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Improvement of starvation resistance via periodic fasting is genetically variable in *Drosophila melanogaster*

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Abstract

Organisms subjected to periodic nutrient limitation early in life exhibit improvements in aspects of survival, including resistance to some environmental stressors. Recent findings indicate that forms of periodic fasting, such as intermittent fasting and time-restricted feeding, can improve starvation resistance. However, it remains unclear to what extent this survival improvement persists across different genetic backgrounds. In this study, we examine fasting-induced starvation resistance across a broad survey of wild-derived lineages and document genetic variation within this trait. We adopt a standard dietary intervention and show improvement in starvation resistance within a common laboratory lineage, replicating previous results. Next, we examine fasting-induced starvation resistance across isofemale lines collected across latitudes and in different seasons, and among inbred lines derived from flies collected on different continents. We discover genetic variation of fasting-induced starvation resistance and show that fasting improved starvation resistance as often as it worsened starvation resistance. Fasted flies generally showed reduced fat concentration, and their starvation survival varied with sex, season of collection and geographic origin. While specific lineages common to the laboratory can show a specific fasting-induced phenotype, we show that this result is not consistent across genetic backgrounds, reinforcing the idea that phenotypes observed in historic laboratory strains may not be conserved across a species.

KEYWORDS
fasting, fat metabolism, natural variation, starvation resistance

INTRODUCTION

Limitations to feeding and nutrient intake can benefit lifespan and survival, and this benefit is broadly conserved across species (Kapahi et al., 2017). Dietary restriction can take several forms. Caloric restriction reduces calorie intake, although it does not necessarily alter food availability (Heilbronn & Ravussin, 2003). Alternatively, dietary restriction assays can function by limiting when food is available via daily or weekly schedules of periodic fasting (Aly, 2014). Short periods of nutrient deprivation are thought to provide physiological resistance to additional stressors (Bubliy et al., 2012), and long-term periodic fasting has recently been shown to benefit starvation resistance (Catterson et al., 2018; Zhang et al., 2018). While these recent studies represent an advancement in our understanding of dietary interventions, it remains unclear to what extent fasting-induced stress resistance is conserved across variable genetic backgrounds.

The well-studied metabolism, powerful genetic tools and broad global presence of *Drosophila melanogaster* make it an ideal model organism for studies seeking to describe genetic variation of dietary responses and metabolic traits (Faria & Sucena, 2017; Lee et al., 2008, p. 200). Fly metabolism is commonly used to gain new insights into broader animal metabolism, as the systems of signalling and regulation

present in flies are broadly homologous to many other animals (Kapahi et al., 2017). Decades of research on dietary restriction using *D. melanogaster* have identified traits impacted by fasting, potential genes involved and shared genetic mechanisms between the model and non-model organisms (reviewed in Krittika & Yadav, 2019). Periodic fasting studies using flies have identified improvements in lifespan, gut health and somatic maintenance (Catterson et al., 2018; Gill et al., 2015; Zhang et al., 2018). Several recent studies have independently noted the ability of periodic fasting to impact fat storage and improve starvation resistance. Catterson et al. (2018) used a 5-day fasted, 2-day fed model to document improved starvation resistance compared with fully fed flies. Zhang et al. (2018) confirmed improvement in starvation resistance with fasting for 8 h of the day, 3 days a week. Increased storage of triglyceride fats is implicated in starvation resistance (Rion & Kawecki, 2007); however, Catterson et al. (2018) and Zhang et al. (2018) report opposing trends in fat storage among fasted flies, indicating variance in this fat-storage trait. However, these studies use common laboratory lines of Canton-S and w1118 and, thus, only examine a fraction of the standing genetic diversity present in *D. melanogaster*.

