficits (B-G) of interests scored as part of the clinical frailty index. (A-G) AL; n = 29, Diluted AL, n = 11, CR, n = 30 biologically independent mice; * symbol represents a significant difference versus AL (CR, $p < 0.0001$); # symbol represents a significant difference versus Diluted AL (CR, $p < 0.0001$), based on Tukey's test post two-way ANOVA. (H-I) A novel object recognition test was performed at 21 months of age to assess (H) short-term (STM) and (I) long-term (LTM) memory. AL; n = 30, Diluted AL, n = 11, CR, n = 30 biologically independent mice; * symbol represents a significant difference versus AL (CR, $p < 0.0001$); # symbol represents a significant difference versus Diluted AL (CR, $p < 0.0001$), based on Tukey's test post two-way ANOVA.(J) Kaplan-Meier plot of the lifespan of mice on the indicated diets starting at 4 months of age; Log-rank test (Mantel-Cox), $p < 0.0001$, AL vs. Diluted AL and AL vs. CR; log-rank test for trend , $p = 0.0001$, AL vs. Diluted AL and AL vs. CR. (K) Tumors observed at the time of necropsy; each dot representing a single mouse. (L) Comparison of phenotypes induced by Diluted AL and CR diets, as compared to AL. All data are represented as mean ± SEM.



Diet Comparison		Slope		Elevation	
		p-Value	Significance	p-Value	Significance
AL	Diluted AL	0.6959	No	<0.0001	Yes
AL	CR	0.8613	No	<0.0001	Yes
CR	Diluted AL	0.6313	No	0.0011	Yes

Diet Comparison		Slope		Elevation	
		p-Value	Significance	p-Value	Significance
AL	Diluted AL	0.2238	No	<0.0001	Yes
AL	CR	0.5741	No	<0.0001	Yes
CR	Diluted AL	0.1229	No	0.4463	No

Figure 22. Additional energy expenditure data for 19-month old C57BL/6J male. A) Energy expenditure vs. time B) Energy expenditure as a function of total body mass was calculated for the 24 hour period following the indicated refeeding time C) Energy expenditure as a function of lean mass was calculated for the 24 hour period following the indicated refeeding time (D) Ambulatory activity vs. time and average sum of ambulatory activity was calculated for the 24 hour period following the indicated refeeding time (n = 14-29 mice per diet, data for each individual mouse is plotted; slopes and intercepts were calculated using ANCOVA). Data are represented as mean ± SEM.

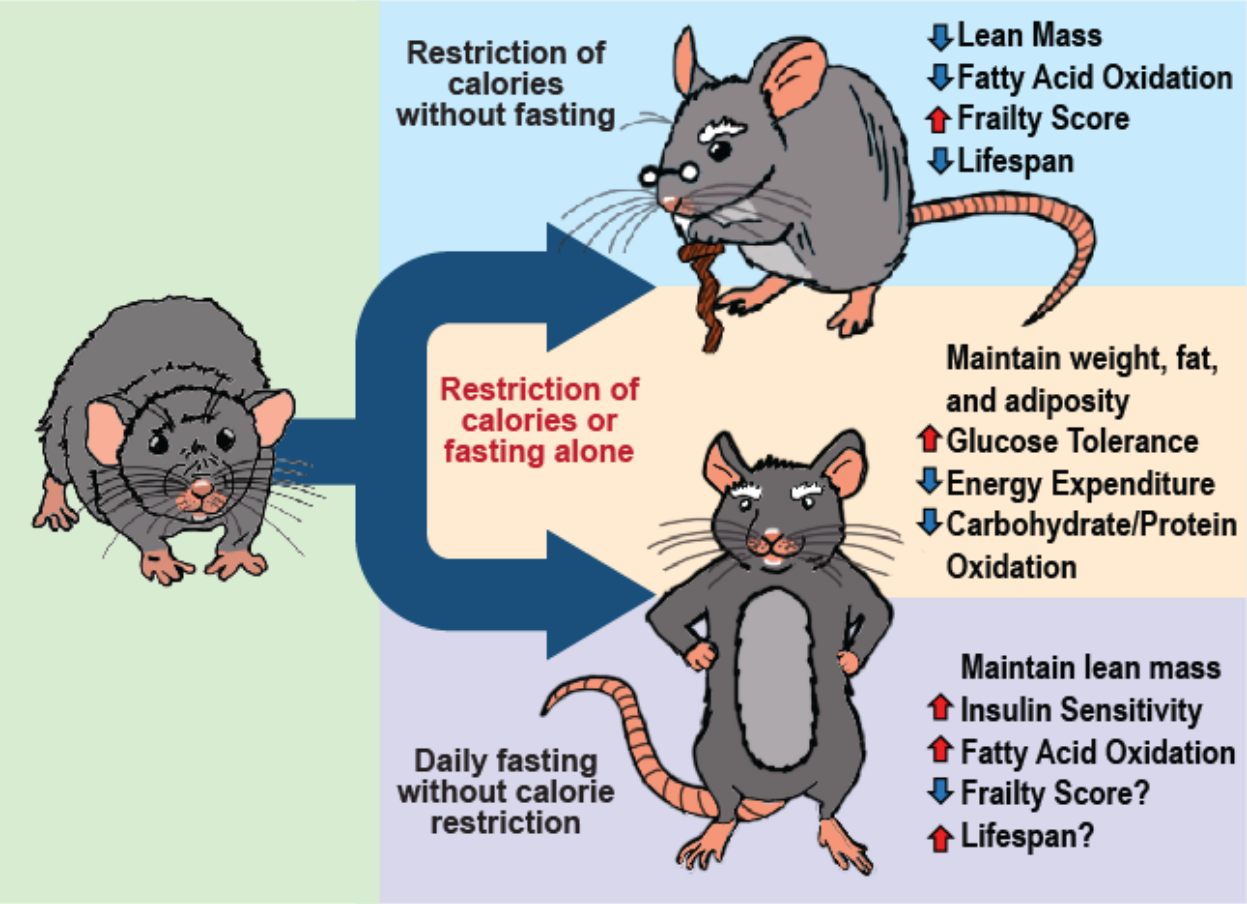


Figure 23. Fasting plays a critical role in the response to a CR diet.

Macronutrients	Teklad Global 2018 (% by kcal)	Teklad Global 2018 with 50% Cellulose (% by kcal) TD.170950
Protein	24.0%	22.9%
Carbohydrate	58.0%	60.5%
Fat	18.0%	16.6%
Kcal/g	3.2	1.6

Table 1. Diet Composition of Envigo Teklad Global 2018 Diet and 50% Diluted Diet

C57BL/6J Males									
WEIGHT		Diet Start		8 Weeks on Diet		12 Weeks on Diet		16 Weeks on Diet	
Group 1	Group 2	Significance	Adjusted P Value	Significance	Adjusted P Value	Significance	Adjusted P Value	Significance	Adjusted P Value
AL	CR	No	0.9299	Yes	0.0031	Yes	0.0016	Yes	0.0361
AL	Diluted AL	No	0.9952	Yes	0.0014	Yes	0.0001	Yes	0.0002
AL	MF.cr	No	0.9996	Yes	0.0001	Yes	<0.0001	Yes	0.0001
CR	Diluted AL	No	0.8017	No	0.9169	No	0.2907	Yes	0.0012
CR	MF.cr	No	0.9811	No	0.0839	Yes	0.0011	Yes	0.0018
Diluted AL	MF.cr	No	0.9893	No	0.2814	Yes	0.0222	No	0.9332
LEAN MASS		Diet Start		8 Weeks on Diet		12 Weeks on Diet		16 Weeks on Diet	
Group 1	Group 2	Significance	Adjusted P Value	Significance	Adjusted P Value	Significance	Adjusted P Value	Significance	Adjusted P Value
AL	CR	No	0.9617	Yes	0.0017	Yes	0.0104	No	0.3627
AL	Diluted AL	No	0.754	Yes	0.009	Yes	0.0005	Yes	0.0009
AL	MF.cr	No	0.9983	Yes	<0.0001	Yes	<0.0001	Yes	0.0001
CR	Diluted AL	No	0.8937	No	0.855	No	0.2177	Yes	0.0029
CR	MF.cr	No	0.9981	Yes	0.0141	Yes	0.0019	Yes	0.0006
Diluted AL	MF.cr	No	0.9338	Yes	0.0033	No	0.0578	No	0.4162
FAT MASS		Diet Start		8 Weeks on Diet		12 Weeks on Diet		16 Weeks on Diet	
Group 1	Group 2	Significance	Adjusted P Value	Significance	Adjusted P Value	Significance	Adjusted P Value	Significance	Adjusted P Value
AL	CR	No	0.0145	Yes	0.0326	Yes	0.0002	Yes	0.034
AL	Diluted AL	No	0.3315	Yes	0.0006	Yes	0.0002	Yes	0.0024
AL	MF.cr	No	0.2589	No	0.1264	Yes	0.0001	Yes	0.0036
CR	Diluted AL	No	0.7373	Yes	0.0118	No	0.9984	Yes	0.0011
CR	MF.cr	No	0.8076	No	0.5475	No	0.6079	Yes	0.02
Diluted AL	MF.cr	No	0.9993	Yes	<0.0001	No	0.7499	No	0.8957
ADIPOSITY		Diet Start		8 Weeks on Diet		12 Weeks on Diet		16 Weeks on Diet	
Group 1	Group 2	Significance	Adjusted P Value	Significance	Adjusted P Value	Significance	Adjusted P Value	Significance	Adjusted P Value
AL	CR	No	0.0237	No	0.1434	Yes	0.0001	Yes	0.039
AL	Diluted AL	No	0.1718	Yes	0.0001	Yes	0.0002	Yes	0.0035
AL	MF.cr	No	0.3652	No	>0.9999	Yes	0.0004	Yes	0.0137
CR	Diluted AL	No	0.8592	Yes	0.0056	No	0.9437	Yes	0.0108
CR	MF.cr	No	0.9267	Yes	0.0347	No	0.7606	No	0.5843
Diluted AL	MF.cr	No	>0.9999	Yes	<0.0001	No	0.9793	No	0.3407

C57BL/6J Females									
WEIGHT		Diet Start		8 Weeks on Diet		12 Weeks on Diet		16 Weeks on Diet	
Group 1	Group 2	Significance	Adjusted P Value	Significance	Adjusted P Value	Significance	Adjusted P Value	Significance	Adjusted P Value
AL	CR	No	>0.9999	No	>0.9999	No	0.0612	No	0.0635
AL	Diluted AL	No	0.9991	No	0.0189	Yes	0.005	Yes	0.0014
AL	MF.cr	No	0.9998	Yes	0.0034	Yes	0.0008	Yes	<0.0001
CR	Diluted AL	No	0.9982	Yes	0.022	No	0.3597	No	0.0596
CR	MF.cr	No	0.9993	Yes	0.0039	Yes	0.0482	Yes	0.0002
Diluted AL	MF.cr	No	>0.9999	No	0.3587	No	0.508	No	0.2847
LEAN MASS		Diet Start		8 Weeks on Diet		12 Weeks on Diet		16 Weeks on Diet	
Group 1	Group 2	Significance	Adjusted P Value	Significance	Adjusted P Value	Significance	Adjusted P Value	Significance	Adjusted P Value
AL	CR	No	>0.9999	Yes	0.0057	Yes	0.0105	Yes	0.0019
AL	Diluted AL	No	0.9996	Yes	0.0197	Yes	0.0041	Yes	0.0004
AL	MF.cr	No	0.9617	Yes	<0.0001	Yes	<0.0001	Yes	<0.0001
CR	Diluted AL	No	0.9994	No	0.8851	No	0.979	No	0.5126
CR	MF.cr	No	0.9572	Yes	<0.0001	Yes	0.0012	Yes	0.0001
Diluted AL	MF.cr	No	0.9361	Yes	<0.0001	Yes	0.0029	Yes	0.0196
FAT MASS		Diet Start		8 Weeks on Diet		12 Weeks on Diet		16 Weeks on Diet	
Group 1	Group 2	Significance	Adjusted P Value	Significance	Adjusted P Value	Significance	Adjusted P Value	Significance	Adjusted P Value
AL	CR	No	0.9957	Yes	0.0018	No	0.3055	No	0.1204
AL	Diluted AL	No	0.9946	No	0.922	Yes	0.0311	Yes	0.0078
AL	MF.cr	No	0.9991	Yes	0.0063	No	0.5586	No	0.2411
CR	Diluted AL	No	>0.9999	Yes	0.0003	Yes	0.003	Yes	0.0072
CR	MF.cr	No	0.9999	No	0.9992	No	0.9193	No	0.909
Diluted AL	MF.cr	No	0.9996	Yes	0.0013	Yes	0.0344	Yes	0.0104
ADIPOSITY		Diet Start		8 Weeks on Diet		12 Weeks on Diet		16 Weeks on Diet	
Group 1	Group 2	Significance	Adjusted P Value	Significance	Adjusted P Value	Significance	Adjusted P Value	Significance	Adjusted P Value
AL	CR	No	0.9944	Yes	<0.0001	No	0.565	No	0.2801
AL	Diluted AL	No	0.9979	No	0.9981	Yes	0.0217	Yes	0.0099
AL	MF.cr	No	0.9974	Yes	<0.0001	No	>0.9999	No	0.9993
CR	Diluted AL	No	>0.9999	Yes	<0.0001	Yes	0.0021	Yes	0.0026
CR	MF.cr	No	>0.9999	No	0.6094	No	0.3387	No	0.1069
Diluted AL	MF.cr	No	>0.9999	Yes	<0.0001	Yes	0.0028	Yes	0.0005

DBA/2J Male									
WEIGHT		Diet Start		8 Weeks on Diet		12 Weeks on Diet		16 Weeks on Diet	
Group 1	Group 2	Significance	Adjusted P Value	Significance	Adjusted P Value	Significance	Adjusted P Value	Significance	Adjusted P Value
AL	CR	No	>0.9999	No	0.1089	Yes	0.0319	Yes	0.0184
AL	Diluted AL	No	0.9997	No	0.1241	Yes	0.0233	Yes	0.0028
AL	MF.cr	No	0.9995	Yes	0.0041	Yes	0.0026	Yes	0.0003
CR	Diluted AL	No	0.9984	No	0.9991	No	0.9845	Yes	0.0144
CR	MF.cr	No	0.9978	Yes	0.0039	Yes	0.0332	Yes	0.0002
Diluted AL	MF.cr	No	>0.9999	Yes	0.0048	Yes	0.0416	No	0.057
LEAN MASS		Diet Start		8 Weeks on Diet		12 Weeks on Diet		16 Weeks on Diet	
Group 1	Group 2	Significance	Adjusted P Value	Significance	Adjusted P Value	Significance	Adjusted P Value	Significance	Adjusted P Value
AL	CR	No	0.997	Yes	0.0233	Yes	0.027	Yes	0.0246
AL	Diluted AL	No	0.995	No	0.3495	No	0.1727	Yes	0.0426
AL	MF.cr	No	0.9814	Yes	0.0004	Yes	0.0003	Yes	0.0001
CR	Diluted AL	No	>0.9999	Yes	0.012	No	0.1296	No	0.8876
CR	MF.cr	No	0.9975	Yes	<0.0001	Yes	0.0003	Yes	<0.0001
Diluted AL	MF.cr	No	0.9961	Yes	<0.0001	Yes	<0.0001	Yes	<0.0001
FAT MASS		Diet Start		8 Weeks on Diet		12 Weeks on Diet		16 Weeks on Diet	
Group 1	Group 2	Significance	Adjusted P Value	Significance	Adjusted P Value	Significance	Adjusted P Value	Significance	Adjusted P Value
AL	CR	No	0.9896	No	0.2264	Yes	0.025	Yes	0.0088
AL	Diluted AL	No	0.97	Yes	0.0299	Yes	0.0008	Yes	0.0005
AL	MF.cr	No	0.8796	No	0.1035	Yes	0.0229	Yes	0.0111
CR	Diluted AL	No	0.9997	No	0.0603	Yes	<0.0001	Yes	<0.0001
CR	MF.cr	No	0.9923	No	0.7241	No	0.9986	No	0.9922
Diluted AL	MF.cr	No	0.9977	No	0.852	Yes	<0.0001	Yes	0.0006
ADIPOSITY		Diet Start		8 Weeks on Diet		12 Weeks on Diet		16 Weeks on Diet	
Group 1	Group 2	Significance	Adjusted P Value	Significance	Adjusted P Value	Significance	Adjusted P Value	Significance	Adjusted P Value
AL	CR	No	0.9562	No	0.7242	Yes	0.0414	Yes	0.0053
AL	Diluted AL	No	0.8894	Yes	0.0135	Yes	<0.0001	Yes	<0.0001
AL	MF.cr	No	0.665	No	0.7633	No	0.2235	No	0.1391
CR	Diluted AL	No	0.9995	Yes	0.0062	Yes	<0.0001	Yes	<0.0001
CR	MF.cr	No	0.9935	No	0.996	No	0.4344	No	0.1532
Diluted AL	MF.cr	No	0.9985	No	0.1825	Yes	<0.0001	Yes	<0.0001

DBA/2J Female									
WEIGHT		Diet Start		8 Weeks on Diet		12 Weeks on Diet		16 Weeks on Diet	
Group 1	Group 2	Significance	Adjusted P Value	Significance	Adjusted P Value	Significance	Adjusted P Value	Significance	Adjusted P Value
AL	CR	No	0.9165	Yes	<0.0001	Yes	0.0007	No	0.1869
AL	Diluted AL	No	0.952	Yes	0.0002	Yes	0.0001	Yes	<0.0001
AL	MF.cr	No	0.9991	Yes	<0.0001	Yes	<0.0001	Yes	<0.0001
CR	Diluted AL	No	>0.9999	No	0.9768	Yes	0.0109	Yes	0.001
CR	MF.cr	No	0.93	No	0.2414	Yes	0.0022	Yes	<0.0001
Diluted AL	MF.cr	No	0.964	No	0.174	No	0.8155	No	0.7556
LEAN MASS		Diet Start		8 Weeks on Diet		12 Weeks on Diet		16 Weeks on Diet	
Group 1	Group 2	Significance	Adjusted P Value	Significance	Adjusted P Value	Significance	Adjusted P Value	Significance	Adjusted P Value
AL	CR	No	0.2903	Yes	<0.0001	No	0.0682	No	0.7191
AL	Diluted AL	No	0.8317	Yes	0.0055	Yes	0.0049	Yes	0.0074
AL	MF.cr	No	0.7226	Yes	<0.0001	Yes	<0.0001	Yes	<0.0001
CR	Diluted AL	No	0.994	No	0.0704	No	0.0894	Yes	0.0216
CR	MF.cr	No	0.76	Yes	<0.0001	Yes	<0.0001	Yes	<0.0001
Diluted AL	MF.cr	No	0.9919	Yes	0.0002	No	0.082	Yes	0.0077
FAT MASS		Diet Start		8 Weeks on Diet		12 Weeks on Diet		16 Weeks on Diet	
Group 1	Group 2	Significance	Adjusted P Value	Significance	Adjusted P Value	Significance	Adjusted P Value	Significance	Adjusted P Value
AL	CR	No	>0.9999	No	0.0859	No	0.2051	No	0.5151
AL	Diluted AL	No	0.951	Yes	0.0223	Yes	0.0002	Yes	0.0007
AL	MF.cr	No	0.9966	No	0.9481	No	0.4703	No	0.9987
CR	Diluted AL	No	0.9423	No	0.4165	Yes	<0.0001	Yes	0.0013
CR	MF.cr	No	0.9961	No	0.0933	No	0.842	No	0.1013
Diluted AL	MF.cr	No	0.8641	Yes	0.0281	Yes	<0.0001	Yes	<0.0001
ADIPOSITY		Diet Start		8 Weeks on Diet		12 Weeks on Diet		16 Weeks on Diet	
Group 1	Group 2	Significance	Adjusted P Value	Significance	Adjusted P Value	Significance	Adjusted P Value	Significance	Adjusted P Value
AL	CR	No	0.9977	No	0.5676	No	0.4596	No	0.6856
AL	Diluted AL	No	0.9749	No	0.0851	Yes	0.0003	Yes	0.0028
AL	MF.cr	No	0.9946	No	0.3903	No	0.961	No	0.0594
CR	Diluted AL	No	0.9202	No	0.2308	Yes	<0.0001	Yes	0.0056
CR	MF.cr	No	>0.9999	Yes	0.0121	Yes	0.024	Yes	0.0003
Diluted AL	MF.cr	No	0.8878	Yes	0.0036	Yes	<0.0001	Yes	<0.0001

Table 2A. Statistics on body composition measurements of C57BL/5J and DBA/2J male and female mice. Tukey's test post one-way ANOVA of weight, fat mass, lean mass and adiposity measured between diet groups.

