Manuscript Details

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Abstract

Mitochondrial genetic variation can have profound effects on fitness, and the mitotype must interact with both the nuclear genes and the environment. We used Drosophila to investigate the extent to which mitotype effects on lifespan and activity are modulated by nucleotype and environmental variation. When nucleotype is varied, mitochondrial effects on lifespan persisted but were relatively small, and still male biased. Varying food as well, mitotype had substantial effects on male climbing speed, modifiable by nucleotype but less so by diet. Finally, mitotype affected fly lifespan much more in a cage environment compared with a vial, also modifiable by nucleotype and diet. The cage may represent a stressful environment. Mitochondrial genotype may affect fitness much more in conditions of stress, which may have implications for human health.

Keywords	mitonuclear; epistasis; GxE interaction; Drosophila; aging; lifespan; stress.

Corresponding Author	David Clancy
Order of Authors	Emma Drummond, Emma Short, David Clancy

Suggested reviewers	Urban Friberg, Kevin Fowler, Neil Gemmell, Alexei Maklakov, Edward Morrow

Submission Files Included in this PDF

File Name [File Type]

cover letter.docx [Cover Letter]

response to reviewers.docx [Response to Reviewers]

highlights.docx [Highlights]

Abstract.docx [Abstract]

Mito GxE ms-revised.docx [Manuscript File]

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Research Data Related to this Submission

Data set	https://data.mendeley.com/datasets/7rnwhcswvb/1

Drosophila mitonuclear GxGxE paper - data

First file, LS.sav, is lifespan data looking at interactions of sex, nuclear and mitochondrial genotypes on lifespan. Second file, Survival Data.sav, is male lifespan data looking at interactions of nuclear and mitochondrial genotypes, food and rearing environments on lifespan. Third file, d14.sav, is climbing data for males (height in 15secs) from Survival Data.sav, measured on day 14 looking at interactions of nuclear and mitochondrial genotypes, and food type. Triplicate (techrep) is nested

Division of Biomedical and Life Sciences | Lancaster University

phone: +44(0)1524 592044
FAX: +44(0)1524 593192
Email: d.clancy@lancaster.ac.uk
Web: https://www.lancaster.ac.uk/fhm/about-us/people/david-clancy

18 March 2019

Cover letter, manuscript submission to Mitochondrion

Title: Mitonuclear gene X environment effects on lifespan and health: how common, how big?

Authors: Emma Drummond, Emma Short, David Clancy

This manuscript, in recognizing the sometimes substantial effects mitochondrial genotypes (mitotypes) can have on health related phenotypes including lifespan, asks how common are these effects and of what magnitude. We use Drosophila as a model because the genetics are well suited to the task, however past work has mostly been done using varied mitotypes on a single homozygous nuclear genetic background (nucleotype). Here we vary mitotype and nucleotype. Because genetic effects on phenotypes are typically modulated by environmental factors, we also simulate non-extreme environmental variations in food and rearing environment to make our survey more comprehensive.

We find that male climbing speed (a measure of functional health) was significantly and substantially affected by mitotype, modified by nucleotype but less so by diet. Lifespan effects of mitotype variation across several nucleotypes are small but significant, and male-biased as predicted. However, compared with these vial-reared flies, mitotype had substantial effects on lifespan of cage-reared flies, also modified by diet and nucleotype. We believe the cages represent a stressful environment and hypothesize that mitochondrial genetic variation may affect health much more under conditions of physiological stress.

This may have implications for human health, particularly the diseases of later life which could impinge on mitochondria in stressful ways, such as circulatory insufficiency, diabetes, pulmonary insufficiency, renal and liver disease.

Yours faithfully,

Lancaster University
Division of Biomedical and
Life Sciences
Faculty of Health and Medicine
Furness Building, Lancaster University
Lancaster, LA1 4YG, UK
T: +44 (0)1524 593 763
www.lancaster.ac.uk/fhm/bls

DAVID J. CLANCY

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Journal: Mitochondrion

Response to reviewers' comments.

We thank the reviewers for their comments. We include those comments here in italics, on which we made changes, and describe the changes made.

Reviewer 1

A major problem with this study is the statistical analyses. As I see it there are two possible approaches a study like this can take: either it tests if interactions between mitotypes and other factors exists, or it quantifies how large these are.

As we see it, we can test if interactions exist, which we did, and once these are demonstrated we were also able to give an idea, not an exact quantification, but a very good idea of relative effect sizes using survival plots and p-values along with chi-square values and degrees of freedom.

When testing if interactions exists a fixed effects model is suitable, but when quantification of the size of interactions a model where the factors and interactions are treated as random effects is needed. In this way the total variation of interest can be partitioned on the various factors and interactions studied, and their relative contribution can be compared. It appears to me as if fixed effect models have been used, which cannot be used to compare the influence of factors and interactions (a comparison of P-values is not valid).

Cox proportional hazards regression analysis does not allow random effects. The purpose of these analyses in this paper is to provide examples of, but not numerically quantify, relative effect sizes. Comparison of p-values will do this, again, not in an analytic way, but they support what can generally be clearly seen in the diagrams.

In the analysis of climbing ability (but not in the analyses of lifespan) there are some random factors included, but it is unclear if the main factors and their interactions are considered random effects.

Table 3, which lists random factors, makes it clear which are random factors. The interaction and main effects are listed together and are not listed as random factors (random factors cannot be given p-values in REML analysis).

The second major statistical problem I see with this study is pseuodreplication. It is never stated what is considered the unit of replication, but given the vary small p-values reported (one as small as 3.11x10^-93!) I can only assume that individual fly is considered the unit of replication. Since flies

within a vial or a cage share the same environment I think vial/cage is the level of replication that should be used.