Exploring natural genetic variation present in model systems can greatly inform the understanding of the relevant genetic loci for any trait of interest (Gasch et al., 2016). Many studies, especially those of model laboratory systems, seeking to understand the genetic basis for a disease, phenotype or pathway would benefit from examining the variation in that trait among naturally occurring populations (Benfey & Mitchell-Olds, 2008). Indeed, several studies have shown that genetic variation of those subjected to dietary interventions significantly alters the success of the interventions (Jin et al., 2020; Liao et al., 2010). For instance, a 40% calorie reduction imposed on a panel of inbred mice reduced lifespan more often than extended it (Liao et al., 2010), and considerable variation in the lifespan and metabolites of fasted *Drosophila* inbred lines has also been observed (Jin et al., 2020). Responses to dietary interventions such as intermittent fasting can vary depending on the presence of specific alleles and epistatic interactions among different genetic backgrounds (Heianza & Qi, 2017). Metabolic studies into *Drosophila* are especially able to assay a diverse range of genetic variations, as *D. melanogaster* exists worldwide within a variety of nutrient-specific environments (Grenier et al., 2015). However, *Drosophila* periodic fasting studies typically employ a select subset of historic laboratory lineages. In addition, there is evidence that after enough generations raised within the lab, lineages will adapt to laboratory conditions, reducing the extent to which lab-adapted flies represent natural physiological responses (Russell & Kurtz, 2012; Linnen et al., 2001; Matos et al., 2000; Sgrò & Partridge, 2000). While there is a rich history of research on established laboratory models, studies into the effect of periodic fasting on starvation resistance would benefit from employing a more diverse representation of the genetic variation present in nature.

Here, we replicate the intermittent fasting work reported by Zhang et al., 2018 and examine the extent to which this dietary regime can alter aspects of survival. Next, we measured the extent of genetic variation of periodic fasting-induced starvation resistance among *D. melanogaster* lineages collected in different seasons, orchards and continents. The fasting protocol outlined in Zhang et al. (2018) can indeed improve the starvation resistance of both male and female *D. melanogaster* when completed in Canton-S/w118 F1 background. However, in more recently sampled lineages with diverse genetic variation, this resistance trait varies significantly, with many lines failing to report any fasting-induced starvation resistance.

MATERIALS AND METHODS

Drosophila stocks and husbandry

Stocks used for this study include an F1 cross of Canton-S and w118, inbred lines obtained from Maine (Behrman et al., 2018), the American southeast, the Bahamas (Kao et al., 2015) and the Netherlands (Grenier et al., 2015), in addition to isofemale lines that were collected at Carter Mountain Orchard in Charlottesville, Virginia (latitude 37.99, longitude −78.47), and the Linvilla Orchard in Media, Pennsylvania (latitude 39.88, longitude −75.41). Flies were kept on a food medium composed of 86% water, 0.574% agar, 6.30% cornmeal, 1.52% yeast, 4.65% molasses, 0.39% propionic acid, 0.15% methylparaben and 0.52% ethanol.

Fasting assay

To perform the fasting treatment, we constructed replicate vials of 30 flies less than 5 days old. Only gravid females were used for the isofemale and inbred line experiments, while the F1 experiment also included separate replicates for males. Four replicates of each genotype were either fasted from 9 AM to 5 PM on a nutrient-less agar or were kept in control conditions on normal media throughout. This fasting protocol was repeated 3 consecutive days out of the week. Starvation resistance was measured at the conclusion of the fasting treatment. For the isofemale line assay, we included additional replicates to measure starvation resistance after each subsequent week of fasting.

Starvation assay

Starvation resistance of flies was measured using the Diamonds method (Seong et al., 2020). Individual flies were transferred to each well of 96-well plates containing 1.5 agar solution. Plates were placed atop flatbed scanners, and images were taken every hour until all flies had stopped moving (5–7 days). Images of the wells were processed using the Sapphire software to estimate the time of death for each fly (Seong et al., 2020) using a neural network trained on fruit fly movement. Sapphire performs semantic segmentation to distinguish between living versus dead flies based on the change-point analysis of location through time. To validate Sapphire's estimates, we performed a test assay in which flies were placed in 96-well plates and starved as described above, with the mortality observed from manual observations of the images confirming the estimates from Sapphire (results not shown).

