TITLE

Modulation of longevity by diet, and youthful body weight, but not by weight gain after maturity

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Abstract

Diet and environmental factors profoundly modulate lifespan. We measured longevity as a function of diet, and weight gain across a large genetically diverse BXD cohort which segregates for over 6 million variants, making it ideal for the analysis of gene-by-diet interactions that modulate lifespan. We followed 1348 females from parental strains, C57BL/6J and DBA/2J, and 76 BXD progeny strains on a standard low fat diet (CD, 18% calories from fat) or a widely used high fat diet (HFD, 60% calories from fat) across their natural life span. A diet rich in saturated fats shortens lifespan by an average of 85 days (HFD 605 ± 6, n = 685; CD 690 ± 8, n = 663), roughly equivalent to a 7-year decrease in humans. This diet is associated with an average two-fold higher age-adjusted risk of death compared to CD. Individual strains show remarkably wide variation in responses to diet, ranging from –54% on HFD in BXD65 to +37% on HFD in BXD8. Baseline weight and early weight gain on HFD associates negatively with longevity, with a gram increase causing lifespan to decrease by 4 days. By 500 days of age, BXDs on HFD gained 4X more weight than those on CD. However, strain-specific variation in the change in body weight does not significantly correlate with strain-specific life span. Major morbidities appear to be influenced by diet, with cases on HFD showing increased prevalence and severity of cardiovascular disease and lesions. Overall, we find that diet significantly impacts longevity even after adjusting for weight gain.

Introduction

Longevity is among the most complex and heterogeneous of traits. Differences in lifespan are dependent on interactions among many genetic and environmental factors, and innumerable gene-by-environmental (GXE) interactions (de Magalhães et al., 2012),(Kuningas et al., 2008), (McDaid et al., 2017), (Hook et al., 2018). Nutrition, of course, has a profound influence on health and lifespan (Fontana and Partridge, 2015). Relative to an ad libitum diet—a condition far from the natural pattern of food consumption—caloric restriction and intermittent fasting improve lifespan and health (reviewed by Heilbronn and Ravussin, 2003; Liang et al., 2018; Speakman et al., 2016). Effects are not entirely dependent on patterns of caloric intake, but depend on dietary macro- and micronutrient composition, the amount of time spent in different metabolic states, age of onset, sex, and perhaps of most importance to us, differences in genotype (Vaughan et al., 2018) and gene-by-diet interactions (Barrington et al., 2018).

The mouse is an excellent mammalian model for research at the interface of metabolism and aging, sharing most protein-coding genes with humans (Pennacchio and Rubin, 2003), but with a much shorter life cycle that enables longevity studies in controlled environments and under various experimental and dietary conditions (Miller et al., 2007), (Yuan et al., 2011), (Strong et al., 2013). However, most rodent studies do not yet incorporate the level of genetic complexity such as is typical of human populations (Saul et al., 2019; Williams, 2006; Williams and Williams, 2017). Effects of DNA variants and dietary, drug, or environmental perturbations are almost inevitably studied on a single genome–often C57BL/6. This narrow focus greatly compromises translational utility of discoveries. To address this problem, we rely on the large family of BXD strains of mice that segregate for over 6 million variants (Peirce et al., 2004), (Wang et al., 2016). Collectively, the family also incorporates an impressive level of variation in phenotypes related to aging, metabolism, expression in liver, muscle, brain, and many other tissues and cell types,(Williams et al., 2014), (Houtkooper et al., 2013), (Andreux et al., 2012), (Houtkooper et al., 2011), (Gelman et al., 1988), (De Haan and Van Zant, 1999).

Studies of BXDs by Gelman, Lang, and colleagues (Gelman et al., 1988; Lang et al., 2010) demonstrate at least two-fold variation in lifespan on a standard diet—from approximately 12–15 months for the shortest lived strains to 30 months for the longest lived strains. In these studies conventional heritabilities of lifespan are as high as 25–45%, but the effective heritabilities (h^2_{RI}) that accounts for the depth of resampling (n = 8 to 12 replicates/genome) are as high as 80% (Belknap, 1998; Hook et al., 2018). The BXD family is particularly well suited to study GXE interactions because diverse but perfectly matched cohorts can be treated in parallel using different diets (Rikke et al., 2010), (Hall et al., 2014), (Andreux et al., 2012), (Williams et al., 2016; Wu et al., 2014). The effect of genetic variation has been well studied in the context

of dietary composition and caloric restriction on life span (Finkel, 2015), (Keipert et al., 2011; Skorupa et al., 2008). However, key results remain controversial. While caloric restriction is undoubtedly advantageous in boosting longevity *on average*, there is good evidence that such effects are not universal, and that certain individuals and genomes do not benefit in all environments (Liao et al., 2010; Mitchell et al., 2016; Rikke et al., 2010).

In this study, we have measured longevity and body weight across a large cohort of fully sequenced, and highly diverse strains of mice that are part of the BXD family (Pierce et al., 2004; Ashbrook et al., 2019). We studied females in a well-controlled environment on two diets that differed greatly in fat content—those on a standard low fat chow diet (18% of calories from fat) and those on a high-fat diet (60% cal from fat). To the best of our knowledge this is the largest GXE experiment on the effects of high fat on longevity and weight changes, and includes matched data for 1348 cases and 76 BXD genotypes.