The reviewer assumes correctly. Individual vials are not used as units of replication in survival analysis. In the approximately 50 survival experiments I have reported across 12 papers I have never done this; no-one uses vial as unit of replication in survival experiments. True, individuals from the same vial cannot be considered completely independent but that's the way the field operates.

Apart from these potential problems I also find the writing very unfocused. I found it very difficult to follow the logical flow of the Introduction and the Methods sections. The Methods further needs a much more detailed description of what was done and how the flies were treated, as well as a through description of the statistical analyses. As currently described, it would not be possible to repeat these experiments for a different lab.

We thank the reviewer for their comments here; re-reading the introduction indicated that it was clear to a worker in the field but less so to a non-specialist. We have rewritten the introduction to improve clarity and logical flow. We have also added to and restructured the methods, and provided subsection headings, to improve clarity. However if someone wished to repeat the entire work from scratch they would have to go back to the papers which describe the original crossing scheme to create isogenic strains. That text is too voluminous, and unnecessary, for the current paper. The methods are also written for someone familiar with fly work, as is typical in all Drosophila papers, and this paper follows a pretty standard level of methodological description.

We describe the statistics used in our results test and in the tables and legend text. They are not out of the ordinary in any way and we are not asking more of the data than it can give, so we feel a full description of statistical methods in the methods section would simply clutter the paper. As it stands, the reader can quite easily view the results and understand how they were arrived at.

In figures 1 & 2 it seems as the proportion of flies alive sometimes increase with time over short periods of time – how is this possible?

In plots where survival appears to increase, this is actually survival remaining unchanged (no mortality) over the short period (one scoring interval), but the way Excel smooths curves can sometimes give a faint impression of increase. Smoothed lines are much easier to look at for comparison purposes, especially when there are lots of plots as in this example, which is why we used them, despite this known issue.

The conclusion focuses on how mito-nuclear interactions may be more pronounced in stressful environments, yet not direct manipulation of stress was undertaken in this study.

Yes, we were not expecting to see an effect of such size. Direct manipulation of stress is our next paper.

Page and line numbers would make commenting easier.

Apologies for this oversight. I assumed the pdf conversion by Elsevier would automatically insert page numbers.

Reviewer 2

We thank the reviewer for their useful comments.

Figure 1and 2 – labels on x and y axes, or give explicitly in the legend

Axes now described in figure legends

Below Table 1. This sentence does not make sense "However, those female flies lived substantially longer than the males whereas we found the reverse, and those lifespans were substantially shorter."

This has been changed to:

"However, in that study all flies lived substantially shorter lifespans compared with ours, and females flies lived substantially longer than the males whereas we found the opposite."

Revise this sentence "in the complete 4-way analysis (not shown) environment was the factor affecting survival by far the most (p=3.11x10-93) " – e.g. environment was the most important factor affecting survival.

We are unwilling to revise this sentence because we cannot see the reason to do so. Revising it as suggested would remove useful information. It is OK to include exact p-values; indeed it should happen more often because they give a useful idea of effect sizes. if the editor requires us to do so we will do so.

"Table 3 shows analysis". Just integrate this into the preceding parentheses e.g. (fig 3, Table 3)

Done

Be consistent with naming lines - Zim and Dah or Zimbabwe and Dahomey?

In methods we have listed mitochondrial lines (Alstonville - Alst, Dahomey - Dah, Japan - Japan) and have continued these throughout the text. Nuclear genotype names we name in full and these have been amended in the text.

Species names in italics.

Fixed.

Ref: MITOCH_2019_53

Title: Mitonuclear gene X environment effects on lifespan and health: how common, how big?

Journal: Mitochondrion

Highlights

- Mitochondrial genetic effects on longevity still show male bias across a range of nuclear genetic backgrounds.
- Nuclear X mitochondrial genetic interactions affect longevity to a modest extent in benign conditions, but affect functional performance to a greater degree.
- Nuclear X mitochondrial genetic interactions affect longevity to a much greater extent in stressful conditions.

Abstract

Mitochondrial genetic variation can have profound effects on fitness, and the mitotype must interact with both the nuclear genes and the environment. We used *Drosophila* to investigate the extent to which mitotype effects on lifespan and activity are modulated by nucleotype and environmental variation. When nucleotype is varied, mitochondrial effects on lifespan persisted but were relatively small, and still male biased. Varying food as well, mitotype had substantial effects on male climbing speed, modifiable by nucleotype but less so by diet. Finally, mitotype affected fly lifespan much more in a cage environment compared with a vial, also modifiable by nucleotype and diet. The cage may represent a stressful environment. Mitochondrial genotype may affect fitness much more in conditions of stress, which may have implications for human health.

Mitonuclear gene X environment effects on lifespan and health: how common, how big?

Emma Drummond, Emma Short, David Clancy*

* To whom correspondence should be addressed: d.clancy@lancaster.ac.uk

Division of Biomedical and Life Sciences, Lancaster University, Lancaster, LA1 4YQ, United Kingdom

Keywords: mitonuclear; epistasis; GxE interaction; *Drosophila*; aging; lifespan; stress.

Abstract

Mitochondrial genetic variation can have profound effects on fitness, and the mitotype must interact with both the nuclear genes and the environment. We used *Drosophila* to investigate the extent to which mitotype effects on lifespan and activity are modulated by nucleotype and environmental variation. When nucleotype is varied, mitochondrial effects on lifespan persisted but were relatively small, and still male biased. Varying food as well, mitotype had substantial effects on male climbing speed, modifiable by nucleotype but less so by diet. Finally, mitotype affected fly lifespan much more in a cage environment compared with a vial, also modifiable by nucleotype and diet. The cage may represent a stressful environment. Mitochondrial genotype may affect fitness much more in conditions of stress, which may have implications for human health.

