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RESEARCH ARTICLE

Dietary restriction extends lifespan across different temperatures in the fly

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Abstract

1. Dietary restriction (DR) has been consistently shown to extend lifespan across a range of taxa. However, it has recently been reported that DR does not extend lifespan at certain, namely lower, temperatures in flies (*Drosophila melanogaster*). Similar to the interpretation of other findings that appear to question DR's universality, this finding has been interpreted as suggesting that lifespan extension in response to DR is an artefact of benign laboratory conditions.
2. We re-test this hypothesis, now using a strain that shows robust lifespan extension at 25°C (across three prior experiments), and using a range of five diets across two temperatures, 18°C and 21°C.
3. We found the DR longevity response to be robust, extending lifespan irrespective of temperature. We measured fecundity as a positive control for the DR phenotype, and found, as predicted, that DR reduces egg laying.
4. We suggest differences in experimental setup, genetic lines used and variation in the diet-lifespan reaction norm are responsible for this discrepancy. In addition, starting with a strain and conditions that show a lifespan extension by DR, as we do here, and then changing environment and/or genotype promises a more robust test of DR modulating factors.
5. In conclusion, it will be important for results that question DR as a phenotype to not be overinterpreted readily, as with a substantially larger sample size and a larger range of diets we were unable to replicate this prior work.

KEYWORDS
aging, diet, evolution, senescence

1 | INTRODUCTION

Dietary restriction (DR) is one of the oldest known and best replicated life extending treatments in animals (Nakagawa et al., 2012; Simons et al., 2013). However, both its physiology and evolutionary biology remain hotly debated (Adler & Bonduriansky, 2014; McCracken, Adams, et al., 2020; Moatt et al., 2020; Piper et al., 2023). Given our incomplete understanding of DR and the intensity of study it continues to receive, it is perhaps unsurprising that with certain regularity studies report that DR is not extending lifespan and attribute this to certain circumstances, either genetic or environmental (Harper et al., 2006; Ja et al., 2009; Liao et al., 2010). Such studies can be interesting, but can also be distracting to the field if overinterpreted.

We recently argued that an absence of a DR response can be due to shift in reaction norm rather than a change in the dose–response (Simons & Dobson, 2023). Although this point has been made by us and others (Flatt, 2014; Tatar, 2011) misinterpretations are still made. If, for example, an environmental condition shifts the overall nutritional requirement of an organisms but does not change the shape of the dose–response, premature death due to malnutrition can readily occur at DR diets that would otherwise extend lifespan. These interactions can also be more complicated with environmental conditions increasing the requirement of a key nutrient that is in short supply in the DR diet, leading to a truncation of lifespan, even though the restriction of this key nutrient is not causal in the DR longevity phenotype. Careful consideration of a range of diets can thus be crucial when concluding environmental or genetic conditions negate the DR response (Simons & Dobson, 2023).

Moreover, DR can arguably only be interpreted as absent when in the same study routine conditions result in a robust and repeatable DR phenotype. A recent example of DR not extending lifespan of flies (*Drosophila melanogaster*) at lower temperatures has been interpreted as DR being a lab artefact with low temperature interpreted as representing stressful conditions (Zajitschek et al., 2023). There are several questions that can be posed to this study, for example: why did DR not extend lifespan at the most regularly used lab temperature of 25°C in females, the most studied sex in this paradigm? Why did lifespan become truncated at low temperatures under DR? Why, if low temperatures induce 'stress', did flies show high levels of fecundity at those temperatures?

These questions largely involve interpretation of these findings. We also sought to question whether a strain that in our hands shows consistent and robust lifespan extension at DR at the arguably standard temperature of 25°C, showed no such response at lower temperatures (18°C and 21°C). We suggest the most reasonable test of the hypothesis posited by this recent work questioning DR, is to start with a strain that reliably and repeatedly shows DR under standard temperatures to then test whether lower temperatures negate the DR response. We use substantially larger sample sizes per treatment group (N is between 253 and 505 females) compared to the work that led to this hypothesis ($N = 100$) (Zajitschek et al., 2023). We further used a broad range of five diets to be able to distinguish a shift from a change in shape of the DR lifespan reaction norm (Simons & Dobson, 2023).