B6M	WEIGHT			LEAN MASS			FAT MASS			ADIPOSITY		
WEEKS	0 vs 8	8 vs 12	12 vs 16	0 vs 8	8 vs 12	12 vs 16	0 vs 8	8 vs 12	12 vs 16	0 vs 8	8 vs 12	12 vs 16
AL	**	*	ns	*	ns	ns	***	ns	ns	***	ns	ns
CR	*	ns	**	ns	ns	**	ns	ns	****	ns	ns	****
Diluted AL	**	ns	ns	ns	*	*	ns	*	ns	ns	*	ns
MF.cr	***	ns	ns	***	ns	ns	ns	***	*	**	***	ns
B6F	WEIGHT			LEAN MASS			FAT MASS			ADIPOSITY		
WEEKS	0 vs 8	8 vs 12	12 vs 16	0 vs 8	8 vs 12	12 vs 16	0 vs 8	8 vs 12	12 vs 16	0 vs 8	8 vs 12	12 vs 16
AL	****	****	ns	****	****	ns	ns	**	ns	ns	***	ns
CR	****	ns	ns	ns	**	ns	****	ns	ns	****	*	ns
Diluted AL	*	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
MF.cr	ns	*	ns	**	***	ns	***	ns	ns	***	*	ns
D2M	WEIGHT			LEAN MASS			FAT MASS			ADIPOSITY		
WEEKS	0 vs 8	8 vs 12	12 vs 16	0 vs 8	8 vs 12	12 vs 16	0 vs 8	8 vs 12	12 vs 16	0 vs 8	8 vs 12	12 vs 16
AL	***	ns	ns	****	ns	ns	ns	ns	ns	ns	ns	ns
CR	ns	ns	ns	ns	**	ns	ns	ns	ns	ns	*	ns
Diluted AL	ns	ns	ns	ns	****	ns	ns	***	ns	ns	****	ns
MF.cr	*	ns	ns	****	***	ns	ns	ns	ns	ns	ns	ns
D2F	WEIGHT			LEAN MASS			FAT MASS			ADIPOSITY		
WEEKS	0 vs 8	8 vs 12	12 vs 16	0 vs 8	8 vs 12	12 vs 16	0 vs 8	8 vs 12	12 vs 16	0 vs 8	8 vs 12	12 vs 16
AL	****	ns	ns	****	ns	ns	***	ns	ns	**	ns	ns
CR	ns	**	**	**	****	ns	**	ns	ns	***	ns	ns
Diluted AL	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
MF.cr	ns	ns	ns	****	**	ns	**	ns	ns	***	ns	ns

Table S2B. Repeated measures of body composition measurements C57BL/5J and DBA/2J male and female mice. Tukey's test post Two-Way ANOVA of weight, fat mass, lean mass and adiposity measured from 0 to 8 weeks, 8 to 12 weeks, and 12 to 16 weeks on diet. Data is represented as level of adjusted p-value: * = $p < 0.05$ vs. AL; ** = $p < 0.01$; *** = $p < 0.001$; **** = $p < 0.0001$; ns = not significant

	Log2 Fold Change		
Liver Metabolites	Diluted AL/AL	Fasted CR/AL	Fed CR/AL
Acetyl Coenzyme A	-0.175	-2.889	0.3828
Adenosine	0.588	1.027	0.3550
ADP	0.194	-0.522	0.0803
Alanine	-0.189	-0.243	
Alpha-ketoglutarate	-0.466	-0.157	-3.0349
AMP	0.247	0.858	0.4237
Aspartic acid	0.053	0.429	-0.4319
ATP	0.151	-1.677	-0.4862
Choline	0.351	1.543	0.3230
Citrate	-0.160	-0.322	-0.4277
CMP	-0.067	0.478	
CTP	-0.074	-2.509	
Cytidine	-0.057	0.377	
FAD	0.297	0.349	0.1705
Fructose 1_6-bisphosphate	0.879	-3.328	
Fumarate	0.194	-0.341	-0.0105
GDP	0.314	-1.007	-0.2782
Gluconate	-0.237	0.710	0.1021
Glucose-6-phosphate	0.303	-4.151	0.0880
glutamate	-0.348	0.656	-0.9865
glutamine	0.029	-0.500	-0.3375
Glutathione (reduced)	0.236	-0.460	
Glutathione disulfide	0.113	-0.074	-0.8126
Glycerol-3-phosphate	0.091	-0.218	
Glycine	0.443	1.620	0.1676
GMP	0.012	1.232	0.9165
GTP	0.136	-2.606	-0.8519
Guanosine	0.022	0.375	0.7409
Histidine	0.300	-0.214	-0.6947
Isoleucine	-0.075	0.388	0.6027
Lactate	-0.177	-0.873	
Leucine	0.010	0.510	0.8951
Lysine	0.171	0.495	1.2245
Malate	0.191	-0.287	0.1662
Methionine	-0.170	1.087	0.4325
NAD+	-0.050	0.270	0.1964
NADH disodium salt hydrate	-0.122	0.696	0.4841
NADP+	0.455	0.618	-0.1632
NADPH	0.167	0.254	1.1234
Nicotinamide	-0.204	0.240	0.4692
Ornithine	0.314	1.368	1.4283
Phenylalanine	-0.152	0.261	0.4095
Phosphoenolpyruvate	-0.075	-2.399	0.3153
Proline	0.001	1.171	0.3549
Propionyl-CoA	-0.718	-0.451	0.4975
Ribulose 5-phosphate	0.042	-0.072	0.6184
S-Adenosyl-L-homocysteine	0.441	1.665	-0.2566
S-Adenosyl-L-methionine	0.853	-1.101	-0.9436
Serine	0.280	0.348	-0.1294
Succinate	-0.254	-0.309	0.0678
Threonine	0.055	0.663	-0.0484
Tryptophan	-0.175	0.218	0.3865
Tyrosine	-0.114	0.322	0.2026
UDP	0.373	-1.510	
UDP-glucuronate	-0.365	0.645	
UDP-N-acetylglucosamine	0.207	-0.110	
Uridine	0.415	0.131	0.1669
UTP	0.289	-2.941	-1.0345
Valine	0.299	0.334	0.2305

Table 3. Log2 fold-change calculations of liver metabolites, related to Extended Figure 2.

	Log2 Fold-Change	
Histone Modification	Diluted AL/AL	CR/AL
H3: K4me1	0.113	0.076
H3: K4me2	-0.144	-0.105
H3: K4me3	-0.153	-0.151
H3: K4un	-0.001	0.000
H3: K9ac K14ac	-0.016	-0.051
H3: K9ac/K14ac	0.119	0.334
H3: K9me1 K14ac	0.100	0.223
H3: K9me1 K14un	0.123	-0.004
H3: K9me2 K14ac	-0.055	0.081
H3: K9me2 K14un	-0.056	-0.195
H3: K9me3 K14ac	0.004	0.075
H3: K9me3 K14un	0.012	-0.040
H3: K9un K14un	0.166	0.056
H3: K18ac K23ac	-0.085	0.118
H3: K18ac/K23ac	-0.005	0.164
H3: K18me1/K23me1	0.184	0.077
H3: K18un K23un	0.002	-0.065
H3: K27me1 K36me2	-0.070	-0.098
H3: K27me1 K36un	-0.318	-0.281
H3: K27me2 K36me1	0.101	0.123
H3: K27me2 K36me2	0.024	-0.157
H3: K27me2 K36un	-0.069	-0.061
H3: K27me3 K36me1	0.067	0.001
H3: K27me3 K36un	0.048	0.085
H3: K27un K36un	-0.341	-0.291
H3: K79me1	0.080	-0.101
H3: K79me2	-0.005	-0.043
H3: K79un	0.016	0.147
H4: 1ac	-0.060	-0.062
H4: 2ac	-0.068	-0.047
H4: 3ac	-0.214	-0.118
H4: 4ac	-0.524	-0.443
H4: unmodified	0.048	0.045

Table 4. Log2 fold-change calculations of histone modifications, related to Extended Figure 2

	Log2 Fold Change	
Skeletal Muscle Metabolites	Diluted AL/AL	CR/AL
2-OHG	1.122	1.362
AKG	1.534	1.334
Arginine	1.844	-0.580
Asparagine	-0.278	-0.172
Aspartic acid	-0.595	0.455
Citrate	1.633	1.800
Citrulline	0.101	-0.297
Fumarate	1.499	2.074
GABA	-0.933	0.181
Glutamine	-0.107	-0.321
Histidine	0.430	-0.258
Isocitrate	1.080	1.125
Isoleucine	-0.399	0.334
Lactate	0.057	0.015
Leucine	-0.226	0.395
Lysine	1.256	-0.879
Malate	0.138	0.595
Methionine	-0.248	-0.665
Ornithine	0.501	0.047
Phenylalanine	-0.132	0.143
Proline	-0.274	-0.075
Pyruvate	0.947	1.183
Serine	-0.034	-0.693
Succinate	-0.224	-0.046
Threonine	0.162	0.282
Tryptophan	0.055	0.517
Tyrosine	-0.121	-0.067
Valine	-0.318	0.215

Table 5. Log2 fold-change calculations of skeletal muscle metabolites.

Condition	B6M vs B6F				D2M vs D2F				B6M vs D2M				B6F vs D2F			
Phenotype	Variable	% of total variation	P value	Significant?	% of total variation	P value	Significant?	Variable	% of total variation	P value	Significant?	% of total variation	P value	Significant?		
Fat (8)	Interaction	26.82	<0.0001	Yes	4.468	0.1115	No	Interaction	1.843	0.377	No	16.72	<0.0001	Yes		
	Sex	0.5328	0.3333	No	9.859	0.0004	Yes	Strain	25.11	<0.0001	Yes	2.696	0.0463	Yes		
	Diet	25.41	<0.0001	Yes	25.68	<0.0001	Yes	Diet	26.63	<0.0001	Yes	23.76	<0.0001	Yes		
Lean (8)	Interaction	1.121	0.0464	Yes	0.405	0.6348	No	Interaction	0.3391	0.8331	No	2.235	0.0341	Yes		
	Sex	63.06	<0.0001	Yes	35.84	<0.0001	Yes	Strain	10.46	<0.0001	Yes	1.471	0.0166	Yes		
	Diet	23.26	<0.0001	Yes	44.28	<0.0001	Yes	Diet	55.27	<0.0001	Yes	75.07	<0.0001	Yes		
Weight (8)	Interaction	8.106	<0.0001	Yes	1.343	0.3118	No	Interaction	0.8531	0.6817	No	12.23	<0.0001	Yes		
	Sex	43.45	<0.0001	Yes	36.7	<0.0001	Yes	Strain	2.261	0.0489	Yes	2.969	0.0141	Yes		
	Diet	23.92	<0.0001	Yes	31.91	<0.0001	Yes	Diet	50.15	<0.0001	Yes	44.03	<0.0001	Yes		
Adiposity (8)	Interaction	16.17	<0.0001	Yes	7.181	0.0272	Yes	Interaction	1.244	0.4156	No	10.64	0.0005	Yes		
	Sex	23.83	<0.0001	Yes	0.7159	0.3303	No	Strain	46.93	<0.0001	Yes	1.241	0.1332	No		
	Diet	31.18	<0.0001	Yes	29.3	<0.0001	Yes	Diet	18.4	<0.0001	Yes	41.6	<0.0001	Yes		
Fat (16)	Interaction	6.291	0.014	Yes	13.84	<0.0001	Yes	Interaction	4.236	0.0239	Yes	3.402	0.2008	No		
	Sex	0.00009684	0.9895	No	7.549	0.0001	Yes	Strain	6.209	0.0003	Yes	0.01587	0.8822	No		
	Diet	46.91	<0.0001	Yes	41.2	<0.0001	Yes	Diet	55.25	<0.0001	Yes	38.02	<0.0001	Yes		
Lean (16)	Interaction	0.847	0.1608	No	3.685	0.0007	Yes	Interaction	4.229	0.0227	Yes	2.892	0.0452	Yes		
	Sex	61.23	<0.0001	Yes	49.55	<0.0001	Yes	Strain	1.702	0.0473	Yes	0.3948	0.2876	No		
	Diet	22.25	<0.0001	Yes	32.03	<0.0001	Yes	Diet	58.3	<0.0001	Yes	68.48	<0.0001	Yes		
Weight (16)	Interaction	1.011	0.401	No	5.899	0.0002	Yes	Interaction	2.044	0.2399	No	2.14	0.2052	No		
	Sex	33.93	<0.0001	Yes	42.25	<0.0001	Yes	Strain	2.464	0.0255	Yes	0.002558	0.9405	No		
	Diet	34.65	<0.0001	Yes	34.7	<0.0001	Yes	Diet	57.24	<0.0001	Yes	60.95	<0.0001	Yes		
Adiposity (16)	Interaction	10.4	0.0007	Yes	10.06	0.0002	Yes	Interaction	7.142	0.0015	Yes	2.837	0.208	No		
	Sex	7.219	0.0005	Yes	0.06476	0.7049	No	Strain	3.647	0.0044	Yes	0.0006684	0.9737	No		
	Diet	36.08	<0.0001	Yes	49.48	<0.0001	Yes	Diet	53.37	<0.0001	Yes	47.38	<0.0001	Yes		
FBG (9)	Interaction	2.346	0.0803	No	7.642	0.0028	Yes	Interaction	4.4	0.0327	Yes	6.88	0.0106	Yes		
	Sex	41.22	<0.0001	Yes	19.82	<0.0001	Yes	Strain	20.1	<0.0001	Yes	14.11	<0.0001	Yes		
	Diet	26.18	<0.0001	Yes	28.47	<0.0001	Yes	Diet	35.08	<0.0001	Yes	28.3	<0.0001	Yes		
FBG (13)	Interaction	8.775	0.004	Yes	5.056	0.057	No	Interaction	1.317	0.4	No	7.194	0.0094	Yes		
	Sex	5.709	0.003	Yes	6.017	0.0031	Yes	Strain	20.07	<0.0001	Yes	28.22	<0.0001	Yes		
	Diet	32.62	<0.0001	Yes	36.1	<0.0001	Yes	Diet	41	<0.0001	Yes	12.88	0.0002	Yes		
GTT (9)	Interaction	1.522	<0.0001	Yes	0.6408	0.6708	No	Interaction	2.274	<0.0001	Yes	8.145	<0.0001	Yes		
	Sex	92.27	<0.0001	Yes	39.68	<0.0001	Yes	Strain	86.6	<0.0001	Yes	47.69	<0.0001	Yes		
	Diet	2.206	<0.0001	Yes	23.51	<0.0001	Yes	Diet	2.683	<0.0001	Yes	16.1	<0.0001	Yes		
GTT (13)	Interaction	10.16	0.0004	Yes	3.92	0.1482	No	Interaction	2.001	0.1009	No	10.55	<0.0001	Yes		
	Sex	20.61	<0.0001	Yes	0.04418	0.8042	No	Strain	53.82	<0.0001	Yes	32.49	<0.0001	Yes		
	Diet	25.15	<0.0001	Yes	36.8	<0.0001	Yes	Diet	16.47	<0.0001	Yes	20.2	<0.0001	Yes		
ITT (10)	Interaction	7.626	0.0038	Yes	2.825	0.1532	No	Interaction	8.904	0.003	Yes	13.6	0.0002	Yes		
	Sex	6.674	0.0006	Yes	52.28	<0.0001	Yes	Strain	45.42	<0.0001	Yes	5.923	0.0028	Yes		
	Diet	46.91	<0.0001	Yes	0.1829	0.9502	No	Diet	2.384	0.2629	No	30.71	<0.0001	Yes		
ITT (14)	Interaction	4.983	0.1475	No	5.4	0.1427	No	Interaction	1.666	0.6612	No	2.439	0.4459	No		
	Sex	12.23	0.0004	Yes	2.256	0.1305	No	Strain	7.046	0.011	Yes	0.002423	0.9589	No		
	Diet	9.489	0.0193	Yes	14.9	0.0027	Yes	Diet	4.909	0.203	No	26.19	<0.0001	Yes		
TEE	Interaction	10.71	0.0001	Yes	3.022	0.4382	No	Interaction	2.855	0.4974	No	3.231	0.1563	No		
	Sex	5.956	0.0006	Yes	1.009	0.3417	No	Strain	0.9397	0.3767	No	0.0304	0.823	No		
	Diet	46.71	<0.0001	Yes	3.022	0.4383	No	Diet	2.853	0.4976	No	44.86	<0.0001	Yes		
FAO	Interaction	7.014	0.061	No	48.12	<0.0001	Yes	Interaction	42.42	<0.0001	Yes	15.57	<0.0001	Yes		
	Sex	0.9971	0.2995	No	4.946	<0.0001	Yes	Strain	26.49	<0.0001	Yes	7.584	0.0002	Yes		
	Diet	19.07	0.0003	Yes	25.38	<0.0001	Yes	Diet	15.18	<0.0001	Yes	33.23	<0.0001	Yes		
CPO	Interaction	11.05	0.0004	Yes	48.12	<0.0001	Yes	Interaction	1.282	0.1152	No	26.83	<0.0001	Yes		
	Sex	5.702	0.0017	Yes	4.946	<0.0001	Yes	Strain	0.5315	0.1153	No	0.1778	0.5908	No		
	Diet	43.09	<0.0001	Yes	25.38	<0.0001	Yes	Diet	80.9	<0.0001	Yes	21.9	<0.0001	Yes		

Table 6. Statistics on sex or strain interaction with diet. Sidak's post Two-Way ANOVA was performed to test for interaction effect between sex or strain with diet.