We address the following questions:

- What is the average impact of a very high fat diet, otherwise well balanced for protein content, on longevity across the entire family?
- To what extent does the genome-type modulate effects of the high fat diet relative to the standard lower fat diet? Put another way: What is the strength of evidence in favor of GXE effects on longevity?
- Does baseline body weight at young adulthood (~120 days) predict longevity or is the change in body weight in response to chronic high fat diet a stronger predictor of longevity?
- To what extent is weight gain *per se* linked to a reduction in longevity and how does weight gain vary among strains?
- Does diet itself modulate longevity after controlling for weight gain across the family or within strain?

Methods

Animals and Diets

Animals were raised and housed in a specific pathogen-free (SPF) facility at UTHSC (Memphis, TN), at 20–24 °C in temperature on a 12-hour light cycle. During the course of our study, serum samples from sentinel mice were tested quarterly for the following pathogens–Ectromelia virus, Epizootic Diarrhea of Infant Mice (EDIM), Lymphocytic Choriomeningitis (LCM), Mycoplasma pulmonis, Mouse Hepatitis Virus (MHV), Murine Norovirus (MNV), Mouse Parvovirus (MPV), Minute Virus of Mice (MVM), Pneumonia Virus of Mice (PVM), Respiratory Enteric Virus III (REO3), Sendai, and Theiler's Murine encephalomyelitis (TMEV GDVII). Semiannual necropsies are performed to test for endoparasites by microscopic examination of intestinal contents and anal tape preparations and ectoparasites by direct pelt microscopic examination. All such tests were negative throughout the experimental course.

The focus of this study is on the overall effects of diet on weight and longevity in the BXD family, rather than on the genetic control of longevity or weight *per se*. In some aspects, our study design is more like an observational prospective cohort rather than a controlled animal experiment—the main reason being that cases were entered into the aging colony and onto the HFD limb of the study at different ages. For this reason, we used methods of observational data analysis with minor modifications.

From October 2011 through to December 2018, animals from both parental strains, C57BL/6J and DBA/2J, and ~76 BXD strains were followed from their move from a large breeding colony into the aging colony (typically around 120 ± 66 days of age but with a wide range, from 26 days to 358 days) until their death. All animals were initially raised by dams on the chow diet. Females were aged in groups of up to 10 in polypropylene cages (935 cm^2) provisioned with Envigo Teklad 7087 soft cob bedding. Animals were provided either a standard low fat chow diet (CD, Envigo Teklad Global 2018, 18.6% protein, 18% calories from fat, 6.2% fat (ether-extractable), 3.1 kcal/g), or a widely used blue high fat diet (HFD, Envigo Teklad TD06414, 18.4% protein, 60.3% calories from lard, 37% saturated, 47% monounsaturated, 16% polyunsaturated fats, 5.1 kcal/g). Animals had *ad libitum* access to food and aquifer-sourced municipal tap water.

We studied a total of 1348 individuals (n = 663 on CD, n = 685 on HFD). Animals were labeled using ear tags, and individuals were randomly assigned to chow or high fat diet. Baseline weight was measured at entry into the study. 77% (n = 527) of animals started on HFD at ages between 50-185 days, but some started on the diet at ages as low as 26 days or as high as 358 days. Fewer than 2% of animals (n = 12) were placed on HFD at an age of greater than 365 days, and these were not included in the analysis. Less than

20% of animals were retired breeders that entered the study at 180+ days of age. Each animal was weighed to the nearest 0.1 gram every other month from start of diet until death. A separate subpopulation of 662 animals (n = 333 on CD, n = 329 on HFD) from matching BXD strains were sacrificed at specific time-points (6, 12, 18 and 24 months-of-age) for tissue collection across both diet cohorts (Williams EG et al., in submission). Organ weight data at 18 months of age from these animals was included in the analysis for this study. The aging colony at UTHSC is still in operation, but for this analysis we only consider animals with deaths between April 2012 and November 2018.

Longevity data from both cohorts in our aging colony (separate, combined and difference scores) are available in GeneNetwork.org (GN) under 'BXD RI Family' in the dataset 'BXD Published Phenotypes' (GN traits #18435, 18441, 19451, 19452, 21302, 21450). Body weight data at 6, 12, 18 and 24 months of age on both diets is also documented in GN (traits #19126, 19130, 19131, 19167, 19168, 19169, 19170, and 19171). Organ weight data on both diets, including liver, heart, kidneys and brain, at 18 months of age, can be found in GN as well (traits #20156, 20157, 20158, 20159, 20353, 20354, 20148, 20149, 20150, 20151, 20146, 20147).