We find that DR extends lifespan consistently across three separate experiments at 25°C. We further find that DR extends lifespan irrespective of temperature (18°C and 21°C) and that its effect is highly quantitatively similar across temperatures even though lower temperatures, as expected, increased lifespan substantially.

2 | METHODS

2.1 | Fly husbandry and diets

The ywR lab strain of *Drosophila melanogaster* was used for all experiments (Wessells et al., 2004). The yellow (y) and white (w) mutants were part of the first studies of cross-over and genetic mapping at the start of the previous century (Morgan, 1915; Muller, 1916; Sturtevant, 1915). This stock itself is thus likely considerably old and inbred. Any genetic stock has the propensity to diverge, however, hence we provide our best provenance of this strain here. We obtained the strain from Marc Tatar, who obtained this strain as detailed in the cited paper (Wessells et al., 2004). All flies were cultured on rich yeast media [8% autolyzed yeast, 13% table sugar, 6% cornmeal, 1% agar, nipagin 0.225% (w/v), propanoic acid 0.4% (v/v)]. Cooked fly media was stored for up to 2 weeks at 4–6°C, and warmed to 25°C before use. For all experimental diets (to which no propanoic acid was added), all components of the fly food media remained consistent but the amount of yeast was varied (2%, 4%, 6%, 8% and 10% yeast), representing a spectrum of rich to restricted diets (McCracken, Buckle, et al., 2020; Simons & Dobson, 2023). For experimental diets, 8% yeast is the standard ad libitum diet used by the laboratory and 2% is the standard restricted diet used. Research conducted with invertebrates such as *Drosophila* does not require ethical approval.

2.2 | Demography protocol

Bottles of 10–12 females and 3–4 males per bottle were set up, allowing for 2 days egg laying before flies were removed to control growing density (Linford et al., 2013). The F1 generation from these bottles were transferred to new mating bottles as they eclosed and left to mate for 2 days. Newly eclosed offspring were transferred every day to generate age matched cohorts. After mating, offspring were anaesthetised using CO_2 (Flystuff Flowbuddy; <5 L/min), females were sorted into groups of 70–100 and put into purpose-built demography cages (Good & Tatar, 2001; McCracken, Adams, et al., 2020), in which experimental diets were started. The cage design allowed for easy removal of dead flies and changing of fly food vials with minimal disruption to the population of flies. Every other day, food vials were replaced for each cage and a census of the flies was conducted. Any dead flies were counted and removed from the cage. Any escaped flies, or flies stuck to the fly food were right censored. Once sorted into cages, flies were housed in temperature-controlled incubators with humidity provided by large trays of water at either 21°C or 18°C (~60% humidity, 12:12 light–dark cycle). For experiments conducted at 25°C, cages were kept in a climate-controlled room (60% humidity, 12:12 light–dark cycle) and the same census protocol was followed.

2.3 | Egg counting

Food vials were taken from demography cages 36 and 40 days after the experiments started, ages determined to be roughly a midpoint in the lifespan of the fly populations at the highest yeast diets. Eggs laid in the vials, constituting 2 days of egg laying, were counted manually under a light microscope.

2.4 | Data analysis

Lifespan data were analysed using time-to-event mixed-effects Cox proportional hazard models, with demography cage as a random term (R package: coxme; function: coxme), to correct for uncertainty of pseudo replicated effects within cages (Therneau et al., 2003). The interaction between temperature and diet was fitted to test for differential effects of diet on mortality depending on temperature. Egg laying was analysed using linear models for each measurement time point separately. Egg counts were divided by the total females in the cage at the time of fecundity measurement to correct for differences in mortality.

2.5 | Sample size and level of inference

We wish to test DR in flies and measured egg laying and lifespan. These experiments were done at large sample sizes and with mated female flies in groups in cages (included as random term in 'coxme', Table 1).