1. Introduction

Model systems have been used to study organism-level effects of mitochondrial genotype variation, showing that mitotype can affect a range of important phenotypes including development time, viability (Mossman et al., 2016a), fertility (Innocenti et al., 2011), longevity (Clancy, 2008), activity (Aw et al., 2017), as well as oxidative phosphorylation, (Dean et al., 2015; Mossman et al., 2016b; Yee et al., 2013). The mitochondrial genome must also interact with the elements of the nuclear genome which are relevant to mitochondrial function as well as the two-way traffic between mitochondria and nucleus and other parts of the cell. Given this extensive requirement for mitonuclear genetic interaction (reviewed in Dobler et al., 2018; Dobler et al., 2014), we might expect some mitonuclear allele combinations to affect phenotypes more than others. These have been demonstrated; significant mitonuclear epistatic interactions exist and have been shown across a range of species, including nematodes (Chang et al., 2016), mice (Nagao et al., 1998), and *Drosophila* (e.g. Zhu et al., 2014). But the frequency and magnitude of nuclear-mitochondrial interaction effects is not well described.

The development of Mitochondrial Replacement Therapies (MRT), which separate previously coexisting nuclear and mitochondrial genomes, increased focus on the ways mitochondrial and nuclear genotypes (mitotype and nucleotype) interact. The tractable model organism *Drosophila melanogaster* has been useful here (e.g. Clancy, 2008; Rand et al., 2016) producing convincing results on a large scale. Since many of these studies compared different mitotypes on a single isogenic (homozygous) nuclear genetic background, the UK Human Fertilization and Embryology Authority (HFEA), while approving MRT, recommended more experiments using outbred nuclear genetic backgrounds. Our current work is partly a response to that recommendation. Fly data remain very relevant to humans; levels of natural genetic variation are similar between the two species (Morrow et al., 2015).

Several studies observe a sex-specific bias with the majority of biases afflicting males at higher frequencies or to a greater extent (Aw et al., 2017; Camus et al., 2012; Innocenti et al., 2011; Mossman et al., 2016b), and this is particularly true in the case of some mitochondrial diseases, such as Leber's Hereditary Optic Neuropathy. Evolutionary theory had predicted this bias due to maternal inheritance of mtDNA protecting male-harming variants from selection (Frank and Hurst, 1996). At this point the magnitude of these sex-specific effects is not clear, particularly for longevity.

Complex traits such as lifespan tend to be modulated by environmental variation, so that phenotypic measurements on a single genotype vary as the environment varies – the norm of reaction of the genotype. An example is the effect on lifespan of varying dietary restriction (Clancy et al., 2002). When the shape of this reaction varies across different genotypes we see a genotype X environment interaction. Mitochondria play a central role in regulating cell metabolism, largely by nutrient and stress sensing, which obviously involves responding to the environment. Therefore we would expect both mitochondrial and mitonuclear genetics to be particularly subject to gene X environment interactions. Here we examine the degree to which environmental variables (food and rearing environment) modify mitonuclear interactions affecting lifespan and locomotor activity.

Zhu et al. (2014) examined GxGxE interactions affecting *Drosophila* lifespan in the context of dietary and caloric restriction as the environmental variable, using combinations of *D. melanogaster* nuclear genotypes with conspecific, as well as *D. simulans*, mitotypes. To make our work more applicable to the degrees of genetic and environmental variation which we might possibly expect to affect

humans, our approach examines within-species genotypes, including outbred nucleotypes, and less extreme dietary variation. We use wild type *D. melanogaster* genomes and investigate two environmental variables: distinct but broadly similarly nutritious food types (sugar-cornmeal-yeast vs sugar-yeast), and rearing environments (cage vs vial). In doing so we aim to describe effects on lifespan and functional health (climbing speed), in type and magnitude, of interactions between environment and mitochondrial and nuclear genotypes.

2. Methods

Here we use three *D. melanogaster* mitotypes crossed with four wildtype nucleotypes to assess how often and to what degree mitonuclear interactions affect longevity and how this differs according to sex (first longevity experiment). We also vary the food type and rearing environments to explore the effects of nutrition and exercise on male lifespan and functional health (second longevity experiment).

For each of the two lifespan experiments parental flies were bred at low density first as individual strains, then as crosses, according to Clancy and Kennington (2001) on sugar yeast food (SY - 8% sugar: 8% yeast: 1.6% agar) before F1 offspring were collected, allowed to mate for 48 hours, then flies collected using light anaesthesia. Food vials were changed, and deaths counted on Monday, Wednesday and Friday, also using brief anaesthesia. All parental and experimental flies were kept in an incubator set to 25 ℃ and 70% relative humidity on a 12:12 light:dark cycle.

The three mitotypes share an isogenic (w1118) nuclear background. There were received from Dr Damian Dowling and had been generated and maintained as described in Yee et al. (2015). Briefly, flies from Alstonville (Australia), Jume (Japan) and Benin (formerly Dahomey) had their nuclear genomes replaced by the isogenic nuclear genome, w^{1118}. They were maintained by regular backcrossing to isogenic w^{1118} which itself had been maintained as isogenic by regular sib mating. Wildtype genotypes were older established lab lines (Athens, Dahomey, Hawaii, Peurto Montt, Red Knight, Zimbabwe) as described in (Clancy, 2008), and Lancaster was mass bred from wild-caught flies from Lancaster, UK, in October 2014. To examine mitonuclear interactions we crossed females of the three mitochondrial genotypes on isogenic nuclear backgrounds with males of the wildtype genotypes to generate a matrix of mitonuclear genotypes of F1 offspring for testing.