The colony was moved to a new vivarium (TSRB) in April 2016. Approximately 60% of the animals lived and died in the original Nash annex vivarium, ~35% were born in the Nash but lived in both vivaria, and ~5% were born and spent their entire lives in the new facility. We carefully evaluated birth and death data over all seasons from both vivaria to successfully rule out any site-specific or seasonal effect on longevity (Supplementary data available). Animals were inspected daily and deaths were recorded for each animal with a precision of one day. Moribund animals (~10%) were euthanized, and those above the age of 200 days were included in longevity calculations. Criteria for euthanasia were based on an assessment by our veterinary staff following AAALAC guidelines.

Most animals were fixed by immersion in 10% neutral buffered formalin within 24 hours after death. The body cavity was opened prior to immersion to improve tissue preservation. Evenly balanced cohorts on the diet were selected based on fixation quality for necropsy with histopathology of tissues. A board-certified veterinary pathologist (RWR) performed necropsies and judged probable cause of death and other morbidities.

All experimental procedures were in accordance with the Guidelines for the Care and Use of Laboratory Animals published by the National Institutes of Health and were approved by the UTHSC institutional Animal Care and Use Committee.

Statistics

Longevity and body weight data were stratified by diet and by strain. Effects of different diets and body weight on longevity were analyzed using a random-effects model in R using the metafor package (Viechtbauer, 2010) and a mixed-effects Cox proportional hazard model using Therneau's *coxme* R package 2.2.-10 (CRAN.R-project.org/package =coxme) (Therneau and Grambsch, 2000). Survival analyses was performed using the survival package for R and the data were right-censored (see Fig 2, censored cases CD $n = 32$, HFD $n = 80$). Survival curves were computed by ANOVA and regression analyses were performed using R. Results were also tested using Wald test, likelihood ratio test, and Wilcoxon test.

Results

High fat diet shortens lifespan but with considerable variation among strains

Sets of females from 76 strains were assigned to HFD diet at an average of 120 days of age (Figure 1A). The HFD has a very significant average effect on lifespan across the family as a whole (p<2.2E-16, r = 0.2). Mean lifespan decreases from 690 ± 8 SE (±199 SD) days on the control diet to 605 ± 6 SE (±169 SD) days on the HFD. Median longevity decreases 77 days—from 703 to 626 (Figure 1B). Assuming linear scaling and that a dietary change was imposed in humans at about 20 years-of-age, the this 77 to 85 days difference would scale roughly to a seven-year loss of longevity in humans (Flurkey K, Currer JM, Harrison DE. 2007).

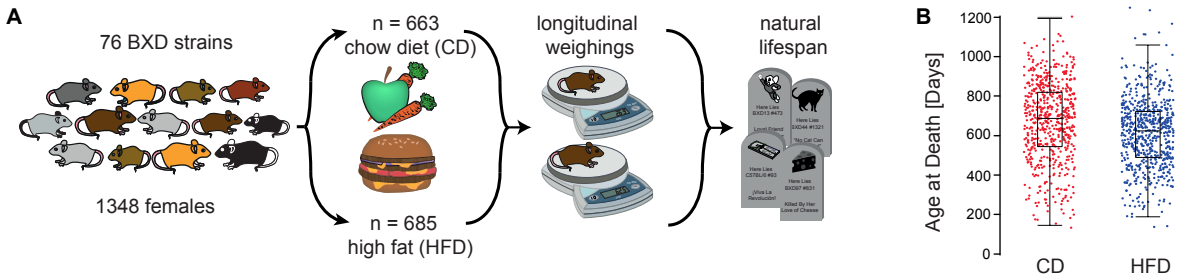

Figure 1 (A) Balanced sets of females from 76 BXD strains were assigned to the low fat chow diet (CD) or the high fat (HFD) diet and weighed every two months. (B) High fat diet modulates longevity. When grouped by diet—and irrespective of genetic background—median lifespan of CD cohort exceeds by 77 days that of the HFD cohort (see *box plot* inset). Red and blue dots represent individual cases on CD and HFD.

Using a mixed-effects Cox model with diet as a fixed effect and strain as random effect, we estimated a hazard ratio of 2.0, indicating that animals on HFD have two-fold higher age-adjusted risk of death after the dietary change compared to CD-fed animals (Figure 2A). The hazards ratio is relatively constant throughout the study and there is no crossing of the two cumulative hazard curves (Figure 2B, p = 0.47).

While HFD decreases lifespan at the family level, individual strains show significant differences in how they react to diet, with 64% heritability. The parents exemplify the difference—the DBA/2J paternal strain shows little or no effect of diet on longevity, whereas the C57BL/6J maternal strain losses 76 days on the HFD (Figure 2C). Some BXD progeny strains even live longer on the HFD, demonstrating that the longevity-diet relation is modulated by a GXE component (Figure 2D). Overall, longevity in 21 strains out of 67 was significantly affected by HFD in either direction at a nominal p threshold of 0.05. Given the large

number of tests (n = 67) we computed FDRs at a q value of 0.1 (see Figure 2D * symbol) and with this correction, only 15 strains are significantly different from the null expectation, including BXD8 and BXD65. BXD8 has significantly improved longevity on a high fat diet, with a median increase in longevity of 208 days (t = 4.0, p = 0.0052, q<0.05, two-tailed), with one other strain trending in the same direction—BXD172 (146 day increase on HFD, p = 0.073), but with unacceptable FDR q value of 0.85. As expected, high fat diet reduces longevity for most strains (Fig 2D, right side)—most prominently for BXD65, with a decrease in median longevity of nearly a year (345 days, t = 9.3 p = 9.0E-7, q = 6.0E-7).