3 | RESULTS

First we wanted to confirm that we used a strain responsive to DR at the lab standard 25°C. In a strain (ywR), we have used extensively before (Drake & Simons, 2023; Gautrey & Simons, 2022) we found a significant DR longevity response in three other separate (before unpublished) experiments we previously conducted ($p < 0.0001$; Table 2). These experiments compared 2% yeast (our standard DR diet) to 8% yeast (the standard ad libitum diet used by our lab) (McCracken, Buckle, et al., 2020) (Figure 1). There is no difference in how we ran these experiments, other than that they were conducted at different times, in the same laboratory, using the same protocol (see methods). The response to DR varied slightly but significantly across these experiments (Chisq = 22.3, df = 2, $p < 0.0001$, Table 2).

We then took this same strain and tested the DR response across five diets (2%, 4%, 6%, 8% and 10% yeast, keeping all other ingredients the same) at two temperatures 18°C and 21°C. We found the DR longevity response across both temperatures ($p < 0.0001$, Figure 2). Furthermore, there was no evidence that the diet effect was influenced by temperature (Chisq = 1.83, df = 4, $p = 0.77$), and effects of diet were largely similar (Table 3; Figure 2a,b). The benefit of using multiple diets here is that it allowed us to see if there was a change in shape of the reaction or merely a shift. The result shows a remarkable similarity in reaction (Table 3; Figure 2a) that is not changed in shape or shifted across the x-plane. The mere difference is an overall effect of temperature that causes a longer lifespan.

TABLE 1 Sample sizes and scale of inference.

Scale of inference	Scale at which the factor of interest is applied	Number of replicates
Individual lifespan	Individuals and Cage	1566 individuals at 18°C across 5 diets with 4 cages per diet 2435 individuals at 21°C across 5 diets with 6 cages per diet 566 individuals at 25°C across 2 diets with 5 cages per diet 934 individuals at 25°C across 2 diets with 5 cages per diet 2420 individuals at 25°C across 2 diets with 14 & 15 cages per diet
Individual egg laying	Cage	49 cages 5 diets 4 cages per diet (3 for one diet) at 18°C 6 cages per diet at 21°C

TABLE 2 DR extended lifespan across three different replicate experiments in ywR flies.

Experiment	logHR of DR (SE)	*p*	*N*
A	−2.68 (0.07)	<0.0001	2420
B	−2.04 (0.13)	<0.0001	566
C	−2.36 (0.09)	<0.0001	934

Note: Log hazard estimates (logHR, with their standard error, SE) reported from separate models for each experiment as we found slight differences in the response to DR across these experiments, which is expected even in replication due to small variations in unknown experimental factors (Simons & Dobson, 2023).

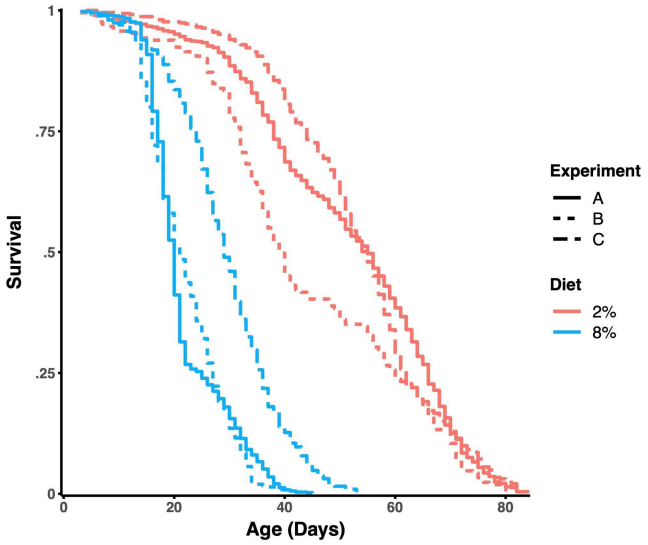

FIGURE 1 Kaplan–Meier plot combining survival curves of three separate experiments, each using ywR flies on 8% and 2% yeast diets and kept at 25°C ($N = 3920$ females total).