2.1. Mitochondrial x Nuclear x Sex effects on lifespan

To determine the frequency and magnitude of mitonuclear interaction effects on lifespan, and differences across sexes, we crossed females of the three isogenic mitochondrial lines (Alstonville - Alst, Dahomey - Dah, Japan - Jap) with males of Athens, Dahomey, Hawaii, and Puerto Montt strains and collected F1 offspring for lifespan assays. This gave three mitotypes X four nucleotypes X two sexes. Each of the 24 treatments had 9 vials containing approximately 20 flies each.

2.2. Mitochondrial x Nuclear x Environmental effects on lifespan

To determine how mitonuclear effects on male lifespan are modulated by environment (GxGxE interactions), we again created mitonuclear genotypes and measured lifespan on two food types (cornmeal, SY) and in two rearing environments (vial, cages). Nutritional differences between diets

are modest whereas the purpose of the cages was to provide a larger living space (~1litre) for the flies to allow increased flying and exercise, which they are otherwise unable to perform within the vials. Cornmeal food contained 13.2% sugar: 3.3% yeast: 5.7% cornmeal: 1% agar, while sugar yeast (SY) food contained 15% sugar: 8% yeast: 1.6% agar. To both foods, 30ml of Nipagin (10% w/v in ethanol) and 5ml of propionic acid per litre were added as a preservative. Food for the cages was prepared identically to that of the vials but was set on a horizontal slope within the vial, increasing the surface area of food available to the flies. These sloped food vials could then be inserted and removed from the side of the cage. Flies in cages were not gassed prior to food changes and dead flies were removed weekly by aspiration.

We crossed females of the three isogenic lines (Alstonville, Dahomey, Japan) with males of Lancaster, Red Knight and Zimbabwe wildtype strains, and collected F1 males into vials containing their allotted experimental food composition. Six vials containing either the cornmeal or SY food type were prepared for each genotype, and approximately 20 males allocated to each using brief anaesthesia, giving a total of approximately 120 flies in each experimental group. 18 cages were used: two for each genotype containing one of the two food types, each containing approximately 120 male flies. Therefore, the fully crossed design had 3 mitotypes X 3 nucleotypes X 2 food types X 2 environments, with approximately 120 males per treatment.

2.3. Mitochondrial x Nuclear x Environmental (food) effects on climbing speed

To determine the frequency and magnitude of mitonuclear X environment (GxGxE) interactions on the functional health of the flies, climbing speed was measured using flies from our second longevity experiment. However only vial-reared flies were used because withdrawing flies from cages and then replacing them is difficult and disruptive to a longevity experiment. So here, the environmental variable tested was solely food type. At day 14 flies were transferred without anaesthesia from their experimental vials into upright acrylic tubes held vertically in a rack, each 50cm long X 1.0cm inner diameter and plugged at each end and allowed 15mins to settle. Flies were tapped to the bottom of the climbing tubes, allowed to climb for 15secs then photographed to allow measurement of height climbed. Triplicate climbs were recorded, with a 2-minute rest period between each. Each treatment combination was represented by 2-3 vials of approximately 20 flies per vial.

3. Results and Discussion

We used three mitotypes on a range of wild type genetic backgrounds to ask three questions. How common and how big are mitonuclear interaction effects on lifespan, and do we see the predicted male-biased mitochondrial effect? To what extent are mitonuclear interaction effects on lifespan modulated by environmental factors? To what extent do mitonuclear variants affect an activity-based measure of health, and to what extent can this modulated by a non-stressful environmental factor (food)?

3.1. Mitochondrial x Nuclear x Sex effects on lifespan

Our first experiment focusses on sex and mito-nuclear interactions. Crossing females from three isogenic lines (Alstonville, Dahomey and Japan) to males from four wildtype lines (Athens, Dahomey,

Hawaii and Puerto Montt) created a matrix of F1 offspring. Male and female lifespans were measured on SY food (figure 1).