Metabolite measurement assay

The triglyceride content of fly samples was estimated using coupled calorimetric determination (Tennessen et al., 2014). Each fly sample was homogenized in Phosphate buffered solution (PBST + 0.05% Tween80) using an electric pestle. The samples were centrifuged, and 100 μL of supernatant was frozen for future use. The rest of the supernatant was separated and heat-fixed for 30 min at 70°C. A total of 20 μL of each sample was added to wells of a 96-well plate. Each sample was paired with a well of 20-μL PBST as a negative control. A total of 20 μL of triglyceride reagent (Sigma; T2449) was added to each sample on a 96-well plate, as well to a coupled set of PBST controls. The plates were incubated at 37°C for 30 min and then centrifuged. Finally, 100 μL of glycerol reagent (Sigma; F6428) was added to each sample and control well and incubated for another 5 min at 35°C. The absorbance of each well at 540 nm was compared to a standard curve to infer fat content. The quantity of protein was measured using the Coomassie blue Bradford assay kit (Fisher 23200). A total of 30 μL of each previously frozen sample was mixed with 1.5 mL of the Coomassie reagent and incubated for 10 min. The absorbance of each sample at 595 nm was compared to a standard curve to infer protein content.

Statistical inferences

In each fasting-induced starvation resistance assay, we measured time to death for control and fasted experimental groups with at least three independent replicate experiments of 20 flies per genotype per treatment group. We assessed the difference in starvation resistance between groups using the Cox proportional hazards test (Coxph), which models the probability of survival as a function of genotype and treatment. For each sex, we built three models and used likelihood ratio tests to assess the statistical significance of each model term using the log-rank test.

Model 1: Survival ~ Time + Treatment.

Model 2: Survival ~ Time + Treatment + Genotype.

Model 3: Survival ~ Time + Treatment + Genotype + Treatment * Genotype.

In addition, we also examined differences in phenotype (survival time, fat concentration) between two sets of observations using *t*-tests. We tested the correlation of fat concentration with survival with a Pearson's product moment test.

RESULTS

Starvation resistance improvement can be replicated within a common lab lineage

To confirm the ability of periodic fasting to promote starvation resistance, we replicated a previously published fasting assay described by Zhang et al., 2018. This experiment used F1 males and females that are a cross between Canton-S and w118, and performed periodic fasting for 3 days out of the week. The fasted male flies experienced significantly higher starvation resistance than the control group (Figure 1a, log-rank test, $\chi^2 = 5.21$, df = 1, $p = 0.02$), confirming the findings of Zhang et al., 2018. The female fasted flies demonstrated even stronger improvement in starvation resistance (Figure 1b; log-rank test, $\chi^2 = 23.51$, df = 1, $p = 1.2e\text{-}06$), as well as higher fasted resistance overall compared with fasted male resistance (Figure 1c; *t*-test, $t = -6.72$, df = 84, $p = 2.06e\text{-}09$).

Genetic variation in fasting-induced starvation resistance

We assayed periodic-fasting-induced starvation resistance across a global panel of inbred lines to gain a broader understanding of the natural variation within this trait. We used a set of inbred lines from the Netherlands, the Bahamas, Maine and the southeastern United States, and fasted them in the same method described above. The inbred panel showed markedly different responses to the fasting regimen compared with the Canton-S/W118 cross. Rather than improving starvation resistance, fasting overall decreased resistance among the inbred lines (Figure 2a, log-rank test, $\chi^2 = 6.86$, df = 1, $p = 0.008$), and we also found significant variation among the lines (Figure 2b, log-rank test, $\chi^2 = 109.2$, df = 7, $p = 2.2e\text{-}16$). While there was no significant gene-by-environment interaction (log-rank test, $\chi^2 = 8.2$, df = 6, $p = 0.22$), different lineages responded in opposing ways to fasting. Most lines (e.g., USA Southeast 2) exhibited a decrease in starvation resistance with fasting, one with an increase in starvation resistance (e.g., Netherlands-2), while others showed no difference between treatments (e.g., Bahamas-2; Figure 2c). Fat concentration (μg triglyceride/μg body protein) also varied across groups. Control treatment flies contained significantly higher triglyceride concentrations than fasted flies after the treatment (*t*-test, $t = 4.59$, df = 61, $p = 2.16e\text{-}05$), with the extent of decrease varying among lineages (Figure 2d). The level of prior-fat concentration was a marker of starvation survival success and positively correlated with hours of survival ($r = 0.246$, df = 323, $p = 6.9e\text{-}06$).