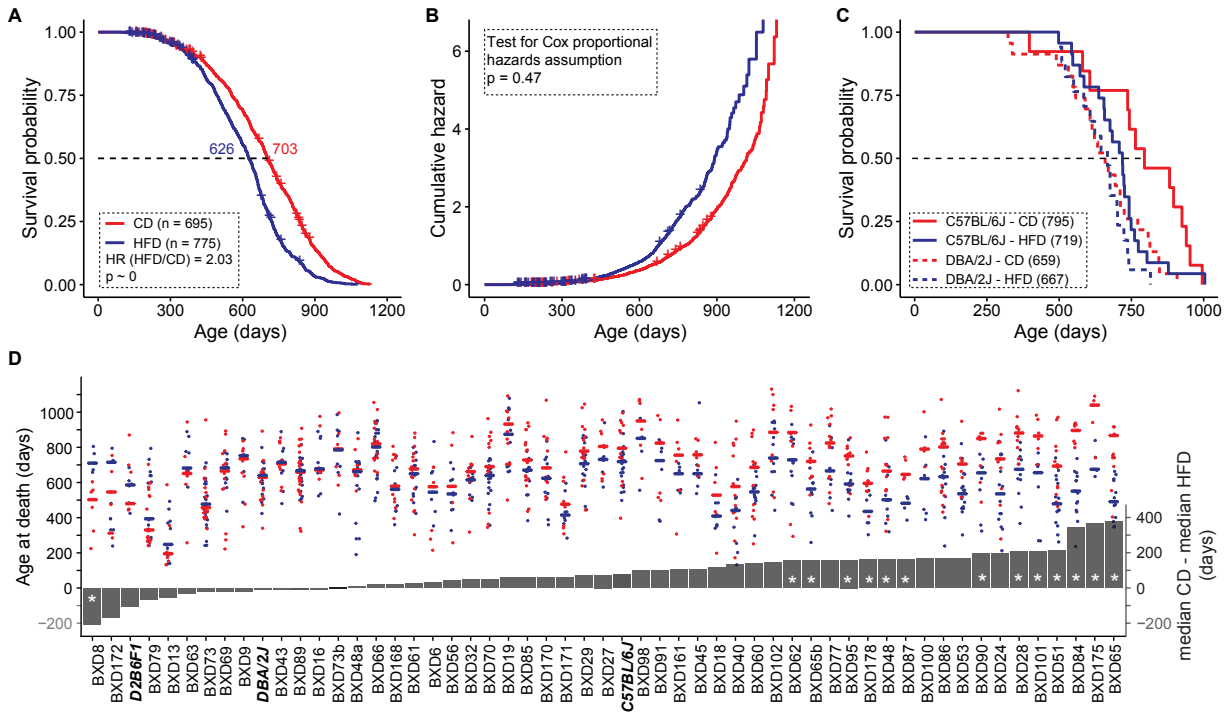

Figure 2 (A) Diet influence on longevity. Cases on the HFD have a two-fold higher risk of death compared to those on the CD. (B) Cumulative hazard curves by diet do not cross and the hazards ratio of 2.0 is relatively constant throughout the study. (C) The lifespan of C57BL/6J, but not DBA/2J, is influenced by diet. Numbers in parentheses are median lifespans in days. (D) Longevity on a high fat diet depends strongly on strain. Red points represent longevities of cases on CD and blue points those on HFD. Lines represent median survival. Grey bars represent the difference in median survival on the diets. The few negative values to the left indicate higher survival on a HFD. Parental strains and F1 are denoted by bold italics font. Asterisks in bars denote significant FDR scores at a q value of 0.1. Censored cases in A–C are still alive and are marked by + signs.

Body weight at young adulthood is a strong predictor of longevity

Body weight at young adulthood (initial baseline weight) measured at entry into the aging colony—typically at 120 days of age on average—has a significant influence on eventual lifespan, after adjusting for differences in age at baseline weight ($p<0.001$, $r = 0.1$) (Figure 3B). Adjusting for strains, for one gram increase in initial baseline weight, longevity decreased by ~5 days, with low heritability (9.6%). At this point in the experiment there is by design no significant weight difference between cases assigned to the HFD (23.11 ± 0.22 SE g, $n = 685$) and those continuing on the CD for the remainder of their lives (23.26 ± 0.22 g, $n = 659$). Of interest, the slope of –5 days/g of body weight at ~120 days is not affected at all by the subsequent diet, indicating that this initial size-longevity relation is relatively insensitive to GXE.