WEIGHT		Diet Start		8 Weeks on Diet		12 Weeks on Diet		16 Weeks on Diet	
Group 1	Group 2	Significance	Adjusted P Value	Significance	Adjusted P Value	Significance	Adjusted P Value	Significance	Adjusted P Value
AL	CR	No	0.9716	Yes	0.0053	Yes	0.0108	Yes	0.0079
AL	TR.al	No	0.9707	No	0.9998	No	0.5267	No	0.9923
TR.al	CR	No	>0.9999	Yes	0.0002	Yes	0.0295	Yes	0.0458
LEAN MASS		Diet Start		8 Weeks on Diet		12 Weeks on Diet		16 Weeks on Diet	
Group 1	Group 2	Significance	Adjusted P Value	Significance	Adjusted P Value	Significance	Adjusted P Value	Significance	Adjusted P Value
AL	CR	No	>0.9999	Yes	<0.0001	Yes	0.0499	Yes	0.0079
AL	TR.al	No	0.9708	No	0.6645	No	0.904	No	0.9923
TR.al	CR	No	0.9725	Yes	0.0020	No	0.1701	Yes	0.0458
FAT MASS		Diet Start		8 Weeks on Diet		12 Weeks on Diet		16 Weeks on Diet	
Group 1	Group 2	Significance	Adjusted P Value	Significance	Adjusted P Value	Significance	Adjusted P Value	Significance	Adjusted P Value
AL	CR	No	0.4145	No	0.3560	Yes	0.0499	Yes	0.0077
AL	TR.al	No	0.4927	No	0.9870	No	0.9040	Yes	0.0260
TR.al	CR	No	0.9328	Yes	0.0002	No	0.1701	No	0.0646
ADIPOSITY		Diet Start		8 Weeks on Diet		12 Weeks on Diet		16 Weeks on Diet	
Group 1	Group 2	Significance	Adjusted P Value	Significance	Adjusted P Value	Significance	Adjusted P Value	Significance	Adjusted P Value
AL	CR	No	0.4013	No	0.7815	Yes	0.0030	Yes	0.0077
AL	TR.al	No	0.4622	No	0.8623	Yes	0.0288	Yes	0.0260
TR.al	CR	No	0.9575	Yes	0.0375	Yes	0.0305	No	0.0646

Table 7. Statistics on body composition measurements of C57BL/5J male mice. Tukey's test post one-way ANOVA of weight, fat mass, lean mass and adiposity measured between diet groups.

WEIGHT		120 Days Old		180 Days Old		270 Days Old		360 Days Old		450 Days Old		510 Days Old	
Group 1	Group 2	Significance	Adjusted P Value	Significance	Adjusted P Value	Significance	Adjusted P Value	Significance	Adjusted P Value	Significance	Adjusted P Value	Significance	Adjusted P Value
AL	CR	No	0.9821	No	0.1189	Yes	<0.0001	Yes	<0.0001	Yes	0.0008	Yes	<0.0001
AL	Diluted AL	No	0.7536	Yes	<0.0001	Yes	<0.0001	Yes	<0.0001	Yes	<0.0001	Yes	<0.0001
Diluted AL	CR	No	0.6778	Yes	<0.0001	Yes	<0.0001	Yes	<0.0001	Yes	<0.0001	Yes	<0.0001
LEAN MASS		120 Days Old		180 Days Old		270 Days Old		360 Days Old		450 Days Old		510 Days Old	
Group 1	Group 2	Significance	Adjusted P Value	Significance	Adjusted P Value	Significance	Adjusted P Value	Significance	Adjusted P Value	Significance	Adjusted P Value	Significance	Adjusted P Value
AL	CR	No	0.8624	Yes	<0.0001	Yes	0.0001	Yes	<0.0001	Yes	0.0008	Yes	<0.0001
AL	Diluted AL	No	0.976	Yes	<0.0001	Yes	<0.0001	Yes	<0.0001	Yes	<0.0001	Yes	<0.0001
Diluted AL	CR	No	0.7094	No	0.6563	Yes	<0.0001	Yes	<0.0001	Yes	<0.0001	Yes	<0.0001
FAT MASS		120 Days Old		180 Days Old		270 Days Old		360 Days Old		450 Days Old		510 Days Old	
Group 1	Group 2	Significance	Adjusted P Value	Significance	Adjusted P Value	Significance	Adjusted P Value	Significance	Adjusted P Value	Significance	Adjusted P Value	Significance	Adjusted P Value
AL	CR	No	0.9909	No	0.3684	Yes	<0.0001	Yes	<0.0001	Yes	<0.0001	Yes	<0.0001
AL	Diluted AL	No	0.9967	Yes	<0.0001	Yes	<0.0001	Yes	<0.0001	Yes	<0.0001	Yes	<0.0001
Diluted AL	CR	No	0.9992	Yes	<0.0001	Yes	<0.0001	Yes	<0.0001	Yes	<0.0001	Yes	<0.0001
ADIPOSITY		120 Days Old		180 Days Old		270 Days Old		360 Days Old		450 Days Old		510 Days Old	
Group 1	Group 2	Significance	Adjusted P Value	Significance	Adjusted P Value	Significance	Adjusted P Value	Significance	Adjusted P Value	Significance	Adjusted P Value	Significance	Adjusted P Value
AL	CR	No	0.9980	No	0.7028	Yes	<0.0001	Yes	<0.0001	Yes	<0.0001	Yes	<0.0001
AL	Diluted AL	No	0.9998	Yes	<0.0001	Yes	<0.0001	Yes	<0.0001	Yes	<0.0001	Yes	<0.0001
Diluted AL	CR	No	0.9991	Yes	<0.0001	Yes	<0.0001	Yes	<0.0001	Yes	<0.0001	Yes	<0.0001

Table 8. Statistics on body composition measurements of C57BL/5J male mice. Tukey's test post one-way ANOVA of weight, fat mass, lean mass and adiposity measured between diet groups.

CHAPTER 4: THE METABOLIC AND MOLECULAR RESPONSE TO CALORIE RESTRICTION IS HIGHLY DEPENDENT ON TEMPORAL CONDITIONS

The following chapter has been prepared for submission as a manuscript

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AUTHOR CONTRIBUTIONS

Experiments were performed in the Lamming Lab at UW-Madison. Heidi Pak and Dudley Lamming contributed in the study conception and design. All authors participated in the performance of the experiments. Heidi Pak and Dudley Lamming prepared the manuscript.

ABSTRACT

Calorie restriction (CR) extends lifespan and healthspan in mammals. Assessing the physiological and molecular differences between *ad libitum* (AL) and CR-fed mice is difficult due to dramatic differences in feeding patterns. Here, we comprehensively determined the physiological and molecular response to fasting duration in AL and CR mice over the course of a 24-hour cycle. We discovered that mice fed a CR diet has a unique response to insulin depending on fasting duration and this response was the same whether fed in the morning or at night. Interestingly, insulin levels for both AL- and CR-fed mice were dependent on time of day, and not fasting duration. We also found that hepatic mTORC1 activity is highly dependent on the length of time since feeding. Our results demonstrate the critical importance of timing and fasting duration since the last meal in how an animal responds to a test.

INTRODUCTION

Fasting has been practiced culturally for over a millennia [1]; however, it has recently become popularized with intermittent fasting regimens. Fasting regimen is often regarded distinct from caloric restriction (CR) [2]. However, we and other have previously demonstrated that one consequence of placing animals on CR animals is that these animals develop a hyperphagic response and rapidly consume their daily portion within ~1-2 hours and fast for the remainder of the day [3, 4], and this prolonged fasting (~22 hours) was necessary and sufficient to elicit the beneficial response from CR in mice [3]. Therefore, the CR response may actually be a fasting driven response.

Now a second consequence of this collaterally imposed fast is that CR animals may have adapted to daily fasting regimen, which adds another layer of complexity when examining between ad libitum fed (AL; non-fasting model) vs CR fed (chronic fasting model) animal. For example, to assess metabolic response to an agonist such as glucose or insulin, animals are fasted somewhere between 4-48 hrs, which has been used to limit the variability and bias in blood glucose measurements [5, 6]. An “overnight fast” disregards the timing and duration of the fast, which significantly complicates the interpretation between studies as in depth understanding of acute changes in metabolic flux during feeding and fasting states are required.

In humans, it is common to fast overnight, and this has been carried over to the laboratory setting where mice are fasted overnight as well; however it has been suggested that an overnight fast is an extreme fast for rodents and fasting for 5-6 hr may be a more proper duration to compare against humans [7]. There is some validity to this timepoint as this is during the post-absorptive state in *ad libitum* fed animals [8];

however, the fasting state is not well defined. Therefore, when interpreting between studies, one must assess if this is a response to fasting or the direct effect of the diet. This becomes particularly important in CR studies as AL and CR animals have drastically different feeding patterns. So far, no study has comprehensively examined the response to fasting duration in dietary restriction models. Most are studied in the context of fasting versus non-fasting, using various cut-points and not taking into account the course of blood glucose and hormonal fluctuation according to duration of fasting status and time of day.

Here we examined the physiological and molecular response to fasting duration during a 24hr diurnal cycle in ad libitum and CR fed mice. We find that CR-fed mice have a unique response to insulin based on fasting duration, and this response is conserved between morning and night fed animals. However, insulin levels were dependent on time of day and not fasting duration. Additionally, we discovered that CR mice suppress hepatic glucose production during a prolonged fast, and this response was highly dependent on insulin signaling. Finally, we show that hepatic mTOR activity is regulated by the fed and fasted state regardless of whether the animal was on an AL or CR diet. Therefore, our study demonstrates the importance of timing and fasting duration since the last meal in how an animal responds to a test.

MATERIALS AND METHODS

Animals, Diets, and Feeding Regimens

All procedures were performed in conformance with institutional guidelines and were approved by the Institutional Animal Care and Use Committee of the William S. Middleton Memorial Veterans Hospital (Assurance ID: D16-00403) (Madison, WI, USA). Male and female C57BL/6J mice (stock number 000664) were purchased from The Jackson Laboratory (Bar Harbor, ME, USA) at 8 weeks of age and acclimated to the animal research facility for at least one week before entering studies. Liver specific *Tsc1* knockout mice (Tsc1-LKO) were generated by crossing mice expressing *Albumin-Cre* (stock number 003574) with mice expressing a conditional allele of *Tsc1* (*Tsc1*$^{LoxP/LoxP}$*),* stock number 005680 as previously described [41, 53, 54]. All animals were housed in a specific pathogen free (SPF) mouse facility with a 12:12 hour light/dark cycle maintained at 20°-22°C. All animals were placed on 2018 Teklad Global 18% Protein Rodent Diet for one week before randomization. Mice were randomized to either AL, *ad libitum* diet or CR, animals in which calories were restricted by 30% and fed once per day. Animals fed an AL and CR were fed 2018 Teklad Global 18% Protein Rodent Diet, Envigo Teklad. A stepwise reduction in food intake by increments of 10% per week, starting at 20% was carried out for mice in the CR group. Body weight and food intake were monitored weekly. Morning-fed CR mice were fed daily at 6:00 - 7:00 a.m. and Night-fed CR mice were fed daily at 6:00 – 7:00 p.m. The caloric intake of the mice in the AL group was calculated weekly to determine the appropriate number of calories to feed the mice in the CR group during the following week.

Metabolic Phenotyping – Morning Fed

Glucose, insulin and alanine tolerance tests (GTT, ITT and ATT) were performed by feeding mice at 6 a.m. and removing food at 9 a.m. We then tested the respective fasting

durations: 4, 8, 12, 16, 20 or 24 hrs. At each time point, we injected a subset of mice with either glucose (1 g/kg), insulin (0.5U/kg) or alanine (2g/kg) intraperitoneally [55]; no mouse was injected twice during the course of the 24 hr cycle. Glucose measurements were taken using a Bayer Contour blood glucose meter and test strips [56]. Meal stimulated insulin tolerance (MSIS) test was performed by feeding mice at 6:00 a.m. and removing food at 9:00 a.m. We then measured and collected blood during their respective fasting durations: 4, 8, 12, 16, 20 or 24 hrs starting at 1 p.m. After the collection of fasted blood mice were allowed to feed for 2 hours which we then measured collected the fed blood. Insulin levels were assessed with Crystal Chem Insulin ELISA. Mouse body composition was determined using an EchoMRI Body Composition Analyzer. For assay of multiple metabolic parameters (O_2, CO_2, food consumption and activity tracking), mice were acclimated to housing in a Columbus Instruments Oxymax/CLAMS metabolic chamber system overnight. AL-fed mice had access to food overnight and morning-fed CR mice were given their daily pellet the following day at 7 a.m. Then we removed food at 10 a.m. for both AL- and CR-fed mice and collected data from a continuous 24-hr period was then recorded and analyzed.

Acute loss of insulin function with diazoxide

Acute diazoxide treatment study was performed by feeding mice at 6:00 a.m. and removing food at 9:00 a.m. We then injected diazoxide (0.75mg/bw) at 5:45 p.m. and then measured and collected fasted blood glucose and allowed access to food for the mice. We initially set-up the experiment for an MSIS (described above); however, the mice did not consume food. Therefore we only measured blood glucose levels after 2 hr, 3hr and 4hr post-feeding.

Metabolic Phenotyping – Night Fed

Glucose, insulin and alanine tolerance tests (GTT, ITT and ATT) was performed by feeding mice at 6:00 p.m. and removing food at 9:00 p.m. We then tested the respective fasting durations: 4, 8, 12, 16, 20 or 24 hrs and then injecting either glucose (1 g/kg), insulin (0.5U/kg) or alanine (2g/kg) intraperitoneally [55]. Glucose measurements were taken using a Bayer Contour blood glucose meter and test strips. Meal stimulated insulin tolerance (MSIS) test were performed by feeding mice at 6:00 p.m. and removing food at 9:00 p.m. We then measured and collected blood during their respective fasting durations: 4, 8, 12, 16, 20 or 24 hrs starting at 1 a.m. After the collection of fasted blood mice were allowed to feed for 2 hours which we then measured and collected the fed blood. Insulin levels were assessed with Crystal Chem Insulin ELISA. Mouse body composition was determined using an EchoMRI Body Composition Analyzer. For assay of multiple metabolic parameters (O_2, CO_2, food consumption and activity tracking), mice were acclimated to housing in a Columbus Instruments Oxymax/CLAMS metabolic chamber system for ~24 hour. During this time AL-fed mice had continual access to food while CR mice were given their daily pellet at 6 p.m. Then we removed food at 9 p.m. for both AL- and CR-fed mice and collected data from a continuous 24 hr period was then recorded and analyzed.

Metabolic Phenotyping – TSCKO

Glucose and insulin tolerance tests (GTT and ITT) were performed by feeding mice at 7 a.m. and removing food at 10 a.m. We then tested at after either 8 or 21 hrs of fasting. At each time point, we injected a subset of mice with either glucose (1 g/kg or 2g/kg) or insulin (0.75U/kg for 8hr fast or 0.5U/kg for 21hr Fast) intraperitoneally [55].Glucose

measurements were taken using a Bayer Contour blood glucose meter and test strips [56]. Mouse body composition was determined using an EchoMRI Body Composition Analyzer. For assay of multiple metabolic parameters (O_2, CO_2, food consumption and activity tracking), mice were acclimated to housing in a Columbus Instruments Oxymax/CLAMS metabolic chamber system overnight. AL-fed mice had access to food overnight and morning-fed CR mice were given their daily pellet the following day at 7 a.m and collected data from a continuous 24-hr period was then recorded and analyzed.

Sacrifice and Collection of Tissues

Mice were sacrificed after 16 weeks on diet. Mice in the morning-fed studies were fed at 6 a.m. then food was removed starting at 9 a.m. and sacrificed in the respective fasting durations: 4, 8, 12, 16, 20 or 24 hrs. Mice in the night-fed studies were fed at 6 p.m. then food was removed starting at 9 p.m. and sacrificed in the respective fasting durations: 4, 8, 12, 16, 20 or 24 hrs. Following blood collection via submandibular bleeding, mice were euthanized by cervical dislocation and tissues (liver, muscle, iWAT, eWAT, BAT, and cecum) were rapidly collected, weighed, and then snap frozen in liquid nitrogen.

Immunoblotting

Tissue samples from liver and muscle were lysed in cold RIPA buffer supplemented with phosphatase inhibitor and protease inhibitor cocktail tablets using a FastPrep 24 (M.P. Biomedicals) with bead-beating tubes and zirconium ceramic oxide bulk beads. Protein lysates were then centrifuged at 13,300 rpm for 10 min and the supernatant was collected. Protein concentration was determined by Bradford (Pierce Biotechnology). 20

mg protein was separated by SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) on 8%, 10%, or 16% resolving gels and transferred to PVDF membrane. To account for the variability between different western runs, we made a stock standard liver lysate sample which was run in duplicate on every gel. Membranes were blocked in 5% non-fat dry milk dissolved in TBST for 10 minutes and were then incubated in primary antibody diluted in 5% BSA (for phospho antibodies) or 5% milk (for non-phospho antibodies) overnight. The following commercial primary antibodies were used for immunoblot analysis p-S473 AKT (1:1,000; Cell Signaling Technology #4060), p-T308 AKT (1:1,000; Cell Signaling Technology # 2965), AKT (1:1,000; Cell Signaling Technology #4691), p-p70 S6K, S6K, p-S757-ULK, ULK, p-AMPK, AMPK, p-eIF2a, eIF2a, ATF4, HSP90 (1:1,000; Cell Signaling Technology #4877), p-S240/244 S6 ribosomal protein (1:1,000; Cell Signaling Technology #2215), S6 ribosomal protein (1:1,000; Cell Signaling Technology #2217), p-T37/S46 4E-BP1 (1:1,000; Cell Signaling Technology #2855), 4E-BP1 (1:1,000; Cell Signaling Technology #9452). Imaging was performed using a GE ImageQuant LAS 4000 imaging station (GE Healthcare). Quantification was performed by densitometry using NIH ImageJ software.

Statistics

Data are presented as mean ± SEM unless otherwise specified. For box-plots, center line represents the median; box limits indicate the upper and lower 25th to 75th percentile, and whiskers extend to the smallest and largest data values. Analyses were performed using Excel (2010 and 2016, Microsoft) or Prism 8 (GraphPad Software). Statistical analyses were performed using one or two-way ANOVA followed by Tukey-Kramer post hoc test specified in the figure legends. Other statistical details can also be

found in the figure legends; in all figures, n represents the number of biologically independent animals. Sample sizes for metabolic studies were determined based on our previously published experimental results with the effects of dietary interventions [57], with the goal of having > 90% power to detect a change in area under the curve during a GTT ($p<0.05$). Data distribution was assumed to be normal, but this was not formally tested.

Randomization All studies were performed on animals or tissues collected from animals. Animals of each sex and strain were randomized into groups of equivalent weight prior to the beginning of the *in vivo* studies.

RESULTS

Time since last feeding impacts the observed physiological response to a CR diet.

CR animals fed once per day consume their food in approximately 2-3 hours; in contrast, AL-fed animals consume small portions of food approximately every 4 hours, with the majority of food consumed at night [3, 9]. The sharp differences in eating behavior of CR and AL-fed mice complicates the interpretation of physiological assays and molecular responses, which are usually examined at a single time point. For example, a sacrifice of CR and AL-fed mice at 6am compares CR-fed mice that have been fasted for up to 21 hours with mice that spent the most recent dark period eating.

To examine how the observed response to a CR diet varies with the length of time since the last meal, we placed male and female C57BL/6J mice on either an AL or CR

diet for 8 weeks, feeding the CR mice each day at the beginning of the light period between 6-7 AM. We then proceeded to perform a series of assays to examine glucose homeostasis. For all of the tests we conducted, both AL and CR-fed mice were provided access to food from 6-9AM, and at the conclusion of this period food was removed from all groups. We then randomized the fasted AL and CR-fed mice into 6 groups and performed metabolic phenotyping on a new group of mice every 4 hours (**Fig. 1A**).

We first performed glucose tolerance tests on C57BL/6J male mice. Due to the differences in fasting blood glucose between diet groups and fasting timepoints, we took the delta of baseline measurements to calculate our values. Both AL and CR-fed male C57BL/6J mice had the lowest area under the curve (AUC) after 4 hrs of fasting as compared to other fasting timepoints, and when mice were fasted between 4-20 hours, CR-fed mice consistently had better glucose tolerance than AL-fed mice (**Fig. 1B-C, Extended Fig. 1A**). Surprisingly, at the 24-hour time point, there was no longer a significant difference in glucose tolerance between diet groups. (**Fig. 1B-C, Fig. 2A**).