Significant variation in fasting-induced starvation also occurs among recently established isofemale lines

To test if fasting-induced starvation resistance could be commonly found in outbred and recently sampled lineages, we examined isofemale lines collected at different times per year in Virginia and Pennsylvania. As with the inbred panel, we do not observe any significant improvement in overall average starvation resistance from the treatment (Figure 3a, log-rank test, $\chi^2 = 1.54$, df = 1, $p = 0.2$). However, we find significant variation in the induced starvation resistance between different isofemale lines (Figure 3b, log-rank test, $\chi^2 = 55.69$, df = 7, $p = 2.33e\text{-}11$). Flies collected during the summer in Pennsylvania showed significant improvement in fasting-induced starvation resistance (Figure 3c, log-rank test, $\chi^2 = 4.85$, df = 1, $p = 0.027$), whereas the other sets of recently collected isofemale lines do not show a change in starvation resistance following periodic fasting. There was no effect of season on starvation

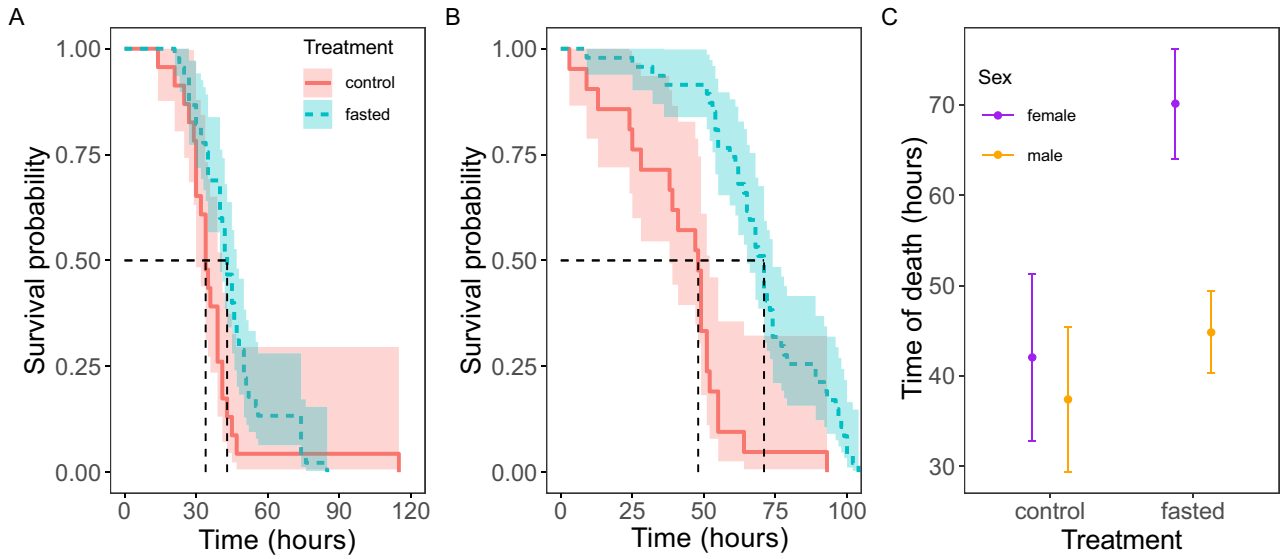

FIGURE 1 Replication of starvation resistance improvement. (a) Comparison of the survival curves of male fasted (blue) and female control (orange) flies. The p value is derived from a log-rank interaction comparing the two groups. (b) Survival curves of fasted and control female flies. (c) The distribution of starvation times across treatments and sexes. Error bars represent 95% standard errors.

resistance among Pennsylvania samples (*t*-test, $t = 1.96$, df $= 136$, $p = 0.052$), whereas Virginia samples showed greater resistance in the fall (*t*-test, $t = -4.99$, df $= 136$, $p = 1.77e\text{-}06$). As an additional assay, we tested starvation resistance after each subsequent week of treatment to explore any age effect on fasting response. While there was no significant effect of fasting on starvation survival at any week(week 1: *t*-test, $t = 0.935$, df $= 302$, $p = 0.350$; week 2: $t = 1.21$, df $= 261$, $p = 0.223$; week 3: $t = -0.135$, df $= 270$, $p = 0.893$), there was a significant effect of age on starvation resistance. One-week old flies of either treatment resisted starvation longer than 3-week-old flies (Figure 3d, *t*-test, $t = -3.68$, df $= 549$, $p = 0.000258$).