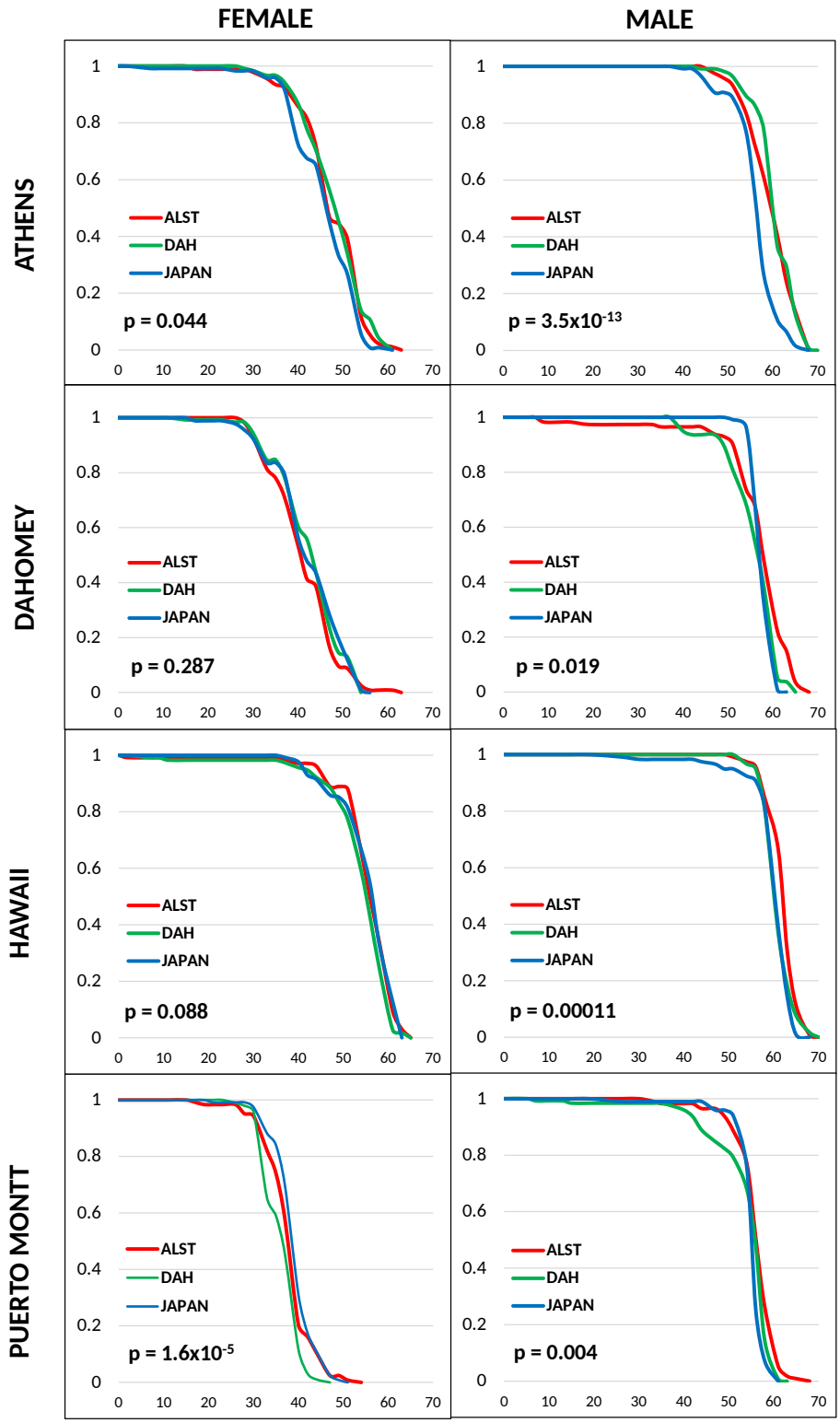

Figure 1: Survival curves of mitotypes by sex and by nucleotype at 27°C. Y-axis is survival probability, x-axis is time (days). Nucleotypes are F1 offspring of crosses between w1118-mitotype females (ALST, DAH, JAPAN) and each of the four wildtype strain males (Athens, Dahomey, Hawaii, Puerto Montt). P-values are from Tarone-Ware tests for survival differences between mitotypes.

In the relatively optimal conditions of SY food in vials, three of four nucleotypes showed more lifespan variation due to mitotype in males compared with females, although in general the magnitude of this mitotype effect was small. Proportional hazards analysis showed a substantial mitotype*sex effect (p=0.0001, analysis not shown). The extent of this difference between sexes is also shown by proportional hazards analyses on the sexes separately (table 1); the mitotype term is strongly significant among males but non-significant among females. Rank orders of lifespan by mitotype differed little between sexes within nucleotype but did change somewhat, as might be expected, between nucleotypes within sexes, as indicated by the significant mito*nuc terms in table 1.

Sex	Factor	d.f.	X^2 *	p
FEMALE				
	nucleotype	3	780.4	<0.0001
	mitotype	2	3.3	0.1941
	nuc*mito	6	21.0	0.0019
MALE				
	nucleotype	3	247.6	<0.0001
	mitotype	2	53.5	$2.4x10^{-12}$
	nuc*mito	6	26.0	0.0002

Table 1: Proportional hazards survival analyses comparing effects on survival of mitotype, nucleotype and mito x nucleotype interaction across sexes. * = likelihood ratio chi-square.

Male bias for mitotype-mediated longevity variation was first observed by Camus et al. (2012) on a single homozygous nuclear background (w^{1118}iso). However, in that study all flies lived substantially shorter lifespans than ours, and females flies lived substantially longer than the males whereas we found the opposite. There is anecdotal and empirical support for increased susceptibility of males to mutations (Linda Partridge, pers. comm., Mallet et al., 2011), so perhaps homozygosity has a similar effect. Our flies here are essentially outbred, being F1 offspring of two distinct strains, and the generally robust lifespans reflect this. Another possibility is that the sexes have differential survival according to food type, despite sharing the same chromosomes.

A male-biased sex X mitotype interaction effect for survival was also seen in Aw et al. (2017) who used the w^{1118}iso nucleotype with two mitotypes. The effect size was substantial with high protein in the food; on food similar to ours, mitotype affected male median survival by about 17%, which disappeared as protein content in food was diluted. Zhu et al. (2014) examined nuclear X mitochondrial combinations in *D. melanogaster* females, using two mitotypes on two nucleotypes, and on food most similar to ours (10% SY vs 8%) saw larger differences in mean lifespans (~8-12% vs 2-7%) between mitotypes. On leaner food (5% SY) these differences disappeared in three of the four cases, so female results in Zhu et al. may be broadly similar to ours. In males here, differences in mean lifespan between mitotypes within nucleotypes were all less than 10%, on a picture of fairly square survival curves and robust lifespans.

3.2. Mitochondrial x Nuclear x Environmental effects on lifespan

Our second experiment aims to examine plasticity of mitotype and mitonuclear interaction effects on lifespan, particularly how affected they were by environment. All flies used were male with the environmental variables being food type (cornmeal food vs sugar-yeast food) and rearing environment (vial vs cage). Figure 2 shows how mitotype affects survival modulated by nucleotype, rearing environment and food type. Relative contributions of these factors are shown in table 2 which provides proportional hazards analyses for cage and vial environment separately; in the complete 4-way analysis (not shown) environment was the factor affecting survival by far the most ($p=3.11x10^{-93}$).