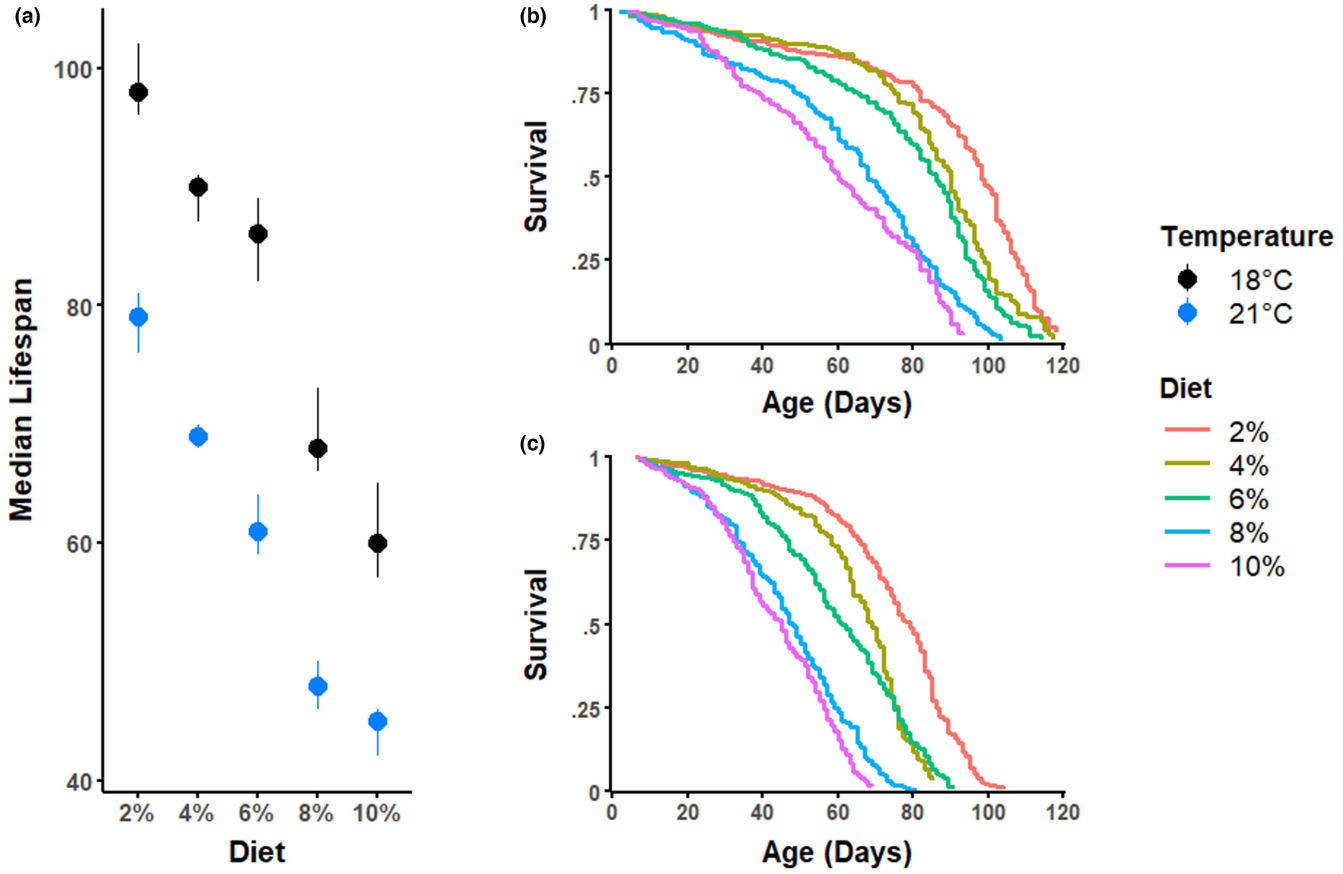

FIGURE 2 (a) Median lifespan and 95% CIs of yw *Drosophila* fed diets with a range of yeast concentrations, kept at 18°C or 21°C. Respective Kaplan–Meier plots, showing survival rates of the different diet treatment groups at (b) 18°C or (c) 21°C. $N = 4001$ females total; 253–505 per diet.