DISCUSSION

Periodic fasting has elicited changes in fitness traits across multiple species (Aly, 2014; Rothschild et al., 2014) and in recent years has been shown to strengthen starvation resistance in fasted *Drosophila* (Catterson et al., 2018; Zhang et al., 2018). However, it remains unclear to what extent these findings are specific to the historic laboratory lineages used and to what extent natural genetic variation will influence these survival improvements. In this work, we leverage the wealth of genetic variation available within *D. melanogaster* to test whether the effects of periodic fasting on survival are conserved within the species.

Periodic fasting promotes starvation resistance in the cantons/w118 background

We replicated previously reported results (Zhang et al., 2018) of increased starvation resistance following periodic fasting of adult flies, compared to a fully fed control flies (Figure 1a,b). This previous work only examined the males of an F1 cross of two historic lab lineages. Here, we show that periodic fasting improves starvation resistance to an even greater extent within females of the same background. Female *Drosophila* has consistently shown greater starvation resistance than males (Rion & Kawecki, 2007; Schwasinger-Schmidt et al., 2012), and the improved starvation resistance of females could be mediated by greater triglyceride stores within the fat body of female flies compared with males (Millington et al., 2021). Taken together, our results confirm that periodic fasting throughout adult life can promote increased starvation resistance.

Periodic fasting's ability to increase starvation resistance significantly varies across genetically diverse lineages

Extending the same fasting model to a global panel of inbred lines revealed significant variation in the effect of fasting on starvation resistance. Lines from as far south as the Bahamas and as far north as the Netherlands exhibited different resistance responses when subjected to periodic fasting (Figure 2b). The general impact of fasting on starvation resistance was detrimental, with control flies out-surviving fasted flies. This finding is surprising given the general view that dietary restriction improves survival (Bubliy et al., 2012; Gill et al., 2015; Kapahi et al., 2017) and seems to contradict our previous results indicating starvation resistance improvement in a historic laboratory lineage (Figure 1a,b). However, other studies have indicated that diverse genetic panels can prove to be exceptions to the commonly held rules of dietary restriction (Gomez et al., 2020). For instance, there is no effect of dietary restriction on lifespan when the feeding of mice with