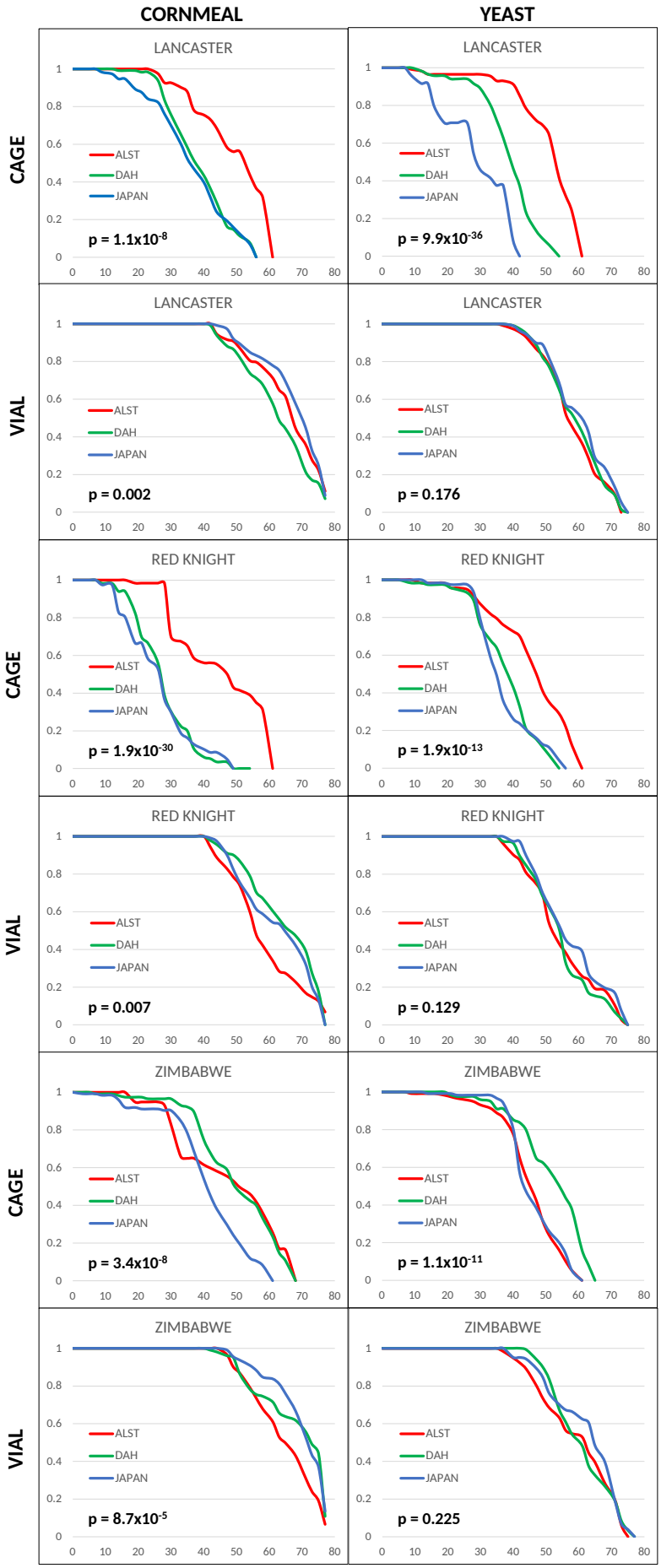
CORNMEAL
YEAST
CAGE
VIAL
CAGE
VIAL
CAGE
VIAL
LANCASTER
RED KNIGHT
ZIMBABWE
ALST
DAH
JAPAN
p = 1.1x10-8
p = 9.9x10-36
p = 0.002
p = 0.176
p = 1.9x10-30
p = 1.9x10-13
p = 0.007
p = 0.129
p = 3.4x10-8
p = 1.1x10-11
p = 8.7x10-5
p = 0.225

Figure 2: Survival curves of male mitotypes by food type (cornmeal vs sugar-yeast - 'yeast'), rearing environment (vial vs cage) and by nucleotype, at 25°C. Y-axis is survival probability, x-axis is time (days). Nucleotypes are F1 offspring of crosses between w1118-mitotype females (ALST, DAH, JAPAN) and each of the three wildtype strain males (Lancaster, Red Knight, Zimbabwe). P-values are from Tarone-Ware tests for survival differences between mitotypes.

Environment	Factor	d.f.	X^2 *	p-value
Cage				
	nucleotype	2	365.4	< 0.0001
	mitotype	2	287.5	$3.7x10^{-63}$
	food	1	0.4	0.4833
	nuc*mito	4	185.2	$5.7x10^{-39}$
	mito*food	2	33.2	$6.2x10^{-8}$
	nuc*food	2	76.0	$3.3x10^{-17}$
	nuc*mito*food	4	35.8	$3.0x10^{-7}$
Vial				
	nucleotype	2	62.2	< 0.0001
	mitotype	2	14.6	0.0007
	food	1	228.2	$1.5x10^{-51}$
	nuc*mito	4	6.9	0.1406
	mito*food	2	1.5	0.4815
	nuc*food	2	0.5	0.7808
	nuc*mito*food	4	9.8	0.0442

Table 2: Proportional hazards survival analyses comparing effects on survival of mitotype, nucleotype, food and two- and three-way interactions across rearing environment (cage vs vial). Exact p-values are supplied to suggest relative effect sizes. * = likelihood ratio chi-square.