We next examined the response to I.P. administration of insulin. While the response of AL-fed male C57BL/6J mice to insulin was similar regardless of the length of the fast, we were surprised to note that the response of CR-fed male C57BL/6J mice to insulin was highly dependent on the length of the fasting period (**Fig. 1D-E, Fig. 2B**). Contrary to our expectations based on the literature, CR-fed mice at the 4-, 8-, and 12-hour time points were insulin-resistant both in absolute terms – blood glucose levels were essentially resistant to insulin administration - and relative to AL-fed mice. CR-fed mice exhibited insulin sensitivity relative to AL-fed mice only after 20 or 24 hours of fasting (**Fig. 1D-E, Fig. 2B**).

It has recently become clear that sex is an important factor in the response to dietary interventions, including CR [10, 11]. To determine if both sexes responded similarly, we also examined the response of AL-fed and CR-fed female C57BL/6J mice to fasting duration (**Fig. 1F-I, Fig. 3**). Similar to males fed a CR diet (MCR), female mice fed a CR diet (FCR) had improved glucose tolerance relative to AL-fed mice at all time points, with both AL-fed and CR-fed females having the lowest AUC after a 4 hour fast (**Fig. 1F-G; Fig. 3A**). Additionally, like MCR, FCR examined following 4, 8, and 12 hours of fasting were insulin resistant both in absolute terms and relative to AL-fed mice and exhibited improved insulin sensitivity relative to AL-fed mice only after 20 or 24 hours of fasting (**Fig. 1H-I Fig. 3B**). Together, these results suggest that glucose homeostasis is rewired in CR mice to adapt to daily fasting, and that this response is conserved between sexes.

Meal stimulated insulin level is not dependent on fasting duration, but dependent on time of feeding.

To investigate the unique response to insulin in CR mice, we conducted a meal-stimulated insulin secretion (MSIS) test. We chose to measure insulin levels after feeding rather than following glucose stimulation in order to examine insulin levels in response to the same diets normally consumed by the animals under natural feeding conditions. We provided both AL and CR-fed C57BL/6J male mice access to food from 6-9AM, and then divided the mice into six groups which were fasted for either 4, 8, 12, 16, 20 or 24 hrs. Following the fast, we collected fasting blood and mice were allowed access to food for two hours, following which we collected fed blood (**Fig. 4A**). Two

hours was chosen as a sufficient amount of time for mice to consume food, while keeping the time between pre-prandial phase and postprandial phase of digestion[12].

We found that CR-fed mice were resistant to fasting-induced changes in blood glucose levels – while AL-fed mice had an approximately 46% decline in fasting blood glucose over the course of 20 hours, CR-fed mice had less than a 15% change over the same time period (**Fig. 4B**). Further, CR-fed mice fasted for 20 hours or less tightly maintained their blood glucose levels post-feeding, while AL-fed mice fasted for 8-20 hours had a 50-100% increase in blood glucose level following feeding (**Fig. 4B-C**). Intriguingly, after 24 hours of fasting – which exceeds the normal length of time between meals CR-fed mice are exposed to – AL-fed and CR-fed mice had almost identical fasting and refed blood glucose levels (**Fig. 4B-C**). Thus, CR-fed mice have adapted their metabolism to better maintain their blood glucose levels during the daily imposed fasting period.

Surprisingly, we discovered that both CR and AL-fed mice had unexpected levels of serum insulin level post-feeding (**Fig. 4D**). Although based on the literature we expected CR-fed mice to have lower fasting and refed insulin levels than AL-fed mice, we observed that this was only true at certain time points – and that CR-fed mice refed after fasting for 4, 20, or 24 hours had insulin levels higher than refed AL-fed mice. Examining the data carefully, we noticed that the pattern of refed insulin levels in CR-fed mice was shifted by 12 hours from that of AL-fed mice. When the CR data was shifted by 12 hrs to match the normal first feeding time of the AL group at the start of the dark cycle at ZT12, AL and CR-fed mice had exactly the same level of refed insulin (**Fig. 4E**).

In order to determine if the differences between AL and CR-fed mice was driven by differences in insulin secretion, we attempted to perform a MSIS test with diazoxide treatment, a well-established drug that inhibits insulin secretion[13-15]. We chose to perform this test after a 12 hr fast because at this timepoint both AL-fed and CR-fed mice had similar fasting blood glucose values and a similar response to insulin administration while having different refed insulin levels. Fifteen minutes prior to the collection of fasting blood, mice were either treated with vehicle or diazoxide, and blood glucose level was measured before and after treatment (**Fig. 4F**). Mice were then provided access to food, but neither diet group consumed their food following AL-fed or CR-fed mice consumed their food after vehicle or diazoxide treatment.

Surprisingly, despite not consuming food, we found that when insulin secretion was inhibited with diazoxide, the blood glucose levels of CR-fed mice climbed rapidly, with levels exceeding 600 mg/dL; in contrast the blood glucose of diazoxide-treated AL-fed mice increased much more modestly (**Fig. 4G**). This data suggested to us that during a fast insulin plays a critical role in inhibiting blood glucose production in CR-fed animals. To test if hepatic gluconeogenesis is suppressed in CR-fed mice, we performed an alanine tolerance test (ATT), allowing us to measure glucose production from this substrate [16], after fasting periods ranging from 4-24 hours (**Fig. 4H-I**). CR-fed mice had improved alanine tolerance relative to AL-fed mice at all time points thru 20 hours (**Fig. 4H-I**).

We next examined fasted and fed blood glucose and serum insulin in female C57BL/6J mice (**Fig. 5A-C**). Just as the males, FCR mice were resistant to fasting-induced changes in blood glucose, and tightly controlled their blood glucose levels

following refeeding (**Fig. 5A-B**). Females had similar trend in circulating insulin as males but did not reach significance between AL and CR (**Fig. 5C**). When comparing males and females fed an AL diet (FAL), females had similar level of fasting blood glucose. However, FCR generally had lower fasting blood glucose than MCR (**Fig. 5D**). On examining insulin levels, both males and females had similar level of insulin in both fasted and fed state (**Fig. 5E**). Additionally, like MCR, FCR showed improved alanine tolerance until the fasting length reached 24 hrs (**Fig. 5F**).

Aged CR mice retain similar response to tests as young CR mice at multiple timepoints.

In our previous work, we observed that aged mice fed a CR diet retained a healthy phenotype [3]. Therefore, we next determined if aged male CR mice (22 months old) displayed similar trend as the young mice at multiple timepoints (**Fig. 6**). Just as the young male mice, aged mice fed a CR diet (OCR) had improved glucose tolerance regardless of the length of the fast (**Fig. 6A**). Similar to young CR mice (YCR), OCR only showed improved insulin sensitivity after a 20 hr fast (**Fig. 6B**). As with YCR, OCR retained tight fasting and meal-stimulated blood glucose levels up to a 20 hr fast (**Fig. 6C-D**), and similar trend in circulating level of insulin **(Fig. 6E**).

Together these data suggest CR mice are resistant to fasting-induced changes in blood glucose and also able to maintain a narrow range in blood glucose following refeeding during a prolonged fast. Additionally, hepatic gluconeogenesis, as assessed by ATT, is suppressed in CR-fed animals and this response is conserved between male and females.

Circulating insulin levels are dependent on time-of-day

Our initial findings that insulin levels in AL-fed and CR-fed appeared to be out of sync by 12 hours (**Fig. 4**) led us to hypothesize that perhaps insulin levels were dependent on the time-of-first feeding. Indeed, previous studies have demonstrated that insulin secretion is highly regulated by circadian controls [17, 18] as well as feeding schedule[19, 20]. Therefore, we repeated our examination of the metabolic response to CR in mice fed not early in the morning, but instead 12 hours later at the start of the dark cycle (6-7 PM) (**Fig. 7A**).

We then proceeded to assess glucose and insulin tolerance in these night-fed mice following fasts ranging from 4-24 hours (**Fig. 7**) We find that just like morning-fed CR mice (MCR), night-fed CR (NCR) mice had improved glucose tolerance relative to AL-fed mice regardless of the length of the fast (**Fig. 7B-C**, **Fig. 8A**). Intriguingly, AL night-fed mice had improved glucose tolerance relative to AL morning-fed mice at most time points, while CR night-fed mice had improved glucose tolerance relative to CR morning-fed mice (**Fig. 9A-B**). Night-fed CR mice did not show improved insulin sensitivity unless fasted for 20 or 24 hours, and there was no significant difference in the response to insulin between morning-fed and night-fed CR mice (**Fig. 7D-E, Extended Fig. 9C-D**).

To address our hypothesis that insulin levels were dependent on time-of-first-feeding, we measured fasted and fed blood glucose and serum insulin levels in the night-fed study (**Fig. 10A-C**). Unlike the AM-fed mice, night-fed mice AL and CR groups were both able to maintain their fasting blood glucose levels over the 24-hour period. Like the MCR group, NCR mice were also able to maintain tight control over their refed

blood levels over the first 16 hour, while night-fed AL mice were not (**Fig. 10A-B**). We observed significant differences between the blood glucose of morning-fed and night-fed animals at couple of the timepoints, but this may be an artifact from the variability between different cohorts of mice (**Fig. 11A**). While we expected to see an alignment in insulin level between AL and CR-fed animals, we observed a 2-fold increase in circulating insulin at the 12 hr 16 hr and 20 hr fast (**Fig. 10C**)., Insulin levels of AL and CR mice did not align when comparing the trend within each diet between the morning-fed and night-fed studies (**Fig. 11B**). However, when shifting the morning-fed data to match that of ZT time of night-fed data and disregarding the fasting duration, AL mice had the same circulating insulin level, with the exception of ZT11 (**Fig. 11C**). This was not true for CR-fed mice, in particular at ZT 3-13 (**Fig. 11C**). Therefore, these results suggest that circulating insulin levels are dependent on time-of-day for an AL-fed mouse, regardless of fasting duration, while fasting duration may have an additive effect to time-of-day on insulin secretion/clearance CR-fed animals.

We observed that both fasted and refed blood glucose were tightly regulated in night-fed CR mice for at least the first 16 hours of a prolonged growing more variable after that (**Fig. 10A-B**). While we expected to see an alignment in insulin level between AL and CR-fed animals, we observed a 2-3-fold increase in circulating insulin at the 12 hr 16 hr and 20 hr fast (**Fig. 10C**). Insulin levels of AL and CR mice did not align when comparing the trend within each diet between the morning-fed and night-fed studies (**Fig. 11B**). However, when shifting the morning-fed data to match that of ZT time of night-fed data and disregarding the fasting duration, AL mice had the same circulating insulin level, with the exception of ZT11 (**Fig. 11C**). This was not true for CR-fed mice,

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We next assessed suppression of hepatic gluconeogenesis following alanine administration in night-fed CR mice. As with morning-fed CR mice, we found significantly improved alanine tolerance in CR-fed mice throughout the duration of the fast (**Fig. 10D-E**) with nearly identical results between morning- and night-fed animals (**Fig. 11D**). Together, these data suggest that the response to IP glucose is regulated by time-of-day; however, the response to insulin and alanine is regulated by fasting duration and not by time of day in CR-fed animals.

Hepatic mTORC1 activity is dictated by feeding and fasting state, not diet.

Genetic or pharmaceutical inhibition of mTOR Complex 1 (mTORC1) in organisms ranging from yeast to mice extends lifespan [21-29], as does genetic inhibition of genes and processes downstream of mTORC1, including S6K1 and protein translation [26, 30, 31]. In yeast, there is an epistatic relationship between CR and TOR signaling [21], and it has since been assumed that CR in higher organisms is likewise mediated at least in part by reduced mTOR signaling [32, 33]. No such analysis has been undertaken in a mammal, but extensive “omics” studies suggest that rapamycin and CR have distinct, largely non-overlapping effects [34-38]. It is also not clear that a CR diet reduces mTORC1 signaling *in vivo*, as investigation of the effects of a CR diet on mTORC1 signaling is substantially complicated by time-of-feeding effects of CR on longevity.

The protein kinase mTORC1 (mechanistic Target Of Rapamycin) is a critical regulator of insulin sensitivity through S6K1 and GRB10 mediated feedback inhibition of insulin receptor substrate [39, 40]. We therefore hypothesized that mTORC1 signaling might not always be reduced in CR-fed mice, and that hyperactivity of mTORC1 following refeeding in CR-fed mice explain the insulin resistance of CR-fed animals following feeding due to the negative feedback regulation of mTORC1 on insulin action. To investigate the role mTORC1 activity on insulin signaling we therefore measured the phosphorylation of mTORC1 substrates and downstream readouts, as well as phosphorylation of the mTORC2 substrate AKT S473 and the PDK1 substrate AKT T308 in both Liver and Muscle of both the night-fed study and morning-fed study (**Fig. 12-13**).

While we expected to see differences in mTORC1 activity between AL and CR-fed mice, we were surprised to see that fluctuations in hepatic mTORC1 activity were independent of diet group at majority of the timepoints in the night-fed study (**Fig. 12C**). As we expected, the phosphorylation of S6K1 and ULK1 S757 decreased as fasting duration increased; however the pattern of phosphorylation was S6 and AKT phosphorylation was more variable (**Fig. 12C**). We observed significant differences in mTORC1 activity at the earliest fasting timepoint (4hrs) for both morning-fed and night-fed studies (**Fig. 12C and Fig. 13A**) which suggested to us that we might see greater differences in mTOR signaling between diet groups immediately after feeding. Therefore, to examine mTORC1 activity post-feeding, we fasted all mice at 6AM in the morning to encourage feeding when the mice were refed (starting at 6PM), and staggered feeding such that we could time collection to the minute when each mice took

its first bite (**Fig. 12D**). When synchronizing the exact feeding time during their natural feeding state, both AL and CR mice exhibited the exact same hepatic mTORC1 activity (**Fig. 12E**). We also examined other nutrient responsive pathway and these may potentially be involved (**Figure 14**); however, at this time, we did not explore further on how these pathways mediate the CR response. These results suggest that contrary to conventional wisdom, mTORC1 activity is not substantially different between AL and CR-fed mice and does not mediate the post-feeding insulin resistance we observe in CR-fed mice.

Suppression of hepatic mTORC1 is required for insulin sensitivity in CR-Fed mice

To directly test if suppression of hepatic mTORC1 activity is required to elicit the CR effect we utilized mice lacking hepatic *TSC1* (TSC1-LKO). TSC1-LKO mice are viable and have constitutively active hepatic mTORC1 signaling, even during fasting [41], and we performed a series of metabolic measurements similar to those performed in morning-fed animals (**Fig. 15**). We performed these tests at two fasting durations (8hr and 21hr) in both sexes.

There were no differences in glucose tolerance between the WT and TSC1-LKO within each respective diet (**Fig. 15A**). However improved insulin sensitivity was only observed in the male WT fed a CR diet after a 21hr fast (**Fig. 15B**). This suggested that hepatic mTORC1 may mediate insulin sensitivity. We next examined if TSC1-LKO mice were able to maintain a tight blood glucose homeostasis as shown previously, and we found that there were no differences between the strains in their respective diets (**Fig. 15C**). Excluding insulin sensitivity, our data suggest that suppression of hepatic mTORC1 signaling is not required to elicit the CR response.

DISCUSSION

For over a century, CR has been studied in rodents and other organisms, and has become the gold standard for geroprotective interventions that promote healthy aging [11]. Throughout this period, many laboratory studies have utilized a CR regimen in which animals are fed once per day; mice fed once per day rapidly consume their daily allotment of food in 2-3 hours, resulting in a collaterally imposed fast of approximately 21 hours [9]. In recent work, our lab has shown that this fasting period is essential for many of the geroprotective effects of a CR diet in mice, including CR-induced improvements in insulin sensitivity, frailty, and lifespan [3].

However, there is a second critical consequence of this collaterally imposed fast that we have not explored; namely, the difficulty in comparing AL-fed and CR-fed mice with rigor. As but one simple example, if CR-fed mice are routinely fed in the morning, but are sacrificed for molecular analysis at around 7am the next day, AL-fed mice which fed overnight are being compared to mice that have fasted for almost 24 hours (if the CR mice are not refed prior to sacrifice) or freshly fed mice (if the CR mice were refed). Metabolic analysis, for instance the performance of GTT and ITT assays, is similarly confounded by both the time of day and how long AL and CR-fed mice have fasted prior to the assay.

To gain insight into how temporal factors contribute to the observed metabolic and molecular response of a CR, we investigated the metabolic and molecular response to fasting duration (every 4 hours) during a diurnal cycle and examined how time-of-day effects the outcome of physiological and molecular parameters. We found that the glucose tolerance of both AL and CR mice was dependent upon fasting duration, with

glucose tolerance decreasing as we prolonged the fast. At all fasting time points from 4-20 hours, CR-fed mice had improved glucose tolerance relative to AL-fed mice in both morning and night-fed studies. Surprisingly, we found that one of the key physiological hallmarks of CR in mammals – improved insulin sensitivity – was only observed in mice that had been fasted for at least 20 hours. In fact, CR-fed animals were insulin resistant as assessed via an ITT when tested after a 4 hr fast, and this response was conserved in both males and females and in both young and old mice. Furthermore, this response to insulin administration was not dependent upon time of day or time of feeding but was specifically dependent on how long the animals were fasted prior to the ITT. ITTs are often performed in CR-fed mice in the morning following an overnight fast; under such conditions, both AL-fed and CR-fed mice are fasted close to 24 hours, and CR-fed mice appear extraordinarily insulin sensitive. These results suggest that although the widely conserved insulin-sensitizing effects of a CR diet are real, they are also an artifact of how long mice have fasted prior to the assay. A long-standing problem in the aging field has been reconciling the lifespan-extending effects of an insulin-sensitizing CR diet with the long lifespan of a number of mouse strains that are insulin resistant [32]. Our results here suggest that in fact, CR mice are not necessarily more insulin sensitive, and under normal housing and feeding conditions may in fact be insulin resistant.

To address this unique response to insulin in CR-fed animals we measured blood glucose and circulating insulin levels with a meal-stimulated insulin secretion (MSIS) test. While we expected to see a linear relationship with fasting duration or specific differences between diet, we observed irregular trends in circulating insulin levels – at some of the timepoints CR mice exhibited higher insulin levels and at other timepoints

AL mice displayed higher insulin levels. This may be a response due to misaligned feeding (i.e., feeding at a time where normally not fed). Indeed, previous studies have shown insulin secretion is highly regulated by circadian controls and this mechanism is gated within a limited timeframe [17, 18]. In addition, this response is altered depending on feeding schedule [19, 20]. However, when assessing blood glucose levels, we show that CR mice were able to maintain tight blood glucose levels up to a 20 hr fast. This suggested that either CR mice were able to maintain this tight blood glucose levels independently of insulin or insulin was involved but circulating insulin levels were dependent on the time of day.

To address if insulin mediated the tight blood glucose levels during a prolonged fast, we used acute diazoxide treatment to suppress insulin secretion during the duration of an MSIS test. To our surprise, CR-mice treated with diazoxide released exorbitant amount of glucose, to the point where our glucometer could not read the blood glucose value, while AL-fed mice only had a moderate increase in glucose as expected. This suggested to us that insulin is essential in regulating blood glucose levels during a prolonged fast, and it may in fact be inhibiting gluconeogenesis during this prolonged fast. Indeed, when measuring gluconeogenic capacity with ATT, glucose production from alanine was limited in CR-fed animals.

Our initial morning-fed CR studies led us to question whether time of day feeding would impact the outcome of the metabolic phenotype we have observed previously. Indeed, there are plethora of studies associating shift-workers with metabolic syndrome [42, 43]. Morning-fed CR mice have often been suggested to be a shift-worker’s feeding regimen and may provide one explanation as to why we observe irregular insulin levels

at different times of the day. However, many of these studies are in the context of an *ad libitum* feeding condition, and there are only a handful of studies which have explored understanding restricted feeding at irregular times [44-47]. While we expected to see an alignment in insulin levels as previously predicted, NCR had 2-3-fold increase in circulating insulin levels during ZT 5, 9 and 13. Surprisingly, at least for AL-fed mice, fed insulin levels were highly dependent on time-of-day and not fasting duration. It is important to note that CR feeding regimen appears to rescue metabolic dysfunction associated with shifted feeding schedule, and in our previous study we have shown that morning-fed CR mice have 30% extension of lifespan [3]. Therefore, perhaps limiting the window of eating could rescue the metabolic dysfunction associated with shift workers.