DR is classically associated with a decline in reproduction and is often used as a convenient readout to distinguish between a rescue from overfeeding and a true DR response (Gautrey & Simons, 2022; Grandison et al., 2009; McCracken, Buckle, et al., 2020). For this reason, we measured egg laying at two time points during middle age (ages 36–41 to 40–45 days). We found no interaction between diet and temperature at either time point ($p = 0.33–0.76$) nor a main effect of temperature ($p = 0.08–0.94$). Higher yeast concentrations were associated with higher egg laying ($p < 0.0001$) and at the lowest yeast diet, on which flies also lived the longest (Figure 2), flies laid the fewest eggs (Figure 3).

4 | DISCUSSION

Our results unambiguously reinforce that DR can reliably increase lifespan in flies at standard laboratory conditions across different temperatures. As such, our work fits with earlier work that used a combination of life-extending treatments, including temperature and DR, and found them to be largely additive (Kim et al., 2020; Shaposhnikov et al., 2022). Our findings and this prior literature contradict those of a recent study (Zajitschek et al., 2023) and counter their argument that DR constitutes a laboratory artefact.

Those authors (Zajitschek et al., 2023) considered 25°C to be a low temperature for flies as it is lower than the ambient temperature in the climate of origin for the species. However, most experimental lines of flies will have been inbred or bottlenecked for many years under laboratory conditions, often at 25°C. The precise original climate of the specific strain used is thus unlikely to still be physiologically relevant. Notably, heatshock protein expression is present at benign temperatures (25°C) in *Drosophila*, with responses differing between lines (Bettencourt et al., 1999) and species (Kristensen et al., 2002; Sørensen et al., 2019). It is unclear therefore why lower temperatures should necessarily be interpreted as stressful (as is done in Zajitschek et al., 2023). For example, low temperatures increase lifespan (Mair et al., 2003) and at very low temperatures diapause is induced which results in mortality amnesia. Flies that are put at 25°C after a period in diapause resume their mortality trajectory as if they were in suspended animation (Tatar & Yin, 2001). In Zajitschek et al. (2023), it is further reported that egg laying is highest at relatively lower temperatures, again suggesting that these temperatures are not necessarily stressful, even under their laboratory conditions, especially because these are also at the temperatures that their flies lived the longest.

Moreover, genetic variation inherent in this 'outbred' line can lead to unexpected variation in the population reaction norm to diet

TABLE 3 DR extends lifespan across both temperatures in ywR flies.

Temp	Log HR of 2% (DR)	Log HR of 4%	Log HR of 6%	Log HR of 10%	*N*
18°C	−1.50 (0.22) $p<0.0001$	−1.06 (0.22) $p<0.0001$	−0.76 (0.21) $p=0.0004$	0.27 (0.21) $p=0.20$	1566
21°C	−2.02 (0.16) $p<0.0001$	−1.16 (0.15) $p<0.0001$	−1.00 (0.15) $p<0.0001$	0.26 (0.15) $p=0.076$	2435

Note: Log hazard estimates are reported compared to the 8% yeast ad libitum diet (reference category). Estimates are largely the same and there is no evidence that the full diet response is modulated substantially across both temperatures. At 2% yeast only there might be a hint of a temperature effect, estimates are strongest at 25°C judging from the replication experiments (Table 2), and decline slightly with decreasing temperature (data presented in this table), but are still highly significant and strong. In linear hazard terms, risk is still lowered by 4.5 fold, and this resulted in an increase in median lifespan to 98 days at 2% yeast from 68 days at 8% yeast at 18°C (Figure 2).