Early body weight gain associated with a reduction in longevity

Animals were weighed regularly throughout their lifespan. As expected, animals on the high fat diet gained more weight on average over time than animals on the chow diet (Figure 3A). Body weight measured at 100 days on both diets correlates negatively with longevity (Figure 3C), with one gram increase in body weight at this early time point corresponding to a decrease in lifespan by 4 days ($r = 0.3$). This effect is observed even after adjusting for strain differences. Overall, 53 out of 67 strains had significant weight gain on the HFD at 100 days ($p <0.05$, $q<0.1$, t values ranging from –3.03 to –13.43). Looking at change in body weight over time, early body weight gain in response to high fat feeding is negatively correlated with longevity ($p = 0.004$, $r = 0.1$) (Figure 3E).

Diet significantly alters longevity after adjusting for weight gain

We chose to focus on two time points for body weight analyses- 100 days (early weight gain on HFD) and 400 days on diet (~highest measured weight point on both diet curves). The mean weight of the population plateaus around 500 days of age and then begins to decline, for both diets. By 500 days of age animals had been on HFD for 400 ± 44 days and gained an average of 29.5 g. Those on the CD gained only 6.2 g (mean weight on CD= 29.7 ± 0.35 SE g, n=447; mean weight on HFD= 52.6 ± 0.63 SE g, n=447). Overall, 45 out of 57 strains had significant weight gain upon eating a high fat diet for 400 days ($p<0.05$, $q<0.1$, t- values ranging from –3.03 to –13.14). Surprisingly, the substantial increase in body weight on the HFD did not significantly correlate with lifespan (Figure 3D). Adjusting for strains, 10% of the effect of diet on longevity is mediated through body weight gain. Mirroring this interesting observation, sustained weight gain after 400 days on high fat diet (Figure 3F) has no predictive value, emphasizing that in our study the diet itself, rather than weight gain, modulates longevity. In both diet cohorts, we calculated a "rollingSlope": body weight change from day x to day (x + 180) reflecting the change in body weight in the next 6 months. In early age, faster growth is associated with shorter lifespan,

while in intermediate and old age, a decrease in body weight (negative slope) is predictive of death (Supplementary figure S2).