We see much more variation across genotypes, particularly mitotypes, in the cage environment vs vial environment. In the cage environment Alst mitotype generally survives better across nucleotypes, except on the Zimbabwe nucleotype, and Japan mitotype survives worst on all foods and nucleotypes. In the vial environment, broadly the reverse seems to be true. Food type itself has no effect on survival in cages but shows significant interactions with nucleotype and mitotype and their GxG interaction. In contrast food is the major factor affecting survival in the vial environment (cornmeal food enhanced survival) but did not interact with genetic factors to affect survival, in agreement with Aw et al. (2017). There is nearly always more variation across mitotypes with cornmeal in both rearing environments, possibly because SY food was used for all larval diets and genotypes may have varied in how they responded to the change in dietary and/or physical environment aspects of the food going from larval to adult stage.

Lifespans are invariably shorter in cages, so, although this was not the intention, it could be considered a stressful environment compared with vials, and it is in this environment that most mitochondrial genetic variation is expressed phenotypically as survival. Food also interacted with mitotype in this environment to affect survival. Flies can fly as well as walk in cages although casual

observation suggested they do not seem to fly much, but they need to walk further for their food compared with the vial environment. Increased activity was the initial intent behind use of cages; houseflies able to fly (cages) lived considerably shorter than those only able to walk (vials), and this was associated with increased mitochondrial protein oxidation (Yan and Sohal, 2000)

Perhaps more importantly here, mitochondria are conduits of stress signals: oxidative, nutritional, and in *Drosophila* desiccation, heat and starvation stresses (Aw et al., 2017; Lasne et al., 2018). Desiccation may have been a factor in the cage environment; the flies were mostly at ambient (70%) humidity whereas the vial microenvironment will have more moisture due to proximity of the food. Starvation may have affected flies at later ages due to reduced locomotor ability requiring more effort to walk to food.

It is difficult to tell what killed cage flies earlier than vial flies, but the environment was certainly relatively stressful, which uncovered substantial mitochondrial and mitonuclear genetic variation for lifespan. This result raises the possibility that mitochondrial and mitonuclear genetic variation for phenotypes related to health may be expressed more dramatically under conditions of stress.

3.3. Mitochondrial x Nuclear x Environmental (food) effects on climbing speed

To examine GxGxE interactions on functional health we measured climbing height in 15secs of flies at 14 days old reared in vials on two food types (fig 3, Table 3). Table 3 shows analysis. Mitotype affected performance (p=0.0006), nucleotype affected rank order of mitotype performance (nuc*mito p=0.001) as expected, but not in the same way across food types. Food type has a generally modest effect on mitotype ranking within each nuclear genotype overall (nuc*mito*food p=0.020) but on the Lancaster and Zimbabwe nucleotypes the food*mito interaction effect is most pronounced. Note that the best performer overall is the Dah mitotype on the Zimbabwe nuclear background; Zimbabwe and Dah mitotypes are very similar (Clancy, 2008) so the two genomes may work best together. The biggest effect size attributable to mitotype was on Zimbabwe nuclear background, cornmeal food: Dah mitotype climbed 49% higher than Alst mitotype (p=0.035), which is a considerable difference.