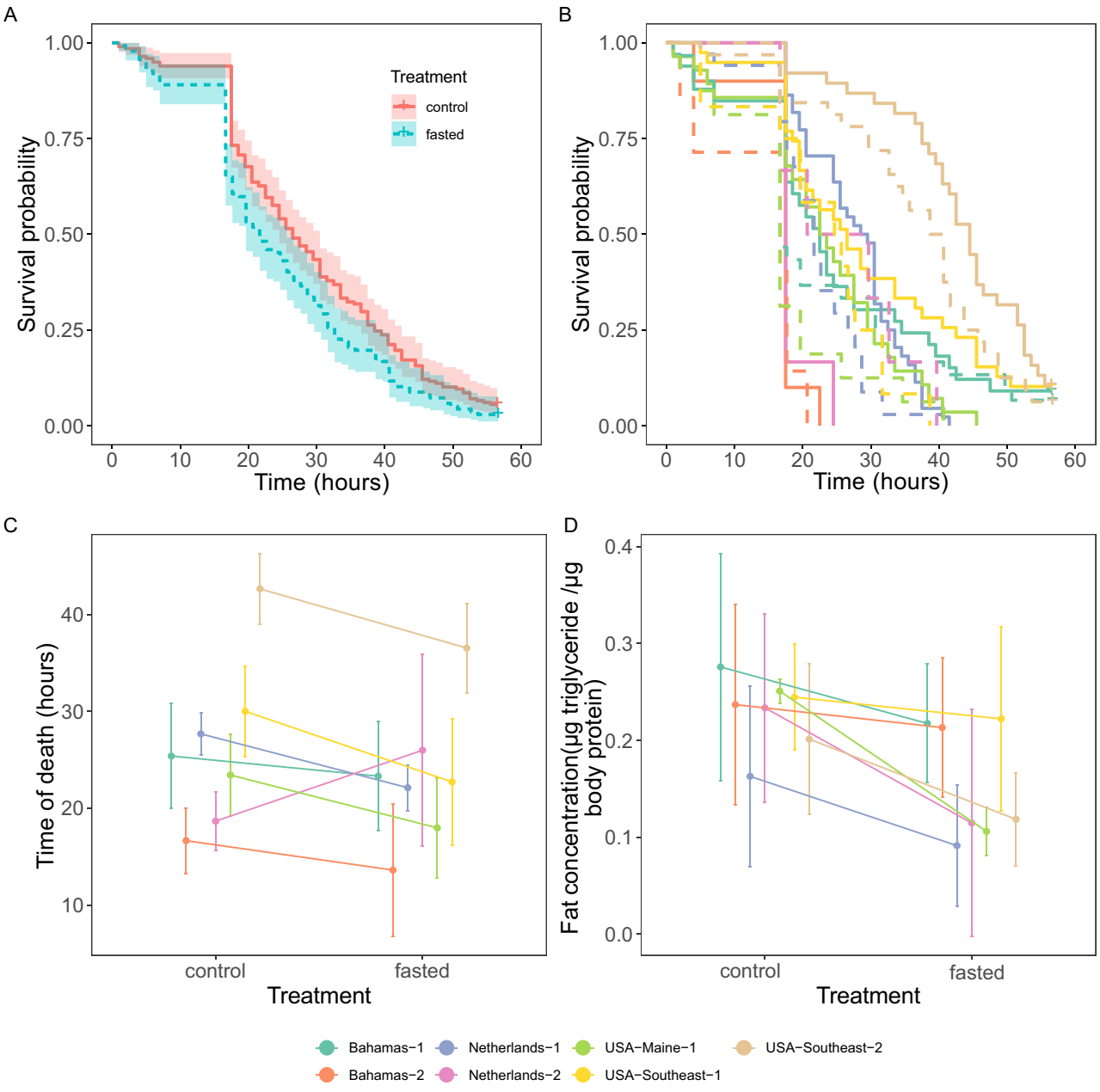

FIGURE 2 Natural variation in the response of a global panel to fasting. (a) Survival curves of control and fasted inbred lines during starvation. (b) Survival curves of individual inbred lineages. Colour is determined by genotype, type determined by treatment. (c) Variation in the impact of fasting on starvation resistance. (d) Variation in the impact of fasting on triglyceride fat concentration (µg triglyceride/µg body protein).

natural genetic backgrounds is restricted (Harper et al., 2006). Additionally, dietary restriction both reduced and improved lifespan among a panel of 161 *D. melanogaster* lineages (Wilson et al., 2020). In a similar manner, we demonstrate natural genetic variation in the response to dietary intervention (Figure 2c). Some lines improved in starvation resistance, some were unaffected and some deteriorated in resistance. The overall mean response trended toward lower resistance in fasted flies. Our metabolite findings could offer insight into this result, as fasting treatment appeared to reduce energy storage among fasted flies. Fat concentration significantly increased in control flies but not in the fasted flies, implying that the periodic fasting regime impaired the buildup of triglycerides that generally occur throughout fly adulthood (Catterson et al., 2018). Post-fasting protein and fat levels among our sampled lines varied, as has been previously reported in fly panels (Figure 2d, Jin et al., 2020). We show that fat storage positively covaried with starvation survival, as is expected (Hoffmann & Harshman, 1999). Additionally, we observe genetic variation in starvation resistance following fasting in a panel of recently caught, outbred isofemale lines (Figure 3b). For example, Pennsylvania summer lines contradicted the general trend and improved starvation resistance with fasting (Figure 3c). Taken together, we