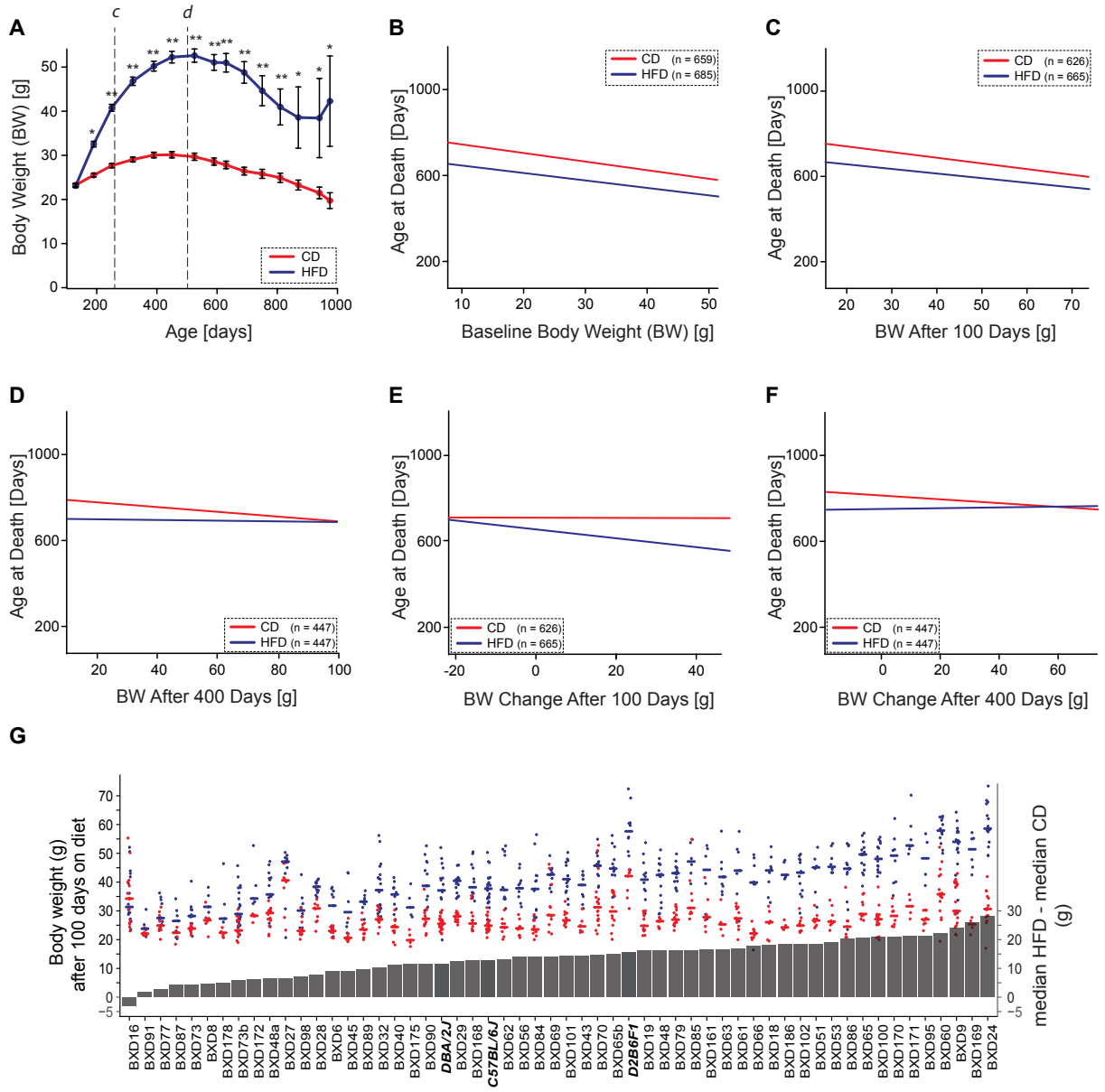

Figure 3 (A) Body weight by diets and age. Asterisks denotes significance at p <0.05 and <0.001. Note the pronounced decline in body weight on both diets after about 500 days. (B) Initial body weight (baseline weight at entry into colony, mean age of ~120 days) has a modest but consistent negative slope

with longevity (–5 days/g, p<0.001) that is not exacerbated by the HFD. (C) Body weight after 100 days in the aging colony on the two diets (~260 days age) still correlates negatively with longevity (–4 days/g, p <0.001, see line labeled c in Panel A). (D) Surprisingly after 400 days on diet (~500 days age) current body weight does not predict variance in longevity (see line labeled d in Panel A). (E) Early weight change in response to HFD (blue line), measured at 100 days on diet, is negatively related with longevity (–4 days/g, p = 0.004), but this is not true of cases remaining on CD. (F) Substantial weight change after 400 days on HFD (blue line) is now not predictive of longevity. (G) Strain-wise changes in median weight after 100 days on diets. Red points represent longevities of cases on CD and blue points those on HFD. Lines represent median survival. Grey bars represent the difference in median survival on the diets. Parental strains and F1 are denoted by bold italics font.

Insufficient evidence of association between longevity and major metabolic organ weight

We compared organ and tissue weights of a separate subsample of animals on the two diets at 500 days. HFD mice (*n* = 63) had 84% greater fat mass, 25% great heart mass, 19% great liver mass, and 18% great kidney mass at 500 days compared CD controls (*n* = 71). However, HFD did not influence brain mass. No significant correlations were found between longevity and any of these organ weights at 18 months in this small subsample (Supplemental figures S1A-F).

Major morbidities contributing to death in the aging colony

We carried out gross necropsy with or without histopathology included for 76 animals (69 with single cause of morbidity and 7 with multiple causes) from 45 strains on CD and 79 animals (71 with single cause of morbidity and 8 with multiple causes) from 43 strains on HFD. In 87% of animals, likely causes of death was clear at necropsy or following histopathology. Hemopoietic neoplasia—lymphomas and histiocytic sarcomas—were a leading cause of death, accounting for ~35% of deaths in both cohorts. Miscellaneous non-neoplastic conditions causing significant morbidity or death were detected in 24% of CD and 32% of HFD-fed animals.

There is some preliminary evidence that causes of morbidity and mortality may have been influenced by diet (Table 1). The high fat diet-fed cohort showed an increased prevalence and severity of cardiovascular disease and lesions (atrial thrombosis, cardiomyopathy and cardiac dilation with hepatic centrilobular atrophy). Nineteen percent of HFD mice (15 out of 79) showed heart pathologies rated moderate to severe whereas only 1 out of 76 CD mice had a heart lesion rated at least moderately severe (2-tail Fisher's exact test p = 0.0003). Four HFD, but no CD, cases had systemic polyarteritis. In contrast, some pathologies appear to be more pervasive in the CD cohort. Thirty-six percent of chow-fed mice displayed non-

hematopoietic malignant neoplasia (27 of 76 CD–11 sarcomas, 15 carcinomas, and 1 teratoma) compared to 13% high fat-fed mice showed non-hemopoietic malignancy (10 of 79 HFD–6 sarcomas and 4 carcinomas); this preliminary finding is nominally significant (Fisher $p = 0.0012$, not corrected for multiple comparisons).

Diet	Morbidity Type	n with single morbidity	n with multiple morbidities
CD	Unnatural causes	7	1
HF		3	0
CD	Non-neoplastic conditions	16	2
HF		22	2
CD	Heart/ cardiovascular	0	1
HF		8	7
CD	Polyarteritis (autoimmune)	0	0
HF		3	1
CD	Lymphoma	25	3
HF		26	1
CD	Sarcoma	9	2
HF		4	2
CD	Carcinoma	12	3
HF		4	0
CD	Renal	0	1
HF		1	3
CD	Teratoma	0	1
HF		0	0

Table 1. Major morbidities contributing to death in the aging BXD colony.

Unnatural causes = flooded cage, foot injury in wire lid, eye abrasion leading to euthanasia etc.