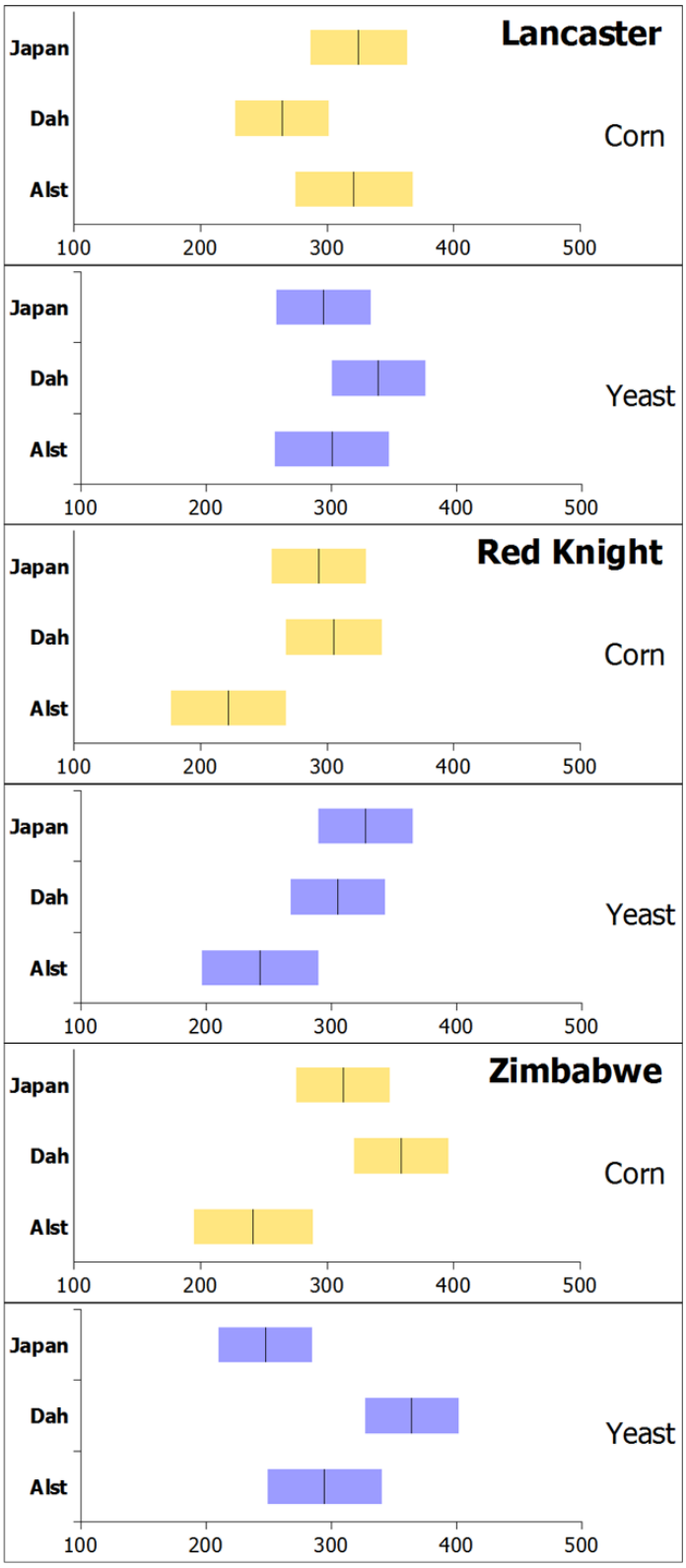

Figure 3: Climbing distance in 15secs (x-axis is millimetres climbed) of mitotype (y-axis) males from vial environments, by food type (cornmeal vs sugar-yeast - 'yeast') and nucleotype. Nucleotypes are F1 offspring of crosses between w1118-mitotype females and each of three wildtype strain males: Lancaster, Red Knight, Zimbabwe. Measures were based on 2-3 tubes per treatment, each with 15-20 flies, measured in triplicate, and are least-squares means estimates with 95% confidence intervals.

Factor	d.f.	F	p
nucleotype	2	2.63	0.0888
mitotype	2	9.60	0.0006
food	1	0.71	0.4064
nuc*mito	4	6.53	0.0007
nuc*food	2	0.40	0.6740
mito*food	2	2.73	0.0811
nuc*mito*food	4	3.44	0.0199
Random factors		**% total variance explained**	
biolrep(nuc*mito*food)			3.58
triplicate (biolrep)			0.16

Table 3: Model fit for climbing speed using restricted maximum likelihood (REML). There were 2-3 tubes (biolreps), each containing 15-20 flies, and these were measured three times in succession (triplicates). Thus the random factor triplicate was nested within biological replicate (biolrep) and biolrep was nested within each combination of nucleotype x mitotype x food type. R-squared for the model = 0.105.

Nuclear background, then, can have substantial effects on expression of mitotype variation for activity. Where nuclear variation has been absent or limited, mitotype effects on activity have been more modest. Physical activity over 24hrs at ages 11 and 32 days of flies bearing a single isogenic (w1118) nuclear background showed a maximum difference between mitotypes of approximately 15% at 11 days old, increasing to 25% at 32 days old (Aw et al., 2011). Distance travelled in 30mins by flies with one of two mtDNA and Y-chromosome genotypes (matched vs mismatched), reared on a rich diet or a starvation diet in solitary or 'social' contexts showed differences attributable to mitotype of ≤10% (Dean et al., 2015); a stressful environment (starvation diet) modulated the effect of mitotype on activity either modestly (social) or not at all (solitary).

Zhu et al. (2014) demonstrated GxGxE interactions affecting *Drosophila* lifespan in the context of dietary and caloric restriction as the environmental variable, and using combinations of *D. melanogaster* nuclear genotypes with conspecific as well as *D. simulans* mitotypes. Our aim was to measure mitonuclear effects on longevity and activity under relatively normal conditions to see the extent to which these effects are modifiable by environment; to show potential effect sizes. In one way our work may underestimate the phenotypic variation possibly generated by mitotype variation because across tested strains half the nuclear genome was the same, coming from the w1118 isogenic nucleotype. Backcrossing repeatedly to outbred genomes may uncover greater effects on phenotype, though these were not especially evident in the case of lifespan in Zhu et al. (2014).

Since the wildtype strains used in our crosses were outbred, with a stronger possibility of MT heteroplasmy, there may be selection post-fertilization for the more development-compatible mitonuclear combinations. However, embryonic or larval lethality did not occur to any obvious degree and, by using F1 flies, we prevent any mitonuclear selection in culture over generations. If anything, however, postfertilization selection would, again, lead to an underestimate of mitonuclear effects.

Between developmental lethality and optimal adult health, there is a spectrum of genetically influenced health. It is along this spectrum that the range of mitonuclear incompatibilities exert their effects on fitness, as has been shown in the *Drosophila* model, in phenotypes beyond survival and activity, including mtDNA copy number and OXPHOS capacity (Camus et al., 2015), fertility, starvation resistance and lipid content (Aw et al., 2011), viability and development time (Mossman et al., 2016a). In Mitochondrial Replacement Therapy poorly coadapted combinations, forced together as they would be, may not survive development, but some negative effects may result due to this intervention, an intervention whose sole purpose is to allow a mother to have a child bearing her own chromosomes, instead of using IVF by donor.

We know interacting gene networks show flexibility (van Swinderen and Greenspan, 2005); stress can increase expression of epistatic genetic variation for developmental fitness traits (Blows and Sokolowski, 1995) and for adult traits (Chirgwin et al., 2016). Overcoming the pressures of selection, recombination and genetic drift, mitonuclear interactions can be strong so that coadaptations can persist in nature (Morales et al., 2018).

4. Conclusions

Overall our data suggest that in benign environments mitonuclear interactions may have mostly minor effects on lifespan yet may have substantial effects on function, but effects on lifespan may be amplified in stressful environments. In this experiment the stress is externally applied, however, under certain conditions of ill-health, stresses can be generated internally, such as inflammation, electrolyte imbalance, osmotic stress, fever, hypoxia and other effects of circulatory or respiratory insufficiency, oxidative stress, hyper- and hypoglycaemia. Given our data and those of others, it is likely that nuclear-mitochondrial interactions modulate the effects of at least some of these stresses. If they can be identified, they can be guarded against.

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Data is available at Mendeley Data. Clancy, David (2019), "*Drosophila* mitonuclear GxGxE paper", Mendeley Data, v1. http://dx.doi.org/10.17632/7rnwhcswvb.1

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