The protein kinase mTOR (mechanistic Target Of Rapamycin) is a critical regulator of insulin sensitivity through S6K1 mediated feedback inhibition of insulin receptor substrate [39, 40]. Therefore, this pathway was the obvious choice to investigate the unique response to insulin in CR-fed mice. Surprisingly, hepatic mTORC1 activity was strictly mediated by fed and fasted state regardless of diet and this activity was perfectly matched when feeding time was initiated around the same time (~ZT 12). Furthermore, it appeared that hepatic mTORC1 activity was more robust in night-fed animals compared to morning-fed animals. Since both AL and CR-fed animals were equally fasted in this study, we initially hypothesized that mTORC1 activity is inhibited daily in CR fed animals (as CR mice are fasted for about 22 hrs), while AL fed animals have a consistent level of mTORC1 activity throughout the day (as AL mice are feeding throughout the day). Therefore, we examined the metabolic outcome of a

strain with hyperactive hepatic mTORC1 activity, TSC1-LKO. However, suppression of hepatic mTORC1 was not required to elicit many of the phenotypes observed in CR animals; with the exception of insulin sensitivity at the later fasting timepoint. These results suggest that inhibition of mTORC1, at least in the liver, is not required to elicit the CR response.

Our study was subject to limitations. First, we assessed insulin sensitivity via I.P. administration of insulin, which most directly measures insulin-stimulated glucose uptake. Future research using clamps may be useful in assessing insulin sensitivity; however, may be difficult to capture the early fasting conditions as clamps require continuous glucose infusion. MSIS test relied on food consumption of the animals within a given time, with CR mice eating more food than the AL mice (CR mice are capable of eating more food than AL animals at each timepoint). While we cannot completely dissociate insulin secretion from food consumption, we did not see a trend in insulin levels based on the amount of food. Additionally, we did not measure insulin levels after 15 minutes post-meal, timepoint based on the biphasic nature of insulin secretion [48]. However, gastric emptying in mice takes about 74 minutes (with the completion of the meal) compared to about 30 minutes in humans [49] and intraduodenal glucose delivery, peak insulin levels were observed after 120 minutes [50]. Therefore, measuring insulin levels after 120 minutes should be sufficient to detect overall insulin secretion after a meal. While our insulin data may not represent peak insulin secretion levels, it reveals the importance of meal-timing and time-of-day in circulating insulin levels.

Our data demonstrate that, under laboratory conditions, it is important to consider timing and duration of a fast when implementing metabolic studies to dissociate the response from a fast. For example, in our initial morning-fed study, we allowed AL-fed mice to feed in their natural state while feeding the CR mice at the start of the light cycle prior to metabolic tests. Under these conditions, we make the assumption that AL-fed animals are fasted equivalently to CR fed animals; however, it is more likely that AL-fed animals have been fasted for longer than CR mice as they finish their last meal before the start of the light cycle [51] , which can explain the differences in mTORC1 activity observed at the earliest fasting duration. Additionally, an overnight fast is ill-defined and if started before the start of their first meal, the fasting duration most likely exceeds 24hrs. This was evident in our AL-fed mice, where the night fasted animals (starting at 9pm) had improved glucose tolerance compared to the morning fasted animals (starting at 9am). Finally, variety of hormones, including insulin and glucocorticoids, are regulated in a circadian manner [52]; therefore, the interaction between these hormones and the time of day should be considered when conducting metabolic studies.

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COMPETING INTERESTS

D.W.L has received funding from, and is a scientific advisory board member of, Aeovian Pharmaceuticals, which seeks to develop novel, selective mTOR inhibitors for the treatment of various diseases. The remaining authors declare no competing interests.

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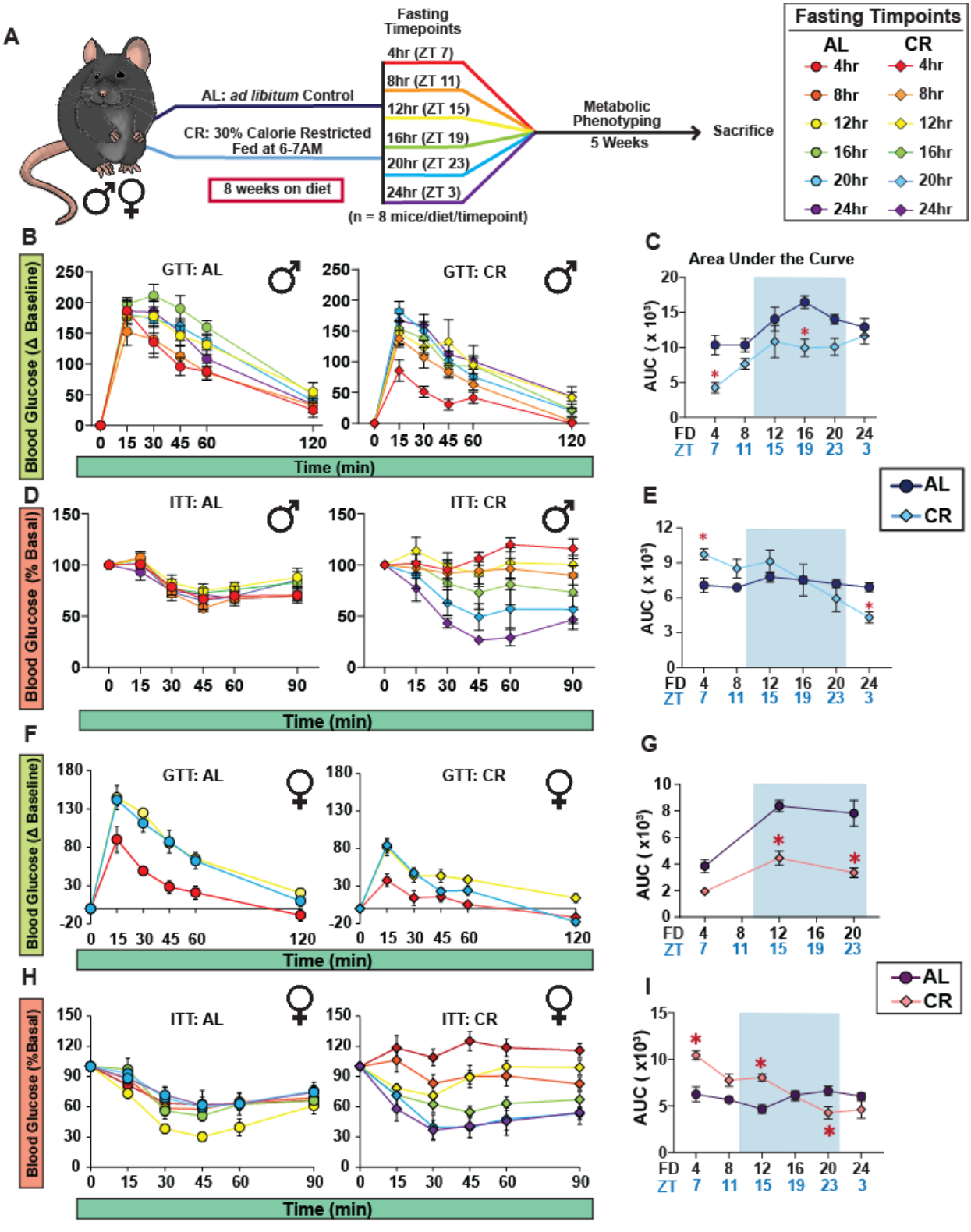
A
Fasting Timepoints
4hr (ZT 7)
8hr (ZT 11)
12hr (ZT 15)
16hr (ZT 19)
20hr (ZT 23)
24hr (ZT 3)
AL: ad libitum Control
CR: 30% Calorie Restricted
Fed at 6-7AM
8 weeks on diet
(n = 8 mice/diet/timepoint)
Metabolic Phenotyping
5 Weeks
Sacrifice
Fasting Timpoints
AL
CR
4hr
8hr
12hr
16hr
20hr
24hr
B
Blood Glucose (Δ Baseline)
GTT: AL
GTT: CR
Time (min)
C
Area Under the Curve
AUC (x 10³)
FD
ZT
D
Blood Glucose (% Basal)
ITT: AL
ITT: CR
Time (min)
E
AUC (x 10³)
AL
CR
F
Blood Glucose (Δ Baseline)
GTT: AL
GTT: CR
Time (min)
G
AUC (x10³)
H
Blood Glucose (%Basal)
ITT: AL
ITT: CR
Time (min)
I
AUC (x10³)
AL
CR

Figure 1: Fasting duration from the time of last feeding impacts how an animal responds to a test. (A) Schematic of experiment design with CR mice fed every morning between 6-7AM. (B-C) Combined glucose (1g glucose/kg body weight) tolerance test of male C57BL/6J mice after during respective fasting timepoints within each diet (AL, n = 7-8 per time point; CR, n = 7-8 per time point biologically independent mice) (B), area under the curve (C). (D-E) Combined insulin (0.5U/kg body weight) tolerance test of male C57BL/6J mice after during respective fasting durations within each diet (AL, n = 7-8 per timepoint; CR, n = 7-8 per timepoint biologically independent mice) tolerance test (D), area under the curve (E). (F-G) Combined glucose (1g glucose/kg body weight) tolerance test of female C57BL/6J mice after during respective fasting durations within each diet (AL, n = 8 per time point; CR, n = 8 per time point biologically independent mice) tolerance test (F), area under the curve (G). (H-I) Combined insulin (0.5U/kg body weight) tolerance test of female C57BL/6J mice after during respective fasting durations within each diet (AL, n = 8 per time point; CR, n = 8 per time point biologically independent mice) tolerance test (H), area under the curve (I). * symbol represents a significant difference versus AL-fed mice based on Šídák's test post two-way ANOVA.

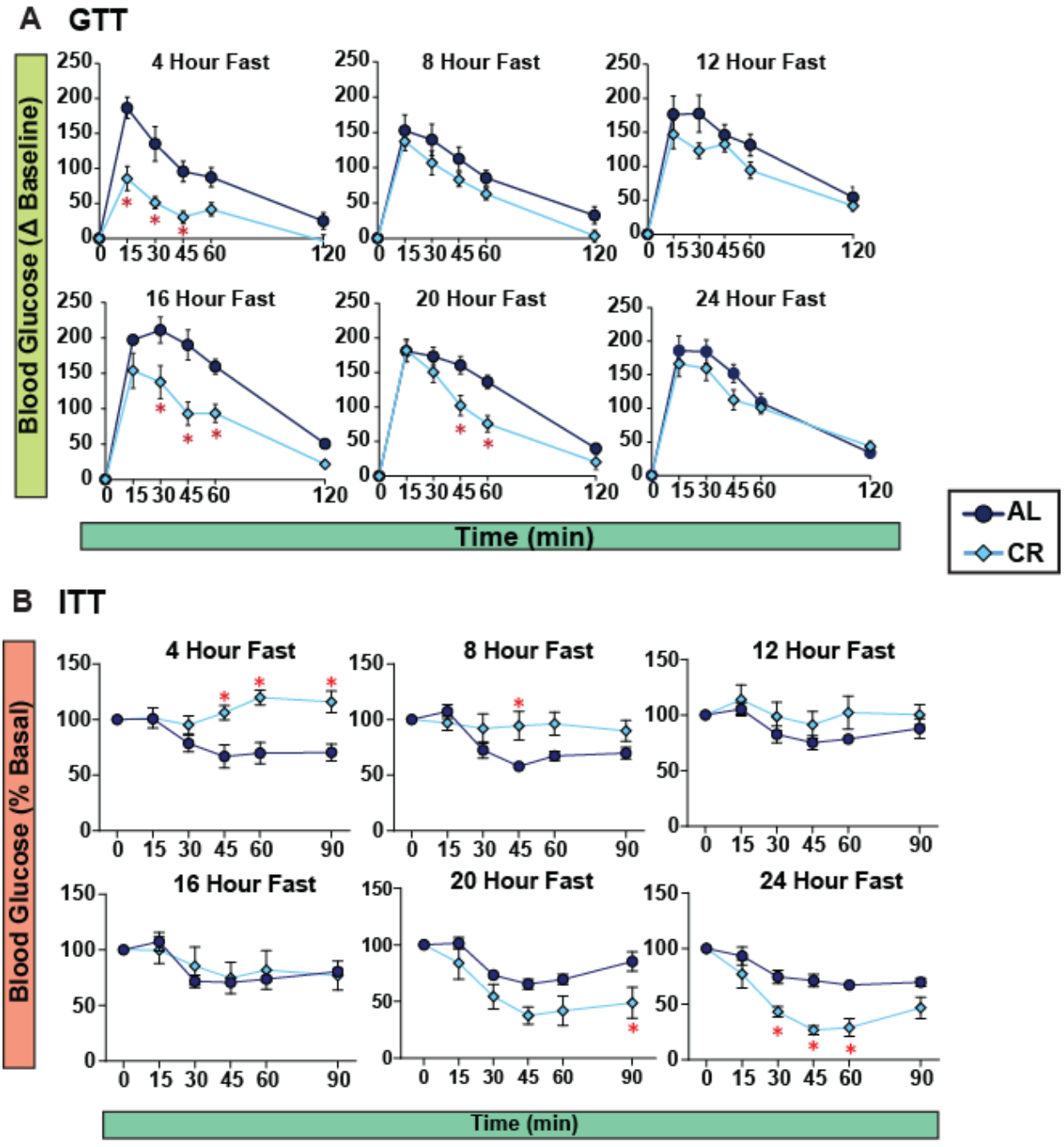
A GTT
Blood Glucose (Δ Baseline)
4 Hour Fast
8 Hour Fast
12 Hour Fast
16 Hour Fast
20 Hour Fast
24 Hour Fast
Time (min)
AL
CR
B ITT
Blood Glucose (% Basal)
4 Hour Fast
8 Hour Fast
12 Hour Fast
16 Hour Fast
20 Hour Fast
24 Hour Fast
Time (min)

Figure 2. Glucose and Insulin tolerance test of male C57BL/6J mice in morning-fed study related to Figure 1. (A) Glucose (1g glucose/kg body weight) tolerance test of male C57BL/6J mice after during respective fasting timepoints within each diet (AL, n = 7-8 per time point; CR, n = 7-8 per time point biologically independent mice) (B) insulin (0.5U/kg body weight) tolerance test of male C57BL/6J mice after during respective fasting durations within each diet (AL, n = 7-8 per timepoint; CR, n = 7-8 per timepoint biologically independent mice) tolerance test. * symbol represents a significant difference versus AL-fed mice based on Šídák's test post two-way repeated measure ANOVA.

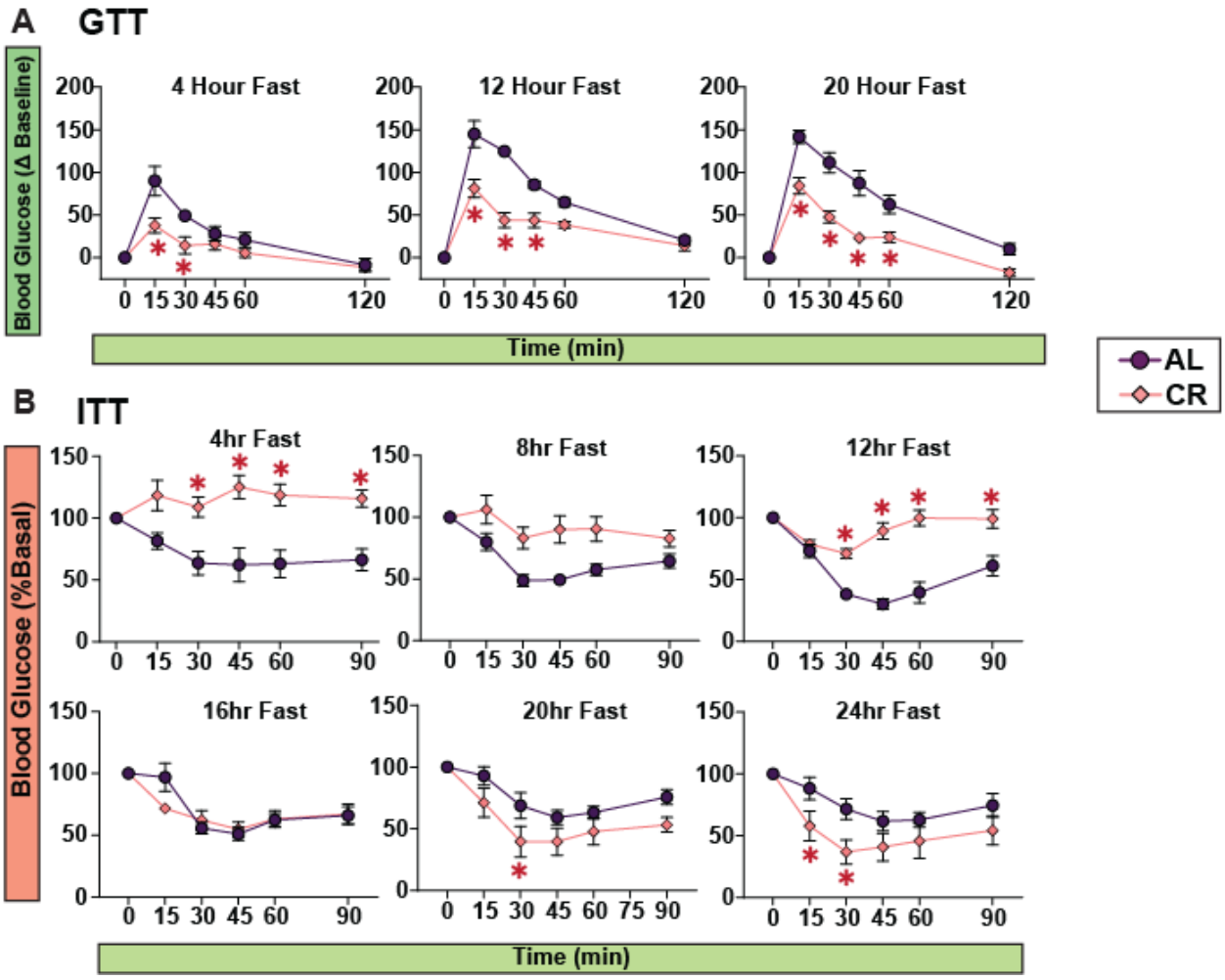

Figure 3. Glucose and Insulin tolerance test of female C57BL/6J mice in morning-fed study related to Figure 1. (A) Glucose (1g glucose/kg body weight) tolerance test of male C57BL/6J mice after during respective fasting timepoints within each diet (AL, n = 7-8 per time point; CR, n = 7-8 per time point biologically independent mice) (B) insulin (0.5U/kg body weight) tolerance test of male C57BL/6J mice after during respective fasting durations within each diet (AL, n = 7-8 per timepoint; CR, n = 7-8 per timepoint biologically independent mice) tolerance test. * symbol represents a significant difference versus AL-fed mice based on Šídák's test post two-way repeated measure ANOVA.