Miscellaneous non- neoplastic conditions = inflammation, infection, amyloidosis, ulcerative dermatitis

Heart = major contribution of cardiovascular failure other than polyarteritis

Polyarteritis = systemic multifocal arteritis suggestive of autoimmune disease

Lymphoma = hematopoietic neoplasia including lymphoma and histiocytic sarcoma

Sarcoma = nonhistiocytic sarcoma, spindle cell, hemangiosacoma, leiomyosarcoma, sarcoma NOS

Carcinoma = carcinomas (hepatocellular, squamous cell)

Renal = renal failure due to severe nephropathy

Discussion

We have studied effects of a high fat diet on aging and lifespan in the BXD family of mice. Our focus here is on overall and strain-specific effects on longevity and weight gain. It is generally accepted that chronically high levels of fat consumption lead to substantial weight gain, associated metabolic disorders, and shortened lifespan, but causality among parameters remains controversial. For example, studies exploiting Mendelian randomization have not shown a compelling causal link between triglyceride levels on longevity in humans (Liu et al., 2017). In general, a diet high in fat leads to obesity and reduced lifespan in diverse species including *Drosophila*, *C. elegans* and mice (Otabe et al., 2007), (Yen and Curran, 2016), (Gáliková and Klepsatel, 2018). However, consumption of high levels of corn oil rich in poly- and mono-unsaturated fats (58.6% fat-derived calories) in C57BL/6 male mice has been shown to improve health and longevity, provided that total calorie consumption stays within normal bounds (Si et al., 2014). Muller and colleagues report that C57BL/6 male mice fed a high fat diet (60% energy from saturated and unsaturated fat– 45% lard and 15% soybean oil) for over two years have decreased life expectancy independent of body weight gain (Muller et al., 2013b). Similarly, the initiation of a HFD in middle-aged mice has a detrimental impact on general health and survival (Baur et al., 2006). In contrast, a low carbohydrate ketogenic diet (89% kcal from fat) initiated in mice at one year of age with control of caloric intake increases life span and improves health (Roberts et al., 2017). Collectively, these studies demonstrate that diet composition, alone and in combination with other environmental factors, modulates lifespan.

In the BXD family, we find that a diet rich in saturated fats shortens lifespan by an average of almost three months, roughly scaling to a 7-year decrease in humans. This diet is associated with an average two-fold higher age-adjusted risk of death compared to the chow-fed controls. Longevity under the two diets correlates moderately (r = 0.60, GN traits #18435 and 18441). However, strains display remarkably wide variation in responses to diet, and despite the strong effect, diet accounts for just 4% of the total variance in longevity. In comparison, strain accounts for 30% of variance. Strain longevity combined across the two diets varies from 307 ± 37 days in BXD13 (n = 21) to 852 ± 33 days in BXD168 (n = 23). Some strains are fully resistant to the negative effects of HFD on body weight and lifespan while others are strongly affected, indicating substantial gene-by-diet interaction effects. While mean longevity on high fat was shortened by an average of 10%, genetic factors accounted for roughly two-fold range in life span. Some strains, such as BXD16 and BXD73, are almost immune to the high fat challenge with respect to change in life span, whereas BXD8 had significantly improved longevity on the HFD (+37% on HF, p = 0.0004) and BXD65 has greatly reduced longevity (-54% on HF, p = 0.0001). Likewise, at least four strains are almost completely resistant to weight gain, including BXD16, BXD77, BXD87, and BXD91, gaining at most two grams over 100 days.

Our findings can be compared to strain variation and GXE effects in response to dietary restriction (DR). DR without malnutrition is regarded as having an almost universal benefit on longevity (Mair and Dillin, 2008; Masoro, 2009; Weindruch et al., 1986). One exception is a pair of studies on the impact of moderately intense restriction—a 40% reduction in caloric intake—across a large family of LXS strains of mice ((Liao et al., 2010; Rikke et al., 2010); *n* of up 44 strains with 10–20 replicates per strain). As expected, these studies demonstrate a gain in life span—an average of ~100 days for the combined data of Liao and colleagues. But the most notable finding is the remarkably high variation in strain-specific effects. Life span is lengthened by more than a year in 10 of 44 LXS strains (maximum of 671-day extension), but shortened by more than 200 days in 5 others (minimum of 300-day loss; GeneNetwork LXS phenotype 10164). Both the Liao and Rikke papers generated substantial controversy (Mattson, 2010). One issue is whether or not fully homozygous loci in this family compromise the generality of effects, as well as whether the DR was associated with excessive metabolic stress or malnutrition in some strains. However, Liao and colleagues provided good counter arguments (Liao et al., 2010b) that their partially dissonant data—a subset of cases with shorter lifespan on DR—is a genuine GXE effect rather than an artifact of design. One of the key arguments is the relatively high heritability of strain differences (that is to say, the low within-strain errors of the DR versus CD effects). This is partially corroborated by the study of Rikke and colleagues who used a similar protocol but in a markedly different vivarium environment. What is concerning is that strain-specific effects in these two LXS studies differ greatly. In fact, the correlation across studies is negative ($r = -0.22$, $n = 29$ strains, see GN LXS phenotypes data set traits 10169 and 10190). This difference may be due to a known hepatitis and norovirus infection that reduced median longevity even on the standard ad libitum diet by about three months (from 879 ± 128 SE in Texas to 788 ± 126 SE days in Colorado; LXS phenotype traits 10156 and 10191) (Rikke et al., 2010). Given this controversy, and a matched controversy in primates (Mattison et al., 2017), it would be worthwhile extending the analysis of HFD to DR in the expanded BXD family (Ashbrook et al., 2019).