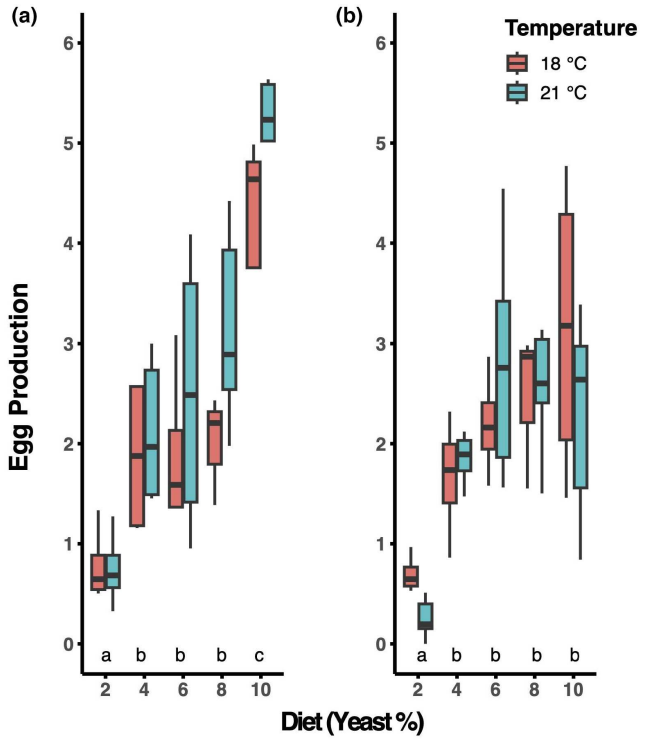

FIGURE 3 Mean egg production per fly across 2 days on different yeast diets, at two different time points [Mean ages of (a) 39 days and (b) 43 days]. $N=49$ demography cages respectively per time point. Letters (a, b and c) indicate significant differences based on post-hoc *t*-tests.

if genotypes present within the stock have different dose-responses to diet (Simons & Dobson, 2023). The simplest form that this can take is that some individuals in the population start to show malnutrition and die prematurely whilst others are at their DR lifespan optimum. Although here we only use a single inbred strain, we have shown previously that reaction norms to DR differ between strains (McCracken, Buckle, et al., 2020). Thus, when an environmental effect on DR is tested it can be argued that a single inbred strain is a more sensitive test, as G×E can cancel out or dampen effects. Such considerations are especially important when DR is not clearly observed in the inbred or outbred strain used at 25°C (the temperature flies are commonly grown at and the temperature at which DR is mostly studied), as in Zajitshek et al., imposing a substantial possible confound to their work.

In conclusion, perhaps certain aspects of the experiment by Zajitschek et al. were not fully permissive of DR, leading to their finding that DR could not be found at certain temperatures. These factors include diet (Simons & Dobson, 2023) and it is worth noting that the diets used by Zajitschek et al. are richer in yeast than those we used here. Their standard diet contains the same amount of yeast as the richest diet used in our study. That a clear positive control, namely DR extending lifespan consistently, is missing, further limits what we can interpret from this prior study.

Our work here, supported by prior published work by others (Kim et al., 2020; Shaposhnikov et al., 2022), does not support the interpretation that DR is a lab artefact and that DR would therefore not extend lifespan in the wild (Zajitschek et al., 2023). Even though conditions, environmental or genetic, that modulate the DR response are a valuable tool toward understanding the DR response and ageing more generally (Simons & Dobson, 2023), it deters progress if such contrary findings are overinterpreted. Such interpretation becomes especially distracting when it includes broader ecological, evolutionary and physiological relevance when these consequences are in fact not directly studied.

AUTHOR CONTRIBUTIONS

EJP and MJPS conceived the ideas and designed methodology; EJP collected the data; EJP and MJPS analysed the data; EJP & MJPS wrote the manuscript. All authors contributed critically to the drafts and gave final approval for publication.

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CONFLICT OF INTEREST STATEMENT

The authors declare no competing interests.

DATA AVAILABILITY STATEMENT

Data available from the Dryad Digital Repository: https://doi.org/10.5061/dryad.fttdz091j.

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