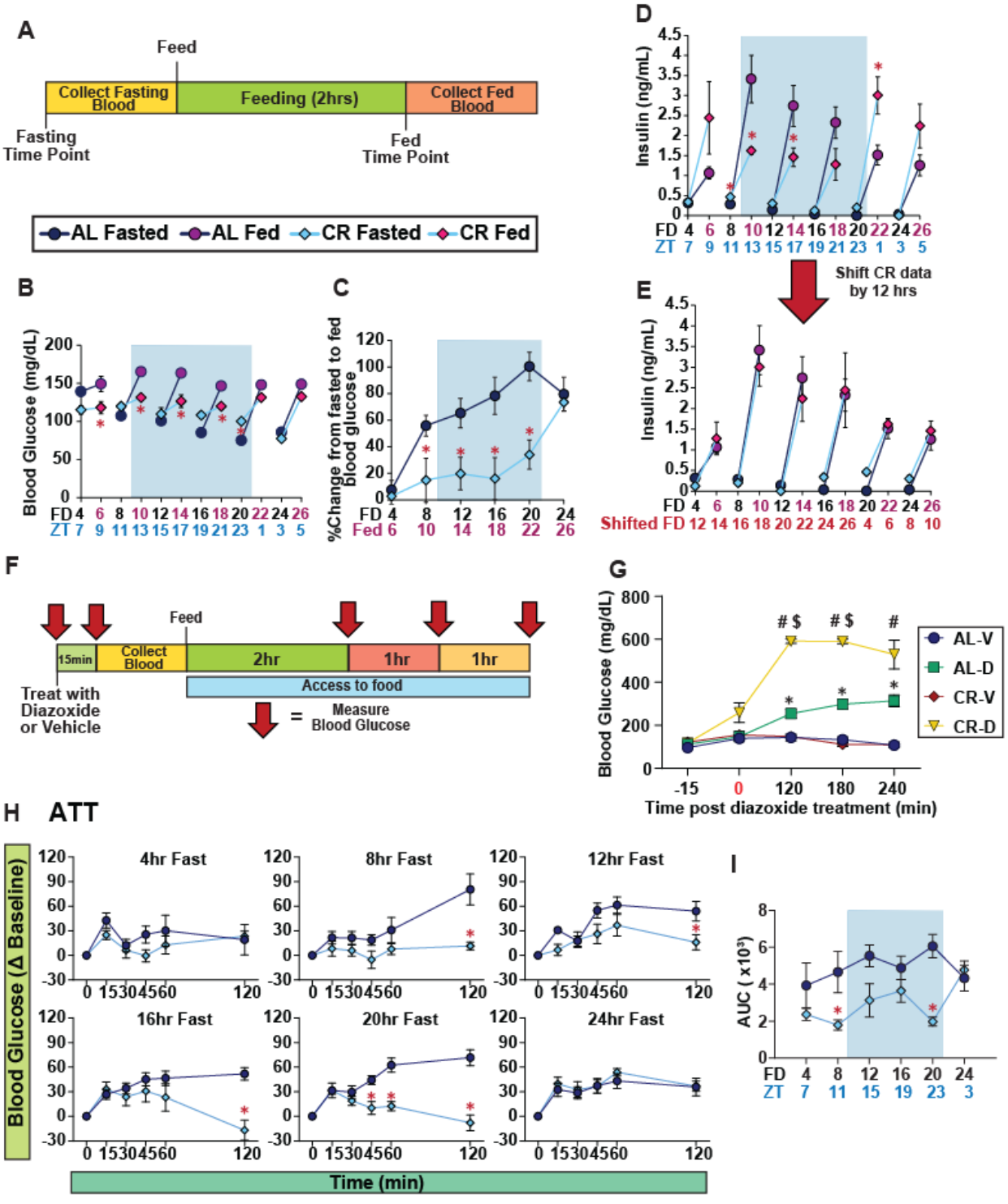
A
Feed
Collect Fasting Blood
Feeding (2hrs)
Collect Fed Blood
Fasting Time Point
Fed Time Point
AL Fasted
AL Fed
CR Fasted
CR Fed
B
Blood Glucose (mg/dL)
C
%Change from fasted to fed blood glucose
D
Insulin (ng/mL)
E
Shift CR data by 12 hrs
Insulin (ng/mL)
Shifted FD 12 14 16 18 20 22 24 26 4 6 8 10
F
Feed
15min
Collect Blood
2hr
1hr
1hr
Access to food
Treat with Diazoxide or Vehicle
= Measure Blood Glucose
G
Blood Glucose (mg/dL)
AL-V
AL-D
CR-V
CR-D
Time post diazoxide treatment (min)
H ATT
Blood Glucose (Δ Baseline)
4hr Fast
8hr Fast
12hr Fast
16hr Fast
20hr Fast
24hr Fast
Time (min)
I
AUC (x10³)

Figure 4. Meal-stimulated insulin level is not dependent on fasting duration, but dependent on time of feeding. (A) Schematic of meal stimulated insulin secretion test. (B-C) Blood glucose level of fasted and fed state during respective fasting timepoints (AL, n = 7-8 per timepoint; CR, n = 7-8 per timepoint biologically independent mice) (B), percent change in blood glucose level from fasted to fed state (C). (D-E) Insulin level of fasted and fed state during respective fasting timepoints (AL, n = 7-8 per timepoint; CR, n = 6-7 per timepoint of biologically independent mice) (D), data presented with CR data shifted by 12 hours (E). (F) Schematic of acute diazoxide treatment experiment. (G) Blood glucose level of mice treated with either vehicle or diazoxide after a 12 hr fast. (H-I) Alanine (2g alanine/kg body weight) tolerance test male C57BL/6J mice after during respective fasting timepoints within each diet (AL, n = 7-8 per time point; CR, n = 7-8 per time point biologically independent mice) (H), area under the curve (I).

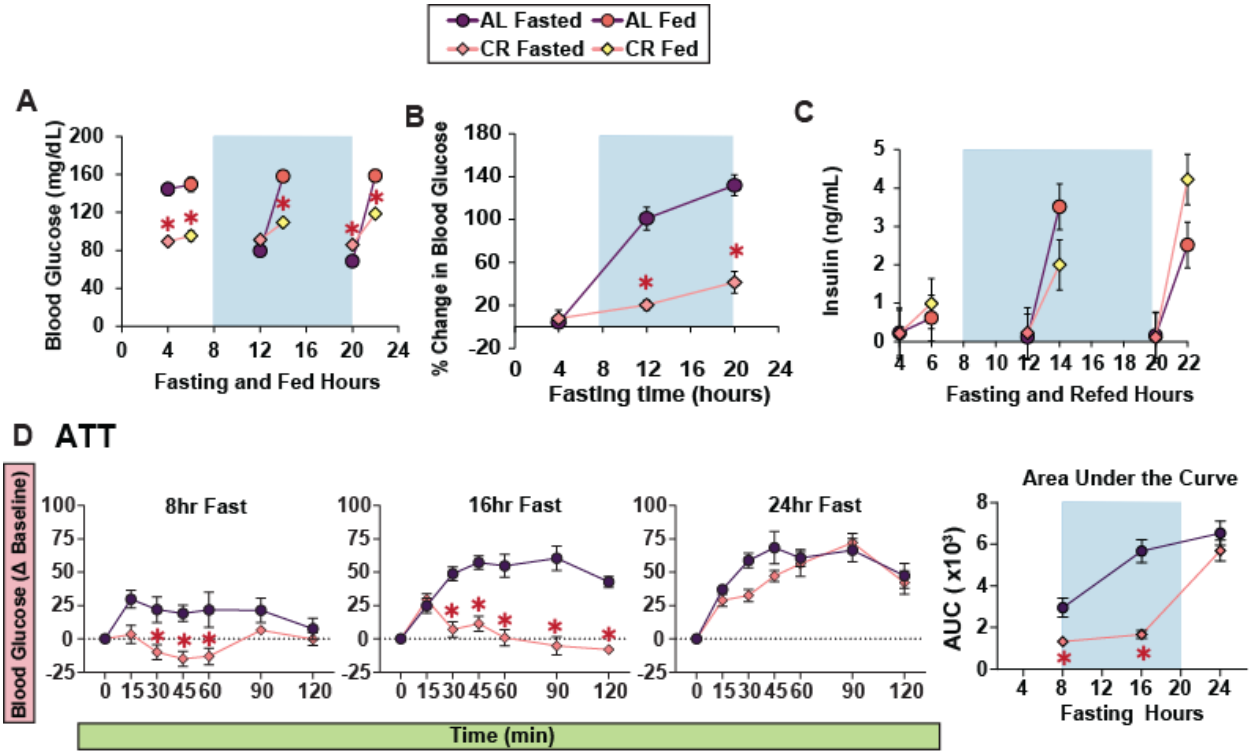

Figure 5. Meal-stimulated insulin level of female C57BL/6J mice in morning-fed study related in to Figure 2. (A-B) Blood glucose level of fasted and fed state during respective fasting timepoints (AL, n = 8 per timepoint; CR, n = 8 per timepoint biologically independent mice) (A), percent change in blood glucose level from fasted to fed state (B). (C) Insulin level of fasted and fed state during respective fasting timepoints (AL, n = 8 per timepoint; CR, n = 8 per timepoint of biologically independent mice). (E) Alanine (2g alanine/kg body weight) tolerance test after respective fasting timepoints within each diet (AL, n = 8 per time point; CR, n = 8 per time point biologically independent mice).

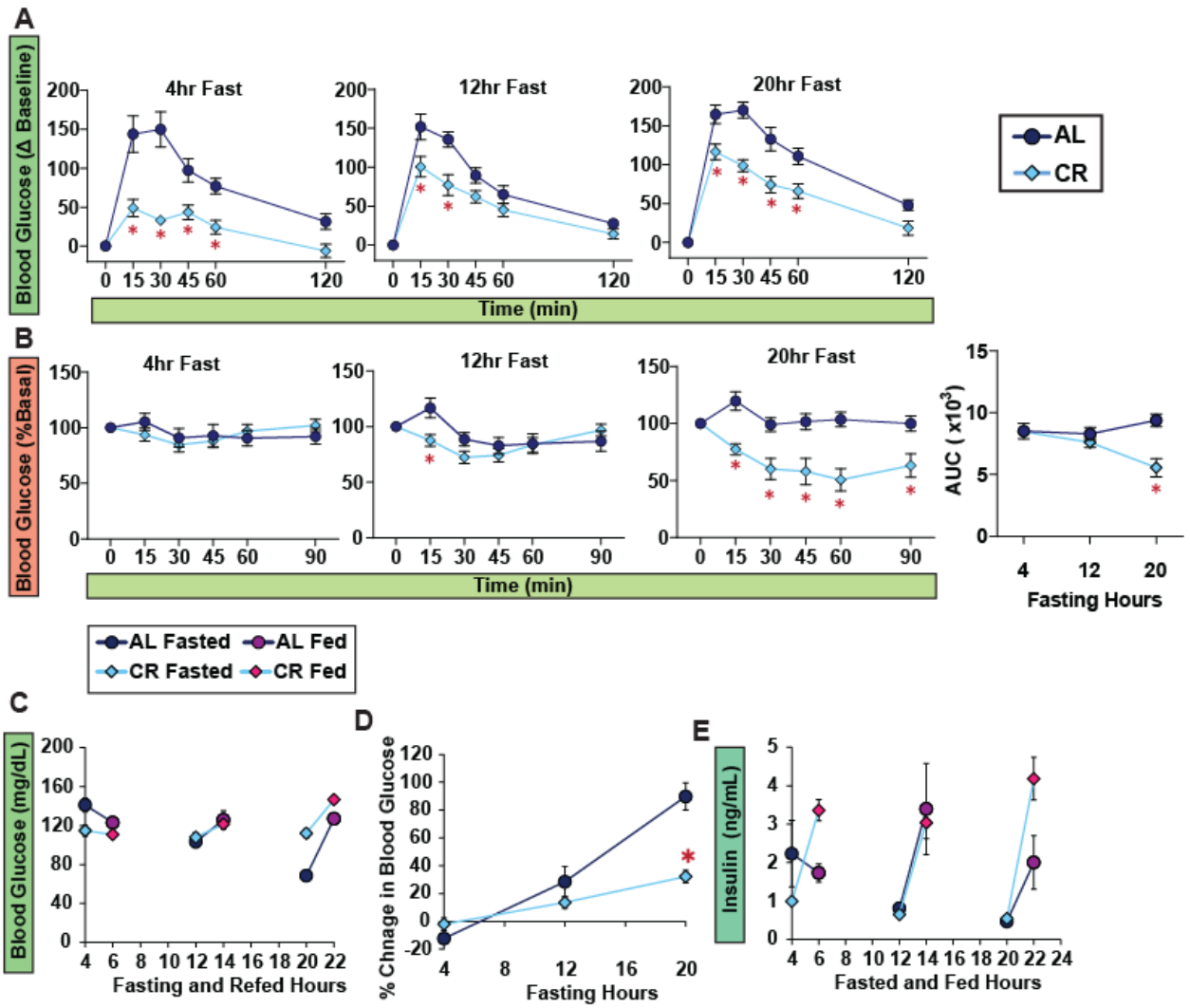
A
Blood Glucose (Δ Baseline)
4hr Fast
12hr Fast
20hr Fast
AL
CR
Time (min)
B
Blood Glucose (%Basal)
AUC (x10³)
Fasting Hours
AL Fasted
AL Fed
CR Fasted
CR Fed
C
Blood Glucose (mg/dL)
Fasting and Refed Hours
D
% Chnage in Blood Glucose
Fasting Hours
E
Insulin (ng/mL)
Fasted and Fed Hours

Figure 6. Metabolic phenotype of aged male C57BL/6J mice in morning-fed study related to Figures 1-2. (A) Glucose (1g glucose/kg body weight) tolerance test of male C57BL/6J mice after during respective fasting timepoints within each diet (AL, n = 8 per time point; CR, n = 8 per time point biologically independent mice) (B) insulin (0.5U/kg body weight) tolerance test of male C57BL/6J mice after during respective fasting durations within each diet (AL, n = 8 per timepoint; CR, n = 8 per timepoint biologically independent mice) tolerance test. (C) Blood glucose level of fasted and fed state during respective fasting timepoints (AL, n = 8 per timepoint; CR, n = 8 per timepoint biologically independent mice) (C), percent change in blood glucose level from fasted to fed state (D). (E) Insulin level of fasted and fed state during respective fasting timepoints (AL, n = 8 per timepoint; CR, n = 8 per timepoint of biologically independent mice). * symbol represents a significant difference versus AL-fed mice based on Šídák's test post two-way repeated measure ANOVA.

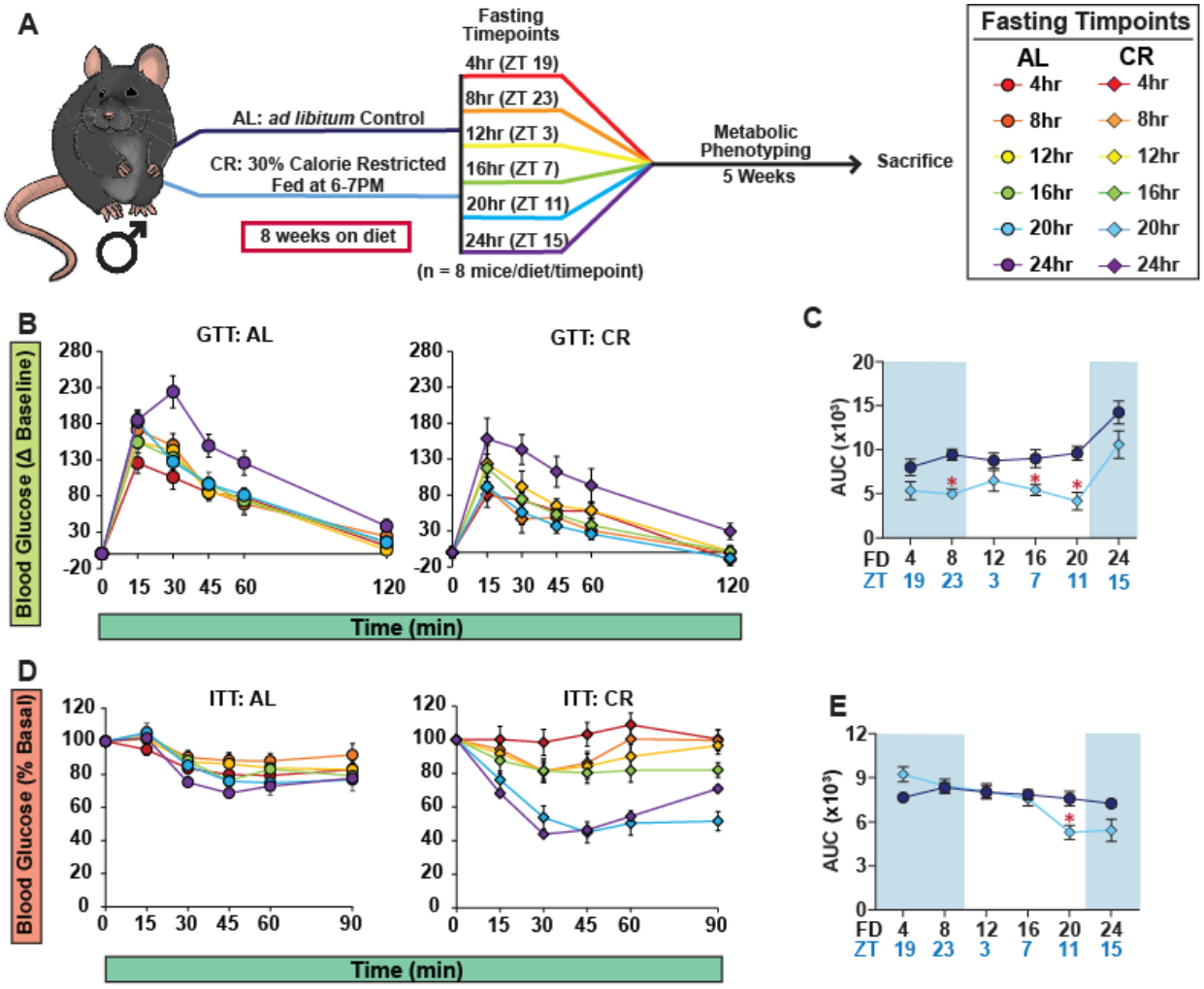

Figure 7. Outcome of glucose and insulin tolerance test is dependent on fasting duration and not time of day. (A) Schematic of experiment design with CR mice fed every morning between 6-7PM. (B-C) Combined glucose (1g glucose/kg body weight) tolerance test of male C57BL/6J mice after during respective fasting timepoints within each diet (AL, n = 7-8 per time point; CR, n = 7-8 per time point biologically independent mice) (B), area under the curve (C). (D-E) Combined insulin (0.5U/kg body weight) tolerance test of male C57BL/6J mice after during respective fasting durations within each diet (AL, n = 7-8 per timepoint; CR, n = 7-8 per timepoint biologically independent mice) tolerance test (D), area under the curve (E).