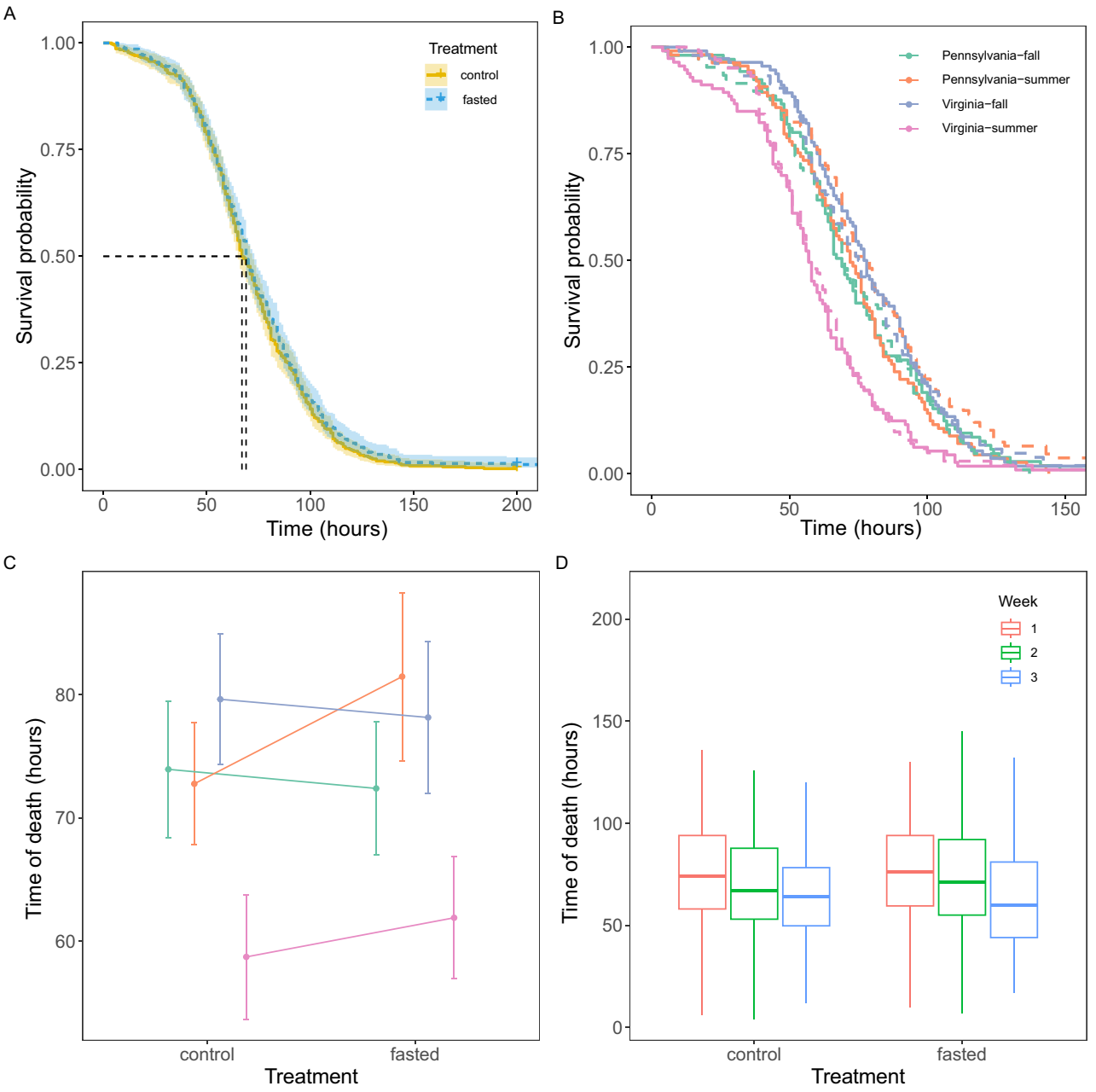

FIGURE 3 Significant variation in the fasting-induced starvation resistance in natural genetic lineages. (a) Survival curves of control and fasted isofemale lines during starvation. (b) Survival curves of individual isofemale lineages. Colour is determined by genotype, type determined by treatment (c) Variation in the impact of fasting on starvation resistance. (d) Box and whiskey plot showing the change in hours of starvation survival across weeks of fasting, with colour representing the weeks spent fasting prior to fasting.

document the large breadth of natural variation in fasting-induced starvation resistance among lineages originating across seasons and across the globe.

By demonstrating significant genetic variation in fasting-induced starvation resistance, we indicate the extent of unreliability in the success of a starvation-hardening treatment. As with cold hardening (Czajka & Lee, 1990) or heat hardening (Sejerkilde et al., 2003), a short duration of starvation-like conditions is thought to promote later resistance to starvation via a shift in gene expression post-treatment (Bubliy et al., 2012). For instance, in *Drosophila* on the Indian subcontinent, there is not only a latitudinal cline in starvation resistance but also sex and latitude-specific effects on the ability of hardening to improve starvation resistance (Aggarwal, 2014). Starvation resistance clines across latitudes indicate different nutritional needs based on the local environment (Rion & Kawecki, 2007). Therefore, fasting-induced responses would also differ based on the environment of origin. We report variation in starvation resistance improvement in lines collected across latitudes and further demonstrate that the genetic variation within the fasted animal has a large impact on subsequent survival.