In our study, higher young adult body weight is associated to reduction in longevity—roughly 4 to 5 days per gram. This corroborates much previous work that also demonstrates that larger body size within a species is typically associated with shorter life span. For example, in outcrossed mice, for each gram increase in body weight at 180 days, longevity is reduced by 10–15 days (Miller et al., 2000, their Figure 1). Among breeds of dog, for each kilogram increase in body weight, longevity is reduced by ~15 days (Kraus et al., 2013). In humans, for each kilogram increase in body weight, longevity is reduced by ~146 days (Samaras et al., 2002). In these three mammals, the loss in longevity amounts 1% to 3% per 5% gain

in young adult body weight, even though the causal mechanisms are quite different among species. Modest correlation between body size and longevity suggests that weight is just one factor that plays a role in determining an individual's longevity (Samaras and Storms, 1992).

As expected, the high fat diet generally causes a marked weight gain, with individuals gaining an average of 1.8-fold relative to the control diet after 400 days. We see substantial gain in 45 strains out of 57, with the rest trending similarly. But as discussed above for longevity, the HFD also has variable effects on weight gain as a function of strain (Figure 3G). For example, even after 400 days on the HFD, BXD16 is only 1.05-fold heavier than control. In contrast BXD24 is 2.1-fold heavier. By 500 days, females on HFD gained four times as much weight as those on CD (29.5 g gain on HFD compared to 6.2 g on CD). Remarkably, 10% of the effect of diet on longevity is mediated through this substantial body weight gain. HFD itself, rather than weight gain *per se*, therefore exerts a stronger direct effect on longevity in the BXD family. Muller and colleagues found similarly that male C57BL/6 mice fed a high fat diet (60% energy from saturated and unsaturated fats) for 27 months were either diet-resistant or developed diet-induced obesity related to body weight gain, and had decreased life expectancy compared to the chow-fed mice. However, the survival rate was not different among the two groups; indicating that a high fat diet decreases survival rate independent of body weight gain (Muller et al., 2013a). This is also shown to be true in BFMI860 mice where feeding a high fat diet showed an increased risk of mortality, but body weight itself had a marginal effect on lifespan within the group of mice on high fat diet (Wagener et al., 2013).

Our exploratory necropsy analysis offers some preliminary evidence that major morbidities and likely causes of death among different members of the BXD family appear to be influenced by diet. Those on HFD have an increased prevalence and severity of cardiovascular disease and lesions. However, the effects of a very high fat diet on cardiovascular disease incidence is quite modest in mice, unlike that observed in human cohorts (Menotti and Puddu, 2015; Sacks Frank M. et al., 2017). Interestingly, incidence of sarcomas and carcinomas are higher in the chow than high fat-diet fed cases. Detailed analyses in large prospective studies have failed to detect strong associations between dietary fat and cancer risk (Willett, 2000). Evaluating a causal role of diet-induced obesity in the etiology of several chronic diseases and cancers has been difficult due to correlations with numerous lifestyle factors and resulting confounding biases. Human epidemiology is increasingly using Mendelian randomization to assess the possible causal associations between risk factors and diseases (Gao et al., 2016). Well-controlled animal experiments could similarly provide additional understanding of causal associations and mechanisms underlying such complex relationships. Our necropsy and histopathology sample size while still modest, indicates both diversity and

regularity in this genetically diverse population with malignant neoplasia and carcinomas contributing to terminal illness in most animals while we also found evidence of miscellaneous non-neoplastic lesions.

Summary

In the introduction, we posed some questions for which we now have good answers: 1. Yes, as expected a diet that is very high in fat (lard, in our case) has a strong negative effect on longevity. The mean reduction is 12% of baseline longevity across 76 strains. And as in humans this diet is associated with higher risk of cardiovascular morbidities. 2. Yes, GXE is detectible, and even after correction for the many comparisons built-in to our study, one family member lives substantially longer on the HFD. 3 We confirm other studies that lower baseline young adult weight is linked to longer longevity, and that this effect is conserved on both diets. 4. There is at best only a modest link between the level of weight gain after maturity and longevity. While the analysis of late-stage weight gain and longevity is complicated by early deaths and late-stage weight loss, we conclude that weight gain between ages of 12 and 18 months accounts for little variation in longevity. In other words, in our study, the high fat diet is a much stronger modulator of longevity that weight gain itself.

Author contributions:

Initial Project Design: EGW, JI, JA, LL, RWW
Aging Colony Management: SR, JI, CJ, MM
Tissue Acquisition: SR, JI, CJ, MM, AC, KM, MM, WZ, JH, SM, LW, TS, CK, LL, RWW
Data Handling: SR, MBS, PJ, EGW, AS, MH, AC, RWR, SS, RWW
Paper: SR, MBS, EGW, RAM, JA, RWW
Companion Web Resources: AC, SR, RWW

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