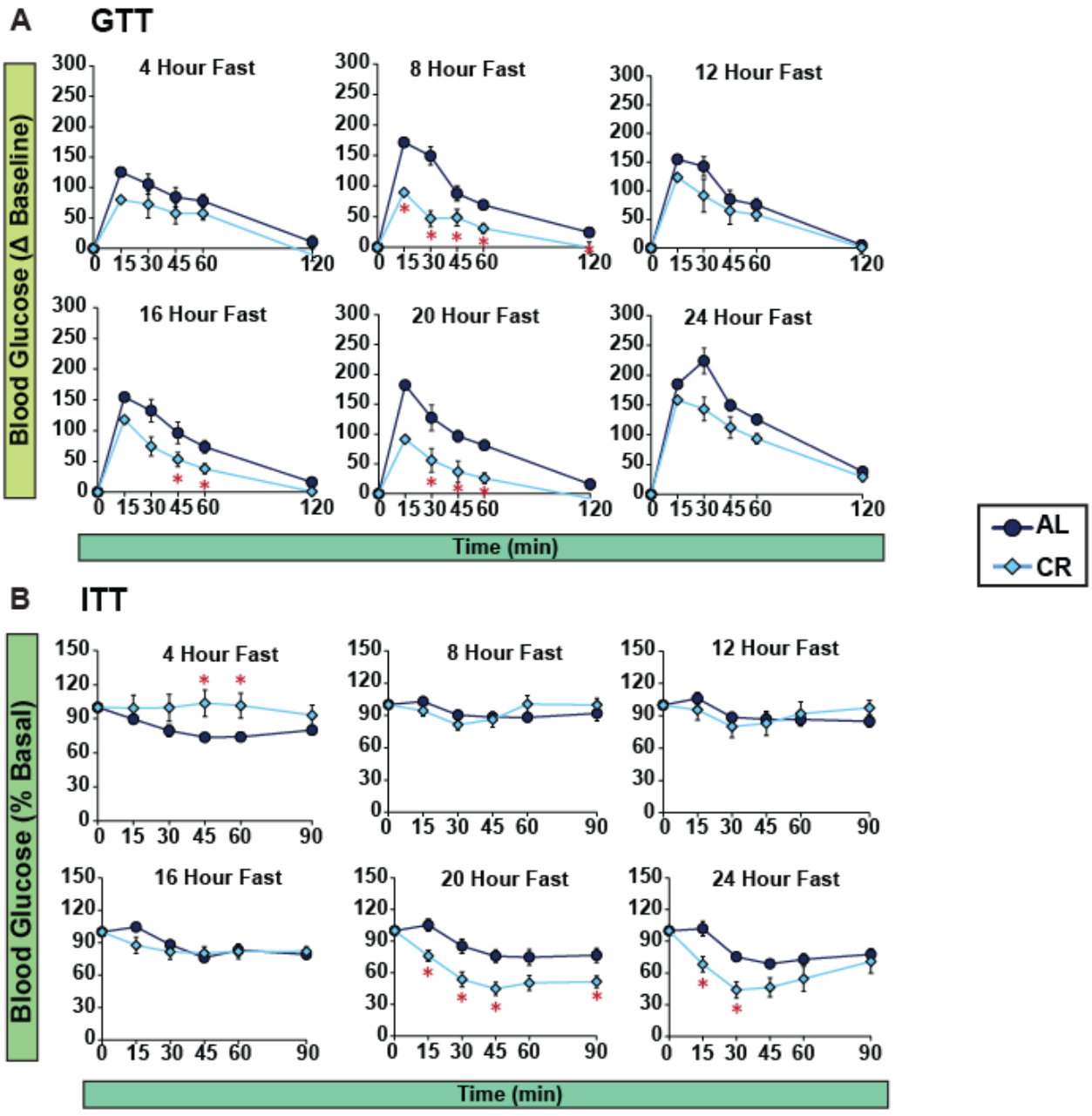
A
GTT
Blood Glucose (Δ Baseline)
4 Hour Fast
8 Hour Fast
12 Hour Fast
16 Hour Fast
20 Hour Fast
24 Hour Fast
Time (min)
AL
CR
B
ITT
Blood Glucose (% Basal)
4 Hour Fast
8 Hour Fast
12 Hour Fast
16 Hour Fast
20 Hour Fast
24 Hour Fast
Time (min)

Figure 8. Glucose and Insulin tolerance test of C57BL/6J mice in night-fed study related to Figure 3. (A) Glucose (1g glucose/kg body weight) tolerance test of male C57BL/6J mice after during respective fasting timepoints within each diet (AL, n = 7-8 per time point; CR, n = 7-8 per time point biologically independent mice) (B) insulin (0.5U/kg body weight) tolerance test of male C57BL/6J mice after during respective fasting durations within each diet (AL, n = 7-8 per timepoint; CR, n = 7-8 per timepoint biologically independent mice) tolerance test. * symbol represents a significant difference versus AL-fed mice based on Šídák's test post two-way repeated measure ANOVA.

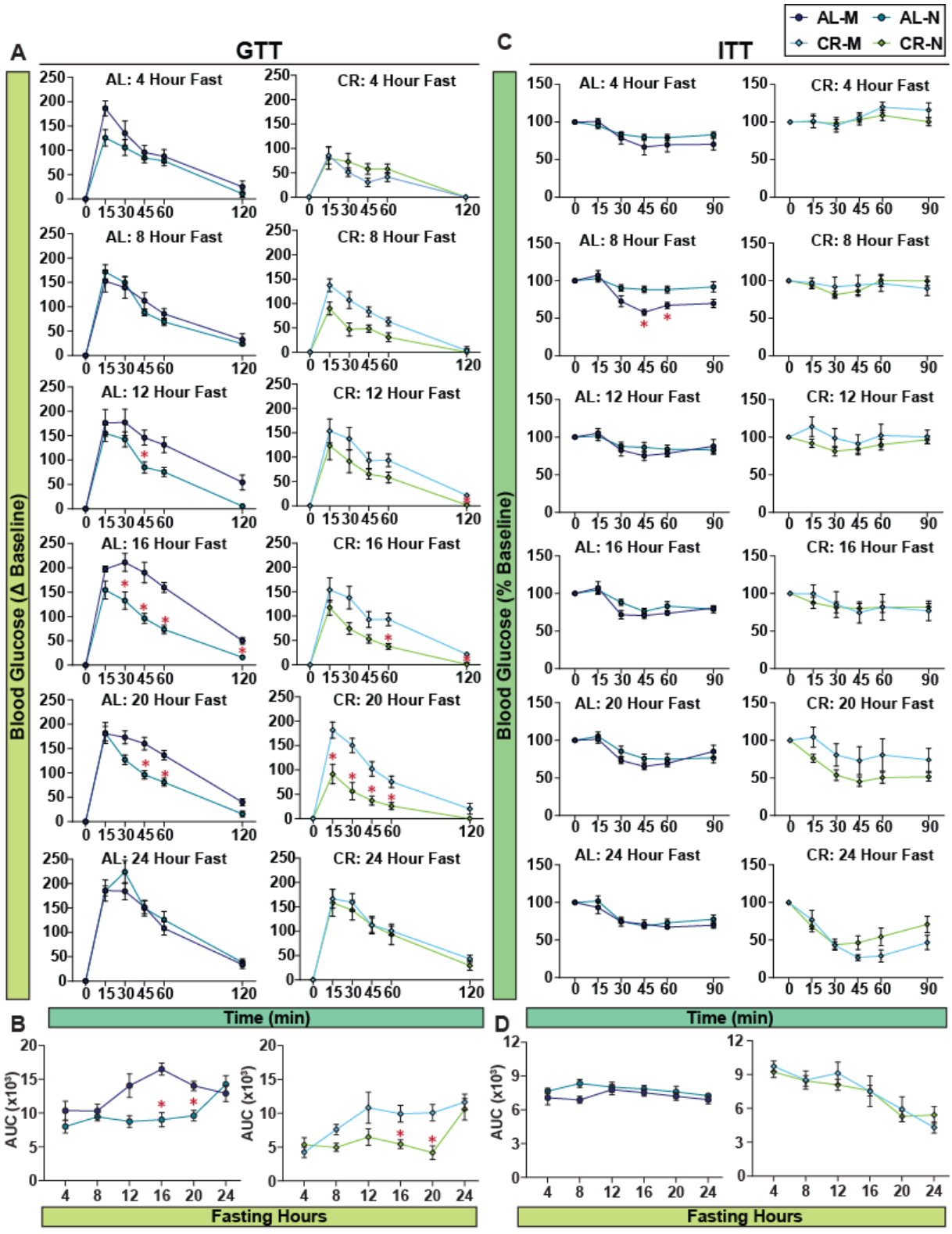

Figure 9. Comparison between morning-fed and night fed study for glucose and insulin tolerance test related to Figures 2 and 8.

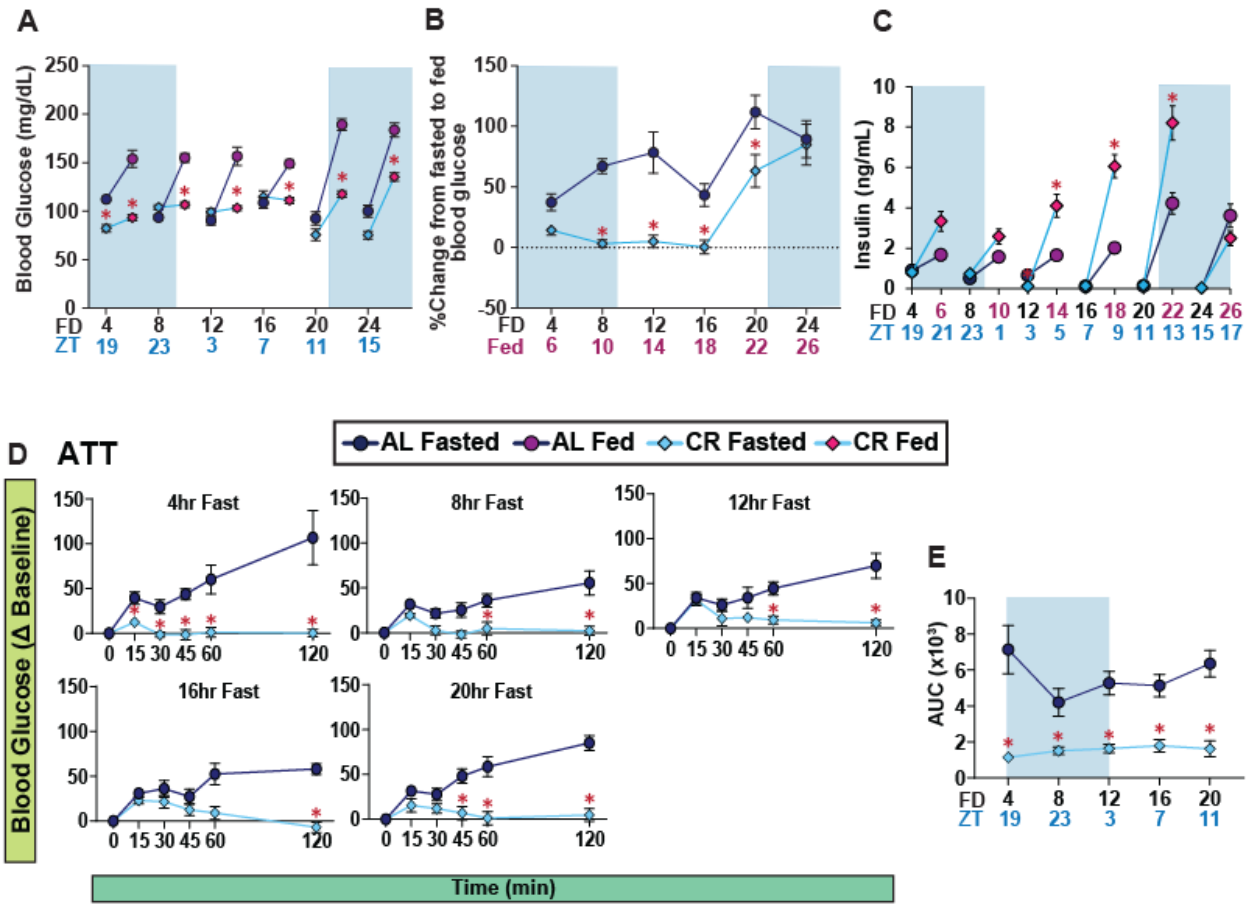

Figure 10. Circulating fed insulin level is dependent on time of day. (A-B) Blood glucose level of fasted and fed state during respective fasting timepoints (AL, n = 7-8 per timepoint; CR, n = 7-8 per timepoint biologically independent mice) (A), percent change in blood glucose level from fasted to fed state (B). (C) Insulin level of fasted and fed state during respective fasting timepoints (AL, n = 7-8 per timepoint; CR, n = 6-7 per timepoint of biologically independent mice) (D-E) Alanine (2g alanine/kg body weight) tolerance test male C57BL/6J mice after during respective fasting timepoints within each diet (AL, n = 7-8 per time point; CR, n = 7-8 per time point biologically independent mice).

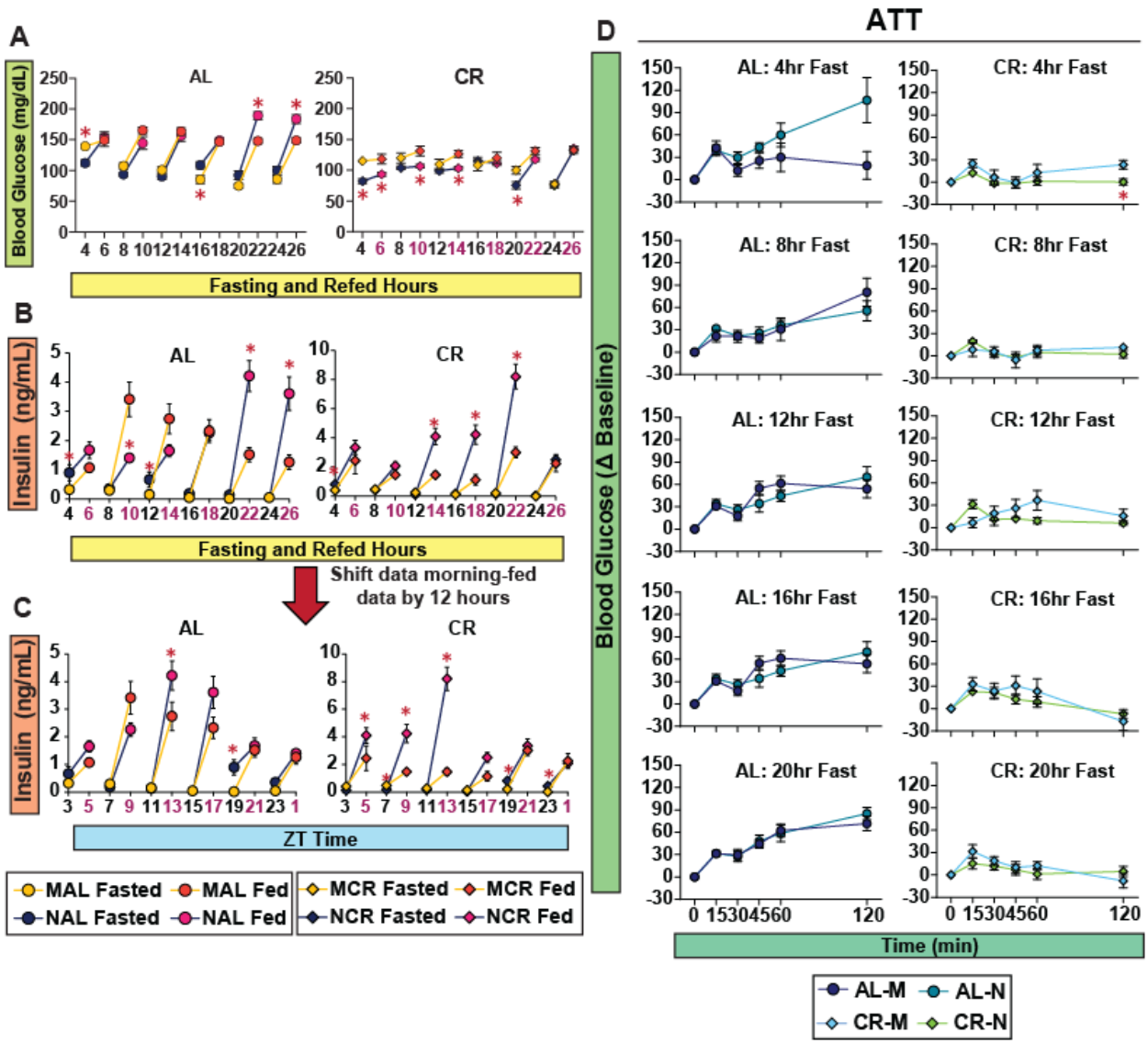

Figure 11. Comparison between morning-fed and night-fed study for meal-stimulated insulin levels and alanine tolerance test related to Figures 4 and 10.

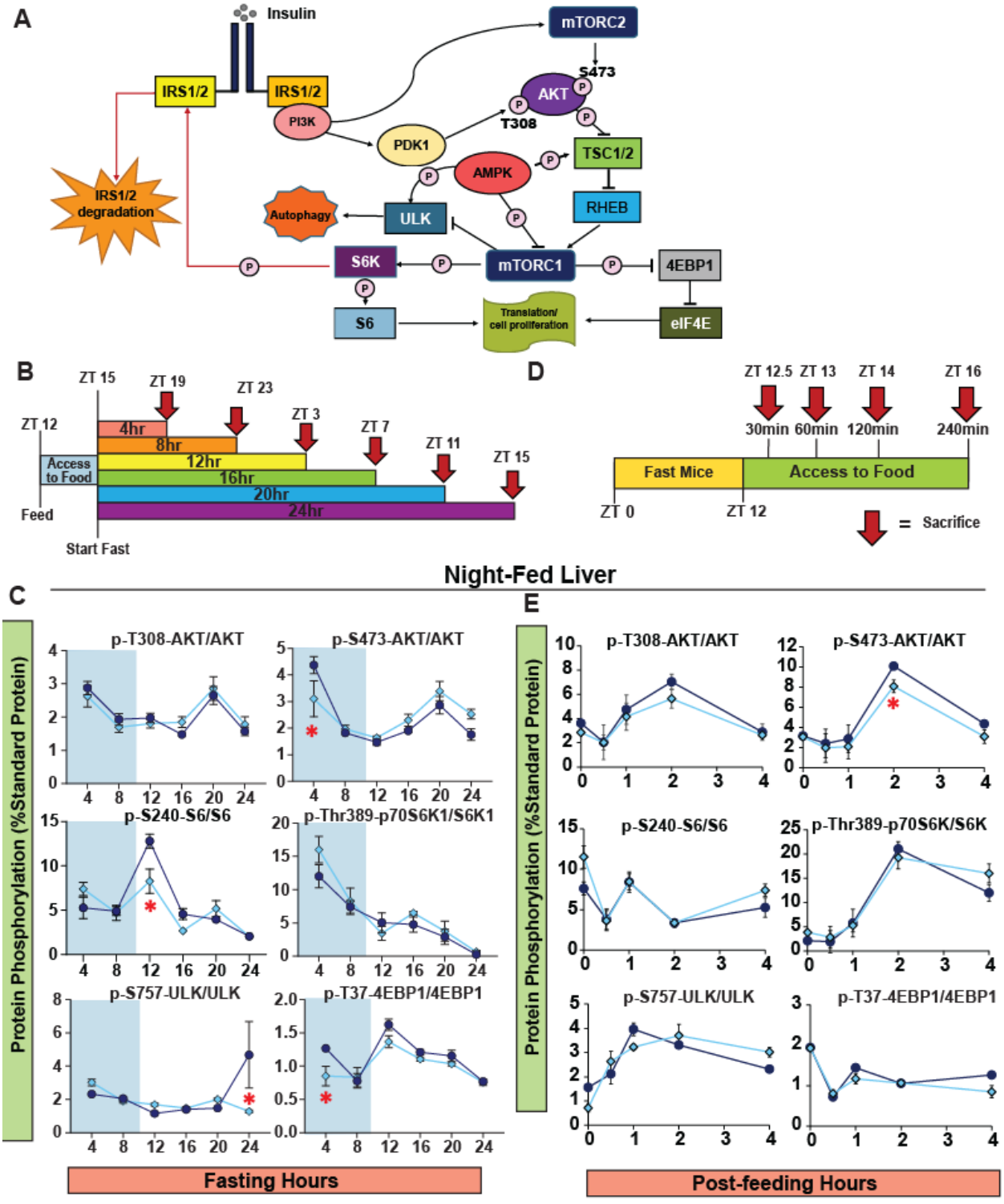
A
Insulin
IRS1/2
IRS1/2
PI3K
mTORC2
S473
AKT
T308
PDK1
TSC1/2
AMPK
IRS1/2 degradation
Autophagy
ULK
RHEB
S6K
mTORC1
4EBP1
S6
Translation/ cell proliferation
eIF4E
B
ZT 15
ZT 19
ZT 23
ZT 12
ZT 3
ZT 7
ZT 11
ZT 15
4hr
8hr
12hr
16hr
20hr
24hr
Access to Food
Feed
Start Fast
D
ZT 12.5
ZT 13
ZT 14
ZT 16
30min
60min
120min
240min
Fast Mice
Access to Food
ZT 0
ZT 12
= Sacrifice
Night-Fed Liver
C
E
Protein Phosphorylation (%Standard Protein)
p-T308-AKT/AKT
p-S473-AKT/AKT
p-S240-S6/S6
p-Thr389-p70S6K1/S6K1
p-S757-ULK/ULK
p-T37-4EBP1/4EBP1
p-T308-AKT/AKT
p-S473-AKT/AKT
p-S240-S6/S6
p-Thr389-p70S6K/S6K
p-S757-ULK/ULK
p-T37-4EBP1/4EBP1
Fasting Hours
Post-feeding Hours

Figure 12. Hepatic mTORC1 activity is mediated by fed and fasted stated. (A) Schematic of mTORC1 and mTORC2 pathway. (B) Schematic of experimental design to collect tissues during the fasted state. (C) Western blot analyses of mTORC1 and mTORC2 signaling in the liver of mice during respective fasting timepoints within each diet (AL, n = 6 per time point; CR, n = 6 per time point biologically independent mice). (D) Schematic of experimental design to collect tissues during the fed state. (E) Western blot analyses of mTORC1 and mTORC2 signaling in the liver of mice during respective fed points (AL, n = 6 per time point; CR, n = 6 per time point biologically independent mice).