CONCLUSION

Genetic variation can influence the success of dietary treatments

A long-term goal of some scientists and nutritionists is to identify the schedule and parameters of dietary restriction, intermittent fasting or nutrition-specific limitation that can consistently improve health across all portions of a population (García-Montero et al., 2021; Stanhope, 2016). But metabolic research, including lineages with genetic variation, indicates an issue with any one-size-fits-all dietary approach. In *Drosophila* studies, there is significant variance in the impact of dietary restriction on survival. Dietary restriction induced both improvement and reduction in lifespan among a *Drosophila* panel and variation in the metabolite profiles of the same panel (Jin et al., 2020; Wilson et al., 2020). This phenomenon extends to other common model organisms. When a panel of wild isolates of *C. elegans* were treated with dietary restriction, their subsequent lifespan considerably varied, with some lineages exhibiting a reduction in lifespan after fasting (Stastna et al., 2015). Among genetically diverse mice, fasting reduced lifespan as often as it extended it (Harper et al., 2006; Liao et al., 2010). Part of the difficulty in identifying a universally beneficial diet is genetic variance among the population. Work presented here and elsewhere (Harper et al., 2006, Liao et al., 2010) demonstrates that the phenotypic response to a dietary treatment can assume different directions when examined across lineages. Mounting evidence indicates that an individual's genetic background impacts aspects of metabolic health, such as obesity and diabetes, and how these phenotypes respond to dietary treatment (Heianza & Qi, 2017, Ortega et al., 2017). As medical concerns increase surrounding the rising level of obesity (Dietz, 2015), it is important to understand how the genetic background of an individual may influence their susceptibility to dietary intervention.

Historic lab lineages are heavily represented in metabolic research but are not always representative of common dietary responses

The genetic variation of fasting-induced starvation resistance that we observe here highlights the importance of exploring the natural variation present within a trait when seeking to understand responses to potential treatments. Over the last 20 years, 16 publications have focused their study on periodic fasting, intermittent fasting or time-restricted feeding in *Drosophila* (Aggarwal, 2014; Catterson et al., 2018; Dissel et al., 2022; Gill et al., 2015; Liu et al., 2021; Livelo et al., 2023; Melkani et al., 2017; Oishi et al., 2004; Ratliff et al., 2016; Salgado-Canales et al., 2023; Ulgherait et al., 2021; Villanueva et al., 2019; Xu et al., 2011; Zhang et al., 2018; Zhang et al., 2023). Only two of these considered a panel of genetic backgrounds (Aggarwal, 2014; Ulgherait et al., 2021), and only one used fly lines maintained in labs for less than 50 years (Aggarwal, 2014). Canton-S, w118, Oregon R and their transgenic variants dominate this area of the literature. There is no question of the value of this work nor the tremendous advancements from using historic laboratory lineages, but this study illuminates the limitations resulting from focusing on a small set of lineages. Here, we observe the Canton-S\w118 fasting response is just one of many possible responses to intermittent fasting. By using fly lines isolated across latitude, season and continent, we were able to demonstrate the considerable variance in how fasting alters protein accumulation, fat concentration and starvation resistance. Subsequent work into periodic fasting across model species can use the toolbox of natural variation to fully characterize the response to fasting as specific to and impacted by the genetic variation present.

AUTHOR CONTRIBUTIONS

Benedict A. Lenhart: Conceptualization; investigation; writing – original draft; methodology; validation; visualization; writing – review and editing; software; formal analysis; data curation; project administration. **Ayesha Ahsan:** Investigation; writing – review and editing; writing – original draft. **Margaret McHaty:** Writing – original draft; writing – review and editing; investigation. **Alan O. Bergland:** Conceptualization; funding acquisition; writing – original draft; writing – review and editing; project administration; supervision; resources; methodology; formal analysis.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are openly available in StarvationResistance2024 at https://github.com/benedictlenhart/StarvationResistance2024/tree/main/data.

ETHICS STATEMENT

The authors declare that this paper is completely original and has not been previously submitted to another journal. The paper reflects the authors' own research and analysis in a truthful and complete manner. The paper properly credits the meaningful contributions of co-authors and co-researchers. The results are appropriately placed in the context of prior and existing research. All sources used are properly disclosed.

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