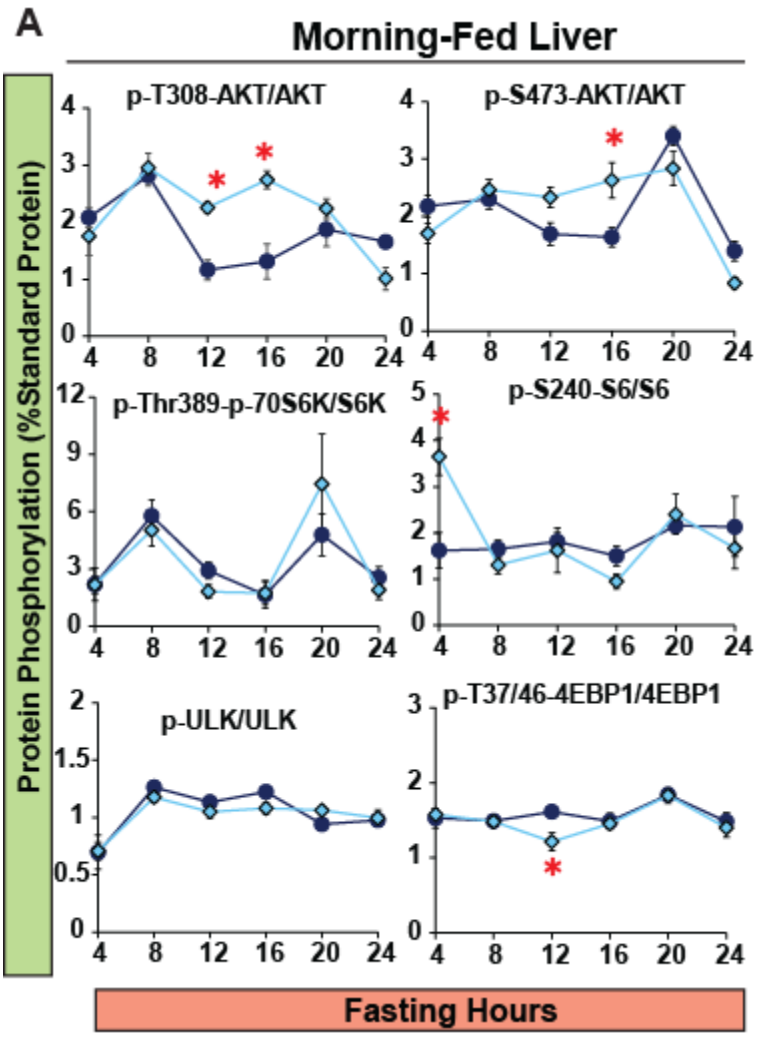

Figure 13. Hepatic mTORC1 activity of male C57BL/6J mice in morning-fed study. (A) Western blot analyses of mTORC1 and mTORC2 signaling in the liver of mice during respective fasting timepoints within each diet (AL, n = 6 per time point; CR, n = 6 per time point biologically independent mice).

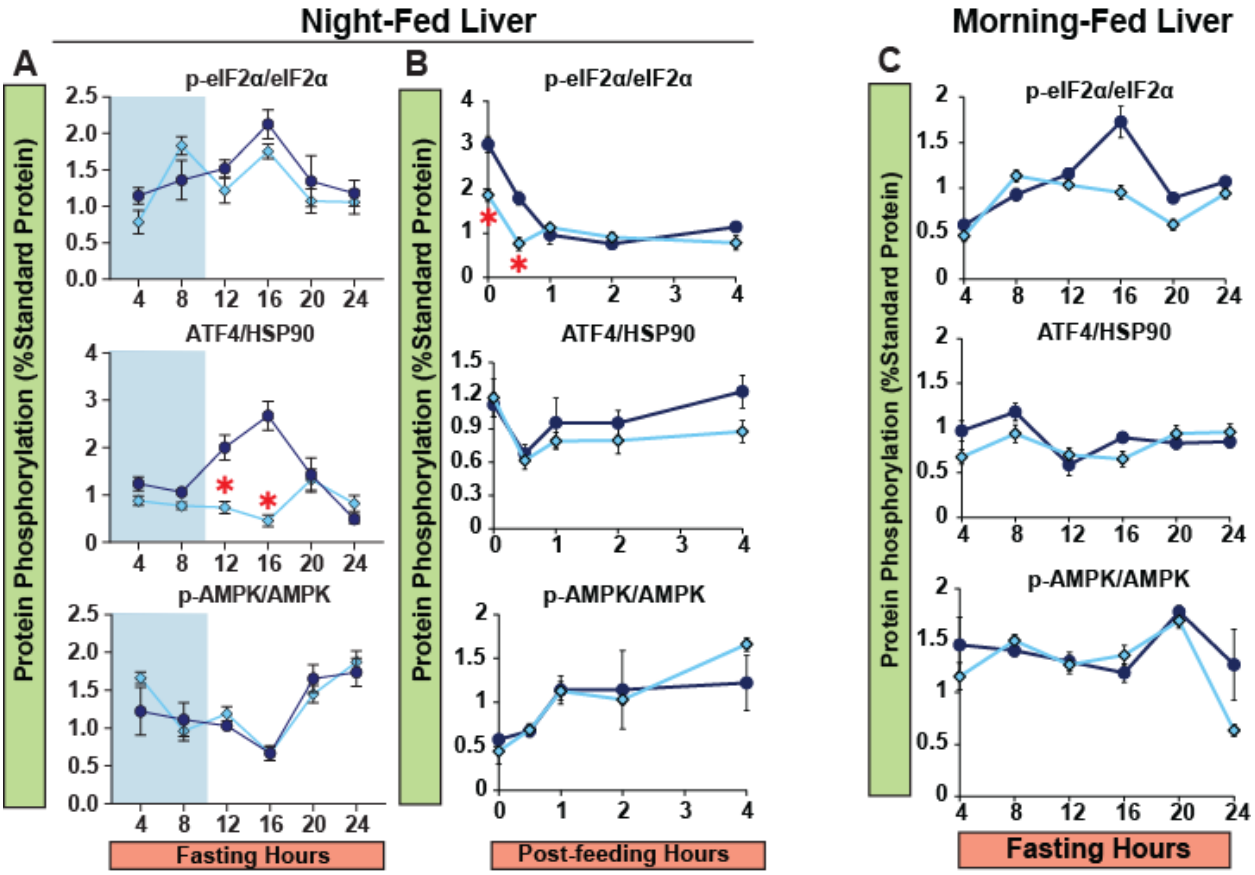

Figure 14. Examination of other nutrient responsive pathways in the liver and muscle. (A) Western blot analyses of GCN2 and AMPK signaling in the liver of mice during respective fasting timepoints within each diet in the night-fed study (B) Western blot analyses of GCN2 and AMPK signaling in the liver of mice during respective fed timepoints within each diet in the night-fed study. (C) Western blot analyses of GCN2 and AMPK signaling in the liver of mice during respective fasting timepoints within each diet in the morning-fed study (AL, n = 6 per time point; CR, n = 6 per time point biologically independent mice).

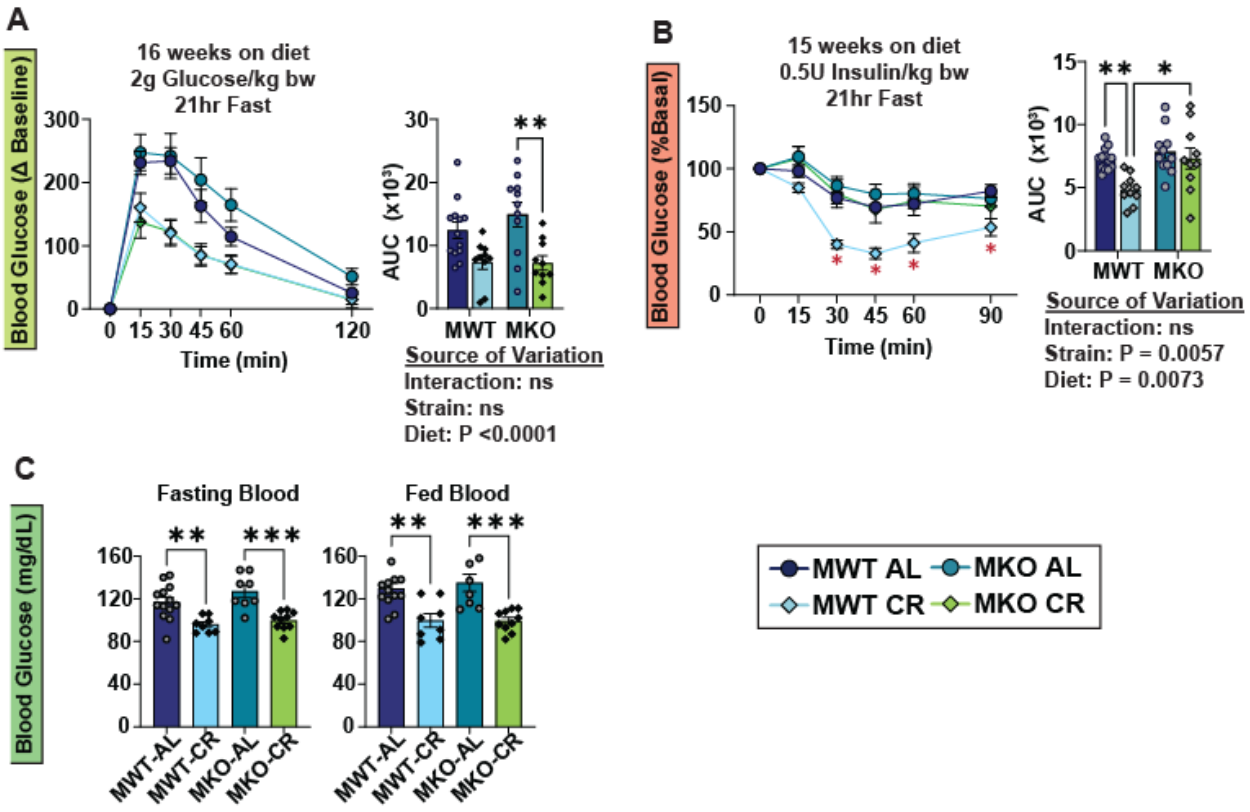

Figure 15. Suppression of hepatic mTORC1 activity is not required to elicit the CR response. (A) Glucose (1g glucose/kg body weight) tolerance test (MWT AL, n = 13; MWT CR, n = 10; MKO AL, n = 11; MKO CR, n = 10 biologically independent mice) (B) insulin (0.5U/kg body weight) tolerance test (MWT AL, n = 13; MWT CR, n = 10; MKO AL, n = 11; MKO CR, n = 10 biologically independent mice). (C) Blood glucose level of 8 hr fasted and 2 hr refed state (MWT AL, n = 13; MWT CR, n = 10; MKO AL, n = 11; MKO CR, n = 10 biologically independent mice). * symbol represents a significant difference versus AL-fed mice based on Šídák's test post two-way repeated measure ANOVA.

CHAPTER 5: CONCLUSIONS AND FUTURE DIRECTIONS

With the dawn of modern medicine, the world's population is living longer than our ancestors have ever. However, lifespan extension has not been accompanied by health, instead, currently increased age is associated with heightened risk of chronic diseases, such as cancer, type 2 diabetes, cardiovascular disorders, and neurodegenerative diseases [1-3]. In the last century, interventions that slow the rate of aging or delay the onset of age associated diseases have been a great interest, and to date, calorie restriction (CR) is the only non-pharmacological intervention known to extend health and lifespan. Unfortunately, the mechanism that mediates health and lifespan from CR still remains to be determined.

One challenge is the poorly defined protocol of CR. Throughout literature, the term "dietary restriction (DR)" is often used interchangeably with CR. However, this is inherently wrong as DR broadly defines many different feeding regimens (e.g. intermittent fasting, alternate day feeding, protein restriction, etc.) [4], while CR refers specifically to the reduction of total calorie intake without malnutrition [5]. Recently, traditional view of DR has been challenged. First, studies in insects and rodents have shown that altering the balance of dietary components an organism ingests, without reducing its overall food intake, can increase lifespan [6-15] implying that specific nutrients, or their ratios, may be important in determining longevity. Second, the genotype of the organism can affect the response to DR, in some cases apparently with complete elimination of lifespan extension [6, 16-19]. Third, fasting duration affects the response to calorie restriction [20]. Finally, body temperature may be an important factor for lifespan extension, at least in small mammals [21]. These new findings clearly

challenge the simple idea that moderately reduced intake of a fixed type of food predictably extends life span.

Implementation of dietary restriction protocols

Systemic monitoring of food consumption behavior of mice placed on a CR diet revealed that CR mice eat all their food within a narrow window; therefore self-imposing a 3 hr time restricted feeding (TRF) [22]. This overlooked feature of CR has only been addressed recently. In Chapter 2, we show that this fasting period is necessary to mediate the beneficial effect of a CR diet and fasting without calorie restriction is sufficient to elicit the same benefit [20]. Similarly, another study demonstrated that life extension was also possible when mice were placed on a 13hr TRF; however, lifespan extension was to a lesser degree than the traditional once-daily fed CR [23]. These results suggest that eating pattern – the time between meals – rather than nutritional value (i.e. calories or diet composition) may dictate lifespan. Also, this could potentially explain why CR and fasting regimen have been reported to have similar benefits [24] while there are non-overlapping molecular mechanisms under certain dietary restriction protocols [25]. Additionally, alternate day feeding (ADF), or every other day (EOD) feeding is a common practice for CR studies. However, a recent study comparing once daily fed CR to an ADF feeding protocol showed that while ADF extended life it did mitigate age-associated decline [26], suggesting that there is an optimum length for a fast between meals.

Conversely, when mice are fed a hypocaloric diet without fasting (diluted diet), life extension was not observed [20, 27]. However, in flies and worms, lifespan extension could be observed even under a diluted diet [28, 29], further complicating the

interpretation of studies between invertebrates to mammals. It is of note, that long-term calorie restriction studies with a fasting component are rarely done in flies [30, 31] or other lower organisms such as yeast and worms, and most studies that examine fasting states in flies are done in the context of a starvation state [32]. Additionally, it is often a difficult task to accurately measure the amount of food eaten by these organisms, which could add onto the confounding factors between CR studies. Therefore, careful consideration should be in place when making conclusions about dietary restriction.

Another key factor to consider when examining metabolic and molecular response to DR is the feeding and fasting methods. For example, mice have a higher metabolic rate than humans [33], yet they are often fasted overnight to mimic the human setting. In Chapter 4, we show that CR animals have differential response to tests based on fasting duration. Additionally, we show that aligning the feeding and fasting schedule have important implications for the molecular outcome. For example, it is often assumed that an *ad libitum* fed mouse is fasted for 16 hrs if fasted prior to the dark cycle. However, as mice are nocturnal animals, these animals most likely have not eaten prior to the fast, and the fasting duration is probably greater than 24 hr fast. This misalignment of fasting schedule could have significant impact on how we interpret molecular markers of DR. This is best exemplified in our study in Chapter 4 where we show in our morning-fed study that CR mice appear to have elevated mTOR activity at the earlier fasting timepoint. However, when re-examining the fed state by accurately measuring the time from when the mouse ate its first bite to the collection of tissue, mTOR activity essentially becomes the same. Therefore, future research should

emphasize the rigor of laboratory conditions when examining both metabolic and molecular response to DR.

Circadian Rhythm and DR

Recent evidence suggests that there is an intimate relationship between CR and biological clocks [34-37]. Circadian rhythm is an evolutionarily conserved biological process which aligns daily metabolic processes to environmental cues, with light/dark and feed/fast cycle as the most potent stimulus [38]. Misalignment of these internal clocks from erratic lifestyle have been shown to adversely impact metabolism [39, 40] and damped with age [41]. Because circadian clock mediates daily metabolism according to cellular needs and nutrient availability, it has been suggested to play a role in in CR-dependent lifespan extension [34, 35]. Indeed, when mice are placed on a CR diet, aged mice on CR retain a robust rhythmic expression of core clock genes [42]. Also, when knocking out Bmal1, a core circadian clock transcription factor, CR failed to extend lifespan in mice [37], suggesting that an intact clock is required for CR-dependent lifespan extension.

Now an important question is how exactly does the circadian clock mediate the beneficial effect of CR? A common theme between CR and circadian rhythm is that the core clock genes directly or indirectly regulate pathways that have been associated with longevity – GH/IGF-1, FOXO, mTOR, AMPK, sirtuins, and NRF2 [43, 44]. Currently, studies on the interaction between circadian clocks and nutrient responsive pathways are limited and more research is required to understand the interaction between the two. In particular, in the context of DR, how nutrient status affects circadian

homeostasis, and ultimately if targeting core clock mechanisms could rescue age-associated decline.

Consideration of other Conditions

Health is influenced by multifactorial phenotype that is also affected by genetic background and environmental factors [45]; therefore, defining healthspan is complex. Also, delaying functional aging in one parameter does not necessarily extend healthspan in a different health parameter [26]. Depending on which health parameter is assessed, healthspan results could be drastically different using the same long-lived mutant animals [46]. Therefore, it will be important for future studies have a uniform definition of health.

Another major complication in understanding the mechanism of DR is interspecies differences in physiology, metabolism and behavior between model organisms, in addition to the non-uniform DR protocol as described above. In some animal models, lifespan is strongly affected by sex and genetic backgrounds [47]. For example, recombinant inbred mouse strains had a huge variation in lifespan in response to both *ad libitum* and CR diet [48, 49]. A simple interpretation would be that CR is not beneficial depending on the genetic variation and sex. One caveat to these studies is that mice were housed five animals per cage which may have caused some animals to be more restricted than others. Of course, there are many more considerations, such as housing temperature, influence of microbiome, diet composition, level of restriction, age of diet implementation, etc. Therefore, it will be important for future studies to have a uniform protocol on implementing DR.

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